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RESEARCH PAPER

Characterization of the vasorelaxant mechanisms of the endocannabinoid anandamide in rat aorta

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Background and purpose: Studies in isolated preparations of vascular tissue (mainly resistance vessels) provide evidence that anandamide exerts vasorelaxation. The aim of the present work was to further characterize the mechanisms involved in the vascular response induced by anandamide in a conduit vessel, rat aorta.
Experimental approach: Isometric tension changes in response to a cumulative concentration–response curve of anandamide (1 nM–100 μM) were recorded in aortic rings from male Wistar rats. The involvement of a number of factors in this relaxation was investigated including endothelium-derived vasorelaxant products, cannabinoid and vanilloid receptors (transient potential vanilloid receptor-1 (TRPV1)), release of calcitonin gene-related peptide (CGRP), anandamide metabolism and the membrane transporter for anandamide.
Key results: Anandamide caused a significant concentration-dependent vasorelaxation in rat aorta. This vasorelaxation was significantly inhibited by *Pertussis* toxin, by a non-CB_1/non-CB_2 cannabinoid receptor antagonist, by endothelial denudation, by inhibition of nitric oxide synthesis or inhibition of prostanoid synthesis via cyclooxygenase-2 (COX-2), by blockade of prostaglandin receptors EP_4 and by a fatty acid amino hydrolase inhibitor. Antagonists for CB_1, CB_2, TRPV1 or CGRP receptors, an inhibitor of the release of endothelium-derived hyperpolarizing factor, and an inhibitor of anandamide transport did not modify the vascular response to anandamide.
Conclusions and implications: Our results demonstrate, for the first time, the involvement of the non-CB1/non-CB2 cannabinoid receptor and an anandamide-arachidonic acid-COX-2 derived metabolite (which acts on EP_4 receptors) in the endothelial vasorelaxation caused by anandamide in rat aorta.
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Keywords: rat aorta; vasorelaxation; anandamide

Abbreviations: AM404, *N*-(4-hydroxy-phenyl)-5,8,11,14-eicosatetraenamine; DFU, 5,5-dimethyl-3-(3-fluorophenyl)-4-(4-methylsulphonyl)phenyl-2(5*H*)-furanone); DMSO, dimethylsulphoxide; EDHF, endothelium-derived hyperpolarizing factor; EP, PGE_2 receptors; FAAH, fatty acid amide hydrolase; GW627368X, *N*-(2-[4-(4,9-diethoxy-1-oxo-1,3-dihydro-2*H*-benzo[f]isoindol-2-yl)phenyl]-acetyl)benzenesulphonamide; L-NAME, *N*ω-nitro-L-arginine methyl ester; O1918, 1,3-dimethoxy-5-methyl-2[(1*R*,6*R*)-3methyl-6-(1-methylethenyl)-2-cyclohexen-1-yl]-benzene; 17-ODYA, 17-octadecynoic acid; PGE_2, prostaglandin E_2; rimonabant, *N*-(piperidin-1-yl)-5-(4-chlorophenyl)-1-(2,4-dichlorophenyl)-4-methyl-1*H*-pyrazole-3-carboxamide; SC-560, 5-(4-chlorophenyl)-1-(4-methoxyphenyl)-3-trifluoromethylpyrazole; SR144528, *N*-((1*S*)-endo-1,3,3-trimethyl bicycle (2.2.1) heptan-2yl)-5-(4-chloro-3-methylphenyl)-1-(4-methylbenzyl)pyrazole-3 carboxamide; URB597, 3′-carbamoyl-biphenyl-3-yl-cyclohexylcarbamate

Introduction

Studies in isolated preparations of vascular tissue and cells provide evidence that anandamide exerts vascular effects. The majority of these studies report that this endogenous cannabinoid elicits vasorelaxation in isolated arteries, although differences between species, as well as in the vascular beds used, should be noted (Ishioka and Bukoski, 1999; Zygmunt *et al*., 1999; Hillard, 2000; Grainger and Boachie-Ansah, 2001; O'Sullivan *et al*., 2004; Randall *et al*., 2004). The studies in resistance vessels show that anandamide induces clear vasodilator responses (Ellis *et al*., 1995; Randall and Kendall, 1997; Pratt *et al*., 1998; Jarai *et al*., 1999; Wagner *et al*., 1999; O'Sullivan *et al*., 2004), but in conduit vessels, different results have been reported. While some authors describe that in the rat hepatic and in guinea-pig basilar arteries (Zygmunt *et al*., 1999) and in rat aorta

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(López-Miranda *et al*., 2004) anandamide produces an important vasorelaxation, other researchers point out that in conduit vessels such as rat carotid and main mesenteric artery and aorta, this cannabinoid does not cause or causes only a slight vasorelaxation (Darker *et al*., 1998; Holland *et al*., 1999; O'Sullivan *et al*., 2004, 2005).

Several putative mechanisms have been put forward to explain the vasorelaxant effect of anandamide, but there is no clear consensus among researchers in this respect. Both extracellular and intracellular mechanisms have been described. With regard to the former, some studies have reported that anandamide acts through the stimulation of cannabinoid CB_1 receptors and/or through a novel and putative endothelial 'anandamide receptor' referred to as a 'non-CB_1/non-CB_2 'cannabinoid receptor (White and Hiley, 1997; Jarai *et al*., 1999; Wagner *et al*., 1999; Offertáler *et al*., 2003), while other authors propose a new mechanism: the stimulation of vanilloid receptors (transient potential vanilloid receptor-1 (TRPV1)) on perivascular sensory nerves, with the subsequent release of the vasodilator neurotransmitter calcitonin gene-related peptide (CGRP) (Zygmunt *et al*., 1999; Ralevic *et al*., 2000, 2002; White *et al*., 2001; Ho and Hiley, 2003). With regard to the latter, the main mechanisms proposed include nitric oxide (NO) release, metabolism to vasoactive arachidonic metabolites or prostanoid analogues, or endothelium-derived hyperpolarizing factor (EDHF) release (Kunos *et al*., 2000; Randall *et al*., 2002). Most of these vasorelaxant mechanisms for anandamide have been established in resistance vessels, but the question of whether or not these mechanisms are same in all the vascular territory and, in particular, in conduit vessels has not been definitively answered.

