British Journal of Pharmacology (1995) 116, 1991-1998 © 1995 Stockton Press All rights reserved 0007-1188/95 $12.00

Mucosa-dependent muscarinic liberation of prostaglandins from rat isolated trachea

Gernot Brunn, *Ignaz Wessler & [1,]†Kurt Racké

Department of Pharmacology, University Hospital of J.W. Goethe-University Frankfurt, Theodor-Stern-Kai 7, D-60590-Frankfurt, Germany; *Department of Pharmacology, Johannes Gutenberg-University Mainz, Obere Zahlbacher Straße 67, D-55101 Mainz, Germany and †Institute of Pharmacology and Toxicology, University of Bonn, Reuterstr. 2b, D-53113 Bonn, Germany

1 The present study examined whether cholinoceptor stimulation modulates the release of arachidonic acid-derived mediators from rat isolated tracheae.

2 Tracheae were preincubated with [^{3}H]-arachidonic acid and the outflow of ^{3}H-compounds was determined. Acetylcholine and the muscarinic agonist, carbachol but not nicotine, increased the rate of tritium outflow maximally by about 30%. The M_3 receptor-preferring antagonist *p*-fluoro-hexahydrosiladiphenidol was more effective than pirenzepine and methoctramine in antagonizing the effect of acetylcholine.

3 High performance liquid chromatography analysis (methanol gradient) of the released ^{3}H-compounds showed that one peak, co-eluting with [^{14}C]-prostaglandin E_2 ([^{14}C]-PGE_2) and [^{3}H]-PGD_2, was enhanced almost 10 fold following muscarinic receptor activation, whereas the outflow of [^{3}H]-arachidonic acid remained unaffected.

4 Using an acetonitril gradient separation it was shown that [^{3}H]-PGE_2, [^{3}H]-PGD_2 and [^{3}H]-$PGF_{2\alpha}$ are released spontaneously, but [^{3}H]-PGE_2 represented the major fraction of ^{3}H-prostaglandins. Acetylcholine enhanced the release of all three ^{3}H-prostaglandins, but the effect on PGE_2 was most pronounced and most consistent.

5 After removal of the mucosa the muscarinic effect of acetylcholine on total tritium and on that of the ^{3}H-prostaglandins ([^{3}H]-PGE_2/PGD_2 peak) was abolished.

6 Acetylcholine also enhanced the outflow of radioimmunologically determined PGE_2 in a mucosa-dependent manner.

7 After inhibition of cyclo-oxygenase by 3 μM indomethacin, the outflow of ^{3}H-prostaglandins was reduced to almost undetectable levels and acetylcholine evoked a marked release of [^{3}H]-arachidonic acid. The phospholipase A_2 inhibitor, quinacrine (up to 100 μM) also blocked the effect of acetylcholine on the outflow of ^{3}H-prostaglandins, but this was not followed by a compensatory increase in the outflow of [^{3}H]-arachidonic acid.

8 In conclusion, activation of muscarinic receptors which have characteristics of the M_3 subtype can evoke release of prostaglandins from the airway mucosa.

Keywords: Arachidonic acid release; airway; epithelium; muscarinic receptors; prostaglandins; PGE_2

Introduction

There is increasing evidence that the airway mucosa can exert paracrine effects. Mucosal mediators appear to modulate the responsiveness of the airway smooth muscle (see Goldie *et al.*, 1990; Hay *et al.*, 1994) as well as neurotransmitter release within the airways (Wessler *et al.*, 1990; Racké *et al.*, 1991). Arachidonic acid metabolites and in particular prostaglandins, appear to be one important group of epithelial mediators (see Eling *et al.*, 1988; Raeburn, 1990). Thus, prostaglandins have been shown to modulate smooth muscle responsiveness (Tschirhart *et al.*, 1987; Farmer *et al.*, 1987) as well as the release of sympathetic (Racké *et al.*, 1992; 1993) and parasympathetic (Deckers *et al.*, 1989; Wessler *et al.*, 1990; 1994) neurotransmitters. So far, there is little known about the mechanisms controlling the liberation of prostaglandins from the airway epithelium.

Binding studies on scraped mucosa of bovine tracheae (Madison *et al.*, 1987) as well as autoradiographical studies on ferret tracheae (Basbaum *et al.*, 1984; Barnes 1993) demonstrated the presence of muscarine receptors on airway mucosal cells, which, among other things, might be linked to prostanoid release. Thus, the muscarinic inhibition of noradrenaline release from rat isolated tracheae (Racké *et al.*, 1993) and that of acetylcholine release from human isolated bronchi (Wessler *et al.*, 1995) was attenuated by indomethacin, suggesting that a muscarinic receptor-mediated liberation of prostanoids may contribute to the inhibition of neurotransmitter release. A muscarine receptor-mediated liberation of prostanoids from tracheal epithelial cells was also suggested by the observation that indomethacin affected the reactivity of guinea-pig tracheae to methacholine in an epithelium-dependent manner (Lamport & Fedan, 1990).

The aim of the present experiments was to test directly whether the liberation of arachidonic acid derived mediators from isolated tracheae is modulated via cholinoceptor stimulation. Therefore rat isolated tracheae were labelled with [^{3}H]-arachidonic acid and the effect of acetylcholine and other muscarinic receptor agonists on the outflow of ^{3}H-compounds was studied. In addition, ^{3}H-compounds were analysed by gradient reverse phase h.p.l.c.

Preliminary accounts of some of the present results have been given (Brunn *et al.*, 1992; 1993; Racké *et al.*, 1995).

Methods

Preparation and incubation of the trachea

Female Sprague-Dawley rats weighing 190-210 g (Charles River Wiga, Sulzfeld, Germany) were used. The animals were kept at a constant temperature (21°C) and a regular light (06 h 30 min-19 h 30 min) dark (19 h 30 min-06 h 30 min) cycle

[1] Author for correspondence.

with food and water *ad lib.* for at least 1 week before use. As described in detail previously (Racké *et al.*, 1991), the animals were killed by stunning followed by exsanguination and the whole tracheae were dissected, opened by a cut along the ventral side, fixed in a glass organ bath and finally incubated in 1.7 ml Krebs-HEPES solution of the following composition (mM): NaCl 118.5, KCl 5.7, $CaCl_2$ 1.25, $MgCl_2$ 1.2, sodium EDTA 0.03, (+)-ascorbic acid 0.06, HEPES 20.0 and phosphate buffer 1.0 (both adjusted to pH 7.4) and D-glucose 11.1. The incubation medium was kept at 37°C and continuously gassed with 100% O_2. After a 30 min equilibration period during which the medium was changed every 10 min the tracheae were incubated for 2 h in medium containing either 46, 185 or 370 kBq [^{3}H]-arachidonic acid depending on the analytical procedure carried out with the incubation media. In most of the experiments in which only tritium outflow was determined, 46 kBq were used, but when ^{3}H-compounds of the incubation media were analysed by h.p.l.c., the tracheae were labelled by incubation with 370 kBq [^{3}H]-arachidonic acid. The tracheae were then washed by changing the incubation medium 10 times within 10 min. Thereafter, 8 consecutive 10 min periods of incubation were carried out. In experiments in which only total tritium was determined the medium was collected directly into counting vials and after addition of a commercial scintillation cocktail the radioactivity was determined (see below). In experiments in which the ^{3}H-compounds were analysed by h.p.l.c., the incubation media were collected into plastic vials, immediately frozen and kept at −60°C until analysed by h.p.l.c. (see below).

