

Bradykinin-Induced Lung Inflammation and Bronchoconstriction: Role in Parainfluenza-3 Virus-Induced Inflammation and Airway Hyperreactivity

Kenneth J. Broadley, Alan E. Blair, Emma J. Kidd, Joachim J. Bugert, and William R. Ford

Division of Pharmacology, Welsh School of Pharmacy (K.J.B., A.E.B., E.J.K., W.R.F.), and Department of Medical Microbiology, School of Medicine (J.J.B.), Cardiff University, Cardiff, United Kingdom

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ABSTRACT

Inhaled bradykinin causes bronchoconstriction in asthmatic subjects but not nonasthmatics. To date, animal studies with inhaled bradykinin have been performed only in anesthetized guinea pigs and rats, where it causes bronchoconstriction through sensory nerve pathways. In the present study, airway function was recorded in conscious guinea pigs by whole-body plethysmography. Inhaled bradykinin (1 mM, 20 s) caused bronchoconstriction and influx of inflammatory cells to the lungs, but only when the enzymatic breakdown of bradykinin by angiotensin-converting enzyme and neutral endopeptidase was inhibited by captopril (1 mg/kg i.p.) and phosphoramidon (10 mM, 20-min inhalation), respectively. The bronchoconstriction and cell influx were antagonized by the B₂ kinin receptor antagonist 4-(S)-amino-5-(4-[2,4-dichloro-3-(2,4-dimethyl-8-quinolyloxymethyl)phenylsulfonamido]-tetrahydro-2H-4-pyranilylcarbonyl)piperazino)-5-oxopentyl(trimethyl)ammonium chloride hydrochloride (MEN16132) when given by inhalation (1 and 10 μM, 20 min) and are

therefore mediated via B₂ kinin receptors. However, neither intraperitoneal MEN16132 nor the peptide B₂ antagonist icatibant, by inhalation, antagonized these bradykinin responses. Sensitization of guinea pigs with ovalbumin was not sufficient to induce airway hyperreactivity (AHR) to the bronchoconstriction by inhaled bradykinin. However, ovalbumin challenge of sensitized guinea pigs caused AHR to bradykinin and histamine. Infection of guinea pigs by nasal instillation of parainfluenza-3 virus produced AHR to inhaled histamine and lung influx of inflammatory cells. These responses were attenuated by the bradykinin B₂ receptor antagonist MEN16132 and *H*-(4-chloro)DPhe-2'-(1-naphthylalanine)-(3-aminopropyl)guanidine (VA999024), an inhibitor of tissue kallikrein, the enzyme responsible for lung synthesis of bradykinin. These results suggest that bradykinin is involved in virus-induced inflammatory cell influx and AHR.

Introduction

In healthy humans, inhalation of bradykinin has little or no effect, but in asthmatics it produces a bronchoconstriction (Polosa et al., 1993). Asthmatic subjects show a greater degree of airway hyperreactivity (AHR) to bradykinin than to methacholine after allergen challenge (Berman et al., 1995). Kinins exert their pharmacological effects through two main kinin receptor subtypes, B₁ and B₂ (Leeb-Lundberg et al., 2005). B₁ receptors are characterized by binding of [des-

Arg⁹]-bradykinin, although there are more selective agonists such as [lys-des-Arg⁹]-bradykinin (Leeb-Lundberg et al., 2005). B₁ receptors are absent in the lungs under normal conditions, but their expression is induced by inflammation (Christiansen et al., 2002). B₂ kinin receptors show high affinity for bradykinin and are constitutively expressed in the airways (Leeb-Lundberg et al., 2005).

In guinea pigs, inhaled bradykinin has been shown to produce bronchoconstriction, which can be blocked by the bradykinin B₂ receptor antagonist icatibant (HOE140) (Wirth et al., 1993; Sakamoto et al., 1994). To date, the effects of bradykinin on the airways have been largely examined after intravenous administration, inhalation, or intratracheal administration to anesthetized animals, such as

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ABBREVIATIONS: AHR, airway hyperreactivity; ACE, angiotensin-converting enzyme; ANOVA, analysis of variance; BALF, bronchoalveolar lavage fluid; DMEM, Dulbecco's modified essential medium; NEP, neutral endopeptidase; PIV-3, parainfluenza-3 virus; sG_{aw}, specific airway conductance; VERO cells, African green monkey kidney epithelial cells; MGTA, DL-2-mercaptopomethyl-3-guanidinoethylthiopropionic acid; MEN16132, 4-(S)-amino-5-(4-[2,4-dichloro-3-(2,4-dimethyl-8-quinolyloxymethyl)phenylsulfonamido]-tetrahydro-2H-4-pyranilylcarbonyl)piperazino)-5-oxopentyl(trimethyl)ammonium chloride hydrochloride; TCID, tissue culture infective dose; VA999024, *H*-(4-chloro)DPhe-2'-(1-naphthylalanine)-(3-aminopropyl)guanidine.

guinea pigs (Ichinose and Barnes, 1990a,b; Wirth et al., 1993; Miura et al., 1994; Sakamoto et al., 1994; Valenti et al., 2005) and rats (Ellis et al., 2004). One study showed that intravenously administered bradykinin caused bronchoconstriction in unanaesthetized guinea pigs (Chodimella et al., 1991). In those earlier studies there are a number of discrepancies in the effects of bradykinin, for example, a lack of blockade of allergen-induced microvascular leakage and bronchoconstriction by the B² antagonist icatibant (Sakamoto et al., 1996) and differences in the sensitivity to bradykinin between bronchoconstriction and microvascular leakage (Ricciardolo et al., 1994). A possible reason for the variable effects of bradykinin in the lung is its rapid breakdown by neutral endopeptidase (NEP) and/or angiotensin-converting enzyme (ACE). There do not seem to have been any studies on the airway effects of inhaled bradykinin in conscious animals, where the effect of anesthetic is eliminated. In this study, we therefore attempted to identify a bronchoconstriction with inhaled bradykinin and examine the effects of phosphoramidon and captopril, inhibitors of NEP and ACE, respectively. Because bradykinin is thought to act on B₂ kinin receptors in the airways (Ellis and Fozard, 2002), we also compared the blockade of bradykinin-induced bronchoconstriction with two distinct B₂ receptor antagonists, icatibant and 4-(S)-amino-5-(4-{4-[2,4-dichloro-3-(2,4-dimethyl-8-quinolyloxymethyl)phenylsulfonamido]-tetrahydro-2H-4-pyranilylcarbonyl}piperazino)-5-oxopentyl(trimethyl)ammonium chloride hydrochloride (MEN16132). Icatibant is a selective peptidic antagonist of B₂ receptors (Leeb-Lundberg et al., 2005), its affinity (pK_i) for displacement of radioligand binding to human B₁ and B₂ receptors expressed in Chinese hamster ovary cells being <5 and 10.2, respectively (Regoli et al., 1998). Icatibant administered intravenously, intratracheally, or by aerosol has been shown to inhibit bronchoconstriction to intravenously administered bradykinin in anesthetized guinea pigs (Tramontana et al., 2001; Valenti et al., 2005). Inhaled icatibant also inhibited bronchoconstriction and microvascular leakage after inhaled bradykinin in anesthetized guinea pigs (Sakamoto et al., 1994). MEN16132 is a nonpeptide-selective antagonist of B₂ kinin receptors, the affinity values (pK_i) of which for B₁ and B₂ receptors are <5 and 10.5, respectively (Cucchi et al., 2005). MEN16132 has been shown to block bronchoconstriction to intravenous bradykinin in anesthetized guinea pigs (Valenti et al., 2005).

In addition to a role for B₂ receptors in bronchoconstriction by bradykinin in asthmatics, kinin levels are increased in the bronchoalveolar lavage fluid (BALF) of asthmatic subjects after allergen challenge (Polosa and Holgate, 1990; Christiansen et al., 1992). Furthermore, B₁, but not B₂, kinin receptor expression is up-regulated in sensitized rat lungs (Huang et al., 1999), murine airways under interleukin-4 stimulation (Bryborn et al., 2004), and asthmatic airway inflammation (Christiansen et al., 2002).

Allergen sensitization and challenge has been repeatedly shown to cause AHR in guinea pigs to a range of spasmogens, including histamine and methacholine (Toward and Broadley, 2004; Smith and Broadley, 2007). AHR to intravenous bradykinin has been demonstrated in anesthetized rats after allergen challenge (Ellis et al., 2004), but there are few studies examining AHR to inhaled bradykinin in conscious animals. In this study, we therefore determined whether ovalbumin exposure in ovalbumin-sensitized guinea pigs produced AHR to inhaled bradykinin. AHR is also a consequence of

viral infection of the airways and has been shown along with an influx of inflammatory cells after inoculation of guinea pigs with parainfluenza-3 virus (PIV-3) (Toward et al., 2005). Folkerts et al. (2000) showed that the bradykinin B₂ antagonist icatibant can inhibit AHR induced by PIV-3 but not the inflammatory cell influx. We therefore determined whether bradykinin was involved in the PIV-3-induced AHR and inflammatory cell influx and whether it was mediated by the B₂ kinin receptor by treating PIV-3-inoculated guinea pigs with the B₂ kinin receptor antagonist MEN16132. Because the primary synthesis of bradykinin in the lung is via tissue kallikrein (Christiansen et al., 1992), we also examined the effects of inhibiting bradykinin production on PIV-3-induced inflammation and AHR by using a potent tissue kallikrein inhibitor, *H*-(4-chloro)DPhe-2'(1-naphthylalanine)-(3-amino-propyl)guanidine (VA999024; also known as FE999024 and CH-2856). VA999024 has previously been shown to be effective in reducing eosinophilia in an ovalbumin challenge model in guinea pigs (Evans et al., 1996).

