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Cannabidiol, a Novel Inverse Agonist for GPR12

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Abstract

GPR12 is a constitutively active, G_s protein-coupled receptor that currently has no confirmed endogenous ligands. GPR12 may be involved in physiological processes such as maintenance of oocyte meiotic arrest and brain development, as well as pathological conditions such as metastatic cancer. In this study, the potential effects of various classes of cannabinoids on GPR12 were tested using a cAMP accumulation assay. Our data demonstrate that cannabidiol (CBD), a major non-psychoactive phytocannabinoid, acted as an inverse agonist to inhibit cAMP accumulation stimulated by the constitutively active GPR12. Thus, GPR12 is a novel molecular target for CBD. The structure-activity relationship studies of CBD indicate that both the free hydroxyl and the pentyl side chain are crucial for the effects of CBD on GPR12. Furthermore, studies using cholera toxin, which blocks G_s protein and pertussis toxin, which blocks G_i protein, revealed that G_s, but not G_i is involved in the inverse agonism of CBD on GPR12. CBD is a promising novel therapeutic agent for cancer, and GPR12 has been shown to alter viscoelasticity of metastatic cancer cells. Since we have demonstrated that CBD is an inverse agonist for GPR12, this provides novel mechanism of action for CBD, and an initial chemical scaffold upon which highly potent and efficacious agents acting on GPR12 may be developed with the ultimate goal of blocking cancer metastasis.

Keywords

GPR12; orphan receptor; cannabidiol; inverse agonist

1. Introduction

G protein-coupled receptor 12 (GPR12) belongs to the rhodopsin families of G protein-coupled receptors (GPCRs), which are important therapeutic targets [1, 2]. GPR12 was first cloned from a mouse cDNA library in 1993 and was originally named GPCR21 [3]. This was followed by cloning of human GPR12, along with the two related orphan receptors GPR3 and GPR6, from a human genomic DNA library [4]. In the brain, GPR12 is located in the medial habenular nucleus, and to a lesser extent in hippocampus, cerebral cortex, olfactory bulb, and striatum [3]. Peripherally GPR12 is found in the testis [3] and oocytes

[5]. GPR12 is involved in several physiological processes. For example, GPR12 plays an important role in meiotic arrest in rat oocytes [5]. In addition, GPR12 participates in the process of neurite outgrowth and may contribute to brain development [6, 7].

GPR12 is constitutively active and couples to both G_s and G_i proteins [7–9]. However, GPR12 is an orphan receptor with no confirmed endogenous ligands. Initially, lysophospholipids sphingosine-1-phosphate (S1P) and sphingosylphosphorylcholine (SPC) were identified as agonists for GPR12 [7, 8]. However, a later study was unable to confirm either S1P or SPC as ligand for GPR12 [10].

In spite of being orphans, GPR12 share about 35% amino acid sequence identity in the transmembrane regions with the CB1 and CB2 cannabinoid receptors [11, 12]. Therefore, it is considered a “cannabinoid receptor-like orphan GPCR” [11]. In the present study, we first tested various classes of cannabinoids for their potential effects on GPR12 using a cAMP accumulation assay. After revealing GPR12 as a novel target for cannabidiol (CBD), we then studied the structure-activity relationship and the involvement of G proteins in the inverse agonistic effects of CBD on GPR12.

2. Materials and Methods

2.1. Materials

Dulbecco’s Modified Eagles’s Medium (DMEM), penicillin/streptomycin, L-glutamine, trypsin, and geneticin were purchased from Mediatech (Manassas, VA). Fetal bovine serum was obtained from Atlanta Biologicals (Lawrenceville, GA). Dichlorodimethylsilane for silanizing glass tubes was purchased from Sigma-Aldrich (St. Louis, Mo). 384-well, round bottom, low volume white plates were purchased from Grenier Bio One (Monroe, NC). The HTRF cAMP Hirange kits were purchased from CisBio International (Bedford, MA). Cannabinoid ligands were purchased from Cayman Chemical (Ann Arbor, MI).

