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Complex regional haemodynamic effects of anandamide in conscious rats

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1 Experiments were carried out in conscious, chronically instrumented, male, Sprague-Dawley rats to delineate the regional haemodynamic effects of the putative endogenous cannabinoid, anandamide, (0.075–3 mg kg^{-1}), and to dissect some of the mechanisms involved.

2 At all doses of anandamide, there was a significant, short-lived increase in mean arterial blood pressure associated with vasoconstriction in renal, mesenteric and hindquarters vascular beds.

3 The higher doses (2.5 and 3 mg kg^{-1}), caused an initial, marked bradycardia accompanied, in some animals, by a fall in arterial blood pressure which preceded the hypertension. In addition, after the higher doses of anandamide, the hindquarters vasoconstriction was followed by vasodilatation.

4 Although some of the effects described above resembled those of 5-HT (25 μg kg^{-1}), the bradycardia and hypotensive actions of the latter were abolished by the $5HT_3$-receptor antagonist, azasetron, whereas those of anandamide were generally unaffected.

5 None of the cardiovascular actions of anandamide were influenced by the CB_1-receptor antagonist, AM 251, but its bradycardic effect was sensitive to atropine, and its hindquarters vasodilator action was suppressed by the β_2-adrenoceptor antagonist, ICI 118551.

6 The results differ, in several aspects, from those previously reported in anaesthetized animals, and underscore the important impact anaesthesia can have on responses to anandamide.

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Introduction

The demonstration of the existence of cannabinoid receptors, and their putative endogenous ligands (see Mechoulam *et al*., 1998, for review), has re-awakened interest in the possibility that cannabinoid analogues might be of therapeutic benefit in certain cardiovascular diseases (see Piomelli *et al*., 2000 for review). This optimism has probably been bolstered by observations indicating that endocannabinoids, such as anandamide, exert hypotensive effects (Varga *et al*., 1995; 1996; Lake *et al*., 1997) through peripheral actions which, largely, have been assumed to involve vasodilatation (see Hillard, 2000; Kunos *et al*., 2000, for reviews).

The results of two recent studies, which examined the haemodynamic effects of anandamide, illustrate the complexity of the published information in this area. Using pentobarbitone-anaesthetized rats, Garcia *et al*. (2001) showed hypotensive responses to anandamide (4 and 10 mg kg^{-1} i.v.). The accompanying falls in systemic and mesenteric vascular resistances to the low, but not the high, dose of anandamide were inhibited by treatment with SR 141716A. Curiously, however, SR 141716A substantially inhibited the hypotensive response to the high dose of anandamide. Very recently, a study published by the same group, but using urethane-anaesthetized rats, provided evidence indicating that the hypotensive effect of anandamide (4 mg kg^{-1}) was associated with vasodilatation in the heart, brain and skeletal muscle. The effects in the heart and brain were sensitive to SR 141716A, but the effects in skeletal muscle were not (Wagner *et al*., 2001). No information on the effects of anandamide on blood pressure in the presence of SR 141716A was provided in that study (Wagner *et al*., 2001), and in contrast to the earlier study (Garcia *et al*., 2001, see above), there was no significant change in mesenteric vascular resistance associated with the hypotension. While it is possible that the choice of different anaesthetics in the two studies influenced the results obtained, it is not obvious how all these findings can be reconciled.

Given this confusion, we considered that an assessment of the regional haemodynamic profile of anandamide in conscious rats would provide the best foundation for dissecting its cardiovascular actions, and for determining the mechanisms involved. Therefore, our first aim was to quantitate the effects of a range of doses of i.v. anandamide on renal, mesenteric, and hindquarters vascular conductances in conscious, unrestrained rats.

In our first studies it soon became apparent that the complex cardiovascular actions of anandamide in the conscious rat model were certainly not consistent with it having a straightforward vasodilator influence. Indeed, the profile of cardiovascular effects of anandamide resembled, in several respects, those of 5-HT, which triggers the von Bezold-Jarisch reflex through stimulating $5\text{-}HT_3$-receptors on vagal afferent fibres, with subsequent vagal efferent activation (Veelken *et al*., 1998). The only vascular bed to show vasodilatation in response to anandamide was the hindquarters, which is consistent with the effects of the synthetic

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cannabinoid, WIN 55212-2, in our model (Gardiner *et al*., 2001; 2002a, b). We have shown that the hindquarters vasodilator responses to WIN 55212-2 are sensitive to CB_1-receptor antagonism, and involve β_2-adrenoceptors (Gardiner *et al*., 2002a, b).

