In the present study, we observed evidence of cross-talk between the cannabinoid receptor CB1 and the orexin 1 receptor (OX1R) using a heterologous system. When the two receptors are co-expressed, we observed a major CB1-dependent enhancement of the orexin A potency to activate the mitogen-activated protein kinase pathway; dose-response curves indicated a 100-fold increase in the potency of orexin-mediated mitogen-activated protein kinase activation. This effect required a functional CB1 receptor as evidenced by the blockade of the orexin response by the specific CB1 antagonist, N-(piperidino-1-yl)-5-(4-chlorophenyl)-1-(2,4-dichlorophenyl)-4-methyl-pyrazole-3-carboxamide (SR141716), but also by pertussis toxin, suggesting that this potentiation is Gi-mediated. In contrast to OX1R, the potency of direct activation of CB1 was not affected by co-expression with OX1R. In addition, electron microscopy experiments revealed that CB1 and OX1R are closely apposed at the plasma membrane level; they are close enough to form hetero-oligomers. Altogether, for the first time our data provide evidence that CB1 is able to potentiate an orexigenic receptor. Considering the anti-obesity effect of SR141716, these results open new avenues to understand the mechanism by which the molecule may prevent weight gain through functional interaction between CB1 and other receptors involved in the control of appetite.

The main active principle of marijuana, Δ9-tetrahydrocannabinol, and endogenous or synthetic cannabinoids exert most of their biological activities through two well characterized receptors named CB1 and CB2. The CB1 receptor is essentially expressed in the brain, whereas CB2 is mainly associated with the immune system (1–5). These receptors belong to the G protein-coupled receptor (GPCR) family and mediate their biological effects via a pertussis toxin (PTX)-sensitive GTP-binding regulatory protein G i. The stimulation of these receptors by an agonist leads to the inhibition of the adenylyl cyclase (6) and to the activation of different members of the mitogen-activated protein kinases (MAPKs) such as the extracellular signal-regulated kinases (p44/p42 ERKs) (7, 8), c-Jun N-terminal kinase and p38K (9), and the immediate-early gene krox24 (7, 8).

The functional effects associated with CB1 receptor activation have been well characterized; they include anti-nociception, decreased spontaneous activity, hypothermia, and impairment of short term memory (10–12). Recently, the endogenous cannabinoid system has emerged as a potential regulator of appetite. Both the endocannabinoids and Δ9-tetrahydrocannabinol have been shown to stimulate food intake in animal models and in humans (13–16). These appetite-stimulating effects are mediated by the CB1 cannabinoid receptor because they are blocked by SR141716, a potent and selective CB1 receptor antagonist (17–25). In addition, genetically CB1 receptor-impaired mice eat less than wild-type mice in response to food deprivation (26). These data provide strong evidence for the involvement of central CB1 receptors as a physiological regulator of eating and suggest that the use of SR141716 could represent a new approach for the treatment of obesity and appetite disorders in human. However, the mechanisms by which SR14176 reduces weight gain remain to be clarified.

Within the brain, CB1 receptor expression is low in the hypothalamus, but higher levels are found in the lateral hypothalamic area (27). CB1 coupling to G proteins was shown to be remarkably more efficient in the hypothalamus than in other CB1 receptor-rich areas (28). Such particularities may be of interest considering that the hypothalamus plays a key role in the regulation of food intake, body mass, and energy balance (29, 30). Specifically, the lateral hypothalamic area is involved in the initiation of food intake, whereas the basomedial hypothalamic nuclei are associated with the cessation of consumption (31). Numerous hypothalamic hormones involved in feeding behaviors have been discovered in the past 10 years; they include appetite-stimulating (orexigenic) neuropeptides, such as neuropeptide Y (NPY), orexins A/B, and melanin-concentrating hormone, or galanin and anorexigenic peptides, such as α-melanocyte-stimulating hormone (32). Those peptides act through GPCRs and participate in neural circuits that operate to control feeding behavior. Further supporting the notion that CB1 may be considered as an orexigenic receptor and belongs to the same neural circuitry regulated by orexigenic mediators, it has been shown that hypothalamic endocannabinoids levels are negatively regulated by leptin and that they maintain food intake through the tonic activation of CB1 receptors in that area (26).

