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G_i Protein Modulation Induced by a Selective Inverse Agonist for the Peripheral Cannabinoid Receptor CB2: Implication for Intracellular Signalization Cross-Regulation.

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ABSTRACT

The peripheral cannabinoid receptor (CB2) is a G protein-coupled receptor that is both positively and negatively coupled to the mitogen-activated protein kinase (MAPK) and cAMP pathways, respectively, through a Bordetella pertussis toxin-sensitive G protein. CB2 receptor-transfected Chinese hamster ovary cells exhibit high constitutive activity blocked by the CB2-selective ligand, SR 144528, working as an inverse agonist. We showed here that in addition to the inhibition of autoactivated CB2 in this model, we found that SR 144528 inhibited the MAPK activation induced by G_i-dependent receptors such as receptor-tyrosine kinase (insulin, insulin-like growth factor 1) or G protein-coupled receptors (lysophosphatidic acid), but not by G_i-independent receptors such as the fibroblast growth factor receptor. We showed that this SR 144528 inhibitory effect on G_i-dependent receptors was mediated by a direct G_i protein inhibition through CB2 receptors. Indeed, we found that through binding to the CB2 receptors, SR 144528 blocked the direct activation of the G_i protein by mastoparan analog in Chinese hamster ovary CB2 cell membranes. Furthermore, we described that sustained treatment with SR 144528 induced an up-regulation of the cellular G_i protein level as shown in Western blotting as well as in confocal microscopic experiments. This up-regulation occurred with a concomitant loss of SR 144528 ability to inhibit the insulin or lysophosphatidic acid-induced MAPK activation. This inverse agonist-induced modulation of the G_i strongly suggests that the modulated protein is functionally associated with the complex SR 144528/CB2 receptors, and that the G_i level may account for the heterologous desensitization phenomena.

To date, two cannabinoid receptors have been characterized: 1) the central cannabinoid receptor (CB1) primarily expressed in central nervous system tissues (Matsuda et al., 1990; Herkenham et al., 1991; Matsuda et al., 1993) and 2) the peripheral cannabinoid receptor (CB2) expressed in the immune system but not in the brain (Munro et al., 1993; Galiegue et al., 1995). The CB1 is a prime target for the psychoactive effect of cannabinoids, whereas cannabinoid-induced immunomodulation is predominantly CB2-mediated. Both CB1 and CB2 receptors belong to the G protein-coupled receptor (GPCR) superfamily, and their stimulation by cannabinoid agonists induced several biological responses among which are the inhibition of adenylyl cyclase (Howlett, 1985; Felder et al., 1995), the activation of the MAPKs (Bouaboula et al., 1995b, 1996), and the in vitro induction of the immediate-early gene Krox 24 (Bouaboula et al., 1995a, 1996), an effect also observed in vivo (Mailleux et al., 1994; Glass and Dragunow, 1995). All of these actions appear to be exerted through one or more members of the Bordetella pertussis toxin (PTX)-sensitive G_i family of GTP-binding regulatory proteins such as G_i and G_o. Although natural (Δ^9-tetrahydrocannabinol) or synthetic (CP-55,940, WIN 55212–2) cannabinoid ligands are not selective for CB1 and CB2 receptors, selective antagonists have recently been developed specifically targeting either CB1 receptor (SR 141716; Rinaldi-Carmona et al., 1994, 1996) or CB2 receptor (SR 144528; Rinaldi-Carmona et al., 1998).

The classical model of GPCR action implies that the binding of an agonist to a receptor is essential for the receptor activation and the transduction of the biological signal across the plasma membrane. However, several studies have revealed that receptor activation may occur spontaneously without any agonist binding. This discovery led to a reclassification of antagonists as neutral antagonists or inverse agonists. Although neutral antagonist blocks agonistic actions being ineffective on the receptor-constitutive activity, inverse agonists not only block the action of agonist, but also suppress constitutive activity (Costa and Herz, 1989;

ABBREVIATIONS: CB1, central cannabinoid receptor; CB2, peripheral cannabinoid receptor; CHO, Chinese hamster ovary; CHO-wt, CHO wild type; FGF-b, basic fibroblast growth factor; GPCR, G protein-coupled receptor; IGF1, insulin-like growth factor 1; LPA, lysophosphatidic acid; MAPKs, mitogen-activated protein kinases; Mas-7, mastoparan analog; PTX, Bordetella pertussis toxin; CTX, cholera toxin; RTK, receptor-tyrosine kinase, Δ^9-THC, delta 9 tetrahydrocannabinol; FCS, fetal calf serum; TBS, Tris-buffered saline.

Lefkowitz et al., 1993; Chidiac et al., 1994; Bond et al., 1995). Inverse agonism was first described as a property of β-carbolines at the γ-aminobutyric acid receptor (Braestrup et al., 1982), and is now identified for numerous GPCRs (Kenakin, 1996).

In this context, we recently demonstrated: 1) an agonist-independent activity for the CB1 receptor when expressed in mammalian cells after transfection and 2) that the CB1-selective antagonist SR141716 not only blocks the actions of cannabinoid agonists but also suppresses the constitutive activity of the receptor, indicating that SR141716 acts as an inverse agonist (Bouaboula et al., 1997). Furthermore, we revealed for the first time a novel and provocative property for this inverse agonist. We indeed demonstrated that SR141716 not only inhibits autoactivated CB1 but also switches off, through CB1, the activation induced by other and unrelated G_i-dependent receptors such as insulin or insulin-like growth factor 1 (IGF1) receptors (Bouaboula et al., 1997). We hypothesized that SR141716 could promote or stabilize CB1-coupled G_i complex, making this protein unavailable for further coupling. According to this model, designated a "three-state receptor model", inverse agonist converted the autoactivated receptor to a suppresser receptor acting in trans by sequestration of the G_i protein, which remains inactive. It is assumed that the receptor is in equilibrium among three conformations: $R°$, R^+, and R^-. In the $R°$ state, the receptor is uncoupled from the G protein, whereas both forms R^+ and R^- are able to bind to the G protein. R^+/G represents the active positive conformation and leads to the classical signal transduction induced by agonists. In contrast, the R^-/G form does not likely induce any signal transduction, but by capturing the G protein, prevents the activation of nonrelated GPCRs localized in its vicinity. Therefore, R^-/G was termed the active negative state. Interestingly, this three-state model fits with the cubic ternary complex model elaborated from thermodynamic calculations predicting the existence of an inactive receptor-G protein complex that is consistent with our biological data (Kenakin, 1996; Weiss et al., 1996a,b).

