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Anandamide acts as a vasodilator of dural blood vessels *in vivo* by activating TRPV1 receptors

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1 Migraine pathophysiology is believed to involve the release of neuropeptides *via* the activation of trigeminal afferents that innervate the cranial vasculature.

2 Anandamide, the endogenous ligand to the cannabinoid receptor, is able to inhibit neurogenic dural vasodilatation, calcitonin gene-related peptide (CGRP)-induced and nitric oxide-induced dural vessel dilation in the intravital microscopy model. In an *in vitro* setting anandamide is also able to activate the vanilloid type 1 (TRPV1) receptor and cause vasodilation, *via* the release of CGRP.

3 In this study we used intravital microscopy to study whether anandamide behaves as a TRPV1 receptor agonist in the trigeminovascular system. We examined if anandamide-induced dural vasodilation involves CGRP release that can be reversed by the CGRP receptor antagonist, $CGRP_{8-37}$, and whether like capsaicin the anandamide effect could be reversed by the TRPV1 receptor antagonist, capsazepine.

4 Anandamide 1 ($19\pm9\%$, $n=12$), 3 ($29\pm5\%$, $n=37$), 5 ($74\pm7\%$, $n=13$) and 10 mg kg^{-1} ($89\pm18\%$, $n=6$) was able to cause a dose-dependent increase in dural vessel diameter. Capsazepine (3 mg kg^{-1}, $t_5=6.2$, $P<0.05$) and $CGRP_{8-37}$ (300 μg kg^{-1}, $t_6=11.1$, $P<0.05$) attenuated the anandamide-induced dural vessel dilation when compared to control (Student's paired *t*-test). AM251 (3 mg kg^{-1}), a cannabinoid type 1 (CB_1) receptor antagonist, was unable to reverse this anandamide-induced dilation.

5 The study demonstrates that anandamide acts as a TRPV1 receptor agonist in the trigeminovascular system, activating TRPV1 receptors that promote CGRP release and cause vasodilation independent of any action at the CB_1 receptor. Anandamide has been shown previously to inhibit trigeminovascular neurons and prevent vasodilation, through an action at CB_1 receptors.
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Abbreviations: AEA, arachidonylethanolamide; AM-251, *N*-(Piperidin-1-yl)-5-(4-iodophenyl)-1-(2,4-dichlorophenyl)-4-methyl-1*H*-pyrazole-3-carboxamide; CB_1, cannabinoid type 1; CGRP, calcitonin gene-related peptide; NO, nitric oxide; TRPV1, vanilloid type 1

Introduction

The pathophysiology of migraine is thought to involve the activation of trigeminal afferents (Goadsby *et al*., 2002). Trigeminal sensory nerve fibres that innervate the cranial vasculature contain the neuropeptides calcitonin gene-related peptide (CGRP), substance P and neurokinin A (Uddman & Edvinsson, 1989). Stimulation of dural sites is painful in humans (Ray & Wolff, 1940), particularly and reliably if electrical stimulation is employed (Wolff, 1948). Experimental animal models exploring the effects of nociceptive activation of the trigeminovascular system have been useful in beginning to understand the pathophysiology of migraine (De Vries *et al*., 1999).

It has been observed that CGRP and neurokinin A levels are increased in the blood plasma taken from patients suffering a migraine attack (Goadsby *et al*., 1990; Gallai *et al*., 1995). Williamson *et al*. (1997a, b) developed a model using intravital microscopy that allows continual observation and measurement of the diameter of dural blood vessels, while activating Aδ-trigeminal nerve fibres (Williamson *et al*., 1997a, b). They showed that activation of these trigeminal nerve fibres by electrically stimulating the surface of a closed cranial window caused release of CGRP from the nerve fibres, which resulted in the vasodilation of dural blood vessels (Williamson *et al*., 1997a). This vasodilation could be blocked by the triptans (Williamson *et al*., 1997b, c), serotonin $5\text{-}HT_{1B/1D}$ receptor agonists that are extremely effective acute antimigraine compounds (Ferrari *et al*., 2002), and by a CGRP receptor antagonist (Williamson *et al*., 1997a), with the latter class of compounds seemingly also having antimigraine effects (Olesen *et al*., 2003). Thus, dural intravital microscopy provides a good window into the physiology and pharmacology of the trigeminovascular system.

