Psychopharmacology (2006) 188:293–305
DOI 10.1007/s00213-006-0506-0

ORIGINAL INVESTIGATION

Effects of endocannabinoid neurotransmission modulators on brain stimulation reward

Styliani Vlachou · George G. Nomikos · George Panagis

Received: 14 February 2006 / Accepted: 4 July 2006 / Published online: 5 September 2006

Abstract
Rationale The endogenous cannabinoid system is responsive to the neurobiological actions of Δ^9-tetrahydrocannabinol (THC) and other cannabinoid ligands. While numerous studies have focused on the behavioral and pharmacological effects of THC and cannabinoid agonists in experimental animals, most recent work focuses on compounds that modulate endocannabinoid neurotransmission. However, the relevant studies concerning the ability of endocannabinoid modulators to modify reward processes in experimental animals remain rather scarce.
Objectives The present study examined the effects of drugs modulating endocannabinoid neurotransmission on brain reward function using the rate–frequency curve shift paradigm of intracranial self-stimulation (ICSS).
Methods Animals were implanted with electrodes into the medial forebrain bundle (MFB). After brain stimulation reward thresholds stabilized, rats received intraperitoneal injections of the fatty acid amide hydrolase (FAAH) inhibitors phenylmethylsulfonyl fluoride (PMSF) (0, 15, 30, and 60 mg/kg) and URB-597 (0, 0.3, 1, and 3 mg/kg) and the selective anandamide reuptake inhibitor OMDM-2 (0, 3, 10, and 30 mg/kg).
Results The highest dose of URB-597 and OMDM-2 significantly increased the threshold frequency required for MFB ICSS, while PMSF increased the threshold frequency in all doses tested. The cannabinoid 1 (CB_1) receptor antagonist SR141716A reversed the actions of URB-597 and OMDM-2, but not PMSF, without affecting reward thresholds by itself.
Conclusions These results indicate that under the present experimental conditions endocannabinoid modulators do not exhibit reinforcing properties, but rather have inhibitory influence on reward processes. The anhedonic effects of URB-597 and OMDM-2, but not PMSF, observed at the highest doses in this study are probably mediated through direct CB_1 receptor stimulation.

Keywords Intracranial self-stimulation · Endogenous cannabinoids · Anandamide transport · Reward · Fatty acid amide hydrolase (FAAH) · Phenylmethylsulfonyl fluoride (PMSF) · OMDM-2 · URB-597 · SR141716A

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Introduction

Within the last 15 years, the discovery of the endogenous cannabinoid system has boosted cannabinoid pharmacology and led to an increasing number of investigations on its role in physiology and pathophysiology and on the therapeutic potential of compounds that modify endocannabinoid neurotransmission. In the brain, this endocannabinoid system is mainly comprised by the cannabinoid 1 (CB_1) receptor and its endogenous ligands with cellular mechanisms for their production, release, and degradation (Ameri 1999; Chaperon and Thiébot 1998; Tanda and Goldberg 2003; Bisogno et al. 2005; Fowler et al. 2005; Pazos et al. 2005). It is generally accepted that the endocannabinoid system plays a modulatory role in the control of motor behavior, learning and memory, nociception, emesis,

appetite, and mood (Inui 2001; Romero et al. 2002; Fowler et al. 2005; Kirkham 2005; Lundqvist 2005; Vickers and Kennett 2005; Viveros et al. 2005). Among the endogenous cannabimimetic substances, anandamide is as yet the best studied and known. Anandamide is released on demand by stimulated neurons; it binds to and activates cannabinoid receptors with relatively high affinity, potency, and efficacy and is rapidly eliminated through a two-step process consisting of a carrier-mediated transport followed by intracellular hydrolysis (Di Marzo et al. 1994; Beltramo et al. 1997; Giuffrida et al. 1999). Recently, an anandamide transport process was identified (Moore et al. 2005), while anandamide hydrolysis is catalyzed by the enzyme fatty acid amide hydrolase (FAAH), a membrane-bound serine hydrolase (Cravatt et al. 1996; Patricelli et al. 1999).

Recent studies have provided strong experimental evidence that endogenous cannabinoids are released from depolarized postsynaptic neurons and act retrogradely onto presynaptic neurons to suppress subsequent neurotransmitter release (Maejima et al. 2001; Wilson and Nicoll 2001; Freund et al. 2003). This neuromodulatory action of endocannabinoids at the synapse is mediated via CB_1 receptors (Schlicker and Kathmann 2001). It is interesting to note that endocannabinoid levels and/or cannabinoid receptor density are purportedly affected in several neuropsychiatric disorders such as Parkinson's disease, Huntington's disease, multiple sclerosis, epilepsy, certain types of pain, and excitotoxicity (Gubellini et al. 2002; Lastres-Becker et al. 2002; Kathuria et al. 2003; Marsicano et al. 2003; Wallace et al. 2003; Fujiwara and Egashira 2004; Lichtman et al. 2004; Ortiz et al. 2004). Therefore, it should be possible to treat these pathological conditions using drugs that modulate endocannabinoid levels (Lambert and Fowler 2005; Mackie 2005). In fact, the knowledge of the mechanisms through which the endogenous levels of endocannabinoids and the activation of cannabinoid receptors are regulated might have an enormous impact on the development of selective compounds with beneficial effects (Martin 2002; Ortega-Gutiérrez 2005; Pertwee 2005).

The development of agonists of the cannabinoid receptors with high potency, efficacy, and selectivity was one of the main targets of the pharmaceutical industry for many years (Howlett et al. 2002). However, activation of the endocannabinoid system through direct agonists was associated with undesirable psychotropic effects (Huestis et al. 2001). Another approach to avoid these unwanted adverse effects is to enhance endogenous cannabinoid tone through inhibition of endocannabinoid degradation. There are experimental and medical interests in studying the effects of molecules that selectively interfere with endocannabinoid neurotransmission. Endocannabinoid neurotransmission enhancers may be used experimentally to uncover the functions of the endocannabinoid system. Furthermore, they may offer a rational approach to various diseases in which elevation of endocannabinoids at their release sites may result in a more selective pharmacological response than the stimulation of CB_1 receptors by direct agonists, as mentioned above. This strategy would lead to enhanced levels of endocannabinoids only "on demand," i.e., when their production and release is recruited. Several compounds that affect endocannabinoid levels were synthesized; while some of these were examined in various behavioral processes (see, e.g., Compton and Martin 1997; Kathuria et al. 2003; de Lago et al. 2004). A few studies have examined their possible reinforcing properties, which seem to be very promising (Gobbi et al. 2005; Hansson et al. 2006; Bortolato et al. 2006), but so far, there is no knowledge about their effects on brain stimulation reward.

