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# The anti-hyperalgesic actions of the cannabinoid anandamide and the putative CB2 receptor agonist palmitoylethanolamide in visceral and somatic inflammatory pain

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**Abstract**

This study assessed the effects of two *N*-acylethanolamides in established rat models of visceral and somatic inflammatory pain. (1) The therapeutic effects of the cannabinoid anandamide and the putative CB2 agonist palmitoylethanolamide were tested in a model of persistent visceral pain (turpentine inflammation of the urinary bladder). Both anandamide (at a dose of 25 mg/kg) and palmitoylethanolamide (at doses of 10–30 mg/kg) were able to attenuate the viscero-visceral hyper-reflexia (VVH) induced by inflammation of the urinary bladder. (2) The effects of the same compounds on the behavioural response to subcutaneous formalin injection were assessed. The characteristic biphasic response was observed in control animals. Anandamide (dose range 5–25 mg/kg) and palmitoylethanolamide (dose range 5–10 mg/kg) both reduced the second phase of the response. The results confirm the analgesic potential of endogenous ligands at cannabinoid receptor sites. The anti-nociceptive effect of the putative CB2 receptor agonist, palmitoylethanolamide, is particularly interesting since it is believed to be a peripherally mediated effect. This observation might be exploited to separate central psychotropic effects from peripheral analgesic actions of the cannabinoids, under inflammatory conditions. 

*Keywords:* Mast cell; Analgesia; Nociception; Autacoid local inflammation antagonism; Nerve growth factor; Rat

## 1. Introduction

The identification and cloning of cannabinoid receptors (Pertwee, 1993; Galiègue et al., 1995; Howlett, 1995) in both brain (CB1) (Matsuda et al., 1990) and peripheral tissues (CB2) (Kaminski et al., 1992; Munro et al., 1993; Bayewitch et al., 1995) has rekindled interest in the analgesic effects of cannabinoids. In addition, putative endogenous cannabinoid ligands have been described for both the central CB1 (Devane et al., 1992; Fride and Mechoulam, 1993) and peripheral CB2 cannabinoid receptors (Facci et al., 1995).

The brain constituent anandamide (ANA), has been shown to be produced by neuronal cells (Di Marzo et al., 1994) and have cannabamimetic effects (Fride and Mechoulam, 1993; Smith et al., 1994; Mechoulam et al., 1996; Stein et al., 1996). Agonists at the CB1 receptor site have been shown to exhibit anti-nociceptive activity in models of acute (Lichtman and Martin, 1991; Welch and Stevens, 1992; Mechoulam et al., 1994; Smith et al., 1994; Welch et al., 1995; Stein et al., 1996) and neuropathic pain (Herzberg et al., 1997). As with the endogenous opioid ligands, the duration of activity of ANA is thought to be short (Fride and Mechoulam, 1993; Smith et al., 1994; Welch et al., 1995; Stein et al., 1996).

Activation of the CB2 receptor appears to be more involved in downregulation of the inflammatory response (Aloe et al., 1993; Facci et al., 1995; Mazzari et al., 1996), although there is some evidence that activation may also inhibit the mechanical hyperalgesia associated with nerve injury (Mazzari et al., 1995). It has recently become clear that CB2 receptors are expressed on cells of immune origin, including lymphocytes, mast cells and macrophages (Facci

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et al., 1995; Galiègue et al., 1995). Palmitoylethanolamide (a candidate for the endogenous ligand at the CB2 receptor) accumulates in inflamed tissue (Natarajan et al., 1982) and has been shown to reduce mast cell degranulation, plasma extravasation and hyperalgesia in a dose dependent manner (Mazzari et al., 1996). It has been proposed that the local production of palmitoylethanolamide may lead to inhibition of both inflammation and the sensitising effects of inflammatory products on nociceptive processes (autacoid local inflammation antagonism; ALIA) (Aloe et al., 1993; Levi-Montalcini et al., 1996); this may be a CB2 receptor mediated effect. Thus, it has been suggested that the unwanted psychotropic effects of cannabinoid agonists may potentially be divorced from the anti-inflammatory and anti-nociceptive effects (Reggio, 1993; Friedman et al., 1994) by exploiting CB2 receptor agonists.

However, the majority of studies which have investigated the antinociceptive effects of cannabinoids has centred on the use of models which examine reflex responses to a single noxious stimulus, such as the tail-flick (Lichtman and Martin, 1991; Welch and Stevens, 1992) and hot-plate (Welch and Stevens, 1992; Stein et al., 1996) tests. Whilst of physiological relevance, these hyper-acute tests have limited relevance to clinical pain. A more persistent model is required to investigate the effects of post-operative and acute inflammatory pain. We have used two established models of persistent inflammatory pain to investigate the effects of cannabinoids in the visceral and somatic domains.

To study the visceral innervation we have utilised a model of chemical cystitis in which intravesical instillation of turpentine results in viscero-visceral hyper-reflexia (VVH) (McMahon and Abel, 1987; McMahon, 1988; Rice and McMahon, 1994; Rice, 1995; Dmitrieva et al., 1997). This is associated with: a referred hyperalgesia to the hind limb (Jaggar and Rice, 1997), sensitisation of both primary afferents in the pelvic nerve (McMahon, 1988; Dmitrieva and McMahon, 1996) and segmental dorsal horn neurones (McMahon, 1988), the recruitment of ‘silent’ afferents (McMahon et al., 1995b), increased expression of the early immediate gene, c-Fos, in the dorsal horn (Birder and De Groat, 1992; Dmitrieva et al., 1996) and an inflammatory response in the bladder tissue (McMahon and Abel, 1987).

