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Effects of fenspiride on human bronchial cyclic nucleotide phosphodiesterase isoenzymes: Functional and biochemical study

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Abstract

We have investigated the role of human bronchial cyclic nucleotide phosphodiesterases in the effects of fenspiride, a drug endowed with bronchodilator and anti-inflammatory properties. Functional studies on human isolated bronchi showed that fenspiride (10^{-6}–3×10^{-3} M, 30 min) induced a shift to the left of the concentration–response curves for isoprenaline and sodium nitroprusside with $-\log EC_{50}$ values of 4.1 ± 0.1 ($n = 7$) and 3.5 ± 0.2 ($n = 8$), respectively. Biochemical studies were carried out on three human bronchi in which separation of cyclic nucleotide phosphodiesterase isoenzymes was performed by ion exchange chromatography followed by determination of phosphodiesterase activity with a radioisotopic method. Phosphodiesterase 4 (cyclic AMP-specific) and phosphodiesterase 5 (cyclic GMP-specific) were the major phosphodiesterase isoforms present in the human bronchial tissue. The presence of phosphodiesterase 1 (Ca^{2+}/calmodulin-stimulated), phosphodiesterase 2 (cyclic GMP-stimulated) and, in two cases, phosphodiesterase 3 (cyclic GMP-inhibited) was also identified. Fenspiride inhibited phosphodiesterase 4 and phosphodiesterase 3 activities with $-\log IC_{50}$ values of 4.16 ± 0.09 and 3.44 ± 0.12, respectively. Phosphodiesterase 5 activity was also inhibited with a $-\log IC_{50}$ value of ~ 3.8. Fenspiride ($\leq 10^{-3}$ M) produced less than 25% inhibition of phosphodiesterase 1 and phosphodiesterase 2 activities. In conclusion, fenspiride is an effective inhibitor of both cyclic AMP and cyclic GMP hydrolytic activity in human bronchial tissues and this action may contribute to its airway effects. © 1998 Elsevier Science B.V.

Keywords: Fenspiride; Phosphodiesterase isoenzyme; Bronchus, human; Smooth muscle, airway

1. Introduction

Several experimental studies have demonstrated that fenspiride is a drug which exerts bronchodilator, antibronchoconstriction and antiinflammatory effects (Carre et al., 1991; Evrard et al., 1991; Cunha et al., 1993; De Castro et al., 1995; Laude et al., 1995). These actions are likely to play an important role in the treatment of a number of respiratory tract diseases (Akoun et al., 1991).

Recently, Girard et al. (1997) have shown that fenspiride (10^{-6} to 10^{-4} M) inhibited the non-adrenergic non-cholinergic component of the contraction of the guinea-pig isolated main bronchus induced by electrical field stimulation. Since fenspiride had no effect on the contractions induced by exogenously added substance P or [Nle^{10}]neurokinin A-(4–10) it was suggested that fenspiride acts at a prejunctional level on sensory nerves. This was supported by data using the guinea-pig perfused lung where fenspiride inhibited low pH-evoked release of calcitonin gene-related peptide, and suggests that fenspiride reduces the release of tachykinins from sensory nerves endings at a prejunctional level.

The non-selective cyclic nucleotide phosphodiesterase inhibitors, theophylline, enprofylline and isbufylline, inhibit non-adrenergic non-cholinergic contractile responses in guinea-pig isolated main bronchus. These findings suggest that cyclic nucleotide phosphodiesterase activity may regulate the release of neuropeptides from sensory nerves (Aikawa et al., 1992; Barlinski et al., 1992; Meini et al., 1993). Furthermore, it has been shown that the selective inhibitor of phosphodiesterase type 4, rolipram, but not the

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selective phosphodiesterase 3 and phosphodiesterase 5 inhibitors, siguazodan and zaprinast, respectively, attenuated the non-adrenergic non-cholinergic contractile response in guinea-pig isolated main bronchus (Qian et al., 1994; Undem et al., 1994; Spina et al., 1995).

In the present work, we have investigated the effects of fenspiride on human bronchial cyclic nucleotide phosphodiesterase activities by functional and biochemical studies. Since the airway relaxant responses to isoprenaline, a β-adrenoceptor agonist, and sodium nitroprusside, a nitrovasodilator, are mediated via activation of adenylyl and guanylyl cyclase, respectively, an inhibitory effect of fenspiride on cyclic AMP or cyclic GMP phosphodiesterase activity would be evidenced by a potentiation of the corresponding effects of these relaxants; hence, in functional experiments we assessed the influence of fenspiride on the concentration–response curves for isoprenaline and sodium nitroprusside in human isolated bronchi. In biochemical studies, fenspiride was tested directly for its inhibitory effect on the activity of cyclic nucleotide phosphodiesterase isoenzymes isolated from human bronchi.

2. Material and methods

2.1. Human bronchial tissue preparation

Bronchial tissues were removed from patients with lung cancer at the time of the surgical operation. All were previous smokers. None was asthmatic. Just after resection, segments of bronchi with an inner diameter of 2 to 4 mm were taken as far away as possible from the malignancy. They were placed in oxygenated Krebs–Henseleit solution (NaCl, 119; KCl, 5.4; $CaCl_2$, 2.5; KH_2PO_4, 0.6; $MgSO_4$, 1.2; $NaHCO_3$, 25; glucose 11.7 mM) and stored overnight at 4°C. For functional studies, four to eight rings of the same bronchus were prepared after removal of adhering fat and connective tissues. Each set of bronchial rings was suspended under an initial tension of 2 g in Krebs–Henseleit solution, bubbled with 95% O_2–5% CO_2 and maintained at 37°C. Force of contraction was measured isometrically with strain gauges UF1 (Pioden, Burkingam, England), amplifiers, and I.O.S.-Moise 3 recorder system (EMKA Technologies, Mitry Mory, France). For biochemical experiments, bronchi from three patients were processed as indicated below.

