6-895X/88/030297-06$02.00/0

)LECULAR PHARMACOLOGY, 33:297-302

Nonclassical Cannabinoid Analgetics Inhibit Adenylate Cyclase: Development of a Cannabinoid Receptor Model

LLYN C. HOWLETT, M. ROSS JOHNSON, LAWRENCE S. MELVIN, and GEORGE M. MILNE

epartment of Pharmacology, St. Louis University Medical School, St. Louis, Missouri 63104 (A.C.H.) and Pfizer Central Research, Groton, onnecticut 06340 (M.R.J., L.S.M., G.M.M.)

eceived October 12, 1987; Accepted December 16, 1987

UMMARY

Extensive structure-activity relationship studies have demonstrated that specific requirements within the cannabinoid structure are necessary to produce potent analgesia. A three-point association between the agonist and the receptor mediating analgesia consists of: 1) the C ring hydroxyl, 2) the phenolic A ring hydroxyl, and 3) the A ring alkyl hydrophobic side chain. Potent tricyclic and bicyclic structures were synthesized as "nonclassical" cannabinoid analgetics that conform to this agonist-receptor three-point interaction model. At the cellular level, centrally active cannabinoid drugs inhibit adenylate cyclase activity in a neuroblastoma cell line. The structure-activity relationship profile for inhibition of adenylate cyclase *in vitro* was consistent with this same three-point association of agonists with the receptor. A correlation exists between the potency of drugs to produce analgesia *in vivo* and to inhibit adenylate cyclase *in vitro*. Enantio- and stereoselectivity were exhibited by the nonclassical cannabinoid compounds for both the analgetic response and the ability to inhibit adenylate cyclase. The magnitude of the enantioselective response was equal for both the biochemical and physiological endpoints. Based on the parallels in structure-activity relationships and the enantioselective effects, it is postulated that the receptor that is associated with the regulation of adenylate cyclase *in vitro* may be the same receptor as that mediating analgesia *in vivo*. A conceptualization of the cannabinoid analgetic receptor is presented.

Folklore medicinal uses of various preparations of *Cannabis sativa*, including marihuana and hashish, have been described (see Refs. 1–3 for recent reviews). Use as an analgetic agent is one potential therapeutic application of cannabinoid drugs in modern medicine (4). Δ^9-THC is the primary centrally active compound isolated from marihuana extracts. The analgetic activity of Δ^9-THC was enhanced by chemical modification of the structure to form HHC (5). Subsequent investigations from Pfizer Central Research laboratories were initiated to define the pharmacophore for analgesia in the Δ^9-THC and HHC structures. A series of structure-activity relationship studies was performed using a battery of *in vivo* tests for analgesia. This work resulted in the development of a model of three-point contact between the agonist and the putative cannabinoid receptor mediating analgesia (6–8). The important functional groups for agonist-receptor interaction were predicted to be: 1) the C ring hydroxyl, 2) the phenolic A ring hydroxyl, and 3) the A ring alkyl side chain (see Fig. 1). Molecules that possess these structural features and retain potent analgetic activity go far beyond the scope of classic ABC tricyclic cannabinoids. Thus, the compounds that exhibit the structural features required for analgesia and lack the ABC tricyclic benzopyran nucleus of cannabinoids are referred to as nonclassical cannabinoid analgetics. This work has recently been reviewed (9).

The mechanism of action of nonclassical cannabinoid analgetics is currently unknown. The demonstration that the opioid antagonist naloxone fails to block analgesia produced by these compounds suggests that opioid receptors are not required for the response (10, 11). The very potent analgetic activity of these drugs, the defined structure-activity relationships, and the enantioselectivity demonstrated for the nonclassical cannabinoid analgetics are properties consistent with the presence of neuronal receptors for this class of drugs. The nature of these receptors has yet to be determined.

