Pulmonary effects of type V cyclic GMP specific phosphodiesterase inhibition in the anaesthetized guinea-pig

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1 We have investigated the bronchodilator potential of type V phosphodiesterase (PDE V) inhibitors in anaesthetized ventilated guinea-pigs using the potent and selective PDE V inhibitor, SK&F 96231. We have compared its activity to that of salbutamol, the PDE III inhibitors, siguazodan and SK&F 95654 and to the PDE IV inhibitor rolipram.

2 Administered as an i.v. infusion SK&F 96231 (0.6 and 1 mg kg⁻¹ min⁻¹, i.v.) caused a slowly developing inhibition of histamine (100 nmol kg⁻¹, i.v.)-induced bronchoconstriction and elevated tracheal cyclic GMP levels in the anaesthetized guinea-pig. SK&F 96231 (0.1 and 0.3 mg kg⁻¹ min⁻¹, i.v.) was without effect on histamine-induced bronchoconstriction. In the presence of a sub-threshold infusion of SNP (0.1 µmol kg⁻¹ min⁻¹, i.v.) there was a marked enhancement of SK&F 96231-induced inhibition of histamine responses such that at infusion rates that were ineffective alone, SK&F 96231 caused a > 50% inhibition of histamine responses. The stimulation of tracheal cyclic GMP accumulation by SK&F 96231 was also potentiated.

3 Administered directly into the airway, SK&F 96231 (300 µg in 5 mg lactose carrier) was largely without effect on histamine-induced bronchoconstriction (4.9 ± 1.9% inhibition). In the presence of SNP (0.1 µmol kg⁻¹ min⁻¹, i.v.) or isosorbide dinitrate (200 µg administered by insufflation into the trachea) there was a marked potentiation of the inhibitory activity of SK&F 96231 (40 ± 4% and 62 ± 1.8% respectively).

4 Salbutamol and rolipram (3–300 µg by insufflation) caused a dose-related inhibition of histamine responses with a maximum of 91 ± 2% and 59 ± 10% respectively. The PDE III inhibitor, siguazodan, was without effect on histamine responses but they were reduced (27.7 ± 4.8% at 300 µg) by SK&F 95654. There was a marked enhancement of the inhibitory activity of rolipram in the presence of SK&F 95654.

5 We conclude that SK&F 96231 has weak anti-spasmodic activity in the guinea-pig *in vivo*, we suggest that this is primarily a consequence of a low endogenous guanylate cyclase activity in the airway. The potentiation of the anti-spasmodic activity of SK&F 96231 by SNP suggests that a combination of PDE V inhibitor and guanylate cyclase agonist might provide significant bronchodilator activity.

6 We have established that PDE IV inhibitors are bronchodilators when administered directly into the airway of anaesthetized guinea-pigs but that PDE III inhibitors are only weakly active. The marked enhancement of the inhibitory activity of rolipram by the PDE III inhibitor, SK&F 95654, indicates that inhibitors of both PDE III and PDE IV might offer greater potential as bronchodilators than inhibitors of either isoenzyme alone.

Keywords: Cyclic nucleotide phosphodiesterases; selective PDE inhibition; SK&F 96231; rolipram; siguazodan; SK&F 95654; SNP; bronchodilatation; anti-spasmodic activity

Introduction

Adenosine 3':5'-cyclic monophosphate (cyclic AMP) and guanosine 3':5'-cyclic monophosphate (cyclic GMP) play an important role in the regulation of airway tone, elevations in either being causally associated with smooth muscle relaxation (Iscii & Murad, 1989; Murray, 1990; Torphy & Undem, 1991).

Intracellular concentrations of cyclic nucleotides are determined by their relative rates of formation, via agonist-induced stimulation of adenylate and guanylate cyclase, and hydrolysis by phosphodiesterase enzymes (PDE). Multiple PDE isoenzymes have been isolated from a variety of tissues and based on their kinetic characteristics, substrate specificity, sensitivity to endogenous regulators and susceptibility to inhibition by selective inhibitors have been divided into five distinct families (PDE I–V; Beavo, 1988; Beavo & Reifsnnyder, 1990). Of these PDE I, II and III can utilise both cyclic AMP and cyclic GMP as substrate, PDE IV is a cyclic

AMP-specific PDE and PDE V a cyclic GMP-specific PDE (Beavo, 1989; Beavo & Reifsnnyder, 1990). Although their relative proportions vary depending on species and airway generation, members of all five families have been detected in smooth muscle from canine (Torphy & Cieslinski, 1990), bovine (Shahid *et al.*, 1991), guinea-pig (Takagi *et al.*, 1992; Turner *et al.*, unpublished observations) and human (de Boer *et al.*, 1992; Giembycz *et al.*, 1992; Cortijo *et al.*, 1993) airway.

