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Interactions between β_2-adrenoreceptor agonist and NO donors in the relaxation of guinea pig trachea in vitro

Kirsi Vaali, Liang Li, Heikki Vapaatalo *

Institute of Biomedicine, Department of Pharmacology and Toxicology, P.O. Box 8, 00014 University of Helsinki, Helsinki, Finland

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Abstract

The effects of interaction between the β_2-adrenoreceptor agonist, salbutamol, and the nitric oxide donors, sodium nitroprusside and 3-morpholinosydnonimine (SIN-1), on guinea pig trachea contraction were studied. Cumulatively increased concentrations (0.1–10 nM) of salbutamol together with a single concentration of SNP (0.33 μM) or with SIN-1 (1 μM) showed significant ($p < 0.001$) synergy for the inhibition of 1 μM metacholine-induced contraction. Significant synergy ($p < 0.05$) was also found for the inhibition of this contraction by cumulatively increased concentrations (0.1–33 μM) of SNP and a single concentration (1 nM) of salbutamol. No synergistic effect was found on the 40 mM KCl-induced contraction. We suggest that the combination of NO donors with salbutamol has a synergistic effect on metacholine contraction.

Keywords: Synergism; Synergy effect; Nitric oxide (NO) donor; β_2-Adrenoreceptor agonist; Smooth muscle; Relaxation; Trachea

1. Introduction

It has been demonstrated that there is an increase in NO levels in the exhaled air of asthmatic patients (Alving et al., 1993). NO has been shown to dilate airways of both experimental animals and humans in vivo (Högman et al., 1993a,b), and NO donors have a bronchodilating action in vitro (Jansen et al., 1992). NO activates soluble guanylate cyclase, which increases cGMP. The β-adrenoreceptor agonists are the most potent bronchodilators (Barnes, 1995), and binding of the agonist to its receptor results in activation of the adenylate cyclase, which in turn increases cellular cAMP. These two cyclic nucleotides are intracellular regulators that phosphorylate their own cAMP- and cGMP-dependent protein kinases. However, there is some recent evidence to suggest that β-adrenoreceptor stimulation in airway smooth muscle directly activates the calcium sensitive K^+ channels (K_{Ca} channels) (Kume et al., 1992). It has also recently been shown that in guinea pig trachea, and using the scorpion toxins, iberiotoxin and charybdotoxin, the relaxation induced by structurally different NO donors is mediated by K_{Ca} channels (Bialecki and Stinson-Fisher, 1995; Vaali et al., 1998). Interestingly, NO has been shown to activate K_{Ca} channels directly in cell-free membrane patches (Bolotina et al., 1994).

Little has been done to investigate the combined effect of the β-adrenoreceptor agonists and NO. The relaxation induced by NO donors in guinea pig trachea could potentiate the effects of salbutamol, i.e., induce a synergistic effect or a potentiating interaction instead of showing no interaction.

If a combination of drugs used is not synergistic, both drugs act at the same 'receptor', (Ariéns et al., 1956) that is, they have a common mechanism of action. The purpose of the present study was to determine whether the combination of β_2-adrenoreceptor agonists and NO donors acted synergistically on relaxation of a contracted guinea pig tracheal preparation.

2. Materials and methods

2.1. Preparation of guinea pig tracheal rings

English short-haired tricolored male guinea pigs (900–1200 g) bred by Harlan, (Winkerlmann, HsdIf:TB), were anesthetized with pentobarbital (75 mg/kg, i.p.) and then decapitated. The tracheas were cut into pieces and mounted in an 8-ml organ chamber containing a Krebs–Ringer

* Corresponding author: Tel.: +358-9-191-8286; Fax: +358-9-191-8200; E-mail: heikki.vapaatalo@helsinki.fi

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Table 1
Relaxant effect of a single concentration of the β_2-adrenoreceptor agonist and NO donors on metacholine- or KCl-contracted guinea pig trachea

