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Investigation into the role of phosphodiesterase IV in bronchorelaxation, including studies with human bronchus

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1 We have investigated the role of cyclic nucleotide phosphodiesterase IV (PDE IV) in the relaxation of human bronchus and guinea-pig trachea *in vitro* and in guinea-pigs *in vivo*.

2 Functional studies showed that the selective PDE IV inhibitors, rolipram and denbufylline, relaxed human and guinea-pig preparations *in vitro*.

3 Two clinically used xanthine non-selective PDE inhibitors, theophylline and pentoxifylline, were also effective in these preparations, but were much less potent than the selective agents used.

4 The rank order of potency for the four PDE inhibitors in both species was similar.

5 Biochemical studies indicated that PDE IV was the major PDE isoform present in the human bronchial tissue. PDEs I, II and V were also identified.

6 Theophylline and pentoxifylline were, as expected, non-selective inhibitors of the human enzymes, but there was a good correlation between PDE IV inhibitory and bronchorelaxation potencies, suggesting that PDE IV inhibition is important for the clinical bronchodilator activities of the two xanthine compounds.

7 We have confirmed the ability of selective PDE IV inhibitors to cause bronchodilatation in guinea-pigs *in vivo*.

8 We conclude that our study has provided further evidence that selective PDE IV inhibitors could act as bronchodilators in the clinic.

Keywords: Phosphodiesterase isoenzymes; PDE IV; human bronchus; theophylline; pentoxifylline; rolipram; denbufylline; bronchorelaxation

Introduction

There is currently interest in the possible use of selective inhibitors of cyclic nucleotide phosphodiesterases (PDEs) in the treatment of asthma (see e.g. Torphy & Undem, 1991; Giembycz & Dent, 1992). There are known to be at least five distinct families of PDE isoenzymes, PDEs I to V, which differ in their substrate specificity and affinity as well as in their regulatory properties and tissue distribution (see Beavo & Houslay, 1990). Of these, PDE IV inhibitors are receiving special interest as anti-asthmatic agents due to evidence that these can act as both anti-inflammatory agents and bronchodilators in animal species (see Nicholson *et al.*, 1991; Giembycz & Dent, 1992). There are, however, few clinical data generally available concerning selective PDE IV inhibitors in asthma.

Theophylline, currently widely used clinically in asthma, is a weak non-selective PDE inhibitor, but it is believed that PDE inhibitory activity (including PDE IV inhibition) may contribute to both its bronchodilator and anti-inflammatory activities in man (see e.g. Torphy & Undem, 1991; Gristwood *et al.*, 1991). Theophylline, therefore, may be an important link compound between *in vitro* human and animal pharmacological studies and the potential clinical efficacy of PDE IV inhibitors.

The purpose of the present investigation was to compare, using human *in vitro* studies, the bronchorelaxant and PDE inhibitory activities of two selective PDE IV inhibitors, rolipram (Reeves *et al.*, 1987) and denbufylline (Nicholson *et al.*, 1989), and two clinically used xanthine non-selective PDE inhibitors, theophylline (Persson, 1986) and pentoxifylline (Ward & Clissold, 1987). Isoprenaline was included as a standard bronchodilator agent.

A further important aim of the study was to compare the human *in vitro* bronchodilator activities of these agents with their *in vitro* and *in vivo* bronchodilator activities in the guinea-pig, a commonly used animal for models of human asthma.

Methods

Relaxant activity in human isolated bronchus

Lung tissue was obtained from 19 patients (18 males and 1 female, 35 to 78 years old, mean age 61.7 ± 2.8 years) who were undergoing surgery (Hospital de La Fe, Valencia, Spain) for lung carcinoma. None of the patients had a history of asthma. After the resection of one or more lung lobes, a piece of macroscopically normal tissue was cut free and submerged in Krebs solution (for composition see drugs and solutions) at 4°C for transport to the laboratory.

Once in the laboratory (Valencia) parts of the bronchus were dissected free from parenchymal lung tissue and preparations cut (3–4 mm length × 2–3 mm internal diameter) as previously described (Cortijo *et al.*, 1992). Preparations were stored in Krebs solution, equilibrated with 5% CO_2 in O_2 at 4°C until used. Experiments were routinely completed within 24 h of initiating storage.

For experiments, the bronchial rings were suspended on tissue hooks in 10 ml organ baths containing Krebs solution, gassed with 5% CO_2 in O_2 at 37°C (pH 7.4). Each preparation was connected to a force displacement transducer (Grass FTO3) and isometric tension changes recorded on a Grass polygraph (model 7P).