Materials and Methods

Animal Welfare and Ethics. Male Dunkin Hartley guinea pigs (250–350 g) were obtained from Harlan UK Limited (Bicester, Oxon, UK). The animals were housed at 20°C ± 2°C with 12-h alternating light/dark cycles at approximately 50% humidity. The animals were fed commercial guinea pig diet pellets (Harlan UK Limited) supplemented with ascorbic acid, and water was allowed ad libitum. The animals were provided with cardboard tubes and received hay every day for environmental enrichment. The animals were acclimatized for at least 1 week before the commencement of any experiments. Animal welfare and experimentation were undertaken in accordance with the Animal Scientific Procedures Act 1986 under Home Office personnel and project licenses. The guinea pigs were without infections of the respiratory airways as evaluated by the health monitoring quality-control report by Harlan UK Limited.

Growth of Virus. African green monkey kidney epithelial (VERO) cells (European Collection of Cell Cultures, Porton Down, Wiltshire, UK) were grown to confluence in a humidified incubator (Sanyo, Osaka, Japan) maintained at 95% with an atmosphere of 95% O₂ and 5% CO₂ at 37°C in T75 culture flasks containing 10 ml of Dulbecco's modified essential medium (DMEM), 10% heat-inactivated fetal bovine serum, and 1% L-glutamate. Antibiotics were not used. The medium was removed, the cells were trypsinized, and 1 ml of fetal calf serum was added to deactivate the trypsin. The cells were then pelleted by centrifugation (Jouan CR412; ThermoFisher Scientific, Waltham, MA) at 1200 rpm for 6 min. The supernatant was removed, and the cells were resuspended in 2 ml of DMEM. Human PIV-3 virus (strain DEL/139/05) (European Collection of Cell Cultures) was added to the cells and mixed by pipetting up and down three times. The cell/viral suspension was then placed in the incubator for 30 min and swirled by hand every 5 min to ensure the virus had access to every cell. Three milliliters of the cell/viral mix was then added to the T75 flask and topped with 7 ml of DMEM, and the flask was replaced in the incubator.

After 4 to 5 days the cytopathic effects of viral infection became obvious, with large syncytia (multinuclear cells) and dead cells floating in the medium. The virus was then harvested. The cells were frozen at –80°C for 5 min and allowed to thaw at room temperature to break open the cell membranes. This freeze–thaw cycle was repeated twice, and the resulting suspension was centrifuged (Jouan CR412) at 4000 rpm (504g) for 5 min to remove cellular debris. The supernatant was then transferred into sterile 1-ml vials and stored at –80°C. For control inoculation uninfected and infected VERO cells were subjected to the same procedure.

Calculation of Viral Titer. The tissue culture infective dose (TCID₅₀/ml) refers to the quantity of virus-producing cytopathic effects in 50% of infected wells. DMEM (450 μ l) was added to 10 wells of a 24-well plate. A vial of frozen virus was thawed, and 50 μ l was added to the first well and mixed by pipetting up and down three times. Serial 1 in 10 dilutions were added to the remaining wells, and the plate was placed on ice. A T75 cell culture flask of confluent VERO cells was trypsinized, and after the addition of fetal calf serum to deactivate the trypsin, the cells were centrifuged (Jouan CR412) at 1000 rpm (224g) for 6 min, the supernatant was removed, and the cells were resuspended in 5 ml of DMEM. Fifty microliters of each viral dilution from the 24-well plate was added to each well of the corresponding column of a 96-well plate. Fifty microliters of the VERO cell suspension was then added to each well, and the plate was placed in the 37°C incubator. The cells were then checked daily for cytopathic effects. After 8 to 10 days, when no additional wells had shown any sign of infection for 2 days, the medium was removed, and 50 μ l of crystal violet was immediately added to each well and left for 30 min. The crystal violet was then washed off with water, and the plate was left to dry. Each well was then examined by manual microscopy for signs of infection. Destroyed cells showed very little staining, but uninfected and control cells showed complete staining. The viral concentration was then determined as the TCID₅₀/ml by using the Karber formula (Reed and Muench, 1938). $TCID_{50}/ml = 10^{\log \text{dilution above 50\% infection} - (I \times \text{Log}H)}$, where I = interpolated value of 50% endpoint (percentage of wells infected above 50% – 50%/percentage of wells infected above 50% – percentage of wells infected below 50%) and H = dilution factor.

Inoculation of Guinea Pigs with Virus. Guinea pigs were inoculated with PIV-3 (3.16×10^8 or 6.32×10^6 infectious units per ml) by intranasal instillation, performed in a class II safety cabinet (Captair Madcap 804; Erlab, Wiltshire, UK). The guinea pigs were held in a supine position with the head firmly supported. The virus or virus-free medium was then pipetted into the nostrils by using a Gilson, Inc. (Middleton, WI) pipette. Each guinea pig was given a 250- μ l inoculation, 125 μ l in one nostril, which was then repeated after 15 min in the other nostril. This procedure was then repeated 24 h later. A fresh vial of virus (or virus-free media) was thawed and warmed by hand to 37°C immediately before inoculation to ensure no decrease in the viral titer and facilitate delivery.

Guinea pigs were treated with a daily 20-min inhalation exposure of the B₂ bradykinin receptor antagonist MEN16132 (10 μ M in saline), sterile 0.9% saline (control), or the tissue kallikrein inhibitor VA999024 (1, 3, or 10 mg/kg in saline) by bilateral subcutaneous injections every 12 h. Dosing with MEN16132 or VA999024 commenced on day 1 and ended on day 8. On days 3 and 4, the animals received the viral inoculations 3 h after drug or saline treatments. On days 2 and 8, histamine reactivity tests were performed, and on day 8, the lungs were lavaged.

Measurement of Respiratory Function. Airway function was monitored in conscious spontaneously breathing animals by whole-body plethysmography and recorded as specific airway conductance (sG_{aw}). Guinea pigs were held by a neckpiece in a restrainer. The animal's snout was covered by a mask with a rubber diaphragm creating an airtight seal. This was attached to a pneumotachograph (Mercury FIL, Glasgow, UK) before the animal, in its restrainer, was placed in the plethysmograph chamber, which was then sealed by a plate secured to the front of the chamber. Before each experiment, the guinea pigs were handled and familiarized with the restrainer and the plethysmograph chamber to reduce stress-related factors.

Pressure transducers (Pioden type 1; Pioden Controls Ltd. Canterbury, UK), UP1 and UP2, attached to the pneumotachograph and plethysmograph chamber, respectively, measured changes in respiratory flow and box pressure. These transducers were attached to a computerized recording system comprised of AcqKnowledge software with a BIOPAC data acquisition system (BIOPAC Systems, Inc., Goleta, CA) as described previously by Smith and Broadley (2007). The resulting waveforms were analyzed by comparing the gradients

of the flow and box pressure at a point where flow tended toward zero, i.e., end tidal volume (at the end of expiration and beginning of inspiration). Each recording period was 5 s long, and from this, a minimum of five breaths was analyzed. Using these values and taking into account air pressure and the weight of each guinea pig, average sG_{aw} was determined. Between recordings, animals were removed from the plethysmograph and returned to the holding cage.

Airway Reactivity to Histamine and Bradykinin. Airway reactivity to aerosolized histamine (1 mM, 20 s) and bradykinin (0.1, 0.3, or 1 mM, 20, 40, or 60 s) delivered to the snout of the guinea pigs was measured by whole-body plethysmography. sG_{aw} was measured before exposure to histamine or bradykinin (baseline), immediately after transferring to the plethysmograph (zero time) and at 5 and 10 min thereafter. From our previous experience, 1 mM histamine causes minimal bronchoconstriction having been found to be a threshold concentration in naive guinea pigs (Smith and Broadley, 2007). Airway reactivity to histamine or bradykinin was measured 24 h before and 24 h after allergen challenge or the day before viral inoculation and on the fourth day after the second viral inoculation.

