

# Noladin ether, a putative endocannabinoid, attenuates sensory neurotransmission in the rat isolated mesenteric arterial bed *via* a non-CB<sub>1</sub>/CB<sub>2</sub> G<sub>i/o</sub> linked receptor

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**1** Noladin ether has recently been reported to be an endocannabinoid, with selectivity for the cannabinoid (CB) CB<sub>1</sub> receptor. In the present study, we investigated the effects of noladin ether in the rat isolated mesenteric arterial bed, cultured dorsal root ganglia (DRG) cells and human vanilloid (TRPV1)-receptor-expressing HEK293 cells (TRPV1-HEK293 cells).

**2** Electrical field stimulation of the mesenteric bed evoked frequency-dependent vasorelaxation due to the action of calcitonin gene-related peptide (CGRP) released from sensory nerves. Noladin ether (0.1–3 μM) attenuated sensory neurogenic relaxation in a concentration-dependent manner. Noladin ether (1 μM) reduced vasorelaxation at a submaximal frequency (8 Hz), from 57.3 ± 6.8 to 23.3 ± 3.8% ( $P < 0.05$ ,  $n = 4$ ).

**3** The inhibitory effects of noladin ether were unaffected by the CB<sub>1</sub> antagonists SR141716A and LY320135, and the CB<sub>2</sub> antagonist SR144528 (1 μM).

**4** Noladin ether had no effect on vasorelaxation elicited by exogenous CGRP or capsaicin. These data suggest that noladin ether is acting at a prejunctional site and no interaction with TRPV1 is involved.

**5** In mesenteric beds from pertussis toxin (PTX)-pretreated rats, the inhibitory actions of noladin ether on sensory neurotransmission were abolished, indicating the involvement of G<sub>i/o</sub> protein-coupled receptors.

**6** Noladin ether evoked a concentration-dependent increase in intracellular Ca<sup>2+</sup> concentration in TRPV1-HEK293 cells at 10 μM (36.5 ± 3.2% of maximal capsaicin-induced response), but it was a less potent agonist than both capsaicin and anandamide and at 1 μM it was essentially inactive. Noladin ether (1 μM) had no effect on capsaicin-evoked Ca<sup>2+</sup> responses in DRG cells, and produced no response alone, indicating it neither modulates nor acts directly on TRPV1 receptors.

**7** These data demonstrate that noladin ether attenuates sensory neurotransmission in rat mesenteric arteries *via* a non-CB<sub>1</sub> non-CB<sub>2</sub> PTX-sensitive prejunctional site, independently of TRPV1 receptors. *British Journal of Pharmacology* (2004) **142**, 509–518. doi:10.1038/sj.bjp.0705789

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**Abbreviations:** abn-CBD, abnormal cannabidiol; ANOVA, analysis of variance; CB, cannabinoid; CGRP, calcitonin gene-related peptide; CPA, cyclopentyladenosine; DRG, dorsal root ganglia; EDHF, endothelium-derived hyperpolarising factor; EFS, electrical field stimulation; THC, Δ<sup>9</sup>-tetrahydrocannabinol; TRPV1, vanilloid receptor

## Introduction

Anandamide and 2 arachidonoylglycerol (2AG) were the first endocannabinoids to be described; they bind to both CB<sub>1</sub> and CB<sub>2</sub> (CB: cannabinoid) receptors and exhibit cannabimimetic actions *in vivo* (Pertwee, 1999). Noladin ether, an ether-linked 2AG analogue, has recently been identified as another endocannabinoid; it was synthesised by Sugiura *et al.* (1999) and Mechoulam *et al.* (1998) and was simultaneously isolated from porcine brain and identified as an endogenous ligand at the CB<sub>1</sub> receptor by Hanuš *et al.*, 2001. Noladin ether binds only weakly to the CB<sub>2</sub> receptor but exerts cannabimimetic

effects in the mouse behavioural tetrad test, and is present in discreet brain regions; the highest levels are reported to be found in the hippocampus and thalamus (Fezza *et al.*, 2002). However, more recent studies in various central nervous system (CNS) tissues indicate that noladin ether might not be present at detectable levels in the brain at least (Oka *et al.*, 2003).

Anandamide has pronounced effects on the cardiovascular system, which are mediated by the activation of both CB<sub>1</sub> and vanilloid (TRPV1) receptors; the latter are nonselective cation channels that are activated by noxious stimuli (Szallasi & Blumberg 1999; Zygmunt *et al.*, 1999; Smart *et al.*, 2000). Some vascular effects of CBs are mediated by sensory nerves. CB receptors are present in dorsal root ganglion (DRG) cells, and are transported to the peripheral terminals of the

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sensory neurones by axonal transport (Hohmann & Herkenham, 1999). Vanilloid receptors (TRPV1) are also present on capsaicin-sensitive sensory nerves and there is considerable overlap in the expression of TRPV1 receptors and CB<sub>1</sub> receptors (Ahluwalia *et al.*, 2000). Synthetic CB agonists such as WIN55,212, CP55,940 and HU210 act at CB<sub>1</sub> and CB<sub>2</sub> but have no activity at TRPV1 receptors. Similarly, the endocannabinoid 2AG has no activity at TRPV1 (Zygmunt *et al.*, 1999).

The expression of functional CB<sub>1</sub> receptors on perivascular sensory nerves has been shown in assays of the rat isolated mesenteric arterial bed, in which WIN55,212 and CP55,940 inhibited sensory neurotransmission and this could be reversed by the CB<sub>1</sub> antagonists SR141716A and LY323501 (Duncan *et al.*, 2001a, b). The inhibitory actions of  $\Delta^9$ -tetrahydrocannabinol (THC) and HU210 in the same preparations could not be reversed by antagonists of CB<sub>1</sub> or CB<sub>2</sub> receptors, indicating that inhibition of sensory neurotransmission by these compounds is *via* a prejunctional non-CB<sub>1</sub>/CB<sub>2</sub> site (Ralevic & Kendall, 2001; Duncan *et al.*, 2003a).

