β -Adrenoceptors mediate inhibition of [3 H]-acetylcholine release from the isolated rat and guinea-pig trachea: role of the airway mucosa and prostaglandins

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1 Rat or guinea pig isolated tracheae were labelled with [3 H]-choline to measure evoked tritium outflow, which reflects neuronal release of [3 H]-acetylcholine. Tritium outflow was evoked either by electrical stimulation of the extrinsic vagal nerve (rat tracheae) or by 27 mM potassium (guinea pig tracheae).

2 In rat tracheae isoprenaline (0.01, 0.1 μ M) inhibited evoked [3 H]-acetylcholine release, whereas β_2 -adrenoceptor-selective agonists (fenoterol, formoterol, salbutamol) were ineffective.

3 The inhibitory effect of isoprenaline was abolished under the following conditions: (i) presence of propranolol (1 μ M) or of the β_1 -selective antagonist CGP 20712 A (0.1 μ M); (ii) removal of the mucosa at the start of the experiments; (iii) blockade of cyclooxygenase activity by 3 μ M indomethacin.

4 In rat isolated tracheae prelabelled with [3 H]-arachidonic acid, isoprenaline (0.1 μ M) but not formoterol (0.01 μ M) enhanced the outflow of [3 H]-prostaglandins (PGD₂, PGE₂). This effect was blocked by 0.1 μ M CGP 20712 A.

5 In guinea pig tracheae electrical stimulation of the extrinsic vagal nerve did not cause a constant release of [3 H]-acetylcholine, but 27 mM potassium elicited a reproducible release of [3 H]-acetylcholine. In this species both isoprenaline (0.1 μ M) and formoterol (0.01 μ M) inhibited evoked [3 H]-acetylcholine release. Inhibition was abolished under the following conditions: (i) presence of propranolol (1 μ M) or of the β_2 -selective antagonist ICI 118551 (0.3 μ M); (ii) removal of the mucosa at the start of the experiments; (iii) blockade of cyclooxygenase activity by 3 μ M indomethacin.

6 In conclusion, the present experiments have demonstrated that activation of β -adrenoceptors localized in the mucosa mediates inhibition of [3 H]-acetylcholine release from the neuroeffector junctions of the pulmonary, parasympathetic nerves most probably by the liberation of inhibitory prostaglandins from the airway mucosa. The adrenoceptor subtype involved differs in rat (β_1 subtype) and guinea pig (β_2 subtype) airways.

Keywords: Rat and guinea pig tracheae; inhibition of [3 H]-acetylcholine release; airway mucosa; β -adrenoceptors; prostaglandins

Introduction

Presynaptic interactions between cholinergic and adrenergic pulmonary nerves are still a matter of debate. The close proximity of adrenergic and cholinergic varicosities found in some species (baboon, cat, dog, guinea pig, monkey) suggests presynaptic communication between both nervous systems (Silva & Ross, 1974; Jones *et al.*, 1980; Knight *et al.*, 1981). In human airways adrenergic innervation is less extensive, but the smaller human bronchi have been found to receive also adrenergic innervation (Daniel *et al.*, 1986). Evidence for a presynaptic communication between the two branches of the autonomic nervous system has been found in functional studies. Using the innervated tube preparation of the guinea pig trachea evidence was found for presynaptic inhibition by acetylcholine or noradrenaline release from adjacent sympathetic nerves (Pendry & MacLagan, 1991). In contrast, β -adrenoceptor agonists have been found to inhibit contractions evoked by vagal stimulation more effectively than those evoked by bath-applied acetylcholine (Vermeire & Vanhoutte, 1979; Danser *et al.*, 1987; Ito, 1988; Aizawa *et al.*, 1991). This observation may indicate a presynaptic inhibitory action of β -adrenoceptor agonists on the release of acetylcholine from the adjacent cholinergic nerves.

However, the only study in which the release of acetylcholine was measured directly (canine trachea) failed to

obtain evidence for an inhibitory action of noradrenaline (Martin & Collier, 1986). In this study, acetylcholine release was measured after blockade of cholinesterase, a condition which is known to affect presynaptic mechanisms (Kilbinger & Wessler, 1980; Pendry & MacLagan, 1991; Wessler, 1992; Wessler *et al.*, 1992). Recently, we have reported a radio-tracer method for the measurement of the release of newly synthesized [3 H]-acetylcholine from the rat or guinea pig isolated trachea in the absence of cholinesterase inhibitors (Wessler *et al.*, 1990; 1991). In an attempt to further elucidate cholinergic–adrenergic interactions, we have investigated the effects of β -adrenoceptor agonists on the release of [3 H]-acetylcholine from isolated rat and guinea pig tracheae. In addition, the effect of β -adrenoceptors on the metabolism of arachidonic acid has been tested. Some of these results have been communicated to the German Society of Pharmacology and Toxicology (Reinheimer *et al.*, 1993).

Materials and methods

Tissue preparation

Rats (Sprague–Dawley, 200–250 g) and guinea pigs (Dunkin–Hartley, 300–400 g) of either sex (Charles River Wiga, Sulzfeld, Germany) were kept at a constant temperature

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(21°C) and a regular light (06.30–19.30 h) dark (19.30–06.30 h) cycle with food and water *ad libitum*. The animals were killed by stunning followed by exsanguination. Tracheae (from the larynx to the carina) together with the left recurrent laryngeal nerve and the right vagal nerve (proximal to the origin of the right recurrent laryngeal nerve) were dissected. The preparation was transferred into a Petri dish filled with a physiological salt solution of the following composition (given in mM): NaCl 125, KCl 2.7, CaCl₂ 1.8, MgCl₂ 1.0, NaH₂PO₄ 0.4; NaHCO₃ 24, D-glucose 5.1, choline 0.00001. The medium was gassed with 5% CO₂ (v/v) in O₂. The pH of the gassed solution was 7.30. The trachea was opened longitudinally by cutting through the cartilage. In some experiments the mucosa was removed mechanically before starting the incubation/perfusion protocol. The opened trachea was fixed by means of small needles with its luminal surface facing upwards. The mucosa was removed by gently rubbing ten times with a pipe cleaner along the whole length of the trachea. Removal of the mucosa was confirmed by microscopic inspection of histological sections stained with haematoxylin and eosin. The trachea was fixed onto an electrode holder vertically under a tension of 1 g by means of cotton threads and the nerves were threaded through two platinum ring electrodes (internal diameter 1 mm). Thereafter the electrode holder together with the tissue was transferred into a 2 ml organ bath containing the salt solution described above, warmed to a constant temperature of 36°C and bubbled with 5% CO₂ in O₂ (v/v).

