British Journal of Pharmacology (2005) 146, 809–816 © 2005 Nature Publishing Group All rights reserved 0007–1188/05 $30.00
www.nature.com/bjp

Anandamide reduces infarct size in rat isolated hearts subjected to ischaemia–reperfusion by a novel cannabinoid mechanism

[1]Nichola J. Underdown, [2]C. Robin Hiley & *,[1]William R. Ford

[1]Welsh School of Pharmacy, Cardiff University, King Edward VII Avenue, Cardiff CF10 3XF and [2]Department of Pharmacology, University of Cambridge, Tennis Court Road, Cambridge CB2 1PD

1 Although the endocannabinoids 2-arachidonoylglycerol (2-AG) and anandamide share a similar pharmacology, 2-AG reportedly limits myocardial ischaemia–reperfusion injury whereas anandamide does not. We therefore investigated whether or not anandamide reduces infarct size and which, if any, of the known cannabinoid-signalling pathways are involved.

2 Rat isolated perfused hearts were subjected to global, no-flow ischaemia (30 min) and reperfusion (1 h). Agonists were present from 5 min before ischaemia until the end of reperfusion. Antagonists, where used, were present throughout the protocol. Recovery of left ventricular developed pressure and coronary flow was incomplete in control hearts and not significantly affected by any drug treatment.

3 In vehicle-treated hearts, $26 \pm 3\%$ ($n = 13$) of the left ventricle was infarcted at the end of reperfusion. Infarction of the left ventricle was significantly reduced after 1 μM anandamide ($10 \pm 1\%$, $n = 7$) or 1 μM methanandamide ($12 \pm 4\%$, $n = 6$) but not 1 μM HU210. Neither ACPA (1 μM; CB_1 receptor agonist) nor JWH133 (1 μM; CB_2 receptor agonist), individually or combined significantly affected infarct size.

4 Anandamide (1 μM) did not reduce infarct size in the presence of the CB_1 receptor antagonist rimonabant (SR141716A, 1 μM) or the CB_2 receptor antagonist, SR144528 (1 μM).

5 Despite sensitivity to CB_1 and CB_2 receptor antagonists, the infarct-limiting action of anandamide was not mimicked by agonists selective for CB_1 or CB_2 receptors suggesting the involvement of a novel cannabinoid site of action.
British Journal of Pharmacology (2005) **146,** 809–816. doi:10.1038/sj.bjp.0706391;
published online 12 September 2005

Keywords: Anandamide; ischaemia; reperfusion; myocardial infarction; cannabinoids; cardioprotection; rat isolated heart

Abbreviations: 2-AG, 2-arachidonoylglycerol; ACEA, arachidonoyl-2-chloroethylamide; ACPA, arachidonoylcyclopropylamide; ANOVA, analysis of variance; DMSO, dimethyl sulfoxide; ECG, electrocardiogram; IR, ischaemia–reperfusion; LVDP, left ventricular developed pressure; PEA, palmitoylethanolamide

Introduction

Endocannabinoids have been indirectly implicated in the mechanisms of protection from cardiac ischaemia–reperfusion (IR) injury due to preconditioning induced by lipopolysaccharide (Lagneux & Lamontagne, 2001) or heat stress (Joyeux *et al*., 2002). In general, these studies suggest that the protection is mediated *via* activation of the CB_2 receptor as the responses are sensitive to the selective CB_2 receptor antagonist, SR144528.

Recently, direct evidence of the cardioprotective properties of endocannabinoids has been published using rat isolated hearts (Lépicier *et al*., 2003). In that study, protection from cardiac IR was observed with the endocannabinoids palmitoylethanolamide (PEA) and 2-arachidonoylglycerol (2-AG) but not anandamide. In addition, infarct size was limited by either a selective CB_1 (arachidonoyl-2-chloroethylamide; ACEA) or a selective CB_2 (JWH015) receptor agonist.

These results are somewhat confusing for a number of reasons. PEA is supposedly inactive at CB_1 and CB_2 receptors (Lambert *et al*., 1999), a fact supported by the finding that PEA was devoid of activity when administered to rat isolated hearts (Ford *et al*., 2002). In contrast, anandamide causes negative inotropy and coronary vasodilatation in rat isolated hearts (Ford *et al*., 2002). It is therefore interesting that anandamide failed to reduce IR injury whereas PEA and 2-AG were effective.

The pharmacology of 2-AG and anandamide is quite similar, in that both can bind to and stimulate CB_1 and CB_2 receptors (Sugiura *et al*., 2000; Steffens *et al*., 2005). Therefore, it is unlikely that the difference between 2-AG and anandamide in the study of Lépicier *et al*. (2003) is due to contrasting affinities or efficacies at cannabinoid receptors, particularly as cardioprotection could apparently be induced by agonists selective for either CB_1 or CB_2 receptors. It was suggested that anandamide might not have been effective because it was rapidly taken up and degraded. However, both 2-AG and anandamide are sensitive to uptake and metabolism and, furthermore, 2-AG is more labile than anandamide (Goparaju *et al*., 1998; Laine *et al*., 2002). It is therefore unlikely that the reason for the differences between 2-AG and anandamide are due to different rates of degradation.

Given the potential confusion about the effectiveness of endocannabinoids in mediating cardioprotection we decided to assess whether or not anandamide could alter infarct size induced by IR, measured directly using triphenyltetrazolium

*Author for correspondence; E-mail: fordwr@cardiff.ac.uk

chloride staining, in rat isolated hearts and identify the pharmacological mechanism. A preliminary report of these findings has been communicated to the British Pharmacological Society (Underdown & Ford, 2004).

Methods

All animals were housed and treated in accordance with Guidance on the Operation of Animals (Scientific Procedures) Act, 1986 (Her Majesty's Stationary Office, U.K.). Male Wistar rats (300–400 g), that had been fed *ad libitum*, were killed with an overdose of sodium pentobarbital (60 mg kg^{-1} i.p, Sagatal; Rhône Mérieux, Harlow, Essex, U.K.) and heparinised (100 U kg^{-1} i.p, CP Pharmaceuticals, Wrexham, Clwyd, U.K.). Hearts were rapidly excised and placed in ice-cooled Krebs–Henseleit solution.

