UC Irvine
UC Irvine Previously Published Works

Title

AM404, an anandamide transport inhibitor, reduces plasma extravasation in a model of neuropathic pain in rat: Role for cannabinoid receptors

Permalink

https://escholarship.org/uc/item/8qt462k1

Journal

Neuropharmacology, 54(3)

ISSN

0028-3908

Authors

La Rana, G
Russo, R
D'Agostino, G
et al.

Publication Date

2008-03-01

DOI

10.1016/j.neuropharm.2007.10.021

Peer reviewed

AM404, an anandamide transport inhibitor, reduces plasma extravasation in a model of neuropathic pain in rat: Role for cannabinoid receptors

G. La Rana [a], R. Russo [a], G. D'Agostino [a], O. Sasso [a], G. Mattace Raso [a], A. Iacono [a], R. Meli [a], D. Piomelli [b], A. Calignano [a],*

[a] *Department of Experimental Pharmacology, University of Naples "Federico II", via D. Montesano 49, 80131 Naples, Italy*
[b] *Department of Pharmacology, University of California, Irvine, CA, USA*

Abstract

Neuropathic pain consequent to peripheral nerve injury has been associated with local inflammation. Following noxious stimulation afferent fibres release substance P (SP) and calcitonin-gene related peptide (CGRP), which are closely related to oedema formation and plasma leakage. The effect of the anandamide transport blocker AM404 has been studied on plasma extravasation after chronic constriction injury (CCI) which consists in a unilateral loose ligation of the rat sciatic nerve (Bennett and Xie, 1988). AM404 ($1-3-10$ mg kg^{-1}) reduced plasma extravasation in the legated paw, measured as μg of Evans Blue per gram of fresh tissue. A strong effect on vascular permeability was also produced by the synthetic cannabinoid agonist WIN 55,212-2 ($0.1-0.3-1$ mg kg^{-1}). Using specific antagonists or enzyme inhibitors, we demonstrate that cannabinoids act at several levels: data on the 3rd day suggest a strong involvement of substance P (SP) and calcitonin gene-related peptide (CGRP) in the control of vascular tone, whereas at the 7th and 14th days the major role seems to be played by prostaglandins (PGs) and nitric oxide (NO). Capsaicin injection in ligated paws of AM404- or WIN 55,212-2-treated rats resulted in an increase of Evans Blue extravasation, suggesting the involvement of the cannabinergic system in the protective effect of C fibres of ligated paws. Taken together, these data demonstrate the efficacy of cannabinoids in controlling pain behaviour through the modulation of several pain mediators and markers of vascular reactivity, such as SP, CGRP, PGs and NO.
© 2007 Elsevier Ltd. All rights reserved.

Keywords: AM404; WIN 55,212-2; Plasma extravasation; Neuropathic pain; Mechanical allodynia; Thermal hyperalgesia

1. Introduction

The endogenous acylethanolamides are a group of substances generated on demand through stimulus-dependent cleavage of membrane phospholipid precursors and, after their release, undergo a rapid inactivation (Di Marzo et al., 1994; Piomelli et al., 1998). Among the acylethanolamides, anandamide (AEA) represents the major endocannabinoid released by neuron stimulation. AEA, acting through cannabinoid receptors-1 and -2 (CB1 and CB2), has been implicated in several physiological and pathological conditions (Di Marzo and Petrosino, 2007). The endocannabinoids are removed from the extracellular space by a high-affinity transport system present both in neural (Beltramo et al., 1997) and non-neural cells, such as J774 macrophages (Bisogno et al., 1997), RBL-2H3 cells (Rakhshan et al., 2000), and endothelial cells (Maccarrone et al., 2000). N-(4-Hydroxyphenyl)-arachidonylamide (AM404), an inhibitor of endocannabinoid uptake, does not directly activate cannabinoid receptors but, protecting AEA from inactivation, increases its circulating levels (Giuffrida et al., 2001). Moreover, AM404 acts at sites other than the endocannabinoid system, including transient receptor potential vanilloid 1 (TRPV1), calcium and sodium channels (Zygmunt et al., 2000; Nicholson et al., 2003; Kelley and Thayer, 2004).

* Corresponding author. Tel.: +39 081 678441; fax: +39 081 678403.
E-mail address: calignan@unina.it (A. Calignano).

Small-diameter primary afferent fibres not only transmit nociceptive messages to central neurons, but are also involved in inflammatory response, such as peripheral neurogenic inflammation (White and Helme, 1985). Following noxious stimulation, these fibres release substance P (SP) and calcitonin gene-related peptide (CGRP), related to oedema and plasma leakage (Basile et al., 1993). Furthermore, it has been observed that there is a decreased reaction to capsaicin in neuropathic animals (Yonehara and Yoshimura, 2001). It has been shown that CB1 expresses dorsal root ganglion (DRG) neurons, co-localised with several other mediators, including CGRP, suggesting a relationship also with the endocannabinoid system (Bridges et al., 2003; Mitrirattanakul et al., 2006). Moreover, Richardson et al. (1998) demonstrated that AEA inhibited capsaicin-induced release of CGRP from isolated hind paw skin through a peripheral mechanism of neurosecretion inhibition via CB1 receptor.

The chronic constriction injury (CCI) is one of the most widely used models for the study of neuropathic pain. It consists of a unilateral loose ligation of the sciatic nerve and shows many of the pathological properties of chronic neuropathic pain in humans (Bennett and Xie, 1988). Peripheral nerve injury has been associated with local release of several mediators including cytokines, such as tumour necrosis factor-α (Wagner and Myers, 1996) and inflammatory enzymes, such as inducible nitric oxide synthase (iNOS) (Levy et al., 1999) and cyclooxygenase-2 (COX-2) (La Rana et al., 2006). Indeed, among the mediators activated in CCI model, a prominent role has been suggested for nitric oxide (NO) and prostaglandins (PGs), involved in the development of neuropathic pain (Ma and Eisenach, 2002; Levy et al., 1999).

In this study we have investigated the effect of daily treatment with the anandamide transport blocker AM404 or the direct cannabinergic drug WIN 55,212-2, on plasma extravasation as a parameter of neurogenic inflammation following CCI, and the involvement of cannabinoid receptors and TRPV1 in this effect. Moreover, we investigated the time-dependent role of SP, CGRP, NO and prostaglandins in vascular permeability in a CCI model.

2. Materials and methods

2.1. Animals

Male Wistar rats (200–220 g) were purchased from Harlan Italy (San Pietro al Natisone, UD, Italy) and housed in stainless steel cages in a room kept at $22 \pm 1\,°C$ with a 12:12 h light/dark cycle. The animals were acclimated to their environment for 1 week and had ad libitum access to standard rodent chow pellets. The same animals were used first for thermal hyperalgesia measurement and 10 min later for mechanical allodynia. Other groups of animals were used for plasma extravasation experiments. All animal experiments complied with the Italian (D.L. no. 116 of January 27, 1992) and associated guidelines in the European Communities Council (Directive of November 24, 1986, 86/609/ECC). All surgical procedures were reviewed and approved by the Ministero della Ricerca Scientifica, and conformed to the guidelines of the International Association for the Study of Pain (Zimmermann, 1983).

