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Effects of isozyme selective phosphodiesterase inhibitors on bovine tracheal smooth muscle tone

Elevation of tissue cyclic AMP levels leads to relaxation of airway smooth muscle preparations [1]. Amongst the potential mechanisms whereby this effect occurs are activation of calcium-dependent K^+ channels [2], reduction in intracellular calcium levels [3], inhibition of inositol phospholipid hydrolysis [4] and alteration in the sensitivity of contractile proteins to calcium [5]. The breakdown of cyclic AMP to 5'AMP within tissues is dependent upon the activity of tissue phosphodiesterases. A range of cyclic nucleotide phosphodiesterase isozymes are present within airway smooth muscle with differing K_m values and selectivities for cyclic AMP and cyclic GMP [6–9]. The aims of this study were to define the relaxant effects of a range of selective inhibitors of phosphodiesterase isozymes in bovine airway smooth muscle and to examine the effects upon airway smooth muscle tone of combinations of these isozyme selective inhibitors with a view to determining the physiologically relevant phosphodiesterase isozymes involved in the control of airway smooth muscle tone. A preliminary account of this work has been presented to the IUPHAR meeting [10].

Materials and Methods

Mechanical responses of strips of bovine tracheal smooth muscle were recorded as described previously [11] after removal of epithelium. Dose–response curves were drawn by inspection and, where appropriate, EC_{30} values were compared by unpaired *t* tests. EC_{30} values were used to compare the effects of relaxant agents routinely causing less than 50% relaxation. Values in the text represent means ± SEM of N separate experiments. The agents studied were the non-selective phosphodiesterase inhibitor IBMX (3-isobutyl-1-methylxanthine), inhibitors of the type III phosphodiesterase isozyme (SK & F 94120, SK & F 94836), an inhibitor of the type IV isozyme (rolipram) and an inhibitor of the type V isozyme (zaprinast)

Chemicals. Gifts of rolipram (Schering AG), zaprinast (M & B 22948, May and Baker), SK & F 94836 (2-cyano-1 - methyl - 3 - [4 - {4 - methyl - 6 - oxo - 1,4,5,6 - tetra - hydropyridine - 3 - yl}phenyl]guanidine) and SK & F 94120 ({5-(4-acetamido-phenyl)pyrazin-2[1*H*]-one}, Smith Kline and French) are gratefully acknowledged. All other agents were obtained from the Sigma Chemical Co. (Poole, U.K.).

Results

Trachealis muscle strips were contracted with 1 or 10 μm methacholine. All experiments were performed using paired control strips to ensure that stable contractile responses were achieved.

Relaxant responses to IBMX, rolipram, SK & F 94120 and SK & F 94836. The ability of the range of phosphodiesterase inhibitors studied to relax bovine trachealis muscle strips precontracted with either 1 or 10 μM methacholine is shown in Fig. 1a–d and Table 1. In four experiments, M & B 22948 (1–100 μM) failed to

