The presence of five cyclic nucleotide phosphodiesterase isoenzyme activities in bovine tracheal smooth muscle and the functional effects of selective inhibitors

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1 The profile of cyclic nucleotide phosphodiesterase (PDE) isoenzymes and the relaxant effects of isoenzyme selective inhibitors were examined in bovine tracheal smooth muscle. The compounds examined were the non-selective inhibitor 3-isobutyl-1-methylxanthine (IBMX), zaprinast (PDE V selective), milrinone and Org 9935 (4,5-dihydro-6-(5,6-dimethoxy-benzo[b]thien-2-yl)-5-methyl-1(2H)-pyridazinone; both PDE III selective), rolipram (PDE IV selective) and Org 30029 (N-hydroxy-5,6-dimethoxy-benzo[b]-thiophene-2-carboximidamide HCl a dual PDE III/IV inhibitor).

2 Ion exchange chromatography showed three main peaks of PDE activity. The first peak was stimulated by Ca^{2+} /calmodulin (PDE I), the adenosine 3':5'-cyclic monophosphate (cyclic AMP) hydrolytic activity of the second peak was stimulated by guanosine 3':5'-cyclic monophosphate (cyclic GMP) (PDE II) whilst that of the third peak was not significantly modified by any regulator (PDE IV). Calmodulin affinity chromatography revealed the additional presence of cyclic GMP-specific PDE (PDE V) in the first peak. A clearly distinct peak of cyclic GMP-inhibited PDE (PDE III) was not observed. However, Org 9935 inhibited the third activity peak more effectively in the presence, than in the absence, of rolipram ($3 \mu\text{mol l}^{-1}$), indicating the presence of PDE III activity.

3 Rolipram was the most potent inhibitor of PDE IV. The mean $-\log_{50} \text{IC}_{50}$ values for rolipram, IBMX, milrinone, Org 30029, Org 9935 and zaprinast were 5.9 ± 0.1 , 4.9 ± 0.1 , 4.7 ± 0.1 , 4.6 ± 0.1 and 4.6 ± 0.1 , respectively.

4 Rolipram was a potent relaxant of both histamine ($1 \mu\text{mol l}^{-1}$) and methacholine ($0.03 \mu\text{mol l}^{-1}$) pre-contracted preparations; (pD_2 values; histamine 7.1 ± 0.1 , methacholine 6.8 ± 0.2 and 4.5 ± 0.1 , biphasic relaxation). IBMX also relaxed all preparations (pD_2 values; histamine 5.6 ± 0.1 , methacholine 5.6 ± 0.1) whilst zaprinast (pD_2 values; histamine 5.2 ± 0.1 , methacholine 4.4 ± 0.3), milrinone (pD_2 values; histamine 5.2 ± 0.1 , methacholine 4.3 ± 0.3) and Org 9935 (pD_2 values; histamine 4.1 ± 0.1 , methacholine 4.1 ± 0.2) did not completely relax preparations at concentrations up to $100 \mu\text{mol l}^{-1}$. Org 30029 (pD_2 values; histamine 6.2 ± 0.1 , methacholine 5.4 ± 0.1) was a more effective relaxant than can be explained on the basis of PDE IV inhibition alone.

5 We conclude that bovine tracheal smooth muscle contains five distinct PDE isoenzymes. PDE IV appears to be more important in the modulation of tissue function than PDE III and PDE V.

Keywords: Cyclic nucleotide phosphodiesterase isoenzymes; bovine tracheal smooth muscle relaxation; rolipram; IBMX; zaprinast; Org 9935; Org 30029; milrinone

Introduction

The presence of multiple cyclic nucleotide phosphodiesterase (PDE) isoenzymes in mammalian cells and their role in the metabolism of adenosine 3':5'-cyclic monophosphate (cyclic AMP) and guanosine 3':5'-cyclic monophosphate (cyclic GMP) has been extensively described (Appleman *et al.*, 1982; Reeves *et al.*, 1987; Beavo, 1988; Nicholson *et al.*, 1991). The identification of five families of PDE in cells has been paralleled by the synthesis of isoenzyme selective inhibitors. By use of these agents as pharmacological tools, the effects of selective isoenzyme inhibition on tissue function have been examined. It has become apparent that the presence of a particular isoenzyme within a cell does not necessarily mean that its inhibition will initiate a response normally associated with cyclic nucleotide elevation (Weishaar *et al.*, 1987; Gristwood *et al.*, 1986; Shahid & Nicholson, 1990). Accordingly, the role played by particular PDE isoenzymes in cells can only be determined by the elucidation of both the isoenzyme profile within a particular tissue as well as the functional activity of isoenzyme selective inhibitors.