2.2. Chronic constriction injury (CCI) model of neuropathic pain

Hyperalgesia was examined in the model of neuropathic pain induced by ligation of the sciatic nerve according to the method described by Bennett

and Xie (1988). Briefly, rats were anaesthetized (100 mg/kg ketamine plus 5 mg/kg xylazine intraperitoneally (i.p.)) and the left sciatic nerve was exposed at the level of the thigh by blunt dissection through the biceps femoris. Proximal to the sciatic nerve trifurcation, about 12 mm of nerve was freed of adhering tissue and four ligatures (silk suture 3-0) were loosely tied around it with about 1 mm spacing so that the epineural circulation was preserved. The length of nerve thus affected was 6–8 mm long. The animals were allowed to recover and used the day after the surgery. In sham-operated rats, the nerve was exposed but not ligated.

2.3. Drugs and treatments

AM404 (Tocris, Cookson Ltd., UK) was dissolved in saline and DMSO (20%) and administered at 1, 3, and 10 mg/kg subcutaneously (s.c.), WIN 55,212-2 (Tocris Cookson Ltd., UK) was dissolved in saline and DMSO (20%) and administered at 0.1, 0.3, and 1 mg/kg s.c. The drugs were administered once a day for 14 days, starting the first day after ligation. Sham (control) and CCI groups received only vehicle in the same condition.

The antagonist of SP, [D-Pro^2, D-Trp^{7,9}]-Substance P (10 ng/10 μl, intraplantarly (i.pl.)), the antagonist of CGRP, Calcitonin Gene Related Peptide fragment 8–37 human (α-CGRP 8–37, 5 μg/10 μl, i.pl.), $N^{ω}$-Nitro-L-arginine methyl ester hydrochloride (L-NAME, 10 μg/10 μl, i.pl.) were all purchased from Sigma–Aldrich (Milan, Italy) and administered 5 min before Evans Blue (EB) intravenous injection (i.v.), in the tail vein. Ketorolac Tris salt (10 mg/kg, i.p.) was purchased from Sigma–Aldrich and administered 1 h before EB injection. Rofecoxib (10 mg/kg, i.p.) was a generous gift from Professor G. Cirino and administered 1 h before EB i.v. injection. Capsaicin (20 μg/10 μl i.pl.) (Tocris, Cookson Ltd., UK) and capsazepine (30 mg/kg i.p.) were administered 30 min before EB i.v. injection. CB1 (SR141716A, SR1), and CB2 (SR144528, SR2) antagonists were a generous gift from Sanofi (France) and were administered (0.1 mg/kg, i.v., acute treatment) 30 min before EB i.v. injection.

2.4. Plasma extravasation

EB i.v. (in the tail vein) injection (75 mg/kg) was used to measure plasma extravasation. On days 3, 7, and 14 after surgery, the rats ($n = 8$ each group) were treated according to the procedure reported in the Section 2.3 with the drugs or vehicle and received i.v. EB injection. One hour later rats were euthanized by CO_2 asphyxiation, and ipsi- (treated) and contralateral (untreated) paws excised. Plasma extravasation was determined as described by Joris et al. (1990) with slight modifications. Briefly, 6 mm punch biopsies were collected from the hind paw plantar skin of both paws and extracted with 1 ml formamide for 72 h and than read on a spectrophotometer at a wavelength of 550 nm to determine optical density. These values were obtained using a standard curve in order to determine the amount of EB dye in each sample.

2.5. Mechanical allodynia

To assess for changes in sensation or in the development of mechanical allodynia, sensitivity to tactile stimulation was measured using the Dynamic Plantar Aesthesiometer (DPA, Ugo Basile, Varese, Italy), which is an automated version of the von Frey hair assessment. Plantar von Frey hairs are used to detect changes in touch sensitivity, in response to mechanical stimulation, resulting from neural damage. The standard von Frey hairs are a set of 20 monofilaments in a linear scale of physical force. The different von Frey hairs are pressed against the skin on the plantar surface of the foot until the animal withdraws its paw. Animals ($n = 8$ for each group) are placed individually in a small enclosed testing arena (20 cm × 18.5 cm × 13 cm, length × width × height) with a wire mesh floor for 5 min. The DPA device is positioned beneath the animal, so that the filament is directly under the plantar surface of the foot to be tested. When a trial is initiated, the device raises the filament to touch the foot and progressively increases force until the animal withdraws its foot, or until it reaches a maximum of 50 g of force. The DPA automatically records the force at which the foot is withdrawn and the withdrawal latency (actually, latency and maximum force are directly related, because the device progressively increases force until withdrawal occurs). Each paw is tested twice per session. This test does not require any special pre-training, just an acclimation period to the

environment and testing procedure. Testing was performed on both the ispsilateral (ligated) and contralateral (unligated) paw before ligation (day 0) and then on the 3rd, 7th and 14th days after ligation. Last treatment with AM404, or WIN 55,212-2, or vehicle was performed 1 h before measuring mechanical allodynia.

2.6. Thermal hyperalgesia

Thermal hyperalgesia was examined by measuring the latency to withdrawal of the hind paws from a focused beam of radiant heat applied to the plantar surface using a Plantar Test (PT) apparatus (Ugo Basile). Two days before experiment, animals ($n = 8$ for each group) were placed in a transparent Perspex box with a thin glass floor and allowed to acclimatise for 10–15 min. Withdrawal latencies to radiant heat were measured on both ipsilateral and contralateral paws on days 0 (before ligation), 3, 7 and 14. Three trials each for the right and left hind paws were performed and for each reading the apparatus was set at a cut-off time of 30 s. Last treatment with AM404, or WIN 55,212-2, or vehicle was performed 1 h before measuring thermal hyperalgesia.

2.7. Rotarod test

Integrity of motor function was assessed in CCI rats using an accelerating Rotarod (Ugo Basile). The animals were acclimated to acceleration in three training runs. Mean performance time (s) determined on the fourth and fifth run served as control value. Performance time was measured every 20 min for a total of 80 min on days 3, 7 and 14 after surgery.

2.8. Statistical analysis

All data are presented as the mean ± S.E.M. Statistical analysis was performed by two-way ANOVA test for multiple comparisons followed by Bonferroni's test. Statistical significance was set at $p < 0.05$.

3. Results

3.1. Effect of AM404 and WIN 55,212-2 on plasma extravasation in CCI animals

A time-dependent increase of plasma leakage was observed in ligated paw compared to those of Sham group. Plasma extravasation was inhibited by chronic administration of AM404: in particular this effect was significant at the doses of 3 and 10 mg/kg ($p < 0.05$) with a more significant effect at day 14 ($p < 0.01$); whereas the dose of 1 mg/kg did not alter plasma extravasation (Fig. 1A). The synthetic cannabinoid agonist WIN 55,212-2 showed the strongest effect, even starting at lower doses (Fig. 1B). Amount of EB was not modified in contralateral paws at all experimental times (data not shown).

