A Selective Inverse Agonist for Central Cannabinoid Receptor Inhibits Mitogen-activated Protein Kinase Activation Stimulated by Insulin or Insulin-like Growth Factor 1

EVIDENCE FOR A NEW MODEL OF RECEPTOR/LIGAND INTERACTIONS

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The mechanism of action of Δ⁹-tetrahydrocannabinol (Δ⁹-THC),¹ the main active principle of marijuana, began to be described only a few years ago. It is now known that Δ⁹-THC, and other potent synthetic cannabinoid receptor agonists as well as anandamide, the putative endogenous ligand (1), bind to specific cannabinoid receptors. The central cannabinoid receptor (CB1) was cloned from both rat and human (2, 3) and was shown to be expressed primarily in brain tissue (4, 5). Although to a lower extent, CB1 mRNA has also been found in testis (3), spleen (6), and leukocytes (7). A second cannabinoid receptor (CB2) has been cloned and recently characterized. CB2 is expressed in macrophages from the marginal zone of spleen, in B lymphocytes, and NK cells but not in brain tissues (8, 9). Both CB1 and CB2 receptors belong to the G protein-coupled receptor (GPCR) superfamily.

CB1 has been associated with several biological responses including inhibition of adenylyl cyclase (AC) and modulation of ion channels (10, 11). More recently, we showed that treatment by cannabinoid agonist of CHO cells expressing CB1 or U373 MG cells induced activation of both mitogen-activated protein kinases (MAPKs) and immediate-early gene Krox 24, also known as NGFI-A, zif/268, egr-1, and TIS 8 (12, 13). Cannabinoid-induced Krox 24 expression was also described in vivo (14, 15). These cannabinoid effects were mediated by a pertussis toxin (PTX)-sensitive guanine nucleotide-binding protein (G-protein) and prevented by the potent CB1-selective antagonist SR 141716A (12, 13, 16, 17).

In the last few years, a new class of antagonist molecules designated as inverse agonists has been identified (18–21). The first to be characterized were the β-carbolines acting toward the ionotropic γ-aminobutyric acid receptor (22). These molecules contrast with classical antagonists in that they exhibit a biological activity by blocking the signal transduction mediated by constitutively activated receptors. Most of the other inverse agonist molecules identified so far are ligands for receptors of the GPCR superfamily (23).

In the present study, we explored in detail the pharmacological and biological properties of the CB1-selective antagonist...
SR 141716A, using CHO cells transfected with human CB1. We here demonstrated that SR 141716A functions not only as an antagonist of cannabinoid-mediated effects but also as an inverse agonist. We next provided evidence for a coupling between CB1 and receptor-tyrosine kinase (RTK) transduction pathways, involving a PTX-sensitive G_i protein. These results suggest that biological functions of inverse agonists have been underestimated and enable us to conceive a new ligand/receptor interaction model.

MATERIALS AND METHODS

Reagents—[^3H]CP-55,940, [^3H]SR 141716A, and [^35S]GTP-S (1250 Ci/mmol) were purchased from DuPont NEN (Paris, France). [^32P]ATP (3000 Ci/mmol) was from Amersham Corp. (Les Ulis, France). Phorbol 12-myristate 13-acetate, forskolin, isobutylmethylxanthine, bovine myelin basic protein (MBP), 5'-driothreohexanocin (5'-DThC), WIN 55212-2, and pertussis toxin (PTX) were purchased from Sigma (Saint-Quentin-Fallavier, France). Wortmannin was from Biomol (Plymouth Meeting, PA). SR 141716A (N-piperidino-1-yl)-5-(4-chlorophenyl)-1-(2,4-dichlorophenyl)-4-methyl-pyrazole-3-carboxamide) and CP-55,940 were synthesized at the Chemistry Department of Sanofi (Montpellier, France). GDP and GTP were from Calbiochem (Meudon, France). G418 was from Life Technologies, Inc. Ro-20–1724 was from Research Biochemicals International (Illkirch, France). GDP and GTP-S were purchased from Boehringer Mannheim (Meylan, France).

Plasmid Constructions—A synthetic oligonucleotide containing six cAMP-responsive elements (CREs) was obtained as described (24). This CRE cassette was placed upstream from the herpes simplex virus-thymidine kinase (tk) promoter and cloned into the pSE1 plasmid (25) in place of the SV40 early enhancer-promoter. The interleukin-2 coding sequence was then replaced with luciferase, and the resulting CRE-luciferase reporter construct was called p661.