We also utilised the sub-cutaneous (s.c.) formalin model described by Dubuisson and Dennis in 1977 (and later improved by others) (Dubuisson and Dennis, 1977; Tjolsen et al., 1992; Watson et al., 1997). Formalin injection results in a characteristic biphasic response of both behavioural (Dubuisson and Dennis, 1977) and electrophysiological (Dickenson and Sullivan, 1987) parameters. Whilst the first phase is a response to the injection process, the second phase is related to the sensitisation of nociceptive neurones at both receptor and spinal cord dorsal horn levels (i.e. primary and secondary hyperalgesia) (Tjolsen et al., 1992).

## 2. Materials and methods

### 2.1. Animal maintenance

All experiments conformed with British Home Office regulations. Bladder experiments were performed on 48 female Wistar rats weighing 180–240 g (mean weight 218 g). Formalin tests were performed on 49 female Wistar rats with a mean weight of 209 g (range 180–245 g).

### 2.2. Bladder inflammation

#### 2.2.1. Animal preparation

The method has been described in detail elsewhere (McMahon and Abel, 1987; Rice and McMahon, 1994; Rice, 1995; Dmitrieva et al., 1997). Animals were anaesthetised with a single dose of 1.25 g/kg intra-peritoneal (i.p.) urethane. Cannulation of the trachea and carotid (nylon catheter o.d. 0.75 mm) was performed. A sterile, lubricated urethral catheter (o.d. 1.02 mm, Portex, UK, 200/300/030) was passed into the bladder and secured using 3-0 silk ties around the external urethral orifice. A free flow of clear urine through the catheter was ensured. This, in conjunction with a mid-line laparotomy to directly visualise the bladder, confirmed correct catheter placement and ensured bladder emptying was complete after each cystometrogram (CMG). Saline soaked swabs over the wounds and a heated operating table protected the rats from hypothermia during the experiments. At the end of each experiment the animals were killed.

#### 2.2.2. Measurement of bladder motility

The CMG (pressure–volume relationship for the urinary bladder) was used to measure reflex bladder activity (McMahon and Abel, 1987; Rice and McMahon, 1994; Rice, 1995; Dmitrieva et al., 1997). CMGs were performed by infusing warm, sterile saline, infused into the bladder at a rate of 0.05 ml/min (via an infusion pump) to a total volume of 0.7 ml. This gradual filling produces a gradual increase in intra-vesical pressure until a critical threshold volume (usually approximately 0.25–0.5 ml) is reached. Once this threshold volume is reached large, regular, active, reflex micturition contractions of the bladder commence. The intra-vesical pressure was monitored via a pressure transducer, attached to a three way tap on the side arm of the infusion system (Neurolog 108T, Digitimer, UK) and then amplified (Neurolog NL108 system, Digitimer). Data were analysed and stored using the MP100 system (Biopac, Santa Barbara, CA, USA) PC and AcqKnowledge software. A single measure of the reflex bladder activity was derived from the CMG: the micturition threshold ($V_{mic}$), which is the intra-vesical volume at which the first micturition contraction occurred. Examples of a CMGs are shown in Fig. 1. CMGs were performed prior to inflammation (baseline), 2 h after induction of inflammation by intra-vesical turpentine oil and 1hr after treatment with the compound under investigation.

*2.2.3. Inflammation of the bladder*

Inflammation of the bladder was achieved by the instillation of 0.5 ml of 50% turpentine in olive oil for 1 h. This produces a sterile inflammatory response in the bladder wall associated with a VVH. VVH produces well-described changes in the post-inflammatory CMG, the most predictable of which is a lowering of $V_{mic}$. This can be observed from as early as 1 h post-turpentine and lasts for at least 24 h (McMahon and Abel, 1987; Rice and McMahon, 1994; Rice, 1995; Dmitrieva et al., 1997).

*2.2.4. Measurement of plasma extravasation into the bladder tissue*

We used a previously described technique to measure plasma extravasation, using Evans Blue dye (EB) as a marker (McMahon et al., 1984; McMahon and Abel, 1987). After completion of CMG recordings, animals were slowly perfused with 50 mg/kg EB via the intra-arterial (i.a.) cannula. Fifteen minutes later the animals were killed and the EB was cleared from the vascular compartment by a terminal trans-cardial perfusion with 50 ml of 0.9% saline. The bladders were then excised and weighed, before storage in 4 ml of filtered extraction mixture (70:30 acetone + 0.5 g/l sodium sulfide) for a minimum of 5 days. The quantity of EB extravasated into bladder tissue was then determined by spectrophotometry at $\lambda = 620$ nm and the tissue concentration of EB calculated.