Recent studies have demonstrated that the N18TG2 neuroblastoma cell in culture provides a suitable model system for the study of cannabinoid drugs at the cellular level. Cyclic AMP accumulation is attenuated by centrally active cannabinoid drugs in this cell line (12). This effect can be attributed to the rapid and reversible inhibition of adenylate cyclase (EC 4.6.1.1) via a G-protein component of the enzyme (13–15). The active cannabinoid compounds fail to interact with the δ-opioid, muscarinic cholinergic, or α_2-adrenergic receptors known to inhibit

This work was supported in part by United States Public Health Service Grants RO1-DA03690 and KO4-NS00868 to A. C. H.

ABBREVIATIONS: THC, tetrahydrocannabinol; HHC, 9-nor-9β-hydroxyhexahydrocannabinol; PDQ, phenylbenzylquinone; MPE, maximum possible effect; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; EDTA, ethylenediaminetetraacetic acid; DALN, desacetyllevonantradol.

adenylate cyclase in membrane fractions from these cells (13, 16). Thus, it may be hypothesized that a unique receptor exists to mediate the effects of centrally active cannabinoid compounds in the adenylate cyclase system.

The goal of the present investigation was to determine if the putative receptor that is associated with inhibition of adenylate cyclase may be the same pharmacological receptor that mediates the analgetic response. Toward this end we have shown that compounds having potent analgetic activity *in vivo* are potent inhibitors of adenylate cyclase *in vitro*. Compounds that fail to meet the structural requirements determined for analgesia are poor or inactive as effectors in the adenylate cyclase assay.

Experimental Procedures

Materials. Levonantradol hydrochloride, *N*-methyllevonantradol hydrochloride, dextronantradol hydrochloride, HHC, CP-42,096, CP-47,497, (−)-CP-55,940, (+)-CP-55,940, (−)-CP-55,244 and (+)-CP-55,244 were synthesized at Pfizer Central Research. Δ^9-THC was obtained from the National Institute on Drug Abuse. Sources of reagents for the adenylate cyclase assay were detailed previously (13, 14).

Analgetic activity. Biological assays have been described previously for the analgetic tests (10). Drugs were administered subcutaneously to mice (for the PBQ-induced writhing, tail flick, and hot plate tests) or rats (tail pinch and flinch jump tests). The PBQ writhing, tail flick, and hot plate tests were performed 1 hr after, and tail pinch and flinch jump were performed 2 hr after drug administration. Data from these standard tests of analgesia were first calculated as percentage MPE (% MPE). Mean % MPE data at various doses were subjected to least squares regression analysis to obtain an MPE_{50} value (i.e., best estimate of the dose at which 50% of the maximum possible effect is observed).

Adenylate cyclase determinations. Cell culture and membrane preparation procedures were as previously described (14). Adenylate cyclase activity was determined in membranes from at least four separate preparations. Incubations were started by the addition of 10 μg of membrane protein to a 100-μl reaction volume containing: 50 mM Na-HEPES, pH 8.0, 1 mM EDTA, 5 mM $MgCl_2$, 100 μM GTP, 100 μM 4-(3-butoxy-4-methoxybenzyl)-2-imidazolidinone (RO 20-1724), 100 μM cyclic [^{3}H]AMP (10 nCi), 100 μg/ml fatty acid-deficient bovine serum albumin, 10 μg/ml pyruvate kinase, 3 mM phosphoenolpyruvate, 500 μM [^{32}P]ATP (0.5 μCi), 500 nM secretin, and compounds to be tested at the indicated concentrations. Assays were performed at 30° for 20 min, and the reaction was stopped and cyclic [^{32}P]AMP isolated according to the method of Salomon *et al.* (17). Triplicate determinations were made for each concentration tested within each experiment. For each experiment, the data were standardized such that the inhibition by 200 nM compound **8** was defined as maximal (100%) inhibition, and the results of multiple experiments were combined for analysis. Values of K_{inh} (inhibition constant) and the slope factor were calculated using the Hill equation (18).

