

Anandamide Is Able to Inhibit Trigeminal Neurons Using an in Vivo Model of Trigeminovascular-Mediated Nociception

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ABSTRACT

Arachidonylethanolamide (anandamide, AEA) is believed to be the endogenous ligand of the cannabinoid CB₁ and CB₂ receptors. CB₁ receptors have been found localized on fibers in the spinal trigeminal tract and spinal trigeminal nucleus caudalis. Known behavioral effects of anandamide are antinociception, catalepsy, hypothermia, and depression of motor activity, similar to Δ^9 -tetrahydrocannabinol, the psychoactive constituent of cannabis. It may be a possible therapeutic target for migraine. In this study, we looked at the possible role of the CB₁ receptor in the trigeminovascular system, using intravital microscopy to study the effects of anandamide against various vasodilator agents. Anandamide was able to inhibit dural blood vessel dilation brought about by electrical stimulation by 50%, calcitonin gene-related peptide (CGRP) by 30%, capsaicin by 45%, and nitric oxide by 40%. CGRP₈₋₃₇ was also able to

attenuate nitric oxide (NO)-induced dilation by 50%. The anandamide inhibition was reversed by the CB₁ receptor antagonist AM251. Anandamide also reduced the blood pressure changes caused by CGRP injection, this effect was not reversed by AM251. It would seem that anandamide acts both presynaptically, to prevent CGRP release from trigeminal sensory fibers, and postsynaptically to inhibit the CGRP-induced NO release in the smooth muscle of dural arteries. CB₁ receptors seem to be involved in the NO/CGRP relationship that exists in causing headache and dural blood vessel dilation. It also seems that some of the blood pressure changes caused by anandamide are mediated by a noncannabinoid receptor, as AM251 was unable to reverse these effects. It can be suggested that anandamide is tonically released to play some form of modulatory role in the trigeminovascular system.

Migraine pathophysiology is beginning to be understood in some detail, and it is likely to involve the activation of trigeminal afferents (Goadsby et al., 2002). Trigeminal sensory nerve fibers that innervate the cranial vasculature contain calcitonin gene-related peptide (CGRP), substance P, and neurokinin A (Uddman and Edvinsson, 1989). Activation of these A δ trigeminal fibers cause the release of CGRP (Goadsby et al., 1988) that in turn causes vasodilation of cranial blood vessels (Williamson et al., 1997c). Recent clinical trial evidence suggests that blockade of CGRP has a potent acute antimigraine effect (Olesen et al., 2003).

Arachidonylethanolamide (anandamide, AEA) is believed to be the endogenous ligand to the cannabinoid CB₁ and CB₂ receptors (Matsuda et al., 1990; Hoehe et al., 1991) that are found in the central and peripheral nervous systems. CB₁ receptors are localized on fibers in the spinal trigeminal tract and spinal trigeminal nucleus caudalis (Tsou et al., 1998),

whereas CB₂ receptors are found predominantly in the immune system (Egertová and Elphick, 2000). The CB₁ and CB₂ receptors are negatively coupled through G proteins to adenylate cyclase and various calcium channel types (Gebremedhin et al., 1999; Hillard, 2000). Anandamide mediates vascular changes via CB₁ receptors found on cerebral artery smooth muscle and the smooth muscle of other vascular beds and modulates cerebral vascular tone (Plane et al., 1997; Gebremedhin et al., 1999). Receptors in the endothelium of blood vessels, responsive to anandamide, also affect the vasculature, although this seems to be mediated by an anandamide receptor distinct from the CB₁ receptor (Pratt et al., 1998; Wagner et al., 1999).

The known behavioral effects of anandamide are similar to that of Δ^9 -tetrahydrocannabinol, the psychoactive constituent of cannabis, being antinociception, catalepsy, hypothermia, and depression of motor activity (Dewey, 1986; Adams et al., 1998). Although there is a history of anecdotal evidence suggesting the use of cannabinoids is effective at reducing headache and providing other pain relief, its potential as an acute migraine treatment and even preventive has never

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ABBREVIATIONS: CGRP, calcitonin gene-related peptide; CB, cannabinoid; NO, nitric oxide; SNP, sodium nitroprusside; L-NAME, N^ω-nitro-L-arginine methyl ester; NOS, nitric-oxide synthase; nNOS, neuronal nitric oxide synthase; AM251, N-(piperin-1-yl)-5-(4-iodophenyl)-1-(2,4-dichlorophenyl)-4-methyl-1H-pyrazole-3-carboxamide; AM404, N-(4-hydroxyphenyl)-52,82,112,142-eicosatetraenamide.

been scientifically studied in animal studies or clinical trial (Russo, 1998). However, one anonymous standardized survey found that of those using cannabis medicinally, over 10% were using it to relieve headache or migraine (Schnelle et al., 1999). Although many aspects of the study are open to debate, such as the highly selected nature of patient group, it is nevertheless an interesting observation.

