Regulation of Peripheral Cannabinoid Receptor CB₂ Phosphorylation by the Inverse Agonist SR 144528

IMPLICATIONS FOR RECEPTOR BIOLOGICAL RESPONSES

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We recently demonstrated that the selective cannabinoid receptor antagonist SR 144528 acts as an inverse agonist that blocks constitutive mitogen-activated protein kinase activity coupled to the spontaneous autoactivated peripheral cannabinoid receptor (CB₂) in the Chinese hamster ovary cell line stably transfected with human CB₂. In the present report, we studied the effect of SR 144528 on CB₂ phosphorylation. The CB₂ phosphorylation status was monitored by immunodetection using an antibody specific to the COOH-terminal CB₂ which can discriminate between phosphorylated and non-phosphorylated CB₂ isoforms at serine 352. We first showed that CB₂ is constitutively active, phosphorylated, and internalized at the basal level. By blocking autoactivated receptors, inverse agonist SR 144528 treatment completely inhibited this phosphorylation state, leading to an up-regulated CB₂ receptor level at the cell surface, and enhanced cannabinoid agonist sensitivity for mitogen-activated protein kinase activation of Chinese hamster ovary-CB₂ cells. After acute agonist treatment, serine 352 was extensively phosphorylated and maintained in this phosphorylated state for more than 8 h after agonist treatment. The cellular responses to CP-55,940 were concomitantly abolished. Surprisingly, CP-55,940-induced CB₂ phosphorylation was reversed by SR 144528, paradoxically leading to a non-phosphorylated CB₂ which could then be fully activated by CP-55,940. The process of CP-55,940-induced receptor phosphorylation followed by SR 144528-induced receptor dephosphorylation kept recurring many times on the same cells, indicating that the agonist switches the system back on. Finally, we showed that autophosphorylation and CP-55,940-induced serine 352 CB₂ phosphorylation involve an acidotropic GRK kinase, which does not use G₁βγ. In contrast, SR 144528-induced CB₂ dephosphorylation was found to involve an okadaic acid and calyculin A-sensitive type 2A phosphatase.

Two cannabinoid receptors have been characterized so far: the central cannabinoid receptor (CB₁)¹ primarily expressed in brain tissue (1–3) and the peripheral cannabinoid receptor (CB₂) expressed in the immune system but not in the brain (4, 5). CB₁ is the prime target, accounting for the psychoactive effects of cannabis, while cannabinoid-induced immunomodulation is mainly CB₂-mediated. Both CB₁ and CB₂ receptors belong to the G-protein-coupled receptor (GPCR) superfamily and their stimulation by cannabinoid agonists induces several biological responses, including inhibition of adenyl cyclase (6, 7), activation of mitogen-activated protein kinases (8, 9), induction of immediate-early gene Krox 24 in vitro (9, 10), the latter has also been observed in vivo (11, 12). All of these actions appear to be exerted through one or more members of the PTX-sensitive Gᵢ family of GTP-binding regulatory proteins that comprises Gᵢα, Gᵢβγ. While synthetic (CP-55,940, WIN55212–2) cannabinoid ligands cannot discriminate between CB₁ and CB₂ receptors, selective antagonists have recently been developed that specifically target either CB₁ (SR141716) (13, 14) or CB₂ receptors (SR 144528) (15).

Several studies revealed that receptor activation can occur spontaneously in the absence of an agonist. This discovery led to a reclassification of antagonists as neutral antagonists or inverse agonists. Neutral antagonists block agonist action without any effect on constitutive activity (16–19), whereas agonists block agonist action but also suppress constitutive activity.

We recently demonstrated the agonist-independent activity of CB₁ and CB₂ receptors expressed in mammalian cells following transfection (20, 21). We also showed that the CB₁ antagonist SR141716 and the CB₂ antagonist SR 144528 not only block the actions of cannabinoid agonists but they also suppress the constitutive activity of these receptors, indicating that these molecules act as inverse agonists. Furthermore, we revealed for the first time a novel property of these inverse agonists. We demonstrated that they also switch off the activation induced by other unrelated Gᵢ-dependent receptors such as insulin or insulin-like growth factor-1 receptors, strongly suggesting that the biological functions of inverse agonists have been underestimated.

