MOLECULAR NEUROBIOLOGY OF THE CANNABINOID RECEPTOR

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Marijuana is currently the most widely abused street drug. However, the functional significance of the cannabinoid receptor system in health and disease includes the use of cannabinoids as analgesics, antiemetics in cancer patients, anticonvulsants for epilepsy, and as antiglaucoma agents as well as immunomodulatory agents. Our knowledge of the mechanisms of action of cannabinoids has increased greatly in the past several years. Two cannabinoid receptors have been identified to date: one is located predominantly in the central nervous system (CB1), whereas the other is expressed in peripheral tissues (CB2). Both are members of the G-protein-coupled receptor family and couple to inhibition of adenylyl cyclase (as well as additional second messenger systems), in transfected cells expressing these receptors, and in the nervous system. An endogenous ligand has been isolated for the CB1 receptor; it is arachidonic acid ethanolamide, or anandamide. Candidate endogenous ligands for the CB2 receptor have also been described. Another development is the discovery of a selective antagonist for the CB1 receptor. The distribution of the cannabinoid receptor subtypes has been mapped by receptor autoradiography, RT–PCR and *in situ* hybridization. These new research tools will aid in the elucidation of the physiological role of the endogenous cannabinoid system.

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(Bidaut-Russell and Howlett, 1991; Schatz *et al.*, 1992). The identity of a cDNA clone as the cannabinoid receptor was confirmed by transfecting the clone into CHO cells and demonstrating CP-55,940-mediated inhibition of adenylyl cyclase (Matsuda *et al.*, 1990). Devane *et al.* (1988) reported that a selected series of analogs exhibited an excellent correlation between antinocicieptive potency and affinity for this site. A recent study by Compton *et al.* (1993) extended this correlation to include 60 cannabinoids and several behavioral measures. A high degree of correlation was found between the K_i values and *in vivo* potency in the mouse. In addition, the rank order of potency for 16 cannabinoid analogs for inhibition of cAMP production and the K_is for inhibition of [^{3}H]CP-55,940 binding in cells transfected with rat and human cannabinoid receptors are nearly identical to those of receptor binding in rat brain, as well as for several behavioral parameters (Felder *et al.*, 1992). Thus, this receptor appears to be sufficient to mediate many of the known pharmacological effects of cannabinoids. However, other behavioral effects of cannabinoids such as anxiolytic, anticonvulsant, and antiemetic effects have not as yet been correlated with binding to [^{3}H]CP-55,940 sites.

The existence of a cannabinoid receptor in the spleen was established next (Kaminski *et al.*, 1992). A second cannabinoid receptor clone (CB2) that has a different sequence but a similar binding profile to the CB1 clone was discovered by a polymerase chain reaction (PCR)-based strategy designed to isolate G protein-coupled receptors in differentiated myeloid cells (Munro *et al.*, 1993). The CB2 receptor, which has been found in the spleen and cells of the immune system, has 44% amino acid identity with the brain clones and thus represents a receptor subtype. The affinities for several cannabinoids are comparable to the brain receptor (Munro *et al.*, 1993). However, a discrepancy arises with cannabinol; although it is only weakly cannabimimetic and binds the brain receptor with tenfold less affinity than does THC, cannabinol and Δ^9-THC have similar affinities for the expressed peripheral clone (Munro *et al.*, 1993).

In addition to the many central actions of cannabinoids, there are also diverse effects on the immune system (reviewed by Friedman *et al.*, 1994). In general, high doses or concentrations (millimolar) of Δ^9-THC and other psychoactive, as well as nonpsychoactive, cannabinoids have been found to produce immunosuppressive effects on lymphocyte function (including proliferation and production of interleukin-2) and macrophage function (Friedman *et al.*, 1994; Kaminski *et al.*, 1992). Moderate doses of Δ^9-THC suppress antibody formation, reduce spleen weight, and inhibit interferon production (Cabral *et al.*, 1986; Friedman *et al.*, 1994). However, at low concentrations, Δ^9-THC produces some immunoenhancing effects—it increases the production of interleukin-1 and tumor necrosis factor (Zhu *et*

al., 1994). Recently, very low (nanomolar) concentrations of Δ^9-THC have been shown to induce B cell proliferation, an effect that is probably due to activation of the CB2 receptor, as it is not inhibited by the CB1 receptor antagonist; CB2 is the predominant subtype expressed in B cells (Derocq *et al.*, 1995).

B. Endogenous Ligands

Once definitive evidence for a cannabinoid receptor was obtained, attention turned to the identification of an endogenous ligand. Although several laboratories were actively pursuing the endogenous cannabinoid, Devane *et al.* (1992) successfully isolated a substance from porcine brain that bound to the cannabinoid receptor and inhibited electrically stimulated contractions of murine vas deferens. By mass spectrometry, the structure of this compound was established to be arachidonic acid ethanolamide and was named anandamide.

