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ACCELERATED COMMUNICATION

Determination and Characterization of a Cannabinoid Receptor in Rat Brain

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SUMMARY

The determination and characterization of a cannabinoid receptor from brain are reported. A biologically active bicyclic cannabinoid analgetic CP-55,940 was tritium-labeled to high specific activity. Conditions for binding to rat brain P_2 membranes and synaptosomes were established. The pH optimum was between 7 and 8, and specific binding could be eliminated by heating the membranes to 60°. Binding to the P_2 membranes was linear within the range of 10 to 50 μg of protein/ml. Specific binding (defined as total binding displaced by 1 μM Δ^9-tetrahydrocannabinol (Δ^9-THC) or 100 nM desacetyllevonantradol) was saturable. The K_d determined from Scatchard analysis was 133 pM, and the B_{max} for rat cortical P_2 membranes was 1.85 pmol/mg of protein. The Hill coefficient for [^{3}H]CP-55,940 approximated 1, indicating that, under the conditions of assay, a single class of binding sites was determined that did not exhibit cooperativity. The binding was rapid ($k_{on} \approx 2.6 \times 10^{-4}$ pM^{-1} min^{-1}) and reversible ($K_{off} \approx 0.016$ min^{-1}) and ($k_{off}' > 0.06$ min^{-1}). The two K_d values estimated from the kinetic constants approximately 55 pM and exceeded 200 pM, respectively. The binding of the agonist ligand [^{3}H]CP-55,940 was decreased by the nonhydrolyzable GTP analog guanylylimidodiphosphate. The guanine nucleotide induced a more rapid dissociation of the ligand from the binding site, consistent with an allosteric regulation of the putative receptor by a G protein. The binding was also sensitive to $MgCl_2$ and $CaCl_2$. Binding of [^{3}H]CP-55,940 was displaced by cannabinoid drugs in the following order of potency: CP-55,940 ≥ desacetyllevonantradol > 11-OH-Δ^9-THC = Δ^9-THC > cannabinol. Cannabidiol and cannabigerol displaced [^{3}H]CP-55,940 by less than 50% at 1 μM concentrations. The (−)-isomer of CP-55,940 displaced with 50-fold greater potency than the (+)-isomer. This pharmacology is comparable to both the inhibition of adenylate cyclase *in vitro* and the analgetic activity of these compounds *in vivo*. The criteria for a high affinity, stereoselective, pharmacologically distinct cannabinoid receptor in brain tissue have been fulfilled.

Various preparations of *Cannabis sativa* (marihuana) have traditionally been used therapeutically and for their psychological manifestations [see reviews by Hollister (1) and Dewey (2)]. Δ^9-THC is the major compound in extracts of cannabis to have effects on the CNS (3). The predominant CNS responses to Δ^9-THC include analgesia and antiemesis, as well as a "psychological high," drowsiness, alterations in cognition and memory, and a decrement in psychomotor performance in humans (1, 2). Animal behavioral patterns associated with cannabinoid drug actions include altered behavior in monkeys, a characteristic static ataxia in dogs, and hypothermia, analgesia, a typical cannabinoid immobility, and a biphasic change in spontaneous locomotor activity in rodents (3). Although extensive structure-activity relationships have been studied in humans and in these animal models (3), the actions of cannabinoid drugs in the brain remain poorly understood. At the present time, very little is known concerning the neuroanatomical location of cells responsive to cannabinoid drugs, the classical neurotransmitter pathways that may interact with cannabinoceptive cells, or the effects that cannabinoid drugs have on neurons in the CNS.

One reason for our lack of insight concerning the actions of cannabinoid drugs in the CNS is that a clearly defined cellular mechanism(s) for this class of drugs has remained elusive [see Ref. 4 for a thorough evaluation). Our recent studies have overcome this obstacle by demonstrating that the centrally active cannabinoid drugs inhibit adenylate cyclase activity in a model neuronal system (5, 6). The ability of cannabinoid drugs

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ABBREVIATIONS: THC, tetrahydrocannabinol; DALN, desacetyllevonantradol; Gpp(NH)p, guanyl (β,γ)-imidodiphosphate; CNS, central nervous system; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

to regulate adenylate cyclase was determined to be related to the ability of these compounds to produce CNS effects in humans and animal models (7, 8). The response could be produced at submicromolar concentrations (7, 8) and thus would be consistent with drug levels that might be expected to be present in the brain during peak activity (9–11). Using a series of cannabinoid compounds, developed at Pfizer Central Research for their analgetic activity (12), enantioselectivity was demonstrated for the inhibition of adenylate cyclase that paralleled the isomeric selectivity exhibited in analgetic tests in animals (8). The inhibition of adenylate cyclase occurred only in certain cell types (13, 14), arguing that the effect on adenylate cyclase was not a universal phenomenon such as would be expected of cannabinoid-induced membrane fluidity changes. Further studies clearly demonstrated the requirement for G_i (6, 13), a guanine nucleotide regulatory protein that mediates the responses of hormone receptors to ultimately decrease adenylate cyclase activity. The evidence accrued from these studies strongly suggested the presence of pharmacologically unique cannabinoid receptors on the cultured neuronal cells. Logically, neurons in the CNS should also possess cannabinoid receptors.

The tools to search for a cannabinoid receptor in the brain were not available until recently. The relatively low potency and tendency to partition into biological membranes suggest that Δ^9-THC is a poor candidate for a radiolabeled ligand for the detection and characterization of cannabinoid receptors. Δ^9-THC was able to inhibit adenylate cyclase with a K_{inh} of 430 nM (7). It might be expected that radioactively labeled Δ^9-THC would have an affinity for cannabinoid receptors in the nanomolar range and would bind to receptors estimated to be present in the brain in the range of fmoles per milligram of tissue. Reports of the membrane/buffer partition coefficient for Δ^9-THC have ranged from 400 (15) to 12,500 (16). It can be calculated that the amount of labeled Δ^9-THC binding to receptors could potentially be 5 or 6 orders of magnitude smaller than the amount that would be expected to partition into membranes.

