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Dual intracellular signaling pathways mediated by the human cannabinoid CB_1 receptor

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Abstract

It has long been established that the cannabinoid CB_1 receptor transduces signals through a pertussis toxin-sensitive G_i/G_o inhibitory pathway. Although there have been reports that the cannabinoid CB_1 receptor can also mediate an increase in cyclic AMP levels, in most cases the presence of an adenylyl cyclase costimulant or the use of very high amounts of agonist was necessary. Here, we present evidence for dual coupling of the cannabinoid CB_1 receptor to the classical pathway and to a pertussis toxin-insensitive adenylyl cyclase stimulatory pathway initiated with low quantities of agonist in the absence of any costimulant. Treatment of Chinese hamster ovary (CHO) cells expressing the cannabinoid CB_1 receptor with the cannabinoid CP 55,940, {(−)-*cis*-3-[2-hydroxy-4-(1,1-dimethylheptyl)phenyl]-*trans*-4-(3-hydroxypropyl) cyclohexan-1-ol} resulted in cyclic AMP accumulation in a dose–response manner, an accumulation blocked by the cannabinoid CB_1 receptor-specific antagonist SR 141716A, {*N*-(piperidin-1-yl)-5-(4-chlorophenyl)-1-(2,4-dichlorophenyl)-4-methyl-1*H*-pyrazole-3-carboxamide hydrochloride}. In CHO cells coexpressing the cannabinoid CB_1 receptor and a cyclic AMP response element (CRE)-luciferase reporter gene system, CP 55,940 induced luciferase expression by a pathway blocked by the protein kinase A inhibitor *N*-[2-(*p*-bromocinnamylamino)ethyl]-5-isoquinolinesulfonamide hydrochloride (H-89). Under the same conditions the peripheral cannabinoid CB_2 receptor proved to be incapable of inducing cAMP accumulation or luciferase activity. This incapacity allowed us to study the luciferase activation mediated by CB_1/CB_2 chimeric constructs, from which we determined that the first and second internal loop regions of the cannabinoid CB_1 receptor were involved in transducing the pathway leading to luciferase gene expression.

Keywords: Cannabinoid; Cannabinoid CB_1 receptor; Cannabinoid CB_2 receptor; Chimeric receptor; Luciferase; Cyclic AMP

1. Introduction

Interest in cannabinoid research has intensified in recent years following the cloning of specific cannabinoid receptor subtypes: the cannabinoid CB_1 receptor from rat (Matsuda et al., 1990), human (Gérard et al., 1991) and mouse (Chakrabarti et al., 1995) brain, and the cannabinoid CB_2 receptor from human (Munro et al., 1993) and mouse (Shire et al., 1996b) peripheral tissues, and following the discovery of endogenous ligands (Devane et al., 1992; Mechoulam et al., 1995) and the highly-specific cannabinoid CB_1 and CB_2 receptor antagonists SR 141716A {*N*-(piperidin-1-yl)-5-(4-chlorophenyl)-1-(2,4-dichlorophenyl)-4-methyl-1*H*-pyrazole-3-carboxamide hydrochloride} (Rinaldi-Carmona et al., 1994) and SR 144528 {*N*-[(1*S*)-endo-1,3,3-trimethyl bicyclo [2.2.1]-heptan-2-yl]-5-(4-chloro-3-methylphenyl)-1-(4-methoxybenzyl)-pyrazole-3-carboxamide} (Rinaldi-Carmona et al., 1998), respectively. Both the central cannabinoid CB_1 and peripheral cannabinoid CB_2 receptors are members of the heptahelical G protein-coupled receptor superfamily. The cannabinoid CB_2 receptor is known to mediate its effects through the pertussin toxin-sensitive $G_{i/o}$ inhibition of adenylyl cyclase (Bayewitch et al., 1995). The cannabinoid CB_2 receptor can also mediate βγ-mediated intermediate-early gene expression through the mitogen-activated protein (MAP) kinase pathway and this is also blocked by pertussis toxin treatment (Bouaboula et al., 1996). The cannabinoid CB_1 receptor, apart from exhibiting all the same functions as the cannabinoid CB_2 receptor, also inhibits N- and Q-type voltage-dependent Ca^{2+} channels

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and stimulates inwardly rectifying K^+ currents (see Pertwee, 1997 for a recent review). In the past, a functional link with the inositol phosphate/Ca^{2+} pathway could not be established despite numerous investigations with diverse tissues containing cannabinoid CB_1 receptors (Howlett, 1995). Recently, however, a rapid, transient elevation of intracellular free Ca^{2+} upon agonist binding to cannabinoid CB_1 receptors in neuroblastoma × glioma NG108-15 and NG18TG2 cells, mediated by pertussis toxin-sensitive G proteins, has been observed (Sugiura et al., 1996, 1997, 1999).