2.2. Functional study

In all experiments, human bronchi were first contracted maximally with acetylcholine (1 mM) and then relaxed with theophylline (1 mM). These concentrations did not alter subsequent responsiveness of the tissue as previously noted (Naline et al., 1996). During the next 60 min, the tissues were washed every 15 min and were equilibrated before beginning the experimental procedure. Experiments were conducted on parallel groups of 4 to 8 rings, one ring serving as control.

Following the resting period, the bronchial rings were incubated (30 min) with or without fenspiride (10^{-6} to 3×10^{-3} M). After the relaxant plateau was reached, bronchial rings were contracted with acetylcholine (3×10^{-5} M). 15 min after, concentration–response curves to isoprenaline (3×10^{-9} to 3×10^{-6} M) or sodium nitroprusside (10^{-8} to 10^{-4} M), were obtained by applying increasing concentrations of drugs, every 5 to 10 min until a plateau was reached. After the maximal effect of each drugs was obtained, theophylline (1 mM) was added to the bath in order to determine the maximal relaxation. Only one concentration–response curve to a relaxant agonist was recorded in each ring.

Since fenspiride ($\geq 10^{-4}$ M) reduced the cholinergic contraction level (see Results), readjusted tone experiments were carried out in which the above protocol was followed except that the concentration of acetylcholine (3×10^{-5} to 2×10^{-4} M) was titrated to produce the same starting level of contraction in the absence (control tissues contracted with 3×10^{-5} M acetylcholine) and presence of fenspiride (10^{-4}, 3×10^{-3} or 10^{-3} M); then relaxant curves for isoprenaline and sodium nitroprusside were obtained.

2.3. Biochemical study

Individual human bronchi were homogenized (Ultraturrax at 9000 r.p.m. for 60 s) 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). The homogenate was centrifuged at $15{,}000 \times g$ 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 phosphodiesterases 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 throughout and fractions of 0.5 ml were collected, analysed and stored as previously described (Gristwood et al., 1992; Cortijo et al., 1993). Cyclic nucleotide phosphodiesterases are classified according to the nomenclature outlined by Beavo et al. (1994).

Cyclic nucleotide phosphodiesterases were assayed following the procedure of Thompson and Strada (1984). The standard incubation mixture contained, in a final volume of 400 μl, 40 mM Tris–HCl, 5 mM $MgCl_2$, 3.75 mM β-mercaptoethanol, 1 μM ^{3}H-labelled/unlabelled cyclic nucleotide ($\sim$ 200,000 d.p.m.) and fenspiride at the concentration tested (see below). The substrate was cyclic AMP or cyclic GMP as appropriate. The standard incubation mixture and the enzyme solution were separately preincubated at 30°C for 2 min. Then, the assay was

initiated by adding 100 μl of the enzyme solution to the standard incubation mixture and the reaction was carried out at 30°C for 20 min. Inhibition assays were run in duplicate in at least two different enzyme preparations. The cyclic AMP phosphodiesterase activity was also determined in the presence of either Ca^{2+} (10 μM)/calmodulin (1.2 μM) or cold cyclic GMP (5 μM) as previously outlined by Cortijo et al. (1993) to look for phosphodiesterase activity stimulated or inhibited by these endogenous regulators.

Fenspiride was tested in preliminary experiments at low concentrations (10^{-8} to 10^{-6} M) to see the magnitude of the inhibition produced on cyclic nucleotide hydrolytic activity. Further experiments were carried out for all fractions in the absence (drug vehicle) or presence of fenspiride (10^{-5}, 10^{-4} or 10^{-3} M). Since co-elution of phosphodiesterase 3 and phosphodiesterase 4 activities has been previously obtained in human airways (De Boer et al., 1992), the contribution of the contaminating isoenzyme was reduced by assessing phosphodiesterase 3 activity in the presence of 10 μM rolipram whereas phosphodiesterase 4 activity was evaluated in the presence of 10 μM SKF94120 as outlined by Torphy et al. (1993) and Cortijo et al. (1996). These concentrations of SKF94120 and rolipram were added to the standard mixture and have been shown previously to be selective for phosphodiesterase 3 and phosphodiesterase 4, respectively. Appropriate controls with drug vehicles were carried out to see whether any alteration of enzyme activities was present.

2.4. Statistical analysis of results. Drugs

The maximal relaxant effects (E_{max}) of isoprenaline or sodium nitroprusside were expressed as a percentage of the relaxation induced by theophylline (1 mM). The $-\log EC_{50}$ (i.e. pD_2) values, defined as the concentration of drug which induces an effect equal to 50% of its own maximal effect, were determined. In biochemical studies, calculation of inhibitory concentration 50% (IC_{50}) of phosphodiesterase isoenzymes activities was derived from the inhibitory effect of fenspiride against phosphodiesterase activity of the relevant column fractions; the $-\log IC_{50}$ values were obtained by non-linear regression using GraphPad software.

