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Molecular Biology of Cannabinoid Receptors

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Abstract To date, two cannabinoid receptors have been isolated by molecular cloning. The CB_1 and CB_2 cannabinoid receptors are members of the G protein-coupled receptor family. There is also evidence for additional cannabinoid receptor subtypes. The CB_1 and CB_2 receptors recognize endogenous and exogenous cannabinoid compounds, which fall into five structurally diverse classes. Mutagenesis and molecular modeling studies have identified several key amino acid

residues involved in the selective recognition of these ligands. Numerous residues involved in receptor activation have been elucidated. Regions of the CB_1 receptor mediating desensitization and internalization have also been discovered. The known genetic structures of the CB_1 and CB_2 receptors indicate polymorphisms and multiple exons that may be involved in tissue and species-specific regulation of these genes. The cannabinoid receptors are regulated during chronic agonist exposure, and gene expression is altered in disease states. There is a complex molecular architecture of the cannabinoid receptors that allows a single receptor to recognize multiple classes of compounds and produce an array of distinct downstream effects.

Keywords Cannabinoid receptor · Mutagenesis · Polymorphism · Gene regulation, binding

1 Introduction

Our knowledge of the mechanism of action of cannabinoids has increased greatly in the past several years due to numerous major discoveries. The development of novel synthetic analogs of (–)-Δ^9-tetrahydrocannabinol (Δ^9-THC), the primary psychoactive constituent in marijuana, played a major role in the characterization and cloning of a neuronal cannabinoid receptor, a member of the G protein-coupled receptor family (GPCR) (Matsuda et al. 1990). The identity of the cDNA clone as the cannabinoid receptor (CB_1) was confirmed by transfection into Chinese hamster ovary (CHO) cells and the demonstration of cannabinoid-mediated inhibition of adenylyl cyclase (Gerard et al. 1991; Matsuda et al. 1990). This receptor can also modulate G protein-coupled Ca^{2+} and K^+ channels (Mackie and Hille 1992; McAllister et al. 1999). Five structurally distinct classes of cannabinoid compounds have now been identified: the classical cannabinoids [Δ^9-THC, Δ^8-THC-dimethylheptyl (HU210)]; non-classical cannabinoids (CP 55,940); indoles (WIN 55,212-2), eicosanoids (anandamide, 2-arachidonoylglycerol) and antagonist/inverse agonists (SR141716A, SR145528) (Devane et al. 1992; Eissenstat et al. 1995; Howlett 1995; Mechoulam et al. 1995; Rinaldi-Carmona et al. 1994; Rinaldi-Carmona et al. 1998a; Xie et al. 1996).

The CB_1 receptor gene has been inactivated in mice (by in-frame deletion of most of the coding region) using homologous recombination in two laboratories (Ledent et al. 1999; Zimmer et al. 1999). Significantly, not only did the CB_1 receptor knockout mice lose responsiveness to most cannabinoids, the reinforcing properties of morphine and the severity of the withdrawal syndrome were strongly reduced (Ledent et al. 1999). The CB_1 receptor appears to play a central role in drug addiction.

The existence of a second type of cannabinoid receptor in the spleen was established (Kaminski et al. 1992). The CB_2 receptor was isolated by a polymerase chain reaction (PCR)-based strategy designed to isolate GPCRs in differentiated myeloid cells (Munro et al. 1993). The CB_2 receptor, which has only been found in the spleen

and cells of the immune system, has 44% amino acid identity with CB_1, and a distinct yet similar binding profile, and thus represents a receptor subtype. The CB_2 receptor gene has been inactivated by homologous recombination in mice (Buckley et al. 2000); the most notable effect was impairment of immunomodulation by helper T cells.

Another major breakthrough in cannabinoid research was the discovery of endogenous ligands for the cannabinoid receptors; this uncovered a novel neurotransmitter/neuromodulatory system. The first ligand, arachidonoyl ethanolamide (anandamide, AEA) was isolated from porcine brain; it competed for binding to the CB_1 receptor and inhibited electrically stimulated contractions of the mouse vas deferens in the same manner as Δ^9-THC (Devane et al. 1992). 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 (Fride and Mechoulam 1993). The biosynthetic pathways of anandamide synthesis, release, and removal are under investigation by several laboratories (Deutsch and Chin 1993; Di Marzo et al. 1994; Hilliard and Campbell 1997; Piomelli et al. 1999; Walker et al. 1999). Additional fatty acid ethanolamides with cannabimimetic properties have been isolated, suggesting the existence of a family of endogenous cannabinoids (Hanus et al. 1993). 2-Arachidonoylglycerol (2AG) in several systems acts as a full agonist, whereas anandamide is a partial agonist, suggesting that the CB_1 receptor may in fact be a 2AG receptor (Stella et al. 1997; Sugiura et al. 1997).

Additionally, virodhamine, arachidonic acid and ethanolamine joined by an ester linkage, has been isolated (Porter et al, 2001). Noladin ether, 2-arachidonyl glyceryl ether, is a potent endogenous agonist at the CB_1 receptor (Hanus et al. 2001). *N*-Arachidonoyl-dopamine (NADA), is primarily a vanilloid receptor agonist, but has some activity at CB_1 receptors as well (Huang et al. 2002). Palmitoylethanolamide (PEA) has been suggested as a possible endogenous ligand at the CB_2 receptor (Facci et al. 1995). However, subsequent studies showed no affinity for palmitoylethanolamide at the CB_2 receptor (Griffin et al. 2000; Lambert et al. 1999; Showalter et al. 1996). Instead, PEA seems to increase the potency of AEA, in part by inhibiting fatty acid amide hydrolase (FAAH), the enzyme responsible for breakdown of AEA (Di Marzo et al. 2001).

In addition to actions at cannabinoid receptors, AEA, 2AG, virodhamine, noladin ether, and NADA also act at the vanilloid receptor (transient receptor potential vanilloid type 1 TRPV1; previously know as VR1), a ligand-gated ion channel that is a member of the transient receptor potential (TRP) ion channel family (recently reviewed by Di Marzo et al. 2002). In addition, Δ^9-THC and cannabinol at high (20 μM) concentrations have recently been identified as agonists at another TRP, the ANKTM1 channel (Jordt et al. 2004). These findings raise the possibility that the TRP channels may be ionotropic cannabinoid receptors.

The existence of a family of endogenous ligands suggests the presence of additional cannabinoid receptor subtypes. In addition, some of the diverse effects may result from different receptor conformations. Experimental evidence from several laboratories suggests that cannabinoid receptor ligands can induce differ-

ent conformations of the CB_1 receptor, which in turn can activate select G proteins (Glass and Northup 1999; Griffin et al. 1998; Kearn et al. 1999; Mukhopadhyay et al. 2000; Selley et al. 1996). This selectivity appears to be driven by distinct molecular interactions that occur between the different classes of cannabinoid compounds and the receptor proteins. These data indicate that receptor "subtypes" may also be observed as a result of activation of distinct second messenger pathways that produce different physiological responses.

This chapter will focus on the molecular biology of the G protein-coupled cannabinoid receptors.

2 General Structure and Distribution

Two cannabinoid receptors have been identified to date; the CB_1 receptor is localized predominantly in the central nervous system (CNS), whereas the CB_2 receptor is located primarily in the immune system. The CB_1 receptor cDNA was isolated from a rat brain library by a homology screen for GPCRs and its identity 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 lacks CNS activity) 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 cell lines previously shown to possess cannabinoid-inhibited adenylyl cyclase activity. Distribution of the expression of CB_1 mRNA also paralleled that of cannabinoid receptor binding in rat brain. Analysis of the primary amino acid sequence of the CB_1 receptor predicts seven transmembrane (TM) domain regions, typical of GPCRs. Bramblett et al. (1995) have constructed a model of the cannabinoid receptor. A representation of the CB_1 receptor based on their model is shown in Fig. 1.