Arachidonylethanolamide (AEA or anandamide) is believed to be the endogenous ligand to the cannabinoid CB_1 and CB_2 receptors (Matsuda *et al*., 1990; Hoehe *et al*., 1991; Devane *et al*., 1992; Munro *et al*., 1993). The known behavioural effects of anandamide are similar to that of Δ^9-tetrahydrocannabinol, the psychoactive constituent of cannabis, being

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antinociception, catalepsy, hypothermia and depression of motor activity (Dewey, 1986; Crawley *et al*., 1993; Smith *et al*., 1994; Adams *et al*., 1998). We have shown recently, using the model of intravital microscopy, that anandamide is able to attenuate both neurogenic dural vasodilation, CGRP-induced and nitric oxide (NO) induced dural vessel dilation (Akerman *et al*., 2004). This trigeminovascular inhibitory property of anandamide has and this is of its effects within the dural circulation interest in terms of understanding primary headache disorders.

Anandamide has been studied as a potential agonist at the vanilloid type 1 (TRPV1) receptor. Anandamide is structurally related to capsaicin and olvanil (*N*-vanillyl-9-oleamide) and both are TRPV1 agonists, having both an amide group and an aliphatic side chain. Zygmunt *et al*. (1999) hypothesised that the hypotensive effects of anandamide and capsaicin have a common mechanism involving excitation of sensory nerves and consequent release of vasodilator peptides. Anandamide appears to induce vasodilation of isolated arteries by activating TRPV1 receptors on perivascular sensory nerves and causing the release of CGRP. This vasodilation is inhibited by a CGRP receptor blocker, $CGRP_{8-37}$, and a TRPV1 receptor antagonist, capsazepine, but not by the CB_1 antagonist, SR141716A, indicating that the cannabinoid receptor plays no part in this interaction (Zygmunt *et al*., 1999). More recently, in support of this hypothesis, it was shown that anandamide also activates the TRPV1 receptor on trigeminal ganglion neurons to promote the release of CGRP, an effect reversed by capsazepine (Michele *et al*., 2002). Therefore, it also appears that anandamide might be acting as an agonist in the vanilloid system as well as an agonist in the cannabinoid system.

We have shown previously using intravital microscopy that capsaicin, acting on the TRPV1 receptor, is able to invoke CGRP release from trigeminal nerve endings and cause dural blood vessel dilation (Akerman *et al*., 2003). It was therefore the aim of this study to determine if anandamide was able to activate trigeminal nerves and cause dural vessel dilation in this model in which it is able to inhibit the activation of trigeminal neurons. We also looked at whether this activation was *via* the cannabinoid system or the vanilloid system, and if it was *via* the vanilloid system, if it was *via* a similar mechanism of action as capsaicin.

Methods

Surgical preparation

All experiments were conducted under U.K. Home Office, Animals (Scientific Procedures) Act (1986). Male Sprague–Dawley rats (180–385 g) were anaesthetised throughout the experiments with sodium pentobarbitone (60 mg kg^{-1} i.p. and then 18 mg^{-1} kg^{-1} h i.v. infusion). The left femoral artery and vein were cannulated for blood pressure recording and intravenous infusion of anaesthetic and test compounds, respectively. Temperature was maintained throughout using a homeothermic blanket system. The rats were placed in a stereotaxic frame and ventilated with oxygen-enriched air, 3–5 ml, 60–80 strokes per minute (Small Rodent Ventilator – Model 683, Harvard Instruments, U.K.). End-tidal CO_2 was monitored (Capstar-100, CWE Inc., U.S.A.) and kept between 3.5 and 4.5%, and blood pressure was monitored continually. This allows one to monitor for changes to respiration and blood pressure due to long-term anaesthetic maintenance. The rats were placed in a stereotaxic frame, the skull exposed and the right or left parietal bone thinned by drilling with a saline-cooled drill until the blood vessels of the dura mater were clearly visible through the intact skull.

Intravital microscopy

The cranial window was covered with mineral oil (37°C) and a branch of the middle meningeal artery was viewed using an intravital microscope (Microvision MV2100, U.K.) and the image was displayed on a television monitor. Dural blood vessel diameter was continuously measured using a video dimension analyser (Living Systems Instrumentation, U.S.A.) and displayed with blood pressure on an online data analysis system (CED spike4 v2 software).