The aim of the present study was to analyse the PDE isoenzymes present in bovine tracheal smooth muscle and to evaluate the functional effects of isoenzyme inhibitors in this

tissue. PDE inhibitors differ in their ability to inhibit contractions of airways preparations induced by different agonists (Heaslop *et al.*, 1986; Torphy *et al.*, 1988). The ability of non-selective PDE inhibitors to relax airways smooth muscle is well known (Fredholm *et al.*, 1979; Lohmann *et al.*, 1977). Thus we investigated the effects of a range of isoenzyme-selective inhibitors on both histamine and methacholine-induced contractions. The inhibitors used were 3-isobutyl-1-methylxanthine (IBMX), a non-selective PDE inhibitor (Davis 1984; Nicholson *et al.*, 1989), the PDE III selective inhibitors milrinone (Bristol *et al.*, 1984; Shahid & Nicholson, 1990) and Org 9935 (4,5-dihydro-6-(5,6-dimethoxybenzo[b]thien-2-yl)-5-methyl-3(2H)-pyridazinone; Shahid *et al.*, 1991), rolipram, a PDE IV selective inhibitor (Reeves *et al.*, 1987), Org 30029 (N-hydroxy-5,6-dimethoxy-benzo[b]thiophene-2-carboximidamide HCl) a dual PDE III/PDE IV inhibitor (Shahid & Nicholson, 1990) and zaprinast, a PDE V selective inhibitor (Weishaar *et al.*, 1986).

Methods

Separation of cyclic nucleotide phosphodiesterase isoenzymes

Fresh bovine tracheae were obtained from a local slaughterhouse and transported to the laboratory in chilled Krebs-Henseleit solution (pH 7.4) pre-gassed with 95% O_2 : 5%

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CO₂. All subsequent steps were performed at 4°C. Tracheal smooth muscle was carefully dissected and cut into smaller strips which were then trimmed free of mucosal, connective and fat tissue in Krebs-Henseleit solution as described by Roffel *et al.* (1988). Two ion-exchange chromatography procedures were used for the separation of PDE isoenzymes.

Ion-exchange chromatography on Q-sepharose fast flow

Bovine tracheal smooth muscle (12–20 g) was chopped into small cubes and homogenized (Polytron: PT20 probe, 2 × 10 s bursts at speed setting 5 and 2 × 10 s bursts at speed setting 7) in 6 volumes of buffer (pH 6.5) containing (mmol l⁻¹): Bistris 20, dithiothreitol 1, benzamidine 2, EDTA 2, sodium acetate 50 and phenylmethanesulphonyl fluoride (PMSF) 0.1. The homogenate was filtered through two layers of cheese-cloth and centrifuged at 12,000 *g* for 10 min. The supernatant fraction (40–80 ml) was applied (60 ml h⁻¹) to a Q-sepharose fast flow column (1 × 28 cm) pre-equilibrated in homogenization buffer. The column was then washed with at least two bed volumes of homogenization buffer to remove unbound material. Proteins bound to the Q-sepharose were eluted (30 ml h⁻¹) with a linear sodium acetate gradient approximately 5 bed volumes in size. Fractions (2 ml) were collected and initial characterization of PDE activity performed within 24 h. Ethylene glycol was added to a final concentration of 30% (v/v) and the active fractions frozen in liquid nitrogen before long term storage at -70°C. Under these conditions, the enzyme activity was stable for at least 3 months.

Ion-exchange chromatography on Mono-Q

This procedure was essentially the same as that described by Shahid & Nicholson (1990). The use of a Mono-Q column attached to a fast protein liquid chromatography system enabled rapid separation of PDE isoenzymes, reduced proteolysis to a minimum and optimised isoenzyme resolution.

Membrane-bound phosphodiesterase

The pellet obtained after the 12,000 *g* spin of the tracheal smooth muscle extract was resuspended (Potter S homogeniser; 4 passes at 400 r.p.m.) in 2–5 ml of homogenization buffer and was used as the source of membrane-bound PDE.

