Modulation of CB1 Cannabinoid Receptor Functions after a Long-Term Exposure to Agonist or Inverse Agonist in the Chinese Hamster Ovary Cell Expression System

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ABSTRACT
We have investigated the adaptive changes of the human central cannabinoid receptor (CB1) stably expressed in Chinese hamster ovary cells (CHO-CB1), after agonist (CP 55,940) or selective CB1 inverse agonist (SR 141716) treatment. CB1 receptor density and affinity constant as measured by binding assays with both tritiated ligands remained essentially unchanged after varying period exposure of CHO-CB1 cells (from 30 min to 72 hr) to saturating concentrations of CP 55,940 or SR 141716. However, using a CMyc-tagged version of the CB1 receptor, FACS analysis and confocal microscopy studies on CB1 expression indicated that the agonist promoted a disappearance of cell surface receptor although inverse agonist increased its cell surface density. Taken together these results suggest that 1) agonist induces internalization of the receptor into a cellular compartment that would be still accessible to both the hydrophobic ligands CP 55,940 or SR 141716; 2) inverse-agonist promotes externalization of the receptor from an intracellular preexisting pool to the cell surface. In parallel, we also investigated the associated effects of CP 55,940 and SR 141716 on CB1 receptor-coupled second messengers. We showed that preexposure of cells to CP 55,940 induced a rapid desensitization of the CB1 to the agonist response. The ability of CP 55,940 to inhibit the forskolin-stimulated adenylyl cyclase and to activate the mitogen-activated protein kinase activity was dramatically reduced. By striking contrast, SR 141716 pretreatment of CHO-CB1 cells not only had no significant effect on the potency of CP 55,940 to inhibit the forskolin-stimulated adenylyl cyclase but also induced a significant enhancement of the CP 55,940 ability to stimulate the mitogen-activated protein kinase activity. These results suggest that the modulation of the number of cell surface receptor could lead to functional desensitization or sensitization of the CB1 receptors.

It is now well established that Δ9-THC (the main active principle of marijuana), anandamide (an endogenous ligand) (Devane et al., 1992) and synthetic cannabinoid receptor agonists mediate their cellular effects through specific cannabinoid receptors belonging to the large multigene superfamly of the GPCR. To date, two human cannabinoid receptor cDNAs have been identified, designated CB1 and CB2 (Matsuda et al., 1990, Munro et al., 1993). Two splice variants of CB1 mRNA (CB1 and CB1A) have been described in human and rat brain (Shire et al., 1995). Although CB1 mRNA is predominantly expressed in the brain (Matsuda et al., 1990, Westlake et al., 1994), it has also been detected in testis (Gérard et al., 1991), spleen cells (Kaminski et al., 1992) and in leukocytes (Bouaboula et al., 1993). By contrast the CB2 subtype has not been detected in the brain, but found remarkably abundant in immune tissues (Galiègue et al., 1995, Deroq et al., 1995), and may account for cannabinoid-mediated immune responses. Both receptors mediate their effects via a pertussis toxin-sensitive GTP-binding regulatory protein Gi. Upon stimulation, both CB1 and CB2 induced the inhibition of AC (Howlett and Fleming, 1984, Slipel et al., 1995, Felder et al., 1995) and the activation of the MAPK (Bouaboula et al., 1995a, 1996). In neurons, CB1 has been found to be associated with the inhibition of N-type (Mackie and Hille, 1992) or P/Q-type (Twitchell et al., 1997) calcium channels. In addition, CB1 activation has recently been shown to induce immediate-early gene expression such as Krox 24 through a cAMP-independent pathway (Bouaboula et al., 1995b). These central cannabinoid receptor-mediated effects were all prevented by the potent and selective CB1 receptor antagonist SR 141716 (Rinaldi-Carmona et al., 1994, 1995, 1996). There is now substantial evidences showing that for some GPCRs, antagonists induce effects that are opposite to those observed by agonists.
thereby displaying negative intrinsic activity. This referring to inverse agonism (Lefkowitz et al., 1993, Kenakin, 1996). Such an effect was recently demonstrated for the selective CB1 receptor antagonist SR 141716 (Landsman et al., 1997, Bouaboula et al., 1997) that is therefore defined as an inverse agonist.

It is clearly established that the functionality and the expression of the GPCRs are dynamically regulated after agonist or antagonist exposure. Indeed it has often been observed that cell exposure to agonist causes the desensitization and a down-regulation of the receptors, whereas antagonist treatment leads to their up-regulation. Several in vivo studies have also demonstrated the same behavior for cannabinoid receptor agonist ligands after chronic treatment that induce tolerance to many pharmacological effects: anti-convulsant activity, catalepsy, depression of locomotor activity, hypothermia, hypotension, immunosuppression and static ataxia (Pertwee, 1991, Pertwee et al., 1993, Fan et al., 1994, Fride, 1995). In vivo, tolerance took place with a significant decrease in the density of cannabinoid receptors in striatal structures (Oviedo et al., 1993, Rodriguez De Fonseca et al., 1994) in the cerebellum (Fan et al., 1996) and in the spleen (Massi et al., 1997) as well as in the levels of cannabinoid receptor mRNA (Rubino et al., 1994).

