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In-vitro Bronchorelaxing Effects of Novel Nitric Oxide Donors GEA 3268 and GEA 5145 in Guinea–pigs and Rats

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Abstract

Endogenously released nitric oxide (NO) in airways might contribute to physiological bronchodilation; induced production of NO might play a role in the pathogenesis of asthma, although it could also be a compensatory mechanism to other factors that cause bronchoconstriction or inflammation. To investigate the efficacy of NO donors on bronchial tone, the bronchorelaxing efficacies of NO donors, new experimental GEA compounds 3268 and 5145 (oxatriazole sulphonylamides) were compared with those of sodium nitroprusside and SIN–1 (3–morpholinosydnonimine) and to the standard β_2–adrenergic agonist, salbutamol, in bronchi of guinea–pigs and rats in-vitro. Their relaxing effects were also studied in rat mesentery arteries to compare the selectivity for airways. The capacity of the NO donors to produce nitrites and nitrates was assayed by the Griess reaction.

The novel NO donors GEA 3268 and GEA 5145 were more potent bronchorelaxing agents than the old NO donors sodium nitroprusside and SIN–1. In guinea–pig bronchi, however, salbutamol was most potent. In rat bronchi the GEA compounds induced the strongest relaxation effect when compared with the old NO donors or with salbutamol. The airway selectivity of the drugs studied decreased in the order of salbutamol, SIN–1, GEA 5145, GEA 3268, sodium nitroprusside. The nitrites and nitrates produced spontaneously did not correlate with the efficacy of the relaxants

The results obtained suggest that NO is only partly responsible for the relaxation and the potency is dependent on the animal species and constricting agents used.

In man the inhibitory nonadrenergic–noncholinergic nerve response is predominantly mediated by nitric oxide in the airways (Barnes 1992; Belvisi et al 1992a; Belvisi et al 1992b; Ellis & Undem 1992; Bai & Bramley 1993); in guinea–pig tracheal smooth muscle it is partly mediated by NO and, probably, partly by vasoactive intestinal peptide (Tucker et al 1990; Li & Rand 1991). In the respiratory tract, moreover, other sources of NO include endothelial cells, vascular and airway smooth muscle cells, many inflammatory cells and the airway epithelium (Wright et al 1989; Barnes & Belvisi 1993; Jain et al 1993). Most of the different effects of NO are mediated by activation of soluble guanylate cyclase, resulting in an increase in cGMP in the target cell. It has long been known that nitrates induce bronchial relaxation. Nitric oxide–containing vasodilators, such as glyceryl trinitrate and sodium nitroprusside, induce relaxation of the isolated airway smooth muscle (Katsuki & Murad 1977; Gruetter et al 1989) and activate guanylate cyclase and raise tissue cGMP levels (Gruetter et al 1989). In the airways, on the other hand, part of the bronchorelaxing effect of NO is mediated independently from cGMP (Zou & Torphy 1991; Ellis & Conanan 1994; Jones et al 1994) through cAMP–dependent pathways (Ellis & Conanan 1995). Accordingly, the synergistic effect of cGMP and cAMP would result in activation of cGMP–kinase and further lead to the opening of large calcium–activated K^+ channels leading ultimately in reduction of cytosolic Ca^{2+} concentration (Ellis & Conanan 1994).

Mesoionic oxatriazole imines (GEA 3162, GEA 5024) and their derivatives, sulphonylamides (GEA 3175, GEA 3268), amides (GEA 3233, GEA 3232) and several urea types of NO donor exhibit anti–platelet, fibrinolytic, thrombolytic and broncholytic properties. They increase cGMP levels in guinea–pig trachea and parenchyma and in human granulocytes in–vitro and protect against bronchoconstriction in–vivo (Corell et al 1994). These novel compounds can inhibit the generation of leucotriene B_4 in formyl–L–methionyl–L–leucyl–L–phenylalanine–stimulated human polymorphonuclear cells (Corell et al 1994). In murine epithelial cell lines mixture of cytokines such as interleukin–1, tumour necrosis factor α and interferon-γ, increase nitrite levels 9–fold and increase of inducible mRNA (Robbins et al 1994). It is, therefore, of interest that GEA 3162 and GEA 3175 have been reported to inhibit the activation of human polymorphonuclear cells by inhibiting leucotriene B_4 synthesis, degranulation, chemotaxis and superoxide anion (O_2^-) release, and may thus act as a local modulator in inflammatory processes (Moilanen et al 1993).