Relaxant drug	Metacholine (1 μM)		KCl (40 mM)	
	% Maximal relaxation	*n*	% Maximal relaxation	*n*
SNP 0.1 μM	5.4±2.2	13	8.2±4.0*	9
SNP 0.33 μM	15.8±3.8**	13	12.7±4.3**	9
SIN-1 0.33 μM	2.3±1.7	10	0.8±0.6	5
SIN-1 1 μM	12.5±3.1**	10	3.0±1.3	5
Salbutamol 0.1 nM	1.2±0.5	7	0.7±0.6	6
Salbutamol 1 nM	1.3±0.6	7	1.8±0.8	8

The data are the mean±S.E.M. ($n = 5$–13) of the maximal percent relaxation.
Asterisk (*) indicates statistically significant difference between the vehicle and a single concentration of relaxant drug (* = $p < 0.05$ and ** = $p < 0.01$).

solution of the following composition (mM): NaCl 119, $NaHCO_3$ 25, glucose 11.1, $CaCl_2 \cdot H_2O$ 1.6, KCl 4.7, KH_2PO_4 1.2, $MgSO_4 \cdot 7H_2O$ 1.2. The pH was adjusted to 7.4. When 40-mM KCl for inducing contraction was prepared, Na^+ was replaced by K^+, to produce K^+-rich isoosmolar modification of the Krebs–Ringer buffer. The solution was aerated with a mixture of 96% O_2 + 4% CO_2 during the experiments, and the initial load (including the spontaneous tension) was set at 1.5 g. The tension changes were recorded with Grass force displacement transducers and amplifiers (FT03, Grass Medical Instruments, Quincy, MA, USA).

The experimental procedure was approved by the Animal Experimental Committee of Institute of Biomedicine, University of Helsinki, Finland.

2.2. Drugs used

Drugs from the following sources were used: SIN-1 (3-morpholino-sydnonimine) was synthethized by GEA Farmaceutisk Fabrik, (Hvidovre, Denmark); salbutamol was a generous gift from Leiras (Turku, Finland). SNP (sodium nitroprusside) from Hoffmann-La Roche, (Basel, Switzerland) and pentobarbital sodium from Danisco Ingredients, (Grinsted, Denmark). Metacholine (acetyl-β-methylcholine chloride) came from Sigma Chemicals, (St. Louis, MO, USA), and reagents for the Krebs–Ringer from Riedel-de Haën, (Seelze, Germany). The Krebs–Ringer solution was prepared in ultrapure water (MilliQ, Millipore, Bedford, MA, USA).

2.3. Determination of synergism, zero interaction and positive cooperativity for the combined 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

were 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 given in a single concentration, then, according to Ariéns et al. (1956) and Pöch and Holzmann (1980), the theoretical interaction can be calcu-

