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# Role of $BK_{Ca}$ channels and cyclic nucleotides in synergistic relaxation of trachea

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**Abstract**

β-Adrenoceptor agonists, nitric oxide (NO), and NO donors have been shown to mediate their effects through large conductance $Ca^{2+}$-activated $K^+$ ($BK_{Ca}$) channels. The mechanism of the synergistic effect of the $\beta_2$-adrenoceptor agonist, salbutamol, and an NO donor, sodium nitroprusside, was studied in guinea pig tracheal preparations. Salbutamol (0.1 nM) and sodium nitroprusside (0.33 μM) alone relaxed the acetyl-β-methylcholine chloride (methacholine)-contracted preparations only by 0.5% and 28%, respectively, but their combination caused a maximum of 60% relaxation (at 3 min), which stabilized to 40% (at 10 min). Iberiotoxin, a selective inhibitor of the $BK_{Ca}$ channels, did not abolish the synergistic effect. 3-isobutyl-1-methylxanthine (IBMX) did not modify relaxation evoked by the drugs. Concentrations of cyclic nucleotides did not correlate with relaxations as a function of time. The mechanism of synergy remains to be clarified. The results show that NO is an important modulator in the relaxation of guinea pig trachea induced by $\beta_2$-adrenoceptor agonists in vitro. 

*Keywords:* Nitric oxide (NO); $\beta_2$-Adrenoceptor agonist; Smooth muscle; cGMP; cAMP; Iberiotoxin

## 1. Introduction

The β-adrenoceptor agonists are the most potent bronchodilators currently available. Binding of the β-adrenoceptor agonists to its receptor ($\beta_1$, $\beta_2$, or to both) results in activation of adenylate cyclase, which, in turn, increases intracellular cyclic AMP levels and induces relaxation. Nitric oxide (NO) is an inflammatory mediator that is increased in the exhaled air of asthmatics (Alving et al., 1993). NO does not act via receptors, instead it penetrates through the cells and activates intracellular soluble guanylate cyclase, which increases intracellular cyclic GMP. Increased production of cyclic AMP and cyclic GMP will ultimately lead to relaxation. Cyclic AMP and cyclic GMP are degraded by endogenous phosphodiesterases.

$\beta_1$- and $\beta_2$-adrenoceptor agonist, isoprenaline, (Kume et al., 1989); NO (Bolotina et al., 1994); and NO donors (Bialecki and Stinson-Fisher, 1995; Vaali et al., 1998a); have been shown to activate the $BK_{Ca}$ channels. These channels are abundant in smooth muscle, 10,000 channels having been estimated to exist on a single cell. Their role can be extensive: when a channel is opened, a large number of ions pass into the cell, thus, these channels are also called maxi-channels. Activation of the $BK_{Ca}$ channels has been suggested to be a very important relaxation factor for smooth muscle tone (Toro et al., 1998). These channels have been studied in smooth muscle preparations by inhibiting their opening with scorpion toxins, with iberiotoxin being the most selective of these toxins.

The combination of two bronchorelaxant drugs, a $\beta_2$-adrenoceptor agonist and an NO donor, produces a synergistic effect in the relaxation of guinea pig tracheal smooth muscle in vitro (Thierstrup et al., 1997; Vaali et al., 1998b). Inhibition of phosphodiesterases has been suggested to explain the synergistic relaxing effect in rat aortic smooth muscle (Maurice et al., 1990), and in guinea pig trachea (Thierstrup et al., 1997; Vaali et al., 1998b), but there is only indirect evidence for the phosphodiesterase inhibition. Synergistic relaxation studies have also been done by combination of β-adrenoceptor agonist or NO donor with a phosphodiesterase inhibitor in guinea pig (Turner et al., 1994) and in canine (Torphy et al., 1990) trachea, in rat aortic smooth muscle (Maurice et al., 1990), and in human conduit arteries (He et al., 1996).

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The aim of the present study was to investigate (a) the importance of the $BK_{Ca}$ channels in the relaxation evoked by the combination of the $\beta_2$-adrenoceptor agonist and an NO donor, (b) whether inhibition of phosphodiesterases could account for this synergistic effect, and (c) to identify any possible correlation with changes in cyclic AMP and cyclic GMP levels and tracheal smooth muscle relaxation induced by the combination of a $\beta_2$-adrenoceptor agonist and the NO donor.

## 2. Materials and methods

### 2.1. Preparation of guinea pig tracheal rings

Tracheal tissues from outbred Dunkin–Hartley male guinea pigs (280–400 g, Mol:DUHA, Møllegaard, Denmark) were kindly provided by Orion Pharma (Espoo, Finland). The guinea pigs were housed in a barrier animal room, $20 \pm 1$°C, in a light cycle of 14:10. They were given a blow to the head and then decapitated. During the 20–40 min transportation to our laboratory, the tracheas were kept in Krebs–Ringer solution that had been aerated with a mixture of 96% $O_2$ + 4% $CO_2$. The Krebs–Ringer solution was prepared in ultrapure water (MilliQ, Millipore, Bedford, MA, USA) of the following composition (mM): NaCl 119, $NaHCO_3$ 25, glucose 11.1, $CaCl_2 \times H_2O$ 1.6, KCl 4.7, $KH_2PO_4$ 1.2, and $MgSO_4 \times 7H_2O$ 1.2, at pH 7.4. Tracheal pieces (2–3 mm) were cut and mounted in an 8-ml organ chamber containing the Krebs–Ringer solution and aerated with a mixture of 96% $O_2$ + 4% $CO_2$ during the experiments; the initial load (including the spontaneous tension) was set at 2.5 g. The preparations were allowed to stabilize for at least 60 min to reach their spontaneous tone before any contracting agent was added. The tension changes were recorded with Grass force displacement transducers and amplifiers (FT03, Grass Medical Instruments, Quincy, MA, USA).

