

RESEARCH PAPER

Capsaicin-induced mucus secretion in rat airways assessed *in vivo* and non-invasively by magnetic resonance imagingH Karmouty-Quintana^{1,2,5}, C Cannet¹, R Sugar³, JR Fozard⁴, CP Page² and N Beckmann¹¹Discovery Technologies, Novartis Institutes for BioMedical Research, Basel, Switzerland; ²Sackler Institute of Pulmonary Pharmacology, School of Biomedical and Health Sciences, King's College, London, UK; ³Novartis Horsham Research Centre, Horsham, UK and ⁴Respiratory Diseases Area, Novartis Institutes for BioMedical Research, Basel, Switzerland

Background and purpose: An up-regulation of the sensory neural pathways in the lung has been implicated in asthma and chronic obstructive pulmonary disease (COPD) and is thought to contribute to mucus hypersecretion, an essential feature of both diseases. The aim of this study was to assess non-invasively the acute effects (up to 60 min) of sensory nerve stimulation by capsaicin in the lung, using magnetic resonance imaging (MRI).

Experimental approach: Male Brown Norway rats were imaged prior to and 10, 30 and 60 min after intra-tracheal challenge with capsaicin (30 μgkg^{-1}) or vehicle (0.5% ethanol solution). In subsequent studies, pre-treatment with the transient receptor potential vanilloid (TRPV)-1 antagonist, capsazepine; the dual neurokinin (NK) 1 and NK2 receptor antagonist, DNK333 and the mast cell stabilizer, di-sodium cromoglycate (DSCG) was used to modulate the effects of capsaicin.

Key results: Diffuse fluid signals were detected by MRI in the lung as early as 10 min after capsaicin, remaining constant 30 and 60 min after treatment. Broncho-alveolar lavage (BAL) fluid analysis performed 60 min after capsaicin revealed increased mucin concentration. Capsazepine (3.5 mgkg^{-1}), DNK333 (10 mgkg^{-1}) but not DSCG (10 mgkg^{-1}) administered prophylactically were able to block the effect of capsaicin in the airways.

Conclusions and implications: These observations suggest that the fluid signals detected by MRI after capsaicin administration reflected predominantly the release of mucus following activation of sensory nerves. They point to the opportunity of non-invasively assessing with MRI the influence of neuronal mechanisms in animal models of asthma and COPD.

British Journal of Pharmacology (2007) 150, 1022–1030. doi:10.1038/sj.bjp.0707168; published online 12 March 2007

Keywords: sensory nerves; tachykinins; non-invasive; spontaneously breathing; TRPV-1; neurokinin

Abbreviations: BAL, bronchoalveolar lavage; CGRP, calcitonin gene related peptide; DMSO, dimethylsulphoxide; DNK333, N-[(R,R)-(E)-1-(3,4-dichlorobenzyl)-3-(2-oxoazepan-3-yl) carbamoyl]allyl-N-methyl-3, 5-bis(trifluoromethyl)benzamide; DSCG, di-sodium-cromoglycate; EPO, eosinophil peroxidase; i.p., intraperitoneally; i.t., intratracheally; MPO, myeloperoxidase; MR, magnetic resonance; MRI, magnetic resonance imaging; NKA, neurokinin A; s.e.m., standard error of the mean; SP, substance P; TRPV1, transient potential receptor vanilloid (subtype)-1

Introduction

Cough and sputum production are characteristic of asthma, a condition linked increased mucus production, goblet cell hyperplasia and submucosal gland hypertrophy (Liu *et al.*, 1998; Rogers, 2001). Chronic mucus hypersecretion is indicative of poor asthma control and is closely associated

with a decline in lung function, increasing the risk of morbidity and mortality (Rogers, 2004). Mucus hypersecretion and a reduced capacity to clear it are also important characteristics of chronic obstructive pulmonary disease (COPD) (Rogers, 2005), contributing to increased morbidity and mortality in COPD patients (Vestbo *et al.*, 1996). Consequently, it is essential to understand the pathophysiology of mucus hypersecretion in asthma (Rogers, 2004) and the component of COPD attributable to impaired mucociliary clearance (Melton, 2002; Rogers, 2005).

An upregulation of sensory-efferent neural pathways is also implicated in COPD and asthma (Rogers, 1997). Airway

Correspondence: Dr N Beckmann, Novartis Institutes for BioMedical Research; Discovery Technologies; Lichstr 35, WSJ-386.2.09, CH-4002 Basel, Switzerland.

E-mail: nicolau.beckmann@novartis.com

⁵Current address: Meakins-Christie Laboratories, Department of Medicine, McGill University, Montreal, Quebec, Canada.

Received 31 August 2006; revised 15 December 2006; accepted 9 January 2007; published online 12 March 2007

sensory nerves release several neuropeptides of which the tachykinins (neurokinin A (NKA) and substance P (SP)) contribute most to the pathophysiology of asthma and COPD (Joos *et al.*, 2003). These neuropeptides activate the NK receptors NK1, NK2 and NK3, thereby inducing a variety of biological effects (Patacchini and Maggi, 1995). In subjects with asthma, increased immunoreactivity to the tachykinin SP has been observed in plasma, induced sputum and nasal lavage. An increase in tachykininergic nerves and upregulation of a tachykinin receptor mRNA has also been observed (Mapp *et al.*, 2000). In COPD, levels of SP are increased in sputum (Mapp *et al.*, 2000). Tachykininergic nerve stimulation, or pollutant and irritant gases that activate sensory nerves (Germonpre *et al.*, 1995), are implicated in the pathophysiology of COPD (Rogers, 2005) and have been shown experimentally to contribute to mucin upregulation (Rogers, 2001).

Activation of sensory nerves in the lung can be induced experimentally by a variety of insults such as electrical stimulation of the vagus nerve or by application of chemical and mechanical irritants, such as capsaicin, hypertonic saline and cigarette smoke (Germonpre *et al.*, 1995). Activation of sensory nerves by capsaicin or other stimuli leads to the release of neuropeptides from nerve terminals either directly or through axonal reflexes (Reynolds *et al.*, 1997). An increase in plasma extravasation from the rat trachea has been shown with capsaicin administered intratracheally (i.t.) at doses between 3 and 30 $\mu\text{g kg}^{-1}$ (Fozard *et al.*, 2001). Tachykinins may be involved in smooth muscle contraction, vasodilatation, edema, mucus secretion and inflammatory cell activation (Reynolds *et al.*, 1997).

The measurement of fluid volumes from the lungs is important for the assessment of mucus plugging and inflammation, particularly in the peripheral compartment of the lung as the diagnostic techniques that are often used are more sensitive to changes in the central conducting airways than to those in the peripheral compartment of the lung (Mead, 1970). Through the assessment of lung fluid signal volumes, proton magnetic resonance imaging (MRI) techniques have been shown to be successful in detecting edema as a result of allergen challenge (Beckmann *et al.*, 2001; 2002) or mucus hypersecretion following treatment with endotoxin.