3.2. Time-dependent role of several mediators on plasma extravasation

In order to evaluate the different role played by neuropeptides and inflammatory mediators in plasma extravasation in CCI paws, we used the CGRP antagonist Frag 8–37-CGRP, the SP antagonist DD-SP, L-NAME, as NO synthesis inhibitor, and selective COX-1 or COX-2 inhibitors ketorolac and rofecoxib, respectively. As shown in Fig. 2A both CGRP and SP played a major role in plasma leakage in early phase of CCI, since both neuropeptides antagonists inhibited significantly plasma extravasation ($p < 0.05$ vs. CCI group) on the 3rd day. Conversely, L-NAME, and COX-1 and -2 inhibitors did
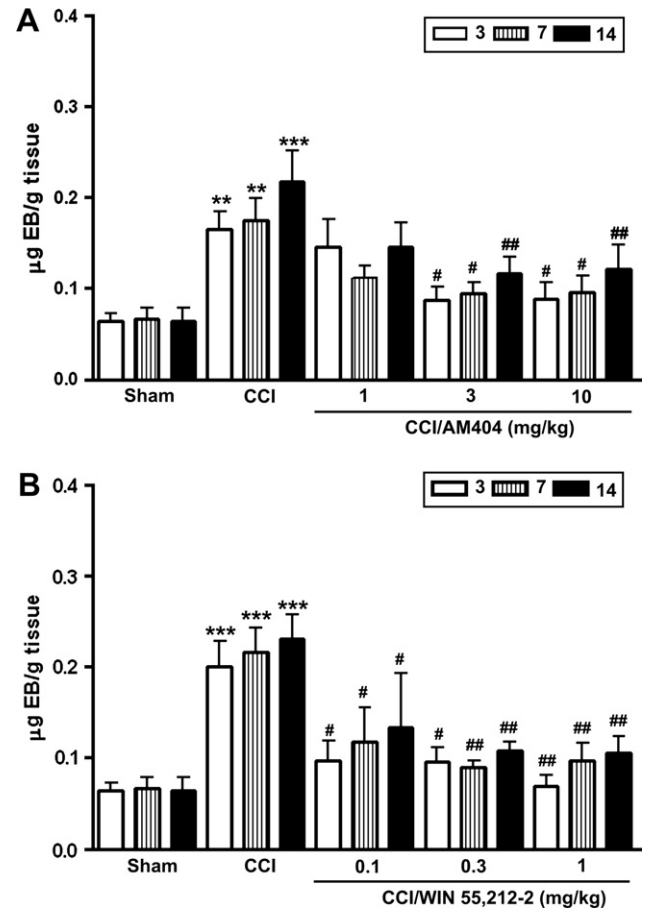

Fig. 1. Effect of repeated treatments of AM404 (1, 3 and 10 mg/kg daily s.c.) (A) or WIN 55,212-2 (0.1, 0.3, and 1 mg/kg daily s.c.) (B) in CCI rats on plasma extravasation evaluated at the 3rd, 7th and 14th day after surgery. This parameter was quantified as μg of EB per gram of tissue. AM404, WIN 55,212-2, or vehicle was administered 1 h before animal death. Data represent mean ± S.E.M. of 8 rats. Statistical significance reported in the graph is relative to comparison of groups belonging to the same experimental time. **$p < 0.01$ and ***$p < 0.001$ vs. Sham; #$p < 0.05$ and ##$p < 0.01$ vs. CCI.

not modify significantly plasma extravasation at the 3rd day, while it was inhibited very significantly at the 7th and 14th days (Fig. 2B), suggesting a pivotal role for NO and PGs in the late phase of CCI.

3.3. Effect of AM404 and WIN 55,212-2 on plasma leakage on ipsilateral and contralateral paws of CCI animals acutely treated with capsaicin or capsazepine

An acute intraplantar injection in both paws of capsaicin (CCI + capsaicin group), an active neuropeptide releasing drug, caused in CCI ipsilateral paw an increase of plasma extravasation only at day 3 ($p < 0.05$), and not at the 7th and 14th day, if compared to the CCI group, due to ligation-induced depletion of neuropeptide vesicle content (Fig. 3A). Conversely, as expected, in contralateral undamaged paw there was an enhanced EB accumulation at all experimental times (3rd, 7th and 14th day; $p < 0.001$, data not shown).

Furthermore, our results showed that capsaicin injection in ligated paws of AM404 (10 mg/kg) treated rats resulted in an
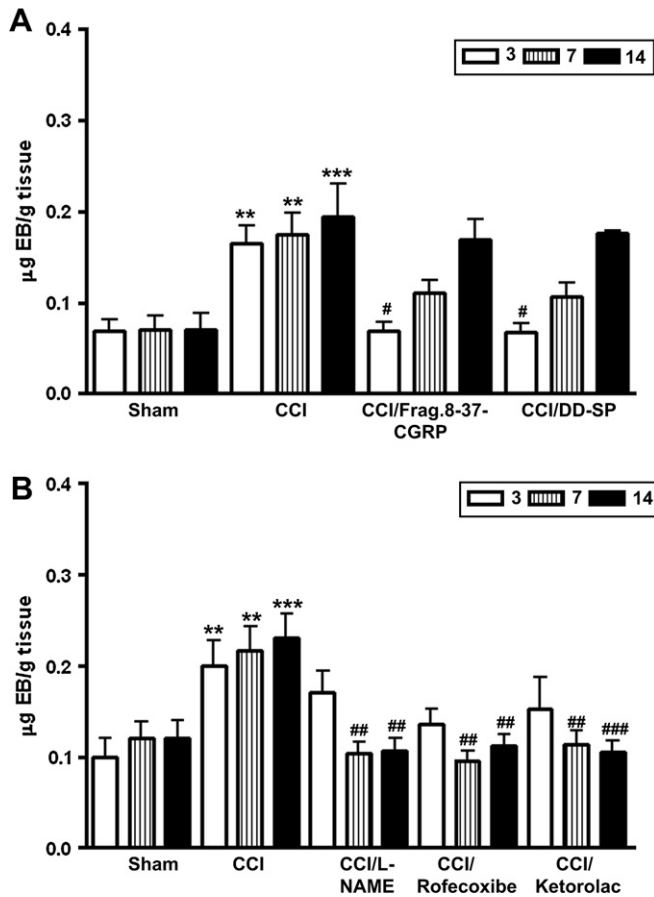
Fig. 2. Time-dependent role of several mediators on plasma extravasation. Effect of the CGRP antagonist Frag 8–37-CGRP (5 µg/10 µl, i.pl.), and the substance P antagonist DD-SP (10 ng/10 µl, i.pl.) (A) or L-NAME (10 µg/10 µl, i.pl.), rofecoxib, COX-2 inhibitor (10 mg/kg, i.p.) and ketorolac, COX-1 inhibitor (10 mg/kg, i.p.) (B) on plasma extravasation, quantified as µg of EB per gram of tissue. Data represent mean ± S.E.M. of 8 rats. Statistical significance reported in the graph is relative to comparison of groups belonging to the same experimental time. $*p < 0.05$, $**p < 0.01$ and $***p < 0.001$ vs. Sham; $^{\#}p < 0.05$ $^{\#\#}p < 0.01$ and $^{\#\#\#}p < 0.001$ vs. CCI.

increase of EB extravasation with respect to the CCI/AM404 group, at all experimental times ($p < 0.001$) (Fig. 3A), suggesting that AM404 preserved the integrity of sensorial fibres (i.e. neuropeptide content) in ligated paws. A similar effect was also obtained with the direct CB1/CB2 agonist WIN 55,212-2, confirming the involvement of the cannabinergic system in the protective effect of C fibres of ligated paws (Fig. 3B). Indeed the acute systemic injection of capsazepine at days 3, 7 and 14 did not alter the effect of AM404 (10 mg/kg) (Fig. 3C).

