Cannabinoids induce complex cardiovascular changes by acting directly or indirectly via various peripheral and/or central receptors: cannabinoid CB\(_1\) and CB\(_2\), vanilloid TRPV1, O-1918-sensitive endothelial or GPR18 cannabinoid receptors as well as thromboxane A\(_2\) (TP), N-methyl-D-aspartate (NMDA) and \(\beta\)-adrenergic receptors (1-4). In humans cannabis preparations including their major psychotropic constituent Δ\(_9\)THC evoke a rapid increase in heart rate (HR), which may be accompanied by a modest increase in blood pressure (BP) (1, 4).

The cardiovascular effects of cannabinoids differ between anaesthetized and conscious animals. Thus, in urethane-anaesthetized rats or pentobarbitone-anaesthetized mice, a prolonged decrease in BP accompanied by a decrease in HR (the so-called phase III) is the most prominent effect of intravenous (i.v.) cannabinoid administration (including Δ\(_9\)-THC, the endocannabinoid anandamide (AEA), its synthetic analogue methanandamide (MethAEA) or synthetic cannabinoid receptor agonists WIN55-212 or CP55940) (5-11). This effect follows phase I (a rapid and pronounced bradycardia and transient drop in BP) and phase II (a pressor response) induced by AEA, MethAEA and Δ\(_9\)-THC (only phase II). On the contrary, in conscious rats the most distinct effect of AEA, MethAEA, Δ\(_9\)-THC and WIN55212-2 is a pressor response (6, 12-14) (phase II). Phase I was also observed in response to higher doses AEA; however, unlike in anaesthetized rats, none of the five cannabinoids induced phase III.

Cannabinoid CB\(_1\) receptors have been proved (with the use of CB\(_1\) receptor antagonists or in CB\(_1\)-/– mice) to be responsible for the prolonged hypotension in anaesthetized (phase III) and for the pressor response in conscious animals (phase II) (5-11). In the case of phase III, the CB\(_1\) receptors are located presynaptically on the sympathetic nerve endings innervating resistance vessels (15, 16) and heart (16, 17). For phase II, a central localization has been suggested. Thus, intracisternal injection of WIN55212-2 and CP55940 to conscious rabbits (18) and rats (19) increased BP, plasma noradrenaline levels and lowered HR in a manner sensitive to CB\(_1\) receptor antagonists. Interestingly, also central administration of cannabinoids induced a pressor effect in anaesthetized animals. Thus, injection
of AEA, WIN55212-2 or HU-210 into the cisternal system (20), the rostral ventrolateral medulla (RVLM) (21) or the dorsal periaqueductal gray (dPAG) (22) of anaesthetized rats increased BP, plasma noradrenaline levels and/or renal sympathetic nerve activity. CB1 receptor antagonists diminished the above effects. Moreover, a pressor response to AEA i.v. was preceded by a transient rise in the activity of the RVLM and a brief rise in splanchnic sympathetic nerve discharge in urethane-anasthetized rats (23).

It has been shown that in anaesthetized rats cannabinoids may also elicit pressor effects in a CB1 receptor-independent manner, do not elicit a pressor response at all or even lead to a depressor effect: (a) WIN55212-2 and CP55940 given i.v. did not increase BP (5); (b) cardiovascular effects of systemically administered Δ9-THC depend on the hindbrain and peripheral activation of cyclooxygenase (24); (c) the pressor response to AEA or MethAEA (i.v.) was not reduced by the CB1 receptor antagonist rimonabant (7); (d) intracerebroventricular (i.c.v.) injection of AEA and MethAEA increased BP only after the combined i.v. administration of a CB2 and TRPV1 receptor antagonist (8); (e) rimonabant given i.c.v. inhibited the endotoxic hypotension evoked by lipopolysaccharide in conscious and anaesthetized rats (25); (f) rimonabant given i.v. elicited a marked increase in BP (but not in HR) in anaesthetized hypertensive (but not normotensive) rats (26); this finding is very interesting in the light of the potential use of CB1 antagonists as antiobesity drugs since drugs increasing BP would create problems in patients suffering from a metabolic syndrome, i.e. a disorder combining increased body weight, high BP, raised fasting blood glucose and dyslipidemia (27).

The central regulation of cardiovascular functions depends upon neuroendocrine and autonomic nerve-mediated mechanisms in specific brain regions. One of them is the paraventricular nucleus of the hypothalamus (PVN), which acts as a major integrative site for metabolic, cardiovascular and respiratory functions (28). Cannabinoid CB1 receptors located in the PVN have been demonstrated to be involved in the regulation of food intake and energy homeostasis (29, 30), stress response (31) or penile erection (32). They may also play a role for the cardiovascular system since the hypertensive effect of angiotensin II microinjected into the PVN of anaesthetized rats is counteracted by the CB1 receptor antagonist AM251; this effect may be related to an increased formation of endogenous cannabinoids (33). Thus, the aims of our study were (a) to further determine the role of the PVN in the cardiovascular effects of cannabinoids in anaesthetized rats and (b) to check the involvement of CB1 receptors.