Research evidence indicates that the endocannabinoid system plays a role in brain reward circuitries, which are activated by different types of reinforcers, and among them, the habit-forming drugs (Arnone et al. 1997; Comings et al. 1997; Ledent et al. 1999; Mascia et al. 1999; Hungund and Basavarajappa 2000; Lallemand et al. 2001). However, it was rather difficult to demonstrate the rewarding properties of cannabis or synthetic cannabinoids in the currently used rodent models of addictive behavior. A number of studies failed to show self-administration of cannabis or Δ^9-tetrahydrocannabinol (THC) in rodents or primates (Corcoran and Amit 1974; Harris et al. 1974; Leite and Carlini 1974; Van Ree et al. 1978; Mansbach et al. 1994). However, some reports indicate a facilitation of brain stimulation reward (Gardner et al. 1988; Lepore et al. 1996), sustained self-administration (Takahashi and Singer 1979; Tanda et al. 2000; Justinova et al. 2003), and conditioned place preference (Lepore et al. 1995; Valjent and Maldonado 2000) by THC in experimental animals. Similarly, sustained self-administration of the selective CB_1 receptor agonist WIN 55,212-2 was reported in drug-naive mice (Martellotta et al. 1998) and rats (Fattore et al. 2001), whereas various CB_1 agonists were shown to establish both place conditioning (Braida et al. 2001) and place aversion (McGregor et al. 1996; Sañudo-Pena et al. 1997; Chaperon et al. 1998; Mallet and Beninger 1998; Cheer et al. 2000; Robinson et al. 2003) or taste aversion (Elsmore and Fletcher 1972; Hunt and Amit 1987; Parker and Gillies 1995; McGregor et al. 1996). Arnold et al. (2001) have reported that the CB_1 receptor agonist CP 55,940 did not affect the reinforcing properties of medial forebrain bundle (MFB) self-stimulation. Recently, we also showed that the CB_1 receptor agonists WIN 55,212-2, CP 55,940, and HU-210 either did not affect or increase intracranial self-stimulation (ICSS) threshold, depending on the dose used (Vlachou et al. 2005), whereas the CB_1 receptor agonist WIN 55,212-2 in a dose that did not affect baseline self-stimulation reduces the reinforcing effects induced by cocaine (Vlachou et al. 2003).

An unresolved issue also exists regarding the direct involvement of endocannabinoids, such as anandamide, in brain reward processes. Thus, Justinova et al. (2005) showed that anandamide is intravenously self-administered by squirrel monkeys, while in the study by Mallet and Beninger (1998), anandamide did not support conditioned place preference. Similarly, Gobbi et al. (2005) showed that URB-597, which increases brain anandamide levels, neither exerted reinforcing properties in the conditioned place preference paradigm nor produced generalization to the discriminative effects of THC in rats. Furthermore, anandamide does not generally produce THC-like effects in drug discrimination studies, although its synthetic analog R(+)-methanandamide shows cross-discrimination with THC in rats (Jarbe et al. 2001; Maldonado and Rodriguez de Fonseca 2002; Tanda and Goldberg 2003; Wiley et al. 2004). On the other hand, Bortolato et al. (2006) showed that the endocannabinoid neurotransmission enhancer AM-404 elicited rewarding effects in the conditioned place preference paradigm in rats housed under enriched conditions, but not in rats kept in standard cages.

Furthermore, it was argued that compounds that increase endocannabinoid neurotransmission may affect the actions of other drugs of abuse both in the acute and the dependence state (Vela et al. 1995; Gallate et al. 1999; Del Arco et al. 2002; Vigano et al. 2004; Yamaguchi et al. 2001; Vlachou et al. 2003; Solinas et al. 2005). Other studies have demonstrated that the pharmacological management of endocannabinoid neurotransmission might influence several aspects of addiction, such as vulnerability, degree of dependence, reinforcement, abstinence, craving, and relapse (Arnold 2005; De Vries and Schofeelmeer 2005; Fattore et al. 2005; Parolaro et al. 2005; Rodriguez de Fonseca et al. 2005; Solinas et al. 2005; Hansson et al. 2006). These observations have contributed to expectations that modulating endocannabinoid levels for therapeutic purposes would have minimal psychotropic effects and abuse liability (Martin 2002; Piomelli 2003, 2004; Di Marzo et al. 2004; Ortega-Gutiérrez 2005; Pertwee 2005).

Against this background, the aim of the present study was to further investigate the influence of the endogenous cannabinoid system on reinforcement processes, using drugs that inhibit endocannabinoid degradation. In particular, we studied the effects of phenylmethylsulfonyl fluoride (PMSF), a nonselective serine protease inhibitor that blocks the activity of FAAH and prevents the hydrolysis of anandamide (Deutsch and Chin 1993; Hillard et al. 1995); URB-597, an inhibitor of intracellular FAAH activity (Kathuria et al. 2003; Fegley et al. 2004, 2005) and OMDM-2, a selective and metabolically stable inhibitor of anandamide cellular uptake that has minimal activity against FAAH (Ortar et al. 2003; de Lago et al. 2004) on reward, using the ICSS paradigm. Because not all of the centrally mediated effects of anandamide occur through CB_1 receptor stimulation, we also studied the ability of the selective CB_1 receptor antagonist SR141716A (Rinaldi-Carmona et al. 1994) to counteract the tentative effects of endocannabinoid modulators on brain stimulation reward.

