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# A possible role of lipoxygenase in the activation of vanilloid receptors by anandamide in the guinea-pig bronchus

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**1** In the absence of indomethacin, anandamide did not contract the guinea-pig bronchus at concentrations up to 100 $\mu$M. In the presence of indomethacin (10 $\mu$M), anandamide induced concentration-related contractions with a $pEC_{50}$ value of $5.18 \pm 0.11$. It was significantly less potent than capsaicin ($pEC_{50}$ $7.01 \pm 0.1$). The anandamide uptake inhibitor AM404, produced only a $14.1 \pm 3.22\%$ contraction at 100 $\mu$M. All experiments were conducted in the presence of PMSF (20 $\mu$M).

**2** The vanilloid receptor antagonist, capsazepine (10 $\mu$M), significantly attenuated the contractile effect of anandamide, the response to 100 $\mu$M anandamide being $40.53 \pm 7.04\%$ in the presence of vehicle and $1.57 \pm 8.93\%$ in the presence of 10 $\mu$M capsazepine. The contractile actions of anandamide and AM404 were markedly enhanced by the peptidase inhibitor thiorphan.

**3** The log concentration-response curve of anandamide was unaltered by the $CB_1$ receptor antagonist, SR141716A. The $pEC_{50}$ values for anandamide were $4.88 \pm 0.08$ and $5.17 \pm 0.19$ in the presence of vehicle and SR141716A (1 $\mu$M) respectively.

**4** The lipoxygenase inhibitors 5,8,11,14-eicosatetraynoic acid (ETYA) and 5,8,11 eicosatriynoic acid (ETI) reduced the effect of 100 $\mu$M anandamide from $34.7 \pm 1.9\%$ (vehicle) to $7.7 \pm 5\%$ (ETYA, 10 $\mu$M) and from $41.85 \pm 4.25\%$ ($n=6$) (vehicle) to $10.31 \pm 3.54$ ($n=6$) (ETI, 20 $\mu$M). Neither inhibitor significantly affected contraction of the tissue by substance P.

**5** This study provides evidence that anandamide acts on vanilloid receptors in the guinea-pig isolated bronchus. These data raise the possibility that the contractile action of anandamide may be due, at least in part, to lipoxygenase metabolites of this fatty acid amide that are vanilloid receptor agonists.

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**Keywords:** Anandamide; bronchus; vanilloid; cannabinoid; lipoxygenase; cyclooxygenase; ETYA; ETI

**Abbreviations:** AM404, (4-hydroxyphenyl arachidonylamide); Anandamide, (arachidonyl ethanolamide); Capsaicin, (3-methoxy-4-hydroxybenzyl-8-methyl-6-nonenamide); $CB_1$, cannabinoid receptor; CZP, capsazepine; DMSO, dimethylsulphoxide; ETI, 5,8,11-eicosatriynoic acid; ETYA, 5,8,11,14-eicosatetraynoic acid; PMSF, phenylmethylsulphonyl fluoride; VR1, vanilloid receptor

## Introduction

There is mounting evidence that anandamide, and its analogue AM404, can activate vanilloid receptors, both in cells transfected with the VR1 receptor (Smart *et al*., 2000; Jerman *et al*., 2000, Ross *et al*., 2001) and in tissues which natively express this receptor (Zygmunt *et al*., 1999; 2000). These studies have shown that AM404, the anandamide transport inhibitor is significantly more potent than anandamide at vanilloid receptors. Capsaicin is known to produce a vanilloid receptor-mediated contractile response in isolated guinea-pig bronchi by releasing sensory neuropeptides (Holzer, 1991). A recent report demonstrated that anandamide induces capsazepine-sensitive contractions of the guinea-pig bronchus (Spina *et al*., 2000). However, there is also evidence of a $CB_1$ receptor mediated contractile action of anandamide in guinea-pig isolated lung parenchyma (Calignano *et al*., 2000). Studies in the isolated pawskin have suggested that anandamide may act on $CB_1$ receptors to attenuate capsaicin-mediated CGRP release from sensory nerves (Richardson *et al*., 1986).

There is evidence that vanilloid receptors may be activated by endogenous products of lipoxygenases (Hwang *et al*., 2000). These compounds include 12- and 15-hydroperoxyeicosatetraenoic acids (HPETE) and 5- and 15-hydroxyeicosatetraenoic acids (HETE) and leukotriene $B_4$. It has also been shown that lipoxygenase products, including lipoxin $A_4$, may be mediators of the excitatory effects of arachidonic acid on capsaicin-sensitive sensory nerves in the guinea-pig bronchus (Manzini & Meini, 1991). These effects were only observed in the presence of the cyclo-oxygenase inhibitor, indomethacin. Some studies have demonstrated lipoxygenase mediated hydroxylations of anandamide analogous to those observed for arachidonic acid (Burstein *et al*., 2000). Thus, it is possible that such compounds may be implicated in the contractile action of anandamide in the guinea-pig bronchus. In this study we investigate the nature of the receptors mediating contraction of the guinea-pig bronchus by anandamide and AM404, and the effects of inhibition of cyclo-oxygenase and lipoxygenase on this action.

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## Methods

### *Drugs and chemicals*

Anandamide (arachidonyl ethanolamide), was obtained from Biomol research laboratories. AM404 (4-hydroxyphenyl arachidonyl amide), capsaicin, capsazepine, and (+)-WIN55212 [(*R*)-(+)-[2,3-dihydro-5-methyl-3-(4-morpholinyl-methyl)pyrrolo-[1,2,3-de]-1,4-benzoxazin-6-yl]-1-naphthale-nyl-methanonemesylate] were obtained from Tocris and SR141716A [N-(piperidin-1-yl)-5-(4-chlorophenyl)-1-(2,4-di-chlorophenyl)-4-methyl-1H-pyrazole-3-carboxamide hydro-chloride] from Sanofi Recherche. Bovine serum albumin (BSA), cell culture media, non-enzymatic cell dissociation solution, 5,8,11-eicosatriynoic acid (ETI), 5,8,11,14-eicosate-traynoic acid (ETYA), G418, L-glutamine, Krebs salts, penicillin with streptomycin, thiorphan, phenylmethylsulpho-nyl fluoride (PMSF), and Triton X-100 were all obtained from Sigma-Aldrich. $^{45}Ca^{2+}$ (5–50 mCi mg$^{-1}$ calcium) were obtained from Amersham Pharmacia Biotech (U.K.). Rat VR1 transfected CHO cells were a gift from Novartis, London.

