A cannabinoid receptor, sensitive to O-1918, is involved in the delayed hypotension induced by anandamide in anaesthetized rats

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Background and purpose: Intravenous injection of the endocannabinoid anandamide induces complex cardiovascular changes via cannabinoid CB₁, CB₂ and vanilloid TRPV1 receptors. Recently, evidence has been accumulating that in vitro, but not in vivo, anandamide relaxes blood vessels, via an as yet unidentified, non-CB₁ vascular cannabinoid receptor, sensitive to O-1918 (1,3-dimethoxy-5-2-[(1R,6R)-3-methyl-6-(1-methylethenyl)-2-cyclohexen-1-yl]-benzene). We here examined whether the anandamide-induced hypotension in urethane-anaesthetized rats was also mediated via a non-CB₁ vascular cannabinoid receptor.

Experimental approach: Effects of two antagonists (O-1918 and cannabidiol) of the non-CB₁ vascular cannabinoid receptor on anandamide-induced changes in mean, systolic and diastolic blood pressure (MBP, SBP, DBP), mesenteric (MBF) and renal (RBF) blood flow and heart rate (HR) in urethane-anaesthetized rats was examined.

Key results: In anaesthetized rats, anandamide (1.5–3 μmol·kg⁻¹) and its stable analogue methanandamide (0.5 μmol·kg⁻¹) caused a delayed and prolonged decrease in MBP, SBP, DBP, MBF and RBF by about 10–30% of the respective basal values without changing HR. In pithed rats, anandamide (3 μmol·kg⁻¹) decreased blood pressure by about 15–20% of the basal value without affecting HR, MBF and RBF. All vascular changes were reduced by about 30–70% by cannabidiol and O-1918 (3 μmol·kg⁻¹, each).

Conclusions and implications: Non-CB₁ cannabinoid vascular receptors, sensitive to O-1918, contribute to the hypotensive effect of anandamide in anaesthetized rats. Activation of these receptors may be therapeutically important as the endocannabinoid system could be activated as a compensatory mechanism in various forms of hypertension.

Keywords: anandamide; methanandamide; cannabinoid receptors; endothelial cannabinoid receptor; cannabidiol; O-1918; haemodynamic responses; anaesthetized rat; pithed rat

Abbreviations: AM 251, N-(piperidin-1-yl)-5-(4-iodophenyl)-1-(2,4-dichlorophenyl)-4-methyl-1H-pyrazole-3-carboxamide; CBD, cannabidiol; CP 55,940, (-)-cis-3-[2-hydroxy-4-(1,1-dimethylheptyl)phenyl]-trans-4-(3-hydroxypropyl)cyclohexanol; DBP, diastolic blood pressure; DMSO, dimethyl sulphoxide; HR, heart rate; MBF, mesenteric blood flow; MBP, mean blood pressure; O-1918, 1,3-dimethoxy-5-2-[(1R,6R)-3-methyl-6-(1-methylethenyl)-2-cyclohexen-1-yl]-benzene; RBF, renal blood flow; SBP, systolic blood pressure
advanced liver cirrhosis. The compound induces complex cardiovascular changes via cannabinoid CB₁, CB₂ and vanilloid TRPV1 receptors (see Malinowska et al., 2008; Pacher et al., 2008; nomenclature follows Alexander et al., 2008). In anaesthetized rats (Varga et al., 1995; Lake et al., 1997; Malinowska et al., 2001a; Kwolek et al., 2005) or mice (Pacher et al., 2004) i.v. injection of anandamide elicits a triphasic response. In the first phase, there is an immediate transient decrease in heart rate (HR) and cardiac contractility that is accompanied by a fall in blood pressure known as the Bezold-Jarisch reflex (phase I), which is mediated via vanilloid TRPV1 receptors located on sensory vagal nerves in the heart (Malinowska et al., 2001a; Pacher et al., 2004). This phase is followed by a brief pressor response and an increase in cardiac contractility (phase II), which involves central and peripheral components, located probably in the medulla oblongata and in blood vessels, respectively (Pacher et al., 2004; Kwolek et al., 2005). Finally, there is a more prolonged decrease in blood pressure accompanied by a marked decrease in cardiac contractility and a slight decrease in total peripheral resistance (phase III) that is mediated via presynaptic cannabinoid CB₂ receptors innervating blood vessels and heart (Malinowska et al., 1997; 2001a; Niederhofer et al., 2003). However, the involvement of TRPV1 receptors in the spinal cord (del Carmen García et al., 2003) and a decrease in cardiac contractility (Bátkai et al., 2004) have also been suggested as additional mechanisms.

On the other hand, in conscious normotensive rats, low doses of anandamide induce a brief pressor response associated with vasoconstriction in reninal, mesenteric and hindquarters vascular beds. At higher doses the vasoconstriction in the hindquarters is followed by a β-adrenoceptor-mediated vasodilatation and an initial bradycardia. A prolonged fall in blood pressure is observed only in hypertensive but not normotensive conscious rats (Lake et al., 1997; Gardiner et al., 2002; 2009; Ho and Gardiner, 2009).

