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### Journal
Journal of Pharmacology and Experimental Therapeutics, 317(3)

### ISSN
0022-3565



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### Publication Date
2006-06-01

### DOI
10.1124/jpet.105.100792

0022-3565/06/3173-1365–1371$20.00
THE JOURNAL OF PHARMACOLOGY AND EXPERIMENTAL THERAPEUTICS Vol. 317, No. 3

100792/3113920
JPET 317:1365–1371, 2006 Printed in U.S.A.



# Modulation of Neuropathic and Inflammatory Pain by the Endocannabinoid Transport Inhibitor AM404 [*N*-(4-Hydroxyphenyl)-eicosa-5,8,11,14-tetraenamide]

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Received December 29, 2005; accepted February 28, 2006

## ABSTRACT

The endocannabinoid system may serve important functions in the central and peripheral regulation of pain. In the present study, we investigated the effects of the endocannabinoid transport inhibitor AM404 [*N*-(4-hydroxyphenyl)-eicosa-5,8,11,14-tetraenamide] on rodent models of acute and persistent nociception (intraplantar formalin injection in the mouse), neuropathic pain (sciatic nerve ligation in the rat), and inflammatory pain (complete Freund's adjuvant injection in the rat). In the formalin model, administration of AM404 (1–10 mg/kg i.p.) elicited dose-dependent antinociceptive effects, which were prevented by the $CB_1$ cannabinoid receptor antagonist rimonabant (SR141716A; 1 mg/kg i.p.) but not by the $CB_2$ antagonist SR144528 (1 mg/kg i.p.) or the vanilloid antagonist capsazepine (30 mg/kg i.p.). Comparable effects were observed with UCM707 [*N*-(3-furylmethyl)-eicosa-5,8,11,14-tetraenamide], another anandamide transport inhibitor. In both the chronic constriction injury and complete Freund's adjuvant model, daily treatment with AM404 (1–10 mg/kg s.c.) for 14 days produced a dose-dependent reduction in nocifensive responses to thermal and mechanical stimuli, which was prevented by a single administration of rimonabant (1 mg/kg i.p.) and was accompanied by decreased expression of cyclooxygenase-2 and inducible nitric-oxide synthase in the sciatic nerve. The results provide new evidence for a role of the endocannabinoid system in pain modulation and point to anandamide transport as a potential target for analgesic drug development.

The endocannabinoids, anandamide and 2-arachidonoylglycerol (2-AG), are removed from the extracellular space by a high-affinity transport system present both in neural and non-neural cells (Beltramo et al., 1997; Hillard et al., 1997). The molecular identity of this putative transporter is still unknown, but some of its biochemical and pharmacological properties have been characterized (for review, see Hillard and Jarrahian, 2003). These include stereoselective substrate recognition and saturation at 37°C, independence from ion gradients, and pharmacological inhibition by agents such as AM404, UCM707, and LY2183240 (Beltramo et al., 1997; Piomelli et al., 1999; Lopez-Rodriguez et al., 2001; Moore et al., 2005). After reuptake, anandamide is hydrolyzed by fatty acid amide hydrolase (FAAH) (Cravatt et al., 1996), an intracellular membrane-bound serine hydrolase whose activity is selectively inhibited by the compounds URB597 and OL-135 (Kathuria et al., 2003; Lichtman et al., 2004) as well as by a variety of nonselective agents (for review, see Piomelli, 2005). Intracellular 2-AG is hydrolyzed by monoacylglycerol lipase (Stella et al., 1997; Dinh et al., 2002), which is inhibited by the compound URB602 (Hohmann et al., 2005; Makara et al., 2005).

Pharmacological blockade of FAAH activity elicits modest but significant antinociceptive effects in rats (Kathuria et al., 2003; Lichtman et al., 2004), whereas mutant mice in which

This work was supported by the National Institute on Drug Abuse (Grants DA-12447 and DA-3412), by the University of California Discovery Program (to D.P.), and by Ministero dell'Istruzione, Università e Ricerca (to A.C.). M.B. was a National Institute on Drug Abuse INVEST fellow.

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Article, publication date, and citation information can be found at http://jpet.aspetjournals.org.
doi:10.1124/jpet.105.100792.

**ABBREVIATIONS:** 2-AG, 2-arachidonoylglycerol; AM404, *N*-(4-hydroxyphenyl)-eicosa-5,8,11,14-tetraenamide; UCM707, *N*-(3-furylmethyl)-eicosa-5,8,11,14-tetraenamide; LY2183240, 5-biphenyl-4-ylmethyl-tetrazole-1-carboxylic acid dimethylamide; FAAH, fatty acid amide hydrolase; URB597, cyclohexylcarbamic acid 3′-carbamoylbiphenyl-3-yl ester; OL-135, 1-oxo-1-[5-(2-pyridyl)oxazol-2-yl]-7-phenylheptane; URB602, (1,1′-biphenyl)-3-yl-carbamic acid, cyclohexyl ester; SR141716A, rimonabant; CCI, chronic constriction injury; CFA, complete Freund's adjuvant; iNOS, inducible nitric-oxide synthase; Cox-2, cyclooxygenase-2; PBS, phosphate-buffered saline; s.c., subcutaneous; SR144528, *N*-[(1*S*)-endo-1,3,3-trimethylbicycloheptan-2-yl]-5-(4-chloro-3-methylphenyl)-1-(4-methylbenzyl)-pyrazole-3-carboxamide.

the *faah* gene has been deleted by homologous recombination ($FAAH^{-/-}$ mice) are less responsive to acute noxious stimuli than are their wild-type littermates (Cravatt et al., 2001), suggesting that endogenously released anandamide may modulate pain sensitivity. This hypothesis is further supported by two observations. First, intraplantar formalin injection and electric foot shock administration stimulate the release of anandamide and 2-AG in the rat periaqueductal gray, a key pain-processing structure of the midbrain (Walker et al., 1999; Hohmann et al., 2005). Second, the $CB_1$ cannabinoid antagonist rimonabant (SR141716A) enhances nocifensive responses in some pain models (Calignano et al., 1998; Strangman et al., 1998), albeit not in others (Beaulieu et al., 2000), and abrogates the nonopioid component of stress-induced analgesia (Hohmann et al., 2005).