The CB_2 receptor was also isolated by its homology to other GPCRs, using a PCR-based approach in myeloid cells (Munro et al. 1993). The human CB_2 receptor cDNA was isolated from the human promyelocytic cell line, HL60. The clone has 44% amino acid sequence identity overall with the CB_1 clone, and percentage similarity rises to 68% in the TM domains. The amino acid residues conserved between CB_1 and CB_2 are shaded in Fig. 1. The localization of the CB_2 receptor appears to be mainly in the periphery: in the spleen and in low levels in adrenal, heart, lung, prostate, uterus, pancreas, and testis and in cells of immune origin, including microglia in the CNS (Munro et al. 1993; Galiegue et al. 1995; Walter et al. 2003). An alignment of human CB_1 and CB_2 is shown in Fig. 2. Using the numbering scheme of Ballesteros and Weinstein (Ballesteros and Weinstein 1995), each amino acid is given a number that begins with the helix number followed by

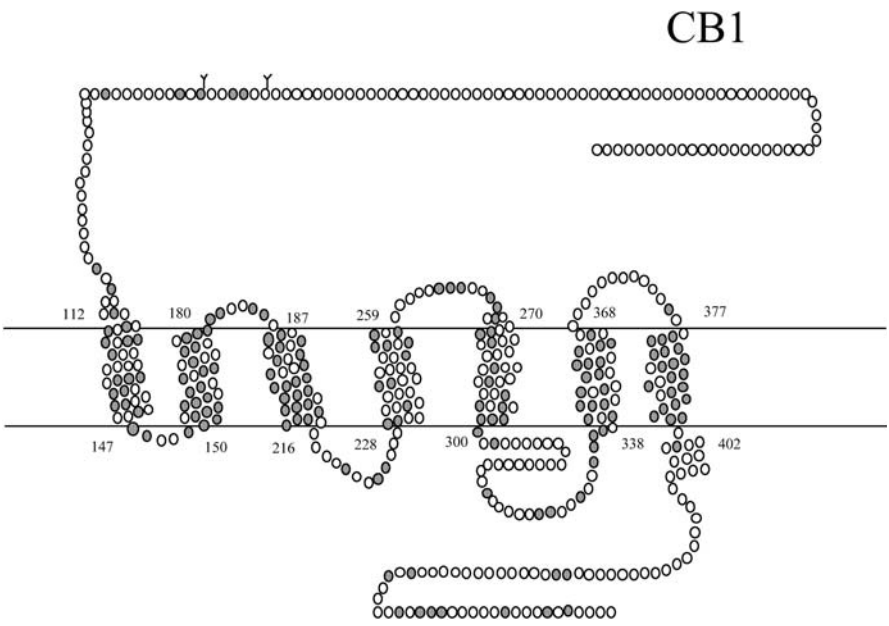

Fig. 1. A helix net representation of the human CB_1 receptor. The amino acids shared with the CB_2 receptor are *shaded*

a two-digit decimal. The most highly conserved residue in each helix is assigned a value of 0.50 and the other residues numbered relative to the conserved residue.

Transfected cell lines expressing the CB_2 receptor have an affinity for CP 55,940 that is similar to those expressing the CB_1 receptor (Felder et al. 1995; Munro et al. 1993; Showalter et al. 1996). Furthermore, the affinities for Δ^9-THC, 11-OH-Δ^9-THC, anandamide and cannabidiol at the CB_2 receptor are comparable to the brain (Showalter et al. 1996) receptor. In contrast, cannabinol (which is known to be ten times less potent than Δ^9-THC at the CB_1 receptor) was found to be equipotent to Δ^9-THC at the CB_2 receptor (Showalter et al. 1996). Based on these binding profiles, it was concluded that the peripheral receptor clone may be a cannabinoid receptor subtype. Indeed, a more extensive characterization of this receptor demonstrates a separation of pharmacological selectivities (Felder et al. 1995; Showalter et al. 1996; Slipetz et al. 1995). The compounds that have been identified as CB_1 and CB_2 selective serve as lead compounds in the design of even more selective ligands. The affinity of SR141716A (the CB_1 receptor antagonist) is at least 50-fold higher at the CB_1 receptor than at the CB_2 receptor (Felder et al. 1995; Rinaldi-Carmona et al. 1994; Showalter et al. 1996) and has provided a starting point for the design of more selective antagonists and agonists.

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CB1   1  MKSILDGLAD TTFRTITTDL LYVGSNDIQY EDIKGDMASK LGYFPQKFPL  50
CB2      .......... .......... .......... .......... ..........

CB1  51 TSFRGSPFQE KMTAGDNPQL VPADQVNITE FYNKSLSSFK ENEENIQCGE  100
CB2   1 .......... .......... ..MEECWVTE IANGSKDGLD SN........   20

                    111111111 1111111111 1111111111 11111    2
                    223333333 3334444444 4455555555 55566    3
                    890123456 7890123456 7890123456 78901    7
CB1 101 NFMDIECFMV LNPSQQLAIA VLSLTLGTFT VLENLLVLCV ILHSRSLRCR  150
CB2  21 ...PMKDYMI LSGPQKTAVA VLCTLLGLLS ALENVAVLYL ILSSHQLRRK   67

        2222222222 2222222222 2222222222     333333 3333333333
        3344444444 5555555555 5566666666     222222 2223333333
        8901234567 8901234567 8901234567     123456 7890123456
CB1 151 PSYHFIGSLA VADLLGSVIF VYSFIDFHVF HRKDSRNVFL FKLGGVTASF  200
CB2  68 PSYLFIGSLA GADFLASVVF ACSFVNFHVF HGVDSKAVFL LKIGSVTMTF  117

        3333333333 3333333333         44 4444444444 4444444444
        3334444444 4445555555         33 4444444444 5555555555
        7890123456 7890123456         89 0123456789 0123456789
CB1 201 TASVGSLFLT AIDRYISIHR PLAYKRIVTR PKAVVAFCLM WTIAIVIAVL  250
CB2 118 TASVGSLLLT AIDRYLCLRY PPSYKALLTR GRGLVTLGIM WVLSALVSYL  167

        4444                5 5555555555 5555555555 5555555555
        6666                3 3333344444 4444455555 5555566666
        0123                4 5678901234 5678901234 5678901234
CB1 251 PLLGWNCEKL QSVCSDIFPH IDETYLMFWI GVTSVLLLFI VYAYMYILWK  300
CB2 168 PLMGWTCC.. PRPCSELFPL IPNDYLLSWL LFIAFLFSGI IYTYGHVLWK  215

                                          666666666 6666666666
                                          222222333 3333333444
                                          456789012 3456789012
CB1 301 AHSHAVRMIQ RGTQKSIIIH TSEDGKVQVT RPDQARMDIR LAKTLVLILV  350
CB2 216 AHQHVASL.. .......... .SGHQDRQVP GMARMRLDVR LAKTLGLVLA  252

        6666666666 6666666666      77777 7777777777 7777777777
        4444444555 5555555666      33333 3334444444 4445555555
        3456789012 3456789012      23456 7890123456 7890123456
CB1 351 VLIICWGPLL AIMVYDVFGK MNKLIKTVFA FCSMLCLLNS TVNPIIYALR  400
CB2 253 VLLICWFPVL ALMAHSLATT LSDQVKKAFA FCSMLCLINS MVNPVIYALR  302

        7777777777 777
        5555666666 666
        7890123456 789
CB1 401 SKDLRHAFRS MFPSCEGTAQ PLDNSMGDSD CLHKHANNAA SV.HRAAESC  449
CB2 303 SGEIR..... ..SSAHHCLA HWKKCVRGLG SEAKEEAPRS SVTETEADGK  345

CB1 450 IKSTVKIAKV TMSVSTDTSA EAL* 472
CB2 346 ITPWPDSRDL DLSDC*.... .... 360
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Fig. 2. An alignment of the human CB_1 and CB_2 receptors. The transmembrane domains are *underlined*. The standard single letter amino acid code is used. The numbering system of Ballesteros and Weinstein (1995) is shown *above* each transmembrane domain

3
Gene Structure and Species Diversity

Shortly after the cloning of the rat cannabinoid receptor, isolation of a human CB_1 receptor cDNA was reported (Gerard et al. 1991). The rat and human receptors are highly conserved, 93% identity at the nucleic acid level and 97% at the amino acid level. There is an excellent correlation between binding affinities at the cloned CB_1 receptor as compared to binding in brain homogenates using [^{3}H]CP 55,940 as the radioligand (Felder et al. 1992).

There is evidence for splice variants of the cannabinoid receptors. A PCR amplification product was isolated that lacked 167 base pairs of the coding region of the human CB_1 receptor (Shire et al. 1995). This alternative splice form (CB_{1A}) is unusual in that it is generated from the mRNA encoding CB_1, and not from a separate exon (Shire et al. 1995). When expressed, the CB_{1A} clone would translate to a receptor truncated by 61 amino acid residues with 28 amino acid residues different at the NH_2-terminal. This might lead to a receptor with altered ligand-binding properties. CB_{1A} expression has been detected in many tissues by RT-PCR (Table 1). It will be important to confirm that the CB_{1A} receptor protein is expressed, since splice variants often arise from incomplete splicing during library construction and RT-PCR techniques. The construction of antibodies selective to CB_1 or CB_{1A} peptides would be useful to detect these proteins. The CB_{1A} splice variant is not present in rat or mouse, because the splice consensus sequence is absent in these genes (the invariant GT of the splice donor site becomes a GA in both the rat and mouse) (Bonner 1996).

