

Sensory neuropeptides induce histamine release from bronchoalveolar lavage cells in both nonasthmatic coughers and cough variant asthmatics

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Summary

Background Sensory neuropeptides have been suggested to play a role in the pathogenesis of a number of respiratory diseases including asthma and chronic non-productive cough.

Objectives To investigate the action of sensory neuropeptides on airway mast cells obtained by bronchoalveolar lavage (BAL).

Methods BAL was performed on 23 nonasthmatic patients with cough (NAC), 11 patients with cough variant asthma (CVA) and 10 nonatopic controls. Washed lavage cells were stimulated (20 min, 37 °C) with calcitonin gene-related peptide (CGRP), neurokinin A (NKA) and substance P (25 and 50 µmol/L).

Results The neuropeptides tested induced histamine release in all groups studied. Only CGRP (50 µmol/L) induced significantly more histamine release from both NAC and CVA patients compared with control subjects ($P = 0.038$ and 0.045 , respectively).

Conclusion Regardless of aetiology, mast cells from patients with chronic cough appear to have an increased responsiveness to CGRP compared with controls. The results of the present study suggest that the role of CGRP in chronic cough should be further investigated.

Keywords: airway inflammation, bronchoalveolar lavage, chronic non-productive cough, mast cells, sensory neuropeptides

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Introduction

Neurogenic inflammation may be important in the pathophysiology of chronic respiratory disease. A population of airway sensory nerves, the C-fibres, contain several neuropeptides including calcitonin gene-related peptide (CGRP), substance P and neurokinin A (NKA) [1], which may be released on retrograde activation of the nerve through a local (axon) reflex mechanism [2]. These sensory neuropeptides mimic many of the pathophysiological features of asthma. NKA is a very potent constrictor of human airways and enhances cholinergic neurotransmission [3,4]. Substance P is a vasodilator, causes microvascular leakage

and stimulates mucus secretion from submucosal glands and epithelial goblet cells [3,4]. Substance P also induces histamine release from mast cells obtained from bronchoalveolar lavage (BAL) fluid [5]. CGRP is a potent and long-lasting vasodilator [6] and an extremely potent bronchoconstrictor of human bronchi *in vitro* [7].

There is evidence that sensory nerve function may be altered in asthma. Substance P-containing nerves are more abundant in lungs obtained at autopsy from asthmatics compared with healthy individuals [8], however, this finding was not confirmed in biopsy material from the lungs of mild asthmatics [9]. Increased levels of substance P-like immunoreactivity (SP-LIR) have been detected in BAL fluid from atopic asthmatics as compared with healthy individuals [10], with an increase following allergen challenge [10]. There have been similar findings in induced sputum from

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asthmatic and chronic bronchitic patients [11]. Despite increasing evidence of a role for sensory neuropeptides and neurogenic inflammation in the pathogenesis of asthma, little is known about the role of neuropeptides in other airway diseases.

Cough is a powerful physiological reflex, essential for removal of airway secretions and protection from foreign body inhalation. Airway disease is frequently manifest by a cough and that which accompanies an upper respiratory tract infection is troublesome but usually self-limiting. When a cough persists for more than 3 weeks it is defined as chronic [12]. A chronic non-productive cough is associated with considerable morbidity and is a common reason why patients seek medical attention [13]. Among non-smokers, it is often due to either one or a combination of asthma, the postnasal drip syndrome (PNDS) and gastro-oesophageal reflux (GOR) [14]. However, in up to 30% cases, the cause of the cough is not clear despite extensive investigation and trials of empirical therapy [15].