So far no detailed insights on the modulation of CB1 functions by cannabinoid ligands have been described in vitro. Therefore, in our report we have explored the adaptive changes in CB1 receptors after CP 55,940 or SR 141716 treatment in vitro. Their effects on CB1 receptor binding properties, CB1 receptor cell surface expression and their associated effects on CB1 receptor-coupled second messenger were investigated in a CHO cell stably expressing the CB1 receptor. Our results show that exposure of CHO-CB1 cells to the agonist CP 55,940 leads to a desensitization of the CB1 receptor functions (forskolin-induced cAMP accumulation and the MAP kinase activity). These effects were associated with agonist-promoted receptor internalization without modification of the total binding site number. By striking contrast MAPK signal transduction pathway was dramatically enhanced in SR 141716-pretreated CHO-CB1 cells. An effect that may be related to the observed receptor externalization to the plasma membrane from an intracellular pool in response to its binding with the inverse agonist SR 141716.

Experimental Procedures

Materials. Fatty acid-free BSA, IBMX and forskolin were from Sigma Chemical Co. (St. Louis, MO). Biofluor liquid scintillant and [3H]-CP 55,940 (103.4 Ci/mmol) were purchased from New England Nuclear Corporation (Paris, France). [3H]-SR 141716A (42 Ci/mmol), γ32P ATP (112.9 Ci/mmol), cAMP scintillant proximity assay, the enhanced chemiluminescence detection system and Biotrack p42/p44 MAPK kits were from Amersham (Les Ulis, France). RO 20-1724{4-[3-butoxy-4-methoxyphenyl)methyl]-2-imidazolidinone} was purchased from Research Biochemicals Incorporated (Illkirch, France). [3H]-CP 55,940 (0.1 to 30 nM) or [3H]-SR 141716A (0.3 to 30 nM) in the presence of 10% FCS for 24 hr before beginning of the treatment with CP 55,940 or SR 141716. For long-time exposure cells were first exposed to the drug for 24 or 48 hr in the presence of 10% FCS and then for 24 hr in the presence of 0.5% FCS.

Binding experiments. For binding assays, treated and untreated CHO-CB1 cells were washed and incubated at 37°C with [3H]-CP 55,940 (0.1 to 30 nM) or [3H]-SR 141716A (0.3 to 30 nM) in the presence of 10% FCS for 24 hr before addition of washing buffer [25 mM HEPES/Tris (pH 7.5), 140 mM NaCl, 5 mM KC1, 1.8 mM CaCl2, 0.9 mM MgCl2, 4.5 g/liter glucose and 0.5% acid-free BSA], then tested for binding or cAMP assays. The washing protocol after incubation of the cells with the ligands, was optimized as checked by binding experiments with [3H]-CP 55,940. For MAPK assays, cells were maintained in culture medium containing 0.5% FCS for 24 hr before treatment of the cells with CP 55,940 or SR 141716. For long-time exposure cells were first exposed to the drug for 24 or 48 hr in the presence of 10% FCS and then for 24 hr in the presence of 0.5% FCS.

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Construction of expression vectors. The CB1 coding sequence (Shire et al., 1995) was amplified by polymerase chain reaction with a sense primer bearing HindIII sites and Kozak consensus sequences 5’-CCACAGGTTTTCCACATTGATCGATCTTATGAGGCC and an antisense primer carrying an EcoRI site, 5’-CCATCGAATTCCTCATACAGAAGCCGCGCAG. The amplicons were digested with HindIII/EcoRI to generate unique cloning sites and inserted into pGEM4Z (Promega) for 30 min at 37°C, then washed and the fluorescence was analyzed by flow cytometry.
on 12-mm glass coverslips (Prolabo, Vaulx-en-Velin, France) and treated with cannabinoid ligands for various periods. Washed cells were incubated with 9E10 monoclonal antibody for 30 min at 4°C washed and incubated with 1/200 dilution of CY3-conjugated anti-mouse 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 anti-bleaching reagent DABCO at 50 ng/ml (Sigma). Fluorescence analyses were performed using a confocal microscope (LSM410, Zeiss, Oberkochen, Germany).

**cAMP measurements.** cAMP measures were carried out as already described (Rinaldi-Carmona et al., 1996). Briefly, cells were incubated for 5 min at 37°C in PBS containing 0.25% acid-free BSA, IBMX (0.5 mM), RO 20-1724 (0.2 mM), with or without CP 55,940. Forskolin was added (5 μM final concentration) and cells were incubated for 20 min at 37°C. The reaction was stopped by rapid aspiration of the assay medium and addition of 1.5 ml of ice-cold 50 mM Tris-HCl, pH 8.4, 4 mM ethylenediamine tetraacetic acid. Dishes were cooled on ice for 5 min and then the extracts were transferred to a glass tube. Extracts were boiled and then centrifuged for 10 min at 3500 × g to eliminate cellular debris. Aliquots from supernatants were dried and the cAMP concentration was determined by radioimmunoassay.