The findings outlined above raise the possibility that inhibitors of anandamide internalization, which prevent the access of anandamide to intracellular FAAH and prolong the duration of action of this endocannabinoid mediator (Beltramo et al., 1997; Kathuria et al., 2003; Fegley et al., 2004), may also have analgesic properties. To test this possibility, in the present study, we have investigated the effects of the anandamide transport inhibitor AM404 in models of acute and persistent pain (mouse formalin test), neuropathic pain (rat CCI test), and inflammatory pain (rat CFA test).

## Materials and Methods

**Animals.** We used male Swiss mice (20–25 g; Charles River Laboratories, Inc., Wilmington, MA) for the formalin test and male Wistar rats (200–220 g; Charles River Laboratories, Inc.) for all other experiments. The procedures met National Institutes of Health guidelines for the care and use of laboratory animals and those of the Italian Ministry of Health (D.L. 116/92) and were approved by the local Institutional Animal Care and Use Committees.

**Drugs.** AM404 and capsazepine were obtained from Tocris Cookson (Avonmouth, UK), UCM707 was from Cayman Chemical (Ann Arbor, MI), rimonabant and SR144528 were from the National Institute on Drug Abuse (Bethesda, MD), and all other chemicals were from Sigma-Aldrich (St. Louis, MO). Fresh drug solutions were prepared immediately before use in a vehicle of sterile saline/dimethyl sulfoxide (80:20 for single administrations) or sterile saline/polyethylene glycol/Tween 80 (90:5:5 v/v for repeated administrations). The i.p. route was selected for single AM404 administrations, and the s.c. route was selected for repeated administrations.

**Formalin Model.** We allowed Swiss mice to acclimate to the testing room for at least 12 h before experiments. We injected formalin (5% formaldehyde in sterile saline, 10 $\mu$l) into the plantar surface of the left hind paw using a 27-gauge needle fitted to a microsyringe and immediately transferred the animals to a transparent observation chamber. Nocifensive behavior (licking and biting of the injected paw) was monitored continuously for a period of 45 min divided into two intervals: phase I, 0–15 min; and phase II, 15–45 min (Dubuisson and Dennis, 1977).

**CCI Model.** The sciatic nerve of Wistar rats was surgically ligated as described previously (Bennett and Xie, 1988). In brief, the animals were anesthetized with ketamine (100 mg/kg i.p) and xylazine (5 mg/kg i.p.). The left sciatic nerve was exposed at midthigh level through a small incision, and one-third to one-half of the nerve thickness was loosely ligated with four silk threads. The wound was closed with muscle suture and skin clips and dusted with streptomycin powder. In parallel surgeries, the nerve was exposed but not ligated (sham-operated rats). Behavioral tests were performed on the day before surgery (day −1) and again on days 7 and 14 after surgery.

**CFA Model.** We administered CFA (Sigma-Aldrich) in a vehicle of paraffin oil/mannide mono-oleate (85:15, v/v; 0.1 ml) by intradermal injection into the left hind paw of Wistar rats using a 27-gauge needle fitted to a microsyringe on 3 separate days (1, 3, and 7) (Billingham, 1990). Behavioral tests were performed before the first CFA injection (day −1) and again on day 14 of treatment.

**Mechanical Hyperalgesia.** We measured mechanical hyperalgesia using a Randall-Selitto analgesimeter (Ugo Basile, Varese, Italy). Latencies of paw withdrawal to a calibrated pressure were assessed on both ligated and controlateral paws on day −1 (before ligation or CFA treatment) and again on days 7 and 14. Cut-off force was set at 150 g.

**Thermal Hyperalgesia.** We measured thermal hyperalgesia using a Hargreaves apparatus (Hargreaves et al., 1988) (Ugo Basile). Two days before the experiment, the animals were placed in a transparent Perspex box with a thin glass floor and allowed to acclimate for 10 to 15 min. A focused beam of radiant heat applied to the plantar surface and latencies of paw withdrawal were assessed on both ligated and controlateral paws on day −1 (before ligation or CFA treatment) and again on days 7 and 14. Cut-off time was set at 1 min.

