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REVIEW

Efficacy in CB_1 receptor-mediated signal transduction

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CB_1 receptor cellular signal transduction is dependent on the expression of G proteins to which the receptor couples, the potential for precoupling of particular G proteins to the receptors either by scaffolding mechanisms or colocalization in lipid raft domains, and the effector mechanisms that these transducer molecules regulate. This discourse will evaluate studies of efficacy for CB_1 receptor-Gi/o activation at the molecular level. Evidence for brain regional differences in CB_1 receptor signal transduction efficacy and agonist selectivity for G proteins will be summarized. The possibility that CB_1 receptors interact with Gs or Gq will be evaluated, and questions with regard to the constitutive activity and G protein sequestration will be posed.
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Abbreviations: A, agonist; B_{max}, maximum binding calculated in a radioligand equilibrium binding assay; EC_{50}, concentration of ligand that produces half of the maximum response; E_{max}, maximum response in a biological assay; G_{GDP} or G_, G protein heterotrimer with a GDP bound or an empty nucleotide binding site, respectively; $G\alpha_{GXP}$, Gα monomer possessing a guanine nucleotide (GDP, GTP, GTPγS) bound to the nucleotide binding site; G$\beta\gamma$, G$\beta\gamma$ dimer; GPCR, G protein-coupled receptor; GppNHp, guanylyl-imidodiphosphate; GTPγS, guanosine 5′-*O*-(3-thio)-triphosphate; I, inverse agonist; MAPK, mitogen-activated protein kinase; PI3K, phosphatidylinositol-3-kinase; R or R*, receptor in ground state or in a conformation which is able to release GDP from G proteins, respectively; SDS–PAGE, sodium dodecylsulfate–polyacrylamide gel electrophoresis; THC, tetrahydrocannabinol

Introduction

Cannabinoid receptors comprise two cloned types of G protein-coupled receptors (GPCR), the CB_1 receptor found predominantly in the brain and other nervous tissue and the CB_2 receptor found predominantly in immune cells. Studies of these receptor types have been recently defined and discussed by the IUPHAR Committee on Nomenclature (Howlett *et al*., 2002). As described in that review (Howlett *et al*., 2002), these receptor types respond to cannabinoid compounds such as Δ^9-tetrahydrocannabinol (Δ^9-THC), its metabolite 11-OH-Δ^9-THC, and synthetic analogs that have been developed by numerous laboratories (e.g. HU210, levonantradol). When the classical A–B(pyran)–C tricyclic ring structure was broken, a series of A–C bicyclic analogs (e.g. CP55940) and more structurally rigid A–C–D tricyclic analogs were created, and these series of compounds have become known as nonclassical cannabinoid compounds. In addition, a series of aminoalkylindole and related agonists for CB_1 and CB_2 receptors have been developed based on a series of drugs designed by Sterling Research Group. WIN55212-2 is the prototype of this series. Finally, a series of arachidonic acid derivatives (arachidonyl ethanolamide (anandamide), 2-arachidonoyl glycerol, noladin ether) have been found in nature to behave as agonists for the cannabinoid receptors, and these endogenously synthesized eicosanoid agonists are referred to as 'endocannabinoids'. Synthetic analogs have been developed for research investigation (e.g. (*R*)-methanandamide).

Considerable evidence exists to support the idea that CB_1 receptors couple through Gi/o proteins to inhibit adenylyl cyclase, regulate ion channels, activate mitogen-activated protein kinase (MAPK), and modulate several other pathways, and these studies have been reviewed recently (Pertwee, 1999; Howlett *et al*., 2002; Mukhopadhyay *et al*., 2002). GPCR-Gi/o activation is generally considered to be due to agonist-stimulated G protein dissociation to allow the release of a G protein subunit to regulate effector proteins. The nature of the effector molecule is important in determining the way a cell will respond to the G protein stimulus. The isoform of adenylyl cyclase determines whether the response will be an inhibition due to interaction with the released αi (isoforms 1,3,8,5,6) or stimulation due to the release of the $\beta\gamma$ dimers (isoforms 2,4,7) (Rhee *et al*., 1998). Only certain types of Ca^{2+} channels respond to CB_1 receptor stimulation (Mackie & Hille, 1992; Mackie *et al*., 1995; Twitchell *et al*., 1997).

Some CB_1 receptor signal transduction pathways utilize Gi/o proteins to serve as scaffolding proteins to localize regulatory enzymes such as phosphatidylinositol-3-kinase (PI3K). This could lead to the regulation of MAPK pathways

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and subsequent regulatory events (Wartmann *et al*., 1995; Bouaboula *et al*., 1997; Sanchez *et al*., 1998; Gomez *et al*., 2000). Other signaling pathways do not utilize G proteins as transducers. Sanchez *et al*. (2001) showed that sphingomyelinase activation by the CB_1 receptor was mediated by the adaptor protein Fan but not by G proteins.

This level of complexity notwithstanding, CB_1 receptor-mediated signal transduction by the endogenous eicosanoid ligands and a variety of plant products and synthetic ligands is generally governed by the pharmacodynamic principles of agonist affinity (ability to bind to the receptor) and efficacy (ability to evoke a functional response). For the purposes of the present review, we will consider the immediate response evoked as the ligand interacts with the receptor, or 'intrinsic efficacy', and the discussion will predominantly be limited to the initiation of the cellular signal transduction pathways mediated by G proteins.