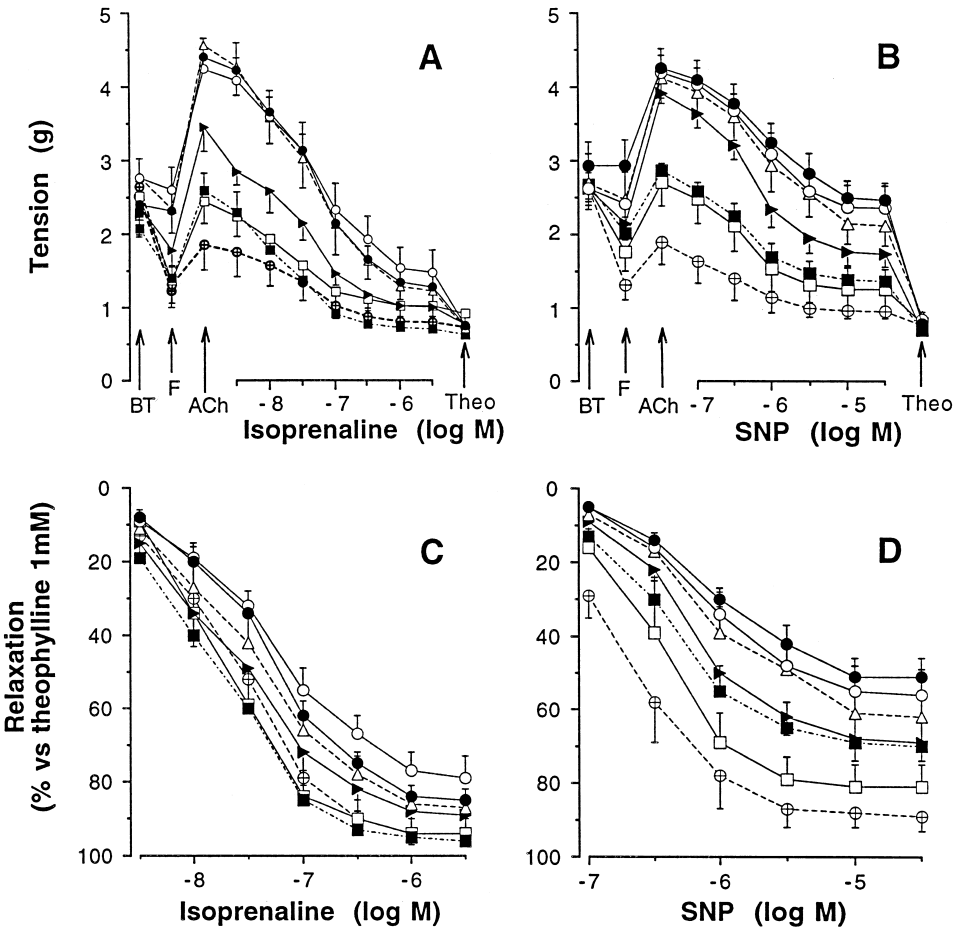

Fig. 1. Influence of fenspiride on the concentration–response curve for isoprenaline (panels A and C) or sodium nitroprusside (panels B and D) in human isolated bronchi. Concentration–response curves for isoprenaline or sodium nitroprusside were obtained after 30 min incubation and in the presence of saline (control, ●) or fenspiride in concentrations of 10^{-6} (○), 10^{-5} (△), 10^{-4} (▶), 3×10^{-4} (■), 10^{-3} (□), or 3×10^{-3} M (⊕). Results are expressed as tension changes (g) (A, B) or as relaxation as percentage of theophylline (1 mM) (C, D). Values are means ± S.E.M.

Data are expressed as mean ± S.E.M. Statistical analysis of the results was performed using analysis of variance and Student's *t*-test for paired or unpaired data. *P*-values lower than 0.05 were considered significant.

The drugs used were: fenspiride (Servier, Courbevoie, France), isoprenaline HCl, sodium nitroprusside (Sigma, St. Louis, MO) and acetylcholine HCl (Pharmacie central des Hôpitaux, Paris, France). Theophylline sodium anisate was used as proprietary injectable solution (Theophylline Bruneau®, Paris, France). Calmodulin was obtained from Boehringer Mannheim (Barcelona, Spain); benzamidine, phenylmethylsulphonylfluoride, cyclic GMP and cyclic AMP were from Sigma-Aldrich Química S.A. (Madrid, Spain). SKF94120 (5-(4-acetimidophenyl)pyrazin-(1H)-one) and rolipram were obtained as a gift from Laboratorios Almirall (Barcelona, Spain). [8-^{3}H]adenosine 3′:5′-cyclic monophosphate and [8-^{3}H]guanosine 3′:5′-cyclic monophosphate were from Amersham International (Madrid, Spain). SKF94120 and rolipram were prepared in 20% polyethyleneglycol 300. Other drugs used were dissolved in distilled water and then diluted in the Krebs solution as appropriate.

Table 1
Shift of the concentration–response curves and E_{max} of isoprenaline and sodium nitroprusside (sodium nitroprusside) in the presence of fenspiride on isolated human bronchus precontracted by acetylcholine (3×10^{-5} M)

		Isoprenaline	Sodium nitroprusside
Control (pD_2, $-\log$ M)		7.41 ± 0.13 (7)	6.11 ± 0.08 (8)
Fenspiride	10^{-6} M	0.05 ± 0.09 (5)	0.01 ± 0.03 (7)
	10^{-5} M	0.08 ± 0.07 (7)	0.00 ± 0.05 (8)
	10^{-4} M	0.22 ± 0.09 (7)[a]	0.14 ± 0.02 (8)[c]
	3×10^{-4} M	0.36 ± 0.09 (7)[b]	0.26 ± 0.06 (8)[b]
	10^{-3} M	0.21 ± 0.09 (6)	0.37 ± 0.08 (8)[b]
	3×10^{-3} M	0.20 ± 0.11 (7)	0.58 ± 0.09 (7)[c]
Control (E_{max}, %)		85 ± 3 (7)	51 ± 5 (8)
Fenspiride	10^{-6} M	−10 ± 5 (5)	+4 ± 5 (7)
	10^{-5} M	+2 ± 4 (7)	+11 ± 7 (8)
	10^{-4} M	+3 ± 4 (7)	+15 ± 4 (8)[a]
	3×10^{-4} M	+11 ± 3 (7)[a]	+16 ± 5 (8)[a]
	10^{-3} M	+11 ± 5 (6)	+30 ± 5 (8)[b]
	3×10^{-3} M	+9 ± 3 (7)[a]	+34 ± 4 (7)[c]

Shifts were calculated as differences between pD_2 or E_{max} values of isoprenaline or sodium nitroprusside in the absence (control) or in the presence of fenspiride. The number of experiments is given in parenthesis. Significant shifts: [a]$P < 0.05$; [b]$P < 0.01$; [c]$P < 0.001$.

