Effects of BRL 38227 on neurally-mediated responses in the guinea-pig isolated bronchus

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1 In guinea-pig isolated bronchus treated with indomethacin (2.8 μ M), electrical field stimulation (EFS; 10 Hz, 0.5 ms, 60–70 V, for 10 s) evoked a tetrodotoxin (3 μ M)-sensitive, biphasic contraction comprising a rapid, atropine (1 μ M)-sensitive cholinergic response succeeded by a slowly developing, capsaicin (10 μ M)-sensitive, non-adrenergic, non-cholinergic excitatory (NANCe) response.

2 BRL 38227 (0.3–3 μ M), salmeterol (0.003–3 μ M) and ketotifen (1.0–300 μ M) each produced concentration-dependent inhibition of both NANCe and cholinergic responses to EFS in guinea-pig isolated bronchus.

3 Substance P (SP; 1 μ M) and neurokinin A (NKA; 0.07 μ M) produced contractions equivalent in magnitude to the NANCe response to EFS, which were inhibited by salmeterol (1 μ M), but not by BRL 38227 (3 μ M) or ketotifen (100 μ M).

4 Acetylcholine (ACh; 6 μ M) was equi-effective with the electrical activation of cholinergic neurones. BRL 38227 (3 μ M) slightly inhibited responses to ACh (6 μ M). Salmeterol (1 μ M) and ketotifen (100 μ M) markedly inhibited responses to ACh (6 μ M).

5 In bronchial rings pre-contracted with ACh (100 μ M), BRL 38227 (0.1–30 μ M), salmeterol (0.001–3 μ M) and ketotifen (0.1–100 μ M) each produced concentration-dependent relaxation. Unlike ketotifen, BRL 38227 and salmeterol only partially ($18.8 \pm 2.1\%$ and $51.8 \pm 3.9\%$ respectively) reversed the ACh-induced contraction.

6 The (+)-analogue of BRL 38227, BRL 38226 (0.3–100 μ M), was without effect on responses to EFS and had no effect on the inhibition caused by BRL 38227. The K⁺-channel activators pinacidil (3.0–30 μ M) and RP 52891 (3.0–30 μ M) exerted similar inhibitory actions on responses to EFS as BRL 38227, but were less potent. Glibenclamide (0.1–1.0 μ M) and phentolamine (3 μ M) antagonized the inhibitory effects of BRL 38227 on responses to EFS.

7 It is concluded that BRL 38227 and ketotifen can inhibit NANCe neuroeffector transmission at concentrations exerting little or no inhibitory effects on responses to exogenously applied tachykinins. By contrast, in addition to suppressing NANCe responses to EFS, salmeterol also markedly inhibits responses to SP and NKA. At concentrations markedly suppressing cholinergic neuroeffector transmission, BRL 38227 has only minor effects on responses to exogenously-applied ACh. Salmeterol and ketotifen both depress responses to ACh within the concentration-range over which they inhibit cholinergic responses to EFS. The inhibitory effects of BRL 38227 on responses to EFS exhibit stereo-specificity and may involve the opening of a neuronal K⁺-channel. This K⁺-channel is glibenclamide- and phentolamine-sensitive and appears similar to the smooth muscle K⁺-channel which is modulated by BRL 38227.

Keywords: BRL 38227; guinea-pig bronchus; cholinergic nerves; NANCe nerves; glibenclamide; phentolamine

Introduction

It is becoming increasingly recognised that, in addition to bronchospastic episodes, chronic inflammatory changes in the airways are likely to be of importance in the pathogenesis of asthma. It has been suggested that the activation of a subset of sensory nerve endings, via an axon reflex, may contribute to the inflammatory reaction in asthmatic airways. In particular, the release of tachykinins such as substance P (SP) and neurokinin A (NKA) from non-adrenergic, non-cholinergic excitatory (NANCe) nerves has been implicated (Barnes *et al.*, 1990). Agents interfering with NANCe nerve activity might, therefore, exert an anti-inflammatory effect in the airways.

The ability of K⁺-channel activators such as cromakalim to relax airways smooth muscle contracted by a variety of mediators is well established (Allen *et al.*, 1986; Arch *et al.*, 1988). More recently, work in guinea-pig airways has suggested that cromakalim might also exert an inhibitory effect on the activity of cholinergic and NANCe nerves (Hall & MacLagan, 1988; McCaig & de Jonckheere, 1989; Ichinose &

Barnes, 1990; Lewis & Raeburn, 1990; Burka *et al.*, 1991). In these studies, cromakalim was more effective in suppressing responses evoked by the electrical activation of neuronal elements, than contractions produced by exogenously applied acetylcholine (ACh) or SP. It was therefore suggested that cromakalim was acting at a prejunctional site, to inhibit neurotransmitter release.