MATERIAL AND METHODS

Animals

Male Wistar normotensive rats (weighing 280 – 350 g) were used in the present experiments. All surgical procedures and experimental protocols were in accordance with European and Polish legislation and were approved by the local Animal Ethic Committee in Bialystok (Poland).

Mounting of cannulae for drug administration into the paraventricular nucleus of the hypothalamus

Rats were anaesthetized by intraperitoneal (i.p.) injection of pentobarbitone sodium (300 µmol/kg) and placed in a stereotaxic instrument (Stoelting WPI, Wood Dale, IL, USA). Stainless steel cannulae (outer and inner diameter of 0.5 and 0.3 mm, respectively) were stereotaxically implanted on the right side (bilaterally in the case of chemical lesion only), using established coordinates obtained from the Paxinos and Watson (34) rat brain atlas (1.5 mm caudal to the bregma; 0.5 mm lateral to the midline and 8 mm below the skull surface).

Anaesthetized rats

At least 7 days later rats were anaesthetized with urethane (14 mmol/kg; i.p.). The trachea was cannulated. The general procedure and the equipment for the measurement of mean BP (MBP), systolic BP (SBP), and diastolic BP (DBP) and heart rate (HR), the intraveneous (i.v.): 0.5 ml/kg) and i.p. (2.0 ml/kg) drug administration, the vasopressin (VP) infusion and the maintenance of constant body temperature were like in our previous studies (e.g. 8, 10).

PVN microinjections were administered slowly in a volume of 100 nl per rat and completed within 1 min. Correct cannula placement was confirmed histologically and analyzed by light microscopy at the end of the experiment. Only animals for which the correct placement of the guide cannula to the PVN was confirmed were included in this study.

Experimental protocol

In most experiments MethAEA (8) or CP55940 (35) were administered into the PVN twice (S1 and S2, 20 min apart). The maximal changes in the particular cardiovascular parameters were recorded. Only one dose of MethAEA or CP55940 was examined in one rat. The CB1 receptor antagonist AM251 (3 µmol/kg) (8), the TRPV1 receptor antagonist ruthenium red (3 µmol/kg) (8), the CB1 receptor antagonist SR144528 (3 µmol/kg) (36) or their solvents were administered i.v. 5 min before S1. The peripherally restricted cannabinoid CB1 receptor antagonist AM6545 (15 µmol/kg, i.p.) (37) was given 90 min before S1. In one series of experiments, AM251 (0.03 µmol/animal) (38) or its solvent was administered into the PVN during S2 together with CP55940 (0.1 nmol/animal) in a total volume of 100 nl.

In additional series of experiments, three increasing doses of CP55940 were administered i.v. with sufficient time for recovery to the preinjection value. The first dose of CP55940 was given 90 min after AM6545 (15 µmol/kg, i.p.) or its solvent or 90 min after chemical lesion of the PVN by bilateral PVN microinjection of kainic acid 2 nmol to each side (39) (in the corresponding control series, the solvent of kainic acid was used instead).

Preparation of paraventricular nucleus of the hypothalamus and cerebral cortex

At the end of the experiment, brains were quickly removed. The cortex and PVN were immediately dissected and analyzed. PVN punches were made from brain sections using a Stoelting brain punch with a diameter of 1.0 mm (Stoelting) according to Du et al. (40). The cortex and PVN were flash frozen in liquid nitrogen and stored at –80°C.

Measurement of anandamide levels

Anandamide was quantified according to the method described by Lam et al. (41) and modified by Marceylo et al. (42), using ultra high performance liquid chromatography-tandem mass spectrometry (UPLC-MS/MS). A Zorbax Extend C18 column (2.1 mm × 150 mm, 1.8 µm, Agilent, Santa Clara, CA, USA) was used and octadeuterated AEA (AEA-d8) served as internal standard. AEA has been isolated from brain tissue using a solid phase extraction method. UPLC-MS/MS analysis
was performed using an Agilent 1290 UPLC system interfaced with an Agilent 6460 triple quadrupole mass spectrometer with electrospray ionization source. The samples were analyzed in the positive-ion mode using multiple reaction monitoring. Transitions of the precursors to the product ions were as follows: m/z 348.3-62.1 (for AEA detection) and 356.3-63.1 (for AEA-d<sub>6</sub> detection).

