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Role of β-adrenoceptors, cAMP phosphodiesterase and external Ca^{2+} on polyamine-induced relaxation in isolated bovine tracheal strips

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Abstract:
Polyamines relax several smooth muscles and elicit cardiotonic effects in the rat heart *via* interactions with β-adrenoceptors. The aim of this work was to establish whether β_2-adrenoceptors were involved in polyamine-relaxation of bovine tracheal strips. A variety of polyamines displaced the specific radioligand, [^{3}H]dihydroalprenolol, but spermine was the most potent. The polyamines elicited an acute transient relaxation, which was independent of β-adrenoceptor activation, followed by a maintained component, which was shown to be dependent on β-adrenoceptor activation because it was antagonized and reversed by propranolol. Polyamines did not alter salbutamol-induced acute relaxation. Polyamines modified the salbutamol-induced long-term effect on airway tone, which was shown by a partial reversal of β-adrenoceptor desensitization. This process was delayed by α-difluoromethylornithine, but spermine increased the latency and time of reversal and decreased receptor desensitization. Putrescine prolonged the time-constant without changes in the desensitization. Spermine, but not putrescine, might block Ca^{2+} channels because it relaxed KCl- or electrical stimulated-contractions, which are related to Ca^{2+} influx, and the inhibition of cAMP phosphodiesterase activity. These differences might explain the functional differences observed between putrescine and spermine. Therefore, polyamines may modulate airway smooth muscle tone and interfere with the mechanism of receptor desensitization *via* several mechanisms involving β_2-adrenoceptors, Ca^{2+} influx and cAMP phosphodiesterase.

Key words:
polyamines, airway smooth muscle, β-adrenoceptors, calcium, phosphodiesterase

Introduction

Polyamines play an important role in cellular growth, differentiation and apoptosis. They have been associated with several pathological conditions, including cancer [32], cardiac and renal hypertrophy [28], asthma [23] and cystic fibrosis [12].

The role of polyamines in asthma may be the consequence of alterations in arginine metabolism through increased arginase expression and activity, which

could potentiate airway reactivity by competing with nitric oxide (NO) synthases for the common substrate, arginine [40]. This effect may cause a relative deficiency of NO or an increase in the production of L-proline and L-ornithine [23]. Subsequently, L-ornithine is metabolized by ornithine decarboxylase to produce the polyamine putrescine, which is converted to spermidine and spermine [38]. The endogenous polyamine metabolites might also be involved in asthma-induced airway remodeling. Ornithine decarboxylase expression, the rate limiting enzyme in the synthesis of polyamines [29], was reported to increase in the airway smooth muscle of smoking asthmatic patients [2]. This enzyme is regulated by androgens [13], which, among other factors, might account for gender differences in the prevalence of asthma [30].

Polyamines might also be involved in the modulation of airway smooth muscle tone. Indeed, polyamines have been shown to relax smooth muscle tone in guinea-pig trachea [10] and cause smooth muscle contraction in rat trachea [7]. The contractile effect was related to an interaction with G-protein coupled receptors in macrophages, which caused the release of histamine and serotonin [7].

Polyamines have also been associated with respiratory diseases, such as cystic fibrosis [12] and infections of *Pneumocystic carinii* [19], where an accumulation of polyamine levels in the lung is related to alveolar apoptosis. It is also possible that different bacterial pathogens may alter polyamine metabolism and levels in the respiratory tree. There are also examples of this phenomenon occurring in other tissues. In addition, bacterial LPS [31] and *Helicobacter pylori* [9] have been shown to induce ornithine decarboxylase, which is associated with compromised host innate immune responses.

The role of polyamines in the modulation of airway smooth muscle tone has not been as widely studied as it has for the modulation of several vascular [24] and nonvascular smooth muscle tones [21, 25–27]. In addition to the effects on smooth muscles, polyamines also modify cardiac contractility. Indeed, spermine and spermidine produced negative inotropism [37], and putrescine elicited a cardiotonic effect [3, 36] in isolated myocardium preparations. It has been reported that the effects of polyamines modulating smooth muscle tone [17, 26] and inducing negative inotropism [37] were related to a decrease in Ca^{2+} influx. Moreover, an interaction with β-adrenoceptors in rat hearts have been reported [3]. Indeed, putrescine has been shown to modulate β-adrenoceptor mediated responses in rat hearts and elicit cardiotonic effects associated with increased intracellular cAMP [3, 36].

The work presented here aimed to establish the possibility of an interaction between polyamines and β_2-adrenoceptors as well as the functional consequences of this interaction. In addition, using bovine tracheal smooth muscle strips as a model, this study investigated the role of phosphodiesterase and external calcium on the spasmolytic effect of polyamines.

Materials and Methods

Preparations of tracheal bovine strips and experimental procedures

Bovine tracheal smooth muscle strips were obtained from healthy male cattle (10–13 months old, breed – Asturiana de los Valles, Asturias, Spain) [5]. Four to six adjacent transversal, similar rectangular segments (approximately 15 × 2.5 mm and 155.14 ± 3.31 mg in weight) were obtained and mounted in a 6-ml organ bath containing Krebs solution (118 mM NaCl; 4.75 mM KCl; 2.5 mM $CaCl_2$; 1.19 mM KH_2PO_4; 25 mM $NaHCO_3$; 1.2 mM $MgSO_4$; and 11 mM glucose) according to a previously described and validated method [5, 16].