Thus the aim of our study was to investigate non-invasively, by the use of MRI to determine lung fluid signal volumes, the acute effects induced in the lungs following the activation of airway sensory nerves by capsaicin in fully anaesthetized Brown Norway (BN) rats. The role of sensory nerves and tachykinins in mediating the effects of capsaicin in the airways was studied using the transient receptor potential vanilloid (TRPV)-1 antagonist capsazepine (3.5 mg kg^{-1} i.t.); and the dual NK1 and NK2 receptor antagonist *N*-[(*R,R*)-(E)-1-(3,4-dichlorobenzyl)-3-(2-oxoazepan-3-yl) carbamoyl]allyl-*N*-methyl-3,5-bis(trifluoromethyl)-benzamide (DNK333) (10 mg kg^{-1} i.p.). As TRPV-1 has also been observed in mast cells (Biro *et al.*, 1998), we have also utilized the drug di-sodium cromoglycate (DSCG), which has been shown to inhibit mast cell activation in the rat (Norris, 1996).

Materials and methods

Experiments were performed with the approval of the Veterinary Authority of the City of Basel licence 567 (Kantonales Veterinaeramt, Basel-Stadt).

Animals

Fifty-three male BN rats, weighing 250–300 g, were supplied by IFFA CREDO (L' Arbresle Cedex, France). The rats were kept at an ambient temperature of $22 \pm 2^\circ\text{C}$ under a 12 h normal phase light–dark cycle and fed NAFAG pellets (Nahr und Futtermittel AG, Gossau, Switzerland). Drinking water was freely available.

Administration of substances

Rats were kept anaesthetized with 2.5% isoflurane (Abbott, Cham, Switzerland) in a mixture of $\text{O}_2/\text{N}_2\text{O}$ (1:2) administered via a face mask, throughout the whole experimental procedure. Animals were killed immediately at the end of each experiment. Following the baseline MRI signal acquisition (see below), animals were taken out of the magnet and a single dose of capsaicin (30 $\mu\text{g kg}^{-1}$, $n = 12$) or vehicle (0.5% alcohol, 0.2 ml, $n = 12$) was administered i.t., at a level above the carina, without letting the animals wake up. The dose was chosen based on the experiments of Fozard *et al.* (2001), where doses ranging from 3 to 30 $\mu\text{g kg}^{-1}$ of capsaicin were shown to cause plasma extravasation in the rat trachea. The rats were repositioned in the magnet immediately after capsaicin administration.

In subsequent studies animals were anaesthetized and, following acquisition of baseline images, pretreated with (i) capsazepine (3.5 mg kg^{-1} i.t.; $n = 5$) or its vehicle (0.2 ml of a 0.5% ethanol solution i.t.; $n = 4$); (ii) DSCG (10 mg kg^{-1} i.t., $n = 5$) or its vehicle (0.1 ml saline i.t., $n = 5$); or (iii) DNK333 (10 mg kg^{-1} intraperitoneally (i.p.), $n = 5$) or its vehicle (2 mg kg^{-1} of a 4% dimethyl sulphoxide (DMSO) solution in Neoral placebo ($n = 5$)). Capsaicin (30 $\mu\text{g kg}^{-1}$ i.t.) was administered i.t. thirty minutes after treatment with capsazepine, DNK333 or their respective vehicles, and immediately following DSCG or vehicle treatment. Following administration of capsaicin, animals were kept anaesthetized with 2.5% isoflurane and imaged 60 min after administration of capsaicin. The dose of capsazepine used was based on experiments by Lee and Lundberg (1994), where doses of 2 to 3.5 mg kg^{-1} i.v. inhibited chemoreflexes (apnea, bradycardia, and hypotension) induced by capsaicin. Oral administration of DNK333 (10 mg kg^{-1}) has been shown to inhibit citric acid-induced cough by 82% and SP-induced bronchoconstriction when administered intraduodenally 30 min before endotoxin challenge in the guinea pig (Lewis *et al.*, 2004). The affinity of DNK333 for the rat NK1 and NK2 receptors is of the same order as that for the guinea pig receptors (unpublished observations, Novartis Pharma Research). The dose of DSCG was based on experiments performed by Dixon and Barnes (1989), showing that inhaled DSCG (10 mg) was able to inhibit the effects of bradykinin-induced bronchoconstriction in mild asthmatics.

MRI

MRI acquisition protocol. During MRI acquisitions rats were placed in a supine position in a cradle made of Plexiglas, in the centre of the magnet. Body temperature was maintained at $37 \pm 1^\circ\text{C}$ using warm air, regulated by a rectal temperature probe (DM 852, Ellab, Copenhagen, Denmark). Rats were anaesthetized with 2.5% isoflurane, in a mixture of $\text{O}_2/\text{N}_2\text{O}$ (1:2), administered via a facemask. All scans were performed on spontaneously breathing animals, and neither cardiac nor respiratory triggering was applied. Measurements were carried out with a Biospec 47/40 spectrometer (Bruker Medical Systems, Ettlingen, Germany) operating at 4.7 T, equipped with an actively shielded gradient system capable of generating a gradient of 200 mTm^{-1} (Figure 1).

The operational software of the scanner was Paravision (version 3.1, Bruker). The parameters of a gradient-echo sequence were chosen with the aim of detecting fluid signals appearing in the lung as observed after an allergen or endotoxin challenge (Beckmann *et al.*, 2001, 2002): repetition time, 5.6 ms; echo time, 2.7 ms; excitation pulse (Gaussian shape) length, 1 ms; flip angle of the excitation pulse approximately 15° ; field-of-view, $6 \times 6\text{ cm}^2$; matrix size, 256×128 ; slice thickness 1.5 mm. A single transverse slice image was obtained by computing the two-dimensional Fourier transform of the averaged signal from 45 individual image acquisitions and interpolating the data set to 256×256 pixels. There was an interval of 530 ms between individual image acquisitions, resulting in a total acquisition time of 56 s for a single slice. Twenty slices were acquired consecutively. Animals were subject to MRI scanning sessions at baseline (before capsaicin or vehicle administration) and at 10, 30 and 60 min after challenge. In experiments involving capsazepine, DSCG or DNK333, animals were imaged at baseline and 60 min after capsaicin ($30\text{ }\mu\text{g kg}^{-1}$ i.t.) challenge.

MRI analysis. The volume of fluid signal was quantified using a semiautomatic segmentation procedure implemented in the Interactive Data Language Research Systems ((IDL)

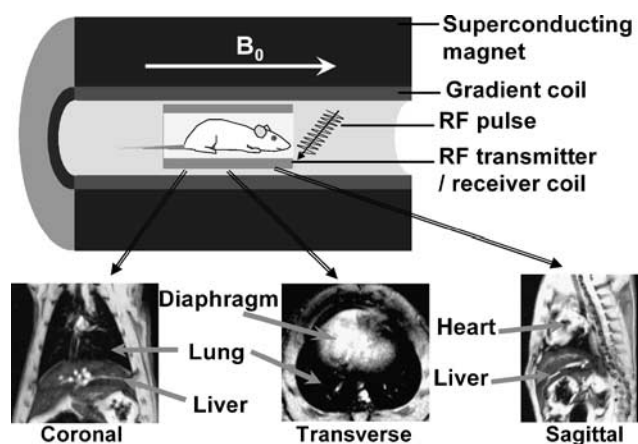


Figure 1 Top: Diagrammatic representation of the components required for an MRI scan. B_0 represents the homogenous magnetic field at 4.7 T. Bottom: Representative coronal, transverse and sagittal MR images of a rat showing the position of the lung, liver, diaphragm and heart in the different slices.

