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SR144528 as Inverse Agonist of CB2 Cannabinoid Receptor

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Abstract

It is now well established that several G protein-coupled receptors can signal without agonist stimulation (constitutive receptors). Inverse agonists have been shown to inhibit the activity of such constitutive G protein-coupled receptor signaling. Agonist activation of the $G_{i/o}$-coupled peripheral cannabinoid receptor CB2 normally inhibits adenylyl cyclase type V and stimulates adenylyl cyclase type II. Using transfected COS cells, we show here that application of SR144528, an inverse agonist of CB2, leads to a reverse action (stimulation of adenylyl cyclase V and inhibition of adenylyl cyclase II). This inverse agonism of SR144528 is dependent on the temperature, as well as on the concentration of the cDNA of CB2 transfected. Pertussis toxin blocked the regulation of adenylyl cyclase activity by SR 144528.

Key Words : Cannabinoids, CB2 cannabinoid receptor, SR144528, Inverse agonism, G protein Adenylyl cyclase.

Introduction

Two cannabinoid receptor subtypes (referred to as CB1 and CB2) have been cloned so far (Matsuda et al., 1990; Munro et al., 1993). Both receptors belong to the heptahelical G protein-coupled receptor (GPCR) family and were found to inhibit and stimulate the activity of certain isozymes of adenylyl cyclase (AC) (Rhee et al., 1998) and to stimulate mitogen-activated protein kinase (MAPK) activity (Bouaboula et al., 1995; Bouaboula et al., 1996). All these actions appear to be exerted through a pertussis toxin (PTX)-sensitive $G_{i/o}$ protein. Receptor-mediated activation of the G protein results in the exchange of tightly-bound GDP for GTP on the G protein a α subunit, followed by dissociation of the GTP-coupled a α subunits from the $\beta\gamma$ dimmers (Birnbaumer, 1990; Bourne, 1997; Clapham and Neer, 1997). The resulting $\beta\gamma$ dimers have been implicated as activators or inhibitors of various effectors, including phospholipase C, inward rectifier potassium channels, MAPK, and certain isoforms of AC (Koch et al., 1994; Federman et al., 1992; Clapham and Neer, 1997; Bayewitch et al., 1998; He et al., 1999).

It has recently been established that some GPCRs can signal spontaneously (without any agonist binding), and not only following agonist binding as previously believed. These receptors are referred to as constitutively active receptors. Inverse agonists are described as ligands with negative efficacies or negative intrinsic activity, which suppress the constitutive activity of the receptor and block the action of agonist(Bond et al., 1995; Kenakin, 1996; Lefkowitz et al., 1993; Milligan et al., 1995). On the other hand, neural antagonists do not alter basal GPCR signaling, but are able to prevent the action of both agonists and inverse agonists by oocupying the GPCR binding site(Kenakin, 1996; Milligan et al., 1995).

The first potent CB2 antagonist, SR 144528 (N-[-(1S)-endo-1,3,3-trimethyl bicyclo [2,2,1] heptan-2-yl]-5-(4-chloro-3-methyl-phenyl)-1-(4methylbenzyl)-pyrazole-3-carboxamide), displayed subnanomolar affinity for both the rat and human CB2 receptor, and was shown to antagonize the inhibitory effect of the cannabinoid agonist CP 55,940 on forskolin (FS)- stimulated AC activity in Chinese hamster ovary (CHO) cells (Rinaldi-Carmona et al., 1998). Thereafter, it was shown that SR 144528 inhibited the p42-p44 MAPK activation induced by G_i-dependent receptors. In addition it also prevented the high constitutive activity of the CB2 receptor on MAPK activation (Bouaboula et al., 1999).

We examined the role of SR 144528 in the modulation of certain AC isoforms in transiently transfected COS-7 cells. We found that CB2 in COS cells has a constitutive activity, and thus leading to inhibition of AC-V activity even in the absence of agonist. In addition, this constitutive modulation of AC is reversed by SR144528.