Incubation and superfusion protocol ([³H]-choline)

The experimental protocol reported previously was used (Wessler *et al.*, 1991; 1993). In short, the tissues were allowed to equilibrate for a 30 min period of superfusion (2 ml min⁻¹). During the last 10 min of this equilibration period the cut ends of the left recurrent laryngeal nerve and of the right thoracic vagal nerve (proximal to the origin of the right recurrent nerve) were stimulated electrically (0.2 ms, 20 V, 20 mA, 1 Hz; current flow was determined by the voltage drop across a resistance by use of an oscilloscope). Thereafter, superfusion was stopped and the trachea was incubated in 1.5 ml of the salt solution containing in addition 0.01 µM [³H]-choline with a specific activity of 1.2 GBq nmol⁻¹. During the incubation with [³H]-choline the cut ends of the nerves were stimulated continuously at 10 Hz. Guinea pig tracheae were stimulated transmurally during the incubation with [³H]-choline (biphasic pulses of 1 ms, 100 V, 250 mA). Labelling was followed by a 75 min washout (perfusion rate 8 ml min⁻¹) to remove excess radioactivity. From the washout period onwards, 10 µM hemicholinium-3 was added to the medium and the concentration of unlabelled choline was raised to 1 µM. At the end of the washout period superfusion was stopped (time zero) and the bath fluid was exchanged every 3 min. Tritium content in each individual sample was measured by liquid scintillation spectrometry (Packard TriCarb 1900 CA); external standardization was used to correct for counting efficiency.

Stimulation of tritium outflow

Release of [³H]-acetylcholine was elicited by two periods (S₁, S₂) of either electrical nerve stimulation (cut ends of both parasympathetic nerves) or chemical stimulation (elevation of extracellular potassium to 27 mM; equimolar reduction of sodium). S₁ and S₂ started 12 and 42 min after the end of the washout period respectively. During each stimulation period (S₁, S₂) four trains of 20 s duration at 15 Hz were applied with 10 s intervals between the trains. β-Adrenoceptor agonists were added 18 min before S₂ to the bath medium; in each experiment the antagonists were present from 30 min before S₁ onwards. When high potassium was used as release stimulus, extracellular potassium was increased from 13–15 min (S₁) and 43–45 min (S₂) after the end of the washout. At

the end of the experiments tracheae were blotted and weighed.

Separation of the radioactive compounds (acetylcholine, choline, phosphorylcholine) by reversed-phase high-performance liquid chromatography (h.p.l.c.)

In the present experiments β₂-adrenoceptor agonists enhanced the basal tritium outflow immediately after application to the organ bath. To identify the underlying radioactive compound responsible for this effect, acetylcholine, choline and phosphorylcholine were separated by reversed-phase h.p.l.c. and quantified by measuring the radioactive content of the collected eluate by β-scintillation spectrometry (see Wessler & Werhand, 1990).

[³H]-Arachidonic acid metabolism in rat isolated tracheae

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 10 µCi of [³H]-arachidonic acid. Thereafter, the tracheae were washed ten times within 10 min followed by eight consecutive 10 min periods of incubation (see Figure 5, upper part). The medium was collected into plastic vials, frozen immediately and kept at –60°C until analysed by h.p.l.c. (see below). Test stimuli were added to the medium from the 40th min of incubation onwards. In the corresponding interaction experiments, the β-adrenoceptor antagonist CGP 20712 A was added to the incubation medium from the onset of incubation. At the end of incubation each trachea was blotted, weighed, minced and extracted in 2 ml of methanol for 1–2 h at 0–4°C. The extracts were centrifuged at 20,000 g × min and the supernatants were stored at –60°C until analysed.

Arachidonic acid and its metabolites were separated by reversed-phase gradient h.p.l.c. The h.p.l.c. system used was from Waters/Millipore (Waters 600 E Powerline), equipped with a Rheodyne injection valve (1000 µl loop) and a reversed-phase column (250 mm length, 4.6 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 into counting vials for the determination of radioactivity (see below). A methanol–glacial acid gradient solvent system which had been shown to allow the separation of all classes of eicosanoids (van Scott *et al.*, 1990) was used. Two solvents, A (100% methanol) and B (10% methanol adjusted 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, starting with 52% and changing at 27, 52 and 77 min). ¹⁴C- or ³H-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 (Zinsser aqua safe) by liquid scintillation spectrometry in a Packard TR 1900a. External standardization was used to correct for counting efficiency and background was subtracted automatically.

Analysis of results

Tritium outflow ([³H]-choline) is expressed as d.p.m. g⁻¹ 3 min⁻¹. Stimulated tritium outflow was calculated by subtracting the basal tritium outflow from the total (see Wessler *et al.*, 1991; 1993). Tritium outflow ([³H]-arachidonic acid) is expressed as a percentage of the corresponding tissue radioactivity or is normalized to the outflow observed during 30–40 min of incubation (i.e. fourth period) in each individual experiment (see Figure 5). The radioactivity of individual peaks in the radiochromatograms were quantified as d.p.m. per incubation samples and were also expressed as percentage of that observed during the fourth incubation period.