Stable Cell Lines and Culture Conditions—

CHO wild-type (CHO-WT) cells were routinely grown as monolayers at 37 °C in a humidified atmosphere containing 5% CO_2 in air with 10% fetal calf serum (FCS) and 300 μg/ml G418. Individual clones were tested for an inducible expression of luciferase following stimulation with forskolin. CHO-CB1 cells were grown in MEM (Life Technologies, Inc.) supplemented with 10% fetal calf serum, 60 μg/ml gentamicin, 5% FCS. Twenty-four hours later, cells were pretreated for 5 min with 50 μM forskolin and then directly lysed in Laemmli’s loading buffer containing 6 m urea. Proteins were heated for 10 min at 95 °C and separated by SDS-PAGE on 11% acrylamide gel. Following gel transfer onto nitrocellulose filters in 25 mM Tris, 0.19 M glycine, 20% methanol, membranes were blotted in TN buffer (20 mM Tris, pH 7.5, 120 mM NaCl) supplemented with 10% dried milk powder. Blots were then incubated with anti-p42 and anti-p44 antibodies (0.25 μg/ml in blotting TN buffer containing 2% dried milk powder) at 4 °C overnight. After extensive washing in TN buffer containing 0.1% Tween 20, a peroxidase-labeled anti-rabbit IgG antibody (Sigma) was added for 45 min at room temperature. After 5 washes, immunostained MAPKs were visualized using the enhanced chemiluminescence detection system according to the supplier’s instructions (Amersham Corp.) and subjected to autoradiography.
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RESULTS

Effect of SR 141716A on MAPK Basal Activity in CHO-CB1 Cells—The biological properties of the CB1 selective antagonist SR 141716A were studied in CHO cells stably expressing the human CB1 (CHO-CB1 cells) in which we measured MAPK activity. MAPK activity was assayed toward MBP as substrate, after specific immunoprecipitation of p42 and p44 MAPK isoforms. We showed that stimulation of CB1 by the synthetic cannabinoid agonist CP-55,940 resulted in a strong activation of MAPKs which is in agreement with our previously described results (12). This effect was not observed in parental cells (CHO-WT) (Fig. 1A) and was completely prevented in CHO-CB1 cells by treatment with SR 141716A (data not shown).

Interestingly, a comparison of CHO-CB1 and CHO-WT cells showed an enhanced basal MAPK activity in CHO-CB1 cells (Fig. 1B). Furthermore, this basal activity was reduced in a dose-dependent manner by SR 141716A with an IC50 of 5 nM (Fig. 1B). A concentration of 30 nM SR 141716A reduced the basal level of MAPKs in CHO-CB1 cells to that observed in CHO-WT cells, whereas SR 141716A treatment had no effect on CHO-WT cells. Pretreatment of CHO-CB1 cells by PTX induced an inhibition of MAPK basal level, whereas it had no significant effect in CHO-WT cells. When CHO-CB1 cells were co-treated with PTX and SR 141716A, no additive inhibitory effects were observed (data not shown). This suggested that the enhanced MAPK activation was related to a CB1-coupled PTX-sensitive G protein. Activation of MAPKs has been described to lead to the appearance of slower migrating forms in SDS-PAGE, resulting from the phosphorylation on specific threonine and tyrosine residues of p42 and p44 MAPK isoforms (29).

In CHO-CB1 cells, the presence of phosphorylated proteins, which was markedly enhanced by treatment with CP-55,940, was clearly apparent in the untreated cells (Fig. 2A). Treatment with SR 141716A abolished the CP-55,940-induced forms and also the constitutively activated forms. Similar results were obtained in an in-gel kinase assay performed with proteins extracted from CHO-CB1 cells and run in SDS-PAGE. Intact MBP protein, trapped in the polyacrylamide gel, was efficiently phosphorylated by p42/44 MAPKs from unstimulated or cells treated with CP-55,940 (Fig. 2B). Again, the intensity of MAPKs was decreased by treatment with SR 141716A in cells stimulated with CP-55,940 as well as in unstimulated cells. In a control experiment, we did not observe any modulation of p42/p44 MAPKs by SR 141716A in either Western blot or in-gel kinase assays on CHO-WT cells (data not shown). These results indicate that the enhanced basal MAPK activity in CHO cells expressing CB1 is related to the autoactivation of CB1 receptors and that it is specifically decreased by SR 141716A which thereby acts as an inverse agonist.