The aim of this study was to compare the efficacy and potency of new and old NO donors on the airway smooth muscle in–vitro using two different animal species. The effects of these cGMP–dependent NO donors were compared with those of the established cAMP dependent anti–asthma drug salbutamol. The possible selectivity of the compounds for bronchial and vascular smooth muscle was also studied.

Materials and Methods

Drugs

GEA 3268 (1,2,3,4–oxatriazolium 3–(3–chloro–2–methylphenyl)–5–[[(4–methoxyphenyl)sulphonyl]amino]-hydroxide

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inner salt) and GEA 5145, (1,2,3,4–oxatriazolium 3–(3–chloro–2–methylphenyl)–5–[(methylsulphonyl)amino] hydroxide inner salt) and SIN–1 (3–morpholinosydnonimine) were synthesised by A/S GEA Farmaceutisk Fabrik, Hvidovre, Denmark. Sodium nitroprusside was from Hoffmann–La Roche (Basle, Switzerland), noradrenaline ([−]–arterenol, bitartrate salt), acetylcholine chloride, acetyl–β–methylcholine chloride and sulphanilamide from Sigma (St Louis, MO, USA). Pentobarbital sodium was from Danisco Ingredients (Grinsted, Denmark), histamine acid phosphate from BDH (Poole, UK) and *N*–napthylethylenediamine from Riedel-de Haën (Seelze, Germany). Salbutamol was a generous gift from Leiras Ltd (Turku, Finland). Nitrate reductase (EC 1.6.6.2; Aspergillus species), NADPH, FAD, rabbit muscle lactate dehydrogenase, sodium pyruvate were from Boehringer–Mannheim, (Mannheim, Germany). GEA 3268 and GEA 5145 were dissolved in DMSO to give a 10 μM stock solution. All other compounds and the Krebs–Ringer solution were dissolved in Milli–Q ultrapure water (Millipore, Bedford, MA, USA).

Isolated bronchi of guinea–pigs and rats

English short–haired three coloured guinea–pigs, 350–450 g, of either sex, bred in the Public Institute of Health, Helsinki, Finland, were anaesthetized with pentobarbital (75 mg kg^{-1}) and decapitated. Male Wistar rats, 250–350 g, bred in the Institute of Biomedicine, University of Helsinki, Finland, were decapitated without anaesthesia. The main bronchus of the guinea–pigs was cut into 3·5–mm–wide rings and those of the rats into 5·0–mm–wide rings; the rings were mounted in an organ chamber containing Krebs–Ringer solution of 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 and the pH was adjusted to 7.4. The solution was oxygenated with 96% O_2 + 4% CO_2 during the experiments. The epithelium of some rings was removed by gentle luminal rubbing. The resting bronchial tension was set at 1 g and the tension changes were recorded with a Grass 7 B polygraph using Grass force displacement transducers (FT03, Grass Medical Instruments, Quincy, MA, USA). The rings were constricted with either 1 μM metacholine, 1 μM histamine or 40 mM KCl, concentrations which were chosen on the basis of submaximum responses. Relaxing effects of 10 nM–10 μM GEA 3268, GEA 5145, sodium nitroprusside and SIN–1, and 0.1 nM–1.0 μM salbutamol were studied cumulatively.

Isolated mesenteric arteries of rat

Rat mesenteric arteries were cut from the same animals as the bronchi and prepared and studied otherwise as above. The endothelium was removed by gently rubbing of the intimate surface. The preparations were constricted with 1 μM noradrenaline and the presence or absence of endothelium was tested with 1 μM acetylcholine (ACh). The relaxing effects of NO donors were studied in cumulative doses after 1 μM noradrenaline constriction.

The experimental design of the study was approved by the Animal Experimentation Committee of the University of Helsinki, Finland.

Determination of nitrite and nitrate (NO_x)

Samples of (NO_2^- and $NO_2^- + NO_3^-$, i.e. NO_x) were collected under conditions identical to those described above (Krebs–Ringer solution at 37°C and with 96% O_2 + 4% CO_2) but without tissue samples. Time points of 0, 5, 10, 15, 30, 60 min were used. The concentration of the NO donors used was 10 μM in order to detect generated nitrite. The detection limit of the assay was 0.5 μM. $NaNO_2$ diluted in water was used to produce the calibration graph; it was linear for NO_2^- in the concentration range 0–20 μM. The assay for NO_x was performed according to Moshage et al (1995) and Schmidt et al (1992).

