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Influence of respiratory tract viral infection on endothelin-1-induced potentiation of cholinergic nerve-mediated contraction in mouse trachea

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1 This study examined the influence of respiratory tract infection with influenza A/PR-8/34 virus on endothelin receptor-mediated modulation of contraction induced by stimulation of cholinergic nerves in mouse isolated trachea.

2 The ET_B receptor-selective agonist, sarafotoxin S6c (30 nM) induced large transient contractions ($118 \pm 5\%$ C_{max}, $n=13$; where C_{max} is the contraction induced by 10 μM carbachol) of isolated tracheal segments from control mice. The peak contractile response to 30 nM sarafotoxin S6c was significantly lower in preparations from virus-inoculated mice at day 2 ($57 \pm 8\%$ C_{max}, $n=3$, $P<0.05$) and 4 post-inoculation ($90 \pm 8\%$ C_{max}, $n=9$, $P<0.05$), consistent with virus-induced attentuation of the ET_B receptor-effector system linked to airway smooth muscle contraction. The mean peak contraction to 30 nM sarafotoxin S6c of preparations from virus-inoculated mice at day 8 post-inoculation ($94 \pm 17\%$ C_{max}, $n=4$) was not significantly different from that of control.

3 Electrical field stimulation (EFS; 90 V, 0.5 ms duration, 10 s train, 0.1-30 Hz) of preparations from control and virus-inoculated mice, caused contractions that were abolished by 0.1 μM atropine or 3 μM tetrodotoxin, indicating that these responses were mediated by neuronally released acetylcholine. Sarafotoxin S6c markedly potentiated contractions induced by a standard stimulus (0.3 Hz, every 3 min) in tracheal segments from control and virus-inoculated mice. In tracheal tissue from control mice, 30 nM sarafotoxin S6c significantly increased a standard EFS-induced contraction of $24 \pm 4\%$ C_{max} by a further $24 \pm 3\%$ C_{max} (i.e. 2 fold increase, $n=11$). Sarafotoxin S6c (30 nM) also markedly potentiated standard EFS-induced contractions in preparations from virus-inoculated mice at day 2 ($17 \pm 2\%$ C_{max}, $n=3$), day 4 ($17 \pm 5\%$ C_{max}, $n=9$) and day 8 ($26 \pm 5\%$ C_{max}, $n=4$) post-inoculation. The level of potentiation of EFS-induced contractions in preparations from virus-inoculated mice was similar to that in tissue from control mice at days, 2, 4 and 8 post-inoculation. In contrast, sarafotoxin S6c (30 nM) did not enhance contractile responses of tracheal segments from control and virus-inoculated mice to exogenously applied acetylcholine ($n=3$).

4 Endothelin-1 (1 nM) caused similar potentiations of standard EFS-induced contractions in tracheal segments from control ($13 \pm 2\%$ C_{max}, $n=23$) and virus-inoculated mice at day 2 ($13 \pm 1\%$ C_{max}, $n=5$), day 4 ($16 \pm 5\%$ C_{max}, $n=6$), and day 8 ($13 \pm 3\%$ C_{max}, $n=8$) post-inoculation. In contrast, 1 nM endothelin-1 did not enhance contractile responses of tracheal segments from control and virus-inoculated mice to exogenously applied acetylcholine ($n=4$). Neither the ET_A receptor-selective antagonist, BQ-123 (3 μM) nor the ET_B receptor-selective antagonist, BQ-788 (1 μM) alone had any significant inhibitory effect on endothelin-1-induced potentiations of tracheal segments from control or virus-inoculated mice at days 2, 4 and 8 post-inoculation. However, simultaneous pre-incubation with BQ-123 (3 μM) and BQ-788 (1 μM) prevented endothelin-1-evoked potentiations, indicative of a role for both ET_A and ET_B receptors in this system.

5 These data clearly demonstrate that respiratory tract viral infection attenuated the function of the postjunctional ET_B receptor-effector system linked directly to airway smooth muscle contraction. However, the function of prejunctional ET_A and ET_B receptor-effector systems linked to augmentation of cholinergic nerve-mediated airway smooth muscle contraction remained unaffected during respiratory tract viral infection in mice.

Keywords: Endothelin-1; sarafotoxin S6c; BQ-788; BQ-123; endothelin receptors; electrical field stimulation; airway smooth muscle; cholinergic nerves; respiratory tract virus; influenza A/PR-8/34 virus

Introduction

Respiratory tract viral infections are frequently associated with exacerbations of pre-existing asthma and with the induction of bronchial hyperresponsiveness in otherwise healthy individuals (Empey *et al.*, 1976; Little *et al.*, 1978; Aquilina *et al.*, 1980; Beasley *et al.*, 1988; Lemanske *et al.*, 1989; Laitinen *et al.*, 1991). The reasons for this association are poorly understood, although a variety of complex mechanisms, including virus-induced damage to the respiratory epithelium, inflammation of the airways and alterations in the neural control of airway smooth muscle tone seem to be involved (Buckner *et al.*, 1985; Jacoby & Fryer, 1990; Hegele *et al.*, 1995; Folkerts & Nijkamp, 1995).