In this study, we used intravital microscopy, which allows continual measurement of dural blood vessel diameter, to examine the effects of anandamide against dural vessel dilation brought about by electrical stimulation, CGRP, capsaicin, and nitric oxide (NO)-induced dural dilation (Williamson et al., 1997a,b; Akerman et al., 2002a, 2003b). We were also able to monitor the mean arterial blood pressure changes caused by the chemical vasodilators, and the effect of anandamide. Neurogenic dural vasodilation identifies potential migraine targets such as sumatriptan (Williamson et al., 1997b) and opioid agonists (Williamson et al., 2001). CGRP injections trigger both migraine (Lassen et al., 2002) and dural vessel dilation (Williamson et al., 1997a) and CGRP antagonists have acute antimigraine properties (Olesen et al., 2003). NO is another known trigger of migraine (Dalsgaard-Nielsen, 1955; Thomsen et al., 1994) and dural vessel dilation (Akerman et al., 2002c). Both NO-induced dural vessel dilation (Akerman et al., 2002a) and headache (Iversen and Olesen, 1996) are affected by the antimigraine compound sumatriptan. Capsaicin has also been shown to cause vasodilation of dural arteries via the release of CGRP from trigeminal nerve ending (Akerman et al., 2002b). We examined whether any of the effects of anandamide were reversed by the CB₁ receptor antagonist AM251. CGRP antagonists have proved successful as a treatment for migraine in patients (Olesen et al., 2003), as well as for neurogenic, CGRP, and capsaicin-induced dilation; therefore, to validate the use of anandamide against NO-induced dilation we wanted see whether NO-induced dilation could be inhibited by CGRP₈₋₃₇, the CGRP receptor blocker, first before using with anandamide.

Materials and Methods

Surgical Preparation

All experiments were conducted under UK Home Office Animals (Scientific Procedures) Act (1986). Male Sprague-Dawley rats (180–385 g) were anesthetized throughout the experiments with sodium pentobarbitone (60 mg kg⁻¹ i.p. and then 18 mg kg⁻¹ h⁻¹ i.v. infusion). The left femoral artery and vein were cannulated for blood pressure recording and intravenous infusion of anesthetic and test compounds, respectively, whereas the carotid artery was cannulated for infusion of NO donor. Temperature was maintained throughout using a homeothermic blanket system. The rats were placed in a stereotaxic frame and ventilated with oxygen-enriched air, 3 to 5 ml, 60 to 80 strokes per minute (small rodent ventilator model 683; Harvard Instruments, Edenbridge, Kent, UK). End-tidal CO₂ was monitored (Capstar-100; CWE Inc., Ardmore, PA) and kept between 3.5 and 4.5%, and blood pressure was monitored continually. This allows one to monitor for changes in respiration and blood pressure due to long-term anesthetic 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 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 viewed using an intravital microscope (MV2100; Microvision, Cambridge, UK) and the image displayed on a television monitor. Dural blood vessel diameter was continuously measured using a video dimension analyzer (Living Systems Instrumentation, Burlington, VT) and displayed with blood pressure on an online data analysis system (CED spike4 version 2 software).

Experimental Protocols

Defining Electrical Stimulation Parameters. Electrical stimulation was used to evoke dilation of the dural blood vessels with a bipolar stimulating electrode (NE 200X; Clark Electromedical, Edenbridge, Kent, UK) placed on the surface of the cranial window approximately 200 μm from the vessel of interest. The surface of the cranial window was stimulated at 5 Hz, 1 ms for 10 s (Grass stimulator S88) with increasing voltage until maximal dilation was observed. Subsequent electrically induced responses in the same animal were then evoked using that voltage (Williamson et al., 1997b; Akerman et al., 2002c).

CGRP, Capsaicin, and NO-Induced Dilation. In the preparations where CGRP was used to dilate dural blood vessels, CGRP was given as an intravenous bolus of 1 μg kg⁻¹, and this has been shown previously to produce a maximal dilation (Williamson et al., 1997a). In those preparations where capsaicin was used to dilate the dural blood vessels, capsaicin was given as an intravenous bolus up to 7 μg kg⁻¹, until a maximal dilation was observed (Akerman et al., 2003b). In preparations where NO was used to dilate the dural blood vessel, sodium nitroprusside, the NO donor, was given as a 10-min infusion at 2 μg kg⁻¹ min⁻¹ via the carotid artery, as to produce maximal dilation (Akerman et al., 2002a).

The reproducibility of the vasodilator challenges has been demonstrated previously using either three or four consecutive saline-controlled stimuli for electrical stimulation and CGRP bolus (Akerman et al., 2002c), capsaicin bolus (Akerman et al., 2003b), and sodium nitroprusside (Akerman et al., 2002a), to test whether there was any systematic effect of test compounds over time in the meningeal circulation. In each case, there was no significant effect across the cohort. In these studies, on each occasion the chemical vasodilators were administered they caused a significant drop in blood pressure (S. Akerman, unpublished data). CGRP and sodium nitroprusside (SNP) injections caused decreases in blood pressure that were not significantly different across the cohorts. Blood pressure changes caused by capsaicin are triphasic, as has been demonstrated previously (Chahl and Lynch, 1987), and the response to second and third injections of capsaicin was reduced compared with the first injection.

Effects of the Cannabinoid Agonist Anandamide on Evoked Dural Vessel Dilation. The effects of the cannabinoid receptor agonist anandamide were studied. A control response to one of the vasodilator challenges, either electrical stimulation, bolus of CGRP or capsaicin was performed and at least 10 min later anandamide (1 mg kg⁻¹) was administered intravenously, and the vasodilator repeated 10 min later. Using the same protocol a second dose of anandamide (3 mg kg⁻¹) was given and a third vasodilator challenge performed. In the series of experiments involving CGRP a third dose of anandamide (10 mg kg⁻¹) was given followed by a fourth bolus of CGRP, all in the same animal.

NO-induced dural vessel dilation was tested with anandamide using a similar protocol. Two control responses to sodium nitroprusside infusion (2 μg kg⁻¹ min⁻¹) for 10 min. Completion of the second infusion was followed, at least 10 min later, by a bolus injection of anandamide (3 mg kg⁻¹), which was followed 10 min later by a repeat of the sodium nitroprusside infusion.