3. Results

3.1. Functional study

Isoprenaline and sodium nitroprusside produced a concentration-related relaxation of acetylcholine-contracted human isolated bronchi (Fig. 1). The potency and E_{max} values for these two relaxants are shown in Table 1. Sodium nitroprusside was less potent than isoprenaline and the maximal relaxation produced by sodium nitroprusside was also smaller than that obtained for isoprenaline (Fig. 1, Table 1). Fig. 1 shows that after 30 min incubation of the human isolated bronchus with fenspiride (10^{-6} to 3×10^{-3} M), the concentration–response curves for isoprenaline and sodium nitroprusside were shifted to the left and the maximal effects were increased. Leftward shifts of the pD_2 values of isoprenaline and sodium nitroprusside were obtained for fenspiride ($\geq 10^{-4}$ M) but the extent of the shift produced by fenspiride was greater for sodium nitroprusside. The augmentation of E_{max} values of sodium nitroprusside was observed with concentrations of fenspiride (10^{-4} M) lower than those (3×10^{-4}–3×10^{-3} M) producing a potentiation of isoprenaline-induced relaxation (Table 1). The maximal augmentation by fenspiride of the effects of sodium nitroprusside was greater than that observed for isoprenaline. The $-\log EC_{50}$ values of fenspiride inducing potentiation of the effects of isoprenaline and sodium nitroprusside were 4.1 ± 0.1 ($n = 7$) and 3.5 ± 0.2 ($n = 8$), respectively.

Since fenspiride ($\geq 10^{-4}$ M) reduced the cholinergic contraction level from which relaxant curves were constructed, readjusted tone experiments were carried out. In control experiments, isoprenaline and sodium nitroprusside relaxed acetylcholine (3×10^{-5} M)-induced contraction with pD_2 and E_{max} values that were 7.39 ± 0.06 and 85 ± 2%, and 5.95 ± 0.04 and 49 ± 2%, respectively ($n = 5$). The contraction produced by acetylcholine (3×10^{-5} to 2×10^{-4} M) in fenspiride (10^{-4} to 10^{-3} M)-treated tissues was not significantly different from the level obtained in control tissues (data not shown). Fenspiride produced significant leftward shifts of the concentration–response curves for isoprenaline without significant changes of E_{max} of isoprenaline. Shifts of pD_2 values were 0.24 ± 0.07, 0.37 ± 0.09 and 0.23 ± 0.09 for 10^{-4}, 3×10^{-4} and 10^{-3} M fenspiride, respectively ($n = 5$). Fenspiride produced significant left and upward shifts of the concentration–response curves for sodium nitroprusside. Shifts of pD_2 and E_{max} values were 0.16 ± 0.05 and +8 ± 2, 0.19 ± 0.05 and +19 ± 5, and 0.33 ± 0.10 and +29 ± 1 for 10^{-4}, 3×10^{-4} and 10^{-3} M fenspiride, respectively ($n = 5$).

3.2. Biochemical study

A representative chromatogram of the phosphodiesterases isolated from human bronchi by ion exchange is shown in Fig. 2. When assayed with 1 μM cyclic AMP as substrate a broad band of cyclic AMP phosphodiesterase activity appeared for low sodium concentrations followed by a large peak (column fraction 48) at high sodium concentration that represented the largest portion of cyclic AMP hydrolytic activity (Fig. 2A). The cyclic AMP hydrolytic activity in fractions 16 (0.20 M sodium acetate) and 24 (0.40 M sodium acetate) was stimulated several-fold by the addition of Ca^{2+}/calmodulin (Fig. 2B) indicating the