Materials and Methods

Materials

[^{3}H-2]adenine (18.0 Ci/mmol) was purchased from American Radiolabeled Chemicals (St. Louis, MO). The

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phosphodiesterase inhibitors, 1-methyl-3-isobutylxanthine (IBMX) and RO-20-1724, were from Calbiochem (La Jolla, CA). Forskolin (FS), cAMP, fatty acid-free bovine serum albumin (FAF-BSA) and thyroid-stimulating hormone (TSH) were from Sigma (St. Louis, MO). Pertussis toxin (PTX) was from List Biological Laboratories (Campbell, CA). The cannabinoid agonist, HU 210, was kindly provided by Dr. R. Mechoulam (Hebrew University, Jerusalem, Israel). The cannabinoid antagonist SR 144528 was kindly obtained from Dr. Rinaldi-Carmona (Sanofi Research Institute, Montepellar, France). Tissue culture reagents were from Life Technologies (Gaithersburg, MD).

Plasmids

Plasmids encoding β-gal, rat wild-type TSH receptor, and AC-II and AC-V were described previously(Avidor-Reiss et al., 1996; Rhee et al., 1998).

Construction of hemaglutinin (HA)-tagged human CB2

The oligonucleotide primers (P1 and P2) were synthesized and used to amplify by PCR an 1100 bp fragment containing the entire human CB2 coding sequence (using the human CB2 cDNA as a template):

P1: 5′-GCGGATCCGAGGAATGCTGGGTG-3′ sense primer

P2: 5′-GCGCGGCCGCTCAGCAATCAGAGAG-3′ antisense primer

P1 is homologous to the cDNA sequence at the CB2 coding start site and was engineered to contain a unique BamH I site (underlined) for subcloning into a Bgl II site at the carboxy end of HA sequence in pcDNA 3. The P2 sequence was designed to allow for the amplification of a unique Not I site (underlined) for ligation into the multiple cloning site of pcDNA 3 following Not I digestion. The PCR reaction was carried out using a Mastercycler 5330 Plus (Eppendorf) programmed for 25 cycles in the following manner: 1-min denaturation at 92° C, 1-min annealing at 45° C, and 1-min extension at 72° C. The 1100 bp PCR product of not I sites of HA pcDNA3 was digested with BamH I and Not I and cloned into Bgl II. The sequence of peripheral cannabinoid receptor was confirmed with the sequencing(Fig. 1).

Transient cell transfection

Twenty-four hr before transfection, a confluent 10 cm plate of COS-7 cells in Dulbecco's modified Eagle's medium (DMEM) supplemented with 5% fetal calf serum, 100 IU/ml penicillin and 100 μg/ml streptomycin in a humidified atmosphere consisting of 5% CO_2 and 95% air at 37° C, was trypsinized and split into five 10-cm plates. The cells were transfected, using the DEAE-dextran chloroquine method (Rhee et al. 1998), with HA-CB2 cDNA (at the indicated concentrations) and AC V or AC II cDNAs (1 μg/plate), or of pXMD1-gal (for mock DNA transfection). Fourty-eight hrs later, the cells were trypsinized and re-cultured in 24-well plates, and after an additional 24 hrs, the cells were assayed for AC activity as described below. Transfection efficiencies were normally in the range of 40-80%, as determined by staining for β-galactosidase activity.

AC activity

The assay was performed in triplicate as described previously (Rhee et al., 1998). In brief, cells cultured in 24 well plates were incubated for 2 hrs with 0.25 ml/well fresh growth medium containing 5 μCi/ml [2-^{3}H]adenine. This medium was replaced with DMEM containing 20 mM HEPES (pH 7.4) and the phosphodiesterase inhibitors RO-20-1724 (0.5 mM) and IBMX (0.5 mM). Cannabinoids diluted in 10 mg/ml FAF-BSA were then added. AC activity was stimulated in the presence or absence of cannabinoids by the addition of either FS or TSH (in the latter case, the assayed cells were transfected with the TSH receptor). After 10 min at 37° C, the medium was removed and the reaction was stopped with perchloric acid, neutralized, and applied to a two-step column separation procedure. The [^{3}H]cAMP was eluted into scintillation vials and counted. In experiments utilizing PTX, it was added, at 100 ng/ml, to the cultures 20 hrs before the addition of [^{3}H]adenine and was replenished upon the addition of [^{3}H]adenine.

Competition binding assay with [3H]HU-243

This assay was performed as described previously (Rhee et al., 1997). In brief, the assay was performed in 1.5 ml Eppendorf tubes in a final volume of 1 ml of 50 mM Tris-HCl, 5 mM $MgCl_2$, 10 mM $CaCl_2$, 2.5 mM EDTA, pH 7.4, and 5 mg/ml FAF-BSA. The protein concentration of cell homogenate (determined by the Bradford method) was 10-20 μg per assay. The reaction was started by adding 300 pM of [^{3}H]HU-243 to each tube. The binding mixture was incubated at 30° C for 90 min with gentle shaking and centrifuged at 14,000 rpm for 10 min. The bottoms of the 1.5 ml tubes were then cut, and counted for radioactivity. Non-specific binding determined in the presence of 1 μM HU 210 was subtracted.