After cannulating the aorta, constant pressure (80 mmHg) Langendorff perfusion was commenced (Ford *et al*., 2001). The perfusate, a modified Krebs–Henseleit solution (NaCl 118 mM, KCl 4.7 mM, KH_2PO_4 1.2 mM, $MgSO_4$ 1.2 mM, 2.5 mM $CaCl_2$, 11 mM glucose, 100 mU l^{-1} insulin), was maintained at 37°C and continuously bubbled with a gas mixture of 95% O_2/5% CO_2. Hearts were immersed in a chamber containing perfusate at a constant 37°C. During periods of perfusion, hearts were electrically paced *via* bipolar platinum electrodes at a frequency of 5 Hz (Stimulator 100; Palmer Bioscience, Sheerness, Kent, U.K.).

Left ventricular-developed pressure (LVDP) was measured by means of a pressurised (10–20 mmHg) balloon inserted into the left ventricle and connected to a pressure transducer (Ohmeda, model P23XL-1). Coronary flow was measured by ultrasonic flow probes (Transonic H4X). The electrocardiogram (ECG) was continuously recorded by means of two stainless-steel needles inserted into the ventricular apex and base, connected to an ECG amplifier (Grass EKG/Tachograph model 7P4). Hearts that failed to respond to electrical pacing during aerobic periods of perfusion for more than 10 min were excluded from all analyses. All parameters were continuously recorded using a PowerLab 800 (ADInstruments, Hastings, East Sussex, U.K.) and stored using a Macintosh PowerPC.

Experimental protocols

Agonist studies

Hearts were randomly assigned to receive the cannabinoid vehicle, Tocrisolve™ 100 (a 1 : 4 soya oil : water mixture emulsified with poloxamer F188, Tocris Cookson Ltd, Avonmouth, Bristol, U.K.), or one of the following; 1 μM anandamide, 1 μM methanandamide, 1 μM arachidonoylcyclopropylamide (ACPA), 1 μM JWH133 or a combination of ACPA and JWH133 (1 μM each).

Antagonist studies

Where used, antagonists or their vehicle dimethylsulfoxide (DMSO; 0.01% vol vol^{-1}, final concentration) were present in the perfusate throughout the protocol at a concentration of 1 μM. Antagonists were prepared in DMSO as 1 mM stock solutions before being added to the perfusate. Hearts were randomly assigned to one of six groups. In the first group, the DMSO was present throughout the protocol and hearts were infused with Tocrisolve™ 100. Hearts assigned to the second group were treated with DMSO and an infusion of anandamide (1 μM final concentration). The third group were treated with 1 μM rimonabant (SR141716A) and infused with Tocrisolve™ 100. In the fourth group, hearts were treated with 1 μM rimonabant and an infusion of anandamide (1 μM final concentration). The fifth group were treated with 1 μM SR144528 and an infusion of Tocrisolve™100. The sixth group received 1 μM SR144528 and an anandamide infusion (1 μM final concentration).

In all groups of hearts, cannabinoid agonists or Tocrisolve™ 100 vehicle were infused at a rate adjusted to be 10% of coronary flow. Drug or vehicle infusion was started 5 min before ischaemia (Figure 1). Ischaemia, induced by clamping the aortic inflow line, was maintained for 30 min. During ischaemia, drug or Tocrisolve™ 100 infusion and electrical pacing were stopped. After 30 min of global, no-flow ischaemia, the clamps were removed and perfusate allowed to flow. Drug infusion and electrical pacing was restarted and continued throughout the 2 h of reperfusion.

Infarct size determination

At the end of reperfusion, hearts were frozen at −4°C and then cut into 3–4 mm slices. The slices were immersed in a 1% solution of triphenyltetrazolium chloride (Sigma Chemical Co., Poole, Dorset, U.K.) in Krebs–Henseleit solution for 10–20 min at room temperature. Stained hearts were stored overnight in 10% formalin solution (VWR, Loughborough, Leicestershire, U.K.) before infarct size was determined by an investigator blinded to the experimental treatment. The right ventricle and connective tissues were removed and the slices compressed between two plates with a spacing of 2.5 mm. The total area of the left ventricle together with areas with an absence of red stain (infarct area) were traced onto acetate and scanned into a personal computer. The total area (in pixels) for each heart and the area of infarction was calculated using SigmaScan Pro 5 software (Systat Software U.K., Hounslow, Middlesex, U.K.). Infarct size for each heart is expressed as a percentage of the total left ventricular area.

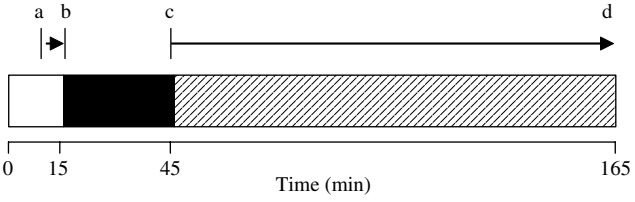

Figure 1 Diagrammatic representation of the ischaemia–reperfusion protocol. Hearts are aerobically perfused for the first 15 min (open box). Baseline values are taken at 10 min (a), pre-ischaemic values are taken just before ischaemia is induced (b). Reperfusion commences after 30 min global, no-flow ischaemia (c). Hearts are reperfused for 2 h (hatched box) and then frozen. Reperfusion values are reported at the end of the protocol (d) before hearts are removed and frozen ready for tetrazolium chloride staining. The arrows indicate the presence of vehicle (Tocrisolve™ 100) or agonist infusion. Antagonists or their vehicle (DMSO 0.01% vol vol^{-1}), where used, were present throughout the protocol.

Drug preparation and administration

The cannabinoids, anandamide, methanandamide, ACPA and JWH133 (Tocris Cookson Ltd, Avonmouth, Bristol, U.K.), obtained as solutions of 5 mg ml^{-1} dissolved in Tocrisolve™ 100 (Tocris Cookson Ltd, Avonmouth, Bristol, U.K.), were diluted in Krebs–Henseleit solution to a concentration of 10^{-5} M such that when infused at a rate equal to 10% of coronary flow a final concentration of 1 μM was obtained. The cannabinoid agonist vehicle, Tocrisolve™ 100 was diluted in Krebs bicarbonate to give a concentration of 0.07% solution that when infused at a rate controlled to be 10% of coronary flow a final concentration of 0.007% was obtained. Rimonabant (SR141716A) and SR144528 (gifts from Sanofi Synthelabo, France) were prepared in DMSO (Sigma Chemical Co., Poole, Dorset, U.K.) as 1 mM stock solutions and added to the perfusate (final concentration was 1 μM).