The mouse CB_1 gene and cDNA sequences have been reported (Abood et al. 1997; Chakrabarti et al. 1995; Ho and Zhao 1996). Sequence analysis of the mouse CB_1 clones also indicates a high degree of conservation among species. The mouse and

Table 1. Amino acid residues important in cannabinoid receptor ligand recognition

CB_1 receptor	**CP 55,940 binding**	**WIN 55,212-2 binding**
SR141716A binding	F3.25(189)	G3.31(195)
K3.28(192)	K3.28(192)	F3.36(201)
F3.36(201)	C174	W5.43(280)
W5.43(280)	C179	V5.46(282)
W6.48(357)		W6.48(357)
Anandamide binding	**CB_2 receptor**	**WIN 55,212-2 binding**
F3.25(190)	SR144528 binding	S3.31(112)
K3.28(192)	S4.53(161)	F5.46(197)
	S4.57(165)	
	C175	
All ligand binding lost (conformational changes)		
Y5.39 (Y275 in CB_1, Y190 in CB_2)	C174 in CB_1	C179 in CB_2
D3.49(130) in CB_2	W4.50(158) in CB_2	W4.64(172) in CB_2
L5.50(201) in CB_2	Y7.53(299) in CB_2	

rat clones have 95% nucleic acid identity (100% amino acid identity). The mouse and human clones have 90% nucleic acid identity (97% amino acid identity). Rat CB_1 probes can be used to detect mouse 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 CB_1 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 CB_1 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 CB_1 gene (Shire et al. 1995). These data suggest the existence of splice variants of the CB_1 receptor as well as possible divergence of regulatory sequences between these genes. A third exon is present in the rat and human genes in their 5′ untranslated regions (Bonner 1996). The reported transcription start sites are consistent with the presence of two promoters for the CB_1 genes (Bonner 1996).

The CB_1 receptor has been studied in a molecular phylogenetic analysis of 64 mammalian species (Murphy et al. 2001). The sequence diversity in 62 species examined varied from 0.41% to 27%. In addition to mammals, the CB_1 receptor has been isolated from birds (Soderstrom et al. 2000b), fish (Yamaguchi et al. 1996), amphibia (Cottone et al. 2003; Soderstrom et al. 2000a), and an invertebrate, *Ciona intestitinalis* (Elphick et al. 2003). This deuterostomian invertebrate cannabinoid receptor contains 28% amino acid identity with CB_1, and 24% with CB_2 (Elphick et al. 2003). Since a CB receptor ortholog has not been found in *Drosophila melanogaster* or *Caenorhabditis elegans*, it has been suggested that the ancestor of vertebrate CB_1 and CB_2 receptors originated in a deuterostomian invertebrate (Elphick et al. 2003).

The CB_2 receptor has also been isolated from mouse (Shire et al. 1996b; Valk et al. 1997), rat (Griffin et al. 2000; Brown et al. 2002), and the puffer fish *Fugu rubripes* (Elphick 2002). The CB_2 receptor shows less homology between species than does CB_1; for instance, the human and mouse CB_2 receptors share 82% amino acid identity (Shire et al. 1996b), and the mouse and rat 93% amino acid identity. The human, rat, and mouse sequences diverge at the C-terminus; the mouse sequence is 13 amino acids shorter, whereas the rat clone is 50 amino acids longer than the human CB_2 (Brown et al. 2002).

There is also an intron in the C-terminus of the CB_2 receptor. This intron is also species-specific; it is only present in the rat CB_2 receptor (Brown et al. 2002). This may give rise to rat-specific pharmacology of the CB_2 receptor. We found differences in ligand recognition with a number of compounds at the rat CB_2 receptor compared to the human CB_2 receptor in transfected cells (Griffin et al. 2000). It is important to note, however, that the clone described in these studies was a genomic clone of rat CB_2 and did not contain the edited C-terminus discovered by Brown et al. (2002).

To date, the complete genetic structure including 5′ and 3′ untranslated regions and transcription start sites of the CB_1 and CB_2 genes have not been mapped. From what we know so far, the diversity in the regulatory regions of the CB_1 and CB_2 genes may provide flexibility in gene regulation.

4
Ligand Recognition at the CB_1 Receptor

4.1
The Aminoalklylindole/SR141716A Binding Region

Mutation studies as well as studies with novel ligands have suggested a separation of the binding site for aminoalkylindoles (typified by WIN 55,212-2) from that of the other three classes of cannabinoid agonist ligands (Table 2) (Chin et al. 1998; Song and Bonner 1996; Tao et al. 1999). A K3.28(192)A mutation of CB_1 results in no loss of affinity or efficacy for WIN 55,212-2, but greater than 1,000-fold loss in affinity and efficacy for HU-210, CP 55,940, and anandamide (Chin et al. 1998; Song and Bonner 1996), and a 17-fold loss for SR141716A (Hurst et al. 2002). The CB_2 selectivity of WIN 55,212-2 (Felder et al. 1995; Showalter et al. 1996) may be due to the presence of an additional TM helix (TMH)5 aromatic residue, F5.46 in the CB_2 receptor (Song et al. 1999). Receptor chimera studies of the CB_1 and CB_2 receptors have demonstrated that the region delimited by the fourth and fifth TM domains of the CB_1 receptor is crucial for the binding of the CB_1 receptor antagonist SR141716A, but not CP 55,940, and that this same region in the CB_2 receptor is crucial for the binding of WIN 55,212-2 and the CB_2 receptor antagonist SR144528 (Shire et al. 1996a, 1999). These results reinforce the hypothesis that the aminoalkylindole-binding region at the CB_1 receptor is in the TMH 3-4-5 region and is not identical to that for other CB agonists. Furthermore, these results suggest that SR141716A binding shares the aminoalkylindole binding region but also interacts with K(3.28)192.

In addition, the carbonyl oxygen as well as the morpholino ring of the aminoalkylindoles can be replaced without affecting affinity; therefore hydrogen bonding may not be the primary interaction of these compounds at the CB_1 receptor (Huffman 1999; Huffman et al. 1994; Kumar et al. 1995; Reggio 1999). Huffman et al. (1994) also reported that the replacement of the naphthyl ring of WIN 55,212-2 with an alkyl or alkenyl group resulted in complete loss of CB_1 receptor affinity (K_i>10,000 nM in both cases). The fact that the carbonyl oxygen or the morpholino ring of the aminoalkylindoles can be removed without significant effect, along with evidence that the presence of the carbonyl and morpholino group (in the absence of an aryl substituent) is insufficient to produce CB_1 affinity, suggests that aromatic stacking, rather than hydrogen bonding, may be the primary interaction for aminoalkylindoles at the CB_1 receptor.

Aromatic–aromatic stacking interactions are significant contributors to protein structure stabilization (Burley and Petsko 1985). Modeling studies indicate that in the active state (R*) model of CB_1, there is a patch of aromatic amino acids in the TMH 3-4-5 region with which WIN 55,212-2 can interact (McAllister et al. 2003). There is an upper (extracellular side) stack formed by F3.25(189 in human CB_1, 190 in mouse CB_1), W4.64(255/256), Y5.39(275/276), and W5.43(279/280). When WIN 55,212-2 is computationally docked to interact with this patch, it also can interact with a lower (towards intracellular side) aromatic residue, F3.36(200/201). In this docking position, WIN 55,212-2 creates a continuous aromatic stack over

several turns of TMHs 3, 4, and 5 that is likely to be energetically favored. Similarly, studies in the Reggio lab suggested that in the inactive (R) state of CB_1 the amide oxygen of SR141716A interacts with a salt bridge formed by K3.28 and D6.58(366), while the dichlorophenyl ring of SR141716A interacts with F3.36 and W6.48 and the monochlorophenyl ring interacts with F3.36 and W5.43 (Hurst et al. 2002).

In a recent study, McAllister et al. tested the hypothesis that a CB_1 TMH3-4-5-6 aromatic microdomain that includes F3.25, F3.36, W4.64, Y5.39, W5.43, and W6.48, constitutes the binding domain of SR141716A and WIN 55,212-2 (McAllister et al. 2003). Stably transfected cell lines were created for single-point mutations of each aromatic microdomain residue to alanine. The binding of SR141716A and WIN 55,212-2 were found to be affected by the F3.36A, W5.43A, and W6.48A mutations, suggesting that these residues are part of the binding site for these two ligands. In particular, the W5.43A mutation resulted in profound loss of affinity for SR141716A. Mutation of W4.64 to A resulted in loss of ligand binding and signal transduction; however, this was shown to be a result of improper cellular localization; the mutant receptor was not expressed on the cell surface.

Anandamide was used as a control in this study, as aromatic stacking interactions are not key to its binding. However, according to the molecular model, F3.25A is a direct interaction site for anandamide. F3.25A had no effect on WIN 55,212-2 or SR141716A binding, but resulted in a sixfold loss in affinity for anandamide (McAllister et al. 2003).

4.2 The Classical/Non-Classical/Endogenous CB Binding Region

As stated above, the mutation studies of CB_1 demonstrated greater than 1,000-fold loss in affinity and efficacy for HU-210, CP 55,940, and anandamide at K3.28(192)A (Chin et al. 1998; Song and Bonner 1996). This indicated that K3.28(192) is a primary interaction site for the phenolic hydroxyl of HU-210 and other classical cannabinoids, as well as the non-classical cannabinoids (e.g., CP 55,940) in the CB_1 receptor (Huffman et al. 1996). Modeling studies suggested that the alkyl side chain of CP 55,940 resides in a hydrophobic pocket (Tao et al. 1999). In CB_1, the primary interaction is between the phenolic hydroxyl of CP 55,940 and K3.28(192). These considerations suggest that the TMH 3-6-7 region is the binding site for classical and non-classical cannabinoids, and presumably the endogenous cannabinoids.