Studies of a repertoire of classical and nonclassical cannabinoid agonists provided a relatively good correlation between affinity for the CB_1 receptor in brain membranes ([^{3}H]CP55940 displacement) and activity in *in vitro* assays (adenylyl cyclase) (Devane *et al*., 1988; Howlett *et al*., 1990) and *in vivo* responses (Compton *et al*., 1993; Melvin *et al*., 1993; 1995). Similarly, within a series of aminoalkylindole compounds, good correlations between ligand binding affinity and ability to produce a biological response were obtained (Compton *et al*., 1992; Shim *et al*., 1998). At the level of adenylyl cyclase regulation, it appeared that the lower potency cannabinoid agonists also exhibited a lower efficacy for the response (see Figure 2c in review by Mukhopadhyay *et al*., 2002). However, a confounding factor in the interpretation of efficacy results that could not be addressed until the advent of an antagonist for the CB_1 receptor was the high lipid solubility and poor aqueous solubility of these compounds that does not correlate with biological activity (Thomas *et al*., 1990) (see also review by Makriyannis & Rapaka, 1990). The resulting high membrane-media partition coefficient drives high concentrations of poorly potent compounds into biological membranes where perturbation of the lipid structure could be expected to alter biological functions (Hillard *et al*., 1985; 1990). When membrane perturbation was examined specifically as a potential negative influence on the signal transduction activity of poorly active cannabinoid agonists, the results were difficult to interpret (Hillard *et al*., 1990; Howlett *et al*., 1989). The eicosanoid endocannabinoid anandamide was shown to behave as a partial agonist in the inhibition of adenylyl cyclase (Childers *et al*., 1994), and this provided the first clear indication that differences in efficacy could be observed for CB_1 receptor agonists to regulate this signal transduction pathway.

Evidence for efficacy differences among CB_1 receptor agonists in the regulation of Ca^{2+} channels was reported for anandamide in the inhibition of N-type Ca^{2+} currents using electrophysiological measures in morphologically differentiated N18 mouse neuroblastoma cells (Mackie *et al*., 1993). In those studies (Mackie *et al*., 1993) and experiments that determined Ca^{2+} influx fluorometrically (Sugiura *et al*., 1997b), maximally active concentrations of anandamide produced less than the maximal response that could be achieved with WIN55212-2. Anandamide was able to antagonize the response to WIN55212-2, indicating that it behaved as a partial agonist (Mackie *et al*., 1993). In contrast, in cultured rat hippocampal neurons, the maximal inhibition of Ca^{2+} currents carried by N- and P/Q-type channels in response to anandamide was similar to that of WIN55212-2 and CP55940 (Twitchell *et al*., 1997). Shen *et al*. (1996) noted that CB_1 receptor-mediated glutamatergic neurotransmission in cultured rat hippocampal neurons was maximally reduced in response to anandamide and WIN55212-2, but that CP55940 behaved as a partial agonist. Anandamide was also as effective as WIN55212-2 in inhibiting the Ca^{2+} current carried by Q-type channels (as well as stimulating the inwardly rectifying K^+ current) in cultured AtT20 mouse pituitary cells expressing exogenous CB_1 receptors (Mackie *et al*., 1995). The differences in anandamide's efficacy between these experimental cell types was postulated to be related to the density of the CB_1 receptors

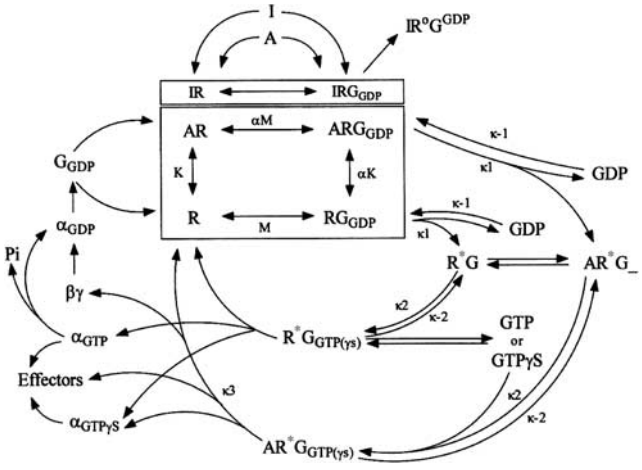

Figure 1 Ternary complex equilibrium model for agonist (A)–receptor (R)–G protein (G) interactions, coupled with the G protein activation cycle. Details with regard to the mechanisms depicted here are provided in the text. According to the ternary complex model, receptors (R), G proteins (G) and ligands can form an equilibrium (depicted within the box). The free CB_1 receptor (R) and the CB_1 receptor-Gα complex (RG_{GDP}) exist in equilibrium in the absence of exogenously added agonist (A) or inverse agonist (I) ligands (central box). This facile association is believed to be responsible for the constitutive activity reported for CB_1 receptors. The influence of agonist ligands (A) or inverse agonist ligands (I) on this equilibrium expands the equilibrium to include the AR and ARG_{GDP} complexes (main box) or the IR and IRG_{GDP} complexes (upper box). Outside the ternary complex equilibrium box, GTP and its analogs exchange for GDP in the absence of agonists (R*G_ and $R^*G_{GTP\gamma S}$) or in the presence of agonists (AR*G_ and $AR^*G_{GTP\gamma S}$. The presence of GTP or its analog facilitates dissociation of the Gαi and G$\beta\gamma$ proteins from the receptors. The activation cycle is reinitiated by the hydrolysis of GTP, and recombination of Gαi and G$\beta\gamma$ proteins to form the heterotrimer (G_{GDP}). GTPγS alone can promote dissociation of the G proteins from the CB_1 receptor, indicating that some RG_{GDP} complexes can spontaneously become activated in the absence of agonists, allowing GDP release (transiently empty G protein R*G_) and GTPγS binding. Once GTPγS binds, the $G\alpha_{GTP\gamma S}$ dissociates irreversibly and the GTPγS cannot be hydrolyzed, such that the $G\alpha_{GTP\gamma S}$ can no longer re-enter the equilibrium reaction. In the presence of an inverse agonist (I), the IR and IRG_{GDP} complexes exist. The IRG_{GDP} is believed to form an inactive complex, which in the figure is depicted as exiting the ternary complex equilibrium box, and sequestering G proteins in an inactive (IR^oG_{GDP}) state. This state was originally proposed by Bouaboula *et al*. (1997) to describe a mechanism for the CB_1 receptor to 'sequester' Gi proteins, thereby explaining their data that basal signal transduction through the MAPK or adenylyl cyclase pathways were blocked in the presence of SR141716 (see text).

