ELSEVIER

European Journal of Pharmacology 298 (1996) 265–270

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Effects of selective and non-selective phosphodiesterase inhibitors on tracheal mucus secretion in the rat

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Received 29 August 1995; revised 14 November 1995; accepted 17 November 1995

Abstract

The present study was designed to characterize the effects of unselective and isoenzyme-selective phosphodiesterase inhibitors on airway mucus secretion. The isolated rat trachea was incubated in a modified Ussing chamber. Mucus macromolecules were metabolically labelled with ^{35}S. The inhibitors were applied at the luminal side. The unselective phosphodiesterase inhibitors theophylline, enprofylline and 3-isobutyl-methylxanthine stimulated mucus secretion in a concentration-dependent manner with half-maximum effects (EC_{50} values) at 690 μM, 400 μM and 46 μM, respectively. The adenosine antagonist 8-phenyltheophylline did not significantly stimulate mucus output, suggesting a negligible role of adenosine in the cellular mechanisms of mucus secretion. Adenosine itself did not increase radiolabel output. Rolipram, an inhibitor of phosphodiesterase isoenzyme IV, and zardaverine, which inhibits the isoenzymes III and IV, increased potently macromolecule output with EC_{50} values of 40 nM and 6 μM, respectively. The selective inhibitors of phosphodiesterase isoenzymes III and V, motapizone and zaprinast, did not influence airway mucus release, suggesting a relatively low activity of isoenzymes III and V in glands of rat trachea. The stimulatory effect of theophylline on airway mucus secretion may contribute to its beneficial action in chronic obstructive airway disease. Our data suggest that this effect is mediated predominantly by phosphodiesterase isoenzyme IV.

Keywords: Mucus secretion; Trachea; Theophylline; 8-Phenyltheophylline; Enprofylline; 3-Isobutyl-methylxanthine; Motapizone; Rolipram; Zaprinast; Zardaverine

1. Introduction

Theophylline (dimethylxanthine) belongs to the methylxanthines, a class of alkaloids widely spread throughout the plant world. For over 50 years it has been used in the treatment of asthma. Today, however, we know that theophylline influences several functions of lung and airways. Some studies demonstrate a significant improvement of mucociliary transport by theophylline, especially an improvement of ciliary beat frequency (Barnes and Pauwels, 1994; Konrad et al., 1994; Serafini et al., 1976; Wanner, 1985). The drug acts as a potent bronchodilator. Theophylline also influences the action of inflammatory cells and macrophages, monocytes and eosinophils. Thus, several mechanisms might contribute to theophylline's beneficial effect in the treatment of obstructive airway diseases. Surprisingly, only little information is available about theophylline's effect on airway mucus secretion (Barbieri and Barsigia, 1985; Barnes and Pauwels, 1994; Torphy and Undem, 1991).

Several molecular mechanisms are suggested to be responsible for the therapeutic effects of theophylline. It is now generally accepted that the drug increases intracellular cAMP levels by an inhibition of phosphodiesterases (Finkbeiner et al., 1992). Other mechanisms include adenosine antagonism (Johnson and McNee, 1985) and an increase in endogenous catecholamine plasma levels. These findings explain the well-known side effects of theophylline. Most importantly, theophylline induces hypertension and tachycardia and these effects are due to inhibi-

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SSDI 0014-2999(95)00794-6

tion of phosphodiesterase in the cardiovascular system. Thus, 'airway-specific' phosphodiesterase inhibitors (Dent et al., 1994; Hoymann et al., 1994; Miyamoto et al., 1994; Quian et al., 1994; Raeburn and Karlsson, 1993; Tokuyama et al., 1994; Torphy and Undem, 1991; Underwood et al., 1994) could be helpful in the treatment of obstructive airway diseases. Consequently in the present study we characterized the effects of three unspecific phosphodiesterase inhibitors (theophylline, 3-isobutyl- methylxanthine, enprofylline) on airway mucus secretion. The use of the phosphodiesterase subtype-specific inhibitors motapizone, rolipram, zardaverine and zaprinast allowed us to determine the participation of phosphodiesterase isoenzymes in the regulation of mucus secretion in rat tracheal mucosa.