Western blots

Routine Western blotting procedures were used, as described previously (43). Briefly, samples of PVN and cortex were homogenized in radioimmunoprecipitation assay buffer containing a cocktail of protease inhibitors (Roche Diagnostics GmbH, Mannheim, Germany). Total protein concentration was determined using the bicinchoninic acid method with bovine serum albumin as a standard. Next, homogenates were transferred onto nitrocellulose membranes. The membranes were immunoblotted with the primary antibodies of interest, followed by incubation with appropriate horseradish peroxidase-labeled secondary antibody. Equal protein concentration loading was controlled by Ponceau S staining. After adding a suitable substrate for horseradish peroxidase (Thermo Scientific, Rockford, IL, USA), protein bands were quantified densitometrically using a ChemiDoc visualization system EQ (Bio-Rad, Warsaw, Poland). The protein expression was standardized to β-tubulin.

Chemicals

Drugs used for the whole animal experiments were AM251, AM6545 (Sigma-Aldrich, St. Louis, MO, USA), CP55940, cremophor El, R-(-)-methanandamide (Tocris Cookson, Bristol, UK), cyclodextrin, ruthenium red, kainic acid, urethane (Sigma, Munchen, Germany), SR144528 (Cayman Chemicals, Ann Arbor, MI, USA), pentobarbitone sodium (Biowet, Pulawy, Poland). Drugs were dissolved in saline with the following exceptions: AM251 was dissolved in a mixture of ethanol, Cremophor El, dimethyl sulphoxide (DMSO) and saline (1:1:1:9.5); methanandamide was purchased from Tocris Cookson as a 10 mg/ml emulsion in soya water (1:4); AM6545 was dissolved in DMSO using gentle heating before being diluted with Tween 80 and saline (4% DMSO; 1% Tween 80; 95% saline) for i.p. injections; CP55940 was dissolved in a 19% w/v solution of cyclodextrin. A stock solution of SR144528 was prepared in a mixture of DMSO, ethanol, and Cremophor El (2:1:1) and further diluted (1:10) in isotonic saline immediately before the experiment. The antibodies used in the Western blots were purchased from Cayman (anti-FAAH antibody, 1:200 dilution, cat. no. 101600), Novus Biological (anti-β-tubulin, 1:1000 dilution, cat. no. NB600-936) and Santa Cruz Biotechnology (anti-rabbit IgG horseradish peroxidase, 1:3000 dilution, cat. no. Sc-2004). The AEA and octa-deuterated anandamide (AEA-d<sub>6</sub>) standards needed for the UPLC-MS/MS determinations were purchased from Cayman Chemical (Ann Arbor, MI, USA), whereas acetonitrile and ethanol were purchased from Sigma Chemical (St. Louis, MO, USA). All the stock solutions of standards were prepared in ethanol and stored at −80°C. Further dilutions were prepared using acetonitrile.

Data analysis

Results are given as means ± S.E.M. (n, number of rats). To quantify the effects of antagonists on the MethAEA- and CP55940-induced changes in cardiovascular parameters, S<sub>1</sub> and S<sub>2</sub> values were calculated as percent of the basal MBP, SBP, DBP and HR immediately before injection of the particular agonist. For comparison of the mean values, the t-test for paired and unpaired data was used, as appropriate.

RESULTS

General

Basal SBP, DBP, MBP and HR measured immediately before the administration of the agonist (S<sub>1</sub> and S<sub>2</sub>) into the PVN are given in Table 1. The basal values of the four cardiovascular parameters determined before S<sub>1</sub> and S<sub>2</sub> did not differ, confirming that the cardiovascular effects induced by agonists during S<sub>1</sub> and S<sub>2</sub> were similar.

Table 1. Basal systolic, diastolic and mean blood pressure (SBP, DBP and MBP) and heart rate (HR) immediately before S<sub>1</sub> or S<sub>2</sub> in urethane-anæsthetized rats.

