Effect of respiratory tract viral infection on murine airway β -adrenoceptor function, distribution and density

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1 The effects of a respiratory tract viral infection on β -adrenoceptor density, distribution and function were investigated in murine airways.

2 Following intranasal inoculation of CBA/CaH mice with influenza A/PR-8/34 virus, the virus proliferated rapidly in trachea (peak titres 2 days post-inoculation) and lung (peak titres 4–6 days post-inoculation). Respiratory tract viral infection was associated with a significant increase in lung weight (88% higher than control mice at day 6 post-inoculation) that was related temporally to the development of peripheral lung inflammation and consolidation.

3 Analysis of specific binding of [¹²⁵I]-cyanopindolol to β -adrenoceptors revealed that on days 2, 4 and 8 post-inoculation with virus, mouse isolated tracheal sections contained, on average, 40% more β -adrenoceptors than tracheal sections from time matched control mice. Subsequent quantitative autoradiographic studies demonstrated that this increase in total tracheal β -adrenoceptors was due primarily to a 90% increase in the density of β -adrenoceptors in the tracheal epithelium in virus-infected mice.

4 In contrast, virus-infection had no significant effect on the density of β -adrenoceptors in tracheal airway smooth muscle, although within 2 days of inoculation with virus, mouse tracheal smooth muscle segments were approximately 2 fold less sensitive to the β -adrenoceptor agonist, noradrenaline (mean $pD_2 = 6.57 \pm 0.04$, $n = 24$) and to the adenylyl cyclase-activator forskolin (mean $pD_2 = 6.78 \pm 0.04$, $n = 12$) compared to segments from control mice (mean $pD_2 = 6.84 \pm 0.06$ for noradrenaline; mean $pD_2 = 7.03 \pm 0.07$ for forskolin). Similar values were obtained 8 days post-inoculation. At day 2, but not day 8 post-inoculation with virus, relaxation responses to theophylline were also marginally attenuated compared with controls.

5 Mouse isolated tracheal segments obtained 2 days after virus inoculation and segments from time-matched control mice were equisensitive to the spasmogenic actions of the muscarinic cholinergic agonist, carbachol. However, tracheal segments from mice inoculated with virus were less responsive to carbachol on day 4 (mean $pD_2 = 6.45 \pm 0.04$, $n = 8$) and day 8 (mean $pD_2 = 6.45 \pm 0.02$, $n = 12$) compared to control preparations (day 4, mean $pD_2 = 6.73 \pm 0.06$, $n = 8$; day 8, mean $pD_2 = 6.65 \pm 0.04$, $n = 12$, $P < 0.05$). In contrast, endothelin-1-induced contractions of tracheal smooth muscle were not affected by virus-infection.

6 These data demonstrate that respiratory tract viral infection can produce significant tissue-selective changes in airway β -adrenoceptor density as well as small reductions in airway smooth muscle muscarinic cholinergic and β -adrenoceptor function.

Keywords: β -Adrenoceptors; influenza A virus; mouse trachea

Introduction

Respiratory tract viral infections in both asthmatic and otherwise healthy individuals are frequently associated with increased airway reactivity (Empey *et al.*, 1976; Little *et al.*, 1978). Virus-induced damage to the airway epithelium may contribute to this hyperreactivity by eliminating epithelium-derived relaxant factors, by removing enzymes such as enkephalinase which metabolize airway smooth muscle spasmogenic peptides including substance P, or by exposing afferent nerve endings (Empey *et al.*, 1976; Jacoby *et al.*, 1988; Jacoby & Fryer, 1990). In addition, increased vagal efferent activity, enhanced mast cell histamine release and impaired β -adrenoceptor function may also be contributing factors (Buckner *et al.*, 1981; 1985; Busse *et al.*, 1983).

Both bacterial and viral respiratory pathogens have been reported to modulate variably the function of airway β -adrenoceptors. Bacterial infections have been shown to attenuate the airway smooth muscle relaxant effects of β -adrenoceptor agonists, possibly by reducing receptor concentration (Schreurs *et al.*, 1980; Norris & Eyre, 1981; Engels *et al.*, 1987). Similarly, exposure of various individual inflammatory cell types including T-lymphocytes (Lee, 1980), neutro-

phils (Lee, 1980; Busse *et al.*, 1979) and pulmonary alveolar macrophages (Ogunbiyi *et al.*, 1988) to respiratory tract viruses is associated with a reduction in cell responsiveness to β -adrenoceptor agonists. Furthermore, whole lung membrane fractions from mice infected intranasally with a respiratory tract virus have also been shown to be hyporesponsive to β -adrenoceptor stimulation (Scarpace & Bender, 1989). The mechanisms responsible for the virus-induced reduction in β -adrenoceptor function is unclear, although altered β -adrenoceptor coupling to adenylyl cyclase and/or reduced adenylyl cyclase activity may be involved (Scarpace & Bender, 1989). In addition, recent evidence suggests that the β_1 -adrenoceptor subtype may be more susceptible to virus-induced impairment than β_2 -adrenoceptors (Ogunbiyi *et al.*, 1988).

