

Effects of Alterations in Cannabinoid Signaling, Alone and in Combination with Morphine, on Pain-Elicited and Pain-Suppressed Behavior in Mice

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Received January 6, 2012; accepted April 17, 2012

ABSTRACT

Inhibitors of fatty acid amide hydrolase (FAAH) and anandamide (AEA) uptake, which limit the degradation of endogenous cannabinoids, have received interest as potential therapeutics for pain. There is also evidence that endogenous cannabinoids mediate the antinociceptive effects of opioids. Assays of pain-elicited and pain-suppressed behavior have been used to differentiate the effects of drugs that specifically alter nociception from drugs that alter nociception caused by nonspecific effects such as catalepsy or a general suppression of activity. Using such procedures, this study examines the effects of the direct cannabinoid type 1 (CB1) agonist (–)-*cis*-3-[2-hydroxy-4-(1,1-dimethylheptyl)phenyl]-*trans*-4-(3-hydroxypropyl)cyclohexanol (CP55940), the FAAH inhibitor cyclohexylcarbamic acid 3'-carbamoylbiphenyl-3-yl ester (URB597), and the AEA uptake inhibitor *N*-(4-hydroxyphenyl)arachidonylamide (AM404). Additional experiments examined these compounds in combination with morphine. CP55940 produced antinociception in assays of pain-elicited, but not

pain-suppressed, behavior and disrupted responding in an assay of schedule-controlled behavior. URB597 and AM404 produced antinociception in assays of pain-elicited and pain-suppressed behavior in which acetic acid was the noxious stimulus, but had no effect on the hotplate and schedule-controlled responding. CP55940 in combination with morphine resulted in effects greater than those of morphine alone in assays of pain-elicited and scheduled-controlled behavior but not pain-suppressed behavior. URB597 in combination with morphine resulted in enhanced morphine effects in assays of pain-elicited and pain-suppressed behavior in which diluted acetic acid was the noxious stimulus, but did not alter morphine's effects on the hotplate or schedule-controlled responding. These studies suggest that, compared with direct CB1 agonists, manipulations of endogenous cannabinoid signaling have enhanced clinical potential; however, their effects depend on the type of noxious stimulus.

Introduction

Agonists at cannabinoid type 1 (CB1) receptors produce behavioral effects that include disruption of locomotor activity (Pascual et al., 2005; Smith et al., 2009), dysregulation of food consumption (Järbe and DiPatrizio, 2005; Li et al., 2006), interference with thermoregulation (Wang et al., 2008; Diaz et al., 2009), and reinforcing effects (Justinova et al., 2008; Negus and Rice, 2009). Research also implicates the endogenous cannabinoid system in the mediation of pain

responses. Genetic (Cravatt et al., 2001; Lichtman et al., 2004b) and pharmacological (Lichtman et al., 2004a; Costa et al., 2010, 2006) inhibition of the activity of fatty acid amide hydrolase (FAAH) or anandamide (AEA) uptake, both of which limit the degradation of endogenous cannabinoids, results in CB1-mediated antinociception. Because these effects are not accompanied by catalepsy, hypothermia, and other effects that are associated with the administration of direct CB1 agonists (Kathuria et al., 2003; Jayamanne et al., 2006), there is interest in the clinical potential of endogenous cannabinoid modulators as analgesics.

In preclinical pain assays, exogenous cannabinoid agonists such as Δ^9 -tetrahydrocannabinol potentiate the effects of morphine in the mouse tail-flick test (Cichewicz and McCarthy, 2003), the rat paw pressure test (Cox et al., 2007), and

This work was supported by the National Institutes of Health National Institute on Drug Abuse [Grants R01-DA002749, T32-DA007244, F31-DA025446].

Article, publication date, and citation information can be found at <http://jpet.aspetjournals.org>.
<http://dx.doi.org/10.1124/jpet.112.191478>.

ABBREVIATIONS: CB1, cannabinoid type 1; FAAH, fatty acid amide hydrolase; AEA, anandamide; MPE, maximum possible effect; FR, fixed ratio; CL, confidence level; CNS, central nervous system; V/S, vehicle/saline; CP55940, (–)-*cis*-3-[2-hydroxy-4-(1,1-dimethylheptyl)phenyl]-*trans*-4-(3-hydroxypropyl)cyclohexanol; URB597, cyclohexylcarbamic acid 3'-carbamoylbiphenyl-3-yl ester; AM404, *N*-(4-hydroxyphenyl)arachidonylamide; SR141716A, 5-(4-chlorophenyl)-1-(2,4-dichlorophenyl)-4-methyl-*N*-1-piperidinyl-1*H*-pyrazole-3-carboxamide; URB937, 3'-carbamoyl-6-hydroxy-[1,1'-biphenyl]-3-yl cyclohexylcarbamate.

the rat formalin test (Finn et al., 2004). Such interactions are of interest because of their therapeutic potential in the treatment of pain (Welch, 2009), but enthusiasm is tempered by the fact that CB1 agonists also potentiate other opioid effects (Finn et al., 2004), including their reinforcing activity (Norwood et al., 2003; Manzanedo et al., 2004; Solinas et al., 2005).

Endogenous cannabinoids also modulate the antinociceptive effects of opioids. For example, CB1 antagonists attenuate morphine-induced antinociception in the hotplate, writhing, and tail-flick tests (Pacheco et al., 2009; Miller et al., 2011) and in models of hyperalgesia (da Fonseca Pacheco et al., 2008). Exogenously administered AEA in combination with the FAAH inhibitor cyclohexylcarbamic acid 3'-carbamoylbiphenyl-3-yl ester (URB597), *N*-(4-hydroxyphenyl)arachidonylamide (AM404) enhances the antinociceptive effects of morphine in the tail-flick test (Haller et al., 2008), and methylarachidonoylfluorophosphate, which inhibits the degradation of the endogenous cannabinoids AEA and 2-arachidonoyl ethanolamine, enhances the antinociceptive effects of morphine in the tail-flick test and in hyperalgesia models (da Fonseca Pacheco et al., 2008; Pacheco et al., 2009).

The vast majority of preclinical pain research uses behavioral models that can be described as assays of pain-elicited behavior. In these types of experiments, a noxious stimulus (e.g., hotplate) evokes a behavioral response (e.g., licking of the hindpaw). Assays of pain-suppressed behavior have been used to complement these models and provide a more complete characterization of candidate analgesics. In assays of pain-suppressed behavior a noxious stimulus suppresses the rate, frequency, or intensity of a particular behavior (Negus et al., 2006). These assays can be used to differentiate the effects of compounds that selectively alter responses to nociceptive stimuli from compounds that produce nonspecific effects (e.g., general suppression of behavior). For example, the clinical analgesic morphine attenuates nociceptive responses and attenuates the suppression of behaviors by noxious stimuli (Stevenson et al., 2006). Compounds that attenuate pain-elicited behavior and also restore behavior that has been suppressed by pain may offer clinical advantages.

The primary purpose of the present study was to compare the effects of the FAAH inhibitor, URB597, and the purported AEA uptake inhibitor, AM404, with a direct CB1 agonist, (-)-*cis*-3-[2-hydroxy-4-(1,1-dimethylheptyl)phenyl]-*trans*-4-(3-hydroxypropyl)cyclohexanol (CP55940), in two assays of pain-elicited behavior (hotplate assay and acetic acid-elicited writhing) and two assays of pain-suppressed behavior (acetic acid-suppressed feeding and wheel running). Morphine, a clinically relevant analgesic, was examined for comparison. In addition, the effects of morphine, URB597, AM404, and CP55940 on food-maintained, schedule-controlled behavior were examined to provide a measure of the nonspecific, rate-decreasing effects of each drug. Based on previous findings of cannabinoid/opioid interactions, the current study also examined the effects of administration of a FAAH inhibitor or an AEA uptake inhibitor in combination with morphine. Previous studies have demonstrated that CB1 agonists enhance a variety of opioid effects. Therefore, assays of pain-elicited, pain-suppressed, and schedule-controlled behavior may be useful in differentiating the effects of combinations of these compounds with morphine.