The bronchodilator activity and anti-asthma potential of xanthines is attributed, at least in part, to non-selective inhibition of multiple PDE activities (Torphy & Undem, 1991). More recently, however, interest has focused on the potential of isoenzyme-selective PDE inhibitors as bronchodilator and anti-inflammatory agents. Selective inhibitors of the PDE III and IV isoenzymes are effective relaxants of guinea-pig trachea (Harris *et al.*, 1989; Tomkinson *et al.*, 1993), canine trachealis (Torphy *et al.*, 1988; Torphy *et al.*, 1991), bovine trachea (PDE III inhibitors are only weakly active, Shahid *et al.*, 1991) and human bronchus (de Boer *et*

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al., 1992; Cortijo *et al.*, 1993) *in vitro* and have bronchodilator activity in the guinea-pig (Harris *et al.*, 1989; Cortijo *et al.*, 1993; Howell *et al.*, 1993) and dog (Heaslip *et al.*, 1991) *in vivo*. In contrast despite the presence of PDE V in the airway of all species so far studied, the PDE V inhibitor zaprinast alone is without effect on canine tracheal tone (Torphy *et al.*, 1991) and at concentrations that retain selectivity for PDE V, is a relatively ineffective relaxant of bovine trachea (Hall *et al.*, 1990; Shahid *et al.*, 1991) and human bronchus (de Boer *et al.*, 1992) *in vitro*. Nevertheless it is generally accepted that increases in intracellular cyclic GMP are causally related to relaxation of airway smooth muscle (Polson *et al.*, 1985; Torphy & Undem, 1991). In this regard both sodium nitroprusside and atrial natriuretic peptide (ANP) increase cyclic GMP levels and potentially relax bovine and canine tracheal smooth muscle (Lorenz & Wells, 1983; Torphy *et al.*, 1985; Ishii & Murad, 1989). Furthermore, zaprinast potentiates both sodium nitroprusside-induced relaxation of canine trachealis and the associated increase in cyclic GMP accumulation (Torphy *et al.*, 1991).

We have previously reported that SK&F 96231 (1,7-dihydro-2-(2-propoxyphenyl)-6H-purin-6-one) is a potent and selective inhibitor of the cyclic GMP PDE V (Murray *et al.*, 1991). SK&F 96231 relaxes guinea-pig trachea precontracted with K^+ , histamine or the thromboxane-mimetic, U46619 and causes a slow time-dependent increase in the cyclic GMP content of guinea-pig lung strips, *in vitro* (Murray *et al.*, 1991). *In vivo*, SK&F 96231 has been shown to produce a modest, short-lived inhibition of U46619-induced bronchoconstriction in anaesthetized guinea-pigs and is approximately 4 fold more potent than zaprinast in this regard (Murray *et al.*, 1991).

In the present study we have used SK&F 96231 to examine the role of PDE V in mediating bronchodilatation in the anaesthetized guinea-pig, a widely employed model of human asthma. In addition we have compared its activity to that of the PDE III inhibitors, siguazodan or SK&F 95654 and the PDE IV inhibitor, rolipram.

Methods

In vivo inhibition of histamine-induced bronchoconstriction in anaesthetized guinea-pigs

Male Dunkin-Hartley guinea-pigs (450–570 g) were anaesthetized with sodium pentobarbitone (Sagatal, 50–60 mg kg^{-1} , i.p.). A carotid artery was cannulated for measurement of blood pressure and a jugular vein for administration of test substances. The trachea was cannulated and the animals mechanically ventilated with room air using a Palmer pump set at a stroke volume of 1 ml $100 g^{-1}$ and a respiratory rate of 50–55 inflations min^{-1} . Pulmonary inflation pressure (PIP) was measured with a Druck PDCR 75 pressure transducer attached to a side arm off the tracheal cannula. After a 15 min equilibration period, histamine 100 nmol kg^{-1} , i.v. was administered at 5 min intervals. When a reproducible increase in PIP had been established, SK&F 96231 or vehicle was administered by constant i.v. infusion (6 ml h^{-1}) for 30 min. In a further series of experiments SK&F 96231, rolipram, siguazodan, SK&F 95654, SNP, isosorbide dinitrate or vehicle were administered directly into the airway. In the latter studies agents were administered as dry powders in 5 mg lactose carrier; animals were temporarily disconnected from the ventilator and the PDE inhibitors introduced into the airway by insufflation into the trachea.