Experimental protocols

Effect of anandamide on dural blood vessel diameter Increasing doses of anandamide were given as an intravenous bolus, 1 ($n=12$), 3 ($n=37$), 5 ($n=13$) and 10 mg kg^{-1} ($n=6$) at least 15 min apart, not all animals received every dose.

Effects of CB_1 receptor antagonist, TRPV1 receptor antagonist and CGRP receptor antagonist on anandamide-induced dural vessel dilation In another series of experiments it was tested whether the CB_1 receptor antagonist, AM251 (*N*-(Piperidin-1-yl)-5-(4-iodophenyl)-1-(2,4-dichlorophenyl)-4-methyl-1*H*-pyrazole-3-carboxamide) ($n=5$), the TRPV1 receptor antagonist, capsazepine ($n=6$), and the CGRP receptor antagonist, $CGRP_{8-37}$ ($n=7$), were able to reverse the vasodilator effects of anandamide. A control response to anandamide (5 mg kg^{-1}) was followed 10 min later by an intravenous bolus injection of either AM251 (3 mg kg^{-1}), capsazepine (3 mg kg^{-1}) or $CGRP_{8-37}$ (300 μg kg^{-1}). Following a further 5 min the anandamide (5 mg kg^{-1}) vasodilator challenge was repeated, in the case of the $CGRP_{8-37}$ series of experiments, the delay was only 2 min, due to the very short half-life of $CGRP_{8-37}$. Also, in the $CGRP_{8-37}$ trial, an additional anandamide (5 mg kg^{-1}) bolus was given after a further 20 min to see if there was any recovery.

Data analysis

The effects of anandamide on dural vessel diameter were calculated as a percentage increase from the prestimulation baseline diameter. The nature of the experimental set-up, where the magnification of the dural vessel visualised was different in each set-up, made it impossible to standardise the dural vessel measurement; therefore, the dural vessel diameter was measured in arbitrary units. Typical vessels measured were between 100 and 200 μm in diameter. All data are expressed as mean ± s.e.m. The statistical analysis for the vasodilator response of anandamide was performed using an exponential regression of dose on dilator response. The analysis for the experiments with antagonist interventions where observations were repeated once was performed with a Student's paired *t*-test. Where there were two observations, with $CGRP_{8-37}$ after the initial anandamide control, an ANOVA for repeated

measure was used, followed by Student's paired *t*-test where appropriate (SPSS v11.5). Observations of blood pressure changes are reported as the maximum actual changes that occurred after drug intervention. Significance was assessed at the $P<0.05$ levels.

Drugs

The infusion of anaesthetic and experimental drugs was all *via* the same femoral catheter; however, the line was always flushed with saline or vehicle first several minutes before administering the different compound. Anandamide (Tocris Cookson Ltd, in a soya oil : water (1 : 4) emulsion that is water soluble) was further diluted in water for injection. $CGRP_{8-37}$ (Sigma-Aldrich, U.K.) was dissolved in deoxygenated water, aliquotted and frozen until required and then re-dissolved in 0.9% NaCl. AM251 and capsazepine (Tocris Cookson, U.K.) were initially dissolved in a couple of drops of DMSO (Sigma-Aldrich, U.K.) and further diluted in a 1 : 1 : 8 solution of Tween 80 (polyoxyethylene-sorbitan mono-oleate, Sigma-Aldrich, U.K.): ethanol: 0.9% NaCl.

Results

Effect of anandamide on dural vessel diameter and blood pressure

Anandamide (1, 3, 5 and 10 mg kg^{-1}) caused a dose-dependent increase in vessel diameter when administered intravenously, $19\pm9\%$ ($n=12$), $29\pm5\%$ ($n=37$), $74\pm7\%$ ($n=13$) and $89\pm18\%$ ($n=6$), respectively ($F_{1,66}=27.0$, $P<0.05$, see Figure 1). Each dose of anandamide bolus injection (1, 3, 5 and 10 mg kg^{-1}) produced a significant maximum drop in mean arterial blood pressure, 30 ± 5, 30 ± 2, 32 ± 4 and 37 ± 7 mmHg in phase III (Chahl & Lynch, 1987), respectively, that was sustained for approximately 2 min. There was no significant difference in the drop in blood pressure across the difference doses ($F_{3,73}=0.57$, $P=0.64$).

