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Effect of BW443C81, a novel opioid, on non-cholinergic bronchoconstrictor responses and neurogenic plasma extravasation in the guinea pig

C. E. Shankley, C. W. P. Anderson[1] and J. J. Adcock[2]

Department of Pharmacology, The Wellcome Research Laboratories, Beckenham, Kent, BR3 3BS, UK and [1] Department of Discovery Biology, Pfizer Central Research, Sandwich, Kent, UK

Abstract

The novel, peripherally acting opioid peptide, BW443C81, which attenuates airway sensory nerve impulses, was examined on non-cholinergic (NC) constrictor responses *in vitro* and *in vivo* and neurogenic plasma extravasation *in vivo* in guinea-pig airways. Non-cholinergic contractions of guinea pig isolated bronchi, evoked by electrical field stimulation, were concentration-dependently inhibited by BW443C81 and morphine (10 nmol/l–100 μmol/l). In anaesthetised, artificially ventilated guinea pigs, frequency-related NC bronchoconstictor responses evoked by antidromic electrical stimulation of the vagus nerves were reduced by BW443C81 (100 μg/kg/min i.v. infusion) and morphine (1 mg/kg i.v.). Neurogenic plasma extravasation produced by bilateral electrical vagal nerve stimulation in spontaneously breathing, anaesthetised guinea pigs was also inhibited by BW443C81 (1 mg/kg i.v.). The inhibitory effects of BW443C81 were reversed/prevented by naloxone.

BW443C81 inhibits NC bronchoconstrictor responses and neurogenic plasma extravasation in guinea pig airways, consistent with its previously described μ-opioid receptor-mediated inhibitory action on airway sensory nerve function.

Introduction

The release of neuropeptides, such as substance P (SP) and neurokinin A (NKA), from lung sensory nerves has been implicated in both non-cholinergic (NC) bronchoconstriction [1] and neurogenic plasma extravasation in the airways [2]. Non-cholinergic bronchoconstrictor responses and neurogenic plasma extravasation are attenuated in animals chronically pretreated with capsaicin [3], a substance known to desensitise primary afferent C-fibres [4]. Acute intravenous administration of capsaicin also induces NC bronchoconstriction in guinea pigs which presumably is due to the initial localised release of neuropeptides from sensory neurones [5].

It has been demonstrated that opioids inhibit electrically evoked NC bronchoconstrictor responses in guinea pig airways *in vitro* and *in vivo* [6, 7] and neurogenic plasma extravasation in the respiratory tract of guinea pigs [8]. Accordingly, it has been suggested that inhibition of the NC effects is due to

[2] Author for correspondence.

an action of opioids, via opioid receptors, on sensory nerve endings in the airways to prevent release of sensory neuropeptides [7].

BW443C81, (H.Tyr.D-Arg.Gly.Phe(4-NO_2).Pro.NH_2), a novel, peripherally acting μ-opioid receptor agonist [9, 10] has previously been shown to inhibit impulse discharges in vagal afferent Aδ- and C-fibres originating from sensory receptors in the respiratory tract of the cat [11, 12]. This action of BW443C81 is consistent with the ability of this compound to inhibit vagal reflexes such as cough and bronchoconstriction in the same species [13, 14]. It has also been demonstrated that BW443C81 inhibits bronchoconstriction evoked by capsaicin aerosol in guinea pigs, a response that consists of two components, a vagal cholinergic reflex and a NC local axon reflex [15].

Since unequivocal evidence supports a direct action of BW443C81 on airway sensory nerves, the purpose of the present study was to investigate if such an action of BW443C81 modifies NC bronchoconstrictor responses and neurogenic plasma extravasation in guinea pig airways.

Part of this work was presented at the December 1988 meeting of the British Pharmacological Society [16].

Methods

Experiments in vitro

Spirally cut main and hilar bronchi (1–2 cm length) prepared from male guinea pigs (Dunkin-Hartley, 300–400 g) (killed by a blow to the head, followed by exsanguination) were mounted between two platinum wire electrodes in 20 ml organ baths containing Krebs buffer, gassed with 95%O_2/5%CO_2 at 37 °C.

