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Time Course of the Effects of Different Cannabimimetics on Prolactin and Gonadotrophin Secretion: Evidence for the Presence of CB_1 Receptors in Hypothalamic Structures and Their Involvement in the Effects of Cannabimimetics

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ABSTRACT. Several reports have demonstrated that (−)-Δ^9-tetrahydrocannabinol (Δ^9-THC) and arachidonylethanolamide [anandamide (AEA)] were able to inhibit prolactin (PRL) secretion from the anterior pituitary gland in male rodents, whereas ovarian phase-dependent effects were seen in females. However, in most of these studies, the analysis of PRL levels was performed at times longer than 30 min after cannabinoid administration. In the present study, we examined the time course of the effects of three different cannabimimetics, Δ^9-THC, AEA, and AM356 (*R*-methanandamide), a more stable analog of AEA, on PRL and gonadotrophin secretion in male Wistar rats. In addition, we characterized the presence of cannabinoid receptors in hypothalamic structures related to neuroendocrine control and studied their potential involvement in the effects of cannabimimetics. We found that the three compounds decreased plasma luteinizing hormone (LH) levels, although only the effects of Δ^9-THC were statistically significant. The inhibitory effect was already apparent at 40 min after administration, but only in the case of Δ^9-THC did it persist up to 180 min after administration. No significant changes were seen in plasma follicle-stimulating hormone (FSH) levels after the administration of any of the three different cannabimimetics at any of the four times analyzed. Both AEA and AM356 produced a significant decrease in plasma PRL levels, which appeared at 20 min after administration and persisted up to 60 min, waning after this time. Interestingly, the time course of the effect of Δ^9-THC resembled that of AEA and AM356 only during the later part of the response, because Δ^9-THC produced a marked increase in plasma PRL levels at 20 min, no changes at 40 min and a decrease from 60 min up to 180 min. In additional experiments, we tried to elucidate which of these two phases observed after Δ^9-THC administration was mediated by the activation of cannabinoid receptors. These receptors are present in hypothalamic structures related to neuroendocrine control, with the highest densities in the arcuate nucleus (dorsal area) and the medial preoptic area, and the lowest in the lateral hypothalamic area, although none of these regions exhibited high densities for this receptor as compared with classical regions containing cannabinoid receptors, such as the basal ganglia. The activation of these receptors by Δ^9-THC seems to be involved in the inhibitory phase of the effect of this cannabinoid on PRL release, but not in the early stimulation; when these receptors were blocked with a specific antagonist, SR141716, the stimulation by Δ^9-THC was still observed, but the late inhibition was abolished. In summary, AEA and AM356 markedly decreased PRL release and slightly decreased LH secretion, with no changes on FSH release. Δ^9-THC also produced a marked inhibition of LH secretion, but its effects on PRL were biphasic with an early stimulation not mediated by the activation of cannabinoid receptors, followed by a late and cannabinoid receptor-mediated inhibition. Their site of action may well be the hypothalamic structures related to neuroendocrine control, which contain a small, but probably very active, population of cannabinoid receptors. BIOCHEM PHARMACOL 53;12:1919–1927, 1997. © 1997 Elsevier Science Inc.

KEY WORDS. prolactin; gonadotrophin; Δ^9-tetrahydrocannabinol; anandamide; methanandamide; cannabinoid receptors

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Abbreviations: AEA, arachidonylethanolamide; AM356, *R*-methanandamide; FSH, follicle-stimulating hormone; LH, luteinizing hormone; PRL, prolactin; RIA, radioimmunoanalysis; Δ^9-THC, (−)-Δ^9-tetrahydrocannabinol.

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The administration to rodents of (−)-Δ^9-tetrahydrocannabinol (Δ^9-THC),# the main psychoactive principle of *Cannabis sativa* derivatives, decreases prolactin (PRL) as well as gonadotrophin release from the anterior pituitary gland at both adult [1–3] and immature ages [4–6]. Moreover, we [7] and others [8] have recently demonstrated that

arachidonylethanolamide (anandamide, AEA), a cannabimimetic substance that has been proposed as the endogenous ligand for the cannabinoid receptor [9], is also able to inhibit PRL secretion.

The site of these inhibitory effects seems to be the hypothalamic structures related to the neuroendocrine control of anterior pituitary secretion rather than the anterior pituitary gland itself. Indeed, Δ^9-THC did not inhibit PRL secretion *in vitro* or from ectopic pituitaries [10], although a certain activity of Δ^9-THC at the anterior pituitary level has recently been suggested [11]. Accordingly, cannabinoid receptors seem to be present, although in a small density, in hypothalamic nuclei [12–14], but they are apparently absent from the anterior pituitary gland [15], clearly supporting the notion that the effects of cannabimimetics are mediated through changes in hypothalamic activity. Specifically, the administration of cannabimimetics was able to alter the activity of certain neurotransmitters, such as serotonin [16] and especially dopamine [3, 6, 17–20], which are involved, either directly or indirectly, in the control of neuroendocrine processes in the medial basal hypothalamus.