In the present study, we investigated the effect of inverse agonists on the desensitization process. It is clearly established that GPCR functionality and expression are dynamically regulated after agonist exposure. Cell exposure to agonists causes the desensitization and sequestration of the receptors. Phosphorylation by serine/threonine kinases and their subsequent binding to members of a family of cytosolic proteins were shown to be key factors for uncoupling the receptor and its cognate G protein and receptor sequestration (22). Here we used the constitutively active CB₂ stably expressed in the CHO cell line as a cellular model to study the effects of inverse agonists on phosphorylation, cell surface receptor modulation, and CB₂ biological responses. The CB₂ phosphorylation status was monitored by immunodetection using a phosphorylation state-spe-
cific antibody. We showed that the inverse agonist SR 144528 inhibits phosphorylation of the constitutively autoactivated CB₂. In addition, we found that SR 144528 induced extensive CB₂ dephosphorylation of agonist-induced CB₂ phosphorylation. These data provide new insight into the relationship between inverse agonists and the phosphorylation/desensitization process.

**EXPERIMENTAL PROCEDURES**

**Reagents**—[γ-³²P]ATP (3000 Ci/mmol) was from Amersham Pharmacia Biotech (Les Ullis, France). Phorbol 12-myristate 13-acetate (PMA), isobutylmethylxanthine, 3-[H]-tetrahydrocannabinol (³H-THC), WIN 55212.2, and pertussis toxin (PTX) were purchased from Sigma (Saint-Quentin-Fallavier, France). SR 144528 ([N-(15)-endo-1,3,3trimethylbicyclo[2.2.1]hepta-2-yl]-5-(4-chloro-3-methylphenyl)-1-(4methylbenzyl)-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 New England Biolabs (Beverly, MA). GF109203X, calycin A, cyclosporin A, okadaic acid, and wortmannin were from Calbiochem (Meudon, France). G418 was from Life Technologies, Inc. (Cergy Pontoise, France); 9E10 antibody specific to c-myc was from Santa Cruz Biotechnology (Santa Cruz, CA). The peptides were synthesized in phosphorylated and non-phosphorylated isoforms (Neosystem, Strasbourg, France).

**Stable Cell Lines and Culture Conditions**—For stable expression, the CHO dihydrofolate reductase-negative cell line was co-transfected using a modified calcium phosphate precipitation method (23) with plasmid p1211 coding for human CB₂ or CB₃, carrying the supplementary 13-amino acid NH₂-terminal c-myc (MEQKLISEEDLRL) (24) and selected for dihydrofolate reductase expression as described previously (9). CHO wild-type cells were routinely grown as monolayers at 37 °C in a humidified atmosphere containing 5% CO₂ in a minimal essential medium (Life Technologies, Inc.) supplemented with 5% dialyzed fetal calf serum, 40 μg/ml l-proline, 1 mm sodium pyruvate, 60 μg/ml tylo- cine, 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 10 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 MgCl₂, 1 mM EDTA, and stored at −80 °C until use.

**Alkaline Phosphatase Treatment**—The membranes were solubilized in 0.1% Triton, 10 mM Tris-HCl, pH 7.5, 1 mM EDTA, 50 μl/mg protease inhibitor mixture at a protein concentration of 1–5 mg/ml, for 1 h at 4 °C. The mixture was centrifuged and the supernatant was diluted (v/v) with the dephosphorylation buffer (150 mM Tris, pH 8.3, 10 mM ZnCl₂, 10 mM MgCl₂) containing 50 units/ml alkaline phosphatase, for 18 h at 37 °C. Samples were then analyzed in Western blot experiments (25).

