

Inhibition of guinea-pig and human sensory nerve activity and the cough reflex in guinea-pigs by cannabinoid (CB₂) receptor activation

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1 There is considerable interest in novel therapies for cough, since currently used agents such as codeine have limited beneficial value due to the associated side effects. Sensory nerves in the airways mediate the cough reflex *via* activation of C-fibres and RARs. Evidence suggests that cannabinoids may inhibit sensory nerve-mediated responses.

2 We have investigated the inhibitory actions of cannabinoids on sensory nerve depolarisation mediated by capsaicin, hypertonic saline and PGE₂ on isolated guinea-pig and human vagus nerve preparations, and the cough reflex in conscious guinea-pigs.

3 The non-selective cannabinoid (CB) receptor agonist, CP 55940, and the selective CB₂ agonist, JWH 133 inhibited sensory nerve depolarisations of the guinea-pig vagus nerve induced by hypertonic saline, capsaicin and PGE₂. These responses were abolished by the CB₂ receptor antagonist SR144528, and unaffected by the CB₁ antagonist SR141716A. Similarly, JWH 133 inhibited capsaicin-evoked nerve depolarisations in the human vagus nerve, and was prevented by SR144528.

4 Using a guinea-pig *in vivo* model of cough, JWH 133 (10 mg kg⁻¹, i.p., 20 min) significantly reduced citric acid-induced cough in conscious guinea pigs compared to those treated with the vehicle control.

5 These data show that activation of the CB₂ receptor subtype inhibits sensory nerve activation of guinea-pig and human vagus nerve, and the cough reflex in guinea-pigs, suggesting that the development of CB₂ agonists, devoid of CB₁-mediated central effects, will provide a new and safe antitussive treatment for chronic cough.

British Journal of Pharmacology (2003) **140**, 261–268. doi:10.1038/sj.bjp.0705435

Keywords: Cannabinoids; airway sensory nerves; cough; human; guinea-pig

Abbreviations: COPD, chronic obstructive pulmonary disease; KHS, Krebs–Henseleit solution; PGE₂, prostaglandin E₂; RARs, rapidly adapting receptors; THC, Δ⁹-tetrahydrocannabinol; TRPV1, vanilloid receptor 1

Introduction

Cough is a dominant and persistent symptom of many inflammatory lung diseases, including asthma, chronic obstructive pulmonary disease (COPD), viral infections, pulmonary fibrosis and bronchiectasis. Chronic cough can also be idiopathic in nature, where no obvious causal mechanism is evident. Cough is the most common complaint for which medical attention is sought, and although effective treatments for cough are not available, narcotic agents, such as the opioid codeine, are often used. However, such agents have only limited beneficial value due to the associated side effects such as constipation, nausea, vomiting and drowsiness. Therefore, the identification of novel therapies, devoid of central activity, for the treatment of chronic cough would be of significant therapeutic benefit and greatly enhance the quality of life of patients who suffer from this condition.

The cough reflex is predominantly under the control of two different classes of sensory afferent nerve fibres, namely the

myelinated, rapidly adapting receptors (RARs or Aδ fibres), and nonmyelinated C-fibres with bronchial or pulmonary endings (Coleridge & Coleridge, 1984; Sant'Ambrogio, 1987; Laloo *et al.*, 1995), activation of which elicits cough *via* an afferent central reflex pathway. Evidence suggests that sensory nerve activity may be enhanced in inflammatory lung diseases such that the normally protective cough reflex becomes exacerbated and deleterious (Carr & Undem, 2001). Hence, theoretically, agents that inhibit sensory nerve activity (i.e. nerve depolarisations) will ultimately lead to a reduction in the cough reflex. In fact, this paradigm exists not only in relation to the cough response (Fox *et al.*, 1997), but also with other sensory nerve-mediated responses such as vagally-induced plasma extravasation into the airways (Birrell *et al.*, 2002). Moreover, such agents would act peripherally and therefore avoid the CNS side effects of centrally acting drugs such as opioids.

Agents such as hypertonic saline (Laloo *et al.*, 1995; Fox *et al.*, 1996; 1997; Pedersen *et al.*, 1998), capsaicin (Laloo *et al.*, 1995; Fox *et al.*, 1996) and the endogenous prostanoid,

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Advance online publication: 4 August 2003

prostaglandin E₂ (PGE₂; Roberts *et al.*, 1985; Smith *et al.*, 1998), are known sensory nerve stimulants. Furthermore, isolated guinea-pig and human vagus nerve preparations have been shown to elicit similar nerve depolarisation responses to these stimulants (Belvisi *et al.*, 1998). Moreover, these agents, along with citric acid, are known tussigenic agents in human (Costello *et al.*, 1985; Stone *et al.*, 1992; Laude *et al.*, 1993) and animal studies (Laloo *et al.*, 1995). Both *in vivo* cough studies (Laloo *et al.*, 1995) and *in vitro* single-fibre recordings of sensory nerves innervating the airways have shown that hypertonic saline excites RARs (conducting in the A δ range) and C-fibres, and that citric acid and capsaicin excite C-fibres based on the sensitivity of these responses to the capsaicin receptor (vanilloid receptor 1 (TRPV1)) antagonist, capsazepine (Fox *et al.*, 1993; 1995). Furthermore, *in vivo* studies suggest that PGE₂ activates C-fibres (Coleridge *et al.*, 1976) and RARs (Mohammed *et al.*, 1993).

There is interest in the therapeutic potential of cannabinoids, including the major active principle of marijuana, Δ^9 -tetrahydrocannabinol (THC). Non-selective cannabinoid receptor agonists have been shown to have therapeutic applications for a number of important medical conditions, including pain, anxiety, glaucoma, nausea, emesis, muscle spasm and wasting diseases (Porter & Felder, 2001). Although non-selective cannabinoids, such as anandamide, have been shown to suppress the cough reflex (Gordon *et al.*, 1976; Calignano *et al.*, 2000), the associated side effects such as sedation, cognitive dysfunction, tachycardia and psychotropic effects have hampered the use of such agonists for treatment purposes (Porter & Felder, 2001). Furthermore, appropriate validation of this hypothesis in the relevant human tissue (i.e. the vagal sensory nerves) or *in vivo* in man has not yet been provided.

