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Anandamide, an Endogenous Cannabinoid, Has a Very Low Physical Dependence Potential[1]

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ABSTRACT

Using N-(piperidin-1-yl)-5-(4-chlorophenyl)-1-(2,4-dichlorophenyl)-4-methyl-1H-pyrazole-3-carboxamide · HCl (SR 141716A), a cannabinoid antagonist, several investigators (de-Fonseca *et al.*, 1997; Aceto *et al.*, 1995, 1996; Tsou *et al.*, 1995) demonstrated physical dependence on THC [Δ^9-tetrahydrocannabinol]. This demonstration prompted us to determine whether anandamide, an endogenous cannabinoid agonist, would also produce physical dependence. A low-dose regimen (10, 20, 40 and 40) or a high-dose regimen (25, 50, 100 and 100) expressed as mg/kg/24 hr was infused i.p. on a continuous basis, from days 1 through 4, respectively. During the infusion, especially at the high-dose regimen, the rats became immobile and developed eyelid ptosis. Abrupt discontinuation of anandamide did not elicit rebound behavioral activity. Neither arachidonic acid, a precursor and metabolite of anandamide (50, 100, 200 and 200 mg/kg/24 hr on days 1 through 4, respectively), nor 2-Me-F-AN [2-methylarachidonyl-(2′-fluoroethyl)-amide], a metabolically stable analog of anandamide (5, 10, 20 and 20 mg/kg/24 hr for 4 days, respectively), had remarkable effects. Notably, groups pretreated with anandamide or 2-Me-F-AN and challenged with SR 141716A did not show significantly elevated behavioral scores when compared with SR 141716A controls. On the other hand, nearly all groups receiving SR 141716A showed significant activation of these behaviors compared with vehicle controls, which suggests that this cannabinoid antagonist itself was activating behavior. We concluded that anandamide has little if any capacity for physical dependence. The finding that SR 141716A activated behavior supports the hypothesis that the cannabimimetic system exerts a depressant effect in the CNS.

The identification of the major active constituent of *Cannabis sativa*, THC, by Gaoni and Mechoulam in 1964, followed by the characterization of the cannabinoid receptor (Howlett *et al.*, 1988; Devane *et al.*, 1988; Matsuda *et al.*, 1990), provided a solid foundation and opened new perspectives for the study of this neurochemical system. Additionally, the isolation of an endogenous ligand designated anandamide (Devane *et al.*, 1992), descriptions of its synthetic and metabolic pathways (Deutsch and Chin, 1993; Devane and Axelrod, 1994) and subsequent synthesis of a competitive antagonist, SR 141716A (Rinaldi-Carmona *et al.*, 1994), furnished compelling evidence for the existence of an endocannabinergic system.

Anandamide and THC have pharmacological properties in common (see review by Di Marzo and De Petrocellis, 1997). For example, both substances produced hypomotility, hypothermia, antinociception and catalepsy in rodents. Based on the results of studies on chemical structure and biological activity, Martin *et al.* (1987) showed that THC derivatives that were active on this tetrad of tests were likely to be psychoactive cannabinoids. Anandamide also produced inhibitory effects on memory (Lichtman *et al.*, 1995), inhibited forskolin-stimulated adenylyl cyclase activity (Felder *et al.*, 1993) and prolactin release (Romero *et al.*, 1994) and stimulated adrenocorticotropic hormone discharge (Weidenfeld *et al.*, 1994). Regulatory effects on dopamine (Schlicker *et al.*, 1996) and GABA neurotransmission (Romero *et al.*, 1995), as well as similar effects on reproductive function (Schuel *et al.*, 1994) and the immune system (Schwarz *et al.*, 1994), were reported.

In terms of the pharmacological determinants of dependence, there is evidence that THC causes tolerance and physical dependence in humans and animals (see reviews by Altman *et al.*, 1996; Pertwee, 1991; Jones and Benowitz, 1976; and studies by de Fonseca *et al.*, 1997; Aceto *et al.*, 1995, 1996; Tsou *et al.*, 1995). Other investigators demonstrated cross-tolerance among THC, anandamide and other cannabimimetics for their inhibitory effects on the twitch response in the vas deferens but not for their hypothermic effects (Pertwee *et al.*, 1993).

The present study was designed primarily to address the

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ABBREVIATIONS: SR 141716A, N-(piperidin-1-yl)-5-(4-chlorophenyl)-1-(2,4-dichloro-phenyl)-4-methyl-1H-pyrazole-3-carboxamide · HCl; THC, Δ^9-tetrahydrocannabinol; 2-Me-F-AN, 2-methylarachidonyl-(2′-fluoroethyl)-amide; ANOVA, analysis of variance.

question of physical dependence on anandamide and to explore the involvement of arachidonic acid, its possible precursor (Devane and Axelrod, 1994) or metabolite (Di Marzo and De Petrocellis, 1997). Because anandamide is rapidly metabolized, we decided to administer this ligand by continuous infusion. Experimental conditions were kept as close as possible to those employed in the THC studies in this laboratory (Aceto *et al.*, 1995, 1996). We also wished to explore further the observation in our laboratory (Compton *et al.*, 1996) and that of others (de Fonseca *et al.*, 1997) that SR 141716A activates behavior. Reconfirmation of this behavioral activation by SR 141716A would support the proposal that anandamide mediates sleep (Mechoulam *et al.*, 1997).

