

## RESEARCH PAPER

# Inhibition of 2-arachidonoylglycerol catabolism modulates vasoconstriction of rat middle cerebral artery by the thromboxane mimetic, U-46619

CJ Hillard<sup>1</sup>, W-SV Ho<sup>1,3</sup>, J Thompson<sup>1</sup>, KM Gauthier<sup>1</sup>, CE Wheelock<sup>2,4</sup>, H Huang<sup>2</sup> and BD Hammock<sup>2</sup>

<sup>1</sup>Department of Pharmacology and Toxicology, Medical College of Wisconsin, Milwaukee, WI, USA and <sup>2</sup>Department of Entomology, Cancer Research Center, University of California at Davis, Davis, CA, USA

**Background and purpose:** Cerebrovascular smooth muscle cells express the CB<sub>1</sub> cannabinoid receptor and CB<sub>1</sub> agonists produce vasodilatation of the middle cerebral artery (MCA). The thromboxane A<sub>2</sub> mimetic, U-46619, increased the content of the endocannabinoid, 2-arachidonoylglycerol (2-AG) in the MCA and 2-AG moderated the vasoconstriction produced by U46619 in this tissue. The purposes of this study were to examine the extent to which 2-AG is catabolized by cerebral arteries and to determine whether blockade of 2-AG inactivation potentiates its feedback inhibition of U-46619-mediated vasoconstriction.

**Experimental approach:** The diameters of isolated, perfused MCA from male rats were measured using videomicroscopy.

**Key results:** Exogenous 2-AG produces a CB<sub>1</sub> receptor-dependent and concentration-related increase in the diameter of MCA constricted with 5-HT. The  $E_{max}$  for 2-AG dilation is increased 4-fold in the presence of the metabolic inhibitors 3-(decylthio)-1,1,1-trifluoropropan-2-one (DETFP), URB754 and URB597. To examine the role of catabolism in the effects of endogenous 2-AG, vasoconstriction induced by U-46619 was studied. DETFP and URB754, but not the fatty acid amide hydrolase inhibitor, URB597, significantly increased the EC<sub>50</sub> for U-46619. These data support a physiological role for endocannabinoid feedback inhibition in the effects of U-46619 and indicate that endogenously produced 2-AG is also efficiently catabolized within the MCA.

**Conclusions and implications:** MCA express mechanisms for the efficient inactivation of 2-AG, providing further support for an endocannabinoid feedback mechanism that opposes thromboxane-mediated vasoconstriction. These data suggest that potentiation of endogenously produced 2-AG could be a novel therapeutic approach to the treatment of thrombotic stroke. *British Journal of Pharmacology* advance online publication, 24 September 2007; doi:10.1038/sj.bjp.0707468

**Keywords:** cannabinoid receptor; CB<sub>1</sub> receptor; endocannabinoid; 2-arachidonoylglycerol; U-46619; monoacylglycerol lipase; fatty acid amide hydrolase; middle cerebral artery; DETFP; URB754

**Abbreviations:** AEA, *N*-arachidonylethanolamine; 2-AG, 2-arachidonoylglycerol; DETFP, 3-(decylthio)-1,1,1-trifluoropropan-2-one; FAAH, fatty acid amide hydrolase; MCA, middle cerebral artery; MGL, monoacylglycerol lipase

## Introduction

Cannabinoids exert significant effects on the cardiovascular system through a variety of mechanisms (Hillard, 2000b; Pacher *et al.*, 2005). In fact, the most common physiological

consequences of marijuana intoxication in humans are cardiovascular in nature (Dewey, 1986). Exposure to cannabinoids via smoking marijuana and oral administration of synthetic and plant extracted compounds results in a spectrum of cardiovascular changes that include tachycardia and orthostatic hypotension that can be accompanied by significant dizziness and syncope upon standing (Mathew *et al.*, 2003).

A variety of studies reveal that cannabinoids exert significant effects on the cerebral circulation. Humans exposed to marijuana exhibit a significant drop in cerebral blood velocity upon standing that is consistent with a loss of cerebral autoregulation (Mathew *et al.*, 1992a, b, 2003;

Correspondence: Professor CJ Hillard, Department of Pharmacology and Toxicology, Medical College of Wisconsin, 8701 Watertown Plank Road, Milwaukee, WI 53226, USA.

E-mail: chillard@mcw.edu

<sup>3</sup>Present address: E Floor, School of Biomedical Sciences, University of Nottingham Medical School, Queen's Medical Center, Nottingham, NG7 2UH, UK.

<sup>4</sup>Present address: Division of Physiological Chemistry II, Department of Medical Biochemistry and Biophysics, Karolinska Institutet, Scheeles vag 2, Se-171 77 Stockholm, Sweden.

Received 13 June 2007; revised 6 August 2007; accepted 28 August 2007

Mathew and Wilson, 1993). Preclinical studies have shown that cerebral arterial vascular smooth muscle cells from cat and rat express the CB<sub>1</sub> cannabinoid receptor protein (Gebremedhin *et al.*, 1999; Ashton *et al.*, 2004; Rademacher *et al.*, 2005). Electrophysiological studies demonstrate that the CB<sub>1</sub> receptor agonist, Win 55212-2, produces potent and nearly complete inhibition of the opening of L-type calcium channels of isolated cat arteriolar vascular smooth muscle cell (Gebremedhin *et al.*, 1999). The EC<sub>50</sub> for this effect of Win 55212-2 is approximately 30 nM and it is blocked by both *Pertussis* toxin and the CB<sub>1</sub> receptor antagonist, rimonabant (also called SR141716). Therefore, cerebral vascular smooth muscle cells express a CB<sub>1</sub> receptor that couples to the inhibition of L-type calcium channel opening via a *Pertussis* toxin-sensitive G protein. Most probably, as a result of this signalling mechanism, agonists of the CB<sub>1</sub> cannabinoid receptor produce vasodilatation of cerebral resistance vessels at nanomolar concentrations (Ellis *et al.*, 1995; Gebremedhin *et al.*, 1999; Wagner *et al.*, 2001; Rademacher *et al.*, 2005).