A collaborative interaction between our laboratories has allowed the investigation of cannabinoid receptors in the brain using a highly potent analgetic bicyclic cannabinoid compound, CP-55,940 (8, 12). This structure is one of a series of compounds that conform to a postulated three-point agonist-receptor interaction model proposed for the cannabinoid association with the CNS receptor that mediates analgesia (12). The important functional groups for agonist-receptor interaction were proposed to be 1) the C-ring hydroxyl, 2) the phenolic A-ring hydroxyl, and 3) the A-ring alkyl side chain. These same functional groups were found to be required for the inhibition of adenylate cyclase *in vitro* (8). The regulation of adenylate cyclase by CP-55,940 was found to exhibit a K_{inh} of 25 nM, and the (−)-isomer was found to be 200-fold more potent than the poorly analgetic (+)-isomer (8). The high affinity and enantioselectivity exhibited by CP-55,940 made it a potentially useful radioligand for binding studies to characterize the cannabinoid receptor. The studies reported here describe the binding site for [^{3}H]CP-55,940 and provide convincing evidence that this binding site is the elusive cannabinoid receptor.

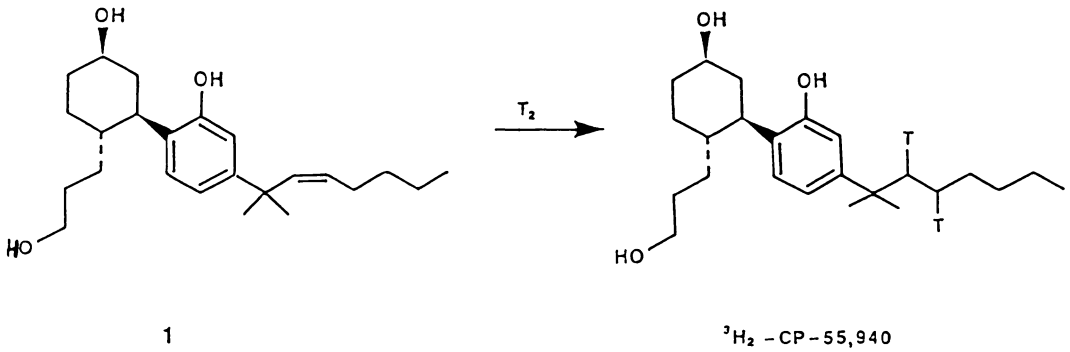
Experimental Procedures

Materials. The natural cannabinoid drugs were provided by the National Institute on Drug Abuse. DALN and the isomers of CP-55,940 were synthesized at Pfizer Central Research. Cannabinoid drugs were stored as 10 mM stock solutions in absolute ethanol at −20°. Drugs were initially diluted to 20 μM in 9.4 mg/ml fatty acid-deficient bovine serum albumin using Regisil-treated glassware. All subsequent dilutions were made into a vehicle containing 5 mg/ml bovine serum albumin.

[^{3}H]CP-55,940 was radiolabeled at DuPont NEN by tritium reduction, in the presence of a Pd catalyst, of a double bond between carbons 2 and 3 of the A-ring alkyl side chain (Fig. 1). Labile tritium was removed by several washes with methanol. Product was purified by high performance liquid chromatography on a 25-cm Zorbax ODS column using the solvent system CH_3CN/25 mM NaH_2PO_4, pH 4.3 (65:35). Purified material was judged by high performance liquid chromatography to be greater than 97% chemically pure. The specific activity was determined to be 93.4 Ci/mmol, using the UV absorbance to quantitate the yield of product. Tritium exchange with labile hydrogens probably accounts for the labeling in excess of the theoretical specific activity. [^{3}H]CP-55,940 was stored at 1 mCi/ml in ethanol at −80° for long term storage and at −20° for routine usage. Purity of the stored material was monitored by thin layer chromatography on silica gel GHLF plates using the solvent system ether/isopropanol (98:2). Biological activity of the radioligand was also monitored. [^{3}H]CP-55,940 was able to inhibit the adenylate cyclase activity of N18TG2 membranes in a dose-dependent manner, using the protocol previously described (6) (data not shown).

Membrane preparations. Male Sprague-Dawley rats weighing 250 to 370 g were decapitated, and the brains were rapidly removed and dissected on ice. Unless indicated, all results reported were obtained with a washed P_2 preparation prepared as follows. The entire cortices of two or three rats were homogenized with a Dounce glass homogenizer in 45 ml of a solution consisting of 320 mM sucrose, 2 mM Tris·EDTA, and 5 mM $MgCl_2$. The homogenate was centrifuged at 1600 × *g* for 10 min. The supernatant was saved, and the pellets were washed twice as above. The combined supernatant fractions were then centrifuged at 39,000 × *g* for 15 min. The pellet was resuspended in 90 ml of buffer A (50 mM Tris·HCl, pH 7.0 at 30°, 2 mM Tris·EDTA, 5 mM $MgCl_2$), incubated at 37° for 10 min, and centrifuged at 23,000 × *g* for 10 min. The membranes were resuspended in buffer A, incubated at 30° for 40 min, and centrifuged at 11,000 × *g* for 15 min. These two washing steps were found to be important for observing a single homogeneous binding site in equilibrium studies (see Results). The final pellet was resuspended in buffer B (50 mM Tris·HCl, pH 7.4 at 30°, 1 mM Tris·EDTA, 3 mM $MgCl_2$) at a protein concentration of 4 to 5 mg/ml and stored at −80°. Storage for up to 6 weeks had no noticeable effect on binding. Protein values were determined by the method of Bradford (17) using bovine γ-globulin as the standard. Some of the studies were also performed using a synaptosomal preparation derived from the hippocampus plus prefrontal cortex of the rat. The synaptosomal preparation was made following the protocol of Dodd *et al.* (18) with several modifications. The initial homogenization was performed using a 50-ml Dounce glass homogenizer and the homogenate was centrifuged in 50-ml tubes at 1600 × *g* for 10 min. The differential sedimentations over 1.2M and 0.8M sucrose were performed at 50,000 rpm in a Beckman Ti50 rotor for 12 min. The final synaptosomal pellet was resuspended to 5 mg/ml protein in a buffer containing 25 mM Tris·HCl, pH 7.4, 1 mM Tris·EDTA, and 16.6 mM sucrose and was stored at −80°.