Respiratory epithelial cells produce and release the 21 amino-acid peptide, endothelin-1 (MacCumber *et al.*, 1989; Black *et al.*, 1989; Rozengurt *et al.*, 1990; Noguchi *et al.*, 1995). Endothelin-1, via the activation of ET_A and/or ET_B receptors exerts numerous actions within the airways. In human subjects, endothelin-1-induced airway smooth muscle contraction is mediated predominantly via ET_B receptors (Goldie *et al.*, 1995), although in other species ET_A receptors may also be involved (Hay *et al.*, 1993; Henry, 1993; Inui *et al.*, 1994; Goldie *et al.*, 1994). In addition to this direct spasmogenic

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action, endothelin-1 has potent neuromodulatory actions on postganglionic cholinergic neurones within the airways (McKay *et al.*, 1993; Henry & Goldie, 1995). Prejunctional ET_B receptors were involved, as the ET_B receptor-selective agonist, sarafotoxin S6c, markedly potentiated contractions induced by cholinergic nerve stimulation, but did not affect contractions to exogenously added acetylcholine (Henry & Goldie, 1995). Thus, the increased levels of endothelin-1 associated with lung diseases such as asthma (Springall *et al.*, 1991), may contribute to elevated airway smooth muscle tone directly, by stimulation of airway smooth muscle endothelin receptors and indirectly by augmentation of cholinergic nerve-mediated airway smooth muscle contraction.

Recent investigations from our laboratory have demonstrated that respiratory tract viral infection is associated with a significant reduction in the density of tracheal airway smooth muscle ET_B sites and in a marked attenuation of contraction mediated via the ET_B receptor-effector system (Henry & Goldie, 1994; Carr *et al.*, 1996). However, the influence of viral infection on the function of endothelin receptor-effector systems other than those linked directly to airway smooth contraction remains unknown. The aim of the current study was to determine the effect of respiratory tract viral infection on the ET_B receptor-effector system responsible for potentiation of cholinergic nerve-mediated contractions in mouse isolated trachea.

Methods

Respiratory tract virus stock

Influenza A/PR-8/34 virus was grown in the allantoic fluid of 10-day-old embryonated chicken eggs at 37°C for 3 days as described previously (Williams & MacKenzie, 1977). The allantoic fluid was harvested and contained 2.7×10^6 egg infectious doses (EID_{50}) of virus ml^{-1} as determined by the method of allantois-on-shell titration for infectivity (Fazekas de St. Groth & White, 1958). The virus stock was stored in 0.5 ml aliquots at −70°C.

Viral inoculation and harvesting of tracheal tissue

Eight week old male CBA/CaH mice, specified pathogen-free, were obtained from the Animal Resources Centre (Perth, Australia), housed in a controlled environment, receiving food and water *ad libitum*. Mice were anaesthetized (50 mg kg^{-1} pentobarbitone sodium, i.p.) and inoculated intranasally with 15 μl of fluid containing 1000 EID_{50} doses of influenza A/PR-8/34 virus or 15 μl of a 1 in 40 dilution of the allantoic fluid from virus-free chicken eggs (control mice). At day 2, 4 and 8 post-inoculation, mice were anaesthetized with halothane (Fluothane, ICI) and killed by cervical dislocation. The trachea was removed and placed in Krebs bicarbonate solution (KBS) of the following composition (in mM): NaCl 117, KCl 5.36, $NaHCO_3$ 25.0, KH_2PO_4 1.03, $MgSO_4.7H_2O$ 0.57, $CaCl_2.2H_20$ 2.5 and glucose 11.1. The cyclo-oxygenase inhibitor, indomethacin (2.5 μM) was present in the KBS throughout all studies.

Isometric tension recording

Two tracheal ring segments (2 mm long) were obtained from each mouse. Each tracheal segment was suspended under 0.5 g tension and placed in a 2 ml organ bath containing KBS at 37°C bubbled continuously with 5% CO_2 in O_2. Changes in isometric tension were recorded via FT03 force-displacement transducers (Grass Instruments). Tracheal segments were allowed to equilibrate for 45 min and during this time preparations were washed every 15 min. During the equilibration period, changes in resting tension were adjusted to 0.5 g. Isolated tracheal preparations were exposed to cumulative additions of 0.2 and 10 μM carbachol to assess the viability of the preparations and then washed and rested for 20 min prior to the application of electrical field stimulation (EFS). A non-cumulative frequency-response curve was then constructed for each preparation (90 V, 0.5 ms duration, 10 s train; 0.1, 0.3, 1, 3, 10 and 30 Hz at 2 min intervals). EFS was delivered by a Grass S44 stimulator connected to a stimulus isolation unit (SIU5, Grass Instruments) and a timing device. Stimuli were applied across tracheal ring preparations by means of two parallel platinum electrodes.

Characterization of EFS-induced contractions

To evaluate the role of cholinergic nerves in the contractile response to EFS we investigated the effects of the muscarinic cholinoceptor antagonist, atropine (0.1 μM), and the neurotoxin, tetrodotoxin (3 μM), on EFS-induced contractions (non-cumulative frequency-response curves, 0.1–30 Hz) in tracheal airway smooth muscle preparations from control and virus-inoculated mice at day 4 post-inoculation. In addition, the influence of these agents on contractile responses of tracheal segments from control and virus-inoculated mice (day 4 post-inoculation) to cumulatively applied acetylcholine (10 nM–1 mM, at 0.5 log concentration increments) was tested. EFS and acetylcholine-induced contractions were recorded in the absence (time controls) or presence of a single agent (atropine or tetrodotoxin).