2.2. Cell-based HTRF cAMP Assay

The HTRF cAMP assay was performed as previously published [13]. In brief, GPR12 HEK293 cells were washed twice with phosphate-buffered saline (8.1 mM NaH_2PO_4, 1.5 mM KH_2PO_4, 138 mM NaCl, and 2.7 mM KCl, pH 7.2), and then dissociated in phosphate-buffered saline containing 1 mM EDTA. Dissociated cells were collected by centrifugation for 5 min at 2000*g*. The cells were resuspended in cell buffer (DMEM plus 0.2 % fatty acid free bovine serum albumin) and centrifuged a second time at 2000*g* for 5 min at 4°C. Subsequently, the cells were resuspended in an appropriate final volume of cell buffer plus the phosphodiesterase inhibitor Ro 20-1724 (2 μM). 5000 cells were added at 5 μl per well into 384-well, round bottom, low volume white plates (Grenier Bio One, Monroe, NC). Compounds were diluted in drug buffer (DMEM plus 2.5 % fatty acid free bovine serum albumin) and added to the assay plate at 5 μl per well. Following incubation of cells with the drugs or vehicle for 1 hour at 37°C and 5% CO_2, d2-conjugated cAMP and Europium cryptate-conjugated anti-cAMP antibody were added to the assay plate at 5 μl per well. After 2 hour incubation at room temperature, the plate was read on a TECAN GENious Pro microplate reader with excitation at 337 nm and emissions at 665 nm and 620 nm.

2.3. Data Analysis

Data analyses for cAMP accumulation assays were performed based on the ratio of fluorescence intensity of each well at 620 nm and 665 nm. Data are expressed as ΔF%, which is defined as [(standard or sample ratio – ratio of the negative control) / ratio of the negative control] × 100. The standard curves were generated by plotting ΔF% versus cAMP concentrations using non-linear least squares fit (Prism software, GraphPad, San Diego, CA). Unknowns are determined from the standard curve as nanomolar concentrations of cAMP. Ligand-induced changes in cAMP accumulation were calculated by dividing cAMP levels in the presence of different concentrations of ligands by basal cAMP levels, times 100. Subsequently, data were subject to non-linear regression analysis using GraphPad Prism (GraphPad Software, San Diego, CA) and the graphs were also generated using GraphPad Prism. Data points shown are presented as mean ± SEM, and were obtained from three independent experiments performed in quadruplicate.

3. Results

3.1. Constitutive activity of GPR12

As shown in Figure 1A, HEK293 cells stably expressing GPR12 exhibited 8.5-fold cAMP levels compared to that of parental HEK293 cells.

3.2. Effects of various classes of cannabinoids on GPR12 mediated cAMP accumulation

To determine whether endocannabinoids are capable of altering cAMP accumulation mediated by GPR12, various concentrations of endocannabinoids 2-arachidonoylglycerol (2-AG), anandamide (AEA), virodhamine, and noladin ether (NE) were tested. As shown in Figure 2A, neither AEA nor NE had any significant effects on cAMP accumulation at any of the concentrations tested. However, 2-AG and virodhamine caused a significant reduction of cAMP accumulation at the highest tested concentration of 100 μM.

To test whether phytocannabinoids are capable of changing cAMP accumulation stimulated by GPR12, various concentrations of phytocannabinoids Δ^9-tetrahydrocannabinol (Δ^9-THC), cannabidiol (CBD), cannabinol (CBN), cannabigerol (CBG), and cannabichromene (CBC) were used. As illustrated in Figure 2B, Most of the phytocannabinoids tested had no significant effects on cAMP accumulation. CBN significantly reduced cAMP accumulation only at the highest tested concentration of 100 μM. Most significantly, CBD reduced cAMP accumulation at both the 10 μM and 100 μM concentrations.

To determine if synthetic cannabinoids are capable of changing cAMP accumulation mediated by GPR12, various concentrations of HU-210, CP55,940, and WIN55,212-2 were tested. As demonstrated in Figure 2C, HU-210, CP55,940 and WIN55,212-2 significantly inhibited cAMP accumulation only at the highest tested concentration of 100 μM.

3.3. CBD structure-activity relationship at GPR12

In the next set of experiments, we performed a structure-activity relationship study of CBD and related compounds. The results showed that cannabidivarin (CBDV) and cannabidiol 2′, 6′-dimethyl ether (CBDD), significantly reduced cAMP accumulation at a concentration of

100 μM, whereas O-1821 did not significantly reduce cAMP accumulation to GPR12 at any of the tested concentrations (Figure 3).

3.4. Involvement of G proteins in GPR12-mediated cAMP accumulation

As shown in Figure 4, after cholera toxin (CTX) pretreatment, CBD lost its ability to inhibit cAMP accumulation. In contrast, after pertussis toxin (PTX) pretreatment, CBD was still effective at reducing cAMP accumulation stimulated by GPR12 at both the 10 μM and 100 μM concentrations.