Materials and Methods

Subjects. All rats received care in accordance with "Guide for the Care and Use of Laboratory Animals," DHHS Publication, revised, 1996. The facilities are certified by the American Association for the Accreditation of Laboratory Care. These studies were approved by the Institutional Animal Care and Use Committee at Virginia Commonwealth University.

Continuous-infusion studies in rats. The experimental procedure was described earlier (Aceto *et al.*, 1996) in a similar study involving THC. Briefly, adult male Sprague-Dawley rats were purchased from Dominion Laboratories (Dublin, VA). Upon arrival, the rats were examined by a licensed veterinarian and placed in quarantine. They were in the weight range of 250 to 280 g when assigned to a study. Once selected, they were housed individually in stainless steel cages. The vivarium was temperature- and humidity-controlled with alternating light-dark cycles (lights on at 06:00 hr and off at 24:00 hr).

The method described by Teiger (1974) was used, with the modifications indicated below. The rats were acclimated to their new surroundings for at least 3 days before i.p. cannulas were implanted. After they were anesthetized with pentobarbital (45 mg/kg i.p.), the lateral side of the lower left abdomen and the back of the neck were shaved, and the exposed skin was cleansed with Povidone-Iodine Solution (Redi-Product, WV). Then each rat was fitted with a cannula (PE90 tubing, Clay Adams, NJ). The cannula passed s.c. from the nape of the neck to the lateral side of the lower abdomen. The peritoneal end of the cannula was enclosed in silastic tubing to prevent foreign body reaction. It was introduced into the peritoneal cavity through a stab-wound entry site. The cannula was secured with sutures at both sites. Next, each rat was fitted with a harness. The harness consisted of a flat stainless steel plate fitted with a shoulder collar, a narrow strip of Velcro and a spring coil. The collar was passed over the head of the rat, and the harness was secured by means of the strip of Velcro, which girdled the chest. The cannula passed through the harness and spring coil. The harness and spring coil protected the cannula. The other end of the enclosed cannula was then attached to a flow-through swivel (Instech Lab., Horsham, PA). The swivel allowed the rat to move about in the cage and to eat and drink normally. An infusion pump (Harvard Apparatus, South Natick, MA, Model-945) delivered the solutions to the swivel in a volume of 8 ml every 24 hr. Fresh solutions were prepared daily after the rats were weighed.

Behavioral ratings. During the infusion of anandamide, the rats were observed daily for 1 hr for overt behavioral signs. In addition, they were observed for withdrawal signs for 1 hr after the abrupt termination of the infusion (pre-challenge) and for 1 hr after the injection of SR 141716A or vehicle (post-challenge). In the abrupt-withdrawal tests, behavior was noted daily for 1 hr. The behavioral signs designated were scratching, wet-dog shakes, head shakes, paw shakes, facial rubbing, chewing, tongue rolling, retropulsion or walking backward, immobility and ptosis (at least 50% closure of both eyelids). These were scored if observed. The signs wet-dog shake and facial rubbing were quantified. All other signs were simply scored once during an observation period. A trained observer who was "blind" regarding the treatment regimens was used to record the behavioral signs.

Statistical analysis. Statistical analysis of the quantified data was performed by ANOVA. *Post-hoc* comparisons were appraised using the conservative Bonferroni/Dunn test. In all cases, significance was at least $P < .05$. The StatView statistical package (Brainpower, Inc., Agoura Hills, CA) was utilized for these analyses.

Chemical supplies. Anandamide and 2-Me-F-AN were synthesized at Organix, Inc. (Woburn, MA), and SR 141716A was prepared at Pfizer Central Research (Groton, CT). THC and naloxone · HCl were obtained from the National Institute on Drug Abuse. Alkamuls EL-620, formerly Emulphor EL-620 or polyoxyethylated castor oil (Rhône-Poulenc, Cranbury, NJ), Encapsin HPB or hydroxypropyl-b-cyclodextrin (Carestar, Hammond, IN), sterile saline (Baxter Healthcare Corp., Deerfield, IL), arachidonic acid (Nu-Chek-Prep, Inc., Elysian, MN) and other necessary supplies were obtained commercially. Anandamide, 2-Me-F-AN and arachidonic acid were first dissolved in a minimal amount of ethanol and added to an aqueous solution of hydroxypropyl-b-cyclodextrin. SR 141716A was dissolved in 1:1:18 (Alkamuls/ethanol/sterile saline) vehicle.

Results

Chronic exposure to anandamide. In a preliminary study (Aceto *et al.*, 1994), we reported that continuous i.p. exposure to anandamide for 4 days produced immobility, body weight loss and eyelid ptosis. The dose regimen, expressed as mg/kg/24 hr, was 50 on day 1, 100 on day 2, 200 on day 3 and 100 on day 4. The dose was reduced on day 4 because one rat died on day 3. Irritability expressed as vocalizing when touched and heightened startle to a gentle puff of air were observed 2 and 3 days after anandamide was abruptly discontinued. Bearing in mind that this dose regimen was in the toxic range, we tentatively concluded at that time that withdrawal after chronic and continuous exposure to anandamide was associated with some rebound activity.