Results

Analgetic activity. The development of tri- and tetracyclic nonclassical cannabinoid analgetic compounds based on the HHC structure is depicted in Fig. 1. Analgetic activity for each of these compounds was determined using several different tests (Table 1). Since these compounds altered the reactions to thermal, mechanical, and chemical discomfort, it cannot be argued that a single sensory modality was affected by these compounds. The modification of the ring A alkyl side chain in

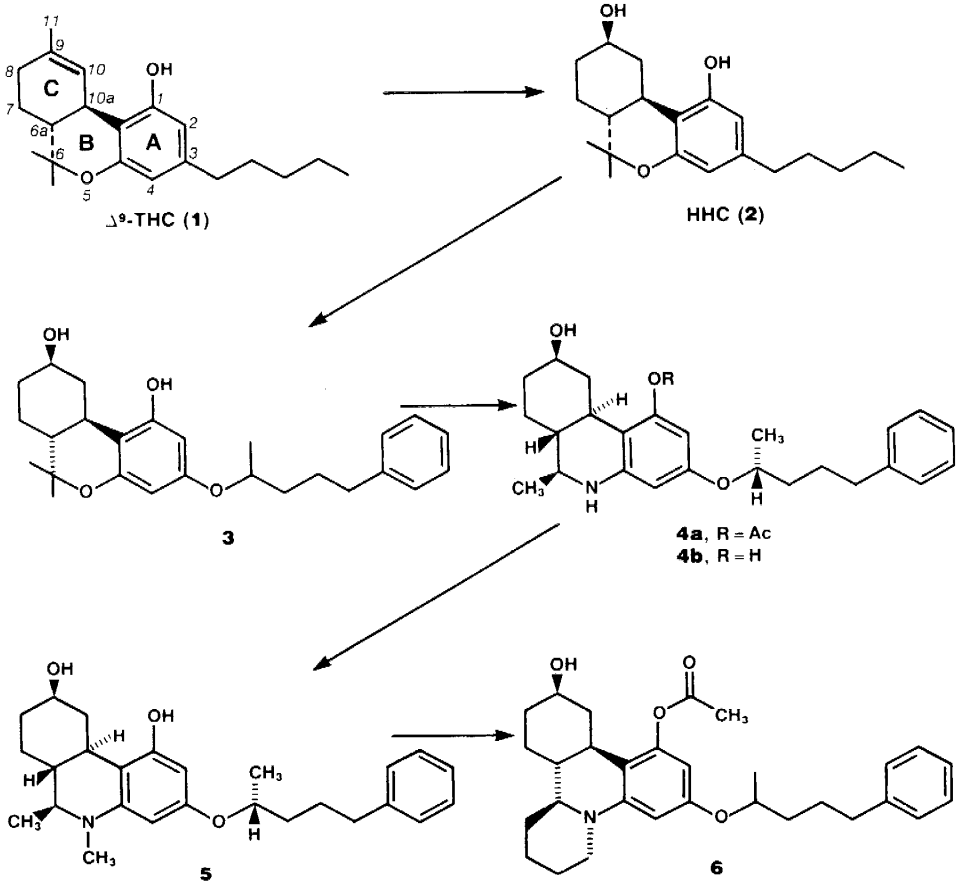

Fig. 1. Evolution of ABCE-tetracyclic cannabinoids.