The pharmacological properties of anandamide are consistent with its initial identification as an endogenous ligand for the cannabinoid receptor(s). *In vivo,* anandamide produces many of the same pharmacological effects as the classical cannabinoid ligands, including hypomotility, antinociception, catalepsy, and hypothermia (Crawley *et al.,* 1993; Fride and Mechoulam, 1993). A careful pharmacological comparison between anandamide and Δ^9-THC revealed that anandamide was 4- to 20-fold less potent in producing these pharmacological effects and had shorter duration of action than Δ^9-THC (Smith *et al.*, 1994). Although the composite of the above behavioral paradigms has been shown to be highly predictive of cannabinoid activity, drug discrimination is considered to be one of the most reliable means for confirming specificity. Wiley *et al.* (1995) demonstrated that rats trained to discriminate between Δ^9-THC and vehicle identified anandamide as Δ^9-THC–like. Wickens and Pertwee (1993) reported similarities between Δ^9-THC and anandamide in that both enhanced the ability of muscimol to induce catalepsy when administered into the globus pallidus of rats. Others have proposed that the nigrostriatal dopaminergic system is involved in the motor effects of both Δ^9-THC and anandamide (Romero *et al.*, 1995). Anandamide also affected the hypothalamic-pituitary-adrenal axis in a manner similar to Δ^9-THC (Weidenfeld *et al.,* 1994). Intracerebroventricular adminsitration of anandamide decreased CRF-41 levels in the median eminence and increased serum ACTH and corticosterone levels.

While the above evidence provides ample support for anandamide producing cannabinoid effects, there are some differences between

Δ^9-THC and anandamide. For instance, κ opioid antagonists block the spinal analgesic effects of Δ^9-THC but not those of anandamide (Smith *et al.*, 1994). There have also been several examples of anandamide's failure to exert full agonist effects (Mackie *et al.*, 1993; Mechoulam and Fride, 1995; Barg *et al.*, 1995), and these observations led Fride (1995) to question whether tolerance would develop to anandamide. Tolerance readily develops to Δ^9-THC following repeated administration. Two weeks of daily injections of anandamide (20 mg/kg, intraperitoneally) resulted in a modest degree of tolerance as well as cross-tolerance with Δ^9-THC. It is somewhat surprising that tolerance developed at all considering the relatively short duration of anandamide's effects following a single injection. However, these results provide additional support for a common action of Δ^9-THC and anandamide.

As discussed earlier, anandamide's abiltiy to bind to the brain cannabinoid receptor provided the first evidence that it was an endogenous ligand. Visual inspection of Δ^9-THC and anandamide suggests little structural similarity between them and raises questions as to how both can interact with the same receptor. Unfortunately, molecular modeling studies provide little insight because of the highly flexible nature of anandamide. However, extensive structure-activity relationship studies of anandamide analogs (Adams *et al.*, 1995b), followed by molecular modeling studies (Thomas *et al.*, 1996), revealed that minimum energy conformations of traditional cannabinoids and anandamide shared similar steric and electrostatic characteristics. Additionally, alignment of Δ^9-THC and anandamide could be made that satisfied the traditional cannabinoid pharmacophore. Structure-activity relationship studies also led to the development of stable and potent anandamide analogs (Abadji *et al.*, 1994; Adams *et al.*, 1995). Addition of methyl substituents in the vicinity of the amide bond increased potency, presumably by retarding metabolism.

Anandamide binds both CB1 and CB2 cannabinoid receptors as has been demonstrated in membrane preparations from brain and in transfected cells (Devane *et al.*, 1992; Felder *et al.*, 1993; Munro *et al.*, 1993; Vogel *et al.*, 1993; Adams *et al.*, 1995a; Slipetz *et al.*, 1995; Showalter *et al.*, in press). Although the initial study suggested that anandamide's affinity for the CB2 receptor was considerably less than that for the CB1 receptor (Munro *et al.*, 1993), subsequent studies have demonstrated that anandamide's affinity for CB2 receptors was approximately fourfold less than that for CB1 receptors in stably transfected cells (Felder *et al.*, 1995; Slipetz *et al.*, 1995; Showalter *et al.*, in press). As with Δ^9-THC, anandamide inhibited adenylyl cyclase activity (Felder *et al.*, 1993) and N-type calcium channels (Mackie *et al.*,1993), the putative second messenger systems for the CB1 cannabinoid receptor. Felder *et al.* (1995) found that anandamide was three

times more potent in inhibiting forskolin-stimulated cAMP accumulation in CHO cells transfected with CB1 receptors as compared to CHO cells transfected with CB2 receptors, results that are commensurate with anandamide's receptor affinities. However, Bayewitch *et al.* (1995) found no effect of anandamide on adenylyl cyclase activity when they examined CHO cells transfected with CB2 receptors; they also found that Δ^9-THC was only marginally effective in these cells.

Also, not all of the effects of anandamide are mediated through the currently defined cannabinoid receptors. Anandamide inhbits gap-junction conductance and intercellular signaling in striatal astrocytes via a CB1-receptor independent mechanism because neither did the cannabimimetic agents CP-55,940 and WIN-55,212 mimic the effect of anandamide nor did the CB1 receptor antagonist SR141716A reverse anandamide's actions (Venance *et al.*, 1995).