Recently it has been demonstrated that the endocannabinoids anandamide and virodhamine and the synthetic cannabinoid ‘abnormal cannabidiol’ (a neuro-behaviourally inactive cannabinoid that does not bind to CB₁ or CB₂ receptors) caused a marked relaxation by an, as yet, unidentified non-CB₁, vascular cannabinoid receptor sensitive to O-1918 (1,3-dimethoxy-5-2-[(1R,6R)-3-methyl-6-(1-methylethenyl)-2-cyclohexen-1-yl]-benzene). This receptor was identified in rat and mouse isolated mesenteric arteries (Járai et al., 1999; Wagner et al., 1999; Ho and Hiley, 2003; 2004; Offertáler et al., 2003), including the mesenteric artery isolated from CB₂ receptor−/− knockout mice (Járai et al., 1999). This novel receptor is also involved in the relaxation of isolated human pulmonary arteries elicited by abnormal cannabidiol (Kozłowska et al., 2007) and virodhamine (Kozłowska et al., 2008). There is some speculation as to whether this receptor also participates in the fall in blood pressure elicited by anandamide. This is possible as anandamide decreased blood pressure (DBP) in conscious hypertensive rats, an effect resistant to the CB₂ receptor antagonist N-(piperidin-1-yl)-5-(4-iodophenyl)-1-(2,4-dichlorophenyl)-4-methyl-1H-pyrazole-3-carboxamide (AM 251) (Ho and Gardiner, 2009). In a study using anaesthetized rats, the hypotensive effect of a high (10 mg·kg⁻¹) but not of lower doses of anandamide was resistant to nimodipine, another CB₂ receptor antagonist (Garcia et al., 2001). Furthermore, abnormal cannabidiol has a hypotensive effect in anaesthetized wild-type (Offertáler et al., 2003) or CB₂−/− knockout mice (Járai et al., 1999). However, anandamide failed to produce a prolonged decrease in blood pressure in conscious normotensive rats (Gardiner et al., 2002; 2009; Ho and Gardiner, 2009) and in CB₂−/− receptor knockout mice (Járai et al., 1999; Ledent et al., 1999).

Therefore, the present study was undertaken in order to examine whether anandamide-induced hypotension in urethane-anaesthetized rats was also mediated via an O-1918-sensitive non-CB₁ vascular cannabinoid receptor. We used two antagonists of this receptor, namely O-1918 and cannabidiol. In our study, not only blood pressure but also mesenteric and renal blood flows (MBF and RBF) were examined as anandamide-related and CB₁ and CB₂ receptor-independent effects were found in the corresponding vascular beds (Járai et al., 1999; Wagner et al., 1999; Ho and Hiley, 2003; 2004; Offertáler et al., 2003; Koura et al., 2004; Ho and Gardiner, 2009). Our results suggest that an O-1918-sensitive non-CB₁ vascular cannabinoid receptor did indeed contribute to the delayed hypotension (phase III) induced by anandamide.

Methods

All animal care, surgical procedures and experimental protocols were approved by the local Animal Ethics Committee in Białystok (Poland). Male Wistar normotensive rats (weighing 200–300 g) with free access to food pellets and water were used.

Anaesthetized rats

Animals were anaesthetized i.p. with urethane (14 mmol·kg⁻¹). The depth of anaesthesia was assessed by monitoring the reflex responses to nociceptive stimuli. The trachea was cannulated. Mean, systolic and diastolic blood pressure (MBP, SBP and DBP, respectively) were measured from the right carotid artery via a transducer (ISOtec; Hugo Sachs Elektronik, March–Hugstetten, Germany). As DBP reflects changes in vascular resistance, we have mainly concentrated on this cardiovascular parameter (e.g. when selecting the dose of vasopressin and of anandamide). Moreover, for the experiments, in which the influence of cannabinoid receptor antagonists on (-)-cis-3-[2-hydroxy-4-(1,1-dimethylheptyl)phenyl]-trans-4-(3-hydroxypropyl)cyclohexanol (CP 55,940)-induced inhibition of neurogenic tachycardia was examined in pithed rats, only DBP is given (see below). HR was measured by a rate–meter triggered from the pressure record. The left femoral vein was cannulated for i.v. injection of drugs administered in a volume of 0.5 mL·kg⁻¹. Body temperature was kept constant at about 37–38°C using a heating pad (Bio-Sys-Tech, Białystok, Poland) and monitored by a rectal probe (Physitemp BAT10, Clifton, NJ, USA). Middle laparotomy was performed using a longitudinal abdominal incision, and the mesenteric and renal arteries were gently isolated from the surrounding tissues and from the underlying veins for about 0.5 cm. At the end of surgical preparation, ultrasound transit time flow probes (0.5 mm, V-series and
Pithed rats

Two types of experiments were performed in pithed rats. (i) The influence of anandamide on basal cardiovascular parameters was examined in rats anaesthetized i.p. with urethane (14 μmol·kg⁻¹); and (ii) the effect of CP 55,940 on the electrically stimulated increase in HR was studied in animals anaesthetized i.p. with pentobarbitone sodium (300 μmol·kg⁻¹). In both cases, atropine (2 μmol·kg⁻¹) was injected i.p. simultaneously with the anaesthetic. After cannulation of the trachea, the animals were pithed by inserting a stainless-steel rod (1.5 mm diameter and 190 mm length) through the right orbit and the foramen magnum and down a lower basal DBP to raise DBP to a level of about 60–70 mmHg. As in an earlier study (Malinowska et al., 2001b), vasopressin was not used in pithed animals in which electrically induced tachycardia was examined.