3.4. Effect of AM404 and WIN 55,212-2 on mechanical allodynia and thermal hyperalgesia

All data presented in Figs. 4 and 5 on mechanical allodynia and thermal hyperalgesia are relative to ipsilateral paw measurements, as no effects were seen in contralateral paws (data not shown). On the 3rd, 7th and 14th day after ligation, CCI animals showed a significant time-dependent reduction of mechanical and thermal threshold. Confirming previous data (Costa et al., 2006; La Rana et al., 2006), repeated treatments
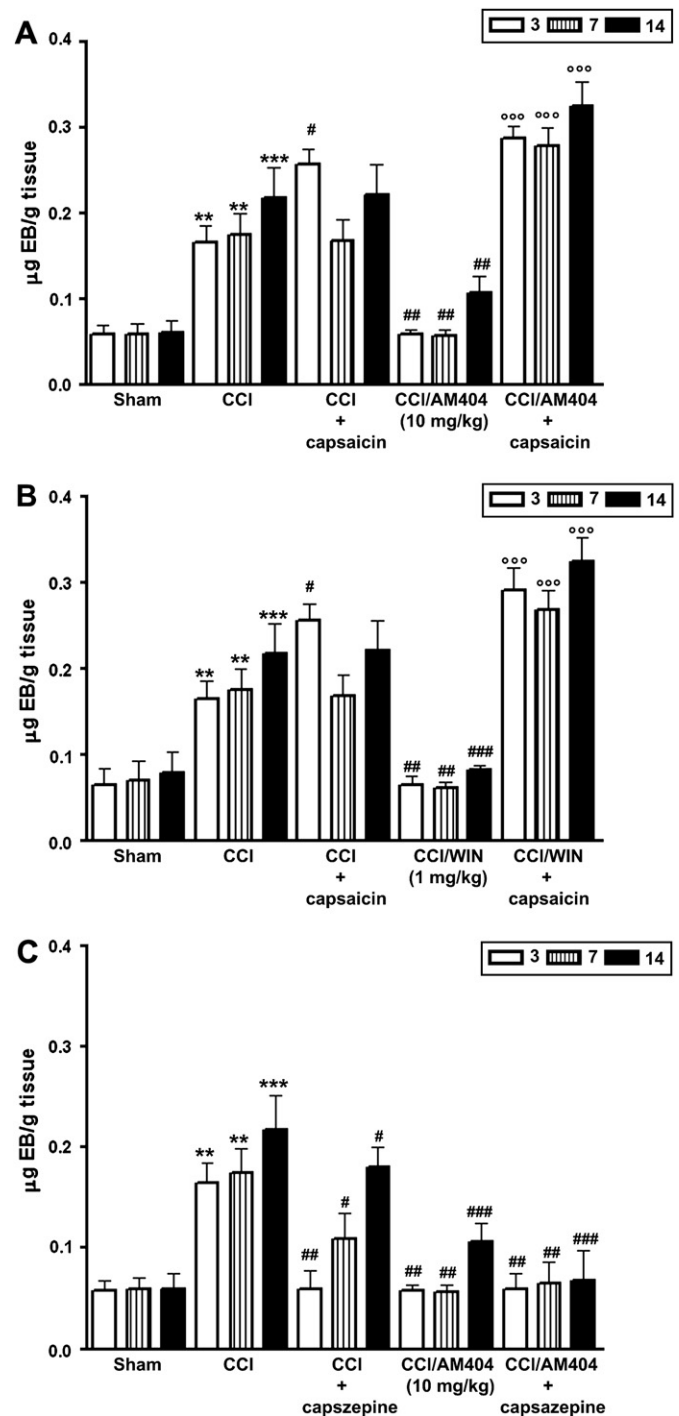

Fig. 3. Effect of AM404 (10 mg/kg s.c.) (A) and WIN 55,212-2 (1 mg/kg s.c.) (B) on plasma extravasation in capsaicin injected paws of CCI animals. Capsaicin (20 µg, acute treatment i.pl.) was injected in CCI animals 30 min before EB determination and evaluated at the 3rd, 7th and 14th day after surgery. (C) Effect of AM404 (10 mg/kg s.c.) on plasma extravasation in capsazepine acutely treated animals (30 mg/kg i.p. 30 min before EB injection). This parameter was quantified as µg of EB per gram of tissue. Data represent mean ± S.E.M. of 8 rats. Statistical significance reported in the graph is relative to comparison of groups belonging to the same experimental time. $**p < 0.01$ and $***p < 0.001$ vs. Sham; $^{\#}p < 0.05$, $^{\#\#}p < 0.01$ and $^{\#\#\#}p < 0.001$ vs. CCI; $^{\circ\circ\circ}p < 0.001$ vs. CCI/AM404 (A) or /WIN 55,212-2 (B).
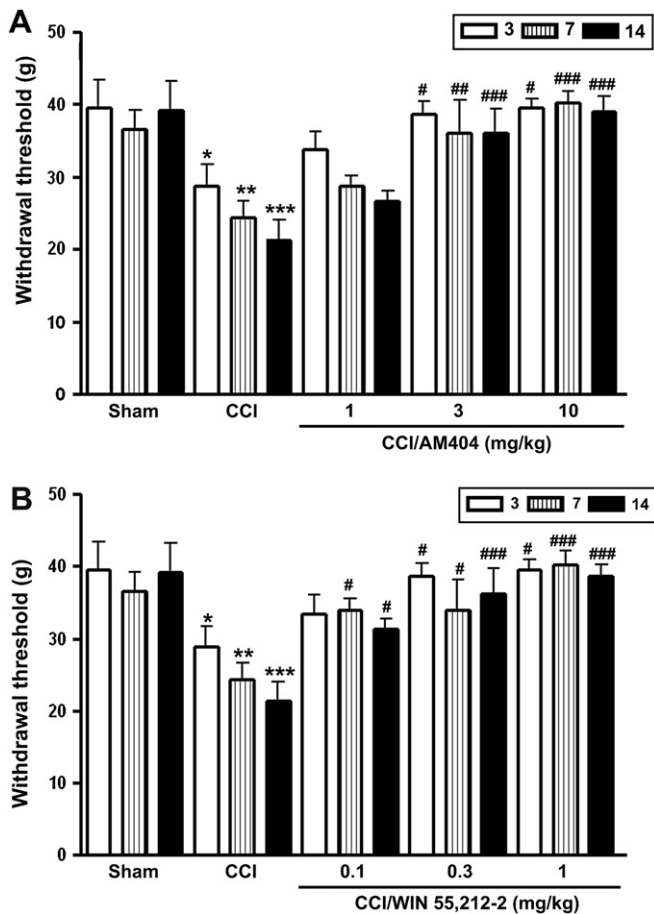
Fig. 4. Effect of repeated treatments with AM404 (1, 3 and 10 mg/kg daily s.c.) (A) or WIN 55,212-2 (0.1, 0.3, and 1 mg/kg daily s.c.) (B) in CCI rats on mechanical allodynia evaluated at the 3rd, 7th and 14th day after surgery. Mechanical withdrawal threshold of the injured paws is expressed as grams. Data represent mean ± S.E.M. of 8 rats. Statistical significance reported in the graph is relative to comparison of groups belonging to the same experimental time. *$p < 0.05$, **$p < 0.01$ and ***$p < 0.001$ vs. Sham; #$p < 0.05$, ##$p < 0.01$ and ###$p < 0.001$ vs. CCI.
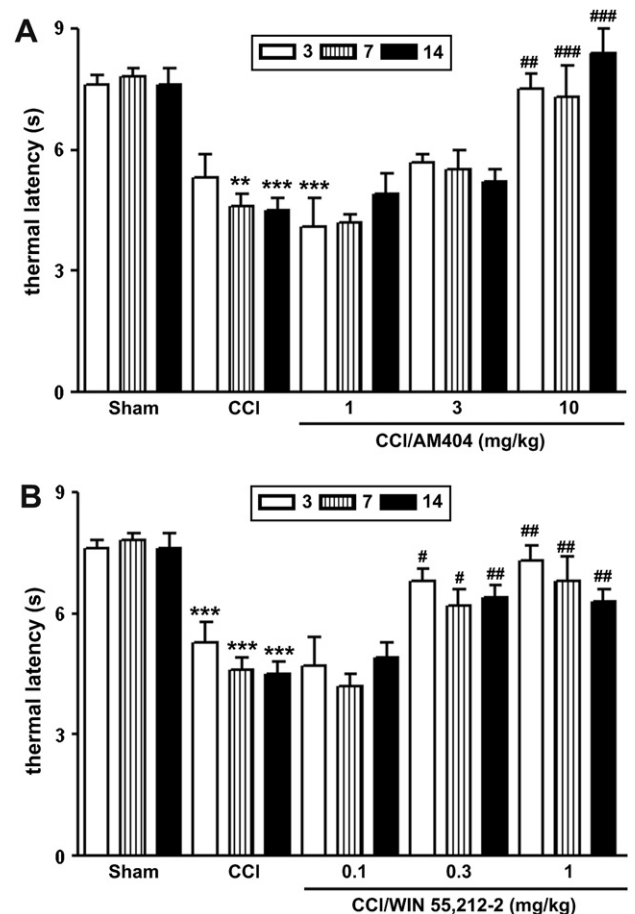
Fig. 5. Effect of repeated treatments with AM404 (1, 3 and 10 mg/kg daily s.c.) (A) or WIN 55,212-2 (0.1, 0.3, and 1 mg/kg daily s.c.) (B) in CCI rats on thermal hyperalgesia evaluated at the 3rd, 7th and 14th day after surgery. Withdrawal latency to heat of the injured paws is expressed in seconds. Data represent mean ± S.E.M. of 8 rats. Statistical significance reported in the graph is relative to comparison of groups belonging to the same experimental time. **$p < 0.01$ and ***$p < 0.001$ vs. Sham; #$p < 0.05$, ##$p < 0.01$ and ###$p < 0.001$ vs. CCI.