The aim of the present study was to determine whether the putative endocannabinoid noladin ether could mimic the actions of synthetic CBs on perivascular sensory neurotransmission in rat mesenteric arteries. To characterise the mode of action, the experiments were carried out in the presence of CB<sub>1</sub> and CB<sub>2</sub> receptor antagonists and in tissues from rats pretreated with pertussis toxin (PTX) to inactivate G<sub>i/o</sub> protein-coupled receptors. To determine pre- or postjunctional actions, the effect of noladin ether on the vasorelaxant response to exogenous calcitonin gene-related peptide (CGRP) was investigated. There have been no reports to date of the action of noladin ether on vanilloid receptors. We have, therefore, also examined the effects of noladin ether on the vasorelaxant actions of capsaicin in the mesenteric bed and on the capsaicin-induced Ca<sup>2+</sup> response in rat DRG cells in primary culture. A comparison was made between anandamide and noladin ether on the Ca<sup>2+</sup> responses in HEK293 cells transfected with human TRPV1 receptors. Preliminary accounts of some of these results have been reported previously (Duncan *et al.*, 2002; 2003a, b, c).

## Methods

### *Mesenteric arterial bed preparation*

Male Wistar rats (250–300 g) were killed by decapitation after exposure to CO<sub>2</sub>. Mesenteric beds were isolated and perfused *via* the superior mesenteric artery. The abdomen was opened and the superior mesenteric artery was exposed and cannulated with a blunted hypodermic needle. The superior mesenteric vein was cut, blood flushed from preparation with 0.5 ml Krebs' solution and the gut dissected away from the mesenteric vasculature. The preparations were mounted on stainless steel grids (7 × 5 cm) in a humid chamber and perfused at a constant flow rate of 5 ml min<sup>-1</sup> using a perfusion pump (model: 7554-30, Cole Parmer Ltd, Chicago, IL, U.S.A.). The perfusate was Krebs' solution of the following composition (mM): NaCl 133, KCl 4.7, NaH<sub>2</sub>PO<sub>4</sub> 1.35, NaHCO<sub>3</sub> 16.3, MgSO<sub>4</sub> 0.61, CaCl<sub>2</sub> 2.52 and glucose 7.8 gassed with 95% O<sub>2</sub>–5% CO<sub>2</sub> and maintained at 37°C. In order to ascertain that the electrical circuit was complete, electrical field

stimulation (EFS) was applied comprising a brief (5 s) stimulus (20 Hz, 90 V, 0.1 ms) *via* the metal grid (negative electrode) and the hypodermic needle (positive electrode), to excite the sympathetic fibres thus eliciting a small vasoconstriction and a transient rise in perfusion pressure. Guanethidine (5  $\mu$ M) was added to block sympathetic neurotransmission, and after 30 min methoxamine (5–100  $\mu$ M) was added to precontract the preparation (30–80 mmHg above baseline). EFS (2–12 Hz, 0.1 ms, 60 V, 30 s) was applied with a Grass S9D stimulator. The resulting vasorelaxation response has been shown to be blocked by tetrodotoxin (1  $\mu$ M) and capsaicin (1  $\mu$ M) (Ralevic *et al.*, 1991). Responses were measured as changes in perfusion pressure (mmHg) with a pressure transducer (model: P23XL, Viggo-Spectramed, Oxnard, CA, U.S.A.), situated on a side arm proximal to the preparation, and recorded using an A to D converter (PowerLab/400, ADI Instruments, Australia). Preparations were allowed to equilibrate for 30 min prior to experimentation.

### *Experimental protocol*

Three consecutive relaxant response curves to EFS at 1–12 Hz, 'EFS control', 'EFS I' and 'EFS II', were generated in the precontracted mesenteric arterial beds. After each stimulus, the tone of the preparation was allowed to return to its precontracted value before the next stimulus was applied. The first response curve acted as a control. The compound under investigation was then added to the perfusate, and after 15 min response curves EFS I and EFS II were generated. Antagonists were added at the start of the equilibration period. Only a single concentration of agonist was used per preparation. In a separate series of experiments, dose–response curves were constructed to CGRP (0.05 pmol–0.5 nmol) and, in separate preparations, capsaicin (0.05 pmol–5 nmol), by applying 50  $\mu$ l bolus injections *via* norprene tubing proximal to the preparation.

### *PTX pretreatment*

Animals were pretreated using 10 mg kg<sup>-1</sup> i.p. PTX 48 h prior to experiment (Schultz *et al.*, 1998). The mesenteric beds were isolated and perfused with Krebs' solution containing guanethidine as previously described. The protocol for the preparations from PTX pretreated rats had to be modified, as methoxamine was unable to maintain tone throughout the experiment. Endothelin (0.2–2 nM) was added to the perfusate after the preparations had equilibrated, which raised the tone to 30–40 mmHg above baseline. With further additions of methoxamine (1–10  $\mu$ M), the tone was sustained at this level for the remainder of the experiment. The preparations were stimulated at two frequencies (8 and 12 Hz), noladin ether was then added for 40 min and the preparations were again stimulated at 8 and 12 Hz. Control experiments were carried out to determine if the G<sub>i/o</sub> proteins had been successfully inactivated by the PTX pretreatment. In these experiments, the adenosine A<sub>1</sub> agonist cyclopentyladenosine (CPA) was used in the place of noladin ether. CPA is known to attenuate capsaicin-sensitive sensory neurotransmission in the rat isolated mesenteric arterial bed *via* G<sub>i/o</sub>-coupled A<sub>1</sub> receptors (Rubino *et al.*, 1993).

### Data analysis – mesenteric arterial beds

Vasorelaxant responses of the mesenteric beds were expressed as a percentage relaxation of the methoxamine-induced increase in tone above baseline. Data were compared by Student's *t*-test and one-way/two-way analysis of variance (ANOVA) with Tukey's *post hoc* test. A value of  $P < 0.05$  was taken to indicate a statistically significant difference. The software package Prism GraphPad (3.0) was used to perform the analyses.  $R_{\text{MAX}}$  indicates maximal relaxation.

### DRG preparation

DRG were isolated from adult Wistar rats (300–350 g) and neurones cultured as described by Lindsay (1988) with minor modifications. Cells were grown on 13 mm glass coverslips for 24 h prior to incubation with Fura 2-AM (5  $\mu\text{M}$ , 30 min, 37°C). The mean diameter of the cells sampled was  $18.6 \pm 0.4 \mu\text{m}$  ( $n = 241$ ). Intracellular  $\text{Ca}^{2+}$  concentrations ( $[\text{Ca}^{2+}]_i$ ) in individual neurones in fields of 241 cells were estimated as the ratios of peak fluorescence intensities (measured at 500 nm) at excitation wavelengths of 340 and 380 nm respectively (Bundey & Kendall, 1999), using an Improvisation imaging system with Ion Vision software.