Although respiratory tract viruses produce β -adrenoceptor hyporesponsiveness in many lung cell types, airway smooth muscle responses to β -adrenoceptor agonists have been reported to be either enhanced (Eyre *et al.*, 1982) or unchanged (Buckner *et al.*, 1981; Lemanske *et al.*, 1989) following respiratory tract virus infection. In the present investigation, we have used functional and autoradiographic techniques to assess further the effects of a respiratory tract viral infection on the density, distribution and function of β -adrenoceptors in murine airways.

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Methods

Virus stock and animal inoculation

The A/PR-8/34 strain (H1N1) of influenza virus was grown in the allantoic fluid of 10-day-old embryonated eggs at 37°C for 3 days as described previously (Williams & Mackenzie, 1977). The crude allantoic fluid was harvested and contained 4×10^7 egg infectious doses (EID₅₀) of virus ml⁻¹ as determined by the method of allantois-on-shell titration for infectivity (Fazekas de St. Groth & White, 1958). Virus-containing allantoic fluid was stored in 0.5 ml aliquots at -70°C.

Four-week-old male CBA/CaH mice, specified pathogen-free, were anaesthetized (pentobarbitone sodium, 80 mg kg⁻¹, i.p.) and inoculated intranasally with 2000 EID₅₀ of virus or 25 μ l phosphate-buffered saline (PBS) (control mice). Otherwise, all mice were housed in the same environment and treated similarly. Mice received food and water *ad libitum*.

Tracheal and lung virus titres

On specified days post-inoculation, mice were killed (pentobarbitone sodium, 200 mg kg⁻¹, i.p.) and exsanguinated by section of the renal artery. The trachea and lungs were excised, separated and placed in sterile PBS on ice. Lungs from each animal were blotted dry, weighed and then immersed in 2 ml PBS. Tracheal segments from 3 animals were dissected free of surrounding tissue and immersed in 1 ml PBS. Tissues were homogenized with motorized glass tissue grinders and the suspension clarified by centrifuging at 2000 *g* for 5 min at 4°C. Infectious virus was assayed by allantois-on-shell titration for infectivity. Briefly, 6 mm $\times$ 6 mm pieces of allantois-on-shell from 11-day-old embryonated chicken eggs were incubated in sterile round bottom tubes containing 0.35 ml of Standard Medium (SM) and 25 μ l aliquots of serial 10 fold dilutions of virus (10^{-1} to 10^{-9}) in SM. Five replicates were used at each dilution. Tubes were sealed, mounted in holders and placed in a horizontal shaker working in a 35°C room for 48 h. The fluid from each tube was transferred to a haemagglutination tray and one drop of 10% washed, goose red blood cells was added to each cell. The trays were shaken briefly and left to stand for 40 min. Positive haemagglutination indicated infection and infectious virus titre (EID₅₀) was calculated by the method of Thomson (1947). The composition of SM (pH 7.0) was (mM): NaCl 137, KCl 8, CaCl₂ 7.2, MgCl₂ 0.52, glucose 1.7, and acid-free gelatin 2.0 g l⁻¹, phenol red 2.5 mg l⁻¹, gentamycin 10 mg l⁻¹ and penicillin 100 mg l⁻¹.

At the same time that mouse lungs and trachea were removed for infectious virus assay, replicate mice were killed and their lungs and trachea fixed in 10% buffered formal saline. After fixation, tissues were embedded in paraffin, cut to a thickness of 5 μ m, stained with haematoxylin and eosin, and viewed by light microscopy.