or the G proteins that transduce the response (Mackie *et al*., 1995).

This review describes the research addressing the molecular mechanism(s) by which CB_1 receptor ligands evoke varying efficacy in their regulation of G proteins. For these purposes, the term efficacy will refer to the maximal functional signal transduction response produced by a particular ligand. Efficacy will be examined based on our current understanding of the G protein activation cycle as developed for other GPCRs (see theoretical discussion by Waelbroeck, 1999). Figure 1 summarizes the 'ternary complex model' of agonist (A)–receptor (R)–G protein (G) distribution in a steady-state model (Leff, 1995), combined with the catalytic pathway by which agonists activate the receptor-G protein (ARG_{GDP}) complex to promote GDP dissociation (AR*G_), receptor–heterotrimer dissociation (to AR, $G\alpha_{GTP}$ (or $G\alpha_{GTP\gamma S}$) and $G\beta\gamma$), and activation of effectors. The model also depicts possible mechanisms for the constitutive activity (RG_{GDP} to R*G_ conversion in the absence of A) and inverse agonist (I) effects to promote an inactive ternary complex ($IR^{o}G_{GDP}$).

Efficacy for CB_1 receptor-Gi/o activation

By examination of the first step in signal transduction after the ligand interacts with the CB_1 receptor, efficacy differences have been characterized for GDP/GTP exchange measured as ^{35}S-labeled guanosine 5′-*O*-(3-thio)-triphosphate ([^{35}S]GTPγS) binding to activated G proteins. This assay determines the ability of the agonist to activate the GPCR-G protein complex to release GDP from the Gα subunit and allow the binding of the nonhydrolyzable GTP analog [^{35}S]GTPγS. Conditions in these assays favored a reduced basal GDP/GTP exchange in order to maximize the response to agonists (150 mM NaCl, 2–9 mM Mg^{2+}, and 10–100 μM GDP) (Selley *et al*., 1996; Breivogel *et al*., 1998). [^{35}S]GTPγS binding to G proteins in rat cerebellar membranes (Selley *et al*., 1996; Breivogel *et al*., 1998; Griffin *et al*., 1998; Kearn *et al*., 1999) and mouse whole brain membranes (Burkey *et al*., 1997) was stimulated to a maximal extent by CP55940, WIN55212-2, HU210, and levonantradol; to a fraction of the maximum by methanandamide and analogs (and CP55940 in some experiments); poorly by anandamide, Δ^9-THC and 11-OH-Δ^9-THC; and not at all by cannabinol or SR141716. A study of rat cerebellar membranes that was performed with lower concentrations of Na^+ and limiting Mg^{2+} yielded similar results (Petitet *et al*., 1997). Disparities between the concentration for half-maximal response (EC_{50}) compared with the inhibition constants in heterologous radioligand competition assays (K_i) as a measure of affinity suggest that efficacy differences could be more complex than can be assessed simply by examination of the maximal response (Griffin *et al*., 1998). Burkey *et al*. (1997) analyzed the data using a formula combining the factors of: (1) maximal effect of the agonist (maximum response in a biological assay, E_{max}) compared with that of CP55940; and (2) the EC_{50} for G protein activation relative to the K_i determined from heterologous displacement of [^{3}H]SR141716. They proposed a relative efficacy for cannabinoid agonists of: CP55940 : HU210 : Δ^9-THC = 1 : 0.5 : 0.27; and anandamide = 0.39.

Childer's laboratory (Sim *et al*., 1996) analyzed their data by comparing the maximal effect (E_{max}) for [^{35}S]GTPγS binding to G proteins with the B_{max} (maximum binding calculated in a radioligand equilibrium binding assay) for high-affinity agonist ([^{3}H]WIN55212-2) binding to CB_1 receptors, as a determinant of the receptor/transducer catalytic amplification ratio. The receptor affinity assays were performed in the absence of guanine nucleotides in order to capture the AR*G_ complex which has a high affinity for agonists (see Figure 1). The G protein activation assays were performed in a reaction mixture containing 100 mM NaCl, 3 mM Mg^{2+}, and 20 μM GDP to maximize the response to the agonist to activate the ARG_{GDP} complex. Examination of rat striatal membranes indicated that the receptor/transducer catalytic amplification ratio was only 3 for CB_1 agonist-mediated G protein activation compared with a ratio of about 20 for μ-opioid and δ-opioid receptors in the same membranes (Sim *et al*., 1996). When similar analyses were performed in membranes from other distinct brain regions, the amplification ratios ranged from 2.5 to 6, with the greatest amplification being in brain regions having relatively sparse CB_1 receptors (Breivogel *et al*., 1997). These studies showed that each agonist-occupied CB_1 receptor was able to activate fewer G proteins compared with an agonist-occupied opioid receptor, particularly if there were excess measurable CB_1 receptors. Thus, the question arises as to the role of the receptors (R and RG_{GDP}) that were not measured in the high-affinity state for agonists.

