Anandamide is able to inhibit trigeminal neurons using an *in vivo* model of trigeminovascular-mediated nociception

S Akerman, H Kaube and PJ Goadsby[*]

Headache Group
Institute of Neurology
and
The National Hospital for Neurology and Neurosurgery
Queen Square
London, UK

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Anandamide and the trigeminovascular system

Correspondence:

Professor P. J. Goadsby
Institute of Neurology
Queen Square
London
WC1N 3BG UK
Telephone: +44 20 7829 8749
Fax: +44 20 7813 0349
Email: peterg@ion.ucl.ac.uk

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Abstract

Arachidonylethanolamide (AEA or anandamide) is believed to be the endogenous ligand of the cannabinoid CB_1 and CB_2 receptors. CB_1 receptors have been found localized on fibers in the spinal trigeminal tract and spinal trigeminal nucleus caudalis. Known behavioural effects of anandamide are anti-nociception, catalepsy, hypothermia and depression of motor activity, similar to Δ^9-tetrahydocannanbinol, 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 CB_1 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%, CGRP by 30%, capsaicin by 45% and nitric oxide by 40%. $CGRP_{8-37}$ was also able to attenuate NO-induced dilation by 50%. The anandamide inhibition was reversed by the CB_1 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 pre-synaptically, to prevent CGRP release from trigeminal sensory fibers, and post-synaptically to inhibit the CGRP-induced NO release in the smooth muscle of dural arteries. CB_1 receptors appear 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 non-cannabinoid 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 Aδ 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 (AEA or anandamide) is believed to be the endogenous ligand to the cannabinoid CB_1 and CB_2 receptors (Hoehe et al., 1991; Matsuda et al., 1990) that are found in the central and peripheral nervous systems. CB_1 receptors are localized on fibers in the spinal trigeminal tract and spinal trigeminal nucleus caudalis (Tsou et al., 1998), whereas CB_2 receptors are found predominantly in the immune system (Egertová and Elphick, 2000). The CB_1 and CB_2 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 CB_1 receptors found on cerebral artery smooth muscle and the smooth muscle of other vascular beds and modulate cerebral vascular tone (Gebremedhin et al., 1999; Plane et al., 1997). 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 CB_1 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 anti-nociception, catalepsy, hypothermia and depression of motor activity (Adams et al., 1998; Dewey, 1986). 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 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, calcitonin gene-related peptide (CGRP), capsaicin and NO-induced dural dilation (Akerman et al., 2002a; Akerman et al., 2003b; Williamson et al., 1997a; Williamson et al., 1997b). We were also able to monitor the mean arterial blood pressure (MABP) changes caused by the chemical vasodilators, and the effect of ananadamide. 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 anti-migraine properties (Olesen et al., 2003). Nitric oxide (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 anti-migraine 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 CB_1 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 if NO-induced dilation could be inhibited by $CGRP_{8-37}$, 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 (180-385g) were anaesthetized throughout the experiments with sodium pentobarbitone (60 mgkg^{-1} i.p. and then 18 mg^{-1}kg^{-1}hr-i.v. infusion). The left femoral artery and vein were cannulated for blood pressure recording and intravenous infusion of anesthetic and test compounds, respectively, while 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, 3-5 mls, 60-80 strokes per minute (Small Rodent Ventilator – Model 683, Harvard Instruments, UK). End-tidal CO_2 was monitored (Capstar-100, CWE Inc., USA) and kept between 3.5–4.5 % and blood pressure was monitored continually. This allows one to monitor for changes to 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 (Microvision MV2100, UK) and the image displayed on a television monitor. Dural blood vessel diameter was continuously measured using a video dimension analyzer (Living Systems Instrumentation, USA) and displayed with blood pressure on an online data analysis system (CED spike4 v2 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) placed on the surface of the cranial window approximately 200μm from the vessel of interest. The surface of the cranial window was stimulated at 5 Hz, 1 ms for 10 seconds (Grass Stimulator S88, Grass Instrumentation) with increasing voltage until maximal dilation was observed. Subsequent electrically induced responses in the same animal were then evoked using that voltage (Akerman et al., 2002c; Williamson et al., 1997b).