Tissues were allowed to stabilize for at least 2 h, and the buffer solution was renewed every 30 min. Afterwards, the preparations were contracted by the addition of carbachol (0.3 μM) to the organ bath, which elicited approximately 50% of the maximal contraction to carbachol, or KCl (80 mM) (using a modified Krebs solution with 43 mM NaCl and 80 mM KCl, instead of 118 mM NaCl and 4.75 mM KCl) [5]. When the contraction became stable, the effects of the polyamines were studied by adding cumulative (1 to 10 mM) or single concentrations (10 mM) to the organ bath.

To avoid potential long-term effects or paradoxical effects of polyamines [10], only one exposure was performed on each preparation. The influence of the epithelium on polyamine-induced relaxation was studied in pairs of strips: one strip had an intact epithelium and the other had the epithelium removed.

Incubation times were 5 to 10 min for the β-adrenoceptor antagonist propranolol (1 μM) and 1 h for tyramine (0.1 mM), an indirect sympathomimetic amine [14]. In some preparations, tyramine (0.1 mM) was administrated 45 min before the polyamines in carbachol-induced contraction. Propranolol (1 μM) was also added once a stable polyamine-induced relaxation was achieved.

The effects of polyamines on β_2-adrenoceptor-mediated acute responses were studied by the addition of α-difluoromethylornithine (3 mM), putrescine (10 mM) or spermine (10 mM) into the organ bath. These compounds were added to the bath 30 min before the relaxation concentration-response curve of salbutamol (1 nM to 30 μM) or salbutamol (3 and 30 μM)-induced relaxation to study long-term activation and functional desensitization of β_2-adrenoceptors.

Additional experiments were performed in preparations whose contractions were elicited by electrical stimulation, applying trains of pulses (0.5 ms, 20 Hz and 10 s) every 120 s using silver wire electrodes and a Grass S11 Stimulator (MA, USA).

The influence of polyamines (10 mM) on carbachol (0.3 μM)-induced contraction was studied by adding various polyamines to the organ bath 20 min before the second contraction elicited by 0.3 μM carbachol.

Binding assay in bovine tracheal membranes

The membranes were prepared from bovine tracheal smooth muscle, with the epithelium and connective tissue removed, following a previously described method [5]. Displacement experiments were performed using increasing concentrations of the polyamines (10 μM to 100 mM) putrescine, spermidine and spermine in the presence of [^{3}H]dihydroalprenolol (1 nM). All experiments were conducted in triplicate and repeated independently at least four times.

Ornithine decarboxylase assay

These assays used bovine tracheal smooth muscle strips from healthy female and male cattle, 30 to 40 months old, with the epithelium and connective tissue removed. The determination of ornithine decarboxylase activity was based on a previously described method [4]. The strips were homogenized in 1 ml of ice-cold buffer containing 10 mM Tris-HCl, 50 μM pyridoxal-5-phosphate and 2 mM dithiothreitol (pH 7.2). The homogenate was centrifuged for 15 min at 26,000 × g at 4°C. Then, 300 μl of the supernatant and 0.25 μCi of L-[1-^{14}C]ornithine (final concentration 20 μM) were incubated for 60 min at 37°C in a closed tube equipped with a filter paper wetted in 50 μl of 10% KOH to trap released $^{14}CO_2$. The incubation was terminated by injecting 150 μl of 10% trichloroacetic acid and incubated for a further 45 min at 37°C to release $^{14}CO_2$, which was measured by liquid scintillation.

The non-specific $^{14}CO_2$ released was measured in blank tubes in which α-difluoromethylornithine was added before the supernatant. Assays were duplicated in each experiment. Specific ornithine decarboxylase activity was expressed as pmol of $^{14}CO_2$ per h per mg of protein.

Phosphodiesterase assay

This assay utilized bovine tracheal strips, with or without the epithelium, contracted by carbachol (0.3 μM). The strips were homogenized in ice-cold lysis buffer as previously described [34]. The insoluble proteins were removed by centrifugation at 16,000 × g for 10 min at 4°C. The supernatant was used to assay phosphodiesterase activity using a two-step procedure in the reaction buffer (10 mM Tris-HCl, pH 8; 0.5 mM $MgCl_2$, 1 μM cAMP and 0.08 μCi of [$^{2,8\text{-}3}$H] cAMP) for 20 min at 30°C in a total volume of 100 μl. Reactions were stopped by boiling the samples and incubating them with 50 μg of snake venom, *Crotalus attrox* (Sigma), at 30°C for 10 min. Following this incubation, we added 0.4 ml of Dowex resin (Sigma 1x8-400) [34]. The radioactivity was determined by liquid scintillation, and the results were expressed in pmol of cAMP hydrolyzed per min per mg protein. To determine the influence of polyamines on cAMP phosphodiesterase activity, the tracheal extracts were incubated with putrescine or spermine (1 to 10 mM) for 30 min.

The protein content was determined by the Bradford procedure.

Drugs and radiochemicals

The following drugs were obtained from Sigma (St. Louis, MO, USA) and used in the present experiments: carbachol (carbamylcholine chloride), putrescine (tetramethylenediamine dihydrochloride), spermidine (N-[3-aminopropyl]-1,4-butanediamine trihydrochloride), spermine (N,N'-bis[3-aminopropyl]-1,4-butane-

diamine tetrahydrochloride), salbutamol (α-[(t-butylamino) methyl]-4-hydroxy-*m*-xylene-α,α'-diol), ICI-118,551 hydrochloride ((±)-1-[(2,3-dihydro-7-methyl-1H-inden-4-yl)-3-[(1-methylethyl) amino]-2-butanol hydrochloride), propranolol (1-[isopropylamino]-3-[1-naphthyloxy]-2-propanolol) and tyramine (4-hydroxyphenethylamine hydrochloride; tyrosamine hydrochloride). α-difluoromethylornithine (DL-α-difluoromethylornithine) was donated by Dr. Wooster (Wayne University, USA). All of these drugs were dissolved in purified water. [^{3}H]dihydroalprenolol and L-[1-^{14}C]ornithine were obtained from Amersham (Munich, Germany), and [$^{2,8\text{-}3}$H] cAMP was obtained from Perkin-Elmer (Waltham, MA, USA).