2. Materials and methods

2.1. Chemicals

Pentobarbital (Nembutal) was used for anaesthesia (70 mg/kg intraperitoneally). The organ culture medium, Medium M-199 in Earle's balanced salt solution, was purchased from Gibco, Eggenstein, Germany. For radiolabelling $Na_2^{35}SO_4$ was obtained from Amersham, Braunschweig, Germany. Dialysis tubing (cellulose dialysis tubing, 12000–14000 Da molecular mass cut-off) was purchased from Serva, Heidelberg, Germany. Sodium azide was obtained from Merck, Darmstadt, Germany (10 mg/l) for prevention of bacterial growth in dialysis fluid. The non-selective phosphodiesterase isoenzyme antagonists theophylline, 8-phenyltheophylline, 3-isobutyl-1- methylxanthine (IBMX), enprofylline (3-propylxanthine) were purchased from Sigma, Deisenhofen, Germany. The selective phosphodiesterase isoenzyme antagonists motapizone, rolipram, zaprinast, zardaverine were gifts from Byk-Gulden, Konstanz, Germany.

2.2. Animals

50 male Sprague-Dawley rats (Zentralinstitut für Versuchstierzucht, Hannover, Germany) with an average body weight of 400 g and free access to food and water and a standard rat diet (Altromin, Lage, Germany) were used in the present study.

2.3. Ussing chamber technique

Rats were anaesthesized with pentobarbital (Nembutal) using dosages of 70 mg/kg intraperitoneally and killed by excision of the tracheae (cranially from the larynx, caudally to the bifurcation) through a ventral collar midline incision and median sternotomy. The trachea was transferred to an organ bath filled with Medium M-199 in Earle's balanced salt solution, equilibrated with 95% oxygen and 5% carbon dioxide. The trachea was opened along the posterior membrane and mounted between the two halves of an Ussing chamber (6 mm long, 3 mm wide with an elliptic profile). The pH was adjusted to 7.41 at 37°C. To the solution bathing the submucosal side 50 μCi $Na_2^{35}SO_4$ was added and allowed to equilibrate with the tissues for the duration of the experiment. After 2 h the release of bound $^{35}SO_4$ to the luminal (= mucosal) side reaches steady state (= basal secretion). Then the perfusate bathing the luminal side (7 ml, according to the volume of the perfusion device) was collected at 15-min intervals.

The perfusate samples from the luminal side were collected in cellulose dialysis tubing (12000–14000 Da molecular mass cut-off) which retains secreted molecules with molecular masses over 12000–14000 Da and dialyzed against distilled water containing unlabelled SO_4, to remove unincorporated $^{35}SO_4$, and sodium azide (10 mg/l) to prevent bacterial degradation. Dialysis was complete when the radioactivity of the dialysis fluid 3 h after the last change was equal to the radioactivity of water used for dialysis. The radioactivity of the samples was then determined using a scintillation counter. The count rates of the labelled macromolecules reflect the mucus secretion rates.

This Ussing chamber method for separating submucosal and mucosal solutions is a well established method to measure macromolecular secretion of glands and epithelium of tracheal preparations. It offers the advantage that the radiolabelled precursor can equilibrate with the submucosal side of the tissue, during which time materials secreted into the lumen can be collected. The method depends on an intact epithelial diffusion barrier, which effectively avoids free diffusion of the precursor. According to the literature (Borson et al., 1988) and our own studies (Wagner et al., 1993, 1995a, b) the choice of sulphate, which is present on many carbohydrate side chains as a terminal residue, as precursor has great advantages over other precursors (e.g. labelled amino acids or sugars), especially because it is not metabolized (Borson et al., 1988). Thus the output of labelled mucins together with proteoglycans can be assessed. Dialysis against distilled water containing excess unlabelled SO_4 effectively removes ionically bound unincorporated $^{35}SO_4$, so that an unintentional study of a mere SO_4 transport can be excluded. Own high-performance liquid chromatography studies of the $^{35}SO_4$-labelled molecules identified them as high-molecular-weight glycoconjugates that are not digested by chondroitinase ABC. Thus these macromolecules are true glycoproteins.