DRG neurones were superfused (2 ml min<sup>-1</sup>) with capsaicin (100 nM) for 60 s followed by a 45 min washout. Noladin ether (1  $\mu\text{M}$ ) was then added for 4 min, and capsaicin (100 nM) in combination with noladin ether (1  $\mu\text{M}$ ) was then added for 60 s. After a washout period of 45 min, capsaicin (100 nM) alone was added. This protocol has previously been used to demonstrate HU210 inhibition of capsaicin-induced increase of  $[\text{Ca}^{2+}]_i$  in DRG cells (Millns *et al.*, 2001).

### Data analysis – DRG calcium responses

Data are expressed as ratios of 340:380 peak fluorescence as a percentage (means  $\pm$  s.e.) of the initial capsaicin response for each cell. Statistical analysis was performed using unpaired Student's *t*-test.

### Cloning and expression of human vanilloid TRPV1 receptors in HEK293 cells

The cloning of the human vanilloid TRPV1 receptor was conducted as described previously (Hayes *et al.*, 2000). Briefly, human vanilloid TRPV1 receptor cDNA was identified using the published rat vanilloid TRPV1 receptor sequence (GenBank accession AF029310) to search public nucleotide databases. Expressed sequence tag T48002 was identified and its sequence extended by rapid amplification of the cDNA ends using cDNA templates from a number of tissue sources. The full cDNA was amplified from brain cDNA, inserted into the expression vector pcDNA3.1, double strand sequenced and stably expressed in HEK293 cells.

### Cell culture – TRPV1-HEK293 cells

Human vanilloid TRPV1 receptor-expressing HEK293 cells were routinely grown as monolayers in minimum essential medium supplemented with non-essential amino acids, 10%

foetal calf serum and 0.2 mM L-glutamine, and maintained under 95%/5% air/CO<sub>2</sub> at 37°C. Cells were passaged every 3–4 days and the highest passage number used was 28.

### Measurement of intracellular $\text{Ca}^{2+}$ concentrations using the FLIPR™

Intracellular  $\text{Ca}^{2+}$  concentrations were monitored using FLIPR™ (Molecular Devices, U.K.) as described previously (Smart *et al.*, 2001). Briefly, human vanilloid TRPV1 receptor-expressing HEK293 cells, seeded into 96-well plates (25,000 cells per well), were incubated with culture medium containing the cytoplasmic  $\text{Ca}^{2+}$  indicator, Fluo-3 (4  $\mu\text{M}$ ; Teflabs, Austin, TX, U.S.A.), at 25°C for 120 min. The cells were then washed four times with Tyrode's medium containing 0.1% BSA, before being incubated for 30 min at 25°C in the presence or absence of various antagonists. The plates were then placed into a FLIPR™ to monitor cell fluorescence ( $\lambda_{\text{EX}} = 488 \text{ nm}$ ,  $\lambda_{\text{EM}} = 540 \text{ nm}$ ) (Wood & Smart, 2001) before and after the addition of various agonists.

### Data analysis – recombinant vanilloid TRPV1-expressing cells

Responses were measured as peak minus basal fluorescence intensity, and where appropriate were expressed as a percentage of a maximum capsaicin-induced response. Data are expressed as mean  $\pm$  s.e.m. unless otherwise stated. Curve-fitting and parameter estimation were carried out using Graph Pad Prism 3.0 (GraphPad Software Inc., California, U.S.A.). Statistical comparisons were made where appropriate using Student's *t*-test.

### Drugs

Noladin ether was a kind gift from Prof. T. Sugiura (Teikyo University, Japan), or was purchased from Tocris U.K. PTX was from Calbiochem, U.K. SR141716A (*N*-piperidino-5-(4-chlorophenyl)-1-(2,4-dichlorophenyl)-4-methyl-3-pyrazole-carboxamide) and SR144528 (*N*-[1*S*]-endo-1,3,3-trimethyl bicyclo [2.2.1]heptan-2-yl]-5-(4-chloro-3-methylphenyl)-1-(4-methylbenzyl)-pyrazole-3-carboxamide) were gifts from Sanofi (Montpellier, France). LY320135 [6-methoxy-2-(4-methoxyphenyl) benzo[*b*]-thien-3-yl][4-cyanophenyl] methanone) was a gift from Eli Lilly. CGRP and capsaicin were from Sigma (Poole, Dorset, U.K.). Guanethidine (Ismelin) was from Alliance Pharmaceuticals (Chippenham, Wiltshire, U.K.).

All CBs and capsaicin were dissolved in ethanol at a stock concentration of 10<sup>-2</sup> M. CBs were added directly to the perfusate reservoir. Further dilutions of capsaicin were made in distilled H<sub>2</sub>O. CGRP was dissolved in distilled water.

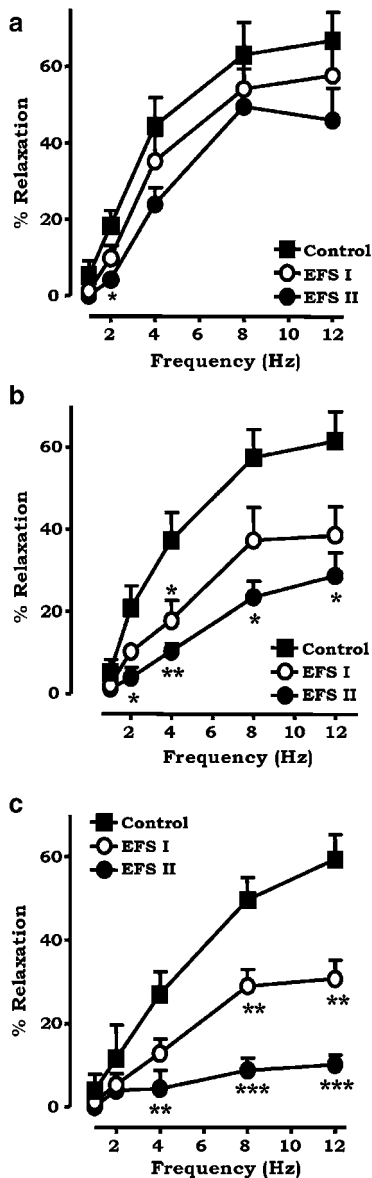
In the DRG experiments, drug dilutions were made in superfusion buffer of the following composition: (mM) NaCl 145; KCl 5; CaCl<sub>2</sub> 2; MgSO<sub>4</sub> 1; HEPES 10; glucose 10; pH 7.4.

## Results

### Effect of noladin ether on vasorelaxant responses to EFS

EFS (1–12 Hz, 0.1 ms, 60 V, 30 s) produced frequency-dependent relaxation of the rat isolated mesenteric arterial bed.

