British Journal of Pharmacology (1996) 117, 1413–1420

Paradoxical facilitation of acetylcholine release from parasympathetic nerves innervating guinea-pig trachea by isoprenaline

[1]Maria G. Belvisi, Hema J. Patel, Tsuneyuki Takahashi, Peter J. Barnes & Mark A. Giembycz

Department of Thoracic Medicine, Royal Brompton National Heart and Lung Institute, London

1 Previous studies have provided evidence that activation of β-adrenoceptors on cholinergic nerve terminals can inhibit neurotransmission in the airways. However, in most cases, this conclusion has been based on indirect evidence obtained from mechanical experiments where changes in airways smooth muscle tone were measured.

2 We have assessed whether modulation of cholinergic neurotransmission by β-adrenoceptor agonists is due to a pre- or post-junctional action by investigating the effect of isoprenaline on contractile responses evoked by exogenous acetylcholine (ACh) and electrical field stimulation (EFS; 4 Hz, 40 V, 0.5 ms pulse width every 15 s), and on EFS-induced ACh release from cholinergic nerves innervating guinea-pig and human trachea. Furthermore, the subtype of β-adrenoceptor which modulates neurotransmission and the potential role of cyclic AMP in this response were evaluated.

3 In guinea-pig trachea, isoprenaline (1 nM – 1 μM) inhibited the contractile response evoked by exogenous ACh (1 μM) to a similar extent to that evoked by EFS (EC_{50} = 19.9 and 23 nM, respectively).

4 In epithelium-denuded guinea-pig strips treated with indomethacin (10 μM), isoprenaline significantly enhanced EFS-induced ACh release from cholinergic nerve terminals (by 36% at 0.3 μM). This effect was blocked by propranolol and ICI 118, 551 (each 0.1 μM). In contrast, isoprenaline failed to affect EFS-induced ACh release from parasympathetic nerves innervating human trachea.

5 To evaluate the role of cyclic AMP in the β-adrenoceptor-induced facilitation of cholinergic neurotransmission, the effects of various cyclic AMP elevating drugs on ACh release were studied. Forskolin (10 μM) significantly augmented (by 17%) EFS-induced ACh release, an effect which was not reproduced by 1,9-dideoxyforskolin (10 μM) which does not activate adenylyl cyclase. Similarly, the cyclic AMP analogue, 8-bromo-cyclic AMP (1 mM) and cholera toxin (1 μg ml^{-1}) facilitated ACh output by 22 and 47% respectively, whereas prostaglandin E_2 (PGE_2, 0.1 nM – 1 μM) *inhibited* this response (by 67% at 1 μM).

6 Zardaverine (10 μM), a dual inhibitor of the phosphodiesterase (PDE)3 and PDE4 isoenzyme families, did not affect EFS-induced ACh release and failed to facilitate the actions of either isoprenaline or PGE_2. Similarly, neither SK&F 94120 (10 μM) nor rolipram (10 μM), selective inhibitors of PDE3 and PDE4 respectively, significantly affected the release of ACh in response to EFS.

7 The result of this study suggests that isoprenaline facilitates cholinergic neurotransmission in guinea-pig, but not human, trachea by activation of pre-junctional β_2-adrenoceptors, an effect that may be mediated via activation of the cyclic AMP/cyclic AMP-dependent protein kinase cascade. Furthermore, the data presented herein illustrate the need to undertake direct measurements of neurotransmitter release when examining the effect of agents purported to act pre-junctionally.

Keywords: Cholinergic neurotransmission; isoprenaline; cyclic AMP; guinea-pig trachea

Introduction

The parasympathetic nervous system plays a dominant role in the control and regulation of the respiratory system in man and in animals. Cholinergic nerves provide the major bronchoconstrictor neural mechanism in all species (Barnes, 1993) whereas the sympathetic innervation to the airways smooth muscle is species-dependent and may be sparse or even absent (Mann, 1971). In cats and guinea-pigs, for example, noradrenergic fibres are more abundant in the airways (Richardson, 1979); however, in man sympathetic nerves innervate bronchial blood vessels, submucosal glands and parasympathetic ganglia and there are few, if any, nerve fibres supplying the airways smooth muscle (Richardson & Béland, 1976). Although noradrenergic nerves do not directly control airways smooth muscle tone they may influence cholinergic neurotransmission via activation of pre-junctional α- or β-adrenoceptors (Barnes, 1994). In fact, even in species that lack a direct sympathetic supply to the airways smooth muscle, cholinergic and noradrenergic varicosities lie in close apposition which has lead to the suggestion that there may be cross-talk between the two nervous systems (Jones *et al.*, 1980b).

Previous studies have provided evidence that activation of β-adrenoceptors on cholinergic nerve terminals can inhibit neurotransmission in the airways. Generally, this conclusion is based on indirect evidence obtained from mechanical experiments where changes in airways smooth muscle tone were measured. Thus, β-adrenoceptor agonists inhibit nerve-induced contractions of canine (Vermeire & Vanhoutte, 1979; Ito & Tajima, 1982; Danser *et al.*, 1987; Ito, 1988) and human airway smooth muscle (Rhoden *et al.*, 1988; Janssen & Daniel, 1990; Aizawa *et al.*, 1991) following electrical field stimulation (EFS) of the parasympathetic supply more effectively than the tension generated by exogenous acetylcholine (ACh). Although these data indicate that the preferential action of β-adrenoceptor agonists is to inhibit cholinergic neurotransmis-

[1] Author for correspondence.

sion, they are difficult to interpret unambiguously since β-adrenoceptor agonists are also potent relaxants of airways smooth muscle making it difficult to differentiate a pre-junctional inhibitory action of these compounds on ACh release from a direct suppressive effect at the level of the airways smooth muscle.

The β-adrenoceptor subtype which modulates cholinergic neurotransmission is apparently species-dependent. Thus while activation of β_1-receptors by noradrenaline or following EFS of the sympathetic nerves inhibits cholinergic contractile responses in canine airways (Danser *et al.*, 1987), the selective β_2-agonist, procaterol, evokes the same response (Ito, 1988) suggesting that receptors of both the β_1- and β_2-subtype co-exist pre-junctionally on the cholinergic nerves which innervate this species (Janssen & Daniel, 1990). In contrast, current evidence suggests that the inhibition of cholinergic neurotransmission in human airways is mediated solely via β_2-adrenoceptors (Rhoden *et al.*, 1988; Aizawa *et al.*, 1991). The discrepancies in pre-junctional adrenoceptor subtype between species may be related to the catecholamine which is predominantly seen by the β-receptors. This interpretation would be consistent with the hypothesis that β_1-receptors are activated by noradrenaline released from noradrenergic nerves (which are abundant in canine airways) while β_2-receptors are activated by circulating adrenaline. However, it is also conceivable that these differences arise artifactually as a result of attributing changes in EFS-induced contractile responses to alterations in ACh output from cholinergic nerve terminals.

In some studies, this problem has been circumvented by measuring directly the output of ACh following EFS of the parasympathetic supply. In canine trachea, for example, noradrenaline has no effect on ACh release evoked by electrical field stimulation (Martin & Collier, 1986) whereas more recent experiments have demonstrated that activation of pre-junctional β-adrenoceptors inhibits cholinergic neurotransmission in rat and guinea-pig airways (Wessler *et al.*, 1994).

