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The endogenous cannabinoid agonist, anandamide stimulates sensory nerves in guinea-pig airways

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1 The endogenous cannabinoid agonist, anandamide produced a modest contractile response in guinea-pig isolated bronchus compared with the vanilloid receptor agonist capsaicin. The contractile response to both anandamide and capsaicin was inhibited by the vanilloid receptor antagonist, capsazepine. Furthermore, the NK_2-selective antagonist, SR48968 but not the NK_1-selective antagonist, SR140333 inhibited contractile responses to anandamide.

2 The contractile response to anandamide was abolished in tissues desensitized by capsaicin. However, anandamide failed to cross-desensitize the contractile response to capsaicin.

3 The contractile response to anandamide was not significantly altered in the presence of the CB_1 receptor antagonist, SR141716A, nor the amidase inhibitor, phenylmethylsulphonyl fluoride (PMSF) but was significantly increased in the presence of the neutral endopeptidase inhibitor, thiorphan.

4 The cannabinoid agonist, CP55,940 failed to significantly attenuate the excitatory non-adrenergic non-cholinergic (eNANC) response in guinea-pig airways. In contrast, the ORL_1 receptor agonist, nociceptin, significantly inhibited this response.

5 The results demonstrate that anandamide induces a modest contractile response in guinea-pig isolated bronchus that is dependent upon the activation of vanilloid receptors on airway sensory nerves. However, cannabinoid receptors do not appear to play a role in this regard, nor in regulating the release of neuropeptides from airway sensory nerves under physiological conditions.
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Abbreviations: ANOVA, analysis of variance; DMSO, dimethylsulphoxide; eNANC, excitatory non-adrenergic non-cholinergic; PMSF, phenylmethylsulphonyl fluoride; PDE, phosphodiesterase; VR, vanilloid receptor

Introduction

The archetypal vanilloid receptor agonist capsaicin is known to activate a ligand-gated channel that was recently cloned (Caterina *et al*., 1997) and the similarity in structure between the cannabinoid receptor agonist anandamide and the vanilloid agonist, olvanil, led to the discovery that anandamide is an activator of vanilloid receptors (Zygmunt *et al*., 1999; Smart *et al*., 2000). This substance is thus a potential candidate for the much sought after endogenous vanilloid receptor agonist (Szallasi & Blumberg, 1999) that joins a growing list of endogenous activators/modulators of the vanilloid receptor, including inflammatory mediators (Stucky *et al*., 1998; Vyklicky *et al*., 1998), hydrogen ions (Caterina *et al*., 1997; Vyklicky *et al*., 1998), heat (Caterina *et al*., 1997; Tominaga *et al*., 1998), arachidonic acid (Manzini *et al*,. 1989; Manzini & Meini, 1991), lipoxin A_4 (Meini *et al*., 1992), products of the lipoxygenase pathway (Hwang *et al*., 2000), and prostacylin (Mapp *et al*., 1991). In contrast, anandamide has recently been shown to inhibit the release of neuropeptides from sensory nerves in the rat *via* a CB_1-dependent mechanism (Richardson *et al*., 1998a,b) indicating that anandamide may have both inhibitory and excitatory actions on sensory nerves.

We have previously shown that bronchial hyperresponsiveness mediated by platelet activating factor (Spina *et al*., 1991) and 15-hydroperoxyeicosatetraenoic acid (Riccio *et al*., 1997) in naïve rabbits and allergen challenge in immunized rabbits (Riccio *et al*., 1993) is abolished following chronic treatment with capsaicin. This is also consistent with numerous studies showing that bronchial hyperresponsiveness induced by toluene diisocyanate, virus, and allergen in guinea-pigs is also attenuated by capsaicin treatment (reviewed in Spina *et al*., 1998). Together these studies indicate that sensory nerves may be a common pathway by which many stimuli can induce bronchial hyperresponsiveness and consistent with the hypothesis that 'hyperalgesia' of airway sensory nerves may contribute toward this phenomenon (Adcock & Garland, 1993; Spina *et al*., 1998). The finding that inflammatory mediators (Stucky *et al*., 1998; Vyklicky *et al*., 1998), hydrogen ions (Caterina *et al*., 1997; Vyklicky *et al*., 1998), heat (Caterina *et al*., 1997; Tominaga *et al*., 1998) and arachidonic acid metabolites (Manzini *et al*., 1989; Manzini & Meini, 1991; Mapp *et al*., 1991; Meini *et al*., 1992; Hwang *et al*., 2000) can activate the vanilloid receptor suggest that these agents may be potential endogenous mediators that are released during an inflammatory response, and can sensitize airway sensory nerves and induce bronchial hyperresponsiveness.

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Guinea-pig isolated bronchus is a useful biological readout of neuropeptide release from airway sensory nerves and we have shown that various pharmacological agents including the phosphodiesterase (PDE)4 inhibitor, Ro-201724 (Spina *et al*., 1995); the phosphatase 1/2A inhibitor, okadaic acid (Harrison *et al*., 1997) and the ORL_1-receptor agonist, nociceptin (Shah *et al*., 1998) attenuated the eNANC response in this preparation. We have therefore investigated whether anandamide stimulates vanilloid receptors on airway sensory nerves and the role of cannabinoid receptors in modulating the eNANC response in guinea-pig airways.

Methods

Tissue preparation

Male albino guinea-pigs (300–400 g) were killed by cervical dislocation and the lungs removed and placed in cold (4°C) Krebs-Henseleit solution aerated with 95% O_2 and 5% CO_2. Main bronchial rings (2 mm) were suspended under 0.5 g tension, in 12 ml organ baths in Krebs-Henseleit solution aerated with 95% O_2 and 5% CO_2 at 37°C, containing the cyclo-oxygenase inhibitor, indomethacin (5 μM). Changes in tension were measured, *via* a FTO3C transducer, and recorded using Maclab (version 3.3.8). Tissues were allowed to equilibrate for 40 min with changes in Krebs-Henseleit solution being made at 10 min intervals. Methacholine (1 and 100 μM) was added cumulatively to the bath to establish the sensitivity of the tissue and after the contractile response had reached plateau, the tissues were washed for 10 min and allowed to equilibrate for a further 30 min.