At the moment, the only exception to this general inhibitory effect of Δ^9-THC and related cannabinoids on PRL secretion has been certain ovarian phases in normal cycling rats. Thus, females were unresponsive to the administration of Δ^9-THC during proestrus or exhibited an increase in plasma PRL levels in parallel to a decrease in tuberoinfundibular dopaminergic activity during the afternoon of estrus [18]. These ovarian phase-dependent changes in responsiveness to Δ^9-THC might be due to the fact that (1) cannabinoid binding sites exhibit several sexual dimorphisms, fluctuate during the ovarian cycle, and are affected by gonadectomy and sex steroid replacement in several brain areas including hypothalamic structures [21]; and (2) the metabolism of cannabinoids to inactive compounds by the action of the P450 cytochrome system in females, because only the pharmacological blockade of this complex allows Δ^9-THC to increase hypothalamic dopaminergic activity and decrease PRL secretion in these unresponsive ovarian phases [19]. In pilot experiments recently performed in our laboratory, we have seen that Δ^9-THC might also produce a stimulatory effect on PRL secretion in male rats. This stimulatory effect was observed at short times after cannabinoid administration, an observation that contrasts with most of the published literature with regard to male rodents, in which the inhibition of PRL secretion by Δ^9-THC was always tested at times longer than 30 min [1, 3, 5, 7, 8, 17–20].

On the basis of these preliminary results, the present work was designed to address three related elements: (1) to examine the time course of the effects of several cannabimimetics, i.e. Δ^9-THC, AEA, and AM356 (*R*-methanandamide), a more stable analog of AEA [22], on PRL and gonadotrophin secretion in male rats, so as to determine the possible existence of biphasic effects (Experiment I); (2) to describe the hypothalamic structures containing cannabinoid receptors using autoradiography (these receptors presumably would be the main site of action of cannabimimetics on neuroendocrine control (Experiment II)); and (3) to characterize the potential involvement of these receptors in the time-dependent effects of Δ^9-THC on PRL release by using a specific antagonist for these receptors [23] (Experiment III).

MATERIALS AND METHODS

Animals, Treatments, and Sampling

Male rats of the Wistar strain were housed from birth in a room with a controlled photoperiod (lights on: 0800–2000 hr) and temperature (23 ± 1°C). They had free access to standard food (Panlab, Barcelona, Spain) and water. At adult age (>8 weeks of life; 250 ± 25 g), animals were used for three different experiments. In Experiment I, they were subjected to a single i.p. injection of well-characterized doses of Δ^9-THC (10 mg/kg weight), generously supplied by the National Institute on Drug Abuse (Rockville, MD), AEA (10 mg/kg weight), purchased from Cayman Chemical Company (Ann Arbor, MI), AM356 (10 mg/kg weight), synthesized by Dr. Makriyannis, as previously described [22], or vehicle (Tween 80-saline solution), and sacrificed at different times after treatment (20, 40, 60, and 180 min). After sacrifice, trunk blood was collected in tubes containing 0.4 mL of 6% EDTA, immediately centrifuged, and the plasma removed and stored frozen at −40°C until the analysis of PRL and gonadotrophin levels. In Experiment II, adult naive animals were sacrificed and their brains quickly and carefully removed and rapidly frozen by immersion in a 2-methyl-butane dry ice bath. Samples were stored at −70°C until processed for autoradiographic analysis. In Experiment III, animals were subjected to a single i.p. injection of SR141716 (3 mg/kg weight), a specific cannabinoid receptor antagonist generously supplied by Sanofi Recherche (Montpellier, France) [23], or vehicle (distilled water with one drop of Tween 80). Thirty minutes later, both SR141716- and vehicle-injected animals were submitted to a new treatment, namely, injections of Δ^9-THC (5 mg/kg weight) or vehicle (Tween 80-saline solution). Animals were sacrificed at 20 or 60 min after the final injection. After sacrifice, trunk blood was collected in tubes containing 0.4 mL of 6% EDTA, immediately centrifuged and the plasma removed and stored frozen at −40°C until analysis of PRL levels.

Autoradiography of Cannabinoid Receptors

TISSUE PREPARATION. Coronal sections 20 μm thick were cut in a cryostat, according to the Paxinos and Watson atlas [24]. For this experiment, sections corresponding to plates 24 and 28 of the Paxinos and Watson atlas were chosen, because they contain an important diversity of hypothalamic structures. Sections were thaw mounted onto gelatin-coated slides and dried briefly at 30°C and stored at −40°C until use. For the identification of the different hypotha-

lamic nuclei, adjacent sections to those used for autoradiographic analysis were stained with cresyl violet and analyzed according to the Paxinos and Watson atlas [24].