Effects of SR 141716A on Adenylyl Cyclase Activity—We examined whether SR 141716A inverse agonist activity was also manifested by other cellular responses. Previous studies have established that cannabinoids alter cAMP production through the GTP-binding protein G, (10, 30). The increase of intracellular cAMP levels leads to the activation of protein kinase A. The catalytic subunit of the activated protein kinase A is translocated to the nucleus where it phosphorylates the cAMP response element binding protein which thereafter binds
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CHO-CB1 cells were either pretreated or not with increasing concentrations of cannabinoids for 5 min before a 20-min stimulation with forskolin (3 μM). The reaction was stopped by addition of 50 mM Tris-HCl, 4 mM EDTA. cAMP levels were determined as described under “Materials and Methods.” The results are means of triplicate determinations (± S.E.). The basal level of cAMP in cells untreated with forskolin is 2.5 ± 1.9 pmol/well.

141716A was observed in the absence of forskolin, excluding a direct stimulation of GTP-binding protein Gi by SR 141716A (data not shown). The effects of SR 141716A per se on CRE-luciferase are consistent with the blockage of AC inhibition mediated by autoactivated CB1 receptors. As an additional control, we observed that when experiments were carried out with CHO cells transfected with CRE-luciferase but lacking CB1 (CHO-CRE cells), neither CP-55,940 nor SR 141716A affected the level of luciferase induced by forskolin (data not shown). These results were confirmed by direct measurement of cAMP levels (Table I), which showed that SR 141716A treatment induced a marked increase in forskolin-stimulated cAMP production in a dose-dependent manner in CHO-CB1 cells. Under the same conditions, CP-55,940 inhibited the forskolin-induced cAMP production in CHO-CB1 cells.

**Pharmacological Properties of 141716A**—It has often been observed that guanidylc nucleotide analogs decrease the binding of agonists while increasing the binding of inverse agonists (32). To determine whether SR 141716A behaved as an inverse agonist, we first compared the capacity of the unlabelled ligands SR 141716A and CP-55,940 to displace the binding of either [3H]CP-55,940 or [3H]SR 141716A on CHO-CB1 membranes, in the presence of GTPγS. As shown in Fig. 4A, the addition of 100 μM GTPγS shifted the displacement curve of [3H]CP-55,940 by SR 141716A to lower concentrations (IC50 = 22.77 ± 6.6 nM; plus GTPγS, IC50 = 4.2 ± 1.4 nM). Fig. 4B indicates a shift of the displacement curves of [3H]SR 141716A by CP-55,940 to higher concentrations (IC50 = 10.37 ± 3.6 nM; plus GTPγS, IC50 = 25.75 ± 5.5 nM). Furthermore, we observed that GTPγS enhanced the binding of [3H]SR 141716A, while decreasing the binding of [3H]CP-55,940 (Fig. 4C). This pharmacology agrees with what has already been described for agonists and inverse agonists.

**Effect of SR 141716A on Receptor-Tyrosine Kinase-mediated MAPK Activation in CHO-CB1 Cells**—The above results provide strong evidence that autoactivation of CB1 is shut off by SR 141716A treatment. We next asked the following question: does SR 141716A-mediated CB1 inactivation affect other signal pathways?

MAPKs can be activated in response to both GPCR or receptor-tyrosine kinase (RTK) stimulation. Insulin receptors belong to the RTK family, and their stimulation by insulin has been shown to activate MAPKs in CHO cells (33). When CHO-CB1 or CHO-WT cells were stimulated with 1 μg/ml insulin, a time-dependent activation of MAPKs (evaluated by phospho-rylation of MBP) was observed (Fig. 5). The maximum level of induction was obtained after a 10-min stimulation and then

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**Table I**

| Cannabinoid receptor ligands (M) | CHO-CRE | CHO-CB1 |
|---------------------------------|---------|---------|
| SR 141716A                     | 9.5     | 7.2     |
| CP-55940                        | 9.61    | 7.1     |
| 6THC                            | 23      | 23      |
| WIN 55212-2                     | 42.75   | 42.75   |
| 10 nM                           | 100 ± 9.5| 100 ± 9.5|
| 10 µM                           | 100 ± 9.5| 100 ± 9.5|
| 100 µM                          | 100 ± 9.5| 100 ± 9.5|
| 1 mM                            | 100 ± 9.5| 100 ± 9.5|

* ND, not determined.
declined with time. Surprisingly, 100 nM SR 141716A completely inhibited insulin-activated MAPK in CHO-CB1 cells (Fig. 5A). On the other hand, SR 141716A had no effect on insulin-stimulated MAPKs in CHO-WT cells (Fig. 5D), establishing that the above effects required the interaction of SR 141716A with CB1 receptors. These results were confirmed by Western blot analysis of p42/44-kDa MAPK proteins (Fig. 6A) as well as by a MAPK renaturation assay (Fig. 6B).

