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Antiinflammatory action of endocannabinoid palmitoylethanolamide and the synthetic cannabinoid nabilone in a model of acute inflammation in the rat

[1]Silvia Conti, [2]Barbara Costa, [2]Mariapia Colleoni, [3]Daniela Parolaro & *,[1]Gabriella Giagnoni

[1]Department of Biotechnology and Bioscience, Faculty of Sciences, University of Milan-Bicocca, Piazza della Scienza 2, 20126 Milan, Italy; [2]Department of Pharmacology, Via Vanvitelli 32, 20129 Milan, Italy and [3]Department of Structural and Functional Biology, Pharmacology Section, University of Insubria, Via Vanvitelli 32, 20129 Milan, Italy

1 The antiinflammatory activity of synthetic cannabinoid nabilone in the rat model of carrageenan-induced acute hindpaw inflammation was compared with that of the endocannabinoid palmitoylethanolamide and the nonsteroidal antiinflammatory drug indomethacin.

2 Preliminary experiments in rats used a tetrad of behavioural tests, specific for tetrahydrocannabinol-type activity in the CNS. These showed that the oral dose of nabilone 2.5 mg kg^{-1} had no cannabinoid psychoactivity.

3 Intraplantar injection of carrageenan (1% w v^{-1}) elicited a time-dependent increase in paw volume and thermal hyperalgesia.

4 Nabilone (0.75, 1.5, 2.5 mg kg^{-1}, p.o.), given 1 h before carrageenan, reduced the development of oedema and the associated hyperalgesia in a dose-related manner. Nabilone 2.5 mg kg^{-1}, palmitoylethanolamide 10 mg kg^{-1} and indomethacin 5 mg kg^{-1}, given p.o. 1 h before carrageenan, also reduced the inflammatory parameters in a time-dependent manner.

5 The selective CB_2 cannabinoid receptor antagonist {N-[(1S)-endo-1,3,3-trimethyl bicyclo [2.2.1]heptan-2-yl]-5-(4-chloro-3-methylphenyl)-1-(4-methylbenzyl)pyrazole-3 carboxamide} (SR 144528), 3 mg kg^{-1} p.o. 1 h before nabilone and palmitoylethanolamide, prevented the anti-oedema and antihyperalgesic effects of the two cannabinoid agonists 3 h after carrageenan.

6 Our findings show the antiinflammatory effect of nabilone and confirm that of palmitoylethanolamide indicating that these actions are mediated by an uncharacterized CB_2-like cannabinoid receptor.

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Keywords: Cannabinoids; nabilone; palmitoylethanolamide; inflammation; carrageenan oedema; cannabinoid receptor

Abbreviations: COX, cyclo-oxygenase; IL, interleukin; PAF, platelet activating factor; PG, prostaglandin; THC, tetrahydrocannabinol; TNF, tumour necrosis factor

Introduction

Δ^9-Tetrahydrocannabinol (Δ^9-THC), the major psychoactive component of marijuana, has a wide range of pharmacological effects, which may have therapeutic value. Despite the potential therapeutic applications of Δ^9-THC, ranging from analgesia, anti-emesis and appetite stimulation, to anti-glaucoma properties, its medical use has been limited by its psychotropic effects (Razdan & Howes, 1983; Mechoulam, 1986). In recent years, much effort has been made to discover synthetic analogues that have the medicinal properties without the psychotropic effects.

Among the synthetic derivatives, Δ^8-THC-11-oic acid (THC-11), orally administered to mice, reduced the oedema caused by topical application of arachidonic acid on the ear or by intraplantar injection of platelet activating factor (PAF) (Burstein *et al*., 1989). This cannabinoid also inhibited the production of 5-hydroxyeicosatetraenoic acid, PGE_2 and $PGF_{2\alpha}$ *in vitro* in mouse peritoneal cells. Its derivative with a dimethylheptyl side chain, 1^1-1^1-dimethylheptyl-Δ^8-THC-11-oic acid (CT-3), was even more potent and showed analgesic activity in the mouse hot-plate and phenylquinone-writhing tests (Burstein *et al*., 1998) and antiinflammatory activity in acute and chronic models of inflammation (Zurier *et al*., 1998). After oral administration CT-3 suppressed acute inflammation induced in mice by interleukin-1β (IL-1β) and tumour necrosis factor α (TNFα) in the subcutaneous air pouch and reduced the severity of adjuvant-induced polyarthritis in the rat. CT-3 suppressed cyclo-oxygenase-2 (COX-2) activity in 3T3 cells in tissue culture (Zurier *et al*., 1998) and, unlike the non-steroidal antiinflammatory drug indomethacin, did not induce gastrointestinal ulcera when administered either acutely or chronically (Dajani *et al*., 1999).