With this in mind, we hypothesized that SR141716 may mediate its effect on weight gain either by directly blocking the...
endogenous tone of anandamide and/or through specific cross-talks between CB1 and other receptors. This study is aimed at documenting the latter hypothesis, which has been supported by numerous observations. First, interactions of the CB1 system with different GPCR systems have been recently suggested. For example, Meschler and Howlett (33) reported signal transduction interactions between CB1 and dopamine receptors in the rat and monkey striatum. An interaction between the cannabinoid and serotonin systems has also been suggested by the modulation of the cannabinoid agonist binding by serotonin in the rat cerebellum (34). Considering food intake control, it was shown that tetrahydrocannabinol-induced hyperphagia was reversed by low doses of naloxone and that the combination of SR141716 and naloxone inhibited food intake elicited by orphenadrine FQ (35). Second, SR141716 has recently been shown to interfere by its inverse agonist properties within receptor cross-talk and inhibit the MAPK response mediated by insulin and insulin-like growth factor 1 receptors in CHO-CB1 cells, whereas the activation of MAPK by fibroblast growth factor b was not altered (19). In this study, we addressed potential cross-talk of CB1 with orexigenic receptors. Specifically, we tested whether functional interactions between CB1 and the orexin system occur, and we analyzed the pertinence of this hypothesis using SR141716. Orexins (orexin A and orexin B) are orexigenic mediators selectively expressed in the hypothalamus, within the lateral hypothalamic area neurons (36). Orexin A is implicated in food intake in satiated rats but also in the regulation of drinking behavior (37), whereas the role of orexin B is not as clear. These neuropeptides bind and activate two closely related GPCRs: orexin 1 receptor, specific for orexin A, and orexin 2 receptor, which is a high affinity receptor for both orexins (36). Taking into account the role of endogenous cannabinoids and orexin A in obesity and given the recently described expression pattern of OX1R, which indicates that CB1 and OX1R receptors are expressed in similar brain regions, specifically in the lateral hypothalamic area (38), we explored the possibility of intracellular interactions between CB1 and OX1R. To this aim, we produced a heterologous system where these two receptors are co-expressed. Our results demonstrate a dramatic increase in the orexin response, which is prevented by SR141716. When tested in similar conditions, CB1 and NPYR5 do not interact. These data open up new insights and lead to the hypothesis that obesity effects observed with SR141716 could be related at least in part to the blockade of CB1/OX1R cross-talk.

**EXPERIMENTAL PROCEDURES**

**Materials—**[γ-32P]ATP (2500 Ci/mmole) was purchased from American Biosciences. PTX was obtained from Sigma, N-(Piperidino-1-yl)-5-(4-chlorophenyl)-1-(2,4-dichlorophenyl)-4-methyl-pyrazole-3-carboxamide (SR141716A) was synthesized at the Chemistry Department of Sanofi-Synthelabo (Montpellier, France). CP-55,940 was from Toecis (Bristol, UK). Human orexin A was from Bachem AG (Budendorf, Switzerland). Mouse anti-c-Myc antibodies (9E10 clone) were produced by our core facilities as ascites fluids. Rabbit polyclonal anti-p44 (C-16, anti-ERK1) and anti-p42 (C-14, anti-ERK2) antibodies were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Rabbit polyclonal anti-CB1 (N-terminal) antibody was purchased from PharMin (San Diego, CA). Mouse anti-phosho-p44/p42 MAPKs (Thr202/Tyr204) E10 monoclonal antibodies were from Cell Signaling Technology (Beverly, MA). Horseradish peroxidase-conjugated secondary antibodies were obtained from Amersham Biosciences. Protease inhibitor mixture (complete EDTA-free) was from Roche Applied Science. All other reagents were of analytical grade and were obtained from various commercial suppliers.

**Stable Cell Lines and Culture Conditions—**CHO cells stably expressing Myc-CB1R were from Sanofi-Synthelabo (Labège, France). Stable CHO cells expressing CB1 were described previously (39). For stable co-expression of Myc-OX1R and CB1 receptor, a CHO dihydrofolate reductase negative cell line was co-transfected with plasmid p2169-

CB1 and OX1R Cross-talk

MCV-1/OX1R and μDNA3-CB1 receptor using LipofectAMINE (Invitrogen) according to the manufacturer’s procedure. Selection was imposed 12 days in modified Eagle’s medium (Invitrogen) without deoxyribo

nucleosides or ribonucleosides supplemented with 5% of dialyzed fetal calf serum (FCS) and 300 μg/ml of G418. Individual cell lines were cloned by limiting dilution, and CB1 and OX1R expression was assessed by fluorescence-activated cell sorting analysis. CHO cells were maintained at 37 °C in a humidified atmosphere containing 5% CO2. CHO-CB1, CHO-Myc-OX1R, and CHO-CB1/Myc-OX1R cell lines were routinely grown in modified Eagle’s medium (Invitrogen) supplemented with 5% dialyzed FCS, 40 μg/ml l-typsin, 1 mM sodium pyruvate, 60 μg/ml tylosin, and 20 μg/ml gentamycin.