### *$^{45}Ca^{2+}$ uptake experiments*

rVR1 transfected CHO cells were maintained in MEM alpha minus media containing 2 mM L-glutamine supplemented with 10% hyclone foetal bovine serum, 350 $\mu$g ml$^{-1}$ G418, 100 u ml$^{-1}$ penicillin and 100 $\mu$g ml$^{-1}$ streptomycin. Cells were maintained in 5% $CO_2$ at 37°C. For $^{45}Ca^{2+}$ uptake assay, cells were plated in 24-well plates at $5\times10^5$ cells ml$^{-1}$. Cells were incubated for 10 min at 37°C with 100 $\mu$l of anandamide and 1 $\mu$Ci $^{45}Ca^{2+}$ in a total volume of 1 ml of assay buffer (Minimum Essential Medium containing 0.25 mg ml$^{-1}$ BSA). A basal stimulation was measured in the presence of the appropriate vehicle equivalent. The cells were incubated with PMSF (100 $\mu$M) and either vehicle or ETYA (10 $\mu$M) for 20 min at 37°C, prior to the addition of anandamide. Following incubation, plates were placed on ice and washed three times with ice-cold assay buffer. Cells were incubated with assay buffer containing 0.5% Triton X-100 at 45°C for 20 min, before a 200 $\mu$l aliquot was removed for scintillation counting. The counts per min above basal for each datum point were expressed as a percentage of the response to 1 nM resiniferatoxin.

### *Guinea-pig bronchus*

Bronchus and lower trachea were obtained from Dunkin-Hartley guinea-pigs weighing 300–800 g. Animals were stunned, exsanguinated and the lungs with attached bronchi and trachea were quickly removed. The main bronchi and lower 1 cm of trachea were dissected and 3 mm rings prepared. Each ring was mounted in a 4 ml organ bath at an initial tension of 0.5 g. The baths contained Krebs solution which was kept at 37°C and bubbled with 95% $O_2$ and 5% $CO_2$. The composition of the Krebs solution was (mM): $MgSO_4.7H_2O$ 1.29, NaCl 118.2, KCl 4.75, $KH_2PO_4$ 1.19, $NaHCO_3$ 25.0, glucose 11.0, $CaCl_2$ $6H_2O$ 2.54. It also contained 10 $\mu$M indomethacin (unless otherwise stated). Contractions were monitored by computer (Apple Macintosh LCIII and Performa 475) using a data recording and analysis system (MacLab) that was linked to either UF1 transducers (Pioden Controls) or Model 1030 transducers (UFI, CA, U.S.A.). All agonist additions were made cumulatively without washout in a volume of 10 $\mu$l. Top stocks of drugs were 10 mM in dimethylsulphoxide (DMSO), except indo-methacin which was a 20 mg ml$^{-1}$ stock in ethanol. In control experiments, DMSO was added instead of agonist or antagonist and it had no contractile action when added alone ($n=6$, data not shown). Antagonists were added 30 min prior to the addition of agonists. Similarly, enzyme inhibitors were added 30 min (PMSF and ETYA) or 1 h (ETI) prior to the addition of agonists. In all experiments with anandamide and AM404 (unless otherwise stated), the tissues were preincu-bated with 20 $\mu$M PMSF for 30 min prior to the addition of the cannabinoid.

### *Analysis of data*

Peak contractions were calculated as a percentage of the contraction induced by 100 $\mu$M histamine added at the end of each experiment. Values have been expressed as means and variability as s.e.mean or as 95% confidence limits. The values for $pEC_{50}$ ($-\log EC_{50}$) are calculated from the effective concentration producing 50% of the maximum response inducible by that compound. $EC_{50}$ and maximal effects ($E_{max}$) and the s.e.mean or 95% confidence limits of these values have been calculated by non-linear regression analysis using the equation for a sigmoid concentration-response curve (Graph-Pad Prism). $K_B$ values for capsazepine and its 95% confidence limits were determined by symmetrical (2+2) dose parallel line assays (see Ross *et al*., 2001). This method was also used to determine whether log concentration-response plots deviated significantly from parallelism.

## Results

### *Log concentration-responses curves*

In some tissues anandamide is rapidly metabolised to arachidonic acid and ethanolamide by the action of fatty acid amide hydrolase (FAAH), this enzyme can be inhibited by phenylmethyl sulphonyl fluoride (PMSF). Unless other-wise stated all the experiments with anandamide were carried out in the presence of 20 $\mu$M PMSF.

### *Indomethacin present*

In the presence of indomethacin, capsaicin and anandamide (20 $\mu$M PMSF) induced concentration-related contractions of the isolated guinea-pig bronchus with $pEC_{50}$ values of $7.01\pm0.01$ ($n=10$) and $5.18\pm0.11$ ($n=9$) respectively. The maximal contraction of $37.02\pm3.91$% induced by ananda-mide was significantly less ($P<0.01$, unpaired *t*-test) than that of $74.81\pm3.69$% which was obtained with capsaicin. In the absence of PMSF the $pEC_{50}$ for anandamide was significantly lower ($4.15\pm0.64$, $n=4$; $P<0.05$, unpaired *t*-test) (Figure 1). The rate of onset of the contractions by anandamide was slow ($26\pm2$ min to reach a plateau, $n=12$) in comparison to capsaicin ($5.42\pm0.34$, $n=19$). The anandamide uptake inhibitor, AM404, has previously been shown to be a full agonist at VR1 receptors (Jerman *et al*.,

2000; Zygmunt *et al.*, 2000). However, AM404 had little effect in the bronchus, producing a maximum contraction of $14.15 \pm 3.22\%$ ($n=7$) at 100 $\mu$M (20 $\mu$M PMSF), which was not significantly different from the baseline tension ($P>0.05$, unpaired $t$-test).

