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Changes in Rat Brain Cannabinoid Binding Sites After Acute or Chronic Exposure to Their Endogenous Agonist, Anandamide, or to Δ^9-Tetrahydrocannabinol

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ROMERO, J., L. GARCÍA, J. J. FERNÁNDEZ-RUIZ, M. CEBEIRA AND J. A. RAMOS. *Changes in rat brain cannabinoid binding sites after acute or chronic exposure to their endogenous agonist, anandamide, or to Δ^9-tetrahydrocannabinol.* PHARMACOL BIOCHEM BEHAV 51(4) 731-737, 1995.—A brain constituent, the *N*-amide derivative of arachidonic acid, termed anandamide, has been recently proposed as a possible endogenous ligand for the cannabinoid receptor. The present study has been designed to examine whether the acute or chronic exposure to anandamide affected the binding of cannabinoid receptors in specific brain areas as occurred with the exogenous cannabinoid agonist, Δ^9-tetrahydrocannabinol (THC). To this end, we measured the maximum binding capacity (B_{max}) and the affinity (K_d) of cannabinoid receptors, by using [^{3}H]CP-55,940 binding assays, in membranes obtained from several brain areas of male rats acutely or chronically treated with anandamide or THC. Results were as follows. The acute administration of either anandamide or THC increased the B_{max} of cannabinoid receptors in the cerebellum and, particularly, in the hippocampus. This effect was also observed after 5 days of a daily exposure to either anandamide or THC. However, whereas the increase in the B_{max} after the acute treatment seems to be caused by changes in the receptor affinity (high K_d), the increase after the chronic exposure may be attributed to an increase in the density of receptors. On the contrary, the [^{3}H]CP-55,940 binding to cannabinoid receptors in the striatum, the limbic forebrain, the mesencephalon, and the medial basal hypothalamus was not altered after the acute exposure to anandamide or THC. However, the chronic exposure to THC significantly decreased the B_{max} of these receptors in the striatum and nonsignificantly in the mesencephalon. This effect was not elicited after the chronic exposure to anandamide and was not accompanied by changes in the K_d. In summary, the response of cannabinoid receptors to the exposure to cannabinoid agonists varied depending on the brain region. Thus, there were brain areas, such as the hippocampus and the cerebellum, where [^{3}H]CP-55,940 binding increased after the acute or chronic exposure to either anandamide or THC. Cannabinoid receptors were unaffected in other regions, such as the limbic forebrain and the medial basal hypothalamus, or downregulated in the striatum and slightly in the mesencephalon, but only after a chronic exposure to THC.

Cannabinoids Cannabinoid receptors Arachidonylethanolamide Anandamide Δ^9-Tetrahydrocannabinol Brain

DEVANE et al. (7) have recently presented evidence of an arachidonic acid derivative—arachidonylethanolamide—called anandamide, that, being synthetized in the brain, might bind to cannabinoid receptors. This constituent has been reported to be capable of: i) displacing the binding of radioactive cannabinoid agonists to brain membranes (7); ii) activating the molecular mechanism coupled to cannabinoid receptors (inhibition of adenylate cyclase) (31); iii) inhibiting the electrically evoked twitch response of the vas deferens (7); iv) producing hypothermia, analgesia, and hypoactivity (11); and v) inhibiting prolactin secretion (29) and stimulating adrenocorticotrophic hormone release (32). All these effects are

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characteristic of the exposure to psychotrophic cannabinoids, mainly Δ^9-tetrahydrocannabinol (THC) (2,5,8–10,18,22,23, 26), the main constituent of *Cannabis sativa* derivatives. Moreover, during the course of this study, Mechoulam and coworkers (13) have demonstrated that there exist other *N*-amide derivatives of unsaturated fatty acids that are able to displace the binding of radioactive cannabinoids to brain membranes, suggesting that the cannabinoid receptor ligand is a family of endogenous compounds with similar chemical characteristics.