Materials and methods

Animals and surgery

Male Sprague–Dawley rats (n=103) weighing 300–350 g at the time of surgery were used. Before surgery they were housed in groups of three under a 12:12-h light–dark cycle with free access to food and water. The animals were anesthetized with intramuscular (im) injection of ketamine hydrochloride (100 mg/kg) and xylazine (10 mg/kg). Atropine sulfate (0.6 mg/kg, im) was injected to reduce bronchial secretion. Moveable monopolar stimulating electrodes (Model SME-01, Kinetrods, Ottawa, Ontario, Canada) were lowered into the MFB at the level of lateral hypothalamus (coordinates anteroposterior: −2.5 mm from bregma, lateral: −1.7 mm from the midline, ventrodorsal: −8.0 from a flat skull) according to Paxinos and Watson (1998).

The electrodes consisted of a plastic guiding base and a 0.25-mm diameter moveable stainless steel wire, which were insulated with Epoxylite except for the conically shaped tip. The anode was an Amphenol pin connected to five miniature skull screws. After implantation and for the entire duration of the experiments, the animals were housed individually.

Animal care and the procedures used were in accordance with NIH public document 85-23 (1985).

Apparatus and procedures for self-stimulation

One week after surgery, the animals were tested for self-stimulation in an operant chamber that was made of transparent Plexiglas (25-cm-wide, 25-cm-deep, and 30-cm-high). A stainless steel rodent lever protruded 2 cm from the left wall at a height of 4 cm from the floor. Each bar press triggered a constant current generator that delivered a 0.4-s train of rectangular cathodal pulses of constant duration (0.1 ms) and intensity (250 μA) and variable frequency (25–125 Hz, i.e., 10–50 number of pulses/0.4 s). The pulse frequency, i.e., the number of pulses within a train, was progressively increased up to 40 per stimulation train until the subject showed vigorous self-stimulation. If the implantation site failed to support self-stimulation, the electrode was lowered by steps of 0.16 mm (one step every 24 h), until a self-stimulation site was found. The electrode position was held unchanged in all

subsequent testing. During the acquisition phase the animals were trained to self-stimulate for at least three consecutive days (1 h daily), using stimulation parameters that maintained near maximal bar pressing rates. After self-stimulation was acquired and stabilized for a given pulse frequency, animals were trained under a protocol in which frequency was systematically manipulated to generate rate–frequency response curves. On this protocol the animals were tested at several stimulation frequencies, beginning with frequencies that sustained responding at maximal rates and descending in frequencies that did not sustain responding. The pulse frequency was varied by steps of approximately 0.1 log units. Fourteen rate–frequency trials were conducted during each session. At the beginning of each trial, the animals received three trains of priming stimulation at the frequency of the stimulation, which was available for that trial. Each frequency was tested within trials of 60 s in duration, followed by an extinction period of 30 s (intertrial interval). A rate–frequency determination (i.e., the entire session) lasted about 45 min. One rate–frequency curve was established daily for 10–12 days, depending on the period when the self-stimulation indices (i.e., shifts in the lateral position of the curve and threshold measure) were stable.

Unequivocally, ICSS behavior has the advantage of not being affected by satiation (factor) or dysphoric effects, which are potentially modulated by cannabinoids. On the other hand, because both endogenous cannabinoids and cannabinoid agonists seem to disrupt motor activity/performance capacity in a dose-dependent manner (Stark and Dews 1980; Chaperon and Thiébot 1998; Romero et al. 2002; Iversen 2003), the use of a rate-free, reward selective measure like the curve shift was requisite. In this method, plotting the responses of the animals against the various pulse frequencies yields a sigmoidal rate–frequency curve as shown in Fig. 4. Shifts in the lateral position of the curve provide selective measure of stimulation-produced reward, as elegantly demonstrated by Edmonds and Gallistel (1974), while vertical shifts provide information on motor/performance capacity. Furthermore, this method offers quantitative scaling of drug-induced changes in reward (see Campbell et al. 1985) that is useful when comparing the effects of different drugs. In other words, the rate–frequency method appears to have reward selectivity that is required in psychopharmacological research (Liebman 1983; Miliaressis et al. 1986; Markou and Koob 1992, 1993).

Drugs

PMSF (Sigma-Aldrich, St. Louis, MO, USA), URB-597 (Cayman Chemical, Ann Arbor, MI, USA), OMDM-2 (Tocris Bioscience, Ellisville, MO, USA), and SR141716A (synthesized by Lilly Research Laboratories, Indianapolis, IN, USA; see Vlachou et al. 2003) were dissolved into a vehicle solution that consisted of 5% dimethylsulfoxide, 5% cremophor EL, and 90% of 0.9% NaCl and were injected intraperitoneally (i.p.) at a volume of 3 ml/kg of body weight. Control animals received i.p. the corresponding vehicle solutions in the same injection volume. The doses of the cannabinoid compounds tested are within the range of doses regularly used in a plethora of functional studies (see, e.g., Compton and Martin 1997; de Lago et al. 2004; Kathuria et al. 2003; Holt et al. 2005), and which most likely result in a substantial increase in concentrations of endocannabinoids in the brain.

Experimental procedures

Drug testing began for each animal when the function relating bar pressing rate to pulse frequency (the rate–frequency function) was stable for at least three consecutive days. The criterion for stability was met when the frequency thresholds did not vary by more than 0.1 log units. Each drug or vehicle self-stimulation test consisted of a baseline and a drug rate–frequency function determination (for 45 min each). After the baseline period, each animal was injected with the drug or its vehicle. The animals were tested 10 min after the last injection. This time interval was also used in self-stimulation studies with other drugs of abuse (see, for example, Maldonado-Irizarry et al. 1994; Ranaldi and Beninger 1994; Vlachou et al. 2003, 2005) and appears to be critical for the observation of other behavioral and physiological effects of cannabinoids (see original studies in Compton and Martin 1997; Chaperon and Thiébot 1998; Kathuria et al. 2003; de Lago et al. 2004).

