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A Peripheral Mechanism for CB1 Cannabinoid Receptor-Dependent Modulation of Feeding

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Recent studies suggest that the endocannabinoid system modulates feeding. Despite the existence of central mechanisms for the regulation of food intake by endocannabinoids, evidence indicates that peripheral mechanisms may also exist. To test this hypothesis, we investigated (1) the effects of feeding on intestinal anandamide accumulation; (2) the effects of central (intracerebroventricular) and peripheral (intraperitoneal) administration of the endocannabinoid agonist anandamide, the synthetic cannabinoid agonist *R*-(+)-(2,3-dihydro-5-methyl-3-[(4-morpholinyl)methyl]pyrol[1,2,3-de]-1,4-benzoxazin-6-yl)(1-naphthalenyl) methanone monomethanesulfonate (WIN55,212-2), and the CB1-selective antagonist *N*-piperidino-5-(4-chlorophenyl)-1-(2,4-dichlorophenyl)-4-methylpyrazole-3-carboxamide (SR141716A) on food intake in rats; and (3) the effects of sensory deafferentation on the modulation of feeding by cannabinoids. Food deprivation produced a sevenfold increase in anandamide content in the small intestine but not in the brain or stomach. Refeeding normalized intestinal anandamide levels. Peripheral but not central administration of anandamide or WIN55,212-2 promoted hyperphagia in partially satiated rats. Similarly, peripheral but not central administration of SR141716A reduced food intake. Capsaicin deafferentation abolished the peripheral effects of both cannabinoid agonists and antagonists, suggesting that these agents modulate food intake by acting on CB1 receptors located on capsaicin-sensitive sensory terminals. Oleoylethanolamide, a noncannabinoid fatty ethanolamide that acts peripherally, prevented hyperphagia induced by the endogenous cannabinoid anandamide. Pretreatment with SR141716A enhanced the inhibition of feeding induced by intraperitoneal administration of oleoylethanolamide. The results reveal an unexpected role for peripheral CB1 receptors in the regulation of feeding.

Key words: anandamide; cannabinoid; capsaicin; cholecystokinin; food intake; rat; satiety; SR141716A; WIN55,212-2

Historical descriptions of the stimulatory effects of *Cannabis sativa* on feeding are now explained by the ability of its psychoactive constituent Δ^9-tetrahydrocannabinol (THC) to interact with CB1 cannabinoid receptors (Williams et al., 1998; Kunos and Batkai, 2001). Both THC and the endogenous cannabinoid anandamide (AEA) (Devane et al., 1992) promote overeating in partially satiated rats (Williams and Kirkham, 1999). Moreover, THC increases fat intake in laboratory animals and stimulates appetite in humans (Sacks et al., 1990; Williams et al., 1998; Koch, 2001). The selective CB1 receptor antagonist SR141716A (Rinaldi-Carmona et al., 1995) counteracts these effects and, when administered alone, decreases standard chow intake and caloric consumption (i.e., sucrose or ethanol intake), presumably by antagonizing the actions of endogenously released endocannabinoids such as anandamide and 2-arachidonoylglycerol (Arnone et al., 1997; Colombo et al., 1998; Simiand et al, 1998; Kirkham and Williams, 2001; Rowland et al., 2001). These results suggest that endocannabinoid substances may play a role in the promotion of food intake, possibly by delaying satiety.

It is generally thought that the hyperphagic actions of cannabinoids are mediated by CB1 receptors located in brain circuits involved in the regulation of motivated behaviors (Herkenham et al., 1991). Thus, infusions of anandamide in the ventromedial hypothalamus were shown to promote hyperphagia (Jamshidi and Taylor, 2001), whereas the anorectic effects of leptin were found to be associated with a decrease in hypothalamic anandamide levels (Di Marzo et al., 2001). Nevertheless, evidence suggests that cannabinoids also may promote feeding by acting at peripheral sites. Indeed, CB1 receptors are found on nerve terminals innervating the gastrointestinal tract (Croci et al., 1998; Hohmann and Herkenham, 1999), which are known to be involved in mediating satiety signals that originated in the gut (Reidelberger, 1992).

To test this hypothesis, in the present study we have examined (1) the impact of feeding on intestinal anandamide accumulation, (2) the effects of central versus peripheral systemic administration of cannabinoid receptor agonists on feeding behavior, and (3) the effects of sensory deafferentation on cannabinoid-induced hyperphagia.

