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In vitro studies on the interactions of β_2-adrenoceptor agonists, methylxanthines, Ca^{2+}-channel blockers, K^+-channel openers and other airway smooth muscle relaxants in isolated guinea-pig trachea

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Abstract

Pharmacodynamic interactions in vitro between different types of airway smooth muscle relaxants were systematically and quantitatively evaluated by using a new methodological technique. Relaxant concentration-effect curves for terbutaline, theophylline, cromakalim, sodium nitroprusside and isradipine were obtained in isolated guinea-pig trachea contracted by histamine (1 μM). The effects of three different fixed concentrations of each airway smooth muscle relaxant were initially attained and concentration-effect curves for combinations with increasing concentrations of either one of the other relaxants were produced. Based on pharmacodynamic parameters obtained by non-linear regression analysis of experimental data for the relaxants alone theoretical concentration-effect curves for predicted additive interaction were constructed by using the isobolic method. Synergistic (over-additive) interaction was defined as existing when data points and derived pharmacodynamic parameters obtained with combinations of the relaxants showed statistically significant deviation from the predicted additive interaction curve and its functional parameters. Significant synergistic interaction with terbutaline was found for both theophylline (70 or 200 μM), cromakalim (0.1, 0.3 or 1 μM), sodium nitroprusside (30 or 100 nM) and isradipine (1, 3 or 10 nM). Theophylline showed synergistic interaction with cromakalim (0.1, 0.3 or 1 μM), sodium nitroprusside (10 nM) and isradipine (1, 3 or 10 nM). Interactions between cromakalim and sodium nitroprusside (10, 30 or 100 nM) were also synergistic, whereas cromakalim and isradipine (1, 3 or 10 nM) produced only additive interaction. Possible mechanisms underlying the interactions are discussed on basis of existing knowledge with special regards to phosphodiesterase isoenzymes, K^+ and Ca^{2+} channels.

Keywords: Asthma; Drug combination; Interaction; Smooth muscle, airway; Trachea; β_2-Adrenoceptor agonist; Methylxanthine; K^+ channel opener; Sodium nitroprusside; Dihydropyridine Ca^{2+} channel blocker

1. Introduction

The goal of drug combination therapy is to obtain therapeutic advantages over therapy with a single drug. Such advantages are most often ascribed to pharmacodynamic or pharmacokinetic interactions. The resulting effect of an interaction can be classified as either less than (antagonistic interaction), more than (synergistic or over-additive interaction) or equal to (additive interaction) the effect that would be expected from the effects of the individual agents used alone (Berenbaum, 1989).

Although asthma is now recognised primarily as an inflammatory condition, bronchodilators are still drugs of choice in first-line therapy of the asthmatic bronchospastic attack in order to improve airflow and produce symptomatic relief (Anonymous, 1992). Inhalation of β_2-adrenoceptor agonists (e.g., salbutamol and terbutaline), usually in combination with corticosteroids, is effective in both chronic and acute treatment of most asthmatics (Ziment, 1995). However, treatment of certain patient subgroups, e.g., those with nocturnal asthma and acute exacerbations, may require additional bronchodilator therapy (Manthous, 1995; Peters, 1995). Combination therapy with additional methylxanthines (e.g., theophylline) has traditionally been employed in these situations (Manthous,

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1995; Peters, 1995; Weinberger and Hendeles, 1996), but other drugs with airway smooth muscle relaxant activity seem needed to improve therapy. K^+-channel openers, Ca^{2+}-channel blockers and nitric oxide (NO) donors have obtained interest as putative antiasthmatics (Barnes, 1992, 1996). The cellular mechanisms underlying the airway smooth muscle relaxation produced by these drugs have recently been reviewed (Knox and Tattersfield, 1995) and are schematically illustrated in Fig. 1.

The aim of the present in vitro study was to obtain a systematic and quantitative evaluation of the pharmacodynamic interactions between different classes of airway smooth muscle relaxants by using a new methodological technique. This was based upon a combination of the isobolic method and iterative non-linear regression analysis of experimental data whereby predicted functional curve parameters for additive interaction were obtained. The relaxant effects of a β_2-adrenoceptor agonist (terbutaline), a methylxanthine (theophylline), a K^+-channel opener (cromakalim), a nitrovasodilator (sodium nitroprusside) and a dihydropyridine Ca^{2+}-channel blocker (isradipine) were studied either alone or in combinations in isolated guinea-pig trachea contracted by histamine. The β_2-adrenoceptor agonist salbutamol was studied alone and in combination with terbutaline as a reference combination expected to show additive interaction. The results were discussed in view of the existing knowledge of cellular mechanisms of action of the drugs.

2. Materials and methods

2.1. Tracheal preparation and measurement of contractile force

Hartley-Dunkin guinea-pigs of either sex (356 ± 12 g; $n = 28$) were stunned by a blow to the neck and exsanguinated. The thorax was opened, the heart removed and the full-length trachea transferred to cold oxygenated Krebs solution. The trachea was carefully cleaned of connective tissue under a dissecting microscope. The mid-trachea was cut into rings comprising two adjoining cartilage segments (length approximately 2 mm). Six rings were each transferred to a 5 ml organ bath containing Krebs solution (37°C; pH = 7.4) continuously gassed with a mixture of oxygen and carbon dioxide (5% CO_2 in 95% O_2). The preparations were mounted in precision myographs for measurement of isometric force (Nielsen-Kudsk et al., 1986) and six experiments were run in parallel. Each ring was suspended at a passive force of 0.6 g, which is optimal for development of contractile force. The amplified transducer signals were recorded on a six-channel recorder (Graphtec WR3101, Japan). The preparations equilibrated for 60 min before start of experiments. The cyclooxygenase inhibitor indomethacin (2 μM) was present throughout the experiments in order to prevent spontaneous tonus oscillations.