In the present work, we investigated whether this property could be extended to another couple receptor/inverse agonist model. We used the novel CB2-selective inverse agonist SR 144528 and the human CB2 stably transfected into Chinese hamster ovary (CHO) cells. We confirmed that inverse agonist may inhibit transduction pathways through a PTX-sensitive G_i protein of other receptors such as receptor-tyrosine kinases (RTKs) and GPCR. In addition, using this property as a starting point, we further demonstrated that inverse agonist induces through CB2 a modulation of the G_i to which it is coupled. These data provide new insights into the relationship between inverse agonist, GPCR, and its cognate G protein.

Materials and Methods

Reagents. [^{35}S]GTPγS (1250 Ci/mmol) was purchased from DuPont-NEN (Paris, France). [γ^{32}P]ATP (3000 Ci/mmol) was purchased from Amersham (Les Ulis, France). Bovine myelin basic protein, Δ^9-tetrahydrocannabinol (Δ^9-THC), and WIN 55212.2 PTX were purchased from Sigma Chemical Co. (Saint-Quentin-Fallavier, France). SR 144528 [*N*-(1*S*)-endo-1,3,3-trimethyl bicyclo [2.2.1] hepta-2-yl]-5-(4-chloro-3-methylphenyl)-1-(4-methylbenzyl)-pyrazole-3-carboxamide) and CP-55,940 were synthesized at the Chemistry Department of Sanofi (Montpellier, France). Phospho-MAPK rabbit polyclonal antibodies were purchased from Biolabs (Hertfordshire, England). Mastoparan analog (Mas-7) was purchased from Calbiochem (Meudon, France). GDP and GTPγS were purchased from Boehringer Mannheim (Meylan, France). Cholera toxin (CTX) was obtained from Calbiochem. Anti-rabbit antibody, $G_i\alpha_3$, $G_i\alpha_{1-2}$, $G_i\alpha_{3-0}$ and Gβ were obtained from Santa Cruz Biotechnology (Santa Cruz, CA).

Stable Cell Lines and Culture Conditions. For stable expression of the human CB2, the CHO dihydrofolate reductase negative cell line was transfected using a modified calcium phosphate precipitation method (Graham and Eb, 1973) with plasmid p1211 coding for human CB2, and was selected for dihydrofolate reductase expression as described previously (Bouaboula et al., 1996). CHO wild-type (CHO-wt) cells were routinely grown as monolayers at 37°C in a humidified atmosphere containing 5% CO_2 in α-modified Eagle's medium (Gibco-BRL) supplemented with 5% dialyzed fetal calf serum (FCS), 40 μg/ml L-proline, 1 mM sodium pyruvate, 60 μg/ml tylocine, and 20 μg/ml gentamycin.

Preparation of Cellular Membranes. Cells grown to confluence were collected by scraping and spun at 200*g* for 10 min at 4°C. Crude membranes were prepared by homogenization of cells in 5 mM Tris-HCl (pH 7.5) and centrifugation at 1000*g* for 5 min. The supernatant was centrifuged at 40,000*g* for 40 min at 4°C, the pellet was resuspended in a buffer consisting of 50 mM Tris-HCl, pH 7.5, 5 mM $MgCl_2$, 1 mM EDTA, and stored at −80°C until use.

[^{35}S]GTPγS Binding. The [^{35}S]GTPγS binding was measured as described by Selley et al. (1996). Briefly, membranes from CHO-CB2 or CHO-wt cells (30 μg protein) were incubated with various drugs for 60 min at 30°C in assay buffer (50 mM Tris-HCl, pH 7.4; 3 mM $MgCl_2$; 0.2 mM EGTA; 100 mM NaCl; 0.1% BSA) in the presence of 0.1 nM [^{35}S]GTPγS and 50 μM GDP, in a final volume of 200 μl. The reaction was carried out in 96-well microtitration plates (Multiscreen FB Glass Fiber, Millipore Corp., Bedford, MA). Nonspecific binding was measured in the presence of 10 μM unlabeled GTPγS. The reaction was terminated by rapid filtration, the microfiltration plates washed five times with ice-cold wash buffer (50 mM Tris-HCl, pH 7.4), and bound radioactivity was determined.

CTX-Catalyzed ADP Ribosylation. 25 μg of CHO-CB2 membranes were treated with dithiothreitol-activated CTX (5 μg) in reaction mixture (100 μl) containing 3 mM [^{32}P]-NAD^+ (5 μCi/tube), 3 mM $MgCl_2$, 1 mM ATP, 10 mM thymidine, 0.2% bovine serum albumin, and 0.1 M potassium phosphate. After incubation for 60 min at 30°C, the reactions were terminated by addition of 20 mM HEPES/NaOH pH 7.4 (4°C), and centrifuged at 12,000*g* for 10 min at 4°C. The membrane pellets were then dissolved in Laemmli's buffer and resolved in 12% sodium dodecyl sulfate polyacrylamide gel electrophoresis. The gel was dried and analyzed by autoradiography. The spot signals in the autoradiography were scanned, and the data was expressed in histogram representation.