Among the questions that arise from these studies is 'What is the role and nature of endogenous CB<sub>1</sub> receptor signalling in the cerebral vasculature?'. Regulation of vascular tone is accomplished through the actions of constricting and dilating factors that arise from a variety of cellular sources, and we hypothesize that the endocannabinoids are one of these physiological regulators of cerebrovascular tone. There is considerable evidence that two arachidonic acid derivatives, *N*-arachidonylethanolamine (AEA or anandamide) and 2-arachidonoylglycerol (2-AG) function as endogenous agonists of CB<sub>1</sub> cannabinoid receptors. Both of these molecules are present in lipid extracts of rat middle cerebral artery (MCA) (Rademacher *et al.*, 2005). Recent work from our laboratory suggests that endocannabinoid signalling in the MCA functions as a feedback mechanism that opposes vasoconstriction produced by thromboxane A<sub>2</sub>. In particular, we have shown that nanomolar concentrations of the thromboxane mimetic, U-46619, produces significant increases in the content of both AEA and 2-AG, in the MCA (Rademacher *et al.*, 2005). Moreover, inhibition of CB<sub>1</sub> receptor activation results in increased U-46619-mediated vasoconstriction. These data are consistent with the hypothesis that thromboxane receptor activation results in both vasoconstriction and in the synthesis of vasodilatory endocannabinoids, and that the endocannabinoids act via the CB<sub>1</sub> receptor to oppose or moderate the vasoconstriction produced by U-46619.

In the present study, we have postulated that if endocannabinoid signalling is physiologically important in the cerebrovasculature, then mechanisms involved in endocannabinoid inactivation should be present and functional. We have carried out two studies to explore this hypothesis: first, we have determined the effects of known inhibitors of 2-AG catabolism on the vasodilatory efficacy of exogenously added 2-AG; and, second, we have determined the effects of these inhibitors on the constrictor response to U-46619. The feedback hypothesis presented above predicts that potentiation of endogenously produced 2-AG (through inhibition of 2-AG catabolism) will reduce the vasoconstriction produced by U-46619.

## Methods

### *Animals*

All experimental protocols were approved by the Institutional Animal Care and Use Committee of The Medical College of Wisconsin approved. All studies were carried out in accordance with the National Institutes of Health 'Guide for the Care and Use of Experimental Animals'.

Male, Sprague-Dawley rats (Harlan, Madison, WI, USA) weighing 175–225 g were used for these studies. Rats were maintained on a 12:12 light/dark schedule (lights on at 0600 hours) with food and water provided *ad libitum*. The rats were acclimated to the Biomedical Resource Center of the Medical College of Wisconsin for at least 3 days prior to use in an experiment.

### *MCA isolation*

Rats were deeply anaesthetized by inhalational exposure to isoflurane (Abbott Laboratories, North Chicago, IL, USA) followed by swift decapitation. Brains were removed and the proximal portion of the MCA was dissected and placed into ice-cold, physiological salt solution containing (mM): NaCl (119); KCl (4.9); CaCl<sub>2</sub> (1.6); MgSO<sub>4</sub> (1.17); glucose (5.5); NaHCO<sub>3</sub> (24); NaH<sub>2</sub>PO<sub>4</sub> (1.18); HEPES (5.8) and EDTA (0.026).

### *MCA vascular reactivity*

MCA were cleaned of adhering fat and connective tissue and the endothelium was removed by the intraluminal introduction of an air bolus. Endothelium removal was verified by the complete elimination of the vasodilator response to acetylcholine. The MCA were cannulated using tapered glass micropipettes that were fixed within a Lucite perfusion and superfusion chamber as described previously (Gauthier-Rein *et al.*, 1997). Arteries were maintained at a perfusion pressure of 60 mm Hg; all solutions were equilibrated with 21% O<sub>2</sub>, 5% CO<sub>2</sub> and 74% N<sub>2</sub>. This gas mixture resulted in a pH of 7.4 and pO<sub>2</sub> of 140 mm Hg. Internal diameters were obtained using a Nikon SMZ-800 inverted microscope coupled to a Spot RT camera (Diagnostic Instruments, Sterling Heights, MI, USA); images were captured and analysed using the Spot/Metaview acquisition and analysis software. Diameters were determined when the vessel reached equilibrium and were calculated as the mean of 12 measurements made along the length of the vessel. At the end of each experiment, the artery was perfused and superfused with Ca<sup>2+</sup>-free buffer that also contained 1 μM nimodipine to determine the maximum vasodilatory capacity for that preparation. In the experiments investigating the vasodilatory effects of the cannabinoid agonists, the vessels were precontracted with 1 μM 5-HT and the cannabinoid agonists were added serially. The per cent maximal dilation was calculated as the [(diameter in the presence of cannabinoid)–(diameter in the presence of 5-HT)]/[(diameter in the presence of nimodipine/Ca<sup>2+</sup>-free buffer)–(diameter in the presence of 5-HT)] × 100. In the second set of studies, the per cent constriction produced by concentrations of U-46619 between 1 and 1000 nM were determined. The per cent

constriction was calculated using the formula: [(predrug diameter–postdrug diameter)/predrug diameter] × 100.

#### Statistical analyses

Data are shown as mean ± s.e.m. The number of determinations (*n*) refers to the number of individual animals. Log concentration–response curves were fitted to a sigmoidal equation and values of log EC<sub>50</sub> and *E*<sub>max</sub> and their 95% confidence intervals were estimated from least squares analyses using GraphPad Prism software (San Diego, CA, USA). The log EC<sub>50</sub> values and confidence intervals were subsequently converted to the natural scale, resulting in a skew in the confidence interval. Differences between *E*<sub>max</sub> and EC<sub>50</sub> values were considered significant if there was no overlap between the 95% confidence intervals for the values. The effects of inhibitors and receptor blockers in single concentration studies were assessed using unpaired Student's *t*-tests. The effects of the inhibitors on the constrictor responses to U-46619 were determined using two-way analyses of variance, with the concentration of U-46619 as one factor and the presence of inhibitor as the second factor.

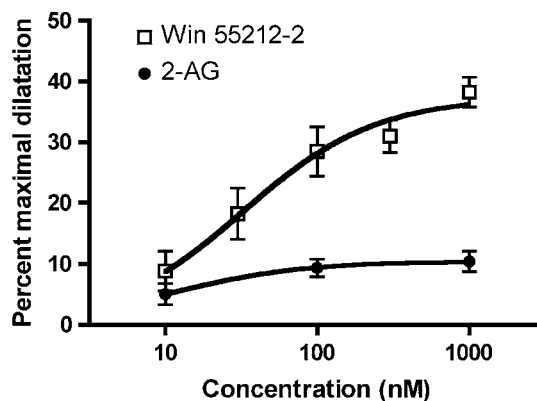
#### Materials

Buffers and salts were purchased from Sigma Chemical Company (St. Louis, MO, USA). U-46619, URB597, URB754, AM1172 and 2-AG were purchased from Cayman Chemical Company (Ann Arbor, MI, USA). Win 55212-2 was purchased from Sigma Chemical Company; AEA and VDM11 were purchased from Tocris Cookson (Ellisville, MO, USA); and rimonabant was obtained from the National Institute on Drug Abuse Drug Inventory Supply Program. 3-(Decylthio)-1,1,1-trifluoropropan-2-one (DETFP) was synthesized using previously described methods (Wheelock *et al.*, 2001; Wadkins *et al.*, 2007).