In addition to the multiple signaling functions implicating the cannabinoid CB_1 receptor, there were several reports prior to the cloning and characterization of the receptor that suggested that under certain conditions cannabinoids could also promote the accumulation of cAMP in cell cultures and tissue homogenates [reviewed in (Pertwee, 1988)]. More recently, it was shown (Glass and Felder, 1997; Felder et al., 1998) that pertussis toxin treatment of the cannabinoid CB_1 receptor attenuated agonist-mediated inhibition of adenylyl cyclase and unmasked an agonist-mediated stimulation of the enzyme. However, the stimulation necessitated the action of a costimulant such as forskolin before the effect could be seen. It was reported (Maneuf and Brotchie, 1997) that high concentrations of the potent cannabinoid WIN 55,212-2 {((*R*)-(+)[2, 3-dihydro-5-methyl- 3-[(4-morpholinyl)methyl]pyrrolo[1, 2, 3-*de*]- 1, 4-benzoxazin- 6-yl](1-naphthale-nyl)methanone}, with an EC_{50} of 32.8 μM, could stimulate basal cAMP accumulation in a slice preparation of globus pallidus in the absence of either costimulant or pertussis toxin and that the effects could be blocked by the antagonist SR 141716A, thereby clearly showing that they were mediated by the cannabinoid CB_1 receptor. More recently, the potency and intrinsic activity of various cannabinoid receptor ligands in stimulating or inhibiting cAMP accumulation were quantified (Bonhaus et al., 1998).

We are at present investigating structural features of the cannabinoid CB_1 receptor implicated in signal transduction using chimeric CB_1/CB_2 and mutated wild type cannabinoid receptors. To study cannabinoid CB_1 receptor modifications that may have repercussions on downstream gene transcription, in addition to direct cAMP assays, we are making use of a reporter gene system consisting of a minimal promoter containing cAMP-response elements (CRE) fused to the firefly luciferase coding region (Bouaboula et al., 1997). We discovered that CP 55,940 could induce cAMP accumulation in both a Chinese hamster ovary (CHO) cell line, CHO-CB_1, and in CHO cells transiently expressing the receptor. We subsequently cotransfected CHO cells with expression vectors for the human cannabinoid CB_1 receptor and the CRE-luciferase fusion and discovered that the basal luciferase activity increased following treatment of the cells with the cannabimimetic CP 55,940 {(−)-*cis*-3-[2-hydroxy-4-(1,1-dimethylheptyl)phenyl]-*trans*-4-(3-hydroxypropyl) cyclohexan-1-ol} in a strictly dose–response manner, or after incubation with pertussis toxin, the two effects being additive. SR 141716A counteracted the effect of the agonist, also in a dose–response manner, showing that the luciferase induction was cannabinoid CB_1 receptor-mediated. Since the cannabinoid CB_2 receptor failed to produce a response after coexpression with the CRE-luciferase cassette, using chimeric CB_1/CB_2 receptor constructs we have been able to investigate the some of the structural features of the cannabinoid CB_1 receptor implicated in initiating this pathway.

2. Materials and methods

2.1. Drugs and chemicals

Forskolin and pertussis toxin were purchased from Sigma (Saint-Quentin-Fallavier, France). CP 55,940 {(−)-*cis*-3-[2-hydroxy-4-(1,1-dimethylheptyl)phenyl]-*trans*-4-(3-hydroxypropyl) cyclohexan-1-ol} and SR 141716A {*N*-(piperidin-1-yl)-5-(4-chlorophenyl)-1-(2,4-dichlorophenyl)-4-methyl-1*H*-pyrazole-3-carboxamide hydrochloride} were synthesized by the Chemistry Department of Sanofi Recherche (Montpellier). Stock solutions of drugs were dissolved in dimethylsulfoxide at 10^{-2} M and stored at −20°C. The concentration of solvent in assays never exceeded 0.1% (v/v); this final concentration had no effect on assays. *N*-[2-(*p*-bromocinnamylamino)ethyl]-5-isoquinolinesulfonamide · 2HCl (H-89), 2-[2-amino-3-methoxyphenyl]-4*H*-1-benzopyran-4-one (PD 98059) and bisindolylmaleimide (GF 109203X) from Biomol Research Laboratories (Plymouth Meeting, PA) and lipofectamine from Gibco/BRL Life Technologies (Paisley, UK) were used according to the manufacturers' recommendations. Luciferase activities were determined using the Luciferase Assay System (Promega).