We next wondered whether this inhibition could be extended to other RTKs. MAPK phosphorylation in response to 10 ng/ml insulin-like growth factor 1 (IGF1) exposure followed a similar time course and was also prevented by SR 141716A in CHO-CB1 but not in CHO-WT (Fig. 5, B and E). Both Western blot analysis and MAPK renaturation assay also confirmed these results (Fig. 6). In contrast, MAPK stimulated by 10 ng/ml basic fibroblast growth factor (FGF-b) was not affected by SR 141716A treatment (Fig. 5, C and F). It is noticeable that the level of MAPK activation induced by FGF-b was much higher than that elicited by insulin or IGF-1. However, this difference could not account for the lack of effect of SR 141716A. Indeed, even when using low concentrations of FGF-b (0.1 ng/ml), for which a 3-fold lower MAPK activation was observed, SR 141716A still did not inhibit this response (data not shown).

Transduction Pathway Elicited by CB1, Insulin, and IGF1 Receptors—Since MAPK activation by CB1, insulin, and IGF1 receptors could be blocked by SR 141716A, an attractive hypothesis was that they shared a common transduction pathway. Several authors have reported that the PTX-sensitive activation of MAPK by GPCR required the activation of a phosphatidylinositol 3-kinase (PI-3K) upstream to the MAPKs (34). Treatment of CHO-CB1 cells with the PI-3K inhibitor wortmannin resulted in a significant inhibition of both CP-55,940 and insulin-mediated MAPK activation, with IC50 values of 26 ± 6 and 38 ± 7 nM, respectively (Fig. 7). Similar results were obtained with IGF1 (data not shown). On the contrary, wortmannin did not affect the FGF-b-mediated effect (Fig. 7). These data show that stimulations of MAPKs by the Gi-coupled CB1 receptor and the insulin receptor are equally sensitive to wortmannin.

We next wondered whether the same molecular entities were committed in the transduction of CB1 and insulin signals. To address this question, we analyzed their costimulatory effects. The results are summarized in Table II. At suboptimal concentrations of GTPγS, CP-55,940 and SR 141716A binding on CHO-CB1 cell membranes. A, displacement of [3H]CP-55,940 binding by SR 141716A. CHO-CB1 cell membranes were incubated with 0.25 nM [3H]CP-55,940 and increasing concentrations of SR 141716A in the absence (●) or presence (○) of 100 μM GTPγS. B, displacement of [3H]SR 141716A binding by CP-55,940. CHO-CB1 cell membranes were incubated with 0.8 nM [3H]SR 141716A and increasing concentrations of CP-55,940 in the absence (●) or presence (○) of 100 μM GTPγS. C, binding of [3H]CP-55,940 and [3H]SR 141716A in the presence of GTPγS. 0.25 nM [3H]CP-55,940 (○) or 0.8 nM [3H]SR 141716A (●) was incubated with CHO-CB1 cell membranes (50 μg of proteins) and increasing concentrations of GTPγS. Values are means ± S.E. of three measurements from three distinct experiments.

Fig. 5. Effect of SR 141716A on growth factor-induced MAPK activation. Growth-arrested CHO-CB1 (A–C) and CHO-WT (D–F) cells were pretreated for 10 min (○) or not (●) with 100 nM SR 141716A prior to stimulation with 1 μg/ml insulin (A and D), 10 ng/ml IGF1 (B and E), or 10 ng/ml FGF-b (C and F). MAPK activity was determined as a function of time as described under “Materials and Methods.” Each value is the mean ± S.D. of duplicate samples, and the experiments were repeated five times. Results are expressed as percent of value from untreated or SR 141716A-treated cells, taken as 100%.
binding of SR 141716A to CB1 interfered with Gi activity. To the early known steps in signaling, we hypothesized that the activation.
suggest the involvement of the same limiting factor for optimal results are in agreement with a common transduction pathway
tive independently of their concentrations (Table II). These
the other hand, the effects of CP-55,940 and FGF-b were addi-
effect with insulin was noted in costimulatory experiments. On
concentrations of 10 nM CP-55,940 were used, no additional
trations of both CP-55,940 and insulin, additive effects on
MAPK activation were observed. In contrast, when saturating
concentrations of 10 nM CP-55,940 were used, no additional
effect with insulin was noted in costimulatory experiments. On
the other hand, the effects of CP-55,940 and FGF-b were addi-
tive independently of their concentrations (Table II). These
results are in agreement with a common transduction pathway
for cannabinoid- and insulin-mediated MAPK activation and
suggest the involvement of the same limiting factor for optimal
activation.