The afferent sensory receptors and nerves, namely the myelinated rapidly adapting (irritant) receptors (RARs) and the unmyelinated C-fibres are thought to participate in the human cough reflex [16]. An upregulation in sensory nerve function in the afferent portion of the cough reflex appears fundamental to the pathophysiology, which underlies chronic cough although the precise mechanisms remain unclear. This concept of sensory hyperresponsiveness is supported by clinical observations of an enhanced tussive response to inhaled capsaicin in a range of subjects with chronic cough, including those with asthmatic cough [17], following a viral infection [18], those taking angiotensin-converting enzyme inhibitors [19], and individuals with idiopathic cough [20]. Capsaicin, the pungent agent of hot peppers, has been demonstrated to selectively excite afferent C-fibres and release tachykinins from these sensory nerve fibres [21]. Capsaicin-induced cough is inhibited in guinea pigs pretreated with a tachykinin receptor antagonist [22]. Substance P when inhaled causes cough in humans with a viral-associated cough [23], contrasting the absence of a tussive response when inhaled by healthy individuals [24]. Recently, increased numbers of CGRP-immunoreactive nerves have been demonstrated in bronchial biopsy samples from idiopathic coughers compared with controls [25]. As a consequence, more attention has been focused on the role of these bioactive peptides in the pathogenesis of chronic cough.

The mast cell is believed to play a pivotal role in interactions between the nervous and immune systems and has been proposed as an essential down-stream component of neurogenic inflammation [26–28]. While there is much evidence for morphological and functional relationships between the mast cell and C-fibres [29,30], less attention has been paid to the potential role of this cell in the pathogenesis of chronic non-productive cough in humans.

To address this issue, the response of BAL mast cells to the sensory neuropeptides CGRP, NKA and substance P was investigated to determine whether mast cell activity is modulated in this disease state.

Materials and Methods

Subjects

Patients referred to the cough clinic at Belfast City Hospital with a non-productive cough persisting for greater than 3 weeks as their sole respiratory symptom were recruited to the study. All subjects were aged between 18 and 80 years, lifetime non-smokers and had a normal chest X-ray and spirometry. Patients with a previous history of any chest disease, any other systemic disease, symptoms of upper respiratory tract infection within the preceding 8 weeks or those taking angiotensin-converting enzyme (ACE) inhibitors were excluded from the study. Healthy subjects served as controls and were also life time non-smokers, nonatopic and had no preceding upper respiratory tract infection. The study was approved by the Research Ethics Committee of the Faculty of Medicine, The Queen's University of Belfast and prior to taking part, written informed consent was obtained from all subjects.

Patient evaluation

The patients were investigated according to a diagnostic protocol described previously [31]. In brief, after history and physical examination including inspection of ear, nose and throat, histamine challenge [32] and computed tomographic scan of the sinuses in the coronal plane were performed. Ambulatory pH monitoring of the oesophagus was performed using a Synectics Digitrapper Mark 11 (Synectics Medical, Enfield, UK).

Cough subgroups

A cough aetiology was only accepted when the patient reported satisfactory improvement or complete resolution of cough after a period of diagnosis-specific therapy. Subjects with a normal response to inhaled histamine (PC_{20} , the provocative concentration of histamine causing a 20% decrease in forced expiratory volume in 1 s (FEV_1) > 8 mg/mL) were classified as nonasthmatic coughers; while those subjects with airway hyperresponsiveness ($PC_{20} \leq 8$ mg/mL histamine [32]) who reported cough resolution or satisfactory improvement after asthma treatment were classified as having cough variant asthma.

The nonasthmatic group were further classified into gastro-oesophageal reflux (GOR), post-nasal drip syndrome (PNDS) and a third subgroup, termed 'idiopathic', comprising

of subjects whose cough resolved spontaneously or persisted despite sustained courses of empirical therapy directed at all possible aetiologies.

Bronchoalveolar lavage procedure

All patients had a standardized lavage performed [33]. Topical lignocaine (4%) was applied to the oropharynx. The bronchoscope was passed through the nose and lignocaine (2%) applied via a cannula to the vocal cords. Further local anaesthesia was achieved with lignocaine (1%) as required. The bronchoscope was wedged in the right middle lobe. The lavage was performed with sterile isotonic saline (3×60 mL), inserted under minimum hand pressure. After minimum dwell time, the fluid was aspirated into a plastic container and the samples pooled into a polypropylene vessel. This was immediately placed on ice and transferred to the laboratory.