Therefore, with this information as a background, the objectives of the present work were to investigate some possible mechanisms underlying the complex haemodynamic effects of anandamide and, specifically, to determine:

(1) The regional haemodynamic effects of anandamide in the absence and presence of the 5-HT_3-receptor antagonist, azasetron (Fukuda *et al*., 1991; Masuda *et al*., 1997). In order to assess the effectiveness of the latter, we measured the haemodynamic effects of 5-HT in its absence and presence.
(2) The haemodynamic effects of anandamide in animals pre-treated with atropine, i.e., under conditions in which the pronounced initial bradycardia would be expected to be blocked (Varga *et al*., 1995).
(3) The effects of the CB_1-receptor antagonist, AM 251 (Gatley *et al*., 1996; 1997), on the regional haemodynamic responses to anandamide.
(4) The effects of the β_2-adrenoceptor antagonist, ICI 1188551 (Bilski *et al*., 1983), particularly on the hindquarters vasodilator response to anandamide.

Some of the results have been presented to the British Pharmacological Society (Liu *et al*., 2000; Gardiner *et al*., 2002a, b).

Methods

Animals and surgical preparation

All experiments were carried out on male, Sprague-Dawley rats (350–450 g; Charles River U.K.). Animals were kept in the Biomedical Services Unit at Nottingham for at least 1 week after delivery, before any procedures were carried out. All surgery was performed under general anaesthesia (fentanyl and meditomidine, 300 μg kg^{-1} of each i.p., reversed with nalbuphine and atipamezole, 1 mg kg^{-1} of each s.c.). Initially, miniaturized pulsed Doppler flow probes were sutured around the left renal and superior mesenteric arteries, and around the distal abdominal aorta (to monitor hindquarters flow). At least 10 days later, under anaesthesia (as above), catheters were implanted in the distal abdominal aorta (*via* the ventral caudal artery), for monitoring arterial blood pressure and heart rate, and in the right jugular vein for the administration of substances.

Following at least 24 h recovery from the procedures for catheterization, when the animals were fully conscious and freely-moving, the following experiments were performed:

Responses to anandamide or 5-HT in the absence or presence of azasetron

These experiments were performed in the same groups of rats ($n=9$) on consecutive days. On day 1, ascending doses of anandamide (75 μg kg^{-1} up to 2.5 mg kg^{-1}) were given as i.v. injections, separated by at least 30 min, to allow variables to return to baseline values before the next dose. Thirty minutes after the highest dose of anandamide, a single i.v. bolus dose of 5-HT (25 μg kg^{-1}) was given. On experimental day 2, this protocol was repeated, beginning at least 30 min after the onset of administration of azasetron (10 μg kg^{-1} bolus, 10 μg kg^{-1} h^{-1} infusion; Fukuda *et al*., 1991). Azasetron administration was continued throughout this protocol.

On the basis of the findings from the first experiment (see Results), we chose to assess the responses to anandamide at doses of 1 and 3 mg kg^{-1} in the additional experimental protocols. It was expected that the blood pressure response to a dose of 3 mg kg^{-1} would be more reproducible than the response to 2.5 mg kg^{-1} (see Results), and 1 mg kg^{-1} was within the range of effective doses.

Responses to anandamide in the absence or presence of AM 251

In one group of rats ($n=7$), bolus injections of anandamide were given starting 30 min after the end of administration of AM 251 (3 mg kg^{-1} infused i.v. over 30 min at 2 ml h^{-1}), or its vehicle (saline containing 5% propoylene glycol and 2% Tween 80). The experiments were run on two days, with an intervening day without treatment, and animals were randomized to receive AM 251 or vehicle on the first day, and the other treatment on the third day.

Responses to anandamide in the absence or presence of saline, atropine, or ICI 118551

These experiments were run on a single day. In one group of rats ($n=6$), we assessed responses to anandamide in the absence and presence of sterile saline (0.1 ml bolus, 0.4 ml h^{-1} infusion), as a time control for the other experiments. The low dose (1 mg kg^{-1}) of anandamide preceded the high dose (3 mg kg^{-1}) and these doses were repeated, starting at least 45 min after administration of saline. In two other groups of rats a similar protocol was followed, except that atropine (1 mg kg^{-1}, 1 mg kg^{-1} h^{-1}; $n=9$; Widdop *et al*., 1992) or ICI 118551 (0.2 mg kg^{-1}, 0.1 mg kg^{-1} h^{-1}; $n=11$; Gardiner *et al*., 1992) were administered at least 45 min before re-challenging with the doses of anandamide.