Endothelin receptor-mediated modulation of EFS-induced contractions

The influence of the ET_B receptor-selective agonist, sarafotoxin S6c and the non-selective ET_A/ET_B receptor agonist, endothelin-1, on EFS-induced contractions was examined. High concentrations of endothelin-1 evoke large sustained contractions of mouse tracheal segments (Henry & Goldie, 1994; 1995) and therefore only the effects of relatively low concentrations of this agonist could be investigated in the current experiments. In contrast, contractions in response to high concentrations (above 10 nM) of sarafotoxin S6c are transient in nature (Henry & Goldie, 1994; 1995); thus the effects of both low and high concentrations of this agonist were investigated. Standard EFS-induced contractions (90V, 0.5 ms duration, 0.3 Hz) were recorded as 10 s trains every 3 min until three consecutive reproducible contractions had been obtained (3–4 trains). EFS-induced responses were then monitored for a further 60 min in the absence (time control) or presence of sarafotoxin S6c (0.3 or 30 nM) or endothelin-1 (1 nM). To determine the effect of endothelin-1 and 0.3 nM sarafotoxin S6c on EFS-induced contractions, the average magnitude of the three EFS-induced contractions obtained at 12, 15 and 18 min after the addition of agonist was calculated and compared with the magnitude of pre-agonist EFS-induced contractions. In the presence of 30 nM sarafotoxin S6c, the average magnitude of the EFS-induced contractions obtained at 30, 33, and 36 min after the addition of sarafotoxin S6c was used.

Secondly, the effects of the ET_A receptor-selective antagonist, BQ-123 and the ET_B receptor-selective antagonist, BQ-788 were examined. In these experiments standard EFS-induced contractions were obtained as described above and preparations were then incubated with BQ-123 (3 μM) and/or BQ-788 (1 μM) for 15 min prior to the addition of agonist. EFS-induced contractions were then monitored for a further 60 min in the presence of these antagonists.

Effect of endothelin-1 and sarafotoxin S6c on contractions induced by exogenously applied acetylcholine

To evaluate the role of postjunctional ET_A and ET_B receptor activation on muscarinic receptor-mediated tracheal airway smooth muscle contraction, we investigated the effects of sarafotoxin S6c and endothelin-1 on contractile responses to exogenously applied acetylcholine. In these experiments, con-

tractile responses to cumulatively applied acetylcholine (10 nM – 1 mM at 0.5 log concentration increments) were recorded in the absence or presence of sarafotoxin S6c (30 nM) or endothelin-1 (1 nM).

Data analysis

Contractile responses to EFS, acetylcholine and peptides have been expressed in terms of the maximum contractile response evoked by 10 μM carbachol (C_{max}) at the beginning of each experiment. In time control (C) and test (T) preparations, three initial standard EFS-induced contractile responses were obtained and mean responses calculated (C_0,T_0) and subtracted from subsequent EFS-induced contractions (C_t,T_t) produced at 3 min intervals over the 60 min period. Thus, at each of these time points, changes in the magnitude of EFS evoked contractions induced as a function of time and by the presence of an endothelin receptor agonist in test preparations ($\Delta T = T_t - T_0$), as well as by time alone in a time control preparation ($\Delta C = C_t - C_0$), were estimated. Mean time-related changes in responses to EFS were subtracted ($\Delta T_{cor} = \Delta T - \Delta C$) to isolate agonist-induced changes at each time point. Agonist-induced changes to EFS-induced contractions were expressed as mean $\Delta T_{cor} \pm$ s.e.mean for preparations from n different mice. The time-related changes in EFS-induced contractions were small and similar in preparations from control and virus-inoculated mice. For statistical comparisons, EC_{70} data (concentration of acetylcholine producing 70% of C_{max}) were log transformed to mean $-\log EC_{70}$. Unless otherwise stated, differences between treatment means were assessed by analysis of variance (SigmaStat) utilizing a modified t statistic (Wallenstein *et al.*, 1980). P values less than 0.05 were considered to be statistically significant.

Drugs

The following were used: endothelin-1, sarafotoxin S6c, BQ-123 (cyclo[D-Trp-D-Asp-L-Pro-D-Val-L-Leu]) (Auspep, Melbourne, Australia), BQ-788 (N-*cis*-2,6-dimethylpiperidinocarbonyl-L-γ-methylleucyl-D-1-methoxycarbonyltryptophanyl-D-norleucine) (gift from Banyu Pharmaceutical Co., Tsukuba, Japan), carbamylcholine chloride (carbachol), acetylcholine chloride, indomethacin, tetrodotoxin (Sigma Chemical Co, St Louis, U.S.A.), atropine sulphate monohydrate (Fluka AG).

Results

EFS-induced contractions in isolated tracheal airway smooth preparations from control and influenza A virus-inoculated mice

The muscarinic cholinoceptor agonist, carbachol (10 μM), evoked similar maximum contractions (C_{max}; calculated from days 2, 4 and 8 post-inoculation) in isolated tracheal airway smooth muscle preparations from control (1.1 ± 0.02 g, $n = 98$) and virus-inoculated mice (0.97 ± 0.04 g, $n = 77$).

EFS (90 V, 0.5 ms duration, 0.1 – 30 Hz, 10 s train every 2 min) caused similar, monophasic, frequency-dependent contractions in isolated tracheal airway smooth muscle preparations obtained from control and virus-inoculated mice at days 2, 4 and 8 post-inoculation (Figure 1). However, at day 8, contractile responses to stimulation at frequencies of 0.3 – 30 Hz were significantly smaller ($P < 0.05$) than those observed in preparations from control mice (Figure 1c).

