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RESEARCH PAPER

'Entourage' effects of *N*-palmitoylethanolamide and *N*-oleoylethanolamide on vasorelaxation to anandamide occur through TRPV1 receptors

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Background and purpose: The endocannabinoid *N*-arachidonoylethanolamide (anandamide) is co-synthesized with other *N*-acylethanolamides, namely *N*-palmitoylethanolamide (PEA) and *N*-oleoylethanolamide (OEA), which have been shown to potentiate anandamide responses (so-called 'entourage effects') in non-vascular tissues. It remains unclear whether such interactions occur in the circulation.
Experimental approach: In rat isolated small mesenteric arteries, the effects of PEA and OEA on relaxation to anandamide and tissue contents of the *N*-acylethanolamides were examined under myographic conditions.
Key results: Anandamide-induced relaxation was potentiated by pretreatment with PEA (10 μM) or OEA (1 μM), or in combination. The potentiation by PEA and OEA was endothelium-independent and abolished by treatment with capsaicin (10 μM), which desensitizes the transient receptor potential vanilloid type 1 (TRPV1) receptor system, or by the TRPV1 receptor antagonist, *N*-(3-methoxyphenyl)-4-chlorocinnamide (SB366791) (2 μM). It was also observed at molar ratios of anandamide and PEA (or OEA) similar to those found in mesenteric arteries. PEA and inhibition of anandamide hydrolysis by 3′-carbamoyl-biphenyl-3-yl-cyclohexylcarbamate (URB597) (1 μM) additively potentiated anandamide responses. On the other hand, PEA and OEA also induced vasorelaxation *per se* (rank order of potency: anandamide > OEA > PEA), but relaxation to the three *N*-acylethanolamides displayed different sensitivity to treatment with capsaicin, SB366791 and URB597. For example, relaxations to anandamide and OEA, but not PEA, were attenuated by both capsaicin and SB366791.
Conclusion and implications: This study shows that PEA and OEA potentiate relaxant responses to anandamide through TRPV1 receptors in rat small mesenteric arteries. The congeners also induce vasorelaxation *per se*, suggesting a function for the *N*-acylethanolamides in vascular control.
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Keywords: anandamide; palmitoylethanolamide; oleoylethanolamide; fatty acid amide hydrolase; TRPV1 receptor; entourage effect; rat mesenteric artery

Abbreviations: FAAH, fatty acid amide hydrolase; OEA, *N*-oleoylethanolamide; PEA, *N*-palmitoylethanolamide; SB366791, *N*-(3-methoxyphenyl)-4-chlorocinnamide; TRPV1, transient receptor potential vanilloid type 1; URB597, 3′-carbamoyl-biphenyl-3-yl-cyclohexylcarbamate

Introduction

The bioactive lipid *N*-arachidonoylethanolamide (anandamide) was identified as an endogenous agonist for cannabinoid receptors (endocannabinoid) in 1992 (Devane *et al.*, 1992). Studies measuring tissue contents or release of anandamide from leukocytes (Bisogno *et al.*, 1997), brain, liver and intestine (Fegley *et al.*, 2005) have since revealed that production of anandamide (which contains an arachidonyl residue derived from a 20-carbon fatty acid with four double bonds, referred to as C20:4) is often accompanied by substantially higher amounts of *N*-acylethanolamides derived from other fatty acids, particularly *N*-palmitoylethanolamide (PEA; C16:0) and *N*-oleoylethanolamide (OEA; C18:1). It is thought that anandamide is primarily produced from hydrolysis of a minor membrane phospholipid derived from arachidonic acid, *N*-arachidonoylphosphatidylethanolamine, and subsequent metabolism through a novel *N*-acylphosphotidylethanolamine-phospholipase D (Schmid *et al.*, 1990; Di Marzo *et al.*, 1994). Similar biosynthetic pathways using the fatty acids, palmitic acid and oleic acid have also been suggested to produce PEA and OEA,

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respectively (Hansen *et al*., 2000; Okamoto *et al*., 2004). Both PEA and OEA are bioactive; indeed, PEA has long been known to act as an anti-inflammatory and antinociceptive agent (Re *et al*., 2007), whereas recent studies indicate that OEA regulates feeding and body weight (Lo Verme *et al*., 2005; Matias *et al*., 2007). However, unlike anandamide, PEA and OEA have no agonist activity at cannabinoid CB_1 and CB_2 receptors (Sheskin *et al*., 1997; Lambert *et al*., 1999; Griffin *et al*., 2000). Thus, the finding that the CB_2 receptor antagonist, SR144528 inhibits some of the analgesic responses to PEA *in vivo* (Calignano *et al*., 1998; Farquhar-Smith *et al*., 2002) has led to the suggestion that PEA either directly activates an unidentified CB_2-like receptor or acts indirectly by potentiation of endocannabinoid actions. The latter case has been referred to as 'entourage' effects, which can be achieved by enhancing the action of endogenous anandamide through an increase in the affinity for receptors and/or a decrease in enzymic degradation of anandamide (Ben-Shabat *et al*., 1998; Mechoulam *et al*., 1998; Lambert and Di Marzo, 1999). Anandamide is primarily degraded by fatty acid amide hydrolase (FAAH) through hydrolysis into arachidonic acid and ethanolamine (Deutsch and Chin, 1993; Cravatt *et al*., 1996) and inhibition of FAAH activity enhances or prolongs responses to anandamide *in vitro* and *in vivo* (Childers *et al*., 1994; Ross *et al*., 2001; Pacher *et al*., 2005; Ho and Randall, 2007). Two human isoforms of FAAH (FAAH-1 and FAAH-2) have recently been identified but only FAAH-1 is expressed in rodents (Wei *et al*., 2006). Both enzymes hydrolyse anandamide, PEA and OEA (with distinct substrate preference) and are sensitive to a commonly used, selective FAAH inhibitor 3′-carbamoyl-biphenyl-3-yl-cyclohexylcarbamate (URB597) (Wei *et al*., 2006). By serving as an alternative substrate for FAAH, PEA could reduce FAAH-mediated catabolism of anandamide (Bisogno *et al*., 1997; Jonsson *et al*., 2001). Similarly, OEA has also been shown to reduce anandamide degradation by substrate competition for FAAH (Maurelli *et al*., 1995; Jonsson *et al*., 2001). However, there is less evidence for OEA acting as an 'entourage' compound as OEA induces opposing effects on food intake and weight control compared with anandamide (Fu *et al*., 2003).

