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Role of the endogenous cannabinoid system in the formalin test of persistent pain in the rat

Pierre Beaulieu [a,1], Tiziana Bisogno [b,1], Shahid Punwar [a], W. Paul Farquhar-Smith [a], Gerolmina Ambrosino [b], Vincenzo Di Marzo [b], Andrew S.C. Rice [a,*]

[a] *Pain Research Group, Department of Anaesthetics, Imperial College School of Medicine, St. Mary's Hospital Campus, Praed Street, London W2 1NY, UK*

[b] *Istituto per la Chimica di Molecole di Interesse Biologico, Consiglio Nazionale delle Ricerche, Via Toiano 6, 80072 Arco Felice (NA), Italy*

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Abstract

It has been suggested that administration of a cannabinoid CB_1 (SR141716A [*N*-(piperidin-1-yl)-5-(4-chlorophenyl)-1-(2,4-dichlorophenyl)-4-methyl-1-*H*-pyrazole-3-carboxamide]) and CB_2 (SR144528 {*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}) receptor antagonists to mice potentiates inflammatory hyperalgesia by removing an endogenous cannabinoid tone. We examined whether the behavioural response to s.c. formalin injection in rats is similarly enhanced. A total of 30 animals received SR141716A (0.5 or 5 mg/kg) or SR144528 (0.3 or 3 mg/kg) 30 min before 1% formalin. Pain behaviour was quantified using the composite weighted pain score technique (CPS-$WST_{0,1,2}$). An overall CPS-$WST_{0,1,2}$ was calculated for each phase and groups were compared (analysis of variance). The results obtained in the control group confirmed the characteristic biphasic behavioural response to formalin injection. None of antagonist groups had a significant *increase* in overall CPS-$WST_{0,1,2}$ compared to the control. Indeed, a significant *decrease* in CPS-$WST_{0,1,2}$ scores for both phases was detected in most of all of the groups, except SR141716A at 5 mg/kg. Levels of endogenous cannabinoids (anandamide, palmitoylethanolamide, 2-arachidonylglycerol) were measured from rats hind-paw skin 1 h after s.c. injection of 0.9% saline (100 μl), 1% (50 μl), 2.5% (50 μl) and 5% (100 μl) formalin. The concentration of endocannabinoids did not differ between control and formalin-induced inflammation groups. The activity of anandamide amidohydrolase in hind-paw skin also did not change after treatment with formalin. In conclusion, cannabinoid antagonists do not enhance formalin-evoked pain behaviour. These results suggest that, in this model, endogenous cannabinoids do not tonically attenuate inflammatory hyperalgesia.

Keywords: Cannabinoid CB_1 receptor; Cannabinoid CB_2 receptor; Cannabinoid receptor antagonist; Hyperalgesia; Anandamide; Palmitoylethanolamide; 2-Arachidonylglycerol; Nociception; Inflammation

1. Introduction

Several recent advances have elucidated the pharmacology of the endogenous cannabinoid system. Two subtypes of cannabinoid receptors have been identified and cloned: CB_1 (Devane et al., 1988; Matsuda et al., 1990) and CB_2 (Kaminski et al., 1992; Munro et al., 1993), and determined to be members of the G-protein-coupled receptor family (Howlett et al., 1986). Cannabinoid CB_1 receptors have been demonstrated both in the central nervous system (CNS), including areas of brain and spinal cord associated with nociception (Devane et al., 1988; Herkenham et al., 1991; Farquhar-Smith et al., 2000), and in certain peripheral tissues (Galiègue et al., 1995). Cannabinoid CB_2 receptors are expressed mainly on cells of immune origin (Kaminski et al., 1992), including mast cells and macrophages (Facci et al., 1995). Furthermore, putative endocannabinoids such as anandamide, 2-arachidonylglycerol and palmitoylethanolamide have been isolated (Di Marzo, 1998, for review). Anandamide was described by Devane et al. (1992) as the endogenous agonist at the

* Corresponding author. Tel.: +44-171-886-6373; fax: +44-171-886-6425.

E-mail address: a.rice@ic.ac.uk (A.S.C. Rice).

[1] These authors contributed equally to this article.