Effect of a CB₁ Receptor Antagonist in Reversing the Actions of Anandamide. In separate series of experiments, the response of the selective CB₁ receptor antagonist AM251 was used to

determine whether it could reverse any of the effects that anandamide had on the vasodilator challenges. Initially, we used a similar protocol to that reported above to determine the effects of anandamide (3 mg kg⁻¹) against either electrical stimulation, CGRP, or sodium nitroprusside. After the observation of the effects of anandamide, AM251 (3 mg kg⁻¹), a selective CB₁ receptor antagonist, was administered intravenously and was followed by another repeat of the vasodilator challenge 5 min later. It has been shown previously that the antinociceptive effects of anandamide, using tail-flick responses, last up to at least an hour (Smith et al., 1994; Adams et al., 1995). We were therefore confident that the effect of anandamide would still be acting when we followed up the initial observation of anandamide with that of the CB₁ antagonist.

Effect of the CGRP Receptor Blocker CGRP₈₋₃₇ on NO-Induced Dilation. The NO donor sodium nitroprusside was infused for 10 min in the rat to provide a control vasodilation. At least 10 min after the dural blood vessel had returned to its original baseline state, a second infusion was begun, after 5 min of infusion CGRP₈₋₃₇ (300 μg kg⁻¹) was given as an intravenous bolus, and the sodium nitroprusside infusion continued for a further 5 min.

Data Analysis. The effects of electrical stimulation, bolus of CGRP or capsaicin, and sodium nitroprusside infusion on dural vessel diameter were calculated as a percent increase from the pre-stimulation baseline diameter. The nature of the experimental setup, where the magnification of the dural vessel visualized was different in each setup by virtue of selecting an appropriate target vessel, made it impossible to standardize the dural vessel measurement; therefore, the dural vessel diameter was measured in arbitrary units. The typical vessel diameter measured ranges from 120 to 150 μm. Data are expressed as mean ± S.E.M. Statistical analysis was initially performed using an analysis of variance for repeated measures with a two-factor (baseline and treatment) model. Main effects were compared after Bonferroni confidence interval adjustment. Between treatment comparisons were made with Student's paired *t* test (SPSS version 10.0) on effects found different in the analysis of variance and are thus reported in detail. Significance was assessed at the *P* < 0.05 level.

Drugs. The infusion of anesthetic and experimental drugs were all via the same femoral catheter, however, the line was always flushed with saline first, several minutes before administering the different compound. Anandamide (Tocris Cookson Ltd., Bristol, UK) in water-soluble emulsion was further diluted in water for injection. It has been shown previously that dosing between 1 and 10 mg kg⁻¹ is antinociceptive in the rat, and this effect can last up to 30 min (Adams et al., 1995, 1998; Stein et al., 1996). CGRP (Sigma Chemical, Poole, Dorset, UK) and the CGRP receptor blocker CGRP₈₋₃₇ (Sigma Chemical) were dissolved in deoxygenated water, aliquoted, and frozen until required and then redissolved in 0.9% NaCl for use. Capsaicin (Sigma Chemical) was dissolved in a 1:1:8 solution of Tween 80 (polyoxyethylene-sorbitan mono-oleate; Sigma Chemical)/ethanol/0.9% NaCl. AM251 (Tocris Cookson Ltd.) was initially dissolved in a couple of drops of dimethyl sulfoxide (Sigma Chemical) and further diluted in the solution of Tween 80, ethanol, and saline. Sodium nitroprusside (Sigma Chemical) was dissolved in 0.9% NaCl.

Results

Effect of Anandamide and Then CB₁ Receptor Antagonism on Electrical Stimulation. In rats treated with anandamide (1 mg kg⁻¹, *n* = 6 and 3 mg kg⁻¹, *n* = 12), the dilation brought about by electrical stimulation was significantly reduced with both doses of anandamide, 125 ± 10% to 72 ± 10% (1 mg kg⁻¹, *t*₅ = 4.3, *P* < 0.05) and 120 ± 8 to 53 ± 8% (3 mg kg⁻¹, *t*₁₁ = 7.6, *P* < 0.05). The reduction of the electrically induced dilation brought about by anandamide (3 mg kg⁻¹) was significantly reversed by the CB₁ receptor antagonist AM251 (3 mg kg⁻¹, *n* = 6, *t*₅ = 4.3), 47 ± 13 to

99 ± 16%, and this return of dilation was not significant from the original control dilation, 115 ± 12 to 99 ± 16% (Figs. 1 and 2).

Effect of Anandamide and Then CB₁ Receptor Antagonism on CGRP-Induced Dilation. Increases in dural blood vessel diameter evoked by CGRP (1 μg kg⁻¹ i.v.) were significantly inhibited when pretreated with anandamide, 120 ± 7 to 89 ± 10% (3 mg kg⁻¹, *n* = 14, *t*₁₃ = 6.6, *P* < 0.05) and 129 ± 11 to 89 ± 14% (10 mg kg⁻¹, *n* = 6, *t*₅ = 5.7, *P* < 0.05), respectively. The reduction of the CGRP-induced dilation brought about by anandamide (3 mg kg⁻¹) was significantly reversed by the CB₁ receptor antagonist AM251 (3 mg kg⁻¹, *n* = 8, *t*₇ = 6.1), 87 ± 7 to 129 ± 11%, this return of dilation was significantly different from the original control dilation, 114 ± 9 to 129 ± 11% (3 mg kg⁻¹, *n* = 8, *t*₇ = 2.6; Figs. 1 and 3).