Experimental protocols

In order to examine the influence of different receptor antagonists on the changes in cardiovascular parameters induced by anandamide or methanandamide (both in anaesthetized and pithed rats) the particular agonist was injected twice (S₁ and S₂, 20 min apart). Maximal changes in particular cardiovascular parameters (S₁ and S₂) are given. As individual differences in responses to anandamide were observed, we applied anandamide at doses of 1.5–3 μmol·kg⁻¹. We have chosen a dose of anandamide that decreased DBP during phase III by about 20–30% of the basal value. Methanandamide was administered at 0.5 μmol·kg⁻¹. S₂ was applied 5 min after injection of saline solution (anaesthetized rats) or ruthenium red 3 (μmol·kg⁻¹ in pithed rats anaesthetized with urethane). Cannabidiol or O-1918 (3 μmol·kg⁻¹, each) or their solvents were administered 10 min before S₂.

Experiments in which the effect of CP 55,940 on the electrically stimulated increase in HR was studied were initiated by the injection of pancuronium (0.8 μmol·kg⁻¹, i.v.) in order to avoid muscle twitches associated with the electrical stimulation. An increase in HR was induced twice (S₁ and S₂, 10 min apart) by electrical stimulation (1 Hz, 1 ms, 50 V for 10 s) of the preganglionic sympathetic nerves. AM 251, cannabidiol or O-1918 (3 μmol·kg⁻¹, each) were administered simultaneously with pancuronium. S₁ was applied 5 min later. CP 55,940 (1 μmol·kg⁻¹) was injected 5 min before S₂.

Data analysis

Results are given as mean ± SEM (n = number of rats). In order to quantify the effects of antagonists on the anandamide- or methanandamide-induced changes in blood pressure, blood flow and HR, values during S₁ and S₂ were expressed as percentages of the basal MBP, SBP, DBP, MBF, RBF (Figures 2–4) and HR (data not shown) immediately before injection of that particular agonist. S₂ values are expressed as percentages of S₁. In order to quantify the effects of drugs on the electrically induced rise in HR, the ratio of the increase in HR induced by S₂ over that induced by S₁ (S₂/S₁) was determined. This ratio was expressed as percentage of the corresponding ratio obtained from vehicle-treated animals. For comparison of the mean values, Student’s t-test for paired and unpaired data was used. When two or more groups were compared with the same control, one-way analysis of variance (ANOVA) followed by Dunnett’s test was used. Differences were considered as significant when P < 0.05.

Materials

AM 251, anandamide, CP 55,940, (+)-cannabidiol, R-(-)-methanandamide (referred to as ‘cannabidiol’ and ‘methanandamide’ in the text; Tocris Cookson, Bristol, UK); atropine sulphate, hydroxypropyl-β-cyclodextrin, pancuronium bromide, ruthenium red, urethane, [Arg⁸]-vasopressin (Sigma, München, Germany); O-1918 (Cayman Chemicals, Ann Arbor, MI, USA); pentobarbitone sodium (Biowet, Pulawy, Poland). Drugs were dissolved in saline with the following exceptions: cannabidiol was dissolved in a mixture of ethanol and saline (1:9), AM 251 in a mixture of ethanol, Cremophor EL, dimethyl sulphoxide (DMSO) and saline (1:1:1:9.5) and CP 55,940 in a 19% w/v solution of cyclodextrin. Anandamide was purchased from Tocris Cookson as a 10 mg·mL⁻¹ emulsion in soya water (1:4). Vasopressin was provided by the manufacturer as an aqueous stock solution (100 IU·mL⁻¹), which was diluted (1:74) in isotonic saline before the experiment. The solvents for O-1918 and cannabidiol produced biphasic changes in cardiovascular parameters in anaesthetized rats, lasting for less than 1 min. Thus, saline first decreased and then increased MBP, SBP and DBP by about 3–5 and 12–15 mmHg respectively. Simultaneously, increases in MBF and RBF (by about 1.4 and 0.8 mL·min⁻¹, respectively) were noticed. In anaesthetized rats the solvents...
The results of the study are as follows:

