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Mechanisms of relaxations of bovine isolated bronchioles by the nitric oxide donor, GEA 3175

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1 The present study was designed to investigate the effects and mechanisms of relaxation induced by the nitric oxide (NO) donor, GEA 3175 (a 3-aryl-substituted oxatriazole derivative) on bovine bronchioles (effective lumen diameter 200–800 μm) suspended in microvascular myographs for isometric tension recording.

2 In segments of bovine bronchioles contracted to 5-hydroxytryptamine, GEA 3175 (10^{-8}–10^{-4} M) induced concentration-dependent reproducible relaxations. These relaxations were slow in onset compared to other NO-donors such as 3-morpholinosydonimine-hydrochloride (SIN-1) and S-nitroso-N-acetylpenicillamine (SNAP).

3 In 5-hydroxytryptamine-contracted preparations the order of relaxant potency (pD_2) was: salbutamol (7.80) > GEA 3175 (6.18) > SIN-1 (4.90) > SNAP (3.55). In segments contracted to acetylcholine, the relaxant responses were reduced and GEA 3175 relaxed the bronchioles with $pD_2 = 4.41 \pm 0.12$ and relaxations of $66 \pm 10\%$ ($n = 4$), while SNAP and salbutamol caused relaxations of $19 \pm 6\%$ ($n = 4$) and $27 \pm 6\%$ ($n = 8$) at the highest concentration used, respectively.

4 Oxyhaemoglobin (10^{-5} M), the scavenger of nitric oxide, caused rightward shifts of the concentration-relaxation curves to GEA 3175 and NO. 1H-[1,2,4]oxadiazolo[4,3,-a]quinoxalin-1-one (ODQ, 3×10^{-6} M) and LY 83583 (10^{-6} M), the inhibitors of soluble guanylate cyclase, also reduced the relaxations induced by GEA 3175 and nitric oxide. However, ODQ did not affect salbutamol-evoked relaxation in the bovine small bronchioles.

5 GEA 3175-induced relaxations were reduced in potassium-rich (60 mmol l^{-1} K^+) solution. Glibenclamide (10^{-6} M) markedly inhibited the relaxations induced by the opener of ATP-sensitive K^+ channels, levcromakalim (3×10^{-8}–10^{-5} M), but it did not modify the relaxations induced by GEA 3175 or salbutamol. Apamin (5×10^{-7} M), a blocker of the small Ca^{2+}-activated K^+-channels did not affect the relaxations to GEA 3175. In contrast, blockers of large Ca^{2+}-activated K^+-channels, charybdotoxin (3×10^{-8}–10^{-7} M) and iberiotoxin (10^{-8} M), did inhibit the relaxations to GEA 3175. The combination of apamin and charybdotoxin did not induce an additional inhibitory effect on the relaxations to GEA 3175 compared to charybdotoxin alone.

6 In preparations where a concentration-response curve to GEA 3175 or NO was first obtained in the presence of LY 83583, incubation with charybdotoxin (10^{-7} M) did produce an additional inhibitory effect of the relaxations. However, in the presence of ODQ (3×10^{-6} M), iberiotoxin (10^{-8} M) did not produce additional reduction of the NO- or GEA 3175-induced relaxations.

7 The present results suggest that the slow-releasing NO-donor GEA 3175 is more potent than the traditional NO donors in inducing relaxations of bovine bronchioles. GEA 3175, as for exogenously added NO, elicits relaxations through a cyclic GMP-dependent mechanism followed by opening of large conductance Ca^{2+}-activated K^+-channels.

Keywords: Bovine bronchioles; GEA 3175; nitric oxide; K^+-channels; ODQ; iberiotoxin; charybdotoxin; glibenclamide; apamin

Introduction

Inhaled NO produces bronchodilatation and protects against cholinergic and histaminergic bronchoconstriction in several animal species (Dupuy *et al*., 1992; Lindeman *et al*., 1995) and man (Ricciardolo *et al*., 1996), but induces only a variable bronchodilatation in asthmatic patients (Högman *et al*., 1993). Moreover, NO-releasing compounds such as nitrates and nitrosothiols relax human airways *in vitro* (Gruetter *et al*., 1989), but exert little bronchodilator or protective effect in asthma (Okayama *et al*., 1989; Barnes & Belvisi, 1993). This lack of bronchodilatation could be due to spontaneous release of NO, short half life and development of nitrate tolerance (Needleman & Johnson, 1973). Therefore, compounds not inducing nitrate tolerance and with a longer duration than classic organic nitrate esters have been developed (Maragos *et al*., 1991; Salas *et al*., 1994; Ferioli *et al*., 1995; Kankaanranta *et al*., 1996). However, the effect on small airways of slow-releasing NO-donors has so far not been evaluated.

NO, either derived from nitrergic nerves (Kannan & Johnson, 1995; Ward *et al*., 1995) or released from an exogenously added NO donor (Bialecki & Stinson-Fisher, 1995), was shown to relax bronchial strips either through a guanylate cyclase-dependent pathway or through opening of large conductance Ca^{2+}-activated K^+-channels. In vascular and colon smooth muscle, the hyperpolarization observed to NO and nitrovasodilators was also shown to be mediated by Ca^{2+}-activated K^+-channels either through a cyclic GMP-dependent or -independent pathway (Robertson *et al*., 1993; Bolotina *et al*., 1994; Koh *et al*., 1995). Thus, the mechanisms by which NO causes relaxation of airway smooth muscle remain to be clarified.

Mesoionic 3-aryl substituted oxatriazole 5-imino derivatives constitute a class of NO donors which are stable and slowly

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release NO and increase cyclic GMP content in guinea-pig trachea and human platelets (Corell *et al*., 1994; Kankaanranta *et al*., 1996). The aim of the present study was to evaluate the relaxing properties of the 3-aryl substituted oxatriazole-5-imine derivative GEA 3175 in bovine peripheral airways, and to compare the effects of this compound with those of two well-characterized NO donors, S-nitroso-N-acetylpenicillamine (SNAP) and morpholino-sydnonimine (SIN-1), and with the β_2-adrenoceptor bronchodilator, salbutamol, which is used in the treatment of bronchospasm.

Methods

Adult cattle of either sex with no lesions in their respiratory tract were selected from the slaughterhouse. Apices of the lungs were removed immediately after the animals were killed and transported to the laboratory in cold (4°C) Ca^{2+}-free physiological salt solution (PSS, see composition below). They were kept in Ca^{2+}-free solution to avoid development of spontaneous tension before mounting.

Experimental procedure

Bovine bronchioles with an internal lumen diameter of 200–800 μm were carefully dissected removing the adhering pulmonary parenchymal tissue and bronchiolar artery. Segments of the bronchioles (2 mm long) were mounted on two wires with a diameter of 40 μm in microvascular myographs for isometric tension recording (Mulvany & Halpern, 1976). One wire was fixed to an isometric transducer and the other to a displacement unit permitting control of the internal circumference of the preparations. The organ bath contained PSS gassed with 5% CO_2 in O_2 giving a final pH of 7.4. The bovine bronchiolar segments were allowed to equilibrate in PSS, 37°C, adjusted to 3.5–4 mN of passive tension depending on segment size during a period of 30–45 min. The viability of the preparations was examined by exposing them twice to potassium-rich PSS (KPSS), which was PSS with KCl exchanged for NaCl on an equimolar basis giving a final concentration of 125 mmol l^{-1} K^+. The rest of the protocol was performed in normal PSS containing the cyclo-oxygenase inhibitor, indomethacin (3×10^{-6} M), with the aim of preventing the development of basal spontaneous tonus due to prostaglandins.

The bronchioles were contracted with 5-hydroxytryptamine (10^{-6} M), histamine (10^{-5} M), acetylcholine (10^{-5} M) or K^+-PSS (60 mM) and, when the tone was stable, concentration-relaxation curves were obtained to the nitric oxide donor 3-aryl-substituted oxatriazole derivative (GEA 3175, 10^{-8}–10^{-4} M), 3-morpholinosydonimine-hydrochloride (SIN-1, 10^{-7}–10^{-4} M) and S-nitroso-N-acetylpenicillamine (SNAP, 10^{-8}–10^{-3} M), and to the β_2-adrenoceptor agonist, salbutamol (10^{-11}–10^{-5} M) or to levcromakalim (10^{-8}–10^{-5} M).