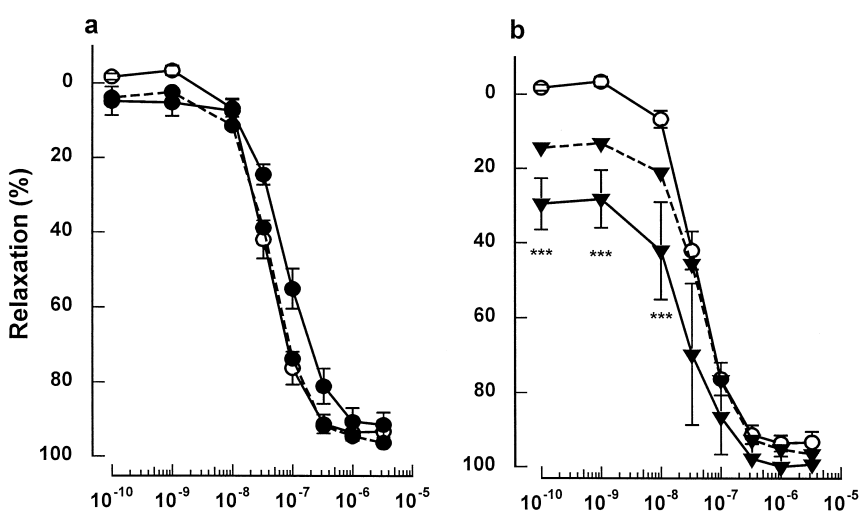

Fig. 1. Cumulative concentration curve of salbutamol relaxation alone (○, $n = 19$) with 1 μM metacholine-induced contraction in guinea pig trachea in vitro. Salbutamol modified by (a) 0.1 μM SNP (●, $n = 9$) or (b) 0.33 μM SNP (▼, $n = 4$). The broken line indicates the theoretical synergy curve for the same quantity of the modifying drug. A significant difference ($p < 0.001$, ***) was found in (b) within the concentration range of 0.1–10 nM when the theoretical synergy curve (▼, broken line), and the control curve (○) were compared; and when the experimental curve (▼, solid line) and the theoretical synergy curve (▼, broken line) are compared.

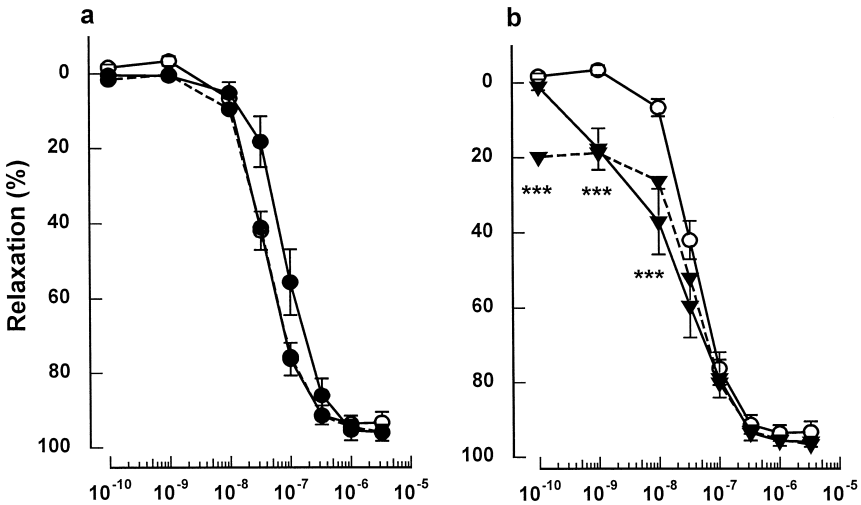

Fig. 2. Cumulative salbutamol relaxation alone with 1 μM metacholine-induced contraction (○, $n = 19$) and modified by (a) 0.33 μM SIN-1 (●, $n = 8$) or 1 μM SIN-1 (▼, $n = 9$). The broken line indicates the theoretical synergy curve for the same quantity of the modifying drug. Synergism was found (b) within the concentration range of 0.1–10 nM. In (b) the theoretical and experimental effects of SIN-1 (▼, broken and solid line, respectively) are both significantly different from the control curve, ($p < 0.001$, ***), but the theoretical and experimental curves did not differ significantly.

lated 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 ($E_A E_B$):

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

when E can be expressed as a fraction of the maximum effect (1.0). Using this equation and the experimentally obtained relaxation for drugs A and B, the theoretical curves for relaxation could be calculated with a spreadsheet. When the experimental curve for drugs A and B shows greater potency than does the theoretical interaction curve, the combination of drugs has positive cooperativity.

2.4. Experimental procedure

The preparations were allowed to stabilize for at least 40 min and to reach their spontaneous tone before any contracting agent was added. Between contractions, the preparations were washed every 15 min with Krebs–Ringer buffer. Submaximal concentration of the contracting agents, 1 μM metacholine and 40 mM KCl, were chosen on the basis of earlier results.