Affinity chromatography on calmodulin-agarose

The first peak of PDE activity obtained after ion-exchange chromatography was further purified on calmodulin-agarose to separate calmodulin-sensitive and insensitive activities. A combined sample (1.3 ml) of fractions representing peak 1 was prepared and the Ca²⁺ and Mg²⁺ concentrations increased to 2 mmol l⁻¹ before application to a calmodulin-agarose column (1.6 × 4 cm) which had been pre-equilibrated in at least 10 bed volumes of buffer A (pH 6.5) containing (mmol l⁻¹): Bistris 20, dithiothreitol 1, benzamidine 2, NaCl 50, MgCl₂ 3, CaCl₂ 0.1 and PMSF 0.1. Penetration into the calmodulin-agarose was ensured by applying 2–4 ml of buffer A after sample loading. To allow complete adsorption, the enzyme sample was left on the column for 30 min before serial stepwise elution with: (a) 20 ml of buffer A, (b) 20 ml of buffer B (buffer A plus 1 M NaCl) and (c) buffer C (buffer B plus 1 mM EGTA). Fractions (1 ml) were collected and assayed for PDE activity within 24 h.

Cyclic nucleotide phosphodiesterase assay

PDE activity was measured as described by Shahid & Rodger (1989). The standard assay mixture contained MgCl₂ 3 mmol l⁻¹, 5'-adenosine monophosphate or 5'-guanosine monophosphate 1 mmol l⁻¹, ³H-labelled/unlabelled cyclic nucleotide (150,000–200,000 d.p.m.) 1 μmol l⁻¹, dithiothreitol 1 mmol l⁻¹, bovine serum albumin 0.05% (w/v) in Tris HCl 50 mmol l⁻¹ (pH 7.4) in a final volume of 200 μl. Inhibitors were tested in the concentration range 0.1–250 μmol l⁻¹ with cyclic AMP (1 μmol l⁻¹) as substrate except for PDE V where

cyclic GMP (1 μmol l⁻¹) was used. Inhibitory effects on PDE I and PDE II activity were examined in the presence of Ca²⁺ (1 mmol l⁻¹)/calmodulin (1.5 μg ml⁻¹) and cyclic GMP (1 μmol l⁻¹), respectively, to enhance basal activity. The drug concentration producing 50% inhibition of PDE activity (IC₅₀ value) was determined with a non-linear regression curve fitting programme. All experiments were repeated between 4 and 12 times with fractions from 2 to 3 preparations.

Milrinone (2.0 mmol l⁻¹), IBMX (4.5 mmol l⁻¹) and zaprinast (1.0 mmol l⁻¹) were dissolved in Tris-HCl 50 mmol l⁻¹ (pH 7.4) whereas Org 30029 (69 mmol l⁻¹) and rolipram (73 mmol l⁻¹) were dissolved in dimethylsulphoxide (DMSO) and subsequently diluted in Tris-HCl 50 mmol l⁻¹. The final concentrations of DMSO, for these compounds, in the assay was less than 0.5% (v/v) and did not significantly affect basal PDE activity. Org 9935 (66 mmol l⁻¹) was dissolved in DMSO and subsequently diluted in 10% DMSO/Tris HCl. The final concentration of DMSO in the assay was 2.4% (v/v) which inhibited PDE activity by 10–20%, control tubes contained the same quantity of DMSO. Protein content was determined by the biuret method.

Determination of kinetic constants

In these experiments, 7 to 9 different substrate concentrations ranging from 0.1 μmol l⁻¹ to 1 mmol l⁻¹ were used. The concentration of radiolabelled cyclic nucleotide was kept constant and the concentration of unlabelled nucleotide varied. Incubation time was also varied (15–45 min) to adjust enzyme activity such that approximately 25% of the substrate was hydrolysed. The data were analysed with a commercial non-linear regression curve fitting programme (ENZFITTER, Elsevier-Biosoft) to determine *K_m* and *V_{max}* values. All experiments were performed in duplicate 2 to 3 times using at least 2 different separations.

Relaxation of tracheal smooth muscle

Bovine tracheal smooth muscle strips (2 × 2 × 10 mm) were mounted for isotonic recording in 20 ml organ baths. A 300 mg load was used which ensured stable and reproducible responses. After equilibration for 30 min in Krebs-Henseleit solution (37°C), (–)-isoprenaline, 0.1 μmol l⁻¹, was applied to establish basal tone. The tissue was washed immediately and later contracted by cumulative administration of methacholine 10 and 90 μmol l⁻¹ followed by 60 min of repeated washings. Subsequently, contraction was induced with either histamine, 1 μmol l⁻¹, or methacholine, 0.03 μmol l⁻¹; 15 min later cumulative relaxation curves were constructed with either IBMX, rolipram, zaprinast, milrinone, Org 30029 or Org 9935. After completion, (–)-isoprenaline, 10 μmol l⁻¹, was added to re-establish basal tone. All experiments were performed 4 to 6 times.