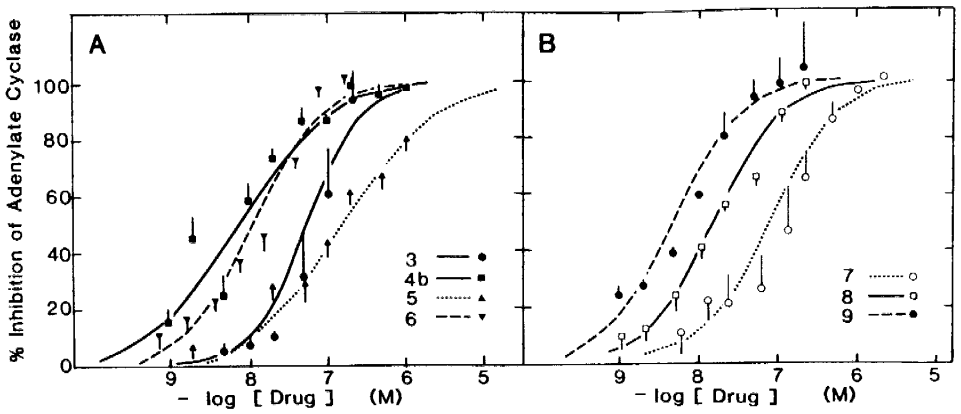

Fig. 3. Log dose response curves for inhibition of adenylate cyclase by nonclassical cannabinoid analgetics. Enzyme activity was determined as described in the text. The percentage of inhibition of secretin-stimulated activity was calculated for each drug at the indicated concentration and standardized such that the inhibition by 200 nM compound **8** was 100% inhibition. The data points are the means from three (compounds **3** and **9**), four (compound **6**,) six (compounds **4b**, **5**, and **8**), or seven (compound **7**) experiments, and the error bars are the standard errors. Secretin-stimulated adenylate cyclase activity was 160 ± 7 pmol/min/mg of protein and the inhibition of activity by 200 nM (compound **8**) was 36 ± 1% for these sets of experiments (mean ± standard error).

TABLE 2

Inhibition of adenylate cyclase by prototypical nonclassical cannabinoid drugs

Compound	K_{inh}
	nM
Δ^9-THC (**1**)	430
(±)-**3**	70
Levonantradol [(−)-**4a**]	100
DALN [(−)-**4b**]	7
(−)-**5**	126
(±)-**6**	10
(−)-**7**	79
(−)-**8**	25
(−)-**9**	5

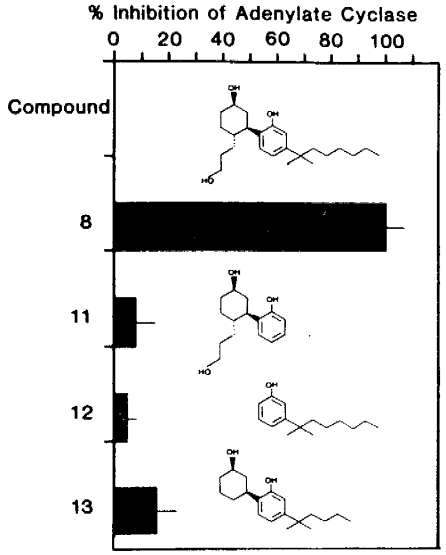

Fig. 4. Structural requirements for inhibition of adenylate cyclase. Inhibition of secretin-stimulated activity was standardized for each experiment such that the inhibition by 200 nM compound **8** was 100% inhibition. Compounds **11**, **12**, and **13** were present at 2 μM. The data are expressed as the mean ± standard error for four (compounds **12** and **13**), five (compound **11**), or six (compound **8**) experiments. Secretin-stimulated adenylate cyclase was 121 ± 11 pmol/min/mg of protein and the inhibition of activity by 200 nM compound **8** was 31 ± 2% for these sets of experiments (mean ± standard error).

To test the hypothesis that a three-point association exists for the drug interaction with the receptor mediating inhibition of adenylate cyclase, the prototype AC-bicyclic compound **7** was examined (Fig. 3B, Table 2). Compound **7** inhibited adenylate cyclase with a K_{inh} of 79 nM. The addition of the side chain on the C ring of **8** increased the potency by greater than 5-fold. The further increase in structural rigidity of **9** enhanced the potency to inhibit adenylate cyclase by another 5-fold. Each of these compounds was as efficacious as DALN. Slope factors for each curve approximated 1.

Compounds that did not conform to the three-point attachment model were not analgetically active (9, 20). Fig. 4 demonstrates that such compounds also were not able to inhibit adenylate cyclase. Compound **11**, lacking the A ring alkyl side chain of **8**, was inactive in analgetic tests. At concentrations up to 10 μM, **11** did not inhibit adenylate cyclase. Compound **12**, lacking the C ring and substituents of **8**, was not analgetic in animal tests and had no ability to inhibit adenylate cyclase at concentrations up to 10 μM. The optimal length of the alkyl side chain was equivalent to 7 carbons for analgetic activity. Structures having shorter alkyl chains extending from the A ring were significantly less potent or inactive as analgetics (20). Compound **13**, having only a 5-carbon alkyl chain, produced only 5% inhibition of adenylate cyclase at 3 μM.