MATERIALS AND METHODS

Animals. Male Wistar rats (350 ± 50 gm) were housed individually with food and water available *ad libitum*, except when restriction was required. All animal procedures met the National Institutes of Health guidelines

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for the care and use of laboratory animals and the European Communities directive 86/609/EEC regulating animal research.

Surgery. For intracerebroventricular injections, stainless steel guide cannulas aimed at the lateral ventricle were implanted in the rats. The animals were anesthetized with equithesin and placed in a David Kopf Instruments (Tujunga, CA) stereotaxic instrument with the incisor bar set at 5 mm above the interaural line. A guide cannula (7 mm, 23 gauge) was secured to the skull by using two stainless steel screws and dental cement and was closed with 30 gauge obturators (Navarro et al., 1996; Rodríguez de Fonseca et al., 2001). The implantation coordinates were 0.6 mm posterior to bregma, ±2.0 mm lateral, and 3.2 mm below the surface of the skull. These coordinates placed the cannula 1 mm above the ventricle. After a 7 d postsurgical recovery period, cannula patency was confirmed by gravity flow of isotonic saline through an 8-mm-long, 30 gauge injector inserted within the guide to 1 mm beyond its tip. This procedure allowed the animals to become familiar with the injection technique.

Chemicals. Capsaicin was purchased from Sigma (St. Louis, MO), and cholecystokinin octapeptide sulfated (CCK-8), *R*-(+)-(2,3-dihydro-5-methyl-3-[(4-morpholinyl)methyl]pyrol[1,2,3-de]-1,4-benzoxazin-6-yl)(1-naphthalenyl) methanone monomethanesulfonate (WIN55, 212-2), and 1,4-dihydro-3-(1,2,3,6-tetrahydro-4-pyridinyl)-5H-pyrrolo[3,2-b]pyridin-5-one (CP93129) were obtained from Tocris Cookson (Bristol, UK). *N*-piperidino-5-(4-chlorophenyl)-1-(2,4-dichlorophenyl)-4-methylpyrazole-3-carboxamide (SR141716A) was a gift from Sanofi Recherche (Montpellier, France). Anandamide and oleoylethanolamide (OEA) were synthesized in the laboratory (Giuffrida et al., 2000). Capsaicin was dissolved in 5% Tween 80, 5% propyleneglycol, and 90% saline. All other drugs were dissolved in dimethylsulfoxide (DMSO) and administered in 70% DMSO in sterile saline.

HPLC/mass spectrometry analyses. Anandamide was solvent-extracted from tissues, fractionated by column chromatography, and quantified by HPLC/mass spectrometry with an isotope dilution method, as described previously (Giuffrida et al., 2000).

Drug treatments. Capsaicin was administered subcutaneously (12.5 mg/ml) (Kaneko et al., 1998) in rats anesthetized with ethyl ether. The total dose of capsaicin (125 mg/kg) was divided into three injections (25 mg/kg in the morning and 50 mg/kg in the afternoon, and then 50 mg/kg on the next day). Control rats received vehicle injections. Experiments were performed 10 d after capsaicin treatment in rats that (1) had lost the corneal chemosensory reflex (eye wiping for 1–3 min after application of 0.1% ammonium hydroxide into one eye), and (2) showed enhanced water intake 10 d after capsaicin treatment. Water intake (in milliliters per 4 hr) was as follows: vehicle, 13.6 ± 1.4; capsaicin rats, 24.0 ± 1.9, $p < 0.01$ ($n = 12$). The associated food intake (grams per 4 hr) was as follows: vehicle, 11.9 ± 0.6; capsaicin rats, 10.9 ± 0.7.

Drugs were administered by intraperitoneal injection 15 min before food presentation in a volume of 1 ml/kg. For intracerebroventricular administration, the obturator was removed from the guide cannula and an 8 mm injector (30 gauge stainless steel tubing) that was connected to 70 cm of calibrated polyethylene-10 tubing was lowered into the ventricle. The tubing was then raised until flow began, and 5 μl of drug solution was infused over a 30–60 sec period. The injector was left in the guide cannula for an additional 30 sec and then removed. The stylet was immediately replaced. Animals were tested 5 min after injections. The intracerebroventricular cannula placements were evaluated after each experiment by dye injection. Only rats with proper intracerebroventricular placements were included in the data analysis.