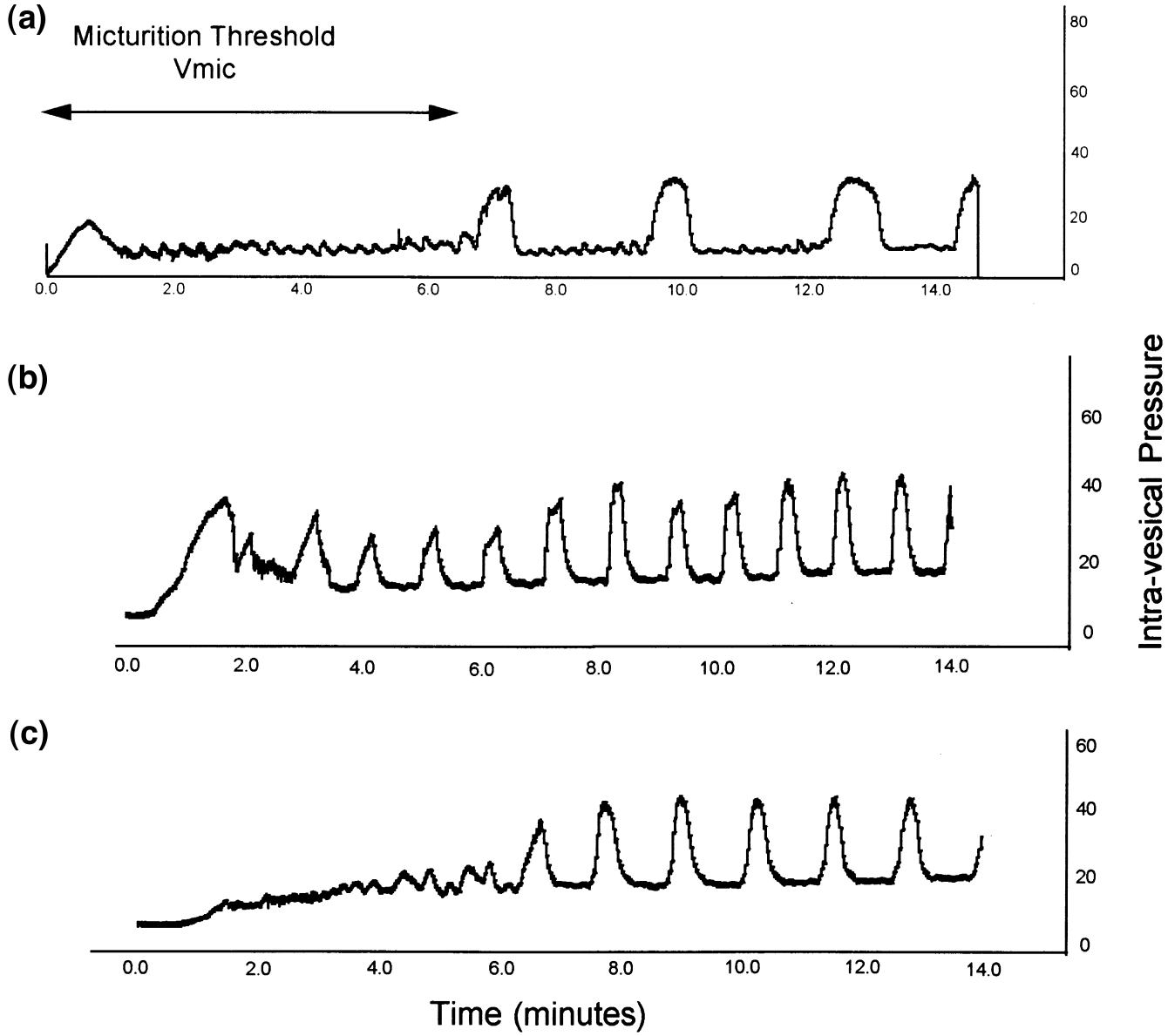


Fig. 1. (a) Example of a baseline cystometrogram (CMG) obtained by continuous intra-vesical pressure monitoring during bladder distension to 0.7 ml with sterile saline. Note the derivation of the micturition threshold ($V_{mic}$), that volume at which the first active micturition contraction occurs. (b) CMG from same animal taken 2 h after instillation of the bladder with 50% turpentine oil. Note the reduction in $V_{mic}$. (c) CMG from same animal obtained 1 h after i.a. treatment with 10 mg/kg palmitoylethanolamide. Note the increase in $V_{mic}$ towards baseline levels.

*2.2.5. Effects of cannabinoid receptor agonist administration*

To investigate the anti-nociceptive potential of two endogenous cannabinoid (CB) agonists, we used the CB1 agonist ANA and the putative CB2 agonist palmitoylethanolamide. These (or a vehicle control) were administered i.a. at the completion of the CMG performed 2 h after instillation of the bladder with turpentine. Doses employed ranged from 10 mg/kg to 25 mg/kg of ANA and 1 to 30 mg/kg of palmitoylethanolamide.

*2.3. Formalin test*

This well-established model of persistent somatic pain was first described by Dubuisson and Dennis in 1977 (Dubuisson and Dennis, 1977) and has since undergone modification (Tjolsen et al., 1992; Watson et al., 1997). Animals were acclimatised to the testing environment (a clear plexiglass box $23 \times 18 \times 14$ cm). Fifty microlitres of 2.5% formalin were administered s.c. into the dorsum of the right hind paw. The animal was then returned to the testing cage and its behaviour was observed. A mirror angled at 45° below the floor of the cage allowed an unobstructed view of the paws. Observations of the animals' behaviour were made in consecutive 5-min periods for the 60 min following formalin injection. The nociceptive response was quantified by measuring the amount of time spent displaying each one of four behaviour patterns, and a weighted score was calculated (Coderre and Melzack, 1992; Watson et al., 1997). The method of Watson et al. (1997) was used to calculate the composite pain score (CPS-$WST_{0,1,2}$) for each of the 5-min observation periods. In addition, an overall CPS-$WST_{0,1,2}$ was calculated for the first (0–15 min) and second (20–50 min) phases of the behavioural response (Watson et al., 1997).

