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Prostanoid receptors of the EP_3 subtype mediate inhibition of evoked [^{3}H]acetylcholine release from isolated human bronchi

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1 The release of neuronal [^{3}H]acetylcholine (ACh) from isolated human bronchi after labelling with [^{3}H]choline was measured to investigate the effects of prostanoids.

2 A first period of electrical field stimulation (S_1) caused a [^{3}H]ACh release of 320 ± 70 and 200 ± 40 Becquerel (Bq) g^{-1} in epithelium-denuded and epithelium-containing bronchi respectively ($P > 0.05$). Subsequent periods of electrical stimulation (S_n, $n = 2$, 3, and 4) released less [^{3}H]ACh, i.e. decreasing S_n/S_1 values were obtained (0.76 ± 0.09, 0.68 ± 0.07 and 0.40 ± 0.04, respectively).

3 Cumulative concentrations (1–1000 nM) of EP-receptor agonists like prostaglandin E_2, nocloprost, and sulprostone (EP_1 and EP_3 selective) inhibited evoked [^{3}H]ACh release in a concentration dependent manner with IC_{50} values between 4–14 nM and maximal inhibition of about 70%.

4 The inhibition of evoked [^{3}H]ACh release by prostaglandin E_2, nocloprost and sulprostone was not affected by the DP-, EP_1- and EP_2-receptor antagonist AH6809 at a concentration of 3 μM, i.e. a 3–30 times greater concentration than its affinity (pA_2 values) at the respective receptors.

5 Circaprost (IP-receptor agonist; 1–100 nM), iloprost (IP- and EP_1-receptor agonist; 10-1000 nM) and U-46619 (TP-receptor agonist; 100–1000 nM) did not significantly affect [^{3}H]ACh release.

6 Blockade of cyclooxygenase by 3 μM indomethacin did not significantly modulate evoked [^{3}H]ACh release in epithelium-containing and epithelium-denuded bronchi. Likewise, the combined cyclo- and lipoxygenase inhibitor BW-755C (20 μM) did not affect evoked [^{3}H]ACh release.

7 In conclusion, applied prostanoids appear to inhibit [^{3}H]ACh release in epithelium-denuded human bronchi under the present *in vitro* conditions, most likely *via* prejunctional prostanoid receptors of the EP_3 subtype.

Keywords: [^{3}H]acetylcholine release; bronchial epithelium; human airways; indomethacin – prostanoid receptors

Introduction

Airway epithelial cells express high levels of cyclooxygenase and lipoxygenases producing eicosanoids with potent paracrine effects on nerves, smooth muscle, glands, and epithelial cells themselves (Holtzman, 1992). In acute and chronic airway inflammation eicosanoids are additionally produced in large quantities by invading immune cells inducing a variety of effects like vasodilatation, oedema and pain (Coleman *et al*., 1990). Epithelial metabolism of arachidonic acid seems to differ species-dependently. For example in rat the cyclooxygenase product prostaglandin E_2 is the major metabolite. In human airway epithelium the main metabolic pathway *via* lipoxygenase results in 15-hydroxyeicosatetraenoic acid (Raeburn, 1990), but human airway epithelial cells produce also prostaglandin E_2 and $F_{2\alpha}$ (Churchill *et al*., 1989; Mitchell *et al*., 1994). Under condition of airway obstruction a shift to constricting leukotrienes is described in patients (Drazen *et al*., 1992). Postjunctional modulation of airway smooth muscle tone is also mediated species-dependently by different subtypes of prostanoid receptors. TP prostanoid receptors seem to play a major role to mediate contraction of human bronchi and parenchymal lung tissue, whereas in guinea-pig trachea also EP_1 receptors are involved (Armour *et al*., 1989; McKenniff *et al*., 1988). In contrast, EP_2 prostanoid receptors produce relaxation in tracheobronchial preparations of human, guinea-pig, and cat (Coleman *et al*., 1990).

Cholinergic neurotransmission is important in regulating airway smooth muscle tone and secretion. However, in human airways the regulation of neuronal ACh release is less well characterized. Besides a number of neurotransmitters and modulators eicosanoids are also expected to exert a prejunctional effect on cholinergic neurotransmission. In canine bronchial tissue prostaglandin E_2 is involved in an inhibitory control mechanism of ACh release (Deckers *et al*., 1989). So far it is unknown whether the neuronal release of ACh from human airways is regulated by prejunctional prostanoid receptors. Thus, the aim of the present study was to elucidate the effect of prostanoids on evoked [^{3}H]ACh release in isolated human bronchi and to characterize the involved receptor subtype.