EFS-induced contractions were prevented by prior incubation with either the muscarinic cholinoceptor antagonist, atropine (0.1 μM) or the neurotoxin, tetrodotoxin (3 μM). Contractions induced by exogenously added acetylcholine were similar in tracheal segments from control and influenza A virus-inoculated mice at day 4 post-inoculation. These contractions were inhibited by atropine but not by tetrodotoxin (Table 1).

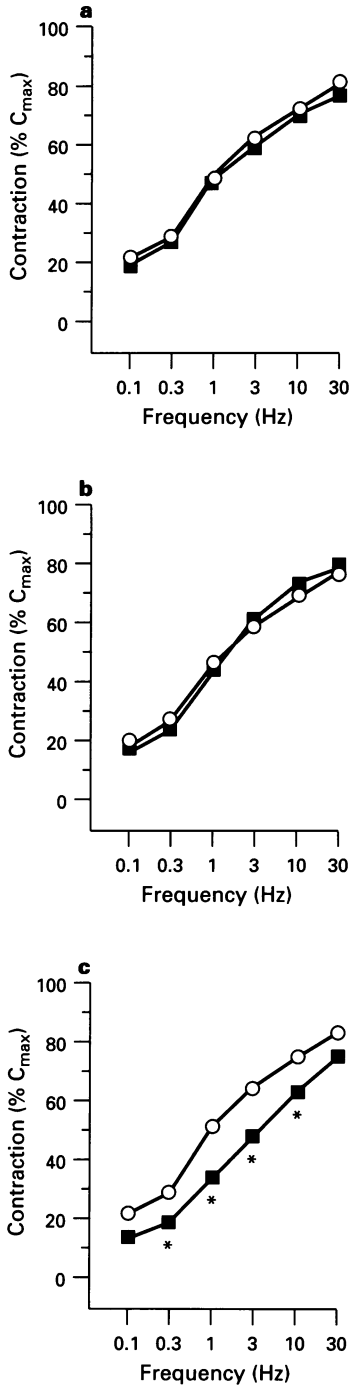

Figure 1 Frequency-response relationship to EFS (90 V, 0.5 ms duration, 10 s train, 0.1 – 30 Hz at 2 min intervals) of isolated tracheal airway smooth muscle preparations from control (○) and virus-inoculated (■) mice at (a) day 2, (b) day 4 and (c) day 8 post-inoculation. Values are the mean ± s.e.mean of $n = 24 - 36$ mice. *$P < 0.05$.

Sarafotoxin S6c-induced potentiation of EFS-evoked contractions

The ET_B receptor-selective agonist, sarafotoxin S6c (30 nM) evoked large but transient contractions of isolated tracheal segments from control mice (Figure 2 and Figure 3a). Contractions in these preparations peaked within 10 min and faded towards baseline over a 40 min period. In contrast, sarafotoxin S6c was a significantly weaker spasmogen in tracheal preparations from virus-inoculated mice. For example, peak contractile responses to 30 nM sarafotoxin S6c were sig-

Table 1 Effect of modulators of cholinergic neurotransmission on the potency of exogenously applied acetylcholine in tracheal preparations from control and virus-inoculated mice

Pretreatment	Control EC_{70} (μM)	n	Virus EC_{70} (μM)	n
None (time control)	0.13 (0.01–1.6)	3	0.16 (0.06–0.44)	3
Atropine (0.1 μM)	100 (59–170)*	4	158 (20–1258)*	4
Tetrodotoxin (3 μM)	0.25 (0.05–4.3)	3	0.32 (0.003–0.5)	3
Endothelin-1 (1 nM)	0.16 (0.03–0.8)	4	0.1 (0.005–2)	4
Sarafotoxin S6c (30 nM)	0.06 (0.004–0.93)	3	0.16 (0.06–0.43)	3

EC_{70} is the mean concentration of acetylcholine that produced 70% of C_{max} (95% confidence limits), n = number of mice. *$P<0.05$ compared to EC_{70} of acetylcholine alone (time control).

nificantly lower in preparations from virus-inoculated mice at days 2 and 4 post-inoculation compared with those in control preparations (Figure 2). Furthermore, the duration of the contractions induced by 30 nM sarafotoxin S6c at days 2, 4 and 8 post inoculation was significantly less than that of control preparations (Figure 2b–d, Figure 3b–d).

In addition to a direct action on tracheal airway smooth muscle tone, sarafotoxin S6c markedly enhanced EFS-induced contractions. Prior to the addition of agonist, standard EFS (90 V, 0.5 ms duration, 0.3 Hz)-induced contractions of control preparations were 24±4% of C_{max} (combined data from days 2, 4 and 8 post-inoculation, $n=11$). During the initial sustained phase of contraction to sarafotoxin S6c (30 nM), standard EFS-induced contractions were suppressed (Figure 3a) as the upper limit for contraction in these preparations was approached. However, as contraction waned towards baseline, EFS-induced contractions were markedly enhanced. Under these conditions, sarafotoxin S6c potentiated EFS-induced contractions of control mouse tracheal segments by 24±4% ($n=11$), i.e. by 2 fold (Figure 3a and Figure 4a).

