BJP BRITISH JOURNAL OF PHARMACOLOGY

BPS BRITISH PHARMACOLOGICAL SOCIETY

British Journal of Pharmacology (2009), 158, 1143–1152

0007-1188/09
www.brjpharmacol.org

RESEARCH PAPER

Factors influencing the regional haemodynamic responses to methanandamide and anandamide in conscious rats

SM Gardiner, JE March, PA Kemp and T Bennett

Centre for Integrated Systems Biology & Medicine, School of Biomedical Sciences, University of Nottingham, Nottingham, UK

Background and purpose: *In vitro* evidence suggests that metabolism of anandamide by cyclooxygenase-2 (COX-2) may be more important when the primary metabolic pathway [i.e. fatty acid amide hydrolase (FAAH)] is inhibited. Thus, the first aim of the present study was to assess the effects of COX-2 and/or FAAH inhibition, on the cardiovascular actions of anandamide. The second aim was to compare the effects of anandamide with those of the metabolically stable analogue (i.e. methanandamide) and investigate mechanisms involved in responses to the latter in conscious rats.
Experimental approach: Rats were chronically instrumented for recording blood pressure, heart rate and renal, mesenteric and hindquarters vascular conductances in the freely moving state.
Key results: Inhibition of FAAH with URB597 (cyclohexycarbamic acid 3′-carbamoyl-biphenyl-3-yl-ester) augmented the haemodynamic actions of anandamide, but there was no effect of COX-2 inhibition with parecoxib, either in the absence or the presence of URB597. Methanandamide caused CB_1 receptor-mediated renal and mesenteric vasoconstriction and evoked β_2-adrenoceptor-mediated hindquarters vasodilatation.
Conclusions and implications: No evidence for an involvement of COX-2 in the systemic cardiovascular actions of anandamide could be demonstrated. Vasoconstrictor actions of methanandamide were shown to involve CB_1 receptors, whereas no involvement of CB_1 receptors in such actions of anandamide has been shown. However, β_2-adrenoceptor-mediated hindquarters vasodilatation, independent of CB_1 receptors, observed here with methanandamide, has previously been seen with anandamide and differs from previous results with other synthetic cannabinoids for which the response was CB_1 receptor-dependent. Thus, mechanisms underlying the cardiovascular actions of endocannabinoids and synthetic analogues appear to be agonist-specific.
British Journal of Pharmacology (2009) **158,** 1143–1152; doi:10.1111/j.1476-5381.2009.00363.x; published online 20 August 2009

Keywords: anandamide; methanandamide; cannabinoid receptors; cyclooxygenase-2; fatty acid amide hydrolase

Abbreviations: AM251, N-(piperidin-1-yl)-5-(4-iodophenyl)-1-(2,4-dichlorophenyl)-4-methyl-1H-pyrazole-3-carboxamide; COX-2, cyclooxygenase-2; FAAH, fatty acid amide hydrolase; ICI118551, (±)-1-[2,3-(dihydro-7-methyl-1H-inden-4-yl)oxy]-3-[(1-methylethyl)amino]-2-butanol) hydrochloride; THC, Δ^9-tetrahydrocannabinol; URB597, cyclohexycarbamic acid 3′-carbamoyl-biphenyl-3-yl-ester

Introduction

The endocannabinoid system is currently considered a potential therapeutic target for a number of conditions, including cardiovascular disease (Pacher *et al.*, 2006; 2008). It is clear that, *in vitro*, endocannabinoids can cause vasodilatation via several different mechanisms (both CB_1 receptor-dependent and independent), and that, *in vivo*, the cardiovascular actions of the endocannabinoid, anandamide, are complex, cannot straightforwardly be explained by CB_1 receptor-mediated vasodilatation and are importantly influenced by the presence of anaesthesia (Randall *et al.*, 2004). If the endocannabinoid system is to be targeted for the treatment of cardiovascular disease, understanding mechanisms involved in the cardiovascular effects of endocannabinoids in the conscious state is of paramount importance.

The first description of the effects of i.v. administration of anandamide in conscious normotensive rats showed a prompt bradycardia associated with hypotension, followed by a short-lived pressor response (Lake *et al.*, 1997). The former was considered to be vagally mediated (as shown under anaesthesia), and, although the mechanism for the latter was not identified, it was augmented by the CB_1 receptor antagonist,

Correspondence: Professor SM Gardiner, Centre for Integrated Systems Biology & Medicine, School of Biomedical Sciences, University of Nottingham, Nottingham, NG7 2UH, UK. E-mail: sheila.gardiner@nottingham.ac.uk
Received 27 April 2009; accepted 12 May 2009

Rimonabant, possibly indicating the presence of an underlying, CB_1 receptor-mediated depressor effect (Lake *et al.*, 1997). To date, to our knowledge, there has been no convincing demonstration of CB_1 receptor-mediated vasodilator effects of anandamide *in vivo* in conscious normal animals. Thus, we have described the complex regional haemodynamic actions of anandamide in conscious rats (Gardiner *et al.*, 2002a; Wheal *et al.*, 2007a,b; Ho and Gardiner, 2009), but have never been able to show inhibition of a vasodilator effect by the CB_1 receptor antagonist, AM251 [N-(piperidin-1-yl)-5-(4-iodophenyl)-1-(2,4-dichlorophenyl)-4-methyl-1H-pyrazole-3-carboxamide]. In our experience, the predominant vasodilator action of anandamide occurs in the hindquarters vascular bed and is inhibited by the β_2-adrenoceptor antagonist, ICI118551 [(±)-1-[2,3-(dihydro-7-methyl-1H-inden-4-yl)oxy]-3-[(1-methylethyl)amino]-2-butanol) hydrochloride], and so is likely to be secondary to adrenaline release (Gardiner *et al.*, 2002a).

In vivo, anandamide is rapidly metabolized, mainly by the fatty acid amide hydrolase (FAAH) pathway (Cravatt *et al.*, 1996), although there is also evidence for an involvement of cyclooxygenase-2 (COX-2), which may become more important when FAAH is inhibited (see review Fowler, 2007). Such rapid metabolism may, in part, explain the complexity of the haemodynamic effects of anandamide. We have recently shown modest effects of FAAH inhibition with URB597 (cyclohexycarbamic acid 3′-carbamoyl-biphenyl-3-yl-ester) on the cardiovascular effects of anandamide *in vivo* (Ho and Gardiner, 2009), although the possible involvement of COX-2 products under those conditions was not explored. Therefore, the first aim of the present study was to evaluate the effects of FAAH inhibition with URB597 (Kathuria *et al.*, 2003; Piomelli *et al.*, 2006) and/or COX-2 inhibition with parecoxib (Talley *et al.*, 2000), on the haemodynamic effects of anandamide.