SDS-PAGE and western immunoblotting

The COS-7 cells transfected with human HA-tagged CB2 cDNA were harvested with cold PBS and spun down at 5000 rpm (at 4° C for 5 min), and the cell pellets were mixed with 100 μl of Laemmli sample buffer, sonicated, and frozen at -20° C before use. Dithiothreitol (0.1 M final) was added and the samples incubated for 5 min at 100° C prior to loading onto 1.5-mm thick 10% polyacrylamide gel. Following electrophoresis, proteins were transferred overnight at room temperature onto nitrocellulose membrane at 100 mA using Bio-Rad Blot cell (Bio-Rad Laboratories). The blot was blocked in PBS containing 5% fat-free milk and 0.5% Tween-20, followed by 1.5 hrs incubation with HA 11 monoclonal antibody diluted 1:1,000 in 5% fat-free milk and 0.5% Tween-20. Blots were washed three times with PBS containing 0.3% Tween-20 and secondary antibodies (horseradish peroxidase (HRP)-coupled rat anti-mouse; Jackson

Immunoresearch Laboratories, Inc.) diluted 1:10,000 in 5% fat-free milk plus 0.5% Tween-20, incubated with the blot for 1 hr, and the blot extensively washed with PBS containing 0.3% Tween-20. Peroxidase activity was observed by the ECL chemiluminescence technique (Amersham)(Fig. 1).

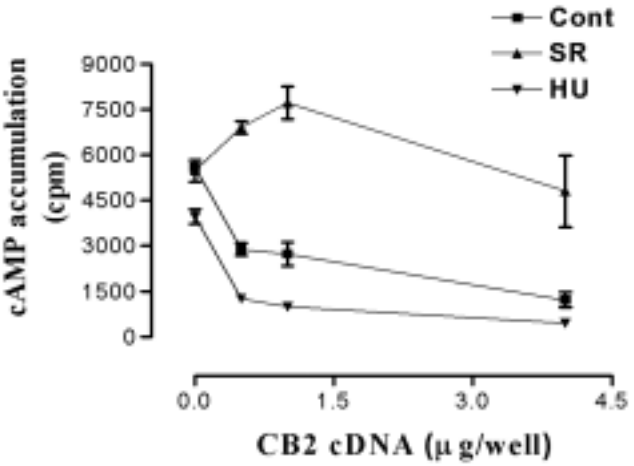

Fig. 1. Constitutively active CB2 receptor inhibits AC-V activity, and SR144528 inverse agonism is dependent on the amount of CB2 cDNA used for transfection. COS cells were cotransfected with the indicated amounts of human CB2 and AC-V cDNAs. AC-V activity was stimulated with 1 μM FS in the presence of either 1 μM SR144528 (SR) or 1 μM HU210 (HU) at 37° C for 10 min. The data represent the means ± SEM of triplicate experiments out of three experiments which gave similar results.

Results

Fusion of HA tag does not alter CB2 cannabinoid receptor CB2 ligand binding or signaling properties

The CB2 receptor cDNA was fused to the HA epitope tag as described above to facilitate detection of CB2 receptor protein expression. [^{3}H]HU-243 binding was measured in membranes prepared from COS-7 cells transiently transfected with wild type or HA-tagged CB2 receptor cDNA. EC_{50} of HU 210 is 654.1 ± 113.2 pM and 635.9 ± 125.1 pM in wild type and HA-tagged CB2-transfected membrane, respectively. In addition, HU 210, an potent agonist, inhibited the AC-V activity stimulated by 1 μM FS with IC_{50} of 1.0 ± 0.1 nM and 1.1 ± 0.3 nM in wild type and HA-CB2, respectively(Table 1).