with AM404 showed in CCI animals a time-dependent anti-allodynic (Fig. 4A) and anti-hyperalgesic (Fig. 5A) effect, as shown from the increased force exerted to induce paw withdrawal in mechanical allodynia and paw withdrawal latency in thermal hyperalgesia, measured at the 3rd, 7th, and 14th day. Moreover, we demonstrated for the first time a time- and dose-dependent effect of chronic administration of WIN 55,212-2 on withdrawal threshold (Fig. 4B) and thermal latency (Fig. 5B).

The anti-allodynic effect of both drugs was statistically significant at 3 and 10 mg/kg for AM404 and at all doses for WIN 55,212-2. Moreover, the anti-hyperalgesic effect of AM404 was significant only at the highest dose, while WIN 55,212-2 had a more effective result. Chronic AM404 or WIN 55,212-2 treatment at the highest dose (10 mg/kg and 1 mg/kg, respectively) did not alter motor function, as assessed in the Rotarod test (data not shown). These results indicate that the anti-hyperalgesic and anti-allodynic effect of AM404 and WIN may not be ascribed to sedation or motor impairment.

3.5. Cannabinergic involvement in the pharmacological effects of AM404

To investigate the involvement of CB receptors in AM404-induced analgesia, we used two specific antagonists of CB1 or CB2 receptor (SR1 and SR2, respectively). These antagonists were administered in acute, i.v. 30 min before plasma extravasation, mechanical allodynia and thermal hyperalgesia measurement. Concerning the effect of AM404 on plasma extravasation, a pivotal role of CB1 receptor was demonstrated (Fig. 6A), without CB2 involvement. In accord with Costa et al. (2006), the CB1 antagonist reversed the AM404 effect (Fig. 6B), whereas CB2 antagonist caused a not statistically significant reduction of anti-allodynic effect of AM404. In Fig. 6C the effect of the antagonists on thermal hyperalgesia is shown. Confirming previous data (Costa et al., 2006), SR1 reduced the AM404 effect at all experimental times. Conversely, in our experimental conditions, CB2 receptor seems not to be involved in the anti-hyperalgesic effect of AM404.
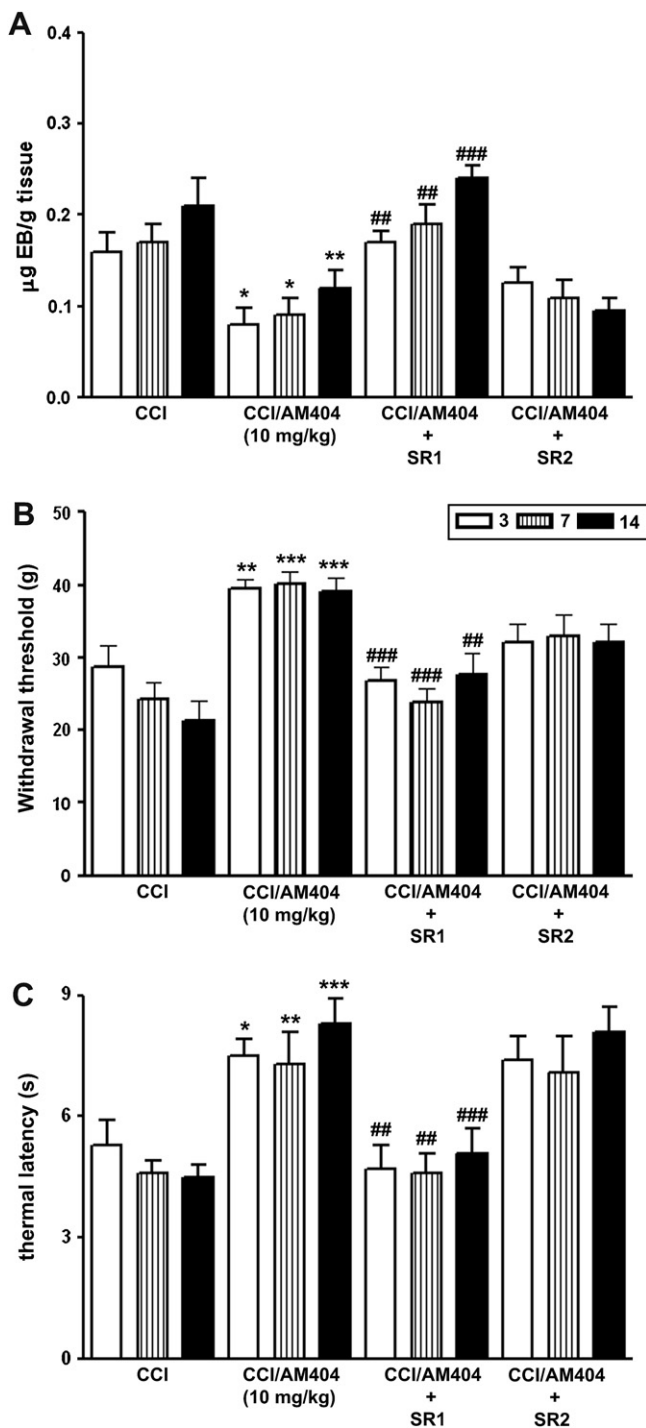
Fig. 6. Effect of SR1, and SR2 (both at 0.1 mg/kg, i.v.) on AM404 (10 mg/kg s.c.) effects on plasma extravasation (A), mechanical allodynia (B), and thermal hyperalgesia (C). Data represent mean ± S.E.M. of 8 rats. Statistical significance reported in the graph is relative to comparison of groups belonging to the same experimental time. *$p < 0.05$, **$p < 0.01$ and ***$p < 0.001$ vs. CCI; ##$p < 0.01$ and ###$p < 0.001$ vs. CCI/AM404.