The procedure of the study was approved by the Animal Experimentation Committee of Orion Pharma, Espoo, Finland.

### 2.2. Determination of synergistic effect and calculation of theoretical effect by combination of drugs

Synergistic effects for drug A and drug B can be defined according to Berenbaum (1989), by the following formula:

$d_a/D_a + d_b/D_b < 1$ synergy effect

$d_a/D_a + d_b/D_b = 1$ zero interaction

where $d_a$ and $d_b$ are the concentrations of drugs A and B used in combination, and $D_a$ and $D_b$ are their single concentrations, which were isoeffective with the combination ($d_a + d_b$) at any specified level of effect.

When drug A is added in cumulative concentrations and the interacting drug B is given in a single concentration, then the theoretical effect can be calculated (Ariëns et al., 1956; Pöch and Holzmann, 1980) for each concentration of A in the presence of B from the respective effects of A ($E_A$) and B ($E_B$) minus the combined effect of A and B ($E_A E_B$):

$$E_{A+B} = E_A + E_B - (E_A E_B)$$

where $E$ can be expressed as a fraction of the maximum effect (1.0). Using this equation and the experimental relaxation results of drugs A and B, the theoretical curves of relaxation were calculated with a spreadsheet. When the experimental curve of drugs A and B is more potent than the theoretical effect of interaction, the combination of drugs has a synergistic effect.

### 2.3. Experimental procedure for the relaxation responses

A submaximal concentration of the contracting agent, acetyl-β-methylcholine chloride (methacholine) at a concentration of 1 μM, was chosen on the basis of our previous studies. Before contractions, the preparations were washed every 15 min with Krebs–Ringer buffer.

The non-selective phosphodiesterase inhibitor, 3-isobutyl-1-methylxanthine (IBMX), was used to inhibit the degradation of cyclic nucleotides. IBMX, ethanol, or iberiotoxin, was added 10 min after methacholine administration. Both IBMX and ethanol were added in a volume of 8 μl (1/1000 volume in organ bath). After methacholine contraction had reached a plateau (35 min incubation time), the cumulative concentration response curve for the relaxing drug was administered: salbutamol (0.1 nM–1 μM), sodium nitroprusside (10 nM–10 μM), all concentrations representing the final concentrations.

The first concentration of the cumulative drug (drug A, salbutamol) was added, after which, the modifying drug (drug B, sodium nitroprusside) or its vehicle was added; then drug A was added cumulatively (see Fig. 1).

Each drug-concentration was allowed to act for 10 min before the next concentration was administered. The relaxation results were always measured 10 min after the added drugs, except when the relaxations were studied in function of time. In the latter case, relaxation was measured at 0, 0.5, 1, 3, 6 and 10 min time points. Only one contraction curve was done for each trachea piece, and time-matched controls were used.

### 2.4. Cyclic GMP and cyclic AMP measurements

Methacholine-induced contraction lasted for 40 min, and IBMX or its vehicle were added 10 min after methacholine, and incubated for 20 min, before salbutamol, sodium nitroprusside, or both were added. In order to be able to release the samples immediately into liquid nitrogen, tension was not applied to these preparations. Only

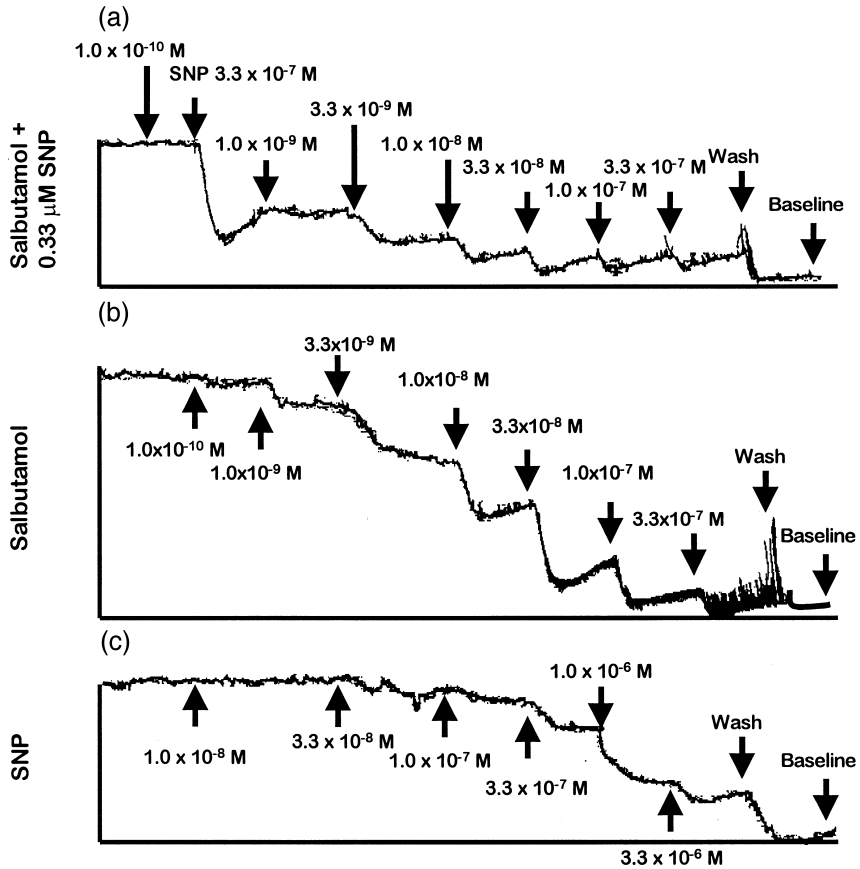


Fig. 1. Typical original recording of a relaxation response of cumulatively added drugs in 1 μM methacholine-induced contraction in the absence of IBMX. SNP: sodium nitroprusside. (a) Combination of the $\beta_2$-adrenoceptor agonist, salbutamol, and the NO donor, sodium nitroprusside (the synergistic curve); (b) salbutamol alone; (c) sodium nitroprusside alone. Arrows indicate time-points of added drug. A single concentration of 0.1 nM salbutamol caused relaxation of only $0.5 \pm 2.3\%$, and 0.33 μM sodium nitroprusside caused only $27.8 \pm 2.3\%$, but in the absence of IBMX, the relaxation by the combination of these compounds at the same concentrations reached $61.9 \pm 11.4\%$ in 3 min, and stabilized at $43.1 \pm 5.4\%$ in 10 min.