Effect of Anandamide on Capsaicin-Induced Dilation. Dural blood vessel dilation brought about by capsaicin showed a significant reduction after pretreatment with anandamide (1 and 3 mg kg⁻¹, *n* = 6) compared with the control dilation, 118 ± 15 to 89 ± 14% (*t*₅ = 4.0) and 65 ± 18% (*t*₅ = 5.4), respectively (*P* < 0.05; Fig. 4).

Effect of Anandamide and Then CB₁ Receptor Antagonism on Sodium Nitroprusside-Induced Dilation. Dural blood vessel dilation brought about by sodium nitroprusside infusion (2 μg kg⁻¹ min⁻¹) showed a significant reduction after pretreatment with a 3 mg kg⁻¹ dose of anandamide, 112 ± 10 to 71 ± 9% (*n* = 14, *t*₁₃ = 6.2, *P* < 0.05). The reduction of the sodium nitroprusside-induced dilation brought about by anandamide (3 mg kg⁻¹) was significantly reversed by the CB₁ receptor antagonist AM251 (3 mg kg⁻¹, *n* = 6, *t*₅ = 3.4), 79 ± 11 to 112 ± 19%, and this return of dilation was not significant from the original control dilation, 123 ± 16 to 112 ± 19%; Figs. 1 and 5.

Effect of AM251 on Dural Blood Vessel Caliber. AM251 caused a nonsignificant 7 ± 6% (*n* = 22, *t*₂₁ = 1.542, *P* = 0.138) decrease in dural blood vessel caliber that was restored to preinjection levels before vasodilator challenge was repeated.

Effect of CGRP₈₋₃₇ on NO-Induced Dural Blood Vessel Diameter. The CGRP receptor blocker CGRP₈₋₃₇ (300 μg kg⁻¹, *n* = 8) was able to inhibit NO-induced dilation, 117 ± 8 to 56 ± 14% (*t*₆ = 4.8, *P* < 0.05). The inhibition was also significant compared with the initial control NO-induced dilation, 104 ± 12 to 56 ± 14% (*t*₇ = 4.1, *P* < 0.05; Fig. 6).

Effect of Anandamide and AM251 on Arterial Blood Pressure. Anandamide caused a significant decrease in mean arterial blood pressure of 30.3 ± 5 mm Hg (1 mg kg⁻¹, *n* = 16, *t*₁₅ = 6.18, *P* < 0.001), 30.4 ± 2 mm Hg (3 mg kg⁻¹, *n* = 38, *t*₃₇ = 14.13, *P* < 0.001) and 30.1 ± 7 (10 mg kg⁻¹, *n* = 5, *t*₄ = 4.59, *P* < 0.05). There was no overall difference between the blood pressure changes across the doses (*F*_{3,68} = 0.05, *P* = 0.985). The changes in blood pressure caused by anandamide were accompanied by a dose-dependent increase in vessel diameter, which is dealt with elsewhere (Akerman et al., 2003a).

AM251 caused a significant reduction in blood pressure 23 ± 4 mm Hg (*n* = 22, *t*₂₁ = 5.431, *P* < 0.0001), which was accompanied by a 7 ± 6% decrease in dural blood vessel diameter, both were restored to preinjection levels before vasodilator challenge was repeated.

