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Inhibition by endothelin-1 of cholinergic nerve-mediated acetylcholine release and contraction in sheep isolated trachea

[1]Peter J. Henry, *Albert Shen, *Fred Mitchelson & Roy G. Goldie

Department of Pharmacology, University of Western Australia, Nedlands, Western Australia, 6009, Australia and *Department of Pharmaceutical Biology and Pharmacology, Victorian College of Pharmacy (Monash University), 381 Royal Parade, Parkville, Victoria, 3052, Australia

1 The relative roles of ET_A and ET_B receptor activation on cholinergic nerve-mediated contraction and acetylcholine (ACh) release were examined in sheep isolated tracheal smooth muscle.

2 Electrical field stimulation (EFS; 90 V, 0.5 ms duration, 1 Hz, 10 s train) applied to sheep isolated tracheal smooth muscle strips induced monophasic contractile responses that were abolished by either 1 μM tetrodotoxin or 0.1 μM atropine, but were insensitive to 10 μM hexamethonium and 100 μM L-NAME. Thus, EFS-induced contractions resulted from the spasmogenic actions of ACh released from parasympathetic, postganglionic nerves.

3 As expected, sheep isolated tracheal smooth muscle preparations did not contract in response to the ET_B receptor-selective agonist, sarafotoxin S6c (0.1 - 100 nM). However, sarafotoxin S6c caused a concentration-dependent and transient inhibition of EFS-induced contractions. The inhibitory effect induced by a maximally effective concentration of sarafotoxin S6c (10 nM; $72.1 \pm 5.7\%$, $n=6$) was abolished in the presence of the ET_B receptor-selective antagonist BQ-788 (1 μM). Contractile responses to exogenously administered ACh (10 nM - 0.3 mM) were not inhibited by sarafotoxin S6c (1 or 10 nM; $n=7$).

4 In contrast to sarafotoxin S6c, endothelin-1 induced marked contractions in sheep isolated tracheal smooth muscle. These contractions were inhibited by BQ-123, consistent with an ET_A receptor-mediated response. In the presence of BQ-123 (3 μM), endothelin-1 produced a concentration-dependent inhibition of EFS-induced contractions (30 nM endothelin-1, $68.9 \pm 10.2\%$ inhibition, $n=5$). These responses were inhibited by 1 μM BQ-788, indicative of an ET_B receptor-mediated process. Endothelin-1 was about 3 fold less potent than sarafotoxin S6c.

5 EFS (90 V, 0.5 ms duration, 1 Hz, 15 min train) induced the release of endogenous ACh (1.94 ± 0.28 pmol mg^{-1} tissue, $n=12$), as assayed by h.p.l.c. with electrochemical detection. EFS-induced release of ACh was inhibited to a similar extent by 100 nM endothelin-1 ($47 \pm 4\%$, $n=9$) and 10 nM sarafotoxin S6c ($46 \pm 9\%$, $n=3$). These effects of endothelin-1 on ACh release were inhibited by 1 μM BQ-788 alone ($n=4$), by BQ-788 in the presence of 3 μM BQ-123 ($n=4$), but not by 3 μM BQ-123 alone ($n=5$).

6 In summary, sheep isolated tracheal smooth muscle contains two anatomically and functionally distinct endothelin receptor populations. ET_A receptors located on airway smooth muscle mediate contraction, whereas ET_B receptors appear to exist on cholinergic nerves that innervate tracheal smooth muscle cells and mediate inhibition of ACh release. The inhibitory effect of ET_B receptor stimulation on cholinergic neurotransmission is in stark contrast to the enhancing effects hitherto described in the airways.

Keywords: Endothelin-1; sarafotoxin S6c; endothelin receptors; electrical field stimulation; acetylcholine release; cholinergic nerves; airway smooth muscle (sheep)

Introduction

Vagal cholinergic nerves innervate airway smooth muscle in many mammalian species and airway regions, and play a predominant role in the control of airways tone. Stimulation of these nerves leads to the release of acetylcholine (ACh) from postganglionic cholinergic nerves which, via activation of M_3 cholinoceptors, induces airway smooth muscle contraction. The release of ACh can be modulated by many endogenous substances. Indeed, ACh inhibits its own release by activating prejunctional M_2 and possibly M_4 cholinoceptors (Fryer & Maclagan, 1984; Kilbinger *et al.*, 1995). Similarly, the contractile responses to cholinergic nerve stimulation can be inhibited by β_2-adrenoceptor agonists (Ito, 1988; Rhoden *et al.*, 1988; Aizawa *et al.*, 1991), histamine (Ichinose & Barnes, 1989), prostaglandin E_2 (Shore *et al.*, 1987), vasoactive intestinal peptide (Ellis & Farmer, 1989; Stretton *et al.*, 1991; Aizawa *et al.*, 1994) and opioid peptides (Belvisi *et al.*, 1990; 1992). These decreased responses are often attributed to the activation of specific prejunctional receptors and to decreased ACh release, although β-adrenoceptor agonists may in fact enhance ACh release (Zhang *et al.*, 1995a,b). In contrast, various autacoids and inflammatory mediators including 5-hydroxytryptamine (Van Oosterhout *et al.*, 1991), thromboxane A_2 (Serio & Daniel, 1988) and substance P (Chung *et al.*, 1985; Tanaka & Grunstein, 1986) can enhance cholinergic nerve-mediated contractions, purportedly via a mechanism involving the increased release of ACh. Although the precise mechanisms underlying agonist-induced modulation of ACh release are not well understood, these processes appear to have an important role for the neurogenic control of airway smooth muscle tone (Barnes, 1992).