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

**Immunoblot Analyses.** CCI, sham-operated, and naive rats were killed, and the sciatic nerves from ligated paws were removed and immediately frozen in liquid $N_2$. Frozen tissue was weighed and homogenized on ice in lysis buffer (20 mM Tris-HCl, pH 7.5, 10 mM NaF, 150 mM, NaCl, 1% Nonidet P-40, 1 mM phenylmethylsulfonyl fluoride, 1 mM $Na_3VO_4$, and 10 $\mu$g/ml leupeptin and trypsin inhibitor; 0.25 ml/50 mg tissue). After 1 h, tissue lysates were centrifuged at 100,000*g* for 15 min at 4°C, and protein content of the supernatant was measured using bovine serum albumin (Sigma-Aldrich) as a standard. Supernatant samples (0.1 mg of protein) were dissolved in Laemmli's buffer, boiled for 5 min, and subjected to SDS-polyacrylamide gel electrophoresis (8% polyacrylamide). Proteins were transferred onto nitrocellulose membranes (Protran nitrocellulose transfer membrane; Schleicher and Schuell Bioscience, Dassel, Germany), blocked with phosphate-buffered saline (PBS) containing 5% nonfat dried milk for 45 min at room temperature, and incubated at 4°C overnight in the presence of commercial antibodies for iNOS (BD Biosciences Transduction Laboratories, Lexington, KY; dilution 1:2000) or Cox-2 (Cayman Chemical; dilution 1:1500) in PBS containing 5% nonfat dried milk and 0.1% Tween 20. The secondary antibody (anti-mouse IgG or anti-rabbit IgG peroxidase conjugate) was incubated for 1 h at room temperature. Blots were washed with PBS, developed using enhanced chemiluminescence detection reagents (Amersham Pharmacia, Piscataway, NJ) following manufacturer's instructions, and exposed to X-Omat film (Eastman Kodak Co., Rochester, NY). Protein bands for iNOS (~130 kDa) and Cox-2 (~72 kDa) were quantified using a model GS-700 imaging densitometer (Bio-Rad, Hercules, CA). An anti-$\alpha$-tubulin antibody (Sigma Aldrich; dilution 1:1000) was used as a control.

**Statistical Analyses.** Results are expressed as the mean ± S.E.M. of $n$ experiments. All analyses were conducted using Statistica (Statsoft, Tulsa, OK). The significance of differences between groups was determined by Student's $t$ test and one- or two-way analysis of variance followed by a Bonferroni post hoc test for multiple comparisons.

## Results

**Antinociceptive Effects of AM404 in the Formalin Model.** Systemic administration of AM404 (1–10 mg/kg i.p,) in Swiss mice produced a dose-dependent reduction in phase

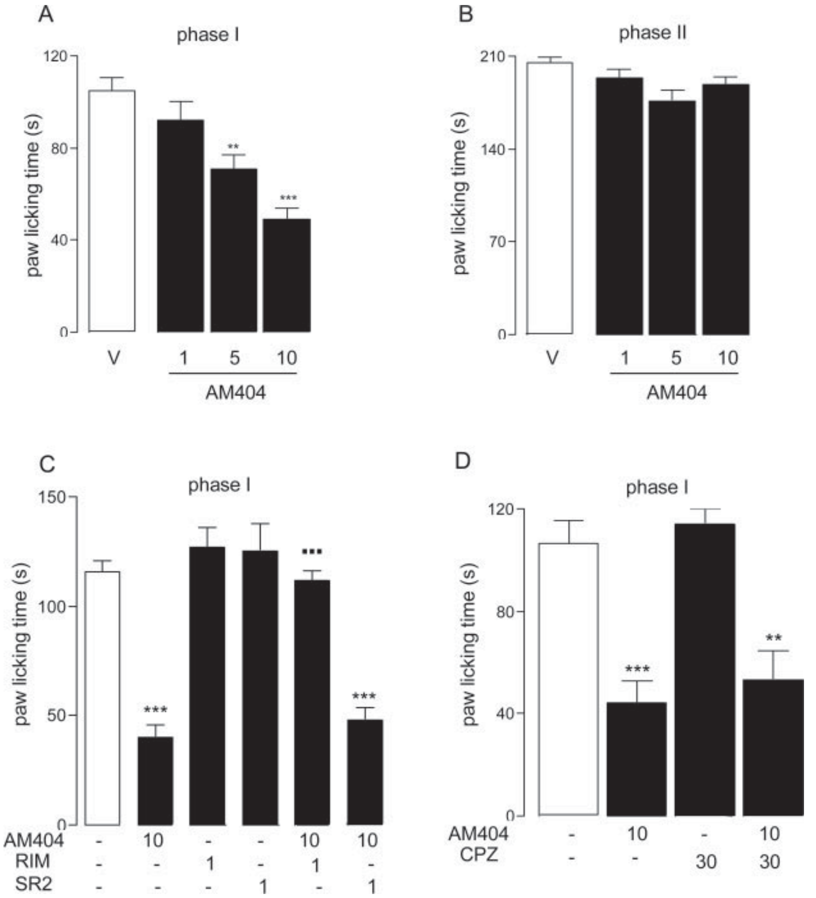


**Fig. 1.** Antinociceptive effects of the endocannabinoid transport inhibitor AM404 on formalin-induced paw licking in mice. AM404 (1–10 mg/kg i.p.) reduced paw licking time in phase I (A) but not phase II (B) (**, $p < 0.01$; ***, $p < 0.001$ compared with vehicle; $n = 8$ per group). This effect was prevented by $CB_1$ antagonist rimonabant (RIM; 1 mg/kg i.p) but not the $CB_2$ antagonist SR144528 (SR2, 1 mg/kg i.p) (C) or the vanilloid antagonist capsazepine (CPZ; 30 mg/kg i.p.) (D) (**, $p < 0.01$; ***, $p < 0.001$ compared with vehicle; ■■■, $p < 0.001$ compared with AM404 alone; $n = 8$ per group).