Methods

Tissue preparation

Macroscopically tumour-free human bronchi of patients with lung cancer and without chronic inflammatory airway diseases were used (Wessler *et al*., 1995). The protocol for obtaining human tissue was proved by the regional ethical committee. Immediately after lobectomy bronchi were longitudinally opened, dissected down to a diameter of 3 mm, and incubated in an ice-cold physiological salt solution of the following composition given in mM: 125 NaCl, 23.8 $NaHCO_3$, 5.05 glucose, 2.68 KCl, 1.80 $CaCl_2$, 1.04 $MgCl_2$, 0.415 NaH_2PO_4,

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0.0567 ascorbic acid, and 0.001 choline chloride. The physiological salt solution was gassed with carbogen, a mixture of 5% (v/v) carbon dioxide in oxygen; pH of the gassed solution was 7.3.

After transport to the laboratory, bronchi were vigorously washed in oxygenated salt solution. Adhering tissue and cartilage were removed as far as possible. Bronchial epithelium either remained or was mechanically removed by rubbing off the luminal surface with a moistened pipe-cleaner before the start of the experiments. This procedure did not penetrate the basal membrane as confirmed by microscopical inspection of histological sections (Reinheimer *et al*., 1996a; Klapproth *et al*., 1997). Bronchi were cut into pieces of about 100 mg and fixed between two platinum wire electrodes in 2 ml organ baths. Experiments started approximately 3 h after surgery.

Experimental protocol

Preparations were 30 min superfused at 37°C with 1.6 ml min^{-1} of warmed and oxygenated physiological salt solution containing in addition 3 μM indomethacin except in experiments where indomethacin was added 18 min before S_2. During the last 15 min of this period bronchial preparations were stimulated transmurally at 1 Hz, 1 ms pulse duration, and 200 mA. Subsequently superfusion was stopped and preparations were radiolabelled by 30 min incubation with 1 μM [^{3}H]choline (specific radioactivity 185 GBq μmol^{-1}) in 2 ml physiological salt solution. Stimulation frequency was increased to 10 Hz augmenting the turnover and labelling of ACh stores.

After labelling the preparations were again superfused at 10 ml min^{-1} for 60 min to remove excess radioactivity. From this time onwards the physiological salt solution contained 10 μM hemicholinium-3 to prevent choline uptake. From the end of the superfusion period onwards, time zero, bath medium was exchanged every 3 min. Samples were collected for measuring [^{3}H]content. Release of newly-synthesized [^{3}H]ACh was evoked by electrical field stimulation. Two or four stimulation periods S_1 - S_2 (- S_4) were applied 12 min and 42 min (72 min and 102 min) from time zero onwards, respectively. Each stimulation period consisted of four 20 s trains at 15 Hz with 5 s intervals. Control experiments were performed in the presence and absence of indomethacin. Test drugs were added at 24 min (54 min and 84 min) from time zero onwards, i.e. 18 min before the respective stimulation period S_n ($n=2$, 3, or 4).

Determination of [^{3}H]radioactivity

A 1 ml portion of each sample was mixed with 2 ml liquid scintillation gel (Aquasafe 300, Zinsser, Frankfurt, Germany). [^{3}H]Radioactivity was measured by β-liquid scintillation spectroscopy (1900 CA Tricarb, Packard, Frankfurt, Germany). Disintegrations were calculated from counts per minute using external standardization to correct for counting efficiency.

Calculations and statistics

Results were expressed as mean value$\pm$s.e.mean of n experiments. Evoked [^{3}H]ACh release was determined from the increase in [^{3}H]efflux above baseline (see Wessler *et al*., 1995). S_n/S_1 values ($n=2$, 3, or 4) were used to compare evoked [^{3}H]ACh release in the presence of test drugs with the individual control [^{3}H]ACh release at S_1. S_n/S_1 values were then compared with the respective control experiments and expressed as % of controls. Statistical comparisons of results were performed by Student's or Welch's t-test. $^*P<0.05$, $^{**}P<0.01$, and $^{***}P<0.001$ were regarded as significant. Graphical fittings and calculation of the respective IC_{50} values were performed using GraphPad Prism®.