The effects of bradykinin were examined 1 h after administration of captopril (1 mg/kg i.p.), phosphoramidon (0.1 mg/kg i.p. or 10 mM by inhalation for 20 min), or combined captopril (10 mg/kg i.p.) and phosphoramidon (10 mM, 20-min exposure). The selective B₂ kinin receptor antagonists, icatibant (10 μ M inhalation exposure) and MEN16132 (30, 100 and 300 nM/kg i.p., or 1 and 10 μ M by 20-min inhalation exposure), were administered 1 h before inhalation exposure to 1 mM bradykinin in guinea pigs treated with captopril (1 mg/kg).

Bronchoalveolar Lavage. Within 30 min of the hyperreactivity test, the guinea pigs were sacrificed with a lethal overdose of the anesthetic (pentobarbital sodium, Euthatal, 400 mg/kg) by bilateral intraperitoneal injection. The trachea was then cannulated by insertion of a nylon intravenous cannula (Sims Portex Ltd, Kent, UK). An incision was made below the level of the diaphragm upward to expose the ribcage. The ribcage was then removed, exposing the lungs and trachea. The trachea and lungs were then removed from the thoracic cavity, and the heart and any fat and connective tissue were then removed. The right bronchi was clamped shut with Spencer-Wells forceps so the lavage could be performed on only the left lung.

Saline (0.9%, 10 ml/kg) was then injected into the left lung with a syringe, via the tracheal cannula. After 3 min the fluid was withdrawn by means of the syringe, while gently massaging the lung. This procedure was repeated, and the two recovered bronchoalveolar lavage fluid samples were combined and placed on ice for total and differential cell counts. Only plasticware was used for the collection process to minimize adherence of the cells to the surface of the tube.

Total and Differential Cell Counts. Total cell counts (cells/ml) were determined by using a hemocytometer (Neubauer, Marienfeld, Germany) viewed under a light microscope (X10; Nikon, Tokyo, Japan). Differential cell counts were then undertaken to determine the levels of alveolar leukocytes. A 100- μ l sample of the BALF was centrifuged by using a cytopsin (ThermoShandon Ltd. Cheshire, UK) at 1000 rpm (112g) for 7 min onto a glass microscope slide and air-dried. The slides were then stained with 1.5% Leishman's stain (Sigma-Aldrich, Dorset, UK) for 7 min. Using a light microscope (X100; Nikon), a minimum of 200 consecutive cells were examined, using standard morphological criteria to determine alveolar macrophages, eosinophils, lymphocytes, and neutrophils. Using the corresponding total cell count, numbers of each subtype of leukocytes were determined.

Ovalbumin Sensitization and Exposure. On days 1 and 5 guinea pigs were sensitized with intraperitoneal injection of 100 μ g of ovalbumin and 100 mg of aluminum hydroxide in 1 ml. The suspension was placed on a magnetic stirrer for at least 2 h before use, and to ensure adequate distribution the dose was divided and administered bilaterally. On day 15 the animals were placed in a steel exposure chamber (40-cm diameter, 15-cm height) and given a

60-min inhalation exposure of ovalbumin (0.01%) dissolved in saline (0.9% NaCl). Any animals that looked to be in respiratory distress were immediately removed from the exposure chamber, and the exposure was considered complete. Lung function measurements were taken by whole-body plethysmography immediately before challenge and at 0, 15, 30, 45, and 60 min after, then hourly up to 12 h and finally at 24 h. Histamine or bradykinin reactivity was determined 24 h before and 24 h after ovalbumin challenge. Histamine reactivity was also tested in saline-challenged guinea pigs. Ovalbumin-sensitized animals were treated with dimethylsulfoxide (50% in saline intraperitoneally) at 24 h before, 30 min before, and 6 h after ovalbumin or saline challenge. This served as a control for drug intervention studies using this solvent not reported in this article.

Data Handling and Statistical Analysis. Baseline sG_{aw} level was determined as the average of two sG_{aw} readings taken before each challenge. Subsequent readings taken after challenge with histamine, bradykinin, or ovalbumin were expressed as a percentage of the baseline value. Statistical comparisons of histamine or bradykinin responses were made between values before and after treatments (e.g., ovalbumin challenge and/or viral inoculation or drug treatment) using Student's paired *t* test.

The ovalbumin model of allergy shows distinct early and late phases of airway reactivity; however, because of natural variation each animal can display these reactions at different time points. The average maximum decrease from baseline in each animal between 0 and 6 h and between 7 and 12 h was determined in a separate graph to demonstrate the early and late phase, respectively alongside the graph showing the time course.

Statistical comparisons between mean values from more than two groups were made by analysis of variance (ANOVA) followed by Dunnett's post hoc test. A *p* value < 0.05 was considered significant. All values are presented as mean $\pm$ S.E.M.

Drugs, Materials, and Solutions. Aluminum hydroxide, bradykinin acetate, captopril, dimethyl sulfoxide, histamine diphosphate, icatibant, ovalbumin, and phosphoramidon disodium salt were obtained from Sigma-Aldrich. Saline was purchased from Baxter Healthcare, Newbury, UK. Fetal bovine serum was obtained from Perbio Science UK Ltd (Chester, UK), and DMEM, L-glutamate, and trypsin were from Invitrogen (Paisley, UK). MEN16132 was kindly provided by Dr. Christopher Fincham (Menarini Ricerche, Pomezia, Italy). VA999024 (Evans et al., 1996) was kindly provided by Dr. D. Michael Evans (Vantia Ltd, Southampton, UK). All drugs were dissolved in saline.

Results

Bradykinin Exposures. After 20-s, nose-only exposures to 0.01, 0.1, and 1 mM bradykinin in guinea pigs, there was no significant decrease in sG_{aw} from baseline (data not shown). Likewise, when guinea pigs were pretreated with captopril (1 mg/kg i.p.), there was no significant reduction in sG_{aw} after exposure to 0.01 and 0.1 mM bradykinin. However, there was a significant decrease in sG_{aw} after exposure to 1 mM bradykinin in captopril-treated guinea pigs (Fig. 1A). After captopril (1 mg/kg) treatment, exposure to 0.3 mM bradykinin produced modest decreases in sG_{aw} after 20-s ($-8.7 \pm 7.0\%$) and 40-s ($-5.9 \pm 6.3\%$) exposures and a significant decrease ($p < 0.05$) after a 60-s exposure ($-17.9 \pm 6.4\%$) (Fig. 1B).

After phosphoramidon (0.1 mg/kg i.p.) there was no significant decrease ($-3.8 \pm 4.4\%$) in sG_{aw} after a 1-mM bradykinin exposure ($p > 0.05$), but after a 20-min inhalation exposure of 10 mM phosphoramidon, bradykinin (1 mM) caused a significant decrease ($21.7 \pm 3.2\%$) in sG_{aw} ($p < 0.05$) (Fig. 1C). After combined phosphoramidon (10 mM, 20-min inhalation exposure) and captopril (1 mg/kg i.p.) there was a

significant $22.3 \pm 3.2\%$ decrease ($p < 0.05$) in sG_{aw} after exposure to 1 mM bradykinin. However, this response was no greater than with the same doses of phosphoramidon or captopril alone (Fig. 1C).

Icatibant (10 μ M) by 20-min inhalation exposure had no effect on the decrease in sG_{aw} seen after exposure to 1 mM bradykinin in captopril-treated (1 mg/kg i.p.) guinea pigs (Fig. 2A). MEN16132 (30, 100, and 300 nM/kg) administered by intraperitoneal injections failed to block the decrease in sG_{aw} seen after exposure to 1 mM bradykinin in captopril-treated (1 mg/kg i.p.) guinea pigs (Fig. 2B). The bronchoconstrictor responses (-11.1 ± 4.3 , -13.6 ± 4.3 , and $-11.2 \pm 7.1\%$) were not significantly different ($p > 0.05$) from the saline control ($-17.3 \pm 2.4\%$). After a 20-min inhalation exposure to MEN16132 (1 and 10 μ M), however, there was complete blockade of the decrease in sG_{aw} seen after exposure to 1 mM bradykinin in captopril-treated (1 mg/kg i.p.) guinea pigs (Fig. 2C).

Bronchoalveolar Lavage. In animals receiving no treatment, the numbers of macrophages, eosinophils, neutrophils, and lymphocytes in the BALF were as follows: total cells, $1.95 \pm 0.09 (\times 10^6/\text{ml})$; macrophages, $1.68 \pm 0.09 (\times 10^6/\text{ml})$; eosinophils, $0.20 \pm 0.04 (\times 10^6/\text{ml})$; neutrophils, $0.04 \pm 0.03 (\times 10^6/\text{ml})$; and lymphocytes, $0.03 \pm 0.01 (\times 10^6/\text{ml})$ (Fig. 3). In animals treated with captopril (1 mg/kg i.p.), which received 1 mM bradykinin before lavage, there was no significant increase ($p < 0.05$) in any of the cell types (Fig. 3). However, in animals treated with captopril (1 mg/kg i.p.) and phosphoramidon (10 mM, 20-min inhalation exposure) that received an inhalation exposure of 1 mM bradykinin before lavage there were significant ($p < 0.05$) increases in total cells, macrophages, eosinophils, and neutrophils compared with the same experiment without phosphoramidon. There was no increase in lymphocytes (Fig. 3).