In some experiments, the effects of a sub-threshold dose of SNP on the SK&F 96231 response was investigated; in these studies a reproducible increase in PIP to histamine was established and SNP infused (10^{-7} mol $kg^{-1} min^{-1}$, i.v.) for 15 min prior to infusion of SK&F 96231 + SNP for 30 min, or administration of SK&F 96231 into the airway.

Histamine-induced bronchoconstriction was assessed as the difference between the basal PIP and the maximum increase in PIP caused by histamine, each animal serving as its own control.

In a separate series of experiments, tracheae were removed at time intervals following infusion of SK&F 96231, SNP or their combination and snap frozen in liquid nitrogen and stored at $-70^{\circ}C$ prior to measurement of cyclic GMP levels. Cyclic nucleotides were extracted by addition of 1 ml cold 0.3 M perchloric acid to the frozen tissues; tissues were then finely chopped, in the perchloric acid, and homogenized (Polytron PTA7KI probe 4×10 s burst, setting 8). Following centrifugation, acid was removed from the supernatant by extraction with a 50/50 (v/v) mixture of tri-*n*-octylamine and freon. Cyclic GMP levels were estimated with a commercially available (Amersham) radioimmunoassay kit.

Protein estimations were determined by the Bio-Rad protein assay based on the method described by Bradford (1976).

Guinea-pig isolated trachea

Male Dunkin-Hartley guinea-pigs weighing from 350–450 g were killed by cervical dislocation. Each trachea was rapidly excised, placed in oxygenated (95% O_2 , 5% CO_2) Krebs-Henseleit solution (composition in mM: NaCl 118, KCl 4.7, $NaHCO_3$ 25, $MgSO_4$ 1.2, KH_2SO_4 1.2, D-glucose 5.5, $CaCl_2$ 2.5) at room temperature and trimmed free of adherent fat and connective tissue.

Each trachea was cut into rings containing 3 adjacent cartilage plates, the rings were opened opposite the trachealis and suspended in 25 ml organ baths containing Krebs solution at $37^{\circ}C$, under an initial load of 2.5 g. Following a 60 min equilibration period, tissues were primed for contractile activity by addition of $1 \mu M$ carbachol (CCh). This procedure was repeated 3 times after washing. Sustained contractions were elicited by addition of 0.1 or $1.0 \mu M$ CCh. When the increase in tension had stabilized, the relaxant activity of SK&F 96231 and sodium nitroprusside (SNP) were investigated by their cumulative addition to the organ bath. Changes in tension were measured with a dynamometer UF1 isometric transducer coupled to an LECTROMED MT8P chart recorder. Relaxations were expressed as a percentage of the maximum relaxation induced by 10^{-4} M SNP. EC_{50} values are expressed as geometric means with 95% confidence limits.

Data analysis

The data are expressed as mean $\pm$ s.e.mean and were compared by Mann Whitney U test for unpaired observations. A two-tailed probability of <0.05 was considered significant. t_1 values for PDE inhibitors and salbutamol represent the time to 50% recovery of histamine responses.

Materials

All drugs and chemicals were obtained from Sigma or BDH Chemicals (both of Poole, Dorset). The cyclic GMP radioimmunoassay kit was from Amersham International (Amersham, Buckinghamshire). SK&F 96231 (1,7-dihydro-2-(2-propoxyphenyl)-6H purin-6-one), siguazodan (SK&F 94836 2-cyano-1-methyl-3-[4-(4-methyl-6-oxo-1,4,5,6-tetrahydropyridazin-3-yl)phenyl]guanidine, SK&F 95654 (R,S-4,5-dihydro-6-[4-(1,4-dihydro-4-oxopyrriin-1-yl)phenyl]-5-methyl-3(2H)-pyridazinone) and rolipram(4-(3-cyclopentyl-4-methoxyphenyl)-2-pyrrolidinone) were synthesized by Dr W.J. Coates of the Department of Medicinal Chemistry, SmithKline Beecham, The Frythe, Welwyn.