**Immunofluorescence Microscopy—**Cells stably expressing either CB1 or OX1R or both were grown on glass coverslips pretreated with a 0.1 mg/ml solution of poly-c-lysine. Washed cells were incubated with either 9E10 mouse monoclonal antibody (anti-c-Myc) or with anti-CB1 rabbit polyclonal antibody or both for 1 h at 4 °C. The cells were washed with cold PBS and incubated with 2000 dilution of Alexa 488-conjugated anti-mouse IgG (Molecular Probes) and/or Cy5-conjugated anti-rabbit IgG (Jackson) for 30 min at 4 °C. After washing with cold PBS, the cells were fixed with paraformaldehyde 1% in PBS and then directly observed. Fluorescence analyses were performed using a confocal microscope (LSM410, Zeiss, Oberkochen, Germany).

**Detection of the Phosphorylated MAPK—**The cells were grown to 80% confluency in 24-well plates then cultured in medium without FCS for 20 h before the assay. After treatment, the cells were washed in cold PBS and directly lysed in 5% of 2× Laemmli buffer containing 120 mM Tris-HCL, pH 6.8, 4% SDS, 20% glycerol, and bromophenol blue. 20 μl of sample were run on 12% polyacrylamide gel and transferred to nitrocellulose. Nonspecific antibody binding was prevented by incubation in Tris-buffered saline (10 mM Tris-HCL, pH 7.6, 150 mM NaCl) containing 0.05% Tween 20 and 5% dried milk. The membranes were incubated with mouse anti-p44/p42 MAPK monoclonal antibodies (1:2000) and then with peroxidase-conjugated goat anti-mouse antibodies (1:10,000). An enhanced chemiluminescence system was used for Western blot development (PerkinElmer Life Sciences). Standardization was achieved using ERK1 and ERK2 measured in the same blots with anti-ERK1 and anti-ERK2 antibodies (1:5000) followed by peroxidase-conjugated goat anti-rabbit antibodies (1:10,000).

**MAPK Assay—**MAPK activity was measured as described previously (7). Briefly, the cells were grown to 80% confluency in 24-well plates then cultured in medium without FCS for 20 h before assay. After treatments, the cells were washed in cold PBS and lysed for 20 min at 4 °C under shaking in 100 μl of lysis buffer (50 mM HEPES, pH 7.4, 150 mM NaCl, 1% v/v Triton X-100) containing 20 mM Na2VO4, 2 mM dithiothreitol, and a protease inhibitor mixture. 17 μl of solubilized cell extracts were analyzed for MAPK activity. The phosphorylation of MAPK-specific peptide substrate was carried out at 30 °C for 30 min with [γ-32P]ATP by using the Biotrack p42/p44 MAPK enzyme system (Amersham Biosciences) according to the manufacturer’s procedure.

**Inositol Phosphate Assay—**The cells were grown for 24 h in 96-well plates, and then the growth medium was replaced by medium supplemented with [3H]myo-inositol (5 μCi/ml) (PerkinElmer Life Sciences). After 20 h, the growth medium was removed, and the cells were washed once with medium without FCS supplemented with 20 μM LiCl for 15 min before treatment with various concentrations of orexin A for 90 min at 37 °C. The reaction was stopped by aspiration of the medium, and the cells were lysed by the addition of 5% trichloroacetic acid for 1 h at 4 °C. The cell lysates were added to 200 μl of Dowex, washed with 60 mM sodium formate, 5 mM sodium tetraborate, and the inositol phosphates eluted using 0.2 mM ammonium formate and 0.1 M formic acid.

**Immunogold Electron Microscopy—**The cells were washed and fixed for 30 min with 1% formaldehyde in PBS and then washed again. Rabbit anti-CB1 antibody and mouse anti-c-Myc antibody were added for 1 h in PBS containing 1% bovine serum albumin. Cell surface-associated antibodies were detected with goat anti-mouse IgG conjugated with 6-nm gold beads and goat anti-rabbit IgG conjugated with 10-nm gold beads. After two washes, the cells were fixed for 1 h with 4% formaldehyde in PBS buffer and then postfixed for 1 h with 1% Oso4. Finally, the cells were dehydrated with ethanol, propylene, and epoxypropylene. Ultrafine sections were contrasted with uranyl acetate. The micrographs were recorded with a JEOL 1010 electron microscope.
CB1 and OX1R Cross-talk

RESULTS

CB1 and OX1R Co-expression Leads to the Sensitization of Orexin A-mediated MAPK Activation