Statistical analysis

All data are expressed as mean ± s.e.m. Multiple comparisons against a single control were made using analysis of variance (ANOVA) supported by Dunnet's *post hoc* test. Comparisons of time course data were analysed using ANOVA with repeated measures. Statistical significance was set at $P<0.05$.

Results

Agonist studies

Diastolic pressure, LVDP and coronary flow did not significantly vary among any of the experimental groups before ischaemia (Table 1).

In hearts treated with the cannabinoid agonist vehicle, Tocrisolve™ 100, diastolic pressure, markedly elevated at the beginning of reperfusion, fell steady throughout reperfusion remaining elevated compared to pre-ischaemic values by the end of reperfusion (Figure 2 and Table 1). LVDP recovered incompletely during reperfusion recovering to a maximum of 40 ± 6% of preischaemic values after 50 min of reperfusion. Thereafter, there was a steady decline in LVDP until the end of the reperfusion period when the values were 29 ± 6% of the preischaemic values (Figure 2). Recovery of coronary flow during reperfusion was incomplete (Table 1) reaching a maximum (37 ± 7% or pre-ischaemic values) after 20 min of reperfusion then declining to be 24 ± 6% of pre-ischaemic values by the end of reperfusion. Infarct size, measured at the end of the reperfusion was 26 ± 3% of the left ventricle (Figure 3).

None of the cannabinoid agonists used in the study had a significant effect on the recoveries of diastolic pressure, LVDP or coronary flow compared to Tocrisolve™ 100-treated hearts (Figure 2 and Table 1). However, infarct size was significantly reduced by the presence of anandamide (10 ± 1% of the left ventricle) and methanandamide (12 ± 4% of the left ventricle) but not HU-210 (19 ± 3% of the left ventricle) compared to hearts that received Tocrisolve™100 (Figure 3). Infarct size in hearts treated with ACPA (26 ± 5% of the left ventricle) or JWH133 (20 ± 2% of the left ventricle) individually or in combination (27 ± 4% of the left ventricle) were not significantly different from those obtained in hearts treated with Tocrisolve™ 100 (Figure 3).

Table 1 Cardiac function and infarct size in hearts treated with cannabinoid agonists

	Perfusion time (min)	n	*Diastolic pressure* (mmHg)	*LVDP* (mmHg)	*Coronary flow* (ml min^{-1})
Vehicle	10		9 ± 1	106 ± 8	15 ± 1
	15	13	8 ± 1	100 ± 6	14 ± 1
	135		41 ± 9	21 ± 3	3 ± 1
Anandamide	10		6 ± 1	104 ± 10	16 ± 1
(1 μM)	15	7	5 ± 2	111 ± 11	18 ± 1
	135		41 ± 13	27 ± 3	5 ± 1
Methanandamide	10		9 ± 2	95 ± 20	12 ± 1
(1 μM)	15	6	10 ± 1	92 ± 16	14 ± 1
	135		27 ± 5	21 ± 5	3 ± 1
HU-210 (1 μM)	10		5 ± 2	104 ± 11	11 ± 1
	15	6	9 ± 2	111 ± 13	15 ± 1
	135		47 ± 6	17 ± 3	3 ± 1
ACPA (1 μM)	10		7 ± 2	91 ± 13	12 ± 1
	15	6	11 ± 2	87 ± 10	13 ± 1
	135		34 ± 12	23 ± 2	4 ± 1
JWH133 (1 μM)	10		8 ± 1	83 ± 10	13 ± 1
	15	8	11 ± 1	89 ± 16	13 ± 1
	135		33 ± 5	20 ± 4	2 ± 1
ACPA + JWH133	10		13 ± 1	93 ± 8	12 ± 1
(1 μM each)	15	9	12 ± 2	94 ± 6	13 ± 1
	135		53 ± 6	16 ± 3	4 ± 1

LVDP = left ventricular developed pressure.
Absolute values are given as mean ± s.e.m. with *n* the number of hearts in the group.

Antagonist studies

Diastolic pressure, LVDP and coronary flow did not significantly vary among any of the experimental groups before ischaemia (Table 2).

In Tocrisolve™ 100-treated hearts with 0.01% vol vol^{-1} DMSO present, diastolic pressure was markedly elevated at the beginning, falling steadily throughout but remaining significantly elevated at the end of the reperfusion period (Figure 4). Recovery of LVDP was incomplete reaching a maximum of 33 ± 8% of pre-ischaemic values after 40 min of reperfusion then falling to 18 ± 4% of preischaemic values by the end of the reperfusion (Figure 4). Coronary flow was impaired during reperfusion (Table 2) recovering to a maximum of 33 ± 7% of pre-ischaemic values after 20 min of the start of reperfusion then falling to 2 ± 1% of pre-ischaemic values by the end of the reperfusion period when 31 ± 4% of the left ventricle was infarcted (Figure 4).

Infusion of anandamide in the presence of 0.01 vol vol^{-1} DMSO had no significant effect on the recovery of diastolic pressure, LVDP or coronary flow during reperfusion compared to vehicle control hearts (Figure 4 and Table 2). Infarct size, measured at the end of reperfusion, was significantly reduced to 12 ± 2% of the left ventricle (Figure 5).

