Cannabinoid Receptor Signaling

A.C. Howlett

Neuroscience/Drug Abuse Research Program, 208 JLC-BBRI, North Carolina Central University, 700 George Street, Durham NC, 27707, USA
ahowlett@nccu.edu

Abstract The cannabinoid receptor family currently includes two types: CB_1, characterized in neuronal cells and brain, and CB_2, characterized in immune cells and tissues. CB_1 and CB_2 receptors are members of the superfamily of seven-transmembrane-spanning (7-TM) receptors, having a protein structure defined by an array of seven membrane-spanning helices with intervening intracellular loops and a C-terminal domain that can associate with G proteins. Cannabinoid receptors are associated with G proteins of the Gi/o family (Gi1,2 and 3, and Go1 and 2). Signal transduction via Gi inhibits adenylyl cyclase in most tissues and cells, although signaling via Gs stimulates adenylyl cyclase in some experimental models. Evidence exists for cannabinoid receptor-mediated Ca^{2+} fluxes and stimulation of phospholipases A and C. Stimulation of CB_1 and CB_2 cannabinoid

receptors leads to phosphorylation and activation of p42/p44 mitogen-activated protein kinase (MAPK), p38 MAPK and Jun N-terminal kinase (JNK) as signaling pathways to regulate nuclear transcription factors. The CB_1 receptor regulates K^+ and Ca^{2+} ion channels, probably via Go. Ion channel regulation serves as an important component of neurotransmission modulation by endogenous cannabinoid compounds released in response to neuronal depolarization. Cannabinoid receptor signaling via G proteins results from interactions with the second, third and fourth intracellular loops of the receptor. Desensitization of signal transduction pathways that couple through the G proteins probably entails phosphorylation of critical amino acid residues on these intracellular surfaces.

Keywords Adenylyl cyclase · Aminoalkylindole · Anandamide · Ca^{2+} · Cannabinoid · Cyclic AMP · Depolarization suppression of inhibition or excitation · Desensitization · Endocannabinoid · G proteins · Ion channels · Mitogen activated protein kinases · Neurotransmission · Nitric oxide · Serine/threonine kinases · Seven-transmembrane spanning receptors · Synaptic plasticity · Tyrosine kinases

1 Introduction

Cannabinoid receptors are members of the rhodopsin-like family of seven-transmembrane-spanning (7-TM) receptors that are formed by the interaction of the seven transmembrane helices, and generally couple to G proteins at their intracellular surface as one mechanism for their signal transduction. The cannabinoid receptor family currently includes two types: CB_1, found in neuronal cells and brain, and CB_2, found in immune cells and tissues (see Howlett et al. 2002 for a comprehensive review of cannabinoid receptor pharmacology). Until the discovery of cannabinoid receptors, the mechanism of action of cannabinoid drugs was generally attributed to their lipid solubility properties, with the membrane/buffer partition coefficients for Δ^9-tetrahydrocannabinol (Δ^9-THC) reported to be in the range of 500 to 12,500 (Seeman et al. 1972; Roth and Williams 1979). Δ^9-THC in the 3 μM to 10 μM range could increase fluidity of synaptic plasma membranes (Hillard et al. 1985). The ability of both psychoactive and inactive cannabinoid drugs to influence ATPase and monoamine oxidase activities, hormone and neurotransmitter binding, and synaptosomal uptake of neurotransmitters in in vitro assays was attributed to their ability to intercalate into cellular membranes (for discussion see Martin 1986; Pertwee 1988). The discovery that sub-micromolar concentrations of psychoactive cannabinoid drugs could attenuate cyclic AMP accumulation in cultured neuronal cells and inhibit adenylyl cyclase activity in membranes (Howlett and Fleming 1984; Howlett 1984, 1985) led to the notion that cannabinoid compounds must be working through signal transduction mechanisms comparable to those defined for hormones and neurotransmitters. The involvement of G proteins in the response to active cannabinoid drugs was demonstrated as the characteristic requirement of sub-millimolar Mg^{2+} concentrations and micromolar guanosine

triphosphate (GTP) concentrations for Gi-mediated inhibition of adenylyl cyclase (Howlett 1985). The elimination of the response to cannabinoid drugs by pretreatment of the neuronal cells or membranes with pertussis toxin confirmed that a member of the pertussis toxin-sensitive Gi/o family mediated the response (Howlett et al. 1986). The observation that the order of potency for this signal transduction pathway paralleled that for in vivo biological responses of antinociception, immobility, and hypothermia (Howlett et al. 1988; Little et al. 1988; Melvin and Johnson 1987; Howlett 1987) led to the understanding that a cellular receptor was responsible for the effects rather than membrane fluidity changes (Howlett et al. 1989; Thomas et al. 1990).

The development of a high-affinity, stereoselective radioligand, [^{3}H]CP55940, led to the pharmacological characterization of a binding site in brain membranes that could be shown to correlate with the pharmacology of in vivo biological responses (Devane et al. 1988; Howlett et al. 1988). [^{3}H]CP55940 was subsequently used to describe structure–activity relationships for the brain cannabinoid receptor (Howlett et al. 1990; Melvin et al. 1993, 1995) and to define brain regional localization of the receptor (Herkenham et al. 1990, 1991). It was soon determined that high-affinity [^{3}H]CP55940 binding could be attributed to two receptor types: the CB_1 receptor cloned from rat and human brain cDNA libraries (Matsuda et al. 1990; Gerard et al. 1990), and the CB_2 receptor cloned from HL60 promyelocytic cells (Munro et al. 1993).

