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Partial inhibition by epithelium of tracheal smooth muscle relaxation induced by the potassium channel activator, BRL 38227

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1 A method is described whereby either the serosal (Out) or epithelial (In) sides of rat isolated tracheae were selectively perfused. Perfusion with BRL 38227 (10^{-8}–5×10^{-6} M; In/Out) of preparations with intact epithelium (+ EP) precontracted with carbachol (10^{-6} M; Out/In) produced complete relaxation. Perfusion with aminophylline (10^{-5}–10^{-3} M; In) of + EP preparations precontracted with carbachol (10^{-6} M; Out) also produced complete relaxation.

2 In preparations precontracted with carbachol (10^{-6} M) epithelium removal (– EP) increased the sensitivity to the relaxant effect of BRL 38227 (In), but not BRL 38227 (Out) [– log EC_{50}, + EP/ – EP; carbachol (In), BRL 38227 (Out): 6.76 ± 0.11 vs 6.67 ± 0.15; carbachol (Out), BRL 38227 (In): 5.93 ± 0.06 vs 6.25 ± 0.07]. Removal of the epithelium increased also the sensitivity to BRL 38227 (In) of preparations precontracted with a lower concentration (5×10^{-7} M) of carbachol (Out). [– log EC_{50}, + EP/ – EP, carbachol (Out), BRL 38227 (In): 6.19 ± 0.14 vs 6.58 ± 0.17].

3 Removal of the epithelium did not affect the sensitivity to BRL 38227 (In) of preparations precontracted with a higher concentration (5×10^{-6} M) of carbachol (Out).

4 In both + EP and – EP preparations precontracted with carbachol (10^{-6} M; Out), BRL 38227 (In) had a more potent relaxant effect than aminophylline (In) (EC_{50}, BRL 38227 vs aminophylline, + EP/ – EP: 5.93 ± 0.06 vs 3.66 ± 0.11/6.25 ± 0.07 vs 3.77 ± 0.11).

5 In preparations precontracted with carbachol (10^{-6} M; Out), removal of the epithelium did not affect the sensitivity to aminophylline (In) but increased the degree of precontraction (T_{max}) following epithelial but not serosal stimulation with carbachol.

6 We conclude that BRL 38227, a K^+ channel activator, is a potent relaxant of rat tracheal smooth muscle precontracted with carbachol, and that the effect can be partially inhibited by the presence of an intact tracheal epithelium, whereas the relaxant effect of aminophylline is not.

Keywords: Smooth muscle; tracheal epithelium; potassium channel activator; BRL 38227 (lemakalim)

Introduction

Potassium (K^+) channels have been associated with the recovery of the resting potential of excitable cells after depolarization. Indeed, drugs that block K^+ channels have been shown to cause an increase in cellular excitability. In airway smooth muscle, application of K^+ channel blocking drugs results in spontaneous action potentials and a reduced threshold of excitation (Davis *et al.*, 1982; Kannan *et al.*, 1983). These changes appear to be similar to the electrophysiological changes described in asthmatic airways (Akasaka *et al.*, 1975). The recent development of drugs that open K^+ channels in smooth muscle reawakened interest in these channels because these drugs relax airway smooth muscle and thus might reduce airway hyperreactivity which is the main feature of asthma.