SR 141716A challenge to anandamide-infused rats. The initial anandamide study provided guidance in choosing infusion regimens for the subsequent anandamide dependence studies. Rats were infused with one of two anandamide regimens. The low-dose regimen was initiated using a dose of 10 mg/kg/24 hr of anandamide on day 1. The dose was doubled on day 2, doubled again on day 3 and maintained at the day-3 level on day 4. The high-dose regimen, expressed as mg/kg/24 hr, was 25 on day 1, 50 on day 2 and 100 on days 3 and 4. The SR 141716A doses were based on those used in the THC studies (Aceto *et al.*, 1995, 1996). A synopsis of this experiment is shown in table 1. For the most part, during the infusion, the rats receiving anandamide became immobile and developed eyelid ptosis, especially at the higher-dose regimen. The other signs observed were scratching, wet-dog shakes, paw shakes, front paw treading, retropulsion, head shakes, tongue rolling, chewing and facial rubbing with front paws. These were associated, on the whole, with SR 141716A challenge in anandamide- and vehicle-treated rats. Wet-dog shakes and facial rubbing were enumerated. These two signs were also quantified in the SR 141716A precipitated-withdrawal studies in THC-dependent rats (Aceto *et al.*, 1995, 1996).

Three anandamide infusion experiments were conducted, and the data were appropriately collated and analyzed as

TABLE 1
Synopsis of anandamide[a] pretreatment dose regimens (mg/kg/24 hr) infused i.p. for 4 days or its vehicle (8 ml/24 hr i.p. for 4 days) and the acute challenge doses of SR 141716A (mg/kg i.p.) or its vehicle[b] (ml/kg i.p.)

Pretreatment Regimen	Day				Challenge Injection (mg/kg)	Number of Subjects[c]
	1	2	3	4		
Anandamide High	25	50	100	100	SR 141716A (10)	11
					Vehicle	11
Anandamide Low	10	20	40	40	SR 141716A (10)	16
					SR 141716A (5)	5
					Vehicle	10
Vehicle					SR 141716A (10)	15
					SR 141716A (5)	15
					Vehicle	15

[a] Anandamide was dissolved in a minimal amount of ethanol and added to an aqueous solution of 5% to 20% hydroxypropyl-β-cyclodextrin (depending on the concentration of anandamide).
[b] SR 141716A was dissolved in 1:1:18 (emulphor/ethanol/sterile saline).
[c] Combined number of subjects in three separate experiments.

indicated below. ANOVA of the pretreatment data for the sign wet-dog shakes in figure 1A indicated no significant differences among treatments ($F = 0.842$, P = .56). ANOVA of the post-treatment challenge shown in figure 1B revealed statistically significant differences among treatments ($F = 3.551$, P = .0022). Comparing all groups with the vehicle-vehicle group reveals that the vehicle-SR5 and An.low-SR10 groups are significantly different ($P < .05$) from the vehicle-vehicle group. Because the vehicle-SR10 group is not significantly different from the vehicle-vehicle group, we can conclude that SR produced more wet-dog shakes in the An.low group than in the vehicle group. Breaking down the analysis to compare only those groups receiving either SR5 or SR10 yielded no other significant differences. Therefore, the important findings are that a dose of 5 mg/kg of SR 141716A produced more wet-dog shakes in the vehicle-treated animals than in the anandamide-treated rats, whereas a higher dose (10 mg/kg) of SR 141716A elicited significantly more wet-dogs shakes in the rats receiving the low-dose regimen of anandamide (fig. 1B).

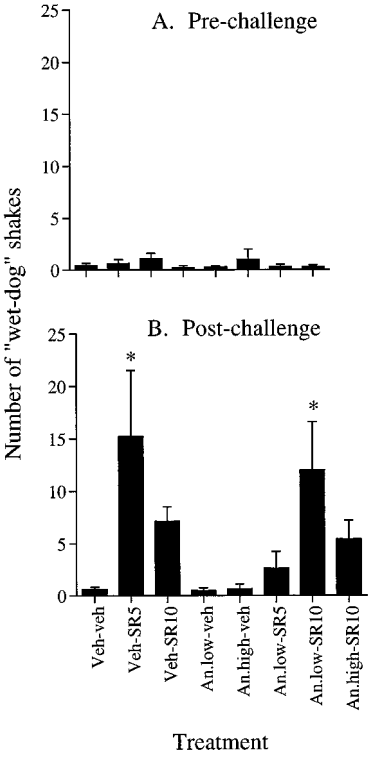

Fig. 1. SR 141716A-elicited wet-dog shakes in rats chronically infused with anandamide or vehicle. Rats were infused with a low- or high-treatment regimen of anandamide for 4 days. At the end of this infusion period, rats were observed for 1 hr before (pre-challenge) and after (post-challenge) challenge either with vehicle or with one of two doses (5 or 10 mg/kg) of SR 141716A. The group names on the abscissa signify infusion regimen/challenge. Additional details of the experimental design, including the number of subjects per treatment regimen, are presented in table 1. The data are expressed as means ± S.E. * Significantly different from vehicle-vehicle group ($P < .05$).