MAPK Assay. MAPK activity was measured as described previously (Bouaboula et al., 1995b). Briefly, cells grown to 80% confluence in 24-well plates were placed in medium containing 0.5% FCS for 24 h (0% FCS when Δ^9-THC was used) before assay. After treatment, cells were washed twice in buffer A (50 mM HEPES, pH 7.4, 150 mM NaCl, 10 mM $Na_4P_2O_7$, 50 mM NaF, 1 mM EDTA, 20 mM glycerophosphate, 1 mM EGTA, 2 mM Na_3VO_4) and lysed for 15 min in 100 μl of buffer A containing 1% (v/v) Triton X-100, 100 U/ml aprotinin, 20 μM leupeptin, 0.2 mg/ml phenylmethylsulfonyl fluoride and 2 mM dithiothreitol. Solubilized cell extracts were centrifuged at 14,000*g* for 15 min and 18 μl of supernatants (20 μg of proteins) were analyzed for MAPK activity. The protein contents in the supernatants were determined using a micro BCA protein assay kit (Pierce Chemical Co., Rockford, IL). The phosphorylation of MAPK-specific peptide substrate was carried out at 30°C for 30 min (linear assay conditions) with [γ^{32}P]ATP by using the Biotrack p42/p44 MAPK enzyme system (Amersham).

Western Blot Analysis. After treatment, cells were phosphate-buffered saline-washed and directly lysed in Laemmli's loading

buffer containing 6 M urea (Laemmli, 1970). Fifty-μg proteins were run on 4 to 20% gradient polyacrylamide gel before being blotted onto nitrocellulose filters. Nonspecific antibodies-binding was prevented by incubating filters in 10% dried milk powder in Tris-buffered saline (TBS) buffer (10 mM Tris-HCl pH 7.6, 150 mM NaCl, and 0.05% Tween 20). Blots were incubated with the anti-phospho-p42 MAPK for 3 h in TBS 1% dried milk. After extensive washes with TBS, the blots were subsequently incubated for 1 h at room temperature with a peroxidase-labeled anti-IgG antibody. After washing, immunostained phospho-MAPKs were visualized using an enhanced chemiluminescence detection system (Amersham).

Immunofluorescence Analysis of G_i. Immunocytochemical fluorescence labeling of G_i was analyzed by confocal laser microscopy. CHO-CB2 cells were grown on 12-mm glass coverslips (Prolabo, Paris, France) and treated with cannabinoid ligands for various periods. Washed cells were incubated with anti-$G_i\alpha_3$ polyclonal antibody for 30 min at 4°C, washed, and incubated with 1/200 dilution of CY3-conjugated anti-rabbit IgG (Sigma Immunochemicals) for another 30 min at 4°C. After washing, coverslips were inverted and mounted on glass microscope slides using glycerol mountant containing the antibleaching reagent DABCO at 50 ng/ml (Sigma). Fluorescence analyses were performed using a confocal microscope (LSM410; Zeiss, Oberkochen, Germany).

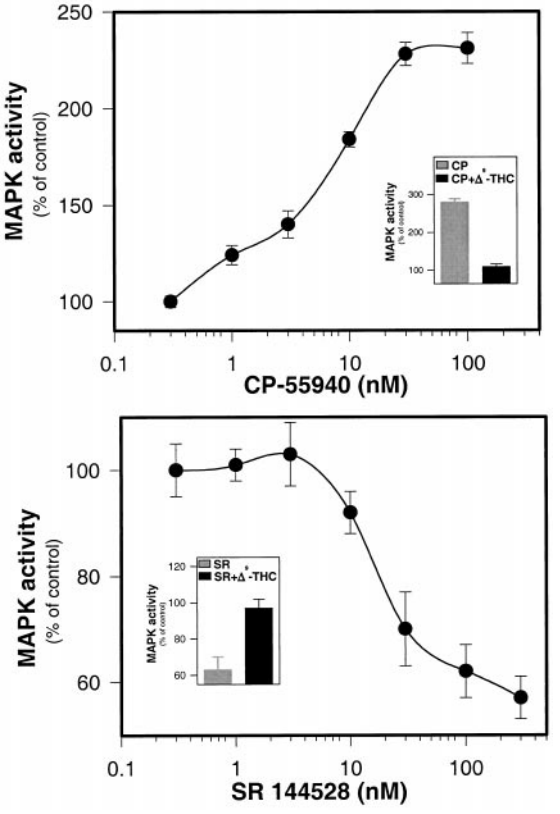

Fig. 1. Effect of cannabinoid receptor ligands on MAPK activity in CHO-wt and CHO-CB2 cells. Growth-arrested CHO-CB2 cells were treated with increasing concentrations of cannabinoid receptor ligands for 10 min. The activities of p42/p44 MAPK were measured in cell lysates as described in *Materials and Methods*. Data points are mean values ± S.D. of duplicate samples and experiments were repeated five times. A, dose-response effects of CP-55,940 on MAPK activity. Dose-response effects of the CB2 selective antagonist SR 144528 on basal MAPK activity in CHO-CB2 cells. In the insets the effects of Δ^9-THC are shown: CHO-CB2 cells were pretreated with 1 μM Δ9-THC for 10 min and exposed to CP-55,940 (inset A) or SR 144528 (inset B) for another 10 min. The activities of p42/p44 MAPK were determined as described below.

Results

SR 144528 Acted as an Inverse Agonist on Autoactivated CB2 Receptor. The biological properties of the CB2-selective antagonist SR 144528 were investigated in CHO cells stably transfected with the human CB2 cDNA (CHO-CB2 cells). Many receptors that couple to heterotrimeric guanine-nucleotide binding proteins (G proteins) have been shown to mediate rapid activation of the MAPK (Van Biesen et al., 1996), a response that is also observed with CB2 (Bouaboula et al., 1996). Stimulation of CB2 by the synthetic cannabinoid agonist CP-55,940 resulted in a dose-dependent activation of MAPKs with an EC_{50} of 8 nM (Fig. 1A), an effect that was completely prevented in CHO-CB2 cells after treatment with SR 144528 (not shown). Comparison of CHO-CB2 and CHO-wt cells showed an enhanced basal MAPK activity in CHO-CB2 cells (not shown); this activity was reduced in a dose-dependent manner by SR 144528 with an IC_{50} of 18 nM (Fig. 1B). Neither the CP-55,940 ability to stimulate MAPK activity nor that of SR 144528 to inhibit this activity are measurable in parental CHO cells (not shown). On the other hand, the natural cannabinoid ligand Δ^9-THC, which acted as a CB2 neutral antagonist (Bayewitch et al., 1996), was unable to modulate MAPK either positively or negatively in these studies. As expected for a competitive interaction, Δ^9-THC was able to block both the stimulating effect of CP 55,940 (Fig. 1A, inset) and the inhibitory effect of SR 144528 (Fig. 1B, inset), thus ruling out the possibility that the constitutive activity of the receptor was due to a putative endogenous cannabinoid ligand carried over from cell culture.