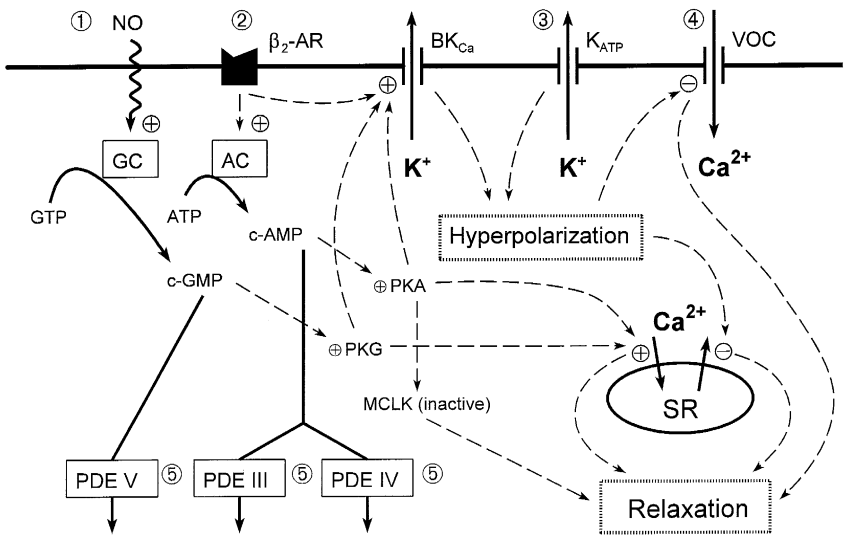

Fig. 1. Main mechanisms involved in drug-induced airway smooth muscle relaxation: (1) increase in intracellular cGMP through stimulation of guanylate cyclase (GC) by nitric oxide (NO) liberated from NO donors (e.g., sodium nitroprusside); (2) increase in intracellular cAMP through stimulation of adenylate cyclase (AC) by β_2-adrenoceptor (β_2-AR) agonists (e.g., terbutaline and salbutamol); (3) plasmalemmal hyperpolarization through opening of ATP-sensitive K^+ channels (K_{ATP}) (e.g., cromakalim); (4) inhibition of Ca^{2+} influx through dihydropyridine-sensitive voltage-operated Ca^{2+} channels (VOC) (e.g., isradipine); and (5) inhibition of cAMP and/or cGMP breakdown through selective or non-selective (e.g., theophylline) phosphodiesterase isoenzyme (PDE) inhibition. BK_{Ca}, large conductance calcium-activated potassium channel; PKA, protein kinase A; PKG, protein kinase G; MCLK, myosin light chain kinase; SR, sarcoplasmic reticulum; ⊕, activation; ⊖, inhibition.

2.2. Experiments

The relaxant effect of terbutaline (10 nM–10 μM), salbutamol (1 nM–1 μM), theophylline (1 μM–1 mM), cromakalim (0.1 μM–30 μM), sodium nitroprusside (1 nM–10 μM) or isradipine (0.1 nM–1 μM) were studied in tracheal preparations precontracted with histamine (1 μM). Drugs were added cumulatively and a stable level of contraction was awaited before increasing drug concentration in the baths.

In order to study the pharmacodynamic interactions between pairs of the aforementioned airway smooth muscle relaxants three concentrations of each compound were selected on the basis of their relaxant action. Concentrations producing less than 10%, between 10% to 20% and between 40% to 60%, respectively, were chosen. Either of these concentrations were established by drug addition to preparations precontracted with histamine (1 μM) which then were allowed to equilibrate until the relaxation had reached a steady state of pretreatment. Subsequently, another airway smooth muscle relaxant was added cumulatively in order to study the relaxant effect of the combination. The concentrations used for pretreatment were: theophylline (30 μM; 70 μM; 200 μM), cromakalim (0.1 μM; 0.3 μM; 1 μM), sodium nitroprusside (10 nM; 30 nM; 100 nM), isradipine (1 nM; 3 nM; 10 nM) and salbutamol (3 nM; 10 nM; 30 nM). Terbutaline was studied in preparations pretreated with either theophylline, cromakalim, sodium nitroprusside, isradipine or salbutamol. Theophylline was studied in preparations pretreated with either cromakalim, sodium nitroprusside or isradipine. Cromakalim was studied in preparations pretreated with either sodium nitroprusside or isradipine, and sodium nitroprusside was studied in isradipine-pretreated preparations.

Experiments involving isradipine or sodium nitroprusside were carried out in dim light to avoid any photodegradation of these drugs.

2.3. Data analysis and statistics

Data are expressed as means ± S.E.M. Relaxant effects are expressed as percentual reduction in histamine-induced tone. Returning to baseline was taken as 100% relaxation. Concentration-relaxation curves are based on mean values for pharmacodynamic parameters obtained by fitting data from single experiments by iterative, non-linear regression analysis to the 'four-parameter logistic equation': $E = (E_{min} + (E_{max} - E_{min}))/(1 + 10^{((\log EC_{50} - \log C) \times S)})$. GraphPad Prism®, version 2.0 (GraphPad Software, USA) was used for the iterative non-linear regression analysis. E_{min} is the bottom value of the concentration-effect curve and E_{max} the theoretically maximal effect. S is equal to the Hill coefficient and related to the slope of the curve (Barlow and Blake, 1989). EC_{50} is the concentration producing an effect half-way between E_{min} and E_{max}. The negative logarithm to EC_{50} is termed pEC_{50} (Jenkinson et al., 1995). E_{min} was held constant in all fitting procedures. In experiments with airway smooth muscle relaxants alone it was set equal to zero, otherwise it was set equal to the relaxant effect induced by the pretreatment.