Although most attention has been focussed on the role of K^Q channels in airway smooth muscle, these channels have also been shown to be present on many different cell types such as nerve terminals, ganglia, macrophages and epithelial cells (Hall *et al.*, 1988; Kakuta *et al.*, 1988; McCaig & Jonckheere, 1989). This may be relevant in airway disease in which these cells have been shown to play an important role in the mechanism of airway hyperreactivity. In this connection, the role played by airway epithelial cells should be pointed out. Indeed, it has been shown that airway epithelium can modulate bronchial smooth muscle contraction (Cuss & Barnes, 1987; Pavlovic *et al.*, 1989, Vanhoutte, 1988). It is therefore possible that K^+ channels on airway epithelial cells may be implicated in the control of airway tone by the bronchial epithelium. Furthermore, in asthma the bronchial epithelium is often damaged which may influence the effect of some bronchodilator compounds and particularly K^+ channel opening agents which have been shown to inhibit the excitatory NANC (non-adrenergic non-cholinergic) response (Ichinose & Barnes, 1990). The aims of this study were, therefore: (i) to test the relaxant effect in airway smooth muscle of a new K^+ channel opening agent BRL 38227 (lemakalim, (–)-enantiomer of cromakalim) on rat trachea *in vitro* and to compare its potency with another smooth muscle relaxant, aminophylline; (ii) to determine whether the relaxant effect of BRL 38227 in this tissue was influenced by the presence of airway epithelium.

Methods

The method used to prepare rat isolated tracheal smooth muscle preparations has been described by Pavlovic *et al.* (1989). Tracheae were taken from male Sprague-Dawley rats (300–350 g body weight) that had been stunned by a blow on the head and quickly exsanguinated. The tracheae were immersed in Krebs solution (composition mM: NaCl 137, KCl 4, $MgCl_2$ 1, KH_2PO_4 1, $NaHCO_3$ 12, $CaCl_2$ 2, glucose 6.5) and cleaned of all surrounding tissue. Proximal ends (10 rings long) were used for the experiments and distal ends

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were discarded. In one-half of the preparations the epithelium was removed (– EP) by gently rubbing with a cotton-wrapped metal stick; in the other half of the preparations the epithelium was left intact (+ EP).

An organ bath was constructed that permitted independent circulation of fluid within the lumen of the tracheal segment (In, epithelial side) or around the exterior (Out, serosal side) of the tracheal segment (Figure 1). A modified 5 ml syringe with top and lateral openings served as the organ bath. The piston served as a support for the tubing system used to intubate and secure the tracheal segments in place. Mounting of tracheal segments involved the following.

Under microscopic control two stainless steel hooks were passed through the tracheal wall around two adjacent cartilaginous rings as close as possible to the muscle insertions. The tracheal segments were then longitudinally connected to the steel tubes built in the piston and firmly tightened with silk thread. The lower hook was attached below, serving as a fixed point. Its length was adjusted such that it did not pull down the tracheal wall.

The piston was then introduced into the syringe and the upper hook connected to a force transducer (UC2, Gould Cleveland, OH, U.S.A.). The latter was attached to a micromanipulator (Prior PO 22, Prior Scientific Instruments, Herts, UK) that enabled the displacement of the upper hook along a strict vertical axis. Any change of tension at the level of the tracheal muscle was registered by the recorder (Gould AT 550) to which the transducer was connected.

Krebs solution (at 37°C, pH 7.4, gassed with 95% O_2:5% CO_2) was perfused at a constant flow rate (2 ml min^{-1}) through the syringe organ bath (outer perfusion) and through the lumen of the tracheal segment (inner perfusion) by using peristaltic pumps (Watson Marlow 5025, Falmouth, Cornwall, UK).

Fluid tightness of preparation

To ensure that the hooks did not induce a fluid leak through the tracheal wall, a solution of methylene blue was perfused into the tracheal lumen or the organ bath in separate experiments. No cross-staining was observed.