Three frequency–response curves were constructed, EFS control, EFS I and EFS II, and were reproducible under control conditions. The equivalent concentration of the drug vehicle (0.01% ethanol) had no effect on the vasorelaxant response (data not shown). The first curve acted as a control. Noladin ether attenuated EFS I and EFS II in a concentration- and time-dependent manner. At the lowest concentration (0.1  $\mu\text{M}$ ,  $n=6$ ), there was a small inhibition of the relaxation response at 2 Hz ( $P<0.05$ ) but no significant difference in  $R_{\text{MAX}}$  (value obtained at 12 Hz) (Figure 1a). At concentrations of 1 and 3  $\mu\text{M}$  noladin ether, the relaxation response was significantly reduced: at a submaximal frequency of 8 Hz, 1  $\mu\text{M}$  reduced EFS control 57.3 $\pm$ 6.8% to EFS II 23.3 $\pm$ 3.8% ( $P<0.05$ ,

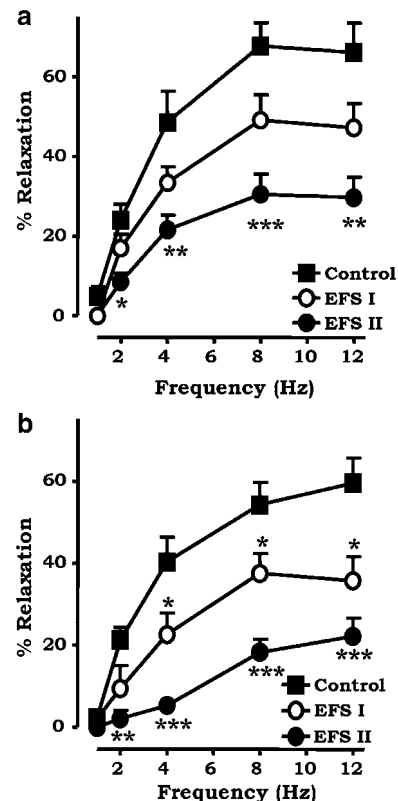


**Figure 1** Effects of (a) 0.1  $\mu\text{M}$  ( $n=6$ ), (b) 1  $\mu\text{M}$  ( $n=4$ ) and (c) 3  $\mu\text{M}$  noladin ether ( $n=5$ ) on frequency-dependent vasorelaxation to EFS in the rat isolated mesenteric arterial bed. Three consecutive curves were constructed to EFS: the first acted as a control, noladin ether was added to the perfusate, then EFS I and EFS II were constructed. Data are presented as means $\pm$ s.e.m. \* $P<0.05$  with respect to control, \*\* $P<0.01$  with respect to control, \*\*\* $P<0.001$  with respect to control.

$n=4$ ) and 3  $\mu\text{M}$  reduced EFS control 49.6 $\pm$ 5.4% to EFS II 8.7 $\pm$ 2.9% ( $P<0.001$ ,  $n=5$ ) (Figure 1b and c).  $R_{\text{MAX}}$  was significantly reduced at both concentrations: at 1  $\mu\text{M}$ , noladin ether reduced EFS control from 61.4 $\pm$ 7.2% to EFS II 28.5 $\pm$ 5.6% ( $P<0.05$ ); at 3  $\mu\text{M}$ , 59.3 $\pm$ 6.0% to EFS II 10.1 $\pm$ 2.2% ( $P<0.001$ ). Noladin ether reduced the tone of the preparation, which was restored and maintained using methoxamine.

#### *Effect of SR141716A and LY320135 on the inhibitory actions of noladin ether on the vasorelaxant response to EFS*

In order to determine if the inhibitory actions exerted by noladin ether were mediated by CB<sub>1</sub> receptors, the experiments in the mesenteric beds were repeated in the presence of 1  $\mu\text{M}$  of the CB<sub>1</sub> selective antagonists SR141716A and LY320135 (Figure 2). The concentration of 1  $\mu\text{M}$  SR141716A was used to minimise nonselective effects, which have been reported at higher concentrations (Ralevic, 2003). SR141716A had no effect on the inhibitory actions of noladin ether on sensory neurogenic vasorelaxation (Figure 2a). At a submaximal frequency of 8 Hz, there was significant inhibition: EFS control 67.8 $\pm$ 5.8% to EFS II 30.5 $\pm$ 5.0%;  $n=6$ ,  $P<0.001$ . LY320135 (1  $\mu\text{M}$ ) also failed to block the inhibitory actions of noladin ether on sensory neurogenic vasorelaxation. At 8 Hz,



**Figure 2** Effect of the CB<sub>1</sub> selective antagonists (a) SR141716A (1  $\mu\text{M}$ ,  $n=6$ ) and (b) LY320135 (1  $\mu\text{M}$ ,  $n=8$ ) on the inhibitory actions of 1  $\mu\text{M}$  noladin ether on frequency-dependent vasorelaxation to EFS in the rat isolated mesenteric arterial bed. Three consecutive curves were constructed to EFS: the first acted as a control, noladin ether was added to the perfusate, then EFS I and EFS II were constructed. Data are presented as means $\pm$ s.e.m. \* $P<0.05$  with respect to control, \*\* $P<0.01$  with respect to control, \*\*\* $P<0.001$  with respect to control.

there was a significant inhibition of neurogenic relaxations by noladin ether: EFS control  $54.2 \pm 5.5\%$  to EFS II  $18.2 \pm 3.1\%$ ;  $n=8$ ,  $P<0.001$  (Figure 2b). SR141716A has previously been reported to augment sensory neurogenic relaxations to EFS in the rat isolated arterial mesenteric bed (Ralevic & Kendall, 2001); however, in control experiments, it had no effect in the present study (data not shown).

#### Effect of SR144528 on the inhibitory actions of noladin ether on the vasorelaxant response to EFS

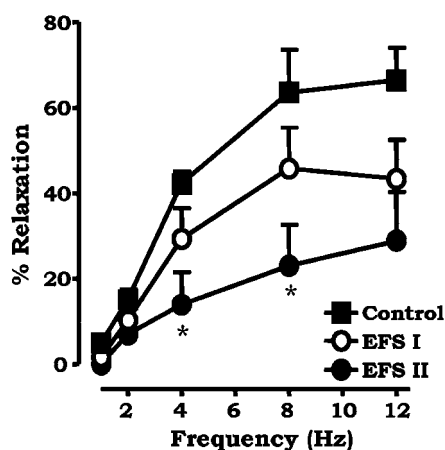
In order to investigate the possibility that noladin ether was acting at CB<sub>2</sub> receptors, we repeated the experiment in the mesenteric beds in the presence of the CB<sub>2</sub> selective antagonist SR144528 ( $1 \mu\text{M}$ ). SR144528 had no effect on the inhibitory actions of noladin ether ( $1 \mu\text{M}$ ) on sensory neurogenic vasorelaxation (Figure 3). At a submaximal frequency of 8 Hz, there was significant inhibition due to noladin ether of EFS control  $68.0 \pm 8.9\%$  to EFS II  $23.9 \pm 7.5\%$ ;  $n=5$ ,  $P<0.05$ .