The preparations were equilibrated for 60–90 min with changes in bath Krebs solution every 20 min before adding drugs.

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A load of 2 g was maintained throughout the equilibrium period and a stable resting level of tone was present immediately prior to drug administration. The effects of test drugs were investigated by adding cumulative concentrations of these to the baths. Responses were allowed time to stabilize prior to increasing the bath drug concentration (usually within 15 min of addition).

Only one concentration-effect curve was constructed with each preparation. Experiments were terminated by the addition of theophylline, 1×10^{-3} M, the effect of which was taken to represent the maximum relaxation possible in the tissue.

Changes in force were measured from isometric recordings and expressed in g. The maximum response induced with a relaxant agent (E_{max}) and the molar concentration required to produce 25% (EC_{25}) and 50% (EC_{50}) of maximal relaxation was calculated by linear regression from the individual concentration-effect curves.

Relaxant activity in guinea-pig isolated trachea

Guinea-pigs (male Dunkin-Hartley) of weight range 400–600 g were maintained without food but with free access to water for 16 h before experimentation. Animals were then killed by cranial percussion, the trachea dissected free and placed into Krebs solution at 4°C. The trachea was cut into preparations consisting of 2 cartilaginous rings which were then cut through the cartilaginous zones.

The preparations were suspended in 30 ml organ baths containing Krebs solution at 37°C gassed with 5% CO_2 in O_2. These were allowed at least 1 h to equilibrate during which time the resting tension was maintained at 1 g. A control response to isoprenaline (1×10^{-7} M) was then obtained, to indicate the maximum relaxation possible in this tissue. The tissues were then washed and allowed 30 min to re-equilibrate before one of the drugs under study was added by a cumulative concentration procedure. Responses were allowed to stabilize (usually within 15 min of drug addition).

Tension was recorded with isometric transducers (Letica TR1 010) onto a Letica polygraph (model 4000). Changes in tension were measured, and EC_{25} and EC_{50} values calculated, as described for human preparations.

Bronchodilator activity in anaesthetized guinea-pigs

Animals of weight range 400–500 g were anaesthetized with sodium pentobarbitone 60 mg kg^{-1}, i.p. A tracheotomy was performed and a polyethylene tube inserted into the cut trachea, connected to a respirator (Ugo Basile 7025) and a side arm connected to a pressure transducer (Letica TRA021). The left carotid artery was cannulated for blood pressure and heart rate recordings and the right jugular vein for drug administration. After a 15 min stabilization period, a continual infusion (Braun infusion pump) of histamine (100 μg ml^{-1}, 2–6 ml h^{-1}) sufficient to elevate insufflation pressure by 150–200% was administered via the left jugular vein and maintained throughout the experiment. The histamine infusion was continued for 45 min, by which time a steady state bronchoconstrictor response had been observed, before one of the test drugs was given by i.v. bolus injections using an ascending dose scheme.

The maximum bronchodilatation produced by each drug was measured and expressed as the % inhibition of the histamine-induced bronchoconstrictor response.

Extraction, separation and characterization of PDE isoenzymes from human bronchus

Individual human bronchi, weighing 0.9–2.7 g were homogenized for 60 s, with an Ultraturrax at 9000 r.p.m. in 5 volumes of ice-cold buffer A (20 mM Bis Tris, pH 6.5, containing 50 mM sodium acetate, 2 mM benzamidine, 2 mM EDTA, 5 mM β-mercaptoethanol and 50 mM phenylmethylsulphonylfluoride (PMSF)). The homogenate was centrifuged at 15000 *g* for 10 min and the clear supernatant was filtered through 0.22 μm Millex filters. The sample was injected into a MONO-Q HR 5/5 column (1 ml of gel bed, Pharmacia) attached to an FPLC chromatography system and equilibrated in the same buffer. After washing with 15 ml of buffer A, the PDEs were eluted by developing a 20 ml linear sodium acetate gradient from 50 to 1000 mM in buffer A. Flow rate was 1 ml min^{-1} throughout. Fractions of 0.5 ml were collected, analyzed and stored as previously described (Gristwood *et al.*, 1992).