Materials and Methods

Subjects

Male C57BL/6 mice purchased from The Jackson Laboratory (Bar Harbor, ME) and bred in-house were approximately 12 weeks of age at the beginning of testing. Mice were group-housed and had free access to food and water except where specified by the experimental protocols below. Lights were programmed on a 12-h light/dark cycle with lights off at 7:00 AM. All experiments were conducted during the dark cycle. Animal protocols were approved by the Institutional Animal Care and Use Committee, and the methods were in accord with the *Guide for the Care and Use of Laboratory Animals* (National Research Council, 2011).

Experimental Procedures

Hotplate. Before testing, mice were habituated to the testing room and handled on two separate occasions. During testing, the mouse was placed onto the $56 \pm 0.1^\circ\text{C}$ surface of the apparatus (25.3×25.3 cm; Columbus Instruments, Columbus, OH), and the latency to lick or flutter the hind paws or jump was recorded. A cutoff time of 20 s was defined as the maximum trial duration.

Responses were measured 30 and 15 min before drug administration, and the latencies from these trials were averaged to yield one baseline value. Separate groups of mice were used to test each drug (groups of 8–10 mice per drug). During dose-effect determination for morphine (0.32–32.0 mg/kg) and CP55940 (0.032–3.2 mg/kg), cumulative doses were administered 30 min apart in half-log increments. The doses and pretreatment times were selected based on previous work in our laboratory. For URB597 (0.1–3.2 mg/kg) and AM404 (0.32–10.0 mg/kg) time courses were determined. Data shown are from the 1-h time point (effects did not differ across time points). In experiments examining the cannabinoids in combination with morphine doses of CP55940, URB597, and AM404 that had no effect on hotplate responses were administered 15 min before the commencement of cumulative dosing of morphine as described above. In all experiments, when CP55940, URB597, or AM404 produced a significant effect or produced changes in the morphine dose-effect curve the effects of pretreatment with the CB1 antagonist 5-(4-chlorophenyl)-1-(2,4-dichlorophenyl)-4-methyl-*N*-1-piperidinyl-1*H*-pyrazole-3-carboxamide (SR141716A) were then determined. In this assay, a dose of 3.0 mg/kg SR141716A, which does not alter hotplate latencies when administered alone (Miller et al., 2011), was administered 75 min before testing. The effect of each treatment is expressed as the percentage of the maximum possible effect (%MPE): [postdrug latency (s) – baseline latency (s)]/[20 – baseline latency (s)].

Acetic Acid-Induced Writhing. Mice were habituated to the testing room and handling on two occasions before testing. Mice were group-housed and had free access to food and water until 23 and 5 h before testing, respectively. Mice were tested in groups of five to eight animals per treatment, with each dose of a given drug tested by using the same number of mice. Before drug testing, baseline assessments were performed in which mice had 30-min access to 32% liquid nutrition (vanilla flavor; CVS, Woonsocket, RI) on the day before testing. During subsequent drug test days, mice were injected with saline or morphine (0.1–3.2 mg/kg) 45 min before testing. CP55940 (0.01–0.32 mg/kg), URB597 (0.32–10.0 mg/kg), and AM404 (1.0–10.0 mg/kg) were injected 1 h before testing, and SR141716A was injected 75 min before testing. For drug combination testing with morphine the pretreatment times above were maintained and combined with doses of CP55940, URB597, and AM404 that had no effect on writhing. Immediately before testing mice were injected with 0.56% acetic acid or saline in control experiments. After this injection mice were immediately placed into clean polycarbonate mouse cages ($11.5 \times 7.5 \times 5$ inches; Allentown Inc., Allentown, NJ) containing dishes of 32% liquid nutrition (vanilla flavor; CVS). Their behavior was videotaped for 30 min for scoring at a later time. The effects of acetic acid were examined once per animal. Writhes were operationally

defined as an elongation of the body with simultaneous extension of the hind limbs. The effect of each treatment is expressed as percentage of inhibition of writhing (% inhibition): $[(\text{writhes in saline-treated mice} - \text{writhes in drug-treated mice}) / \text{writhes in saline-treated mice}] \times 100$.

Acetic Acid-Suppressed Feeding. The data for the acetic acid-suppressed feeding assay were collected in the same mice, during the same session as the acetic acid-induced writhing assay. As a result, the descriptions of the treatment of the mice in the previous section apply.

Consumption of liquid nutrition was quantified by subtracting the weight of the liquid-containing dishes after the session from the weight obtained before the session. This value was divided by the animals' weight, yielding grams of liquid consumed per gram of body weight. In the event of spillage, the protocol was to discard those data, but this did not occur during these experiments. The effect of each treatment on pain-suppressed consumption is expressed as percentage of nonsuppressed consumption (% control): $[(\text{acetic acid-suppressed consumption (g/g)} / \text{nonsuppressed consumption (g/g)}) \times 100]$.

Acetic Acid-Suppressed Wheel Running. At the start of the experiment, mice were individually housed in polycarbonate cages ($14 \times 10.5 \times 5.5$ inches; Tecniplast USA Inc., Exton, PA) containing running wheels (ENV-044; MED Associates, St. Albans, VT). Testing occurred after 2 weeks of habituation to handling and stabilization of wheel-running behavior. Separate groups of mice were used to test each drug (5–9 mice per drug). On the day before testing (control session), mice were injected with saline followed 45 min later by a second injection of saline after which wheel running (revolutions) was recorded for 30 min. The next day (test session) mice were injected with morphine (0.32–3.2 mg/kg), CP55940 (0.01–0.1 mg/kg), URB597 (0.32–10.0 mg/kg), AM404 (1.0–10.0 mg/kg), SR141716A (3.0 mg/kg), or vehicle, alone or in combination by using pretesting injection times identical to those described for the writhing assay. Immediately before the test, mice were injected with 0.56% acetic acid or saline, and wheel running was recorded for 30 min. Acetic acid-suppressed wheel running was examined up to four times in each mouse with at least 1 week between exposures to acetic acid. This schedule of exposure has no effect on wheel running beyond the test day and does not produce long-term disruptions of other behaviors, such as feeding (Stevenson et al., 2006). The effect of each treatment on pain-suppressed wheel running is expressed as percentage of nonsuppressed wheel running (% control): $[(\text{acetic acid-suppressed running} / \text{nonsuppressed running}) \times 100]$.

Schedule-Controlled Behavior. Mice were group-housed in polycarbonate mouse cages ($11.5 \times 7.5 \times 5$ inches; Allentown Inc.) and had free access to food and water except for the 2.5 h before test days. Mice were habituated to the testing room and handling for 2 days before testing. Response rates in the assay of schedule-controlled behavior were determined in standard mouse operant chambers ($8.5 \times 7.0 \times 5.0$ inches; ENV-307W-CT; MED Associates).

During experimental sessions mice were placed into the darkened chambers for 10 min. After this acclimation period, the ventilator fan was activated, the left nose-poke hole was illuminated, and left nose-poke responses were counted toward completion of a fixed ratio (FR) response requirement (right nose pokes had no scheduled consequences). After an initial training period, the FR value was increased from 1 to the terminal ratio of 4, and completion of the FR resulted in the light within the left nose-poke hole being turned off, access to a liquid reinforcer (32% liquid nutrition; CVS), and activation of the house light for 8 s. Once the reinforcer delivery period elapsed, the dipper was lowered, the house light was turned off, and the left nose-poke hole was illuminated, signaling the onset of the next response period. Separate groups of mice were used to test each drug (6–7 mice per drug). Testing occurred 5 days per week, and sessions ended after 30 min elapsed or when 100 reinforcers were earned.