Handling of data

The results are expressed as mean ± s.e.m. of three samples for NO_x assays and of 6–8 preparations for all the other experiments.

Results

Guinea–pig bronchi

After metacholine pre-constriction, the maximum relaxing effects of 1 μM salbutamol and 10 μM GEA 3268, GEA 5145 and sodium nitroprusside were approximately 90%, whereas that of 10 μM SIN–1 was only 35% (Fig. 1a). The EC50 for

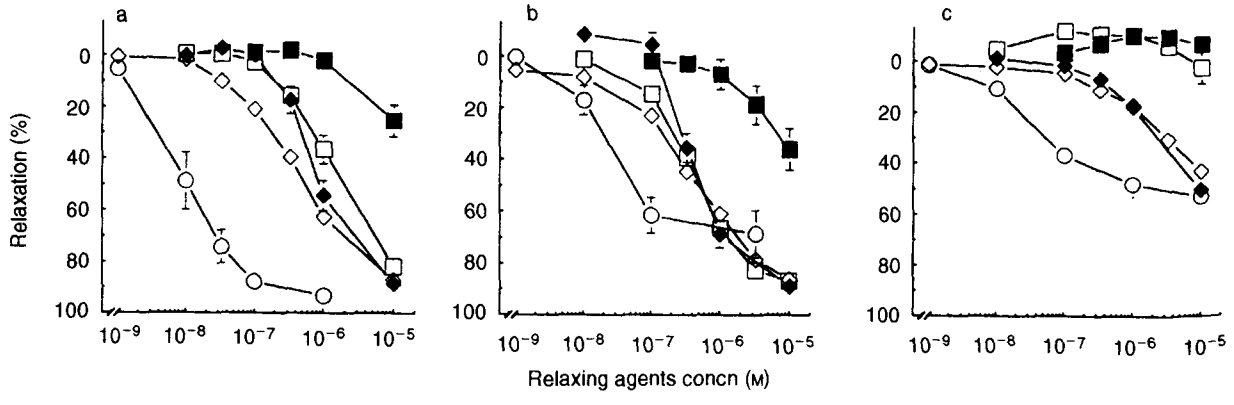

FIG. 1. Dose–response curves for bronchorelaxing action of salbutamol (○), GEA 3268 (◆), GEA 5145 (◇), sodium nitroprusside (□) and SIN-1 (■) on guinea-pig bronchus after precontraction with 1 μM metacholine (a), 1 μM histamine (b) and 40 mM KCl (c).

Table 1. EC50 values of NO donors and salbutamol in guinea-pig and rat bronchi after precontraction with 1 μM metacholine or 1 μM histamine, and EC30 after precontraction with 40 mM KCl.

Relaxant drug	Guinea-pig Metacholine EC50 (μM)	Histamine EC50 (μM)	KCL EC30 (μM)	Rat Metacholine EC50 (μM)	KCl EC30 (μM)
SIN-1	n.d.	n.d.	n.d.	49	30
Sodium nitroprusside	2·1	0·5	n.d.	12	6·5
GEA 3268	0·9	0·5	2	2·5	2·5
GEA 5145	0·56	0·4	3	11	0·6
Salbutamol	0·011	0·06	0·05	n.d.	n.d.

salbutamol was the lowest, about 1/50 to 1/200 those for the NO donors except for SIN–1 for which EC50 could not be determined (Table 1).

After histamine pre–constriction the responses were similar in terms of maximum relaxations for 10 μM sodium nitroprusside, GEA 3268 and GEA 5145 (85–90%). Salbutamol in lower concentration (3.3 μM) induced 70% relaxation and 10 μM SIN–1 was again the weakest (35%). The EC50 values for the NO donors were nearly the same except for that for SIN–1, which could not be determined. The EC50 for salbutamol was nearly 1/10 those for the NO donors.

After KCl pre–constriction, sodium nitroprusside and SIN–1 were ineffective even at the highest concentration (10 μM), whereas 10 μM GEA 3268, GEA 3175 and salbutamol induced 45–50% relaxation. Because of the poor relaxation, EC30 values could be calculated for salbutamol and the GEA compounds only; salbutamol was at less than 1/10 of the concentration of the GEA compounds.