AUTORADIOGRAPHIC DEVELOPMENT. The protocol used is basically that described by Jansen *et al.* [25], with slight modifications. Briefly, slide-mounted brain sections were preincubated for 20 min at 30°C in a buffer containing 20 mM HEPES with 0.5% bovine serum albumin (fatty acid-free) at pH 7.0. Slides were then incubated for 80 min at 30°C with 1 nM [^{3}H]-WIN 55,212-2 (an aminoalkylindole analog that binds to cannabinoid receptor with high affinity [25]) prepared in the same buffer, in the absence or presence of 10 μM nonlabeled WIN 55,212-2, to determine the total and nonspecific binding, respectively. Following this incubation, slides were washed in buffer four times (10 min each) at room temperature, dipped in ice-cold distilled water, and then dried under a stream of cool dried air. Autoradiograms were generated by apposing the labeled tissues, together with autoradiographic standards ([^{3}H] microscales, Amersham Ibérica, Madrid, Spain), to tritium-sensitive film ([^{3}H]-Hyperfilm, Amersham Ibérica) for a period of 3–4 weeks, and developed (D-19, Kodak) for 4 min at 20°C. Developed films were analyzed and quantitated in a computer-assisted videodensitometer (Image Quant 3.3, Molecular Dynamics, Krefeld, Germany) using the standard curve generated from [^{3}H]-standards.

Plasma PRL, LH, and FSH Determinations

Plasma PRL, luteinizing hormone (LH), and follicle-stimulating hormone (FSH) levels were measured by specific double antibody radioimmunoanalysis (RIA) systems using materials generously supplied by the National Hormone and Pituitary Program (NIH, Bethesda, MD). Details of these methods have previously been published [26, 27]. To avoid possible interassay variations, all the samples were assayed in a single RIA for each pituitary hormone. Plasma PRL, LH, and FSH levels were expressed as ng/mL of rPRL-RP3, rLH-RP3, and rFSH-RP2, respectively. The limits of detection were 0.025 ng/mL, 0.02 ng/mL, and 0.3 ng/mL, respectively. Because data for PRL levels varied in vehicle-injected animals between Experiments I and III due to variation in the optimal dilution of the antibody supplied by NIH, data were normalized by transforming to percentage of change over the mean value of each control. Statistical analysis of these data was carried out with both raw and transformed data (see Table 1); similar results were obtained fro both analyses, thus supporting the use of transformed data. Raw data for each control (the different vehicle-injected groups) are included in the legends for Figs. 1 and 3.

Statistics

Data were analyzed using two-way analysis of variance (time × treatment) followed by the Student–Newman–Keuls test.

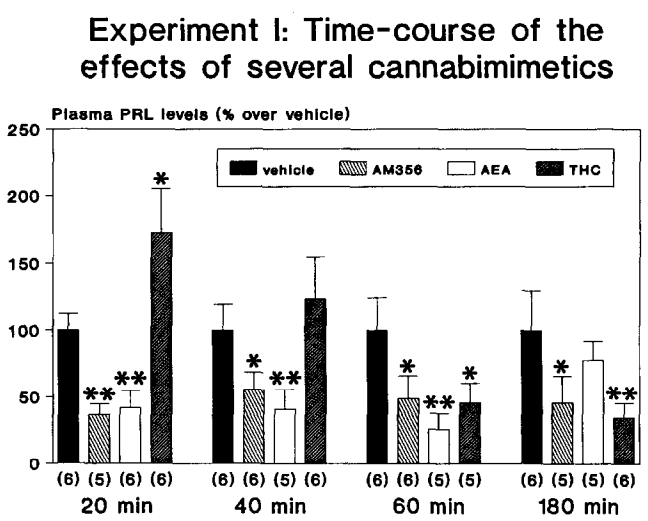

FIG. 1. Plasma PRL levels in male rats subjected to an i.p. injection of R-methanandamide (AM356), anandamide (AEA), Δ^9-tetrahydrocannabinol (Δ^9-THC) or vehicle (Tween 80-saline) and sacrificed at different times (Experiment I) (details in the text). Values are means ± SEM of % of change in each experimental group versus the mean of each vehicle-injected group (20 min: 6.53 ± 0.86 ng/mL; 40 min: 4.70 ± 0.41; 60 min: 4.87 ± 0.78; 180 min: 5.37 ± 1.25). The number of determinations per group is indicated at the bottom of the bar. Data were assessed by two-way analysis of variance (time × treatment; see Table 1) followed by the Student–Newman–Keuls test (*$P < 0.05$; **$P < 0.005$).