SR 141716A Inhibits G_i-mediated MAPK Activation in CHO-
CB1 Cells—It has already been shown that the G_i protein, which is involved in the CB1 response, could also be required for
MAPK activation induced by insulin and IGF1 but not by
FGF-b (35). Our results shown on Fig. 8 indicate similar results.
The MAPK activation induced by insulin or IGF-1 was markedly inhibited by PTX, whereas induction of MAPK by
FGF-b was unaffected by PTX. Since the G_i component is one of
the early known steps in signaling, we hypothesized that the
binding of SR 141716A to CB1 interfered with G_i activity. To
test this hypothesis, we examined the effect of SR 141716A on
MAPK activation induced by an analog of mastoparan. Masto-
paran is a direct activator of G protein with a reported selec-
tivity for G_i/Go (36), and Mas-7 is an analog exhibiting a 5-fold
greater potency than mastoparan (37). Exposure of CHO-CB1
or CHO-WT cells to 3 µM Mas-7 for 10 min resulted in a marked
increase in MAPK activity (Fig. 9A). In agreement with a G_i
coupling, this increase was completely prevented by pretreat-
ment with PTX. Interestingly, 100 nM SR 141716A completely
inhibited the Mas-7-induced MAPK activation in CHO-CB1
cells but not in CHO-WT cells. We next examined whether SR
141716A could act directly at the G_i level. This was investi-
gated by measuring the binding of [35S]GTPyS to G protein.
Indeed, this binding is regulated by the receptor and provides
direct information about the interaction between receptors and
G protein activation (38, 39). Fig. 9B clearly indicates that 20
nM CP-55,940 and 30 µM Mas-7 treatment stimulated [35S]
GTPyS binding to the CHO-CB1 cell membranes. Conversely,
SR 141716A was able to induce a marked inhibition of consti-
tutive as well as CP-55,940- and Mas-7-induced [35S]GTPyS
binding to CHO-CB1 cell membranes. This effect was not ob-
served in CHO-WT cell membranes, indicating that the SR
141716A-induced inhibition is not a nonspecific direct interac-
tion with the G_i protein but a receptor-mediated response.

FIG. 6. Effect of SR 141716A on MAPK activation induced by insulin or IGF1. Quiescent CHO-CB1 cells were either pretreated or not with SR 141716A for 5 min and stimulated with insulin or IGF1. Ten minutes after stimulation MAPK activity was determined by EMSA (A) and in-gel kinase assay (B) as described under “Materials and Methods.” Lane 1, untreated cells, or cells treated with the following: lanes 2 and 3, 1 µg/ml insulin; lanes 4 and 5, 1 µg/ml insulin + 100 nM SR 141716A; lanes 6 and 7, 10 ng/ml IGF1; lanes 8 and 9, 10 ng/ml IGF1 + 100 nM SR 141716A.

FIG. 7. Effect of wortmannin on MAPK activation in CHO-CB1 cells. Growth-arrested CHO-CB1 cells were pretreated for 15 min with increasing concentrations of wortmannin before stimulation for another 10 min with either CP-55,940 (●), insulin (○), or FGF-b (▲). Each data point is the mean ± S.D. of duplicate samples, and experiments were repeated three times. Results are expressed in percentage of values from cells untreated with wortmannin.

Table II
Effect of costimulation by growth factor and CP-55,940 on MAPK activation in CHO-CB1 cells

| Stimulus          | MAPK activity |
|-------------------|---------------|
|                   | Control       | Insulin (5 ng/ml) | Insulin (1 µg/ml) | FGF-b (0.1 ng/ml) | FGF-b (10 ng/ml) |
|                   | Solvent       | CP-55,940 (0.5 nM) | CP-55,940 (10 nM) | Solvent       | CP-55,940 (0.5 nM) | CP-55,940 (10 nM) |
|                   | % of control  | 100 ± 4           | 140 ± 6           | 410 ± 9       | 175 ± 3           | 250 ± 4           | 397 ± 7       |
|                   |               | 270 ± 9           | 320 ± 4.5         | 425 ± 4       | 170 ± 7           | 350 ± 3           | 475 ± 6       |
|                   |               | 430 ± 5           | 498 ± 12          | 620 ± 11      |                 |                 |              |

