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CB₁ cannabinoid receptor-mediated increases in cyclic AMP accumulation are correlated with reduced Gi/o function

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Abstract

Introduction—CB₁ cannabinoid receptors (CB₁Rs) stimulate Gi/o-dependent signaling pathways. CB₁R-mediated cAMP increases were proposed to result from G_s activation, but CB₁R-stimulated GTPγS binding to G_s has not heretofore been investigated.

Methods—Three models of CB₁R-stimulated cAMP production were tested: Pertussis toxin disruption of Gi/o in N18TG2 cells; L341A/A342L-CB₁R expressed in CHO cells; and CB₁ and D₂ dopamine receptors endogenously co-expressed in MN9D cells. cAMP was assayed by [³H]cAMP binding competition. G protein activation was assayed by antibody-targeted Scintillation Proximity Assay (SPA).

Results—In L341A/A342L-CB₁-CHO cells, cannabinoid agonists significantly stimulated cAMP accumulation over vehicle; CP55940-stimulated [³⁵S]GTPγS binding to Gi1/2/3 was reversed, whereas binding to G_s was not different from CB₁R. In MN9D cells, CB₁ agonist HU210 or D₂ agonist quinpirole alone inhibited forskolin-activated cAMP accumulation, whereas HU210 plus quinpirole increased cAMP accumulation above basal. HU210 alone stimulated [³⁵S]GTPγS binding to Gi1/2/3, whereas co-stimulation with quinpirole reversed HU210-stimulated [³⁵S]GTPγS binding to Gi1/2/3.

Conclusions—CB₁R couples to G_s but with low efficacy compared to Gi/o. The L341A/A342L mutation in CB₁R reversed CP55940 activation of Gi to an inhibition, but had no effect on G_s. Combined CB₁ plus D₂ agonists in MN9D cells converted the CB₁ agonist-mediated activation of Gi to inhibition of Gi. In these models, the CB₁ agonist response was converted to an inverse agonist response at Gi activation. Cannabinoid agonist-stimulated cAMP accumulation can be best explained as reduced activation of Gi, thereby attenuating the tonic inhibitory influence of Gi on the major isoforms of adenylyl cyclase.

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Conflict of Interest

The authors have no conflicts of interest to disclose.

Keywords

adenylyl cyclase; biased signaling; cannabinoids; D₂ dopaminergic receptors; G protein coupled receptor (GPCR); inverse agonism

Introduction

The CB₁ cannabinoid receptor (CB₁R) is highly expressed in the central nervous system and other tissues [1], and is an important therapeutic target for neuropathic pain, multiple sclerosis, appetite modulation, curtailing nausea in cancer chemotherapy, cardiovascular and inflammatory diseases and neurodegenerative diseases (see reviews [2–5]). The CB₁R is a G protein coupled receptor (GPCR) [6–9] coupled to G proteins in signal transduction pathways that inhibit adenylyl cyclase activity, regulate ion channels, activate mitogen-activated protein kinase and focal adhesion kinase, and regulate expression of immediate early genes [10]. The CB₁R selectively interacts with Gi/o proteins in the absence of exogenous agonists, such that a CB₁R agonist can activate the effector by promoting dissociation of the G protein subunits, measured either by direct association in detergent solution or by determining the accumulation of the GTP analog [³⁵S]GTPγS bound to the Gα subunit [11–13]. A CB₁R-Gs interaction has been suggested by results observed in three model systems. First, pertussis toxin-treatment which precludes Gαi interaction with GPCRs, increased cAMP accumulation, proposed to be due to Gαs interaction [14–16]. A second experimental system that investigated a CB₁R activation of Gs was developed by the Kendall laboratory [17], who noted that the CB₁R contains a Leu341, Ala342 sequence in the third intracellular loop (IL3) which, when transposed, yielded the motif shown to mediate β-adrenergic receptor coupling to Gs. The Kendall laboratory developed a L341A/A342L mutated CB₁R to test the interaction with Gs in this model [17]. Third, when CB₁R_s and D₂ dopamine receptors (D₂R_s) are co-expressed, co-stimulation with agonists for the two Gi/o-coupled receptors led to an increase in cAMP production in striatal neurons [14] or HEK293 cells expressing recombinant receptors [15;16]. All of those studies investigated cAMP accumulation as an indicator of second messenger differences that might occur if adenylyl cyclase were stimulated by Gs. However, those studies did not provide evidence to support a direct CB₁R-Gs interaction by equilibrium association or by G protein activation.

In the present study, we have determined cAMP changes and [³⁵S]GTPγS binding using an antibody-targeted GTPγS scintillation proximity assay (SPA) approach [18;19] to explore the CB₁R-Gs interaction. This method determines activation of each G protein individually, thereby overcoming issues related to activation or inhibition of multiple G proteins and variability in sensitivity by different G proteins (see [20;21]). Using the previously reported models, we show that CB₁R agonists promote [³⁵S]GTPγS binding to Gs to a much lesser extent than to Gi/o family, but that the increased cAMP accumulation fails to correlate with increased Gs activation. Rather, these manipulations reverse Gi/o family activation, leading us to conclude that an attenuated inhibitory influence of Gαi on adenylyl cyclase is responsible for the increased production of cAMP.