Another possible point of interaction between anandamide and its congeners is the transient receptor potential vanilloid type 1 (TRPV1) receptor. The TRPV1 receptor, a non-selective cation channel expressed in small diameter sensory neurons, is activated by noxious heat, low pH and capsaicin, the pungent ingredient of chilli peppers (Caterina *et al*., 1997). It is now known that anandamide is also an agonist for TRPV1 receptors (Zygmunt *et al*., 1999; Smart *et al*., 2000), leading to the suggestion that it is both an endocannabinoid and an endovanilloid. Activation of TRPV1 receptors on perivascular sensory nerves leads to the release of vasodilator neuropeptides such as calcitonin gene-related peptide, which in turn causes vasorelaxation (Franco-Cereceda and Rudehill, 1989; Zygmunt *et al*., 1999). This mechanism has been shown to mediate partly the relaxant responses to anandamide in certain vascular regions, particularly in mesenteric arteries (Jarai *et al*., 1999; Zygmunt *et al*., 1999; White *et al*., 2001), and contribute to some of the hypotensive effects of anandamide *in vivo* (Wang *et al*., 2005). Thus, anandamide-induced relaxation is attenuated by TRPV1 receptor antagonists or by prolonged treatment with capsaicin. Capsaicin is commonly used to cause functional desensitization of the TRPV1 receptor system owing to rapid receptor desensitization, depletion of neuropeptide stores and/or possibly irreversible damage to neurons resulting from the excessive cation influx through the TRPV1 receptor (for review, see Szallasi and Blumberg, 1999). There is, however, some controversy over the function of anandamide as an endovanilloid, mainly owing to its relatively low potency in expression systems (EC_{50}: 1–4 μM; Di Marzo *et al*., 2001). Nonetheless, a higher potency of anandamide towards TRPV1 receptors has been observed in native tissues. This effect is possibly due to the presence of a larger receptor reserve and/or a potential involvement with endogenous regulators of channel activity, some of which could act through protein kinases C and A and subsequent TRPV1 receptor phosphorylation (Premkumar and Ahern, 2000; De Petrocellis *et al*., 2001b; Vellani *et al*., 2001). Of particular interest are the observations that PEA and OEA potentiate the effects of anandamide at TRPV1 receptors in expressed cells (Smart *et al*., 2000, 2002; De Petrocellis *et al*., 2001a; Vandevoorde *et al*., 2003). Interestingly, OEA, but not PEA, is also a TRPV1 receptor agonist with potency similar to that of anandamide (Ahern, 2003; Movahed *et al*., 2005). Taken together, these findings raise the possibility that endogenously produced anandamide, PEA and OEA act in concert as direct, or indirect, activators of TRPV1 receptors.

Emerging evidence suggests that anandamide is an effective modulator of vascular tone and has a function in cardiovascular regulation (for review, see Randall *et al*., 2004). However, the vascular actions of its congeners, particularly their potential interaction with anandamide, remain unclear. Recently, we have found that FAAH inhibitors including URB597 potentiate relaxant responses to anandamide in rat small mesenteric arteries, suggesting a role for local metabolism in the regulation of endocannabinoid signalling in the circulation (Ho and Randall, 2007). Moreover, given that mesenteric relaxation to anandamide is partly mediated by TRPV1 receptors, PEA and OEA could act as 'entourage' compounds by interacting with TRPV1 receptors as well as FAAH. Therefore, using the rat isolated small mesenteric artery, this study aimed to examine (a) the influence of PEA and OEA on anandamide-induced relaxation; (b) the involvement of FAAH and TRPV1 receptors in the interaction between anandamide and its congeners and (c) the vascular content of *N*-acylethanolamides.

Methods

Myographic studies

All animal care and use was in accordance with the UK Animal (Scientific Procedures) Act 1986. Male Wistar rats (200–350 g; Charles River UK Ltd, Kent, UK) were stunned by a blow to the back of their neck and killed by cervical dislocation. The third-order branches of the superior mesenteric artery were removed and cleaned of adherent tissue. Segments (2 mm in length) were mounted in a Mulvany–

Halpern type wire myograph (Model 610 M; Danish Myo Technology, Aarhus, Denmark) and maintained at 37 °C in gassed (95% O_2/5% CO_2) modified Krebs–Henseleit solution of the following composition (mM): NaCl 118, KCl 4.7, $MgSO_4$ 1.2, KH_2PO_4 1.2, $NaHCO_3$ 25, $CaCl_2$ 2, D-glucose 10) as previously described (Ho and Hiley, 2003). Vessels were equilibrated and set to a basal tension of 2–2.5 mN. The integrity of the endothelium was assessed by precontracting the vessel with 10 μM methoxamine (an α_1-adrenoceptor agonist), followed by relaxation with 10 μM carbachol (a muscarinic ACh receptor agonist); vessels showing relaxations of greater than 90% were designated as endothelium intact. When endothelium was not required, it was removed by rubbing the intima with a human hair; carbachol-induced relaxation of less than 10% indicated successful removal.

Nomenclature of molecular targets, including receptors and ion channels, is used in accordance with the Guide to Receptor and Channels, *British Journal of Pharmacology* (Alexander *et al*., 2008).

Experimental protocols

After the test for endothelial integrity, vessels were left for 30 min and then precontracted with 10 μM methoxamine. This procedure was then followed by construction of cumulative concentration–relaxation curves to anandamide, PEA or OEA. In this study, most experiments were performed in matched vessels; effects of putative modulators or endothelial removal were compared with the control responses obtained in separate vessels taken from the same rat.

To investigate the influence of *N*-acylethanolamide congeners on anandamide, PEA (at 10 μM) or OEA (at 1 or 3 μM), or in combination, was added to the myograph bath 5 min before, and was maintained during the construction of the concentration–response curve of an endocannabinoid. In some experiments, an FAAH inhibitor (URB597; 45 min incubation) or a TRPV1 receptor antagonist (capsazepine or *N*-(3-methoxyphenyl)-4-chlorocinnamide (SB366791); 20 min incubation) was also added before determination of relaxant responses to anandamide, PEA or OEA. In cases where capsaicin was used to desensitize the TRPV1 receptor system, it was incubated with the vessels for 60 min followed by a wash-out period (White *et al*., 2001).