Our histologic studies have demonstrated that the Sprague-Dawley rats used for these experiments have large submucosal glands embedded between the cartilage rings, with the highest density in the ventral midline. They extend from the larynx to at least the trachea bifurcation, decreasing in size distalwards. Therefore, our Ussing chambers are positioned just in between this region of highest submucosal gland density over the ventral midline

in a longitudinal direction in a well-standardized manner from the 2nd to the 8th cartilage ring. These specific pathogen-free rats nearly completely lack goblet cells in their superficial epithelium, so that the almost exclusive source of mucus production is the submucosal glands and not cells in the superficial epithelium. Moreover, autoradiographic studies done with ^{35}S radiolabel demonstrate the accumulation of the label in the submucosal glands, especially the acini. Nearly no label was found in the cells of the superficial epithelium, which furthermore suggests the origin of mucin to be the submucosal glands (Wagner et al., 1993, 1995a, b).

2.4. Experimental design

First, we characterized the effects of non-selective phosphodiesterase inhibitors on airway mucus secretion. Theophylline ($n = 15$) was added to the luminal side in increasing concentrations. After an equilibration period of 2 h, four fractions were collected (the average was set 100%). Theophylline, 10 μM, 100 μM, 1 mM and 10 mM, was added to the luminal side, and four fractions (each 15 min) were collected between each stimulation to allow the system to recover and reach basal values. Finally acetylcholine 1 mM was given to test the viability of the system. 3-Isobutyl-1-methylxanthine (IBMX) ($n = 6$) (1 μM–1 mM) and enprofyllin ($n = 6$) (3-propyl-xanthine) (100 μM–1 mM) were given according to the same time schedule. In addition we studied the effect of adenosine ($n = 4$) (100 μM–1 mM) and the adenosine receptor antagonist 8-phenyltheophylline ($n = 4$) (1 μM–1 mM) according to the same time schedule.

Second, the selective phosphodiesterase inhibitors motapizone ($n = 6$) (100 nM–0.1 mM), rolipram ($n = 6$) (1 μM–100 μM), zaprinast ($n = 6$) (1 μM–100 μM), and zardaverine ($n = 6$) (1 nM–100 μM) were applied luminally in increasing steps of factor 10 according to the above-mentioned design. In order to test the viability of the system, each experiment was concluded with a stimulation with acetylcholine 1 mM.

2.5. Statistical analysis

Values are expressed in percentage of basal secretion. Data are presented as ±S.E.M. Statistical analysis was performed with Student's t-test for unpaired samples. 5–15 experiments for each experimental protocol were performed.

The EC_{50} values were calculated graphically from the curves at those points where half-maximum effects were reached. As far as theophylline, 3-isobutyl-methylxanthine and enprofylline are concerned, values were assessed in the same way from each single dose-response curve, but in order to use comparable scales in our synoptic graphs it was not possible to show all values in these graphs. Therefore, the values are given in the text.

3. Results

First, we characterized the effects of non-selective phosphodiesterase inhibitors on mucus secretion from isolated rat trachea. All substances were applied at the luminal side. Theophylline stimulated mucus secretion concentration dependently: 10 μM 96.6 ± 4.7%; 100 μM 121.2 ± 6.4%; 1 mM 165.7 ± 10.7% and 10 mM 205.4 ± 16.0%. The half-maximum effect (EC_{50}) was calculated at 690 μM (Fig. 1). 3-Isobutyl-methylxanthine and enprofylline also stimulated macromolecule output. 3-Isobutyl-methylxanthine elicited the following response: 1 μM 111.4 ± 5.8%; 10 μM 122.3 ± 7.2%; 100 μM 207.1 ± 9.6% and 1 mM 223.6 ± 29.6%; EC_{50} at 46 μM (Fig. 1). Enprofylline showed the following effects: 100 nM 106.4 ± 10.9%; 1 μM 101.3 ± 3.0%; 10 μM 110.3 ± 1.7%; 100 μM 120.7 ± 8.8%; 1 mM 197.5 ± 27.7%. EC_{50} at 400 μM (Fig. 1).