*2.3.1. Formalin test: effects of cannabinoid receptor agonist administration*

ANA and the putative CB2 agonist palmitoylethanolamide were administered intra-peritoneally (i.p.) immediately prior to the s.c. formalin. The doses ranged from 5 mg/kg to 25 mg/kg for ANA and 1 mg/kg to 10 mg/kg for palmitoylethanolamide. There were two control groups, a formalin alone group ($n = 5$) and a solvent control group ($n = 6$).

*2.4. Materials*

ANA was purchased from Sigma and palmitoylethanolamide was purchased from Tocris Cookson. Both were both diluted in dimethyl sulfoxide (DMSO) and saline in a ratio of 1:19.

*2.5. Statistical comparison*

All statistical tests were performed using Sigmastat, Jandel Scientific Software. Significance level was taken at $P < 0.05$. Variability is expressed as standard error of the mean (SEM).

*2.5.1. Bladder reflex activity*

To confirm that the reduction in $V_{mic}$ associated with turpentine instillation was reversed by a treatment, the $V_{mic}$ post-inflammation (CMG2) was compared (paired *t*-test) to the $V_{mic}$ derived from post-drug (CMG3).

*2.5.2. Bladder: tissue inflammation*

EB tissue concentrations were compared using analysis of variance (ANOVA).

*2.5.3. Formalin test*

The overall CPS-$WST_{0,1,2}$ (Watson et al., 1997) for the first and second phases of the behavioural response were compared using ANOVA. Dunnett's test against the saline control group was then performed, as the post-hoc test (Sigmastat, Jandel Software).

## 3. Results

*3.1. Reflex bladder responses*

Mean baseline $V_{mic}$ ($n = 40$) was 0.45 (±0.02) ml. Tur-

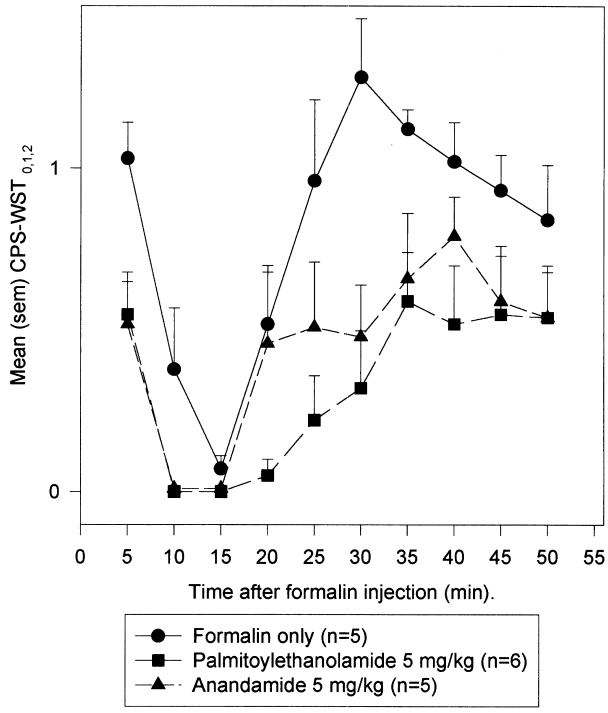


Fig. 2. Example of the behavioural response to s.c. injection of formalin (2.5%, 0.5 ml), showing the composite pain score (CPS-$WST_{0,1,2}$; see text for explanation) plotted against time. Note the biphasic nature of the response and the reduction in CPS-$WST_{0,1,2}$ during the late phase in animals which had been pre-treated with ANA (5 mg/kg) or palmitoylethanolamide (5 mg/kg).

pentine inflammation of the bladder resulted in a significant VVH as demonstrated by a reduction in $V_{mic}$. Data to establish this was pooled from all animals that had a CMG performed at 2 h post-turpentine (CMG2) prior to treatment ($n = 40$). The mean reduction in $V_{mic}$ was to 43.27 ± 2.74% of baseline values (paired $t$-test, $P < 0.001$).

### 3.2. Effect of cannabinoids on reflex bladder responses (Figs. 3 and 4)

In the control group, DMSO + saline ($n = 6$) administration did not result in a reversal of the inflammation-associated reduction in $V_{mic}$.

The CB1 agonist ANA only reversed the inflammation associated decline in $V_{mic}$ at a the highest dose of 25 mg/kg ($n = 6$) (paired $t$-test). There was no significant effect at doses of 10 mg/kg ($n = 6$) or 15 mg/kg ($n = 6$).

Palmitoylethanolamide showed no significant effect at a dose of 1 mg/kg ($n = 6$) although there appeared to be a trend towards reversal of the inflammation associated decline in $V_{mic}$. The fact that this trend does not reach significance is possibly due to inter-animal variability in the response at this dose level. This may reflect the fact that the threshold of effects may lie in the region of 1 mg/kg. However, at doses of 10 mg/kg, 20 mg/kg ($n = 6$) and 30 mg/kg ($n = 5$), there was a significant reversal in the $V_{mic}$ towards baseline values.

### 3.3. Bladder: tissue inflammation

The inflammation-associated plasma extravasation of EB was not significantly different when all three groups were compared. The mean EB concentration was 0.62 (±0.08) mg/mg for the DMSO group, 0.82 (±0.13) mg/mg for the maximum CB1 agonist dose examined, and 0.58 (±0.15) mg/mg for the maximum palmitoylethanolamide dose examined.

### 3.4. Formalin test

The control group ($n = 5$) confirmed the previously described characteristic biphasic behavioural response to s.c. formalin. There was no significant difference in the overall CPS-$WST_{0,1,2}$ between the formalin only control group and the DMSO (solvent) control group for either phase.