The mechanisms involved in the GEA 3175-induced relaxations were investigated in preparations contracted with 5-hydroxytryptamine (10^{-6} M). A first concentration-response curve was constructed, the bath solution was changed several times, and the preparations were allowed to equilibrate for 30 min before they were incubated with either superoxide dismutase (SOD, 100 u ml^{-1}), oxyhaemoglobin (10^{-5} M), the guanylate cyclase enzyme inhibitors, methylene blue (10^{-5} M), ODQ (3×10^{-6} M) or LY 83583 (10^{-6} M), the blocker of ATP-sensitive K^+-channels, glibenclamide (10^{-6} M) or the small and large conductance Ca^{2+}-activated K^+ channels blockers, apamin (5×10^{-7} M) and charybdotoxin (3×10^{-8}–10^{-7} M) or iberiotoxin (10^{-8} M), respectively. The preparations were incubated with the respective drugs for 30 min before they were contracted to 5-hydroxytryptamine and a second concentration-response curve was obtained. The effect of GEA 3175 in the absence and presence of the respective blocker was tested in the same preparation. The stability of responses to GEA 3175 was checked by the use of time-matched control tissues that were not exposed to modifying agents.

Drugs and solutions

The bronchioles were dissected, mounted, and if not indicated otherwise, held relaxed in PSS of the following composition (mmol l^{-1}): NaCl 119, KCl 4.7, $MgSO_4$ 1.17, $NaHCO_3$ 25, KH_2PO_4 1.18, glucose 5.5, $CaCl_2$ 2.5 and ethylenediaminetetraacetic acid (EDTA) 0.026. Ca^{2+}-free solution had the same composition as PSS except that $CaCl_2$ was replaced with EGTA (0.1 mM). KPSS was PSS in which NaCl was exchanged for KCl on an equimolar basis.

The following drugs were used: acetylcholine hydrochloride, apamin, charybdotoxin, glibenclamide, histamine dihydrochloride, iberiotoxin, indomethacin, 5-hydroxytryptamine hydrochloride, salbutamol hemisulphate, superoxide dismutase phosphate (SOD) were obtained from Sigma (U.S.A.); 3-morpholinosydnonimine hydrochloride (SIN-1) and S-nitroso-N-acetylpenicillamine (SNAP) from GEA Ltd. (Copenhagen, Denmark); 1H - [1,2,4] - oxadiazolo [4,3, - a] quinoxalin - 1 - one (ODQ) was supplied by Tocris Cookson (MO, U.S.A.); levcromakalim was a gift from Smith Kline Beecham (U.K.) (6-anilino-5,8-quinol-inedione) LY 83583 was obtained from Lilly (France). Indomethacin and levcromakalim were dissolved in 96% and 70% ethanol, respectively. GEA 3175 (1,2,3,4-oxatriazolium, 3-(3-chloro-2-methylphenyl)-5-[[(4-methylphenyl)sulphonyl]amino]-)hydroxide), ODQ and glibenclamide were dissolved in dimethyl sulphoxide (DMSO). The other compounds were dissolved in distilled water. The solvents, ethanol and DMSO, did not in the concentrations used influence the contractile state of the preparations.

NO solutions were prepared by a modification of the method described by Garland & McPherson (1992). Sealed vials containing 20 ml of twice distilled water at room temperature (20°C) were bubbled with nitrogen for 60 min. Then one of the vials was exposed to a stream of NO for 5 min, enough to produce a saturated solution. The resulting NO concentration of the saturated solution was calculated from the solubility constant for NO in water at 1 atm and 20°C (4.6 ml 100 ml^{-1} H_2O) giving a final concentration of 1.9 mM. Gas-tight syringes were used to apply NO to the organ baths. The pH in the organ bath was controlled when adding NO, but only changed (from 7.4 to 7.3) at concentrations of NO exceeding 3×10^{-5} M.

Oxyhaemoglobin (oxyHb) was prepared from 1 mM solution of commercial haemoglobin (bovine haemoglobin, Sigma) by addition of 10 mM sodium dithionite ($Na_2S_2O_4$, Sigma) as previously described (Martin *et al*., 1985). $Na_2S_2O_4$ was removed by dialysis in distilled water that was gassed with N_2 at 4°C. The purity of the solutions of oxyhaemoglobin was determined spectrophotometrically giving a final concentration of $7–8 \times 10^{-4}$ M.

Calculations

The mechanical responses were measured as force and expressed as wall tension ΔT, which is the increase in measured force, ΔF, divided by twice the segment length (Mulvany & Halpern, 1976). Relaxations are expressed as a percentage of the response to the preconstrictor just before construction of the concentration-relaxation curves. For each concentration-

response curve, the concentration in the absence or presence of blocking agent required to give half-maximal relaxation (EC_{50}) was determined by a computer programme (Graph Pad 4.1, San Diego, California, U.S.A.) fitting the responses and logarithmic concentrations to the Hill equation: $E/E_{max} = A(M)^{n_H}/(A(M)^{n_H} + EC_{50}(M)^{n_H})$, where E/E_{max} is the relative response to the effective concentration of drug, A(M), E_{max} is the maximal response, EC_{50} (M) is the concentration of agonist required to give half-maximal response, when A(M) and EC_{50}(M) are given in molar concentrations, and n_H is a curve fitting parameter or Hill coefficient. The sensitivity and maximal relaxations of the drugs are expressed in terms of pD_2 and E_{max}, respectively, where pD_2 is defined as the negative logarithm of EC_{50} ($pD_2 = -\log EC_{50}(M)$).

Histology

After completion of some of the concentration-response studies, the bath solution was changed to Ca^{2+}-free PSS, and the tissues were prefixed (2% glutaraldehyde in Sørensen buffer, pH = 7.4, Mulvany & Korsgaard, 1983) while still in the myograph. The preparations were demounted after at least 2 h on the myograph and kept in prefix (4°C) until the remaining fixation procedure could be carried out. The segments were processed and embedded in paraffin and cut transversely on a microtome in 10 μm sections, stained with Giemsa, and examined in a light microscope.

Statistics

The results are expressed as mean ± s.e.mean. The significance of differences between means were assessed by a paired *t* test. When several treatments were compared to the same control or responses of more than two drugs were compared, the results were analysed according to one-way analysis of variance (ANOVA) and Bonferroni method as an *a posterio* test (Wallenstein *et al*., 1980). Probability levels less than 5% were considered significant.

Results

Histology

Transverse sections of the bovine small bronchioles ($n = 4$) showed that the epithelium represented a major component. In the media, sparse circularly-oriented smooth muscle cells were observed. In bronchioles with an internal diameter less than 500 μm neither longitudinally orientated smooth muscle, cartilage nor glands were observed. In bronchioles with a diameter of 500 – 800 μm, small islets of cartilage were observed in the outer adventitia, but no glands were found.

Functional responses

After equilibration of the bovine bronchioles to a passive tension of 4.6 ± 0.7 Nm^{-1} ($n = 66$) in Ca^{2+}-free PSS, changing the bathing solution to Ca^{2+}-containing PSS evoked an increase in resting tone of the preparations of 3.2 ± 0.7 Nm^{-1} ($n = 66$). KPSS (120 mM) further contracted the preparations by 10.4 ± 1.0 Nm^{-1} ($n = 66$). 5-Hydroxytryptamine (5-HT, 10^{-6} M) induced contractions in bovine small bronchioles of 4.8 ± 0.7 Nm^{-1} ($n = 66$) corresponding to 46.1 ± 7.2% of the contraction induced by KPSS.