**Results**

**Anaesthetized rats**

In urethane-anaesthetized rats, the basal cardiovascular parameters MBP, SBP and DBP, MBF and RBF, and HR, measured immediately before the administration of the first dose of agonist, are given in Table 1. If DBP was lower than 60 mmHg, vasopressin (0.04–0.4 IU·kg⁻¹·min⁻¹) was used to increase DBP to a level of 60–70 mmHg. Cannabidiol (3 µmol·kg⁻¹) decreased HR, blood pressure (MBP, SBP and DBP) and MBF by about 5–9% of the respective basal values, that is, by about 15 beats·min⁻¹ (MBF) and a fall by about 0.05 was followed by an increase by about 0.7 mL·min⁻¹ (RBF). In pithed rats, the solvent for CBD first decreased SBP, DBP and MBP by about 7–10 mmHg and then increased these cardiovascular parameters by about 6–10 mmHg. All biphasic changes lasted for less than 1 min. Cyclodextrin solution caused small and short-lasting decreases in HR and DBP followed by more pronounced increases in both parameters (by about 65 beats·min⁻¹ and 10 mmHg, respectively), which returned to basal values within 1–2 min.

The first (S₁) i.v. administration of anandamide (1.5–3 µmol·kg⁻¹) induced triphasic changes in cardiovascular parameters in urethane-anaesthetized rats (Figure 1; Table 1). The initial phase I consisted of a rapid, short-lasting fall in MBP, SBP, DBP and a bradycardia, accompanied by very low and, occasionally occurring, decreases in MBF and RBF. The subsequent phase II showed increases in MBP, SBP, DBP, MBF and RBF; the latter alterations lasted for about 30–60 s. Phase III lasted for up to 10 min and encompassed decreases in MBP, SBP, DBP, MBF and RBF and only sporadically a very small fall in HR.

Similar changes were elicited by methanandamide (0.5 µmol·kg⁻¹) (Malinowska et al., 2001a; Table 1). Thus, phase I included rapid decreases in MBP, SBP, DBP and HR; simultaneous decreases in MBF and RBF were very small and occurred only sporadically. In phase II, methanandamide increased MBP, SBP, DBP, MBF and RBF. The long-lasting hypotension (phase III) was accompanied by falls in MBF and RBF but not in HR.

The second administration of anandamide (S₂), 20 min after S₁ and 10 min after injection of the respective solvents for the antagonists under study, produced comparable triphasic changes in cardiovascular parameters (not shown). During phase I this could be studied best for the decrease in HR, which proved to be most robust among the four cardiovascular components of this phase (Malinowska et al., 2001a). The ratios of the decrease in HR evoked by S₁ over that evoked by S₂ (S₂/S₁ × 100) were close to 100% when the solvents of the two antagonists were given; these ratios were not affected by cannabidiol or O-1918 (3 µmol·kg⁻¹ each) (n = 5–10, results not shown).

With respect to the increases in MBP, SBP, DBP, MBF and RBF during phase II, virtually identical values occurred after S₁ and, following administration of the solvents for the two antagonists, after S₂; in other words, the ratios S₂/S₁ × 100 were close to 100%. The ratios were not modified by cannabidiol and O-1918 (n = 8–10, results not shown). O-1918 and its solvent did also not influence the methanandamide-stimulated bradycardia (phase I) and the subsequent increases in MBP, SBP, DBP and RBF (phase II) (n = 6, data not shown).

The solvents for cannabidiol and O-1918 did not affect or slightly increased the phase III stimulated by anandamide or methanandamide. Thus, the decreases in MBP, SBP, DBP, MBF and RBF obtained by the second administration (S₂) were about 90–130% of the respective changes observed during S₁ (see respective controls in Figures 2 and 3). By contrast, cannabidiol markedly reduced the anandamide-elicted falls in SBP and DBP by 44 and 32%, respectively, and tended to diminish the decrease in MBP by 21% (Figure 2). It also reduced the fall in MBF and RBF elicited by anandamide by 54 and 50% respectively (Figure 3). O-1918 caused an inhibition of the anandamide-stimulated decreases in MBP, SBP and DBP by 55, 36 and 42% respectively (Figure 2). Similarly, this antagonist reduced the methanandamide-stimulated fall in SBP and DBP by 44 and 32%, respectively, and tended to diminish the decrease in MBP by 28% (Figure 2). It also diminished the fall in MBF and RBF induced by anandamide by 61 and 67% and that induced by methanandamide by 74 and 49% respectively (Figure 3).

**Pithed rats**

Two experimental series were carried out on pithed rats. In the first series, rats were anaesthetized with urethane, bilaterally vagotomized and treated with ruthenium red (3 µmol·kg⁻¹); the basal cardiovascular parameters are given in Table 1. As DBP was lower than 60 mmHg, infusion of vasopressin (0.04–0.4 IU·kg⁻¹·min⁻¹) was used to increase DBP to a level of 60–70 mmHg. Thus, basal cardiovascular values were similar in anaesthetized and pithed rats. Cannabidiol did not affect basal cardiovascular parameters whereas O-1918 decreased HR by about 15 beats·min⁻¹ and SBP, DBP and MBP by about 15 mmHg. The modifications of cardiovascular parameters were rapid and short-lasting (about 30 s).