Boulder, CO, USA) environment (version 5.1) on a Linux platform. Images were first weakly lowpass filtered with a Gaussian profile filter and then transformed into a set of four gray level classes using adaptive Lloyd–Max histogram quantization. This method avoids operator bias owing to the arbitrary choice of threshold levels on each image (Beckmann *et al.*, 2001). As the fluid comprised high signal intensities in the original images, it was represented by the highest gray level class in the transformed images. This class could be extracted interactively by use of a region grower. Because of the unknown extent of the fluid, no morphology parameters were incorporated into the region growing process. Instead, a contour serving as a growing border was drawn to control region growing manually. The segmentation parameters were the same for all the analyzed images, chosen to segment regions corresponding to high intensity signals. Twenty individual images covering the thorax were analyzed; the thickness of each individual image was 1.5 mm, thus permitting the software to determine the volume of signal present in each individual slice. The total volume corresponding to the sum of 20 consecutive slices per time point was calculated. Because the fluid signals and those from vessels were of comparable intensities, the volume corresponding to the vessels was assessed on baseline images and then subtracted from the total volume determined on post-treatment time points.

Post-mortem analyses

Following the MRI session (60 min after challenge) the animals were killed with an overdose of pentothal (250 mgkg^{-1} i.p.) for bronchoalveolar lavage (BAL) fluid ($n = 12$; six capsaicin and six vehicle treated) and histological analysis ($n = 12$; six capsaicin and six vehicle treated). In experiments involving capsazepine, DSCG and DNK333, only BAL fluid analysis was performed.

BAL fluid analysis. A detailed description of the procedure to obtain BAL fluid and the analysis of the parameters of inflammation herein has been provided earlier (Beckmann *et al.*, 2002, Tigani *et al.*, 2002). Briefly, after killing the animals with an overdose of pentothal, the lungs were lavaged three times with 4 ml of ethylenediaminetetraacetic acid + phosphate-buffered saline (PBS) (solution A) recovering between 10 and 11 ml of BAL fluid that was made up to a volume of 12 ml with solution A. For leukocyte numbers and cell differentiation, approximately 4 ml of BAL fluid was used by an automatic cell analyzing system used for the analysis (Cobas Helios 5Diff, Hoffmann-La Roche, Axon Laboratory, Switzerland). Determination of eosinophil peroxidase was based on the oxidation of *o*-phenylenediamine by eosinophil peroxidase in the presence of hydrogen peroxide. Myeloperoxidase activity was measured in a photometric assay based on the oxidation of *O*-dianiside dihydrochloride by myeloperoxidase in the presence of hydrogen peroxide. The level of protein in the BAL fluid supernatants was measured by a photometric assay, based on the reaction of protein with an alkaline copper tartrate solution and Folin reagent. Mucus in BAL fluid was assessed from 1 ml of BAL fluid per animal, retrieved before lavage for cell count and differentiation. The

sandwich enzyme-linked lectin assay as described earlier (Beckmann *et al.*, 2002) was used to determine the quantity of mucus by measuring the optical density of flat-bottomed Coastr plates coated with Ulex europaeus agglutinin-1 (Sigma) at a wavelength of 492 nm using a SpectraMax 250 plate reader (Molecular Devices, Surrey, UK).

Histological analysis. Lungs were inflated via a cannula inserted into the trachea with 5 ml of 10% PBS neutral formalin. The lungs were then removed from the thorax and immersed in formalin for at least 24 h but no longer than 72 h. The lung tissue was then sectioned transversally through the left lobe, the right apical, the right median and the right caudal lobes so as to include the main bronchi as well as the pulmonary alveoli. After processing to paraffin wax, sections were embedded in blocks. Slices of 3 μm thickness cut from these blocks were stained using Alcian blue-periodic acid Schiff (AB/PAS) for the detection of goblet cells acid and neutral mucus. Staining using Verhoeff's reaction was performed for the demonstration of elastic fibers and for the subsequent quantification of perivascular edema.

Quantification of goblet cells. Three to seven pictures of bronchi from the apical, median and caudal lobes of each lung were captured at $\times 10$ magnification on AB/PAS-stained slides. Morphometric analyses were performed with the software 'Image Access 4.0' (Imagic, Glattbrugg, Switzerland) connected to a video camera 'Prog/Res/3008' (Jenoptik LOS, Eching, Germany). The circumference of each bronchus was manually delineated and the number of goblet cells was counted. Results were expressed as numbers of goblet cells per mm length bronchus. Only bronchi with a diameter between 800 and 2300 μm were measured. The total goblet cell numbers (average from the apical, median and caudal lobes) are presented.

Quantification of perivascular edema. Three to seven pictures of arterioles from each section of each lobe (apical, median and caudal) were captured at $\times 10$ magnification on Verhoeff-stained slides. Morphometric analyses were performed with the software 'Image Access 4.0' connected to a video camera 'Prog/Res/3008'. As described by Quintana *et al.* (2006), the areas of external edema and external elastica lamina were manually circumscribed, and perivascular edema was calculated as a percentage of external elastica lamina area. Only vessels of diameter comprised between 20 and 200 μm were taken into account. Approximately 20 vessels were measured per animal. The total edema determined histologically (average of the edema in the apical, median and caudal lobes) is shown.

Statistics

A multiple comparisons analysis of variance (ANOVA) test (with the Holm-Sidak method) was applied for data relating to MRI fluid signal volume from vehicle- and capsaicin-treated rats imaged at baseline, 10, 30 and 60 min after treatment. Student's *t*-tests (two-tailed) were performed to

assess differences in histological and BAL fluid parameters between capsaicin- and vehicle-treated animals and for MRI fluid signal volumes between vehicle- and drug-treated groups. The Mann-Whitney *U*-test was chosen for analysis of non-parametric histological data relating to goblet cell numbers. The significance levels are $*P < 0.05$, $**0.05 < P < 0.01$ and $***P < 0.001$.

Materials

The suppliers for the drugs used in these experiments were as follows: capsaicin and capsazepine (Sigma-Aldrich, Buchs, Switzerland); di-sodium cromoglycate (DSCG) and DNK333 (Novartis Pharma, Basel, Switzerland) isoflurane (Abbott, Cham, Switzerland).