<table>
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<tr>
<th>Additional antagonist</th>
<th>AM251 (i.v.)</th>
<th>Dose&lt;sup&gt;1&lt;/sup&gt;</th>
<th>n</th>
<th>Before S&lt;sub&gt;1&lt;/sub&gt;</th>
<th>Before S&lt;sub&gt;2&lt;/sub&gt;</th>
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<tr>
<td></td>
<td>SBP (mmHg)</td>
<td>DBP (mmHg)</td>
<td>MBP (mmHg)</td>
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<tr>
<td>-</td>
<td>100 ± 8&lt;sup&gt;a&lt;/sup&gt;</td>
<td>54 ± 6&lt;sup&gt;a&lt;/sup&gt;</td>
<td>56 ± 6&lt;sup&gt;b&lt;/sup&gt;</td>
<td>69 ± 8&lt;sup&gt;a&lt;/sup&gt;</td>
<td>575 ± 16&lt;sup&gt;a&lt;/sup&gt;</td>
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<tr>
<td>-</td>
<td>102 ± 12&lt;sup&gt;b&lt;/sup&gt;</td>
<td>60 ± 9&lt;sup&gt;b&lt;/sup&gt;</td>
<td>70 ± 9&lt;sup&gt;b&lt;/sup&gt;</td>
<td>363 ± 14&lt;sup&gt;b&lt;/sup&gt;</td>
<td>71 ± 8&lt;sup&gt;b&lt;/sup&gt;</td>
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<td>+</td>
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<td>64 ± 5&lt;sup&gt;a&lt;/sup&gt;</td>
<td>59 ± 4&lt;sup&gt;b&lt;/sup&gt;</td>
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<td>4</td>
<td>4</td>
<td>117 ± 9&lt;sup&gt;a&lt;/sup&gt;</td>
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<td>4</td>
<td>4</td>
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<tr>
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<td>4</td>
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<td>51 ± 1&lt;sup&gt;a&lt;/sup&gt;</td>
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<td>62 ± 1&lt;sup&gt;a&lt;/sup&gt;</td>
<td>62 ± 1&lt;sup&gt;a&lt;/sup&gt;</td>
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<td>88 ± 7&lt;sup&gt;a&lt;/sup&gt;</td>
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<td>52 ± 2&lt;sup&gt;a&lt;/sup&gt;</td>
<td>52 ± 2&lt;sup&gt;a&lt;/sup&gt;</td>
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<td>306 ± 35&lt;sup&gt;a&lt;/sup&gt;</td>
<td>306 ± 35&lt;sup&gt;a&lt;/sup&gt;</td>
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<tr>
<td>solvent for AM251 (PVN)</td>
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<td>3</td>
<td>4</td>
<td>86 ± 5&lt;sup&gt;a&lt;/sup&gt;</td>
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<td>54 ± 4&lt;sup&gt;a&lt;/sup&gt;</td>
<td>54 ± 4&lt;sup&gt;a&lt;/sup&gt;</td>
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<td>312 ± 25&lt;sup&gt;a&lt;/sup&gt;</td>
<td>312 ± 25&lt;sup&gt;a&lt;/sup&gt;</td>
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Methanandamide or CP55940 was injected into the PVN twice (S<sub>1</sub>-S<sub>2</sub>), 20 min apart. Antagonists (AM251 and/or additional) were given 5 min before S<sub>1</sub>. In some experiments, AM251 or its solvent was administered into the PVN together with CP55940. Data are given as the means ± S.E.M. of n experiments. There are no significant differences between the respective cardiovascular parameters before S<sub>1</sub> and S<sub>2</sub>.<sup>1</sup>Doses of antagonists are given in µmol/kg;<sup>2</sup>the dose of AM251 administered into the PVN was 0.03 µmol/rat; Ruth. red - ruthenium red. Basal parameters in the group treated with methanandamide<sup>1</sup> or CP55940<sup>0</sup>.
ceased before $S_2$. Basal values were not altered by the CB$_1$ receptor antagonist AM251 (given i.v. or into the PVN), the peripherally restricted CB$_1$ receptor antagonist AM6545 (i.p.), the CB$_2$ receptor antagonist SR144528 (i.v.) and/or the TRPV1 receptor antagonist ruthenium red (i.v.) (Table 1). In the experiments related to Fig. 6 (see later), basal DBP and HR before PVN lesion were $72 \pm 9$ mmHg and $355 \pm 19$ beats/min ($n = 4$). Bilateral microinjection of 2 nmol kainic acid into the PVN resulted in immediate and marked increases in the baseline BP and HR (maximally by about 56 and 34% of basal values, respectively; $P < 0.001$), which gradually recovered within 40 – 60 min and 90 min, respectively; immediately before injection of CP55940, basal DBP and HR were $76 \pm 9$ mmHg and $378 \pm 20$ beats/min ($n = 4$), respectively. The above values of basal cardiovascular parameters were not different from the respective values obtained in control groups which have received the solvent for kainic acid (data not shown).

**Cardiovascular effects of methanandamide given into the paraventricular nucleus of the hypothalamus**

As shown in Fig. 1, the first microinjection ($S_1$) of MethAEA (0.01 µmol/rat) into the PVN decreased SBP, DBP, MBP and HR by 15, 24, 15 and 4% of basal values (i.e. by 17 ± 4, 15 ± 3, 11 ± 2 mmHg and 13 ± 3 beats/min; $n = 4$), respectively. The second administration of the agonist ($S_2$) into the PVN of the same rat induced comparable decreases. On the contrary, the cannabinoid CB$_1$ antagonist AM251 3 µmol/kg given i.v. 5 min before $S_2$ reversed the depressor and bradycardic effects of MethAEA; thus, SBP, DBP, MBP and HR were higher by 11, 15, 12 and 6% than the respective basal values. In contrast to AM251, i.v. injection of ruthenium red 3 µmol/kg 5 min before $S_2$ did not modify the depressant effects of MethAEA on BP and HR. Both MethAEA-induced decreases and increases in cardiovascular parameters lasted for about 90 s.