As shown in Figure 1B, i.v. injection of anandamide did not produce phase I in pithed rats. As we have shown previously, phase II in these animals is higher and longer than in non-pithed urethane-anaesthetized rats (Kwolek et al., 2005). The pronounced phase II automatically reduces the magnitude of phase III (additionally diminished by the abolition of an
Table 1  Basal cardiovascular parameters and effects of the first injection (S₁) of anandamide and methanandamide on basal cardiovascular parameters in anaesthetized and pithed rats

<table>
<thead>
<tr>
<th>Group</th>
<th>Agonist</th>
<th>Parameter</th>
<th>Units</th>
<th>Basal</th>
<th>Changes in cardiovascular parameters noticed in</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Phase I</td>
</tr>
<tr>
<td>Anaesthetized rats (anaesthesia with urethane, basal DBP brought to 60–70 mmHg when necessary)</td>
<td>Anandamide (n = 32)</td>
<td>MBP</td>
<td>mmHg</td>
<td>79.8 ± 1.7</td>
<td>-4.2 ± 1.9</td>
</tr>
<tr>
<td></td>
<td></td>
<td>SBP</td>
<td>mmHg</td>
<td>112.0 ± 2.5</td>
<td>-2.7 ± 0.9</td>
</tr>
<tr>
<td></td>
<td></td>
<td>DBP</td>
<td>mmHg</td>
<td>62.8 ± 1.5</td>
<td>-10.5 ± 2.3</td>
</tr>
<tr>
<td></td>
<td></td>
<td>MBF</td>
<td>mL·min⁻¹</td>
<td>4.84 ± 0.23</td>
<td>-0.11 ± 0.04</td>
</tr>
<tr>
<td></td>
<td></td>
<td>RBF</td>
<td>mL·min⁻¹</td>
<td>2.86 ± 0.18</td>
<td>-0.09 ± 0.06</td>
</tr>
<tr>
<td></td>
<td></td>
<td>HR</td>
<td>beats·min⁻¹</td>
<td>367.8 ± 9.2</td>
<td>-56.8 ± 13.1</td>
</tr>
<tr>
<td>Methanandamide (n = 13)</td>
<td>MBP</td>
<td>mmHg</td>
<td>78.6 ± 2.8</td>
<td>-1.2 ± 0.9</td>
<td>14.8 ± 1.0</td>
</tr>
<tr>
<td></td>
<td>SBP</td>
<td>mmHg</td>
<td>117.3 ± 5.3</td>
<td>-1.0 ± 0.7</td>
<td>11.8 ± 1.5</td>
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<tr>
<td></td>
<td>DBP</td>
<td>mmHg</td>
<td>61.7 ± 2.4</td>
<td>-8.0 ± 1.2</td>
<td>17.1 ± 1.6</td>
</tr>
<tr>
<td></td>
<td>MBF</td>
<td>mL·min⁻¹</td>
<td>4.51 ± 0.36</td>
<td>-0.06 ± 0.04</td>
<td>2.21 ± 0.15</td>
</tr>
<tr>
<td></td>
<td>RBF</td>
<td>mL·min⁻¹</td>
<td>2.74 ± 0.32</td>
<td>-0.05 ± 0.05</td>
<td>0.88 ± 0.10</td>
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<tr>
<td></td>
<td>HR</td>
<td>beats·min⁻¹</td>
<td>354.6 ± 20.6</td>
<td>-29.2 ± 5.5</td>
<td>NA</td>
</tr>
<tr>
<td>Pithed rats (anaesthesia with urethane, basal DBP brought to 60–70 mmHg, treatment with ruthenium red 3 μmol·kg⁻¹)</td>
<td>Anandamide (n = 21)</td>
<td>MBP</td>
<td>mmHg</td>
<td>81.1 ± 1.3</td>
<td>NA</td>
</tr>
<tr>
<td></td>
<td>SBP</td>
<td>mmHg</td>
<td>95.6 ± 1.7</td>
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<td>16.1 ± 5.6</td>
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<tr>
<td></td>
<td>DBP</td>
<td>mmHg</td>
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<td></td>
<td>HR</td>
<td>beats·min⁻¹</td>
<td>372.3 ± 5.0</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>Methanandamide (n = 20)</td>
<td>DBP</td>
<td>mmHg</td>
<td>51.6 ± 1.4</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td></td>
<td>HR</td>
<td>beats·min⁻¹</td>
<td>327.6 ± 3.9</td>
<td>ND</td>
<td>ND</td>
</tr>
</tbody>
</table>

Basal parameters were determined immediately prior to the first i.v. administration (S₁) of anandamide or methanandamide. In pentobarbitone-anaesthetized rats basal cardiovascular parameters immediately before the first electrical stimulation are given. Mean ± SEM of the number of rats given in the second column.