**MAPK activity.** MAPK activity was measured as described (Bouaboula et al., 1995a). Briefly, 80% confluence grown cells were maintained in culture medium containing 0.5% FCS for 24 hr before the addition of ligands. Ligand-treated and vehicle-treated CHO-CB1 cells were washed, then incubated at 37°C with or without CP 55,940 or SR 141716 for 10 min. Cells were then washed at 4°C with 0.5 ml of buffer A (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1 mM ethyleneglycol-bis-(β-aminoethyl ether) N,N,N',N'-tetraacetic acid, 1 mM NaN3, VO43-) and lysed for 15 min in buffer supplemented with 1% triton X-100, 10 μg/ml aprotinin, 10 μg/ml leupeptin, 1 mM dithiothreitol and 1 mM phenylmethylsulfonyl fluoride. Solubilized cell extracts were then clarified by centrifugation at 14,000 × g for 15 min at 4°C. Aliquots (15 μl) were removed and stored at −80°C until use. Phosphorylation assays were carried out at 30°C for 30 min (linear assay conditions) with γ32P ATP using the Biotrack p42/p44 MAPK enzyme system. The radioactivity incorporated was determined by liquid scintillation counting.

**Western-blot analysis.** After stimulation, cells were washed and lysed in Laemmli’s loading buffer containing 6 M urea. Proteins were run on sodium dodecyl sulfate/polyacrylamide gel electrophoresis and blotted onto nitrocellulose filters. Nonspecific binding of antibodies was prevented by incubating filters in 5% dried milk powder in buffer A saturated with 5% BSA. After extensive washings, the blot was subsequently incubated for 45 min at room temperature with a mouse IgG (Sigma Immunochemicals) for another 30 min at 4°C. After washing, immunostained MAPKs were visualized using the enhanced chemiluminescence detection system according to the supplier’s instructions and subjected to autoradiography.

**Data analysis.** EC50, IC50, Kd, Bmax, Ki and Hill coefficient (nH) were analyzed using a nonlinear curve fitting (Marquardt-Levenberg least-squares method) on a Compaq Desk Pro 4/66i computer. Experiments were all performed in duplicate (binding assays) or in triplicate (cAMP and MAPK activity measures).

**Results**

**Binding and functional properties of CP 55,940 and SR 141716 in CHO-CB1 cells.** Specific binding of [3H]-CP 55,940 and [3H]-SR 141716A performed on whole CHO-CB1 cells at 37°C was time-dependent and reached a steady state within a 20-min incubation (data not shown). Binding of these two radioligands was concentration dependent, and the nonspecific binding, measured in the presence of 1 μM of their respective unlabeled ligand, was linearly dependent on the concentration of the radioligand used (10–20 and 45–50% of the total binding at 0.1 and 30 nM, respectively). The specific binding was saturable and reached a maximum of 10 to 15 nM for both ligands (fig. 1, A and B). The nonlinear regression analysis of the saturation curves revealed the presence of one class of binding sites exhibiting high affinity for both ligands. An apparent equilibrium dissociation constant (Kd) value of 5.47 ± 0.28 nM (13 experiments) and 4.84 ± 0.66 nM (10 experiments) was found for [3H]-CP 55,940 and [3H]-SR 141716A, respectively. The total binding site number (Bmax) varied from 1.5 to 3.5 × 106 receptors/cell. The specific [3H]-CP 55,940 binding on whole CHO-CB1 cells was displaced in a concentration-dependent manner by unlabeled CP 55,940 (Kd = 16.9 ± 1.1 nM; nH = 1.06 ± 0.11, 7 experiments) and SR 141716 (Kd = 11.5 ± 1.3 nM; nH = 1.13 ± 0.15, 14 experiments). Similar results were obtained for [3H]-SR 141716A (SR 141716, Kd = 9.0 ± 1.1 nM; nH = 0.66 ± 0.07). Specific binding was defined as the amount of radioligand bound in the presence of 1 μM of their respective unlabeled ligand subtract from total radioligand bound at each concentration. Inset, Represents Scatchard plots of [3H]-CP 55,940 (A) or [3H]-SR 141716A (B) as described in “Experimental Procedure.” Specific binding was defined as the amount of radioligand bound in the presence of 1 μM of their respective unlabeled ligand subtract from total radioligand bound at each concentration. Inset, Represents Scatchard plots of [3H]-CP 55,940 (A) or [3H]-SR 141716A (B) specific binding. Data are from one out of ten to thirteen experiments performed in duplicate.

![Equilibrium binding of [3H]-CP 55,940 or [3H]-SR 141716A to CHO-CB1 cells. Confluent cells (106 cells/well) were incubated for 30 min at 37°C with increasing concentrations of [3H]-CP 55,940 (A) or [3H]-SR 141716A (B) as described in “Experimental Procedure.” Specific binding was defined as the amount of radioligand bound in the presence of 1 μM of their respective unlabeled ligand subtract from total radioligand bound at each concentration. Inset, Represents Scatchard plots of [3H]-CP 55,940 (A) or [3H]-SR 141716A (B) specific binding. Data are from one out of ten to thirteen experiments performed in duplicate.](image-url)
1.15 ± 0.12, 5 experiments, CP 55,940, $K_i = 39.2 \pm 5.6$ nM; nH = 0.80 ± 0.01, 6 experiments).