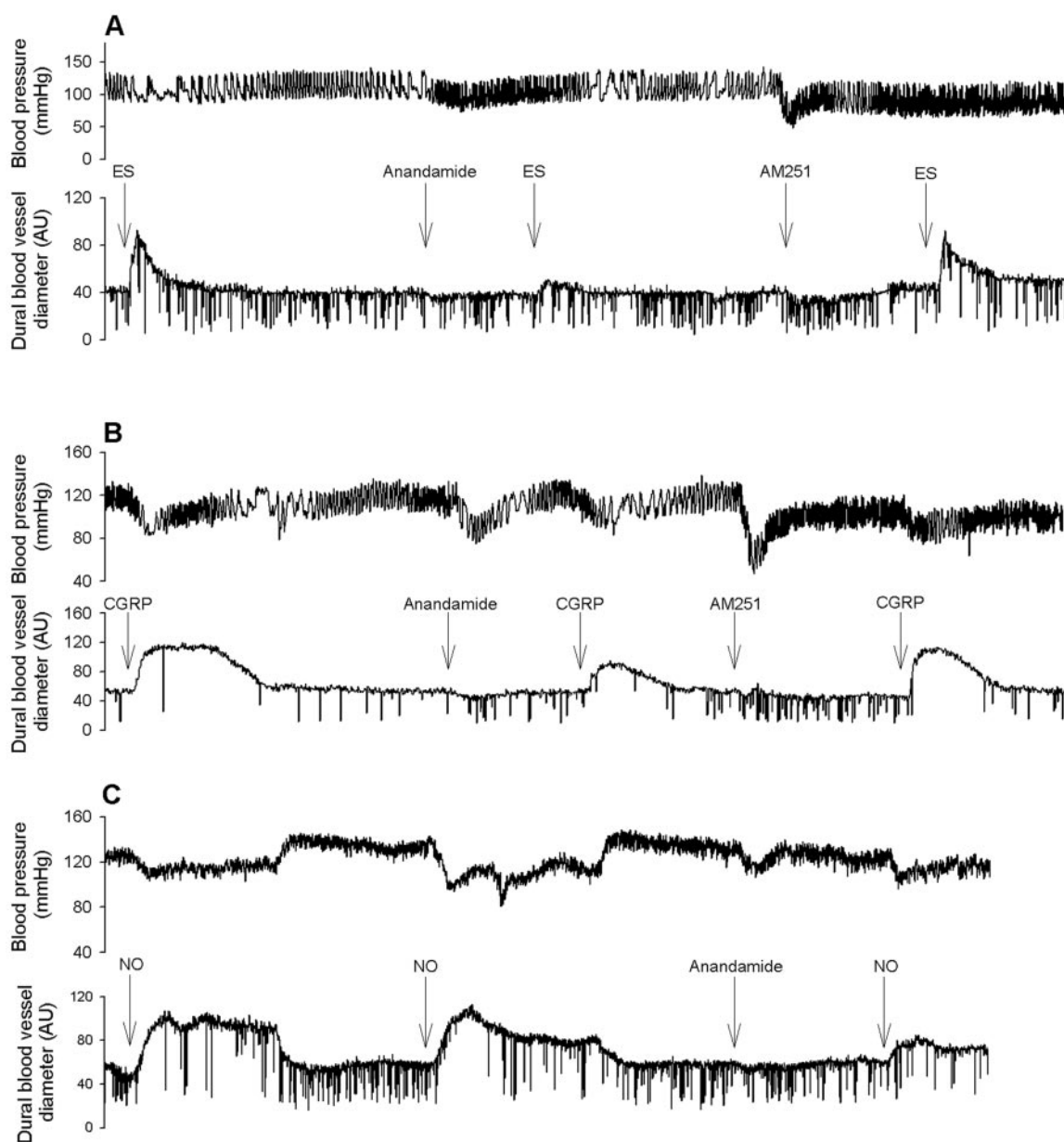


Fig. 1. Sample traces of intravital videomicroscopy with anandamide. Traces show the inhibitory effects of anandamide (3 mg kg^{-1}) on neurogenic dural vasodilation (A), CGRP-induced dural vessel dilation (B), and sodium nitroprusside-induced dural vessel dilation (C). Traces A and B also highlight the effects of the CB₁ receptor antagonist AM251 (3 mg kg^{-1}), in reversing anandamide induced changes. ES, electrical stimulation.

Effect of Anandamide and AM251 on Hypotensive Changes Caused by CGRP, Capsaicin, and Sodium Nitroprusside.

On each occasion CGRP injections caused a significant drop in blood pressure (Tables 1 and 2); however, in the case of the 3 mg kg^{-1} dose of anandamide this drop was significantly reduced, $48 \pm 5 \text{ mm Hg}$ drop compared with $29 \pm 3 \text{ mm Hg}$ (3 mg kg^{-1} , $n = 11$, $t_{10} = 4$, $P < 0.05$). When AM251 was used to reverse the effects of anandamide, it was unable to reverse the reduced blood pressure change found with the 3 mg kg^{-1} dose (Table 2).

The blood pressure response to capsaicin was significantly decreased after each injection; however, the level of drop was reduced after anandamide treatment $42 \pm 3 \text{ mm Hg}$ compared with $35 \pm 3 \text{ mm Hg}$ (1 mg kg^{-1} , $n = 6$, $t_5 = 2.87$, $P < 0.05$) and $29 \pm 4 \text{ mm Hg}$ (3 mg kg^{-1} , $n = 6$, $t_5 = 4.19$, $P <$

0.05 ; Table 1). There was no significant difference in the blood pressure change between the anandamide doses.

Each infusion of sodium nitroprusside caused a significant reduction in blood pressure (Table 1). The blood pressure changes with sodium nitroprusside alone were not significant compared with the SNP changes with anandamide ($F_{2,27} = 0.348$, $P = 0.71$) or AM251 ($n = 6$, $F_{2,10} = 0.35$, $P = 0.71$).

The blood pressure did not change significantly when the CGRP receptor antagonist CGRP₈₋₃₇ was given during a sodium nitroprusside infusion, 102 ± 10 to $103 \pm 10 \text{ mm Hg}$.

Discussion

Anandamide was able to inhibit significantly neurogenic dural vasodilation, CGRP-, capsaicin-, and NO-induced dural

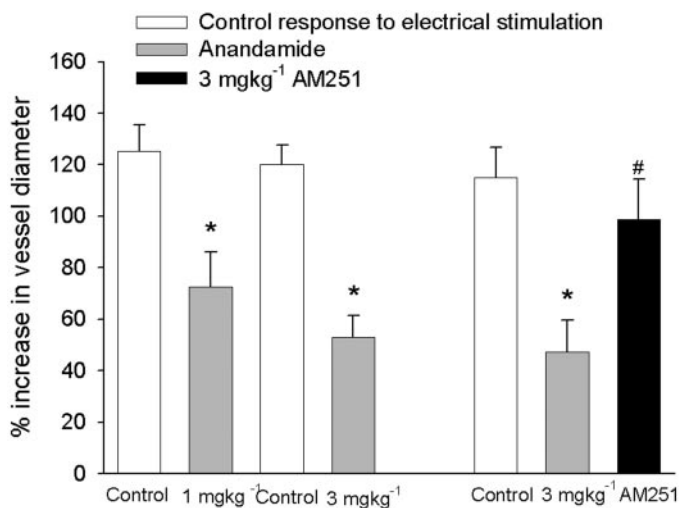


Fig. 2. Effects of repeated electrical stimulation with anandamide treatment (gray column) and the effect of AM251, a CB₁ receptor antagonist in reversing the anandamide response (black column) on dural blood vessel diameter. After control responses rats were injected with either anandamide (1 and 3 mg kg⁻¹) or anandamide (3 mg kg⁻¹) and then AM251 (3 mg kg⁻¹) and electrical stimulation repeated after each separate drug intervention. *, *P* < 0.05 significance compared with the control response. #, *P* < 0.05 significance compared with the inhibitory response of anandamide.