To investigate CB1 and OX1R functional interactions, we generated CHO cells stably expressing the CB1 cannabinoid receptor (CHO-CB1), the orexin 1 receptor (CHO-OX1R), or both (CHO-CB1/OX1R). The OX1R was tagged at the N terminus with the c-Myc epitope to monitor its expression using mouse monoclonal anti-Myc antibodies, whereas specific rabbit polyclonal anti-CB1 antibodies directed against the N-terminal domain of the human receptor were used to detect CB1. The expression of CB1 and OX1R receptors was examined using immunofluorescence confocal microscopy. As shown in Fig. 1A, when expressed alone, CB1 was found at the cell surface as a punctate immunostaining. In CHO-OX1R cells, c-Myc-tagged OX1R was also expressed at the plasma membrane, but the staining appeared more homogeneous (Fig. 1B). In the double expression cell line, CB1 and OX1R were mostly co-localized at the cell surface (Fig. 1C). The specificity of this labeling was verified using CHO-wt cells (data not shown).

To characterize the impact of CB1 and OX1R co-expression on both receptor signaling, we examined the MAPK activation. The time course of MAPK activation induced by CP55,940 in CHO-CB1 or CHO-CB1/OX1R was transient with a maximal activation between 5 and 10 min and returned to a basal level by 30–60 min (Fig. 2A). Comparable kinetics were observed with 50 nM orexin A in both CHO-OX1R and CHO-CB1/OX1R cells (Fig. 2B), except that orexin A-induced MAPK activation remained elevated in CHO-CB1/OX1R compared with CHO-OX1R cells at 60 min (46.0 ± 2.8% versus 23.7 ± 1.7% at 60 min). As a control, CP55,940 did not induce MAPK activation in CHO-OX1R cells, and conversely orexin A did not stimulate CB1 receptors in CHO-CB1 cells (Fig. 2).

We further evaluated the potency of these agonists in dose-response curves. In cells expressing CB1 alone or in combination with OX1R, the EC50 values of CP-55,940-mediated MAPK activation were comparable (3 nM) (Fig. 3A). These data were confirmed in Western blot experiments using an anti-phospho-p42/p44 MAPK antibody (Fig. 3B). In CHO-OX1R cells, orexin A induced MAPK activation with a EC50 of 0.9 nM (Fig. 3C), a result that is consistent with the previously published ones (36). In sharp contrast, co-expression of CB1 and OX1R resulted in a dramatic increase of the potency of orexin-mediated MAPK activation by 100-fold (from EC50 = 9 nM to EC50 = 0.1 nM). The hypersensitization of orexin-induced MAPK phosphorylation was confirmed in Western blot experiments (Fig. 3D). A similar shift of EC50 value was reproducibly observed in the three other independent CHO-CB1/OX1R clones tested (data not shown). When tested at their maximal activating doses, the combination of orexin A with CP-55,940 did not show additivity, indicating that the MAPK responses we measured represented a common responding element within the cell where the two receptor signaling converge (data not shown). Altogether these data indicated that when CB1 and OX1R receptors were co-expressed, a major enhancement in orexin A potency to activate MAPK pathway was observed, whereas the efficacy of CP-55,940 to induce the MAPK signaling remained unaffected.

CB1 Receptor Does Not Sensitize NPY5R-mediated MAPK Activation

To examine whether the synergistic response observed between CB1 and OX1R could be extended to other GPCRs, we selected NPY5R, which is known to play a key role in the regulation of appetite (40) and studied the effect of CB1 and NPY5R co-expression using CHO cells overexpressing NPY5R alone or in combination with CB1. A treatment with increasing

Fig. 1. Cell surface expression of Myc-OX1R and CB1. The cell surface expression of CB1 and Myc-OX1R receptors on CHO-CB1, CHO-OX1R, and CHO-CB1/OX1R was assessed by confocal immunofluorescence microscopy. Intact cells were incubated with mouse anti-Myc and with rabbit anti-CB1 antibodies then with anti-mouse Alexa 488- and anti-rabbit Cy5-conjugated secondary antibodies. Three different views of CHO-CB1 (A) or CHO-OX1R (B) and two different views of CHO-CB1/OX1R (C) are presented. C, left panels, CB1, red; middle panels, Myc, green; right panels, merge, yellow. The figures shown are representative of three independent experiments.