Cannabinoid pharmacology progressed with the discovery of a number of potent ligands; however, until recently little pharmacological specificity for CB_1 and CB_2 receptors was identified. Increased potency and efficacy for both receptors was found for HU210, a dimethylheptyl analog of Δ^9-THC (Howlett et al. 1990; Felder et al. 1995). A number of non-classical AC-bicyclic (e.g., CP55940) and ACD-tricyclic cannabinoid (e.g., CP55244) compounds also exhibited high potency but limited receptor specificity (Johnson et al. 1981). This class of compounds resembles the classical cannabinoid ABC-tricyclic ring structures with the exception that the pyran "B" ring is eliminated in these structures. WIN55212-2, an aminoalkylindole compound, was discovered as a highly potent, full agonist for both cannabinoid receptor types (Compton et al. 1992; Pacheco et al. 1991). The endogenous agonists for cannabinoid receptors are arachidonic acid metabolites, including arachidonylethanolamide (anandamide), 2-arachidonoylglycerol (2-AG), and 2-arachidonylglyceryl ether (noladin ether) (see Di Marzo et al. 1999; Freund et al. 2003; Giuffrida et al. 2001; Howlett and Mukhopadhyay 2000; Martin et al. 1999; Schmid 2000; Sugiura and Waku 2000; Reggio and Traore 2000 for review). The first specific antagonist for the CB_1 cannabinoid receptor was SR141716 (rimonabant), an aryl pyrazole compound discovered at Sanofi Recherche (Rinaldi-Carmona et al. 1994; Barth and Rinaldi-Carmona 1999). A specific CB_2 receptor antagonist, SR144528, has structural similarities to the CB_1 receptor antagonist (Rinaldi-Carmona et al. 1998). These compounds have been the prevalent ligands utilized in studies of signal transduction pathways for cannabinoid receptors.

2
The Cyclic AMP and Protein Kinase A Signal Transduction Pathway

2.1
Cannabinoid Receptor-Mediated Inhibition of Cyclic AMP Production

Cannabinoid receptor-regulated signal transduction through the cyclic AMP system has been reviewed (Howlett 1995; Pertwee 1997, 1999). For the CB_1 receptor, inhibition of cyclic AMP production is the characteristic response to cannabinoid agonists in brain tissue (Bidaut-Russell and Howlett 1991; Childers et al. 1994). Pharmacological studies have been performed using N18TG2 neuroblastoma cells expressing endogenous CB_1 receptors (Howlett et al. 1988; Pinto et al. 1994) and cell lines expressing recombinant CB_1 receptors (Matsuda et al. 1990; Felder et al. 1993, 1995; Vogel et al. 1993). CB_1 receptor-mediated inhibition of adenylyl cyclase is pertussis toxin-sensitive, indicating the requirement for Gi/o proteins (Howlett et al. 1986; Pacheco et al. 1993; Vogel et al. 1993).

Regulation of cellular activities by cyclic AMP-dependent protein kinase (PKA) is a critical pathway in neuronal responses via the potassium channel A-current (Childers and Deadwyler 1996). In rat hippocampal cells, PKA phosphorylation of the potassium channel produced a negative shift in the voltage-dependence (Deadwyler et al. 1995). CB_1 receptor stimulation resulted in a decrease in intracellular cyclic AMP, net dephosphorylation of the channels, activation of the A-type potassium currents, and hyperpolarization of the membrane (Deadwyler et al. 1995; Hampson et al. 1995). The significance of cannabinoid-mediated hyperpolarization of the axon terminals is that it can cause a depression in the response to depolarizing stimuli and failure in neurotransmitter release at the synapse (Childers and Deadwyler 1996).

Synaptic plasticity and neuronal remodeling can be modified by cannabinoid receptors via the cyclic AMP/PKA pathway. CB_1 receptor agonists induced neurite retraction in a neuroblastoma cell model (Zhou and Song 2001) and inhibition of nerve growth factor (NGF)-induced neurite extension in neural progenitor cells or PC12 pheochromocytoma cells transfected with the CB_1 receptor (Rueda et al. 2002). CB_1 receptors could attenuate the NGF-mediated signaling through p42/p44 MAPK (see below). A cannabinoid receptor-mediated decrease in cyclic AMP and PKA activity was demonstrated to be the mechanism from evidence that this response could be reversed by forskolin or hormone-stimulated cyclic AMP production (Rueda et al. 2002). Cannabinoid receptor-stimulation led to Tyr-phosphorylation of focal adhesion kinase (pp125 FAK) in hippocampal slices, and this response was blocked by SR141716 and pertussis toxin, demonstrating its mediation by CB_1 receptors and Gi/o (Derkinderen et al. 1996; Derkinderen et al. 2001b). Evidence demonstrating that Gi-mediated inhibition of adenylyl cyclase is integral to this pathway comes from studies in which Tyr-phosphorylation of both FAK in brain slices (Derkinderen et al. 1996) and FAK-related non-kinase (FRNK) (Zhou and Song 2002) were reversed by 8-Br-cyclic AMP, and mimicked by PKA inhibitors.

Inhibition of forskolin-stimulated cyclic AMP production has been pharmacologically characterized in human lymphocytes and mouse spleen cells expressing endogenous CB_2 receptors, and in CHO cells expressing recombinant CB_2 receptors (Felder et al. 1995; Gonsiorek et al. 2000; Slipetz et al. 1995). This response was blocked by pertussis toxin, indicating the involvement of Gi/o proteins (Felder et al. 1995). The ramifications of the cellular response to a CB_2 receptor-mediated decrease in cyclic AMP have not been fully characterized in immune cells.

2.2 Cannabinoid Receptor-Mediated Stimulation of Cyclic AMP Production

In contrast to the above studies, stimulation of cyclic AMP production has also been observed in response to cannabinoid drugs. Cannabinoid receptor agonists produced an increase in basal cyclic AMP production in globus pallidus slice preparations (Maneuf and Brotchie 1997). Evidence that the same (CB_1) receptor type mediates both the inhibitory and stimulatory components stems from findings that the order of potency for various agonists was the same, and SR141716 was a competitive inhibitor for both components (Bonhaus et al. 1998). Several mechanisms have been reported that might explain this response. One mechanism might be the cellular production of an endogenous stimulator of adenylyl cyclase. The cannabinoid-mediated production of prostaglandins has been reported (Burstein et al. 1986, 1994), and prostaglandin synthesis has been implicated in cannabinoid-mediated cyclic AMP production (Hillard and Bloom 1983).