Results

Instillation of capsaicin into the trachea resulted in the appearance of irregular fluid signals in MR images as early as 10 min after challenge, and which remained constant 30 and 60 min after treatment (Figures 2 and 3a). BAL fluid analysis performed 60 min after challenge revealed an increased

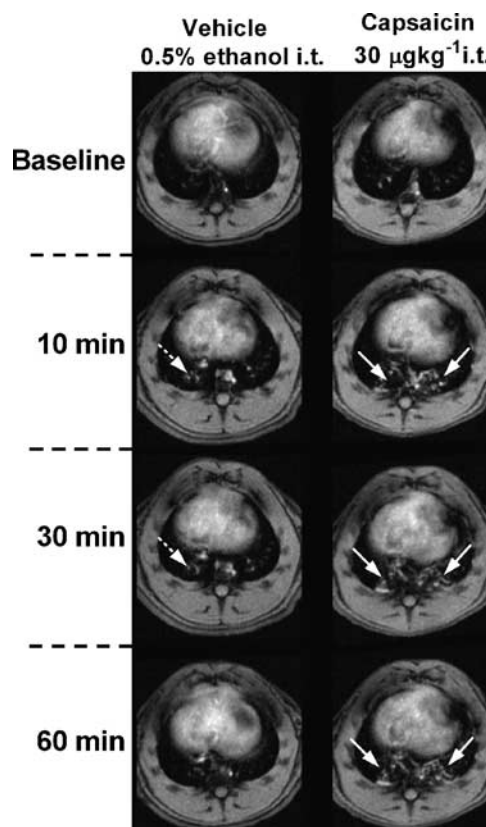


Figure 2 Transverse sections through the thorax of rats treated with either vehicle (0.5% ethanol; 0.2 ml i.t.) or capsaicin ($30 \mu\text{g kg}^{-1}$ i.t.). The time points are relative to the i.t. challenges. The white arrows denote diffuse signals related to capsaicin-induced mucus release as shown by post-mortem BAL fluid and histological analyses. The dotted white arrows show the appearance of a diffuse signal as a result of a volume of fluid being instilled in the lungs of the vehicle-treated animal.

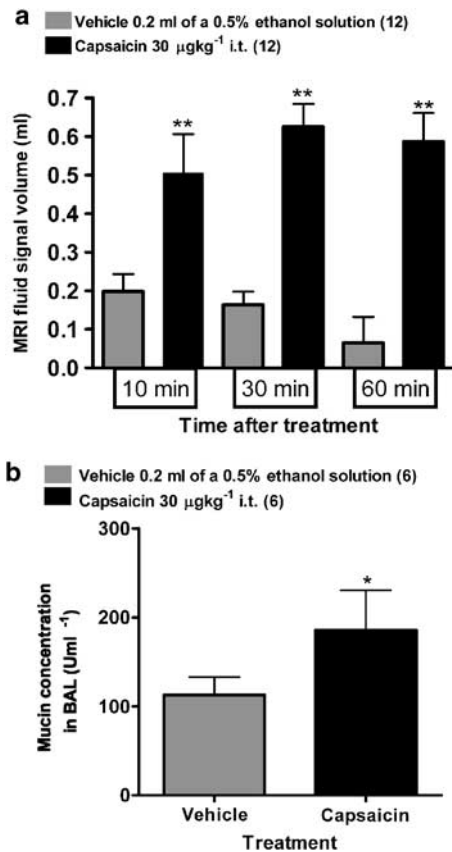


Figure 3 (a) Lung fluid signal volumes (means \pm s.e.m., $n = 12$ per group) determined from MR images at baseline, 10, 30 and 60 min after challenge in rats treated with vehicle or capsaicin. The significance level $**0.001 < P < 0.01$ refers to ANOVAs (Holm–Sidak method) between capsaicin and vehicle treatment at each time point. (b) Mucin concentration (means \pm s.e.m., $n = 6$ per group) in BAL removed 60 min after challenge with vehicle or capsaicin. Higher levels of mucin concentration were present in capsaicin-treated rats compared with vehicle-treated animals ($*P < 0.05$; Student's *t*-test).

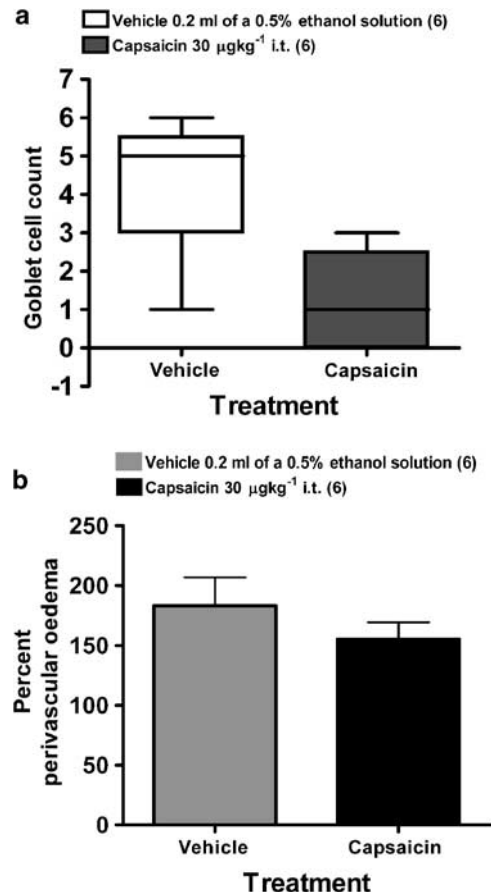


Figure 4 (a) Goblet cell number (means \pm s.e.m., $n = 6$ rats per group) in individual bronchi determined from the apical, median and caudal lobes taken 60 min after challenge from rats treated with vehicle (0.5% ethanol; 0.2 ml i.t.) or capsaicin (30 $\mu\text{g kg}^{-1}$ i.t.). The Mann–Whitney *U*-test revealed no difference between the treatment groups. (b) Perivascular edema assessed from apical, median and caudal lobes. Values (means \pm s.e.m. for six animals in each group) represent total edema values expressed as a percentage.

Table 1 Inflammatory cell infiltration and activation (mean values \pm s.e.m., $n = 6$ animals per group) in BAL fluid of BN rats treated with vehicle (0.5% ethanol; 0.2 ml i.t.) or capsaicin (30 μgkg^{-1} i.t.)

	Vehicle (0.5% ethanol) 0.2 ml i.t. ($n = 6$)	Capsaicin 30 μgkg^{-1} i.t. ($n = 6$)
Neutrophils $\times 10^6$	0.18 \pm 0.03	0.29 \pm 0.02*
MPO (mU ml ⁻¹)	52.1 \pm 10.0	94.9 \pm 10.3*
Eosinophils $\times 10^6$	0.93 \pm 0.24	1.09 \pm 0.05
EPO (mU ml ⁻¹)	2.0 \pm 0.2	2.2 \pm 0.3
Protein ($\mu\text{g ml}^{-1}$)	0.21 \pm 0.03	0.26 \pm 0.03
Macrophages ($\times 10^6$)	1.99 \pm 0.25	4.14 \pm 0.33**
Lymphocytes ($\times 10^6$)	0.39 \pm 0.10	0.44 \pm 0.05
Total cells ($\times 10^6$)	3.60 \pm 0.44	5.96 \pm 0.40**

Abbreviations: BN, Brown Norway; EPO, eosinophil peroxidase; MPO, myeloperoxidase.