Given the inherent difficulties of assessing the ability of agents to modulate neurotransmission (either pre- or post-junctionally) by indirect methods we have compared the effect of isoprenaline on contractile responses evoked by exogenous ACh with its effect on EFS-induced cholinergic contractile responses. Furthermore, ACh release was measured directly from guinea-pig trachea to assess if indirect mechanical measurements provide a reliable indication of ACh output from cholinergic nerves. In addition, the subtype of β-adrenoceptor which modulates cholinergic neurotransmission and the potential role of cyclic AMP in the genesis of this response was evaluated. For comparison, similar studies were performed with human airway smooth muscle.

A premilinary account of some of these data has been presented to the American Thoracic Society (Belvisi *et al.*, 1994).

Methods

Preparation of guinea-pig and human trachea

Male Dunkin-Hartley guinea-pigs (Harlan-Olac) (300–500 g) were killed by cervical dislocation. The lungs, with trachea and bronchi attached, were rapidly removed and placed in oxygenating Krebs-Henseleit solution (KHS) of the following composition (in mM): NaCl 118, KCl 5.9, $MgSO_4$ 1.2, $CaCl_2$ 2.5, NaH_2PO_4 1.2, $NaHCO_3$ 25.5 and glucose 5.6. The trachea was dissected away from the lungs and main bronchi and opened longitudinally by cutting through the cartilage; the epithelium was subsequently removed by careful dissection, minimizing damage to the smooth muscle. Indomethacin (10 μM) was present throughout to prevent the formation of endogenous prostaglandins which are known to affect cholinergic neurotransmission and ACh release *per se* (Walters *et al.*, 1984; Deckers *et al.*, 1989; Wessler *et al.*, 1994).

ACh release from human tracheal strips obtained from 7 donor patients for heart/heart-lung transplantation (12–49 years, 5 male) was studied. There was no evidence of long-term lung disease or airway inflammation in the donors. The lung tissues were immediately placed into oxygenated KHS, cooled to 4°C, and transported to the laboratory. Indomethacin (10 μM) was present throughout to prevent the formation of endogenous prostaglandins. The time elapsed from harvest of the lungs from the donor to the start of experiments was generally 6–8 h. Strips of tracheal smooth muscle were taken from the trachea and all cartilage and connective tissue was dissected away. The epithelium was then removed by careful dissection minimizing damage to the smooth muscle; this was subsequently confirmed by histology.

Measurement of EFS-induced cholinergic contractile responses in guinea-pig trachea

Eight transverse segments of trachea, each containing 3–4 cartilaginous rings, were prepared and suspended between parallel platinum wire electrodes in 10 ml organ baths containing KHS supplement with indomethacin (10 μM) at 37°C which was continually gassed with a 95% O_2/5% CO_2 mixture. The tissues were allowed to equilibrate for 1 h with frequent washing under a resting tension of 1 g which was optimal for determining changes in tension. Isometric contractile responses were measured with force-displacement transducers (model FT-03; Grass Instruments, Quincy, MA, U.S.A.) connected to a polygraph (Model 7D; Grass Instruments, Quincy, MA, U.S.A.). EFS was delivered by two platinum wire field electrodes inserted in parallel (10 mm apart) with the tissue suspended between them. A stimulator (model D345; Digitimer Ltd., Welwyn Garden City, Hertfordshire) provided biphasic square wave pulses of supramaximal voltage (40 V) at source of 0.5 ms duration.

For experiments involving cholinergic contractile responses, EFS was applied for 15 s every 4 min at a frequency of 4 Hz, which evoked rapid increases in tone that were approximately 50% of the maximal EFS-induced contraction. After at least four stable responses of equal magnitude were obtained, isoprenaline (0.1 nM–1 μM) was added and EFS-induced cholinergic contractile responses evoked until the maximal effect of the drug was observed. One concentration of isoprenaline was tested per tissue. Contractile responses obtained following EFS under the aforementioned conditions were abolished by atropine (1 μM) and tetrodotoxin (3 μM), indicating that tension generation was due solely to the release of ACh from parasympathetic nerves.

Measurement of contractile responses evoked by exogenous ACh in guinea-pig trachea

In a separate series of experiments the effect of isoprenaline (1 nM–1 μM) was investigated on post-junctional contractile responses evoked by exogenous ACh (1 μM) after three control responses had been obtained. This concentration of ACh (1 μM) elicited a contraction that was similar in magnitude to that evoked by EFS (40 V, 0.5 ms pulse width, 4 Hz for 15 s). All experiments were performed in the presence of indomethacin (10 μM).

Measurement of ACh release from parasympathetic nerves in guinea-pig and human trachea

The release of ACh from cholinergic nerves was measured as previously described (Ward *et al.*, 1993). Briefly, eight strips of smooth muscle with the cartilage and epithelium removed were studied in parallel. Each tissue was connected top and bottom with silver wire and mounted in a jacketed chamber. Tissues were superfused (Watson-Marlow model 503S; Smith and Nephew, Falmouth), at a rate of 1 ml min^{-1} throughout the experiment with oxygenated KHS maintained at 37°C (pH 7.4). The tissues were allowed to equilibrate for 30 min during which time they were continuously superfused with KHS solution. EFS (40 V; 0.5 ms pulse width, 4 Hz) was applied

continuously for the last 10 min delivered via the silver wire electrodes. Tissues were then placed in vials containing 1.5 ml of oxygenated KHS supplemented with [^{3}H]-choline (67 nM; specific radioactivity: 2.78 TBq mmol^{-1}) and EFS was applied (40 V; 0.5 ms pulse width, 4 Hz) for 45 min in order to facilitate uptake of [^{3}H]-choline into cholinergic nerve terminals. At the end of this period, tissues were superfused with KHS containing hemicholinium-3 (10 μM) to prevent the re-uptake of unlabelled choline into the nerves. Preparations were washed for 2 h in order to achieve a stable baseline of tritium release. The superfusate collected during this period was discarded.

It has been shown previously that most of the tritium outflow evoked by EFS of epithelium-containing trachea is [^{3}H]-phosphorylcholine in addition to [^{3}H]-ACh, whereas EFS of epithelium-denuded tracheal preparations does not elicit significant release of [^{3}H]-phosphorylcholine (Wessler *et al.*, 1990a). Furthermore, the release of ACh following EFS of guinea-pig trachea is better maintained in the presence of indomethacin (D'Agnostino *et al.*, 1990). Accordingly, in the studies described here epithelium-free tissue preparations were used and indomethacin (10 μM) was present throughout to ensure that the EFS evoked release of tritium was almost entirely composed of [^{3}H]-ACh (Ward *et al.*, 1993; Patel *et al.*, 1995).

EFS (0.5 ms pulse width, 4 Hz for 1 min) was applied to each tissue at supramaximal voltage (40 V) and 1 ml samples were collected every minute for 3 min before, 1 min during and 3 min after stimulation and at 5 min intervals outside these times. Previous studies in the author's laboratory have confirmed that the tritium released during EFS under the aforementioned conditions is frequency-dependent and tetrodotoxin-sensitive and is, therefore, neuronal in origin (Ward *et al.*, 1993). Furthermore ACh, at a concentration (10 μM) that evoked a contraction of a similar magnitude to that elicited by EFS using similar stimulation parameters, does not evoke the release of [^{3}H]-ACh by merely contracting the tissue (Ward *et al.*, 1993). Isoprenaline (3 nM – 10 μM), PGE_2 (0.1 nM – 1 μM), zardaverine (10 μM), SK&F 94120 (10 μM), rolipram (10 μM), forskolin (10 μM), 1,9-dideoxyforskolin (10 μM), 8-bromo cyclic AMP (1 mM), propranolol (0.1 μM), ICI 118 551 (0.1 μM) or the appropriate vehicles were added to the KHS superfusing each tissue after one control EFS as detailed in the text and figure legends. A test EFS was then applied 15 min and, in some cases, 30 min after addition of the drugs as indicated. In some experiments, tracheal strips were superfused with cholera toxin (1 μg ml^{-1}), which ADP-ribosylates and therefore irreversibly activates Gs, after one control EFS and a test EFS was then applied 120 min later.