Results

Anti-spasmogenic activity of SK&F 96231 *in vivo*

SK&F 96231 administered as an i.v. infusion at 0.6 and 1.0 mg kg⁻¹ min⁻¹ caused a slowly developing inhibition of histamine (100 nmol kg⁻¹, i.v.)-induced bronchoconstriction which reached a maximum (ca 40% and 90% respectively) 30 min after commencing the infusion. SK&F 96231 infused at 0.1 and 0.3 mg kg⁻¹ min⁻¹ was without effect on histamine-induced bronchoconstriction (Figure 1a). In the presence of SNP (0.1 µmol kg⁻¹ min⁻¹) which alone was without effect on the histamine-induced increase in PIP, there was a pronounced enhancement of the anti-spasmogenic activity of

Figure 1 Effect of SK&F 96231 infused i.v. in anaesthetized ventilated guinea-pigs on histamine-induced bronchoconstriction. The increase in pulmonary inflation pressure to histamine (100 nmol kg⁻¹, i.v.) was measured at a tidal volume of 1 ml 100 g⁻¹ and a ventilation rate of 50–55 breaths min⁻¹. Histamine responses were obtained at 5 min intervals. SK&F 96231 alone (a) or in the presence of 0.1 µmol kg⁻¹ min⁻¹ sodium nitroprusside (SNP) (b) was infused over 30 min at a rate of 0.1 (●), 0.3 (▲), 0.6 (▼) or 1 (■) mg kg⁻¹ min⁻¹; (○) responses to histamine in vehicle control animals and; (□) effect of SNP alone. SNP was infused for 15 min prior to commencing infusion of SK&F 96231. Each point is the mean ± s.e.mean of 3 experiments.

Figure 2 Inhibition of histamine-induced (100 nmol kg⁻¹, i.v.) bronchoconstriction in anaesthetized guinea-pigs by SK&F 96231 administered by insufflation into the airway. Open columns are responses to SK&F 96231 (300 µg or 1 mg, *n* = 5) alone and (hatched) in the presence of SNP (0.1 µmol kg⁻¹ min⁻¹, i.v., *n* = 5) or (diagonal lines) isosorbide dinitrate (200 µg by insufflation, *n* = 3). The solid column is the response to isosorbide alone (*n* = 4). SK&F 96231 was administered in 5 mg lactose carrier, which alone caused a small potentiation of the response to histamine (5.1 ± 3.2%, *n* = 5). The SNP infusion also caused a small increase in the bronchoconstriction elicited by histamine (7.4 ± 5.9%, *n* = 5). **P* < 0.05 compared to SK&F 96231 alone.

Figure 3 The effect of salbutamol and rolipram (both 3, 30 and 300 µg), administered by insufflation into the airway, on histamine-induced (100 nmol kg⁻¹, i.v.) bronchoconstriction in the anaesthetized guinea-pig. Compounds were administered directly into the airway in 5 mg lactose carrier. Results are expressed as % inhibition of the histamine response (*n* = 3).

SK&F 96231 (Figure 1b). Under these latter conditions the inhibition of histamine-induced bronchoconstriction was rapid in onset, achieving a maximum within 5 min of starting the SK&F 96231 infusion; in addition doses which previously were devoid of inhibitory activity were found to cause > 50% inhibition of histamine bronchoconstriction in the presence of SNP.

Administered directly into the airway, SK&F 96231 (300 µg or 1 mg) caused a small, short-lived ($4.9 \pm 1.9\%$, t_1 6.6 ± 1.9 min and $9.9 \pm 10\%$, t_1 4.7 ± 2.3 min respectively) inhibition of the increase in pulmonary inflation pressure elicited by bolus injection of histamine (100 nmol kg^{-1} , i.v.). SK&F 96231 however significantly inhibited the histamine-induced bronchoconstriction ($40 \pm 4\%$ at $300 \mu\text{g}$ and $59 \pm 7\%$ at 1 mg , $n = 5$, $P < 0.05$) in the presence of SNP (infused i.v. at a dose that alone was without effect on the histamine induced bronchoconstriction: $0.1 \mu\text{mol kg}^{-1} \text{ min}^{-1}$). There was however no effect of SNP on the duration of the inhibition.

In contrast to the potentiation of the antispasmodic activity of SK&F 96231 by SNP, when the latter was administered by i.v. infusion, there was no potentiation of the effects of SK&F 96231 by SNP ($300 \mu\text{g}$) when both were administered locally. In contrast whilst isosorbide dinitrate ($200 \mu\text{g}$) administered directly into the airway caused only a $15 \pm 5\%$ inhibition of the responses to histamine its combination with SK&F 96231 ($300 \mu\text{g}$) reduced the histamine-induced bronchoconstriction by $62 \pm 1.8\%$ (Figure 2), the duration of the inhibition however was not affected.