### 3.5. Effect of cannabinoids on response to s.c. formalin (see Table 1)

The overall CPS-$WST_{0,1,2}$ for each phase of the behavioural response to formalin were compared to the values obtained from the formalin control group. Neither ANA nor palmitoylethanolamide attenuated the first phase of the behavioural response to formalin injection at any of the

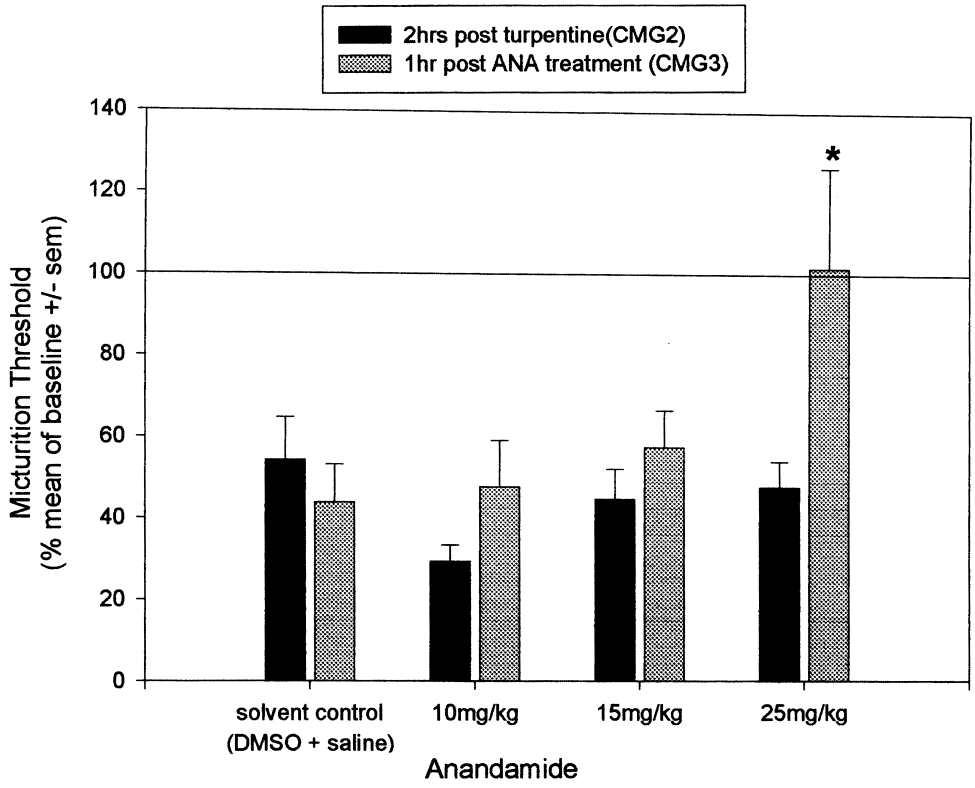


Fig. 3. The effect of post-inflammation administration of DMSO + saline and anandamide on the micturition threshold of the urinary bladder. There is a clear inflammation-associated decline in $V_{mic}$ and reversal of this effect by treatment with the CB1 receptor agonist, anandamide, at a dose of 25 mg/kg. *$P < 0.05$, ANOVA (Dunnett's) for 1 h post-drug administration (CMG3) with respect to post-turpentine (CMG2).

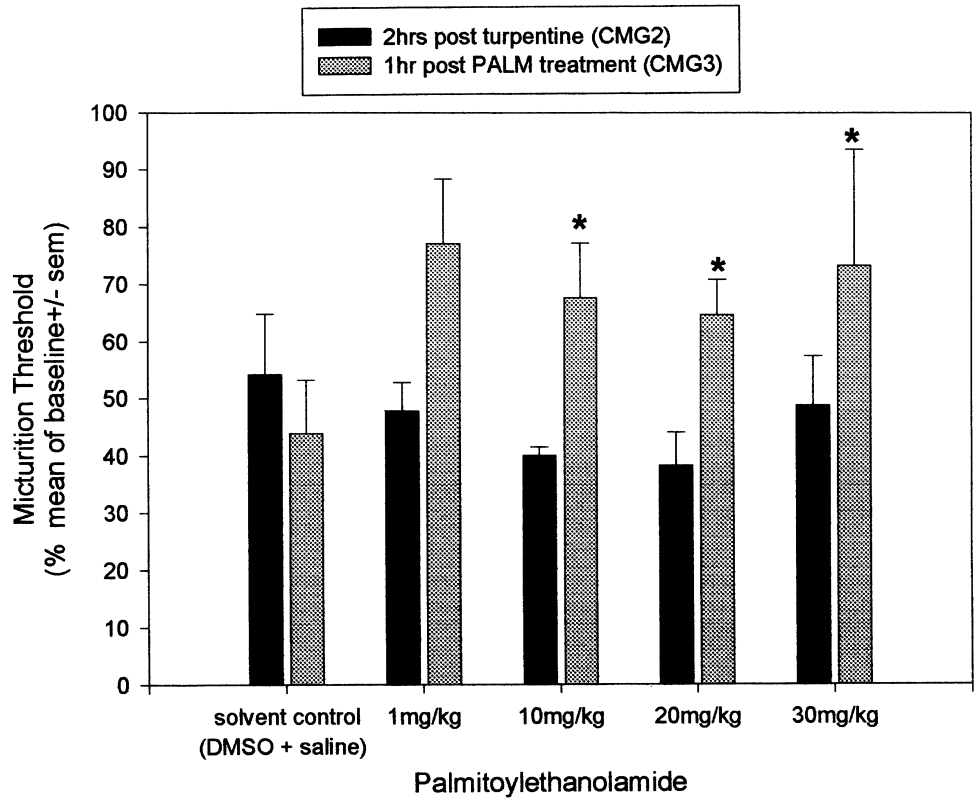


Fig. 4. The effect of post-inflammation administration of DMSO + saline and palmitoylethanolamide on the micturition threshold of the urinary bladder. The inflammation associated decline in $V_{mic}$ is reversed by treatment with the palmitoylethanolamide at doses of 10–30 mg/kg. *$P < 0.05$, ANOVA (Dunnett's) for 1 h post-drug administration (CMG3) with respect to post-turpentine (CMG2).