Cannabinoids mediate their effects *via* CB₁ and CB₂ receptor subtypes (Matsuda *et al.*, 1990; Munro *et al.*, 1993). CB₁ receptors are predominantly distributed throughout the brain and spinal cord, and are also expressed at low levels in several peripheral tissues. In contrast, CB₂ receptors have not, to date, been found to be expressed in the CNS (Munro *et al.*, 1993). In this study, we determined whether activation of specific cannabinoid receptor subtypes could inhibit sensory nerve activity (i.e. nerve depolarisations) in the airways and the cough reflex. Guinea-pig vagus preparations were used to characterise the cannabinoid receptor subtype involved, as pharmacological profiling *in vitro* is often more straightforward when drug action is not complicated by pharmacokinetic issues. Finally, human vagus preparations were used to confirm observations generated in the guinea-pig, to provide the appropriate validation of the target in man and confirm clinical relevance.

In these experiments, we have used the non-selective agonist CP 55940, the CB₂-selective agonist JWH 133, the CB₁ receptor antagonist SR141716A and the CB₂-receptor antagonist SR144528 as pharmacological tools with which to characterise the cannabinoid receptor subtype involved in this response. CP 55940 has essentially the same affinity for CB₁ and CB₂ receptors. The affinities for both receptors are in the nanomolar range, and this agonist exhibits relatively high efficacy at both these receptor types (Pertwee, 1999). JWH133 is the most selective CB₂ receptor agonist that is currently available commercially. Its binding affinities (K_i) for CB₂ and CB₁ receptors are 3.4 ± 1.0 and 677 ± 132 nM, respectively

(Huffman *et al.*, 1999). The diarylpyrazole SR141716A was developed by Sanofi, and is a highly potent and selective CB₁ receptor antagonist ($K_i = 5.9$ nM for CB₁ and > 1 μ M for CB₂; pA₂ for SR141716A at CB₁ receptors = 7.9; Rinaldi-Carmona *et al.*, 1994). SR144528 is also a diarylpyrazole developed by Sanofi that binds with markedly higher affinity to CB₂ than CB₁ receptors ($K_i = 0.6$ nM for CB₂ and 437 nM for CB₁; pA₂ for SR144528 at CB₂ = 6.3; Rinaldi-Carmona *et al.*, 1998).

Methods

Measurement of sensory nerve depolarisation in isolated vagus nerve preparations

Male Dunkin–Hartley guinea-pigs (300–350 g) were housed in a temperature-controlled (21°C) room with food and water freely available. Guinea-pigs were killed by cervical dislocation and the vagus nerves, caudal to the nodose ganglion, were carefully removed and placed in Krebs–Henseleit solution (KHS) of the following composition (mM): NaCl – 118; KCl – 5.9; MgSO₄ – 1.2; NaH₂PO₄ – 1.2; CaCl₂ – 2.5; glucose – 6.6; NaHCO₃ – 25.5, and bubbled with 95% O₂/5% CO₂. Human trachea, with branches of the cervical vagus still attached, was obtained from a donor patient (male, 45 years) for a heart or heart/lung transplant. Relevant approvals were obtained from the Royal Brompton and Harefield Trust Ethics Committee. Segments of human and guinea-pig vagus nerve (40–50 mm) were cleared of connective tissue, and carefully desheathed under a dissecting microscope. Throughout, care was taken to ensure that the nerve trunks remained in oxygenated KHS, and that they were not stretched or damaged in any way. The desheathed nerve trunk was mounted in a 'grease-gap' recording chamber as previously described (Rang & Ritchie, 1988; Birrell *et al.*, 2002). Briefly, the nerve was drawn longitudinally through a narrow channel (2 mm diameter, 10 mm length) in a Perspex block. The centre of the channel was filled with petroleum jelly, injected through a side arm when the nerve was in place, onto the middle of the vagus, creating an area of high resistance, and electrically isolating the extracellular space between the two ends of the nerve. One end of the nerve emerged into a wider channel, and was constantly superfused with KHS at a flow rate of approximately 2 ml min⁻¹. The other nerve ending remained in a second, smaller chamber containing oxygenated KHS throughout the experiments. Ag/AgCl electrodes (Mere 2 Flexible reference electrodes, World Precision Instruments (WPI)), filled with KHS, made contact at either end of the nerve trunk and recorded DC potential *via* a DAM 50 differential amplifier (WPI). DC voltages were amplified $\times 10$, filtered at 1000 Hz, and sampled at 5 Hz. During each experiment, simultaneous recordings were made from two nerves. The temperature of the perfusate was maintained at 37°C by means of a water bath. The pen recorder was calibrated such that 1 mm was equivalent to 10 mV (incorporating the $\times 10$ amplification using a DAM 50 amplifier). The superfusing Krebs solution could be quickly changed by means of a tap, with little artefact, and the new solution reaching the vagus with a delay of approximately 10 s. Drugs were applied at known concentrations into the perfusing solution of the first channel only, and depolarising responses recorded onto a chart recorder (Lectromed Multi-Trace 2).

Sensory nerve activity, that is, nerve depolarisations, were induced by perfusion of the vagus nerve with pre-established (data not shown) submaximal concentrations of either hypertonic saline (2%), capsaicin (1 μM) or PGE₂ (1 μM). The stimulants were applied for a period of 4 min, after which the tissue was washed until the baseline response of the nerve was regained. After two reproducible responses to the nerve stimulants, the non-selective cannabinoid agonist CP 55940 or the CB₂ receptor agonist JWH 133 were added to the KHS, perfusing the nerves for 20 min prior to a subsequent administration of stimulant, while still in the presence of the agonist. In separate experiments, the CB₁ receptor antagonist SR141716A or the CB₂ receptor antagonist SR144528 were added 10 min prior to application of the agonist, and were also present for the duration of the experiment. Only one concentration of one agonist and/or antagonist was tested per vagus preparation. For each experimental condition using guinea-pig vagus preparations, $n=4$ determinations were performed. Due to the limited availability of human vagus nerve, only key experiments were performed.