Calculation and statistical analysis

The peak of the acute spasmolytic effect was expressed as a percentage of relaxation to carbachol (0.3 μM) or KCl (80 mM)-induced contractions. If the whole raised tone was relaxed, it was considered 100% relaxation. The reversal of the relaxation was expressed as the percentage of recovery of polyamine-induced peak relaxation, which was measured when the steady state of long-term tension was reached.

The effect of polyamines on electrically-induced contractions in bovine trachea was expressed as the percentage of inhibition of the contraction.

To plot the influence of polyamines on salbutamol (1 nM to 30 μM)-induced relaxation, the effects of the polyamines were subtracted so that we were only measuring the effect of salbutamol. To analyze the time constants (τ) and the time to reach the maximum relaxation after the addition of the drug to the organ bath, or the partial restoration of tension after the beginning of this effect, the recordings were scanned, digitalized (GetData Graph Digitizer 2.22) and fitted to a single exponential equation (Igor Pro V5.0, WaveMetrics Inc., Oregon, USA). The latency of reversal of polyamine relaxation was measured as the time elapsed from the peak of the relaxation to the beginning of the recovery, which was when the tone started to increase.

The K_D and B_{max}, which were related to the number of receptors, were determined using the computer radioligand program RADLIG (Biosoft). We used a Scatchard plot to graphically view the results.

The results of the phosphodiesterase assay were normalized to the enzymatic activity of the extract in the absence of polyamines.

The data obtained were expressed as the mean ± SEM for a number (*n*) of at least four different preparations obtained from different animals. Statistical significance of differences between means was calculated by Student's *t* test and analysis of variance followed by Bonferroni's test. Values of $p \leq 0.05$ were considered to be significant.

Results

Effects of putrescine, spermidine and spermine on [^{3}H]dihydroalprenolol binding to bovine trachea membranes

Binding of the radioligand to trachea membranes was saturable (0.1 to 10 nM) and was displaced by a selective β_2-adrenoceptor antagonist, ICI-118,551. Under these experimental conditions, the Scatchard plot showed a receptor density of 0.3 pmol/mg protein and a K_D of 0.75 nM.

The competition assay with putrescine, spermidine and spermine (10 μM to 100 mM) showed displacement of [^{3}H]dihydroalprenolol, and the most potent and effective polyamine was spermine (K_D: 3.73 ± 0.41 mM), which fully displaced the radioligand at the 100 mM concentration. Putrescine and spermidine (100 mM) only displaced approximately 50% of [^{3}H]dihydroalprenolol binding (Fig. 1).

Effects of spermine and putrescine on bovine tracheal strips precontracted by carbachol and the influence of the epithelium

Putrescine and spermine (1 to 10 mM) elicited a concentration-dependent relaxation of bovine trachea precontracted by carbachol (0.3 μM). The effect had two components: an acute transient stage of partial restoration of tension (Fig. 2A) followed by a persistent, long-lasting relaxation. The acute effect was slightly slower for spermine (4.09 ± 0.46 min) than putrescine (2.19 ± 0.20 min), and spermine was more effective than putrescine at the 10 mM concentration (Fig. 2B). This acute transient relaxation was concentration dependent when the polyamines were added cumulatively to the organ bath (Fig. 2C). The percentages of recovery of spermine- and putrescine (10 mM)-induced relaxation, respectively, were 54.40 ± 3.63% and 66.53 ± 6.07%, which occurred 21.38 ±