Drugs, chemicals and analytical reagents

The following drugs were obtained from the Sigma Chemical Company (Poole, Dorset, U.K.): indomethacin, tetrodotoxin, atropine sulphate, forskolin, (±)-propranolol, (±)-isoprenaline hydrochloride, ACh chloride, PGE_2, hemicholinium-3, 1,9-dideoxyforskolin, cholera toxin, and DMSO. 8-Bromo cyclic AMP was from Semat Technical Ltd (St Albans, Hertfordshire) and ICI 118,551 (*erythro*-(±)-1-(7-methylindan-4-yloxy)-3-(isopropylaminobutan-2-ol) hydrochloride), zardaverine (6-(4-difluoromethyoxy-3-methoxyphenyl)-3[2H]-pyridazinone), SK&F 94120 (5-(4-acetamidophenyl)pyrazin-2(1H)-one, rolipram (**RS**-4-(3′-cyclopentyloxy-4′-methoxyphenyl)-2-pyrrolidone) were generously donated by Zeneca plc (Macclesfield, Cheshire), Byk-Gulden Pharmazeutica (Konstanz, Germany), SmithKline Beecham (Welwyn, Hertfordshire) and Schering GmbH (Berlin, Germany) respectively. Methyl-[^{3}H]-choline chloride (37 Ci mmol^{-1}) was purchased from Amersham International (Amersham, Buckinghamshire).

All drugs were made up daily and dissolved in distilled water except the following: isoprenaline (all dilutions in 10 mg ml^{-1} ascorbic acid): PGE_2 and rolipram (10 mM stock in 100% ethanol); forskolin, 1,9-dideoxyforskolin and zardaverine (10 mM stock in 100% DMSO); SK&F 94120 (10 mM stock in 0.1 M NaOH); indomethacin (made up at 1 mg ml^{-1} in phosphate buffer (in mM): KH_2PO_4 20, Na_2HPO_4 120, pH 7.8).

Statistical analysis

Data are expressed as mean ± standard error of the mean (s.e.mean) of n independent observations. Contractile responses are expressed as absolute values in mg tension generated before and after drug additions and then normalized as a percentage change. In all experiments each tissue acted as its own control and results obtained before and after drug treatment were compared by Wilcoxon's rank order test for paired data. EC_{50} values (concentration of drug required to elicit 50% of the maximal response) were calculated by using non-linear iterative regression with the 'PRISM' curve fitting programme (GraphPad Software, San Diego, CA, U.S.A.). The null hypothesis was rejected when $P<0.05$.

Results

Effect of isoprenaline on cholinergic contractile responses evoked by EFS in guinea-pig trachea

EFS (40 V, 0.5 ms, 4 Hz for 15 s every 4 min) of guinea-pig trachea evoked rapid cholinergic contractile responses. To evaluate the effects of isoprenaline (0.1 nM – 1 μM) on cholinergic neurotransmission, contractile responses evoked by EFS and by the addition of an equi-effective concentration of ACh (1 μM) were compared in the presence of isoprenaline. Pretreatment of guinea-pig tracheal strips for 15 min with isoprenaline produced a concentration-dependent inhibition of contractile responses evoked by EFS with an EC_{50} of 23 nM; complete inhibition was achieved at 1 μM isoprenaline (Figures 1a, 2). Isoprenaline also inhibited the contractile response evoked by the application of exogenous ACh (1 μM) with similar potency (EC_{50} = 19.9 nM) (Figure 1b and Figure 2).

Effects of isoprenaline on EFS-induced ACh release from cholinergic nerves innervating guinea-pig trachea

Pre-treatment of guinea-pig trachea with isoprenaline elicited a concentration-dependent facilitation of ACh output (Figure 3). However, the concentration-response curve which described this effect was bell-shaped: lower concentrations (0.3 to 3 μM) of isoprenaline markedly augmented cholinergic neurotransmission whereas at higher concentrations of the β-

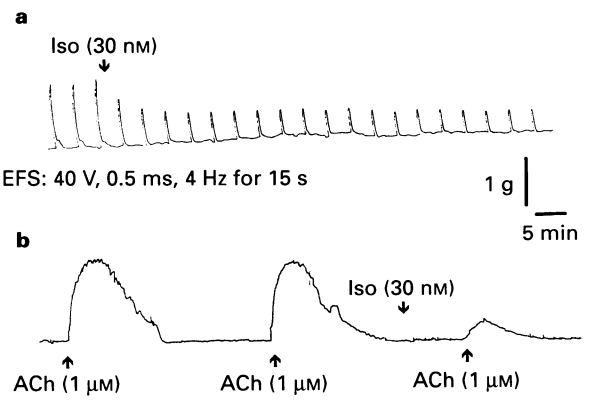

Figure 1 Representative trace illustrating the effect of isoprenaline (Iso, 30 nM) on (a) contractile responses evoked by EFS (40V, 0.5 ms pulse width, 4 Hz for 15 s) of cholinergic nerves and (b) on the application of exogenous ACh (1 vM) in guinea-pig tracheal strips.

adrenoceptor agonist, the facilitation of ACh release was diminished or absent (Figure 3). Thus, at 0.3 μM isoprenaline, EFS-induced ACh output was enhanced by 35.6±10% and 24.6±6.5% when administered 15 min and 30 min prior to the test EFS respectively (Figure 3); the vehicle for isoprenaline, ascorbic acid (10 mg ml^{-1}), had no significant effect on ACh release (0.19±4.9% inhibition, $n=5$, NS) under identical experimental conditions.

To establish the subtype of β-adrenoceptor mediating the facilitation of ACh release from cholinergic nerve terminals, tissues were pretreated with propranolol (0.1 μM) and ICI 118,551 (0.1 μM) for 15 min prior to the third EFS. As shown in Figures 4 and 5 both antagonists prevented the enhancement of ACh release by isoprenaline (1 μM) indicating that this phenomenon was mediated by adrenoceptors of the β_2-subtype. Neither propranolol nor ICI 118,551 affected the basal output of ACh.