Adenosine did not stimulate secretion. It rather slightly,

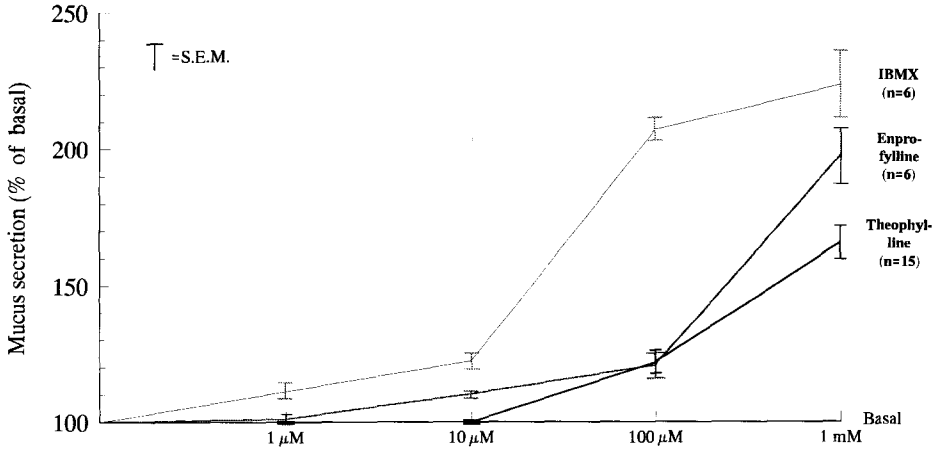

Fig. 1. Concentration-effect correlations (1 μM–1 mM) of 3-isobutyl-methylxanthine, enprofylline and theophylline on tracheal mucus secretion in the rat.

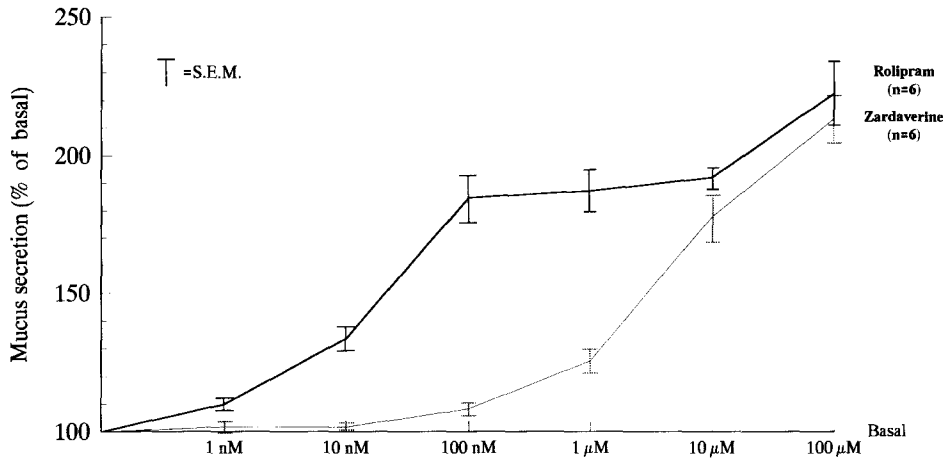

Fig. 2. Concentration-effect correlations (1 nM–100 μM) of rolipram and zardaverine on tracheal mucus secretion in the rat.

but not significantly, decreased secretory activity (100 μM 86.5 ± 1.7%; 1 mM 93.5 ± 11.2%). The adenosine receptor antagonist 8-phenyltheophylline did not alter radiolabel output (100 μM: 126.8 ± 13.7%). Even higher concentrations of this substance did not result in an additional increase. Thus, there was no concentration-response correlation.