CP 55,940 has been shown to inhibit AC and to be a potent stimulator of MAPK. These effects were sensitive to pertussis toxin and were not observed in wild-type CHO cells (Bouaboula et al., 1995a, 1996). SR 141716, which was developed as a potent and selective CB1 antagonist (Rinaldi-Carmona et al., 1994), was able to block both inhibition of AC and MAPK activation induced by CP 55940 (Bouaboula et al., 1995a, 1996, Rinaldi-Carmona et al., 1996). In addition, SR 141716 was also able to induce a marked enhancement of cAMP accumulation and inhibition of basal MAPK activity (fig. 2, A and B). Specific CB1 mediation was demonstrated with SR 141716 being effectless in wild-type CHO cells (data not shown). These effects of SR 141716 per se are consistent with 1) the blockage of AC inhibition and MAPK stimulation mediated by autoactivated CB1 receptor and 2) the inverse agonist status of SR 141716 in agreement with previous observations (Bouaboula et al., 1997).

**Effect of receptor ligands on modulation of CB1 expression.** We used two approaches to examine the effects of CP 55,940 or SR 141716 exposure on CB1 expression: by measuring the ability of CHO-CB1 cells to bind either [3H]-CP 55,940 or [3H]-SR 141716 and by analyzing CB1 cell surface expression. The effects of treatment by CP 55,940 and SR 141716 on binding parameters ($K_d$, $B_{max}$ and $K_i$) of [3H]-CP 55,940 and [3H]-SR 141716A were evaluated after exposure of confluent CHO-CB1 cells to vehicle, CP 55,940 or SR 141716. To guarantee a complete removal of all drugs, we limited to 30 and 10 nM the maximum agonist (CP 55,940) or inverse agonist (SR 141716) concentration, respectively, and we optimized the washing protocol after incubation of the cells with the ligands, as checked by binding experiments with [3H]-CP 55,940. Figure 3 shows that no significant differences ($P < .05$) in the specific binding, measured with [3H]-CP 55,940 or [3H]-SR 141716A, were observed in CHO-CB1 cells exposed to either CP 55,940 or SR 141716 for

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**Fig. 2.** Inverse agonist properties of SR 141716. A, Effect of SR 141716 on forskolin-stimulated cAMP production. CHO-CB1 cells (10^5 cells/well) were pretreated with increasing concentrations of SR 141716 for 10 min before a 20-min stimulation with forskolin (3 μM) at 37°C. cAMP levels were measured as described in “Experimental Procedure.” The data are expressed as the percentage of values from cells stimulated with forskolin alone (control cells, 100%). Data represent mean values ± S.E.M. of one determination performed in triplicate. The basal level of cAMP in control cells was 2.5 ± 1.9 pmol of cAMP/well. Forskolin-stimulated cAMP levels in control cells were 50 ± 2.4 pmol of cAMP/well. B, Effect of SR 141716 on basal MAPK activity in CHO-CB1 cells. Growth-arrested cells (10^5 cells/well) were treated for 10 min with increasing concentrations of SR 141716 at 37°C. The mitogen-activated protein kinase activity was measured in cell lysates as described in “Experimental Procedure.” Results are expressed as percentage of basal MAPK activity in the vehicle-treated cells (100%). The basal activity of the MAP kinase in control cells was 2400 ± 210 cpm/well. Data represent mean values ± S.E.M. of one determination performed in triplicate.

**Fig. 3.** Effect of pretreatment with CP 55,940 or SR 141716 on binding of [3H]-CP 55,940 and [3H]-SR 141716A to CHO-CB1 cells. Confluent cells (10^5 cells/well) were incubated with 30 nM CP 55,940 (●) or 10 nM SR 141716 (■) at 37°C as described in “Experimental Procedure.” After the indicated periods of time, cells were washed with washing buffer and 10 nM of [3H]-CP 55,940 (A) or [3H]-SR 141716A (B) was added for 30 min at 37°C. Specific binding was defined as the amount of radioligand bound in the presence of 1 μM of their respective unlabeled ligand substrate from total radioligand bound at 10 nM concentration. The data are expressed as the mean ± S.D. from two independent determinations performed in triplicate. Results are expressed as the percentage of the binding measured in untreated cells taken as 100%.
different periods ranging from 30 min to 72 hr, compared to untreated control cells. The results obtained for the $K_d$ and $B_{max}$ of $[^{3}H]$-CP 55,940 and $[^{3}H]$-SR 141716A, after short or long exposure with CP 55,940 or SR 141716 were reported in table 1. The $K_d$ values were unchanged after treatment of CHO-CB1 cells with CP 55,940 or SR 141716. Similarly, the $K_d$ value and the $n_H$ values of CP 55,940, measured at 0.4 nM $[^{3}H]$-CP 55,940 in washed CHO-CB1 cells, were unchanged after 1 hr exposure with unlabeled CP 55,940 or SR 141716 (CP 55,940-treated cells, $K_d$ of CP 55,940 = 0.41 ± 0.01 nM, $n_H$ = 0.85 ± 0.02; SR 141716-treated cells, $K_d$ of CP 55,940 = 1.48 ± 0.15 nM, $n_H$ = 0.92 ± 0.08 vs. control cells, $K_d$ of CP 55,940 = 1.20 ± 0.51 nM, $n_H$ = 1.07 ± 0.03, three experiments).

