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THE HUMAN CANNABINOID CB₁ RECEPTOR STABLY EXPRESSED IN
CHINESE HAMSTER OVARY CELLS PROVIDES A MODEL SYSTEM TO
PREDICT THE PHARMACOLOGICAL EFFECTS OF CANNABINOIDS IN MAN

by

Robert Shawn Landsman

A Dissertation Submitted to the Faculty of the
COMMITTEE ON PHARMACOLOGY AND TOXICOLOGY (GRADUATE)

In Partial Fulfillment of the Requirements
For the Degree of

DOCTOR OF PHILOSOPHY

In the Graduate College

THE UNIVERSITY OF ARIZONA

1999
As members of the Final Examination Committee, we certify that we have read the dissertation prepared by Robert Shawn Landsman entitled

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SIGNED: [Signature]
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I was fortunate to have four grandparents when I first moved out here. Unfortunately, I lost one of them in 1995. However, I continue to keep in touch with the others. I know it is difficult for them to travel to Arizona so I am unable to see them as much as I would like, but I still know they are proud of my accomplishments.

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DEDICATION

I would like to dedicate this degree, all it stands for and, most importantly all that I am, to my family and friends. I would especially like to dedicate this period of my life to my late grandfather Daniel Arnold Brown. The choice that I made in attending a University so far from home led to the possibility that you may not be around in life when I returned home. My greatest fears came true approximately 8 a.m. EST on January 29, 1995. My daily concern of possibly losing my dog, Widget, while I have been in Arizona has brought me equal concern. My concerns came to an end when we lost her on December 1, 1997. I have been fortunate thus far in having the rest of my family alive and in good health. Such is life. Finally, I would like to dedicate this degree to myself for managing to put up with five years of graduate school. It was an adventure. Thank me.
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ABSTRACT

Since the cloning of the only known cannabinoid receptor in the human brain, CB₁, scientists have been trying to elucidate the mechanisms of action of this receptor. The possibility of yet unidentified cannabinoid receptors in human brain makes it important to be certain that any results obtained from studies of the CB₁ receptor are due strictly to this receptor. Therefore, the cloned human cannabinoid CB₁ receptor has been stably expressed in Chinese Hamster Ovary (CHO/CB₁) cells, which do not endogenously express and known cannabinoid receptors. Saturation binding studies using [³H]SR141716A and [³H]CP55,940 determined that this system expresses the human cannabinoid CB₁ receptor at approximately 1.8 pmol of receptor/mg protein and that this expression system is able to differentially bind agonists (CP55,940) and antagonists (SR141716A). In the [³⁵S]GTPγS binding assay, CP55,940, WIN 55,212-2 and HU-210 are full agonists, methanandamide and 11-OH-Δ⁹-THC are partial agonists, Δ⁹-THC is a competitive antagonist and SR141716A and AM630 are inverse agonists. The results for the full and partial agonists are consistent with those reported for these compounds in mammalian brain. The findings that Δ⁹-THC is a competitive antagonist and that SR141716A and AM630 are inverse agonists in the [³⁵S]GTPγS assay are novel. CP55,940 and WIN 55,212-2 were also shown to be full agonists by maximally inhibiting forskolin-stimulated cAMP formation in CHO/CB₁ cells. The finding that SR141716A is an inverse agonist was further supported by its ability to augment forskolin-stimulated cAMP formation. Interestingly, Δ⁹-THC was a partial agonist in this assay since it inhibited forskolin-stimulated cAMP formation, but to a lesser extent
than either CP55,950, or WIN 55,212-2. This difference in the activity of $\Delta^9$-THC warrants further study. Pertussis toxin abolished the effects of cannabinoid ligands in these functional assays, confirming that the effects of these compounds are mediated through inhibitory $G_{i/o}$ proteins. The data obtained from radioligand binding and functional assays with the human cannabinoid CB$_1$ receptor stably expressed in Chinese hamster ovary cells conclude that this is a model system to study the pharmacological effects of cannabinoids and to predict the actions of cannabinoids in man.
CHAPTER 1
Introduction, Hypothesis and Aims

1.1 INTRODUCTION

1.1.1 The History of Marijuana

\textit{Cannabis sativa}, otherwise known as marijuana, is believed by historians to have been used for its healing powers as early as the twenty-eighth century B.C. However, the earliest documentation of medical uses of \textit{Cannabis} dates back only to the first century where the Chinese described using a mixture of \textit{Cannabis} and wine as a means of inducing anesthesia (Roffman, 1982). The Romans were the first in the Western world to document the medicinal properties of \textit{Cannabis sativa}. A report, dated 70 A.D., stated that \textit{Cannabis} is effective in treating both earaches and "diminishing sexual desires." Other reports from Rome a century later described marijuana's ability to control pain (Roffman, 1982).

During the 15\textsuperscript{th} and 16\textsuperscript{th} centuries, reports from Europe and Asia claimed that \textit{Cannabis} could be used to enhance speech, to stimulate mental powers and could be used as an astringent. The number of references to \textit{Cannabis} in the medical texts continued to increase during the 17\textsuperscript{th} and 18\textsuperscript{th} centuries. Though the analgesic property of the plant was well known by this time, other uses such as the treatment of depression, cough, jaundice, inflammation, tumors, deposits in the joints, and venereal disease were also reported. The use of \textit{Cannabis} in the treatment of venereal disease was due to the belief that the plant had antibiotic properties. Publications from the early 1800's claimed that \textit{Cannabis} could also be used to treat parasitic infections (Roffman, 1982).
More than 100 reports discussing the use of *Cannabis* in medicine were published between the years 1839 and 1900 as the interest in marijuana continued to grow. Muscle relaxant and anticonvulsant properties were added to the list of therapeutic effects of marijuana. During this time, Squire’s Extract, a preparation of *Cannabis*, was developed and commonly used as a standard treatment for pain and to increase appetite. Concurrently, it was also noticed that even though the thought processes of individuals consuming large doses of hashish were similar to the patterns found in people diagnosed as psychotic, the plant could still be used to treat numerous mental illnesses (Roffman, 1982).

Despite the ever-growing number of medical benefits of marijuana there were still shortcomings which limited its use. One of these was the varying potency of the drug from one source to the other which made it difficult to record consistent results between patients and to effectively dose the drug. Some patients would feel no effect of the marijuana from one source while patients using a different source would experience stronger than desired effects. Another problem was that the drug is not water soluble which meant that it could not be administered i.v. and the onset of action after oral administration could be hours (Roffman, 1982).

Interest in the chemistry and pharmacology of the oil derived from *Cannabis sativa* increased throughout the 1930's and 1940's (Wollner et al., 1942). Numerous advances in the cannabinoid field were made in the 1940’s with the structural determination of cannabinol (Adams et al., 1940a), the isolation of cannabidiol (Adams et al., 1940b) and the synthesis of tetrahydrocannabinol (THC) analogs with marijuana-like activities and other similarly
constituted compounds which have cannabimimetic (marijuana-like) effects in dogs (Adams et al., 1941). Despite this, growing public concern regarding the effects of marijuana called for reform, and the prosecution of users and dealers. The introduction of injectable opiates and synthetic analgesics around the 1900's decreased the use of cannabinoid products and, by the time the Marijuana Tax Act was passed by Congress in 1937, all of the states in the U.S. prohibited the plant. This act still allowed doctors to prescribe the drug, but now they needed to keep strict records and pay a registration tax. In 1941 the drug was removed from the U.S. Pharmacopoeia and the National Formulary. It was not until the 1960's when the increased use of this drug by America's youth (Roffman, 1982) and the discovery and isolation of (-)-\(\Delta^9\)-tetrahydrocannabinol (\(\Delta^9\)-THC), the plant's major active ingredient, (Gaoni and Mechoulam, 1964) garnered the attention of scientists and lawmakers and had them reconsidering the use of this plant for medical purposes.

Despite this increased awareness of the potential medical uses of marijuana the plant was still regulated in the United States. Drug laws were amended in 1970 by the passing of the Comprehensive Drug Abuse Prevention and Control Act. Under this act, Title II, the Controlled Substances Act, not only established which drugs should be controlled, but also what measures should be taken to limit the availability of these substances and how illicit distributors and users of these substances should be penalized. After this, marijuana, hashish and tetrahydrocannabinol were, and still are, categorized as Schedule I drugs. This category, which also includes heroin, d-lysergic acid diethylamide (d-LSD), mescaline, peyote and other hallucinogens, states that these substances: have a high potential for abuse; are not
accepted for medical use in the United States; and have not been proven safe for use even under medical supervision (Roffman, 1982).

Toward the end of the 1970's the general public was beginning to hear about the benefits of marijuana in controlling nausea associated with cancer treatment (Sallan et al., 1975). Though these compounds were still illegal, the public wanted to change the existing laws despite the lack of adequate research. In 1979, a cancer specialist published an editorial recommending the immediate approval of THC by the federal government for cancer patients (Laszlo, 1979). That same year, under a new program in New Mexico, some cancer patients were permitted to receive marijuana or THC. By mid-1981, 31 states enacted the controlled substance therapeutic research acts. By the end of that year, 14 of these states had obtained federal approval and initiated research on the use of this substance (Roffman, 1982).

Further support for the legalization of marijuana has come from numerous lobby groups. Both the Alliance for Cannabis Therapeutics (ACT) and the National Organization for the Reform of Marijuana Laws (NORML) have aided in the easing of marijuana laws. In 1996, voters in Arizona and California overwhelmingly decided to allow doctors to prescribe marijuana and other controlled substances to seriously ill patients. Even though these state laws passed, the federal government did not support these decisions and warned doctors that if they prescribed these drugs then they would be breaking federal law and could be prosecuted. The belief by lawmakers that the voters in these states did not understand what they were voting on called for another vote in 1998 whether or not to revoke the law passed in 1996. Again, voters supported the prescription of certain controlled substances by
medical doctors.

1.1.2 Classification of Cannabinoids

To date, the number of compounds identified from the marijuana plant continues to increase. Currently, approximately 480 chemicals in 18 different chemical classes have been identified from *Cannabis*. Sixty-six of these, the cannabinoids, are found in no other genus of plants (Voth and Schwartz, 1997). There are currently three definitions of the term cannabinoid. Botanical definitions include inactive plant substances such as cannabigerol, as well as alkaloids and other secondary constituents, but exclude synthetic compounds. Chemical definitions include active and inactive analogues of Δ⁹-THC, but exclude a growing number of compounds structurally distinct from Δ⁹-THC, but that share its pharmacologic actions. Pharmacological definitions include any compound which mimics Δ⁹-THC in various *in vivo* and *in vitro* studies (Weissman, 1981).

1.1.3 The Effects of Cannabinoids in Animals and Man

Administration of low doses of cannabinoids in animals result in combined depressant and stimulant effects while high doses result mainly in CNS depression (Dewey, 1986). Various tests for studying cannabinoid-induced psychotomimetic effects in animals have been developed. The corneal areflexia test in rabbits was performed by repeatedly injecting rabbits with a predetermined dose of a cannabinoid until the blink reflex, upon stimulation with a calibrated (horse) hair, was abolished (Loewe, 1945; Valle et al., 1966).
This test has been largely replaced by other animal models, including the dog static ataxia assay, which was first developed by Walton (Walton et al., 1938) and later modified by Dewey (Dewey, 1986) and Martin and colleagues (Martin et al., 1984). This test measures the degree of motor coordination in dogs after cannabinoid administration. Endpoints consist of only a slight depression in motor function to the inability to rise from a prone position. CNS depression, prancing, hyperreflexia and tail-tucking have also been used as endpoints (Martin et al., 1984). Rhesus monkeys have also been used to differentiate cannabinoid doses producing psychotomimetic effects from those producing general CNS depressant effects (Edery et al., 1972). Drowsiness, decreased motor activity, stupor and postural changes were used as endpoints in these studies.

Mice exposed to cannabinoids exhibit a "popcorn" effect in which the animals are in a sedated state with little movement until an outside stimulus causes one or more mice to jump. This startles other mice which causes them to jump. This unique hyperreflexive state is useful in predicting which cannabinoids would have cannabimimetic activity in man (Dewey, 1986). In addition, the mouse multiple evaluation procedure is used to determine the effects of cannabinoids in vivo. This widely-used procedure measures four parameters: locomotor activity (hypokinesia), rectal temperature (hypothermia), performance in the tail flick test (antinociception) and performance in the ring immobility (catalepsy) test (Pertwee, 1993). This important bioassay is faster, less costly and more acceptable than those requiring the use of dogs or monkeys.

Numerous objective and subjective effects from Cannabis use have been recorded
in man. Subjective effects in man during Cannabis use include euphoria, sleepiness, depersonalization, difficulty in concentrating and thinking, dream-like states (Hollister, 1971) as well as a reduction in physical strength (Hollister et al., 1968). In addition, performance on left lateralized, verbal-analytic tests was significantly impaired while performance on right hemisphere, nonverbal-holistic tasks was significantly improved (Cohen, 1976). Objective effects consist of a narrowing of the airways, a decrease in intraocular pressure, a decrease in testosterone and plasma luteinizing hormone, and an increase in cardiac output (Cohen, 1976). Despite numerous detrimental effects, many people knew that cannabinoids could be beneficial. Although nobody knew how these compounds were acting, a few ideas were tested.

1.1.4 Membrane Perturbation Theory of Cannabinoid Actions

One hypothesis as to the mechanism of action of cannabinoids was that these compounds act through non-receptor mechanisms, mainly through inducing alterations in the lipid membrane, to produce their effects. This concept was supported by the knowledge that cannabinoids are lipid-soluble neutral compounds with a very high membrane/aqueous solution partition coefficient (Seeman et al., 1972; Roth and Williams, 1979). This means these compounds are highly capable of inserting into the lipid bilayer of cell membranes and causing perturbation of ordered phospholipid regions (Bach et al., 1976a).

Based on the observation that the clinical potency of phenothiazine tranquilizers correlated with their stabilizing potency against hypotonic hemolysis of red blood cells
(Seeman and Weinstein, 1966), Raz et al. (1972) were interested in studying the ability of cannabinoids to similarly stabilize membranes against osmotic rupture. They have shown that both $\Delta^9$-THC and cannabidiol (CBD) stabilized human erythrocytes against hypotonic hemolysis. This effect, however, depended on the system investigated since, as with many lipid-soluble compounds, $\Delta^9$-THC and CBD have the potential to show biphasic interaction patterns. For example, when Raz et al. (1972) used human erythrocytes, high concentrations of $\Delta^9$-THC consistently stabilized the cells whereas CBD eventually lysed the erythrocytes. However, when the same group looked at the ability of these compounds to stabilize rat liver lysosomes against rupture, both $\Delta^9$-THC and CBD initially stabilized the lysosomes whereas higher doses labilized these membranes. (Raz et al., 1973). Similarly, Bach et al. (1976b) have shown that $\Delta^1$-THC ($\Delta^9$-THC) and CBD are potent disorganizers of phospholipid bilayer membranes.

Cannabinoids are highly lipophilic and, as a result, undergo a high degree of non-specific binding not only to tissue, but to materials including glass, plastic and rubber (Garrett and Hunt, 1974). Though cannabinoids were isolated 40 or more years ago (Adams et al., 1940a; Adams et al., 1940b; Gaoni and Mechoulam, 1964), the relatively low potency of these naturally-derived compounds, such as $\Delta^9$-THC, makes them poor candidates to use as radioligands for the detection and characterization of cannabinoid receptors. Synthesized $\Delta^9$-THC, however, was radiolabeled and this allowed for in vitro studies in numerous animal species. Unfortunately, radioligand binding studies using $[^3H]\Delta^9$-THC led to the conclusion that this compound only bound to hydrophobic regions of cell membranes and that this
binding was non-specific. This led investigators to believe that Δ⁹-THC acts via non-specific interactions (Roth and Williams, 1979). In addition, the high lipid solubility of Δ⁹-THC and its high octanol:water partition coefficient also suggested that this compound interacts with hydrophobic regions in the cell membrane. These non-specific chemical characteristics are common to many anaesthetics and led these investigators to believe that Δ⁹-THC acts similar to anaesthetics. However, the apparent inability of Δ⁹-THC to produce complete surgical anaesthesia in animals has led to the conclusion that this compound is only a partial anaesthetic (Lawrence and Gill, 1975).

In trying to observe specific cannabinoid binding to cell membranes [³H]Δ⁹-THC, which had higher specific activity (70 Ci/mmol) and was more stable than [³H]Δ⁸-THC, was used. However, this compound was displaced by only about 55% by cold Δ⁸-THC in hepatoma cells. Furthermore, the IC₅₀ of Δ⁸-THC in displacing [³H]Δ⁸-THC was high, approximately 1 μM. In rat brain homogenate it was shown that the binding of [³H]Δ⁸-THC was dependent on protein concentration and temperature, but was not saturable (Harris et al., 1978).

In micromolar concentrations the synthetic cannabinoid ligand, (-)-5-((1,1-dimethylheptyl)-2-[5-hydroxy-2-(3-hydroxypropyl)cyclohexyl]-phenol (CP55,940) stimulated the release of arachidonic acid (Felder et al., 1992). Another synthetic cannabinoid, (-)-11-OH-Δ⁴-tetrahydrocannabinol-dimethylheptyl (HU-210), and its less active enantiomer, HU-211, increased intracellular calcium in CHO cells transfected with the rat cannabinoid CB₁ receptor, as well as in the non-transfected wild-type CHO cells (Felder
et al., 1992). From these data it is evident that cannabinoids do interact with cell membranes. It is possible, therefore, that at least one mechanism of action is non-specific perturbation of these membranes.

1.1.5 The Discovery and Cloning of Cannabinoid Receptors

An early belief of the molecular mechanism of cannabinoid action was that these compounds acted through a central prostanoid mechanism (Milne and Johnson, 1981). Levonantradol, a synthetic analog of Δ⁹-THC, shows remarkable stereotopography to prostaglandin E₂ (PGE₂). This, in addition to the analgesic and antiemetic properties of cannabinoids, suggested that cannabinoids may exert some of their effects by interfering with prostaglandin-induced pain and emetic pathways (Milne and Johnson, 1981).

Levonantradol and Δ⁹-THC blocked abstinence signs in morphine-dependent dogs (Gilbert, 1981) while Δ⁹-THC and other cannabinoids have been shown to block these signs in mice (Bhargava, 1976) and rats (Hine et al., 1975). However, the effects of these compounds were not sensitive to general opioid receptor antagonists and, therefore, do not act through opioid receptors. Alternatively, it had been proposed that opioids, which also produce analgesia, act through stereospecific inhibition of prostaglandin-stimulated adenylyl cyclase. In fact, Collier and Roy (1974) showed that morphine, at concentrations obtainable in vivo, inhibited prostaglandin-induced cyclic adenosine monophosphate (cAMP) formation in rat brain homogenate. Since levonantradol has a morphine-like analgesic profile in animals, but no activity at the opiate receptor, it stood to reason that direct inhibition of this
prostaglandin-induced accumulation of cAMP could be one mechanism of action of cannabinoids (Milne and Johnson, 1981).

To test the hypothesis that cannabinoids inhibited prostaglandin-induced accumulation of cAMP, the effects of Δ⁹-THC and levonantradol on prostanoid-stimulated adenylyl cyclase activity in N18TG2 neuroblastoma cells were investigated (Howlett, 1984). It was shown that these compounds decreased cAMP accumulation in response to prostacyclin in both intact cells as well as membranes prepared from these cells. Interestingly, this inhibition was not competitive with prostaglandin E₁ or prostacyclin, implying a separate, non-prostanoid mechanism of action. Further, secretin and vasoactive intestinal peptide stimulation of adenylyl cyclase was also inhibited by cannabinoid and nantradol-related compounds signifying that these drugs act through a mechanism independent of prostanoids (Howlett and Fleming, 1984). The hypothesis was then tested that these compounds were acting either through α-adrenergic, or muscarinic cholinergic receptors, which inhibit adenylyl cyclase activity. However, it was shown that neither yohimbine, an adrenergic inhibitor, nor atropine, a muscarinic inhibitor, was able to block this inhibition by cannabinoids (Howlett and Fleming, 1984).

Even though cannabinoids had not been shown to act through existing receptors the possibility still remained that these drugs were acting through a distinct, yet unidentified, receptor. The observation that psychoactive cannabinoid drugs and their nantradol analogs could inhibit adenylyl cyclase activity in N18TG2 cells and in the neuroblastoma-glioma hybrid cells, NG108-15, but not in rat sperm, C6 glioma or S49 lymphoma cells provided
further evidence that these compounds act through a specific cannabinoid receptor (Howlett et al., 1986).

It was also shown that cannabinoid inhibition of adenylyl cyclase in N18TG2 and NG108-15 cells is pertussis toxin-sensitive (Howlett et al., 1986). This means that receptor-mediated inhibition of adenylyl cyclase requires the presence of either a $G_1$ or $G_2$ guanine nucleotide-binding protein ($G$ protein) complex. $G$ proteins are heterotrimeric proteins which associate with the plasma membrane and couple numerous receptors with downstream effector (second messenger) systems (Birnbaumer et al., 1970). The $G$ protein heterotrimer consists of an $\alpha$, $\beta$- and $\gamma$- subunit. The $\alpha$ subunit contains a single high-affinity binding site for guanine nucleotides (Casey and Gilman, 1988). When the receptor is inactive, guanine diphosphate (GDP) is bound to the $\alpha$-subunit. Upon receptor stimulation, either by ligand binding, or from a constitutively active receptor, GDP dissociates and is replaced by guanine triphosphate (GTP). Though there is some debate, it is believed that the nucleotide-bound (GDP or GTP) form of the receptor-$G$ protein complex represents the low-affinity state of the receptor, whereas the unbound state represents the high-affinity state (Helmreich and Hofmann, 1996). In the GTP-bound state, the heterotrimer dissociates from the receptor. The $\alpha$-subunit then dissociates from the $\beta\gamma$-subunits. Both the $\alpha$- and the $\beta\gamma$-subunits can mediate downstream effector systems (Hamm, 1998). The $\alpha$-subunit has intrinsic GTPase activity so the effects, unless the $\alpha$-subunit protein is mutated, are self-limiting. The $\alpha$-subunit family consists of $\alpha_x$, $\alpha_{i/o}$, $\alpha_{q}$, $\alpha$, and $\alpha_{12}$ (Hamm, 1998). The involvement of either $G_{ia}$, or $G_{oa}$ in a cellular process can be identified based on their sensitivity to inhibition by pertussis toxin.
whereas $G_{ia}$ proteins are sensitive to cholera toxin. Pertussis toxin (islet-activating protein) ribosylates both $G_{ia}$ and $G_{oa}$ which impairs the ability of the G proteins to interact with the receptor. ADP-ribosylation by cholera toxin irreversibly activates $G_{ia}$ (Casey and Gilman, 1988).

In order to support the hypothesis that a given drug acts through a receptor, a number of criteria have to be met (Pertwee, 1993). These criteria state that: a clear relationship should exist between chemical structure and pharmacological activity; enantiomeric compounds should show stereoselectivity; the potencies of these compounds should be in the same concentration range as other types of drugs which act at receptors and significantly higher than those drugs which act through non-receptor-mediated effects; tissues in which these drugs act should contain the receptors, the necessary signal transduction mechanisms and the genetic material capable of expressing these receptors; and antagonists should be able to inhibit these drugs from interacting with the receptor and reverse any effects elicited by this interaction.

The first criterion has been addressed by Razdan (1986). He demonstrated that slight alterations in the chemical structure of cannabinoids can greatly affect the activity of these compounds. It appears that cannabinoids require a benzopyran ring for activity, since compounds such as cannabidiol, which is a ring-opened compound (Figure 1.1), have little activity in animals and no activity in humans. In addition, changing the position of the double bond may strongly affect the potency of these drugs in some species.
In addressing the second criterion, it has been shown that, for the most part, the (-)-enantiomers of cannabinoids are considerably more potent than their corresponding (+)-enantiomers \textit{in vivo} in both man (Hollister et al., 1987) and animals (Pertwee, 1993) as well as \textit{in vitro} (Pertwee, 1993; Herkenham et al., 1990; Devane et al., 1988). The fact that cannabinoids produce maximal effects at µg/kg doses in man and µmol/kg doses in animals is consistent with the properties of other drugs which act through specific receptors such as benzodiazepines and opioids. In contrast, ethanol, which is believed to act through non-receptor-mediated effects, is needed in gram amounts to exert pharmacologic effects (Pertwee, 1993).

The development of the highly potent, radiolabeled synthetic cannabinoid analog, $[^3]$HCP55,940, facilitated the identification of a specific cannabinoid receptor. Saturation binding isotherms showed that $[^3]$HCP55,940 binding was saturable and binding occurred to a single class of sites in rat cortical P2 membranes with a $K_d$ of 133 pM and a $B_{max}$ of 1.9 pmol/mg protein (Devane et al., 1988). Specific $[^3]$HCP55,940 binding was rapid, reversible
and displaced by numerous other cannabinoid compounds. Specific \(^{3}H\)CP55,940 binding was decreased by the addition of the nonhydrolyzable GTP analog guanylylimidodiphosphate as well as by the omission of Mg\(^{2+}\) from the incubation buffer suggesting that the putative receptor couples to G proteins (Devane et al., 1988). These data supported the existence of a high affinity, pharmacologically distinct cannabinoid receptor in rat brain tissue.

Further pharmacologic evidence for the existence of cannabinoid receptors was shown by Herkenham et al. (1990). They demonstrated that various synthetic and natural cannabinoids inhibited \(^{3}H\)CP55,940 binding in whole rat brain "sausage" sections (brain homogenate placed into a tube, frozen and cryostat-cut to make sections of uniform composition and size) and the ability of these compounds to inhibit \(^{3}H\)CP55,940 binding correlated with their ability to produce behavioral and pharmacological effects in animals and humans. These studies showed that \(^{3}H\)CP55,940 binding was saturable and that competitive inhibition curves using various cannabinoids were best fit by a single-site kinetic model. This suggests that the receptor defined by the specific binding of \(^{3}H\)CP55,940 is the same receptor which mediates these effects, including the subjective "high" described by humans.

The idea that the specific binding sites labeled by \(^{3}H\)CP55,940 represent a distinct, novel receptor mediating the action of cannabinoids is supported by the fact that numerous pharmacological agents which bind to other known receptor types failed to displace specific \(^{3}H\)CP55,940 binding (Bidaut-Russell et al., 1990). These compounds include adrenergics,
dopaminergics, serotonergics, muscarinics, opioids, sigmaoids, hormones, eicosanoids and peptides. Further evidence that the specific $[^3H]$CP55,940 binding sites observed were functional cannabinoid receptors was that the highest concentration of these sites occurred in those brain areas in which psychotropic cannabinoids are thought to produce many of their characteristic effects (Pertwee, 1993). In the rat, the largest number of $[^3H]$CP55,940 binding sites were located in the cerebral cortex, cerebellum, hippocampus and corpus striatum with smaller, but significant, binding in the hypothalamus, brainstem and spinal cord (Bidaut-Russell et al., 1990). Similar results were seen using $[^3H]$SR141716A (Breivogel et al., 1997). Cannabinoid receptors in the cerebral cortex and hippocampus likely account for cannabinoid effects on perception, cognition, learning and short-term memory while those in the basal ganglia and cerebellum are likely responsible for motor function and movement, respectively (Herkenham et al., 1990). This idea is further supported by the fact that dogs administered cannabinoids present with static ataxia whereas humans, who have a lower density of cannabinoid receptors in the basal ganglia, show much less motor depression (Razdan, 1986; Herkenham et al., 1990). Receptors in the hypothalamus may be responsible for alterations in body temperature whereas those in the brainstem (periaqueductal grey) and spinal cord may be involved in the antinociception properties of cannabinoids (Lichtman and Martin, 1991).