FIG. 8. Effect of PTX treatment on MAP kinase activation by insulin, IGF-1, and FGF-b. Growth-arrested CHO-CB1 cells were incubated 4 h in the presence or absence of PTX (100 ng/ml) prior to stimulation with insulin (1 µg/ml), IGF-1 (10 ng/ml), or FGF-b (10 ng/ml). Ten minutes after stimulation, MAPK activity was determined as described under “Materials and Methods.” Results are expressed in percentage of values from stimulated cells untreated with PTX. Data points are mean values ± S.D. of duplicate samples, and experiments were repeated three times.
In summary, we demonstrated that the CB1-selective antagonist SR 141716A functions as an inverse agonist for the autoactivated receptor, and this increase was reversed by treatment with SR 141716A. Moreover, it is noticeable that GTPγS was more potent at blocking [3H]CP-55,940 than at potentiating [3H]SR 141716A binding. Assuming that the proportion of sites for agonists that are inhibited by guanine nucleotides is an indication of the receptor status in a precoupled autoactivated form, this correlated well with 1) the marked autoactivation of MAPK in CHO-CB1 cells and 2) the degree of negative and positive effects of SR 141716A on MAPK and AC activities, respectively.

An often raised alternative interpretation of the inhibition of autoactivated receptor by inverse antagonists could be a blockage of endogenous agonists present in culture medium or produced by the cells. We ruled out such a possibility for the following reasons. First, the human astrocytoma cell line U373-MG, which expresses CB1, was not affected in its cAMP metabolism when exposed to culture supernatants from CHO-CB1 cells, whereas in these cells AC is exquisitely sensitive to minute amounts of cannabinoid receptor agonist (data not shown). Second, the dose-response curves for SR 141716A modulation of MAPK and AC activities showed an EC50 in the nM range, which fits with the binding property of [3H]SR 141716A (16, 17, 40). A significantly higher EC50 would be expected if endogenous agonist molecules were acting in competition with SR 141716A. Indeed, we observed that the presence of as low as 10 nM CP-55,940 decreased the potency of SR 141716A on cAMP metabolism by 20-fold (Fig. 3B). Together, these results support the notion that the observed effects of the CB1 antagonist SR 141716A reflect the direct consequences of its binding to unoccupied receptors and the notion that it acts as an inverse agonist.

The properties of autoactivated receptor have already been described in heterologous expression systems for a variety of GPCRs including dopamine receptors D1A and D1B, 5HT2C receptor, δ opioid receptor, and β-adrenergic receptor (20, 39, 41, 42). Our results provide an additional example of such autoactivated receptors.

**SR 141716A Inhibits MAPK Activation Mediated by PTX-sensitive RTK**—One of the most important and provocative findings of this paper, developed in its second part, describes a novel property for the inverse agonist SR 141716A. This concerns the link between CB1 and some growth factor receptors belonging to the RTK family. CHO cells naturally express RTKs such as those for insulin, IGF-1, and FGF. The specific stimulation of these receptors by their natural ligands has been shown to lead to MAPK activation (35). We demonstrated here that the inverse agonist not only inhibits autoactivated CB1 but also switches off MAPK activation from some RTKs, including insulin and IGF1 receptors. This was shown by measuring phosphorylation of MBP in MAPK immunoprecipitates, by EMSA, and by in-gel kinase assays. The contrary, SR 141716A did not affect MAPK activation induced by FGF-b in CHO-CB1 cells. These effects were related to CB1 since they were not observed in CHO-WT cells. We concluded from these results that the binding of SR 141716A to CB1 induced biological responses that negatively interfered with particular RTK pathways. Moreover, exposure of cells to a saturating concentration of SR 141716A, followed by its removal by washing, did not prevent MAPK stimulation by CP-55,940 or insulin, indicating that the effect of SR 141716A was reversible and that the binding of SR 141716A to CB1 was required to mediate its inhibitory effect.