Molecular weights of purified PDEs were determined by gel filtration on a Superose 12 HR 10/30 column (Pharmacia) attached to an FPLC system. The column was equilibrated at room temperature in 20 mM Bis Tris, 150 mM sodium acetate, 2 mM EDTA, 5 mM β-mercaptoethanol, 2 mM benzamidine pH 6.5 buffer. The column was calibrated with proteins of known molecular weight by running 0.1 ml samples at a flow rate of 0.4 ml min^{-1}. Elution volumes were determined by following the absorbance at 280 nm on line, and the obtained K_{av} values were correlated with the logarithm of their molecular weights. Column void volume was determined with Blue Dextran 2000 under the same conditions as the standards. Phosphodiesterase samples obtained from the ion exchange chromatography were also run in a similar manner and 0.20 ml fractions were collected. Elution volumes for the different enzymes were determined by assaying the enzyme activity in the fractions. Molecular weights were calculated by interpolation of the K_{av} values on the regression line obtained for the standards.

Cyclic nucleotide PDEs were assayed following the procedure of Thompson & Strada (1984). Inhibition assays were run in duplicate at 30°C for 20 min at a substrate concentration of 0.25 μM. The substrate was adenosine 3′:5′-cyclic monophosphate (cyclic AMP) unless otherwise stated. PDE II was assayed in the presence of 5 μM unlabelled guanosine 3′:5′-cyclic monophosphate (cyclic GMP). For each of the assayed drugs, 6 to 8 different concentrations were tested in at least two different enzyme preparations. IC_{50} values were obtained by non-linear regression using InPLot, GraphPad Software on an IBM computer.

Drugs, reagents and solutions

The following drugs were used: zaprinast (a gift from Rhône-Poulenc Rorer U.K.), SK&F 94120 (a gift from SK&B Ltd, Welwyn, U.K.), isoprenaline sulphate, obtained from Boehringer Ingelheim (Germany) and theophylline, purchased from Sigma-Aldrich Química S.A. (Madrid, Spain). Denbufylline, rolipram and pentoxifylline were synthesized in the Department of Chemical Synthesis, Almirall.

Calmodulin was obtained from Boehringer Mannheim (Barcelona, Spain). Benzamidine, histamine, PMSF, cyclic GMP and cyclic AMP were from Sigma-Aldrich Química, S.A. (Madrid, Spain). The low and high molecular weight markers and Blue Dextran 2000 were from Pharmacia Iberica (Barcelona, Spain). [8-^{3}H]-adenosine 3′:5′-cyclic monophosphate and [8-^{3}H]-guanosine 3′:5′-cyclic monophosphate were from Amersham International (U.K.).

The drugs used for the biochemical studies were dissolved in dilute NaOH, dimethylsulphoxide (DMSO) or water. Drug vehicles at concentrations employed did not affect enzyme activities.

For the pharmacological studies, isoprenaline was dissolved in distilled water containing 0.57 mM ascorbic acid. Stock solutions of denbufylline were prepared in 40% polyethyleneglycol 300 and rolipram in 20% polyethyleneglycol 300. Theophylline and pentoxifylline were dissolved in water. Subsequent dilutions of drugs were made in the Krebs solution which had the following composition (mM): NaCl 118, KCl 4.7, $CaCl_2$ 2.5, $MgSO_4$ 1.6, $NaHPO_4$ 1.2, $NaHCO_3$ 25 and glucose 11.

Statistics

Values are given as mean ± s.e. mean. Statistical analysis of results was carried out by analysis of variance (ANOVA) followed by Duncan's test or by Student's t test for paired or unpaired data as appropriate.

Results

Bronchorelaxant activity in human bronchus

All drugs tested caused concentration-dependent inhibition of the spontaneous tone of human bronchi as shown in Figure 1. All produced full relaxation, i.e. the maximal relaxation was not significantly different from that obtained with theophylline, 1×10^{-3} M, see Table 1.

The threshold concentrations for isoprenaline, rolipram, pentoxifylline and denbufylline were similar at between 10^{-9} M and 10^{-8} M, whereas the threshold for theophylline was higher, as shown in Figure 1. Considering both EC_{25} and EC_{50} values, the potency order of drugs for relaxation was isoprenaline > rolipram = denbufylline ≫ pentoxifylline > theophylline, as indicated in Table 1.

One further important observation was that in preparations with spontaneous tone, rolipram, denbufylline, and pentoxifylline all produced clear biphasic concentration-response curves (see Figure 1).

Bronchorelaxant activity in guinea-pigs in vitro

All drugs produced concentration-dependent relaxation of the guinea-pig tracheal preparations, as shown in Figure 2. The maximum observed relaxations were as follows; isoprenaline 0.67 ± 0.03 g, rolipram 0.44 ± 0.04 g, denbufylline 0.40 ± 0.04 g, theophylline 0.66 ± 0.03 g and pentoxifylline 0.34 ± 0.07 g, $n = 5-12$.