On the other hand, Herkenham and coworkers (24), using autoradiographic techniques, and we (27), using binding of [^{3}H]CP-55,940 to brain membranes, have recently demonstrated that cannabinoid receptors downregulate after a chronic cannabinoid exposure in rats. This fact seems to underlie the tolerance phenomena observed at the neurobehavioral level after prolonged treatments with cannabinoids (24,27), suggesting that tolerance to cannabinoids is pharmacodynamic in nature (24). On the contrary, Martin and coworkers (1) also recently showed that the behavioral tolerance to THC after prolonged treatments was not accompanied by changes in cannabinoid receptor binding and mRNA levels, although this study was carried out in whole brain from mice. No evidence exists that the endogenous agonist of cannabinoid receptors, anandamide, might also produce a similar downregulation, or even upregulation, of these receptors in response to prolonged treatments. Solely, Pertwee et al. (25) have demonstrated the existence of cross-tolerance between THC and anandamide in their inhibitory effects on the twitch response of vas deferens.

The present study has been designed to elucidate this question. To this end, we analyzed the maximum binding capacity (B_{max}) and affinity (K_d) of cannabinoid receptors, by using CP-55,940 binding assays in membranes obtained from specific brain regions (cerebellum, hippocampus, striatum, mesencephalon, limbic forebrain, and medial basal hypothalamus) of male rats after a chronic exposure to anandamide or THC, at doses similar to those reported to produce neurobehavioral effects in previous dose-response studies (6,11,26). The brain areas were selected because they either display a high density of cannabinoid receptors (14–16,18) or are the areas where important neurobehavioral processes affected by the cannabinoid exposure are located (5,8,9,11). The analysis of binding parameters of cannabinoid receptors was also done after the acute exposure to anandamide or THC to control whether, as reported by Oviedo et al. (24), the administered drug prebound to the receptors might compete for binding of [^{3}H]CP-55,940, originating a K_d change.

METHOD

Animals, Treatments, and Sampling

Male Wistar rats were housed from birth in a room with controlled photoperiod (0800–2000 h light) and temperature (23 ± 1°C). They had free access to standard food (Panlab, Barcelona, Spain) and water. Animals were used for experimental purposes at adult age (>8 weeks of age). In the acute experiment, animals were submitted to a single IP administration of anandamide (3 mg/kg body weight), purchased from Cayman Chemical Company (Ann Arbor, MI), THC (3 mg/kg body weight), kindly supplied from the National Institute on Drug Abuse (USA), or vehicle (Tween-saline solution). Twenty minutes later, animals were sacrificed. The dose of anandamide used (3 mg/kg) was chosen because it was very effective in altering motor behavior and dopaminergic indices in previous and unpublished dose–response studies performed in our laboratory (Romero, García, Fernández-Ruiz, and Ramos, unpublished results). Other authors also found motor effects with this dose or similar dose in mice (6,11), although, recently, Smith et al. (30) did not find any behavioral effects with this dose. We interpret this controversy as related to differences in the methodology employed for behavioral testing. In the chronic experiment, anandamide (3 mg/kg body weight), THC (3 mg/kg body weight), or vehicle were IP injected to male rats daily during 5 days. Twenty minutes after the last injection, the animals were sacrificed. In both experiments, brains were quickly removed after sacrifice and the medial basal hypothalamus, the striatum, the limbic forebrain, the mesencephalon, the hippocampus, and the cerebellum were dissected (12), weighed, and immediately frozen at −70°C until assayed.

Membrane Preparation

On the day of the analysis, tissues were thawed and homogenized for 20 s with a Polytron at speed 2–3 in 5 ml of ice-cold 50 mM Tris-HCl buffer at pH 7.4. The homogenates were centrifuged at 40,000 × *g* for 10 min at 4°C. After one wash, the pellets were resuspended in a volume of the same buffer (variable as a function of the desired protein concentration) and used for the binding assay. An aliquot of membrane fraction was used for determining the protein concentration by using the Lowry method (19). This was approximately 2–3 mg/ml.

