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Effects of inhibitors of phosphodiesterase, on antigen-induced bronchial hyperreactivity in conscious sensitized guinea-pigs and airway leukocyte infiltration

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1 The aim of this study was to determine the effects of inhibitors of phosphodiesterase (PDE) on the early and late phase bronchoconstriction in sensitized, conscious guinea-pigs and the subsequent development of acute airway hyperreactivity to the inhaled thromboxane mimetic, U46619, and leukocyte infiltration following ovalbumin (OvA) challenge.

2 Following an inhalation challenge with OvA, there was an early bronchoconstriction which peaked at 15 min with recovery after 3–4 h. A late phase bronchoconstriction occurred between 17 and 24 h after challenge. The PDE 4 inhibitors, Ro 20-1724 (3 mg kg^{-1}, i.p.) and rolipram (1 mg kg^{-1}, i.p.) administered 30 min before and 6 h after antigen challenge (double dosing regimen), did not affect the development of the early or late phase responses.

3 Seventeen to twenty four hours following an acute OvA or saline challenge, a consistently greater bronchoconstrictor response to inhaled U46619 was observed in the OvA challenged group. This increase in responsiveness was significantly attenuated by the administration of Ro 20-1724 and rolipram 30 min before and 6 h after antigen challenge ($P<0.05$); this was not attributable to a residual bronchodilator effect of these compounds. There was a trend towards inhibition of the hyperreactivity to U46619 by aminophylline but not by the PDE3 inhibitors, siguazodan or SKF 95654.

4 Aminophylline, rolipram and Ro 20-1724 when administered as the double dose regimen attenuated the rise in macrophages, eosinophils and neutrophils recovered in bronchial lavage fluid 17 to 24 h after antigen challenge.

5 The dose of Ro 20-1724 given at 6 h post challenge was essential for attenuation of airway hyperreactivity and to protect against leukocyte influx.

6 In summary, aminophylline, rolipram and Ro 20-1724 have anti-inflammatory effects against antigen-induced airway leukocyte infiltration. Rolipram and Ro 20-1724 additionally attentuated the development of acute airway hyperreactivity, effects which are probably mediated through inhibition of PDE type 4. A dose of PDE inhibitor 6 h after the antigen challenge appears to be essential to achieve this protection. Inhibitors of PDE type 3 were generally without effect. However, there was no effect of rolipram or Ro 20-1724 on the development of either the early or late phase type responses.

Keywords: Airway hyperreactivity; ovalbumin sensitization; conscious guinea-pigs; phosphodiesterase inhibitors; ovalbumin challenge; leukocyte infiltration; rolipram; Ro 20-1724; siguazodan; SKF 95654; aminophylline; U46619 bronchoconstriction

Introduction

Non-specific bronchial hyperreactivity and airway eosinophilia often accompany the development of a late asthmatic response following exposure to antigen, and are two of the classic features of bronchial asthma (reviewed by Town & Holgate, 1991). Anti-inflammatory steroids are the current mainstay of treatment (Djukanovic *et al*., 1992; British Thoracic Society, 1993). Theophylline, a non-specific inhibitor of phosphodiesterase (PDE), has also been used for a number of years although its initial benefit was considered to be as a result of bronchodilatation (Persson, 1986). More recently it has been suggested that theophylline may also possess anti-inflammatory activity, which is perhaps of greater significance to therapy (Ward *et al*., 1993; Sullivan *et al*., 1994). Unfortunately, the use of theophylline is limited by its narrow therapeutic window and prevalence of side effects, which has led to the current evaluation of selective inhibitors of PDE as potential anti-anaphylactic agents.

At present, seven PDE isoenzyme families have been identified (Beavo & Reifsnyder, 1990; Nicholson *et al*., 1991; Beavo *et al*., 1994; Loughney & Ferguson, 1994). Of particular interest is the adenosine 3′:5′-cyclic monophosphate (cyclic AMP)-specific PDE, PDE 4, which is expressed in a variety of pro-inflammatory cells. If the anti-inflammatory effects of theophylline are indeed due to the inhibition of PDE in these cells then selective inhibition of PDE 4 would be of potential benefit in terms of activity and a reduction in side effects may follow. The former has been borne out in practice in a number of animal models of inflammation (Howell *et al*., 1993; Underwood *et al*., 1993; 1994; Teixeira *et al*., 1994; Turner *et al*., 1994; Banner & Page, 1995; Danahay & Broadley, 1995).

Eosinophils are of particular interest in models of airway inflammation since they are the predominant inflammatory cell type in the airways of asthmatic subjects, and are linked with late phase asthmatic responses and with airway hyperreactivity (Frigas & Gleich, 1986; Metzger *et al*., 1986; Wardlaw & Kay, 1987; Hargreave, 1989; Kay, 1991). They express PDE 4 as their major PDE, inhibition of which has been shown to reduce respiratory burst activity and the production of pro-inflammatory mediators such as superoxide, major basic protein (MBP), eosinophil cationic protein and eosinophil derived neurotoxin (Souness *et al*., 1991; 1995; Dent *et al*., 1991; Hatzelmann *et al*., 1995) *in vitro*. Recent work has demonstrated that the direct instillation of human MBP and other highly charged cations into the airways of rats can induce a hyperreactive state (Coyle *et al*., 1994), and it has been recognised for some time that levels of MBP are elevated in the sputum of asthmatics (Frigas *et al*., 1981).

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The aim of this study was to evaluate any anti-inflammatory activity of selective and non-selective inhibitors of PDE. Aminophylline, the ethylenediamine salt of theophylline was used as a non-selective inhibitor in current therapeutic use, siguazodan and SKF 95654 as selective inhibitors of PDE3 and rolipram and Ro 20-1724, as selective inhibitors of PDE 4 (Beavo & Reifsnyder, 1990; Nicholson *et al*., 1991). The effects of these compounds were examined on early and late phase-like asthmatic responses of conscious guinea-pigs induced by a single antigen challenge, the development of airway hyperreactivity and leukocyte influx into the airways by methods previously used in this laboratory (Lewis & Broadley, 1995a,b).