There is a growing body of evidence indicating that cholinergic neurotransmission is also enhanced by endothelin-1 and

[1] Author for correspondence.

related peptides. McKay and coworkers (1993) reported that endothelin-3 augmented the contractile response to electrical field stimulation (EFS) of postganglionic cholinergic nerves in rabbit isolated bronchus. Endothelin-1 and related peptides, via activation of ET_B receptors, have also been demonstrated to enhance cholinergic nerve-mediated contractions in murine airways (Henry & Goldie, 1995). In both studies, indirect evidence was provided to indicate that the enhanced responses resulted from the activation of prejunctional, rather than postjunctional endothelin receptors. These conclusions are consistent with autoradiographic studies in primary cultures of guinea-pig tracheal smooth muscle demonstrating the existence of endothelin receptors on the cell bodies, axonal processes and varicosities of cholinergic and adrenergic intramural neurones (Takimoto *et al.*, 1993). Activation of these receptors induced a tetrodotoxin-sensitive increase in intracellular calcium and contraction of neighbouring smooth muscle cells. Clearly, if similar mechanisms exist in the human respiratory tract, the elevated levels of endothelin-1 observed in asthmatic airways (Springall *et al.*, 1991; Redington *et al.*, 1995) may contribute to bronchoconstriction through at least two different mechanisms, namely direct contraction of airway smooth muscle and facilitation of cholinergic neurotransmission.

Functional studies investigating the influence of endothelin-1 and related peptides on cholinergic nerve-mediated contractions have been somewhat complicated by the dual effects of endothelin-1 on airway smooth muscle tone and cholinergic nerve function (McKay *et al.*, 1993; Henry & Goldie, 1995). Endothelin-1 is a potent spasmogen of airway smooth muscle *per se*, and thus it is not always possible to separate clearly the neuromodulatory effects of endothelin-1 on EFS-induced contractions from the underlying endothelin-1-induced tone. Similar problems have been encountered with other agents such as substance P (Tanaka & Grunstein, 1986), thromboxane A_2 (Serio & Daniel, 1988) and prostaglandin D_2 (Tamaoki *et al.*, 1987) which, like endothelin-1, possess both spasmogenic and neuromodulatory actions in the airways. In an effort to characterize the ET_B receptor-mediated, neuromodulatory effects of endothelin-1 on cholinergic nerve function in isolation from its direct effects on airway smooth muscle tone, the current study was performed in sheep tracheal smooth muscle in which endothelin-1-induced contractions are mediated via activation of ET_A but not ET_B receptors (Goldie *et al.*, 1994). Furthermore, the influence of endothelin-1 and related peptides on cholinergic nerve function was assessed directly by measuring the effects of these peptides on the release of ACh from electrically-stimulated nerves (Shen *et al.*, 1995).

Methods

Contraction studies

Tracheae were obtained at a local abattoir from freshly slaughtered lambs (4–9 months of age) and transferred to our laboratory in ice-cold Krebs-bicarbonate solution (KBS). The composition of the KBS was in mM: NaCl 117, KCl 5.36, $NaHCO_3$ 25, KH_2PO_4 1.03, $MgSO_4.7H_2O$ 0.57, $CaCl_2$ 2.5, glucose 11.1, indomethacin 0.0025 and propranolol 0.001. For contraction studies, longitudinal strips of epithelium-denuded tracheal smooth muscle were prepared as previously described (Goldie *et al.*, 1994). Strips were suspended under 500 mg weight tension in 3 ml organ baths containing KBS at 37°C, bubbled with 5% CO_2 in O_2. Two platinum electrodes were positioned 4–5 mm apart on either side of the tracheal strip for application of electrical field stimulation (EFS) as required. Following a 45 min equilibration period, during which time the KBS was replaced at 15 min intervals, preparations were exposed to the depolarizing spasmogen KCl, at a final bath concentration of 40 mM KCl in KBS. Upon reaching a steady level of tension, preparations were washed repeatedly with KBS and rested for a further 30 min. Experiments were then performed to establish the effects of endothelin-1 and related peptides on cholinergic nerve-mediated responses in these preparations.

First, experiments were performed to establish whether EFS-induced contractions originated from the actions of ACh released from postganglionic, cholinergic nerves. Baseline EFS-induced contractions (90 V, 0.5 ms duration, 1 Hz) were recorded as 10 s trains every 3 min until three consecutive, reproducible contractions had been obtained (3–5 trains). EFS-induced responses were then monitored for a further 15 min in the absence (time control) or presence of tetrodotoxin (1 μM), atropine (0.1 μM), hexamethonium (10 μM) or Nω-nitro-L-arginine methyl ester L-NAME, 100 μM).