The antiinflammatory properties of CT-3 were shared by the endogenous ligand at the CB_2 receptor, palmitoylethanolamide (Showalter *et al*., 1996; Lambert & Di Marzo, 1999) which has been proposed as an autacoid to locally reduce mast cell degranulation, plasma extravasation and hyperalgesia (Aloe *et*

*Author for correspondence; E-mail: gabriella.giagnoni@unimib.it

al., 1993; Mazzari *et al*., 1996). Mazzari *et al*. (1996) reported that orally palmitoylethanolamide reduced carrageenan, formalin and dextran-induced oedema, in a time- and dose-dependent manner, and significantly reduced the mechanical hyperalgesia provoked by carrageenan. Unlike palmitoylethanolamide, peripheral but not systemic administration of anandamide, an endogenous agonist at the cannabinoid CB_1 receptor isolated from porcine brain (Devane *et al*., 1992), inhibited carrageenan-induced oedema and the associated thermal hyperalgesia in the rat through an interaction with a peripheral CB_1 receptor (Richardson *et al*., 1998). The promising results in inflammation animal models, suggest palmitoylethanolamide may offer a new approach in the treatment of inflammation, but to date the only cannabinoids that have obtained drug approval are: Δ^9-THC, and a synthetic derivative of CT-3 nabilone. The former shows analgesic effects in post-operative pain, antiemetic effects in cancer patients and is used to combat weight loss in the acquired immunodeficiency syndrome (AIDS) by stimulating appetite (Pace *et al*., 1999). Nabilone is mainly used against nausea induced by anticancer drugs, and recently to produce pain relief in multiple sclerosis (Notcutt *et al*., 1999; Martyn *et al*., 1995; Hamann & Di Vadi, 1999). Nabilone consists of the basic cannabinoid nucleus of Δ^9-THC, with a ketone function in the A ring and a dimethylheptyl side chain; however, unlike CT-3, the two methyl groups are attached to the same carbon (Lemberger, 1999). In view of the similarity with the structure of CT-3, the present study was designed to verify a possible antiinflammatory and antihyperalgesic role of this compound in carrageenan-induced paw oedema, a rat model of inflammation.

Methods

Animals

Male Wistar rats (100–120 g, Harlan, Italy) were used. Animals were housed in a room with controlled temperature ($22\pm1^{\circ}C$), humidity ($60\pm10\%$) and light (12 h per day) for at least 1 week before being used. Food and water were available *ad libitum*. Before the experiments, rats were fasted overnight, with free access to water. Animal care was in accordance with the Italian State regulations governing the care and treatment of laboratory animals (Permission no. 94/2000A).

Drugs

Drugs were obtained from the following companies: λ-carrageenan (Sigma-Aldrich, Milan, Italy), nabilone (kindly supplied by Eli Lilly and Co.Ltd., Basingstoke, Hampshire, U.K.), palmitoylethanolamide (Cayman Chemical, Ann Arbor, MI, U.S.A.), indomethacin (Sigma-Aldrich, Milan, Italy) and {N-[(1S)-endo-1,3,3-trimethyl bicyclo [2.2.1] heptan-2-yl]-5-(4-chloro-3-methylphenyl)-1-(4-methylbenzyl)-pyrazole-3-carboxamide} (SR 144528) (kindly supplied by Sanofi Recherche, Montpellier, France).