The results are given as means $\pm$ s.e.mean. The significance of differences was evaluated by Student's *t*-test (for paired and unpaired data); for multiple comparison the modified *t*-test according to Bonferroni was used (see Wallenstein *et al.*, 1980). *P*-values < 0.05 were regarded as significant. To evaluate the effects of test compounds on evoked [³H]-acetylcholine release the stimulated tritium outflow elicited by S_2 was related to that elicited by S_1 and the S_2/S_1 ratio was then compared with the control ratio obtained in the absence of test compounds.

Drugs and special chemicals

The following drugs were used: [³H]-arachidonic acid (Amersham Buchler, Frankfurt, Germany; 5.5–8 TBq mmol⁻¹); bradykinin (Sigma Chemie, Munich, Germany); CGP 20712 A ($\pm$ [2-(3-carbamoyl-4-hydroxyphenoxy)ethylamino]-3[4-(1-methyl-4-trifluoromethyl-2-imidazolyl)phenoxy]-2-propanol-methanesulphonate, Ciba-Geigy, Basle, Switzerland); choline chloride (Sigma Chemie); fenoterol hydrobromide (Boehringer Ingelheim, Germany); formoterol hydrochloride (Ciba-Geigy); hemicholinium-3 bromide (Sigma Chemie); methyl-[³H]-choline (3 TBq mmol⁻¹; NEN, Dreieich, Germany); ICI-118551 ($\pm$ 1[(2,3-dihydro-7-methyl-1H-inden-4-yl)oxy]-3[(1-methylethyl)amino]-2-butanol hydrochloride, RBI, Natick, U.S.A.); isoprenaline hydrochloride (Boehringer Ingelheim); neostigmine methylsulphate (Merck, Darmstadt, Germany); propranolol hydrochloride (Sigma Chemie); and salbutamol hemisulphate (Sigma Chemie).

Results

Effects of β -adrenoceptor agonists on evoked tritium outflow ([³H]-choline) from mucosa-intact and mucosa-denuded rat tracheae

Effect of isoprenaline in the absence of indomethacin Tritium outflow from radiolabelled tracheae was measured during 3 min periods (see Figure 1). In the absence of test substances (control, mucosa-intact) two subsequent periods of electrical stimulation of the extrinsic vagal nerves caused a similar increase in tritium outflow, resulting in a ratio S_2/S_1 of 1.00 ± 0.07 ($n = 10$). The electrically stimulated increase in tritium outflow was abolished by removal of extracellular calcium (Wessler *et al.*, 1991), whereas this manipulation did not reduce tritium outflow stimulated by high potassium (27 mM) in mucosa-intact rat tracheae (S_2/S_1 ratio, 1.10 ± 0.10 ; $n = 4$; experiments not shown). For this reason, electrical stimulation was used in all succeeding experiments in the rat trachea.

Isoprenaline, added 18 min before S_2 , reduced the evoked tritium outflow in a concentration-related manner (Figures 1 and 2). A concentration as low as 0.01 μ M isoprenaline was effective. The maximal effect (54% inhibition) occurred in the presence of 0.1 μ M isoprenaline, whereas a 10-fold higher concentration was ineffective, i.e. a bell-shaped concentration-response curve was found (see Figure 2). The non-selective β -adrenoceptor antagonist propranolol (1 μ M) and the β_1 -selective antagonist CGP 20712 A (0.1 μ M) prevented the inhibitory effect of 0.1 μ M isoprenaline (Figure 1 and Table 1); the antagonists were applied from 30 min before S_1 onwards. Propranolol alone, added 18 min before S_2 to the bath medium, did not significantly affect the stimulated tritium outflow (Wessler *et al.*, 1993). When, in the present experiments, propranolol was present in the medium throughout S_1 and S_2 , a small not significant reduction of the ratio S_2/S_1 was obtained (Table 1).

In mucosa-denuded tracheae electrical stimulation of the vagal nerves caused a substantially higher increase in tritium outflow during S_1 and the control ratio S_2/S_1 was lower than in mucosa-intact tracheae (Table 1). Isoprenaline (0.1 μ M),

however, did not inhibit the stimulated tritium outflow in mucosa-denuded preparations, even when a very high concentration of 1 μ M was used (Table 1).

Figure 1 Rat, mucosa-intact trachea: effect of isoprenaline, alone or in the presence of propranolol, on basal and electrically stimulated tritium outflow. After the labelling and washout period (time zero) tritium outflow was measured in 3 min intervals. The extrinsic vagal nerves were electrically stimulated (four 20 s trains at 15 Hz with 10 s intervals) at the times indicated (S_1 , S_2). Isoprenaline (0.1 μ M) was added 15 min before S_2 and when indicated the β -adrenoceptor antagonist propranolol 30 min before S_1 . The filled columns represent evoked tritium outflow. Given are the means of 5–10 experiments (for each series), s.e.mean was 6–15%.

Figure 2 Concentration response curve for isoprenaline on the stimulated tritium outflow from rat isolated tracheae with intact mucosa. The experimental protocol is shown in Figure 1. Given are the means $\pm$ s.e.mean of the number of experiments indicated. Significance of difference from control: * $P < 0.05$, ** $P < 0.01$.