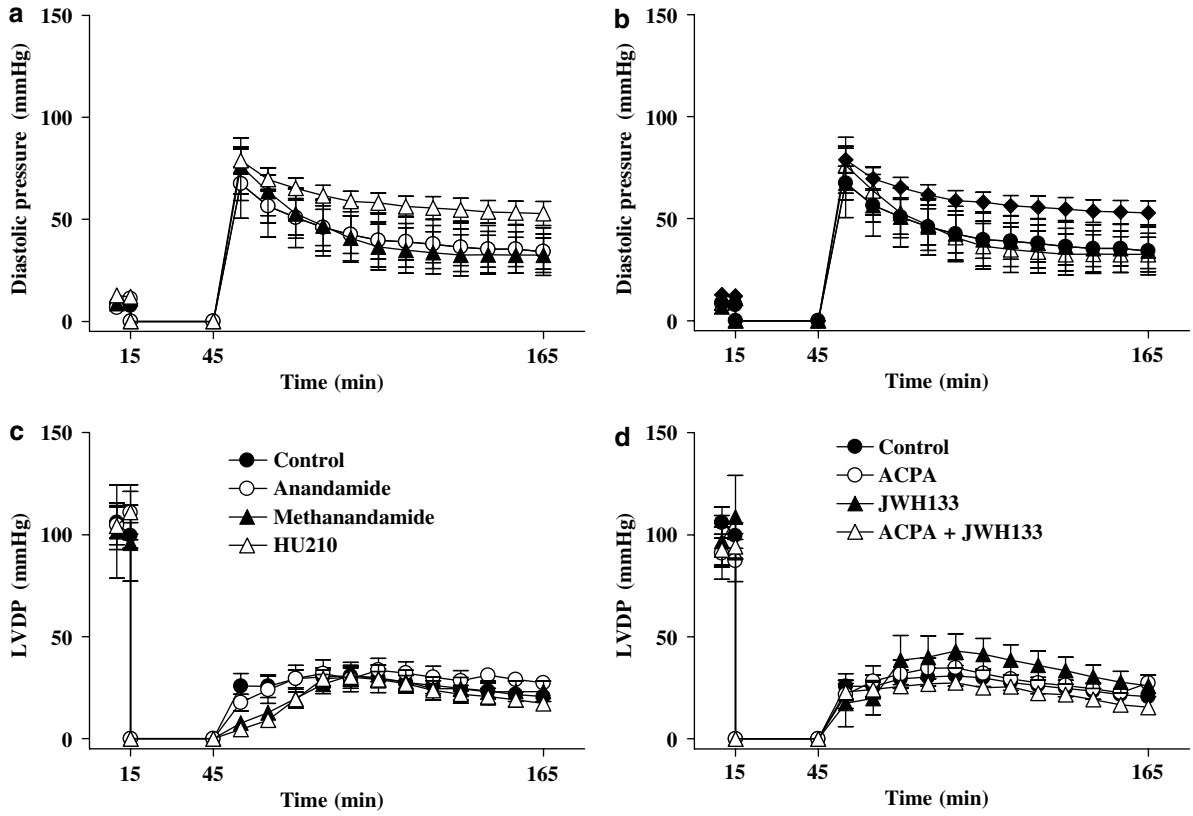

Figure 2 Diastolic pressure (panels a and b) and left ventricular developed pressure (LVDP, panels c and d) measured during periods of perfusion for hearts treated with vehicle (Tocrisolve™ 100), 1 μM anandamide, 1 μM methanandamide, 1 μM HU-210, 1 μM JWH133, or a combination of 1 μM ACPA + 1 μM JWH133. The same control group appears in panels a–d. Diastolic pressure and LVDP did not significantly vary among any of the experimental groups (ANOVA with repeated measures).

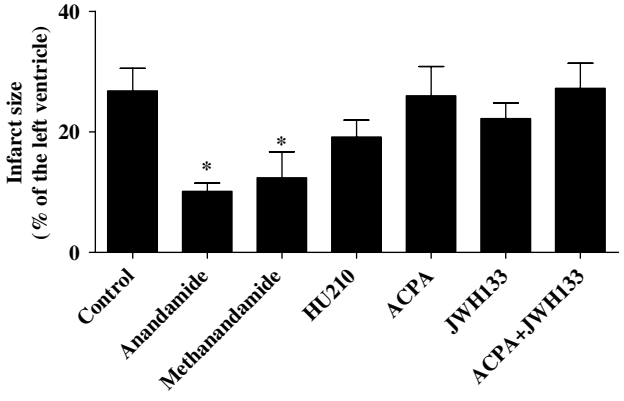

Figure 3 Infarct sizes for individual hearts treated with Tocrisolve™ 100, anandamide, methanandamide, HU-210, ACPA, JWH133 and a combination of ACPA + JWH133. * = $P<0.05$ vs Control (ANOVA supported by Dunnett's *post hoc* test).

The recoveries of diastolic pressure, LVDP and coronary flow were not significantly different from Tocrisolve™ 100-treated hearts with 0.01% vol vol^{-1} DMSO present to hearts where rimonabant (1 μM) or SR144528 (1 μM) was present (Figure 5). Infarcts obtained in hearts where rimonabant or SR144528 was present (Figure 5) were similar to those treated with Tocrisolve™ 100 in the presence of 0.01% vol vol^{-1} DMSO.

Table 2 Cardiac function and infarct size in hearts treated with cannabinoid agonists

	Time (min)	n	*Diastolic pressure* (mmHg)	*LVDP* (mmHg)	*Coronary flow* (ml min^{-1})
Vehicle	10		10±1	101±10	13±2
	15	7	9±2	96±9	13±1
	135		41±11	18±5	2±1
Anandamide	10		7±1	100±11	15±1
(1 μM)	15	7	7±2	103±11	17±1
	135		51±9	22±3	4±1
Rimonabant	10		13±2	88±6	12±2
(1 μM)	15	6	13±2	82±5	12±2
	135		56±6	15±3	2±1
Rimonabant +	10		12±1	81±3	13±1
anandamide	15	6	12±1	81±5	13±1
(1 μM each)	135		53±6	22±1	3±1
SR144528	10		9±2	113±9	15±1
(1 μM)	15	6	12±1	108±8	14±1
	135		58±9	14±3	5±2
SR144528 +	10		9±1	98±5	18±1
anandamide	15	8	8±1	100±5	18±1
(1 μM each)	135		22±8	26±5	5±1

LVDP = left ventricular developed pressure.
Absolute values are given as mean ± s.e.m. with *n* the number of hearts in the group.