Animals were killed 60 min after challenge with either substance. The significance levels $*P < 0.05$, and $**0.001 < P < 0.01$ refer to comparisons between vehicle- and capsaicin-treated animals.

mucin concentration in the fluid removed from capsaicin-treated animals (Figure 3b). Furthermore, an increase in the number of neutrophils, macrophages and myeloperoxidase

(MPO) activity (indicating neutrophil activation) was observed in the BAL fluid (Table 1). Interestingly, no significant differences were observed by histology in goblet cell number between capsaicin- and vehicle-treated groups (Figure 4a). Also, no differences were found at this time point in protein concentration in the BAL fluid or in perivascular edema between capsaicin- and vehicle-treated groups (Table 1 and Figure 4b).

Intratracheal administration of the vehicle for capsaicin (0.2 ml of a 0.5% ethanol solution) resulted in the appearance of diffuse MRI fluid signals of lower magnitude than those following capsaicin. These signals were short-lived, lasting no more than 30 min and are assumed to reflect the introduction of a volume of fluid (0.2 ml) into the lungs of the rat.

Both the TRPV-1 antagonist (capsazepine; 3.5 mgkg^{-1} i.t.) and the dual NK1 and NK2 receptor antagonist (DNK333; 10 mgkg^{-1} i.p.) were able to inhibit the response to capsaicin in the airways when compared with their respective vehicle groups, in terms of MRI fluid signal volumes (Figure 5a).

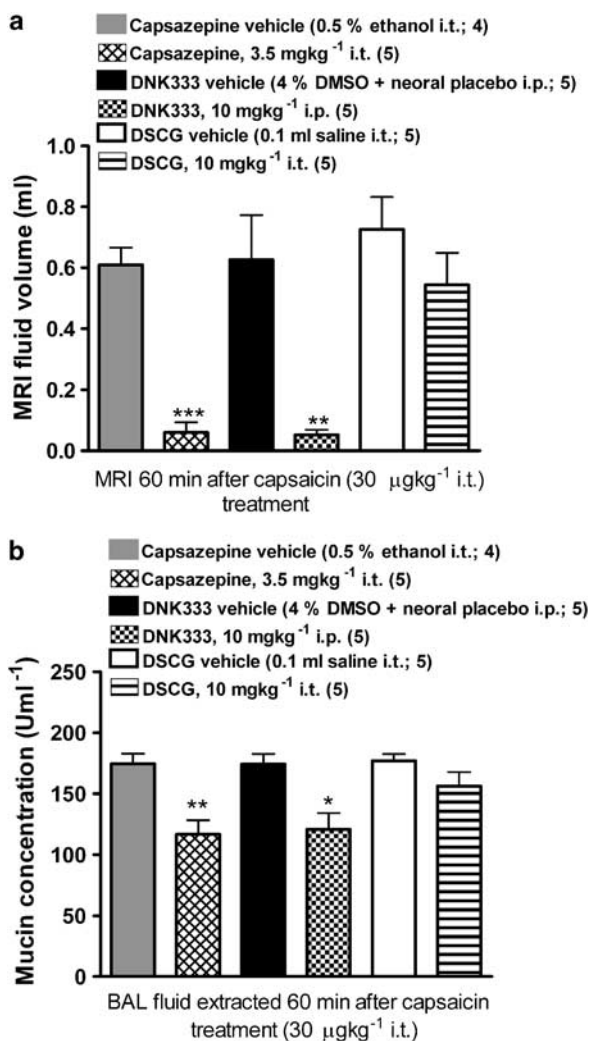


Figure 5 (a) Lung fluid signal volumes (means \pm s.e.m., $n=5$ per group) determined from MR images at baseline, and 60 min after capsaicin challenge in rats pre-treated with (i) capsazepine (3.5 mg kg^{-1} i.t.) or its vehicle (0.2 ml of a 0.5% ethanol solution i.t.); (ii) DNK333 (10 mg kg^{-1} i.p.) or its vehicle (4% DMSO in Neoral placebo i.p.); or (iii) DSCG (10 mg kg^{-1} i.t.) or its vehicle (0.2 ml saline i.t.). Capsaicin was administered 30 min after capsazepine or DNK333 treatment, and immediately after DSCG. Significance levels $**0.001 < P < 0.01$ and $***P < 0.001$ refer to Student's *t*-test comparisons between each treatment regimen and its respective vehicle. (b) Mucin concentration (means \pm s.e.m.) in BAL fluid removed 60 min after capsaicin challenge, immediately following an MRI acquisition, from rats that were submitted to the treatment regimens specified in (a). Both capsazepine and DNK333 resulted in lower concentrations of mucin in BAL fluid when compared with their respective vehicle groups ($*0.01 < P < 0.05$ and $**0.001 < P < 0.01$; Student's *t*-tests).

Mucin concentrations in BAL fluid were also reduced in capsazepine- and DNK333-treated rats compared with their respective vehicle groups (Figure 5b). Treatment with DSCG (10 mg kg^{-1} i.t.) immediately before capsaicin challenge did not have any effect on MRI fluid signal volumes (Figure 5a) or in mucin concentration in BAL fluid when compared with its vehicle group (Figure 5b). Pre-treatment with capsazepine, DNK333, or DSCG did not reduce the infiltration of

inflammatory cells present in BAL fluid and did not inhibit MPO activity in BAL fluid (Table 2).

Discussion and conclusions

The major finding of the present study was that local administration of capsaicin ($30 \mu\text{g kg}^{-1}$ i.t.) resulted in the appearance of diffuse fluid signals in MR images of the lung at 10 min after treatment which were still present 30 and 60 min after administration of the pungent chili pepper extract, capsaicin. The changes correlated with an increase of mucin concentration in the BAL fluid, and a tendency towards a reduction of goblet cells in histological slides from capsaicin-treated, compared with vehicle-treated groups. These results suggest that mucus release from goblet cells induced by capsaicin was the principal contributor to the MRI signals. Pre-treatment with capsazepine, a TRPV-1 antagonist, or with DNK333, an NK1 and NK2 receptor antagonist, prevented the appearance of fluid signals in MR images acquired following capsaicin challenge. Consistent with these observations, a reduced level of mucin concentration was found in capsazepine- and DNK333-treated animals compared with their corresponding vehicle groups.

Capsaicin excites a subset of primary sensory neurons with somata in the dorsal root ganglia or the trigeminal ganglion (Szallasi and Blumberg, 1999). Airway sensory nerves effects are induced via the jugular ganglion of the vagus or the rostral portion of the nodose ganglion (McDonald *et al.*, 1988). Capsaicin-induced effects are mediated through the activation of the vanilloid receptor TRPV-1, a heat-sensitive cation channel on nociceptor terminals (Caterina *et al.*, 1997; Szallasi and Blumberg, 1999). In the present studies, capsazepine (3.5 mg kg^{-1} i.t.), a TRPV-1 antagonist (Bevan *et al.*, 1992), administered 30 min before capsaicin was able to fully inhibit the effects of the chili pepper extract in the lungs as detected by MRI and BAL fluid analysis. This finding further supports a role for the vanilloid receptor, TRPV-1, in mediating capsaicin-induced mucus release in the lungs. However, expression of TRPV-1 is not confined to sensory nerves; evidence suggests that this receptor is also present in mast cells (Biro *et al.*, 1998) and that in the presence of capsaicin, the level of intracellular calcium is raised (Biro *et al.*, 1998). In the present investigation, inhibition of mast cell degranulation by DSCG did not inhibit mucus release induced by capsaicin. This supports the role of TRPV-1 expressed in sensory nerves and not mast cells in mediating the effects of capsaicin challenge in the airways.