Effects of isoprenaline on EFS-induced ACh release from cholinergic nerves innervating human trachea

In human tracheal strips, isoprenaline (0.1, 1 and 10 μM) administered 15 min prior to test EFS had no effect on ACh release (0.36±17.4% facilitation, $n=8$, NS, 4.5±13.4% facilitation, $n=6$, NS and 11.4±17.8% inhibition, $n=6$, NS respectively) when compared with the release of tritium from time-matched, vehicle (10 mg ml^{-1} ascorbic acid)-treated control tissues (11.3±19.3% inhibition, $n=6$, NS)

Effect of atropine on isoprenaline-induced enhancement of EFS-induced ACh release in guinea-pig trachea

The presence of muscarinic M_2 autoreceptors has been demonstrated on cholinergic nerve terminals innervating guinea-pig trachea (D'Agnostino *et al.*, 1990; Kilbinger *et al.*, 1991;

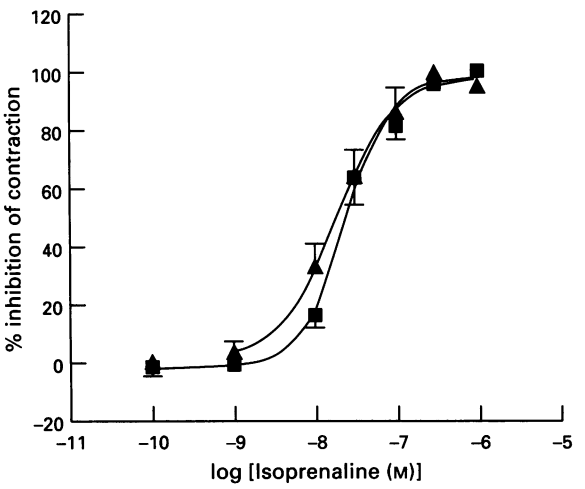

Figure 2 Effect of isoprenaline (0.1 nM–1 μM) on cholinergic contractile responses evoked by EFS (40V, 0.5 ms pulse width, 4 Hz for 15 s; ■) of guinea-pig tracheal strips and on contractile responses evoked by exogenous ACh (1 μM; ▲). Data points represent the mean±s.e.mean of four to six independent observations.

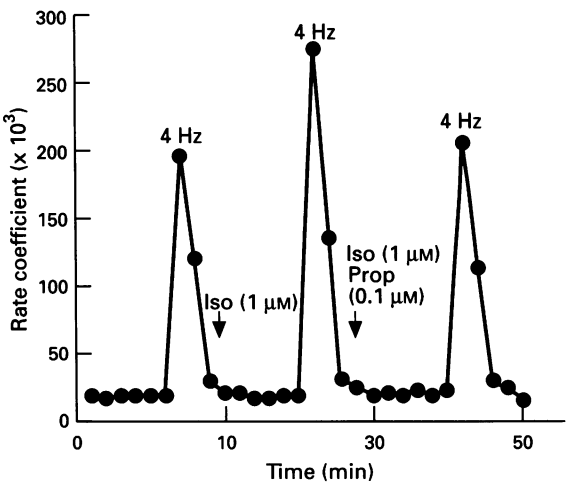

Figure 4 Facilitation by isoprenaline (Iso, 1 μM) of EFS (40V, 0.5 ms pulse width, 4 Hz for 1 min)-induced ACh release from an individual tracheal strip and antagonism of this response by propranolol (Prop, 0.1 μM). The results are expressed as a rate coefficient which is a measure of the fractional ^{3}H-release plotted against time.

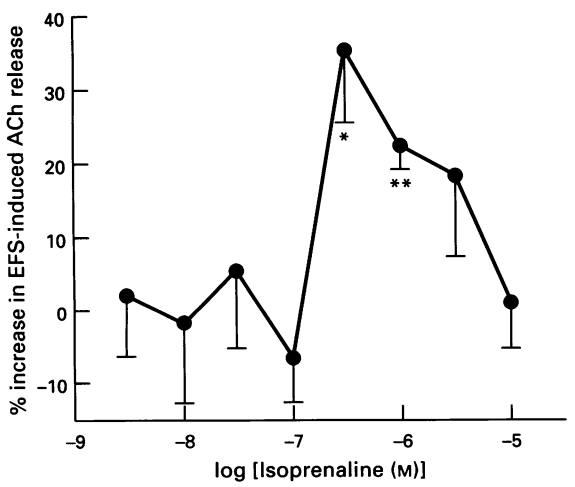

Figure 3 Concentration-response curve for isoprenaline (3 nM–10 μM; administered 15 min prior to the test stimulation) on ACh release evoked by EFS (40V, 0.5 ms pulse width, 4 Hz for 1 min) from guinea-pig trachea. Data points represent the mean±s.e.mean of six to eight independent determinations. *$P<0.05$, **$P<0.01$ compared with values preceding drug administration.

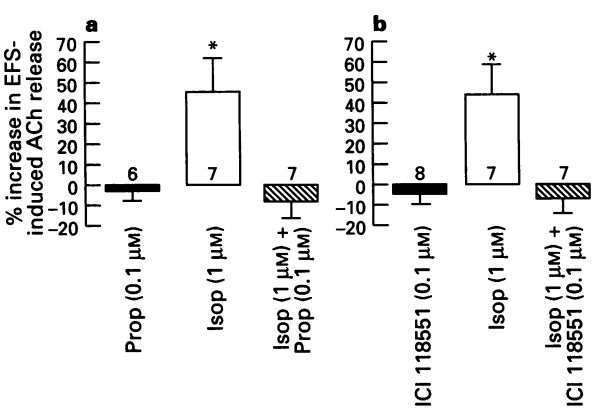

Figure 5 Antagonism by propranolol (0.1 μM; a) and the selective β_2-adrenoceptor antagonist, ICI 118 551 (0.1 μM; b) of the facilitation of isoprenaline-induced ACh release evoked by EFS (40V, 0.5 ms pulse width, 4 Hz for 1 min) from guinea-pig trachea. Each column shows the percentage enhancement of the response after drug administration compared to the first control stimulation and represents the mean of six to eight independent determinations. *$P<0.05$ compared with control values preceding drug administration.

Patel *et al.*, 1995). These pre-junctional muscarinic receptors can be activated by endogenous ACh under physiological conditions which results in a reduction of ACh output. Since isoprenaline failed to enhance ACh release detectably when relatively high concentrations (>3 μM) were used, it was reasoned that this may be due to the increased activation of these pre-junctional muscarinic M_2-autoreceptors (by the ACh released by isoprenaline) which would tend to oppose the increase in neurotransmitter output. To test this possibility the facilitatory effect of isoprenaline was investigated after M_2-autoreceptor blockade. Consistent with the established mechanism of action of M_2-autoreceptors, pretreatment of tracheal strips with atropine (0.1 μM) enhanced EFS-induced ACh release by 79.9±18.4% ($n=6$, $P<0.05$) compared to that released from vehicle-treated control tissues (Figure 6). In the presence of atropine, however, a high concentration (10 μM) of isoprenaline, which did not facilitate cholinergic neurotransmission under control conditions, similarly failed to augment ACh release (74.6±18.1% enhancement, $n=8$, NS). However, when a lower concentration of isoprenaline (1 μM) was examined ACh output was enhanced in an apparently supra-additive manner (171.9±19.7% enhancement, $n=6$, $P<0.05$) (Figure 6).

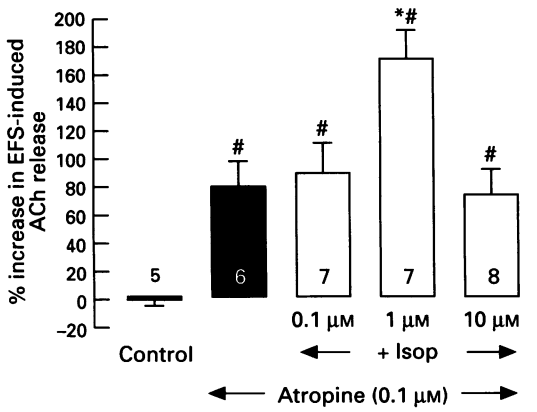

Figure 6 Effect of M_2-muscarinic autoreceptor blockade with atropine (0.1 μM) in the absence and presence of isoprenaline (0.1, 1, 10 μM) on ACh release from guinea-pig trachea evoked by EFS (40 V, 0.5 ms, 4 Hz for 1 min). Columns represent the mean±s.e.mean of five to eight independent determinations. *$P<0.05$ compared with control values preceding drug administration in the same tissue. #$P<0.05$ compared with vehicle control values.