Measurement of cough in conscious guinea-pigs

Male Dunkin–Hartley outbred guinea-pigs (300–350 g) were housed in a temperature-controlled (21°C) room with food and water freely available for at least 1 week before the commencement of experiments. The procedure for measuring cough in conscious guinea-pigs was as previously described (Laloo *et al.*, 1995). Cough sounds were amplified and recorded concurrently *via* a microphone placed inside the cough chamber, and recorded as spikes on a chart recorder. Solutions were delivered by aerosol *via* a nebuliser (De Vilbiss, Somerset, PA, U.S.A.). Coughs were counted by a trained observer and recognised from the characteristic opening of the mouth and posture of the animal, the sound produced, and the sound and airflow recordings. Using these criteria together, cough was easily distinguished from sneezes and augmented breaths. All animals were treated with terbutaline sulphate (0.05 mg kg⁻¹, i.p.) 10 min before the cough challenge, to minimise respiratory distress due to bronchoconstriction. JWH 133 (10 mg kg⁻¹, i.p., $n=8$) or vehicle (0.5% methyl cellulose with 0.2% Tween 80 in saline, i.p., $n=8$) was administered 20 min prior to exposure to the tussive agent citric acid (0.3 M) for 10 min, during which time the number of coughs were counted.

Materials

All Krebs compounds were obtained from BDH (Dorset, U.K.), and KHS was made fresh on a daily basis. SR141716A and SR144528 were kind gifts from Novartis Institute, London, U.K. Cannabinoid agonists were obtained from Tocris Cookson Ltd (Bristol, U.K.). All other chemicals were obtained from Sigma Aldrich. Stock concentrations of PGE₂ and CP 55940 were diluted in 100% ethanol and stock concentrations of capsaicin, SR141716A, SR144528 and JWH 133 were made in 100% DMSO. Further dilutions of all compounds were such that a final concentration of 0.1% of the diluent was always achieved. For the *in vivo* experiments, all drug solutions were freshly prepared on the day of each experiment. JWH 133 was suspended 0.5% methyl cellulose with 0.2% Tween 80 in saline (vehicle).

Statistical analysis

All the values in the figures and text are expressed as mean \pm s.e.m. For the *in vitro* studies, two vagal preparations were obtained from each animal. Only one concentration of one agonist was tested per vagus nerve preparation, and experiments were randomised; hence, different concentrations of different drugs were tested on vagi from the same animal on the same day. Nerve depolarisation responses were measured in mm after 4 min from the time of stimulant addition, and then expressed as mV depolarisation. In experiments where tissues were treated with test compounds, responses were expressed as mV before (control response) and after drug additions, and then expressed as a percentage change from control. In these experiments, the data were subjected to a paired two-tailed *t*-test (significance is denoted by $P<0.05^*$, $P<0.01^{**}$, $P<0.001^{***}$), since the response to a stimulant was measured before and after drug intervention within the same nerve. An unpaired *t*-test ($P<0.001$ denoted by ###) was used, where two different treatment groups were compared. For the *in vivo* cough experiments, the numbers of coughs during the 10 min exposure to citric acid were compared between treated and control groups using an unpaired *t*-test ($P<0.01^{**}$). pD_2 values ($-\log$ of the EC₅₀ defined as the concentration of drug required to elicit 50% of the maximum inhibition) and statistical significance were calculated using 'GraphPad Instat™' (© GraphPad software).

Results

In individual guinea-pig nerve preparations, control responses were obtained to hypertonic saline (2%), capsaicin (1 μM) or PGE₂ (1 μM). These stimuli elicited nerve depolarisations of 0.61 ± 0.04 , 0.34 ± 0.03 and 0.18 ± 0.01 mV, respectively.

Perfusion of the vagus preparations with the non-selective cannabinoid agonist CP 55940 (0.03–3 μM) inhibited capsaicin-induced nerve depolarisation in a concentration-dependent manner ($pD_2=6.2$). CP 55940 (concentrations between 0.03 and 100 μM) also inhibited, in a concentration-dependent manner, depolarisation of the guinea-pig vagus elicited by PGE₂ and hypertonic saline (2%) (pD_2 values of 6.0 and 5.55, respectively). Complete inhibition was observed in each case (Figure 1a–c). Similarly, nerve preparations treated with the selective CB₂ receptor agonist JWH 133 (Huffman *et al.*, 1999; Pertwee, 1999) (concentrations between 0.3 and 100 μM) markedly reduced sensory nerve depolarisation induced by capsaicin, PGE₂ and hypertonic saline (2%) in a concentration-dependent manner, with pD_2 values of 5.5, 5.4 and 5.1. Maximal inhibition was achieved at concentrations of 10 ($97.5 \pm 2.5\%$), 30 (100%) and 30 μM ($91.6 \pm 0.7\%$), respectively (Figure 1d–f).

The involvement of CB₂ receptors, and not CB₁ receptors, in mediating the inhibitory action of CP 55940 or JWH 133 was confirmed in experiments where the CB₁-selective antagonist SR141716A (0.01 μM ; Rinaldi-Carmona *et al.*, 1994) or the CB₂-selective antagonist SR144528 (0.01 μM ; Rinaldi-Carmona *et al.*, 1998) was perfused 10 min prior to application of a submaximal concentration of either CP 55940 or JWH 133. Submaximal concentrations of CP 55940 (1 μM) or JWH 133 (3 μM , where the stimulus was capsaicin, and 10 μM , where the