Exogenous administration of substances

Tissues were incubated in the presence of the neural endopeptidase inhibitor, thiorphan (10 μM) for a period of 30 min. Bronchomotor tone was measured following administration of anandamide, *R*-(+)-methanandamide (1–100 μM), 2-arachidonylglycerol (0.1–100 μM) and capsaicin (0.01–10 μM). In other experiments, the contractile response to anandamide was evaluated in the absence or presence of the vanilloid receptor agonist, capsazepine (10 μM), the CB_1 receptor antagonist SR141716A (1 μM), the amidase inhibitor, PMSF (50 μM), the NK_1-receptor antagonist, SR140333 (1 μM) and the NK_2-receptor antagonist, SR48968 (100 nM). In other experiments, the response to anandamide was evaluated in the absence or presence of the neutral endopeptidase inhibitor, thiorphan (10 μM). Responses to the cannabinoid agonists were expressed as a percentage of the maximal response to capsaicin (10 μM).

Desensitization studies

In other experiments, tissues were exposed to capsaicin (10 μM) in the absence of thiorphan (to facilitate removal of endogenously released neuropeptides from the biophase) and the contractile response allowed to achieve plateau. The tissues were incubated with capsaicin for a period of 20 min and then repeatedly washed over a 45–60 min period. A second response to capsaicin was performed in order to confirm that desensitization had occurred. Tissues were then incubated in the presence of thiorphan and then anandamide was added to the bath in increasing concentrations, 30 min later. Following completion of the dose response curve to anandamide, capsaicin (10 μM) was again added. Control tissues were treated in a similar fashion except that they were exposed to ethanol (0.01%).

In further experiments, tissues were treated with anandamide (100 μM) in the absence of thiorphan for a period of 50 min. The tissues were washed repeatedly over a 30–60 min period and a second application of anandamide was then performed. Using this protocol, the tissues failed to respond to anandamide (100 μM). Tissues were then incubated with thiorphan for a 30 min period and a concentration-response curve to capsaicin was then performed.

Electrical field stimulation studies

Guinea-pig isolated main bronchi were placed between two platinum electrodes and electrically stimulated (3 Hz, 15 s, 0.5 ms pulse width, 40 V, $I = 750$ mAmp). Tissues were incubated for 30 min in the presence of atropine (1 μM), the non-selective β-antagonist, propranolol (0.1 μM) and the neutral endopeptidase inhibitor, thiorphan (10 μM), and electrically stimulated (S1). The resulting eNANC response returned to baseline after 30 min. Tissues were incubated with the cannabinoid receptor agonist, CP55,940 (1 and 10 μM) and the ORL_1 receptor agonist, nociceptin (1 μM) 10 min prior to the second electrical stimulation (S2). The effect of agonist or vehicle on the eNANC response is expressed as per cent control (i.e. S2/S1).

Analysis of results

Results from all experiments are expressed as mean ± standard error of mean where n denotes the number of animals. Where appropriate, differences between mean values were assessed using Student's paired or non-paired t-test. Differences between treatments were also assessed using analysis of variance (ANOVA). Differences between mean values were considered significant if $P < 0.05$.

Drugs

Atropine, indomethacin, methacholine, (-)-propranolol, thiorphan (Sigma-Aldrich Chemical Co., Dorset, U.K.); anandamide, R-(+)-methanandamide, CP55,940, phenylmethylsulphonyl fluoride (PMSF) (Tocris Cookson, Bristol, U.K.); SR141716A, SR140333, SR48968 (Sanofi Recherche, Montpellier Cedex, France); 2-arachidonylglycerol (RBI, St Louis, Missouri, U.S.A.). Composition of Krebs-Henseleit solution (mM): NaCl 117.6, $NaHCO_3$ 25, Glucose 11.1, KH_2PO_4 1.03, $MgSO_4.7H_2O$ 0.57, KCl 5.4 and $CaCl_2$ 2.5. Unless otherwise specified all drugs were prepared in Krebs-Henseleit solution. Stock concentrations of indomethacin (0.01 M) and thiorphan (0.01 M) were prepared in 0.5% Na_2CO_3 and 5% Na_2CO_3 respectively. Anandamide obtained from Tocris Cookson came prepared as a 10 mg ml^{-1} emulsion in soya oil/water (1 : 4) and dilutions made in Krebs-Henseleit solution. Stock concentrations of CP55,940, SR141716A and PMSF (0.01 M) and dilutions were prepared in

dimethylsulphoxide (DMSO) such that bath concentrations of DMSO did not exceed 0.2%. Stock concentrations of capsaicin (0.1 M), SR48968 and SR140333 (1 mM) were prepared in ethanol. The resulting bath concentration of ethanol did not exceed 0.1%.