## Results

#### Exogenously added 2-AG produces modest cerebrovasodilatation

In agreement with previous studies (Gebremedhin *et al.*, 1999; Rademacher *et al.*, 2005), incubation of 5-HT-constricted, endothelium-denuded rat MCA with the CB receptor agonist Win 55212-2 resulted in significant vasodilatation (Figure 1). The EC<sub>50</sub> value for Win 55212-2 was 33 nM (95% confidence interval: 28–38 nM) and the *E*<sub>max</sub> is 37% (36–39%) of the increase in diameter produced by nimodipine/Ca<sup>2+</sup>- buffer. While 2-AG also produced significant dilatation of the MCA at nanomolar concentrations, the *E*<sub>max</sub> for 2-AG was only 10.5% (10.4–10.5%) of the maximal dilatation. To examine the question of the efficacy of 2-AG, we determined whether it can also act as an antagonist. A fully efficacious concentration of Win 55212-2 (1 μM) produced approximately 25% of the maximum diameter; the combination of 1 μM 2-AG and 1 μM Win 55212-2 was not significantly different from that produced by Win 55212-2 alone (Supplementary Figure 1), indicating that 2-AG is not acting as a partial agonist/antagonist. The vasodilatation produced by 100 nM 2-AG was significantly



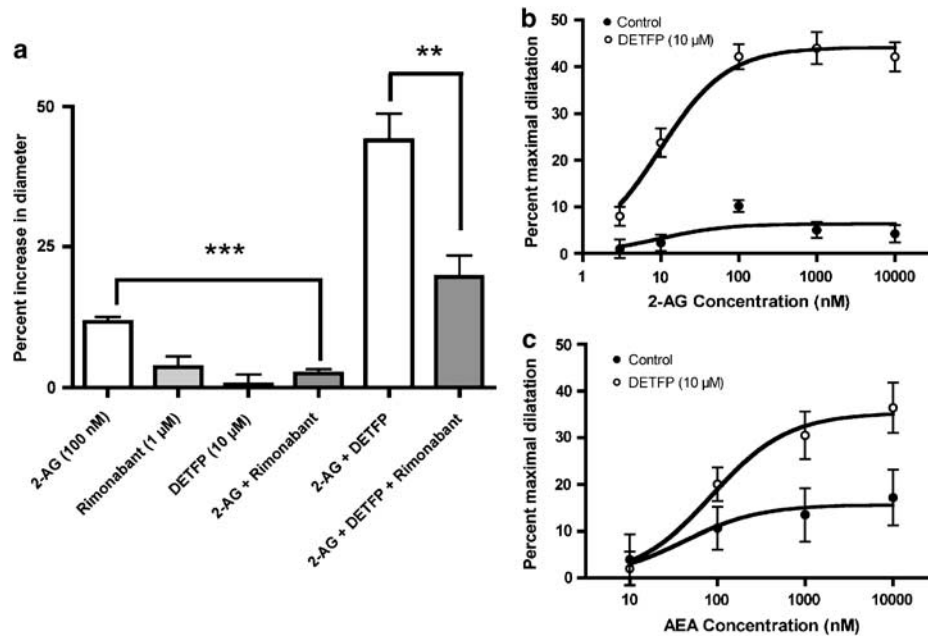
**Figure 1** Comparison of the vasodilatory effects of Win 55212-2 and 2-AG on endothelium-denuded, 5-HT (1 μM) constricted MCA. Vasodilatation was elicited by Win 55212-2 or 2-AG. Values shown are the means and vertical lines represent the s.e.m.; *n* = 5 (Win 55212-2) and *n* = 3 (2-AG). Lines shown are the least squares best fit of the data to a single site, log concentration effect curve using the equation supplied by Prism (GraphPad). 2-AG, 2-arachidonoylglycerol; MCA, middle cerebral artery.

reduced in the presence of the selective CB<sub>1</sub> receptor antagonist, rimonabant (1 μM) (Figure 2a).

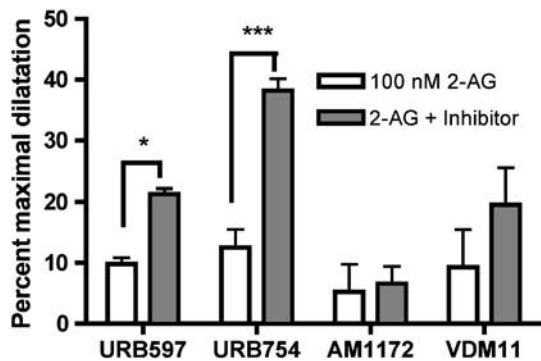
#### Cerebrovasodilatation produced by 2-AG is enhanced by inhibitors of its catabolism

DETFP is a nonselective inhibitor of mammalian carboxyl-esterases (Wheelock *et al.*, 2001; Wadkins *et al.*, 2007) and is also a potent inhibitor of fatty acid amide hydrolase (FAAH) and monoacylglycerol lipase (MGL) (CJ Hillard, unpublished data). All of these enzymes can hydrolyse 2-AG. Coincubation of the MCA with DETFP (10 μM) produced a large increase in the vasodilatory efficacy of 2-AG (Figures 2a and b). The EC<sub>50</sub> value for 2-AG was not changed significantly in the presence of DETFP. DETFP also increased the efficacy of AEA to produce vasodilatation of the MCA (Figure 2c), but DETFP had no effect on the vasodilatation produced by 30 nM Win 55212-2 (22 ± 3% in the presence of dimethyl sulphoxide vehicle and 23 ± 1% in the presence of 10 μM DETFP). The vasodilatation produced by 2-AG in the presence of DETFP was significantly reduced, but not completely inhibited, by coincubation with 1 μM rimonabant (Figure 2a).