3.6. CB1 or CB2 antagonist modulates the pharmacological effects of WIN 55,212-2

In these experiments the antagonists (SR1 and SR2) were administered i.v. 30 min before all measurements. As shown in Fig. 7A, the WIN 55,212-2 effect on plasma extravasation

was related to the involvement of both CB receptors, since SR1 and SR2 significantly inhibited this effect. Our results indicated that the WIN 55,212-2 effect on mechanical allodynia and thermal hyperalgesia was mediated by CB1 receptor at all experimental times, as shown by the reversal effect of SR1 (Fig. 7B and C). Conversely, SR2 reverted the WIN 55,212-2 effect significantly only at the 7th and 14th day, when inflammatory cells expressing CB2 receptors are locally recruited (Fig. 7B and C).

4. Discussion

Fatty acid ethanolamides represent a recent class of lipid mediators involved in several physiological processes. Among them, the endogenous cannabinoid AEA has been well characterised (Cravatt and Lichtman, 2004). The anti-nociceptive effect of AEA is mediated by CB1 receptor activation (Richardson et al., 1998), where its action is terminated by removal from the synaptic space by a high-affinity endocannabinoid transport system (Beltramo et al., 1997). Thus the pharmacological modulation of the cannabinergic system could be obtained either using direct CB agonists, such as WIN 55,212-2, or increasing the endogenous levels of AEA through inhibition of AEA transport with a specific inhibitor, such as N-(4-hydroxyphenyl)-arachidonamide, AM404 (Giuffrida et al., 2001).

Recently we have demonstrated that repeated treatment with AM404 dose-dependently inhibited pain behaviour in the formalin test and the expression of the most powerful inflammatory enzymes involved in tissue damage in CCI model of neuropathic pain, such as COX-2 and iNOS (La Rana et al., 2006), suggesting that NO and COX-2 metabolites play a crucial role in pain signalling in this model (Levy et al., 1999).

Indeed, the endocannabinoids could be considered a precious tool in the pharmacotherapy of neuropathy, not only for their analgesic effect that appears even after a single administration, but also for the control of several pain mediators, such as SP and CGRP. It is known that capsaicin, a naturally derived activator of vanilloid receptor, initially leads to excitation of the nociceptive neurons by selectively activating nociceptors and the consequent perception of pain and local release of inflammatory mediators, such as SP and CGRP, and histamine (Lewin and Mendell, 1993; Dray et al., 1994).

Previously, Richardson et al. (1998) have reported that peripheral administration of AEA inhibited the induction of hyperalgesia, after capsaicin stimulation, through the inhibition of neurosecretion from the peripheral terminals of nociceptive primary afferent fibres. Yonehara and Yoshimura (2001) have also confirmed that capsaicin stimulates the release of SP in a model of neuropathic pain.

Here, we demonstrated that both AM404 and WIN55,212-2 inhibited plasma extravasation of the paws from CCI animals at all experimental times examined. CCI-induced plasma extravasation in the early days after ligature is caused by the release of neuroactive peptides at peripheral levels, whereas other inflammatory mediators (such as NO and PGs) are involved subsequently.
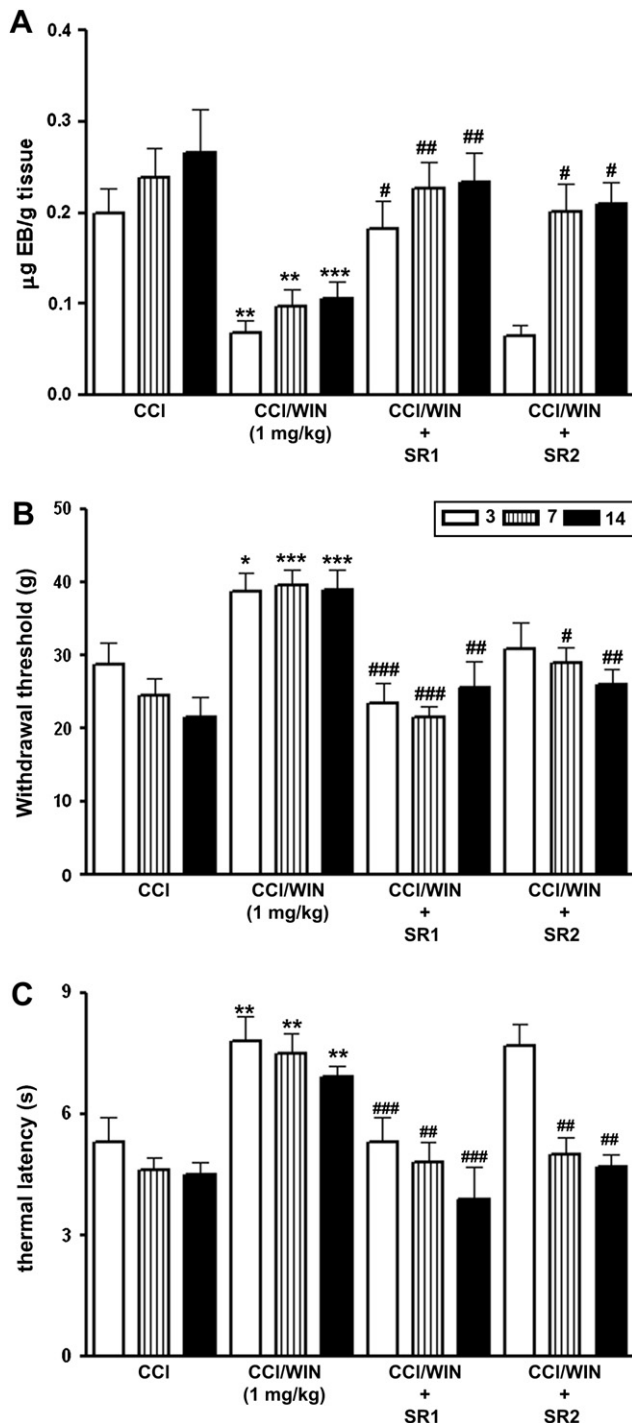
Fig. 7. Effect of SR1, and SR2 (both at 0.1 mg/kg, i.v.) on WIN 55,212-2 (1 mg/kg s.c.) effects on plasma extravasation (A), mechanical allodynia (B), and thermal hyperalgesia (C). Data represent mean ± S.E.M. of 8 rats. Statistical significance reported in the graph is relative to comparison of groups belonging to the same experimental time. $*p < 0.05$, $**p < 0.01$ and $***p < 0.001$ vs. CCI; $^{\#}p < 0.05$, $^{\#\#}p < 0.01$ and $^{\#\#\#}p < 0.001$ vs. CCI/WIN.

Using specific antagonists of SP and CGRP ([D-Pro[2], D-Trp[7,9]]-Substance P and α-CGRP fragment 8–37), we demonstrated that these neurogenic peptides modulate plasma extravasation only during the early days after ligature (early-phase 3rd day), whereas PGs and NO are involved in sustaining the middle and late phase (7th and 14th day).