Materials and methods

Cell culture

N18TG2 neuroblastoma and MN9D hybrid neuron-neuroblastoma cells (a gift from Rong Chen, Univ. Michigan stock) were maintained as previously described [22]. Chinese hamster ovary (CHO) parental and stably transfected with either CB₁R or L341A/A342L mutant CB₁R (gifts from Debra Kendall, University of Connecticut) were grown at the same conditions as the neuronal cells, except that transfected cells were maintained under selection using 0.2 µg/mL G418 (Gemini Bio-Products, CA). Cells were as low passage as possible to obtain. Although we characterized the cells for mRNA and protein, we did not verify the sequence of the CB₁R or G proteins from these cell lines. Quantitative reverse transcriptase polymerase chain reaction (qPCR) methods and data analyses have been previously described [22]. For immunocytochemistry, cells were plated at a density of 25,000 cells on 0.2 mg/ml poly-D-lysine-coated coverslips (Fisher Scientific, PA). Media was removed and cells rinsed twice in cold PBS. Cells were fixed with 4% formaldehyde for 15 min, and then permeabilized with 0.1% triton-X100 in PBS. Coverslips were incubated with primary antibodies (rabbit anti-CB₁ (Cayman Chemical, Ann Arbor, MI) and mouse anti-D₂ (Santa Cruz, Santa Cruz, CA)) at a dilution of 1:50 in 4% normal donkey serum in PBS at room temperature for 90 min, and then rinsed four times in PBS. Secondary antibodies (goat anti-rabbit and donkey anti-mouse) were added in 4% normal donkey serum in PBS at a dilution of 1:150 and incubated at room temperature for 50 min. Secondary antibodies (Alexa Fluor405 mouse anti-rabbit and Alexa Fluor488 donkey anti-mouse) were added in 4% normal donkey serum in PBS at a dilution of 1:150 and incubated at room temperature for 50 min. Coverslips were rinsed four times in PBS, mounted on slides with Prolong Gold, and stored for 24 h before acquiring images with an Olympus IX71 (40X/0.6Ph2, 0.55 NA) equipped with a Hamamatsu Digital CCD C8484-03G02 camera and digital image CellSensTM software.

cAMP assay

The indicated cells were grown to 90% confluence in 24-well plates, cell media was removed and cells were washed with warm (37 °C) physiologic saline solution-HEPES-bovine serum albumin (137 mM NaCl, 10 mM glucose, 5 mM KCl, 1 mM MgSO₄, 1 mM CaCl₂, 10 mM NaHEPES pH 7.4, and 0.5 mg/mL fatty acid-free bovine serum albumin) and incubated with phosphodiesterase inhibitors (100 µM 3-isobutyl-1-methylxanthine and 100 µM rolipram) plus cannabinoid or D₂ agonists or antagonists for 15 min at 37 °C. The adenylyl cyclase activator forskolin (Tocris Bioscience, Minneapolis, MN) or secretin (Sigma, St. Louis, MO) were added at the indicated concentrations for an additional 4 min. cAMP was determined as previously described [22].

[³⁵S]GTPγS-Binding and G protein antibody capture Scintillation Proximity Assay (SPA)

N18TG2, MN9D or stably-transfected CHO cells were homogenized with a glass-glass homogenizer, and a post-nuclear (2,000 X g supernatant) membrane fraction was prepared by sedimentation at 40,000 X g. The protein concentration was determined by the BCA kit (Pierce, Rockford, IL) with bovine serum albumin as standard. [³⁵S]GTPγS binding reactions were performed as previously described [22]. Cell membranes (5 µg) were

incubated for 1 h at 30 °C with 500 pM [³⁵S]GTPγS (PerkinElmer, Billerica, MA), 10 μM GDP and CB₁R or D₂R ligands in 20 mM NaHepes, pH 7.4, 100 mM NaCl, 5 mM MgCl₂, and 1 mM dithiothreitol. To determine binding to total G proteins, the reaction was terminated by filtration using a cell harvester and washing, followed by adding scintillation cocktail to the UniFilter plate. To determine specific binding to G proteins defined by antibody-targeted SPA, membranes were solubilized with 3% IGEPAL CA-630 (30 min, 0–4 °C) and incubated with primary antibodies (anti-Gα_o, anti-Gα_{i1}, anti-Gα_{i2}, anti-Gα_{i3}, anti-Gα_{i1/2/3} or anti-Gα_s (Santa Cruz, Santa Cruz, CA) for 1 h. SPA beads coated with secondary IgG and the fluor (PerkinElmer) were added for 30 min, the plates were centrifuged, and radioactivity was detected on a Top-Count microplate scintillation counter (PerkinElmer). Non-specific binding (determined by adding 10 μM GTPγS to the assay mix) was subtracted from total binding to obtain specific binding. Basal binding was defined as specific binding in the absence of stimulating agonists, and agonist-stimulated values were normalized to basal as 100%.

Statistical analysis

Graphs and statistical analyses were generated using Prism V software (GraphPad, La Jolla, CA). For log dose-response experiments, EC₅₀ values were determined by non-linear regression analysis. All data are expressed as the mean ± SEM, and were considered significantly different when the p value < 0.05.