RESULTS

Experiment I: Time Course Effects of Different Cannabimimetics on Plasma PRL, LH, and FSH Levels

Both AEA and AM356 produced a significant decrease in plasma PRL levels, which appeared early, at 20 min after administration, and persisted up to 60 min before waning (Fig. 1). However, the time course of the effect of Δ^9-THC resembled that of AEA and AM356 only during the later part of the response, because Δ^9-THC produced a marked increase at 20 min, no changes at 40 min, and a decrease from 60 min up to 180 min (Fig. 1). The observation of statistical differences in the interaction between time and treatment in the two-way analysis of variance of data on plasma PRL levels (see Table 1) supports the existence of time-dependent effects of cannabimimetics on the secretion of this hormone.

These differences between Δ^9-THC and AEA and its analog concerning PRL secretion were not observed with regard to their effects on plasma gonadotrophin levels. In the case of LH and FSH levels, the interaction between time and treatment in the two-way analysis of variance was not statistically significant (Table 1). Thus, the three compounds decreased plasma LH levels (Table 2), although only the effects of Δ^9-THC were statistically significant at most of the times studied (see statistics for treatment in Table 1). The effect appeared at 40 min after administration, but only the effect of Δ^9-THC persisted up to 180 min after administration (Table 2). It should be noted that, in this study, a single analysis of LH levels was done at each

TABLE 1. *F*-values, degrees of freedom, and probability levels obtained after applying the two-way analysis of variance (time × treatment) to data on PRL, LH, and FSH levels from Experiments I and III

Experiment	Parameter	Data	Time	Treatment	Time × Treatment
I	PRL	raw data	$F(3, 75) = 13.80$, $P < 0.0001$	$F(3, 75) = 6.80$, $P < 0.0005$	$F(9, 75) = 3.21$, $P < 0.005$
		% of change	$F(3, 74) = 1, 27$, NS	$F(3, 74) = 4.94$, $P < 0.005$	$F(9, 74) = 1.96$, $P = 0.0567$
	LH	raw data	$F(3, 98) = 9.89$, $P < 0.00005$	$F(3, 98) = 11.19$, $P < 0.00005$	$F(9, 98) = 0.79$, NS
	FSH	raw data	$F(3, 107) = 0.63$, NS	$F(3, 107) = 1.36$, NS	$F(9, 107) = 0.69$, NS
III	PRL	raw data	$F(1, 40) = 1.28$, NS	$F(3, 40) = 3.84$, $P < 0.05$	$F(3, 40) = 1.81$, $P = 0.16$
		% of change	$F(1, 40) = 15.47$, $P < 0.0005$	$F(3, 40) = 3.61$, $P < 0.05$	$F(3, 40) = 1.91$, $P = 0.14$

NS = not significant.

TABLE 2. Plasma LH and FSH levels in male rats subjected to an i.p. injection of *R*-methanandamide (AM356), anandamide (AEA), Δ^9-tetrahydrocannabinol (Δ^9-THC) or vehicle (Tween 80-saline solution) and sacrificed at different times (Experiment I) (details in the text)

Time (min)	Treatment	LH (ng/mL)	FSH (ng/mL)
20	+ vehicle	0.60 ± 0.06	5.3 ± 0.8
	+ AM356	0.51 ± 0.05	4.6 ± 0.5
	+ AEA	0.48 ± 0.08	3.9 ± 0.5
	+ Δ^9-THC	0.49 ± 0.07	3.8 ± 0.7
40	+ vehicle	0.56 ± 0.09	4.7 ± 0.4
	+ AM356	0.37 ± 0.07*	5.0 ± 0.9
	+ AEA	0.42 ± 0.07	4.7 ± 0.5
	+ Δ^9-THC	0.23 ± 0.02**	4.2 ± 0.5
60	+ vehicle	0.47 ± 0.08	4.7 ± 0.6
	+ AM356	0.26 ± 0.12	5.2 ± 0.6
	+ AEA	0.39 ± 0.08	5.0 ± 0.4
	+ Δ^9-THC	0.16 ± 0.06**	5.3 ± 0.5
180	+ vehicle	0.40 ± 0.09	5.7 ± 1.0
	+ AM356	0.28 ± 0.05	4.8 ± 0.6
	+ AEA	0.31 ± 0.08	4.8 ± 0.7
	+ Δ^9-THC	0.05 ± 0.01**	3.7 ± 0.4

Values are means ± SEM of more than six determinations per group. Data were assessed by two-way analysis of variance (time × treatment; see Table 1) followed by the Student-Newman-Keuls test (*$P < 0.05$; **$P < 0.005$).

time studied; hence, it was not possible to specify possible changes in the pulsatility for this hormone. As for plasma FSH levels, no significant changes were seen after the administration of any of the three different cannabimimetics at any of the four times analyzed (Table 2).