Data analysis

All data were collected using the Haemodynamics Data Acquisition System designed and built at the University of Maastricht. Offline, data were analysed using software (Datview) from the same source, which provides electronically-averaged values over time intervals selected by the analyst. The system sampled every 2 ms, and averaged each cardiac cycle. In order to track the very rapid, transient changes, particularly in heart rate, following the higher doses of anandamide, and of 5-HT (see Results), data were stored to disc on a beat-by-beat basis for the first 120 s following anandamide or 5-HT administration and, thereafter, at 5 s intervals.

Within-group analyses were carried out using a non-parametric equivalent of ANOVA (Friedman's test). The experimental protocols were run such that only single paired comparisons (Wilcoxon's test) were made. A P value $\leqslant 0.05$ was taken as significant.

Drugs

Fentanyl citrate was obtained from Martindale; meditomidine hydrochloride (Domitor) and atipamezole hydrochloride (Antisedan) were obtained from Pfizer; nalbuphine hydrochloride (Nubain) was obtained from DuPont. Anandamide, AM 251 (N-(Piperidin-1-yl)-5-(4-iodophenyl)-1-(2,4-dichlorophenyl)-4-methyl-1H-pyrazole-3-carboxamide), azasetron (Y-25130, N-(1-Azabicyclo[2.2.2]oct-3-yl)-6-chloro-4-methyl-3-oxo-3,4-dihydro-2H-1,4-benzoxazine-3-carboxamide) and ICI 118551 hydrochloride were obtained from Tocris (U.K.). Atropine methylnitrate and 5-HT creatine sulphate were from Sigma (U.K.). Anandamide was supplied in a soya oil/water (1 : 4) emulsion and diluted in sterile saline, AM 251 was dissolved in sterile saline containing 5% propylene glycol (Sigma) and 2% Tween 80 (B.D.H.), ICI 118551 and azasetron were dissolved in sterile water, atropine and 5-HT were dissolved in sterile saline. Bolus injections were given in a volume of up to 0.12 ml and infusions were at a rate of 0.4 ml h^{-1}, except in the case of AM 251, which was infused at 2 ml h^{-1} for 30 min.

Results

Responses to anandamide or 5-HT in the absence or presence of azasetron

Baseline variables on day 1, prior to administration of the first dose of anandamide in this group of animals were: heart rate, 347 ± 11 beats min^{-1}; mean arterial blood pressure, 105 ± 2 mmHg; renal, mesenteric and hindquarters vascular conductances, 77 ± 7, 82 ± 6 and 37 ± 4 (kHz $mmHg^{-1}$) 10^3, respectively. In the absence of azasetron, anandamide (75 to 1250 $\mu g\ kg^{-1}$) had pressor effects, accompanied by renal and mesenteric vasoconstrictions; there was a biphasic change in hindquarters vascular conductance, with a fall being followed by a rise (Figure 1). The lower doses of anandamide (75–750 $\mu g\ kg^{-1}$) caused tachycardia whereas at the 1250 $\mu g\ kg^{-1}$ dose, the heart rate response was more variable and non-significant (Figure 1). At the highest dose of anandamide administered (2.5 mg kg^{-1}), there was an initial bradycardia (Figure 2) which, in some animals, was accompanied by a very short-lived fall in mean arterial blood pressure, generally lasting for no longer than 1–2 cardiac cycles. Because of the variability in the blood pressure response, the group mean data showed no significant change, but there were significant falls in conductance in all vascular beds (Figure 2). Thereafter, the pattern of haemodynamic changes was qualitatively similar to that seen with the lower doses of anandamide, although the effects were more marked (Figure 2). Administration of azasetron had no consistent cardiovascular effects and, hence, there were no differences in the resting cardiovascular variables immediately prior to administration of the first dose of anandamide on day 2 (i.e., in the presence of azasetron: heart rate, 335 ± 9 beats min^{-1}; mean arterial pressure, 106 ± 3 mmHg; renal, mesenteric and hindquarters vascular conductances, 68 ± 6, 79 ± 11 and 34 ± 4 (kHz $mmHg^{-1}$) 10^3, respectively) compared to those measured at that juncture on day 1 (see above). In the presence of azasetron, the integrated increases in hindquarters vascular conductance in response to anandamide at doses of 750 and 1250 $\mu g\ kg^{-1}$ were significantly attenuated, but no other actions of anandamide were affected (Figures 1 and 2).