The anandamide analogue, methanandamide (Abadji *et al.*, 1994), is metabolically stable because it does not undergo rapid metabolism, although, interestingly, both anandamide and methanandamide can induce COX-2 through CB_1 receptor-dependent (Gardner *et al.*, 2003; Chen *et al.*, 2005) and independent (Hinz *et al.*, 2004; Rösch *et al.*, 2006) mechanisms. We have previously described regional haemodynamic actions of methanandamide (Wheal *et al.*, 2007a), but have not identified any mechanisms involved in those effects. One of the means by which anandamide causes vasodilatation *in vitro* is activation of $TRPV_1$ receptors on sensory nerves causing release of vasodilator peptides (Zygmunt *et al.*, 1999). Although effects of the $TRPV_1$ receptor antagonist, capsazepine, on different components of the blood pressure and heart rate response to anandamide and methanandamide have been reported in anaesthetized normotensive rats (Malinowska *et al.*, 2001) and in conscious, hypertensive rats (Li *et al.*, 2003; Wang *et al.*, 2005), $TRPV_1$ receptor-mediated vasodilatation induced by anandamide *in vivo* has, as far as we are aware, only been shown once (Akerman *et al.*, 2004), and that study was in pentobarbital-anaesthetized rats. Hence, the second aim of this study was to compare the regional haemodynamic effects of methanandamide with those of anandamide and to evaluate the effects of capsazepine thereon. Furthermore, against the background of our previous findings with anandamide (see above), we also assessed the effects of AM251 and ICI118551 on the cardiovascular responses to methanandamide.

Against the background of existing information in the literature, the novel information in this study includes an assessment of the effects of COX inhibition, in the absence and presence of FAAH inhibition, on the regional haemodynamic effects of anandamide; a dose–response comparison of anandamide and methanandamide in the same animals; evaluation of the effects of $TRPV_1$ receptor antagonism on the regional haemodynamic responses to anandamide and methanandamide; and eluciduation of the mechanisms underlying the regional vascular responses to methanandamide.

Methods

Animals and surgical preparation

Adult, male, Sprague-Dawley rats were purchased from Charles River (Margate, UK), and housed in groups of four, with free access to normal rat chow (Teklad Global 18% Protein Rodent Diet, Harlan, Madison, WI, USA) and water for at least a week after arrival, before undergoing any surgical procedure. Thereafter, rats underwent a two-stage surgical procedure for chronic implantation of flow probes (stage 1) and intravascular catheters (stage 2). At the first stage, rats (380–450 g) were anaesthetized (fentanyl and medetomidine, 300 $\mu g \cdot kg^{-1}$ of each i.p.) and, through a midline laparotomy, had miniature, pulsed Doppler flow probes (Crystal Biotech, Holliston, MA, USA) sutured around the left renal artery, superior mesenteric artery and distal aorta (hindquarters). The wires from the probes were taped together and anchored at the nape of the neck. After surgery, anaesthetic reversal and analgesia was achieved with s.c. atipamezole ($1 mg \cdot kg^{-1}$) and buprenorphine ($0.02 mg \cdot kg^{-1}$), and the animals were housed individually. Prior to the second surgical stage, the fitness of the animals was certified by the Named Veterinary Surgeon. At the second stage, animals were anaesthetized (as above) and had an intra-arterial catheter implanted in the distal abdominal aorta via the ventral caudal artery to allow monitoring of arterial blood pressure and heart rate. In addition, three i.v. catheters were implanted in the right jugular vein for administration of substances. The wires from the pulsed Doppler probes were soldered into a microconnector (Microtech Inc., Boothwyn, PA, USA), which was clamped into a harness worn by the rat. The catheters, which emerged from the same site on the nape of the neck as the probe wires, ran through a counter-balanced flexible spring, which was attached to the harness. Following catheterization, the anaesthetic was reversed and analgesia provided (as above), and animals were housed individually in the cages in which they remained for the duration of the experimental protocol, unrestrained, and with free access to food and water. The arterial catheters were connected to fluid-filled swivels for infusion of saline containing heparin ($15 units \cdot mL^{-1}$) to ensure patency. All procedures were approved by the Local Ethical Review Committee and were performed under Home Office Licence authority.

Cardiovascular recordings
The experimental protocols started around 07.00 h on the day following catheterization, when continuous recordings of cardiovascular variables [heart rate, blood pressure, renal, mesenteric and hindquarters Doppler shifts (flow)] were made using a customized, computer-based system [Haemodynamics Data Acquisition System (HDAS), University of Limburg, Maastricht, the Netherlands] connected to a transducer amplifier (Gould model 13-4615-50) and a Doppler flow meter [Crystal Biotech VF-1 mainframe (pulse repetition frequency 125 kHz) fitted with high-velocity (HVPD-20) modules]. Raw data were sampled by HDAS every 2 ms, averaged every cardiac cycle and stored to disk every cardiac cycle for the first 2 min following anandamide or methanandamide administration (Experiments 1 and 2) and at 5 s intervals thereafter.

Experimental protocols
Experiment 1. Cardiovascular responses to anandamide in the absence and presence of URB597 and/or parecoxib. One group of animals ($n = 8$) was used in this part of the study, and the experiments ran over 4 days. On days 1 and 2, all animals were given vehicle for parecoxib (0.1 mL saline, i.v.) and were randomized to receive either URB597 (3 mg·kg^{-1}; Ho and Gardiner, 2009) or vehicle (1 mL sterile saline containing 5% propylene glycol and 2% Tween 80 infused over 30 min) on day 1 and vice versa on day 2. On days 3 and 4, all animals were given parecoxib (10 mg·kg^{-1}, i.v.; Padi *et al.*, 2004; Gardiner *et al.*, 2006) followed by either URB597 or vehicle on day 3 and vice versa on day 4. Parecoxib or the vehicle was given 30 min before the onset of infusion of URB597 or vehicle, and anandamide (3 mg·kg^{-1}, i.v.) was given to all animals 30 min after the end of the URB597 or vehicle infusion.

Experiment 2. Dose–response comparison of the effects of anandamide and methanandamide. One group of rats ($n = 9$) was used in this part of the study, which ran over 4 days. On day 1, animals were given the vehicle control (0.2 mL Tocrisolve) and either anandamide ($n = 4$) or methanandamide ($n = 5$) at doses of 0.5, 1 and 5 mg·kg^{-1} in ascending order and separated by at least 2 h (all in a total volume of 0.2 mL, diluted in Tocrisolve). On day 2, the protocol was repeated with the other cannabinoid being administered. On day 3, a high dose (10 mg·kg^{-1}) of anandamide or methanandamide or the vehicle control (0.4 mL Tocrisolve) was administered but only to a small numbers of animals ($n = 2$ in each case) due to the extreme circulatory effects and marked respiratory depression evoked by anandamide, and to the substantial behavioural effects of methanandamide. On day 4, all animals were given 3 mg·kg^{-1} anandamide and methanandamide in random order separated by at least 2 h.

Experiment 3. Effects of capsazepine on the regional haemodynamic responses to anandamide or methanandamide. One group of rats ($n = 8$) was used in this part of the study, which ran over 4 days. Animals were given anandamide (3 mg·kg^{-1}, i.v.) or methanandamide (3 mg·kg^{-1}, i.v.), 10 min after administration of capsazepine (3 mg·kg^{-1}, i.v.; Akerman *et al.*, 2004) or vehicle (0.1 mL of sterile saline containing 5% propylene glycol and 2% Tween 80). The sequence of experiments across the 4 days was randomized between animals.

Experiment 4. Effects of parecoxib, AM251 or ICI118551 on the cardiovascular responses to methanandamide. Three groups of animals were used in this part of the study. One group ($n = 10$) was given methanandamide (3 mg·kg^{-1}, i.v.) alone on day 1 and methanandamide (3 mg·kg^{-1}, i.v.) 90 min after administration of parecoxib (10 mg·kg^{-1}) on day 3, with no substance administration on day 2. A second group ($n = 8$) was given methanandamide (3 mg·kg^{-1}, i.v.) 30 min after the end of infusion of AM251 (3 mg·kg^{-1} in 1 mL sterile saline containing 5% propylene glycol and 2% Tween 80 infused over 30 min). A third group ($n = 8$) was given methanandamide (3 mg·kg^{-1}, i.v.) 90 min after the onset of primed infusion of ICI118551 (0.2 mg·kg^{-1} bolus followed by 0.1 mg·kg^{-1}·h^{-1} infusion at 0.4 mL·h^{-1}).