Methods

Sensitization

Male Dunkin-Hartley guinea-pigs (200–250g) Halls, Staffs, U.K.) were sensitized to ovalbumin (OvA 1 ml i.p. of a suspension containing 10 μg OvA and 100 mg aluminium hydroxide in normal saline). All procedures were then begun 14 days later.

Administration of PDE inhibitors

Aminophylline (30 mg kg^{-1}, i.p.), siguazodan (3 mg kg^{-1}, i.p.), SKF 95654 (3 mg kg^{-1}, i.p.), rolipram (1 mg kg^{-1}, i.p.), and Ro 20-1724 (1 and 3 mg kg^{-1}, i.p.) were administered both 30 min before and 6 h after an OvA challenge. Doses were selected to compare with those used by others previously (Gristwood *et al*., 1991; Underwood *et al*., 1993,1994; Banner & Page, 1995). In additional groups of guinea-pigs, Ro 20-1724 (3 mg kg^{-1}, i.p.) was administered separately at each of the time points, with the specified volume of vehicle (50% DMSO in saline) being administered *in lieu* of the other Ro 20-1724 dose. The two control groups, which received either saline or OvA challenges, without PDE inhibitor, were dosed with vehicle 30 min before and 6 h after the appropriate inhalation exposure.

Inhalation exposures

OvA exposures Sensitized animals received a 10 min inhalation exposure of either saline or a 0.5% solution of OvA in saline, 30 min after an i.p. dose of mepyramine maleate (30 mg kg^{-1}) to protect against fatal anaphylaxis. A Wright nebulizer, suppled with air at a pressure of 20 lb p.s.i., was used to deliver the antigen or vehicle into a sealed, perspex chamber ($350 \times 200 \times 150$ mm) at 0.5 ml min^{-1}. Any animal appearing to be in respiratory distress during the exposure was immediately removed and the exposure was considered complete.

U46619 exposures To study the development of airway hyperreactivity at 17–24 h after OvA challenge, a further challenge with a dose of inhaled U46619 (30 ng ml^{-1} in saline) for 60 s was made. This has previously been found to be a threshold dose (Lewis & Broadley, 1995b) for a small bronchoconstriction.

To determine the degree of any residual bronchodilator effect of the PDE inhibitors at the time of the U46619 exposure, a larger dose of U46619 (1 μg ml^{-1}, 60 s) was used to induce a reproducible bronchoconstriction in sensitized, but non-antigen challenged, conscious guinea-pigs. Animals were dosed with the double dose PDE inhibitor regimen but received no antigen challenge. U46619 (1 μg ml^{-1}) was then administered 17 h after the first dose of PDE inhibitor and airway function of the conscious animals monitored.

Measurement of respiratory function

Whole body plethysmography of the conscious guinea-pig was used to monitor airway function, recorded as specific airways conductance (sG_{aw}). The groups used for the U46619 exposures were monitored before and at regular intervals for the 20 min after the challenge. Recordings were taken at 0, 2, 5, 10 and 20 min with the animals being removed from the box between all readings except for the 2 and 5 min times. The method was as described by Griffiths-Johnson and co-workers (1988), although a computerized data acquisition system replaced the original oscilloscope and angle resolver. Animals used to study the early and late phase responses to antigen were monitored over the 72 h following exposure.

Animals were fitted with a face mask and placed in a restrainer which was then slid into the plethysmograph chamber. The computer ran AcqKnowledge software with a Biopack data acquisition system. This system was capable of acquiring and storing data referring to the air flow across a pneumotachograph (Mercury FIL) as the animal breathed. The resulting change in box volume (pressure) was simultaneously measured. Changes in air flow and box pressure were measured by two pressure transducers, UP1 and UP2, respectively. The resulting waveforms could then be rapidly analysed by comparing the gradients of the flow and the box pressure waves at a point where flow tended towards zero, i.e. in the first 30 ms of a breath commencing. A function of these two parameters, allowing for air pressure and the weight of the animal, gives the resulting value for sG_{aw}. A minimum of 5 breaths were analysed for each animal at each time point. Before all experiments, animals were handled and familiarized with the apparatus to reduce stress.

Broncho-alveolar lavage

Within 30 min of the U46619 exposure, all animals underwent broncho-alveolar lavage. The guinea-pigs were killed with an overdose of pentobarbitone (200 mg kg^{-1}, i.p., Euthatal) and the trachea was cannulated. Hanks Balanced Salt Solution (1 ml 100 g^{-1} guinea-pig weight) (free of Ca^{2+}, Mg^{2+} and phenol red), was injected through the cannula into the lungs and recovered after 3 min. This was repeated and a total cell count (cells ml^{-1}) performed with a Neubauer haemocytometer. Two drops of the lavage suspension were then centrifuged by a Shandon Cytospin at 1000 r.p.m. for 10 min. The resulting cells were stained with Giemsa/May-Grunwald stains (50:50) for 2 min and then a further 5 min after dilution with phosphate buffer (pH 7.4). A differential cell count was then performed on a minimum of 500 cells to identify eosinophils, macrophages and neutrophils.

Expression of results and statistical analysis

Because of the inter-subject variability of sG_{aw} values, they are expressed as the % change from a baseline value of sG_{aw} taken immediately before the start of a procedure. Absolute values of sG_{aw} are also presented for the baseline and the 15 min and 17–24 h responses after OvA challenge and 2 min after U46619 challenge. Because there were no significant differences between the volumes of lavage fluid recovered from any of the groups studied (70–80% recovery), values are expressed as cells ml^{-1}. All results were compared by use of ANOVA with ad-hoc Neuman-Keuls test for multiple comparisons.