We examined the effects of several other inhibitors of endocannabinoid inactivation on the vasodilatory effects of 2-AG (Figure 3). The FAAH inhibitor, URB597 (1 μM) produced a significant increase in the maximal dilatation produced by 2-AG; however, this inhibitor was not as effective as DETFP. URB754 (10 μM) increased the per cent maximal dilatation in response to 2-AG to greater than 40%, which is comparable to the effect of DETFP. Two inhibitors of cellular accumulation of anandamide, AM1172 (Fegley *et al.*, 2004) and VDM11 (De Petrocellis *et al.*, 2000), did not affect 2-AG-mediated vasodilatation when added 5–10 min prior to the addition of 2-AG. Since VDM-11 is also a substrate for FAAH, we examined the effect of simultaneous addition of VDM-11 and 2-AG. VDM-11 produced a small, but



**Figure 2** Effects of DETFP on the vasodilatation produced by 2-AG and AEA. Endothelium denuded MCA were constricted with 1  $\mu$ M 5-HT. (a) 100 nM 2-AG was added in the presence or absence of rimonabant (1  $\mu$ M) and DETFP (10  $\mu$ M) as indicated. Each bar is the mean of 3–7 determinations, lines represent s.e.m. \*\*A significant difference between the groups indicated with  $P < 0.01$ ; \*\*\*A significant difference between the groups indicated with  $P < 0.001$ . Although not indicated, there were also significant differences between 2-AG and 2-AG + DETFP and between 2-AG + rimonabant and 2-AG + DETFP + rimonabant. (b) Vasodilatation was elicited by 2-AG with DMSO vehicle ( $n = 4$ ) or in the presence of 10  $\mu$ M DETFP ( $n = 4$ ). (c) Vasodilatation was elicited by AEA with DMSO vehicle ( $n = 3$ ) or in the presence of 10  $\mu$ M DETFP ( $n = 4$ ). Values shown are the means and vertical lines represent the s.e.m. 2-AG, 2-arachidonoylglycerol; AEA, *N*-arachidonylethanolamine; DETFP, 3-(decylthio)-1,1,1-trifluoropropan-2-one; DMSO, dimethyl sulphoxide; MCA, middle cerebral artery.



**Figure 3** Effects of inhibitors on the vasodilatation produced by 2-AG. Endothelium denuded MCA were constricted with 1  $\mu$ M 5-HT. 2-AG was added alone and the per cent maximal dilation determined; after washing and return of the diameter to resting levels, the inhibitor was added (10  $\mu$ M) for 5–10 min followed by the addition of 100 nM 2-AG. Each bar is the mean of three determinations; vertical lines represent s.e.m. \*A significant difference between the groups indicated with  $P < 0.05$ ; \*\*\*A significant difference between the groups indicated with  $P < 0.005$ . 2-AG, 2-arachidonoylglycerol; MCA, middle cerebral artery.

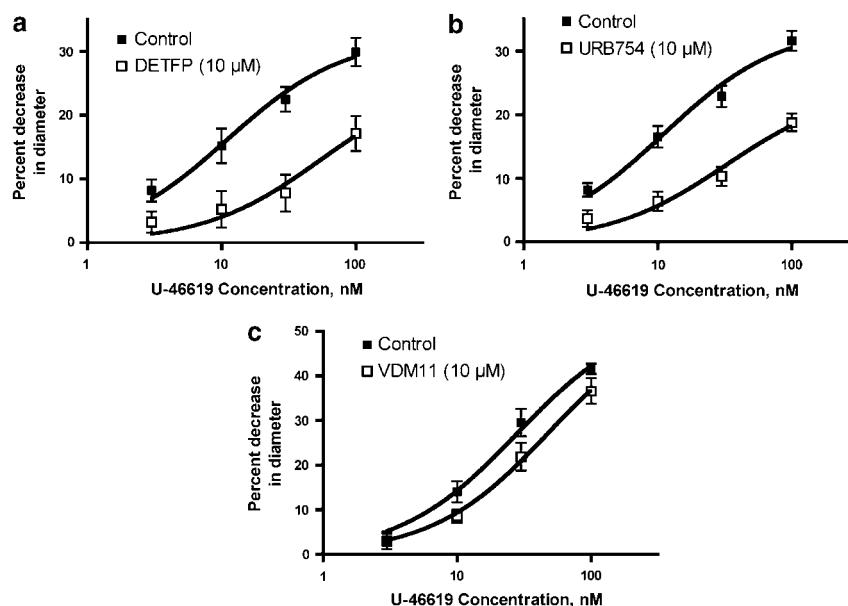
insignificant increase in the effect of 2-AG. None of the inhibitors examined significantly affected the diameter of 5-HT-constricted MCA when added alone (data not shown).

These data indicate that 2-AG is hydrolysed by the MCA preparation to arachidonic acid. We have examined the

effects of the same concentration range of arachidonic acid to explore the contribution of this metabolite to the effects of exogenous 2-AG. Arachidonic acid did not produce a significant vasodilatation of the 5-HT-constricted MCA (Supplementary Figure 2a). However, arachidonic acid produced a concentration-related decrease in MCA diameter; the  $E_{max}$  obtained was 13% (12.5–14%) and the  $EC_{50}$  value was 4.0 nM (2.8–6.0 nM) (Supplementary Figure 2b).

#### *U-46616 cerebrovasoconstriction is blunted by inhibitors of 2-AG catabolism*

U-46619 produced concentration-dependent constriction of perfused, pressurized and endothelium-denuded MCA (Figure 4). The  $EC_{50}$  values for U-46619 varied between 11 and 28 nM and  $E_{max}$  values were between 30 and 65% of predrug MCA diameter (Table 1). Two-way analyses of variance were used to determine whether the presence of inhibitor significantly altered the response of the MCA to U-46619. Addition of dimethyl sulphoxide vehicle to the bath (in the amount used to deliver each of the inhibitors) did not affect the response to U-46619 (Table 1). Three of the inhibitors studied produced significant effects on the U-46619 contractile effect: DETFP, URB754 and VDM11. DETFP (10  $\mu$ M) and URB754 (10  $\mu$ M) produced significant increases in the  $EC_{50}$  value for U-46619 (Figures 4a and b, Table 1). Both compounds also reduced the  $E_{max}$  values for U-46619, although this change was significant only for URB754.



**Figure 4** Effects of inhibitors of 2-AG catabolism on the constrictor response to U-46619. Rat MCA were denuded and serial concentrations of U-46619 were added in the presence of DMSO (control). After washout and re-equilibration, the inhibitors were added and the U-46619 concentration–response curve was re-determined. The inhibitors used were: DETFP (a; 10 μM;  $n = 4$ ); URB754 (b; 10 μM;  $n = 4$ ) and VDM11 (c; 10 μM;  $n = 5$ ). 2-AG, 2-arachidonoylglycerol; DETFP, 3-(decylthio)-1,1,1-trifluoropropan-2-one; DMSO, dimethyl sulphoxide; MCA, middle cerebral artery.