Previous studies carried out in our laboratory showed that anandamide is able to produce a noteworthy vasorelaxation in rat aorta (López-Miranda *et al*., 2004). The aim of the present work was to further characterize the mechanisms involved in the vascular response induced by anandamide in this conduit vessel.

Methods

Tissue preparation

This study was carried out in accordance with the European Community Guidelines for the use of experimental animals. Male Wistar rats (250–300 g body weight) were anaesthetized with sodium pentobarbital (50 mg kg^{-1}, intraperitoneal (i.p.)). The abdomen was opened by a midline incision, and the aorta was carefully excised and placed in ice-cold Krebs–Henseleit (K-H) solution with the following composition (mM): 118 NaCl; 4.75 KCl; 1.2 $MgSO_4$; 1.19 KH_2PO_4; 2.54 $CaCl_2$; 25 $NaHCO_3$; 11 glucose (pH 7.4). All connective and perivascular adipose tissues were removed, with caution taken not to disrupt the endothelium. Transverse vascular rings 3–4 mm long were prepared. Some of the rings were deliberately denuded by rubbing and rolling them around stainless steel forceps before being mounted.

Afterwards, the rings were fixed vertically between two stainless steel hooks and suspended in a 5-ml jacketed glass organ bath, containing K-H solution at 37°C and continuously bubbled with 95% O_2 and 5% CO_2. The upper wire was connected to an isometric force transducer (Grass FT07), and tension measurements were recorded in a computer (PowerLab/4e program). The rings were mounted with a resting tension of 2 g. Tissues were equilibrated for 90 min, during which time the medium was replaced every 15 min.

Experimental protocol

In the first series of experiments, the effect of anandamide on submaximal phenylephrine (1 μM)-induced tone in endothelium intact and denuded aorta rings were examined in the following manner. After the equilibration period, arteries were precontracted with phenylephrine and when a stable level of tone was established, cumulative concentration–response curves to anandamide were constructed (1 nM–100 μM). Control rings were similarly treated with phenylephrine, but corresponding vehicle additions (dimethylsulphoxide, DMSO 0.5%) (López-Miranda *et al*., 2004) were made. Only one experiment was carried out in each aorta ring. At the end of each cannabinoid concentration–response curve, carbachol (10 μM) was added to verify the existence of functional endothelium in the corresponding preparation. Arteries that relaxed to carbachol more than 70% were designated as endothelium-intact preparations and the preparations that relaxed to carbachol less than 10% were designated as endothelium-denuded preparations. Rings with intermediate responses were discarded. All subsequent series of experiments were carried out in intact arteries.

To identify the receptors involved in the vascular effects of anandamide, the following experiments were performed: (1) to evaluate the implication of $G_{i/0}$ protein-coupled receptors, aorta rings were pretreated with 300 ng ml^{-1} of *Pertussis* toxin for 3 h (Petitcolin *et al*., 2001); (2) to test the involvement of classical cannabinoid receptors (CB_1 and CB_2), aorta preparations were pretreated with (a) the selective CB_1 receptor antagonist, *N*-(piperidin-1-yl) – 5-(4-chlorophenyl)-1-(2,4-dichlorophenyl)-4-methyl-1*H*-pyrazole-3-carboxamide (rimonabant) for 15 min at two different concentrations (1 and 3 μM) (Rinaldi-Carmona *et al*., 1994) and (b) the selective CB_2 receptor antagonist, *N*-((1*S*)-endo-1,3,3-trimethyl bicycle (2.2.1) heptan-2yl)-5-(4-chloro-3-methylphenyl)-1-(4-methylbenzyl)pyrazole-3 carboxamide (SR144528) for 15 min at the same concentrations (1 and 3 μM) (Rinaldi-Carmona *et al*., 1998); (3) to test the involvement of the nonclassical and newly proposed non-CB_1/non-CB_2 endothelial cannabinoid receptor, aorta preparations were pretreated for 15 min with an antagonist of this receptor, O1918 (1,3-dimethoxy-5-methyl-2[(1*R*,6*R*)-3methyl-6-(1-methylethenyl)-2-cyclohexen-1-yl]benzene) (10 μM) (Offertáler *et al*., 2003); and (4) to investigate the involvement of TRPV1, two different protocols were performed: (a) arteries were pretreated with the TRPV1 blocker capsazepine 100 nM for 30 min (Jakab *et al*., 2005) and (b) arteries were pretreated with the inhibitor of CGRP receptors $CGRP_{8-37}$ (3 μM) for 30 min (Ralevic *et al*., 2002). The vehicle used to dissolve the antagonists, ethanol, was also evaluated.

The contribution of the main vasorelaxant endothelial mediators was also investigated: (1) the involvement of NO

and of EDHF in the vascular response elicited by anandamide was examined by pretreatment of aorta rings for 30 min with $N\omega$-nitro-L-arginine methyl ester (L-NAME) 100 μM (Engler *et al*., 2000) or apamin plus charybdotoxin (10 nM each) (Perez-Vizcaino *et al*., 1999), respectively; (2) the involvement of vasorelaxant arachidonic acid-derived products was investigated by pretreatment of the preparations for 30 min with (a) 3′-carbamoyl-biphenyl-3-yl-cyclohexylcarbamate (URB597) 1 μM, a fatty acid amide hydrolase (FAAH) inhibitor (Bátkai *et al*., 2004), which converts anandamide to arachidonic acid plus ethanolamine, (b) 17-octadecynoic acid (17-ODYA) 5 μM (cytochrome *P*450 epoxygenase and ω/ω-1-hydroxylase inhibitor) (Grainger and Boachie-Ansah, 2001), (c) indomethacin 10 μM (cyclooxygenase (COX) inhibitor) (Tepareenan *et al*., 2003); (d) 5-(4-chlorophenyl)-1-(4-methoxyphenyl)-3-trifluoromethylpyrazole (SC-560) 1 μM (a selective COX-1 inhibitor, Bolla *et al*., 2004) and (e) 5,5-dimethyl-3-(3-fluorophenyl)-4-(4-methylsulphonyl)phenyl-2(5*H*)-furanone) (DFU) 0.1 μM (a selective COX-2 inhibitor, Riendeau *et al*., 1997). Prostaglandin E_2 (PGE_2) and analogues (PGE_2 ethanolamide) have been identified as derived from anandamide metabolism pathways (Sugimura *et al*., 2002). These products act on PGE_2 receptors (EP) to exert vascular effects. The involvement of the vasorelaxant EP_4 has also been studied by pretreating rat aorta rings with GW627368X (*N*-(2-[4-(4, 9-diethoxy-1-oxo-1,3-dihydro-2*H*-benzo[f]isoindol-2-yl)phenyl]-acetyl)benzenesulphonamide) (1 and 3 μM), a selective antagonist of EP4 receptors (Wilson *et al*., 2006).