Enantiospecificity. Drugs that are active as the result of a molecular interaction with a specific cellular receptor should be topologically complimentary to the stereochemical nature of the receptor. We sought to strengthen our structural arguments concerning the receptor site for cannabinoid analgetics by correlating enantiospecificity of analgetic action and inhibition of adenylate cyclase activity (Table 3). The simplest flexible system **7** exhibited a low enantioselectivity for analgetic action as well as for adenylate cyclase regulation. However, for the more analgetically active compounds **4a** and **8**, the receptors mediating both analgetic and adenylate cyclase-regulatory responses favor an active enantiomer by at least 2 orders of magnitude. The conformationally more rigid compound **9**, exhibiting the greatest potency both *in vivo* and *in vitro*, proved to be the ligand having the greatest degree of enantioselectivity (>2000-fold).

Discussion

Although various preparations of *Cannabis sativa*, including marihuana and hashish, have been used for analgesia for many

TABLE 3
Enantioselectivity demonstrated for analgetic activity *in vivo* and adenylate cyclase activity *in vitro*

Compound	PBQ-induced writhing MPE_{50}				Adenylate cyclase K_{inh}		
	(−)	(+)	(±)	Ratio (+/−)	(−)	(+)	Ratio (+/−)
	mg/kg				*nM*		
Nantradol (**4a**)	0.07	6.5	0.43	81	100	>5000	>50
7	0.55	1.5	1.00	3	79	135	1.7
8	0.059	14.6	0.29	209	25	>5000	>200
9	0.018	>100	0.054	>6300	5	[a]	>2000

[a] No inhibition at 10 μM.