Once response rates were stable, the effects of morphine (1.0–10.0 mg/kg; 45-min pretreatment), CP55940 (0.01–1.0 mg/kg; 1-h pretreatment), and URB597 (1.0–17.0 mg/kg; 1-h pretreatment) were

examined. After the determination of the effects of these drugs alone, the mice did not receive drugs for 2 weeks before being redistributed into separate groups to determine the effects of AM404 (1.0–17.0 mg/kg; 1-h pretreatment) and various drug combinations. Drug effects were assessed on Tuesdays and Fridays. Drug data are expressed as a percentage of control response rates (% control): $[(\text{responses per min on test day} / \text{responses per min on control day}) \times 100]$.

Drugs

Morphine sulfate, URB597, and SR141716A were provided by the National Institute on Drug Abuse (Bethesda, MD). AM404 was purchased from Tocris Bioscience (Ellisville, MO), and CP55940 was purchased from Sigma-Aldrich (St. Louis, MO). Acetic acid was purchased from Thermo Fischer Scientific (Waltham, MA) and diluted in 0.9% saline for intraperitoneal administration (0.1 ml/10 g). All drugs except morphine (administered in saline) were administered in a vehicle of saline, ethanol, and emulpher in a ratio of 18:1:1. Morphine, CP55940, URB597, AM404, and SR141715A were injected subcutaneously at a volume of 0.1 ml/10 g.

Data Analysis

Data are expressed as mean (\pm S.E.M.) percentage of MPE, percentage of inhibition, percentage of control feeding, or percentage of control wheel running, depending on the assay (see above). The dose required to produce a 50% maximal effect (ED_{50}) was derived by using linear regression when possible, and differences in potency were determined and expressed as a potency ratio with 95% confidence limits. In instances in which determination of ED_{50} values was not possible, one-way analysis of variance was used to determine treatment effects. In these instances, comparisons were conducted by using Dunnett's test. The effects of pretreatment with SR141716A on the effects of CP55940, URB597, and AM404 were analyzed with unpaired *t* tests.

In experiments in which ED_{50} values could be determined isobolographs or pseudo-isobolographs were constructed to determine whether drug interactions were additive, supra-additive, or infra-additive. In instances when the values obtained from drug combinations did not deviate significantly from a diagonal line connecting the ED_{50} of morphine and the drug with which it was combined (or a vertical line arising from the ED_{50} value of morphine alone when the ED_{50} of the combined drug could not be determined), the interaction was defined as additive. When the ED_{50} value obtained from a drug combination fell to the left or right of the line of additivity, the interaction was supra-additive or infra-additive, respectively. In these studies, deviation from the line of additivity was defined by determining whether the error bars of the combination ED_{50} overlapped the error bars of the drug-alone error bars (Li et al., 2010). All statistical analyses were conducted with an α level of significance set at $p < 0.05$.

Results

Morphine and CP55940 produced dose-dependent increases in percentage of MPE in the hotplate with maximal effects obtained at the highest doses tested, whereas URB597 and AM404 did not produce antinociception on the hotplate (Fig. 1a). In contrast to the effects obtained on the hotplate, morphine, CP55940, URB597, and AM404 all inhibited writhing in the acetic acid writhing assay (Fig. 1b). Injection of acetic acid that was preceded by saline or the cannabinoid vehicle resulted in mean (\pm S.E.M.) writhes of 29.67 (2.96) and 25.88 (2.72), respectively. Morphine, CP55940, and URB597 all produced more than 75% inhibition of writhing. At peak effect, morphine and CP5594 eliminated the writhing response entirely, and URB597 produced a marked decrease in writhing [mean (\pm S.E.M.) writhes of 2.63 (1.10)].

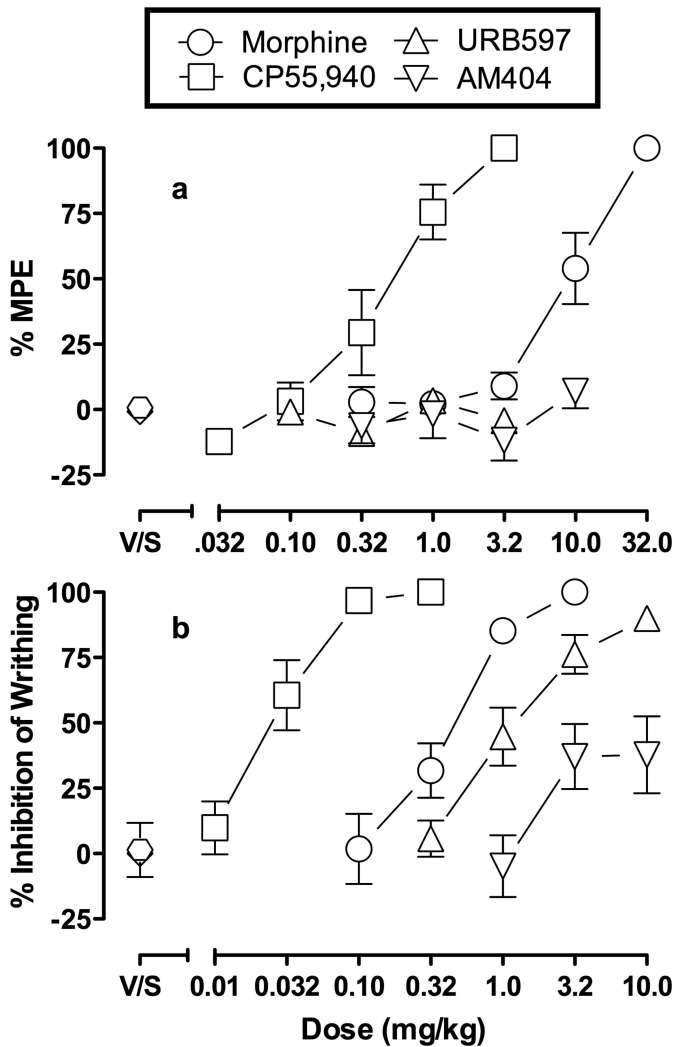


Fig. 1. Effects of morphine, URB597, CP55940, and AM404 in the hotplate (a) and writhing (b) assays. Abscissae, doses in milligrams/kilograms. Ordinate in a, percentage of maximum possible effect. Ordinate in b, percentage of inhibition of writhing. The hexagon and diamond above V/S represent the effects of saline and the cannabinoid vehicle, respectively.

No dose of AM404 fully eliminated writhing, but doses of 1–10 mg/kg significantly inhibited the writhing response ($F_{4,35} = 22.88; p < 0.05$). At peak effect, AM404 decreased the mean (\pm S.E.M.) number of writhes to 16.33 (3.87). The

TABLE 1

Mean (S.E.M.) effect of selected doses of CP55940, URB597, and AM404 alone and in combination with 3.0 mg/kg SR141716A. SR141716A significantly attenuated the effects of each drug in each assay (determined by unpaired *t* tests with an α level of significance of 0.05).