Experiment II: Presence of Cannabinoid Receptors in Hypothalamic Structures

Using autoradiography with [^{3}H]-WIN55,212-2 in slide-mounted brain sections, we were able to demonstrate the presence of specific binding for cannabinoid receptors in several hypothalamic structures (Table 3 and Fig. 2), as previously reported by others [12–14]. Specific binding was

TABLE 3. Specific binding (fmol/mg tissue) for cannabinoid receptors measured by autoradiography in several hypothalamic structures, and in two basal ganglia as reference, of adult male rats (details in the text)

	Specific binding (fmol/mg tissue)	% of specific binding
Hypothalamic structures		
Arcuate nucleus (dorsal area)	41.99 ± 7.78	60.6
Ventromedial hypothalamic nucleus	34.69 ± 6.96	52.9
Lateral hypothalamic area	29.51 ± 4.20	52.7
Periventricular nucleus	37.12 ± 5.86	56.3
Paraventricular nucleus	31.88 ± 3.63	50.4
Medial preoptic area	49.19 ± 5.76	67.1
Dorsal hypothalamic area	37.17 ± 3.91	51.9
Reference structures		
Globus pallidus	203.59 ± 10.84	92.2
Entopeduncular nucleus	192.97 ± 15.46	87.1

Values are means ± SEM of 5 animals analyzed.