Although a weaker spasmogen of airway smooth muscle segments from virus-inoculated mice, sarafotoxin S6c (30 nM) evoked marked potentiations of standard EFS (0.3 Hz)-induced contractions in these preparations (Figure 3b–d). Furthermore, the level of potentiation of EFS-induced contractions was similar to that observed in preparations from control mice. As shown in Figure 4 (b–d) this was a consistent finding at days, 2, 4 and 8 post-inoculation.

As expected, contractions induced by sarafotoxin S6c (30 nM) in tracheal segments from control and virus-inoculated mice were significantly inhibited by pre-incubation with the ET_B receptor-selective antagonist BQ-788 (1 μM) (Figure 2a). In addition, BQ-788 (1 μM) inhibited 0.3 nM sarafotoxin S6c-induced potentiation of standard EFS (0.3 Hz)-induced contractions (Figure 4). However, BQ-788 (1 μM) did not inhibit potentiation of EFS (0.3 Hz)-induced contractions evoked by 30 nM sarafotoxin S6c, in tracheal segments from either control or virus-inoculated mice (Figure 4).

Contribution of ET_A and ET_B receptor-effector systems in endothelin-1-induced potentiation of EFS-induced contractions

At a concentration of 1 nM endothelin-1 induced similar small increases in tracheal airway smooth muscle tone (mean increase calculated from days 2, 4 and 8 post-inoculation) in preparations from control (18±3% C_{max}, $n=19$) and virus-inoculated mice (19±5% C_{max}, $n=16$). Furthermore, at this low concentration, endothelin-1 cause marked, similar potentiations of standard EFS (0.3 Hz)-induced contractions of tracheal segments from control and virus-inoculated mice (Figure 5). Pretreatment with either the ET_A receptor-selective antagonist, BQ-123 (3 μM) or the ET_B receptor-selective antagonist, BQ-788 (1 μM) had no significant inhibitory effect on endothelin-1 evoked potentiation of standard EFS (0.3 Hz)-induced contractions. In contrast, simultaneous treatment with BQ-123 (3 μM) and BQ-788 (1 μM) markedly inhibited such potentiations in tracheal segments from control mice and mice inoculated 2, 4 and 8 days previously with influenza A virus (Figure 5).

Effect of sarafotoxin S6c and endothelin-1 on contractions induced by exogenously added acetylcholine

Acetylcholine caused similar concentration-dependent contractions of tracheal airway smooth muscle preparations from control and influenza A virus-inoculated mice (day 4). However, neither sarafotoxin S6c (30 nM) nor endothelin-1 (1 nM) had any significant effect on acetylcholine-induced contractions in tracheal tissue from either source (Table 1).

Discussion

This study has clearly demonstrated that respiratory tract viral infection is associated with a reduction in the function of the ET_B receptor-effector system linked directly to airway smooth muscle contraction but paradoxically, the neuronal ET_B receptor-effector system linked to augmentation of cholinergic nerve-mediated contractions was unaffected by viral infection.

EFS evoked monophasic contractions of isolated tracheal airway smooth muscle segments from both control and virus-inoculated mice and these contractions were abolished by atropine or the sodium channel blocker, tetrodotoxin. Thus, contractions in these preparations were due to the activation of muscarinic cholinoceptors by neuronally released acetylcholine, consistent with recent investigations of nerve-mediated contractions in mouse trachea (van Oosterhout *et al.*, 1991; Larsen *et al.*, 1992; Garssen *et al.*, 1993; Henry & Goldie, 1995).

Cholinergic nerve-mediated contractions in preparations from control mice were markedly enhanced by sarafotoxin S6c and concentrations of endothelin-1 that had only a small direct contractile effect *per se*. This potentiation was presumably due to enhanced release of acetylcholine via the activation of prejunctional endothelin receptors, since these peptides did not affect contractions evoked by exogenous acetylcholine. The finding that endothelin-1-induced potentiation was not inhibited in the presence of the ET_A receptor-selective antagonist, BQ-123 and that sarafotoxin S6c was also a potent agonist in this system, provides strong evidence for the involvement of ET_B receptors and is consistent with other recent findings from our laboratory (Henry & Goldie, 1995). That ET_A receptors were also involved in this response is suggested by the fact that endothelin-1-induced effects were inhibited only in the combined presence of BQ-123 and the ET_B receptor-selective antagonist, BQ-788, but not in the presence of BQ-788 alone. We have recently reported that airway smooth muscle ET_A and ET_B receptor-effector systems are linked to contraction of tracheal segments from control mice (Carr *et al.*, 1996) and the present study provides strong evidence for the involvement of neuronal ET_A and ET_B receptor-effector systems in mediating enhanced responses to cholinergic nerve-mediated contractions in this preparation.