In vessels pretreated with a single, micromolar concentration of PEA or OEA, an increased concentration (20 μM) of methoxamine was used, where required, to obtain a similar level of tone to that evoked in the absence of the two *N*-acylethanolamides. The tension generated in the test for endothelial integrity was 10.9 ± 0.4 mN, as compared with 11.8 ± 0.4 mN (88 vessels) in the presence of PEA or OEA ($P > 0.05$, unpaired Student's *t*-test).

Detection of anandamide, PEA and OEA

The second- and third-order branches of the superior mesenteric artery were obtained from male Wistar rats as described above. Vessel segments (approximately 5 mm in length) from the same rats were incubated with either URB597 or its vehicle for 45 min at 37 °C in gassed (95% O_2/5% CO_2) modified Krebs–Henseleit solution, then blotted dry and kept at −80 °C until further analysis. The samples were thawed on ice and weighed (1.82 ± 0.21 mg, $n = 10$) immediately before analysis. Vessels were homogenized in ethyl acetate/hexane (9:1 v/v, 1.2 mL) with internal standard (0.42 nmol [$^2H_8$]-anandamide; Axxora, Nottingham, UK), then vortexed for 10 min and centrifuged at 7000 *g* for 10 min at 4 °C. The extraction process was repeated twice, and the supernatant was pooled and dried under vacuum evaporator at room temperature (25 °C). Once dried, samples were reconstituted in 60 μL of acetonitrile. Anandamide, PEA and OEA were quantified by liquid chromatography/electrospray ionization tandem mass spectrometry as described previously (Richardson *et al*., 2007).

Data and statistical analysis

All relaxant responses are expressed as percentage relaxation of the tone induced by methoxamine. Values are given as mean ± s.e.mean and *n* represents the number of animals used. E_{max} represents the maximum effect and pEC_{50} represents the negative logarithm of the concentration of relaxant giving half the maximal relaxation; these values were determined directly from individual log concentration–response curves. Statistical comparisons of concentration–response curves were made by two-way ANOVA (Prism 4; GraphPad Software Inc., San Diego, CA, USA) of the whole data set. Tissue contents of *N*-acylethanolamides in vehicle- vs URB597-treated vessels were compared by Wilcoxon signed rank, paired tests (Prism 4; GraphPad Software Inc.). $P \leqslant 0.05$ was taken as statistically significant.

Drugs

Methoxamine and carbachol (Sigma Chemical Co., Poole, UK) were dissolved in deionized water. Anandamide (*N*-arachidonoylethanolamide; Tocris Bioscience, Bristol, UK) was supplied in Tocrisolve 100 (1:4 soya/water emulsion) and diluted with deionized water. PEA and OEA (Tocris) were dissolved in 100% ethanol and diluted with deionized water. URB597 (Cayman Chemical, Ann Arbor, MI, USA), capsazepine, SB366791 and capsaicin (Sigma Chemical Co.) were dissolved in 100% ethanol.

Results

Relaxation to PEA and OEA

PEA induced a concentration-dependent relaxation of rat isolated mesenteric arteries ($pEC_{50} = 5.3 \pm 0.2$, $E_{max} = 72 \pm 10\%$, $n = 5$). Removal of the endothelium substantially reduced the relaxations ($E_{max} = 18 \pm 4\%$, $n = 5$; $P < 0.01$). OEA also induced mesenteric relaxation, and endothelium removal significantly reduced the potency but not the maximal effect of OEA-induced relaxations (with endothelium, $pEC_{50} = 5.7 \pm 0.1$, $E_{max} = 100 \pm 1\%$, $n = 6$; without endothelium, $pEC_{50} = 5.1 \pm 0.1$, $E_{max} = 91 \pm 3\%$, $n = 5$; $P < 0.01$).

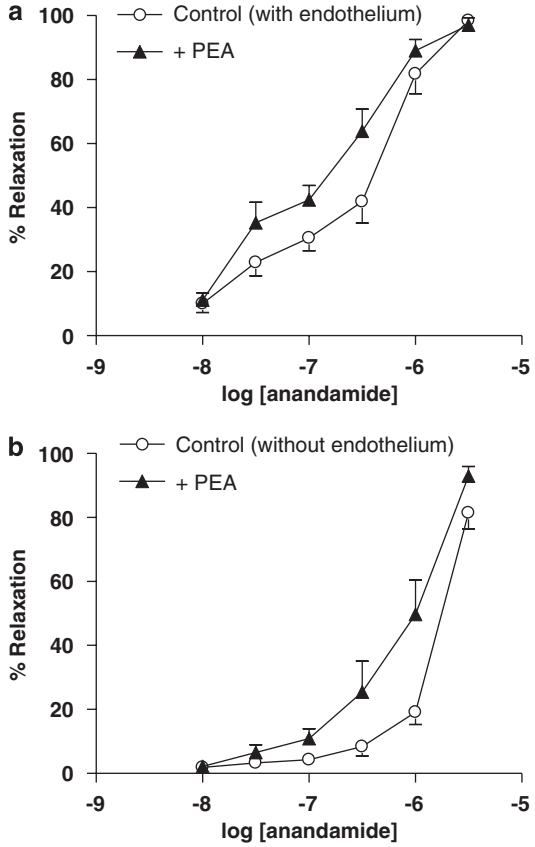

Figure 1 Effects of *N*-palmitoylethanolamide (PEA) (10 μM) on relaxation to anandamide in endothelium-intact (**a**) or endothelium-denuded (**b**) mesenteric arteries. $n=5$–6. Values are shown as means and vertical bars represent s.e.mean.

Effects of PEA on relaxation to anandamide and the involvement of FAAH

PEA (10 μM) significantly potentiated relaxation to anandamide in endothelium-intact (control, $pEC_{50}=6.5.\pm0.1$, $E_{max}=98\pm1\%$, $n=6$; with PEA, $pEC_{50}=6.9\pm0.2$, $E_{max}=97\pm2\%$, $n=6$; $P<0.01$; Figure 1a) and endothelium-denuded vessels (control, $pEC_{50}=5.8\pm0.1$, $E_{max}=81\pm5\%$, $n=5$; with PEA, $pEC_{50}=6.1\pm0.1$, $E_{max}=93\pm3\%$, $n=6$; $P<0.01$; Figure 1b). As reported previously (Ho and Randall, 2007), the FAAH inhibitor, URB597 (1 μM) potentiated anandamide relaxations in endothelium-intact vessels (control, $pEC_{50}=6.4\pm0.1$, $E_{max}=98\pm1\%$, $n=6$; with URB597, $pEC_{50}=6.9\pm0.2$, $E_{max}=93\pm6\%$, $n=7$; $P<0.01$). Interestingly, further addition of PEA caused an additive, potentiation effect (with URB597 and PEA, $pEC_{50}=7.2\pm0.2$, $E_{max}=99\pm1\%$, $n=5$; $P<0.01$ vs control or with URB597 alone; Figure 2).