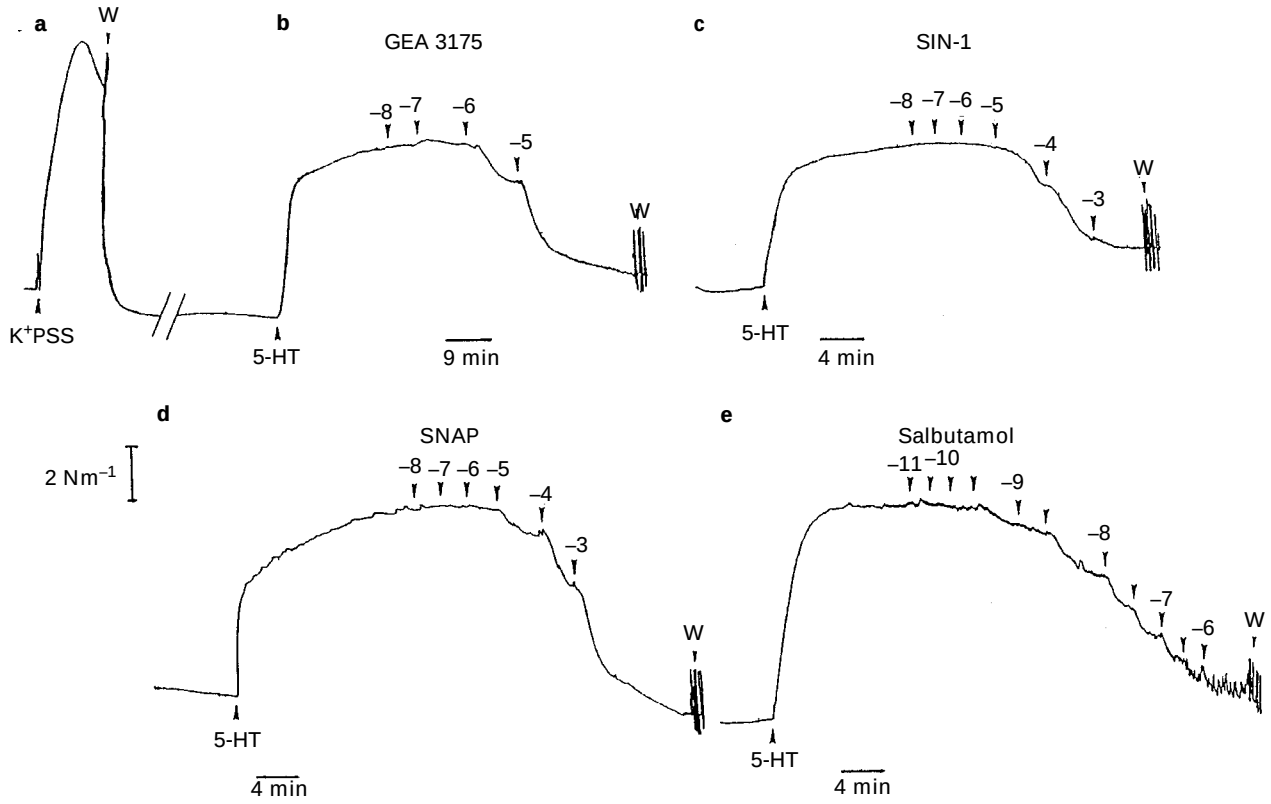

Figure 1 Isometric force recordings showing the contractile response (a) to 120 mM K^+-PSS, and relaxations to (b) GEA 3175 ($10^{-8} - 10^{-5}$ M), (c) SIN-1 ($10^{-8} - 10^{-3}$ M), (d) SNAP ($10^{-8} - 10^{-3}$ M), and (e) the β_2-adrenoceptor agonist, salbutamol ($10^{-11} - 10^{-6}$ M) in 5-hydroxytryptamine (5-HT, 10^{-6} M)-contracted bovine bronchiole with an effective lumen diameter of 541 μm. Vertical bar shows tension in Nm^{-1} and horizontal bar time in min. W: wash out.

GEA 3175 reduced the resting tension by 0.6 ± 0.1 Nm^{-1} with pD_2 of 4.84 ± 0.27 in 11 preparations, while it did not affect the resting tension in 3 preparations. In preparations where tone was raised with 5-HT, GEA 3175 caused relaxations of $97.2 \pm 2.3\%$ with a pD_2 value of 6.18 ± 0.07 ($n = 66$). The relaxations to GEA 3175 (10^{-7}–10^{-4} M) were slow reaching a plateau after 3–4 min, in contrast, to relaxations of the other NO-donors SIN-1 and SNAP, where a plateau was reached after 20–30 s (Figure 1). However, in the 5-HT-contracted preparations, GEA 3175 was more potent than SIN-1 and SNAP. GEA 3175, SIN-1 (10^{-8}–10^{-3} M) and SNAP (10^{-8}–10^{-3} M), and the β_2-adrenoceptor agonist, salbutamol (10^{-11}–10^{-6} M) induced concentration-dependent relaxations with the following rank order of potency: salbutamol > GEA 3175 > SIN-1 > SNAP (Figure 2a). There were no significant differences in maximum responses to the drugs. The relaxations to GEA 3175, salbutamol and SNAP were also dependent on the agonist used as constrictor, since they were more potent against 10^{-6} M 5-HT than against 10^{-5} M acetylcholine (Figure 2b, Table 1). Furthermore, GEA 3175 relaxed histamine (10^{-5} M)-contracted preparations with a pD_2 value of 5.69 ± 0.14 and, at the highest concentration applied (10^{-4} M), the relaxation evoked was equivalent to $84.8 \pm 4.7\%$ ($n = 4$) of the tone induced by histamine.

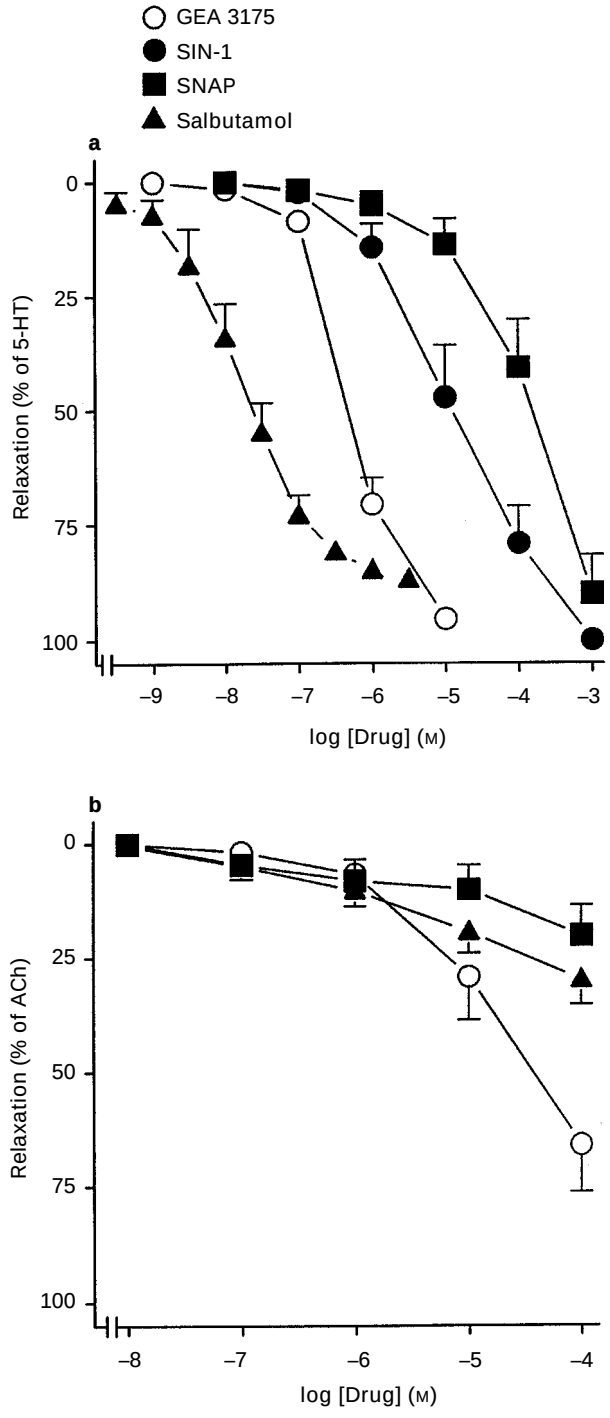

Figure 2 Log concentration-relaxation curves to GEA 3175, SIN-1, SNAP and salbutamol in bovine small bronchioles contracted (a) to 5-hydroxytryptamine (5-HT, 10^{-6} M) or (b) acetylcholine (ACh, 10^{-5} M). Relaxations are expressed as a percentage of the agonist-induced contraction. Results represent means and vertical lines s.e.mean of 5–6 preparations.