Table 1 Effects of isoprenaline (iso), formoterol (for) and salbutamol (sal) on the stimulated tritium outflow from rat isolated tracheae

Agonist (μM)	Antagonist (μM)	S_1 (d.p.m. g^{-1})	S_2/S_1	n
<i>Mucosa-intact</i>				
—	—	$10,000 \pm 1500$	1.00 ± 0.07	10
—	propranolol (1)	$10,100 \pm 2100$	0.85 ± 0.08	4
—	CGP 20712A (0.1)	$12,100 \pm 3000$	0.88 ± 0.10	5
iso (0.01)	—	8500 ± 1600	$0.58 \pm 0.11^*$	4
iso (0.1)	—	$17,800 \pm 5200$	$0.46 \pm 0.07^{**}$	5
iso (0.1)	propranolol (1)	$11,600 \pm 1900$	0.92 ± 0.10	4
iso (0.1)	CGP 20712A (0.1)	$14,300 \pm 24200$	0.85 ± 0.06	6
fen (0.1)	—	$15,100 \pm 1700$	0.95 ± 0.12	4
for (0.01)	—	$12,100 \pm 3100$	1.14 ± 0.07	6
sal (0.1)	—	$12,100 \pm 2400$	0.93 ± 0.13	7
sal (1)	—	$11,500 \pm 1400$	0.86 ± 0.35	5
<i>Mucosa-denuded</i>				
—	—	$23,200 \pm 3500$	0.74 ± 0.04	7
iso (0.1)	—	$18,700 \pm 3000$	0.67 ± 0.04	7
iso (1)	—	$20,000 \pm 3000$	0.68 ± 0.03	4
for (0.001)	—	$35,600 \pm 3100$	0.95 ± 0.11	6
for (0.01)	—	$25,800 \pm 4300$	0.65 ± 0.06	7
for (0.1)	—	$26,800 \pm 9000$	0.89 ± 0.08	4

After the labelling and washout period tritium outflow was measured in 3 min periods. In each experiment mucosa was removed before the start of the perfusion/incubation protocol. Tritium outflow was elicited by electrical nerve stimulation (four 20-s trains at 15 Hz with a 10 s interval). The β -receptor agonists were added 18 min before the second stimulation period (S_2) and the antagonist 30 min before S_1 . Given are the means $\pm$ s.e. mean of the number of experiments indicated. Significance of differences from the respective control: $^*P < 0.05$, $^{**}P < 0.01$.

Effect of isoprenaline in the presence of indomethacin Indomethacin ($3 \mu\text{M}$) was added 30 min before S_1 to block cyclo-oxygenase activity and left in contact. In the presence of indomethacin isoprenaline ($0.1 \mu\text{M}$) lost its inhibitory effect on stimulated tritium outflow (see Figure 3).

Effects of β_2 -adrenoceptor agonists Neither the short-acting β_2 -adrenoceptor agonists fenoterol ($1 \mu\text{M}$) and salbutamol (0.1 and $1 \mu\text{M}$) nor the long-acting β_2 -agonist formoterol ($0.01 \mu\text{M}$) modified evoked tritium outflow from mucosa-intact rat tracheae (Table 1). Also in mucosa-denuded rat tracheae formoterol failed to modify stimulated tritium outflow significantly; the ratios S_2/S_1 are given in Table 1.

Effect of β -adrenoceptor agonists on basal tritium outflow from rat tracheae Formoterol enhanced the basal tritium outflow after its application to the organ bath (Figure 4). Likewise, fenoterol and salbutamol facilitated the basal tritium outflow (experiments not shown). In contrast, in most experiments, isoprenaline did not affect basal tritium outflow (see Figure 1); however, in some experiments ($1 \mu\text{M}$ isoprenaline) a slight increase was also observed (experiments not shown).

The facilitatory effect of formoterol on basal tritium outflow was (i) more pronounced in mucosa-denuded than in mucosa-intact preparations (Table 2), (ii) abolished by propranolol ($1 \mu\text{M}$) in the rat (Figure 4 and Table 2) and by the β_2 -selective antagonist ICI 118551 ($0.3 \mu\text{M}$) in the guinea pig (see Figure 7), (iii) unaffected by a 40 min pre-exposure of rat tracheae with $0.1 \mu\text{M}$ isoprenaline (to induce receptor desensitization; $n = 4$, experiments not shown) and (iv) unaffected by removal of extracellular calcium (incubation in nominally calcium-free medium containing in addition $1000 \mu\text{M}$ EDTA for 30 min before the addition of formoterol; experiments not shown; $n = 4$). Analysis of the tritium outflow by reversed-phase h.p.l.c. showed that the formoterol-induced increase in the basal tritium outflow was caused by an enhanced outflow of [^3H]-phosphorylcholine and to a smaller extent by that of [^3H]-choline, whereas the outflow of [^3H]-acetylcholine remained unchanged (Table 2).

Figure 3 Effect of indomethacin on the isoprenaline-induced inhibition in mucosa-intact rat trachea. The experimental protocol is shown in Figure 1. In each experiment indomethacin was present from 30 min before S_1 onwards and isoprenaline from 18 min before S_2 . Given are means $\pm$ s.e. mean of the number of experiments indicated. Significance of difference (comparison with the untreated control): $P < 0.01$.

Effect of β -adrenoceptor agonists on [^3H]-arachidonic acid metabolism in rat tracheae Total tritium outflow from rat tracheae labelled with [^3H]-arachidonic acid amounted to $0.134 \pm 0.003\%$ 10 min^{-1} of the tissue radioactivity ($n = 30$) during the first 10 min incubation period (starting after the washout; see Methods) and slightly, but continuously declined during the subsequent 70 min of incubation (Figure 5, upper tracing). Application of $0.1 \mu\text{M}$ isoprenaline prevented the decline of the outflow of total tritium, i.e. it had a marginal stimulatory effect on total tritium outflow (Figure 5, upper tracing).