In the present study we used a mixed design, i.e., some animals received only one treatment, whereas other animals received all doses for only one drug treatment tested. All animals took part in only one experiment, either by receiving only one drug treatment or by receiving all drug treatments of the experiment. An initial analysis not presented in the paper showed no difference in the reward and performance measurements of the animals used in both designs (within- and between-subjects design). The reason why we used animals that received all drug treatments and animals that received only one drug treatment in each experiment is because, as it is already known, cannabinoids seem to have some “carry-over” effects due to their lipophilicity. We tried to control these effects by allowing a 3-day period between injections (this period is considered sufficient for the behavior of the animals to return to stable, pretreatment levels, and not being affected by prior cannabinoid administration) and by using animals that would receive only one drug treatment

in one experiment. In fact, the use of animals that received only one treatment gave us confidence that the obtained results were not confounded by such a carry-over effect, and because there was no statistical difference in the responses between the group of animals with different treatment history (see below), the data were pooled and presented together. In the case of animals receiving more than one drug injection, the sequence of injections for the different drug doses was counterbalanced with respect to order and a 3-day period was allowed between injections. As we have observed in previous studies (Vlachou et al. 2003, 2005), this period is considered sufficient for the behavior of the animals to return to stable, pretreatment levels, and not being affected by prior cannabinoid administration, i.e., no carry-over effects of the cannabinoids were detected.

Study 1

Experiment 1: effects of systemically administered PMSF on brain stimulation reward

Sixteen rats were used. Four of them received all doses of PMSF (15, 30, and 60 mg/kg, i.p.) or its vehicle in a randomized order, while 12 received only one drug treatment.

Experiment 2: effects of SR141716A on PMSF-induced changes in brain stimulation reward

Twenty-nine rats were used. Five of them received all different doses of SR141716A (0, 0.02, 0.3, and 1 mg/kg, i.p.) followed 5 min later by PMSF (60 mg/kg, i.p.) or its vehicle in a randomized order, while 24 received only one combination of SR141716A and PMSF.

Study 2

Experiment 1: effects of systemically administered URB-597 on brain stimulation reward

Sixteen rats were used. Four of them received all doses of URB-597 (0.3, 1, and 3 mg/kg, i.p.) or its vehicle, while 12 received only one drug treatment.

Experiment 2: effects of SR141716A on URB-597-induced changes in brain stimulation reward

Eleven rats were used. Three of them received SR141716A (0.02 mg/kg, i.p.) or its vehicle followed 5 min later by URB-597 (3 mg/kg, i.p.) or its vehicle in a randomized order, while eight received only one combination of SR141716A and URB-597.

Study 3

Experiment 1: effects of systemically administered OMDM-2 on brain stimulation reward

Eleven rats were used. Three of them received all doses of OMDM-2 (3, 10, and 30 mg/kg, i.p.) or its vehicle in a randomized order, while eight received only one drug treatment.

Experiment 2: effects of SR141716A on OMDM-2-induced changes in brain stimulation reward

Twenty rats were used. Four of them received SR141716A (0.02 mg/kg, i.p.) or its vehicle followed 5 min later by OMDM-2 (30 mg/kg, i.p.) or its vehicle in a randomized order, while 16 received only one combination of SR141716A and OMDM-2.

Data analysis and statistics

Two aspects of the rate–frequency data were considered for analysis: the lateral position of the rate–frequency function on the frequency axis and the maximal rate. These aspects were analyzed by fitting the rate frequency data to the following variant of the Gompertz sigmoid model (Coulombe and Miliaressis 1987):

$$f(X) \propto e^{-e^{b(x_i - x)}}$$

When this equation is used to fit the rate–frequency function, α represents the maximum rate (asymptote), whereas x_i (X at inflection) represents the threshold frequency. The latter is the pulse number producing 36.7% of the asymptotic rate, i.e., the rate lying on the fastest-accelerating region of the curve. Parameter b represents an index of the slope, whereas e is the base of natural logarithms.

The preinjection session measurement for each animal is considered baseline in the ICSS procedure used. Data gathered for each animal from pre- and postinjection portions of each session are curve-fitted. The posttreatment threshold and asymptote values are expressed as percentage of predrug values. Considering all the above, one-way analysis of variance (ANOVA) was used in all experiments where there was only one drug administered (effects of cannabinoid agonists alone), while two-way ANOVA was used when two drugs were administered (effects of combined administration of cannabinoid agonists and antagonists). In the first case (one-way ANOVA), the cannabinoid agonist administration was the independent variable and the threshold and asymptotic rate of responding were the dependent variables. In the second case, the two compounds administered (antagonist and agonist) were

the two independent variables and the threshold and asymptotic rate of responding were the dependent variables. In all experiments, all statistically significant results were further evaluated by using the least significant difference (LSD) test for multiple contrasts to determine differences between groups.

Histology

At the end of the experiment, the animals were given a lethal dose of sodium Pentothal. The location of the terminal stimulation site was then marked according to the following procedure: a direct anodal current of 0.1 mA and 15-s duration was passed through the electrode tip. The animals were perfused intracardially with 0.9%, NaCl that was followed by a 50-cc solution of potassium ferrocyanide (3%) and trichloroacetic acid (0.5%) in 10% formalin. The brains were then removed and stored in 10% formalin for 3 days, and 2 days in a 30% sucrose solution. Finally, the brains were sliced in a cryostat microtome and the sections containing the electrode tract were mounted on slides and stained with cresyl violet. Only the rats in which tracks from the electrode were verified to be located in the MFB were included in this study. Electrode tips were examined in all animals tested.

Results

Study 1

Experiment 1: effects of systemically administered PMSF on brain stimulation reward

The changes of self-stimulation threshold and asymptotic rate of responding after systemic injection of the FAAH inhibitor PMSF are presented in subpanels a and b in Fig. 1, respectively. PMSF (15, 30, and 60 mg/kg, i.p.) significantly increased self-stimulation thresholds [$F(3,24)=6.845$, $P=0.002$] and decreased the asymptotic rate of responding [$F(3,24)=5.998$, $P=0.003$]. Post hoc analysis with the LSD test showed that these effects on the self-stimulation thresholds were significant at all doses tested (15, 30, and 60 mg/kg, i.p.), compared with the vehicle group, while they were significant for the asymptote only at the highest dose tested (60 mg/kg, i.p.), compared with the vehicle group ($P=0.023$).