In order to evaluate the pharmacodynamic interaction between pairs of airway smooth muscle relaxants analytically predicted pharmacodynamic parameters and corresponding concentration-effect curves for additive interaction of the combination were produced. This was based on a new methodological technique combining two methods. The 'isobole method' (1), which has been extensively reviewed by Berenbaum (1989), is a general valid method where an equation for additive interaction between two agents, A and B, is expressed as: $d_a/D_a + d_b/D_b = 1$. The concentrations of A and B used in combination are termed d_a and d_b, whereas concentrations of either A or B producing an effect equal to the combination are denoted D_a or D_b, respectively. Using this equation and the logistic equation (2) with substitution of the relevant pharmacodynamic parameters derived from analysis of experiments with airway smooth muscle relaxants alone, it was possible to construct a predicted concentration-effect curve for one airway smooth muscle relaxant in the presence of a fixed concentration of another showing additive interaction. The spreadsheet QuatroPro®, version 5.0 (Borland International, USA), was used for this operation. Data points for observed effects of a combination lying statistically significantly above this curve indicates synergistic interaction, whereas points lying significantly under the curve indicates antagonistic interaction.

Statistical comparison was made using the two-tailed one-sample *t*-test with a significance level of 95%.

2.4. Drugs and solutions

Histamine dihydrochloride, indomethacin and sodium nitroprusside were obtained from Sigma-Aldrich UK. Salbutamol was obtained as Ventolin® respirator solution 1 mg/ml (Glaxo-Wellcome, UK). The following drugs were donated by the producer: terbutaline and theophylline (Astra-Draco, Sweden), cromakalim (SmithKline Beecham, UK) and isradipine (Sandoz, Switzerland).

Stock solutions used were: histamine (10 mM), terbutaline (1 mM) and sodium nitroprusside (10 mM) in distilled water, indomethacin (8.38 mM) in 5% $NaHCO_3$, salbutamol (1 mM) in saline 0.9%, theophylline (0.1 M) in one part 0.5 M NaOH plus 4 parts saline 0.9%, cromakalim (10 mM) in 70% ethanol and isradipine (1 mM) in 96% ethanol. The final maximum bath concentration of ethanol in experiments involving cromakalim or isradipine was 2‰ and 0.9‰ respectively. In control experiments these concentrations only produced temporary oscillations in histamine-induced tone. Stock solutions were kept at −70°C until use and then further diluted in saline 0.9%. The composition of the Krebs solution (in mM) was: NaCl 118.0, KCl 4.6, $CaCl_2$ 2.5, $MgSO_4$ 1.15, $NaHCO_3$ 24.9, KH_2PO_4 1.15 and glucose 5.5.

3. Results

3.1. Histamine-induced tracheal tone and relaxation produced by airway smooth muscle relaxants alone

Histamine (1 μM) produced a monophasic and sustained contractile response (2.71 ± 0.04 g; $n = 209$). Each airway smooth muscle relaxant produced concentration-dependent relaxation of histamine-contracted preparations. Terbutaline, salbutamol, theophylline and sodium nitroprusside each were able to produce complete relaxation, whereas cromakalim and isradipine, at the highest concentrations studied, produced 77.7 ± 4.2% ($n = 5$) and 76.1 ± 6.6% ($n = 2$) relaxation, respectively. Derived pharmacodynamic parameters are stated in Table 1 and concentration-effect curves shown in Fig. 2A.

3.2. Relaxation of precontracted trachea produced by combinations of airway smooth muscle relaxants

3.2.1. Terbutaline effects influenced by:

3.2.1.1. Salbutamol. Concentration-effect curves for tracheal relaxation produced by terbutaline in the presence of salbutamol either 3 nM, 10 nM or 30 nM did not deviate statistically significantly from the expected additive curves and the relaxant effect of terbutaline was not altered at any concentration studied. Concentration-effect curves are shown in Fig. 2B and pharmacodynamic parameters for the combinations are found in Table 2.

3.2.1.2. Theophylline. Pretreatment with theophylline 30 μM did not cause any significant alteration in the pharmacodynamic parameters for the combination in comparison with those for the expected additive effect (Table 2); however, terbutaline 0.3 μM produced a significant greater relaxation than expected (Fig. 3A). Pretreatment with theophylline either 70 or 200 μM resulted in significant leftward displacement of the concentration-effect curves away from the calculated additive curves (Table 2 and Fig. 3A).

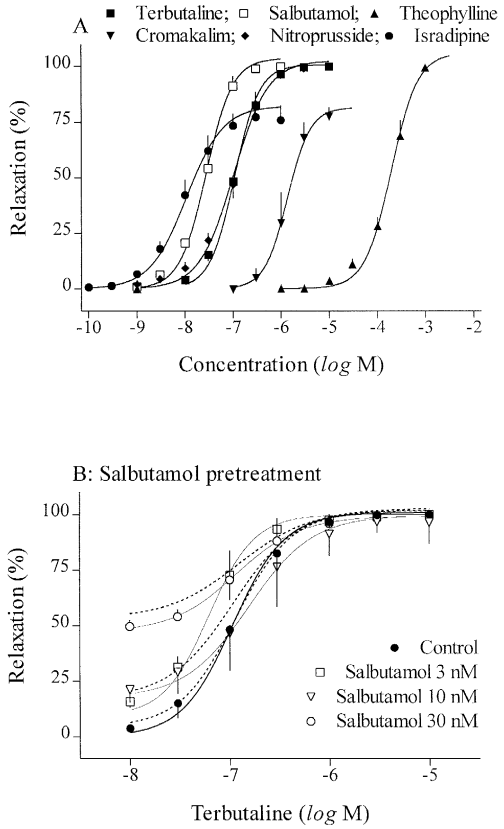

Fig. 2. Isolated guinea-pig trachea contracted by histamine (1 μM). A: Concentration-effect curves for the relaxant action of the drugs stated. B: Concentration-effect curves for the relaxant action of terbutaline alone or in preparations pretreated with the stated concentrations of salbutamol. Hatched curves indicate analytically predicted additive interaction for these combinations. Error bars represent S.E.M.