Results

Effect of anandamide on bronchomotor tone

The cannabinoid agonist anandamide, produced a concentration-dependent contraction of guinea-pig isolated bronchus (Figure 1). The contractile response to anandamide ($pD_2 = 5.26 \pm 0.05$, per cent capsaicin $E_{max} = 41.6 \pm 5.8$, $n = 8$) was significantly less than that observed for capsaicin (Figure 2, $P < 0.05$ ANOVA). Capsaicin induced a concentration-dependent contraction of guinea-pig bronchial tissue yielding a contractile potency ($pD_2 = -\log_{10}$ EC50) of 6.594 ± 0.174, $n = 7$) and maximum response (g tension) of 1.13 ± 0.11. The synthetic analogue *R*-(+)-methanandamide ($pD_2 = 4.84 \pm 0.05$, per cent capsaicin Emax $= 32.2 \pm 7.2$, $n = 7$) also induced a concentration-dependent contraction of guinea-pig isolated bronchus that was also significantly less potent than capsaicin ($P < 0.05$, Figure 2). In contrast, the cannabinoid agonist, 2-arachidonylglycerol failed to significantly increase baseline tone in guinea-pig bronchial preparations ($n = 3$). The vehicle for anandamide (soya oil/water, 0.1%) or methanandamide (ethanol, 0.1%) failed to alter baseline tone in guinea-pig isolated bronchus.

The vanilloid receptor antagonist capsazepine (10 μM) significantly attenuated the contractile response to capsaicin (pD_2 control, 6.88 ± 0.88 vs capsazepine 5.97 ± 0.11, $n = 6$ each, $P < 0.05$; Figure 3a) which yielded an apparent K_B value of -5.85 ± 0.12. Similarly, the contractile response to anandamide was also antagonized by capsazepine (pD_2 control, 5.21 ± 0.14 vs capsazepine 4.64 ± 0.18, $n = 5$, $P < 0.05$, Figure 3b), yielding an apparent K_B value of -5.42 ± 0.18.

Acute desensitization of guinea-pig isolated bronchus with capsaicin, abolished the contractile response to anandamide (Figure 4a) and capsaicin (Figure 4b). In contrast, repeated application of anandamide (100 μM), while inducing desensitization to itself, failed to significantly alter the contractile potency to capsaicin (pD_2; control; 6.83 ± 0.11 vs anandamide treated 6.79 ± 0.06, $n = 5$ each, $P > 0.05$).

The CB_1 selective antagonist, SR141716A (1 μM; Figure 5a) failed to significantly alter the contractile potency to anandamide (control: $pD_2 = 5.25 \pm 0.10$, per cent capsaicin $E_{max} = 49.6 \pm 8.3$, $n = 5$ vs SR141716A: $pD_2 = 5.24 \pm 0.24$, per cent capsaicin $E_{max} = 46.7 \pm 9.4$, $n = 5$, $P > 0.05$). Similarly, the amidase inhibitor PMSF (50 μM; Figure 5b) failed to significantly augment the contractile response to anandamide (control: $pD_2 = 5.21 \pm 0.08$, per cent capsaicin $E_{max} = 37.4 \pm 83.7$, $n = 8$ vs PMSF: $pD_2 = 5.23 \pm 0.10$, per cent capsaicin $E_{max} = 46.5 \pm 6.0$, $n = 8$, $P > 0.05$). In contrast, the neutral endopeptidase inhibitor, thiorphan (10 μM), significantly augmented the contractile response to anandamide (Figure 5c, $P < 0.01$, ANOVA) (anandamide 100 μM; per cent capsaicin $E_{max} = 19.6 \pm 2.8$, $n = 6$ vs thiorphan; 34.0 ± 4.0, $n = 6$, $P < 0.05$).

The NK_1-receptor antagonist, SR140333 (1 μM) failed to significantly alter the contractile response to capsaicin (pD_2, per cent methacholine E_{max}; control, 7.31 ± 0.13, 74.6 ± 7.9, $n = 6$ vs SR140333, 7.34 ± 0.16, 68.4 ± 2.6, $n = 6$, $P > 0.05$, Figure 6a). In contrast, the NK_2-selective antagonist, SR48968 (100 nM) significantly reduced the contractile response to capsaicin (per cent methacholine E_{max}, capsaicin 10 μM; 41.6 ± 10.8, $n = 6$, $P < 0.05$ of control, Figure 6b). In further experiments, SR140333 failed to alter the contractile response to anandamide (pD_2, per cent capsaicin E_{max}; control, 5.71 ± 0.11, 37.0 ± 4.3, $n = 6$ vs SR140333; 5.71 ± 0.16, 37.6 ± 6.4, $n = 6$, $P > 0.05$, Figure 6c). In the presence of SR48968, the contractile response to anandamide was significantly reduced (per cent capsaicin E_{max}, anandamide 100 μM; 4.1 ± 1.7, $n = 6$, $P < 0.05$ *c.f.* control, Figure 6d).

Electrical field stimulation

EFS (3 Hz) of guinea-pig isolated main bronchi induced a contractile response, $35 \pm 3\%$ ($n = 9$) of the maximum response to methacholine (100 μM; 1.24 ± 0.13 g tension). In vehicle controls, no significant reduction in the contractile

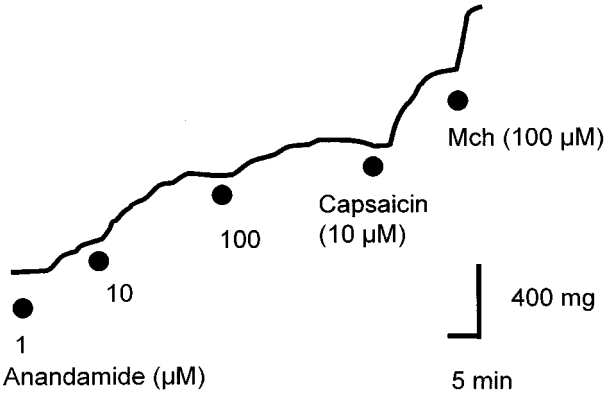

Figure 1 Line representation of the contractile response to increasing concentrations of anandamide. Mch; Methacholine.