In order to address the question of the role of the receptors that were not occupied by agonists in a high-affinity state, further analyses required an examination of receptor/transducer amplification ratios based on the total receptors (R, RG_{GDP}, and R*G_). Several studies have reported that modifiers of the G protein allosteric regulation of the receptor's affinity for the agonist (nonhydrolyzable GTP analogs and NaCl) had no effect on the affinity for the radiolabeled antagonist in rat brain membranes (Rinaldi-Carmona *et al*., 1996; Houston & Howlett, 1998; Kearn *et al*., 1999). The assumption from these studies was that [^{3}H]SR141716 would bind to uncoupled receptors devoid of G proteins (R) as well as to coupled RG_{GDP} or R*G_ states (Breivogel *et al*., 1997). The amplification ratios obtained by comparing E_{max} for G protein activation with the B_{max} for antagonist binding ranged from 2 to 8 for different brain regions. Those regions that exhibited low amplification ratios based on the B_{max} of high-affinity agonist states (AR*G_) exhibited similarly low amplification ratios when the B_{max} of 'total' receptors (AR, ARG_{GDP}, and AR*G_) was used for the calculations. For many brain regions, the high-affinity B_{max} determined by agonist binding was similar to the total receptor B_{max} determined by antagonist binding, suggesting that under the equilibrium binding conditions chosen, the CB_1 receptors were well coupled to G proteins (RG_{GDP} and R*G_). Exceptions occurred in those regions of very sparse density of receptors, in which the fraction of receptors in the high-affinity state was only about half the total estimated receptors.

Childer's laboratory noted that the amplification ratios for G protein exchange failed to correlate with the fraction of receptors in high-affinity (AR*G_) states (Breivogel *et al*., 1997). To understand this, these researchers performed a more detailed kinetic analysis of the mechanism of CB_1 receptor activation of G proteins through an analysis of rates of [^{35}S]GTPγS binding to G proteins (Breivogel *et al*., 1998). In the absence of agonist, the rate of association of [^{35}S]GTPγS decreased with the concentration of GDP (from 0 to 30 μM),

indicating that GDP and GTPγS were competing for the guanine nucleotide binding site. The CB_1 agonist WIN55212-2 functioned to overcome the low rate of association at higher GDP concentrations, consistent with a role for the agonist to destabilize the GDP interaction with the $ARG\alpha_{GDP}$ complex. This would be consistent with the WIN55212-2-occupied CB_1 receptor serving as the guanine nucleotide exchange factor to destabilize the $G\alpha_{GDP}$ complex. The important conclusion from these experiments was that the E_{max} values for various CB_1 agonists obtained from equilibrium [^{35}S]GTPγS binding in the presence of 30 μM GDP correlated well with low-affinity K_i values for GDP competition with [^{35}S]GTPγS. Thus, the differences in efficacy for various agonists could be attributed to the ability of the agonist to destabilize GDP binding.

Hillard's laboratory (Kearn *et al*., 1999) performed an analysis of CB_1 receptor agonist affinity states and efficacy, interpreting their data according to a two-state equilibrium ternary complex model of agonist binding and receptor activation (Leff, 1995). In their analysis, the CB_1 receptor was considered to exist in a state having either a low affinity for agonist (R) or a high affinity for agonist (R*). The R* state would be preferentially stabilized when occupied by the agonist (Leff, 1995). According to this scenario, the AR* complex would interact with G_{GDP} leading to activation. Ligand binding of a radiolabeled agonist, for example, [^{3}H]CP55940 at its K_d concentration, would detect the R* state exclusively, and thus provide a measure of the high-affinity $K_{d(high)}$ for agonists. The assumption was made that the radiolabeled antagonist [^{3}H]SR141716 would have the same affinity for both R and R* states, and thus heterologous competition could be used to detect the two affinity states for agonists. When the GTP analog GppNHp was added to the equilibrium mixture, the binding of agonist A to R^*G_{GDP} would promote dissociation of GDP and association of GppNHp, with dissociation of $G\alpha_{GppNHp}$ from AR* as a consequence. Uncoupled R* or AR* would isomerize to the ground state R and AR, and would accumulate as such because this analog of GTP is not subject to the hydrolysis necessary to form the G protein heterotrimer and reinitiate the cycle. Thus, heterologous competition with [^{3}H]SR141716 in the presence of GppNHp could be used to determine the high-affinity $K_{d(high)}$, low-affinity $K_{d(low)}$, and the percent of receptors in the high-affinity state for different agonists. It should be noted here that these assumptions might not be strictly correct if the radiolabeled [^{3}H]SR141716 is able to bind with high affinity to a novel receptor state, R^o, envisioned as an 'inverse agonist' state. Using these equilibrium assumptions, the fraction of receptors in the high agonist affinity R* state at the maximally effective concentration for each of six agonist ligands was found to correlate well with the maximal activity for [^{35}S]GTPγS binding to the G protein (Kearn *et al*., 1999). How these receptor affinity states exist in association with G proteins was further analyzed by Breivogel & Childers (2000)).