We confirmed the above effects of SR 144528 on the G protein activity in cellular membranes from CHO-CB2 cells by the use of [^{35}S]GTPγS binding. The comparison of basal [^{35}S]GTPγS binding in CHO-wt and CHO-CB2 cellular membranes showed an enhanced G protein activity in membranes from CHO-CB2 cells (not shown). The treatment with CP-55,940 induced a stimulation of [^{35}S]GTPγS binding to the CHO-CB2 but not to the CHO-wt cellular membranes in a dose-dependent manner with a ED_{50} of 5 ± 1.5 nM (Table 1). In contrast, as shown in Table 1, the basal [^{35}S]GTPγS binding was reduced in a dose-dependent manner by SR 144528 with an IC_{50} of 3.1 ± 1.2 nM. SR 144528 treatment had no effect in membranes from nontransfected parental CHO-wt cells (not shown).

In the experiments described below, we further examined whether the G protein involved in this process belongs to the

TABLE 1

Effect of CP-55940 or SR 144528 on the binding of [^{35}S]-GTPγS binding to CB2

CHO-CB2 cell membranes were treated with increasing concentrations of cannabinoid receptor ligands for 60 min. The [^{35}S]GTPγS binding was determined as described in *Materials and Methods*. Data are means ± S.D. of duplicate samples and expressed as a percentage of values from untreated cell membranes.

	[^{35}S]GTPγS binding	
nM	CP-55940	SR 144528
	%	
Control	100 ± 8	100 ± 7
1	115 ± 4	97 ± 5
3	128 ± 2	73 ± 8
10	145 ± 7	52 ± 4
30	165 ± 5	41 ± 3
100	170 ± 4	49 ± 6

G_i subclass. Thiol-activated CTX was shown to catalyze the [^{32}P]ADP ribosylation of $G_s\alpha$ (43–45 Kda) but also that of $G_i\alpha$ (40–41 Kda) when the assay was performed in the absence of guanine nucleotides and when the G_i-coupled GPCR is stimulated by its specific agonist (Milligan et al., 1991). In the absence of exogenously added nucleotides, CTX catalyzed the labeling of a 45-Kda protein that corresponds to the stimulatory $G_s\alpha$, and of another substrate of 40–41 Kda that is attributed to $G_i\alpha$ because, when a similar experiment was carried out with membrane prepared from cells previously treated with PTX, CTX could no longer label the 40–41 Kda band (data not shown). As illustrated in Fig. 2, the quantification of the $G_i\alpha$ [^{32}P]ADP ribosylation showed that the CP-55,940 treatment of CHO-CB2 cellular membranes markedly enhanced the $G_i\alpha$ labeling, whereas SR 144528 provoked a strong inhibition of $G_i\alpha$ labeling. In addition, neither CP-55,940 nor SR 144528 treatment affected the [^{32}P]ADP ribosylation of the $G_s\alpha$ (data not shown).

Taken together, these results suggested that the expression of CB2 results in agonist-independent activation of $G_i\alpha$ protein and MAPK, and that these spontaneous activities can be drastically attenuated by addition of SR 144528, which acted as an inverse agonist.

SR 144528 Inhibits G_i Activity in CHO-CB2 Cells. We have recently demonstrated that an inverse agonist could inactivate MAPK activation induced by other receptors (Bouaboula et al., 1997). We examined whether such a property could also be observed in our present model. To question this, MAPK was stimulated with one of three different ligands such as insulin, basic fibroblast growth factor (FGF-b), or lysophosphatidic acid (LPA), in the presence or absence of SR 144528. MAPK activation in response to insulin or LPA exposure was prevented by SR 144528 in CHO-CB2 cells. In contrast, MAPK stimulated by FGF-b was not affected by SR 144528 treatment. These results, obtained by the detection of the active p42-isoform of MAPK proteins in Western blot experiments (Fig. 3), were confirmed by the measure of MAPK activities in cell lysates as described in the Fig. 1 legend (data not shown). Furthermore, SR 144528 had no effect on LPA- or insulin-stimulated MAPKs in CHO-wt cells (not shown), establishing that the above effects did require the interaction of SR 144528 with CB2 receptors.

It has already been shown that G_i protein, which is the cognate G protein for physiological signaling by CB2, is involved also in MAPK activation induced by insulin, IGF1, and LPA, but not by FGF-b (Luttrell et al., 1995). We indeed observed that the MAPK activation induced by insulin, IGF-1, or LPA was markedly inhibited by PTX, whereas induction of MAPK by FGF-b was unaffected by PTX (data not shown). Because the G_i protein is the key element coincident with both RTK and GPCR and that can be affected by SR 144528, one may assume that the binding of SR 144528 to CB2 interfered with G_i activity. To test this possibility, the effect of SR 144528 on G_i or MAPK activation induced by an analog of mastoparan was studied. Mastoparan is a direct activator of G protein with a reported selectivity for G_i/G_o (Gil et al., 1991). Mas-7 is an analog exhibiting a 5-fold greater potency than does mastoparan (Leyte et al., 1992).

We measured the effect of SR 144528 on G_i activation induced by Mas-7 in cellular membranes from CHO-CB2 cells by the use of [^{35}S]GTPγS binding. Figure 4 clearly indicates that a 3 μM Mas-7 treatment stimulated [^{35}S]GTPγS binding to CHO-CB2 cell membranes. SR 144528 was able to induce a marked inhibition of constitutive as well as Mas-7-induced [^{35}S]GTPγS binding to CHO-CB2 cell membranes. This effect was not observed in CHO-wt cell membranes, indicating that the SR 144528-induced inhibition is not a nonspecific direct interaction with the G_i protein, but a receptor-mediated response. These results suggest that the inhibition of the G_i protein might be the key feature that controls signal inhibition.