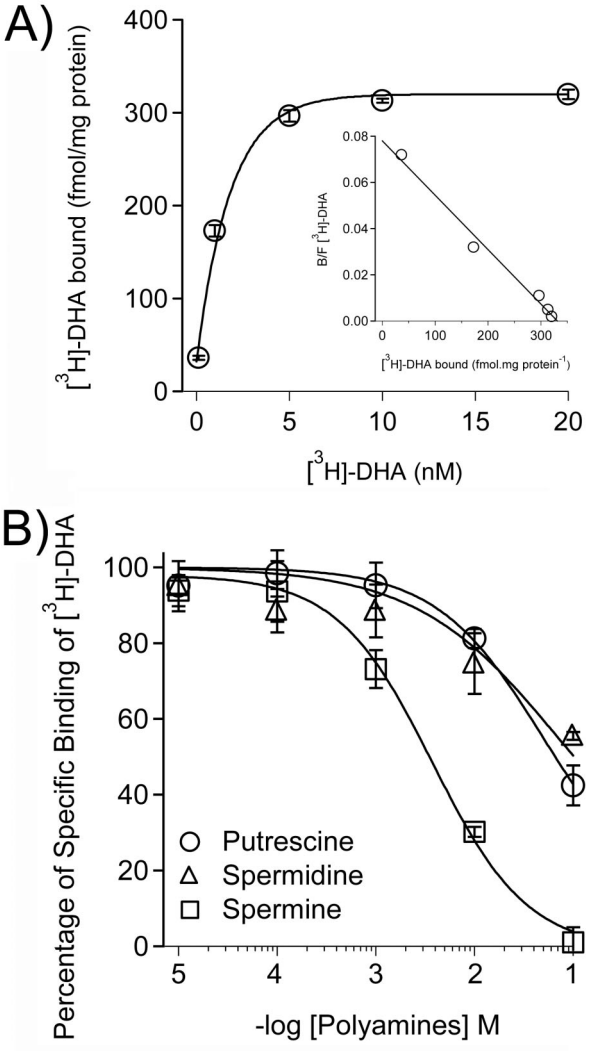

Fig. 1. A) Specific binding of [^{3}H]dihydroalprenolol (DHA) to bovine trachea smooth muscle membranes as a function of the concentration of radioligand. The specific binding of radioligand was defined as the portion displaceable by ICI-118,551 (0.5 μM). This panel shows a Scatchard plot obtained from the same data. *B*: specific radioligand binding; *B*/*F*: ratio of specific binding to the concentration of free radioligand. **B**) Competition for specific [^{3}H]DHA (1 nM) binding to bovine trachea membranes by the polyamines putrescine, spermidine and spermine (10 μM to 100 mM). Values represent the mean ± SEM of 4 different experiments performed in triplicate

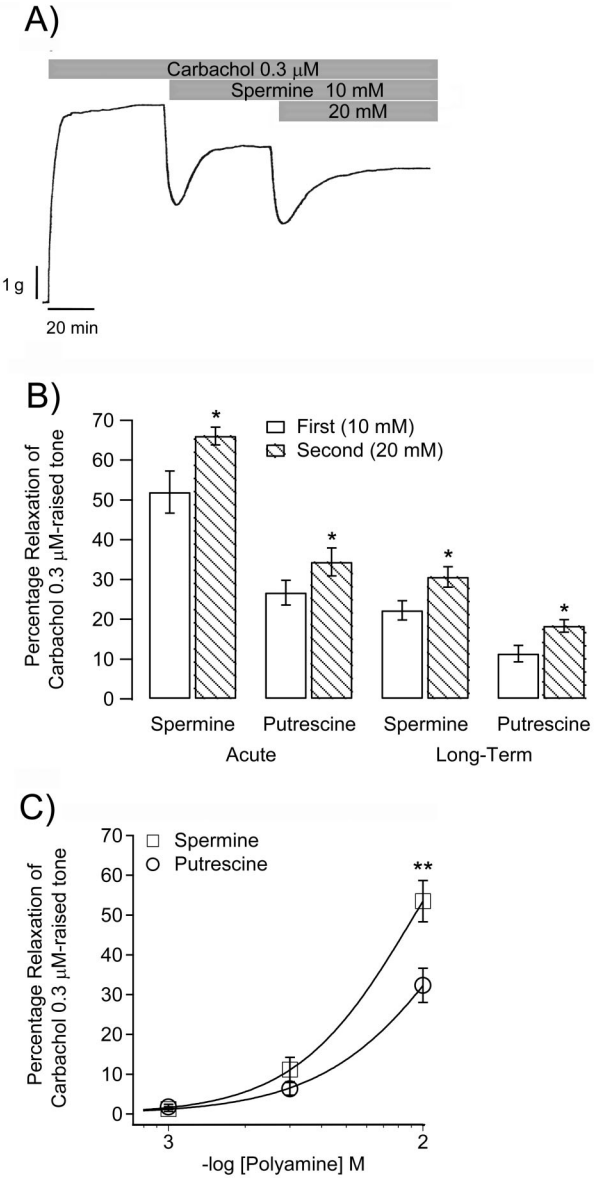

Fig. 2. A) Recording of spermine (10 mM)-induced relaxation and further relaxation by the cumulative addition of another 10 mM of spermine (20 mM in the organ bath) in bovine tracheal strips precontracted by carbachol 0.3 μM. **B**) Histogram of spermine- and putrescine (10 and 20 mM)-induced acute and long-term relaxation. **C**) Acute concentration-response relaxation by spermine and putrescine (1 to 10 mM) in bovine tracheal strips in carbachol (0.3 μM)-elicited raised tone. Each point represents the mean ± SEM; * $p < 0.05$ by comparing the effect of first (10 mM) and the second addition (20 mM), and ** $p < 0.01$ by comparing the effect of spermine and putrescine (10 mM) by means of Bonferroni's test for at least 7 different animals

1.07 min and 19.06 ± 1.29 min after the peak relaxation was reached.

The recovery was followed by a second component of a maintained long-lasting relaxation (Figs. 2A and 2B), which lasted throughout the 2 to 3 h of recording. When long-term relaxation was stable, further addition of a polyamine elicited relaxation of the preparations with the same characteristics, but both relaxation components had bigger effects (Figs. 2A and 2B).

The removal of the epithelium did not modify spermine or putrescine (10 mM)-induced relaxation in carbachol (0.3 μM)-raised tone.