Breivogel & Childers (2000) analyzed the relationship between receptor occupancy and activation of G proteins using identical assay components for both assays: 100 mM NaCl, 3 mM $MgCl_2$, 0.5 nM SR141716, 0.05 nM GTPγS, and 50 μM GDP. They examined membranes from rat cerebellum, hippocampus, and hypothalamus for the activities of five agonists (WIN55212-2, levonanatradol, CP55940, methanandamide, and Δ^9-THC) to bind to the CB_1 receptor assessed by competition with [^{3}H]SR141716 and to stimulate [^{35}S]GTPγS binding. The cerebellum and hippocampus, having similar receptor densities, exhibited similar efficacy profiles determined by E_{max} for stimulation of [^{35}S]GTPγS binding. When the agonist radioligand competition curves were analyzed, three affinity states (high, intermediate, and low) were recognized for agonists (except methanandamide which exhibited two: intermediate and low). Each agonist was characterized by a different fraction of receptors in each state. When activation of G proteins was examined, two apparent K_{act} values could be discerned that generally corresponded to the fractions of receptors exhibiting intermediate and low affinity for agonist, respectively. In this analysis, the fraction of receptors in the high-affinity state for agonists corresponded to the basal [^{35}S]GTPγS binding activity. Within the scheme depicted in Figure 1, it can be speculated that a considerable fraction of the receptor exists coupled with G proteins (RG_{GDP}), and that a certain fraction of these could spontaneously achieve a conformation that allows dissociation of GDP (R*G_). If agonists were introduced into this equilibrium mixture, this state would be characterized by high affinity for those agonists that exhibit high efficacy (AR*G_). The dissociation of GDP from the guanine nucleotide binding site allows facile association of [^{35}S]GTPγS without the necessity of agonists triggering GDP release. The majority of the precoupled receptors remain as the RG_{GDP} form, which exhibits an intermediate affinity for agonists, and requires the stimulus of the agonist interaction to promote isomerization to the AR^*G_{GDP} state that triggers dissociation of GDP and allows subsequent association of [^{35}S]GTPγS. Those receptors that remain uncoupled from G proteins (R) bind the agonist with low affinity. However, when agonist is bound (AR), these receptors can subsequently couple to G_{GDP}, leading to the dissociation of GDP and subsequent association of [^{35}S]GTPγS. For a low efficacy agonist such as (*R*)-methanandamide, the agonist interaction with the receptor exhibited little difference in affinity for either the uncoupled R or precoupled RG_{GDP} receptor states, and so both pathways could be taken to build the ARG_{GDP} complex, and these would appear to exhibit the same agonist affinity and ability to stimulate [^{35}S]GTPγS binding.

Brain regional differences in CB_1 receptor signal transduction efficacy

CB_1 agonist WIN55212-2 stimulation of [^{35}S]GTPγS binding exhibited a range of amplification ratios across various regions of the brain even when adjusted for the density of receptors in those individual brain regions (Sim *et al*., 1996; Breivogel *et al*., 1997). One might look to the different types of G proteins within the Gi/o family that are available to interact with CB_1 receptors within brain membranes for further explanation. Studies of Gi-mediated stimulation of GTPase activity and inhibition of adenylyl cyclase demonstrated brain regional differences in modulation by Na^+, suggesting that the receptor-G protein coupling could be under different regulatory control in different neuronal types (Pacheco *et al*., 1994). Little or no inhibition of adenylyl cyclase could be detected in certain brain regions in which the CB_1 receptor could nevertheless activate G proteins, suggesting that Gi proteins coupled to CB_1 receptors are coupled to selective effectors or

that their coupling efficiency is reduced (Breivogel & Childers, 2000). These findings might be attributed to differential profiles of G proteins (availability of subtypes, or receptor-G protein sequestration) to which the CB_1 receptor couples in different brain regions.

The possibility that brain regional efficacy differences can be related to CB_1 receptor density has been examined in a study of transgenic heterozygote CB_1 (+/−) compared with wild-type CB_1 (+/+) mice (Selley *et al*., 2001). In those studies, the receptor B_{max} was reduced by half in each of the brain regions tested, but the E_{max} of WIN55212-2 to stimulate [^{35}S]GTPγS binding was reduced by only 25% in most regions except for the striatum. The EC_{50} for WIN55212-2 stimulation was significantly increased only in the cerebellum and striatum. This meant that the reduction of CB_1 receptor density in the heterozygote led to an increase in amplification ratio by two-fold in most brain regions, indicative of an increase in the receptor-G protein coupling efficiency. The relative efficacies of the partial agonists (*R*)-methanandamide and Δ^9-THC did not change with the decrement in receptor density, suggesting that these ligands were not able to influence the receptor-G protein equilibrium to overcome the receptor loss (Selley *et al*., 2001).

A credible alternative explanation for regional differences in the response to ligands is that an alternative receptor in addition to the CB_1 receptor recognizes these agonists. Studies of the transgenic CB_1 (−/−) knockout mouse have indicated that anandamide and WIN55212-2, but not classical or nonclassical cannabinoid ligands, stimulate [^{35}S]GTPγS binding in the absence of CB_1 receptors, and this response was not specifically antagonized by SR141716 (Breivogel *et al*., 2001). Although the CB_1 (−/−) knockout response to WIN55212-2 was small compared with that of the CB_1 receptor in wild-type CB_1 (+/+) mice, it was nevertheless significant in certain brain regions (Breivogel *et al*., 2001). The possibility for an alternative receptor is consistent with the biological responses to anandamide that can be observed in the presence of SR141716 (Adams *et al*., 1998) or these CB_1 (−/−) animals (DiMarzo *et al*., 2000; Baskfield *et al*., 2004). Additional evidence for an alternative receptor for WIN55212-2 is that [^{3}H]WIN55212-2 binding sites exist in certain brain regions in the CB_1 (−/−) knockout mouse (Breivogel *et al*., 2001) and might also exist in cultured NG108-15 cells (Stark *et al*., 1997). The pharmacology of this novel non-CB_1, non-CB_2 receptor for anandamide and WIN55212-2 has not been characterized, and signal transduction beyond that of G protein activation has not been reported. Until further studies elucidate the genotypic and phenotypic properties that distinguish this alternative receptor from the CB_1 receptor, interpretations that attribute differences in pharmacological responses to G proteins profiles must be made with caution.