Drugs and solutions

Ovalbumin, Hanks Balanced Salt Solution (free of Ca^{2+}, Mg^{2+} and phenol red), dimethylsulphoxide (DMSO) and U46619 (9,11-dideoxy-11α,9α-epoxymethano-prostaglandin $F_{2\alpha}$) were purchased from Sigma (Poole, Dorset, U.K.) Ro 20-1724 (4-(3-butoxy-4-methoxybenzyl)-2-imidazolidinone) was purchased from R.B.I. (St. Albans, Hertfordshire, U.K.). Siguazodan (**R**,**S**-2-cyano-1-methyl-3-[4-(1,4,5,6-tetrahydro-4-methyl-6-oxo-3-pyridazinyl)phenyl]guanidine) and SKF 95654 (**R**,**S**-4,5-dihydro-6-[4-(1,4-dihydro-4-oxopyridin-1-y1)phenyl]-5-methyl-3(2H)-pyridazinone) were gifts from SmithKline Beechams (Welwyn, U.K.). Rolipram was supplied by Glaxo-

Wellcome (Stevenage, U.K.). Saline 0.9% was purchased from Baxter Healthcare U.K. Aluminium hydroxide ($Al(OH)_3$) and mepyramine maleate were gifts from Rhone-Poulenc-Rorer (Dagenham, Essex). Aminophylline was administered as a commercially available injection (25 mg ml^{-1}, Evans, U.K.). Ro 20-1724, rolipram, SKF 95654 and siguazodan were dissolved in DMSO (50%) in saline.

Results

Effect of Ro 20-1724 and rolipram on early and late phase responses

All animals, untreated and Ro 20-1724 (3 mg kg^{-1}) or rolipram (1 mg kg^{-1}) treated, exhibited an early and a late phase response (Figure 1a,b and Table 1) as a result of the OvA challenge. The early phase response peaked at 15 min and animals had recovered to basal values after 3 to 4 h. Predosing with the PDE 4 inhibitors had no significant effect on the magnitude or duration of this phase. The late phase response occurred between 17 and 24 h in all of the animals studied. The mean maximum fall in sG_{aw} between 17 and 24 h was not significantly affected by treatment with Ro 20-1724 or rolipram. There were no other changes in airway function noted at 48 or 72 h post-challenge.

Airway responsiveness to inhaled U46619

Between 17 and 24 h after either saline or antigen exposure, guinea-pigs were challenged with inhaled U46619 (30 ng ml^{-1}, 60 s). A consistently greater bronchoconstrictor response to U46619 was observed in the antigen challenged group (Figure 2a and Table 2). This bronchoconstriction was maximal at 2 min after the U46619 exposure, with most animals recovering by 10 min. There were no statistically significant differences between starting baseline values of sG_{aw} for any of the groups of animals studied (Table 2).

Effects of PDE inhibitors on increased responsiveness to U46619

When administered 30 min before and 6 h after the antigen challenge, the increased responsiveness to U46619 was significantly attenuated by the higher dose of Ro 20-1724 (3 mg kg^{-1}) (Table 2 and Figure 2b) and rolipram (1 mg kg^{-1}) (Figure 3a) ($P<0.05$) but not the lower dose of Ro 20-1724 (1 mg kg^{-1}). There was a trend towards an attenuation of this increased responsiveness with aminophylline (30 mg kg^{-1}) (Figure 3a) ($P<0.10$). Siguazodan (3 mg kg^{-1}) and SKF 95654 (3 mg kg^{-1}) (Figure 3b) were without significant effect ($P>0.10$).

When Ro 20-1724 (3 mg kg^{-1}) was administered as a single dose 6 h after the antigen challenge, there was no significant attenuation of the development of the hyperresponsiveness although there was a trend towards protection ($P<0.10$) (Table 2 and Figure 2c). A single dose of Ro 20-1724 (3 mg kg^{-1}), administered 30 min before challenge was without significant protection against the increased responsiveness to inhaled U46619.

U46619, at the higher dose of 1 μg ml^{-1}, induced a reproducible bronchoconstriction in sensitised but non-OvA challenged guinea-pigs, which peaked at 2 min post-exposure ($-27.3\pm3.8\%$). This response was unaffected by aminophyl-

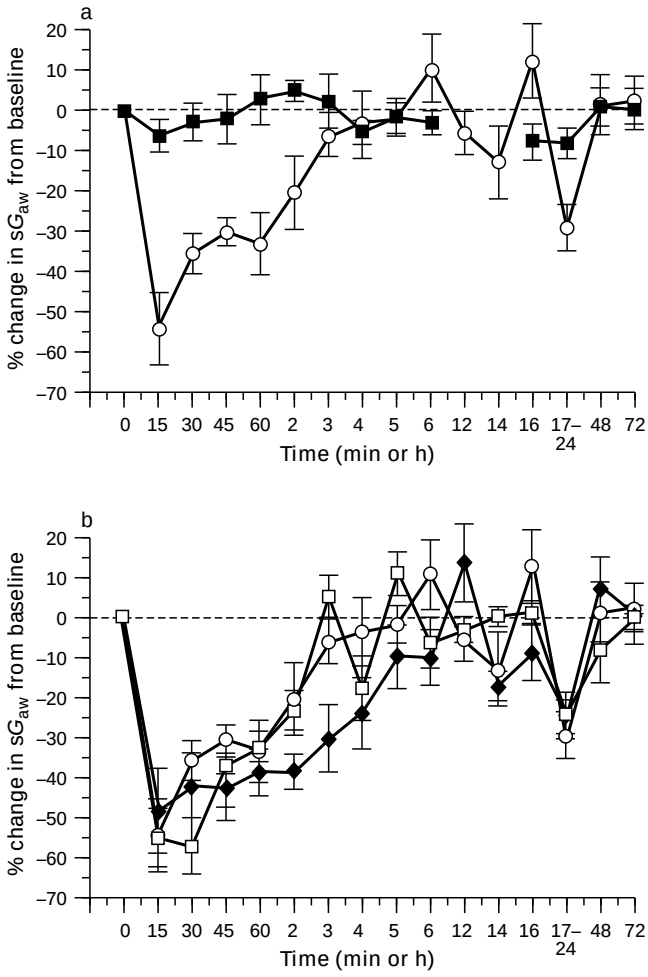

Figure 1 (a) The effects of inhaled saline (■) or OvA (○) challenge on the airway function of sensitized guinea-pigs. (b) The effects of Ro 20-1724 (3 mg kg^{-1}, ◆) or rolipram (1 mg kg^{-1}, □) given at 30 min before and 6 h after the OvA challenge on the airways response. Mean responses (with vertical lines indicating s.e.mean) measured as the % change in sG_{aw} from baseline at regular intervals after the OvA exposure are shown ($n=6-11$). Negative values represent bronchoconstriction.