Effect of superoxide dismutase, oxyhaemoglobin, methylene blue, ODQ and LY 83583 on the response to GEA 3175

In 5-HT-contracted bronchioles, GEA 3175 induced reproducible relaxations with pD_2 values and maximal relaxations of 6.32 ± 0.08 and $95.4 \pm 2.1\%$, and 6.42 ± 0.09 and $94.5 \pm 2.9\%$ ($n = 6$) in a first and second concentration-response curve, respectively.

The free radical scavenger, superoxide dismutase (SOD, 100 u ml^{-1}) did not change either the relaxations to GEA 3175 or those to SIN-1 in the bovine small bronchioles. Thus, the pD_2 values and maximal responses to GEA 3175 were 5.62 ± 0.08 and $100 \pm 0\%$, and 5.68 ± 0.17 and $95.5 \pm 1.9\%$ ($n = 7$) in the absence and presence of SOD, respectively. The pD_2 values and maximal responses to SIN-1 were 4.55 ± 0.31 and $72.3 \pm 3.1\%$, and 4.50 ± 0.39 and $68.7 \pm 4.9\%$ ($n = 6$) in the absence and presence of SOD, respectively.

Oxyhaemoglobin (10^{-5} M), a scavenger of nitric oxide, reduced the relaxations caused by GEA 3175 (Figure 3a) and NO (Figure 3b), but it did not modify the salbutamol-induced relaxation (Figure 3c, Table 2).

The inhibitor of NO-sensitive guanylate cyclase, methylene blue (10^{-5} M), increased resting tone (0.66 ± 0.16 Nm^{-1}, $n = 6$) of the bovine small bronchioles, but although 5-HT induced contractions in the presence of methylene blue, these were unstable and did not allow the construction of concentration-response curves for GEA 3175. Therefore, another guanylate cyclase inhibitor, LY 83583 (10^{-6} M), was applied. This compound at 10^{-6} M did not modify the basal tone of the bronchiolar smooth muscle, but significantly reduced relaxations to GEA 3175 and NO (Table 2). However, higher concentrations of LY 83583 (10^{-5} M) were also associated with unstable 5-HT-induced contractions. ODQ, considered a more selective inhibitor of guanylate cyclase, did not change resting tension, 5-HT-induced contractions or relaxations caused by salbutamol, but significantly reduced relaxations evoked by GEA 3175 or NO (Figure 3, Table 2).

Effects of glibenclamide, apamin, charybdotoxin and iberiotoxin on relaxations evoked by GEA 3175

Sustained contractions to 60 mmol l^{-1} KPSS could only be obtained in the presence of the nitric oxide synthase inhibitor, N^G-nitro-L-arginine (L-NOARG, 10^{-4} M), and therefore this inhibitor was present throughout experiments where the relaxations to GEA 3175 on contractions to 5-hydroxytryptamine and KPSS were compared, L-NOARG increased the contractions evoked by 5-hydroxytryptamine and the concentration applied had to be reduced to 5×10^{-7} M to obtain

contractions comparable to those in untreated preparations. In 5-HT contracted preparations, the relaxations to GEA 3175 were similar in the absence and presence of L-NOARG, However, GEA 3175 was less potent in relaxing KPSS-contracted preparations compared with 5-HT-contracted preparations. Thus, after the addition of 5-HT or 60 mmol l^{-1} KPSS as contractile agonist, the pD_2 values and relaxations obtained to the highest concentration (10^{-4} M) of GEA 3175 were 5.96 ± 0.08 and $98.5 \pm 1.1\%$, and 4.02 ± 0.17 ($P<0.05$) and $49.4 \pm 3.7\%$ ($P<0.05$, paired t test, $n=4$), respectively.

Table 1 Relaxations induced by GEA 3175, SIN-1, SNAP or salbutamol in bovine small bronchioles contracted to a comparable level with either 5-hydroxytryptamine (5-HT, 3×10^{-7}–10^{-6} M) or acetylcholine (Ach, 3×10^{-6}–10^{-5} M)

	n	*Precontraction* (Nm^{-1})	pD_2	R (%)
5-HT contracted				
GEA 3175	11	4.2 ± 0.9	6.24 ± 0.14	98.3 ± 4.4
SIN-1	6	2.4 ± 1.7	4.90 ± 0.27^{a}	100.0 ± 0.0
SNAP	6	2.5 ± 1.7	3.55 ± 0.24^{ab}	90.0 ± 8.3
Salbutamol	4	3.3 ± 0.7	7.80 ± 0.16^{abc}	87.0 ± 1.0
ACh contracted				
GEA 3175	4	2.9 ± 0.5	- - - - - - -	66.0 ± 10.2*
SNAP	4	3.7 ± 0.8	- - - - - - -	18.8 ± 6.0*^{a}
Salbutamol	8	3.4 ± 0.8	- - - - - - -	27.0 ± 6.3*^{a}

Values are mean $\pm$ s.e.mean of n number of preparations examined. *$P<0.05$, parameter significantly different compared with those obtained with the same agonist in 5-HT-contracted preparations (t test). Differences were evaluated by one-way analysis of variance (ANOVA) followed by *a posterio* Bonferroni t test in case of significance: $^{a}P<0.05$ compared to GEA 3175, $^{b}P<0.05$ compared to SIN-1, $^{c}P<0.05$ compared to SNAP. $pD_2 = -\log EC_{50}$, where EC_{50} is the concentration of agonist producing 50% of the maximum relaxation. R: relaxation obtained at the highest concentration applied of each drug: GEA 3175, 10^{-4} M; SIN-1, 10^{-3} M and salbutamol, 10^{-4} M.