The characterization of the synthetic and degradative pathways for anandamide is essential for understanding the role of the endogenous cannabinoid system. Two alternative routes for anandamide synthesis have been proposed, one via condensation of arachidonic acid and ethanolamide, the other via hydrolytic cleavage of *N*-arachidonylethanolamine. Deutsch and Chin (1993) showed that anandamide was rapidly taken up by neuroblastoma and glioma cells and degraded by a cytosolic amidase. Degradation also occurred in brain, heart, kidney, and lung tissues. When brain tissue was incubated with an excess of arachidonic acid and ethanolamine, anandamide was formed. These researchers found that the enzyme inhibitor phenymethylsulfonyl fluoride (PMSF) prevented degradation, but not synthesis, of anandamide, whereas, Devane and Axelrod (1994) found that PMSF did inhibit anandamide synthesis in bovine brain. Interestingly, lower levels of synthetic anandamide activity were found in the cerebellum, which contains a very high density of receptors. The synthesis of anandamide has also been proposed to occur via a novel eicosanoid pathway that is coenzyme A (CoA)-and adenosine triphosphate (ATP)-independent (Kruszka and Gross, 1994). DiMarzo *et al.* (1994) questioned the relevance of the above condensation reaction because the required substrate concentrations far exceeded those normally present in tissues. They proposed that the condensation reaction may be the enzymatic route for anandamide breakdown in reverse. Furthermore, they demonstrated that under the conditions employed by Deutsch and Chin an artifactual adduct of PMSF and ethanolamine is formed that is undistinguishable from authentic anandamide by thin-layer chromatography and normal-phase HPLC (Fontana *et al.*, 1995). Proposing an alternative to the condensation pathway, they provided evidence that anandamide could be formed in cultured neurons through phosphodiesterase-mediated cleavage of *N*-arachidonyl-phosphatidyletha-

nolamine (DiMarzo *et al.*, 1994). Which of these pathways is most relevant to the synthesis of anandamide remains to be established.

Additionally, anandamide may only be representative of a family of endogenous compounds. Two other fatty acid derivatives, homo-γ-linolenylethanolamide and docosatetraenylethanolamide, were isolated from bovine brain and found to compete for binding to the cannabinoid receptor (Hanus *et al.*, 1993; Mechoulam *et al.*, 1995). In addition, Mechoulam *et al.* (1995) identified a mono-glycerol derivative of arachidonic acid (2-arachidonyl glycerol) that they isolated from canine gut, which was found to have weak cannabinoid activity. It binds to both the CB1 and the CB2 cannabinoid receptor, inhibits adenylyl cyclase activity in mouse splenocytes, and exhibits pharmacological effects similar to those of Δ^9-THC.

Palmitoylethanolamide has also been suggested as a possible endogenous ligand at the CB2 receptor. Facci *et al.* (1995) found that although both anandamide and palmitoylethanolamide were able to displace cannabinoid binding in a rat mast cell line (RBL-2H3) that expresses the CB2 receptor, only palmitoylethanolamide produced a functional response, namely, inhibition of antigen-evoked [^{3}H]serotonin release. This is in contrast to the finding that anandamide can inhibit adenylyl cyclase in CHO cells that have been transfected with the human CB2 receptor [palmitoylethanolamide was not examined in these studies performed by Felder *et al.* (1995)]. Future research must address numerous questions in order to advance our understanding of the physiological and neurochemical relevance of the endogenous cannabinoid system.

C. Antagonist

The recent identification of the first selective cannabinoid receptor antagonist SR141716A [*N*-(piperidin-1-yl)-5-(4-chlorophenyl)-1-(2,4-dichlorophenyl)-4-methyl-1H-pyrazole-3-carboxamidehydrochloride] provides a much needed tool for the cannabinoid field (Rinaldi-Carmona *et al.*, 1994). SR141716A is a highly potent, orally active, and selective antagonist at the CB1 receptor. Its affinity for the CB1 receptor is approximately 2 n*M* and for the CB2 receptor, greater than 700 n*M* (Rinaldi-Carmona *et al.*, Felder *et al.*, 1995; 1994; Showalter *et al.*, in press). SR141716A antagonizes the inhibitory effects of cannabinoid receptor agonists (including anandamide) on both mouse vas deferens contractions and adenylyl cyclase activity (Rinaldi-Carmona *et al.*, 1994). *In vivo,* SR141716A antagonizes the hypothermia, antinociception, catalepsy, and drug discrimination produced by cannabinoids (Rinaldi-Carmona *et al.*, 1994; Wiley *et al.*, 1995). Because it antagonizes the effects of anandamide, SR141716A provides a

pharmacological tool to evaluate endogenous cannabimimetic activity. In addition, SR141716A can precipitate physical withdrawal symptoms in rodents made tolerant to Δ^9-THC, providing the first unequivocal demonstration of dependence produced by cannabinoids (Aceto *et al.*, 1995; Tsou *et al.*, 1995).

III. Characterization of Brain and Peripheral Cannabinoid Receptor Subtypes

A. Cloning and Molecular Pharmacology

To reiterate, two cannabinoid receptors have been identified to date—one is localized predominantly in the central nervous system (CB1), whereas the other is located primarily in the immune system (CB2). The CB1 receptor cDNA was isolated from a rat brain library by a homology screen for G protein-coupled receptors, and its identity was confirmed by transfecting the clone into CHO cells and demonstrating cannabinoid-mediated inhibition of adenylyl cyclase (Matsuda *et al.*, 1990). Initial identification of the ligand for this "orphan receptor" involved the screening of many candidate ligands, including opioids, neurotensin, angiotensin, substance P, and neuropeptide Y, among others, until cannabinoids were found to act via this molecule. In cells transfected with the clone CP-55,940, Δ^9-THC and other psychoactive cannabinoids, but not cannabidiol (which is inactive), were found to inhibit adenylyl cyclase, whereas in untransfected cells no such response was found. Furthermore, the rank order of potency for inhibition of adenylyl cyclase in transfected cells correlated well with that of cell lines previously shown to possess cannabinoid-inhibited adenylyl cyclase activity. Distribution of the expression of CB1 mRNA also paralleled that of cannabinoid receptor binding in rat brain. Analysis of the primary amino acid sequence of the CB1 receptor predicts seven transmembrane domain regions, typical of G protein-coupled receptors. Bramblett *et al.* (1995) have constructed a model of the cannabinoid receptor. A representation of the CB1 receptor based on their model is shown in Fig. 1.