Table 1 The effects of PDE4 inhibitors Ro 20-1724 and rolipram on the early and late phase responses of conscious sensitized guinea-pigs to inhaled ovalbumin (OvA)

	Baseline	*15 min response*		*17–24 h response*	
Treatment	(absolute)	(absolute)	(%Δ)	(absolute)	(%Δ)
Untreated (Sal)	0.512 ± 0.010	0.481 ± 0.026	$-6.2\pm3.9\%$**	0.469 ± 0.017	$-8.3\pm3.7\%$**
Untreated (OvA)	0.510 ± 0.021	0.241 ± 0.055	$-54.3\pm9.0\%$	0.354 ± 0.018	$-29.3\pm5.7\%$
Ro (OvA)	0.434 ± 0.026	0.192 ± 0.045	$-48.3\pm10.7\%$	0.370 ± 0.018	$-24.4\pm4.3\%$
RP (OvA)	0.693 ± 0.024	0.315 ± 0.057	$-55.0\pm7.3\%$	0.527 ± 0.050	$-24.1\pm5.9\%$

Ro 20-1724 (3mg kg^{-1}, Ro) and rolipram (1 mg kg^{-1}, RP) were administered at 30 min before and 6 h after the inhalation of OvA (0.5%). Values are expressed as absolute sG_{aw} (s^{-1} cmH_2O^{-1}) and as % change from baseline for the early (15 min) and late (17–24 h peak) antigenic-responses ($n=6-11$). Significant difference from the untreated (OvA) group indicated by $^{**}P<0.05$ or $^{*}P<0.10$ by ANOVA followed by Neuman-Keuls test. Negative values represent bronchoconstriction.

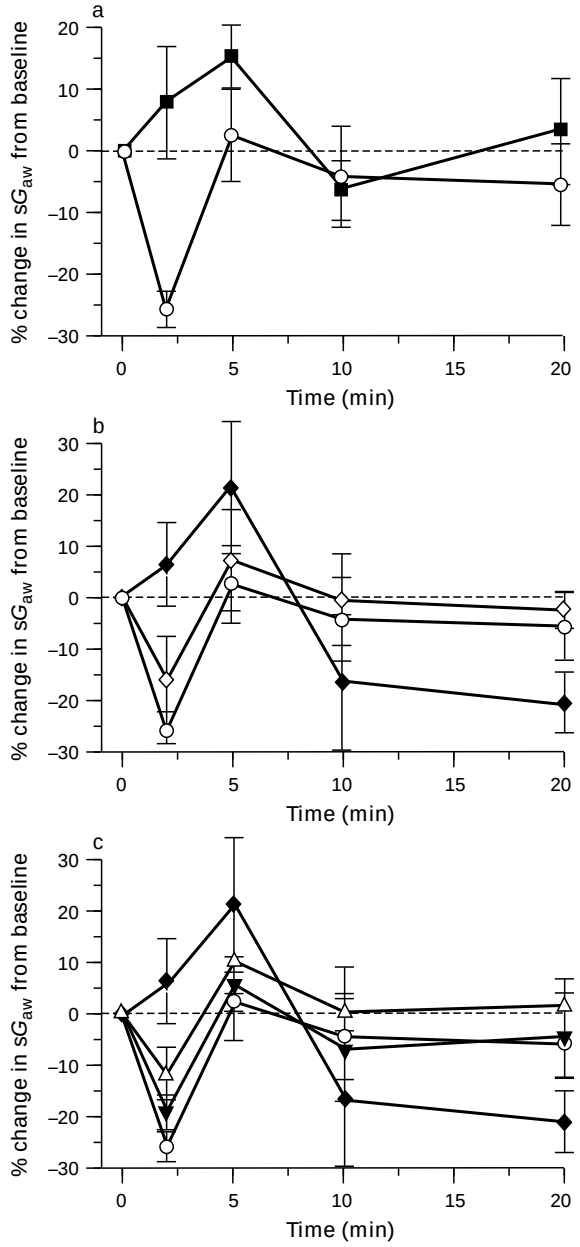

Figure 2 (a) The responsiveness to inhaled U46619 (30 ng ml^{-1} for 60 s) 17–24 h after either saline (■) or OvA (○) challenge of sensitized guinea-pigs, and its modulation by: (b) Ro 20-1724 (1 (◇) and 3 (◆) mg kg^{-1}), double dosing at 30 min before and 6 h after OvA challenge. (c) Ro 20-1724 (3 mg kg^{-1}) double dosing (◆), single pre-challenge dose (▼) or single post challenge dose (△). Mean responses (with vertical lines showing s.e.mean) measured as the % change in sG_{aw} from baseline at regular intervals after the U46619 exposure are shown ($n = 5-7$). Negative values represent bronchoconstriction.

line (30 mg kg^{-1}) ($-38.7 \pm 8.2\%$), rolipram (1 mg kg^{-1}) ($-38.4 \pm 9.7\%$) or Ro 20-1724 (3 mg kg^{-1}) ($-31.5 \pm 5.3\%$) at 17 h after commencement of the double dosing regimen. However, siguazodan treatment did significantly attenuate the response to this dose of U46619, the net response being a small bronchodilatation ($+4.9 \pm 7.0\%$) (Figure 4).