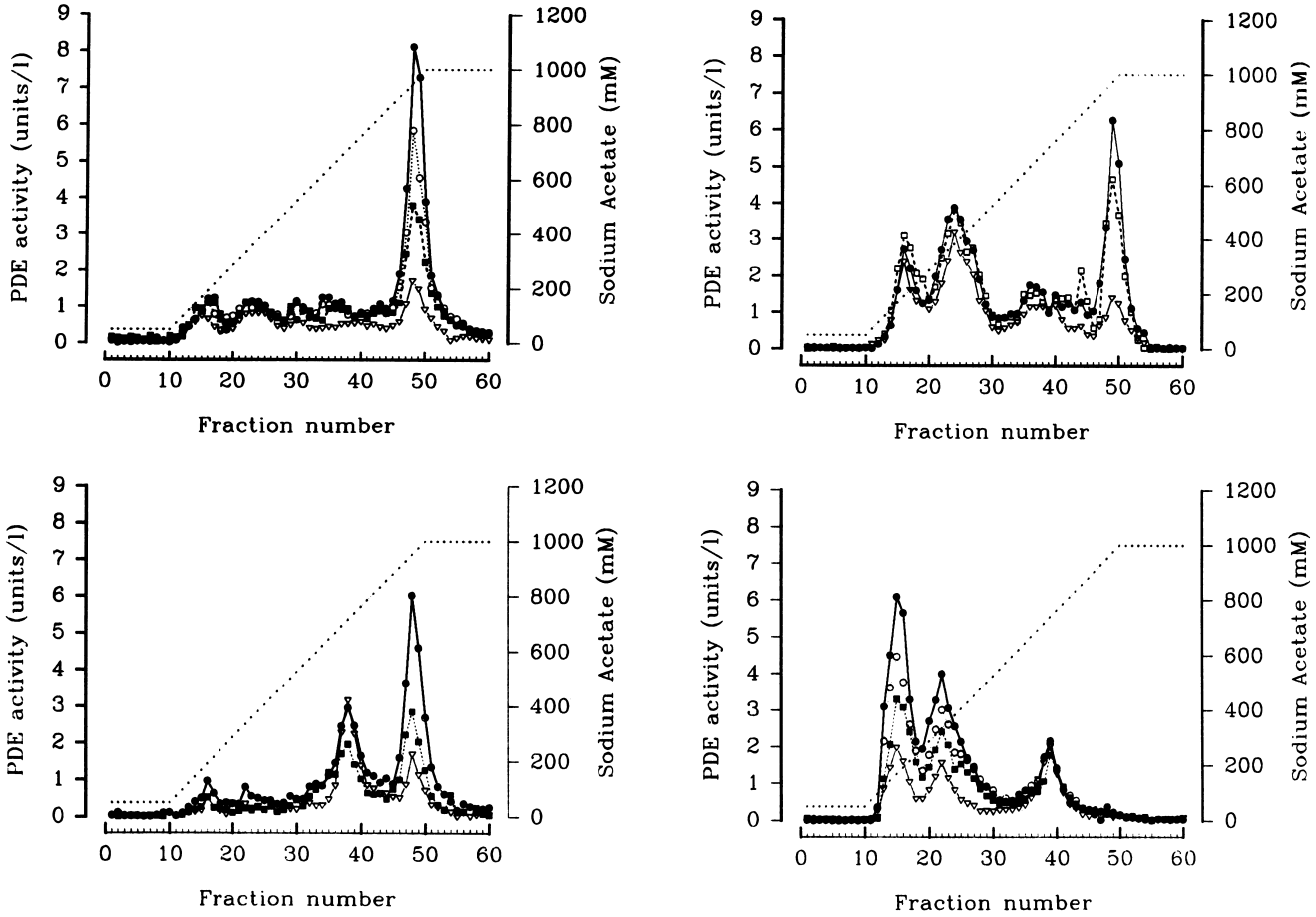

Fig. 2. Representative elution profile of cyclic nucleotide phosphodiesterase (phosphodiesterase) activities from human bronchus on a Mono Q ion exchange column. The low speed centrifugation supernatant from each individual sample was chromatographed as described in the text. Fractions (0.5 ml) were collected and assayed for phosphodiesterase activity in the following conditions: panel A, 1 μM cyclic AMP; panel B, 1 μM cyclic AMP in the presence of Ca^{2+}/calmodulin; panel C, 1 μM cyclic AMP plus 1 μM cold cyclic GMP and panel D, 1 μM cyclic GMP. The phosphodiesterase activities were measured in the absence (●) and in the presence of fenspiride 10^{-5} (○), 10^{-4} (■) or 10^{-3} (▽) M. For the ordinate scale, 1 unit of enzyme activity is defined as the amount hydrolysing 1 μmol of substrate per min.

presence of phosphodiesterase 1. Activity in fraction 38 (0.70 M sodium acetate) was stimulated by cyclic GMP thus revealing the presence of phosphodiesterase 2 (Fig. 2C). The fraction 48 (0.90 M sodium acetate) was very sensitive to rolipram (data not shown) indicating the presence of phosphodiesterase 4 activity. In two patients, the cyclic AMP phosphodiesterase activity in this peak was partly inhibited (~25%) in the presence of cyclic GMP, thus indicating that phosphodiesterase 3 co-eluting with phosphodiesterase 4 contributed to a small portion of cyclic AMP hydrolytic activity of this peak. The cyclic GMP hydrolytic activity eluted in three peaks corresponding to fractions 15 (0.17 M sodium acetate), 22 (0.32 M sodium acetate) and 39 (0.74 M sodium acetate) (Fig. 2D). The first two peaks contained a mixture of phosphodiesterase 5 (sensitive to zaprinast; data not shown) and phosphodiesterase 1 whereas the third peak corresponded to phosphodiesterase 2 activity.

Preliminary experiments showed that concentrations of fenspiride in the range 10^{-8} to 10^{-6} M were producing nil

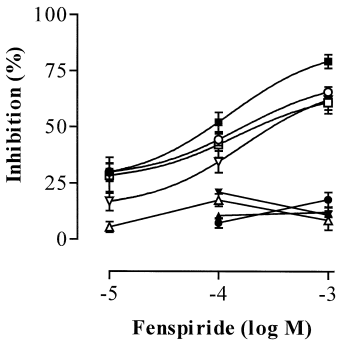

Fig. 3. Inhibition by fenspiride of cyclic AMP and cyclic GMP hydrolytic activities isolated from human bronchi. The inhibitory effect of fenspiride (10^{-5} to 10^{-3} M) is shown for the cyclic nucleotide phosphodiesterase activities of different fractions (fraction numbers as shown in Fig. 2). Fenspiride inhibited, in a concentration-related manner, the cyclic AMP hydrolytic activity of fraction 48 in the presence of either SKF94120 (■) or rolipram (▽) and the cyclic GMP hydrolytic activities of fractions 15 (○) and 22 (□). Fenspiride produced weak inhibition of the cyclic AMP hydrolytic activities of fractions 16 (▲), 24 (▼), and 38 (●) as well as of the cyclic GMP hydrolytic activity of fraction 39 (△). Points are mean ± S.E.M. of data obtained from three patients.