It should be noted that the two binding regions identified (i.e., TMH 3-4-5 for aminoalkylindoles and TMH 3-6-7 for other agonist classes) overlap spatially such that the binding of a ligand in one region would preclude binding in the other region. This would be detected as competitive inhibition in a binding assay.

Residues in the N-terminus as well as in and near extracellular loop 1 have been shown to be important for binding of CP 55,940 (Murphy and Kendall 2003). Loss of affinity for CP 55,940 was seen when dipeptide insertions were made at residues 113, 181, and 188. Six substitution mutants (to alanine) were constructed around these residues; they showed weaker affinity than the wild-type (WT) receptor, but

less of a loss than observed with the corresponding insertion mutant. This pattern suggests that the loop structure itself is important for recognition of CP 55,940.

Interestingly, F189(3.25)A in human CB_1 results in a dramatic reduction of CP 55,940 affinity (Murphy and Kendall 2003), but in mouse CB_1, CP 55,940 binding is not affected, and instead anandamide's affinity is lowered (McAllister et al. 2003). This suggests the minor sequence variation in mouse vs human CB_1 can result in structural differences in ligand recognition.

5
Ligand Recognition at the CB_2 Receptor

5.1
Identification of Amino Acids Which Discriminate CB_1 and CB_2 Receptor Subtypes

The CB_1 and CB_2 receptors (Fig. 2) share only 44% overall amino acid identity, which rises to 68% in the TM domains (Munro et al. 1993). However, most cannabinoid receptor agonists do not discriminate between the receptor subtypes (Felder et al. 1995; Pertwee 1997). There are several ligands which are CB_1- or CB_2-selective (5- to 60-fold), and a few ligands with a greater separation of activity at each receptor (100- to 1,000-fold) (Griffin et al. 1999, 2000; Hanus et al. 1999; Huffman et al. 1996, 1999; Ibrahim et al. 2003; Showalter et al. 1996; Tao et al. 1999). For example, 1-deoxy-Δ^8-THC showed no affinity for the CB_1 receptor but has good affinity (K_i=32 nM) for the CB_2 receptor (Huffman et al. 1999). However, there is a need for more selective agonists to produce specific receptor-mediated effects for in vivo studies.

Structure–activity relationships of Δ^9-THC analogs have revealed three critical points of attachment to a receptor: (1) a free phenolic hydroxyl group; (2) an appropriate substituent at the C9 position and (3) a lipophilic side chain (Howlett et al. 1988). However, compounds with a dimethylheptyl side chain retain affinity for both CB_1 and CB_2 receptors even when they lack a phenolic hydroxyl (Gareau et al. 1996; Huffman et al. 1996). Moreover, these ligands are CB_2-selective (Huffman et al. 1996, 1999).

An alternative approach to traditional structure–activity relationships with synthetic ligands is to map the ligand binding sites of the receptors using in vitro mutagenesis of receptor cDNAs. For example, the lysine residue in the third TM domain of the cannabinoid receptors, which is conserved between the CB_1 and CB_2 receptors, appears to mediate different functional roles in the receptor subtypes. K3.28(192) in the CB_1 receptor is critically important for ligand recognition for several agonists (CP 55,940, HU-210, Δ^9-THC, and anandamide) but not for WIN 55,212-2 (Chin et al. 1998; Song and Bonner 1996). Mutation of the analogous residue in the CB_2 receptor (K109) to alanine or arginine resulted in fully functional CB_2 receptors with all ligands tested (Tao et al. 1999). In this same study a molecular model was generated in order to explain these findings. The model suggested an alternative binding mode could be achieved in the K109A CB_2 mutant in contrast to K192A CB_1. Assuming that ligand binding occurs within the pore

formed by the TMH bundle, and the hydrophobic cluster of amino acids on helices 6 and 7 form the hydrophobic pocket with which the dimethylheptyl side chain of CP 55,940 interacts, receptor docking studies indicated that CP 55,940 is oriented differently in the binding pocket in CB_1 vs CB_2. A unique feature identified in the CP 55,940/CB_2 binding site was a hydrogen bonding cluster formed by a serine, threonine, and an asparagine. In the CP 55,940/CB_1 docking studies this cluster is not present. This suggested that when CB_2 K109 was mutated to A, the hydrogen bonding cluster could compensate for receptor binding to CP 55,940, whereas when CB_1 K192 was mutated to A this compensation did not occur. To test this hypothesis the CB_2 hydrogen-bonding cluster was disrupted by generating the double-mutant K109AS112G. When the serine in the hydrogen-bonding cluster was replaced with a glycine, the receptor was not able to recognize several cannabinoid agonists excluding WIN 55,212-2. This was reminiscent of the findings of CB_1 K192A, except only 10% vs full inhibition of cyclic 3′,5′-adenosine monophosphate (cAMP) accumulation could be produce even in the presence of 10 μM WIN 55,212-2. Receptor expression was determined by immunofluorescence. The WT CB_2 protein was expressed in approximately 90% of the cells. Only 30% of the cells expressed the double-mutant K109AS112G, and the pattern of staining exhibited entrapment of the receptor within the perinuclear region. Interestingly, even the expression of the K109A mutant receptor, which exhibited WT receptor characteristics, was expressed less than the WT receptor (50% vs 90% of the cells expressed the protein, respectively). The reduced expression of the double-mutant K109AS112G could explain why only 10% inhibition of cAMP accumulation was observed in the presence of WIN 55,212-2. Regardless, the serine in combination with the lysine in the CB_2 receptor appears to play a crucial role in determining proper function of the receptor.

The K3.28 mutation studies demonstrated that a separate but overlapping receptor binding site must occur with WIN 55,212-2 compared to other cannabinoid ligands in the CB_1 receptors. Another important feature of WIN 55,212-2 is that it has a higher affinity for the CB_2 receptors, albeit only five- to tenfold higher (Showalter et al. 1996). Two groups sought to discover critical residues in the cannabinoid receptors that impart this agonist selectivity. The first used a molecular modeling approach; it indicated that aromatic stacking interactions are important for aminoalkylindole binding (Song et al. 1999). There is a phenylalanine at position 5.46(F197) in CB_2 vs a valine (V282) in CB_1, which could provide greater aromatic stacking and may impart the selectivity of WIN 55,212-2 for CB_2. Therefore, valine and phenylalanine were switched between the receptors. The CB1V282F mutant bound WIN 55,212-2 in a similar fashion to WT CB_2, whereas the CB_2 F197V mutant adopted CB_1 receptor binding affinity for WIN 55,212-2. This data strongly favored the hypothesis that a phenylalanine at position 5.46 is crucial for WIN 55,212-2 selectivity.

At the same time, the role of TM3 in WIN 55,212-2 selectivity was reported (Chin et al. 1999). In this investigation, a CB_1/CB_2 chimera was constructed, $CB_{1/2}$(TM3), in which the TM3 of CB_1 was replaced with the corresponding region of CB_2. The $CB_{1/2}$(TM3) mutant bound WIN 55,212-2, and the other related aminoalkylindole analogs (JWH015 and JWH018) with WT CB_2 affinities. These results suggested

that the TM3 of the cannabinoid receptor imparts selectivity of aminoalkylindoles to CB_2. When individual amino acid changes were evaluated, S112(3.31) in CB_2, which corresponded to G195 in CB_1, was the amino acid responsible for CB_2 selectivity of aminoalkylindoles. Tao et al. (1998) also reported that mutation of S112 in the K109AS112G mutation resulted in dramatic effects on ligand binding.

Key differences in the ligand recognition sites of the CB_1 and CB_2 receptors were identified using a combination of receptor chimeras and site-directed mutagenesis (Shire et al. 1996a). This study focused on the SR141716A (CB_1-selective) and CP 55,940 (non-selective) binding sites. Replacing the CB_1 receptor with up to the seventh TM region of the CB_2 receptor, including the third extracellular loop, resulted in a receptor that still exhibited CB_1 receptor properties. Further extending the CB_2 structure into the sixth TM region of the CB_1 altered receptor expression; the mutant was sequestered in the intracellular compartment of the cell and could not be analyzed. Further extending the CB_2 structure into the fifth and then fourth TM region of the CB_1 receptor systematically resulted in a $CB_{1/2}$ chimera that acted like a CB_1 receptor. The fifth TM $CB_{1/2}$ chimera acted as a $CB_{1/2}$ hybrid and the reciprocal mutation fifth TM $CB_{2/1}$ chimera had almost identical properties. The fourth TM $CB_{1/2}$ chimera was similar to the WT CB_2 receptor.

A sandwich chimera was next constructed where the CB_1 receptor TM4-e2-TM5 region was replaced with the CB_2 receptor regions (Shire et al. 1996a). This chimera resembled the WT CB_2 receptor, strengthening the findings that these regions are important for CB_1 receptor selectivity of SR 141716A. A sandwich chimera was then created in which just the CB_1 receptor e2 region was replaced with the CB_2 receptor e2 region; SR141716A binding was almost identical to the WT CB_2, but in this case CP 55,940 binding was lost. A smaller sandwich chimera was also created in which just the CB_1 receptor e2 region between conserved cysteines was replaced with the corresponding CB_2 receptor regions; this mutation resulted in a sequestration of the receptor.