Food intake studies. The effects of drugs on feeding behavior were analyzed in animals deprived of food for 24 hr and habituated to handling (Navarro et al., 1996; Rodríguez de Fonseca et al., 2001) or in partially satiated animals (i.e., 24 hr food-deprived animals allowed to eat for 60 min before drug testing) (Williams et al., 1998). To this end, 48 hr before testing, the bedding material was removed from the cage and a small can containing food pellets was placed inside the cage for 4 hr. The animals were then food-deprived for 24 hr, with access to water *ad libitum*. The animals were returned to their home cage 15 min after drug administration; there, a can with a measured amount of food (usually 30–40 gm) and a bottle containing 250 ml of fresh water were placed. Food pellets and food spillage were weighed at 60, 120, and 240 min after starting the test, and the amount of food eaten was recorded. At the end of the test, the amount of water consumed was also measured. For partial satiation of animals, 24 hr food-deprived rats were allowed to eat from the can for 1 hr. The can was replaced and intake was recorded. Fifteen minutes after drug injections, the food was again presented, and the amount consumed was recorded hourly for the next 4 hr.

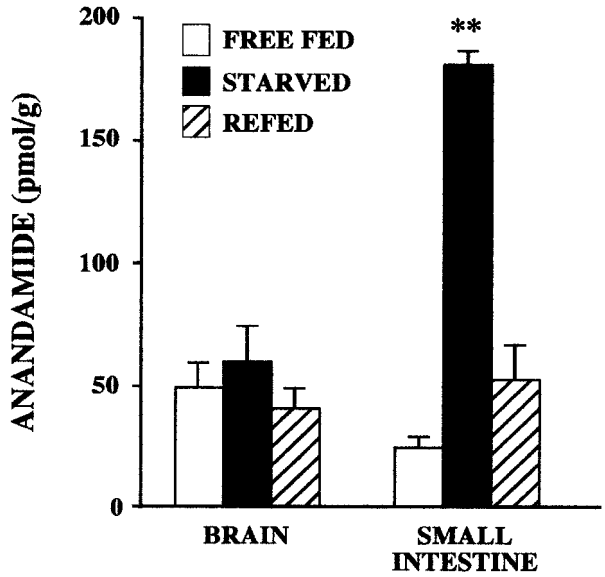

Figure 1. Effects of starvation and feeding on anandamide levels in the brain and small intestine. Starvation promoted the accumulation of anandamide in the small intestine. Data are the means ± SEM of at least five determinations per group. $^{**}p < 0.01$, fed versus starved group; Newman–Keuls.

Open-field test. Motor behaviors in the open field were studied in an opaque open field ($100 \times 10^0 \times 40$ cm) as described previously (Beltramo et al., 2000). The field was illuminated using a ceiling halogen lamp regulated to yield 350 lux at the center of the field. Rats were habituated to the field for 10 min the day before testing. On the experimental day, the animals were treated and placed in the center of the field, and locomotor activity (number of lines crossed) and rearing and grooming behavior (number of rearings and time spent in the center of the field) were scored for 5 min at 5, 30, 60, and 120 min after drug injection. Behavior was scored by trained observers who were unaware of the experimental conditions.

Statistics. Statistical significance was assessed by one-way or multifactorial ANOVA, as required. After a significant *F* value, *post hoc* analysis (Student–Newman–Keuls test) was performed. Calculations were done using the BMDP statistical package (SPSS Inc., Chicago, IL).

RESULTS

Effects of feeding on anandamide levels

We first investigated whether starvation and refeeding affect anandamide content in intestinal tissue, where various intrinsic signals modulating food intake, such as CCK (Reidelberger, 1992) and OEA (Rodríguez de Fonseca et al., 2001), are generated. As shown in Figure 1, food deprivation (24 hr) was accompanied by a sevenfold increase in anandamide content in the small intestine, an effect that was reversed on refeeding. In contrast, no such increase was observed in brain or stomach tissues (Fig. 1) (data not shown). The change in intestinal anandamide did not result from the inhibition of anandamide degradation. Indeed, fatty acid amidohydrolase activity, which catalyzes the deactivating hydrolysis of anandamide, was not affected by the feeding status (data not shown).