4. Discussion

GPR12 is an orphan GPCR with no confirmed endogenous ligands. Since GPR12 is phylogenetically related to cannabinoid receptors, in this study we tested various classes of cannabinoids on GPR12.

First we sought to confirm the constitutive activity of GPR12. Our data showed that HEK293 cells stably expressing GPR12 exhibit 8.5-fold cAMP levels compared to parental HEK293 cells. This confirms the previous reports of GPR12 constitutively activating adenylate cyclase [8, 9].

We then tested several endocannabinoids for their potential effects on GPR12. We used two well-established endocannabinoids, 2-AG and AEA. We also tested NE and virodhamine, which are considered to be putative cannabinoids. A key difference between the structures of NE and 2-AG is that NE contains glycerol and arachidonic acid joined by an ether linkage, whereas 2-AG contains an ester linkage. Likewise, virodhamine and AEA are very similar in structure as both contain ethanolamide and arachidonic acid. However, these are joined by an ester linkage in the virodhamine structure, whereas the AEA structure contains an amide linkage. In our experiments, neither AEA nor NE had any effects on cAMP accumulation. Both 2-AG and virodhamine showed a decrease in cAMP accumulation only at the highest tested concentration of 100 μM. Overall, our results suggest that none of the tested endocannabinoids behave as ligands for GPR12 at nM to low μM concentrations.

Next, we tested five phytocannabinoids, Δ9-THC, CBD, CBN, CBG, and CBC, for their potential in acting on GPR12. Among these phytocannabinoids, CBD significantly decreased cAMP accumulation mediated by GPR12 at concentrations of both 10 μM and 100 μM. CBN significantly reduced cAMP accumulation only at the highest tested concentration of 100 μM. On the other hand, neither Δ9-THC nor CBG significantly affected cAMP accumulation at any of the tested concentrations. These data demonstrate that CBD works on GPR12 as an inverse agonist at μM concentration, and GPR12 is a novel molecular target for CBD. To our knowledge, CBD is the first inverse agonist that has been discovered for GPR12.

In the next set of experiments, we tested three prototypical synthetic cannabinoids, HU-210, CP55,940 and WIN55,212-2, for their potential effects on GPR12. These synthetic cannabinoids significantly inhibited cAMP accumulation mediated by GPR12 only at the

highest tested concentration of 100 μM. Thus, none of the synthetic cannabinoids tested behave as ligands for GPR12 at nM to low μM concentrations.

We then proceeded to investigate the structure-activity relationship of CBD as an inverse agonist of GPR12. In contrast to the pentyl side chain that is seen in CBD, CBDV is a CBD derivative that contains a propyl side chain whereas O-1821 is a CBD derivative that has a methyl side chain. Our data demonstrated that the shortening of the side chain resulted in a decreased ability to inhibit cAMP accumulation mediated by GPR12. Cannabidiol-2′,6′-dimethyl ether (CBDD) is a CBD analogue in which the 2′,6′-hydroxyl groups are substituted with methoxyl groups. Our results showed that blocking the free hydroxyl groups had reduced ability to inhibit cAMP accumulation mediated by GPR12. Overall, our data suggests that both pentyl side chain and the free hydroxyl groups located on the benzene ring are critical for CBD to exhibit its inverse agonistic activity on GPR12.

In the last set of experiments, cholera toxin (CTX) and pertussis toxin (PTX) were utilized to investigate the involvement of G proteins in GPR12 mediated cAMP accumulation. Using CTX to block G_s signaling, we observed an abolishment of CBD inverse agonism. However using PTX to block G_i signaling, CBD inverse agonism on GPR12 was unaffected. These results demonstrate that G_s, not G_i, protein is involved in the inverse agonistic effects of CBD on GPR12 mediated cAMP signaling.

In summary, the key finding of this study is that we have identified, for the first time, CBD to be a novel inverse agonist of GPR12. CBD is a major non-psychoactive component of marijuana that has therapeutic potentials for a variety of illness, including cancer [14, 15]. Our discovery that GPR12 is a novel target for CBD has important implications. For example, previously GPR12 has been suggested to be relevant to cancer metastasis [16]. Specifically, it has been shown that silencing of GPR12 led to a reduction of phosphorylation and reorganization of keratin 8 (K8) filaments, which modulate the viscoelasticity of metastatic cancer cells. In contrast, GPR12 overexpression stimulated K8 phosphorylation and reorganization [16]. These findings indicate that GPR12 may be a potential target for a novel class of therapeutic agents that are able to adjust viscoelasticity of cancerous cells, thus preventing tumor metastasis. Since we have demonstrated that CBD is a novel, inverse agonist for GPR12, this provides the initial chemical scaffolds upon which highly potent and efficacious agents acting on GPR12 may be developed with the ultimate goal of preventing cancer metastasis.