In the next set of experiments selective inhibitors of phosphodiesterase isoenzymes were used to characterize which phosphodiesterase isoenzymes are present in mucus-secreting cells. When rolipram, a selective inhibitor of phosphodiesterase IV, was applied, mucus output was concentration dependently increased (1 nM 109.8 ± 5.3%; 10 nM 133.5 ± 11.1%; 100 nM 184.7 ± 19.5%; 1 μM 187.3 ± 15.7%; 10 μM 192.1 ± 8.4%; 100 μM 222.6 ± 23.9%) with an EC_{50} at 40 nM (Fig. 2). Zardaverine, which inhibits phosphodiesterase III and IV, also stimulated airway mucus secretion dose dependently (1 nM 101.7 ± 4.7%; 10 nM 101.7 ±2.9%; 100 nM 108.2 ± 5.9%; 1 μM 125.5 ± 9.1%; 10 μM 178.0 ± 19.6%; 100 μM 213.6 ± 18.8%) with a calculated EC_{50} at 6 μM (Fig. 2). Motapizone, a phosphodiesterase III inhibitor, did not significantly stimulate secretion (100 nM up to 100 μM). Also zaprinast, a phosphodiesterase V inhibitor, tested in increasing concentrations (1 μM–100 μM), did not alter basal secretion (neither stimulate nor decrease secretory activity). These data show that phosphodiesterase IV is the major phosphodiesterase isoenzyme in mucus-secreting cells in airways.

4. Discussion

The present study demonstrates that inhibition of phosphodiesterase results in an increased mucus secretion from isolated rat trachea. These investigations are in good agreement with studies in which an increased mucus output was observed after stimulation with agents (e.g. peptides of the vasoactive intestinal polypeptide/glucagon family (Wagner et al., 1993, 1995a, b)) that increase intracellular cAMP levels via activation of adenylate cyclase (Adler et al., 1981). However, cAMP is not the only stimulant of airway mucus secretion, since agents that activate the Ca^{2+}/inositol 1,4,5-triphosphate system are also potent mucus secretagogues (Barnes and Pauwels, 1994; Richter et al., 1993). Several aspects should be discussed in greater detail.

4.1. Effects of theophylline on mucus secretion and other airway functions

Our results show that theophylline is a potent stimulator of airway mucus secretion. Theophylline is widely used in the treatment of obstructive airway diseases. It is generally believed that its most important beneficial effects are bronchodilation and a modulation of inflammatory cell function (Barnes and Pauwels, 1994; Dent et al., 1994; Miyamoto et al., 1994; Raeburn and Karlsson, 1993). This study demonstrates that theophylline in therapeutic concentrations is also a potent stimulator of airway mucus secretion. Thus, besides the well-known improvement of ciliary activity (Konrad et al., 1994; Serafini et al., 1976; Wanner, 1985), the secretomotor effect of theophylline could contribute to an increase in mucociliary clearance, which is impaired in obstructive airway disease. We suggest that this represents an important additional effect of this drug.

Concerning smooth muscle relaxation, the extent of adenosine antagonistic activity besides inhibition of phosphodiesterase is not clearly defined, for theophylline is also known as a potent adenosine receptor antagonist (both A_1 and A_2 receptors). As our data suggest, adenosine receptor antagonism, a problem controversely discussed in the literature (Johnson and McNee, 1985), is not involved in the stimulation of airway mucus secretion. However, species-specific differences must be considered.