In addition, the possible involvement of the anandamide transporter in its vascular effect on rat aorta was examined by pretreating the preparations with *N*-(4-hydroxy-phenyl)-5,8,11,14-eicosatetraenamine (AM404, anandamide transporter inhibitor) 10 μM for 30 min (Grainger and Boachie-Ansah, 2001).

Data analysis

Relaxation responses are expressed as the percentage of relaxation of the tone induced by phenylephrine (1 μM). R_{max} is the maximal response obtained when the anandamide concentration–response curve is carried out.

pEC_{50} values refer to the negative logarithm of the concentration of anandamide that produced half (50%) of the maximal response obtained (EC_{50}). EC_{50} values were obtained from individual concentration–response curves by fitting the data to a logistic equation (GraphPad PRISM V2.01).

Data are expressed as the mean $\pm$ s.e.m. The number of animals in each group is expressed by *n*. R_{max} values for anandamide in the different experimental groups were compared using analysis of variance (ANOVA), followed by Bonferroni/Dunn *post hoc* test, as appropriate. pEC_{50} values were compared by a Student's unpaired *t*-test. *P*-values <0.05 were considered significant.

Drugs

Phenylephrine carbachol, L-NAME, indomethacin, apamin, charybdotoxin, 17-ODYA and capsazepine were obtained from Sigma (Sigma Chemical Company, Poole, Dorset, UK), and URB597 was supplied from Cayman Chemical Company (Ann Arbor, MI, USA). *Pertussis* toxin was supplied by Research Biochemicals International (RBI, Natick, MA, USA). DMSO was supplied by Panreac Química S.A, Barcelona, Spain. Anandamide, $CGRP_{8-37}$ (rat), AM 404 and O1918 were supplied by Tocris Cookson (Bristol, UK). SC-560 and DFU were obtained courtesy of Menarini Laboratorios (Barcelona, Spain) and Merck Sharp & Dohme (Madrid, Spain), respectively. Rimonabant and SR144528 were obtained from Sanofi Recherche (Montpellier, France). GW627368X was obtained courtesy of GlaxoSmithKline (Stevenage, UK).

Phenylephrine, carbachol, L-NAME, $CGRP_{8-37}$ (rat), apamin and charybdotoxin were dissolved in distilled water. Indomethacin, capsazepine, rimonabant, SR144528, O1918, DFU, AM 404, URB597, SC-560, 17-ODYA and GW627368X were dissolved in ethanol (Panreac Química S.A.).

A cannabinoid stock solution (0.01 M) was prepared daily in DMSO 0.5% (v/v). Additional dilutions were made by mixing 1 volume of stock solution with up to 9 volumes of distilled water.

Results

Phenylephrine (1 μM) caused a submaximal increase in arterial tone that was similar in all the experimental groups (data not shown).

None of the antagonists or inhibitors, at the concentrations used, modified the vascular function in phenylephrine-precontracted intact arteries (data not shown). When ethanol was used as vehicle, the maximum volume administered in the organ bath was 5 μl to prevent any modification of the functionality of the preparation.

Vasorelaxation caused by anandamide in rat aorta rings

Anandamide caused a concentration-dependent vasorelaxation in intact rat aorta rings, resulting in an R_{max} value of $51 \pm 9\%$ and a pEC_{50} value of 5.92 ± 0.04 ($n = 6$) (Figure 1). This vasorelaxation was gradual in onset and took 7–10 min to reach plateau at each concentration step.

Involvement of $G_{i/o}$ protein-coupled receptors in vasorelaxation caused by anandamide in rat aorta rings

Incubation of the rat aorta ring preparations with pertussis toxin, 300 ng ml^{-1} for 3 h, significantly reduced the vasorelaxation caused by anandamide (R_{max} of $24 \pm 4\%$ and pEC_{50} value of 3.87 ± 0.10 ($n = 4$), $P < 0.001$) (Figure 2).

Involvement of CB_1, CB_2 and non-CB_1/non-CB_2 receptors in vasorelaxation caused by anandamide in rat aorta rings

The vasorelaxation caused by anandamide in endothelium-intact aorta rings was not affected by either the pretreatment with the CB_1 antagonist, rimonabant (rimonabant 1 μM: R_{max} of $42 \pm 5\%$ and pEC_{50} of 5.90 ± 0.02 ($n = 6$); rimonabant 3 μM: R_{max} of $42 \pm 7\%$ and pEC_{50} of 6.01 ± 0.04 ($n = 8$)) (Figure 3a) or the pretreatment with the CB_2 antagonist, SR144528

(SR144528 1 μM: R_{max} of 46±8% and pEC_{50} of 6.10±0.1 ($n=5$); SR144528 3 μM: R_{max} of 42±5% and pEC_{50} of 5.98±0.07 ($n=5$)) (Figure 3b).

The influence of the combination of rimonabant and SR144528 on anandamide-induced vasorelaxation was also evaluated. Pretreatment with both antagonists simultaneously, at a concentration of 1 μM, did not cause any modification of the vascular effect of anandamide (R_{max} of 45±7% and pEC_{50} of 6.01±0.04 ($n=4$)) (Figure 3c).

When aortic preparations were pretreated with O1918 at 10 μM, a significant right-ward shift in the concentration–response curve to anandamide without affecting the maximal response was observed (R_{max} of 52±8%, $P>0.05$ and pEC_{50} of 5.3±0.12 ($n=4$), $P<0.001$) (Figure 3d).

