

Endocannabinoid Receptor Pharmacology

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Abstract This chapter will review the basic pharmacology of endocannabinoid receptors. As the best-described cannabinoid receptors are G-protein-coupled receptors (GPCRs), those will be the focus of this chapter. We will start with a basic review of GPCR signaling, as these concepts are critical to understanding the function of cannabinoid receptors. Next, several features of cannabinoid receptor signaling will be presented, with an emphasis on the effectors modulated by cannabinoid receptors. Finally, we will finish with a discussion of cannabinoid receptor agonists and antagonists and future directions. The aim of this chapter is to introduce the cannabinoid receptor pharmacology that will be necessary to appreciate the intricacies of endocannabinoid signaling presented in later chapters.

Keywords Allosteric modulator • Efficacy • Potency • Protean agonism • Radioligand binding

Abbreviations

2AG	2-Arachidonylethanolamine
AEA	Anandamide
GIRK	G-protein-coupled inwardly rectifying potassium channels
GPCR	G-protein-coupled receptor
RTK	Receptor tyrosine kinase

1 GPCR Overview

G-protein-coupled receptors (GPCRs) are membrane receptor proteins, whose primary function is to transduce extracellular stimuli (communicated as ligands) into intracellular signals. GPCRs comprise the largest protein family with 1,000–2,000 members (>1% of the mammalian genome), of which most encode receptors for odorants and pheromones. Natural ligands for GPCRs are stimuli characterized by their diversity, from photons, ions and amino acid derivatives to large protein

hormones. Based on their homology with rhodopsin, a photon receptor, GPCRs are predicted to contain an extracellular N-terminus, an intracellular C-terminus and seven membrane-spanning helices (TMs), the latter giving them the designation of 7-TM receptors. Stimulation of GPCRs by a ligand induces conformational changes, an event that initiates intracellular signal transduction cascades through the interaction of GPCR intracellular domains with heterotrimeric (comprised of α and $\beta\gamma$ subunits) G proteins (Palczewski et al. 2000) as well as via other protein–protein interactions (Sun et al. 2007).

GPCRs are classified into three main receptor families based on their structural characteristics. Family 1 is by far the largest, and contains characteristic amino acid signatures conserved across members, such as an aspartate in TM2 that has been proposed to be an important amino acid required for receptor activation, a DRY (or ERW) motif immediately C-terminal to TM3, and cysteine residue(s) C-terminal to TM7 serving as a palmitoylation site(s) that plays an important role for G protein coupling and receptor desensitization (Morello and Bouvier 1996). Based on the nature of receptor/ligand interactions, family 1 GPCRs are further divided into three subfamilies: family 1a composed of receptors for small ligands such as odorants, histamine and anandamide (AEA), family 1b for short peptides and cytokines, and family 1c for large glycoproteins and hormones. Family 2 GPCRs are receptors for large peptides such as glucagons and calcitonin, and family 3 are receptors for glutamate, GABA, pheromones, etc. Family 3 GPCRs contain unique, large N-terminal domains often described as a Venus flytrap (Bockaert and Pin 1999). Cannabinoid receptors CB₁, CB₂, and GPR55 all belong to family 1a, and have the basic characteristics of this family, the significance of which will be discussed below.

2 Receptor Pharmacology

An appreciation of the fundamentals of GPCR signaling is essential to understanding cannabinoid receptor signaling, so these concepts will be reviewed in this section. Modern receptor pharmacology is currently based on in vitro pharmacological assays and then their extension to the organism. Both native systems and recombinant receptor expression systems are used, and both come with their limitations.

2.1 GPCR Signaling

In their inactive state GPCRs are associated with quiescent heterotrimeric G proteins. The inactive G protein consists of a GDP-bound α subunit together with its β and γ subunits. Agonist binding to the receptor catalyzes the exchange of GTP for GDP on the α subunit. The binding of GTP prompts the dissociation of the α subunit from the $\beta\gamma$ subunits and the receptor. Both the GTP-bound α subunit (G_{α}) and the $\beta\gamma$ subunits ($G_{\beta\gamma}$, which remain together) modulate an array of

signaling pathways. After a variable period of time, signal transduction is terminated by the hydrolysis of GTP to GDP catalyzed by G_{α} . GDP-bound G_{α} protein re-associates with $G_{\beta\gamma}$, as the GDP-bound form exhibits higher affinity for $G_{\beta\gamma}$ than the GTP-bound form (Selinger 2007). It should be emphasized that these processes are highly regulated by a number of other proteins and factors and the above is only the simplest description of the G protein cycle.

2.2 Radioligand Binding

Key characteristics of a receptor are its affinity for a ligand (a ligand being a molecule that binds with high affinity to a receptor) and the number of receptors in a cell. Both of these parameters can be determined by radioligand binding assays (saturation and competition binding assays). To initially characterize a receptor, a saturation binding assay is performed with increasing concentrations of ligand in order to determine the affinity (K_D) of the radioligand for the receptor, as well as the density of receptor sites (B_{\max}) in the preparation. The K_D value (the equilibrium dissociation constant) is an intrinsic property of the radioligand at the receptor and is defined as the free ligand concentration at which 50% receptor occupancy is achieved. Radioligands that have been employed to study CB_1 and CB_2 receptor pharmacology include non-selective agonist ligands [3H] CP55,940, [3H] WIN55,212-2, [3H] HU243 and [3H] BAY387271 (Mauler et al. 2002), the CB_2 receptor-selective inverse agonist [^{35}S] SCH225336 and the CB_1 receptor-selective inverse agonist [3H] rimonabant. Although extensively used in studying cannabinoid receptor pharmacology, non-selective radioligands need to be employed with caution when assays are performed using native tissues that express both CB_1 and CB_2 receptors. Inverse agonist radioligands also have limitations, as studies have shown that although inverse agonist ligands compete efficiently with both agonist and inverse agonist radioligand, agonist ligands are less efficient in competing with an inverse agonist radioligand than with an agonist radioligand (Thomas et al. 1998).

Radioligand binding assays are usually performed in membranes prepared from either native tissues, such as the spleen for CB_2 or brain for CB_1 , or recombinant cell lines heterologously expressing cannabinoid receptors. CB_1 receptor binding sites are highly abundant in brain (Govaerts et al. 2004; Mauler et al. 2002), exemplified by high B_{\max} values (1–5 pmol mg⁻¹ protein), comparable to the expression levels of recombinant systems (B_{\max} = 1–5 pmol mg⁻¹) (McAllister et al. 2002; Tao and Abood 1998). These high levels of CB_1 expression in native tissues potentially have considerable significance in CB_1 signaling, which will be discussed below. In contrast, the level of CB_2 receptor (Govaerts et al. 2004) binding sites are significantly lower in native tissues (B_{\max} = 697 fmol mg⁻¹ in spleen; and 100–300 binding sites per splenic T cell) compared with the level of CB_1 in the brain or the levels that can be achieved when CB_2 is heterologously expressed (Tao and Abood 1998). The density of receptors impacts downstream signaling (Tao and Abood 1998). This is important to keep in mind when evaluating

the results of experiments examining GPCR signaling in cells heterologously expressing high levels of GPCRs.

Only a small number of cannabinoid ligands are available in a radiolabeled form. Thus, the binding affinities of non-radiolabeled ligands are usually determined indirectly in radioligand competition binding assays, which determine their ability to compete with a radioligand at the receptor binding site. In radioligand competition binding assays, IC_{50} values, defined as the concentration of non-radiolabeled ligand displacing 50% of the bound radioligand at equilibrium for a given concentration of the radioligand used, are obtained. The dissociation constant (K_i) for a non-radiolabeled ligand can be calculated based on the Cheng–Prusoff equation $K_i = \frac{IC_{50}}{1 + \frac{[R]}{K_D}}$ using the IC_{50} value experimentally measured and the radioligand's K_D and concentration ($[L]$) (Tao and Abood 1998). Although IC_{50} values will vary depending upon the concentrations of the radioligand used, the K_i value (like K_D) of a ligand represents an intrinsic property of the ligand—its affinity for the receptor.