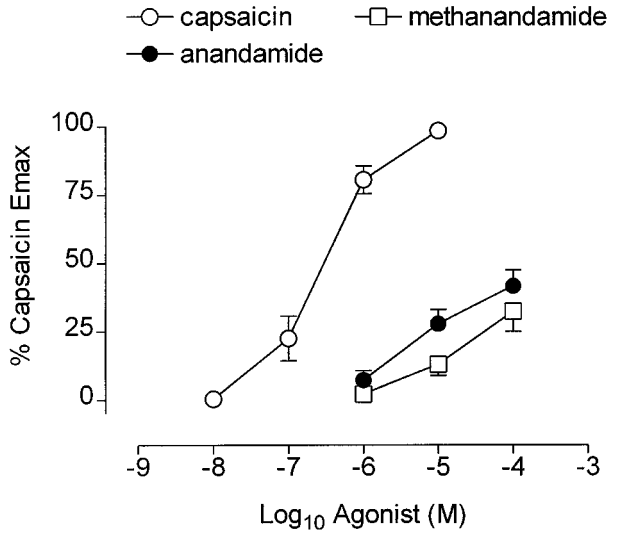

Figure 2 Cumulative concentration effect curves to capsaicin ($n = 7$), anandamide ($n = 8$) and *R*-(+)-methanandamide ($n = 7$). Each point represents the mean and vertical lines represent standard error of the mean.

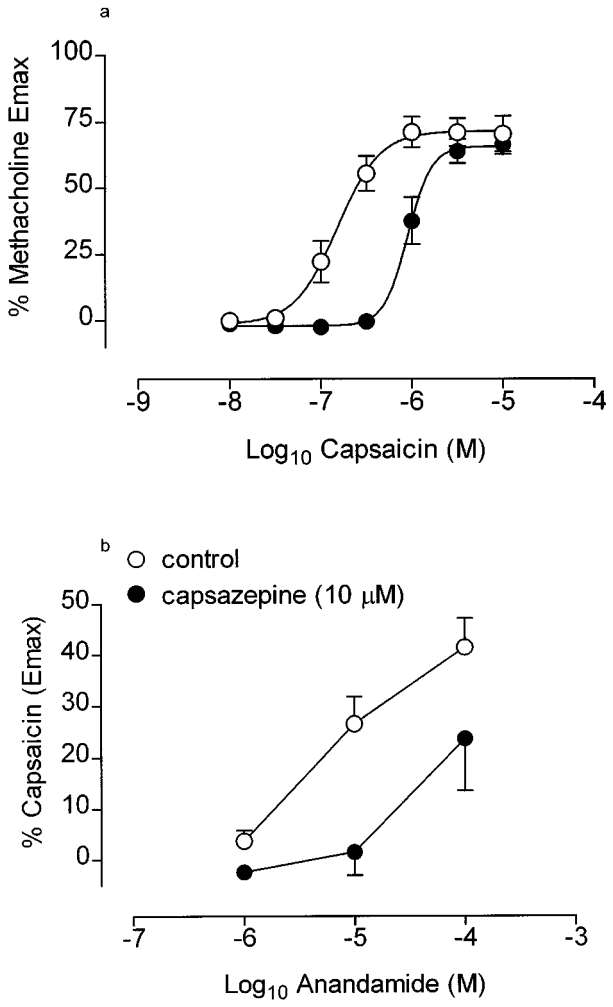

Figure 3 Cumulative concentration effect curves to (a) capsaicin ($n=6$ each) and (b) anandamide ($n=6$ each) in the absence or presence of the vanilloid receptor antagonist, capsazepine (10 μM). Each point represents the mean and vertical lines represent standard error of the mean.

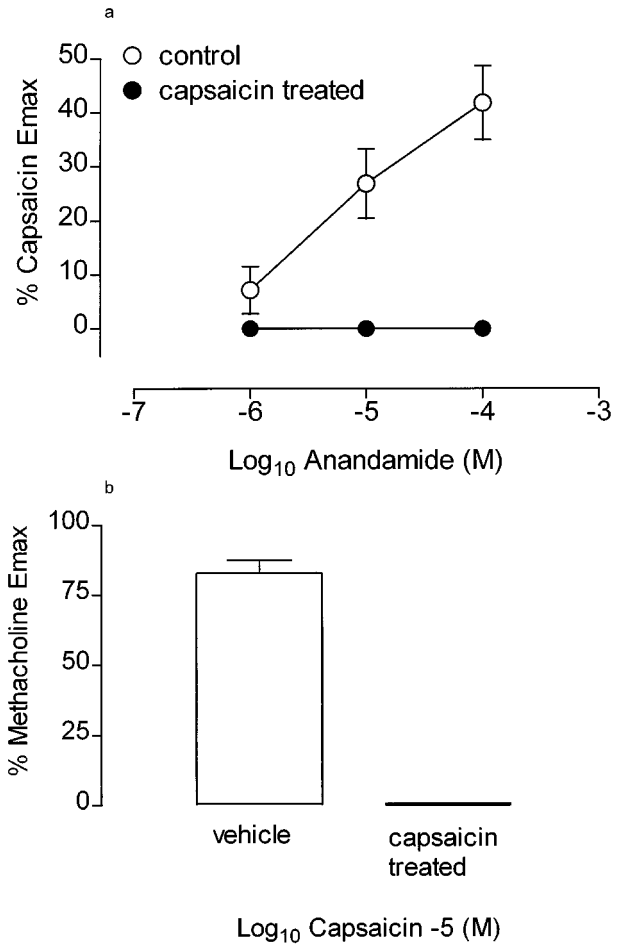

Figure 4 (a) Cumulative concentration effect curves to anandamide in vehicle treated ($n=4$) and capsaicin-desensitized tissues ($n=4$). (b) Bar graph representing the contractile response to maximum concentration of capsaicin (10 μM) in vehicle treated and capsaicin-desensitized tissue ($n=4$ each) following the anandamide concentration-effect curve. Each point represents the mean and vertical lines represent standard error of the mean.

response (eNANC) to repeated EFS was observed (per cent methacholine E_{max}; 35 ± 4, $n=9$, $P>0.05$ *c.f.* control). The cannabinoid receptor agonist CP55,940 (1 and 10 μM) did not attenuate the eNANC contractile response (Figure 7). In contrast, the ORL_1-receptor agonist, nociceptin (1 μM) significantly inhibited the eNANC response in this preparation ($P<0.05$ *c.f.* control; Figure 7). Neither CP55,940 (1 and 10 μM) or vehicle (DMSO, 0.1% and 0.2% respectively) induced contraction of guinea-pig bronchial preparations.