After metacholine- or KCl-induced contraction had reached the plateau (at least 35 min incubation time), the relaxant drug (drug A) was added cumulatively: salbutamol (0.1 nM–33 μM), SNP (10 nM–10 μM), SIN-1 (0.1 μM–0.1 mM), all the concentrations are the final concentrations of drugs. After the first concentration of the cumulative drug (drug A), the modifying drug (drug B) or its vehicle was added, then drug A was added cumulatively. A concentration of drug B that would have affect the relaxation only slightly was chosen. Each concentration of drugs was allowed to take effect for 6–8 min before the next concentration was added. The first contracting agent was always metacholine, and if this contraction was relaxed with drug A, the next contraction (also metacholine-induced) was relaxed with drugs A + B. In every experiment this procedure was carried out with half the organ baths, the order of the relaxing drugs was reversed with the other half, i.e., drug A + drug B in the first contraction, drug A alone for the next contraction. All experiments were carried out for the same length of time. When the first two contractions were obtained with metacholine, the next two were obtained with KCl.

Table 2
Effects of SNP and SIN-1 on salbutamol-induced relaxation of 40-mM KCl-induced contractions

	Relaxation (%)	EC_{50} (nM)	n
Salbutamol			
Exper.	81.7 ± 3.5	205 ± 45	19
SNP (0.1 μM) + salbutamol			
Theor.	83.2 ± 3.2	194 ± 48	19
Exper.	92.1 ± 3.0	96 ± 34 *	14
SNP (0.33 μM) + salbutamol			
Theor.	84.0 ± 3.1	173 ± 39	19
Exper.	80.4 ± 1.9	129 ± 30	6
SIN-1 (0.33 μM) + salbutamol			
Theor.	81.5 ± 3.5	214 ± 45	19
Exper.	92.5 ± 3.1	87 ± 21 *	11
SIN-1 (1 μM) + salbutamol			
Theor.	82.3 ± 3.4	192 ± 40	19
Exper.	71.6 ± 7.6	390 ± 157	6

The data are the mean ± S.E.M. ($n = 6$–19) of maximum percent relaxation and EC_{50} (nM). Theoretical (Theor.) values for synergy effects were calculated according to the theory presented above and compared to the experimental effects (Exper.).
Asterisk (*) indicates significance of $p < 0.05$.

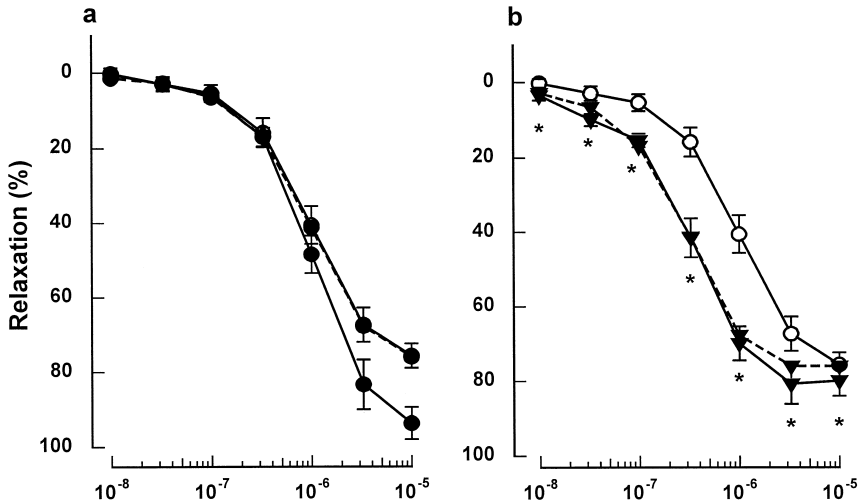

Fig. 3. NO donor-induced relaxation in 1 μM metacholine contraction. The cumulative curve of SNP (○, $n = 13$) modified by (a) 0.1 nM salbutamol (●, $n = 7$) or (b) 1 nM salbutamol (▼, $n = 7$). The broken line indicates the theoretical synergy curve for the same quantity of the modifying drug. Synergism was found in (b) within the concentration range of 0.1–3.3 μM. In (b) the experimental curve of SIN-1 (▼, smooth line) was significantly different from the control curve, (○, $p < 0.05$, *).