Functional studies

BAL fluid was centrifuged (200 g, 10 min 4°C) washed twice in Tyrode's buffer and resuspended in a volume appropriate for the experiment. The cells were prewarmed (37°C , 5 min) and challenged with the secretagogues substance P, NKA and CGRP (Bachem UK, Saffron Walden, UK; 25 and $50\text{ }\mu\text{mol/L}$) and A23187 (Calbiochem-Novabiochem, Nottingham, UK; $1\text{ }\mu\text{mol/L}$). The reactions were stopped by the addition of ice-cold Tyrode's buffer and the cells and supernatants separated by centrifugation. Histamine release was measured in the cell pellets and the supernatants using an automated fluorometric assay based on that originally described by Shore *et al.* with a detectability of $200\text{ pg histamine/mL}$ [34,35]. Histamine release was expressed as a percentage of the total cellular histamine content corrected for spontaneous release (i.e. that occurring in the absence of any stimulus).

Statistical methods

All values are given as mean $\pm$ SEM unless otherwise stated. Comparisons of the magnitude of histamine release between different secretagogues on the same cell sample were made using the paired Student's *t*-test. The Kruskal–Wallis analysis of variance was used to examine for significant inter-group differences and if significant the Mann–Whitney *U*-test was used for between group comparisons. Correlation coefficients were calculated using the Spearman's rank method. A *P*-value of less than 0.05 was considered statistically significant.

Results

Subjects

Forty-two subjects were recruited to the study, 21

Table 1. General characteristics of subjects

Group	Age (years)	Sex (M:F)	Cough duration (m)
NAC ($n=21$)	45.1 ± 2.8	8:13	74.7 ± 18.7
PNDS ($n=7$)	45.0 ± 4.9	4:3	117.2 ± 36.9
GOR ($n=8$)	49.3 ± 3.7	3:5	87.1 ± 35.4
Idiopathic ($n=6$)	46.5 ± 5.0	1:5	73.3 ± 3.3
CVA ($n=11$)	53.0 ± 4.5	2:9	85.1 ± 24.6
Control ($n=10$)	36.1 ± 4.2	7:3	–

NAC – nonasthmatic cough, PNDS – post-nasal drip syndrome, GOR – gastro-oesophageal reflux, CVA – cough variant asthma

had nonasthmatic cough, 11 cough variant asthma and 10 control subjects (Table 1). Subdividing the nonasthmatic cough group according to diagnosis resulted in seven post-nasal drip syndrome, eight gastro-oesophageal and six idiopathic subjects (Table 1). There was no significant difference in the mean age of the groups investigated in this study. Similarly there was no significant difference in the mean duration of cough between the nonasthmatic cough and cough variant asthmatic groups (Table 1).

Spontaneous and A23187-induced histamine release

There was no significant difference in spontaneous histamine release from BAL mast cells derived from nonasthmatic cough ($22.2 \pm 2.6\%$), cough variant asthmatic ($22.9 \pm 2.4\%$) and control ($23.5 \pm 2.1\%$) subjects. Similarly there was no significant difference in the corrected histamine release induced by the calcium ionophore A23187 ($1\text{ }\mu\text{mol/L}$) in BAL mast cells derived from nonasthmatic cough ($20.4 \pm 2.4\%$, $n=20$), cough variant asthmatic ($19.7 \pm 3.1\%$, $n=11$) or control ($16.6 \pm 3.9\%$, $n=10$) subjects (Fig. 1).

Substance P

Substance P induced histamine release from BAL mast cells in all subject groups investigated. Histamine release induced by $25\text{ }\mu\text{mol/L}$ substance P did not differ significantly from that induced by $50\text{ }\mu\text{mol/L}$ substance P in any subject group. Histamine release induced by either 25 or $50\text{ }\mu\text{mol/L}$ substance P did not differ significantly between nonasthmatic coughers ($25\text{ }\mu\text{mol/L}$, $12.2 \pm 1.8\%$; $50\text{ }\mu\text{mol/L}$, $16.8 \pm 1.9\%$, $n=20$), cough variant asthmatics ($25\text{ }\mu\text{mol/L}$, $9.1 \pm 2.0\%$; $50\text{ }\mu\text{mol/L}$, $14.1 \pm 3.4\%$, $n=11$) and controls ($25\text{ }\mu\text{mol/L}$, $12.1 \pm 3.1\%$; $50\text{ }\mu\text{mol/L}$, $20.9 \pm 3.0\%$, $n=10$) (Fig. 2a). There was no correlation between histamine release induced by substance P ($50\text{ }\mu\text{mol/L}$) and either spontaneous