#### Effect of noladin ether on the vasorelaxant response to exogenous CGRP

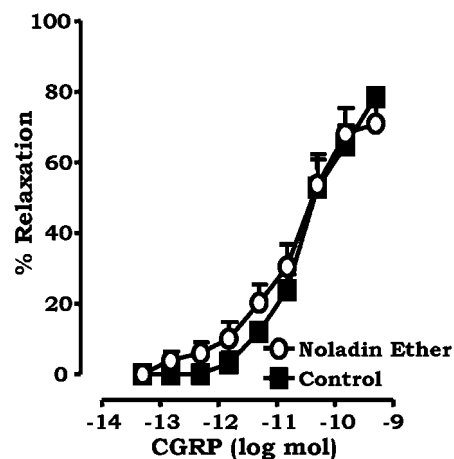
To determine if the inhibitory actions on sensory neurotransmission by the endocannabinoid noladin ether are *via* a pre- or postjunctional site, dose-response curves to CGRP, the principal motor neuropeptide involved in sensory neurogenic relaxation, were constructed in the mesenteric beds, in the absence and presence of  $1 \mu\text{M}$  noladin ether (Figure 4). The  $pD_2$  value was unaltered:  $10.7 \pm 0.2$  and  $10.5 \pm 0.1$  in the presence and absence, respectively, of  $1 \mu\text{M}$  noladin ether ( $P>0.05$ , unpaired *t*-test).

#### Effect of noladin ether on the vasorelaxant response to capsaicin

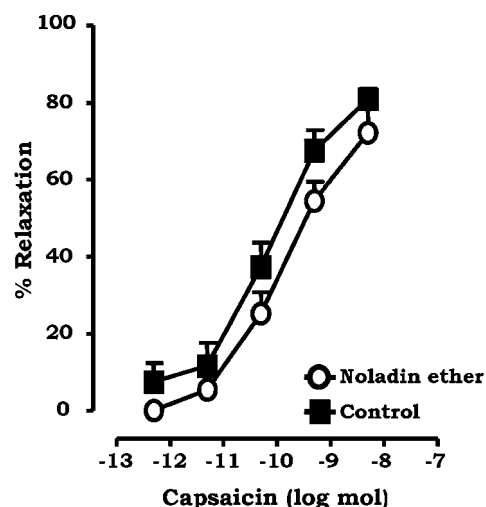
Vanilloid receptors are abundant in perivascular sensory nerves in the rat mesenteric arterial bed, and their activation



**Figure 3** Effect of the CB<sub>2</sub> selective antagonist SR144528 ( $1 \mu\text{M}$ ,  $n=5$ ) on the inhibitory actions of  $1 \mu\text{M}$  noladin ether on frequency-dependent vasorelaxation to EFS in the rat isolated mesenteric arterial bed. Three consecutive curves were constructed to EFS: the first acted as a control, noladin ether was added to the perfusate, then EFS I and EFS II were constructed. Data are presented as means  $\pm$  s.e.m. \* $P<0.05$  with respect to control.



**Figure 4** Effect of  $1 \mu\text{M}$  noladin ether ( $n=6$ ) on vasorelaxant responses of rat isolated mesenteric arterial beds to CGRP ( $0.05 \text{ pmol}$ – $0.5 \text{ nmol}$ ). Data are presented as means  $\pm$  s.e.m.



**Figure 5** Effect of  $1 \mu\text{M}$  noladin ether ( $n=8$ ) on vasorelaxant responses of rat isolated mesenteric arterial beds to capsaicin ( $0.05 \text{ pmol}$ – $5 \text{ nmol}$ ). Data are presented as means  $\pm$  s.e.m.

by capsaicin results in CGRP release and vasodilatation. To determine if noladin ether is acting at TRPV1 receptors or modulating the TRPV1 response, dose-response curves to the TRPV1 agonist capsaicin were constructed, in the presence of  $1 \mu\text{M}$  noladin ether or vehicle (ethanol 0.01%) (Figure 5). The  $pD_2$  value was unaltered:  $9.8 \pm 0.2$  and  $10.1 \pm 0.2$  in the presence and absence, respectively, of  $1 \mu\text{M}$  noladin ether ( $P>0.05$ , unpaired *t*-test).

#### Effects of PTX in vivo pretreatment on the inhibitory actions of noladin ether

Both CB receptors that have been isolated to date (CB<sub>1</sub> and CB<sub>2</sub>) are G<sub>i/o</sub> protein-linked receptors. In order to determine if the inhibitory actions of noladin ether are G<sub>i/o</sub> protein-mediated, rats were pretreated with PTX ( $10 \text{ mg kg}^{-1}$ ;

Schultz *et al.*, 1998) 48 h prior to the experiment. Mesenteric beds were isolated and perfused and the tone was raised to 30–40 mmHg above baseline using endothelin and methoxamine (see Methods). Electrical stimulation at 8 and 12 Hz was then applied. Noladin ether (1  $\mu\text{M}$ ) was added to the perfusate and left for 40 min and the stimulation was then repeated.

In the control animals, 1  $\mu\text{M}$  noladin ether significantly attenuated the sensory neurogenic vasorelaxant response at both frequencies (Figure 6a). The neurogenic response to 8 Hz was reduced from  $39.0 \pm 4.4$  to  $22.9 \pm 6.1\%$  ( $P < 0.01$ ,  $n = 10$ ) and at 12 Hz the response was reduced from  $35.1 \pm 4.9$  to  $21.0 \pm 3.1\%$  ( $P < 0.01$ ,  $n = 10$ ). In the tissues from PTX-pretreated animals, there was no significant difference in the sensory neurogenic relaxant response in the presence of 1  $\mu\text{M}$  noladin ether (Figure 6b). At 8 Hz, the response was unaffected,  $36.2 \pm 5.9$  to  $41.8 \pm 5.6\%$  ( $n = 8$ ,  $P > 0.05$ ), and at 12 Hz,  $40.1 \pm 6.1$  to  $36.5 \pm 6.8\%$  ( $n = 8$ ,  $P > 0.05$ ).