Effects of other cyclic AMP elevating drugs on EFS-induced ACh release in guinea-pig trachea

In many cells and tissues β_2-adrenoceptor agonists elicit their functional effects by activating the cyclic AMP/cyclic AMP-dependent protein kinase cascade. To gain further information on the biochemical mechanism(s) by which isoprenaline enhanced cholinergic neurotransmission in guinea-pig trachea, the effect of a number of cyclic AMP-elevating drugs was evaluated on the release of [^{3}H]-ACh in response to EFS.

Pretreatment of guinea-pig tracheal strips with forskolin (10 μM for 15 min), a direct activator of adenylyl cyclase, significantly augmented (by 17.1±5.9%, $n=8$, $P<0.05$) the EFS-induced output of ACh from cholinergic nerves innervating guinea-pig trachea when compared to vehicle-treated tissues (Figure 7b). In contrast, 1,9-dideoxyforskolin (10 μM), an analogue of forskolin that does not activate adenylyl cyclase, did not significantly alter EFS-induced ACh release (19.8±8.7% inhibition, $n=7$, NS) (Figure 7b).

Consistent with the results obtained with forskolin, pretreatment of guinea-pig tracheal strips with the cyclic AMP analogue, 8-bromo-cyclic AMP (1 mM for 15 min) or cholera toxin (1 μg ml^{-1} for 2 h) similarly facilitated EFS-induced ACh output by 22.2±7.7% ($n=7$, $P<0.05$) and 46.5±15.5% ($n=8$, $P<0.05$) respectively compared to time-matched, vehicle-treated control tissues (Figure 7).

Intriguingly, pretreatment of guinea-pig trachea with PGE_2 (0.1 nM–1 μM for 15 min) evoked a concentration-dependent *inhibition* of EFS-induced ACh release. At the maximally effective concentration of PGE_2 (1 μM), EFS-induced ACh output was inhibited by 67.4±6.2% ($n=6$, $P<0.01$) with respect to the release of neurotransmitter from vehicle (0.1% v/v ethanol)-treated tissues (5.7±5.6% inhibition, $n=6$, NS; Figure 8).

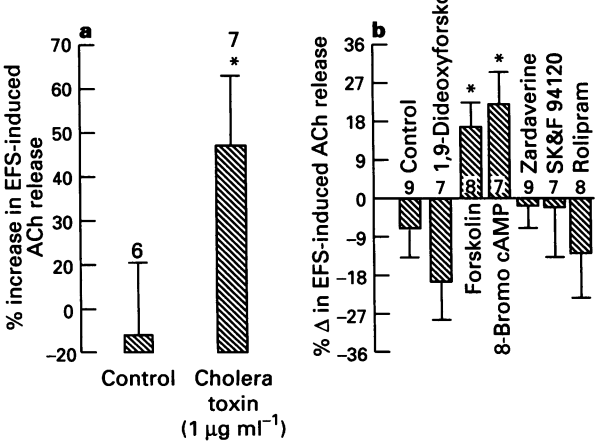

Figure 7 Effect of cholera toxin, forskolin, 1,9-dideoxyforskolin, zardaverine, SK&F 94120 and rolipram on EFS-induced ACh release from guinea-pig trachea. In (a), are shown the effects of the Gs activator, cholera toxin (1 μg ml^{-1}) and an equivalent volume of its vehicle, on [^{3}H]-ACh release evoked by EFS (40V, 0.5 ms pulse width, 4 Hz for 1 min) from guinea-pig trachea. In (b) the effect of forskolin (10 μM), 1,9-dideoxyforskolin (10 μM), 8-bromo-cAMP (1 mM), zardaverine (10 μM), SK&F 94120 (10 μM) and rolipram (10 μM) on EFS-induced ACh release is shown. Columns represent mean±s.e.mean of six to nine determinations. *$P<0.05$ compared with control values preceding drug administration in the same tissue.

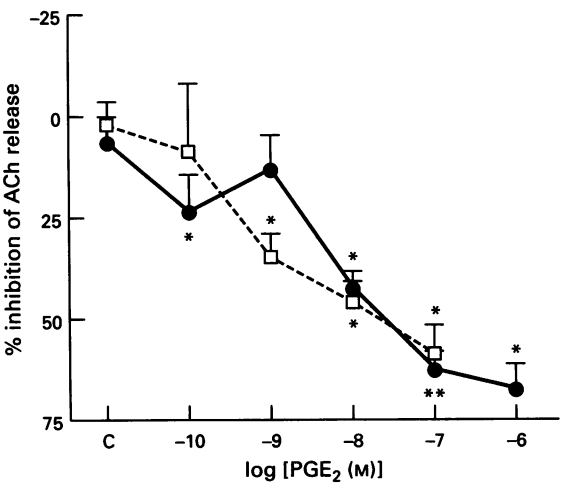

Figure 8 Effect of PGE_2 alone, and in combination with the phosphodiesterase inhibitor zardaverine, on EFS-induced ACh release from guinea-pig trachea. Concentration-response curves were constructed to PGE_2 (0.1 nM–1 μM) in the absence (●) and presence (□) of the mixed PDE3/PDE4 inhibitor zardaverine (10 μM), on ACh release evoked by EFS (40V, 0.5 ms, 4 Hz for 1 min) from guinea-pig trachea. C denotes the effect of the vehicle (0.1% ethanol which is the diluent for the highest concentration of PGE_2) on ACh release. Values represent mean±s.e.mean of six to nine determinations. *$P<0.05$, **$P<0.01$ compared with control values preceding drug administration in the same tissue.

Table 1 Effect of zardaverine, a dual inhibitor of PDE3 and PDE4, on the ability of isoprenaline to facilitate ACh release from parasympathetic nerves innervating guinea-pig trachea

	Isoprenaline (0.1 μM)	*Isoprenaline* (1 μM)	*Isoprenaline* (10 μM)
Vehicle	−6.55± 6.1%(11)	+22.4± 3.2%(9)**	+0.98± 6.4%(9)
Zardaverine (10 μM)	−3.60± 8.5%(8)	+29.1± 6.6%(10)*	+5.94± 5.7%(8)

Data represent the mean±s.e.mean of n independent determinations shown in parentheses.
*$P<0.05$; **$P<0.01$; significant facilitation in [^{3}H]ACh output

Zardaverine (10 μM for 15 min), a mixed inhibitor of the PDE3 and PDE4 isoenzyme families which selectively hydrolyse cyclic AMP (Galvan & Schudt, 1990), had no significant effect on EFS-evoked ACh release (Figure 8). Moreover, zardaverine (10 μM) did not potentiate the facilitatory effect of isoprenaline (0.1, 1 and 10 μM) or the inhibitory action of PGE_2 (0.1 nM to 1 μM) on cholinergic neurotransmission at any concentration studied (Table 1; Figure 8). Similarly, neither SK&F 94120 nor rolipram (each at 10 μM for 15 min) affected EFS-induced ACh release under identical experimental conditions (Figure 7b).