**Table 1**  $EC_{50}$  and  $E_{max}$  values for U-46619-induced constriction of MCA after treatment with inhibitors of endocannabinoid inactivation

Inhibitor	$EC_{50}$ , nM (95% CI)		$E_{max}$ <sup>a</sup> (95% CI)		P-value for the effect of inhibitor <sup>b</sup>
	Control	+ Inhibitor	Control	+ Inhibitor	
DMSO (1 μM; $n = 6$ ) <sup>c</sup>	26.6 (15–47)	21.4 (8–59)	58.3 (46–71)	53.8 (34–74)	0.71
DETFP (10 μM; $n = 4$ )	11.1 (10–13)	54.3 (33–90)	32.4 (31–34)	25.9 (20–32)	<0.0001
URB754 (10 μM; $n = 4$ )	11.1 (9–13)	32.3 (23–46)	33.9 (32–36)	24.3 (21–28)	<0.0001
URB597 (1 μM; $n = 4$ )	21.3 (16–28)	20.3 (16–25)	30.2 (27–33)	26.6 (25–29)	0.12
VDM11 (10 μM; $n = 5$ )	27.8 (17–47)	48.4 (25–95)	54.0 (43–65)	54.7 (38–72)	0.008
AM1172 (10 μM; $n = 6$ )	22.0 (14–34)	18.2 (9–36)	63.5 (54–73)	57.7 (45–71)	0.77

Abbreviations: ANOVA, analyses of variance; DETFP, 3-(decylthio)-1,1,1-trifluoropropan-2-one; DMSO, dimethyl sulphoxide; MCA, middle cerebral artery.

<sup>a</sup>Units are per cent decrease in diameter.

<sup>b</sup>P-values were determined using 2-way ANOVA with U-46619 concentration and the presence of inhibitor as the two factors; in all cases the effect of U-46619 concentration was significant at  $P < 0.0001$  and there were no significant interactions. The P-values for the 'presence of inhibitor' factor are shown.

<sup>c</sup>DMSO equivalent to that added with each inhibitor.

Two-way analyses of variance indicated that VDM11 also had a significant effect on the response to U-46619; however, VDM11 did not significantly affect the  $EC_{50}$  for U-46619 as the 95% confidence intervals for the composite  $EC_{50}$  values in the absence and presence of VDM11 were overlapping (Figure 4c and Table 1). Neither the FAAH inhibitor, URB597, nor the anandamide accumulation inhibitor, AM1172, affected the contractile response to U-46619 in rat MCA (Table 1).

## Discussion and conclusions

These data support and extend our previous observations that  $CB_1$  receptor agonists are potent and efficacious dilators of the rat MCA (Gebremedhin *et al.*, 1999; Rademacher *et al.*,

2005). These results demonstrate that the isolated MCA expresses the enzymatic machinery for the efficient degradation of 2-AG. Finally, these findings provide further support for the hypothesis that activation of thromboxane receptors in the MCA mobilizes the endocannabinoids and that these lipid modulators act via  $CB_1$  receptor activation to dampen thromboxane-mediated vasoconstriction. These data suggest that a function of endocannabinoid/ $CB_1$  receptor signalling in the MCA is to prevent excessive vasoconstriction during thrombosis.

These studies were carried out with the endothelium removed from the MCA. The endothelial cells of the MCA express the  $CB_1$  cannabinoid receptor (Chen *et al.*, 2000; Rademacher *et al.*, 2005) and FAAH (Rademacher *et al.*, 2005) and are likely important contributors to the overall effects of endogenous and exogenous endocannabinoids. In our

earlier study (Rademacher *et al.*, 2005), we found that the effect of U-46619 on endocannabinoid content was not affected by the presence of the endothelium, suggesting that the vascular smooth muscle is the primary site of endocannabinoid production in the MCA. However, the endothelium could play a role in the catabolism of the endocannabinoids. In addition, the endothelial cells are likely to exert a modulatory influence on the vasodilatory effects of the endocannabinoids, if not directly via CB<sub>1</sub> receptor activation, then indirectly via the release of non-endocannabinoid mediators of vascular tone. Therefore, while the present studies demonstrate that the vascular smooth muscle catabolizes the endocannabinoids, it is likely that the MCA endothelial cells also contribute to this process.

The EC<sub>50</sub> value for the cannabinoid receptor agonist, Win 55212-2, to produce vasodilatation of the MCA is 30 nM, which correlates remarkably well with the IC<sub>50</sub> value for the same agonist to inhibit the opening of L-type calcium channels in isolated cat arterial smooth muscle cells (Gebremedhin *et al.*, 1999). In previous studies, we have shown that the vasodilatation produced by Win 55212-2 was inhibited by coinubation with the CB<sub>1</sub> receptor-selective antagonist, rimonabant (SR141716) (Gebremedhin *et al.*, 1999). We have extended those findings in this study and demonstrate that two endocannabinoids 2-AG and AEA also vasodilate rat MCA at nanomolar concentrations. However,  $E_{\max}$  for 2-AG was less than one-quarter of the  $E_{\max}$  for Win 55212-2 in the same assay. This result is inconsistent with the high efficacy of 2-AG in assays of CB<sub>1</sub> receptor-induced GDP/GTP exchange (Hillard, 2000a) and calcium mobilization (Sugiura *et al.*, 1997), where 2-AG acts as a full agonist of the CB<sub>1</sub> receptor. Interestingly, the  $E_{\max}$  for 2-AG was also significantly less than the  $E_{\max}$  for AEA, a ligand that generally acts as a partial agonist of the CB<sub>1</sub> receptor (Kearn *et al.*, 1999). Since 2-AG did not reduce the efficacy of a maximally effective concentration of Win 55212-2, it is not likely to be acting as a partial agonist of the CB<sub>1</sub> receptor in the MCA.