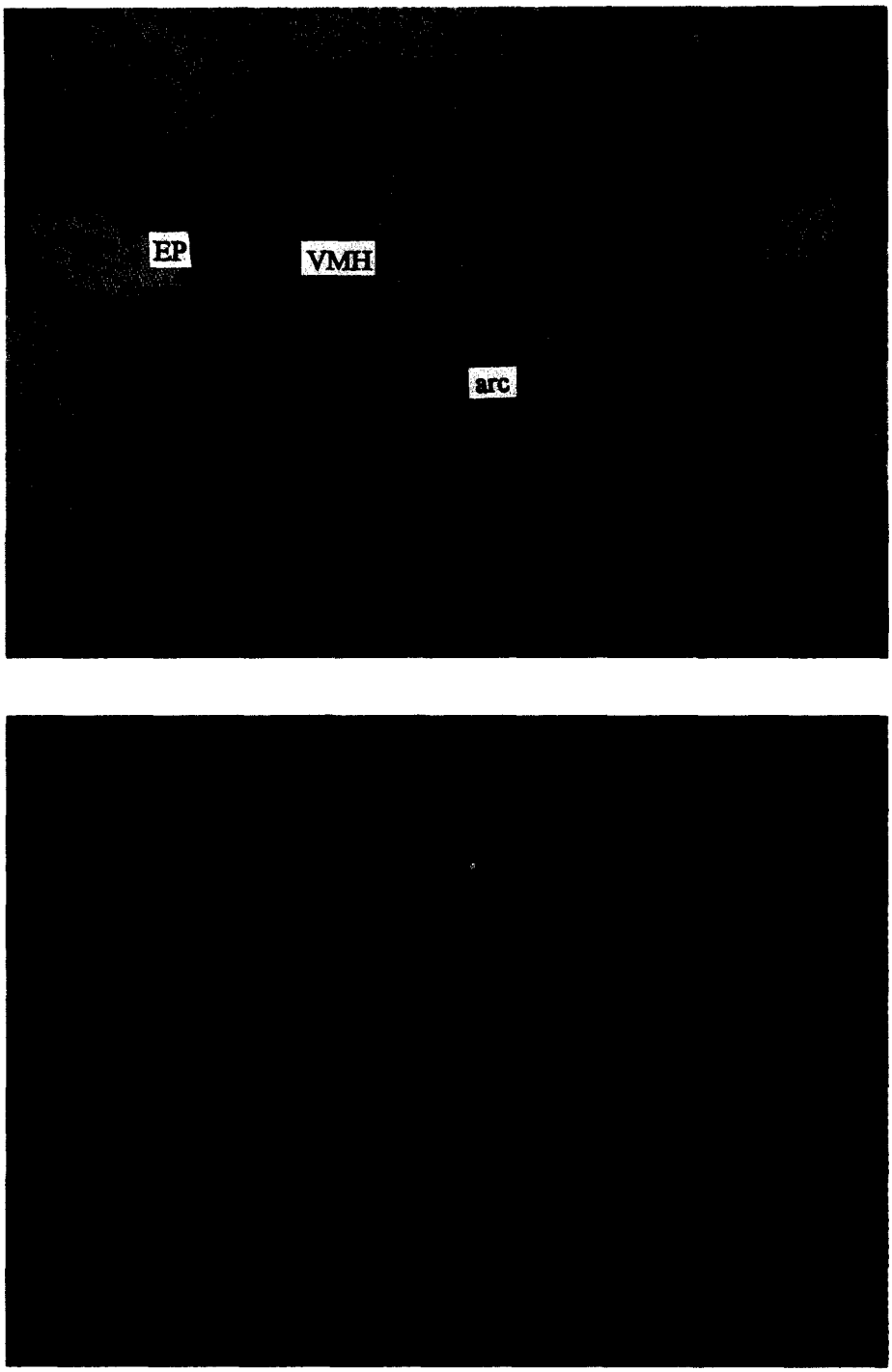

FIG. 2. Representative autoradiograms of plate 28, according to the Paxinos and Watson atlas (this plate was chosen for its diversity of anatomical structures), obtained from slide-mounted sections of adult male rats. Autoradiograms were processed according to the conditions described in Materials and Methods. Top panel is total binding and bottom panel is nonspecific binding. EP: entopeduncular nucleus; VMH: ventromedial hypothalamic nucleus; arc: arcuate nucleus.

very sparse, with the presence of cannabinoid receptors throughout the entire hypothalamus, in particular the dorsal area (see Fig. 2), but apparently without exhibiting a concrete location in specific nuclei. However, we attempted to analyze the densities present in classical hypothalamic nuclei. Thus, the highest densities (>40 fmol/mg tissue) were seen in the dorsal area of the arcuate nucleus and the medial preoptic area. Densities of approximately 30–40 fmol/mg tissue were seen in the ventromedial hypothalamic, periventricular and paraventricular nuclei, as well as the dorsal hypothalamic area. Finally, densities lower than 30 fmol/mg tissue were observed in the lateral hypothalamic area. None of the above regions exhibited high densities for this receptor compared with classical

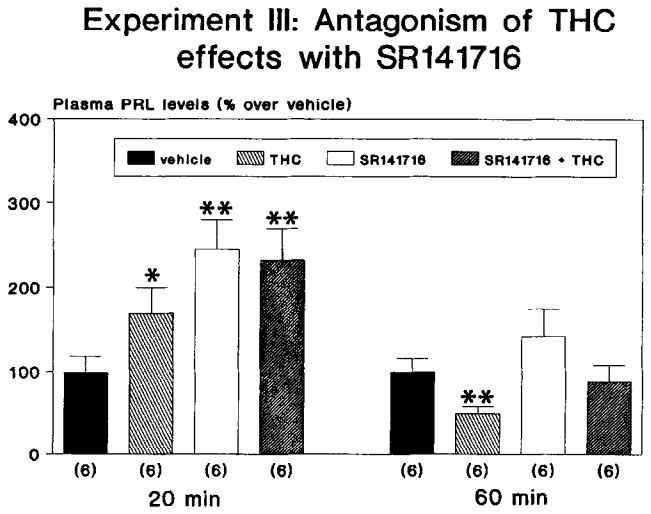

FIG. 3. Plasma PRL levels in male rats subjected to an i.p. injection of Δ^9-tetrahydrocannabinol (Δ^9-THC) or SR141716 or both and sacrificed at 20 or 60 min after the final treatment (Experiment III) (details in the text). Values are means ± SEM of % of change in each experimental group vs. the mean of each vehicle-injected group (20 min: 0.19 ± 0.04 ng/mL; 60 min: 0.31 ± 0.05). The number of determinations per group is indicated at the bottom of the bar. Data were assessed by two-way analysis of variance (time × treatment; see Table 1) followed by the Student–Newman–Keuls test (*$P < 0.05$; **$P < 0.01$).

regions known to contain cannabinoid receptors, such as the basal ganglia; however, in all cases, the % of specific binding was always higher than 50%, supporting a relatively higher contribution of specific binding (receptor-mediated) than nonspecific binding (index of lipophilia, deposition and sequestration [15]). As a reference in the present study, we quantitated the densities for cannabinoid receptors in the globus pallidus and the entopeduncular nucleus, which appear in the same sections used for analyzing the hypothalamic structures. As can be seen in Table 3, specific binding in these two classical regions was considerably higher (fivefold) than in hypothalamic structures.