Rat bronchi

After 1 μM metacholine pre–constriction (Fig. 2), GEA 3268 induced the strongest relaxation amounting to 80% at 10 μM (EC50 3·0 μM, Table 1). Relaxations after 10 μM GEA 5145 and sodium nitroprusside were both approximately 50%; those of salbutamol and SIN–1 were 40 % and 20%, respectively. EC50 values could not be calculated for salbutamol or SIN–1.

After pre–constriction with 40 mM KCl the maximum relaxations induced by GEA 5145 and GEA 3268, i.e. 60% and 50%, respectively, were the strongest. The corresponding EC30 values were 0·6 and 2·5 μM, respectively. The maximum responses after sodium nitroprusside and salbutamol were only

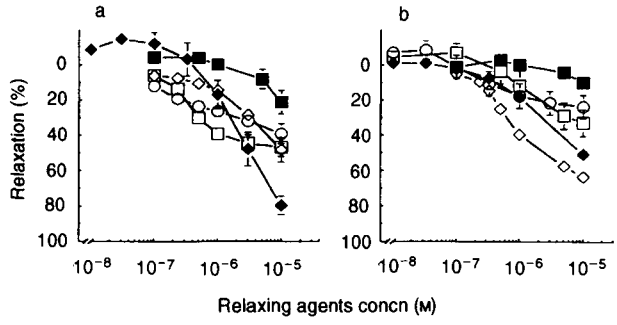

FIG. 2. Dose-response curves for bronchorelaxing action of salbutamol (○), GEA 3268 (◆), GEA 5145 (◇), sodium nitroprusside (□) and SIN-1 (■) on rat bronchus after precontraction with 1 μM metacholine (a) and 40 mM KCl (b).

Table 2. EC50 values and maximum relaxation induced in the endothelium-intact rings of the rat mesenteric artery by sodium nitroprusside, GEA 3268 and GEA 5145 after precontraction with 1 μM noradrenaline.

	EC50 (μM)	Maximum relaxation (%)
SIN-1	0·12	85
Sodium nitroprusside	0·008	96
GEA 3268	0·024	97
GEA 5145	0·1	96
Salbutamol	n.d.	30

from 30 to 25% and that of SIN–1 was 10%. Because of the small maximum responses, EC30 values could be calculated for some the drugs only (Table 1).

Rat mesenteric arteries

In the rat mesenteric artery with intact endothelium and pre–constricted with 1 μM noradrenaline the relaxation potencies (Fig. 3) were in the decreasing order sodium nitroprusside > GEA 3268 > GEA 5145 > SIN–1 > salbutamol. The maximum effects and the EC50 values in the presence of endothelium are given in Table 2. Removal of the endothelium changed the relaxations to NO donors only negligibly whereas the salbutamol induced relaxations were slightly increased.

Determination of nitrite and nitrate

SIN–1 and GEA 3268 generated NOx spontaneously in the solvent whereas that generated by sodium nitroprusside and GEA 5145 rarely exceeded the detection limit. SIN–1 was the most potent producer of NO_2^- and $NO_2^- + NO_3^-$, generating 18.5 and 33·7 μM, respectively, within 5 min. GEA 5145 produced 7 μM NO_2^- + NO_3^- and 0·8 μM NO_2^- within 5 minutes. sodium nitroprusside produced the lowest amounts in NO_x (Fig. 4).

Discussion

In this study, the in-vitro bronchorelaxing efficacies of the two novel NO donors GEA 3268 and GEA 5145 were compared with those of the standard NO donors SIN–1 and sodium nitroprusside and to that of salbutamol, a β_2–adrenergic agonist.