	Drug Alone	+ 3.0 mg/kg SR141716A
Hotplate, %MPE		
1.0 mg/kg CP55940	100 (0.00)	33.39 (8.30)
Acetic acid-elicited writhing, % inhibition		
0.32 mg/kg CP55940	99.98 (0.02)	4.76 (15.57)
10.0 mg/kg URB597	90.00 (4.19)	13.02 (19.06)
10.0 mg/kg AM404	37.78 (14.74)	3.50 (3.06)
Acetic acid-suppressed feeding, % control		
10.0 mg/kg URB597	72.48 (11.90)	5.58 (1.09)
Acetic acid-suppressed running, % control		
10.0 mg/kg URB597	54.08 (12.73)	9.94 (5.82)
10.0 mg/kg AM404	31.18 (9.87)	5.09 (2.73)
Schedule-controlled behavior, % control		
0.1 mg/kg CP55940	1.06 (0.66)	62.61 (17.61)

maximal effects produced by CP55940, URB597, and AM404 in these assays were attenuated by 3.0 mg/kg of the cannabinoid antagonist SR141716A (Table 1).

Pretreatment with a dose of CP55940 (0.1 mg/kg) that was ineffective when administered alone on the hotplate produced a significant leftward shift in the morphine dose-effect curve (Fig. 2; see Table 2 for ED₅₀ values and potency ratios), and this effect was attenuated by the cannabinoid antagonist SR141716A. Isobolographic analyses suggest that 0.032 and 0.1 mg/kg CP55940 interacted with morphine in an additive and supra-additive manner, respectively. Doses of URB597 and AM404 that had no effect when administered alone failed to alter morphine's antinociceptive effects in the hotplate.

Pretreatment with 0.01 mg/kg CP55940 and 0.32 mg/kg URB597, neither of which was effective in the writhing assay when administered alone, produced significant leftward shifts in the morphine dose-effect curve (Fig. 3; see Table 2 for ED₅₀ values and potency ratios), and isobolographic analyses indicated that interactions between morphine and both CP55940 and URB597 are additive. The effect of combined administration of these compounds was attenuated by 3.0 mg/kg of the cannabinoid antagonist SR141716A. AM404 did not alter morphine's effects in this assay.

Injection of acetic acid resulted in a significant suppression in feeding ($t_{10} = 11.32; p < 0.05$) with mean consumption (\pm S.E.M.) of 0.01 (0.003) g/g body weight after acetic acid, relative to control values of 0.11 (0.01) g/g body weight. As shown in Fig. 4a, only URB597 attenuated the suppression of feeding produced by an injection of acetic acid ($F_{4,35} = 11.48; p < 0.05$). The peak attenuation, after 10.0 mg/kg URB597, resulted in mean percentage control consumption (\pm S.E.M.) of 72.47 (11.90). This effect was antagonized by 3.0 mg/kg SR141716A (Table 1). Although there was some indication that morphine attenuated the suppression in feeding produced by the injection of acetic acid, these effects were not statistically significant. URB597 at 3.2 mg/kg, a dose that attenuated the suppression of feeding by acetic acid, and 3.0 mg/kg SR141716A did not alter nonsuppressed feeding under these conditions (Table 3).

Under control conditions, mean wheel-running revolutions per min (\pm S.E.M.) were 1101 (210). Running was completely eliminated ($t_{14} = 4.04; p < 0.05$) after administration of acetic acid. Figure 4b shows that URB597 ($F_{4,35} = 4.03; p < 0.05$), morphine ($F_{4,34} = 4.82; p < 0.05$), and AM404 ($F_{3,28} =$

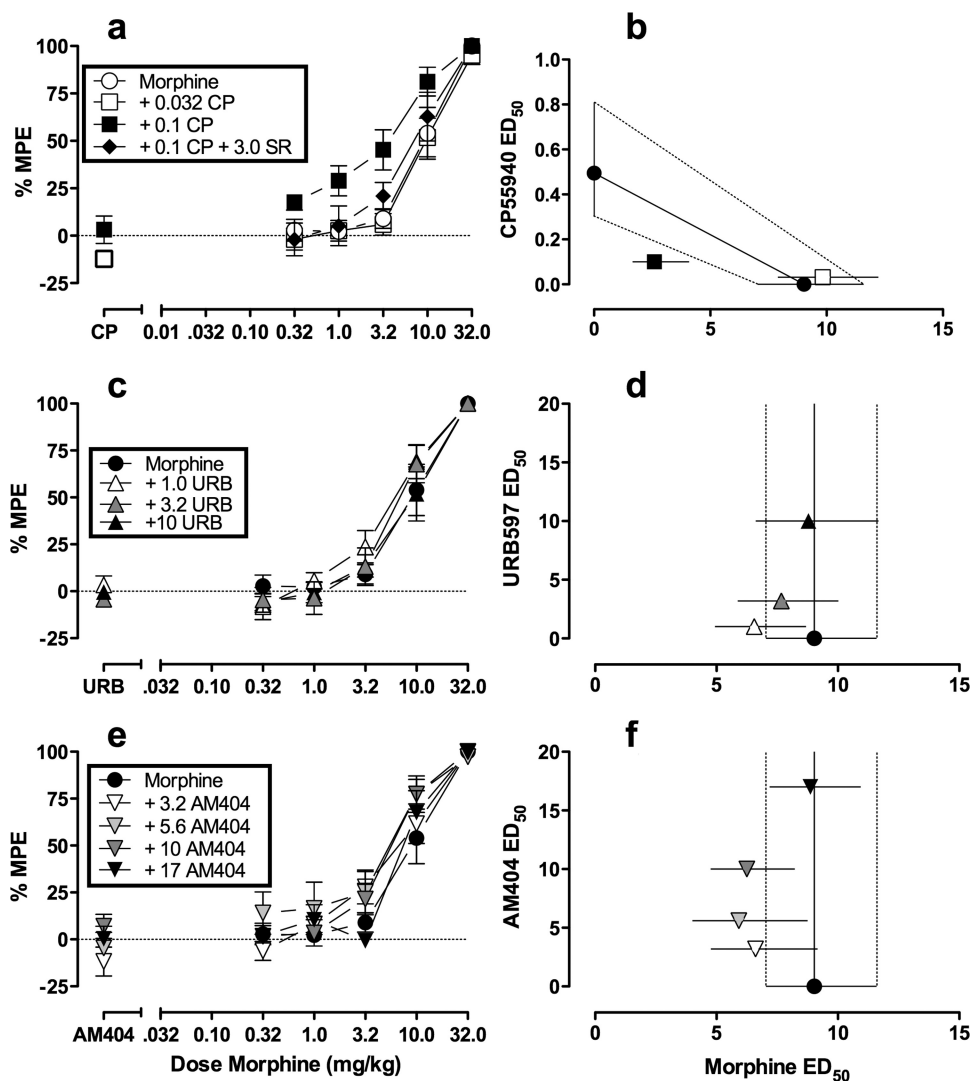


Fig. 2. Effects of morphine alone and in combination with CP55940 (CP), URB597 (URB), and AM404 in the hotplate. a, c, and e, dose-effect curves. Abscissae, doses of morphine in milligrams/kilograms. Ordinates, percentage of maximum possible effect. SR, SR141716A. b, d, and f, isobolographs. Abscissae, morphine ED₅₀ in milligrams/kilograms. Ordinates, CP5594, URB597, or AM404 ED₅₀ in milligrams/kilograms. Symbols above CP, URB, and AM404 show the effects of each drug alone.

TABLE 2

ED₅₀ (95% CL) values and potency ratios obtained from combined administration of morphine with CP55940, URB597, and AM404 in the hotplate assay

Blank spaces indicate that these values could not be determined. Asterisks indicate a significant shift in the morphine dose-effect curve, defined as a potency ratio with 95% confidence limits that do not include 1.