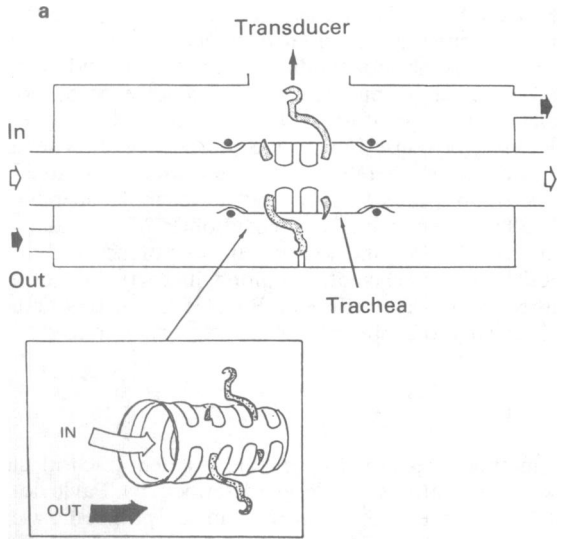

Figure 1 (a) Schematic representation of the experimental apparatus. Inner (In) and outer (Out) perfusions are maintained at 37°C, bubbled with 95% O_2–5% CO_2 and a constant flow rate of 2 ml min^{-1} is maintained.

Procedure

The tracheal muscle was stretched transversely to its optimal length which had been established in preliminary experiments. After a period of stabilization (40–50 min) the preparations were precontracted by perfusing either the epithelial (In) or the serosal (Out) surface of the trachea with a solution of carbachol at a concentration (10^{-6} M) corresponding to the EC_{50}, determined in a preliminary series of experiments (data not shown). In the other series of experiments, the tracheae were perfused with lower (5×10^{-7} M) or higher (5×10^{-6} M) concentrations of carbachol. When the response to carbachol reached a plateau, in a first series of experiments (Table 1), cumulative concentrations (10^{-8} to 5×10^{-6} M) of BRL 38227 were administered either outside or inside the trachea. In this series of experiments, concentration-effect curves for BRL 38227 were constructed. The order of perfusion was such that the bronchoconstrictor agent (carbachol) was always perfused from one side and the bronchodilator agent (BRL 38227) from the other side. To determine whether the relaxant effect was reversible, when the effect of the relaxant agent was maximal, the side where the relaxant agent had been perfused was washed with Krebs solution and then, when the tracheal muscle regained tension, the other side of the trachea which had been perfused with carbachol was washed with Krebs solution until the preparation relaxed completely.

In a second series of experiments (also shown in Table 1) a similar protocol was used. Preparations, precontracted with carbachol, 10^{-6} M (Out), were perfused from the epithelial side (In) with cumulative concentrations (10^{-5} to 10^{-3} M) of aminophylline to compare the relaxant effect of BRL 38227 with that of another smooth muscle relaxant compound. These experiments were performed in preparations with or without epithelium.

After completion of the experiments, 10 tracheal segments without epithelium (– EP) and 10 with epithelium (+ EP) taken at random, were removed from the organ bath and fixed in 2.5% glutaraldehyde. Semi-thin sections from plastic-embedded blocks were then prepared, stained with toluene blue and examined microscopically for the presence of epithelium and/or possible damage of the tracheal wall and epithelium caused by the hooks. A quantification of the epithelium present was performed by estimating the number of nuclei of epithelial cells in the whole circumference of the cross-section of the tracheal segment (intact circumference was taken to be 100%) (Pavlovic *et al.*, 1989).

Materials

The following substances were used: carbachol (carbamylcholine chloride, Sigma Chimie S.a.r.l., 38299 St. Quentin Fallavier, France), BRL 38227 (Beecham Pharmaceuticals, Brockham Park, Betchworth, Surrey), aminophylline (theophylline-ethylene-diamine, Pharmacie Centrale des Hôpitaux, Paris, France). Stock solutions were prepared just before the experiments in distilled water while final dilutions were made with Krebs solution. An initial stock solution (10^{-1} M) of BRL 38227 was made in 70% ethanol.

Analysis of results

The data (changes in tension) are expressed as a percentage of maximal response and in absolute values (g). The results are given as means ± s.e.mean. Half maximal concentrations (EC_{50} values) were calculated from regression analysis of probit-transformed data, and results are given as geometric means of the log EC_{50} obtained. Statistical analysis was conducted by use of Student's t test for paired or unpaired samples. A probability value less than 0.05 was regarded as being statistically significant.