Discussion

In this paper an inhibitory action of the β-adrenoceptor agonist, isoprenaline, on cholinergic contractile responses evoked by EFS in guinea-pig trachea is documented. Although these data are in agreement, to some extent, with other studies reported in the literature a fundamental difference is apparent. Thus in many species (e.g. canine, feline, human airways) exogenous noradrenaline, isoprenaline and endogenously released catecholamines inhibit EFS-induced tension generation to a greater extent than an equivalent contraction induced by the application of exogenous ACh. Based upon these indirect data, most investigators have concluded that the reduction in EFS-induced contraction amplitude is due to a pre-junctional effect at the level of cholinergic nerve terminals (Vermeire & Vanhoutte, 1979; Danser *et al.*, 1987; Ito, 1988; Janssen & Daniel, 1990; Baker & Don, 1989; Aizawa *et al.*, 1991; Rhoden *et al.*, 1988). In this study, however, the EC_{50} value for inhibition of nerve-induced contractions was similar to that obtained for the suppression of contractile responses evoked by exogenous ACh applied directly to the tissue which may indicate that, in contrast to other species, isoprenaline inhibits EFS-induced contractile responses by interacting with β-adrenoceptors located *post-junctionally* on the airways smooth muscle. Furthermore, these results might suggest that there are no, functionally relevant, inhibitory β-adrenoceptors present on the vagus nerve endings that innervate guinea-pig trachea which would corroborate the results obtained in other studies which suggest that post-junctional β-adrenoceptors are responsible for the inhibition of EFS-evoked cholinergic contractions in guinea-pig airways (Kamikawa & Shimo, 1986; Martin *et al.*, 1993; Ten Berge *et al.*, 1995).

To elucidate unequivocally the role of β-adrenoceptor agonists in the control of cholinergic neurotransmission it is necessary to compare functional experiments with direct measurements of neurotransmitter release. Paradoxically, when the effect of isoprenaline on the output of ACh evoked by EFS was investigated, a significant (~30%) facilitation of neurotransmitter output was observed that was not predicted by the results obtained in functional experiments. The increase in ACh release following EFS was prevented in tissues pretreated with propranolol and ICI 118,551 indicating that this phenomenon was mediated through adrenoceptors of the β_2-subtype. The marked discrepancy between pre- and post-junctional measures of neurotransmitter release may indicate that despite the facilitation of neurotransmission, isoprenaline acts predominantly at a post-junctional level to prevent contraction of airways smooth muscle which masks the more subtle enhancement of ACh output. Alternatively, this paradox could equally reflect a modulatory action of isoprenaline on ACh release from nerves that synapse with effector cells other than airways smooth muscle such as submucosal glands and tracheobronchial blood vessels. A final methodological consideration that should not be excluded relates to differences in the electrode configuration used for the functional and neurotransmitter release experiments. Theoretically, this could result in the tissue being subjected to different currents and therefore the potential activation of varying numbers or populations of varicosities following EFS leading to a differential action of isoprenaline. In any event the ability of β-adrenoceptor agonists to facilitate cholinergic neurotransmission in parasympathetic nerves is entirely consistent with what is established in motor nerves (Wessler & Anschutz, 1988; Wessler *et al.*, 1990b).

Intriguingly, the concentration-response curve which described the effect of isoprenaline on cholinergic neurotransmission was bell-shaped; at concentrations of isoprenaline between 0.3 and 3 μM ACh output evoked by EFS was markedly augmented whereas at higher concentrations the effect was diminished or absent. One explanation for the inability of isoprenaline to increase EFS-induced ACh release when relatively high concentrations were employed is that the increased release of ACh evoked by isoprenaline may have activated the pre-junctional M_2-muscarinic autoreceptors which would lead subsequently to a reduction of ACh output (Kilbinger *et al.*, 1990; D'Agnostino *et al.*, 1991; Patel *et al.*, 1995). This possibility was excluded, however, since isoprenaline still elicited a bell-shaped concentration-response curve in atropine-treated tissues. A more likely explanation for these data is that isoprenaline rapidly desensitized the pre-junctional β-adrenoceptors which would be consistent with results obtained from experiments with motor nerves. Indeed, Wessler and colleagues (Wessler & Anschutz, 1988; Wessler *et al.*, 1990b) have reported that the enhancement of neuromuscular transmission at the rat phrenic nerve diaphragm by a relatively low concentration of isoprenaline prevents facilitation of ACh release when higher concentrations of the β-adrenoceptor agonist are studied.

The finding that isoprenaline facilitated EFS-evoked ACh release from guinea-pig trachea conflicts with data recently reported by other investigators. In particular, Wessler *et al.* (1994) found that isoprenaline *inhibited*, rather than enhanced, the EFS-induced output of ACh from parasympathetic nerves that innervate both guinea-pig and rat airways. Significantly, however, the inhibition of neurotransmitter output by isoprenaline was not apparent in epithelium-denuded preparations or when cyclo-oxygenase was inhibited (Wessler *et al.*, 1994). Since β-adrenoceptor agonists enhance phospholipase A_2 activity in guinea-pig lung (Blackwell *et al.*, 1978; Suzuki *et al.*, 1987) and generate PGD_2 and PGE_2 from airway epithelial cells, Wessler *et al.* (1994) concluded that stimulation of β-adrenoceptors on airway epithelial cells leads to the liberation of prostanoids which then act to inhibit EFS-evoked ACh release. This is a plausible theory and satisfactorily explains the differences between the two studies. Indeed, the inhibitory influence of endogenously released PGE_2 on the release of ACh is substantiated by our findings that exogenous PGE_2 evoked a concentration-dependent inhibition of cholinergic neurotransmission from guinea-pig trachea. These data are also in agreement with the inhibitory action of PGE_2 on EFS-evoked ACh release and on contractile responses elicited by EFS of cholinergic nerves in other species (Nakanishi *et al.*, 1978;

Jones *et al.*, 1980a; Walters *et al.*, 1984; Inoue *et al.*, 1984; Deckers *et al.*, 1989; DeLisle *et al.*, 1992) although a direct inhibitory effect of PGE_2 on smooth muscle is also likely to contribute to this response.

In contrast to the results obtained in guinea-pig airways, isoprenaline failed to affect detectably EFS-evoked ACh release from human trachea despite the fact that we and others have previously reported that EFS-induced cholinergic contractions are *more* potently inhibited by β-adrenoceptor agonists than are contractions evoked by exogenous ACh (Rhoden *et al.*, 1988; Aizawa *et al.*, 1991). The reason underlying the discrepancy between the mechanical and ACh release studies is unclear but may simply reflect a lack of functional pre-junctional β-adrenoceptors on parasympathetic nerves innervating human airways. If true, then these data imply changes in EFS-induced contractile responses in airways smooth muscle are an unreliable measure of neurotransmitter output. It is important to emphasize, however, that these data do not preclude the possibility that the methods we have employed are not sufficiently sensitive to detect changes in ACh output in human airways.