Chemicals

The following substances were used: 3-isobutyl-1-methylxanthine, calmodulin, calmodulin-agarose (Sigma, Poole, Dorset), rolipram (a gift from Schering, Berlin, F.R.G.), milrinone, Org 30029 and Org 9935 (Chemistry Department, Organon Laboratories Ltd, Newhouse, Lanarkshire), [³H]-cyclic AMP, [³H]-cyclic GMP (Amersham International, Bucks) and Q-sepharose (fast-flow), Mono Q (Pharmacia, Milton Keynes). All other chemicals were obtained either from Sigma Chemical Co or BDH Chemicals (both of Poole, Dorset) and were of Analar or equivalent grade. Water purified on a Milli-Q (Millipore, Herts) system was used throughout.

Results

Analysis of PDE activity in bovine tracheal homogenate (H), supernatant (S) and pellet (P) showed that 2.3% and 0.60% of

Figure 1 The hydrolysis of cyclic AMP (a) and cyclic GMP (b) by homogenate, supernatant and pellet fractions prepared from bovine tracheal smooth muscle in the absence (open columns) and presence of either Ca^{2+} ($20 \mu\text{mol l}^{-1}$)/calmodulin ($1.5 \mu\text{g ml}^{-1}$) (solid columns) or cyclic GMP ($1 \mu\text{mol l}^{-1}$) (hatched columns). Supernatant and particulate fractions were obtained by centrifugation from the homogenate (values are means with s.e.mean shown by vertical bars, $n = 3-6$; see text for details). $** P < 0.01$ (paired t test).

the total cyclic AMP- and cyclic GMP-hydrolytic activity was present in the membrane fraction. The effects of the regulators Ca^{2+} /calmodulin and cyclic GMP on H, S and P fractions are shown in Figure 1. The cyclic AMP-hydrolytic activity of all three fractions was stimulated by Ca^{2+} /calmodulin. Cyclic GMP hydrolysis tended towards an increase but this did not reveal the level of statistical significance. EGTA (1 mmol l^{-1}) inhibited the basal PDE activity of H, S and P fractions by 33%, 41% and 18%, respectively, indicating activation by endogenous Ca^{2+} /calmodulin. Cyclic GMP did not significantly affect the cyclic AMP-hydrolytic activities of any fraction.

Separation of phosphodiesterase isoenzymes

Elution profiles obtained with both Q-sepharose and Mono-Q FPLC were similar. The resolution was superior with the latter method, but greater sample capacity was achieved with Q-sepharose. Approximately 82–93% of the applied PDE activity was recovered. Three major peaks of PDE activity eluting at 0.23 M, 0.34 M and 0.44 M NaCl were identified (Figure 2). According to the nomenclature of Beavo & Reifsnnyder (1990), these peaks could be tentatively designated PDE I, PDE II and PDE IV, respectively. The cyclic AMP-hydrolytic activity of PDE I and PDE II was stimulated by Ca^{2+} /calmodulin and cyclic GMP, respectively. The hydrolytic activity of the third activity peak was not significantly modulated by either regulator (Figure 2). A further minor activity peak eluting at 0.69 M NaCl was also observed. As analysed in one separation, cyclic AMP-hydrolytic activity was not inhibited by cyclic GMP but was potentially inhibited by rolipram ($-\log \text{IC}_{50} 6.52$) suggesting that it may be an isoform of PDE IV. This peak was of low activity and although reproducibly obtained, it was not further character-

Figure 2 Elution profiles of cyclic nucleotide phosphodiesterase (PDE) activities from bovine tracheal smooth muscle after separation on a Mono-Q ion-exchange column. Low speed (12,000 g) supernatant fractions from fresh bovine tracheal smooth muscle (4–6 g) were prepared as described for the Q-sepharose method, except that the sodium acetate in the homogenization buffer was replaced with NaCl, 50 mmol l^{-1} . Each sample was filtered ($0.22 \mu\text{m}$ filters) and 10–27 ml applied (1 ml min^{-1}) to a 1 ml Mono-Q column, pre-equilibrated in homogenization buffer. The column was washed with 6 ml of homogenization buffer to remove unbound material. PDE activity was then eluted at 1 ml min^{-1} with a 32 ml gradient of NaCl (0.05–1.0 M) in homogenization buffer kept on ice. Fractions (1 ml) were collected in tubes kept on ice and assayed for cyclic AMP ($1 \mu\text{mol l}^{-1}$) PDE activity either in the absence of any known regulator ($\bullet$) or in the presence of cyclic GMP ($1 \mu\text{mol l}^{-1}$; a; $\circ$) or Ca^{2+} ($20 \mu\text{mol l}^{-1}$)/calmodulin ($1.5 \mu\text{g ml}^{-1}$; b; $\circ$).

ized. The elution profile in the absence and presence of cyclic GMP did not reveal PDE III activity.