Among the radioligands described above, [3H] CP55,940 and [3H] WIN55,212-2 are the most widely used to characterize cannabinoid receptor pharmacology. Although in general most cannabinoid receptor ligands displace both radioligands in a similar fashion in recombinant cell lines, some discrepancies of receptor binding properties have been observed for the two (radio)ligands. For example, in 2001, Breivogel et al. demonstrated that WIN55,212-2 activates a GPCR in the brain of CB₁ knockout mice with a pharmacology consistent with a non-CB₁, non-CB₂ receptor (Breivogel et al. 2001). Reyes et al. (SFN poster, 2007) reported the presence of a high affinity and saturable binding site for [3H] WIN55,212-2 on HEK cell membranes. Since these cells do not express CB₁ or CB₂ receptors, this indicates that WIN55,212-2 has binding sites besides those of CB₁ and CB₂ receptors.

Binding kinetics have been performed for at the CB₂ receptor. [3H] CP55,940 has demonstrated a fast on-rate ($0.263 \text{ nM}^{-1} \text{ min}^{-1}$) and a slower off-rate ($0.041 \text{ nM}^{-1} \text{ min}^{-1}$) with a calculated K_D value of 0.156 nM , consistent to those derived from saturation binding analysis. On-rates are similarly fast and off-rates similarly slow for CP55,940 and rimonabant binding to CB₁ receptors (Herkenham et al. 1991; Rinaldi-Carmona et al. 1996).

Cannabinoid ligands in general are highly lipophilic. Receptor mutation studies suggest cannabinoid ligands interact with the hydrophobic TM domains of cannabinoid receptors. Consistent with this site of interaction, it has been proposed that cannabinoid ligands approach their receptors by fast lateral diffusion within the cell membrane (Tian et al. 2005).

2.3 GTP γ S Binding as a Measure of GPCR Function

Although radioligand binding assays are widely used to determine the affinities of ligands for a receptor and the number of receptors in a cell, they reveal little information about how ligands modulate receptor activity. Thus, functional receptor assays are required in order to evaluate the properties of a ligand (most

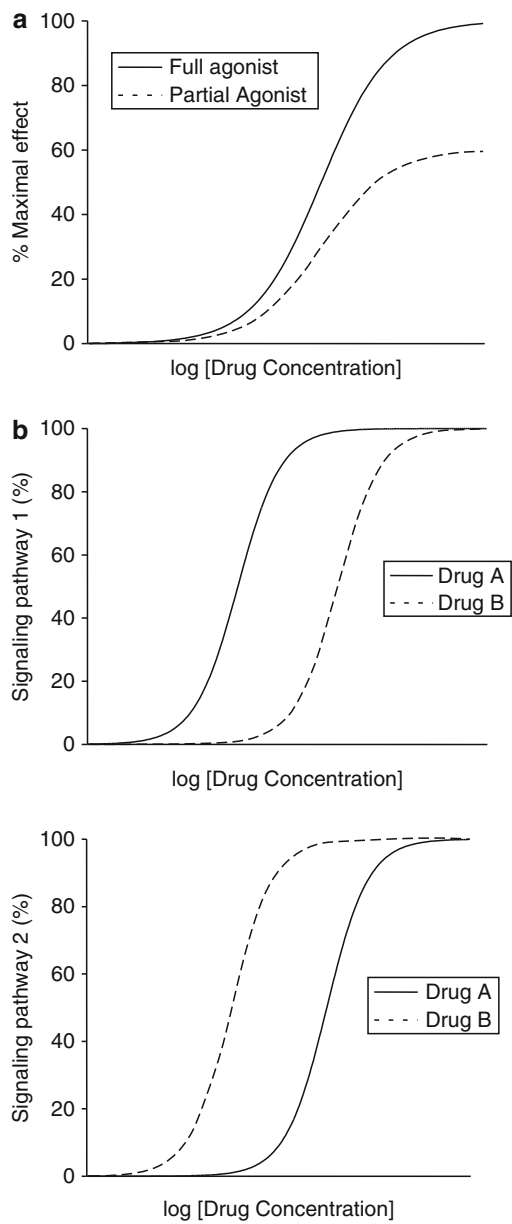
fundamentally, is it an agonist, neutral antagonist or inverse agonist?) and the receptor. GTP γ S binding assays are often employed to determine the receptor activation level by measuring the binding of GTP γ S (a GTP analog that is resistant to hydrolysis) to the receptor/G protein complex. Active GPCRs will catalyze the exchange of GDP for GTP γ S. Since the GTP γ S is not hydrolyzed, it will remain associated with the G protein α subunit and, if the GTP γ S is labeled with ^{35}S , GTP γ S can be detected by standard radiochemical assays. Thus, the amount of ^{35}S incorporated into the G protein α subunit pool will be proportional to the number of G proteins activated. Like radioligand binding assays, [^{35}S] GTP γ S assays are typically performed using membrane preparations. However, like radioligand receptor binding assays, this technique can also be adapted to tissue sections (Sim et al. 1995).

Several useful concepts relevant for receptor signaling emerge from [^{35}S] GTP γ S binding experiments. The most important of these for understanding cannabinoid receptor signaling is intrinsic efficacy (Galandrin et al. 2007). The concept of intrinsic efficacy is that all agonists are not equal – some will more strongly activate receptors than others. Thus, at full receptor occupancy agonist A might stimulate signaling substantially more than agonist B (Fig. 1a). In this case, agonist B is said to have a lower intrinsic efficacy. One way of conceptualizing intrinsic efficacy is that different agonists will favor distinct receptor conformations and some of these receptor conformations will more vigorously activate G proteins than others. It is important to note that potency and efficacy are independent concepts: Efficacy is a measure of the consequence of receptor activation. Potency is a measure of the concentration of agonist required to achieve certain levels of efficacy. For example, the concentration required to achieve 50% of the full efficacy is defined as EC₅₀. It is entirely possible to have a very potent compound which is highly efficacious and vice versa. Examples of low efficacy cannabinoid receptor agonists include anandamide and $\Delta^9\text{THC}$, while WIN55,212-2, HU210, and 2AG are high efficacy agonists (Luk et al. 2004). A low intrinsic efficacy agonist may show partial agonism; however this will depend on receptor and downstream effector density. Specifically, partial agonism will be favored by low receptor density and/or less efficient effector coupling.

An important corollary of intrinsic efficacy is that different agonists acting at the same receptor (by inducing distinct receptor conformations) may activate different repertoires of G proteins. This is known as functional selectivity, biased agonism, or agonist-induced trafficking (Fig. 1b) (Schonbrunn 2007; Urban et al. 2007). This is a very important concept with significant therapeutic ramifications. It emphasizes the principle that all agonists are not equal and different agonists (which may appear identical based on binding affinities and stimulation of GTP γ S binding) may produce very different signaling, cellular, and physiological effects. Functional selectivity is relevant for both CB₁ and CB₂ signaling (Bonhaus et al. 1998; Lauckner et al. 2005; Shoemaker et al. 2005).

Another concept that emerges with GTP γ S studies is that of spare receptors. Evidence for “spare receptors” in a system comes when maximal signaling is observed despite submaximal receptor occupancy. GTP γ S binding can also measure

Fig. 1 *Partial agonism and functional selectivity.*
(a) *Partial agonism.* Two hypothetical drugs have similar potencies (concentration eliciting half maximal effect). However, the partial agonist only has 60% of the efficacy of the full agonist at its maximal effective concentration.
(b) *Drugs may have different potencies for activating different signaling pathways (functional selectivity).* In this example, for signaling pathway 1 (for example, inhibition of adenylyl cyclase) drug A is more potent. For signaling pathway 2 (for example, stimulation of MAP kinase) drug B is more potent than drug A



the efficiency of G protein activation by GPCRs. In these experiments, the number of G proteins activated by a single receptor is calculated. As an example, CB₁ receptors are inefficient in activating G proteins relative to opioid receptors (Sim et al. 1996).