Ligand binding assays. Ligand binding assays were performed in Regisil-treated test tubes in a volume of 1 ml containing buffer B, radioligand, and cannabinoid drugs as specified. The incubation was started by the addition of 20 to 50 μg of membrane protein. With the exception of the kinetic experiments, all reactions were carried out at 30° for 50 min. After incubation, the samples were transferred to 1.5-ml polypropylene microfuge tubes and immediately centrifuged for 9 min at 13,000 × *g*. After centrifugation, the supernatant was aspirated and counted to determine the concentration of free [^{3}H]CP-55,940. The microfuge tubes were drained on drying pins for 30 min, after



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$^3H_2$ -CP-55,940

Fig. 1. The synthesis of [^{3}H] CP-55,940 by the tritium reduction of compound 1.

which the tips of the tubes were sliced, using a heated spatula and a cutting block designed to ensure that the cut tips were of identical size. The tips were then placed in scintillation vials and submersed in 2 ml of a solubilizing solution (5% ethanol, 5% Triton X100, 0.2 N NaOH). The vials were then shaken for at least 4 hr in order to dissolve the pelleted membranes. Scintillation cocktail (10 ml) was added to each vial and the radioactivity was determined using a Beckman LS1800 with an efficiency for tritium of 30%. Nonspecific binding to the microfuge tip was assessed in control tubes having radioligand but no protein (typically 2% to 3% of the total radioactivity available in the incubation medium). Subtracting this value from total binding gave the total binding in the pelleted membranes. Specific binding was defined as the difference between total binding to the membranes in the absence and presence of either 100 nM DALN or 1 μM Δ^9-THC. Nonspecific binding to the membranes was typically 15% to 30% of the total binding to the membranes, dependent upon the membrane and ligand concentrations (see Fig. 2). Assays were carried out in triplicate with an average coefficient of variation for the samples of 2.5%, and experiments were repeated at least three times.

Metabolism of [^{3}H]CP-55,940. To determine whether [^{3}H]CP-55,940 was metabolized during the binding assay, 40 μg of membrane protein were incubated with 70 pM [^{3}H]CP-55,940 for 90 min at 30° in the standard assay buffer described above. After centrifugation, the supernatant and the pellet were separated and the radioactivity was extracted from each using ether. After drying with N_2 gas, the samples and an unincubated [^{3}H]CP-55,940 control were resuspended in absolute ethanol, and thin layer chromatography was performed using the procedure described above. The plate was sprayed with EN^{3}HANCE (NEN, Boston, MA), placed against Kodak X-Omat film and stored at −80° for 6 days. Upon developing, a single band was observed for the samples which comigrated with the control. These studies demonstrated that neither the free nor bound [^{3}H]CP-55,940 was metabolized or chemically modified during the assay procedure.

Results

Conditions for cannabinoid receptor binding. Initial studies addressed the separation of unbound ligand from bound radiolabeled ligand. In agreement with the experience of Roth and Williams (16) and Harris *et al.* (19) using THC as the labeled ligand, separation of free [^{3}H]CP-55,940 using a filtration technique met with little success. The binding of ligand to glass fiber and cellulose nitrate or cellulose acetate filters was excessive and varied with the concentration of radioligand added. Treatment of the filters with various organic solvents, detergents, polyethyleneimine, or bovine serum albumin did not provide acceptable conditions for separation. Separating the free [^{3}H]CP-55,940 by adsorption onto dextran-coated charcoal was also unsuccessful (see also Ref. 19). Optimal conditions were achieved using the sedimentation procedure described above. The incubations were performed in Regisil-treated glass tubes in an effort to minimize the adsorption of cannabinoid compounds to the surface (20). Similarly, the presence of bovine serum albumin in the incubation mixture would also effectively decrease the amount of cannabinoid drug bound to the glassware (16). Any alterations in the free concentration of [^{3}H]CP-55,940 resulting from adherence to the glassware during the incubation were accounted for by determining the exact amount of radiolabeled ligand in the supernatant and adhering to the microfuge tube after the sedimentation for each assay.

To determine the optimal incubation conditions for binding, several different buffers were tried at various concentrations and pH values. Of the buffers tested, including K^+ HEPES, K^+*N-tris[Hydroxymethyl]methyl-2-aminoethanesulfonate, K^+ phosphate, and imidazole Cl^-, none performed any better than 50 mM Tris Cl^-. Optimal binding was observed between pH 7 and 8, with specific binding of only 60% of optimal at pH 6 or 9 (data not shown). The phenolic moiety of [^{3}H]CP-55,940 might be expected to have a pK in the vicinity of pH 10. The 40% loss of specific binding observed between pH 8 and 9 cannot be entirely accounted for by a modification of the ligand. The pH requirement can be postulated to also reflect the optimal pH for amino acids that would interact with the ligand or be required to maintain the optimal protein conformation.*

Under the experimental conditions used, linear binding extended from 10 to 50 μg/ml of protein for the P_2 membrane preparation (data not shown). Experiments were routinely conducted using 20 to 40 μg/ml P_2 protein, and in this range specific binding typically represented 85% of the total binding. Specific binding was linear through 120 to 150 μg/ml of protein for the synaptosomal preparation (data not shown). As the concentration of membranes was increased, there was a decline in the percentage of the total binding that could be described as specific.

Thermolability of the specific binding was demonstrated. Membrane preparations that were incubated at 60° for 12 min before assay failed to show significant specific binding (data not shown). These findings would be consistent with the binding of [^{3}H]CP-55,940 to a protein component of the membranes that is subject to thermal denaturation.

Characterization of the [^{3}H]CP-55,940 binding site. [^{3}H]CP-55,940 binding to cortical membranes was saturable, whereas nonspecific binding continued to increase with increasing concentrations of [^{3}H]CP-55,940 (Fig. 2A, *inset*). An example of a saturation binding isotherm and the Scatchard plot obtained therefrom are depicted in Fig. 2. A K_d of 133 ± 11 pM was obtained by Scatchard transformation (21) of the data from four experiments (mean ± standard error). The density of binding sites for the P_2 cortical preparation was 1.85 ± 0.26 pmol/mg of protein (four experiments). The data were analyzed by the Hill transformation (21), yielding a straight line (Fig.