A more likely explanation for these data is that exogenously added 2-AG is rapidly inactivated by the isolated MCA which results in a reduction in its efficacy. This hypothesis is supported by recent data from Ho and Randall (2007) demonstrating significant metabolism of the endocannabinoids in small mesenteric arteries. However, our data suggest that the low efficacy of 2-AG results from more than just catabolic inactivation. If this were the case, then high concentrations of 2-AG should saturate the catabolic processes and result in higher efficacy. If anything, very high concentrations of 2-AG either had no effect or induced vasoconstriction (data not shown). Instead, it is likely that 2-AG acts as both an agonist of the CB<sub>1</sub> receptor and as a precursor for arachidonic acid, which, in turn, is converted to vasoconstrictor eicosanoids, such as 20-hydroxyeicosatetraenoic acid (Roman *et al.*, 2006). In fact, we have found that arachidonic acid produces significant vasoconstriction of the endothelium-denuded MCA, but does not produce significant vasodilatation. Therefore, we hypothesize that as greater concentrations of 2-AG are added and hydrolysed to arachidonic acid, greater amounts of an arachidonate-

derived vasoconstrictor are produced which acts as a functional antagonist of intact 2-AG.

A primary mechanism of 2-AG inactivation is hydrolysis to glycerol and arachidonic acid, since neither of these metabolites bind to the CB<sub>1</sub> receptor (Bisogno *et al.*, 1997; Dinh *et al.*, 2002). 2-AG hydrolysis can be accomplished through several enzymatic pathways, including MGL (Dinh *et al.*, 2004), FAAH (Di Marzo *et al.*, 1999b), and other uncharacterized esterases (Nithipatikom *et al.*, 2005). In the brain, the primary mechanism for the catabolism of AEA is hydrolysis by FAAH. In fact, there is no detectable AEA hydrolysis in membranes prepared from FAAH<sup>-/-</sup> mice (Patel *et al.*, 2005).

A series of trifluoromethylketone-containing compounds have been developed and found to act as a tight binding, reversible and potent (nM) inhibitors of mammalian carboxyl-esterases (Wheelock *et al.*, 2001; Wadkins *et al.*, 2007). Nithipatikom *et al.* (2005) have reported that members of this series are potent inhibitors of exogenous and endogenous 2-AG inactivation in cells. We have studied these compounds *in vitro* and found that the decyl derivative, DETFP, is a potent inhibitor of FAAH and the hydrolysis of 2-acylglycerols by brain cytosolic preparations with IC<sub>50</sub> values of 5 and 800 nM, respectively.

Our initial approach to examine the role of catabolism in the low efficacy of 2-AG to vasodilate the MCA was to use the trifluoromethylketone series of inhibitors, since they inhibit all of the known pathways of 2-AG metabolism. The coinubation of the MCA with DETFP resulted in a large increase in the  $E_{\max}$  for 2-AG to produce vasodilatation. These data are consistent with the hypothesis that the majority of exogenously added 2-AG is hydrolysed by the MCA preparation and indicate that DETFP inhibits that metabolism. DETFP did not affect the EC<sub>50</sub> value for 2-AG, suggesting that the pool of 2-AG that is not metabolized acts via the CB<sub>1</sub> receptor to produce vasodilatation. This conclusion is supported by the complete blockade by rimonabant of 2-AG vasodilatation in the absence of DETFP. DETFP also increased the  $E_{\max}$  for AEA in the MCA. Since AEA is not a substrate for MGL (Goparaju *et al.*, 1999) and does not contain an ester moiety, it is possible that the effect of DETFP on AEA  $E_{\max}$  is via inhibition of FAAH. This hypothesis is in agreement with our finding that DETFP is a very potent inhibitor of FAAH (IC<sub>50</sub> value of 5 nM). Finally, DETFP did not affect Win 55212-2-mediated vasodilatation of the MCA, which provides evidence that DETFP does not affect the signalling cascade initiated by CB<sub>1</sub> receptor activation. Interestingly, the CB<sub>1</sub> receptor antagonist, rimonabant, did not completely inhibit the vasodilatory effect of 2-AG in the presence of DETFP. These data suggest that, at high concentrations, 2-AG either acts itself via a non-CB<sub>1</sub> receptor mechanism to produce vasodilatation or is converted to a metabolite that vasodilates via another mechanism. With regard to the second of these possibilities, we would expect DETFP to completely inhibit 2-AG conversion to arachidonic acid at this concentration, so it is not likely that arachidonic acid is an intermediate in the production of active metabolites.

We have identified two other inhibitors that also increase the  $E_{\max}$  for 2-AG to produce vasodilatation: URB754 and

URB597. We have also examined the effects of URB754 on the ability of 2-AG to produce vasodilatation of the MCA. URB754 was originally described as an inhibitor of MGL (Makara *et al.*, 2005), although the inhibition was subsequently attributed to a contaminant (Makara *et al.*, 2007). Others report that the inhibitor has no effect on the hydrolysis or signalling capacity of 2-AG in brain (Saario *et al.*, 2006; Vandevoorde *et al.*, 2007). In our assay of 2-acylglycerol hydrolysis by brain cytosol (Rademacher *et al.*, 2007), URB754 is an effective although not very potent inhibitor, exhibiting an  $IC_{50}$  value of  $42 \mu\text{M}$  and inhibiting approximately 90% of 2-oleoylglycerol hydrolysis. Based upon its relatively potent effect in the current study (URB754 produced a threefold increase in the maximal dilation by 2-AG at a concentration of  $10 \mu\text{M}$ ), it is possible that a primary mechanism for the hydrolysis of 2-AG in the MCA is an as-yet uncharacterized hydrolase that can be inhibited by URB754 and DETFP. While further studies are needed to characterize the second pathway, the relatively high potency of URB754 compared to its potency to inhibit MGL in brain suggests that the target of URB754 is not MGL. It is our current hypothesis that URB754 and DETFP, which have very different structures, inhibit a common, as yet uncharacterized, hydrolase.

URB597 is an inhibitor of FAAH that has very little effect on 2-acylglycerol hydrolysis in brain cytosol (Saario *et al.*, 2004) or in membranes from FAAH<sup>-/-</sup> mice (CJ Hillard, unpublished data). Although neither pharmacologic inhibition nor complete ablation of FAAH affect total brain 2-AG levels (Patel *et al.*, 2005), there is clear evidence from *in vitro* studies that 2-AG is a substrate for FAAH (Di Marzo *et al.*, 1999a). Therefore, the URB597 data could reflect hydrolysis of 2-AG by FAAH in the MCA. However, two other inhibitors of FAAH, AM1172 (CJ Hillard, unpublished data) and VDM11 (Vandevoorde and Fowler, 2005) did not significantly enhance 2-AG vasodilatory efficacy. Therefore, it is possible that the effect of URB597 on 2-AG is not due to inhibition of FAAH. We have also examined the effects of two inhibitors of AEA accumulation in neurons, AM1174 (Fegley *et al.*, 2004) and VDM11 (De Petrocellis *et al.*, 2000) on 2-AG efficacy. Neither of these inhibitors produced a significant effect on 2-AG-mediated vasodilatation, which indicates that re-uptake processes do not affect the ability of 2-AG to reach its metabolic enzymes in the MCA.