While [³⁵S] GTPγS binding is a useful way to assess GPCR signaling some caveats must be kept in mind. The first is that [³⁵S] GTPγS binding preferentially identifies activation of the most abundant G proteins (and/or those that are most efficiently activated by the receptor). In brain, the most abundant G proteins are those of the G_{i/o} class. Thus activation of other G proteins, such as G_{q/11}, might be overlooked in [³⁵S] GTPγS binding studies. Also, the development of GTPγS binding assays requires considerable optimization, thus it can be difficult to compare results between different laboratories. In addition, GTPγS binding assays measure the first step of the signal transduction pathway, and lack the signal amplification inherent in other functional assays such as those measuring changes of cAMP levels, calcium responses, and transcriptional activity, therefore assay windows and signal-to-noise ratios are sometimes low. In addition, GTPγS binding assays give little information on the spectrum of G proteins coupling to the receptor.

3 CB₁ Receptor Gene Structure

CB₁ receptor cDNA was originally cloned from rat using a homology approach to identify orphan GPCRs (Matsuda et al. 1990). Subsequently, it has been found in all vertebrates and several invertebrates. CB₁ phylogeny is the topic of several excellent reviews (Anday and Mercier 2005; Elphick and Egertova 2005; McPartland et al. 2007).

3.1 Chromosomal Structure, Potential Alternative Splicing

The genes for human, rat, and mouse CB₁ receptors (*CNR1*) are found on chromosomes 6, 5, and 4, respectively. While the translated regions of rodent CB₁ appear to be intronless, two splice variants of human CB₁ have been described. While they may vary in their pharmacology (Ryberg et al. 2005), both variants are found in low abundance and their physiological significance remains to be elucidated (Ryberg et al. 2005; Shire et al. 1995). The potential splice donor sites present in the coding regions of human CB₁ receptors are absent in rodent CB₁ receptors (Howlett et al. 2002).

3.2 *CNR1* Polymorphisms

As discussed elsewhere in this volume, substantial evidence suggests that endocannabinoids play a major role in metabolic regulation and psychiatric disorders. A logical extension of this relationship is to determine if mutations in the *CNR1*

locus are associated with human diseases or responsiveness to endocannabinoid-based therapies. A number of studies examining single nucleotide polymorphisms and other *CNR1* variants have been conducted. Several of these are limited by small sample size and other methodological constraints. Because of the involvement of the endocannabinoid system in various aspects of drug dependence, several studies have searched for associations of *CNR1* polymorphisms with drug dependence. Two studies have reported associations between the intronic *CNR1* SNPs rs64,54,674 and rs8,06,368 with increased substance dependence (Ehlers et al. 2007; Zuo et al. 2007). Epidemiological and animal studies have proposed a link between schizophrenia and cannabis use. Linkage analysis studies so far have failed to find a strong link between *CNR1* SNPs and susceptibility to schizophrenia, but one report suggests that the exonic 1,359G/A SNP was associated with responsiveness to atypical antipsychotics, with an improved response in individuals with the “A” allele (Hamdani et al. 2008; Seifert et al. 2007). In addition to SNP analysis, variations in trinucleotide repeats with *CNR1* have been associated with a form of anorexia (Siegfried et al. 2004), aspects of polysubstance abuse (Hoenicka et al. 2007), and a subtype of schizophrenia (Ujike et al. 2002). Clearly, much work remains to be done to determine the contributions of these variations of *CNR1* to human disease and response to endocannabinoid system-based therapeutics, but this is an area of active research and interesting discoveries are likely to be forthcoming.

4 CB₂ Receptor Gene Structure

CB₂ receptor cDNA was originally cloned from the HL60 human promyelocytic leukemic cell line in 1992 (Munro et al. 1993). Subsequently, CB₂ receptor cDNAs have been isolated from various species including rat, mouse, zebra fish, and domestic cattle.

4.1 *CB₂ Receptor Chromosomal Localization and Potential Alternative Splicing*

The human CB₂ gene (*CNR2*) is located at p36.11 on chromosome 1. Other than an intron present in the 5'-untranslated region (5'-UTR), the coding sequence is intronless (Valk et al. 1997). The mouse CB₂ gene, located on chromosome 4, is also intronless in its coding region. In contrast, two variants have been reported in the literature for the rat CB₂ receptor an intronless isoform with identical length of coding sequence to the human CB₂ receptor (Griffin et al. 2000), and a longer isoform (Brown et al. 2002). The human CB₂ receptor and the short isoform of rat CB₂ receptors contain 360 amino acids, and they share 82% sequence identity and 88% sequence homology in their overall sequence. The long isoform of rat CB₂ receptors contains of a total 410 amino acids, of which the N-terminal 343 residues are identical to the short isoform. The sequence from amino acids 343–410 is unique

to the long isoform, resulting from two additional splicing events – an excision of two introns of 1,239 and 143 bp respectively plus an addition of two exons encoding for 45 and 39 amino acids, respectively. The genomic DNA at the junction of 5' and 3' end of the first intron in the coding sequence of the long isoform receptor contains 5' AG/GTGA 3' and 5' CAG/A 3', respectively, consistent with the consensus sequences that often serve as splicing donor and acceptor sites.

4.2 *CNR2* Polymorphisms

Three non-synonymous single nucleotide polymorphisms (SNPs) have been identified for the human CB₂ receptor: 63Q/R, 316H/Y and 342A/T. Both 63Q and 63R SNPs are prevalent with 46:54 ratio in the Caucasian population (Sipe et al. 2005). Thus far, three haploids (concomitant occurrence of more than one SNP in the same protein), 63Q/316H, 63Q/316Y and 63R/316H, have been reported in humans. Haploid 63R/316H has been shown to have a significantly high linkage to the occurrence of osteoporosis and autoimmune disease (Karsak et al. 2005).

5 Structural Characteristics of the CB₁ Receptor

CB₁ receptors belong to the family 1a of the GPCR superfamily. Remarkable features for CB₁ receptors include a relatively long (about 100 residues) amino terminus in the absence of a signal sequence (Andersson et al. 2003) and an unusually high degree of primary sequence identity across species (Fig. 2a). Considerable effort has been directed towards identifying residues important in binding CB₁ agonists and antagonists. Noteworthy residues identified include K192 (important for binding of agonists, except those of the aminoalkylindole class), as well as rimonabant (Song and Bonner 1996), Y275 and W255 (aromatic stacking, important for recognition of multiple cannabinoid ligands) (McAllister et al. 2003), F170 and F189 (interactions with the double bonds in the arachidonoyl component of endocannabinoids), and a cluster of hydrophobic amino acids in TMs 3, 5, and 6 (McAllister et al. 2003). A disulfide bond between cysteines 257 and 264 in the second extracellular loop also appears critical for receptor trafficking and activity (Fay et al. 2005; Shire et al. 1996). Several domains have been identified to be important for regulation of CB₁ receptor signaling. Regulation by phosphorylation appears to involve (residues are numbered according to rat CB₁) S317 (protein kinase C phosphorylation and uncoupling from G protein signaling) (García et al. 1998) and S426 and S430 (desensitization of CB₁ activation of ERK1/2 and inwardly rectifying potassium channels) (Jin et al. 1999). The distal C-terminus appears to be involved in ligand-induced internalization of CB₁ receptors and its interactions with CRIP1a (cannabinoid receptor interacting protein 1a) and GASP1 (a protein involved in the endosomal targeting of ligand-bound GPCRs) (Hsieh et al. 1999; Martini et al. 2007; Niehaus et al. 2007).