Experiment 2: effects of SR141716A on PMSF-induced changes in brain stimulation reward

Figure 1c,d presents the changes in self-stimulation threshold and asymptotic rate of responding after systemic injection of SR141716A or its vehicle and PMSF or its vehicle. Two-way ANOVA showed that PMSF (60 mg/kg) produced an increase in self-stimulation

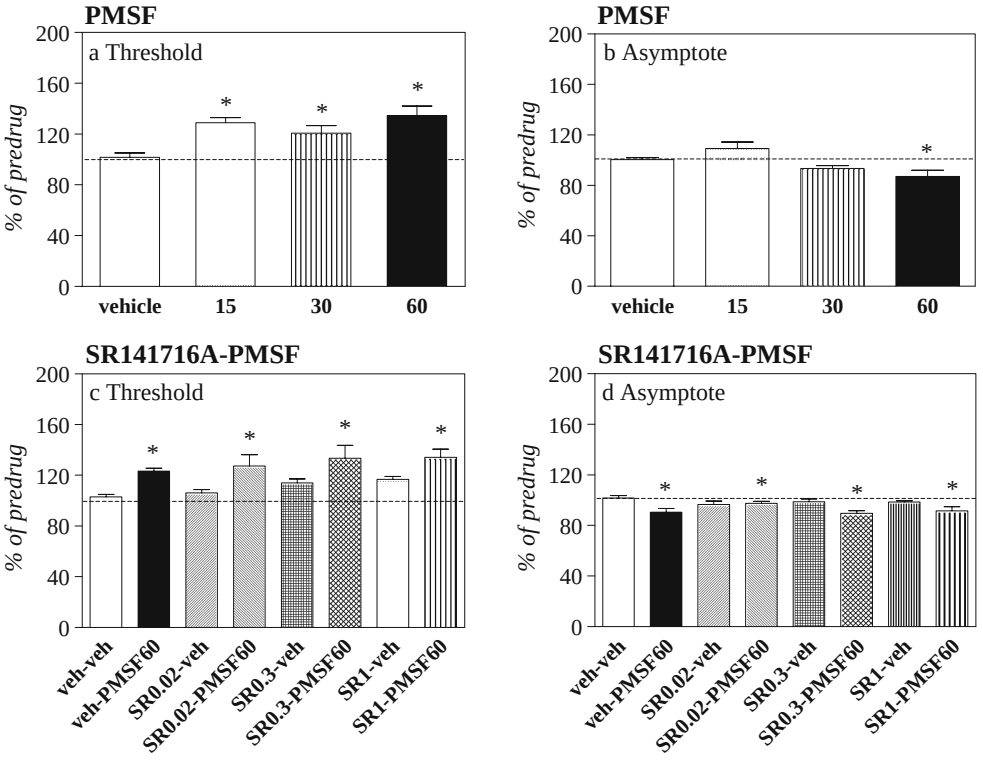

Fig. 1 Changes in self-stimulation threshold (**a**, **c**) and asymptotic rate (**b**, **d**) of responding (expressed as percentage of predrug values) after PMSF (0, 15, 30, and 60 mg/kg, i.p.) and SR141716A (0, 0.02, 0.3, and 1 mg/kg, i.p.) + PMSF (0 and 60 mg/kg, i.p.) treatments. *Vertical bars* represent the standard errors of the mean. The *asterisk* signifies an ICSS threshold and asymptote value significantly different from the control condition

threshold [$F(1,56)=23.872$, $P<0.0001$], while the administration of SR141716A did not block this effect [$F(3,56)=0.042$, $P=0.988$] in any of the doses tested (0.02, 0.3, and 1 mg/kg). SR141716A did not have any effect per se in the self-stimulation threshold [$F(3,56)=2.116$, $P=0.108$]. Two-way ANOVA also showed that PMSF (60 mg/kg) reduced the asymptotic rate of responding [$F(1,56)=15.643$, $P<0.0001$]. The effect of SR141716A on blocking the decreased asymptotic rate of responding, as induced by PMSF, did not reach statistical significance, although a tendency was observed [$F(3,56)=2.372$, $P=0.080$].

Study 2

Experiment 1: effects of systemically administered URB-597 on brain stimulation reward

The changes of self-stimulation threshold and asymptotic rate of responding after systemic injection of the FAAH inhibitor URB-597 are presented in subpanels a and b in Fig. 2, respectively. As it can be seen, URB-597 (0.3, 1, and 3 mg/kg, i.p.) significantly increased self-stimulation thresholds [$F(3,24)=5.084$, $P=0.007$], whereas it did not affect the asymptotic rate of responding [$F(3,24)=1.833$, $P=0.168$]. Post hoc analysis with the LSD test showed that the effects on the self-stimulation threshold were significant at the two highest doses tested (1 and 3 mg/kg), compared with the vehicle group ($P=0.008$ and 0.002, respectively).

Experiment 2: reversal of the action of URB-597 by SR141716A

Figure 2c,d shows the changes in self-stimulation threshold and asymptotic rate of responding after systemic injection of SR141716A or its vehicle and URB-597 or its vehicle. URB-597 (3 mg/kg, i.p.) produced a statistically significant increase in self-stimulation threshold [$F(1,16)=17.362$, $P=0.001$]. Administration of SR141716A (0.02 mg/kg, i.p.) significantly blocked this effect [$F(1,16)=9.723$, $P=0.007$]. Also, URB-597 [$F(1,16)=0.474$, $P=0.501$], SR141716A [$F(1,16)=0.899$, $P=0.357$], or their coadministration did not affect the asymptotic rate of responding [$F(1,16)=1.613$, $P=0.222$].