Psychoactive effects of nabilone

A series of four consecutive observations were conducted on each rat, following a standard procedure employed to evaluate psychoactive cannabinoid-induced effects (Compton *et al*., 1993). Briefly, before oral doses of nabilone (2.5 or 5 mg kg^{-1}) or its vehicle (carboxymethylcellulose 1.5% w v^{-1} in saline), then every hour for 3 h, rats were consecutively tested for body temperature, nociceptive threshold, spontaneous locomotor activity and ring immobility. All these measures were made in a quiet room at a temperature of $22\pm1^{\circ}C$. Body temperature was measured with a rectal thermistor probe (Ellab, Roedrove, Denmark) inserted to a constant depth of 5 cm. To determine thermal nociception, rats were tested for responsiveness to radiant heat with a tail-flick analgesia meter (D'Amour & Smith, 1941) and the latency to remove the tail from the path of the stimulus (expressed in seconds) was recorded automatically by a photoelectric cell. Spontaneous locomotor activity was counted for 5 min by an Animex activity meter type S (LKB, Farad Electronics, Hagerstein, Sweden), which recorded the number of crossings on the magnetic solenoids. The apparatus was calibrated so it was sensitive to horizontal movements but not to stereotypic movements (sensitivity 5 μA; intensity of magnetic field 40 μA). Immobility time was measured by the 'ring test' described by Pertwee (1972) with modifications for the rat. The apparatus consisted of a wire ring, 12 cm in diameter, fixed horizontally to a ring stand at a point 38 cm above the desk. The experimenters placed the rat across the ring, so that it was supported only by its front and rear paws. Its forepaws were placed on diametrically opposite points on the ring. The number of seconds the rat remained motionless on the ring (except for breathing movements) was recorded. The test was conducted for 5 min and catalepsy was measured as the percentage of the total time spent on the ring during which the rat remained immobile.

Antiinflammatory and antihyperalgesic assays

Carrageenan-induced oedema and hyperalgesia The antiinflammatory activity of nabilone, was compared with palmitoylethanolamide and indomethacin, by measuring the oedema induced by intraplantar (i. pl.) injection of 0.1 ml carrageenan (1% w v^{-1} in saline) into the right paw.

Nabilone, at non-psychoactive doses (0.75, 1.5 and 2.5 mg kg^{-1}), palmitoylethanolamide (10 mg kg^{-1}) and indomethacin (5 mg kg^{-1}), or an appropriate volume of vehicle (carboxymethylcellulose 1.5% w v^{-1} in saline) were administered orally 1 h before carrageenan. The palmitoylethanolamide and indomethacin doses were those Mazzari *et al*. (1996) reported were effective in reducing carrageenan oedema. The paw volume was measured with a plethysmometer (Ugo Basile, Va, Italy) immediately before and 1, 2, 3 and 10 h after the intraplantar carrageenan injection. For all animals both the ipsilateral (injected) and controlateral (non-injected) hindpaw volume was measured. Oedema was expressed as the increase in paw volume (ml).

Thermal hyperalgesia was evaluated on the same animals used to determine the antiinflammatory effects. Briefly, paw withdrawal latencies were recorded in both hindpaws using the radiant heat method (Hargreaves *et al*., 1988). Animals were placed in a clear acrylic box on a glass platform (Plantar Test, Ugo Basile, Va, Italy). A beam of radiant heat was applied through the platform to the plantar surface of the hindpaw. A photocell detected paw withdrawal and the

latency was recorded. After paw baseline withdrawal latencies (s) had been recorded, thermal hyperalgesia was estimated for each animal 1, 2, 3 and 10 h after carrageenan injection.

Antagonism studies

The selective CB_2 cannabinoid receptor antagonist SR 144528, 3 mg kg^{-1} (an oral dose which totally displaced the *ex vivo* [^{3}H]-CP55940 binding to the mouse spleen membrane CB_2 receptor, but had no effect on the binding of [^{3}H]-CP55940 to its specific sites in the brain: Rinaldi-Carmona *et al*., 1998) or its vehicle (10% Tween 80, 20% DMSO in H_2O) was administered orally 1 h before the injection of nabilone (2.5 mg kg^{-1}), palmitoylethanolamide (10 mg kg^{-1}) and indomethacin (5 mg kg^{-1}). One hour later the animals received an intraplantar injection of 1% carrageenan and the oedema and thermal hyperalgesia were measured after 3 h.

Statistical analysis

The results were expressed as the means $\pm$ s.e.mean. The data were analysed using one way analysis of variance (ANOVA) followed by Tukey's test. Differences were considered significant at $P<0.05$. Linear regression analysis was calculated using GraphPAD Software, San Diego.