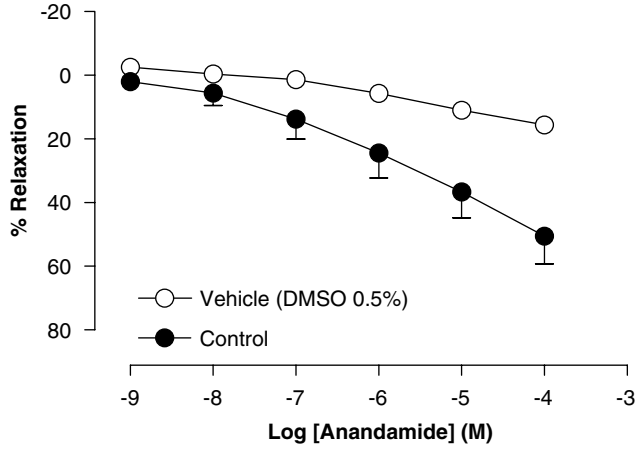

Figure 1 Concentration–response curve for the relaxation of isolated rat aorta rings, precontracted by phenylephrine (1 μM), by anandamide. Values are expressed as mean±s.e. for 4–8 animals. The dimethylsulphoxide 0.5% curve represents the effect of the vehicle alone.

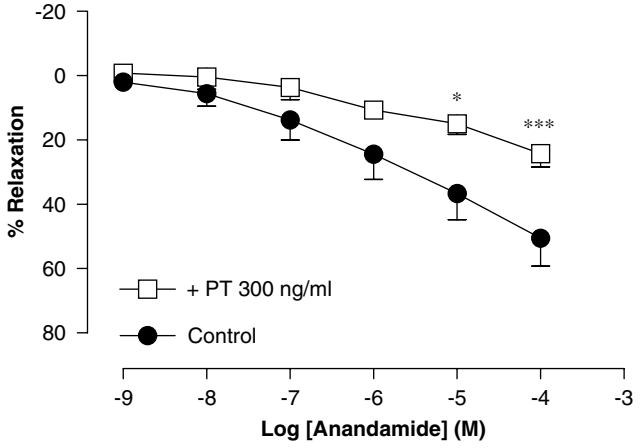

Figure 2 Effect of pretreatment (3 h) with *Pertussis* toxin (PT) on the relaxation produced by anandamide in intact rat aorta rings precontracted by phenylephrine (1 μM). Values are expressed as mean±s.e. for 4–8 animals. A two-way analysis of variance followed by Bonferroni/Dunn *post hoc* test was used for statistical analysis (*$P<0.05$, **$P<0.01$, ***$P<0.001$ vs control).

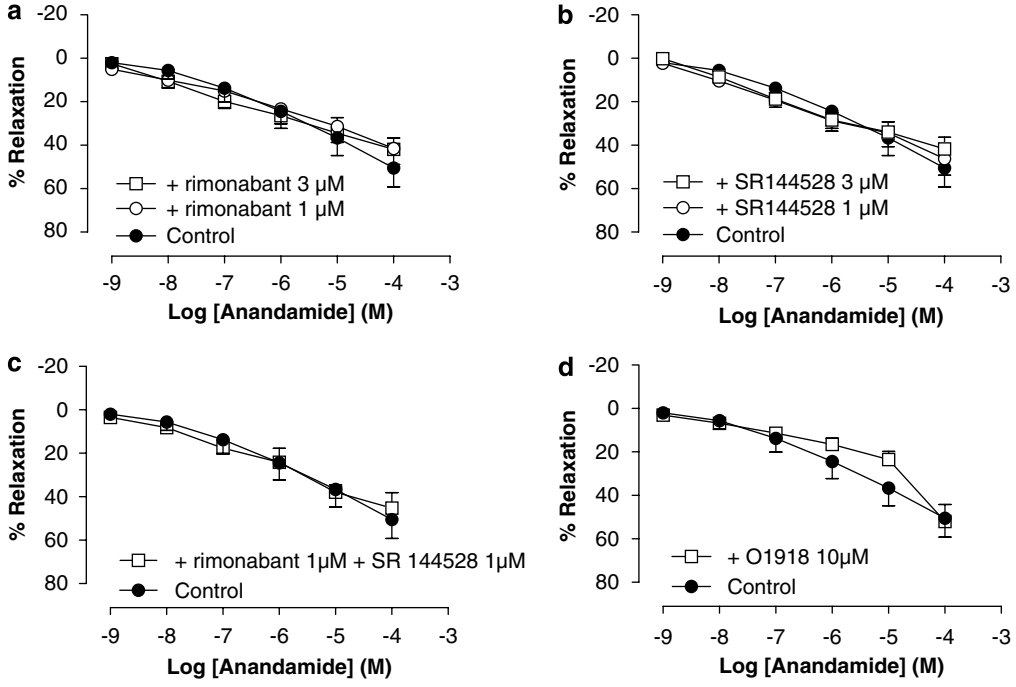

Figure 3 Effect of rimonabant (**a**), SR144528 (**b**), rimonabant plus SR144528 (**c**) and O1918 (**d**) on the relaxation produced by anandamide in intact rat aorta rings precontracted by phenylephrine (1 μM). Values are expressed as mean±s.e. for 4–8 animals. A two-way analysis of variance followed by Bonferroni/Dunn *post hoc* test was used for statistical analysis. (*$P<0.05$, **$P<0.01$, ***$P<0.001$ vs control). O1918, 1,3-dimethoxy-5-methyl-2[(1*R*,6*R*)-3methyl-6-(1-methylethenyl)-2-cyclohexen-1-yl]benzene; SR144528, *N*-((1*S*)-endo-1,3,3-trimethyl bicycle (2.2.1) heptan-2yl)-5-(4-chloro-3-methylphenyl)-1-(4-methylbenzyl)pyrazole-3 carboxamide.