2.2. Expression vectors

The construction of the p658-derived vectors (Miloux and Lupker, 1994) expressing chimeric cannabinoid CB_1/CB_2 receptors was described previously (Shire et al., 1996a), as was the p661 vector for the CRE-luciferase system (Bouaboula et al., 1997). The coding sequence for the corticotrophin releasing factor receptor was inserted into the pSE1 expression vector (Minty et al., 1993) in place of the interleukin-2 coding sequence.

2.3. Cyclic AMP assays

Cells grown to confluence in 24-well plates were washed and incubated for 30 min in serum-free medium containing 0.1 mM isobutylmethylxanthine. Cells were treated with CP 55,940 for the periods stated in Section 3. The reaction was stopped by aspiration of the medium and addition of

65% ethanol. The determination of cAMP levels was performed by a radioimmunoassay (Immunotech, France) according to the manufacturer's instructions. Each value is the mean of triplicate determinations ± S.E.M. from three independent experiments.

2.4. Firefly luciferase activity

Cells were seeded into 96-well plates, 30,000 cells/well, and cotransfected with receptor (10 μg/plate) and CRE-luciferase (5 μg/plate) vectors using lipofectamine. After overnight incubation the cells were treated with the appropriate quantity of CP 55,940 or forskolin (10^{-6} M) for 4 h, then washed before adding luciferin. Quantification of light emission was measured using a Hamamatsu MTP Reader and mean values from triplicate samples were expressed in relative light units divided by basal values. Where SR 141716A and pertussis toxin (100 ng/ml) were used these were added 10 min and 60 min, respectively, before applying the agonist.

2.5. Coexpression of cannabinoid receptors and the corticotrophin releasing factor receptor

CHO cells were seeded into 24-well plates, 1.25×10^5 cells/well and cotransfected with vectors for the cannabinoid receptors (24 μg/plate) and corticotrophin releasing factor receptor (6 μg/plate) using lipofectamine. After overnight incubation the cells were treated with the appropriate concentration of CP 55,940 for 5 min then corticotrophin releasing factor was added at a final concentration of 10^{-9} M and the plates were incubated at 37°C with 5% CO_2 for 25 min. The reaction was stopped and the cAMP measured as above.

3. Results

3.1. Cannabinoid CB_1 receptor-mediated cAMP accumulation

We first carried out a time course study of the effect of a single concentration of CP 55,940 on a CHO cell line permanently expressing the cannabinoid CB_1 receptor or with CHO cells transiently expressing the receptor. The results presented in Fig. 1a and b show that the results were the same in both expression systems. The agonist at 10^{-6} M induced cAMP accumulation that quickly rose to a peak within 30 min, before falling slightly to a plateau where it remained for at least 4 h. As shown in the figures the presence of the phosphodiesterase inhibitor isobutylmethylxanthine (IBMX) was essential. The cAMP accumulation induced by CP 55,940 was blocked by SR 141716A and was, therefore, clearly mediated by the cannabinoid CB_1 receptor. In mock-transfected control cells and in cells expressing the cannabinoid CB_2 receptor no accumulation was detectable with the CP 55,940 treatment. In the transient expression system the cAMP accumulation was dependent on agonist concentration (Fig. 2). Although pertussis toxin treatment did not appear to augment basal cAMP levels, an additive response of CP 55,940 and pertussis toxin was obtained. Mock transfected cells and cells transiently expressing the cannabinoid CB_2 receptor