**MAPK Assay**—MAPK activity was measured as described previously (8). Briefly, cells grown to 80% confluence in 24-well plates were placed in medium containing 0.5% fetal calf serum for 24 h (0% fetal calf serum for 14 h at 37 °C). The cells were washed in phosphate-buffered saline and harvested with 20% trichloroacetic acid at 4 °C for 10 min. The supernatants were collected for dihydrofolate reductase expression as described previously (9). CHO wild-type cells were routinely grown as monolayers at 37 °C in a humidified atmosphere containing 5% CO₂ in a minimal essential medium (Life Technologies, Inc.) supplemented with 5% dialyzed fetal calf serum, 40 μg/ml l-proline, 1 mm sodium pyruvate, 60 μg/ml tylo- cine, and 20 μg/ml gentamycin.

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**MAPK Assay**—MAPK activity was measured as described previously (8). Briefly, cells grown to 80% confluence in 24-well plates were placed in medium containing 0.5% fetal calf serum for 24 h (0% fetal calf serum when ³H-THC was used) before assay. After treatment, cells were washed twice and lysed. 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 the micro-BCA protein assay kit (Pierce). These data provide new insight into the relationship between inverse agonists and the phosphorylation/desensitization process.

**Western Blot Analysis**—Following treatment, cells were washed in phosphate-buffered saline and directly lysed in Laemmli’s loading buffer containing 6 μL urea (26). Fifty micrograms of proteins were run on a 4–20% gradient polyacrylamide gel before being blotted onto nitrocellulose filters. Nonspecific binding of antibodies was inhibited by incubating filters in 10% dried milk powder in TBST buffer (10 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.05% Tween 20). The blots were incubated with the 4P anti-CB 2 rabbit antibody or with anti-phospho-rylated MAPK isoforms, for 3 h at TBST with 1% dried milk. After extensive washings with TBST, the blots were subsequently incubated for 1 h at room temperature with a peroxidase-labeled anti-IgG antibody. After washing, immunostained CB₂ was visualized using an enhanced chemiluminescence detection (ECL) system (Amersham Pharmacia Biotech).
Mained at this level during CP-55,940 treatment (Fig. 3). No variation in the signal intensity was observed when the anti-c-myc antibody was used instead (data not shown). Altogether, these results indicated that: 1) the agonist induced Ser352 phosphorylation, 2) a fraction of the CB2 receptor was phosphorylated in the absence of an agonist, likely due to an autoactive receptor, and 3) by blocking auto-activated CB2, SR 144528 treatment induced the appearance of the CB2 unphosphorylated form.

We then attempted to determine which kinase(s) were involved in Ser352 CB2 phosphorylation by testing the effect of various drugs that either activated or inhibited protein kinases. None of the PKC- or PKA-specific molecules affected the phosphorylation status of Ser352 CB2 in the control or in CP-55,940-treated cells (Fig. 4, D and E). We also investigated whether Ser352 CB2 phosphorylation was dependent on signal transduction. As the G protein is the cognate G protein for physiological signaling by CB2, we tested the effect of PTX on the phosphorylation status of CB2. As shown in Fig. 4B, treatment of CHO-CB2 cells with 100 ng/ml PTX did not affect the phosphorylation of Ser352 CB2 in control or CP-55,940-treated cells. Our results showed that the appearance of the CB2 unphosphorylated form following SR 144528 treatment was inde-
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SR 144528 Induced Up-regulation of CB2 Cell Surface Expression—Receptor phosphorylation leads to the uncoupling of receptors from G-proteins and also increases the affinity of arrestin (or dynemin, clathrin) binding to the phosphorylated COOH-terminal receptor promoting receptor internalization in endocytosis vesicles (22). As SR 144528 induced enhancement of non-phosphorylated CB2 in the absence of agonist, we also examined, whether SR 144528 could increase the CB2 cell surface density. Cell surface CB2 expression was quantitated by flow cytometry using the monoclonal antibody 9E10 (anti-c-myc tag). As shown in Fig. 5, a rapid and marked decrease in cell surface CB2 expression was observed in CHO-myc-CB2 treated with CP-55,940. This CP-55,940-induced internalization of CB2 was rapid, with a maximum reached 30 min after treatment, and partial since 50% of the CB2 was detected at the cell surface. In striking contrast, a time-dependent 40% up-regulation of CB2 expression was observed in CHO-myc-CB2 treated with SR 144528 instead (Fig. 5). Because we showed that the total amount of CB2 remains constant, this result suggested that the variation of the level of the receptor on cell surface is very likely related to an alteration of the cellular distribution.