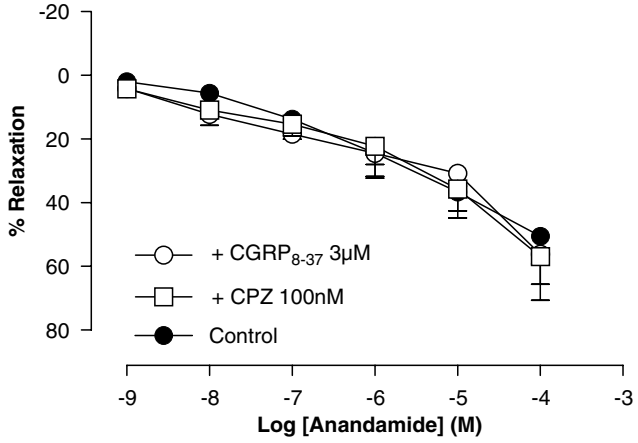

Figure 4 Effect of capsazepine (CPZ) and $CGRP_{8-37}$ on the relaxation produced by anandamide in intact isolated rat aorta rings precontracted by phenylephrine (1 μM). Values are expressed as mean $\pm$ s.e. for 4–8 animals. A two-way analysis of variance followed by Bonferroni/Dunn *post hoc* test was used for statistical analysis (*$P<0.05$, **$P<0.01$, ***$P<0.001$ vs control). CGRP, calcitonin gene-related peptide.

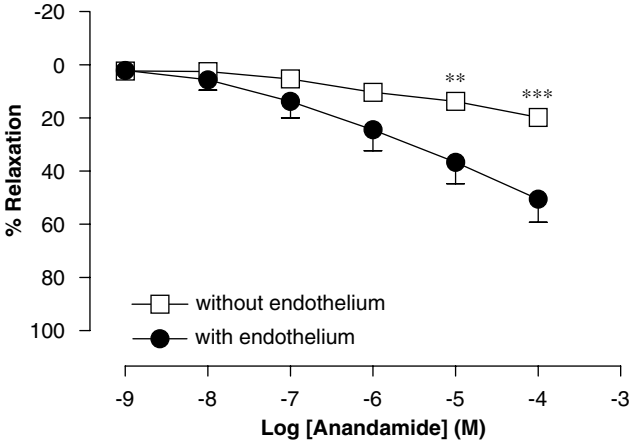

Figure 5 Concentration–response curve for the relaxation of isolated rat aorta rings, precontracted by phenylephrine (1 μM), by anandamide in the presence or absence of functional endothelium. Values are expressed as mean $\pm$ s.e. for 4–8 animals. A two-way analysis of variance followed by Bonferroni/Dunn *post hoc* test was used for statistical analysis (*$P<0.05$, **$P<0.01$, ***$P<0.001$ vs control).

Involvement of TRPV-1 and CGRP in vasorelaxation caused by anandamide in rat aorta rings

Neither pretreatment with capsazepine (100 nM) nor with $CGRP_{8-37}$ (3 μM) modified the vasorelaxation caused by anandamide in intact rat aorta ring preparations (R_{max} of $57\pm9\%$ and pEC_{50} of 5.99 ± 0.13 ($n=4$) and R_{max} of $56\pm15\%$ and pEC_{50} of 6.09 ± 0.16 ($n=4$), respectively) (Figure 4).

Involvement of endothelium in the vasorelaxation caused by anandamide in rat aorta rings

Endothelial denudation resulted in a complete inhibition of the vasorelaxation caused by anandamide, obtaining a R_{max} value of $20\pm1\%$ ($n=5$), similar to that obtained in the vehicle (DMSO 0.5%) group (R_{max} of $16\pm2\%$ ($n=5$)) (Figure 5).

Involvement of NO and EDHF in vasorelaxation caused by anandamide in rat aorta rings

Pretreatment with L-NAME (100 μM) for 30 min significantly inhibited the vasorelaxation produced by anandamide in rat aorta rings (R_{max} of $14\pm3\%$ and pEC_{50} of 2.56 ± 0.23 ($n=5$), $P<0.001$) (Figure 6). Pretreatment with apamin plus charybdotoxin (10 nM each) did not modify the endothelium-dependent vasorelaxation elicited by anandamide in isolated rat aorta rings (R_{max} of $49\pm6\%$ and pEC_{50} of 6.2 ± 0.10 ($n=4$)) (Figure 6).

Involvement of metabolism-derived products in vasorelaxation caused by anandamide in rat aorta rings

Inhibition of FAAH by pretreatment with URB597 (1 μM) caused a significant reduction of the vasorelaxant effect of anandamide in rat aorta (R_{max} of $34\pm5\%$ and pEC_{50} of 4.50 ± 0.04, $P<0.001$ ($n=4$)) (Figure 7a).

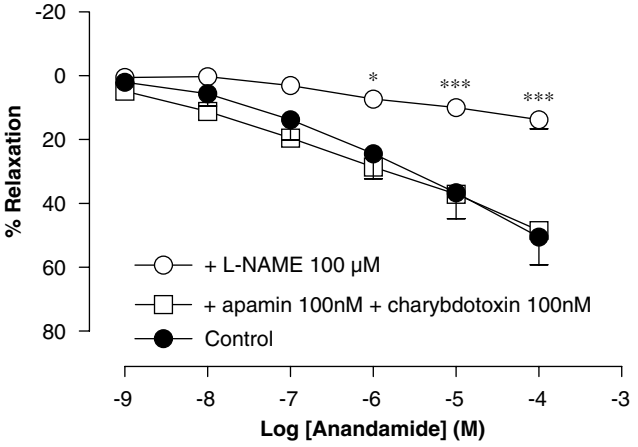

Figure 6 Effect of $N\omega$-nitro-L-arginine methyl ester (L-NAME) and apamin plus charybdotoxin on the relaxation produced by anandamide in intact isolated rat aorta rings precontracted by phenylephrine (1 μM). Values are expressed as mean $\pm$ s.e. for 4–8 animals. A two-way analysis of variance followed by Bonferroni/Dunn *post hoc* test was used for statistical analysis (*$P<0.05$, **$P<0.01$, ***$P<0.001$ vs control).

Pretreatment with the cytochrome *P*450 epoxygenase and ω/ω-1-hydroxylase inhibitor, 17-ODYA (5 μM), did not modify the vasorelaxation induced by anandamide in rat aorta rings (R_{max} of $43\pm5\%$ and pEC_{50} of 6.01 ± 0.03 ($n=9$), $P>0.05$) (Figure 7b), while pretreatment with indomethacin 10 μM for 30 min significantly reduced the vasorelaxation produced by anandamide in rat aorta rings (R_{max} of $16\pm6\%$ ($n=8$) and pEC_{50} of 3.07 ± 0.09 ($n=5$), $P<0.001$) (Figure 7b).