	Hotplate		Writhing	
	ED ₅₀ (95% CL)	Potency Ratio (95% CL)	ED ₅₀ (95% CL)	Potency Ratio (95% CL)
URB597			1.30 (0.92–1.84)	
AM404				
CP55940	0.49 (0.30–0.81)		0.03 (0.01–0.08)	
Morphine	9.03 (7.04–11.59)		0.42 (0.28–0.65)	
+ 0.032 mg/kg CP55940	9.83 (7.90–12.23)	1.09 (0.80–1.49)		
+ 0.1 mg/kg CP55940	2.60 (1.65–4.08)	3.56 (2.14–5.92)*	0.16 (0.09–0.29)	2.77 (1.62–4.82)*
+ 0.32 mg/kg URB597			0.17 (0.10–0.28)	2.44 (1.49–4.02)*
+ 1.0 mg/kg URB597	6.55 (4.94–8.69)	1.31 (0.93–1.85)		
+ 3.2 mg/kg URB597	7.67 (5.87–10.02)	1.17 (0.83–1.64)		
+ 10.0 mg/kg URB597	8.79 (6.62–11.67)	1.02 (0.72–1.45)		
+ 1.0 mg/kg AM404			0.38 (0.19–0.76)	1.12 (0.64–1.95)
+ 3.2 mg/kg AM404	6.61 (4.77–9.16)	1.28 (0.89–1.85)		
+ 5.6 mg/kg AM404	5.92 (4.01–8.75)	1.41 (0.96–2.08)		
+ 10.0 mg/kg AM404	6.25 (4.76–8.22)	1.38 (0.99–1.94)		
+ 17.0 mg/kg AM404	8.87 (7.19–10.94)	1.03 (0.75–1.41)		

6.82; $p < 0.05$), but not CP55940, significantly attenuated the suppression of wheel running after an injection of acetic acid. Although these drugs significantly attenuated the suppression of wheel running, the mean percentage of control run-

ning (\pm S.E.M.) obtained after morphine [maximum effect: 49.7 (17.1)], URB597 [maximum effect: 54.0 (12.7)], and AM404 [maximum effect: 32.4 (9.79)] shows that none of the compounds restored wheel running to control levels. In this

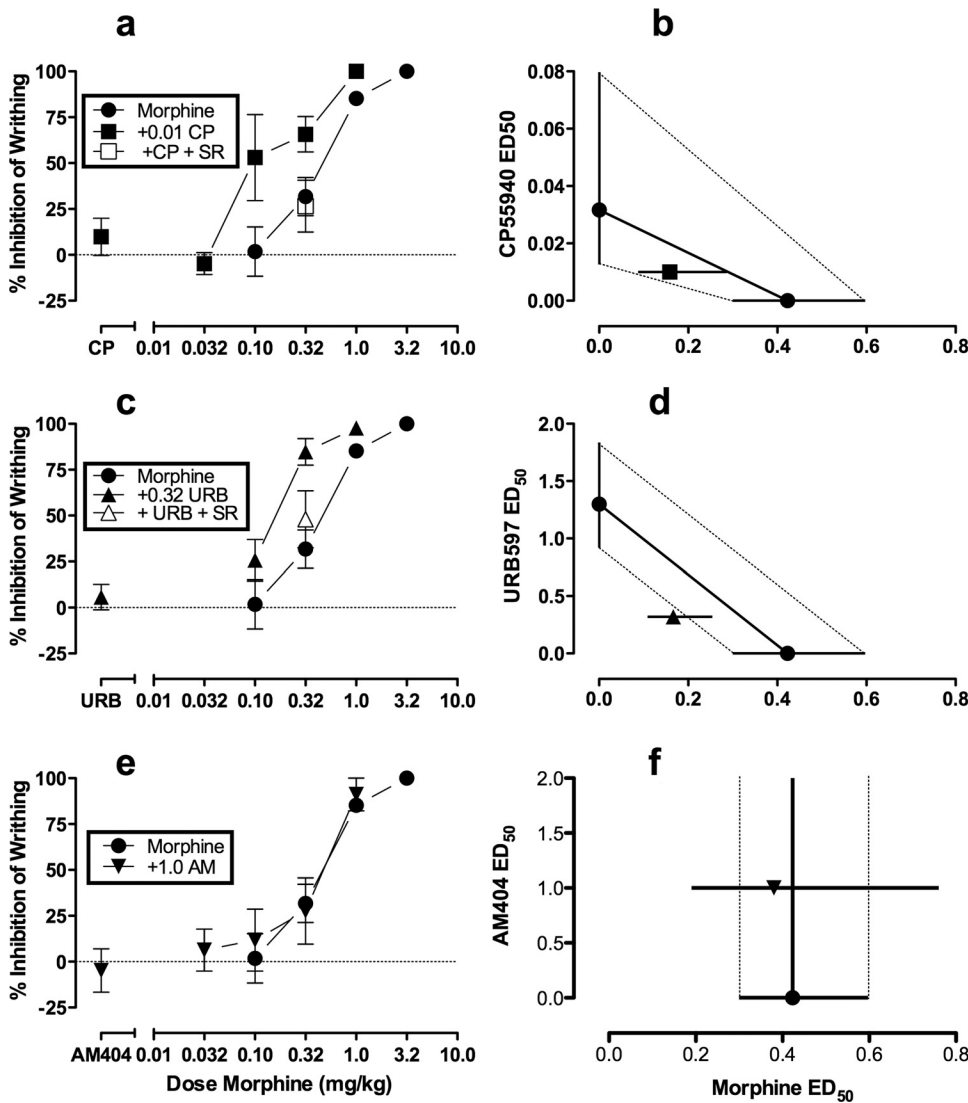


Fig. 3. Effects of morphine alone and in combination with CP55940 (CP), URB597 (URB), and AM404 in the writhing assay. a, c, and e, dose-effect curves. Abscissae, doses of morphine in milligrams/kilograms. Ordinate, percentage of maximum possible effect. SR, SR141716A. b, d, and f, isobolographs. Abscissae, morphine ED₅₀ in milligrams/kilograms. Ordinate, CP55940, URB597 or AM404 ED₅₀ in milligrams/kilograms. Symbols above CP, URB, and AM404 show the effects of each drug alone.

assay, the effects of 10 mg/kg URB597 and 10 mg/kg AM404 were antagonized by 3.0 mg/kg SR141716A (Table 1). Although 1.0 mg/kg morphine, 10.0 mg/kg URB597, and 10.0 mg/kg AM404 attenuated the suppression of wheel running by acetic acid, these treatments and 3.0 mg/kg SR141716A had no effect on nonsuppressed running (Table 4).

Morphine [ED₅₀ (95% CL) = 4.36 (3.47–5.48)] and CP55940 [0.05 (0.05–0.06)] dose-dependently decreased response rates in the assay of schedule-controlled behavior, whereas URB597 and AM404 had no effect on responding in this assay (Fig. 4c). The cannabinoid antagonist SR141716A attenuated the rate-decreasing effects of 0.1 mg/kg CP55940 (Table 1).

CP55940 and AM404 did not alter morphine's effects on pain-suppressed feeding (Fig. 5). Although morphine had no effect on pain-suppressed feeding when administered alone, when morphine was combined with an ineffective dose of URB597 (0.32 mg/kg) suppression of feeding by acetic acid injection was attenuated ($F_{3,22} = 49.15; p < 0.05$), and this effect was antagonized by 3.0 mg/kg SR141716A. Likewise, although CP55940 and AM404 did not alter morphine's effects in the wheel-running assay, when 0.32 mg/kg URB597, which had no effect when administered alone, was adminis-

tered in combination with morphine the increase in wheel running was greater than that obtained with morphine alone ($F_{1,46} = 6.53; p < 0.05$). This effect was attenuated by 3.0 mg/kg SR141716A.