Cannabinoid Receptor Binding Assay

The measurement of the B_{max} and the K_d of cannabinoid binding sites was performed by using a novel filtration method, based on the procedures described by Bridgen et al. (4) and Houston et al. (17) with slight modifications previously published (27). Assays were always performed in borosilicate tubes silanized with sigmacote (Sigma, St. Louis, MO). The radioactive ligand was [^{3}H]CP-55,940 (104.0 Ci/mmol) purchased from NEN (Boston, MA). This was used at a range of concentrations of 0.125–2.5 nM (six to seven different concentrations). THC was used as displacer at a concentration of 5 μM. THC solution was prepared the day of the assay by diluting a 10^{-4} M stock solution (THC was dissolved in absolute ethanol and diluted in 5 mg/ml fatty acid-free bovine serum albumin). Both radioactive ligand and THC were diluted at the above-mentioned concentrations in incubation buffer. This consisted of 50 mM Tris-HCl (pH 7.4) containing 1 mM EDTA, 3 mM $MgCl_2$, and 5 mg/ml bovine serum albumin. The aliquot of membrane fraction was also diluted in the incubation buffer until a final protein concentration in the incubation volume of 0.2–0.3 mg/ml. The final incubation volume was 0.5 ml for all the studies. Incubation was allowed for 60 min at 30°C and finished by rapid filtration through Whatman GF/C glass fiber filters, presoaked in 1 mg/ml bovine serum albumin. Filters were washed twice with 5 ml of ice-cold Tris-HCl buffer (pH 7.4) containing 1 mg/ml bovine serum albumin. Radioactivity bound to membranes was determined by liquid scintillation counting. Specific [^{3}H]CP-55,940 binding was calculated as the difference between binding in the presence or absence of THC. Data were analyzed by Scatchard transformations. A simplified method was applied to measure the relative binding capacity of individual medial basal hypothalami for cannabinoid receptor, due to the limited amount of tissue available. This procedure analyzes the total specific binding with a saturating concentration of ligand

(10 nM) that ensures more than 90% receptor occupancy. This procedure exclusively allows the calculation of the maximum bound to the medial basal hypothalamic membranes with no indications about affinity (28).

Statistics

Data were assessed by two-way (acute treatment × chronic treatment) analysis of variance (ANOVA).

RESULTS

The administration of a single IP dose of either anandamide or THC increased the B_{max} of cannabinoid receptors in the cerebellum (Fig. 1) and, particularly, in the hippocampus (Fig. 2). These increases still remained after 5 days of a daily exposure to either anandamide or THC in both brain areas (Figs. 1, 2). However, the increase in the B_{max} after the acute treatment in both brain areas appears to be related to changes in the cannabinoid receptor affinity, as revealed by the increase in the K_d after the administration of anandamide (cerebellum: 0.90 ± 0.07 nM, trend; hippocampus: 1.42 ± 0.10 nM, $p < 0.05$) or THC (cerebellum: 1.20 ± 0.10 nM, $p < 0.05$; hippocampus: 1.32 ± 0.15 nM, $p < 0.05$) vs. their respective controls (cerebellum: 0.73 ± 0.10 nM; hippocampus: 0.91 ± 0.07 nM). In contrast, the increase in the B_{max} after the chronic exposure appears to exclusively reflect an increase in the number of receptors because no changes in the receptor affinity were seen in the cerebellum (vehicle: 0.81 ± 0.21 nM; anandamide: 0.85 ± 0.09 nM; THC: 1.18 ± 0.25 nM) and the hippocampus (vehicle: 1.04 ± 0.18 nM; anandamide: 1.06 ± 0.11 nM; THC: 1.12 ± 0.15 nM). Representative saturation curves and Scatchard plots showing the effects of acute or chronic administration of anandamide in the hippocampus have been included in Figs. 3 and 4.