Generation of functional CB_2/CB_1 chimeras proved to be more difficult when trying to study the TM4-e2-TM5 regions. When the CB_2 receptor TM4-e2-TM5 region was replaced with the CB_1 or a sandwich chimera was created in which just the CB_2 receptor e2 region was replaced with CB_1 e2, the receptors were expressed but could not bind CP 55,940 or SR141716A (Shire et al. 1996a).

One notable difference between cannabinoid receptors and many other GPCRs is the lack of conserved cysteines in the second extracellular (EC) domain. However, the third EC domain of both cannabinoid receptors does contain two or more cysteines. These cysteines are thought to form sulfhydryl bonds with cysteines in neighboring TM domains and to stabilize the receptor. When C257 and C264 in the third EC domain of the CB_1 receptor were replaced with serine residues, the mutant receptors were sequestered (Shire et al. 1996a). These residues were then replaced with alanine. In this case the receptors were expressed normally but failed to bind CP 55,940. When cysteine residues (C174 and C179) in the third EC domain of the CB_2 receptor were replaced with serine residues, the mutant receptor, although expressed normally on the cell surface, could not bind CP 55,940. Disruption of a disulfide bridge with the two cysteines in the amino-terminal region of the CB_1 receptor was not the explanation, because the double mutant C98,107S resulted in

a receptor with WT properties. Overall, these results suggest the e2 domain and corresponding cysteines are important for CP 55,940 ligand recognition, but not for SR141716A.

5.2 The SR14428 Binding Site

The SR144528 binding site (Table 1) on CB_2 has been analyzed by a combination of site-directed mutagenesis and molecular modeling (Gouldson et al. 2000). Mutation of C175 (in the third EC loop) to serine resulted in a receptor with normal affinity for [^{3}H]CP 55,940, but loss of recognition of SR144528. Consequently, SR144528 did not act as an antagonist at this mutant. An eightfold loss of affinity for WIN 55,212-2 was observed with the C175S mutant. Mutation of S4.53(161) and S4.57(165) to alanines also resulted in the loss of SR144528 binding and functional activity. These serines are alanines in the CB_1 receptor, which supports a direct ligand–residue interaction at CB_2. Several other mutations were analyzed that did not affect SR144528 binding. In the corresponding molecular model of CB_2, SR144528 interacts with residues in TM 3,4, and 5 through a combination of hydrogen bonds and hydrophobic interactions (Gouldson et al. 2000). In particular, W4.64(172) and W5.43(194) form an aromatic stack similar to that proposed for WIN 55,212-2 in the CB_2 receptor (Song et al. 1999) and WIN 55,212-2 and SR141716A in the CB_1 receptor (McAllister et al. 2003).

6 Receptor Conformation

In addition to specific ligand–receptor interactions, several residues have been shown to be keys to maintaining proper receptor conformation for ligand recognition. For example, at the top of the TMH 3-4-5 aromatic cluster in both the CB_1 [Y5.39(275)] and CB_2 [Y5.39(190)] receptors is a tyrosine residue. Creating a tyrosine-to-phenylalanine mutation in both CB_1 and CB_2 resulted in subtle alterations in receptor affinity and signal transduction. In contrast, a tyrosine-to-isoleucine mutation in CB_1 and CB_2 led to receptors that lost ligand-binding capability (McAllister et al. 2002). Evaluation of receptor expression revealed no significant differences between the Y5.39I mutant and the WT receptor. Mutation of Y5.39(275) to A resulted in a receptor which failed to be expressed at the cell surface (Shire et al. 1999). Monte Carlo/stochastic dynamics studies suggested the hypothesis that aromaticity at position 5.39(275) in CB_1 and 5.39(190) in CB_2 is essential to maintain cannabinoid ligand WT affinity; while the CB_1 Y5.39(275)F mutant was very similar to WT, the Y5.39(275)I mutant showed pronounced topology changes in the TMH 3-4-5 region (McAllister et al. 2002).

Two conserved tryptophan residues, W4.50(158) and W4.64(172), are required for proper ligand recognition and signal transduction (Rhee et al. 2000a). W4.50 is conserved among most GPCRs, whereas W4.64 is conserved between CB_1 and CB_2

receptors. Substitutions to aromatic residues phenylalanine or tyrosine as well as to leucine and alanine were evaluated. For both tryptophan residues, the W-to-F mutant retained WT binding and signaling properties and the L and A mutations resulted in loss of ligand binding and signal transduction. In this study, expression of protein was assessed by Western analyses; however, cellular localization was not examined (Rhee et al. 2000a). W4.64 has been suggested to be an interaction site for the aminoalkylindoles and pyrazole antagonists, and in CB_1, the W4.64A mutation resulted in a receptor that did not localize to the cell surface (McAllister et al. 2002).

Absence of a conserved proline is crucial for proper function of the CB_2 receptor (Song and Feng 2002). In most GPCRs, there is a proline residue in the middle of TM5, but in the cannabinoid receptors this residue is a leucine. Substitution of L5.50(201) to proline caused a complete loss of ligand binding and function, probably due to an overall conformational change in the mutant receptor (Song and Feng 2002).

The highly conserved tyrosine in the $NP(X)_nY$ motif in TM7 also plays an important role in the CB_2 receptor's proper conformation for ligand recognition and signal transduction (Feng and Song 2001). The Y7.53(299)A mutation produced a receptor that was correctly targeted to the cell membrane, yet led to a complete loss of ligand binding and functional coupling to adenylyl cyclase. Since the location of Y299 is very close to the cytoplasmic face, it is not postulated to be directly involved in ligand binding; instead these results are probably due to conformational changes in the receptor protein (Feng and Song 2001).

7
CB_1 Receptor Activation

7.1
Constitutive Activity

Overexpression of many GPCRs leads to some degree of constitutive (agonist-independent) activity (Lefkowitz et al. 1993). Experimental evidence for constitutively active CB_1 receptors was first noted when SR141716A, initially described as a CB_1 antagonist, was found to have inverse agonist properties (Bouaboula et al. 1997). In transfected CHO cells expressing CB_1, cannabinoid agonists activated mitogen-activated protein kinase (MAPK) activity (Bouaboula et al. 1997). However, basal MAPK activity was higher in CB_1-transfected cells as compared to untransfected cells, suggesting the presence of autoactivated CB_1 receptors. SR141716A not only antagonized the agonist effect on MAPK, but also reduced basal MAPK activity in CB_1-transfected but not untransfected cells. Similarly, basal cAMP levels were reduced, and SR141716A raised basal cAMP levels in transfected cells. The EC_{50} for SR141716A was similar to its IC_{50}, suggesting that these effects are a result of direct binding to unoccupied (precoupled) CB_1 receptors and not due to the presence of endogenous ligands in the cultures. A significantly higher EC_{50} would be predicted if endogenous agonists were competing with SR141716A. Sub-

sequent studies extended these findings to CB_1 receptor-activated guanosine-5′-*O*-(3-thiotriphosphate) (GTPγS) binding (Landsman et al. 1997) and inhibition of calcium conductance (Pan et al. 1998). Additionally, CB_1 receptors can sequester G proteins, making them unavailable to couple to other receptors (Vasquez and Lewis 1999). SR141716A is also an inverse agonist when CB_1 receptors are co-expressed with G protein-coupled potassium channels in *Xenopus* oocytes (McAllister et al. 1999).

Previously, inverse agonist effects had not been observed in cell lines possessing native CB_1 receptors (Bouaboula et al. 1995), or in primary neuronal cultures (Jung et al. 1997). However, a study in primary cultures of rat cerebellar granule neurons presented evidence for inverse agonism by SR141716A on nitric oxide synthase activity (Hillard et al. 1999). Evidence for inverse agonism was also reported in the guinea pig small intestine (Coutts et al. 2000).

Constitutively active GPCRs can arise from mutations (either naturally occurring or engineered), presumably as a result of transforming the receptor to a constitutively active state. Mutations that result in constitutive activity may provide clues to the key amino acids involved in receptor activation. Generally, constitutively active receptors are also constitutively phosphorylated and desensitized, providing support for a model where a single active state conformation is the target for phosphorylation, internalization and desensitization (Leurs et al. 1998). However, a recent study on the angiotensin II receptor and a series of studies on the CB_1 receptor suggest that GPCRs may possess several transition states, each associated with conformationally distinguishable states of receptor activation and regulation (Houston and Howlett 1998; Hsieh et al. 1999; Jin et al. 1999; Roche et al. 1999; Thomas et al. 2000).