MAPKs represent a point of convergence of mitogenic signals emerging from several distinct types of GPCRs and RTKs, suggesting the integration of redundant information. Although these interactions remain poorly understood, recent findings...
support such a network, which may function as a mechanism of enhancement or counter-regulation. For instance, tyrosine phosphorylation of the β2-adrenergic receptor by insulin has been shown to produce supersensitization of adrenergic signaling (43), whereas protein kinase A activation by β-adrenergic receptor leads to an attenuation of insulin and EGF-stimulated MAPK activity (44). More recently, lysophosphatidic acid (LPA) and thrombin, two GPCR ligands, were shown to mediate cell proliferation through ligand-independent induction of tyrosine phosphorylation of the EGF receptor belonging to RTK (45). For the first time, we demonstrate an original situation where an inverse agonist specific for GPCR may induce an RTK inhibition through an intracellular signal cross-talk. Although the mechanism by which SR 141716A counteracts insulin or IGF receptors remains to be elucidated, several points can be raised. It has been suggested that the responses mediated by RTKs segregate into two groups, one PTX-sensitive, resembling the Gi-coupled LPA receptor, and one PTX-insensitive (35). Unlike the FGF receptor pathway, the insulin or IGF-1 signal can be blocked by either PTX treatment or wortmannin, an inhibitor of PI-3K, which is an early intermediate of Gβγ-mediated MAPK signaling pathway (34). This latter pathway has been implicated in MAPK activation mediated by classical Gi-coupled receptors such as CB1. The mechanism whereby insulin or IGF1 receptors can induce the generation of free Gβγ subunits is unclear, although indirect evidence suggests a direct protein-protein interaction between receptor and G protein (46). It has been reported that insulin attenuates the sensitivity of a 40-kDa Gβγ-like protein toward PTX in hepatocyte membranes (47, 48). Jo et al. (49) reported that the insulin receptor is directly associated with two proteins of 40 and 67 kDa that bind GTP. Recently, peptides derived from the insulin receptor β subunit have been shown to directly activate Gγ in phospholipid vesicles (50). Whatever the molecular mechanism, the physiological importance of the Gγ protein in insulin receptor signaling is supported by recent results from Moxham and Malbon (51) who established that Gγ deficiency in liver and adipose tissues of transgenic mice impaired glucose tolerance and induced insulin resistance.

However, it is noteworthy that the RTK segregation as described above cannot be considered as a stringent rule. Indeed in some cells, FGF receptors have been reported to be PTX-sensitive (52). Conversely, in other cases, PTX-sensitive G proteins are not involved in MAPK activation by insulin or IGF-1 (32, 53). More recently, Harada et al. (54) have demonstrated that insulin exhibits multiple signal transduction pathways that vary from cell to cell. For instance, they showed that, in the mouse myeloid cells 32D, insulin-stimulated MAP kinase did not involve PI 3-kinase activation (54), which differs from our observation.

Using CHO-transfected cells, we showed that CB1, insulin, and IGF shared a PTX-sensitive signaling pathway. We have shown here that when CHO-CB1 cells were co-stimulated with saturating concentrations of insulin and CP-55,940, the MAPK response was not cumulative, suggesting that a pool of common proteins or key enzymes is used downstream from both CB1 and the insulin receptor. Mitogenic signals originating from insulin, IGF1, and CB1 receptors converge at a point upstream of p21, probably represented by the Gγ protein. We thus hypothesized that Gγ represents the limiting factor for optimal stimulation and that the binding of an inverse agonist to CB1 may inactivate the Gγ protein. The two following observations argue in favor of this: (i) SR 141716A bound to CB1 receptor prevents the activation of MAPKs induced by Mas-7, which acts directly at the Gγ level; (ii) the effects of SR 141716A 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 Gγ by PTX.

The effect of SR 141716A on PTX-sensitive RTK was shown in CHO-CB1 cells, but it is likely to be a general phenomenon since we could extend our observations to Rat-1 fibroblast cells (data not shown). These cells naturally express insulin, IGF1, FGFb, EGF, as well as LPA receptors. When transfected with CB1, SR 141716A blocked the PTX-sensitive-MAPK activation induced by insulin, IGF1, or LPA, whereas the PTX-insensitive MAPK activation induced by EGF or FGF-b was not altered by SR 141716A. Thus, the SR 141716A-CB1 complex, acting as a reversible negative dominant of the Gγ, can be considered as a potent and valuable tool to study the signal transduction pathway involving Gγ.

Predictive Model of Receptor Activation of G Proteins—It is tempting to hypothesize that sequestration of CB1-coupled Gγ protein makes this protein unavailable for coupling to insulin or IGF1 receptors. According to this model, which we designate a “three-state receptor model” as illustrated in Fig. 10, SR 141716A converted the CB1 autoactivated receptor to a suppressor receptor acting in trans by sequestration of the Gγ protein, which very likely remains inactive under a GDP-bound form. One possibility could be that the SR 141716A-CB1-Gγ complex blocks the GDP-GTP exchange. We assume that the receptor could be in equilibrium between three conformations: Rα, Rβ, Rγ. In the Rα state, the receptor is uncoupled to the G

![Fig. 10. Schematic illustration of receptor activation of G proteins: a three-state receptor model.](image-url)
protein, whereas the two forms R+ and R− are able to bind 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 induce signal transduction, but by capturing the G protein, this state prevents the activation of nonrelated G protein-coupled receptors localized in its vicinity. We therefore term R−/G the active negative state. To activate the G protein the receptor should deliver two simultaneous pieces of information, one for the formation of R−/G complex and the other to catalyze guanine nucleotide exchange by dramatically increasing the GDP dissociation rate. A study of structure-activity by site-directed mutagenesis of both R and G components would help to elucidate the molecular nature of these two distinct signals involved in G activation.