In endothelium-intact vessels, URB597 (1 μM) also potentiated PEA-induced relaxation (control, $pEC_{50}=5.3\pm0.2$, $E_{max}=76\pm9\%$, $n=6$; with URB597, $pEC_{50}=5.7\pm0.3$, $E_{max}=81\pm5\%$, $n=6$; $P<0.05$). However, URB597 (1 μM) had no effect on the residual relaxations to PEA in endothelium-denuded vessels (with URB597, $E_{max}=26\pm3\%$, $n=4$).

Effects of OEA on relaxation to anandamide

In endothelium-intact vessels, anandamide-induced relaxation, notably at concentrations <300 nM, were potentiated

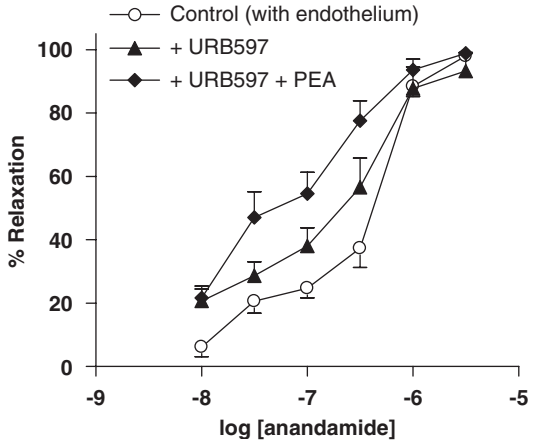

Figure 2 Effects of combined addition of 3′-carbamoyl-biphenyl-3-yl-cyclohexylcarbamate (URB597) (1 μM) and *N*-palmitoylethanolamide (PEA) (10 μM) on relaxation to anandamide in endothelium-intact mesenteric arteries. $n=5$–7. Values are shown as means and vertical bars represent s.e.mean.

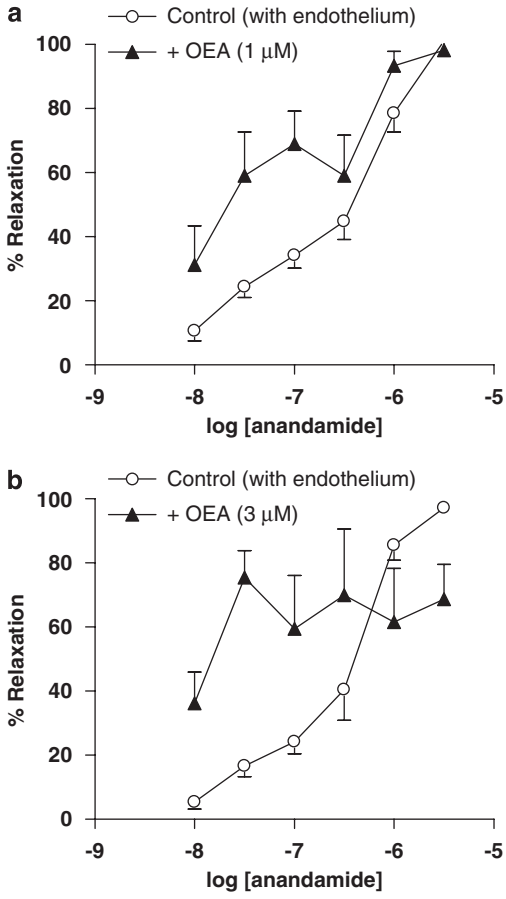

Figure 3 Effects of *N*-oleoylethanolamide (OEA) on relaxation to anandamide in endothelium-intact mesenteric arteries. Relaxation was elicited by anandamide alone, or in the presence of 1 μM OEA (**a**) or 3 μM OEA (**b**). $n=6$ for all. Values are shown as means and vertical bars represent s.e.mean.

by 1 μM OEA (control, $pEC_{50}=6.5.\pm0.1$, $E_{max}=101\pm1\%$, $n=7$; with OEA, $pEC_{50}=7.4\pm0.3$, $E_{max}=98\pm2\%$, $n=7$; $P<0.01$; Figure 3a). A higher concentration of OEA (3 μM)

also potentiated relaxation to (<300 nM) anandamide but, in two out of seven vessels, tachyphylaxis occurred at 100 nM anandamide (control, $pEC_{50} = 6.4 \pm 0.1$, $E_{max} = 97 \pm 1\%$, $n = 6$; with OEA, $pEC_{50} = 7.7 \pm 0.3$, $E_{max} = 98 \pm 1\%$, $n = 6$; $P < 0.01$; Figure 3b). Interestingly, in endothelium-denuded vessels, 1 μM OEA potentiated relaxation at lower concentrations and attenuated maximal relaxation to anandamide (control, $pEC_{50} = 6.0 \pm 0.1$, $E_{max} = 97 \pm 3\%$, $n = 5$; with OEA, $pEC_{50} = 7.0 \pm 0.2$, $E_{max} = 70 \pm 9\%$, $n = 5$; $P < 0.01$ interaction of OEA treatment and anandamide concentrations as analysed by two-way ANOVA).

Relaxations induced by the TRPV1 receptor agonist capsaicin were slightly potentiated by OEA (1 μM) but not PEA (10 μM; data not shown).

Involvement of TRPV1 receptors in the effects of PEA

Prolonged treatment with capsaicin (10 μM), which causes functional desensitization of the TRPV1 receptor system, significantly reduced the maximal relaxation to PEA (with endothelium: control, $EC_{50} = 5.3. \pm 0.2$, $E_{max} = 84 \pm 6\%$, $n = 5$; after capsaicin, $pEC_{50} = 5.3 \pm 0.1$, $E_{max} = 53 \pm 8\%$, $n = 5$; $P < 0.01$; Figure 4a), but the TRPV1 receptor antagonist, SB366791 (2 μM) had no significant effect (with SB366791, $E_{max} = 78 \pm 10\%$, $n = 5$).