A second mechanism for cannabinoid receptor-mediated stimulation of cyclic AMP production could depend upon which isoform of adenylyl cyclase is expressed in target cells and the way that the particular isoform responds to Gi/o-mediated regulation. Inhibition of adenylyl cyclase by recombinant CB_1 or CB_2 receptors was observed in cells that co-express either the isoform 5/6 family or the 1/3/8 family (Rhee et al. 1998) as a result of inhibition by Gi (α subunit). On the other hand, stimulation of adenylyl cyclase was observed in cells coexpressing cannabinoid receptors and the adenylyl cyclase isoform 2/4/7 family, as a result of augmentation of a Gs response by the G$\beta\gamma$ dimers released from Gi due to cannabinoid receptor stimulation (Rhee et al. 1998).

A third mechanism could be the direct interaction between CB_1 receptors and Gs. Evidence for this mechanism has come from findings that pertussis toxin treatment of neurons and CHO cells expressing recombinant CB_1 receptors resulted in cannabinoid agonist stimulation of cyclic AMP accumulation (Glass and Felder 1997; Felder et al. 1998; Bonhaus et al. 1998). In cultured striatal cells, stimulation by combinations of dopamine and cannabinoid agonists resulted in an increase in cyclic AMP production (Glass and Felder 1997). To further investigate this phenomenon, Jarrahian and colleagues (2004) transfected recombinant D_2 dopamine and CB_1 receptors into HEK293 cells, and found that the expression of D_2 dopamine receptors was sufficient to convert the inhibition of forskolin-stimulated cyclic AMP production by CP55940 to a stimulation of cyclic AMP production. Pertussis toxin attenuated the inhibition but not the stimulation of cyclic AMP production,

consistent with Gi mediation of the inhibition component and Gs mediation of the stimulation component. The finding that overexpression of Gαi1 could overcome the stimulatory component led these researchers to suggest that the D_2 dopamine receptors could sequester Gi proteins, resembling the response to pertussis toxin treatment, and thereby preclude their coupling to the CB_1 receptors (Jarrahian et al. 2004). The CB_1 receptor-mediated stimulation of cyclic AMP production required greater concentrations of CP55940 than did inhibition (Jarrahian et al. 2004). The efficacies of cannabinoid receptor agonists for regulation of Gs were not as great as for regulation of Gi (Bonhaus et al. 1998). HU210, CP55940, and WIN55212-2 were full agonists to inhibit forskolin-stimulated cyclic AMP accumulation by Gi, and Δ^9-THC and anandamide were partial agonists. Following pertussis toxin treatment, WIN55212-2 was a full agonist to stimulate cyclic AMP accumulation, but HU210, CP55940, Δ^9-THC, and anandamide behaved as partial agonists for this response.

3 Cannabinoid Receptor-Mediated Ca^{2+} Fluxes and Phospholipases C and A

Cannabinoid and endocannabinoid compounds increased intracellular free Ca^{2+} as determined by fura-2 fluorescence in undifferentiated N18TG2 neuroblastoma and NG108-15 neuroblastoma-glioma hybrid cells (Sugiura et al. 1996, 1997a, 1999). The CB_1 receptor and Gi/o proteins were implicated because this response was blocked by SR141716 and pertussis toxin (Sugiura et al. 1996, 1999). From studies directly measuring isotopic Ca^{2+} influx into N18TG2 neuroblastoma cells, the evidence suggests that desacetyllevonantradol stimulated Ca^{2+} uptake via CB_1 receptor coupling to Gs, cyclic AMP production, and PKA activation (Bash et al. 2003). Further evidence suggested that a second component of Ca^{2+} influx was due to CB_1 receptor coupling to Gi/o, leading to receptor Tyr kinase transactivation, PKC phosphorylation, and regulation of MAPK (Rubovitch et al. 2004). Evidence for a CB_1 receptor-mediated Tyr phosphorylation of N18TG2 cell proteins that can be immunoprecipitated with the CB_1 receptor has been reported (Peterson et al. 2004).

Some controversy exists regarding the ability of cannabinoid receptors to signal through the inositol 1,4,5-triphosphate (IP_3)-Ca^{2+} mobilization pathway. In studies using Ca^{2+} reporter fura-2 fluorometry, Ca^{2+} mobilization in N18TG2 neuroblastoma cells was blocked by a phospholipase C (PLC) inhibitor, indicating that PLCβ could be the effector (Sugiura et al. 1996, 1997a). CB_1 receptor activation in cultured cerebellar granule cells resulted in an augmented Ca^{2+} signal in response to depolarization by glutamate receptors or high K^+ (Netzeband et al. 1999). In these cells, Ca^{2+} was mobilized from a caffeine-sensitive and IP_3 receptor-sensitive pool. This Ca^{2+} signal was attenuated by SR141716, pertussis toxin, and a PLC inhibitor (Netzeband et al. 1999), indicative of a CB_1 receptor-mediated PLC mechanism for Ca^{2+} mobilization from endoplasmic reticulum stores.

The primary evidence against a PLC-mediated pathway is that agonist-stimulated CB_1 receptors that were heterologously expressed in competent CHO cells failed to

couple to IP_3 or phosphatidic acid release (Felder et al. 1992, 1995). Furthermore, cannabinoid compounds inhibited (rather than augmented) neurotransmitter-stimulated inositol phospholipid production in hippocampal preparations (Nah et al. 1993). Anandamide and WIN55212-2 both failed to activate PLC in competent CHO cells expressing recombinant CB_2 receptors (Felder et al. 1992, 1993, 1995).

Some evidence exists for phospholipase A_2 (PLA_2) activity that could be regulated by cannabinoid receptors. Cannabinoid-induced arachidonic acid release has been observed in several cell culture systems, and this is believed to be mediated both by phospholipase activity and G proteins (Burstein 1991; Burstein et al. 1994; Shivachar et al. 1996).

4
Cannabinoid Receptor-Mediated Regulation of Ion Channels

Studies of the effects of cannabinoid drugs on neurophysiological responses in the years prior to the elucidation of the existence of a cannabinoid receptor were targeted at investigating a mechanism for the anticonvulsant properties of cannabidiol and mixed excitatory properties of Δ^9-THC (for a description and other original references see Karler and Turkanis 1981; Turkanis and Karler 1981). The laboratory of Karler and Turkanis used an in vivo model of cat spinal motor neurons to observe changes in amplitude of excitatory post-synaptic potentials evoked by these cannabinoid compounds (Turkanis and Karler 1983, 1986). These researchers also used cultured neuroblastoma cells to identify Δ^9-THC and 11-OH-Δ^9-THC-induced depression of inward Na^+ currents, suggesting a possible mechanism for CNS depression by these compounds (Turkanis et al. 1991).