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cannabinoid CB_1 receptor and was isolated from porcine brain. Palmitoylethanolamide is a putative endogenous agonist of the cannabinoid CB_2 receptor (Facci et al., 1995). The enzyme anandamide amidohydrolase is responsible for the hydrolysis of anandamide amide bond, and was later shown to also recognise palmitoylethanolamide and 2-arachidonylglycerol as substrates (Di Marzo, 1998). These endogenous ligands exhibit actions similar to those of classical cannabinoid receptor agonists (e.g., increased appetite, catalepsy, analgesia, etc.) (Di Marzo, 1998). Recently cannabinoid CB_1 (Rinaldi-Carmona et al., 1994) and CB_2 (Rinaldi-Carmona et al., 1998) receptor antagonists have been produced. Finally, genetically modified animals have been developed and the function of the cannabinoid CB_1 receptor has been investigated in mice by invalidating its gene (Ledent et al., 1999; Zimmer et al., 1999).

Central antinociceptive effects of cannabinoids have been well documented (Lichtman and Martin, 1991; Meng et al., 1998), however, much less is known about the peripheral effects of the cannabinoids in inflammatory hyperalgesia. Peripheral cannabinoid CB_2 receptors have been suggested to modulate inflammation (Aloe et al., 1992; Mazzari et al., 1996; Hanus et al., 1999). One potential mechanism for the anti-hyperalgesic effect of palmitoylethanolamide is the inhibition of mast cell degranulation (Facci et al., 1995; Mazzari et al., 1996; Jaggar et al., 1998) and, therefore, down-regulation of the pro-hyperalgesic actions of the neurotrophin nerve growth factor (Farquhar-Smith et al., 1999). Additionally, Richardson et al. (1998) have shown that anandamide produces anti-hyperalgesic effects via an interaction with peripheral cannabinoid CB_1 receptors, and suggested that cannabinoids inhibit neurosecretion (calcitonin gene-related peptide release) from capsaicin-sensitive primary afferent fibres. Furthermore, Calignano et al. (1998) have demonstrated in mice that peripheral cannabinoid CB_1-like and CB_2-like receptors participate in the control of pain initiation and have suggested that locally generated anandamide and palmitoylethanolamide are present in concentrations enough to mediate this effect. In the same study, the authors showed that cannabinoid CB_1 and CB_2 receptor antagonists, when given systemically to mice and locally (for the cannabinoid CB_1 receptor antagonist), prolong and enhance the pain behaviour produced by tissue damage. The intrinsic pro-hyperalgesic effects of SR141716A, a cannabinoid CB_1 receptor antagonist, given by intrathecal injection or using an antisense technique targeted against cannabinoid CB_1 receptor mRNA, have also been reported in untreated mice (Richardson et al., 1997, 1998). Also, Herzberg et al. (1997) in the chronic constriction injury model of neuropathic pain, and Strangman et al. (1998) using the formalin test in rats, demonstrated the pro-hyperalgesic effect of SR141716A. Recently, Martin et al. (1999) have shown that intrathecal injection of SR141716A increased the number of Fos-immunoreactive neurones in the spinal cord of both normal and inflamed (using complete Freund's adjuvant) rats. Finally, Chapman (1999) showed that SR141716A, but not SR144528, selectively facilitated nociceptive responses of dorsal horn neurones in the rat. Thus, it has been suggested that the cannabinoid system, unlike the endogenous opioid system, tonically regulates inflammatory hyperalgesia.

These reports contrast with the findings from the original publication by Rinaldi-Carmona et al. (1994) on the development of SR141716A where the cannabinoid CB_1 receptor antagonist when given alone failed to produce any effect in pharmacological and behavioural models (e.g., tail-flick latency) in mice. Their results have been since confirmed by several other authors (Richardson et al., 1998; Welch et al., 1998; Hohmann et al., 1999; Hanus et al., 1999). Although the hyperalgesic effects of cannabinoid receptor antagonists may be due to the inhibition of the actions of tonically released endogenous cannabinoids, it is also possible that these compounds act as inverse agonists (Landsman et al., 1997; MacLennan et al., 1998; Pan et al., 1998; Rinaldi-Carmona et al., 1998). Furthermore, the two studies in mice in which the gene encoding the cannabinoid CB_1 receptor has been deleted have shown that, although it seems possible that the endogenous cannabinoid system plays a role in the modulation of pain sensitivity, it remains unclear to what extent the cannabinoid mechanisms are activated under normal resting conditions (Ledent et al., 1999; Zimmer et al., 1999).