When the radioactivity of the incubation samples (collected during 30–40 min and 40–50 min) was separated by h.p.l.c.,

two peaks were consistently observed (see examples of radiochromatograms in Figure 6), one co-eluting with arachidonic acid and the other with the prostaglandins PGE₂ and PGD₂ (which were not separated by the current conditions). In the sample collected during 30–40 min of incubation in the absence of drugs the [³H]-prostaglandin peak amounted to 237 ± 33 d.p.m. and the [³H]-arachidonic acid peak to 2870 ± 380 d.p.m. ($n = 18$). In control experiments

the outflow of prostaglandins determined in the subsequent (40–50 min) sample was reduced by about 35% (Figure 5, lower part). However, isoprenaline (0.1 μ M) caused a clear increase in the outflow of [³H]-prostaglandins (Figure 5), without affecting that of [³H]-arachidonic acid (not shown). For comparison, the effect of bradykinin, a mediator which is well known to cause release of prostaglandins in the airways (Bramley *et al.*, 1990), was also tested. In the present

Figure 4 Effect of formoterol on the basal tritium outflow in the absence and presence of propranolol. Formoterol (10 nmol l^{-1}) was added to mucosa-intact, isolated rat tracheae preincubated with [³H]-choline. In one group of experiments propranolol was present 42 min before the addition of formoterol (bars indicate the presence of the test compounds). Formoterol enhanced the basal tritium outflow (middle part) and the effect was abolished by propranolol (lower part). Propranolol by itself did not affect the basal tritium outflow (not shown). Given are the means $\pm$ s.e.mean of 4–10 experiments (for each series). Significance of difference: $P < 0.01$ (paired *t*-test)

Figure 5 Effects of isoprenaline on the outflow of total tritium (upper part) and effects of 0.1 μ M bradykinin (Brad), 0.1 μ M isoprenaline in the absence (Iso) and presence of 0.1 μ M CGP 20712 A (Iso + CGP) or 0.01 μ M formoterol (For) on the outflow of [³H]-prostaglandins (lower part) from isolated rat tracheae preincubated with [³H]-arachidonic acid for 2 h. Upper part, ordinate: Outflow of total tritium, expressed as a percentage of that observed during 30–40 min of incubation in the individual experiments, means $\pm$ s.e.mean of three (filled symbols, isoprenaline) or 12 (open symbols, controls) experiments. Lower part, height of columns: Outflow of [³H]-prostaglandins observed during 40–50 min of incubation expressed as a percentage of that observed during 30–40 min of incubation in the individual experiments, means $\pm$ s.e.mean of 3–6 experiments. Significance of differences: * $P < 0.01$ from the controls (Ctr.); † $P < 0.01$ from the experiments 'Iso' alone

Table 2 Effect of formoterol (0.01 μ M) on the basal tritium outflow (d.p.m. 3 min^{-1} per trachea) in mucosa-intact and mucosa-free rat tracheae

	Total tritium	PCH	CH	ACH
<i>Mucosa-free</i>				
before	2800 ± 300	1000 ± 100	1300 ± 150	240 ± 100
after	$3600 \pm 160^*$	$1650 \pm 60^{**}$	1600 ± 240	230 ± 50
<i>Mucosa-intact</i>				
before	2600 ± 120	1470 ± 220	800 ± 140	120 ± 50
after	2900 ± 120	1700 ± 170	880 ± 140	70 ± 20

After the labelling and washout period (time zero), tritium outflow was measured in 3 min periods. Aliquots (200 μ l) of the incubation medium obtained before (24–26 min) and after (27–29 min) the application of formoterol were injected into the h.p.l.c. system to separate phosphorylcholine (PCH), choline (CH) and acetylcholine (ACH). Given are means $\pm$ s.e.mean of four experiments in each series. Significance of difference from the value obtained before the application of formoterol: * $P < 0.05$; ** $P < 0.01$.

experiments $0.1 \mu\text{M}$ bradykinin also enhanced the outflow of the $[^3\text{H}]$ -prostaglandins, and the effect was slightly larger than that of isoprenaline (Figure 5). In addition, bradykinin increased the outflow of other metabolites (see Figure 6) which appeared earlier in the chromatogram and which might represent 6-keto-PGF $_{1\alpha}$ (see van Scott *et al.*, 1990). The stimulatory effect of isoprenaline on the outflow of $[^3\text{H}]$ -prostaglandins was prevented by the β_1 -selective antagonist CGP 20712 A (Figure 5). When the concentration of isoprenaline was enhanced to $1 \mu\text{M}$ the stimulatory effect on the outflow of $[^3\text{H}]$ -prostaglandins was vanished: after application of $1 \mu\text{M}$ isoprenaline the outflow of $[^3\text{H}]$ -prostaglandins amounted to $86 \pm 15\%$ ($n = 3$) of the predrug period only, a value which did not differ from the control (see Figure 5, lower part), whereas $0.1 \mu\text{M}$ isoprenaline increased the $[^3\text{H}]$ -prostaglandin outflow to $205 \pm 24\%$ of the predrug level (Figure 5, lower part). Correspondingly, evoked $[^3\text{H}]$ -acetylcholine release was not inhibited in the presence of $1 \mu\text{M}$ isoprenaline (see Figure 2). In rat tracheae formoterol ($0.01 \mu\text{M}$) had no effect on the outflow of total tritium (not shown) or on the outflow of $[^3\text{H}]$ -prostaglandins (Figure 5).

Effects of isoprenaline and formoterol on evoked tritium outflow ($[^3\text{H}]$ -choline) from mucosa-intact and mucosa-denuded guinea pig tracheae

In mucosa-intact guinea pig tracheae electrical stimulation of the extrinsic, parasympathetic nerves caused a small and variable increase in tritium outflow, even when the stimulation period was prolonged (1800 instead of 1200 pulses; experiments not shown; $n = 15$). Transmural stimulation of mucosa-intact tracheae has already been found to trigger

mainly an outflow of $[^3\text{H}]$ -phosphorylcholine (Wessler *et al.*, 1990). Thus, it was not possible to investigate the effect of β -adrenoceptor agonists on electrically-stimulated release of $[^3\text{H}]$ -acetylcholine from guinea pig tracheae with intact mucosa. Therefore high potassium was used as the release stimulus in the following experiments with the guinea pig trachea.