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 μgkg^{-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 μgkg^{-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 minute infusion at 2 $\mu gkg^{-1}min^{-1}$ 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), in order 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 (unpublished data). CGRP and 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 to 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 minutes later anandamide (1 mgkg^{-1}) was administered intravenously, and the vasodilator repeated 10 minutes later. Using the same protocol a second dose of anandamide (3 mgkg^{-1}) was given and a third vasodilator challenge performed. In the series of experiments involving CGRP a third dose of anandamide (10 mgkg^{-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 μgkg^{-1}min^{-1}) for ten minutes. Completion of the second infusion was followed, at least ten minutes later, by a bolus injection of anandamide (3 mgkg^{-1}) which was followed ten minutes later by a repeat of the sodium nitroprusside infusion.

Effect of a cannabinoid type 1 (CB$_1$) receptor antagonist in reversing the actions of anandamide

In separate series of experiments, the response of the selective cannabinoid type 1 (CB$_1$) 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 mgkg^{-1}) against either electrical stimulation, CGRP or sodium nitroprusside. After the observation of the effects of anandamide, AM251 (3 mgkg^{-1}), a selective cannabinoid type 1 (CB$_1$) receptor antagonist was administered intravenously and was followed by another repeat of the vasodilator challenge five minutes later. It has been shown previously that the anti-nociceptive affects of anandamide, using tail-flick responses, last up to at least an hour (Adams et al., 1995; Smith et al., 1994). 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 CB$_1$ antagonist.

Effect of the CGRP receptor blocker, CGRP$_{8-37}$, on NO-induced dilation.

The NO donor, sodium nitroprusside was infused for ten minutes in the rat to provide a control vasodilation. At least ten minutes after the dural blood vessel had returned to its original baseline state a second infusion was begun, after 5 minutes of infusion CGRP$_{8-37}$ (300 μgkg^{-1}) was given as an intravenous bolus, and the sodium nitroprusside infusion continued for a further five minutes.

Data Analysis

The effects of electrical stimulation, bolus of CGRP or capsaicin, and sodium nitroprusside infusion on dural vessel diameter were calculated as a percentage

increase from the pre-stimulation baseline diameter. The nature of the experimental set-up, where the magnification of the dural vessel visualised was different in each set-up by virtue of selecting an appropriate target vessel, made it impossible to standardise the dural vessel measurement, therefore, the dural vessel diameter were measured in arbitrary units. The typical vessel diameter measured ranges from 120 - 150 μm. Data are expressed as mean ± SEM. Statistical analysis was initially performed using an ANOVA 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 v10.0) on effects found different in the ANOVA 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, in water soluble emulsion) was further diluted in water for injection. It has been shown previously that dosing between $1 - 10$ mgkg^{-1} is anti-nociceptive in the rat, and this effect can last up to 30 minutes (Adams et al., 1998; Adams et al., 1995; Stein et al., 1996). CGRP (Sigma-Aldrich, UK) and the CGRP receptor blocker, CGRP$_{8-37}$ (Sigma-Aldrich, UK) were dissolved in deoxygenated water, aliquotted and frozen until required and then redissolved in 0.9% NaCl for use. Capsaicin (Sigma-Aldrich) was dissolved in a 1:1:8 solution of Tween 80 (polyoxyethylene-sorbitan mono-oleate, Sigma-Aldrich, UK): ethanol: 0.9% NaCl. AM251 (Tocris Cookson, UK) was initially dissolved in a couple of drops of DMSO (Sigma-Aldrich, UK) and further

dilated in the solution of Tween 80, ethanol and saline. Sodium nitroprusside (Sigma-

Aldrich) was dissolved in 0.9% NaCl.

Results

Effect of anandamide and then CB$_1$ receptor antagonism, on electrical stimulation

In rats treated with anandamide (1 mgkg^{-1}, $n = 6$ and 3 mgkg^{-1}, $n = 12$) the dilation brought about by electrical stimulation was significantly reduced with both doses of anandamide, $125 \pm 10\%$ to $72 \pm 10\%$ (1 mgkg^{-1}, $t_5 = 4.3$, $P < 0.05$) and $120 \pm 8\%$ to $53 \pm 8\%$ (3 mgkg^{-1}, $t_{11} = 7.6$, $P < 0.05$). The reduction of the electrically induced dilation brought about by anandamide (3 mgkg^{-1}) was significantly reversed by the CB$_1$ receptor antagonist AM251 (3 mgkg^{-1}, $n = 6$, $t_5 = 4.3$), $47 \pm 13\%$ to $99 \pm 16\%$, this return of dilation was not significant from the original control dilation, $115 \pm 12\%$ to $99 \pm 16\%$ (figures 1 and 2).