Following application of a 1 g initial tension, the tissues were left to stabilise for a period of 1 h. Contractions were induced by electrical field stimulation (EFS): 40 V (supramaximal voltage), 0.5 ms pulse width, 32 Hz frequency for 10 s (Grass S44/S88 stimulators) [6]. One EFS was given at the start of the experiment to check for viability of the preparation. Non-cholinergic contractions were examined after the addition of atropine (1 μmol/l) to the baths at the start of the experiment. Responses were measured isometrically (Grass FT.03 transducers connected to Gould BS-272 2-channel recorders). In some experiments, tetrodotoxin (TTX) (300 nmol/l) was added to the baths before stimulation to confirm that responses were neurogenically-mediated.

Preliminary experiments revealed that a maximum of six stimulations could be performed before the contractions decreased in magnitude (data not shown).

One control response to EFS was obtained before a single concentration of BW443C81 (10 nmol/l–100 μmol/l) or morphine (10 nmol/l–100 μmol/l) was added to the bath. Following a 20 min incubation, the bronchi were stimulated again. Naloxone (10 μmol/l, 20 min incubation) was added 10 min later and EFS repeated.

In a separate series of experiments, the effect of BW443C81 (10 μmol/l) on SP-induced contractions of the guinea pig bronchus was determined. Tissues were set up as described above and acetylcholine (Ach) (100 μmol/l) was administered to maximally contract the tissue. Following washout, atropine (1 μmol/l) was added to the baths. 20 min later BW443C81 (10 μmol/l) was added and incubated for 20 min before a single cumulative concentration–response curve to SP (1 nmol/1–3 μmol/l) was constructed. Control curves (without BW443C81) were also performed.

Experiments in vivo

NC bronchoconstriction. Male guinea pigs (Dunkin-Hartley, 300–500 g) were anaesthetised with 2.5% halothane (carrier gas: O_2, 2 l/min). Following cannulation of the right jugular vein, anaesthesia was maintained with α-chloralose 100–150 mg/kg i.v.). The trachea was cannulated and the animal artificially ventilated (Palmer Bioscience pump, 50 strokes/min of 1 ml laboratory air/100 g body weight). Pulmonary inflation pressure (PIP, cmH_2O) was measured from the side arm of the tracheal cannula with a Gould P23XL pressure transducer. The right carotid artery was cannulated to record arterial blood pressure (BP, mmHg) and heart rate (HR, beats/min) was derived from the arterial pulse via a cardiotachograph. Variables were recorded in analogue form on a Grass 7D polygraph. Both the cervical vagus nerves were carefully isolated, sectioned, the cut ends placed on silver bipolar electrodes (Harvard Instruments Ltd.) and immersed in light mineral oil. Body temperature (35–37 °C) was maintained with a heated table (Palmer Bioscience). The vagi were

stimulated (Grass S88) at 10 V, 5 ms pulse width for 30 s at 1, 3 and 6 Hz, with 15 min between each frequency. Typical resting baseline values were as follows: mean BP, 32 ± 3.5 mgHg; HR, 209 ± 17.8 beats/min; PIP, 5.4 ± 0.66 cmH_2O ($n=6$). Atropine (1 mg/kg i.v.) was administered at the start of the experiment. BW443C81 was administered 15 min before and continuously throughout the second frequency–response curve by infusion (100 μg/kg/min i.v., total dose administered 3 mg/kg over 30 min). Morphine was administered as a bolus (1 mg/kg i.v.) 15 min before stimulation. In some experiments naloxone was administered by infusion (100 μg/kg/min i.v., total dose 3 mg/kg over 30 min) at the same time as BW443C81.

Neurogenic plasma extravasation. Male guinea pigs (Dunkin-Hartley, 400–525 g) were anaesthetised with urethane (2 g/kg i.p). A jugular vein was cannulated for administration of drugs and a carotid artery cannulated to record BP and HR. Animals were spontaneously breathing. The vagi were prepared as before. Atropine and propranolol were administered (both at 1 mg/kg i.v.) 5 and 6 min, respectively, after surgery. After a further 19 min either saline (1 ml/kg i.v.) or BW443C81 (1 mg/kg i.v.) was administered followed 9 min later by Evans blue dye (30 mg/kg i.v.) The vagi were stimulated 1 min later at the following parameters: 7 Hz, 5 ms, 5 V for 5 min. Control animals (sham) were set up as described above but without stimulation. In some experiments naloxone (1 mg/kg i.v.) was administered 18 min before saline or BW443C81.

The concentration of dye in the tissue after stimulation was determined after perfusing the systemic circulation via the aorta with 40 ml saline. The trachea and main bronchi were removed, cleaned, lightly blotted on filter paper and weighed before placing in formamide. After 48 h at room temperature, the resulting solution was read at the absorbance of 620 nm wavelength and the tissue content [ng dye/mg tissue (wet wt.)] calculated from a standard curve in the range 0.3125–12.5 μg/ml.