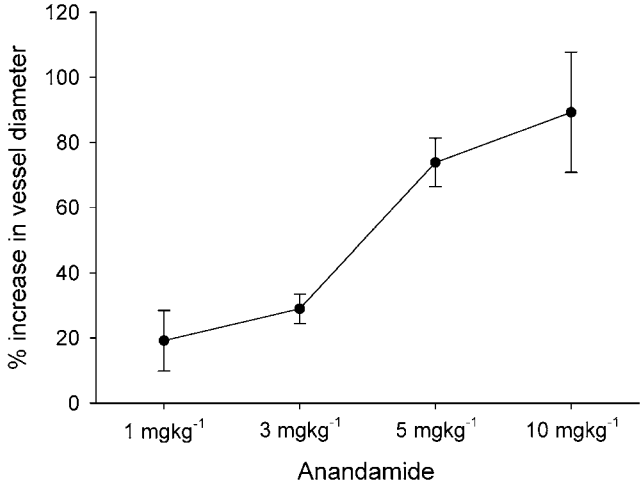

Figure 1 Effects of increasing dose of anandamide (1, 3, 5 and 10 mg kg^{-1}) on dural blood vessel diameter.

Effect of CB_1, TRPV1 and CGRP receptor antagonists on the anandamide-induced dural vessel dilation

In rats pretreated with AM251 (3 mg kg^{-1}), the CB_1 receptor antagonist, the dilation brought about by anandamide (5 mg kg^{-1}) was unaltered from 54 ± 6 to $57\pm4\%$ ($n=6$, $t_5=0.8$, $P=0.46$). AM251 ($n=5$) produced a significant drop in mean arterial blood pressure of 30 ± 11 mmHg ($P<0.05$), which was accompanied by a nonsignificant drop in dural blood vessel diameter. Both were naturally restored to preinjection levels prior to repeat administration of anandamide, within the time constraints of the experimental protocol. In rats treated with capsazepine, the TRPV1 receptor antagonist, the dilation brought about by anandamide (5 mg kg^{-1}) was significantly reduced from 57 ± 4 to $21\pm6\%$ ($n=6$, $t_5=6.2$, $P<0.01$; see Figure 2). Capsazepine ($n=5$) produced a significant increase in mean arterial blood pressure of 8 ± 2 mmHg ($P<0.05$), which was accompanied by a significant drop in dural blood vessel diameter of $56\pm7\%$ ($P<0.05$). Both were naturally restored to preinjection levels prior to repeat administration of anandamide. When analysed using ANOVA for repeated measures, anandamide-induced (5 mg kg^{-1}) dilation was significantly affected by $CGRP_{8-37}$ intervention ($F_{2,10}=37.39$, $P<0.05$, $n=6$). When analysed using a Student's paired *t*-test the anandamide-induced (5 mg kg^{-1}) dilation was significantly reduced by pretreatment with the CGRP receptor antagonist, $CGRP_{8-37}$, from 91 ± 9 to $14\pm5\%$ (300 μg kg^{-1}, $n=7$, $t_6=11.1$, $P<0.05$). After 20 min the vasodilator response to anandamide significantly returned, 15 ± 5 to $56\pm11\%$ ($n=6$, $t_5=4.9$, $P<0.05$), although this vasodilator increase was still significantly lower than initial anandamide response, 88 ± 10 to $56\pm11\%$ ($n=6$, $t_5=3.1$, $P<0.05$; Figure 3). $CGRP_{8-37}$ had no effect on either mean arterial blood pressure or dural blood vessel diameter.