Data analysis
Data were analysed off-line using software (Datview, University of Limburg, Maastricht, the Netherlands), which interfaced with HDAS. Because not all the data were normally distributed, a non-parametric, two-way analysis of variance (Friedman's test; Theodorsson-Norheim, 1987) was used for within-group comparisons, and the Kruskal–Wallis test was used for between-group comparisons. Vascular conductances were calculated from the mean arterial blood pressure and Doppler shift (flow) data. $P \leq 0.05$ was taken as significant.

Materials
AM251, ICI118551, anandamide and methanandamide (in Tocrisolve) were obtained from Tocris (Avonmouth, UK). Parecoxib sodium (*N*-[[(5-methyl-3-phenylisoxazol-4-yl)-phenyl] sulphonyl] propanamide sodium) was purchased from Sequoia Research Products (Oxford, UK), and URB597 was purchased from Sigma (Poole, UK).

Fentanyl citrate was from Janssen-Cilag (High Wycombe, UK); medetomidine hydrochloride (Domitor) and atipamezole hydrochloride (Antisedan) were from Pfizer (Sandwich, Kent, UK), buprenorphine (Vetergesic) was from Alstoe Animal Health (York, UK).

Drug/molecular target nomenclature conforms with the British Journal of Pharmacology Guide to Receptors and Channels (Alexander *et al.*, 2008).

Results

Experiment 1. Cardiovascular responses to anandamide in the absence and presence of URB597 and/or parecoxib
Cardiovascular variables prior to any intervention, following vehicle or parecoxib administration, and after subsequent administration of URB597 or vehicle are shown in Table 1. There were no differences between the four conditions with the exception of heart rates, which were significantly reduced ($P < 0.05$, Friedman's test) by administration of URB597 (Table 1).

Table 1 Cardiovascular variables during the pretreatment period prior to administration of anandamide (Experiment 1)

	Control	*30 min*	*60 min*	*90 min*
Heart rate				
Vehicle/vehicle	357 ± 20	356 ± 24	354 ± 17	359 ± 19
Vehicle/URB597	365 ± 18	357 ± 19	341 ± 14*	342 ± 11*
Parecoxib/vehicle	348 ± 15	346 ± 15	342 ± 13	348 ± 16
Parecoxib/URB597	350 ± 10	347 ± 12	326 ± 8*	337 ± 9*
Blood pressure				
Vehicle/vehicle	106 ± 3	108 ± 4	109 ± 3	108 ± 3
Vehicle/URB597	105 ± 2	101 ± 3	103 ± 2	106 ± 3
Parecoxib/vehicle	109 ± 3	107 ± 2	108 ± 3	105 ± 3
Parecoxib/URB597	105 ± 2	105 ± 2	103 ± 1	100 ± 2
Renal VC				
Vehicle/vehicle	67 ± 12	66 ± 11	64 ± 8	68 ± 9
Vehicle/URB597	70 ± 7	71 ± 7	73 ± 7	71 ± 7
Parecoxib/vehicle	70 ± 9	66 ± 9	70 ± 10	70 ± 10
Parecoxib/URB597	66 ± 10	67 ± 10	69 ± 8	69 ± 10
Mesenteric VC				
Vehicle/vehicle	66 ± 9	59 ± 8	57 ± 7	64 ± 9
Vehicle/URB597	72 ± 9	71 ± 8	65 ± 6	68 ± 5
Parecoxib/vehicle	65 ± 8	64 ± 8	61 ± 4	66 ± 6
Parecoxib/URB597	69 ± 8	66 ± 6	64 ± 5	65 ± 5
Hindquarters VC				
Vehicle/vehicle	46 ± 5	42 ± 4	42 ± 3	43 ± 4
Vehicle/URB597	44 ± 3	46 ± 3	44 ± 2	42 ± 2
Parecoxib/vehicle	41 ± 3	44 ± 4	42 ± 3	42 ± 3
Parecoxib/URB597	48 ± 3	43 ± 1	44 ± 2	44 ± 2

Measurements were made before any intervention (control), 30 min after administration of vehicle (0.1 mL saline) or parecoxib (10 mg·kg^{-1}) (30 min), at the end of infusion of URB597 (3 mg·kg^{-1}) or vehicle (1 mL 5% propylene glycol, 2% Tween 80 in saline) (60 min) and immediately prior to administration of anandamide (3 mg·kg^{-1}) (90 min). The results are presented as mean ± SEM. *P < 0.05 versus control (Friedman's test). Units are heart rate, beats·min^{-1}; blood pressure, mmHg; vascular conductance (VC), (kHz·mmHg^{-1})10^3.
URB597, cyclohexycarbamic acid 3'-carbamoyl-biphenyl-3-yl-ester.

Intravenous administration of anandamide in the presence of vehicle controls (Figure 1) caused a complex haemodynamic response as described previously (Gardiner *et al.*, 2002a; Wheal *et al.*, 2007a,b; Ho and Gardiner, 2009). There was a prompt and marked fall in heart rate (P < 0.05 vs. baseline from 5–300 s), a fall in blood pressure (P < 0.05 at 5 s) followed by a rise (P < 0.05 from 20–300 s), falls in renal and mesenteric vascular conductances (P < 0.05 from 5–240 s and 5–600 s respectively) and an initial fall in hindquarters vascular conductance (P < 0.05 from 5–20 s) followed by a rise (P < 0.05 from 50–120 s).

Pretreatment with parecoxib and the vehicle for URB597 did not affect the cardiovascular response to anandamide (Figure 1A). Pretreatment with URB597 and the vehicle for parecoxib (saline) did not affect the initial (at 5 s) cardiovascular effects of anandamide, but the mesenteric vasoconstriction and hindquarters vasodilatation were prolonged, such that the integrated (0–10 min) changes were significantly (P < 0.05, Friedman's test) different (Figure 1B). Combined pretreatment with parecoxib and URB597 had no additional effects to those seen with URB597 in the presence of the vehicle for parecoxib (Figure 1C).

Experiment 2. Dose–response comparison of the effects of anandamide and methanandamide

For this experiment, the maximum changes in the different components of the haemodynamic responses to the cannabinoids at each dose have been analysed separately, that is, the fall in heart rate (at 5 s), the fall and subsequent rise in blood pressure (at 5 s and 10–20 s respectively), the falls in renal and mesenteric vascular conductances (at 5–10 s) and the fall and subsequent rise in hindquarters vascular conductance (at 5–10 s and 90–120 s respectively). Because only two animals were given the 10 mg·kg^{-1} doses, no statistical comparisons have been performed on those data.

Heart rate (Figure 2A)

At the low doses (0.5 and 1 mg·kg^{-1}), neither anandamide nor methanandamide had any significant effect on heart rate, whereas at the higher doses (3–10 mg·kg^{-1}) both cannabinoids caused marked bradycardia, the magnitude of which was not dose-dependent. The bradycardic effects of anandamide (3 and 5 mg·kg^{-1}) were significantly greater than those of methanandamide (P < 0.05, Wilcoxon's test).