Stimulation of sensory nerves by capsaicin results in the release of neuropeptides such as SP, calcitonin gene-related peptide (CGRP) and NKA (Szallasi and Blumberg, 1999). Among these sensory neuropeptides, release of SP shows the best correlation with activation of TRPV-1 (Buck and Burks, 1986; Holzer 1991). SP and NKA act mainly through NK1 receptors in secretory glands and in the respiratory epithelium mediating mucus hypersecretion, edema formation, vasodilatation, and release of prostaglandins and pro-inflammatory cytokines (Rogers, 1995; Reynolds *et al.*, 1997). Activation of NK1 receptors is strongly linked to

Table 2 Inflammatory cell infiltration and activation (mean values \pm s.e.m.) in BAL fluid of BN rats treated with (i) capsaizepine (3.5 mg kg^{-1} i.t.; $n=5$) or its vehicle (0.2 ml of a 0.5% ethanol solution i.t.; $n=4$); (ii) DSCG (10 mg kg^{-1} i.t., $n=5$) or its vehicle (0.1 ml saline i.t., $n=5$); or (iii) DNK333 (10 mg kg^{-1} i.p., $n=5$) or its vehicle (2 mg kg^{-1} of a 4% DMSO solution in Neoral placebo, $n=5$). Capsaicin was administered 30 min after treatment with capsaizepine, DNK333 or their respective vehicles, and immediately following administration of DSCG or its vehicle

	Vehicle (0.5% ethanol)	Capsaizepine (3.5 mg kg^{-1} i.t.)	Vehicle (4% DMSO in Neoral placebo)	DNK333 (10 mg kg^{-1} i.p.)	Vehicle saline 0.2 ml i.t.	DSCG (10 mg kg^{-1} i.t.)
Neutrophils ($\times 10^6$)	0.49 ± 1.58	0.37 ± 0.09	0.25 ± 0.04	0.25 ± 0.04	0.49 ± 1.58	0.17 ± 0.03
MPO mU mL^{-1}	46 ± 3	35 ± 8	43 ± 5	40 ± 2	46.8 ± 4	41 ± 1
Eosinophils ($\times 10^6$)	1.22 ± 0.32	$0.80 \pm 0.1^*$	0.94 ± 0.31	0.83 ± 0.39	1.17 ± 0.12	0.92 ± 0.16
Macrophages ($\times 10^6$)	5.10 ± 0.76	4.60 ± 0.49	3.73 ± 0.64	3.03 ± 0.69	4.77 ± 0.82	5.62 ± 0.48
Lymphocytes ($\times 10^6$)	1.30 ± 0.56	0.83 ± 0.06	0.68 ± 0.30	0.26 ± 0.06	1.28 ± 0.37	$0.28 \pm 0.05^*$
Total cells ($\times 10^6$)	8.10 ± 1.66	6.10 ± 0.63	5.81 ± 0.91	4.37 ± 1.12	7.70 ± 1.39	6.98 ± 0.48

Abbreviations: BAL, bronchoalveolar lavage; DMSO, dimethyl sulfoxide; DSCG, di-sodium cromoglycate; MPO, myeloperoxidase.

Animals were killed 60 min after capsaicin administration for BAL fluid analysis. Substances were administered i.t., with the exception of DNK-33 and its vehicle, which were applied i.p. The significance levels $*P < 0.05$ refer to comparisons between vehicle-treated and capsaizepine or DSCG-treated animals.

neurogenic airway secretor processes that comprise mucus secretion and ion transport (Rogers, 1995). *In vitro* studies showed that SP stimulates mucus secretion from submucosal glands in ferret and in human airways (Rogers *et al.*, 1989; Meini *et al.*, 1993; Ramnarine *et al.*, 1994), as well as in the guinea pig trachea (Kuo *et al.*, 1990). Further evidence of the role of sensory nerves in mucus secretion comes from studies where sensory nerves have been de-sensitized. De-sensitization of sensory nerves by sub-chronic administration of capsaicin either in neonatal animals (resulting in degeneration of nerve fibers) or in adult animals (resulting in depletion of sensory neuropeptides) leads to inhibition of goblet cell discharge and in a reduction of microvascular leakage (Solway and Leff, 1991; Barnes, 2001). In our study, pre-treatment with the dual NK1 and NK2 receptor antagonist DNK333 (Gerspacher *et al.*, 2003) fully inhibited mucus release provoked by capsaicin. These results support a role for neuropeptide release following stimulation of sensory nerves leading to NK1 and/or NK2-mediated mucus release.

In addition to the increase in mucin concentration, raised levels of macrophages, neutrophils, and MPO concentration (closely linked to neutrophil activation) were found in BAL fluid 60 min following capsaicin-treatment (Table 1). Neutrophils and macrophages have been implicated in promoting mucus hypersecretion via the release of neutrophil elastase, a potent goblet cell degranulating agent, and through the release of tumor-necrosis factor- α (Delgado *et al.*, 2003), leading to the expression and activation of epithelial growth factor receptors (Kim and Nadel, 2004). However, despite a reduction in MRI fluid signals and a decline in mucin concentration in BAL fluid following therapy with capsaizepine or DNK333, we observed no concomitant changes in MPO activity or in macrophage and neutrophil influx. Taken together, these findings suggest that capsaicin challenge results in the activation of sensory nerves leading to the release of tachykinins that induce mucus secretion via direct activation of secretory glands, through activation of NK receptors.

It is interesting to note that changes in MRI fluid signal volume were much greater in magnitude than the changes in mucin levels in BAL fluid. An explanation for this may be that mucus is a visco-elastic fluid composed of glycoproteins,

proteins (such as mucin), and lipids in a watery mixture (King, 2005). Assessment of mucin concentration in BAL fluid by the sandwich enzyme-linked lectin assay is likely to detect only a single component of mucus. However, MRI is able to detect the entire visco-elastic fluid, predominantly the water component of mucus. This together with the fact that the lung is practically devoid of signals (it appears black in MR images) provides a good contrast for the detection of mucus (water is rich in protons and thus will lead to a signal of high intensity) that could explain the increased sensitivity of the MRI technique compared with assays of mucin or protein from the BAL fluid. Despite the fact that there is an increase in mucin (and other mucus-related proteins) following capsaicin treatment, these are not detected by the Folin protein assay. However, it is important to consider that there are numerous proteins in BAL fluid that would be measured by a non-specific protein assay. This could mask a small change in mucus-related proteins, which would be detected with a more selective assay such as the enzyme-linked lectin assay for mucin.