Fig. 2. Time course of MAPK activation by CP55,940 or by orexin A. Serum-deprived CHO-CB1, CHO-OX1R, and CHO-CB1/OX1R were treated with CP55,940 (50 nM) (A) or were stimulated with orexin A (50 nM) (B) for up to 60 min. After drug incubation, the activities of p42/p44 MAPK were determined by using the Biotrack p42/p44 MAPK enzyme system. The data are the mean values ± S.D. of duplicate samples, and the experiments were repeated three times.
concentrations of NPY resulted in a similar dose-dependent response in MAPK activity in both CHO-NPY5R or CHO-CB1/NPY5R and CHO-CB1/OX1R cells (EC50 5 nM) (Fig. 4A). Similarly, CP55,940-induced MAPK activation was not different in CHO-CB1/NPY5R when compared with CHO-CB1 (data not shown). Moreover, a treatment with PTX abolished both CP55,940 and NPY responses regardless of the cell line tested (Fig. 4B), consistent with the Gi/Go coupling of CB1 and NPY5R receptors. Thus co-expression of NPY5R with CB1 in CHO cells did not result in a synergistic MAPK response.

Need for a Functional CB1 Receptor to Sensitize Orexin A-mediated MAPK Activation

Inhibition of Orexin A-mediated MAPK Activation by SR141716—To assess the contribution of each receptor in the hypersensitization of OX1R responses, we examined the effect of the specific CB1 antagonist, SR141716, on CB1-mediated orexin A sensitization. As expected, a treatment of CHO-CB1 or CHO-CB1/OX1R cells with SR141716 completely abrogated the MAPK signal induced by CP55,940 (Fig. 5, A and B), whereas SR141716 did not affect orexin A-mediated MAPK activation in CHO-OX1R (Fig. 5C). By contrast, the molecule completely prevented the shift of EC50 that we previously observed in CHO-CB1/OX1R (Fig. 5D). With SR141716 restoring the initial orexin response, these data suggested that an active CB1 receptor form was required to enhance the MAPK signal induced by orexin.

Effect of PTX—Contrasting with OX1R, CB1 is PTX-sensitive. Therefore we assessed the effect of a PTX treatment (50 ng/ml) on the orexin A-induced responses in the different cell lines included in our study. As observed with SR141716, a PTX pretreatment completely abrogated the EC50 shift of orexin-mediated MAPK response in CHO-CB1/OX1R cells (Fig. 6C) and restored the orexin A response identical to that observed for OX1R alone. As a control, a similar PTX pretreatment fully inhibited the CP55,940-mediated response in CHO-CB1 cells and in CHO-CB1/OX1R cells (Fig. 6D) and was inactive in CHO-OX1R cells (Fig. 6D). These data strengthened the implication of an active CB1 receptor in the enhancement of MAPK activation induced by orexin A in CHO-CB1/OX1R cells.

CB1 Receptor Does Not Alter the Gq-dependent Signaling of OX1R

To further analyze the effect of CB1 and OX1R co-expression on the OX1R effector systems, we compared the production of inositol phosphate stimulated by orexin A in CHO-OX1R and in CHO-CB1/OX1R cells (Fig. 7). The dose-dependent potency of orexin A to stimulate inositol phosphate production (EC50 = 5

**FIG. 3.** Dose-response of MAPK activation by CP55,940 or by orexin A. Serum-deprived CHO-CB1 or CHO-OX1R and CHO-CB1/OX1R cells were treated with increasing concentrations (5 pM to 100 nM) of CP55,940 (A and B) or orexin A (C and D) for 7 min. The activities of p42/p44 MAPK were measured in cell lysates by using the Biotrack p42/p44 MAPK enzyme system (A and C) or phospho-MAPK (Thr202/Tyr204) level (pMAPK) was measured by Western blotting with anti-phospho-MAPK antibody (B and D). Standardization was with ERK1/2 kinase (MAPK) measured in the same blots, with the use of anti-ERK1/2 antibodies. The data are the mean values ± S.D. of duplicate samples, and the experiments were repeated three times. Western blot experiments are representative of three independent experiments.

**FIG. 4.** Dose-response of MAPK activation by NPY. Serum-deprived CHO-NPY5R and CHO-CB1/NPY5R cells were treated with increasing concentrations (10 pM to 50 nM) of NPY for 7 min (A) or pretreated with PTX (50 ng/ml) then with CP55,940 (50 nM) (B). After drug incubation, phospho-MAPK (Thr202/Tyr204) level (pMAPK) was measured in cell lysates by Western blotting with anti-phospho-MAPK antibody. Standardization was with ERK1/2 kinase (MAPK) measured in the same blots, with the use of anti-ERK1/2 antibodies. The figure shown is representative of three independent experiments.
with SR141716 (500 nM) for 20 min and then stimulated with increasing CP55,940 (100 n M) did not stimulate inositol phosphate concentrations of CP55,940 (preparation A and B) or orexin A (C and D) for 7 min. The activities of p42/p44 MAPK were measured in cell lysates by using the Biotrack p42/p44 MAPK enzyme system. The data are the mean values ± S.D. of duplicate samples, and the experiments were repeated three times.