Agonist selectivity for G proteins

Evidence for differential regulation of different Gi subtypes by different agonists was provided by Houston & Howlett (1998), who examined rat brain membrane CB_1 receptors for multiple agonist affinity states detected by competition for [^{3}H]SR141716 binding. Under standard assay conditions (4 mM Mg^{2+}, no guanine nucleotides), the cannabinoid desacetyllevonantradol and the aminoalkylindole WIN55212-2 both bound in two discrete affinity states, representing approximately 30% of the receptors in a high-affinity state for the agonist, and the remainder in a low-affinity state. A difference in the affinity states for the two agonists was obvious in the ratios of the two K_i values and in the modulation by Na^+ and GTP analogs. Under conditions of Na^+ regulation (100 mM NaCl), both affinity components for desacetyllevonantradol were reduced in affinity, particularly the component representing receptors in the low-affinity state. However, the addition of NaCl had little influence on WIN55212-2 binding affinities. The addition of GTPγS reduced affinity in the high-affinity state, indicative of an accumulation of $G\alpha_{GTP\gamma S}$ and increase in the population of receptors in the uncoupled R state. This shift of equilibrium was complete for WIN55212-2, but not for desacetyllevonantradol. This finding might suggest that two or more different populations of G proteins that couple to the CB_1 receptor are modulated differently, with WIN55212-2 modulating all G protein subtypes and desacetyllevonantradol affecting only a subset of G proteins.

Glass & Northup (1999) found differences in the way that CB_1 receptor agonists could regulate purified Gi *versus* Go proteins. They studied CB_1 receptors that were expressed in Sf9 insect cells by isolating the membranes and treating them with the chaotrope urea in order to uncouple and remove G proteins. By this means, they were able to reconstitute receptor-G protein coupling by adding purified G protein subunits to the membranes. The activation of specific G proteins by agonist-occupied CB_1 receptors was monitored by determining [^{35}S]GTPγS binding to the Gα proteins in the presence of saturating concentrations of $\beta\gamma$ proteins. These assays were performed in the presence of an excess of $\beta\gamma$ proteins, 100 mM NaCl and 4 μM GDP and <1 nM concentrations of [^{35}S]GTPγS, conditions that limited the amount of GDP-[^{35}S]GTPγS exchange that would occur in the absence of agonists. In these studies, both Gi and Go proteins were activated most efficaciously by HU210 and least efficaciously (60%) by Δ^9-THC. Significant G-protein-specific differences were observed for WIN55212-2 and anandamide, which exhibited maximal or near-maximal efficacy for Gi, but only about 70% maximal efficacy for Go. The potency order remained unchanged, suggesting that the binding to the receptor remained the same irrespective of the G protein being activated.

Studies of Prather *et al*. (2000) demonstrated differences in Gi/o-subtype activation in response to a single cannabinoid receptor agonist, WIN55212-2. These studies utilized GDP-[^{32}P]azidoanilidoGTP exchange. The covalent binding of this GTP analog to the G protein would preclude any reverse reaction, so that the labeled proteins could be isolated by immunoprecipitation or separation and identification after sodium dodecyl sulfate–polyacrylamide electrophoresis (SDS–PAGE). In those studies, Gαi1/ i3, Gαi2, Gαo1, Gαo2, and Gαo3 were distinguishable bands on immunoblots and autoradiographs, and could be quantitated densitometrically. Using this measure of G protein activation, a maximally active concentration of WIN55212-2 stimulated greater incorporation of [^{32}P]azidoanilidoGTP into Gαo1 compared with Gαo2 or Gαo3 for all areas of the rat brain examined. However, the EC_{50} for WIN55212-2 to activate the various G protein subtypes differed by 36-fold, from 100 nM for Gαi1 and Gαo3 to 3.7 μM for Gαo2. Thus, it could be con-

cluded that low concentrations of the CB_1 agonist WIN55212-2 can activate certain Gα subtypes without activating others.

Mukhopadhyay & Howlett (2000) studied selective receptor-Gi-subtype interactions by quantitating the Gαi and Gβγ proteins that co-immunoprecipitate with the CB_1 receptor from a detergent extract of N18TG2 membranes in the presence of ligands. An equilibrium dissociation between ARG_{GDP} and AR plus G_{GDP} was observed in response to the aminoalkylindole WIN55212-2 for all three RGαi complexes, the cannabinoid desacetyllevonantradol for Gαi1 and Gαi2, and the eicosanoid (*R*)-methanandamide for Gαi3. However, desacetyllevonantradol maintained RGαi3 complexes and (*R*)-methanandamide maintained RGαi1 and RGαi2 complexes even in the presence of GTPγS. The biaryl pyrazole SR141716 maintained all three RGαi complexes, but supported some equilibrium mixtures in the presence of GTPγS. Gβ proteins exhibited the same association/dissociation pattern as the Gα proteins. These results can be explained by invoking the existence of an inverse agonist (I)-supported inactive state (IR^oG_{GDP}), in the ternary complex equilibrium model (see Figure 1). In this model, WIN55212-2 behaves as an agonist for all three Gi subtypes, and SR141716 behaves as an inverse agonist for all three Gi subtypes. However, desacetyllevonantradol behaves as an agonist for Gi1 and Gi2 and an inverse agonist for Gi3, and (*R*)-methanandamide behaves as an inverse agonist for Gi1 and Gi2 and an agonist for Gi3. These ligand-selective G protein responses imply that multiple conformations of the receptor could be evoked by ligands in order to regulate individual G proteins.