Elevation of extracellular potassium (27 mM) caused a substantial and reproducible increase in tritium outflow from mucosa-intact guinea pig tracheae (see Figure 7). The potassium-evoked increase in tritium outflow was nearly abolished by removal of extracellular calcium (S_2/S_1 ratio: 0.16 ± 0.12 , $n = 4$; experiments not shown) indicating a calcium-dependent release mechanism. Isoprenaline ($0.1 \mu\text{M}$) or formoterol ($0.01 \mu\text{M}$) reduced the potassium-stimulated tritium outflow significantly (see Figure 7 and Table 3). Salbutamol also reduced the stimulated tritium outflow, but a considerably higher concentration was required ($3 \mu\text{M}$, see Table 3; $P < 0.1$). The inhibitory action of formoterol was prevented by propranolol or by ICI 118551, whereas formoterol maintained its inhibitory effect in the presence of the β_1 -selective antagonist CGP 20712 A (Table 3). Formoterol

Figure 6 Examples of radiochromatograms of incubation samples of the experiments described in Figure 5. Each tracing shows two chromatograms superimposed: one collected during 30–40 min of incubation in the absence and the other during 40–50 min of incubation in the presence of the test drug. For further details see Figure 5 and Methods.

Figure 7 Guinea pig, mucosa-intact trachea: effect of formoterol, alone or in the presence of ICI 118551, on basal and high potassium-stimulated tritium outflow. After the labelling and washout period (time zero) tritium outflow was measured in 3 min intervals. Tritium outflow was stimulated by high potassium: extracellular potassium was increased to 27 mM (equimolar reduction of sodium chloride) for two (S_1 , S_2) 3 min periods as indicated. Formoterol was added 18 min before S_2 and ICI 118551 30 min before S_1 . Given are the means of 5–7 experiments (for each series), s.e.mean was 6–10% of the means. The filled columns represent evoked tritium outflow. Isoprenaline did not enhance the basal tritium outflow but caused similar inhibition of the stimulated tritium outflow to the data seen with formoterol (see Table 2)

Table 3 Effects of isoprenaline (iso), formoterol (for) and salbutamol (sal) on the stimulated tritium outflow from guinea pig isolated tracheae

Agonist (μM)	Antagonist (μM)	S_1 (d.p.m. g ⁻¹)	S_2/S_1	n
<i>Mucosa-intact</i>				
—	—	10,000 $\pm$ 2300	1.04 $\pm$ 0.13	7
—	propranolol (0.1)	16,000 $\pm$ 1100	0.69 $\pm$ 0.10	6
—	ICI 118551 (0.3)	13,400 $\pm$ 2500	0.60 $\pm$ 0.06*	6
—	CGP 20712 (0.3)	11,800 $\pm$ 900	0.86 $\pm$ 0.03	4
iso (0.01)	—	17,700 $\pm$ 3200	0.53 $\pm$ 0.09*	6
sal (0.1)	—	14,100 $\pm$ 2500	0.85 $\pm$ 0.13	6
sal (1)	—	18,000 $\pm$ 5000	0.82 $\pm$ 0.13	4
sal (3)	—	12,000 $\pm$ 4500	0.58 $\pm$ 0.30	4
for (0.01)	—	13,700 $\pm$ 1400	0.52 $\pm$ 0.09*	8
for (0.01)	propranolol (0.1)	12,300 $\pm$ 5700	1.11 $\pm$ 0.29	4
for (0.01)	ICI 118551 (0.3)	10,800 $\pm$ 1700	0.94 $\pm$ 0.13	4
for (0.01)	CGP 20712 (0.3)	13,300 $\pm$ 1700	0.60 $\pm$ 0.07†	4
<i>Mucosa-denuded</i>				
—	—	27,300 $\pm$ 5200	0.81 $\pm$ 0.09	4
for (0.01)	—	26,800 $\pm$ 8000	0.85 $\pm$ 0.04	4

After the labelling and washout period tritium outflow was measured in 3 min periods. Tritium outflow was elicited by 27 mM potassium. The β -receptor agonists were added 18 min before the second stimulation period (S_2) and the antagonist 30 min before S_1 . Given are the means $\pm$ s.e.mean of the number of experiments indicated. Significance of differences from the controls: control experiments without test compounds, * $P < 0.01$; control experiments with CGP 20712 A alone, † $P < 0.01$.

failed to cause inhibition in mucosa-denuded tracheae (see Table 3), whereas the agonist still enhanced the basal tritium outflow in mucosa-denuded tracheae (see Table 2). As before, indomethacin prevented the inhibitory effect of β -adrenoceptor activation as shown by the following data: in control experiments (indomethacin present throughout) a ratio S_2/S_1 of 0.70 ± 0.06 ($n = 4$) was found and a similar ratio was obtained, when $0.01 \mu\text{M}$ formoterol was additionally added 18 min before S_2 (0.72 ± 0.09 ; $n = 5$).

When the β -receptor antagonist propranolol or ICI 118551 was added already 30 min before S_1 , considerably lower ratios S_2/S_1 were obtained in control experiments (see Table 2), indicating a slowly developing, depressant effect on evoked [³H]-acetylcholine release. The underlying mechanism is unknown, but a similar effect was not observed when electrical nerve stimulation was used to selectively excite the extrinsic vagal nerves in the rat trachea (see Table 1).