Blood pressure (Figure 2B)

There was an initial hypotensive response to high (3–10 mg·kg^{-1}) but not to low (0.5 and 1 mg·kg^{-1}) doses of anandamide, and a subsequent pressor response at all doses. The magnitude of the hypotensive response to anandamide was not dose-dependent (Figure 2B1), and there was no hypotensive response to vehicle or methanandamide at any dose given. Administration of vehicle caused a small rise in blood pressure, but anandamide and methanandamide caused further rises in blood pressure, the magnitude of which increased with increasing dose. The pressor responses to the cannabinoids were generally similar, except at the 5 mg·kg^{-1}

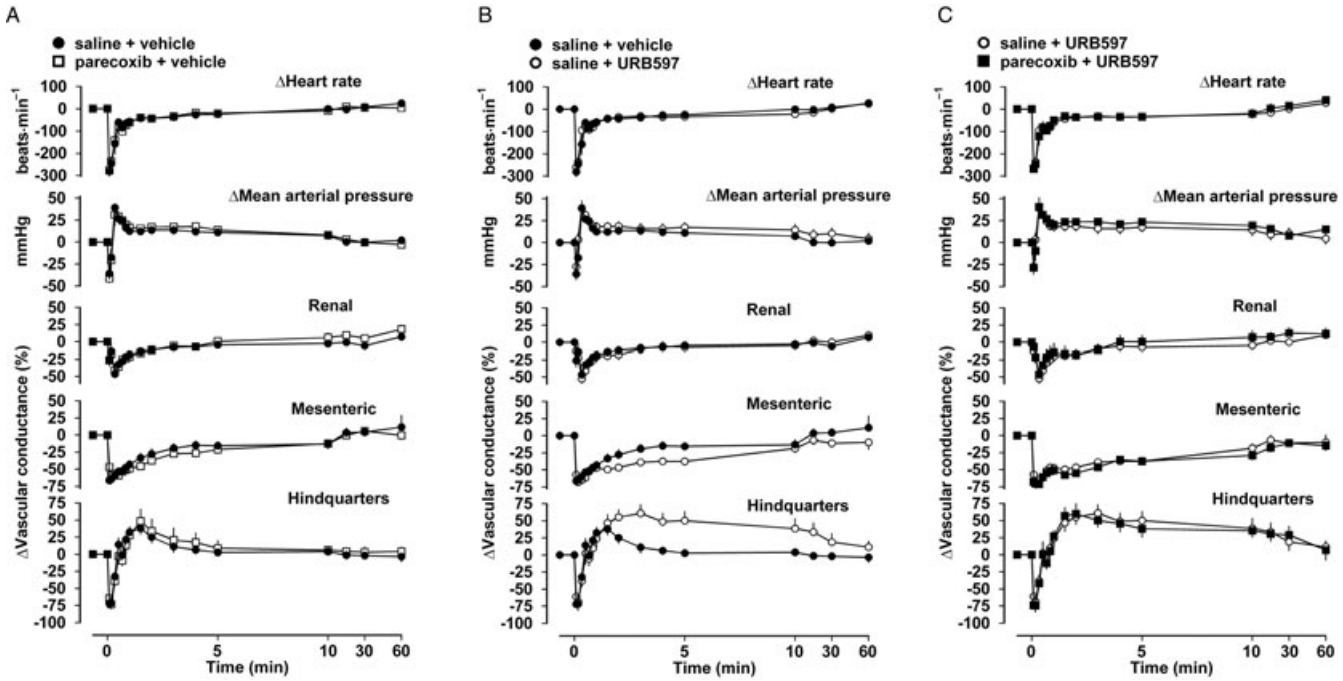

Figure 1 Cardiovascular responses to anandamide (3 mg·kg^{-1}) following pretreatment with (A) saline (0.1 mL) or parecoxib (10 mg·kg^{-1}) together with vehicle for URB597 (cyclohexycarbamic acid 3′-carbamoyl-biphenyl-3-yl-ester) (5% propylene glycol, 2% Tween 80 in saline), (B) saline (0.1 mL) together with vehicle or URB597 (3 mg·kg^{-1}) or (C) saline or parecoxib together with URB597 (C). Values are mean and vertical bars indicate SEM; $n = 8$. Between-group and within-group differences are given in the text.

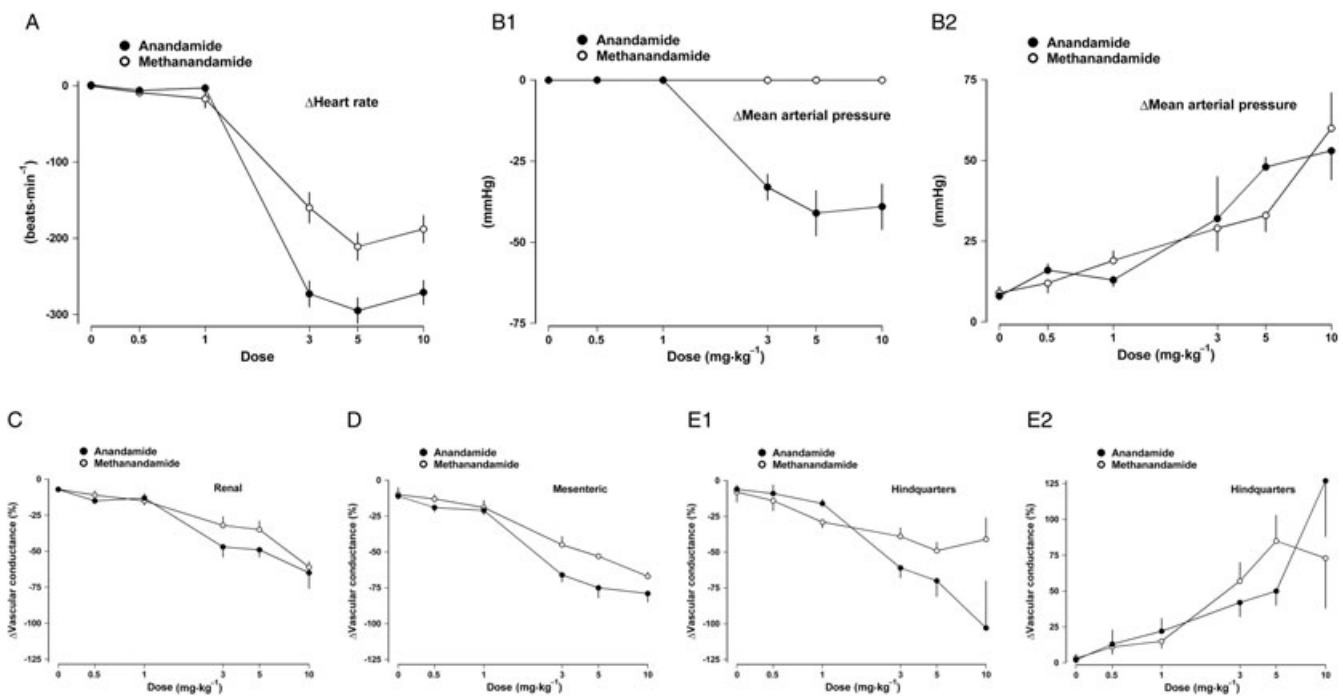

Figure 2 Maximum changes in cardiovascular variables in response to increasing doses of anandamide and methanandamide. The falls in heart rate (A) and blood pressure (B1) were measured at 5 s, the rise in blood pressure (B2) was measured at 10–20 s, the falls in renal (C) mesenteric (D) and hindquarters vascular conductance (E1) were measured at 5–10 s, and the subsequent rise in hindquarters vascular conductance (E2) was measured at 90–120 s. Values are mean and vertical bars indicate SEM; $n = 9$ except in the case of the highest dose where $n = 2$ in each case. Statistical comparisons are given in the text.