Sustained Treatment of the CB2 with Inverse Agonist Causes a Cellular G_i Protein Up-Regulation. It is tempting to hypothesize that G_i would be physically linked to CB2, making this protein unavailable for further coupling to GPCR or RTK. If so, we speculated that prolonged exposure to SR 144528 may provoke a cellular response that affects the cellular level of the G_i protein to which the receptor is coupled. The analysis of cellular content of the G_i subunit in CHO-wt or CHO-CB2, measured immunologically by West-

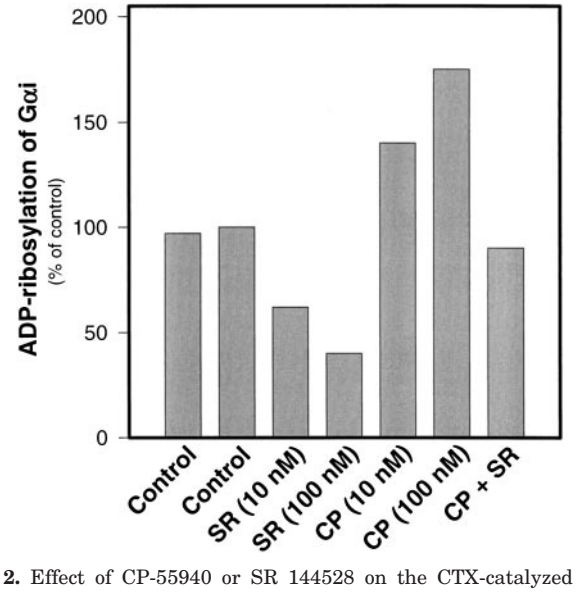

Fig. 2. Effect of CP-55940 or SR 144528 on the CTX-catalyzed ADP ribosylation of the $G_i\alpha$-protein in membranes of CHO-CB2 cells. Membranes (25 μg) from CHO-CB2 cells were incubated with [^{32}P]NAD$^+$ and thiol-activated CTX (5 μg) in the absence of ligand or in the presence of varying concentrations of CP-55,940 or SR 144528 for 60 min as described in *Materials and Methods*. The samples were resolved on sodium dodecyl sulfate-polyacrylamide gel electrophoresis and subjected to autoradiography. The signal spot of [^{32}P]ADP ribolysation of $G_i\alpha$ were quantified and expressed as histogram.

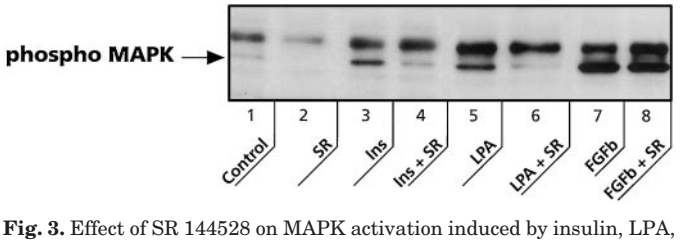

Fig. 3. Effect of SR 144528 on MAPK activation induced by insulin, LPA, and FGF-b. Quiescent CHO-CB2 cells were either pretreated or not with SR 144528 for 10 min and stimulated with insulin, LPA, or FGF-b. Ten minutes after stimulation, phosphorylated isoforms of MAPK were determined by Western blot using an anti-phospho p42/p44 MAPK. Lane 1, untreated cells. Cells treated with: lane 2, 100 nM SR 144528; lane 3, 1 μg/ml insulin; lane 4, 1 μg/ml insulin + 100 nM SR 144528; lane 5, 1 μg/ml LPA; lane 6, 1 μg/ml LPA + 100 nM SR 144528; lane 7, 10 ng/ml FGF-b; lane 8, 10 ng/ml FGF-b + 100 nM SR 144528.

ern blot analysis, revealed the presence of the following G protein subunits: Gα (αi0/1, αi1/2, αi3) and Gβ. As shown in Fig. 5A, a 24-h treatment of CHO-CB2 cells with the agonist CP-55,940 significantly decreased the abundance of the G_i protein. In striking contrast, similar treatment with SR 144528 induced a completely opposite scenario by up-regulating the G_i protein levels (αi3, αi0, αi1/2, and β). Interestingly, when SR 144528-treated cells were washed and further exposed for another 5-h time period to CP-55,940, the SR 144528-induced enhancement is shown to be reversible (Fig. 5B). Neither CP-55,940 nor SR 144528 affected $G_s\alpha$. On the other hand, treatment of parental nontransfected CHO cells with either CP-55,940 or SR 144528 had no effect on levels of G_i (results not shown).

To examine this further, we carried out immunocytochemical analyses of G_i protein expression in CHO-CB2 cells stimulated or not with either CP-55,940 or SR 144528 (Fig. 6). In untreated cells, confocal images show that G_i was distributed throughout the cell except in the nucleus (Fig. 7). Activation of the cell with CP-55,940 induced both a redistribution from the plasma membrane to endocytic vesicles as visualized by a brightly stained punctuate accumulation in the cytoplasm together with a profound down-regulation of the protein. In agreement with Western blot analysis, a clear up-regulation of G_i was observed after SR 144528 treatment. In addition, in SR 144528-treated cells, G_i was confined along the edge of the cells instead of localizing in a diffuse distribution throughout the cytoplasm.

The discovery that G_i proteins may be up-regulated by inverse agonist led us to examine whether, under such conditions, desensitization of the functional response of RTK or GPCR could still be observed after long-term inverse agonist exposure. As shown in Fig. 7 after a 24-h treatment, SR 144528 was unable to inhibit neither insulin- nor LPA-activated MAPK any longer. We ruled out the possibility that insulin or LPA may use another signalization pathway that could bypass SR 144528 inhibition. Indeed, PTX treatment inhibited both insulin- and LPA-induced MAPK even after 20 h of SR 144528 treatment, indicating that insulin and LPA signaling remained a G_i-dependent process. We concluded from these experiments that the up-regulation of G_i overcame the negative control exerted by CB2-bound SR 144528 on RTK or GPCR signalization.