In the absence of azasetron, 5-HT (25 $\mu g\ kg^{-1}$) caused a bradycardia followed by a tachycardia, and a biphasic fall in mean arterial blood pressure (Figure 2). All three vascular beds showed initial falls in conductance and subsequent vasodilatations; the mesenteric bed showed a delayed vasoconstriction (Figure 2).

Azasetron blocked the bradycardic and initial depressor effects of 5-HT, but the tachycardia and delayed fall in mean arterial blood pressure were unaffected, as were the changes in regional vascular conductances (Figure 2).

Responses to anandamide in the absence or presence of AM 251

The patterns of response to the two doses of anandamide chosen for this part of the experiment (1 and 3 mg kg^{-1}) were generally similar to those described above for 1.25 and 2.5 mg kg^{-1}. Thus, both doses had a transient pressor effect associated with vasoconstriction and, at the high dose, there was also an initial, brief bradycardia, and a delayed vasodilatation in the hindquarters (data for the higher dose are shown in Figure 3). Administration of AM 251 had no significant cardiovascular actions and, hence, resting cardiovascular variables prior to administration of anandamide in the presence of AM 251 (heart rate, 334 ± 11 beats min^{-1}; mean arterial blood pressure, 100 ± 3 mmHg; renal, mesenteric and hindquarters vascular conductances, 92 ± 3, 117 ± 9 and 43 ± 5 (kHz $mmHg^{-1}$) 10^3, respectively) were not different from the corresponding values in the presence of vehicle (heart rate, 342 ± 15 beats min^{-1}; mean arterial blood pressure, 101 ± 4 mmHg; renal, mesenteric and hindquarters vascular conductances, 86 ± 4, 108 ± 9 and 36 ± 2 (kHz $mmHg^{-1}$) 10^3, respectively). There were no significant effects of AM 251 on the cardiovascular responses to anandamide (Figure 3).

Responses to anandamide in the absence or presence of saline, or atropine, or ICI 118551

In the time-control experiment (i.e. saline infusion) there was a fall in mesenteric vascular conductance (Table 1) which we have noted on previous occasions (e.g., Gardiner *et al*., 1995), and which is probably due to the waning of postprandial hyperaemia. The cardiovascular responses to anandamide were as described above, and were reproducible, following repeated administration in the presence of saline (Figure 3).

After atropine there was a significant increase in resting heart rate, consistent with abolition of vagal tone. There was a mesenteric vasoconstriction, as seen in the time controls, and there were no other cardiovascular changes (Table 1). In the presence of atropine (Figure 3), the bradycardic action of the highest dose of anandamide was inhibited substantially but, thereafter, the changes in blood pressure, and renal and mesenteric vascular conductances were not significantly affected. However, the hindquarters vasodilator response to the higher dose of anandamide was enhanced in the presence of atropine (Figure 3).

Administration of ICI 118551 caused a significant reduction in resting hindquarters vascular conductance; there was