Table 2 Cardiovascular variables immediately prior to administration of anandamide (A, 3 mg·kg^{-1}) or methanandamide (MethA, 3 mg·kg^{-1}) following pretreatment with vehicle or capsazepine (Capz, 3 mg·kg^{-1}) (Experiment 3)

	Vehicle/A	*Vehicle/MethA*	*Capz/A*	*Capz/MethA*
Heart rate	353 ± 16	343 ± 11	341 ± 8	354 ± 11
Blood pressure	108 ± 4	107 ± 2	106 ± 2	110 ± 4
Renal VC	70 ± 4	68 ± 5	71 ± 6	68 ± 6
Mesenteric VC	67 ± 5	66 ± 8	71 ± 5	65 ± 7
Hindquarters VC	40 ± 5	45 ± 6	43 ± 5	41 ± 5

The results are presented as mean ± SEM. The vehicle consisted of 0.1 mL 5% propylene glycol, 2% Tween 80 in saline. Units are heart rate, beats·min^{-1}; blood pressure, mmHg; vascular conductance (VC), (kHz·mmHg^{-1})10^3.

dose, where the effects of anandamide were significantly greater than those of methanandamide (Wilcoxon's test) (Figure 2B2).

Renal vascular conductance (Figure 2C)
Administration of vehicle caused a small fall in renal vascular conductance, but anandamide and methanandamide caused further falls in renal vascular conductance with increasing dose. The renal vasoconstrictor effects of anandamide (3 and 5 mg·kg^{-1}) were significantly greater than those of methanandamide.

Mesenteric vascular conductance (Figure 2D)
There was a small mesenteric vasoconstrictor response to vehicle administration, but anandamide and methanandamide caused additional, dose-dependent decreases in mesenteric vascular conductance, which differed from the vehicle for all doses of anandamide and for doses of 1 mg·kg^{-1} and higher of methanandamide. The mesenteric vasoconstrictor effects of anandamide (3 and 5 mg·kg^{-1}) were significantly greater than those of methanandamide.

Hindquarters vascular conductance (Figure 2E)
There was no significant effect of vehicle administration on hindquarters vascular conductance. Anandamide and methanandamide both caused initial, dose-related falls in hindquarters vascular conductance, but the hindquarters vasoconstrictor effects of anandamide (1, 3 and 5 mg·kg^{-1}) were greater (Wilcoxon's test) than those of methanandamide (Figure 2E1). Following the initial fall in hindquarters vascular conductance, anandamide and methanandamide both caused dose-dependent rises in hindquarters vascular conductance; the hindquarters vasodilator effects of methanandamide (3 and 5 mg·kg^{-1}) were greater (Wilcoxon's test) than those of anandamide (Figure 2E2).

Experiment 3. Effects of capsazepine on the regional haemodynamic effects of anandamide and methanandamide
Pretreatment with capsazepine had no significant cardiovascular effects; hence, immediately prior to administration of anandamide or methanandamide, there was no difference in cardiovascular variables on the four occasions (Table 2).

The cardiovascular effects of anandamide in this group of animals in the presence of vehicle were as described above (Experiments 1 and 2), that is, bradycardia, hypotension followed by hypertension, renal and mesenteric vasoconstriction and hindquarters vasoconstriction followed by vasodilatation (Figure 3A). Pretreatment with capsazepine had no significant effect on any component of the response to anandamide (Figure 3A).

Following pretreatment with vehicle, the cardiovascular effects of methanandamide were as described above (Experiment 2), that is, a prompt bradycardia that rapidly resolved ($P < 0.05$ from 5–30 s); no initial hypotension, but a rapid rise in blood pressure (+30 ± 7 mmHg at 10 s) that reversed but stayed significantly above baseline for 600 s (Figure 3B); falls in vascular conductance in the renal ($P < 0.05$ from 5–300 s) and mesenteric ($P < 0.05$ from 5–600 s) vascular beds; and in the hindquarters, an initial short-lived fall in vascular conductance ($P < 0.05$ from 5–10 s) followed by a more prolonged vasodilatation ($P < 0.05$ from 20–1200 s). In animals pretreated with capsazepine, the only minor difference in the cardiovascular response to methanandamide was a more rapid recovery in mesenteric vascular conductance, but the integrated (0–30 min) cardiovascular responses to methanandamide were not significantly affected by capsazepine (Figure 3B).

Experiment 4. Effects of parecoxib, AM251 or ICI118551 on the cardiovascular responses to methanandamide
Following pretreatment with parecoxib, AM251 or ICI118551 there were no consistent cardiovascular changes and, immediately prior to administration of methanandamide, cardiovascular variables in rats pretreated with parecoxib, AM251 or ICI118551 were not different from those in the control group (Table 3).

In this series of experiments, the initial (5–30 s) cardiovascular responses to methanandamide were not analysed as comparisons were not being made with anandamide. Thus, cardiovascular responses to methanandamide in the control group (Figure 4) comprised bradycardia ($P < 0.05$ from 1–10 min), a rise in blood pressure ($P < 0.05$ from 1–10 min), falls in renal vascular conductance ($P < 0.05$ from 1–5 min) and mesenteric vascular conductance ($P < 0.05$ from 1–40 min) and a rise in hindquarters vascular conductance ($P < 0.05$ from 1–20 min). In animals pretreated with parecoxib compared with control, there were no significant differences between the integrated (0–60 min) falls in heart rate (–864 ±

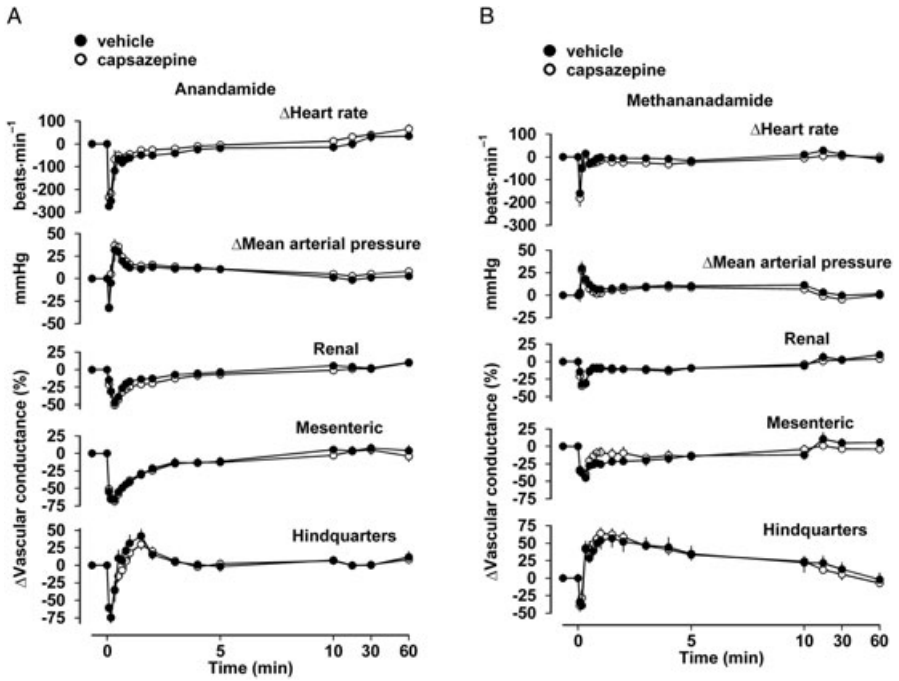

Figure 3 Cardiovascular responses to 3 mg·kg^{-1} anandamide (A) or methanandamide (B) following pretreatment with vehicle (0.1 mL 5% propylene glycol, 2% Tween 80 in sterile saline) or capsazepine (3 mg·kg^{-1}). Values are mean and vertical bars indicate SEM.; n = 8. Between-group and within-group differences are given in the text.