Such a model is not consistent with the theoretical framework of the “two-state model” currently in use. This latter model postulates that an antagonist with negative intrinsic activity stabilizes the inactivated form of the receptor that is uncoupled to the G protein. One of the main arguments for this is based on the increase in affinity of inverse agonists in the presence of guanine nucleotides, which are assumed to increase the level of uncoupled receptors. Thus, antagonists with negative activity and guanine nucleotides are both believed to promote or stabilize the inactivated form of the receptor that is uncoupled to the G protein. This is one possible interpretation for these effects, but several observations make that interpretation questionable.

We show here that the addition of guanine nucleotide is indeed accompanied by a decrease in agonist binding and a reciprocal enhancement in antagonist binding. However, these effects do not occur at equivalent concentrations of nucleotides, as opposed to what could be expected. In our case, the concentration of GTPγS needed to produce a half-maximal effect on SR 141716A binding is 20 times greater than that required to produce a half-maximal effect on CP-55,940 binding (Fig. 4C). Similar observations have already been described for the 5-HT2C receptor (32, 55).

Thus, nucleotide-induced changes in binding do not necessarily indicate a dissociation of receptor-G protein complexes. Indeed, guanylate imidodiphosphate has been shown to reduce agonist binding to solubilized cardiac muscarinic acetylcholine receptors under conditions that did not prevent G protein co-immunoprecipitation (56). As already raised by Chidich (57), this indicates that there is no obvious relationship between inverse agonist nucleotide-induced changes in binding and the stability of receptor-G protein complexes. On the contrary, we think from these observations, together with the results presented here, that the inverse agonist promotes or stabilizes an active negative state R−/G. The enhancement of both binding and affinity of the inverse agonist, which occurred only at very high nucleotide concentrations, requires further studies to be correctly interpreted.

Interestingly the three-state model that we propose fits with the cubic ternary complex model elaborated from thermodynamic calculations that predict the existence of an inactive receptor-G protein complex that is consistent with our biological data (23, 58, 59).

One important question that arises from a stoichiometric point of view is how the SR 141716A-CB1 complexes can alter most of the cellular G protein responses, as manifested here by the inhibition of Mas-7-mediated G activation. Two possibilities may account for such an observation as follows: 1) although G protein/receptor-coupled systems often seem to have much more G proteins than receptors (60), in our case the very high CB1 expression level per cell (around 2 pmol/mg proteins) could be in excess when compared with the G protein level; 2) as proposed by Schlegel et al. (61) and by Jahangeer and Rodbell (62) in the GDP-bound state, G proteins are likely complexed as multimeric structures, estimated as dodecamers. During the activation process, activated monomers are released from multimers. In that context, one may envision that one SR 141716A molecule, which inhibits G protein activation, prevents the dynamic progression of several G proteins. A precise measurement of the CB1/G,G stoichiometry may favor one of these alternatives; in addition, it would provide an interesting opportunity for validating the existence of such a G protein multimeric structure.

Concluding Remarks—If the above-mentioned hypothesis is valid, then the full range of properties and therapeutic potential of inverse agonists remain to be explored. Moreover, the interpretation of biological effects observed in the presence of any antagonists has to be re-examined since some of them may be related to negative effects rather than zero efficacy.

Similar observations remain to be extended to cells naturally expressing CB1 receptors to assess their physiological relevance. However, one can anticipate that such observations may be difficult to obtain due to compartmentalization. Indeed, it is very likely that, in intact cells, some sets of receptors, G proteins, and effectors may be organized into separated microdomains and do not have direct access to other sets. In contrast, transfected cells may not faithfully mirror the organization of wild-type cells, because overexpression may saturate normal compartments and artificially introduce signaling components into abnormal sites. In our case, CHO cells that expressed a high level of CB1 per cell may account for the observed exacerbated trans-signal responses.

Additionally, our observations were made on an autoactivated receptor. We do not know yet whether they are also valid on receptors not constitutively activated and therefore in normal physiology. However, before this important point has been clarified, the new interaction described here deserves further exploration for therapeutic strategies. Indeed, our results could be important in pathologies where point mutations (63, 64) as well as overexpression of receptors (23, 20) are known to promote such autoactivated receptors and are often associated with human diseases (65, 66). Tumors expressing high levels of autoactivated receptors are an obvious example in which inverse agonists would offer a considerable therapeutic benefit by trans-inactivation of a variety of growth factors involved in cell proliferation.

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