2.5. Statistical analysis

The data are presented as mean ± S.E.M. of the number of experiments shown. Analysis of variance (ANOVA/MANOVA) was studied with Statistica program, release 4.5, 1993 (Statsoft, Tulsa, OK, USA), followed by the Newman–Keuls test for multiple comparisons.

3. Results

3.1. Relaxation induced by β_2-adrenoreceptor agonist and NO donors

Salbutamol (0.1 nM or 1 nM) caused less than 3% relaxation of the trachea contracted by metacholine (1 μM) or KCl (40 mM). SNP (0.1 μM and 0.33 μM) and SIN-1 (0.33 μM and 1 μM) caused less than 20% relaxation of the trachea contracted by metacholine or KCl (Table 1).

3.2. Cumulative relaxation of the β_2-adrenoreceptor agonist in the presence of an NO donor

Cumulatively increased concentrations of the β_2-adrenoreceptor agonist, salbutamol (0.1–33 μM) relaxed the metacholine-contracted trachea concentration-dependently. The maximum relaxation was 96.1 ± 1.8%. This relaxation was not significantly affected by a low concentration of SNP (0.1 μM, Fig. 1a). When the higher concentration of SNP (0.33 μM) was added, the relaxing effect of salbutamol increased significantly ($p < 0.001$) within the range of 0.1–10 nM, but when the salbutamol concentra-

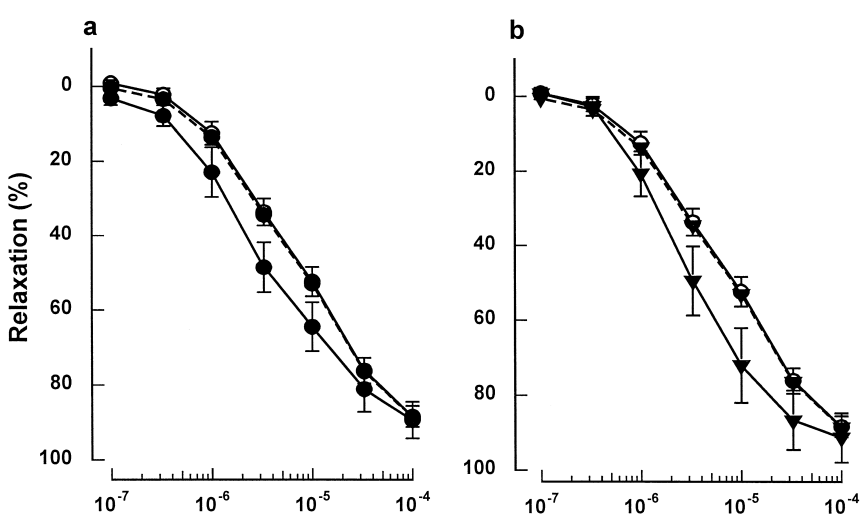

Fig. 4. NO donor-induced relaxation in 1 μM metacholine contraction. Cumulative curve for SIN-1 relaxation (○, $n = 10$) modified by salbutamol (a) 0.1 nM (●, $n = 5$) or (b) 1 nM (▼, $n = 6$). The broken line indicates the theoretical synergy curve for the same quantity of the modifying drug.

tion was increased by over 100 nM, no modifying effect was seen (Fig. 1b). The effect seen between the range of 0.1–10 nM was also significantly different from the theoretical synergy curve ($p < 0.001$), suggesting positive cooperativity.

The relaxation with salbutamol was not significantly affected by a low concentration of SIN-1 (0.33 μM, Fig. 2a). When the higher concentration of SIN-1 (1 μM) was added, the relaxing effect of salbutamol increased significantly ($p < 0.001$) within the concentration range of 0.1–10 nM but, again, when the salbutamol concentration was increased by over 100 nM, no modifying effect was seen (Fig. 2b).