Study 3

Experiment 1: effects of systemically administered OMDM-2 on brain stimulation reward

The changes of self-stimulation threshold and asymptotic rate of responding after systemic injection of the selective anandamide reuptake inhibitor OMDM-2 are presented in subpanels a and b in Fig. 3, respectively. As it can be seen, OMDM-2 (3, 10, and 30 mg/kg, i.p.) produced a significant increase in self-stimulation threshold [$F(3,16)=9.932$, $P=0.001$], while it did not affect the asymptotic rate of responding [$F(3,16)=0.483$, $P=0.699$]. Post hoc analysis with the LSD test showed that the increase on the self-stimulation threshold was significant at the highest dose tested (30 mg/kg) ($P<0.0001$), compared with the vehicle group.

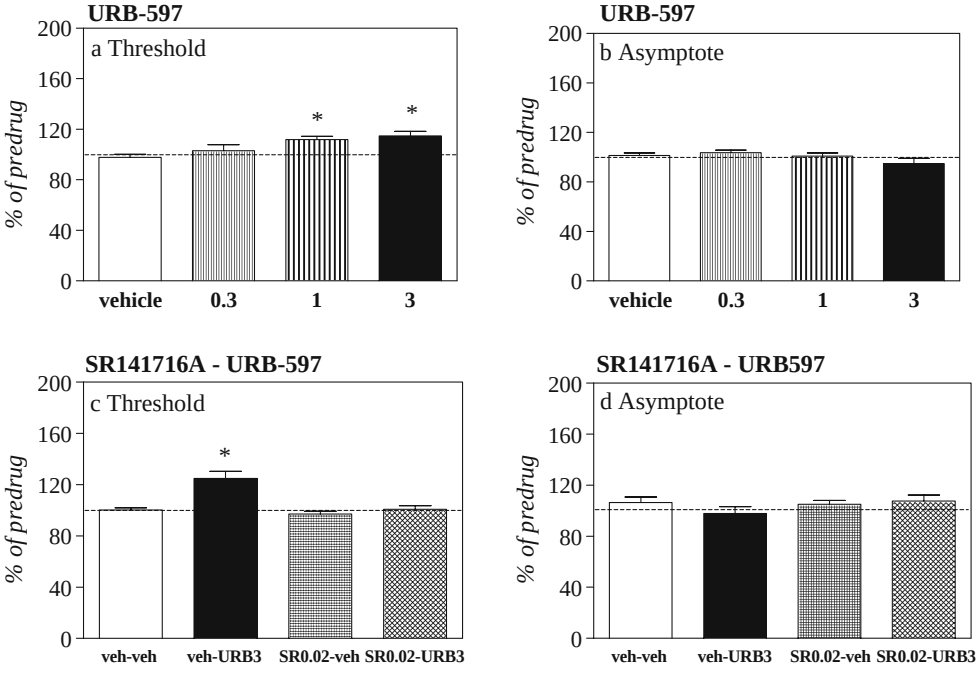

Fig. 2 Changes in self-stimulation threshold (**a**, **c**) and asymptotic rate (**b**, **d**) of responding (expressed as percentage of predrug values) after URB-597 (0, 0.3, 1, and 3 mg/kg, i.p.) and SR141716A (0, 0.02 mg/kg, i.p.) + URB-597 (0 and 3 mg/kg, i.p.) treatments. *Vertical bars* represent the standard errors of the mean. The *asterisk* signifies an ICSS threshold significantly different from the control condition

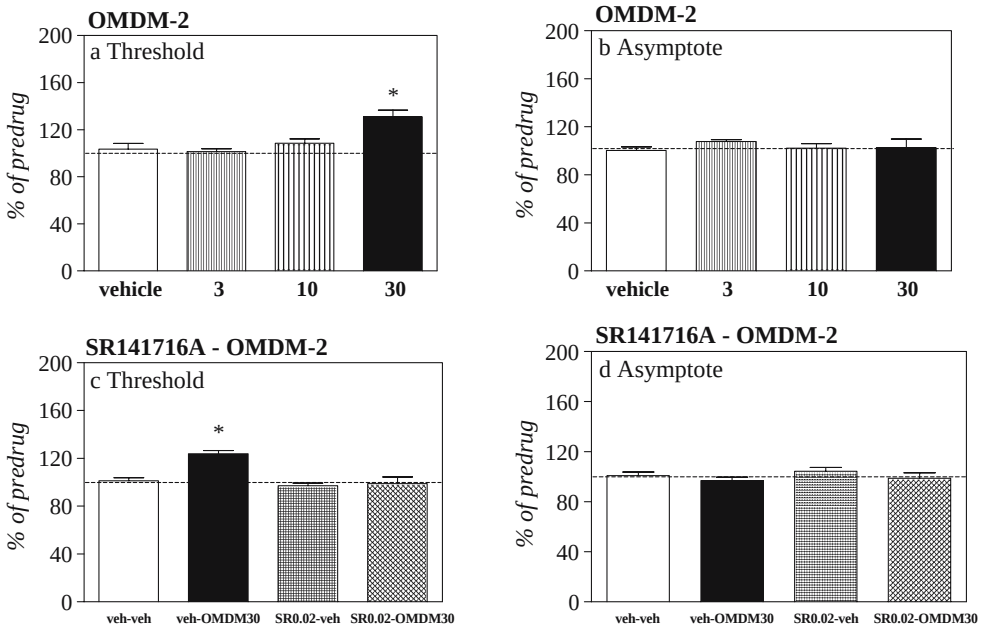

Fig. 3 Changes in self-stimulation threshold (**a**, **c**) and asymptotic rate (**b**, **d**) of responding (expressed as percentage of predrug values) after OMDM-2 (0, 3, 10, and 30 mg/kg, i.p.) and SR141716A (0 and 0.02 mg/kg, i.p.) + OMDM-2 (0 and 30 mg/kg, i.p.) treatments. *Vertical bars* represent the standard errors of the mean. The *asterisk* signifies an ICSS threshold significantly different from the control condition

Experiment 2: reversal of the action of OMDM-2 by SR141716A

Figure 3c,d presents the changes in self-stimulation threshold and asymptotic rate of responding after systemic injection of SR141716A or its vehicle and OMDM-2 or its vehicle. Two-way ANOVA showed that OMDM-2 (30 mg/kg) produced an increase in self-stimulation threshold [$F(1,40)=7.722$, $P=0.008$]. The administration of SR141716A (0.02 mg/kg, i.p.) blocked this effect [$F(3,40)=5.804$, $P<0.0001$]. Two-way ANOVA also showed that OMDM-2 (30 mg/kg) [$F(1,40)=2.819$, $P=0.101$], SR141716A (0.02 mg/kg) [$F(3,40)=0.784$, $P=0.510$], or their coadministration [$F(3,40)=0.130$, $P=0.942$] did not affect the asymptotic rate of responding.