Discussion

We have demonstrated that the cannabinoid agonist, anandamide induces contraction of guinea-pig isolated bronchus *via* activation of airway sensory nerves, a response that was abolished by acute desensitization with capsaicin, antagonized by the vanilloid receptor antagonist, capsazepine and the NK_2-receptor antagonist, SR48968. In contrast, the cannabinoid receptor agonist, CP55,940 and 2-arachidonyl-glycerol failed to increase bronchomotor tone suggesting that activation of cannabinoid receptors *per se*, does not induce the release of sensory neuropeptides in this preparation. Furthermore, cannabinoid receptors do not appear to play a role in modulating the release of sensory neuropeptides under physiological conditions in this preparation.

With the recent cloning of the vanilloid receptor (Caterina *et al*., 1997) there is considerable interest in the discovery of endogenous ligands for this receptor which has important implications for the development of novel analgesic agents (Szallasi & Blumberg, 1999). It has been shown that various inflammatory mediators including bradykinin, serotonin, prostaglandin E_2 (Stucky *et al*., 1998; Vyklicky *et al*., 1998); heat (Caterina *et al*., 1997; Tominaga *et al*., 1998) and arachidonic acid metabolites (Mapp *et al*., 1991; Meini *et al*., 1992; Hwang *et al*., 2000) can activate the vanilloid receptor. Furthermore, acid pH appears to augment the ability of various agents including capsaicin, certain inflammatory mediators and heat to activate this receptor (Caterina *et al*., 1997; Tominaga *et al*., 1998; Vyklicky *et al*., 1998). It has recently been demonstrated that the endogenous cannabinoid,

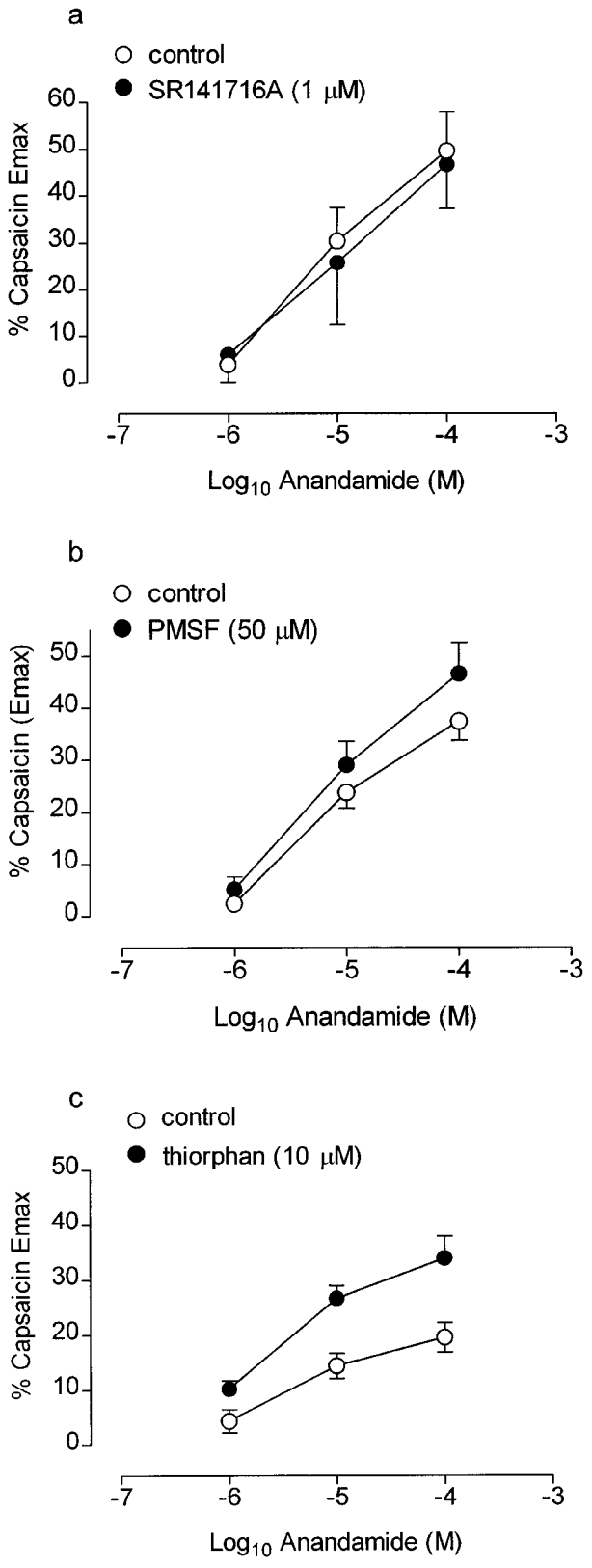

Figure 5 Cumulative concentration effect curves to anandamide in the absence or presence of (a) SR141716A ($n=5$ each), (b) phenylmethylsulphonyl fluoride (PMSF, $n=8$ each) and (c) thiorphan ($n=6$ each). Each point represents the mean and vertical lines represent standard error of the mean.

anandamide, induced vasodilation that was not antagonized by the CB_1 receptor antagonist SR141716A (Wagner *et al*., 1999; Zygmunt *et al*., 1999). Furthermore, anandamide which has structural similarities to a vanilloid receptor agonist, olvanil, provided the impetus to the discovery that this endogenous cannabinoid agonist stimulates the release of neuropeptides from rat blood vessels and activates the cloned vanilloid receptor (VR)-1 receptor (Zygmunt *et al*., 1999; Smart *et al*., 2000). Our findings add further weight to this notion since anandamide stimulated contraction of guinea-pig isolated bronchus by a vanilloid receptor- and neuropeptide-dependent mechanism.