Our group has shown that anandamide and palmitoylethanolamide prevent nociceptive behaviour induced by s.c. formalin injection into the hind-paw of the rat (Jaggar et al., 1998). The aim of the present study was first to assess whether the behavioural response to s.c. formalin injection in the rat is altered by cannabinoid CB_1 (SR141716A) and CB_2 (SR144528) receptor antagonists and then to quantify the production of the endogenous cannabinoids (anandamide, palmitoylethanolamide and 2-arachidonylglycerol) and the activity of anandamide amidohydrolase in control and formalin-induced inflamed tissues, thus elucidating the physiological role of endogenous cannabinoids in this scenario.

2. Materials and methods

2.1. Drug preparation

SR141716A [*N*-(piperidin-1-yl)-5-(4-chlorophenyl)-1-(2,4-dichlorophenyl)-4-methyl-1-*H*-pyrazole-3-carboxamide] was a gift from Research-Biochemicals-International/NIMH Chemical Synthesis Program (USA), while SR144528 {*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} was donated by Sanofi Recherche (Montpellier,France). Both compounds were dissolved in dimethyl sulfoxide (DMSO), and further di-

luted with saline 0.9% in a ratio of 1:19. The final volume of the saline or various doses of antagonists administered i.p. was of 1–2 ml.

2.2. Animal maintenance and preparation

All experiments conformed with British Home Office regulations. The animals were housed in colony cages and maintained on a 12 h light/dark cycle with free access to food and water prior to the experiments. Formalin tests were performed on 30 male Wistar rats weighing 210–330 g (mean weight 274 g). The levels of endogenous cannabinoids and anandamide amidohydrolase activity were measured in the paw skin of 30 animals.

2.3. Formalin test

The formalin test is a well-established rat model of persistent somatic pain (Dubuisson and Dennis, 1977) which was refined by Tjølsen et al. (1992) and Watson et al. (1997). Animals were acclimatised to the testing environment (clear plexiglass box 23 × 18 × 14 cm) for 10–15 min until explorative behaviour ceased. Care was taken while handling the animals in order to minimise stress. Pain scores, which are low under vehicle conditions, increase dose-dependently to 2.5% formalin concentration, where it reaches asymptote (Dubuisson and Dennis, 1977). Therefore, to avoid any ceiling effect and increase the possibility of observing either an increase or an attenuation of the response we used a 1% concentration of formalin. Animals were allocated to different experimental groups (see below). Fifty microlitres of 1% formalin were administered s.c. into the dorsum of the right hind-paw (the plantar aspect of the right paw was also marked with a pen). Following the s.c. injection, the animal was returned to the chamber, and its subsequent nociceptive behaviour observed. A mirror angled at 45° below the observation chamber allowed the experimenter an unobstructed view of the marked paw. Observation of the animal's behaviour was made in consecutive 5-min periods for 60 min following formalin injection. The total time the animal spent in three distinct behavioural categories in each 5-min period was recorded: (1) injected paw had little or no weight on it; (2) injected paw was elevated, not in contact with any surface; (3) injected paw was licked, bitten or shaken. Nociceptive behaviour was quantified using the composite pain score — weighted scores technique (CPS-$WST_{0,1,2}$) as proposed by Watson et al. (1997) where behaviour (1) is discarded, behaviour (2) weighted times one, and behaviour (3) weighted times two. 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). The animals were culled at the end of each experiment.

2.4. Experimental formalin groups

All animals ($n = 6$ per group) received a formalin concentration of 1% and also an i.p. injection 30 min before the formalin test of either 1 ml of normal saline in DMSO (1:19) (control group), SR141716A at 0.5 mg/kg or 5 mg/kg, SR144528 at 0.3 mg/kg or 3 mg/kg. The doses of antagonists administered were based on reported ex vivo ED_{50} values for the cannabinoid CB_1 and CB_2 receptor antagonists (Rinaldi-Carmona et al., 1995, 1998).