SR 144528 Induced an Enhanced CB2-coupled Cellular Response—The above results indicated that, in the absence of agonist, a fraction of CB2 was constitutively phosphorylated and internalized. As SR 144528 blocked this process it could be expected that inverse agonist treatment would further enhance CB2 biological responses to an agonist. CHO-CB2 cells were first incubated with SR 144528 for 1 h, washed, and then stimulated with CP-55,940, and MAPK activity was analyzed as described under “Experimental Procedures.” SR 144528 pre-treatment led to an increase in CB2-coupled MAPK activation, which was enhanced 3-fold compared with the control, without altering the EC50 (5–8 nM) (Fig. 6A). These results were also observed in kinetic analyses (Fig. 6A, inset). These results, obtained by measuring MAPK activity, were confirmed by Western blot detection of the active p42 isoform of MAPK proteins (Fig. 6B). We previously described that CB2-induced MAPK activation was PTX-sensitive and partially protein kinase C-dependent. PTX treatment completely abolished CP-55,940-induced MAPK activation, while the PKC inhibitor GF109203X partially inhibited this response, regardless of whether cells were pretreated or not with SR 144528, indicating that the same transduction pathway was used in both cases. These results indicated that the constitutively active CB2 was constitutively phosphorylated, internalized, and desensitized. The inverse agonist, by up-regulating CB2, converted into greater maximal stimulation by the agonist.

SR 144528 Could Regenerate Desensitized CB2 Receptors—We next wondered whether SR 144528 could modulate the phosphorylation state of the receptor after being extensively phosphorylated by agonist exposure. In these experiments, cells were first exposed to the agonist to induce phosphorylation, then treated with SR 144528, and both Ser352 CB2 was phosphorylated, internalized, and desensitized. The inverse agonist, by up-regulating CB2, converted into greater maximal stimulation by the agonist.
remained phosphorylated even 8 h after the treatment (Fig. 7B). Surprisingly, CB₂ became slightly unphosphorylated when cells were treated with SR 144528 for 30 min after the first CP-55,940 stimulation. By contrast, 1 or 7 h later, CB₂ became entirely unphosphorylated (Fig. 7B). We therefore examined whether SR 144528-induced CB₂ dephosphorylation could be associated with CB₂ resensitization. Parallel examination of

the biological response triggered by a second agonist challenge showed that the MAPK-CB₂-mediated response was fully recovered. These results showed that SR 144528 at any time led to a reversion of desensitization, i.e. resensitized the receptor.

The next prevailing question to address was: how many
times can the receptor be repetitively switched off and on by alternative treatment with the agonist and the inverse agonist, respectively? As shown in Fig. 8, alternation of CP-55,940 and SR 144528 came with a CB2 phosphorylation/desensitization and CB2 dephosphorylation/resensitization processes, which could be repeated at least three times without any changes in the cellular responses.