The present study was undertaken to assess the effects of BRL 38227, the active (–)-enantiomer of cromakalim (Buckingham *et al.*, 1986), on NANCe and cholinergic nerve-mediated responses to electrical field stimulation (EFS) in the guinea-pig isolated bronchus. The anti-asthma drugs ketotifen and the long-acting β_2 -adrenoceptor agonist salmeterol were also included in this study in view of their suggested anti-inflammatory effects in the airways (Kamikawa, 1989; Grant *et al.*, 1990; Twentyman *et al.*, 1990; Whelan & Johnson, 1990). We have sought to establish whether BRL 38227, salmeterol and ketotifen inhibit excitatory neuroeffector transmission in the guinea-pig bronchus, and if so, whether this is mediated at a pre- or post-junctional site. In addition, we have attempted to characterize the mechanism by which BRL 38227 is producing its effects. A preliminary account of

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this work has previously been communicated to the British Pharmacological Society (Good & Hamilton, 1991).

Methods

Male, Dunkin-Hartley guinea-pigs (300–500 g) were killed by stunning and exsanguination. Ring segments 2–3 mm in length were prepared from the main bronchi. Each segment was suspended for the recording of isometric tension changes, under a resting tension of 0.5–1.0 g. The Krebs solution bathing the tissues contained indomethacin (2.8 μM). The tissues were allowed a half hour equilibration period.

Field stimulation experiments

Bronchial rings were mounted between pairs of platinum electrodes, 1 cm apart. NANCe and cholinergic nerve-mediated responses were evoked by the application of 10 s trains of 60–70 V, biphasic, 0.5 ms pulses at a frequency of 10 Hz. A minimum of 15 min was allowed between successive stimuli to allow baseline levels of tension to be regained. Before the start of an experiment, 2–3 responses to electrical field stimulation (EFS) were obtained to establish their reproducibility.

Cumulative concentration-effect curves for one of BRL 38227 (0.3–1.0 μM), BRL 38226 (0.3–100 μM), pinacidil (0.3–10 μM) RP 52891 (0.3–10 μM), ketotifen (1.0–300 μM), salmeterol (0.03–3.0 μM), or the appropriate vehicle were constructed. Each concentration of drug was allowed a 10 min equilibration period before the tissues were subjected to EFS. An exception was salmeterol, where a 20 min pre-incubation was used. Response were expressed as a % of the response to EFS immediately prior to drug exposure.

The ability of glibenclamide (0.1–1.0 μM), phentolamine (3.0–30 μM) or BRL 38226 (0.5, 1.5, 5.0 and 30 μM) to antagonize the actions of BRL 38227 was investigated. Tissues were pre-incubated with a single concentration of glibenclamide, phentolamine or BRL 38226, or the relevant vehicle, for 30 min. EFS was then applied. Cumulative concentration-effect curves for BRL 38227 were then constructed as described above. Responses were expressed as a % of the response to EFS obtained following exposure to antagonist, but before exposure to BRL 38227.

Exogenously-applied spasmogens

The effects of pretreatment with BRL 38227 (3 μM), salmeterol (1 μM) or ketotifen (100 μM) on contractions evoked by ACh, SP and NKA were examined. Preliminary experiments established that ACh (6 μM) evoked contractions equivalent in magnitude to the cholinergic response following EFS (~30% histamine (1 mM) maximum). Similarly SP (1 μM) and NKA (0.07 μM) were equi-effective with the activation of NANCe neurones by EFS (~20% histamine (1 mM) maximum).

Bronchial rings were initially challenged with one of SP (1 μM), NKA (0.07 μM) or ACh (6 μM). Once the evoked spasm had developed, the tissues were washed at 15 min intervals until baseline levels of tension were regained. Following incubation with BRL 38227 (3 μM for 10 min), ketotifen (100 μM for 10 min) or salmeterol (1 μM for 20 min) or their relevant vehicles, the tissues were re-challenged with SP, NKA or ACh, as appropriate. Responses are expressed as a % of the response to spasmogen before exposure to BRL 38227, salbutamol or ketotifen.