Central cannabinoid administration does not affect food intake

As reported previously (Williams et al., 1998), intraperitoneal administrations of the endogenous cannabinoid anandamide or the synthetic cannabinoid agonist WIN55,212-2 (0.1–2 mg/kg)

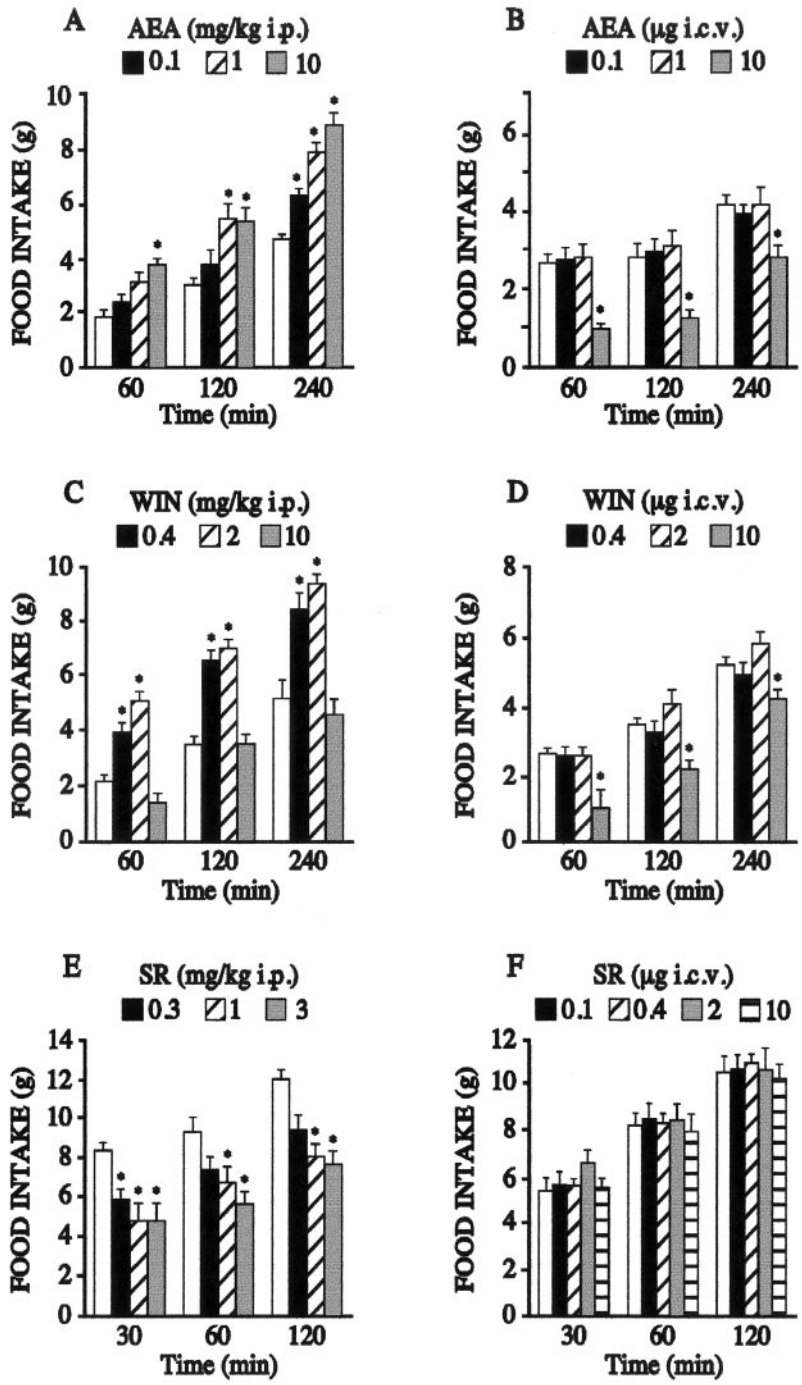

Figure 2. Peripheral effects of cannabinoids on food intake. *A*, AEA elicited hyperphagia in partially satiated animals when injected after a 60 min meal. *B*, Anandamide has no effect after intracerebroventricular administration. *C*, Acute intraperitoneal injection of WIN55,212-2 (*WIN*) promoted hyperphagia in partially satiated animals. *D*, WIN55,212-2 had no effect after intracerebroventricular injection. *E*, Acute intraperitoneal injection of SR141716A (*SR*) reduced food intake in food-deprived rats during the 240 min testing period. *F*, The intracerebroventricular administration of SR141716A did not affect food intake in food-deprived animals. Data are means ± SEM of at least 10 determinations per group. $^*p < 0.01$ versus vehicle-treated group (*white bars*); Newman–Keuls.

had no effect on food intake in food-deprived rats (data not shown). Nevertheless, when administered to partially satiated animals, these drugs elicited significant and prolonged hyperphagia (Fig. 2*A,C*). At a dose of 10 mg/kg, WIN55,212-2 also produced profound immobility, which interfered with feeding behavior (Fig. 2*C*). In contrast, central injections of anandamide and WIN55,212-2 had no effect on feeding, except at the highest dose (10 μg), which resulted in motor impairment (Fig. 2*B,D*) (data not shown).