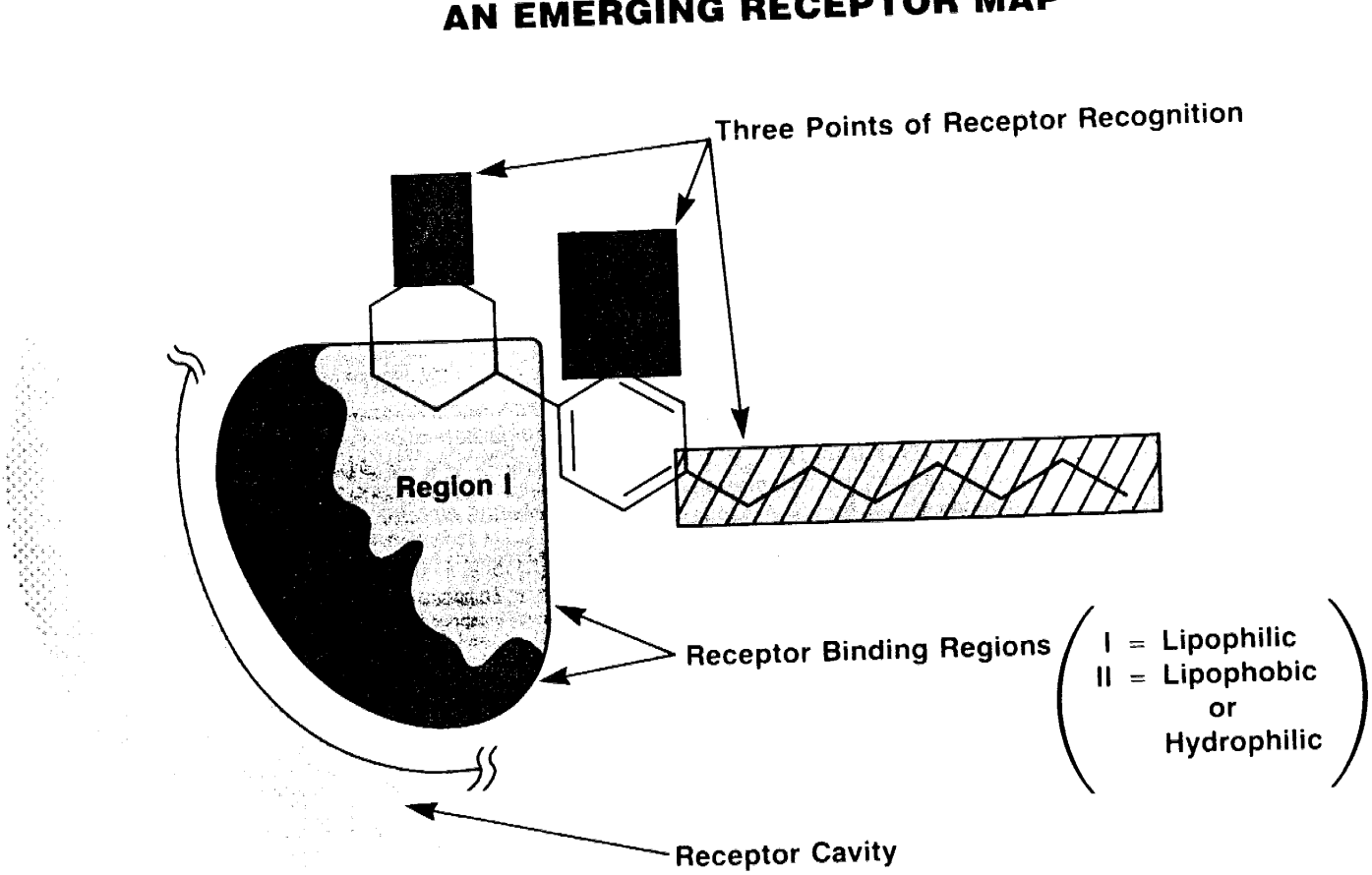

Fig. 5. Conceptualization of the analgetic cannabinoid agonist-receptor interaction. A prototypical agonist would be expected to minimally consist of the bicyclic structure shown. Elaborations of the structure in the *yellow* region must interact with lipophilic sites and those in the *green* region must interact with hydrophilic sites on the putative receptor. The three minimal points of agonist-receptor interaction are denoted by *slashes*.

centuries (2, 3), a good medicinal chemical rationale for the action of Δ^9-THC has only recently been investigated. Based on a molecular hypothesis explaining the analgetic activity of HHC, a synthetic program probing new structure-activity relationships was evolved. Initial studies revealed that a new grouping, the 1-methyl-4-phenylbutyloxy C-3 side chain, elaborates a unique lipophilic region (e.g. compound **3**), and conferred increased potency as an analgetic. Introduction of a weakly basic nitrogen at C-5 and deletion of the axial methyl group in the B ring resulted in a unique family of phenanthridines having potent analgetic activity. The prototype of this series, levonantradol (**4a**), exhibited potent analgetic activity (Table 1). *N*-alkylation of DALN led to an even more analgetically potent compound **5**, which in turn led to the more structurally rigid but highly potent new ring system exemplified by compound **6**.

These early studies demonstrated that the alkylated pyran ring of HHC was not required for analgetic activity. However, the C ring hydroxyl, the phenolic A ring hydroxyl, and the A ring hydrophobic side chain were recognized to be of significant importance for analgetic activity. Thus, it was hypothesized that the agonist-receptor interaction must minimally require these three points of contact. In order to test this three-point receptor-binding model, the nonclassical AC-bicyclic cannabi-

noids, devoid of the HHC pyran ring, were synthesized (8). Compound **7** is the simplest prototypical AC-bicyclic compound to comprise the lipophilic side chain, phenol and alcohol. This compound was shown to possess a biological profile and potency similar to those of HHC (Table 1). As was the case for the tricyclic compounds, such activity and potency were highly dependent on the side chain but were not influenced to as great an extent by substitution in the cyclohexanol ring. Further structural elaboration and development of structure-activity relationship studies around compound **7** led to the more potent AC-bicyclic derivative **8**. The hydroxypropyl analog **8** is shown in Table 1 to be significantly more potent than the tricyclic HHC or the simplest AC-bicyclic **7**.