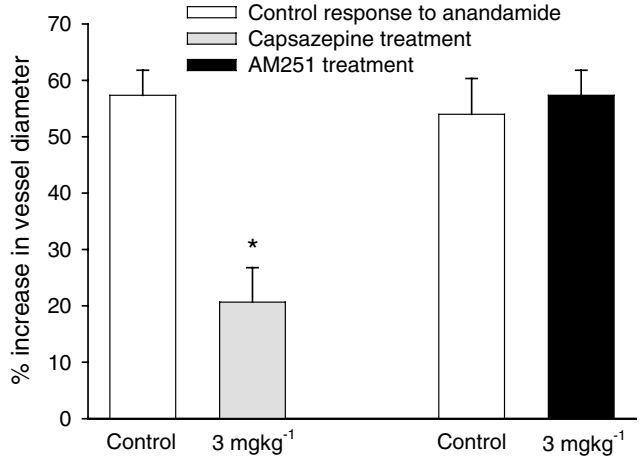

Figure 2 Effect of anandamide response on dural blood vessel diameter with capsazepine or AM251 intervention. Following control responses to anandamide (5 mg kg^{-1}) rats were injected with either capsazepine (3 mg kg^{-1}) or in a separate series of experiments, AM251 (3 mg kg^{-1}) and anandamide injection repeated after 5 min. *$P<0.05$ significance when compared to the control dilation of anandamide using Student's *t*-test.

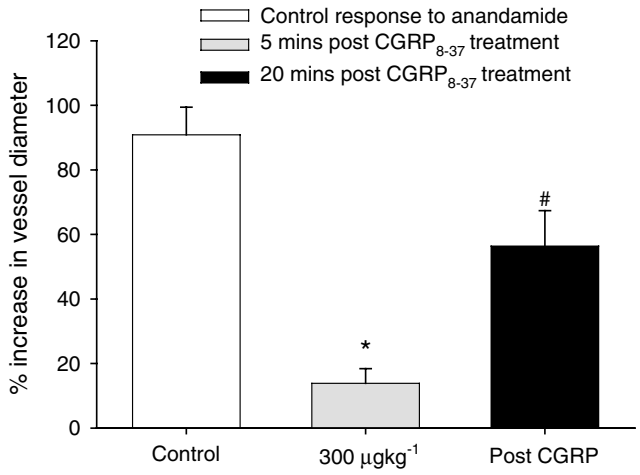

Figure 3 Effect of anandamide response on dural blood vessel diameter with $CGRP_{8-37}$ intervention. Following control responses to anandamide ($5\,mg\,kg^{-1}$), rats were injected with either $CGRP_{8-37}$ ($300\,\mu g\,kg^{-1}$) and anandamide injection repeated after 5 and 20 min. $^{*}P<0.05$ significance when compared to the control dilation of anandamide using Student's *t*-test. $^{\#}P<0.05$ significance when compared to $CGRP_{8-37}$ induced inhibition of anandamide using Student's *t*-test.

The effect of AM251, capsazepine and $CGRP_{8-37}$ on the hypotensive changes caused by anandamide

The blood pressure reduction that accompanied anandamide ($5\,mg\,kg^{-1}$) bolus was significantly reduced after pretreatment with AM251, 34 ± 8 mmHg compared to 5 ± 3 mmHg ($n=5$, $t_4=4.7$, $P<0.05$). Likewise, capsazepine was able to significantly reduce the phase I response of anandamide on blood pressure (Chahl & Lynch, 1987), 34 ± 8 mmHg compared to 6 ± 1 mmHg ($n=5$, $t_4=3.3$, $P<0.05$). Traces of blood pressure and vessel diameter changes with anandamide, AM251 and capsazepine are presented in Figure 4. There was a significant reduction in mean arterial blood pressure after each bolus of anandamide in the trial where $CGRP_{8-37}$ was used as a pretreatment, and there was no significance between the changes in blood pressure with the control anandamide bolus and after pretreatment with $CGRP_{8-37}$, 30 ± 5 mmHg compared to 32 ± 4 mmHg ($n=7$, $t_6=0.82$, $P=0.44$, Table 1).

Discussion

Anandamide was able to dose-dependently dilate dural arteries by an action not blocked by the CB_1 receptor antagonist AM251. The anandamide dural vasodilator effect was attenuated by the specific TRPV1 receptor antagonist capsazepine and involved CGRP release, just as does the effect of capsaicin in the same model (Akerman *et al*., 2003). Given that the hypotensive effect of anandamide was almost completely abolished by AM251, and that the dose given was able to reverse the anandamide induced inhibition of neurogenic dural vasodilation (Akerman *et al*., 2004), we feel the antagonist was present in a biologically active dose, and therefore suggest that the substantive effect of anandamide on dural vessel vasodilation is mediated by TRPV1 receptor-mediated release of CGRP.