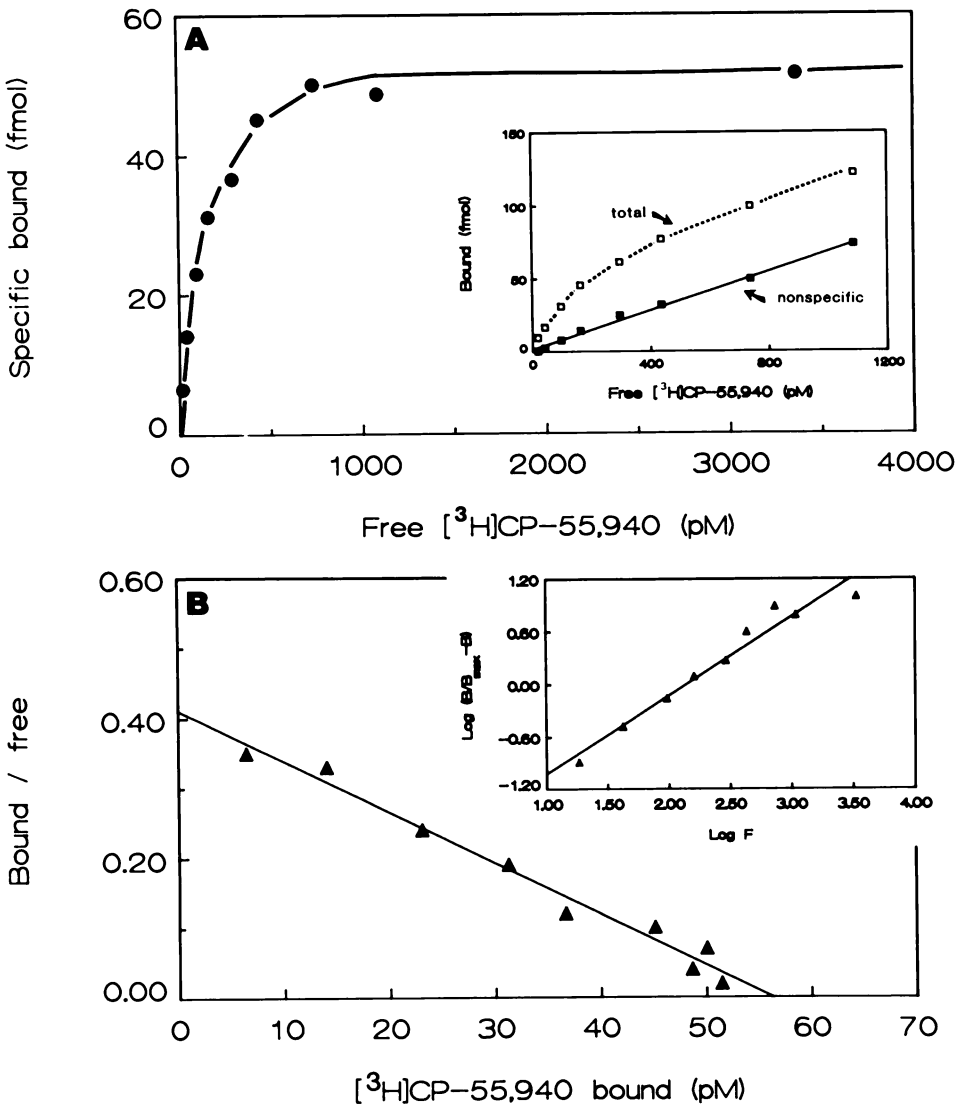

Fig. 2. Equilibrium binding of [^{3}H]CP-55,940. Membranes (43 μg of protein) were incubated with various concentrations of [^{3}H]CP-55,940. A, The saturation isotherm of specific binding. *Inset;* binding of [^{3}H]CP-55,940 in the absence (□) or presence (■) of 1 μM DALN. B, Scatchard transformation of [^{3}H]CP-55,940 binding data from A with the bound ligand being expressed in terms of concentration (pM). This experiment exhibited K_d and B_{max} values of 139 pM and 1.3 pmol/mg of protein, respectively. *Inset;* The Hill transformation of data from A. *F*; free drug concentration; *B*; specifically bound drug. The Hill coefficient (n_H) was calculated to be 0.90 for this experiment. The *lines* drawn represent the best fit as determined by least squares linear regression analysis.

2B, *inset*). The K_d derived from such analysis was 116 ± 12 pM and the n_H was 0.88 ± 0.08 (four experiments). The observation that the n_H approaches one suggests that a single class of binding sites is being labeled by [^{3}H]CP-55,940 under the assay conditions described and that no significant cooperativity exists among binding sites.

Kinetic analysis of the binding of [^{3}H]CP-55,940 to P_2 membranes indicates a rapid association of the ligand with the receptor (Fig. 3A). Equilibrium was reached rapidly, with greater than 90% of maximal specific binding attained within 50 min at 30°. The binding plateau remained stable for at least 2 hr. This is consistent with the determination that the radioligand is not being metabolized or chemically altered during the incubation (see Experimental Procedures). The nonspecific binding component reached steady state at the earliest time point measurable and underwent no further change through 3 hr.

The dissociation of the [^{3}H]CP-55,940-receptor complex initiated by the addition of 100 nM DALN is depicted in Fig. 3B. These studies were performed by establishing equilibrium directly in the microfuge tubes rather than by transferring the reaction mixture before sedimentation. Semilog plots suggest that dissociation was not monophasic (Fig. 3B). When the microfuge tubes were centrifuged immediately upon addition of 100 nM DALN, 20% of the specific binding at equilibrium was already displaced. It should be noted that the time for complete sedimentation is 10 min. However, one would expect that the major fraction of membranes would have sedimented within the first 3 min of centrifugation. Thus, the data obtained for the earliest time points depicted may represent the displacement occurring during the period of manipulation. Assuming first-order dissociation (21), the k_{-1} for the slower component was 0.016 ± 0.001 min^{-1} (three experiments) ($t_{½}$ = 45 min). A more rapid dissociation could also be discerned, having at $t_{½} \leq$ 11 min ($K_{-1} \geq$ 0.06 min^{-1}). It is possible that these two kinetic states may represent interchangeable forms of the receptor. One mechanism for this may be the transient interaction of the receptor with G proteins either possessing tightly bound GDP or free of guanine nucleotides. Evidence for such an interaction is described below. It is of interest that two binding affinities were not discernible in the equilibrium binding studies. In preliminary studies using unwashed P_2 membranes, it was observed that multiple affinity states could be discerned in equilibrium binding studies (data not shown). An explanation