Capsaicin in CCI animals induces a significant increase of plasma extravasation at the 3rd day but not at the 7th and 14th, confirming that CCI causes the release of neuropeptide vesicles that could require several days to be reconstituted, and thus the major contribution of the neuroactive peptides to the early phase. Conversely, in AM404 treated rats capsaicin-induced plasma extravasation was also active on the 7th and 14th day in CCI rats. The finding that these animals were still sensitive to capsaicin even 7 and 14 days after ligature suggests that AM404 exerts a protective effect, preserving the integrity of sensorial fibres and reducing neuropeptide release from C fibres.

The elevation of endocannabinoid concentration in the sciatic nerve (Witting et al., 2004; Bilsland et al., 2006), as well as in the spinal cord and in some supraspinal areas involved in nociception (Petrosino et al., 2007) after constriction in rats would explain why inhibitors of endocannabinoid uptake, which are expected to be active only in the presence of enhanced turnover of endocannabinoids, could reduce plasma leakage and pain behaviour in this experimental model of pain.

Although AM404 could interact with TRPV1 receptor (Zygmunt et al., 2000) at concentrations similar to those necessary to inhibit endocannabinoid transport, its effect on plasma leakage in neuropathic rats does not involve the vanilloid system. In fact, our results show that acute treatment with capsazepine, a TRPV1 antagonist, produces a similar inhibition of EB amount measured at all experimental times, as in the CCI/AM404 group, suggesting no involvement of TRPV1 in AM404 effect on plasma leakage.

However, it is conceivable that the protective effect of AM404, through endocannabinoid level increase, in reducing neuropeptide release from C-fibres, should be related to its effect on several channels. It has been reported that cannabinoids reduce neuropeptide release and cellular firing by the blockage of Ca^{2+} (Caulfield and Brown, 1992) and K^+ channel opening and subsequent hyperpolarisation (Henry and Chavkin, 1995).

COXs and iNOS play a pivotal role in inducing and sustaining the neuropathy in the CCI model (Dudhgaonkar et al., 2007). Our results indicate that these enzymes mainly contribute to the middle and late phase, as suggested by the reduction obtained by ketorolac, rofecoxib and L-NAME. AM404 treatment resulted in a significant reduction of COX-2 and iNOS expression that contributes to down-regulation of neurodegenerative process induced primarily by SP and CGRP (La Rana et al., 2006). From this point of view the indirect (AM404) or direct (WIN 55,212-2) cannabinoids agonist does not act as a symptomatic drug but is able to control the neuropathological process from the beginning of the pathology.

In accord with previous data (Costa et al., 2006; La Rana et al., 2006), we demonstrate here that the effect of AM404 on plasma extravasation is also evident on mechanical allodynia and thermal hyperalgesia, the main signs of neuropathy. The AM404 effect involved mainly the CB1 receptor. In fact, SR141716A completely reversed AM404 effects, while in our experimental conditions CB2 antagonist had no effect. Furthermore, even repeated treatments with WIN 55,212-2 led to similar anti-hyperalgesic and anti-allodynic effects,

confirming previous reports on the anti-hyperalgesic effect of acute treatment of WIN 55,212-2 (Herzberg et al., 1997; Bridges et al., 2001). Our results show that SR1 reverse significantly the analgesic effect and plasma extravasation reduction of WIN 55,212-2 at all experimental times, while SR2 partially modifies these parameters only at the 7th and 14th day, indicating the major, but not exclusive, role for CB1 receptor in the effect of WIN 55,212-2 on neuropathic pain control and plasma leakage reduction.

While CB1 receptors are known to modulate transmission in sensory neuron pathways, it is not clear how CB2 receptors can affect pain response. Recently, it was demonstrated that a selective CB2 receptor agonist inhibited neuropathic pain in rats and CB1-deficent mice, defining a role for CB2 receptor in modulating neuropathic pain (Ibrahim et al., 2003). These receptors were found on mast cells and macrophages that, releasing endogenous inflammatory agents, could be involved in peripheral nerve stimulation (Nackley et al., 2003). A strong recruitment of these cells at the ligation site participates in the subsequent neurogenic inflammation evoked by CCI of sciatic nerve (Moalem and Tracey, 2006). Thus, the stimulation of peripheral CB2 receptors (Elmes et al., 2004) could result in the inhibition of the sensitizing substances that leads to reduction of pain threshold and vascular active neurokinins.

Furthermore, here we confirm that repeated treatment with AM404 causes an analgesic effect, affecting either thermal hyperalgesia or mechanical allodynia which was observed at all time determinations. These findings extend our knowledge on the role of endocannabinoids in the development of pathological modification that occurs during the evolution of neuropathy.

Taken together, our results demonstrate that during the development of neuropathic pain in rats, cannabinergic drugs exert a protective role in preserving the integrity and functionality of peripheral sensory neurons and should be considered not only as possible analgesic drugs but also as endogenous substances that repair a pathological lesion.

Our results show that AM404 and WIN 55,212-2 reduce pain behaviour, plasma extravasation and inflammatory enzyme production at non-psychoactive doses. In fact, neither drug modified motor coordination, basal withdrawal thresholds and thermal latency measured in contralateral paws (data not shown). Moreover, even after 14 days of treatment, the animals did not show any changes in motility or body weight, revealing that the dose used was well tolerated (data not shown). In conclusion, our findings highlight the therapeutic potential of AM404 and WIN 55,212-2 as drugs able to reduce peripheral inflammation and alleviate neuropathic pain, modulating the endogenous cannabinoid system at non-psychoactive doses that represent the limiting factor for their therapeutic use.

Acknowledgements

References

Basile, S., Khalil, Z., Helme, R.D., 1993. Skin vascular reactivity to the neuropeptide substance P in rats with peripheral mononeuropathy. Pain 52, 217–222.

Beltramo, M., Stella, N., Calignano, A., Lin, S.Y., Makriyannis, A., Piomelli, D., 1997. Functional role of high-affinity anandamide transport, as revealed by selective inhibition. Science 277, 1094–1097.

Bennett, G.J., Xie, Y.K., 1988. A peripheral mononeuropathy in rat that produces disorders of pain sensation like those seen in man. Pain 33, 87–107.

Bilsland, L.G., Dick, J.R., Pryce, G., Petrosino, S., Di Marzo, V., Baker, D., Greensmith, L., 2006. Increasing cannabinoid levels by pharmacological and genetic manipulation delay disease progression in SOD1 mice. FASEB J. 20, 1003–1005.

Bisogno, T., Maurelli, S., Melck, D., De Petrocellis, L., Di Marzo, V., 1997. Biosynthesis, uptake, and degradation of anandamide and palmitoylethanolamide in leukocytes. J. Biol. Chem. 272, 3315–3323.

Bridges, D., Ahmad, K., Rice, A.S., 2001. The synthetic cannabinoid WIN 55,212-2 attenuates hyperalgesia and allodynia in a rat model of neuropathic pain. Br. J. Pharmacol. 133, 586–594.

Bridges, D., Rice, A.S., Egertova, M., Elphick, M.R., Winter, J., Michael, G.J., 2003. Localisation of cannabinoid receptor 1 in rat dorsal root ganglion using in situ hybridisation and immunohistochemistry. Neuroscience 119, 803–812.

Caulfield, M.P., Brown, D.A., 1992. Cannabinoid receptor agonists inhibit Ca^{++} current in NG108-neuroblastoma cells via a pertussis toxin-sensitive mechanism. Br. J. Pharmacol. 106, 231–232.