a

		1		80
human CB1	(1)	-----MKSILDLGLADTTFRITITD	LLVGSNDIQYEDIKGDMASKLG	YFPQKFLP
rat CB1	(1)	-----MKSILDLGLADTTFRITITD	LLVGSNDIQYEDIKGDMASKLG	YFPQKFLP
dog CB1	(1)	-----MKSILDLGLADTTFRITITD	LLVGSNDIQYEDIKGDMASKLG	YFPQKFLP
mouse CB1	(1)	-----MKSILDLGLADTTFRITITD	LLVGSNDIQYEDIKGDMASKLG	YFPQKFLP
chicken CB1	(1)	-----MKSILDLGLADTTFRITITD	LLVGSNDIQYEDIKGDMASKLG	YFPQKFLP
zebra finch CB1	(1)	-----MKSILDLGLADTTFRITITD	LLVGSNDIQYEDIKGDMASKLG	YFPQKFLP
edible frog CB1	(1)	-----MKSILDLGLADTTFRITITD	LLVGSNDIQYEDIKGDMASKLG	YFPQKFLP
newt CB1	(1)	-----MKSILDLGLADTTFRITITD	LLVGSNDIQYEDIKGDMASKLG	YFPQKFLP
zebrafish CB1	(1)	MLFPASKSDVKSVLDGVAETTFRTIT	SGLQYIGSNDIGYDTHIDGDF	SKSGYSPADKVADEELV
Consensus	(1)	MKSILDLGLADTTFRITITD	LLVGSNDIQYEDIKGDMASKLG	YFPQKFLP
		81		160
human CB1	(72)	PA--DQVNITEFYNKSLS	SFKNEENIQCGENFMD	IECFMVLNPSQQLAIAVLSL
rat CB1	(72)	PAG-DTTNITEFYNKSLS	SFKNEENIQCGENFMD	IECFMVLNPSQQLAIAVLSL
dog CB1	(72)	PA--DQVNITEFYNKSLS	SFKNEENIQCGENFMD	IECFMVLNPSQQLAIAVLSL
mouse CB1	(72)	PAG-DTTNITEFYNKSLS	SFKNEENIQCGENFMD	IECFMVLNPSQQLAIAVLSL
chicken CB1	(72)	IIPSDQINITEFYNKSLS	SFKNEENIQCGENFMD	IECFMVLNPSQQLAIAVLSL
zebra finch CB1	(72)	IIPSDQINITEFYNKSLS	SFKNEENIQCGENFMD	IECFMVLNPSQQLAIAVLSL
edible frog CB1	(66)	DS----FNATEFYNKSIT	TFKDGDNICQGNFMD	IECFMVLNPSQQLAIAVLSL
newt CB1	(72)	FYPLDQFNITEFYNKSLS	SFKNEENIQCGENFMD	IECFMVLNPSQQLAIAVLSL
zebrafish CB1	(81)	GLFFPYTNSDSVPGN---N	SHSADDSIQCGENFMD	IECFMVLNPSQQLAIAVLSL
Consensus	(81)	A DQ NITEFYNKSLS	SFKNEENIQCGENFMD	IECFMVLNPSQQLAIAVLSL
		161		240
human CB1	(150)	RPSYHFIGSLAVAL	LLGSVIFVYSFIDPHV	FHRKDSRNVFLPKLGGV
rat CB1	(151)	RPSYHFIGSLAVAL	LLGSVIFVYSFIDPHV	FHRKDSRNVFLPKLGGV
dog CB1	(150)	RPSYHFIGSLAVAL	LLGSVIFVYSFIDPHV	FHRKDSRNVFLPKLGGV
mouse CB1	(151)	RPSYHFIGSLAVAL	LLGSVIFVYSFIDPHV	FHRKDSRNVFLPKLGGV
chicken CB1	(152)	RPSYHFIGSLAVAL	LLGSVIFVYSFIDPHV	FHRKDSRNVFLPKLGGV
zebra finch CB1	(152)	RPSYHFIGSLAVAL	LLGSVIFVYSFIDPHV	FHRKDSRNVFLPKLGGV
edible frog CB1	(142)	RPSYHFIGSLAVAL	LLGSVIFVYSFIDPHV	FHRKDSRNVFLPKLGGV
newt CB1	(152)	RPSYHFIGSLAVAL	LLGSVIFVYSFIDPHV	FHRKDSRNVFLPKLGGV
zebrafish CB1	(158)	RPSYHFIGSLAVAL	LLGSVIFVYSFIDPHV	FHRKDSRNVFLPKLGGV
Consensus	(161)	RPSYHFIGSLAVAL	LLGSVIFVYSFIDPHV	FHRKDSRNVFLPKLGGV
		241		320
human CB1	(230)	RPKAVAFCLMWTIA	IAIVAVLPLLGWNCKLQ	SVCSDFPLIDETYL
rat CB1	(231)	RPKAVAFCLMWTIA	IAIVAVLPLLGWNCKLQ	SVCSDFPLIDETYL
dog CB1	(230)	RPKAVAFCLMWTIA	IAIVAVLPLLGWNCKLQ	SVCSDFPLIDETYL
mouse CB1	(231)	RPKAVAFCLMWTIA	IAIVAVLPLLGWNCKLQ	SVCSDFPLIDETYL
chicken CB1	(232)	RPKAVAFCLMWTIA	IAIVAVLPLLGWNCKLQ	SVCSDFPLIDETYL
zebra finch CB1	(232)	RPKAVAFCLMWTIA	IAIVAVLPLLGWNCKLQ	SVCSDFPLIDETYL
edible frog CB1	(232)	RPKAVAFCLMWTIA	IAIVAVLPLLGWNCKLQ	SVCSDFPLIDETYL
newt CB1	(232)	RPKAVAFCLMWTIA	IAIVAVLPLLGWNCKLQ	SVCSDFPLIDETYL
zebrafish CB1	(238)	RTKAVIAFCMMWAI	SIIIAVAVLPLLGWNCKLQ	SVCSDFPLIDETYL
Consensus	(241)	RPKAVAFCLMWTIA	IAIVAVLPLLGWNCKLQ	SVCSDFPLIDETYL
		321		400
human CB1	(310)	QRGTQKSI	IIHTSDEGKVQVTRPDQARMDIR	LAKTLVLILVLLICWGP
rat CB1	(310)	QRGTQKSI	IIHTSDEGKVQVTRPDQARMDIR	LAKTLVLILVLLICWGP
dog CB1	(310)	QRGTQKSI	IIHTSDEGKVQVTRPDQARMDIR	LAKTLVLILVLLICWGP
mouse CB1	(311)	QRGTQKSI	IIHTSDEGKVQVTRPDQARMDIR	LAKTLVLILVLLICWGP
chicken CB1	(312)	QRGTQKSI	IIHTSDEGKVQVTRPDQARMDIR	LAKTLVLILVLLICWGP
zebra finch CB1	(312)	QRGTQKSI	IIHTSDEGKVQVTRPDQARMDIR	LAKTLVLILVLLICWGP
edible frog CB1	(302)	QRGTQKSI	IIHTSDEGKVQVTRPDQARMDIR	LAKTLVLILVLLICWGP
newt CB1	(312)	QRGTQKSI	IIHTSDEGKVQVTRPDQARMDIR	LAKTLVLILVLLICWGP
zebrafish CB1	(318)	RTSQKSLVHSAD	TKVQTRPDQARMDIRLAKTLVLILVLLICWGP	LAIIMVYDVF
Consensus	(321)	QRGTQKSI	IIHTSDEGKVQVTRPDQARMDIR	LAKTLVLILVLLICWGP
		401		480
human CB1	(390)	STVNPFLIALRSKDL	RHAFRSMFPSCG---	TAQPLD
rat CB1	(391)	STVNPFLIALRSKDL	RHAFRSMFPSCG---	TAQPLD
dog CB1	(390)	STVNPFLIALRSKDL	RHAFRSMFPSCG---	TAQPLD
mouse CB1	(391)	STVNPFLIALRSKDL	RHAFRSMFPSCG---	TAQPLD
chicken CB1	(392)	STVNPFLIALRSKDL	RHAFRSMFPSCG---	TAQPLD
zebra finch CB1	(392)	STVNPFLIALRSKDL	RHAFRSMFPSCG---	TAQPLD
edible frog CB1	(382)	STVNPFLIALRSKDL	RHAFRSMFPSCG---	TAQPLD
newt CB1	(382)	STVNPFLIALRSKDL	RHAFRSMFPSCG---	TAQPLD
zebrafish CB1	(398)	STVNPFLIALRSKDL	RHAFRSMFPSCG---	TAQPLD
Consensus	(401)	STVNPFLIALRSKDL	RHAFRSMFPSCG---	TAQPLD
		481		
human CB1	(467)	TSAAEL		
rat CB1	(468)	TSAAEL		
dog CB1	(467)	TSAAEL		
mouse CB1	(468)	TSAAEL		
chicken CB1	(468)	TSAAEL		
zebra finch CB1	(468)	TSAAEL		
edible frog CB1	(448)	-----		
newt CB1	(468)	TSGEAV		
zebrafish CB1	(470)	TSAAEV		
Consensus	(481)	TSAAEL		