Results

CB₁R synthetic and endocannabinoid agonists inhibit forskolin- and G_s-stimulated cAMP accumulation

The ability of cannabinoid agonists to inhibit cAMP accumulation in a G_{i/o}-sensitive manner in different cell models and in brain membranes became one of the most widely-recognized signaling mechanisms for the CB₁R (see reviews [23;24]). The CB₁R response to agonists in the neuronal model N18TG2 neuroblastoma was determined in the pre-equilibrium, linear accumulation phase (four-min) (Fig. 1). Incubation with 1 μM forskolin significantly increased cAMP accumulation in N18TG2 cells 10-fold over vehicle (Fig. 1A). When the cells were incubated with 1 μM of endocannabinoid agonist 2-arachidonoylglycerol (2-AG), the fatty acid amide hydrolase-resistant methyl-anandamide (mAEA), the bicyclic cannabinoid CP55940, or the aminoalkylindole WIN55212-2, prior to forskolin, these agonists were able to significantly inhibit forskolin-activated cAMP accumulation.

Evidence suggesting coupling of CB₁R to G_s in the brain was reported in the absence of forskolin: i.e., in globus pallidus slice preparations, WIN55212-2 inhibited cAMP production under conditions of forskolin-treatment, but increased cAMP production under basal conditions [25;26]. In contrast, in the N18TG2 cells in the absence of forskolin, the CB₁R agonists (1 μM) failed to produce a significant increase in cAMP accumulation compared with vehicle (Fig. 1B). The small increases in cAMP accumulation were less than 5% of forskolin-activated accumulation, and these increases were not reliably observed. The dose-response curve for cannabinoid agonists indicated that forskolin-activated cAMP

accumulation was inhibited by CP55940 ($EC_{50} = 3.11$ nM), and WIN55212-2 ($EC_{50} = 7.4$ nM) with no evidence for an increase in cAMP levels at any concentration of agonist (Fig. 1C). These studies demonstrate that when forskolin is used to reversibly activate adenylyl cyclase, there is no evidence for a stimulatory response to these full agonists at any concentration. The studies also demonstrate that there is no apparent “inverse agonist” effect of SR141716 alone (1 μ M) under forskolin activation (Fig. 1A) or basal (Fig. 1B) conditions.

In order to demonstrate the effects of G_i on G_s -stimulated adenylyl cyclase, we stimulated the endogenous secretin receptor in the N18TG2 cells (Fig. 1D). The CB_1 agonists inhibited secretin-stimulated adenylyl cyclase, as expected if G_i is acting to counteract the effects of G_s on adenylyl cyclase. In order to determine the effect that desensitization of the CB_1 R exerts on cannabinoid inhibition of cAMP accumulation, we treated N18TG2 cells with vehicle (control) or the full agonist CP55940 for 1 h, and then stimulated cAMP accumulation with secretin in the presence of vehicle or the CB_1 R agonists CP55940 or WIN55212-2. We observed a desensitization of the CB_1 R-mediated inhibition of cAMP accumulation in the secretin-stimulated cells (Fig. 1D). This can be interpreted to mean that removing the inhibition by G_i allowed G_s to stimulate adenylyl cyclase without restraint. Desensitization of the WIN55212-2-mediated G_i response on basal adenylyl cyclase was also observed.

Preventing G_i/o coupling to CB_1 R does not augment CB_1 R activation of G_s

Evidence that the CB_1 R is a GPCR linked to G_i/o came from studies using pertussis toxin-treatment to ADP-ribosylate G_i/o family proteins and thereby prevent these G proteins from interacting with their GPCRs [27]. The suggestion of CB_1 R- G_s coupling is based on the notion that G_s binding will be augmented if G_i/o coupling is precluded by pertussis toxin, and is based solely on observations of increased cAMP production by CB_1 R agonists in pertussis toxin-treated neurons and CB_1 -CHO cells [14;26;28]. To investigate this mechanism, we used the SPA assay with N18TG2 cell membranes, and found that CP55940 was able to stimulate [35 S]GTP γ S binding to G_s (Fig. 2A). The stimulation above basal for G_s was compared with that of the G_i/o proteins and found to be approximately 10% of that for total G_i/o activation both in terms of percent above basal (Fig. 2B) and raw CPMs (data not shown). After cells were treated with pertussis toxin (100 ng/ml for 18 h), CP55940 stimulated [35 S]GTP γ S binding to the G_i/o proteins was reduced to basal or below (Fig. 2B). However, G_s was unchanged compared with untreated controls. The lack of a stoichiometric increase in G_s activation associated with pertussis toxin-mediated loss of G_i/o activation suggests that either the intrinsic efficacy of CB_1 R to activate G_s is small compared with G_i/o proteins, or that the population of CB_1 Rs capable of being coupled to G_i/o is not in a location that is able to be coupled to G_s .