Leukocyte infiltration

There was a large rise in the numbers of leukocytes recovered in lavage fluid at 17 to 24 h after a single OvA exposure, all of which were significantly greater than the saline challenged group (Table 3). Ro 20-1724 (1 and 3 mg kg^{-1}) attenuated the rises in macrophages and eosinophils, with the higher dose also attenuating the neutrophilia, when administered at 6 h after the challenge. There appeared to be no additional protection afforded by pre-dosing. When only the pre-challenge dose was administered, Ro 20-1724 failed to attenuate the rises in macrophages and eosinophils although a significant reduction in the recovery of neutrophils was observed.

Aminophylline or rolipram treatment at 0 and 6 h also protected against the elevated leukocyte numbers. Siguazodan and SKF 95654 were without significant effect on either eosinophils or macrophages, although both did attenuate the rise in neutrophil numbers.

Discussion

Non-specific bronchial hyperreactivity and airway eosinophilia are two classic hallmarks of bronchial asthma and clinical evidence suggests that the two are linked (Frigas & Gleich, 1986; Metzger *et al*., 1986; Wardlaw & Kay, 1987; Hargreave, 1989; Kay, 1991). It has been proposed that toxic eosinophil metabolites damage the airways and lead to increased airway reactivity by exposing sensory nerve endings (Barnes, 1986) or through the loss of an epithelial derived relaxant factor (Flavahan *et al*., 1988). Animal models have been developed to study antigen-induced airway hyperreactivity (Howell *et al*., 1993; Turner *et al*., 1994; Lewis & Broadley, 1995b). Most studies have utilised anaesthetized animals to determine lung function. However, in the present study we have used a modification of the conscious animal model previously developed in this laboratory (Lewis & Broadley, 1995b). The major advantage of this paradigm is that any influence of vagal tone or sensory reflexes would not be abolished by the anaesthetic. Additionally, it could be argued that U46619, being a thromboxane-mimetic, parallels the action of an endogenous inflammatory mediator and is hence a more relevant bronchoprovocator than methacholine, for instance.

Ro 20-1724 and rolipram are highly selective inhibitors of PDE4 (Beavo & Reifsnyder, 1990; Nicholson *et al*., 1991). Type 4 PDE has been identified in a number of relevant cell types including: eosinophils, basophils, mast cells, neutrophils and T-lymphocytes (Souness *et al*., 1991; Giembycz, 1992), where it exerts a regulatory role (Averill & Kammer, 1985; Mary *et al*., 1987; Peachell *et al*., 1990; Giembycz, 1992; Hatzelmann *et al*., 1995).

The present results show that rolipram and Ro 20-1724 can inhibit the development of acute antigen-induced airway hyperreactivity to the thromboxane mimetic U46619. This effect was not due to a non-specific bronchodilating effect of these two inhibitors, which persisted for 17 h until the U46619 challenge, since the bronchoconstriction induced by the thromboxane mimetic was not reduced. However, in contrast to Howell and co-workers (1993), who used rolipram as the selective PDE4 inhibitor, we were unable to prevent the development of hyperreactivity with a single dose of Ro 20-1724 administered before antigen challenge. This discrepancy may be due to differences in the methods of sensitization and antigen challenge. In the model presented here, a dose 6 h after the antigen challenge was essential to attenuate the development of hyperreactivity. When Ro 20-1724 was administered both before and after challenge, the attenuation of the development of the hyperreactivity tended to be greater than when it was administered as a single dose at 6 h. This suggests that a pre-challenge dose does influence the development of the hyperreactivity in some way. That the 6 h dose is essential indicates that the primary site of action of this compound is an inflammatory event, or events, well after the initiation of the inflammatory response, presumably after mast cell degranulation is complete (Town & Holgate, 1991).

There was also a trend, although not significant, towards siguazodan attenuating the responsiveness of antigen chal-

Table 2 The effects of PDE inhibitors on the bronchoconstrictor responses to inhaled U46619 (30 ng ml^{-1} for 60 s) at 17–24 h after OvA challenge of sensitized guinea-pigs

	Dose	*Baseline*	*2 min response*		
Treatment	(mg kg^{-1})	(absolute)	(absolute)	(%Δ)	n
Untreated (Sal)	Vehicle	0.627±0.047	0.662±0.029	+7.9±9.2%**	5
Untreated (OvA)	Vehicle	0.577±0.045	0.423±0.026	−25.8±2.9%	7
Ro $t=0$ & 6 (OvA)	1	0.503±0.048	0.413±0.040	−16.0±8.3%	5
Ro $t=0$ & 6 (OvA)	3	0.474±0.047	0.487±0.016	+6.4±8.2%**	6
Ro $t=0$ (OvA)	3	0.572±0.029	0.460±0.019	−19.1±3.4%	6
Ro $t=6$ (OvA)	3	0.470±0.026	0.417±0.036	−11.6±5.1%	6
RP $t=0$ & 6 (OvA)	1	0.460±0.047	0.461±0.027	+3.4±8.4%**	5
Am $t=0$ & 6 (OvA)	30	0.484±0.020	0.428±0.012	−13.0±3.5%*	7
Sig $t=0$ & 6 (OvA)	3	0.480±0.011	0.429±0.021	−16.9±3.7%	9
SKF $t=0$ & 6 (OvA)	3	0.525±0.044	0.371±0.070	−27.6±9.5%	4

The PDE inhibitors were: Ro 20-1724 (1 and 3 mg kg^{-1}, Ro), rolipram (1 mg kg^{-1}, RP), siguazodan (3 mg kg^{-1}, Sig), SKF 95654 (3 mg kg^{-1}, SKF) and aminophylline (30 mg kg^{-1}, Am) which were administered at 30 min before ($t=0$) and/or 6 h after ($t=6$) the inhalation of OvA (0.5%). Values are expressed as both absolute sG_{aw} (s^{-1} cmH_2O^{-1}) and as % change from baseline for the 2 min peak constrictor response to U46619. Significant difference from the untreated (OvA) group indicated by $^{**}P<0.05$ or $^{*}P<0.10$ by ANOVA followed by Neuman-Keuls test. Negative values represent broncohconstriction.