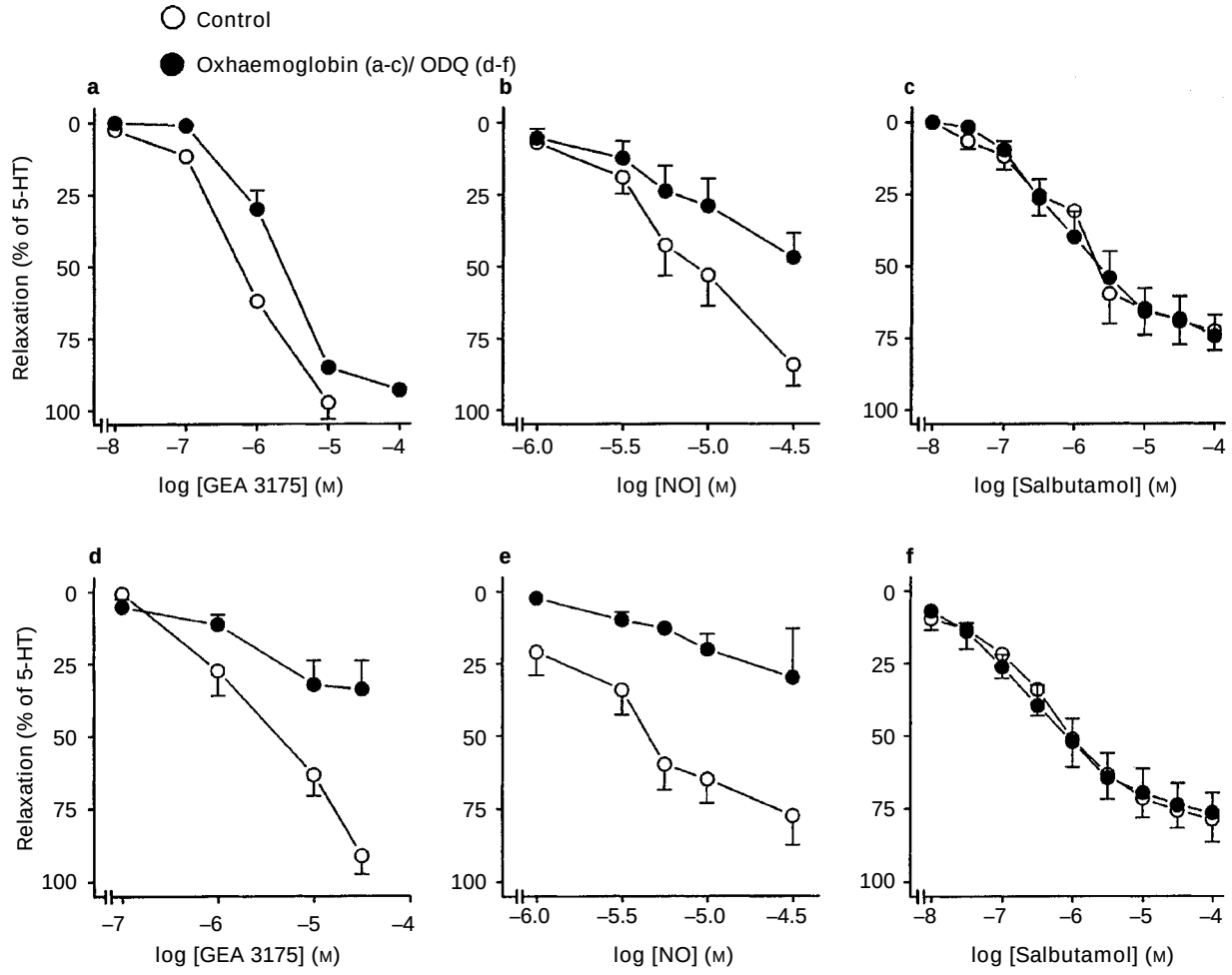

Figure 3 Log concentration-relaxation curves to (a,d) GEA 3175, (b,e) nitric oxide (NO) and (c,f) salbutamol in bovine small bronchioles contracted to 5-hydroxytryptamine (5-HT, 10^{-6} M) in control conditions and in the presence of oxyhaemoglobin (10^{-5} M) (a,b,c) or of ODQ (3×10^{-6} M) (d,e,f). The relaxations are expressed as a percentage of 5-hydroxytrypamine-induced contraction. Results represent means and vertical lines s.e.mean of 5–7 preparations.

Table 2 Effect of oxyhaemoglobin (10^{-5} M), LY 83583 (10^{-6} M), ODQ (3×10^{-6} M), charybdotoxin (ChTX, 10^{-7} M), iberiotoxin (IbTX, 10^{-8} M), LY 83583 plus ChTX, and ODQ plus IbTX on relaxations to GEA 3175 or exogenously added NO of bovine small bronchioles contracted to 5-HT (3×10^{-7}–10^{-6} M)

	GEA 3175			*NO*			*Salbutamol*		
	n	pD_2	R (%)	n	pD_2	R (%)	n	pD_2	R (%)
Control	6	6.20±0.05	97.3±5.8	8	5.12±0.14	84.3±7.2	12	5.98±0.18	72.7±6.7
OxyHb	6	5.76±0.09[a]	95.2±6.1	7	5.11±0.12	47.1±8.4[a]	9	5.83±0.19	74.3±7.2
Control	21	5.80±0.15	98.4±4.9	12	5.21±0.18	80.5±7.7	–	- - - - - - -	- - - - - - -
LY 83583	9	5.34±0.11[a]	76.5±5.0[a]	6	4.68±0.25	50.7±7.2[a]	–	- - - - - - -	- - - - - - -
ChTx	6	4.30±0.47[a]	53.2±13[a]	5	- - - - - - -	21.7±8.3[a]	–	- - - - - - -	- - - - - - -
LY 83583+ChTX	10	5.14±0.18[a]	46.0±7.4[a,b]	6	- - - - - - -	23.6±2.6[a,b]	–	- - - - - - -	- - - - - - -
Control	16	5.53±0.11	94.5±4.2	7	5.46±0.17	77.5±9.9	10	6.48±0.29	77.1±8.3
ODQ	7	5.48±0.13	34.0±9.8[a]	6	- - - - - - -	30.0±16.9[a]	4	6.40±0.23	76.5±6.8
IbTX	6	5.36±0.06	50.9±7.8[a]	–	- - - - - - -	- - - - - - -	6	6.49±0.17	75.9±8.7
ODQ+IbTX	6	4.98±0.11[a]	37.1±4.7[a]	6	- - - - - - -	38.8±15.1[a]	–	- - - - - - -	- - - - - - -

Values are mean±s.e.mean of n number of preparations examined. Differences were evaluated by one-way analysis of variance (ANOVA) followed by *a posterio* Bonferroni t test in the case of significance: [a]$P<0.05$ compared to control, [b]$P<0.05$ compared to relaxations in the presence of LY83583 or ODQ, respectively. $pD_2=-\log EC_{50}$, where EC_{50} is the concentration of the agonist producing 50% of the maximum relaxation. R: relaxation obtained at the highest concentration used: GEA 3175, 10^{-4} M; NO, 3×10^{-5} M and salbutamol 10^{-4} M.

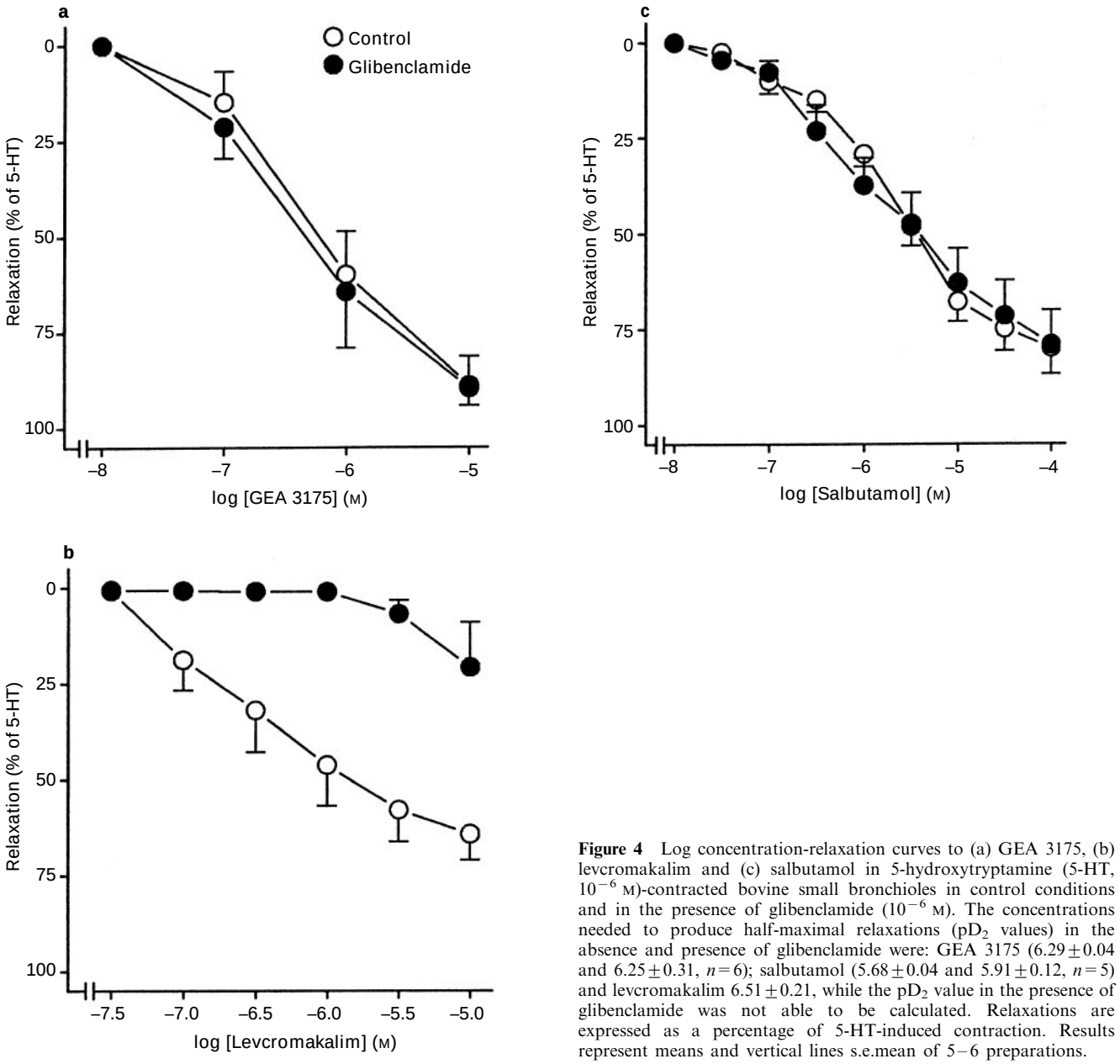

Figure 4 Log concentration-relaxation curves to (a) GEA 3175, (b) levcromakalim and (c) salbutamol in 5-hydroxytryptamine (5-HT, 10^{-6} M)-contracted bovine small bronchioles in control conditions and in the presence of glibenclamide (10^{-6} M). The concentrations needed to produce half-maximal relaxations (pD_2 values) in the absence and presence of glibenclamide were: GEA 3175 (6.29±0.04 and 6.25±0.31, $n=6$); salbutamol (5.68±0.04 and 5.91±0.12, $n=5$) and levcromakalim 6.51±0.21, while the pD_2 value in the presence of glibenclamide was not able to be calculated. Relaxations are expressed as a percentage of 5-HT-induced contraction. Results represent means and vertical lines s.e.mean of 5–6 preparations.