Salbutamol and rolipram ($3\text{--}300 \mu\text{g}$) caused dose-related inhibitions of histamine-induced bronchoconstriction (Figure

3, Table 1); at $300 \mu\text{g}$, histamine responses were reduced by $91 \pm 2\%$ and $59 \pm 10\%$ respectively. The PDE III inhibitor, siguazodan ($300 \mu\text{g}$) was without effect on histamine responses but they were reduced $28 \pm 5\%$ by the PDE III inhibitor, SK&F 95654 ($300 \mu\text{g}$). There was a marked potentiation of the effects of rolipram in the presence of SK&F 95654 (Figure 4, Table 1).

Mean resting blood pressure in these experiments was $43.3 \pm 1.4 \text{ mmHg}$ systolic, $28.0 \pm 1.14 \text{ mmHg}$ diastolic ($n = 55$). Administration of salbutamol, SK&F 96231, SK&F 95654 and rolipram (or the combination of the latter two) into the airways was accompanied by a small (up to 10 mmHg), transient fall in blood pressure associated with disconnection of the animal from the ventilator. On reconnection of ventilation this was followed by a return to pre-dosing levels ($n = 23$) or by a small ($11.5 \pm 1.5 \text{ mmHg}$) but sustained increase in blood pressure ($n = 16$). The pressor response however was unrelated to either compound or dose, suggesting that it was a nonspecific event related to the dosing. Infusion

Table 1 Duration of the anti-spasmodic activity of salbutamol, rolipram and SK&F 95654 when given directly into the airway of anaesthetized guinea-pigs

	Inhibition of histamine induced bronchoconstriction		
	3 µg	(t_1 min) 30 µg	300 µg
Salbutamol	8.7 ± 1.7	17 ± 2.9	$> 30 \text{ min}$
Rolipram	4.7 ± 2.3	8.7 ± 1.7	13.7 ± 4.4
SK&F 95654		3.7 ± 1.7	7.0 ± 0.0
Rolipram (30 µg) + SK&F 95654	12 ± 0.0	15.3 ± 3.3	23.7 ± 1.7

Histamine (100 nmol kg^{-1} , i.v.) was administered at 5 min intervals. When a reproducible increase in pulmonary inflation pressure had been established, compounds were administered directly into the airway, by insufflation on 5 mg lactose carrier, and their effect on histamine responses followed for the following 30 min. Duration of action (t_1) is expressed as the time to 50% recovery of the increase in pulmonary inflation pressure induced by histamine. The magnitude of the inhibition of histamine responses are as shown in Figures 3 and 4 ($n = 3$ in all cases).

Figure 4 Potentiation of the inhibition of histamine-induced (100 nmol kg^{-1} , i.v.) bronchoconstriction by rolipram in the presence of SK&F 95654. Rolipram ($30 \mu\text{g}$) was administered by insufflation into the airway alone (solid column, $n = 3$) or in combination with SK&F 95654 ($3, 30, 300 \mu\text{g}$, hatched columns, $n = 3$). Open columns are the inhibitory effects of SK&F 95654 administered alone ($n = 3$). * $P < 0.05$ compared to rolipram alone.

Table 2 Effect of SK&F 96231 on *in vitro* tracheal cyclic GMP levels

	Cyclic GMP (pmol mg ⁻¹ protein, $0.1 \mu\text{M}$ CCh)		Cyclic GMP (pmol mg ⁻¹ protein, $1 \mu\text{M}$ CCh)	
	1 µM	10 µM	1 µM	10 µM
Carbachol alone	$0.71 \pm 0.12^*$		$0.65 \pm 0.01^*$	
+ SK&F 96231	0.62 ± 0.07	0.88 ± 0.07	0.64 ± 0.08	0.61 ± 0.14
		$1.49 \pm 0.28^\dagger$		$1.39 \pm 0.11^\dagger$

Guinea-pig tracheal tubes were incubated, free floating in Krebs-Henseleit at 37°C , containing either $0.1 \mu\text{M}$ or $1.0 \mu\text{M}$ carbachol (CCh) alone or in combination with SK&F 96231 ($1\text{--}100 \mu\text{M}$), for 15 min. Tissues were frozen by immersion in liquid nitrogen prior to assay for cyclic GMP levels by radioimmunoassay (Amersham). Results are the mean $\pm$ s.e.mean of 3 observations. Unstimulated levels of cyclic GMP in control tissues were $0.37 \pm 0.08 \text{ pmol mg}^{-1} \text{ protein}$. * $P < 0.05$ compared to unstimulated levels; $^\dagger P < 0.05$ compared to carbachol alone.

of SNP however caused a sustained 24.7 ± 1.55 mmHg fall in systolic blood pressure. Neither SK&F 96231 nor isosorbide dinitrate alone had any effect on resting blood pressure, when administered directly into the airways, their combination, however, caused a transient fall (14.7 ± 1.76 mmHg).