To study the membrane localization of the CB1, we designed a chimeric cDNA of the coding region of the C-myc fused to the full coding region of the CB1. The fusion of the tag C-myc to the N-terminus of the CB1 did not alter receptor ligand binding affinity as well as signal transduction (Rinaldi-Carmona et al., 1996). The effect of agonist or inverse agonist treatment on cell surface CB1 expression was determined by immunostaining using a C-myc epitope-tagged version of CB1 receptor and a specific anti-C-myc monoclonal antibody followed by flow cytometric analyses. As shown in figure 4 a rapid and marked decrease in cell surface CB1 expression was observed in CHO-CO-myc-CB1 treated with CP 55,940. This CP 55,940-induced down-regulation of CB1 was apparent at 30 min up to 20 hr. By striking contrast a slight but significant upregulation of the receptor was observed when cells were treated with SR 141716 instead (fig. 4A). Here again this SR 141716-induced upregulation of CB1 was apparent at 30 min and lasted at least up to 20 hr. To further document the above findings the cellular distribution of CB1 was visualized by confocal laser microscopy. Figure 4B shows in a representative confocal section the cellular localization of immuno fluorescently labeled C-myc-CB1 in vehicle-treated cells in comparison with cells exposed to CP 55,940 or SR 141716 for 1 hr. In vehicle-treated cells, labeled receptors were abundant on the cell surface, appearing as a bright ring of fluorescence. After sustained exposure to CP 55,940, the ring pattern of fluorescence labeling turned into a discontinue and less intense one indicating that the agonist promoted a disappearance of cell surface CB1. Conversely, a clear increase in cell surface CB1 density was observed in cell exposed to SR 141716. These data are in total agreement with our flow cytometry observations.

**Effect of agonist or inverse agonist exposure on cAMP accumulation.** We examined the effect of a permanent presence of agonist or inverse agonist on the coupling efficiency of CB1 to AC. First of all, we checked whether such a treatment per se affected the forskolin-induced cAMP production. Confluent CHO-CB1 cells were exposed to either vehicle, CP 55,940 or SR 141716 for variable periods of time ranging from 15 min to 72 hr. After treatment, the cells were extensively washed, and forskolin-stimulated adenylyl cyclase activity was determined. Pretreatment of CHO-CB1 cells with CP 55,940 led to a significant decrease in the activity of the adenylyl cyclase to be stimulated by forskolin. This effect which reflected the agonist effect of CP 55,940, began at 15 min (54% of inhibition), leveled off within 1 hr (69% of inhibition) and lasted at least 3 hr (33% of inhibition). After 6 hr, the stimulation exerted by forskolin was equivalent to that reached in untreated cells. In contrast, in SR 141716-treated CHO-CB1 cells a marked increase in the level of activation of the forskolin-stimulated adenylyl cyclase activity was observed in agreement with the blockage of autoactivated CB1 receptors by SR 141716. This effect was transient and was observed within the first 30 min of incubation with SR 141716 (56% of stimulation). Thereafter, the level of activation of forskolin was similar to that obtained for untreated cells.

We next determined the ability of the cannabinoid receptor agonist CP 55,940 to inhibit the forskolin-stimulated adenylyl cyclase activity in cells pretreated with vehicle, CP 55,940 or SR 141716. As reported in table 2 and shown in figure 5, the ability of CP 55,940 to inhibit the forskolin-stimulated cAMP production was dramatically impaired upon CP 55,940 exposure. This loss of responsiveness was time dependent; it was significant (P < .05) within the first 15 min after the addition of the agonist and was maximum at 30 min up to 6 hr. Thereafter, the forskolin-stimulated adenylyl cyclase became slightly sensitive to the inhibition by the cannabinoid agonist. As shown in figure 6, the agonist-induced desensitization was concentration dependent, with a maximal effect measured at 30 nM. By striking contrast, under the same experimental conditions, exposure of CHO-CB1 cells to 10 nM SR 55,940 had no effect on the ability of CP 55,940 to inhibit cAMP accumulation in these cells whatever long the treatment was from 30 min to 72 hr (figs. 5 and 6). Both potency and the maximal inhibition in SR 141716-treated cells were similar as into control cells (table 2).

**Effect of agonist or inverse agonist treatment on MAP kinase activation.** We also studied the effect of sustained CP 55,940 or SR 141716 treatment of CHO-CB1 on MAPK activity. We first examined the effect of such a treatment on the basal level of MAPK. Growth arrested CHO-CB1 cells were pretreated for different periods ranging from 30 min to 72 hr in the presence of vehicle, CP 55,940 or SR 141716. After treatment, cells were extensively washed, and the basal MAPK activity was determined. As shown in figure

### Table 1

Effects of exposure with CP 55,940 or SR 141716 on binding parameters of $[^{3}H]$-CP 55,940 and $[^{3}H]$-SR 141716A to CHO-CB1 cells