one concentration of any of the relaxant drug was added to the organ baths before each sample was taken. When low concentrations of drugs were studied, 0.1 nM salbutamol, 0.33 μM sodium nitroprusside, or the combination of these were used; and when higher concentrations were studied, 1 μM salbutamol, 10 μM sodium nitroprusside were used. Samples at 0.5, 1, 3, 6 and 10 min were taken so that the correlation to the relaxation response (from separate experiments) could be evaluated. Cyclic nucleotide concentration and the relaxation results were compared to the results at 0 min.

The samples were stored in −20°C until the cyclic nucleotides were extracted and measured. During the homogenizing procedure, all the solutions and equipment were cooled to and kept at +4°C to prevent degradation by phosphodiesterases. The tracheal rings were homogenized with an ultraturrax (Ultra-Turrax T8, Ika Labortechnik, Janke and Kunkel, Staufen, Germany), in 6% trichloroacetic acid. The homogenates were centrifuged at $10{,}000 \times g$, and the precipitated proteins were dissolved in 1 ml of 1 M NaOH +37°C overnight and assayed (Lowry et al., 1951).

The supernatants were acidified with HCl (25 mM final concentration) and extracted at room temperature five times with two volumes of diethylether (water saturated) and lyophilized. Acetylated cyclic GMP was determined by radioimmunoassay (Axelsson et al., 1988). Acetylated cyclic AMP was assayed with a commercial [$^{125}$I] radioimmunoassay kit (Amersham).

Duplicate and single determinations were performed for cyclic GMP and cyclic AMP, respectively; the number of tissues used differed among experiments and is indicated in each figure.

### 2.5. Drugs and chemicals

Drugs from the following sources were used: salbutamol came from Leiras LTD (Turku, Finland); IBMX was provided by Dr. Eeva Moilanen of the University of Tampere, Medical School, Tampere, Finland. Sodium nitroprusside was from Hoffmann-La Roche (Basel, Switzerland). Methacholine, iberiotoxin, bovine gamma globulins Cohn fraction II and III, bovine serum albumin, and unlabelled cyclic GMP came from Sigma (St. Louis, MO,

USA). Reagents for the Krebs–Ringer and trichloroacetic acid were from Riedel-de Haën (Seelze, Germany); diethylether is from Lab-Scan (Dublin, Ireland); sodium acetate and Folin–Ciocalteau's phenol reagent are from Merck (Darmstadt, Germany). The cyclic GMP measurements were performed with [$^{125}$I] cyclic GMP and in-house prepared polyclonal rabbit antibody that cross-reacted less than 0.001% with related compounds (Lähteenmäki et al., 1998) and 0.03% with cyclic AMP. Cyclic AMP was determined with [$^{125}$I] cyclic AMP radioimmunoassay kit Amersham International (Little Chalfont, Buckinghamshire, England).

Of the drugs used, only IBMX was dissolved in 96% ethanol and, therefore, vehicle used for IBMX was ethanol.

### 2.6. Statistical analysis

The data are presented as mean ± S.E.M. of the indicated number of experiments. Analysis of variance (ANOVA/MANOVA) was studied with the program Statistica, release-date 4 May, 1993 (Statsoft, Tulsa OK, USA), followed by the Newman–Keuls test for multiple comparisons.

## 3. Results

### 3.1. Relaxation induced by salbutamol or sodium nitroprusside and inhibiting effect of iberiotoxin

In methacholine-contracted trachea, salbutamol (0.1 nM–1 μM) caused a concentration-dependent relaxation, the maximum being 81.4 ± 2.5%. Sodium nitroprusside (10 nM–10 μM) also evoked a concentration-dependent relaxation; its maximum was 69.8 ± 5.9% (Fig. 2). The highest concentration of iberiotoxin (100 nM) that selectively inhibits the $BK_{Ca}$ channels was used and this reduced the salbutamol- and sodium nitroprusside-induced relaxations to 25.0 ± 3.8% and to 23.3 ± 8.5%, respectively, (Fig. 2).

### 3.2. Synergistic relaxation by salbutamol in the presence of sodium nitroprusside and inhibiting effect of iberiotoxin

The cumulative relaxation by salbutamol was significantly ($P < 0.001$) increased in the presence of a small concentration of sodium nitroprusside (0.33 μM), when the concentration of salbutamol ranged from 0.1 nM to 10 nM (Fig. 3a). The experimental curve of combination of drugs was more potent than the theoretical, calculated effect of the combination of drugs and, therefore, the combination of drugs produced a synergistic effect (Fig. 3a). The maximum cumulative relaxation of the combination was 95.1 ± 2.3%, which is significantly more than the maximum relaxation by salbutamol (Fig. 2a) or sodium nitroprusside (Fig. 2b). The selective $BK_{Ca}$ channel inhibitor, iberiotoxin (33 nM and 100 nM), concentration-dependently inhibited the relaxation induced by the combination, for which the maximum cumulative relaxations were 62.8 ± 7.9% and 46.4 ± 3.7%, respectively (Fig. 3b). There are no statistical differences in the increase of 100 nM iberiotoxin-induced contractions of salbutamol (33.0 ± 4.2%), sodium nitroprusside (32.4 ± 2.7%), or the combination (29.2 ± 2.2%) in the presence of iberiotoxin. There-