4.1
Voltage-Gated Ca^{2+}-Channels

The first reports of the CB_1 receptor and Gi/o protein regulation of Ca^{2+} currents described a cannabinoid agonist-mediated inhibition of N-type voltage-gated Ca^{2+} channels in differentiated N18 neuroblastoma and NG108-15 neuroblastoma-glioma hybrid cells (Caulfield and Brown 1992; Mackie and Hille 1992; Mackie et al. 1993; Priller et al. 1995; Pan et al. 1996). WIN55212-2 and CP55940 elicited a maximal response, anandamide produced agonist/antagonist actions, and SR141716 antagonized this response (Mackie et al. 1993). In studies using fura-2 fluorescence to measure intracellular Ca^{2+} levels, 2-AG and anandamide inhibited the depolarization-evoked intracellular Ca^{2+} increase in differentiated NG108-15 cells (Sugiura et al. 1997b). Further investigations on the mechanism of inhibition of N-type currents have been carried out using neuronal expression systems (Priller et al. 1995; Pan et al. 1996, 1998; Vasquez and Lewis 1999; Guo and Ikeda 2004).

Q-type Ca^{2+} currents were inhibited by WIN55212-2 and anandamide in AtT-20 pituitary cells expressing recombinant CB_1, but not CB_2 receptors (Mackie et al. 1995). Pertussis toxin-sensitivity indicated that Gi/o proteins mediated the

response. P/Q-type Ca^{2+} fluxes, detected by fura-2 fluorescence in rat cortical and cerebellar preparations, were inhibited by anandamide (Hampson et al. 1998). This response was blocked by SR141716 and pertussis toxin, indicating mediation by CB_1 receptors and Gi/o proteins.

L-type Ca^{2+} currents were inhibited by anandamide and WIN55212-2 in cat brain arterial smooth muscle cells that endogenously express the CB_1 receptor (Gebremedhin et al. 1999). This response was blocked by SR141716 and pertussis toxin, indicating a critical role for CB_1 receptors and Gi/o. Regulation of L-type Ca^{2+} channels in these smooth muscle cells could be pharmacologically correlated with vascular relaxation in cat cerebral arterial rings (Gebremedhin et al. 1999).

4.2 G Protein-Coupled Inwardly-Rectifying K^+ Channels

In AtT-20 pituitary tumor cells exogenously expressing CB_1 receptors, cannabinoid receptor agonists anandamide and WIN55212-2 activated the inwardly rectifying K^+ currents (K_{ir}). This was a pertussis toxin-sensitive response, indicating the mediation by Gi/o proteins (Mackie et al. 1995; Henry and Chavkin 1995; McAllister et al. 1999). A reduction in cyclic AMP and PKA activity was not required, providing evidence that a direct interaction exists between G protein subunits and the ion channel proteins. Cannabinoid receptor-mediated regulation of these channels was also demonstrated in *Xenopus laevis* oocytes (McAllister et al. 1999) and rat sympathetic neurons (Guo and Ikeda 2004) coexpressing the CB_1 receptor and G Protein-Coupled Inwardly-Rectifying K^+ (GIRK1) and GIRK4 channels.

4.3 Depolarization-Induced Suppression of Inhibition and Excitation

The above-described neurophysiological mechanisms of CB_1 receptor signaling permit a critical function for endocannabinoids as retrograde regulators of neuronal excitability via a mechanism referred to as depolarization-induced suppression of inhibition (DSI) or excitation (DSE) (Wilson and Nicoll 2001, 2002; Wilson et al. 2001). DSI, or DSE, is the feedback mechanism by which a depolarized postsynaptic cell can release a neuromodulator that diffuses to neighboring neurons in the synaptic network to block release of an inhibitory, or excitatory, neurotransmitter (Alger 2002). Wilson, Nicoll, and colleagues showed that DSI could be blocked by the CB_1 antagonist SR141716 and was absent in CB_1 receptor (–/–) knock-out mice, implicating release of endocannabinoids and participation of presynaptic CB_1 receptors (Wilson and Nicoll 2001; Wilson et al. 2001). According to the proposed schema, endocannabinoid production and diffusion from the postsynaptic cell would stimulate CB_1 receptors on presynaptic terminals of a subclass of interneurons in the hippocampus, leading to decreased release of γ-aminobutyric acid (GABA) (Wilson and Nicoll 2001, 2002; Wilson et al. 2001). In addition to hippocampal circuits (Hoffman et al. 2003; Ohno-Shosaku et al. 2002b;

Misner and Sullivan 1999), other brain areas in which neurotransmission appears to be modulated by endocannabinoid release include basal forebrain (Harkany et al. 2003; Steffens et al. 2003), striatum (Gerdeman et al. 2002), and cerebellum (Breivogel et al. 2004; Kreitzer et al. 2002; Maejima et al. 2001b).

In the hippocampus, depolarization-induced opening of pyramidal cell N-type voltage-gated Ca^{2+} channels (Wilson and Nicoll 2002) would lead to release of endocannabinoid neuromodulators (Piomelli 2003). This response did not occur with a high probability in hippocampal cells firing under normal conditions, leading some researchers to suggest that high frequency discharges would be more likely to evoke elevated intracellular Ca^{2+} levels via activated voltage-gated Ca^{2+} channels (Hampson et al. 2003; Zhuang et al. 2003; Alger et al. 1996; Beau and Alger 1998; Morishita et al. 1998). Other synaptic events that might occur concurrently to promote endocannabinoid release in DSI or DSE include convergence of multiple signals that increase intracellular Ca^{2+} (Kim et al. 2002; Brenowitz and Regehr 2003), signal transduction directed by metabotropic glutamate receptors (Galante and Diana 2004; Maejima et al. 2001a; Morishita et al. 1998; Ohno-Shosaku et al. 2002a; Varma et al. 2001), and regulation of post-synaptic transport mechanisms for these retrograde modulators (Ronesi et al. 2004).