In terms of threshold concentrations there was a clear order of isoprenaline ≪ denbufylline = rolipram ≪ pentoxifylline = theophylline.

EC_{25} and EC_{50} values for the compounds on guinea-pig trachea are shown in Table 2. For EC_{25} values the potency order was isoprenaline ≫ rolipram = denbufylline ≫ pentoxifylline > theophylline. For EC_{50} values the order was now isoprenaline ≫ denbufylline = rolipram > pentoxifylline > theophylline.

Of all agents tested only rolipram produced a clear biphasic concentration-response curve (Figure 2).

Bronchodilator activity in anaesthetized guinea-pigs

All drugs produced dose-dependent inhibitions of the histamine-induced bronchospasm, as shown in Figure 3. In terms of threshold doses the order was isoprenaline > rolipram > denbufylline ≫ pentoxifylline = theophylline. In order to compare drug potencies, doses causing a mean decrease of 50% of the histamine-induced bronchoconstriction were cal-

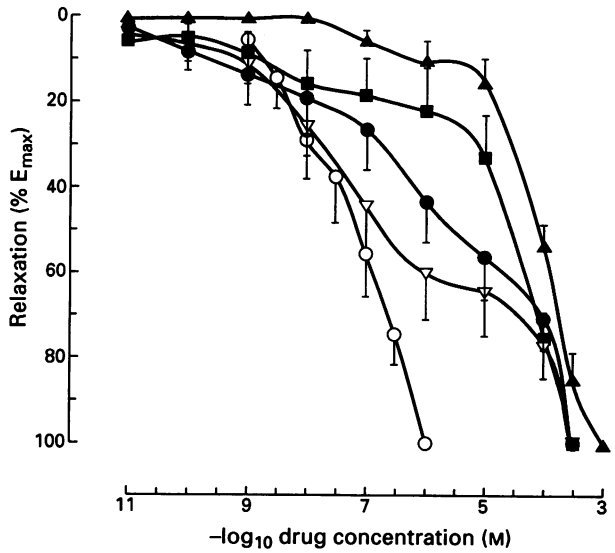

Figure 1 Relaxant effects of theophylline (▲), pentoxifylline (■), denbufylline (●), rolipram (▽) and isoprenaline (○) in human bronchi with spontaneous tone. Points are means ± s.e.mean (vertical bars). n values are indicated in Table 1.

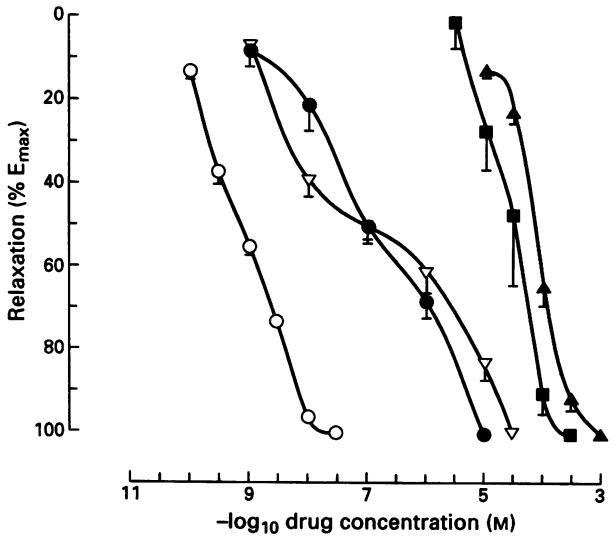

Figure 2 Relaxant effects of theophylline (▲), pentoxifylline (■), denbufylline (●), rolipram (▽) and isoprenaline (○) in guinea-pig tracheal ring preparations. Points are means ± s.e.mean (vertical bars). n values are indicated in Table 2.