Drugs and special chemicals

AH6809 (6-isopropoxy-9-oxoxanthen-2-carbonic acid, gift of Glaxo, Greenford, Great Britain), L(+)ascorbic acid (Merck, Darmstadt, Germany), BW-755C (3-amino-1-[m-(trifluoromethyl)phenyl]-2-pyrazoline, gift of Wellcome, Beckenham, Great Britain), choline chloride (Sigma, Deisenhofen, Germany), [^{3}H]choline chloride (Du Pont, Dreieich, Germany), circaprost (gift of Schering, Berlin, Germany), D(+)glucose (Merck), hemicholinium-3 (Sigma), iloprost (gift of Schering), indomethacin (Sigma), nocloprost clathrat (gift of Schering), prostaglandin E_2 (SPI-bio, Gif sur Yvette, France), sulprostone (gift of Schering), and U-46619 (9,11-dideoxy-9α,11α-methanoepoxy prostaglandin $F_{2\alpha}$, SPI-bio). AH6809 was diluted in a solution containing 0.1% $NaHCO_3$ in 0.4% saline; circaprost was diluted in saline. Indomethacin, iloprost, prostaglandin E_2, sulprostone, nocloprost and U-46619 were made up in a 10 mM stock solution in 100% ethanol; the control experiments were performed in the highest final ethanol concentration (0.03%).

Results

Evoked release of [^{3}H]acetylcholine in control experiments

Electrical field-stimulation of epithelium-denuded human bronchi caused a reproducible release of newly-synthesized [^{3}H]ACh (Figure 1; see also Wessler *et al*., 1995). In the presence of 3 μM indomethacin throughout the experiments evoked [^{3}H]ACh release at S_1 amounted to 320 ± 70 Bq g^{-1} bronchi ($n=10$), and S_n/S_1 values of 0.76 ± 0.09, 0.68 ± 0.07, and 0.40 ± 0.04 were found (Figure 1). In the absence of indomethacin S_1 caused the release of 300 ± 80 Bq [^{3}H]ACh g^{-1} bronchi, and a S_2/S_1 value of 0.78 ± 0.10 was obtained ($n=8$). In epithelium-containing bronchi (presence of 3 μM indomethacin) evoked [^{3}H]ACh release of the first stimulation was 200 ± 40 Bq g^{-1}, and S_n/S_1 values of 0.83 ± 0.09, 0.57 ± 0.07, and 0.49 ± 0.09 ($n=6$) were found.

Effect of applied prostanoids on evoked [^{3}H]acetylcholine release

Cumulative concentrations of prostaglandin E_2 inhibited evoked [^{3}H]ACh release in epithelium-denuded bronchi, S_n/S_1 values of 0.53 ± 0.10, 0.30 ± 0.07, and 0.18 ± 0.05 ($n=8$) were obtained at concentrations of 10 nM, 100 nM, and 1 000 nM prostaglandin E_2, respectively (Figure 1). The concentration-response curve of prostaglandin E_2 is given in Figure 2, the calculated IC_{50} value was about 14 nM. Also nocloprost, a stable prostaglandin analogue, and sulprostone, an EP_1- and EP_3-receptor agonist, caused a similar inhibition of evoked [^{3}H]ACh release (Figure 2). The maximally inhibitory effect of all three agonists was about 70% compared to control. Nocloprost and sulprostone, the synthetic prostanoids, showed a somewhat higher inhibitory potency than prostaglandin E_2, the calculated IC_{50} value for both compounds was about 4 nM.