The mechanism by which cannabinoid receptors modulate neurotransmitter release is not understood. Some evidence suggests that this could involve K^+ channels (Daniel et al. 2004; Kreitzer et al. 2002). Alternatively, regulation of N or P/Q voltage-gated Ca^{2+} channels might be the mechanism for endocannabinoid agonist action (Shen and Thayer 1998; Guo and Ikeda 2004). Synergism between endocannabinoid-stimulated cellular responses and signal transduction pathways initiated by other synaptic events might be important in the regulation of neurotransmitter release (Netzeband et al. 1999).

5
Cannabinoid Receptor-Mediated Signal Transduction to the Nucleus

5.1
p42/p44 Mitogen-Activated Protein Kinases (Extracellular Signal-Regulated Kinase 1 and 2)

Although in vivo administration of Δ^9-THC can activate brain p42/p44 mitogen-activated protein kinases (MAPK), also known as extracellular signal-regulated kinase 1 and 2 (ERK1 and ERK2), it is likely that this response could reflect multisynaptic cellular events involving multiple neuromodulators, including dopamine (Valjent et al. 2004). Thus, signal transduction studies have been performed using cultured cell model systems. p42/p44 MAPK activation by an SR141716-sensitive and pertussis toxin-sensitive pathway was first identified in several cell types, including WI-38 fibroblasts, U373MG astrocytic cells, C6 glioma cells and primary astrocytes, and various host cells expressing recombinant CB_1 receptors (Bouaboula et al. 1995b; Guzman and Sanchez 1999; Sanchez et al. 1998; Wartmann et al. 1995).

One mechanism for p42/p44 MAPK activation by CB_1 receptors coupled to Gi/o could utilize the G$\beta\gamma$ dimer to provide a scaffold for proteins in the MAPK activation complex. According to this schema, recruitment of phosphatidylinositol-3-kinase (PI3K) and phosphorylation of membrane inositol phospholipids would recruit protein kinase B (PKB, also known as Akt). This would result in the sequential phosphorylation and activation of the three-kinase module consisting of Raf-1, MAP-ERK kinase (MEK) and p42/p44 MAPK. Evidence for this mechanism comes from studies in which CB_1 receptor-mediated signaling via p42/p44 MAPK was blocked by the PI3K inhibitors wortmannin and LY294002 (Bouaboula et al. 1995b; Galve-Roperh et al. 2002; Wartmann et al. 1995). Δ^9-THC, HU210, and CP55940 produced an SR141716-sensitive activation of the PKB isoform I_B in the human astrocytoma cell line U373MG and in CHO cells expressing recombinant CB_1 receptors (Galve-Roperh et al. 2002; Gomez et al. 2000). Δ^9-THC promoted PI3K and tyrosine phosphorylation of Raf-1 and its translocation to the membrane in rat cortical astrocytes (Sanchez et al. 1998).

An alternative mechanism for regulation of p42/p44 MAPK could be the release of the inhibitory regulation of c-Raf that results from the phosphorylation of Raf by PKA. CB_1 receptor/Gi-mediated inhibition of cyclic AMP production and reduction of PKA activity would promote a net dephosphorylation of c-Raf, thereby permitting the Raf kinase to serve as an activator of MEK in the p42/p44 MAPK activation module. Evidence for this pathway has been described for WIN55212-2-stimulated N1E-115 neuroblastoma cells (Davis et al. 2003) and hippocampal slice preparations (Derkinderen et al. 2003).

Activation of p42/p44 MAPK can be linked to expression of immediate early genes, as has been demonstrated for krox-24 expression induced by CB_1 receptors in U373MG human astrocytoma cells (Bouaboula et al. 1995a). Administration of Δ^9-THC to mice led to the p42/p44 MAPK-dependent expression of c-fos and zif268 in the hippocampus (Derkinderen et al. 2003). These transcription factors modulate the gene expression pattern for proteins involved in cellular functions associated with synaptic plasticity, cell survival, and differentiation.

CB_2 receptors promoted the phosphorylation of 42/p44 MAPK in cultured human promyelocytic-HL60 cells, and in CHO cells expressing recombinant CB_2 receptors (Bouaboula et al. 1996). The mediation by pertussis toxin-sensitive G proteins was demonstrated for HL60 cells (Kobayashi et al. 2001). A PI3K pathway was not the mechanism for regulation by CB_2 receptors in HL60 cells inasmuch as cannabinoid agonists failed to activate PKB/Akt (Gomez del Pulgar et al. 2000). Stimulation of CB_2 receptors by 2-AG in rat RTMGL1 microglial cells led to p42 MAPK activation and cell proliferation (Carrier et al. 2004). It should be pointed out that regulation of p42/p44 MAPK signaling is often by a complex network involving multiple stimuli. For example, sustained p42/p44 MAPK phosphorylation in mouse splenocytes resulted from stimulation of PKC by phorbol esters in addition to calmodulin kinase by Ca^{2+} ionophores (Faubert Kaplan and Kaminski 2003). Under these conditions, cannabinoid compounds were able to block the response (Faubert Kaplan and Kaminski 2003).

Krox-24 expression was induced by CB_2 receptors in HL60 promyelocytes (Bouaboula et al. 1996). A gene expression profile for CB_2 receptor-activated HL60

cells showed an induction of genes involved in cytokine synthesis, regulation of transcription, and cell differentiation (Derocq et al. 2000).

5.2
p38 MAPK and Jun N-Terminal Kinases

p38 MAPK was activated by cannabinoid receptor agonists in CHO cells expressing recombinant CB_1 receptors (Rueda et al. 2000) and in human vein endothelial cells possessing endogenous CB_1 receptors (Liu et al. 2000). Anandamide, 2-AG, and Δ^9-THC activated p38 MAPK via the CB_1 receptor in mouse hippocampal slices (Derkinderen et al. 2001a).

Jun N-terminal kinases (JNK1 and JNK2) were activated in response to cannabinoid receptor agonists in CHO cells expressing recombinant CB_1 receptors, and this was mediated through a pathway that included Gi/o, PI3K and Ras (Rueda et al. 2000). In the CHO cells (a fibroblast cell line), the transactivation of platelet-derived growth factor receptor was implicated in the JNK activation mechanism (Rueda et al. 2000).