Test stimuli were added to the medium from 50 min of incubation onwards. In the respective interaction experiments, muscarinic receptor antagonists, indomethacin, quinacrine or calmidazolium were added to the incubation medium from the onset of incubation.

In some experiments the mucosa was removed prior to start of the equilibration period (i.e. before labelling) or after the end of the labelling period (i.e. before washing period) by gently rubbing the luminal surface of the trachea with a pipe cleaner. Removal of the mucosa by this procedure has previously been confirmed by light microscopy after staining with haematoxylin and eosin (Racké *et al.*, 1991; 1992).

At the end of incubation the tracheae were blotted, (part of them also weighed), minced and extracted in 2 ml methanol for 24 h at 0–4°C. The extracts were centrifuged at 10,000 *g* for 2 min and the supernatants were stored at −60°C until analysed. The mean weight of mucosa-intact tracheae was 44 ± 1 mg (n = 85) and that of mucosa-denuded tracheae 41 ± 1 mg (n = 16).

In a few experiments, in which the outflow of endogenous prostaglandin E_2 (PGE_2) was determined by a radioimmunoassay, the incubation protocol started immediately after the tissue had been dissected. The incubation media were changed every 10 min, the samples of the first 60 min discarded, then collected into plastic vials, immediately frozen and stored at −60°C until analysed.

Analytical procedure

Arachidonic acid and its metabolites were separated by reverse phase gradient h.p.l.c. The h.p.l.c. systems used were either from Waters/Millipore (Waters 600 E Powerline) or from Varian (Model 5000), each equipped with a Rheodyne injection valve (1000 µl loop) and a reverse phase column (250 mm length, 46 mm inner diameter, prepacked with Shandon ODS-Hypersil 5 µm). The mobile phase was continuously pumped through the column at a rate of 1 ml min^{-1}. The effluent was collected in 1 min fractions directly in counting vials for the determination of radioactivity (see below). Two different solvent systems were used: the first, a methanol/glacial acid gradient which had been shown to allow the separation of all classes of eicosanoids (Henke *et al.*, 1984; van Scott *et al.*, 1990). Two solvents, A (100% methanol) and B, (10% methanol buffered to pH 5.05 with glacial acetic acid and ammonium hydroxide) were mixed to yield four gradients over 120 min (52, 62, 71 and 100% solvent A, changing at 27, 52 and 77 min). Since this gradient system gives only partial resolution between the different cyclo-oxygenase products, in particular it does not allow the separation between PGE_2 and PGD_2, a second gradient system was used in a selected series of experiments. Here, 100% acetonitrile (solvent A) and distilled water buffered to pH 3.5 with glacial acetic acid and ammonium hydroxide (solvent B) were mixed to yield three gradient steps of 30, 32 and 100% acetonitrile over a 70 min period, changing at 25 and 50 min. ^{14}C- or ^{3}H-labelled standards were run consecutively with samples to identify specific peaks.

Radioactivity in the incubation media, tissue extracts or h.p.l.c. fractions was determined after addition of a commercial scintillation cocktail (Luma Safe, Canberra Packard) by liquid scintillation spectrometry in a Packard TR 1900a. External standardization was used to correct for counting efficency and background was subtracted automatically.

In some experiments, PGE_2 was determined by a specific, commercially available radioimmunoassay (NEN, Dreieich, Germany).

Calculations and statistical analysis

The outflow of tritium is expressed as a percentage of the respective tissue radioactivity. In addition, tritium outflow is also expressed as % of outflow observed between 40 and 50 min of incubation in each individual experiment. The radioactivity of individual peaks in the radiochromatograms was quantified as d.p.m./incubation samples. EC_{50} values were calculated with the help of a computer programme (Tallarida & Murray, 1988). Antagonism was quantified by calculating $-\log K_B$ values from EC_{50} values according to equation (4) given by Furchgott (1972). Mean values of n observations are given ± s.e.means. The significance of differences was evaluated by Student's t test. For the comparison of one control with several experimental groups, the significance of differences was evaluated by the modified t test according to Bonferroni (see Wallenstein *et al.*, 1980).

Drugs and special chemicals

The following was used: A 23187 (Calbiochem, Bad Soden, Germany); acetylcholine chloride (Sigma, München, Germany); [5,6,8,9,11,12,14,15-^{3}H] arachidonic acid, (specific activity: 7.59 TBq mmol^{-1}, Amersham Buchler, Braunschweig, Germany); bradykinin (Sigma); calmidazolium (RBI, Natick, MA, U.S.A.); carbachol (carbamylcholine chloride, Sigma); *p*-fluoro-hexahydrosiladiphenidol hydrochloride (gift of Prof. Lambrecht, Frankfurt, Germany); indomethacin (Sigma); methoctramine tetrahydrochloride, (RBI); nicotine bitartrate (Sigma); oxotremorine sesquifumarate (Sigma); pirenzepine dihydrochloride (RBI); [5,6,8,9,12,14,15(n)-^{3}H]-prostaglandin D_2, (specific activity 6.73 TBq mmol^{-1}, Amersham Buchler); [1-^{14}C]-prostaglandin E_2, (specific activity 2.21 GBq mmol^{-1}, Amersham Buchler); quinacrine dihydrochloride (Sigma).

Stock solutions (10 mM) were prepared in dimethyl sulphoxide (DMSO) for A 23187 and calmidazolium, in ethanol for indomethacin and in water for all other drugs. None of the solvents alone affected the spontaneous outflow of [^{3}H]-arachidonic acid or of any metabolite when tested in the concentration which was applied with the highest concentration of the respective test drug (each n = 2–3, data not shown).

Results

Effects of removal of mucosa on accumulation of [^{3}H]-arachidonic acid in rat isolated tracheae and on spontaneous tritium outflow

Incubation of isolated tracheae with medium containing [^{3}H]-arachidonic acid (46, 185 or 370 kBq, corresponding to about

4, 15 and 30 μM, respectively) resulted in a concentration-dependent accumulation of radioactivity in the tissue (Table 1). When the mucosa was removed after the tracheae had been incubated with [^{3}H]-arachidonic acid (46 and 370 kBq) the tissue tritium content was reduced by about 30% and 55%, respectively. Removal of the mucosa before the incubation with [^{3}H]-arachidonic acid resulted in a slight increase in tissue radioactivity, when incubated with 46 kBq, but not with 370 kBq (Table 1).

The spontaneous rate of tritium outflow expressed as a percentage of the respective tissue radioactivity was very similar, independent of the [^{3}H]-arachidonic acid concentration offered during the labelling period. The pooled value of the spontaneous rate of tritium outflow from mucosa containing tracheae (determined between 30–40 min incubation) amounted to $0.113 \pm 0.002\% \times 10\ min^{-1}$ of the tissue radioactivity ($n = 185$). As shown in Figure 1a, in control experiments the rate of spontaneous tritium outflow declined slighty during the observation period. After removal of the mucosa the spontaneous rate of tritium outflow was reduced by about 30%, independent of whether the mucosa had been removed before or after the labelling period (0.076 ± 0.003 and $0.078 \pm 0.004\% \times 10\ min^{-1}$, respectively, each $n = 12$ and $P < 0.001$ vs control values given above).