Shortly after the cloning of the rat cannabinoid receptor, isolation of a human CB1 receptor cDNA was reported (Gerard *et al.*, 1991). The rat and human receptors are highly conserved, with 93% identity at the nucleic acid level and 97% homology at the amino acid level. Finally, there is an excellent correlation between binding affinities at the clones CB1 receptor and binding in brain homogenates using [^{3}H]CP-55,940 as the radioligand (Felder *et al.*, 1992).

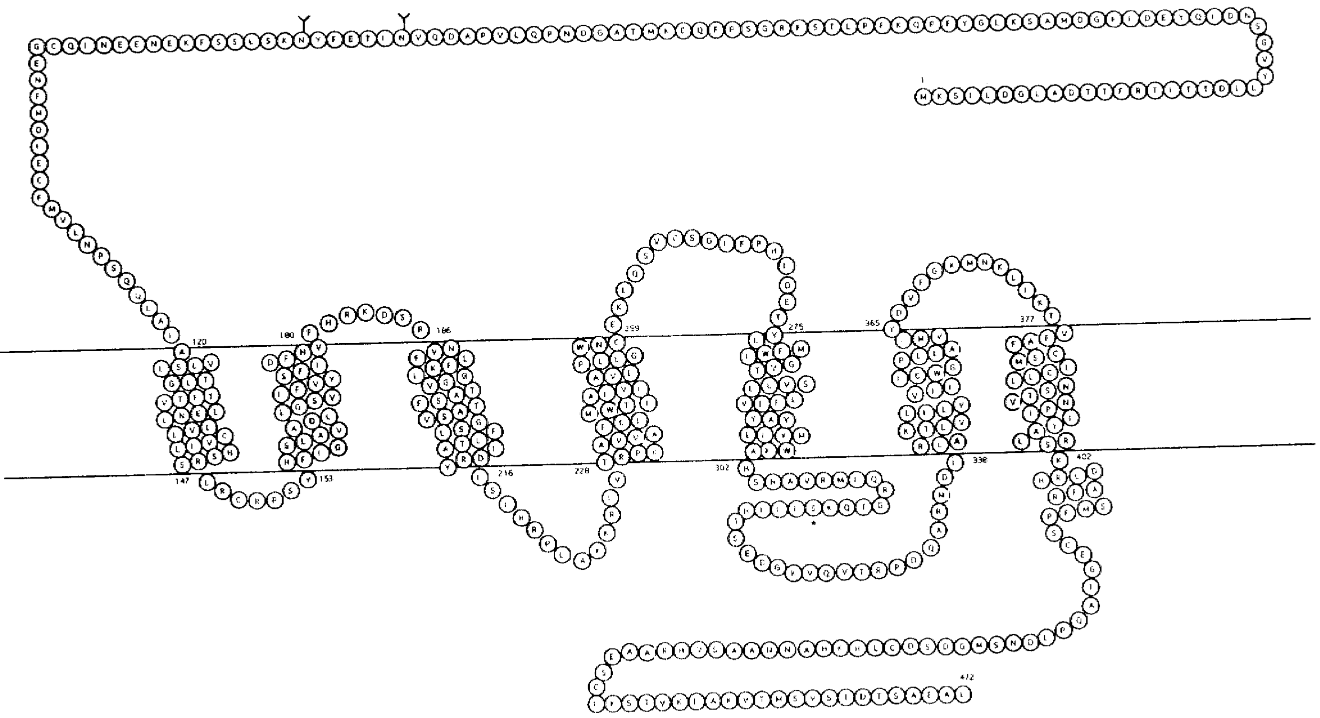

FIG. 1. Proposed membrane topography of the human CB1 cannabinoid receptor. Membrane spanning domains are defined according to hydropathy analysis and determination of helix ends (Bramblett *et al.*, 1995). Shaded circles indicate amino acids that are conserved between the CB1 and CB2 receptors. Y, potential N-linked glycosylation sites; *, potential phosphorylation sites.

The CB2 receptor was also isolated by its homology to other G protein-coupled receptors, using a PCR-based approach in myeloid cells (Munro *et al.*, 1993). The human CB2 receptor cDNA was isolated from the human promyelocytic cell line HL60. The clone has 44% amino acid sequence identity overall with the CB1 clones, and identity rises to 68% in the transmembrane domains. The amino acid residues conserved between CB1 and CB2 are shown shaded in Fig. 1. The localization of the CB2 receptor appears to be exclusively in the periphery—in the spleen, in hematopoietic cell lines (Munro *et al.*, 1993), and in mast cells (Facci *et al.*, 1995).