On the contrary, the B_{max} and the K_d of cannabinoid receptors was not altered after either the acute exposure or the chronic treatment with anandamide or THC in other brain regions, such as the limbic forebrain and the medial basal hypothalamus (Table 1). Similarly, the acute administration of either anandamide or THC did not alter the [^{3}H]CP-55,940 binding to cannabinoid receptors in the striatum (Fig. 5) and the mesencephalon (Table 1). However, the chronic exposure to THC, but not to anandamide, elicited a significant decrease in the B_{max} of these receptors in the striatum (Fig. 5), with no changes in their affinity (vehicle: 1.09 ± 0.18 nM; THC: 0.93 ± 0.14 nM). This decrease was also statistically significant compared with the acute THC-treated rats. A nonsignificant decrease in the B_{max} was also seen in the mesencephalon (Table 1). A representative saturation curve and Scatchard plot showing the effects of chronic administration of THC in the striatum has been included in Fig. 6.

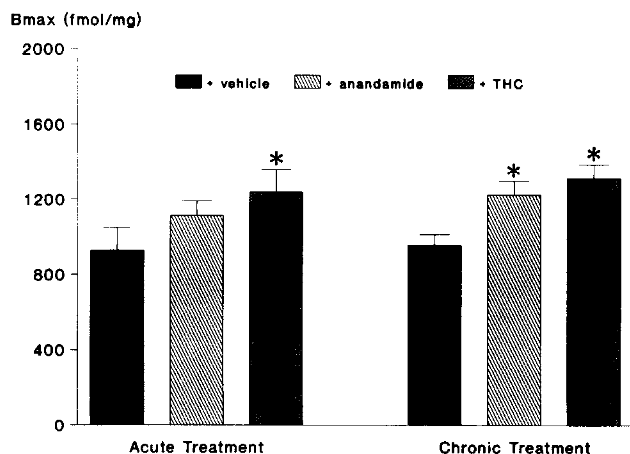

FIG. 1. Maximum binding capacity (B_{max}) of cannabinoid receptors in the cerebellum of male rats acutely or chronically exposed to either anandamide or Δ^9-tetrahydrocannabinol (THC). Details in the text. Values are means ± SEM of six to eight determinations per group. Data were analyzed by two-way ANOVA (*$p < 0.05$ vs. the corresponding control).

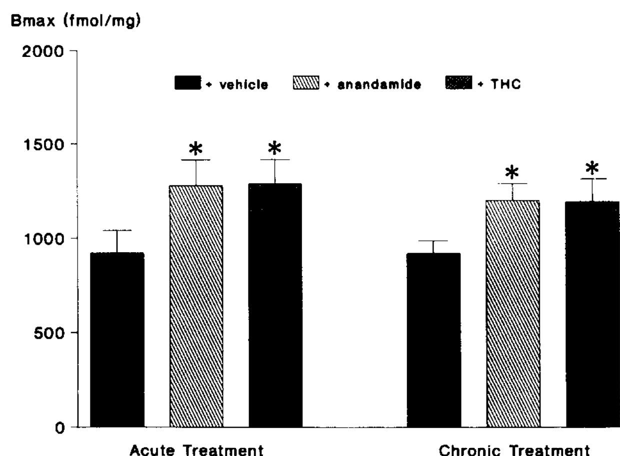

FIG. 2. Maximum binding capacity (B_{max}) of cannabinoid receptors in the hippocampus of male rats acutely or chronically exposed to either anandamide or Δ^9-tetrahydrocannabinol (THC). Details in the text. Values are means ± SEM of six to eight determinations per group. Data were analyzed by two-way ANOVA (*$p < 0.05$ vs. the corresponding control).

DISCUSSION

In the present study, we tried to examine whether the administration of the recently described endogenous agonist of cannabinoid receptors, anandamide (7), affected the binding characteristics of these receptors in specific brain areas, as previously described with THC (24,27). Collectively, our results support the notion that cannabinoid receptors respond to the exposure of their endogenous agonist, although this response, as well as their response to THC, varied as a function of the brain region. Thus, cannabinoid receptors in the hippocampus and cerebellum had a similar response to the administration of either anandamide or THC. Both cannabimimetic agents increased the B_{max} of these receptors in both regions, a priori suggesting an upregulatory response, similar to the response of other drug receptors, such as opiate (3) and nicotinic receptors (20), when chronically exposed to their pharmacological agonists. Moreover, the effects reported for opiates and nicotine were small and region specific, as occurred in our present study.