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Abbreviations

GPCR	G protein-coupled receptor
GPR12	G protein-coupled receptor 12
CBD	cannabidiol

AEA	anandamide
2-AG	2-arachidonoyl glycerol
NE	noladin ether
Δ^9-THC	Δ^9-tetrahydrocannabinol
CBN	cannabinol
CBG	cannabigerol
CBC	cannabichromene
HTRF	homogenous time-resolved fluorescence
CTX	cholera toxin
PTX	pertussis toxin

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Highlights

- GPR12 is a novel molecular target for CBD.
- CBD acts as a new inverse agonist on GPR12.
- Both free hydroxyl and the pentyl side chain are crucial for the effects of CBD
- G_s, but not G_i is involved in inverse agonistic activity of CBD on GPR12.
- Potent and efficacious ligands for GPR12 may be developed based on CBD scaffold.

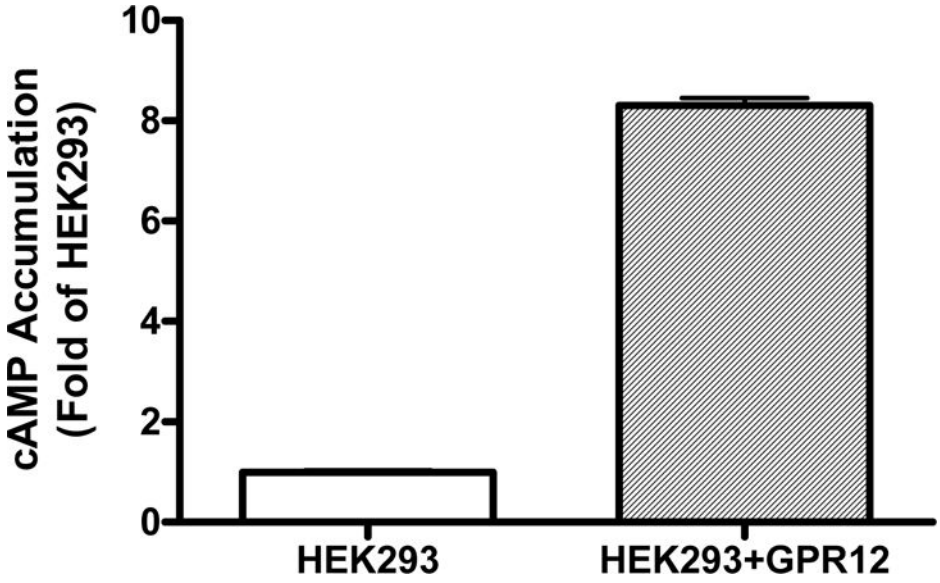

Figure 1. Constitutive activity of GPR12

HEK293 parental cells and HEK293 cells stably expressing GPR12 were incubated for one hour, and cAMP accumulation assays were performed. The open bar represents parental HEK293 cells, while the striped bar represents HEK293 cells stably expressing GPR12. Data shown represent the mean ± SEM of three independent experiments performed in quadruplicate.