In a further set of experiments, the spasmolytic properties of BRL 38227, salmeterol and ketotifen in the guinea-pig bronchus were examined. Tissues were precontracted with ACh (100 μM ; EC_{70}). Cumulative concentration-effect curves for BRL 38227 (0.1–30 μM), ketotifen (0.1–100 μM) and salmeterol (0.001–3 μM) were then constructed. Tissue contact times were 10 min for BRL 38227 and ketotifen and

20 min for salmeterol. Time-matched control tissues received the appropriate concentration of vehicle. Since BRL 38227 and salmeterol failed to produce full reversal of the tension increment induced by ACh, responses were expressed as % relaxation of the initial spasmogen-induced tone.

Drugs and solutions

The physiological salt solution used in the present experiments was a Krebs solution of the following composition (mM): Na^+ 143, K^+ 5.9, Mg^{2+} 1.2, Ca^{2+} 2.5, Cl^- 123, SO_4^{2-} 1.2, $\text{H}_2\text{PO}_4^{4-}$ 1.2, HCO_3^- 25, and glucose 10.1. Drugs used, together with their sources are listed below. Acetylcholine chloride (Sigma), aminophylline (Sigma), atropine hydrobromide (Sigma), capsaicin (Sigma), glibenclamide (Roussel), indomethacin (Sigma), ketotifen (Sandoz), neurokinin A (Sigma), phentolamine mesylate (Ciba), RP 52891 (Rhône-Poulenc Rorer), substance P (Sigma), tetrodotoxin (Sigma), BRL 38227, BRL 38226, pinacidil and salmeterol were synthesized by the laboratories of SmithKline Beecham Pharmaceuticals.

Stock solutions of acetylcholine, indomethacin and capsaicin were prepared in absolute ethanol. Glibenclamide was dissolved in dimethylsulphoxide (DMSO). Stock solutions of salmeterol and pinacidil were prepared in N/10 HCl. BRL 38227, BRL 38226, RP 52891 and ketotifen were each dissolved in polyethylene glycol (PEG) and diluted as appropriate with twice distilled water. All other stock solutions were prepared in twice distilled water.

Analysis of results

Data are presented as mean values $\pm$ s.e.mean. Potency estimates represent the concentration of drug required to produce a 50% (NANCe) or 30% (cholinergic) decrease in the response to EFS. These were calculated as geometric means $\pm$ s.e.mean of values derived from individual log concentration-effect curves. The significance of differences between mean values was assessed by a two-way, unpaired Student's *t* test, Duncan's multiple range test, or Dunnett's test. Differences were considered significant when calculated probability (*P*) values were less than 0.05.

Time- and vehicle-matched control experiments suggested that there was a progressive reduction in the size of the response of bronchial rings to repeated field stimulation, or successive challenges with spasmogenic agents. In order to prevent these changes influencing the inhibitory effects of any drugs tested, all responses in these experiments were corrected for changes in their individual time- and vehicle-matched controls. This procedure involved calculating the fractional change in the response of the individual time- and vehicle-matched control tissues and using this value to adjust the corresponding response of the test tissues.

Results

General features of the response to EFS

Field stimulation of guinea-pig isolated bronchial rings treated with indomethacin (2.8 μM) evoked a biphasic, contractile response (Figure 1a,b). This was abolished by tetrodotoxin (3 μM), suggesting it was neurogenic in origin (data not shown). The initial (cholinergic) component developed and decayed rapidly and was abolished by atropine (1 μM ; Figure 1a). This was succeeded by a secondary component which developed over a period of 1–2.5 min and thereafter declined to baseline levels within 10–15 min (Figure 1a(i) and b(i)). This latter response persisted in the presence of atropine (Figure 1a(ii)), but was abolished by prior exposure to capsaicin (10 μM ; Figure 1b(ii)), suggesting the involvement of NANCe nerves (Andersson & Grundstrom, 1983; Lundberg *et al.*, 1983). The magnitude of the cholinergic response was

reduced following exposure to capsaicin (10 μM ; Figure 1b(ii)).

Effects of potassium channel activators on responses to EFS

BRL 38227 (0.1–3.0 μM), pinacidil (3.0–30 μM) and RP 52891 (3.0–30 μM) each produced a concentration-dependent suppression of EFS-evoked responses in guinea-pig isolated bronchus treated with indomethacin (2.8 μM) (Figure 2a,c,d). The rank order of potency for the inhibition of both the NANCe and the cholinergic components of the response was BRL 38227 > pinacidil $\geq$ RP 52891 (Table 1). None of the K⁺-channel activators tested completely suppressed contractions of the bronchial smooth muscle evoked by EFS (Figure 2a,c,d). However, each drug was more effective as an inhibitor of NANCe, than of cholinergic responses (Table 1, Figure 2a,c,d). Over the range 0.3–100 μM , BRL 38226, the (+)-isomer of BRL 38227, failed to produce significant suppression of either NANCe or cholinergic responses to EFS (Figure 2b).