In the second set of studies, we examined the effects of the inhibitors on the response of the MCA to the vasoconstrictor, U-46619. Our earlier studies demonstrated that U-46619 increases the MCA contents of both 2-AG and AEA and that the endocannabinoids oppose U-46619-induced vasoconstriction via activation of the CB<sub>1</sub> receptor (Rademacher *et al.*, 2005). In particular, we found that rimonabant and AM251 enhanced U-46619 constrictions. A prediction of the hypothesis derived from these earlier studies is that inhibition of endocannabinoid hydrolysis should reduce U-46619 vasoconstrictions. Our results are consistent with this prediction: both DETFP and URB754 produced a significant rightward shift in the U-46619 constriction curve. This shift was reflected in an increase in the  $EC_{50}$  value for U-46619. Although URB597 potentiated the vasodilatory effect of exogenous 2-AG, it did not affect the response to U-46619.

These data indicate that although FAAH is present in the MCA, it does not play a significant role in the regulation of endocannabinoid that is synthesized in response to U-46619. Interestingly, VDM11, an uptake inhibitor that also inhibits FAAH and MGL (Vandevoorde and Fowler, 2005), also produced a significant change in the U-46619 response as revealed by two-way analyses of variance. However, although the  $EC_{50}$  for U-46619 was increased in the presence of VDM11, it was not significantly different from the control value. The significance of these data is not clear at present; however, the lack of effect of either AM1172 or URB597 suggests that it is the ability of VDM11 to inhibit 2-oleoylglycerol hydrolysis that is responsible for its effect.

These findings support the hypothesis that vascular smooth muscle cells of the cerebral circulation use endocannabinoid signalling to produce local changes in vessel tone. In addition to the expression of the CB<sub>1</sub> cannabinoid receptor (Gebremedhin *et al.*, 1999) and processes for the synthesis of the endocannabinoids (Rademacher *et al.*, 2005), the current studies demonstrate that the MCA also has intrinsic processes for the catabolism of the endocannabinoids. Furthermore, because thromboxane A<sub>2</sub> is released by activated platelets and contributes to cerebral vasospasm (von Holst *et al.*, 1982), our data suggest that the endocannabinoid signalling system plays a very important and significant role in the negative regulation of vasoconstriction during thrombosis. As such, inhibitors of endocannabinoid inactivation could represent a novel class of agents for the treatment of thrombotic disorders, particularly those occurring within the cerebral circulation.

## Acknowledgements

This work was supported by the National Institutes of Health Grant R01-NS41314. Partial support was provided by NIEHS Grant R37 ES02710 and the NIEHS Superfund Basic Research Program Grant P42 ES04699 (BDH). The authors acknowledge the technical assistance of Amanda M Savoie.

## Conflict of interest

The authors state no conflict of interest.

## References

- Ashton JC, Zheng Y, Liu P, Darlington CL, Smith PF (2004). Immunohistochemical characterisation and localisation of cannabinoid CB<sub>1</sub> receptor protein in the rat vestibular nucleus complex and the effects of unilateral vestibular deafferentation. *Brain Res* 1021: 264–271.
- Bisogno T, Sepe N, Melck D, Maurelli S, De Petrocellis L, Di Marzo V (1997). Biosynthesis, release and degradation of the novel endogenous cannabimimetic metabolite 2-arachidonoylglycerol in mouse neuroblastoma cells. *Biochem J* 322: 671–677.
- Chen Y, McCarron RM, Ohara Y, Bemby J, Azzam N, Lenz FA *et al.* (2000). Human brain capillary endothelium: 2-arachidonoglycerol (endocannabinoid) interacts with endothelin-1. *Circ Res* 87: 323–327.
- De Petrocellis L, Bisogno T, Davis JB, Pertwee RG, Di Marzo V (2000). Overlap between the ligand recognition properties of the anandamide transporter and the VR1 vanilloid receptor: inhibitors of