Transfected cell lines expressing the CB2 receptor have an affinity for CP-55,940 that is similar to that of cell lines expressing the CB1 receptor Munro *et al.*, 1993; Felder *et al.*, 1995; Showalter *et al.*, in press). Furthermore, the affinities for Δ^9-THC, 11-OH-Δ^9-THC, anandamide, and cannabidiol at the CB2 receptor are comparable to their affinities at the brain receptor. In contrast, cannabinol (which is known to be 10 times less potent than Δ^9-THC at the CB1 receptor) was found to be equipotent to Δ^9-THC at the CB2 receptor. Based on these binding profiles, it was concluded that the peripheral receptor clone may be a cannabinoid receptor subtype. We (and others) have conducted a more extensive characterization of this receptor and can indeed demonstrate a separation of pharmacological selectivities (Felder *et al.*, 1995; Slipetz *et al.*, 1995; Showalter *et al.*, in press). The compounds that have been identified as being either CB1- or CB2-selective serve as lead compounds in the design of even more selective ligands. The affinity of SR141716A (the CB1 receptor antagonist) is at least 50-fold higher at the CB1 receptor than at the CB2 receptor (Rinaldi-Carmona *et al.*, 1994; Felder *et al.*, 1995; Showalter *et al.*, in press) and thus provides a starting point for the design of more selective antagonists and agonists.

At least two separate second messenger systems mediate the effects of cannabinoids via the CB1 receptor. In neuronal and transfected cell lines, both Howlett and Fleming (1984) and Matsuda *et al.* (1990) have shown that the potency of a series of cannabinoid analogs to inhibit cAMP accumulation correlates with their ability to displace [^{3}H]CP-55,940 binding. However, in the same neuronal cell line, others (Mackie and Hille, 1992; Felder *et al.*, 1993) have demonstrated a G protein-mediated inhibition of Ca^{2+} channels that was not cAMP dependent. Furthermore, AtT20 pituitary cells transfected with CB1 receptor cDNA exhibited cannabinoid-mediated inhibition of Q-type Ca^{2+} channels and activation of an inwardly rectifying K^+ channel, as well as inhibition of adenylyl cyclase (Mackie *et al.*, 1995). Thus, even in a single cell line, coupling between a single cannabinoid receptor and distinct second messenger

systems has been demonstrated. Interestingly, transfection of the CB2 receptor into AtT20 cells conferred cannabinoid-mediated inhibition of adenylyl cyclase activity but not modulation of Ca^{2+} or K^+ channels (Felder *et al.*, 1995).

In addition to modulation of cAMP levels and channels, other possible second messenger systems for cannabinoids have been investigated. There has been long standing interest in the role of prostaglandins in cannabinoid action, with evidence that cannabinoids can either stimulate or inhibit arachidonic acid release (Burstein, 1987). However, definitive evidence that eicosanoids play a direct role in the actions of cannabinoids has been elusive. The identification of an arachidonic acid derivative as an endogenous cannabinoid ligand has renewed interest in this area. Felder *et al.* (1995) demonstrated that anandamide stimulated arachidonic acid release from CHO cells transfected with the CB1 (or CB2) receptor, as well as from nontransfected cells and concluded that this effect of anandamide occurred via a receptor-independent mechanism. On the other hand, Shivachar *et al.* (1996) recently showed that anandamide and Δ^9-THC stimulated the release of arachidonic acid in primary cultures of rat brain cortical astrocytes (which express CB1 but not CB2) by a receptor-mediated mechanism.

Cannabinoid agonists have previously been shown to have effects on intracellular Ca^{2+} (Martin, 1986). In CHO cells, cannabinoid agonists were shown to induce a nonspecific release of intracellular Ca^{2+} (Felder *et al.*, 1992; Felder *et al.*, 1995). Both the untransfected, as well as the CB1- or CB2- transfected, CHO cells were able to release calcium when HU-211 or HU-210 were used as agonists. These data suggest that the transfected cannabinoid receptor clones do not mediate these effects and therefore a nonreceptor mechanism was postulated.

Investigation of tertiary messenger systems for the cannabinoids has recently been described. Glass and Dragunow (1995) reported that 2 h after administration of the cannabinoid agonist CP 55,940 to rats, expression of the immediate early gene *krox*-24 (also known as NGFI-A, *zif*/268, *egr*-1, and TIS8) was induced in striosomes obtained from these animals. Bouaboula *et al.* (1993) also found that stimulation of the CB1 receptor induced expression of *krox*-24 in human astrocytoma cells, as well as in CB1-transfected CHO cells. That this effect was receptor mediated was demonstrated by blockade of the response by SR141716A and the inability of CP 55,940 to induce expression in untransfected CHO cells. In addition, they examined expresssion of several other immediate early genes and showed that *jun*-B and *krox*-20 were also induced by cannabinoids but that c-*fos* was not. Induction of *krox*-24 expression was mediated by a pertussis toxin sensitive

G protein and probably not via cAMP. Whether activation of the CB2 receptor leads to induction of immediate early genes remains to be determined.

B. Distribution and Expression

The distribution of the cannabinoid receptors has been mapped using various techniques, including receptor binding and autoradiography, northern blot analysis, *in situ* hybridization, and reverse-transcription polymerase chain reaction (RT-PCR). Very recently, localization using an antibody to the CB2 receptor has been described (Galiegue *et al.*, 1995). In general, all the techniques show the CB1 receptor to be most abundantly expressed in the central nervous system, whereas the CB2 receptor is restricted to cells and tissues derived from the immune system (Table I).