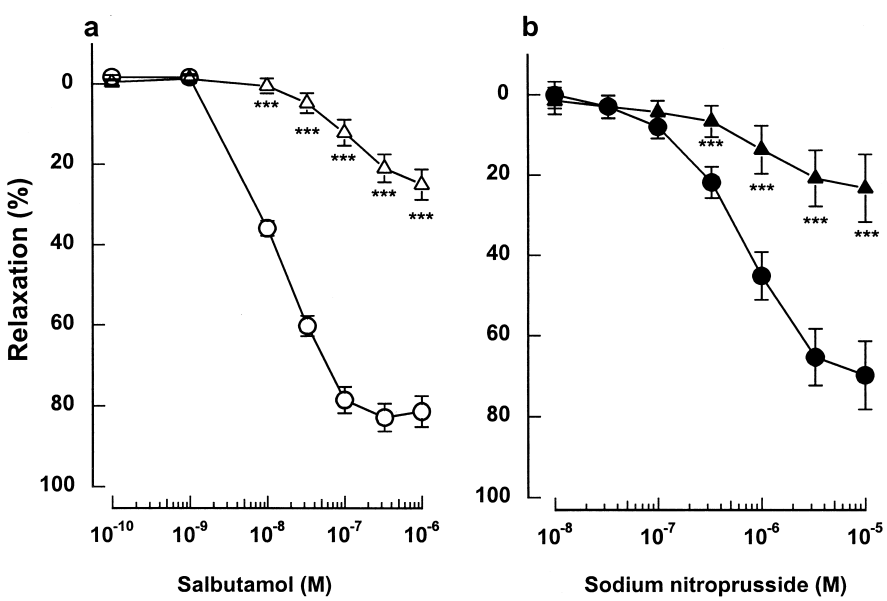


Fig. 2. Effect of the selective $BK_{Ca}$ channel-inhibitor, iberiotoxin, on relaxants in 1 μM methacholine-induced contraction. (a) Control relaxation curve for salbutamol in the absence (○, $N = 10$), and in the presence (△, $N = 14$) of 100 nM iberiotoxin. (b) Control relaxation curve for NO donor sodium nitroprusside in the absence (●, $N = 5$) and presence (▲, $N = 4$) of 100 nM iberiotoxin.

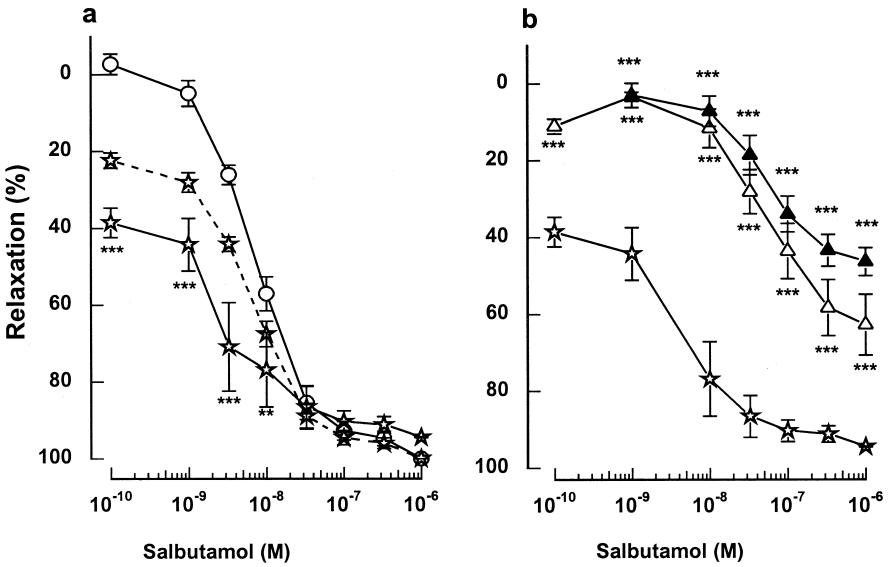


Fig. 3. Experimental synergistic relaxation curve of cumulative concentration of salbutamol in the presence of 0.33 μM sodium nitroprusside (☆, solid line, $N = 15$) in 1 μM methacholine-induced contraction. (a) The calculated theoretical synergistic effect for the cumulative concentration of salbutamol in the presence of sodium nitroprusside (0.33 μM), (☆, broken line, $N = 5$). Cumulative concentration–response curve of salbutamol alone (○, $N = 5$) presented as a reference. (b) The same experimental synergistic relaxation curve (☆) in the presence of 33 nM iberiotoxin (△, $N = 9$) or 100 nM iberiotoxin (▲, $N = 5$). The difference between the experimental synergistic relaxation curve (☆, solid line) and the relaxation curve of salbutamol (○) is compared, and at each concentration point, significance is marked. * Indicates statistically significant difference when compared to the combination curve (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$).

fore, the surplus of contraction cannot explain differences among the three drug groups studied.