After systemic administration, the selective CB1 antagonist SR141716A elicited a dose-dependent reduction of food intake in both 24 hr food-deprived rats (Fig. 2*E*) and partially satiated rats (data not shown). However, the drug had no effect after central administration (Fig. 2*F*). Regardless of the administration route, SR141716A reduced rearing behavior and increased grooming (Table 1) in the open field, indicating that the drug effectively interacted with brain cannabinoid receptors (Navarro et al., 1997). The results suggest that the hyperphagia evoked by cannabinoid receptor agonists, as well as the anorexia elicited by the CB1 antagonist SR141716A, may be dependent on the interaction of these agents with peripheral cannabinoid receptors. Additional experiments were done with the CB2 receptor antagonist *N*-[(1*S*)-endo-1,3,3-trimethyl bicyclo[2.2.1]heptan-2-yl]-5-(4-chloro-3-methylphenyl)-1-(4-methylbenzyl)-pyrazole-3-carboxamide (SR144528). As reported previously (Rodríguez de Fonseca et al., 2001), blockade of CB2 receptors did not affect feeding. Moreover, pretreat-

Table 1. Effects of either central (intracerebroventricular) or peripheral (intraperitoneal) administration of vehicle and the CBl cannabinoid receptor antagonist SR141716A on motor behaviors measured in the open field

	Crossings	Rearings	Time spent grooming
Vehicle, (intracerebroventricular)	160.6 ± 20.9	46.0 ± 6.2	27.8 ± 8.5
SR141716A, (5 μg/5 μl, i.c.v.)	120.8 ± 18	20.2 ± 5.9*	84.5 ± 14.5*
Vehicle, (intraperitoneal)	143.3 ± 0.7	30.9 ± 4.1	8.8 ± 4.2
SR141716A, (3 mg/kg, i.p.)	156.9 ± 24.7	9.0 ± 2.7*	25.4 ± 10.6*

*$p < 0.05$; Newman–Keuls.

ment with SR144528 did not affect WIN55,212-2-induced hyperphagia (data not shown).

Sensory deafferentation prevents cannabinoid effects on feeding

Treatment with the neurotoxin capsaicin abolished the anorexic response elicited by the peptide CCK-8 (10 μg/kg, i.p.) but not that induced by the centrally acting 5-HT-1_B agonist CP93129 (1 mg/kg, i.p.) (Fig. 3*A*), indicating that sensory terminals innervating the gut had been destroyed. The treatment also resulted in a loss of the hyperphagic effects of either WIN55,212-2 (2 mg/kg, i.p.) (Fig. 3*B*) or anandamide (2 mg/kg, i.p.) (data not shown) and of the hypophagic effects of SR141716A (3 mg/kg, i.p.) (Fig. 3*C*).

SR141716A and OEA synergistically inhibit feeding

The small intestine produces both anandamide, which stimulates food intake (Williams and Kirkham, 1999), and OEA, which inhibits food intake by acting on peripheral sensory fibers (Rodríguez de Fonseca et al., 2001). However, the intestinal levels of the two compounds appear to be reciprocally regulated. Thus, the OEA content decreases (Rodríguez de Fonseca et al., 2001), whereas the anandamide content increases (present study) during starvation. To examine the possible interaction of these fatty acid ethanolamides on feeding, we studied (1) whether OEA blocks AEA-induced hyperphagia and (2) whether blockade of CB1 receptors with a low, subthreshold dose of SR141716A potentiates the inhibitory actions of OEA on food intake. The results, illustrated in Figure 4*A*, indicate that pretreatment with OEA inhibits AEA-induced hyperphagia in partially satiated rats, whereas SR141716A and OEA act synergistically to decrease eating in food-deprived animals (Fig. 4*B*). The effects were observed during the 240 min period of testing. The inhibitory actions of combined SR141716A and OEA lasted for at least 24 hr (data not shown), a prolonged effect that these drugs do not elicit separately.