Cellular kinase activation and sequelae in the absence of evidence of cannabinoid receptor participation should be interpreted with caution. Mechanisms other than cannabinoid receptor-mediated signal transduction could be possible. For example, anandamide stimulated p38 MAPK and JNK activation in PC12 pheochromocytoma cells (Sarker et al. 2003) and human umbilical vein endothelial cells (Yamaji et al. 2003), and these activated kinases were associated with triggering processes leading to apoptotic cell death. Further investigation indicated that activation of these kinases, leading to apoptosis in a number of cultured cell models (PC12, C6 glioma, Neuro-2A, CHO, HEK, Jurkat, and HL60), is a non-CB_1, non-CB_2 receptor-mediated process that involves anandamide and membrane lipids (Sarker and Maruyama 2003). In a second example, cannabinol and Δ^9-THC at high micromolar concentrations activated p42/p44 MAPK, leading to inhibition of gap junction function in a liver epithelial cell line by an undefined non-CB_1, non-CB_2 receptor-mediated process (Upham et al. 2003).

6
Cannabinoid Receptor-Mediated Nitric Oxide Production

Cannabinoid receptor agonists stimulate the production and release of nitric oxide (NO) by a CB_1 receptor-mediated mechanism utilizing one of the NO synthase (NOS) isoforms in neuronal tissues and model cells (see Fimiani et al. 1999a for review). The signal transduction pathway between CB_1 receptors and neuronal NOS (nNOS) regulation is believed to be important for mediating the effects of Δ^9-THC on hypothermia and locomotor activity (but not antinociception), as determined by the absence of these responses in nNOS (–/–) knock-out mice (Azad et al. 2001). NO production was stimulated by anandamide via SR141716-sensitive CB_1 receptors in rat median eminence slices, but it was not clear from these studies

whether NOS in neurons was responsible (Prevot et al. 1998). The presence of Ca^{2+}-dependent constitutive NOS in N18 neuroblastoma homogenates was inferred from a cyclic guanosine monophosphate (cGMP) reporter assay (Simmons and Murphy 1992), and demonstrated by Western blot identification (Mukhopadhyay et al. 2002b; Norford et al. 2002). NO production was stimulated by anandamide and CP55940 in leech or mussel ganglia by an SR141716-sensitive mechanism, implicating the involvement of a CB_1-like receptor (Stefano et al. 1997a,b). Antagonism by the NOS inhibitor L-*N*-arg-methyl ester is evidence that this CB_1-like receptor initiates a signal transduction pathway leading to regulation of one of the isoforms of NOS (Prevot et al. 1998).

It is possible that the CB_1-mediated NO signal transduction pathway may play a role in inhibition of neurotransmitter release by cannabimimetic agonists. Both anandamide and the NO generating agent *S*-nitroso-*N*-acetyl-penicillamine could inhibit the release of preloaded radiolabeled dopamine from invertebrate ganglia, leading Stefano and coworkers to postulate a role for NO in mediating anandamide's effects on neurotransmitter release (Stefano et al. 1997a). Glutamate release from neurons in the rat medulla was blocked by NO donors SIN-1 and spermine NONOate (Huang et al. 2004). This response was blocked by a peroxynitrite decomposition catalyst but not by an NO-stimulated guanylyl cyclase inhibitor, indicating that generation of peroxynitrite was the mechanism (Huang et al. 2004). Further studies indicated that adenosine released in response to the peroxynitrite might mediate the inhibition of glutamatergic neurotransmission (Huang et al. 2004).

In non-neuronal cells, anandamide and HU210 stimulated NO production in human saphenous vein segments (Stefano et al. 1998), cultured human arterial endothelial cells (Fimiani et al. 1999b; Mombouli et al. 1999), cultured human umbilical vein endothelial cells (Maccarrone et al. 2000), and human monocytes (Stefano et al. 1996) in an SR141716-sensitive manner, implicating CB_1 receptors. NO production in cultured human arterial endothelial cells followed a rapid intracellular Ca^{2+} mobilization (Fimiani et al. 1999b; Mombouli et al. 1999). The generation of NO in saphenous vein endothelial cells required extracellular Ca^{2+} (Stefano et al. 1998). Although the isoform(s) of NOS was not identified in these cell lines, these characteristics of NO production are consistent with the stimulation of a Ca^{2+}-regulated constitutive NOS, perhaps endothelial NOS (eNOS).

NO and peroxynitrite in human endothelial cells, human embryonic kidney (HEK) cells, and C6 glioma cells promoted activation of the anandamide and 2-AG transporter(s) (Maccarrone et al. 2000; Bisogno et al. 2001; De Petrocellis et al. 2001). This phenomenon may have ramifications for cellular mechanisms that require anandamide as a regulator. For example, indomethacin is thought to augment anandamide's stimulation of CB_1 receptors in a model of inflammatory hyperalgesia by reducing spinal NO and relieving the activation of the anandamide transporter (Guhring et al. 2002). The net result would be increased extracellular concentrations of anandamide with decreased concentrations of NO, producing an antinociceptive response that was not reversed by prostaglandin E_2 (Guhring et al. 2002). Another example is the potential for NO to activate the anandamide transporter leading to increased intracellular accumulation of anandamide where

it can serve as a regulator of transient receptor potential vanilloid type 1 (TRPV1, formerly VR1) (De Petrocellis et al. 2001).

Inhibition of iNOS induction is an important function of cannabinoid receptor agonists in inflammatory reactions, and may be a critical contributor to their anti-inflammatory and neuroprotective effects. Lipopolysaccharide plus interferon-γ induced iNOS expression in saphenous vein endothelium, and this was inhibited by anandamide (Stefano et al. 1998). A similar phenomenon was reported for CP55940 in rat microglial cells (Cabral et al. 2001) and mouse astrocytes (Molina-Holgado et al. 1997; Molina-Holgado et al. 2002), and for WIN55212-2 in rat C6 astrocytoma cells (Esposito et al. 2002). The mechanism could involve feedback by NO, inasmuch as it could be mimicked by NO donors (Esposito et al. 2002; Stefano et al. 1998). The mechanism also appears to involve stimulation of the CB_1 receptor and a reduction in cellular cyclic AMP, presumably via production of NO (Esposito et al. 2002; Molina-Holgado et al. 2002; Stefano et al. 1998). Δ^9-THC inhibited iNOS induction in RAW264.7 macrophage cells by a mechanism that involves CB_2 receptors and a reduction in cyclic AMP (Jeon et al. 1996). A final common pathway for the CB_1- and CB_2-mediated responses is the release of the cytokine interleukin-1 receptor antagonist (IL-1ra), which suppresses iNOS expression (Molina-Holgado et al. 2003).