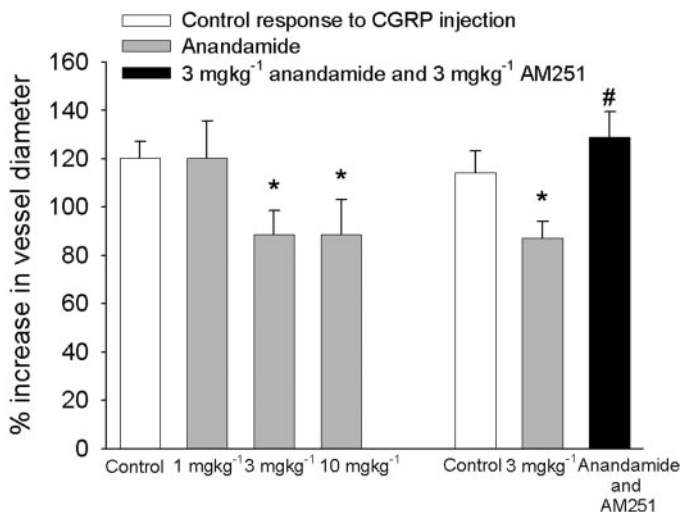


Fig. 3. Effects of repeated CGRP injection with anandamide treatment (gray column) and the effect of AM251, a CB₁ receptor antagonist in reversing the anandamide response (black column) on dural blood vessel diameter. After control responses rats were injected with either anandamide (1 and 3 mg kg⁻¹) or anandamide (3 mg kg⁻¹) and then AM251 (3 mg kg⁻¹) and CGRP injection repeated after each separate drug intervention. *, *P* < 0.05 significance compared with the control response. #, *P* < 0.05 significance compared with the inhibitory response of anandamide.

vessel dilation found in the rat intravital microscopy model of trigeminovascular activation. The CB₁ receptor antagonist AM251 was able to reverse the inhibition of dural vessel dilation mediated by anandamide for the neurogenic, CGRP-, and NO-induced dilation. CGRP₈₋₃₇ was also able to attenuate the NO-induced dilation.

Neurogenic dural vasodilation is likely to be a result of CGRP release via activation of perivascular trigeminal sensory nerve fibers. Anandamide was able to attenuate the neurogenic dural vasodilation by over 50%, and the CB₁ receptor antagonist AM251 reversed this. This effect of anan-

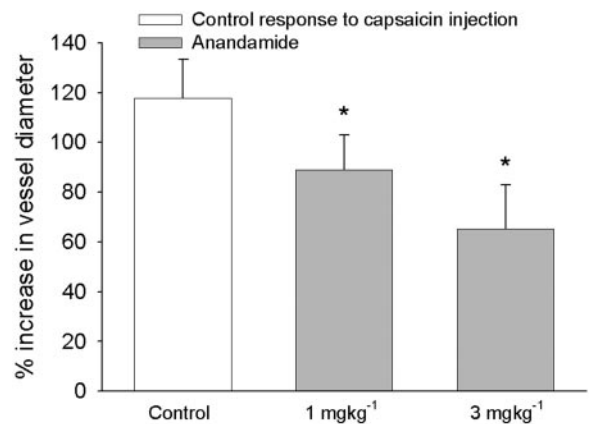


Fig. 4. Effects of repeated capsaicin injection with anandamide treatment (gray column) on dural blood vessel diameter. After control responses rats were injected with anandamide (1 and 3 mg kg⁻¹) and capsaicin injection repeated. *, *P* < 0.05 significance compared with the control response.

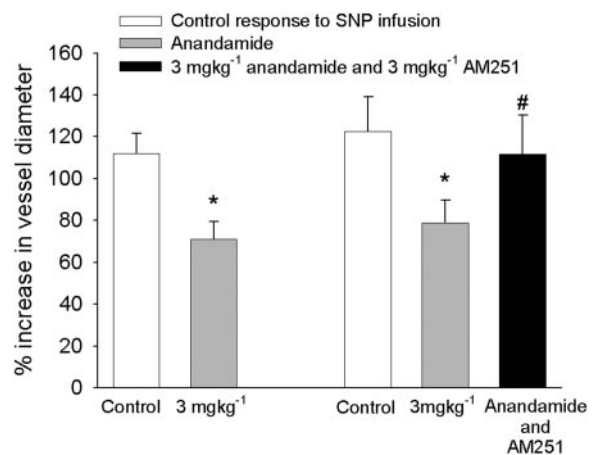


Fig. 5. Effects of SNP infusion with anandamide treatment (gray column) and the effect of AM251, a CB₁ receptor antagonist in reversing the anandamide response (black column) on dural blood vessel diameter. After control responses rats were injected with just anandamide (3 mg kg⁻¹) or anandamide (3 mg kg⁻¹) and then AM251 (3 mg kg⁻¹) and SNP infusion repeated after each separate drug intervention. *, *P* < 0.05 significance compared with the control response. #, *P* < 0.05 significance compared with the inhibitory response of anandamide.

damide may involve either pre- or postsynaptic receptors, or both, to inhibit dural vasodilation. In contrast, we have reasoned that exogenous CGRP is acting directly on postsynaptic CGRP receptors in the smooth muscle of dural arteries. Anandamide is also able to attenuate the CGRP-induced dural blood vessel dilation by just over 30%, an effect reversed by the CB₁ receptor antagonist, AM251. Therefore, it would seem that anandamide is at least acting postsynaptically to inhibit dural blood vessel dilation, and may, in addition, be acting presynaptically to account for the greater attenuation of the response. Because the CB₁ receptor antagonist AM251 was able to reverse the effects of anandamide, it seems likely that the responses reported are due to activation of the CB₁ receptor. Anandamide was also able to inhibit capsaicin-induced dilation. Capsaicin evokes CGRP release from trigeminal sensory nerves (Flores et al., 2001) and more generally from sensory nerve terminals (Saito and Goto, 1986; Maggi et al., 1988; Martling et al., 1988). We have also previously shown (Akerman et al., 2003b) using this model