Table 1 Relaxant effects of cyclic nucleotide phosphodiesterase (PDE) inhibitors on spontaneous tone of human bronchial preparations

	p/n	$-log\ EC_{50}$ (M)	$-log\ EC_{25}$ (M)	E_{max} (g)	*Maximal relaxation* (g) *induced by theophylline* (1×10^{-3} M)
Theophylline	10/6	4.11 ± 0.13*	4.98 ± 0.30‡	1.53 ± 0.25	-
Pentoxifylline	11/6	5.30 ± 0.53	6.10 ± 0.70	1.31 ± 0.17	1.52 ± 0.19
Denbufylline	9/5	5.94 ± 0.62	7.21 ± 0.74	1.23 ± 0.09	1.74 ± 0.13
Rolipram	9/6	6.23 ± 0.54	7.75 ± 0.59	1.34 ± 0.14	1.56 ± 0.16
Isoprenaline	8/6	7.35 ± 0.30†	7.80 ± 0.20†	1.35 ± 0.22	1.72 ± 0.17

Values are means ± s.e.mean. p represents the number of preparations and n the number of patients.
*$P < 0.05$ from the other agents; †$P < 0.05$ from theophylline and pentoxifylline; ‡$P < 0.05$ from the other agents except pentoxifylline.

Table 2 Relaxant effects of cyclic nucleotide phosphodiesterase (PDE) inhibitors on spontaneous tone of guinea-pig tracheal preparations

	n	$-log\ EC_{50}$ (M)	$-log\ EC_{25}$ (M)
Theophylline	8	4.15 ± 0.15	4.51 ± 0.06
Pentoxifylline	6	4.71 ± 0.17*	5.03 ± 0.20*
Denbufylline	5	6.91 ± 0.13*	8.01 ± 0.24*
Rolipram	12	6.68 ± 0.23*	8.07 ± 0.17*
Isoprenaline	8	9.13 ± 0.04*	9.75 ± 0.05*

*$P < 0.05$ compared with theophylline.

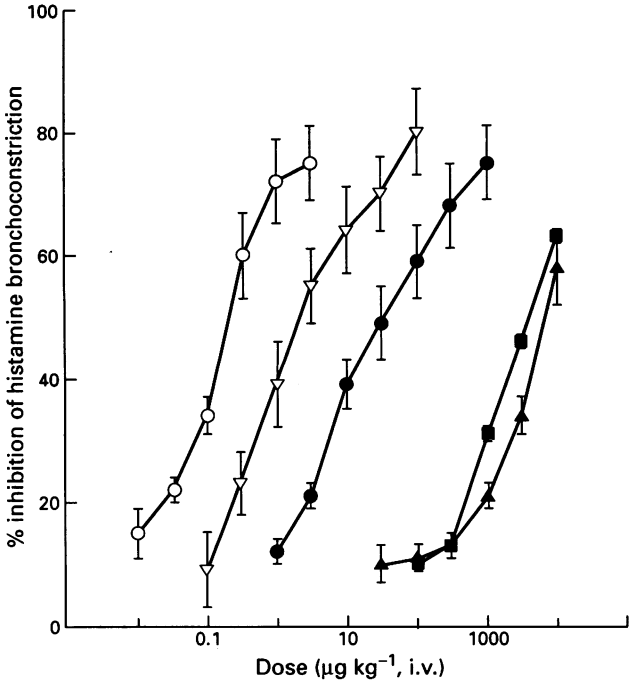

Figure 3 Inhibition of histamine-induced bronchoconstriction in anaesthetized guinea-pigs by isoprenaline (○), rolipram (▽), denbufylline (●), pentoxifylline (■) and theophylline (▲). Points are means ± s.e.mean (vertical bars). $n = 4-6$.

culated. These were as follows in μg kg^{-1} i.v.: isoprenaline 0.45 ± 0.1, rolipram, 4 ± 1, denbufylline 69 ± 20, pentoxifylline 4534 ± 860 and theophylline 5842 ± 911 ($n = 4-6$).

Isolation and characterization of the human bronchial phosphodiesterases

A representative chromatogram of the PDEs isolated from human bronchi by ion exchange is shown in Figure 4. In 5 out of 6 individual preparations, 4 peaks of PDE activity were observed.

The characterization of the peaks is shown in Table 3. When assayed with 1 μM cyclic AMP as substrate, peak A was the most variable in size, ranging from 8 to 21% of total PDE activity. When submitted to gel filtration, peak A was resolved into two distinct peaks. This, taken together with its differential sensitivity to zaprinast when cyclic GMP was used as substrate instead of cyclic AMP and its marginal activation by Ca^{2+}/calmodulin (Table 3) indicates that peak A is a mixture of PDE I and PDE V.