When the selective COX-1 inhibitor, SC-560 (1 μM), was added, the vasorelaxation caused by anandamide was not affected (R_{max} of $55\pm12\%$ and pEC_{50} of 5.81 ± 0.11 ($n=5$)). However, when the selective COX-2 inhibitor, DFU (0.1 μM)

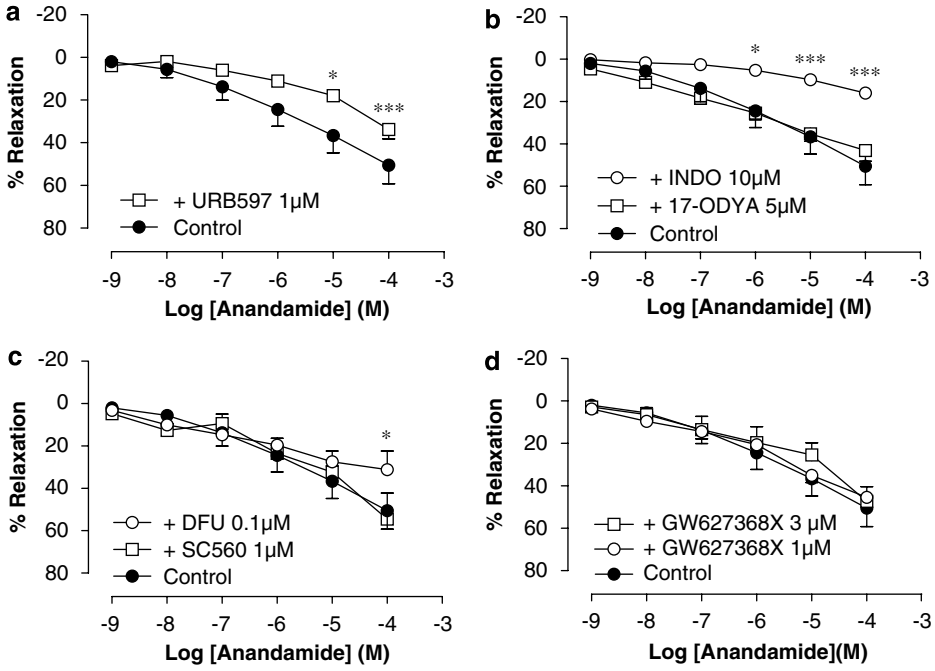

Figure 7 Effect of the FAAH inhibitor, URB597 (**a**), the cytochrome *P*450 epoxygenase and ω/ω-1-hydroxylase inhibitor, 17-ODYA and the COX inhibitor indomethacin (INDO) (**b**), the selective COX-1 inhibitor, SC560, and the selective COX-2 inhibitor, DFU (**c**) and the selective EP_4 receptor antagonist GW627368X (**d**) on the relaxation produced by anandamide in intact rat aorta rings precontracted by phenylephrine (1 μM). Values are expressed as mean$\pm$s.e. for 4–8 animals. A two-way ANOVA followed by Bonferroni/Dunn *post hoc* test was used for statistical analysis (*$P<0.05$, **$P<0.01$, ***$P<0.001$ vs control). FAAH, fatty acid amino hydrolase; URB597, 3′-carbamoyl-biphenyl-3-yl-cyclohexylcarbamate.

was used, a significant inhibition of the vasorelaxant response provoked by anandamide in rat aorta was observed (R_{max} of $31\pm9\%$, $P<0.05$, and pEC_{50} of 4.97 ± 0.07, $P<0.001$ ($n=4$)) (Figure 7c).

GW627368X, the selective inhibitor of EP_4 receptors, did not cause a modification in the maximal vasorelaxation caused by anandamide at the lower concentration (1 μM) tested (R_{max} of $46\pm1\%$ ($n=4$) and pEC_{50} of 5.77 ± 0.08 ($n=4$)). However, this antagonist at the higher concentration (3 μM) caused a significant rightward shift in the concentration–response curve to anandamide without affecting the maximal response (R_{max} of $47\pm7\%$, $P>0.05$ and a pEC_{50} of 5.43 ± 0.10, $P<0.001$ ($n=4$)) (Figure 7d).

Involvement of the anandamide transporter in vasorelaxation caused by anandamide in rat aorta rings

Blockade of the anandamide membrane transporter by pretreatment with AM404 (10 μM) did not modify the vascular response of anandamide in rat aorta rings (R_{max} of $48\pm10\%$ and pEC_{50} of 6.1 ± 0.10 ($n=6$) (Figure 8).

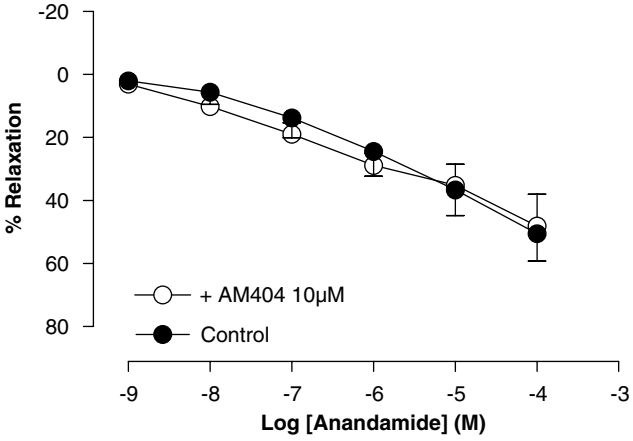

Figure 8 Effect of the anandamide transporter inhibitor AM404 on the relaxation produced by anandamide in intact rat aorta rings precontracted by phenylephrine (1 μM). Values are expressed as mean$\pm$s.e. for 4–8 animals. A two-way analysis of variance followed by Bonferroni/Dunn *post hoc* test was used for statistical analysis (*$P<0.05$, **$P<0.01$, ***$P<0.001$ vs control). AM404, *N*-(4-hydroxy-phenyl)-5,8,11,14-eicosatetraenamine.