Results

Psychoactive effects of nabilone

In order to select a dose of nabilone with no psychoactive effects, which could be used to study its antiinflammatory and antihyperalgesic activity, rats were tested in the tetrad of assays employed to evaluate cannabinoid psychoactive effects (Compton *et al*., 1993). One, two and three hours after oral doses of nabilone (2.5 and 5 mg kg^{-1}) each rat's body temperature, nociceptive threshold, locomotor activity and ring immobility were consecutively measured. The behavioural evaluations are given in Figure 1. Rats given 2.5 mg kg^{-1} of nabilone did not show any of these effects, whereas the higher dose (5 mg kg^{-1}) caused decreases in body temperature and motor activity with catalepsy and analgesia. These effects were time-dependent: 3 h after the dose of nabilone rectal temperature had dropped 2.6°C and motor activity by 69%, ring immobility increased by 71% and tail-flick latency increased by 612%.

Antiinflammatory and antihyperalgesic effects

Before carrageenan injection there was no significant difference in volume and withdrawal latencies between ipsilateral and controlateral hindpaws (data not shown). Intraplantar injection of carrageenan 1% w v^{-1} resulted in a time-related increase in ipsilateral hindpaw volume. One hour after carrageenan the hindpaw oedema was (0.87 ± 0.15 ml), at 3 h it peaked and at 10 h it was still present (Figure 2a). Controlateral hindpaw volume did not change significantly throughout the experiment (data not shown). Withdrawal latencies of the ipsilateral hindpaw 1, 2, 3 h after carrageenan injection were significantly lower than the baseline value (6.42 ± 0.43 s). This hyperalgesia had disappeared 10 h after carrageenan, when the latencies were not significantly different from baseline value (Figure 2b). No differences in paw withdrawal latencies were found in the controlateral hindpaw at any time after carrageenan (data not shown).

Nabilone (2.5 mg kg^{-1}, p.o.) was then employed for a time-course study in comparison to palmitoylethanolamide (10 mg kg^{-1}, p.o.) and indomethacin (5 mg kg^{-1} p.o.). The three drugs administered 1 h before carrageenan inhibited paw oedema at all time points considered; this inhibition was maximal 3 h after carrageenan injection when the oedema was reduced by 40, 47 and 60% respectively (Figure 2a). Hyperalgesia observed at 1, 2, 3 h after carrageenan was also abolished by all drugs. In rats treated with vehicle the withdrawal latency for the ipsilateral hindpaw 3 h after carrageenan injection was 3 ± 0.1, compared to 6.4 ± 0.3, 6.3 ± 0.3 and 6.5 ± 0.3 s in nabilone, palmitoylethanolamide and indomethacin treated rats (Figure 2b).

Dose-related anti-oedema and antihyperlagesic effects of nabilone

The anti-oedema and antihyperalgesic effect of nabilone was evaluated at the peak time of inflammatory response, 3 h after carrageenan injection. Nabilone (0.75, 1.5 and 2.5 mg kg^{-1}, p.o.) caused a dose-related inhibition of carrageenan-induced oedema and hyperalgesia (Figure 3a,b). Oedema was reduced by 8, 28 and 42% respectively and hyperalgesia was decreased, in fact the paw withdrawal latencies of the inflamed paw were increased by 27, 71 and 116%, respectively.

Antagonism studies

To elucidate whether the anti-oedema and antihyperalgesic effects of nabilone and palmitoylethanolamide were mediated by the peripheral CB_2 cannabinoid receptor, rats were orally treated with the selective CB_2 receptor antagonist SR 144528 (3 mg kg^{-1}) 1 h before oral administration of these drugs. To verify the specificity of this antagonism, the influence of SR 144528 on the antiinflammatory effect of indomethacin was also evaluated. Pretreatment with SR 144528 blocked the anti-oedema and antihyperalgesic effects of nabilone and palmitoylethanolamide but, as expected, had no effect on those evoked by indomethacin (Figures 4 and 5). SR 144528 on its own did not affect carrageenan-induced oedema and hyperalgesia and oedema and paw withdrawal latency in the ipsilateral hindpaw did not differ in vehicle treated animals (Figures 4 and 5).