3.2.1.3. Cromakalim. Pretreatment with cromakalim either 0.1, 0.3 or 1 μM significantly displaced the concentration-effect curves for terbutaline to the left compared to curves for additive interaction (Fig. 3B and Table 2). As a result of this, the relaxant effects of terbutaline 30 nM, 0.1 and 0.3 μM were significantly potentiated by each of the three concentrations of cromakalim and pretreatment with cromakalim 1 μM also potentiated the relaxant effect of terbutaline 10 nM (Fig. 3B).

3.2.1.4. Sodium nitroprusside. Combining sodium nitroprusside 10 nM with terbutaline did not produce any significant displacement of the concentration-effect curve in comparison with the additive curve for this combination (Table 2). However, a small but significant potentiation of the relaxant effect of terbutaline 30 nM and 0.3 μM were seen (Fig. 3C). Pretreatment with either sodium nitroprusside 30 or 100 nM showed synergistic interaction with terbutaline. In both situations it was indicated by a significant leftward displacement of the EC_{50} and a steeper concentration-effect curve (indicated by a significantly greater *S*-value) compared to curves for additive interaction (Table 2).

Table 1
Pharmacodynamic parameters ($pEC_{50} = -\log(EC_{50})$, E_{max} and S) and S.E.M. for the relaxant action of terbutaline, salbutamol, theophylline, cromakalim, sodium nitroprusside or isradipine in rings of isolated guinea-pig trachea contracted by histamine (1 μM)

	pEC_{50}	E_{max} (%)	S	n
Terbutaline	6.97 ± 0.08	100.9 ± 0.5	1.67 ± 0.17	7
Salbutamol	7.56 ± 0.07	103.1 ± 0.8	1.57 ± 0.13	6
Theophylline	3.71 ± 0.06	106.3 ± 1.4	1.56 ± 0.13	5
Cromakalim	5.87 ± 0.02	81.7 ± 0.7	1.92 ± 0.13	5
Sodium nitroprusside	6.99 ± 0.07	102.8 ± 0.8	1.21 ± 0.14	5
Isradipine	7.97 ± 0.12	82.3 ± 3.6	1.14 ± 0.09	6

Mean parameters were derived by iterative, non-linear regression analysis of data from single experiments.

Table 2
Pharmacodynamic parameters ($pEC_{50} = -\log(EC_{50})$, E_{max} and S) and S.E.M. for the relaxant action of terbutaline in tracheal preparations contracted by histamine (1 μM) and pretreated with different concentrations (as indicated) of either salbutamol, theophylline, cromakalim, sodium nitroprusside (SNP) or isradipine

	pEC_{50}		E_{max} (%)		S		n
Terbutaline							
Salbutamol							
3 nM	7.21 ± 0.11	(6.94)	99.6 ± 0.7	(101.7)	1.90 ± 0.24	(1.54)	4
10 nM	6.81 ± 0.12	(6.97)	99.6 ± 1.7	(102.7)	1.36 ± 0.10	(1.35)	7
30 nM	6.94 ± 0.01	(6.91)	99.3 ± 0.4	(102.2)	1.33 ± 0.04	(1.23)	3
Theophylline							
30 μM	7.10 ± 0.09	(6.95)	101.5 ± 0.3	(102.0)	1.86 ± 0.22	(1.49)	6
70 μM	7.40 ± 0.10 [a]	(6.97)	100.2 ± 0.5 [a]	(102.6)	2.39 ± 0.32 [a]	(1.36)	5
200 μM	7.75 ± 0.03 [a]	(6.92)	100.5 ± 0.8	(102.3)	1.62 ± 0.17	(1.23)	5
Cromakalim							
0.1 μM	7.44 ± 0.07 [a]	(6.93)	99.0 ± 0.5 [a]	(102.2)	2.47 ± 0.40	(1.55)	7
0.3 μM	7.61 ± 0.12 [a]	(6.95)	99.8 ± 0.4 [a]	(104.0)	2.39 ± 0.49	(1.37)	6
1 μM	7.88 ± 0.08 [a]	(6.94)	101.5 ± 1.3	(102.1)	1.19 ± 0.24	(1.12)	5
SNP							
10 nM	7.08 ± 0.17	(6.92)	102.9 ± 1.6	(101.7)	1.99 ± 0.21	(1.55)	5
30 nM	7.24 ± 0.06 [a]	(6.91)	100.4 ± 0.5 [a]	(102.2)	2.37 ± 0.21 [a]	(1.45)	5
100 nM	7.42 ± 0.07 [a]	(6.85)	100.2 ± 0.1 [a]	(102.4)	2.46 ± 0.20 [a]	(1.28)	4
Isradipine							
1 nM	7.22 ± 0.06 [a]	(6.90)	101.4 ± 0.5 [a]	(103.2)	1.58 ± 0.14	(1.55)	6
3 nM	7.16 ± 0.06 [a]	(6.86)	100.9 ± 0.8	(107.1)	1.67 ± 0.20	(1.36)	6
10 nM	7.29 ± 0.10 [a]	(6.73)	100.7 ± 0.5 [a]	(109.7)	1.49 ± 0.15	(1.19)	5

The mean parameters were derived by iterative, non-linear regression analysis of data from single experiments. Expected pharmacodynamic parameters for an additive interaction are stated in parentheses. [a] $P < 0.05$.