Muscarinic liberation of ^{3}H-compounds from ^{3}H-arachidonic acid labelled tracheae

Acetylcholine (3–300 μM) caused a transient, concentration-dependent increase in the outflow of tritium (Figures 1 and 2). This effect was highly significant, although the absolute magnitude of the maximum effect, an increase of 30% at a concentration of 300 μM, was only moderate. An EC_{50} of 9 μM was calculated. For comparison Figure 1 shows also that bradykinin (100 nM), which is known to cause prostaglandin release within the airways (Bramley *et al.*, 1990), caused a similar transient increase in tritium outflow, whereas the ionophore A 23187 (10 μM) induced a substantially higher increase in tritium outflow. Nicotine (up to 100 μM) had no effect on the outflow of tritium (data not shown), but the muscarinic receptor agonist, carbachol, caused an increase in tritium outflow which was similar in magnitude to that caused by acetylcholine, and carbachol was apparently more potent than acetylcholine (Figure 2a). The muscarinic receptor agonist, oxotremorine, also increased the outflow of tritium but appears to be only a partial agonist, as the maximal effect produced by oxotremorine was only about 60–70% of the maximal effect of acetylcholine or carbachol (Figure 2a). The effect of acetylcholine was antagonized by *p*-FHHSiD in a competitive manner. The concentration-response curve of acetylcholine was shifted to the right by 1 μM *p*-FHHSiD (Figure 2a) resulting in a $-\log K_B$ value of 7.1 for this an-

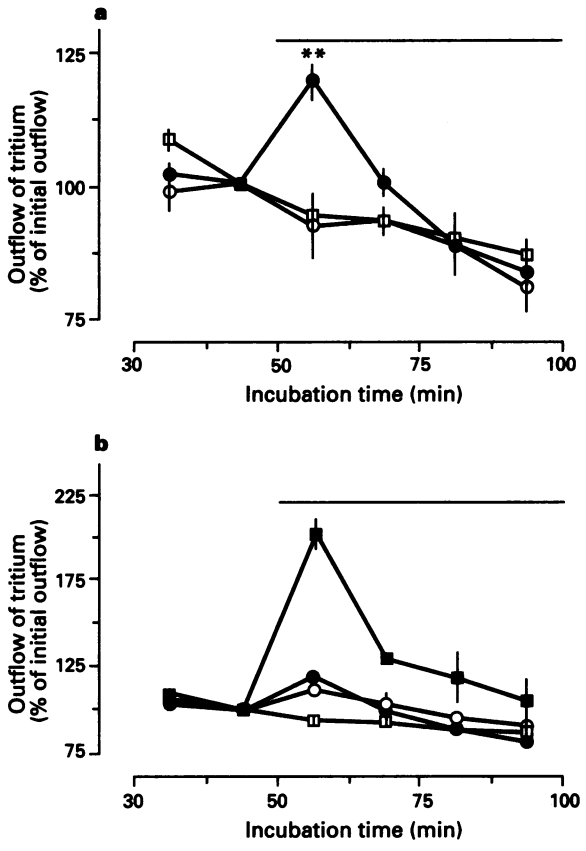

Figure 1 (a) Effects of 300 μM acetylcholine on the outflow of ^{3}H-compounds from mucosa-intact (●, $n = 22$) or mucosa-denuded (○, $n = 7$) rat isolated tracheae labelled with [^{3}H]-arachidonic acid. (b) Comparison of the effects of 300 μM acetylcholine (●), 0.1 μM bradykinin (◇, $n = 3$) and 10 μM of the ionophore A 23187 (■, $n = 4$) on the outflow of ^{3}H-compounds from mucosa-containing tracheae. Tracheae were preincubated for 30 min in Krebs-HEPES solution followed by 2 h incubation in medium containing 46 kBq (≈4 μM) [^{3}H]-arachidonic acid. After washing (10 min), tracheae were incubated for 8 consecutive 10 min periods and the radioactivity in the medium was determined by liquid scintillation spectrometry. Test drugs were added as indicated by the horizontal bar, except in controls where no drug was added (a, □, $n = 7$). In the respective experiments, the mucosa was removed prior to the start of preincubation period (i.e. before the labelling with [^{3}H]-arachidonic acid). *Abscissae*: time of incubation (after the labelling period). *Ordinates*: fractional rate of tritium outflow expressed as percentage of the outflow observed between 40–50 min of incubation in the individual experiment. Means ± s.e.means of 7–22 experiments. Significance of the differences from the control experiments (no test drug): $^{**}P < 0.01$.

Table 1 Concentration-dependent accumulation of radioactivity during incubation with [^{3}H]-arachidonic acid of rat isolated tracheae and effects of removal of the mucosa

	[^{3}H]-arachidonic acid concentration			
	46 kBq (≈4 μM)	*185 kBq (≈15 μM)*	*370 kBq (≈30 μM)*	
Mucosa intact	1.04 ± 0.025 (111)	2.43 ± 0.106 (64)	6.95 ± 0.279 (38)	d.p.m. × 10^6
Mucosa removed after labelling	0.72 ± 0.077* (8)	–	3.12 ± 0.150* (4)	d.p.m. × 10^6
Mucosa removed before labelling	1.28 ± 0.034* (8)	–	6.46 ± 0.519 (4)	d.p.m. × 10^6

Rat isolated tracheae were incubated for 2 h in medium containing [^{3}H]-arachidonic acid at the concentrations indicated. Thereafter, the tracheae were washed and incubated as described in Figure 1. At the end of the incubation protocol the tracheae were extracted for 24 h in 2 ml methanol and the radioactivity of the extracts was determined. Values represent radioactivity in the trachea at the beginning of the incubation protocol (i.e. sum of radioactivity released during incubation and of that found in extracts at the end of the experiments), mean ± s.e.means of *n* experiments. Significance of differences from the respective value, mucosa intact: $^*P < 0.01$.

tagonist. In addition, the effect of 300 μM acetylcholine, which was not significantly affected by 1 μM *p*-FHHSiD, was antagonized by 10 μM *p*-FHHSiD (Figure 2b). On the other hand, methoctramine (up to 10 μM) did not affect the stimulatory effect of 300 μM acetylcholine (Figure 2b). The effect of 300 μM acetylcholine was not affected by 1 μM, but was partially antagonized by 10 μM pirenzepine (Figure 2b). None of the antagonists alone affected the spontaneous outflow of tritium (not shown).

In mucosa-denuded preparations, acetylcholine (300 μM) failed to affect the outflow of tritium, independently of whether the mucosa had been removed prior to the labelling period with [^{3}H]-arachidonic acid (Figure 1a) or after the labelling period (not shown).