Surprisingly, the increases in the B_{max} of cannabinoid receptors in the cerebellum and the hippocampus occurred not only after 5 days of a daily exposure to either anandamide or THC, but also after a single dose of each agonist. The analysis of the affinity of these receptors revealed that the changes in the [^{3}H]CP-55,940 binding to cannabinoid receptors in both brain areas after the acute exposure seem to be originated

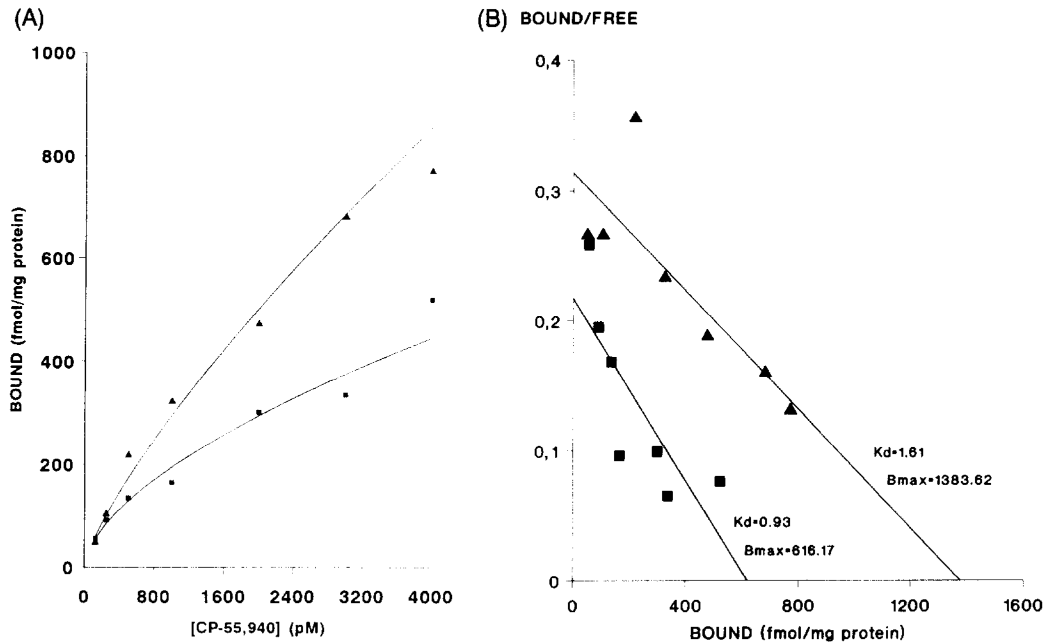

FIG. 3. Saturation curve (A) and Scatchard plot (B) of a representative [^{3}H]CP-55,940 binding assay using membranes of the hippocampus of male rats acutely exposed to anandamide (▲) or vehicle (■). Details in the text.

more by changes in the K_d rather than in the density of receptors. It is true that the increases in the K_d are very small (less than 60% of increase over the controls) and, probably, irrelevant from the view of affinity, but they seem to affect the calculation of the B_{max}. This did not occur after the chronic exposure, where the increases in B_{max} likely reflect increases in the number of receptors, and, then, in their de novo synthesis, because they were not accompanied by changes in K_d.

Hence, a priori it is likely that the rapid response of cannabinoid receptors observed after acute THC or anandamide