Concerning the sign designated facial rubbing, no significant differences were detected among treatments ($F = 0.663$, P = .703) by ANOVA in the 1-hr period before antagonist challenge, as shown in figure 2A. However, in the post-treatment phase illustrated in figure 2B, significant differences among treatments were calculated ($F = 9.088$, P = .0001). All groups except An.low-veh and An.high-veh are statistically different from Veh-veh. It is clear that neither dose of SR 141716A produced greater rubbing behavior in the anandamide-treated groups.

Anandamide abrupt withdrawal and SR 141716A challenge. In one of the anandamide-infusion experiments, separate groups of rats were followed for 144 hr after the SR 141716A or vehicle challenge. The results are shown in figure 3. ANOVA repeated-measures analysis indicated that statistically significant treatment differences existed for wet-dog shakes ($F = 2.112$, P = .002) and for facial rubbing ($F = 4.433$, P = .0001). *Post-hoc* analysis revealed no statistically significant differences among the treatment groups during the period 24 to 144 hr for either sign. These results provided strong evidence that abrupt withdrawal of anandamide after chronic administration was not associated with rebound increases for the signs wet-dog shakes and facial rubbing. However, evaluation of the scores obtained during the 1-hr observation period immediately after SR 141716A challenge revealed statistically significant differences. For the sign wet-dog shakes, ANOVA yielded $F = 4.407$ (P = .0058). *Post-hoc* analysis showed that the scores of the vehicle-pretreated and SR 141716A-challenged groups were significantly different from those of the vehicle-vehicle group. The scores in the groups receiving the high- and low-dose regimes of anandamide and challenged with SR 141716A were elevated, but this effect did not achieve statistical significance.

For the sign facial rubbing, significant differences among treatment regimens were documented only for the 1-hr period after challenge SR 141716A ($F = 9.379$, P = .05). *Post-hoc* analysis indicated that the scores of the SR 141716A-

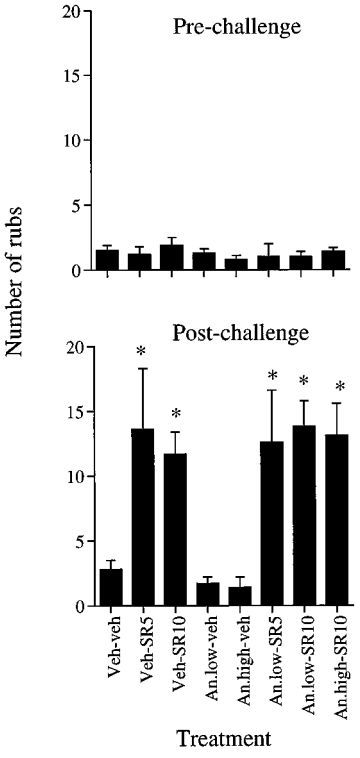

Fig. 2. SR 141716A-elicited facial rubbing behavior in rats chronically infused with anandamide or vehicle. Rats were infused with a low- or high-treatment regimen of anandamide for 4 days. At the end of this infusion period, rats were observed for 1 hr before (pre-challenge) and after (post-challenge) challenge either with vehicle or with one of two doses (5 or 10 mg/kg) of SR 141716A. The Bonferroni/Dunn test was used for *post-hoc* comparisons after challenge with SR 141716A. * Significantly different from vehicle-vehicle group ($P < .05$). No significant differences were calculated among SR 141716A-treated groups. Additional details of the experimental design, including the number of subjects per treatment regimen, are presented in table 1.