Figure 4 depicts rate–frequency functions from representative animals obtained before and after antagonist–endocannabinoid enhancer injections. As indicated in the figure, each of the endocannabinoid enhancers produced a parallel curve shift to the right, indicating a clear decrease in the rewarding efficacy of the stimulation. On the other hand, we can see that this effect was reversed by coadministration of SR141716A (for URB-597 and OMDM-2).

Discussion

Systemic administration of the endocannabinoid neurotransmission modulators PMSF, URB-597, and OMDM-2, depending on the dose administered, either did not affect or decrease brain reward function, a finding reflected in elevated brain reward thresholds. Most of the administered doses did not affect the asymptotic rate of responding. These results indicate that the effects of endocannabinoid modulators on reward thresholds were not confounded by performance effects and are consistent with previous reports on homologous findings with direct CB_1 receptor agonists (Antoniou et al. 2005; Vlachou et al. 2005). It should be emphasized that the endocannabinoid modulators used might increase extracellular concentrations of not only anandamide, but also of other endocannabinoids, which exert their neurobiological actions through CB_1 or non-CB_1 receptors (for a recent review, see Lambert and Fowler 2005). Solely based on the obtained data, we cannot exclude that PMSF has other endocannabinoid-independent effects or that URB-597 and OMDM-2 in high doses act by directly stimulating the CB_1 receptors.

PMSF is a nonselective serine protease inhibitor that blocks the activity of FAAH and prevents the hydrolysis of anandamide (Deutsch and Chin 1993; Hillard et al. 1995). Animals receiving PMSF exhibit cannabinoid effects, i.e., antinociception, hypothermia, and immobility. In our study, PMSF increased brain reward threshold, independently of the dose tested. This effect was not blocked or diminished by the CB_1 receptor antagonist SR141716A. This implies that the observed activity of PMSF is not likely caused by actions at the CB_1 receptors. Indeed, PMSF acts as a nonspecific inhibitor of various proteases and enzymes, which may be responsible for its effects on reward thresholds of ICSS (Compton and Martin 1997). Furthermore, it was demonstrated that anandamide is an endogenous ligand also for vanilloid receptors (see, e.g., De Petrocellis and Di Marzo 2005; Ross 2003; Van der Stelt and Di Marzo 2004). Thus, the ICSS effects of PMSF might be attributed to elevated anandamide levels resulting in stimulation of those

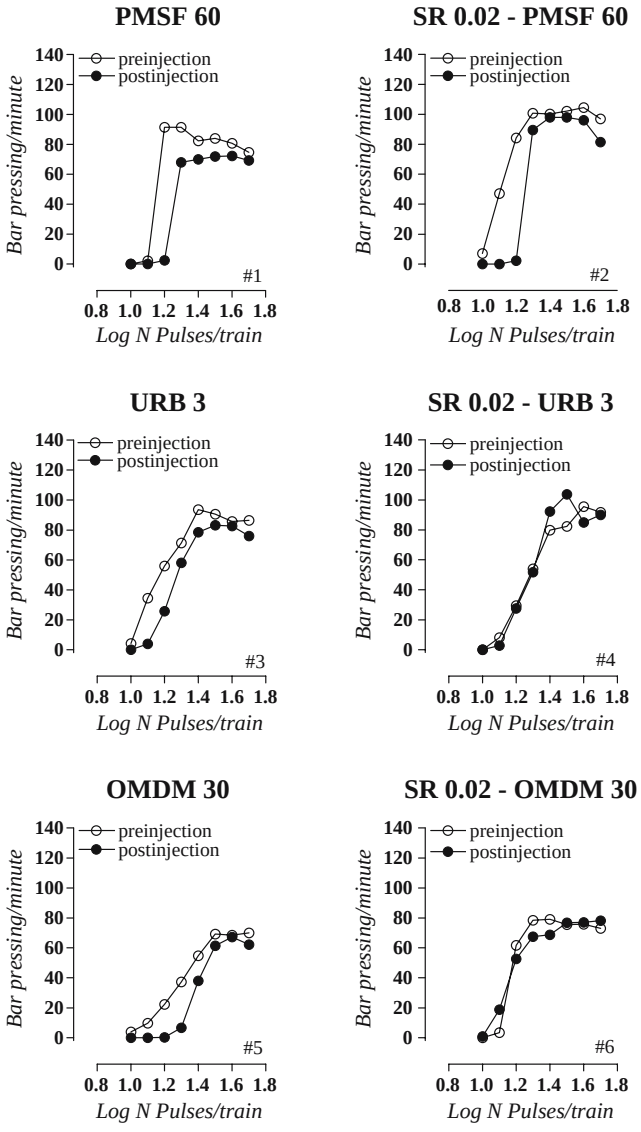

Fig. 4 Rate–frequency functions (rate of lever pressing as a function of stimulation frequency) taken from representative animals for each drug treatment. Each plot represents data from a single animal under predrug and drug conditions. Rate–frequency functions were obtained by logarithmically decreasing the frequency of the stimulation pulses from a value that sustained maximal lever pressing to one that failed to sustain lever pressing

receptors. Alternatively, the anhedonic effects of PMSF might be a result of increasing levels of endogenous substances, other than cannabinoids, which activate non-cannabinoid receptors or via an altogether endocannabinoid-independent mechanism. It is worth noting that this is not the first study in which behavioral actions of PMSF were not antagonized by SR141716A. Compton and Martin (1997) showed that SR141716A did not block the antinociceptive, hypothermic, and hypolocomotive actions of PMSF. It is interesting that PMSF decreased the asymptotic rate of responding for ICSS only at the highest dose tested (60 mg/kg). Detailed locomotor activity studies showed that PMSF reduces spontaneous activity at doses higher than 100 mg/kg (Compton and Martin 1997).