Table 1 Experimental protocol

Preparation	n	*Protocol* (time →)	
+EP	10	C ——— in (10^{-6})	P - - - - - - - - W BRL, out (10^{-8} to 5×10^{-6})
+EP	10	C ——— out (10^{-6})	P - - - - - - - - W BRL, in (10^{-8} to 5×10^{-6})
−EP	10	C ——— in (10^{-6})	P - - - - - - - - W BRL, in (10^{-8} to 5×10^{-6})
−EP	10	C ——— out (10^{-6})	P - - - - - - - - W BRL, in (10^{-8} to 5×10^{-6})
+EP	10	C ——— out (5×10^{-6})	P - - - - - - - - W BRL, in (10^{-8} to 5×10^{-6})
+EP	10	C ——— out (5×10^{-7})	P - - - - - - - - W BRL, in (10^{-8} to 5×10^{-6})
−EP	9	C ——— out (5×10^{-6})	P - - - - - - - - W BRL, in (10^{-8} to 5×10^{-6})
−EP	10	C ——— out (5×10^{-7})	P - - - - - - - - W BRL, in (10^{-8} to 5×10^{-6})
+EP	9	C ——— out (10^{-6})	P - - - - - - - - W Aminophylline, in (10^{-5} to 10^{-3})
−EP	10	C ——— out (10^{-6})	P - - - - - - - - W Aminophylline, in (10^{-5} to 10^{-3})

+EP = with epithelium; −EP = without epithelium; C = carbachol; W = wash, in = inside (epithelial side); out = outside (serosal side); P = plateau; (- - -) = carbachol perfusion; (———) = BRL 38227 or aminophylline perfusion (see text for details).

Results

Histology

Twenty preparations, of which ten had been rubbed for epithelium removal, were selected at random for histological evaluation. In (− EP) preparations 60–80% of the epithelium was removed without any obvious damage to the underlying submucosa or muscle layer. After 3 h in the organ bath, 60–80% of the epithelium remained intact in the control tissues (+ EP).

Contractility

In + EP preparations, BRL 38227 (In) produced a concentration-dependent relaxation of rat tracheal muscle precontracted with 10^{-6} M carbachol (Out). Complete relaxation was obtained with 5×10^{-5} M BRL 38227 (Figure 2). This figure also shows that under the same precontraction conditions, BRL 38227 (In) was much more potent ($P < 0.001$) than aminophylline (In), for which complete relaxation was observed with a concentration of 10^{-3} M (EC_{50}, BRL 38227 vs aminophylline, +/− epithelium: 5.93 ± 0.06 vs 3.66 ± 0.11/6.25 ± 0.07 vs 3.77 ± 0.11, $P < 0.001$).

As shown in Figure 3a and Table 2, in + EP preparations the relaxant effect of BRL 38227 was signficantly more pronounced ($P < 0.001$) when the agent was perfused outside rather than inside the trachea. However, in − EP preparations this difference was abolished (Figure 3b, Table 2).

Removal of the epithelium also increased the sensitivity to BRL 38227 of the preparations precontracted with a lower concentration (5×10^{-7} M) of carbachol but not of preparations precontracted with a higher concentration (5×10^{-6} M) of carbachol (Figure 4, Table 3).

By contrast, as shown in Figure 5, epithelium removal had no effect on the relaxant action of aminophylline, no significant difference in potency being observed with or without epithelium when the drug was perfused inside the trachea.