Table 3 Cardiovascular variables immediately prior to administration of methanandamide (3 mg·kg^{-1}) in control rats and in rats pretreated with parecoxib (10 mg·kg^{-1}), AM251 (3 mg·kg^{-1}) or ICI118551 (0.2 mg·kg^{-1} bolus, 0.1 mg·kg^{-1}·h^{-1} infusion)

Pretreatment	*Control*	*Parecoxib*	*AM251*	*ICI118551*
Heart rate	356 ± 8	332 ± 11	360 ± 13	338 ± 12
Blood pressure	112 ± 4	108 ± 4	110 ± 3	111 ± 5
Renal VC	77 ± 6	73 ± 4	77 ± 10	87 ± 10
Mesenteric VC	68 ± 6	78 ± 6	73 ± 6	67 ± 7
Hindquarters VC	43 ± 4	40 ± 3	38 ± 5	35 ± 5

The results are presented as mean ± SEM. Units are heart rate, beats·min^{-1}; blood pressure, mmHg; vascular conductance (VC), (kHz·mmHg^{-1})10^3.
AM251, N-(piperidin-1-yl)-5-(4-iodophenyl)-1-(2,4-dichlorophenyl)-4-methyl-1H-pyrazole-3-carboxamide; ICI118551, (±)-1-[2,3-(dihydro-7-methyl-1H-inden-4-yl)oxy]-3-[(1-methylethyl)amino]-2-butanol) hydrochloride.

278 vs. –979 ± 244 beats), rises in blood pressure (497 ± 143 vs. 395 ± 96 mmHg·min), falls in renal vascular conductance (–472 ± 182 vs. 375 ± 91%·min), falls in mesenteric vascular conductance (–1054 ± 246 vs. 980 ± 186%·min) and rises in hindquarters vascular conductance (+1154 ± 204 vs. 1030 ± 178%·min) in response to methanandamide.

In animals pretreated with AM251, the bradycardic and hindquarters vasodilator responses to methanandamide were not significantly affected, but the renal and mesenteric vasoconstrictions were changed to vasodilatations (P < 0.05 from 2–40 min and 3–5 min for renal and mesenteric vascular conductances respectively), and the accompanying rise in blood pressure was converted to a fall in blood pressure (P < 0.05 from 2–20 min) (Figure 4A). In contrast, in animals pretreated with ICI118551, although the heart rate response to methanandamide was unaffected, the rise in blood pressure was enhanced [P < 0.05 for integrated (0–60 min) change] together with enhanced renal vasoconstriction [P < 0.05 for integrated (0–60 min) change] and a total abolition of the hindquarters vasodilatation (Figure 4B).

Discussion

The present study contains several novel observations regarding the cardiovascular actions of anandamide and methanandamide in conscious rats. First, while there is a clear role for FAAH in the breakdown of anandamide, there was no evidence of an involvement of COX-2, even under conditions in which FAAH was inhibited. Second, by comparing different doses of anandamide and methanandamide in the same animals, it was clear that the initial bradycardic action was only triggered at doses of 3 mg·kg^{-1} and higher, was not dose-dependent and was more marked with anandamide. Even at the highest dose used (10 mg·kg^{-1}), the bradycardic effect of methanandamide was not accompanied by hypotension. Third, there was no evidence for an involvement of $TRPV_1$ receptors in the cardiovascular responses to anandamide or methanandamide at the doses used. Finally, any vasodilator effect of methanandamide, like that of anandamide (Gardiner *et al*., 2002a), was mainly localized to the hindquarters vascular bed and was sensitive to β_2-adrenoceptor antagonism, but not to CB_1 receptor antagonism. However, the pressor and vasoconstrictor effects of methanandamide, unlike those of anandamide (Gardiner *et al*., 2002a; Ho and Gardiner, 2009), were CB_1 receptor-mediated, as has been previously reported for such actions of other synthetic cannabinoids (Gardiner *et al*., 2002b) and Δ^9-tetrahydrocannabinol (THC) (O'Sullivan *et al*., 2007).

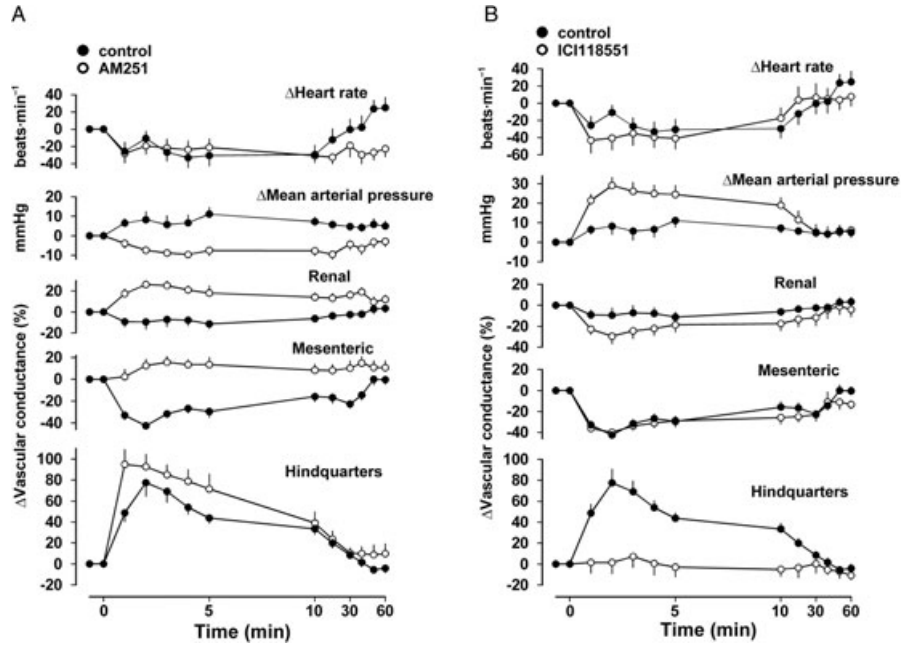

Figure 4 Cardiovascular responses to methanandamide (3 mg·kg^{-1}) under control conditions (n = 10) and in the presence of either (A) AM251 [N-(piperidin-1-yl)-5-(4-iodophenyl)-1-(2,4-dichlorophenyl)-4-methyl-1H-pyrazole-3-carboxamide] (3 mg·kg^{-1}; n = 8) or (B) ICI118551 [(±)-1-[2,3-(dihydro-7-methyl-1H-inden-4-yl)oxy]-3-[(1-methylethyl)amino]-2-butanol) hydrochloride] (0.2 mg·kg^{-1} bolus, 0.1 mg·kg^{-1}·h^{-1} infusion; n = 8). Values are mean and vertical bars indicate SEM. Between-group and within-group differences are given in the text.