To analyse further the PDE isoenzymes present in peak 1, the combined fractions of this peak obtained with Q-sepharose were applied to a calmodulin-agarose column. As shown in Figure 3, when Ca^{2+} containing wash buffer was used, the PDE activity obtained in the eluate was not stimulated by Ca^{2+} /calmodulin and was specific for cyclic GMP and hence could be classified as PDE V (Beavo & Reifsnnyder, 1990). The Ca^{2+} /calmodulin dependent PDE could be released from the column by buffer containing EGTA.

Kinetic parameters of phosphodiesterase isoenzymes

The kinetic characteristics (K_m and V_{max} values) of bovine tracheal PDE isoenzymes are presented in Table 1. PDE V had a high affinity and activity for cyclic GMP. PDE I had a high affinity for cyclic AMP. PDE II shows a low affinity but high activity against both cyclic nucleotides; cyclic GMP hydrolysis by this enzyme displayed positive cooperativity. In the presence of cyclic GMP, cyclic AMP hydrolysis followed Michaelis-Menten kinetics. The kinetics of cyclic AMP hydrolysis in the absence of cyclic GMP were not evaluated, but it is known that the presence of cyclic GMP reduces the degree of cooperativity in the kinetics of cyclic AMP hydrolysis by PDE II from canine trachealis (Torphy & Cieslinski, 1990). PDE IV had a high affinity for cyclic AMP and a low cyclic GMP hydrolytic activity. The kinetic parameters of cyclic AMP for PDE V and cyclic GMP for PDE I and IV were not precisely determined, as basal activity was low.

Figure 3 Subfractionation of cyclic nucleotide phosphodiesterase (PDE) activities contained within the first activity peak obtained after ion-exchange chromatography of the supernatant fractions from bovine tracheal smooth muscle. PDE activity was eluted from a calmodulin-agarose column with either Ca^{2+} or EGTA containing buffers (see text for details). Fractions were assayed for cyclic GMP (a) or cyclic AMP (b) hydrolytic activities ($1\text{ }\mu\text{mol l}^{-1}$ substrate concentration) in the absence ($\bullet$) or presence of Ca^{2+} (1 mmol l^{-1})/calmodulin ($1.5\text{ }\mu\text{g ml}^{-1}$; $\circ$).

Figure 4 The inhibition of the third peak of cyclic AMP hydrolytic activity obtained from the supernatant fraction of bovine tracheal smooth muscle by Org 9935 ($\circ$), rolipram (Δ) and Org 9935 in the presence of $3\text{ }\mu\text{mol l}^{-1}$ rolipram ($\square$). Rolipram $3\text{ }\mu\text{mol l}^{-1}$ inhibited activity by $52 \pm 7\%$; in experiments examining PDE inhibition by Org 9935 in the presence of rolipram, the activity remaining in the presence of rolipram was normalised to 100%. All points represent mean (with s.e.mean shown by vertical bars) of at least six determinations performed with 2–3 separations. Asterisks denote significant difference (Student's *t* test, **P* < 0.05, ***P* < 0.01) from inhibition produced by Org 9935 alone.

The effects of phosphodiesterase inhibitors on phosphodiesterase isoenzyme activity

The effects of PDE isoenzyme inhibitors on bovine tracheal PDE isoenzymes are summarized in Table 2. IBMX non-specifically inhibited all isoenzymes. Zaprinast showed

Table 1 Kinetic characteristics of bovine tracheal phosphodiesterase (PDE) isoenzymes

PDE	Isoenzyme family	K_m ($\mu\text{mol l}^{-1}$)		V_{max} ($\text{nmol min}^{-1}\text{ ml}^{-1}$)	
		Cyclic AMP	Cyclic GMP	Cyclic AMP	Cyclic GMP
I	Ca^{2+} /calmodulin-dependent	2.9^1	ND	4.0^1	ND
II	Cyclic GMP-stimulated	105^2	85^3	131^2	72
IV	Cyclic GMP-insensitive	2.9	ND	4.1	ND
V	Cyclic GMP-specific	ND	2.6	ND	694

ND, Low activity at $1\text{ }\mu\text{mol l}^{-1}$, precise value not determined.
¹ In the presence of Ca^{2+} (1 mmol l^{-1}) and calmodulin ($1.5\text{ }\mu\text{g ml}^{-1}$).
² In the presence of cyclic GMP ($1\text{ }\mu\text{mol l}^{-1}$).
³ Non linear kinetics (value given is the substrate concentration giving 50% of maximal activity).
All values are means of 2–3 determinations performed in duplicate from at least 2 different separations.