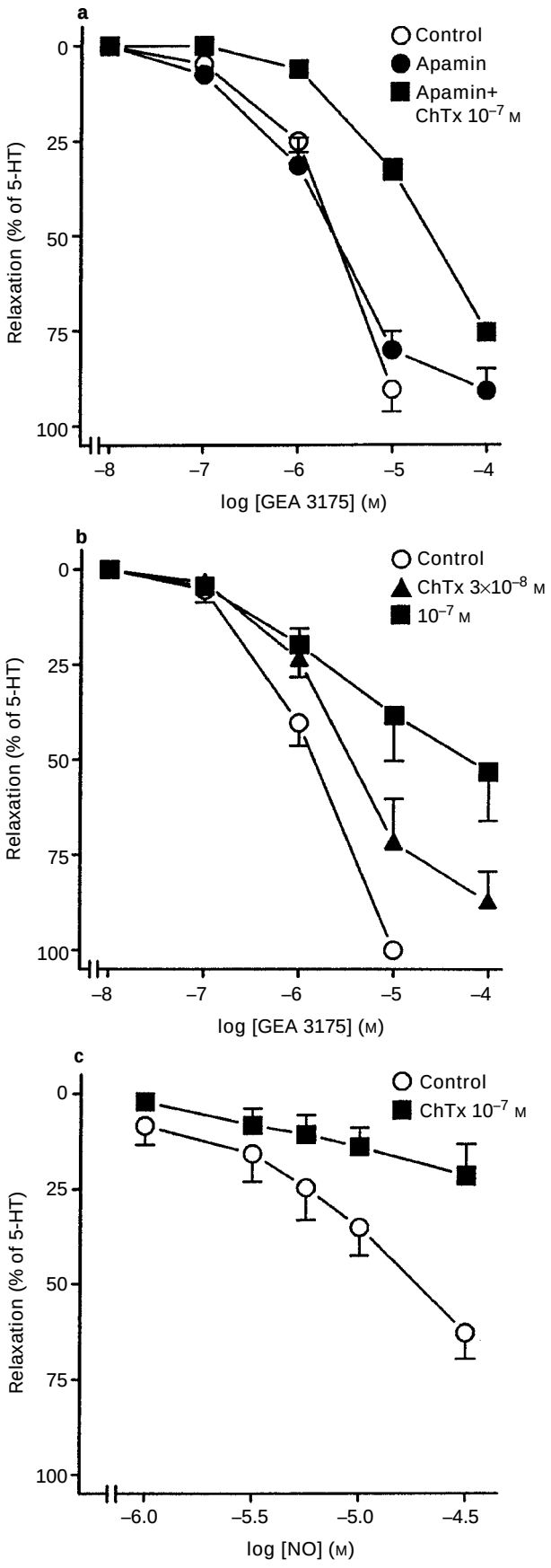

Figure 5 Log concentration-relaxation curves to (a,b) GEA 3175 and (c) nitric oxide (NO) in 5-hydroxytryptamine (5-HT, 10^{-6} M)-contracted bovine small bronchioles preparations in control conditions, and the presence of the inhibitor of (a) small conductance Ca^{2+}-activated K^+-channels, apamin (5×10^{-7} M), and apamin plus the inhibitor of large conductance Ca^{2+}-activated K^+ channels, charybdotoxin (ChTx, 10^{-7} M), (b) charybdotoxin (3×10^{-8}–10^{-7} M), and (c) charybdotoxin (10^{-7} M). Relaxations are expressed as a percentage of 5-HT-induced contractions. Results represent means and vertical lines s.e.mean of 6–15 preparations.

The ATP-sensitive K^+ channel blocker, glibenclamide (10^{-6} M), did not influence basal tension or the relaxations induced by GEA 3175 or salbutamol in bovine small bronchioles, but it reduced both the sensitivity and maximal relaxant effect induced by the opener of ATP-sensitive K^+ channels, levcromakalim (3×10^{-8}–10^{-5} M) (Figure 4).

Incubation of bronchiolar segments with the blocker of small Ca^{2+}-activated K^+ channels, apamin (5×10^{-7} M) did not change the concentration-response curves for GEA 3175. The pD_2 values and maximal relaxations of GEA 3175 were 5.69 ± 0.12 and $90.4\pm5.8\%$, and 5.46 ± 0.14 ($n=9$) and $90.8\pm5.8\%$ ($n=9$) in the absence and presence of 5×10^{-7} M apamin, respectively (Figure 5a). The inhibitors of large conductance Ca^{2+}-activated K^+ channels, charybdotoxin and iberiotoxin reduced the relaxations induced by both GEA 3175 and NO in the bovine small bronchioles. In contrast to iberiotoxin, charybdotoxin (3×10^{-8} M) increased both resting tension and contractions to 5-hydroxytryptamine, and the concentration of 5-hydroxytryptamine had to be reduced to 3×10^{-7} M to obtain precontracting levels comparable (3.6 ± 0.9 Nm^{-1}, $n=9$, in the presence of 10^{-7} M charybdotoxin) to those observed in the absence (3.8 ± 1.2 Nm^{-1}, $n=15$) of the toxin. Charybdotoxin concentration-dependently (3×10^{-8} and 10^{-7} M) reduced the relaxations to GEA 3175 in bovine small bronchioles contracted to 5-hydroxytryptamine (Figure 5b). GEA 3175 relaxed bovine small bronchioles with pD_2 values and effect at the highest concentration applied (10^{-4} M) of 5.92 ± 0.07 and $100\pm0\%$ in the absence of charybdotoxin, and with 5.46 ± 0.30 ($P<0.05$, $n=6$, Bonferroni t test) and $87.2\pm8.0\%$ in a second concentration-response curve in the presence of 3×10^{-8} M charybdotoxin (Figure 5b). Charybdotoxin 10^{-7} M induced additional rightward shifts of the concentration-response curves to GEA 3175 (Figure 5b, Table 2). A combination of apamin (5×10^{-7} M) and charybdotoxin (10^{-7} M) did not produce additional inhibition of the relaxations to GEA 3175 compared to charybdotoxin alone (Figure 5a). Charybdotoxin (10^{-7} M) also reduced the relaxation to NO. The pD_2 and effect at the highest concentration (3×10^{-5} M) applied in control conditions were 5.15 ± 0.11 and $63\pm7\%$, and in the presence of 10^{-7} M charybdotoxin $22\pm8\%$ ($P<0.05$, $n=5$, paired t test) (Figure 5c.) Iberiotoxin (10^{-8} M) reduced the relaxations to GEA 3175 without altering the salbutamol-induced relaxations (Figure 6, Table 2).

Incubation with both LY 83583 and 10^{-7} M charybdotoxin had an additional inhibitory effect on the relaxations to GEA 3175 or NO compared to LY 83583 alone (Table 2), but not when they were compared with the effect of charybdotoxin alone on these relaxations. Moreover, the treatment with ODQ (3×10^{-6} M) and iberiotoxin (10^{-8} M) did not exert an additional inhibitory effect with respect to those exhibited by ODQ or iberiotoxin alone on relaxations induced by GEA 3175 or NO (Figure 6a, Table 2).