A dose of CP55940 (0.032 mg/kg) that had no effect when administered alone produced a significant leftward shift in the morphine dose-effect curve in the assay of schedule-controlled behavior (Fig. 6) [ED₅₀ (95% CL) = 1.05 (0.81–1.45)], yielding a potency ratio (95% CL) of 4.16 (3.01–5.75). Isobolographic analysis suggests that this interaction was additive. Pretreatment with the CB1 antagonist SR141716A (3.0 mg/kg), which had no effect on response rates when administered alone, attenuated the rate-decreasing effects of the CP55940/morphine combination. URB597 and AM404 did not alter morphine's rate-decreasing effects in this assay.

Discussion

Table 5 summarizes the primary findings of this study. Specifically, the FAAH inhibitor URB597, the purported AEA uptake inhibitor AM404, and morphine were the only drugs to produce antinociception as operationally defined in at least one assay of pain-elicited behavior and assays of

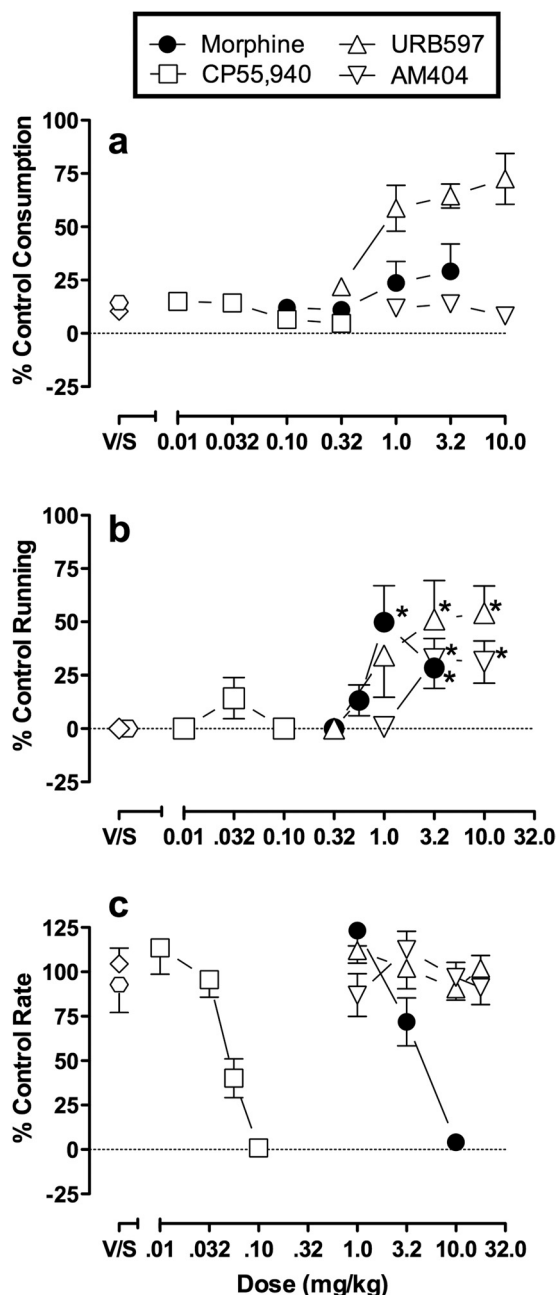


Fig. 4. Effects of morphine, URB597, CP55940, and AM404 on pain-suppressed feeding (a), pain-suppressed wheel running (b), and schedule-controlled behavior (c). Abscissae, doses in milligrams/kilograms. Ordinate in a, percentage of control consumption. Ordinate in b, percentage of control running. Ordinate in c, percentage of control response rate. The hexagon and diamond above V/S represent the effects of saline and the cannabinoid vehicle, respectively. Vehicle and drug data presented for the feeding and running assays were collected in the presence of acetic acid and are expressed as a percentage of vehicle data collected in the absence of acetic acid.

pain-suppressed behavior. CP55940 was effective only in the assays of pain-elicited behavior, and none of the drugs tested here were effective in all of the assays. These experiments also show that URB597 and AM404 did not disrupt food-maintained operant behavior at any dose tested, including those that produced antinociception, whereas morphine and CP55940 dose-dependently decreased response rates in this assay. Finally, although the combination of CP55940 and

TABLE 3

Liquid nutrition consumption (grams consumed/gram of body weight) by control mice, mice receiving 0.56% acetic acid in the absence of drug treatments, and mice receiving drug treatments in the absence of acetic acid

Asterisk indicates a significant difference in consumption compared with animals that did not receive acetic acid (determined by an unpaired *t* test with an α level of significance of 0.05).

Acetic Acid Concentration	Drug Condition	Mean (S.E.M.) Consumption
0	Saline	0.11 (0.01)
0.56%	Saline	0.01 (0.003)*
0	3.0 mg/kg SR1417116A	0.10 (0.01)
0	3.2 mg/kg URB597	0.09 (0.01)
0	0.32 mg/kg URB597 + 0.32 mg/kg morphine	0.09 (0.02)

TABLE 4

Wheel running (revolutions) by control mice, mice receiving 0.56% acetic acid in the absence of drug treatments, and mice receiving drug treatments in the absence of acetic acid

Asterisk indicates a significant difference in wheel running compared with animals that did not receive acetic acid (determined by an unpaired *t* test with an α level of significance of 0.05).

Acetic Acid Concentration	Drug Condition	Mean (S.E.M.) Revolutions
0	Saline	1101.75 (210.30)
0.56%	Saline	0.00 (0.00)*
0	3.0 mg/kg SR1417116A	775.38 (178.04)
0	1.0 mg/kg morphine	998.63 (210.30)
0	10.0 mg/kg URB597	945.17 (242.83)
0	10.0 mg/kg AM404	961.00 (266.01)
0	0.32 mg/kg URB597 + 0.56 mg/kg morphine	794.17 (104.56)

morphine produced greater antinociceptive effects than morphine alone in both assays of pain-elicited behavior, this combination also enhanced rate-decreasing effects in the assay of schedule-controlled behavior. In contrast, the combination of URB597 and morphine resulted in enhanced antinociceptive effects in the writhing assay and the two assays of pain-suppressed behavior, but did not enhance rate-decreasing effects on schedule-controlled behavior.

Research has shown that pharmacological manipulations that inhibit AEA degradation (Lichtman et al., 2004a; Kinsey et al., 2009; Costa et al., 2010) or limit AEA uptake (Costa et al., 2006) are efficacious in preclinical pain models. Furthermore, the blockade of endocannabinoid degradation does not produce many of the nonantinociceptive effects that are associated with CB1 agonists, such as catalepsy and disruption of locomotor activity (Cravatt et al., 2001; Moore et al., 2005; Russo et al., 2007). The results of the present experiments are consistent with these findings. First, the results obtained from the assays of pain-suppressed behavior suggest that URB597 and, to a lesser extent, AM404 produce antinociception at doses that do not produce nonspecific, behavioral suppressant effects. Effective doses of these compounds did not produce nonspecific increases in the behaviors measured by these models. In addition, a range of doses of URB597 and AM404, including doses that are effective in numerous pain models and have been shown to increase AEA levels (Fegley et al., 2004, 2005), failed to decrease rates of schedule-controlled responding. In contrast, the CB1 agonist CP55940 only produced maximum antinociception at doses that also resulted in decreases in schedule-controlled behavior.