Table 2 Inhibition of bovine tracheal smooth muscle phosphodiesterase (PDE) isoenzymes (I, II, IV, V) and rabbit heart PDE III

	Ca^{2+} /calmodulin ² -dependent PDE I	Cyclic GMP ³ -stimulated PDE II	$-\log IC_{50}^1$ Cyclic GMP ⁴ -insensitive PDE IV	Cyclic GMP ⁵ -specific PDE V	Cyclic GMP ^{4,6} -inhibited PDE III
IBMX	4.7 ± 0.1	4.3 ± 0.1	4.9 ± 0.1	4.5 ± 0.1	5.1 ± 0.1
Zaprinast	4.8 ± 0.1	4.5 ± 0.2	4.6 ± 0.1	6.6 ± 0.1	3.9 ± 0.1
Rolipram	<3.60 (24)	<3.60 (11)	5.9 ± 0.1	<3.60 (23)	4.3 ± 0.2
Milrinone	<3.60 (48)	<3.60 (22)	4.7 ± 0.1	<3.60 (34)	5.5 ± 0.1
Org 30029	<3.60 (32)	<3.60 (14)	4.6 ± 0.1	<3.60 (19)	4.5 ± 0.1
Org 9935	<3.60 (39)	<3.60 (6)	4.6 ± 0.1	5.01 ± 0.15	7.0 ± 0.2

¹ Mean $\pm$ s.e.mean of 4–12 assays performed with 2–3 separations; figures in parentheses indicate % inhibition at $250\text{ }\mu\text{mol l}^{-1}$.
² Cyclic AMP ($1\text{ }\mu\text{mol l}^{-1}$) as substrate and in the presence of Ca^{2+} (1 mmol l^{-1}) and calmodulin ($1.5\text{ }\mu\text{g ml}^{-1}$).
³ Cyclic AMP ($1\text{ }\mu\text{mol l}^{-1}$) as substrate and in the presence of cyclic GMP ($1\text{ }\mu\text{mol l}^{-1}$).
⁴ Cyclic AMP ($1\text{ }\mu\text{mol l}^{-1}$) as substrate.
⁵ Cyclic GMP ($1\text{ }\mu\text{mol l}^{-1}$) as substrate.
⁶ Inhibition of rabbit heart PDE III (Shahid & Nicholson, 1990).
IBMX = 3-isobutyl-1-methylxanthine.

approximately 100 fold selectivity for the inhibition of PDE V. Milrinone, Org 30029, Org 9935 and rolipram inhibited PDE IV, rolipram being the most potent inhibitor. Comparison with cardiac PDE III data shows rolipram to be the sole truly selective PDE IV inhibitor, milrinone and Org 9935 are both more potent PDE III inhibitors, with Org 9935 showing the greater selectivity. Org 30029 has a similar ability to inhibit both PDE III and PDE IV.

To evaluate further the third peak of PDE activity, the ability of cyclic GMP and Org 9935 to attenuate hydrolytic activity was examined in the presence and absence of rolipram ($3 \mu\text{mol l}^{-1}$). Org 9935 was chosen for these experiments because it has the greatest selectivity for PDE III amongst the compounds examined in the present study. Rolipram ($3 \mu\text{mol l}^{-1}$) produced a $52 \pm 7\%$ inhibition of total activity in these assays. Part of the remaining activity was potently inhibited by Org 9935 (Figure 4). The concentration-inhibition curve was clearly biphasic, the $-\log \text{IC}_{50}$ for the initial section of the curve (7.4 ± 0.3) was similar to the $-\log \text{IC}_{50}$ for cardiac PDE III (Table 2). Cyclic GMP ($1 \mu\text{mol l}^{-1}$) inhibited 7% and 12% of the hydrolytic activity in the absence and presence of rolipram, respectively.