Radioligand binding and quantitative autoradiography

On days 2, 4 and 8 post-inoculation, frozen tissue blocks of mouse trachea were prepared as described previously (Henry *et al.*, 1990). Serial frozen transverse sections (10 μ m) were cut at -20°C and thaw-mounted onto gelatin/chrom alum-coated glass slides. Each slide contained 18 tracheal sections from different mice. For radioligand binding experiments, slides were incubated with the β -adrenoceptor radioligand (-)-[¹²⁵I]-cyanopindolol (I-CYP, 10-240 pM) in Tris-HCl buffer (170 mM, pH 7.6, 22°C) containing the protease inhibitor phenylmethylsulphonyl fluoride (10 μ M). Non-specific binding was estimated in the presence of 1 μ M (-)-propranolol. After a 150 min incubation period, slides were washed for 2 $\times$ 15 min in Tris-HCl buffer to remove unbound I-CYP. Tissue sections were wiped onto GF/A glass fibre filter papers (Whatman) and counted in a gamma counter (Packard). Specific binding for trachea was expressed as fmol/tracheal section.

For autoradiographic studies, slides were incubated with I-CYP and washed in Tris-buffer as described above for the radioligand binding studies. Slides were then briefly rinsed in distilled water and rapidly dried by a stream of cold, dry air. Emulsion-coated coverslips (Kodak NTB-2) were cemented to the non-tissue end of the slide and preparations exposed for 41 h at 4°C in X-ray cassettes. The emulsion was developed (Kodak Dektol; 1:1, 3 min), rinsed briefly in dilute acetic acid (2%) containing 2.5% hardener (Ilford Hypam Hardener) and fixed (Ilford Hypam; 1:4, 2.5 min). Tissue sections were stained with haematoxylin, dehydrated in ethanol, cleared in xylene and mounted for light microscopy. The relative densities of β -adrenoceptors over tracheal epithelium and tracheal smooth muscle from virus-inoculated and control mice were determined by a quantitative autoradiographic technique as described previously (Henry *et al.*, 1990). In these experiments, 8 fields were viewed from each tracheal section; 4 over the epithelial layer, 3 over the smooth muscle band and 1 over non-tissue (background). Thus approximately 4000 fields were analyzed (8 fields from each of 6 tracheal rings on 84 slides). Grain densities were expressed as grains 1000 μ m⁻².

Functional studies

Isolated tracheal smooth muscle segments from virus-inoculated and control mice were prepared for isometric tension recordings as described previously (Henry & Goldie, 1990). The epithelium was not removed intentionally from any tracheal segment. In each experiment, a total of 4 segments were prepared; 2 tracheal segments from a virus-inoculated mouse and 2 tracheal segments from a control mouse. All preparations were suspended under 500 mg resting tension in a 3 ml organ bath containing Krebs-Henseleit solution at 37°C bubbled with 5% CO₂ in O₂. Following a 45 min equilibration period, the tissues were primed by cumulative addition of 0.2 and 10 μ M carbachol. Tissues were washed and rested for 40 min and a concentration-effect curve subsequently obtained to carbachol (0.1-30 μ M).

Mouse isolated tracheal preparations do not exhibit intrinsic tone (Henry *et al.*, 1990) and therefore all concentration-effect curves to relaxant agonists were constructed in preparations pre-contracted with carbachol. In preliminary experiments, the influence of the initial level of carbachol-induced tone on the subsequent relaxant potency of noradrenaline was assessed in tracheal segments from virus-inoculated and control mice. In the functional antagonism studies, preparations were precontracted with carbachol to produce specified levels of induced tone and cumulative concentration-effect curves obtained to noradrenaline (0.01-10 μ M). In all other studies, preparations were contracted with carbachol (EC₅₀ concentration estimated for that particular preparation) and cumulative concentration-effect curves obtained to one of the relaxant agonists, noradrenaline, forskolin or theophylline. Another 2 concentration-effect curves to the relaxants were subsequently constructed in each preparation. Cocaine (10 μ M) and 10 μ M phentolamine were added to the bath 30 min before the start of all concentration-effect curves to inhibit α -adrenoceptors and neuronal and extra-neuronal uptake systems (Goldie & Paterson, 1982). Relaxation responses were expressed as % reversal of the carbachol-induced tone and the relaxation response induced by the highest concentration of agonist was taken as the maximal response (E_{max}). Agonist potency, defined as the concentration of agonist that produced 50% E_{max} (EC₅₀ value), was estimated by fitting the concentration-effect data to a logistic function by computer-assisted nonlinear least squares regression analysis.

Drugs

Drugs used were: (-)-noradrenaline bitartrate, carbamylcholine chloride (carbachol), cocaine hydrochloride, forskolin, theophylline (Sigma Chemical Company, St. Louis, U.S.A.); endothelin-1 (Auspep, Melbourne, Australia); phen-

tolamine mesylate (Ciba-Geigy, Sydney, Australia). The composition of the Krebs Henseleit solution was (in mM): NaCl 117, KCl 5.36, NaHCO₃ 25.0, KH₂PO₄ 1.03, MgSO₄ · 7H₂O 0.57, CaCl₂ · 2H₂O 2.5 and glucose 11.1.