The neuronal distribution of the CB1 cannabinoid receptor has been reported by several laboratories (Herkenham *et al.*, 1991a; Mailleux and Vanderhaeghen, 1992; Matsuda *et al.*, 1993). The pattern of neuronal expression of this receptor is conserved among species (Herkenham *et al.*, 1990). Receptor autoradiographic studies utilizing [^{3}H] CP-55,940 binding reveal dense binding in the basal ganglia (lateral caudate putamen, globus pallidus, entopeduncular nucleus, substantia nigra pars reticulata), the molecular layer of the cerebellum, the innermost layers of the olfactory bulb, and the hippocampus (dentate gyrus molecular layer and the CA3 regions); moderately dense binding in the remaining forebrain; and sparse binding in the brain stem and spinal cord (Herkenham *et al.*, 1991b; Mailleux and Vanderhaeghen, 1992).

Determinations of the tissue distribution of the CB1 receptor mRNA by *in situ* hybridization show general agreement between expression of this mRNA and CP-55,940 binding sites (Herkenham *et al.*, 1991b; Mailleux and Vanderhaeghen, 1992; Matsuda *et al.*, 1993). Most of the discrepancies can be attributed to expression of the CB1 gene in projection neruons (mRNA in cell bodies distant from receptors at axon terminals); for example, in the cerebellum, in which granule cell axons (containing CB1 mRNA) project to the molecular layer (containing binding sites). *In situ* hybridization studies on the CB1 receptor mRNA revealed a subset of neurons that express very high levels of mRNA in addition to cells expressing mRNA at moderate levels (Matsuda *et al.*, 1990, 1993; Mailleux and Vanderhaeghen, 1992). Whether the neurons that express substantially more CB1 mRNA also produce a greater number of receptors remains to be determined.

TABLE I
DISTRIBUTION OF CANNABINOID RECEPTORS

Tissue/cell	CB1	CB1A	CB2	Reference
Whole brain	*[a]	+[b]	–[c]	(Matsuda *et al.*, 1990; Shire *et al.*, 1995)
Cerebellum	*	+	–	[Matsuda *et al.*, 1990; Shire *et al.*, 1995(*)]
Cortex	*	+	–	(Matsuda *et al.*, 1990; Shire *et al.*, 1995)
Adrenal gland	*	+	–	(Shire *et al.*, 1995)
Heart	+	+	–	(Shire *et al.*, 1995)
Lung	*	+	+	(Shire *et al.*, 1995)
Kidney	+	+	–	(Shire *et al.*, 1995)
Liver	+	+	–	(Shire *et al.*, 1995)
Bile duct	+	+	–	(Shire *et al.*, 1995)
Muscle	+	+	–	(Shire *et al.*, 1995)
Stomach	+	+	–	(Shire *et al.*, 1995)
Colon	+	+	–	(Shire *et al.*, 1995)
Prostate	+	+	–	(Shire *et al.*, 1995)
Uterus	*	+	+	(Das *et al.*, 1995; Shire *et al.*, 1995)
Placenta	+	+	–	(Shire *et al.*, 1995)
Testes	*		–	(Galiegue *et al.*, 1995)
Ovary	+		–	(Galiegue *et al.*, 1995)
Pancreas	+	+	+	(Shire *et al.*, 1995)
Bone marrow	+	+	+	(Galiegue, 1995)
Thymus	+	+	+	(Galiegue *et al.*, 1995)
Spleen	+		*	(Kaminski *et al.*, 1992; Munro *et al.*, 1993)
Tonsils	+		*	(Galiegue *et al.*, 1995)
T cells (CD4⁺)	+		+	(Galiegue *et al.*, 1995)
T cells (CD8⁺)	+		+	(Galiegue *et al.*, 1995)
PMN	+*		+	(Galiegue *et al.*, 1995)
Monocytes	+		+	(Galiegue *et al.*, 1995)
NK cells	+		+	(Galiegue *et al.*, 1995)
B cells	+		+	(Galiegue *et al.*, 1995)
Mast cells			+	(Facci *et al.*, 1995)
Cell lines				
N18 neuroblastoma	*			(Matsuda *et al.*, 1990)
U373 astrocytoma	+	+		(Bouaboula *et al.*, 1993; Shire *et al.*, 1995)

(continues)

TABLE I (*Continued*)

Tissue/cell	CB1	CB1A	CB2	Reference
U937 myeloma	+	+	+	(Galiegue *et al.*, 1995; Shire *et al.*, 1995)
HL60 myeloma			*	(Munro *et al.*, 1993)
Molt-4 (T cell leukemia)			+	(Galiegue *et al.*, 1995)
DAUDI (B cell leukemia)	+		+	(Galiegue *et al.*, 1995)
THP-1 (monocyte)	+			(Bouaboula *et al.*, 1993; Daaka *et al.*, 1995)

[a] * = detected by northern blot analysis or *in situ* hybridization
[b] + = detected by RT-PCR
[c] − = not detected
PMN, polymorphonuclear neutrophils; NK cells, natural killer cells

The dense localization of cannabinoid receptors in the hippocampus and forebrain provide a basis for interpreting previous pharmacological data implicating cannabinoids in learning and memory, as well as for the anticonvulsant effects of these agents. Similarly, the cerebellar distribution of these receptors indicates a role in movement, for instance, ataxia during intoxication. Finally, the low densities of receptors in the brain stem areas controlling respiratory functions may explain why cannabinoids lack respiratory depressant effects (Herkenham *et al.*, 1990).