Effects of salmeterol and ketotifen on responses to EFS

Salmeterol (0.03–3 μM) produced a concentration-dependent suppression of responses to EFS (Figure 3a). It inhibited

Figure 1 Responses to electrical field stimulation (EFS) in guinea-pig isolated bronchus, before (i) and after (ii) exposure to (a) atropine (1 μM) or (b) capsaicin (10 μM). In both (a) and (b), panels (i) and (ii) show responses of the same bronchial ring.

Table 1 Effects of potassium channel activators, salmeterol and ketotifen on responses to electrical field stimulation (EFS) in guinea-pig isolated bronchus

Drug		NANCe response	Cholinergic response
BRL 38227	^a potency	6.09 $\pm$ 0.09	6.12 $\pm$ 0.16
(n = 6)	^b maximum	80.9 $\pm$ 3.8%	57.1 $\pm$ 6.0%
Pinacidil	^a potency	5.45 $\pm$ 0.15*	5.50 $\pm$ 0.02*
(n = 5)	^b maximum	85.6 $\pm$ 4.8%	53.5 $\pm$ 5.9%
RP 52891	^a potency	5.38 $\pm$ 0.11*	5.39 $\pm$ 0.13*
(n = 6)	^b maximum	73.4 $\pm$ 11.2%	48.0 $\pm$ 6.9%
Salmeterol	^a potency	7.22 $\pm$ 0.22*	6.82 $\pm$ 0.10*
(n = 6)	^b maximum	97.5 $\pm$ 2.5%*	81.2 $\pm$ 3.6%*
Ketotifen	^a potency	3.84 $\pm$ 0.16*	4.62 $\pm$ 0.11*
(n = 6)	^b maximum	98.1 $\pm$ 1.9%*	98.6 $\pm$ 1.4%*

Values are means $\pm$ s.e.mean. ^aPotency estimates represent $-\log \text{IC}_{50}$ values for NANCe responses and $-\log \text{IC}_{30}$ values for cholinergic responses. ^b% reduction of initial response to EFS.

Significant difference from the response to BRL 38227:

* $P < 0.05$.

NANCe responses more potently than cholinergic responses (Figure 3a, Table 1). Salmeterol was the most potent inhibitor of electrically-evoked responses tested (Table 1) and, in a concentration of 1 μM , virtually abolished the response to EFS (Figure 3a).

Over the range 30–300 μM , ketotifen produced a concentration-dependent suppression of responses to EFS (Figure 3b), but was the least potent agent tested (Table 1). In 5 out of the 6 tissues, the highest concentration of ketotifen tested (300 μM), evoked a marked contraction of the bronchial smooth muscle. Like salmeterol, ketotifen, in adequate concentrations, abolished completely EFS-evoked contractions (Figure 3b). However, unlike salmeterol and the K⁺-channel activating agents, it inhibited cholinergic responses more potently than NANCe responses (Table 1).

Effects of BRL 38227, salmeterol and ketotifen on responses to exogenously-applied spasmogens

BRL 38227 (3 μM), a concentration which was maximally effective in causing suppression of NANCe responses to EFS (Figure 2a), had no significant effect on responses to exogenously-applied SP (1 μM) or NKA (0.07 μM) (Table 2). BRL 38227 (3 μM) produced a small, but nevertheless significant inhibition of responses to ACh (6 μM). However, this was less than its inhibitory effect on cholinergic responses to EFS (Table 2).

Ketotifen (100 μM) suppressed NANCe responses to EFS, but had no significant effect on contractions evoked by SP (1 μM) or NKA (0.07 μM) (Table 2). By contrast, responses to exogenously-applied ACh (6 μM) were almost completely abolished by ketotifen (100 μM). Indeed, ketotifen (100 μM) inhibited responses to ACh (6 μM) to a greater extent than the cholinergic component of the response to EFS (Table 2). Salmeterol (1 μM) markedly reduced responses to each of SP (1 μM), NKA (0.07 μM) and ACh (6 μM) (Table 2).

Spasmolytic effects of BRL 38227, salmeterol and ketotifen in ACh-precontracted bronchial rings

In bronchial rings precontracted with ACh (100 μM), BRL 38227, salmeterol and ketotifen each evoked concentration-dependent relaxation (Figure 4). However, there were marked differences in the maximal effects of these three agonists, with only ketotifen causing a full suppression of ACh-induced tension (Figure 4). Salmeterol exhibited a greater potency than BRL 38227 and ketotifen. The latter two agents produced relaxation over a similar concentration range (Figure 4).