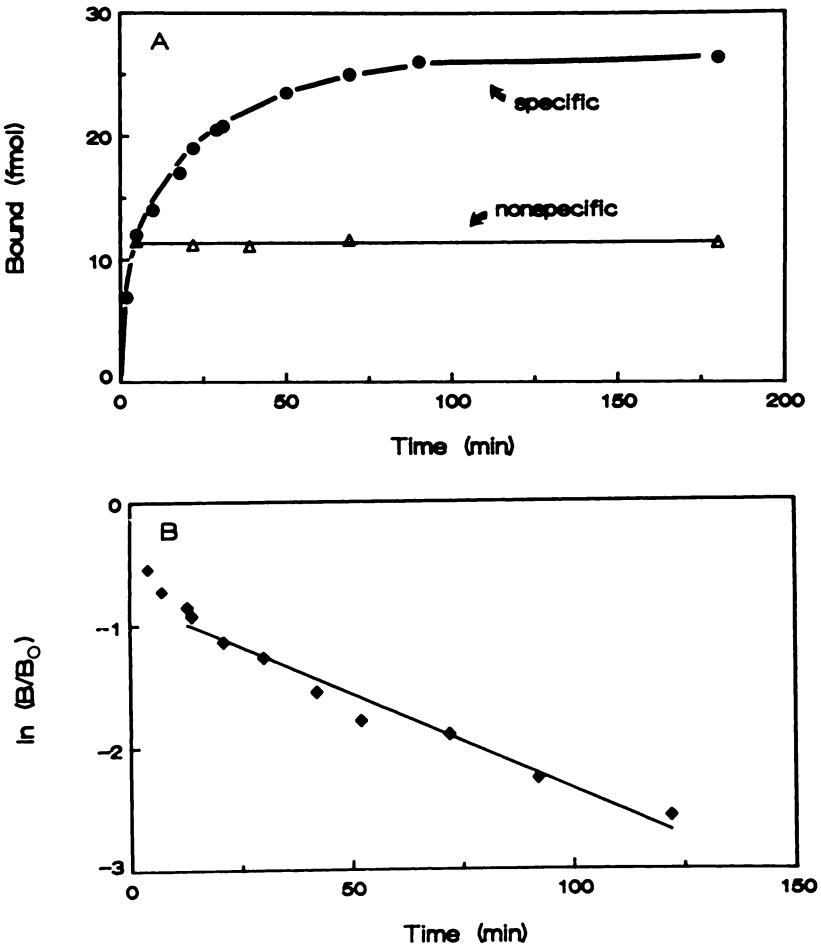

Fig. 3. Time course of association (A) and dissociation (B) of [^{3}H]CP-55,940. [^{3}H]CP-55,940 (81 pM) was incubated with 28 μg of P_2 membranes at 30°. A, The times indicated are those that elapsed between the addition of protein (start of the reaction) and the start of centrifugation of the microfuge tubes. Specific and nonspecific binding were determined with 100 nM DALN as described in Experimental Procedures. B, After equilibrium binding of [^{3}H]CP-55,940 had been reached (70 min), 100 nM DALN was added (t = 0) and dissociation was monitored. Data presented are a first-order representation with B/B_0 denoting the specific binding at the time indicated/specific binding at t = 0. The results are means of triplicate determinations from a single representative experiment, which was performed three times.

for these results might be that the population of G proteins possessing tightly bound GDP could be greater in unwashed membranes.

The K_d for binding may be calculated from the association and dissociation rates. The initial rate of association was estimated by assuming that pelleting of the membranes required 3 min and that the dissociation would not contribute appreciably to the reaction until after 5 min of incubation. The k_{+1} estimated from the initial rate of binding (22) was 3.4 ± 0.76 × 10^{-4} pM^{-1} min^{-1} (three experiments). Using this value, the K_d calculated for the slowly displacing site was 47 pM. The K_d for the rapidly dissociating site would have to exceed 180 pM. An alternative treatment of the data would be to estimate a k_{obs} as the reaction proceeds to equilibrium using a pseudo-first-order method (21, 22). Using the slower dissociation rate, the k_{+1} may be calculated to be 2.6 ± 0.2 × 10^{-4} pM^{-1} min^{-1} and the K_d would be 62 pM. Although both of these methods for estimating the k_{+1} (and thus the K_d values) have theoretical and methodological limitations, the kinetic estimates of the K_d are similar to the values calculated using linear transformation of the equilibrium binding data. Thus, an internal consistency for the methodology has been demonstrated.

Allosteric regulation of binding. One would hypothesize that a receptor that transmits its signal to the adenylate cyclase system via a G protein would be regulated by allosteric mechanisms similar to those that have been demonstrated for other functionally homologous receptors. Our current understanding of the influence of G proteins on agonist-receptor interactions has been reviewed by Birnbaumer and colleagues (23) and Casey and Gilman (24). To summarize briefly, G proteins are believed to exist in a heterotrimer form ($\alpha\beta\gamma$) possessing tightly bound GDP in the presence of Mg^{2+}. Upon interaction with a receptor-agonist complex, a conformational change confers sufficient energy to the system such that the GDP dissociates. In the absence of guanine nucleotides, the receptor-hormone-G protein intermediate complex exhibits a relatively high affinity for the agonist. Upon binding of GTP or a nonhydrolyzable analog to this complex, the affinity of the agonist ligand for the receptor is decreased, and the G protein dissociates from the receptor and separates into α and $\beta\gamma$ subunits. The effector (e.g., adenylate cyclase) interacts with the GTP-bound α subunit. Dissociation of the hormone from the receptor and hydrolysis of the GTP on the α subunit allow the system to perpetually respond to altered concentrations of hormone.