Effect of SK&F 96231 infusion on tracheal cyclic GMP levels *in vitro* and *in vivo*

Previous observations indicate that SK&F 96231 elevates total cyclic GMP levels in guinea-pig lung strips *in vitro* (Murray *et al.*, 1991). Since interpretation of these data is complicated by the highly vascularized nature of this preparation, we have carried out studies to determine if SK&F 96231 also increases cyclic GMP levels in tracheal tissue *in vitro* and *in vivo*. The results are summarized in Tables 2 and 3. In the isolated trachea, cyclic GMP levels were elevated above control (in the presence of carbachol) only at concentrations of SK&F 96231 that caused maximum relaxations of carbachol elevated tone. When administered by i.v. infusion in the anaesthetized guinea-pig, SK&F 96231 caused a dose- and time-dependent increase in tracheal cyclic GMP levels that paralleled the time-course of inhibition of histamine-induced contractions. Table 4 shows the results of a study of the interaction of SK&F 96231 and SNP. When administered alone there was no effect of SNP ($0.1 \mu\text{mol kg}^{-1} \text{min}^{-1}$) on either the histamine responses or on tracheal cyclic GMP levels (measured at 5 min post-dose). Similarly SK&F 96231 ($0.6 \text{ mg kg}^{-1} \text{min}^{-1}$) by itself did not alter histamine-induced

bronchoconstriction but caused a small non-significant increase in cyclic GMP content. In contrast, the combination of SK&F 96231 and SNP at the same doses caused a 68% inhibition of histamine-induced bronchoconstriction and a 4 fold increase in tracheal cyclic GMP content.

Relaxant activity of SK&F 96231 in guinea-pig isolated trachea

SK&F 96231 (0.1 – $100 \mu\text{M}$) and SNP (0.01 – $10 \mu\text{M}$) caused concentration-related relaxation of tissues precontracted with $0.1 \mu\text{M}$ CCh, EC_{50} 's $10.5 \mu\text{M}$, (95% confidence limits 6.79 to $16.22 \mu\text{M}$) and $0.33 \mu\text{M}$, (95% confidence limits 0.23 to $0.46 \mu\text{M}$) respectively; the spasmolytic activity of both SK&F 96231 and SNP was reduced in tissues precontracted with $1 \mu\text{M}$ CCh (Figure 5). The functional antagonism of the

Table 3 Time-course of SK&F 96231-induced cyclic GMP accumulation in anaesthetized guinea-pigs

SK&F 96231	Cyclic GMP (pmol mg^{-1} protein)		
	5 min	15 min	30 min
$0.6 \text{ mg kg}^{-1} \text{min}^{-1}$	0.33	0.45	0.74
$1.0 \text{ mg kg}^{-1} \text{min}^{-1}$	0.56	1.07	1.17

SK&F 96231 was infused i.v. for 30 min at 0.6 or $1.0 \text{ mg kg}^{-1} \text{min}^{-1}$. Bronchoconstriction was induced by i.v. injection of histamine (100 nmol kg^{-1}) at 5 min intervals beginning 15 min prior to infusion of SK&F 96231, as in the functional studies. Tracheae were removed at time intervals during the infusion, snap frozen in liquid nitrogen and assayed for cyclic GMP levels by radioimmunoassay. Assays were performed in triplicate and values are the mean of data from two separate experiments. Cyclic GMP levels in animals not receiving SK&F 96231 were 0.19 ± 0.032 pmol mg^{-1} protein ($n = 3$).

Figure 5 Functional antagonism of the spasmolytic activity of sodium nitroprusside (SNP) (Δ) and SK&F 96231 ($\circ$). Tissues were pre-contracted with $0.1 \mu\text{M}$ carbachol (open symbols) or $1.0 \mu\text{M}$ carbachol (closed symbols). When the increase in tension had reached a plateau, concentration-response curves to SNP or SK&F 96231 were constructed by their cumulative addition to the bath. Each point is the mean $\pm$ s.e. mean of 5 or 6 observations.