In 1990, Matsuda et al. attempted to clone novel receptors from a rat cerebral cortex cDNA library using an oligonucleotide probe derived from the sequence of the bovine substance-K receptor (Matsuda et al., 1990). In the process, they isolated a cDNA (SKR6)
which coded for a 473-amino-acid protein having seven hydrophobic domains and several potential glycosylation sites. This receptor also had numerous amino acids which are highly conserved among the G protein-coupled family of receptors and these investigators, therefore, realized this newly discovered receptor was a member of that family. When this cDNA was transfected into either CHO cells or *Xenopus* oocytes, numerous non-cannabinoid compounds failed to interact with the expressed receptor either in radioligand binding or in functional assays. The belief that cannabinoid-mediated adenylyl cyclase inhibition in N18TG2 cells occurred through a pharmacologically specific receptor (Howlett and Fleming, 1984), along with Northern-blot analysis revealing that SKR6 mRNA was also evident in N18TG2 as well as in NG108-15 cells (Devane et al., 1986; Matsuda et al., 1990), led Matsuda et al. to investigate cannabinoid drugs as ligands for this receptor. Furthermore, this group found high levels of SKR6 mRNA in the hippocampus and cerebral cortex of rat brain which was in agreement with Herkenham et al. (1990) and Bidaut-Russell et al. (1990) where *in situ* hybridization using [*H]CP55,940 identified specific cannabinoid binding sites in this tissue. In CHO-K1 cells stably transfected with SKR6 cDNA, Δ⁹-THC and CP55,940 dose-dependently inhibited forskolin-stimulated cAMP accumulation. This response was stereoselective whereas the (+) enantiomers of Δ⁹-THC and CP55,940 were 50- and 100-fold less potent, respectively, in this assay (Matsuda et al., 1990). The inhibition of cAMP accumulation by (-)-Δ⁹-THC and CP55,940 was abolished by pretreatment of the cells with pertussis toxin, showing that these receptors are coupled to G₁₅ proteins.

The following year, using degenerate nucleotide primers corresponding to conserved
sequences of known G protein-coupled receptors, Gerard et al. (1991) cloned a cDNA encoding a G protein-coupled receptor from a human brainstem cDNA library (Figure 1.1). The deduced amino acid sequence encodes a protein of 472 residues which is 97.3% identical with the rat receptor cloned by Matsuda et al. (1990). The intracellular loops, which are believed to interact with G proteins, are also conserved among these proteins. Also, sequences and transmembrane segments, which are implicated in the ligand binding properties of various G protein-coupled receptors, are identical. For example, the identical first half of the amino-terminus (the first 58 amino acids) and the 3 possible glycosylation sites at the N-terminal extracellular domain in both species are implicated in ligand binding. The greatest differences were observed in the N-terminal extracellular and the C-terminal intracellular domains (Figure 1.2). Regardless, the high conservation of the cannabinoid receptor between the two species implicates a crucial role in the nervous system (Gerard et al., 1991).
Though the majority of the transcripts of this human cannabinoid receptor were found in the brain, lower amounts were also found in the testis. Specific $[^{3}H]CP55,940$ binding was observed in membranes of COS-7 cells transiently transfected with the cDNA. In CHO-K1 cells stably expressing this receptor, various cannabinoids, including CP55,940 and $\Delta^{9}$-THC, dose-dependently inhibited forskolin-stimulated cAMP formation. This inhibition was stereoselective (Gerard et al., 1991).

In 1993, Munro et al. attempted to identify novel G protein-coupled receptors expressed in myeloid cells (Munro et al., 1993). They used the polymerase chain reaction
(PCR) along with degenerate primers to screen a cDNA library prepared from a human promyelocytic leukemia HL60 cell line. Amplification products from cells treated with dimethylformamide (DMF), to induce granulocyte differentiation, were cloned and sequenced. Of the six classes of clones showing homology to the G protein-coupled receptor family, only one, CX5, showed particular homology to a published receptor, the rat brain cannabinoid receptor. When the CX5 insert was used to screen the HL60 library, one clone (CX5.36) was isolated showing 44% overall amino acid identity to the human cannabinoid CB₁ receptor with 68% identity for those transmembrane residues proposed to confer ligand specificity. This receptor is called CB₂ (Figure 1.3).
When membranes from COS cells expressing this receptor were prepared, saturable binding of both $[^3H]WIN\ 55,212-2$ and $[^3H]CP55,940$ was seen with affinities similar to those reported for the brain receptor. The mRNA for this "peripheral" cannabinoid receptor, $\text{CB}_2$, was also found in macrophages/monocytes in the marginal zones of rat (Munro et al., 1993) and mouse (Das et al., 1995) spleen. $\text{CB}_2$ receptor mRNA was also found in human B-lymphocytes, natural killer cells, monocytes, polymorphonuclear cells, T8 cells (suppressor T-cells) and T4 cells (helper T-cells) at 10 - 100-fold higher levels than $\text{CB}_1$ mRNA (Galiegue et al., 1995). Human pancreas also showed low levels of $\text{CB}_2$ mRNA.
expression (Galiegue et al., 1995).

Expression of CB₂ protein in human tonsil was demonstrated using immunohistological analysis with anti-human CB₂ IgG (Galiegue et al., 1995) and supported by the fact that the CB₂-selective antagonist, SR144528 (Rinaldi-Carmona et al., 1998), antagonized the stimulating action of CP55,940 on human tonsillar B-cell proliferation. The inability of the CB₁-selective antagonist, SR141716A, to displace [³H]CP55,940 from rat spleen membranes demonstrated that, in addition to CB₂ mRNA, CB₂ receptors are expressed in this tissue (Rinaldi-Carmona et al., 1994).

To better understand the pharmacology and signal transduction of the two cannabinoid receptors, Felder et al. (1995) produced stably transfected cell lines of the human cannabinoid CB₁ and CB₂ receptors. In competitive binding experiments using AtT-20 cells, Δ⁹-THC, CP55,940 and anandamide were all equipotent at both receptor types. WIN 55,212-2 and cannabinol bound with higher affinity to the CB₂ receptor while HU-210 had higher affinity for the CB₁ receptor. Except for the higher affinity of WIN 55,212-2 and cannabinol for the CB₂ receptor, all cannabinoid agonists tested displayed an identical rank order of potency for both receptor types. Similar results were observed for the functional inhibition of adenylyl cyclase activity in CB₂-transfected CHO cells.

The only major pharmacological or biochemical difference noted between these two receptor types is that, unlike the CB₁ receptor, the CB₂ receptor, when expressed in AtT-20 cells, did not inhibit Q-type calcium channels, or activate inward-rectifying potassium channels (Felder et al., 1995). The observation that CB₂ inhibited adenylyl cyclase, but did
not activate the potassium channels, is intriguing since both of these events are believed to
be mediated by G/Q. Furthermore, neither receptor type activated phospholipase A, C, or
D, or mobilized intracellular free Ca\(^{2+}\) (Felder et al., 1995). This latter result conflicts with
a recent report which shows that cannabinoids do increase intracellular free Ca\(^{2+}\) in NG108-
15 cells through a CB\(_1\)-dependent mechanism (Sugiura et al., 1999).

In an attempt to answer the questions arising from the apparent redundancy of the
CB\(_1\) and CB\(_2\) receptors, Shire et al. (1995) cloned the full-length human cannabinoid CB\(_1\)
receptor from a human lung cDNA library. In the process, they discovered the presence of
two introns in the CB\(_1\) gene. One of these occurred in the 5' untranslated region (UTR) while
the other occurred in the coding region of the receptor. They showed that the translation of
this coding region produced a splice variant of the human cannabinoid CB\(_1\) receptor. They
referred to this as the cannabinoid CB\(_{1A}\) receptor (Figure 1.4). This alternatively spliced
form translates into an NH\(_2\)-modified isoform of the CB\(_1\) receptor which is 61 amino acids
shorter and with the first 28 amino acids being completely different and more hydrophobic
than those of the CB\(_1\) receptor. Using PCR, the investigators also showed that CB\(_1\) and CB\(_{1A}\)
mRNAs are found in the majority of the central and peripheral tissues with CB\(_{1A}\) mRNA
present at approximately 20% of the CB\(_1\) mRNA levels. In most of the peripheral tissue,
CB\(_{1A}\) mRNA was consistently found at approximately 10% of the CB\(_1\) levels and only at
about 1% of CB\(_1\) in the kidney (Shire et al., 1995).
Figure 1.4 - Comparison of the amino acid (a.a.) sequences of the human cannabinoid CB₁ and CB₁A receptors. The amino acid sequence for the CB₁ receptor (top) for the CB₂ receptor (bottom) are shown to the right of the receptor. The amino terminus for each receptor sequence identifies the amino terminus of the receptor.

When Rinaldi-Carmona et al. (1996a) compared the potencies of cannabinoid ligands at the human CB₁ and CB₁A receptors expressed in CHO cells they discovered that the cannabinoid agonists, CP55,940, WIN 55,212-2 and Δ⁹-THC, bound to CB₁A with approximately 3-fold lower affinity than to CB₁. However, the EC₅₀'s of these agonists in inhibiting forskolin-stimulated adenylyl cyclase were similar at both receptors. This inhibition was blocked by pertussis toxin pretreatment in both CHO/CB₁ and CHO/CB₁A cell lines. The cannabinoid receptor antagonist, SR141716A, had approximately a 9-fold
lower affinity for CB₁A. SR141716A reversed the inhibition of forskolin-stimulated cAMP accumulation by CP55,940 in both CHO/CB₁ and CHO/CB₁A cell lines (Rinaldi-Carmona et al., 1996a).

The potential benefits of cannabinoid compounds in the treatment of a variety of diseases has long been known. However, one major limitation of the use of these compounds in medicine is that it has not been possible to separate the desired pharmacologic effects from the associated changes that impair cognitive function. Therefore, either new classes of compounds need to be developed which do not produce the associated cognitive impairment and other side-effects, or new cannabinoid receptor types and/or subtypes need to be discovered which do not produce these psychoactive effects. Unfortunately, no major differences in activity between CB₁ and CB₁A have been seen in the limited studies done (Rinaldi-Carmona et al., 1996a). Cloning and expressing these receptors, especially the human receptor, in artificial systems, such as mammalian cell lines, should aid in the development of more selective ligands which are devoid of undesired effects.

1.1.6 Classification of Cannabinoid Receptor Ligands

The discovery and cloning of cannabinoid receptors allowed for further classification of the cannabinoids pharmacologically. Cannabinoid receptor agonists are cannabinoids which mimic the effects of Δ⁹-THC. Currently, there are four classes of cannabinoid agonists (Figure 1.5). Naturally occurring compounds from the plant Cannabis sativa, including Δ⁹-THC, and their derivatives, including HU-210 (Mechoulam et al., 1987) and nabilone (Eli
Lilly and Company, Indianapolis, IN), are termed classical cannabinoids. Synthetic structural analogs of Δ⁹-THC, which include CP55,940 (Devane et al., 1988) and other compounds developed by Pfizer Central Research (Groton, CT; Johnson and Melvin, 1986), are considered non-classical cannabinoids. For both of these classes of cannabinoids, the (-)-enantiomers have greater binding affinity at cannabinoid receptors as well as greater pharmacologic activity. A third class of cannabinoid agonists, developed by the Sterling Research Group (Sterling Drug Inc., Rensselaer, NY), are the aminoalkylindoles, which includes R(+)-[2,3-dihydro-5-methyl-3-(morpholinyl) methyl] pyrrolo[1,2,3-de]-1,4-benzo- xazin-yl]-(1-naphthalenyl)methanone mesylate (WIN 55,212-2) (Compton et al., 1992). For this class, the (+)-enantiomers are more active. The final class of cannabinoids is the eicosanoids (animal-derived cannabinoids and their analogs). In 1992, Devane et al. isolated anandamide (N-arachidonylethanolamide) from porcine brain (Devane et al., 1992). This compound is an arachidonic acid derivative which was identified while screening for endogenous cannabinoid ligands. Anandamide was able to inhibit specific binding of radiolabeled cannabinoids to synaptosomal membranes in a competitive and dose-dependent manner. This compound was also able to inhibit the electrically evoked twitch response of the mouse vas deferens in a concentration-dependent manner, a response characteristic of psychotropic cannabinoids (Devane et al., 1992).

Based on the observation that N-acylethanolamine phosphate is a potent agonist at the lysophosphatidic acid receptor on human platelets, Sugiura et al. (1995) examined whether analogues of anandamide containing a glycerol backbone are active at the
cannabinoid receptor. They reported that, despite the fact that 2-arachidonoylglycerol (2-AG) is present in rat brain at levels about 1000 times higher than anandamide, it binds to the cannabinoid receptor in rat brain synaptosomes with much lower affinity than anandamide (48 μM vs 89 nM for anandamide; Sugiura et al., 1995). Though the affinity of 2-AG was still lower than anandamide, the affinities of both were markedly increased (2.4 μM vs 100 nM for anandamide) in the presence of the general serine esterase inhibitor, diisopropyl fluorophosphate (DFP; Sugiura et al., 1995). See Figure 1.5.
**Figure 1.5** - *Structures of various classes of cannabinoid ligands.* Structures of classical cannabinoids ($\Delta^9$-THC, HU-210, nabilone), non-classical cannabinoids (CP55,940), aminoalkylindoles (WIN 55,212-2) and eicosanoids (anandamide, 2-arachidonoylglycerol).
Later studies showed that 2-AG can induce a rapid transient elevation of intracellular free Ca^{2+} in NG108-15 cells with an EC_{50} value of 150 nM (Sugiura et al., 1996). In this study, anandamide was a partial agonist in its ability to increase intracellular free Ca^{2+} compared to 2-AG. The most recent report from Sugiura et al. (1999) showed that 2-AG produced a larger maximal response than either CP55,940 or HU-210. Therefore, they concluded that 2-AG, and not anandamide, is the endogenous ligand for the cannabinoid CB_{1} receptor.

By 1994, cannabinoids had been isolated from *Cannabis sativa* (Adams et al., 1940b), various synthetic cannabinoid agonists developed (Devane et al., 1988; Compton et al., 1992) and endogenous cannabinoid ligands isolated (Devane et al., 1992; Sugiura et al., 1995). However, since cannabinoids were believed to act through non-receptor mechanisms (Seeman et al., 1972; Roth and Williams, 1979), a selective cannabinoid receptor antagonist needed to be developed in order to determine that these cannabinoid agonists were indeed acting through a distinct cannabinoid receptor. In addition, since two types of cannabinoid receptors had been identified (Matsuda et al., 1990; Munro et al., 1993), it was also necessary to develop an antagonist that was selective for one or the other cannabinoid receptor types. This would allow researchers to identify which of the known receptor types was responsible for the observed effects in experimental animals. Receptor antagonists are compounds which interact with specific receptors, but do not produce any change in receptor conformation. Therefore, these compounds do not produce any effect on effectors coupled to these receptors. Additionally, receptor antagonists block, or attenuate the effects of agonists at
these receptors. In general, a selective cannabinoid antagonist would provide a powerful tool for studying the \textit{in vivo} functions of the cannabinoid system.

In 1994, a potent and selective antagonist of the brain cannabinoid (CB\textsubscript{1}) receptor was developed (Rinaldi-Carmona et al., 1994). The compound, [N-(piperidin-1-yl)-5-(4-chlorophenyl)-1-(2,4-dichlorophenyl)-4-methyl-1H-pyrazole-3-carboxamide hydrochloride], SR141716A, is a novel, highly potent orally active antagonist which has 1000-fold higher affinity for the CB\textsubscript{1} receptor than for the CB\textsubscript{2} receptor (Figure 1.6). This compound displaced \textsuperscript{[3]H}CP55,940 from rat brain synaptosomal membranes in a concentration-dependent manner while having no affinity for various other receptors including those for histamine, dopamine, adrenergics, adenosine, opioids and serotonin (Rinaldi-Carmona et al., 1994). This compound also antagonized the inhibitory effects of cannabinoid receptor agonists on both mouse vas deferens contractions and forskolin-stimulated adenylyl cyclase activity in rat brain membranes (Rinaldi-Carmona et al., 1994). It also prevented the cannabinoid agonist-induced inhibition of acetylcholine release in the rat hippocampus (Gessa et al., 1997). Orally administered SR141716A fully inhibited \textit{ex vivo} \textsuperscript{[3]H}CP55,940 binding to rat cerebral membranes (Rinaldi-Carmona et al., 1995). The synthesis of the radiolabeled compound, \textsuperscript{[3]H]SR141716A, which demonstrated saturable and reversible binding in rat whole brain membranes, provides a useful research tool for the labeling of cannabinoid CB\textsubscript{1} receptors both \textit{in vitro} and \textit{in vivo} (Rinaldi-Carmona et al., 1996b).

Recently, a selective antagonist for the cannabinoid CB\textsubscript{2} receptor has been developed (Rinaldi-Carmona et al., 1998). This compound, N-[(1S)-endo-1,3,3-trimethyl bicyclo [2,2,1]
heptan-2-yl]-5-(4-chloro-3-methylphenyl)-1-(4-methylbenzyl)-pyrazole-3-carboxamide (SR144528), displays nanomolar affinity for both rat spleen and cloned human CB$_2$ receptors and has 700-fold lower affinity for both rat brain and cloned human CB$_1$ receptors (Figure 1.6). Since CB$_2$ receptors are largely isolated in cells involved in the immune response, this compound should provide a powerful tool in the investigation of the in vivo effects of the cannabinoid system on immune function.

Figure 1.6 - Structures of SR141716A (left) and SR144528 (right).
1.1.7 The Discovery of Inverse Cannabinoid Agonists

Until the early 1980's, compounds which interact with receptors were characterized as either agonists, if they produced a given response upon interaction with its associated receptor, or as antagonists, if they produced no effect at the receptor. However, in 1982, Braestrup et al. discovered that, whereas traditional benzodiazepine agonists acting at the benzodiazepine receptor had anticonvulsant activity in mice and rats, some compounds acting at this receptor produced opposite (convulsant) effects. This "inverse agonist" effect at the benzodiazepine ion channel was later well documented for the G protein-coupled δ-opioid receptor (Costa and Herz, 1989; Mullaney et al., 1996; Szekeres and Traynor, 1997). Whereas the δ-opioid agonist, DADLE, produced dose-dependent stimulation of basal GTPase activity at the cloned mouse δ-opioid receptor expressed in rat 1 fibroblast cells (Mullaney et al., 1996), and at the δ-opioid receptor expressed in NG108-15 cells (Costa and Herz, 1989), the opioid ligand, ICI 174864, inhibited basal GTPase activity in both cell lines in these studies. ICI 174864 also demonstrated inverse activity in both rat 1 fibroblast (Mullaney et al., 1996) and NG108-15 (Szekeres and Traynor, 1997) cells by inhibiting basal [35S]GTPγS binding in these membranes. Inverse agonism was also shown for the serotonin 5-HT1A receptor where spiperone decreased basal [35S]GTPγS binding in CHO cell membranes expressing this receptor (Newman-Tancredi et al., 1997).

Evidence for inverse cannabinoid agonists has also recently been demonstrated. In contrast to cannabinoid receptor agonists, SR141716A alone stimulated locomotor activity
(Compton et al., 1996) and evoked a significant thermal hyperalgesia in mice (Richardson et al., 1997). Terranova et al. (1996) have shown that, whereas cannabinoid agonists inhibited short term memory, SR141716A, when given alone, enhanced short-term olfactory memory in the social recognition test in rodents, and reduced memory deficit in aged rats. Navarro et al. (1997) reported that acute administration of SR141716A induced defensive responses in two anxiety tests, whereas cannabinoid agonists usually produce a placid relaxation. Bouaboula et al. (1997) reported that SR141716A, in contrast to cannabinoid agonists, inhibited mitogen-activated protein kinase activation stimulated by insulin or insulin-like growth factor in CHO cells transfected with the human cannabinoid CB₁ receptor. In addition, this compound also inhibited basal [³⁵S]GTPγS binding in membranes prepared from these cells as well as prevented the inhibition of adenylyl cyclase mediated by autoactivated CB₁ receptors in intact cells. Landsman et al. also showed that SR141716A (Landsman et al., 1997), as well as AM630 (Landsman et al., 1998) were inverse agonists at the human cannabinoid CB₁ receptor expressed in CHO cells. These compounds were shown to inhibit basal [³⁵S]GTPγS binding in membranes of these cells. Using a similar system, MacLennan et al. (1998) confirmed the previous findings of inverse agonism of SR141716A in the [³⁵S]GTPγS binding assay. Further studies need to be done to determine the full potential of inverse cannabinoid agonists for therapeutic use in man.
1.2 GENERAL HYPOTHESIS AND AIMS

It is the general hypothesis of this dissertation that the cloned human cannabinoid CB₁ receptor stably expressed in CHO cells provides a model system to predict the pharmacological effects of cannabinoids in man. The development of a reliable *in vitro* system to study the human cannabinoid CB₁ receptor will facilitate cannabinoid research and the data obtained will help to support the use of cannabinoids in medicine. The goal of producing a model system was accomplished by completing three specific aims. The first aim was to stably express the human cannabinoid CB₁ receptor in a Chinese hamster ovary (CHO) cell line. The second aim was to characterize this receptor by using radioligand binding and functional assays. The final specific aim was to begin to elucidate the molecular mechanisms involved in the actions of cannabinoids.

The first aim, as presented in chapter two of this dissertation, was to produce a mammalian cell line which stably expresses the human cannabinoid CB₁ receptor. The cDNA received was first sequenced to verify that it was that of the human cannabinoid CB₁ receptor. Once this was done, the cDNA was then transfected into a mammalian (CHO) cell line and cells were grown until isolated colonies appeared. These colonies were then tested to see if they expressed functional human cannabinoid CB₁ receptors.

Chapters three, four and five address specific aims two and three. Chapter three describes the procedure used to identify the clone to be chosen for the studies used in this dissertation. A clone (CHO/CB₁f) which showed high specific radiolabeled cannabinoid ([^3H]SR141716A) binding was chosen for the remaining studies. This clone was then further
characterized using other radioligand binding techniques. First, saturation binding studies using radiolabeled cannabinoid ligands were done to determine the total number of cannabinoid binding sites ($B_{max}$) as well as the dissociation constants ($K_d$) for these ligands. The affinities of these radioligands were compared to those reported for these compounds in the literature to add support that this system was appropriate. Competitive binding studies were also done in which various non-radiolabeled cannabinoid ligands competed for specific $[^3H]SR141716A$ binding in CHO/CB1 f cell membranes. This allowed for the determination of the inhibition constants of these non-radiolabeled cannabinoids. Again, comparison of these values to those in the literature further supported the relevance of this clone as a model for the study of the human cannabinoid CB1 receptor and cannabinoid ligands.

Chapter four discusses the functional studies performed in characterizing this clone. Two widely used assays were implemented. The $[^35S]GTP\gamma S$ binding assay measures the effect of various cannabinoid ligands at the first step of receptor activation. Ligands are classified based on their ability to mediate $[^35S]GTP\gamma S$ binding to the G proteins associated with the receptor. Further characterization of the CHO/CB1 f clone was done by observing the effects of a cannabinoid agonist after altering the conditions of the $[^35S]GTP\gamma S$ assay. The cAMP assay measures the ability of these ligands to mediate forskolin-stimulated cAMP formation, which involves the downstream effector, adenylyl cyclase (AC). Ligands are classified based on their ability to mediate $[^35S]GTP\gamma S$ binding and adenylyl cyclase activity and are generally classified as agonists, antagonists, or inverse agonists.

Chapter five was written based on data published in two peer-reviewed journals. The
effects of SR141716A, a compound initially thought to be an antagonist (Rinaldi-Carmona et al., 1994), and AM630, previously reported to be either an antagonist (Pertwee et al., 1995; K. Hosohata et al., 1997; Y. Hosohata et al., 1997), or an agonist (Pertwee et al., 1996), depending on the system, were investigated. The data presented in chapter five demonstrate the novel finding of inverse agonism at the human cannabinoid CB₁ receptor expressed in CHO cells. Explanations as to the possible mechanism of action as well as future experimental procedures to be used to determine this mechanism(s) are given.

Chapter six discusses the effects of Δ⁹-THC in both the [³⁵S]GTPγS binding assay and the cAMP assay. The data presented here demonstrate the novel finding that Δ⁹-THC is a competitive antagonist in the [³⁵S]GTPγS assay. In contrast, Δ⁹-THC is a partial agonist in its ability to inhibit forskolin-stimulated cAMP formation. Possible explanations for these differential effects are given.

The final chapter, seven, summarizes the objectives and findings of this dissertation and identifies questions which need to be answered in future studies. The findings reported in this dissertation, coupled with future studies using this and other cannabinoid systems, should give scientists a clearer understanding of the molecular and biochemical mechanisms of action of cannabinoids in hopes of one day widely using these compounds for medicinal purposes.
CHAPTER 2

Production of a Stable Mammalian Cell Line Expressing the Cloned Human Cannabinoid CB₁ Receptor

2.1 INTRODUCTION

The goal of cannabinoid pharmacology is to ultimately be able to use cannabinoid ligands therapeutically in animals and man. Not only do federal regulations make it difficult to perform \textit{in vivo} cannabinoid trials in man, but the effects of these drugs can vary greatly between subjects. An alternative means of studying the functional aspects of cannabinoids is to produce a cell line which stably expresses the receptor of interest. This method is reliable and more reproducible than using individual human subjects. The cloning of the human cannabinoid CB₁ receptor (Gerard et al., 1991) made it possible to develop a stable cell line expressing this receptor. The amino acid sequence and proposed conformation of the human cannabinoid CB₁ receptor is shown in figure 2.1. Chinese hamster ovary (CHO) cells were used to produce the stable clone utilized for the studies in this dissertation. The main reason for choosing this cell line was that these cells do not endogenously express cannabinoid receptors so any effects seen in the transfected cells not seen in wild-type CHO cells can be attributed to the expression of the CB₁ receptor. In addition, these cells are easy to transfect and maintain. Furthermore, this cell line has already been used to study the cloned rat cannabinoid CB₁ receptor (Matsuda et al., 1990) and the cloned human cannabinoid CB₁ receptor (Gerard et al., 1991; Rinaldi-Carmona et al., 1994; Rinaldi-Carmona et al., 1996a).
Figure 2.1 - Serpentine structure (top) and amino acid sequence (bottom) of the human cannabinoid CB₁ receptor. The above figure represents the secondary amino acid structure of the cloned human cannabinoid CB₁ receptor. The rectangle represents the plasma membrane and the shaded regions represent the transmembrane domains. The amino terminus (NH₂-) and carboxy terminus (COOH-) are labeled. The lower figure represents the primary amino acid sequence of the cloned human cannabinoid CB₁ receptor. The underlined amino acids represent the predicted transmembrane domains. The carboxy terminus (COOH) is labeled. The amino acid sequence is taken from Gerard et al. (1991).
2.2 MATERIALS AND METHODS

2.2.1 Transformation of Competent Cells

E. coli DH5α competent cells (50 µl; Gibco BRL, Life Technologies, Gaithersburg, MD) were placed into a microcentrifuge tube along with plasmid (pSVL; 50 ng; Figure 2.2) containing the human cannabinoid CB₁ cDNA insert (a gift from Dr. Marc Parmentier, Bruxelles, Belgium). The mixture was incubated on ice for 30 minutes and then heat shocked for 2 minutes in a 42°C water bath. The tube was placed back on ice for 2 minutes.