Secondly, the influence of ET_B receptor stimulation on cholinergic nerve-mediated contractions was examined by use of various endothelin receptor agonists and antagonists. In these experiments, baseline EFS-induced contractions were obtained at 3 min intervals (as described above) and then monitored for a further 30 min in the absence (time control) or presence of an ET_B receptor-selective agonist, sarafotoxin S6c (0.1, 1, 10 or 100 nM). Likewise, the influence of ET_B receptor activation on EFS-induced contractions was assessed by examining the effects of endothelin-1 in the presence of an ET_A receptor-selective antagonist, BQ-123. In these experiments, baseline EFS-induced responses were obtained and the preparations were then incubated for 15 min with 3 μM BQ-123 prior to a further 30 min incubation with endothelin-1 (1, 3, 10 or 30 nM) in the continued presence of BQ-123. EFS-induced responses were monitored at 3 min intervals throughout the procedure. To confirm that any modulation of these effects resulted from the activation of ET_B receptors, the experiments were repeated in the presence of an ET_B receptor-selective antagonist, BQ-788 (1 μM for 15 min prior to addition of 30 nM endothelin-1 or 10 nM sarafotoxin S6c).

Finally, the influence of ET_B receptor activation on postjunctional responses to ACh was examined. Preparations were incubated for 5 min with sarafotoxin S6c (1 or 10 nM). A cumulative concentration-effect curve to ACh (10 nM–0.3 mM, 0.5 log-concentration increments) was then completed in the uninterrupted presence of sarafotoxin S6c. In all of these experiments, each preparation was exposed to a single concentration of endothelin-1 or sarafotoxin S6c.

ACh release studies

Epithelium-denuded strips of tracheal smooth muscle (10 mm long × 4 mm wide) were obtained from sheep trachea. The ends were connected with cotton thread to form a ring preparation, which was suspended under 2 g tension in a tissue bath containing 1.5 ml KBS at 37°C bubbled with 5% CO_2 in O_2. Following a 45 min equilibration period, the viability of the preparations was tested by the addition of 40 mM KCl (usual response 50–70 g tension). Preparations were washed and a series of standard EFS responses obtained (90 V, 1 Hz, 0.5 ms duration, 10 s train every 3 min for 10–15 min). After a 15 min wash period, preparations were washed three times (over 30 s) with a modified KBS (termed KBS+) that contained 10 μM physostigmine (to inhibit the breakdown of released ACh by acetylcholinesterases) and 1 μM atropine (to inhibit activation of prejunctional auto-inhibitory cholinoceptors) and incubated for a further 15 min in KBS+. At the end of this period, the bath contents were discarded and the preparations washed 3 times with KBS+ over 30 s. EFS (90 V, 1 Hz, 0.5 ms, continuous train) was then applied to the preparation for 15 min. At the completion of this first stimulation period, the bath contents were collected (S1) and replaced with KBS+. Following a 15 min rest period, preparations were washed 3 times with KBS+ and 15 min later, the bath contents were collected (no EFS, basal ACh release, C) and the preparations washed and rested for another 15 min in KBS+. In control experiments, preparations were washed 3 times over 30 s, EFS applied as for S1 for a final 15 min period and the bath contents collected (S2). Thus, during the course of the experiment 3 × 1.5 ml samples were

collected from each preparation (S1, C, S2). Experiments were completed in duplicate (neighbouring tissue baths) and the samples combined to give 3 × 3 ml samples from which ACh was extracted and assayed, as described below. To examine the effect of sarafotoxin S6c or endothelin-1 on ACh release, a peptide was added 2 min prior to the start of the second period of EFS (S2). In selected studies, endothelin receptor antagonists, BQ-123 and/or BQ-788 were administered 10 min prior to the agonist (endothelin-1 or sarafotoxin S6c) and were present throughout S2. Collected samples were frozen (−20°C) until assayed. Tissues were blotted dry and weighed.

ACh and choline were extracted from the samples by the method of Goldberg & McCaman (1974). Briefly, each 3 ml sample was added to 1.5 ml of tetraphenylboron in 3-heptanone (10 mg ml^{-1}), vortexed and centrifuged. A 0.5 ml aliquot of the upper organic phase was mixed with an equal volume of 0.4 M HCl. After further vortexing and centrifugation, 0.4 ml of the acid phase was evaporated to dryness and stored at −20°C until assayed.

ACh was assayed by h.p.l.c. with electrochemical detection. The h.p.l.c. apparatus consisted of a Bioanalytical Systems (BAS) CC4 liquid chromatograph with a BAS ACh/choline assay kit (MF-8910) containing two cartridges which consisted of a polymeric analytical column followed by an immobilised enzyme reactor column. The columns were stationed in an oven maintained at 28°C by a BAS LC-22A temperature controller. The apparatus was coupled to a BAS PM-60 pump set at a flow rate of 1 ml min^{-1}.

Dried samples were reconstituted in 125 μl mobile phase and 50 μl was injected onto the column via a Rheodyne 7125 injection valve fitted with a 20 μl sample loop. A BAS LC-4B amperometric detector containing a Ag/AgCl reference electrode and platinum electrode set at +500 mV was used. Mobile phase consisted of 0.025 M $NaHPO_4$ in h.p.l.c. grade water at pH of 8.5. Kathon was added as a bacteriostatic.