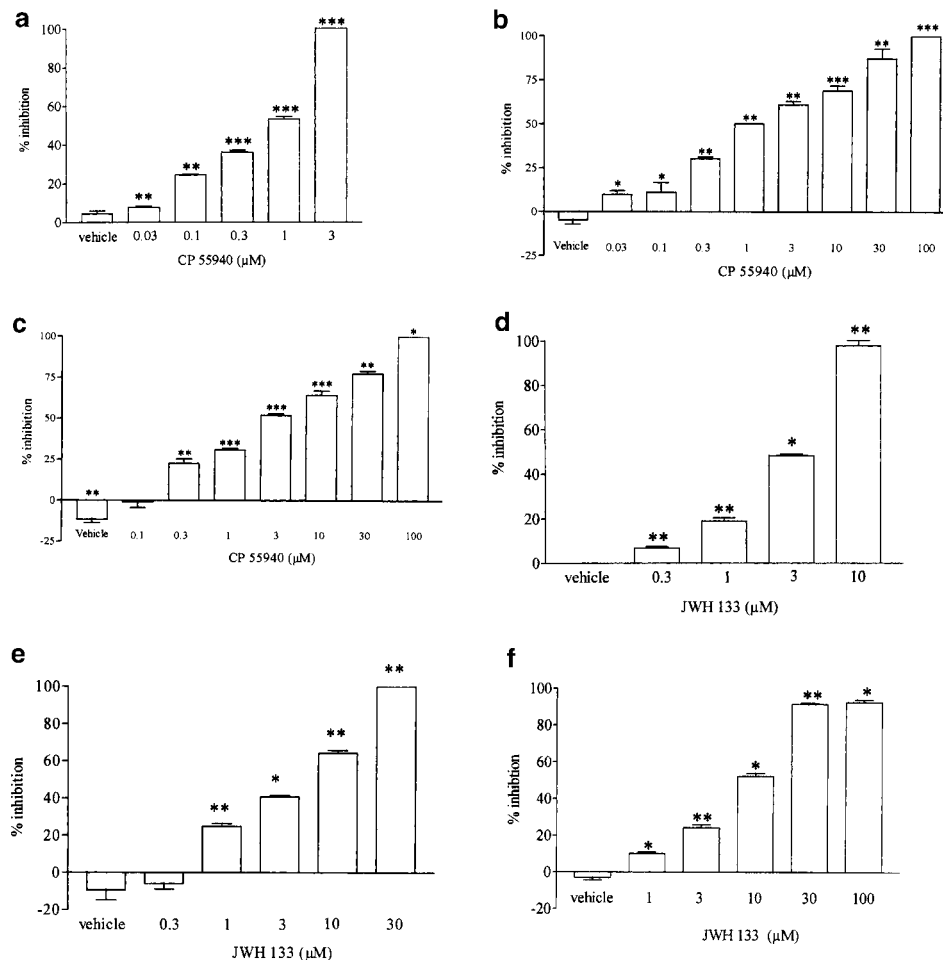


Figure 1 Inhibition of nerve depolarisation by cannabinoids. The nonselective cannabinoid agonist CP 55940 inhibits (a) capsaicin (1 μM), (b) PGE₂ (1 μM) and (c) hypertonic saline (2%)-induced depolarisation of the guinea-pig vagus nerve. Similarly, the CB₂-selective receptor agonist JWH 133 inhibits (d) capsaicin (1 μM), (e) PGE₂ (1 μM) and (f) hypertonic saline (2%)-induced depolarisation of the guinea-pig vagus nerve. Nerve depolarisation responses were expressed as absolute values in mV depolarisation before and after drug additions, and then expressed a percentage change. The data were subjected to a paired two-tailed *t*-test, since the response to a stimulant was measured before and after drug treatment within the same nerve. **P* < 0.05, ***P* < 0.01 and ****P* < 0.001 denote statistical significance. Values are presented as the mean ± s.e.m. percentage change of *n* = 4 determinations. *pD*₂ values (−log of the EC₅₀ defined as the concentration of drug required to elicit 50% of the maximum response) and statistical significance were calculated using 'GraphPad Instat™', (© GraphPad Software).

stimulus was hypertonic saline or PGE₂) were selected based on their respective concentration–response curves. CP 55940 (1 μM) inhibited capsaicin-induced nerve depolarisations of guinea-pig vagus nerve (before: 0.39 ± 0.04 mV; after: 0.21 ± 0.02 mV; *n* = 4; *P* < 0.01). This effect was completely blocked in the presence of SR144528 (before: 0.38 ± 0.05 mV; after: 0.53 ± 0.1 mV; *n* = 4), and was unaffected by SR141716A (before: 0.5 ± 0.05 mV; after: 0.23 ± 0.03 mV; *n* = 4; *P* < 0.01). The vehicles for these agents (0.1% DMSO for SR141716A and SR144528 or 0.1% ethanol for CP 55940) had no significant effect on capsaicin-induced nerve depolarisations, either alone or in combination. Inhibitory responses induced by JWH 133 were also completely blocked by SR144528 and unaffected by SR141716A in all cases, regardless of the stimulus used (Figure 2). Experiments performed on the human vagus nerve confirm a similar inhibitory effect of JWH 133 (40% inhibition at 10 μM) against nerve depolarisations induced by capsaicin (1 μM), which was prevented in the presence of SR144528 (Figure 3).

Based on these observations, experiments were performed using an *in vivo* model of cough, in order to determine if the inhibitory actions of JWH 133 on sensory nerves *in vitro* are also seen *in vivo*. Indeed, administration of JWH 133 (10 mg kg^{−1}, i.p.) 20 min prior to exposure to the commonly used tussive agent citric acid (0.3 M, 10 min) significantly reduced cough in conscious guinea-pigs (0.94 ± 0.24 cough min^{−1}), compared to those treated with the vehicle control (2.05 ± 0.24 cough min^{−1}; Figure 4). No sedation was observed in the guinea-pigs treated with JWH 133.

Discussion

In this study, we have shown for the first time that activation of the CB₂ receptor subtype inhibits both guinea-pig and human airway sensory nerve activity and the cough reflex in guinea-pigs. The isolated vagus preparation was used to characterise the cannabinoid receptor subtype involved in this