Fig. 2 Alignments of *CB₁* and *CB₂* protein sequences from representative vertebrates. Transmembrane domains are indicated in gray and conserved motifs discussed in the text are highlighted in green, *a. CB₁*. *b. CB₂*

b

	1	80
human CB2	(1) MEECVTEIANGSKDGLDSNPMKDYMLSGPQKTAVALCTLLGLLSALENVAVLVYLILSSHQLRKRPYSYLFITGSLAGA	
rat CB2S	(1) MECCRELELTNGSNGGLEFNPMEKYMILSDAQIAVAVLCTLMGLLSALENVAVLVYLILSSQRLRRKPSYLFITGSLAGA	
rat CB2L	(1) MAGCRELELTNGSNGGLEFNPMEKYMILSDAQIAVAVLCTLMGLLSALENVAVLVYLILSSQRLRRKRPYSYLFITGSLAGA	
mouse CB2	(1) MEGCRETEVTNGSNGGLEFNPMEKYMILSSGQOIATAVAVLCTLMGLLSALENVAVLVYLILSSRRRLRRKPSYLFITGSLAGA	
cow CB2	(1) MEICLKTEAANGSSDGLNFPMEKYMILSGPQKTAIAVLCTLLGLLSALENVAVLVYLILSSRRRLRRKRPYSYLFITGSLAGA	
Consensus	(1) MEGCRELELTNGSNGGLEFNPMEKYMILS AQQIAVAVLCTLMGLLSALENVAVLVYLILSS RLRRKPSYLFITGSLAGA	
	81	160
human CB2	(81) FLASVVFACSFVNFHFVFGVDSKAVFLFKIGSVTMTPTASVGSLLLTAVLCLCRYPPTPKALVTRGRALVALGVMVVL	
rat CB2S	(81) FLASVIFACNFVIFHFVFGVDSDNFIPLKIGSVTMTPTASVGSLLLTAVLCLCRYPPTPKALVTRGRALVALGVMVVL	
rat CB2L	(81) FLASVIFACNFVIFHFVFGVDSDNFIPLKIGSVTMTPTASVGSLLLTAVLCLCRYPPTPKALVTRGRALVALGVMVVL	
mouse CB2	(81) FLASVIFACNFVIFHFVFGVDSDNFIPLKIGSVTMTPTASVGSLLLTAVLCLCRYPPTPKALVTRGRALVALGVMVVL	
cow CB2	(81) FLASVVFACSFVNFHFVFGVDSDNFIPLKIGSVTMTPTASVGSLLLTAVLCLCRYPPTPKALVTRRRALVTLGIMVVL	
Consensus	(81) FLASVIFACSFVIFHFVFGVDSDNFIPLKIGSVTMTPTASVGSLLLTAVLCLCRYPPTPKALVTRGRALVALGVMVVL	
	161	240
human CB2	(161) SALVSYLPLMGWTCPP--PCSELPFLIPNDYLLSWLLFIAFLFSGIITYTVGHVWKAHQHVASLSGHQDR-----	
rat CB2S	(161) SALISYLPLMGWTCPPS--PCSELPFLIPNDYLLGWLLFIAFLFSGIITYTVGVWKAHQHVASLTETHLDR-----	
rat CB2L	(161) SALISYLPLMGWTCPPS--PCSELPFLIPNDYLLGWLLFIAFLFSGIITYTVGVWKAHQHVASLTETHQDR-----	
mouse CB2	(161) SALISYLPLMGWTCPPS--PCSELPFLIPNDYLLGWLLFIAFLFSGIITYTVGVWKAHRHVAETAEHQDR-----	
cow CB2	(161) AALVSYLPLMGWTCPP--PCSELPFLIPNDYLLGWLLFIAFLFAGIITYTVAHVWKAHQHVASLAHRDR-----	
Consensus	(161) SALISYLPLMGWTCPPS PCSELPFLIPNDYLLGWLLFIAFLFSGIITYTVGVWKAHQHVASLTETHQDR	
	241	320
human CB2	(230) ----QVPGIARMRLDVLRAKTLGLVLAFLICWFPFALALMGHSLVTTLSQVKEAFACSMCLCLVNSMVPETIALRSGE	
rat CB2S	(230) ----QVLIARMRLDVLRAKTLGLVMAFLICWFPFALALMGHSLVTTLSQVKEAFACSMCLCLVNSMVPETIALRSGE	
rat CB2L	(230) ----QVPGIARMRLDVLRAKTLGLVMAFLICWFPFALALMGHSLVTTLSQVKEAFACSMCLCLVNSMVPETIALRSGE	
mouse CB2	(230) ----QVPGIARMRLDVLRAKTLGLVLAFLICWFPFALALMGHSLVTTLSQVKEAFACSMCLCLVNSMVPETIALRSGE	
cow CB2	(230) ----HLSGIARMRLDVLRAKTLGMLLAFLIFWFPFALALMVYSLGARLSQVKEAFACSMCLCLVNSMVPETIALRSGE	
Consensus	(241) QVPGIARMRLDVLRAKTLGLVLAFLICWFPFALALMGHSLVTTLSQVKEAFACSMCLCLVNSMVPETIALRSGE	
	321	400
human CB2	(306) IRSSAHHLAHWKKEVRLGSEAKEEAPRSSVTETADGKITPWPDRDLDSLC-----	
rat CB2S	(306) IRSSAAQHLLGWKKYLGGLGSEGKEEAPKSSVTEAEVKTTPGRLRPSCSNC-----	
rat CB2L	(306) IRSSAAQHLLGWKKYLGGLGSEGKEEAPKSSVTEAEVLTVDKPELGGLGGLRTSSIHSPMLSLADSANRQDVRPHCP	
mouse CB2	(306) IRSSAAQHLLIGWKYLGGLGSEGKEEAPKSSVTEAEVKTTPGRLRPSCSNC-----	
cow CB2	(306) IRSSAHHRLARWKKVRLGSGEGKEEAPRSSVTETADVKTTPGLDRELSPDEL-----	
Consensus	(321) IRSSAAQHLL GWKKYLGGLGSEGKEEAPRSSVTETADVKTTPGLDRELSPDEL	
	401	425
human CB2	(361) -----	
rat CB2S	(361) -----	
rat CB2L	(386) EELTWWCSSVRRPISLPNKAQGSTLL	
mouse CB2	(348) -----	
cow CB2	(362) -----	
Consensus	(401) -----	

Fig. 2 (continued)

6 Structural Characteristics of the CB₂ Receptor

CB₂ receptors also belong to GPCR family 1a. Site-directed mutagenesis and receptor modeling studies suggested that, unlike other GPCRs, where the DRY motif and A244 in TM6 are important for receptor activation and where mutation of these residues lead to constitutive activity, mutagenesis of D130 in the DRY motif and A244 of the CB₂ receptor only abolishes ligand binding and no constitutive activity was observed (Feng and Song 2003). In contrast, C313 and C320 in the human CB₂ receptor are important for functional receptor coupling to adenylyl cyclase but not for ligand binding affinity. In addition, Y299 in the NPVIY motif of TM7 appears to be important for ligand binding and receptor function. It has also been demonstrated that the human CB₂ receptor undergoes agonist-induced phosphorylation of S352, which promotes its desensitization and internalization (Bouaboula et al. 1999b). Interestingly, this residue is lacking in mouse CB₂.

CB₂ receptor sequences are less conserved throughout evolution than those of CB₁ receptors, with the overall sequence homology between mammals including human, cattle, rat (short isoform) and mouse about 70% (Fig. 2b). The mouse and

rat CB₂ receptors are 90% identical, but they are less homologous to the human CB₂ receptor, sharing 80% and 81% identity with the human receptor, respectively.