Anandamide is rapidly hydrolysed to arachidonic acid, a process that is predominantly mediated by FAAH (Cravatt *et al.*, 1996), so it has been suggested that FAAH could be a potential therapeutic target for manipulating endocannabinoid activity in disease states (Cravatt and Lichtman, 2003; Pacher *et al.*, 2008). In the rabbit isolated lung preparation, it has been shown that a vasoconstrictor action of anandamide is insensitive to CB_1 and $TRPV_1$ receptor antagonism, but depends on FAAH and is inhibited by COX-2 inhibition, suggesting that FAAH-mediated hydrolysis of anandamide leads to the formation of arachidonic acid products that are metabolized by COX-2 to produce vasoconstrictor prostanoids (Wahn *et al.*, 2005). If this mechanism was manifest *in vivo* and in the systemic circulation, then the vasoconstrictor effect of anandamide should be inhibited by URB597 and be sensitive to COX-2 inhibition. However, that was not the case, inasmuch as there was enhancement of the vasoconstrictor effects of anandamide in the presence of URB597 and no effect of parecoxib. It has recently been reported that COX-2 inhibition caused a slight enhancement of anandamide-induced relaxation in isolated small mesenteric arteries, but this was not seen in the combined presence of COX-2 and FAAH inhibitors, again suggesting a role for COX-2 products downstream of FAAH-induced hydrolysis of anandamide (Ho and Randall, 2007). If such mechanisms were apparent *in vivo*, then any vasodilator effect of anandamide should have been enhanced or uncovered by parecoxib, but this was not observed.

It has been shown that anandamide itself is a substrate for COX-2, and that the contribution of COX-2 to the metabolism and action of anandamide is increased in the presence of FAAH inhibition (for review see Fowler, 2007). If this mechanism was active *in vivo* and in the systemic circulation, then an effect of COX-2 inhibition on the cardiovascular effects of anandamide should be more apparent in the presence of FAAH inhibition, but there was no effect of parecoxib, either in the absence, or in the presence of URB597. Thus, we found no evidence of a role for COX-2 metabolites in the cardiovascular actions of anandamide in conscious, normotensive rats.

Comparison of the effects of different doses of anandamide with those of methanandamide in the same animals (Experiment 2) showed smaller effects of methanandamide on heart rate with no initial hypotension at any dose used, and generally smaller vasoconstrictor effects but greater delayed hindquarters vasodilator effects of methanandamide. In a previous study using methanandamide in the same strain of rat (Wheal *et al.*, 2007a), the initial cardiovascular effects were more modest than seen here and, surprisingly, there was a second phase of response, starting approximately 40 min after cannabinoid administration. In that study, we speculated about a possible involvement of COX-2 induction in the second phase of response. However, in the present studies, there was no second phase of response to methanandamide (data collection continued for 3 h post-dosing at 3 and 5 mg·kg^{-1}), and treatment with parecoxib had no effect on the cardiovascular response to methanandamide. We have no explanation for this, other than to suggest that it may be attributable to different batches of methanandamide. The present study used three different batches of methanandamide, all of which were other than that used in the experiments of Wheal *et al.* (2007a).

While there is good *in vitro* evidence for an involvement of $TRPV_1$ receptors in the vascular effects of anandamide (see Randall *et al.*, 2004), the *in vivo* evidence using capsazepine as

a $TRPV_1$ receptor antagonist is less clear-cut. Thus, Malinowska *et al.* (2001) showed that capsazepine (~1 mg·kg^{-1}) caused some inhibition of the initial bradycardic response to anandamide (and methanandamide) in urethane-anaesthetized rats, and, although Akerman *et al.* (2004) were able to show inhibition by capsazepine (3 mg·kg^{-1}) of the hypotensive response to anandamide (5 mg·kg^{-1}) in pentobarbital-anaesthetized rats, the main component of the blood pressure response being measured in that study was the third phase, that is, the phase that does not occur in conscious animals. In conscious, normotensive rats capsazepine had no effect on responses to methanandamide (Li *et al.*, 2003; Wang *et al.*, 2005).

The clearest evidence of a role for $TRPV_1$ receptors in the cardiovascular effects of anandamide comes from a study using anaesthetized, $TRPV_1$ receptor knockout mice (Pacher *et al.*, 2004), where the initial hypotensive and bradycardic effects of anandamide were absent, and the subsequent short-lived pressor response was reduced, whereas the more prolonged hypotension was not different from the control. On the basis of that study, we expected to see inhibition of the initial cardiovascular responses to anandamide and methanandamide by capsazepine. Furthermore, from the findings of Li *et al.* (2003), Akerman *et al.* (2004) and Wang *et al.* (2005), we hypothesised that capsazepine might inhibit a vasodilator action of the cannabinoids, or enhance a vasoconstriction. However, we were unable to demonstrate any effect of capsazepine on any component of the cardiovascular actions of either anandamide or methanandamide. On the basis of our findings we have to conclude that, either there is no $TRPV_1$ receptor involvement in the cardiovascular actions of anandamide or methanadamide in conscious, normotensive rats, or that the dose of capsazepine was inadequate under the conditions of our experiment.

In the final series of experiments, we sought to establish the role of CB_1 receptors and β_2-adrenoceptors in the cardiovascular actions of methanandamide. We have previously reported that the renal and mesenteric vasoconstrictor effects of synthetic cannabinoids (Gardiner *et al.*, 2002b) and THC (O'Sullivan *et al.*, 2007) were susceptible to antagonism by AM251, whereas those effects of anandamide were not (Gardiner *et al.*, 2002a; Wheal *et al.*, 2007a; Ho and Gardiner, 2009). The present results showed a very clear antagonism, by AM251, of the renal and mesenteric vasoconstrictor action of methanandamide, such that the modest pressor effect was changed to a depressor effect. We suggested previously that this pressor effect may have been due to centrally mediated sympathoexcitation, and the present results indicate that such a putative action may be confined to the more stable cannabinoids. With regard to the hindquarters vasodilatation, we have consistently shown an effect of β_2-adrenoceptor antagonism on this component of the response to both anandamide (Gardiner *et al.*, 2002a) and synthetic cannabinoids (Gardiner *et al.*, 2002b), and the present study now shows the same result with methanandamide. However, in our previous experiments, the involvement of CB_1 receptors in cannabinoid-induced hindquarters vasodilatation has not been uniform. Thus, for WIN 55212-2 and HU210 (Gardiner *et al.*, 2002b) and THC (O'Sullivan *et al.*, 2007), the hindquarters vasodilatation is also blocked by AM251, whereas there was no effect of AM251 on anandamide-induced hindquarters vasodilatation (Gardiner *et al.*, 2002a). The present results show that, for this component of the response, methanandamide resembled anandamide, inasmuch as there was no effect of AM251, raising the possibility of these compounds having a direct, non-CB_1 receptor-mediated stimulating effect on adrenal chromaffin cells to cause adrenaline release (Gardiner *et al.*, 2002a).

In conclusion, although FAAH inhibition enhanced the cardiovascular actions of anandamide, we could find no evidence that COX-2 inhibition influenced cardiovascular responses to the endocannabinoid, either in the absence or presence of FAAH inhibition. Thus, it appears that there is no role for COX-2-derived products in the cardiovascular actions of anandamide (or methanandamide) *in vivo*. Furthermore, we could see no signs of $TRPV_1$ receptor-mediated vasodilatation in the cardiovascular actions of anandamide or methanandamide, although methanandamide caused CB_1 receptor-mediated renal and mesenteric vasoconstriction and β_2-adrenoceptor-mediated vasodilatation in the hindquarters.

Conflicts of interest

None.

References

Abadji V, Lin S, Taha G, Griffin G, Stevenson LA, Pertwee RG *et al.* (1994). R)-methanandamide: a chiral novel anandamide possessing higher potency and metabolic stability. *J Med Chem* **37**: 1889–1893.