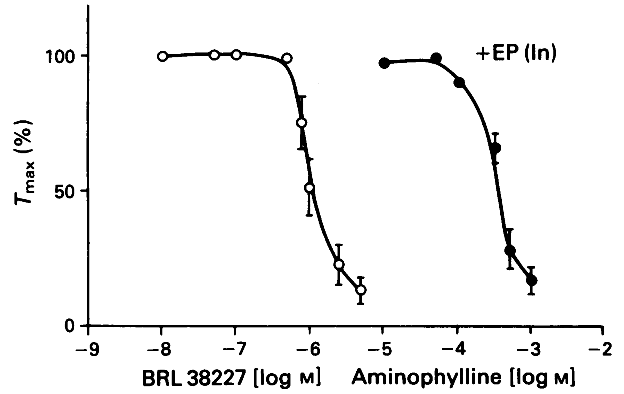

Figure 2 Cumulative concentration-response curves for relaxation induced by either BRL 38277 (○, $n = 10$) or aminophylline (●, $n = 10$) perfused from the epithelial side (In) of the trachea with epithelium precontracted with 10^{-6} M carbachol solution. Tension is expressed as a percentage of the maximal tension (T_{max}) obtained with 10^{-6} M carbachol before administration of BRL 38277 or aminophylline and presented as mean data. Values are mean ± 1 s.e.mean. The two curves represent the results obtained in two different sets of experiments.

The degree of precontraction (T_{max}) obtained by perfusing carbachol 10^{-6} M was not affected by the presence or absence of the epithelium in the preparations exposed to carbachol (Out) but was significantly increased in the preparations exposed to carbachol (In) in − EP preparations (Table 4).

Discussion

The results of the present study indicate that BRL 38227, a K^+ channel activator, is a potent relaxant of rat tracheal smooth muscle precontracted with carbachol and that this effect is partially inhibited by the tracheal epithelium.

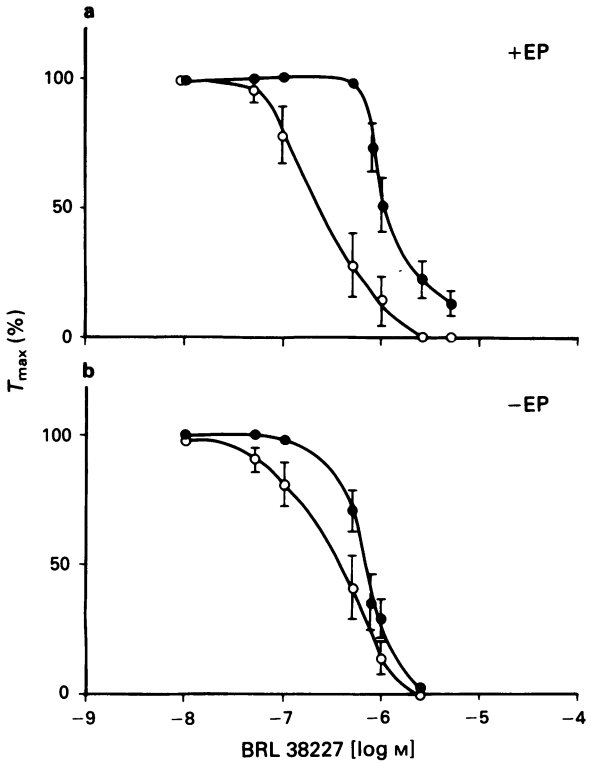

Figure 3 Cumulative concentration-responses curves constructed after administration of BRL 38277 in rat isolated trachea with (a; +EP) and without epithelium (b; −EP). In both preparations BRL 38277 was administered inside (In) (●, $n = 10$) or outside (Out) (○, $n = 10$) the trachea. Tension is expressed as a percentage of the maximal tension (T_{max}) obtained with 10^{-6} M carbachol before administration of BRL 38277 and presented as mean data ± s.e.mean.

Table 2 EC_{50} values for BRL 38227

	BRL In	*BRL Out*	P *(BRL In/BRL Out)*
+EP	5.93 ± 0.06	6.76 ± 0.11	0.001
−EP	6.25 ± 0.07	6.67 ± 0.15	0.01
P (+EP/−EP)	0.005	NS	

Geometric means (± s.e.means) of −log EC_{50} values for relaxation obtained in preparations with (+EP) and without epithelium (−EP) following perfusion from epithelial (In) or serosal sides (Out) with cumulative concentrations of BRL 38227 (BRL). The preparations were precontracted with carbachol 10^{-6} M (NS = non significant).