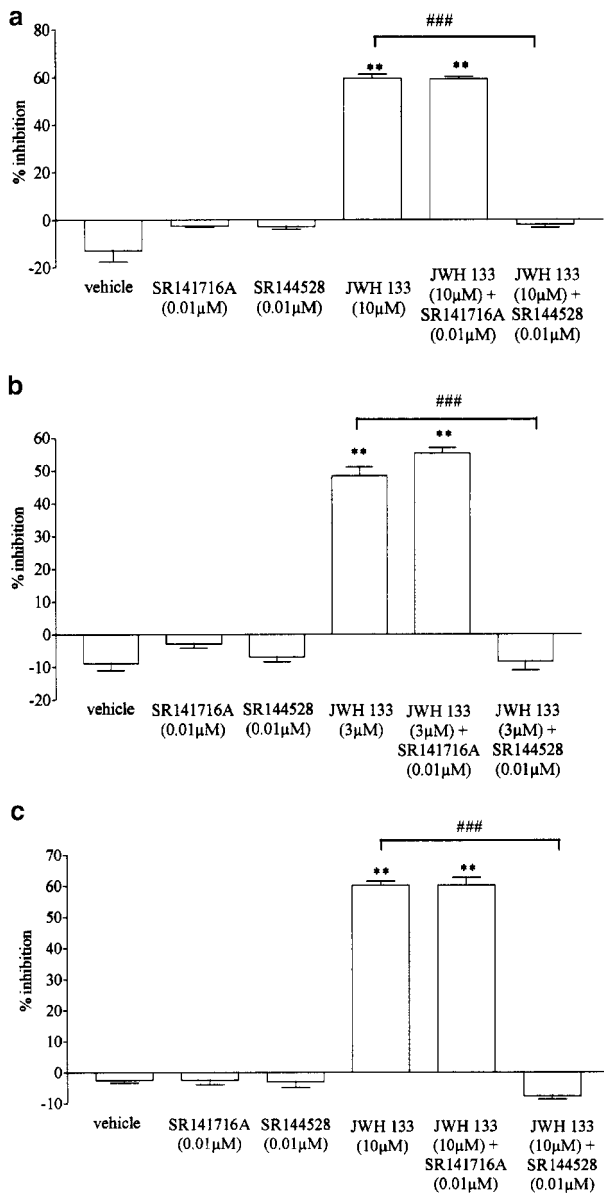


Figure 2 JWH 133-mediated inhibitory responses on nerve depolarisations are SR144528 sensitive. The inhibitory action of JWH 133 on guinea-pig vagus nerve depolarisations induced by (a) hypertonic saline (2%), (b) capsaicin (1 μM) and (c) PGE₂ (1 μM) is abolished in the presence of the CB₂ receptor antagonist SR144528, and is unaffected by the CB₁ receptor antagonist SR141716A. Nerve depolarisation responses were expressed as absolute values in mV depolarisation before and after drug additions, and then expressed a percentage change. The data were subjected to a paired two-tailed *t*-test, since the response to a stimulant was measured before and after drug treatment within the same nerve. ***P* < 0.01 denotes the statistical significance compared to control responses in the same tissue prior to drug treatment and ###*P* < 0.001 denotes the statistical significance between two different treatment groups using an unpaired *t*-test. Values are presented as the mean ± s.e.m. percentage change of *n* = 4 determinations.

response, as pharmacological profiling *in vitro* is often more straightforward as pharmacokinetic issues do not complicate the interpretation of the drug action. However, although the isolated vagus preparation presents us with the ideal opportunity to conduct a comprehensive pharmacological assess-

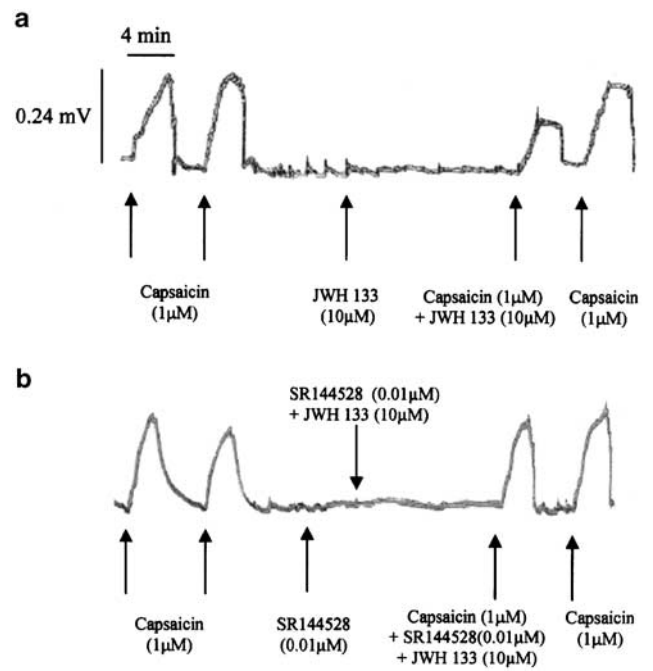


Figure 3 Effect of cannabinoid ligands on nerve depolarisations of isolated human vagus. Traces showing (a) the inhibitory effect of JWH 133 on nerve depolarisations induced by capsaicin, and (b) the blockade of this response by the CB₂ receptor antagonist SR144528 from human vagus nerve.

ment, data using this preparation should be interpreted with some caution since the pharmacological agents are applied to the axon of the isolated vagus nerve *in vitro*. Thus, the depolarisation signal recorded extracellularly represents a summation of the changes in membrane potential of all the axons *via* activation of receptors expressed in the neuronal membrane of the axon. Furthermore, the receptor expression and signal transduction mechanisms in the axon may not necessarily represent the behaviour of those elements in the peripheral endings.

Capsaicin, PGE₂ and hypertonic saline-induced nerve depolarisations of the guinea-pig vagus nerve were inhibited by the non-selective cannabinoid agonist CP 55940 in a concentration-dependent manner. Similarly, the CB₂-selective agonist JWH 133 (Huffman *et al.*, 1999; Pertwee, 1999) reduced responses to hypertonic saline, capsaicin and PGE₂ in a concentration-dependent manner. Furthermore, the inhibitory responses induced by CP 55940 on depolarisation responses evoked by capsaicin were not affected when vagus preparations were pretreated with the selective CB₁ receptor antagonist SR141716A (*K*_i = 5.9 nM for CB₁ and > 1 μM for CB₂; Rinaldi-Carmona *et al.*, 1994), but were completely abolished by the selective CB₂ receptor antagonist SR144528 (*K*_i = 0.6 nM for CB₂ and 437 nM for CB₁; Rinaldi-Carmona *et al.*, 1998). In addition, the inhibitory action of JWH 133 on nerve depolarisations induced by any of the stimuli was completely abolished by SR144528 and unaffected by SR141716A. Similarly, the inhibitory effect of JWH 133 on capsaicin-induced nerve depolarisation of the human vagus was abolished in the presence of SR144528. Antagonist affinity is the key factor when assessing receptor selectivity. The concentration of the antagonists used (0.01 μM) is similar to the