The observation that a unique pattern of functional interaction is ligand specific (i.e. the behavior as an agonist or inverse agonist at a given Gi subtype is not the same for each ligand) has great biological significance because it implies that ligands can direct cellular signal transduction pathways *via* one Gi subtype at the expense of inactivation of another. The functional consequences of CB_1 receptor stimulation within cells possessing multiple Gi/o subtypes would be both ligand- and Gi/o-subtype specific.

Evidence that CB_1 receptors interact with Gq or Gs

Several studies have suggested that phospholipase C can be stimulated by CB_1 receptors (Sugiura *et al*., 1996; 1997a; Netzeband *et al*., 1999), which might suggested the possibility for a coupling to Gq, were it not for the observation that these responses were blocked by pertussis toxin. Furthermore, recombinant CB_1 receptors failed to alter the production of inositol phospholipids when expressed in host cells that would have allowed for stimulation of phospholipase C (Felder *et al*., 1992; 1995). In a hippocampal preparation, cannabinoid compounds inhibited neurotransmitter-stimulated inositol phospholipid production (Nah *et al*., 1993). Thus, CB_1 receptor coupling to the Gq family is not well supported by the available research findings.

The question of whether CB_1 receptors can interact with Gs has been suggested from findings that, under conditions of pertussis toxin treatment that prevents the receptor's interaction with Gi/o proteins, a stimulation of cyclic AMP accumulation was observed in cultured neurons and CHO cells expressing recombinant CB_1 receptors (Glass & Felder, 1997; Felder *et al*., 1998). In striatal cell cultures, combinations of dopaminergic and cannabinergic stimulation resulted in an increase in cyclic AMP (Glass & Felder, 1997) and WIN55212-2 produced an increase in basal cyclic AMP production in globus pallidus slice preparations (Maneuf & Brotchie, 1997).

A study using a recombinant model system of HEK293 cells stably transfected with the D_2 dopamine receptor and transiently transfected with the human CB_1 indicated that the expression of D_2 dopamine receptors was sufficient to convert the inhibition of forskolin-stimulated cyclic AMP production by CP55940 to a stimulation of cyclic AMP production (Jarrahian *et al*., 2004). This would be consistent with the uncovering of a cryptic ability of the CB_1 receptor to couple to Gs in addition to Gi. Evidence supporting this notion included the observation within this experimental model that pretreatment with pertussis toxin eliminated the component of inhibition of cyclic AMP accumulation but did not affect the stimulation of cyclic AMP. Interestingly, the converse did not occur: the D_2 dopaminergic inhibition of forskolin-stimulated cyclic AMP accumulation was not affected by the expression of CB_1 receptors (Jarrahian *et al*., 2004). However, stimulation of the CB_1 receptors by CP55940 did increase the net cyclic AMP accumulation, consistent with a stimulation of Gs by the agonist-stimulated CB_1 receptor. The mechanism for this response could be explained by invoking the ability D_2 dopamine receptors to sequester Gi proteins such that these transducers would no longer be available to couple to the CB_1 receptors, leaving the CB_1 receptors to couple to Gs proteins, which would be readily available endogenously in this model cell system. Evidence in support of this explanation is that overexpression of Gαi1 but not Gαo allowed the inhibition of cyclic AMP accumulation by CP55940-stimulated CB_1 receptors to prevail (Jarrahian *et al*., 2004). Additional evidence was that when the D_2 receptor coupling to Gi was compromised by persistent agonist stimulation (18 h treatment of the cells with the D_2 agonist quinpirole), the CB_1 receptor-Gi inhibition was the prevalent response (Jarrahian *et al*., 2004). The CB_1 receptor-Gs stimulation was not robust, being only 30% of forskolin's maximal response (Jarrahian *et al*., 2004). The Gs response required greater occupancy of the CB_1 receptors than did the Gi response, inasmuch as inhibition of cyclic AMP accumulation occurred at 10–1000 nM CP55940, but stimulation of cyclic AMP accumulation required 0.1–10 μM (Jarrahian *et al*., 2004).

The CB_1 receptor interaction with Gs has also been demonstrated in CHO cells stably expressing recombinant human CB_1 receptors (Bonhaus *et al*., 1998). In order to observe receptor coupling to Gs, the cells were pretreated with pertussis toxin such that the Gi/o proteins were unable to interact with the receptor. The agonists exhibited a different order of efficacies when tested for Gi *versus* Gs regulation of forskolin-stimulated cyclic AMP production (Bonhaus *et al*., 1998). The inhibition of the forskolin-stimulated cyclic AMP accumulation by Gi was maximal in response to full agonists, HU210, CP55940, and WIN55212-2 (in order of potency), and only 50 and 75% of maximal by partial agonists, Δ^9-THC, and anandamide, respectively. Following pertussis toxin treatment, stimulation of cyclic AMP accumulation, presumably by Gs, was increased by 100% above the forskolin-stimulated value

by WIN55212-2. Cyclic AMP accumulation was increased by only about 50% by HU210 and CP55940, and about 35% by Δ^9-THC and anandamide, suggesting that these compounds behave as partial agonists for this response. The potency order was the same whether a decrease or an increase in cyclic AMP was being measured, even though HU210 and CP55940 exhibited relatively lower maximal activities when coupled to Gs. The CB_1 receptor antagonist SR141716 behaved as a competitive inhibitor with equal ability to antagonize both responses.