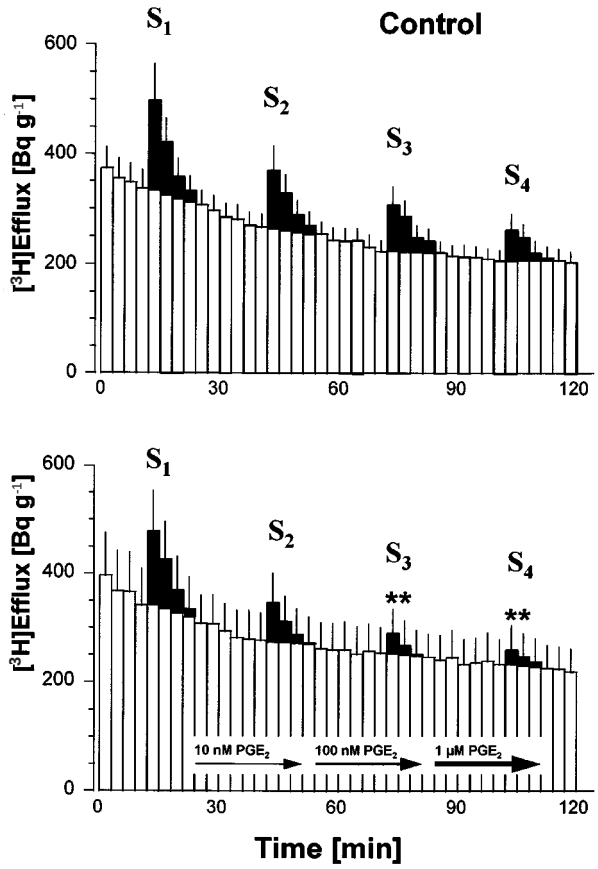

Figure 1 Inhibition of electrically evoked [^{3}H]ACh release from isolated human bronchi by cumulative concentrations of prostaglandin E_2. After the labelling and washout period tritium overflow was measured in 3 min intervals. S1–S4 indicate the periods of transmural electrical stimulation (four 20 s trains at 15 Hz with 5 s intervals). Filled part of the columns indicate stimulated tritium efflux, i.e. evoked release of [^{3}H]ACh. Given are the means ± s.e.mean of 10 (control) and 8 experiments. Release of [^{3}H]ACh during by S1 (individual control) amounted to 320 ± 70 and 290 ± 58 Bq g^{-1} in control and prostaglandin E_2 experiments. Significance of differences from the respective control: $^{**}P<0.01$.