DBP, diastolic blood pressure; HR, heart rate; MBF, mesenteric blood flow; MBP, mean blood pressure; NA, not applicable; ND, not determined; RBF, renal blood flow; SBP, systolic blood pressure.
influence of presynaptic CB₁ receptors on sympathetic nerve endings in pithed rats; e.g. Malinowska et al. 1997). Thus, all experiments in this experimental model were performed in the presence of a blocker of phases I and II, ruthenium red, which was administered 5 min before the first administration of anandamide. Under these conditions anandamide (3 μmol·kg⁻¹) caused a biphasic change in MBP, SBP and DBP (increase followed by decrease; Figure 1B; Table 1). The anandamide-stimulated decrease in DBP in pithed rats was lower (by 27%) than the corresponding change observed in

![Figure 1](image_url)
non-pithed animals (Table 1, \(P < 0.001\)). The fall in MBP and SBP tended to be lower (by about 11 and 21%, respectively; see Table 1) in pithed than in non-pithed animals. In pithed rats anandamide (3 \(\mu\)mol·kg\(^{-1}\)) caused only an increase (without subsequent decrease) in MBF and RBF (Figure 1B). Thus, we did not measure MBF and RBF in further interaction experiments.

Cannabidiol, O-1918 and their solvents did not affect the anandamide-stimulated increases in MBP, SBP and DBP (\(n = 4–6\), results not shown). The anandamide-induced long-lasting fall in MBP, SBP and DBP was reduced by cannabidiol by 40–46% and by O-1918 by 55, 64 and 35, respectively, but not affected by the respective solvents (Figure 4).

In the second experimental series on pithed rats, the animals were anaesthetized with pentobarbitone, bilaterally vagotomized and treated with pancuronium (0.8 \(\mu\)mol·kg\(^{-1}\)); basal DBP and HR are given in Table 1. Electrical stimulation (1 Hz, 1 ms, 50 V for 10 s) of the preganglionic sympathetic nerve fibres innervating the heart (S\(_1\) and S\(_2\), 10 min apart) increased HR (increase during S\(_2\) by 69.9 ± 3.9 beats·min\(^{-1}\); \(n = 20\)). CP 55,940, 1 \(\mu\)mol·kg\(^{-1}\), administered 5 min prior to S\(_2\) reduced the neurogenic tachycardia (S\(_2\)/S\(_1\)) by about 25%. 

\[\text{Figure 2 Influence of cannabidiol (CBD, 3 \(\mu\)mol·kg\(^{-1}\)) and 1,3-dimethoxy-5-2-[(1R,6R)-3-methyl-6-(1-methylethenyl)-2-cyclohexen-1-yl]-benzene (O-1918) (3 \(\mu\)mol·kg\(^{-1}\)) on the decreases in mean (A; MBP), systolic (B; SBP) and diastolic (C; DBP) blood pressure (phase III) induced by anandamide (AEA; 1.5–3 \(\mu\)mol·kg\(^{-1}\)) and methanandamide (MethAEA; 0.5 \(\mu\)mol·kg\(^{-1}\)) in urethane-anaesthetized rats. AEA and MethAEA were given twice (S\(_1\) and S\(_2\), 20 min apart). S\(_1\) and S\(_2\) were calculated as percentages of the respective basal values. CBD or O-1918 or their solvents were administered 10 min before S\(_2\). All drugs were administered i.v. Means ± SEM of 5–10 rats. *\(P < 0.05\), **\(P < 0.01\), ***\(P < 0.001\) compared with the respective control (CON).}

\[\text{Figure 3 Influence of cannabidiol (CBD, 3 \(\mu\)mol·kg\(^{-1}\)) and 1,3-dimethoxy-5-2-[(1R,6R)-3-methyl-6-(1-methylethenyl)-2-cyclohexen-1-yl]-benzene (O-1918) (3 \(\mu\)mol·kg\(^{-1}\)) on the decrease in (A) mesenteric blood flow (MBF) and (B) renal blood flow (RBF) (phase III) induced by anandamide (AEA; 1.5–3 \(\mu\)mol·kg\(^{-1}\)) and methanandamide (MethAEA; 0.5 \(\mu\)mol·kg\(^{-1}\)) in urethane-anaesthetized rats. AEA and MethAEA were given twice (S\(_1\) and S\(_2\), 20 min apart). S\(_1\) and S\(_2\) were calculated as percentages of the respective basal values. CBD or O-1918 or their solvents were administered 10 min before S\(_2\). All drugs were administered i.v. Means ± SEM of 5–10 rats. *\(P < 0.05\), **\(P < 0.01\), ***\(P < 0.001\) compared with the respective control (CON).}
As shown in Figure 5, the inhibitory effect of CP 55,940 was not modified by cannabidiol and O-1918 but it was strongly attenuated by the cannabinoid CB1 receptor antagonist AM 251 (3 μmol·kg⁻¹ each) (administered 5 min prior to S1). The cannabinoid receptor antagonists did not affect basal HR or the electrically stimulated increase in HR (S1) by themselves.