In some preliminary experiments, animals were prepared using the same anaesthetic regime and method as described for the non-cholinergic bronchoconstriction experiments. Stimulation parameters were: 10 V, 5 ms, 10 Hz for 5 min.

In all *in vivo* experiments the animals were killed painlessly without recovery by 1–2 ml euthesate i.v. (200 mg/ml pentobarbitone solution).

Data analysis

Experiments in vitro. Contractions were measured in mm and converted to grams of tension. Means $\pm$SEM were calculated for each treatment. Statistical significance was evaluated by Student's *t*-test for raw paired data. Results are expressed as the mean percentage inhibition of control response for each concentration of BW443C81 or morphine. Contractions to SP at each concentration in the absence and presence of BW443C81 were expressed as a percentage of acetylcholine response. Statistical significance was calculated using Student's *t*-test for unpaired data.

Experiments in vivo. NC bronchoconstriction: Results were expressed as a change in PIP (cmH_2O). Means $\pm$SEM were calculated before and after BW443C81 or morphine at each frequency. Statistical significance was calculated using Student's *t*-test for paired data.

Neurogenic plasma extravasation: Results were expressed as ng dye/mg tissue (wet wt.) for trachea, left bronchus and right bronchus for each treatment. Means $\pm$SEM were calculated for each treatment and statistical significance was calculated using Student's *t*-test for unpaired data.

In all data analysis, p values < 0.05 were considered significant.

Drugs and solutions. Krebs buffer composition: 118 m*M* NaCl, 11 m*M* glucose, 25 m*M* $NaHCO_3$, 4.75 m*M* KCl, 0.93 m*M* NaH_2PO_4, 2.55 m*M* $CaCl_2$ and 1.16 m*M* $MgCl_2$.

The chemicals used were: atropine sulphate, acetylcholine bromide, formamide and light mineral oil (BDH Chemicals Ltd.); morphine hydrochloride and Urethane® (Aldrich Ltd.); naloxone hydrochloride (DuPont); halothane (May and Baker); propranolol hydrochloride, tetrodotoxin and Evans blue (Sigma Ltd.); α-chloralose (Koch-Light Ltd.); substance P (Cambridge Research Biochemicals Ltd.); Euthasate® (Willows Francis Veterinary Ltd.) and BW443C81, H.Tyr.D-Arg.Gly.Phe (4-NO_2).Pro.NH_2 diacetate (original synthesis Wellcome Research Labs., Baehem batch).

Drugs for *in vitro* experiments were dissolved in distilled water. Drugs for *in vivo* experiments were dissolved in 0.85% saline and expressed as free base.

Results

Experiments in vitro

Electrical field stimulation of the bronchus produced a biphasic response consisting of a fast followed by a slow contraction of the tissue. Both types of response were blocked by TTX (300 nmol/l, $n=3$) indicating that the contractions were neuronally mediated. Atropine (1 μmol/l) abolished the fast contraction confirming that this was cholinergically mediated. BW443C81 (10 nmol/l–100 μmol/l) inhibited the NC-mediated contraction in a concentration-dependent manner with a maximum inhibition of 73%; $n=6$, ($p<0.05$), as shown on Fig. 1. Morphine (10 nmol/l–100 μmol/l) inhibited the NC-mediated contraction with a maximum inhibition of 57.3%; $n=6$, ($p<0.01$) (Fig. 1). Naloxone (10 μmol/l; $n=6$) reversed both the inhibition by morphine (morphine 100 μmol/l: 57.3% inhibition; morphine + naloxone: 19.9% inhibition) and BW443C81 (BW443C81 100 μmol/l: 73.7% inhibition; BW443C81 + naloxone: 17.3% inhibition). Both morphine (100 μmol/l) and BW443C81 (100 μmol/l) caused a slight (10–100 mg) contraction of the bronchi.

Although full concentration–response curves to SP could not be obtained on the bronchus, BW443C81 (10 μmol/l) had no significant effect on SP-induced contractions at the concentrations used. SP at the highest concentration used, 3 μmol/l, elicted a contraction of 56.8% of the Ach response. In the presence of BW443C81 the contraction was 47% of the Ach response (NS, $n=10$).