2.5. Identification and quantification of endogenous cannabinoids and anandamide amidohydrolase activity

Skin was excised from 30 male Wistar rats (immediately after the animals were culled) 1 h after 0.9% saline solution injection (100 μl), or after formalin injection s.c. into the right hind-paw at concentrations of 1% (50 μl), 2.5% (50 μl) or 5% (100 μl). Tissue specimens were immediately weighted, immersed into liquid nitrogen, then stored at −70°C until analysis. Tissue was then extracted with chloroform/methanol (2:1, by volume) containing 5 nmol each of d_8-anandamide, d_4-palmitoylethanolamide and d_8-2-arachidonylglycerol synthesised as described previously from either the d_8- or d_4-deuterated fatty acids and either ethanolamine or glycerol (Bisogno et al., 1997a,b). The lipid extracts were purified by silica column chromatography and normal phase high pressure liquid chromatography (NP-HPLC), carried out as described previously glycerol (Bisogno et al., 1997a,b), and the fractions corresponding to either anandamide or palmitoylethanolamide (retention time 26–27 min) or 2-arachidonylglycerol (retention time 18–22 min) were derivatised and analysed by isotope dilution gas chromatography-mass spectrometry (GC-MS) carried out in the selected monitoring mode as described in detail elsewhere (Calignano et al., 1998; Bisogno et al., 1999). Results were expressed as pmol/mg extracted lipids to keep into account possible dilution effects due to the injection of 50–100 μl formalin solution or vehicle into rat paws. On average 1 g of wet tissue yielded 45–90 mg of extracted lipids.

To measure anandamide amidohydrolase activity, [^{14}C]anandamide (5 mCi/mmol), synthesised as described previously from [^{14}C]ethanolamine and arachidonic acid (Bisogno et al., 1997a), was used as the radioligand at a 10 μM concentration. Membrane fractions prepared from either vehicle- or formalin-injected skin as described previously acid (Bisogno et al., 1997a) were assayed. The assay was carried out in 50 mM Tris–HCl, pH = 9, at 37°C for 30 min. [^{14}C]Ethanolamine produced from the reaction was quantified as described previously (Bisogno et al., 1997a), and the activity expressed as pmol of [^{14}C]ethanolamine produced/min/mg protein.

2.6. Statistical analysis

All comparisons were performed using SigmaStat, Jandel Scientific Software, version 1.0. The overall CPS-$WST_{0,1,2}$ for the first and the second phases of the behavioural response were compared using analysis of variance with Bonferroni *t*-test as post-hoc analysis. The critical level of significance was set at $P < 0.05$. Data are expressed as mean ± standard error of the mean (S.E.M.).

3. Results

The control group confirmed the previously described characteristic biphasic behavioural response to s.c. formalin. There is a significant *decrease* in CPS-$WST_{0,1,2}$ scores for both early and late phases in all the animals, which were given the antagonists except in the high dose (5 mg/kg) SR141716A group (Figs. 1 and 2). The time course of nociceptive activity (CPS-$WST_{0,1,2}$ against time) for all group treatments is also shown in the figures. Thus, there is no increase in the behavioural response to formalin injection associated with the cannabinoid receptor antagonists.