A F3.36/W6.48 interaction is proposed to be key to the maintenance of the CB_1 inactive state (Singh et al. 2002). Previous modeling studies have suggested that a F3.36/W6.48 interaction requires a F3.36 *trans* χ1/W6.48 g+ χ1 rotameric state. SR141716A stabilizes this F3.36/W6.48 aromatic stacking interaction, while WIN55,212-2 favors a F3.36 g+ χ1/W6.48 *trans* χ1 state (Singh et al. 2002). Cannabinoid receptor activation of GIRK1/4 channels in *Xenopus* oocytes was used to assess functional characteristics of the mutant proteins (McAllister et al., 2004). Of five mutant receptors tested, only the F3.36(201)A demonstrated a limited activation profile in the presence of multiple agonists. Ligand-independent receptor activation of GIRK1/4 channels showed that the F3.36A mutant had statistically higher

Table 2. Amino acids important in signal transduction

CB_1 receptor	**CB_2 receptor**
D2.50(163/164)	D2.50(80)
F3.36(201)	R3.50(131)
L6.34(341) and A6.35(342)	Y2.51(132)
C-terminus (401–417)	Y5.58(207)
	A6.34(244)
	C313
	C320

levels of constitutive activity compared to WT CB_1. This result supports the hypothesis of a $\chi 1$ rotamer "toggle" switch (W6.48 $\chi 1$ g+, F3.36 $\chi 1$ *trans*) → (W6.48 $\chi 1$ *trans*, F3.36 $\chi 1$ g+) for activation of CB_1.

7.2 Residues Involved in Activation of CB_1

Studies to date have indicated that not only are sets of different amino acids involved in the binding of several cannabinoid ligands, but that these ligands promote interactions with different G proteins (Bonhaus et al. 1998; Glass and Northup 1999; Griffin et al. 1998; Kearn et al. 1999; Mukhopadhyay et al. 2000; Selley et al. 1996; Tao et al. 1999). The different sites of ligand–receptor interaction may promote different receptor conformations, which in turn result in selective interaction with different G proteins. Evidence that different receptor conformations can promote distinct G protein interactions is provided by a study in which a mutation produced a constitutively active CB_1 receptor that coupled to G_s in preference to G_i (Abadji et al. 1999). The predominant coupling of the WT CB_1 receptor is to G_i; coupling to G_s can usually only be demonstrated in the presence of pertussis toxin, which uncouples receptors from $G_{i/o}$ proteins (Glass and Felder 1997). A swap of two adjacent residues in the carboxyl terminus of the third intracellular loop/bottom of helix 6, L6.34(341)A/A6.35(342)L, resulted in a receptor that produced minimal inhibition of adenylyl cyclase in the presence of agonist, but instead showed increased basal levels of cAMP in the absence of agonist (Abadji et al. 1999).

Using synthetic peptides derived from the CB_1 receptor, Howlett's laboratory has demonstrated that the amino terminal side of the intracellular (i3) loop can interact with G_i, leading to the inhibition of adenylyl cyclase and that the juxtamembrane portion of the C-terminus is critical for G protein activation (Howlett et al. 1998). As in many other GPCRs, the CB_1 receptor C terminal region may assume a helical structure. In fact, this helical segment is quite clear in the Rho crystal structure (Palczewski et al. 2000). Synthetic peptides derived from this region can autonomously inhibit adenylyl cyclase by regulation of G_i and G_o proteins (Mukhopadhyay et al. 1999, 2000). Residues R400, K402, and C415 have been implicated as potential sites for G protein activation (Mukhopadhyay et al. 1999). Interestingly, the analogous region of CB_2 does not activate G_i (Mukhopadhyay et al. 1999, 2000).

Residues in the C-terminus have also been shown to be important in G protein coupling and sequestration (Nie and Lewis 2001a,b). Truncation of the CB_1 receptor at residue 417 attenuates G protein coupling, and truncation at residue 400 abolishes the inhibition of calcium channels produced by CB_1 receptors expressed in superior cervical ganglia neurons (Nie and Lewis 2001a). Truncation at residue 417 also enhances constitutive activity and G protein sequestration of receptors (Nie and Lewis 2001b). These mutations did not affect trafficking of the receptor to the cell surface.

In contrast, mutation of D2.50(164) to N abolished G protein sequestration and constitutive activity without disrupting agonist activity of CB_1 receptors expressed

in neurons (Nie and Lewis 2001b). The consequences of mutation of D2.50, a highly conserved residue present in most GPCRs, appear to depend on the system in which the mutant receptor is expressed. Mutation of human CB_1 D2.50(163) to glutamine or glutamate disrupted G protein coupling but allowed the receptors to retain high affinity for cannabinoid compounds when the mutant receptors were expressed in human embryonic kidney (HEK) 293 cells (Tao and Abood 1998). A subsequent study by Roche et al. (1999) found that rat CB_1 D164N expressed in AtT20 cells retained coupling to adenylyl cyclase and inhibition of calcium currents, but did not couple to GIRK channels internalized following cannabinoid exposure. Interestingly, this same disparity had previously been observed with the α-adrenergic receptor, in that transfection of D2.50N mutant receptors into fibroblasts lacked adenylyl cyclase coupling, but those expressed in AtT20 pituitary cells coupled to adenylyl cyclase (Surprenant et al. 1992). Thus, the cellular background into which the mutant receptors are introduced is also an important determinant of functional coupling. It is possible that this is due to differential localization of the transfected receptors or differential G protein expression.

8
CB_2 Receptor Activation and Constitutive Activity

8.1
Constitutive Activity

The CB_2 receptor has also been shown to be constitutively active (Bouaboula et al. 1999a). Furthermore, CB_2 receptors expressed in CHO cells also sequester G_i proteins; the CB_2 inverse agonist SR144528 inhibits basal G protein activity as well as switching off MAPK activation from receptor tyrosine kinases and the GPCR lysophosphatidic acid (LPA) receptor (Bouaboula et al. 1999a). CB_2 receptors are constitutively phosphorylated and internalized (Bouaboula et al. 1999b). Autophosphorylation as well as agonist-induced phosphorylation occurs on S352 and involves a GPCR kinase (GRK) (Bouaboula et al. 1999b).

8.2
CB_2 Receptor Activation

As with the CB_1 receptor, mutation of the highly conserved aspartate residue in the second TM domain of the CB_2 receptor, D2.50(80) to glutamine or glutamate, disrupted G protein coupling without affecting high-affinity agonist binding (Tao and Abood 1998).

The DRY motif has been shown to be important for activation of a number of GPCRs. This motif has been examined in two separate studies of the CB_2 receptor, with different results (Feng and Song 2003; Rhee et al. 2000b). Both investigations found that mutation of D3.49(130) to A resulted in loss of ligand binding and subsequent signal transduction (Feng and Song 2003; Rhee et al. 2000b). This was

proposed to be due to a conformational change in the CB_2 receptor, rather than a direct effect on ligand binding, since this residue is at the cytoplasmic end of TM3. Mutation of Y2.51(132) to A resulted in a loss of signal transduction without affecting ligand recognition (Rhee et al. 2000b). However, Rhee et al. (2000a) demonstrated that mutation of R3.50(131) to A resulted in a slight reduction of signal transduction, whereas Feng and Song (2003) found no evidence for G protein coupling in the mutant receptor, including an abolition of constitutive activity in the mutant cell line. In one case, transient transfection into COS cells was employed (Rhee et al. 2000b), in the other, stable transfection into HEK 293 cells was used (Feng and Song 2003), again suggesting the cellular background plays an important role in the function of these GPCRs. Coupling to different G proteins is one explanation for the disparate results. In fact, a recent study found that 2AG induced a pertussis toxin-sensitive response, whereas CP 55,940 functional responses were unaffected by treatment with pertussis toxin; mutation of R3.50(131) to A resulted in reduction of the 2AG but not the CP 55,940-mediated responses (Alberich Jorda et al. 2004).

Mutation of A6.34(244) to glutamate resulted in a loss of ligand binding, signal transduction and constitutive activity (Feng and Song 2003). The location of this amino acid, at the bottom of helix 6, suggests that it may be important in receptor conformation. Highlighting the differences between CB_1 and CB_2 receptors, this amino acid in the CB_1 receptor was partly responsible for enhancing G protein coupling to G_s (Abadji et al. 1999).

The presence of a tyrosine residue conserved between CB_1 and CB_2,Y5.58(207), is critical for signal transduction in the CB_2 receptor (Song and Feng 2002). The Y5.58A mutant receptor retained ligand binding, albeit with an eightfold reduced affinity for [^{3}H]WIN 55,212-2, and fivefold reduction in HU-210 and anandamide binding. This residue resides at the cytoplasmic end of helix 5, an area which has been demonstrated to be involved in G protein coupling; therefore this conserved tyrosine may play a role in propagation of agonist-induced conformational changes for signal transduction (Song and Feng 2002).

Cysteine residues in the C-terminal domains have been shown to be important in functional coupling in several GPCRs. Mutation of C313 or C320 to alanine in the CB_2 receptor resulted in a mutant that retained WT ligand recognition properties but loss of functional coupling to adenylyl cyclase (Feng and Song 2001). In several other GPCRs, C-terminal cysteine mutations also led to lack of desensitization; this was not the case with the CB_2 receptor (Feng and Song 2001). These data demonstrate the importance of residues in the C-terminal domain to functional coupling in the CB_2 receptor.