doses examined. ANA significantly ($P < 0.05$) reduced the CPS-$WST_{0,1,2}$ of the second phase of the behavioural response at all the doses tested between 5 and 25 mg/kg. Similarly, palmitoylethanolamide significantly ($P < 0.05$) reduced the second phase behavioural response, but only at the 5 and 10 mg/kg dose levels. An example plot of the CPS-$WST_{0,1,2}$ against time is given in Fig. 2.

## 4. Discussion

In the visceral pain (bladder) model the results confirm previous work, showing that the effect of turpentine instillation into the urinary bladder of the rat is to produce a VVH. The reduction in $V_{mic}$ 2 h after instillation of turpentine (McMahon and Abel, 1987; Jaggar et al., 1997) is related to an increased state of excitability of both primary afferents and dorsal horn neurones (McMahon, 1988; Dmitrieva and McMahon, 1996). This is associated with an increased expression of c-*fos* in the spinal cord (Birder and De Groat, 1992; Dmitrieva et al., 1996) and a referred hyperalgesia to the skin (Jaggar and Rice, 1997). In the somatic pain (formalin) model our results substantiate the typical biphasic behavioural response seen after s.c. formalin injec-

Table 1
Effect of anandamide and palmitoylethanolamide on the overall composite pain score (CPS-$WST_{0,1,2}$) for the first and second phases of the nociceptive response to formalin

| Drug | Dose (mg/kg) administered i.p. | CPS-$WST_{0,1,2}$ (0–15 min); mean (SEM) | CPS-$WST_{0,1,2}$ (20–50 min); mean (SEM) |
|---|---|---|---|
| Formalin ($n = 5$) | | 0.49 (0.11) | 0.96 (0.09) |
| Anandamide ($n = 5$) | 5 | 0.18 (0.04) | 0.58 (0.08)* |
| Anandamide ($n = 5$) | 10 | 0.30 (0.05) | 0.58 (0.06)* |
| Anandamide ($n = 6$) | 15 | 0.36 (0.99) | 0.53 (0.10)* |
| Anandamide ($n = 5$) | 25 | 0.36 (0.99) | 0.54 (0.16)* |
| Palmitoylethanolamide ($n = 5$) | 1 | 0.34 (0.05) | 0.77 (0.11) |
| Palmitoylethanolamide ($n = 6$) | 5 | 0.19 (0.04) | 0.40 (0.11)* |
| Palmitoylethanolamide ($n = 6$) | 10 | 0.32 (0.08) | 0.61 (0.08)* |

*$P < 0.05$, ANOVA (Dunnett's) for drug-treated animals in comparison to formalin alone (control) animals.

tion (see Fig. 2) (Dubuisson and Dennis, 1977; Coderre and Melzack, 1992; Tjolsen et al., 1992; Watson et al., 1997). High nociceptive scores were recorded during the first 5 min after s.c. formalin administration and were followed by a reduction in scores for 10–15 min. A later elevation was maintained until the end of the test period. The initial rise in nociceptive behaviour is in response to the injection of the noxious chemical itself, whilst the second phase response depends upon sensitisation of both peripheral and central (dorsal horn) neurones (Dickenson and Sullivan, 1987; Coderre and Melzack, 1992).

These results demonstrate that the endogenous cannabinoid, ANA, reverses the inflammation induced VVH and the second phase of the behavioural response to formalin injection. Palmitoylethanolamide, similarly attenuates both the vesical hyper-reflexic response and the behavioural response during the second phase of the formalin test.

It is now clear that at least two cannabinoid receptors are present in animal tissues (Howlett, 1995; Abood and Martin, 1996). The CB1 cannabinoid receptor is constitutive and its expression is predominantly localised to the tissues of the central nervous system. Binding studies have shown that although it is distributed throughout the brain and spinal cord, the highest concentrations are found in areas of the brain associated with higher cognitive functions and movement control (Glass et al., 1997). Although low levels of the CB1 receptor are also observed in tissues of the immune system and other peripheral tissues (including autonomic neurones), much higher concentrations of the CB2 cannabinoid receptor are observed at these peripheral sites (Galiègue et al., 1995; Abood and Martin, 1996; Pertwee and Fernando, 1996). Expression of the CB2 receptor is induced under conditions of immune cell activation (Abood and Martin, 1996).