Table 1. HA-tagging does not significantly affect the receptor binding affinity and the inhibition of adenylyl cyclase activity

	Binding affinity(EC_{50})	AC inhibition (IC_{50})
HA-tagged CB2	635.9 ±125.1 pM	1.1 ±0.3 nM
CB2	654.1 ±113.2 pM	1.0 ±0.1 nM

* COS cells were transfected with human CB2 cDNA (for receptor binding affinity) or were cotransfected with cDNAs of human CB2 and adenylyl cyclase type V (for adenylyl cyclase). The classical potent cannabinoid agonist, HU210, was used for the assays of binding affinity and adenylyl cyclase. The assays of binding affinity and adenylyl cyclase activity were performed as described in Materials and Methods. The data represent the means ± SEM of three expreiments.

Expression of HA-tagged CB2 cannabinoid receptor

We have analyzed expression of the CB2 receptor by both quantitative and functional methods, namely, Western blotting and inhibition of AC-V activity (Fig. 2 and 3). Fig. 2 shows the Western blot using HA antibody (HA 11) in homogenates of whole cells transiently transfected with CB2 cDNA. The specific immunoreactive species had a relative molecular mass of ~ 40 kDa, which is consistent with that predicted for the human CB2 receptor protein (Nowell et al., 1998). The potent cannabinoid agonist, HU 210, inhibited the activity of AC-V with an IC_{50} of 1.1 ± 0.3 nM in COS cells transiently cotransfected with CB2 and AC-V, but in COS-7 cells transfected with only AC-V, HU210 was without inhibitory effect, indicating that this model system is functionally active (Fig. 3).

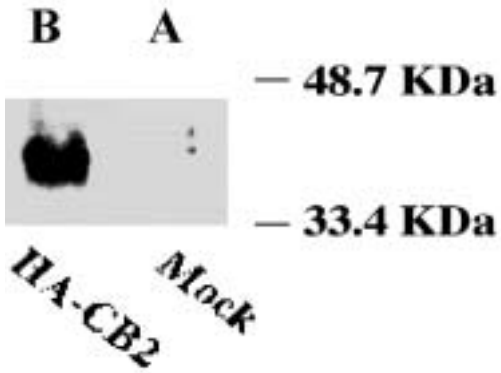

Fig. 2. Western immunoblotting of HA-tagged human CB2. Whole homogenates of COS cells transfected with human HA-tagged CB2, reacted with HA antibody (HA11), exhibit an immunoreactive band of 40 KDa relative molecular weight(lane B). No immunoreactive band was found in mock transfection (i.e., without HA-tagged CB2, lane A).

Inverse agonism of SR 144528 in COS cells transiently co-transfected with CB2 and AC-V

The first selective antagonist for the CB2 peripheral cannabinoid receptor, SR 144528, was shown to act as an inverse agonist in the MAPK pathway (Bouaboula et al., 1999). In agreement with that, SR 144528 enhanced FS-stimulated AC-V activity in COS-CB2 cells (Fig. 3) in a concentration-dependent manner. As expected, COS-7 cells transfected with only AC-V were not affected by treatment with SR 144528, indicating that the inverse agonism of SR 144528 occurs through the CB2 receptor. The interaction of a ligand with a G protein-coupled receptor leads to the exchange of GDP (bound to the G protein α subunit) for

GTP, which causes the subsequent dissociation of the heterotrimer into α and $\beta\gamma$ subunits (Gilman, 1987; Birnbaumer, 1990). PTX covalently modifies certain $G\alpha$ subunits so that ligand-induced exchange of GDP for GTP on the $G\alpha$ subunit is blocked. The toxin completely blocks the activation of AC-V by SR 144528, by interfering with the release of the $\beta\gamma$ subunits from the heterotrimeric G proteins (Fig. 5). In addition, the toxin increases the basal and FS-stimulated activity of AC-V in CB2-transfected COS-7 cells, whereas it decreases the basal and FS-stimulated activity of AC-V in mock-transfected COS-7 cells (Fig. 5).