The effects of URB597 and AM404 in these studies seem to be CB1-mediated given their attenuation by the CB1 antag-

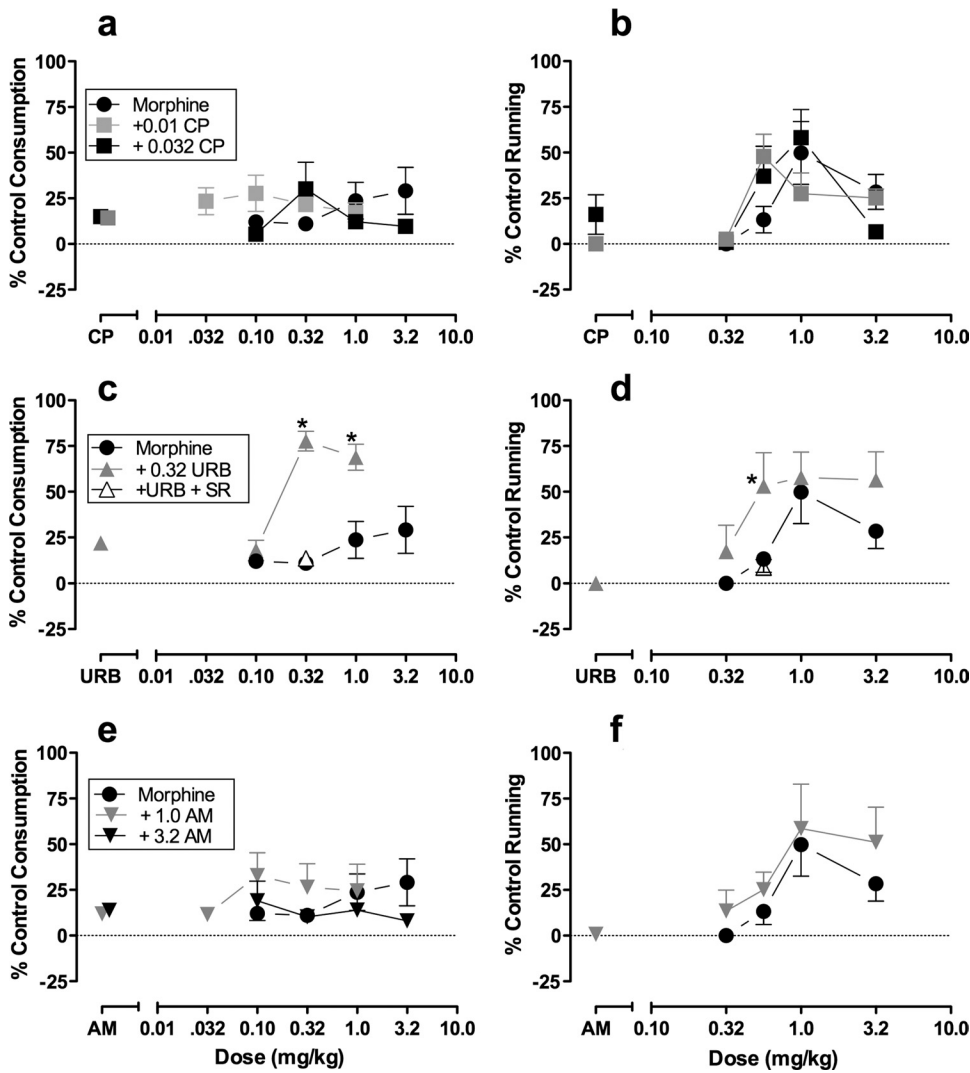


Fig. 5. Effects of morphine alone and in combination with CP55940 (CP; a and b), URB597 (URB; c and d), and AM404 (AM; e and f) in the pain-suppressed feeding assay (a, c, and e) and pain-suppressed wheel-running assay (b, d, and f). Abscissae, doses of morphine in milligrams/kilograms. Ordinates in a, c, and e, percentage of control consumption. Ordinates in b, d, and f, percentage of control running. Symbols above CP, URB, and AM show the effects of each drug alone. SR, SR141716A.

onist SR141716A. This finding is consistent with a body of literature suggesting that cannabinoid antinociception occurs largely via activity at CB1 receptors, although a complete description of the mechanisms that underlie these effects remains unidentified. There is evidence that disruption of endogenous cannabinoid metabolism could produce effects at other receptors such as CB2 (Booker et al., 2012) and transient receptor potential cation channel subfamily V member 1 (Howlett et al., 2002). In addition, inhibition of FAAH enhances AEA levels and those of other fatty acid amides (Clement et al., 2003), and, as a result, the mechanisms that mediate the effects seen here warrant further examination.

Previous studies have examined interactions between the cannabinoid and opioid systems by administering CB1 agonists in combination with morphine and have consistently shown that CB1 agonists enhance the antinociceptive effects of morphine (Smith and Martin, 1992; Welch and Stevens, 1992; Smith et al., 1994; Welch et al., 1995; Massi et al., 2001). More recent studies using CB1 antagonists also provide evidence that endogenous cannabinoids mediate the antinociceptive effects of morphine (da Fonseca Pacheco et al., 2008; Pacheco et al., 2009; Miller et al., 2011). Moreover, morphine's antinociceptive effects are enhanced when AEA is

protected from metabolism (da Fonseca Pacheco et al., 2008; Haller et al., 2008; Pacheco et al., 2009).

The present study demonstrates that the combination of an ineffective dose of URB597 with morphine produced greater antinociception than morphine alone in the writhing assay, the pain-suppressed feeding assay, and the pain-suppressed wheel-running assay. The CB1 antagonist SR141716A attenuated the effects of this combination, indicating a role for CB1 receptors, but it should be noted that this antagonist also attenuates the effects of URB597 alone (present study) as well as those of morphine alone (Miller et al., 2011). Thus SR141716A effects on URB597 and morphine may have contributed to the attenuation of the effects of URB597 and morphine in combination. URB597 did not alter morphine's effects in the hotplate assay or the assay of schedule-controlled behavior. Because URB597 did not enhance morphine's effects on schedule-controlled behavior, it is unlikely that the enhancement in morphine's antinociceptive effects is caused by an increase in its behavioral suppressant effects. This is in contrast to the CB1 agonist CP55940, which enhanced morphine antinociception in both assays of pain-elicited behavior (hotplate and writhing), but also enhanced morphine's rate-decreasing effects. Therefore, it is possible that the differences in experimental outcomes for the direct