In animals treated with captopril (1 mg/kg i.p.) and phosphoramidon (10 mM, 20-min inhalation exposure), the increases in cells caused by inhalation of 1 mM bradykinin were blocked by MEN16132 (10 μ M, 20-min inhalation exposure). Total cells, eosinophils, neutrophils, and lymphocytes were significantly reduced ($p < 0.05$), with a modest decrease in macrophages, compared with bradykinin exposure without MEN16132 (Fig. 3).

Ovalbumin-Sensitized Animals. In ovalbumin-sensitized guinea pigs, ovalbumin exposure caused an immediate $25.5 \pm 4.5\%$ decrease in sG_{aw} from baseline. After 4 h there was a partial recovery, which was followed by a secondary $19.6 \pm 8.3\%$ decrease in sG_{aw} at 8 h (Fig. 4A). In ovalbumin-sensitized guinea pigs before ovalbumin exposure, there was no significant decrease in sG_{aw} from baseline levels after exposure to 1 mM bradykinin (Fig. 4B). Twenty four hours after ovalbumin exposure of sensitized guinea pigs, however, there was a significant decrease in sG_{aw} after exposure to 1 mM bradykinin ($p < 0.05$) (Fig. 4B).

In these animals, when inhaled bradykinin (1 mM, 20 s) was used to test for reactivity 24 h after ovalbumin challenge, there were significant increases ($p < 0.05$) in total cells, macrophages, eosinophils, neutrophils, and lymphocytes compared with saline-challenged, ovalbumin-sensitized, and histamine-exposed guinea pigs (Fig. 4C). The numbers of macrophages, eosinophils, neutrophils, and lymphocytes in the BALF of saline-challenged animals were as follows: total cells, $4.38 \pm 0.42 (\times 10^6/\text{ml})$; macrophages, $2.31 \pm 0.22 (\times 10^6/\text{ml})$.

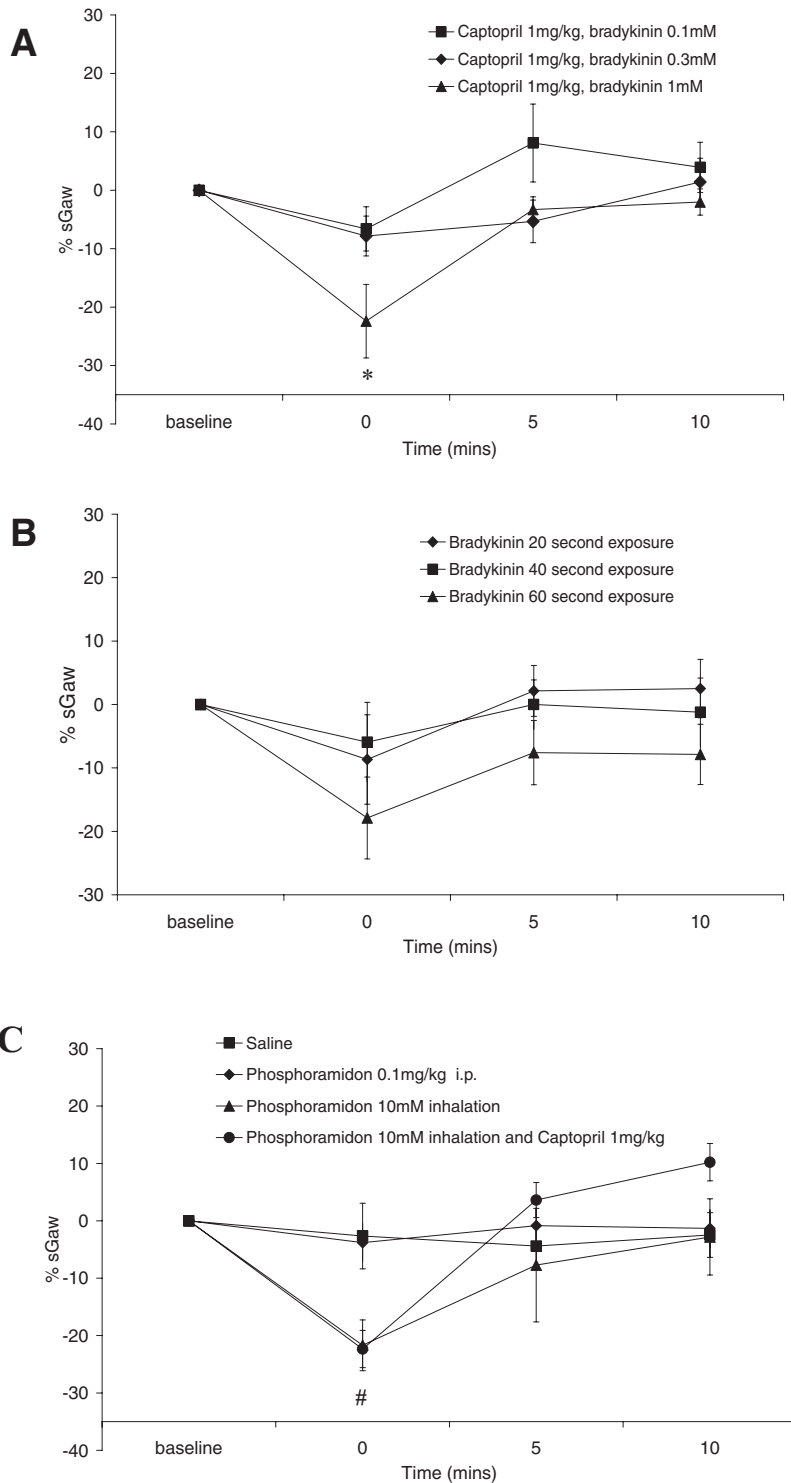


Fig. 1. Effects of inhaled bradykinin on sG_{aw} in conscious guinea pigs after treatment with captopril or phosphoramidon. A, effects of exposure to 0.1, 0.3, and 1 mM bradykinin for 20 s after treatment with captopril (1 mg/kg i.p.). B, effects of exposure to 0.3 mM bradykinin for 20, 40, and 60 s after treatment with captopril (1 mg/kg i.p.). C, effect of phosphoramidon (10 mM, 20-min inhalation exposure) and combining phosphoramidon (10 mM 20-min inhalation) with captopril (1 mg/kg i.p.) on bradykinin (1 mM, 20-s exposure). Each point represents the mean $\pm$ S.E.M. ($n = 6$) change in sG_{aw} expressed as a percentage of the baseline sG_{aw} values. Negative values represent bronchoconstriction. * indicates a significant ($p < 0.05$) difference from baseline sG_{aw} after bradykinin exposure, and # indicates a significant ($p < 0.05$) difference between the changes in sG_{aw} values with saline and phosphoramidon alone and with captopril treatment as determined by ANOVA followed by Dunnett's post hoc test.

ml); eosinophils, $1.95 \pm 0.34 (\times 10^6/\text{ml})$; neutrophils, $0.03 \pm 0.01 (\times 10^6/\text{ml})$; and lymphocytes, $0.1 \pm 0.02 (\times 10^6/\text{ml})$ (Fig. 4C). Compared with guinea pigs in which histamine was used to test for reactivity 24 h after the ovalbumin challenge, the levels of macrophages after the bradykinin reactivity test were significantly less ($29.9 \pm 10.1\%$) with modest decreases in total cells and eosinophils and small increases in neutrophils and lymphocytes (Fig. 4C).

Effects of PIV-3 Inoculation. Before inoculation with virus, there was no significant decrease in sG_{aw} after inha-

lation exposure to histamine (1 mM, 20 s, nose only) (Fig. 5A). After viral inoculation (3.16×10^8 infectious units per ml), in the saline-treated guinea pigs, there was a significant reduction ($31.5 \pm 6.3\%$) ($p < 0.05$) in sG_{aw} value immediately after histamine exposure compared with the baseline value. sG_{aw} recovered after 5 and 10 min (Fig. 5A). In animals receiving no virus and medium only the numbers of macrophages, lymphocytes, eosinophils, and neutrophils in the BALF were as follows: total cells, $1.54 \pm 0.17 (\times 10^6/\text{ml})$; macrophages, $1.37 \pm 0.15 (\times 10^6/\text{ml})$; eosinophils, $0.12 \pm 0.01 (\times 10^6/\text{ml})$;