Discussion

The airway mucosa of mammals contains a dense population of β -adrenoceptors of unknown physiological functions (Xue *et al.*, 1983; Davis *et al.*, 1990; see also Nijkamp *et al.*, 1992). In the present experiments β -adrenoceptors mediate inhibition of the stimulated release of [³H]-acetylcholine in isolated rat and guinea pig tracheae. This effect was prevented by propranolol, indicating a specific, receptor-mediated effect. Most importantly, the inhibition could not be demonstrated in mucosa-denuded tracheae or when cyclooxygenase activity was blocked by indomethacin. Isoprenaline stimulated the liberation of prostaglandins (PGD₂, PGE₂) from the isolated trachea, and β -adrenoceptor agonists have already been reported by others to enhance phospholipase A₂ activity in guinea pig lung (Blackwell *et al.*, 1978; Suzuki *et al.*, 1987). Our data suggest the existence of a previously uncharacterized regulatory mechanism controlling cholinergic transmission in the airways: β -adrenoceptors localized at the mucosa mediate the liberation of prostanoids inhibitory to the release of newly synthesized acetylcholine (see scheme in Figure 8). Although less likely, a permissive role of the mucosa and of prostaglandins on neuronal effects of presumably presynaptically localized β -adrenoceptors cannot be excluded. However, classical presynaptic receptors (for

instance α_2 - or muscarinic receptors) produce their effects directly at nerve terminals without the involvement of surrounding tissue, whereas in the present study isoprenaline was ineffective in mucosa-denuded preparations. In rat cava an indirect regulatory pathway similar to the present one has been described: β_2 -adrenoceptors assumed to be localized on subendothelial cells facilitate noradrenaline release by the generation of angiotensin II, which activates facilitatory angiotensin receptors present on the adrenergic nerves (Göthert & Kolleck, 1986).

In the airways an inhibitory role of β -adrenoceptors on cholinergic neurotransmission has already been postulated on the basis of functional experiments (for references see Barnes, 1992; Nijkamp *et al.*, 1992). β -Adrenoceptor agonists inhibited nerve-evoked contractions in the canine and human bronchial tissue more effectively than those evoked by bath-applied acetylcholine; this difference was considered to indicate a neuronal effect (Vermeire & Vanhoutte, 1979; Danser *et al.*, 1987; Ito, 1988; Rhoden *et al.*, 1988; Janssen & Daniel, 1990; Aizawa *et al.*, 1991). β -Adrenoceptor agonists, however, have direct effects on smooth muscle tone, and it may be difficult to discriminate between both sites of action. The present release experiments clearly support the concept that β -adrenoceptors mediate inhibition of cholinergic transmission. Inhibition was observed after preganglionic stimulation (rat) or after chemical stimulation with 27 mM potassium (guinea pig). Thus, the post-ganglionic neuro-effector junction is the target of the inhibitory, mucosa-dependent mechanism. This conclusion is in line with the results drawn from functional experiments (guinea pig trachea) in which transmural electrical stimulation was applied (Ito, 1988; Aizawa *et al.*, 1991).

There are, however, important differences between the present results and those obtained in functional experiments. For instance, Ito (1988) reported the 'presynaptic' inhibitory effect of procaterol to occur in the presence of indomethacin. Danser *et al.* (1987) found 'prejunctional' β_1 -adrenoceptors to inhibit canine bronchial contraction evoked by electrical transmural stimulation in the absence of the airway mucosa. In this study tyramine was used to displace noradrenaline from neuronal stores and tyramine was found to inhibit contractile responses evoked by transmural stimulation of canine bronchial tissue even in epithelium-denuded preparations and in the presence of $10 \mu\text{M}$ indomethacin. Species

Figure 8 Scheme of β -adrenoceptor-mediated inhibition of cholinergic transmission in rat or guinea pig tracheae. Varicosities of post-ganglionic parasympathetic and sympathetic pulmonary nerves are localized close to the airway mucosa. Mucosally localized β -adrenoceptors may be activated by neuronally released noradrenaline (depending on species), by circulating hormones (noradrenaline and adrenaline) and by inhaled drugs. Upon stimulation prostaglandins are liberated from the mucosa and inhibit release of acetylcholine from neuroeffector junctions. Evidence for a direct, β -adrenoceptor-mediated inhibition of ganglionic transmission has not been obtained in the present experiments, because isoprenaline did not inhibit [^3H]-acetylcholine release evoked by stimulation of extrinsic vagal nerve in mucosa-denuded rat tracheae.

differences may account for the differences between these results and the present study. In addition, one cannot exclude the possibility that there are different regulatory mechanisms for newly-synthesized [^3H]-acetylcholine and endogenous, stored acetylcholine. Nevertheless, even in the study reported by Danser *et al.* (1987), removal of the mucosa nearly halved the inhibitory action of tyramine, an observation that suggests a mucosa-dependent pathway, as concluded from the results of the present study.

In control experiments lower S_2/S_1 ratios were obtained in mucosa-denuded than in mucosa-intact tracheae (see Table 1 and 3), indicating some fading of evoked [^3H]-acetylcholine release in the former. It had been shown that mechanical removal of the mucosa, as applied in the present study, disrupts mast cells and causes release of their content (Francconi *et al.*, 1990). Possibly, mast cell products are inhibiting [^3H]-acetylcholine release over the time. Alternatively, fading of [^3H]-acetylcholine release may also be explained by the limitation of the releasable [^3H]-acetylcholine pool; in mucosa-denuded tracheae release of [^3H]-acetylcholine evoked by S_1 was doubled and therewith the amount of [^3H]-acetylcholine available for S_2 reduced.

β -Receptor subtype

In rat tracheae isoprenaline but not β_2 -adrenoceptor-selective agonists inhibited evoked [^3H]-acetylcholine release, indicating involvement of a β_1 -adrenoceptor subtype. This con-

clusion is confirmed by the effect of the antagonist CGP 20712 A, which prevented the isoprenaline-mediated inhibition at a concentration which is known to be β_1 -subtype specific. Corroborative evidence was found in the experiments in which [^3H]-arachidonic acid metabolism was studied: isoprenaline but not formoterol caused enhanced liberation of prostaglandins and the effect was prevented by a subtype-specific concentration of CGP 20712 A. Also, binding studies have confirmed the existence of β_1 -receptors in rat lung (Rugg *et al.*, 1978). In conclusion, in rat tracheae adrenoceptors of the β_1 -subtype are involved in the liberation of prostaglandins and the inhibition of [^3H]-acetylcholine release.