Statistical analyses

All data are presented as arithmetic mean $\pm$ s.e.mean. Estimates of the dissociation constant (K_d) and maximum binding capacity (B_{max}) of specific I-CYP binding were obtained by non-linear least squares regression analyses of data fitted to a single site model. Estimates were also obtained by Scatchard analysis. The nature of the binding was determined by Hill plot analysis. Differences between means were established by Student's two-tailed, non-paired *t* test and were considered significant if $P < 0.05$.

Results

General effects of influenza A/PR-8/34 virus infection

Following intranasal inoculation, the virus proliferated rapidly in the trachea during the initial 2 days, then declined progressively to undetectable levels by day 8 (Figure 1a). In contrast, lung virus titres peaked later (between days 4 and 6) and then fell rapidly to levels that could not be detected by day 10 (Figure 1a). As shown in Figure 1b, virus infection was associated with marked increases in lung weight which peaked at day 6 post-inoculation (88% higher than control mice) and was still elevated by 46% on day 21. The significant increase in lung weight from day 4 onwards was apparently due to the

Figure 1 (a) Influenza A/PR-8/34 virus titres in mouse trachea (□) and lungs (○) at various time points post-inoculation. Data are presented as log EID₅₀/lung or /trachea (mean; $n = 3$ animals per time point, vertical bars show s.e.mean). Detection limits of virus titres were 1.1 log EID₅₀/trachea and 1.9 log EID₅₀/lung. No virus was detected in the trachea or lungs of control mice. The shaded area indicates the period over which some animals died. (b) Lung weights of mice inoculated with virus (○) or sterile PBS (●) at various time points up to 21 days post-inoculation. Data are presented as mean ($n = 3$ mice per time point).

development of peripheral lung inflammation and consolidation which was observed macroscopically and microscopically. The percentage survival rates for mice were 100% up to day 7, 86.7% at day 8, 66.1% at day 9 and 57.8% at day 10. No further deaths were observed between day 10 and 21.

Radioligand binding studies

Specific I-CYP binding to mouse tracheal sections obtained from virus-inoculated and control mice was saturable, involving a single population of non-interacting sites ($nH = 0.96 \pm 0.01$, $n = 6$ experiments). The mean density of specific binding sites for I-CYP in tracheal sections obtained from control mice was 23 ± 6 amols/tracheal section, which is similar to the density previously reported in control mice from this laboratory (22 ± 3 amol/tracheal section, Henry *et al.*, 1990). As shown in Figure 2 and Table 1, the density of specific binding sites for I-CYP (B_{max}) was higher in tracheal sections obtained from virus-inoculated mice than from control mice. On average, tracheal sections from virus-inoculated mice had $41 \pm 12\%$ more specific binding sites for I-CYP than control mice (mean increase calculated over the 3 days tested, i.e. days 2, 4 and 8). There was no significant difference between the estimates obtained for the dissociation constant

Figure 2 Concentration-dependence of [¹²⁵I]-cyanopindolol (I-CYP) binding to 10 μ m frozen sections of (a) control and (b) virus-inoculated mouse trachea (8 days post-inoculation) showing specific (closed symbols) and non-specific (open symbols) binding. Each point represents the mean data from a single experiment conducted using triplicate slides. Also shown is Scatchard analysis (c) of specific I-CYP binding for control (●) and virus-inoculated (■) mouse trachea. Estimates of B_{max} and K_d for days 2, 4 and 8 are presented in Table 1.

Table 1 Binding parameter estimates for $B_{\max}$ and K_d obtained from analysis of [125 I]-cyanopindolol (I-CYP) saturation binding curves in mouse tracheal sections

Days post-inoculation	Treatment	K_d (pM)	$B_{\max}$ (amol/tracheal section)
Day 2	Virus	117.2	51
	Control	112.3	33
	*Ratio	1.04	1.57
Day 4	Virus	45.9	22
	Control	52.6	18
	*Ratio	0.87	1.23
Day 8	Virus	53.8	25
	Control	53.2	17
	*Ratio	1.01	1.44

*Ratio = Virus: Control, for respective K_d and $B_{\max}$ values.

(K_d) of specific I-CYP binding in tracheal sections from virus-inoculated and control mice (Table 1).