I nociceptive behavior (Fig. 1A) ($F_{3,32} = 15.08$, $p < 0.0001$; $n = 8$), which was probably dependent on endocannabinoid-mediated activation of $CB_1$ receptors since it was prevented by the $CB_1$ antagonist rimonabant (1 mg/kg i.p.) but not by the $CB_2$ antagonist SR144528 (1 mg/kg i.p.) (Fig. 1C) ($F_{5,50} = 31.72$, $p < 0.0001$; $n = 8$) or the vanilloid antagonist capsazepine (30 mg/kg i.p.; all drugs given 30 min before AM404, $n = 8$) (Fig. 1D) ($F_{3,23} = 16.06$, $p < 0.0001$; $n = 8$). In contrast with its ability to reduce formalin-induced phase I nociception, AM404 failed to alter phase II nociception at any of the doses tested (Fig. 1B). This lack of effect might be accounted for by enzymatic degradation (Fegley et al., 2004). Consistent with this possibility, we found that UCM707 (10 mg/kg i.p.), an anandamide transport inhibitor that is partially resistant to enzymatic hydrolysis (Lopez-Rodriguez et al., 2001), significantly reduced both phase I and phase II nociception (Fig. 2) ($t = 6.016$, $p < 0.0001$ and $t = 2.553$, $p < 0.05$; $n = 8$).

**Antihyperalgesic Effects of AM404 in the CCI Model.** The ability of AM404 to reduce nociception in the formalin test prompted us to investigate the impact of this agent in the CCI model of neuropathic pain (Bennett and Xie, 1988). In an initial test, we measured the effects of a single injection of AM404 on the nocifensive response to thermal stimuli. When administered on day 7 after ligation of the sciatic nerve, AM404 (1–10 mg/kg s.c.) produced a modest antihyperalgesic effect, which was significant at the dose of 10 mg/kg (Fig. 3A)

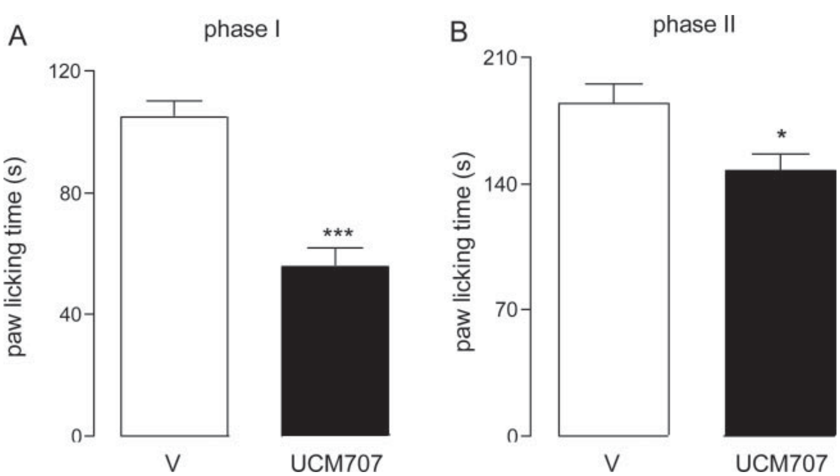


**Fig. 2.** Antinociceptive effects of the endocannabinoid transport inhibitor UCM707 on formalin-induced paw licking. UCM707 (10 mg/kg i.p.) reduced licking time in both phase I (A) and phase II (B) (*, $p < 0.05$; ***, $p < 0.01$ compared with vehicle; $n = 8$ per group).

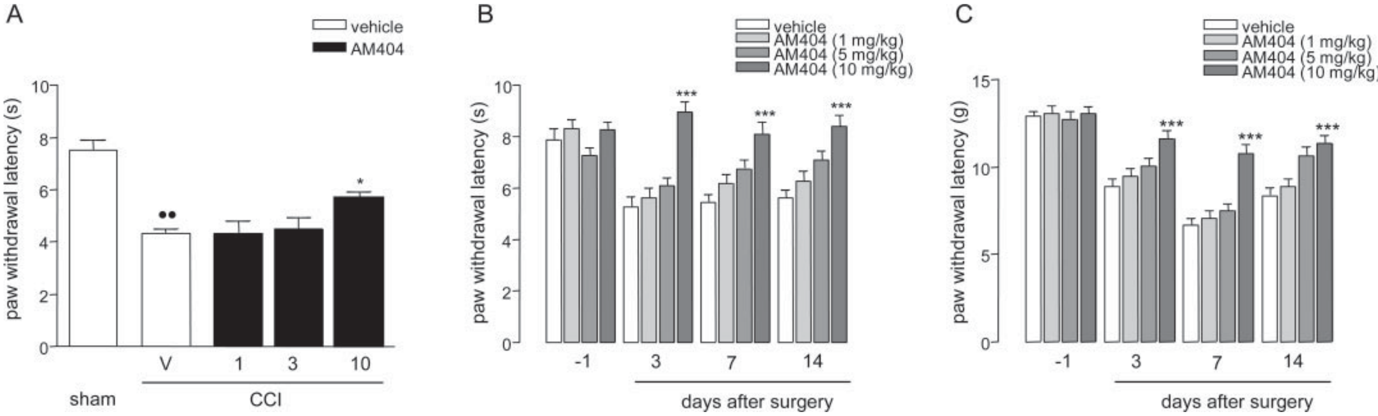


**Fig. 3.** Antihyperalgesic effects of AM404 in the rat CCI model of neuropathic pain. A, effects of single AM404 injections (1–10 mg/kg i.p.) on thermal hyperalgesia. B and C, effects of repeated AM404 injections (1–10 mg/kg s.c. once daily for 3–14 days) on thermal and (B) mechanical hyperalgesia (C) (●●, $p < 0.01$ versus sham; ∗, $p < 0.05$; ∗∗∗, $p < 0.001$ versus CCI-vehicle; $n = 12$–32 per group).