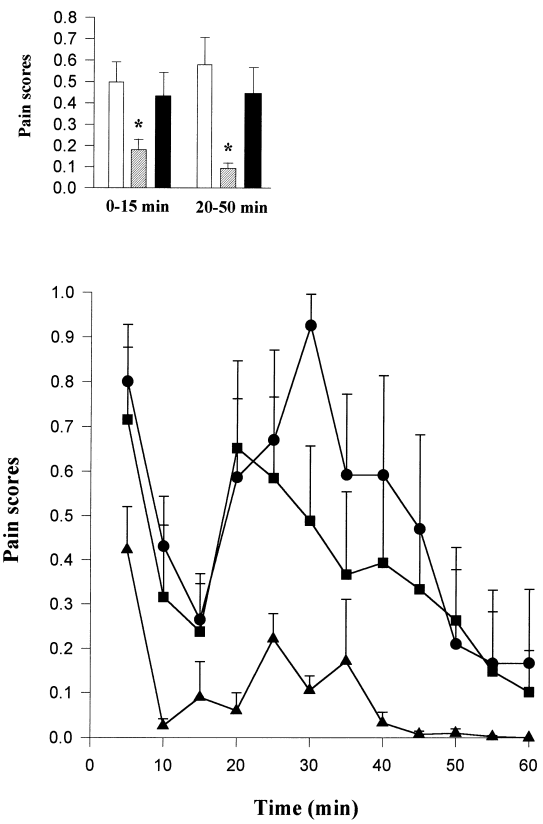

Fig. 1. Time course of nociceptive activity (mean composite weighted pain score technique (CPS-$WST_{0,1,2}$) ± S.E.M.) for 1% formalin (50 μl; s.c. into hind paw) treatments groups in rats. Filled circles represent control animals ($n = 6$). Filled squares identify animals ($n = 6$) which received the cannabinoid CB_1 receptor antagonist SR141716A (5 mg/kg; i.p.) 30 min before the formalin injection. Filled triangles represent animals ($n = 6$) which received SR141716A (0.5 mg/kg; i.p.) 30 min before the formalin injection. There is a statistically significant reduction in CPS-$WST_{0,1,2}$ in animals pre-treated with low dose (0.5 mg/kg) SR141716A. Mean pain scores ± S.E.M. for both early (0–15 min) and late (20–50 min) phases are given in the upper panel. Open columns represent control animals, grey-shaded columns identify animals which received low dose of SR141716A (0.5 mg/kg) and filled columns those which received high dose of SR141716A (5 mg/kg). Asterisk indicates $P < 0.05$.

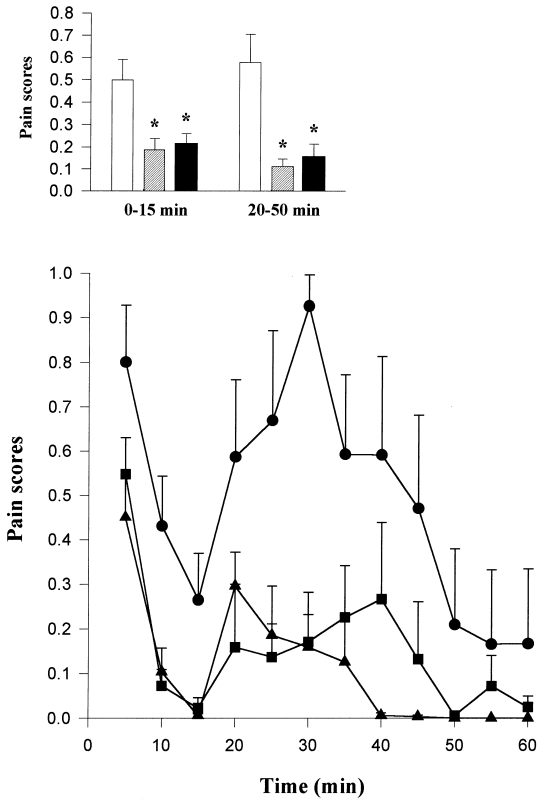

Fig. 2. Time course of nociceptive activity (mean composite weighted pain score technique (CPS-$WST_{0,1,2}$) ± S.E.M.) for 1% formalin (50 μl; s.c. into hind paw) treatments groups in rats. Filled circles represent control animals ($n = 6$). Filled squares identify animals ($n = 6$) which received the cannabinoid CB_2 receptor antagonist SR144528 (3 mg/kg; i.p.) 30 min before the formalin injection. Filled triangles represent animals ($n = 6$) which received SR144528 (0.3 mg/kg; i.p.) 30 min before the formalin injection. There is a statistically significant reduction in CPS-$WST_{0,1,2}$ in animals pre-treated with SR144528 at the two doses used. Mean pain scores ± S.E.M. for both early (0–15 min) and late (20–50 min) phases are given in the upper panel. Open columns represent control animals, grey-shaded columns identify animals which received low dose of SR144528 (0.3 mg/kg) and filled columns represent those which received high dose of SR144528 (3 mg/kg). Asterisk indicates $P < 0.05$.