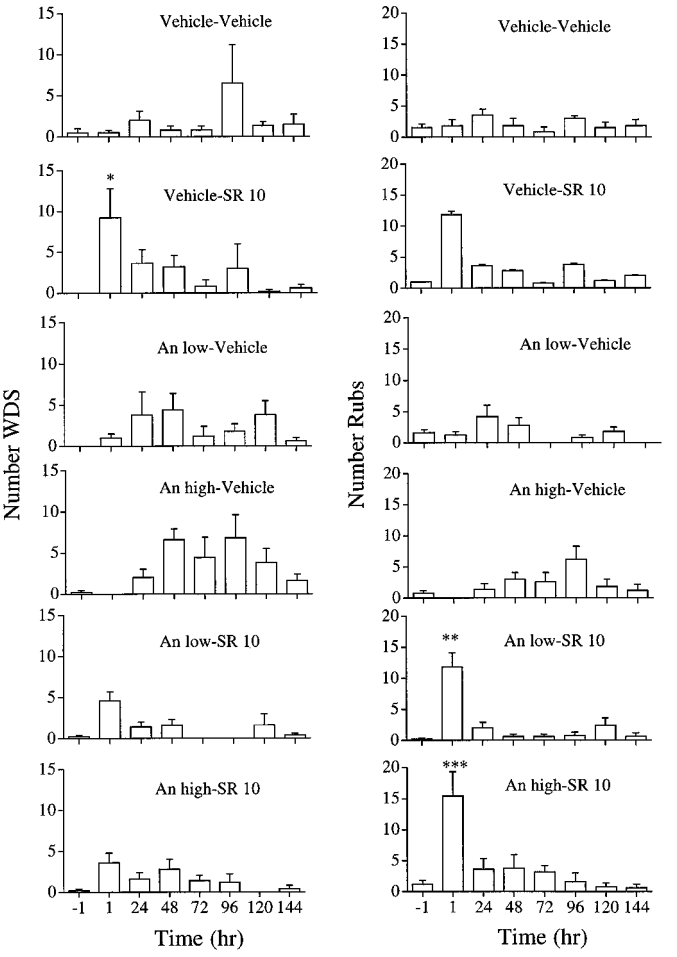

Fig. 3. Duration of wet-dog shakes or facial rubbings observed in rats continuously exposed to anandamide for 4 days and then challenged with either vehicle or SR 141716A. The times expressed as hours are relative to the administration of SR 141716A. All groups contained five rats except the vehicle-vehicle group, which contained four animals. The data are expressed as means ± S.E. for each behavioral sign. The Bonferroni/Dunn test was used for *post-hoc* comparisons. * Significantly different from the vehicle-vehicle group as well as the anandamide-infused groups challenged with SR 141716A. ** Significantly different from the anandamide low-dose infusion group challenged with vehicle. *** Significantly different from vehicle-vehicle controls and the high-dose anandamide group challenged with vehicle. Significance was set at $P < .05$.

challenged groups pretreated with the high-dose anandamide infusion were significantly different. Moreover, neither the high-dose nor the low-dose anandamide pretreated group challenged with SR 141716A had scores that were significantly different from those of the vehicle group challenged with SR 141716A. Elevated but nonsignificant scores were observed for the vehicle-SR 141716A-challenged group.

Body weight was monitored during the infusion period and for 6 days thereafter. The results are depicted in figure 4. Repeated-measures ANOVA indicated a significant treatment effect ($F = 3.137$, $P = .0265$). In addition, there was a significant interaction between treatment and time ($F = 4.673$, $P = .0001$). *Post-hoc* analysis indicated that the rats receiving the high-dose anandamide regimen and challenged with either vehicle or SR 141716A had body weights that were significantly different from those of the vehicle-vehicle group. None of the body weights of the other treatment groups differed significantly from vehicle control. These results underscore the fact that the anandamide treatment regimens elicited pharmacological effects. We also carried out a two-factor ANOVA of the data for days 4 and 5. ANOVA revealed both within-subject ($F = 33.207$, $P = .05$) and between-subjects or between-treatments differences ($F = 11{,}867$, $P = .05$). Subsequent *post-hoc* analysis of days 4 and 5 revealed that none of the body weights in the different treatment groups on day 4 was significantly different from that of the vehicle-vehicle group. On day 5, the body weights of both the low- and high-dose anandamide groups challenged with SR 141716A were significantly different from those of the vehicle-vehicle group, but not significantly different from those of the vehicle-SR 141716A group.

Chronic exposure to arachidonic acid. Because arachidonic acid is associated with the synthesis and degradation of anandamide (Devane and Axelrod, 1994), a control experiment similar to that conducted with anandamide was conducted with arachidonic acid. Expressed as mg/kg/24 hr, the doses were 50, 100, 200 and 200 on days 1 through 4, respectively. A control group was infused with the vehicle. Six rats were used for each treatment group. When compared with vehicle, no differences were apparent during its administration or after it was abruptly withdrawn (data not shown), which indicates that arachidonic acid was devoid of behavioral effects and was well tolerated.

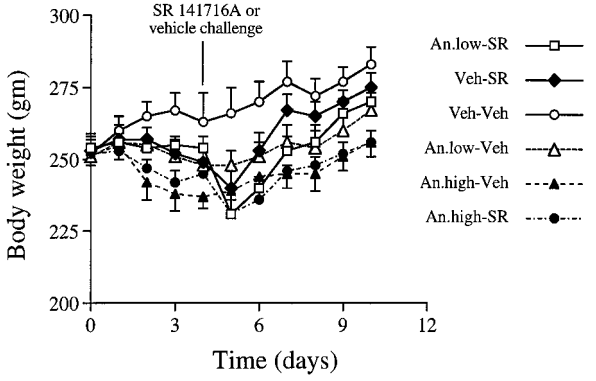

Fig. 4. Effects on body weight of various treatment regimens illustrated in figure 3. The results are expressed as means ± S.E. The Bonferroni/Dunn test was used for *post-hoc* comparisons ($P < .05$). Analysis indicated that the rats receiving the high-dose anandamide regimen and challenged with either vehicle or SR 141716A had body weights that were significantly different from the vehicle-vehicle group. On day 5, the body weights of both the low- and high-dose anandamide groups challenged with SR 141716A were significantly different from those of the vehicle-vehicle group, but they were not significantly different from those of the vehicle-SR 141716A group.