High performance liquid chromatography separation of ^{3}H-compounds

Figure 3 shows examples or radiochromatograms (obtained by glacial acid/methanol gradient separation) from samples of the different experimental conditions. In each tracing two chromatograms, one showing the resting sample and the other one showing the sample collected during exposure to acetylcholine, are superimposed. Under all conditions, most of the radioactivity was found in the solvent front and in a peak co-eluting with [^{3}H]-arachidonic acid. In mucosa-containing preparations an additional peak, co-eluting with

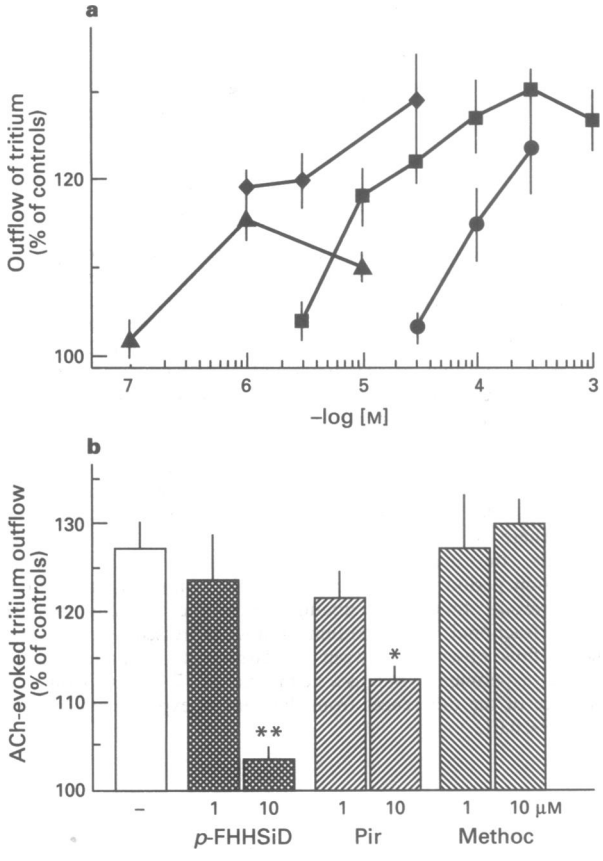

Figure 2 (a) Concentration-dependent effects of acetylcholine in the absence (■) and presence (●) of 1 μM *p*-fluoro-hexahydrosiladiphenol (*p*-FHHSiD), and of carbachol (◆) and oxotremorine (▲) on the outflow of ^{3}H-compounds from rat isolated tracheae. Tracheae were labelled with [^{3}H]-arachidonic acid and incubated as described in Figure 1. The agonists (at the concentrations indicated by the abscissa scale) were added from 40 min of incubation onwards, *p*-FHHSiD was present from the onset of incubation. (b) Concentration-dependent effects of *p*-FHHSiD, pirenzepine (Pir) and methoctramine (Methoc) on the release of ^{3}H-compounds evoked by 300 μM acethylcholine (ACh, as described in a). The antagonists were present from the onset of incubation at the concentrations indicated below the columns. *Ordinate or height of columns*: fractional rate of tritium outflow observed between 50–60 min of incubation expressed as a percentage of the mean outflow observed in the absence of test drug, means ± s.e.means of 5–22 experiments. Significance of differences from the experiments in the absence of antagonists: $*P<0.05$, $**P<0.01$.

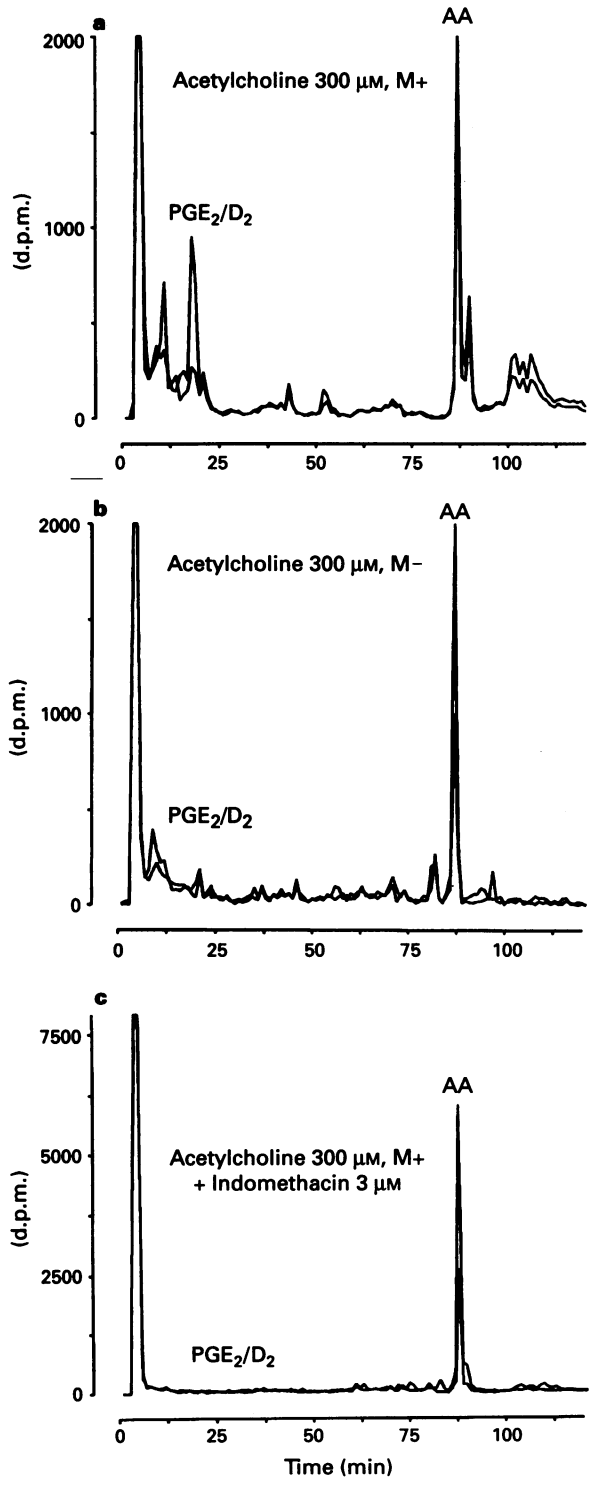

Figure 3 Examples of h.p.l.c. radiochromatograms of incubation samples obtained using a glacial acid/methanol gradient system as mobile phase (for details see methods). Each tracing shows 2 chromatograms superimposed, one collected during rest (40–50 min of incubation) and the other one collected during exposure to acetylcholine (50–60 min); further details see Figure 1. (a) Mucosa-intact trachea (M+); (b) mucosa-denuded trachea (M–); (c) mucosa-intact tracheae and presence of 3 μM indomethacin.

[^{3}H]-PGE_2/D_2 was consistently observed, and this peak (subsequently referred to as ^{3}H-prostaglandins) markedly increased when acetylcholine was added to the incubation medium. In the absence of test drugs, the outflow of ^{3}H-prostaglandins declined by about 40% between 40–50 and 50–60 min of incubation, and compared to this control condition, acetylcholine (300 μM) caused an almost 10 fold increase in the outflow of ^{3}H-prostaglandins (Figures 3 and 4b), without affecting significantly the outflow of [^{3}H]-arachidonic acid (Figures 3 and 4d). Similar changes in the outflow of ^{3}H-compounds were observed (7 fold increase of ^{3}H-prostaglandins, no change in [^{3}H]-arachidonic acid) in experiments with 30 μM carbachol (not shown). After removal of the mucosa the spontaneous outflow of ^{3}H-prostaglandins and [^{3}H]-arachidonic acid was not significantly altered (Figures 3 and 4a,c), but the effect of acetylcholine on the outflow of ^{3}H-prostaglandins was abolished (Figures 3 and 4b). In the presence of 3 μM indomethacin, the spontaneous outflow of ^{3}H-prostaglandins was reduced to almost undetectable levels (Figures 3 and 4a), whereas that of [^{3}H]-arachidonic acid was unaffected (Figures 3 and 4b). In the presence of indomethacin, acetylcholine (300 μM) instead of increasing the outflow of ^{3}H-prostaglandins evoked a marked rise in the outflow of [^{3}H]-arachidonic acid (Figures 3 and 4b,d). On the other hand, quinacrine (10 and 100 μM) concentration-dependently also inhibited the acetylcholine-evoked increase in the outflow of ^{3}H-prostaglandins, but this effect was not accompanied by a compensatory increase in the outflow of [^{3}H]-arachidonic acid. The spontaneous outflow of ^{3}H-prostaglandins and [^{3}H]-arachidonic acid was not significantly altered by quinacrine (Figure 4b,d). Finally, the calmodulin antagonist, calmidazolium (10 μM) did not affect the spontaneous outflow of ^{3}H-prostanglandins and [^{3}H]-arachidonic acid (Figure 4a,c) and had also no significant effect on the acetylcholine-induced increase in the outflow of ^{3}H-prostaglandins (Figure 4b,d).