Salbutamol (0.1–33 μM) relaxed KCl-contracted trachea concentration-dependently, the maximum being 91.8 ± 2.3%. This relaxation was potentiated ($p < 0.05$) by SNP (0.1 μM) or SIN-1 (0.33 μM), whereas SNP (0.33 μM) or SIN-1 (1 μM) had no effect (Table 2).

3.3. Cumulative relaxation of NO donors in the presence of the β_2-adrenoreceptor agonist

SNP (10 nM–10 μM) and SIN-1 (0.1 μM–0.1 mM) relaxed the metacholine-contracted trachea concentration-dependently. The maximal relaxation was 76.8 ± 6.5% and 84.9 ± 3.5%, respectively (Table 1). Salbutamol (0.1 nM) did not modify SNP-induced relaxation, but the relaxation was modified significantly by the higher concentration of salbutamol (1 nM) ($p < 0.05$, Fig. 3b). Salbutamol (0.1 or 1 nM) did not alter SIN-1-induced relaxation (Fig. 4a and b).

SNP and SIN-1 relaxed KCl-contracted trachea concentration-dependently. The maximum relaxations with SNP and SIN-1 were 64.2 ± 7.1% and 70.7 ± 10.1%, respectively. Salbutamol (0.1 nM or 1 nM) did not modify SNP or SIN-1-induced relaxations, (Table 3).

Table 3
Relaxant effect of salbutamol on NO donor-induced relaxation of 40-mM KCl-induced contractions

	SNP			SIN-1		
	Relaxation (%)	EC_{50} (μM)	*n*	Relaxation (%)	EC_{50} (μM)	*n*
Exper.	58.0 ± 6.6	5.2 ± 0.9	6	64.2 ± 6.9	38.4 ± 8.8	7
Salbutamol (0.1 nM) + SNP				Salbutamol (0.1 nM) + SIN-1		
Theor.	58.3 ± 6.6	5.1 ± 0.9	6	64.5 ± 6.8	45.1 ± 10.5	7
Exper.	71.2 ± 6.7	3.9 ± 1.2	6	78.1 ± 8.7	33.3 ± 14.3	5
Salbutamol (1 nM) + SNP				Salbutamol (1 nM) + SIN-1		
Theor.	58.8 ± 6.5	5.6 ± 1.0	6	65.6 ± 7.5	43.1 ± 10.0	7
Exper.	45.2 ± 4.5	n.d.	7	58.9 ± 7.9	36.3 ± 13.0	5

Data are the mean ± S.E.M., ($n = 6$–19) of maximum percent relaxation and EC_{50} (μM), n.d. = not determined.
Theoretical (Theor.) results of synergy effects were calculated according to the theory presented above and compared to the experimental effects (Exper.).

4. Discussion

β-Adrenoreceptor agonists have been shown to activate K_{Ca} channels by phosphorylation of a channel, or channel-related protein(s) in rabbit trachea, and also independently of cAMP-dependent protein phosphorylation (Kume et al., 1989, 1992). NO has been shown directly to activate K_{Ca} channels in cell-free membrane patches without requiring cGMP, and to relax rabbit aortic rings (Bolotina et al., 1994). The relaxation of NO donors in guinea pig trachea can be inhibited by the toxins selective for K_{Ca} channels (Bialecki and Stinson-Fisher, 1995; Johansson-Rydberg et al., 1997; Vaali et al., 1998). Thus, on the basis of the theory of the interaction of compounds on receptor systems (Ariéns et al., 1956), there should be no interaction if both drugs mediate their relaxing effects through K_{Ca} channels.

We found significant synergy for the cumulative relaxation of the β_2-adrenoreceptor agonist, salbutamol (0.1–10 nM), in the presence of a small concentration of the NO donor, in the case of metacholine-induced contraction. Weaker synergy was also observed with metacholine contraction when the cumulatively added drug was SNP and the modifying drug was salbutamol, but this was not the case when the cumulatively added drug was SIN-1.