Discussion

The main findings of the present study are that the 3-aryl-substituted oxatriazole derivative, GEA 3175, potently induces slowly developing relaxations and inhibits contractions to

several agonists in bovine small bronchioles. These relaxations appear to be guanylate cyclase-dependent and involve opening of large conductance Ca^{2+}-activated K^+ channels.

The preparations examined were isolated from the peripheral airways, which contain only few smooth muscle fibres. The orientation of the smooth muscle was circular making it suitable to suspend the preparations as rings in microvascular myographs, and the present approach thus constitutes a supplement to the perfusion technique in the study of small airways (Burgaud & Oudart, 1993).

Cholinergic nerves cause bronchoconstriction through activation of a variable portion of muscarinic receptors in the airways (Roffel *et al*., 1988; Yang *et al*., 1991). In proximal airways, β-adrenoceptor agonists induce less relaxation of acetylcholine-contracted preparations compared to preparations contracted to other agonists (Fernandes *et al*., 1992). In the present study, the β_2-adrenoceptor agonist, salbutamol, which is widely used in treatment of bronchospasm, relaxed 5-hydroxytryptamine-contracted bovine small bronchioles, but it had almost no effect in preparations contracted to acetylcholine although the contractions were matched to the same level. Thus, it appears that the functional antagonism exerted by muscarinic receptor activation against β-adrenoceptor relaxations is also present in small peripheral bronchioles.

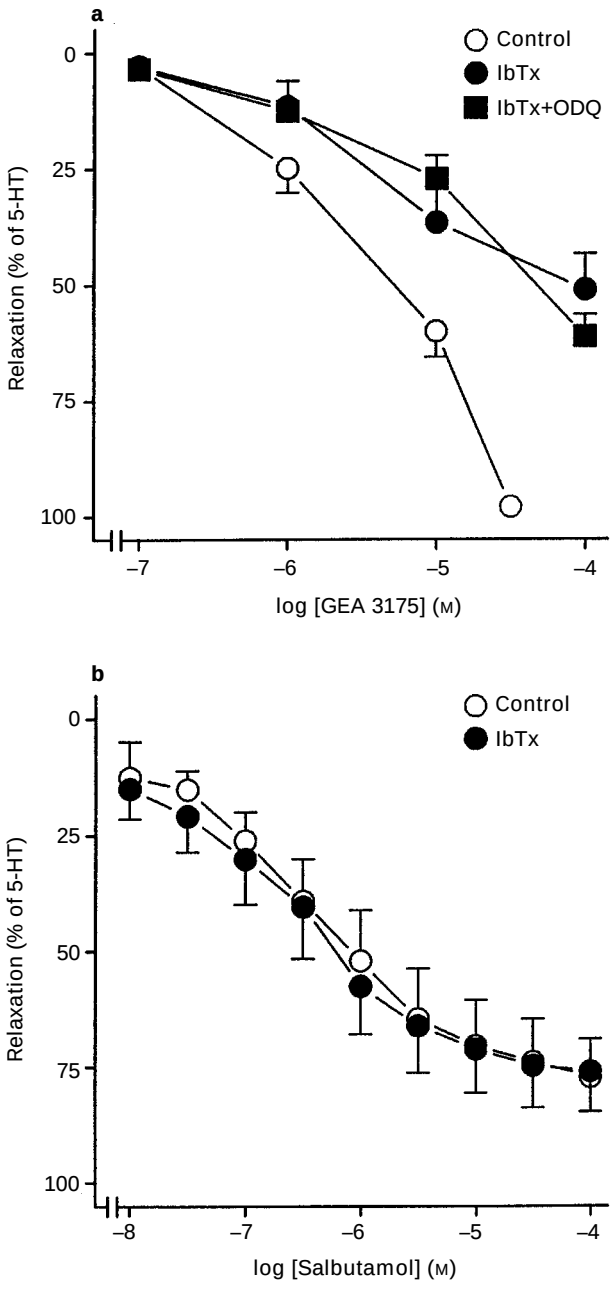

Figure 6 The relaxations to (a) GEA 3175 and (b) salbutamol in 5-hydroxytryptamine (5-HT, 10^{-6} M)-contracted bovine small bronchioles in control conditions, in the presence of iberiotoxin (IbTx, 10^{-8} M), and in the presence of both iberiotoxin (10^{-8} M) and ODQ (3×10^{-6} M). Relaxations are expressed as a percentage of 5-HT-induced contractions. Results represent mean and vertical lines s.e.mean of 4–6 preparations.

Cholinoceptor agonists might also be relatively resistant to the relaxant effects of NO-donors. Thus, Jansen and colleagues (1992) observed that nitroso-bronchodilators were more potent in relaxing guinea-pig bronchus contracted to leukotriene D_4 compared to methacholine-contracted preparations, and in guinea-pig tracheal rings GEA 3175 was also found to be less potent in relaxing preparations contracted to methacholine compared to histamine (Corell *et al*., 1994). In the present study, the relaxations to both SNAP and GEA 3175 were less pronounced against acetylcholine-induced contractions compared to either 5-HT- or histamine-induced contractions. Thus, it appears that acetylcholine is relatively resistant to the relaxant effects of both NO donors. However, in contrast to salbutamol, GEA 3175 still inhibited most of the contraction induced by acetylcholine suggesting the extent of cholinoceptor functional antagonism against GEA 3175 is less pronounced than against β-adrenoceptor agonists in bovine small bronchioles.

The short acting NO donors, like SIN-1 and SNAP, spontaneously break down and release NO and nitrite (NO_2^-) in aqueous solution (Feelisch *et al*., 1989; Kankaanranta *et al*., 1996). In contrast, no formation of nitrite was measured in phosphate buffer during a 40 min incubation period with GEA 3175, but GEA 3175 released large quantities of NO in human diluted plasma (Kankaanranta *et al*., 1996). It has been suggested that an enzymatic degradation of the sulphonamide moiety has to take place before NO can be released (Karup *et al*., 1994). Therefore, GEA 3175 probably needs to come in contact with the tissue to release NO and this could explain the observation that GEA 3175 was more potent than SIN-1 and SNAP in relaxing guinea-pig tracheal smooth muscle and rat bronchial smooth muscle (Corell *et al*., 1994; Paakkari *et al*., 1995). In the present study, GEA 3175 was more potent than SIN-1 and SNAP. The relaxations evoked by GEA 3175 were also slower to develop than those evoked by the short acting NO donors, SIN-1 and SNAP.

The effect of NO donors acting through sulphydryl groups of low molecular weight is less sensitive to NO-scavenging than NO donors releasing NO spontaneously in aqueous solution. Thus, it was observed in rabbit aorta rings that the furoxan derivatives which react with sulphydryl groups of low molecular weight thiols were less potent in the presence of the NO-scavenger oxyhaemoglobin, but the maximal relaxations were unaltered (Ferioli *et al*., 1995). Moreover, it was found that the formation of NO from the nitrosothiols were undetectable in the presence of the extracellular NO-scavenger, oxyhaemoglobin, but the nitrosothiols were still able to elicit bronchodilatation in the guinea-pig lung (Bannenberg *et al*., 1995). Thus, the rightward shifts of the relaxation curve to GEA 3175 and inhibition of NO-induced relaxations of bovine small bronchioles in the presence of oxyhaemoglobin provide support in favour of a NO-mediated action. However, it is not possible from these results to deduce whether the relaxations to GEA 3175 exclusively can be ascribed to release of NO.