Effect of acetylcholine on release of radioimmunologically determined PGE_2

The spontaneous outflow of PGE_2 from rat isolated tracheae with intact mucosa was 410 ± 22 pg 10 min^{-1} $n = 8$, determined between 60–70 min of incubation. In control experiments it did not change significantly during a 40 min period of observation. Addition of 300 μM acetylcholine caused a marked increase in the outflow of PGE_2 (Figure 5). After removal of the mucosa the spontaneous outflow of PGE_2 was not significantly altered, but acetylcholine failed to evoke a significant increase (Figure 5).

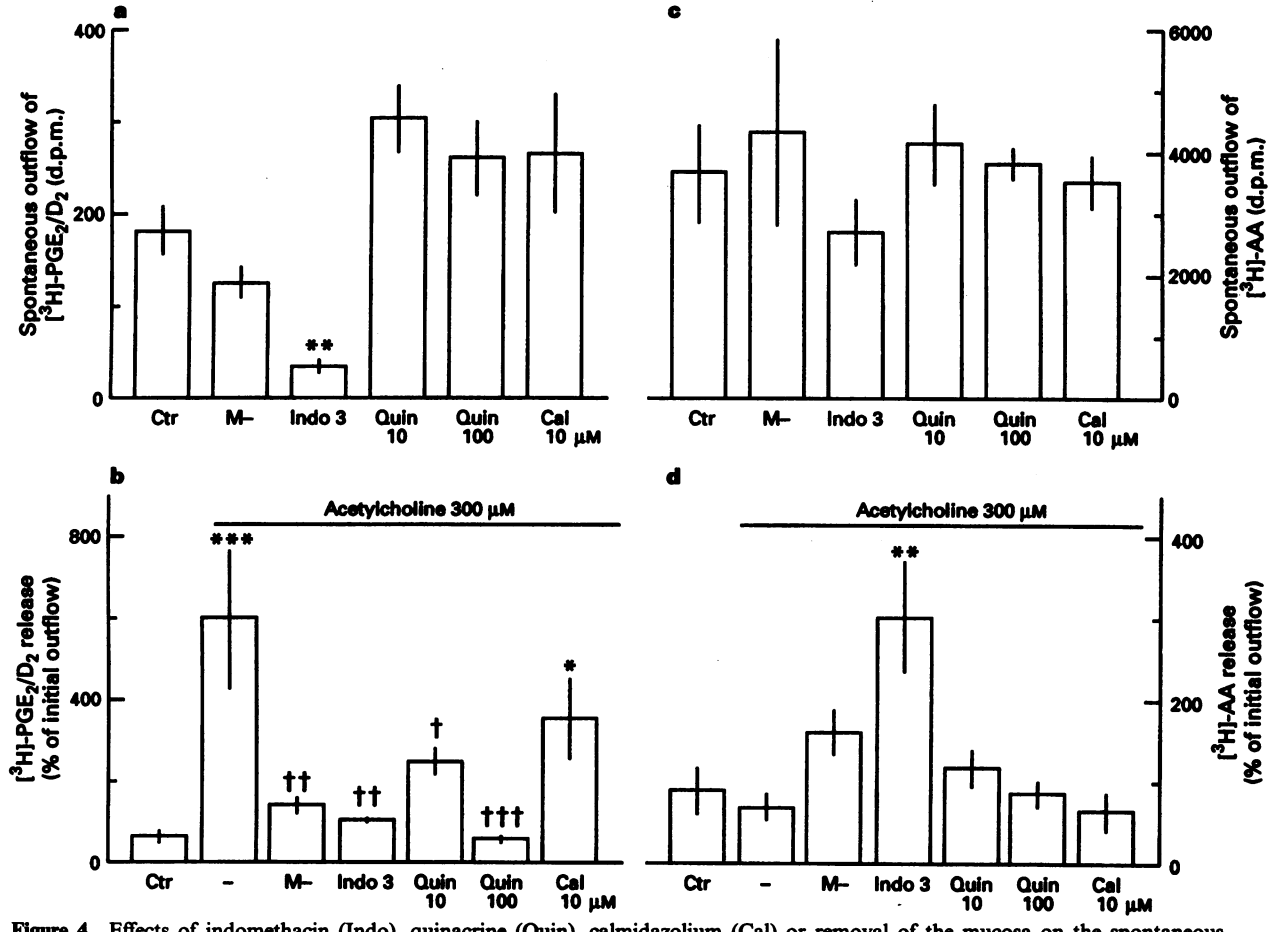

Figure 4 Effects of indomethacin (Indo), quinacrine (Quin), calmidazolium (Cal) or removal of the mucosa on the spontaneous (a,c) and acetylcholine-evoked (b,d) outflow of ^{3}H-prostanoids (PGE_2/D_2) and [^{3}H]-arachidonic acid ([^{3}H]-AA) from rat isolated tracheae. Tracheae were labelled with [^{3}H]-arachidonic acid and incubated as described in Figure 1. In the respective experiments (M–), the mucosa was removed mechanically before the start of the preincubation period (i.e. before labelling). Acetycholine (300 μM) was added from 50 min of incubation onwards, the other drugs (at the concentrations indicated below the columns) were present from the start of incubation. *Height of columns*, (a,c): total radioactivity in the respective h.p.l.c. peak (see Figure 5), expressed as d.p.m./sample; (b,d): fractional rate of tritium outflow observed between 50–60 min of incubation expressed as a percentage of the outflow observed between 40–50 min of incubation in the individual experiments. Means ± s.e.means of 3–5 experiments. Significance of differences from experiments in the absence of test drugs (Ctr): $^*P<0.05$; $^{**}P<0.01$; $^{***}P<0.001$; from the corresponding value, mucosa-intact tracheae and presence of acetylcholine alone: $\dagger P<0.05$; $\dagger\dagger P<0.01$; $\dagger\dagger\dagger P<0.001$.