Discussion

The endocannabinoid anandamide induces triphasic changes in cardiovascular parameters in anesthetized rats, and the aim of the present study was to investigate whether the putative non-CB1 vascular cannabinoid receptor contributes to the third phase, that is, a delayed and prolonged decrease in blood pressure. Rats were anesthetized with urethane as, in our previous study (Kwolek et al., 2005), pentobarbitone reduced the cardiovascular effects of anandamide. Also as the vasopressor/vasodepressor effects were more marked at higher levels of blood pressure (Malinowska and Schlicker, 1993), we increased DBP to about 60 mmHg by infusion of vasopressin in anaesthetized animals with low DBP and in pithed rats (series shown in Figure 4). An influence of vasopressin on our results is unlikely as the vasopressin V1a receptor antagonist d(CH2)5[Tyr(Me)2Arg8]-vasopressin failed to affect the cardiovascular responses to anandamide (Kwolek et al., 2005).

Our experiments on anaesthetized rats show that the anandamide-induced prolonged decrease in SBP, DBP and MBP (phase III) was strongly counteracted by two antagonists at the non-CB1 vascular cannabinoid receptor, that is, cannabidiol and O-1918, whereas phases I and II were not affected. These results suggest that the latter receptor did indeed contribute to phase III but not to phases I and II. Admittedly, the...
two antagonists decreased the cardiovascular parameters under study by themselves. These effects, which were very short-lived and had recovered 9 min prior to the administration of anandamide, may be non-specific in nature and do not suggest a tonic activation of the novel receptors. In this case, one would expect an increase rather than decrease of cardiovascular parameters.

Four series of experiments were carried out in order to support the view that a non-CB1, vascular cannabinoid receptor was involved in the hypotensive effect of anandamide in phase III. First, the possibility had to be considered that anandamide, which is rapidly degraded for example by the fatty acid amide hydrolase (Giufrida et al., 2001), partially or solely acted via metabolites. In order to exclude this possibility we used methanandamide, a metabolically stable analogue of anandamide. The effect of methanandamide on DBP in phase III was affected by O-1918 in a manner similar to that shown for anandamide. As expected, the hypotensive effect of methanandamide occurred at a somewhat lower dose than that of anandamide (Malinowska et al., 2001a). On the other hand, in conscious rats, the inhibitor of fatty acid hydrolase URB597 augmented the haemodynamic effects of anandamide, but no evidence for an involvement of cyclooxygenase-2 in these actions was found (Gardiner et al., 2009).

Second, cannabidiol and O-1918 have very low affinities for the classical CB1 receptor (Offertáler et al., 2003; Thomas et al., 2007); nonetheless, cannabidiol proved to be a potent non-competitive antagonist against CB1 receptor-mediated effects in vitro (Thomas et al., 2007). The possibility had to be considered that such a mechanism might also occur in vivo (and perhaps also with O-1918); in other words, the effect of both antagonists on the anandamide- and methanandamide-induced phase III might be related to the blockade of CB1 receptors, which play an important role in this phase (Malinowska et al., 1997; 2001a; Niederhoffer et al., 2003). In order to exclude this possibility, experiments were performed on pithed rats in which the inhibition of the neurogenic tachycardia via presynaptic CB1 receptors was considered (Malinowska et al., 2001b). Because urethane strongly attenuates effects mediated via presynaptic CB1 receptors (Kurz et al., 2009), in these particular experiments pentobarbitone was used as anaesthetic; vasopressin was omitted, as in the study by Malinowska et al. (2001b). As expected, the effect of the cannabinoid receptor agonist CP 55,940 was counteracted by the CB1 receptor antagonist AM 251; however, cannabidiol and O-1918 at the doses used in the present study failed to influence the effect of CP 55,940.

Third, the question arises as to whether the hypotensive effect caused by anandamide (phase III) and related to a non-CB1, vascular cannabinoid receptor involves the CNS. For this reason, experiments were performed on pithed rats (anaesthetized with urethane), in which the brain and the spinal cord can no longer influence the responses. As expected, phase I of the cardiovascular responses to anandamide, which is related to the Bezold-Jarisch reflex (Malinowska et al., 2001a), was absent under these experimental conditions, whereas phases II and III persisted. The increase in blood pressure in phase II is, however, particularly marked in pithed rats (Kwolek et al., 2005) and it may potentially diminish the magnitude of phase III. Thus, in order to have conditions comparable with those in anaesthetized rats, experiments were carried out in the presence of ruthenium red known to attenuate the increase in blood pressure during phase II without affecting its decrease during phase III (Kwolek et al., 2005). The phase III hypotensive effect of anandamide was somewhat less pronounced in pithed when compared with anaesthetized rats but the extent of the antagonistic effect of cannabidiol and O-1918 against anandamide was comparable. These data suggest that the hypotensive effect of anandamide related to the O-1918-sensitive cannabinoid receptor has, at least for the major part, a peripheral site of action. The data on pithed rats support the role of a non-CB1, vascular cannabinoid receptor also indirectly, as they exclude two alternative explanations involving the CNS (Malinowska et al., 1997; 2001b; del Carmen Garcia et al., 2003; Niederhoffer et al., 2003). Thus, CB1 receptors on sympathetic neurones do not function spontaneously in the pithed rat model (and can be studied only if the sympathetic outflow is stimulated electrically) and TRPV1 receptors on sensory vagal nerves cannot have an influence on the CNS.