Effect of anandamide and then CB$_1$ receptor antagonism on CGRP-induced dilation

Increases in dural blood vessel diameter evoked by CGRP (1 μgkg^{-1}, i.v.) were significantly inhibited when pre-treated with anandamide, $120 \pm 7\%$ to $89 \pm 10\%$ (3 mgkg^{-1}, $n = 14$, $t_{13} = 6.6$, $P < 0.05$) and $129 \pm 11\%$ to $89 \pm 14\%$ (10 mgkg^{-1}, $n = 6$, $t_5 = 5.7$, $P < 0.05$), respectively. The reduction of the CGRP-induced dilation brought about by anandamide (3 mgkg^{-1}) was significantly reversed by the CB$_1$ receptor antagonist AM251 (3 mgkg^{-1}, $n = 8$, $t_7 = 6.1$), $87 \pm 7\%$ to $129 \pm 11\%$, this return of dilation was significantly different from the original control dilation, $114 \pm 9\%$ to $129 \pm 11\%$ (3 mgkg^{-1}, $n = 8$, $t_7 = 2.6$; figures 1 and 3).

Effect of anandamide on capsaicin-induced dilation

Dural blood vessel dilation brought about by capsaicin showed a significant reduction after pre-treatment with anandamide (1 and 3 mgkg^{-1}, $n = 6$) when compared to the control dilation, $118 \pm 15\%$ to $89 \pm 14\%$ ($t_5 = 4.0$) and $65 \pm 18\%$ ($t_5 = 5.4$), respectively ($P < 0.05$; figure 4).

Effect of anandamide and then CB$_1$ receptor antagonism on sodium nitroprusside-induced dilation

Dural blood vessel dilation brought about by sodium nitroprusside infusion (2 μgkg^{-1} min^{-1}) showed a significant reduction after pre-treatment with a 3 mgkg^{-1} dose of anandamide, $112 \pm 10\%$ to $71 \pm 9\%$ ($n = 14$, $t_{13} = 6.2$, $P < 0.05$). The reduction of the sodium nitroprusside-induced dilation brought about by anandamide (3 mgkg^{-1}) was significantly reversed by the CB$_1$ receptor antagonist AM251 (3 mgkg^{-1}, $n = 6$, $t_5 = 3.4$), $79 \pm 11\%$ to $112 \pm 19\%$, this return of dilation was not significant from the original control dilation, $123 \pm 16\%$ to $112 \pm 19\%$, see figures 1 and 5.

Effect of AM251 on dural blood vessel caliber

AM251 caused a non-significant $7 \pm 6\%$ ($n = 22$, $t_{21} = 1.542$, $P = 0.138$) decrease in dural blood vessel caliber that was restored to pre-injection levels before vasodilator challenge was repeated.

Effect of CGRP$_{8-37}$ on NO-induced dural blood vessel diameter

The CGRP receptor blocker, CGRP$_{8-37}$ (300 μgkg^{-1}, $n = 8$) was able to inhibit NO-induced dilation, $117 \pm 8\%$ to $56 \pm 14\%$ ($t_6 = 4.8$, $P < 0.05$). The inhibition was also significant when compared to the initial control NO-induced dilation $104 \pm 12\%$ to $56 \pm 14\%$ ($t_7 = 4.1$, $P < 0.05$; figure 6.

Effect of anandamide and AM251 on arterial blood pressure

Ananadamide caused a significant decrease in mean arterial blood pressure of 30.3 ± 5 mmHg (1 mgkg^{-1}, $n = 16$, $t_{15} = 6.18$, $P < 0.001$), 30.4 ± 2 mmHg (3 mgkg^{-1}, $n = 38$, $t_{37} = 14.13$, $P < 0.001$) and 30.1 ± 7 (10 mgkg^{-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 mmHg ($n = 22$, $t_{21} = 5.431$, $P < 0.0001$), which was accompanied by a 7 ± 6 % decrease in dural blood vessel diameter, both were restored to pre-injection levels before vasodilator challenge was repeated.