The concentration of anandamide required to elicit activation of the vanilloid receptor in the guinea-pig isolated bronchus was considerably greater than those required to activate CB_1-receptors in mouse vas deferens (Devane *et al*., 1992; Pertwee *et al*., 1995; Lay *et al*., 2000). A number of factors could account for this difference including active metabolism of anandamide by different tissues (Pertwee *et al*., 1995), although we can rule out this possibility since in the presence of the amidase inhibitor PMSF, there was no significant augmentation of the contractile response to anandamide. Furthermore, *R*-(+)-methanandamide which is more resistant to enzymatic degradation (Pertwee *et al*., 1995) was not a more potent contractile agonist compared with anandamide, although it is less active at the vanilloid receptor (Smart *et al*., 2000). Alternatively, significant differences in the pharmacological activity of anandamide at CB_1-receptors has been observed between species (Lay *et al*., 2000). This species difference in activity of anandamide also extends to its effects on vanilloid receptors (Zygmunt *et al*., 1999). We have shown that anandamide is significantly less potent than capsaicin in eliciting contractile responses in guinea-pig isolated bronchus. This is consistent with findings in guinea-pig basilar artery where anandamide is one to two orders of magnitude less potent than capsaicin in mediating relaxation of these vessels (Zygmunt *et al*., 1999) and at least one to two orders of magnitude less potent in activating the vanilloid receptor expressed in HEK293 cells or in rat DRG neurones compared with capsaicin (Zygmunt *et al*., 1999; Smart *et al*., 2000; Hwang *et al*., 2000). However, anandamide appears to behave as a partial agonist in guinea-pig isolated bronchus, consistent with the data observed for activation of vanilloid receptors in rat DRG neurones (Zygmunt *et al*., 1999; Smart *et al*., 2000; Hwang *et al*., 2000), but not it would appear, in rat vasculature (Zygmunt *et al*., 1999) or HEK293 cells expressing the human vanilloid receptor (Smart *et al*., 2000). While it is possible that the actual concentrations of anandamide that are achieved in the biophase may be a limiting factor in some studies, in view of the lipophilicity of this agonist (Smart *et al*., 2000), this is clearly not a significant factor when studying the effects of anandamide at CB_1-receptors in isolated tissues (Pertwee *et al*., 1995; Lay *et al*., 2000). Our data support the view that anandamide is less efficacious than capsaicin in activating the vanilloid receptor (Zygmunt *et al*., 1999; Hwang *et al*., 2000). Furthermore, we show that the contractile response to capsaicin was unaffected by exposure to anandamide, despite producing desensitization to the contractile response to anandamide itself. In contrast, anandamide did induce cross desensitization to capsaicin in HEK293 cells expressing the human vanilloid receptor,