The levels of endogenous cannabinoids and anandamide amidohydrolase activity in control or formalin-induced inflamed rat skin paws are given in the Table 1. None of the mean levels of anandamide, palmitoylethanolamide and 2-arachidonylglycerol in inflamed tissues, after both 1, 2.5 and 5% formalin, were statistically different from control.

Table 1
Levels of anandamide, palmitoylethanolamide (PEA), 2-arachidonylglycerol (2-AG) and anandamide amidohydrolase (AAH) activity in rat paw skin during control and formalin-induced inflammation conditions. Results (mean ± S.E.M.) are expressed in pmol/mg of extracted lipids for endogenous cannabinoids and in pmol/mg protein^{-1} min^{-1} for anandamide amidohydrolase activity. On average, 1 g wet tissue yields 45–90 mg extracted lipids

	Control ($n = 12$)	Formalin 1% ($n = 6$)	Formalin 2.5% ($n = 6$)	Formalin 5% ($n = 6$)
Anandamide	0.69 ± 0.14	0.68 ± 0.1	0.53 ± 0.19	0.81 ± 0.22
PEA	5.60 ± 1.0	5.0 ± 0.9	3.64 ± 0.52	3.82 ± 0.67
2-AG	51.1 ± 9.0	69.5 ± 10.9	79.8 ± 12.3	77.8 ± 22.3
AAH	19.4 ± 5.2	–	15.1 ± 2.5	15.0 ± 1.0

Similarly, there was no significant difference in anandamide amidohydrolase activity between control and inflamed tissues. This indicates that the levels of endogenous cannabinoids measured were not influenced by changes in hydrolytic metabolism.

4. Discussion

In the present study we have reported that the cannabinoid CB_1 and CB_2 receptor selective antagonists SR141716A and SR144528 do not induce hyperalgesia and, in fact, can decrease pain behaviour (CPS-$WST_{0,1,2}$) scores in the rat formalin test. In addition, skin levels of endogenous cannabinoids (anandamide, palmitoylethanolamide and 2-arachidonylglycerol) are not increased during formalin-induced inflammation compared to control levels. These data do not support the hypothesised endogenous cannabinoid analgesic tone during the inflammatory response produced by s.c. formalin injection in rats. However, there have been reports providing evidence for the existence of such an endogenous cannabinoid analgesic tone: (i) by using the formalin test in mice, cannabinoid CB_1 and CB_2 receptor antagonists were shown to evoke hyperalgesia (Calignano et al., 1998); (ii) the cannabinoid CB_1 receptor antagonist SR141716A or a cannabinoid CB_1 receptor anti-sense oligonucleotide were found to induce hyperalgesia in rats via spinal mechanisms (Richardson et al., 1997, 1998); (iii) SR141716A exhibits pro-hyperalgesic properties in the rat in a model of neuropathic pain (Herzberg et al., 1997), and the in the formalin test (Strangman et al., 1998); (iv) SR141716A also facilitates nociceptive responses of dorsal horn neurones of the spinal cord (Chapman, 1999) and when injected intrathecally increases the number of Fos-immunoreactive neurones in the spinal cord of both normal and inflamed (injection of complete Freund's adjuvant in hind paw) rats (Martin et al., 1999). However, there are important experimental differences between the models used in these studies and the current study. For example: (i) species, strain, body temperature and other experimental variables may influence the levels at which the endocannabinoid system is activated in a behaviourally active animal, and (ii) both the protocol used in the present study, which is different from the one employed in other studies using the formalin test (especially regarding the scoring system used), and recent data which appeared in the literature may explain some of the differences found here. Thus, Richardson et al. (1997) have reported that SR141716A produces hyperalgesia in mice using the hot-plate test. Although these results are perfectly valid, the model used is of more physiological than clinical relevance. Indeed, the formalin test, that measures nociceptive behaviour after s.c. injection of dilute formalin into one of the two hind-paws (Dubuisson and Dennis, 1977), has been shown to be a better reflection of clinical inflammatory pain than ephemeral physiological stimuli such as the hot-plate and tail-flick tests (Tjølsen and Hole, 1997). Even in those previous studies that have used the formalin test and have described analgesic effects of cannabinoids, somewhat inconsistent results on the hyperalgesic action of cannabinoid receptor antagonists have been reported. Thus, Calignano et al. (1998), when showing that SR141716A and SR144528 produced hyperalgesia in mice, used the unconventionally high concentration of 5% formalin and measured the duration of paw licking. Furthermore, in this study, the systemic administration of the cannabinoid CB_2 receptor antagonist SR144528 caused a selective enhancement only of the early-phase, and not of late-phase, responses. Also in the study by Strangman et al. (1998), although the authors showed an enhancement of the response to formalin injection with SR141716A compared to control rats, the authors could have used the validated CPS-$WST_{0,1,2}$ scoring system which is now recommended (Watson et al., 1997). Finally, in a recent study Hanus et al. (1999) reported analgesia in the mice formalin test with a novel cannabinoid CB_2 receptor agonist, but were unable to show a significant hyperalgesic effect with SR144528. Studies pointing to the existence of interactions between anandamide and agonists of capsaicin VR_1 receptors (Di Marzo et al., 1998; Zygmunt et al., 1999; Smart et al., 2000) may also explain the hyperalgesic effects of cannabinoid receptor antagonists under particular conditions. In fact, Zygmunt et al. (1999) showed that anandamide can induce the release of calcitonin gene-related peptide from perivascular sensory fibres by activating capsaicin (vanilloid) receptors. The authors also found that anandamide could activate the VR_1 subtype of these receptors, which is involved in the