2.2.2 Plasmid Amplification

One ml of SOC (Sambrook et al., 1989) medium was added to the above tube and the mixture was incubated for 1 hour at 37°C while shaken at 150 rpm. SOC medium is made by dissolving bacto-tryptone (20 g), bacto-yeast extract (5 g), NaCl (0.5 g), KCl (10 ml of a 250 mM solution) in 1 L of distilled water, adjusting the pH to 7.0 with NaOH and autoclaving for 20 minutes. Just before use, MgCl₂ (5 ml of 2 M solution) is added and autoclaved an additional 20 minutes. After the solution has cooled, glucose (20 ml of a 1 M solution sterilized by filtration through a 0.22 micron filter) is added. The culture medium (100 µl or 900 µl) was then spread on separate LB Medium (Sambrook et al., 1989) plates containing 100 µg/ml ampicillin (Sigma Chemical Company, St Louis, MO). The plates were incubated in an inverted position at 37°C. The next day, well isolated colonies were removed from the plates using a sterile loop and incubated separately overnight in sterile culture tubes containing 5 ml of 2 x YT media (Gibco BRL, Life Technologies,
Gaithersburg, MD) containing 100 μg/ml ampicillin. Sterile filters and all above reagents except bacto-tryptone and bacto-yeast extract (DIFCO Laboratories, Detroit, MI) were supplied by Sigma (St. Louis, MO).

2.2.3 Isolation of Plasmid from Competent Cells

DNA was isolated and purified using the Wizard Miniprep DNA Purification System (Promega, Madison, WI). Culture tubes containing transformed competent E. coli DH5α cells (ampicillin-resistant) incubated as described in Sections 2.2.1 and 2.2.2 were centrifuged at 1520 × g for 5 minutes at 4°C. Cells were then resuspended in resuspension solution (200 μl; 50 mM Tris, 10 mM EDTA, 100 mg/ml RNAse A, pH 7.5) and transferred to separate microcentrifuge tubes. Cell lysis solution (200 μl; 0.2 M NaOH, 1% SDS) was added and the samples were gently mixed by inversion until the solution was clear. Neutralization solution (200 μl; 1.32 M KOAc, pH 4.8) was then added and samples were mixed by inversion. Samples were centrifuged at 14,000 rpm for 5 minutes and the supernatants were transferred to clean microcentrifuge tubes. DNA purification resin (1 ml) was added to each of the tubes and the samples were vortexed. The DNA/resin mixtures were pipetted into separate syringe barrels attached to minicolumns placed on a vacuum manifold. A vacuum was applied until the mixtures were pulled through the minicolumn. Column wash (2 ml; 200 mM NaCl, 20 mM Tris-HCl, 5 mM EDTA, 55% ethanol, pH 7.5) was then added to each syringe barrel while the vacuum was being applied. After the solutions were pulled through the minicolumns, the columns were allowed to dry under vacuum for 30 seconds.
Minicolumns containing DNA/resin were placed in clean microcentrifuge tubes and centrifuged at 14,000 rpm for 2 minutes. Sterile, distilled water (50 µl) was added to each minicolumn and after 1 minute the DNA was eluted into clean microcentrifuge tubes by centrifuging at 14,000 rpm for 20 seconds. Plasmid DNA was stored at -20°C until use.

2.2.4 Confirming the Sequence of the Human Cannabinoid CB₁ Receptor cDNA

The human cannabinoid CB₁ cDNA sample (5 µg) was brought to 50 µl using distilled water. A mixture (5 µl) of NaOH (2 M) and EDTA (2 mM) was added and the sample was mixed and incubated at 37°C for 30 minutes. Sodium acetate (NaOAc; 0.1 volume) and ethanol (2 volumes of 100% ethanol) were then added. The sample was then incubated at -70°C for 15 minutes and centrifuged at 14,000 for 15 minutes. The pellet was rinsed with ethanol (70%; 4°C) and centrifuged again for 5 minutes. The pellet was air dried and resuspended in distilled water (6 µl).

Sequencing was performed using the Sequenase 2.0 kit (Amersham, Cleveland, OH). A 15-base oligonucleotide primer (5'-CAT CAT CAT CCA CAC-3'; Integrated DNA Technologies, Inc., Coralville, IA) corresponding to bases 948-962 of the human cannabinoid CB₁ cDNA inserted into the pSVL vector was used. Sequenase buffer (2 µl) and primer (2 µl; 10 ng/ml) were added to the sample and the mixture was incubated for 2 minutes at 65°C. The sample was then allowed to slowly cool to 35°C in a water bath, briefly centrifuged and then chilled on ice.

The labeling mixture (dCTP, dGTP, dTTP; 7.5 µM each) was diluted 1:5 with
distilled water. Sequenase enzyme was diluted 1:8 with enzyme buffer. Dithiothreitol (DTT; 1 μl), labeling mixture (2 μl) and [35S] dATP (0.5 μl) were added to a clean microcentrifuge tube. Tubes containing 2.5 μl of one of the four dideoxynucleotides (ddA, ddC, ddG, ddT) were prepared ("ACGT" tubes) and incubated for 2 minutes at 37°C. Labeling reaction mixture (3.5 μl) and sequenase (2 μl) were added to the DNA samples on ice and the mixture was incubated at room temperature for 5 minutes. A portion of each sample (3.5 μl) was then transferred to the prewarmed "ACGT" tubes and incubated for 5 minutes. Stop solution (4 μl; 95% formamide, 20 mM EDTA, 0.05% bromophenol blue, 0.05% xylene cyanol FF) was then added and the samples were stored on ice.

To prepare the sequencing gel, two glass plates were washed twice with detergent and rinsed with tap water. The glass was then washed with distilled water and the gel contact sides were cleaned with ethanol (100%) and dried. One gel contact side was then siliconized (Sigmacote, Sigma, St Louis, MO). The two plates of glass were separated by spacers and taped to make a water-tight seal. The gel was prepared by first mixing urea (31.5 g; Fisher Scientific, Fair Lawn, NJ), 10x Tris Borate-EDTA (TBE; 9 ml; Boehringer Mannheim, Indianapolis, IN) and acrylamide gel solution (Long Ranger; 7.5 ml; J.T. Baker, Phillipsburg, NJ) and adjusting the volume to 75 ml with distilled water. Ammonium peroxydisulfate (375 μl of a 10% solution; Sigma, St. Louis, MO) and TEMED (37.5 μl; Sigma, St. Louis, MO) were then added and the mixture was immediately poured into the gap between the two plates of glass. Plastic combs were placed at the top portion of the gel to produce wells in which to load the sample. Large binder clips were placed around the perimeter of the plates
and the gel was allowed to cool (polymerize) for two hours.

Before loading the gel, the sequencing apparatus (Model S2, Bio-Rad Laboratories, Hercules, CA) was heated to approximately 50°C. The gel was then inserted into the sequencing apparatus. A 0.6x TBE buffer was prepared using distilled water and poured into the upper and lower portions of the sequencing apparatus. Using a syringe, both the top and bottom sections of the gel were then rinsed with the TBE buffer. After the reaction samples were warmed to 75°C for 2 minutes, 5 μl of each sample were then loaded into the gel. The gel was run at 60 watts and 1800 volts for 1.5 hours, or until the first line of dye (from the Stop solution) was gone. Another 4 μl of sample was then added and run until the second dye line from the first samples disappeared. The gel was then removed from the glass, placed on filter paper, covered with Saran wrap (Dow Chemical Company, El Paso, TX) and vacuum dried for 30 minutes. The Saran wrap was then removed and the gel was exposed to autoradiographic film (Hyperfilm MP, Amersham, Cleveland, OH) at room temperature for 2-3 days.

2.2.5 Transfer of the Human Cannabinoid CB, Receptor cDNA into a Stable Expression Vector

The human cannabinoid CB, receptor cDNA in the pSVL vector (10 μg) was cut with Xba I (10 units) and Bam HI (10 units) in buffer containing 50 mM Tris-HCl, 10 mM MgCl₂ and 50 mM NaCl, pH 8.0, brought to a final volume of 20 μl with distilled water and incubated for 2 hours at 37°C. The cDNA was then inserted into the corresponding site of the
shuttle vector, pGEM-3Z(-) (Promega, Madison, WI), which was digested by the same method (Figure 2.2). The CB, cDNA and pGEM-3Z(-) were ligated by incubating this mixture overnight at 4°C with T4 DNA ligase (1 unit) in buffer containing 50 mM Tris-HCl, 10 mM MgCl₂, 1 mM ATP and 1 mM DTT in 25% (w/v) polyethylene glycol-8000, pH 7.6 and brought to a final volume of 40 µl with distilled water. The cDNA was then cut with Sal I (10 units) and Bam HI (10 units) by incubating the mixture in buffer containing 50 mM Tris-HCl, 6 mM MgCl₂, 50 mM NaCl and 50 mM KCl, pH 7.4 for 2 hours at 37°C. The mixture was brought to a final volume of 20 µl by using distilled water. The cDNA was then inserted into the Sal I - Bam HI site of the pHβAPr-1-neo vector (LK444, a gift from Dr. L. Kedes, Stanford University) which was digested by the same method. The CB, cDNA and LK444 were ligated as described for pGEM-3Z(-) (Figure 2.2). All enzymes and buffers were obtained from Gibco BRL (Gaithersburg, MD). Amplification and isolation of plasmid were performed as described in Sections 2.2.1 - 2.2.3.
Figure 2.2 - Production of a stable LK444/CB₁ expression vector. The human cannabinoid CB₁ receptor cDNA insert in the pSVL vector was cut with the enzymes Xba I and Bam HI and placed into the corresponding site in the pGEM-3Z(-) vector. The CB₁ cDNA was then excised using Sal I and Bam HI and placed into the corresponding site of the LK444 vector to produce the LK444/CB₁ mammalian expression vector. Arrows represent the direction of the transcription of the CB₁ insert.

2.2.6 Transfection of the CB₁ Receptor cDNA into a Mammalian Cell Line

DNA (5 µg; LK444/CB₁) was diluted to 0.1 µg/µl in 20 mM HEPES buffer (sterile, cell culture grade). Separately, the cationic lipid, DOTAP (30 µl; N-[1-(2,3-Dioleoyloxy)propyl]-N,N,N-trimethylammonium methylsulfate, Boehringer Mannheim, Indianapolis, IN) was mixed with HEPES buffer (70 µl). The DNA solution was added to
the DOTAP solution and gently mixed. The transfection mixture was incubated for 15 minutes at room temperature then added, along with fresh medium (6 ml), to the CHO cells. The medium used for the CHO cells was Ham's F12 medium containing fetal bovine serum (10%), penicillin (100 U/ml) and streptomycin (100 µg/ml). Cells were incubated for approximately 6 hours and the medium was then replaced with fresh medium (6 ml). Cells were then grown for an additional 48 hours. All cell incubations were done at 37°C in a humidified environment containing 5% CO₂ and 95% air.

After the 48 hour incubation, the medium was aspirated and the cells were rinsed with 3 ml of PD (Ca²⁺- and Mg²⁺-free phosphate-buffered saline). Trypsin (2 ml; 0.25%, 1x Versene in PD) was added to the cells and the excess was aspirated. After 30 seconds, the cells were dislodged by striking the flask. CHO medium (10 ml) containing G418 (500 µg/ml; geneticin; selection medium) was then added. The cell suspension was transferred equally to two 100 mm plates. The flask was washed with an additional 10 ml of medium and also transferred equally to the plates. Cells were incubated at 37°C in a humidified environment containing 5% CO₂ and 95% air for 10-14 days until G418-resistant colonies were seen (Figures 2.3 and 2.4).
Add:

\[ 5 \mu g \text{DNA} + 30 \mu l \text{DOTAP/70} \mu l \text{HEPES} \]

incubate mixture for 15 minutes

transfer mixture to a 25 cm² culture flask containing CHO cells and 6 ml Ham's F12 medium

- Incubate for 6 hrs
- Replace with 6 ml fresh medium containing G418
- Incubate cells for 48 hrs at 37°C
- Detach cells with trypsin/ED
- Split suspension into 2 Petri dishes
- Wash flask and transfer medium
- Wait until colonies develop
- Isolate and expand single colonies for radioligand binding and functional assays

Figure 2.3 - Lipid-mediated transfection of the human cannabinoid CB₁ receptor cDNA into CHO cells. See text (Section 2.2.6) for details.

Figure 2.4 - Mechanism of lipid-mediated transfection. The positive charge of the lipid (DOTAP) molecule interacts with the negative charge of the CB₁ cDNA. The remaining positive charge of DOTAP interacts with the CHO cell membrane, allowing for the uptake of the DOTAP/CB₁ receptor cDNA complex into the cell.
2.3 RESULTS

2.3.1 Verification of the Human Cannabinoid CB₁ Receptor cDNA Sequence

Dideoxynucleotide sequencing of the human cannabinoid CB₁ receptor cDNA in the pSVL vector confirmed that the cDNA received was a 1508 base pair fragment of the human cannabinoid CB₁ receptor. This contains the 1416 bases of the CB₁ cDNA as well as upstream and downstream bases. The upstream region includes a Sac I restriction site. The downstream region includes a Bam HI restriction site. The sequence is read from bottom (NH₂-terminus) to top (COOH-terminus). A segment starting at base # 1059 (C; -NH₂) and ending at base # 1097 (A; -COOH) is shown in figure 2.5. This sequence, which corresponds to the one published by Gerard et al. (1991) is as follows:

5' - CATCTGCTGGGCCCCTGCTTGCAATCATGGTGATG -3'
Figure 2.5 - Verification of the human cannabinoid CB₁ receptor cDNA sequence. Four CB₁ DNA samples were run simultaneously on the gel. The sequence shown represents a portion of the amino terminus. Sequence is read as thymidine (T; left), guanine (G), cytosine (C) and adenine (A; right). See Section 2.3.1 for details.
2.3.2 Production of a Suitable Expression Vector Containing the Human Cannabinoid CB₁ Receptor cDNA

The human cannabinoid CB₁ receptor cDNA was isolated from the pSVL vector by cutting with Xba I and Bam HI. This Xba I - Bam HI fragment included the CB₁ receptor cDNA as well as an upstream and downstream region of the CB₁ receptor and part of the polycloning region of the pSVL vector. The cDNA was then inserted into the corresponding site of the shuttle vector pGEM-3Z(-) (Promega, Madison, WI). The cDNA was then inserted into the Sal I - Bam HI site of the LK444 for stable expression in CHO cells. Figure 2.2 shows a cartoon of this procedure. Figure 2.6 shows the agarose gels which were used to isolate the appropriate DNA fragments in order to prepare pGEM-3Z(-)/CB₁ and LK444/CB₁. A restriction map of pSVL/CB₁ is shown in figure 2.7.
Figure 2.6 - Production of a suitable expression vector containing the human cannabinoid CB₁ receptor cDNA. The human cannabinoid CB₁ receptor cDNA in the pSVL vector (10 μg) was cut with Xba I (10 units) and Bam HI (10 units) in buffer containing 50 mM Tris-HCl, 10 mM MgCl₂ and 50 mM NaCl, pH 8.0, brought to a final volume of 20 μl with distilled water and incubated for 2 hours at 37°C. The cDNA was then inserted into the corresponding site of the shuttle vector pGEM-3Z(-) which was digested by the same method. The CB₁ cDNA and pGEM-3Z(-) were ligated by incubating this mixture overnight at 4°C with T4 DNA ligase (1 unit) in buffer containing 50 mM Tris-HCl, 1 mM ATP and 1 mM DTT in 25% (w/v) polyethylene glycol-8000, pH 7.6 brought to a final volume of 40 μl with distilled water. The cDNA was then cut with Sal I (10 units) and Bam HI (10 units) by incubating the mixture in buffer containing 50 mM Tris-HCl, 6 mM MgCl₂, 1 mM ATP and 1 mM DTT in 25% (w/v) polyethylene glycol-8000, pH 7.4 for 2 hours at 37°C. The mixture was brought to a final volume of 20 μl by using distilled water. The cDNA was then inserted into the Sal I - Bam HI site of the pHßAPr-1-neo vector (LK444) which was digested by the same method. The CB₁ cDNA and LK444 were ligated as described for pGEM-3Z(-). DNA standards are shown on the left of each gel.

Figure 2.7 - Restriction map of the pSVL/CB₁ vector. pSVL/CB₁ cut with Sac I/Eco RI (left) produced expected bands of 2.8 kb, 2.0 kb and 1.5 kb. Bam HI/Xba I (center) produced the expected 4.9 kb and 1.55 kb bands. Xba I/Apa I (right) produced the expected 5.25 kb and 1.1 kb bands. The DNA standard is shown at the far left of the gel.
2.4 DISCUSSION

Though cannabinoids appear to be beneficial in the treatment of numerous diseases in man and animals, the inability to separate the desired effects from the undesired psychoactive effects limits their use. The current, yet limited, use of Δ⁹-THC in man and the potential widespread benefits of Δ⁹-THC and other cannabinoids make it important to more fully understand the molecular mechanisms of their actions. Though only one type of cannabinoid receptor (CB₁) and its splice variant (CB₁A) have been discovered in the human brain, the possibility remains that others may have yet to be discovered. One method employed to be certain that only the receptor of interest is being studied is to transfect the cDNA encoding the receptor of interest into a cell line which does not endogenously express this receptor. This technique was applied to the study of the human cannabinoid CB₁ receptor in this research by stably expressing the CB₁ cDNA in CHO cells.

The main advantage of a stable cell line compared to a transiently transfected cell line is that the results obtained from repeated studies are more consistent and, therefore, easier to interpret. In addition, these results can be more easily compared to those in which assay conditions are altered. This is due to the constant number of receptors per cell in the stably transfected cell line as opposed to a cell line which has been transiently transfected. In addition, transient transfections must be performed before each experiment. This could lead to a wide variation in the number of receptors expressed per cell as well as among experiments. For example, using a stably transfected cell line, not only will the effects of ligand "A" be more consistent among experiments, but can be more easily compared to the
effects of ligand "B", or to those results in which ligand "A" has been investigated using various assay conditions.

The human cannabinoid CB₁ receptor cDNA which was obtained in the pSVL vector was excised and placed into a shuttle vector, pGEM-3Z(-), and finally into LK444, a vector suitable for stable expression in mammalian (CHO) cells. The presence of G418 (500 µg/ml) in the medium ensured that the surviving cells contained the LK444 vector with the G418-resistant gene. Since it is possible that the LK444 vector introduced into the cells does not contain the CB₁ receptor cDNA insert, the presence of G418-resistant clones does not guarantee that the cells contain the CB₁ receptor cDNA. Even if the cells incorporated a LK444 vector containing the CB₁ insert, it does not ensure that the CHO cells will express this receptor. For this reason, it was necessary to perform radioligand binding studies with a radiolabeled cannabinoid ligand. This issue is addressed in Chapter 3.
CHAPTER 3

Characterization of the Cloned Human Cannabinoid CB₁ Receptor Using Radioligand Binding Assays

3.1 INTRODUCTION

The idea that specific cannabinoid binding sites existed was first demonstrated in the murine C1300 spontaneous peripheral neuroblastoma, N18TG2, cell line in which cannabinoids inhibited forskolin-stimulated adenylyl cyclase (Howlett, 1984). Radioligand binding techniques first helped to identify specific binding sites for cannabinoid ligands in rat brain (Devane et al., 1988). Specific [³H]CP55,940 binding determined the maximal level of binding sites (Bₘᵢₓ) in rat cortical P₂ membranes to be 1.85 pmoles of binding sites/mg of protein. The Kₐ value (dissociation constant) of [³H]CP55,940 in these membranes was 133 pM. However, it was not until 1990 when Matsuda et al. cloned and expressed the first cannabinoid receptor, the rat CB₁ receptor (Matsuda et al., 1990). The cloning of the first human cannabinoid CB₁ receptor occurred the following year (Gerard et al., 1991). Transient expression of this receptor in African Green Monkey Kidney (COS-7) cells produced a clone which had a maximum number of specific [³H]CP55,940 binding sites of approximately 200 fmol/mg of protein and a Kₐ value of approximately 1 nM. Stable expression of the human cannabinoid CB₁ receptor in CHO cells produced maximum specific [³H]CP55,940 binding of 2.43 pmol/mg of protein and a Kₐ value of 1.39 nM (Rinaldi-Carmona et al., 1996a). A stable cell line expressing the human cannabinoid CB₁ receptor needed to be developed for this dissertation in order to further understand the mechanisms of action of cannabinoids at
this receptor. CHO cells were chosen since they do not endogenously express any known cannabinoid receptors. Before functional tests were performed, it was important to determine the maximum number of binding sites as well as the affinity of various cannabinoid ligands at the CHO/CB₁ clone. Therefore, both the cannabinoid receptor agonist, [³H]CP55,940, and the cannabinoid receptor antagonist, [³H]SR141716A, were used to initially characterize this system.

3.2 MATERIALS AND METHODS

3.2.1 General Methods

3.2.1.a Drugs

[³H]SR141716A (52.0 Ci/mmol) was purchased from Amersham Life Sciences (Arlington Heights, IL). [³H]CP55,940 (104 Ci/mmol, or 165 Ci/mmol) was obtained from DuPont NEN (Boston, MA). WIN 55,212-2 and Δ⁹-THC were purchased from Research Biochemicals International (Natick, MA). SR141716A was a gift from Gerard Le Fur (Sanofi Recherche, Montpellier, France). AM630 was synthesized in Professor Alexandros Makriyannis’ laboratory (University of Connecticut, Storrs, CT). All non-radiolabeled cannabinoid drugs, except Δ⁹-THC were initially dissolved in 100% ethanol to produce 2.0 mM stock solutions. Δ⁹-THC was initially dissolved in 100% ethanol to produce a 1 mM stock solution. Further dilutions of all drugs were done using the appropriate assay buffer. The assay buffer for tissue linearity and saturation binding studies was 50 mM Tris-HCl, 5.0 mM MgCl₂, 1.0 mg/ml bovine serum albumin (BSA), 30 μM bacitracin, 30 μM bestatin, 10
μM captopril and 0.1 mM phenylmethylsulfonyl fluoride, pH 7.4. The assay buffer for drug competition binding studies was 25 mM Tris-HCl, 150 mM NaCl, 2.5 mM MgCl₂, 1.0 mM EDTA, 0.25% BSA, 50 μM GDP, 30 μM bestatin, 10 μM captopril and 0.1 mM PMSF, pH 7.4.

3.2.1.b Expansion and Maintenance of Cell Lines

To expand the geneticin-resistant colonies, the medium was aspirated from the 100 mm plates and rinsed with 5 ml of Ca²⁺- and Mg²⁺-free phosphate-buffered saline (PD). Sterile, trypsin-soaked filter paper (approximately 3 mm x 3 mm) was placed on each colony. After 2 minutes the paper was lifted and each was placed in a separate well of a 24 well plate and 1 ml of selection medium was added. The forceps used to handle the filter papers were dipped in ethanol (100%) and flame sterilized between each use. Cells were incubated approximately 3-5 days until the wells were confluent.

Upon reaching confluency, the colonies were further expanded into 25 cm² flasks. Wells were rinsed with PD (0.5 ml) and trypsin (125 μl; 0.25%, 1x Versene in PD) was then added. After 1 minute, selection medium (0.5 ml) was added to each well and the suspension was transferred to a 25 cm² flask along with an additional 5 ml of selection medium. Clones not immediately screened were prepared and stored in liquid nitrogen. The remaining clones were expanded by aspirating the medium, rinsing with PD (3 ml) and then adding trypsin (0.25%, 1x Versene in PD). After 30 seconds the cells were dislodged by striking the flask. Selection medium (10 ml) was added and the suspension was transferred to 162 cm² flasks.
Additional selection medium (20 ml) was added to the 162 cm$^2$ flasks. Cells were placed in an incubator and reached confluency within 3-5 days. Radioligand binding and functional assays were then performed as discussed in Chapters 3 and 4. A 25 cm$^2$ flask of these expanded clones was also used to prepare the cells for storage in liquid nitrogen.

3.2.1.c General Membrane Preparation

The CHO/CB$\_f$ cells used in this study (clone "f") were passaged approximately 60-70 times. Confluent monolayers of CHO/CB$\_f$ cells were washed once with PD and incubated for 5 minutes at 37°C with PD containing 0.02% EDTA. Cells were then harvested and centrifuged at 1520 x g for 5 minutes at 4°C. The pellet was resuspended by pipetting in 20 volumes of 50 mM Tris-HCl buffer containing 5.0 mM MgCl$_2$, pH 7.4, and centrifuged again at 1520 x g for 5 minutes at 4°C. The supernatant was decanted and pellets were stored at -70°C until use. All membrane preparations were used within 4 weeks.

3.2.2 Tissue Preparation for Radioligand Binding Studies

On the day of use, pellets were homogenized in 20 volumes of the appropriate assay buffer (see Section 3.2.1.a) using 10 strokes of a glass/teflon homogenizer. Cells were then centrifuged at 40,000 x g for 15 minutes and homogenized as above in the appropriate assay buffer to give the appropriate tissue concentration.
3.2.3 General Incubation and Filtering Conditions for Radioligand Binding Assays

Unless otherwise specified, assay tubes (polypropylene culture test tubes, Fisher Scientific, Pittsburgh, PA) were brought to a final volume of 1 ml by the addition of assay buffer (50 mM Tris-HCl, 5.0 mM MgCl₂, 1.0 mg/ml BSA, 30 μM bacitracin, 30 μM bestatin, 10 μM captopril and 0.1 mM phenylmethylsulfonyl fluoride, pH 7.4). Tubes were incubated at 25°C for 90 minutes. At the end of the incubation the reaction was stopped by rapid filtration, using a tissue harvester (Brandel Inc., Gaithersburg, MD) onto GF/B glass microfiber filters (Whatman International Ltd., Maidstone, England) presoaked for 90 minutes in polyethylenimine (PEI; 0.5%). Filters were washed 3 times with ice-cold normal (0.9%) saline. Liquid scintillation cocktail (Ecolite, ICN Biomedical, Irvine, CA) was added and samples were stored at 4°C overnight and counted in a Beckman LS 6000SC Scintillation counter.

3.2.4 Radioligand Binding Studies

3.2.4.a Tissue Linearity Studies

CHO/CB₁ membranes at concentrations of 0.0316, 0.063, 0.125, 0.25 and 0.5% weight to volume (w/v) were incubated in duplicate along with [³H]SR141716A (0.5 nM) to give total binding. Non-specific binding was determined with [³H]SR141716A (0.5 nM) in the presence of WIN 55,212-2 (10 μM). Assay buffer was 50 mM Tris-HCl, 5.0 mM MgCl₂, 1.0 mg/ml BSA, 30 μM bacitracin, 30 μM bestatin, 10 mM captopril and 0.1 mM phenylmethylsulfonyl fluoride, pH 7.4. Incubation and filtration of samples were performed
as described in Section 3.2.3. The clone demonstrating the highest specific $[^3]$H]SR141716A binding, CHO/CB$_1$f, was used for all future studies.

3.2.4.b Saturation Binding Isotherms

CHO/CB$_1$f membranes at a final concentration of 0.1% w/v were incubated with 0.05 nM - 20 nM of either $[^3]$H]SR141716A, or $[^3]$H]CP55,940 at 25°C for 90 minutes to determine total binding. In addition, non-specific binding tubes contained WIN 55,212-2 (10 μM). Assay buffer was 50 mM Tris-HCl, 5.0 mM MgCl$_2$, 1.0 mg/ml BSA, 30 μM bacitracin, 30 μM bestatin, 10 mM captopril and 0.1 mM phenylmethylsulfonyl fluoride, pH 7.4. Incubation and filtration of samples were performed as described in Section 3.2.3. Two direct counts were taken for each radioligand concentration and were used to calculate the total radioligand concentration added to the tubes.