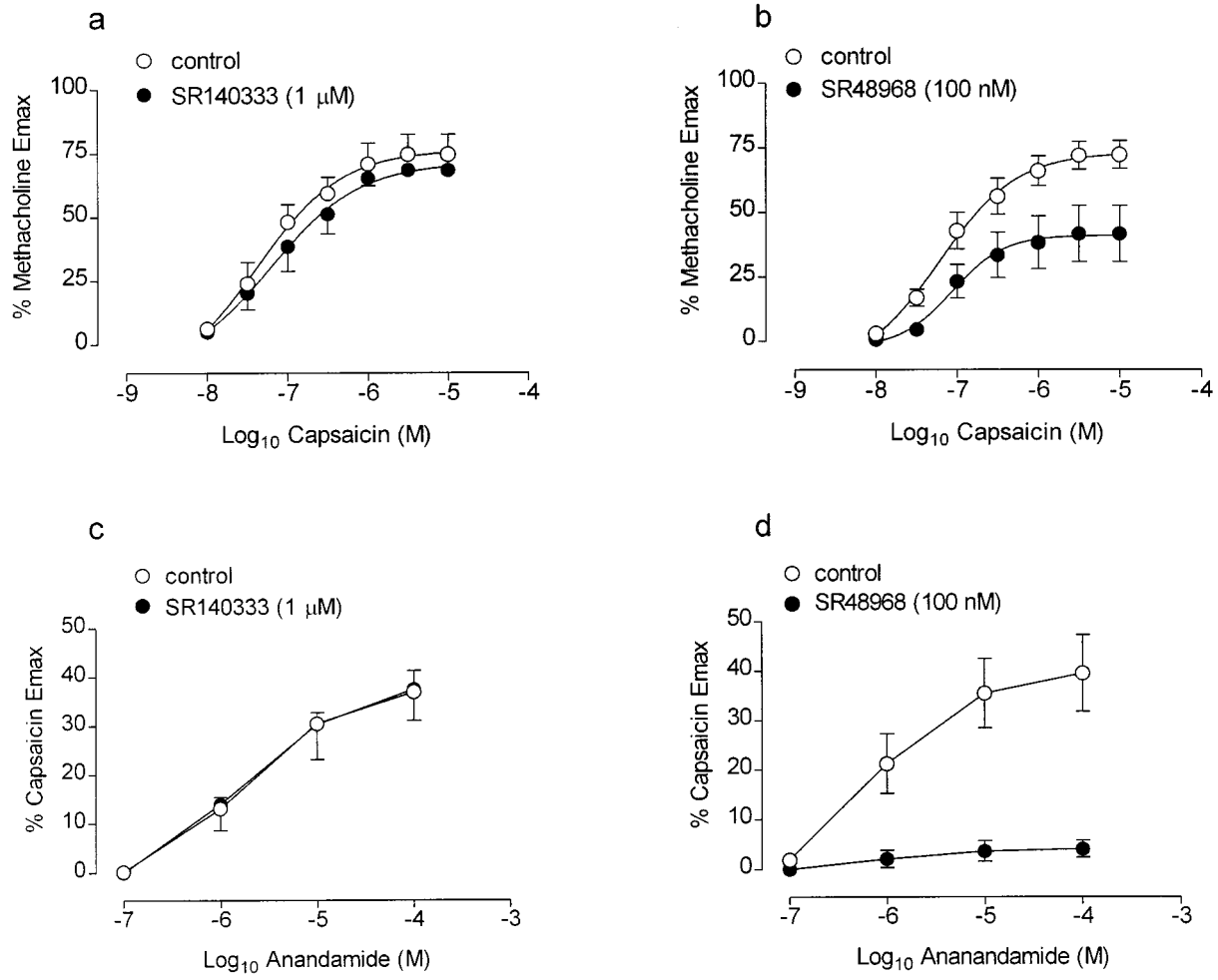

Figure 6 Cumulative concentration effect curves to capsaicin (a,b, $n=5-6$) and anandamide (c,d, $n=6$) in the absence or presence of the NK_1- (a,c) and NK_2- (b,d) selective antagonist, SR140333 (1 μM) and SR48968 (100 nM), respectively. Each point represents the mean and vertical lines represent standard error of the mean.

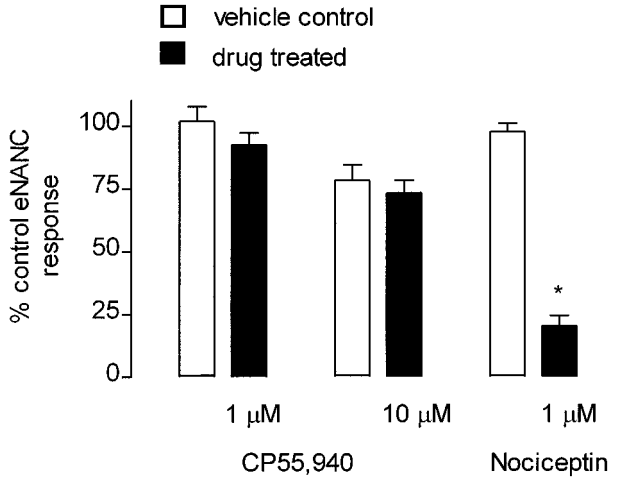

Figure 7 Bar graph representing the eNANC response as a percentage of control values. Tissues were either treated with vehicle or the CB_1-selective agonist CP55,940 (1 μM $n=9$; 10 μM $n=10$) or the ORL_1-receptor agonist, nociceptin ($n=4$). Vertical lines represent standard error of the mean. *$P<0.05$ *c.f.* vehicle control.

although, this was not an unexpected finding in view of the fact that anandamide behaves as a full agonist in this preparation (Smart *et al.*, 2000). It is clear that different stimuli induce the release of different quantities of neuropeptide from the pool of releasable peptide within sensory nerves (Hakanson *et al.*, 1987). Therefore, it is likely that under the current experimental conditions, anandamide is not a sufficient stimulus to promote complete desensitization of the vanilloid receptor and/or deplete airway sensory nerves of their releasable pool of neuropeptides and to suppress the response to a full agonist like capsaicin. Consistent with this view is the observation that compared with capsaicin, the vanilloid receptor agonist olvanil, is less able to induce desensitization to responses evoked by capsaicin in some preparations (Dray *et al.*, 1990; Wardle *et al.*, 1996).

The finding that the vanilloid receptor antagonist, capsazepine, inhibited the contractile response to anandamide is further evidence that this substance activates the vanilloid receptor in this preparation. The apparent $-pK_B$ for capsazepine against capsaicin-induced contraction was 5.85 (approximately 1.4 μM) which is lower than that previously reported in this tissue ($-pK_B=5.12$) (Belvisi *et al.*, 1992) but

higher than that reported in HEK293 cells (7.31) (Smart *et al*., 2000) and rat hepatic arteries (6.24) (Zygmunt *et al*., 1999). The apparent pK_B for capsazepine against anandamide-induced contraction was also greater than that reported in HEK293 and rat hepatic arteries and this may reflect species differences. We also investigated the role of sensory neuropeptides in the contractile response to anandamide following activation of the vanilloid receptor. The NK_2-selective antagonist, SR48968 significantly attenuated the contractile response to capsaicin, while the NK_1-receptor antagonist was without effect, consistent with a previous study (Ellis & Undem, 1994). The combination of NK_1 and NK_2-receptor antagonist resulted in almost complete suppression of the contractile response to capsaicin, thereby implicating a role for both neurokinin A and substance P in the contractile response to capsaicin (Ellis & Undem, 1994). Similarly, the contractile response to anandamide was nearly abolished in the presence of SR48968, a response typical of indirect acting agonists (Black *et al*., 1980) and supports the view that anandamide is less effective than capsaicin in stimulating neuropeptide release in this preparation.