cAMP accumulation and G protein activation in the L341A/A342L-mutated CB_1 R

Additional evidence linking CB_1 R and G_s came from studies in the Kendall laboratory using a CB_1 R mutant in which Leu341, Ala342 was replaced with Ala341, Leu342, resulting in the IL3 signature motif ALKT identified in the G_s -coupled β_2 -adrenergic receptor [17]. Abadji and colleagues [17] characterized this mutant as having a nearly two-fold greater

forskolin-activated cAMP accumulation compared with WT CB₁R, and the CB₁ agonist-mediated inhibition was attenuated. Upon pertussis toxin-treatment which occludes Gi/o proteins from interacting with their receptors, the mutant CB₁R exhibited a three-fold increase compared with WT in both forskolin-activated cAMP accumulation alone and the CP55940-mediated augmentation of forskolin-activated cAMP accumulation, leading the authors to suggest that “the mutant receptor couples to Gs more strongly than the WT receptor” and that the explanation for the attenuated inhibition of cAMP accumulation in the mutant receptor in the presence of agonist is that “the increased coupling to Gs partially obscured the inhibitory effect of agonists due to Gi coupling”. We found that CB₁R full agonists inhibited forskolin-activated cAMP accumulation in CHO cells expressing WT or L341A/A342L-mutated CB₁R, (Fig. 3A, whereas in L341A/A342L-mutated CB₁-CHO cells in the absence of forskolin, cannabinoid agonists significantly stimulated cAMP accumulation over the vehicle control (Fig. 3B). At maximally-effective concentrations, CP55940, WIN55212-2 and 2-AG stimulated approximately 5-fold above basal, whereas mAEA stimulated only 2.5-fold. Cannabinoid agonists were not able to stimulate above basal cAMP levels in either parental CHO or CB₁R-expressing CHO cells. These data are consistent with the explanation of Abadji and colleagues that the L341A/A342L-mutated CB₁R couples to Gs in CHO cells, and in addition, indicate that mAEA is a partial agonist compared with the other ligands in this response.

To test this hypothesis, we investigated the effect of this mutation on G protein coupling using the SPA [³⁵S]GTPγS binding assay to measure activation of specific G proteins. High concentrations of CP55940 (>30 nM) stimulated [³⁵S]GTPγS binding to Gs similarly in membranes from CHO cells expressing WT or L341A/A342L-mutated CB₁R (Fig. 3C). CP55940 stimulated [³⁵S]GTPγS binding to Gi1/2/3 in membranes from WT (two-fold above basal at 1 nM; four-fold above basal at 300 nM) (Fig. 3D). Notably, 1 nM CP55940 failed to stimulate [³⁵S]GTPγS binding to Gi1/2/3 by the L341A,A342L-mutated CB₁R, and CP55940 at >1 nM inhibited, dropping below basal (100%) in the 10 nM to 1 μM range (Fig. 3D). These data suggest that CP55940 behaves as an inverse agonist for the L341A/A342L-mutated CB₁R coupling to Gi1/2/3.

Effect of CB₁R-D₂R interaction on cAMP accumulation

CB₁R-D₂R co-activation led to an increased cAMP production that could be interpreted as an enhancement of CB₁R-Gs interaction [14–16]. We examined CB₁R-D₂R interaction using a mesencephalic-derived neuron-N18TG2 hybrid cell line, MN9D, which has gained popularity as a model of CNS dopaminergic neurons in studies related to neurotoxicity, oxidative stress, and neurodegenerative diseases [29–33]. The endogenous expression of CB₁R and D₂R is comparable in MN9D cells as determined by qPCR analysis (Fig. 4A) and immunocytochemical studies (Fig. 4B). cAMP production could depend upon which isoform of adenylyl cyclase is expressed in the MN9D cells, as each isoform family responds to Gi/o regulation in a different pattern [34;35]. Using qPCR, we found that MN9D cells express AC6 at levels three-fold greater than AC3, and 10-fold greater than AC1 or AC4, and a similar pattern exists for N18TG2 cells (Fig. 4C).

In MN9D cells, CB₁ agonist HU210 inhibited forskolin-activated cAMP accumulation in a concentration-dependent fashion (Fig. 5A). Quinpirole (100 nM) alone inhibited cAMP accumulation, and augmented the inhibition at all concentrations of HU210 (Fig. 5A). SR141716A (1 μ M) reversed the inhibition mediated by 100 nM HU210 or CP55940 (data not shown), demonstrating the requirement for CB₁R for this response. Forskolin activated adenylyl cyclase in a concentration-dependent fashion. Importantly, as the forskolin concentration diminished (approaching basal), the inhibition by combined CB₁R-D₂R agonists converted to a stimulation (Fig. 5B). This phenomenon is readily observed when the data are redrawn as a percent of the activation at each forskolin concentration (Fig. 5C). Quinpirole-HU210 combination increased cAMP accumulation in the presence of low concentrations of forskolin (50 nM) (Fig. 5D), vehicle (basal) (Fig. 5E) or secretin (30 nM) (Fig. 5F). To understand the role of D₂Rs in stimulated cAMP accumulation, cells were incubated with HU210 (1 μ M) plus D₂ agonists quinpirole or sumanirole (100 nM). Both agonists inhibited maximal forskolin (1 μ M)-activated cAMP production, but stimulated sub-maximal forskolin (50 nM) or basal responses (Fig. 6A, B). D₂ antagonist raclopride reversed the stimulation of cAMP accumulation by combined CB₁R-D₂R agonists to below 100% levels, resulting in the HU210-inhibited response.