Figure 2 Effects of (a) BRL 38227, (b) BRL 38226, (c) pinacidil and (d) RP 52891 on non-adrenergic, non-cholinergic excitatory (●) and cholinergic (▲) responses to electrical field stimulation (EFS) in guinea-pig isolated bronchus. Data are means of values from 6 tissues, s.e.means are shown by the vertical bars. *Significant suppression of the response to EFS ($P < 0.05$).

Effects of BRL 38226, glibenclamide and phentolamine on BRL 38227-induced suppression of responses to EFS

By itself, glibenclamide (0.1 – $1 \mu\text{M}$) had no significant effect on responses to EFS (Table 3), but caused a concentration-dependent rightward shift of the log concentration-effect curve for BRL 38227 in field stimulated, guinea-pig isolated bronchus (Figure 5a,b). In that the shifts of the curves appeared parallel, and the maximum response to BRL 38227 was not significantly reduced, the antagonistic effect of glibenclamide appeared to be competitive.

Phentolamine ($3 \mu\text{M}$) had no effect on the response of guinea-pig isolated bronchus to EFS (Table 3), but reduced

the suppressant effects of BRL 38227 (Figure 6a,b). Higher concentrations of phentolamine (10 – $30 \mu\text{M}$) caused no further reduction in responses to BRL 38227, but there was a tendency for the log concentration-effect curve to become flattened and to shift back towards the control curve (Figure 6a,b). However, at concentrations greater than $3 \mu\text{M}$, phentolamine itself caused a concentration-dependent suppression of responses to EFS (Table 3).

BRL 38226 (0.5 , 1.5 , 5 and $30 \mu\text{M}$) itself had no effect on responses to EFS (Table 3) and, furthermore, did not modify the inhibition of responses to EFS produced by BRL 38227 (Figure 7a,b).

Table 2 Comparative effects of BRL 38227, salmeterol and ketotifen on responses to electrical field stimulation, substance P (SP), neurokinin A (NKA) and acetylcholine (ACh)

	% initial response to spasmogenic stimulus				
	NANC	SP ($1 \mu\text{M}$)	NKA ($0.07 \mu\text{M}$)	Cholinergic	ACh ($6 \mu\text{M}$)
BRL 38227 ($3 \mu\text{M}$)	$13.0 \pm 2.5^*$	64.7 ± 10.0	64.3 ± 6.0	$36.5 \pm 3.6^*$	$76.4 \pm 6.5^*$
Vehicle	69.8 ± 2.2	80.8 ± 7.1	81.2 ± 7.6	87.9 ± 4.2	95.2 ± 5.0
Salmeterol ($1 \mu\text{M}$)	$7.3 \pm 3.9^*$	$45.8 \pm 3.6^*$	$36.6 \pm 8.5^*$	$28.4 \pm 5.5^*$	$47.7 \pm 3.3^*$
Vehicle	86.5 ± 4.5	99.2 ± 8.0	101.7 ± 4.2	97.8 ± 2.8	96.8 ± 5.4
Ketotifen ($100 \mu\text{M}$)	$54.0 \pm 4.9^*$	79.3 ± 4.4	92.9 ± 4.9	$41.3 \pm 8.9^*$	$2.3 \pm 1.1^*$
Vehicle	84.7 ± 3.7	75.2 ± 3.3	105.5 ± 9.8	97.1 ± 2.9	96.8 ± 5.4

Values are means $\pm$ s.e.mean, $n = 6$ – 12 .

*Significant difference from vehicle control ($P < 0.05$).

Figure 3 Effects of (a) salmeterol and (b) ketotifen on non-adrenergic, non-cholinergic excitatory (●) and cholinergic (▲) responses to electrical field stimulation (EFS) in guinea-pig isolated bronchus. Data are means of values from 6 tissues, s.e.means are shown by the vertical bars. *Significant suppression of the response to EFS ($P < 0.05$).

Discussion

NANCe responses to EFS: pre- or post-junctional site of action?