Quantitative autoradiography

As shown in Figures 3 and 4, significant densities of autoradiographic grains were located over the tracheal smooth muscle band and epithelial layer in tracheal sections from virus-inoculated and control mice. Quantitative analysis of saturation binding curves constructed from autoradiographic grain counting data demonstrated that there was no significant difference between tracheal sections from virus-inoculated and control mice with respect to the density of specific I-CYP binding sites associated with the smooth muscle band (Figure 3 and Table 2). In sharp contrast, the density of specific grains over the epithelial layer was, on average, $95 \pm 13\%$ higher in tracheal sections from virus-inoculated mice than from control mice (Figures 3 and 4). Similar results were obtained at each time point examined (days 2, 4 and 8 post-inoculation; Table 2). Further analysis of saturation binding curves indicated that the apparent dissociation constant for I-CYP binding to the epithelial layer was consistently lower in tracheal sections from virus-inoculated mice than in control mice (Table 2).

Functional studies

The effects of influenza A/PR-8/34 virus inoculation on mouse isolated tracheal smooth muscle responses to carbachol are shown in Figure 5. Carbachol-induced contractions of tracheal smooth muscle preparations from control mice (mean pD_2 value = 6.62 ± 0.03 ; mean maximum contraction ($C_{\max}$), 1.29 ± 0.06 g; $n = 32$ preparations) and from mice 2-days

Figure 3 Concentration-dependence of [125 I]-cyanopindolol (I-CYP) binding to airway smooth muscle (a,b) and epithelium (c,d) of control (a,c) and virus-inoculated (b,d) mouse trachea showing specific (●) and non-specific (○) binding. I-CYP binding is presented in terms of grain densities estimated from quantitative autoradiography (see Methods). Each curve represents the mean responses of preparations from 6 mice; s.e.mean shown by vertical bars. Trachea were obtained 8 days post-inoculation. Estimates of $B_{\max}$ and K_d for days 2, 4 and 8 are presented in Table 2.

post-inoculation (mean pD_2 value = 6.64 ± 0.05 ; $C_{\max} = 1.35 \pm 0.10$ g; $n = 12$) were not significantly different. However, smooth muscle responsiveness to carbachol was significantly lower in trachea from virus-inoculated mice at both 4 days (virus-inoculated, mean pD_2 value = 6.45 ± 0.04 ; control, mean pD_2 value = 6.73 ± 0.06 , $n = 8$, $P < 0.05$) and 8-days post-inoculation (virus-inoculated, mean pD_2 value = 6.45 ± 0.02 ; control, mean pD_2 value = 6.65 ± 0.04 , $n = 12$, $P < 0.05$). In addition, the mean maximum contraction produced by carbachol was marginally, but statistically significantly lower on day-8 post-inoculation ($C_{\max} = 1.11 \pm 0.06$ g; $P < 0.05$) than in preparations from day-matched control mice. In contrast, the contractile responses of mouse isolated

Table 2 Binding parameter estimates of $B_{\max}$ and K_d obtained from analysis of [125 I]-cyanopindolol (I-CYP) saturation binding curves constructed from autoradiographic grain (G) counting data

Days post-inoculation	Treatment	Smooth muscle		Epithelium	
		K_d (pM)	$B_{\max}^a$ (G $1000 \mu m^{-2}$)	K_d (pM)	$B_{\max}$ (G $1000 \mu m^{-2}$)
Day 2	Virus	113.5	159.5	84.2	186.9
	Control	146.4	159.3	130.8	104.7
	*Ratio	0.78	1.00	0.64	1.79
Day 4	Virus	56.3	119.8	34.4	194.8
	Control	53.9	120.0	61.0	90.4
	*Ratio	1.04	1.00	0.56	2.15
Day 8	Virus	49.9	84.1	53.1	110.8
	Control	89.2	90.8	62.4	57.9
	*Ratio	0.56	0.93	0.85	1.91

*Ratio = Virus: Control, for respective K_d and $B_{\max}$ values.

^a $B_{\max}$ values are presented as G $1000 \mu m^{-2}$. Note that differences in $B_{\max}$ values for control tissues between days 2, 4 and 8 do not represent real differences in maximum β -adrenoceptor densities but simply reflect differences in the specific activity of I-CYP on each of these days.