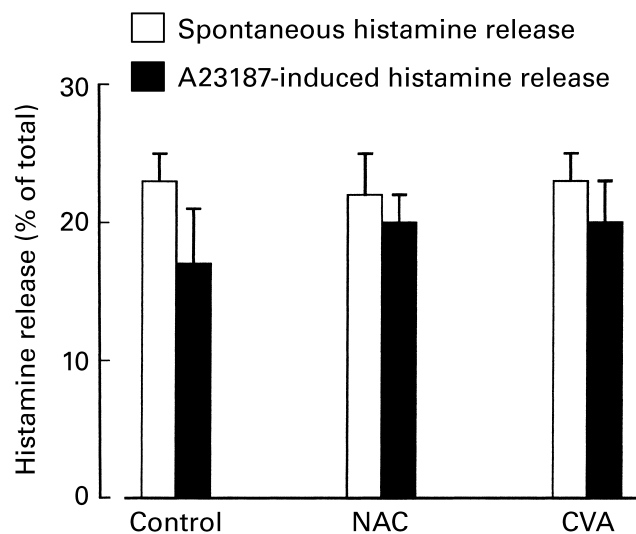


Fig. 1. Spontaneous and A23187 (1 $\mu\text{mol/L}$) induced histamine release from BAL mast cells. Data are presented as mean $\pm$ SEM ($n=10-20$). Spontaneous release has been subtracted from the values for A23187 induced release. NAC – nonasthmatic cough, CVA – cough variant asthma

($P=0.64$, $r=-0.07$, $n=42$) or A23187- (1 $\mu\text{mol/L}$) ($P=0.7$, $r=0.054$, $n=40$) induced histamine release.

Neurokinin A

NKA-induced histamine release from BAL mast cells in all subject groups investigated. In the control group histamine release induced by 25 and 50 $\mu\text{mol/L}$ NKA did not differ significantly ($P=0.077$). However, in the nonasthmatic cough ($P=0.02$) and cough variant asthmatic ($P=0.0015$) groups, NKA induced significantly more histamine release at the higher concentration (Fig. 2b). Histamine release induced by either 25 or 50 $\mu\text{mol/L}$ NKA did not differ significantly between nonasthmatic coughers (25 $\mu\text{mol/L}$, $12.7 \pm 2.2\%$; 50 $\mu\text{mol/L}$, $21.2 \pm 2.7\%$, $n=20$), cough variant asthmatics (25 $\mu\text{mol/L}$, $8.6 \pm 2.1\%$; 50 $\mu\text{mol/L}$, $22.5 \pm 2.1\%$, $n=11$) and controls (25 $\mu\text{mol/L}$, $11.1 \pm 2.5\%$; 50 $\mu\text{mol/L}$, $18.6 \pm 2.8\%$, $n=10$) (Fig. 2b). There was no correlation between histamine release induced by NKA (50 $\mu\text{mol/L}$) and either spontaneous ($P=0.35$, $r=-0.15$, $n=39$) or A23187- (1 $\mu\text{mol/L}$) ($P=0.9$, $r=0.006$, $n=38$) induced histamine release.

Calcitonin gene-related peptide

CGRP induced histamine release from BAL mast cells in all subject groups investigated. In the control group histamine release induced by 25 and 50 $\mu\text{mol/L}$ CGRP did not differ significantly ($P=0.06$). However, in the nonasthmatic cough ($P=0.009$) and cough variant asthmatic ($P=0.018$)