In addition to the identification of cannabinoid receptors, putative endogenous ligands at these receptors have recently been identified. ANA is an endogenous, unsaturated *N*-acylethanolamide which is produced by cells of the central nervous system (Devane et al., 1992; Di Marzo et al., 1994). It fulfils criteria suggesting it is an endogenous agonist at the CB1 receptor site (Mechoulam et al., 1994). These include its isolation from brain tissue, demonstration of pharmacological cannabamimetic activity (which shows a structure activity relationship) and competitive displacement of the radio-labelled CB1 receptor probe HU-210 from binding sites in brain tissue (Devane et al., 1992). In animal models it has been shown to produce a wide variety of central effects also known to occur on the administration of classical cannabinoid compounds (Devane, 1994; Smith et al., 1994; Stein et al., 1996). These effects include: a reduction in spontaneous motor activity, hyper-responsiveness to sensory stimuli, hypothermia and antinociceptive effects in acute, somatic pain models such as the tail-flick (Smith et al., 1994; Welch et al., 1995) and hot-plate tests (Stein et al., 1996). In addition to binding to the CB1 receptor site, ANA shows a lesser degree of binding affinity to the CB2 receptor (Facci et al., 1995; Felder et al., 1995; Shire et al., 1996), but appears to have no functional agonist activity at the mast cell CB2 receptor (Facci et al., 1995).

The saturated *N*-acylethanolamide, palmitoylethanolamide accumulates in inflamed tissue (Natarajan et al., 1982) and in an inflammatory model decreases oedema formation and hyperalgesia in a mast cell dependent process (Mazzari et al., 1996). This phenomenon has been termed autacoid local inflammation antagonism (ALIA) (Aloe et al., 1993; Levi-Montalcini et al., 1996). There is some evidence that the actions of palmitoylethanolamide are mediated by agonist activity at CB2 receptors preventing antigen evoked release of 5-HT from mast cells. Displacement studies show that palmitoylethanolamide binds to CB2 receptors located on mast cells, in that it displaces a CB1/CB2 probe ([$^3$H]WIN-55,212-2) from mast cell membranes which express CB2, but not CB1, receptors (Facci et al., 1995). Evidence of palmitoylethanolamide agonist activity at CB2 receptors has also been shown for granule cells of the central nervous system (Skaper et al., 1996a). However, Showalter et al. have produced contradictory evidence that palmitoylethanolamide only weakly displaces the same CB1/CB2 probe from binding sites on membranes of CB2 receptor transfected cells (Showalter et al., 1996). Therefore the weight of evidence so far suggests that the anti-inflammatory actions of palmitoylethanolamide are possibly mediated via CB2 receptors expressed on mast cells and that palmitoylethanolamide is a candidate for the endogenous ligand at the CB2 receptor. However, it will not be possible to fully resolve the issue of whether the anti-hyperalgesic effects of palmitoylethanolamide are indeed mediated via CB2 receptors, until either selective CB2 antagonists, or transgenic animals which do not express the CB2 receptor, are generally available. Palmitoylethanolamide has no significant affinity for the CB1 receptor (Felder et al., 1993).

In our experiments ANA only reversed the visceral responses at the highest doses examined (which lie near the dose range shown to produce psychotropic effects) (Fride and Mechoulam, 1993; Stein et al., 1996) but attenuated the hyperalgesic behaviour in the formalin test at lower doses. Whilst brain CB1 receptors are the most likely site of action of the analgesic effect of ANA, other sites, such as the spinal cord or periphery, may contribute. CB1 receptors are present in the spinal cord (Howlett et al., 1990; Abood and Martin, 1996) and the VVH following turpentine inflammation is mediated via a spinal reflex (McMahon and Abel, 1987) which is influenced by spinal glutaminergic (NMDA) (Rice and McMahon, 1994) and nitric oxide systems (Rice, 1995). Likewise, there is evidence that the second phase of the formalin response is, at least in part, a spinal phenomenon, driven by on-going afferent activity also involving spinal NMDA receptors and nitric oxide (Haley et al., 1990; Malmberg and Yaksh, 1993). Whilst peripheral expression (under basal conditions) of the CB1 receptor is low (Bouaboula et al., 1993; Galiègue et al.,

1995; Abood and Martin, 1996), there is some evidence that peripheral CB1 receptor expression may be upregulated during inflammation (Abood and Martin, 1996) and their effects may participate in anti-nociceptive processes (Richardson et al., 1998).

The dose of ANA required to attenuate VVH was greater than that which has been required to demonstrate anti-nociception both here in the formalin test and in other somatic pain models (Smith et al., 1994; Stein et al., 1996). This is not without precedent, however, as we have previously shown that the doses of bradykinin antagonists needed to reverse the VVH effects following inflammation of the bladder are greater than those needed to reduce hyperalgesia in the somatic domain (Jaggar et al., 1997). This may reflect a more intense inflammatory stimulus in the bladder model, as opposed to the formalin test.

The response to ANA in the formalin model would appear to support experiments utilising hyper-acute somatic nociceptive models such as the tail-flick (Smith et al., 1994; Welch et al., 1995) and hot-plate (Stein et al., 1996) tests. In these models a dose-related antinociceptive response has been demonstrated at doses ranging from 10 to 100 mg/kg. In addition, it has previously been shown that the prototypical cannabinoid $\Delta^9$-tetrahydrocannabinol (when delivered by inhalation) can reduce nociceptive behaviour in both phases of the formalin response (Roach et al., 1996). The hyperalgesia associated with persistent neuropathic pain also appears to be attenuated by the CB1 receptor agonists (Herzberg et al., 1997), although ANA itself has not been assessed in this model.