### *Indomethacin absent*

In this series of experiments the effect of capsaicin and anandamide were compared in the presence of 10 $\mu$M indomethacin or the vehicle equivalent (Figure 2). The $pEC_{50}$ and $E_{max}$ values were not significantly different ($P>0.05$, unpaired $t$-test) for capsaicin in the presence of vehicle or indomethacin (10 $\mu$M). The $pEC_{50}$ values were $7.33 \pm 0.08$ (vehicle) and $7.19 \pm 0.04$ (indomethacin), and the $E_{max}$ values were $74.18 \pm 5.01$ (vehicle) and $82.30 \pm 6.31$ (indomethacin). In the absence of indomethacin (vehicle equivalent present), anandamide (20 $\mu$M PMSF) did not contract the tissue, a concentration of 100 $\mu$M produced a relaxation of the tissue of $23.8 \pm 8.8\%$ ($n=6$).

### *Effect of SR141716A*

The $CB_1$ receptor antagonist, SR141716A (1 $\mu$M) did not significantly affect the contractions induced by capsaicin (Figure 3a), the $pEC_{50}$ values being $7.39 \pm 0.12$ and $7.42 \pm 0.12$ ($P>0.05$, unpaired $t$-test, $n=5$) in the presence of vehicle and SR141716A respectively. Similarly, the contractions induced by anandamide were unaffected by the $CB_1$ receptor antagonist (Figure 3b), the $pEC_{50}$ values being $4.88 \pm 0.08$ and $5.17 \pm 0.19$ ($P>0.05$, unpaired $t$-test, $n=6$) in the presence of vehicle and SR141716A respectively.

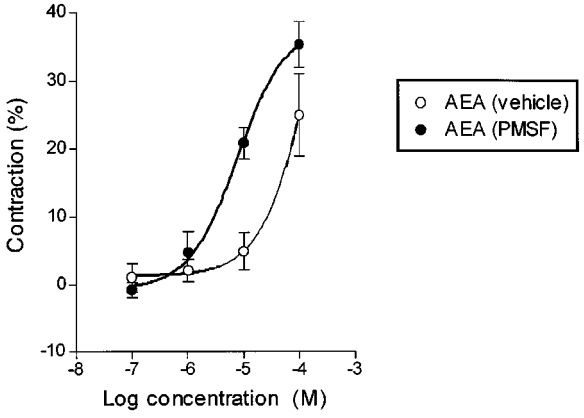


**Figure 1** Log concentration-response curve for contraction of the guinea-pig isolated bronchus by anandamide in the presence of either PMSF vehicle or 20 $\mu$M PMSF. The experiments were carried out in the presence of indomethacin (10 $\mu$M). Each symbol represents the contraction calculated as a percentage of the maximum contraction induced by 100 $\mu$M histamine $\pm$ s.e.mean.

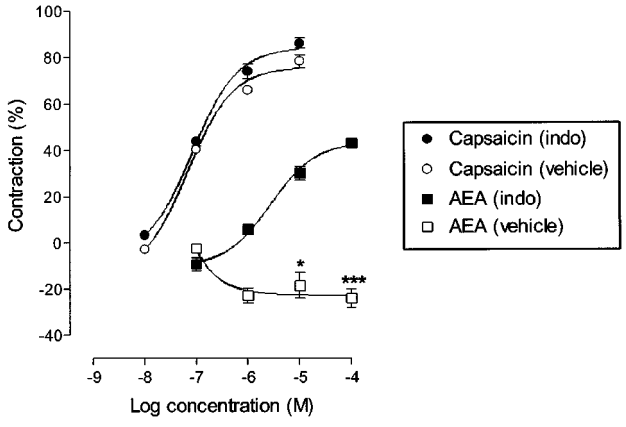


**Figure 2** Log concentration-response curves for contraction of the isolated guinea-pig bronchus by capsaicin and anandamide (20 $\mu$M PMSF) in the presence of either indomethacin vehicle or indomethacin (10 $\mu$M). Each symbol represents the contraction calculated as a percentage of the maximum contraction induced by 100 $\mu$M histamine $\pm$ s.e.mean. The action of anandamide was significantly attenuated by indomethacin, $***P<0.001$, $*P<0.05$, Student's unpaired $t$-test.