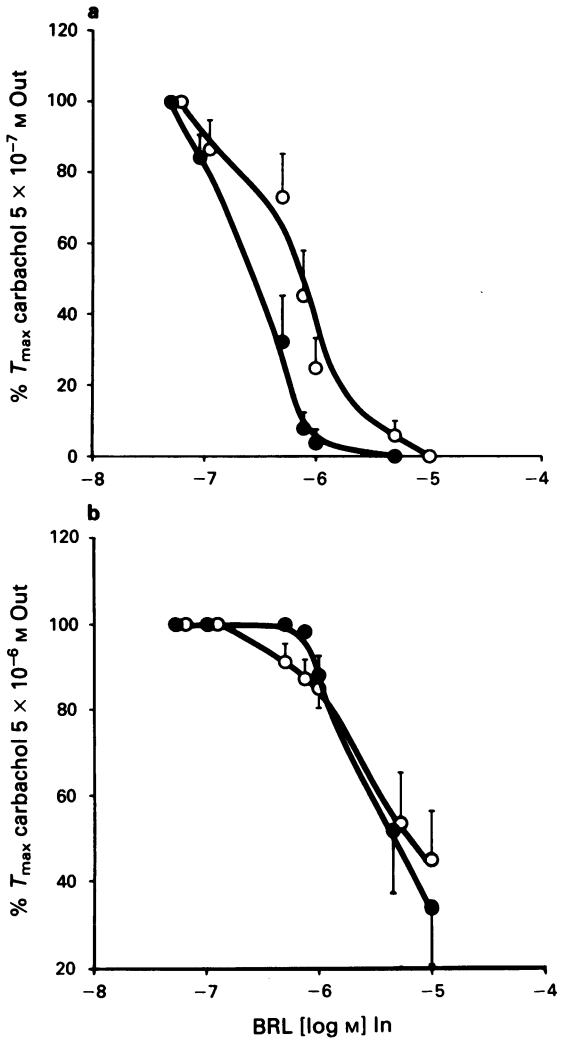

Figure 4 Cumulative concentration-response curves for the relaxant effect of BRL 38227 administered from epithelial side (In) in rat isolated tracheal preparations with (+EP, ○) and without epithelium (−EP, ●). Tension is expressed as a percentage of the maximal (T_{max}) obtained with 5×10^{-7} M carbachol ($n = 9$) (a), or 5×10^{-6} M carbachol ($n = 10$) (b), perfused from the serosal side (Out) and presented as mean data ± s.e.mean.

Table 3 EC_{50} values for BRL 38227 at different carbachol concentrations

	Carbachol Out	
BRL In	5×10^{-7}	5×10^{-6}
+EP	6.19 ± 0.14	5.27 ± 0.18
−EP	6.58 ± 0.07	5.18 ± 0.17
P	<0.03	NS

Geometric means (± s.e.mean) of −log EC_{50} values obtained in preparations with (+EP) and without epithelium (−EP) following epithelial (In) perfusion with cumulative concentrations of BRL 38227. The preparations were precontracted with carbachol 5×10^{-7} and 5×10^{-6} M from the serosal side (Out) (NS = not significant).