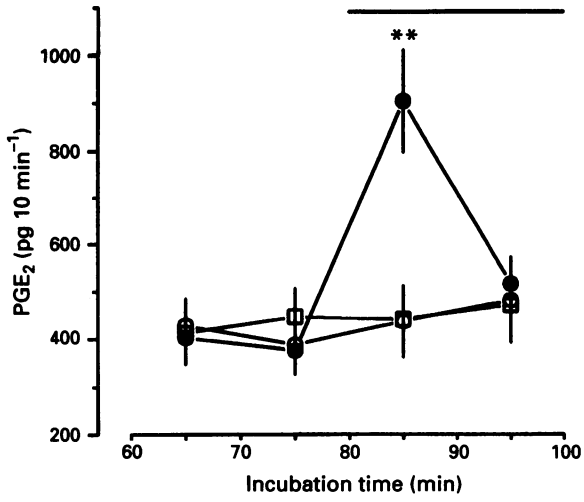

Figure 5 Effects of acetylcholine on the outflow of prostaglandin E_2 (PGE_2) from mucosa-containing (●) or mucosa-denuded (○) rat isolated tracheae. Tracheae were incubated in Krebs-HEPES solution and PGE_2 in the medium was determined by radioimmunoassay. Acetylcholine (300 μM) was added as indicated by the horizontal bar. In the respective experiments, the mucosa was removed prior to the start of the incubation period. Control experiments (no acetylcholine) of mucosa-containing tracheae are given by (□). *Abscissa* scale: time of incubation. *Ordinate* scale: outflow of PGE_2. Means ± s.e.means of 4 experiments. Significance of the differences from the respective predrug value: $^{**}P<0.01$.

Discussion

Muscarinic liberation of [^{3}H]-arachidonic acid

As outlined in the *Introduction*, the airway mucosa appears to exert several paracrine functions and the prostanoids are one group of epithelial mediators. The present experiments showed that a radio-tracer technique could be used to study the release of arachidonic acid-derived mediators from rat isolated tracheae and that muscarinic receptor-mediated mechanisms are involved in the control of prostanoid release.

Acetylcholine evoked the release of ^{3}H-compounds from isolated tracheae labelled with [^{3}H]-arachidonic acid and this effect was antagonized by *p*-FHHSiD in a manner suggesting a competitive interaction, indicating a specific muscarinic receptor-mediated mechanism. Currently, five different muscarinic receptors can be differentiated (Bonner *et al.*, 1987; 1988; Kerlavage *et al.*, 1987), and there is evidence that at least four of them (M_1-M_4) are of functional significance within the airways (see Barnes, 1993). In the present study, three different subtype preferring antagonists were tested. The M_3/M_4 preferring antagonist *p*-FHHSiD (Lambrecht *et al.*, 1988; Dörje *et al.*, 1991) was the most potent antagonist. The involvement of M_4 and also that of M_2 receptors may be excluded because the M_2/M_4 preferring antagonist methoctramine (Melchiorre, 1988; Dörje *et al.*, 1988) was ineffective. Since pirenzepine, which has a higher potency on M_1 receptors than *p*-FHHSiD on M_3 receptors (Lambrecht *et al.*, 1988; Dörje *et al.*, 1991) was less potent in the present experiments than *p*-FHHSiD, the involvement of M_1 receptors is also unlikely. Thus, the muscarinic receptors mediating liberation of arachidonic acid in the rat trachea have characteristics of the M_3 subtype. The muscarinic receptor agonists carbachol and oxotremorine mimicked the effect of acetylcholine, although oxotremorine was apparently a partial agonist. Partial agonist properties of oxotremorine have also been observed for other M_3 receptor-mediated responses such as inositol phosphate accumulation in airway and intestinal smooth muscle (Meurs *et al.*, 1988; Konno & Takayanagi, 1989) or the rise in intracellular calcium in Jurkat cells (Kaneda *et al.*, 1993).

The increase in [^{3}H]-arachidonic acid release following muscarinic receptor activation was transient, despite the continuous presence of the agonists. An exhaustion of the available [^{3}H]-arachidonic acid pool is unlikely, as A 23187 evoked a much larger release of ^{3}H-compounds than the muscarinic agonists. On the other hand, it is not unusual that muscarinic receptor-mediated cellular responses show a fatigue within minutes. Thus, in Jurkat cells the muscarinic-increased cytoplasmatic calcium levels returned to baseline within 1–2 min (Kaneda *et al.*, 1993) and the muscarinic-stimulated neurotransmitter release from PC12 cells terminated within about 10 min (Rabe *et al.*, 1987). Whether this fatigue reflects receptor desensitization and/or an additional activation of inhibitory pathways remains unclear at present.

Separation of [^{3}H]-arachidonic acid metabolites

For the separation of ^{3}H-compounds two different reverse phase h.p.l.c. gradient systems, as described by van Scott *et al.* (1990) were applied. In a first set of experiments a methanol/glacial acetic acid gradient was used. This technique has the advantage that in one run all major classes of arachidonic acid metabolites (prostanoids, leukotrienes and the different HETE metabolites) can be separated. In the present experiments, the spontaneous outflow of ^{3}H-prostaglandin ([^{3}H]-PGE_2 and [^{3}H]-PGD_2 were not separated under these h.p.l.c. conditions) amounted to about 5% of that of intact [^{3}H]-arachidonic acid. However, activation of muscarinic receptors caused an almost 10 fold increase in this ^{3}H-prostaglandin fraction without affecting the release of intact [^{3}H]-arachidonic acid. Further separation of the ^{3}H-prostaglandin fraction showed that several prostaglandins are released, but the [^{3}H]-PGE_2 fraction represented the major prostaglandins, being substantially higher than [^{3}H]-PGD_2; and [^{3}H]-$PGF_{2\alpha}$ was found only in traces. Moreover, acetylcholine appeared to enhance the outflow of all three ^{3}H-prostaglandins, but the effect on the outflow of [^{3}H[-PGE_2 was the most pronounced and most consistent.

Role of mucosa for [^{3}H]-arachidonic acid release

Although mechanical removal of the mucosa resulted in a loss of tissue of less than 10%, tissue radioactivity was reduced by 30–55% when the mucosa had been removed after the labelling procedure, indicating that [^{3}H]-arachidonic acid was particularly enriched in the airway mucosa, most likely in the epithelial cells which represent the main population of mucosal cells. Furthermore, the mucosa appears to be the compartment from which [^{3}H]-arachidonic acid is preferentially released as indicated by the observation that the spontaneous rate of tritium release from the mucosa-denuded preparation was reduced by about 30%, independent of whether the mucosa had been removed prior to or after the labelling procedure. Finally, the muscarinic receptor-evoked liberation of [^{3}H]-arachidonic acid appears to be confined to the mucosa, as acetylcholine failed to affect tritium outflow and [^{3}H]-PGE_2 release from mucosa-denuded tracheae, again independent of whether the mucosa had been removed prior to or after the labelling procedure. In agreement with these observations, acetylcholine also evoked a clear release of endogenous PGE_2 (determined radioimmunologically) and this effect showed a similar dependency on an intact mucosa. The present experiments do not allow the identification of the type of mucosal cells from which the prostaglandins are released. However, epithelial cells (ciliated cells, goblet cells and basal cells) are the major cell types within the tracheal mucosa and it is known that airway epithelial cells are endowed with a cyclo-oxygenase system and can generate different prostaglandins (see Eling *et al.*, 1988; Raeburn, 1990). When rat tracheal cells in primary culture were labelled with [^{3}H]-arachidonic acid and the release of ^{3}H-

compounds was studied as in the present experiments, ^{3}H-prostaglandins (the PGE_2/D_2 fraction) were the major fraction (Reimann *et al.*, 1995).