For the cannabinoid receptor, equilibrium binding studies indicated that the presence of 100 μM Gpp(NH)p resulted in a 40% decrease in specific binding of [^{3}H]CP-55,940 (data not shown). The kinetics of dissociation were analyzed in the presence or absence of guanine nucleotide (Fig. 4). The addition of Gpp(NH)p reduced the $t_{1/2}$ from 45 min to 12 min. The k_{-1} calculated for dissociation in the presence of Gpp(NH)p was 0.059 ± 0.009 min^{-1} (three experiments) and the K_d was 176 pM. This value is similar to the K_d estimated for the rapidly dissociating component described above. An in-depth analysis

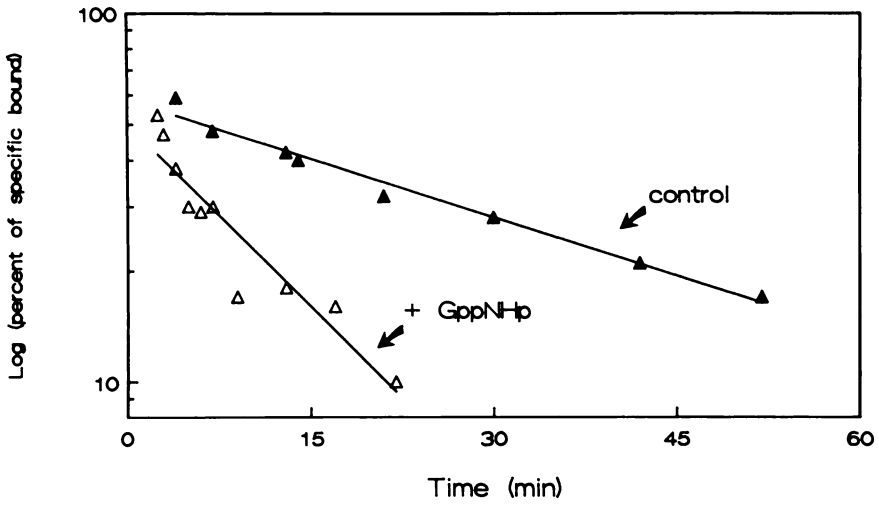

Fig. 4. Effect of Gpp(NH)p on the dissociation rate of [^{3}H]CP-55,940. [^{3}H]CP-55,940 (81 pM) was incubated with 28 μg of P_2 preparation membranes for 70 min at 30° and dissociation was monitored after addition of 100 nM DALN (t = 0). *Control*, addition of 100 nM DALN; *+Gpp(NH)p*, simultaneous addition of 100 nM DALN plus 100 μM Gpp(NH)p. The x axis indicates the time that elapsed between the addition of the above compounds and 2 min after the start of centrifugation. The y axis is a log-scale presentation of the percentage of specific binding at the indicated time/specific binding at t = 0. The data are the means of triplicate determinations from a single representative experiment, which was repeated three times.

of the interaction of the [^{3}H]CP-55,940 binding site with G proteins in various states is beyond the scope of this study. The most facile interpretation of the results presented here is that the [^{3}H]CP-55,940 binding site can be influenced by guanine nucleotides in a manner consistent with the interaction of a receptor with a G protein.

Divalent cations have been reported to influence the affinity of agonists for their receptors. It is believed that the G protein possesses at least one site for Mg^{2+} (23, 24). A role for this divalent cation has been shown for the dissociation of GDP in the presence of the receptor-hormone complex, in addition to other functions associated with a site having a much higher affinity for Mg^{2+} (23, 24). The effects of Mg^{2+} to increase the affinity of agonist ligands for receptors associated with adenylate cyclase have been discussed by Maguire (25). In the present investigation, concentrations of $MgCl_2$ as low as 1 mM stimulated specific binding of the agonist ligand [^{3}H]CP-55,940 by greater than 50% (Fig. 5). Qualitatively similar effects were observed with $CaCl_2$. $MnCl_2$ also stimulated specific binding in a manner similar to $MgCl_2$ (data not shown).

Studies of the opioid receptor, which is coupled to adenylate cyclase in an inhibitory manner, indicated that Na^+ may act as an allosteric regulator (26, 27). Na^+ has been demonstrated to decrease the affinity of agonist ligands for the opioid receptor (see Ref. 26 and references therein). In an effort to determine whether regulation of the binding of [^{3}H]CP-55,940 by monovalent cations could be observed, the effects of various chloride salts were determined (Fig. 5). Monovalent cations were tested at concentrations that might be expected to be present intracellularly or extracellularly. At 20 mM, Na^+ reduced specific binding by about 40%. Low concentrations of K^+ had minimal effects. Concentrations of 120 mM NaCl and 100 mM KCl inhibited specific binding by about 80%. The selectivity of this response to Na^+ does not appear to be great, suggesting that this inhibition may not be the result of a specific interaction with a Na^+ site. It is possible that a chaotropic effect of higher salt concentrations is altering the ability of the ligand to bind to the receptor.

Pharmacology of the cannabinoid receptor. The specificity of [^{3}H]CP-55,940 binding was established by determining the ability of related synthetic compounds and several natural cannabinoid compounds to compete with [^{3}H]CP-55,940 for occupancy of the specific binding sites in the cortical membranes (Fig. 6). Unlabeled CP-55,940 had a K_i of 68 ± 6.2 pM and B_{max} of 1.75 ± 0.20 pmol/mg protein (three experiments) as determined by computer analysis of homologous displacement data using the LIGAND program (version 2.3.11; May, 1987) (28). A one-site model fit the data better than a two-site model for both CP-55,940 and other cannabinoid drugs tested using an F test criterion on the residual variances at the level

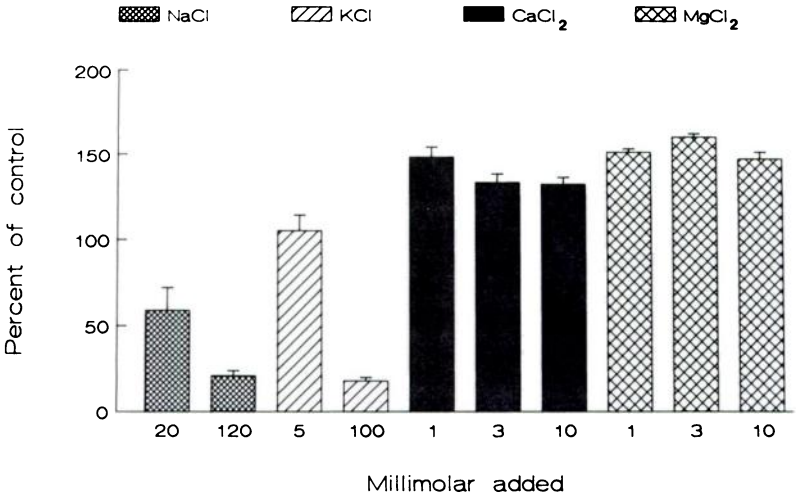

Fig. 5. The influence of cations on specific binding of [^{3}H]CP-55,940. Control samples contained 50 mM Tris·HCl, 1 mM Tris·EDTA, and 0.1 mM $MgCl_2$. Experimental samples contained the same buffer plus the indicated concentrations of salts. The data are the means ± standard error of triplicate determinations from a single representative experiment. All values were different from control at $p < 0.05$ except 20 mM NaCl and 5 mM KCl. Similar results were observed in two other experiments.