The maximum cumulative relaxation with the combination, in the presence of 100 nM iberiotoxin (Fig. 3b), was clearly greater than the relaxation achieved in the presence of 100 nM iberiotoxin, and in only one relaxant, either salbutamol or sodium nitroprusside (Fig. 2a and b, respectively). However, the quantity of inhibition was the same: 100 nM iberiotoxin inhibited the maximum relaxing effect of the combination of drugs only by 48% (Fig. 3b), whereas it inhibited single drugs, i.e. salbutamol- or sodium nitroprusside-induced maximum relaxation response, by

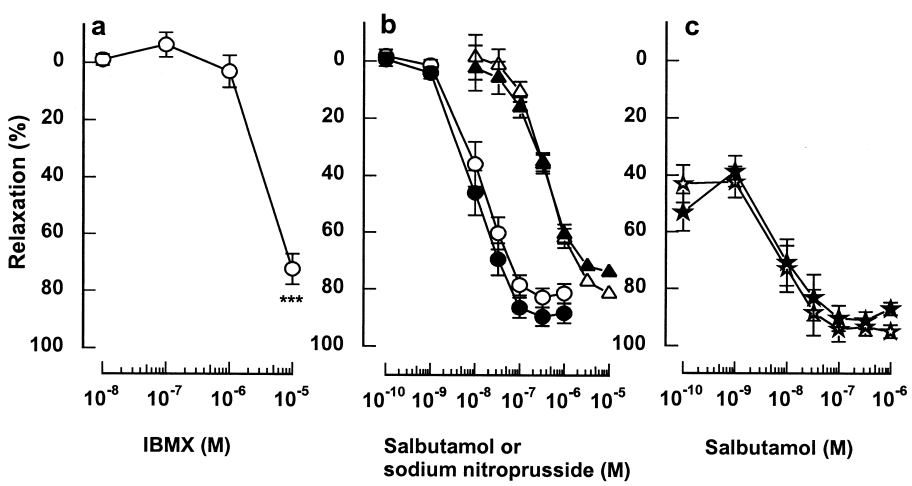


Fig. 4. Pretreatment effect of the non-selective phosphodiesterase inhibition in cumulative relaxation of salbutamol or sodium nitroprusside or their combination on 1 μM methacholine-induced contraction. (a) Concentration-dependent effect of IBMX on methacholine-contracted guinea pig trachea (○, $N = 10$). (b) Pretreatment-effect of IBMX on the cumulative relaxation by the drugs alone. Control relaxation curve of salbutamol (○, $N = 10$) and sodium nitroprusside (△, $N = 8$), and the same in the presence of 1 μM IBMX (●, $N = 4$ and ▲, $N = 7$, respectively). (c) Cumulative synergistic relaxation curve of the combination of salbutamol and 0.33 μM sodium nitroprusside in the presence (☆, $N = 9$) and in the absence (★, $N = 6$) of IBMX. Footnote to the figure: *** indicates significance of $P < 0.001$ when compared to methacholine contraction in the absence of IBMX.

56% or 47% (Fig. 2). Therefore, it is proposed that the interactive effect was not abolished by blockade of the $BK_{Ca}$ channels.

### 3.3. Concentration-dependent effect of IBMX alone on methacholine contraction

The non-selective phosphodiesterase inhibitor, IBMX, did not induce relaxation of the methacholine-induced contraction at concentrations of 0.01 μM–1 μM. However, a 10 μM concentration of IBMX induced relaxation up to 72.5 ± 5.1%, (Fig. 4a). Relaxation had to be studied at a submaximal contraction level, since IBMX at 1 μM relaxed only at 3.7 ± 5.0%, 1 μM was chosen as the pretreatment concentration to inhibit the degradation of cyclic nucleotides in the following experiments.

### 3.4. Pretreatment-effect of IBMX on cumulative relaxation

IBMX (1 μM) induced no significant effect on the potency or efficacy of concentration-dependent relaxation by salbutamol, sodium nitroprusside, or their combination (Fig. 4b,c).

### 3.5. Relaxation as a function of time

When the effect of the combination of salbutamol (0.1 nM) and sodium nitroprusside (0.33 μM) was studied in cumulative relaxation response, the relaxation effect reached the plateau by 10 min, but the most potent relax-