We explored the G protein activation in CB₁R-D₂R co-stimulated membranes from MN9D cells. HU210 stimulated [³⁵S]GTP γ S binding to total G proteins, which could be blocked by SR141716A (1 μ M) (Fig. 7A). Addition of quinpirole (100nM) did not change the HU210-stimulated [³⁵S]GTP γ S binding in the combined G protein pool. [³⁵S]GTP γ S binding to G_s was increased above basal at high HU210 concentrations (>100nM). Quinpirole increased [³⁵S]GTP γ S binding to G_s above that of HU210 (Fig. 7B), suggesting that the D₂R could also couple to G_s, thereby resulting in the additive response. HU210 stimulated [³⁵S]GTP γ S binding to Gi1/2/3 over a broad concentration range (Fig. 7C). The curve shown begins at 1 nM HU210, which is two-fold above basal level (expressed as 100%). Combination with quinpirole reversed the low-affinity component of HU210-stimulated [³⁵S]GTP γ S specific binding (>100nM HU210). Quinpirole also attenuated HU210-stimulated [³⁵S]GTP γ S specific binding to G_o (data not shown).

Discussion

Experimental models of CB₁R-stimulated cAMP production: Gi/o activation is attenuated

The goal of these studies was to address the question of whether the CB₁R could couple to G_s. We found that G_s could be activated by CB₁R stimulation, but with poor efficacy compared with Gi/o proteins (Fig. 2B). We postulated that in a state of fewer Gi/o proteins, the CB₁R might favor an interaction with G_s. However, this did not appear to be the case: treatment of cells with pertussis toxin, a well-known tool to abolish the ability of Gi/o proteins to interact with GPCRs, failed to significantly affect CB₁R-mediated G_s activation (Fig. 2B). Furthermore, cannabinoid agonists failed to activate Gi via a CB₁R mutated to a dysfunctional G protein regulatory motif, whereas there was no effect on G_s activation (Fig. 3). Finally, co-stimulation of endogenously expressed CB₁R and D₂R resulted in increased cAMP production (Fig. 5, 6) concurrently with reduced Gi/o activation (Fig. 7). Thus,

instead of biasing the signaling to an alternative G protein, these manipulations reduced the availability of functional G α i/o during CB₁R signal transduction.

Following the identification of dysfunctional signal transduction resulting from the L341A/A342L mutation [17], investigations of the structure of the CB₁R IL3 by the Kendall and Mierke laboratories indicated that the mutation resides within the juxtamembrane C-terminus region of the IL3 at the first helical turn of Helix 6 [36]. Peptides that mimicked the IL3 domain could compete for the association of G α i1 and G α i2 with the CB₁R [12;37]. An IL3 C-terminus peptide, but not the L341A, A342L-mutated homologous peptide, could stimulate GTPase activity of purified G α i1[38]. This was attributed to the requirement for a helical structure of the IL3 C-terminus peptide to interface with G α i1, which is not formed by the mutant peptide [38]. These studies provide evidence to support a function for this CB₁R domain in optimal Gi protein activation.

Consistent with the proposal of reduced functional Gi/o, Jarrahian and colleagues [16] examined a model of HEK293 cells in which the D₂R was stably expressed and the CB₁R was transiently transfected into the cell line. In their HEK293-D₂R-CB₁R cells, CP55940 increased forskolin-stimulated cAMP accumulation to nearly 150% compared with forskolin alone. Because this stimulation could be reversed by over-expression of G α i1, it was postulated that the presence of D₂Rs reduced Gi protein availability to interact with the CB₁R [16]. The mechanism for the CB₁R-D₂R regulation of G protein activation is not entirely clear. However, evidence for heterodimerization has been provided by Kearn and colleagues, who demonstrated co-immunoprecipitation when the HEK293 cells exogenously expressing both receptors were treated with agonists for each receptor [15].

It should be noted that the data presented herein do not address constitutive activity of CB₁ or D₂ receptors in the absence of exogenous ligands. To define the “basal” stimulation of GTP γ S binding or cAMP accumulation as “constitutive”, we rely on the reduction of the response by an inverse agonist (e.g., SR141716) [39–41]. In any biological system, the abundance of endogenously-produced endocannabinoids is an important determinant of whether the CB₁R is under the influence of high “endocannabinoid tone” that could be reversed by a competitive antagonist (for discussion, see [42]). CHO cells express phospholipase C and diacylglycerol lipase [43;44], as do neuronal cell lines (Howlett, unpublished observations), enzymes that govern production of the endocannabinoid 2-AG. This suggests the necessity of testing responses to a diacylglycerol lipase inhibitor to demonstrate whether forskolin-activated cAMP accumulation can be influenced by endocannabinoids produced by the cultured cells.