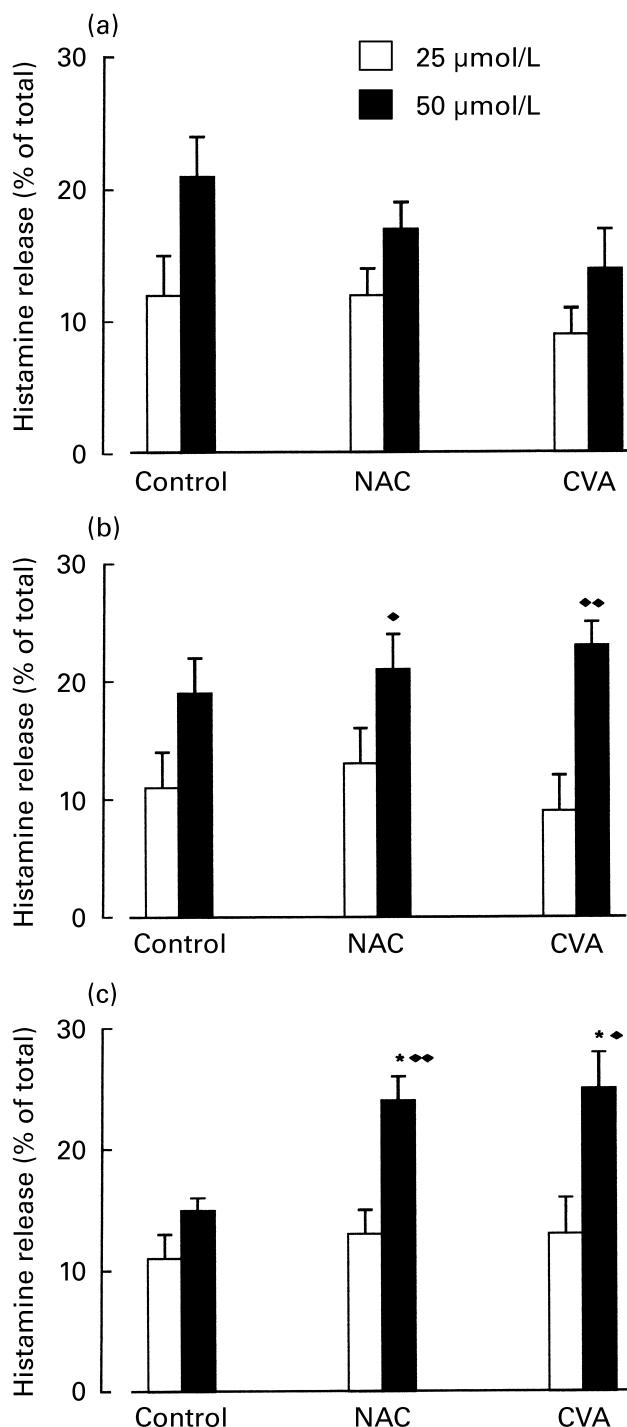


Fig. 2. Histamine release from BAL mast cells induced by 25 $\mu\text{mol/L}$ and 50 $\mu\text{mol/L}$ of (a) substance P, (b) NKA and (c) CGRP. The data are expressed as mean $\pm$ SEM ($n=10-20$). Histamine release is expressed as a percentage of total cellular histamine content and corrected for spontaneous release. NAC – nonasthmatic cough, CVA – cough variant asthma. (♦ $P < 0.05$, ♦♦ $P < 0.01$ compared with release induced by 25 $\mu\text{mol/L}$; * $P < 0.05$ compared to release in control subjects)

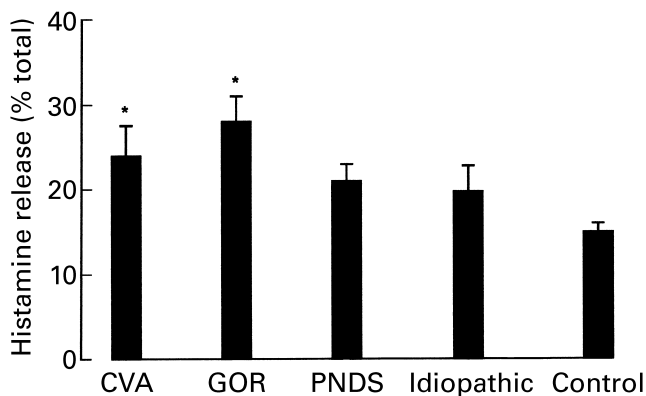


Fig. 3. Histamine release induced by 50 µmol/L CGRP from BAL mast cells in cough diagnostic subgroups. The data are expressed as mean $\pm$ SEM ($n=6-10$). Histamine release is expressed as a percentage of total cellular histamine content and corrected for spontaneous release ($*P<0.05$ compared with control). CVA – cough variant asthma, GOR – gastro-oesophageal reflux, PNDS – post-nasal drip syndrome