**Fig. 1.** Influence of methanandamide (MethAEA) microinjected into the PVN on systolic, diastolic and mean blood pressure (SBP, DBP, MBP) and heart rate (HR) and its interaction with AM251 or ruthenium red (Ruth. red) in urethane-anaesthetized rats. MethAEA was administered twice ($S_1$ and $S_2$, 20 min apart). AM251 or Ruth. red was given intravenously (i.v.) 5 min before $S_2$. Results are calculated as percent of basal values determined immediately before $S_1$ and $S_2$. Means ± S.E.M. of four rats. ***$P < 0.001$ compared to the corresponding $S_1$. All percentage changes obtained in $S_1$ and $S_2$ are significantly different with $P < 0.05$ compared to the respective basal values immediately before $S_1$ and $S_2$.

**Fig. 2.** Influence of CP55940 microinjected into the PVN on systolic, diastolic and mean blood pressure (SBP, DBP, MBP) and heart rate (HR) and its interaction with AM251 in urethane-anaesthetized rats. CP55940 was administered twice ($S_1$ and $S_2$, 20 min apart). AM251 was given intravenously (i.v.) 5 min before $S_2$. Results are calculated as percent of basal values determined immediately before $S_1$ and $S_2$. Means ± S.E.M. of 25 (control) and 4 rats (AM251). ***$P < 0.001$ compared to the corresponding $S_1$. All percentage changes obtained in $S_1$ and $S_2$ are significantly different with $P < 0.05$ compared to the respective basal values immediately before $S_1$ and $S_2$. 

The first microinjection of CP55940 (0.1 nmol/rat) into the PVN decreased DBP by about 25% and MBP and SBP by about 15% of basal values; the hypotensive effect lasted for 118 ± 8 s (n = 4). A marginal bradycardia also occurred (Fig. 2). Almost identical changes in BP and HR were observed under control conditions during S1. Similarly to MethAEA, entirely different responses to CP55940 were observed 5 min after the i.v. administration of AM251. Thus, under CB1 receptor blockade, administration of CP55940 into the PVN increased SBP, DBP, MBP and HR by 20, 28, 24 and 5% of the basal value, respectively.

Cardiovascular effects of CP55940 given into the paraventricular nucleus of the hypothalamus

The first microinjection of CP55940 (0.1 nmol/rat) into the PVN decreased DBP by about 25% and MBP and SBP by about 15% of basal values; the hypotensive effect lasted for 118 ± 8 s (n = 4). A marginal bradycardia also occurred (Fig. 2).
Since the depressor and pressor effects of MethAEA and CP55940 were highest in the case of DBP, we have concentrated on the changes in DBP and HR in all further experiments. Fig. 3 shows the dose-response curves for CP55940 (0.01 – 1 nmol/animal). Most of its effects (determined before and after i.v. administration of AM251, respectively) were dose-dependent although its depressor effect was not. The maximal pressor response (about 30% of the basal value) was obtained for 0.1 nmol per animal. It lasted for 87 ± 9 s (n = 4) and was accompanied by an increase in HR by about 5% of the basal value. Thus, this dose was chosen for the subsequent studies.

**Influence of CB\(_1\), CB\(_2\) and TRPV1 receptor antagonists on the pressor response to CP55940 given into the paraventricular nucleus of the hypothalamus**

In the experiments of this section, the pressor effect of CP55940 microinjected into the PVN at a dose of 0.1 nmol/animal was examined in the presence of AM251 (3 µmol/kg; i.v.) given 5 min before S\(_1\).

The potential involvement of central CB\(_1\) receptors in the pressor effect of CP55940 was examined in two series of experiments. Firstly, we microinjected AM251 (0.03 µmol/rat) into the PVN during S\(_1\) 5 min after its i.v. application (Fig. 4A). Secondly, we administered the peripherally restricted cannabinoid CB\(_1\) receptor antagonist AM6545 (15 µmol/kg, i.p.) 90 min before S\(_1\) (Fig. 4B). (As an exception, AM251 was not given i.v. and CP55940 was microinjected only once in the latter experiments). We found that the additional application of AM251 into the PVN reversed the pressor response to CP55940 microinjected into the PVN into a depressor one and reduced its tachycardic effect by about one third (P < 0.01). However, the CP55940-induced decrease in DBP was by about 40% lower in the presence of AM251 (S\(_2\)) injected into the PVN than in its absence (S\(_1\); P < 0.05). On the contrary, after blockade of peripheral CB\(_1\) receptors by AM6545, microinjection of CP55940 into the PVN increased DBP and HR by 43 and 7% of the respective basal values (Fig. 4B); the duration of the pressor effect was 356 ± 24 s (n = 4).