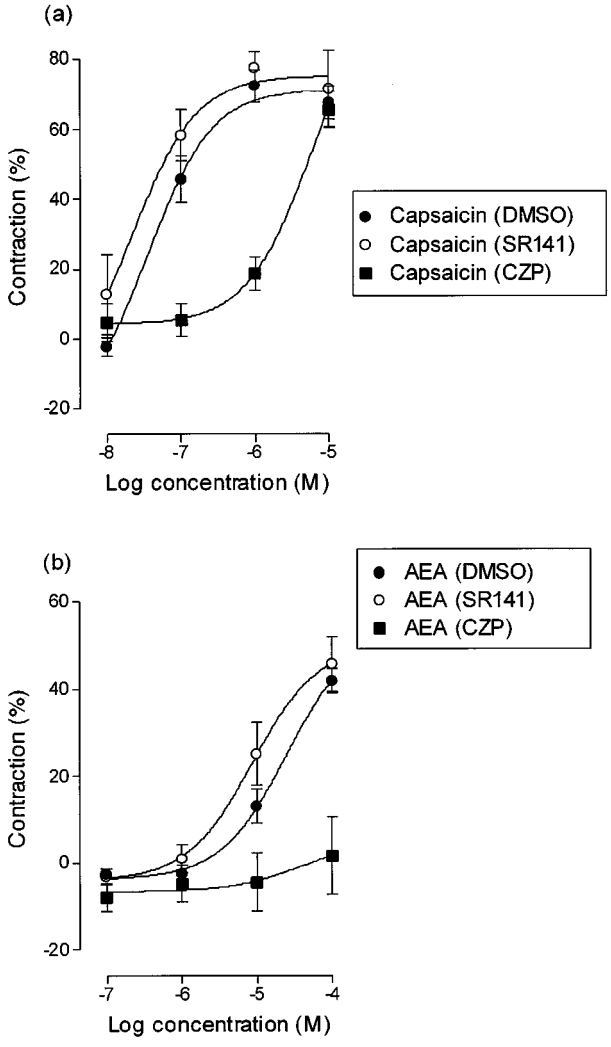


**Figure 3** Log concentration-response curves for contraction of the guinea-pig isolated bronchus by (a) capsaicin in the presence of vehicle, SR141716A (SR141, 1 $\mu$M) and capsazepine (CZP, 10 $\mu$M) or (b) by anandamide (with 20 $\mu$M PMSF) in the presence of vehicle, SR141716A (1 $\mu$M) and capsazepine (10 $\mu$M). The experiments were carried out in the presence of indomethacin (10 $\mu$M). Each symbol represents the contraction calculated as a percentage of the maximum contraction induced by 100 $\mu$M histamine $\pm$ s.e.mean.

*Effect of cannabinoid receptor agonist, CP55940*

The cannabinoid receptor ligand, CP55940 failed to induce contractions of the tissue at concentrations of 10 nM – 10 $\mu$M. Ten micro molar produced a relaxation of the tissue of $4.68 \pm 5.64\%$ ($n=8$), which was not significantly different from the baseline values. Pre-treatment of the tissue with CP55940 caused a slight rightward shift in the log concentration response curve to capsaicin, but the $pEC_{50}$ values were not significantly different, being $7.15 \pm 0.17$ in the presence of vehicle and $6.88 \pm 0.12$ in the presence of 100 nM CP55940 ($P > 0.05$, unpaired $t$-test, $n=5$).

*Effect of capsazepine*

The VR1 receptor antagonist, capsazepine (10 $\mu$M) induced a parallel rightward shift in the log concentration-response curve of capsaicin (Figure 3a), the $pEC_{50}$ values being $7.39 \pm 0.12$ and $5.35 \pm 0.16$ ($n=5$) in the presence of vehicle and capsazepine respectively. The $pK_B$ value for capsazepine was 6.62 (95% confidence limits 6.84 – 6.37). At the same concentration, capsazepine significantly inhibited the contractions induced by anandamide (Figure 3b). The response to 100 $\mu$M was $40.53 \pm 7.04\%$ in the presence of vehicle and $1.57 \pm 8.93\%$ in the presence of 10 $\mu$M capsazepine ($P < 0.01$, unpaired $t$-test, $n=6$). Measurement of $K_B$ values for capsazepine against anandamide was not possible because the low potency of anandamide would require using inappropriately high concentrations at which this compound may have non-specific effects.

*Desensitization experiments*

In these experiments, tissues were exposed to a 10 $\mu$M concentration of capsaicin and left for 2 h, at which point the contraction had returned to baseline. After desensitization of the tissues with 10 $\mu$M capsaicin, 100 $\mu$M anandamide produced a relaxation of $8.64 \pm 2.84\%$ ($n=6$), which was not significantly different from the baseline values ($P > 0.05$, unpaired $t$-test). Desensitized tissues were unresponsive to capsaicin (data not shown).

*Effect of thiorphan*

Thiorphan is a peptidase inhibitor, which has been shown to markedly enhance the response to capsaicin. The response to 100 $\mu$M anandamide in the isolated bronchus was significantly enhanced ($P < 0.01$, unpaired $t$-test) when thiorphan (10 $\mu$M) was present, contractions being $29.92 \pm 9.2$ ($n=4$) and $62.36 \pm 12.2$ ($n=4$) in the presence of vehicle and thiorphan respectively (Figure 4c). The response to AM404 was significantly ($P < 0.05$, unpaired $t$-test) enhanced by thiorphan from $2.82 \pm 5.86$ (vehicle) to $33.13 \pm 10.7$ ($n=4$) (thiorphan) (Figure 4b).

*Effect of SC-51089*

SC51089 is an $EP_1$ receptor antagonist with a $pA_2$ value of 6.5 (Hallinan *et al*, 1993). In the guinea-pig bronchus this compound markedly attenuated the contractile action of $PGE_2$. 100 nM $PGE_2$ elicited a contraction of $51.9 \pm 5.66\%$ ($n=5$) in the presence of vehicle and $4.55 \pm 6.31\%$ ($n=5$) in the presence of the SC51089 (10 $\mu$M). The contractile effect of anandamide in this tissue was unaffected by SC51089. One hundred micro molar anandamide induced a $43.53 \pm 5.91\%$ ($n=4$) contraction in the presence of vehicle and $42.40 \pm 7.81\%$ ($n=4$) in the presence of the antagonist (10 $\mu$M) (Figure 4c).