Discussion

The experiments described here confirmed the antiinflammatory activity of the endocannabinoid palmitoylethanolamide (10 mg kg^{-1}, p.o.) and for the first time, demonstrated that the synthetic cannabinoid nabilone, at oral doses with no psychoactive effects, inhibits rat paw carrageenan-induced oedema in a dose-dependent fashion. The efficacy of palmitoylethanolamide in reducing oedema in different models of acute inflammation was already known (Benvenuti *et al*., 1968; Mazzari *et al*., 1996). We showed that the anti-

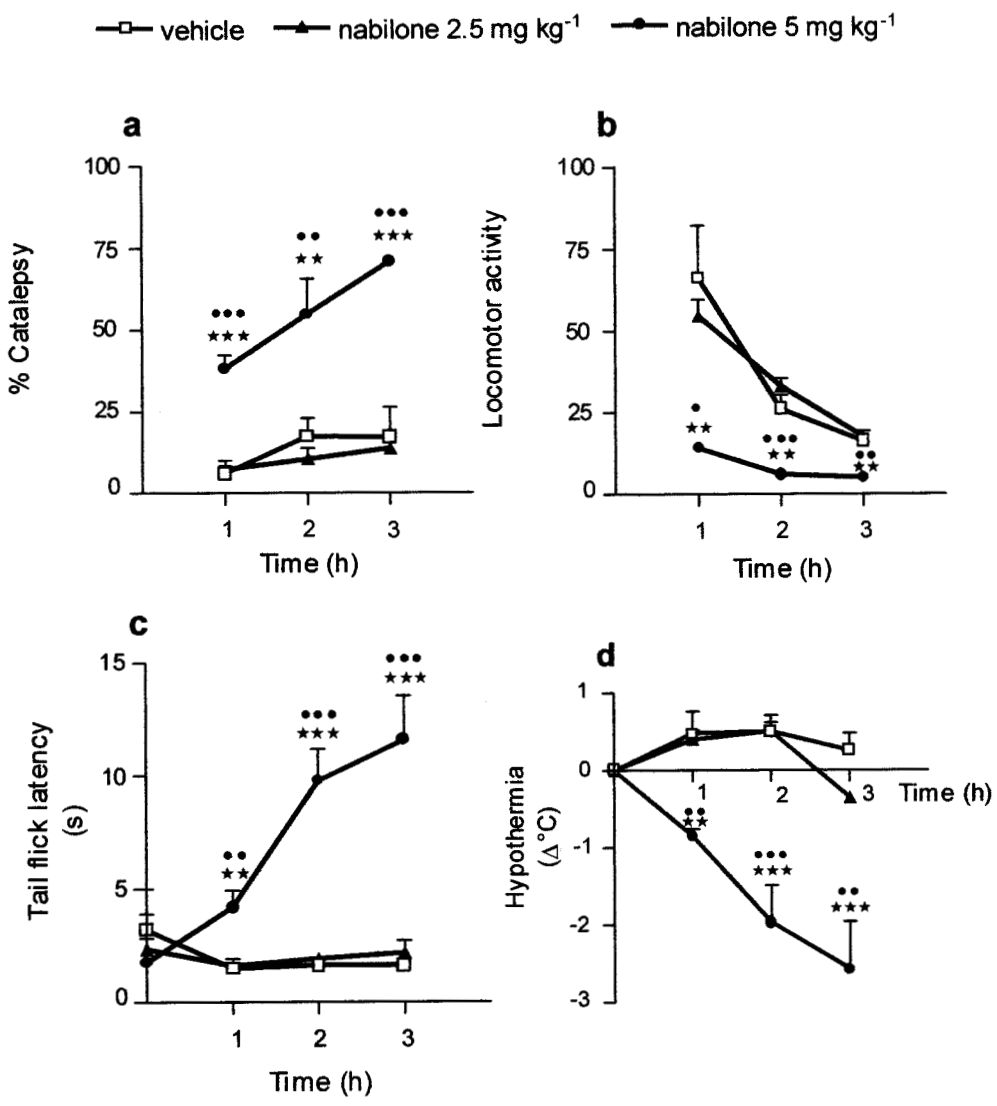

Figure 1 Time course of behavioural effects induced by oral nabilone in the rat. Each point represents the mean ± s.e.mean of 3–4 animals. ***$P<0.001$, **$P<0.01$ vs vehicle; •••$P<0.001$, ••$P<0.01$, •$P<0.05$ vs nabilone 2.5 mg kg^{-1}.