groups CGRP induced significantly more histamine release at the higher concentration (Fig. 2c). Histamine release induced by 25 µmol/L CGRP did not differ significantly between nonasthmatic coughers ($13.1 \pm 2.3\%$, $n=20$); cough variant asthmatics ($13.0 \pm 3.4\%$, $n=11$) and controls ($11.2 \pm 2.3\%$, $n=10$). However, 50 µmol/L CGRP induced significantly greater histamine release from BAL mast cells in cough variant asthmatic ($24.9 \pm 2.3\%$, $P=0.045$, $n=11$) and nonasthmatic cough ($22.5 \pm 2.2\%$, $P=0.038$, $n=19$) subjects compared with controls ($15.4 \pm 2.2\%$, $n=10$) (Fig. 2c). On examining nonasthmatic cough diagnostic subgroups, only BAL mast cells from the gastro-oesophageal reflux subgroup released significantly more histamine than control subjects (28.2 ± 2.7 , $P=0.019$, $n=6$) (Fig. 3). There was no correlation between CGRP- (50 µmol/L) induced histamine release from BAL mast cells and either spontaneous ($P=0.55$, $r=-0.095$, $n=40$) or A23187- (1 µmol/L) ($P=0.37$, $r=0.15$, $n=39$) induced release. There was a significant positive correlation between histamine release induced by NKA (50 µmol/L) and by substance P (50 µmol/L; $P=0.005$, $r=0.438$, $n=38$) and CGRP (50 µmol/L; $P=0.013$, $r=0.400$, $n=37$). However, there was no correlation between histamine release induced by CGRP and substance P ($P=0.326$, $r=0.16$, $n=39$) (Fig. 4).

Discussion

All three of the major neuropeptides colocalized in C-fibres (substance P, NKA and CGRP) induce histamine release from BAL mast cells. In control subjects increasing the concentration of these neuropeptides from 25 to 50 µmol/L did not cause significant enhancement of histamine release.