The relaxation of rat tracheal smooth muscle that we observed following BRL 38227 administration was similar to that previously described for this compound in guinea-pig (Ichinose & Barnes, 1990) and human isolated bronchi (Black *et al.*, 1990). In the latter study, BRL 38227 was equally effective against contractions induced by carbachol, histamine, or neurokinin A. The maximal relaxation obtained in human bronchi *in vitro* with BRL 38227 amounted to 60–80% of that induced by a maximal concentration of isoprenaline (Black *et al.*, 1990). In our study, the magnitude of the relaxant effect of BRL 38227 was compared to that of aminophylline. However, when compared to aminophylline, BRL 38227 was much more potent, whether the agent was administered inside or outside the trachea. A xanthine compound was used instead of a stimulator of adrenoceptors because rat tracheal smooth muscle has very few β-adrenoceptors (O'Donnell *et al.*, 1987). Indeed, in previous experiments using the same preparation as in the present study we were unable to obtain with salbutamol, (a β_2-agonist) a significant relaxation in rat trachea precontracted with carbachol (unpublished data).

The relaxant effect of BRL 38227 in our model was significantly influenced by the presence of the tracheal epithelium and, when the epithelium was intact, by the route

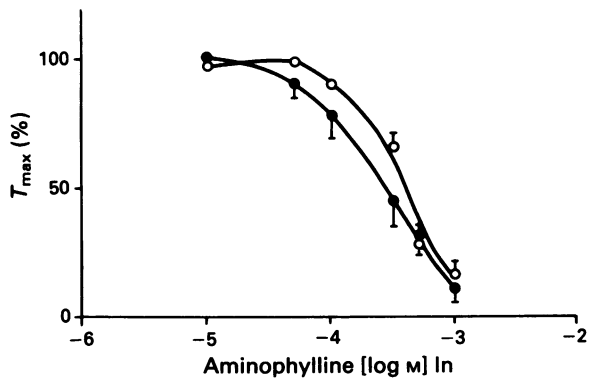

Figure 5 Cumulative concentration-response curve for the relaxant effect of aminophylline perfused from the epithelial side (In) ($n = 10$) in rat isolated tracheal preparations with epithelium (+EP, ○) and without epithelium ($n = 10$) (−EP, ●). Tension is expressed as a percentage of the maximal tension (T_{max}) obtained with 10^{-6} M carbachol before administration of aminophylline and presented as mean data ± s.e.mean.

Table 4 Effects of epithelial removal on carbachol T_{max}

Carbachol (M)	5×10^{-7} Out ($n = 9$)	10^{-6} Out ($n = 10$)	10^{-6} In ($n = 10$)	5×10^{-6} Out ($n = 10$)
−EP	1.1 ± 0.1	1.14 ± 0.08	1.62 ± 0.19	1.4 ± 0.12
+EP	1.32 ± 0.09	1.36 ± 0.12	1.03 ± 0.11	1.68 ± 0.17
P	NS	NS	<0.03	NS

Maximal tension (T_{max}) in g obtained following stimulation with low (5×10^{-7} M), intermediate (10^{-6} M, equivalent to EC_{50}), and high concentrations (5×10^{-6} M) of carbachol perfused from the serosal side (Out) or (for carbachol 10^{-6} M only) also from epithelial (In) side in tracheal preparations with or without epithelium (+EP, −EP) (mean ± s.e.mean, NS = non significant).

of BRL 38227 administration (inside or outside the trachea). Indeed, when the drug was perfused inside the trachea, the relaxant effect of BRL 38227 in tracheae precontracted with carbachol was much less potent in the preparations with epithelium than in those without epithelium. Furthermore, it was also found that BRL 38227 was more effective if administered outside (serosal side) than inside (epithelial side) the trachea with the epithelium intact.