The 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, see tables 1 and 2, however in the case of the 3 mgkg^{-1} dose of anandamide this drop was significantly reduced, 48 ± 5 mmHg drop compared to 29 ± 3 mmHg (3 mgkg^{-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 mgkg^{-1} dose, see 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 ± 3

mmHg compared to 35 ± 3 mmHg (1 mgkg^{-1}, $n = 6$, $t_5 = 2.87$, $P < 0.05$) and 29 ± 4 mmHg (3 mgkg^{-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 when compared to 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, CGRP$_{8-37}$ was given during a sodium nitroprusside infusion, 102 ± 10 mmHg to 103 ± 10 mmHg.

Discussion

Anandamide was able to inhibit significantly neurogenic dural vasodilation, calcitonin gene-related peptide (CGRP), capsaicin and NO-induced dural vessel dilation found in the rat intravital microscopy model of trigeminovascular activation. The CB_1 receptor antagonist, AM251, was able to reverse the inhibition of dural vessel dilation mediated by anandamide for the neurogenic, CGRP and NO-induced dilation. $CGRP_{8-37}$ 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 CB_1 receptor antagonist, AM251, reversed this. This effect of anandamide may involve either pre or post-synaptic receptors, or both, to inhibit dural vasodilation. In contrast, we have reasoned that exogenous CGRP is acting directly on post-synaptic 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 CB_1 receptor antagonist, AM251. Therefore, it would appear that anandamide is at least acting post-synaptically to inhibit dural blood vessel dilation, and may, in addition, be acting pre-synaptically to account for the greater attenuation of the response. Since the CB_1 receptor antagonist, AM251, was able to reverse the effects of anandamide it seems likely that the responses reported are due to activation of the CB_1 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 (Maggi et al., 1988; Martling et al., 1988; Saito and Goto, 1986). We have also previously shown (Akerman et al., 2003b) using this model 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 post-junctionally 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 (a 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 methylester (L-NAME), a NO synthase inhibitor, were all able to reduce pain related behavior, and these effects were reversed by the CB_1 receptor antagonist, AM251(Guhring et al., 2002). It is interesting from a translational and head pain perspective that AM251 is able to reverse the anti-nociceptive 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 anti-nociceptive 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, $CGRP_{8-37}$, 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 $CGRP_{8-37}$ blocks the response post-synaptically. We have demonstrated previously that endothelial NO synthase inhibitors are able attenuate CGRP-induced dilation (Akerman et al., 2002c). Given that anandamide is able to attenuate both CGRP and NO-induced dilation, the CB_1 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 CB_1 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 eNOS inhibitors in the same model (Akerman et al., 2002c). Given the CB_1 receptor is present in the smooth muscle of cerebral arteries and other vascular beds (Gebremedhin et al., 1999; Hillard, 2000; Plane et al., 1997), it is possible that there are CB_1 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 eNOS.

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 pre-synaptic terminals of 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 appears to be acting on a CB_1 receptor as the highly specific CB_1 receptor antagonist, AM251, was able to reverse this effect. There is evidence that the CB_1 receptor plays a part in modulating neuronal type (N-type) calcium channel currents, in that, activation of the CB_1 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 pre-synaptic trigeminal sensory nerve terminals.

Anandamide was able to attenuate the NO-induced dural vessel dilation by nearly 40%, $CGRP_{8-37}$ by over 50%. Indometacin also attenuates NO-induced dural vessel dilation perhaps by inhibiting the activity of trigeminal neurons (Akerman et al., 2002a). Pestonjamasp 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 anti-nociceptive effects (Guhring 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. $CGRP_{8-37}$ is likely to be blocking trigeminal sensory nerve

fiber activated NO-induced CGRP release post-synaptically. Similar findings have been found in cats, in that NO-induced cerebral vasodilation was reduced by $CGRP_{8-37}$ (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 (VR1) rather than the CB_1 receptor as anandamide has been found to act as an agonist at the VR1 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 appeared to be reduced after anandamide treatment. Neither anandamide nor $CGRP_{8-37}$ 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 CB_1 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 antagonist, 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 CB_1 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 post-synaptic 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, whilst maintaining an anti-nociceptive effect. It will be necessary to use more specific CB_1 receptor antagonists to dissect fully the role of the CB_1 receptor on blood pressure.