The activation state of the adenylyl cyclase determines the outcome of inhibition or stimulation

Using the N18TG2 model, our laboratory has not been able to observe a stimulation of cAMP accumulation by any class of CB₁R agonists when the adenylyl cyclase is activated by forskolin (Fig. 1A) or under conditions of stimulation by a Gs-coupled receptor (Fig. 1D). Increases in basal adenylyl cyclase activity by CB₁R agonists are small when observed, and are not reproducible between experiments. For this reason, we chose to investigate

models in which CB₁R-mediated stimulation of cAMP production had been reliably demonstrated.

We noted that stimulation of CB₁R in L341A, A342L-CB₁-CHO cells in the basal state resulted in an augmentation of cAMP accumulation in cells. In our studies, cannabinoid agonists elicited an inhibition of forskolin-activated adenylyl cyclase in the mutated as well as the WT CB₁R. These data diverge from the original report in which the L341A/A342L-CB₁-CHO cells incurred a significant attenuation of cannabinoid inhibition of forskolin-activated cAMP production (from about 50% in WT to 20% in mutated) [17]. The Abadji report demonstrated that following treatment with pertussis toxin, the forskolin-activated cAMP accumulation could be increased approximately two-fold in WT and five-fold in mutated L341A, A342L-CB₁-CHO cells, indicating that the forskolin had not attained a full activation of adenylyl cyclase in those studies [17]. This suggests that the Kendall assays more closely resembled the partially activated adenylyl cyclase state that our studies show in Fig. 5C.

The adenylyl cyclase could be activated by G_s rather than forskolin to observe inhibition by cannabinoid agonists. The Abadji report demonstrated that after cholera toxin pre-treatment to convert G_s to a constitutively active state, CP55940 promoted an inhibition of cAMP accumulation in cells expressing either WT or mutated CB₁R [17]. Thus, irrespective of the source of the activated G_s, the CB₁R-mediated G_αi release attenuated the highly G_s-stimulated adenylyl cyclase activity. Glass and Felder showed that in CB₁-CHO cells that had been treated with cholera toxin, HU210 at low concentrations (0.01 to 1 nM) produced an inhibition of cAMP production, whereas this curve was reversed to a limited extent at higher HU210 concentrations (10 nM to 1 μM) [14]. It would be difficult to envision that this could be attributed to G_s activation above that which had already been activated by cholera toxin. It could be that at these high concentrations of cannabinoid agonist, other influences can affect the receptor-G protein-adenylyl cyclase complex.

The same requirement for an activated adenylyl cyclase was observed when we studied MN9D cells, a model of interacting CB₁R and D₂R. At higher levels of activation by forskolin, dual stimulation of CB₁R and D₂R using HU210 plus quinpirole resulted in an inhibition of cAMP accumulation. In contrast, simultaneous stimulation of both receptors in the presence of low concentrations, or in the absence of forskolin, resulted in an augmentation of cAMP accumulation in MN9D cells. These results are consistent with the Glass and Felder data [14], showing the stimulatory effect with a low concentration (50 nM) of forskolin. However, our findings differ from those of Jarrahian and colleagues, who reported increased cAMP accumulation by 1 or 10 μM CP55940 in HEK293-D₂R-CB₁R cells in the presence of forskolin (10 μM) even in the absence of a D₂ agonist [16]. It is not clear how much these two receptors had been over-expressed, compared to the MN9D cells which express these receptors endogenously. If receptors in HEK293-D₂R-CB₁R cells were expressed in great enough abundance to activate G_i or G_s proteins in the absence of agonist-stimulation, the stoichiometry of signaling proteins would be very different between this model and the MN9D cells.

Distinctly different responses to cannabinoid agonists were demonstrated in a systematic analysis of recombinant adenylyl cyclase isoforms and CB₁R co-expressed in COS-7 cells [45]. HU210 or WIN55212-2 inhibited cAMP accumulation by > 50% in cells that co-expressed either AC5 or AC6 isoforms stimulated by either 1 μ M forskolin or thyrotropin (via exogenously expressed Gs-coupled thyrotropin receptor). A similar 50% inhibition was observed for the Ca²⁺-calmodulin-stimulated AC1 or AC8 isoforms [45]. This is in contrast to the augmentation of cAMP production observed in cells that co-expressed any of the AC2/AC4/AC7 family members. For those adenylyl cyclase isoforms, the stimulation by HU210 or WIN55212-2 was only about 10%-20% above that stimulated by the Gs-coupled TSH receptor or a constitutively active G α s [45]. This augmentation could be attributed to an amplification of the G α s response by the G $\beta\gamma$ released from Gi [34].