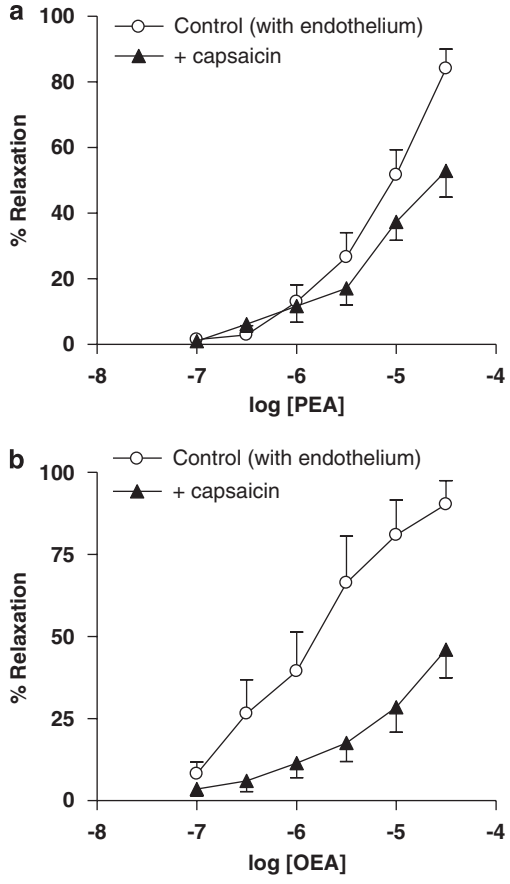

Figure 4 Effects of capsaicin (10 μM) on relaxation to *N*-palmitoylethanolamide (PEA) (**a**) and *N*-oleoylethanolamide (OEA) (**b**) in endothelium-intact mesenteric arteries. $n = 5$–6. Values are shown as means and vertical bars represent s.e.mean.

However, treatment with capsaicin (10 μM) greatly inhibited anandamide-induced relaxation (with endothelium: control, $pEC_{50} = 6.6 \pm 0.1$, $E_{max} = 101 \pm 1\%$, $n = 4$; after capsaicin, $pEC_{50} = 6.9 \pm 0.4$, $E_{max} = 44 \pm 15\%$, $n = 4$; $P < 0.01$; Figure 5a). Application of PEA (10 μM) after capsaicin treatment failed to potentiate relaxations to anandamide (with PEA and capsaicin, $pEC_{50} = 6.8 \pm 0.3$, $E_{max} = 45 \pm 6\%$, $n = 4$; $P < 0.01$ vs control; Figure 5a). Similarly, SB366791 (2 μM) abolished the potentiation by PEA of anandamide responses (with endothelium: control, $EC_{50} = 6.8 \pm 0.1$, $E_{max} = 99 \pm 1\%$, $n = 6$; with SB366791, $pEC_{50} = 6.4 \pm 0.2$, $E_{max} = 80 \pm 7\%$, $n = 6$; $P < 0.01$; with PEA and SB366791, $pEC_{50} = 6.4 \pm 0.2$, $E_{max} = 82 \pm 9\%$, $n = 6$; $P < 0.01$ vs control; Figure 5b). Interestingly, we found that capsazepine (3 μM), another TRPV1 receptor antagonist, attenuated anandamide-induced relaxations but not the potentiation effect of PEA (with endothelium: control, $pEC_{50} = 6.5 \pm 0.1$, $E_{max} = 100 \pm 2\%$, $n = 6$; with capsazepine, $pEC_{50} = 6.2 \pm 0.1$,

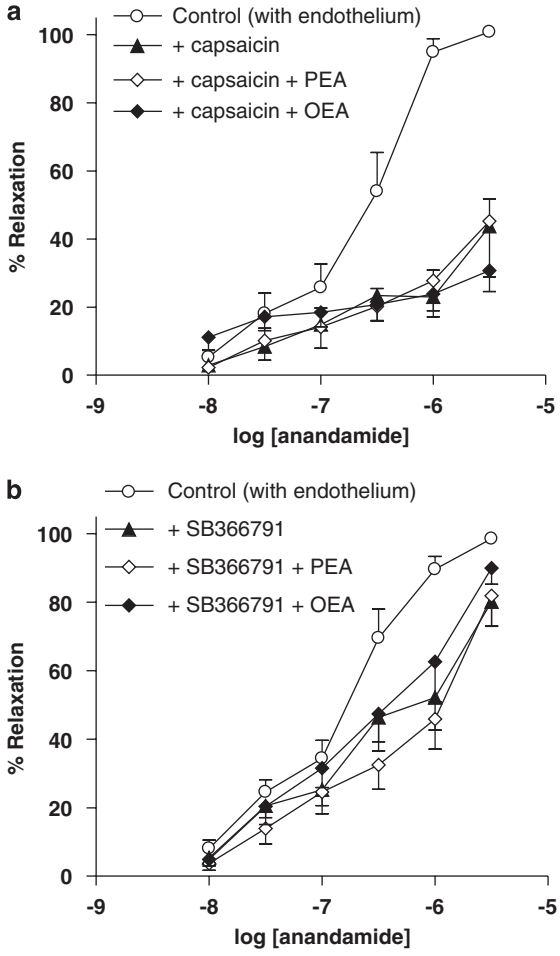

Figure 5 Effects of transient receptor potential vanilloid type 1 (TRPV1) receptor inhibitors on interactions between anandamide and its congeners in endothelium-intact mesenteric arteries. (**a**) Relaxation was elicited by anandamide alone, or after treatment with capsaicin (10 μM), or capsaicin plus *N*-palmitoylethanolamide (PEA) (10 μM) or *N*-oleoylethanolamide (OEA) (1 μM). $n = 4$ for all. (**b**) Relaxation was elicited by anandamide alone, or with *N*-(3-methoxyphenyl)-4-chlorocinnamide (SB366791) (2 μM), or SB366791 plus PEA (10 μM) or OEA (1 μM). $n = 6$ for all. Values are shown as means and vertical bars represent s.e.mean.

$E_{max} = 98 \pm 1\%$, $n = 4$; $P < 0.01$; with PEA and capsazepine, $pEC_{50} = 6.5 \pm 0.2$, $E_{max} = 91 \pm 4\%$, $n = 5$).