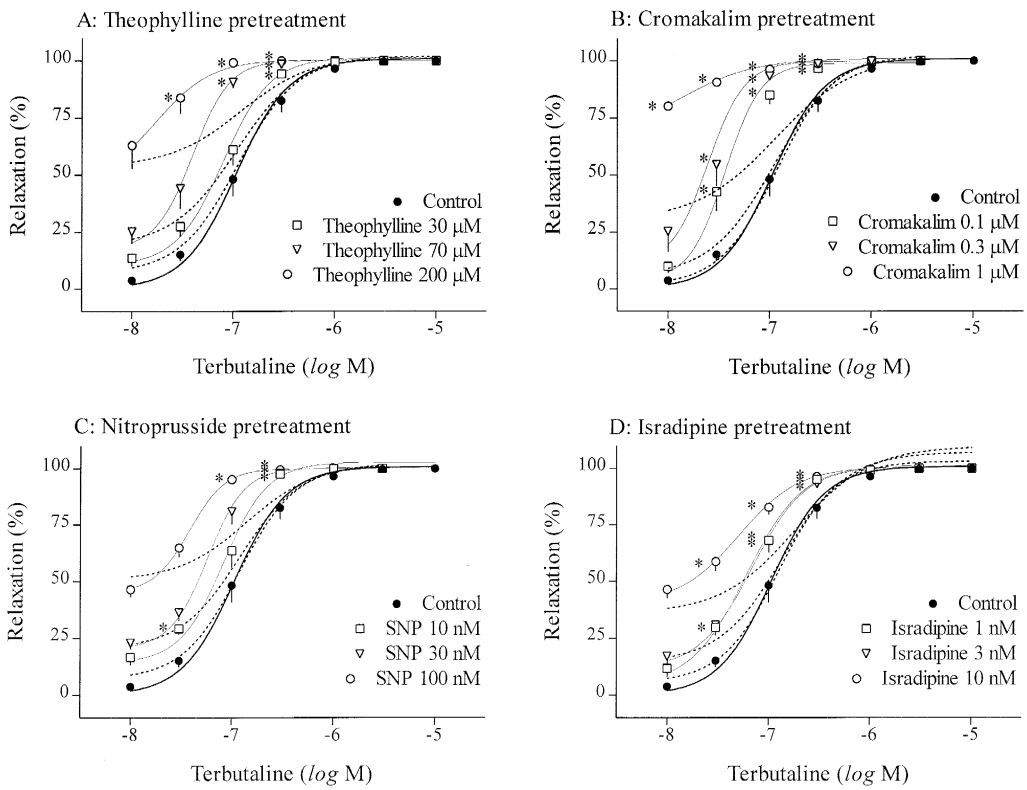

Fig. 3. Isolated guinea-pig trachea contracted by histamine (1 μM). Concentration-effect curves for the relaxant action of terbutaline alone and in preparations pretreated with the stated concentrations of either (A) theophylline, (B) cromakalim, (C) sodium nitroprusside (SNP) or (D) isradipine. Hatched lines indicate concentration-effect curves for analytically predicted additive interactions. * $P < 0.05$ compared to additive interaction. Error bars represent S.E.M.

3.2.1.5. Isradipine. The concentration-effect curves for terbutaline in combination with either of the three concentrations of isradipine were significantly leftward displaced compared to curves for expected additive interaction (Fig. 3D and Table 2). All three concentrations of isradipine were able to significantly potentiate the relaxation produced by terbutaline 0.1 and 0.3 μM, whereas the relaxation produced by terbutaline 30 nM was potentiated by isradipine 3 and 10 nM, respectively (Fig. 3D).

3.2.2. Theophylline effects influenced by:

3.2.2.1. Cromakalim. Pretreatment with cromakalim either 0.1, 0.3 or 1 μM significantly displaced the concentration-effect curves for theophylline to the left compared to curves for additive interaction (Table 3). Despite of this, only pretreatment with cromakalim 1 μM resulted in significant potentiation of the relaxation produced by theophylline 0.1 and 0.3 mM (Fig. 4A).

3.2.2.2. Sodium nitroprusside. Pretreatment with sodium nitroprusside 10 nM produced a slight by significant leftward displacement of the concentration-effect curve for theophylline compared to the additive curve for this combination (Table 3). The concentration-effect curves produced by theophylline in preparations pretreated with either sodium nitroprusside 30 or 100 nM showed concordance with the expected additive curves for the corresponding combinations (Fig. 4B and Table 3).

3.2.2.3. Isradipine. The concentration-effect curves for theophylline in combination with isradipine 1, 3 or 10 nM were all significantly leftward displaced compared to the calculated expected additive curves (Table 3). However, pretreatment with either of the three concentrations of isradipine was unable to significantly potentiate the relaxant effect of theophylline at any concentration studied (Fig. 4C).

3.2.3. Cromakalim effects influenced by:

3.2.3.1. Sodium nitroprusside. Pretreatment with either of the three concentrations of sodium nitroprusside (10, 30 or 100 nM) resulted in statistically significant leftward displacement of the concentration-effect curves (Fig. 5A) and significant elevation of E_{max} values compared to calcu-

Table 3
Pharmacodynamic parameters ($pEC_{50} = -\log(EC_{50})$, E_{max} and S) and S.E.M. for the relaxant action of airway smooth muscle relaxant combinations with either theophylline, cromakalim or sodium nitroprusside (SNP) in tracheal preparations contracted by histamine (1 μM)