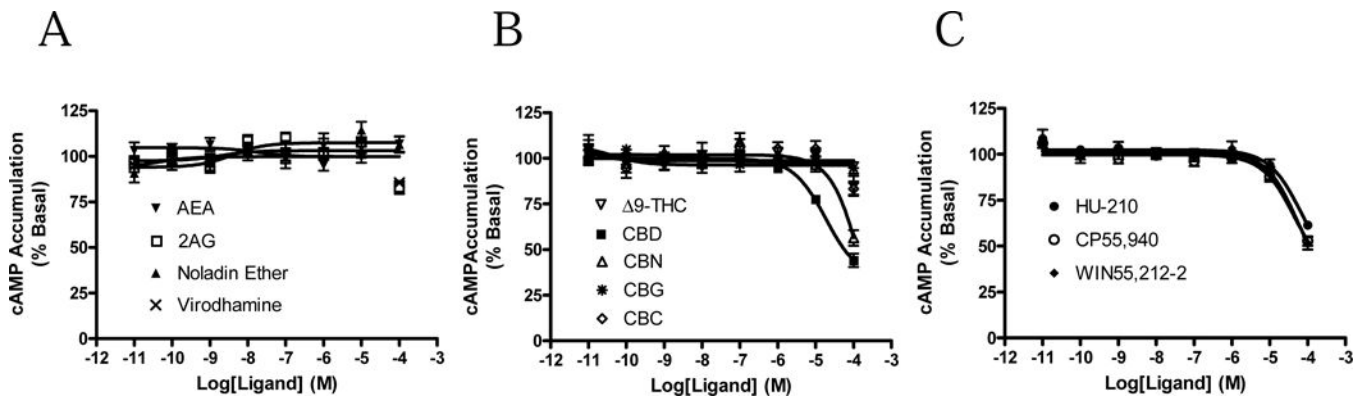

Figure 2. Effects of various classes of cannabinoids on cAMP accumulation mediated by GPR12

(A) Effects of endocannabinoids. (B) Effects of phytocannabinoids (C) Effects of synthetic cannabinoids. HEK293 cells stably expressing GPR12 were treated with different concentrations of the various classes of cannabinoids for one hour. Data shown represent the mean ± SEM of three independent experiments performed in quadruplicate.

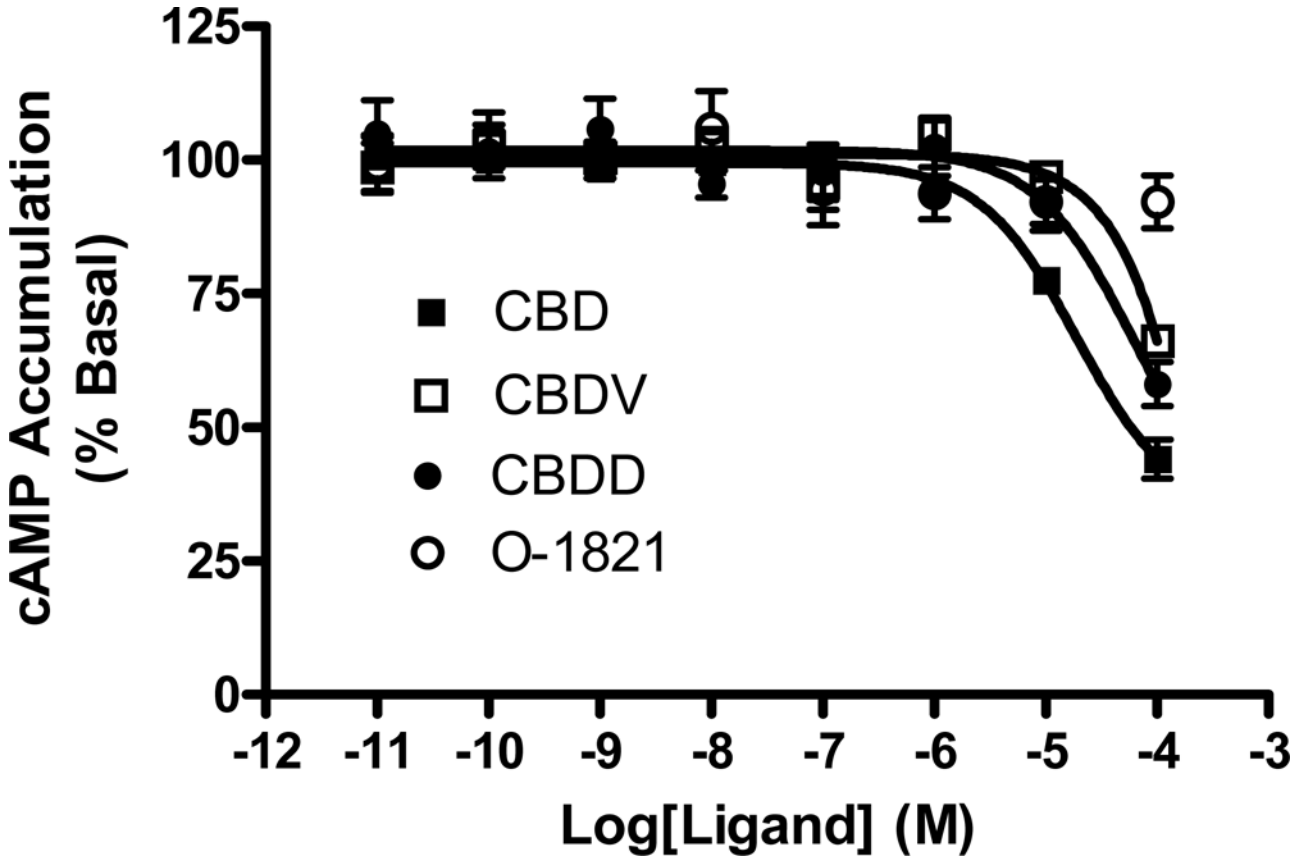

Figure 3. Cannabidiol structure-activity relationship at GPR12

HEK293 cells stably expressing GPR12 were treated with different concentrations of CBD derivatives for one hour. Data shown represent the mean ± SEM of three independent experiments performed in quadruplicate.