Effects of different enzyme inhibitors

When the cyclo-oxygenase was inhibited by indomethacin the spontaneous outflow of ^{3}H-prostaglandins decreased to almost undetectable levels. Since the spontaneous outflow of ^{3}H-prostaglandins accounted for only about 5% of the spontaneous outflow of [^{3}H]-arachidonic acid, a significant shift to an enhanced basal release of intact [^{3}H]-arachidonic acid after indomethacin was not expected and was also not observed. In contrast, such a shift was seen for the stimulatory effect of acetylcholine. After inhibition of cyclo-oxygenase, acetylcholine caused a marked increase in the outflow of [^{3}H]-arachidonic acid, whereas in the absence of indomethacin acetylcholine evoked selectively the release of ^{3}H-prostaglandins without affecting the outflow of [^{3}H]-arachidonic acid. These observations indicate that activation of muscarinic receptors results in an activation of phospholipase A_2, but normally all of the arachidonic acid liberated enters immediately the cyclo-oxygenase pathway and is metabolized to prostaglandins. The conclusion that muscarinic receptors mediated an activation of phospholipase A_2 is finally supported by the observation that the phospholipase A_2 inhibitor quinacrine (Blackwell *et al.*, 1978) also inhibited the muscarinic receptor-mediated liberation of ^{3}H-prostaglandins, but without a compensatory increase in [^{3}H]-arachidonic acid release. Several mechanisms appear to be possible, by which muscarinic receptors may cause activation of phospholipase A_2. Since the cellular phospholipase A_2 can be activated by a rise in intracellular calcium, a liberation of intracellular calcium via phospholipase C-inositol triphosphate could be one pathway, particularly as the M_3 receptors are generally linked to these intracellular signal transduction mechanisms. On the other hand, in A9 L cells, stably transfected with m_1-m_4 muscarinic receptors, m_1 and m_3 receptor activation caused a liberation of arachidonic acid and evidence was provided that this effect was mediated by liberation of intracellular calcium, independent of the phospholipase C-inositol triphosphate pathway (Conklin *et al.*, 1988). Finally, there is good evidence that a receptor-mediated activation of phospholipase A_2 can also occur via direct interaction with G proteins (see Axelrod *et al.*, 1988; Axelrod, 1990). However, the pathway responsible for the muscarinic receptor mediated activation of phospholipase A_2 described here, remains to be determined. A role of calmodulin-dependent mechanisms may be excluded since calmidazolium did not significantly affect the acetylcholine evoked prostanoid release.

The present experiments showed that activation of muscarinic receptors can evoke the liberation of prostaglandins from rat isolated tracheae in a mucosa-dependent manner. Since prostaglandins have been shown to inhibit the release of acetylcholine in the airways it is suggested that such a cholinergic liberation of prostaglandins may be part of neuro-paracrine feedback mechanisms. Since mucosal damage is frequently observed in obstructive airway diseases (e.g. Dunnill, 1960; Boushey & Holtzman, 1985; Laitinen *et al.*, 1985), it is interesting to hypothesize, that disturbance of such neuro-paracrine feedback mechanisms may contribute to the pathogenesis of the broncho-obstruction. However, it has to be tested in future experiments whether the mucosal muscarinic receptors described here are indeed a target for neuronally released acetylcholine.

This paper contains part of the Dr rer.nat. thesis of G.B. This study was supported by the Deutsche Forschungsgemeinschaft (Ra 400/3-2) and by Arthur and Margarete Ebert-Stiftung.

References

AXELROD, J. (1990). Receptor-mediated activation of phospholipase A_2 and arachidonic acid release in signal transduction. *Biochem. Soc. Trans.*, **18**, 503–507.

AXELROD, J., BURCH, R.M. & JELSEMA, C.L. (1988). Receptor-mediated activation of phospholipase A_2 via GTP-binding proteins: arachidonic acid and its metabolites as second messengers. *Trends Neurosci.*, **3**, 117–123.

BARNES, P.J. (1993). Muscarinic receptor subtypes in airways. *Life Sci.*, **52**, 521–527.

BASBAUM, C.B., GRILLO, M.A. & WIDDICOMBE, J.H. (1984). Muscarinic receptors: evidence for a nonuniform distribution in tracheal smooth muscle and exocrine glands. *J. Neurosci.*, **4**, 508–520.

BLACKWELL, G.J., FLOWER, R.J., NIJKAMP, F.P. & VANE, J.R. (1978). Phospholipase A_2 activity of guinea-pig isolated perfused lungs: stimulation, and inhibition by anti-inflammatory steroids. *Br. J. Pharmacol.*, **62**, 79–89.

BONNER, T.I., BUCKLEY, N.J., YOUNG, A.C. & BRANN, M.R. (1987). Identification of a family of muscarinic acetylcholine receptor genes. *Science*, **237**, 527–533.

BONNER, T.I., YOUNG, A.C., BRANN, M.R. & BUCKLEY, N.J. (1988). Cloning and expression of the human and the rat m_5 muscarinic acetylcholine receptor genes. *Neuron*, **1**, 403–410.

BOUSHEY, H.A. & HOLTZMAN, M.J. (1985). Experimental airway inflammation. *Am. Rev. Respir. Dis.*, **131**, 312–313.

BRAMLEY, A.M., SAMHOUN, M.N. & PIPER, P.J. (1990). The role of the epithelium in modulating the responses of guinea-pig trachea to bradykinin *in vitro*. *Br. J. Pharmacol.*, **99**, 762–766.

BRUNN, G., WESSLER, I. & RACKE, K. (1992). Muscarinic liberation of ^{3}H-arachidonic acid from epithelium-containing, but not from epithelium-denuded isolated rat tracheae. *Naunyn-Schmied. Arch. Pharmacol.*, **345**, R47.

BRUNN, G., WESSLER, I. & RACKE, K. (1993). Characterization of ^{3}H-arachidonic acid metabolites, liberated by muscarinic receptor activation from isolated rat tracheae. *Naunyn-Schmied. Arch. Pharmacol.*, **348**, R146.

CONKLIN, B.R., BRANN, M.R., BUCKLEY, N.J., MA, A.L., BONNER, T.I. & AXELROD, A. (1988). Stimulation of arachidonic acid release and inhibition of mitogenesis by cloned genes for muscarinic receptor subtypes stably expressed in A9 L cells. *Proc. Natl. Acad. Sci. U.S.A.*, **85**, 8698–8702.

DECKERS, I.A., RAMPART, M., BULT, H. & HERMAN, A.G. (1989). Evidence for the involvement of prostaglandins in modulation of acetylcholine release from canine bronchial tissue. *Eur. J. Pharmacol.*, **167**, 415–418.

DÖRJE, F., WESS, J., LAMBRECHT, G., TACKE, R., MUTSCHLER, E. & BRANN, M.F. (1991). Antagonist binding profiles of five cloned human muscarinic receptor subtypes. *J. Pharmacol. Exp. Ther.*, **256**, 727–733.

DUNNILL, M.S. (1960). The pathology of asthma, with special reference to changes in the bronchial mucosa. *J. Clin. Pathol.*, **167**, 27–33.

ELING, T.E., HENKE, D. & DANILOWICZ, R. (1988). Arachidonic acid metabolites in respiratory epithelial cells. *Gen. Pharmacol.*, **19**, 313–316.

FARMER, S.G., HAY, D.W.P., RAEBURN, D. & FEDAN, J.S. (1987). Relaxation of guinea-pig tracheal smooth muscle to arachidonate is converted to contraction following epithelium removal. *Br. J. Pharmacol.*, **92**, 231–236.