SR 144528-induced CB2 Dephosphorylation Requires Phosphatase Involvement and CB2 Internalization—It was of interest to investigate the mechanism by which SR 144528 enhances the appearance of the unphosphorylated form. One possible interpretation for the above results is that, even after extensive washing, a minute amount of agonist (CP-55,940 or endogenous agonist produced by the cells) could still remain bound to the receptor and keep it phosphorylated. The effect of SR 144528 could therefore merely involve agonist displacement, allowing receptor resensitization by the classical dephosphorylation/recycling process. This is the case if similar effects could likely be obtained with the neutral CB2 antagonist Δ⁹-THC. As shown in Fig. 7C, Δ⁹-THC was unable to modulate CB2 phosphorylation, but it inhibited SR 144528-induced CB2 dephosphorylation. Similar effects were obtained when CHO-CB2 cells were pretreated with cycloheximide, indicating that SR 144528 effect was not due to the increase of neosynthetized CB2. Altogether these results suggest that SR 144528 alone induces a dephosphorylation process. As receptor dephosphorylation should involve phosphatases (28–30), we investigated the role of these enzymes. Cells were exposed to CP-55,940 for 1 h, then treated with SR 144528 in the presence or absence of protein phosphatase inhibitors, and the phosphorylation state was tested by immunoblot. Inhibition of the phosphatase PP2B by

FIG. 8. The effect of alternation of agonist and inverse agonist on the CB2 phosphorylation state and CB2-induced MAPK. Confluent CHO-CB2 cells were submitted to repeated alternation of a pulse of 10 nM CP-55,940 wash-out and a pulse of 50 nM SR 144528 wash-out (A). After each step, CHO-CB2 cells were either lysed in Laemmli’s buffer and immunoblotted with 4P to determine CB2 phosphorylation (A) or stimulated with 50 nM CP-55,940 for various times and MAPK activity was determined as described under “Experimental Procedures” (B). The results are expressed as a percentage of unstimulated cells, and the values are means ± S.E. of duplicates.

FIG. 9. The effect of phosphatase inhibitors and internalization inhibitor on SR 144528-induced CB2 dephosphorylation. A, quiescent CHO-CB2 cells were incubated or not with increasing concentrations of okadaic acid for 15 min and then treated with 50 nM SR 144528 for 1 h before immunodetection of CB2. B, CHO-CB2 cells were pretreated with 10 nM CP-55,940 for 45 min and then incubated or not with either 2 nM calyculin A (Caly A) or 1 μM cyclosporin A (Cyclosp A) for 15 min. The cells were then washed to remove CP-55,940 and then exposed to 50 nM SR 144528 in the presence or absence of calyculin A or cyclosporin A. One hour later, cells were lysed and CB2 immunoblotted with 4P. C, to block CB2 internalization, CHO-CB2 cells were pretreated or not with concanavalin A (Con A) at 0.25 or 0.5 mg/ml for 30 min and then exposed to 50 nM SR 144528 for another 1 h before CB2 immunoblotting.
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cyclosporin A at 1 μM failed to alter SR 144528-induced CB₂
dephosphorylation (Fig. 9B). By contrast, the okadaic acid that
inhibits phosphatase 2A blocked SR 144528-induced dephosphorylation (Fig. 9A). In agreement with these results, strong
inhibition was also observed with another PP2A inhibitor, i.e.
calystatin A at 2 nM (Fig. 9B).

A question raised by these results is whether the phosphatase,
which induced CB₂ dephosphorylation, dephosphorylated
the CB₂ on cell surface or after internalization in the intracel-
lar compartment. As shown in Fig. 9C, the blockade of CB₂
internalization with concanavalin A (29) inhibited the SR
144528-induced CB₂ dephosphorylation indicating that the SR
144528 induced CB₂ dephosphorylation when it was internal-
ized into endosomes.