oedema effect was already evident 1 h after carrageenan, when oedema formation is dependent on the release of mast cell mediators (Di Rosa *et al*., 1971; Al-Haboubi & Zeitlin, 1983); the anti-oedema effect was still present at 10 h, suggesting a lasting efficacy of this cannabinoid. The anti-oedema activity of nabilone (2.5 mg kg^{-1}, p.o.), paralleled that of palmitoylethanolamide and the classic nonsteroidal antiiinflammatory drug indomethacin (5 mg kg^{-1}, p.o.). This effect was already evident 1 h after carrageenan injection, was higher at 3 h and was still present at 10 h. So far we have only tested the antiinflammatory activity of nabilone in the model of acute carrageenan induced inflammation so we do not known whether nabilone, as already demonstrated for palmitoylethanolamide (Mazzari *et al*., 1996), reduces formalin and dextran oedema, which like carrageenan inflammation, mainly depend on release of mast cell mediators. The natural compound Δ^9-THC, at doses ranging from 3.75–100 mg kg^{-1}, p.o., given to rats 1 h before carrageenan, always showed an anti-oedema effect (Sofia *et al*., 1973) whereas Kosersky *et al*. (1973) observed this effect only at the high dose of 100 mg kg^{-1}, given orally to rats.

Burstein *et al*. (1992) synthesized a cannabinoid with analgesic and antiinflammatory activities: CT-3. This compound is chemically similar to THC-11, differing only in the substitution of dimethylheptyl chain on C3 instead of the n-pentyl radical. CT-3 is more than two orders of magnitude more potent than the template molecule THC-11 in the rodent paw oedema and hot plate tests (Burstein *et al*., 1992). Thus it seems that the dimethylheptyl chain increases the antiinflammatory and analgesic potency of THC. Nabilone shares a similar structure, differing only for the presence of a ketonic group on C9. Thus it is likely that the dimethylheptyl chain is also responsible for the anti-oedema effect of nabilone in the model of carrageenan inflammation. Nabilone, like palmitoylethanolamide, inhibited antigen-induced secretion of serotonin from rat mast cells and RBL-2H3 cells (Facci *et al*., 1995). Mast cells release inflammatory mediators such as histamine and serotonin,

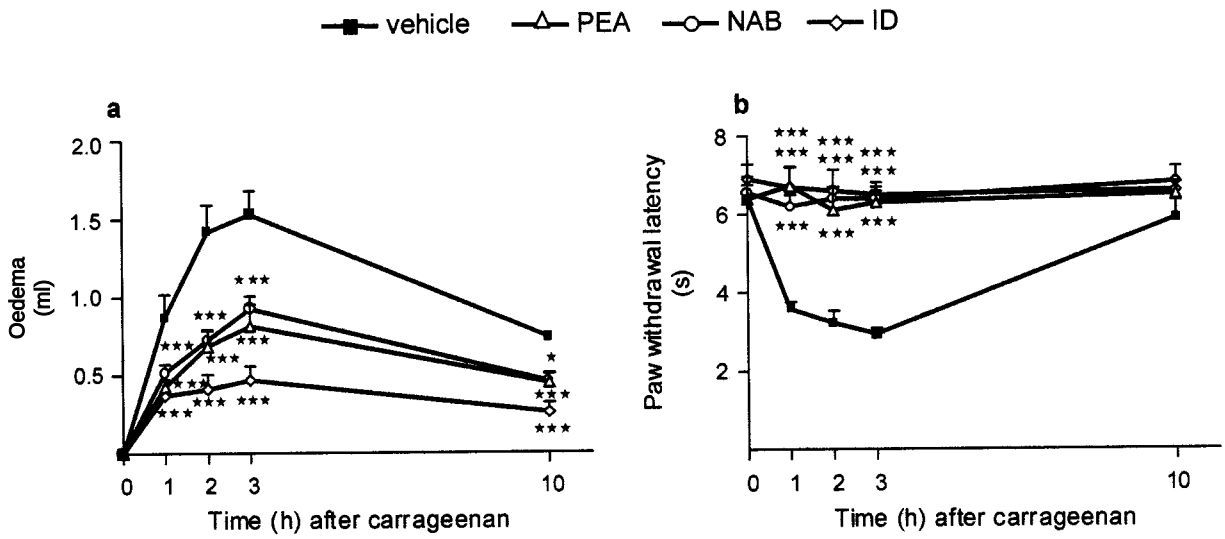

Figure 2 Effect of palmitoylethanolamide (PEA) 10 mg kg^{-1}, nabilone (NAB) 2.5 mg kg^{-1} and indomethacin (ID) 5 mg kg^{-1} given orally to the rat 1 h before carrageenan, on oedema (a) and ipsilateral hindpaw withdrawal latency (b) 1, 2, 3 and 10 h after injection of carrageenan. Each point represents the mean ± s.e.mean of 6–9 animals. ***$P<0.001$, *$P<0.05$ vs vehicle.