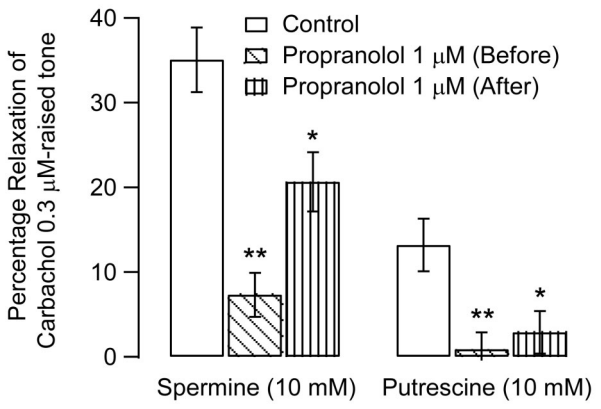

Fig. 3. Effects of propranolol (1 μM), added to the organ bath before the polyamines or after a stable long-lasting relaxation was reached (after), on spermine- and putrescine (10 mM)-induced relaxations in bovine tracheal strips precontracted by carbachol (0.3 μM). Values represent the mean ± SEM; * $p < 0.05$ and ** $p < 0.01$ by comparing the effects of the polyamines in the absence (control) and the presence of propranolol by Bonferroni's test for at least 7 different animals

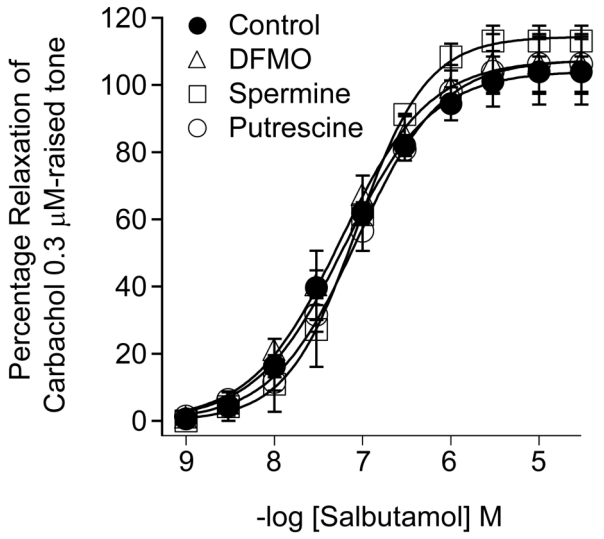

Fig. 4. Effects of -difluoromethylornithine (DFMO, 3 mM), spermine (10 mM) and putrescine (10 mM) on salbutamol (1 nM to 30 μM)-induced relaxation in bovine tracheal strips precontracted by carbachol (0.3 μM). Values represent the mean ± SEM for at least 6 different preparations, and the solid lines are the data fit to the Hill equation

Effects of propranolol (1 μM) on spermine- and putrescine (10 mM)-induced relaxation on bovine tracheal strips precontracted by carbachol (0.3 μM)

A 10-min incubation with propranolol (1 μM), a β-adrenoceptor antagonist, in tracheal bovine strips precontracted with carbachol (0.3 μM), antagonized the salbutamol (10 μM)-induced relaxation. However, neither propranolol significantly modified the percentage of the acute relaxation nor the time for spermine (10 mM) and putrescine (10 mM) to reach the maximum effects. The percentage of relaxation of the long-term component, however, was significantly decreased for both polyamines (Fig. 3).

When a stable relaxation caused by spermine (10 mM) or putrescine (10 mM) was elicited in carbachol (0.3 μM)-raised tone, the administration of propranolol (1 μM) significantly reversed their relaxation (Fig. 3), which was similar to its effect on salbutamol (10 μM)-induced relaxation.

Effect of tyramine (0.1 mM) on polyamine (10 mM)-induced relaxation of bovine tracheal strips precontracted with carbachol (0.3 μM)

The addition of tyramine (0.1 mM) to the organ bath elicited a transient relaxation (21.04 ± 5.52%) of bovine tracheal strips precontracted by carbachol (0.3 μM), but further addition of tyramine (0.1 mM) was ineffective. Tyramine (0.1 mM)-induced relaxation was reversed by propranolol (1 μM).

Compared with the control conditions in the absence of tyramine, a 45-min preincubation with tyramine (0.1 mM) after carbachol (0.3 μM)-raised tone did not alter the acute (57.81 ± 5.12% and 34.29 ± 6.32%) or the long-term relaxation (20.36 ± 2.57% and 7.86 ± 1.5%) of spermine (10 mM) or putrescine (10 mM), respectively.

Effects of α-difluoromethylornithine and polyamines on salbutamol-induced acute and long-term relaxation of bovine tracheal strips precontracted by carbachol (0.3 μM)

A 30-min incubation with α-difluoromethylornithine (3 mM) or the polyamines spermine or putrescine (10 mM) did not significantly modify the salbutamol (1 nM to 30 μM) relaxation concentration-response curve of carbachol (0.3 μM)-raised tone (Fig. 4).

Long-term incubation with salbutamol (30 μM) led to a spontaneous reversion of the relaxation (Fig. 5A). The tone was 51.87 ± 4.10% with respect to the contraction elicited by carbachol (0.3 μM). The reversion was produced with a latency of 1.05 ± 0.56 min after the peak relaxation was reached, with a τ of 25.97 ±