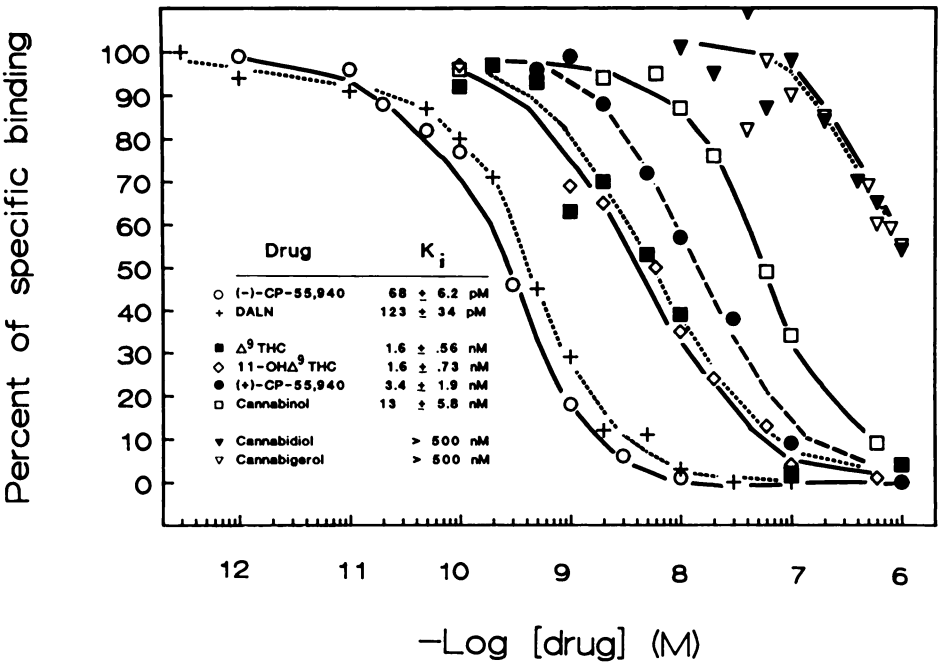

Fig. 6. Competitive inhibition of [^{3}H]CP-55,940 binding by various synthetic and natural cannabinoid drugs. [^{3}H]CP-55,940 (50–70 pM) was incubated with P_2 membranes (16–30 μg) for 50 min at 30° with either the indicated concentrations of drug or vehicle alone. The results were normalized to 100% of specific binding, which was determined with 100 nM DALN as described in Experimental Procedures. Data points represent the averages of triplicate determinations from single representative experiments. The K_i values listed in the *inset table* were determined using the LIGAND program and represent the mean ± standard error of three independent experiments for each drug.

of $p = 0.05$. Using 68 pM as the K_d for [^{3}H]CP-55,940, the K_i values of the various compounds were determined by LIGAND analysis of heterologous displacement data. The (+)-isomer of CP-55,940 was 50-fold less potent than the (−)-isomer, having a K_i of 3.4 nM. DALN was nearly equipotent with CP-55,940, having a K_i of 123 pM. Δ^9-THC and 11-OH-Δ^9-THC both exhibited high affinity binding with K_i values of 1.6 nM. Cannabinol was 8-fold less potent than Δ^9-THC, having a K_i of 13 nM. This order of potency generally parallels the order of potency for both CNS activity *in vivo* (1, 3, 12, 29) and inhibition of adenylate cyclase *in vitro* (7, 8).

Cannabidiol and cannabigerol were much less potent, binding the [^{3}H]CP-55,940 site with K_i values estimated to be greater than 500 nM. Cannabidiol and cannabigerol were unable to fully displace the specifically bound [^{3}H]CP-55,940 at the highest concentrations tested (1 μM). Concentrations greater than this were not tested due to the limited solubility of cannabinoid drugs above 1 μM and the confounding aspects of increasing the solvent or bovine serum albumin concentration, which would be necessary to maintain higher concentrations of cannabinoid compounds in solution. These latter two compounds fail to exhibit cannabinoid activity in humans or animal models (1–3). One of several explanations for the binding results could apply. 1) The two compounds could bind to the receptor with low affinity, but the concentrations required to observe a biological response may not be possible to achieve *in vivo*. 2) The cannabinoid response to these two compounds may be masked by drug effects at high concentrations, such as membrane perturbation in *in vitro* studies and CNS depression in *in vivo* studies. 3) The observed binding displacement may be the result of a contaminant in the drug preparation. The latter explanation is a possible artifact that must be dealt with in future investigations. These drugs were isolated from organic extracts of cannabis that originally contained a variety of cannabinoid compounds, including Δ^9-THC.

Discussion

Studies suggestive of a cannabinoid receptor were based on the ability of centrally active cannabinoid drugs to interact with a well characterized, cellular second messenger system. The ability of cannabinoid compounds to inhibit adenylate cyclase in a reversible, cell type-specific, potent, and enantioselective manner (5–8) would support the hypothesis that these compounds interact with a biological membrane-bound receptor. Additional arguments in favor of a receptor mediating the interaction of cannabinoid drugs with adenylate cyclase are the characteristic guanine nucleotide and divalent cation requirements for this interaction and the demonstrated pertussis toxin sensitivity characteristic of G_i-linked receptors (6, 13).

The findings presented in this study provide the strongest argument currently available for a cannabinoid receptor. The binding site described here is entirely consistent with a receptor that would be associated with a second messenger system via a G protein. The pH sensitivity and thermolability are consistent with a protein structure for this binding site. The rapid and reversible binding are properties expected of a neuromodulator receptor. The binding saturability and the B_{max} determined in the rat cortex are consistent with values reported for CNS neuromodulator receptors (30). The K_d for binding of [^{3}H]CP-55,940 derived from the kinetic constants agrees remarkably with the K_d obtained from equilibrium binding studies. The affinity determined for this agonist ligand is consistent with what would be expected for a neuromodulatory receptor in the CNS (30).