Costa, B., Siniscalco, D., Trovato, A.E., Comelli, F., Sotgiu, M.L., Colleoni, M., Maione, S., Rossi, F., Giagnoni, G., 2006. AM404, an inhibitor of anandamide uptake, prevents pain behaviour and modulate cytokine and apoptotic pathways in a rat model of neuropathic pain. Br. J. Pharmacol. 148, 1022–1032.

Cravatt, B.F., Lichtman, A.H., 2004. The endogenous cannabinoid system and its role in nociceptive behaviour. J. Neurobiol. 61, 149–160.

Di Marzo, V., Fontana, A., Cadas, H., Schinelli, S., Cimino, G., Schwartz, J.C., Piomelli, D., 1994. Formation and inactivation of endogenous cannabinoid anandamide in central neurons. Nature 372, 686–691.

Di Marzo, V., Petrosino, S., 2007. Endocannabinoids and the regulation of their levels in health and disease. Curr. Opin. Lipidol 18 (2), 129–140. Review.

Dray, A., Urban, L., Dickenson, A., 1994. Pharmacology of chronic pain. Trends Pharmacol. Sci. 15, 190–197.

Dudhgaonkar, S.P., Tandan, S.K., Kumar, D., Naik, A.K., Raviprakash, V., 2007. Ameliorative effect of combined administration of inducible nitric oxide synthase inhibitor with cyclooxygenase-2 inhibitors in neuropathic pain in rats. Eur. J. Pain 11, 528–534.

Elmes, S.J., Jhaveri, M.D., Smart, D., Kendall, D.A., Chapman, V., 2004. Cannabinoid CB2 receptor activation inhibits mechanically evoked responses of wide dynamic range dorsal horn neurons in naive rats and in rat models of inflammatory and neuropathic pain. Eur. J. Neurosci. 20, 2311–2320.

Giuffrida, A., Beltramo, M., Piomelli, D., 2001. Mechanisms of endocannabinoid inactivation: biochemistry and pharmacology. J. Pharmacol. Exp. Ther 298 (1), 7–14.

Henry, D.J., Chavkin, C., 1995. Activation of inwardly rectifying potassium channels (GIRK1) by co-expressed rat brain cannabinoid receptors in Xenopus oocytes. Neurosci. Lett. 186, 91–94.

Herzberg, U., Eliav, E., Bennett, G.J., Kopin, I.J., 1997. The analgesic effects of R(+)-WIN 55,212-2 mesylate, a high affinity cannabinoid agonist, in a rat model of neuropathic pain. Neurosci. Lett. 221, 157–160.

Ibrahim, M.M., Deng, H., Zvonok, A., Cockayne, D.A., Kwan, J., Mata, H.P., Vanderah, T.W., Lai, J., Porreca, F., Makriyannis, A., Malan Jr., T.P., 2003. Activation of CB2 cannabinoid receptors by AM1241 inhibits experimental neuropathic pain: pain inhibition by receptors not present in the CNS. Proc. Natl. Acad. Sci. U.S.A. 100, 10529–10533.

Joris, J., Costello, A., Dubner, R., Hargreaves, K.M., 1990. Opiates suppress carrageenan-induced edema and hyperthermia at doses that inhibit hyperalgesia. Pain 43 (1), 95–103.

Kelley, B.G., Thayer, S.A., 2004. Anandamide transport inhibitor AM404 and structurally related compounds inhibit synaptic transmission between rat hippocampal neurons in culture independent of cannabinoid CB1 receptors. Eur. J. Pharmacol. 496, 33–39.

La Rana, G., Russo, R., Campolongo, P., Bortolato, M., Manieri, R.A., Cuomo, V., Iacono, A., Mattace Raso, G., Meli, R., Piomelli, D., Calignano, A., 2006. Modulation of neuropathic and inflammatory pain by the endocannabinoid transport inhibitor AM404. J. Pharmacol. Exp. Ther 317, 1365–1371.

Levy, D., Hoke, A., Zochodne, D.W., 1999. Local expression of inducible nitric oxide synthase in an animal model of neuropathic pain. Neurosci. Lett. 260, 207–209.

Lewin, G.R., Mendell, L.M., 1993. Nerve growth factor and nociception. Trends Neurosci. 16, 353–359.

Ma, W., Eisenach, J.C., 2002. Morphological and pharmacological evidence for the role of peripheral prostaglandins in the pathogenesis of neuropathic pain. Eur. J. Neurosci. 15, 1037–1047.

Maccarrone, M., Bari, M., Lorenzon, T., Bisogno, T., Di Marzo, V., Finazzi-Agró, A., 2000. Anandamide uptake by human endothelial cells and its regulation by nitric oxide. J. Biol. Chem. 275, 13484–13492.

Mitrirattanakul, S., Ramakul, N., Guerriero, A.V., Matsuka, Y., Ono, T., Iwase, H., Mackie, K., Faull, K.F., Spigelman, I., 2006. Site-specific increases in peripheral cannabinoid receptors and their endogenous ligands in a model of neuropathic pain. Pain 126, 102–114.

Moalem, G., Tracey, D.J., 2006. Immune and inflammatory mechanisms in neuropathic pain. Brain Res. Rev. 51, 240–264.

Nackley, A.G., Makriyannis, A., Hohmann, A.G., 2003. Selective activation of cannabinoid CB(2) receptors suppresses spinal fos protein expression and pain behavior in a rat model of inflammation. Neuroscience 119, 747–757.

Nicholson, R.A., Liao, C., Zheng, J., David, L.S., Coyne, L., Errington, A.C., Singh, G., Lees, G., 2003. Sodium channel inhibition by anandamide and synthetic cannabimimetics in brain. Brain Res. 978, 194–204.

Petrosino, S., Palazzo, E., de Novellis, V., Bisogno, T., Rossi, F., Maione, S., Di Marzo, V., 2007. Changes in spinal and supraspinal endocannabinoid levels in neuropathic rats. Neuropharmacology 52, 415–422.

Piomelli, D., Beltramo, M., Giuffrida, A., 1998. Endogenous cannabinoid signaling. Neurobiol. Dis. 5, 462–473.

Rakhshan, F., Day, T.A., Blakely, R.D., Barker, E.L., 2000. Carrier-mediated uptake of the endogenous cannabinoid anandamide in RBL-2H3 cells. J. Pharmacol. Exp. Ther. 292, 960–967.

Richardson, J.D., Kilo, S., Hargreaves, K.M., 1998. Cannabinoids reduce hyperalgesia and inflammation via interaction with peripheral CB1 receptors. Pain 75 (1), 111–119.

Wagner, R., Myers, R.R., 1996. Schwann cells produce tumor necrosis factor alpha: expression in injured and non injured nerves. Neuroscience 73, 625–629.

White, D.M., Helme, R.D., 1985. Release of substance P from peripheral nerve terminals following electrical stimulation of the sciatic nerve. Brain Res. 336, 27–31.

Witting, A., Weydt, P., Hong, S., Kliot, M., Moller, T., Stella, N., 2004. Endocannabinoids accumulate in spinal cord of SOD1 G93A transgenic mice. J. Neurochem. 89, 1555–1557.

Yonehara, N., Yoshimura, M., 2001. Influence of painful chronic neuropathy on neurogenic inflammation. Pain 92, 259–265.

Zimmermann, M., 1983. Ethical guidelines for investigations of experimental pain in conscious animals. Pain 16, 109–110.

Zygmunt, P.M., Chuang, H., Movahed, P., Julius, D., Hogestatt, E.D., 2000. The anandamide transport inhibitor AM404 activates vanilloid receptors. Eur. J. Pharmacol. 396, 39–42.