($F_{4,55} = 14.68$, $p < 0.0001$; $n = 12$). At this dose, AM404 did not change nocifensive responses to thermal stimuli applied to contralateral, nonoperated paws (vehicle, 6.9 ± 0.3 s; AM404, 7.2 ± 0.4 s; $n = 6$). Next, to determine whether the antihyperalgesic effect of AM404 could be enhanced by repetitive administration, we subjected neuropathic or sham-operated rats to a 14-day regimen with AM404 (1–10 mg/kg s.c. once daily) or vehicle. AM404 produced a reduction in both thermal (Fig. 3B) and mechanical (Fig. 3C) hyperalgesia, compared with vehicle ($F_{3,372} = 18.08$ for treatment, $F_{3,372} = 17.71$ for time and thermal hyperalgesia; $F_{3,372} = 13.20$ for treatment, $F_{3,372} = 123.8$ for time and thermal hyperalgesia; $p < 0.0001$, no significant treatment × time interactions, $F_{9,372} = 5.410$; $n = 32$). These effects were significant at 10 mg/kg (Fig. 3, B and C) and were prevented by a single injection of the $CB_1$ antagonist rimonabant but not the $CB_2$ antagonist SR144528 (Fig. 4) (each drug at 1 mg/kg i.p. 30 min before AM404) ($F_{3,324} = 31.84$ for treatment, $F_{3,324} = 14.13$ for time and thermal hyperalgesia; $F_{3,324} = 21.75$ for treatment, $F_{3,324} = 71.55$ for time thermal hyperalgesia; $p < 0.0001$; $n = 24$–32).

As expected, CCI rats displayed a marked disruption in motor function, as assessed in the Rotorod test (Fig. 5) ($F_{2,33} = 14.77$; $p < 0.0001$; $n = 12$). This deficit was significantly ameliorated either by acute AM404 administration (10 mg/kg s.c.) (Fig. 5A) or subchronic AM404 treatment (10 mg/kg s.c. once daily for 7 or 14 days) (Fig. 5B) ($F_{2,33} = 54.63$ for treatment, $p < 0.0001$, $F_{1,33} = 0.9888$, $p > 0.05$ for time, $n = 12$). In contrast, AM404 (10 mg/kg s.c. once daily for 7 or 14 days) exerted no significant effect in sham-operated rats (Rotorod latency; day 7, vehicle, 102.5 ± 5.9 s; AM404, 97.1 ± 4.6 s; day 14, vehicle, 103 ± 4.7 s; AM404, 104 ± 4 s). These results indicate that the antihyperalgesic actions of this agent may not be ascribed to sedation or motor impairment.

**Effects of AM404 on Cox-2 and iNOS Expression in the CCI Model.** To further investigate the antihyperalgesic properties of AM404, we asked whether this endocannabinoid transport inhibitor alters the expression of Cox-2 and iNOS, two enzymes that have been implicated in the pathogenesis of neuropathic pain (Bingham et al., 2005; De Alba et al., 2005). As reported previously (Levy et al., 1999), immunoblot analyses revealed that sciatic nerve extracts from CCI rats contained significantly higher levels of immunoreactive Cox-2 and iNOS than did extracts from either naive or sham-operated animals (Fig. 6). Importantly, daily administration of AM404 (3 and 10 mg/kg s.c.) for 7 or 14 days decreased levels of both proteins in a dose-dependent manner (Fig. 6). In particular, the 14-day regimen with AM404 almost normalized Cox-2 and iNOS expression levels, reducing them to values comparable with those measured in sham-operated rats (Fig. 6).

**Antihyperalgesic Effects of AM404 in the CFA Model.** Finally, we examined the ability of AM404 to attenuate hy-

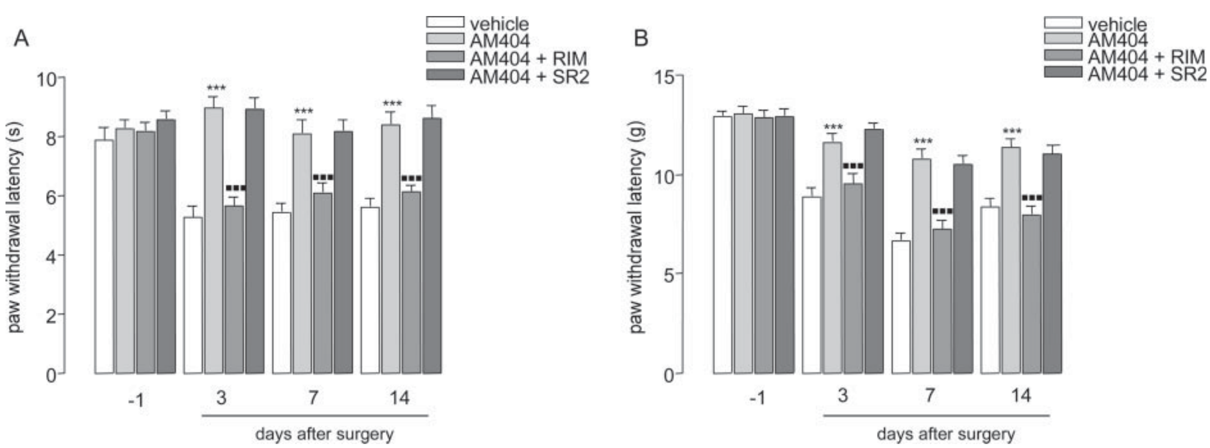


**Fig. 4.** The $CB_1$ antagonist rimonabant prevents the effects of AM404 in the rat CCI model. Effects of single injections of rimonabant (RIM; 1 mg/kg i.p.) or SR144528 (SR2; 1 mg/kg i.p.) on the effects of subchronic AM404 (10 mg/kg s.c. once daily for 3–14 days) on thermal (A) and mechanical (B) antihyperalgesia (∗∗∗, $p < 0.001$ versus CCI vehicle; ■■■, $p < 0.01$ versus AM404 alone; $n = 24$–32 per group).