The CB2 receptor is the most abundantly expressed cannabinoid receptor subtype in the immune system; it can be readily detected by northern blot analysis and is probably the species visualized by [^{3}H]CP-55,940 autoradiography (Lynn and Herkenham, 1994). However, low levels of CB1 mRNA are also present, which can be detected by RT-PCR techniques. Kaminski *et al.* (1992) first reported the expression of a cannabinoid receptor in murine splenocytes, using RT-PCR to amplify CB1 mRNA, as well as [^{3}H]CP-55,940 binding and functional assays. CB1 mRNA expression has been found in both murine and human leukocytes (Bouaboula *et al.*, 1993; Daaka *et al.*, 1995). Furthermore, the level of CB1 expression appears to be increased during immune cell activation (Daaka *et al.*, 1995). This is also the case with CB2 and mRNA expression (Munro *et al.*, 1993). The tight regulation of the cannabinoid receptors suggests that these receptors play a significant functional role in the immune system.

C. Evidence for Additional Cannabinoid Receptor Subtypes

Splice variants of the cannabinoid receptor occur. A PCR amplification product was isolated that lacked 167 base pairs of the coding region of the CB1 receptor (Shire *et al.*, 1995). This alternative splice form (CB1A) is unusual because it is generated from the mRNA encoding CB1 and not from a separate exon (Shire *et al.*, 1995). When expressed, the CB1A clone would translate to a receptor truncated by 61 amino acid residues with 28 amino acid residues different at the NH_2-terminal (Fig. 2). This might lead to a receptor with altered ligand-binding properties. CB1A expression has been detected in many tissues by RT-PCR (Table I). It will be important to confirm that the CB1A receptor protein is expressed because splice variants often arise from incomplete splicing during library construction and when using RT-PCR techniques. The construction of antibodies selective to CB1 or CB1A peptides would be useful to detect these proteins.

The mouse CB1 gene and cDNA sequences have been reported (GenBank accession numbers U22948 and U17985). Sequence analysis of the mouse CB1 clones indicates a high degree of conservation among species. The mouse and rat clones have 95% nucleic acid identity (99.5% amino acid identity). The mouse and human clones have 90% nucleic acid identity (97% amino acid identity). Rat CB1 probes can be used to detect mouse

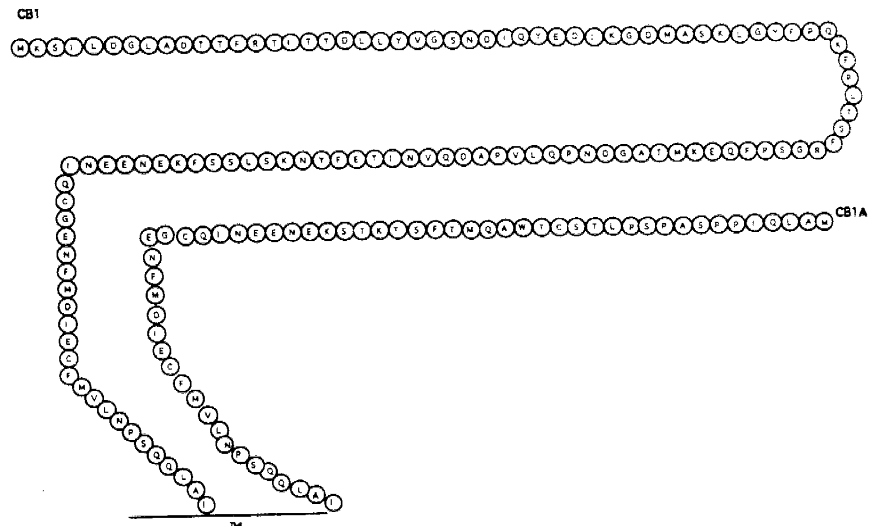

Fig. 2. Proposed amino terminal domains of the human CB1 and CB1A receptors.

cannabinoid receptor mRNA (Abood *et al.*, 1993), again indicating conservation among species. However, the human and rat sequences diverge about 60 bp upstream of the translation initiation codon. Furthermore, we have isolated a rat CB1 clone that is identical to the published sequence in the coding region, but diverges about 60 bp upstream of the translation codon (unpublished data). Examination of the 5′ untranslated sequence of the mouse CB1 genomic clone indicates a splice junction site approximately 60 bp upstream from the translation start site. This splice junction site is also present in the human CB1 gene (Shire *et al.*, 1995). These data suggest the existence of splice variants of the CB1 receptor, as well as possible divergence of regulatory sequences between these genes.

D. Regulation of Receptor Expression

Alterations in cannabinoid receptor (CB1) gene expression occur during early development. The CB1 receptor is expressed as early as postnatal day 3 in rat brain; both CB1 receptor mRNA and binding sites can be detected (McLaughlin and Abood, 1993; McLaughlin *et al.*, 1994; Rodriguez-de-Fonseca *et al.*, 1993; Belue *et al.*, 1995). Relative CB1 mRNA expression steadily increases in the cerebellum and brain stem until postnatal days 18–21, whereas expression in the forebrain does not change (McLaughlin and Abood, 1993). In addition to these studies using northern blot analysis, *in situ* hybridization data from our laboratory also suggest that there are regional differences in the relative expression of CB1 mRNA that may parallel cerebellar proliferation and organization or may reflect unique tissue-specific expression of the cannabinoid receptor (McLaughlin *et al.*, 1992).