SR 141716A challenge in rats chronically infused with 2-Me-F-AN. To investigate whether the rapid degradation of anandamide was a significant factor in the failure of anandamide to produce physical dependence, the metabolically stable anandamide analog 2-Me-F-AN was infused (5, 10, 20 and 20 mg/kg/24 hr on days 1, 2, 3 and 4, respectively). The results are summarized in figure 5. Few wet-dog shakes or facial rubbings were observed during the 1-hr period preceding the SR 141716A challenge. However, after challenge with SR 141716A (10 mg/kg i.p.) ANOVA indicated significant treatment effects for the sign designated wet-dog shakes ($F = 5.995$, $P = .0061$). On the basis of the results of the *post-hoc* comparisons, we concluded that the number of wet-dog shakes in the 2-Me-F-AN-SR 141716A-challenged group was significantly greater than that in the vehicle-vehicle group and the 2-Me-F-AN-vehicle group but did not differ from that in the vehicle-SR 141716A-challenged group, a result that implicates the cannabinoid antagonist as the source of this effect. There were relatively few wet-dog shakes elicited during the next 6 days in any of the groups, except for the 2-Me-F-AN-infused group that was challenged with vehicle at the 72-hr interval ($F = 8.582$, $P = .0013$). Thus there was a progressive increase in the number of wet-dog shakes that reached statistical significance at 72 hr only. *Post-hoc* analysis revealed that the number of wet-dog shakes in this group was significantly greater than that in all other groups. Elevated scores continued for the duration of the 6-day withdrawal period. They approached, but did not achieve, statistical significance. Regarding rubbing behavior, ANOVA was significant for treatment effects ($F = 6.008$, $P = .0061$). *Post-hoc* analysis showed that the 2-Me-F-AN group challenged with SR 141716A produced a significantly greater number of facial rubs compared with its vehicle-vehicle control group. Also, the vehicle group challenged with SR 141716A showed significantly elevated scores ($P < .05$) when compared with the vehicle-vehicle controls. Finally, the score of the 2-Me-F-AN group challenged with SR 141716A was significantly greater than either that of the vehicle-vehicle group or that of the 2-Me-F-AN-treated group challenged with vehicle.

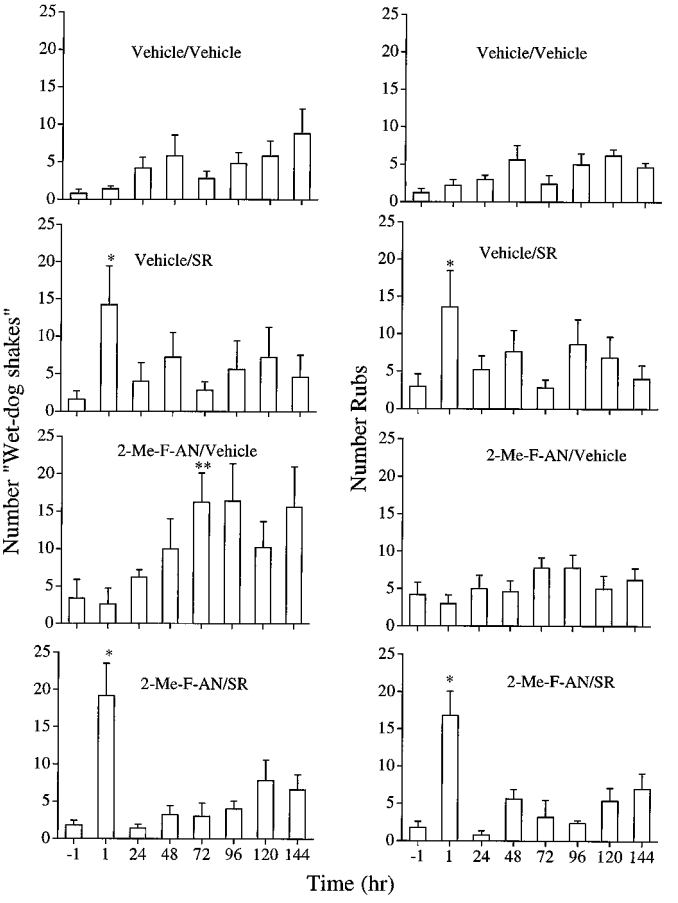

Fig. 5. Incidence of wet-dog shakes or facial rubbings during and after chronic exposure to 2-Me-F-AN followed by abrupt withdrawal of 2-Me-F-AN or SR 141716A challenge. The infusion was terminated after 4 days, and the rats were observed 1 hr before and after SR 141716A (10 mg/kg) at the times indicated on the abscissa. The data are expressed as means ± S.E. for the behavioral signs designated wet-dog shakes and facial rubbing. Five rats per group were tested. The Bonferroni/Dunn test was used for *post-hoc* comparisons for each time period. * Significantly different from the vehicle-vehicle and 2-Me-F-AN-vehicle groups. ** Significantly different from all other groups ($P < .05$).

SR 141716A dose-response study. To examine further the extent to which SR 141716A produced behavioral effects on its own, SR 141716A was given i.p. at doses of 0.3, 1, 10 and 30 mg/kg to rats infused with vehicle for 4 days. We performed the experiment twice, using 5 to 6 rats in each group for each experiment. The results are depicted in figure 6. Adhering to the anandamide protocol, the infusion was terminated, and the rats were observed for behavioral signs for a 1-hr intervals before and after SR 141716A administration. ANOVA indicated that the scores for the signs wet-dog shakes ($F = .860$, $P = .4947$) and rubbing ($F = 321$, $P = .8624$) were not significantly elevated before SR 141716A challenge.