Importantly, the pressor effect of the same dose of CP55940 lasted four times longer (P < 0.001) and was stronger by 60% (P < 0.05) in the presence of AM6545 (Fig. 4B) than after AM251 (Fig. 2).

As shown in Fig. 5, the non-selective TRPV1 receptor antagonist ruthenium red (Ruth. red) on the increase in diastolic blood pressure (DBP) and heart rate (HR) induced by CP55940 microinjected into the PVN in urethane-anaesthetized rats. CP55940 was administered twice (S\(_1\) and S\(_2\); 20 min apart). SR144528 or Ruth. red was administered intravenously (i.v.) together with AM251, 5 min before S\(_2\). Results are calculated as percent of basal values determined immediately before S\(_1\) and S\(_2\). Means ± S.E.M. of 4 rats. ***P < 0.001 compared to the corresponding S\(_1\). All percentage changes obtained in S\(_1\) and S\(_2\) are significantly different with P < 0.05 compared to the respective basal values immediately before S\(_1\) and S\(_2\).
Anandamide levels and expression of fatty acid amide hydrolase protein in the rat cerebral cortex and paraventricular nucleus of the hypothalamus

The AEA level in the rat cerebral cortex and PVN was quantified using UPLC-MS/MS and amounted to 22 ± 1 and 3 ± 0.2 pmol/g tissue (n = 8), respectively (Fig. 7). The expression of fatty acid amide hydrolase (FAAH) (an enzyme responsible for AEA degradation) was analyzed by Western blotting (Fig. 7). Western blot analysis showed a single immunoreactive band of the molecular size expected for FAAH (63 kDa) in cortex and PVN.

Fig. 6. Influence of AM6545 (15 µmol/kg) and chemical lesion of the PVN on the changes in systolic, diastolic and mean blood pressure (SDB, DBP, MBP) and heart rate (HR) induced by intravenous (i.v.) injection of CP55940. Three increasing doses of CP55940 were injected to one rat with sufficient time for the recovery to the preinjection value. The first dose of CP55940 was given 90 min after AM6545 (15 µmol/kg, intraperitoneally; i.p.) or its vehicle (veh.; “control”) or 90 min after chemical lesion of the PVN (by bilateral injection of kainic acid 2 nmol/rat into the PVN). One group of rats (‘control lesion + veh. AM6545”) was treated with the vehicles of AM6545 and kainic acid. All cardiovascular effects (SBP, DBP, MBP and HR) of CP55940 in the presence of AM6545 and after chemical lesions (with and without of AM6545) are significantly different from the respective controls (with at least P < 0.05). Means ± S.E.M. of 4 rats. With the exception of CP55940 given after chemical lesions all percentage changes induced by CP55940 (10 and 100 nmol/kg) are significantly different with P < 0.05 compared to the respective basal values immediately before its administration.
DISCUSSION

We examined the potential involvement of the PVN, a key area integrating sympathetic outflow (28), in the cannabinoid-induced changes of cardiovascular parameters in anaesthetized rats. For this purpose, we microinjected directly into the PVN (a) MethAEA, which given i.v. mimics the well-known triphasic changes in cardiovascular parameters induced by AEA (i.v.) (7), and (b) CP55940, a synthetic agonist of cannabinoid receptors (44), which decreases BP after its i.v. injection only (5). We preferred urethane over pentobarbitone anesthesia since pentobarbitone reduces the AEA-stimulated increase in BP (10) and under urethane anaesthesia the autonomic nervous system remains tonically active in the control of the cardiovascular parameters (45). Moreover, urethane (but not pentobarbitone) increases the resting sympathetic tone, since its i.v. administration enhances plasma adrenaline and noradrenaline concentrations (46, 47) and noradrenaline release from the PVN (47). With the exception of AM6545 (see later) all receptor ligands given i.v. in the present study penetrate the blood-brain barrier (48-50).