nociception associated with thermal and inflammatory stimuli (Tominaga et al., 1998). Smart et al. (2000) showed that anandamide activates vanilloid receptors also in dorsal root ganglia. If this effect also occurs in non-cannabinoid CB_1 receptor-containing sensory afferent neurones (where VR_1 receptors are present), anandamide could also exert a pro-hyperalgesic action, which might be unmasked in the presence of SR141716A (which would only block the cannabinoid CB_1-dependent anti-hyperalgesic effect of endocannabinoids). Thus, in these conditions treatment with SR141716A could lead to hyperalgesia.

Another major difference between Calignano et al. (1998) study and ours is in the quantification of endogenous cannabinoids in rat paw skin. Although these authors studied the effects of SR141716A and SR144528 in the mouse formalin test, they quantitated anandamide and palmitoylethanolamide in *rat* paw skin and found concentrations 5- to 10-fold higher than those observed in rat brain and plasma, and suggested that these levels are sufficient to cause tonic activation of local cannabinoid receptors. However, no data on the possible stimulation of anandamide and palmitoylethanolamide levels during the formalin-induced behavioural response was presented. In our study, we used isotope-dilution GC-MS to measure the levels not only of anandamide and palmitoylethanolamide but also of 2-arachidonylglycerol in both vehicle- and formalin-treated rat paws, and found no statistically significant difference from controls at either of the three formalin concentrations (1%, 2.5% and 5%) used. In all samples the amounts of anandamide and palmitoylethanolamide were similar to those described by Calignano et al. (1998), while the levels of 2-arachidonylglycerol were usually about 100-fold higher than those of anandamide. The fact that we could not observe any variation in endocannabinoid levels was not due to a possible up-regulation of anandamide amidohydrolase, since we have also showed here that the activity of this enzyme did not change on treatment with formalin.

Apart from the present study, the arguments against a tonic activation of the endocannabinoid system come from three lines of evidence: (i) previous reports from the literature, (ii) the widely reported inverse agonist properties of cannabinoid antagonists, and (iii) results obtained in studies carried out with the cannabinoid CB_1 knockout mice. (i) In their original publication on the development of a cannabinoid CB_1 receptor antagonist, Rinaldi-Carmona et al. (1994) showed that SR141716A administered alone failed to produce any effect on behavioural responses to noxious heat, and concluded that the response to this ephemeral stimulus was not under the tonic influence of an endogenous cannabinoid CB_1 receptor ligand. Welch et al. (1998) also using the tail-flick latency test in mice reported that SR141716A at any dose tested failed to produce either antinociceptive or hyperalgesic effects. There are published reports showing that: (1) the effects of SR141716A are not different from vehicle when measuring rat paw withdrawal latency during radiant heat stimulus (Richardson et al., 1998); (2) the cannabinoid CB_1 receptor antagonist alone does not alter spontaneous or evoked firing rate of wide dynamic range neurones of the lumbar dorsal horn of rats (Hohmann et al., 1999).