7 CB₁ and CB₂ Receptor Localization

The distribution and subcellular localization of CB₁ receptors are discussed at length in the chapter “Endocannabinoid Receptors: CNS Localization of the CB₁ Cannabinoid Receptor” by István Katona in this volume and so will not be further considered here. The CB₂ receptor was originally described as a “peripheral” cannabinoid receptor and was found at the highest levels in tissues of the immune system, such as spleen, tonsil, thymus and lymphoid tissues (Galiegue et al. 1995). Accurate assessment of CB₂ expression has been hampered by non-selective antibodies and by the fact that CB₂ expression is highly inducible, for example in cell culture. That is, the presence of CB₂ in a cultured cell does not necessarily imply that CB₂ receptors are found at signaling relevant levels in the native tissue. Thus studies purporting to show the presence of CB₂ by a single technique, particularly in the absence of appropriate controls, must be treated with skepticism. Preferable are studies that show (functional) expression by multiple approaches, for example by antibodies, rt-PCR, in situ hybridization, and/or pharmacological tools. With these caveats in mind, CB₂ mRNA is present in immune cells with a rank order of expression as follows: B cells > macrophage/monocytes > NK cells > T cells (Galiegue et al. 1995). Recently, CB₂ expression has been reported in keratinocytes (Ibrahim et al. 2005), gut neurons (Wright et al. 2008), and brainstem (Van Sickle et al. 2005). In addition, CB₂ receptors have been shown to be expressed or up-regulated under pathological states; examples include spinal cord and DRG tissues of animal pain models (Jhaveri et al. 2008; Wotherspoon et al. 2005; Zhang et al. 2003) and human multiple sclerosis CNS tissues (Benito et al. 2007). Evidence has been presented for both a neuronal and microglial localization of these induced CB₂ receptors.

8 Cellular Signaling of CB₁ and CB₂ Receptors

8.1 *Inhibition of Adenylyl Cyclase – G_{i/o} Coupling of CB₁ and CB₂ Receptors*

CB₁ and CB₂ are both G_{i/o}-coupled GPCRs, and their activation leads to the inhibition of adenylyl cyclase and reduction in the production of cAMP (Howlett et al. 2002). If adenylyl cyclase activity is high prior to the activation of cannabinoid receptors, this will result in a decrease in cAMP levels. In practice for adenylyl cyclase assays measuring the activity of G_{i/o}-coupled GPCRs, the intracellular

cAMP level is first raised by forskolin, an adenylyl cyclase activator, or a G_s -receptor agonist such as secretin or isoproterenol, allowing a sufficient assay window for measuring reduction of cAMP levels upon the activation of a $G_{i/o}$ -coupled GPCRs.

8.2 *Cannabinoid Receptor Activation of MAP Kinases*

Activation of CB_1 and CB_2 receptors reliably leads to the activation of mitogen-activated protein kinases, particularly the extracellular signal-regulated kinases (ERK1/2) through a pertussis toxin-sensitive $G_{i/o}$ pathway (Howlett et al. 2002). In addition, Jnk and p38 MAP kinases are activated by these receptors (Howlett 2005).

8.3 *Crosstalk Between Cannabinoid and Other Receptors*

Crosstalk between the MAP kinase signaling pathways mediated by CB_2 receptor activation and MAP kinase activity evoked by other $G_{i/o}$ -dependent receptors has been observed, as the CB_2 inverse agonist SR1,44,528 has been shown to inhibit the MAP kinase activity induced by other $G_{i/o}$ -dependent receptors, such as a lysophosphatidic acid receptor (Bouaboula et al. 1999a). It is hypothesized that crosstalk between distinct signaling pathways that convergent to the activation of MAP kinase is possibly achieved by altering the stoichiometry of $G_{i/o}$ proteins that are available to other GPCRs when the $CB_2/G_{i/o}$ complex is promoted and stabilized by CB_2 receptor inverse agonists. Over-expression of CB_2 receptors can also alter modulation of ion channels by other $G_{i/o}$ -linked GPCRs (Felder et al. 1995). Similar phenomena have been observed for CB_1 receptor attenuating modulation of calcium channels and MAP kinase by other $G_{i/o}$ -linked receptors (Canals and Milligan 2008; Vasquez and Lewis 1999).

8.4 *Transactivation Between Cannabinoid Receptors and Tyrosine Kinase Receptors*

Transactivation of receptor tyrosine kinases (RTKs) is a frequent point of crosstalk between GPCR and RTK signaling and might be responsible for some of the growth-promoting effects of GPCR agonists. CB_1 receptors have been reported to transactivate TrkB (BDNF) receptors. CB_1 /TrkB transactivation mediates

endocannabinoid-induced chemotaxis in the absence of BDNF (Berghuis et al. 2005). Transactivation between CB₂ receptors and RTKs has not been reported, but likely occurs.

8.5 Cannabinoid Receptor-Mediated Modulation of Ion Channels

Most G_{i/o}-coupled receptors also inhibit a subset of voltage-gated calcium channels and activate inwardly rectifying potassium (GIRK) channels. CB₁ receptors follow this paradigm (Mackie et al. 1995). The marked presynaptic localization of CB₁ receptors in close proximity to voltage-gated calcium channels suggests that a major mode of action of CNS CB₁ receptors is the modulation of synaptic transmission (Nyiri et al. 2005). As discussed in the chapter “Endocannabinoid Signaling in Neural Plasticity” by Alger in this volume, this appears to be the case. CB₁ activation of GIRK channels is observed in heterologous expression systems (Mackie et al. 1995) and is likely in at least some neurons (Bacci et al. 2004; Kreitzer et al. 2002). The situation is more complicated with CB₂ receptors. One report examining transfected CB₂ receptor modulation of endogenous calcium and GIRK channels in AtT20 cells did not find effects of CB₂ agonists on these channels (but expression of CB₂ receptors did disrupt signaling of other GPCRs, the latter effect consistent with G protein sequestering (see above)) (Felder et al. 1995). However, another report examining over-expression of both CB₂ receptors and GIRKs in *Xenopus* oocytes did find CB₂-mediated activation of GIRK currents (Ho et al. 1999), suggesting that under some conditions CB₂ is capable of activating GIRK channels. With the likely presence of CB₂ in some neurons under some conditions, it will be important to determine if CB₂ can directly modulate ion channels.

9 Implications of Constitutive Receptor Activity, Protean Agonism, and Inverse Agonism

Receptor constitutive activity refers to the ability of a receptor to activate G proteins and downstream signaling pathways in the absence of agonist. It is generally believed that constitutive activity is due to receptors spontaneously assuming an active conformation in the absence of an agonist. However, one needs to keep in mind that endogenous ligands, if present in the tissues studied, will produce a similar effect in the absence of added ligand, an issue particularly relevant for lipid receptors where their ligands may be continuously produced in the course of membrane turnover or remodeling (Gbahou et al. 2003). Thus, constitutive activity means that a fraction of receptors are actively signaling in the absence

of an agonist. The level of receptor constitutive activity is dependent upon the system, including factors such as receptor expression levels, cellular environment and the conditions of cell growth (Yao et al. 2006). The high levels of CB₁ expression in a variety of neurons means that constitutive activity of this receptor may be relevant in the clinical use of CB₁ inverse agonists. Receptor constitutive activity can be revealed by the use of inverse agonist ligands, as these ligands