Figure 4 (a) Dark-field photomicrograph of a tracheal section from a control mouse showing the distribution of autoradiographic grains derived from [125 I]-cyanopindolol (I-CYP) binding (20 pM). (b) Bright-field photomicrograph of the above section. SM = smooth muscle, C = cartilage, E = epithelium. Bar = 100 μ m. (c) Dark-field photomicrograph showing the distribution of non-specific autoradiographic grains in a neighbouring section incubated with 20 pM I-CYP and 1 μ M (–)-propranolol. (d), (e) and (f) are the corresponding micrographs obtained from a virus-inoculated mouse (4 days post-inoculation).

tracheal smooth muscle preparations to endothelin-1 were not significantly attenuated by virus inoculation (Day 2 post-inoculation, mean control $pD_2 = 7.99 \pm 0.05$, mean virus-inoculated $pD_2 = 8.07 \pm 0.15$, $n = 6$; Day 4 and 8 post-inoculation, mean control $pD_2 = 7.99 \pm 0.17$, mean virus-inoculated $pD_2 = 8.00 \pm 0.17$, $n = 6$).

In view of (a) the variable changes in the potency of carbachol observed during the time-course of virus-infection (Figure 5) and (b) the likelihood that β -adrenoceptor agonist relaxant potency will vary depending on the level of carbachol-induced tone (Buckner & Saini, 1975), a series of preliminary experiments was completed to investigate the respective influences of virus-infection and functional antagonism in determining noradrenaline relaxant potency (Figure 6). As early as 2 days post-inoculation with virus, mouse tracheal segments pre-contracted with 0.5 μ M carbachol were hyporesponsive to noradrenaline (mean pD_2 ; control, 6.84 ± 0.06 versus virus-inoculated 6.57 ± 0.04 , $n = 24$, $P < 0.05$, Figure 6a). On the other hand, at 8 days post-inoculation, mouse tracheal segments from virus-inoculated and control mice precontracted with 0.5 μ M carbachol were

equiresponsive to noradrenaline (Figure 6b). However, at this later time point, the level of tone induced by 0.5 μ M carbachol was significantly lower in preparations from virus-inoculated mice ($43 \pm 2\%$ C_{max}) compared with controls ($53 \pm 2\%$ C_{max}). Subsequent functional antagonism experiments in virus-inoculated preparations (and control preparations, data not shown) revealed that noradrenaline was significantly less potent in relaxing preparations precontracted to 53% C_{max} than to 43% C_{max} (mean pD_2 values, 6.36 ± 0.05 and 6.61 ± 0.06 respectively, $n = 12$, $P < 0.05$; Figure 6c). Indeed, when the influence of functional antagonism was reduced (by precontracting day 8 virus-infected and control preparations to similar levels of carbachol-induced tone), noradrenaline was less potent in relaxing tracheal preparations from virus-infected mice (Figure 6d), as previously observed on day 2 post-inoculation (Figure 6a). In all subsequent experiments investigating the influence of virus-inoculation on the relaxant potencies of noradrenaline, forskolin and theophylline, tracheal preparations were precontracted with an EC_{50} concentration of carbachol, rather than a preselected concentration of carbachol (Figure 7).

Figure 5 Mean concentration-effect curves to carbachol for contraction of tracheal segments obtained from control mice (○) and virus-inoculated mice at 2 (●), 4 (□) and 8 (■) days post-inoculation. As expected, there was no significant difference between the mean concentration-effect curves to carbachol in tracheal segments from control mice at days 2, 4 and 8 post-inoculation. Thus, for clarity, the control concentration-effect curve shown represents the combined control data from days 2, 4 and 8 ($n = 32$). Also shown are the maximal levels of carbachol-induced contraction (C_{max}) produced in control preparations (C, $n = 32$) and at 2, 4 and 8 days post-inoculation with virus ($n = 8-12$). Data are presented as mean $\pm$ s.e.mean (* $P < 0.05$ compared to controls).

At both 2 and 8 days post-viral inoculation, mouse tracheal segments were significantly less responsive to noradrenaline and forskolin than were trachea from control animals ($P < 0.05$; Figure 7 and Table 3). At 2 days, but not 8 days post-inoculation, tracheal smooth muscle sensitivity to theophylline was also marginally attenuated compared to control preparations. The maximum relaxation responses (E_{max}) produced by noradrenaline, forskolin and theophylline in tracheal preparations from virus-inoculated mice were not significantly different from the respective E_{max} values obtained in control preparations at 2, 4 or 8 days post-inoculation.