	pEC_{50}		E_{max} (%)		S		n
Theophylline							
Cromakalim							
0.1 μM	3.93 ± 0.06 [a]	(3.70)	103.9 ± 4.7	(107.5)	1.35 ± 0.22	(1.45)	4
0.3 μM	3.88 ± 0.05 [a]	(3.72)	101.7 ± 2.7	(109.0)	1.55 ± 0.25	(1.30)	7
1 μM	4.39 ± 0.02 [a]	(3.72)	100.5 ± 0.5 [a]	(104.8)	1.34 ± 0.08	(1.09)	7
SNP							
10 nM	3.87 ± 0.03 [a]	(3.69)	101.7 ± 1.4 [a]	(107.7)	1.36 ± 0.01	(1.45)	5
30 nM	3.74 ± 0.02	(3.67)	101.0 ± 0.8 [a]	(108.6)	1.73 ± 0.11 [a]	(1.34)	5
100 nM	3.81 ± 0.10	(3.57)	103.6 ± 1.1 [a]	(109.5)	2.04 ± 0.41	(1.18)	5
Isradipine							
1 nM	3.85 ± 0.03 [a]	(3.67)	101.2 ± 1.9 [a]	(108.8)	1.68 ± 0.19	(1.46)	5
3 nM	3.93 ± 0.02 [a]	(3.62)	101.2 ± 0.9 [a]	(112.9)	1.40 ± 0.09	(1.32)	5
10 nM	3.99 ± 0.07 [a]	(3.49)	101.7 ± 1.9 [a]	(115.1)	1.17 ± 0.21	(1.16)	5
Cromakalim							
SNP							
10 nM	6.14 ± 0.03 [a]	(5.88)	94.9 ± 1.1 [a]	(82.8)	3.29 ± 0.54	(1.73)	5
30 nM	6.24 ± 0.07 [a]	(5.90)	99.3 ± 0.5 [a]	(82.9)	4.84 ± 1.04 [a]	(1.55)	5
100 nM	6.39 ± 0.02 [a]	(5.89)	99.9 ± 0.7 [a]	(83.7)	1.97 ± 0.12 [a]	(1.31)	6
Isradipine							
1 nM	6.06 ± 0.02	(5.87)	81.5 ± 1.5	(82.3)	2.80 ± 0.53	(1.80)	5
3 nM	5.97 ± 0.04	(5.86)	90.4 ± 2.7	(83.4)	2.22 ± 0.54	(1.62)	6
10 nM	5.77 ± 0.06	(5.82)	87.7 ± 2.1	(83.9)	1.57 ± 0.29	(1.39)	6
SNP							
Isradipine							
1 nM	6.90 ± 0.09	(6.95)	103.1 ± 0.8	(103.8)	1.08 ± 0.15	(1.17)	5
3 nM	7.22 ± 0.12	(6.91)	102.3 ± 1.3	(105.0)	1.05 ± 0.10	(1.12)	5
10 nM	7.52 ± 0.23 [a]	(6.78)	101.2 ± 0.8 [a]	(104.9)	0.91 ± 0.16	(1.06)	5

Theophylline was studied in preparations pretreated with different concentrations (as indicated) of either cromakalim, sodium nitroprusside (SNP) or isradipine. Cromakalim was studied in preparations pretreated with different concentrations (as indicated) of either SNP or isradipine, whereas SNP was studied in preparations pretreated with isradipine (concentrations as indicated). The mean pharmacodynamic parameters were derived by iterative, non-linear regression analysis of data from single experiments. Expected parameters for an additive interaction are stated in parentheses. [a] $P < 0.05$.

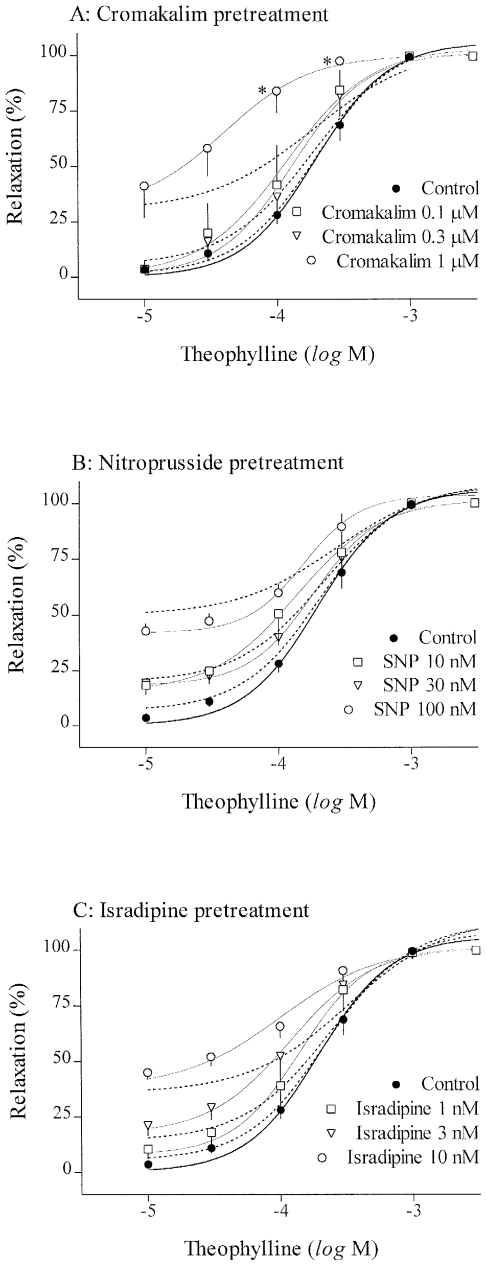

Fig. 4. Isolated guinea-pig trachea contracted by histamine (1 μM). Concentration-effect curves for the relaxant action of theophylline alone and in combination with stated concentrations of (A) cromakalim, (B) sodium nitroprusside (SNP) or (C) isradipine. Hatched lines indicate concentration-effect curves for analytically predicted additive interactions. $^{*}P < 0.05$ compared to additive interaction. Error bars represent S.E.M.

lated additive curves (Table 3). The elevation of E_{max} was also reflected in significant potentiation of the relaxations produced by cromakalim 3, 10 and 30 μM in the presence of either of the three concentrations of sodium nitroprusside and the potentiation of cromakalim 1 μM in preparations pretreated with sodium nitroprusside 30 nM. The combination of sodium nitroprusside 100 nM with cromakalim 0.1 μM produced a lower relaxant effect than expected, indicating a possible antagonistic interaction.