9
CB_1 Receptor Polymorphisms in Addiction and Disease

The CB_1 receptor has been shown to regulate cocaine and heroin reinforcement as well as opioid dependence (De Vries et al. 2001; Ledent et al. 1999). When the CB_1 receptor was knocked out by homologous recombination, not only did

the mutant mice lose responsiveness to cannabinoids, the reinforcing properties of morphine and the severity of the withdrawal syndrome were strongly reduced (Ledent et al. 1999). Several laboratories have demonstrated that CB_1 receptors regulate mesolimbic dopaminergic transmission in brain areas known to be involved in the reinforcing effects of morphine, and it has now been shown that the CB_1 receptor is critical for this μ-opioid receptor effect (Chen et al. 1990; Mascia et al. 1999; Tanda et al. 1997). In addition to increasing mesolimbic dopamine, Δ^9-THC facilitates brain stimulation reward, an animal model for abuse liability (Gardner and Lowinson 1991). Moreover, genetic variations in the response have been clearly demonstrated in three strains of rats (Lepore et al. 1996). Lewis rats showed the most pronounced Δ^9-THC-induced enhancement of brain reward functions. Sprague-Dawley rats showed an enhancement that was approximately half that seen in Lewis rats and, at the dose tested, brain reward functions in Fischer 344 rats were unaffected. A subsequent study also found a strain-specific facilitatory effect on dopamine efflux in nucleus accumbens (Chen et al. 1991). These data demonstrate that genetic variations to cannabinoid effects exist and suggest that genetic variation influences drug abuse vulnerability. Indeed, differential sensitivity to Δ^9-THC in the elevated plus-maze test of anxiety was also shown in three mouse strains (Onaivi et al. 1995). Two different doses of Δ^9-THC induced aversion to the open arms of the maze in ICR mice, but not in DBA/2 and C57BL/6 mice. Basal locomotor activity was significantly different in the three strains of mice, and may be related to differences in CB_1 receptor function (Basavarajappa and Hungund 2001).

The CB_1 receptor has been cloned and sequenced from two strains of mice, C57BL/6 (Chakrabarti et al. 1995) and 129SJ (Abood et al. 1997) as well as from NG108-15 cells (Ho and Zhao 1996). Additional mouse genomic sequence information has been deposited at NCBI. However, the additional full-length sequences are also from the 129SJ strain. Sequence analysis of the C57BL/6 CB_1 receptor cDNA (accession No. U17985), indicates three amino acid differences compared to that obtained from the 129SJ strain (genomic clones, accession No. U22948 and Abood et al. 1997) and NG108-15 (cDNA clone, accession No. U40709). One of them, T210R, is in the third TM domain, an area found to be critical for ligand recognition in the CB_1 receptor (Chin et al. 1998, 1999; Song and Bonner 1996; Tao et al. 1999). CB_1 receptor polymorphisms may underlie differential sensitivity to Δ^9-THC. In addition, a recent report showed distinct differences in CB_1 receptor binding properties in the brains of C57Bl/6 and DBA/2 mice (Hungund and Basavarajappa 2000). It is possible that naturally occurring mutations confer functional differences in CB_1 responses.

Human CB_1 receptor polymorphisms have been identified. One study found a positive association between a microsatellite polymorphism in the CB_1 gene and intravenous drug abuse (Comings et al. 1997). The initial polymorphism found was a restriction fragment length polymorphism (RFLP) in the intron preceding the coding exon of the receptor (Caenazzo et al. 1991). The CB_1 receptor gene is intronless in its coding region, but possesses an intron 5′ to the coding exon with three putative upstream exons (Abood et al. 1997; Bonner 1996). The first polymorphism in the coding exon was recently reported by Gadzicki et al. (1999).

They identified a silent mutation in T453 (G to A)—a conserved amino acid present in the C-terminal region of the CB_1 and CB_2 receptors—that was a common polymorphism in the German population. While this mutation is silent, analysis of several human sequences present in the database reveals that CB1K5 (accession No. AF107262), a full-length sequence, contains five nucleotide changes, three of which result in amino acid differences. Coincidentally, two amino acid differences are in the third TM domain, F200L and I216V. The third variant is in the fourth TM domain, V246A. A recent report by the group that submitted the sequence to the database revealed that this was a somatic mutation in an epilepsy patient; i.e., DNA obtained from his or her blood was unaltered, but DNA from the hippocampus showed the mutation (Kathmann et al. 2000). The presence of a somatic mutation rather than a polymorphism is generally indicative of the disease process in cancers [e.g. mutant p53 or APC expression in tumors but not normal tissues (Baker et al. 1989; Lamlum et al. 2000)]. CB_1 receptor polymorphisms may affect responsiveness to cannabinoids.

10
The Role of Receptor Regulation in the Development of Cannabinoid Tolerance

Cannabinoid tolerance develops in the absence of pharmacokinetic changes (Martin et al. 1976); therefore, biochemical and/or cellular changes are responsible for this adaptation. The production of tolerance can be associated with a drug's abuse potential (O'Brien 1996); therefore receptor mechanisms contributing to cannabinoid tolerance are of significant interest. One hypothesis for tolerance development is that receptors lose function during chronic agonist treatment, leading to diminished biological responses. Potential cellular mechanisms that might play important roles in tolerance include receptor desensitization, internalization, and downregulation.

Current theories for GPCR regulation predict that activated receptors are phosphorylated by GRKs and/or second messenger-activated kinases (Garcia et al. 1998; Leurs et al. 1998). β-Arrestins bind to phosphorylated receptors and sterically hinder further association of the receptor with G protein, terminating signaling. For some GPCRs, arrestins can serve as adapters to target the receptors for clathrin-mediated internalization and to promote coupling to tyrosine kinase signaling pathways (Luttrell et al. 1999). Also, in the continued presence of agonist, receptors are targeted to lysosomes for degradation (Zastrow and Kobilka 1992). It is this last event that is detected as decreased surface receptor binding.

Early studies of cannabinoid receptor downregulation at the mRNA level in conjunction with ligand binding did not detect changes in either receptor number or mRNA levels in whole brains from mice tolerant to Δ^9-THC (Abood et al. 1993). However, in mice tolerant to CP 55,940, cannabinoid receptor downregulation in cerebella is concomitant with increased levels of receptor mRNA, without alteration of the inhibitory effect of cannabinoid agonists on cAMP accumulation (Fan et al. 1996). Extensive downregulation in cerebellar membranes without any effect on

receptor-G protein coupling was subsequently confirmed (Breivogel et al. 1999). Brain region specificity of receptor downregulation has also been demonstrated by several laboratories (Breivogel et al. 1999; Oviedo et al. 1993; Rodriguez-de-Fonseca et al. 1994; Romero et al. 1997). A comprehensive study examining the time course of changes in cannabinoid-stimulated [^{35}S]GTPγS binding and cannabinoid receptor binding in both rat brain sections and membranes, following daily Δ^9-THC treatments for 3, 7, 14, and 21 days, found time-dependent decreases in both [^{35}S]GTPγS and [^{3}H]WIN 55212-2 and [^{3}H]SR141716A binding in cerebellum, hippocampus, caudate-putamen, and globus pallidus, with regional differences in the rate and magnitude of downregulation and desensitization (Breivogel et al. 1999). In a parallel study, the time course and regional specificity of expression of the CB_1 receptor was examined (Zhuang et al. 1998). They found that CB_1 mRNA levels were increased above vehicle control animals at 7 days of treatment (Fan et al. 1996). However, another laboratory found some regions which showed no changes in receptor binding, [^{35}S]GTPγS activation, or mRNA levels following chronic cannabinoid administration (Romero et al. 1998a,b).

Several recent studies in transfected cell systems have implicated regions of the CB_1 receptor involved in receptor regulation following chronic agonist exposure. Rapid internalization of CB_1 receptors was observed after agonist exposure (Hsieh et al. 1999; Rinaldi-Carmona et al. 1998b). In contrast, chronic treatment of cells with the inverse agonist SR141716A caused upregulation of cell surface receptors (Rinaldi-Carmona et al. 1998b). As in other GPCRs, the C-terminal domain is critical for receptor internalization; truncation of the terminal 14 amino acids eliminates receptor internalization (Hsieh et al. 1999). Truncation of the C-terminus at residue 418 abolished desensitization, as did deletion of residues 418–439 (Jin et al. 1999).

On the other hand, phosphorylation of S426 and S430 (tail region) or S317 (third intracellular loop) resulted in CB_1 receptor desensitization; however, these sites had no influence on internalization (Garcia et al. 1998; Jin et al. 1999). While receptor internalization was not affected when G protein signaling was disrupted by treatment with pertussis toxin, a mutation of the highly conserved aspartate residue in the second TM domain in which G protein coupling is altered did block CB_1 receptor internalization (Roche et al. 1999).

Both in vivo and in vitro, different cannabinoid compounds can produce various degrees of tolerance and desensitization, suggesting their actions at cannabinoid receptors may not be identical (Dill and Howlett 1988; Fan et al. 1994). In a comparison of three cannabinoid agonists, the most potent compound (CP 55,940)

Table 3. Amino acids important for desensitization and internalization

Desensitization	Internalization
S317 in CB_1	D2.50(164) in CB_1
S426 in CB_1	C-terminus 458–472 in CB_1
S430 in CB_1	
S352 in CB_2	
C-terminus 418–439	

produced the most tolerance in vivo (Fan et al. 1994). In most in vitro studies, a single cannabinoid agonist has been used; so the cellular basis for this differential tolerance has yet to be determined.