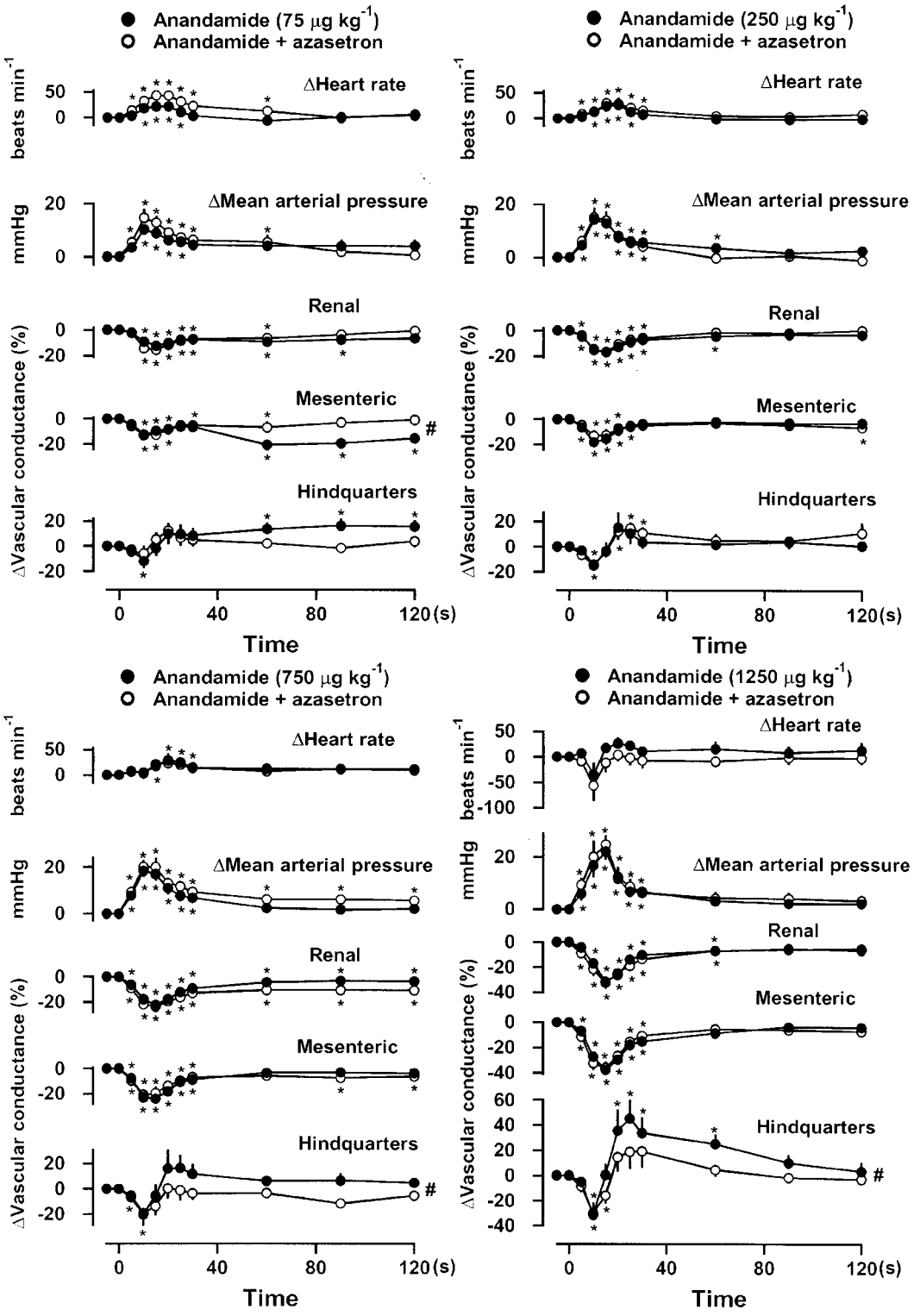

Figure 1 Cardiovascular changes elicited by different doses of anandamide in the same group ($n=9$) of rats in the absence or presence of azasetron (10 μg kg^{-1} bolus, 10 μg kg^{-1} h^{-1} infusion). Values are mean and vertical bars show s.e.mean; $^{*}P<0.05$ versus baseline (Friedman's test); $\#P<0.05$ for integrated responses in the absence and presence of azasetron (Wilcoxon's test).

also a fall in mesenteric vascular conductance, but this was similar to that seen in the time control group (Table 1). In the presence of ICI 118551, the hindquarters vasodilator effect of the highest dose of anandamide was substantially inhibited, and the pressor response, bradycardia, and renal vasoconstriction were prolonged (Figure 3).