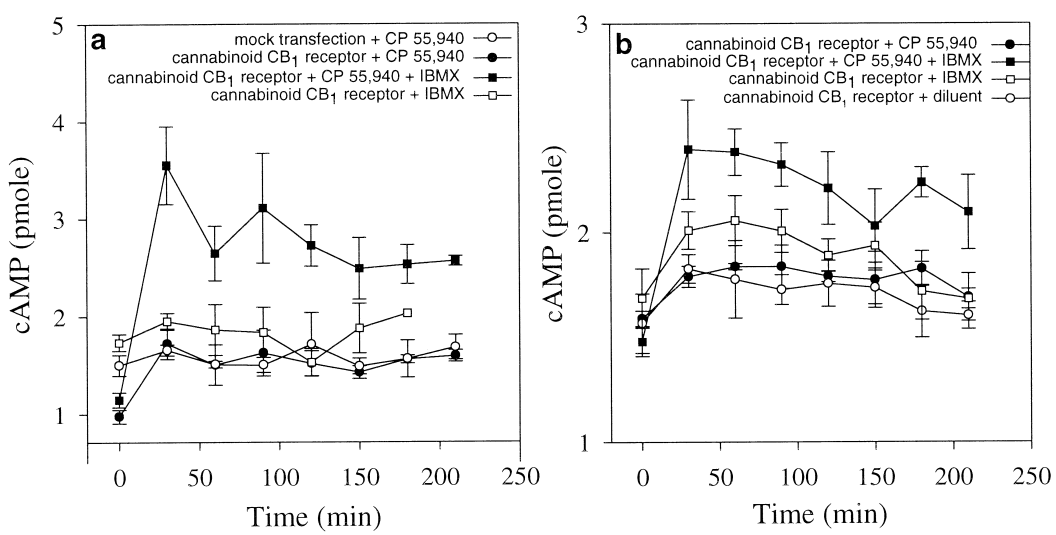

Fig. 1. Time course of CP 55,940-induced cAMP accumulation in CHO cells expressing the wild type cannabinoid CB_1 receptor. The accumulation of cAMP was measured by radioimmunoassay as described in Section 2 in CHO cells (a), transiently expressing the cannabinoid CB_1 receptor and (b), stably expressing the cannabinoid CB_1 receptor. Cells were treated with 10^{-6} M CP 55,940 in the presence or absence of 0.1 mM isobutylmethylxanthine (IBMX). The figure represents data ± S.E.M. from one of three independent experiments performed in triplicate.

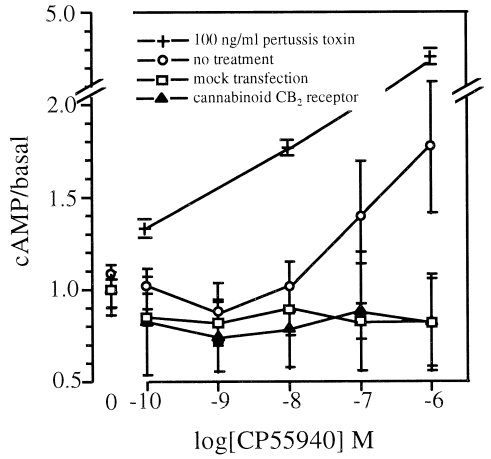

Fig. 2. Concentration dependence of CP 55,940-induced cAMP accumulation in CHO cells transiently expressing cannabinoid CB_1 or CB_2 receptors. The figure represents data ± S.E.M. from one of three independent experiments performed in triplicate.

remained at baseline levels and the specific cannabinoid CB_1 receptor antagonist SR 141716A at 10^{-6} M completely inhibited CP 55,940-induced cAMP accumulation in these experiments (data not shown).

3.2. Cannabinoid CB_1 receptor-mediated effects on a CRE-luciferase reporter gene system

Following the finding that the cannabinoid CB_1 receptor could stimulate cAMP accumulation, we investigated the effect of the accumulation on gene expression using a previously-described reporter gene system consisting of six cyclicAMP response elements (CRE) upstream from a minimal thymidine kinase promoter fused to the firefly luciferase coding sequence (Bouaboula et al., 1997). Transient coexpression of wild type cannabinoid CB_1 receptor and CRE-luciferase in CHO cells followed by treatment with CP 55,940 for 4 h resulted in an increase in luciferase induction and activity in a dose–response manner (Fig. 3a) with an EC_{50} of 7 nM. Maximal induction of the luciferase response was obtained at 10^{-6} M CP 55,940, the highest concentration tested, and was similar to that obtained by stimulation with 10^{-6} M forskolin. SR 141716A at 10^{-7} M had no effect by itself on luciferase activity (not shown), as would be expected since forskolin directly stimulates adenylyl cyclase. However, at this concentration the antagonist was able to block the effect of CP 55,940 below and at 10^{-8} M, but not at 10^{-6} M, results compatible with their respective cannabinoid CB_1 receptor binding affinities (Fig. 3a). A dose–response curve (Fig. 4) showed that CP 55,940 stimulated the luciferase response with an EC_{50} of 5×10^{-9} M, in line with its nanomolar binding affinity for the cannabinoid CB_1 receptor in CHO cell membranes (Bouaboula et al., 1995a,b; Showalter et al., 1996). SR 141716A blocked 10^{-8} M CP 55,940-induced luciferase activity in a dose–response manner with an IC_{50} of 5×10^{-8} M (Fig. 4) providing compelling evidence that the luciferase induction was a cannabinoid CB_1 receptor-mediated effect. SR 141716A alone at 10^{-7} M had no effect on the forskolin-induced response (not shown).