4.2. Effects of selective phosphodiesterase inhibitors on airway mucus secretion

As mentioned above, especially cardiovascular side effects often limit the therapeutic use of theophylline. These

Table 1
Comparison of EC_{50} and maximum values of secretomotor effects

Substance	EC_{50}	Maximum average response [% of basal secretion]	PDE isoform
Nonselective phosphodiesterase (PDE) inhibitors			
Theophylline	690 μM	205 ± 16 (at 10 mM)	
Enprofylline	400 μM	197 ± 27 (at 1 mM)	
IBMX	46 μM	223 ± 29 (at 1 mM)	
Selective phosphodiesterase (PDE) inhibitors			
Zardaverine	6 μM	213 ± 19 (at 100 μM)	III and IV
Rolipram	40 nM	223 ± 24 (at 100 μM)	IV
Motapizone		No effect	III
Zaprinast		No effect	V

effects are due to inhibition of phosphodiesterases in the cardiovascular system. Accumulating evidence exists that there are several phosphodiesterase isoforms, which are expressed in a tissue-specific manner (Dent et al., 1994). Which phosphodiesterase isoforms are expressed in mucus-secreting cells of the airways is so far unknown. During the last few years specific inhibitors of the individual phosphodiesterase isoforms have been developed (Hoymann et al., 1994; Quian et al., 1994; Raeburn and Karlsson, 1993; Tokuyama et al., 1994; Underwood et al., 1994). Therefore the second part of this study was designed as a first attempt to characterize the pattern of expression of phosphodiesterase isoforms in airway mucus-secreting cells. Our data demonstrate that phosphodiesterase isoenzyme IV, but not the isoforms III and V, plays a major role in mucus-secreting cells of airways (Table 1). As known from the literature (Barnes and Pauwels, 1994; Dent et al., 1994), phosphodiesterase IV has been detected in airway smooth muscle, vascular smooth muscle, inflammatory cells (e.g. mast cells, macrophages, monocytes, eosinophils, neutrophils, CD^{4+} lymphocytes, endothelial cells) and phosphodiesterase III has been also found in most of these cells except monocytes, eosinophils and neutrophils. Phosphodiesterase IV or combined phosphodiesterase III/IV inhibitors are now considered as 'the most promising type of anti-asthmatic drug' (Barnes and Pauwels, 1994), because of their widespread anti-inflammatory as well as smooth muscle relaxant potency. In addition, our data, which suggest an important role of phosphodiesterase IV (and obviously to a negligible extent phosphodiesterase III) in airway mucus-secreting cells, fit well in this pharmacologic concept. They even strengthen the concept of airway-specific phosphodiesterase inhibitors. The comparison of the maximal effects shows that higher concentrations (about 100 μM) of 3-isobutyl-methylxanthine and zardaverine lead to effects of similar potency as those of rolipram, which, however, reaches its half-maximum effect at a concentration as low as 40 nM. This reflects the high affinitiy of rolipram for the phosphodiesterase isoform IV. The observation that the non-selective phosphodiesterase inhibitor 3-isobutyl-methylxanthine did not have a greater maximal effect than rolipram speaks in favour of the predominant or even exclusive involvement of the phosphodiesterase isoenzyme IV in cAMP hydrolysis. The latter is emphasized by the observation that zardaverine, an exclusive inhibitor of phosphodiesterase isoforms III and IV, did not have a stronger maximal effect. An involvement of phosphodiesterase III seems not likely, because the exclusive inhibition of phosphodiesterase III by motapizone did not have any effect on mucus secretion. The suggestion of a predominant role of phosphodiesterase isoenzyme IV in tracheal mucus secretion gains additional support from our findings that even higher concentrations of the non-selective phosphodiesterase inhibitor theophylline, as high as 10 mM, did not further increase mucus secretion above the level produced by rolipram at 100 μM.

Taken together, this study demonstrates that inhibition of phosphodiesterase results in an increase in airway mucus secretion. The effect is mediated mainly via phosphodiesterase isoform IV. Thus, our data provide good experimental evidence that phosphodiesterase isoform IV and combined phosphodiesterase III/IV inhibitors might be useful in the treatment of obstructive airway disease. Our data might be helpful to develop 'airway-specific' phosphodiesterase inhibitors to improve the treatment of obstructive airway diseases with minimal extrapulmonary side effects.

Acknowledgements

This study was supported by the Deutsche Forschungsgemeinschaft (Wa 844/3-1).

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