After SR 141716A, ANOVA indicated that significant differences existed among treatment groups for the sign facial rubbing ($F = 5.211$, $P = .0014$) and for the sign wet-dog shakes ($F = 3.473$, $P = .0143$). Application of the Bonferroni/Dunn test for the sign facial rubbing showed that SR 141716A challenges at doses of 10 and 30 mg/kg were significant compared with vehicle. It is important to note that these doses were not significantly different from one another; that is, they were not dose-responsive.

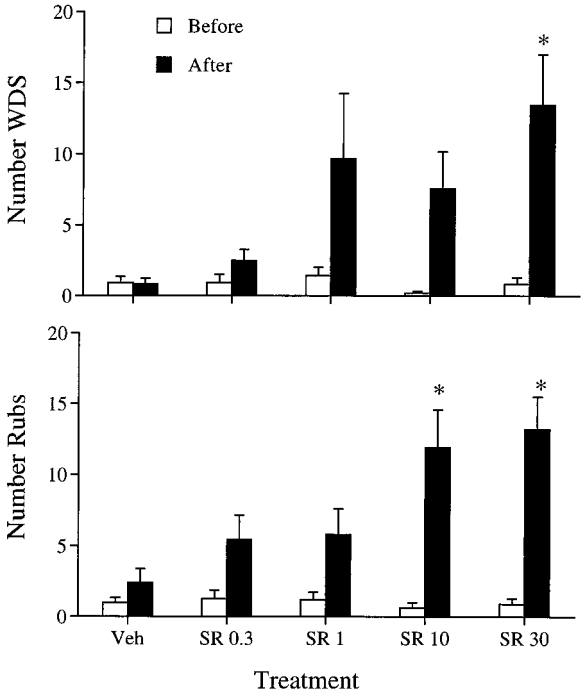

Fig. 6. SR 141716A-induced wet-dog shakes (upper panel) and facial rubbing (lower panel) in rats infused for 4 days with vehicle. Behavior was scored 1 hr before and 1 hr after the i.p. injection of SR 141716A at the doses (mg/kg) indicated in the x-axis. This experiment was performed twice using 5 to 6 rats in each treatment regimen for each experiment. * Significantly different from the vehicle-vehicle group. The Bonferroni/Dunn test was used for *post-hoc* comparisons for each time period ($P < .05$).

For the sign wet-dog shakes, the comparison SR30-vehicle was significant. Still and all, for both signs, the response was constrained. That is, it produced a seemingly limited effect, as was noted by Compton *et al.* (1996).

Discussion

A number of animal models have been utilized for the study of the chronic effects and physical dependence potential of abused substances. Intermittent parenteral injections, administration of test drugs in food or water, depot preparations such as pellets or tablets and implantation of osmotic pumps and continuous infusion methods have been reported (Aceto, 1990). Administration of drugs in food or water is limited by considerations such as stability, palatability, feeding and drinking cycles and dosing frequencies. Depot methods also present some difficulties, including stability of drugs at body temperature and lack of flexibility regarding daily adjustments of dose regimens. Because of anandamide's purported short duration of action (Deutsch and Chin, 1993), we deemed it prudent to infuse it continuously. Continuous exposure of receptors to an agonist is more likely to maximize the development of dependence. The continuous infusion method described by Teiger (1974) addressed these issues and was applied.

Even with the use of a continuous infusion procedure and at pharmacologically active doses, we were unable to demonstrate physical dependence on anandamide either by abrupt withdrawal or by means of the cannabinoid receptor antagonist SR 141716A. It should be emphasized that the results obtained for abrupt withdrawal were in accordance with those reported by us for THC (Aceto *et al.*, 1996). Nonetheless, we fully expected that the cannabinoid receptor antagonist SR 141716A would promptly displace anandamide from its receptor site and precipitate a robust withdrawal syndrome, as was reported with THC (de Fonseca *et al.*, 1997; Aceto *et al.*, 1995, 1996; Tsou *et al.*, 1995).

There are several possible explanations for the failure of anandamide to display a withdrawal syndrome after the administration of SR 141716A. One of the most obvious questions is whether sufficient anandamide was administered to produce physical dependence. The doses of anandamide used in the present study compare favorably with those used in previous THC studies (Aceto *et al.*, 1995, 1996) when considering differences in pharmacological potency between the two agents. A THC dosage regimen as low as 2.5, 5, 10 and 20 mg/kg/24 hr resulted in robust and significant increase of withdrawal signs when the animals were challenged with SR 141716A. Notably, the increase was substantially greater than that observed in the vehicle-infused rats when challenged with SR 141716A. The observation that the high-dose regimen of anandamide produced behavioral effects and weight loss suggested that a pharmacologically relevant regimen was employed. Furthermore, the fact that a higher-dose regimen produced toxicity in the preliminary study precluded escalation of the dosing regimen. However, we cannot exclude the possibility that anandamide is rapidly metabolized to metabolites that contribute to toxicity and that, as a consequence, pharmacologically relevant concentrations are not attained.