**OXIR and CB1 Receptors Are Closely Localized in CHO Cells**

The above findings suggested a close interaction of CB1 and OX1R. Therefore, we examined whether these two receptors are closely associated and searched for receptor complexes; we favored microscopic techniques because they do not require cell disruption and avoid biochemical artifacts. As shown by double-label immunofluorescence (Fig. 1), CB1 and OX1R receptors were co-localized at the cell surface of CHO-CB1/OX1R cells. However, the resolution provided by light microscopy limits the conclusions that can be drawn at the molecular level. Thus cell surface expression of CB1 and OX1R receptors was more precisely assessed by electronic microscopy techniques (Fig. 8) using anti-c-Myc and anti-CB1 antibodies revealed with 6- and 10-nm immunogold anti-IgG for the detection of OX1R and CB1 receptors, respectively. Bead sizes were in the similar range to guarantee homogenous labeling property of the antibodies and limit physical hampering but are different enough to easily distinguish both receptors. In addition the immuno-electronic microscopy study was performed using prefixed cells to ensure that the receptor distribution was not altered by antibody incubation. The fixation does not alter the antibody labeling specificity (data not shown). Double-label experiments were carried out in CHO-CB1, CHO-OX1R, and CHO-CB1/OX1R cells. The high magnification images we obtained (×125,000) revealed that CB1 receptors (10-nm gold particles) were expressed at the cell surface of CHO-CB1 as microclusters (Fig. 8A). OX1R receptors (6-nm gold particles) were also detected in microdomains on the plasma membrane in CHO-OX1R cells (Fig. 8B). Double labeling of CHO-CB1/OX1R cells showed that some CB1 and OX1R receptors are observed independently in CHO-CB1/OX1R, indicating that these receptors could be expressed as homo-oligomers on the plasma membrane (data not shown). Interestingly, we observed that many particles corre-
receptor, and the close localization of OX1R with an auto-activated CB1 would enhance OX1R-dependent responses. Indeed, in our system, CB1 is auto-activated as demonstrated by the basal MAPK activation in both CHO-CB1 and CHO-CB1/OX1R, which is blocked by SR141716 (7, 8). This property is in agreement with in vitro and in vivo studies indicating that SR141716 behaved as an inverse agonist on CB1 receptors (7, 8, 50–52).

The biological significance of CB1/OX1R cross-talk remains to be explored. Our observations were made in a heterologous system, and we do not know yet whether they are also valid in vivo, either in normal physiology or in pathological processes. Such a functional interaction requires 1) that the two receptors are co-expressed in identical cell populations, 2) that the two receptors form hetero-oligomers, and 3) that CB1 is in an activated form. First, some brain regions are known to express both receptors, but further studies need to verify whether the two receptors are expressed on the same target neurons. The latter two issues could be considered in the context of obesity. Indeed, obesity is now recognized as a pathological state. GPCR hetero-oligomerizations have been observed in normal conditions and have also been described in human pathologies (53). On the other hand autoactivated receptors have also been associated with different pathological conditions (54). Considering CB1, the receptor may be in an active form in vivo as a consequence of its activation by endogenous cannabinoids, whose expression levels are increased in obesity. But CB1 receptors have also been described to exist spontaneously “pre-coupled” to G proteins in the cell membrane (55). This may account for an activated form of CB1 in vivo. Thus one can assume that the heterodimerization of the CB1/OX1R receptors combined with an activated CB1 may lead to a hypersensitization to the orexin stimulus and consequently be a specific event responsible for a hyperphagic behavior.

Such a scenario would explain the very potent anti-hyperphagic property of SR141716, which blocked, on the one hand, the orexigenic pathway mediated by endogenous cannabinoids and, on the other hand, the CB1-mediated hypersensitization of orexin A responses. This is highly consistent with 1) the quasi-minimal effect of the molecule on food intake in nonobese animals and 2) the fact that CB1 knockout mice are

---

**Fig. 6.** Effect of PTX on CP55,940-and orexin A-mediated MAPK activation in CHO-CB1, CHO-OX1R, and CHO-CB1/OX1R. Serum-deprived CHO-CB1 and CHO-CB1/OX1R or CHO-OX1R and CHO-CB1/OX1R were pretreated (open circles) or not (filled circles) with PTX (50 ng/ml) for 20 h and then stimulated with increasing concentrations of CP55, 940 (A and B) or orexin A (C and D) for 7 min. The activities of p42/p44 MAPK were measured in cell lysates by using the Biotrack p42/p44 MAPK enzyme system. The data are the mean values ± S.D. of duplicate samples, and the experiments were repeated three times.