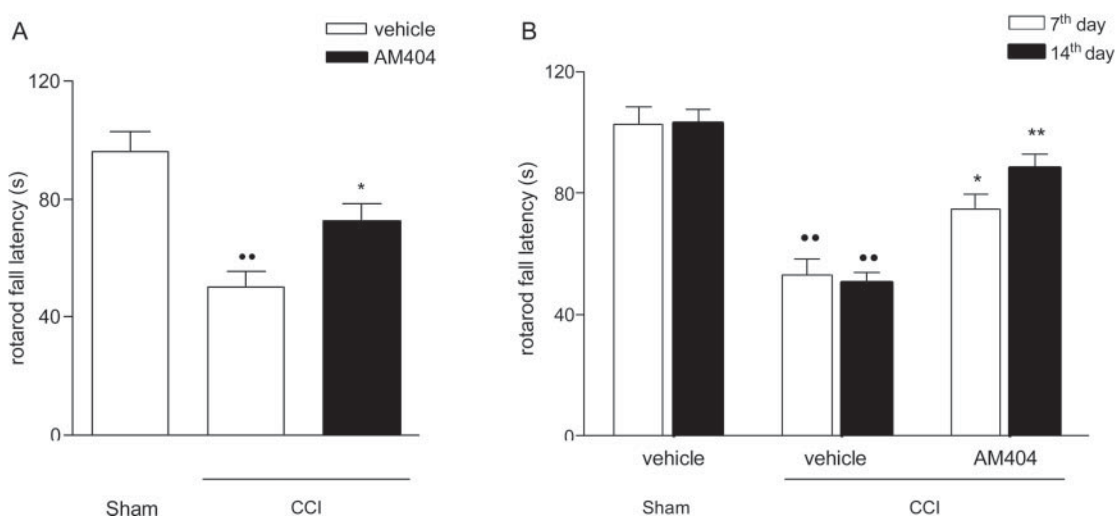


**Fig. 5.** Effects of AM404 on motor function in CCI rats. A, effect of single injections of AM404 (10 mg/kg i.p.) or vehicle. B, effect of repeated injections of AM404 (10 mg/kg i.p. once daily for 7 or 14 days) or vehicle (●●, $p < 0.01$ versus sham; ∗, $p < 0.05$; ∗∗, $p < 0.01$ versus CCI vehicle; $n = 12$ per group).