Involvement of TRPV1 receptors and FAAH in the effects of OEA
Relaxation induced by OEA was greatly inhibited by treatment with capsaicin (control, $pEC_{50} = 5.8. \pm 0.2$, $E_{max} = 90 \pm 7\%$, $n = 6$; after 10 μM capsaicin, $pEC_{50} = 5.3 \pm 0.2$, $E_{max} = 46 \pm 9\%$, $n = 6$; $P < 0.01$; Figure 4b) or SB366791 (control, $pEC_{50} = 6.0 \pm 0.1$, $E_{max} = 101 \pm 1\%$, $n = 5$; with 2 μM SB366791, $pEC_{50} = 5.6 \pm 0.3$, $E_{max} = 93 \pm 6\%$, $n = 5$; $P < 0.01$). However, unlike the case for anandamide, URB597 (1 μM) had no significant effect on relaxations to OEA in either endothelium-intact (with URB597, $pEC_{50} = 5.8 \pm 0.3$, $E_{max} = 98 \pm 1\%$, $n = 5$) or endothelium-denuded vessels (with URB597, $pEC_{50} = 5.3 \pm 0.1$, $E_{max} = 80 \pm 11\%$, $n = 4$).

The potentiation effect of OEA on anandamide-induced relaxation was blocked by treatment with capsaicin (endothelium-intact vessels: with capsaicin and OEA, $pEC_{50} = 7.6 \pm 0.6$, $E_{max} = 31 \pm 6\%$, $n = 4$; $P < 0.01$ vs control; Figure 5a) or SB366791 (endothelium-intact vessels: with SB366791 and OEA, $pEC_{50} = 6.5 \pm 0.2$, $E_{max} = 90 \pm 5\%$, $n = 6$; $P < 0.01$ vs control; Figure 5b). Another TRPV1 receptor antagonist, capsazepine also attenuated the potentiation induced by OEA (data not shown).

Effects of combined additions of PEA and OEA on relaxation to anandamide
In endothelium-intact vessels, the combination of PEA (10 μM) and OEA (1 μM) significantly potentiated anandamide-induced relaxation (control, $pEC_{50} = 6.7. \pm 0.2$, $E_{max} = 97 \pm 1\%$, $n = 5$; with PEA and OEA, $pEC_{50} = 7.7 \pm 0.2$, $E_{max} = 96 \pm 1\%$, $n = 5$; $P < 0.01$; Figure 6a). Similar observation was obtained for endothelium-denuded vessels (control, $pEC_{50} = 6.4 \pm 0.2$, $E_{max} = 98 \pm 1\%$, $n = 4$; with PEA and OEA, $pEC_{50} = 7.6 \pm 0.2$, $E_{max} = 89 \pm 6\%$, $n = 5$; $P < 0.01$; Figure 6b). It was noted that the effects of PEA and OEA were additive, at least in denuded vessels ($P < 0.01$ vs with PEA or OEA alone).

Vascular content of anandamide, PEA and OEA
Tissue content of anandamide, PEA and OEA were detected in isolated mesenteric arteries (Table 1). Treatment with URB597 (1 μM) significantly increased the vascular content of anandamide ($P = 0.05$), but not that of PEA or OEA.

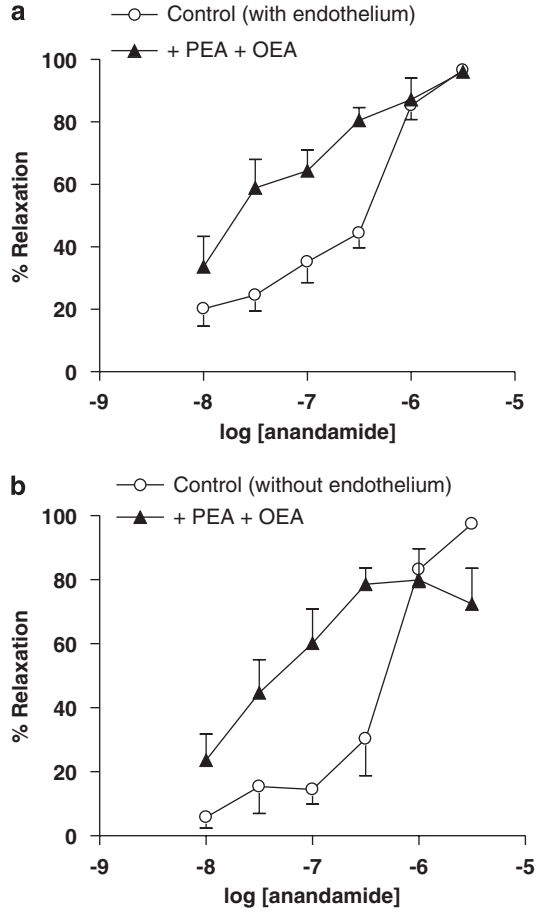

Figure 6 Effects of combined addition of *N*-palmitoylethanolamide (PEA) (10 μM) and *N*-oleoylethanolamide (OEA) (1 μM) in endothelium-intact (**a**) or endothelium-denuded (**b**) mesenteric arteries. $n = 4$–5. Values are shown as means and vertical bars represent s.e.mean.

Table 1 Content of anandamide, PEA and OEA in rat mesenteric arteries

	Anandamide (pmol g^{-1})	*PEA (nmol g^{-1})*	*OEA (nmol g^{-1})*
Vehicle	23.7 ± 6.1	4.1 ± 0.9	2.2 ± 1.1
URB597	54.8 ± 19.8*	5.0 ± 0.8	2.9 ± 1.5

Abbreviations: OEA, *N*-oleoylethanolamide; PEA, *N*-palmitoylethanolamide; URB597, 3′-carbamoyl-biphenyl-3-yl-cyclohexylcarbamate.
*$P = 0.05$ significantly different from data in vehicle-treated vessels.
Vascular contents of the *N*-acylethanolamides were determined after incubation with URB597 (1 μM) or its vehicle (see Methods). $n = 10$ for all.

Discussion and conclusions

The major finding of this study is that PEA and OEA, which are more abundant than anandamide, enhance anandamide-induced relaxation through TRPV1 receptors in rat isolated small mesenteric arteries. The *N*-acylethanolamide congeners also exert direct relaxant effects, although with lower potencies than anandamide.