Discussion

In the current study, quantitative autoradiographic analyses of I-CYP binding to mouse tracheal β -adrenoceptors revealed that infection with the respiratory pathogen, influenza A/PR-8/34, was not associated with significant changes in either the density or binding characteristics of tracheal smooth muscle β -adrenoceptors. However, functional studies showed that relaxation responses to the β -adrenoceptor agonist noradrenaline were depressed in tracheal segments from virus-infected mice. These data suggest a functional lesion distal to the β -adrenoceptor moiety. This conclusion is supported by the fact that relaxation responses to the adenylyl cyclase-activator forskolin, were also impaired following virus infection. Although direct measures of adenylyl cyclase activity in tracheal smooth muscle have not been attempted in the current study, it appears that a component of the virus-induced lesion involves processes initiated following β -adrenoceptor activation. There

is other evidence which also suggests that adenylyl cyclase activity may be reduced by respiratory tract viral infection (Halbach & Koschel, 1979). In addition, Scarpace & Bender (1989) found that isoprenaline- and NaF-stimulated adenylyl cyclase activity of lung membranes obtained from mice infected with influenza A/Port Chalmers/73 virus was significantly lower than that observed in membranes from control mice. These findings are consistent with the earlier observation of Busse and coworkers (1979, 1980) showing that the inhibition of β -glucuronidase release from granulocytes exerted by agonists acting via adenosine 3':5'-cyclic monophosphate (cyclic AMP) generation (isoprenaline, histamine and prostaglandin E_2 (PGE_2)) was impaired following infection with Rhinovirus or bivalent (A + B) influenza vaccine. In none of these studies was impaired adenylyl cyclase activity associated with any significant alteration in the number of β -adrenoceptors (Busse *et al.*, 1980; Scarpace & Bender, 1989). At present the mechanism of virus-induced attenuation of relaxation responses to noradrenaline and forskolin is not fully resolved, although several general mechanisms have previously been proposed to explain virus-induced attenuation of adenylyl cyclase activity (e.g. localized physical insult, locally released and/or systemic mediators, direct effect of the virus) (Scarpace & Bender, 1989). The focus of the lesion may be at the level of adenylyl cyclase activity. However, other more distal processes may also be impaired following virus infection, since relaxation responses to theophylline were marginally, but significantly, attenuated at day 2, although no change was seen at day 8 post-inoculation.

Radioligand binding experiments revealed that tracheal sections obtained from mice inoculated with influenza A/PR-8/34 virus, contained 40% more β -adrenoceptors than tracheal sections from control mice. Subsequent quantitative autoradiographic analyses revealed that this increase in total tracheal β -adrenoceptors was due to a 90% increase in the density of β -adrenoceptors in the epithelial layer. The reason for this large increase in tracheal epithelial β -adrenoceptor density following virus inoculation is not clear, although we are currently investigating the possibility that the presence of damaged or regenerating epithelial cells, the influx of inflammatory cells or virus-activated β -adrenoceptor synthesis contribute to this effect. The observation that the density of β -adrenoceptors within different tracheal structures may be variably modified following respiratory tract virus infection is in accord with the recent report of Engels and coworkers (1989) showing that another respiratory pathogen, *Haemophilus influenzae*, significantly changed the density of β -adrenoceptors over many lung structures (alveolar septa, bronchial epithelia etc.) but not airway smooth muscle. In the current study, epithelial function was not assessed. Thus, it is not clear whether the virus-induced increase in epithelial β -adrenoceptor density is linked to changes in epithelial function, e.g. β -adrenoceptor-mediated increases in ciliary beat frequency (Lopez-Vidriero *et al.*, 1985), ion/water secretion (Davis *et al.*, 1979). It has been well established that the epithelium can alter airway smooth muscle reactivity to some bronchodilator and bronchoconstrictor agents. For example, studies of guinea-pig isolated

Table 3 Mean pD_2 values of noradrenaline, forskolin and theophylline for relaxation of tracheal smooth muscle preparations obtained from control and virus-inoculated mice 2 and 8-days post-inoculation

Days post-inoculation	Treatment	Noradrenaline	Forskolin	Theophylline
Day 2	Control	6.84 ± 0.06	7.03 ± 0.07	3.78 ± 0.04
	Virus	$6.57 \pm 0.04^*$	$6.78 \pm 0.04^*$	3.63 ± 0.04
	<i>n</i>	24 (12)	12 (6)	12 (6)
Day 8	Control	6.81 ± 0.04	6.91 ± 0.04	3.69 ± 0.04
	Virus	$6.54 \pm 0.04^*$	$6.70 \pm 0.04^*$	3.63 ± 0.03
	<i>n</i>	12 (6)	12 (6)	12 (6)

* n = number of preparations (number of animals).

* $P < 0.05$ compared to Control value.