Table 4 Effect of SK&F 96231 alone and in combination with sodium nitroprusside (SNP) on histamine induced bronchoconstriction and tracheal cyclic GMP levels in anaesthetized guinea-pigs

	Increase in pulmonary inflation pressure (mmHg)	Tracheal cyclic GMP (pmol mg^{-1} protein)
Control	30 ± 3.9	0.19 ± 0.03
SNP ($0.1 \mu\text{mol kg}^{-1} \text{min}^{-1}$)	38 ± 3.9	0.24 ± 0.03
SK&F 96231 ($0.6 \text{ mg kg}^{-1} \text{min}^{-1}$)	29 ± 6.4	0.33 ± 0.12
SNP ($0.1 \mu\text{mol kg}^{-1} \text{min}^{-1}$) + SK&F 96231 ($0.6 \text{ mg kg}^{-1} \text{min}^{-1}$)	$9.5 \pm 2.9^*$	$0.68 \pm 0.22^*$

Bronchoconstriction was induced by i.v. injection of histamine (100 nmol kg^{-1}) at 5 min intervals. SNP was infused for 15 min prior to infusion of SK&F 96231 as in the functional studies. Effects of agents on the increase in pulmonary inflation pressure were measured 5 min after commencing the infusion of SK&F 96231. In separate experiments tracheae were removed at this time point, frozen in liquid nitrogen and assayed for cyclic GMP levels by radioimmunoassay. Assays were performed in triplicate and values are the mean of data from three separate experiments. $^*P < 0.05$ compared to control.

relaxant activity of SK&F 96231 was not accompanied by a concomitant reduction in tracheal cyclic GMP levels (Table 2). The spasmolytic activity of SK&F 96231 was unaffected by epithelium removal or by 10 μ M methylene blue.

Discussion

Both SNP and SK&F 96231 elevate cyclic GMP levels in airway smooth muscle *in vitro* (Katsuki & Murad, 1977; Torphy *et al.*, 1985; Ishii & Murad, 1989; Murray *et al.*, 1991); however, whilst SNP stimulates the soluble guanylate cyclase directly (Waldman & Murad, 1987) the ability of PDE V inhibitors to elevate smooth muscle cyclic GMP levels, *in vivo*, might be expected to be dependent on the level of endogenous guanylate cyclase activity.

Consistent with this hypothesis we have shown that SK&F 96231, infused i.v. at high doses, causes a slowly developing inhibition of histamine-induced bronchoconstriction which was accompanied by a parallel increase in tracheal cyclic GMP content. We consider therefore that the effects of SK&F 96231 on airway tone *in vivo* are likely to be a consequence of cyclic GMP accumulation. This conclusion is supported by the observation that the SK&F 96231 inhibition of histamine-induced bronchoconstriction and increase in tracheal cyclic GMP content was significantly enhanced in the presence of a sub-threshold infusion of SNP. Moreover at infusion rates of SK&F 96231 and SNP which alone were devoid of any inhibitory activity, their combination elicited a marked inhibition of histamine responses. Similarly, administered directly into the airway SK&F 96231 was largely without effect on the bronchoconstriction to histamine. This is consistent with its poor anti-spasmogenic activity when administered by i.v. bolus injection (32% inhibition of histamine induced bronchoconstriction at 1 mg kg⁻¹ i.v.; unpublished observation). When coupled with a subthreshold i.v. infusion of SNP however, SK&F 96231 administered directly into the airways caused significant inhibition (40% at 300 μ g, 62% at 1 mg). In addition, although SNP failed to potentiate the effects of SK&F 96231 when both agents were delivered by the inhaled route the less labile nitrovasodilator, isosorbide dinitrate, significantly potentiated its bronchorelaxant activity. This combined action, of agents that stimulate guanylate cyclase directly and SK&F 96231, particularly at doses, systemically or locally, that alone are without any anti-spasmogenic activity strongly suggests that even at mechanically silent doses of SK&F 96231, PDE V activity is inhibited. That this is only uncovered in the presence of guanylate cyclase stimulation therefore suggests that the anti-spasmogenic and spasmolytic activity of PDE V inhibitors, *in vivo*, is limited by guanylate cyclase activity.

Isoenzyme profiling of guinea-pig trachea (Turner, unpublished information; Tagaki *et al.*, 1992) has shown that it contains at least two cyclic GMP hydrolytic activities, the Ca²⁺-calmodulin stimulated PDE I and the cyclic GMP-specific PDE V. We cannot rule out therefore the possibility that the poor spasmolytic activity of SK&F 96231 is due to hydrolysis of cyclic GMP by PDE's other than PDE V. However, since coupling SK&F 96231 with an exogenous stimulus to guanylate cyclase activity uncovered significant inhibition of bronchoconstriction, we consider it more likely that it is a consequence of low endogenous guanylate cyclase activity in the airway, perhaps due to the absence of an endogenous agonist.