It may be hypothesized that the binding site for [^{3}H]CP-55,940 is linked to adenylate cyclase in the brain. Previous investigations of the inhibition of adenylate cyclase by cannabinoid drugs have utilized a cloned neuroblastoma cell model system. To strengthen the putative association of the cannabinoid receptor with adenylate cyclase in the CNS, we now have evidence using brain slice preparations. Cyclic AMP production in several rat brain regions is decreased in response to cannabinoid drugs (31, 32). The affinity of the agonist [^{3}H]CP-55,940 for its cortical binding site in the absence of guanine nucleotides was more than 2 orders of magnitude greater than its K_{inh} for regulation of adenylate cyclase in the neuronal cell

model (8). However, the affinity state promoted by the addition of Gpp(NH)p would be the prevalent state concurrent with adenylate cyclase regulation (23, 24). The order of potencies for ligand interaction and the enantioselectivity described for this binding site are consistent with previously reported data for the inhibition of adenylate cyclase (7, 8).

One of the responses that the [^{3}H]CP-55,940 receptor site may regulate *in vivo* is analgesia. This ligand was specifically designed to possess potent analgetic activity (12). The analgetic activity for CP-55,940 was demonstrated in the tail flick, hot plate, phenylbenzylquinone writhing, tail clamp, and flinch jump tests in rodents (8, 12). The ratio of the activities of the (−)- to the (+)-isomer in the analgetic tests was 200-fold. This agrees reasonably well with the 50-fold enantioselectivity demonstrated here for the [^{3}H]CP-55,940 binding site. The order of potency for analgetic activity is mimicked by the order of potency reported here for the binding to the receptor. Other functions typical of the cannabinoid class of drugs, including changes in spontaneous locomotor activity, hypothermia, and immobility, have also been demonstrated for CP-55,940 and have been shown to be enantioselective (29). Thus, this receptor site appears to be associated with certain of the typical cannabinoid responses observed in animals in addition to analgesia.

Previous attempts to find and characterize a cannabinoid receptor associated with *in vitro* or *in vivo* functions have not met with success. Harris and colleagues (19) and Roth and Williams (16) demonstrated binding of [^{3}H]Δ^8-THC and [^{3}H]Δ^9-THC, respectively, to crude or purified synaptosomal membranes from rat brains. The former group were able to displace up to 10% of the binding with 1 μM Δ^8-THC; however, the binding was not saturable and pharmacological displacement by other cannabinoid ligands was not performed (19). The latter investigators were unable to discern a high affinity component of binding other than membrane adsorption, which was not dependent upon the concentration of free Δ^9-THC (16).

A high affinity binding site in brain membranes was described by Nye and colleagues (33, 34) using the [^{3}H]5′-trimethylammonium analog of Δ^8-THC. The ligand used for binding to this site does not exhibit biological activity in typical animal behavioral models of cannabinoid action, with the exception of CNS depression (35). This poor biological activity is consistent with our previous demonstration of the importance of maintaining the hydrophobic nature of the alkyl side chain extending from the A-ring (8). The pharmacological profile for displacement of [^{3}H]5′-trimethylammonium Δ^8-THC indicated that cannabinoid compounds having greatest affinity in several brain regions (e.g., cannabigerol and cannabidiol) are neither agonists nor antagonists in *in vivo* animal models or in humans (33). Thus, the pharmacological relevance of this binding site to cannabinoid effects *in vivo* may be questioned. The selectivity of the [^{3}H]trimethylammonium Δ^8-THC binding site for stereoisomers of Δ^9-THC and Δ^8-THC was less than 2-fold, and no stereoselectivity was observed for levonantradol and dextronantradol (33). The kinetic constants derived for the binding of [^{3}H]trimethylammonium Δ^8-THC yielded a K_d that was 3 orders of magnitude lower than the K_d determined by Scatchard analysis of the equilibrium binding data (34). This unusual finding may in part be explained by the observation that, throughout these experiments, the aqueous solubility and adsorption to glass of the ligands were not considered (33). Evidence suggests that this binding site is not linked to a G protein inasmuch as binding of [^{3}H]trimethylammonium Δ^8-THC was enhanced rather than decreased by nonhydrolyzable analogs of guanine nucleotides (34). It is clear that the [^{3}H]trimethylammonium Δ^8-THC binding site is definitely different from the cannabinoid receptor described here using [^{3}H]CP-55,940 as the ligand.

Other laboratories have suggested that cannabinoid drugs alter the binding of other neuromodulators to their receptors. Hillard and Bloom (36) reported that concentrations in excess of 3 μM Δ^9-THC or 11-OH-Δ^9-THC in the presence of a detergent vehicle increased the specific binding of the β-adrenergic antagonist ligand [^{3}H]dihydroalprenolol in mouse cortical homogenates. The interpretation of this finding was that the cannabinoid drugs altered membrane properties such that the binding behavior was modified (36). This mechanism is supported by additional studies from that laboratory, which demonstrated that similarly high concentrations of cannabinoid drugs altered fluidity of synaptic plasma membranes as detected by fluorescence polarization (37). Vaysse and colleagues (38) reported a similar interference with certain binding assays for opioid receptors by addition of high concentrations of several cannabinoid drugs in the presence of 100 mM ethanol. We have previously demonstrated that the site of cannabinoid action in neuroblastoma cells is not related to the binding of agonists to the δ opioid receptor or to subsequent signal transduction (14). The studies reported in the present work clearly indicate the presence of a pharmacologically selective, high affinity binding site for cannabinoid drugs. However, membrane perturbation may be the mechanism by which high concentrations of cannabinoid drugs may interfere with binding determinations made for a variety of other receptor types.

The development of a ligand binding assay for the cannabinoid class of drugs will allow investigations of cannabinoid actions that have previously not been possible. Pathways in the brain that may be involved in cannabinoid action can be examined. The cellular regulation of the receptor can be more fully characterized and its interaction with alternative second messenger systems can be assessed. Perhaps an antagonist for the cannabinoid drugs can be developed now that a binding site has been found that correlates with a cellular function (inhibition of adenylate cyclase). Furthermore, efforts to search for a putative endogenous ligand can now proceed. Thus, the importance of the characterization of a cannabinoid receptor will make a major impact on research in this field.

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