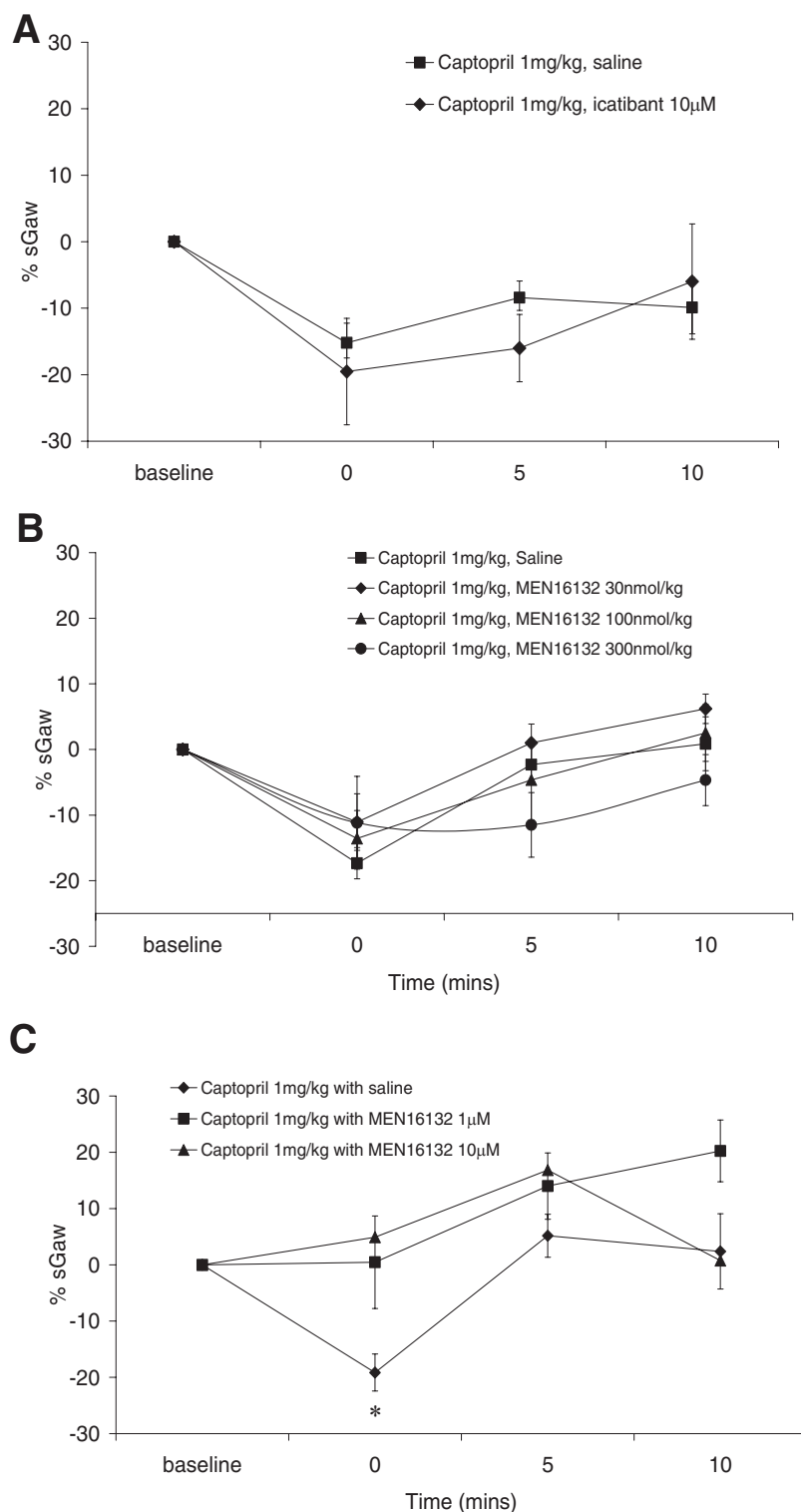


Fig. 2. Effects of bradykinin B₂ antagonists icatibant and MEN16132 on bronchoconstrictor responses to bradykinin (1 mM, 20-s exposures) after treatment with captopril (1 mg/kg i.p.). A, effects of icatibant (10 μ M, 20-min inhalation exposure) or saline (20-min inhalation exposure). B, effects of MEN16132 (30, 100, and 300 nmol/kg i.p.) or saline (intraperitoneally). C, effects of MEN16132 (1 and 10 μ M by 20-min inhalation exposures) or saline (20-min inhalation exposure). Each point represents the mean $\pm$ S.E.M. ($n = 6$) change in sG_{aw} expressed as a percentage of the baseline sG_{aw} values. Negative values represent bronchoconstriction. * denotes a significant ($p < 0.05$) difference between the changes in baseline sG_{aw} value with and without MEN16132 treatment as determined by ANOVA followed by a Dunnett's post hoc test.

lymphocytes, 0.06 ± 0.02 ($\times 10^6$ /ml); and neutrophils, 0.03 ± 0.01 ($\times 10^6$ /ml) (Fig. 6). In PIV-3-inoculated guinea pigs treated with saline (daily for 8 days, 20-min exposures), there were significant increases ($p < 0.05$) in total cells ($55.0 \pm 6.4\%$), macrophages ($51.3 \pm 6.7\%$), eosinophils ($69.1 \pm 7.3\%$), and neutrophils ($66.3 \pm 8.8\%$) and a modest increase in lymphocytes compared with medium controls.

Effects of MEN16132 and VA999024 on PIV-3 Responses. Treatment of guinea pigs with MEN16132 (10 μ M

daily for 8 days, 20-min inhalation exposures) abolished the bronchoconstriction to histamine after viral inoculation because there was only a $-5.6 \pm 4.6\%$ reduction in sG_{aw} after histamine exposure (Fig. 5B). After MEN16132 (10 μ M daily for 8 days, 20-min inhalation exposures), there were significant decreases ($p < 0.05$) in total cells ($29.3 \pm 5.5\%$), macrophages ($29.5 \pm 7.3\%$), neutrophils ($42.1 \pm 25.2\%$), and lymphocytes ($43.9 \pm 8.4\%$) and a modest decrease in eosinophils (Fig. 6).

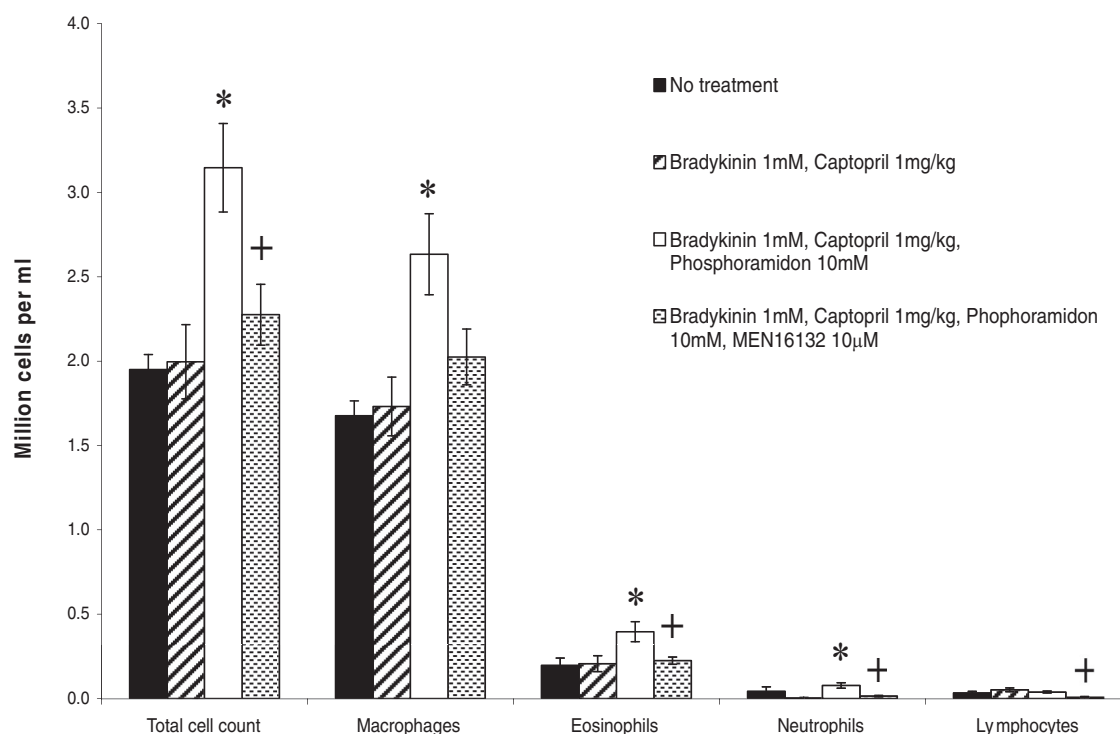


Fig. 3. Total and differential cell (macrophage, eosinophil, neutrophil, and lymphocyte) counts of BALF removed from guinea pigs receiving no treatment or receiving bradykinin (1 mM, 20-s exposure) with captopril (1 mg/kg i.p.) with or without 10 mM phosphoramidon. The final group received phosphoramidon and 10 μ M MEN16132. Each bar represents the mean $\pm$ S.E.M. ($n = 6$) of total or differential count of cells ($\times 10^6$ per ml). * denotes a significant ($p < 0.05$) difference compared with the corresponding group without phosphoramidon. + denotes a significant ($p < 0.05$) difference compared with the group without MEN16132 as determined by ANOVA followed by Dunnett's post hoc test.