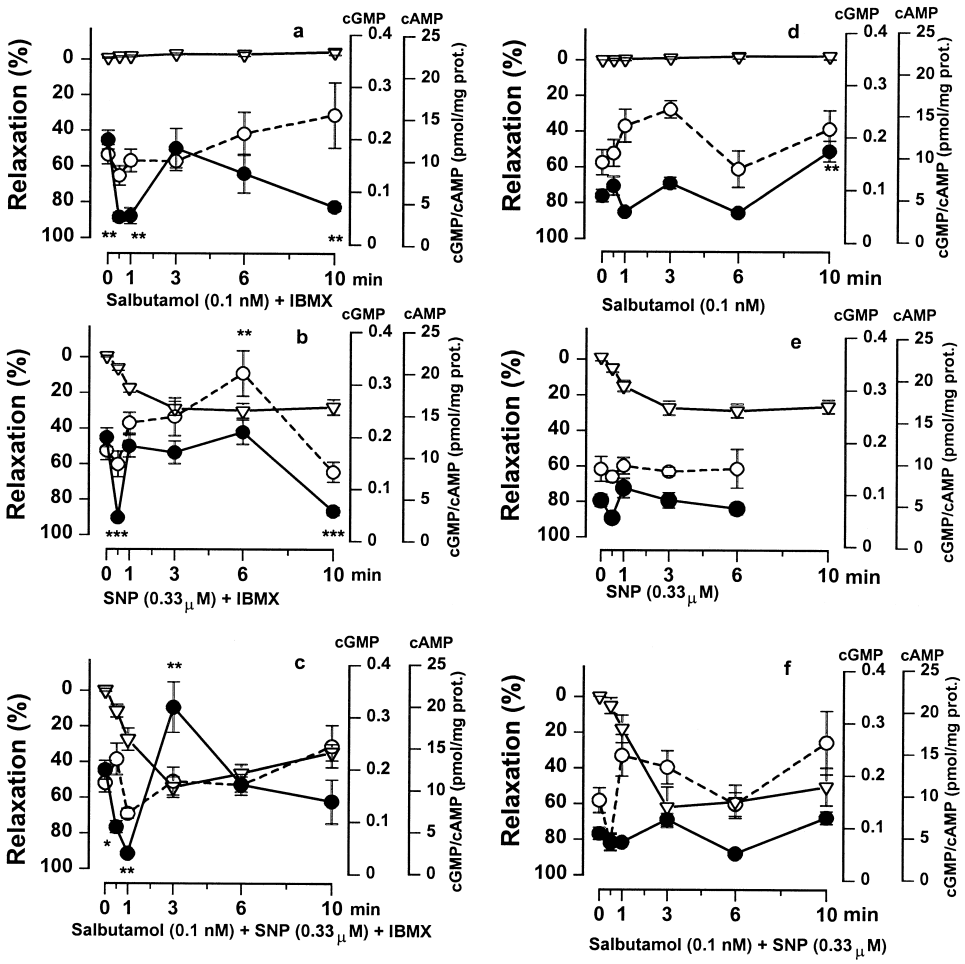


Fig. 5. Relaxation curves of cyclic nucleotides and drugs in function of time. Concentrations of cyclic GMP (●, solid lines) and cyclic AMP (○, broken line), relaxation curves (▽, solid lines). Samples are presented in the presence of 1 μM IBMX (right, a, b, c), or in the presence of its vehicle (left, c, d, f). The *X*-axis indicates time in minutes. The preparations were contracted with methacholine (1 μM) for 10 min, and IBMX or ethanol was added and, after 20 min pretreatment, single concentrations of relaxant drugs (salbutamol, sodium nitroprusside or their combination) were added and after the time point indicated in *X*-axis, the preparations were immediately frozen in liquid nitrogen. SNP: sodium nitroprusside. (a and d) Salbutamol (0.1 nM); (b and e) sodium nitroprusside (0.33 μM); and (c and f) the combination of salbutamol (0.1 nM) and sodium nitroprusside (0.33 μM). Footnote to the figure: salbutamol ($N = 5–6$) in the relaxation response, cyclic AMP and cyclic GMP ($N = 5–6$). Sodium nitroprusside ($N = 7–14$) in the relaxation response, cyclic AMP and cyclic GMP ($N = 5–8$). With the combination of drugs, relaxation response ($N = 5–6$), and cyclic AMP and cyclic GMP ($N = 5–16$). * Indicates statistically significant difference in cyclic nucleotide concentrations, when compared to the control value of time point 0 min (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$).

Table 1
Cyclic GMP and cyclic AMP concentrations in the absence (ethanol) and presence of 1 μM IBMX in guinea pig trachea in vitro with high concentrations of salbutamol (1 μM) and sodium nitroprusside (10 μM). The preparations were contracted with methacholine (1 μM) for 10 min, and IBMX or its vehicle ethanol was added, and after 20 min incubation, single concentrations of relaxant drugs (salbutamol or sodium nitroprusside) were added, and after the time point indicated, the preparations were immediately frozen in liquid nitrogen

| Pretreatment | Min | Ethanol | | | | IBMX (1 μM) | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | cyclic GMP | | cyclic AMP | | cyclic GMP | | cyclic AMP | |
| | | *N* | Mean ± S.E.M. | *N* | Mean ± S.E.M. | *N* | Mean ± S.E.M. | *N* | Mean ± S.E.M. |
| Control | 0 | 13 | 0.089 ± 0.012 | 13 | 9.5 ± 1.5 | 9 | 0.198 ± 0.018 | 10 | d10.8 ± 1.1 |
| Salbutamol | 0.5 | – | N.D. | – | N.D. | 5 | 0.123 ± 0.032 * | 6 | 17.5 ± 2.2 * * |
| | 1 | – | N.D. | – | N.D. | 6 | 0.140 ± 0.031 | 5 | 19.7 ± 2.4 * * |
| | 3 | 3 | 0.101 ± 0.018 | 3 | 9.9 ± 1.9 | 6 | 0.285 ± 0.033 * | 6 | 24.0 ± 5.6 * * |
| | 6 | 5 | 0.060 ± 0.018 | 5 | 8.9 ± 0.8 | 4 | 0.238 ± 0.025 | 4 | 23.8 ± 9.4 * |
| | 10 | 8 | 0.061 ± 0.008 | 7 | 8.2 ± 0.9 | 5 | 0.250 ± 0.039 | 5 | 15.5 ± 4.9 |
| | 60 | 6 | 0.145 ± 0.018 * * | 7 | 20.5 ± 3.0 * * | – | N.D. | – | N.D. |
| Sodium nitroprusside | 1 | 3 | 0.117 ± 0.039 | 3 | 10.0 ± 1.4 | 6 | 0.111 ± 0.025 * | 7 | 12.9 ± 1.9 |
| | 3 | 4 | 0.154 ± 0.013 * | 3 | 9.5 ± 4.0 | 10 | 0.408 ± 0.066 * * | 10 | 17.6 ± 3.0 * |
| | 6 | 4 | 0.128 ± 0.006 | 4 | 8.6 ± 1.8 | 6 | 0.315 ± 0.024 * | 6 | 23.3 ± 4.4 * * |
| | 10 | – | N.D. | – | N.D. | 7 | 0.442 ± 0.089 * * | 7 | 17.6 ± 4.8 * |

N.D., not determined.
* Indicates statistically significant difference when compared to the control value of time point 0 min ($P < 0.05$).
* * Indicates statistically significant difference when compared to the control value of time point 0 min ($P < 0.01$).

ation could be seen at 3 min (Fig. 1). With the 0.33 μM sodium nitroprusside-induced relaxation, there was plateau at 3 min, but salbutamol (0.1 nM) did not induce relaxation. In the presence and absence of IBMX at the 3-min time-point, when salbutamol (0.1 nM) and sodium nitroprusside (0.33 μM) in combination were evaluated, the achieved relaxations were 54.2 ± 5.9% and 61.9 ± 11.4%, respectively. A concentration of 0.1 nM salbutamol alone caused only 0.5 ± 2.3% relaxation, and 0.33 μM sodium nitroprusside relaxed tissue by 27.8 ± 2.3% on its own (Fig. 5).