3.2.4.c Drug Competition Binding Studies

CHO/CB$_1$f membranes at a final concentration of 0.1% w/v were incubated with $[^3]$H]SR141716A (0.5 nM) and either WIN 55,212-2 (1 nM - 10 μM), Δ9-THC (1 pM - 10 μM), SR141716A (1 pM - 1 μM), or AM630 (0.1 nM - 10 μM). Assay buffer was 25 mM Tris-HCl, 150 mM NaCl, 2.5 mM MgCl$_2$, 0.25% BSA, 50 μM GDP, 30 μM bestatin, 10 μM captopril and 0.1 mM PMSF, pH 7.4. The lowest concentration of unlabeled ligand was used to determine non-specific binding. Incubation and filtration of samples were performed as described in Section 3.2.3.
3.3 RESULTS

3.3.1 Tissue Linearity Studies

The introduction of the human cannabinoid CB₁ receptor into Chinese hamster ovary cells produced multiple stable clones in which specific binding increased with increasing tissue concentrations (Table 3.1). However, the one clone displaying the highest amount of specific [³H]SR141716A binding (1.82 pmol binding sites/mg protein), CHO/CB₁f, was chosen to be used in subsequent binding and functional assays (Figure 3.1). Since the specific binding of this clone was linear from a tissue concentration of 0.0316 % - 0.125 % weight of tissue/assay volume (w/v), a final tissue concentration of 0.1% w/v was chosen for all future assays.

Table 3.1 - Comparison of specific [³H]SR141716A binding for selected stable CHO/CB₁ clones

<table>
<thead>
<tr>
<th>Clone (CHO/CB₁)</th>
<th>Specific [³H]SR141716A Binding (% tissue; w/v; dpm)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0.0316</td>
</tr>
<tr>
<td>a</td>
<td>198</td>
</tr>
<tr>
<td>d</td>
<td>N/A</td>
</tr>
<tr>
<td>f</td>
<td>987</td>
</tr>
<tr>
<td>g</td>
<td>242</td>
</tr>
<tr>
<td>h</td>
<td>651</td>
</tr>
<tr>
<td>r</td>
<td>253</td>
</tr>
</tbody>
</table>

CHO/CB₁ clones (0.0316% - 0.5% tissue, w/v) were incubated with [³H]SR141716A (0.5 nM). WIN 55,212-2 (10 μM) was used to determine non-specific binding.
Figure 3.1 - Tissue linearity in CHO/CB,f cell membranes using [3H]SR141716A. CHO/CB,f cell membranes (0.0316% - 0.5% tissue weight/assay volume) were incubated in the presence of [3H]SR141716A (0.5 nM) to determine specific binding. Non-specific binding was determined in the presence of WIN 55,212-2 (10 μM). WIN 55,212-2 was initially dissolved in 100% ethanol to produce a 2 mM stock solution. Further dilutions of both drugs were done using assay buffer. Specific [3H]SR141716A binding in CHO/CB,f membranes was determined by subtracting the total amount of [3H]SR141716A bound in the presence of WIN 55,212-2 (10 μM) from the total amount of [3H]SR141716A bound. A representative experiment is shown.
3.3.2 Saturation Binding Isotherms

Receptor expression levels of the CHO/CB₃ clone needed to be determined. Both the high affinity radiolabeled cannabinoid receptor agonist, [³H]CP55,940, and the high affinity CB₁ receptor-selective antagonist, [³H]SR141716A, were used. Non-specific binding was determined in the presence of WIN 55,212-2 (10 μM). Saturation binding isotherms using [³H]SR141716A yielded a receptor concentration of 1.82 picomoles of binding sites/mg of protein and a Kᵅ value of [³H]SR141716A of 2.85 nM (Figure 3.2a; n=3). [³H]CP55,940 yielded a receptor expression level of 0.47 picomoles of binding sites/mg of protein and a Kᵅ value of 4.14 nM (n=3; Figure 3.2b). Protein concentration of approximately 0.21 pmol protein/tube was determined using the method developed by Lowry (1951). The observation that the radiolabeled antagonist bound more sites than the radiolabeled agonist was expected since, in the absence of guanine nucleotides, agonists label only the high-affinity sites of the receptor.
Figure 3.2a - Saturation binding isotherm with CHO/CB,f membranes using [3H]SR141716A. Specific [3H]SR141716A binding in CHO/CB,f membranes (0.1% tissue, w/v; n=3) was determined by subtracting the total amount of [3H]SR141716A bound in the presence of WIN 55,212-2 (10 μM) from the total amount of [3H]SR141716A bound. Final [3H]SR141716A concentrations of 0.05 nM - 20 nM were used. The incubation was performed at 25°C for 90 minutes. A receptor expression level of 1.82 pmol/mg of protein and a K_d value of 2.85 nM was determined. A representative experiment is shown.

Figure 3.2b - Saturation binding isotherm with CHO/CB,f membranes using [3H]CP55,940. Specific [3H]CP55,940 binding in CHO/CB,f membranes (0.1% tissue, w/v; n=3) was determined by subtracting the total amount of [3H]CP55,940 bound in the presence of WIN 55,212-2 (10 μM) from the total amount of [3H]CP55,940 bound. Final [3H]CP55,940 concentrations of 0.05 nM - 20 nM were used. The incubation was performed at 25°C for 90 minutes. A receptor expression level of 0.47 pmol/mg of protein and a K_d value of 4.14 nM was determined. A representative experiment is shown.
3.3.3 Drug Competition Binding Studies

To further characterize the transfected receptor, drug competition binding studies between [3H]SR141716A and various cannabinoid ligands were performed. WIN 55,212-2 (1 pM - 30 μM; n=5), Δ⁹-THC (1 pM - 10 μM; n=2), SR141716A (0.01 nM - 1 μM; n=2) and AM630 (0.1 nM - 10 μM; n=1) all dose-dependently competed for [3H]SR141716A (0.5 nM) binding to the transfected CB₁ receptor. WIN 55,212-2 maximally inhibited [3H]SR141716A binding by 86.9% with an IC₅₀ value of 1.18 μM (Figure 3.3a). Δ⁹-THC maximally inhibited [3H]SR141716A binding by 93.5% with an IC₅₀ value of 75 nM (Figure 3.3a). SR141716A maximally inhibited [3H]SR141716A binding by 94.8% with an IC₅₀ value of 1.3 nM (Figure 3.3b). AM630 maximally inhibited [3H]SR141716A binding by 91.8% with an IC₅₀ value of 816 nM (Figure 3.3b). The Kᵢ values were determined to be 1.0 μM for WIN 55,212-2, 63.8 nM for Δ⁹-THC, 1.1 nM for SR141716A and 694 nM for AM630 (Kᵢ = IC₅₀/(1+[radioligand]/Kₐ); Cheng and Prusoff, 1973).
Figure 3.3a - Drug competition binding studies with WIN 55,212-2 and Δ⁹-THC vs.[³H]SR141716A in CHO/CB₁f cell membranes. The effect of WIN 55,212-2 (n=5; squares) and Δ⁹-THC (n=2; triangles) on [³H]SR141716A (0.5 nM) binding to CHO/CB₁f membranes was determined. WIN 55,212-2 maximally inhibited [³H]SR141716A binding by 86.9% with an IC₅₀ value of 1.18 μM. Δ⁹-THC maximally inhibited [³H]SR141716A binding by 93.5% with an IC₅₀ value of 75 nM. Symbols represent the mean response and the vertical lines represent the S.E.M. WIN 55,212-2 was initially dissolved in 100% ethanol to produce a 2 mM stock solution. Δ⁹-THC was initially dissolved in 100% ethanol to produce a 1 mM stock solution. Further dilutions were done using assay buffer to give final WIN 55,212-2 concentrations of 1 pM - 30 μM and final Δ⁹-THC concentrations of 1 pM - 10 μM.
Figure 3.3b - Drug competition binding studies with SR141716A and AM630 vs. [3H]SR141716A in CHO/CB, f cell membranes. The effect of SR141716A (n=2; squares) and AM630 (n=1; triangles) on [3H]SR141716A (0.5 nM) binding to CHO/CB, f membranes. SR141716A maximally inhibited [3H]SR141716A binding by 94.8% with an IC50 value of 1.3 nM. AM630 maximally inhibited [3H]SR141716A binding by 91.8% with an IC50 value of 816 nM. Symbols represent the mean response and the vertical lines represent the S.E.M. SR141716A and AM630 were initially dissolved in 100% ethanol to produce 2 mM stock solutions. Further dilutions were done using assay buffer to give final SR141716A concentrations of 1 pM - 1 μM and final AM630 concentrations of 0.1 nM - 10 μM.
3.4 DISCUSSION

To study the actions of cannabinoid ligands at the cloned human cannabinoid CB₁ receptor, the cDNA was stably transfected into a Chinese hamster ovary cell line (see also Landsman et al., 1997; Landsman et al., 1998). Tissue linearity studies using [³H]SR141716A (0.5 nM) in the absence or presence of WIN 55,212-2 (10 μM) revealed a clone (CHO/CB₁f) in which [³H]SR141716A binding was linear from 0.0316% - 0.125% tissue (w/v; final concentration) when incubated at 25°C for 90 minutes. Therefore, a final tissue concentration of 0.1% (w/v) was chosen for all remaining binding and functional assays. No specific [³H]SR141716A binding was seen in wild-type CHO cells (data not shown). CHO cell membranes were used in all studies in this chapter.

Saturation binding experiments using [³H]SR141716A (0.05 nM - 20 nM) and [³H]CP55,940 (0.05 nM - 20 nM) were done to determine the density of human cannabinoid CB₁ receptor expressed in the CHO/CB₁f cell line. Saturation binding studies using [³H]SR141716A (0.05 nM - 20 nM) either in the absence or presence of WIN 55,212-2 (10 μM) showed that this cell line expressed the human cannabinoid CB₁ receptor at 1.82 pmoles of binding sites/mg protein. The Kᵅ value of [³H]SR141716A at the human cannabinoid receptor in this system was 2.85 nM. The Kᵅ and Bₘₐₓ values of [³H]SR141716A in CHO/CB₁f membranes is similar to that first reported for [³H]SR141716A in whole rat brain (minus cerebellum) synaptosomes (Kᵅ = 0.61 nM; Bₘₐₓ = 0.72 pmol/mg of protein; Rinaldi-Carmona et al., 1996b). Further support that this receptor concentration is relevant can be seen from the studies by Breivogel et al. (1997) in which they determined receptor levels in
membranes from various rat brain regions. They obtained $B_{\text{max}}$ values for $[^3H]SR141716A$ ranging from 2.47 pmol/mg protein to 6.85 pmol/mg protein.

Saturation binding studies using the cannabinoid agonist, $[^3H]CP55,940$ (0.05 nM - 20 nM), in either the absence or presence of WIN 55.212-2 (10 $\mu$M) showed that this cell line expressed the human cannabinoid CB$_1$ receptor at 0.47 picomoles of binding sites/mg protein. The $K_d$ value of $[^3H]CP55,940$ at the human cannabinoid receptor in this system was 4.14 nM. This $K_d$ value in CHO/CB$_1$ f cells is in agreement with previous reports by Rinaldi-Carmona et al. (1996a) where they calculated the $K_d$ value of $[^3H]CP55,940$ to be 1.39 nM at membranes of CHO cells expressing the human cannabinoid CB$_1$ receptor. Similarly, in membranes of COS-7 cells expressing the human cannabinoid CB$_1$ receptor, Gerard et al. (1991) obtained a $K_d$ value for $[^3H]CP55,940$ of approximately 1 nM. In addition, Breivogel et al. (1997) obtained $B_{\text{max}}$ values for the cannabinoid agonist, $[^3H]WIN 55,212-2$, ranging from 1.23 pmol/mg protein to 6.17 pmol/mg protein.

The larger number of binding sites determined by $[^3H]SR141716A$ compared to that of $[^3H]CP55,940$ in the current studies and compared to $[^3H]WIN 55,212-2$ in the studies by Breivogel et al. (1997) is due to the belief that G protein-coupled receptors exist in more than one activation state, at least one which has low affinity for ligands and one which has high affinity for ligands. In the absence of guanine nucleotides, agonists label only the high-affinity sites of the receptor.

To further verify that the human cannabinoid CB$_1$ receptor has been expressed, competition binding between the CB$_1$-selective ligand, $[^3H]SR141716A$, and various
cannabinoid ligands was performed. WIN 55,212-2 maximally inhibited \(^{3}H\)SR141716A (0.5 nM) binding in CHO/CB\(_{1}\)f cell membranes by 86.9% with an IC\(_{50}\) value of 1.18 \(\mu\)M. Δ\(^{9}\)-THC inhibited \(^{3}H\)SR141716A (0.5 nM) binding in this system by 93.5% with an IC\(_{50}\) value of 75 nM. SR141716A inhibited \(^{3}H\)SR141716A binding by 94.8% with an IC\(_{50}\) value of 1.3 nM. AM630 inhibited \(^{3}H\)SR141716A binding by 91.8% with an IC\(_{50}\) value of 816 nM. These data are in agreement with those reported by Rinaldi-Carmona et al. (1996b). In their report they obtained IC\(_{50}\) values of 2.35 nM for SR141716A, 68.5 nM for WIN 55,212-2 and 70.5 nM for Δ\(^{9}\)-THC in inhibiting \(^{3}H\)SR141716A (0.45 nM) binding in whole rat brain (minus cerebellum) synaptosomes. These values correspond to \(K_{i}\) values of approximately 1.35 nM for SR141716A, 39.4 nM for WIN 55,212-2 and 40.6 nM for Δ\(^{9}\)-THC (Cheng and Prusoff, 1973). \(K_{i}\) values of 0.89 nM for SR141716A, 28 nM for WIN 55,212-2 and 35 nM for Δ\(^{9}\)-THC for the inhibition of \(^{3}H\)SR141716A were obtain by Petitet et al. (1996). The \(K_{i}\) values reported in the literature are similar to the current experiments which the \(K_{i}\) values were determined to be 1.0 \(\mu\)M for WIN 55,212-2, 63.8 nM for Δ\(^{9}\)-THC, 1.1 nM for SR141716A and 694 nM for AM630.

One difference between the assay conditions used by both Rinaldi-Carmona et al. (1996b) and Petitet et al. (1996) as compared to the assay conditions in the current studies is that the latter used high concentrations of both sodium and GDP. The presence of these compounds in the assay buffer shifts the IC\(_{50}\) and \(K_{i}\) values of agonists, but not antagonists, to lower potency. The consistency of the IC\(_{50}\) and \(K_{i}\) values for the cannabinoid antagonist, SR141716A, among Rinaldi-Carmona et al. (1996b), Petitet et al. (1996) and the data in the...
current studies supports this observation. Interestingly, while the addition of sodium and GDP in the current studies shifted the IC$_{50}$ and K$_i$ values of the cannabinoid agonist, WIN 55,212-2, to lower potency as expected, the IC$_{50}$ and K$_i$ values for the cannabinoid agonist, Δ$^9$-THC, among these three studies, does not change. Sodium and GDP were used in the competition binding assays in order to keep the conditions consistent with the [${}^{35}$S]GTP$\gamma$S binding assay (see Chapter 4). This allowed for the comparison of the K$_i$ values (competition binding) to the EC$_{50}$ values ([${}^{35}$S]GTP$\gamma$S assay).

The results of the above studies support the existence of an expressed human cannabinoid CB$_1$ receptor in a CHO cell line. Saturation binding studies have shown that this receptor binds cannabinoid ligands with an affinity similar to that reported from previous studies (Breivogel et al., 1997). In addition, the IC$_{50}$ and K$_i$ values for various cannabinoid ligands in inhibiting the binding of the CB$_1$-selective ligand, [${}^3$H]SR141716A, is also in agreement with other studies (Rinaldi-Carmona et al., 1996b).
CHAPTER 4

Characterization of the Human Cannabinoid CB₁ Receptor Using Functional Assays

4.1 INTRODUCTION

In studying the cannabinoid system, two functional assays have been widely used. These are the $[^{35}S]GTP\gamma S$ assay (Traynor and Nahorski, 1995) and the cAMP assay (Gilman, 1970; Howlett, 1984). The $[^{35}S]GTP\gamma S$ assay uses the principle that G protein-coupled receptors, when activated by ligand, cause the release of prebound GDP from the $G_\alpha$ subunit of the G protein which is then replaced with GTP. This activated $G_\alpha$ subunit of the G protein is then released from both the receptor and from the associated $\beta\gamma$ subunits of the G protein and is free to interact with effector systems. The activity of the $G_\alpha$ subunit of the G protein is halted when the GTP is cleaved to GDP by the intrinsic GTPase activity of the $G_\alpha$ subunit. Since GTP binding is one of the initial steps in receptor activation, it is a logical point in which to measure receptor activation. The use of a non-hydrolyzable GTP analog (GTPγS) inhibits the $G_\alpha$ subunit of the G protein from breaking down the GTP, and radiolabeling this analog ($[^{35}S]GTP\gamma S$) will allow one to measure the extent of receptor activation upon ligand binding (Figure 4.1). The cAMP assay measures the effect of receptor activation at one such effector, adenylyl cyclase, which produces cAMP from adenosine triphosphate (ATP) in response to forskolin.
Figure 4.1 - Ternary complex model of cannabinoid receptor activation. $G_{\alpha_{GDP}}{\beta\gamma}$ binds cannabinoid agonist (CB,A)-activated cannabinoid receptor (CB,R). Nucleotide exchange occurs and dissociated $G_{\alpha_{GTP}}$ and $\beta\gamma$ subunits mediate downstream effector (E) systems. Inactivation of $G_{\alpha}$ occurs through its GTPase activity, liberating GDP and inorganic phosphate ($P_{i}$). $E^*$ = activated effector.

The results of these assays are more easily interpreted with confidence when the system studied is one in which the receptor is expressed in its native tissue. For example, Sim et al. (1996) and Burkey et al. (1997) demonstrated that cannabinoid agonists stimulate basal $[^{35}S]GTP\gamma S$ binding in rat brain membranes and mouse brain membranes, respectively. The endogenous expression of these receptors in these tissues makes it certain that the components naturally coupled to the receptor are present. However, in using heterologous expression systems, such as CHO cells, it is possible that the host cell does not contain the appropriate components to couple to the introduced receptor. It is also possible that the non-native membrane environment of the host cell alters the receptor conformation in such a way
as to not alter the radioligand binding properties of the receptor, but to affect the receptor's ability to couple to intracellular effector mechanisms. However, CHO cells do appear to allow the transfected cannabinoid receptor to be expressed and functional. The expression of the rat CB₁ cannabinoid receptor in CHO cells produced a functional receptor. In this system, Δ⁹-THC inhibited forskolin-stimulated cAMP formation by 39% with an EC₅₀ value of 13.5 nM (Matsuda et al., 1990). Similarly, CP55,940 inhibited the forskolin-stimulated cAMP response by 56% in this cell line with an EC₅₀ value of 0.87 nM (Matsuda et al., 1990). Both the percent inhibition of cAMP formation and the EC₅₀ values for Δ⁹-THC and CP55,940 were as expected. In CHO cells expressing the cloned human cannabinoid CB₁ receptor, CP55,940 inhibited forskolin-stimulated cAMP formation by approximately 80% and with an EC₅₀ value of 1 nM (Gerard et al., 1991). As with the data from Matsuda et al., the percent of forskolin-stimulated cAMP inhibition and the EC₅₀ value were as expected (Matsuda et al., 1990). Furthermore, others demonstrated that the EC₅₀ values for various cannabinoid ligands in inhibiting forskolin-stimulated cAMP formation, including WIN 55,212-2, HU-210, CP55,940 and Δ⁹-THC, agree with previously reported values (Felder et al., 1995; Rinaldi-Carmona et al., 1996a). In order to support the hypothesis that CHO cells are an adequate model to study the effects of the human cannabinoid CB₁ receptor, functional assays needed to be done to determine that these receptors were functional. Both the [³⁵S]GTPγS binding assay and the cAMP assay were used to help characterize the CHO/CB₁ clone. Furthermore, the effects of altering the parameters of the [³⁵S]GTPγS binding assay were also investigated.
4.2 MATERIALS AND METHODS

4.2.1 Drugs

See Section 3.2.1.a. In addition, CP55,940 was generously supplied by Pfizer (Groton, CT). R-(+)-methanandamide was purchased from Research Biomedical International (Natick, MA). HU-210 was supplied by Professor Raphael Mechoulam (Hebrew University, Israel), or purchased from Tocris (Ballwin, MO). 11-OH-Δ⁹-THC was supplied by the National Institutes of Health (Bethesda, MD). Forskolin (7β-deacetyl-7β-(γ-N-methylpiperazino)-butyryl, dihydrochloride) was purchased from Calbiochem (La Jolla, CA). [³H]cAMP was purchased from New England Nuclear (Boston, MA). Activated charcoal (Norit Ultra C) was purchased from Norit (Amersfoort, Netherlands). cAMP-dependent protein kinase and 3-isobutyl-1-methylxanthine (IBMX) were purchased from Sigma Chemical Company (St. Louis, MO). For the [³S]GTPγS assay all non-radiolabeled cannabinoid drugs, except methanandamide and Δ⁹-THC, were initially dissolved in 100% ethanol to produce 2.0 mM stock solutions. Methanandamide and Δ⁹-THC were initially dissolved in 100% ethanol to produce 1 mM stock solutions. For the cAMP assay, all non-radiolabeled cannabinoid drugs were initially dissolved in 100% ethanol to produce 1 mM stock solutions. Further dilutions of all drugs were done using the appropriate assay buffer. For the [³S]GTPγS assay the buffer consisted of 25 mM Tris-HCl, 150 mM NaCl, 2.5 mM MgCl₂, 0.25% BSA, 50 μM GDP, 30 μM bestatin, 10 μM captopril and 0.1 mM PMSF, pH 7.4. Drug dilutions for the cAMP assay were done using Iscove’s modified Dulbecco’s medium (IMDM).
4.2.2 Tissue Preparation for the \([^{35}S]GTP\gamma S\) Assay

On the day of the assay, frozen pellets were slowly thawed on ice and homogenized in 20 volumes of assay buffer containing 25 mM Tris-HCl, 150 mM NaCl, 2.5 mM MgCl₂, 1.0 mM EDTA, 0.25% BSA, 50 μM GDP, 30 μM bestatin, 10 μM captopril and 0.1 mM PMSF, pH 7.4, using 10 strokes of a glass/teflon homogenizer and incubated for 30 minutes at 30°C. The cell lysates were then centrifuged at 40,000 x g for 15 minutes then homogenized as above in assay buffer to give a stock tissue concentration of 1.0% (w/v). One hundred μl of tissue were used in each assay tube. This gave a final CB₁ receptor concentration of approximately 0.21 pmol protein/tube (0.1% w/v) as determined by the Lowry assay (1951).

4.2.3 Tissue Preparation for the cAMP Assay

CHO cells were plated in 24-well plates at 30,000 cells/well and allowed to grow for 48 hours before the assay. Wild-type CHO cells were grown in Ham’s F12 medium containing 10% fetal bovine serum, 100 U/ml penicillin and 100 μg/ml streptomycin. CHO/CB₁ cells were grown in the same medium with the addition of 500 μg/ml G418. Cells pretreated with pertussis toxin 24 hours before performing the assay were plated at 50,000 cells/well. This was done since the medium was changed to serum-free Ham’s F12 containing 100 U/ml penicillin and 100 μg/ml streptomycin. Serum-free medium does not promote growth of these cells.
4.2.4 Activation of $[^{35}]$GTP$\gamma$S Binding in CHO/CB$_4$ Cell Membranes by Cannabinoid Ligands

Unless otherwise specified, assay tubes contained final concentrations of 0.1 nM $[^{35}]$GTP$\gamma$S, 0.1% tissue (w/v) and various concentrations of cannabinoid ligands and were brought to a final volume of 1.0 ml in assay buffer (25 mM Tris-HCl, 150 mM NaCl, 2.5 mM MgCl$_2$, 1.0 mM EDTA, 0.25% BSA, 50 µM GDP, 30 µM bestatin, 10 µM captopril and 0.1 mM PMSF, pH 7.4). The amount of $[^{35}]$GTP$\gamma$S bound at the lowest concentration of each ligand was referred to as basal binding for that ligand and was assigned a value of 100%. Tubes containing the reaction mixture were incubated at 30°C for 90 minutes and filtered using a tissue harvester (Brandel Inc., Gaithersburg, MD) onto GF/B glass microfiber filters (Whatman International Ltd., Maidstone, England). Filters were washed 4 times with ice-cold GTP$\gamma$S wash buffer (25 mM Tris-HCl, 120 mM NaCl, pH 7.4). Scintillation cocktail (Ecolite, ICN Biomedical, Irvine, CA) was added and samples were stored at 4°C overnight and then counted in a Beckman LS 6000SC Scintillation counter.

4.2.5 Alteration of $[^{35}]$GTP$\gamma$S Binding Parameters

To be certain that the concentration of ethanol used to dissolve the cannabinoid ligands did not affect the $[^{35}]$GTP$\gamma$S binding assay, CHO/CB$_4$ membranes were incubated with ethanol (0.1% - 25%) in the presence of WIN 55,212-2 (10 µM). All other experiments performed to investigate various binding parameters used WIN 55,212-2 (1.0 nM - 100 µM).
CHO/CB, f membranes were also incubated in the presence or absence of NaCl (0 or 150 mM), MgCl₂ (0, 0.1, 1, 2.5, 5, 7.5, or 10 mM), or GDP (0, 1, 5, 10, 20, 30, 40, or 50 μM), in either the absence or presence of WIN 55,212-2 to determine the concentration of these compounds necessary to observe substantial WIN 55,212-2-mediated [³⁵S]GTPγS binding.

4.2.6 Pretreatment of CHO/CB, f Cells with Pertussis Toxin Prior to the [³⁵S]GTPγS Binding Assay

The medium from confluent (90-95%) plates of CHO/CB, f cells was aspirated. Cells were washed once with IMDM (5 ml) and the medium aspirated. Fresh IMDM (25 ml) was then added along with pertussis toxin (6.25 μl of 200 μg/ml; Sigma, St. Louis, MO). Cells were then incubated 24 hours at 37°C in a humidified environment containing 5% CO₂ and 95% air. Cells were then harvested as described in Section 3.2.1c.

4.2.7 cAMP Formation Studies

The cAMP assay was performed according to a method modified from Gilman (1970). Cells stably expressing the human cannabinoid CB₁ receptor were plated in 24 well plates as described in Section 4.2.3. Experiments using pertussis toxin are described in Section 4.2.8.

On the day of the experiment, cells were rinsed once with IMDM (1 ml) and then stimulated with the appropriate concentration of forskolin in the absence or presence of cannabinoid ligands. All experiments were performed in the presence of the
phosphodiesterase inhibitor, 3-isobutyl-methylxanthine (IBMX; 5 mM), for 20 minutes at 37°C. The reaction was terminated by aspirating the medium and adding ice-cold Tris ethylenediaminetetraacetic acid (EDTA) buffer (150 μl; 50 mM Tris HCl/Trisma base, 4 mM Na₂ EDTA, pH 7.5, 25°C) to the wells and placing the plates on ice. The cells were dislodged from the wells using a Costar cell scraper and were transferred to plastic microcentrifuge tubes (Bio-Rad, Hercules, CA) and boiled for 10 minutes.

Tubes were centrifuged at 5600 x g for 2 minutes and an aliquot (50 μl) of the supernatant was removed and analyzed for cAMP content using a protein binding assay. The supernatant and 50 μl of a standard ([cAMP] of 0.125 pM - 128 pM) were separately incubated with [³H]cAMP (50 μM of 0.9 pmol/50 μl) and cAMP-dependent protein kinase solution (100 μl of 60 μg of protein kinase/ml in a 50 mM Tris HCl, 4 mM EDTA, 0.1% BSA buffer, pH 7.4, 25°C) on ice for 2 hours.