Discussion

The aim of the present study was to further characterize the vasorelaxant mechanisms of anandamide in rat aorta. Currently, there are only two studies that have evaluated the vasorelaxant effect of anandamide in conduit vessels. One describes a clear vasorelaxant effect of anandamide, around 60%, in rabbit aorta (Mukhopadhyay *et al*., 2002) and the other refers to no more than a slight vasorelaxation (around 20%) in rat aorta (O'Sullivan *et al*., 2005). The present study shows a slowly developing concentration-dependent relaxation induced by anandamide in intact

isolated rat aorta rings, reaching a maximal value of around 35% (versus vehicle). Although these results are not in agreement with those obtained by O'Sullivan *et al*. (2005) in spite of using the same animal species, it is possible that methodological differences could explain these differing findings: O'Sullivan's group used ethanol as anandamide solvent, while in the present study, the vehicle is DMSO 0.5%. We have previously demonstrated that the vascular responses of anandamide can be lost depending on the solvent/vehicle used (López-Miranda *et al*., 2004).

Anandamide can exert its vascular actions through three different $G_{i/0}$-regulatory protein-coupled cannabinoid receptors: CB_1, CB_2 and the non-CB_1/non-CB_2 cannabinoid receptors (Bouaboula *et al*., 1999; Holland *et al*., 1999; Jarai *et al*., 1999; Randall *et al*., 2002; Begg *et al*., 2003; Offertáler *et al*., 2003). In our experimental conditions, the involvement of $G_{i/0}$-protein-coupled receptors in the vasorelaxation caused by anandamide has been confirmed because of the significant reduction of anandamide's effects in the presence of *Pertussis* toxin. But, is a cannabinoid receptor responsible for the vascular effect? If this were the case, which is/are the subtype/s involved?

To answer these questions, the vasorelaxation of anandamide in the presence of CB_1, CB_2 and non-CB_1/non-CB_2 receptor antagonists, rimonabant, SR144528 and O1918, respectively, was evaluated. The rimonabant and SR144528 concentrations used in the present study (1 and 3 μM) were chosen taking into account the results obtained by White and Hiley (1998). They concluded that concentrations of rimonabant higher than 3 μM are not appropriate for the investigation of cannabinoid receptor-dependent processes in vascular tissue. Due to the lack of similar studies with SR144528, we decided to use the same range of concentrations as for the CB_1 antagonist. In the present study, anandamide vasorelaxation was insensitive to CB_1 and CB_2 receptor antagonists at the different concentrations tested, either when both antagonists were administered separately or simultaneously. In studies in which anandamide exerts only a modest vasorelaxation in rat aorta, other authors have also suggested CB_1-and CB_2-independent vasorelaxant mechanisms for anandamide (O'Sullivan *et al*., 2004). We used O1918 at 10 μM, a concentration which does not bind to cloned CB1 and CB2 receptors and which has been shown to cause a 10-fold rightward shift of the concentration–relaxation curve for abnormal cannabidiol in rat mesenteric arteries (Offertáler *et al*., 2003). In our experiments with O1918, a significant rightward shift of the concentration–relaxation curve of anandamide was obtained, suggesting that this non-CB_1/non-CB_2 receptor may be involved in the vascular effect of anandamide in rat aorta. Other researchers have also described similar results in rat mesenteric and coronary vessels (Jarai *et al*., 1999; Ford *et al*., 2002; Offertáler *et al*., 2003; O'Sullivan *et al*., 2004), and some of them have even suggested that this receptor is specific for anandamide (Offertáler *et al*., 2003).

Anandamide binds to TRPV-1 with micromolar affinity, and this interaction was shown to result in the release of the potent vasodilator peptide CGRP from sensory nerve endings, provoking vasorelaxation (Jarai *et al*., 1999; Zygmunt *et al*., 1999; Mukhopadhyay *et al*., 2002). In the present study, neither the inhibition of the TRPV-1 by capsazepine nor the inhibition of CGRP receptors by $CGRP_{8-37}$ modified the vasorelaxant effect of anandamide, which rules out the involvement of this mechanism in the vascular effect of anandamide in rat aorta. Although it has not been discussed in the present study, the vasorelaxation caused by anandamide in rat aorta is endothelium-dependent. There are studies which have demonstrated that only the endothelium-independent component in the vasorelaxation induced by anandamide is blocked by capsazepine or by CGRP inhibitors (Jarai *et al*., 1999; Zygmunt *et al*., 1999; Mukhopadhyay *et al*., 2002), while the endothelium-dependent vasodilator effect of anandamide is unaffected by these antagonists (Jarai *et al*., 1999; Mukhopadhyay *et al*., 2002; Ho and Hiley, 2003; Offertáler *et al*., 2003). This may explain the absence of TRPV-1 and CGRP involvement in our preparation.

Other important mechanisms proposed for the vasorelaxant effect of anandamide are endothelial intracellular pathways (Kunos *et al*., 2000; Randall *et al*., 2002). Our results show that the vasorelaxation provoked by anandamide in rat aorta is endothelium-dependent. For this reason, a more detailed investigation of the implication of the different endothelial vasorelaxant pathway/s was carried out. The production of NO, the release of EDHF and the production of prostanoids have been investigated.

The results of the present study show that while the production of NO is a pivotal step in the vasorelaxation caused by anandamide in rat aorta, the contribution of a hyperpolarizing mechanism (via EDHF) is not involved. These data are in agreement with those obtained by other authors (Mukhopadhyay *et al*., 2002; O'Sullivan *et al*., 2004).

With regard to prostanoids, it has been known that anandamide can be converted to arachidonic acid plus ethanolamine through its hydrolysis via FAAH (Deutsch *et al*., 1997). Besides, there is evidence from some resistance vessels that anandamide vasorelaxation is dependent on arachidonic acid metabolism via COX and/or epoxygenase pathways (Pratt *et al*., 1998; Fleming *et al*., 1999; Grainger and Boachie-Ansah, 2001). To date, there is no study that has assessed the involvement of arachidonic acid metabolites in the anandamide vasorelaxant effect in conduit vessels, but, since it is a possible mechanism, we investigated this option.