It is important to emphasize that neither determination of protein concentration in BAL fluid nor histological assessment of perivascular edema revealed significant differences between capsaicin- and vehicle-treated groups. Activation of sensory nerves by capsaicin is known to cause plasma protein extravasation (Szallasi and Blumberg, 1999). Thus we suppose that the extent of edema was too low to be detected by the Folin protein assay and by histological assessment. A more sensitive method such as tagging plasma albumin with Evan's Blue may have demonstrated plasma protein extravasation in the airways. It is plausible that a small amount of edema could have been detected by MRI following capsaicin administration, but was not detected by the Folin protein assay or by histology. Nevertheless; the fact that (i) increased mucin content was observed in the BAL fluid of capsaicin-treated animals and (ii) the number of goblet cells detected by histology appeared to decrease following capsaicin-treatment compared with vehicle-challenged rats supports the view of mucus release as the primary contributor to MRI signals detected after capsaicin challenge.

Fozard *et al.* (2001) demonstrated an increased extravasation of plasma protein tagged with Evans Blue in the rat

trachea (from the larynx to the bifurcation) 10 min after i.t. administration of capsaicin ($3\text{--}30\ \mu\text{g kg}^{-1}$). In our studies, MRI signals following capsaicin administration were observed in lower parts of the lung that are associated with an increase of mucin concentration in BAL fluid. An explanation for the discrepancy between the current results and those of Fozard *et al.* (2001) could be the greater density of sensory nerves in upper airways compared to lower ones as demonstrated by Larson *et al.* (2003). Thus it cannot be excluded that capsaicin induces different responses in the tracheal region and in more distal sections of the lung. In support of this argument, studies performed by McDonald *et al.* (1988) in rats demonstrated that electrical stimulation of the superior laryngeal nerves leads to an increase in permeability of venules in the trachea, but not the bronchi. Additionally, all nerves studied seemed to have bilateral effects in the trachea but vagus nerves appeared to have a unilateral effect in the bronchi (McDonald *et al.*, 1988). Moreover, it has been shown that mast cells are present in close proximity to SP and CGRP containing sensory nerves in the trachea of mammals (Lundberg *et al.*, 1984, Uddman *et al.*, 1985). SP released by sensory nerves in the trachea activates local mast cells (Maggi, 1997), leading to plasma protein extravasation. Therefore, it is possible that activation of sensory nerves by capsaicin in the trachea and in the peripheral airways may lead to distinct outcomes within 60 min after administration of the compound.

DSCG has been shown to inhibit rat mast cell activation (Norris, 1996). However, DSCG also possesses an inhibitory effect upon sensory nerves as reviewed by Page (1994). Our observations support those of Ray *et al.* (1991) in the rat trachea where DSCG did not inhibit SP release induced by capsaicin. They are also in line with the fact that DSCG did not inhibit hypotension and bronchospasm induced by capsaicin administration to guinea pigs (Biggs and Goel, 1985) as well as with clinical studies performed by Collier and Fuller (1984) showing that DSCG pre-treatment did not alter cough induced by nebulized capsaicin. Taken together, these studies suggest that DSCG does not inhibit capsaicin-sensitive sensory nerve terminals and the responses that we observe by capsaicin are unlikely to be due to activation of mast cells.

In summary, our data indicate that stimulation of sensory nerves by capsaicin leads to a TRPV1-dependent response that promotes the release of neuropeptides resulting predominantly in the release of mucus through NK1 and/or NK2 receptors. The current study points to the opportunity of non-invasively assessing, with MRI, the influence of neuronal mechanisms in animal models of asthma and COPD.

Acknowledgements

NB acknowledges the 3R Research Foundation, Muesingen, Switzerland for supporting this work (Project 82/02).

Conflict of interest

The authors state no conflict of interest.

References

- Barnes PJ (2001). Related Neurogenic inflammation in the airways. *Respir Physiol* **125**: 145–154.
- Beckmann N, Tigani B, Ekatodramis D, Borer R, Mazzoni L, Fozard JR (2001). Pulmonary edema induced by allergen challenge in the rat: noninvasive assessment by magnetic resonance imaging. *Magn Reson Med* **45**: 88–95.
- Beckmann N, Tigani B, Sugar R, Jackson AD, Jones G, Mazzoni L, Fozard JR (2002). Noninvasive detection of endotoxin-induced mucus hypersecretion in rat lung by MRI. *Am J Physiol Lung Cell Mol Physiol* **283**: L22–L30.
- Bevan S, Hothi S, Hughes G, James IF, Rang HP, Shah K, Walpole CS, Yeats JC (1992). Capsazepine: a competitive antagonist of the sensory neurone excitant capsaicin. *Br J Pharmacol* **107**: 544–552.
- Biggs DF, Goel V (1985). Mechanisms of action of sodium cromoglycate. *Can J Physiol Pharmacol* **63**: 760–765.
- Biro T, Maurer M, Modarres S, Lewin NE, Brodie C, Acs P, Paus R, Blumberg PM (1998). Characterization of functional vanilloid receptors expressed by mast cells. *Blood* **91**: 1332–1340.
- Buck SH, Burks TF (1986). The neuropharmacology of capsaicin: review of some recent observations. *Pharmacol Rev* **38**: 179–226.
- Caterina MJ, Schumacher MA, Tominaga M, Rosen TA, Levine JD, Julius D (1997). The capsaicin receptor: a heat-activated ion channel in the pain pathway. *Nature* **389**: 816–824.
- Collier JG, Fuller RW (1984). Capsaicin inhalation in man and the effects of sodium cromoglycate. *Br J Pharmacol* **81**: 113–117.
- Delgado AV, McManus AT, Chambers JP (2003). Production of tumor necrosis factor- α , interleukin 1- β , interleukin 2, and interleukin 6 by rat leukocyte subpopulations after exposure to substance P. *Neuropeptides* **37**: 355–361.
- Dixon CM, Barnes PJ (1989). Bradykinin-induced bronchoconstriction: inhibition by nedocromil sodium and sodium cromoglycate. *Br J Clin Pharmacol* **27**: 831–836.
- Fozard JR, Baur F, Wolber C, Collingwood SP (2001). Inhibition by viozan of extravasation induced in rat trachea by capsaicin is mediated exclusively by beta 2-adrenoceptors. *Naunyn Schmiedeberg Arch Pharmacol* **364**: 570–572.
- Germonpre PR, Joos GF, Everaert E, Kips JC, Pauwels RA (1995). Characterization of neurogenic inflammation in the airways of two highly inbred rat strains. *Am J Respir Crit Care Med* **152**: 1796–1804.
- Gerspacher M, Lewis C, Ball HA, Howes C, Subramanian N, Ryffel K, Fozard JR (2003). Stereoselective preparation of N-[(R,R)-(E)-1-(3,4-dichlorobenzyl)-3-(2-oxoazepan-3-yl)carbamoyl]allyl-N-methyl-3,5 bis(trifluoromethyl)benzamide, a potent and orally active dual neurokinin NK(1)/NK(2) receptor antagonist. *J Med Chem* **46**: 3508–3513.
- Holzer P (1991). Capsaicin: cellular targets, mechanisms of action, and selectivity for thin sensory neurons. *Pharmacol Rev* **43**: 143–201.
- Joos GF, De Swert KO, Schelfhout V, Pauwels RA (2003). The role of neural inflammation in asthma and chronic obstructive pulmonary disease. *Ann N Y Acad Sci* **992**: 218–230.
- Kim S, Nadel JA (2004). Role of neutrophils in mucus hypersecretion in COPD and implications for therapy. *Treat Respir Med* **3**: 147–159.
- King M (2005). Mucus and its role in airway clearance and cytoprotection. In: Qutayba H, Shannon J, Martin JG (eds) *Physiologic Basis of Respiratory Disease*. BC Decker Inc: Hamilton, pp 409–416.
- Kuo H, Rhode JAL, Tokuyama K, Barnes PJ, Rogers DF (1990). Capsaicin and sensory neuropeptide stimulation of goblet cell secretion in guinea pig trachea. *J Physiol (Lond)* **431**: 629–641.
- Larson SD, Schelegle ES, Hyde DM, Plopper CG (2003). The three-dimensional distribution of nerves along the entire intrapulmonary airway tree of the adult rat and the anatomical relationship between nerves and neuroepithelial bodies. *Am J Respir Cell Mol Biol* **28**: 592–599.
- Lee LY, Lundberg JM (1994). Capsazepine abolishes pulmonary chemoreflexes induced by capsaicin in anesthetized rats. *J Appl Physiol* **76**: 1848–1855.
- Lewis CA, El-Hashim AZ, Gerspacher M, Hoshiko K, Mazzoni L, Pfannkuche HJ, Subramanian N, Fozard JR (2004). The airways