Akerman S, Kaube H, Goadsby PJ (2004). Anandamide acts as a vasodilator of dural blood vessels *in vivo* by activation TRPV1 receptors. *Br J Pharmacol* **142**: 1354–1360.

Alexander SPH, Mathie A, Peters JA (2008). Guide to receptors and channels (GRAC), 3rd edn. *Br J Pharmacol* **153** (Suppl. 2): S1–S209.

Chen P, Hu S, Yao JY, Moore SA, Spector AA, Fang X (2005). Induction of cyclooxygenase-2 by anandamide in cerebral microvascular endothelium. *Microvasc Res* **69**: 28–35.

Cravatt BF, Lichtman AH (2003). Fatty acid amide hydrolase: an emerging therapeutic target in the endocannabinoid system. *Curr Opin Chem Biol* **7**: 469–475.

Cravatt BF, Giang DK, Mayfield SP, Boger DL, Lerner RA, Gilula NB (1996). Molecular characterization of an enzyme that degrades neuromodulatory fatty-acid amides. *Nature* **384**: 83–87.

Fowler CJ (2007). The contribution of cyclooxygenase-2 to endocannabinoid metabolism and action. *Br J Pharmacol* **152**: 594–601.

Gardiner SM, March JE, Kemp PA, Bennett T (2002a). Complex regional haemodynamic effects of anandamide in conscious rats. *Br J Pharmacol* **135**: 1889–1896.

Gardiner SM, March JE, Kemp PA, Bennett T (2002b). Influence of the CB1 receptor antagonist, AM 251, on the regional haemodynamic effects of WIN-55212-2 or HU 210 in conscious rats. *Br J Pharmacol* **136**: 581–587.

Gardiner SM, March JE, Kemp PA, Bennett T (2006). Effects of nitric oxide synthase inhibition with or without cyclooxygenase-2 inhibition on resting haemodynamics and responses to exendin-4. *Br J Pharmacol* **149**: 802–809.

Gardner B, Zhu LX, Sharma S, Tashkin DP, Dubinett SM (2003). Methanandamide increases COX-2 expression and tumor growth in murine lung cancer. *FASEB J* **17**: 2157–2159.

Hinz B, Ramer R, Eichele K, Weinzierl U, Brune K (2004). R(+)-methanandamide-induced cyclooxygenase-2 expression in H4 human neuroglioma cells: possible involvement of lipid rafts. *Biochem Biophys Res Commun* **324**: 621–626.

Ho W-SV, Gardiner SM (2009). Acute hypertension reveals depressor and vasodilator effects of cannabinoids in conscious rats. *Br J Pharmacol* **156**: 94–104.

Ho W-SV, Randall MD (2007). Endothelium-dependent metabolism by endocannabinoid hydrolases and cyclooxygenases limits vasorelaxation to anandamide and 2-arachidonoylglycerol. *Br J Pharmacol* **150**: 641–651.

Kathuria S, Gaetani S, Fegley D, Valino F, Duranti A, Tontini A *et al*. (2003). Modulation of anxiety through blockade of anandamide hydrolysis. *Nat Med* **9**: 76–81.

Lake KD, Martin BR, Kunos G, Varga K (1997). Cardiovascular effects of anandamide in anesthetized and conscious normotensive and hypertensive rats. *Hypertension* **29**: 1204–1210.

Li J, Kaminski NE, Wang DH (2003). Anandamide-induced depressor effect in spontaneously hypertensive rats: role of the vanilloid receptor. *Hypertension* **41** (Pt 2): 757–762.

Malinowska B, Kwolek G, Göthert M (2001). Anandamide and methanandamide induce both vanilloid VR1- and cannabinoid CB_1 receptor-mediated changes in heart rate and blood pressure in anaesthetised rats. *Naunyn Schmiedebergs Arch Pharmacol* **364**: 562–569.

O'Sullivan SE, Randall MD, Gardiner SM (2007). The *in vitro* and *in vivo* cardiovascular effects of Δ^9-tetrahydrocannabinol in rats made hypertensive by chronic inhibition of nitric oxide synthase. *J Pharmacol Exp Ther* **321**: 663–672.

Pacher P, Bátkai S, Kunos G (2004). Haemodynamic profile and responsiveness to anandamide of $TRPV_1$ receptor knock-out mice. *J Physiol* **558** (Pt 2): 647–657.

Pacher P, Bátkai S, Kunos G (2006). The endocannabinoid system as an emerging target of pharmacotherapy. *Pharmacol Rev* **58**: 389–462.

Pacher P, Mukhopadhyay P, Mohanraj R, Godlewski G, Bátkai S, Kunos G (2008). Modulation of the endocannabinoid system in cardiovascular disease. *Hypertension* **52**: 601–607.

Padi SSV, Jain NK, Singh S, Kulkarni SK (2004). Pharmacological profile of parecoxib: a novel, potent injectable selective cyclooxygenase-2 inhibitor. *Eur J Pharmacol* **491**: 69–76.

Piomelli D, Tarzia G, Duranti A, Tontini A, Mor M, Compton TR *et al*. (2006). Pharmacological profile of the selective FAAH inhibitor KDS-4103 (URB597). *CNS Drug Rev* **12**: 21–38.

Randall MD, Kendall DA, O'Sullivan S (2004). The complexities of the cardiovascular actions of cannabinoids. *Br J Pharmacol* **142**: 20–26.

Rösch S, Ramer R, Brune K, Hinz B (2006). R(+)-methanandamide and other cannabinoids induce the expression of cyclooxygenase-2 and matrix metalloproteinases in human nonpigmented ciliary epithelial cells. *J Pharmacol Exp Ther* **316**: 1219–1228.

Talley JJ, Bertenshaw SR, Brown DL, Carter JS, Graneto MJ, Kellogg MS *et al*. (2000). N-[[(5-methyl-3-phenylisoxazol-4-yl)-phenyl]sulfonyl] propanamide, sodium salt, parecoxib sodium: a potent and selective inhibitor of COX-2 for parenteral administration. *J Med Chem* **43**: 1661–1663.

Theodorsson-Norheim E (1987). Friedman-Quade tests: BASIC computer program to perform nonparametric two-way analysis of variance and multiple comparisons on ranks of several related samples. *Comput Biol Med* **17**: 85–99.

Wahn H, Wolf J, Kram F, Frantz S, Wagner JA (2005). The endocannabinoid arachidonyl ethanolamide (anandamide) increases pulmonary arterial pressure via cyclooxygenase-2 products in isolated rabbit lungs. *Am J Physiol Heart Circ Physiol* **289**: H2491–H2496.

Wang Y, Kaminski NE, Wang DH (2005). VR1-mediated depressor effects during high salt intake: role of anandamide. *Hypertension* **46** (Pt 2): 986–991.

Wheal AJ, Bennett T, Randall MD, Gardiner SM (2007a). Effects of chronic nitric oxide synthase inhibition on the cardiovascular responses to cannabinoids *in vivo* and *in vitro*. *Br J Pharmacol* **150**: 662–671.

Wheal AJ, Bennett T, Randall MD, Gardiner SM (2007b). Cardiovascular effects of cannabinoids in conscious spontaneously hypertensive rats. *Br J Pharmacol* **152**: 717–724.

Zygmunt PM, Petersson J, Andersson DA, H-h C, Sørgård M, Di Marzo V *et al*. (1999). Vanilloid receptors on sensory nerves mediate the vasodilator action of anandamide. *Nature* **400**: 452–457.