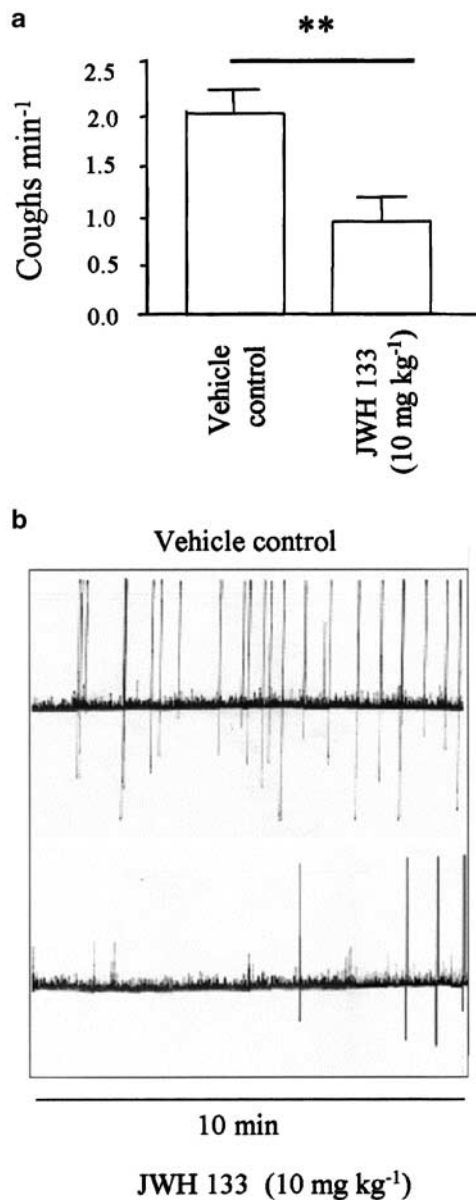


Figure 4 JWH 133 inhibits citric acid-induced cough in guinea-pigs. Histogram describing the mean data (a) and a representative trace (b) showing the effect of the CB₂-selective agonist JWH 133 (10 mg kg⁻¹, i.p., 20 min, *n* = 8) on citric acid (0.3 M, 10 min)-induced cough in conscious guinea-pigs compared to that of vehicle control-treated animals (0.5% methyl cellulose with 0.2% Tween 80 in saline, i.p., 20 min, *n* = 8). The number of cough sounds detected by a microphone and recorded on a chart recorder (indicated by spikes) during the 10 min exposure period to citric acid (0.3 M) were compared between treated and control groups using an unpaired *t*-test (***P* < 0.01).

*p*A₂ values for SR141716A at the CB₁ receptor (*p*A₂ = 7.9; Rinaldi-Carmona *et al.*, 1994), and less than the *p*A₂ for SR144528 at the CB₂ receptor (*p*A₂ = 6.3; Rinaldi-Carmona *et al.*, 1998). Hence, the data presented here clearly demonstrate that activation of CB₂ receptors mediates the inhibitory action of cannabinoids on sensory nerve depolarisation. This study is unique given the opportunity we have to validate the target, in this case the CB₂ receptor, in the relevant human

tissue involved in evoking a tussive response, that is, human vagal sensory nerves.

We have previously demonstrated that agents shown to directly inhibit sensory nerve depolarisations *in vitro* also inhibit sensory nerve-mediated responses *in vivo*, such as plasma exudation (Birrell *et al.*, 2002) and cough (Fox *et al.*, 1997). Based on the observation that JWH 133 inhibits guinea-pig sensory nerve depolarisations induced by hypertonic saline, capsaicin and PGE₂, it is probable that JWH 133 inhibits the activity of both subpopulations of sensory nerves involved in the cough reflex (i.e. RARs and C-fibres). Indeed, the *in vivo* activity of JWH 133 was demonstrated by an inhibition of citric acid-induced cough in conscious guinea-pigs compared to those treated with vehicle control. No sedation was observed in guinea-pigs treated with the CB₂ agonist as measured by lack of activity, nonsupine position and responses to external environment, thus indicating that no central effects of this agent were observed, suggesting that this response is mediated *via* peripheral CB₂ receptor activation. Contrary to this, it has been suggested that the nonselective endocannabinoid anandamide suppresses cough in conscious guinea-pigs *via* activation of CB₁ receptors and not CB₂ (Calignano *et al.*, 2000). However, such agents have sedative effects *via* activation of central CB₁ receptors and, therefore, the suppressive effect of anandamide on the cough reflex through sedation cannot be excluded (Lichtman *et al.*, 1998; Manzanares *et al.*, 1999). More recently, however, anandamide has been shown to increase the cough reflex *via* activation of TRPV1 receptors (Jia *et al.*, 2002), and is consistent with data suggesting that anandamide (at high concentrations) activates rat and guinea-pig pulmonary vagal C fibres *via* TRPV1 receptor activation (Kagaya *et al.*, 2002; Lin & Lee, 2002). The contrasting data from these studies may be due to the different experimental conditions employed, and in particular the doses of anandamide used, as this agent is considerably less potent than capsaicin at the TRPV1 receptor (Szallasi & Di Marzo, 2000; Ralevic *et al.*, 2001).

The cough reflex is thought to be initiated *via* the activation of either RAR or C-fibre afferents. However, it has been suggested that effects such as bronchospasm, mucus secretion and plasma extravasation due to the release of neuropeptides following C-fibre activation may indirectly lead to RAR activation and the initiation of the cough reflex (Canning, 2002). Consistent with the notion of C-fibre-mediated RAR activation, capsaicin-induced cough can virtually be abolished by neurokinin receptor antagonists. Such effects of inhaled neurokinin antagonists (Girard *et al.*, 1995; Xiang *et al.*, 1998), and systemically administered, low CNS-penetrant compounds (Hay *et al.*, 2002), in the guinea-pig cough model may argue for an indirect role of C-fibres in cough. Hence, it is possible that the inhibitory action of the CB₂ agonist on sensory nerve function may be due to a prejunctional effect on neurokinin release from airway C-fibres. However, contrary to this, Bolser *et al.* (1997) have demonstrated that neurokinin receptor antagonists inhibit cough in guinea-pigs and cats, solely *via* an effect on the central nervous system. On this basis, a central action of neurokinin receptor antagonists cannot be ruled out. Therefore, the suggestion that the CB₂ agonist directly inhibits airway C-fibres to inhibit cough cannot be excluded, and is consistent with the *in vitro* data presented on the isolated nerve preparation.