Although PEA and OEA do not activate cannabinoid receptors (Sheskin *et al*., 1997; Lambert *et al*., 1999; Griffin *et al*., 2000), they have been shown to exert pharmacological effects such as antinociception (PEA: Calignano *et al*., 1998) and satiety (OEA: Fu *et al*., 2003). In rat isolated small mesenteric arteries, PEA and OEA induced concentration-dependent vasorelaxation. The rank order of potency for relaxation in endothelium-intact mesenteric arteries is anandamide (pEC_{50}: 6.5) > OEA (pEC_{50}: 5.7) > PEA (pEC_{50}: 5.2). Interestingly, PEA (10 μM) also potentiated the relaxant response to anandamide, resulting in a leftward shift of the concentration–response curve to anandamide. As PEA is a substrate for the primary degradation enzyme for anandamide, FAAH, it could compete with anandamide for FAAH

leading to a reduction in anandamide hydrolysis. Indeed, 10 μM PEA has been found to inhibit anandamide hydrolysis by 40–50% in rat brain membranes (Jonsson *et al*., 2001; Smart *et al*., 2002). In this study, we confirmed that mesenteric relaxation to anandamide is potentiated by the FAAH inhibitor, URB597 (this study; Ho and Randall, 2007), indicative of local FAAH activity (presumably FAAH-1) in the vascular wall. Similarly, URB597 slightly enhanced relaxation to PEA and thus FAAH-mediated metabolism also limits the vascular action of PEA. However, it seems unlikely that FAAH mediates the interaction between PEA and anandamide. First, URB597 potentiated the relaxations to anandamide (Ho and Randall, 2007) and PEA (this study) in endothelium-intact but not endothelium-denuded mesenteric arteries, pointing to FAAH activity in the endothelium. In contrast, the potentiation effect of PEA was endothelium-independent. Second, the combination of URB597 and PEA elicited a larger potentiation than URB597 alone. An alternative target for PEA is TRPV1 receptors on perivascular sensory nerves, which are found at high density in the mesenteric artery (Zygmunt *et al*., 1999). It is now established that anandamide is an agonist of TRPV1 receptors, activation of which partly mediate mesenteric relaxations to anandamide (Zygmunt *et al*., 1999; White *et al*., 2001). Thus, we found that prolonged treatment with capsaicin, which causes functional desensitization of the TRPV1 receptor system (Szallasi and Blumberg, 1999), and the presence of TRPV1 receptor antagonists attenuated anandamide relaxations in rat small mesenteric arteries. Previous studies have suggested that although PEA has no (Zygmunt *et al*., 1999) or small direct effects on TRPV1 receptors in expressed cells (at 10 μM: approximately 15% of maximal response to anandamide; De Petrocellis *et al*., 2001a; Smart *et al*., 2002; Vandevoorde *et al*., 2003), it could increase the potency of anandamide at TRPV1 receptors. Indeed, treatment with capsaicin and the TRPV1 receptor antagonist, SB366791 had minimal effect on the relaxation to PEA but abolished the potentiation by PEA on anandamide-induced relaxation. These results strongly support the involvement of TRPV1 receptors in the interaction between anandamide and PEA, even though PEA causes very little direct, activation of TRPV1 receptors. One possible explanation is that PEA acts as an allosteric modulator, enhancing the binding of anandamide at TRPV1 receptors in mesenteric arteries. This hypothesis is supported by radioligand binding data, suggesting that PEA binds to an allosteric site on TRPV1 receptors that is distinct from the intracellular binding site for anandamide and capsaicin, and thereby enhances the binding of anandamide to the receptor (De Petrocellis *et al*., 2001a; Ross *et al*., 2001). It was noted that capsazepine, another antagonist for TRPV1 receptors had no effect on the potentiation induced by PEA even though it significantly attenuated anandamide relaxations. This effect could be due to the lower affinity of capsazepine than SB366791 for TRPV1 receptors (Gunthorpe *et al*., 2004), or the allosteric action of PEA as capsazepine has been shown to compete for the binding site for anandamide (De Petrocellis *et al*., 2001a; Ross *et al*., 2001) and show modality-dependent antagonist activity at TRPV1 receptors (Savidge *et al*., 2001).

In contrast to PEA, OEA has been shown to activate TRPV1 receptors directly, though it might require protein kinase C activity in some tissues (Ahern, 2003; Movahed *et al*., 2005). Thus, capsaicin treatment and SB366791 greatly reduced mesenteric relaxation to OEA, as they did for anandamide. So perhaps it is not surprising that OEA (at 1 or 3 μM) also potentiated responses to anandamide in a capsaicin- and SB366791-sensitive manner, possibly by increasing the excitability of TRPV1 receptor-expressing perivascular sensory nerves. Interestingly, the potentiation by OEA was more notable at lower concentrations of anandamide and, in two vessels, pretreatment with 3 μM OEA also promoted tachyphylaxis of anandamide responses. This bidirectional influence of OEA on anandamide responses was also evident in endothelium-denuded vessels, where OEA tended to augment relaxations to anandamide at lower concentrations and reduced the maximal relaxation achieved. At present, the significance of these observations remains unclear. It is possible that high concentrations of OEA could limit the extent to which anandamide responses are potentiated by PEA and OEA. Importantly, however, the combination of PEA (10 μM) and OEA (1 μM) also potentiated relaxation to anandamide in endothelium-intact and -denuded vessels. Given that OEA, but not PEA, also significantly enhanced relaxation induced by capsaicin, we conclude that different mechanisms underlie the potentiation effect of OEA and PEA through TRPV1 receptors. It is speculated that the effect of PEA might be selective for a restricted number of endovanilloids, depending on their binding sites on the receptor, whereas OEA can potentiate responses to other TRPV1 receptor agonists. In fact, OEA might also potentiate responses to agents that activate TRPV1 receptor-expressing nerves by non-TRPV1 receptors. This is because we have found that OEA also slightly potentiated mesenteric relaxation to 2-AG, another major endocannabinoid that caused capsaicin-sensitive relaxation but has negligible activity at TRPV1 receptors (W-SV Ho, unpublished results).