- anandamide uptake with negligible capsaicin-like activity. *FEBS Lett* **483**: 52–56.
- Dewey WL (1986). Cannabinoid pharmacology. *Pharmacol Rev* **38**: 151–178.
- Di Marzo V, Bisogno T, De Petrocellis L, Melck D, Orlando P, Wagner JA *et al.* (1999a). Biosynthesis and inactivation of the endocannabinoid 2-arachidonoylglycerol in circulating and tumoral macrophages. *Eur J Biochem* **264**: 258–267.
- Di Marzo V, De Petrocellis L, Bisogno T, Melck D (1999b). Metabolism of anandamide and 2-arachidonoylglycerol: an historical overview and some recent developments. *Lipids* **34** (Suppl): S319–S325.
- Dinh TP, Carpenter D, Leslie FM, Freund TF, Katona I, Sensi SL *et al.* (2002). Brain monoglyceride lipase participating in endocannabinoid inactivation. *Proc Natl Acad Sci USA* **99**: 10819–10824.
- Dinh TP, Kathuria S, Piomelli D (2004). RNA interference suggests a primary role for monoacylglycerol lipase in the degradation of the endocannabinoid 2-arachidonoylglycerol. *Mol Pharmacol* **66**: 1260–1264.
- Ellis EF, Moore SF, Willoughby KA (1995). Anandamide and delta 9-THC dilation of cerebral arterioles is blocked by indomethacin. *Am J Physiol* **269**: H1859–H1864.
- Fegley D, Kathuria S, Mercier R, Li C, Goutopoulos A, Makriyannis A *et al.* (2004). Anandamide transport is independent of fatty-acid amide hydrolase activity and is blocked by the hydrolysis-resistant inhibitor AM1172. *Proc Natl Acad Sci USA* **101**: 8756–8761.
- Gauthier-Rein KM, Bizub DM, Lombard JH, Rusch NJ (1997). Hypoxia-induced hyperpolarization is not associated with vasodilation of bovine coronary resistance arteries. *Am J Physiol* **272**: H1462–H1469.
- Gebredemhin D, Lange AR, Campbell WB, Hillard CJ, Harder DR (1999). Cannabinoid CB1 receptor of cat cerebral arterial muscle functions to inhibit L-type Ca<sup>2+</sup> channel current. *Am J Physiol* **276**: H2085–H2093.
- Goparaju SK, Ueda N, Taniguchi K, Yamamoto S (1999). Enzymes of porcine brain hydrolyzing 2-arachidonoylglycerol, an endogenous ligand of cannabinoid receptors. *Biochem Pharmacol* **57**: 417–423.
- Hillard CJ (2000a). Biochemistry and pharmacology of the endocannabinoids arachidonyl ethanolamide and 2-arachidonoylglycerol. *Prostaglandins Other Lipid Mediat* **61**: 3–18.
- Hillard CJ (2000b). Endocannabinoids and vascular function. *J Pharmacol Exp Ther* **294**: 1–6.
- Ho WS, Randall MD (2007). Endothelium-dependent metabolism by endocannabinoid hydrolases and cyclooxygenases limits vasorelaxation to anandamide and 2-arachidonoylglycerol. *Br J Pharmacol* **150**: 641–651.
- Kearn CS, Greenberg MJ, DiCamelli R, Kurzawa K, Hillard CJ (1999). Relationships between ligand affinities for the cerebellar cannabinoid receptor CB1 and the induction of GDP/GTP exchange. *J Neurochem* **72**: 2379–2387.
- Makara JK, Mor M, Fegley D, Szabo SI, Kathuria S, Astarita G *et al.* (2005). Selective inhibition of 2-AG hydrolysis enhances endocannabinoid signaling in hippocampus. *Nat Neurosci* **8**: 1139–1141.
- Makara JK, Mor M, Fegley D, Szabo SI, Kathuria S, Astarita G *et al.* (2007). Selective inhibition of 2-AG hydrolysis enhances endocannabinoid signaling in hippocampus (Corrigendum). *Nat Neurosci* **10**: 134.
- Mathew R, Wilson W (1993). Acute changes in cerebral blood flow after smoking marijuana. *Life Sci* **52**: 757–767.
- Mathew RJ, Wilson WH, Davis R (2003). Postural syncope after marijuana: a transcranial Doppler study of the hemodynamics. *Pharmacol Biochem Behav* **75**: 309–318.
- Mathew RJ, Wilson WH, Humphreys D, Lowe JV, Wiethe KE (1992a). Middle cerebral artery velocity during upright posture after marijuana smoking. *Acta Psychiatr Scand* **86**: 173–178.
- Mathew RJ, Wilson WH, Humphreys DE, Lowe JV, Wiethe KE (1992b). Regional cerebral blood flow after marijuana smoking. *J Cereb Blood Flow Metab* **12**: 750–758.
- Nithipatikom K, Endsley MP, Isbell MA, Wheelock CE, Hammock BD, Campbell WB (2005). A new class of inhibitors of 2-arachidonoylglycerol hydrolysis and invasion of prostate cancer cells. *Biochem Biophys Res Commun* **332**: 1028–1033.
- Pacher P, Batkai S, Kunos G (2005). Cardiovascular pharmacology of cannabinoids. *Handb Exp Pharmacol* **186**: 599–625.
- Patel S, Carrier EJ, Ho WS, Rademacher DJ, Cunningham S, Reddy DS *et al.* (2005). The postmortal accumulation of brain N-arachidonyl ethanolamine (anandamide) is dependent upon fatty acid amide hydrolase activity. *J Lipid Res* **46**: 342–349.
- Rademacher DJ, Meier SE, Shi L, Ho W-SV, Jarrahan A, Hillard CJ (2007). Effects of acute and repeated restraint stress on endocannabinoid content in the amygdala, ventral striatum and medial prefrontal cortex in mice. *Neuropharmacol* (in press).
- Rademacher DJ, Patel S, Ho WS, Savoie AM, Rusch NJ, Gauthier KM *et al.* (2005). U-46619 but not serotonin increases endocannabinoid content in middle cerebral artery: evidence for functional relevance. *Am J Physiol Heart Circ Physiol* **288**: H2694–H2701.
- Roman RJ, Renic M, Dunn KM, Takeuchi K, Haccin-Bey L (2006). Evidence that 20-HETE contributes to the development of acute and delayed cerebral vasospasm. *Neuro Res* **28**: 738–749.
- Saario SM, Palomaki V, Lehtonen M, Nevalainen T, Jarvinen T, Laitinen JT (2006). URB754 has no effect on the hydrolysis or signaling capacity of 2-AG in the rat brain. *Chem Biol* **13**: 811–814.
- Saario SM, Savinainen JR, Laitinen JT, Jarvinen T, Niemi R (2004). Monoglyceride lipase-like enzymatic activity is responsible for hydrolysis of 2-arachidonoylglycerol in rat cerebellar membranes. *Biochem Pharmacol* **67**: 1381–1387.
- Sugiura T, Kodaka T, Kondo S, Nakane S, Kondo H, Waku K *et al.* (1997). Is the cannabinoid CB1 receptor a 2-arachidonoylglycerol receptor? Structural requirements for triggering a Ca<sup>2+</sup> transient in NG108-15 cells. *J Biochem (Tokyo)* **122**: 890–895.
- Vandevoorde S, Fowler CJ (2005). Inhibition of fatty acid amide hydrolase and monoacylglycerol lipase by the anandamide uptake inhibitor VDM11: evidence that VDM11 acts as an FAAH substrate. *Br J Pharmacol* **145**: 885–893.
- Vandevoorde S, Jonsson KO, Labar G, Persson E, Lambert DM, Fowler CJ (2007). Lack of selectivity of URB602 for 2-oleoylglycerol compared to anandamide hydrolysis *in vitro*. *Br J Pharmacol* **150**: 186–191.
- von Holst H, Granstrom E, Hammarstrom S, Samuelsson B, Steiner L (1982). Effect of leucotrienes C4, D4, prostacyclin and thromboxane A2 on isolated human cerebral arteries. *Acta Neurochir (Wien)* **62**: 177–185.
- Wadkins RM, Hyatt JL, Edwards CC, Tsurkan L, Redinbo MR, Wheelock CE *et al.* (2007). Analysis of mammalian carboxylesterase inhibition by trifluoromethylketone-containing compounds. *Mol Pharmacol* **71**: 713–723.
- Wagner JA, Jarai Z, Batkai S, Kunos G (2001). Hemodynamic effects of cannabinoids: coronary and cerebral vasodilation mediated by cannabinoid CB(1) receptors. *Eur J Pharmacol* **423**: 203–210.
- Wheelock CE, Severson TF, Hammock BD (2001). Synthesis of new carboxylesterase inhibitors and evaluation of potency and water solubility. *Chem Res Toxicol* **14**: 1563–1572.

Supplementary Information accompanies the paper on British Journal of Pharmacology website (<http://www.nature.com/bjp>)