DISCUSSION

4P Is a CB₂ Phosphorylation State-specific Antibody—
Among 18 peptides corresponding to different intracellular or
extracellular loops of the CB₂ receptor, only the peptide corre-
sponding to the extreme carboxyl-terminal end led to the
ontention of a specific anti-CB₂ receptor antibody. The specificity
of this antibody (named 4P) has already been demonstrated by
immunoblotting, flow cytometry, and confocal analysis (5). We
here showed that 4P is an antibody directed against the pe-
ipheral cannabinoid receptor CB₂ whose binding site was lost
upon receptor phosphorylation at the Ser³⁵² of the CB₂ COOH-
terminus. This conclusion drawn on the basis of the two
following observations: (i) CB₂ phosphorylation by alkaline
phosphatase enhanced the 4P signal; (ii) in competition binding
experiments with synthetic peptides corresponding to the CB₂
COOH terminus, which were phosphorylated at each potential
phosphorylation site, Ser³⁵² phosphorylation only rendered the
peptide unable to compete with the antibody labeling. To our
knowledge, 4P is the first known antibody with this property.
Although this precludes the use of this antibody for studying
overall CB₂ modulation, this phosphorylation state-specific an-
tibody is a very appropriate tool for studying site-specific phos-
phorylation of the G-protein-coupled receptor CB₂, which
was the focus of the present study. Most studies of GPCR phos-
phorylation/dephosphorylation processes have been based on
quantification of ³²P incorporation in the whole protein. How-
ever, this method is rather insensitive and cannot discriminate
between the different specific phosphorylation sites. In our
study using the 4P antibody, focusing on the Ser³⁵² phos-
phorylation/dephosphorylation state made the signal detection
very sensitive and specific.

CP-55,940-induced CB₂ Phosphorylation, Internalization,
and Desensitization of the Receptor—Using the 4P antibody, we
showed that treatment of CHO-CB₂ cells with CP-55,940 in-
duced a time- and dose-dependent phosphorylation of Ser³⁵² of
the CB₂. Two classes of serine/threonine kinases could be in-
volved in agonist-induced GPCR phosphorylation: a second
message-regulated kinase PKC or PKA and a GRK-type ki-
nase (31). The possible involvement of PKC or PKA is very
likely, as no effect on the CP-55,940-induced CB₂ phospho-
rylation was observed in the presence of GF10920X or
Ro31-8220 (a PKC-selective inhibitor), phorbol 12-myristate 13-
acetate (a PKC activator), or forskolin (a PKA activator). This
is consistent with sequence analysis in which Ser³⁵² at the CB₂
COOH terminus was not a phosphorylation consensus site for
PKC or PKA. Another possibility could be the involvement of a
GRK-type kinase. Among the known GRKs that phosphorylate
agonist occupied GPCRs, two classes have been identified. The
first includes GRK2 and GRK3, which have domains for bind-
ing to βγ subunits of G-protein, and their enzymatic activity is
potentiated by the βγ subunit upon activation and dissociation
of the heterotrimeric G protein (32, 33); and the second includes

GRK1, GRK4, GRK5, and GRK6, which do not have sites for
binding to βγ subunits (34). The PTX treatment did not affect
CP-55,940-induced Ser³⁵² CB₂ phosphorylation, indicating that
there is no involvement of the first class of GRK. On the other
hand, it was shown that both GRK1 and GRK2 are acidotropic
kinases, preferring acidic amino acids in proximity of phospho-
rylatable residues (35–37). As the COOH-terminal CB₂ is an
aspartic amino acid-rich region, a GRK1-like kinase (very
likely distinct to the GRK1 because of its restricted expression
to retina and pineal gland; Ref. 31) could be a potential can-
didate in CP-55,940-induced Ser³⁵² phosphorylation in CHO-CB₂
cells.

SR 144528 Enhanced the CB₂ Unphosphorylated Form, Cell
Surface Receptor Expression, and Supersensitization of the
CB₂-coupled MAPK Response—We clearly showed that the con-
stitutively active CB₂ was constitutively phosphorylated, and
internalized: two characteristics of desensitized receptors. We
showed that the inverse agonist inhibits both phosphorylation
and internalization of CB₂ to the cell surface, leading to rapid
recycling of internalized receptors to the cell surface accompa-
nied by supersensitization to agonists. Indeed, we showed that
SR 144528 pretreatment induced an enhancement of CP-
55,940-induced MAPK activation. We also demonstrated that
SR 144528 sensitized other CB₂-coupled cellular responses
such as immediate early gene expression (Krox 24, c-fos) (data
not shown).