**Fig. 7.** Dose response of inositol phosphate production by orexin A. CHO-OX1R (filled circles) and CHO-CB1/OX1R (open circles) cells labeled with [3H]myo-inositol were treated with increasing concentrations (0.1 nM to 100 nM) of orexin A for 90 min. After drug incubation, the ligand-stimulated increase of the inositol phosphate levels was determined as described under “Experimental Procedures.” The data are the mean values ± S.D. of duplicate samples, and the experiments were repeated three times.

**Fig. 8.** Immuno-electronic microscopy evidenced heterogeneous clusters of CB1 and OX1R receptors. Cell surface expression of CB1 and OX1R on CHO-CB1 (A), CHO-Myc-OX1R (B), and CHO-CB1/Myc-OX1R (C and D) cells was assessed by immuno-electronic microscopy. The cells were fixed, embedded, and double-labeled using mouse anti-Myc and rabbit anti-CB1 antibodies. Primary antibodies bound were detected using anti-mouse 6-nm colloidal gold conjugate and anti-rabbit antibodies 10-nm colloidal gold conjugate. The arrowheads indicate CB1 receptors closely apposed to OX1R at the cell surface membrane. Bar, 100 nm.
not obese even when subjected to a high fat diet (56). Altogether, this may indicate that targeting the cannabinoid system is highly relevant in the context of obesity because it may impact a pathway that is activated only in pathological conditions.

In conclusion, for the first time, we provide biochemical, pharmacological, and functional evidence for cross-talk between CB1 and OX1R, a receptor implicated in the regulation of feeding behavior. Taken together, our data obtained in an overexpression system support the hypothesis that heterodimerization may be a mechanism for the regulation of CB1 and OX1R receptor functions. The present study suggests that SR141716 may be a potent modulator of the orexin A system; however, future work will have to verify whether SR141716 could block orexin A response in vivo and to determine whether SR141716 is still active in OX1R knockout mice fed a high fat regimen. In addition, it should be investigated whether other systems controlling the feeding behavior are affected through cross-talk with CB1.

Acknowledgments—We are grateful to Géraldine Périard for help in fluorescence-activated cell sorting and confocal microscopy experiments. We also thank Régine Foulard for excellent technical support.