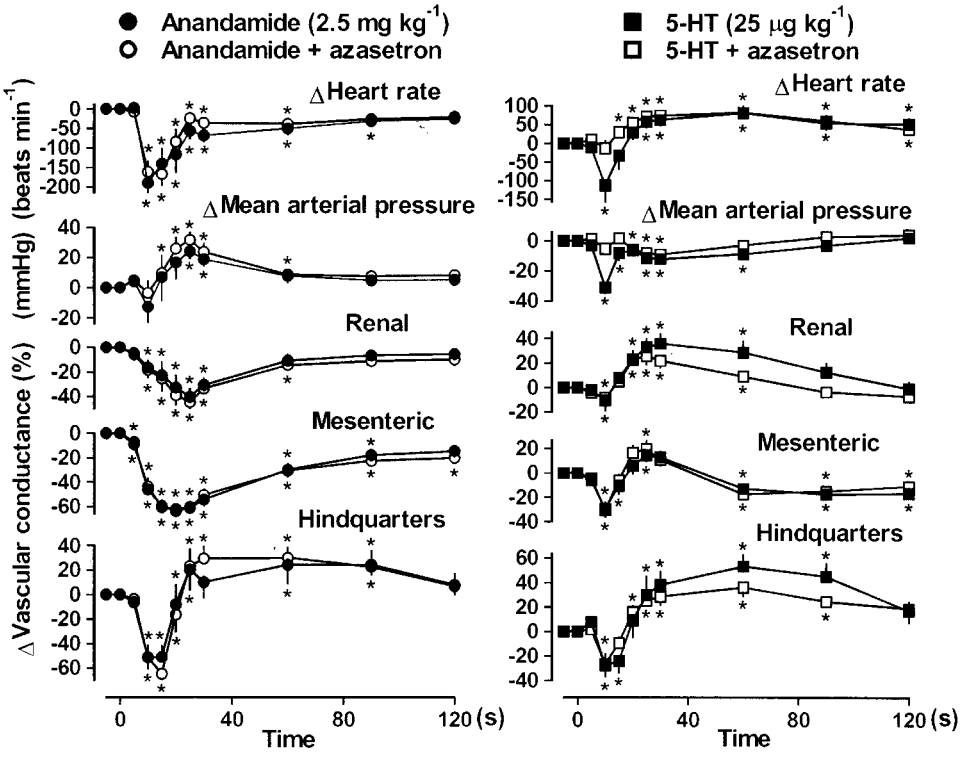

Figure 2 Cardiovascular changes elicited by anandamide or 5HT in the same group of rats ($n=9$, i.e., those in Figure 1) in the absence or presence of azasetron. Values are mean and vertical bars show s.e.mean; $^*P<0.05$ versus baseline (Friedman's test).

Discussion

The primary objectives of this work were, firstly, to establish the regional haemodynamic effects of a range of doses of anandamide in conscious rats, and, secondly, to attempt to identify some of the possible mechanisms involved in those actions by examining the effects of antagonism of 5-HT_3 receptors (using azasetron), muscarinic receptors (using atropine), cannabinoid (CB_1) receptors (using AM 251), or β_2 adrenoceptors (using ICI 118551).

Our results clearly show a complex regional haemodynamic response to anandamide, some components of which are consistent with those reported previously, and some which are not. Thus, the initial, marked, but short-lived bradycardic response, which we observed at the highest dose of anandamide, has been reported by others in conscious and anaesthetized rats (Varga *et al*., 1995; 1996; Stein *et al*., 1996; Lake *et al*., 1997). Lake *et al*. (1997) found, as we did, that the bradycardia was unaffected by cannabinoid receptor antagonism, and Varga *et al*. (1995) showed it was sensitive to atropine. Because of the extent and rapidity of the initial bradycardia, the accompanying cardiovascular changes were difficult to assess. In some animals, there was a very short-lived fall in blood pressure, lasting for only 1–2 cardiac cycles. However, in all animals, blood flows fell dramatically, and the proportional falls in blood flow were greater than those in blood pressure, indicating that there were transient falls in regional vascular conductances. In the presence of atropine, when the bradycardia was substantially blocked, there was no initial hypotension in any animal; rather, there was a consistent early rise in arterial blood pressure. It appears, therefore, that any early fall in blood pressure, when it occurs, is due to an atropine-sensitive (probably rate-dependent) fall in cardiac output, and, when this is antagonized, an underlying vasoconstriction causes blood pressure to rise.

From our experiments we cannot determine the mechanism(s) responsible for the acute vasoconstrictor responses to anandamide. Others have shown, in anaesthetized rats, that there is a transient pressor response which is not sympathetically-mediated, and does not involve cannabinoid receptors (Lake *et al*., 1997). We tested the hypothesis that at least some component of the haemodynamic response to anandamide might involve 5-HT_3-receptors, since we saw certain similarities between the effects of anandamide and aspects of the Bezold–Jarisch reflex, and it has been reported that the latter is 5-HT_3-receptor-mediated (Veelken *et al*., 1998). However, it is clear from our results that the 5-HT_3-receptor antagonist, azasetron, at a dose that effectively blocked the early bradycardic and hypotensive actions of 5-HT, had no effect on the initial bradycardic response to anandamide.