FURCHGOTT, R.F. (1972). The classification of adrenoceptors (adrenergic receptors). An evaluation from the standpoint of receptor theory. In *Handbook Exp. Pharmacol.*, Vol. 33. ed. Blaschko, H. & Muscholl, E. pp. 283–335. Berlin: Springer.

GOLDIE, R.G., FERNANDES, L.B., FARMER, S.G. & HAY, D.W.P. (1990). Airway epithelium-derived inhibitory factors. *Trends Pharmacol. Sci.*, **11**, 67–70.

HAY, D.W.P., FARMER, S.G. & GOLDIE, R.G. (1994). Inflammatory mediators and modulation of epithelial/smooth muscle interactions. In *Handbook of Immunopharmacology: Immunopharmacology of Epithelial Barriers*. ed. Goldie, R.G. pp. 119–146, London: Academic Press.

HENKE, D.C., KOUZAN, S. & ELING, T.E. (1984). Analysis of leukotriens prostaglandins and other oxygenated metabolites of arachidonic acid by high-performance liquid chromatography. *Anal. Biochem.*, **140**, 87–94.

KANEDA, T., KITAMURA, Y. & NOMURA, Y. (1993). Presence of m_3 subtype muscarinic acetylcholine receptors and receptor-mediated increases in the cytoplasmic concentration of Ca^{2+} in Jurkat, a human leukemic helper T-lymphocyte line. *Mol. Pharmacol.*, **43**, 356–364.

KERLAVAGE, A.R., FRASER, C.M. & VENTER, J.C. (1987). Muscarinic cholinergic receptor structure: molecular biological support for subtypes. *Trends Pharmacol. Sci.*, **8**, 426–431.

KONNO, F., TAKAYANAGI, I. (1989). Relationship between the contractile responses and their coupling second messenger systems for muscarinic drugs in the guinea-pig ileal longitudinal muscle. *Arch. Int. Pharmacodyn.*, **301**, 15–29.

LAITINEN, L.A., HEINO, M., LAITINEN, A., KAVA, T. & HAAHTELS, T. (1985). Damage of airway epithelium and bronchial reactivity in patients with asthma. *Am. Rev. Respir. Dis.*, **131**, 599–606.

LAMBRECHT, G., FEIFEL, R., FORTH, B., STROHMANN, C., TACKE, R. & MUTSCHLER, E. (1988). p-Fluoro-hexahydro-sila-difenidol: The first $M_{2\beta}$-selective muscarinic antagonist. *Eur. J. Pharmacol.*, **152**, 193–194.

LAMPORT, S.J. & FEDAN, J.S. (1990). Modulation of reactivity of guinea-pig isolated trachealis by respiratory epithelium: effects of cooling. *Br. J. Pharmacol.*, **99**, 369–373.

MADISON, J.M., JONES, C.A., TOM-MOY, M. & BROWN, J.K. (1987). Affinities of pirenzepine for muscarinic cholinergic receptors in membranes isolated from bovine tracheal mucosa and smooth muscle. *Am. Rev. Respir. Dis.*, **135**, 719–724.

MELCHIORRE, C. (1988). Polymethylene tetramines: a new generation of selective muscarinic antagonists. *Trends Pharmacol. Sci.*, **9**, 216–220.

MEURS, H., ROFFEL, A.F., POSTEMA, J.B., TIMMERMANS, A., ELZINGA, C.R.S., KAUFFMAN, H.F. & ZAAGSMA, J. (1988). Evidence for a direct relationship between phosphoinositide metabolism and airway smooth muscle contraction induced by muscarinic agonists. *Eur. J. Pharmacol.*, **156**, 271–274.

RABE, C.S., DELORME, E. & WEIGHT, F.F. (1987). Muscarine-stimulated neurotransmitter release from PC12 cells. *J. Pharmacol. Exp. Ther.*, **243**, 534–541.

RACKE, K., BÄHRING, A., BRUNN, G., ELSNER, M. & WESSLER, I. (1991). Characterization of endogenous noradrenaline release from intact and epithelium-denuded rat isolated trachea. *Br. J. Pharmacol.*, **103**, 1213–1217.

RACKE, K., BÄHRING, J., LANGER, C., BRÄUTIGAM, M. & WESSLER, I. (1992). Prostanoids inhibit release of endogenous norepinephrine from rat isolated trachea. *Am. Rev. Respir. Dis.*, **146**, 1182–1186.

RACKE, K., BRUNN, G., ELSNER, M. & WESSLER, I. (1993). Effects of indomethacin on muscarinic inhibition of endogenous noradrenaline release from rat isolated trachea. *Naunyn-Schmied. Arch. Pharmacol.*, **348**, 21–27.

RACKE, K., BRUNN, G. & WESSLER, I. (1995). Mucosa-dependent release of prostaglandins from isolated rat trachea by muscarine receptor activation. *Life Sci.*, **56**, 1016.

RAEBURN, D. (1990). Eicosanoids, epithelium and airway reactivity. *Gen. Pharmacol.*, **21**, 11–16.

REIMANN, A., BRUNN, G., HEY, C., WESSLER, I. & RACKE, K. (1995). Arachidonic acid liberation from rat tracheal epithelial cells by alveolar macrophages. In *Advances in Prostaglandin, Thromboxane and Leukotriene Research.*, Vol. 23, pp. 357–359. New York: Raven Press.

TALLARIDA, R.J. & MURRAY, R.B. (1988). *Manual of Pharmacological Calculation.* Berlin: Springer.

TSCHIRHART, E., FROSSARD, N., BERTRAND, C. & LANDRY, Y. (1987). Arachidonic acid metabolites and airway epithelium-dependent relaxant factor. *J. Pharmacol. Exp. Ther.*, **243**, 310–316.

VAN SCOTT, M.R., MCINTIRE, M.R. & HENKE, D.C. (1990). Arachidonic acid metabolism and regulation of ion transport in rabbit Clara cells. *Am. J. Physiol.*, **259**, L213–L221.

WALLENSTEIN, S., ZUCKER, C.L. & FLEISS, J.L. (1980). Some statistical methods useful in circulation research. *Circ. Res.*, **47**, 1–9.

WESSLER, I., BENDER, H., HÄRLE, P., HÖHLE, K.-D., KIRDORF, G., KLAPPROTH, H., REINHEIMER, T., RICNY, J., SCHNIEPP-MENDELSSOHN, K.E. & RACKE, K. (1995). Release of [^{3}H]acetylcholine in human isolated bronchi. Effect of indomethacin on muscarinic autoinhibition. *Am. J. Respir. Crit. Care Med.*, **151**, 1040–1046.

WESSLER, I., HELWIG, D. & RACKE, K. (1990). Epithelium-derived inhibition of [^{3}H]acetylcholine release from the guinea-pig trachea. *Naunyn-Schmied. Arch. Pharmacol.*, **342**, 387–393.

WESSLER, I., REINHEIMER, T., BRUNN, G., ANDERSON, G.P., MACLAGAN, J. & RACKE, K. (1994). β-Adrenoceptors mediate inhibition of [^{3}H]-acetylcholine release from isolated rat and guinea-pig trachea: role of airway mucosa and prostaglandins. *Br. J. Pharmacol.*, **113**, 1221–1230.

(Received May 30, 1995
Accepted June 20, 1995)