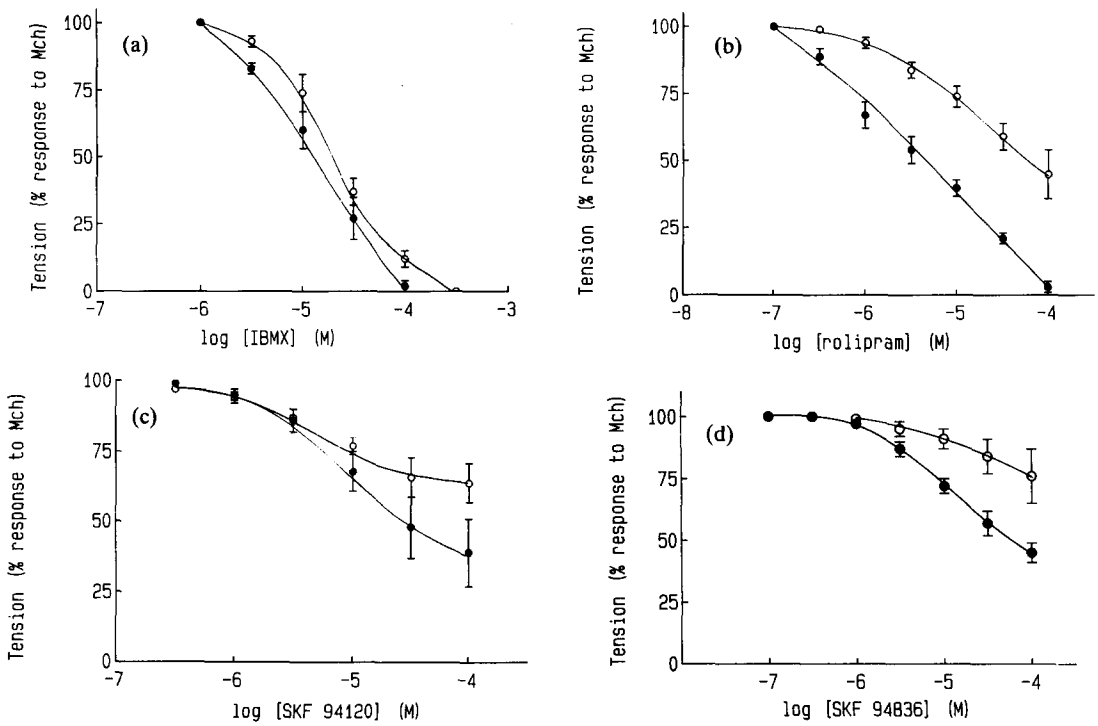

Fig. 1. Relaxant response curves for the non-selective phosphodiesterase inhibitor IBMX (a), the type IV isozyme-selective phosphodiesterase inhibitor rolipram (b) and type III selective phosphodiesterase inhibitors SK & F 94120 (c) and SK & F 94836 (d) performed following precontraction of bovine tracheal smooth muscle strips with either 1 (filled circles) or 10 μM (open circles) methacholine. Each data point represents the mean (± SEM) of four to eight experiments. Relaxant responses are expressed as percentage of contractile response above baseline tissue tension.

Table 1. Comparison of relaxant effects of phosphodiesterase inhibitors at two concentrations of the contractile agonist methacholine

	Concentration of agent causing 30% relaxation (μM)	
	Methacholine (1 μM)	Methacholine (10 μM)
IBMX	8.8 ± 2.4 (4)	32 ± 10 (5)‡
Rolipram	1.2 ± 0.4 (4)	29 ± 13 (7)*‡
SK & F 94120	6.3 ± 0.5 (4)*	35 ± 22 (4)†‡
SK & F 94836	12.0 ± 2.0 (4)	NA

* In one further experiment for both rolipram and SK & F 94120, 30% relaxation was not attained at concentrations of agent selective for the respective isozyme (<0.1 mM).

† In two further experiments with SK & F 94120 30% relaxation was not attained.

‡ Significantly different from EC_{30} value when 1 μM methacholine used as agonist ($P < 0.05$).

NA, 30% relaxation not achieved.

produce relaxation of tissue precontracted with either 1 or 10 μM methacholine.

Additivity of effects of rolipram, SK & F 94120 or SK & F 94836, and IBMX. The effects of combinations of phosphodiesterase inhibitors were examined by contracting tissue with 10 μM methacholine and adding a maximal concentration of SK & F 94120, which remained selective for the type III isozyme (0.1 mM, [7, 12]), before a maximally selective concentration of the type IV isozyme-selective inhibitor rolipram was added. Finally, 0.1 mM IBMX was added in order to maximally inhibit tissue phosphodiesterase activity. SK & F 94120 (100 μM) produced 36 ± 7% relaxation. Addition of rolipram resulted in a further 54 ± 10% relaxation, with IBMX (100 μM) producing a further 10 ± 4% (mean data from N = 6 animals). When 1 μM methacholine was used as the contractile agonist, following the addition of a maximally selective dose of SK & F 94120 or SK & F 94836, rolipram was able to completely relax tissue to baseline tension. As described above, under these conditions rolipram alone was able to relax tissue to baseline tension (Fig. 1b).

Discussion

At least five distinct phosphodiesterase isozymes are present in mammalian airway smooth muscle, each having different selectivities and K_m values for cyclic AMP and cyclic GMP [7, 9]. Of these, both the type III (low K_m, cyclic AMP selective) and type IV (high K_m, cyclic AMP selective) isozymes appear to be important for the regulation of cyclic AMP breakdown in intact airway smooth muscle from canine or bovine trachealis [4, 6, 7, 13] although in canine airway smooth muscle these isozymes account for only 10 and 5% of total tissue phosphodiesterase activity when measured in homogenates [6]. Inhibition of the type III isozyme by either SK & F 94120 [6, 14] or SK & F 94836 [7] results in a relaxation response in canine trachealis muscle precontracted with methacholine. Inhibition of the type IV isozyme with rolipram relaxes precontracted bovine airway smooth muscle strips *in vitro* and elevates tissue cyclic AMP content [4, 11]. However, in one previous study, no type III phosphodiesterase activity was demonstrated in bovine trachealis although only the soluble fraction of the smooth muscle homogenate was examined [15].