Assuming the optimum substitution, hydroxypropyl, at C-4 of the cyclohexane ring of **7** had been found, the potential for stereochemical selectivity of this new primary alcohol group was explored. To this end, a more complex ring system, **9**, which incorporates a rigidly positioned hydroxypropyl moiety, was prepared (21). From Table 1, it is seen that the rigid decalin **9** is 2- to 10-fold more potent than the less rigid structure **8**. A suggestion that the axial hydroxymethyl group of **9** is optimally oriented comes from the lessened (52×) potency observed for the equatorial isomer **10**. The potency and enantioselectivity of these synthetic, nonclassical cannabinoids, and their retained activity at the extremes of structural elaboration, are evidence to support the existence of a pharmacologically distinct receptor mediating the analgetic response *in vivo*.

As was the case for analgetic activity, a decrease in K_{inh} for adenylate cyclase activity occurred in going from HHC to the more potent side chain of **3**. Further potent inhibition occurs with the nantradol series, compounds **4b, 5,** and **6**. A similar trend is apparent in the series **7–9** wherein increasing analgetic potency is accompanied by increasingly potent inhibition of adenylate cyclase. Enantiospecificity is evident for the inhibition of adenylate cyclase by these highly potent compounds. Thus, it can be tentatively concluded that the receptor mediating the inhibition of adenylate cyclase *in vitro* is similar or identical to the receptor mediating analgesia *in vivo*.

The extensive body of structure-activity relationship studies developed in this series of compounds leads us to propose the conceptual model of the cannabinoid receptor illustrated in Fig. 5. The three points of contact that are believed to be minimally necessary for receptor recognition of the agonist are the hydrophilic C ring hydroxyl and the phenolic A ring hydroxyl, and the hydrophobic alkyl chain extending from the A ring. Optimal chain length for this group appears to be equivalent to a 7-carbon alkyl unit. More potent agonist activity can be obtained from compounds having a hydrophobic nature in the area, referred to in Fig. 5 as Region I (compounds **5** and **6**). However, extensions beyond Region I that possess hydrophilic functional groups (Region II) confer the greatest potency to compounds **8, 9,** and **10**. Steric barriers exist to prevent further extensions of agonist molecules beyond Region II.

These studies have provided a pharmacologic picture of the cannabinoid receptor mediating analgesia and inhibition of adenylate cyclase. Such a receptor would be predicted to be present on neurons in the brain and/or spinal cord in pathways expected to integrate responses to pain. One way to address this question would be to detect receptors present in specific peripheral and central nervous system regions using a radioligand binding assay. Current studies are underway to develop a radioligand binding assay using these potent, stereoselective cannabinoid analgetics. It is expected that these future studies will advance our understanding of pain mechanisms at the organismal as well as at the cellular level.

Acknowledgments

The excellent technical assistance by Gerald Wilken and maintenance of cell cultures by Jill Dill are greatly appreciated. The assistance of Linda Russell with word processing is also appreciated.