The present study indicates, with the use of pharmacological tools, the existence of neuronal CB₂ receptors in the airways. These data show, for the first time, that activation of the CB₂ receptor subtype inhibits both A δ and C-fibre activation and the cough reflex in guinea-pigs. Moreover, the inhibitory action of the CB₂ receptor agonist and the prevention of this effect by the CB₂ receptor antagonist on human vagus nerve provides proof of concept for the mechanism in man, and is strong evidence to suggest that the development of CB₂

agonists, devoid of central effects, will provide a new and safe antitussive treatment for chronic cough.

This work was supported by a grant from the National Asthma Campaign. Professor Maria Belvisi is supported by the Harefield Research Foundation. SR144528 and SR141716A were kind gifts provided by Dr Alyson J. Fox, Novartis Institute for Medical Sciences, London.

References

- BELVISI, M.G., VENKATESAN, P., BARNES, P.J. & FOX, A.J. (1998). A comparison of the chemosensitivity of the isolated guinea-pig and human vagus nerves. *Am. J. Respir. Crit. Care Med.*, **157**, A487.
- BIRRELL, M.A., CRISPINO, N., HELE, D.J., PATEL, H.J., YACOUB, M.H., BARNES, P.J. & BELVISI, M.G. (2002). Effect of dopamine receptor agonists on sensory nerve activity: possible therapeutic targets for the treatment of asthma and COPD. *Br. J. Pharmacol.*, **136**, 620–628.
- BOLSER, D.C., DE GENNARO, F.C., O'REILLY, S., MCLEOD, R.L. & HEY, J.A. (1997). Central anti-tussive activity of the NK₁ and NK₂ tachykinin receptor antagonists, CP 99, 994 and SR 48968, in the guinea pig and cat. *Br. J. Pharmacol.*, **1221**, 165–170.
- CALIGNANO, A., CATONA, I., DESARNAUD, F., GIUFFRIDA, A., LA RANA, G., MACKIE, K., FREUND, T.F. & PIOMELLI, D. (2000). Bidirectional control of airway responsiveness by cannabinoids. *Nature*, **408**, 96–101.
- CANNING, B.J. (2002). Interactions between vagal afferent nerve subtypes mediating cough. *Pulm. Pharmacol. Ther.*, **15**, 187–192.
- CARR, M.J. & UNDEM, B.J. (2001). Inflammation induced plasticity of the afferent innervation of the airways. *Environ. Health Persp.*, **109**, 567–571.
- COLERIDGE, H.M., COLERIDGE, J.C., GINZEL, C.H., BAKER, D.G., BANZETT, R.B. & MORRISON, M.A. (1976). Stimulation of 'irritant' receptors and afferent C-fibres in the lungs by prostaglandins. *Nat. Lond.*, **264**, 451–453.
- COLERIDGE, J.C. & COLERIDGE, H.M. (1984). Afferent vagal C fibre innervation of the lungs and airways and its functional significance. *Rev. Physiol. Biochem. Pharmacol.*, **99**, 1–110.
- COSTELLO, J.F., DUNLOP, L.S. & GARDINER, P.J. (1985). Characteristics of prostaglandin induced cough in man. *Br. J. Clin. Pharmacol.*, **20**, 355–359.
- FOX, A.J., BARNES, P.J., URBAN, L. & DRAY, A. (1993). An *in vitro* study of the properties of single vagal afferents innervating guinea-pig airways. *J. Physiol.*, **469**, 21–35.
- FOX, A.J., BARNES, P.J., VENKATESAN, P. & BELVISI, M.G. (1997). Activation of large conductance potassium channels inhibits the afferent and efferent function of airway sensory nerves in the guinea pig. *J. Clin. Invest.*, **99**, 513–519.
- FOX, A.J., LALLOO, U.G., BELVISI, M.G., BERNAREGGI, M., CHUNG, K.F. & BARNES, P.J. (1996). Bradykinin-evoked sensitisation of airway sensory nerves: a mechanism for ACE-inhibitor cough. *Nat. Med.*, **2**, 814–817.
- FOX, A.J., URBAN, L., BARNES, P.J. & DRAY, A. (1995). Effects of capsazepine against capsaicin and proton-evoked excitation of single C-fibres and vagus nerve from the guinea-pig. *Neuroscience*, **67**, 741–752.
- GIRARD, V., NALINE, E., EDMONDS-ALT, X. & ADVENIER, C. (1995). Effect of two tachykinin antagonists, SR 48968 and SR140333, on cough induced by citric acid in the unanaesthetised guinea pig. *Eur. Resp. J.*, **8**, 1110–1114.
- GORDON, R., GORDON, R.J. & SOFIA, D. (1976). Antitussive activity of some naturally occurring cannabinoids in anesthetized cats. *Eur. J. Pharmacol.*, **35**, 309–313.
- HAY, D.W.P., GIARDINA, G.A.M., GISWOLD, D.E., UNDERWOOD, D.C., KOTZER, C.J., BUSH, B., POTTS, W., SANDHU, P., LUNDBERG, D., FOLEY, J.J., SCHMIDT, D.B., MARTIN, L.D., KILIAN, D., LEGOS, J.J., BARONE, F.C., LUTTMANN, M.A., GRUGNI, M., RAVEGLIA, L.F. & SARAU, H.M. (2002). Nonpeptide tachykinin receptor antagonists. III. SB 235375, a low central nervous system-penetrant, potent and selective neurokinin-3 receptor antagonist, inhibits citric acid-induced cough and airways hyperreactivity in guinea pigs. *J. Pharmacol. Exp. Ther.*, **300**, 314–323.
- HUFFMAN, J.W., LIDDLE, J., YU, S., AUNG, M.M., ABOOD, M.E., WILEY, J.L. & MARTIN, B.R. (1999). 3-(1',1'-Dimethylbutyl)-1-deoxy-D⁸-THC and related compounds: synthesis of selective ligands for the CB₂ receptor. *Bioorg. Med. Chem.*, **7**, 2905–2914.
- JIA, Y., MCLEOD, R.L., WANG, X., PARRA, L.E., EGAN, R.W. & HEY, J.A. (2002). Anandamide induced cough in conscious guinea-pigs through VR1 receptors. *Br. J. Pharmacol.*, **137**, 831–836.
- KAGAYA, M., LANB, J., ROBBINS, J., PAGE, C.P. & SPINA, D. (2002). Characterisation of the anandamide induced depolarisation of guinea pig isolated vagus nerve. *Br. J. Pharmacol.*, **137**, 39–48.
- LALLOO, U.G., FOX, A.J., BELVISI, M.G., CHUNG, K.F. & BARNES, P.J. (1995). Capsazepine inhibits cough induced by capsaicin and citric acid but not by hypertonic saline in guinea pigs. *J. Appl. Physiol.*, **79**, 1082–1087.
- LAUDE, E.A., HIGGINS, K.S. & MORICE, A.H. (1993). A comparative study of the effects of citric acid, capsaicin and resiniferatoxin on the cough challenge in guinea-pig and man. *Pulm. Pharmacol.*, **6**, 171–175.
- LICHTMAN, A.H., WILEY, J.L., LAVECCHIA, K.L., NEVIASER, S.T., ARTHUR, D.B., WILSON, D.M. & MARTIN, B.R. (1998). Effects of SR141716A after acute or chronic cannabinoid administration in dogs. *Eur. J. Pharmacol.*, **357**, 139–148.
- LIN, Y.S. & LEE, L.Y. (2002). Stimulation of pulmonary vagal C-fibres by anandamide in anaesthetized rats: role of vanilloid type 1 receptors. *J. Physiol.*, **538**, 947–955.
- MANZANARES, J., CORCHERO, J., ROMERO, J., FERNANDEZ-RUIZ, J.I., RAMOS, J.A. & FUENTES, J.A. (1999). Pharmacological and biochemical interactions between opioids and cannabinoids. *Trends Pharmacol. Sci.*, **20**, 287–294.
- MATSUDA, L.A., LOLAIT, S.J., BROWNSTEIN, M.J., YOUNG, A.C. & BONNER, T.I. (1990). Structure of a cannabinoid receptor and functional expression of the cloned cDNA. *Nature*, **346**, 561–564.
- MOHAMMED, S.P., HIGENBOTTAM, T.W. & ADCOCK, J.J. (1993). Effects of aerosol-applied capsaicin, histamine and prostaglandin E₂ on airway sensory receptors of anaesthetised cats. *J. Physiol.*, **469**, 51–66.
- MUNRO, S., THOMAS, K.L. & ABU-SHAAR, M. (1993). Molecular characterisation of a peripheral receptor for cannabinoids. *Nature*, **365**, 61–65.
- PEDERSEN, K.E., MEEKER, S.N., RICCIO, M.M. & UNDEM, B.J. (1998). Selective stimulation of jugular ganglion afferent neurons in guinea pig airways by hypertonic saline. *J. Appl. Physiol.*, **84**, 499–506.
- PERTWEE, R.G. (1999). Pharmacology of cannabinoid receptor ligands. *Curr. Med. Chem.*, **6**, 635–664.
- PORTER, A.C. & FELDER, C.C. (2001). The endocannabinoid nervous system: unique opportunities for therapeutic intervention. *Pharmacol. Ther.*, **90**, 45–60.