DISCUSSION

The present results suggest, first, that systemically administered cannabinoid agents (both agonists and antagonists) affect food intake predominantly by engaging peripheral CB1 receptors localized to capsaicin-sensitive sensory terminals and, second, that intestinal anandamide is a relevant signal for the regulation of feeding.

Two observations support the idea that cannabinoid agents modulate feeding through a peripheral mechanism. First, the lack of effect of central administration of cannabinoid antagonists such as SR14116A (present data) and 6-iodo-2-methyl-1-[2-(4-morpholinyl)ethyl]-[1H]-indol-3-yl (4-methoxyphenyl) methanone

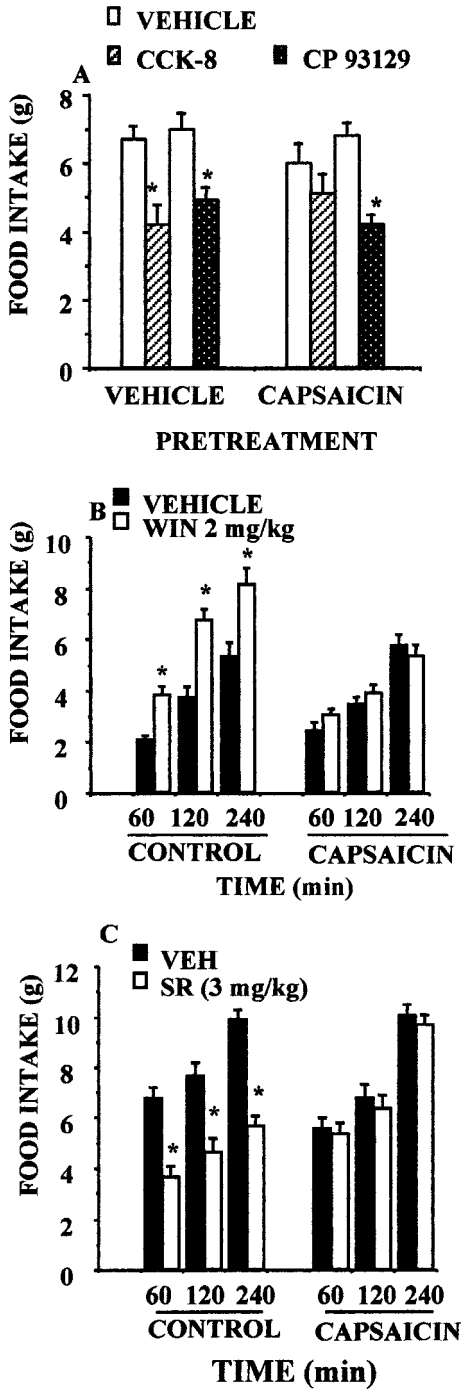

Figure 3. *A*, Capsaicin treatment abolished the anorexic effect of CCK-8, which acts peripherally, but not that of the 5-HT-1_B agonist CP93129, which acts centrally. *B*, WIN55,212-2 (*WIN*) did not produce hyperphagia in capsaicin-treated rats. *C*, Capsaicin treatment abolished the reduction of food intake elicited by SR141716A (*SR*) in food-deprived rats. *VEH*, Vehicle. Data are the means ± SEM of at least 10 determinations per group. *$p < 0.01$ versus vehicle-treated group; Newman–Keuls.

(Koch and Werner, 2000) on food intake in food-deprived animals and, second, the ability of capsaicin-induced deafferentation to prevent changes in feeding elicited by the peripheral administration of cannabinoid drugs. Moreover, the similar pattern of expression of the early gene c-*fos* on hypothalamic and brainstem areas regulating food intake after both the peripheral adminis-