References

1. Bhargava N. N. Potential therapeutic applications of naturally occurring and synthetic cannabinoids. *Gen. Pharmacol.* **9**:195–213 (1978).
2. Mechoulam, R. The pharmacohistory of *Cannabis sativa*, in *Cannabinoids as Therapeutic Agents* (R. Mechoulam, ed.). CRC Press, Boca Raton, FL, 1–20 (1986).
3. Lemberger, L. Potential therapeutic usefulness of marijuana. *Annu. Rev. Pharmacol. Toxicol.* **20**:151–172 (1980).
4. Segal, M. Cannabinoids and analgesia, in *Cannabinoids as Therapeutic Agents* (R. Mechoulam, ed.). CRC Press, Boca Raton, FL, 105–120 (1986).
5. Wilson, R. S., and E. L. May. Analgesic properties of the tetrahydrocannabinols, their metabolites, and analogs. *J. Med. Chem.* **18**:700–703 (1975).
6. Johnson, M. R., L. S. Melvin, and G. M. Milne. Prototype cannabinoid analgetics, prostaglandins and opiates—a search for points of mechanistic interaction. *Life Sci.* **31**:1703–1706 (1982).
7. Milne, G. M., and M. R. Johnson. Levonantradol: a role for central prostanoid mechanisms. *J. Clin. Pharmacol.* **21**:367S–374S (1981).
8. Melvin, L. S., M. R. Johnson, C. A. Harbert, G. M. Milne, and A. Weissman. A cannabinoid derived prototypical analgetic. *J. Med. Chem.* **27**:67–74 (1984).
9. Johnson, M. R., and L. S. Melvin. The discovery of nonclassical cannabinoid analgetics, in *Cannabinoids as Therapeutic Agents* (R. Mechoulam, ed.). CRC Press, Boca Raton, FL, 121–145 (1986).
10. McIlhenny, H. M., R. W. Mast, M. R. Johnson, and G. M. Milne. Nantradol hydrochloride: pharmacokinetics and behavioral effects after acute and chronic treatment. *J. Pharmacol. Exp. Ther.* **219**:363–369 (1981).
11. Yaksh, T. L. The antinociceptive effects of intrathecally administered levonantradol and desacetyllevonantradol in the rat. *J. Clin Pharmacol.* **21**:334S–340S (1981).
12. Howlett, A. C. Inhibition of neuroblastoma adenylate cyclase by cannabinoid and nantradol compounds. *Life Sci.* **35**:1803–1810 (1984).
13. Howlett, A. C., and R. M. Fleming. Cannabinoid inhibition of adenylate cyclase. Pharmacology of the response in neuroblastoma cell membranes. *Mol. Pharmacol.* **26**:532–538 (1984).
14. Howlett, A. C. Cannabinoid inhibition of adenylate cyclase. Biochemistry of the response in neuroblastoma cell membranes. *Mol. Pharmacol.* **27**:429–436 (1985).
15. Howlett, A. C., J. M. Qualy, and L. L. Khachatrian. Involvement of G_i in the inhibition of adenylate cyclase by cannabimimetic drugs. *Mol. Pharmacol.* **29**:307–313 (1986).
16. Devane, W. A., J. W. Spain, C. J. Coscia, and A. C. Howlett. An assessment of the role of opioid receptors in the response to cannabimimetic drugs. *J. Neurochem.* **46**:1929–1935 (1986).
17. Salomon Y., C. Londos, and M. Rodbell. A highly sensitive adenylate cyclase assay. *Anal. Biochem.* **58**:541–548 (1974).
18. Cornish-Bowden, A., and D. E. Koshland. Diagnostic uses of the Hill (logit and Nernst) plots. *J. Mol. Biol.* **95**:201–212 (1975).
19. Howlett, A. C. Cannabinoid inhibition of adenylate cyclase: relative activities of marihuana constituents and metabolites of marihuana. *Neuropharmacology* **26**:507–512 (1987).
20. Melvin, L. S., M. R. Johnson, and G. M. Milne. Cannabinoid receptor site models, in Abstracts of Papers, 14th Northeast Regional Meeting of the American Chemical Society, Fairfield, CT, June 1984. American Chemical Society, Washington, D.C., Abstr. MEDI 81 (1984).
21. Melvin, L. S., M. R. Johnson, and G. M. Milne. A cannabinoid derived analgetic, in Abstracts of Papers, 186th American Chemical Society National Meeting, Washington, D. C., August 1983, American Chemical Society, Washington, D.C., Abstr. MEDI 22 (1983).

Send reprint requests to: Dr. Allyn C. Howlett, Department of Pharmacology, St. Louis University School of Medicine, 1402 South Grand Boulevard, St. Louis, MO 63104.