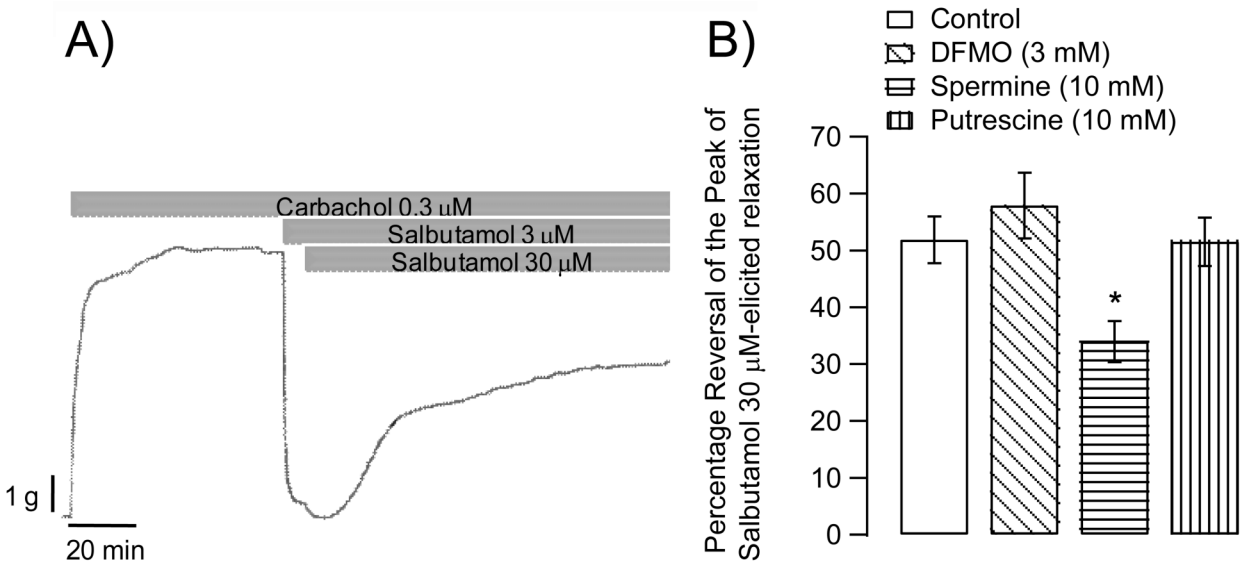

Fig. 5. A) Recording of the long-term effect of salbutamol (30 μM, control) in bovine tracheal strips precontracted by carbachol (0.3 μM), and **B**) the effects of previous incubation with -difluoromethylornithine (DFMO, 3 mM), spermine (10 mM) or putrescine (10 mM) on the percentage of reversion. Values represent the mean ± SEM; * $p < 0.05$ by comparing the effect of spermine with the control, by Bonferroni's test for at least 7 different animals

2.17 min, and was stable after 91.07 ± 5.21 min of the maximum relaxation to salbutamol (30 μM) (Fig. 5B).

Although the result was not significant, the 30-min incubation with α-difluoromethylornithine (3 mM) tended to slow the reversion of salbutamol (30 μM)-induced relaxation (τ = 31.99 ± 3.47 min and the time to the maximum reversal was 107 ± 8.60 min) and increase the percentage of reversion (Fig. 5B).

The incubation with spermine (10 mM) increased the latency of reversion (8.84 ± 2.33 min, $p < 0.01$), which was faster than the control (τ = 17.42 ± 2.30 min, $p < 0.01$), increased the time taken to reach the maximum reversal (57.34 ± 7.70 min, $p < 0.001$) and significantly diminished the percentage of reversion (Fig. 5B).

The incubation with putrescine (10 mM) significantly slowed the reversion of the peak relaxation (τ = 44.47 ± 6.71 min, $p < 0.01$) and the time taken to reach the maximum reversal (118.59 ± 9.36 min, $p < 0.01$) without changes in the percentage of reversion (Fig. 5B).

Ornithine decarboxylase activity in female and male bovine tracheal strips

The determination of basal ornithine decarboxylase activity in tracheal smooth muscle from 30- to 40-month-old female and male bovines showed that the enzymatic activity was not significantly different between gender (1.40 ± 0.38 and 2.01 ± 0.83 pmol/h/mg protein (n = 4) in females and males, respectively).

Effects of spermine and putrescine (10 mM) on bovine tracheal strips precontracted by KCl (80 mM)

In bovine tracheal strips whose tone was raised by KCl (80 mM), spermine and putrescine (10 mM) relaxed the preparations, but the time-course was slower than it was for carbachol (0.3 μM)-raised tone. In addition, the relaxation caused by putrescine was significantly smaller than the response to putrescine obtained after carbachol (0.3 μM) (Fig. 6A). The spasmolytic effect of spermine (10 mM) was reversed by the administration of propranolol (1 μM) in the organ bath.

Effects of spermine (3 and 10 mM) and putrescine (10 and 20 mM) on bovine tracheal strips contracted by electrical stimulation

Spermine (3 and 10 mM) inhibited (47.50 ± 9.61 and 91.85 ± 1.99%, respectively), the contractions of bovine tracheal strips induced by electrical stimulation in a concentration dependent manner. This effect was

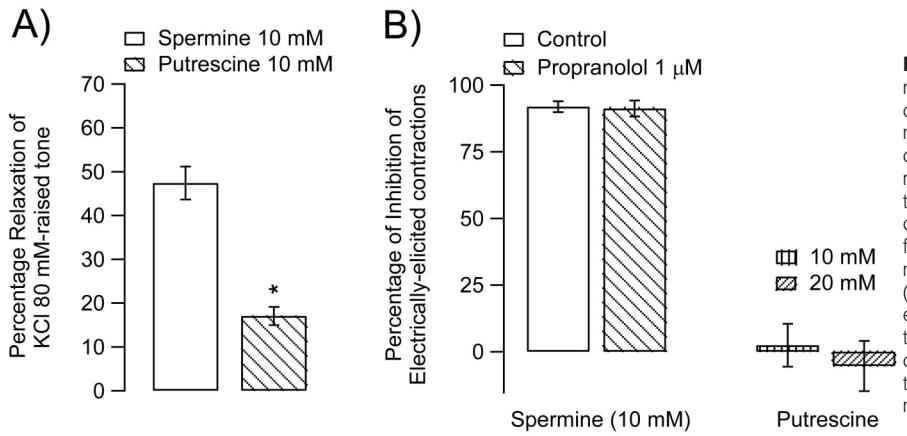

Fig. 6. A) Spermine and putrescine (10 mM)-induced relaxation in bovine tracheal strips precontracted by KCl (80 mM); * $p < 0.05$ by comparing the percentage of relaxation to putrescine (10 mM) in KCl-precontracted tissues with the percentage in carbachol-precontracted tissues by means of Bonferroni's test for at least 7 different animals. **B**) Inhibitory effect of spermine (10 μM), in the absence or the presence of propranolol (1 μM), and of putrescine (10 and 20 mM) on electrically-induced contractions in bovine tracheal strips. Values represent the mean ± SEM