<table>
<thead>
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<th>$[^{3}H]$-SR 141716</th>
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<td></td>
<td>$K_d$ (nM)</td>
<td>$B_{max}$ (%)</td>
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<tr>
<td>Untreated cells</td>
<td>5.47 ± 0.70</td>
<td>100</td>
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<tr>
<td>CP 55,940 treated cells</td>
<td>6.33 ± 0.68</td>
<td>87 ± 7</td>
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<tr>
<td>SR 141716 treated cells</td>
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<tbody>
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<td></td>
<td>$K_d$ (nM)</td>
<td>$B_{max}$ (%)</td>
</tr>
<tr>
<td>Untreated cells</td>
<td>4.84 ± 0.66</td>
<td>100</td>
</tr>
<tr>
<td>CP 55,940 treated cells</td>
<td>4.70 ± 1.40</td>
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<tr>
<td>SR 141716 treated cells</td>
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<td>91 ± 9</td>
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Confluent cells (10⁵ cells/well) were incubated with vehicle, 30 nM CP 55,940 or 10 nM SR 141716 at 37°C for 1 or 18 hr. Cells were washed with washing buffer and 10 increasing concentrations of $[^{3}H]$-CP 55,940 (0.1–30 nM) or $[^{3}H]$-SR 141716A (0.3–30 nM) were added for 30 min at 37°C. Binding assays were performed as described in "Experimental Procedure." Data are expressed as the mean ± S.E.M. from three independent determinations performed in duplicate.
pretreatment of CHO-CB1 cells with 30 nM CP 55,940 causes a significant 2-fold enhancement of the basal activity of the enzyme. This effect was maximal at 1 hr and lasted 3 hr. This reflects the transient activation of MAPK by the cannabinoid agonist. In contrast, the treatment of the cells by 10 nM of SR 141716 had no significant effect on the basal activity of the enzyme. Then we assessed the effect of agonist on receptor responsiveness to stimulate MAPK on cells that have been pretreated with CP 55,940 or SR 141716. As reported in table 3, the cannabinoid receptor agonist CP 55,940 is able to increase the MAP kinase activity by about 3-fold in untreated cells with an EC50 value varying from 2.3 to 4.7 nM. Exposure of the cells to CP 55,940 resulted in a marked decrease (P < .05) in CP 55,940 efficiency to stimulate the MAP kinase activity (no stimulation at 10^-7 M). This effect was time-dependent with the addition of the agonist, and maximal 1 hr after the beginning of the experiment and maintained unchanged after up to 72 hr. By contrast incubation of cells with SR 141716 led to sensitization of CB1-coupled MAPK as indicated by an increase in maximal activities. This effect which was maximal 1 hr after the treatment by SR 141716, was maintained up to 72 hr. This result suggested an enhancement of CB1 receptor response by the agonist (table 3). As shown in figure 7 changes in CP 55,940 or SR 141716-modulated MAPK activation in CHO-CB1 cells were concentration dependent, with a maximal effect measured at 30 nM for CP 55,940 (fig. 7B) and 10 nM for SR 141716 (fig. 7C). The activated forms of MAPKs were phosphorylated on both Tyr and Thr residues (Anderson et al., 1990) and were readily detectable on immunoblotting using
TABLE 2

Effects of pretreatment with CP 55,940 or SR 141716 on CP 55,940-induced inhibition of the forskolin-stimulated adenylyl cyclase activity in CHO-CB1 cells

<table>
<thead>
<tr>
<th>Time (Hr)</th>
<th>IC50 (nM) of CP 55,940</th>
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<td>Vehicle-treated cells</td>
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<tr>
<td>0.25</td>
<td>1.37</td>
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<td>0.35</td>
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<tr>
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<td>0.33</td>
</tr>
</tbody>
</table>

Confluent cells (10^5 cells/well) were incubated with vehicle, 30 nM CP 55,940 or 10 nM SR 141716 at 37°C. After the indicated periods of time, cells were washed with washing buffer and incubated with CP 55,940 (10^-11^-10^-8 M) for 5 min, then forskolin was added for 20 min. cAMP levels were measured as described in "Experimental Procedure." Results are from one experiment performed in triplicate. The IC50 values were derived from the concentration-response curves. Six concentrations ranging from 0.1 to 10 nM were used to determine the IC50 values. ND, Not determined.

* IC50 value incalculable (> 1000 nM).

In this report, we studied CB1 receptor regulation induced by the agonist CP 55,940 or the antagonist SR 141716 in stably transfected CHO cells expressing CB1. Direct binding experiments of [3H]-CP 55,940 and [3H]-SR 141716A to CHO cells expressing the human CB1 (CHO-CB1 cells), showed that both ligands bind to one class of specific binding sites (Bmax = 1.5 to 3.5 x 10^6 receptors/cell) in a saturable manner with high affinity (Kd = 5.47 ± 0.28 nM and 4.84 ± 0.66 nM for [3H]-CP 55,940 and [3H]-SR 141716A, respectively). Exposure of the cloned receptor CB1 in CHO cells results in agonist-independent activation that is decreased by the antagonist SR 141716 that has negative intrinsic activity and is referred to as an inverse agonist. These conclusions emerged from observation on two independent-signaling pathways: G<sub>βγ</sub>-mediated MAPK and G<sub>ai</sub>-mediated AC responses. First, we showed that the basal level of MAPK activity was enhanced in CHO cells after transfection of CB1 receptors (Bouaboula et al., 1997) and this increase was reversed by treatment with SR 141716. Second, we showed that SR 141716 prevented the inhibition of AC mediated by autoactivated CB1 receptors. In addition, we observed that the coupling of the CB1 receptors with the adenylyl cyclase through G<sub>ai</sub> is more efficient than the coupling with the MAP kinase signaling pathway depending on G<sub>βγ</sub>.

**Exposure to CP 55,940 induced CB1 desensitization.**

To study receptor localization we used a chimeric protein designed with the CB1 and a peptide derived from C-myc. We verified that the presence of fusion of the N-terminus of the CB1 to C-myc peptide did not alter receptor ligand binding affinity and signal transduction (Rinaldi-Carmona et al., 1996).