During the preparations for the present study, Thierstrup et al. (1997) reported that in histamine-contracted guinea pig trachea, significant potentiation of the relaxant effect of the cumulatively added drug, the β_2-adrenoreceptor agonist, terbutaline (30 nM–0.3 μM), with a single modifying concentration of SNP (30 and 100 nM). Inhibition of endogenous phosphodiesterases (PDE) was suggested as the reason for the potentiation.

Maurice et al. (1990) reported that SNP and SIN-1 in combination with isoprenaline did not act synergistically on the relaxation of rat aortic smooth muscle, but did show synergy for inhibition of the contraction. They proposed that the synergism resulted from the inhibition of cAMP breakdown by a cGMP-inhibited low K_m cAMP phosphodiesterase (phosphodiesterase type III).

Miura et al. (1992) could not demonstrate that the relaxation induced by theophylline, a non-selective phosphodiesterase inhibitor, was significantly affected by the K_{Ca} channel inhibitor, charybdotoxin, although there was a tendency to an effect. In the same study, the relaxation induced by the β-adrenoreceptor agonist, isoproterenol, was significantly and concentration-dependently inhibited. The authors suggested that activation of any of the several types of K^+ channels would produce relaxation of the tracheal smooth muscle and showed that the relaxation induced by phosphodiesterase inhibition was not directly mediated through the K_{Ca} channels. Thus the combination of activation of the K_{Ca} channels and inhibition of phosphodiesterases should be synergistic. Also, inhibition of phosphodiesterase types III and IV was shown to augment

the actions of isoprenaline in airways (Raeburn and Advenier, 1995).

It is obvious that inhibition of the endogenous phosphodiesterases would potentiate the relaxation induced by NO or a β-adrenoreceptor agonist. The combined effect of the β-adrenoreceptor agonist and an NO donor would most probably mimic phosphodiesterase type III-induced inhibition; phosphodiesterase type III being one of the most potent phosphodiesterases for relaxation of guinea pig trachea (Raeburn and Advenier, 1995). Also, the facts that phosphodiesterases are known to exist in guinea pig trachea and that they were probably inhibited during the experimental procedure, suggest that one ought to find this kind of activity. According to Pöch and Holzmann (1980), positive cooperativity can be termed sequential synergism. In the present study, this was manifested as the modifying drug (SNP) inhibiting (through phosphodiesterase inhibition) the degradation of the other drug's (salbutamol) mediator, cAMP. The significantly greater synergism found in the experimental curve for salbutamol with the modifying concentration of SNP (0.33 μM) as compared to that of the theoretical curve could represent positive cooperativity. We cannot explain why salbutamol cannot modify the relaxation of SIN-1.

The lowered relaxation efficacy of NO donors in 40 mM KCl than in the Krebs–Ringer buffer (K^+ concentration 5.9 mM), may be explainable by the reduced membrane potential. If the extracellular concentration of K^+ is high, opening of the K^+ channels does not allow as great a K^+ flux through as when the membrane potential is close to its physiological level. In metacholine-induced contraction NO donors mediate the relaxing effect through the K_{Ca} channels, but these play a minor role in the relaxation of NO donors in the case of KCl-induced contraction (Vaali et al., 1998). If K_{Ca} channels are rate-limiting in the system, then if the synergy is studied with high-KCl, the combination of NO donors and salbutamol cannot be as effective as it is with metacholine-induced contraction. Thus the decreased synergy with NO donors and salbutamol on KCl-induced contraction could be explained by reduced flux of K^+ ions through the K_{Ca} channels.

It can be concluded that the synergism can be seen with nanomolar concentrations of salbutamol, when the modifying drug is an NO donor, if metacholine-induced contraction is studied. The same is true when the modifying drug is salbutamol, with micromolar concentrations of SNP. If these drugs are studied using KCl contraction, the synergism is less pronounced.

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