Peak B was always the smallest of all and could not be clearly identified in one of the samples. The characterization

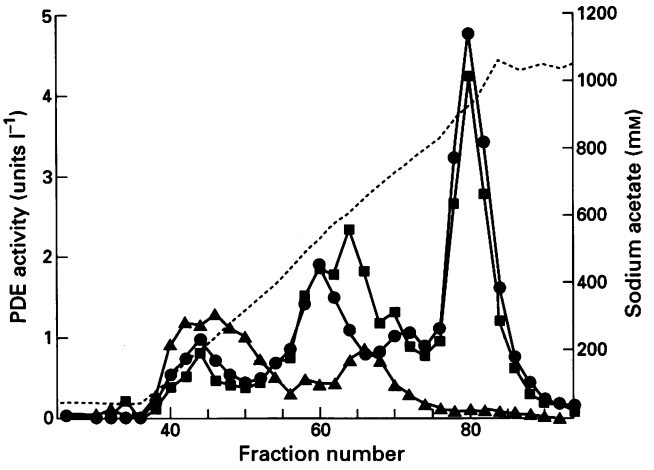

Figure 4 Representative elution profile of cyclic nucleotide phosphodiesterase (PDE) activities from human bronchus on a MONO Q ion exchange column. The low speed centrifugation supernatant (10 ml) from one individual sample was chromatographed as described in the text. Fractions (0.5 ml) were collected and assayed for PDE activity in the following conditions: 1 μM cyclic AMP (●), 1 μM cyclic AMP plus 1 μM cold cyclic GMP (■) and 1 μM cyclic GMP (▲). The 4 peaks collected were: fractions 37 to 53 (A), fractions 56 to 62 (B), fractions 64 to 68 (C) and fractions 76 to 85 (D). For the ordinate scale, 1 unit of enzyme activity is defined as the amount hydrolysing 1 μmol of substrate per min.

of its activity using kinetic and regulatory properties suggested that peak B corresponded to a form of PDE IV. IC_{50} values of 1.1 and 1.3 μM were obtained for this peak using denbufylline and rolipram respectively.

The low molecular weight obtained for peak B suggested that it could have been derived from native PDE IV by limited proteolysis. This, and the small amounts of peak B obtained, led us to exclude this enzyme from the biochemical studies with the rest of inhibitors.

Peak C represented 16.7 ± 3.4% of the total activity measured with 1 μM cyclic AMP as substrate and the data presented in Table 3 indicate that this enzyme is the cyclic GMP stimulated form (PDE II).

Peak D was the major peak in all samples (56 ± 1.7% of total). Its characterization, shown in Table 3, indicates that this peak is also PDE IV. Gel filtration studies indicated a molecular weight of 82300 ± 5600, as well as the homogeneity of the enzyme, with little contamination by other PDE isoenzymes (see Figure 5).

No clear evidence of cyclic GMP inhibited PDE (PDE III) was obtained either from the chromatograms or from the use of the selective PDE III inhibitor SK&F 94120 (Gristwood *et al.*, 1986) on selected PDE fractions.

Effects of the drugs on the isolated isoenzymes

The effects of the PDE inhibitors on the three major peaks of human bronchial PDE activities are shown in Table 4. As expected, rolipram and denbufylline displayed selectivity for Peak D, whereas theophylline and pentoxifylline inhibited all three peaks. The potency order of the inhibitors on peak D was denbufylline = rolipram ≫ pentoxifylline > theophylline.

Discussion

The results from this study have confirmed the relaxant activity of theophylline in human respiratory muscle *in vitro*. This is in agreement with previous findings (Cortijo *et al.*, 1992). Furthermore, we have shown that pentoxifylline, another clinically used xanthine, also has relaxant properties

Table 3 Characterization of the human bronchial phosphodiesterases separated by ion exchange chromatography

	Peak A	*Peak B*	*Peak C*	*Peak D*
K_m cyclic AMP	1.3	ND	77*	1.15
K_m cyclic GMP	0.8	>500	9.6**	>500
Ca^{2+}/Calmodulin (% activation)	31	NE	NE	NE
Cyclic GMP 5 μM (% activation)	NE	NE	87	NE
Mol. weight (KD)	ND	27.8	ND	82.3
Zaprinast[a] (IC_{50})	53	50	55	61
Zaprinast[b] (IC_{50})	0.32	ND	ND	ND
SK&F 94120 (IC_{50})	>500	>500	>500	>500

All concentrations are in μM. [a]Cyclic AMP as substrate; [b]Cyclic GMP as substrate. ND, not determined. NE, no effect. * indicates $S_{0.5}$ because of non hyperbolic kinetics. **K_m value for cyclic AMP in the presence of cyclic GMP. Dispersion values, lower than 10% in all cases, have been omitted for clarity.