Cell surface CB1 density was measured using a monoclonal antibody for the C-myc epitope of C-myc tagged CB1 and immunochemical labeling imaged by flow cytometry as well...
Cells were washed with washing buffer and the effect of CP 55,940 (30 nM SR 141716) for 10 min. Reaction was ended by the addition of CP 55,940 or SR 141716 for 10 min. CP 55,940 a marked decrease in plasma membrane receptor was observed. Agonist-induced drop and redistribution of surface CB1 was distributed to many small micropatches as confocal laser scanning microscopy. In the absence of treatment, CB1 was distributed to many small micropatches in the periphery of the cells. A similar distribution, which has been found to be associated to caveole, has been described for endothelin receptor (Chun et al., 1994). After exposure to CP 55,940 a marked decrease in plasma membrane receptor was observed. Agonist-induced drop and redistribution of surface CB1 from a diffuse pattern to aggregates were revealed. The rate of internalization of CB1 is rapid because a maximal reduction was observed after 30 min. Under these conditions we could not detect any difference in the total cell CB1 density or the affinity constant of the CB1 receptor measured with either [3H]-CP 55,940 or [3H]-SR 141716. From these results we concluded for an agonist-induced receptor internalization in a cellular compartment that can be reached by the cannabinoid hydrophobic ligands. This rapid internalization occurs without any decrease in receptor number, suggesting that receptor degradation does not occur. In addition, we demonstrated a decreased responsiveness of the signaling system to agonist. We showed that in a time-dependent manner, sustained agonist exposure desensitizes both responses of MAPK activation and AC inhibition to agonist stimulation. The agonist-induced desensitization was concentration-dependent with an IC50 = 2.5 nM which is close to the Kd found for agonist binding to CHO-CB1 cells. After a long exposure to CP 55,940 (48 hr) a partial resensitization of CB1 was noted. We do not have any sure interpretation for this resensitization but similar observation was made with bradykinin B2 receptor under continuous exposure to bradykinin, also in murin 3T3 (Blaikut et al., 1996). Also in F442A adipocytes the β3 AR is less prone to the down-regulation that occurs after a long-term exposure to the agonist (Thomas et al., 1992).

Such an agonist-mediated endocytosis that contributes to the desensitization response to agonist stimulation has already been observed for many GPCRs, including adrenergic receptors, muscarinic receptor, peptide receptor and proteinase receptor (Von Zastrow and Krohlik, 1992, Koenig and Edwardson, 1994, Grady et al., 1995 a and b). A prominent feature of these proteins is their rapid loss of responsiveness despite the presence of receptor. Phosphorylation of the receptor by serine/threonine kinases and subsequent binding of members of a family of cytosolic proteins has been shown to be a critical event in uncoupling the receptor and its cognate G protein (Attalamadai et al., 1992, Lohse et al., 1990). Such a phosphorylation of CB1 is very likely to occur because the cDNA sequence predicts several potential phosphorylation sites that fit the consensus sequences of various protein kinases, in its intracellular domains (Palczewski et al., 1989, Benovic et al., 1990, Onorato et al., 1991).

![Fig. 7. Effect of pretreatment with CP 55,940 and SR 141716 on the basal MAPK activity in CHO-CB1 cells. A, Effect on the basal MAPK activity. Growth-arrested cells (105 cells/well) were pretreated with vehicle (○), 0.3 (■), 3 (▲), 10 (●) nM of CP 55,940 or 1 (▲), 3 (●), 10 (●) nM of SR 141716 at 37°C. After 1 hr, cells were washed with washing buffer and the effect of CP 55,940 (3 × 10–10 to 10–7 M) on the MAPK activity was determined in cell lysates as described in “Experimental Procedure.” Data for vehicle and drug conditions are from one of two independent determinations performed in triplicate. B and C, Concentration effects on CP 55,940-modulated MAPK activation in CHO-CB1 cells. Growth-arrested cells (105 cells/well) were pretreated with vehicle (○), 0.3 (■), 1 (▲), 3 (●), 10 (●) nM of CP 55,940 or 1 (▲), 3 (●), 10 (●) nM of SR 141716 at 37°C. After 1 hr, cells were washed with washing buffer and the effect of CP 55,940 (3 × 10–10 to 10–7 M) on the MAPK activity was determined in cell lysates as described in “Experimental Procedure.” Data for vehicle and drug conditions are from one of two independent determinations performed in triplicate. D, Western-blot analysis of phosphorylated MAPK isoforms. Growth-arrested cells (3 × 105 cells/well) were pretreated with vehicle (lanes 1, 2 and 4) or 30 nM (lane 3) of CP 55,940 or 10 nM (lane 5) of SR 141716. After 1 hr, cells were washed with washing buffer (four times) and treated with 10 nM of CP 55,940 (lanes 2, 3 and 5) or 10 nM of SR 141716 (lanes 2 and 4) for 10 min. Reaction was ended by the addition of Leammil’s buffer and protein extracts were processed as described in “Experimental Procedure.”]
to the reduction of spontaneous locomotor activity. However, in contrast to our observations these in vivo studies (Oviedo et al., 1993, Rodriguez De Fonseca et al., 1994, Fan et al., 1996) have reported that chronic treatment with cannabinoid receptor agonists results in a marked loss (50–70%) of the number of cannabinoid receptors, as measured by autoradiographic or binding studies with [3H]-CP 55,940, in different rodent brain structures including the striatum and the cerebellum. It has also been shown that chronic treatment with CP 55,940 lowers cannabinoid receptor mRNA in the caudate-putamen and in the cerebral cortex (Rubino et al., 1994). This phenomenon of receptor down-regulation that is believed to be at least in part the causal effect of tolerance, has been observed in many brain receptor systems including α- and β-adrenergic (Scarpace and Abram, 1982), muscarinic cholinergic (Creese and Sibley, 1981), dopaminergic (Creese and Sibley, 1981), benzodiazepine (Tietz et al., 1986) and opioid receptor (Werling et al., 1989).