Figure 6 Mean concentration-effect curves to noradrenaline for relaxation of mouse isolated tracheal preparations. Each curve shows mean response of 12 preparations from 6 mice; vertical bars show s.e.mean. (a) Tracheal segments obtained from virus-inoculated (●) and control (○) mice 2 days post-inoculation. All preparations were precontracted with $0.5 \mu\text{M}$ carbachol which produced the same level of tone (% C_{max}) in virus-inoculated (filled column) and control (open column) preparations. (b) Tracheal segments obtained from virus-inoculated (●) and control (○) mice 8 days post-inoculation. All preparations were precontracted with $0.5 \mu\text{M}$ carbachol. (c) Tracheal segments obtained from virus-inoculated mice 8 days post-inoculation. Preparations were precontracted with carbachol to produce an initial tonus level of 55% (●) or 43% (■) C_{max} . (d) Tracheal segments obtained from virus-inoculated (●) and control (○) mice 8 days post-inoculation. All preparations were precontracted with carbachol to produce a similar level of induced tone (56–59% C_{max}).

trachea indicate that removal of the epithelium is associated with either small increases in smooth muscle sensitivity to β -adrenoceptor agonists (Hay *et al.*, 1986; Holroyde, 1986) or no change in sensitivity (Goldie *et al.*, 1986). However, at present, the influence of the epithelium on β -adrenoceptor-mediated relaxation of isolated tracheal smooth muscle from either virus-infected or control mice is not known. Thus, the

Figure 7 Mean concentration-effect curves to noradrenaline, forskolin and theophylline for relaxation of carbachol-contracted tracheal segments obtained from virus-inoculated (●) and control (○) mice. Tracheal preparations were used at either (a) 2 days or (b) 8 days post-inoculation. Response data are expressed in terms of mean $\pm$ s.e.mean ($n = 12$ preparations).

extent to which virus-induced changes in the tracheal epithelium contributed to the observed reduction in β -adrenoceptor agonist relaxant potency is not clear, but warrants further investigation.

Although β -adrenoceptor-mediated responses in many preparations are impaired following infection with respiratory tract viruses (Busse *et al.*, 1979; 1980; Lee, 1980; Ogunbiyi *et al.*, 1988; Scarpace & Bender, 1989), attenuation of β -adrenoceptor-mediated relaxation of airway smooth muscle has not previously been reported. Indeed, Eyre and coworkers (1979) found that guinea-pig isolated tracheal strips from animals inoculated with equine influenza virus were 10 fold more sensitive to the relaxant actions of isoprenaline than were non-infected controls. Furthermore, tracheal strips obtained from control guinea-pigs or from animals infected with parainfluenza-3 virus were equiresponsive to the relaxant actions of the β -adrenoceptor agonist salmeterol (Buckner *et al.*, 1981). The reason for these differences is unclear, but may reflect differences between animal species, virus strains or the severity of infection. Whether the small reductions in β -adrenoceptor-mediated relaxations of isolated tracheal smooth muscle observed in the current study would be associated with a significant reduction *in vivo* in airway responsiveness to β -adrenoceptor bronchodilators is not known.

In the present murine model, virus infection was associated with attenuated tracheal smooth muscle responsiveness to the muscarinic agonist carbachol. However, no direct temporal relationship was observed between periods of maximal virus growth (day 2) and attenuated tracheal responsiveness to carbachol (days 4 and 8), suggesting the involvement of subsequent inflammatory processes in this effect, rather than the presence of the virus *per se*. Furthermore, these results are consistent with recent reports that, compared to muscarinic M_2 -cholinoceptors, the M_3 -cholinoceptors of airway smooth muscle are resistant to the neuraminidase, sialic acid-cleaving actions of respiratory tract viruses such as influenza and parainfluenza (Fryer *et al.*, 1990; Jacoby & Fryer, 1990).

Although a small reduction (17%) was observed in the magnitude of the maximum carbachol-induced contraction in trachea taken from mice at day 8 post-inoculation, the mechanism responsible is not clear. It cannot be discounted that differences in either smooth muscle mass or in the length-tension relationship may have contributed to this reduction. However, the attenuated responses to carbachol did not appear to be the result of a generalized effect on the airway smooth muscle since contractile responses to endothelin-1 were not affected by virus-infection.

The present study has clearly demonstrated that influenza A virus infection in mouse trachea resulted in significant attenu-

ation of both airway smooth muscle β -adrenoceptor and muscarinic cholinergic function. Additionally, in airway epithelial cells, which act as the airway site of virus replication, major changes in the density of β -adrenoceptors were apparent. The possibility that these effects were primarily related to inflammatory processes associated with viral infection is under investigation.

This research was supported by the National Health and Medical Research Council of Australia and the Clive and Vera Ramaciotti Foundations (Australia).

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(Received April 9, 1991
Revised August 5, 1991
Accepted August 8, 1991)