Separation of bound and free [³H]cAMP was obtained by adding ice-cold activated charcoal in 50 mM Tris HCl, 4 mM EDTA, 2% bovine serum albumin (100 μl) to each tube. The samples were mixed and centrifuged at 5600 x g for 45 seconds. An aliquot (200 μl) of each sample supernatant was transferred to a scintillation vial and mixed with Ecolite (4 ml; ICN Biomedical, Irvine, CA) and the radioactivity was counted.
4.2.8 Pretreatment of CHO/CB,f Cells with Pertussis Toxin Prior to the cAMP Assay

CHO/CB,f cells were grown in 24 well plates 48 hours before the experiment. Twenty four hours before the experiment, the medium (Ham's F12) was aspirated from each well of CHO/CB,f cells to be used for pertussis toxin studies. Cells were washed once with IMDM (1 ml) and replaced with serum-free Ham's F12 medium (1 ml) containing 100 U/ml penicillin, 100 μg/ml streptomycin and 50 ng/ml pertussis toxin. Cells were allowed to grow for an additional 24 hours. Cells used in the pertussis toxin studies were plated at 50,000 cells per well instead of 30,000 cells per well since the absence of serum inhibits cell growth. Forskolin was initially dissolved in distilled water. Further dilutions of forskolin were done using IMDM to give a final forskolin concentration of 100 μM. Pertussis toxin was bought in 50% glycerol/50 mM Tris.

4.2.9 Data Analysis

Data from all radioligand binding and functional assays were analyzed by non-linear regression analysis using GraphPad Prism (GraphPad Software, Inc., San Diego, CA). Both one-site and two-site binding analyses were performed to determine best-fit. Significant (p<0.05) stimulation, or inhibition of basal [35S]GTPγS binding was determined using One-Way Analysis of Variance (ANOVA, SigmaStat, SPSS, Chicago, IL) followed by the Tukey test (SigmaStat, SPSS, Chicago, IL).
4.3 RESULTS

4.3.1 The Influence of Changing Parameters on Basal and WIN 55,212-2-Stimulated $[^{35}]$GTPγS Binding in CHO/CB,f Cell Membranes

4.3.1.a Ethanol on WIN 55,212-2-Stimulated $[^{35}]$GTPγS Binding in CHO/CB,f Cell Membranes

Ethanol at concentrations up to 5.0% altered WIN 55,212-2-stimulated $[^{35}]$GTPγS binding by less than 5% in membranes of CHO/CB,f cells incubated at 30°C for 90 minutes (Figure 4.2). WIN 55,212-2-stimulated $[^{35}]$GTPγS binding was decreased more than 20% at ethanol concentrations of 10% or greater (Figure 4.2). For this reason, ethanol concentrations of 5% or less were generally used.

![Figure 4.2 - Ethanol on WIN 55,212-2-stimulated $[^{35}]$GTPγS binding in CHO/CB,f cell membranes. CHO/CB,f membranes (0.1% w/v) were incubated with ethanol (0% - 25%; n=1) in the presence of WIN 55,212-2 (10 µM) at 30°C for 90 minutes. WIN 55,212-2 was initially dissolved in 100% ethanol to produce a 2 mM stock solution. Further dilutions were done using assay buffer. Ethanol at concentrations up to 5.0% affected WIN 55,212-2-stimulated $[^{35}]$GTPγS binding by less than 5.0%. WIN 55,212-2-stimulated $[^{35}]$GTPγS binding was decreased more than 20% at ethanol concentrations of 10% or greater.](image-url)
4.3.1.b Sodium on Basal and WIN 55,212-2-Stimulated $[^{35}S]$GTPγS Binding in CHO/CB₁f Cell Membranes

The effect of sodium (Na⁺) on basal (Figure 4.3a) and WIN 55,212-2-stimulated (Figure 4.3b) $[^{35}S]$GTPγS binding in CHO/CB₁f cell membranes incubated at 30°C for 90 minutes was determined. The level of WIN 55,212-2-stimulated $[^{35}S]$GTPγS binding is dependent on the concentration of Na⁺ in the assay so one experiment was run to determine the optimal concentration of sodium needed to achieve maximal WIN 55,212-2-stimulated $[^{35}S]$GTPγS binding in CHO/CB₁f cell membranes. The absence of sodium in the assay produced higher basal $[^{35}S]$GTPγS binding (4294 disintegrations per minute, dpm) than when 150 mM sodium was added (1618 dpm; Figure 4.3a). The lower baseline in the presence of 150 mM sodium allowed for a larger percent stimulation than when no sodium was present. In the absence of sodium, WIN 55,212-2 stimulated basal $[^{35}S]$GTPγS binding by 40.8% with an EC₅₀ value of 28.6 nM. WIN 55,212-2-stimulated $[^{35}S]$GTPγS binding in the presence of 150 mM Na⁺ was 113% above basal with an EC₅₀ value of 606 nM (Figure 4.3b).
Figure 4.3a - Sodium on basal $[^{35}\text{S}]\text{GTP}^\gamma\text{S}$ binding in CHO/CB,$f$ cell membranes. The effect of sodium (150 mM; $n=1$) on basal $[^{35}\text{S}]\text{GTP}^\gamma\text{S}$ binding in CHO/CB,$f$ cell membranes (0.1% w/v) was determined. Membranes were incubated at 30°C for 90 minutes. The absence of sodium in the assay produced basal $[^{35}\text{S}]\text{GTP}^\gamma\text{S}$ levels of 4294 dpm. The presence of 150 mM sodium produced a basal level of 1618 dpm.

Figure 4.3b - Sodium on WIN 55,212-2-stimulated $[^{35}\text{S}]\text{GTP}^\gamma\text{S}$ binding in CHO/CB,$f$ cell membranes. The effect of sodium (150 mM; $n=1$) on WIN 55,212-2-stimulated $[^{35}\text{S}]\text{GTP}^\gamma\text{S}$ binding in CHO/CB,$f$ cell membranes (0.1% w/v) was determined. Membranes were incubated with Mg$^{2+}$ (2.5 mM) and GDP (30 μM) at 30°C for 90 minutes. In the absence of sodium (squares), WIN 55,212-2 stimulated basal $[^{35}\text{S}]\text{GTP}^\gamma\text{S}$ binding by 40.8% with an EC$_{50}$ value of 28.6 nM. In the presence of 150 mM Na$^+$ (triangles), WIN 55,212-2-stimulated $[^{35}\text{S}]\text{GTP}^\gamma\text{S}$ binding 113% above basal levels with an EC$_{50}$ value of 606 nM.
4.3.1.c Magnesium on Basal and WIN 55,212-2-Stimulated \[^{35}\text{S}]\text{GTP}\gamma\text{S} Binding in CHO/CB, f Cell Membranes

The effect of magnesium (Mg\(^{2+}\)) on basal (Figure 4.4a) and WIN 55,212-2-stimulated (Figure 4.4b) \[^{35}\text{S}]\text{GTP}\gamma\text{S} binding in CHO/CB, f cell membranes incubated at 30\(^\circ\)C for 90 minutes was determined. The level of WIN 55,212-2-stimulated \[^{35}\text{S}]\text{GTP}\gamma\text{S} binding is dependent on the concentration of Mg\(^{2+}\) in the assay so one experiment was run to determine the optimal concentration of magnesium needed to achieve substantial WIN 55,212-2-stimulated \[^{35}\text{S}]\text{GTP}\gamma\text{S} binding in CHO/CB, f cell membranes. Almost no stimulation was seen with WIN 55,212-2 at 0 or 0.1 mM Mg\(^{2+}\). The percent stimulation at 0 mM Mg\(^{2+}\) was 4.6% above basal with an EC\(_{50}\) value of 2.67 \(\mu\)M whereas 0.1 mM Mg\(^{2+}\) in the assay buffer showed a 15.3% increase in WIN 55,212-2-stimulated binding with an EC\(_{50}\) value of 19.1 nM. WIN 55,212-2 produced a 29.1% increase in basal \[^{35}\text{S}]\text{GTP}\gamma\text{S} binding at a concentration of 1 mM Mg\(^{2+}\) and an EC\(_{50}\) value of 132 nM. A 78.6% increase and an EC\(_{50}\) value of 455 nM was seen at 2.5 mM Mg\(^{2+}\). A concentration of 5 mM Mg\(^{2+}\) produced maximum WIN 55,212-2-stimulated \[^{35}\text{S}]\text{GTP}\gamma\text{S} binding levels 85.5% above basal with an EC\(_{50}\) value of 181 nM whereas 7.5 mM Mg\(^{2+}\) produced a 101% increase and an EC\(_{50}\) value of 306 nM. The highest concentration of Mg\(^{2+}\) used, 10 mM, produced maximum WIN 55,212-2-stimulated \[^{35}\text{S}]\text{GTP}\gamma\text{S} binding levels 93.7% above basal with an EC\(_{50}\) value of 235 nM. Basal \[^{35}\text{S}]\text{GTP}\gamma\text{S} binding was also affected by the concentration of magnesium in the assay since higher magnesium concentrations producing higher levels of basal binding. The data are summarized in Table 4.1.
Figure 4.4a - Magnesium on basal $[^{35}S]$GTPyS binding in CHO/CB,f cell membranes. The effect of magnesium (0 mM - 10 mM; n=1) on basal $[^{35}S]$GTPyS binding in CHO/CB,f cell membranes (0.1% w/v) was determined. Membranes were incubated with Na$^+$ (150 mM) and GDP (30 μM) at 30°C for 90 minutes. WIN 55,212-2 was initially dissolved in 100% ethanol to produce a 2 mM stock solution. Further dilutions were done using assay buffer.

Figure 4.4b - Magnesium on WIN 55,212-2-stimulated $[^{35}S]$GTPyS binding in CHO/CB,f cell membranes. The effect magnesium on WIN 55,212-2-stimulated (1 nM - 100 μM) $[^{35}S]$GTPyS binding in CHO/CB,f cell membranes (0.1% w/v) was determined. Membranes were incubated with Na$^+$ (150 mM) and GDP (30 μM) at 30°C for 90 minutes in either the absence (squares; n=1), or presence of Mg$^{2+}$ (2.5 mM; circles; n=1). WIN 55,212-2 was initially dissolved in 100% ethanol to produce a 2 mM stock solution. Further dilutions were done using assay buffer.
Table 4.1 - The effect of magnesium on basal and WIN 55,212-2-stimulated $[^{35}\text{S}]$GTP$\gamma$S binding in CHO/CB,f cell membranes

<table>
<thead>
<tr>
<th>[Mg$^{2+}$] (mM)</th>
<th>WIN 55,212-2-stimulated</th>
<th>basal $[^{35}\text{S}]$GTP$\gamma$S bound (dpm)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$E_{\text{max}}$ (% above basal)</td>
<td>$EC_{50}$ (nM)</td>
</tr>
<tr>
<td>0</td>
<td>4.60</td>
<td>2670</td>
</tr>
<tr>
<td>0.1</td>
<td>15.3</td>
<td>19.1</td>
</tr>
<tr>
<td>1</td>
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</tr>
<tr>
<td>2.5</td>
<td>78.6</td>
<td>455</td>
</tr>
<tr>
<td>5</td>
<td>85.5</td>
<td>181</td>
</tr>
<tr>
<td>7.5</td>
<td>101</td>
<td>306</td>
</tr>
<tr>
<td>10</td>
<td>93.7</td>
<td>235</td>
</tr>
</tbody>
</table>

CHO/CB,f cell membranes (0.1% w/v) were incubated with magnesium chloride (0 mM - 10 mM) in the absence (basal) or presence (WIN 55,212-2-stimulated) of WIN 55,212-2 (1 nM - 100 μM).
4.3.1.d GDP on Basal and WIN 55,212-2-Stimulated [\textsuperscript{35}S]GTP\gamma S Binding in CHO/CB\textsubscript{1}f Cell Membranes

CHO/CB\textsubscript{1}f cell membranes were incubated with various concentrations of GDP at 30\textdegree C for 90 minutes. As can be seen in figure 4.5a, the concentration of GDP in the assay affected basal [\textsuperscript{35}S]GTP\gamma S binding with higher GDP concentrations lowering the baseline. These data are summarized in Table 4.2. The effect of WIN 55,212-2-stimulated [\textsuperscript{35}S]GTP\gamma S binding was also dependent on the concentration of GDP in the assay (Figure 4.5b). While no effect of WIN 55,212-2 was seen at 0 \textmu M GDP, the addition of GDP (50 \textmu M) produced a 71.0\% increase in basal [\textsuperscript{35}S]GTP\gamma S binding in the presence of WIN 55,212-2 and an EC\textsubscript{50} value of 433 nM.

![Figure 4.5a](image)

**Figure 4.5a - GDP on basal [\textsuperscript{35}S]GTP\gamma S binding in CHO/CB\textsubscript{1}f cell membranes.** The effect GDP (0 \textmu M - 50 \textmu M) on basal [\textsuperscript{35}S]GTP\gamma S binding in CHO/CB\textsubscript{1}f cell membranes (0.1\% w/v) was determined (n=1). Membranes were incubated at 30\textdegree C for 90 minutes with 150 mM Na\textsuperscript{+} and 2.5 mM Mg\textsuperscript{2+}. The 0 \textmu M GDP point (43788 dpm) was removed to show better separation of other data points.
Table 4.2 - The effect of GDP on basal $[^{35}]$GTP$\gamma$S binding in CHO/CB,f cell membranes

<table>
<thead>
<tr>
<th>[GDP], $\mu$M</th>
<th>Basal $[^{35}]$GTP$\gamma$S Bound (dpm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>43788</td>
</tr>
<tr>
<td>1</td>
<td>18146</td>
</tr>
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<td>5</td>
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</tr>
<tr>
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<td>4018</td>
</tr>
<tr>
<td>50</td>
<td>2786</td>
</tr>
</tbody>
</table>

CHO/CB,f cell membranes (0.1% w/v) were incubated with GDP (0 $\mu$M - 50 $\mu$M) in the absence of WIN 55,212-2.

Figure 4.5b - GDP on WIN 55,212-2-stimulated $[^{35}]$GTP$\gamma$S binding in CHO/CB,f cell membranes. The effect GDP (50 $\mu$M) on WIN 55,212-2-stimulated (1 nM - 30 $\mu$M) $[^{35}]$GTP$\gamma$S binding in CHO/CB,f cell membranes (0.1% w/v) was determined (n=1). Membranes were incubated at 30°C for 90 minutes with 150 mM Na$^+$ and 2.5 mM Mg$^{2+}$. No stimulation was seen on the absence of GDP (squares). WIN 55,212-2 produced a 71% increase in basal $[^{35}]$GTP$\gamma$S binding in the presence of GDP (50 $\mu$M; triangles) with an EC$_{50}$ value of 433 nM. WIN 55,212-2 was initially dissolved in 100% ethanol to produce a 2 mM stock solution. Further dilutions were done using assay buffer.
4.3.2 Other Cannabinoids on Basal \[^{35}\text{S}]\text{GTP} \gamma \text{S} Binding in CHO/CB, Cell Membranes

4.3.2.a HU-210, CP55,940 and WIN 55,212-2 on Basal \[^{35}\text{S}]\text{GTP} \gamma \text{S} Binding in CHO/CB, Cell Membranes

HU-210 (0.01 nM - 1 \mu M) dose-dependently increased basal \[^{35}\text{S}]\text{GTP} \gamma \text{S} binding by 60.8\% with an EC\textsubscript{50} value of 3.34 nM (n=3; Figure 4.6) in CHO/CB, cell membranes incubated at 30\(^\circ\)C for 90 minutes with Na\(^+\) (150 mM), Mg\(^{2+}\) (2.5 mM) and GDP (50 \mu M). CP55,940 (0.01 nM - 10 \mu M) dose-dependently increased basal \[^{35}\text{S}]\text{GTP} \gamma \text{S} binding by 64.5\% with an EC\textsubscript{50} value of 92.4 nM (n=3; Figure 4.6) WIN 55,212-2 (1 nM - 100 \mu M), shown for comparison, increased basal \[^{35}\text{S}]\text{GTP} \gamma \text{S} binding by 73.2\% with an EC\textsubscript{50} value of 649 nM (n=3; Figure 4.6). Non-linear regression analysis determined that HU-210, CP55,940 and WIN 55,212-2 stimulation of \[^{35}\text{S}]\text{GTP} \gamma \text{S} binding were mediated through a single high affinity site. The Paired t-test showed that there was no significant difference in the maximal stimulation among these three compounds (p<0.05). Data are summarized in Table 4.3.
Figure 4.6 - HU-210, CP55,940 and WIN 55,212-2 on basal [\( ^{35} \)S]GTP\( \gamma \)S binding in CHO/CB, f cell membranes. CHO/CB, f cell membranes (0.1% w/v) were incubated with HU-210 (0.01 nM - 1 µM; circles; n=3), CP55,940 (0.01 nM - 10 µM; triangles; n=3), or WIN 55,212-2 (1 nM - 100 µM; squares; n=3) at 30°C for 90 minutes with Na\(^+\) (150 mM), Mg\(^{2+}\) (2.5 mM) and GDP (50 µM). HU-210 increased basal [\( ^{35} \)S]GTP\( \gamma \)S binding by 60.8% with an EC\(_{50}\) value of 3.34 nM. CP55,940 increased basal [\( ^{35} \)S]GTP\( \gamma \)S binding by 64.5% with an EC\(_{50}\) value of 92.4 nM. WIN 55,212-2 increased basal [\( ^{35} \)S]GTP\( \gamma \)S binding by 73.2% with an EC\(_{50}\) value of 649 nM. Symbols represent the mean response and the vertical lines represent the S.E.M. HU-210, CP55,940 and WIN 55,212-2 were initially dissolved in 100% ethanol to produce 2 mM stock solutions. Further dilutions were done using assay buffer.
4.3.2.b WIN 55,212-2 and Methanandamide on Basal $[^{35}\text{S}]\text{GTP} \gamma \text{S}$ Binding in CHO/CB,f Cell Membranes

Methanandamide (1 nM - 30 μM) increased basal $[^{35}\text{S}]\text{GTP} \gamma \text{S}$ binding by 20.4% with an EC$_{50}$ value of 709 nM (n=3; Figure 4.7) in CHO/CB,f cell membranes incubated at 30°C for 90 minutes with Na$^+$ (150 mM), Mg$^{2+}$ (2.5 mM) and GDP (50 μM). WIN 55, 212-2 (1 nM - 100 μM), shown for comparison, increased basal $[^{35}\text{S}]\text{GTP} \gamma \text{S}$ binding by 73.2% with an EC$_{50}$ value of 649 nM (n=3; Figure 4.7). Non-linear regression analysis determined that methanandamide-stimulated $[^{35}\text{S}]\text{GTP} \gamma \text{S}$ binding was mediated through a single high-affinity site. Data are summarized in Table 4.3.

![Figure 4.7 - WIN 55,212-2 and methanandamide on basal $[^{35}\text{S}]\text{GTP} \gamma \text{S}$ binding in CHO/CB,f cell membranes. CHO/CB,f cell membranes (0.1% w/v) were incubated with methanandamide (1 nM - 30 μM; n=3) at 30°C for 90 minutes with Na$^+$ (150 mM), Mg$^{2+}$ (2.5 mM) and GDP (50 μM). Methanandamide increased basal $[^{35}\text{S}]\text{GTP} \gamma \text{S}$ binding by 20.4% with an EC$_{50}$ value of 709 nM. WIN 55,212-2 increased basal $[^{35}\text{S}]\text{GTP} \gamma \text{S}$ binding by 73.2% with an EC$_{50}$ value of 649 nM. Symbols represent the mean response and the vertical lines represent the S.E.M. WIN 55,212-2 was initially dissolved in 100% ethanol to produce a 2 mM stock solution. Methanandamide was initially dissolved in 100% ethanol to produce 1 mM stock solution. Further dilutions were done using assay buffer.](image-url)
4.3.2.c WIN 55,212-2 and 11-OH-Δ⁹-THC on Basal [³⁵S]GTPγS Binding in CHO/CB,f Cell Membranes

11-OH-Δ⁹-THC (1 nM - 100 μM) stimulated basal [³⁵S]GTPγS binding by 9.4% with an EC₅₀ value of 3.50 μM (n=3; Figure 4.8) in CHO/CB,f cell membranes incubated at 30°C for 90 minutes with Na⁺ (150 mM), Mg²⁺ (2.5 mM) and GDP (50 μM). WIN 55,212-2 (1 nM - 100 μM), showed for comparison, increased basal [³⁵S]GTPγS binding by 73.2% with an EC₅₀ value of 649 nM (n=3; Figure 4.8). Non-linear regression analysis determined that 11-OH-Δ⁹-THC stimulation of [³⁵S]GTPγS binding was through a single high affinity site. Data are summarized in table 4.3.

Figure 4.8 - WIN 55,212-2 and 11-OH-Δ⁹-THC on basal [³⁵S]GTPγS binding in CHO/CB,f cell membranes. CHO/CB,f cell membranes (0.1% w/v) were incubated with 11-OH-Δ⁹-THC (1 nM - 100 μM; n=3) at 30°C for 90 minutes with Na⁺ (150 mM), Mg²⁺ (2.5 mM) and GDP (50 μM). 11-OH-Δ⁹-THC stimulated basal [³⁵S]GTPγS binding by 9.4% with an EC₅₀ value of 3.50 μM. WIN 55,212-2 stimulated basal [³⁵S]GTPγS binding by 73.2% with an EC₅₀ value of 649 nM. Symbols represent the mean response and the vertical lines represent the S.E.M. WIN 55,212-2 and 11-OH-Δ⁹-THC were initially dissolved in 100% ethanol to produce 2 mM stock solutions. Further dilutions were done using assay buffer.
Table 4.3 - $EC_{50}$ and $E_{max}$ values of various cannabinoid agonists in stimulating basal $[^{35}S]GTP\gamma S$ binding

<table>
<thead>
<tr>
<th>Cannabinoid Agonist</th>
<th>$E_{max}$ (% above basal)</th>
<th>$EC_{50}$ value (nM)</th>
<th>Agonist Classification</th>
</tr>
</thead>
<tbody>
<tr>
<td>WIN 55,212-2</td>
<td>72.3</td>
<td>649</td>
<td>full</td>
</tr>
<tr>
<td>CP55,940</td>
<td>64.5</td>
<td>92.4</td>
<td>full</td>
</tr>
<tr>
<td>HU-210</td>
<td>60.8</td>
<td>3.34</td>
<td>full</td>
</tr>
<tr>
<td>Methanandamide</td>
<td>20.4</td>
<td>709</td>
<td>partial</td>
</tr>
<tr>
<td>11-OH-$\Delta^9$-THC</td>
<td>9.4</td>
<td>3500</td>
<td>weak partial</td>
</tr>
</tbody>
</table>

CHO/CB, f cell membranes (0.1% w/v) were incubated with Na+ (150 mM), Mg$^{2+}$ (2.5 mM) and GDP (50 μM) at 30°C for 90 minutes in the presence of various cannabinoid ligands. Agonist classification based on the current studies.
4.3.3 Pertussis Toxin Pretreatment on WIN 55,212-2-Stimulated [³⁵S]GTPγS Binding in CHO/CB,f Cell Membranes

In the absence of pertussis toxin, WIN 55,212-2 (1 nM - 100 μM) maximally stimulated basal [³⁵S]GTPγS binding 41.6% with an EC₅₀ value of 302 nM. The pretreatment (24 hours) of whole CHO/CB,f cells with pertussis toxin (50 ng/ml) abolished WIN 55,212-2-stimulated basal [³⁵S]GTPγS binding (n=1). The [³⁵S]GTPγS assay was performed by incubating CHO/CB,f cell membranes (0.1% w/v) at 30°C for 90 minutes as described in Section 4.2.4. Na⁺ (150 mM), Mg²⁺ (2.5 mM) and GDP (50 μM) were used. These data are shown in Figure 4.9.

![Figure 4.9 - Pertussis toxin pretreatment on WIN 55,212-2-stimulated [³⁵S]GTPγS binding in CHO/CB,f cell membranes. CHO/CB,f cell membranes (0.1% w/v) either without (squares), or with (triangles) pretreatment with pertussis toxin (50 ng/ml; n=1) were stimulated with WIN 55,212-2 (1 nM - 100 μM) and incubated at 30°C for 90 minutes with Na⁺ (150 mM), Mg²⁺ (2.5 mM) and GDP (50 μM). Pertussis toxin was added to whole CHO/CB,f cells 24 hours before the experiment. WIN 55,212-2 was initially dissolved in 100% ethanol to produce a 2 mM stock solution. Further dilutions were done using assay buffer.](image-url)
4.3.4 Forskolin on cAMP Formation in Whole Wild-Type CHO and CHO/CBf Cells

In both whole wild-type CHO and CHO/CBf cells, forskolin (1 nM - 300 μM) dose-dependently stimulated cAMP formation (n=3; Figure 4.10). Forskolin produced a maximal cAMP level in wild-type CHO cells of 579 pmol/10^6 cells with an EC_{50} value of 31.5 μM. The maximal forskolin-stimulated cAMP level in CHO/CBf cells was calculated to be 1231 pmol/10^6 cells with an EC_{50} value of 54.0 μM.

4.3.5 CP55,940 and WIN 55,212-2 on Forskolin-Stimulated cAMP Formation in Whole Wild-Type CHO and CHO/CBf Cells

CP55,940 (0.1 nM - 10 μM) inhibited forskolin-stimulated (10 μM)cAMP formation by 95.1% with an EC_{50} value of 14.7 nM (Figure 4.11) in whole CHO/CBf cells. WIN 55,212-2 (1 nM - 30 μM) inhibited forskolin-stimulated (10 μM) cAMP formation by 98.7% with an EC_{50} value of 143 nM (Figure 4.11). WIN 55,212-2 had no effect on forskolin-stimulated cAMP formation in wild-type CHO cells (data not shown).

4.3.6 Methanandamide and 11-OH-Δ^9-THC on Forskolin-Stimulated cAMP Formation in Whole Wild-Type CHO and CHO/CBf Cells

Methanandamide (1 nM - 30 μM) inhibited forskolin-stimulated (10 μM)cAMP formation by 97.7% with an EC_{50} value of 432 nM (Figure 4.12) in whole CHO/CBf cells. 11-OH-Δ^9-THC (1 nM - 30 μM) inhibited forskolin-stimulated (10 μM) cAMP formation by 88.5% with an EC_{50} value of 140 nM (Figure 4.12).
Figure 4.10 - Forskolin dose-response curve in whole wild-type CHO and CHO/CB,t cells. Whole wild-type CHO (n=3; squares) and CHO/CB,t (n=3; triangles) cells were incubated with forskolin (1 nM - 300 μM). For clarity, the graph was cut at 100 nM forskolin. Forskolin produced a maximal cAMP level in wild-type CHO cells of 579 pmol/10^6 cells with an EC_{50} value of 31.5 μM. The maximal forskolin-stimulated cAMP levels in CHO/CB,t cells was 1231 pmol/10^6 cells with an EC_{50} value of 54.0 μM. Symbols represent the mean response and the vertical lines represent the S.E.M. Forskolin was initially dissolved in water. Further dilutions were done using IMDM.
Figure 4.11 - Inhibition of forskolin-stimulated (10 μM) cAMP formation in whole CHO/CB,f cells by CP55,940 and WIN 55,212-2. Whole CHO/CB,f cells were incubated with forskolin (10 μM) in the presence of either CP55,940 (0.1 nM - 10 μM; inverted triangles; n=3), or WIN 55,212-2 (1 nM - 30 μM; squares; n=3). CP55,940 dose-dependently inhibited forskolin-stimulated cAMP formation by 95.1% with an EC_{50} value of 14.7 nM in whole CHO/CB,f cells. WIN 55,212-2 dose-dependently inhibited forskolin-stimulated cAMP formation by 98.7% with an EC_{50} value of 143 nM. Symbols represent the mean response and the vertical lines represent the S.E.M. Forskolin was initially dissolved in water. CP55,940 and WIN 55,212-2 were initially dissolved in 100% ethanol to produce 1 mM stock solutions. Further dilutions of all drugs were done using IMDM.