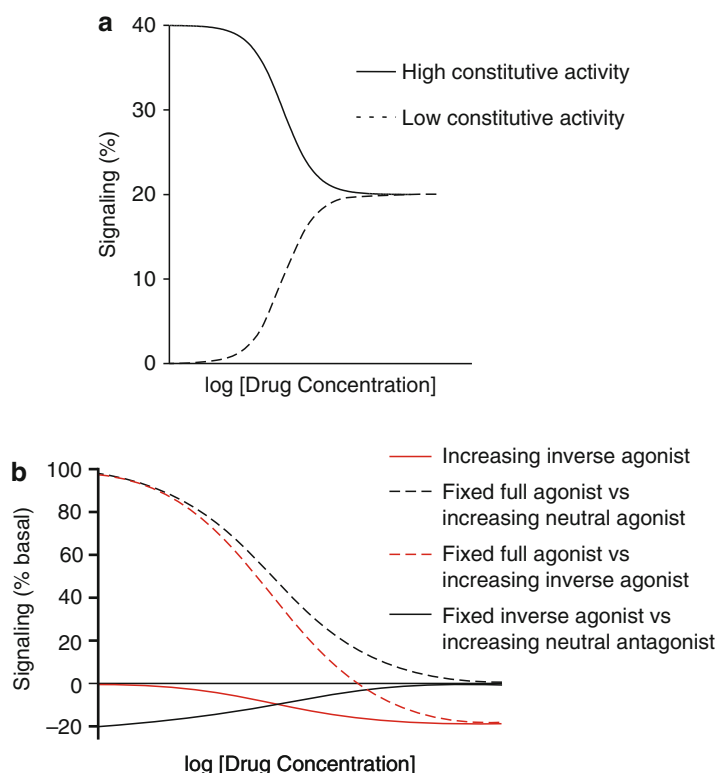


Fig. 3 *Protean agonism and inverse agonism.* (a) *Protean agonism.* In this example, the protean agonist is assumed to have an efficacy of 20%. In the case of high constitutive activity (40%), increasing concentrations of the protean agonist will decrease the observed signaling, appearing to be an inverse agonist. If the constitutive activity is low (0%) increasing concentrations of the protean agonist will increase the signaling, appearing to be an (partial) agonist. Note that if the baseline constitutive activity is 20%, the protean agonist will behave as a neutral antagonist. (b) *Interactions between a full agonist, neutral antagonist, and inverse agonist.* Increasing concentrations of a neutral antagonist will reverse the positive efficacy of an agonist (dashed black line) or the negative efficacy of an inverse agonist (solid black line), returning the system to its basal level. Increasing concentrations of an inverse agonist (dashed red line) will reverse the effect of a full agonist, eventually leading to negative efficacy. Increasing concentrations of an inverse agonist in the absence of other ligands (solid red line) will inhibit basal signaling activity, causing negative efficacy

stabilize a receptor conformation that promotes a lower activation state than the resting state, resulting in an apparent negative efficacy. It is worth noting that the apparent efficacy of a ligand is dependent upon the level of receptor constitutive activity of the receptor in the assay system used. Therefore, a partial agonist in one system can behave as an antagonist or an inverse agonist in others (that is, it can be a protean agonist – Fig. 3a). GTP γ S and adenylyl cyclase assays are often used to evaluate receptor constitutive activity and for characterization of inverse agonists. A true neutral antagonist will block both agonist as well as inverse agonist activity, independent of the level of receptor constitutive activity (Fig. 3b). It has been speculated that there are very few true neutral antagonists for GPCRs (Kenakin 2004). Most apparent neutral antagonists are low affinity inverse agonists or their neutral antagonism is specific to the assay system in which they are characterized (Bond and Ijzerman 2006). Thus, it is important when characterizing a putative neutral antagonist that a variety of different conditions (that is, varying levels of receptor expression and second messenger systems) are evaluated.

Receptor constitutive activity is a physiologically and/or pathologically important phenomenon. The constitutive activity for the CB₂ receptor, although not extensively studied in tissues, has been demonstrated in recombinant cell lines expressing the CB₂ receptor (Yao et al. 2006). For example, SR1,44,528 has been shown to potentiate the gene expression induced by forskolin-induced cAMP responsive element (Portier et al. 1999), and in addition, AM630 produced a further increase in the forskolin-induced cAMP level (Ross et al. 1999), indicating constitutive activity of CB₂ in these recombinant systems that is readily reversed by the inverse agonists SR1,44,528 and AM630.

As mentioned above, protean agonists describe a group of ligands that behave as agonists in one system but as inverse agonists or neutral antagonists in another. For example, AM1,241 behaves as a partial agonist, neutral antagonist or inverse agonist at CB₂ receptors depending on the assay systems employed and assay conditions used (Yao et al. 2006).

10 Cannabinoid Receptor Ligands

10.1 Non-Selective CB₁/CB₂ Receptor Agonists

There are four well-developed classes of cannabinoid receptor agonist (Howlett et al. 2002): the classical cannabinoids, non-classical cannabinoids, aminoalkylindoles and eicosanoids. Classical cannabinoids are ABC-tricyclic benzopyrans. Classical cannabinoids may be found in nature, such as Δ^9 -tetrahydrocannabinol (Δ^9 THC) or may be synthetic, such as HU210 (11-hydroxy- Δ^8 -tetrahydrocannabinol-dimethylheptyl) or DALN (desacetyl-levo-nantradol). Non-classical cannabinoids arose from extensive SAR work conducted at Pfizer thirty years ago. These compounds are characterized by the opening of the dihydropyran ring. Many of

these compounds have high affinity for both CB₁ and CB₂ receptors. Of these, CP55,940 has played a major role in defining CB₁ receptor localization and function (Herkenham et al. 1991). Recently, compounds in the naphthalene class have reported to be potent agonists at CB₁ and CB₂ receptors with limited brain penetration (Dziadulewicz et al. 2007).

The classical and non-classical cannabinoids are structurally related to Δ^9 THC, varying primarily in side chain modifications, some of which substantially increase receptor affinity (for example, the 3-dimethyl heptyl analogs). However, the next class of cannabinoid receptor ligands, the aminoalkylindoles, were developed as anti-inflammatory drugs and analgesics, and were only subsequently found to be cannabinoid receptor agonists (at both CB₁ and CB₂ receptors) (Compton et al. 1992). Of the aminoalkylindoles, WIN55,212-2 is the most frequently encountered. As discussed below, aminoalkylindoles have provided a route towards the synthesis of relatively selective CB₂ agonists. Not unexpectedly, given their structural differences from other cannabinoid receptor agonists, aminoalkylindoles bind to CB₁ receptors in a slightly different fashion (but still in a displaceable manner) than the other well-characterized CB₁ receptor agonists (Song and Bonner 1996). Consistent with this, WIN55,212-2 activation of CB₁ receptors promotes a different repertoire of cellular events (Compton et al. 1992), a fact that must be kept in mind when evaluating experiments performed with (high concentrations of) this agonist.

The final group of CB₁ receptor ligands are the eicosanoids. These eicosanoid derivatives collectively form the group of compounds known as endogenous cannabinoids (endocannabinoids) (Freund et al. 2003). The synthesis and degradation of the endocannabinoids is discussed in the chapter “The Life Cycle of the Endocannabinoids: Formation and Inactivation” by Alexander and Kendall, this volume. There are two major classes of endocannabinoids, the acylethanolamides and the acylesters. The prototypic member of the acylethanolamide family is *N*-arachidonylethanolamine (anandamide, AEA) (Devane et al. 1992). However, a number of additional acylethanolamides, varying in chain length or extent of acyl chain saturation are found in vivo and have activity at CB₁ receptors (Felder et al. 1993). A hallmark of the acylethanolamides is that they have relatively low intrinsic efficacy at CB₁ receptors. An extensive literature exists on the SAR of acylethanolamides for CB₁ (Lin et al. 1998; Reggio 2002; Ryan et al. 1997). In general, a shorter acyl chain and decreasing degree of saturation leads to lower affinity. 2-Arachidonoyl glycerol (2AG) (and its 1/3 isomer) is the only acylester extensively studied (Stella et al. 1997; Sugiura et al. 1995). While 2AG's affinity for CB₁ receptors is similar to that of AEA, it is consistently found to have a higher intrinsic efficacy (Luk et al. 2004). In addition to the acylamides and esters, additional eicosanoid compounds have been reported to be endogenous CB₁ agonists. Two of these, virodhamine and noladin ether, were initially reported to be present in brain; however later studies have failed to consistently verify these initial reports (Richardson et al. 2007). In addition, there are a large number of acyl amino acid conjugates that have been reported to have varying efficacy at CB₁ receptors (Bradshaw and Walker 2005).

10.2 *CB₁ Receptor Antagonists*

The first and most extensively studied class of CB₁ antagonists is the 1,5-diarylpyrazoles, typified by rimonabant (SR141,716A) (Howlett et al. 2002). Other widely encountered members of this family include AM251 and AM281 (Howlett et al. 2002). These compounds generally show 100–1000-fold selectivity for CB₁ over CB₂ (depending on the assay system). They are also inverse agonists. Another early CB₁ antagonist is the substituted benzofuran, LY320,135. While much less studied than rimonabant, it has a lower affinity for CB₁ than rimonabant, but like rimonabant it shows strong selectivity for CB₁ and is an inverse agonist (Felder et al. 1998).