3.2.3.2. Isradipine. The concentration-effect curves produced by cromakalim in preparations pretreated with either isradipine 1, 3 or 10 nM showed concordance with the expected additive curves for the corresponding combina-

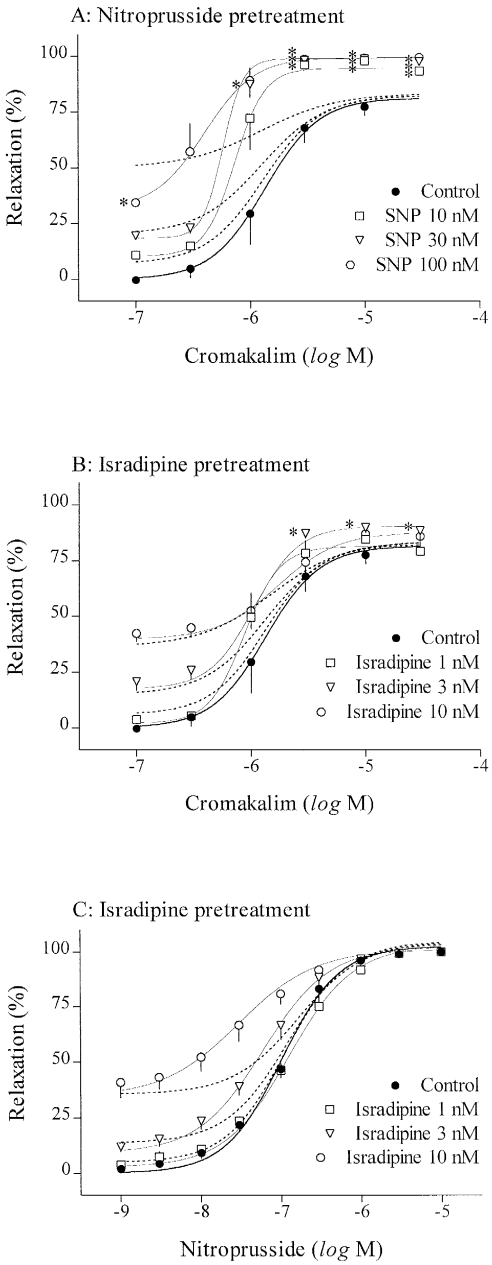

Fig. 5. Isolated guinea-pig trachea contracted by histamine (1 μM). Panels A and B show concentration-effect curves for the relaxant action of cromakalim alone and in combination with stated concentrations of (A) sodium nitroprusside (SNP) or (B) isradipine. Panel C shows concentration-effect curves for the relaxant action of sodium nitroprusside alone and in combination with stated concentrations of isradipine. Hatched lines indicate concentration-effect curves for analytically predicted additive interactions. $^{*}P < 0.05$ compared to additive interaction. Error bars represent S.E.M.

tions (Table 3). However, pretreatment with isradipine 3 nM significantly potentiated the relaxant effect of cromakalim 3, 10 and 30 μM (Fig. 5B).

3.2.4. Sodium nitroprusside and isradipine

Pretreatment with either isradipine 1 or 3 nM neither potentiated the relaxant effect of any concentration of sodium nitroprusside studied nor caused any alteration in the pharmacodynamic parameters for these combinations compared to data for expected additive interaction (Fig. 5C and Table 3). The concentration-effect curve for the combination of isradipine 10 nM with sodium nitroprusside was significantly leftward displaced compared to the curve for expected additive interaction, but isradipine 10 nM did not significantly potentiate the relaxation produced by any concentration of sodium nitroprusside (Table 3 and Fig. 5C).

4. Discussion

Only few in vitro studies dealing with the pharmacodynamic effects of airway smooth muscle relaxant combinations in isolated airway tissue have been reported. In some of these studies interaction is defined as additive when the effect of the combination equals the sum of the effects produced by each relaxant alone. This view is not generally valid and is based on an assumption of linear concentration-effect relationship (Berenbaum, 1989). Other authors (Taylor, 1987; Löfdahl et al., 1985) have used the 'isobole method' or an equivalent method (Pöch and Holzmann, 1980), or have investigated the effect of one airway smooth muscle relaxant in the presence of a sub-relaxant concentration of another (Sarria et al., 1994; Shikada and Tanaka, 1992). To our knowledge this is the first study systematically investigating the pharmacodynamic interaction between five different classes of airway smooth muscle relaxants. In order to validate our new methodological technique combining the isobolic method with iterative non-linear regression analysis of experimental data whereby predictions of additive functional parameters were obtained we investigated the pharmacodynamic interaction between two selective β_2-adrenoceptor agonists, terbutaline and salbutamol, which was expected to be additive. Fig. 2B and the functional pharmacodynamic parameters stated in Table 2 clearly demonstrate an additive interaction between these two drugs.

In addition to additive interaction with salbutamol, terbutaline showed synergistic interaction with all other drugs investigated. Synergistic interaction between terbutaline and theophylline is in agreement with a previous report (Taylor, 1987). This interaction is presumably due to inhibition of cAMP breakdown through nonselective phosphodiesterase (PDE) isoenzyme inhibition by theophylline (cf., Fig. 1) (Barnes and Pauwels, 1994), although other not yet identified molecular mechanisms involving increased intracellular Ca^{2+} sequestration may underlie the action of theophylline against various bronchoconstrictor stimuli (Weinberger and Hendeles, 1996). The synergism was first evident at the two highest concentrations of theophylline studied (70 μM and 200 μM) which could be explained by the finding that theophylline is a rather weak inhibitor of these PDE isoenzymes (Raeburn and Advenier, 1995).