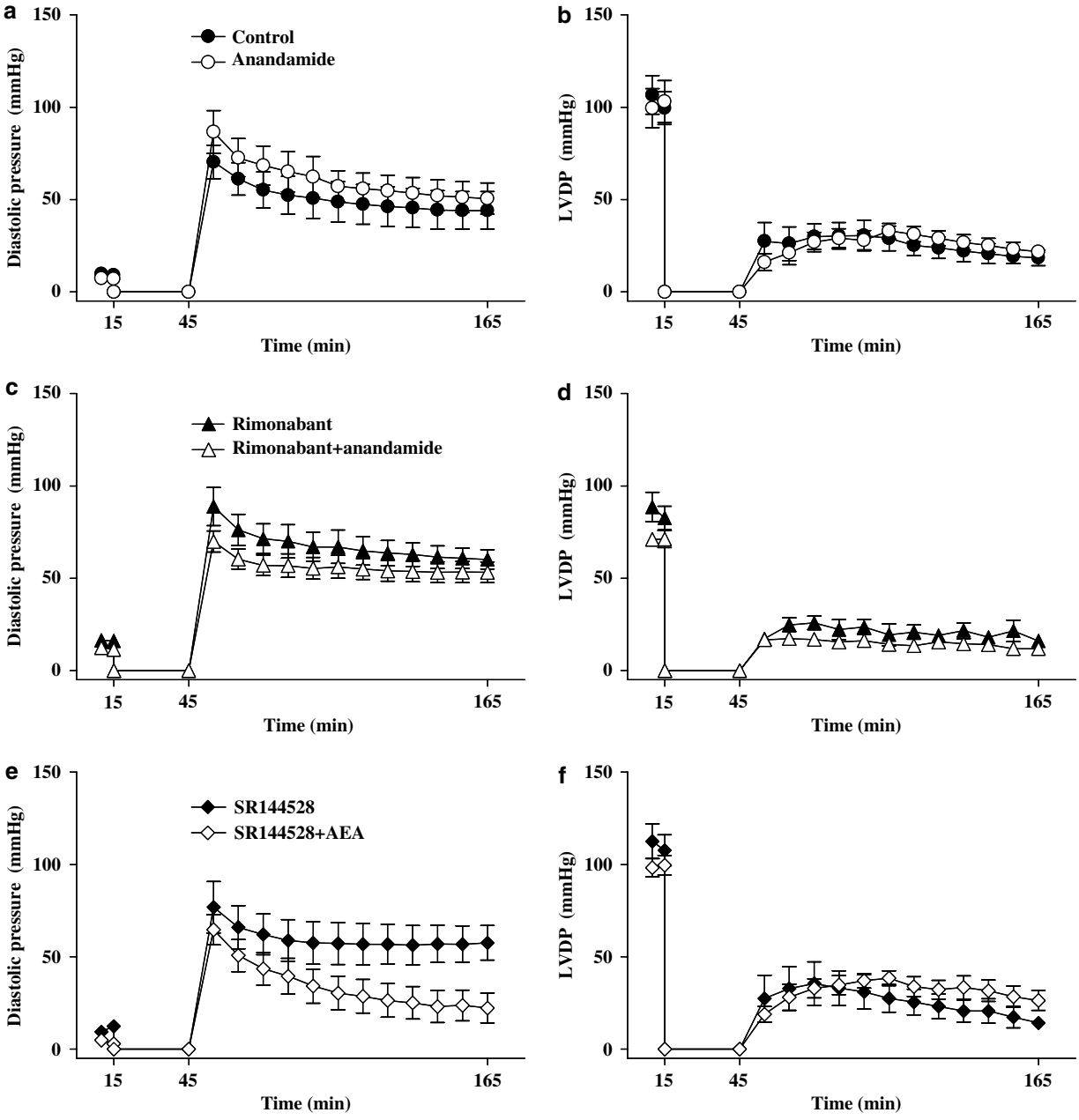

Figure 4 Diastolic pressure (panels a, c and e) and left ventricular developed pressure (LVDP, panels b, d and f) for hearts treated with Tocrisolve™ 100 or 1 μM anandamide in the absence (panels a and b) or presence of 1 μM rimonabant (panels c and d) or 1 μM SR144528 (panels e and f). There were no significant differences in recovery of diastolic pressure or LVDP among any of the groups (ANOVA with repeated measures).

The combination of either rimonabant or SR144528 with anandamide infusion had no significant effect on the recoveries of diastolic pressure, LVDP or coronary flow during reperfusion compared to hearts treated with rimonabant or SR144528 alone (Figure 5). However, the infarct size reduction observed when anandamide was infused alone was lost in the presence of either antagonist (Figure 5).

Discussion

The main finding of this study is that exogenous addition of the endocannabinoid, anandamide, limits infarct size induced by IR. This infarct-limiting action was not mimicked by CB_1 or CB_2 receptor-selective agonists either used individually or in combination. Furthermore, the infarct-limiting action of anandamide was blocked by the presence of either rimonabant or SR144528, which are regarded as CB_1 and CB_2 receptor selective antagonists, respectively. These results therefore suggest that anandamide acts either by action at both CB_1 and CB_2 receptors or that it limits the cardiac infarction associated with IR by activation of one or more novel cannabinoid sites of action. However, since neither ACPA (a selective CB_1 receptor agonist) nor JWH133 (which is selective for CB_2 receptors), alone or in combination, affected infarct size the involvement of a novel site seems to be the most likely explanation.

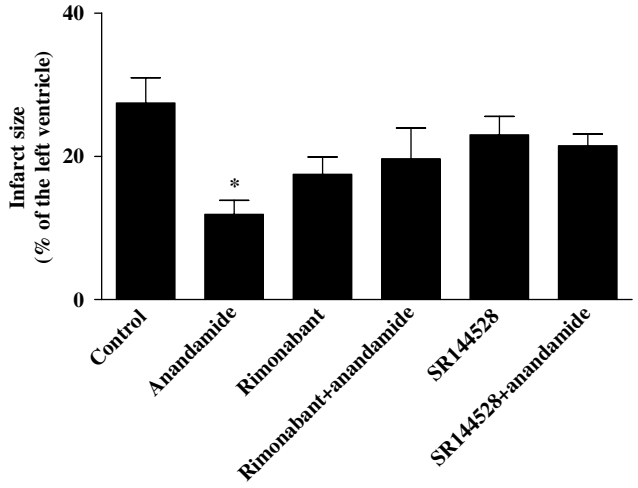

Figure 5 Infarct sizes for individual hearts treated with Tocrisolve™ 100 in the absence (control) and presence of 1 μM rimonabant or 1 μM SR144528, or 1 μM anandamide in the absence or presence of rimonabant or SR144528. *$P<0.05$ *versus* control (ANOVA supported by Dunnett's *post hoc* test).

The first indication that endocannabinoids might play a role in reducing cardiac IR injury came from indirect evidence concerning lipopolysaccharide- (Lagneux & Lamontagne, 2001) and heat stress-induced (Joyeux *et al*., 2002) preconditioning. Both of these types of preconditioning were sensitive to the selective CB_2 receptor antagonist, SR144528, but not to rimonabant, leading the authors of both studies to conclude that the cardioprotection is mediated through activation of the CB_2 receptor.