Furthermore, a recent study in non-arthritic and arthritic rats showed that SR141716A failed to block the antinociceptive effects of anandamide in either group of rats (Smith et al., 1998). The authors suggested that the effects of anandamide did not result from cannabinoid CB_1 receptor stimulation and that they may be due to the interaction with endogenous opioids since naloxone significantly blocked anandamide effect. Finally, Lichtman et al. have recently found that SR141716A does not exert any consistent hyperalgesic action in several models of pain, including the mouse formalin test (personal communication by Dr. A. Lichtman). (ii) SR141716A and SR144528 have a complex pharmacological profile and are not pure antagonists (Compton et al., 1996; Landsman et al., 1997; Barth, 1998; MacLennan et al., 1998; Pan et al., 1998; Rinaldi-Carmona et al., 1998). Thus, the hyperalgesic effects reported in the literature following administration of the cannabinoid antagonists could be explained by their inverse agonist properties in the various models of pain used and not by the existence of an analgesic tone exerted by endocannabinoids. (iii) Cannabinoid CB_1 receptor knockout studies in mice do not support the existence of an endogenous cannabinoid tone. Ledent et al. (1999) found no change in pain thresholds in their $CB_1 -/-$ mice using heat, mechanical pressure or chemical irritants as pain stimuli. These animals also showed no analgesic responses at all to Δ^9-tetrahydrocannabinol in the hot-plate test. Zimmer et al. (1999) also found that the analgesic responses to Δ^9-tetrahydrocannabinol in the hot-plate test were abolished in the $CB_1 -/-$ mice, but, in agreement with part of the data described herein, reported hypoalgesia in the $CB_1 -/-$ mice when tested in the hot-plate or formalin paw models. Together these results show no evidence of increased inflammatory hyperalgesia in $CB_1 -/-$ mice, although they do indicate that *exogenous* cannabinoids can be analgesic through a CB_1-mediated mechanism.

On the basis of these observations, how is it then possible to explain our finding of an analgesic action of SR141716A and SR144528 in the rat formalin test? First of all it should be pointed out that SR141716A can also exhibit a weak agonist activity (anti-proliferative action against human breast cancer cells (De Petrocellis et al., 1998) and anti-inflammatory action in the arachidonate-induced ear swelling model (Hanus et al., 1999)). This could explain why SR141716A behaves in our study as an analgesic. Another, more intriguing explanation is based on the observation that anandamide exerts a biphasic effect on nociception, consisting of hyperalgesic effects at low concentrations and analgesic actions at high doses (Mechoulam and Fride, 1995; Sulcova et al., 1998). A

possible biochemical explanation to this phenomenon was recently provided by Calandra et al. (1999) who showed that the cannabinoid CB_1 receptor can couple to either inhibition or stimulation of adenylyl cyclase, via $G_{i/o}$ and G_s, respectively. Therefore, the anti-nociceptive action of the two cannabinoid receptor antagonists, as well as the hypoalgesia observed in CB_1 knockout mice by Zimmer et al. (1999), may be explained by a counteraction of a tonic stimulation of nociceptors by endogenous cannabinoids, likely to be present in vivo at low concentrations.

In conclusion, we have shown that SR141716A and SR144528, two cannabinoid CB_1 and CB_2 receptor antagonists, respectively, decrease the nociceptive behaviour during both phases of the formalin test when administered i.p. in rats. Furthermore, no significant increase in skin levels of endogenous cannabinoids were observed in formalin-induced inflamed conditions. These data argue against the presence of an endogenous cannabinoid system tonically inhibiting nociception in this model. Even though our results and those of the cannabinoid CB_1 receptor knockout studies (Ledent et al., 1999; Zimmer et al., 1999) cannot support the concept of an endogenous anti-hyperalgesic cannabinoid tone in inflammation, they do not lessen the case for exogenous cannabinoids behaving as analgesics.

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