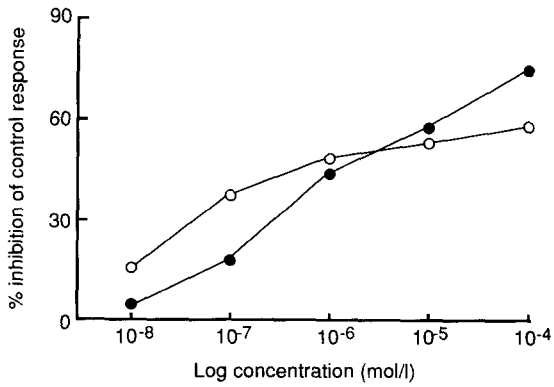

Figure 1
Inhibition by BW443C81 and morphine of EFS-evoked NC contraction of the guinea pig isolated bronchus (EFS parameters: 40 V, 32 Hz, 0.5 ms, 10 s). Results are expressed as percentage inhibition of control response plotted against log concentration. Control contractions were in the range 0.07–0.35 g. (○) morphine; (●) BW443C81. Each point represents the mean of 3–6 tissues.

Experiments in vivo

NC bronchoconstriction in anaesthetised, artificially ventilated guinea pigs. Preliminary experiments showed that two frequency–response curves (1, 3 and 6 Hz) could be obtained in the same animal without a decrease in magnitude of the bronchoconstriction (before saline 1 ml/kg i.v.: 8.5 ± 2.1, 23.5 ± 3.5 and 26.9 ± 3.0 cmH_2O change, respectively; after saline: 7.2 ± 1.8, 16.3 ± 3.8 and 21.2 ± 3.5 cmH_2O change, respectively; $n=6$, $p>0.05$). A frequency–response curve to bilateral vagal stimulation was obtained before and after atropine (1 mg/kg i.v.) treatment. Atropine caused a significant inhibition of the bronchoconstriction at 3 and 6 Hz (Table 1).

After atropine treatment, morphine (1 mg/kg i.v.) significantly ($p<0.01$ and $p<0.05$, $n=6$) inhibited the NC bronchoconstrictor responses elicited by vagal stimulation at 3 and 6 Hz. Similarly, BW443C81 (100 μg/kg/min i.v. infusion) significantly ($p<0.05$ and $p<0.01$, $n=5$) inhibited the NC bronchoconstrictor responses at 3 and 6 Hz (Table 1).

Naloxone (100 μg/kg/min i.v.) when administered by infusion, at the same time as BW443C81 antagonised the inhibitory effect of BW443C81 (Table 1). Neither morphine nor BW443C81 had any effect on base-line PIP at the doses used ($n=5/6$).

Neurogenic plasma extravasation in anaesthetised, spontaneously breathing, guinea pigs. In both the trachea and bronchi, vagal stimulation after treatment with atropine and propranolol, each at 1 mg/kg i.v., evoked a significant plasma extravasation, as defined by an increase of Evans blue dye in the tissue, compared to controls ($n=6$–8) (Fig. 2). Following BW443C81 (1 mg/kg i.v.) treatment the amount of extravasation (in both trachea and bronchi) due to vagal stimulation was significantly ($p<0.05$) reduced (Fig. 2). Naloxone (1 mg/kg i.v.) antagonised the inhibitory effect of BW443C81 (Fig. 2). BW443C81 had no effect on dye content of tissue in control animals (Fig. 2).

It was noted that during the course of these experiments BW443C81 caused an increase in BP

Table 1
Effect of various treatments on NC bronchoconstriction evoked by electrical vagal stimulation in the anaesthetised, artificially ventilated guinea pig.

Frequency (Hz)	Change in PIP	Change in PIP	(*n*)
	Control	Atropine	
1	12.0±1.8	7.6±0.9	6
3	26.5±3.5	15.8±1.6*	6
6	27.3±2.2	19.2±1.6*	6
	Atropine	Morphine	
1	3.0±1.4	3.6±1.5	6
3	17.2±3.2	7.9±3.1**	6
6	23.9±2.4	16.2±1.4*	6
	Atropine	BW443C81	
1	5.1±3.1	2.8±1.0	5
3	15.2±3.0	3.1±1.0**	5
6	23.0±2.0	11.9±3.7*	5
	Atropine	BW443C81 + naloxone	
1	3.0±1.2	5.2±1.2**	5
3	11.1±2.5	11.2±2.7**	5
6	13.8±1.6	12.7±2.8	5

Data are expressed as change in PIP (pulmonary inflation pressure; cmH_2O) from baseline. Mean ± SEM for each frequency. Doses of drugs administered are as follows: atropine 1 mg/kg i.v., morphine 1 mg/kg i.v. BW443C81 100 μg/kg/min i.v. infusion, naloxone 100 μg/kg i.v. infusion. Statistical significance was determined by Student's *t*-test for paired data; * $p<0.05$, ** $p<0.01$. Statistical significance for a comparison of BW443C81 alone vs. BW443C81 + naloxone was determined by Student's *t*-test for unpaired data; ** $p<0.05$.