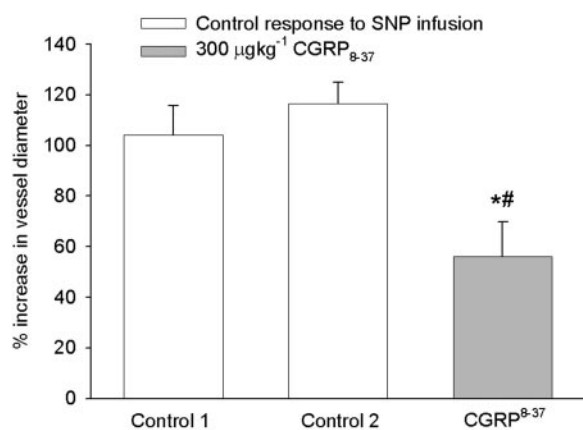


Fig. 6. Effects of repeated SNP infusion with CGRP receptor blocker (gray column) on dural blood vessel diameter. During the second sodium nitroprusside infusion, which acts as control 2, rats were injected with CGRP₈₋₃₇ (300 $\mu\text{g kg}^{-1}$) and sodium nitroprusside infusion continued. *, $P < 0.05$ significant change in dural vessel diameter compared to control 1; #, $P < 0.05$ significant change in dural vessel diameter compared to control 1.

that the capsaicin-mediated dilation is inhibited by a CGRP blocker and therefore it is likely that capsaicin-induced dilation is mediated by CGRP release. Given that anandamide is able to inhibit CGRP induced dilation acting postjunctionally, it seems reasonable to conclude that it is also inhibiting the capsaicin-induced dilation via this same mechanism.

Anandamide was able to attenuate the dural blood vessel increases caused by sodium nitroprusside (an NO donor) infusions by 37%. Anandamide is able to inhibit nitric oxide production in macrophage and microglial cells (Coffey et al., 1996). In our experimental design, anandamide-mediated alterations in NO generation are unlikely given that we are providing the NO exogenously. When a formalin test was performed in spinally microdialysed mice to promote nociception, anandamide, indomethacin, and L-nitroarginine methyl ester (L-NAME), an NO synthase inhibitor, were all able to reduce pain-related behavior, and these effects were reversed by the CB₁ receptor antagonist AM251 (Guhring et al., 2002). It is interesting from a translational and head pain perspective that AM251 is able to reverse the antinociceptive effects of both L-NAME and indomethacin in the spinal dorsal horn

(Guhring et al., 2002). This is especially so given that L-NAME is able to attenuate both neurogenic and CGRP-induced dural vessel dilation (Akerman et al., 2002c) and that indomethacin attenuates both NO-induced headache (Castellano et al., 1998) and NO-induced dural vessel dilation (Akerman et al., 2002a). They also found that the endocannabinoid reuptake blocker AM404 reduced pain-related behavior, indicating that endocannabinoids are either naturally released in response to pain, or that they provide tonic antinociceptive effects (Guhring et al., 2002). The latter may be altered by rates of degradation, which have recently been reported to be abnormal in migraineurs (Cupini et al., 2003).

We also observed that the CGRP receptor blocker CGRP₈₋₃₇ attenuated the sodium nitroprusside-induced blood vessel dilation by over 50% when applied during the infusion. This suggests that NO may activate trigeminal neurons to release CGRP and CGRP₈₋₃₇ blocks the response postsynaptically. We have demonstrated previously that endothelial NO synthase inhibitors are able to attenuate CGRP-induced dilation (Akerman et al., 2002c). Given that anandamide is able to attenuate both CGRP and NO-induced dilation, the CB₁ receptor may also play a role in the NO/CGRP relationship.

In the context of our model of trigeminovascular activation, how is anandamide and more specifically the CB₁ receptor involved? Considering CGRP-induced dural vessel dilation first, anandamide was able to attenuate it by 30%, this is similar to the effect of specific endothelial NOS inhibitors in the same model (Akerman et al., 2002c). Given the CB₁ receptor is present in the smooth muscle of cerebral arteries and other vascular beds (Plane et al., 1997; Gebremedhin et al., 1999; Hillard, 2000), it is possible that there are CB₁ receptors in the smooth muscle of dural arteries. These receptors would mediate the post-synaptic inhibition of CGRP-induced dilation perhaps by interfering with the CGRP-induced NO production that takes place via the activation of endothelial NOS.

Neurogenic dural vasodilation is attenuated by anandamide by over 50%, and this is similar to the attenuation brought about by specific neuronal NOS (nNOS) inhibitors (Akerman et al., 2002c). The data presented here indicate that anandamide may be acting on presynaptic terminals of

TABLE 1

Summary of blood pressure changes with vasodilators and anandamide

	Blood Pressure Decrease (mm Hg)			
	Control	Anandamide (1 mg kg ⁻¹)	Anandamide (3 mg kg ⁻¹)	Anandamide (10 mg kg ⁻¹)
CGRP (1 $\mu\text{g kg}^{-1}$)	47.9 \pm 5*	35.2 \pm 6*	29.5 \pm 3*#	20.7 \pm 2*
Capsaicin (7 $\mu\text{g kg}^{-1}$)	41.5 \pm 3*	35.4 \pm 3*#	28.7 \pm 4*#	
Sodium nitroprusside (2 $\mu\text{g kg}^{-1} \text{ min}^{-1}$)	43.9 \pm 5*		39.6 \pm 5* and 38.6 \pm 3*	

* $P < 0.05$ significant drop in blood pressure compared with preinjection level.