The ability of CB₁ antagonists to depress food consumption and promote weight loss has lead to robust efforts among pharmaceutical companies to develop additional CB₁ antagonists (Black 2004). Other than rimonabant, the compound furthest along in clinical development is Merck's substituted acyclic sulfonamide, taranabant or MK0364 (Addy et al. 2008). A Pfizer compound, CP945,598, has also been used in multiple clinical trials. Another antagonist that has been tested in man is the 3,4-diaryl-4,5-dihydropyrazole (SLV-319) (Foloppe et al. 2008).

The compounds discussed above all show inverse agonism under appropriate assay conditions and it has been hypothesized that some of the adverse effects of rimonabant and taranabant might be mediated by inverse agonism. In this regard it is interesting that a pyrazole analog, AM4113, which has high affinity for CB₁ receptors, does not show inverse agonism in the adenylyl cyclase assay but does suppress food intake and may have a lower incidence of pro-emetic behaviors (Bergman et al. 2008; Chambers et al. 2007; Sink et al. 2008). Whether neutral antagonists of CB₁ will have a therapeutic advantage over CB₁ inverse agonists is speculative and remains to be determined.

All of the CB₁ ligands described above are small, lipophilic molecules. However, a recent report identified the endogenous peptide, hemopressin, to be a novel CB₁ receptor inverse agonist (Heimann et al. 2007). The implications of this observation are profound and if these findings are confirmed they will force a re-thinking of the control CB₁ receptor function.

10.3 *CB₂ Receptor Agonists*

Significant efforts have focused on generating CB₂ receptor selective agonists as potential therapeutic agents, as it is believed that selective activation of CB₂ receptors will produce anti-inflammation, analgesia and other therapeutic benefits without the undesirable CNS side effects thought to be mainly mediated by the activation of CB₁ receptors. Many synthetic CB₂ receptor agonists have been developed with significant (but not absolute) selectivity over the CB₁ receptor. They can be divided into several classes according to their structures. Indoles

represented by AM1,241 are thoroughly studied, and have been characterized in a variety of in vivo animal models to demonstrate CB₂-mediated efficacies. Although AM1,241-evoked analgesic efficacy has been reported to involve the μ -opioid receptor (Ibrahim et al. 2005), this phenomenon is not a consistent characteristic of CB₂-mediated analgesia, as other CB₂ receptor selective agonists in the class (A-796,260, A-836,339 (Yao et al. 2008; Yao et al. 2009) and L-768,242, as well as GW405,833) do not share this property (Whiteside et al. 2005). A class of synthetic Δ^9 THC derivatives that is quite selective for CB₂ receptors emerged from SAR-based structural design. One of the well-characterized ligands in this class is JWH-133 (Marriott and Huffman 2008). JWH-133 has been shown to have anti-spasticity efficacy in animal models of multiple sclerosis (Baker et al. 2000). However, due to less than perfect selectivity, the effects are likely to be at least partly mediated by CB₁ receptors (Pryce and Baker 2007). Thiazolyldine compounds, such as the Taisho compounds (Ohta et al. 2008), and A-8,36,339 demonstrated excellent selectivity over the CB₁ receptor and have been shown to be efficacious in in vivo analgesic models (Yao et al. 2009).

10.4 CB₂ Receptor Antagonists

The most widely used CB₂ receptor selective antagonists are SR1,44,528, a pyrazole, and AM630, an indole. In in vitro pharmacological studies SR1,44,528 and AM630 have been shown to block CB₂ receptor activation by selective agonists (Rinaldi-Carmona et al. 1998; Shire et al. 1999). In addition, in in vivo studies these antagonists block CB₂ receptor-mediated actions (Ibrahim et al. 2005; Yao et al. 2008). JTE-907, a quinolinone-3-carboxamide, has been shown to be an inverse agonist at the CB₂ receptor (Ueda et al. 2005). The triaryl bis-sulfones (SCH2,26,336) are a new class of CB₂ antagonist (Lavey et al. 2005). Both JTE-907 and SCH2,26,336 have been shown to have anti-inflammatory effects (Lavey et al. 2005; Ueda et al. 2005). SCH2,26,336 has been radiolabeled and [³⁵S] SCH2,26,336 has been used in in vitro pharmacological characterization of the CB₂ receptor, as well as localization of CB₂ receptors by autoradiography in tissue sections (Gonsiorek et al. 2006).

10.5 Allosteric Modulators of Cannabinoid Receptors

The preceding discussion has focused on orthosteric ligands of the cannabinoid receptor. These are ligands that interact directly with the binding site whose occupancy activates the receptor. Another class of molecules that interact with receptors are allosteric modulators. These compounds bind to sites on the receptor distinct from the orthosteric binding site but induce conformational changes in the receptor that alter the properties of orthosteric ligands. A well-known example of an allosteric modulator would be a benzodiazepine acting on the GABA_A receptor. Allosteric

modulators of receptor function are potentially exciting therapeutic targets as they alter the function of endogenous ligands and may bypass some of disadvantages of orthostatic ligands (desensitization, up-regulation, etc.). Two families of allosteric modulators have been described for CB₁ receptors (Horswill et al. 2007; Price et al. 2005). No allosteric modulators of CB₂ receptors have been published. This is an active area of research and advances can be expected over the next few years.

11 Non-CB₁/Non-CB₂ Receptors

11.1 GPR55

The persistence of cannabinoid effects in CB₁ and/or CB₂ knockout mice suggests the existence of additional cannabinoid receptors (Begg et al. 2005). In addition, strong pharmacological evidence supports the presence of a vascular cannabinoid receptor distinct from CB₁ or CB₂ (Begg et al. 2005). Evidence has emerged over the past several years that GPR55 may be one such receptor. Although some controversy remains, this receptor can be formally considered a cannabinoid receptor based on its activation by anandamide and Δ^9 THC at low micromolar concentrations (Lauckner et al. 2008; Ryberg et al. 2007; Waldeck-Weiermair et al. 2008). In addition, lysophosphatidylinositol (LPI), an endogenous lipid mediator, also activates this receptor (Lauckner et al. 2008; Oka et al. 2007; Waldeck-Weiermair et al. 2008). However, LPI is not a specific GPR55 agonist as it also activates TRPM8 at concentrations reported to activate GPR55 (Andersson et al. 2007). GPR55 stimulation releases calcium from intracellular stores via phospholipase C (Lauckner et al. 2008; Waldeck-Weiermair et al. 2008) and, in some cases, activates ERK1/2 MAP kinase (Oka et al. 2007; Waldeck-Weiermair et al. 2008). GPR55 mRNA is widely distributed at moderate to low levels in the CNS and is also found in the vasculature and other peripheral tissues (Ryberg et al. 2007). While GPR55 appears to fulfill the criteria of a cannabinoid receptor, its pharmacology is inconsistent with several of the non-CB₁/non-CB₂ effects mentioned above. Therefore, additional cannabinoid receptors clearly remain to be identified.

11.2 Interactions of Cannabinoids with Ion Channels

Numerous cannabinoids and cannabinoid receptor ligands have been found to interact with various ligand-gated and voltage-gated ion channels, typically in the low micromolar range (Akopian et al. 2008; Barann et al. 2002; Maingret et al. 2001; Oz et al. 2004; Poling et al. 1996; Ross 2003). While these interactions may have physiological relevance under some conditions, this topic is beyond the scope of the current review and the interested reader is referred to an excellent recent review (Oz 2006).

12 Conclusions

The most direct route to manipulate the endocannabinoid system is by engaging cannabinoid receptors with agonists or antagonists. However, in order to understand and interpret these interactions, a basic familiarity with the principles of receptor pharmacology, including selectivity, efficacy, functional selectivity, and allosteric modulation, is necessary. The past thirty years have seen a proliferation of CB₁ and CB₂ agonists and antagonists. A few of these, for example mixed CB₁/CB₂ agonists (Δ^9 THC) and CB₁ antagonists, have therapeutic efficacy in man. Others, such as CB₂ agonists, have considerable therapeutic promise based on preclinical studies. Finally, non-orthosteric ligands, such as allosteric modulators, offer intriguing therapeutic possibilities. Certainly, the next few years will be a rich and exciting time for cannabinoid receptor pharmacology.

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