7
Mechanisms by Which the CB_1 Receptor Signals Through G Proteins

Studies from our own laboratory have investigated domains of the CB_1 receptor that are important for activating selective Gi/o proteins, using strategies that include use of peptides that mimic intracellular domains and co-immunoprecipitation of G proteins to determine selectivity of protein–protein associations. When the CB_1 receptor was immunoprecipitated from detergent-solubilized rat brain membranes, Gαo and various Gαi subtypes were found to be associated with the CB_1 receptor (Houston and Howlett 1998; Mukhopadhyay et al. 2000; Mukhopadhyay and Howlett 2001). Similar immunoprecipitation of CB_1 receptors solubilized from N18TG2 neuroblastoma cell membranes revealed an association with Gαi1, Gαi2, and Gαi3. Pertussis toxin treatment disrupted the CB_1 receptor-Gα association, demonstrating that these complexes represent a functional equilibrium with the receptor–G protein complex as the preferred state (Howlett et al. 1999; Mukhopadhyay et al. 2000).

The domains of the CB_1 receptor that interact with G proteins were studied using peptides representing the juxtamembrane C-terminal region or a series of peptide analogs (Howlett et al. 1998; Mukhopadhyay et al. 1999). Palmitoylation of a *cys* residue anchors the C-terminal domain to the plasma membrane distal to the putative helical intracellular domain (Mukhopadhyay et al. 2002a). Thus, this region is also referred to as the fourth intracellular loop (IC4). The peptide mimicking the juxtamembrane C-terminal domain promoted G protein activation in rat brain membranes and the inhibition of Gs-stimulated or forskolin-activated adenylyl cyclase in N18TG2 membranes (Howlett et al. 1999; Mukhopadhyay et

al. 1999; Howlett et al. 1998). In solubilized brain or N18TG2 membrane preparations, the juxtamembrane C-terminal peptide competed for the protein–protein association of the CB_1 receptor with Gαo or Gαi3 (Mukhopadhyay et al. 2000; Mukhopadhyay and Howlett 2001). Because this peptide failed to disrupt the CB_1 receptor interaction with Gαi1 or Gαi2, it is believed that the C-terminal IC4 domain interacts primarily with Gαo or Gαi3 proteins. The IC4 peptide was able to form a helical structure only in a negatively charged environment (Mukhopadhyay et al. 1999), suggesting that changes in the seventh transmembrane helix (TM7) that would alter the positions of critical amino acids could promote activation of Gαo or Gαi3. CB_1 receptor mutants that are truncated two residues distal to the palmitoylated *cys* showed perturbed regulation of Ca^{2+} currents (Nie and Lewis 2001). However, mutants truncated such that the entire IC4 region was deleted were devoid of Ca^{2+} channel regulation (Nie and Lewis 2001), as would be expected if this region were critical for interaction with Go as the transducer of this response.

Three peptides comprising the third intracellular loop (IC3) of the CB_1 receptor were able to disrupt the CB_1 receptor association with Gαi1 or Gαi2 in solubilized preparations of rat brain or N18TG2 membranes (Mukhopadhyay et al. 2000; Mukhopadhyay and Howlett 2001). The C-terminal side of IC3 was considered to be most important for the activation of G proteins, presumed to be Gαi1 or Gαi2 (Howlett et al. 1998). In support of this, a nine-amino acid peptide, mimicking the C-terminal side of IC3 at the membrane-cytosol interface, promoted GTPase activity of a pure preparation of Gαi1 (Ulfers et al. 2002b). The structure of a larger peptide comprising the entire IC3 loop was shown by nuclear magnetic resonance (NMR) analysis to be helical at the N-terminal side distal to TM5 (Ulfers et al. 2002a). The peptide appeared to be amorphous at the middle third except for a turn occurring at an intracellular Gly residue, and exhibited helical structure beginning within the C-terminal third approximately two turns proximal to TM6 (Ulfers et al. 2002a). NMR analysis of a peptide representing this C-terminal region indicated that this peptide was also helical in the presence of Gαi1 (Ulfers et al. 2002b). A Leu-Ala-Lys-Thr sequence at the membrane interface may be critical to Gi interaction because reversal of this Leu-Ala sequence to Ala-Leu in a mutated CB_1 receptor resulted in a loss of coupling to Gi, thereby attenuating inhibition of cyclic AMP production (Abadji et al. 1999). This mutation also promoted coupling to Gs when Gi proteins were inactivated by pertussis toxin (Ulfers et al. 2002b).

Computational modeling studies have made some predictions regarding how the movement of transmembrane helices might be associated with activation of the CB_1 receptor. Shim and colleagues (Shim et al. 2003) have developed a CB_1 cannabinoid receptor homology model based upon the ground-state structure of rhodopsin. A docking site for non-classical cannabinoid ligands was deduced, and included interactions with multiple amino acid residues, including a hydrophobic binding pocket that would accommodate the aromatic A ring and the alkyl side chain of non-classical cannabinoid ligands (Shim et al. 2003). Assuming that the conformation of the ligand that is necessary to conform to the ground-state receptor was not the lowest energy conformation, Shim and Howlett (Shim and Howlett 2004) predicted potential ligand conformations that would release the constrained energy. As the ligand achieved lower free energy states, steric clash with amino

acid residues in TM3 and TM6 would be predicted, which may release inter-helical bonds and trigger a conformational change in the CB_1 receptor. Reggio's laboratory has envisioned that helical translocation may occur in a manner similar to what has been predicted for rhodopsin and the β-adrenergic receptor. Starting with a model based on the ground state of rhodopsin, these researchers have modified helical structure to predict a receptor conformation that could represent one of the agonist-activated states of the receptor–G protein cycle (Singh et al. 2002). These modeling studies envision changes in the TM3 and TM6 that might be directed at regulation of movement of the IC3. Future studies will be necessary to test these hypotheses, and to extend them to other intracellular domains that could be important for G protein coupling.