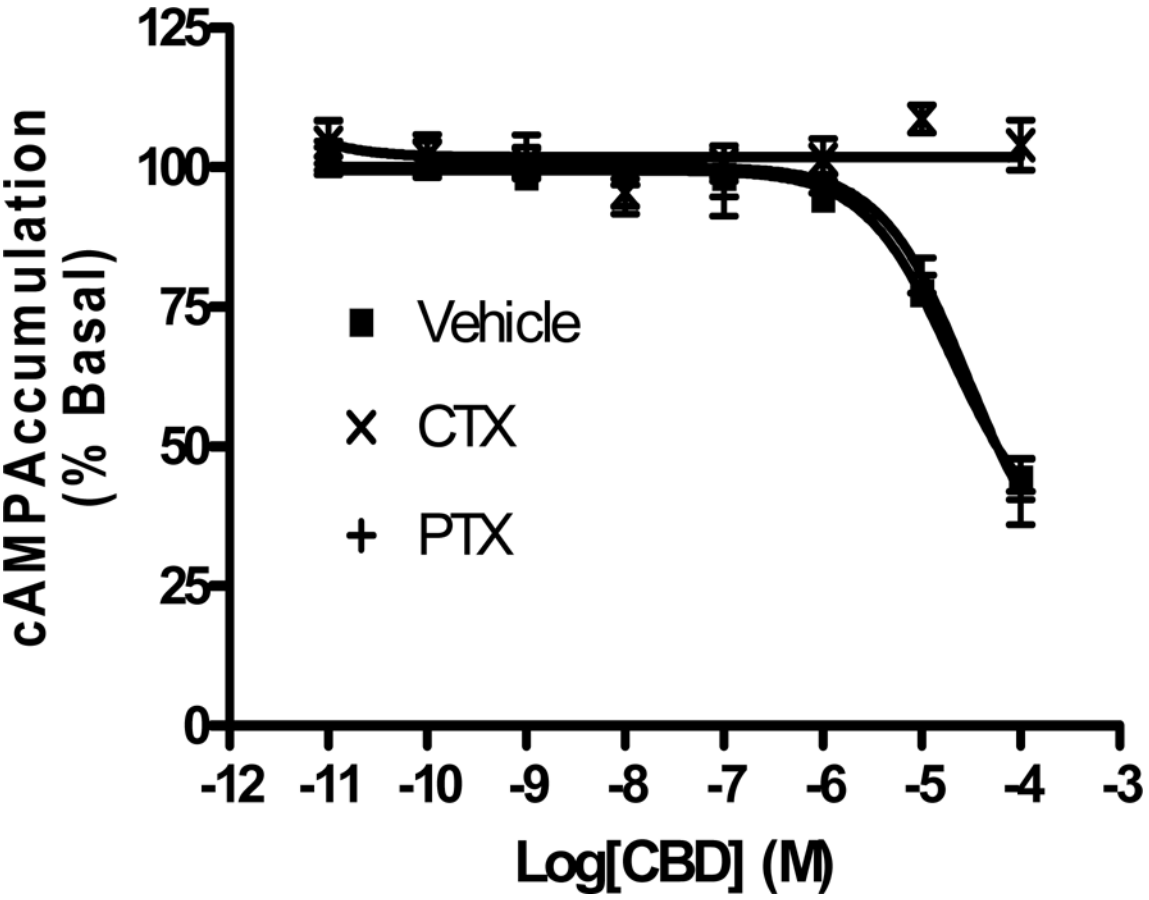

Figure 4. Involvement of G protein in cAMP accumulation mediated by GPR12

HEK293 cells stably expressing GPR12 were treated with vehicle, CTX or PTX overnight prior to stimulation with different concentrations of CBD for one hour. Data shown represent the mean ± SEM of three independent experiments performed in quadruplicate.