Functional activity of phosphodiesterase inhibitors

The concentrations of histamine ($1 \mu\text{mol l}^{-1}$) and methacholine ($0.03 \mu\text{mol l}^{-1}$) employed produced $55.8 \pm 7.0\%$ and $57.7 \pm 2.3\%$ of the methacholine maximum contractions, respectively. All PDE inhibitors tested produced concentration-dependent relaxant responses (Figure 5); the $-\log \text{EC}_{50}$ values are summarised in Table 3. The most effective relaxant of both histamine and methacholine-induced

Table 3 Relaxation of bovine tracheal smooth muscle by cyclic nucleotide inhibitors in the presence of methacholine ($0.03 \mu\text{mol l}^{-1}$) or histamine ($1.0 \mu\text{mol l}^{-1}$)

		Methacholine	Histamine
IBMX		5.6 ± 0.1	5.6 ± 0.1
Zaprinast		4.4 ± 0.3	5.2 ± 0.1
Rolipram	1st phase	6.8 ± 0.2	7.1 ± 0.1
	2nd phase	4.5 ± 0.1	
Milrinone		4.1 ± 0.2	4.5 ± 0.1
Org 30029		5.4 ± 0.1	6.2 ± 0.1
Org 9935		4.1 ± 0.2	4.1 ± 0.2

Data represent $-\log \text{EC}_{50} \pm \text{s.e. mean}$ from 4–6 experiments. IBMX = 3-isobutyl-1-methylxanthine.

contractions was rolipram; with methacholine as the agonist, the relaxation curve was biphasic. IBMX and Org 30029 were active but less potent. Zaprinast, milrinone and Org 9935 were not effective at concentrations maintaining inhibitory selectivity for PDE V and PDE III, respectively, although Org 9935 did produce a small relaxation of methacholine-induced contractions at low concentrations.

Discussion

The present study demonstrated bovine tracheal smooth muscle to contain representatives of all five presently identifiable PDE isoenzymes in the cell cytosol. As such it is qualitatively comparable to canine tracheal smooth muscle (Torphy & Cieslinski, 1990). The finding of three major peaks of PDE activity in the supernatant of bovine tracheal smooth muscle homogenates is in agreement with published data (Elliott *et al.*, 1991). As previously described for canine (Torphy & Cieslinski, 1990), but not for bovine preparations, PDE V coelutes with PDE I, hence calmodulin affinity chromatography is necessary to separate these isoenzymes.

The high affinity of PDE I in bovine trachealis for cyclic AMP indicates that this isoform of PDE I is different from that found in cerebral tissue (Strada *et al.*, 1984) and is similar to that found in cardiac muscle (Reeves *et al.*, 1987) and canine tracheal smooth muscle (Torphy & Cieslinski, 1990). However, in these latter tissues PDE I has a comparable activity against cyclic GMP, therefore, the identity of PDE I in bovine tracheal smooth muscle awaits further clarification. PDE V in bovine tracheal smooth muscle has characteristics similar to those previously reported for this isoenzyme in lungs (Beavo, 1988) and canine tracheal smooth muscle (Torphy & Cieslinski, 1990). It is a different isoform from that found in the retina of the eye which has a lower affinity for cyclic GMP (Beavo, 1988). The characteristics of PDE II were similar to those observed in canine tracheal smooth muscle (Torphy & Cieslinski, 1990) and other tissues such as cardiac muscle (Reeves *et al.*, 1987; Nicholson *et al.*, 1989) and brain (Nicholson *et al.*, 1989).

The major Ca^{2+} /calmodulin-independent low K_m cyclic AMP PDE activity peak initially appeared to meet the criteria (Beavo, 1988) for being PDE IV-like in character. However, previous studies have found PDE III and PDE IV to co-elute from guinea-pig (Harris *et al.*, 1989) and canine (Torphy & Cieslinski, 1990) trachea. The inhibitory activity of Org 9935 and cyclic GMP in the presence of rolipram revealed the presence of PDE III within the third major activity peak, accounting for 12–18% of activity. The activity of PDE III relative to PDE IV was less than that found in guinea-pig (Harris *et al.*, 1989) and canine (Torphy & Cieslinski, 1990) tracheal smooth muscle. The importance of PDE IV for cyclic AMP hydrolysis in bovine trachea has previously been suggested by Hall *et al.* (1989) who found rolipram to elevate cyclic AMP more effectively than the PDE III selective inhibitor, SK & F 94120.

Figure 5 Relaxation of histamine- (a; $1 \mu\text{mol l}^{-1}$) and methacholine- (b; $0.03 \mu\text{mol l}^{-1}$) contracted bovine tracheal smooth muscle by cyclic nucleotide phosphodiesterase inhibitors (O, rolipram; ■, Org 30029; △, isobutylmethylxanthine; ●, zaprinast; ▲, milrinone; □, Org 9935). Data are the mean of between 4 and 6 experiments; s.e. mean shown by vertical bars.