### *3.6. Cyclic GMP and cyclic AMP concentrations in the presence and absence of IBMX*

In the presence of 1 μM IBMX, the control level of cyclic GMP was higher than in the presence of vehicle (Table 1), but IBMX also induced clearer decreases in cyclic GMP levels at 0.5 and 1 min than the vehicle (Fig. 5, Table 1). These decreases in cyclic GMP at 0.5 and 1 min were significant ($P < 0.01$, $P < 0.001$ and $P < 0.01$) for all three cases (0.1 nM salbutamol, 0.33 μM sodium nitroprusside, and their combination), respectively, (Fig. 5a–c). During the studied time, only the combination of these two drugs produced a significant increase ($P < 0.01$) in cyclic GMP at the 3-min time-point (Fig. 5c).

IBMX did not affect the control level of cyclic AMP (Table 1). No significant changes in cyclic AMP, in the presence of IBMX, were induced by salbutamol (0.1 nM) or by the combination of salbutamol (0.1 nM) and sodium nitroprusside (0.33 μM; Fig. 5a,c). Unexpectedly, the only significant change from the baseline in cyclic AMP was seen with sodium nitroprusside (0.33 μM) at 6 min ($P < 0.01$; Fig. 5b).

Between the time-points 1–3 min, the combination of drugs (salbutamol 0.1 nM and sodium nitroprusside 0.33 μM) or salbutamol (0.1 nM) alone, tended to produce more cyclic AMP when IBMX was absent (Fig. 5f or d) compared to its presence (Fig. 5c or a). Although the increase in production was not significant, it appeared in both cases at the same time-points. In the absence of IBMX, the only significant ($P < 0.01$) increase in cyclic AMP was at the 10-min time-point with salbutamol (0.1 nM) on its own.

When higher concentrations of drugs were studied, salbutamol (1 μM) or sodium nitroprusside (10 μM), the fluctuation in cyclic nucleotides concentrations as a function of time could be observed again (Table 1). In the presence of IBMX, there was a significant decrease in the cyclic GMP concentration at 0.5-min time-point with salbutamol (1 μM), but cyclic AMP concentrations increased up to 3 min. Also, when IBMX was present, there was a decrease in cyclic GMP levels after sodium nitroprusside (10 μM) at 1 min. One cannot state, whether a higher concentration of sodium nitroprusside would have produced a steady increase in cyclic GMP in the presence of IBMX.

## 4. Discussion

The mechanisms responsible for the synergism between a $\beta_2$-adrenoceptor agonist, salbutamol, and an NO donor, sodium nitroprusside, to cause relaxation of guinea pig trachea in vitro were studied. The role of the $BK_{Ca}$ channels could be shown to affect the relaxation partially, but inhibition of cyclic nucleotide degradation by IBMX did not modify the relaxation responses, although phosphodi-

esterase inhibition for more than 3 min in the presence of the relaxing drugs did increase the concentrations of cyclic nucleotides. Furthermore, the cyclic nucleotide levels did not correlate with the relaxation response as a function of time.

### *4.1. Role of the $BK_{Ca}$ channels in relaxation induced by a single drug or drug combination*

Iberiotoxin, the selective $BK_{Ca}$ channel inhibitor, reduced approximately to half the maximum relaxation effect of the single drugs or their combination, indicating that in all the three cases, $BK_{Ca}$ channel inhibition plays an equal part. The inhibition of the guinea pig tracheal relaxations by salbutamol or sodium nitroprusside in the presence of iberiotoxin (100 nM) was also in accordance with the findings of Corompt et al. (1998). However, in the presence of iberiotoxin, the relaxation effect of the combination of salbutamol and sodium nitroprusside, in comparison to that of a single drug, was more marked, suggesting that some other relaxing mechanism(s) is(are) involved.

### *4.2. Phosphodiesterase inhibition*

We have suggested earlier that the combined effect of $\beta_2$-adrenoceptor agonist and NO donor, through increased production of cyclic AMP and cyclic GMP, respectively, mimics the effects of phosphodiesterase type III inhibition (Vaali et al., 1998b). The type III enzyme is inhibited by cyclic GMP, but it degrades cyclic AMP. Therefore, one could anticipate there an increase in cyclic AMP levels and in the extent of relaxation. IBMX, at a concentration of 1 μM in our experiments, increased the tissue cyclic AMP and cyclic GMP levels. The concentration of IBMX was 1/1000 — 1/100 of that used in cell culture conditions, in which the cyclic nucleotides have been measured (Billington et al., 1999; Hamad et al., 1997), but in cell culture, the relaxation response cannot be studied, thus, there are no practical limits to the use of high concentrations. It was not possible to use higher concentrations of IBMX for pretreatment, because at a concentration of 10 μM, IBMX markedly relaxed the methacholine-contracted preparations. Fredholm et al. (1979) have shown in guinea pig tracheal preparation that IBMX is equipotent for inhibiting cyclic AMP and cyclic GMP hydrolysis and that the $IC_{50}$ values for cyclic nucleotides hydrolysis are in the micromolar range. Lavan et al. (1989) reported that IBMX inhibits all phosphodiesterases, except type VII. Also, when higher concentrations of salbutamol or sodium nitroprusside were used, the 1 μM concentration of IBMX increased the cyclic nucleotide levels significantly over the studied 10-min time. The phosphodiesterase inhibition of IBMX, in combination with salbutamol, would have been anticipated to potentiate the relaxant effect. However, this was not the case; IBMX did not affect either the cumulative relaxation curve of sodium nitroprusside on its own or when it was used in the combination of salbutamol (0.1 nM) and sodium nitroprusside (0.33 μM). Therefore, the observed synergistic effect cannot be explained by decreased cyclic nucleotide degradation; some other reason for the potentiating effect of the relaxation must exist.