REFERENCES

1. Devane, W. A., Hanus, L., Breuer, A., Pertwee, R. G., Stevenson, L. A., Griffin, G., Gibson, D., Mandelbaum, A., Etinger, A., and Mechoulam, R. (1992) Science 258, 1946–1949
2. Herkenham, M., Lynn, A. B., Johnson, M. R., Melvin, L. S., de Costa, B. R., and Ruiz, R. C. (1991) J. Neurosci. 11, 563–573
3. Matsuda, L. A., Bonner, T. J., and Lollai, S. J. (1993) J. Comp. Neurol. 327, 535–550
4. Munro, S., Thomas, K. L., and Abu-Shaar, M. (1993) Nature 365, 61–65
5. Galiegue, S., Mary, S., Marchand, J., Dussossoy, D., Carrière, D., Carayon, P., Bouaboula, M., Shire, D., Le Fur, G., and Casellas, P. (1995) Eur. J. Biochem. 232, 54–61
6. Howlett, A. C., and Fleming, R. M. (1984) Mol. Pharmacol. 26, 532–538
7. Bouaboula, M., Pizot-Chazel, C., Bourréi, B., Canat, X., Calandra, B., Rinaldi-Carmona, M., Le Fur, G., and Casellas, P. (1995) Biochem. J. 312, 637–641
8. Bouaboula, M., Bourréi, B., Rinaldi-Carmona, M., Shire, D., Le Fur, G., and Casellas, P. (1995) J. Biol. Chem. 270, 13973–13980
9. Rueda, D., Guille-Roperbe, I., Haro, A., and Guzmán, M. (2000) Mol. Pharmacol. 58, 814–820
10. Hollister, L. E. (1986) PharmacoL Rev. 38, 1–20
11. Dewey, W. L. (1986) PharmacoL Rev. 38, 151–178
12. Howlett, A. C., Barth, F., Bencherif, T. I., Cabral, G., Casellas, P., Devane, W. A., Felder, C. C., Herkenham, M., Mackie, K., Martin, B. R., Mechoulam, R., and Pertwee, R. G. (2003) Pharmacol. Rev. 54, 161–202
13. Williams, C. M., Rogers, P. J., and Kirkham, T. C. (1998) J. Mol. Med. 76, 157–159
14. Williams, C. M., and Kirkham, T. C. (1998) Trends Pharmacol. Sci. 19, 247–254
15. Meschler, J. P., and Howlett, A. C. (2001) Neuropharmacol. 40, 918–926
16. Devlin, M. G., and Christopoulos, A. (2002) J. Neurochem. 82, 1095–1102
17. Pietras, T. A., and Howland, N. E. (2002) Eur. J. Pharmacol. 442, 237–238
18. Bouaboula, M., Ishii, M., Matsuoki, I., Chemelli, R. M., Tamakka, H., Williams, S. C., Richardson, J. A., Kozlovski, G. P., Wilsen, S., Arch, J. R. S., Buckingham, R. E., Haynes, A. C., Carr, S. A., Annan, R. R., McNulty, D. E., Liu, W. S., Tettar, J. A., Elsebouragey, N., Bergoma, D. J., and Yanagisawa, M. (1998) Cell 92, 573–585
19. de Lecea, L., Kilduff, T. S., Peyron, C., Gao, X.-B., Foye, P. E., Danielson, P. E., Fukuhara, C., Battenberg, E. L. F., Gauthier, V. T., Bartlett, F. S., Frankel, W. N., van den Pol, A. N., Bloom, F. E., Gautvik, K. M., and Sutcliffe, J. G. (1998) Proc. Natl. Acad. Sci. U. S. A. 95, 322–327
20. Hervieu, G. J., Cluverud, J. E., Harrison, D. C., Roberts, J. C., and Leslie, R. A. (1993) Nature 365, 380–383
21. Rinaldi-Carmona, M., Le Duigou, A., Oustrie, D., Barth, F., Bouaboula, M., Carayon, P., Casellas, P., and Le Fur, G. (1998) J. Pharmacol. Exp. Ther. 287, 1038–1047
22. Cabrera, C., Langer, M., Bader, W., Wieland, H. A., Doods, H. N., Berze, O., and Beck-Siegen, A. G. (2000) J. Biol. Chem. 275, 36404–36408
23. Bouvier, M. (2001) Nat. Rev. Neurosci. 2, 274–286
24. Jordan, B. A., and Devi, L. A. (1999) Nature 399, 697–700
25. White, J. H., Wise, A., Main, M. J., Green, A., Fraser, N. J., Disney, G. H., Barnes, A. A., Emson, P., Fair, S., and Marshall, P. H. (1996) Nature 380, 679–682
26. Maggio, R., Barbier, P., Colletti, A., Salvadori, F., Demontis, G., and Corsini, G. (1999) J. Pharmacol. Exp. Ther. 291, 251–257
27. Rocheville, M., Lange, D. C., Kumar, U., Patel, S. C., Patel, R. C., and Patel, Y. C. (2000) Science 288, 154–157
28. Génesis S., Hillion, J., Törvén, M., Le Crem, S., Cassadó, V., Canela, E. I., Rondón, S., Lew, J. Y., Watson, S. Z., Zoli, M., Agnati, L. F., Vernier, P., Lluis, C., Fèvre, S., Fuxe, K., and Franço, R. (2000) Proc. Natl. Acad. Sci. U. S. A. 97, 8860–8861
29. Abdalla, S., Loher, H., and Quitterer, U. (2000) J. Neurosci. 20, 7617–7622
30. MacEwen, D. J., Kim, G. D., and Miller, G. (1995) Mol. Pharmacol. 48, 316–325
31. Sim-Selley, L. J., Brunk, L. K., and Sellely, D. E. (2001) Eur. J. Pharmacol. 414, 135–141
32. de Ligt, R. A. F., Kounnas, A. P., and Eijman, A. P. (2000) Br. J. Pharmacol. 130, 1–12
33. Meschler, J. P., Kräuchi, D. M., Wilken, G. H., and Howlett A. C. (2000) Biochem. Pharmacol. 69, 1515–1523
34. Abdalla, S., Loher, H., Massier, A., and Quitterer, U. (2001) Nat Med. 7, 1003–1009
35. Parma, J., Duprez, L., Van Sande, J., Cochoax, P., Gervy, C., Mocell, K., Dumont, J., and Vassart, G. (1993) Nature 365, 649–651
36. Mühlenpfordt, S., McIntosh, H. H., Houston, D. B., and Howlett, A. C. (2000) Mol. Pharmacol. 57, 163–170
37. Raven-Tillou, C., Aronne, M., Delgorce, C., Gonadal, N., Keane, P., Maffrand, J. P., and Soubré, P. (2003) Am. J. Physiol. 284, R345–R353