Different results about the adrenoceptor subtype involved were obtained in guinea pig trachea. In this species both isoprenaline and formoterol inhibited potassium-evoked release of [^3H]-acetylcholine, an effect that was prevented by the β_2 -selective antagonist ICI 118551 but maintained in the presence of CGP 20712 A. These results confirm that a β_2 -subtype is involved in guinea pig airways. However, as different stimuli were used in the two species studied in these experiments, one might argue that acetylcholine released by different stimuli may be regulated by different receptor subtypes. Unfortunately, it was not possible to use the same stimuli in both species because in rat tracheae high potassium caused a calcium-independent increase in tritium outflow which made it impossible to measure potassium-induced [^3H]-acetylcholine release in the rat. Nevertheless, in rat tracheae β_2 -adrenoceptors cannot inhibit high potassium-evoked [^3H]-acetylcholine release in a way similar to that found with electrical nerve stimulation, because formoterol did not trigger the liberation of prostaglandins and the effect of isoprenaline was exclusively mediated by β_1 -adrenoceptors.

A bell-shaped concentration-response curve was obtained for isoprenaline; $0.1 \mu\text{M}$ isoprenaline caused a maximal inhibition, whereas $1 \mu\text{M}$ was ineffective. The same concentration dependency was found for the effect of isoprenaline to mediate the liberation of prostaglandins. So far, a conclusive explanation cannot be given. It is, however, known that β_1 -adrenoceptor-mediated effects can rapidly desensitize. Possibly, the high concentration of $1 \mu\text{M}$ isoprenaline may have desensitized the mucosally localized β -adrenoceptors and therewith cut the response.

Effect on basal tritium outflow

The β_2 -selective adrenoceptor agonists formoterol, salbutamol and fenoterol, immediately after their application to the organ bath, enhanced the basal tritium outflow, whereas isoprenaline was rather ineffective. The facilitatory effect was prevented by propranolol or by the β_2 -selective antagonist ICI 115881, i.e. was related to the binding at β_2 -adrenoceptors. Separation of the radioactive compounds by reversed-phase h.p.l.c. showed that formoterol increased mainly the outflow of [^3H]-phosphorylcholine and to a smaller extent that of [^3H]-choline. This observation suggests a breakdown of phosphatidylcholine by activation of phospholipase C, however the effect was maintained in the absence of extracellular calcium. Therefore, it is also possible that β_2 -adrenoceptor agonists displace [^3H]-phosphorylcholine and [^3H]-choline from the outer leaflet of the cell membrane when penetrating and binding to the β_2 -receptor (see also Anderson, 1992). The difference between β_1 - and β_2 -adrenoceptor agonists may indicate a difference of the microenvironment in which both receptor subtypes are embedded.

Formoterol failed to cause inhibition of evoked [^3H]-acetylcholine release in mucosa-denuded tracheae, whereas the agonist still enhanced the basal tritium outflow in mucosa-denuded tracheae (see Tables 2 and 3). This clearly indicates that both the enhancement of basal tritium outflow and the inhibition of evoked [^3H]-acetylcholine release are independent mechanisms. The same conclusion can also be drawn from the experiments with isoprenaline which did not

enhance basal tritium efflux but caused inhibition of evoked [³H]-acetylcholine release (see Figure 1).

Underlying inhibitory mechanism

Blockade of cyclooxygenase prevented the inhibitory effect of isoprenaline on evoked [³H]-acetylcholine release, indicating involvement of prostaglandins either directly or indirectly in the inhibitory pathway. The present experiments have also shown that isoprenaline liberates prostaglandins from the rat airways. Prostaglandin E₂ inhibits cholinergic transmission in canine trachea (Deckers *et al.*, 1989) and indomethacin enhances evoked [³H]-acetylcholine in guinea pig, mucosa-intact tracheae (Wessler *et al.*, 1990). Thus, it is suggested that mucosally localized β -adrenoceptors stimulate the liberation of prostanoids, which diffuse to the submucosa and mediate inhibition of acetylcholine release (see proposed scheme in Figure 8). A prior condition of the proposed regulatory pathway is that prostanoids released from the mucosa can gain access to cholinergic nerve endings. Indirect experimental evidence for this assumption has already been obtained as mentioned above: in guinea pig tracheae indomethacin enhances evoked [³H]-acetylcholine release only in mucosa-intact but not in mucosa-denuded preparations (Wessler *et al.*, 1990). In addition, tissue content of endogenous acetylcholine is markedly reduced by removal of

the mucosa (rat trachea, I. Wessler, unpublished observation), indicating cholinergic nerve terminals to be located very close to or even within the airway mucosa, at least in the rat trachea.

It is an undecided question whether mucosally localized β -adrenoceptors are innervated. In rat tracheae the tissue content of noradrenaline was substantially lower in mucosa-denuded than in mucosa-intact preparations (Racké *et al.*, 1992), an observation which favours a localization of adrenergic nerve fibres very close to the airway mucosa. If this is so, activation of mucosally localized β -adrenoceptors could also occur in response to the transmitter noradrenaline in the rat trachea. The airway mucosa is also an important target for therapeutic interventions; mucosally localized β -adrenoceptors can be very effectively activated by inhaled β -adrenoceptor agonists and can thus inhibit evoked acetylcholine release by the indirect pathway which is shown in this study. Such a mechanism would have a beneficial effect in the treatment of bronchial asthma. Most importantly, the inhibitory effect depends on the presence of an intact airway mucosa and the generation of prostaglandins.

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