Data analysis

Contractile responses to EFS, ACh and peptides were expressed as a percentage of the response obtained to 40 mM KCl at the beginning of the experiment. Agonist-induced effects on EFS-mediated contractions were compared to those induced by vehicle in time-matched preparations taken from the same animal. Thus, in time-control (C) and test (T) preparations, the mean of the three initial standard EFS-induced contractions was estimated (C_0, T_0) and subtracted from the subsequent EFS-induced contractions (C_t, T_t) produced at three min intervals over the 30 min period. Thus, at each of these time-points, changes in the magnitude of EFS-induced contractions induced as a function of time and by an endothelin receptor agonist in a test preparation ($\Delta T = T_t - T_0$) and by time alone in a control preparation ($\Delta C = C_t - C_0$) were estimated. Time-related changes in EFS responses were subtracted ($\Delta T_{cor} = \Delta T - \Delta C$) to isolate agonist-induced changes at each time-point. Agonist-induced changes were expressed as mean $\Delta T_{cor} \pm$ s.e.mean of n different preparations. The time-related changes in EFS-induced contractile responses (ΔC) were small and consistent; an increase of 5.2 ± 2.8% was observed at 15 min and of 10.0 ± 3.9% at 30 min ($n = 26$ control preparations).

ACh release is expressed as pmol mg $tissue^{-1}$ or as a ratio of S2/S1, as indicated. Grouped data are expressed as mean ± s.e. mean. Differences between means were assessed by a one-way ANOVA. The Student-Newman-Keuls method was used when multiple comparisons were made (SigmaStat). Probability (P) values of less than 0.05 were considered to be statistically significant.

Drugs

Endothelin-1, sarafotoxin S6c and BQ-123 (cyclo[D-Trp-D-Asp-L-Pro-D-Val-L-Leu]) were purchased from Auspep (Melbourne, Australia). BQ-788 (N-cis-2,6-dimethylpiperidinocarbonyl-L-γ-methyleucyl-D-methoxycarbonyltryptophanyl-D-norleucine) was a generous gift from the Banyu Pharmaceutical Co. Ltd (Tsukuba, Japan). Endothelin-1 and sarafotoxin S6c were stored as 50 μM solutions in 0.1 M acetic acid at −20°C. BQ-123 was stored as a 10 μM solution in 100 mM Na_2CO_3 at −20°C. BQ-788 was stored as 1 mM solutions in 10% dimethylsulphoxide (DMSO). Other drugs used were acetylcholine chloride, propranolol hydrochloride, physostigmine salicylate, indomethacin, tetrodotoxin, hexamethonium chloride and N^{ω}-nitro-L-arginine methyl ester hydrochloride (L-NAME) (Sigma Chemical Co, St Louis, U.S.A.), and atropine sulphate monohydrate (Fluka AG). All dilutions were made in saline, stored on ice and protected from light.

Results

Contractile studies

As illustrated in Figure 1a, EFS applied to sheep isolated tracheal smooth muscle induced a monophasic contractile response. The magnitude of the standard EFS-induced contraction (90 V, 0.5 ms, 1 Hz, 10 s train) was 49.6 ± 5.6% of the response to 40 mM KCl ($n = 21$). EFS-induced contractions were significantly inhibited by 0.1 μM atropine (97.5 ± 1.0% reduction in contraction by 15 min, $n = 5$, $P < 0.01$; Figure 1b) and by 1 μM tetrodotoxin (97.4 ± 2.7% reduction in contraction by 15 min, $n = 5$, $P < 0.01$; Figure 1c), but were not significantly affected by either 10 μM hexamethonium (4.5 ± 8.8% increase, $n = 5$) or 100 μM L-NAME (2.3 ± 5.5% decrease, $n = 5$) (data not shown).

The ET_B receptor-selective agonist, sarafotoxin S6c, did not alter baseline tone (Figure 1d) but markedly inhibited EFS-induced contractions (Figures 1d and 2). The inhibitory effects induced by 10 nM sarafotoxin S6c were transient; peak in-

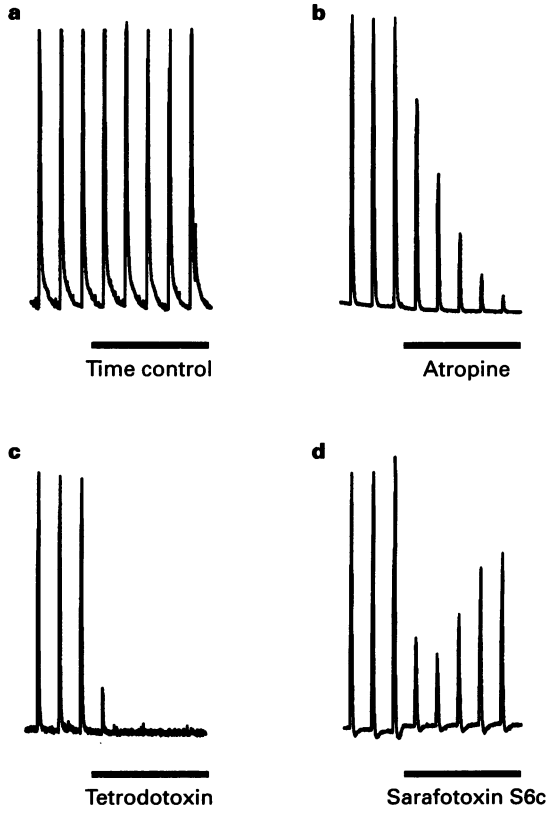

Figure 1 Representative isometric tension recordings of contractions in sheep isolated tracheal smooth muscle preparations induced by EFS (90 V, 0.5 ms, 1 Hz, 10 s trains) applied at 3 min intervals in the absence (a, time control) and presence of (b) 0.1 μM atropine, (c) 1 μM tetrodotoxin and (d) 10 nM sarafotoxin S6c. Similar recordings were observed in at least four other experiments.