Experiment III: Involvement of Cannabinoid Receptors in the Biphasic Effects of Δ^9-THC on PRL Secretion

This experiment was aimed at elucidating which of the two effects of Δ^9-THC on PRL secretion observed in Experiment I is mediated by the activation of cannabinoid receptors. Thus, the blockade of these receptors with a specific antagonist, SR141716, only abolished the inhibitory effect observed at 60 min after administration of Δ^9-THC (Fig. 3), but not the early stimulation observed at 20 min after treatment, revealed by the plasma levels of PRL in animals administered both SR141716 and Δ^9-THC as compared with the animals treated with Δ^9-THC alone at the two time points (Fig. 3; see statistics in Table 1). The administration of SR141716 alone usually produced increases in plasma PRL levels (Fig. 3) and was more pronounced in animals sacrificed at 20 min after the final administration, presumably due to the blockade of the endogenous anandamidergic tone.

DISCUSSION

The present study confirms previous observations from us [3, 5–7, 17–20] and others [1, 2, 4, 8, 10, 16, 28, 29], which demonstrated that the administration of Δ^9-THC and related cannabinoids inhibits PRL and LH secretion. The new findings provided by this study are twofold, serving to demonstrate: (1) that the effect of Δ^9-THC on PRL release is really biphasic, with an early stimulation that precedes the classical inhibitory effect (this did not occur after the administration of AEA and AM356); and (2) that only the inhibitory effect is mediated by the activation of cannabinoid receptors, presumably those located in hypothalamic nuclei involved in neuroendocrine control of anterior pituitary hormone secretion.

At present, the studies performed on this topic have supplied two lines of evidence. First, as mentioned above, most of the literature has shown that Δ^9-THC and AEA inhibit PRL secretion in immature and adult male rodents [1–8, 16–20]. The only exception to this classically accepted inhibitory effect of cannabimimetic compounds on PRL secretion was our observation that normal cycling rats were unresponsive to the administration of Δ^9-THC during proestrus and that an increase in plasma PRL levels was even observed when the cannabinoid was administered during the afternoon of estrus [18]. Furthermore, our present results demonstrate the appearance of stimulatory effects of Δ^9-THC on plasma PRL levels in male rats as well. Thus, the administration of this cannabinoid produced an unexpected early stimulation on plasma PRL levels (approximately 20 min after administration) that precedes the classical inhibitory effect, which appeared from 60 min up to 180 min after treatment. It is important to note that most of the studies published on this topic had examined PRL secretion at times longer than 30 min after administration of Δ^9-THC [1–8, 16–20], but not at earlier times. This biphasic pattern was specific for Δ^9-THC, because it did not occur after the administration of AEA and AM356. Both compounds produced a decrease in plasma PRL levels, which appeared early, at 20 min after administration (perhaps much earlier than 20 min), and persisted up to 60 min before waning. At the same time, this biphasic effect produced by Δ^9-THC was specific for PRL release, because this did not occur with regard to its effects on LH and FSH levels. These effects were similar to those produced by the other cannabimimetics, with slight differences that can be attributed to their different potencies or half-lives rather than to a possible activation of different mechanisms. Thus, AEA, AM356 and, particularly, Δ^9-THC decreased LH secretion, with no changes in FSH secretion, in concordance with the results of other authors [1, 2, 8]. The cannabimimetic-induced inhibition of LH secretion has been proposed to be mediated through changes in the hypothalamic mechanisms controlling

GnRH release to the portal blood [2, 29], namely opioid, noradrenergic and dopaminergic influences. In this respect, we have recently found that Δ^9-THC decreases hypothalamic noradrenergic activity while increasing dopaminergic tone in parallel to decreased LH levels [17], whereas Murphy *et al.* [29] have demonstrated that Δ^9-THC interferes with the noradrenergic response involved in the LH surge that occurs during sexual motivation behavior.

The second line of evidence has demonstrated that cannabinoid receptors are present, although in a small density, in important areas of neuroendocrine control, such as several hypothalamic structures [12–14]; however, they are apparently absent from the anterior pituitary gland [15], thus indicating that the effects of cannabinoids on anterior pituitary hormone release would presumably be mediated through the hypothalamus. The absence of specific antagonists for these receptors until recently prevented the possibility of verifying the involvement of cannabinoid receptors by using pharmacological blocking strategies. However, 3 years ago, Rinaldi-Carmona *et al.* [23] described a specific antagonist for these receptors, which is now available for studies of this type. Thus, in the present study, we have used two complementary strategies.