Pretreatment of aorta preparations with the selective and potent FAAH inhibitor, URB597, caused a significant reduction of the anandamide vasorelaxation, suggesting an involvement of anandamide metabolism in its vascular effect. Some experiments were carried out to further investigate this possibility. As cyclooxygenase and/or epoxygenase pathways have been implicated in vasorelaxation of anandamide in resistance vessels, these metabolism routes were tested in the present study. Our data showed that, while the epoxygenase pathway was not involved in the anandamide vasorelaxant response in rat aorta, the COX metabolism pathway was important in the vasodilatory response in this blood vessel, as there was no inhibition of this vascular effect after pretreatment with 17-ODYA and but inhibition after indomethacin. In addition, the selective inhibition of

COX-1 and COX-2 separately showed that the vasorelaxation induced by anandamide was only affected by the presence of the selective COX-2 inhibitor (DFU). So, we can propose that, at least, a vasodilator metabolite of arachidonic acid derived via COX-2 is involved in the vasorelaxation caused by anandamide in rat aorta. Recently, other authors have described that vasorelaxation caused by anandamide in rat mesenteric vessels is limited by its catabolism by FAAH and COX-2 metabolites (Ho and Randall, 2007).

The most important arachidonic acid metabolite related to anandamide pharmacology *via* COX-2 metabolism is prostaglandin E_2 (Kozak and Marnett, 2002). PGE_2 exerts its vascular actions through EPs: EP_1, EP_2, EP_3 and EP_4. The EP_2 and EP_4 receptors mediate vasorelaxant responses (Davis *et al*., 2004; Wilson and Giles, 2005) and, while EP_2 receptors are the least abundant among the EP receptors, EP_4 receptors are widely distributed throughout the body (Narumiya *et al*., 1999). For this reason, in the present study, the involvement of EP_4 receptors in anandamide vasorelaxation was evaluated. In the presence of GW627368X (an EP_4 selective blocker), a rather small but statistically significant inhibitory effect was observed. This indicates that this receptor may be involved in the vasorelaxant effect of anandamide in rat aorta.

At this time, it is important to mention that anandamide can also be metabolized directly by COX-2 leading to a major product, the prostamide, PGE_2-ethanolamide (Yu *et al*., 1997; Burstein *et al*., 2000) and PGE_2-ethanolamide acts directly on EP_4 receptors and causes vasorelaxant responses in blood vessels (Ross *et al*., 2002). So, it could be possible that PGE_2-ethanolamide can act on EP_4 receptors, causing vasorelaxation in rat aorta. However, if data obtained with URB597 and DFU are compared, it can be observed that the magnitude of the anandamide vasorelaxation inhibition is very similar to both inhibitors (R_{max} of $34 \pm 5\%$ ($n = 4$) and R_{max} of $31 \pm 12\%$ ($n = 5$), respectively). This leads us to suggest that the 'PGE_2 like' product proposed should be PGE_2, because if PGE_2-ethanolamide were the compound responsible for the anandamide vasorelaxation, the inhibition with URB597 would not have been obtained.

Anandamide is readily taken up into cells by a process of facilitated diffusion (Hillard and Jarrahian, 2000; Fowler and Jacobsson, 2002). Some studies have demonstrated that vascular responses to anandamide are affected by an inhibitor of carrier-mediated anandamide uptake, AM404 (Calignaro *et al*., 1997; Chaytor *et al*., 1999). The results obtained in the present study show that the vasorelaxation caused by anandamide was not affected by the presence of AM404. This finding suggests that anandamide does not need a specific transporter protein to access the cytosol to exert its vasorelaxant effect, which would agree with data described by Glaser *et al*. (2003) in neuronal cells. Alternatively, the anandamide transporter may not exist or operate in this particular conduit vessel. When this transporter protein is definitively identified, these questions can be clearly answered. It is important to note that, although AM404 can exert other actions (agonist at TRPV-1 (Zygmunt *et al*., 2000; Ralevic and Kendall, 2001) and inhibitor of FAAH in neuronal cells (Glaser *et al*., 2003)), in the present study, the AM404 concentration used ($10\,\mu M$) did not modify vascular function or interfere with results obtained with the FAAH inhibitor, discounting the possibility of these other effects.

In summary, the present study demonstrates, for the first time, a clear and important endothelium-mediated vasorelaxant effect of anandamide in rat aorta mainly mediated by two mechanisms: (a) anandamide may act on the endothelial non-CB_1/non-CB_2 cannabinoid receptor promoting NO synthesis and the subsequent vasorelaxation; (b) anandamide could be metabolized by FAAH resulting in arachidonic acid formation, which is a substrate of COX-2 leading to the formation of a 'PGE_2 like' product, which binds to the EP_4 receptor in vascular smooth muscle and causes vasorelaxation.

We propose that when anandamide acts on non-CB_1/non-CB_2 cannabinoid receptors, it promotes the release of NO in rat aorta. Our study does not demonstrate this proposal, but it is in agreement with the signalling cascade of endothelial non-CB_1/non-CB_2 cannabinoid receptor proposed by Begg *et al*. (2003) in human umbilical vein endothelial cells. Besides, recently, McCollum *et al*. (2007) have demonstrated that the synthetic analogue of anandamide, methanandamide, promotes NO production acting through the novel non-CB_1/non-CB_2 anandamide receptor in rabbit aortic endothelial cells (McCollum *et al*., 2007). A detailed investigation when this receptor is cloned should be performed to definitively test this hypothesis.

We would also like to point out that, although either NO (via non-CB_1/non-CB_2 cannabinoid receptor) or prostaglandin (via a 'PGE_2 like' product which binds to the EP_4 receptor) is involved in the vasorelaxant effect of anandamide in rat aorta, these endothelial vasorelaxant pathways need not be mutually exclusive. Indeed, there is much evidence for 'crosstalk' between endothelial NO and COX pathways. NO regulates COX activity in normal and inflamed tissues, and it has been demonstrated that NO can modulate the synthesis of PGE_2 via activation of the COX enzymes. On the other hand, there is also much evidence for the action of prostaglandins on the L-arginine:-NO pathway (Di Rosa *et al*., 1996.). Such interactions would explain the similar inhibition of anandamide's vasorelaxant effects when L-NAME or indomethacin were used in our study.

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Conflict of interest

The authors state no conflict of interest.

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