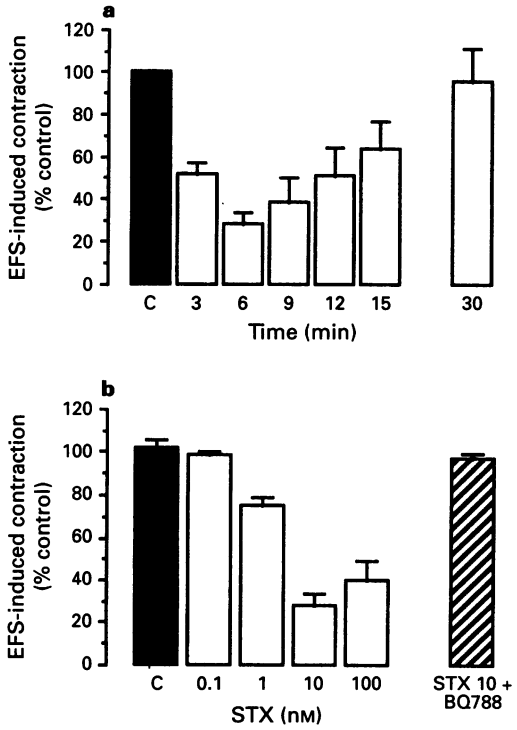

Figure 2 (a) EFS-induced responses in sheep isolated tracheal smooth muscle preparations obtained prior to (C, control) and at various times following the administration of 10 nM sarafotoxin S6c. Data shown are the mean ± s.e.mean of 6 separate experiments. (b) Concentration-dependent inhibition of EFS-induced responses produced by sarafotoxin S6c (STX) and prevention of responses by the ET_B receptor antagonist, BQ-788 (1 μM). EFS-induced responses were obtained 6 min after addition of sarafotoxin S6c (maximum effect) and represent the mean ± s.e.mean of 6 separate experiments.

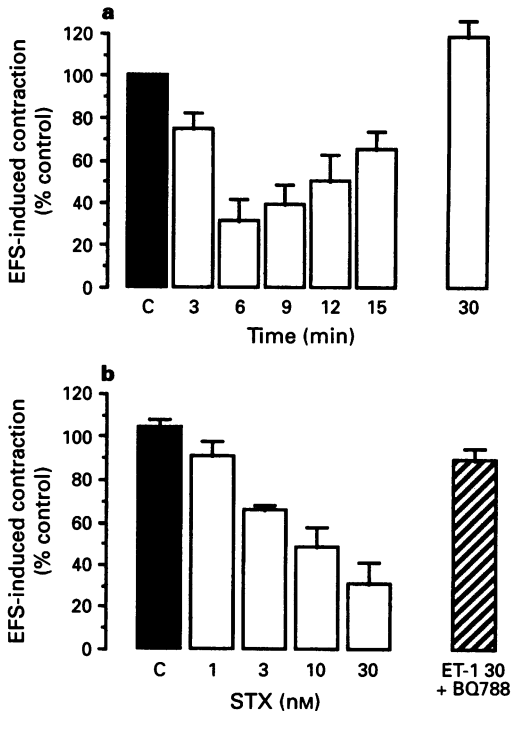

Figure 3 (a) EFS-induced responses in sheep isolated tracheal smooth muscle preparations obtained prior to (C, control) and at various times following the administration of 30 nM endothelin-1. Data shown is the mean ± s.e.mean of 5 separate experiments. (b) Concentration-dependent inhibition of EFS-induced responses produced by endothelin-1 (in the uninterrupted presence of BQ-123) and prevention of these responses by the ET_B receptor antagonist, BQ-788 (1 μM). EFS-induced responses were obtained 6 min after addition of endothelin-1 (maximum effect) and represent the mean ± s.e.mean of 5 separate experiments.

hibitory responses were observed at 6 min (72.1 ± 5.7% inhibition, $n=6$) and by 30 min contractile responses were not significantly lower than control values (4.4 ± 15.5% inhibition, $n=6$; Figure 2a). In addition to this time-dependency, the inhibitory effects produced by sarafotoxin S6c were concentration-dependent (Figure 2b). Moreover, the effects produced by a maximally effective concentration of sarafotoxin S6c (10 nM) were abolished by the ET_B receptor-selective antagonist, BQ-788 (1 μM, Figure 2b).

As expected (Goldie *et al.*, 1994), endothelin-1 induced potent and concentration-dependent contractions of sheep isolated tracheal smooth muscle (30 nM endothelin-1 produced 73.2 ± 4.1% of the contractile response to 40 mM KCl, $n=4$). However, in the presence of the ET_A receptor-selective antagonist, BQ-123 (3 μM), endothelin-1 did not induce any significant contraction (30 nM, 2.3 ± 1.6%, $n=5$). In the presence of BQ-123, endothelin-1 produced a transient, concentration-dependent inhibition of EFS-induced contractions (Figure 3). The profile of inhibition produced by endothelin-1 was similar to that of sarafotoxin S6c, although endothelin-1 was approximately 3 fold less potent. The inhibitory effects produced by 30 nM endothelin-1 (68.9 ± 10.2%, $n=5$) were significantly reduced by the ET_B receptor-selective antagonist, BQ-788 (1 μM; 10.5 ± 5.3%, $n=5$; Figure 3b). BQ-788 (1 μM) had no effect on the magnitude of EFS-induced contractions *per se* ($n=5$, data not shown).