Thus in stark contrast with previous in vivo report, in vitro desensitization 1) does not modify the binding sites population of the CB1 receptor and additionally 2) is a very fast process although in vivo tolerance required several days (Fan et al., 1996). This suggests that the rapid internalization process is distinct from the slower process of receptor down-regulation.

**Exposure to SR 141716 induced CB1 sensitization.** Incubation of cells expressing CB1 with SR 141716 did not affect CB1 receptor density and affinity constant measured by binding assays. Despite this similarity with CP 55,940, treatment with SR 141716 induced a completely different scenario. In CHO cells expressing the epitope-tagged CB1, exposure to inverse agonist causes a substantial alteration in the cellular distribution profile of the receptor by up-regulating the density of cell surface receptors. The rate of externalization of CB1 is rapid with a maximal enhancement by about 30 min, as determined by the increase in mean surface receptor fluorescence intensity measured using flow cytometry. Such an increase in receptor density at the cell surface requires either synthesis of new receptors or translocation of intracellular pools of intact receptors. We favored this latter possibility because treatment with SR 141716 had no significant effect on the total population of CB1. We also showed that exposure of cells to SR 141716 resulted in a rapid and pronounced sensitization of the agonist-induced MAPK activity whereas the ability of the agonist-inhibited AC was not affected. The inverse agonist-induced sensitization was concentration-dependent with an EC_{50} = 2 nM. This demonstrated that 1) up-regulation of the G protein-coupled receptor can occur in response to binding with inverse agonist and 2) inverse agonist can enhance subsequent responsiveness to agonist stimulation. The explanation why sensitization was only apparent on MAPK and not on AC may be that maximal effect was already reached in control CHO-CB1 cells in which agonist induced 100% inhibition in cAMP accumulation. Another possibility that remains to be explored could be that inverse agonist pretreatment switches the coupling of the receptor to a different G protein leading to a stronger response through the β2 subunit.

Our results are in agreement with previous observations that showed that incubation of cells expressing a constitutively activated β2-adrenergic receptor with the inverse agonist betaxolol led to both up-regulation and sensitization of the receptor (Mac Ewan and Milligan, 1996). However, such a property may not be common for all inverse agonists. For instance, studies of the 5-hydroxytryptamine 2C receptors have indicated that ligands that have been shown to exert inverse agonist activity such as mianserin caused a down-regulation of the receptor on chronic exposure in vitro (Barker and Sanders-Bush, 1993), whereas the neutral antagonist treatment did not result in the same modulation (Barker et al., 1994, Labrecque et al., 1995).

An interesting question is to understand the mechanism by which SR 141716 enhances the agonist response. Lefkowitz (1993) demonstrated that GPCRs such as β2-adrenergic receptor can be not only constitutively active but also constitutively desensitized. Thus constitutively active receptor recruits known elements of the cellular desensitization machinery. Indeed such a receptor, in the absence of agonist was phosphorylated by G protein-coupled receptor kinase in a way similar to the agonist-occupied receptor. They showed that the rate and extent of the agonist-independent phosphorylation of CAM β2 adrenergic receptor were comparable to the agonist-dependent phosphorylation of the receptor. We have previously and rigorously demonstrated (Bouaboula et al., 1997) and here further confirmed the inverse agonist status of SR 141716. The inverse agonist-induced receptor up-regulation may result from an inhibition of autoactivated receptor and thus their spontaneous desensitization and internalization, and this led to an increase in the number of receptor molecules on the cell surface (Milligan et al., 1995).

We report that a reduction of total cell surface receptor induced by agonist seems to be the predominant mechanism causing functional desensitization of CB1. Conversely the enhancement of membrane receptor number induced by inverse agonist resulted in sensitization. Similar observations were reported with mu opioid receptors (Pak et al., 1996) suggesting a link between the number of membrane receptors regulation and sensitization. Although quantitative as well as qualitative discrepancies in the desensitization of endogenous vs. recombinant receptors may exist (Ozcelebi et al., 1996), the super sensitization of G protein-coupled receptor that occurs in response to binding with inverse agonist might have therapeutic implications and awareness of this possibility would affect the establishment of dosage schedules for an inverse agonist.

As a final comment, it is noteworthy to add that, although the CHO transfected system is a very appropriate tool to specifically analyze CB1 mediated signaling, we are fully aware that such a system exhibits a greatly higher CB1 expression level than primary cells. We therefore intend to perform further studies to transpose this analysis in primary cells naturally expressing CB1.

**References**


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