Proposal of a model

The G α i/o released by CB₁R stimulation acts at the G α i binding site to suppress the activation of adenylyl cyclase (see Fig. 8). Basal, as well as G α s-stimulated and forskolin-activated adenylyl cyclase, can be attenuated in the presence of G α i. When Gi/o signaling is reduced by G α i/o dysfunction (pertussis toxin) or CB₁R dysfunction (IL3 mutation or D₂R influence), this G α i/o-mediated suppression of basal as well as low activity Gs-stimulated adenylyl cyclase is now relieved. The greater the activation by forskolin (which facilitates the juxtaposition of the intracellular catalytic domains of the adenylyl cyclase enzyme, thereby activating the enzyme) or G α s, the less relief of the G α i suppression will be evident. The GPCR(s) responsible for releasing G α s are cell-type specific and would depend on the local scaffolding of GPCRs, G proteins and adenylyl cyclases. Alternative mechanisms by which the CB₁R could stimulate adenylyl could include: direct coupling to and activation of Gs, or coupling to and activation of Gi/o to release G $\beta\gamma$, thereby augmenting AC2/AC4/AC7. In the MN9D (shown here) and N18TG2 (data not shown) cells, 1) CB₁R is coupled predominantly to activate Gi/o proteins and the conditions that increase cAMP also attenuate Gi/o activation; 2) the activation of Gs is not augmented under conditions that increase cAMP production; and 3) AC6 predominates over other isoforms of adenylyl cyclase, thereby providing the mechanism by which reduced availability of G α i could allow G α s-stimulated enzyme activity.

Implications for biased signaling

Much attention has been given to biased signaling by the 7-TM receptors, and particularly with respect to the development of agonists that can select for either a G protein response or a beta-arrestin response [46;47]. However, the emphasis on distinguishing between G protein versus beta-arrestin may have turned our attention away from the implications of biased signaling within the G proteins associated with the GPCR. It appears that the currently available agonists for CB₁R promote a preference for coupling to Gi/o family proteins (Eldeeb and Howlett, unpublished observations). However, within the Gi/o family, it appears that these agonists can distinguish which Gi/o subtypes are effectively activated and dissociated from the CB₁R [48;49]. Using the present technology, it is now possible to search among the current orthosteric agonists to determine if any of these have the potential to promote coupling and/or activation of G proteins other than the Gi/o family. We recently reported that over-expression of the small CB₁R-associated Cannabinoid Receptor

Interacting Protein 1a (CRIP1a) was able to diminish the activation of Gi3, but promote the activation of Gi1 and Gi2 in the N18TG2 model system [50]. Thus, it is possible that interacting proteins and small molecule allosteric modulators can bias the CB₁R signaling for Gi/o family or non-Gi/o proteins. Perhaps orthosteric ligands or allosteric modulators that select for unique G protein or beta-arrestin subtypes can be developed for pharmacotherapeutic interventions of the future.

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Abbreviations

2-AG	2-arachidonoylglycerol
CB₁R	CB ₁ cannabinoid receptor
CHO	Chinese hamster ovary
CP55940	(-)-3-[2-hydroxyl-4-(1,1-dimethylheptyl)phenyl]-4-[3-hydroxyl propyl] cyclohexan-1-ol
CPM	counts per min
CREB	cAMP Response Element Binding protein
CRIP1a	Cannabinoid Receptor Interacting Protein 1a
D₂R	D ₂ dopamine receptor
GPCR	G protein coupled receptor
HEK293	human embryonic kidney
IL3	intracellular loop 3
mAEA	methyl-anandamide
PKA	protein kinase A
SPA	scintillation proximity assay
SR141716A	N-(piperidin-1-yl)-5-(4-chlorophenyl)-1-(2,4-dichlorophenyl)-4-methyl-1H-pyrazole-3-carboxamide
WIN55212-2	[2,3-dihydro-5-methyl-3-[(4-morpholinyl)methyl]pyrrolo[1,2,3-de]-1,4-benzoxazin-6-yl](1-naphthyl)methanone

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Fig. 1.

CB₁ agonist, endocannabinoid and antagonist effects on forskolin-stimulated compared with basal adenylyl cyclase. A,B: N18TG2 cells were pre-incubated in PBS-HEPES-bovine serum albumin plus phosphodiesterase inhibitors plus the indicated CB₁ agonists (CP55940, WIN55212-2, 2-arachidonoylglycerol (2-AG), and methyl-anandamide (mAEA) (1 μ M) or CB₁ antagonist SR141716A (1 μ M). After 15 min, cells were incubated for an additional 4 min with forskolin (1 μ M) (A) or vehicle (B). C: Log dose-response curves were generated over a range of 5-orders of magnitude for CP55940 or WIN55212-2, with 4 min stimulation with forskolin (1 μ M). D: Cells were pre-stimulated with CP55940 (1 μ M) or vehicle for 1 h, and washed before initiating cAMP accumulation by treating cells with or without 30 nM secretin in the presence of vehicle, CP55940 (1 μ M) or WIN55212-2 (1 μ M) for 4 min. cAMP levels were determined as described in Materials and methods. Data are presented as the mean $\pm$ SEM from four experiments (A,B) and three experiments (D). Data were analyzed by one-way analysis of variance (ANOVA) followed by Dunnett's Multiple Comparison post hoc test. A. ***significantly different from forskolin alone, $p < 0.001$; B. *** significantly different from vehicle alone, $p < 0.001$; C. data analyzed as a non-linear least squares regression analysis for one-site binding and EC₅₀ values were obtained using Prism V software; D. data analyzed by unpaired, one-way Students t-test, significantly different from control, * $p < 0.05$, ** $p < 0.01$.

Fig. 2.