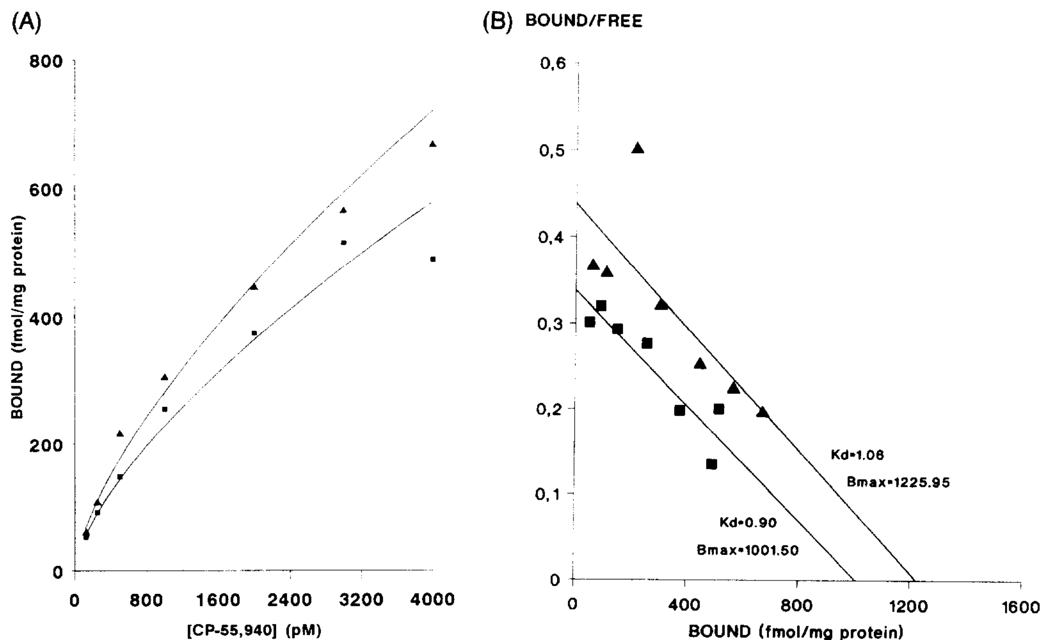

FIG. 4. Saturation curve (A) and Scatchard plot (B) of a representative [^{3}H]CP-55,940 binding assay using membranes of the hippocampus of male rats chronically exposed to anandamide (▲) or vehicle (■). Details in the text.

TABLE 1

MAXIMUM BINDING CAPACITY (B_{MAX}) OF CANNABINOID RECEPTORS IN THE MEDIAL BASAL HYPOTHALAMUS, THE LIMBIC FOREBRAIN AND THE MESENCEPHALON OF MALE RATS ACUTELY OR CHRONICALLY EXPOSED TO EITHER ANANDAMIDE OR Δ^9-TETRAHYDROCANNABINOL (THC)

		B_{max} (fmol/mg protein)		
Brain Region	Treatment	+ Vehicle	+ Anandamide	+ THC
Hypothalamus	Acute	607.4 ± 98.8	551.6 ± 99.9	677.9 ± 75.1
	Chronic	517.7 ± 96.0	718.5 ± 87.0	629.2 ± 97.4
Limbic forebrain	Acute	740.5 ± 89.1	696.3 ± 99.2	805.3 ± 95.6
	Chronic	807.6 ± 97.9	711.6 ± 96.3	885.8 ± 97.7
Mesencephalon	Acute	460.1 ± 10.0	518.4 ± 39.0	472.5 ± 77.6
	Chronic	506.1 ± 87.2	521.6 ± 59.2	372.9 ± 80.5

Affinity (K_d) was not changed in the limbic forebrain and the mesencephalon (affinity was not measured in the medial basal hypothalamus) by each treatment (control values in the acute treatment were: 1.22 ± 0.15 and 1.08 ± 0.10 nM, respectively, and in the chronic treatment: 1.05 ± 0.31 and 1.23 ± 0.29 nM, respectively). Details in the text. Values are means ± SEM of six to eight determinations per group. Data were analyzed by two-way ANOVA.