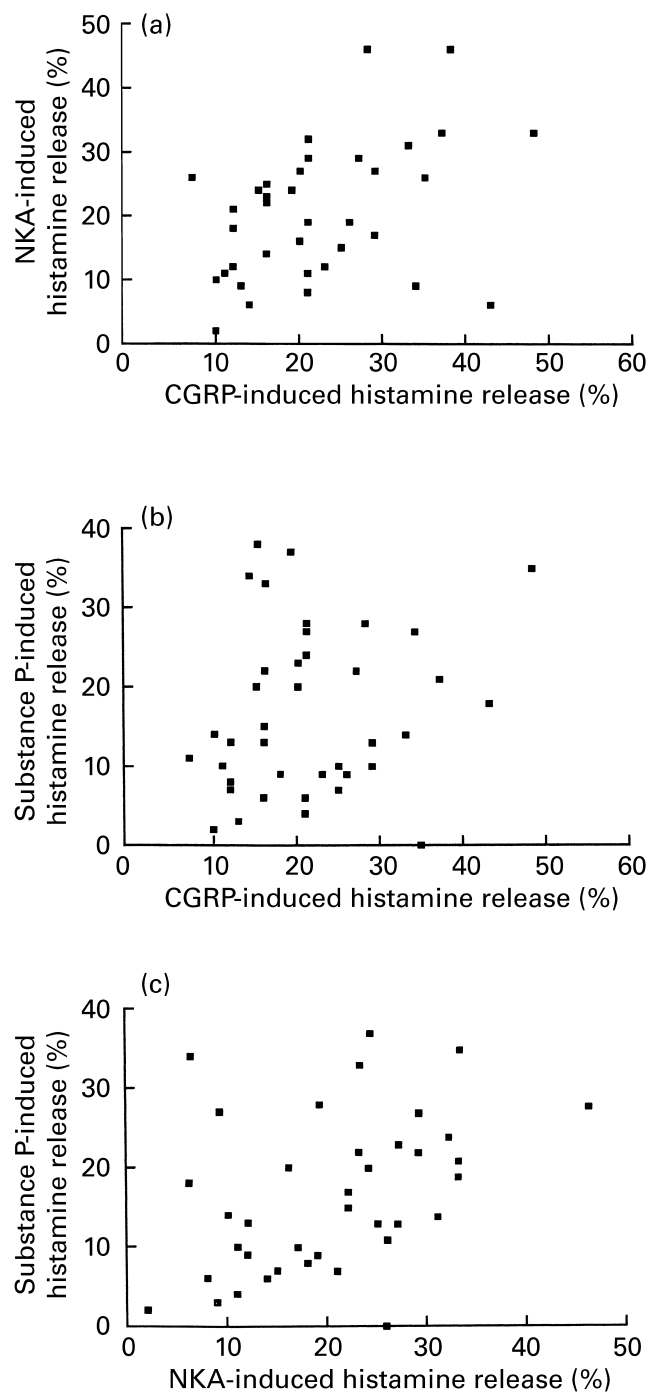


Fig. 4. Correlation between histamine release induced by neuropeptides (50 µmol/L). (a) CGRP vs NKA ($P=0.0129$, $r=0.40$, $n=37$), (b) CGRP vs substance P ($P=0.326$, $r=0.16$, $n=39$) and (c) NKA vs substance P ($P=0.005$, $r=0.438$, $n=38$)

It may be speculated that a close-to-maximal response was attained with the lower concentration of all three peptides, although priming and lower peptide concentrations were not investigated. However, in both asthmatic and nonasthmatic

coughers, increasing the concentration of NKA and CGRP induced significantly more histamine release. Thus, chronic cough modulates the ability of lavage cells to respond to neuropeptides. A correlation between spontaneous and substance P-induced histamine release from human BAL mast cells has been reported previously [5]. However, the present study found no correlation between spontaneous histamine release and that induced by substance P, NKA or CGRP. A correlation between NKA and substance P-induced histamine release first reported by Cross *et al.* [36] was supported by the present study. Correlations between histamine release induced by NKA and both CGRP and substance P suggest that there may be some commonality in the mechanism of mast cell response to these peptides.

BAL mast cells from both asthmatic and nonasthmatic coughers exhibited an increased response to CGRP compared with controls. However, in contrast with findings with atopic and asthmatic subjects [37], there was no enhancement of response to substance P or indeed NKA. This suggests that changes in the airway mediator milieu produced by various disease processes may act to selectively upregulate the response of mast cells to a particular neuropeptide. The selective nature of the upregulation of mast cell response to CGRP is further emphasized by the observation that histamine release induced by the calcium ionophore A23187 did not differ among the subject groups. This indicates that there is not a general enhancement of mast cell activity. Increased spontaneous histamine release from BAL mast cells in asthmatic subjects has been reported [38,39] and it was suggested that increased basal histamine release in the disease state may be due to the mast cell being more 'leaky'. Evidence for the ability of the allergic state to enhance the leakiness of cells was provided by Aloui *et al.* who demonstrated increased membrane fluidity of blood leucocytes from allergic subjects [40]. This increase in membrane fluidity could be induced in leucocytes from nonallergic subjects by the addition of leukotriene B₄ [40]. In contrast with findings in symptomatic asthmatics, there was no increase in spontaneous release from nonasthmatic coughers or cough variant asthmatics. This finding further suggests that the increased mediator release in the subjects with cough are due to a specific stimulation of the mast cell rather than a more general, ongoing release of mediators, as has been proposed for symptomatic asthmatics [39].

Previous reports have indicated a potential role for CGRP in the enhancement of C-fibre responsiveness seen in chronic non-productive cough. Intermittent exposure of guinea pigs to cigarette smoke for 14 days produced both an enhanced cough response to capsaicin, and increased CGRP immunoreactivity in tracheal tissue [41]. Further, O'Connell *et al.* have demonstrated an increased density of intraepithelial nerves containing CGRP in the airways of subjects with chronic non-productive cough [25]. These

studies do not provide evidence of a cause and effect relationship between increased levels of CGRP and sensory hyperresponsiveness. However, the demonstration that CGRP induces mast cell degranulation, and that this response is enhanced in patients with chronic non-productive cough, provides a potential mechanism by which this might occur. In addition to histamine, which has been shown to induce cough and increase afferent nerve activity, mast cells also secrete other mediators, such as tumour necrosis factor- α (TNF α) and nerve growth factor (NGF), which have modulatory effects on afferent nerve activity. TNF α has been reported to increase the capsaicin sensitivity of rat sensory neurones and also to potentiate the capsaicin evoked release of CGRP in isolated rat trachea [42,43]. NGF has been implicated as a regulatory factor in the increased substance P and CGRP content of sensory neurones during inflammation [44].