In contrast to previously reported experiments with only one sub-relaxant concentration of cromakalim that produced no synergistic effect (Shikada and Tanaka, 1992), our study showed that terbutaline and cromakalim displayed clear synergistic interaction. This could in theory be caused by phosphodiesterase inhibition by cromakalim, but guinea-pig tracheal cAMP phosphodiesterase is only inhibited by cromakalim concentrations greater than 0.1 mM, and at these concentrations inhibition is still less than that produced by equivalent concentrations of theophylline (Berry et al., 1991). Interaction at different K^+ channels seems more likely. Hyperpolarization through activation of large-conductance calcium-activated K^+ channels (BK_{Ca}) is now an established cellular action of β_2-adrenoceptor agonists (cf., Fig. 1), although its functional importance to smooth muscle relaxation is still controversial (Kotlikoff and Kamm, 1996). However, the definite opening of ATP-sensitive K^+ channels (K_{ATP}) by cromakalim (cf., Fig. 1) could presumably augment BK_{Ca}-induced hyperpolarization caused by terbutaline thereby making it of functional importance.

The finding of a synergistic interaction between terbutaline and sodium nitroprusside has not previously been reported in isolated airway tissue. Interaction between different isoenzyme-selective PDE inhibitors in guinea-pig trachea (Turner et al., 1994) and between isoprenaline and nicorandil (Satake et al., 1995) or NO donors (Maurice et al., 1991) in aortic smooth muscle indicates an inhibitory action of intracellular cGMP on cAMP-specific PDE isoenzyme type III (PDE III) (cf., Fig. 1). A similar mechanism most likely underlies the observed synergistic interaction between terbutaline and sodium nitroprusside.

Isradipine in all three concentrations studied potentiated the relaxant action of terbutaline. Although dihydropyridine Ca^{2+}-channel blockers are considered poor tracheal relaxants (Knox and Tattersfield, 1995) because agonist-induced airway smooth muscle contraction is more dependent on Ca^{2+} release from intracellular stores than on Ca^{2+} influx through voltage-gated Ca^{2+} channels, our findings are consistent with some functional importance of dihydropyridine-sensitive Ca^{2+} channels (cf., Fig. 1). Another mechanism could, however, be involved. Both nicardipine and nifedipine produce synergistic interactions with isoprenaline and the adenylate cyclase activator forskolin in human and guinea-pig airways in vitro, and inhibition of PDE III and PDE IV may be possible mechanisms (Sarria et al., 1994).

Theophylline displayed synergistic interaction with cro-

makalim and isradipine, but only additive interaction with sodium nitroprusside. As seen with terbutaline and cromakalim a synergistic interaction between theophylline and cromakalim could be expected since tracheal relaxation induced by theophylline, like that induced by β_2-adrenoceptor agonists, involves activation of BK_{Ca} (Jones et al., 1990). Although the functional importance of BK_{Ca} activation by theophylline seems less than that involved in β_2-adrenoceptor agonist-induced relaxation (Jones et al., 1990) an interaction at different K^+ channels (BK_{Ca} and K_{ATP}) could explain the observed synergistic interaction. The weak phosphodiesterase inhibition produced by theophylline and the relatively little functional importance of cGMP-specific phosphodiesterase (PDE V) in guinea-pig trachea (Raeburn and Advenier, 1995) are possible explanations for the observed additive interaction between theophylline and sodium nitroprusside. Synergistic interaction has, however, been reported (Sarria et al., 1994). Since interaction between two non-selective cAMP phosphodiesterase inhibitors is expected to be additive, the observed synergistic interaction between theophylline and all three concentrations of isradipine is in disagreement with the postulated phosphodiesterase inhibition by Ca^{2+}-channel blockers (Sarria et al., 1994) and this points at a functional importance of dihydropyridine-sensitive Ca^{2+} channels.

In addition to the interactions discussed above, cromakalim showed significant synergistic interaction with sodium nitroprusside and additive interaction with isradipine. The interaction with sodium nitroprusside has been shown to be dependent on activation of K_{ATP} located in the tracheal epithelium (Shikada and Tanaka, 1992), and interaction at different K^+ channels, as suggested for the interaction between cromakalim and terbutaline or theophylline, is plausible since sodium nitroprusside-induced tracheal relaxation also involves activation of BK_{Ca} (Jones et al., 1990). The additive interaction with isradipine further supports functional importance of dihydropyridine-sensitive Ca^{2+} channels since hyperpolarization brought about by activation of K_{ATP} reduces Ca^{2+} influx through voltage-operated Ca^{2+} channels (VOC; cf., Fig. 1), some of them being sensitive to dihydropyridines (Small et al., 1993).

In vivo studies discriminating between additive or synergistic pharmacodynamic interactions of airway smooth muscle relaxants are sparse. Both additive (Chow and Fung, 1989; Leopold and Handslip, 1979) and synergistic (Laursen et al., 1985; Wolfe et al., 1978) interactions between β_2-adrenoceptor agonists and methylxanthines have been reported. This discrepancy can be explained by in vivo findings in animals that the type of interaction depends not only on the doses of airway smooth muscle relaxants but also on the site and degree of airway obstruction (Salonen, 1985). Also the dihydropyridine Ca^{2+}-channel blockers and β_2-adrenoceptor agonists have been shown to exhibit synergistic interaction in vivo (Sharma et al., 1990; Lever et al., 1984).

This study comprises a systematic and quantitative evaluation of pharmacodynamic interactions between five different airway smooth muscle relaxants in vitro obtained by using a new methodological technique. Possible cellular mechanisms underlying these interactions have been discussed on basis of existing knowledge of molecular drug actions. Other in vitro receptor studies are required to depict the exact mechanisms responsible for the interactive effects observed. Results of the study may provide a basis for future in vivo investigations on the effects and clinical usefulness of new bronchodilator combinations.

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