NANCe responses of the guinea-pig isolated bronchus to EFS are believed to reflect the antidromic activation of a subset of intramural sensory neurones (Andersson & Grundstrom, 1987). The ability of capsaicin to abolish NANCe responses (Andersson & Grundstrom, 1983; Lundberg *et al.*, 1983; Present Study, Figure 1b), suggests that the transmitter(s) in this pathway is a sensory neuropeptide (Buck & Burks, 1986), although its identity remains to be established. SP has previously been considered a strong candidate (Lundberg *et al.*, 1983). However, it has recently been suggested that NKA may in fact mediate NANCe nerve-mediated contractions. Thus, dactinomycin inhibited contractions of guinea-pig bronchus evoked by NANCe nerve stimulation and NKA, but had no effect on responses to SP (Lundberg & Lou, 1991).

Figure 4 Spasmolytic effects of BRL 38227 (●), salmeterol (▲) and ketotifen (■) in acetylcholine (ACh, 100 μM)-precontracted guinea-pig isolated bronchus. Data are means of values from 6 tissues, s.e.means are shown by the vertical bars. *Significant suppression of ACh-induced tone ($P < 0.05$).

BRL 38227 (3 μM) and ketotifen (100 μM) each attenuated NANCe responses to EFS, but had no significant effect on the spasm evoked by an equi-effective challenge with SP (1 μM) or NKA (0.07 μM). This shows good agreement with previous reports, where cromakalim was tested in guinea-pig isolated trachea (Burka *et al.*, 1991) and ketotifen in guinea-pig bronchus (Kamikawa, 1989). These findings may suggest that the inhibitory effects of BRL 38227 (3 μM) and ketotifen (100 μM) on NANCe responses to EFS are unlikely to result from inhibition of the response of the bronchial smooth muscle to the NANCe transmitter, but, instead, may reflect a pre-junctional effect on NANCe nerve neurotransmission. One possibility is that BRL 38227 and ketotifen may reduce the amount of transmitter released in response to EFS (Kamikawa, 1989; Burka *et al.*, 1991). However, this requires confirmation, for instance by direct measurement of tachykinin release from guinea-pig bronchus in response to EFS.

The ability of salmeterol (1 μM) to attenuate markedly responses to SP and NKA suggests that, by contrast, a large component of its inhibitory effects on NANCe responses to EFS can be attributed to depression of bronchial smooth muscle responsiveness. Similar conclusions were reached when another β -adrenoceptor agonist, isoprenaline, was tested in the guinea-pig bronchus (Kamikawa & Shimo, 1990).

Cholinergic responses to EFS: pre- or postjunctional site of action?

That the initial, rapid response to EFS was mediated by cholinergic neurones is suggested by its sensitivity to atropine (1 μM) (Figure 1a). The present findings that capsaicin reduces the magnitude of cholinergic-nerve mediated responses agrees well with previous work by Stretton *et al.* (1989), and may suggest that NANCe nerves exert a facilitory effect on cholinergic neurotransmission in the guinea-pig bronchus.

The cholinergic component of the response to EFS was inhibited by BRL 38227, albeit less potently and effectively than were NANCe responses (Figure 2a). By contrast with its effect on nerve-mediated responses, BRL 38227 was relatively ineffective as a relaxant of ACh-induced tone in the guinea-pig bronchus (Figure 4), and caused only a small reduction in the response to a challenge with ACh (6 μM) (Table 2). This suggests that BRL 38227 may also modulate cholinergic

Figure 5 Effects of glibenclamide 0.1 μM (▲), 0.3 μM (■), 1.0 μM (◆) or dimethylsulphoxide (---○---) on responses to BRL 38227 in field stimulated, guinea-pig isolated bronchus. (a) Non-adrenergic, non-cholinergic excitatory responses to electrical field stimulation (EFS), (b) cholinergic responses to EFS. Data are means of values from 5–6 tissues, s.e.means are shown by the vertical bars.

Table 3 Effects of glibenclamide, phentolamine and BRL 38226 on responses to electrical field stimulation (EFS) in guinea-pig isolated bronchus

		% Initial response to EFS	
		NANC responses	Cholinergic responses
Vehicle		87.8 ± 4.1	100.7 ± 2.3
Glibenclamide	0.1 μM	101.4 ± 6.9	104.2 ± 2.7
	0.3 μM	93.9 ± 7.1	107.9 ± 3.7
	1.0 μM	95.9 ± 5.6	109.1 ± 3.3
Vehicle		100.5 ± 4.1	102.5 ± 2.9
Phentolamine	3 μM	99.3 ± 2.5	99.9 ± 2.9
	10 μM	76.6 ± 10.4*	88.8 ± 5.8*
	30 μM	43.8 ± 7.6*	54.6 ± 4.1*
	100 μM	13.6 ± 7.2*	22.7 ± 6.9*
Vehicle		95.6 ± 4.3	99.6 ± 3.7
BRL 38226	0.5 μM	88.6 ± 5.4	98.9 ± 3.8
	1.5 μM	80.2 ± 9.2	91.5 ± 6.2
	5.0 μM	94.4 ± 6.3	102.9 ± 4.2
	30 μM	95.9 ± 4.9	100.3 ± 4.2

Values are means ± s.e.mean, $n = 4-6$.