We have shown previously that anandamide acting *via* the CB_1 receptor is able to inhibit neurogenic dural vasodilation, CGRP- and NO-induced dural vessel dilation *via* inhibition of trigeminal neurons (Akerman *et al*., 2004). As well as acting at the CB_1 receptor, anandamide has been found to act as an agonist at the vanilloid type 1 (TRPV1) receptor (Zygmunt *et al*., 1999), and it is able to activate sensory neurons *via* the TRPV1 receptor. It is paradoxical that anandamide stimulates sensory nerve activation as well as inhibiting neuronal transmission in the brain, and alters neuronal excitability. In the new experiments we have shown that anandamide is also able to cause dural vasodilation. It would appear that this is not *via* a cannabinoid receptor as the CB_1 receptor antagonist, AM251, which was able to reverse the anandamide-induced inhibition of neurogenic dural vasodilation (Akerman *et al*., 2004), was unable to reverse this anandamide-induced dilation.

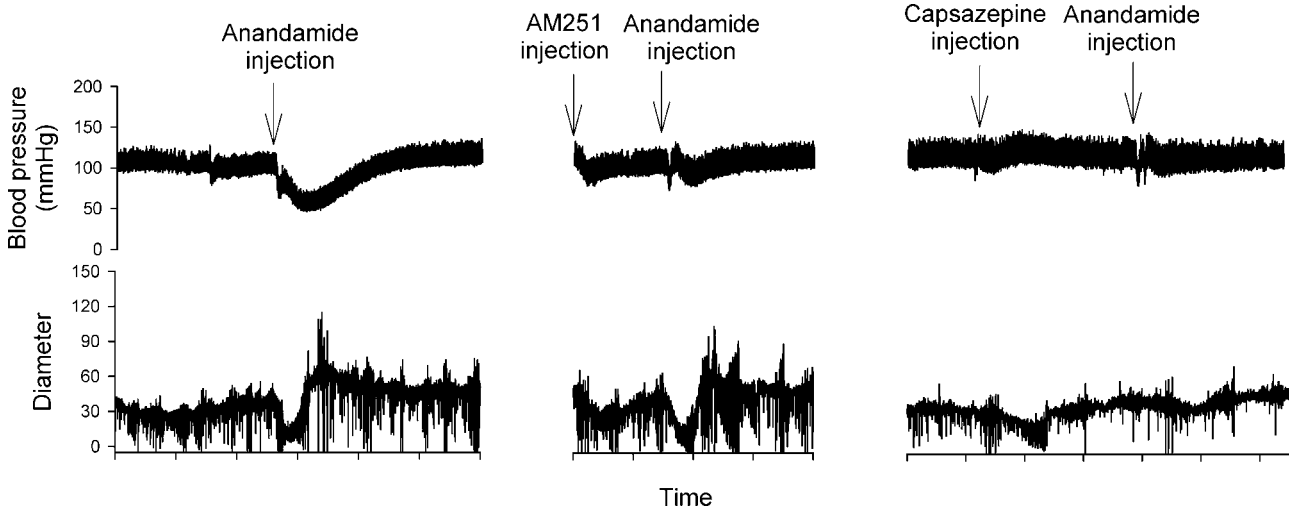

Figure 4 Example traces showing the effects of anandamide on arterial blood pressure and dural blood vessel diameter, as well as the effects of AM251 and capsazepine on the effects of anandamide. Anandamide given was alone, and then anandamide was preceded by either AM251 or capsazepine.

Table 1 Summary of maximum blood pressure changes of anandamide challenged with different drug regimens

Anandamide injection (5 mg kg^{-1})	*AM251 intervention (maximum change in BP)*	*Capsazepine intervention (maximum in BP)*	*$CGRP_{8-37}$ intervention (maximum in BP)*
1. Control response to anandamide (no antagonist)	(Control) drop by 34±8 mmHg*	(Control) drop by 34±8 mmHg*	(Control) drop by 30±5 mmHg*
2. Anandamide response after antagonist intervention	Drop by 5±3 mmHg**	Drop by 6±1 mmHg*·**	Drop by 32±5 mmHg*
3. Anandamide response 20 min after antagonist intervention	Not tested	Not tested	Drop by 51±4 mmHg*

BP = blood pressure.
Control represents the initial Anandamide injection prior to any drug intervention.*$P<0.05$ significance when compared to the blood pressure prior to intravenous anandamide injection.**$P<0.05$ significance when compared to the control drop in blood pressure.