This study extends previous observations on the relevance of the inhibition of individual phosphodiesterase isozymes in airway smooth muscle to airway smooth muscle relaxation and suggests that both the type III and type IV isozymes are physiologically relevant in controlling cyclic AMP breakdown in bovine trachealis muscle in that inhibitors of these isozymes were able to produce trachealis muscle relaxation at concentrations selective for action upon the respective isozyme alone. The effects upon smooth muscle tone of inhibition of the type III and type IV isozymes appeared to be additive. In contrast, inhibition of the type V phosphodiesterase isozyme in bovine trachealis muscle with M & B 22948 failed to produce a relaxant response in bovine airway smooth muscle, in agreement with previous reports in canine and guinea pig airway preparations [6, 16]. The relaxant effects of all the phosphodiesterase isozymes were reduced in the presence of high concentrations of methacholine.

The observation that the majority of cyclic AMP hydrolytic activity present in homogenates is present in the type I isozyme peak [6] whilst this isozyme appears to be physiologically unimportant in controlling cyclic AMP levels in whole cell preparations has still not been explained. One possible explanation is that a degree of structural or kinetic compartmentalization of cyclic AMP and/or phosphodiesterase isozymes exists within cells. As discussed above, cyclic AMP is likely to initiate airway smooth muscle relaxation through a range of different mechanisms. Hence, compartmentalization of phosphodiesterase isozymes within the cell would be a physiological mechanism for controlling the metabolism of different intracellular pools of cyclic AMP.

In conclusion, this study shows that the inhibition of both type III and type IV phosphodiesterase isozymes in precontracted bovine airway smooth muscle with SK & F 94120 or SK & F 94836, and rolipram respectively leads to relaxation, and that under conditions where one isozyme is maximally inhibited but where a maximal relaxant response has not been attained, inhibition of the other isozyme produces a further increment of relaxation. These results suggest that isozyme-selective phosphodiesterase inhibitors, either singly or in combination, may have a useful role as bronchodilator agents. In addition, these agents are useful tools for the study of cyclic AMP-dependent mechanisms in airway smooth muscle preparations.

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Inhibitory action of the potassium channel opener BRL 38227 on agonist-stimulated phosphoinositide metabolism in bovine tracheal smooth muscle

Agents which increase the probability of potassium channel opening have been shown to cause hyperpolarization and relaxation of a variety of smooth muscles [1, 2]. The prototypic potassium channel opener cromakalim has been shown to be an effective inhibitor of agonist-induced bronchospasm in guinea-pigs *in vivo* [3], and *in vitro* [4] and *in vivo* [5] studies suggest that this class of agent also exhibits similar anti-spasmogenic properties in human subjects.

Spasmogenic agonists, such as acetylcholine and histamine, initiate airway smooth muscle contraction by activation of phosphoinositidase C to increase the rate of inositol phospholipid hydrolysis [6, 7]. The initial transient increase in inositol 1,4,5-trisphosphate has been causally related to initiation of contraction, whilst the sustained increase in phosphoinositide turnover, and consequent production of 1,2-diacylglycerol, may play an important role in the maintenance of the contractile response [6, 7]. Despite the wealth of functional data on the relaxant properties of potassium channel openers, little is known about the molecular mechanisms by which they bring about this action. In the present study, the consequences of potassium channel opening on spasmogen-stimulated phosphoinositide turnover has been investigated in bovine tracheal smooth muscle.

* Abbreviations: BTSM, bovine tracheal smooth muscle; [^{3}H]InsP$_x$, [^{3}H]inositol phosphates.

Materials and Methods

Cromakalim was synthesized in SmithKline Beecham Laboratories and was resolved into (−)- and (+)-enantiomers (BRL 38227 and 38226, respectively), as described previously [8]. All reagents were obtained from the same suppliers as previously [9, 10]. In addition, [2,8-^{3}H]adenosine 3′,5′-cyclic monophosphate was purchased from Amersham International (Amersham, U.K.) and glibenclamide was purchased from the Sigma Chemical Co. (Poole, U.K.)

Incubation techniques. Bovine tracheal smooth muscle (BTSM*) slices (300 μm × 300 μm) were prepared and incubated, and tissue inositol phospholipids were labelled with 1 μCi/mL [^{3}H]inositol, as described previously [9]. Aliquots of gravity-packed BTSM slices (75 μL) were incubated in a final volume of 500 μL of Krebs–Henseleit buffer containing 1 μCi/mL [^{3}H]inositol and 5 mM LiCl for 30 min, with regular gassing of vials with O_2/CO_2 (19:1). Additions of BRL 38227, BRL 38226 and/or glibenclamide were made 15 min before those of carbachol or histamine, and unless otherwise stated incubations were then continued for 30 min. All incubations were terminated by addition of 500 μL ice-cold 1 M trichloroacetic acid.

Measurement of [^{3}H]*InsP$_x$ and* [^{3}H]*inositol phospholipids.* After 20 min on ice, samples were centrifuged, the supernatant neutralized by repeated extraction with water-saturated diethylether and total [^{3}H]InsP$_x$ separated on Dowex 1-×8 (100–200 mesh, Cl^- form) columns [9, 11].