A more recent study has examined the direct effects of endocannabinoid administration on the severity of cardiac IR injury (Lépicier *et al*., 2003). In that study, the endocannabinoids PEA and 2-AG were shown to reduce infarction associated with low-flow ischaemia in rat isolated hearts. Although infarct size was not directly measured, 0.3 μM anandamide, a relatively nonselective endocannabinoid (Glass & Northup, 1999), was ineffective at reducing leakage of creatine kinase or lactate dehydrogenase into the coronary effluent (biochemical markers of cellular damage). In our study, we have shown that anandamide infusion (final concentration 1 μM) significantly reduced infarct size by more than 50% compared to vehicle-treated controls. Clearly this is not concordant with the previous investigation. However, there are a number of methodological differences between the two studies. One potentially critical difference is that the vehicles used to deliver anandamide differ. Lépicier *et al*. (2003) used propylene glycol (1 : 9 vol vol^{-1} with Krebs–Henseleit buffer) to deliver anandamide whereas Tocrisolve™ 100 (from Tocris Cookson, Bristol, U.K.) was used here. Responses to anandamide in Tocrisolve™ 100 have previously been reported (Begg *et al*., 2002; Ford *et al*., 2002; Kwolek *et al*., 2005) whereas we are unaware of any reports where anandamide responses have been observed with propylene glycol used as the vehicle. Given the recent report that responses to anandamide can be lost depending upon the solvent/vehicle used (López-Miranda *et al*., 2004), this is a major consideration.

In their study, Lépicier *et al*. (2003) found that ACEA, a selective CB_1 receptor agonist or JWH015, a selective CB_2 receptor agonist, were able to reduce infarct size to a similar degree as either PEA or 2-AG. However, in the present study, neither ACPA, a selective CB_1 receptor agonist (Hillard *et al*., 1999), nor JWH133, a selective CB_2 receptor agonist (Huffman *et al*., 1999), reduced infarct size when used individually or in combination. At the present time, we are unable to account for this difference between the two studies. The differences in pharmacology between the two sets of selective CB_1 and CB_2 receptor agonists are not sufficient to explain the disparity in results. Indeed the selective agonists in our study were used at a higher concentration than those used by Lépicier *et al*. (2003). However, the choice of vehicle for the selective agonists again differed in the two sets of experiments with Lépicier *et al*. (2003) using ethanol in contrast to the Tocrisolve™ 100 used here. Therefore, it could be argued that ACPA and JWH133 may be biologically inactive when delivered in Tocrisolve™100 as these preparations have not been extensively tested as yet.

Cannabinoids are known to mediate responses *via* a number of different mechanisms, one of which is uptake and metabolism to form products of the arachidonic acid cascade (Ellis *et al*., 1995; Kozak *et al*., 2002). As methanandamide, a nonhydrolysable analogue of anandamide (Abadji *et al*., 1994), also reduced infarct size, it is unlikely that the mechanism of cannabinoid-induced cardioprotection involves uptake, metabolism and release of arachidonic acid metabolites.

Both anandamide (Smart *et al*., 2000) and methanandamide (Malinowska *et al*., 2001) are known to be able to activate vanilloid VR_1 receptors. However, rimonabant does not seem to block VR_1 receptor-mediated responses (del Carmen García *et al*., 2003). As rimonabant was effective in blocking the reduction in infarct size induced by anandamide, it is unlikely that this process is mediated *via* activation of the vanilloid VR_1 receptor.

Cannabinoid responses can also be mediated by two receptor subtypes that have been identified and cloned, namely the CB_1 (Matsuda *et al*., 1990) and the CB_2 receptors (Munro *et al*., 1993). Anandamide binds and activates both CB_1 (Vogel *et al*., 1993) and CB_2 receptors (Zoratti *et al*., 2003) although there are also examples in the literature where it does not activate CB_2 receptors (Bayewitch *et al*., 1995; Facci *et al*., 1995). Methanandamide, on the other hand, is a relatively selective agonist for the CB_1 receptor (Mechoulam *et al*., 1998). Therefore, it would appear that a likely candidate receptor for mediating the infarct size limiting actions of anandamide and methanandamide in our study is the CB_1 receptor. However, the infarct-limiting response to anandamide is blocked by the selective CB_2 receptor antagonist, SR144528 as well rimonabant, which is widely used as a selective CB_1 receptor antagonist. There are two possible explanations for these data. Firstly, that the infarct-limiting action of anandamide requires costimulation of CB_1 together with CB_2 receptors such that if one or other is blocked the cardioprotective response is lost. Secondly, the response to anandamide is mediated by a receptor distinct from either the CB_1 or CB_2 receptor but which is sensitive to the two cannabinoid receptor antagonists.

We found that, individually, neither the selective CB_1 agonist, ACPA (Hillard *et al*., 1999) nor the selective CB_2 agonist JWH133 (Pertwee, 1999) reduced infarct size. This would be expected if the former hypothesis, that stimulation of both receptor subtypes is required in order to observe infarct

size reduction, is true. However, when the two selective cannabinoid agonists are used in combination, the size of infarction should be reduced. Our data demonstrates that this is not the case, indicating that the mechanism of anandamide-induced infarct size limitation is not mediated by synergistic activation of CB_1 and CB_2 receptors, favouring the hypothesis that the response is mediated at a site distinct from these receptor subtypes.

The presence of a cannabinoid receptor which is distinct from either the CB_1 or CB_2 receptor and is located on endothelial cells has recently been proposed (Mo *et al*., 2004). Anandamide is thought to be able to activate this receptor and therefore activation of the putative endothelial cannabinoid receptor (Járai *et al*., 1999) might play a role in the mechanism of anandamide infarct size limitation. However, the fact that the response to anandamide was abolished by SR144528 would indicate that the putative endothelial cannabinoid receptor is not involved as it is reportedly insensitive to this antagonist (Mo *et al*., 2004).

We have previously reported that anandamide mediates cardiac responses such as negative inotropy and coronary vasodilatation by interaction with a novel site distinct from any of the pathways already known to mediate cannabinoid responses (Ford *et al*., 2002). The pharmacological profile of the limitation in infarct size by anandamide observed in this study is the same as that we reported in our previous study (Ford *et al*., 2002). Therefore, it would appear that in addition to the previously reported cardiac responses, anandamide reduces infarct size by interaction with one or more novel mechanisms of cannabinoid signal transduction similar to those that mediate negative inotropy and coronary vasodilatation.