The constitutive active mutant β₂-adrenergic receptor was
shown to be constitutively phosphorylated (38), internalized
(39), and desensitized (40). Pei et al. demonstrated that GPCRs
such as β₂-adrenergic receptors are both constitutively active and
constitutively desensitized. Constitutively active receptors thus
recruit known elements of cellular desensitization machin-
ery. In the absence of agonist, such receptors were found to be
phosphorylated by G protein-coupled receptor kinase in a
way similar to the agonist-occupied receptor. They showed that
the rate and extent of agonist-independent phosphorylation of
constitutive active mutant β₂-adrenergic receptor were compa-
rable to agonist-dependent phosphorylation. We previously
observed that the expression of cloned CB₂ receptor in CHO cells
results in agonist-independent activation, which can be de-
creased by the inverse agonist SR 144528. The simplest expla-
nation for our results is that SR 144528 blocks the auto-acti-
vated receptor and consequently desensitization does not occur.
Autoactivation of CB₂ likely induces its phosphorylation and
internalization in endocytic vesicles where it is dephosphory-
ated. The receptor is recycled to the surface for another run.
Treatment with SR 144528 blocks CB₂ activation, phosphory-
lation, and internalization. The intracellular CB₂ pool once
dephosphorylated accumulated on the cell surface, which
enhanced the amount of cell surface CB₂. Thus, the inverse
agonist could not only block the constitutively active CB₂ but
also inhibit constitutive phosphorylation, desensitization, and
internalization of the receptor. Inhibition of autoactivated re-
ceptors by inverse agonists is also often interpreted as being
due to blockage of endogenous agonist present in the culture
medium or produced by the cells. However, this could be ruled
out as the natural cannabinoid ligand THC acted as a neutral
agonist in our assay. Overall, these results indicate that the
observed effects of the CB₂ antagonist SR 144528 are the direct
consequences of its binding to unoccupied receptors and sup-
port the notion that it acts as an inverse agonist with high
intrinsic activity.

A similar receptor regulation pattern was recently noted for
inverse agonists at a constitutive active mutant of the β₂-
adrenergic receptor, with betaxolol and sotalol causing marked
increases in the levels of this receptor after its stable expres-
sion in NG 108.15 cells (41). Furthermore, Smit et al. (42) also reported an up-regulation of histamine A_{3} receptors in response to long term treatment with inverse agonists but not neutral antagonists.

**SR 144528-induced Regulation of CP-55,940-inactivated CB_{2} and Recovery of CB_{2}-coupled Cellular Responses**—We further demonstrated that after a single CP-55,940 pulse CB_{2} remained phosphorylated and inactivated as long as 8 h after agonist wash-out. We ruled out that this persistent effect could be related to the presence of residual CP-55,940 in the medium as Δ^{9}-THC, which is a neutral antagonist, had only a slight effect on CB_{2} phosphorylation (Fig. 7C).

The time required for receptor recovery varies according to the receptors and cells involved. Bradykinin, β_{2}-adrenergic, and C5a receptors were shown to be resensitized 10, 25, or 60 min, respectively, after agonist removal (28, 43, 44). On the other hand, it was found that the recovery period for the thrombin receptor is 1 h in endothelial cells, but 16–18 h in megacaryoblastic cell lines (45). The m3-cholinergic receptor transfected in HEK 293 cells remained fully desensitized at 4 h and only partially desensitized 24 h after a short carbachol treatment (2 min) (46).

During this time period, SR 144528 generated a dephosphorylated CB_{2} and fully restored the CB_{2}-coupled cellular responses. This effect was not observed with the neutral antagonist Δ^{9}-THC, which otherwise could block the SR 144528 effects (Fig. 7C). The cycloheximide did not affect SR 144528-induced CB_{2} dephosphorylation, indicating that this effect was not due to the neosynthesis of CB_{2} (Fig. 7C, lower).