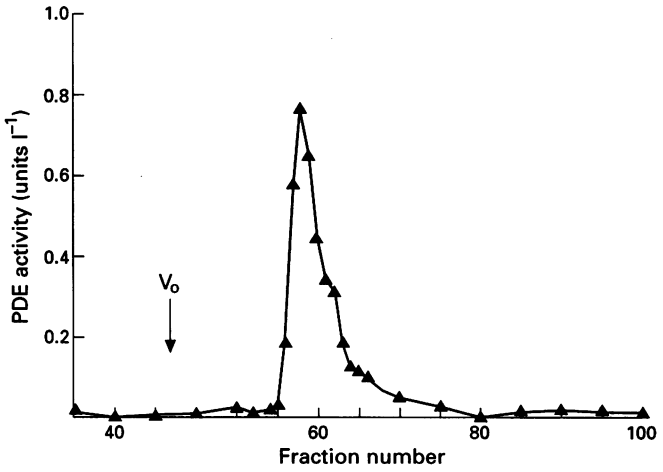

Figure 5 Gel filtration profile on Superose 12 of an aliquot of the 4th peak obtained from the ion exchange chromatography (peak D). The arrow indicates column void volume. See text for details. For the ordinate, 1 unit of enzyme activity is defined as the amount hydrolysing 1 μmol of substrate per min.

in human bronchial tissue *in vitro*. Although pentoxifylline is currently used clinically as a haemorheological agent (Ward & Clissold, 1987), clinical studies have shown that the drug has bronchodilator activity in man (Nolte, 1971).

We consider that the concentrations of both theophylline and pentoxifylline used in this study are relevant to the plasma concentrations of these drugs achieved clinically. Thus, in the case of theophylline, therapeutic plasma concentrations are between 50 and 100 μM, 60% of which is protein bound (Svedmyr, 1988). Therefore, free plasma concentrations would not be very different from the *in vitro* EC_{25} (≈10 μM) and EC_{50} (≈80 μM) values found in this study. For pentoxifylline, mean peak plasma concentrations in man (following a standard 400 mg dose) are approximately 5 μM (plasma binding is low, see Ward & Clissold, 1987), again a value close to the *in vitro* EC_{25} (≈1 μM) and EC_{50} (≈5 μM) found for pentoxifylline in this study.

Considering responses in human preparations at the EC_{50} level, both theophylline and pentoxifylline were relatively weakly active on the tissues, whereas the selective PDE IV inhibitors tested, rolipram and denbufylline, were much more potent relaxants. Indeed rolipram, at the response level of EC_{25}, was close to isoprenaline in its potency.

These data confirm the bronchodilator potential of PDE IV inhibitors in man, particularly in terms of potency and maximal effect.

Consistent with the bronchorelaxant activities of denbufylline and rolipram, our biochemical studies confirmed the presence of PDE IV in human bronchi and furthermore, indicated that it is the major PDE isoform present in this tissue. There were in fact two peaks of activity that could be characterized as PDE IV, (Peaks B and D) although we believe, based on their properties, that Peak B could be a proteolysed form of the enzyme. Thus, whereas peak D eluted in the position typical for PDE IV both from human (Reeves *et al.*, 1987) and animal sources (Gristwood *et al.*, 1992) and had a molecular weight close to that of cloned human PDE IV (Livi *et al.*, 1990) peak B was smaller and eluted before PDE II in the chromatogram.

A comparison of the inhibitory potencies of the four PDE inhibitors on PDE IV (Peak D activity) and relaxant activity in human bronchi (Tables 1 and 4) indicated a good correlation, and this suggests that PDE IV inhibitory activity for both theophylline and pentoxifylline is at least partly responsible for their *in vitro* bronchorelaxant activities in human tissue.

We cannot, of course, exclude the possibility that for the xanthine compounds, inhibition of the other PDE isoenzymes present, contributed to their relaxant activities, but no correlation could be found for the tested compounds between inhibition of the other peaks and their relaxant potencies, particularly in the case of rolipram.