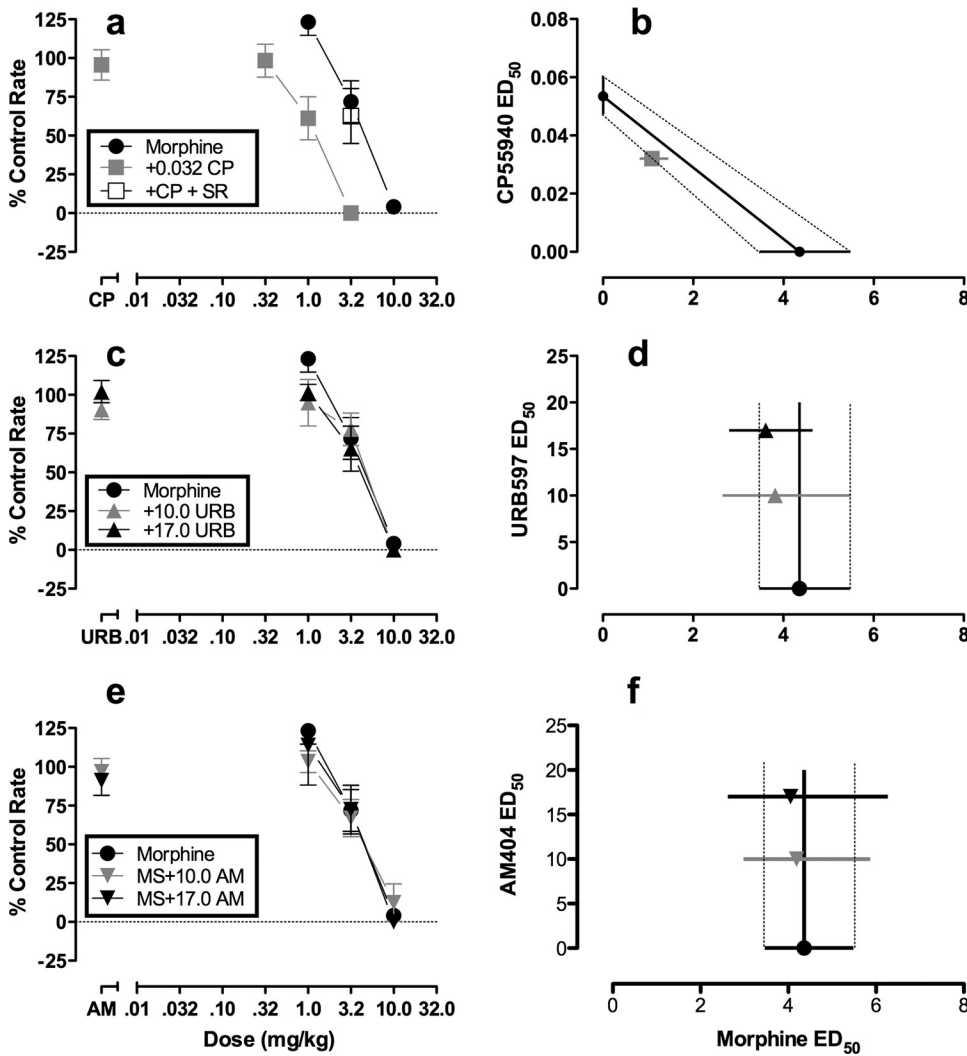


Fig. 6. Effects of morphine alone and in combination with CP55940 (CP), URB597 (URB), and AM404 (AM) in the assay of schedule-controlled behavior. a, c, and e, dose-effect curves. Abscissae, doses of morphine in milligrams/kilograms. Ordinates, percentage of control rate. b, d, and f, isobolographs. Abscissae, morphine ED₅₀ in milligrams/kilograms. Ordinates, CP5594, URB597, or AM404 ED₅₀ in milligrams/kilograms. Symbols above CP, URB, and AM show the effects of each drug alone. SR, SR141716A.

TABLE 5

Summary of drug effects in all five assays

Yes and No indicate whether the drug produced antinociception in the pain assays. For the assay of schedule-controlled behavior, Yes and No indicate whether the drug produced rate-decreasing effects. Arrows indicate that the combination of the compound with morphine produced effects that were greater than morphine alone.

	Hotplate	Writhing	Feeding	Running	Schedule-Controlled Behavior
Morphine	Yes	Yes	No	Yes	Yes
CP55940	Yes ↑	Yes ↑	No	No	Yes ↑
URB597	No	Yes ↑	Yes ↑	Yes ↑	No
AM404	No	Yes	No	Yes	No

agonist and the FAAH inhibitor could be caused in part by enhancement of morphine’s behavioral suppressant effects by the former and a lack of behavioral suppression by the latter.

Previous studies have demonstrated supra-additive interactions between opioids and CB1 agonists, and the data reported here for CP55940 and morphine in the hotplate procedure support those findings. The current experiments also indicate that combinations of morphine and CP55940 produce additive effects in the writhing and schedule-controlled behavior assays. Taken together, these findings suggest that CB1 agonists are not likely to enhance morphine’s antinociceptive effects without also producing other unwanted effects and that there may be limited clinical utility for cannabinoid agonist/morphine combinations. URB597

and morphine also interacted in an additive manner in the preclinical pain assays, but in contrast to CP55940, there was no enhancement of morphine’s rate-decreasing effects.

The underlying mechanisms of the differing behavioral effects of URB597 and CP55940 are not entirely clear, but there is evidence that tonic exposure to noxious stimuli elevates endocannabinoid levels in regions associated with pain processing (Mitrirattanakul et al., 2006; Agarwal et al., 2007). Furthermore, the endogenous cannabinoid system modulates a variety of CNS functions, and there is evidence that certain neurotransmitter systems may be more sensitive to endocannabinoid control than others (Adermark and Lovinger, 2009). Relative to exogenous agonists, the effects of drugs such as FAAH inhibitors, which enhance the activity of endogenous ligands, are probably influenced to a large extent

by the role of endocannabinoids under basal conditions and in response to environmental changes. Drugs such as URB597 may enhance endocannabinoid levels in a manner that reveals regional and/or temporal selectivity, and this selectivity may explain the relative absence of other CNS effects (e.g., locomotor effects, reinforcing effects) after administration of these compounds compared with direct CB1 agonists. Such evidence is compelling, comparing the effects and potential clinical utility of cannabinoid agonists and drugs that alter endogenous cannabinoid signaling, but other variables warrant examination. For instance, CP55940 is a full CB1 agonist whereas AEA is a partial CB1 agonist (Pertwee et al., 2010), but the effects of lower-efficacy exogenous CB1 agonists have not been determined.

URB597 and AM404 resulted in antinociception only when acetic acid was used as a noxious stimulus. A peripherally acting FAAH inhibitor, 3'-carbamoyl-6-hydroxy-[1,1'-biphenyl]-3-yl cyclohexylcarbamate (URB937), produces CB1-dependent antinociception in a variety of pain models that involve tonic exposure to noxious stimuli, including acetic acid (Clapper et al., 2010). In addition, peripheral FAAH inhibition produces CB1-dependent suppression of dorsal horn responses to formalin injection in the hind paw of rats (Clapper et al., 2010). However, like the results obtained with URB597 and AM404 in the present study, URB937 was not effective on the hotplate. Although URB597 and AM404 are not restricted to the periphery, taken together, these data suggest that elevations of anandamide in the periphery are sufficient to produce antinociception in assays of pain-elicited and pain-suppressed behavior depending on the noxious stimulus used in a particular assay. Therefore, examination of peripherally restricted inhibitors of endogenous cannabinoid degradation and noxious stimuli other than intraperitoneal acetic acid, in assays of pain-elicited and pain-suppressed behavior, may provide valuable information about the role of the endogenous cannabinoid system in pain processing.

It should be noted that, although the assays of pain-elicited and pain-suppressed behavior were useful in differentiating the effects of CP55940 from URB597 and AM404, the combination of drug effects observed across the assays of pain-suppressed behavior in this study were not entirely predictive of clinical utility. Specifically, morphine, an opioid that is widely used clinically, did not significantly attenuate the suppression of feeding by acetic acid. Others have demonstrated the effectiveness of morphine in assays of pain-suppressed feeding (Stevenson et al., 2006) as well as other behaviors such as wheel running (present study), locomotor activity (Stevenson et al., 2009), and intracranial self-stimulation (Pereira Do Carmo et al., 2009). Thus, a growing body of literature supports the potential for assays of pain-suppressed behavior to complement traditional preclinical pain assays.

Taken together, these experiments demonstrate that administration of URB597 and, to a lesser extent, AM404, result in CB1-mediated antinociception in assays in which the operational definition of antinociception is a decrease in pain-elicited behaviors as well as in assays in which antinociception is defined as a decrease in the suppression of behavior by noxious stimuli. In addition, these experiments suggest that combinations of URB597 and morphine result in enhanced antinociceptive effects without recruiting nonspecific effects. These studies, together with other recent find-

ings, suggest that compounds that enhance endogenous cannabinoid signaling may have potential as therapeutics, whereas CNS effects associated with direct CB1 agonists seem to pose a significant obstacle to their clinical utility.

Acknowledgments

We thank Dr. Steve Negus for important comments and advice before the initiation of these studies.

Authorship Contributions

Participated in research design: Miller, Picker, Schmidt, and Dykstra.

Conducted experiments: Miller, Umberger, and Schmidt.

Performed data analysis: Miller.

Wrote or contributed to the writing of the manuscript: Miller, Picker, and Dykstra.

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