Although there is no direct evidence for the novel site of cannabinoid action in the heart being a receptor, it is the most likely explanation for our observations as responses to anandamide were mimicked by methanandamide, a nonhydrolysable analogue, and antagonised by rimonabant and SR144528 at concentrations below those reported to have nonspecific actions. The sensitivity of the novel cardiac cannabinoid site to SR144528 or rimonabant excludes the possibility that it shares identity with either the putative endothelial cannabinoid receptor (Mo *et al*., 2004) or the putative CB_3 receptor identified in the CNS (Fride *et al*., 2003). The orphan, G-protein-coupled receptor, GPR55, can be activated by anandamide but is unlikely to be responsible for mediating the responses observed in the heart as rimonabant acts as a GPR55 agonist (Brown & Wise, 2001; Drmota *et al*., 2004; Brown *et al*., 2005) whereas responses to anandamide were antagonised in our study. Although the literature contains evidence supporting the existence of a number of cannabinoid receptors distinct from CB_1, CB_2 or VR_1, responses to anandamide have a unique pharmacological profile in that they are sensitive to both rimonabant and SR144528.

In summary, anandamide and its stable analogue *R*-(+)-methanandamide, limit infarct size induced by IR in rat isolated hearts. The pharmacological profile of this response is the same as that previously reported for anandamide-induced cardiac responses (Ford *et al*., 2002) and fails to match with any of the previously known mechanisms of cannabinoid action. We therefore conclude that anandamide reduces infarct size in rat isolated hearts by interaction with one or more novel sites of cannabinoid action that might involve a new cannabinoid receptor subtype.

References

ABADJI, V., LIN, S., TAHA, G., GRIFFIN, G., STEVENSON, L.A., PERTWEE, R.G. & MAKRIYANNIS, A. (1994). (*R*)-methanandamide: a chiral novel anandamide possessing higher potency and metabolic stability. *J. Med. Chem.,* **37,** 1889–1893.

BAYEWITCH, M., AVIDOR-REISS, T., LEVY, R., BARG, J., MECHOULAM, R. & VOGEL, Z. (1995). The peripheral cannabinoid receptor: adenylate cyclase inhibition and G protein coupling. *FEBS Lett.,* **375,** 143–147.

BEGG, M., DALE, N., LLAUDET, E., MOLLEMAN, A. & PARSONS, M.E. (2002). Modulation of the release of endogenous adenosine by cannabinoids in the myenteric plexus-longitudinal muscle preparation of the guinea-pig ileum. *Br. J. Pharmacol.,* **137,** 1298–1304.

BROWN, A.J. & WISE, A. (2001). Identification of modulators of GPR55 activity. *Patent number,* WO0186305.

BROWN, A.J., UENO, S., SUEN, K., DOWELL, S.J. & WISE, A. (2005). Molecular identification of GPR55 as a third G protein-coupled receptor responsive to cannabinoid ligands. *ICRS Symposium on the cannabinoids*, www.cannabinoidsociety.org.

DEL CARMEN GARCÍA, M., ADLER-GRASCHINSKY, E. & CELUCH, S.M. (2003). Hypotensive effect of anandamide through the activation of CB1 and VR1 spinal receptors in urethane-anesthetized rats. *Naunyn Schmiedebergs Arch. Pharmacol.,* **368,** 270–276.

DRMOTA, T., GREASLEY, P. & GROBLEWSKI, T. (2004). Screening assays for cannabinoid-ligand type modulators of GPR55. *Patent number,* WO2004074844.

ELLIS, E.F., MOORE, S.F. & WILLOUGHBY, K.A. (1995). Anandamide and delta 9-THC dilation of cerebral arterioles is blocked by indomethacin. *Am. J. Physiol.,* **269,** H1859–H1864.

FACCI, L., DAL TOSO, R., ROMANELLO, S., BURIANI, A., SKAPER, S.D. & LEON, A. (1995). Mast cells express a peripheral cannabinoid receptor with differential sensitivity to anandamide and palmitoylethanolamide. *Proc. Natl. Acad. Sci. U.S.A.,* **92,** 3376–3380.

FORD, W.R., CLANACHAN, A.S., HILEY, C.R. & JUGDUTT, B.I. (2001). Angiotensin II reduces infarct size and has no effect on post-ischaemic contractile dysfunction in isolated rat hearts. *Br. J. Pharmacol.,* **134,** 38–45.

FORD, W.R., HONAN, S.A., WHITE, R. & HILEY, C.R. (2002). Evidence of a novel site mediating anandamide-induced negative inotropic and coronary vasodilatator responses in rat isolated hearts. *Br. J. Pharmacol.,* **135,** 1191–1198.

FRIDE, E., FOOX, A., ROSENBERG, E., FAIGENBOIM, M., COHEN, V., BARDA, L., BLAU, H. & MECHOULAM, R. (2003). Milk intake and survival in newborn cannabinoid CB_1 receptor knockout mice: evidence for a 'CB_3' receptor. *Eu. J. Pharmacol.,* **461,** 27–34.

GLASS, M. & NORTHUP, J.K. (1999). Agonist selective regulation of G proteins by cannabinoid CB_1 and CB_2 receptors. *Mol. Pharmacol.,* **56,** 1362–1369.

GOPARAJU, S.K., UEDA, N., YAMAGUCHI, H. & YAMAMOTO, S. (1998). Anandamide amidohydrolase reacting with 2-arachidonoylglycerol, another cannabinoid receptor ligand. *FEBS Lett.,* **422,** 69–73.

HILLARD, C.J., MANNA, S., GREENBERG, M.J., DICAMELLI, R., ROSS, R.A., STEVENSON, L.A., MURPHY, V., PERTWEE, R.G. & CAMPBELL, W.B. (1999). Synthesis and characterization of potent and selective agonists of the neuronal cannabinoid receptor (CB_1). *J. Pharmacol. Exp. Ther.,* **289,** 1427–1433.

HUFFMAN, J.W., LIDDLE, J., YU, S., AUNG, M.M., ABOOD, M.E., WILEY, J.L. & MARTIN, B.R. (1999). 3-(1′,1′-Dimethylbutyl)-1-deoxy-delta8-THC and related compounds: synthesis of selective ligands for the CB_2 receptor. *Bioorg. Med. Chem.,* **7,** 2905–2914.

JÁRAI, Z., WAGNER, J.A., VARGA, K., LAKE, K.D., COMPTON, D.R., MARTIN, B.R., ZIMMER, A.M., BONNER, T.I., BUCKLEY, N.E., MEZEY, E., RAZDAN, R.K., ZIMMER, A. & KUNOS, G. (1999). Cannabinoid-induced mesenteric vasodilation through an endothelial site distinct from CB_1 or CB_2 receptors. *Proc. Natl. Acad. Sci. U.S.A.,* **96,** 14136–14141.