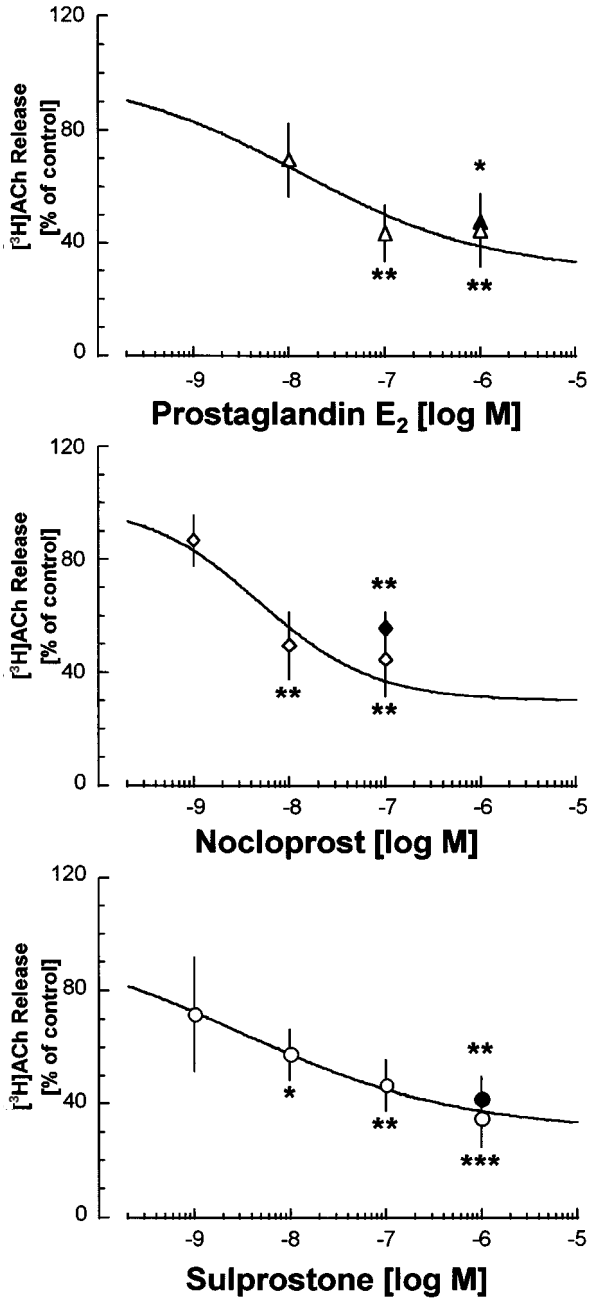

Figure 2 Concentration-response curves of prostaglandin E_2, nocloprost, and sulprostone on evoked [^{3}H]ACh release from isolated human bronchi (open symbols). Filled symbols indicate the effect of the applied prostanoids in the presense of 3 μM AH6809. [^{3}H]ACh release evoked by S1 (individual control) amounted to 290 ± 58 Bq g^{-1} (prostaglandin E_2), 470 ± 120 Bq g^{-1} (nocloprost) and 350 ± 100 Bq g^{-1} (sulprostone). Given are the means ± s.e.mean; for each individual concentration 4–14 experiments were performed.

Characterization of the involved prostanoid receptor subtype

Sulprostone does not discriminate between EP_1- and EP_3-receptor subtypes, therefore experiments with AH6809, an EP_1-/EP_2-/DP-receptor antagonist, were performed. AH6809 (3 μM) did not affect the inhibitory effects of prostaglandin E_2, nocloprost, and sulprostone (Figure 2, filled symbols).

Circaprost (1 nM – 100 nM), an IP-receptor agonist, had no effect on evoked [^{3}H]ACh release (Table 1). Also in the presence of iloprost (10 – 1000 nM), an EP_1- and IP-receptor agonist, evoked [^{3}H]ACh release was not modified. Finally, U-46619 (0.1 and 1 μM), a TP-receptor agonist, did not affect evoked [^{3}H]ACh release.

Effects of indomethacin and BW-755C on evoked [^{3}H]acetylcholine release

In epithelium-containing bronchi indomethacin (3 μM) added 18 min before S_2 did not affect [^{3}H]ACh release; also in epithelium-denuded bronchi [^{3}H]ACh release was not significantly modulated by indomethacin (Table 2). BW-755C (20 μM), a cyclo- and lipoxygenase inhibitor, did not modulate evoked [^{3}H]ACh release in epithelium-containing and epithelium-denuded bronchi (Table 2).

Discussion

Evoked release of newly-synthesized [^{3}H]ACh

Electrically-evoked release of newly-synthesized [^{3}H]ACh from isolated human bronchi can be measured over two or up to

Table 1 Lack of effect of circaprost, iloprost, and U-46619 on evoked [^{3}H]ACh release from isolated human bronchi

Substance	*[^{3}H]ACh Release [% of control]*	n
1 nM Circaprost	105±5	6
10 nM Circaprost	91±6	6
100 nM Circaprost	108±8	5
10 nM Iloprost	91±12	8
100 nM Iloprost	104±9	9
1000 nM Iloprost	110±25	8
100 nM U-46619	106±11	4
1000 nM U-46619	107±22	7

After the labelling and washout period tritium efflux from epithelium-denuded human bronchi was measured in 3 min intervals. Indomethacin (3 μM) was present throughout the experiments, and transmural electrical stimulation was applied as shown in Figure 1. Test substances were applied 18 min before the respective stimulation period. The evoked [^{3}H]ACh release obtained in the presence of test substances is given as percentage of the respective control. Neither circaprost, nor iloprost nor U-46619 affected evoked [^{3}H]ACh release. Given are the means±s.e.mean of the number of experiments indicated.

Table 2 Effect of BW-755C and indomethacin on evoked [^{3}H]ACh release from epithelium-containing and epithelium-denuded human bronchi

Inhibitor	*Epithelium*	*[^{3}H]ACh Release [% of control]*	n
20 μM BW-755C	+	105±11	7
20 μM BW-755C	–	119±23	4
3 μM Indomethacin	+	88±10	6
3 μM Indomethacin	–	133±18	6

After the labelling and washout period tritium efflex was measured in 3 min intervals. Two periods (S_1, S_2) of the transmural electrical stimulation were applied. Test substances were given 18 min before S_2, and the S_2/S_1 ratios were compared with respective control experiments which were carried out in the absence of the test substances (control S_2/S_1 ratio in epithelium-containing bronchi: 0.84±0.07 (12); control S_2/S_1 ratio in epithelium-denuded bronchi: 0.78±0.10 (8)). Given are the means±s.e.mean of the number of experiments indicated.

four stimulation periods (Wessler *et al*., 1995). Electrically-evoked tritium overflow depended on extracellular calcium and was inhibited by the neurotoxin tetrodotoxin, i.e. stimulated tritium overflow represents the release of neuronal [^{3}H]ACh (Wessler *et al*., 1995). Nevertheless, the labelling of non-neuronal ACh stores (Klapproth *et al*., 1997) cannot fully excluded. Therefore, the present experiments addressed to characterize prejunctional prostanoid receptors were performed with epithelium-denuded bronchi eliminating epithelial ACh and eicosanoids of epithelial origin (Holtzman, 1992). Moreover, experiments were carried out in presence of indomethacin to inhibit the formation of endogenous prostanoids.