These results suggest that the epithelium may act as a diffusion barrier, limiting access of the drug to the smooth muscle. Indeed, in the same model, we have previously shown that the time course of tension development was longer when carbachol was administered inside the trachea rather than outside, an effect that was abolished when the epithelium was removed (Pavlovic *et al.*, 1989). In the present study, the relaxant effect of BRL 38227 was more pronounced when the epithelium had been removed; no difference was observed when BRL 38227 was administered inside or outside the trachea, contrary to what was noted in the intact preparations. This suggests that the epithelium could act as a diffusion barrier when BRL 38227 was perfused inside the trachea in the preparations with intact epithelium. This hypothesis is further supported by the results obtained in experiments where we tested the effects of BRL 38227 in preparations precontracted with different (higher and lower) concentrations of carbachol. Indeed, it has been suggested (Stuart-Smith & Vanhoutte, 1990) that the type of contractile agent and the level of the excitation might be important in explaining the modulatory effects of bronchial epithelium on airway smooth muscle tone. However, we found that maximal tension of precontraction was changed (increased) only in preparations without epithelium when carbachol was perfused from the epithelial side and BRL 38227 from the serosal side. In this series of experiments we did not observe an effect of epithelium removal on the sensitivity to BRL 38227. Indeed, in the preparations precontracted with low concentrations of carbachol (5×10^{-7} M) the sensitivity to BRL 38227 (In) was strongly influenced by the presence of the bronchial epithelium. By contrast, in the preparations precontracted with the high concentration of carbachol (5×10^{-6} M), the sensitivity to BRL 38227 was not affected by the epithelium removal. The fact that we did not find a difference in sensitivity to BRL 38227 in preparations with or without epithelium precontracted with higher concentrations of carbachol (5×10^{-6} M) further supports our hypothesis that the tracheal epithelium could play the role of a diffusion barrier. At a high level of contractile excitation such as that obtained with a high carbachol concentration, higher concentrations of BRL 38227 are also necessary to obtain the relaxant effect. Assuming that the epithelium has a limited capacity to act as a diffusion barrier, in the presence of high concentrations of BRL 38227 which largely overcome the capacity of the epithelium layer, the effect of a diffusion barrier would be practically unobservable.

Another explanation underlying the modulation of the drug effect by the tracheal epithelium could be a direct effect of BRL 38227 on the epithelial cells or on the release of neuropeptides from sensory nerve terminals. In the latter case, it has been shown that another potassium channel activator, cromakalim, inhibited the excitatory NANC response in guinea-pig airways, probably by reducing the release of neuropeptides from sensory nerve terminals (Ichinose & Barnes, 1990). This effect may be more pronounced when the epithelium had been removed, facilitating the access of BRL 38227 to the sensory nerve terminals. Finally, BRL 38227 may stimulate tracheal epithelial cells to produce some unknown constricting factor. Or, increased permeability of the cell membrane could affect production of the putative EpDIF (epithelium-derived inhibitory factor). Indeed, it has been suggested that potassium exchange at the cell membrane level could be important for the regulation of tracheal smooth muscle tone through various mechanisms which are still not fully elucidated (Fedan *et al.*, 1988; Black & Barnes, 1990).

The present results are difficult to compare with experiments performed earlier by other investigators who used different *in vitro* models, such as bronchial rings (Stuart-Smith & Vanhoutte, 1990) or tracheal spirals (Arch *et al.*, 1988). In these models both sides of the bronchial smooth muscle (epithelial and serosal) were perfused simultaneously. The specific effect of epithelium removal may have been missed by the presence of the agent not only on the epithelial but on the serosal side as well. In our experiments the effect of agents that were examined were perfused exclusively from either the epithelial or serosal side.

By contrast to what was observed with BRL 38227, the relaxant effect of aminophylline was not influenced by the removal of the epithelium. Thus although it has been previously suggested that the relaxant effect of aminophylline depends on the presence of epithelium (Papadimikiou *et al.*, 1988) our results do not confirm this observation. This discrepancy may be explained by the preparation we used in which we could separate the application of aminophylline to the epithelial and serosal sides of the trachea.

Whatever the mechanisms by which the relaxant effect of BRL 38227 is influenced by the tracheal epithelium, this observation may be important in asthma since it has been shown that in these patients, airway epithelium is damaged (Laitinen *et al.*, 1985). Our finding that the relaxant effect of BRL 38227 is more potent after epithelium removal may therefore have a therapeutic interest, particularly if the compound is given by inhalation.

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