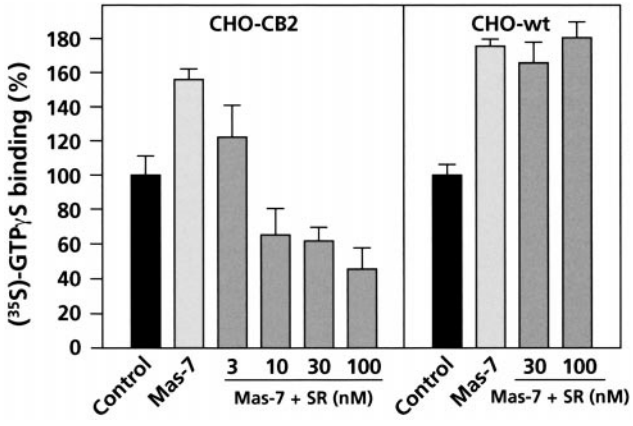

Fig. 4. Effect of SR 144528 on Mas-7-induced [^{35}S]GTPγS binding in CHO-CB2 cell membranes. CHO-CB2 or CHO-wt cell membranes were pretreated or not with increasing concentration of SR 144528 for 10 min before stimulation with 3 μM Mas-7 for 60 min. [^{35}S]GTPγS binding was determined as described in *Materials and Methods*. Data are means ± S.D. of duplicate samples and expressed as a percentage of values from untreated cell membranes.

Discussion

SR 144528 is an Inverse Agonist. In the first part of this paper, we demonstrated that the CB2-selective antagonist SR 144528 functions as an inverse agonist for the autoactivated CB2. These conclusions emerged from observations on two independent biological responses. First, we showed that the basal level of MAPK activity was enhanced in CHO cells after transfection of CB2 receptors, and that this increase

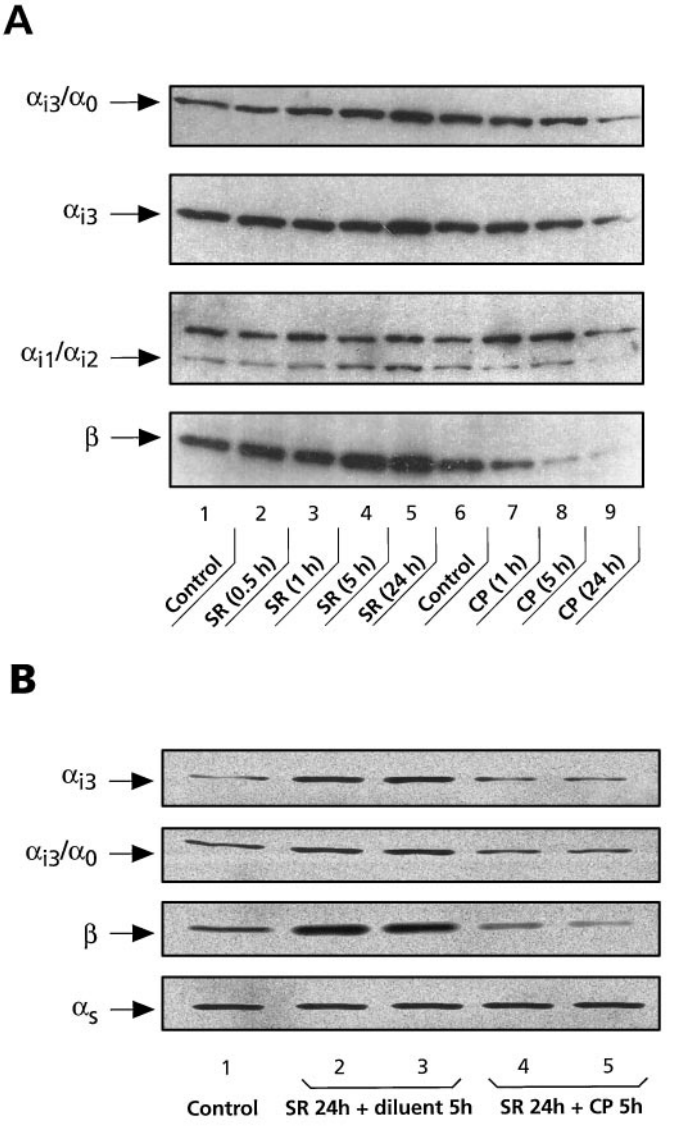

Fig. 5. G_i up- and down-regulation in CHO-CB2 cells exposed to CP-55,940 or SR 144528. A, growth-arrested CHO-CB2 cells were exposed to 30 nM CP-55,940 (7–8-9) or 50 nM SR 144528 (2–3-4–5) for varying time periods. Reaction was ended by the addition of Laemmli's buffer and the G_i protein level was determined by Western blot analysis using an antibody directed against one or two G_i-protein subunits. B, growth-arrested CHO-CB2 cells were incubated with 50 nM SR 144528 (2–3-4–5) for 24 h and then washed three times to remove the residual SR 144528, before being treated with diluent (2–3) or 50 nM CP-55940 (4–5) for another 5 consecutive hours. The G_i-protein content was determined by Western blot as described below.

was reversed by treatment with SR 144528. Second, we showed that SR 144528 inhibited the basal level of G protein activity. This was demonstrated by [^{35}S]GTPγS binding to G proteins that are directly regulated by the receptor. We showed that membranes derived from CHO-CB2 displayed a basal binding activity stimulated markedly by CP-55,940 but inhibited by SR 144528 in a dose-dependent manner. We also used an indirect test in which the G_i protein (PTX-sensitive) becomes substrate of CTX-catalyzed ADP ribosylation only when it is activated by a receptor (Milligan et al., 1991; Mullaney et al., 1996). This assay has previously been used to examine the coupling of the delta opioid receptor to G_i-like protein in NG 108–15 (Roerig et al., 1992). In membranes of CB2 cells, in the absence of receptor ligand, in addition to the expected [^{32}P]ADP ribosylation of $G_s\alpha$, CTX was able to catalyze the [^{32}P]ADP ribosylation of a 40-Kda $G_i\alpha$. The treatment of CHO-CB2 cell membranes with CP-55,940 markedly enhanced the $G_i\alpha$ labeling, whereas the [^{32}P]ADP ribosylation of the $G_s\alpha$ isoforms was unaffected. Conversely, addition of SR 144528 provoked a strong inhibition of $G_i\alpha$ labeling with no effect on the radioactivity incorporation into the other bands (data not shown).