MethAEA given into the PVN decreased BP by 20% and HR by 5%. Our data with the TRPV1 receptor antagonist ruthenium red and the slow administration of all compounds into the PVN argue against the involvement of the TRPV1 receptor-mediated Bezold-Jarisch reflex, which is induced by rapid i.v. injection only (7). By contrast, the i.v. injection of the CB₁ receptor antagonist AM251 not only reduced but even reversed the depressor effect of MethAEA into a pressor one. Similarly, we had previously shown for AEA that combined i.v. administration of AM251 and ruthenium red reversed its (i.c.v.) hypotensive response into a pressor effect (8). The pressor effect of MethAEA cannot be a reflex response to the hypotension during S₁, because it was induced 20 min after S₁. Moreover, we have excluded previously that the pressor effect of AEA is just a reflex response to a preceding hypotension since it was not modified by vagotomy (10).

In conscious rats (12) and rabbits (18), CB₁ receptors are postulated to modulate the pressor effects of the cannabinoids CP55940, WIN55212-2 and HU-210. Thus, we decided to examine the cardiovascular effects of CP55940 given into the PVN. We chose CP55940 (a high-efficacy agonist of CB₁ and CB₂ receptors; (44)) for our studies since its central and peripheral cardiovascular effects have been described in detail (1). Similarly to MethAEA, CP55940 caused a dose-dependent fall in BP (which was strongest in the case of DBP; expressed in % of basal values) and a modest decrease in HR. We have concentrated on the changes in DBP which reflect changes in the peripheral resistance. Importantly, after AM251 (i.v.) a dose-dependent pressor effect and slight tachycardia occurred. These effects were not further modified by SR144528 suggesting that CP55940 does not act via CB₂ receptors in our experimental model.

In previous studies, administration of AEA, WIN55212-2 or HU-210 i.c.v. (20) into the RVLM (21) or dPAG (22) of anaesthetized rats increased BP and/or renal sympathetic nerve activity in a manner sensitive to CB₁, antagonists. By contrast, in our paper microinjection of MethAEA and CP55940 decreased BP. The above hypotensive effects of both agonists were reversed into pressor responses by the CNS-penetrating CB₁ antagonist AM251 and/or the peripherally restricted CB₁ antagonist AM6545. One might assume that CP55940 injected into the PVN produces a sympathetic activation accompanied by an increased formation of endocannabinoids or an increase in the constitutive activity of the CB₁ receptors in the vascular sympathetic nerve endings. In the absence of an inverse CB₁ agonist/antagonist the endocannabinoids and/or the high constitutive activity may override the central pressor effect of CP55940, thereby leading to hypotension.

The shift from the hypo- to a hypertensive response of the cannabinoids might, however, also be related to the fact that the final integration of the sympathetic outflow by the PVN results from the balance between stimulatory and inhibitory inputs depending on glutamate and GABA, respectively (28, 51). Presynaptic CB₁ receptors inhibit the release of either transmitter

**Fig. 7.** Content of anandamide (AEA) and Western blots of fatty acid amide hydrolase (FAAH) protein in the cerebral cortex and the PVN of rats. On the bottom of the right panel, representative Western blots for FAAH and β-tubulin (which served as loading control) are given. Means ± S.E.M. of 8 rats (AEA content) and 4 rats (Western blots).
In the conscious rat with a normal sympathetic tone, CB1 receptor-related inhibition of GABA release leads to sympathoexcitation and a BP increase (19). However, under urethane anaesthesia a high resting sympathetic tone occurs (46) leading to a higher inhibitory effect of cannabinoids on glutamate than on GABA release associated with a hypotensive effect. It is an intriguing idea that blockade of peripheral CB1 receptors via an afferent reflex leads to a shift from the predominance of CP55940 at CB1 receptors on glutamatergic to those at GABAergic neurons leading to hypertension. Admittedly, the question whether the cardiovascular effects of the two cannabinoids are related to the latter phenomena or to a more peripheral mechanism as outlined in the previous paragraph cannot be decided so far.

In the present paper, we have concentrated on the hypertensive effects of cannabinoids. The increase in BP induced by CP55940 was further studied in experiments in which we microinjected CP55940 into the PVN together with AM251 and 5 min after AM251 (i.v.). Combined topical plus systemic administration of AM251 counteracted the pressor effect of CP55940 suggesting that CB1 receptors in the PVN are implicated in its pressor effect. These CB1 receptors may be the target of endogenously formed endocannabinoids, as postulated by Gyömberkői et al. (35). The occurrence of one of the endocannabinoids, i.e. AEA, in the PVN has been proven by us with the UPLC-MS/MS method. The enzyme involved in the degradation of AEA, fatty acid amide hydrolase, has been identified as well, using the Western blot technique. The fact that CP55940, after topical and i.v. administration of AM251, even led to a depressor response (which was, however, less pronounced than that obtained in the absence of AM251) points to an additional, CB1 receptor-independent hypotensive effect of CP55940.