Prostanoids inhibit evoked [^{3}H]ACh release

Prostaglandin E_2, nocloprost, and sulprostone inhibited evoked [^{3}H]ACh release from human bronchi in a concentration-dependent manner with a maximal inhibition of about 70%. IC_{50} values for the prostanoids investigated were between 4–14 nM. Previously, in a functional study it was already demonstrated that prostaglandin E_2 concentration-dependently inhibited the cholinergic response in isolated human bronchi (Ellis & Conanan, 1996). Furthermore it was concluded that low concentrations of prostaglandin E_2 suppress excitatory junction potentials in human bronchi indicating inhibition of acetylcholine release (Ito *et al*., 1990). Both latter observations agree with the inhibitory effect of prostaglandin E_2 on electrically-evoked [^{3}H]ACh release as observed in the present study. Likewise, in the guinea-pig trachea prostaglandin E_2 inhibited electrically-evoked [^{3}H]ACh release by about 70% (DeLisle *et al*., 1992; Spicuzza *et al*., 1998), and also in isolated canine airways exogenous and endogenous prostanoids mediated inhibition of endogenous ACh release (Inoue *et al*., 1984; Shore *et al*., 1987; Deckers *et al*., 1989). A recent study in which the effects of prostanoid receptor agonists on the release of [^{3}H]ACh from the guinea-pig trachea were investigated obtained similar results as found in the present experiments (Spicuzza *et al*., 1998); the degree of inhibition (74%), the potency of prostaglandin E_2 (7.6 nM) as well as the receptor subtype involved (EP_3 subtype) agreed excellently with the results of the present study. Whether the inhibitory effects of the prostanoids are of biological significance remains an open question depending on the amount and localization of endogenous prostanoid synthesis. However, the lacking effect of indomethacin in epithelium-containing bronchi might argue against a prominent action of prostanoids on the release of newly-synthesized [^{3}H]ACh. In equine airways neither endogenous nor exogenous prostanoids had any effect on the release of endogenous ACh (Wen-Wang *et al*., 1994).

Prostanoid receptors of the EP_3 subtype mediate inhibition of [^{3}H]ACh release

Prostaglandin E_2 mediates its agonistic activity in various smooth muscle preparations with EC_{50} values between 5 and 10 nM (Dong *et al*., 1986; Coleman *et al*., 1994). Related to prostaglandin E_2, sulprostone, an EP_1- and EP_3-receptor agonist, shows a 5 fold lower and roughly a 10 fold higher potency at EP_1- and EP_3-receptors respectively (Coleman *et al*., 1994). Iloprost, an EP_1- and IP-receptor agonist, appears equieffective to prostaglandin E_2 at EP_1-receptors (Coleman *et al*., 1994).

In the present experiments the EP-receptor mediating inhibition of [^{3}H]ACh release could be characterized as an EP_3 subtype because of the following reasons. Firstly, the inhibition by prostaglandin E_2, nocloprost and sulprostone, the latter was somewhat more potent than prostaglandin E_2 (see preceding paragraph), was not affected by 3 μM AH6808. The concentration of AH6808 is 3–30 times higher than its pA_2 value at EP_1-/EP_2-/DP-receptors (Coleman *et al*., 1994; Spicuzza *et al*., 1998). Secondly, iloprost even at a concentration of 100 or 1000 nM had no influence on [^{3}H]ACh release; such high concentrations of iloprost mediate sufficient stimulation of EP_1-receptors (Coleman *et al*., 1994; see also preceding paragraph). Thirdly, neither circaprost, an IP-receptor agonist, nor U-46619, a thromboxane A_2 mimetic (TP-receptor agonist), modulated [^{3}H]ACh release. Hence it can additionally concluded that IP- and TP-receptors do not affect [^{3}H]ACh release in the present model.