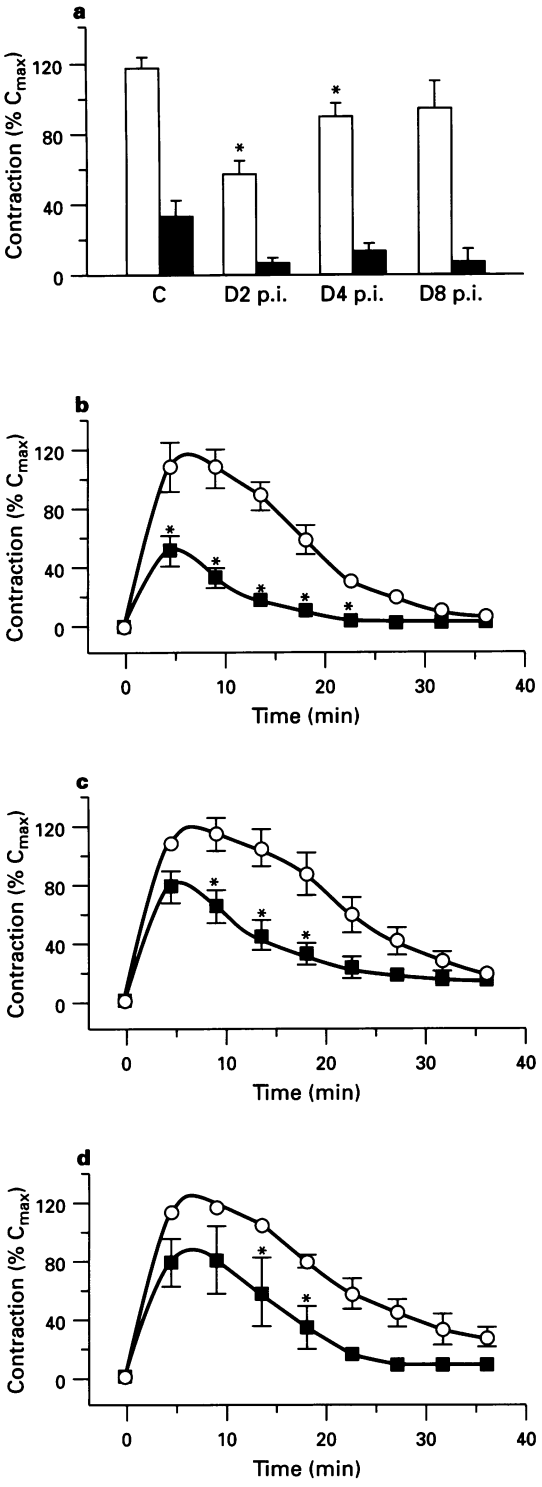

Figure 2 Magnitude and time course of contractions in isolated tracheal airway smooth preparations evoked by 30 nM sarafotoxin S6c. (a) Magnitude of contractions to 30 nM sarafotoxin S6c in the absence (open columns) and presence of the ET_B receptor-selective antagonist, BQ-788 (1 μM, solid columns) in preparations from control (C, mean data from day 2, 4 and 8 post-inoculation, $n=10-13$ mice) and virus-inoculated mice at day 2 (D2 p.i., $n=3$ mice), day 4 (D4 p.i., $n=8-9$ mice) and day 8 (D8 p.i., $n=3-4$ mice) post inoculation. (b–d) Time course of contractions to sarafotoxin S6c in tracheal airway smooth muscle in preparations from control (○) and virus-inoculated mice (■) at day 2 (b, $n=3$ mice), day 4 (c, $n=6-9$ mice) and day 8 (d, $n=4$ mice) post-inoculation. Values are the mean ± s.e.mean. Differences in the duration of contractions to 30 nM sarafotoxin S6c were assessed by analysis of variance for repeated measures. $^*P<0.05$.

A major finding of the current study was that the neuromodulatory actions of sarafotoxin S6c and endothelin-1 were similar in preparations obtained from both virus-inoculated and control mice. Furthermore, results with receptor-selective antagonists suggest that the augmentary effects of endothelin-1 on cholinergic nerve-mediated contractions in preparations from virus-inoculated mice were also mediated via the activation of neuronal ET_A and ET_B receptors. These findings are in stark contrast to the effects of respiratory tract viral infection on endothelin receptors linked directly to airway smooth muscle contraction. For example, in the current study, sarafotoxin S6c induced large contractions of tracheal segments from control mice, however sarafotoxin S6c was a weak spasmogen in tracheal segments from virus-inoculated mice. These latter findings are consistent with recent reports that viral infection was associated with decreased density and function of the ET_B receptor-effector system linked directly to airway smooth muscle contraction (Henry & Goldie, 1994; Carr *et al.*, 1996). Thus, although we have not measured endothelin receptor densities on cholinergic nerve varicosities, nor have we investigated the effects of sarafotoxin S6c or endothelin-1 over their entire concentration-effect range, it would appear that respiratory tract viral infection does not significantly modulate the function of ET_A and ET_B receptor-effector systems linked to augmented acetylcholine release.

A variety of circumstantial evidence suggests that increased levels of endothelin-1 may be responsible for the down-regulation of airway smooth muscle ET_B receptors during viral infection. Firstly, various inflammatory mediators known to be released during respiratory tract viral infection (Hennet *et al.*, 1992) caused enhanced release of endothelin-1 from airway epithelial cells (Endo *et al.*, 1992). Secondly, prolonged exposure to elevated levels of endothelin-1 or sarafotoxin S6c