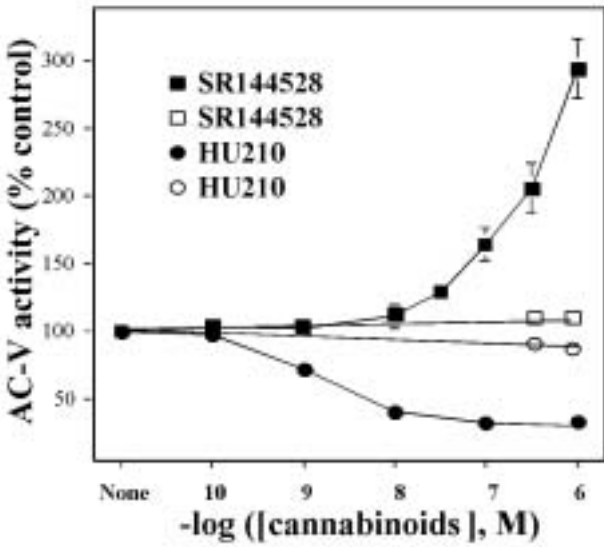

Fig. 3. Effect of the inverse agonist SR144528 and the agonist HU210 on the activity of adenylyl cyclase type V. COS cells were transfected with cDNAs of either CB2 and AC-V (■, □), or AC-V alone (●, ○). The activity of AC-V was stimulated by 1 μM FS with or without the indicated concentration of cannabinoids. The data represent the means ± SEM of three experiments.

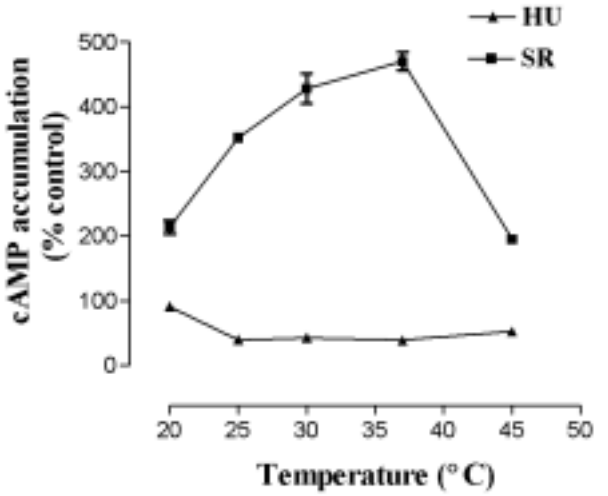

Fig. 4. SR144528 inverse agonism depends on the temperature. COS cells were cotransfected with cDNAs of CB2 and AC-V. The cells were treated with 1 μM SR144528 (SR) or HU210 (HU) at the indicated temperatures for 10 min, followed by the addition of 1 μM FS. The data represent the means ± SEM of three experiments.

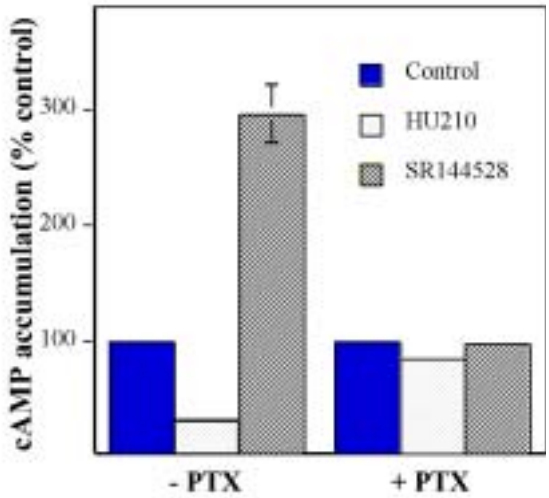

Fig. 5. PTX treatment abolishes the inverse agonism and increases the FS-stimulated AC activity. COS cells co-transfected with either cDNA of AC-V or cDNAs of CB2 and AC-V, were treated with 100 ng/ml PTX for 18 h, where indicated. The cells were stimulated with 1 μM FS in the presence of 1 μM SR144528 at 37° C for 10 min. The data represent the means ± SEM of two experiments.

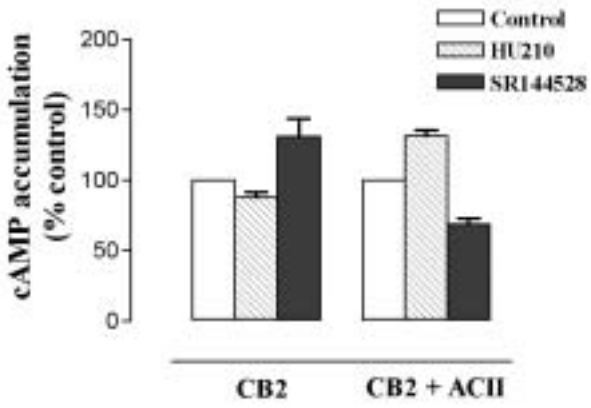

Fig. 6. SR144528 inhibited the activity of AC-II. COS cells were transfected with or without TSH receptor cDNA in the presence of cDNA of AC-II (AC-II). The activity of AC-II was stimulated with 0.1 mM TSH for 10 min. The data represent the means ± SEM of three experiments.