Figure 4.12 - Inhibition of forskolin-stimulated (10 μM) cAMP formation in whole CHO/CB,f cells by Methanandamide and 11-OH-Δ²-THC. Whole CHO/CB,f cells were incubated with forskolin (10 μM) in the presence of either Methanandamide (1 nM - 30 μM; triangles; n=3), or 11-OH-Δ²-THC (1 nM - 30 μM; squares; n=3). Methanandamide dose-dependently inhibited forskolin-stimulated cAMP formation by 97.7% with an EC_{50} value of 432 nM in whole CHO/CB,f cells. 11-OH-Δ²-THC dose-dependently inhibited forskolin-stimulated cAMP formation by 88.5% with an EC_{50} value of 140 nM. Symbols represent the mean response and the vertical lines represent the S.E.M. Forskolin was initially dissolved in water. Methanandamide and 11-OH-Δ²-THC were initially dissolved in 100% ethanol to produce 1 mM stock solutions. Further dilutions of all drugs were done using IMDM.
4.3.7 Pertussis Toxin Pretreatment on WIN 55,212-2 Inhibition of Forskolin-Stimulated cAMP Formation in Whole CHO/CB,f Cells

The effects of either 0, 0.1, 1, 10, 50, 100 ng/ml, or 1 μg/ml pertussis toxin pretreatment on forskolin-stimulated (10 μM) cAMP production were investigated. At all pertussis toxin concentrations, cells were viable and produced cAMP (Figure 4.13). For all future experiments, 50 ng/ml of pertussis toxin was used. In the absence of pertussis toxin, WIN 55,212-2 inhibited forskolin-stimulated (10 μM) by 89.0% with an EC₅₀ value of 165 nM (Figure 4.14). In the presence of pertussis toxin (50 ng/ml) the WIN 55,212-2 dose-response curve was shifted to the right 83.6-fold to produce an EC₅₀ value of 13.8 μM. The extrapolated maximal percent inhibition by WIN 55,212-2 in the presence of pertussis toxin was 72.6% (Figure 4.14).

Figure 4.13 - Pertussis toxin pretreatment on forskolin-stimulated (100 μM) cAMP formation in whole CHO/CB,f cells. The effect of pertussis toxin on basal (left side of graph) and forskolin-stimulated (right side of graph; 100 μM forskolin) cAMP formation was examined (n=3). CHO/CB,f cells were incubated for 24 hours in either the absence (single dots), or presence of either 0.1 ng/ml (light grey), 1 ng/ml (dark grey), 10 ng/ml (black), 100 ng/ml (horizontal lines), or 1 μg/ml (vertical lines) pertussis toxin.
Figure 4.14 - Pertussis toxin pretreatment on WIN 55,212-2 inhibition of forskolin-stimulated (10 μM) cAMP formation in whole CHO/CB1f cells. WIN 55,212-2 inhibition of forskolin-stimulated (10 μM) cAMP formation in whole CHO/CB1f cells (squares) was inhibited by 24 hour pretreatment with pertussis toxin (50 ng/ml; triangles). The experiment was done three times. Symbols represent the mean response and the vertical lines indicate the S.E.M. Forskolin was initially dissolved in distilled water. WIN 55,212-2 was initially dissolved in 100% ethanol to produce a 2 mM stock solution. Further dilutions of both drugs were done using IMDM to give a final forskolin concentration of 10 μM and final WIN 55,212-2 concentrations of 1.0 nM - 30 μM. Pertussis toxin was bought as a 50% glycerol/50 mM Tris solution.
4.4 DISCUSSION

To verify that the expressed cannabinoid receptors are functionally coupled to downstream effector systems, various cannabinoid receptor ligands were used to attempt to stimulate $[^{35}\text{S}]\text{GTP} \gamma \text{S}$ binding in CHO/CB$_1$ f cell membranes. The use of the CHO/CB$_1$ f cell line to study various cannabinoid ligands makes it important to characterize the $[^{35}\text{S}]\text{GTP} \gamma \text{S}$ binding assay under various conditions. The most important consideration in characterizing this assay is not to choose the assay conditions which produce the maximal agonist-stimulated $[^{35}\text{S}]\text{GTP} \gamma \text{S}$ binding, but, since cannabinoid ligands exist which are full agonists, partial agonists and antagonists, it is more important to choose conditions in which the separation of full and partial agonists from each other and from antagonists can be seen.

The first thing which needed to be done was to confirm that the concentration of ethanol needed to dissolve the cannabinoid ligands for the assay did not affect cannabinoid-stimulated $[^{35}\text{S}]\text{GTP} \gamma \text{S}$ binding. Cannabinoids are highly lipophilic and it was, therefore, necessary to initially dissolve the majority of these compounds in 100% ethanol to produce a 2 mM stock solution and then to dilute this solution 1:1 in assay buffer to give a final stock concentration of 1 mM drug (50% final ethanol concentration) since most ligands dissolved in less than a 50% final ethanol concentration at 1 mM began to precipitate. Δ$_9$-THC and methanandamide needed to be dissolved to 1 mM in 100% ethanol to avoid precipitation. The concern of ligand precipitation was a limiting factor in determining the highest concentration of ligand to use in the functional assays. Therefore, the highest concentration of ethanol which could be used in the assays was determined by incubating CHO/CB$_1$f
membranes with WIN 55,212-2 (10 μM) and a final concentration of 0.1% - 25% ethanol. In the presence of WIN 55,212-2 (10 μM), ethanol concentrations of up to and including 5.0% decreased WIN 55,212-2-stimulated [35S]GTPγS binding by less than 5% whereas WIN 55,212-2-stimulated [35S]GTPγS binding was reduced more than 20% at ethanol concentrations of 10% or greater. For this reason, the concentration of ethanol used in all the remaining [35S]GTPγS binding assays discussed in this chapter was no greater than 5.0%. This limited the concentration of cannabinoid ligands in the assay to no greater than 100 μM.

Once the maximal ethanol concentration to be used in these assays was determined, it was then necessary to characterize various other parameters. Sodium (Na+) has been shown to allosterically regulate G protein-coupled receptors by interacting with the aspartate residue in the second transmembrane domain (Hortsman et al., 1990). As previously described, the addition of Na+ to the assay is necessary in order to see agonist-stimulated [35S]GTPγS binding in this adenosine receptor system (Lorenzen et al., 1993). In other cases, sodium was not necessary, but decreased basal [35S]GTPγS binding to such an extent that agonist-stimulated binding as a percent increase above basal levels was greatly increased (Selley et al., 1996). The present data demonstrate that, in the absence of sodium, WIN 55,212-2 stimulated basal [35S]GTPγS binding by 40.8% whereas, in the presence of 150 mM sodium, WIN 55,212-2 stimulated basal [35S]GTPγS binding by 113%. From these data alone it can be seen that the high sodium concentration produces a higher percent of WIN 55,212-2-stimulated [35S]GTPγS binding. In addition, basal [35S]GTPγS binding was approximately 4300 dpm in the absence of sodium, whereas it was approximately 1600 dpm in the presence
of 150 mM sodium. This high level of basal $[^{35}S]$GTP\textgamma S binding in the absence of sodium would make it extremely difficult to differentiate full agonists, partial agonists and antagonists. Therefore, a higher concentration of sodium (150 mM) is necessary to substantially decrease basal $[^{35}S]$GTP\textgamma S binding levels in order to observe a large increase in basal $[^{35}S]$GTP\textgamma S binding by cannabinoids.

High concentrations of magnesium (Mg$^{2+}$) are also necessary to measure agonist-stimulated $[^{35}S]$GTP\textgamma S binding to G proteins (Lorenzen et al., 1993). In order to obtain reasonable ratios between basal and agonist-stimulated $[^{35}S]$GTP\textgamma S binding at the A$_1$ adenosine receptor, Mg$^{2+}$ concentrations of 100 $\mu$M or greater were required. Hilf et al. (1989) demonstrated that appreciable muscarinic acetylcholine receptor-stimulated binding of guanosine 5'-O-(3-$[^{35}S]$thio)triphosphate to G proteins in cardiac membranes was not seen at Mg$^{2+}$ concentration less than 10 $\mu$M. The requirement for high magnesium concentrations was also the case to observe appreciable WIN 55,212-2-stimulated $[^{35}S]$GTP\textgamma S binding in CHO/CB$_1$ cell membranes. In the presence of 150 mM NaCl and 50 $\mu$M GDP, the absence of Mg$^{2+}$ produced maximal WIN 55,212-2-stimulated (100 $\mu$M) $[^{35}S]$GTP\textgamma S binding levels only 4.6% above basal binding with an EC$_{50}$ value of 2.67 $\mu$M. WIN 55,212-2-stimulated $[^{35}S]$GTP\textgamma S binding continued to increase up to 7.5 mM Mg$^{2+}$ to a maximum of 101% above basal. The substantial increase in WIN 55,212-2-stimulated $[^{35}S]$GTP\textgamma S binding in the presence of 2.5 mM Mg$^{2+}$ compared to 1 mM, along with the lower baseline in the presence of 2.5 mM Mg$^{2+}$ compared to higher Mg$^{2+}$ concentrations led to the decision to use 2.5 mM Mg$^{2+}$ in the remainder of the $[^{35}S]$GTP\textgamma S binding assays. In addition, this magnesium
concentration is consistent with that used to study cannabinoid-mediated $[^{35}S]$GTP$\gamma$S binding in rodent brain membranes in our laboratory (Burkey et al., 1997; K. Hosohata et al., 1997; Y. Hosohata et al., 1997).

The final parameter optimized was the concentration of GDP to use in the $[^{35}S]$GTP$\gamma$S binding assay. Though increasing the GDP concentration dose-dependently decreased the absolute amount of $[^{35}S]$GTP$\gamma$S binding in the presence of the agonist, carbachol, to porcine atrial membranes, it dose-dependently increased the $[^{35}S]$GTP$\gamma$S stimulation by the agonist relative to basal with a maximum carbachol-induced increase in $[^{35}S]$GTP$\gamma$S binding occurring between 0.1 $\mu$M - 1.0 $\mu$M GDP (Hilf et al., 1989). A minimum of 300 nM GDP was needed to observe fentanyl-stimulated $[^{35}S]$GTP$\gamma$S binding to human neuroblastoma SH-SY5Y cell membranes, while a maximal signal-to-noise ratio was achieved at 3 $\mu$M GDP (Traynor and Nahorski, 1995). Similarly, Selley et al. (1996) reported that GDP dose-dependently inhibited both basal and WIN 55,212-2-stimulated $[^{35}S]$GTP$\gamma$S binding to rat cerebellar membranes. However, the percent stimulation of $[^{35}S]$GTP$\gamma$S binding by WIN 55,212-2 increased with increasing concentrations of GDP, producing greater than a 150% stimulation above basal levels at 30 $\mu$M GDP. Similar results were observed by Lorenzen et al. (1993) where the optimal percent increase of agonist-induced $[^{35}S]$GTP$\gamma$S binding was not seen until 10 $\mu$M GDP.

In optimizing the $[^{35}S]$GTP$\gamma$S assay for CHO/CB1 cells, GDP concentrations ranging from 0 $\mu$M - 50 $\mu$M were used. It can be seen that increasing concentrations of GDP substantially lower the basal level of $[^{35}S]$GTP$\gamma$S binding. In addition, the absence of GDP
in the assay produced no observable WIN 55,212-2-stimulated \([^{35}S]\)GTP\(\gamma\)S binding, whereas maximum WIN 55,212-2-stimulated \([^{35}S]\)GTP\(\gamma\)S binding in the presence of GDP (50 \(\mu\)M) was 71% above basal levels, giving an EC\(_{50}\) value of 433 nM.

Once the \([^{35}S]\)GTP\(\gamma\)S binding assay was optimized for WIN 55,212-2, the determination that this system is sensitive enough to differentiate full agonists, partial agonists and antagonists was made by using other cannabinoid ligands in this assay. When run in parallel, WIN 55,212-2 produced a larger percent (73.2%) stimulation of basal \([^{35}S]\)GTP\(\gamma\)S binding than CP55,940 (64.5%; Figure 4.6). WIN 55,212-2 also produced a larger percent stimulation of basal \([^{35}S]\)GTP\(\gamma\)S binding than HU-210 (60.8%; Figure 4.6). However, the differences between maximal \([^{35}S]\)GTP\(\gamma\)S binding by WIN 55,212-2, CP55,940 and HU-210 were not statistically different (p<0.05) using the Paired t-test. Therefore, all three of these compounds were considered full agonist in the \([^{35}S]\)GTP\(\gamma\)S assay using CHO/CB\(_1\)f cell membranes. The EC\(_{50}\) values of WIN 55,212-2, CP55,940 and HU-210 were 649 nM, 92.4 nM and 3.34 nM, respectively. The EC\(_{50}\) value of HU-210 is in agreement with previous findings by Howlett et al. (1990) where they demonstrated that HU-210 inhibited forskolin-stimulated cAMP formation with an EC\(_{50}\) value of 1.8 nM in whole N18TG2 cells.

Methanandamide was a partial agonist in the \([^{35}S]\)GTP\(\gamma\)S assay, producing an \(E_{\text{max}}\) of 20.4% above basal levels and an EC\(_{50}\) value of 704 nM. 11-OH-\(\Delta^9\)-THC also was a partial agonist in this system, producing an \(E_{\text{max}}\) of \([^{35}S]\)GTP\(\gamma\)S binding 9.4% above basal levels and an EC\(_{50}\) value of 3.50 \(\mu\)M. These data show that the conditions used for the \([^{35}S]\)GTP\(\gamma\)S binding assay are suitable to observe partial agonism in CHO/CB\(_1\)f cell membranes.
Interestingly, WIN 55,212-2 only stimulated basal $[^{35}S]GTP\gamma S$ binding 41.6% above basal when the assay was performed in the presence of IMDM (Figure 4.9), as compared to 113% above basal when the cells were grown in Ham's F12 medium (Figure 4.3b). A possible explanation for this difference is that IMDM is not the required medium for CHO cells.

The reason the $[^{35}S]GTP\gamma S$ binding assay was performed first is that it is a simple and convenient way to initially characterize a G protein-coupled system. This assay measures the first step in G protein-coupled receptor activation. However, it was important to characterize this system using another assay, preferably one which measures activation of a downstream effector. For this reason, the ability of cannabinoids to mediate forskolin-stimulated cAMP formation was investigated. Another reason this assay was performed was that, unlike the $[^{35}S]GTP\gamma S$ binding assay which uses cell membranes, the cAMP assay uses intact cells. This procedure may give more reliable results since the preparation of cell membranes may disrupt both the cell membrane as well as internal structures and proteins. The first cAMP experiment was to study the effect of forskolin on both whole wild-type CHO cells and CHO/CB, f cells.

As expected, forskolin (1 nM - 300 µM) dose-dependently stimulated cAMP formation in both cell lines. The maximal forskolin-stimulated (300 µM) cAMP level in wild-type CHO cells was 579 pmol/10^6 cells. The corresponding EC_{50} value was 31.5 µM. The maximal forskolin-stimulated (300 µM) cAMP level in CHO/CB, f cells was 1231 pmol/10^6 cells with a corresponding EC_{50} value of 54.0 µM. A likely explanation for the
increased forskolin-stimulated cAMP formation in CHO/CB₁,f cells is that wild-type CHO cells have constitutively active G_0/G_1 (G_{i/o}) proteins. These constitutively active inhibitory proteins modulate adenylyl cyclase activity in wild-type CHO cells. However, the transfected cannabinoid receptors, which are coupled to G_{i/o}, sequester a percentage of the inhibitory G protein pool, removing a portion of the inhibitory effect on adenylyl cyclase and increasing the ability of this enzyme to produce cAMP in the presence (and absence) of forskolin.

Since cannabinoid receptors are coupled to G_{i/o} proteins (Howlett and Fleming, 1984; Gerard et al., 1991), activation of these receptors by cannabinoid agonists should inhibit forskolin-stimulated cAMP formation. The fact that WIN 55,212-2 and CP55,940 inhibited forskolin-stimulated (10 μM) cAMP formation in CHO/CB₁,f cells whereas WIN 55,212-2 had no effect in wild-type CHO cells provides additional support that these ligands are acting through the transfected cannabinoid CB₁ receptor. WIN 55,212-2, CP55,940, 11-OH-Δ⁹-THC and methanandamide were all full agonists in the cAMP assay. One reason 11-OH-Δ⁹-THC and methanandamide were full agonists in the cAMP assay, but partial agonist in the [³⁵S]GTPγS assay is that the relatively small response measured at the receptor ([³⁵S]GTPγS) is amplified downstream (cAMP). Unlike 11-OH-Δ⁹-THC and methanandamide, WIN 55,212-2 and CP55,940 maximally stimulate both systems.

One noticeable difference regarding WIN 55,212-2 is that the EC₅₀ value in the [³⁵S]GTPγS assay is 649 nM (Figure 4.6) whereas it is only 143 nM in the cAMP assay (Figure 4.11). A possible explanation for this is that the [³⁵S]GTPγS binding assay is done in the presence of 150 mM sodium which shifts the dose-response curve of the agonist to the
right (low affinity). This explanation is supported by the fact that WIN 55,212-2 is more potent in the \[^{35}\text{S}]\text{GTP} \gamma \text{S} \text{assay in the absence of sodium (28.6 nM)} \). The same rationale can be used for the difference in the effect of CP55,940 in the two assays. Both WIN 55,212-2 and CP55,940 are approximately 5 to 6-fold more potent in the cAMP assay. The similarity of the \(K_i \) (1 \( \mu \text{M} \)) and \(EC_{50} \) (649 nM) values for WIN 55,212-2 shows that there are few spare receptors in the CHO/CB, \(f\) cell line.

It was important to determine if the cannabinoid inhibition of forskolin-stimulated cAMP formation in CHO/CB, \(f\) cells was also coupled to \(G_{i\alpha} \) proteins. This was done by incubating these cells for 24 hours with pertussis toxin (50 ng/ml). Since pertussis toxin is toxic to cells, it was important to first determine a concentration of pertussis toxin which could prevent cannabinoid-mediated inhibition of forskolin-stimulated adenylyl cyclase without killing the cells. Cells were pretreated with 0 to 1 \( \mu \text{g/ml} \) pertussis toxin. At all concentrations of pertussis toxin chosen, CHO/CB, \(f\) cells stimulated with forskolin (100 \( \mu \text{M} \)) actually produced higher levels of cAMP to those not pretreated with pertussis toxin. A possible explanation for this is similar to that for why CHO/CB, \(f\) cells produce higher forskolin-stimulated cAMP levels than wild-type CHO cells. In this case, pertussis toxin is inactivating a large portion of the \(G\) protein pool and, since \(G_{i\alpha} \) proteins may be constitutively inhibiting adenylyl cyclase, the inhibition of \(G_{i\alpha} \) activity by the addition of pertussis toxin may allow for increased cAMP formation in the presence of forskolin.

In fact, it was shown that WIN 55,212-2 inhibition of forskolin-stimulated (10 \( \mu \text{M} \)) cAMP formation was sensitive to pertussis toxin (50 ng/ml). Though this concentration of
pertussis toxin was able to completely abolish WIN 55,212-2-stimulated $[^{35}\text{S}]\text{GTP}\gamma\text{S}$ binding (Figure 4.9) it did not completely abolish the inhibition of forskolin-stimulated cAMP formation by WIN 55,212-2 (Figure 4.13). In the absence of pertussis toxin, WIN 55,212-2 inhibited forskolin-stimulated (10 μM) by 89.0% with an EC$_{50}$ value of 165 nM. In the presence of pertussis toxin (50 ng/ml) the WIN 55,212-2 dose-response curve was shifted to the right 83.6-fold to produce an EC$_{50}$ value of 13.8 μM. The percent inhibition by WIN 55,212-2 in the presence of pertussis toxin was 72.6%. It is possible that the ethanol concentration (5%) used for the highest WIN 55,212-2 concentration (30 μM) decreased cAMP production which made it appear that pertussis toxin did not fully abolish this inhibition by WIN 55,212-2 since, at times, 5% ethanol did appear to have an effect in both the $[^{35}\text{S}]\text{GTP}\gamma\text{S}$ and cAMP assays. The reason pertussis toxin abolished the augmentation of cAMP formation by SR141716A (Figure 5.5) is likely due to the lower concentration of ethanol used (1%) at the highest SR141716A concentration (10 μM). The lower maximal SR141716A concentration compared to WIN 55,212-2 is due to the higher potency of SR141716A in this system. However, the small decrease in the curve of SR141716A in the presence of pertussis toxin (50 ng/ml; Figure 5.5) supports the belief that ethanol is responsible for this decrease since, if pertussis toxin was not totally abolishing the augmentation of forskolin-stimulated cAMP formation by SR141716A, an increase, rather than a decrease, would be seen. Therefore, it can be concluded that the cannabinoid CB$_1$ receptors in these CHO cells couple to G$_{i/o}$ proteins.
CHAPTER 5

The Discovery of Inverse Cannabinoid Agonists at the Human Cannabinoid CB₁ Receptor in CHO Cells

5.1 INTRODUCTION

For a couple of years after its synthesis SR141716A was believed to be a true receptor antagonist (Rinaldi-Carmona et al., 1994). This CB₁ receptor-selective compound antagonized the inhibitory effects of cannabinoid receptor agonists on both mouse vas deferens contractions and on adenylyl cyclase activity in rat brain membranes (Rinaldi-Carmona et al., 1994). SR141716A alone had no effect on basal or forskolin-stimulated adenylyl cyclase activity in either rat substantia nigra synaptosomes or at the human CB₁ receptor expressed in CHO cells. However, SR141716A reversed the inhibition of forskolin-stimulated adenylyl cyclase activity produced by the cannabinoid agonist, WIN 55,212-2, in rat brain synaptosomes with an IC₅₀ value of 48 nM, and by CP55,940 at the human CB₁ receptor with an IC₅₀ of 5.6 nM (Rinaldi-Carmona et al., 1994).

Intravenous injection of SR141716A in mice inhibited Δ⁹-THC-induced hypoactivity, hypothermia and antinociception at doses well below 3 mg/kg. However, above this dose, SR141716A stimulated locomotor activity (Compton et al., 1996). Richardson et al. (1997) have shown that intrathecal injection of 1.0 pM SR141716A evoked a significant thermal hyperalgesia in mice. In the above studies, SR141716A was suggested to be either inhibiting an endogenous cannabinoid system or acting as an inverse agonist. Inverse agonists are compounds which act opposite to those defined as agonists. Terranova
et al. (1996) have shown that, whereas cannabinoid agonists inhibited short term memory, SR141716A, when given alone, enhanced short-term olfactory memory in the social recognition test in rodents, and reduced memory deficit in aged rats. Navarro et al. (1997) reported that acute administration of SR141716A induced defensive responses in two anxiety tests, whereas cannabinoid agonists usually produce a placid relaxation. These findings also suggest that either the brain cannabinoid receptor is constitutively active, or that SR141716A is inhibiting an endogenous cannabinoid system. A receptor is constitutively active when it mediates downstream events, such as activating effectors, in the absence of ligand.

Evidence also exists showing that SR141716A may act as an inverse agonist at the human cannabinoid CB₁ receptor. Bouaboula et al. (1997) reported that SR141716A, in contrast to cannabinoid agonists, inhibited mitogen-activated protein kinase activation stimulated by insulin or insulin-like growth factor in CHO cells transfected with the human cannabinoid CB₁ receptor. In addition, this compound also inhibited basal [³⁵S]GTPγS binding in membranes prepared from these cells as well as prevented the inhibition of adenylyl cyclase mediated by autoactivated CB₁ receptors in intact cells. Using a similar system, MacLennan et al. (1998) confirmed the previous findings of inverse agonism of SR141716A in the [³⁵S]GTPγS binding assay. They also showed that this compound is an inverse agonist at the human cannabinoid CB₂ receptor.

AM630 (6-iodopravadoline) is a synthetic compound which binds to cannabinoid receptors (Pertwee et al., 1995). Interestingly, AM630 has different pharmacological actions when tested in different biological systems. Initial studies by Pertwee et al. (1995) have
shown that AM630 is an antagonist in the mouse isolated vas deferens as it attenuated the ability of the cannabinoid agonist, WIN 55,212-2, to inhibit electrically-evoked contractions in this tissue. The $K_a$ value of AM630 in antagonizing WIN 55,212-2 in this assay was calculated to be 36 nM. However, later studies by Pertwee et al. (1996) showed that AM630 is an agonist in the guinea pig myenteric plexus with an IC$_{50}$ value of 1.9 $\mu$M in inhibiting electrically-evoked contractions. Interestingly, additional studies by K. Hosohata et al. (1997) demonstrated that AM630 is an antagonist in the guinea pig brain with a $K_e$ value of 9.3 $\mu$M ($K_e$ = antagonist dissociation constant = [antagonist]/(dose-ratio)-1; Tallarida and Murray, 1987). The potential therapeutic benefits of cannabinoids in man make it important to study the effects of these compounds at human cannabinoid receptors. The present investigation examines the effects of WIN 55,212-2, SR141716A and AM630 in CHO/CB$_1$ membranes in the [$^{35}$S]GTP$_\gamma$S binding assay and of WIN 55,212-2 and SR141716A in the cAMP assay.

5.2 MATERIALS AND METHODS

5.2.1 Drugs

See Sections 3.2.1 and 4.2.1.

5.2.2 Production of a Stable CHO/CB$_1$ Cell Line

The CHO/CB$_1$f cell line was used for these studies. The procedure is described in Sections 2.2.5 and 2.2.6.
5.2.3 Mediation of $[^{35}S]$GTP$\gamma$S Binding in CHO/CB$_1$ Cell Membranes by Cannabinoid Ligands

Unless otherwise specified, assay tubes contained final concentrations of 0.1 nM $[^{35}S]$GTP$\gamma$S (1250 Ci/mmol, DuPont NEN, Boston, MA), 0.1% tissue (w/v) and either WIN 55,212-2 (0.1 nM - 100 $\mu$M; Research Biochemicals International, Natick, MA), SR141716A (0.1 pM - 1 $\mu$M; a gift from Gerard Le Fur, Sanofi Recherche, Montpellier, France), or AM630 (0.1 nM - 100 $\mu$M; a gift from Alexandros Makriyannis, Storrs, CT) and were brought to a final volume of 1 ml using assay buffer (25 mM Tris-HCl, 150 mM NaCl, 2.5 mM MgCl$_2$, 1.0 mM EDTA, 0.25% BSA, 50 $\mu$M GDP, 30 $\mu$M bestatin, 10 $\mu$M captopril and 0.1 mM PMSF, pH 7.4). WIN 55,212-2, SR141716A and AM630 (Figure 5.1) were initially dissolved in 100% ethanol to produce 2.0 mM stock solutions. Further dilutions were done using assay buffer. The amount of $[^{35}S]$GTP$\gamma$S bound at the lowest concentration of each ligand was referred to as basal binding for that ligand and was assigned a value of 100%. Tubes were incubated at 30°C for 90 minutes and filtered using a tissue harvester (Brandel Inc., Gaithersburg, MD) onto GF/B glass microfiber filters (Whatman International Ltd., Maidstone, England) presoaked for 90 minutes in assay buffer. Filters were washed 4 times with ice-cold GTP$\gamma$S wash buffer (25 mM Tris-HCl, 120 mM NaCl, pH 7.4). Scintillation cocktail (Ecolite, ICN Biomedical, Irvine, CA) was added and samples were stored at 4°C overnight and counted in a Beckman LS 6000SC Scintillation counter.
5.2.4 Pretreatment of CHO/CBF Cells with Pertussis Toxin Prior to the cAMP Assay

This procedure was the same as described in section 4.2.7 except for the substitution of SR141716A for WIN 55,212-2. Forskolin was initially dissolved in distilled water. SR141716A was initially dissolved in 100% ethanol to produce a 2 mM stock solution. Further dilutions of both drugs were done using IMDM to give a final forskolin concentration of 10 μM and final SR141716A concentrations of 0.1nM - 10 μM. Pertussis toxin was initially prepared in 50% glycerol/50 mM Tris.