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Table 1 Summary of blood pressure changes with vasodilators and anandamide

	Blood pressure decrease (mmHg)			
	Control	**Anandamide (1 mgkg⁻¹)**	**Anandamide (3 mgkg⁻¹)**	**Anandamide (10 mgkg⁻¹)**
CGRP (1 µgkg⁻¹)	47.9 ± 5*	35.2 ± 6*	29.5 ± 3*#	20.7 ± 2*
Capsaicin (7 µgkg⁻¹)	41.5 ± 3*	35.4 ± 3*#	28.7 ± 4*#	-
Sodium nitroprusside (2 µgkg⁻¹min⁻¹)	43.9 ± 5*	-	39.6 ± 5* and 38.6 ± 3*	-

CGRP – calcitonin gene-related peptide
* $P < 0.05$ significant drop in blood pressure compared to pre-injection level.
# $P < 0.05$ significance compared to control drop in blood pressure

Table 2 Summary of vasodilator induced blood pressure changes with anandamide and AM251

	Blood pressure decrease (mmHg)		
	Control	**Anandamide (3 mgkg^{-1})**	**AM251 (3 mgkg^{-1})**
CGRP (1 µgkg^{-1})	54.9 ± 4*	31.7 ± 5*	21.8 ± 3*
		#($n = 6$, t$_5$ = 3.27)	#($n = 6$, t$_5$ = 5.85)
Sodium nitroprusside **(2 µgkg^{-1}min^{-1})**	51.5 ± 7*	49.4 ± 6*	55.6 ± 4*

CGRP – calcitonin gene-related peptide

* P < 0.05 significant drop in blood pressure compared to pre-injection level.

 # P < 0.05 significance compared to control drop in blood pressure

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Figure 1. Sample traces of intravital videomicroscopy with anandamide

Traces show the inhibitory effects of anandamide (3 mgkg^{-1}) on A). neurogenic dural vasodilation B). CGRP-induced dural vessel dilation and C). sodium nitroprusside-induced dural vessel dilation. Traces A) and B) also highlight the effects of the CB$_1$ receptor antagonist, AM251 (3 mgkg^{-1}), in reversing anandamide induced changes. ES – electrical stimulation, CGRP – calcitonin gene-related peptide, NO – nitric oxide donor, sodium nitroprusside.

Figure 2. Effects of repeated electrical stimulation with anandamide treatment (grey bar) and the effect of AM251, a CB$_1$ receptor antagonist in reversing the anandamide response (black bar) on dural blood vessel diameter. Following control responses rats were injected with either anandamide (1 and 3mgkg^{-1}) or anandamide (3mgkg^{-1}) and then AM251 (3mgkg^{-1}) and electrical stimulation repeated after each separate drug intervention. * $P < 0.05$ significance compared to the control response. # $P < 0.05$ significance compared to the inhibitory response of anandamide.

Figure 3. Effects of repeated CGRP injection with anandamide treatment (grey bar) and the effect of AM251, a CB$_1$ receptor antagonist in reversing the anandamide response (black bar) on dural blood vessel diameter. Following control responses rats were injected with either anandamide (1 and 3mgkg^{-1}) or anandamide (3mgkg^{-1}) and then AM251 (3mgkg^{-1}) and CGRP injection repeated after each separate drug intervention. * $P < 0.05$ significance compared to the control response. # $P < 0.05$ significance compared to the inhibitory response of anandamide.

Figure 4. Effects of repeated capsaicin injection with anandamide treatment (grey bar) on dural blood vessel diameter. Following control responses rats were injected with anandamide (1 and 3mgkg^{-1}) and capsaicin injection repeated. * $P < 0.05$ significance compared to the control response.

Figure 5. Effects of sodium nitroprusside (SNP) infusion with anandamide treatment (grey bar) and the effect of AM251, a CB$_1$ receptor antagonist in reversing the anandamide response (black bar) on dural blood vessel diameter. Following control responses rats were injected with just anandamide (3mgkg^{-1}) or anandamide (3mgkg^{-1}) and then AM251 (3mgkg^{-1}) and SNP infusion repeated after each separate drug intervention. * $P < 0.05$ significance compared to the control response. # P < 0.05 significance compared to the inhibitory response of anandamide.