A prejunctional prostanoid receptor of the EP_3 subtype has also been found to inhibit the release of noradrenaline in the rat trachea (Racké *et al*., 1992). Likewise, it has recently been shown that prostaglandin E_2 inhibits [^{3}H]ACh release from the guinea-pig trachea via stimulation of EP_3-receptors (Spicuzza

et al., 1998). These latter authors used the same radiotracer technique and applied the same compounds at similar concentrations as tested in the present study. The inefficiency of AH6809 to antagonize the inhibitory effects of prostaglandin E_2, nocloprost and sulprostone indicates the involvement of EP_3-receptors. In this context it is noteworthy that the binding affinity of AH6809 at the EP_2-subtype has been estimated in human tissue only (Woodward *et al*., 1995). The present experiments had been performed with human tissue which gives additional support to conclude that EP_2-receptors are not involved.

In canine airways the release of ACh appears to be enhanced by leukotriens C_4 and D_4 and by U-46619, the thromboxane A_2 mimetic (Chung *et al*., 1985; Abela & Daniel, 1994). Also in guinea-pig trachea endogenous prostanoids were proposed to facilitate cholinergic transmission *via* thromboxane receptors on nerve terminals (Jacques *et al*., 1992). Furthermore, U-46619 can increase airway smooth muscle tone in asthmatic subjects, an effect that was assumed to be at least in part mediated by releasing ACh from cholinergic nerves (Saroea *et al*., 1995). In the present experiments U-46619 at concentrations of 0.1 and 1 μM did not facilitate evoked [^{3}H]ACh. This discrepancy may originate from the different methodological approaches applied in the various studies (end-organ response *vs* release; endogenous ACh *vs*. radiolabelled ACh; species-differences). In the guinea-pig myenteric plexus prostaglandin E_2 and prostacyclin mediated facilitatory effects on ACh release (Gaion & Trento, 1983; Takeuchi *et al*., 1992; Fukunaga *et al*., 1993; Mulholland & Simeone, 1993).

Effect of indomethacin and BW-755C on evoked [^{3}H]ACh release

In epithelium-containing human bronchi indomethacin and BW-755C, a combined cyclo- and lipoxygenase inhibitor, had no influence on evoked [^{3}H]ACh release. This is a surprising finding because the airway epithelium represents the major compartment in which prostanoids like prostaglandin E_2 are synthesized (Raeburn, 1990; Holtzman, 1992). In agreement with this observation indomethacin nearly doubled ACh release in epithelium-intact but not in epithelium-denuded guinea-pig trachea indicating spontaneous liberation of inhibitory prostanoids from the airway epithelium in this species (Wessler *et al*., 1990). Thus, the present observation with indomethacin points to an important species difference. In epithelium-denuded bronchi indomethacin showed some tendency to increase [^{3}H]ACh release but this effect was statistically not significant (see Table 2). Indirect evidence for an inhibitory action of endogenous prostanoids on ACh release from human bronchi was reported recently (Wessler *et al*., 1995). A low concentration of oxotremorine, a muscarinic receptor agonist, reduced [^{3}H]ACh release and this inhibition was prevented by indomethacin. The authors concluded that muscarinic receptor stimulation mediates the liberation of inhibitory prostanoids acting on neurons to inhibit ACh and noradrenaline release (Racké *et al*., 1993; Wessler *et al*. 1995). A mucosa-dependent liberation of prostaglandins triggered by stimulation of muscarinic receptors has been demonstrated in the rat isolated trachea (Brunn *et al*., 1995). Likewise, β-adrenoceptor stimulation can mediate the liberation of inhibitory prostanoids from the airway epithelium of rat and guinea-pig trachea (Wessler *et al*., 1994).

Taken together, important species- and organ-differences appear to exist with respect to the functional role of prostanoids. The present experiments demonstrate that applied prostanoids inhibit evoked [^{3}H]ACh release in human bronchi, most likely by the stimulation of prejunctional EP_3-receptors.

The present work contains parts of the MD thesis of EH. Parts of the study have already been presented at the autumn meeting of the German Society for Experimental and Clinical Pharmacology and Toxicology (Reinheimer *et al*., 1996b). We thank Glaxo-Wellcome and Schering for the generous gifts of the above-mentioned compounds.

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