Neuropeptides including substance P and neurokinin A which are released into the airways from sensory nerves are subject to degradation by neutral endopeptidase in this preparation (Maggi *et al*., 1990). The contractile response induced by anandamide in bronchial preparations was augmented by the neutral endopeptidase inhibitor, thiorphan. Together these data are consistent with the view that contraction of guinea-pig bronchial preparations is attributable to the release of neuropeptides following activation of the vanilloid receptor on airway sensory nerves. We can rule out a role for cannabinoid receptors in this response since the synthetic agents, WIN55,212-2 and CP55,940 failed to activate vanilloid receptors expressed in HEK293 cells (Smart *et al*., 2000). Similarly, neither CP55,940 (non-selective) and 2-arachidonylglycerol, recently shown to activate CB_2-receptors (Sugiura *et al*., 2000), did not induce contraction of guinea-pig bronchial preparations, thereby ruling out a role for these receptors in the contractile response to anandamide. Furthermore, we were unable to demonstrate an inhibitory effect of a CB_1-selective antagonist on anandamide-induced contraction in guinea-pig isolated bronchus and we employed a concentration of SR141716A (1 μM) that would be sufficient to effectively antagonise (K_i of 12 nM) responses at CB_1 receptors (Felder *et al*., 1998). Whilst it has recently been shown that high concentrations of SR141716A induced a non-specific relaxation of vascular smooth muscle (White & Hiley, 1998), at the concentration employed in the present study, SR141716A was without an inhibitory effect.

An important area of cannabinoid pharmacology is the role played by these agonists as analgesic agents, and in this regard, there is convincing evidence that endogenous cannabinoids alleviate pain perception *via* stimulation of CB_1 and CB_2 receptors (Calignano *et al*., 1998) and that anandamide inhibits neuropeptide release from spinal cord and rat skin (Richardson *et al*., 1998a,b). The effect of anandamide in the respiratory system is controversial, with one study reporting that intravenous administration of anandamide failed to alter respiratory mechanics in conscious guinea-pigs (Stengel *et al*., 1998). In contrast, anandamide appeared to elicit a dual response in the airways dependent on an intact vagus nerve. Thus, intravenously administered anandamide inhibited bronchoconstriction induced by capsaicin in anaesthetized guinea-pigs, but induced bronchoconstriction in guinea-pigs that had been vagotomized and treated with atropine. Both these effects were mediated *via* a CB_1-receptor dependent mechanism (Calignano *et al*., 2000). The mediators responsible for the bronchoconstrictor response was not identified, but the role of vanilloid receptors was ruled out as capsazepine did not attenuate the bronchoconstrictor response to intravenously administered anandamide. This finding contrasts with reports showing that anandamide stimulates vanilloid receptors (Zygmunt *et al*., 1999; Smart *et al*., 2000; Hwang *et al*., 2000). The inability to document a neuropeptide-dependent bronchoconstrictor response to anandamide *in vivo*, is most likely due to degradation of endogenously release neuropeptides by neural endopeptidase, which would tend to limit the concentration of neuropeptides at the level of airway smooth muscle, as observed in this study.

In further experiments, we were unable to demonstrate that cannabinoid receptors are functionally linked to inhibition of neuropeptide release in guinea-pig bronchial preparations. The synthetic cannabinoid agonist CP55,940 did not significantly attenuate the eNANC contractile response in guinea-pig isolated bronchus an observation consistent with the lack of effect of this agonist on contractile responses elicited by parasympathetic stimulation in the trachea of this species (Spicuzza *et al*., 2000). The concentrations of CP55,940 employed in this study were chosen on the basis that they cause near maximal inhibition of the cholinergic contractile response in guinea-pig small intestine (Pertwee *et al*., 1996). The reasons for this discrepancy remain to be established, although it is clear that there is a paucity of CP55,940 binding sites in rat and guinea-pig airways (Lynn & Herkenham, 1994; Spicuzza *et al*., 2000). However, CB_1-receptor binding sites may be localized to discrete regions within the lung including nerves (Calignano *et al*., 2000) and lymphoid tissues (Lynn & Herkenham, 1994). Furthermore, these receptors may only be expressed on a sub-population of nerves as illustrated by a recent study showing co-expression of cannabinoid receptors on 13% of substance *P* mRNA-containing sensory nerves in the rat dorsal ganglia (Hohmann & Herkenham, 1999). Consistent with this view is the finding that inhibition of the eNANC response in the ileum by cannabinoid agonists was less efficacious and between 10–100-fold less potent compared with the μ-opioid selective agonist D-[Ala^2-,N-methyl-Phe^4,Gly^5-ol]enkephalin, suggesting that CB_1-receptor density and/or distribution on sensory nerves is less than for μ-opioid receptors in this preparation (Izzo *et al*., 1998). Similary, in the present study we have confirmed our previous findings that the ORL_1-receptor agonist, nociceptin inhibited the eNANC response (Shah *et al*., 1998), which serves as a positive control.

It has yet to be established whether endogenous cannabinoids are released in sufficient quantities to activate vanilloid receptors in the airways, although macrophages and neuronal cells are potential sources of this putative endogenous vanilloid receptor agonist (diMarzo *et al*., 1996). However, under the current experimental conditions, the eNANC response was not modified in the presence of the CB_1-selective antagonist, SR141716A suggesting that insufficient

quantities, if any, of anandamide are released under physiological conditions in the airways. However, quantifiable amounts of anandamide have been measured in the periaqueductal gray during an inflammatory insult following subcutaneous injection of formalin into the rat hindpaw (Walker *et al*., 1999), and a significantly lower level of anandamide was detected following removal of calcium from guinea-pig lung membranes (Calignano *et al*., 2000).

In conclusion, we have demonstrated that the endogenous cannabinoid receptor agonist, anandamide, stimulates eNANC contraction secondary to activation of vanilloid receptors on airway sensory nerves. However, unlike capsaicin, it is a modest activator of this receptor in guinea-pig isolated bronchus.

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