In contrast to the regulation of cyclic AMP production, no evidence has been found to support a direct CB_1 receptor-Gs or Gq interaction *in vitro* in equilibrium association or G protein activation studies. In studies of solubilized CB_1 receptor from brain or N18TG2 cell membranes, no indications have been found that Gs or Gq could be stably associated with the receptor, as has been shown to be the case for the Gi/o family (Mukhopadhyay *et al*., 2000). In studies of membranes from Sf9 cells expressing CB_1 receptors, no activation of reconstituted Gαq was found under conditions in which the activation of reconstituted Gi and Go was quite robust (Glass & Northup, 1999).

A mechanism for the activation of Gs by the CB_1 receptor has been explored. An *Ala-Leu* sequence in IL3 is believed to interact with Gs, but does not form the appropriate helical structure to interact with Gi (Ulfers *et al*., 2002). To demonstrate this, stimulation of cyclic AMP production was observed when host cells bearing the mutant CB_1 receptor *Ala-Leu* sequence were treated with pertussis toxin to block interaction of Gi proteins with the receptor. In the absence of functional Gi proteins after pertussis toxin pretreatment, the mutant CB_1 receptor was able to couple to Gs (Abadji *et al*., 1999), suggesting that the mutant CB_1 receptor could associate with Gs as might be pred icted from sequence homologies with Gs-coupled receptors.

Cannabinoid receptor constitutive activity and inverse agonism of SR141716

The constitutive activity for GPCRs in the absence of an agonist has been noted in recombinant expression systems. This is believed to be due to the high concentrations of exogenously expressed receptor that drive the R plus G_{GDP} equilibrium reaction in the forward direction, thereby increasing the concentration RG_{GDP} and any R*G_ (see Figure 1) that would be generated due to spontaneous isomerization in the absence of an agonist (Kenakin, 1997). Constitutive activity for exogenously expressed CB_1 receptors has been demonstrated (Bouaboula *et al*., 1997; Landsman *et al*., 1997; MacLennan *et al*., 1998; Pan *et al*., 1998), indicating that activity in the absence of stimulation by an exogenously applied agonist is possible. Evidence that native CB_1 receptors exhibit constitutive activity is relatively sparse, but alteration of experimental conditions can allow such observations (Meschler *et al*., 2000; Sim-Selley *et al*., 2001). Studies from Sim-Selley's laboratory have further explored this 'inverse agonist' component of the action of SR141716 (Sim-Selley *et al*., 2001). Relatively high concentrations of SR141716 were required to observe the inverse agonist behavior in [^{35}S]GTPγS binding assays, even under assay conditions made more appropriate to observe constitutively activated receptors (Sim-Selley *et al*., 2001). Further evidence for constitutive activity in endogenously expressed CB_1 receptor systems will be necessary to support the notion that this is a phenomenon that occurs in neurons in the body.

Bouaboula *et al*. (1997) proposed that the mechanism of the inverse agonist ability of SR141716 was a stabilization of the receptor-G protein complex that prevents G protein activation (IR^oG_{GDP} as depicted in Figure 1). They further proposed that SR141716-occupied CB_1 receptors could sequester Gi/o proteins away from other signal transduction pathways that are presumed to share G proteins. Studies from Lewis' laboratory on the regulation of Ca^{2+} channels by CB_1 receptor and Gi/o proteins have provided evidence favoring the sequestration of G proteins (Pan *et al*., 1998; Vasquez & Lewis, 1999). In superior cervical ganglia neurons expressing recombinant CB_1 receptors, the responses to norepinephrine or somatostatin *via* their endogenous receptors were tempered by the presence of CB_1 receptors, suggesting a shared pool of Gi/o proteins (Vasquez & Lewis, 1999). Additional support for the sequestration of G proteins needs to be demonstrated for natively expressed receptors for ion channel regulation and for other effector responses. These studies should be forthcoming now that demonstrations in recombinant systems have opened the possibility that constitutive activity and G protein sequestration exists.

Goals for studies of efficacy

This review has concentrated on describing potential explanations for differences in intrinsic efficacy for ligands that interact with the CB_1 cannabinoid receptor type. Much less information is available for a comparable analysis of the CB_2 cannabinoid receptor. Intrinsic efficacy is a property of the ligand itself, and reflects the ability of the ligand to interact with the receptor and produce a response. The molecular mechanism for this response would have to involve conformational changes in the receptor protein that lead to conformational activation of the G protein heterotrimer. The nature of the conformational changes induced by ligands for GPCRs is a subject of great research activity. Efficacy to produce a biological response in *in vivo* systems involves determinants other than the intrinsic efficacy of the ligand. Efficacy in *in vivo* systems also includes parameters that define the ability of the particular *in vivo* system to produce a maximum stimulus for the response. Factors that influence the stimulus efficacy are related to the involvement of multiple cell types in a pathway toward the end point of the response, as well as environmental factors associated with the response mechanism and pharmacokinetic parameters of drug distribution and metabolism within the *in vivo* system. The goal of research activities in the area of pharmacodynamics is to discover drugs that can produce a maximal therapeutic response while at the same time evoking a minimum of untoward side effects. Unfortunately, we are currently suffering from an inadequate understanding of all the factors involved in the stimulus efficacy in *in vivo* systems, and are only beginning to translate our understanding of intrinsic efficacy to the development of novel ligands having therapeutic value in CNS pharmacology.

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