It was a surprise to us that the cannabinoid, CB_1-receptor antagonist, AM 251 (Gatley *et al*., 1997), had no effect whatsoever on the cardiovascular actions of anandamide, since we have shown that the dose of AM 251 we used inhibits the haemodynamic responses to the synthetic cannabinoid, WIN 55212-2 (Gardiner *et al*., 2002a). We must, therefore, conclude that, in our conscious rat model, at the doses used, exogenous anandamide does not act at CB_1-receptors to exert any of its haemodynamic influences. According to the literature on anaesthetized rats (Lake *et al*., 1997; Garcia *et al*., 2001; Wagner *et al*., 2001), the only component of the cardiovascular response to anandamide which is sensitive to CB_1-receptor antagonism (with SR

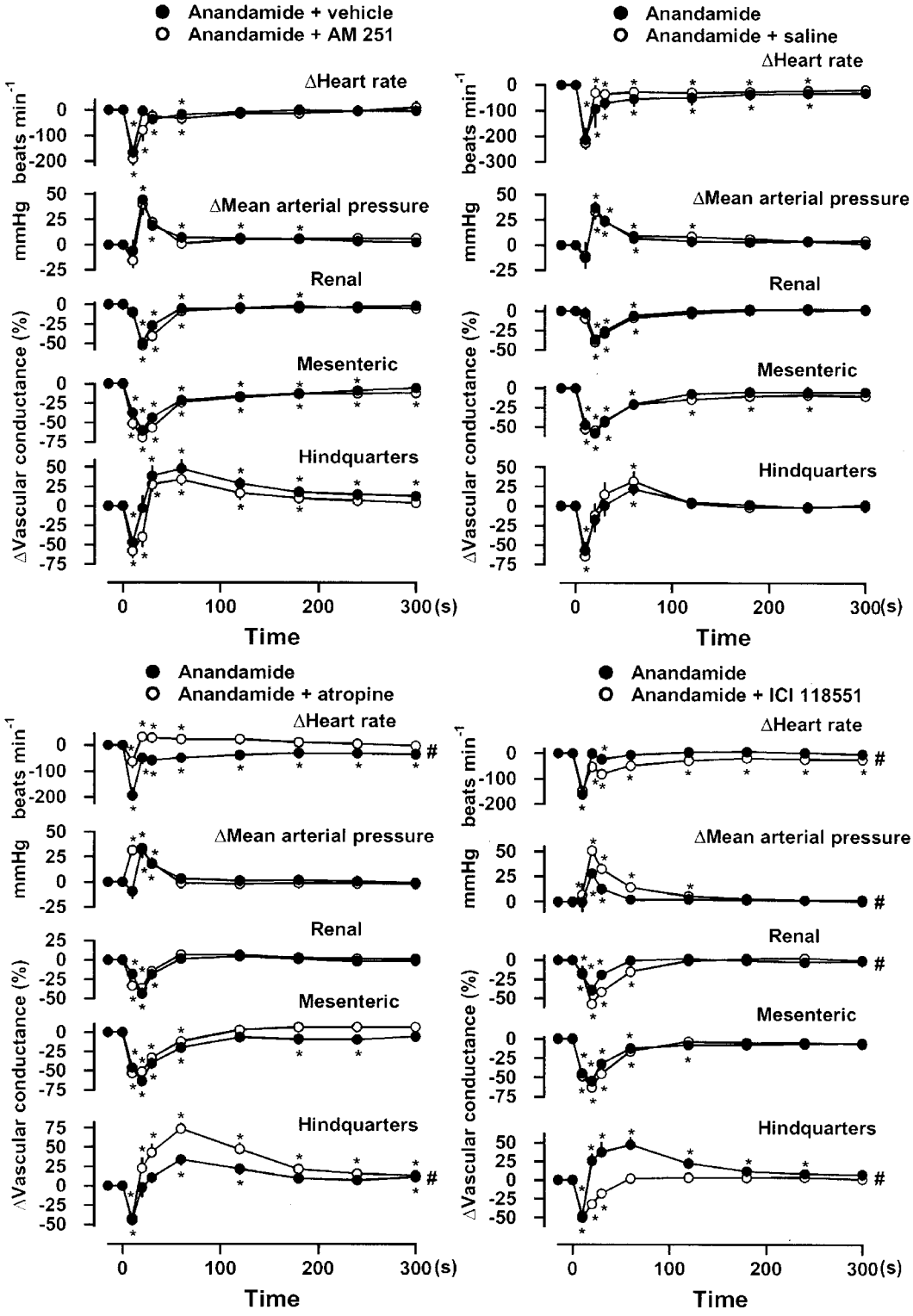

Figure 3 Cardiovascular responses to anandamide (3 mg kg^{-1}) in the presence of vehicle or AM 251 ($n=7$), or to anandamide alone, or in the presence of saline ($n=6$), or in the presence of atropine ($n=9$), or in the presence of ICI 118551 ($n=11$). Values are mean and vertical bars show s.e.mean; *$P<0.05$ versus baseline (Friedman's test); #$P<0.05$ for integrated areas in the two conditions (Wilcoxon's test).

141716A), is a delayed fall in blood pressure. This event has been attributed to sympathetic withdrawal, and may not be seen in conscious rats, possibly because of their relatively low level of resting sympathetic tone (Lake *et al*., 1997), although the experiments of Stein *et al*. (1996) did show a delayed hypotension in conscious rats after anandamide at a dose of 10 mg kg^{-1}. Although there is some evidence to suggest that there are CB_1-receptor-mediated regional vasodilatations

Table 1 Resting cardiovascular variables in conscious, Sprague–Dawley rats prior to administration of anandamide

	Group 1 (n=6)		*Group 2* (n=9)		*Group 3* (n=11)	
	Control	*Saline*	*Control*	*Atropine*	*Control*	*ICI 118551*
Heart rate (beats min^{-1})	348±13	354±15	339±6	434±9*	322±9	326±11
Mean blood pressure (mmHg)	109±6	110±6	99±3	104±4	106±4	113±5
Renal vascular conductance ((kHz mmHg)10^3)	103±10	107±11	93±5	96±6	87±7	84±8
Mesentric vascular conductance ((kHz mmHg)10^3)	84±11	64±9*	101±5	85±6*	94±5	78±5*
Hindquarters vascular conductance ((kHz mmHg)10^3)	46±5	44±5	41±2	46±4	43±5	36±4*