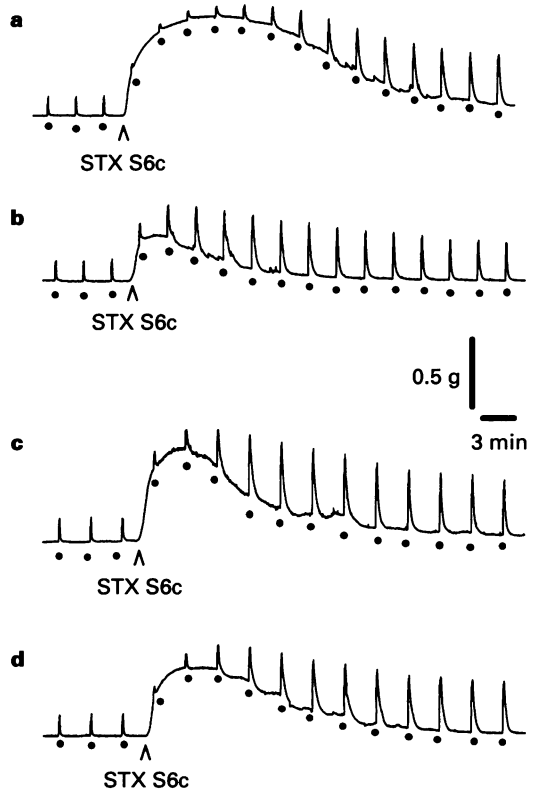

Figure 3 Typical isometric tension recordings demonstrating the effects of the ET_B receptor-selective agonist, sarafotoxin S6c (30 nM) on isolated tracheal airway smooth muscle tone and EFS-induced contractions (90 V, 0.5 ms duration, 0.3 Hz, 10 s train every 3 min) in preparations from control (a) and virus-inoculated mice at day 2 (b), day 4 (c) and day 8 (d) post-inoculation. EFS was applied (●) prior to and after the addition of 30 nM sarafotoxin S6c (STX S6c).

caused marked down-regulation of the airway smooth muscle ET_B receptor-effector system (Henry, 1993; Henry & Goldie, 1994). In contrast, the prejunctional ET_B receptor-effector system is relatively resistant to tachyphylaxis as potentiation of EFS-induced contractions were maintained well after sarafotoxin S6c-evoked airway smooth muscle contractions had subsided (current study; Henry & Goldie, 1995). Thus, the resistance of the prejunctional ET_B receptor-effector system to desensitization may be at least partially responsible for the

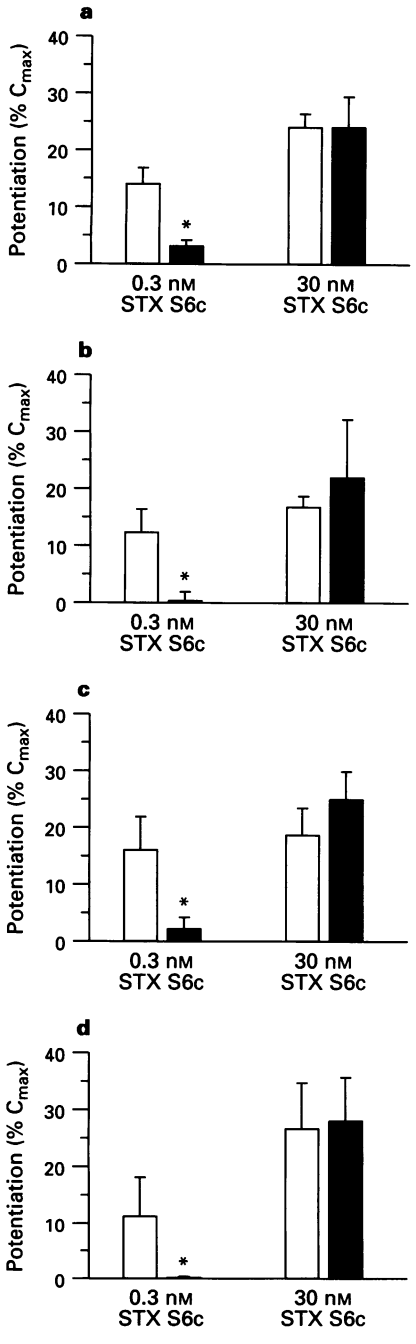

Figure 4 Effects of the ET_B receptor-selective agonist, sarafotoxin S6c (0.3 and 30 nM) on EFS-induced (90 V, 0.5 ms duration, 0.3 Hz, 10 s train every 3 min) contractions in isolated tracheal airway smooth muscle preparations from (a) control (combined data from day 2, day 4 and day 8 post-inoculation, $n=9-14$ mice) and (b–d) virus-inoculated mice at day 2 (b, $n=3-5$ mice), day 4 (c, $n=5-8$ mice) and day 8 (d, $n=3-4$ mice) post-inoculation, in the absence (open columns) and presence (solid columns) of the ET_B receptor-selective antagonist, BQ-788 (1 μM). Values are the mean ± s.e.mean. *$P<0.05$.