At least two biosynthetic pathways have been proposed for the synthesis of anandamide *in vivo*. The first pathway involves the reaction of high mM concentrations of arachidonic acid and ethanolamine by the enzyme designated anandamide synthase (Devane and Axelrod, 1994). In addition, there is evidence that anandamide is synthesized through a D- or C-type phosphodiesterase-mediated cleavage of a membrane precursor, N-archidonoyl-phosphatidylethanolamine, that undergoes hydrolytic degradation to phosphatidylethanolamine and arachidonic acid (Di Marzo and De Petrocellis, 1997). Thus arachidonic acid could be involved in the synthesis and/or degradation of anandamide. It is also possible that anandamide and THC do not have identical mechanisms of action, despite the fact that both are capable of binding to cannabinoid receptors. It should be noted that anandamide and THC have been reported to release arachidonic acid independently of their activation of the cannabinoid receptor (Felder *et al.*, 1992). Accordingly, arachidonic acid was tested under the same conditions reported above for anandamide. The dose regimen mimicked the levels of arachidonic acid anticipated assuming that the metabolism of anandamide was brisk. No remarkable changes in behavior were recorded either during its administration or after its abrupt withdrawal. This is somewhat noteworthy, because the arachidonic acid cascade serves several biochemical pathways from which many potent modulators of cellular activity originate. Our results indicated that continuous and prolonged exposure to high doses of arachidonic acid neither spurred anandamide-associated behaviors nor produced evidence for physical dependence. These results suggest that neither anandamide conversion to arachidonic acid nor the reverse was a significant factor in its behavioral effects and that a non–receptor-mediated effect was not involved.

In another effort to address the possible confound of metabolic inactivation, we evaluated the infusion of a putative metabolically stable anandamide analog, 2-Me-F-AN. This analog has been shown to bind avidly to the cannabinoid receptor *in vitro* even in the absence of metabolic inhibitors and to exhibit pharmacological potency somewhat less than that of THC (Adams *et al.*, 1995). Failure of this analog to produce a dependence syndrome is in agreement with the other evidence discussed above—specifically, that metabolism is not a factor in anandamide's failure to induce physical dependence. However, it is interesting to note that a small but statistically significant number of wet-dog shakes appeared 72 hr after the vehicle challenge in the 2-Me-F-AN-treated rats. Although it is tempting to attribute these effects to delayed withdrawal, it should be pointed out that they did not occur in the SR 141716A-challenged rats.

Anandamide is generally regarded as having many pharmacological and biochemical properties in common with THC (see the introduction), but many differences have also been reported. Some investigators have reported that anandamide and other members of that family can act as partial agonists compared with THC (Fride *et al.*, 1995; Mechoulam and Fride, 1995). They also showed that low doses of anandamide inhibited the characteristic THC-induced pharmacological effects on psychomotor activity, analgesia, immobility and body temperature. Welch and her collaborators (1995) found that alterations in cAMP levels, as well as nor-binaltorphimine pretreatment, influenced THC antinociception at the spinal level, whereas these manipulations had no effect on anandamide-induced antinociception. Recently, it was reported that SR 141716A was unable to block the behavioral effects of anandamide in mice (Adams *et al.*, 1998). Finally, a difference between the discriminative stimulus effects of anandamide and THC was reported. Although anandamide substituted for THC in rats trained to discriminate THC from vehicle, it did so only at a dose that was associated with a decreased response rate (Wiley *et al.*, 1995). To this list of results that suggest a lack of correspondence between anandamide and THC, we add our findings.

Compton and co-workers (1996) reported that SR 141716A itself stimulated locomotor activity in mice at more than 200% above control levels. Regarding these results with SR 141716A, de Fonseca and his group (1997) noted in rats a mild SR 141716A-induced activation of cannabinoid behavioral withdrawal signs in vehicle controls. In the present study, we demonstrated variable and limited increases in the number of facial rubbings and wet-dog shakes in all rats receiving SR 141716A. In addition, there was a subjective impression of psychomotor activation in all SR 141716A-treated rats. One plausible explanation for these behavioral effects is that SR 141716A blockade of the cannabinoid receptor disrupts a tonic inhibitory action of the endogenous system. In this regard, Mechoulam *et al.* (1997) recently provided evidence that anandamide mediates sleep induction. It is well known that chronic administration of THC produces CB 1 receptor down-regulation that is probably responsible for the withdrawal syndrome that follows SR 141716A challenge in THC-treated animals. Failure of SR 141716A to precipitate withdrawal in anandamide-treated animals suggests that anandamide is incapable of producing comparable receptor down-regulation. Consistent with this notion is the fact that studies conducted so far reveal only a modest development of tolerance to anandamide (Welch, 1997). On the other hand, there has been a recent suggestion that SR 141716A may also act as an inverse agonist (Richardson *et al.*, 1997). However, the modest effects produced by SR 141716A alone, compared with the robust effects produced in THC-treated animals, do not provide a compelling argument for agonistic activity for SR 141716A.

In conclusion, the evidence suggests that anandamide, unlike THC, has a low capacity, if any, to produce physical dependence. Apparently, obvious metabolic factors are not involved. That behavioral activation was nearly always associated with SR 141716A suggests that the cannabimimetic system may normally exert a depressant effect on the CNS.

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