If anandamide is acting at the TRPV1 receptor as has been reported in other systems (Zygmunt *et al*., 1999), it may behave similarly to the effects of capsaicin in the trigeminovascular system. We have shown previously that capsaicin activates TRPV1 receptors, causing the release of CGRP and subsequent dural vessel dilation (Akerman *et al*., 2003). The capsaicin-induced dilation is inhibited by capsazepine, a TRPV1 receptor antagonist, and $CGRP_{8-37}$, the CGRP receptor antagonist. Dural blood vessels are densely innervated by trigeminal nerves that release vasodilatory neuropeptides when activated (Uddman & Edvinsson, 1989). It seems likely that the mechanism by which capsaicin is able to cause dural vasodilation is activation of TRPV1 receptors presynaptically on trigeminal neurons. This in turn causes release of vasodilatory neuropeptides, including CGRP activating its receptors on dural vessels and thus causing dural vasodilation. Anandamide-induced dilation was attenuated by both capsazepine and $CGRP_{8-37}$; therefore, it appears that anandamide behaves similar to capsaicin to induce dural vessel dilation. It is important to note that the vehicle used for both capsazepine and AM251 was the same, given that AM251 was unable to inhibit the ananadamide-induced vasodilation, the effects of capsazepine cannot be explained by the vehicle. Interestingly, it has been recently reported in an *in vitro* setting that anandamide-activated TRPV1 receptors on trigeminal ganglion neurons promote CGRP release (Michele *et al*., 2002). The data presented here, and in our previous study, cannot confirm that this is the mechanism of action of both capsaicin and anandamide, but suggests this possibility needs examination. We would need to repeat the experiments and use immunohistochemistry to look at whether the trigeminovascular neurons are activated, perhaps using a double staining for *c-fos*, the immediate early gene that indicates cell activation and a probe directed at the TRPV1 receptor.

Anandamide produces blood pressure effects that are similar to capsaicin, with a triphasic effect (Chahl & Lynch, 1987) that accompanies the vasodilation, with a sharp decrease (Phase I), then increase (Phase II) followed by a decrease again (Phase III). The main and more sustained change is the final (Phase III) drop in blood pressure that is reported in the results for anandamide. However, the blood pressure drop with anandamide was not dose-dependent. In our previous study with capsaicin, the blood pressure effects were also studied, and showed that capsazepine was able to reverse the Phase I effects, while $CGRP_{8-37}$ had little effect (Akerman *et al*., 2003). In the present study, capsazepine was able to significantly reverse the effects of anandamide on blood pressure in phase I and reduced phase III changes, while again $CGRP_{8-37}$ had little or no effect. This is an interesting finding given that in a previous study capsazepine was only able to inhibit the phase I changes, being unable to alter the blood pressure changes in phase III of both anandamide and methanandamide (Malinowska *et al*., 2001). They also found that a CB_1 receptor antagonist was only able to inhibit phase III changes. The phase III response was also inhibited by a CB_1 receptor antagonist in our study, which was however unable to inhibit the phase I and II changes as observed in Figure 4. It seems anandamide behaves in a similar way to capsaicin on blood pressure, or as a TRPV1 receptor agonist, as has been previously reported. Interestingly, AM251 was also able to reverse the blood pressure effects of anandamide, thus confirming that it was present in a biologically active dose in our experiments. This is likely to be because anandamide acts on CB_1 receptors both on trigeminal neurons and in the periphery, and it is believed that anandamide is present to provide tonic antinociception (Pan *et al*., 1998). A discrepancy between our data and that of Malinowska *et al*. (2001) study was some inhibition of phase III. Upon closer observation of the methodologies, the TRPV1 agonists were given 2 min after antagonist treatment in the Malinowska study, but after 10 min in the present study. We cannot be sure if this time interval is important: does capsazepine take longer to act on the TRPV1 receptor for Phase III than it does for Phase I? More studies are required. Another observation is the doses of drugs used. In total, 1 mg kg^{-1} anandamide was used in the Malinowska study, and 5 mg kg^{-1} was used in the present study. The dose of antagonists differ, in our study we used 3 mg kg^{-1} of AM251 and capsazepine, while in the Malinowska study they used 1 μmol kg^{-1}, which equates to approximately 0.3 mg kg^{-1}, a 10-fold difference. It is possible that this greater concentration of capsazepine was able to affect the blood pressure as a whole. We can reasonably say that the blood pressure effects of anandamide are due to CB_1-mediated activity, and may also be due to TRPV1 mediated activity, and antagonists at each of these receptors are able to reverse this effect.