VA999024 (1, 3, and 10 mg/kg s.c.) was also examined in animals inoculated with PIV-3 (6.32×10^6 infectious units per ml). Before inoculation of guinea pigs with PIV-3, there was no significant decrease in sG_{aw} after histamine inhalation (Fig. 7A). In PIV-3-inoculated guinea pigs treated with saline (subcutaneously) for 8 days, there was a significant bronchoconstriction to histamine seen as a significant reduction in the peak sG_{aw} value at 10 min ($-13.5 \pm 3.5\%$) after histamine exposures (Fig. 7A). After treatment of PIV-3-inoculated animals with VA999024 (1 mg/kg s.c.), there was still a significant bronchoconstriction ($p < 0.05$) to histamine ($-19.5 \pm 4.2\%$ reduction in sG_{aw}). However, after treatment with higher doses of 3 and 10 mg/kg there was no significant bronchoconstriction to histamine (Fig. 7A). The bronchoalveolar lavage of animals receiving no virus (medium controls) showed the numbers of macrophages, lymphocytes, eosinophils, and neutrophils in the BALF as follows: total cells, $1.54 \pm 0.17 (\times 10^6/\text{ml})$; macrophages, $1.37 \pm 0.15 (\times 10^6/\text{ml})$; eosinophils, $0.12 \pm 0.01 (\times 10^6/\text{ml})$; lymphocytes, $0.06 \pm 0.02 (\times 10^6/\text{ml})$; and neutrophils, $0.03 \pm 0.01 (\times 10^6/\text{ml})$ (Fig. 7B). In guinea pigs inoculated with PIV-3 (6.32×10^6) and treated with saline for 8 days, there were significant increases ($p < 0.05$) in total cells, macrophages, eosinophils, and lymphocytes (Fig. 7B). These increases in leukocytes were inhibited in a dose-related manner by treatment with VA999024, the highest dose producing significant reductions in total cells, macrophages, and lymphocytes (Fig. 7B).

Discussion

Bradykinin inhalation exposures up to 1 mM produced no significant bronchoconstriction in conscious guinea pigs. This

contrasts with observations made in anesthetized guinea pigs where intravenous (Ichinose and Barnes, 1990a; Wirth et al., 1993; Tramontana et al., 2001), intratracheal (Ichinose and Barnes, 1990b), or inhaled (Sakamoto et al., 1994) bradykinin exerted bronchoconstriction. The lack of effect of inhaled bradykinin in conscious guinea pigs seems to be caused by the rapid breakdown of bradykinin because there was a significant bronchoconstriction after treatment with captopril, an ACE inhibitor. ACE (also known as kininase II) and NEP catalyze the breakdown of bradykinin into inactive metabolites, whereas kininase I produces the active metabolite [des-Arg⁹]-bradykinin (Decarie et al., 1996). [des-Arg⁹]-bradykinin has approximately 4-fold selectivity of binding to B_1 receptors over B_2 receptors (Leeb-Lundberg et al., 2005) but does not cause any bronchoconstriction in asthmatic or normal subjects (Polosa and Holgate, 1990), suggesting that B_1 kinin receptors are not involved in bronchoconstriction. ACE mediates the breakdown of bradykinin in the circulation, and the lung endothelial cells are a rich source of this enzyme, as are the airways (Dusser et al., 1988a).

Intraperitoneal treatment with phosphoramidon had no effect, but after inhalation exposure there was a bronchoconstriction to bradykinin similar to that seen after captopril treatment. NEP is expressed in the respiratory epithelium (Baraniuk et al., 1995), so inhalation exposure would enable phosphoramidon to have an instant effect, whereas after intraperitoneal injection the drug may not be reaching the lung in a concentration sufficient to inhibit NEP. Captopril and phosphoramidon were shown to potentiate the bronchoconstriction by airway-instilled bradykinin in anesthetized guinea pigs (Ichinose and Barnes, 1990b) and intravenous

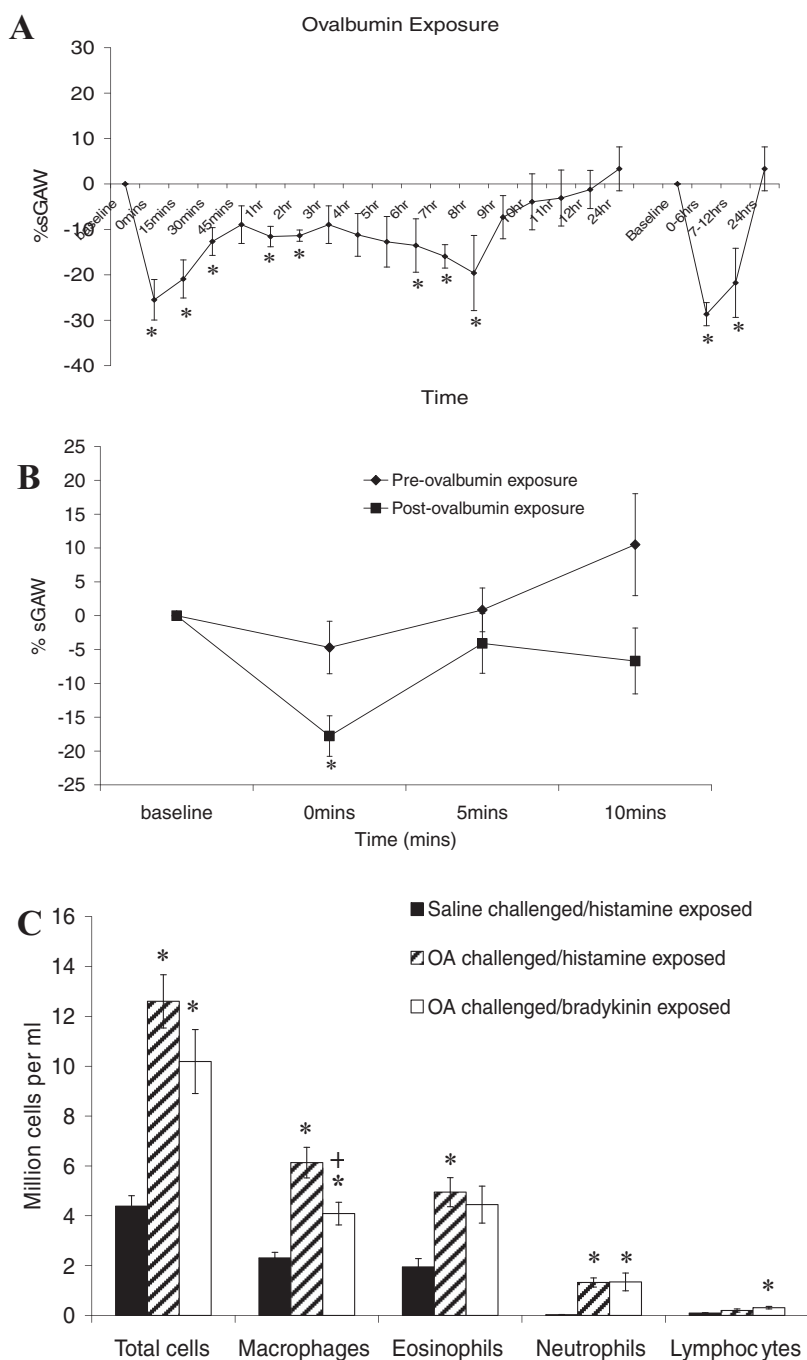


Fig. 4. Effect of bradykinin in ovalbumin-sensitized guinea pigs. A, guinea pigs were exposed to ovalbumin (60 min, 0.01%), and changes in sG_{aw} were monitored for 12 h and at 24 h. B, changes in sG_{aw} in response to bradykinin (1 mM, 20-s exposure) measured 24 h before and 24 h after the inhalation exposure to ovalbumin. * denotes a significant ($p < 0.05$) difference between the changes to baseline sG_{aw} values before and after ovalbumin exposure, as determined by Student's *t*-paired test. In A and B, each point represents the mean $\pm$ S.E.M. ($n = 6$) change in sG_{aw} expressed as a percentage of the baseline sG_{aw} values. Negative values represent bronchoconstriction. In A, the graph on the right shows the mean $\pm$ S.E.M. peak falls in sG_{aw} occurring between 0 and 6, 7 and 12, and at 24 h. C, total and differential cell (macrophage, eosinophil, lymphocyte, and neutrophil) counts in BALF removed from ovalbumin-sensitized and -challenged animals. Each point represents the mean $\pm$ S.E.M. ($n = 6$) of total or differential count of cells ($\times 10^6$ per ml). * denotes a significant ($p < 0.05$) difference compared with the corresponding saline-challenged and histamine-exposed group. + denotes a significant ($p < 0.05$) difference compared with the ovalbumin-challenged and histamine-exposed group, as determined by ANOVA followed by Dunnett's post hoc test.

bradykinin in conscious guinea pigs (Chodimella et al., 1991). The kininase I inhibitor DL-2-mercaptopomethyl-3-guanidinoethylthiopropionic acid (MGTA) failed to alter the bronchoconstriction in both of these studies. This suggested that both ACE and NEP degrade bradykinin but kininase I is not involved. In the present study, when capropril and phosphoramidon treatment were combined the bronchoconstriction by bradykinin was similar to that seen with treatment with each drug individually. A similar observation was made by Ichinose et al. (1990). This could be because the maximum bronchoconstriction to bradykinin has been reached. It was interesting, however, that there was also no prolongation of the bronchoconstriction when the two inhibitors were combined.