The inhibitory activity of SR 144528 against AC-II

It is now well established that AC-II activation can be induced by G protein $\beta\gamma$ subunits (Bayewitch et al., 1998; Chen et al., 1995; Clapham, 1997; Rhee et al., 1998, see Fig. 6), although stimulation by $G\beta\gamma$ subunits was initially

thought to be conditional on concurrent stimulation by $G\alpha_s$ (Federman et al., 1992; Tang and Gilman, 1991). Therefore, we attempted to show whether SR 144528 affects the activity of AC-II. Surprisingly, SR 144528 was found to inhibit the activity of AC-II (by 32%) stimulated with 0.1 μ M of TSH in COS cells cotransfected with the cDNAs of CB2, TSH receptor and AC-II, whereas activation of the CB2 cannabinoid receptor with HU 210 stimulated the activity of AC-II by ~ 35%, in agreement with previous reports (Avidor-Reiss et al., 1997; Federman et al., 1992; Rhee et al., 1998, see Fig. 6).

Discussion

In this report, we show that SR 144528 acts as an inverse agonist in the AC-cAMP pathway, which differs from the MAPK pathway for the CB2 cannabinoid receptor (Bouaboula et al., 1996). The CB2 cannabinoid receptor activates mainly the AC-cAMP and MAPK pathways. The latter is dependent on the small G-protein Ras and both pathways are coupled to PTX-sensitive-$G\alpha_{i/o}$. The CB2 receptor has been shown to be constitutively active (Bouaboula et al., 1999). In line with that, constitutively active CB2 receptor was found here to strongly inhibit AC-V activity (Fig. 2 and 5).

It is now well established that effectors (e.g., AC) are regulated by different types of G proteins ($G\alpha_i$ or $G\alpha_s$). There is increasing evidence that chronic receptor activation can not only affect the receptor but can also have an effect at the G-protein-effector level. The mutant of $G\alpha_s$ (α_s-Q227L), which abolishes GTP hydrolysis (albeit not completely), constitutively activates the AC (Masters et al., 1989). If inverse agonism occurs through the activation of $G\alpha_s$, this action might be significantly impaired. However, inverse agonism by SR144528 on COS cells co-transfected with cDNAs of CB2, α_s-Q227L and AC-V or AC-II still remains (data not shown). Consequently, these results show that we can rule out the possibility of $G\alpha_s$ mediation, or of interaction between $G\alpha_s$ and $\beta\gamma$ subunits. Moreover, in line with a previous report (Bouaboula et al., 1999), PTX treatment abolished SR144528-induced inverse agonism (Fig. 5), indicating that the action is mediated via $G\alpha_i$ protein but is not via $G\alpha_s$ subtypes. In addition, the toxin treatment increased AC-V activity in CB2-transfected COS cells, but decreased AC-V activity in mock-transfected cells. Therefore, we conclude that the CB2 cannabinoid receptor is constitutively active and that SR144528 reversed this activity by modulation of $G\alpha_i$ or $G_{\beta\gamma}$ dimers.

It has recently been reported that activation of the CB2 receptor enhanced MAPK activity (Derocq et al., 1998; Bouaboula et al., 1996; Bouaboula et al., 1999; Makda et al., 1997; Rinaldi-Carmona et al., 1998). In addition, several G protein-coupled receptors that interact with PTX-sensitive heterotrimeric G proteins were found to mediate Ras-dependent activation of MAPK, which is mediated through $\beta\gamma$ subunits (Crespo et al., 1994; Coso et al., 1996; van Biesen et al., 1995). To assess the role of Ras on SR144528-induced inverse agonism, we introduced dominant negative mutants of Ras (N17-Ras). Expressing N17-Ras did not interfere with the inverse agonism (data not shown). As suggested previously (Bouaboula et al., 1996), in AC-cAMP signaling, the pathway of inverse agonism of SR 144528 modulated by the G protein $\beta\gamma$ subunits is probably different from that of MAPK depending on the PKC isoforms.

In summary, we have shown here that SR144528, known as a selective antagonist of the CB2 peripheral cannabinoid receptor, acts as an inverse agonist in AC-cAMP and inverse agonism by SR144528 is independent of Ras pathways.

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