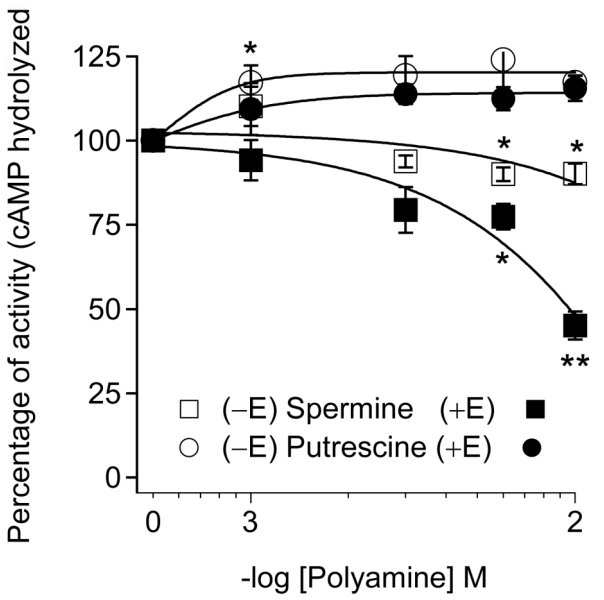

Fig. 7. Percentage of modifications of hydrolyzed cAMP (pmol/min/mg of protein) in extracts of bovine trachea with and without epithelium (in the absence or the presence of spermine and putrescine (1 to 10 mM)). Values represent the mean ± SEM for 4 different animals; * $p < 0.05$ and ** $p < 0.01$ by comparing the effect of spermine or putrescine to the control by Bonferroni's test

neither antagonized by previous incubation with propranolol (1 μM) nor reversed by the addition of propranolol in the organ bath when the inhibition of the contraction was produced. Putrescine (10 and 20 mM) did not modify electrically stimulated contractions (Fig. 6B).

Salbutamol (10 μM) or a calcium-free media suppressed 90% of the contractions. Salbutamol-induced effect was reversed by propranolol (1 μM) (data not shown).

Effects of spermine and putrescine (10 mM) on 0.3 μM carbachol-induced contractions in bovine tracheal strips

Preincubation with spermine or putrescine (10 mM) 30 min prior to the second cumulative contraction with carbachol did not significantly modify the contractile response (110.17 ± 7.34% and 108.61 ± 8.85%, respectively).

Effects of spermine and putrescine (1 to 10 mM) on cAMP phosphodiesterase activity in bovine tracheal extracts

The cAMP phosphodiesterase activity was 15.35 ± 0.51 pmol of hydrolyzed cAMP/min/mg protein when the epithelium was intact and 18.21 ± 0.377 pmol in its absence. Spermine (1 to 10 mM) decreased cAMP phosphodiesterase activity, and the highest levels of inhibition occurred in the extracts prepared with the epithelium (55% *vs.* 10% at 10 mM). Putrescine (1 to 10 mM) also increased the enzymatic activity of the phosphodiesterase (Fig. 7).

Discussion

The study showed that polyamines bound to β_2-adrenoceptors in bovine tracheal membranes, which suggested that the spasmolytic effects of polyamines might be due to a β-adrenoceptor-mediated response. However, the pharmacological characterization showed a more complex model than should be expected based

on the molecular interaction of polyamines with β_2-adrenoceptors.

The study of the modulation of airway smooth muscle tone was focused on the effect of the endogenous polyamine spermine because it was more potent than the other polyamines in the binding assay. We also studied putrescine because it has been reported to be a low affinity agonist of β-adrenoceptors in the rat heart [3].

Previous studies in guinea-pig trachea [10] reported that spermine was more effective than putrescine in eliciting relaxation by a direct effect on airway smooth muscle. There are two components in the relaxation response: an acute transient relaxation followed spontaneously by a partial recovery and a long-lasting relaxation. The functional characterization of the role of β-adrenoceptors in polyamine-induced relaxation showed that propranolol, a β-adrenoceptor antagonist, did not modify the percentage of the acute transient relaxation or the time required to reach the maximum effect. These results excluded the possibility of a functional interaction between polyamines and β-adrenoceptors in the transient component of the relaxation, in agreement with previous reports [10]. However, propranolol significantly antagonized polyamine-induced long-lasting relaxation. Moreover, propranolol reversed this component of the response to polyamines after the steady state was reached, which was similar to the results observed in the salbutamol-induced relaxation. These findings suggested that β-adrenoceptors were not involved in the induction of polyamine-induced relaxation but were important in the maintenance of the effect.