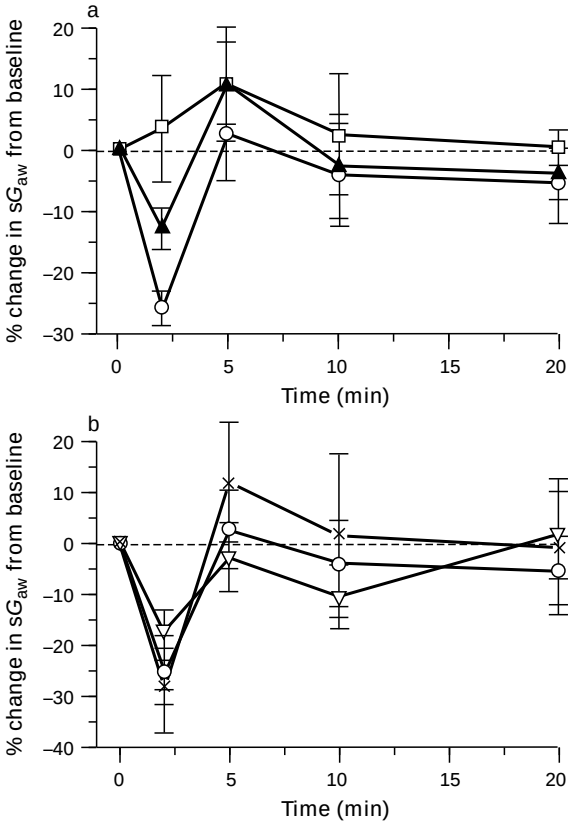

Figure 3 The effects of dosing at 30 min before and 6 h after OvA challenge with (a) rolipram (1 mg kg^{-1}, □) or aminophylline (30 mg kg^{-1}, ▲) and (b) siguazodan (3 mg kg^{-1}, ▽) or SKF 95654 (3 mg kg^{-1}, X) on the increased responsiveness to inhaled U46619 (30 ng ml^{-1} for 60 s) 17–24 h after OvA (○) challenge of sensitized guinea-pigs. Mean responses (with vertical lines showing s.e.mean) measured as the % change in sG_{aw} from baseline at regular intervals after the U46619 exposure are shown ($n=4-9$). Negative values represent bronchoconstriction.

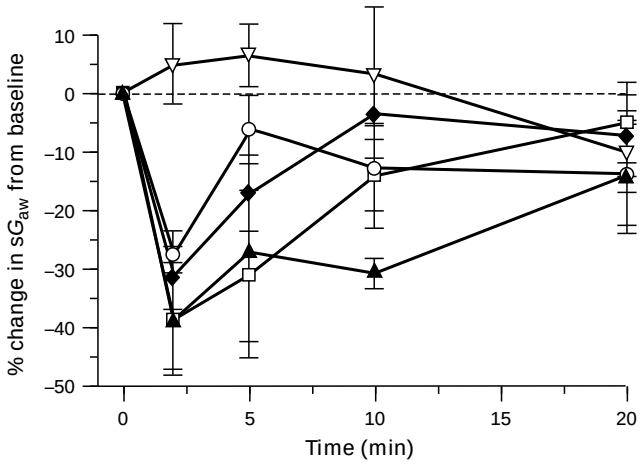

Figure 4 The responsiveness of sensitized, but non-antigen-challenged guinea-pigs to inhaled U46619 (1 μg ml^{-1} for 60 s) 17 h after the first of a double-dose of vehicle (○), Ro 20-1724 (3 mg kg^{-1}, ◆), rolipram (1 mg kg^{-1}, □), siguazodan (3 mg kg^{-1}, ▽) or aminophylline (30 mg kg^{-1}, ▲). Mean responses (with vertical lines showing s.e.mean) measured as the % change in sG_{aw} from baseline at regular intervals after U46619 exposure are shown ($n=4-5$). Negative values represent bronchoconstriction.

lenged animals to U46619. However, this was likely to be as a result of a persistent bronchodilator effect of the compound, since it was able to attenuate the response to a higher, suprathreshold dose of U46619, when administered 17 and 11 h beforehand. Howell and co-workers (1993) demonstrated a similar persistent bronchodilator effect with the PDE3 inhibitor CI-930, administered 24 h before a spasmogen challenge. SKF 95654 was without effect on the development of the hyperresponsive state.

Studies concerning leukocyte infiltration in inflammation and the potential role for selective inhibitors of PDE are numerous (Gristwood *et al*., 1991; Teixeira *et al*., 1994; Underwood *et al*., 1993; 1994; Sullivan *et al*., 1994; Turner *et al*., 1994; Banner & Page, 1995; Howell *et al*., 1995). In general, it is agreed that non-selective inhibitors of PDE and selective inhibitors of PDE4 do attenuate eosinophil recruitment into the airways. Some have achieved this with a single, acute pretreatment dose (Underwood *et al*., 1993; 1994; Turner *et al*., 1994) whilst others required chronic pretreatment (Banner & Page, 1995R). That the observed neutrophilia may be as a consequence of LPS contamination of the OvA cannot be ignored, since LPS has been demonstrated to induce 400 fold increases in lavage neutrophil counts (Howell *et al*., 1995).