We have shown that palmitoylethanolamide attenuates responses in both the visceral and somatic models. In the visceral model this was evidenced by a reduction in the VVH by administration of doses of 10–30 mg/kg, whilst in the formalin test, doses of 5–10 mg/kg reduced the CPS-$WST_{0,1,2}$ of the second phase of the test (the first phase was uneffected at any of the doses used). Palmitoylethanolamide has been shown to bind to CB2 receptors on mast cells and downregulate their activity (Aloe et al., 1993; Facci et al., 1995), thus attenuating the inflammatory response and reducing the hyperalgesic behaviour observed after carrageenan injection (Facci et al., 1995; Mazzari et al., 1996).

There is increasing evidence that the interaction between mast cells and the neurotrophin NGF is pivotal to the hyperalgesia which accompanies inflammation. Mast cells express the NGF *trkA* receptor, but also produce NGF (in addition to other sensitising molecules, for example serotonin and histamine) themselves, thus amplifying the sensitising effect of NGF on 1° afferent neurones (Aloe et al., 1993; Leon et al., 1994; Levi-Montalcini et al., 1996). The effects of palmitoylethanolamide could therefore be explained by the inhibition of mast cell activation reducing peripheral nociceptor sensitisation. Similar inhibition of VVH has been shown after the administration of locally acting compounds such as HOE 140 (an antagonist at the bradykinin-2 receptor) (Jaggar et al., 1997), locally delivered opioids (Andreev et al., 1995) and an NGF sequestering molecule (Dmitrieva et al., 1997). Mast cells are extensively distributed in the body and are most commonly located perivascularly and perineurally (Lundeberg et al., 1993; Theoharides, 1996). Since mast cells are important not only in generating the hyperalgesia associated with acute inflammation, but also in more chronic conditions such as interstitial cystitis (a common, chronic inflammatory condition of the bladder, most prevalent in females) (Letourneau et al., 1992; Lundeberg et al., 1993; Theoharides, 1996), cannabinoids acting at the CB2 receptor (with a longer duration of action than the endogenous ligands) may find a use in the treatment of acute and chronic inflammatory pain.

Failure of either ANA or palmitoylethanolamide to reduce oedema formation in bladder tissue (despite a reduction in VVH) implies a differential effect on hyperalgesia and tissue inflammation responses. Whilst this contradicts studies examining responses in the skin (Mazzari et al., 1996) where both the oedema and hyperalgesic responses to carrageenan were shown to be decreased by oral administration of palmitoylethanolamide, it is not without precedent. Our data regarding the effects of two bradykinin receptor antagonists on the turpentine inflamed bladder model show a similar separation of effects (Jaggar et al., 1997). In addition, evidence from the somatic domain has shown a differential response of hyperalgesia and oedema to paracetamol treatment (Bianchi and Panerai, 1996).

The ability of palmitoylethanolamide to reduce inflammation induced nociceptive responses, which we have shown in both the visceral and somatic domain, provides further evidence for the importance of ALIA. This is a mechanism by which it is postulated that the inflammatory response is modulated at the level of the mast cell (Levi-Montalcini et al., 1996). Mast cells are known to produce a wide variety of pro-inflammatory mediators, including the neurotrophin nerve growth factor (NGF) (Leon et al., 1994). NGF mRNA levels have been shown to be increased in inflamed tissue (Aloe et al., 1992; Andreev et al., 1994; Oddiah et al., 1995) and mast cells participate in the process (Lewin et al., 1994; McMahon et al., 1997). Since it can also stimulate mast cell activity (via its interaction with *trkA* receptors), it is able to amplify the inflammatory process. In the visceral pain model we used here, the essential role of NGF has already been documented (Dmitrieva et al., 1997) and in addition, in somatic models of inflammation, the sequestration of endogenous NGF has been shown to prevent hyperalgesia (Woolf et al., 1994; McMahon et al., 1995a).

The ability of putative CB2 agonists such as palmitoylethanolamide to downregulate mast cell activity provides a potential, controlling, negative effect on mast cell activity, during such inflammatory conditions. A similar effect has been shown to occur in cerebellar granule cells, where palmitoylethanolamide affords protection against glutamate induced neurotoxicity (Skaper et al., 1996a).

An analogous proposition of peripherally mediated analgesia has been made for endogenous opioids under inflammatory conditions (in a similar fashion to ALIA) (Stein, 1993). Endogenous opioid ligands are known to be produced by immune cells present in inflamed tissue, including lymphocytes, monocytes and macrophages. Since opioid receptors are located on peripheral immune cells (in addition to primary afferent neurones, sympathetic neuronal terminals and dorsal root ganglia), it is possible to envisage a local modulatory function of peripheral opioids. Additionally, endogenous opioids have been shown to provide analgesia both in animal models (Stein et al., 1989, 1990a,b; Schafer et al., 1994) and humans (Kalso et al., 1997).

In conclusion, our findings are that the cannabinoid ANA and the putative CB2 agonist palmitoylethanolamide are able to reverse both the VVH (but not the tissue oedema) induced by turpentine instillation into the bladder and the nociceptive behaviour induced by s.c. formalin injection in the hind paw of the rat. Since the weight of available evidence supports the fact that palmitoylethanolamide is a CB2 receptor agonist and is devoid of activity at brain CB1 cannabinoid receptors, it may be possible to divorce the unwanted central effects of cannabinoids from the peripheral analgesic effects during inflammation, thus leading the way for the development of novel cannabinoid analgesic agents devoid of psychotropic effects (and with longer duration of action than the endogenous ligands). It has also been suggested that this may also be an achievable goal using the effects of cannabinoids in the treatment of multiple sclerosis (Skaper et al., 1996b) and neuropathic pain (Mazzari et al., 1995; Herzberg et al., 1997).

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