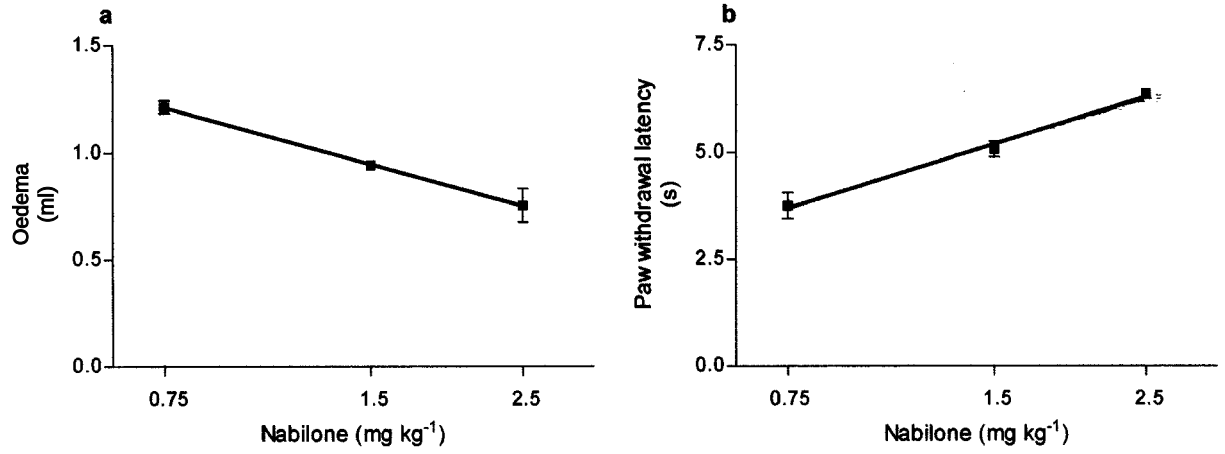

Figure 3 Dose-response relationships for anti-oedema (a) and antihyperalgesic effect (b) of nabilone orally administered in the rat 1 h before carrageenan. The response was measured at 3 h after injection of carrageenan. Each symbol represents the mean ± s.e.mean of four animals.

involved in vasodilatation, increased vascular permeability and activation of nociceptors. It remains to be ascertained whether nabilone reduces carrageenan-induced oedema by modulating mast cell degranulation.

We demonstrated here that nabilone has dose-dependent action in reducing not only oedema but also hyperalgesia, supporting the clinical data showing that this compound relieves the severe and intractable pain of muscle spasm in multiple sclerosis (Hamann & Di Vadi, 1999; Martyn *et al*., 1995). These findings are consistent with the ability of palmitoylethanolamide and other cannabinoids to reduce hyperalgesia in rodent models of acute inflammation induced by various agents, such as carrageenan, formalin, kaolin, acetic acid and magnesium sulphate (Mazzari *et al*., 1996; Jaggar *et al*., 1998; Richardson *et al*., 1998; Hanus *et al*., 1999; Calignano *et al*., 1998; 2001). In our hands palmitoylethanolamide also abolished the thermal hyperalgesia evoked by carrageenan.

The activation of CB_2 receptor appears to be involved in downregulation of the inflammatory response (Aloe *et al*., 1993; Facci *et al*., 1995; Mazzari *et al*., 1996). This is supported by experimental evidence that the CB_2 cannabinoid receptor antagonist SR 144528 reduced the antiinflammatory and analgesic effects of the selective CB_2 cannabinoid receptor agonist HU-308 (Hanus *et al*., 1999) and also prevented palmitoylethanolamide-evoked antihyperalgesia (Calignano *et al*., 1998; 2001).