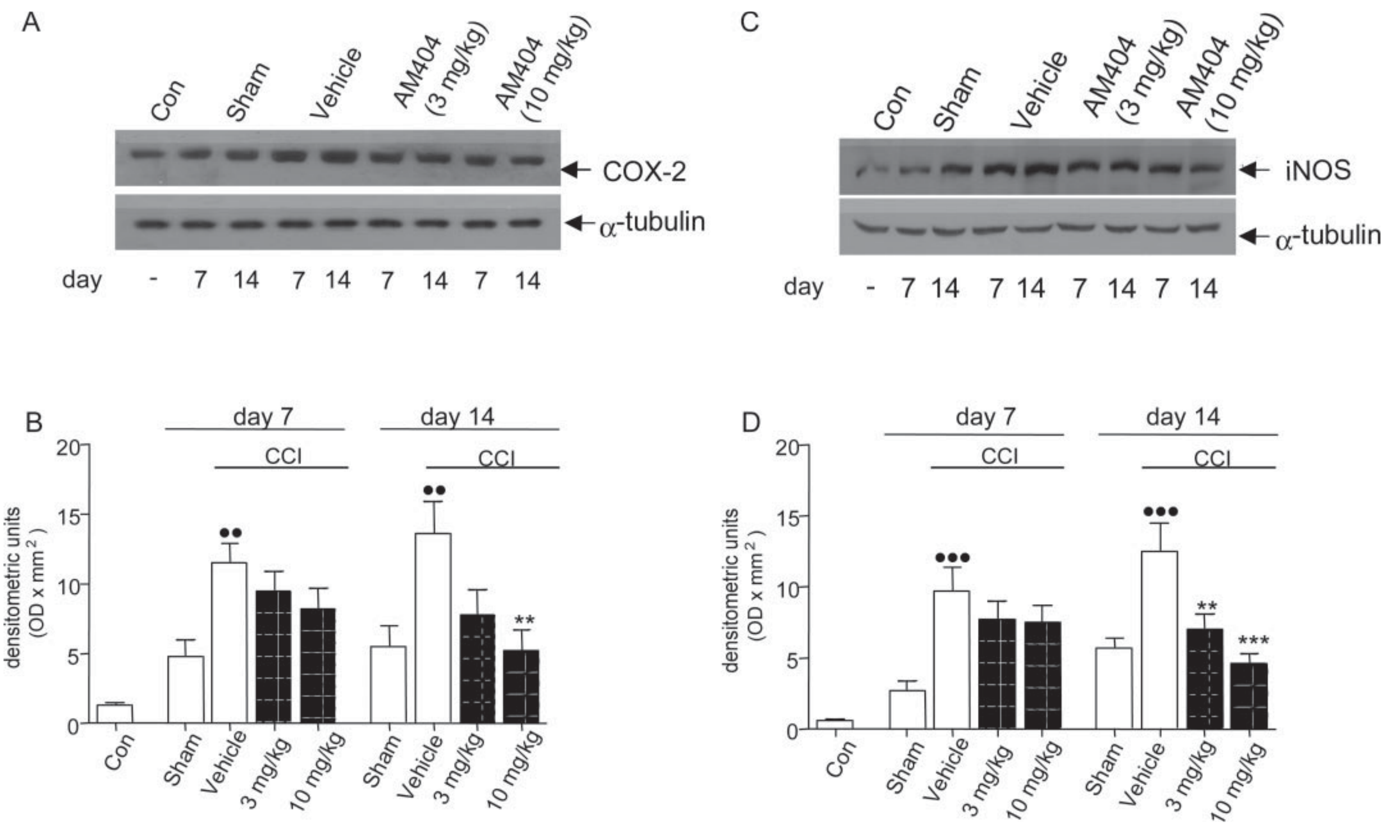


**Fig. 6.** Effects of AM404 on immunoreactive Cox-2 and iNOS in the sciatic nerve of CCI rats. A and B, effects of subchronic treatment with AM404 (3 or 10 mg/kg s.c. once daily for 7 or 14 days) on Cox-2 levels: A, representative immunoblots; B, densitometric quantification (filled bars represent AM404-treated). C and D, effects of subchronic treatment with AM404 (3 or 10 mg/kg s.c. once daily for 7 or 14 days) on iNOS levels: C, representative immunoblots; D, densitometric quantification (filled bars represent AM404-treated). Equal loading was confirmed by α-tubulin staining (●●, $p < 0.01$; ●●●, $p < 0.001$ versus sham; ∗∗, $p < 0.01$; ∗∗∗, $p < 0.001$ versus CCI vehicle; $n = 6$ rats per group).

peralgesic responses in the CFA model of arthritis (Billingham, 1990). We treated rats with CFA and then subjected them to a 14-day regimen with AM404 (1–10 mg/kg s.c. once daily) or vehicle. AM404 produced a dose-dependent reduction in both thermal (Fig. 7A) and mechanical (Fig. 7B) hyperalgesia, compared with vehicle. These effects were prevented by a single injection of rimonabant but not SR144528 (each drug at 1 mg/kg i.p. 30 min before AM404) ($F_{8,263}$ = 12.19, $p < 0.0001$ for thermal hyperalgesia; $F_{8,271} = 6.373$, $p < 0.0001$ for mechanical hyperalgesia; $n = 24$–32).

## Discussion

The main finding of the present study is that the endocannabinoid transport inhibitor AM404 exerts significant antinociceptive and antihyperalgesic effects in three mechanis-

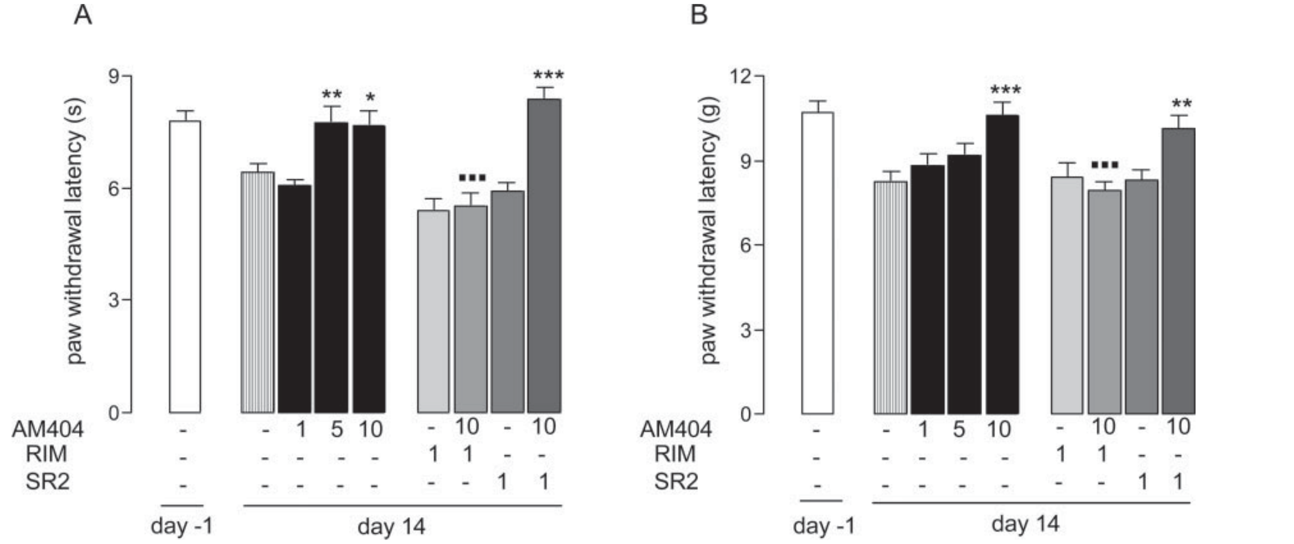


**Fig. 7.** Antihyperalgesic effects of AM404 in the rat CFA model of arthritic pain. Effects of subchronic treatment with AM404 (1–10 mg/kg s.c. once daily for 14 days) on thermal (A) and mechanical (B) hyperalgesia. Rimonabant (RIM; 1 mg/kg i.p.), but not SR144528 (SR2; 1 mg/kg i.p.), prevented these effects (*, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$ compared with vehicle; ■■■, $p < 0.001$ compared with AM404 alone; $n = 12$ per group).

tically distinct rodent models of pain: the formalin model of acute and persistent nociception (Dubuisson and Dennis, 1977), the CCI model of neuropathic pain (Bennett and Xie, 1988), and the CFA model of arthritis-induced pain (Billingham, 1990). These effects are prevented by the $CB_1$ antagonist rimonabant, but not by $CB_2$ and vanilloid antagonists, suggesting that they are caused by endocannabinoid-mediated activation of $CB_1$ receptors. Notably, the analgesic actions of AM404 cannot be attributed to sedation since they are accompanied by an improvement rather than an impairment of motor activity in the Rotorod test and are associated with a marked reduction in the expression of Cox-2 and iNOS, two proteins that are involved in the pathogenesis of neuropathic pain and inflammation (Levy et al., 1999; De Alba et al., 2005).