Figure 6. The effect of repeated sodium nitroprusside (SNP) infusion with CGRP receptor blocker (grey bar) on dural blood vessel diameter. During the second sodium nitroprusside infusion, which acts as control 2, rats were injected with CGRP$_{8-37}$ (300μgkg^{-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.
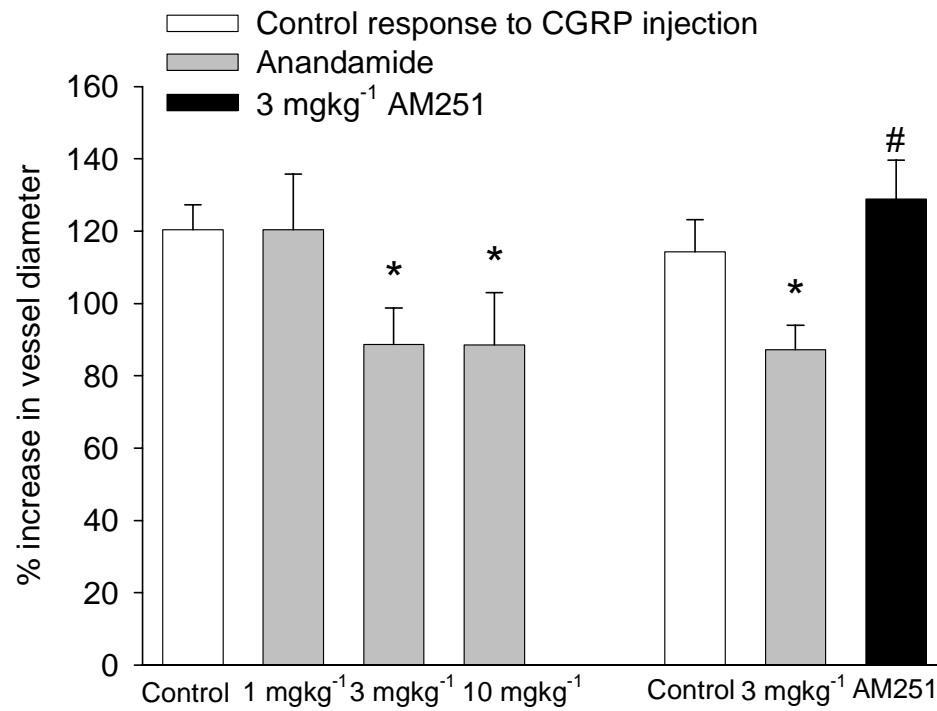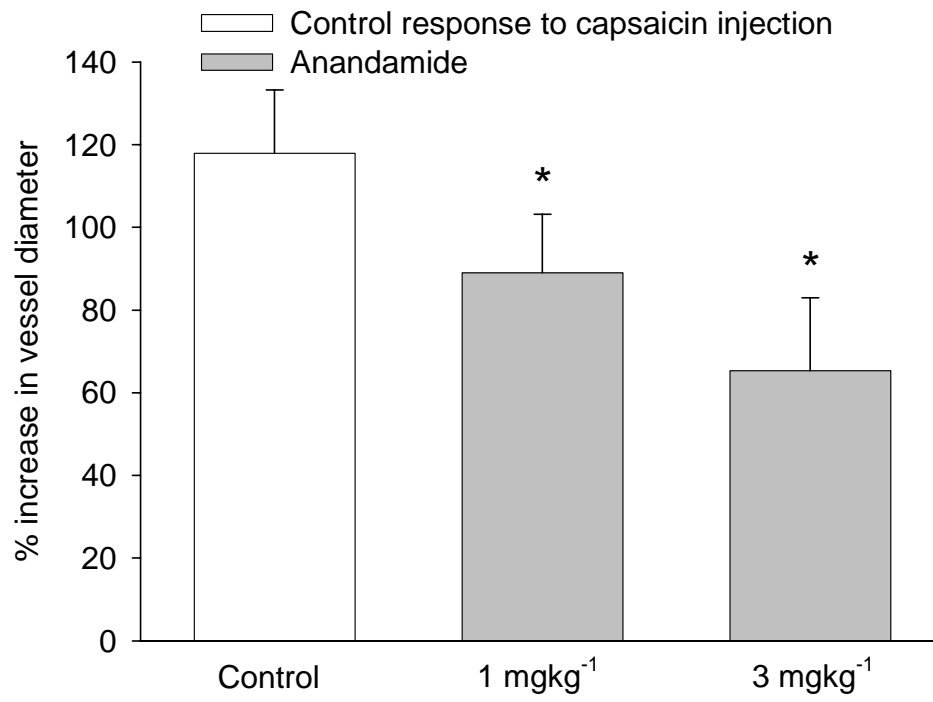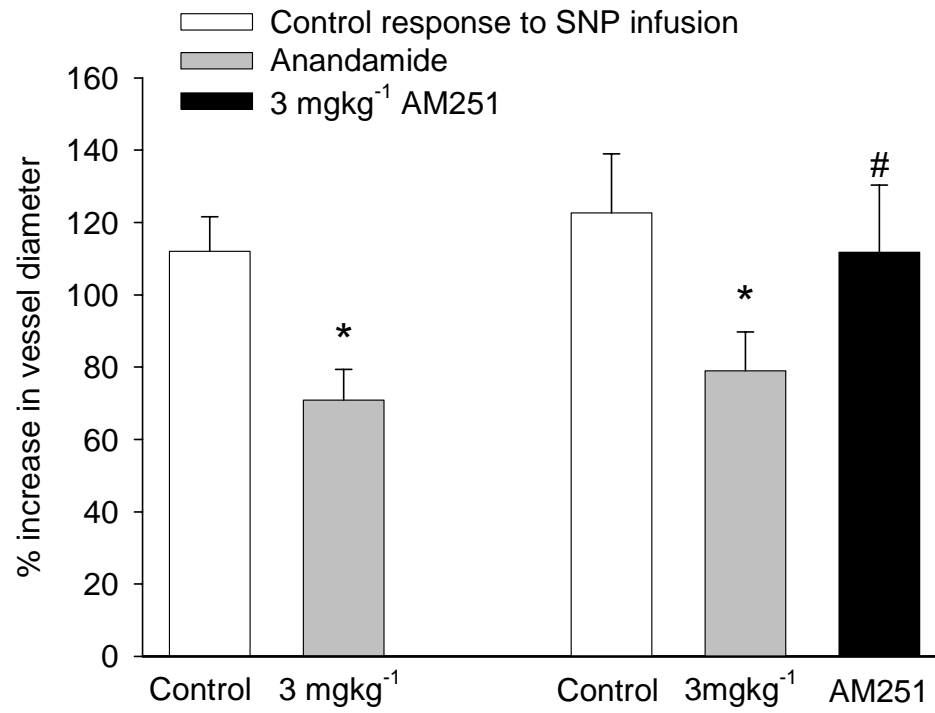