An often raised alternative interpretation of the inhibition of autoactivated receptor by inverse agonists would be the blockade of endogenous agonists present in culture medium or produced by the cells. As the natural cannabinoid ligand Δ^9-THC acts as a neutral CB2 antagonist in our assay, such a possibility could be ruled out. Together, these results support the notion that the observed effects of the CB2 antagonist SR 144528 reflect the direct consequences of its binding to unoccupied receptors and the notion that it acts as an inverse agonist with high intrinsic activity.

SR 144528-Induced Heterologous Desensitization. We demonstrated here that the inverse agonist SR 144528 not only inhibits autoactivated CB2, but also switches off MAPK activation from RTKs, including insulin and IGF1 receptors or the GPCR LPA receptor. By contrast, SR 144528 did not affect MAPK activation induced by FGF-b in CHO-CB2 cells. These effects were CB2-mediated because they were not observed in the parental CHO cells. These results indicated that SR 144528 binding to CB2 induced biological responses that negatively interfered with particular RTK or GPCR pathways. We have already described this novel property for the central cannabinoid receptor inverse agonist, SR 141716 (Bouaboula et al., 1997). The present results extended this property to another couple inverse agonist/receptor model.

How could the inhibition of CB2 receptor autoactivation by the inverse agonist intercept the MAPK activation triggered by either RTK or GPCR? Signal transduction between receptors on the plasma membrane and MAPK in the cytosol requires a series of events leading to the assembly of an activated Ras-Raf complex on the membrane, followed by initiation of the cytosolic MEK-MAPK cascade. A high degree of parallelism has been described among GPCR- and RTK-mediated mitogenic signal transduction pathways to MAPK (Van Biesen et al., 1996). Both receptor types used many of the same intermediates and the upstream point at which all these mitogenic signals, originating from either insulin, IGF1, or LPA and CB2 receptors, converge is very likely the G_i protein. The following observations argue for the G_i protein being this key element: 1) in CHO-transfected cells, we showed that CB2, insulin, IGF1, and LPA shared a PTX-sensitive signaling pathway, 2) FGF-b-stimulated MAPK phosphorylation, which is not G_i-mediated, is not affected by the presence of the inverse agonist, 3) SR 144528 bound to CB2 receptor prevents the activation of both G_i protein and MAPKs induced by Mas-7, which acts directly at the G_i level,

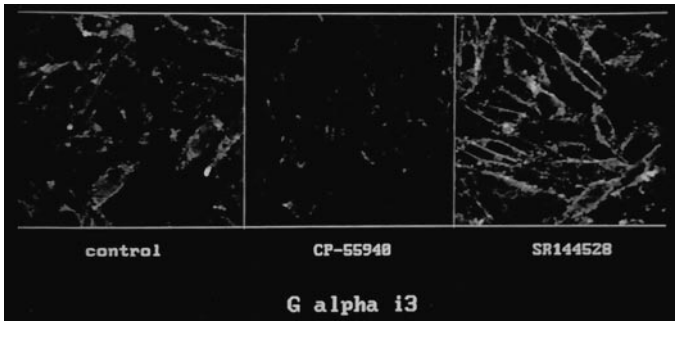

Fig. 6. Effect of CP-55940 and SR 144528 on the immunohistolocalization of the G_i in CHO-CB2 cells. Growth-arrested CHO-CB2 cells were left inactive (left) or stimulated for 20 h with 30 nM CP-55940 (middle) or 50 nM SR 144528 (right), and fixed then probed with an anti $G_i\alpha 3$ protein as described in *Materials and Methods*.

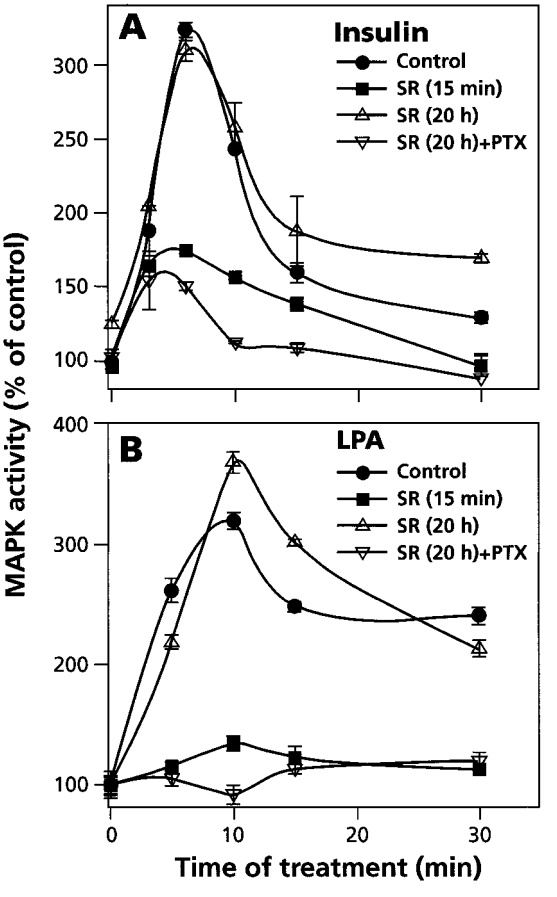

Fig. 7. Effect of sustained treatment with SR 144528 on growth factor-induced MAPK activation. Growth-arrested CHO-CB2 cells were incubated with 50 nM SR 144528 for 15 min or 20 h and then stimulated with 1 μg/ml insulin (A) or 1 μg/ml LPA (B) in the absence or presence of 100 ng/ml PTX for various time periods. The activities of p42/p44 MAPK were determined as described in the Fig. 1 legends. The results are expressed as a percentage of value from untreated cells, taken as 100%. Data points are mean values ± S.D. of duplicate samples and experiments were repeated three times.

and 4) the effects of SR 144528 were abolished in parallel to those of the agonist when the ability of the receptor to couple to G protein was lost by ADP ribosylation of the G_i after treatment by PTX (data not shown).