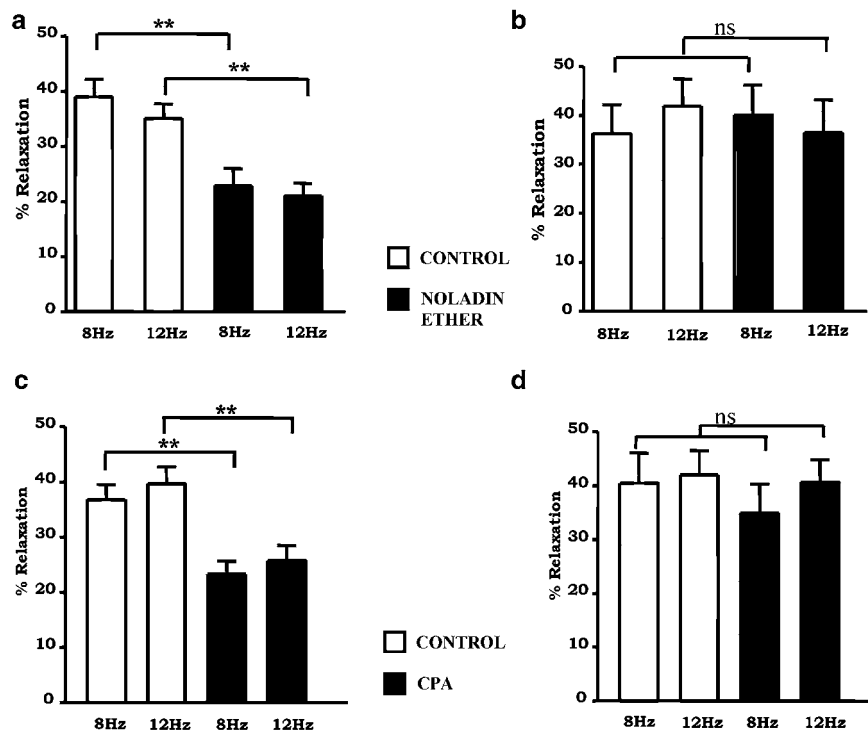
The control compound CPA, an adenosine  $A_1$  receptor agonist, significantly reduced sensory neurogenic relaxations in control tissues (Figure 6c): at 8 Hz the response was reduced from  $36.8 \pm 2.7$  to  $23.3 \pm 2.3\%$  ( $P < 0.01$ ), and at 12 Hz the response was reduced from  $39.7 \pm 2.3$  to  $25.8 \pm 2.7\%$  ( $n = 10$ ,  $P < 0.01$ ). In the tissues from PTX-pretreated animals, the inhibitory actions of CPA were abolished (Figure 6d). The response at 8 Hz was unaffected,  $40.0 \pm 7.0$  to  $34.9 \pm 6.9\%$  ( $P > 0.05$ ), and at 12 Hz,  $42.0 \pm 5.9$  to  $40.7 \pm 4.0\%$  ( $n = 7$ ,  $P > 0.05$ ).

### Effects of noladin ether on capsaicin-evoked calcium response in cultured DRG cells

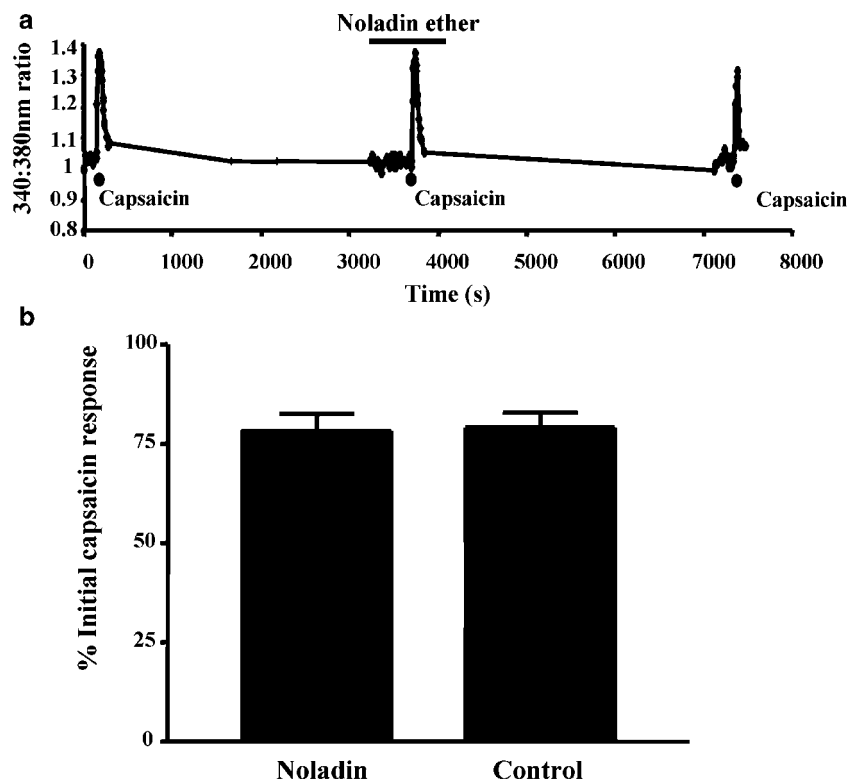
HU210 has been reported to inhibit the capsaicin-induced  $\text{Ca}^{2+}$  response in DRG neurones *via* a functional CB receptor sensitive to SR141716A (Millns *et al.*, 2001). We investigated whether noladin ether also acts at  $\text{CB}_1$  receptors in DRG cells. A total of 48% of the cells examined (mean diameter  $18.5 \pm 0.5 \mu\text{m}$ ,  $n = 155$ ) responded to capsaicin. The initial capsaicin response (100 nM, 60 s) was taken as control (100%) response. The responses were then calculated as a percentage of the initial capsaicin response. In untreated cells, the mean 340:380 ratio (basal  $[\text{Ca}^{2+}]_i$ ) was  $1.1 \pm 0.01$  ( $n = 52$ ). The capsaicin-evoked calcium response in the presence of 1  $\mu\text{M}$  noladin ether was  $78.3 \pm 4.3\%$  ( $n = 52$ ), compared to  $79.1 \pm 3.8\%$  ( $n = 35$ ) in the separate control experiments (Figure 7). Noladin ether alone had no effect:  $6.4 \pm 1.35\%$  of the initial capsaicin response.