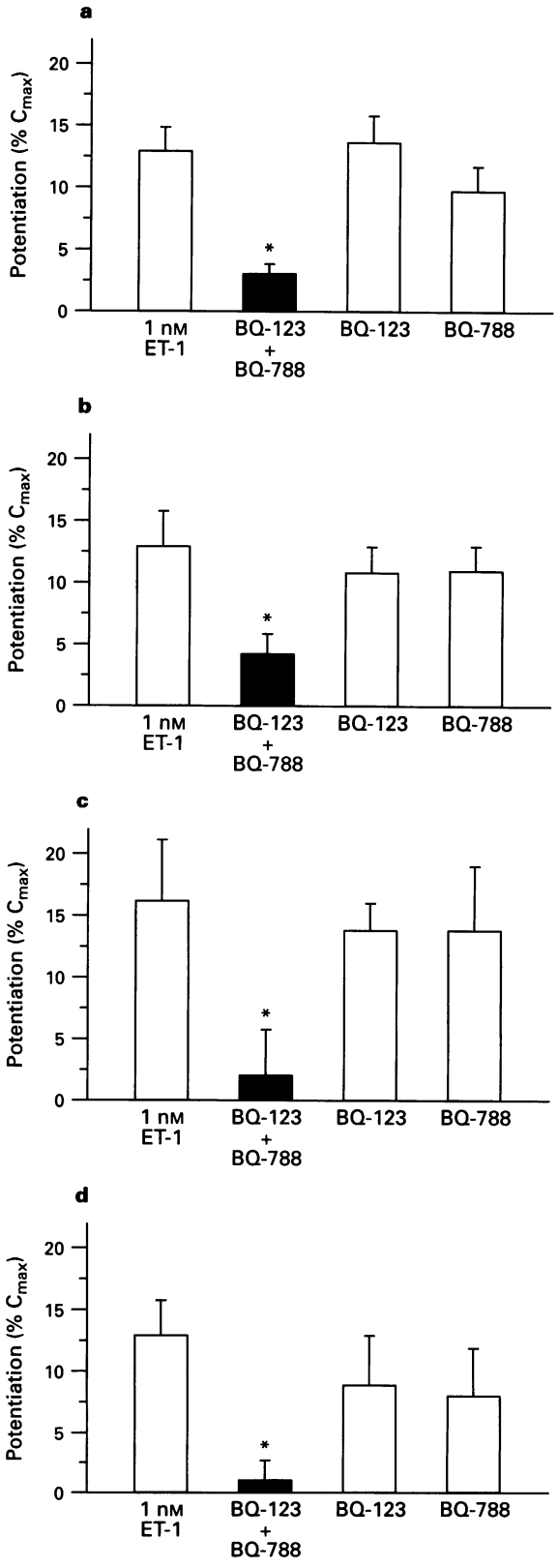

Figure 5 Effect of the ET_A receptor-selective antagonist, BQ-123 (3 μM) and the ET_B receptor-selective antagonist, BQ-788 (1 μM), on endothelin-1 (1 nM)-evoked enhancement of EFS-induced (90 V, 0.5 ms duration, 0.3 Hz, 10 s train every 3 min) contractions in tracheal airway smooth muscle preparations from (a) control (combined data from days 2, 4 and 8 post-inoculation, $n=21-23$ mice) and (b–d) virus-inoculated mice at day 2 (b, $n=6$ mice), day 4 (c, $n=5-6$ mice) and day 8 (d, $n=5-8$ mice) post-inoculation. Values are the mean ± s.e.mean. *$P<0.05$.

maintenance of its function during respiratory tract viral infection. Thus, if endothelin-1 levels are indeed elevated during respiratory tract viral infection, they may contribute to enhanced airway smooth muscle contraction directly, via stimulation of ET_A receptors and indirectly via the activation of neuronal ET_A and ET_B receptors linked to augmentation of cholinergic tone.

As expected, the direct contractile actions of sarafotoxin S6c on tracheal segments from control and virus-inoculated mice were significantly inhibited by pre-incubation with the ET_B receptor-selective antagonist BQ-788. In addition, 0.3 nM sarafotoxin S6c evoked BQ-788-sensitive potentiations of cholinergic nerve-mediated airway smooth muscle contractions. In contrast, potentiation of EFS-induced contractions, evoked by sarafotoxin S6c at the higher concentration of 30 nM, in tracheal segments from control and virus-inoculated mice were not inhibited by pre-incubation with BQ-788. The precise reason for these disparate findings is unknown and in view of the potent spasmogenic actions of endothelin receptor agonists, further investigations directed towards the pharmacological characterization of prejunctional ET_B receptors in this preparation should consider the direct measurement of transmitter release.

Acetylcholine release upon stimulation of parasympathetic nerves within the airways is subject to modulation by the activation of prejunctional autoinhibitory muscarinic M_2 receptors (Watson, 1994). Because of this, studies directed towards examining the effects of various factors enhancing the release of acetylcholine from pulmonary cholinergic neurones are complicated by autoinhibition of transmitter release. In addition, the neuraminidase activity of viruses such as influenza and parainfluenza, results in cleavage of sialic acid residues from muscarinic M_2 receptors causing receptor dysfunction (Fryer *et al.*, 1990; Jacoby & Fryer, 1990). However, it is unlikely that these factors played a significant role in the findings of the current study, as EFS-induced contractions of mouse tracheal airway smooth muscle preparations are not subject to modulation by autoinhibitory muscarinic M_2 receptors (Garssen *et al.*, 1993). In line with these findings, contractile responses to EFS of tracheal segments from virus inoculated-mice were not larger than those in control tissue. Indeed, at day 8 post-inoculation, responses to stimulation frequencies between 0.3 and 10 Hz were slightly, but significantly lower in preparations from virus-inoculated mice. The precise reason for this is unknown, but it may be related to decreased sensitivity of the postjunctional muscarinic M_3 receptor-effector system in murine airways that is observed during the later stages of respiratory tract viral infection (Henry *et al.*, 1991).

In summary, the present study has clearly demonstrated that during respiratory tract viral infection in mice, the function of prejunctional ET_A and ET_B receptor-effector systems linked to augmentation of cholinergic nerve-mediated airway smooth muscle contraction remains intact. In contrast, viral infection clearly attenuated the function of the postjunctional ET_B receptor-effector linked directly to airway smooth muscle contraction.

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