5.2.5 Data Analysis

Data were analyzed by non-linear regression analysis using GraphPad Prism (GraphPad Software, Inc., San Diego, CA). Both one-site and two-site binding analysis were performed to determine best-fit. Significant (p<0.05) stimulation, or inhibition of basal \[^{35}S\]GTPγS binding was determined using One-Way Analysis of Variance (ANOVA) followed by the Newman-Keuls’ Multiple Range test (SigmaStat, SPSS, Chicago, IL).
Figure 5.1 - Structures of WIN 55,212-2 (top left), SR141716A (top right) and AM630 (bottom).
5.3 RESULTS

5.3.1 Cannabinoid-Mediated \(^{[35]}\text{S}\)GTP\(\gamma\)S Binding in CHO/CB\(_1\) Cell Membranes

WIN 55,212-2 dose-dependently stimulated basal \(^{[35]}\text{S}\)GTP\(\gamma\)S binding in CHO/CB\(_1\) cell membranes 76% above basal with an EC\(_{50}\) of 473 nM (Figure 5.2). In contrast, SR141716A dose-dependently inhibited basal \(^{[35]}\text{S}\)GTP\(\gamma\)S binding by 22.3% with an EC\(_{50}\) value of 0.82 nM (Figure 5.2). Non-linear regression analysis determined that both WIN 55,212-2 stimulation and SR141716A inhibition of basal \(^{[35]}\text{S}\)GTP\(\gamma\)S binding in CHO/CB\(_1\) cell membranes was best fit by a one-site model (Prism, GraphPad Software Inc., San Diego, CA). One-Way ANOVA and the Newman-Keuls’ Multiple-Range test showed that maximal \(^{[35]}\text{S}\)GTP\(\gamma\)S binding using 1 \(\mu\)M SR141716A was significantly lower than basal \(^{[35]}\text{S}\)GTP\(\gamma\)S binding (\(p<0.05\)). No effect of WIN 55,212-2 or SR141716A was seen in wild-type CHO cells (data not shown).

In a separate experiment, the effects of WIN 55,212-2 and AM630 at the human cannabinoid CB\(_1\) receptor in the \(^{[35]}\text{S}\)GTP\(\gamma\)S assay were determined (Figure 5.3). WIN 55,212-2 dose-dependently stimulated \(^{[35]}\text{S}\)GTP\(\gamma\)S binding 77.9% above basal levels with an EC\(_{50}\) value of 360 nM. In contrast, AM630 dose-dependently inhibited basal \(^{[35]}\text{S}\)GTP\(\gamma\)S binding by 20.9% with an EC\(_{50}\) value of 900 nM. Non-linear regression analysis determined that all data best fit a one-site model (Prism, GraphPad Software Inc., San Diego, CA). One-Way ANOVA and the Newman-Keuls’ Multiple-Range test showed that \(^{[35]}\text{S}\)GTP\(\gamma\)S binding using 10 \(\mu\)M and 100 \(\mu\)M AM630 was significantly different than lower \(^{[35]}\text{S}\)GTP\(\gamma\)S binding
These results show that WIN 55,212-2 is an agonist (positive intrinsic activity) and AM630 is an inverse agonist (negative intrinsic activity) at the human cannabinoid CB₁ receptor in CHO cells. No effect of WIN 55,212-2 or AM630 was seen in wild-type CHO cells (data not shown).

**Figure 5.2** - WIN 55,212-2 and SR141716A on basal [³⁵S]GTPyS binding in CHO/CB₁ cell membranes. The effect of WIN 55,212-2 (n=4; squares) and SR141716A (n=4; triangles) on [³⁵S]GTPyS (0.1 nM) binding to membranes prepared from CHO cells stably expressing the human cannabinoid CB₁ receptor. Membranes were incubated at 30°C for 90 minutes. WIN 55,212-2 stimulated [³⁵S]GTPyS binding 76% above basal levels with an EC₅₀ value of 473 nM. SR141716A inhibited basal [³⁵S]GTPyS binding by 22.3% with an EC₅₀ value of 0.82 nM. Symbols represent the mean response and the vertical lines indicate the S.E.M. Drugs were initially dissolved in 100% ethanol to produce 2 mM stock solutions. Further dilutions were done using assay buffer to give final WIN 55,212-2 concentrations of 1 nM - 100 µM and final SR141716A concentrations of 0.1 pM - 1 µM.
Figure 5.3 - WIN 55,212-2 and AM630 on basal [$^{35}$S]GTPyS binding in CHO/CB$_1$ cell membranes. The effects of WIN 55,212-2 (squares) and AM630 (triangles) on 0.1 nM [$^{35}$S]GTPyS binding to membranes prepared from CHO cells stably expressing the human cannabinoid CB$_1$ receptor. Membranes were incubated at 30°C for 90 minutes. WIN 55,212-2 stimulated [$^{35}$S]GTPyS binding 77.9% above basal levels with an EC$_{50}$ value of 360 nM. In contrast, AM630 inhibited basal [$^{35}$S]GTPyS binding by 20.9% with an EC$_{50}$ value of 900 nM. Symbols represent the mean response and the vertical lines indicate the S.E.M. Drugs were initially dissolved in 100% ethanol to produce 2 mM stock solutions. Further dilutions were done using assay buffer to give final WIN 55,212-2 and AM630 concentrations of 0.1 nM - 100 μM. Experiments were done at least 6 times.
5.3.2 SR141716A on Forskolin-Stimulated cAMP Formation in Whole CHO/Cbf Cells

The effect of SR141716A (0.1 nM - 3 μM) on forskolin-stimulated cAMP formation was examined. SR141716A augmented forskolin-stimulated (0.1 μM; n=2) cAMP formation 176% above that seen with forskolin alone (basal) with an EC$_{50}$ value of 167 nM (Figure 5.4a) whereas SR141716A augmented forskolin-stimulated (1 μM; n=2) cAMP formation 233% above basal with an EC$_{50}$ value of 112 nM (Figure 5.4b). In addition, SR141716A augmented forskolin-stimulated (10 μM; n=3) cAMP formation by 172% with an EC$_{50}$ value of 109 nM (Figure 5.4), whereas SR141716A augmented forskolin-stimulated (100 μM; n=4) cAMP formation by 59.1% with an EC$_{50}$ value of 52.3 nM (Figure 5.4d). SR141716A had no effect on forskolin-stimulated cAMP formation in wild-type CHO cells (data not shown). The effect of the agonist, WIN 55,212-2, is shown for comparison. WIN 55,212-2 inhibited forskolin-stimulated (10 μM; n=3) cAMP formation by 98.7% with an EC$_{50}$ value of 143 nM (Figure 5.4c).

Figure 5.4a - SR141716A augmentation of forskolin-stimulated (0.1 μM) cAMP formation in whole CHO/CB,f cells. Whole CHO/CB,f cells were incubated with SR141716A (0.1 nM - 3 nM) in the presence of forskolin (0.1 μM; squares; n=2). SR141716A augmented forskolin-stimulated cAMP formation by 176% with an EC_{50} value of 167 nM. Symbols represent the mean response and the vertical lines represent the S.E.M. Forskolin was dissolved in water. SR141716A was initially dissolved in 100% ethanol to produce a 1 mM stock solution. Further dilutions of both drugs were done using IMDM.

Figure 5.4b - SR141716A augmentation of forskolin-stimulated (1 μM) cAMP formation in whole CHO/CB,f cells. Whole CHO/CB,f cells were incubated with SR141716A (0.1 nM - 3 μM) in the presence of forskolin (1 μM; squares; n=2). SR141716A augmented forskolin-stimulated cAMP formation by 233% with an EC_{50} value of 112 nM. Symbols represent the mean response and the vertical lines represent the S.E.M. Forskolin was dissolved in water. SR141716A was initially dissolved in 100% ethanol to produce a 1 mM stock solution. Further dilutions of both drugs were done using IMDM.
Figure 5.4c - SR141716A and WIN 55,212-2 on forskolin-stimulated (10 μM) cAMP formation in whole CHO/CB1f cells. Whole CHO/CB1f cells were incubated with forskolin (10 μM) in the presence of either SR141716A (squares; 0.01 nM - 3 μM; n=3) or WIN 55,212-2 (triangles; 1 nM - 30 μM; n=3). SR141716A augmented forskolin-stimulated cAMP formation 172% above basal with an EC50 value of 109 nM. WIN 55,212-2 inhibited forskolin-stimulated cAMP formation by 98.7% with an EC50 value of 143 nM. Symbols represent the mean response and the vertical lines represent the S.E.M. Forskolin was dissolved in water. SR141716A and WIN 55,212-2 were initially dissolved in 100% ethanol to produce 1 mM stock solutions. Further dilutions of all drugs were done using IMDM.

Figure 5.4d - SR141716A augmentation of forskolin-stimulated (100 μM) cAMP formation in whole CHO/CB1f cells. Whole CHO/CB1f cells were incubated with forskolin (100 μM) in the presence of SR141716A (0.01 nM - 3 μM; n=3). SR141716A augmented forskolin-stimulated cAMP formation 59.1% above basal with an EC50 value of 52.3 nM. Symbols represent the mean response and the vertical lines represent the S.E.M. Forskolin was dissolved in water. SR141716A was initially dissolved in 100% ethanol to produce a 1 mM stock solution. Further dilutions of both drugs were done using IMDM.
5.3.3 Pertussis Toxin Pretreatment on the SR141716A Augmentation of Forskolin-Stimulated cAMP Formation in Whole CHO/CB,f Cells

SR141716A increased forskolin-stimulated (10 μM) cAMP levels 265% above basal with an EC₅₀ value of 38.3 nM in whole CHO/CB,f cells (Figure 5.5). This effect was inhibited by 24 hour pretreatment with pertussis toxin (50 ng/ml; Figure 5.5).

![Graph](image)

Figure 5.5 - Pertussis toxin pretreatment on SR141716A augmentation of forskolin-stimulated (10 μM) cAMP formation in whole CHO/CB,f cells. SR141716A increased forskolin-stimulated (10 μM) cAMP levels 265% above basal (squares) with an EC₅₀ value of 38.3 nM in whole CHO/CB,f cells. This effect was inhibited by 24 hour pretreatment with pertussis toxin (50 ng/ml; triangles). Experiment was done three times. Symbols represent the mean response and the vertical lines indicate the S.E.M. Forskolin was initially dissolved in distilled water. SR141716A was initially dissolved in 100% ethanol to produce a 1 mM stock solution. Further dilutions of both drugs were done using IMDM to give a final forskolin concentration of 10 μM and final SR141716A concentrations of 0.1nM - 10 μM. Pertussis toxin was initially prepared in 50% glycerol/50 mM Tris.
5.4 DISCUSSION

The major findings of this study are that, in the \([^{35}\text{S}]\text{GTP}\gamma\text{S}\) assay, SR141716A and AM630 are inverse agonists in membranes of CHO cells expressing the human cannabinoid CB₁ receptor. Incubation of CHO/CB₁ cell membranes with WIN 55,212-2 demonstrated a dose-dependent increase in basal \([^{35}\text{S}]\text{GTP}\gamma\text{S}\) binding. In contrast, SR141716A and AM630 dose-dependently decreased basal \([^{35}\text{S}]\text{GTP}\gamma\text{S}\) binding in these membrane preparations. Furthermore, SR141716A is also an inverse agonist in the cAMP assay in this cell system.

The finding that WIN 55,212-2 is a cannabinoid agonist is in agreement with other reports in the literature (Pacheco et al., 1991; Compton et al., 1992). Our discovery that SR141716A and AM630 are inverse cannabinoid agonists is novel. However, other investigators have recently reported that SR141716A is an inverse agonist (Bouaboula et al., 1997; MacLennan et al., 1998). These results differ from earlier findings in the literature where SR141716A has been shown to be a CB₁ receptor-selective antagonist in vitro (Rinaldi-Carmona et al., 1994). In mice, the ability of SR141716A to increase locomotor activity (Compton et al., 1996) and to produce hyperalgesia (Richardson et al., 1997) suggests that these two responses may occur via inverse agonism. Furthermore, our data for SR141716A in the cAMP assay agree with those of Bouaboula et al. (1997) who have shown that SR141716A (100 nM) augmented forskolin-stimulated (3 \(\mu\text{M}\)) cAMP production 76% above basal in CHO cells stably expressing the human cannabinoid CB₁ receptor.

Interestingly, the \(E_{\text{max}}\) and \(EC_{50}\) values of SR141716A in the cAMP assay do not correspond to the \(E_{\text{max}}\) and \(EC_{50}\) values for SR141716A in the \([^{35}\text{S}]\text{GTP}\gamma\text{S}\) binding assay. The
$E_{\text{max}}$ value for SR141716A in the cAMP assay is approximately 10-fold higher. SR141716A only inhibited basal $[^{35}\text{S}]\text{GTP} \gamma \text{S}$ binding approximately 22% whereas it increased cAMP formation by forskolin (10 $\mu$M) by 265% in the current study. One explanation for this difference in the $E_{\text{max}}$ values of SR141716A in the two assays is that the 22% inhibition by SR141716A in the $[^{35}\text{S}]\text{GTP} \gamma \text{S}$ assay is the largest inhibition possible under the assay conditions tested, the remaining measurable radioactivity being non-specific binding. Therefore, a higher baseline (in dpm) would show a larger percent decrease. Additionally, since the cAMP assay measures activity downstream from that of the $[^{35}\text{S}]\text{GTP} \gamma \text{S}$ assay, the signal may be amplified allowing a larger effect of SR141716A to be seen in the cAMP assay. The observation that SR141716A can maximally stimulate cAMP formation 172% - 265% in the presence of lower forskolin concentrations (0.1 $\mu$M - 10 $\mu$M) compared to the 59% increase seen with 100 $\mu$M forskolin is likely due to the possibility that higher forskolin concentrations already stimulate near maximal cAMP formation, diminishing the ability of SR141716A to increase cAMP production further.

Similarly, the $E_{50}$ value is approximately 200-fold higher (lower affinity) for SR141716A in the cAMP assay compared to that for the $[^{35}\text{S}]\text{GTP} \gamma \text{S}$ assay. One explanation for this difference in the $E_{50}$ values for SR141716A in these two functional assays is that while the $[^{35}\text{S}]\text{GTP} \gamma \text{S}$ binding assay measures radioligand binding to the $\alpha$ subunit of the G protein, the activation of adenylyl cyclase may involves the $\beta \gamma$ subunits. In fact, it was shown that $G_{\alpha \alpha}$ from rabbit liver activated adenylyl cyclase in the 0.1 nM - 1 nM range, whereas effects of $\beta \gamma$ required concentrations in the 1 nM - 20 nM range (Tang and Gilman, 1991).
Despite these interesting findings the question remains whether SR141716A and AM630 are acting as inverse agonists by inhibiting the constitutively active receptor or are simply inhibiting an endogenous cannabinoid ligand in a dose-dependent manner. Using the $[^{35}S]$GTP$\gamma$S assay MacLennan et al. (1998) provided evidence that SR141716A is an inverse agonist inhibiting a constitutively active human cannabinoid CB$_1$ receptor by showing that another cannabinoid ligand, cannabinol, had no effect in this assay at a concentration 100-fold higher than its binding affinity. They, therefore, concluded that endogenous cannabinoid receptor agonist were not a significant factor in CHO cells stably expressing the human cannabinoid CB$_1$ receptor. Since, if endogenous cannabinoid ligands did exist, there would likely be no neutral antagonists, such as cannabinol, only inverse agonists since these "antagonists" would be dose-dependently inhibiting the actions of the endogenous ligands, appearing to be inverse agonists.

Further evidence that the human cannabinoid CB$_1$ receptor is constitutively active is seen by the fact that 24 hour pretreatment of CHO/CB$_1$f cells with pertussis toxin, which uncouples G proteins from receptors without altering the ability to bind GTP (or $[^{35}S]$GTP$\gamma$S), decreased basal levels of $[^{35}S]$GTP$\gamma$S binding (2376 dpm vs 1620 dpm). If the cannabinoid receptor was not constitutively interacting with G proteins then pertussis toxin should have no effect on basal $[^{35}S]$GTP$\gamma$S binding. This effect of pertussis toxin in lowering basal $[^{35}S]$GTP$\gamma$S binding provided evidence for constitutive activity of another G$_{\text{o}}$ protein-coupled receptor, the $\delta$-opioid receptor (Costa and Herz, 1989; Szekeres and Traynor, 1997).
Various models have been proposed to attempt to explain drug-receptor interactions (Bond et al., 1995; Bouaboula et al., 1997). A two-state model has been proposed for inverse agonists which states that a receptor can exist in either an active ($R^*$) or inactive ($R$) state (Figure 5.6; Bond et al., 1995). In the absence of a ligand these two states are in equilibrium (not necessarily 50:50), producing some level of basal activity. Agonists are able to preferentially bind to $R^*$, producing a shift in this equilibrium. In order to maintain the equilibrium of $R$:$R^*$, a percentage of $R$ is converted to $R^*$, resulting in less inactive ($R$) receptor and subsequent activation of the system. Conversely, according to this model, inverse agonists, such as SR141716A and AM630, are believed to preferentially bind to the inactive receptor ($R$), again shifting the equilibrium. A percentage of $R^*$ is then converted to $R$ to maintain equilibrium. This results in a larger amount of $R$ and, therefore, inhibition of the original basal activity.
Figure 5.6 - **Two-state model of receptor activation.** Ligand (L) can bind either to inactive (R) or active (R*) receptor conformations. $K_r$ represents the equilibrium constant between R and R* in the absence of ligand. $K_L$ and $K_{L*}$ are the dissociation equilibrium constants for the inactive and active receptor conformations, respectively. $K_r = [R]/[R^*]$; $K_L = [L][R]/[LR]$; $K_{L*} = [L][R^*]/[LR^*]$. Agonists promote receptor activation (LR*) whereas inverse agonists stabilize the inactive receptor conformation (LR). Reproduced from Bond et al. (1995).

In 1997, Bouaboula et al. discussed a ternary complex model to explain the inverse agonist properties of SR141716A (Figure 5.7). This model considered an inactive state of the receptor (R°), a positive state (R+) and a negative state (R-) as well as an associated G protein. In the R° state, the receptor is uncoupled from the G protein, whereas R+ and R- are able to couple to G proteins. Antagonists are believed to stabilize the R° state, agonists stabilize the R+ state, and inverse agonists preferentially stabilize the R- state. R+/G represents the active positive conformation and leads to agonist-induced activation of the system by classical substitution of GTP for prebound GDP on the G protein. In contrast, R-/G prevents the associated G protein to release GDP and bind GTP, stabilizing the inactive form of the receptor.
This leads to the question of how can SR141716A, which acts at the inhibitory ($G_{\text{o/o}}$-coupled) cannabinoid CB₁ receptor, *increase* forskolin-stimulated cAMP formation? The observation that the human cannabinoid CB₁ receptor expressed in CHO cells is constitutively active, since SR141716A inhibited basal [³²P]GTPγS binding in CHO/CB₁ cell membranes, likely means that the interaction of SR141716A with the human cannabinoid CB₁ receptor decreases the amount of active $G_\alpha$ protein associated with this receptor. Therefore, if constitutive $\beta\gamma$ subunits endogenously inhibit adenylyl cyclase then
the increased "free" $G_\alpha$ subunits liberated by SR141716A binding to the CB$_1$ receptor, by decreasing constitutive $G_\alpha$ binding to the CB$_1$ receptor, would be able to sequester a larger portion of "free" $\beta\gamma$ subunits which would then allow for increased activity of adenylyl cyclase.

An initial attempt to determine if $\beta\gamma$ was involved in the SR141716A-induced increase in forskolin-stimulated cAMP formation in CHO/CB$_1$f cells was to repeat the experiment after pretreatment of cells with pertussis toxin. Though this experiment would not clearly demonstrate the involvement of $\beta\gamma$, it would rule out the possibility that SR141716A, either directly, or indirectly, produced this effect through activation of $G_\gamma$. Since pertussis toxin pretreatment abolished the increase in cAMP formation by SR141716A, $G_\gamma$ was ruled out as a possible mechanism of the augmented forskolin-stimulated adenylyl cyclase activity. Since ADP-ribosylation of the $G_\alpha$ subunit by pertussis toxin inhibits the dissociation of and, therefore, the effects of both $G_\alpha$ and $\beta\gamma$ subunits of inhibitory $G$ protein-coupled receptors, the possibility still remains that $\beta\gamma$ is involved in the observed increase in adenylyl cyclase activity in the presence of SR141716A.

Various approaches exist which should help to determine the role of $\beta\gamma$ in the increase in forskolin-stimulated cAMP formation by SR141716A. Of the nine isoforms of mammalian adenylyl cyclases cloned, CHO cells were shown to have adenylyl cyclases VI and VII (Varga et al., 1998). An early report stated that AC V and AC VI appeared to be immune to the regulatory effects of $\beta\gamma$ (Iyengar, 1993). However, a recent report by Bayewitch et al. (1998) stated that $\beta\gamma$ can inhibit both AC types V and VI. This inhibition
was demonstrated by cotransfecting COS-7 cells with the cDNA's for both AC V and the α
subunit of transducin, αr. αr acts to scavenge free βγ subunits. Bayewitch et al. (1998)
showed that the addition of αr to AC V-transfected COS-7 cells augments forskolin-
stimulated (1 μM) cAMP accumulation.

SR141716A may also be producing its effect on cAMP formation by regulating AC
VII. In fact, it has been shown that AC VII and AC II, which is closely related to type VII,
contain the QXXER (Gln-X-X-Glu-Arg) motif. These amino acids have been shown to be
involved in βγ interaction with AC II (Chen et al., 1995; Tang and Gilman, 1991). This βγ
binding motif is also conserved in regions of potassium channels and β-adrenergic receptor
kinases that participate in βγ interactions. AC VII, which contains the closely related
RXXER motif, may also be susceptible to stimulation by βγ subunits (Chen et al., 1995).

Other approaches, which are based on a "scavenging" concept similar to that of αr,
have also been reported and may be helpful in elucidating the mechanism of this SR141716A
augmentation of forskolin-stimulated cAMP formation. The first method is to express the
carboxy terminus of the β-adrenergic receptor kinase in CHO/CB1f cells. This 195 amino
acid βARK1 "minigene", which binds βγ, has been shown to markedly inhibit
phosphoinositol hydrolysis in response to activation of α2-adrenergic and M2-muscarinic
receptors, which stimulate phospholipase C via Gβγ (Koch et al., 1994). Again, if SR141716A
is acting through βγ, then the expression of the βARK1 minigene should attenuate this
response.
QEHA27, which also contains the QXXER βγ binding motif found in AC II and several other effector proteins for βγ, has also recently been used in understanding the role of βγ on calcium current mediation by serotonin (Chen and Penington, 1997). Retinal phosducin (Bauer et al., 1992; Schulz et al., 1996) and phosducin-like protein (Schroder and Lohse, 1996) which also bind βγ, have been used.

In conclusion, the data presented here show that WIN 55,212-2 is an agonist whereas SR141716A and AM630 are inverse agonists in membranes of CHO cells expressing the human cannabinoid CB₁ receptor. Furthermore, the consistency of the current data with other reports in the cannabinoid literature supports the use of transfected CHO cells in studying the human cannabinoid CB₁ receptor. It is important to note that CHO cells do not endogenously express cannabinoid receptors. Therefore, it is not known if the receptor's tertiary conformation in the plasma membrane and its coupling mechanisms mimic that of endogenously expressed cannabinoid CB₁ receptors in human neurons. Further studies need to be done to determine the full potential of inverse cannabinoid agonists for use in man. Uses of inverse cannabinoid agonists, as seen in studies by Terranova et al. (1996), may be beneficial in the treatment of memory disorders in humans. Nevertheless, the development of other cannabinoids with greater negative intrinsic activity can be investigated using SR141716A and AM630 as prototypes.
CHAPTER 6

The Differential Effects of Δ⁹-THC at the Human Cannabinoid CB₁ Receptor in Two Functional Assays

6.1 INTRODUCTION

Δ⁹-tetrahydrocannabinol (Δ⁹-THC; Figure 6.1), the major psychoactive compound of the plant Cannabis sativa, is capable of producing maximal responses in certain in vivo bioassays similar to those for the more potent cannabinoids. For example, whereas mice treated with Δ⁹-THC experienced less inhibition of motor activity and a smaller temperature decrease than those treated with WIN 55,212-2, both groups experienced 100% of the maximum possible antinociception (Compton et al., 1992). Therefore, it can be concluded that Δ⁹-THC can act as either a partial or full agonist.

However, in vitro, Δ⁹-THC has recently been shown to be both a partial agonist and an antagonist. Δ⁹-THC only partially stimulated basal [³⁵S]GTPγS binding compared to the full agonist, WIN 55,212-2, in both rat cerebellar (Sim et al., 1996) and mouse whole brain (Burkey et al., 1997) membranes. Similarly, Δ⁹-THC was a partial agonist compared to WIN 55,212-2 in inhibiting glutamate-mediated synaptic transmission between cultured rat hippocampal neurons (Shen and Thayer, 1999). However, Δ⁹-THC is also a partial antagonist since it partially reversed the inhibition of excitatory postsynaptic current amplitude produced by WIN 55,212-2 (Shen and Thayer, 1999). Δ⁹-THC was also shown to dose-dependently antagonize WIN 55,212-2-stimulated [³⁵S]GTPγS binding in rat cerebellar membranes (Sim et al., 1996). Currently, Δ⁹-THC has not been shown to be an antagonist
in vivo. One possibility as to why a full agonist effect of Δ9-THC was seen in vivo, but not in vitro could be due to differences in either G protein coupling or the composition of the G protein pool among the various systems examined.

The current study shows that, depending on the functional assay, Δ9-THC can act either as a competitive antagonist, or as a partial agonist at the human cannabinoid CB1 receptor expressed in CHO cells. This compound acted as a neutral antagonist by producing no effect in the [35S]GTPγS assay when given alone, but shifted the dose-response curves of the agonist, WIN 55,212-2, and of the inverse agonist, SR141716A, to the right in a parallel manner. Δ9-THC, however, is a partial agonist in the cAMP assay where it inhibited forskolin-stimulated cAMP formation significantly less than the full agonists, WIN 55,212-2 and CP55,940 (Paired t-test; p<0.05).

Figure 6.1 - Structure of Δ9-tetrahydrocannabinol (Δ9-THC).
6.2 MATERIALS AND METHODS

6.2.1 Drugs

See Sections 3.2.1 and 4.2.1.

6.2.2 Tissue Preparation for the \[^{35}S\]GTP\gamma S Assay

On the day of the assay, frozen pellets were slowly thawed in ice and homogenized in 20 volumes of assay buffer containing 25 mM Tris-HCl, 150 mM NaCl, 2.5 mM MgCl\(_2\), 1.0 mM EDTA, 0.25% BSA, 50 \(\mu\)M GDP, 30 \(\mu\)M bestatin, 10 \(\mu\)M captopril and 0.1 mM PMSF, pH 7.4, using 10 strokes of a glass/teflon homogenizer and incubated for 30 minutes at 30°C. Cells were then centrifuged at 40,000 x g for 15 minutes then homogenized as above in assay buffer to give a stock tissue concentration of 1.0% (w/v). One hundred \(\mu\)l of tissue were used in each assay tube. This gave a final CB\(_1\) receptor concentration of approximately 0.21 pmol protein/tube (0.1% w/v) as determined by the Lowry assay (1951).