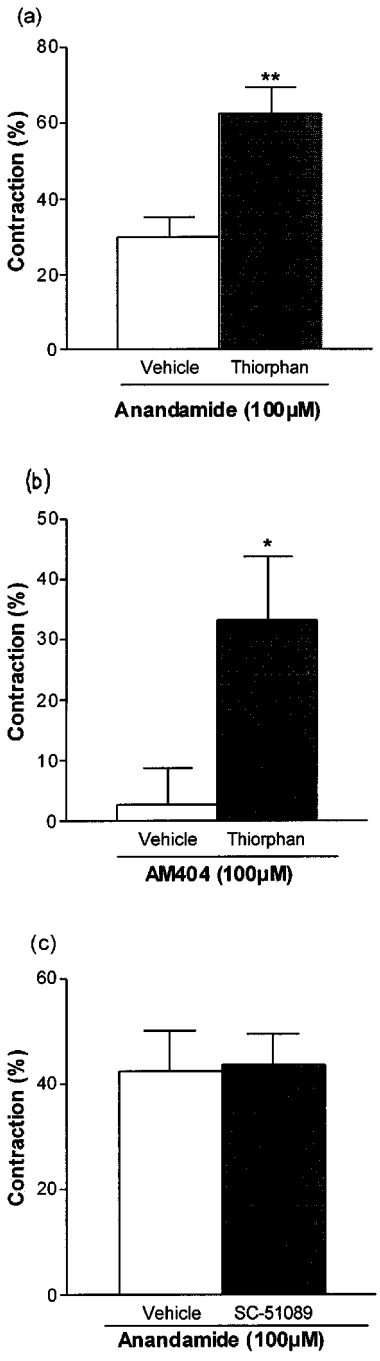


**Figure 4** Contraction of the guinea-pig bronchus by (a) 100 $\mu$M anandamide (20 $\mu$M PMSF) in the presence of vehicle or thiorphan (10 $\mu$M), (b) 100 $\mu$M AM404 (20 $\mu$M PMSF) in the presence of vehicle or thiorphan (10 $\mu$M) and (c) 100 $\mu$M anandamide in the presence of vehicle or the $EP_1$ receptor antagonist, SC-51089 (10 $\mu$M). The experiments were carried out in the presence of indomethacin (10 $\mu$M). The bars represent the contraction calculated as a percentage of the maximum contraction induced by 100 $\mu$M histamine $\pm$ s.e.mean. Anandamide and AM404 were significantly enhanced by thiorphan, $^{**}P < 0.01$, $^{*}P < 0.05$, Student's unpaired $t$-test.

### *Effect of ETYA*

ETYA is an inhibitor of all lipoxygenase and cyclo-oxygenase enzymes (Tobias & Hamilton, 1979) with $ID_{50}$ values of 12 and 4 $\mu$M respectively. A 30-min pre-treatment with the ETYA (10 $\mu$M) did not affect the contractile response to substance P (Figure 5a). However, it did produce a modest (Figure 5b), but significant, attenuation of the $pEC_{50}$ values for capsaicin ($P<0.05$, unpaired $t$-test), but the $E_{max}$ values were not significantly different ($P>0.05$, unpaired $t$-test). The $pEC_{50}$ and $E_{max}$ values were $7.16\pm0.05$ and $71.48\pm3.14\%$ ($n=5$) in the presence of vehicle and $6.66\pm0.07$ and $64.36\pm5.81\%$ ($n=7$) in the presence of ETYA. After treatment with ETYA the response to 100 $\mu$M anandamide (Figure 5c) was significantly reduced from $34.74\pm1.9\%$ in the presence of vehicle to $7.68\pm5\%$ ($P<0.01$, unpaired $t$-test, $n=7$) in the presence of the enzyme inhibitor. ETYA did not significantly affect the maximum contraction due to histamine (data not shown). $^{45}Ca^{2+}$ uptake experiments in rVR1 transfected cells were carried out to check whether this compound has a direct action on VR1 receptors. In rVR1 transfected CHO cells, pretreatment with ETYA did not affect the basal levels of $^{45}Ca^{2+}$ uptake and had no significant effect on the log concentration-response curve for stimulation of $^{45}Ca^{2+}$ uptake by anandamide (Figure 5d).

### *Effect of ETI*

ETI is a non-selective inhibitor of lipoxygenasese (Hammarstrom, 1977) with an $ID_{50}$ of 24 $\mu$M. A 1 h incubation with 20 $\mu$M ETI caused a rightward shift in the log concentration-response curve for anandamide (Figure 6b). The responses to 10, 30 and 100 $\mu$M anandamide were significantly reduced ($P<0.001$, unpaired $t$-test) from $21.32\pm2.74\%$, $40.33\pm4.55\%$ and $41.85\pm4.25\%$ to $-4.80\pm3.86\%$, $5.82\pm5.57\%$ and $10.31\pm3.54\%$ after pretreatment with ETI. ETI had no effect on the contractile action of substance P in this tissue (Figure 6a).