(baseline BP: 39±1 mmHg; + BW443C81 1 mg/kg i.v.: 54±0.6 mmHg; $n=11$) the duration of which was 7–8 min. The BP returned to baseline values before stimulation.

Throughout these experiments in which the animals were not artificially ventilated, respiration became severely compromised (and in some cases stopped) and BP and HR were extremely low during electrical vagal stimulation.

In experiments using the method described for NC bronchoconstriction of the airways in which animals were artificially ventilated, the amount of dye content in the tissues following electrical vagal stimulation was not significantly different compared to controls [sham, trachea: 28.9 ±1.8 ng dye/mg tissue (wet wt.); stimulated, trachea: 24.2±3 ng dye/mg tissue (wet wt.), $n=8$]. In these experiments, however, a significant increase in PIP was evident (sham: change in PIP: 0.01±0.1 cmH_2O; stimulated: change in PIP 32.9 ±3.3 cmH_2O).

Both BP and HR remained relatively stable during stimulation compared to experiments where animals were spontaneously breathing.

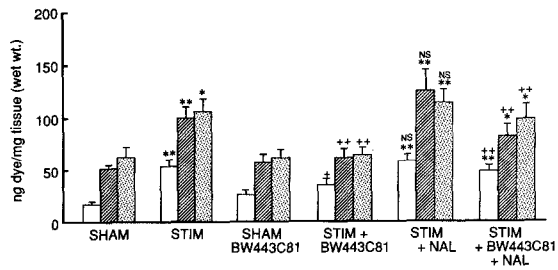

Figure 2
Inhibition by BW443C81 (1 mg/kg i.v.) ± naloxone (nal; 1 mg/kg i.v.) of plasma extravasation evoked by electrical stimulation of the vagus nerves (7 Hz, 5 ms, 5 V for 5 min), in the urethane anaesthetised, spontaneously breathing guinea pig. All experiments conducted in the presence of atropine and propranolol (1 mg/kg i.v.). Results expressed as ng dye/mg tissue (wet wt.). Open columns = trachea, hatched = right bronchus, speckled = left bronchus. Sham = not stimulated, stim. = stimulated; $n=6$–8 animals per treatment. Vertical bars represent SEM. Statistical significance determined by Student's *t*-test for unpaired data, compared to sham; * $p<0.05$, ** $p<0.01$. Other statistical comparisons made were: stim. vs. stim. + BW443C81, stim. + BW443C81 vs. stim. + BW443C81 + nal and stim. vs. stim. + nal and are shown by NS = not significant, † $p<0.05$, †† $p<0.01$.

Discussion

We have demonstrated that the novel, peripherally acting μ-opioid receptor agonist, BW443C81, inhibits NC bronchoconstrictor responses *in vitro* and *in vivo* and neurogenic plasma extravasation in guinea pig airways. The inhibitory effects of BW443C81 were blocked by naloxone confirming that BW443C81 acts via opioid receptors.

Previous studies have shown that opioids attenuate NC bronchoconstrictor responses and plasma extravasation in guinea pig airways [6–8]. Contractions of guinea pig airway tissue *in vitro* provoked by EFS non-cholinergic non-adrenergic (NANC) nerves were inhibited by morphine and the μ-opioid receptor agonist DAMGO ([D-Ala^2, *N*-Me-Phe^4, Glyol]enkephalin). This effect was not seen with the κ-opioid agonist dynorphin and only a weak effect was observed with δ-opioid agonists [6]. Similar observations with μ-, κ- and δ-opioid agonists have been reported in guinea pigs *in vivo* when NANC bronchoconstrictor responses were evoked by electrical stimulation of the cut vagus nerves [7]. Morphine was also found to inhibit the neurogenic plasma extravasation evoked by electrical stimulation of the cut vagus nerves [8]. It was suggested that inhibition by opioids of NANC responses may be due to inhibition of the release of neuropeptides via activation of prejunctional μ-opioid receptors on sensory nerves. Indeed, Lembeck and Donnerer [17] demonstrated that SP release from sensory nerves in the rat hind paw was inhibited by opioids. The lack of effect of opioids, including BW443C81, on the contractile responses of the guinea pig bronchus to exogenously administered neuropeptides such as SP support this hypothesis. Moreover, opioid receptors have been localised to sensory fibres of the vagus nerves [18] and it has also been suggested that opioid receptors are present on a capsaicin-sensitive population of sensory nerves [19].