### *4.3. Changes in the concentrations of cyclic GMP and cyclic AMP in function of time*

In bovine coronary arteries, Kukovetz et al. (1979) used four different nitro compounds and demonstrated that there was a very good correlation between the increase in cyclic GMP and in the maximum relaxation at 20 min, but the increase in cyclic GMP exceeded the relaxation response between 1 and 5 min, with all the studied compounds. Again, no decrease in cyclic GMP was reported, which may be due to the different tissues and nitrovasodilators potency in vascular smooth muscle preparations. It is possible that vascular smooth muscle should be studied with even lower concentrations of NO donors to be comparable to tracheal smooth muscle.

In the absence of IBMX, it is not surprising that the low concentration of the $\beta_2$-adrenoceptor agonist salbutamol did not increase cyclic AMP concentrations. Isoprenaline, a $\beta_1$- and $\beta_2$-adrenoceptor agonist, did not increase cyclic AMP between time points of 2 and 6 min in bovine retractor penis, in concentrations that induced complete relaxation (Gillespie and Sheng, 1988). Also, Katsuki and Murad (1977) showed in bovine trachea that increases in cyclic AMP do not necessarily correlate with relaxation, and vice versa.

In the presence of IBMX, fluctuations in the cyclic GMP concentration were most evident and occurred with low and high concentrations of salbutamol or sodium nitroprusside. Salbutamol (0.1 nM) alone, and in combination with sodium nitroprusside (0.33 μM) at 1 and 3 min, increased cyclic AMP, more in the absence of IBMX than in its presence. A possible explanation for this has been presented recently by Pyne et al. (1996): after the initial increase in cyclic GMP, there is an inhibition of phosphodiesterase type III, which reduces cyclic AMP degradation. This increase in cyclic AMP would activate protein kinase A. Since protein kinase A activates phosphodiesterase type V (Burns et al., 1992), which is most selective for cyclic GMP degradation, some feedback mechanism may also be involved downstream to the cyclase.

We could not apply tension to the preparations that were used for the studies of cyclic nucleotide concentrations. This is because it is very difficult to take a preparation out of a hook for cyclic nucleotide measurements as quickly as needed without producing harm to the transducers recording the preparations tone. Of course, when longer incubation time is used, this is possible and the time needed for detaching the sample will not produce too big variation to the results. However, it must be reminded that most of the previous studies did not use such short incuba-

tion times, very often, 10, 20 or 30 min were used in order to get the cyclic nucleotides to accumulate to the samples. Even though the different procedures of the smooth muscle relaxation when compared to the cyclic nucleotide samples were used, the relative changes can be compared within each procedure. Therefore, in the presence of IBMX, the unexpected drop of cyclic GMP concentrations at 0.5–1 min by all the studied groups, which could not be found in the absence of IBMX, cannot be explained by the procedural difference. This drop can be repeatedly and significantly found only during the very first minute of relaxation when high or low concentrations of drugs are used.

### 4.4. Cyclic nucleotide-independent relaxation

In most of the studies, increases in cyclic GMP or cyclic AMP have been assessed with the maximum increases being regarded as important. If activation of the $BK_{Ca}$ is one of the most important determinants of relaxation of airway smooth muscle, then its phosphorylation would be critical for channels activation. Our results show that there was a significant decrease at 0.5–1 min in the amount of cyclic GMP produced with either salbutamol (0.1 nM) or sodium nitroprusside (0.33 μM), as well as their combination. We suggest that this lowering of cyclic GMP level could reflect a cyclic GMP signal that is transduced further into phosphorylations of different proteins, leading to consumption of the intracellular cyclic GMP pool.

In the presence of IBMX, salbutamol (0.1 nM), at a concentration which did not affect the methacholine-induced contraction, could still induce a decrease in cyclic GMP level at 0.5–1 min that was similar in magnitude to that of the combination of drugs. In the presence and absence of IBMX, salbutamol alone could not evoke relaxation, but the combination of drugs caused up to 55% and 60% relaxation at 3 min, respectively, that stabilized to a somewhat lower level (40% and 50%, respectively) several minutes later. These results imply that there is a threshold level that is not reached with 0.1 nM salbutamol or 0.33 μM sodium nitroprusside alone, but their combination produces the surplus force needed to achieve a strong relaxation signal.

Other investigators have shown that isoprenaline, $\beta_1$- and $\beta_2$-adrenoceptor agonist, (Kume et al., 1994) and NO (Bolotina et al., 1994) can activate the $BK_{Ca}$ channels without production of the cyclic nucleotides. Also, in the perfusion of guinea pig trachea, Sadeghi-Hashjin et al. (1996) have shown that sodium nitroprusside-induced relaxation is not mediated by cyclic GMP. These and several other results, together with our results, suggest the correlation of cyclic nucleotide concentrations, probably to be overestimated with smooth muscle relaxation in the literature.

### 4.5. Conclusion

We have shown that the positive synergistic interaction of a nanomolar concentration of a $\beta_2$-adrenoceptor agonist, salbutamol, and a micromolar concentration of the NO donor, sodium nitroprusside, in the relaxation of guinea pig trachea is not due exclusively to activation of the $BK_{Ca}$ channels. Furthermore, the synergistic effect is not attributable to the inhibition of phosphodiesterases, but must be mediated through some other mechanism(s). The synergistic relaxation, produced by combined salbutamol and sodium nitroprusside, seems to be independent of cyclic nucleotide production. On the basis of this study, we suggest that NO donors bring a marked benefit in the relaxation of guinea pig tracheal smooth muscle in vitro, when combined with $\beta_2$-adrenoceptor agonist.

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