A G_i-dependent cross-talk between cannabinoid receptors and other receptors was shown for CB1 and dopamine D2 (receptors in striatal neuron by analyzing the other cannabinoid cellular response, G_i-dependent cAMP production (Glass and Felder, 1997).

SR 144528 Induces G_i Protein Modulation. How did SR144528 mediate G_i protein inhibition? Inverse agonist may induce or stabilize a CB2/G_i protein complex that remains inactive. Initial experiments where we attempted to visualize the CB2/G_i complex by immunoprecipitation and Western blotting were unsuccessful very likely due to the high detergent concentration needed for the receptor solubilization, which could be incompatible with maintaining a putative CB2/G_i complex interaction. We therefore addressed this question from another point of view. As prolonged cell exposure to a GPCR agonist can result in a down-regulation of cellular levels of the G protein to which the receptor is normally coupled (Milligan, 1993), we reasoned that, as a corollary, if indeed CB2 stably interacts with G_i in the presence of SR 144528, then sustained treatment with inverse agonist is expected to affect the cellular level of G_i protein.

We here provide evidence that maintained cell exposure to inverse agonist can result in a time-dependent enhancement in cellular levels of α subunit of $G_i\alpha_1$, $G_i\alpha_2$, and $G_i\alpha_3$ without altering levels of $G_s\alpha$, an effect that was observed together with a redistribution of G_i proteins from the cytosol to the plasma membrane. Remarkably, we showed that chronic exposure to CP-55,940 produces a time-dependent down-regulation of the same G-protein subunits. This effect indicated that SR 144528 enhanced the G-protein subclasses, which are precisely those coupled to the receptor in the presence of the agonist. This point was further supported by the ability of CP-55,940 to reduce the same G-protein subunit levels that have been increased by SR 144528. Although it has not been formally demonstrated, from these results it is reasonable to surmise that the binding of SR 144528 to the receptor promotes or stabilizes the SR 144528/CB2/G_i complex that controls signal transduction. A predicted result from this model is that, if the G_i stoichiometry increases when compared to the receptor CB2 load, then the capacity of SR 144528 to inhibit RTK or GPCR signaling should be lost. This is in agreement with our observations of the G_i protein being up-regulated by the inverse agonist.

The G_s protein down-regulation by an agonist treatment has already been reported. For instance, sustained treatment of NG 108–15 cells transfected to express the human β2-adrenoreceptor with isoprenaline resulted in a decrease in membrane-associated levels of the α subunit of the G_s protein that interacts with the receptor (Kim and Milligan, 1994). However, sustained treatment with inverse agonist failed to cause any alteration in levels of this G_s protein in this model (MacEwan and Milligan, 1996), indicating that the property of SR 144528 we described here may not be common for all inverse agonists.

Predictive Model for Receptor Activation of G Proteins. The "two-state model" currently in use suggests that the mode of action of inverse agonists can be explained by a higher affinity of these compounds for the inactive uncoupled form of the receptor. Binding of a compound preferentially to this form leads to a reduction in constitutive activity by shifting the equilibrium from the active form to the inactive uncoupled form. Our results cannot be adequately interpreted in this theoretical framework.

An agonist-bound receptor activates an appropriate G protein that promotes dissociation of GDP. Although the interactions between receptor and G protein are poorly understood, both mutagenesis and biochemical experiments with a variety of GPCR suggest that, first of all, the receptor activation by ligand binding causes a change in the relative orientations of the transmembrane helices 3 and 6. This modification then affects the conformation of the G protein-interacting intracellular loop of the receptor and thus uncovers previously masked G protein-binding sites (Wess, 1997). Secondly, as GDP is buried within the Gα protein, the receptor-G protein interaction must, in addition, promote changes in interdomain interactions necessarily. Thus, to activate the G protein, the receptor had to deliver two pieces of information-one for the formation of R/G complex and the other to induce the exchange of bound GDP to GTP on heterotrimeric G proteins, resulting in the dissociation of the G protein into active Gα-GTP and G$\beta\gamma$ subunits.

Although the physical process involved in the stabilization and inactivation of the complex remains undefined, we suggest that inverse agonist may trigger only the first part of this activation process. Another alternative would be the involvement of the recently described regulators of G protein signaling or RGS proteins (De Vries et al., 1996; Druey et al., 1996). Given the evidence that RGS proteins are negative regulators of G protein signaling that act at the level of the dissociated Gα subunit or above, one may hypothesize that RGS proteins would be recruited in the presence of an inverse agonist, thus acting as inhibitors of GDP dissociation and blocking G protein activation. This possibility could be addressed by analyzing whether RGS interacts with $G_i\alpha3$ in the presence of SR 144528. The detailed mechanisms responsible for inverse agonist-induced G protein inhibition are the basis of ongoing studies.

The data provided herein demonstrate that inverse agonist occupation of CB2 receptor can selectively regulate both the activity and the cellular level of G_i. The inverse agonist-induced modulation of the G_i strongly suggested that the modulated protein must be physically associated with the complex SR 144528/CB2, which accounts for the heterologous desensitization phenomena. The results presented in this study demonstrate a complex pattern of cellular G protein regulation after inverse agonist that may be as complex as that associated with the agonist-induced activation of the receptor.

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