In our *in vitro* experiments, it was noted that the concentration–response curve to BW443C81, compared to morphine, spanned a greater number of log units and a clear maximum was not reached. This may be due to a number of reasons. Firstly, morphine may have a greater inhibitory effect against the relatively high frequency used (32 Hz) than BW443C81. Secondly, the incubation time of 20 min may not have been long enough for BW443C81 to reach equilibrium at the lower concentrations. Thirdly, that a removal process becomes saturated at the higher concentrations of BW443C81.

The results in the present study with BW443C81 add considerable support to the argument that the inhibitory effects of opioids on NANC excitatory responses are mediated by activation of μ-opioid receptors on sensory nerves. Firstly, BW443C81 is a novel, peripherally acting opioid peptide which acts predominantly via μ-opioid receptors in a number of experimental models involving sensory nerves [9, 10]. Secondly, and more importantly, it has been demonstrated that BW443C81 inhibits vagally mediated reflex bronchoconstriction in the anaesthetised cat [14]. This effect of BW443C81 is due to the inhibition of lung irritant and C-fibre receptor discharges, since unequivocal evidence shows that BW443C81 modulates, via activation of μ-opioid receptors, spontaneous and stimulated impulse activity in vagal Aδ- and C-fibres originating from these receptors in cats [11, 12]. These findings thus highlight a direct effect of an opioid agonist on sensory nerves. Thirdly, it has also been demonstrated that BW443C81 inhibits bronchoconstriction evoked by capsaicin aerosol in guinea pigs, a response that consists of two components: a vagal cholinergic reflex and a non-cholinergic local axon reflex [15]. Thus, BW443C81 may have two mechanisms of action on sensory nerves to inhibit neuropeptide release. Firstly, by inhibition of the activation of sensory nerve endings in response to a stimulus and secondly, by acting prejunctionally to inhibit neuropeptide release. In the present experiments the latter is more likely, since the sensory nerves were activated by electrical antidromic stimulation, which would presumably bypass the normal mechanism of activation of a sensory receptor by chemical or physical means.

The exact mechanism of action of BW443C81 is not known. It has been shown that the release of sensory neuropeptides is dependent on calcium entry into the sensory neurones [20]. In addition, it has been demonstrated that μ- and δ-opioid receptor agonists reduce calcium-dependent action potential duration by increasing potassium conductance [21]. Hence, BW443C81 may act via opioid receptors to increase potassium conductance and/or decrease calcium conductance and thereby inhibit the release of neuropeptides involved in NANC constrictor responses and plasma extravasation. However, further detailed electrophysiological experiments are required to support and confirm this.

During the course of our studies of NC extravasation in the artificially ventilated, chloralose anaesthetised guinea pig it was observed that al-

though electrical vagal stimulation of the vagus nerves provoked a significant bronchoconstriction, extravasation above that in control animals did not occur. This could be due to the different anaesthetic used, since in the spontaneously breathing animals anaesthetised with urethane, significant plasma extravasation occurred after electrical stimulation of the vagus nerves. In support of this argument Martling [3] demonstrated that plasma extravasation could be evoked in the airways of artificially ventilated guinea pigs anaesthetised with sodium pentobarbital. This suggests, therefore, that chloralose may inhibit plasma extravasation, although NC bronchoconstrictor responses were apparently unaffected. However, since NC bronchoconstrictor responses were not measured in the spontaneously breathing animals a direct comparison cannot be made and it may be argued that NC bronchoconstrictor responses are less in the chloralose-anaesthetised animals. Nevertheless, these differences highlight a need for caution when choosing the anaesthetic for such experiments.

The present study with BW443C81 in particularly relevant since it has been postulated that neurogenic mechanisms may be involved in inflammation and hyperreactivity of the airways that occur in diseases of the airway such as asthma in man [22, 23]. Thus, BW443C81 or agents with a similar peripheral mechanism of action may be useful probes to investigate the contribution, if any, of neurogenic inflammation to airway disease in man.

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