- pharmacology of DNK333, a potent, selective, non-peptide dual NK1/NK2 receptor antagonist. *Drug Dev Res* **63**: 161–173.
- Liu Y-C, Khawaja AM, Rogers DF (1998). Pathophysiology of airway mucus secretion in asthma. In: Barnes PJ, Rodger IW, Thomson NC, (eds.), Third Edition *Basic Mechanisms and Clinical Management*. Academic Press: San Diego, pp 205–227.
- Lundberg JM, Hokfelt T, Martling CR, Saria A, Cuello C (1984). Substance P-immunoreactive sensory nerves in the lower respiratory tract of various mammals including man. *Cell Tissue Res* **235**: 251–261.
- Maggi CA (1997). The effects of tachykinins on inflammatory and immune cells. *Regul Pept* **7**: 75–90.
- Mapp CE, Miotto D, Braccioni F, Saetta M, Turato G, Maestrelli P, Krause JE, Karpitskiy V, Boyd N, Geppetti P, Fabbri LM (2000). The distribution of neurokinin-1 and neurokinin-2 receptors in human central airways. *Am J Respir Crit Care Med* **161**: 207–215.
- McDonald DM, Mitchell RA, Gabella G, Haskell A (1988). Neurogenic inflammation in the rat trachea II. Identity and distribution of nerves mediating the increase in vascular permeability. *J Neurocytol* **17**: 605–628.
- Mead J (1970). The lung's 'quiet zone'. *N Engl J Med* **282**: 1318–1319.
- Meini S, Mak JCW, Rohde JAL, Rogers DF (1993). Tachykinin control of ferret airways: Mucus secretion, bronchoconstriction and receptor mapping. *Neuropeptides* **24**: 81–89.
- Melton L (2002). Does mucus hypersecretion matter in airway disease? *Lancet* **359**: 1924.
- Norris AA (1996). Pharmacology of sodium cromoglycate. *Clin Exp Allergy* **26**: 5–7.
- Page C (1994). Sodium cromoglycate, a tachykinin antagonist? *Lancet* **343** (8889): 70.
- Patacchini R, Maggi CA (1995). Tachykinin receptors and receptor subtypes. *Arch Int Pharmacodyn Ther* **329**: 161–184.
- Quintana HK, Cannet C, Schaeublin E, Zurbrugg S, Sugar R, Mazzoni L, Page CP, Fozard JR, Beckmann N (2006). Identification with MRI of the pleura as a major site of the acute inflammatory effects induced by ovalbumin and endotoxin challenge in the airways of the rat. *Am J Physiol Lung Cell Mol Physiol* **291**: L651–L657.
- Ramnarine SI, Hirayama Y, Barnes PJ, Rogers F (1994). Sensory-efferent neural control of mucus secretion: Characterization using tachykinin receptor antagonists in ferret trachea *in vitro*. *Br J Pharmacol* **113**: 1183–1190.
- Ray NJ, Jones AJ, Keen P (1991). Morphine, but not sodium cromoglycate, modulates the release of substance P from capsaicin-sensitive neurones in the rat trachea *in vitro*. *Br J Pharmacol* **102**: 797–800.
- Reynolds PN, Holmes MD, Scicchitano L (1997). Role of tachykinins in bronchial hyper-responsiveness. *Clin Exp Pharmacol Physiol* **24**: 273–280.
- Rogers DF, Aursudkij B, Barnes PJ (1989). Effects of tachykinins on mucus secretion on human bronchi *in vitro*. *Eur J Pharmacol* **174**: 283–286.
- Rogers DF (1995). Neurokinin receptors subserving airways secretion. *Can J Physiol Pharmacol* **73**: 932–939.
- Rogers DF (1997). Neurogenic inflammation in lung disease: burn out? *Inflammo Pharmacol* **5**: 319–329.
- Rogers DF (2001). Motor control of airway goblet cells and glands. *Resp Physiol* **125**: 129–144.
- Rogers DF (2004). Airway mucus hypersecretion in asthma: an undervalued pathology? *Curr Opin Pharmacol* **4**: 241–250.
- Rogers DF (2005). Mucociliary dysfunction in COPD: effect of current pharmacotherapeutic options. *Pulm Pharmacol Ther* **18**: 1–8.
- Solway J, Leff AR (1991). Sensory neuropeptides and airway function. *J Appl Physiol* **71**: 2077–2087.
- Szallasi A, Blumberg PM (1999). Vanilloid (Capsaicin) receptors and mechanisms. *Pharmacol Rev* **51**: 159–212.
- Tigani B, Schaeublin E, Sugar R, Jackson AD, Fozard JR, Beckmann N (2002). Pulmonary inflammation monitored noninvasively by MRI in freely breathing rats. *Biochem Biophys Res Commun* **292**: 216–221.
- Uddman R, Luts A, Sundler F (1985). Occurrence and distribution of calcitonin gene-related peptide in the mammalian respiratory tract and middle ear. *Cell Tissue Res* **241**: 551–555.
- Vestbo J, Prescott E, Lange P (1996). Association of chronic mucus hypersecretion with FEV1 decline and chronic obstructive pulmonary disease morbidity. Copenhagen City Heart Study Group. *Am J Respir Crit Care Med* **153**: 1530–1535.