URB-597 is reported to be a potent inhibitor of the enzyme FAAH that degrades anandamide (Kathuria et al. 2003). Experimental data suggest that URB-597 is rather selective and does not affect the activities of other serine hydrolases (Kathuria et al. 2003). The dose of URB-597 at which inhibition of the FAAH enzyme is maximal is 0.3 mg/kg (Kathuria et al. 2003). It is surprising that 0.3 mg/kg of URB-597, which increases anandamide levels in the brain, did not affect brain stimulation reward, while higher doses (1 and 3 mg/kg) even increased brain reward thresholds. This effect was completely abolished by pretreatment with the CB_1 receptor antagonist SR141716A. Although, the increase in brain reward threshold was slight, compared to the one observed after PMSF administration, it still might be attributed to other unselective actions of this drug, such as direct stimulation of the CB_1 receptors. This is supported further by the finding that the anhedonic effects of URB-597 appear to be CB_1-receptor-dependent. It is interesting to note that URB-597 did not affect maximal rates of responding even at the highest doses used that could potentially result in a direct CB_1 receptor stimulation. In the study by Kathuria et al. (2003), administration of URB-597 did not produce catalepsy, hypothermia, and hyperphagia, three typical signs of CB_1 receptor activation, although exerted mild analgesic actions at doses that were at the lower end of those used here.

OMDM-2 is a rather selective and metabolically stable inhibitor of anandamide cellular reuptake that has minimal activity against FAAH (Ortar et al. 2003; de Lago et al. 2004). Administration of OMDM-2 at low doses (3 and 10 mg/kg), which were shown to increase anandamide levels (de Lago et al. 2004), did not affect ICSS behavior, whereas at the highest dose it increased brain reward thresholds. This effect of OMDM-2 was completely abolished by pretreatment with the CB_1 receptor antagonist SR141716A. However, because it is not clear whether OMDM-2 at the dose of 30 mg/kg can still selectively block anandamide reuptake, the observed effects could be due to a nonselective increase of extracellular concentrations of endocannabinoids other than anandamide (see above). Similar to URB-597, but not to PMSF, the anhedonic effects of OMDM-2 are seemingly CB_1-receptor-mediated. This is an indication that the anhedonic effects of OMDM-2 observed at higher doses could be a result of direct CB_1 receptor stimulation. OMDM-2 did not affect maximal rates of responding. This confirms previous studies showing the lack of significant activity of the same compound on motor performance (de Lago et al. 2004).

The present data should be viewed with regard to the results of previous studies on the rewarding/reinforcing

effects of cannabinoid ligands. To extend that the endocannabinoid modulators we studied affect cannabinoid receptor neurotransmission, our work confirms previous findings that direct cannabinoid agonists at low doses did not affect ICSS behavior, whereas at higher doses, increased brain reward thresholds (Antoniou et al. 2005; Vlachou et al. 2005), which is consistent with other reports indicating that cannabinoid agonists do not have direct reinforcing properties in experimental animals (Corcoran and Amit 1974; Harris et al. 1974; Leite and Carlini 1974; Van Ree et al. 1978; Mansbach et al. 1994; Arnold et al. 2001; Braida et al. 2001; see also "Introduction"). It should be noted that our study provides clear evidence that compounds modulating endocannabinoid neurotransmission in the brain do not activate brain reward processes in experimental animals. Our results are also in agreement with the study by Mallet and Beninger (1998) in which administration of the endogenous cannabinoid anandamide did not produce any significant effect in place conditioning using male Wistar rats and the study by Gobbi et al. (2005) in which URB-597 neither exerted reinforcing properties in the conditioned place preference paradigm nor produced generalization to the discriminative effects of THC in rats. However, it should be noted that it was recently reported that both the endogenous cannabinoid anandamide and its synthetic analog R(+)-methanandamide are intravenously self-administered by squirrel monkeys (Justinova et al. 2005). It is interesting to note that in a very recent study, Bortolato et al. (2006) showed that the endocannabinoid transport inhibitor AM-404 elicited rewarding effects in the conditioned place preference paradigm in rats housed under enriched conditions, but not in rats kept in standard cages. Furthermore, in a recent study by Solinas et al. (2005), administration of AM-404 or URB-597 did not enhance but rather reduced the reinforcing efficacy of heroin, whereas both THC and WIN 55212-2 have the opposite effect. These seemingly contrasting results could be attributed to differences in the animals used and the different experimental paradigms followed.

These findings may change our views of cannabinoids in relation to their therapeutic actions and dependence liabilities. The possible therapeutic applications of THC or its synthetic analogs that directly activate the CB_1 receptors are hindered by their psychotropic side effects. For this reason, substances that activate CB_1 receptors indirectly, i.e., by enhancing endocannabinoid levels, might offer a new therapeutic target. Indeed, to date several examples of the use of endocannabinoid enhancers with beneficial effects in animal models of various human diseases were reported (Ortega-Gutiérrez 2005).

In summary, the present study clearly shows that the endocannabinoid neurotransmission modulators PMSF, URB-597, and OMDM-2, administered at pharmacologically effective doses (which also substantially increase endocannabinoid levels), do not exhibit reinforcing properties in the ICSS paradigm. These compounds might increase brain stimulation reward threshold by enhancing the brain levels of endogenous compounds that do not bind to cannabinoid CB_1 receptors (for example PMSF), or when administered at higher unselective doses, which are above those necessary to obtain their known pharmacological activity (for example URB-597 and OMDM-2), might directly activate CB_1 cannabinoid receptors. The latter is supported by the fact that the anhedonic actions of these compounds observed after administration of higher, unselective doses were sensitive to SR141716A pretreatment.

Acknowledgements This study was supported by a grant from the Research Committee (KA 2303) and the Department of Psychology of the University of Crete. Styliani Vlachou was supported by a scholarship from PROPONTIS Foundation.

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