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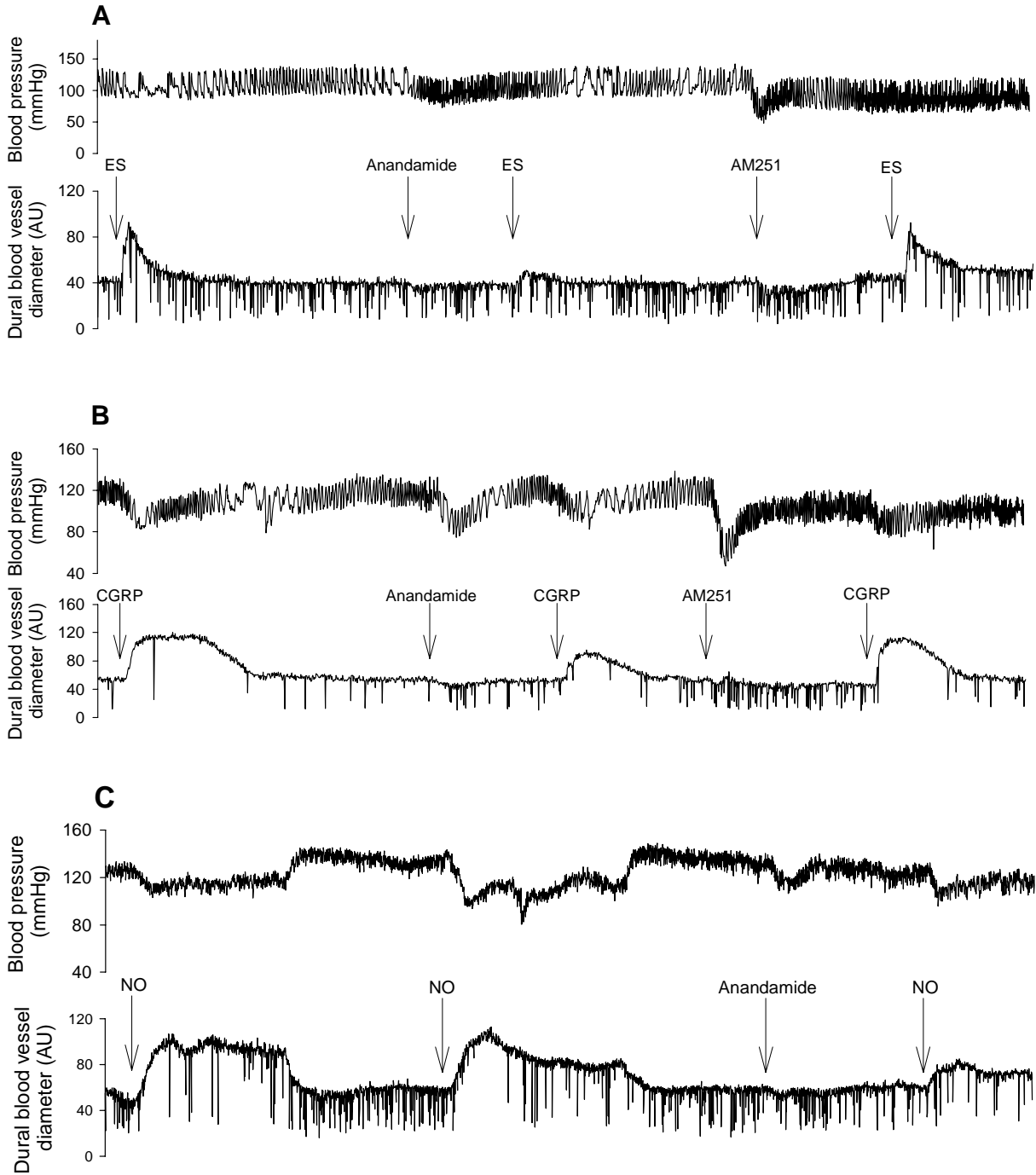
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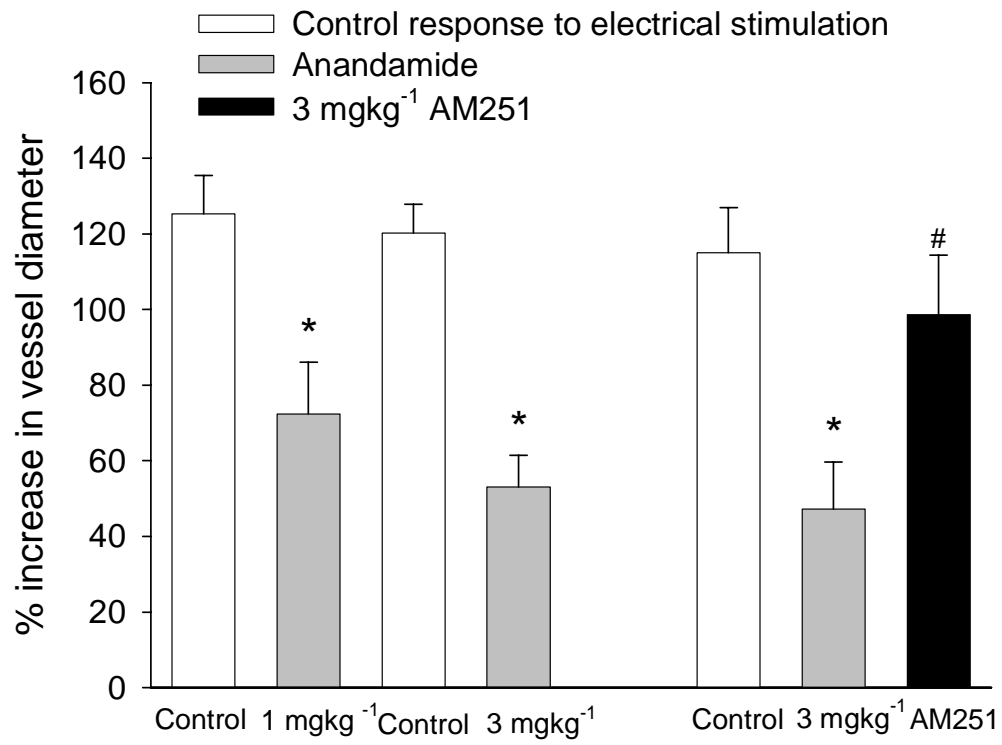
Figure 3

Figure 4

Figure 5

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