The present results with Ro 20-1724 show a similar pattern against leukocyte infiltration, to that observed with the hyperreactivity. A single dose administered before the antigen

Table 3 The effects of PDE inhibitors on the recovery of leukocytes from the airways of sensitized guinea-pigs 17–24 h after OvA challenge

Treatment	*Dose* (mg kg^{-1})	*Macrophages*	*Eosinophils*	*Neutrophils*	*Total cell count*
Untreated (Sal)	Vehicle	12.2±1.9**	1.0±0.4**	0.0±0.0**	13.2±2.3**
Untreated (OvA)	Vehicle	41.8±6.2	19.2±2.9	7.3±1.1	72.3±7.3
Ro $t=0$ & 6	1	21.9±5.3**	11.5±2.4**	4.7±2.4	38.1±9.3**
Ro $t=0$ & 6	3	22.0±3.7**	8.4±1.6**	1.3±0.4**	31.7±5.2**
Ro $t=0$	3	34.1±5.1	21.1±2.9	3.9±0.9**	59.1±8.1
Ro $t=6$	3	23.8±5.2**	8.4±1.9**	1.2±0.3**	33.4±7.2**
RP $t=0$ & 6	1	19.7±4.2**	7.5±2.1**	1.2±0.9**	28.4±6.7**
Am $t=0$ & 6	30	22.2±2.1**	9.6±1.3**	3.3±0.6**	35.1±3.2**
Sig $t=0$ & 6	3	29.9±2.3	21.2±2.6	4.1±0.6**	55.2±4.1
SKF $t=0$ & 6	3	26.4±2.1**	16.0±3.2	3.9±1.0**	38.9±8.51**

The PDE inhibitors were: Ro 20-1724 (1 and 3 mg kg^{-1}, Ro), rolipram (1 mg kg^{-1}, RP), siguazodan (3 mg kg^{-1}, Sig), SKF 95654 (3 mg kg^{-1}, SKF) and aminophylline (30 mg kg^{-1}, Am) which were administered at 30 min before ($t=0$) and/or 6 h after ($t=6$) the inhalation of OvA (0.5%). Values are the mean cell counts ($\times 10^5$ cells ml^{-1}) for macrophages, eosinophils and neutrophils ($n=4-9$). Significant difference from the untreated (OvA) group indicated by $^{**}P<0.05$ or $^{*}P<0.10$ by ANOVA followed by Neuman-Keuls test. Negative values represent bronchoconstriction.

exposure was without effect on the eosinophil infiltration, in agreement with Banner & Page (1995), but in contrast to the observations of Underwood *et al.* (1993) and Turner *et al.* (1994). However, the influx of neutrophils was significantly attenuated. In common with the hyperreactivity study, we have observed that the 6 h dose of Ro 20-1724 is essential to attenuate the eosinophil infiltration. This 6 h dose significantly attenuates the total rise in numbers of all of the leukocytes studied. An additional pre-challenge dose appears to be of no further benefit. Gristwood and co-workers (1991) observed similar effects with theophylline administered both pre and post challenge, although they did not study these doses separately. In the present study, rolipram and aminophylline also protected against the antigen-induced leukocyte influx into the airways, in agreement with Gristwood *et al.* (1991).

The mechanisms by which aminophylline, Ro 20-1724, rolipram and other selective inhibitors of PDE4 attenuate antigen-induced airway eosinophilia are presently unknown. It would be expected that aminophylline also inhibits PDE4, albeit less potently and that it acts on similar mechanisms. There are several possible sites of action of these compounds including effects on the development of eosinophils from their stem cells, the permeability of the vascular endothelium to leukocytes, the production and release of inflammatory mediators and cytokine synthesis.

It has been shown that rolipram can inhibit the development of eosinophil stem cells in mice (DeBrito *et al.*, 1991), and hence reduce the number of circulating eosinophils, but this required chronic drug treatment and is probably of little relevance in this acute model.

The pulmonary vasculature and endothelium are another potential site of action. Endothelial cells express PDE4 (Lugnier & Schini, 1990) and increased levels of cyclic AMP in these cells have been shown to reduce the expression of the adhesion molecules ELAM-1 and VCAM-1 (Pober *et al.*, 1992). PDE4 inhibitors can also inhibit the release of tumour neurosis factor α (TNFα) which could indirectly reduce the expression of ICAM-1 and ELAM-1 (Wellicombe *et al.*, 1990; Thornhill & Haskard, 1990). Rolipram has also been shown to reduce platelet-activating factor (PAF)-induced microvascular leakage in guinea-pig airways (Raeburn & Karlsson, 1992).

A variety of chemical signals generated by inflammatory cells can act as chemoattractants and activators of eosinophils, such as PAF, leukotriene (LTB_4), complement and cytokines (Nourshargh, 1993). In view of the fact that most inflammatory cells express PDE4, it is very difficult to establish which cell, or what combination of cells and mediators a PDE4 inhibitor may be acting upon. An action on mast cells would seem unlikely since pretreatment was of no benefit, and degranulation would be expected to be complete by 6 h. The other potential sites of action would therefore appear to be on the cells present and active 6 h after the challenge. Work in this laboratory, in which a similar exposure method is used (Lewis & Broadley, 1995a), has demonstrated high levels of neutrophils present in the airways at this time. Neutrophils can synthesize and release PAF, LTB_4 (Barnes *et al.*, 1988) and interleukin (IL)-8 (Bazzoni *et al.*, 1991), another eosinophil attractant, and it is possible that inhibition of the activity of this cell could attenuate the eosinophilia by blocking the release of these mediators.

Another potential target for PDE inhibition is the T-lymphocyte which orchestrates the inflammatory response. T-lymphocytes can produce, amongst others: IL-2, IL-3, IL-4, IL-5 and IL-8, all of which are relevant to the recruitment of leukocytes to the airways (Nakajima *et al.*, 1992; Selig & Tocker, 1992; Smith *et al.*, 1992; Nourshargh, 1993; Pretolani *et al.*, 1993; Watson *et al.*, 1993) and some also appear to be involved in the development of airway hyperreactivity (Selig & Tocker, 1992; Smith *et al.*, 1992; Pretolani *et al.*, 1993; Watson *et al.*, 1993). Inhibitors of PDE4, including 3-isobutylmethylxanthine, theophylline, Ro 20-1724 and other agents that regulate cyclic AMP, have been shown to inhibit IL-2 synthesis in T-lymphocytes (Averill & Kammer, 1985; Mary *et al.*, 1987). IL-2 is a T-lymphocyte autocrine, so inhibition of its synthesis would dampen all T-cell mediated events including those linked to the induction of eosinophilia and possibly hyperreactivity.