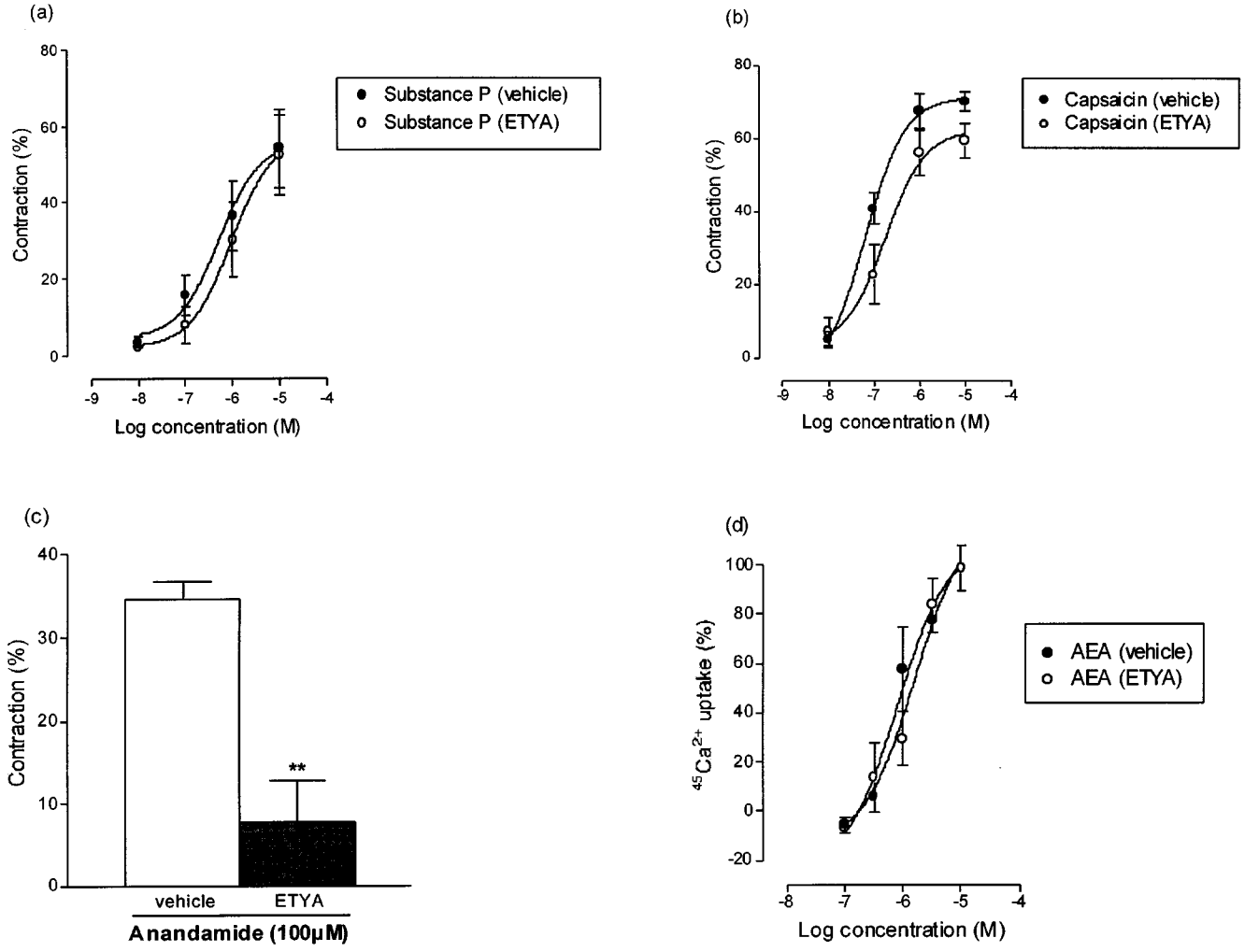


**Figure 5** The effects of the lipoxygenase and cyclooxygenase inhibitor, ETYA (10 $\mu$M) on: (a) the contractile action of substance P in the bronchus, (b) the contractile action of capsaicin in the bronchus, (c) the contractile action of 100 $\mu$M anandamide (20 $\mu$M PMSF) in the bronchus and (d) the stimulation of $^{45}Ca^{2+}$ uptake in VR1 transfected CHO cells by anandamide. The symbols/bars represent the contraction calculated as a percentage of either the maximum contraction induced by 100 $\mu$M histamine (bronchus) or the maximum $^{45}Ca^{2+}$ uptake by 10 nM RTX (VR1 transfected cells)$\pm$s.e.mean. The contractile action of anandamide was significantly attenuated by ETYA $^{**}P<0.01$, Student's unpaired $t$-test.

## Discussion

The data presented confirm the findings of Spina *et al.* (2000) that the contractile action of anandamide in the isolated guinea-pig bronchus is mediated by vanilloid receptors. We have extended these findings to investigate the role of cyclo-oxygenase and lipoxygenase in the contractile action of anandamide. Evidence for vanilloid receptor activation by anandamide is threefold. Firstly, the log concentration-response curve of anandamide is shifted markedly to the right in the presence of the vanilloid receptor antagonist, capsazepine. Secondly, anandamide is inactive in tissues in which the vanilloid receptors have been desensitized by pre-treatment with capsaicin. Thirdly, the contractile action of anandamide is markedly enhanced by the peptidase inhibitor thiorphan, thus implicating the release of neuropeptides in the contractile action of the endogenous cannabinoid. We found no evidence of a $CB_1$ receptor mediated action in the isolated guinea-pig bronchus. The $CB_1$ receptor antagonist, SR141716A, did not shift the log-concentration response curve of anandamide to the right. In addition, the $CB_1/CB_2$ receptor agonist CP55940 did not contract this preparation. During the preparation of this paper Tucker *et al.* (2001) have also reported results implicating the vanilloid receptor in the contractile action of anandamide in the guinea-pig bronchus.

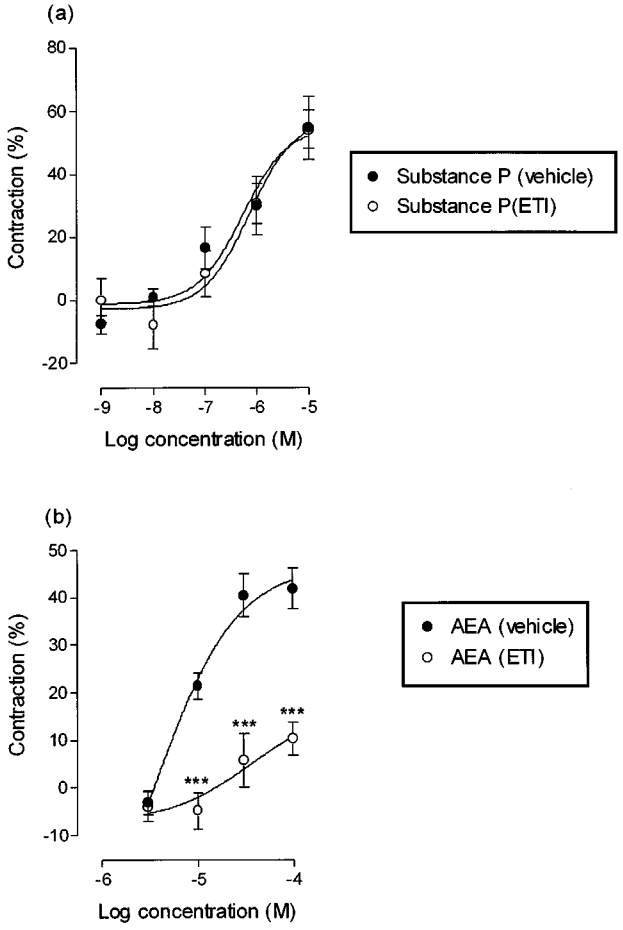


**Figure 6** The effect of the lipoxygenase inhibitor, ETI (20 $\mu$M) on the log concentration-response curves for (a) substance P and (b) anandamide (20 $\mu$M PMSF) in the guinea-pig bronchus. The experiments were carried out in the presence of indomethacin (10 $\mu$M). The symbols/bars represent the contraction calculated as a percentage of the maximum contraction induced by 100 $\mu$M histamine $\pm$ s.e.mean. The contractile action of anandamide was significantly attenuated by ETI, $^{***}P<0.001$, Student's unpaired *t*-test.