- RALEVIC, V., KENDALL, D.A., JERMAN, J.C., MIDDLEMISS, D.N. & SMART, D. (2001). Cannabinoid activation of recombinant and endogenous vanilloid receptors. *Eur. J. Pharmacol.*, **424**, 211–219.
- RANG, H.P. & RITCHIE, J.M. (1988). Depolarisation of nonmyelinated fibres of the rat vagus nerve produced by activation of protein kinase C. *J. Neurosci.*, **8**, 2606–2617.
- RINALDI-CARMONA, M., BARTH, F., HEAULME, M., SHIRE, D., CALANDRA, B., CONGY, C., MARTINEZ, S., MARUANI, J., NELIAT, G., CAPUT, D., FERRARA, P., SOUBRIE, P., BRELIERE, J.C. & LE FUR, G. (1994). SR141716A, a potent and selective antagonist of the brain cannabinoid receptor. *FEBS Lett.*, **350**, 240–244.
- RINALDI-CARMONA, M., BARTH, F., MILLAN, J., DEROCQ, J.M., CASELLAS, P., CONGY, C., OUSTRIC, D., SARRAN, M., BAUBOULA, M., CALANDRA, B., PORTIER, M., SHIRE, D., BRELIERE, J.C. & LE FUR, G.L. (1998). SR144528, the first potent and selective antagonist of the CB₂ cannabinoid receptor. *J. Pharmacol. Exp. Ther.*, **284**, 644–650.
- ROBERTS, A.M., SCHULTZ, H.D., GREEN, J.F., ARMSTRONG, D.J., KAUFMAN, M.P., COLERIDGE, H.M. & COLERIDGE, J.C. (1985). Reflex tracheal contraction evoked in dogs by bronchodilator prostaglandins E₂ and I₂. *J. Appl. Physiol.*, **58**, 1823–1831.
- SANT'AMBROGIO, G. (1987). Nervous receptors of the tracheobronchial tree. *Ann. Rev. Physiol.*, **49**, 611–627.
- SMITH, J.A., AMAGASU, S.M., EGLEN, R.M., HUNTER, J.C. & BLEY, K.R. (1998). Characterisation of prostanoid receptor-evoked responses in rat sensory neurones. *Br. J. Pharmacol.*, **124**, 513–523.
- STONE, R., BARNES, P.J. & FULLER, R.W. (1992). Contrasting effects of prostaglandins E₂ and F₂ alpha on sensitivity of the human cough reflex. *J. Appl. Physiol.*, **73**, 649–653.
- SZALLASI, A. & DI MARZO, V. (2000). New perspectives on enigmatic vanilloid receptors. *Trends Neurosci.*, **23**, 491–497.
- XIANG, A., UCHIDA, Y., NOMURA, A., IJHIMA, H., DONG, F., ZHANG, M.-J. & HASEGAWA, S. (1998). Effects of airway inflammation on cough response in the guinea pig. *J. Appl. Physiol.*, **85**, 1847–1854.

(Received March 27, 2003

Revised June 13, 2003

Accepted June 25, 2003)