GEA 3175 has been shown to increase cyclic GMP content of guinea-pig trachea and human platelets (Corell *et al*., 1994; Kankaanranta *et al*., 1996). Therefore, to investigate whether GEA 3175-induced relaxations of bovine small bronchioles were mediated through activation of guanylyl cyclase, the tissues were incubated with methylene blue. However, the effect of methylene blue could not be examined, as the tissues did not maintain their tone for a period long enough to construct a concentration-response curve for GEA 3175. A similar effect of methylene blue has been observed in pig tracheal smooth muscle (Kannan & Johnson, 1995). Therefore, another putative inhibitor of guanylyl cyclase, LY 83583 (Mülsch *et al*., 1988), was applied and it did cause significant inhibition of the relaxations to GEA 3175. Recently, 1H-[1,2,4]oxadiazolo[4,3,-a]quinoxalin-1-one (ODQ), was shown to be a more selective inhibitor of NO-stimulated guanylyl cyclase activity (Garthwaite *et al*., 1995; Brunner *et al*., 1996; Schrammel *et al*., 1996). In the bovine small bronchioles, ODQ inhibited the relaxations induced by GEA 3175 and NO, but did not alter the salbutamol-evoked relaxation. These results suggest that GEA 3175 induces relaxation of the bovine small bronchioles through activation of soluble guanylyl cyclase. However, neither LY 83583 nor ODQ caused total inhibition of the relaxations to GEA 3175 suggesting that an additional mechanism also contributes to these relaxations.

Recent studies have suggested that endogenously released NO and the NO donors SNAP and SIN-1 can induce relaxations of proximal airway smooth muscle through increases in cyclic GMP and opening of K^+-channels (Kannan & Johnson, 1995; Bialecki & Stinson-Fisher, 1995; Yamakage *et al*., 1996). GEA 3175 was less effective in relaxing bovine bronchiole rings contracted by increasing the extracellular K^+-concentration compared with preparations contracted by 5-HT. Such an effect could be ascribed to functional antagonism, since K^+-rich medium by causing membrane depolarization opens voltage-dependent Ca^{2+}-channels and enhances Ca^{2+}-influx, thereby interfering with the ability of relaxant agents such as β-adrenoceptor agonists to relax airway smooth muscle (Huang *et al*., 1993; Cook *et al*., 1995). The possibility that increasing the K^+ concentration is also associated with an increased release of excitatory neurotransmitters such as acetylcholine cannot be excluded. However, in our experiments where bovine bronchioles were contracted to the same level by K^+-rich medium or 5-HT, GEA 3175 caused less relaxation of the K^+-contracted preparations. Therefore, although K^+-rich medium can activate additional mechanisms (Huang *et al*., 1993; Cook *et al*., 1995), these findings suggest that the relaxations induced by GEA 3175 might be mediated through hyperpolarization and opening of K^+ channels.

The ATP-sensitive K^+ channel openers, cromakalim and pinacidil, have been demonstrated to relax guinea-pig and human isolated tracheal smooth muscle, and these relaxations were inhibited in the presence of a blocker of ATP-sensitive K^+-channels, glibenclamide (Mellemakjær *et al*., 1989; Longmore *et al*., 1991). Glibenclamide also inhibited the relaxations evoked by the selective opener of ATP-sensitive K^+-channels, levcromakalim, suggesting the presence of these channels in bovine small bronchioles. However, glibenclamide did not modify the relaxations evoked by GEA 3175 or by salbutamol. These results suggest that ATP-sensitive K^+ channels are not involved in the GEA 3175-induced relaxations of bovine small bronchioles.

Opening of Ca^{2+}-activated small conductance K^+ channels has earlier been described to mediate the hyperpolarizations and relaxations to nitrovasodilators in ileum (Osthaus & Galligan, 1992). However, in the present study the antagonist of the small Ca^{2+}-activated K^+ channels, apamin, did not change the concentration-relaxation curves to GEA 3175, suggesting that opening of these channels does not mediate the relaxation to GEA 3175 in bovine small bronchioles. Electrophysiological studies of porcine isolated tracheal smooth muscle cells demonstrated that sodium nitroprusside activates large conductance Ca^{2+}-activated K^+-channels (Yamakage *et al*., 1996). Moreover, mechanical studies of bovine, guinea-pig, and porcine isolated proximal airway segments have indicated that large conductance Ca^{2+}-activated K^+-channels were involved in the relaxations induced by endogenously released NO and several NO-donors (Jones *et al*., 1990; Hamaguchi *et al*., 1992; Bialecki & Stinson-Fisher, 1995; Kannan & Johnson, 1995). The inhibitory effect of charybdotoxin on the relaxations to the GEA 3175 suggests that this compound can also activate Ca^{2+}-activated large conductance K^+-channels in bovine small bronchioles. NO has been earlier described to activate both small and large K^+-channels in colonic smooth muscle (Koh *et al*., 1995). However, the combination of apamin and charybdotoxin did not produce additional inhibition compared to charybdotoxin alone of the relaxations to GEA 3175, and this suggests that small conductance Ca^{2+}-activated K^+-channels are not involved in the relaxations induced by this NO donor.

In addition to inhibition of large conductance Ca^{2+}-activated K^+-channels, charybdotoxin, is also found in some preparations to block Ca^{2+}-independent voltage-gated K^+-channels and to have prejunctional effects on the release of neurotransmitters (Garcia *et al*., 1991; De Man *et al*,. 1993; Robitaille *et al*., 1993). In our experiments, the toxin-induced contractions of bovine bronchioles and the concentrations of 5-hydroxytryptamine were adjusted to obtain a matched preconstriction level, before the NO-donors were added. It is unlikely that the increases of resting tone caused by charybdotoxin are due to inhibition of prejunctional Ca^{2+}-activated K^+-channels and release of excitatory neurotransmitters, since iberiotoxin, which is considered to be selective for the large Ca^{2+}-activated K^+-channels (Garcia *et al*., 1991), did not change the resting tension of the preparation. Studies of isolated segments of guinea-pig trachea and human bronchial segments have suggested the involvement of large conductance Ca^{2+}-activated K^+ channels in the relaxations induced by β-adrenoceptor agonists (Miura *et al*., 1992; Jones *et al*., 1993). In contrast, iberiotoxin did not change the relaxations induced by salbutamol in bovine small bronchioles. Further studies may show whether such differences can be ascribed to heterogeneity in relaxation mechanisms of the β-adrenoceptor agonists in proximal versus distal parts of the airways. However, iberiotoxin like charybdotoxin also reduced the relaxations to GEA 3175. These results suggest that the activation of large conductance Ca^{2+}-activated K^+ channels is involved in the relaxant response of bovine small bronchioles to GEA 3175.

In smooth muscle, different mechanisms have been proposed for hyperpolarization through Ca^{2+}-activated K^+ channels by NO. Thus, NO can open Ca^{2+}-activated K^+ channels through a cyclic GMP-independent mechanism, probably by direct activation of the K^+ channels (Bolotina *et al*., 1994; Vanheel *et al*., 1994; Koh *et al*., 1995), or opening of these channels can take place through cyclic GMP-dependent mechanisms by activation of cyclic GMP-dependent protein kinase (Tare *et al*., 1990; Robertson *et al*., 1993). In bovine small bronchioles, where the soluble guanylyl cyclase sensitive to NO was inhibited by LY 83583, charybdotoxin produced an additional inhibition of the relaxations induced by GEA 3175 and NO suggesting that these relaxations in part

should be guanylate cyclase-independent. On the other hand, LY 83583 was less potent than ODQ suggesting that the inhibition of the guanylate cyclase was not complete. Moreover, the combination of ODQ and iberiotoxin did not produce an additional inhibitory effect of the relaxations evoked by GEA 3175 compared to either blocker alone. This finding suggests that the guanylate cyclase-dependent relaxations induced by GEA 3175 are associated with opening of large Ca^{2+}-activated K^+-channels.

Recently, Takeuchi and colleagues (1996) observed that NO induced relaxations of rat proximal colon by an unknown mechanism which was not associated with changes in cyclic GMP content and membrane potential of the smooth muscle. In the present study, there was persistence of a minor part of the relaxations to both GEA 3175 and exogenous NO in the presence of LY 83583 and charybdotoxin. This suggests that other mechanisms, as observed in the rat proximal colon (Takeuchi *et al*., 1996), independent of both guanylate cyclase activation and K^+ channels might also contribute to the relaxations obtained to GEA 3175 and NO in bovine small bronchioles.

In summary, the present study suggests that the new slow-releasing NO-donors such as GEA 3175 are more potent than the NO donors presently available in inducing relaxations of bovine small bronchioles. GEA 3175 elicits relaxations in part though a guanylate cyclase-dependent mechanism involving opening of large conductance Ca^{2+}-activated K^+ channels similar to exogenously added NO.

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