To ascertain whether the anti-oedema and antihyperalgesic effects of nabilone and palmitoylethanolamide were mediated by a peripheral cannabinoid receptor, we employed the CB_2

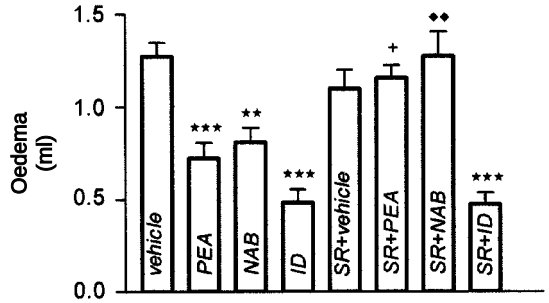

Figure 4 Effect of SR 144528 (SR) 3 mg kg^{-1}, p.o. in the rat 1 h before palmitoylethanolamide (PEA) 10 mg kg^{-1} p.o., nabilone (NAB) 2.5 mg kg^{-1} p.o., indomethacin (ID) 5 mg kg^{-1} p.o. or vehicle, on oedema 3 h after injection of carrageenan. Each point represents the mean ± s.e.mean of 10–15 animals. ***$P<0.001$, **$P<0.01$ vs vehicle; +$P<0.05$ vs PEA; ◆◆$P<0.01$ vs NAB.

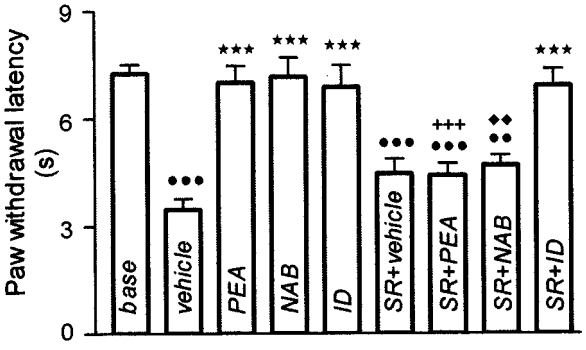

Figure 5 Effect of SR 144528 (SR) 3 mg kg^{-1}, p.o. in the rat 1 h before palmitoylethanolamide (PEA) 10 mg kg^{-1} p.o., nabilone (NAB) 2.5 mg kg^{-1} p.o., indomethacin (ID) 5 mg kg^{-1} p.o. or vehicle, on ipsilateral hindpaw withdrawal latency 3 h after injection of carrageenan. Baseline hindpaw withdrawal latencies measured before carrageenan were not different from each other. Thus in the figure the base indicates the baseline ipsilateral hindpaw withdrawal latencies of vehicle-treated rats. Each point represents the mean ± s.e.mean of 10–15 animals. ***$P<0.001$ vs vehicle; ●●●$P<0.001$, ●●$P<0.01$ vs base; + + +$P<0.001$ vs PEA; ◆◆$P<0.01$ vs NAB.

cannabinoid receptor antagonist SR 144528 (Rinaldi-Carmona *et al*., 1998). This antagonist completely prevented the anti-oedema and antihyperalgesic effects of nabilone and palmitoylethanolamide but did not affect oedema and hyperalgesia. It has been reported that systemically administered SR 144528 enhanced formalin-induced pain responses in mice only in the early phase (Calignano *et al*., 1998). In agreement with Calignano *et al*. (1998), we found that 5 min after carrageenan, SR 144528 by itself evoked a pain behaviour (licking and flexing of the treated limb) (data not shown). Even so, the production of hyperalgesia by SR 144528 is unlikely to account for its ability to prevent the cannabinoid-induced antihyperalgesic effect, 3 h after carrageenan.

Nabilone has the same affinity for human CB_1 and CB_2 receptors (Gareau *et al*., 1996). Theoretically, the drug could therefore alleviate pain by acting at peripheral CB_1 receptors on nociceptive neurons, without any recruitment of brain CB_1 receptors, since at 2.5 mg kg^{-1} it did not have central effects. However, our findings seem to exclude CB_1 receptor involvement and point more to a role of CB_2 receptors, since SR 144528 completely prevented nabilone-induced antihyperalgesia. It remains to be seen how a dose unable to activate CB_1 receptors can exclusively bind the CB_2 receptor. At the moment, we do not understand why this happens, but one possible explanation is that nabilone may bind and activate a not-yet characterized CB_2-like receptor, reported by Calignano *et al*. (1998) to be sensitive to SR 144528; this receptor may also mediate the antiinflammatory effects of palmitoylethanolamide (Calignano *et al*., 1998; 2001), which does not bind with high affinity to either of the two cannabinoid receptor subtypes (Lambert & Di Marzo, 1999).

In conclusion, our findings indicate that nabilone and palmitoylethanolamide have a peripheral antiinflammatory effect mediated by an uncharacterized CB_2-like receptor. However, the biochemical mechanisms underlying the antiinflammatory action of palmitoylethanolamide and nabilone and whether these cannabinoids are also active once inflammation has started remains to be clarified.

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