$P < 0.05$ significance compared with control drop in blood pressure.

TABLE 2

Summary of vasodilator induced blood pressure changes with anandamide and AM251

	Blood Pressure Decrease (mm Hg)		
	Control	Anandamide (3 mg kg ⁻¹)	AM251 (3 mg kg ⁻¹)
CGRP (1 $\mu\text{g kg}^{-1}$)	54.9 \pm 4*	31.7 \pm 5* ($n = 6$, $t_5 = 3.27$)#	21.8 \pm 3* ($n = 6$, $t_5 = 5.85$)#
Sodium nitroprusside (2 $\mu\text{g kg}^{-1} \text{ min}^{-1}$)	51.5 \pm 7*	49.4 \pm 6*	55.6 \pm 4*

* $P < 0.05$ significant drop in blood pressure compared with preinjection level.

$P < 0.05$ significance compared with control drop in blood pressure.

trigeminal neurons to prevent CGRP release, similar to previous findings that nNOS inhibitors seem to prevent NO production from trigeminal terminals and thus, reduce CGRP release. Anandamide seems to be acting on a CB₁ receptor because the highly specific CB₁ receptor antagonist AM251 was able to reverse this effect. There is evidence that the CB₁ receptor plays a part in modulating neuronal type (N-type) calcium channel currents, in that, activation of the CB₁ receptor in neuroblastoma cells was shown to inhibit the N-type calcium channel, via a pertussis-sensitive G protein (Caulfield and Brown, 1992). Inhibiting the N-type calcium channel thus reduces calcium transport into cells and, therefore, reduces the likelihood of cells firing (Pan et al., 1998). It is possible that anandamide is decreasing the likelihood of cell firing and therefore, reducing the likelihood of CGRP release and nNOS activity in presynaptic trigeminal sensory nerve terminals.

Anandamide was able to attenuate the NO-induced dural vessel dilation by nearly 40%, CGRP₈₋₃₇ by over 50%. Indometacin also attenuates NO-induced dural vessel dilation perhaps by inhibiting the activity of trigeminal neurons (Akerman et al., 2002a). Pestonjamas and Burstein (1998) suggested that indometacin may actually protect anandamide from metabolism into prostaglandin ethanolamine, thus allowing more anandamide to be present in the system. Others have suggested that increasing levels of anandamide by prolonging its time at the synapse, helps exert its antinociceptive effects (Guhning et al., 2002). It is likely then that anandamide is inhibiting the NO-induced dilation in a similar way to that described with neurogenic dural vasodilation, by decreasing the likelihood of trigeminal neurons firing, and inhibiting CGRP release. CGRP₈₋₃₇ is likely to be blocking trigeminal sensory nerve fiber activated NO-induced CGRP release postsynaptically. Similar findings have been found in cats, in that NO-induced cerebral vasodilation was reduced by CGRP₈₋₃₇ (Wei et al., 1992).

Blood Pressure Effects. Anandamide and AM251 were both found to have an effect on blood pressure when given intravenously. Anandamide reduced the blood pressure, and this was accompanied by a dose-dependent increase in dural blood vessel diameter. It is believed that this effect may reflect activity at the vanilloid type 1 receptor rather than the CB₁ receptor as anandamide has been found to act as an agonist at the vanilloid type 1 receptor (Zygmunt et al., 1999). Further research into this area needs to be done before we can conclude that anandamide is able to activate trigeminal neurons and causes dural vessel dilation. AM251 also reduced blood pressure with a slight decrease in dural vessel diameter.

The profound blood pressure effects of CGRP and capsaicin seemed to be reduced after anandamide treatment. Neither anandamide nor CGRP₈₋₃₇ was able to alter the blood pressure changes produced by sodium nitroprusside. It has been shown previously that the effects of capsaicin on blood pressure are reduced over consecutive injections; therefore, it seems likely that a similar reaction is occurring here rather than an effect of anandamide. There are other known vascular changes caused by anandamide that are endothelial, NO-dependent that are mediated by an anandamide receptor distinct from the CB₁ receptor (Pratt et al., 1998; Wagner et al., 1999). It has been shown that the hypotensive effects of CGRP can be reduced with the use of a β -adrenergic antag-

onist, without affecting the brain blood flow (Shen et al., 2001) indicating that the blood flow changes are in some part not simply due to cerebral CGRP receptors. These data suggest that the vasodilator effects of CGRP and capsaicin are not directly related to their hypotensive effects.

Anandamide or the CB₁ receptor site represents a potential therapeutic target for migraine, given anandamide's ability to attenuate neurogenic, CGRP, and NO-induced dural vessel dilation. It has been shown for the first time that anandamide is able to inhibit the neurons in the trigeminovascular system when activated by various approaches. This inhibition is likely to involve both pre- and postsynaptic mechanisms. The data also suggest that anandamide may tonically inhibit neuronal firing in the trigeminovascular system, and manipulation of its transport reuptake, with endocannabinoid transporter blockers, may provide a method of reducing the known psychoactive effect of anandamide that limit its use in the clinic, whereas maintaining an antinociceptive effect. It will be necessary to use more specific CB₁ receptor antagonists to dissect fully the role of the CB₁ receptor on blood pressure.

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