It is notable that Richardson *et al.* (1998a) found that anandamide attenuated capsaicin-evoked neuropeptide release from spinal cord of hyperalgesic animals in an SR141716A-sensitive manner. In the bronchus however, there is no evidence of $CB_1$ receptors on primary afferent fibres as CP55940 did not significantly attenuate the contractile action of capsaicin. Finally, we have also excluded the possibility that anandamide may be acting on $EP_1$ prostanoid receptors to contract the bronchus, the $EP_1$ receptor antagonist, SC-51089 having no effect on the contractile action of anandamide.

The lipoxygenase and cyclo-oxygenase inhibitor ETYA, has been shown to inhibit the contraction of the guinea-pig trachea induced by arachidonic acid (Mitchell, 1982). The inhibition of the contractile action of anandamide by ETYA suggests that the action of anandamide may be due, at least in part, to lipoxygenase metabolites of this fatty acid amide that are vanilloid receptor agonists. A direct action of ETYA on vanilloid receptors is unlikely as it had no effect either alone or on the $^{45}Ca^{2+}$ uptake stimulated by anandamide in rVR1 transfected cells. A second lipoxygenase inhibitor, ETI also markedly attenuated the contractile action of anandamide in this tissue. It is important to note that both the lipoxygenase inhibitors used may have non-selective actions and the possibility remains that the inhibition of anandamide-induced contraction of this tissue by ETI and ETYA is not due to prevention of the formation of lipoxygenase products. A non-specific effect of ETYA or ETI seems unlikely in that neither of these compounds altered the contractile action of substance P or histamine in this preparation. However, both substance P and neurokinin A are released from capsaicin-sensitive nerves in the bronchus (Maggi, 1995) and it is possible that the lipoxygenase inhibitors may have a non-specific effect on the contractile action of neurokinin A.

Thus our data suggest that, in this tissue, anandamide may be metabolized to hydroperoxyeicosatetraenoyl ethanolamides and lipoxin ethanolamides that, like the hydroperoxy-derivatives of arachidonic acid (HPETEs) and lipoxin $A_4$, may be vanilloid receptor agonists. Earlier studies have suggested that the presence of the cyclo-oxygenase inhibitor, indomethacin, may encourage arachidonic acid to pass through the lipoxygenase pathway, and aspirin has been shown to trigger the biosynthesis of lipoxins (Serhan, 1997). Such biosynthetic processes may also be applicable to anandamide. It is notable that the rate of onset of the vanilloid receptor mediated action of anandamide (26 min) in this tissue was markedly slower than that previously observed (5 min) in the mouse vas deferens (Ross, *et al.*, 2001). This may be indicative of the conversion of anandamide to active metabolites in the guinea-pig bronchus. In this study we found that, similar to earlier findings with arachidonic acid, anandamide did not contract the tissue in the absence of indomethacin. The contractile action of capsaicin was unaffected by the exclusion of the cyclo-oxygenase inhibitor.

In the absence of indomethacin, anandamide appears to cause a relaxation of the tissue, the nature of this effect and the receptors involved is the subject of ongoing investigations. It is, of course, possible that anandamide is being metabolized to arachidonic acid which, in turn, is metabolised to activators of vanilloid receptors. However, this appears to be unlikely because inhibition of FAAH metabolism of anandamide by PMSF caused a modest enhancement of the contractile action of anandamide.

It would appear that ETYA also attenuates the contractile action of capsaicin, although the effect was modest in comparison to that of the lipoxygenase inhibitor on anandamide. This raises the possibility that the increase in intracellular calcium caused by VR1 receptor activation leads to the release of arachidonic acid and/or anandamide, whose hydroxylation by lipoxygenase may lead to the formation of compounds which are themselves vanilloid receptor agonists.

We have extended investigations in the bronchus to study the anandamide uptake inhibitor AM404. It is surprising that, in this tissue, AM404 is significantly less potent than anandamide. In a number of previous investigations AM404 has been shown to be significantly more potent as a vanilloid receptor agonist than anandamide (Jerman *et al*., 2000; Zygmunt *et al*., 2000; Ross *et al*., 2001). In the bronchus, the fact that thiorphan enhances the contractile action of AM404 implicates vanilloid receptor activation in the contractile action of this compound. However, the fact that anandamide is significantly more potent than AM404 in the guinea-pig bronchus preparation is further evidence that metabolism to active products may amplify its contractile action. Recent findings suggest that both anandamide and AM404 may have low efficacy at the VR1 receptor (Ross *et al*., 2001). The low potency of anandamide and AM404 at vanilloid receptors in this study is in line with this hypothesis and may reflect a low vanilloid receptor reserve in the guinea-pig bronchus. There are numerous examples of the potency/$E_{max}$ of a low efficacy agonist being markedly affected by receptor reserve differences between native tissues and high expression transfected cell lines. In addition, there is evidence of considerable species variability in vanilloid receptor pharmacology (Szallasi & Blumberg, 1999). Such differences may account for the low potency of AM404 and anandamide at VR1 receptors in the guinea-pig bronchus as compared with that observed in cells transfected with the rat and human VR1 receptor.

The agonist action of anandamide in this tissue is only evident at high concentrations (10 and 100 $\mu$M). The physiological relevance of the activation of VR1 receptors by anandamide is the subject of debate. There is, however, evidence that the ligand binding site on the vanilloid receptor may be intracellular (Jung *et al*., 1999). The release of anandamide at or near this binding site, and indeed its rapid hydroxylation to HPETE ethanolamides and other lipoxygenase products, may be a physiologically relevant means of vanilloid receptor activation.