Recently Shahid *et al.* (1992) have shown that up to six different peaks of PDE activity could be resolved by ion exchange chromatography in human bronchi. Although the chromatographic profile described by these authors appears to be more complex than that found in the present paper, no major discrepancies can be perceived. As with our samples, PDE IV was the main activity hydrolyzing cyclic AMP in the study of Shahid *et al.* (1992). PDE V and two slightly

Table 4 Effect of the compounds under study on the phosphodiesterase peaks isolated by ion exchange chromatography

	Peak A	*Peak C*	*Peak D*
Theophylline	3.75 ± 0.01	3.26 ± 0.15	3.82 ± 0.02
Pentoxifylline	4.19 ± 0.02	4.01 ± 0.04	4.35 ± 0.06
Denbufylline	4.57 ± 0.01	4.13 ± 0.06	6.33 ± 0.14
Rolipram	3.46 ± 0.05	3.59 ± 0.04	6.21 ± 0.10

For each drug 6–8 concentrations were tested in duplicate for are at least two different samples. Values are $-\log IC_{50}$ in molar concentration ± s.e.mean.

different forms of PDE I could be detected by the authors eluting at low salt concentrations. These match the enzymes that were identified in our peak A. PDE II was also detected. The main difference found was the presence of a minor form of PDE IV eluting at a different position from that of peak B in the study of Shahid *et al.* (1992).

It was interesting that another major PDE isoenzyme, PDE III, was not identified in our biochemical experiments. This is in agreement with recent data (Shahid *et al.*, 1992). Consistent with this, it has recently been shown that selective PDE III inhibitors, unlike selective PDE IV inhibitors, did not induce increases in intracellular cyclic AMP content in human cultured tracheal smooth muscle cells (Hall *et al.*, 1992). These data taken together suggest that PDE III inhibitors may not have important bronchodilator actions in man.

As in human preparations, all PDE inhibitors induced concentration-dependent relaxation of guinea-pig tracheal preparations with similar maximal effects. Furthermore, the rank order of potency of the drugs was similar for the two species. The only major quantitative difference between guinea-pig and human tissues was that isoprenaline was almost 2 orders of magnitude more potent on guinea-pig preparations. The potencies of the PDE inhibitors, however, appeared similar.

We did not investigate PDE enzymes in guinea-pig trachea in the present study. Biochemical studies have been previously reported in the literature and it is known that PDE IV is present in this tissue (Silver *et al.*, 1988; Takagi *et al.*, 1992). Unlike human bronchus, guinea-pig trachea also has PDE III present (Silver *et al.*, 1988), and PDE III inhibitors are effective bronchodilators in the guinea-pig both *in vitro* (Bryson & Roger, 1987) and *in vivo* (Gristwood & Sampford, 1987). It is therefore, possible that both PDE III and IV inhibition contributed to the bronchorelaxant activity of theophylline and pentoxifylline in the guinea-pig.

Rolipram and denbufylline both demonstrated potent dose-related bronchodilator activity in anaesthetized guinea-pigs. This confirms that selective PDE IV inhibitors can act as bronchodilators *in vivo*. In this *in vivo* preparation there was a difference in potency between rolipram and denbufylline that was not indicated by the guinea-pig *in vitro* studies. The reason for this is not known, but may have been due to pharmacokinetic considerations. Nevertheless, it is clear that both selective PDE IV inhibitors were much more potent than either pentoxifylline or theophylline *in vivo*.

The consistency of the bronchorelaxant efficacy of the PDE IV inhibitors and the xanthines in human bronchus and guinea-pig trachea *in vitro* coupled with their efficacy in the guinea-pig *in vivo*, and evidence that theophylline and pentoxifylline act as bronchodilators in man, all lead us to the conclusion that selective PDE IV inhibitors will have bronchodilator activity in man *in vivo*.

One additional finding of interest was that in human preparations, rolipram, denbufylline, pentoxifylline and theophylline all produced concentration-response curves which appeared somewhat biphasic in nature. This was particularly so for the rolipram curve which had evidence of a plateau between 10^{-6} M and 10^{-5} M.

A biphasic concentration-response curve for rolipram was also evident in guinea-pig tracheal preparations, where there was evidence of a plateau between 10^{-8} and 10^{-6} M.

Although a definite explanation for these findings cannot be given, the possibility of the involvement of another PDE isoenzyme or isoenzymes in the relaxation of human tissue cannot be excluded. If that were the case, the most selective compound of those tested (rolipram) might be expected to have the most evident plateau, as indeed is the case.

In conclusion, our study has indicated the importance of PDE IV in the relaxation of human bronchial smooth muscle *in vitro*. The data obtained indicate that the inhibition of this isoenzyme is important for the bronchorelaxation activities of rolipram, denbufylline as well as theophylline and pentoxifylline both in guinea-pig and man and, further, that selective PDE IV inhibitors could be clinically useful bronchodilators in man.

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