### Effects of noladin ether on intracellular $\text{Ca}^{2+}$ concentrations in hTRPV1-HEK293 cells

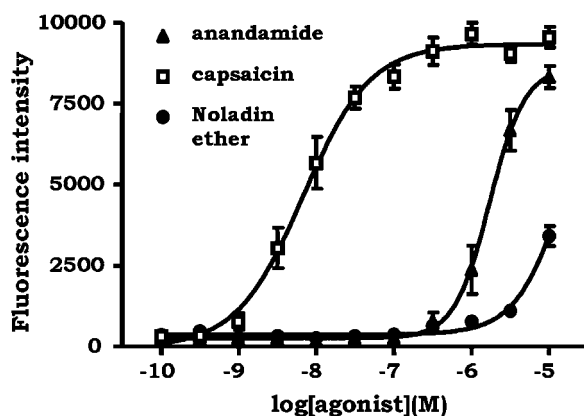
Capsaicin and anandamide (100 pM–10  $\mu\text{M}$ ) evoked concentration-dependent  $\text{Ca}^{2+}$  responses in hTRPV1-HEK293 cells (Figure 8), with  $\text{pEC}_{50}$  values of  $8.16 \pm 0.05$  and  $5.75 \pm 0.02$  respectively, consistent with previous reports (Smart *et al.*, 2000). Noladin ether (1–10  $\mu\text{M}$ ) also evoked a concentration-related  $\text{Ca}^{2+}$  response in hTRPV1-HEK293 cells (Figure 8),



**Figure 6** Effect of 1  $\mu\text{M}$  noladin ether on sensory neurogenic relaxation to EFS in mesenteric beds from (a) control ( $n = 10$ ) and (b) PTX-pretreated rats ( $n = 8$ ) and the effect of 3 nM CPA on sensory neurogenic relaxation to EFS in mesenteric beds from (c) control ( $n = 10$ ) and (d) PTX-pretreated rats ( $n = 7$ ). Two frequencies of EFS, 8 and 12 Hz, were applied. Noladin ether and CPA were added to the perfusate for 40 min, and then stimulation was repeated. Data are presented as means  $\pm$  s.e.m. \*\* $P < 0.01$  with respect to control; ns = not significant.



**Figure 7** (a) A representative trace showing the 340:380nm ratios in a single DRG neurone, in response to capsaicin in the presence of  $1\ \mu\text{M}$  noladin ether. The cell is initially exposed to capsaicin ( $100\ \text{nM}$ ) for 60 s; after a 45 min washout period, noladin ether was applied alone for 4 min; a combination of capsaicin and noladin ether was then applied for 60 s. After a further 45 min, the cell was challenged with capsaicin alone. (b) Effect of  $1\ \mu\text{M}$  noladin ether on capsaicin-induced calcium response in individual DRG cells ( $n=52$ ), compared with the response to capsaicin alone in separate control experiments ( $n=35$ ). Results are expressed as percentages of the responses to  $100\ \text{nM}$  capsaicin alone.



**Figure 8** Noladin ether is a weak agonist at hTRPV1. Intracellular  $\text{Ca}^{2+}$  concentration was monitored using Fluo-3 in human vanilloid TRPV1 receptor-expressing HEK293 cells before and after the addition of capsaicin ( $100\ \text{pM}$ – $30\ \mu\text{M}$ ), anandamide ( $100\ \text{pM}$ – $10\ \mu\text{M}$ ) or noladin ether ( $100\ \text{pM}$ – $10\ \mu\text{M}$ ). Responses were measured as peak increase in fluorescence minus basal and are given as mean  $\pm$  s.e.m.  $n=3$ .

but was a much weaker agonist ( $36.5 \pm 3.2\%$  of a maximal capsaicin-induced response at  $10\ \mu\text{M}$ ). At  $1\ \mu\text{M}$  noladin ether, the concentration used in the vasorelaxation studies in the mesenteric beds, there was no significant response. The responses to all three ligands in hTRPV1-HEK293 were

blocked by capsazepine ( $1\ \mu\text{M}$ ), and were absent in wild-type HEK293 cells (data not shown), confirming that all three were acting as TRPV1 agonists.

## Discussion

The data presented clearly show that noladin ether attenuates capsaicin-sensitive sensory neurotransmission in the rat isolated mesenteric arterial bed *via* a PTX-sensitive prejunctional site. However, the inhibitory actions could not be attenuated by selective antagonists for  $\text{CB}_1$  and  $\text{CB}_2$  receptors at concentrations previously shown to block the actions of synthetic CBs (Duncan *et al.*, 2001a, b). Postjunctional actions and modulation of TRPV1 receptors were ruled out as potential mechanisms of action of noladin ether by the finding that dose–response curves to CGRP and capsaicin in the mesenteric bed were unaltered in the presence of noladin ether.

The inhibitory actions of noladin ether on the sensory neurogenic vasorelaxant response in the rat isolated mesenteric arterial bed are comparable to our previously published findings for HU210 and THC (Ralevic & Kendall, 2001; Duncan *et al.*, 2003a). Neurogenic vasorelaxation mediated by the actions of CGRP release from sensory nerves was inhibited by HU210 ( $0.1$ – $3\ \mu\text{M}$ ) and these responses were unaffected by selective antagonists for  $\text{CB}_1$  and  $\text{CB}_2$  receptors. Likewise, HU210 had

no effect on the relaxation response to CGRP, indicating a prejunctional site of action (Ralevic & Kendall, 2001). The inhibitory actions of THC are also resistant to CB<sub>1</sub> and CB<sub>2</sub> antagonists, and have no effect on the relaxation response to CGRP (Duncan *et al.*, 2003a). It is therefore conceivable that the actions of HU210, THC and noladin ether are mediated by a non-CB<sub>1</sub> CB<sub>2</sub> receptor on sensory nerves in rat mesenteric arteries.

There have been several proposed novel CB receptors, although to date only two putative novel CB receptors have been characterised in the vascular system. One of these is an endothelial 'anandamide receptor' (Di Marzo *et al.*, 2002), through which anandamide mediates vasodilatory effects in mesenteric artery preparations; abnormal cannabidiol (abn-CBD) also activates this novel receptor (Járai *et al.*, 1999). The actions of anandamide, releasing endothelium-derived hyperpolarising factor (EDHF) to the underlying smooth muscle, at this novel receptor are blocked by PTX pretreatment (Offertaler *et al.*, 2003). Since the actions of both anandamide and abn-CBD are antagonised by SR141716A, it is therefore unlikely that the SR141716A-insensitive noladin ether responses that we have observed could be mediated by the endothelial anandamide receptor. Moreover, the endothelial anandamide receptor studies were performed in perfused rat mesenteric beds, where SR141716A was maximally effective at 0.5  $\mu$ M (Wagner *et al.*, 1999). Therefore, the concentration of SR141716A used in these experiments would rule out the novel anandamide receptor involvement. The effects of noladin ether do appear to be mediated by a G protein-coupled receptor since they were absent in preparations from animals treated with the G<sub>i/o</sub> protein inactivator PTX.