SR 144528, by dephosphorylating CB_{2}, thus accelerated the recovery of desensitized receptors. The process of CP-55,940-induced receptor phosphorylation followed by SR 144528-induced receptor dephosphorylation kept recurring many times on the same cells, indicating that the agonist switches the system off but the inverse agonist switches the system back on. This property has never been described before. In CB_{2}, it therefore seems that receptor phosphorylation/dephosphorylation of Ser^{352} could be a critical factor governing receptor sequestration/recycling/resensitization. The construction of mutant CB_{2} (Ser^{352}) could help to accurately determine the role of Ser^{352} in this process. There are eight Ser/Thr residues in the COOH-terminal cytoplasmic domain of CB_{2}. Among them, three are clustered in the cytoplasmic tail. In addition to COOH-terminal cytoplasmic domain, other Ser/Thr residues in the intracellular loop (I_{1}, I_{3}, I_{5}, and I_{6}) of CB_{2} are also candidate substrates for kinases and could be involved in the desensitization/resensitization process. Moreover, the CB_{2} have also a “NPVY” motif (for “NPXY” consensus) in the seventh transmembrane domain that has been suggested as playing a key role for internalization in other GPCR (47).

By what mechanism does SR 144528 induce CB_{2} dephosphorylation and resensitization? Some GPCR internalized receptors return to the cell surface after agonist treatment in a process called recycling (Fig. 10). While many molecular mechanisms are proposed to be involved in receptor internalization, the mechanisms that trigger receptor recycling are still not well understood. Krueger et al. (30) showed that agonist-occupied β_{2}-adrenergic receptors were phosphorylated and internalized in endocytic vesicles, where acidic pH induced a change of receptor conformation, becoming substrate of phosphatase 2A. Dephosphorylation and re-expression of receptors on the cell surface induced resensitization of receptor-coupled cellular responses. Receptor dephosphorylation is thus the major mechanism that triggers resensitization.

In the presence of concanavalin A (0.25 mg/ml), an inhibitor of receptor internalization (29), SR 144528 could not dephosphorylate the receptor (Fig. 9C). These results suggested that SR 144528 dephosphorylated CB_{2} only when it was internalized. This agrees with the observation that dephosphorylation initiation was rapid after inverse agonist treatment, but an apparent lag period was observed between agonist stimulation and the onset of inverse agonist dephosphorylation.

We showed that SR 144528-induced CB_{2} dephosphorylation was independent of CB_{2} signaling transduction (PTX-, GF109203X-, and wortmannin-insensitive) but involved serine/threonine PP2A-type phosphatase, which were sensitive to okadaic acid and to calyculin A but not to cyclosporin A. The involvement of PP2A-type phosphatase has already been described for cholecystokinin receptors, rhodopsin, and β_{2}-adrenergic receptors (30, 48, 49). We do not yet know whether SR 144528 induces a conformational change of CB_{2}, making it a better phosphatase-specific substrate or if binding of SR 144528 activates specific phosphatases. Such dephosphorylation generation has already been described for rhodopsin receptors. Indeed, it was recently shown that orange light converts rhodopsin from the active to the inactive state by dephosphorylating the rhodopsin COOH-terminal tail, which involves the retinal degeneration C phosphatase (49).

An important question that should be addressed is: can these observations be reproduced in cells that normally express CB_{2} receptors? If so, it could be expected that although SR 144528 initially blocks the endogenous ligand, in a second step, after its withdrawal, it could intensify the effect of the endogenous agonist. This could have major pharmacological implications. This point remains unanswered, as the effects we described have not been observed in cells naturally expressing the receptor which are not autoactivated. However, this point deserves further investigation.

**Conclusion**—We showed that CB_{2} is phosphorylated at Ser^{352} under basal conditions and phosphorylation is increased by agonist treatment, conditions that resulted in desensitization of receptor signaling. On the other hand, treatment with the inverse agonist dephosphorylated CB_{2} under both basal and agonist-phosphorylated CB_{2} conditions, resulting in CB_{2}
resensitization. Hence, by using agonists and inverse agonists, the phosphorylation status of the receptor can be manipulated along with its response potential.

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