It is a limitation of the model used that any changes in blood vessel size may be explained by changes in systemic blood pressure, rather than a drug action at receptors on the

blood vessels, or nerve fibres that innervate the blood vessels. Taken together the effects of the antagonists in this study would indicate that any blood vessel changes caused by anandamide can be explained by an action at the site of the blood vessel rather than the blood pressure effects. Capsazepine and AM251 act in reducing the blood pressure effects of anandamide at different phases. Only capsazepine is able to inhibit the blood vessel changes of anandamide, indicating that these changes are likely to be caused by anandamide acting at the blood vessel itself. Likewise, $CGRP_{8-37}$ does not cause any blood pressure changes, nor is it able to reduce the blood pressure changes caused by anandamide. It is likely that $CGRP_{8-37}$ is acting postsynaptically, directly on the blood vessels, preventing vasodilation, and not on the nerves that maintain haemodynamic changes.

In our new study we noted that the doses of anandamide required to produce a maximal dilator response, $10\,mg\,kg^{-1}$, was much higher than the maximal dose necessary to inhibit neurogenic dural vasodilation (Akerman *et al*., 2004). Higher concentrations of anandamide were needed to activate the TRPV1 receptor than at the CB_1 receptor *in vitro* (Zygmunt *et al*., 1999). Conversely, another study found that only a small increase from the lowest effective dose at the CB_1 receptor was able to activate CGRP release from the TRPV1 receptor, and that anandamide was equipotent with capsaicin in activating CGRP release (Ahluwalia *et al*., 2003). It is possible, however, that higher dosing of anandamide will produce even greater vasodilator effects, but unfortunately a limitation of the technique is that the impact of intravenous anandamide on the blood pressure with increased dosing meant that it was difficult to interpret reliably the data. It would be interesting to explore further the dose-dependence of this effect and any shift in the dose–response curve that capsazepine might cause. Indeed, in the Ahluwalia *et al*. (2003) study when anandamide was applied the response was potentiated with a CB_1 antagonist. We did not find any potentiation when AM251 was applied in our study.

Several explanations could account for this discrepancy between anandamide doses that activate CB_1 and TRPV1 receptors and the differential results found regarding the potency of anandamide. Recent findings have shown that *in vitro* anandamide is less effective when applied extracellularly when acting on the TRPV1 receptor than intracellularly (Evans *et al*., 2004). The TRPV1 binding site is thought to be intracellular (Jordt & Julius, 2002) and therefore the potency of exogenously applied anandamide may be dependent on its ability to enter the cell. This may explain why in the Ahluwalia *et al*. experiment anandamide was equipotent at both CB_1 and TRPV1 receptors, while with the data presented here and in the Zygmunt study the CB_1 antagonist was not able to potentiate the response of anandamide. Other possible explanations for this discrepancy are that anandamide has a much lower affinity at the TRPV1 receptor as compared to its affinity at the CB_1 receptor; therefore, a much higher concentration of anandamide is needed to have a response at the TRPV1 receptor. It has been reported that anandamide has similar kinetic and electrophysiological properties to capsaicin, but is markedly less potent (Smart *et al*., 2000). Another potential explanation is that anandamide acts only as a partial agonist at the TRPV1 receptor, although there are reports that anandamide does in fact act as a full agonist at the TRPV1 receptor (Smart *et al*., 2000), and therefore can be discounted. All of these explanations may explain why a greater dose of anandamide was needed against the TRPV1 receptor to exact a response.

In summary, we have shown for the first time in an *in vivo* setting that anandamide, the CB_1 receptor agonist, can have TRPV1 receptor-mediated agonist actions as demonstrated by the dural vasodilation, which we believe to be *via* an action at the dural trigeminovascular system. This is in marked contrast to its inhibitory effects on neurogenic trigeminovascular dural vasodilation. Anandamide activates the TRPV1 receptor, in a similar way to capsaicin, promoting the release of CGRP and causing dural vasodilation. This is antagonised by both TRPV1 receptor and CGRP receptor antagonists. Exploring the role of endogenous cannabinoids will be an important part of understanding the pharmacology of the trigeminovascular system.

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