Fourth, as the non-CB1, vascular cannabinoid receptor has been shown to occur in mesenteric and renal vessels (for references see Introduction), it was plausible to assume that the decrease in DBP is associated with an increase in the MBF and RBF. For this reason, MBF and RBF were measured simultaneously with DBP and HR in our experiments but, surprisingly, both parameters were decreased rather than increased in anaesthetized rats (not affected in pithed rats), suggesting that the hypotensive effect of anandamide related to the O-1918-sensitive non-CB1, cannabinoid receptor involves other vascular beds. One candidate is the vasculature of the hindquarters, the conductance of which is strongly increased in response to the hypotensive effect of anandamide in the conscious hypertensive rat; this hypotensive effect is CB1 receptor-independent and possibly involves a non-CB1, vascular cannabinoid receptor (Ho and Gardiner, 2009). Our results related to the MBF and RBF are in line with data obtained in conscious normotensive (Gardiner et al., 2002; 2009) and hypertensive rats (Ho and Gardiner, 2009), in which anandamide caused vasoconstriction of the mesenteric and renal vascular bed; nonetheless, additional experiments, in which the resistance in the renal and mesenteric vascular bed is determined directly, will be necessary to finally prove the role of either vascular bed in the effect of anandamide in our experimental model.

Although our data suggest that a vascular non-CB1 receptor related to, or identical with, the endothelial cannabinoid receptor (‘abnormal cannabinoid receptor’) originally described by Járai et al. (1999) is implicated in the effect of anandamide and methanandamide, three alternative possibilities had to be considered as well. First, O-1918 has been recently shown to inhibit large-conductance, Ca2+-activated, potassium (BKCa) channels (Godlewski et al., 2009), and anandamide is known to activate these channels (e.g. Kozlowska et al., 2007; 2008). We cannot exclude the possibility that this mechanism also participates in the hypotensive influence of the endocannabinoid. However, it should be noted that the vasodepressor influence of anandamide was also counteracted
by cannabidiol. Second, a decrease in cardiac contractility (Bátkai et al., 2004) has been suggested as an additional mechanism of the hypotensive effect of anandamide in rats. We cannot exclude the possible participation of this mechanism in our study, in which cardiac contractility was not determined directly. Third, the potential involvement of the recently characterized GPR55 receptor in the effect of anandamide had to be considered, as it is an agonist at this receptor (Pertwee, 2007). However, this possibility is very unlikely as recent experiments with a GPR55 receptor knockout mouse clearly showed that this receptor does not lead to a vasodilator response (Johns et al., 2007).

In conclusion, our data based on two antagonists, two antagonists and two experimental models show that part of the decrease in blood pressure during the third phase of the cardiovascular effect of anandamide is related to the activation of an O-1918-sensitive non-CB1 cannabinoid receptor. The data show that this receptor, which has been identified in some vascular beds (for references, see Introduction), also plays a role in a very complex phenomenon such as blood pressure regulation. The fact that the non-CB1 vascular cannabinoid receptor is already the fourth mechanism contributing to the anandamide-related delayed decrease in blood pressure in the anaesthetized rat is not so surprising as anandamide is well-known for its pleiotropic effects. Our results are compatible with studies on conscious hypertensive rats (Ho and Gardiner, 2009) and anaesthetized rats (Garcia et al., 2001), in which anandamide caused a CB1 and/or CB2 receptor-independent hypotensive effect although the antagonism by cannabidiol or O-1918 has not been examined. Our data are also compatible with studies on wild-type (Offertáler et al., 2003) and CB2 receptor knockout mice (Járai et al., 1999), in which abnormal cannabidiol, an agonist at the endothelial cannabinoid receptor, showed a hypotensive effect that was counteracted by O-1918 and cannabidiol respectively.

It has been suggested that the endocannabinoid system may be activated as a compensatory mechanism in various forms of hypertension (Bátkai et al., 2004). In this context, the non-CB1 vascular cannabinoid receptor, which like CB1 receptors, spinal TRPV1 receptors and a decrease in cardiac contractility contributes to the hypotensive effect of anandamide in the anaesthetized rat, may be of particular interest. This receptor has been identified in vessels of humans (Kozłowska et al., 2007; 2008) and a hypotensive effect of anandamide, most likely mediated via this receptor, occurred in hypertensive but was absent in normotensive conscious rats (Ho and Gardiner, 2009).

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Conflicts of interest

None.

References


Johns DG, Behm DJ, Walker DJ, Ao Z, Shapland EM, Daniels DA et al. (2007). The novel endocannabinoid receptor GPR55 is activated by atypical cannabinoids but does not mediate their vasodilator effects. Br J Pharmacol 152: 825–831.