An ideal bronchodilating drug should show selectivity to the airway smooth muscle over the vascular smooth muscle in order to minimize cardiovascular adverse effects. Airway selectivity of the drugs studied decreased in the order salbu-

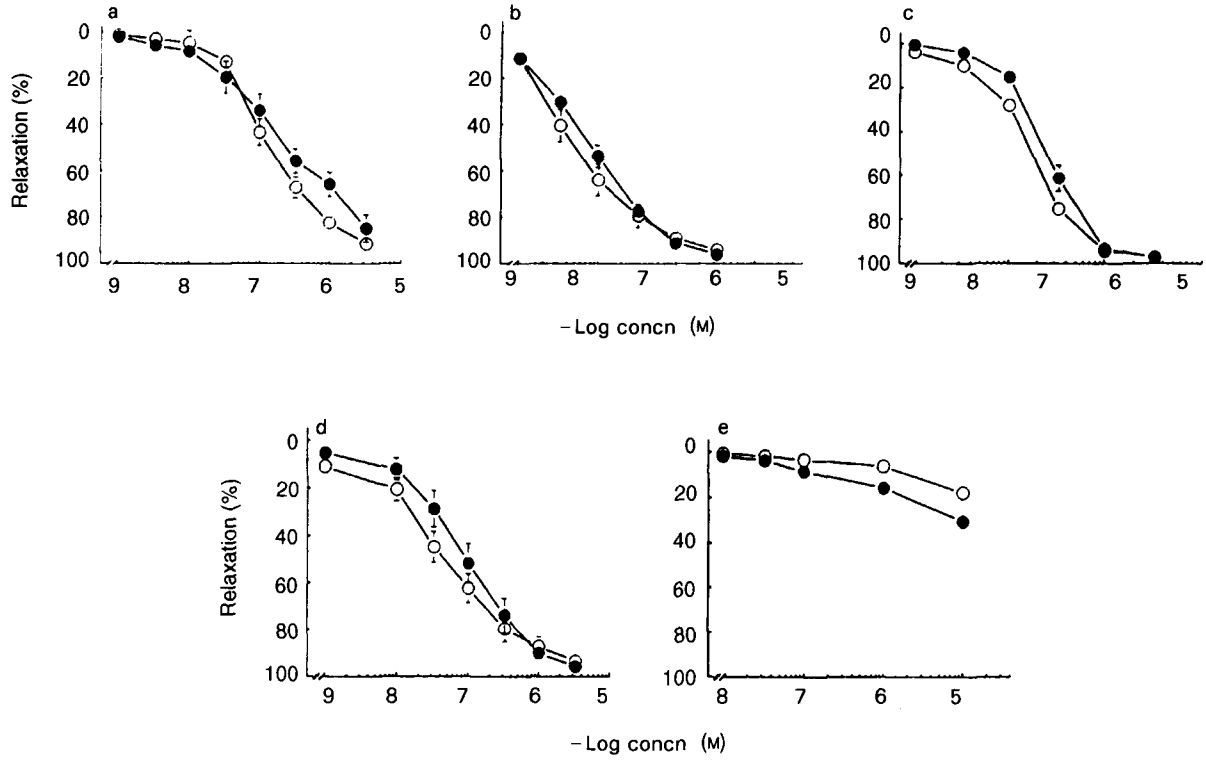

FIG. 3. Effects of relaxation agents on rat mesentery artery in the presence (●) and absence (○) of endothelium after precontraction with 1 μM noradrenaline. SIN-1(a), sodium nitroprusside (b), GEA3268 (c), GEA5145 (d) and salbutamol (e).

tamol < GEA 5145 < GEA 3268 < sodium nitroprusside < SIN–1. Our findings on the selectivity of sodium nitroprusside are in accordance with those of O'Donnell et al (1991) who reported that sodium nitroprusside was more potent in relaxing the rat pulmonary artery than the guinea–pig trachea. Our findings show, moreover, that sodium nitroprusside was more potent in relaxing the rat mesenteric artery than the GEA compounds. As could be expected, the relaxing effects of GEA 3268, GEA 5145 and sodium nitroprusside were independent of the vascular endothelium.

In the rat bronchi the GEA compounds were more potent than SIN–1, sodium nitroprusside or salbutamol under all the experimental conditions used. In the guinea–pig bronchi, moreover, both GEA compounds were more potent than sodium nitroprusside and SIN–1. The bronchorelaxing effects of all the NO donors studied were, however, clearly inferior to that of salbutamol in the guinea–pig bronchi.