The CB_2 receptor is also desensitized and internalized following agonist treatment in vitro (Bouaboula et al. 1999b). These studies, conducted in CB_2-transfected CHO cells, demonstrated that phosphorylation at S352 appears to play a key role in the loss of responsiveness of the CB_2 receptor. Furthermore, SR144528 could regenerate the desensitized CB_2 receptors by activating a phosphatase that dephosphorylated the receptor. Hence the pharmacological properties and phosphorylation state of the CB_2 receptor can be regulated by both agonists and antagonists.

11
Physiological Receptor Regulation and Disease

Early studies investigated cannabinoid receptor mRNA levels using in situ hybridization (Mailleux and Vanderhaeghen 1993; Mailleux and Vanderhaeghen 1993; Mailleux and Vanderhaeghen 1994). Following adrenalectomy, CB_1 mRNA levels in the striatum increased 50% as compared to control rats (Mailleux and Vanderhaeghen 1993). This increase could be counteracted by dexamethasone treatment, suggesting glucocorticoid downregulation of cannabinoid receptor gene expression in the striatum. A negative dopaminergic influence on CB_1 gene expression has been suggested by studies in which a unilateral 6-hydroxydopamine lesion was associated with 45% increase in mRNA levels in the ipsilateral side; furthermore, treatment with dopamine receptor antagonists mimicked the effect (Mailleux and Vanderhaeghen 1993). Previous experiments had documented the disappearance of CP 55,940 binding following an ibotenic acid lesion of the striatum, but not following a 6-hydroxydopamine lesion, indicating that cannabinoid receptors are not co-localized 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 30% decrease in mRNA levels, and treatment with the *N*-methyl-D-aspartate (NMDA) receptor antagonist MK-801 resulted in an approximate 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.

CB_1 receptors are drastically reduced in substantia nigra and lateral globus pallidus in Huntington's disease (Glass et al. 1993; Richfield and Herkenham 1994). The CB_1 receptor agonist nabilone significantly reduced L-dopa-induced dyskinesia in an animal model of Parkinson's disease as well as in Parkinson's patients (Sieradzan et al. 2001; Fox et al. 2002). CB_1 receptor knockout mice displayed increased neuropeptide expression in striatal output pathways and were severely hypoactive in an exploratory test, although their motor coordination was unaltered, suggesting these receptors may be important for initiation of movement (Steiner et al. 1998).

The first report of alteration of CB_2 receptor expression was in the original cloning paper; CB_2 was isolated as a result of its differential expression following treatment with dimethylformamide to produce granulocyte differentiation in the human promyelocytic leukemia line HL60 (Munro et al. 1993). CB_2 transcripts are also elevated when HL60 cells are induced to differentiate into macrophages by tetradecanoylphorbol acetate treatment (Munro et al. 1993). The chromosomal location of CB_2 is in a common virus integration site, and it is overexpressed in retrovirally transformed mouse myeloid leukemias (Valk et al. 1997). Furthermore, CB_2 is aberrantly expressed in several human myeloid cell lines and primary acute myeloid leukemia samples, whereas normal bone marrow precursor cells do not express CB_2 (Alberich Jorda et al. 2004).

Evidence for CB_2 receptor expression has not been found in normal human CNS; however, CB_2 has been found in Alzheimer's brains (Benito et al. 2003). CB_2 immunoreactivity was selectively expressed in microglia associated with neuritic plaques, suggesting that modulation of their activity may have therapeutic implications (Benito et al. 2003).

12
Evidence for Additional Cannabinoid Receptor Subtypes

Not all of the effects of anandamide are mediated through the currently defined cannabinoid receptors. Anandamide inhibits gap-junction conductance and intercellular signaling in striatal astrocytes via a CB-receptor independent mechanism, since the cannabimimetic agents CP 55,940 and WIN 55,212-2 did not mimic the effect of anandamide, nor did the CB_1 receptor antagonist SR141716A reverse anandamide's actions (Venance et al. 1995). Additional fatty acid ethanolamides have been isolated, as well as a 2-arachidonoyl glycerol with cannabimimetic properties, suggesting the existence of a family of endogenous cannabinoids that may interact with additional cannabinoid receptor subtypes (Mechoulam et al. 1995; Mechoulam et al. 1994).

CB_1 receptor knockout mice have now been constructed in four laboratories (Ibrahim et al. 2003; Ledent et al. 1999; Marsicano et al. 2002; Zimmer et al. 1999). In one strain, although CB_1 receptor knockout mice lost responsiveness to most cannabinoids, Δ^9-THC still produced antinociception in the tail-flick test of analgesia (Zimmer et al. 1999). Further characterization of this non-CB_1 Δ^9-THC response suggests the presence of a novel cannabinoid receptor/ion channel in the pain pathway (Zygmunt et al. 2002).

Anandamide produces the full range of behavioral effects (antinociception, catalepsy, and impaired locomotor activity) in CB_1 receptor knockout mice (Di Marzo et al. 2000). Furthermore, anandamide-stimulated GTPγS activity can be elicited in brain membranes from these mice (Breivogel et al. 2001). These effects were not sensitive to inhibition by SR141716A. Interestingly, of all cannabinoid ligands tested, only WIN 55,212-2 elicited GTPγS activity in CB_1 knockout mice. This same phenomenon has also been demonstrated in a second strain of CB_1 receptor knockout mice (Monory et al. 2002).

A cannabinoid receptor subtype has been found in the hippocampus that is responsive to WIN 55,212-2 and CP 55,940 and blocked by capsazepine (Hajos et al. 2001). These receptors are found on excitatory (pyramidal) axon terminals and have been shown to suppress glutamate release in CB_1 receptor knockout animals.

An "abnormal cannabidiol receptor" has also been characterized. Cannabinoids, including anandamide, elicit cardiovascular effects via peripherally located CB_1 receptors (Ishac et al. 1996; Jarai et al. 1999; Wagner et al. 1999). Abnormal cannabidiol (abn-cbd), a neurobehaviorally inactive cannabinoid that does not bind to CB_1 receptors, caused hypotension and mesenteric vasodilation in WT mice and in mice lacking CB_1 receptors or both CB_1 and CB_2 receptors (Jarai et al. 1999). In contrast to the studies described above, these cardiovascular and endothelial effects were SR141716A-sensitive. A stable analog of AEA (methanandamide) also produced SR141716A-sensitive hypotension in CB_1/CB_2 knockout mice. These effects were not due to activation of vanilloid receptors, which also interact with AEA (Zygmunt et al. 1999). A selective antagonist, O-1918, has recently been developed; it inhibits the vasorelaxant effects of abn-cbd and anandamide (Offertaler et al. 2003).

Signal transduction pathways for the abn-cbd receptor have been studied in human umbilical endothelial cells (HUVEC) (Offertaler et al. 2003). Abn-cbd induces phosphorylation of extracellular signal-regulated kinase (ERK) and protein kinase B/Akt via a PI3 kinase-dependent pertussis toxin-sensitive pathway; these effects were blocked by O-1918 (Offertaler et al. 2003). The abn-cbd receptor subtype also appears to be present in microglia (Walter et al. 2003). Anandamide and 2AG triggered migration in BV-2 cells, a microglial cell line; their effects were blocked with O-1918. 2AG also induced phosphorylation of ERK1/2 in BV-2 cells (Walter et al. 2003). These data suggest a common signaling pathway for the abn-cbd receptor in endothelial cells and microglia.

Palmitoylethanolamide has been suggested as a possible endogenous ligand at the CB_2 receptor (Facci et al. 1995). However, it has a low affinity for the cloned human CB_2 receptor (Showalter et al. 1996). This difference suggested that there may be species differences with the CB_2 receptor, as have been found with other GPCRs, but the cloned rat and mouse CB_2 receptors also showed low affinity for palmitoylethanolamide (Griffin et al. 2000). Palmitoylethanolamide has recently been shown produce to a G protein-mediated response in microglial cells that was not affected by CB_1, CB_2, or abn-cbd antagonists, suggesting it acts via its own GPCR (Franklin et al. 2003).

In summary, there is compelling evidence for the existence of additional cannabinoid receptor subtypes. Proof of their existence awaits molecular cloning and expression studies.

13 Conclusion

It is apparent from the growing number of mutagenesis investigations, synthesis of CB_1- and CB_2-selective compounds, and discovery of multiple endogenous

agonists, that there is a complex molecular architecture of the cannabinoid receptors. This arrangement allows for a single receptor to recognize multiple classes of compounds and produce an array of distinct downstream effects. Natural polymorphisms and alternative splice variants may also contribute to the pharmacological diversity of the cannabinoid receptors. As our knowledge of the distinct differences grows, we may be able to target select receptor conformations and their corresponding pharmacological responses. Importantly, the basic biology of the endocannabinoid system will continue to be revealed by ongoing investigations.

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