Pertussis toxin treatment fails to affect CB₁R-stimulated [³⁵S]GTPγS specific binding to Gs in N18TG2 membranes. A. Membranes were incubated with [³⁵S]GTPγS and full agonist CP55940 at the indicated concentrations, and subjected to SPA using anti-Gα_s antibody. Data are shown as the specific binding in counts per min (CPM) as mean ± SEM of three independent experiments. B. N18TG2 cells were treated with vehicle or pertussis toxin (100 ng/ml for 18 h), harvested and membranes fractions isolated. Membranes were incubated with [³⁵S]GTPγS and CP55940 (100 nM), and SPA performed using the indicated anti-Gα antibody. Data are shown as the specific binding as a percent over basal, and are the mean ± SEM of three or four independent experiments, and analyzed using unpaired, one-way Student's t-test; **significantly different from untreated controls, p< 0.001.

Fig. 3.

CB₁ agonists inhibit forskolin-activated, but stimulate basal cAMP accumulation in L341A/A342L-mutated CB₁R; [³⁵S]GTPγS specific binding in L341A/A342L-mutated CB₁R. A,B. Parental (black stippled), CB₁-CHO (red checkered) or L341A,A342L-CB₁-CHO (blue lined) cells were incubated (15 min) with phosphodiesterase inhibitors plus CP55940, WIN55212-2, 2-AG, or mAEA (1 μM). Cells were incubated for an additional 4 min with (A) or without (B) forskolin (1 μM). 100% represents the amount of cAMP produced by forskolin alone (A) or vehicle (B). Data are the mean and SEM from three independent experiments, and were analyzed by one-way analysis of variance (ANOVA) followed by Dunnett's multiple comparison post hoc test. B.***significantly different from vehicle alone, $p < 0.001$. C,D. [³⁵S]GTPγS binding in response to CP55940 was determined by SPA for Gs (C), or Gi1/2/3 (D) in membranes from CB₁-CHO (red squares) or L341A,A342L-CB₁-CHO (blue circles) cells. Data are presented as the mean ± SEM from three or four independent experiments, where the percent of specific binding is reported as the percent above basal (100%).

Fig. 4.

MN9D cells express both CB₁R and D₂R, and AC6 and other isotypes of adenylyl cyclase.

A. CB₁R and D₂R expression determined by qPCR using actin and GAPDH as reference genes. B. Immunocytochemical analyses of CB₁R and D₂R expression in MN9D cells using receptor-specific antibodies and fluorescence imaging as described in the Materials and methods section. C, D. Adenylyl cyclase expression was determined by qPCR using actin and GAPDH as reference genes. Data are expressed as a percent of AC6 as 100%.

Fig. 5.

CB₁R-D₂R interaction affects cAMP accumulation is biased by the activation state. MN9D cells were incubated with phosphodiesterase inhibitors plus the indicated concentrations of HU210 in the presence (blue squares) or absence (red circles) of quinpirole (100 nM) (A,D,E,F), or quinpirole alone (A, black bar); B,C: 1 μM HU210 plus 100 nM quinpirole (black checkered) or vehicle (red stippled). After 15 min, cells were incubated for an additional 4 min with the indicated concentration of forskolin (FSK) (B,C); 1 μM (A) or 50 nM (D) forskolin; vehicle (E); or 30 nM secretin (F). cAMP accumulation was determined as described in the Materials and methods section. 100% represents the amount of cAMP produced in cells stimulated by 1 μM (A) or 50 nM (D) forskolin, vehicle (E), or 30 nM secretin (F). The data in C are redrawn from B as a percentage of FSK stimulation. Data are presented as the mean ± SEM from three independent experiments.

Fig. 6.

Effect of D₂R on CB₁R responsive cAMP accumulation in MN9D cells. MN9D cells were incubated with phosphodiesterase inhibitors plus CB₁ agonist HU210 (1 μM), D₂ agonists quinpirole or sumanirole (100 nM), and D₂ antagonist raclopride (10 μM), as indicated. After 15 min, cells were incubated for an additional 4 min with or without forskolin (1 μM or 50 nM). cAMP levels were determined as described in the Materials and methods section. 100% represents the amount of cAMP produced by vehicle or forskolin, presented as the mean ± SEM from three independent experiments.

Fig. 7.

Effect of combined D_2R agonist quinpirole and CB_1R agonist HU210 on $[^{35}\text{S}]\text{GTP}\gamma\text{S}$ specific binding to total G proteins (A), G_s (B) or $G_{i1/2/3}$ (C) in MN9D cells. Membranes (5 μg) from MN9D cells were incubated with 500 pM $[^{35}\text{S}]\text{GTP}\gamma\text{S}$ and HU210 as indicated, plus: vehicle (green squares); 100 nM quinpirole (red circles); or 1 μM SR141716A (blue diamond, dashed line) for 1 h at 30 $^{\circ}\text{C}$, and binding determined as described in the Materials and methods section. Data are presented as the percentage of specific binding over basal (100%) (mean $\pm$ SEM) from three or four experiments.

Fig. 8.

Gi suppression of forskolin-activated or Gs-stimulated adenylyl cyclase enzyme activity. As Gai levels are reduced, the enzymatic activity increases, leading to increased concentration of cAMP. The images of the catalytic domain of adenylyl cyclase with Gαs and Gαi are taken from Sunahara and Taussig, 2002 [34].