Exogenously applied ACh induced marked concentration-dependent contractions in sheep isolated tracheal smooth muscle (Figure 4). Despite its marked inhibitory effects on the ACh-mediated contractions induced by EFS (see Figure 2b), sarafotoxin S6c (1 or 10 nM) did not inhibit the contractions induced by exogenously applied ACh (Figure 4, $n=7$).

ACh release studies

As expected, the application of a 15 min period of EFS (90 V, 1 Hz, 0.5 ms) was associated with significantly enhanced ACh release (S1; 1.94 ± 0.28 pmol mg^{-1} tissue, $n=12$) above basal ACh release (C, 0.06 ± 0.03 pmol mg^{-1} tissue, $P<0.01$). In control preparations, similarly elevated levels of ACh release were detected following a second period of EFS (S2; 2.11 ± 0.28 pmol mg^{-1} tissue; ratio of S2/S1, 1.08 ± 0.05, $n=12$). Incubation with tetrodotoxin (1 μM) for 15 min prior to, and also during S2 abolished ACh release (ratio S2/S1, 0.04 ± 0.04, $n=3$), indicating that EFS-induced ACh release was neural in origin (Figure 5a).

As shown in Figure 5a, ACh release was significantly attenuated by endothelin-1. Application of 100 nM endothelin-1 immediately prior to the second stimulation period (S2) resulted in a 47 ± 4% reduction in the S2/S1 ratio (from 1.08 ± 0.05 in control preparations to 0.57 ± 0.04 in the presence of endothelin-1, $n=9$, $P<0.01$). A similar 46 ± 9% reduction in S2/S1 ratio was observed in the presence of 10 nM sarafotoxin S6c (S2/S1 reduced to 0.58 ± 0.09, $n=3$, Figure 5a).

The effects of 100 nM endothelin-1 on ACh release were inhibited by the ET_B receptor antagonist, BQ-788 (1 μM; $n=4$, $P<0.05$), but not by the ET_A receptor antagonist, BQ-123 (3 μM; $n=5$, Figure 5b). The inhibitory effects observed in the combined presence of BQ-788 and BQ-123 ($n=4$, $P<0.05$) were not significantly different from those produced in the presence of BQ-788 alone (Figure 5b). The effects of 10 nM sarafotoxin S6c on ACh release were significantly inhibited in the presence of BQ-788 (ratio S2/S1, 0.90 ± 0.04, $n=3$, $P<0.05$) (data not shown).

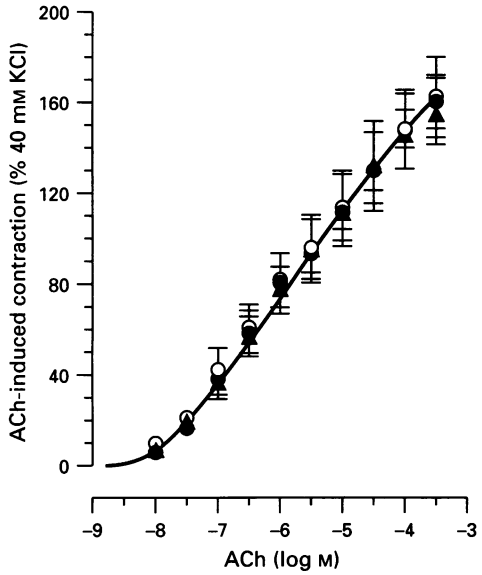

Figure 4 Cumulative concentration-effect curves obtained to exogenously administered ACh in the absence (○) and presence of 1 nM (●) or 10 nM (▲) sarafotoxin S6c. Data shown are the mean ± s.e. mean of 7 separate experiments.