might reflect changes at the level of the pool of synthesized cannabinoid receptors rather than an increase of the receptor synthesis. For instance, receptors incorporated into the membrane would not be active to recognize their ligands but might be rapidly activated (i.e., by covalent modifications leading to changes in the binding site affinity) by the presence of an agonist. An alternative explanation for the increases in the [^{3}H]CP-55,940 binding after the acute cannabinoid exposure derives from the studies of Herkenham and coworkers (24), as has been mentioned in the Introduction. These authors also found that the K_d increased after the acute cannabinoid exposure and suggested that the changes in affinity were apparently due to the administered drug, which would be prebound to the cannabinoid binding site and would compete with the radioactive ligand in the in vitro assay because competition for binding sites would be reflected as a K_d change. Hence, our increases in the K_d of cannabinoid receptors in the cerebellum and, particularly, in the hippocampus after the acute exposure to THC or anandamide might be due to a remaining drug prebound to the receptor during the in vitro assay. On the contrary, this prebound competing drug did not have a particular effect in the animals chronically exposed to THC or anandamide. In our opinion, this was because the increase in the [^{3}H]CP-55,940 binding in the chronic state was presumably due to an increase in the synthesis of receptors, which would diminish the changes in the affinity originated by the prebound drug. Finally, another interesting question was whether the effect of the "on-board" competing drug during the acute situation was region specific because it was only circumscribed to the cannabinoid receptors in the cerebellum and hippocampus. This probably would be related to the differences in the response to the cannabimimetic drugs regarding the brain area.

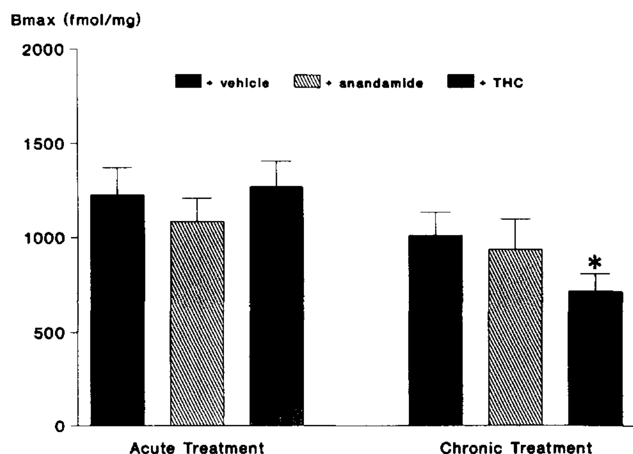

FIG. 5. Maximum binding capacity (B_{max}) of cannabinoid receptors in the striatum of male rats acutely or chronically exposed to either anandamide or Δ^9-tetrahydrocannabinol (THC). Details in the text. Values are means ± SEM of six to eight determinations per group. Data were analyzed by two-way ANOVA (* $p < 0.05$ vs. the corresponding control).

Contrary to the above brain areas, the [^{3}H]CP-55,940 binding to cannabinoid receptors in the medial basal hypothalamus and the limbic forebrain was not altered after either the acute exposure or the chronic treatment with anandamide or THC, supporting the notion of regional differences in the responsiveness of cannabinoid receptors to their natural agonists. Other brain regions, such as the striatum and the mesencephalon, also did not respond to the acute administration of either anandamide or THC. However, the chronic exposure to THC, but not to anandamide, elicited a significant decrease in the B_{max}, with no changes in the K_d of these receptors in the striatum and a trend to decrease in the mesencephalon. Several points deserve to be discussed from these last results.

First, the chronic THC-induced decreases in the density of cannabinoid receptors in these areas had been observed in our earlier study (27) in relation to an attenuation of motor disturbances caused by this cannabinoid. Moreover, Oviedo et al. (24) reported a decrease in the density of cannabinoid receptors in the striatum, measured by autoradiography, after chronic treatment with THC and other cannabinoid agonists. Furthermore, there exists a point of controversy because downregulation of cannabinoid receptors by chronic THC had also been found in the limbic forebrain in our earlier study (27), but it did not occur in the present study. We do not have a plausible explanation for this disagreement besides the