or marginal inhibitions of phosphodiesterase activity (data not shown). The concentration-dependent inhibitory effects of fenspiride (10^{-5}, 10^{-4} and 10^{-3} M) are shown in Fig. 2 for cyclic AMP phosphodiesterase activity (in the absence and presence of endogenous regulators, i.e. Ca^{2+}/calmodulin and cyclic GMP) and cyclic GMP phosphodiesterase activity for all column fractions. The $-\log IC_{50}$ value for fenspiride against phosphodiesterase 4 was calculated to be 4.16 ± 0.09 (Fig. 3). In two patients in which phosphodiesterase 3 was co-eluting with phosphodiesterase 4, fenspiride inhibited phosphodiesterase 3 activity with $-\log IC_{50}$ value of 3.44 ± 0.12 ($P < 0.05$ compared to value for phosphodiesterase 4). Fenspiride inhibited cyclic GMP phosphodiesterase activity of fractions 15 and 22 with $-\log IC_{50}$ values of 3.80 ± 0.10 and 3.61 ± 0.23, respectively (Fig. 3). The inhibitory effect of fenspiride against cyclic AMP phosphodiesterase activity of fractions 16 and 38 and against cyclic GMP phosphodiesterase activity of fraction 39 was very weak with values below 25% inhibition (Fig. 3).

4. Discussion

4.1. Functional study

Phosphodiesterase activity is responsible for the cellular breakdown of cyclic AMP and cyclic GMP. At least five phosphodiesterase isoenzyme families have been identified and evidence exists of differences in the tissue distribution of these isoenzymes (Beavo and Reifsnyder, 1990; Beavo, 1995). Recently, phosphodiesterase 6 and 7 have also been identified (Beavo et al., 1994) but their relevance in airways is yet uncertain. It appears that inflammatory cells contain mainly phosphodiesterase 4 while airway smooth muscle contains phosphodiesterases 1 to 5 (Torphy and Undem, 1991; Nicholson and Shahid, 1994; Raeburn and Advenier, 1995). However, from a functional point of view, phosphodiesterase 4 seems predominant in human airway smooth muscle (De Boer et al., 1992; Qian et al., 1993; Cortijo et al., 1993). Consequently, selective inhibition of phosphodiesterase 4 which combines antiinflammatory and bronchodilator properties appears of interest in the treatment of airway diseases.

Our results (Fig. 1, Table 1) demonstrate that fenspiride potentiates the effects of both isoprenaline and sodium nitroprusside. However, in these experiments fenspiride ($\geq 10^{-4}$ M) reduced the starting level of cholinergic contraction from which isoprenaline and sodium nitroprusside relaxant curves were constructed and therefore a lessened degree of functional antagonism (Van Amsterdam et al., 1990) may have contributed to the potentiation produced by fenspiride. In additional experiments, the decrease in tone was fully corrected by increasing the acetylcholine concentration within limits that reasonably exclude the risk of interfering the agonists signalling system (Roffel et al., 1995). In readjusted tone experiments, fenspiride potentiated also the relaxant responses to isoprenaline and sodium nitroprusside. The shifts of the pD_2 values found in the present study for fenspiride are in the range of those reported in other studies for the potentiation of the relaxant curves of isoprenaline and sodium nitroprusside by non-selective and selective phosphodiesterase inhibitors (Qian et al., 1993; Zhang et al., 1993; Naline et al., 1996).

The potentiation by fenspiride of the responses to isoprenaline and sodium nitroprusside is exerted at similar concentrations though the potentiation of the relaxant effects of sodium nitroprusside was greater than that observed for isoprenaline in terms of both leftward shift of concentration–response curve and augmentation of maximal effect. However, the assignment of any preferential inhibitory effect of fenspiride against particular isoenzymes remains speculative in the absence of data generated under the same experimental conditions by selective phosphodiesterase inhibitors.

4.2. Biochemical study

The predominant cyclic AMP and cyclic GMP hydrolytic activities found in human bronchus were phosphodiesterase 4 and phosphodiesterase 5 although other phosphodiesterase activities (phosphodiesterase 1, phosphodiesterase 2 and in two cases phosphodiesterase 3) were also identified. These results are consistent with those reported for human airway smooth muscle in this (Cortijo et al., 1993, 1996) and other laboratories (De Boer et al., 1992; Rabe et al., 1993; Torphy et al., 1993).

We have shown that fenspiride inhibits cyclic AMP phosphodiesterase 4 and cyclic GMP phosphodiesterase 5 activities with similar $-\log IC_{50}$ values (~ 4.1 and ~ 3.8, respectively, i.e. circa 10^{-4} M). It is interesting to compare the $-\log IC_{50}$ values of fenspiride as inhibitor of human bronchial cyclic AMP and cyclic GMP specific phosphodiesterase activities with the $-\log EC_{50}$ values of the same drug as relaxant of human isolated bronchus. Data from Advenier (1988) showed that the $-\log EC_{50}$ values for fenspiride as relaxant of human isolated bronchus pre-contracted by different spasmogens were situated around 4 (4.49 ± 0.14, 3.76 ± 0.10, 2.91 ± 0.16 and 3.15 ± 0.18 for histamine-, carbachol-, KCl- and leukotriene D_4-induced tone, respectively). These results would be also consistent with data showing that concentrations of fenspiride in the same range (i.e. around 10^{-4} M) potentiate the relaxation produced by both isoprenaline and sodium nitroprusside in guinea-pig isolated trachea (unpublished results) and human isolated bronchus (this study) since this potentiation would have its basis in the inhibitory effect exerted by fenspiride against cyclic AMP (potentiation of isoprenaline-induced relaxation) and cyclic GMP (potentiation of sodium nitropruside-induced relaxation) phosphodiesterase activities.