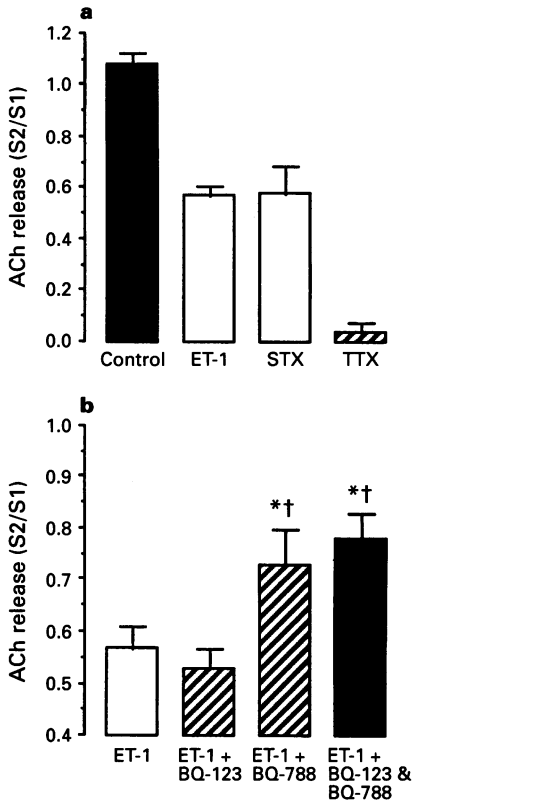

Figure 5 (a) Inhibitory effects of endothelin-1 (ET-1), sarafotoxin S6c (STX) and tetrodotoxin (TTX) on EFS-induced ACh release from sheep isolated tracheal smooth muscle preparations. The ratio (S2/S1) expresses the amount of ACh released during the second period of EFS (S2) in terms of the amount of ACh released during the first period of EFS (S1). During S2, preparations were exposed to saline (control, solid column, $n=12$), 100 nM endothelin-1 (open column, $n=9$), 10 nM sarafotoxin S6c (open columns, $n=3$) or 1 μM tetrodotoxin (hatched column, $n=3$). Vertical bars represent the s.e.mean of n experiments. (b) Endothelin-1-induced inhibition of EFS-mediated ACh release in the absence (open column, $n=9$) or presence of the ET_A receptor-selective antagonist, BQ-123 (3 μM, hatched column, $n=5$), the ET_B receptor-selective antagonist, BQ-788 (1 μM, hatched column, $n=4$) or the combined presence of BQ-123 and BQ-788 (solid columns, $n=4$). *Significantly different from ET-1; †significantly different from ET-1 + BQ-123.

Discussion

Stimulation of parasympathetic postganglionic cholinergic nerves within the airway wall leads to the release of ACh and contraction of airway smooth muscle. In the current study, cholinergic nerve-mediated increases in ACh release and smooth muscle contraction were both diminished following stimulation of prejunctional ET_B receptors. These findings were in stark contrast to previous studies in rabbit and murine airways which demonstrated that stimulation of prejunctional ET_B receptors by endothelin-1 and related peptides markedly enhanced cholinergic nerve-mediated contractions (McKay *et al.*, 1993; Henry & Goldie, 1995).

Stimulation of postganglionic cholinergic nerves within sheep isolated tracheal smooth muscle was achieved via the application of an electrical field across the muscle band. The resultant contractions were unaffected by the ganglion blocking agent, hexamethonium, but were fully inhibited by either the muscarinic cholinoceptor antagonist, atropine or the neurotoxin, tetrodotoxin, indicating that the contractions were mediated by activation of postganglionic cholinergic nerves. The lack of any residual contraction in the presence of atropine and the monophasic nature of the contraction argued against the existence of a significant e-NANC component in the response. In addition, an i-NANC relaxant component mediated by NO was not evident in the current study, since the application of the NO synthase inhibitor L-NAME did not potentiate the monophasic, EFS-induced contractions. Furthermore, any potential influences that cyclo-oxygenase products may have had on EFS-induced contractions (Inoue *et al.*, 1984; Shore *et al.*, 1987) were precluded by the inclusion of indomethacin in the physiological solution. Thus, the observed contractile responses to EFS in the current study were primarily the result of the release of ACh from postganglionic cholinergic nerves.

In the current study, endothelin-1 and sarafotoxin S6c markedly suppressed cholinergic nerve-mediated contractions. The ET_B receptor-selective agonist, sarafotoxin S6c, caused a potent, concentration-dependent inhibition of EFS-induced contractions. Confirmation of the involvement of ET_B receptors in this inhibitory effect was obtained from studies using the ET_B receptor-selective antagonist, BQ-788 (Ishikawa *et al.*, 1994), which abolished the inhibitory effects of sarafotoxin S6c. Moreover, these ET_B receptors appeared to be located prejunctionally, rather than postjunctionally, since sarafotoxin S6c inhibited contractions induced by EFS, but not those induced by exogenously applied ACh. Indeed, this is consistent with the findings that sarafotoxin S6c inhibited the release of ACh from EFS-stimulated tracheal smooth muscle. Endothelin-1, in the presence of an ET_A receptor-selective antagonist, BQ-123, produced a similar profile of effects; concentration-dependent inhibition of EFS-induced contraction and ACh release. Together, these data indicate that endothelin-1 and sarafotoxin S6c inhibited cholinergic neurotransmission via ET_B receptor-mediated inhibition of ACh release from postganglionic cholinergic nerves.

Although the inhibitory effects of ET_B receptor activation were clearly demonstrable in functional studies, the effects of possible neuronal ET_A receptor activation on EFS-induced contractions could not be clearly established in the face of the marked direct spasmogenic actions exerted by endothelin-1 via activation of the ET_A receptor population on tracheal smooth muscle. This raises the question as to whether ET_A receptor activation influences cholinergic neurotransmission in sheep isolated trachea. In the absence of any currently available ET_A receptor-selective agonists, the possible neuromodulatory influences of ET_A receptor activation were investigated by examining the effects of endothelin-1 on EFS-induced ACh

release in the presence and absence of the receptor-selective antagonists, BQ-123 and BQ-788. In the current study, endothelin-1-induced attenuation of ACh release was inhibited by the ET_B receptor-selective antagonist, BQ-788, but not by the ET_A receptor-selective antagonist, BQ-123, indicating that ET_A receptors probably exert only minor modulatory influences on cholinergic neurotransmission in sheep trachea.