The ability of the selective PDE3 inhibitors, siguazodan and SKF 95654, to attenuate selectively the influx of neutrophils into the airways is in contrast to the work of Howell *et al.* (1995), which failed to demonstrate any protection against an LPS-induced neutrophilia in guinea-pigs with selective inhibitors of PDE3 and PDE4.

In our study, it is interesting to note that the single dose of Ro 20-1724 administered before the antigen challenge had a similar effect to the PDE3 inhibitors against the neutrophil influx. Also, Church and co-workers (1993) showed that pretreatment of conscious guinea-pigs with a β_2-agonist had a similar inhibitory action on neutrophil influx without an effect on the eosinophilia following antigen challenge. This suggests that some component early on in the mechanism responsible for selective neutrophil recruitment into the airways following antigen challenge, is readily inhibited by agents eliciting a rise in cyclic AMP. The effects of siguazodan and SKF 95654 could be through an action on a mechanism involved in neutrophil recruitment which is sensitive to inhibition of PDE3. The location of such a putative site could be the T-lymphocyte which expresses PDE3 as well as PDE4. As mentioned above,

T-lymphocytes are capable of synthesizing and releasing a number of cytokines which induce and regulate leukocyte recruitment and it is possible that inhibition of PDE3 could regulate this in some way. Alternatively, initial mast cell degranulation, which has been demonstrated to be regulated by cyclic AMP (Frossard *et al*., 1981; Undem *et al*., 1988; Gentilini *et al*., 1993) could be directly responsible for neutrophil recruitment. Human mast cells have been shown to store IL-8 and TNFα (Bradding *et al*., 1994; 1995) both of which are relevant to the recruitment of neutrophils. This might also explain the pattern of protection seen with the single pre-challenge dose of Ro 20-1724.

In the present study, all animals exhibited both an early (0 to 3 h) and late phase response (between 17 and 24 h) to inhaled antigen. The times of the appearance of these phases is in agreement with several other studies (Hutson *et al*., 1988; Lewis & Broadley, 1995a). The appearance of a late phase response is often associated with a non-specific bronchial hyperreactivity and is often used as a marker for clinical asthma (Hargreave, 1989). However, in human asthmatics the late phase response appears to present sooner after the early phase, usually between 8 and 10 h (Robertson *et al*., 1974; Hargreave, 1989). Leukocyte influx, particularly of eosinophils and neutrophils, is also apparent during this phase, in animal models of asthma (Hutson *et al*., 1988; Abraham *et al*., 1992; Lewis & Broadley, 1995a) and human asthmatics (Robertson *et al*., 1974; Hargreave, 1989). However, in this study neither phase was significantly affected by treatment with Ro 20-1724 or rolipram. A similar lack of effect of rolipram on the acute response to antigen was observed by Turner *et al*. (1994) in atopic monkeys. However, other studies of the effects of PDE inhibitors on early asthmatic responses in animals (Howell *et al*., 1993; Underwood *et al*., 1993; 1994) have shown protection against the acute antigenic response. It should be borne in mind that in these studies antigen challenges not requiring anti-histamine protection were used, either because the animals were anaesthetized or because the challenge was not severe, in contrast to the study presented here. We have demonstrated that *in vitro* protection against the acute response to antigen, by inhibition of PDE4 in airway perfused lungs from sensitized guinea-pigs, is as a consequence of inhibition of histamine release (unpublished observations). However, Underwood *et al*. (1993) attributed protection to the inhibition of the release of PGD_2 but in guinea-pig isolated superperfused trachea. In the study presented here, the lack of effect of inhibition of PDE4 on the early response to antigen may be due to the presence of mepyramine masking any effect on histamine release. Alternatively, the OvA-challenge used in the present study produced a maximal bronchoconstriction, so any protection afforded by the PDE4 inhibitors was overwhelmed.

The exact determinants of the late phase response are presently unknown. Sodium cromoglycate, a putative mast cell stabilising agent, has been shown to inhibit the development of the late phase response in a similar guinea-pig model (Hutson *et al*., 1988), suggesting a role for the mast cell. The eosinophil is also consistently found in the airways of atopic asthmatics (Frigas & Gleich, 1986; Metzger *et al*., 1986; Wardlaw & Kay, 1987; Hargreave, 1989; Kay, 1991). Two of the major products of activated eosinophils are LTB_4 and LTD_4, both potent bronchoconstrictors, which can also enhance mucus secretion and increase vascular permeability (Wardlaw & Kay, 1987). Further evidence supporting a role for leukotrienes in the late phase response, is the ability to attenuate it with the 5-lipoxygenase inhibitor, zileuton (Abraham *et al*., 1992). With the observed anti-inflammatory effects of these PDE inhibitors on the development of hyperreactivity and inflammatory cell influx at the time of the appearance of the late phase, it is surprising that Ro 20-1724 was without effect on this phase and would seem to dissociate the theory of a common denominator. However, the present method involved a large antigen challenge, requiring the concomitant administration of a high dose of an H_1-antagonist to prevent fatal anaphylaxis. It is believed that the magnitude of the late phase response is linked to that of the early phase (O'Byrne *et al*., 1987). It is possible that the dose of antigen used triggered the development of leukocyte influx and acute hyperreactivity which are sensitive to the dose of Ro 20-1724 used, but its effect on the late phase response was overwhelmed by its magnitude.

With these observed similarities between the effects of Ro 20-1724, rolipram and aminophylline on the development of hyperreactivity and the attenuation of eosinophilia, it is tempting to suggest that, in this model at least, there is a link. As already mentioned, clinical evidence supports a link between late-phase asthmatic responses, airway eosinophilia and bronchial hyperreactivity. The lack of effect of siguazodan or SKF 95654 on either of these inflammatory events is supported by other workers, who suggest that inhibition of PDE3 is more effective as a bronchodilator mechanism (Howell *et al*., 1993).

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