In vitro studies also showed that the SK&F 96231- and SNP-induced relaxations of guinea-pig trachea were functionally antagonized by high concentrations of carbachol (but not histamine, data not shown). Functional antagonism of relaxant responses by high concentrations of muscarinic agonists have previously been reported in canine and bovine tracheal smooth muscle for agents which elevate cyclic AMP:- prostaglandin E₂, isoprenaline, forskolin, SK&F 94836, AH21-132; but not cyclic GMP (Torphy *et al.*, 1983;

1985; 1988; Giembycz & Barnes, 1991). The results of the present study demonstrated that, unlike canine trachea where the concentration of muscarinic agonist is reported to have little effect on the mechanical responses to SNP or 8-bromo cyclic GMP (Torphy *et al.*, 1985), in the guinea-pig trachea, the spasmolytic effects of SK&F 96231 and SNP are modified by the degree of muscarinic tone. In contrast the increase in intracellular cyclic GMP, stimulated by SK&F 96231, was unaffected; indeed, consistent with previous findings in canine tracheal smooth muscle (Katsuyama *et al.*, 1990) carbachol alone stimulated a 2 fold increase in tracheal cyclic GMP levels. The attenuation of the spasmolytic activity of SK&F 96231 by high concentrations of carbachol therefore cannot be explained by a muscarinic receptor modulation of guanylate cyclase activity or phosphodiesterase activation. Torphy *et al.* (1988) have reported that methacholine suppresses the ability of SK&F 94836 to activate the cyclic AMP-dependent protein kinase (Torphy *et al.*, 1985; 1988). Our present results suggest that the functional antagonism of the relaxant activity of agents that elevate cyclic GMP may similarly operate at or beyond the level of the cyclic GMP-dependent protein kinase.

We have also shown that when administered directly into the airway, rolipram inhibits histamine-induced bronchoconstriction demonstrating that selective PDE IV inhibitors can act as bronchodilators when inhaled. The bronchodilator activity of rolipram however was only 60% of that to an equivalent dose of salbutamol (300 μ g) and was of a shorter duration. In contrast Underwood *et al.* (1993) have reported that rolipram fails to affect significantly histamine-induced contraction of guinea-pig isolated trachea at concentrations up to 1 μ M. Furthermore, the same authors showed that rolipram only reduced histamine-induced bronchoconstriction at high doses (3–10 mg kg⁻¹, i.v.; Underwood *et al.*, 1993). Similarly, rolipram (3 mg kg⁻¹, i.v.) has been reported to elicit only a weak inhibition of the bronchoconstriction induced by aerosolized histamine (Howell *et al.*, 1993). Our present study therefore indicates that the anti-spasmogenic potency of rolipram, when delivered directly into the airway is approximately 30 fold greater than when it is given i.v.

The results of the present study also suggest that PDE III inhibitors, when administered into the airway are relatively ineffective in inhibiting histamine-induced bronchoconstriction. Nevertheless, the combination of rolipram and the PDE III inhibitor SK&F 95654, at doses that alone had only minimal effects on histamine responses, caused a marked inhibition of histamine-induced bronchoconstriction. This combined action of inhaled PDE III and IV inhibitors has, to our knowledge, not previously been reported *in vivo* but is in agreement with their reported interaction in guinea-pig isolated trachea (Harris *et al.*, 1989). The results suggest that both PDE III and PDE IV are involved in hydrolysis of cyclic AMP in the guinea-pig airway, *in vivo*, but that when only one isoenzyme is inhibited its ability to elevate intracellular cyclic AMP levels and thence to cause bronchodilatation is restricted by cyclic AMP hydrolysis through other PDE isoforms. The data suggest therefore that inhibitors of both PDE III and PDE IV may offer greater potential as bronchodilators in asthma than inhibitors of either isoenzyme alone.

In conclusion PDE V inhibitors alone are relatively weak inhibitors of histamine-induced bronchoconstriction in the anaesthetized guinea-pig. The enhancement of the inhibition of bronchoconstriction by coupling SK&F 96231 with either SNP or isosorbide dinitrate suggests that the poor activity of SK&F 96231 alone is secondary to low guanylate cyclase activity in the airway. The combination of PDE V inhibitor and guanylate cyclase agonist might therefore provide significant bronchodilator potential in asthma.

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