Regulation of cannabinoid receptor mRNA levels has been investigated using *in situ* hybridization (Mailleux and Vanderhaeghen, 1993a,b; Mailleux and Vanderhaeghen, 1994). Following adrenalectomy, CB1 and mRNA levels in the striatum increased approximately 50% compared to control rats (Mailleux and Vanderhaeghen, 1993b). This increase could be counteracted by dexamethasone treatment, suggesting glucocorticoid downregulation of cannabinoid receptor gene expression in the striatum. A negative dopaminergic influence on CB1 gene expression has been suggested by studies in which induction of unilateral lesions by 6-hydroxydopamine was associated with an approximately 45% increase in mRNA levels in the ipsilateral side; furthermore, treatment with dopamine receptor antagonists mimicked the effect (Mailleux and Vanderhaeghen, 1993a). Previous experiments had documented the disappearance of CP 55,940 binding following ibotenic acid-induced injury to the striatum, but not following

6-hydroxydopamine-induced injury, indicating that cannabinoid receptors are not colocalized with dopamine-containing neurons but are probably on axonal terminals of striatal intrinsic neurons (Herkenham *et al.*, 1991). Glutamatergic regulation of cannabinoid receptor mRNA levels in the striatum has also been reported (Mailleux and Vanderhaeghen, 1994). Unilateral cerebral decortication resulted in an approximately 30% decrease in mRNA levels, and treatment with the NMDA receptor antagonist MK-801 resulted in an approximately 52% decrease, as compared to control. These data suggest an NMDA receptor-mediated upregulation of cannabinoid receptor mRNA levels. The mechanisms by which these changes occur are not known.

Alterations in CB1 receptor expression have also been examined following chronic exposure to cannabinoids. Tolerance develops to most pharmacological effects of Δ^9-THC after a period of chronic exposure in laboratory animals (McMillan *et al.*, 1971; Compton *et al.*, 1990; Pertwee, 1991). These effects include anticonvulsant activity, catalepsy, depression of locomotor activity, hypothermia, hypotension, immunosuppression, and static ataxia. Tolerance develops not only to Δ^9-THC but to other psychoactive cannabinoids, such as Δ^8-THC, 11-OH-Δ^9-THC, and 11-OH-Δ^8-THC and CP 55,940 (Pertwee, 1991). Furthermore, a much greater degree of tolerance develops to CP 55,940 than to Δ^9-THC in several murine pharmacological assays (Fan *et al.*, 1994).

Cannabinoid tolerance develops in the absence of pharmacokinetic changes (Martin *et al.*, 1976); therefore, biochemical or cellular changes are responsible for this adaptation. One hypothesis for tolerance development is that receptors lose function during chronic agonist treatment leading to diminished biological responses. The phenomenon of receptor downregulation has been observed in many brain receptor systems including α- and β-adrenergic (Scarpace and Abrass, 1982), dopaminergic (Creese and Sibley, 1981), and opioid receptors (Law *et al.*, 1982; Werling *et al.*, 1989). Initial studies from our laboratory failed to detect changes in either receptor number or mRNA levels in *whole* brains from mice tolerant to Δ^9-THC (Abood *et al.*, 1993). However, we have recently found, in mice tolerant to CP 55,940, that cannabinoid receptor downregulation in *cerebella* is concomitant with increased levels of receptor mRNA (Fan *et al.*, in press). Similarly, development of tolerance to Δ^9-THC and CP 55,940 in rats was accompanied by decreases in receptor density in striatum (Oviedo *et al.*, 1993). Rodriguez-de-Fonseca *et al.* (1994) also observed region-specific differences in rats made tolerant to Δ^9-THC; receptor downregulation was observed in striatum and limbic forebrain, but not in ventral mesencephalon.

In other G protein-coupled receptor systems, downregulation is preceded by desensitization [for example, adrenergic receptors (Collins *et al.*, 1992; Lohse, 1993) and opioid receptor (Law *et al.*, 1983)]. In addition, transcriptional regulation of G protein-coupled receptors modulates the response to chronic agonist exposure (reviewed by Hadcock and Malbon, 1991; Morris, 1993). In N18TG2 cells exposed to Δ^9-THC for 24 h, cannabinoid-inhibited adenylyl cyclase activity was attenuated (Dill and Howlett, 1988). In cerebella from mice chronically treated with CP 55,940, receptor downregulation was observed following 7 days of treatment, but no change in adenylyl cyclase was found at that time (Fan, 1996). However, since desensitization is expected to precede downregulation, it is possible that alterations in receptor coupling to adenylyl cyclase may have occurred earlier. Alterations in receptor number or coupling to signal transduction systems are only one set of mechanisms by which a system responds to repeated drug administration. It will be important to determine the other biochemical changes that take place in the cannabinoid system during the development of tolerance.

IV. Future Directions

The major challenge facing researchers is to elucidate the physiological role of the endogenous cannabinoid system, which may provide insight into the mechanism by which cannabinoids produce their unique behavioral effects. One of the most fundamental questions is whether the cannabinoid system is an integral part of cognitive processes, the mechanisms (direct versus indirect) whereby exogenous cannabinoids disrupt cognition can now be examined. Similarly, it will be important to determine the relationship between the cannabinoid system and central control of motor function. A critical question is whether the endogenous ligands act as neurotransmitters or neuromodulators, an issue that can be clarified by elucidation of synthesis, degradation, storage, and stimulated release. Methods for quantitation of endogenous anandamide release *in vivo* are not currently available. Other approaches to determining the role of the endogenous cannabinoid system are via chronic administration of receptor agonists or antagonists, construction of knockout transgenic mouse models, or pharmacological knockouts of the receptors using antisense oligonucleotides. These approaches may lead to an understanding of the pathological consequences of a dysfunctional cannabinoid system and, consequently, to novel therapies.

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