JOYEUX, M., ARNAUD, C., GODIN-RIBUOT, D., DEMENGE, P., LAMONTAGNE, D. & RIBUOT, C. (2002). Endocannabinoids are implicated in the infarct size-reducing effect conferred by heat stress preconditioning in isolated rat hearts. *Cardiovasc. Res.,* **55,** 619–625.

KOZAK, K.R., CREWS, B.C., MORROW, J.D., WANG, L.H., MA, Y.H., WEINANDER, R., JAKOBSSON, P.J. & MARNETT, L.J. (2002). Metabolism of the endocannabinoids, 2-arachidonylglycerol and anandamide, into prostaglandin, thromboxane, and prostacyclin glycerol esters and ethanolamides. *J. Biol. Chem.,* **277,** 44877–44885.

KWOLEK, G., ZAKRZESKA, A., SCHLICKER, E., GOTHERT, M., GODLEWSKI, G. & MALINOWSKA, B. (2005). Central and peripheral components of the pressor effect of anandamide in urethane-anaesthetized rats. *Br. J. Pharmacol.,* **145,** 567–575.

LAGNEUX, C. & LAMONTAGNE, D. (2001). Involvement of cannabinoids in the cardioprotection induced by lipopolysaccharide. *Br. J. Pharmacol.,* **132,** 793–796.

LAINE, K., JARVINEN, K., MECHOULAM, R., BREUER, A. & JARVINEN, T. (2002). Comparison of the enzymatic stability and intraocular pressure effects of 2-arachidonylglycerol and noladin ether, a novel putative endocannabinoid. *Invest. Ophthalmol. Vis. Sci.,* **43,** 3216–3222.

LAMBERT, D.M., DIPAOLO, F.G., SONVEAUX, P., KANYONYO, M., GOVAERTS, S.J., HERMANS, E., BUEB, J., DELZENNE, N.M. & TSCHIRHART, E.J. (1999). Analogues and homologues of *N*-palmitoylethanolamide, a putative endogenous CB_2 cannabinoid, as potential ligands for the cannabinoid receptors. *Biochim. Biophys. Acta,* **1440,** 266–274.

LÉPICIER, P., BOUCHARD, J.F., LAGNEUX, C. & LAMONTAGNE, D. (2003). Endocannabinoids protect the rat isolated heart against ischaemia. *Br. J. Pharmacol.,* **139,** 805–815.

LÓPEZ-MIRANDA, V., HERRADON, E., DANNERT, M.T., ALSASUA, A. & MARTÍN, M.I. (2004). Anandamide vehicles: a comparative study. *Eur. J. Pharmacol.,* **505,** 151–161.

MALINOWSKA, B., KWOLEK, G. & GOTHERT, M. (2001). Anandamide and methanandamide induce both vanilloid VR1- and cannabinoid CB_1 receptor-mediated changes in heart rate and blood pressure in anaesthetized rats. *Naunyn Schmiedebergs Arch. Pharmacol.,* **364,** 562–569.

MATSUDA, L.A., LOLAIT, S.J., BROWNSTEIN, M.J., YOUNG, A.C. & BONNER, T.I. (1990). Structure of a cannabinoid receptor and functional expression of the cloned cDNA. *Nature,* **346,** 561–564.

MECHOULAM, R., FRIDE, E. & DI MARZO, V. (1998). Endocannabinoids. *Eur. J. Pharmacol.,* **359,** 1–18.

MO, F.M., OFFERTÁLER, L. & KUNOS, G. (2004). Atypical cannabinoid stimulates endothelial cell migration *via* a Gi/Go-coupled receptor distinct from CB1, CB2 or EDG-1. *Eur. J. Pharmacol.,* **489,** 21–27.

MUNRO, S., THOMAS, K.L. & ABU-SHAAR, M. (1993). Molecular characterization of a peripheral receptor for cannabinoids. *Nature,* **365,** 61–65.

PERTWEE, R.G. (1999). Pharmacology of cannabinoid receptor ligands. *Curr. Med. Chem.,* **6,** 635–664.

SMART, D., GUNTHORPE, M.J., JERMAN, J.C., NASIR, S., GRAY, J., MUIR, A.I., CHAMBERS, J.K., RANDALL, A.D. & DAVIS, J.B. (2000). The endogenous lipid anandamide is a full agonist at the human vanilloid receptor (hVR1). *Br. J. Pharmacol.,* **129,** 227–230.

STEFFENS, M., ZENTNER, J., HONEGGER, J. & FEUERSTEIN, T.J. (2005). Binding affinity and agonist activity of putative endogenous cannabinoids at the human neocortical CB_1 receptor. *Biochem. Pharmacol.,* **69,** 169–178.

SUGIURA, T., KONDO, S., KISHIMOTO, S., MIYASHITA, T., NAKANE, S., KODAKA, T., SUHARA, Y., TAKAYAMA, H. & WAKU, K. (2000). Evidence that 2-arachidonoylglycerol but not *N*-palmitoylethanolamine or anandamide is the physiological ligand for the cannabinoid CB_2 receptor. Comparison of the agonistic activities of various cannabinoid receptor ligands in HL-60 cells. *J. Biol. Chem.,* **275,** 605–612.

UNDERDOWN, N.J. & FORD, W.R. (2004). Anandamide reduces infarct size in rat isolated hearts by a mechanism independent of CB_1 or CB_2 receptor activation. *Proc. Br. Pharmacol. Soc.,* at http://www.pa2online.org/Vol2Issue2abst091P.html.

VOGEL, Z., BARG, J., LEVY, R., SAYA, D., HELDMAN, E. & MECHOULAM, R. (1993). Anandamide, a brain endogenous compound, interacts specifically with cannabinoid receptors and inhibits adenylate cyclase. *J. Neurochem.,* **61,** 352–355.

ZORATTI, C., KIPMEN-KORGUN, D., OSIBOW, K., MALLI, R. & GRAIER, W.F. (2003). Anandamide initiates Ca^{2+} signaling *via* CB_2 receptor linked to phospholipase C in calf pulmonary endothelial cells. *Br. J. Pharmacol.,* **140,** 1351–1362.

(Received July 28, 2005
Revised August 3, 2005
Accepted August 11, 2005
Published online 12 September 2005)