*Significant difference from vehicle-treated tissues ($P < 0.05$).

nerve activity in the guinea-pig bronchus, by an action at a prejunctional site. These findings show good agreement with previous studies where BRL 38227 was tested in the electrically stimulated, guinea-pig isolated trachea (Cooper *et al.*, 1991) and cromakalim was tested against cholinergic nerve- and ACh-induced bronchoconstriction in the anaesthetized guinea-pig (Ichinose & Barnes, 1990; Lewis & Raeburn, 1990). However, the present study contrasts with the work of McCaig & de Jonckheere (1989), who reported that cromakalim failed to affect responses to EFS in the guinea-pig trachea, although responses to selective, preganglionic, stimulation of vagal nerves were inhibited.

Again, quantification of the effects of BRL 38227 on the amount of transmitter released by bronchial cholinergic nerves in response to EFS is required to establish its site of action. It is noteworthy that investigations of the effects of a K^+ -channel activator on [3H]-ACh release caused by field stimulation of cholinergic nerves in the guinea-pig trachea have failed to demonstrate any inhibition (Wessler *et al.*, 1991).

By contrast with BRL 38227, ketotifen (100 μM) and salmeterol (1 μM) each markedly inhibited responses to

Figure 6 Effects of phentolamine 3 μM (▲), 10 μM (■), 30 μM (◆), or twice distilled water (---○---) on responses to BRL 38227 in field stimulated guinea-pig isolated bronchus. (a) Non-adrenergic, non-cholinergic excitatory responses to electrical field stimulation (EFS), (b) cholinergic responses to EFS. Data are means of values from 5–6 tissues, s.e.means are shown by the vertical bars. *Significant difference from tissues treated with twice distilled water ($P < 0.05$).

Figure 7 Effects of BRL 38227 0.5 μM (▲), 1.5 μM (■), 5 μM (◆), 30 μM (●) or polyethylene glycol (—○—) on responses to BRL 38227 in field stimulated, guinea-pig isolated bronchus. (a) Non-adrenergic, non-cholinergic excitatory responses to electrical field stimulation (EFS), (b) cholinergic responses to EFS. Data are means of values from 5–6 tissues, s.e. means are shown by the vertical bars.

exogenously applied ACh (6 μM ; Table 2). Moreover, these agonists were more effective than BRL 38227 at producing relaxation of ACh-induced spasm of the bronchial smooth muscle (Figure 4). These findings show good agreement with previous work using ketotifen and isoprenaline (Kamikawa, 1989; Kamikawa & Shimo, 1990), and suggest that the inhibitory effects of ketotifen and salmeterol on cholinergic responses to EFS can largely be attributed to a postjunctional action at the level of the bronchial smooth muscle.

Mechanism of action of BRL 38227 on responses to EFS

BRL 38227, pinacidil and RP 52891, the active (–)-enantiomer of RP 49356 (Mondot *et al.*, 1990), are each thought to cause smooth muscle relaxation by opening K^+ -channels in the plasmalemma of smooth muscle cells (Bray *et al.*, 1987; Arch *et al.*, 1988; Mondot *et al.*, 1988). The very similar profiles of action of BRL 38227, pinacidil and RP 52891 on responses to EFS may therefore suggest that K^+ -channel activation is a common underlying mechanism for their suppressant effects on nerve-mediated contractions.

The sulphonylurea glibenclamide is considered to be a

relatively specific blocker of the ATP-dependent K^+ -channel in the pancreas (Ashcroft, 1988). At higher concentrations, it has been shown to block an ATP-dependent K^+ -channel opened by cromakalim in rabbit mesenteric arterial smooth muscle cells (Standen *et al.*, 1989). Over the range 0.1–1 μM , glibenclamide selectively antagonized the tracheal smooth muscle relaxant actions of cromakalim in an apparently competitive manner (Murray *et al.*, 1989) and prevented the hyperpolarization of guinea-pig trachealis cells caused by cromakalim (Murray *et al.*, 1989).