The role of phosphodiesterase 3 in the maintenance of human bronchial tone is uncertain. Unlike in guinea-pig trachea (Harris et al., 1989), the cyclic AMP hydrolysing isoenzyme that mainly regulates tone in human bronchus is the type 4 but type 3 contributes also. Thus, selective as well as mixed inhibitors of phosphodiesterase types 4 and 3 fully relaxed spontaneous and pharmacologically supported tone in human isolated bronchus (De Boer et al., 1992; Cortijo et al., 1993; Qian et al., 1993; Naline et al., 1996) although others found weak relaxations for rolipram (Rabe et al., 1993; Torphy et al., 1993). However, phosphodiesterase 3 activity is not always separated from human bronchus (De Boer et al., 1992; Cortijo et al., 1993) and when isolated co-elutes with phosphodiesterase 4 and represents only a small percentage ($\leq 25\%$) of total cyclic AMP phosphodiesterase activity (De Boer et al., 1992; present study). Furthermore, selective phosphodiesterase 3 inhibitors failed to induce increases in intracellular cyclic AMP content in human cultured tracheal smooth muscle cells (Hall et al., 1992). Therefore, since fenspiride inhibits phosphodiesterase 3 with a potency close to that found for inhibition of phosphodiesterases 4 and 5, it is possible that these three isoenzymes contribute to the pulmonary effects of this drug in a measure proportional to their relative functional relevance. Fenspiride inhibited also calmodulin-stimulated phosphodiesterase (type 1) and cyclic GMP-stimulated phosphodiesterase (type 2) activities isolated from human bronchi but its effectiveness and potency to this respect was much less than that showed against the other phosphodiesterases studied.

In conclusion, the closeness of the EC_{50} values of fenspiride in functional studies (bronchodilation and potentiation of relaxant agents) with the IC_{50} values found in biochemical studies (inhibition of relevant cyclic AMP and cyclic GMP hydrolytic activities) suggests the participation of phosphodiesterase inhibition in the pulmonary effects of this compound. However, the clinical relevance of this effect remains to be established. Airway tissue levels after repeated administration of fenspiride (oral dose of 1.1 mg/kg/day) are unknown but plasma levels are about 3×10^{-6} M (unpublished data), a concentration which produces only marginal inhibition of phosphodiesterase activity. For comparison, the therapeutic plasma concentrations of unbound theophylline (oral dose of 10 mg/kg/day) are around 3×10^{-5} M which produces $\leq 20\%$ inhibition of cyclic nucleotide phosphodiesterase activity in airway muscle (Small et al., 1989).

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References

Advenier, C., 1988. Fenspiride et relaxation de la musculature trachéo-bronchique Mécanisme daction. Rhinology 4 (Suppl.), 67–74.

Aikawa, T., Sekiza, W.A., Morikawa, M., Itabashi, S., Sasaki, H., Takishimi, T., 1992. The role of cyclic AMP in non-adrenergic non-cholinergic contraction in guinea-pig bronchi. Br. J. Pharmacol. 105, 609–612.

Akoun, G., Arnaud, F., Blanchon, F., Botto, J.M., Cerf, C., Clavier, J., Derenne, J.P., Leophonte, P., Nouvet, G., Paramelle, B., Patron, P., Prefaut, C., Verlardocchio, J.M., 1991. Effects of fenspiride on airway function and blood gases in stable COPD patients. Eur. Respir. Rev. 1, 111–125.

Barlinski, J., Lockhart, A., Frossard, N., 1992. Modulation by theophylline and enprofylline of the excitatory non-cholinergic transmission in guinea-pig bronchi. Eur. Respir. J. 5, 1201–1205.

Beavo, J.A., 1995. Cyclic nucleotide phosphodiesterases: Functional implications of multiple isoforms. Physiol. Rev. 75, 725–748.

Beavo, J.A., Reifsnyder, D.H., 1990. Primary sequence of cyclic nucleotide phosphodiesterase isoenzymes and the design of selective inhibitors. Trends Pharmacol. Sci. 11, 150–155.

Beavo, J.A., Conti, M., Heaslip, R.J., 1994. Multiple cyclic nucleotide phosphodiesterases. Mol. Pharmacol. 46, 399–405.

Carre, P.H., Pipy, B., Pinelli, E., Forgue, M.F., Beraud, M., Didier, A., Leophonte, P., 1991. In vitro effect of fenspiride on the production of free oxygen radicals, prostaglandins and leukotrienes by guinea-pig alveolar macrophages. Eur. Respir. Rev. 1, 79–85.

Cortijo, J., Bou, J., Beleta, J., Cardelús, I., Llenas, J., Morcillo, E., Gristwood, R.W., 1993. Investigation into the role of phosphodiesterase IV in bronchorelaxation, including studies with human bronchus. Br. J. Pharmacol. 108, 562–568.

Cortijo, J., Villagrasa, V., Navarrete, C., Sanz, C., Berto, L., Michel, A., Bonnet, P.A., Morcillo, E.J., 1996. Effects of SCA40 on human isolated bronchus and human polymorphonuclear leukocytes: Comparison with rolipram, SKF94120 and levcromakalim. Br. J. Pharmacol. 119, 99–106.

Cunha, F.Q., Boukili, M.A., Braga da Motta, J.I., Vargaftiig, B.B., Ferreira, S.H., 1993. Blockade by fenspiride of endotoxin-induced neutrophil migration in the rat. Eur. J. Pharmacol. 238, 47–52.

de Boer, J., Philpott, A.J., Van Amsterdam, R.G.M., Shahid, M., Zaagsma, J., Nicholson, C.D., 1992. Human bronchial cyclic nucleotide phosphodiesterase isoenzymes: Biochemical and pharmacological analysis using selective inhibitors. Br. J. Pharmacol. 106, 1028–1034.

de Castro, C.M.M.B., Nahori, M.A., Dumarey, C.M.H., Vargaftig, B.B., Bachelet, M., 1995. Fenspiride: An anti-inflammatory drug with potential benefits in the treatment of endotoxemia. Eur. J. Pharmacol. 294, 669–676.