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## References


BURSTEIN, S.H., ROSSETTI, R.G., YAGEN, B. & ZURIER, R.B. (2000). Oxidative metabolism of anandamide. *Prostaglandins and other Lipid Mediators,* **61,** 29–41.

CALIGNANO, A., KATONA, I., DESARNAUD, F., GIUFFRIDA, A., LA RANA, G., MACKIE, K., FREUND, T.F. & PIOMELLI, D. (2000). Bidirectional control of airway responsiveness by endogenous cannabinoids. *Nature,* **408,** 96–101.

HALLINAN, E.A., HAGEN, T.J., HUSA, R.K., TSYMBALOV, S., RAO, S.N., VANHOECK, J.P., RAFFERTY, M.F., STAPELFELD, A., SAVAGE, M.A. & REICHMAN, M. (1993). N-substituted dibenzoxazepines as analgesic $PGE_2$ antagonists. *J. Med. Chem.,* **36,** 3293–3299.

HAMMARSTROM, S. (1977). Selective inhibition of platelet n-8 lipoxygenase by 5,8,11-eicosatriynoic acid. *Biochim. Biophys. Acta.,* **487,** 517–591.

HOLZER, P. (1991). Capsaicin: cellular targets, mechanisms of action, and selectivity for thin sensory neurones. *Pharmacol. Rev.,* **43,** 143–201.

HWANG, S.W., HAWOON, C., KWAK, J., LEE, S.-Y., KANG, C.-J., JUNG, J., CHO, S., MIN, K.H., SUH, Y.-G., KIM, G. & OH, U. (2000). Direct activation of capsaicin receptors by products of lipoxygenase: endogenous capsaicin-like substances. *Proc. Nat. Acad. Sci. (U.S.A.),* **97,** 6155–6160.

JERMAN, J.C., BROUGH, S.J., DAVIS, J.B., MIDDLEMISS, D.N. & SMART, D. (2000). The anandamide transport inhibitor AM404 is an agonist at the rat vanilloid receptor (VR1). *Br. J. Pharmacol. (Proc. Suppl.),* **129,** 73P.

JUNG, J., HWANG, S.W., KWAK, J., LEE, S.Y., KANG, C.J., KIM, W.B., KIM, D. & OH, U. (1999). Capsaicin binds to the intracellular domain of the capsaicin-activated ion channel. *J. Neurosci.,* **19,** 529–538.

MAGGI, C.A. (1995). The mammalian tachykinin receptors. *Gen. Pharmacol.,* **26,** 911–944.

MANZINI, S. & MEINI, S. (1991). Involvement of capsaicin-sensitive nerves in the bronchomotor effects of arachidonic acid and melittin: a possible role for lipoxin A4. *Br. J. Pharmacol.,* **103,** 1027–1032.

MITCHELL, H.W. (1982). Effect of ETYA and BW 755c on arachidonate-induced contractions in the guinea-pig isolated trachea. *Br. J. Pharmacol.,* **76,** 527–529.

RICHARDSON, J.D., AANONSEN, L. & HARGREAVES, K.M. (1998a). Antihyperalgesic effects of spinal cannabinoids. *Eur. J. Pharmacol.,* **345,** 145–153.

RICHARDSON, J.D., KILO, S. & HARGREAVES, K.M. (1998b). Cannabinoids reduce hyperalgesia and inflammation via interaction with peripheral $CB_1$ receptors. *Pain,* **75,** 111–119.

ROSS, R.A., BROCKIE, H.C., GIBSON, M., CRAIB, S.J., LESLIE, M., PASHMI, G., DI MARZO, V. & PERTWEE, R.G. (2001). Structure-activity relationship for the endogenous cannabinoid, anandamide and certain of its analogues at vanilloid receptors in transfected cells and vas deferens. *Br. J. Pharmacol.,* **132,** 631–640.

SERHAN, C.N. (1997). Lipoxins and novel aspirin-triggered 15-epi-lipoxins: a jungle of cell-cell interactions or a therapeutic opportunity? *Prostaglandins,* **53,** 107–137.

SMART, D., GUNTHORPE, M.J., JERMAN, J.C., NASIR, S., GRAY, J., MUIR, A.I., CHAMBERS, J.K., RANDALL, A.D. & DAVIS, J.B. (2000). The endogenous lipid anandamide is a full agonist at the human vanilloid receptor (hVR1). *Br. J. Pharmacol.,* **129,** 227–230.

SPINA, D., TUCKER, R.C. & PAGE, C.P. (2000). Effect of the putative vanilloid receptor agonist, anandamide on the baseline tone in the guinea-pig bronchus. *Proc. Aust. Soc. Clin. Exp. Pharmacol. Toxicol.,* **7,** 60.

SZALLASI, A. & BLUMBERG, P.M. (1999). Vanilloid (capsaicin) receptors and mechanisms. *Pharmacol. Rev.,* **51,** 159–211.

TOBIAS, L.D. & HAMILTON, J.G. (1979). The effect of 5,8,11,14-eicosatetraynoic acid on lipid metabolism. *Lipids,* **14,** 181–193.

TUCKER, R.C., KAGAYA, M., PAGE, C.P. & SPINA, D. (2001). The endogenous cannabinoid agonist, anandamide stimulates sensory nerves in guinea-pig airways. *Br. J. Pharmacol.,* **132,** 1127–1135.

ZYGMUNT, P.M., CHUANG, H.-H., MOVAHED, P., JULIUS, D. & HÖGESTÄTT, E.D. (2000). The anandamide transport inhibitor AM404 activates vanilloid receptors. *Eur. J. Pharmacol.,* **396,** 39–42.

ZYGMUNT, P.M., PETERSSON, J., ANDERSSON, D.A., CHUANG, H., SORGÅRD, M., DI MARZO, V., JULIUS, D. & HÖGESTÄTT, E.D. (1999). Vanilloid receptors on sensory nerves mediate the vasodilator action of anandamide. *Nature,* **400,** 452–457.

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