Given that β-adrenoceptor agonists activate adenylyl cyclase in essentially all tissues including the vagus nerve (Roch & Salamin, 1977), the possible involvement of cyclic AMP in isoprenaline-induced facilitation of ACh release from guinea-pig trachea was investigated. Evidence to support a role of cyclic AMP in this response was the finding that forskolin, cholera toxin and 8-bromo cyclic AMP each mimicked the effect of isoprenaline. These results are in complete agreement with the effect of cyclic AMP-elevating drugs on neuromuscular transmission (Breckenridge *et al.*, 1967; Goldberg & Singer, 1969; Wilson, 1974; Standaert & Dretchen, 1979; Hattori & Maehashi, 1987; Wessler & Anschutz, 1988; Dryden *et al.*, 1988; Wessler *et al.*, 1990b) and suggest that cyclic AMP may be intimately involved in regulating the biosynthesis, storage and exocytosis of ACh (Wilson, 1974). It is not yet known how an increase in cyclic AMP levels could lead to facilitation of ACh release but it has been suggested that phosphorylation of proteins associated with synaptic vesicles increases the availability of the transmitter for exocytotic release (Dryden *et al.*, 1988). Furthermore, these stimulating effects could also arise secondarily as a result of increases in intracellular calcium in response to an elevated level of cyclic AMP (Tomlinson *et al.*, 1985).

A particularly perplexing observation was the finding that PGE_2, which like isoprenaline stimulates cyclic AMP biosynthesis in the vagus (Roch & Salamin, 1977; Kalix, 1979), inhibited rather than potentiated the release of EFS-induced ACh release from cholinergic nerve terminals. The explanation for this unexpected finding remains elusive but indicates that PGE_2 and isoprenaline mediate their effects, at least in part, via the activation of different signal transduction pathways. It is similarly unclear how 1,9-dideoxyforskolin, which does not activate adenylyl cyclase, tended to decrease (albeit not statistically significantly) EFS-induced ACh output from cholinergic nerves although, in this case, it is tempting to speculate that its effects are mediated through inhibition of voltage-gated ion channels (Laurenza *et al.*, 1989).

If isoprenaline and/or PGE_2 modulate cholinergic neurotransmission via a receptor-mediated activation of adenylyl cyclase, then an inhibitor of the enzyme(s) which hydrolyses cyclic AMP should theoretically potentiate their effects. To assess this possibility the effect of zardaverine, a dual inhibitor of the PDE3 and PDE4 isoenzyme family which selectively hydrolyses cyclic AMP (Glavan & Schudt, 1990), was examined on the modulation of EFS-induced ACh release by isoprenaline and PGE_2. Surprisingly, zardaverine was inactive by itself and, furthermore, failed to interact with either agonist in a synergistic manner. A similar result was documented by Chiou & Chang (1988) who reported that the PDE4 inhibitor Ro 20-1724 did not modulate the quantal release of ACh from the phrenic nerve terminals innervating the mouse diaphragm. A number of explanations could account for these results. Perhaps the most plausible possibility is that representatives of PDE3 and PDE4 are absent from vagal cholinergic nerve varicosities in guinea-pig trachea and that cyclic AMP is either inactivated by another PDE isoenzyme family(s) or is simply extruded from the cell (Barber & Butcher, 1981). Support for this contention was the finding that neither SK&F 94120 nor rolipram enhanced EFS-induced ACh output at concentrations which significantly inhibit PDE3 and PDE4 respectively. Alternatively, a more heretical, although entirely feasible hypothesis given the opposing effects of isoprenaline and PGE_2 on EFS-induced ACh release, is that these agents may modulate cholinergic neurotransmission via cyclic AMP-independent mechanisms. Clearly, further studies are required to assess these possibilities.

In conclusion, the results of this study demonstrate that isoprenaline facilitates cholinergic neurotransmission in guinea-pig, but not human, trachea by interacting with pre-junctional β_2-adrenoceptors. The molecular mechanism(s) responsible for this effect is currently unclear but may involve the activation, at least in part, of the cyclic AMP/cyclic AMP-dependent protein kinase cascade. Finally, these data highlight the need to undertake direct measurements of neurotransmitter release when examining the effect of agents that are purported to act pre-junctionally.

M.G.B., H.J.P., T.T. and M.A.G. are supported by a Wellcome Trust Post-doctoral Fellowship, the National Asthma Campaign (UK), Fisons/Fujisawa (Japan) and the Medical Research Council (UK) respectively.

References

AIZAWA, H., INOUE, H., IKEDA, T., HIROSE, T. & ITO, Y. (1991). Effects of procaterol beta-2-adrenoceptor stimulant, on neuroeffector transmission in human bronchial tissue. *Respiration*, **58**, 3–166.

BAKER, D.G. & DON, U. (1987). Catecholamines abolish vagal but not acetycholine tone in the cat trachea. *J. Appl. Physiol.*, **63**, 2490–2498.

BARBER, R. & BUTCHER, R.W. (1981). The quantitative relationship between intracellular concentration and egress of cyclic AMP from cultured cells. *Mol. Pharmacol.*, **19**, 8–43.

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

BARNES, P.J. (1994). Modulation of neurotransmitter release from airways nerves. In *Airways Smooth Muscle: Structure, Innervation and Neurotransmission*. ed. Raeburn, D. & Giembycz, M.A. pp. 209–259. Basel: Birkhauser Verlag AG.

BELVISI, M.G., PATEL, H., TAKAHASI, T., BARNES, P.J. & GIEMBYCZ, M.A. (1994). β-Adrenoceptor agonists facilitate cholinergic neurotransmission in guinea-pig trachea. *Am. J. Respir. Crit. Care. Med.*, **149**, A480.

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.

BRECKENRIDGE, B.M., BURNS, J.H. & MATSCHINSKY, F.M. (1967). Theophylline, epinephrine and neostigmine facilitation of neuromuscular transmission. *Proc. Natl. Acad. Sci. U.S.A.*, **57**, 1893–1897.

CHIOU, L.C. & CHANG, C.C. (1988). A selective inhibitor of cyclic AMP phosphodiesterase, Ro 20-1724, has no effect on the quantal release of acetycholine from mouse phrenic nerve. *J. Pharm. Pharmacol.*, **40**, 148–149.

D'AGNOSTINO, G., CHIARI, M.C., GRANA, E., SUBISSI, A. & KILBINGER, H. (1990). Muscarinic inhibition of acetycholine release from a novel in vitro preparation of the guinea-pig trachea. *Naunyn-Schmied. Arch. Pharmacol.*, **342,** 141–145.

DANSER, A.H.J., VAN DER ENDE, R., LORENZ, R.R., FLAVAHAN, N.A. & VANHOUTTE, P.M. (1987). Pre-junctional β_1-adrenoceptors inhibit cholinergic transmission in canine bronchi. *J. Appl. Physiol.*, **62,** 785–790.

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.

DELISLE, S., BIGGS, D., WANG, A. & MARTIN, J.G. (1992). Effects of prostaglandin E_2 on ganglionic transmission in the guinea-pig trachea. *Respir. Physiol.*, **87,** 131–139.

DRYDEN, W.F., SINGH, Y.N., GORDON, T. & LAZARENKO, G. (1988). Pharmalogical elevation of cyclic AMP and transmitter release at the mouse neuromuscular junction. *Can. J. Physiol. Pharmacol.*, **66,** 207–212.

GALVAN, M. & SCHUDT, C. (1990). Actions of the phosphodiesterase inhibitor zardaverine on guinea-pig ventricular muscle. *Naunyn. Schmied. Arch. Pharmacol.*, **342,** 221–227.