6.2.3 Assay Conditions for Cannabinoid-Mediated \[^{35}S\]GTP\gamma S Binding in CHO/CB\(_1\)f Cell Membranes

Assay tubes contained final concentrations of 0.1 nM \[^{35}S\]GTP\gamma S, 0.1% tissue (w/v), and either 1 nM - 100 \(\mu\)M WIN 55,212-2, 1 \(p\)M - 100 \(\mu\)M SR141716A, or 0.01 nM - 30 \(\mu\)M \(\Delta^9\)-THC and were brought to a final volume of 1.0 ml in assay buffer (25 mM Tris-HCl, 150 mM NaCl, 2.5 mM MgCl\(_2\), 1.0 mM EDTA, 0.25% BSA, 50 \(\mu\)M GDP, 30 \(\mu\)M bestatin, 10 \(\mu\)M captopril and 0.1 mM PMSF, pH 7.4). Another experiment was done in which WIN
55,212-2 was incubated alone, or in the presence of Δ⁹-THC (100 nM or 1 μM). Similarly, SR141716A was also incubated alone, or in the presence of Δ⁹-THC (30 μM). The amount of [³⁵S]GTPγS bound at the lowest concentration of each ligand was referred to as basal binding for that ligand and was assigned a value of 100%. Tubes were incubated at 30°C for 90 minutes and filtered using a tissue harvester (Brandel Inc., Gaithersburg, MD) onto GF/B glass microfiber filters (Whatman International Ltd., Maidstone, England). Filters were washed 4 times with ice-cold GTPγS wash buffer (25 mM Tris-HCl, 120 mM NaCl, pH 7.4). Scintillation cocktail (Ecolite, ICN Biomedical, Irvine, CA) was added and samples were stored at 4°C overnight and counted in a Beckman LS 6000SC Scintillation counter. WIN 55,212-2 and SR141716A were initially dissolved in ethanol (100%) to produce 2 mM stock solutions. Δ⁹-THC was dissolved in ethanol (100%) to produce a 1 mM stock solution. Further dilutions for all drugs were done using assay buffer.

6.2.4 Tissue Preparation for the cAMP Assay

CHO cells were plated in 24-well plates at 30,000 cells/well and allowed to grow for 48 hours before the assay. Wild-type CHO cells were grown in Ham’s F12 medium containing 10% fetal bovine serum, 100 U/ml penicillin and 100 μg/ml streptomycin. CHO/CB₁ cells were grown in the same media with the addition of 500 μg/ml G418.
6.2.5 cAMP Formation Studies

Cells stably expressing the human cannabinoid CB₁ receptor were plated in 24 well plates as described in section 6.2.4. See Section 4.2.7 for complete procedure.

6.2.6 Data Analysis

Data from all radioligand binding and functional assays were analyzed by non-linear regression analysis using Prism (GraphPad Software, Inc., San Diego, CA). One- and two-site binding analyses were performed to determine best-fit. Significant (p<0.05) stimulation, or inhibition of basal [³⁵S]GTPγS binding was determined using One-Way Analysis of Variance (ANOVA) followed by the Tukey test (SigmaStat, SPSS, Chicago, IL). Determination of full and partial agonists in both functional assays was made by comparing maximal percent effect among cannabinoids using the Paired t-test. Full and partial agonists were separated at p<0.05.
6.3 RESULTS

6.3.1 WIN 55,212-2, 11-OH-Δ⁹-THC and Δ⁹-THC on Basal [³⁵S]GTPγS Binding in
CHO/CB₁f Cell Membranes

WIN 55,212-2 dose-dependently stimulated [³⁵S]GTPγS binding 76.8% above basal
levels with an EC₅₀ value of 553 nM (Figure 6.2). 11-OH-Δ⁹-THC dose-dependently
stimulated [³⁵S]GTPγS binding 9.4% above basal levels with an EC₅₀ value of 3.5 μM
(Figure 6.2) Interestingly, Δ⁹-THC did not significantly affect basal [³⁵S]GTPγS binding
(Figure 6.2).

6.3.2 Δ⁹-THC on WIN 55,212-2-Stimulated and SR141716A-Inhibited [³⁵S]GTPγS Binding
in CHO/CB₁f Cell Membranes

Δ⁹-THC (100 nM) produced a parallel rightward shift (4.8-fold) in the WIN 55,212-2
dose-response curve, giving a dissociation constant (Kᵅ value) of 26.3 nM (Figure 6.3),
whereas Δ⁹-THC (1 μM) shifted the WIN 55,212-2 dose-response curve to the right 13-fold
in a competitive manner giving a Kᵅ value of 83.3 nM (Figure 6.3). Δ⁹-THC (30 μM) also
produced a parallel rightward shift (781-fold) in the SR141716A dose-response curve, giving
a Kᵅ value of 38.4 nM (Figure 6.4). The Kᵅ values were determined by dividing the
concentration of Δ⁹-THC used in each experiment by the EC₅₀ values of WIN 55,212-2, or
SR141716A in the presence and absence of Δ⁹-THC (dose-ratio; "DR") minus 1 for the
respective experiment (Kᵅ = [antagonist]/DR-1; Tallarida and Murray, 1987).
Figure 6.2 - WIN 55,212-2, 11-OH-Δ⁹-THC and Δ⁹-THC on basal [³²P]GTPyS binding in CHO/CB₁ cell membranes. CHO/CB₁ cell membranes (0.1% w/v) were incubated with either WIN 55,212-2 (1 nM - 30 μM; squares; n=4), 11-OH-Δ⁹-THC (1 nM - 100 μM; circles; n=3) or Δ⁹-THC (0.01 nM - 30 μM; triangles; n=16). WIN 55,212-2 dose-dependently stimulated [³²P]GTPyS binding 76.8% above basal levels with an EC₅₀ value of 553 nM. 11-OH-Δ⁹-THC dose-dependently stimulated [³²P]GTPyS binding 9.4% above basal levels with an EC₅₀ value of 3.5 μM. Δ⁹-THC did not significantly affect basal [³²P]GTPyS binding. Symbols represent the mean response and the vertical lines represent the S.E.M. WIN 55,212-2 and 11-OH-Δ⁹-THC were initially dissolved in 100% ethanol to produce 2 mM stock solutions. Δ⁹-THC was initially dissolved in 100% ethanol to produce a 1 mM stock solution. Further dilutions were done using assay buffer.
Figure 6.3 - $\Delta^9$-THC on WIN 55,212-2-stimulated $[^35]S$GTPyS binding in CHO/CB1 cell membranes. Squares (n=7) represent the effect of WIN 55,212-2 alone. Triangles (n=4) represent the effect of WIN 55,212-2 in the presence of 100 nM $\Delta^9$-THC. Inverted triangles (n=5) represent the effect of WIN 55,212-2 in the presence of 1 $\mu$M $\Delta^9$-THC. $\Delta^9$-THC (100 nM) produced a parallel rightward shift (4.8-fold) in the WIN 55,212-2 dose-response curve, giving a dissociation constant (K<sub>e</sub> value) of 26.3 nM whereas $\Delta^9$-THC (1 $\mu$M) shifted the WIN 55,212-2 dose-response curve to the right 13-fold in a competitive manner giving a K<sub>e</sub> value of 83.3 nM. Symbols represent the mean response and the vertical lines indicate the S.E.M. WIN 55,212-2 was initially dissolved in 100% ethanol to produce a 2 mM stock solution. $\Delta^9$-THC was initially dissolved in 100% ethanol to produce a 1 mM stock solution. Further dilutions were done using assay buffer to give final WIN 55,212-2 concentrations of 1 nM - 100 $\mu$M and final $\Delta^9$-THC concentrations of 100 nM and 1 $\mu$M. K<sub>e</sub> = [agonist]/DR-1 (Tallarida and Murray, 1987).
Figure 6.4 - Δ⁹-THC on SR141716A-inhibited [³⁵S]GTPγS binding in CHO/CB₁ cell membranes. Effect of SR141716A on basal [³⁵S]GTPγS binding in membranes of CHO cells stably expressing the human cannabinoid CB₁ receptor in the absence (squares; n=4) and presence (triangles; n=4) of Δ⁹-THC (30 µM). Δ⁹-THC (30 µM) shifted the SR141716A dose-response curve to the right 781-fold giving a Kᵦ value for Δ⁹-THC of 38.4 nM. Symbols represent the mean response and the vertical lines indicate the S.E.M. SR141716A was initially dissolved in 100% ethanol to produce a 2 mM stock solution. Δ⁹-THC was initially dissolved in 100% ethanol to produce a 1 mM stock solution. Further dilutions were done using assay buffer to give final SR141716A concentrations of 1 pM - 100 µM and a final Δ⁹-THC concentration of 30 µM. Kᵦ = [antagonist]/DR-1 (Tallarida and Murray 1987).
6.3.3 WIN 55,212-2, CP55,940 and Δ²-THC on Forskolin-Stimulated cAMP Formation in Whole CHO/CB₁ Cells

The effects of WIN 55,212-2 (1 nM - 30 μM), CP55,940 (1 nM - 30 μM) and Δ²-THC (1 nM - 30 μM) on forskolin-stimulated (10 μM) cAMP formation were examined. CP55,940 inhibited forskolin-stimulated cAMP formation by 95.1% with an EC₅₀ value of 14.7 nM (Figure 6.5) in whole CHO/CB₁ f cells. WIN 55,212-2 inhibited forskolin-stimulated cAMP formation by 98.7% with an EC₅₀ value of 143 nM (Figure 6.5) Δ²-THC dose-dependently inhibited forskolin-stimulated cAMP formation by 72.4% with an EC₅₀ value of 28.1 nM (Figure 6.5). Δ²-THC had no effect on forskolin-stimulated cAMP formation in wild-type CHO cells (data not shown).
Figure 6.5 - WIN 55,212-2, CP55,940 and Δ⁹-THC inhibition of forskolin-stimulated (10 μM) cAMP formation in whole CHO/CB₁ cells. Whole CHO/CB₁ cells were incubated with forskolin (10 μM) in the presence of CP55,940 (1 nM - 30 μM; inverted triangles; n=3), WIN 55,212-2 (1 nM - 30 μM; squares; n=3), or Δ⁹-THC (1 nM - 30 μM; triangles; n=3). CP55,940 inhibited forskolin-stimulated cAMP formation by 95.1% with an EC₅₀ value of 14.7 nM. WIN 55,212-2 inhibited forskolin-stimulated cAMP formation by 98.7% with an EC₅₀ value of 143 nM. Δ⁹-THC dose-dependently inhibited forskolin-stimulated cAMP formation by 72.4% with an EC₅₀ value of 28.1 nM. Symbols represent the mean response and the vertical lines represent the S.E.M. Forskolin was initially dissolved in water. CP55,940, WIN 55,212-2 and Δ⁹-THC were initially dissolved in 100% ethanol to produce 1 mM stock solutions. Further dilutions of all drugs were done using IMDM.
6.4 DISCUSSION

The major finding of this study is that, in CHO cells expressing 1.82 pmol receptor/mg protein of the human cannabinoid CB₁ receptor, Δ⁹-THC can behave either as a neutral (showing no preference for agonists or inverse agonists) competitive antagonist, or as a partial agonist, depending upon the functional assay. The fact that WIN 55,212-2 is an agonist at the human cannabinoid CB₁ receptor is in agreement with previous results from our laboratory (Landsman et al., 1997; Landsman et al., 1998). WIN 55,212-2 has also been shown to be an agonist in other systems including rat cerebellar membranes (Selley et al., 1996; Sim et al., 1996) and mouse whole brain membranes (Burkey et al., 1997). However, our studies, which find that Δ⁹-THC is a neutral competitive antagonist at the human cannabinoid CB₁ receptor in the [³⁵S]GTPγS assay is novel.

The data from the present study offer some interesting findings, and bring up some intriguing questions. First, though our finding that Δ⁹-THC is a neutral competitive antagonist in the [³⁵S]GTPγS assay is novel, an antagonistic property of Δ⁹-THC could be interpreted in the study by Selley et al. (1996) using rat cerebellar membranes where they stated that “[Δ⁹-THC] was too weak to provide reproducible stimulation of [³⁵S]GTPγS binding until concentrations were attained at which the vehicle (ethanol) began to produce non-specific effects on [³⁵S]GTPγS binding." In a later study, Sim et al. (1996) showed that Δ⁹-THC was a partial antagonist in rat cerebellar membranes by its ability to antagonize WIN 55,212-2-stimulated [³⁵S]GTPγS binding.
Δ⁹-THC also partially reversed the inhibition of excitatory postsynaptic current amplitude produced by WIN 55,212-2 (Shen and Thayer, 1999). Δ⁹-THC was concluded not to be a neutral antagonist in these studies, but rather a partial antagonist. However, Δ⁹-THC has been shown to be a partial agonist in some systems. Sim et al. (1996) showed that Δ⁹-THC stimulated basal [³⁵S]GTPγS binding by approximately 20% in rat cerebellar membranes. Burkey et al. (1997) reported similar findings (25% stimulation) in mouse brain membranes. Regardless of whether Δ⁹-THC is a neutral antagonist, or a weak partial agonist in the [³⁵S]GTPγS binding assay, the results are surprising since Δ⁹-THC is the main psychoactive ingredient in marijuana (Gaoni and Mechoulam, 1964), so it would be expected that this compound would produce a larger effect in this assay. To further show that Δ⁹-THC is a neutral antagonist in CHO/CB₁ cell membranes, the effect of Δ⁹-THC on WIN 55,212-2- and SR141716A-mediated [³⁵S]GTPγS binding was examined. Δ⁹-THC shifted the dose-response curves of both WIN 55,212-2 and SR141716A to the right in a competitive fashion, as would be expected from a neutral competitive antagonist. Δ⁹-THC (100 nM) produced a parallel rightward shift (4.8-fold) in the WIN 55,212-2 dose-response curve, giving a Kᵦ value of 26.3 nM, whereas Δ⁹-THC (1 μM) shifted the WIN 55,212-2 dose-response curve to the right 13-fold in a competitive manner giving a Kᵦ value of 83.3 nM. Δ⁹-THC (30 μM) also produced a parallel rightward shift (781-fold) in the SR141716A dose-response curve, giving a Kᵦ value of 38.4 nM. Kᵦ = [antagonist]/DR-1; Tallarida and Murray, 1987). From these data it appears that Δ⁹-THC is a good antagonist in the current system.
In addition to the $[^{35}S]GTP\gamma S$ data, this study finds that $\Delta^9$-THC is a partial agonist in the cAMP assay. WIN 55,212-2 and CP55,940 both completely inhibited the forskolin-stimulated (10 μM) response with EC$_{50}$ values of 143 nM and 14.7 nM, respectively. In contrast, $\Delta^9$-THC significantly inhibited the forskolin-stimulated (10 μM) response by 72.4% with an EC$_{50}$ value of 28.1 nM. These data agree with those of Matsuda et al. (1990) who found that $\Delta^9$-THC (100 nM) inhibited forskolin-stimulated (500 nM) cAMP formation by approximately 40% with an EC$_{50}$ value of 13 nM at the rat cannabinoid CB$_1$ receptor expressed in CHO cells whereas CP55,940 inhibited forskolin-stimulated (500 nM) cAMP formation by 60% in this system. From these data it appears that $\Delta^9$-THC is a partial agonist in the system developed by Matsuda et al. (1990).

One possibility for this difference in the effect of $\Delta^9$-THC between the $[^{35}S]GTP\gamma S$ assay and the cAMP assay is that $\Delta^9$-THC is a very weak partial agonist at the human cannabinoid CB$_1$ receptor and the $[^{35}S]GTP\gamma S$ assay is not sensitive enough to detect any basal stimulation. This is a reasonable assumption since nucleotide exchange after receptor activation is the first step in the signal transduction process of G protein-coupled receptors. On the other hand, the further down the transduction pathway one measures, the more amplification one sees and the effects of weaker agonists are easier to observe. Therefore, since the cAMP assay measures an effect downstream from $[^{35}S]GTP\gamma S$ binding, this minute undetectable signal produced from initial receptor activation (as seen with $\Delta^9$-THC) can be measured at the level of adenylyl cyclase inhibition. This can be seen with the inverse
agonist SR141716A. Landsman et al. (1997) have shown that SR141716A inhibits basal 
[^35S]GTPγS binding at the human cannabinoid CB₁ receptor by approximately 22% whereas 
this compound has been shown to dose-dependently augment forskolin-stimulated cAMP 
formation 59% at 100 μM forskolin and 265% at 10 μM forskolin (Section 5.3.2).

Another possibility is that one or more proteins, perhaps G proteins, responsible for 
producing the agonist effects of Δ⁹-THC are washed out in the preparation of the CHO cell 
membranes. However, this does not appear to be the case since Δ⁹-THC behaves the same 
in intact digitonin-permeabilized CHO/CB₁f cells (personal communication, Chen and 
Yamamura). However, the possibility remains that a soluble protein, which may be depleted 
from permeabilized cells, is involved in producing the agonist effects of Δ⁹-THC seen in the 
cAMP assay. Further studies are required to determine if this is the case.

One final explanation as to why Δ⁹-THC shows no activity in CHO/CB₁f cell 
membranes could be that the CB₁ receptor density is too low. The calculated density of 1.82 
pmol/mg protein for CHO/CB₁f cells is lower than those values reported for various rat brain 
regions (Breivogel et al., 1997). Specific[^3H]SR141716A binding determined Bₘₐₓ values 
ranging from 2.48 pmol/mg protein in the hypothalamus to 6.85 pmol/mg protein in the 
striatum. However, the lower receptor density determined for the CHO/CB₁f cells does not 
appear to be a concern since there is enough receptor in this system to show that Δ⁹-THC is 
able to inhibit forskolin-stimulated cAMP formation by 72.4%.

Separately, there has not been a cannabinoid described which is a neutral antagonist 
in all systems tested. SR141716A was initially found to be a neutral antagonist at the human
cannabinoid CB₁ receptor expressed in CHO cells in the cAMP assay (Rinaldi-Carmona et al., 1994). However, it was later shown to be an inverse agonist in the same system in the [³⁵S]GTPγS binding assay (Bouaboula et al., 1997; Landsman et al., 1997) so the possibility remained that this receptor is either constitutively active or that there is a basal level of production of one or more endogenous cannabinoid ligands. The discovery that Δ⁸-THC is a neutral antagonist at this receptor in CHO cells ([³⁵S]GTPγS assay) supports the existence of a constitutively active receptor. If endogenous ligands were present in these membrane preparations then one would expect to see no neutral antagonists, only inverse agonists, as these "antagonists" would actually be inhibiting receptor activation by endogenous ligand in a dose-dependent manner.

Δ⁸-THC is not the only antagonist discovered for the human cannabinoid CB₁ receptor in CHO cells. MacLennan et al. (1998) showed that cannabinol, which dose-dependently inhibited [³H]CP55,940 (1 nM) binding to CHO cells expressing the human cannabinoid CB₁ receptor with a Kᵣ of approximately 100 nM, also had no effect on basal [³⁵S]GTPγS binding at this receptor expressed in CHO cells at concentrations up to 10 μM whereas SR141716A was an inverse agonist in the [³⁵S]GTPγS assay in this cell line. It is still possible that endogenous ligands are activating the receptor and that Δ⁸-THC is a true antagonist which binds to a different location on the CB₁ receptor than the endogenous compounds in a noncompetitive, nonallosteric manner. However, this does not appear to be the case since it has been shown that Δ⁸-THC analogs and one putative endogenous ligand,
anandamide, likely bind, at least in part, to the same location (Devane et al., 1992; Song and Bonner, 1996). Of course, other endogenous ligands such as 2-arachidonoylglycerol (2-AG) exist which may not compete for Δ⁹-THC binding sites (Sugiura et al., 1995). The use of washed cell membranes instead of intact cells in the current study, along with the fact that the potencies of these cannabinoids are as expected, also help argue against any endogenous ligand remaining in the assay.

In summary, both WIN 55,212-2 and SR141716A behave as expected in this system. The finding that Δ⁹-THC can be a neutral competitive antagonist in the [³⁵S]GTPγS assay, but a partial agonist in the cAMP assay is likely due to amplification of downstream events. It is clear that more research needs to be done to elucidate the mechanisms of these differential effects of Δ⁹-THC.
CHAPTER 7

Summary and Conclusion

The goals of these studies were to stably express the human cannabinoid CB₁ receptor in Chinese hamster ovary cells (CHO) and to characterize this system using a variety of radioligand binding and functional assays. This heterologous expression system has the advantage of not containing any known cannabinoid receptors. Therefore, the transfected human cannabinoid CB₁ receptor should be the only one responsible for mediating the actions of the cannabinoid ligands in this system. Furthermore, since ethical issues limit experimentation in humans, and extrapolating animal data to humans is not always reliable, the expression of the human cannabinoid CB₁ receptor in a mammalian cell line, though having its limitations, allows us to more confidently interpret the results with regards to human relevance.

The first aim was to stably express the human cannabinoid CB₁ receptor in a Chinese hamster ovary (CHO) cell line. The second aim was to characterize this receptor by using radioligand binding and functional assays. The final specific aim was to begin to elucidate the molecular mechanisms involved in the actions of cannabinoids. All three of these aims were accomplished. Under the conditions used for both the [³⁵S]GTPγS and cAMP assays, it was possible to separate full agonists, partial agonist, antagonists and inverse agonists (Tables 7.1 and 7.2).
Table 7.1 - The effects of cannabinoid ligands in the \(^{35}\text{S}\)GTP\(\gamma\)S binding assay

<table>
<thead>
<tr>
<th>Cannabinoid Ligand</th>
<th>EC(_{50}) value (nM) for (^{35}\text{S})GTP(\gamma)S Assay</th>
<th>% of Basal (^{35}\text{S})GTP(\gamma)S Binding</th>
<th>Ligand Classification</th>
</tr>
</thead>
<tbody>
<tr>
<td>WIN 55,212-2</td>
<td>649</td>
<td>172.3</td>
<td>full agonist</td>
</tr>
<tr>
<td>CP55,940</td>
<td>92.4</td>
<td>164.5</td>
<td>full agonist</td>
</tr>
<tr>
<td>HU-210</td>
<td>3.34</td>
<td>160.8</td>
<td>full agonist</td>
</tr>
<tr>
<td>Methanandamide</td>
<td>709</td>
<td>120.4</td>
<td>partial agonist</td>
</tr>
<tr>
<td>11-OH-(\Delta^9)-THC</td>
<td>3500</td>
<td>109.4</td>
<td>weak partial agonist</td>
</tr>
<tr>
<td>(\Delta^9)-THC</td>
<td>N/A</td>
<td>N/A</td>
<td>weak partial agonist</td>
</tr>
<tr>
<td>AM630</td>
<td>900</td>
<td>79.1</td>
<td>inverse agonist</td>
</tr>
<tr>
<td>SR141716A</td>
<td>0.82</td>
<td>77.7</td>
<td>inverse agonist</td>
</tr>
</tbody>
</table>

Table 7.2 - The effects of cannabinoids in the cAMP assay

<table>
<thead>
<tr>
<th>Cannabinoid Ligand</th>
<th>EC(_{50}) value (nM) for cAMP Assay</th>
<th>% of Forskolin-Stimulated (10 (\mu)M) cAMP Formation</th>
<th>Ligand Classification</th>
</tr>
</thead>
<tbody>
<tr>
<td>WIN 55,212-2</td>
<td>143</td>
<td>1.3</td>
<td>full agonist</td>
</tr>
<tr>
<td>Methanandamide</td>
<td>432</td>
<td>2.3</td>
<td>full agonist</td>
</tr>
<tr>
<td>CP55,940</td>
<td>14.7</td>
<td>4.9</td>
<td>full agonist</td>
</tr>
<tr>
<td>11-OH-(\Delta^9)-THC</td>
<td>140</td>
<td>11.5</td>
<td>full agonist</td>
</tr>
<tr>
<td>(\Delta^9)-THC</td>
<td>28.1</td>
<td>27.6</td>
<td>partial agonist</td>
</tr>
<tr>
<td>SR141716A</td>
<td>109</td>
<td>272</td>
<td>inverse agonist</td>
</tr>
</tbody>
</table>

Some of the problems with using this artificial expression system are that, first of all, Chinese hamster ovary cells are not the native cells which express the human cannabinoid CB\(_1\) receptor. This probably means that the lipid bilayer, which likely influences the ternary structure of the receptor, is different between CHO cells and human neurons. Even though the binding affinities and potencies (including rank order) were as expected, which, in fact,
adds strength to the use of CHO cells as a model, other factors, such as exactly which G proteins are coupling to the CB₁ receptor, either constitutively or upon receptor activation, may be altered. This may be due to either an altered receptor conformation in the non-native cell membrane, or to the likelihood that the G protein pool is different in either the types or quantity of particular G proteins.

Another problem in using a cell line is that, while it is relatively easy to study the effects of drugs on an isolated cell line, it is much more difficult in predicting what effects these ligands would have in a more complex, multi-cell type, *in vivo* system, namely man. However, an isolated, mammalian cell line is a logical place to start and CHO cells, when compared to studies in animals and man, appear to be a good predictor of the pharmacological actions of cannabinoid ligands.

The study of the CHO/CB₁ f cell line has produced many results which are similar to those reported in other human cannabinoid CB₁ cell lines as well as those reported in animals. These include the findings that WIN 55,212-2 and CP55,940 are full agonists in both the ${}^{[35]}$GTPγS assay and the cAMP assay. Interestingly, Δ⁹-THC was shown to be a partial agonist in the cAMP assay whereas it was a neutral competitive antagonist in the $[^{35}]$GTPγS assay. The reason for this is that Δ⁹-THC may be too weak of an agonist in the $[^{35}]$GTPγS assay to be measurable. However, the likelihood that the signal is amplified downstream may account for the observed partial agonist effect of Δ⁹-THC in the cAMP assay. The same can be said for methanandamide and 11-OH-Δ⁹-THC which are partial agonists in the $[^{35}]$GTPγS assay, but a full agonists in the cAMP assay. The ability of
pertussis toxin to abolish the effects of cannabinoid ligands in both the \([^{35}S]GTP\gamma S\) assay and cAMP assay demonstrates that these effects occur through a G\(_{ij}\)-coupled receptor.

Along with finding that \(\Delta^9\)-THC is an antagonist, SR141716A was also shown to be an inverse agonist in both the \([^{35}S]GTP\gamma S\) and cAMP assays. The major future study using this human cannabinoid CB\(_1\) receptor clone will be to attempt to understand the mechanism of inverse agonism in this system by using one or more approaches discussed in chapter 5. In addition, the fact that \(\Delta^9\)-THC is an antagonist in the \([^{35}S]GTP\gamma S\) assay suggests that the CB\(_1\) receptor may not be the major site of action for this cannabinoid. Therefore, it will be of interest to continue attempting to clone other cannabinoid receptors in the central nervous system.

In conclusion, upon analyzing the data presented in this dissertation, it appears that the human cannabinoid CB\(_1\) receptor expressed in Chinese hamster ovary cells represents a model system for predicting the pharmacological effects of cannabinoids in man.
APPENDIX A

Abstracts and Publications

Abstracts


Publications


**Publications in Progress**

Landsman RS, Waite S, Burkey TH, Varga E, Consroe P, Roeske WR and Yamamura HI. The inverse agonist effect of SR141716A at the human cannabinoid CB₁ receptor in CHO cells is G_{i;0} mediated. *Biochem. Pharmacol.*


Chen L, Burkey TH, Landsman RS, Consroe P, Roeske WR and Yamamura HI. Uncoupling of the human cannabinoid CB₁ receptor from G proteins is dependent on agonist efficacy. *Life Sci.*
REFERENCES


Bouaboula, M., Perrachon, S., Milligan, L., Canat, X., Rinaldi-Carmona, M., Portier, M.,


Cheng, Y.-C., and Prusoff, W.H., (1973) Relationship between the inhibition constant (Ki) and the concentration of inhibitor which causes 50 per cent inhibition (IC50) of an enzymatic reaction. Biochem. Pharmacol. 22: 3099-3108.