To study the possibility of an indirect sympathomimetic effect of polyamines on airway smooth muscle, we examined the influence of a tyramine-sensitive pool of intracellular catecholamines [14] on polyamine-induced relaxation. Tyramine produced a transient relaxation of bovine tracheal strips that was reversed by propranolol, which was compatible with the release of catecholamines from nerve endings. Similar to reports for other preparations [1], a rapid tachyphylaxis phenomenon was produced, which was shown by the absence of an effect of a subsequent dose of tyramine. Incubation with tyramine prior to polyamine exposure, either before the contraction with carbachol or after carbachol-raised tone, did not modify the acute or long-term effects of spermine or putrescine. This result excluded the existence of catecholamine release from a tyramine-sensitive pool as a mechanism involved in polyamine-induced relaxation. In addition, the binding data supported the possibility that polyamines may act as non-selective, low affinity agonists at β-adrenoceptors. However, putrescine has been shown to have a greater affinity at β_1-adrenoceptors [3], and, in the present study, spermine has been shown to have greater affinity at β_2-adrenoceptors. An interaction of polyamines with G-protein coupled receptors has also been reported in rat trachea where a release of mast cell mediators have been shown to increase smooth muscle tone [7].

Endogenous polyamines did not mediate acute β-adrenoceptor-mediated relaxation because incubation with α-difluoromethylornithine, an inhibitor of ornithine decarboxylase [22], did not modify salbutamol-induced relaxation in bovine tracheal strips. These results suggested that naturally occurring polyamines were not modulators of airway smooth muscle tone. In addition, the results of the present study suggested that there were no gender differences because ornithine decarboxylase activity was similar in adult female and male bovine trachea smooth muscle. However, a different situation could exist in respiratory diseases, such as asthma, where gender differences in prevalence have been shown [30]. Indeed, sex hormone regulation of ornithine decarboxylase has been reported in other tissues [6, 13].

Polyamines may interfere with the mechanisms related to β-adrenoceptor desensitization. This was shown as the loss of effect of salbutamol at high concentrations and long-term exposure, which was recorded as a partial reversal of the bovine trachea relaxation. α-Difluoromethylornithine decreased the time-constant in the loss of β-adrenoceptor-mediated relaxation. The increase of extracellular spermine delayed the onset and increased the time-constant of desensitization associated with a decrease in the magnitude of β-adrenoceptor desensitization. Polyamine-induced relaxation may be a pharmacological effect produced under pathological conditions when polyamine levels increase in tissues, such as in cystic fibrosis [12], respiratory infections [19], human asthmatics [18] and a mouse model of allergic asthma [39]. An increase in ornithine decarboxylase activity in the epithelium and smooth muscle of the airways has been reported in asthmatic patients who smoke [2].

Additional mechanisms independent of β-adrenoceptor activation exist in polyamine-induced relaxation. Indeed, modifications in Ca^{2+} and K^+ channel permeability may be relevant mechanisms in the spas-

molytic effects reported in different smooth muscles. These possibilities were also studied in bovine airway smooth muscle by contracting the preparations with KCl or electrical stimulation. The results suggested different mechanisms of action for spermine and putrescine. Spermine relaxed both types of contractions, which suggested the possibility of a Ca^{2+} entry blockade. This mechanism has been suggested to be responsible for the relaxation of most smooth muscles [17, 25, 26] and spermine-induced negative inotropism [37]. This mechanism is unlikely for putrescine, however, because putrescine lacked an effect in electrically stimulated preparations and was diminished when the tone was raised by KCl. However, putrescine relaxed carbachol-induced contractions, which suggested that the increase in K^+ permeability played a role in its mechanisms of relaxation. In addition to the mechanisms mentioned, β-adrenoceptor-dependent mechanisms may also be involved in the spasmolytic effect of polyamines in KCl-induced contractions. Indeed, propranolol partially reversed spermine- and putrescine-induced relaxation after KCl-induced contractions but not when the preparations were contracted by electrical stimulation.

We also explored the effect of polyamines on cAMP phosphodiesterase of bovine airway extracts because it has been reported that polyamines modulate the basal activity of this enzyme [11] as well as the activity once it is activated [15]. Tracheal epithelium and smooth muscle showed a spermine-sensitive cAMP phosphodiesterase activity that could modify intracellular cAMP. The inhibitory effect on smooth muscle might be important to spermine-induced relaxation and the synergism with the long-term spasmolytic effect of salbutamol. However, putrescine may activate the enzyme. These differences observed on cAMP phosphodiesterase activity might explain the functional differences observed between putrescine and spermine on airway smooth muscle. We concluded that the effect of polyamines on epithelial cAMP phosphodiesterase did not contribute to the modulation of smooth muscle tone because the removal of the epithelium did not modify the spasmolytic effect. However, the epithelium could be responsible for the effects of polyamines in the physiology and pathophysiology of epithelial ionic secretion *via* cAMP-dependent mechanisms [20, 33].

The spasmolytic effects of polyamines were not due to the modulation of carbachol-induced contractions because the contractions were not significantly modified. In addition, spermine produced spasmolytic effects even when the tone was raised by KCl or electrical stimulation. Other studies have reported that spermine and putrescine inhibited muscarinic receptor-operated cation current in guinea pig ileal smooth muscle myocytes [35], and spermine facilitated carbachol-induced airway smooth muscle contraction in type-2 cationic amino acid transporter (CAT-2)-deficient mice [8].

In summary, polyamines may modulate airway smooth muscle tone *via* several mechanisms that are partially related to β_2-adrenoceptor activation, modulation of functional responses caused by the activation of these receptors, an interaction with Ca^{2+} influx and cAMP phosphodiesterase activity. These mechanisms may be relevant in situations that result in an increase of endogenous polyamines, such as increased arginase and ornithine decarboxylase activity and/or expression (e.g., asthma [2, 23] and respiratory infections [19]). Further studies should be performed to establish the role of polyamines in respiratory physiology and diseases.

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