GOLDBERG, A.L. & SINGER, J.J. (1969). Evidence for a role of cyclic AMP in neuromuscular transmission. *Proc. Natl. Acad. Sci. U.S.A.*, **64,** 134–141.

HATTORI T. & MAEHASHI, H. (1987). Facilitation of frog neuromuscular transmission by sodium fluoride. *Br. J. Pharmacol.*, **92,** 513–519.

INOUE, T., ITO, Y. & TAKEDA, K. (1984). Prostaglandin-induced inhibition of acetylcholine from neuronal elements of dog tracheal tissue. *J. Physiol.*, **349,** 553–570.

ITO, Y. (1988). Pre- and post-junctional actions of procaterol, a β_2-adrenoceptor stimulant, on dog tracheal tissue. *Br. J. Pharmacol.*, **95,** 268–274.

ITO, Y. & TAJIMA, K. (1982). Dual effects of catecholamines on pre- and post-junctional membranes in dog trachea. *Br. J. Pharmacol.*, **75,** 433–440.

JANSSEN, L.J. & DANIEL, E.E. (1990). Characterisation of the prejunctional beta adrenoceptors in canine bronchial smooth muscle. *J. Pharmacol. Exp. Ther.*, **254,** 741–749.

JONES, T.R., HAMILTON, J.T. & LEFCOE, N.M. (1980a). Pharmacologic modulations of cholinergic neurotransmission in guinea-pig trachea *in vitro*. *Can. J. Physiol. Pharmacol.*, **58,** 810–822.

JONES, T.R., KANNAN, M.S. & DANIEL, E.E. (1980b). Ultrastructural study of guinea-pig tracheal smooth muscle and its innervation. *Can. J. Physiol. Pharmacol.*, **58,** 974–983.

KALIX, P. (1979). Prostaglandin E_1 raises the cAMP content of peripheral nerve tissue. *Neurosci. Letts.*, **12,** 361–364.

KAMIKAWA, Y. & SHIMO, Y. (1986). Inhibitory effects of sympathomimetic drugs on cholinergically mediated contractions of guinea-pig isolated tracheal muscle. *J. Pharmac. Pharmacol.*, **38,** 742–747.

KILBINGER, H.R., SCHNIEDER, H., SIEFKEN, D., WOLF, D. & D'AGNOSTINO, G. (1991). Characterization of prejunctional muscarinic autoreceptors in the guinea-pig trachea. *Br. J. Pharmacol.*, **103,** 1757–1763.

LAURENZA, A., MCHUGH SUTKOWSKI, E. & SEAMON, K.B. (1989). Forskolin: a specific stimulator of adenylyl cyclase or a diterpene with multiple sites of action. *Trends Pharmacol. Sci.*, **10,** 442–447.

MANN, S.P. (1971). The innervation of mammalian bronchial smooth muscle, the localisation of catecholamines and cholinesterases. *Histochem.*, **3,** 319–331.

MARTIN, C.A., NALINE, E., MANARA, L. & ADVENIER, C. (1993). Effects of two β_3-adrenoceptor agonists, SR 58611 A and BRL 37344, and of salbutamol on cholinergic and NANC neural contraction in guinea-pig bronchi *in vitro*. *Br. J. Pharmacol.*, **110,** 1311–1316.

MARTIN, J.G. & COLLIER, B. (1986). Acetycholine release from canine isolated airway is not modulated by noradrenaline. *J. Appl. Physiol.*, **61,** 1025–1030.

NAKANISHI, H., YASHIDA, H. & SUZUKI, T. (1978). Inhibitory effects of prostaglandins on excitatory transmission in isolated canine tracheal muscle. *Jpn. J. Pharmacol.*, **28,** 883–889.

PATEL, H.J., BARNES, P.J., TAKAHASHI, T., TADJKARIMI, S., YACOUB, M.H. & BELVISI, M.G. (1995). Evidence for prejunctional muscarinic autoreceptors in human and guinea-pig trachea. *Am. J. Respir. Crit. Care. Med.*, **152,** 872–878.

RHODEN, K.J., MELDRUM, L.A. & BARNES, P.J. (1988). Inhibition of cholinergic neurotransmission in human airways by β_2-adrenoceptors. *J. Appl. Physiol.*, **65,** 700–705.

RICHARDSON, J. (1979). Nerve supply to the lung. *Am. Rev. Respir. Dis.*, **119,** 785–802.

RICHARDSON, J. & BELAND, J. (1976). Non-adrenergic inhibitory nervous system in human airways. *J. Appl. Physiol.*, **41,** 764–771.

ROCH, P. & SALAMIN, A. (1977). Effect of β-adrenergic drugs on adenosine 3',5'-monophosphate in rabbit vagus nerve. *J. Neurochem.*, **28,** 947–950.

STANDAERT, F.G. & DRETCHEN, K.L. (1979). Cyclic nucleotides and neuromuscular transmission. *Fed. Proc.*, **38,** 2183–2192.

SUZUKI, K., SUGIYAMA, S., TAKAGI, K., SATAKE, T. & OZAWA, T. (1987). The role of phospholipase in β-agonist-induced down regulation in guinea-pig lungs. *Biochem. Med. Metab. Biol.*, **37,** 157–166.

TEN BERGE, R.E.J. WEENING, E.C., ROFFEL, A.F. & ZAAGSMA, J. (1995). β_2- but not β_3-adrenoceptors mediate pre-junctional inhibition of non-adrenergic non-cholinergic contraction of guinea-pig main bronchi. *Eur. J. Pharmacol.*, **275,** 199–206.

TOMLINSON, S., MACNEIL, S. & BROWN, B.L. (1985). Calcium, cyclic AMP, and hormone action. *Clin. Endocrinol.*, **23,** 595–610.

VERMEIRE, P.A. & VANHOUTTE, P.M. (1979). Inhibitory effects of catecholamines in isolated canine bronchial smooth muscle. *J. Appl. Physiol.*, **46,** 787–791.

WALTERS, E.H., O'BYRNE, P.M., FABBRI, L.M., GRAF, P.D., HOLTZMAN, M.J. & NADEL, J.A. (1984). Control of neurotransmission by prostaglandins in canine trachealis smooth muscle. *J. Appl. Pharmacol.* **57,** 129–184.

WARD, J.K., BELVISI, M.G., FOX, A.J., TADJKARIMI, S., YACOUB, M.H. & BARNES, P.J. (1993). Modulation of cholinergic neural bronchoconstriction by endogenous nitric oxide and vasoactive intestinal peptide in human airways *in vitro*. *J. Clin. Invest.*, **92,** 736–743.

WESSLER, I. & ANSCHUTZ, S. (1988). β-Adrenoceptor stimulation enhances transmitter output from the rat phrenic nerve. *Br. J. Pharmacol.*, **94,** 669–674.

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

WESSLER, I., HOLZER, G. & SZARMA, E. (1990b). Stimulation of β_1-adrenoceptors enhances electrically evoked [^{3}H]acetylcholine release from rat phrenic nerve. *Clin. Pharmacol. Physiol.*, **17,** 23–32.

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

WILSON, D.F. (1974). The effects of dibutyryl cyclic adenosine 3,5-monophosphate, theophylline and aminophylline on neuromuscular transmission in the rat. *J. Pharmacol. Exp. Ther.*, **188,** 447–452.

(Received September 19, 1995
Revised November 21, 1995
Accepted December 6, 1995)