Sheep isolated tracheal tissue contains at least two distinct endothelin receptor-effector systems capable of modulating airway smooth muscle tone. Firstly, an ET_A receptor-effector system in airway smooth muscle appears to mediate the direct spasmogenic actions of endothelin-1. This conclusion is based on the results of functional studies which demonstrated that endothelin-1-induced contractions in sheep isolated airway preparations were inhibited by the ET_A receptor antagonist, BQ-123 and on allied autoradiographic studies which identified a homogeneous population of ET_A receptors in this tissue (Goldie *et al.*, 1994). Consistent with this, BQ-123 also blocked the bronchoconstrictor response to inhaled endothelin-1 in allergic sheep (Noguchi *et al.*, 1995). The inability of autoradiographic techniques to identify a significant population of ET_B receptors in sheep tracheal smooth muscle, coupled with the inactivity of ET_B receptor agonists as spasmogens argues against any notable ET_B receptor-effector system existing in these smooth muscle cells (Goldie *et al.*, 1994). Despite the absence of ET_B receptors on tracheal smooth muscle, the current study has identified a population of ET_B receptors located prejunctionally on the postganglionic cholinergic nerves which innervate the tracheal smooth muscle cells. Activation of these ET_B receptors inhibits cholinergic neurotransmission by inhibiting acetycholine release. However, the physiological role or functional significance of these ET_B receptors is not clear. Recent data indicate that endothelins may be released from nerves and act as neuromodulators of cholinergic nerve function. For example, Shinkai and coworkers (1994) have suggested that endothelin released from a tetrodotoxin-sensitive site is involved in the modulation of acetylcholine release in the rat iris sphincter preparation. Furthermore, Eaker *et al.* (1995) have demonstrated high levels of endothelin-1 expression in myenteric neurones cultured from rat small intestine. In the current study of ovine trachea, neither endothelin content nor endothelin expression was measured. Nevertheless, the finding that the magnitude of EFS-induced contractions in ovine trachea were not affected by the ET_B receptor-selective antagonist, BQ-788, provides some indirect evidence that even if endothelin is released by EFS it then exerts negligible neuromodulatory effects on cholinergic nerve function. However, this does not preclude the possibility that cholinergic nerve function is modulated by endothelin released from neighbouring, non-neuronal sources such as the tracheal epithelium. It is apparent though that tracheal tone is differentially modulated by endothelin-1 in sheep airways; increased by ET_A receptor-mediated contraction, but inhibited by ET_B receptor-mediated inhibition of cholinergic neurotransmission.

The profile of effects produced by endothelin-1 in the current study of sheep trachea, namely ET_A receptor-mediated contraction and ET_B receptor-mediated inhibition of cholinergic neurotransmission, is markedly different from that hitherto described in airways from other animal species. In murine trachea and rabbit bronchus (McKay *et al.*, 1993; Henry & Goldie, 1995), activation of prejunctional ET_B receptors caused potentiation of EFS-induced, cholinergic nerve-mediated contractions. Such frank interspecies differences with respect to the modulatory effects of an agonist on cholinergic neurotransmission are somewhat unusual. For example, muscarinic (M_2) cholinoceptor agonists, β_2-adrenoceptor agonists, histamine (H_3) receptor agonists, prostaglandin E_2 and the opioids characteristically inhibit cholinergic neurotransmission in the airways of many different laboratory animal species (for review see Barnes, 1992). On the other hand, tachykinins and 5-hydroxytryptamine generally potentiate cholinergic neurotransmission in the airways (Tanaka & Grunstein, 1986; Hall *et al.*, 1989; Black *et al.*, 1990; Van Oosterhout *et al.*, 1991; Belvisi *et al.*, 1994; Takahashi *et al.*, 1995). In the light of the marked interspecies differences observed with endothelin-1, a comprehensive assessment of the influence of endothelin receptor activation on cholinergic neurotransmission in human airways would seem warranted. A preliminary study indicates that endothelin-1 may not exert prominent neuromodulatory effects on cholinergic nerves within human airways (Black *et al.*, 1995). However, until a more complete evaluation of the effects of endothelin-1 and related peptides on cholinergic neurotransmission in human bronchus is conducted, the usefulness of sheep tracheal tissue as a model remains uncertain.

Interestingly, although the distribution and function of ET_A and ET_B receptors in sheep trachea is markedly different from that observed in the airways of other species so far examined, the profile is similar to that recently described in guinea-pig ileal tissue (Guimaraes & Rae, 1992; Warner *et al.*, 1993). Endothelin-1 exerts a dual effect on the guinea-pig field-stimulated ileum, characterized by an ET_B receptor-mediated suppression of cholinergic neurotransmission and an ET_A receptor-mediated slowly developing tonic contraction (Guimaraes & Rae, 1992; Warner *et al.*, 1993). Furthermore, Guimaraes & Rae (1992) reported that the inhibitory effects of endothelin-1, -2 and -3 on nerve-mediated twitch contractions were rather transient, which is consistent with the transient nature of ET_B receptor-mediated responses observed in the current study of sheep isolated trachea. The time-profile of the inhibitory effects induced by sarafotoxin S6c (or by endothelin-1 in the presence of BQ-123) on cholinergic nerve-mediated contractions in the current study was similar to that previously observed for ET_B-receptor-mediated contraction of rat and mouse trachea (Henry, 1994; Henry & Goldie, 1995). The underlying mechanisms responsible for the transiency of ET_B receptor-mediated responses in sheep isolated trachea were not investigated, although the transient responses reported in aforementioned studies were associated with marked and rapid desensitization of the ET_B receptor-effector system (Guimaraes & Rae, 1992; Henry, 1994; Henry & Goldie, 1995).

In summary, cholinergic nerve-mediated contractions of sheep isolated trachea were inhibited by endothelin-1 through a mechanism involving the activation of prejunctional ET_B receptors. Activation of these neuronal ET_B receptors was associated with a marked reduction in ACh release from cholinergic nerves. Thus, the current investigation provides further compelling evidence that the endothelin family of peptides exert potent neuromodulatory actions on cholinergic, postganglionic nerves within the airways.

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