Initial data obtained from rat peritoneal mast cell studies suggested that substance P-induced histamine release was not mediated via activation of the NK1 receptor [45]. However, pharmacological investigations of tachykinin-induced histamine release from mast cells in the trachea of guinea pigs and rats have indicated a role for NK1 and NK2 receptors in mast cell activation [46,47]. These pharmacological findings are supported by the recent discovery of NK1 receptors on the RBL-2H3 mast cell line [48].

The mechanism by which CGRP stimulates BAL mast cell secretion is unknown, indeed there have been few reports of CGRP-induced histamine release from any population of mast cells. Lowman *et al.* demonstrated that CGRP induced histamine release from human skin mast cells but was four times less potent than substance P in this respect [49]. Only recently has there been an unequivocal demonstration of CGRP-induced histamine release from mast cells [50]. Ottosson and Edvinsson reported CGRP induced histamine release from rat dural mast cells which was greater than that induced by substance P [50]. Although the issue of whether mast cells have receptors for neuropeptides has yet to be resolved, CGRP-induced histamine release from rat dural mast cells was inhibited by the CGRP antagonist CGRP₈₋₃₇, which suggests this peptide activates rat dural mast cells via a receptor coupled mechanism [50].

The concentration of neuropeptides used (25–50 $\mu\text{mol/L}$) are similar to those used in studies of substance P-induced activation of human skin and rodent mast cells [51,52]. The close apposition of mast cells and nerve fibres has been observed in a range of tissues and such intimate contact has been proposed to provide a basis for communication between the nervous and immune systems [30]. Within this framework, activation of mast cells with micromolar concentrations of sensory neuropeptides may be physiologically relevant since such concentrations may occur at presynaptic terminals.

The weal and flare response occurring in human skin following injection of substance P occurs at picomolar concentrations and is attenuated by an H_1 -receptor antagonist [26]. Therefore, the micromolar concentrations required for *in vitro* activation of mast cells may reflect a physiological response which is triggered by picomolar concentrations *in vivo*.

Substance P induces an immediate release of $TNF\alpha$ from rat mast cells at concentrations below those required to induce histamine release [29]. Thus even at low concentrations of substance P, the mast cell can release a potent pro-inflammatory cytokine without classical degranulation occurring. The ability of other neuropeptides, such as CGRP, to induce cytokine production by mast cells has yet to be explored. However, neuropeptides may influence both the extent and profile of cytokine production by mast cells. It is conceivable that the cytokine profile induced by CGRP favours the development of sensory hyperresponsiveness, as seen in patients with chronic non-productive cough.

It is well established that mast cells from different species and from different anatomical sites in the same species are histologically and functionally heterogeneous. The results of the present study suggest that the development of airway epithelial mast cells may be influenced by the local environment to produce a specialized population of cells which respond to sensory neuropeptides and mediate neuro-immune interactions. Therefore unlike mast cells of the lung, adenoids, tonsils and intestine, which do not respond to neuropeptides [53], and whose primary role is thought to be immunoglobulin E-mediated host defence, mast cells retrieved at BAL most likely fulfil a homeostatic role. In this respect BAL mast cells are similar to human skin mast cells. However, the neuropeptide response profiles of these two cell types are quite distinct. CGRP and NKA are much less potent secretagogues of human skin mast cells than substance P [53] while NKA and CGRP are at least as potent as substance P in inducing histamine release from BAL mast cells. This emphasizes the delicate control of the local environment on mast cell phenotype and highlights how changes in the microenvironment caused by inflammatory infiltrates might selectively upregulate the response of BAL mast cell to neuropeptides.

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