Thus, either ACE or NEP had to be inhibited to prevent bradykinin breakdown before a bronchoconstriction to inhaled bradykinin could be observed in conscious guinea pigs. Kininase I probably is not involved in the metabolism of inhaled bradykinin because this enzyme is localized to the blood (Proud and Kaplan, 1988). However, a second kininase I-type enzyme, carboxypeptidase M, which is also inhibited by MGTA, is highly expressed in the lungs (Nagae et al., 1993), including guinea pig lungs (Chodimella et al., 1991). The lack of effect of MGTA alone, however, suggests that neither of these enzymes is involved in the breakdown of bradykinin. The mechanism of the bronchoconstriction by the inhaled route is probably via a neural mechanism. Atropine, to block parasympathetic muscarinic pathways, and capsa-

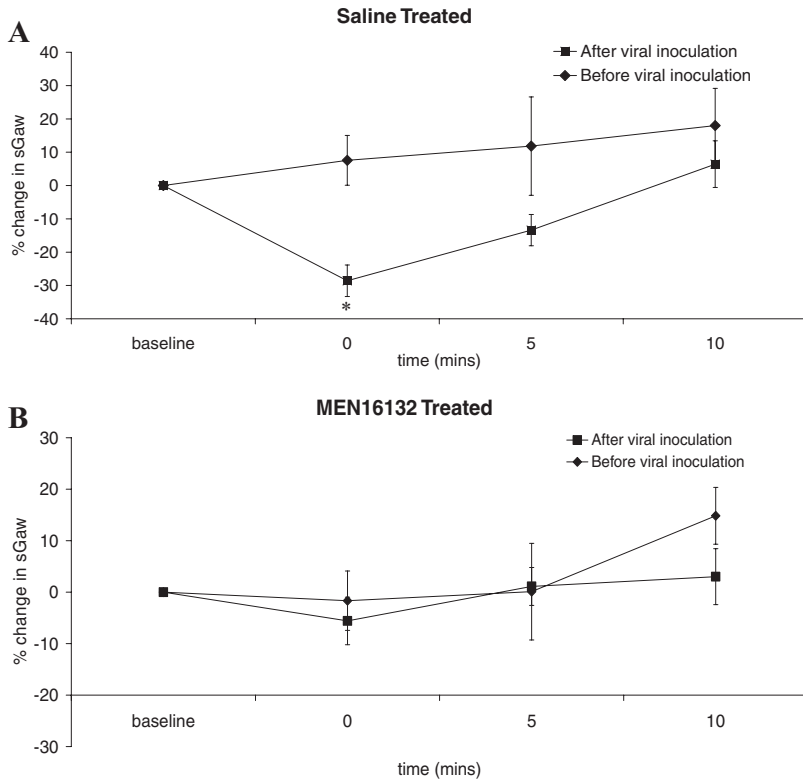


Fig. 5. Effect of histamine (1 mM, 20-s nose-only exposure) on airway function before and after instillation of PIV-3 virus (3.16×10^6 infectious units per ml) in guinea pigs treated with saline (20-min inhalation exposures) (A) and MEN16132 (10 μ M, 20-min inhalation exposures) (B). Each point represents the mean $\pm$ S.E.M. ($n = 6$) change in sGaw expressed as a percentage of the baseline sGaw values. Negative values represent bronchoconstriction. * denotes a significant ($p < 0.05$) difference between the changes from baseline sGaw values before and after virus instillation, as determined by Student's paired t test.

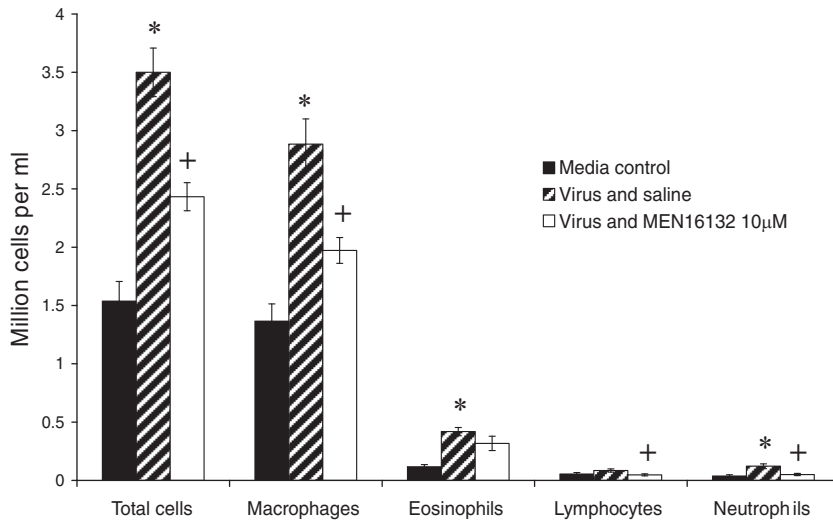


Fig. 6. Total and differential cell (macrophage, eosinophil, lymphocyte, and neutrophil) count in BALF removed from virus-free medium-inoculated animals or PIV-3-inoculated animals treated with saline or MEN16132 (10 μ M, 20-min exposure for 4 days). Each point represents the mean $\pm$ S.E.M. ($n = 6$) of total or differential count of cells ($\times 10^6$ per ml). * denotes a significant ($p < 0.05$) difference compared with the corresponding no virus control. + denotes a significant ($p < 0.05$) difference compared with the corresponding saline group, as determined by ANOVA followed by a Dunnett's post hoc test.

icin, to deplete sensory neuron tachykinins, blocked the responses in anesthetized guinea pigs to instilled bradykinin but not intravenous bradykinin (Ichinose et al., 1990). Thus, it is possible that the potentiation of inhaled bradykinin by ACE and NEP inhibitors was also caused by enhancement of tachykinins, because these are also susceptible to breakdown by these enzymes (Dusser et al., 1988b). Intravenous bradykinin mediates bronchoconstriction mainly by release of cyclooxygenase products, because it was attenuated by indomethacin (Ichinose et al., 1990).

The B_2 kinin receptor antagonist MEN16132 (1 and 10 μ M inhalation exposure) blocked the bronchoconstriction to exposure to 1 mM bradykinin, indicating the involvement of B_2 kinin receptors rather than B_1 receptors. Icatibant (10 μ M inhalation exposure), however, had no effect. These results

are similar to those by Valenti et al. (2005), who have shown that MEN16132 is more potent and longer lasting than icatibant in inhibiting bradykinin-induced bronchoconstriction and microvascular leakage in anesthetized guinea pigs. Our result differs from those of Wirth et al. (1993), however, who found that both aerosolized and intravenous icatibant inhibited the bronchoconstriction to bradykinin in anesthetized guinea pigs. It is difficult to compare our dose of icatibant with those used by Wirth et al. (1993) because they delivered directly into the lungs, whereas our delivery was via box exposure. Thus, mode of delivery and the fact that our animals were conscious may explain the discrepancy. MEN16132 administered intraperitoneally had no effect in our study, possibly because the drug was not absorbed by this route and did not reach the lungs in enough concentration to have an effect,