Evrard, Y., Kato, G., Bodinier, M.C., Chapelain, B., 1991. Fenspiride and inflammation in experimental pharmacology. Eur. Respir. Rev. 1, 93–100.

Girard, V., Naline, E., Crambes, O., Malbezin, M., Malmström, R.E., Lundberg, J.M., Advenier, C., 1997. Pre- and postjunctional inhibitory effects of fenspiride on guinea-pig bronchi. Eur. Respir. J. 10, 1015–1020.

Gristwood, R.W., Beleta, J., Bou, J., Cardelus, I., Fernandez, A.G., Llenas, J., Berga, P., 1992. Studies on the cardiac actions of flosequinan in vitro. Br. J. Pharmacol. 105, 985–991.

Hall, I.P., Townsend, P., Daykin, K., Widdop, S., 1992. Control of tissue cyclic AMP content in primary cultures of human airway smooth muscle cells. Br. J. Pharmacol. 105, 71P.

Harris, A.L., Connell, M.J., Ferguson, E.W., Wallace, A.M., Gordon, R.J., Pagani, D.E., Silver, P.J., 1989. Role of low k_m cyclic AMP phosphodiesterase inhibition in tracheal relaxation and bronchodilation in the guinea-pig. J. Pharmacol. Exp. Ther. 251, 199–206.

Laude, E.A., Bee, D., Crambes, O., Howard, P., 1995. Antitussive and antibronchoconstriction actions of fenspiride in guinea-pigs. Eur. Respir. J. 8, 1699–1704.

Meini, S., Ballati, L., Evangelista, S., Manzini, S., 1993. Isbufylline, a xanthine derivative, inhibits bronchoconstrictor responses produced by stimulation of capsaicin-sensitive sensory nerves in guinea-pig: 'In vitro' and 'in vivo' evidence. Pulmon. Pharmacol. 6, 279–286.

Naline, E., Qian, Y., Advenier, C., Raeburn, D., Karlsson, J.A., 1996. Effects of RP 73401, a novel, potent and selective phosphodiesterase type 4 inhibitor, on contractility of human, isolated bronchial muscle. Br. J. Pharmacol. 118, 1939–1944.

Nicholson, C.D., Shahid, M., 1994. Inhibitors of cyclic nucleotide phosphodiesterase isoenzymes. Their potential utility in the therapy of asthma. Pulmon. Pharmacol. 7, 1–18.

Qian, Y., Naline, E., Karlsson, J.A., Raeburn, D., Advenier, C., 1993. Effects of rolipram and siguazodan on the human isolated bronchus and their interaction with isoprenaline and sodium nitroprusside. Br. J. Pharmacol. 109, 774–778.

Qian, Y., Girard, V., Martin, C.A.E., Molimard, M., Advenier, C., 1994. Rolipram, but not siguazodan or zaprinast, inhibits the excitatory noncholinergic neurotransmission in guinea-pig bronchi. Eur. Respir. J. 7, 306–310.

Rabe, K.F., Tenor, H., Dent, G., Schudt, C., Liebig, S., Magnussen, H., 1993. Phosphodiesterase isozymes modulating inherent tone in human airways: Identification and characterization. Am. J. Physiol. 264, L458–L464.

Raeburn, D., Advenier, C., 1995. Isoenzyme-selective cyclic nucleotide phosphodiesterase inhibitors: Effects on airways smooth muscle. Int. J. Biochem. Cell Biol. 27, 29–37.

Roffel, A.F., Meurs, H., Elzinga, C.R.S., Zaagsma, J., 1995. No evidence for a role of muscarinic M_2 receptors in functional antagonism in bovine trachea. Br. J. Pharmacol. 115, 665–671.

Small, R.C., Boyle, J.P., Duty, S., Elliott, K.R.F., Foster, R.W., Watt, A.J., 1989. Analysis of the relaxant effects of AH21–132 in guinea-pig isolated trachealis. Br. J. Pharmacol. 97, 1165–1173.

Spina, D., Harrison, S., Page, C.P., 1995. Regulation by phosphodiesterase isoenzymes of non-adrenergic non-cholinergic contraction in guinea-pig isolated main bronchus. Br. J. Pharmacol. 116, 2334–2340.

Thompson, W.J., Strada, S.J., 1984. Cyclic nucleotide phosphodiesterase (PDE). In: Bergmayer, H.U. (Ed.), Methods of Enzymatic Analysis, vol. IV, 3rd ed. Verlag Chemie, Weinheim, pp. 127–134.

Torphy, T.J., Undem, B.J., 1991. Phosphodiesterase inhibitors: New opportunities for the treatment of asthma. Thorax 46, 512–523.

Torphy, T.J., Undem, B.J., Cieslinski, L.B., Luttmann, M.A., Reeves, M.L., Hay, D.W.P., 1993. Identification, characterization and functional role of phosphodiesterase isozymes in human airway smooth muscle. J. Pharmacol. Exp. Ther. 265, 1213–1223.

Undem, B.J., Meeker, S.N., Chen, J., 1994. Inhibition of neurally mediated nonadrenergic noncholinergic contractions of guinea-pig bronchys by isozyme-selective phosphodiesterase inhibitors. J. Pharmacol. Exp. Ther. 271, 811–817.

van Amsterdam, R.G.M., Meurs, H., Ten Berge, R.E.J., Veninga, N.C.M., Brouwer, F., Zaagsma, J., 1990. Role of phosphoinositide metabolism in human bronchial smooth muscle contraction and in functional antagonism by beta-adrenoceptor agonists. Am. Rev. Respir. Dis. 142, 1124–1128.

Zhang, Y., Palette-Pays, C., Naline, E., Varoquaux, O., Advenier, C., 1993. Effect of molsidomine and linsidomine on the human isolated bronchus and the guinea-pig isolated trachea. J. Pharm. Pharmacol. 45, 280–285.