In further experiments, CP55940 administered via the i.v. route induced the well-known, dose-dependent fall in all cardiovascular parameters (5) without any pressor effect but, after AM6545 i.p., elicited a very strong and prolonged increase in BP. These results confirm that peripheral CB1 receptors are responsible for prolonged hypotension (1). Moreover, bilateral PVN chemical lesion with kainic acid abolished the pressor and depressor responses to CP55940 both in the absence or presence of AM6545. These results again suggest the important role of the PVN in the depressor and pressor effects of cannabinoids. But why did CP55940 (i.v.) not decrease BP in the PVN-lesioned rats? One might have expected a depressor effect since CP55940 should activate the CB1 receptors on the sympathetic nerve endings in the cardiovascular system in this experimental series; moreover, basal cardiovascular parameters had fully recovered after the lesion at the time point of CP55940 administration. The explanation may be that the PVN lesion has such a fundamental effect on the sympathetic outflow that its peripheral modulation via CB1 receptors is lost. Similarly, chemical PVN lesion completely abolished the increases in renal sympathetic activity and BP induced by the adipose afferent reflex (39).

Why did intravenous injection of AM251 block the depressor but not the pressor response to MethAEA and CP55940 given into the PVN? We suppose that AM251 (i.v.) could not reach the PVN in sufficient amounts. Studies with [125I]AM251 performed on baboons have demonstrated a poor brain penetration of this antagonist (48) attributed to its extremely high lipophilicity (c.LogP 7.36) or to a tight, specific association to a plasma protein. Nonetheless, a partial inhibition of central CB1 receptors is suggested by the fact that the pressor response to CP55940 after i.v. AM251 was smaller and shorter than that after i.p. AM6545.

The depressor and pressor responses induced by PVN microinjection of MethAEA and CP55940 were accompanied by only slight bradycardia and tachycardia, respectively. Changes in HR were modified by various pharmacological tools employed in our study usually in the same way like the changes in BP and are probably mediated by the same mechanisms.

The PVN is a site in which signals associated with the body energy status determine the direction of the effects of endocannabinoids on food intake via CB1 receptors (hyper- or hypophagia) (29). Similarly, the sympathetic tone may determine the blood pressure response to cannabinoids via CB1 receptors in the PVN. According to anaesthetized spontaneously hypertensive rats (SHR) with a higher sympathetic tone than in normotensive rats, a longer hypotensive effect of AEA or AM3506 (an inhibitor of FAAH) was observed (6, 53). A hypotensive response was also reported in acute hypertensive conscious rats (54) whereas an increase in BP occurred in normotensive rats that exhibit a lower sympathetic tone (12, 54).

In conclusion, our results show that in addition to its well-known hypotensive effect related to the stimulation of peripheral CB1 receptors, CP55940 injected into the PVN of urethane-anaesthetized rats can induce a depressor and pressor effect accompanied with a less marked bradycardia or tachycardia, respectively. The direction of the response is probably dependent on the sympathetic tone. Importantly, the pressor effect of cannabinoids is unmasked and strongly enhanced in the presence of peripheral CB1 receptor blockade. Consequently, one has to consider that peripherally restricted CB1 receptor antagonists, which may become new therapies for controlling obesity and related disorders (27, 37, 55), may increase blood pressure; particularly in patients with a metabolic syndrome, the latter effect may outweigh the beneficial effect on body weight.

Abbreviations: AEA, anandamide; AM251, (1S)-endo-1,3,3-trimethylbicyclo[2.2.1]heptan-2-yl)-5-(4-chloro-3-methylphenyl)-1-(4-methylbenzyl)-pyrazole-3-carboxamide; AM6545, 5-(4-(4-cyanobutyl-1-yl)phenyl)-1-(2,4-dichlorophenyl)-4-methyl-1H-pyrazole-3-carboxamide; BP, blood pressure; CP55940, (-)-cis-3-[2-hydroxy-4-(1,1-dimethylheptyl)phenyl]-trans-4-(3-hydroxypropyl)cyclohexanol; DBP, diastolic blood pressure; DMSO, dimethyl sulfoxide; dPAG, dorsal periaqueductal gray; FAAH, fatty acid amide hydrolase; HR, heart rate; MBP, mean blood pressure; MethAEA, methanandamide; PVN, paraventricular nucleus of the hypothalamus; RVL, rostral ventrolateral medulla; SBP, systolic blood pressure; SRI44528, N-[(1S)-endo-1,3,3-trimethylbicyclo[2.2.1]heptan-2-yl]-5-(4-chloro-3-methylphenyl)-1-(4-methylbenzy1)-pyrazole-3-carboxamide; Dα-THC, Dα-tetrahydrocannabinol; UPLC-MS/MS, ultra-high-performance liquid chromatography-tandem mass spectrometry

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