The potentiation effects of PEA and OEA on anandamide responses reported herein might be referred to as 'entourage' effects, as they were observed at molar ratios of anandamide/PEA and anandamide/OEA (between 1:3 and 1:333; see Figures 1 and 5) similar to those found in isolated mesenteric arteries (anandamide: PEA, approximately 1:173; anandamide: OEA, approximately 1:93). The *N*-acylethanolamide contents are also similar to those found in isolated middle cerebral artery (Rademacher *et al*., 2005; CJ Hillard and W-SV Ho, unpublished observations) and in broad agreement with data obtained from the whole mesenteric arterial bed (Movahed *et al*., 2005), intestine (Fegley *et al*., 2005) and brain (Richardson *et al*., 2007), confirming that PEA and OEA are more abundant than anandamide in vascular tissues. It is acknowledged that the absolute content of *N*-acylethanolamide in mesenteric arteries cannot be easily extrapolated to conditions *in vivo*. To minimize variation, isolated vessels for *N*-acylethanolamide detection and tension recording were prepared under similar experimental conditions (see Methods) in this study. If we assume 1 g wet weight corresponds to 1 ml volume, then the apparent, basal concentrations are 24 nM for anandamide, 4 μM for PEA and 2 μM for OEA in mesenteric arteries. These estimates are in the same

Table 2 Sensitivity of *N*-acylethanolamide-induced relaxation to inhibitors of the TRPV1 receptor system and FAAH

Treatment	*Anandamide*	*PEA*	*OEA*
Capsaicin	−	(−)	−
SB366791	−	X	−
URB597	+	+	X

Abbreviations: FAAH, fatty acid amide hydrolase; OEA, *N*-oleoylethanolamide; PEA, *N*-palmitoylethanolamide; TRPV1, transient receptor potential vanilloid type 1.
+, significant potentiation; −, significant inhibition; (−), small reduction in the maximum relaxation achieved; X, no significant effect.

concentration range as the concentrations of exogenous PEA and OEA used, providing further evidence that the 'entourage' effects are achievable *in vivo*. On the other hand, the finding that URB597 significantly increased mesenteric content of anandamide provides further support for a role for FAAH in the regulation of endocannabinoid signalling in the vasculature. URB597 slightly potentiated PEA relaxations and also tended to increase the vascular content of PEA. However, the FAAH inhibitor had no effect on both the relaxant effect and tissue content of OEA. These data are consistent with our proposal that, at least in the mesenteric arteries, competition with anandamide for FAAH is unlikely to explain the 'entourage' effects of PEA and OEA and that FAAH has relatively little function in the vascular actions of OEA. It should be pointed out that inhibition of FAAH by URB597 might be expected to increase mesenteric content of all three *N*-acylethanolamides, as has been shown in the brain (Fegley *et al*., 2005). It is, however, possible that enzymes other than FAAH predominate in the degradation of PEA and OEA in mesenteric arteries. Indeed, it has recently been shown that URB597 effectively inhibits FAAH activity in rat brain and intestine, but it modifies neither the intestinal content of OEA nor hypophagic response to OEA *in vivo* (Fegley *et al*., 2005).

This study has shown that, in addition to enhancing responses to exogenous anandamide through TRPV1 receptors, OEA and, to a lesser extent, PEA also caused mesenteric vasorelaxation *per se*. It is possible that PEA and OEA resulted in significant vasorelaxation through the effect of endogenous anandamide (at nanomolar range) present in the mesenteric arteries. However, the different pharmacological profiles of relaxation induced by the three *N*-acylethanolamides (summarized in Table 2) indicate direct relaxant actions of PEA and OEA, independent of their potentiation effects. For example, relaxations to OEA and anandamide were greatly inhibited by capsaicin or TRPV1 receptor antagonists, indicating that activation of TRPV1 receptors accounts for a large component of their relaxant responses. However, unlike anandamide, OEA-induced relaxation was not significantly potentiated by the FAAH inhibitor, URB597, consistent with direct activation by OEA of TRPV1 receptors. On the other hand, although our data strongly suggest that TRPV1 receptors mediate the potentiating effects of PEA, an endothelium-dependent relaxant mechanism(s) through non-TRPV1 receptors has a predominant role in PEA-induced relaxation. Further experimentation is required to examine the precise signalling pathways involved and whether the same endothelium-dependent mechanism(s) also underlie the residual relaxation induced by OEA after capsaicin treatment. Recent reports suggest that although the congeners are not agonists for cannabinoid receptors, they can activate the peroxisome proliferator-activated receptor (PPAR)-α, which mediates some of their anti-inflammatory and satiety actions (Fu *et al*., 2003; Lo Verme *et al*., 2005). However, the acute relaxant and potentiation effects (minutes) of PEA and OEA reported herein seem inconsistent with a significant contribution from the activation of PPARα, a ligand-regulated transcription factor that controls gene expression ($\geqslant$1 h). The PPARα receptor agonist, bezafibrate also has no vasorelaxant effect (O'Sullivan *et al*., 2005). Nevertheless, in view of the finding that anandamide, which might also activate PPARα receptors at micromolar concentrations, and PPARα receptor agonists induce synergistic antinociceptive effect (Russo *et al*., 2007), further investigation is warranted to test whether the PPARα receptor modulates the interaction between anandamide and the congeners in the vasculature.

Significantly, our data corroborate and extend previous observations indicating that anandamide might act as an endogenous agonist for the TRPV1 receptor. Although micromolar concentrations of anandamide are often required to activate TRPV1 receptors in expressed cells, a higher potency of anandamide might be achieved in native tissues, for instance due to TRPV1 receptor phosphorylation by protein kinases C and A (Premkumar and Ahern, 2000; De Petrocellis *et al*., 2001b; Vellani *et al*., 2001) and receptor upregulation in inflammation (Ji *et al*., 2002) and salt-sensitive hypertension (Wang *et al*., 2005). Results from this study indicate that an increased production of PEA and OEA in physiological and pathophysiological conditions, for instance myocardial infarction (Epps *et al*., 1979) and inflammation (Kondo *et al*., 1998), could also augment the action of anandamide through perivascular TRPV1 receptors. In fact, it is feasible that vascular actions of anandamide and their final outcome are dependent on the relative tissue contents of PEA and OEA. Future studies will aim to evaluate the importance of interactions among endocannabinoids and their congeners in the cardiovascular system. In this regard, it is interesting to note that some preliminary experiments have indicated that potentiation of anandamide relaxations by PEA might be altered in hypertensive states (W-SV Ho, unpublished observations).

In conclusion, this study demonstrates that the congeners, PEA and OEA, potentiate the vasorelaxant responses to anandamide. This 'entourage' effect is independent of the endothelium but requires an intact TRPV1 receptor system, providing further support for an important function of TRPV1 receptors in anandamide signalling in the circulation. Furthermore, PEA and OEA, which are present at higher concentrations than anandamide, also elicit vasorelaxation *per se* and thus might represent important vasomodulators.

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Conflict of interest

The authors state no conflict of interest.

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