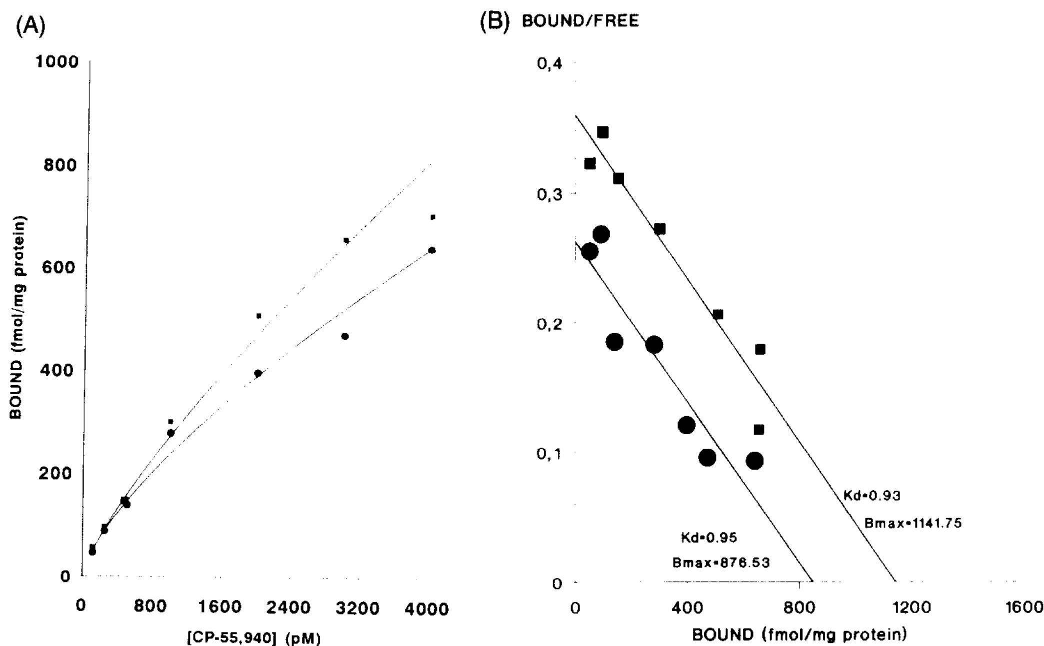

FIG. 6. Saturation curve (A) and Scatchard plot (B) of a representative [^{3}H]CP-55,940 binding assay using membranes of the striatum of male rats chronically exposed to Δ^9-tetrahydrocannabinol (●) or vehicle (■). Details in the text.

possible methodological differences in the length of the period of chronic exposure, in the dose of THC, and in the time after the last injection in which the analyses were done.

The second question deals with the differences between the response of striatal and mesencephalic cannabinoid receptors to chronic THC and the absence of response after chronic anandamide. The downregulation of these receptors after chronic THC was an expected result, not only by the preceding studies (24,27), but also because it was the current response to a chronic exposure with a pharmacological agonist. However, the endogenous agonist of these receptors clearly failed to elicit a response similar to that produced by the exogenous agonist. We do not have a plausible explanation for this observation, although some points might be mentioned for supporting these differences as well as for the regional differences in the response to the agonists. First, Pertwee et al. (25) recently found cross-tolerance between THC and anandamide for the inhibition of the twitch response of the vas deferens but not for their hypothermic effect. Second, it is possible different subtypes of cannabinoid receptors exist in the brain, which has been suggested but has not been demonstrated. These subtypes of receptors could have different affinities or sensitivities for the different cannabinoid agonists and/or have a different distribution in the brain. In this sense, the existence of a peripheral receptor different to the brain receptor has been recently reported by Munro et al. (21). Third, it could be possible that there exist regional differences (metabolizing enzymes that can inactivate or potentiate the agonist effect; different microenvironments, etc.) in the in vivo availability of anandamide, the endogenous ligand, compared with the availability of a pharmacological agonist such as THC, leading to differences in the response to each cannabinoid agonist.

In summary, the response of cannabinoid receptors to the exposure to cannabinoid agonists varied depending on the brain area. Thus, there were brain areas, such as the hippocampus and the cerebellum, where [^{3}H]CP-55,940 binding to cannabinoid receptors increased after the acute or chronic exposure with either anandamide or THC, although the changes in the acute state probably result from an increase in the K_d. Cannabinoid receptors were unaffected by cannabinoid exposure in other regions, such as the limbic forebrain and the medial basal hypothalamus, or downregulated in the striatum and slightly in the mesencephalon, but only after a chronic exposure to the exogenous cannabinoid, THC. The reason(s) for these regional differences remains to be determined.

ACKNOWLEDGEMENTS

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