Another, different, non-CB<sub>1</sub>/CB<sub>2</sub> receptor also appears to be present in perivascular sensory nerves (Zygmunt *et al.*, 2002); it is activated by THC and cannabidiol, resulting in an endothelium-independent release of CGRP. This effect is resistant to the TRPV1 antagonist capsazepine, and is blocked by the CGRP antagonist 8-37 CGRP and the nonselective channel blocker ruthenium red. The effect is also observed in TRPV1 knockout mice, and the ability of ruthenium red to block the action implies that an ion channel is involved. However, as the inhibitory actions of noladin ether on sensory neurotransmission were found to be resistant to ruthenium red (Duncan *et al.*, 2003b), this suggests that it is unlikely that noladin ether is acting at the putative sensory nerve ionotropic receptor.

Other novel CB receptors have been described in the brain and central nervous system. A receptor activated by WIN55,212 and anandamide has been described in the mouse brain; both of these compounds cause enhanced [<sup>35</sup>S]GTP $\gamma$ S binding in CB<sub>1</sub> knockout (CB<sub>1</sub><sup>-/-</sup>) mice but this effect is not produced by THC, HU210 or CP55,940. High levels of binding were reported in the cerebral cortex, midbrain, hippocampus and brain stem (Di Marzo *et al.*, 2000; Breivogel *et al.*, 2001). A presynaptic CB receptor has been described on central glutamate terminals, activated by WIN55,212 in CB<sup>-/-</sup> mice, in electrophysiological experiments using mouse hippocampal slices (Hájos *et al.*, 2001). This is, however, SR141716A-sensitive, again indicating that it is unlikely that this is same as the putative receptor activated by noladin ether.

Capsaicin excites initially, *via* the ionotropic receptor TRPV1, and then attenuates the action of sensory nerves in the rat

mesenteric arterial bed. Thus, the ability of noladin ether to induce Ca<sup>2+</sup> responses in hTRPV1-HEK293 cells was studied as a possible mechanism involved in its inhibitory action on sensory neurotransmission. Noladin ether was a weak agonist at TRPV1 receptors in hTRPV1-HEK293 cells. At a concentration of 10  $\mu$ M, noladin ether only produced 36.5  $\pm$  3.2% of the maximal capsaicin-induced response compared with approximately 80% for anandamide, and at 1  $\mu$ M it was essentially inactive at TRPV1. Noladin ether (30 nM) has been reported to induce rapid transient [Ca<sup>2+</sup>]<sub>i</sub> fluxes in NG 108-15 (neuroblastoma  $\times$  glioma hybrid cells), but this action was reversed by 1  $\mu$ M SR141716A (Sugiura *et al.*, 1999), indicating CB<sub>1</sub> involvement. In addition to its agonist actions at TRPV1 (Zygmunt *et al.*, 1999; Smart *et al.*, 2000), anandamide (1  $\mu$ M) evokes an increase in intracellular Ca<sup>2+</sup> concentrations in DRG cells, which is unaffected by capsazepine but blocked by 1  $\mu$ M SR141716A (Millns *et al.*, 2002). However, in the cultured DRG cells, noladin ether (1  $\mu$ M) had no effect alone, and so does not appear to interact with CB<sub>1</sub> or TRPV1 receptors.

HU210 has been reported to inhibit the capsaicin-evoked Ca<sup>2+</sup> response *via* a CB<sub>1</sub>-like receptor in cultured DRG cells (Millns *et al.*, 2001); to determine if noladin ether modulates TRPV1 in a similar manner to HU210, the effects of noladin ether on the capsaicin-evoked Ca<sup>2+</sup> response in DRG cells were studied. Noladin ether, however, had no significant effect on the capsaicin-evoked calcium response. Noladin ether also had no significant effect on TRPV1-mediated relaxation to capsaicin in the mesenteric bed. Thus, while noladin ether mimics the antagonist-resistant actions of HU210 on capsaicin-sensitive sensory nerves in the mesenteric bed, it does not interact with the HU210-sensitive CB receptor in DRG cells.

Noladin ether-activated [<sup>35</sup>S]GTP $\gamma$ S binding in rat brain membranes indicated the presence of CB receptors in the hippocampus, globus pallidus, substantia nigra and the molecular layer of the cerebellum (Savinainen *et al.*, 2001). Binding in all brain regions was reversible by SR141716A, indicating that, in the brain, noladin ether acts at CB<sub>1</sub> rather than novel CB receptors (Savinainen *et al.*, 2001). Noladin ether has been reported to occur naturally in the pig brain (0.6 nmol g<sup>-1</sup> brain; Hanuš *et al.*, 2001) and rat brain (25.4 pmol g<sup>-1</sup> brain; Fezza *et al.*, 2002). However, Oka *et al.* (2003) recently reported that no appreciable amounts of noladin ether (<0.2 pmol g<sup>-1</sup> brain) could be detected in the rat, mouse, hamster, guinea pig and porcine brain, casting doubt on its role as an endocannabinoid. Levels of noladin ether in the periphery have not been measured, but the presence of the noladin ether-sensitive CB-like receptors that we have pharmacologically characterised in perivascular capsaicin-sensitive sensory nerves in the rat mesentery indicates a possible role in physiological or pathophysiological conditions. However, a more definitive characterisation of noladin ether as a member of the endocannabinoid messenger family must await analytical confirmation of its presence in the peripheral and/or central nervous system.

Our previous studies have demonstrated that WIN55,212 and CP55,940 inhibit sensory neurotransmission in the rat isolated mesenteric arterial bed, in an SR141716A- and LY320135-sensitive manner (Duncan *et al.*, 2001a, b), indicating the presence of a CB<sub>1</sub>-like receptor. Noladin ether, THC and HU210 also act at CB<sub>1</sub> receptors, and there is molecular



and pharmacological evidence for CB<sub>1</sub> receptors in sensory nerves; however, the evidence suggests that these CBs are acting at a novel receptor present in the sensory nerves. The

data from the present study suggest that noladin ether exerts inhibitory actions *via* a non-CB<sub>1</sub>/CB<sub>2</sub>, PTX-sensitive prejunctional receptor in the rat mesenteric bed.

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