First, we verified the presence of cannabinoid receptors by using autoradiographic analysis in hypothalamic structures in which the neuronal influences controlling PRL and gonadotrophin secretion reside. It is important to note that, as mentioned in the Results, the specific binding was very sparse and not particularly located in classical hypothalamic nuclei, thus producing relatively similar values of specific binding in most of the hypothalamic nuclei. However, specific binding can be differentiated over levels of binding in the adjacent thalamus [30]. We have observed the presence of specific binding for cannabinoid receptors in the arcuate nucleus (mainly in the dorsal area), where tuberoinfundibular dopaminergic neurons, the main hypothalamic input controlling PRL release, are located [31]. The activation of these neurons has been reported to be implicated in the inhibitory effects of classical cannabinoids on PRL secretion [5–7, 17]. Specific binding was also seen in the medial preoptic area, an area of importance in the control of gonadotrophin secretion [32]. Other related hypothalamic nuclei, such as the ventromedial, periventricular, and paraventricular nuclei, also contain cannabinoid receptors, but their densities were lower than in the arcuate nucleus and the medial preoptic area. In general, the presence of cannabinoid receptors in these hypothalamic structures is clearly lesser than in classical regions, such as the cerebellum, hippocampus, and basal ganglia. In the present study, we have used two of these areas as a basis for quantitating receptor density, i.e. the entopeduncular nucleus and the globus pallidus, which are present in the two sections used for analysis of hypothalamic structures. Indeed, the specific binding found in these areas is markedly higher than in the hypothalamic structures. Nevertheless, the activation of cannabinoid receptors in hypothalamic structures seems to be associated with a marked effect at the neuroendocrine level. It is, therefore, reasonable to assume that the population of these receptors, although sparsely distributed, should be located on hypothalamic neurons, which play key roles in the control of anterior pituitary hormone release. For example, concerning the inhibition of LH release discussed above, it might be tentatively assumed that cannabinoid receptors could be located presynaptically on opioidergic, noradrenergic and/or dopaminergic neurons controlling the activity of GnRHergic neurons in adult rats. Their presence in noradrenergic and/or dopaminergic neurons could also be related to the effects of cannabimimetics on PRL secretion. We have recently observed that the specific binding for the cannabinoid receptor in hypothalamic nuclei was not altered in animals with hypothalamic deafferentation, although these studies were conducted in a different strain of rats (Sprague–Dawley) than in our present study (Romero, Wenger, Ramos and Fernández-Ruiz, unpublished results). This observation, in addition to the fact that Δ^9-THC was also effective in acutely suppressing LH and PRL release in these animals, as previously reported by Puder *et al.* [33], suggests that cannabinoid receptors could be located in neurons intrinsic to the hypothalamus and that cannabimimetics would act locally within the medial basal hypothalamus to suppress LH and PRL release. This is concordant with Mailleux and Vanderhaeghen [30], who found that neurons containing cannabinoid receptors in the hypothalamus have their cell bodies mainly in the ventromedial hypothalamic nucleus, as revealed by the location of mRNA levels for cannabinoid receptor by *in situ* hybridization.

With the second strategy used in the present study, we have tried to better understand the involvement of these receptors in the neuroendocrine effects of cannabinoids. Thus, we used coadministration of the cannabinoid receptor antagonist SR141716 [23] and Δ^9-THC to block these receptors and prevent the effects of this cannabinoid on PRL release. In particular, we have attempted to elucidate which of the two effects produced by Δ^9-THC on PRL release is cannabinoid receptor mediated. Our results clearly suggest that only the late inhibitory effect was mediated through the activation of cannabinoid receptors, because this effect disappeared after coadministration of Δ^9-THC and SR141716. This did not occur with the stimulatory effect observed at 20 min, which would be produced by a cannabinoid receptor-independent mechanism. Tentatively, we assume that this stimulatory effect might be related either to a nonspecific effect of Δ^9-THC, probably at the membrane level, or to its previously proposed estrogenic activity [15, 18, 27] because of certain chemical similarities between tricyclic cannabinoids and estrogens (for review, see [6]). Further research will be needed to clarify this hypothesis.

It is also noteworthy that SR141716 itself was able to produce increases in plasma PRL levels, more marked at 20 min after the second treatment in Experiment III. This stimulatory effect was concordant with the inhibition

observed in Experiment I at similar times after the administration of AEA or AM356, suggesting that it might be related to the blockade of endogenous anandamidergic tone in SR141716-treated animals. This again supports the existence of an inhibitory action on PRL secretion associated with the activation of hypothalamic cannabinoid receptors and, indirectly, the presence of an endogenous anandamidergic influence, which would play a physiological role in the neuroendocrine control of the release of anterior pituitary hormones.

In summary, AEA and AM356 markedly decreased PRL release and slightly decreased LH secretion, with no changes on FSH release, whereas Δ^9-THC also produced a marked inhibition of LH secretion. However, its effects on PRL were biphasic, with an early stimulation not mediated by the activation of cannabinoid receptors, followed by a late and cannabinoid receptor-mediated inhibition. The sites of action of these cannabimimetics in all likelihood are the hypothalamic structures associated with neuroendocrine control, which contain a small, but probably very active, population of cannabinoid receptors.

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