A large body of evidence indicates that direct-acting cannabinoid agonists reduce nocifensive behaviors in animals and alleviate pain in humans by activating cannabinoid receptors. In animals, systemic or intracerebral administration of cannabinoid agonists produces profound antinociceptive effects and suppresses activity in $CB_1$-expressing nociceptive neurons of the thalamus, midbrain, and brainstem (Hohmann et al., 1995, 1999, 2005; Meng et al., 1998). Likewise, neuropathic pain patients orally treated with the synthetic cannabinoid agonist CT-3 reported significantly less pain than did placebo-treated patients (Karst et al., 2003). In addition to these central effects, cannabinoid agonists may also prevent formalin-evoked pain responses in mice (Calignano et al., 1998) and capsaicin-evoked pain in monkeys and human volunteers (Ko and Woods, 1999; Rukwied et al., 2003) presumably by interacting with $CB_1$ or $CB_2$ receptors localized on sensory neuron terminals or resident non-neural cells (Hohmann and Herkenham, 1999; Nackley et al., 2004; Ibrahim et al., 2005).

The results outlined above suggest that AM404 may produce its analgesic effects by blocking endocannabinoid transport, thus magnifying endocannabinoid-mediated modulation of pain sensitivity. Three lines of evidence provide further support for this possibility. First, $CB_1$ receptor blockade enhances pain behaviors in several pain models (Calignano et al., 1998; Strangman et al., 1998; for contrasting results, see Beaulieu et al., 2000) and abrogates nonopioid stress-induced analgesia (Hohmann et al., 2005), suggesting the existence of an analgesic endocannabinoid tone. Second, administration of AM404 increases endogenous anandamide levels in the mouse brain (Fegley et al., 2004) and decreases cFOS immunoreactivity in the dorsal superficial laminae of the spinal cord in CCI rats (Rodella et al., 2005). Third, genetic deletion or pharmacological inhibition of FAAH elevates brain anandamide levels and reduces nocifensive behaviors in mice and rats (Cravatt et al., 2001; Kathuria et al., 2003; Lichtman et al., 2004). Finally, the nonaliphatic anandamide transport inhibitor LY2183240, although structurally unrelated to AM404 and UCM707, exerts significant antinociceptive effects in the formalin model (Moore et al., 2005). However, it is worth noting that although LY2183240 and UCM707 inhibit both first and second phase of formalin nociception, AM404 only inhibits the former. This discrepancy might be accounted for by the known sensitivity of AM404 to enzymatic degradation (Fegley et al., 2004), but current information on the pharmacological properties of UCM707 and LY2183240 is too limited to allow us to exclude the possibility that these compounds may act through alternative noncannabinoid mechanisms.

Previous studies have shown that AM404 does not closely reproduce the pharmacological profile of direct-acting cannabinoid agonists (Beltramo et al., 2000; Fegley et al., 2004). This difference has been attributed to the ability of AM404 to inhibit endocannabinoid transport without directly activating $CB_1$ receptors (Beltramo et al., 1997; 2000). Recently, the existence of endocannabinoid transport has been questioned in favor of a simple diffusion mechanism, whereby anandamide accumulation may be solely driven by an inward concentration gradient maintained by FAAH-mediated hydrolysis (Glaser et al., 2003). In this context, the actions of AM404 have been ascribed to its ability to serve as a FAAH substrate and to compete with anandamide for FAAH activity. However, the discovery that pharmacological or genetic inactivation of FAAH does not affect anandamide internalization in neurons strongly argues against this possibility and in favor of the transporter hypothesis (Kathuria et al., 2003; Fegley et al., 2004; Ortega-Gutierrez et al., 2004). Additional support to this hypothesis comes from the recent discovery of potent nonaliphatic inhibitors of endocannabinoid transport, which has led to the identification of a high-affinity binding site presumably involved in the transport process (Moore et al., 2005).

In addition to its inhibitory action on anandamide transport, AM404 binds in vitro to several unrelated pharmacological targets, such as vanilloid TRPV1 receptors and sodium channels (Piomelli, 2005). Although the in vivo significance of these effects is still unclear, we cannot rule out that some of the observed effects might depend on nonspecific actions of AM404. Nevertheless, the ability of the $CB_1$ antagonist rimonabant, but not the vanilloid antagonist capsazepine, to prevent the actions of AM404 suggests that such actions can be ascribed to endocannabinoid-mediated activation of $CB_1$ receptors.

Several questions remain unanswered. For example, the identity of the endocannabinoid modulator(s) affected by AM404 treatment and the brain region(s) implicated in its effects are still unknown. Nevertheless, our results do suggest that endocannabinoid transport may provide a valuable target for analgesic drugs, underscoring the need to molecularly characterize this system and to further current efforts to develop a second generation of potent and selective transport inhibitors.

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