As previously reported for rat kidney (Hoey & Houslay, 1990) and liver (Lavan *et al.*, 1989), the present study indicated the presence of two distinct PDE IV isoforms in bovine trachea that could be separated by ion-exchange chromatography. Whether one has been displaced from membranes during homogenization or if the two isoforms represent distinct cytosolic activities, as opposed to one being a proteolytic breakdown product, requires clarification.

As in canine trachea (Torphy & Cieslinski, 1990), the membrane-bound PDE activity observed in bovine tracheal smooth muscle contains PDE I. Cyclic GMP neither inhibited nor stimulated cyclic AMP hydrolysis, hence, neither PDE III nor PDE II activity seem to be concentrated in a particulate fraction, although PDE IV may be present.

The most significant finding of the functional experiments of the present study was that rolipram was the most effective relaxant of both histamine and methacholine-induced tone. The selective inhibition of PDE IV by rolipram indicates that PDE IV is of great functional significance in bovine trachealis. However, a high affinity binding site has been identified at which rolipram has a higher affinity than for the catalytic site of PDE IV (Schneider *et al.*, 1986). Until the nature of rolipram binding site is clarified, all the pharmacological activity of rolipram cannot unequivocally be attributed to PDE inhibition. The rolipram-induced relaxation of methacholine-induced tone was clearly biphasic. In guinea-pig trachealis, rolipram produces a biphasic relaxation of carbachol and histamine-induced contractions (Harris *et al.*, 1989), which was converted into a monophasic relaxation in the presence of a PDE III inhibitor. Apparently, combined PDE III and PDE IV inhibition more effectively relaxes guinea-pig trachea than selective inhibition of either of these isoenzymes alone. As described above, in bovine trachea PDE IV constitutes a greater proportion of the Ca^{2+} /calmodulin independent low K_m cyclic AMP PDE than in guinea-pig tracheal smooth muscle. This may explain why rolipram is a more effective relaxant of histamine-induced contractions in bovine trachea. PDE III is not only present in small quantities in bovine tracheal smooth muscle it also appears to be functionally relatively unimportant. PDE III inhibitors do not effectively elevate cyclic AMP in bovine trachea (Hall *et al.*, 1989) and the present study has shown them to be singularly ineffective in causing relaxation. The small relaxation of methacholine-induced contractions produced by low concentrations of Org 9935 may be a reflection of the presence of some PDE III activity.

Zaprinast was weakly active as a relaxant, indicating the relative lack of functional significance of PDE V, at least when guanylate cyclase tone is low, in bovine tracheal smooth muscle. Previously, zaprinast has been reported to be completely ineffective in relaxing methacholine-induced contractions of bovine tracheal smooth muscle preparations (Hall *et al.*, 1990). However, in this earlier study a higher concentration of methacholine was used to contract the preparations than in the present study. With the exception of Org 9935 and IBMX, all the PDE inhibitors tested more effectively relaxed histamine – than methacholine – contracted preparations. The preferential relaxation of histamine-contracted preparations is in agreement with previous findings (Torphy *et al.*, 1988). The ability of PDE inhibitors to relax tracheal preparations is crucially dependent upon the degree of muscarinic agonist-induced tone (Torphy *et al.*, 1988). This may explain the activity of zaprinast and also why Org 9935 and IBMX were equieffective relaxants of both methacholine and histamine in the present study.

Org 30029, despite showing similar potency to milrinone as a PDE IV inhibitor, was more effective as a tracheal relaxant. This compound is not only a dual PDE III/IV inhibitor (Shahid & Nicholson, 1990) it also produces Ca^{2+} sensitization in skinned cardiac muscle (Miller & Steele, 1990). Additional properties may explain the potency of this compound as a tracheal relaxant.

In summary, the present study has shown bovine tracheal smooth muscle to contain representatives of all five presently identifiable PDE isoenzyme families. In comparison with guinea-pig and canine trachealis, PDE III has a very low activity in bovine trachealis. Whereas the PDE IV inhibitor rolipram is a potent relaxant of bovine tracheal preparations, PDE III inhibitors are relatively inactive. This contrasts with tracheal preparations from other species in which PDE III and PDE IV inhibitors are approximately equieffective relaxants. In view of these species differences and because PDE inhibitors have potential utility as drugs for the treatment of asthma, clarification of both the relative activity levels and functional significance of PDE isoenzymes in human airways is important.

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