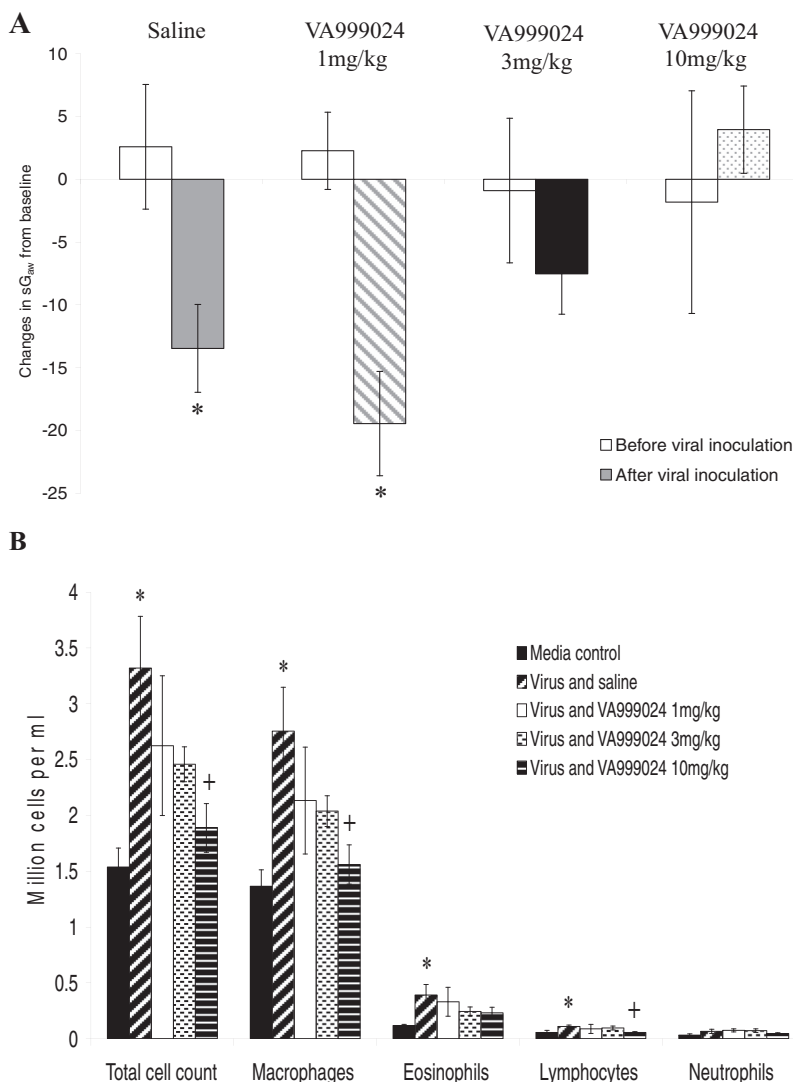


Fig. 7. Effects of treatment of guinea pigs with VA999024 (1, 3, and 10 mg/kg s.c.) or saline on airway responses to histamine (1 mM, 20-s nose-only exposure) before and after instillation of PIV-3 virus (6.32×10^6 infectious units per ml) (A) and total and differential cell (macrophage, eosinophil, lymphocyte, and neutrophil) counts in BALF removed from virus-free medium-inoculated animals or PIV-3-inoculated animals treated with saline or VA999024 (B). Responses to histamine are the mean $\pm$ S.E.M. ($n = 6$) peak changes in sG_{aw} expressed as a percentage of the baseline sG_{aw} values. Negative values represent bronchoconstriction. * denotes a significant ($p < 0.05$) difference between the changes in the maximum decrease in sG_{aw} from baseline before and after viral inoculation, as determined by a Student's paired t test. Total or differential cell counts are the mean $\pm$ S.E.M. ($n = 6$) cells ($\times 10^6$ per ml) in medium-treated controls and virus-inoculated guinea pigs treated with saline or VA999024. * denotes a significant ($p < 0.05$) difference compared with the corresponding medium control. + denotes a significant ($p < 0.05$) difference compared with the corresponding saline group, as determined by ANOVA followed by Dunnett's post hoc test.

although in the study of Valenti et al. (2005) it was effective by intravenous administration.

After bradykinin inhalation exposure with captopril treatment there was no significant increase in inflammatory cells in the lung but after inhalation exposure with both captopril and phosphoramidon there were significant increases in total cells, macrophages, eosinophils, and neutrophils. This suggests that the threshold for leukocyte influx was higher than for bronchoconstriction, because both degradative enzymes were required to be blocked. After treatment with the B_2 receptor antagonist MEN16132 (10 μ M inhalation exposure), the increases in total cells, eosinophils, neutrophils, and lymphocytes were significantly inhibited and there was a modest inhibition of the macrophage increase. This study shows that bradykinin can produce an influx of inflammatory cells but only after its metabolism in the lung is prevented by inhibition of both ACE and NEP. Although bradykinin is not thought to have much direct effect on activation and recruitment of inflammatory cells, it is thought to work indirectly through the release of monocyte and neutrophil chemotactic activity from the airway epithelial cells (Koyama et al., 1995) to cause release of neutrophil, monocyte, and eosinophilic factors from alveolar macrophages (Sato et al., 1996) and stimulate eotaxin release from human lung fibroblasts (Sato

et al., 2000). The inhibition of the bradykinin-induced inflammatory cell influx by MEN16132 indicates that it is mediated through the bradykinin B_2 receptor.

Bradykinin exposure had no effect in the absence of enzyme inhibitors in guinea pigs that were sensitized but not challenged with ovalbumin. Thus, sensitization alone is not sufficient to induce a bronchoconstriction to bradykinin. This contrasts with another indirect bronchoconstrictor, adenosine, which shows no bronchoconstriction in normal subjects (Cushley et al., 1983) and unsensitized guinea pigs (Smith and Broadley, 2008) but produces a bronchoconstriction in asthmatic subjects and sensitized guinea pigs. However, in sensitized guinea pigs that received an ovalbumin exposure at 24 h after challenge there was a significant bronchoconstrictor response to bradykinin. This was achieved without inhibition of ACE or NEP and indicates that ovalbumin challenge induces airway hyperreactivity to bradykinin. Because ovalbumin challenge causes airway hyperreactivity to other directly acting spasmogens, including histamine and methacholine (Toward and Broadley, 2004; Smith and Broadley, 2007), these results demonstrate that the hyperreactivity is extended to the indirect spasmogen, bradykinin. Whether this is because of a common mechanism cannot be deduced from the present study. It is probably caused by epithelial

damage caused by the ovalbumin challenge. This would expose the sensory nerves through which bradykinin acts after inhalation (Ichinose et al., 1990) or result in loss of the epithelium-derived NEP and ACE. The hyperreactivity could also arise from a loss of an epithelial-derived relaxing factor, such as NO, which can be released by bradykinin (Ricciardolo et al., 1994). If this opposing relaxing component is lost, it would allow the bronchoconstrictor effects of bradykinin to emerge. Allergen-induced airway hyperreactivity to inhaled bradykinin does not seem to have been demonstrated previously in guinea pigs, although it has been demonstrated in brown Norway rats after intravenous administration (Ellis et al., 2004). These results therefore provide a basis for the appearance of bronchoconstriction in asthmatics but not in normal subjects after bradykinin inhalation (Polosa and Holgate, 1990).

Inoculation of guinea pigs with parainfluenza-3 virus caused AHR to inhaled histamine and inflammatory cell influx into the BALF, in agreement with previous studies (Folkerts et al., 2000; Toward et al., 2005). The B₂ receptor antagonist MEN16132 and the tissue kallikrein inhibitor VA999024 (Evans et al., 1996) abolished these effects after PIV-3 inoculation. The inhibition of the PIV-3-induced inflammatory cell influx with MEN16132 and VA999024 shows that bradykinin is involved in the virus-induced inflammation and is mediated through B₂ kinin receptors. Folkerts et al. (2000) also showed that the B₂ receptor antagonist icatibant administered subcutaneously inhibited the AHR to intravenously administered histamine in anesthetized guinea pigs treated with PIV-3. However, they did not show any reduction in cell influx after icatibant. This discrepancy could be caused by the different route of delivery of MEN16132 in our study or the fact that this compound has greater efficacy when administered locally to the lungs. In their study, there was a tendency for an increase in bradykinin levels in BALF after PIV-3 infection, which supports the idea that bradykinin is involved in the AHR and cell influx caused by PIV-3 infection. In experiments using histamine to test for AHR, it is of interest that the histamine challenge may itself release bradykinin. Thus, inhibition of the AHR to histamine by MEN16132 could have been caused by blockade of released bradykinin rather than the underlying bradykinin-mediated AHR. However, this would require that the bronchoconstrictor responses to histamine were entirely caused by bradykinin release. Clearly this is very unlikely because inhaled bradykinin does not cause bronchoconstriction unless its breakdown is inhibited.

In summary, this study has demonstrated a bronchoconstriction and influx of inflammatory cells in conscious guinea pigs after inhaled bradykinin. However, these responses only occur when breakdown of bradykinin is inhibited by blockade of ACE and NEP. These responses are mediated via B₂ kinin receptors because they were antagonized by inhaled MEN16132. Neither intraperitoneal MEN16132 nor inhaled icatibant antagonized bradykinin, indicating the importance of the route of injection of these agents in bradykinin research. Sensitization of guinea pigs is not sufficient to induce airway hyperreactivity to the bronchoconstriction by inhaled bradykinin. However, ovalbumin challenge of sensitized guinea pigs causes AHR to bradykinin and to histamine and methacholine. Viral infection of guinea pigs with PIV-3 also produces AHR to inhaled histamine and influx of inflamma-

tory cells. These responses are attenuated by the B₂ receptor antagonist MEN16132 by inhalation and the tissue kallikrein inhibitor VA999024, suggesting that bradykinin is involved in virus-induced inflammatory cell influx and AHR.

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Address correspondence to: Kenneth J. Broadley, Division of Pharmacology, Welsh School of Pharmacy, Cardiff University, King Edward VII Avenue, Cardiff CF10 3NB, UK. E-mail: broadleykj@cardiff.ac.uk