8
Cellular Changes in Signal Transduction upon Chronic Exposure to Agonists

Chronic exposure to Δ^9-THC and other cannabinoid receptor agonists generally leads to biological adaptive mechanisms that may be related to the phenomenon of tolerance. Cellular modifications in response to chronic agonist stimulation have included cannabinoid receptor down-regulation, as well as desensitization of signal transduction pathways. These effects have been recently reviewed in detail (Sim-Selley 2003).

CB_1 cannabinoid receptor numbers in the brain have been reported to decrease after prolonged treatment of animals with agonist drugs (Fan et al. 1996; Oviedo et al. 1993; Rodriguez de Fonseca et al. 1994; Romero et al. 1997). In other studies that used different drugs, concentrations and times of exposure, this decline in CB_1 receptor levels was not observed (Romero et al. 1995; Abood et al. 1993). Differences in the rates and magnitudes of receptor down-regulation across brain regions have been demonstrated (Breivogel et al. 1999). Chronic Δ^9-THC treatment abrogated G protein activation by cannabinoid receptors ($[^{35}S]GTP\gamma S$ binding) in a number of rat brain regions that are expected to be important for cannabinoid effects (Sim et al. 1996). The time course of the decrease in cannabinoid-stimulated $[^{35}S]GTP\gamma S$ binding to G proteins differed between brain regions (Breivogel et al. 1999). More distal responses may not be obviously correlated with the changes in receptor number and coupling to G proteins. Chronic treatment of animals with CP55940 did not produce a measurable change in adenylyl cyclase in cerebellar membranes even though cannabinoid receptor numbers were reduced (Fan et al. 1996). Chronic exposure of rodents to Δ^9-THC increased the MAPK pathway that signals to phosphorylated cyclic AMP response element binding protein (phosphoCREB) and FosB transcription factors in the nucleus (Rubino et al. 2004). These researchers reported evidence that sustained stimulation of the MAPK pathway could be coupled to the development of tolerance to the antinociception and hypomobility responses (Rubino et al. 2004).

Studies of cellular adaptation to cannabinoid drugs have identified cellular changes that could predict the mechanism of synaptic plasticity. Homologous desensitization of adenylyl cyclase inhibition was observed within minutes of ex-

posure to Δ^9-THC and levonantradol in cultured neuroblastoma cells (Dill and Howlett 1988; Shapira et al. 1998). One-way cross-desensitization has been reported, in that chronic exposure to morphine in cultured N18TG2 or NG108-15 cells caused a reduction in the response to acute stimulation of the cannabinoid receptor (Shapira et al. 1998; Eisinger et al. 2002).

8.1 Phosphorylation of the Cannabinoid Receptors as a Mechanism for Desensitization

Phosphorylation of Ser residues on the IC3 and C-terminal of the CB_1 receptor is important for regulation of coupling to G proteins and subsequent signaling. A critical Ser_{317} on the IC3 could be phosphorylated by activation of PKC in a recombinant model system (Garcia et al. 1998). This modification might serve as a heterologous desensitization mechanism by which activation of PKC could lead to the failure of CB_1 receptors to regulate GIRK channels and inhibit P/Q-type Ca^{2+} channels (Garcia et al. 1998). Studies of site-mutations of Ser_{426} and Ser_{430} indicated that these residues were required for desensitization, suggesting the importance of this domain for G protein receptor kinase-3 phosphorylation, and perhaps, association with β-arrestin 2 (Jin et al. 1999). CB_1 receptor mutants that are truncated two residues distal to the palmitoylated *cys* of the C-terminal failed to desensitize the GIRK channel activation response to agonist stimulation of the receptor, demonstrating the importance of the C-terminal tail for desensitization (Jin et al. 1999).

The role of protein kinases in maintaining the tolerant state in rodents was examined by the Welch laboratory (Lee et al. 2003). In those studies, animals were chronically exposed to Δ^9-THC, and then tested for their antinociceptive response to a dose of Δ^9-THC. The tolerance to Δ^9-THC was reversed by prior administration of a PKA inhibitor and a Src family tyrosine kinase inhibitor. These studies suggested that PKA and a tyrosine kinase could be important in maintaining the tolerant state. It is intriguing to speculate on what these findings might imply regarding the signal transduction pathways that might include cannabinoid receptors. However, the complexity of the intact brain and spinal cord in the nociceptive response makes it difficult to assign any particular substrate for PKA, or Src tyrosine kinases, as the target(s) for these phosphorylation-dependent changes.

9 Summary and Predictions

The CB_1 and CB_2 cannabinoid receptors in nervous, immune, and other tissues of the body participate in G protein-mediated signal transduction pathways. Particularly well characterized are those that regulate the second messengers cyclic AMP, Ca^{2+}, and perhaps IP_3. CB_1 receptors are modulators of ion channels, which makes them key players in the control of neurotransmission. These receptors also partic-

ipate in signal transduction via scaffolding mechanisms, including regulation of MAPK signaling to the nucleus via transcription factors. These receptors promote intercellular signaling via NO, a diffusible ligand that can impact properties of neighboring cells. Chronic administration of cannabinoid receptor agonists can orchestrate pleiotropic changes in cellular signal transduction that contribute to synaptic plasticity in the processes of learning and memory, cognition, nociception, and other responses to CB_1 receptor stimulation.

Future studies should elucidate additional signal transduction pathways in which the cannabinoid receptors can participate. G proteins other than Gi, Go, Gs, and Gq may be important in initiating signal transduction pathways that have not yet been considered for these receptors. Transactivation of alternative signal transduction pathways, with or without the participation of G proteins, may be discovered to be important for cannabinoid receptor-mediated responses. Non-G protein-mediated signal transduction mechanisms may represent alternative cellular signaling pathways. As we continue to learn more about other cellular proteins with which the cannabinoid receptors can potentially interact, we will have a better appreciation of both physiological and pathological processes mediated by endocannabinoid compounds.

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