In the present study, glibenclamide (0.1–1 μM) also antagonized the actions of BRL 38227 on responses to EFS in guinea-pig bronchus in a competitive manner. Similarly, previous studies have reported that glibenclamide antagonized the inhibition of NANCe and cholinergic nerve-mediated responses produced by cromakalim in the guinea-pig isolated trachea (Burka *et al.*, 1991; Cooper & MacLagan, 1990) and anaesthetized guinea-pig (Ichinose & Barnes, 1990). These findings lend support to the proposition that the effects of BRL 38227 on responses to EFS are mediated by K^+ -channel activation, and may further suggest the involvement of an ATP-dependent K^+ -channel (Quast & Cook, 1989). Nevertheless, the relatively high concentrations of glibenclamide required to inhibit responses to BRL 38227 suggest that the channel involved is unlikely to be identical with that found in pancreatic β cells (Schmid-Antomarchi *et al.*, 1987).

It has recently been suggested that phentolamine is also a blocker of ATP-dependent K^+ -channels. Thus, in mouse pancreatic β cells and RINm5F insulin-secreting cells, phentolamine (5–100 μM) reduced diazoxide-induced $^{86}\text{Rb}^+$ efflux (Plant & Henquin, 1990) and inhibited ATP-sensitive K^+ -currents (Plant & Henquin, 1990; Dunne, 1991). In vascular and tracheal smooth muscle, phentolamine (1.0–100 μM) specifically inhibited the relaxant effects of cromakalim, by a mechanism unrelated to blockade of α -adrenoceptors (McPherson & Angus, 1989; Murray *et al.*, 1989). Furthermore, phentolamine (100 μM) prevented the hyperpolarization of rat mesenteric arterial smooth muscle and of guinea-pig trachealis caused by cromakalim (Murray *et al.*, 1989; McPherson & Angus, 1991).

In the present study, the use of phentolamine as an analytical tool was weakened by the finding that, at concentrations greater than 3 μM , phentolamine itself caused inhibition of responses to EFS. Nevertheless, at a concentration exerting no inhibitory effects on responses to EFS (3 μM), phentolamine antagonized the actions of BRL 38227 on nerve-mediated responses in the guinea-pig bronchus (Figure 5a,b). In agreement with Murray *et al.* (1989), we found that the inhibition of responses to BRL 38227 by phentolamine (3 μM) was non-competitive in nature (Figure 5a,b). However, the present findings contrast with the report of Ichinose & Barnes (1990) that, although phentolamine antagonized the blood pressure lowering effects of cromakalim in the anaesthetized guinea-pig, it had no effect on the ability of cromakalim to cause suppression of NANCe nerve-mediated bronchoconstriction.

The inhibition of nerve-mediated responses in guinea-pig bronchus by BRL 38227 is observed over a concentration-range similar to that at which it causes relaxation of spontaneous tone in tracheal smooth muscle (0.3–3 μM ; Arch *et al.*, 1988). Furthermore these actions exhibit stereo-specificity (Figures 1a,b), and can be blocked by known antagonists of the actions of K^+ -channel activators on airways smooth muscle (Figures 5 and 6). If BRL 38227 is inhibiting NANCe and cholinergic nerve activity in the guinea-pig bronchus by a mechanism involving K^+ -channel activation, then these findings suggest that the channel involved has characteristics similar to that mediating airways smooth muscle relaxation.

The (+)-isomer of BRL 38227, BRL 38226 is generally considered to be essentially devoid of activity (Buckingham *et al.*, 1986; Arch *et al.*, 1988). The possibility of an interaction between the two enantiomers was investigated in view of a recent report that, in guinea-pig tracheal cholinergic

nerves, BRL 38226 itself caused a slight suppression of responses to electrical stimulation and reduced the inhibitory effects of BRL 38227 on these nerves (Cooper *et al.*, 1991). However, we obtained no evidence for an interaction between BRL 38226 and BRL 38227 in guinea-pig bronchial cholinergic or NANCe nerves (Figure 7a,b). Our findings do not support the suggestion of Cooper *et al.* (1991) that BRL 38226 may be a partial agonist for the neuronal K⁺-channel mediating the effects of BRL 38227.

It has been suggested that neural mechanisms are impor-

tant in the pathogenesis of asthma (Barnes, 1986). In particular, it is thought that peptidergic NANCe neurones may be involved in the generation and propagation of inflammatory changes in the airways of asthmatic patients (Barnes *et al.*, 1990). Accordingly, the present findings that BRL 38227 interferes with NANCe neuroeffector transmission may suggest that, in addition to providing direct bronchodilatation, BRL 38227 may also exert an anti-inflammatory effect in the airways.

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