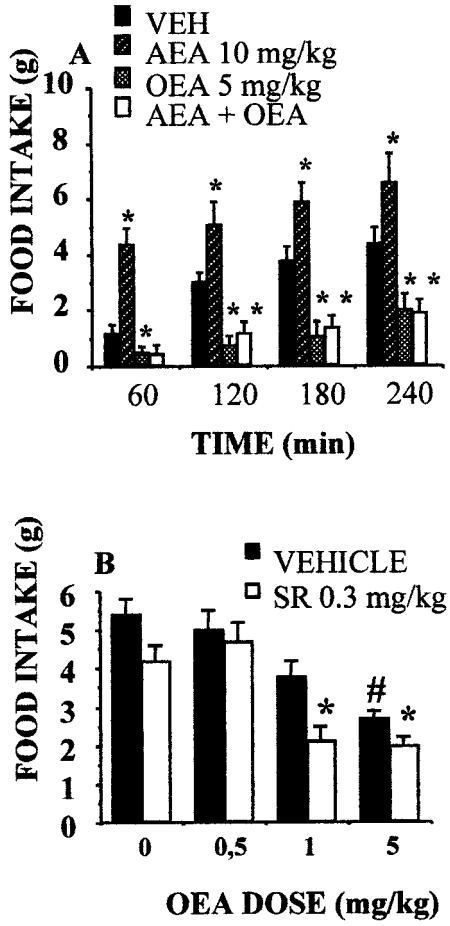

Figure 4. *A*, OEA blocked hyperphagia elicited by AEA (10 mg/kg) when injected 30 min before the endogenous cannabinoid in partially satiated rats. *VEH*, Vehicle. *B*, SR141716A (*SR*) potentiates the feeding suppression induced by OEA. The effects of a subthreshold dose of SR141716A (0.3 mg/kg, i.p.) on OEA (0.5, 1, and 5 mg/kg, i.p.) induced feeding suppression on food intake in 24 hr food-deprived rats 1 hr after the injection of OEA. Either vehicle (*open bars*) or SR141716A (*black bars*) was injected 30 min before OEA. Similar results were obtained 4 and 24 hr after the administration of drugs (data not shown). Data are the means ± SEM of at least 10 determinations per group. $^{*}p < 0.01$ versus vehicle-treated group; Newman–Keuls. $^{\#}p < 0.01$ versus 0 dose.

tration of either CB1 agonists and antagonists (Rodríguez de Fonseca et al., 1997) and the acute administration of peripherally acting satiety modulators such as gastrointestinal hormones (Turton et al., 1996) or feeding inhibitors such as OEA (Rodríguez de Fonseca et al., 2001) further support the peripheral actions of cannabinoids on food intake. Finally, the fact that the CB1 receptor antagonist SR141716A was active only after intraperitoneal or oral administration but not after subcutaneous injection (Rowland et al., 2001) further supports this hypothesis. These results do exclude the possibility that peripheral anandamide also modulates feeding by acting on specific hypothalamic areas involved in caloric homeostasis (such as the ventromedial, arcuate, or paraventricular hypothalamic nuclei) (Di Marzo et al., 2001; Jamshidi and Taylor, 2001). However, they do suggest that the predominant effects of systemically administered SR141716A are mediated by peripheral CB1 receptors, which may thus represent a potential target for anorexic agents.

The concentration of anandamide in intestinal tissue increases during food deprivation, reaching levels that are threefold greater than those needed to half maximally activate CB1 receptors (Devane et al., 1992). This surge in anandamide levels, the mechanism of which is unknown, may serve as a short-range hunger signal to promote feeding. This idea is supported by the ability of SR141716A to reduce food intake after systemic but not central administration. Locally produced anandamide also may be involved in the regulation of gastric emptying and intestinal peristalsis, two processes that are inhibited by this endocannabinoid (Calignano et al., 1997; Izzo et al., 1999). Thus, intestinal anandamide appears to serve as an integrative signal that concomitantly regulates food intake and gastrointestinal motility.

The predominant peripheral component of feeding suppression induced by SR141716A led us to analyze whether the modulation of food intake derived from CB1 receptor stimulation/blockade may interact with that produced by the noncannabinoid anandamide analog OEA (Rodríguez de Fonseca et al., 2001). Our results indicate that the hyperphagic effects elicited by CB1 receptor stimulation were counteracted by the administration of OEA, whereas CB1 receptor blockade potentiates the suppression of feeding evoked by OEA. Because the intestinal levels of anandamide and OEA are inversely correlated (OEA increases after a meal, which results in a decrease in anandamide levels; anandamide increases during starvation, associated with a profound decrease in intestinal OEA) (Rodríguez de Fonseca et al., 2001; and present data), it is tempting to speculate that both compounds act in a coordinated manner to control feeding responses through their opposing actions on sensory nerve terminals within the gut.

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