There are several possible explanations for the differences between the potencies of the compounds studied in the guinea–pig and rat. Rat lungs do not have any adrenergic nerve supply to the bronchial muscle (Bivin et al 1979). The lower density of β_2–receptors in the rat bronchi compared with the guinea–pig bronchi could explain the weaker β–adrenergic relaxation in the former. The number of β–receptors in the rat lung (Rugg et al 1978) is 3–fold lower than in that of the guinea–pig (Barnes 1980) and 75% of rat adrenoceptors are β_2–subtype (Rugg et al 1978). Vornanen (1982) showed that in the rat β–receptors are not equally distributed and there are fewer β–receptors in the bronchi.

Exogenous NO donors are different in their half–lives and in their capacity to produce NO (Karup et al 1994). Some release NO slowly and continuously; others release the compound in a bolus fashion; yet others need thiol groups to be effective. Extensive spontaneous production of NO_x by SIN–1 has been reported previously (Kita et al 1995; Kankaanranta et al 1996). The low production of NO_x by sodium nitroprusside in the absence of thiol groups has also been reported by Kita et al (1995). The release of NO_x by GEA 3175, a compound very similar to both GEA 3268 and GEA 5145, is enhanced by the thiol groups of L–cysteine (Cohen et al 1995). In the present study nitric oxide could, to some extent, be released from GEA 5145 without the presence of thiol groups. The level of NO_x generated in the Krebs–Ringer solution did not correlate with the level of relaxation produced, suggesting either that activation of the molecule by the target tissue is needed for NO_x release or that the relaxation was produced by a property of the drug other than NO-release.

The weak cGMP–mediated NO relaxation effect could also be explained by the finding of Katsuki & Murad (1977) that constrictions induced by 1 μM carbachol and 0.1–10 μM histamine resulted in a significant increase in the cGMP and cAMP content of bovine tracheal smooth muscle. Both increases could be abolished with atropine and diphenhydramine. As the increase in cGMP induced by the constricting agents was bigger than that of cAMP it could be suggested that this effect would generally explain the low potency of a cGMP–mediated NO donor compared with that of the cAMP–mediated β_2-agonist.

In conclusion, in–vitro tests show that the new NO donors GEA 3268 and GEA 5145 are more potent bronchorelaxing

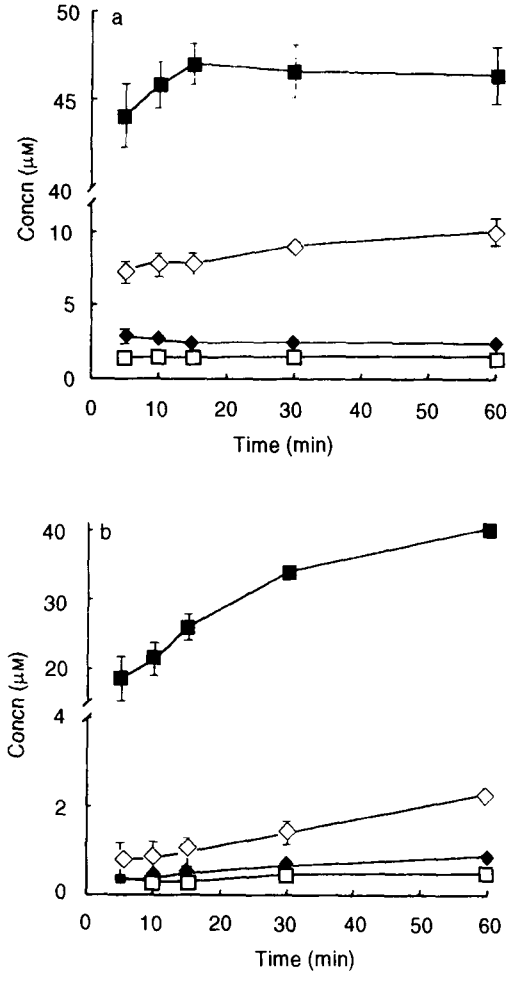

FIG. 4. Total nitrite and nitrate (NO_2^- + NO_3^-) production (a) and nitrite (NO_2^-) production (b) assayed by the Griess reaction in the absence of tissue samples. GEA 3268 (◆), GEA 5145 (◇), sodium nitroprusside (□) and SIN-1 (■).

agents than the old donors sodium nitroprusside and SIN–1. In guinea–pig bronchi salbutamol was generally most potent in all the relaxations, whereas in the rat bronchi the GEA compounds induced the strongest relaxation effect. When compared with a β_2–agonist the efficacy of an NO donor in relaxing airway smooth muscle is dependent on the animal species used.

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