Control values were obtained prior to the first dose of anandamide; values for saline, atropine or ICI 118551 were measured at least 45 min after onset of their infusion. Values are mean±s.e.mean. *$P<0.05$ *vs* corresponding control value (Wilcoxon's test).

accompanying the hypotensive effects of anandamide in anaesthetized rats (Garcia *et al*., 2001; Wagner *et al*., 2001), the data from the published studies are not entirely consistent, possibly because different anaesthetics were used (see Introduction).

Interestingly, Wagner *et al*. (2001) found anandamide caused non-CB_1-receptor-mediated vasodilatation in the hindquarters, which does concur with our findings, although those authors did not comment on that observation. Our studies showed that the hindquarters vasodilator response to anandamide was inhibited by ICI 118551, and we have recently observed a similar phenomenon with HU 210 and with WIN 55212-2 (Gardiner *et al*., 2002b). However, in those cases, the hindquarters vasodilator effects were also inhibited by AM 251. Furthermore, at least with WIN 55212-2, the hindquarters vasodilatation was insensitive to ganglion blockade (Gardiner *et al*., 2001), leading us to speculate on the possibility of involvement of CB_1-receptor-mediated release of adrenaline from chromaffin cells (Gardiner *et al*., 2002b). In contrast, in the case of anandamide, it appears that CB_1-receptors are not involved. However, it is possible that vanilloid (VR_1)-receptors, for which anandamide is an agonist (Zygmunt *et al*., 1999), exist on chromaffin cells and influence adrenaline release. Alternatively, since there is evidence that capsaicin-sensitive nerves modulate adrenaline release from the adrenal medulla, (Zhou & Livett, 1991), it is feasible that anandamide acts on these to trigger adrenaline release.

In conclusion, the present results in conscious rats, viewed against previously published findings in anaesthetized animals, indicate that the haemodynamic effects of anandamide are significantly influenced by anaesthesia. Moreover, at the doses of anandamide used, there is no evidence for anandamide exerting any cardiovascular actions involving 5-HT_3-receptors or CB_1-receptors, in conscious rats. However, it is possible that direct and/or indirect VR_1-receptor-mediated adrenomedullary adrenaline release is involved in anandamide-induced hindquarters vasodilatation.

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