In addition to its inactivation of the adenylyl cyclase pathway, the autoactivated cannabinoid CB_1 receptor has been shown to initiate a pathway leading to mitogen-activated protein kinase phosphorylation and *Krox24* gene expression, presumably mediated by G protein βγ-subunits (Bouaboula et al., 1995a,b). Pertussis toxin treatment blocks both pathways through covalent modification and inactivation of $G_{i/o}$ α-subunits. However, although its direct effect on cAMP accumulation was marginal at best (Fig. 1), here pertussis toxin treatment increased the basal luciferase response in the absence of agonist (Fig. 3a), presumably through a pathway favored by the uncoupling of $G_{i/o}$ from the receptor. As with cAMP accumulation, this pertussis toxin-enhanced response was additive to the

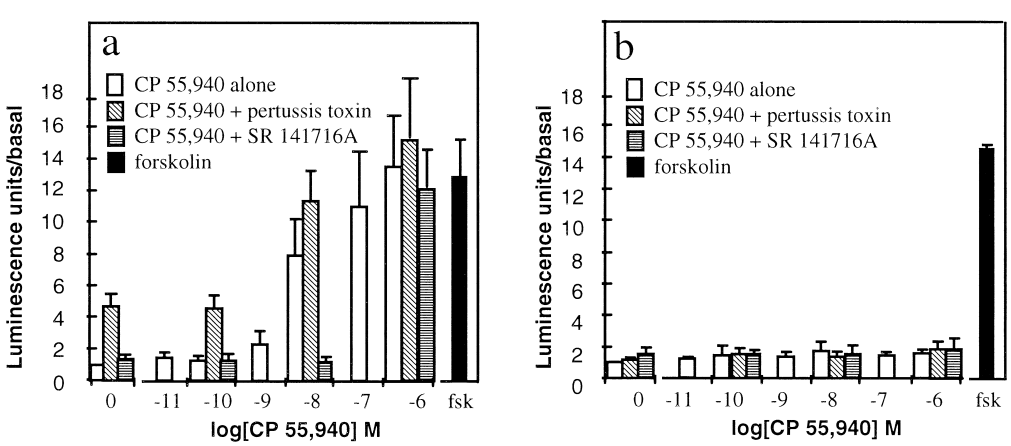

Fig. 3. Induction of luciferase activity through the mediation of wild type cannabinoid receptors. Luminescence was measured in CHO cells cotransfected with expression vectors for CRE-luciferase and (a), wild type cannabinoid CB_1 receptor; (b), wild type cannabinoid CB_2 receptor. Pertussis toxin was used at 100 ng/ml and forskolin at 10^{-6} M. The results are the means ± S.E.M. of three separate experiments carried out in triplicate.

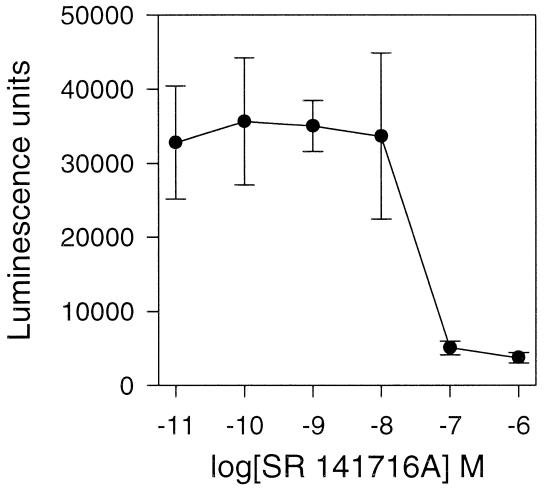

Fig. 4. Inhibition by SR 141716A of luciferase activity induced by 10^{-8} M CP 55,940 in CHO cells transiently coexpressing CRE-luciferase and the wild type CB_1 cannabinoid receptor. The results are the means ± S.E.M. of two separate experiments carried out in triplicate.

CP 55,940-induced response (Fig. 3a). The replacement of the cannabinoid CB_1 receptor by the cannabinoid CB_2 receptor in the coexpression system showed that the cannabinoid CB_2 receptor failed to mediate a detectable luciferase response above the basal level, either with agonist or with pertussis toxin (Fig. 3b). The transfected cells responded well to forskolin (Fig. 3b), showing that the CRE-luciferase system was functional and, in addition, positive [^{3}H]CP 55,940 binding confirmed the presence of the cannabinoid CB_2 receptor (not shown). This result may indicate a fundamental difference between the cannabinoid CB_1 and the cannabinoid CB_2 receptors with respect to their signaling properties in CHO cells or merely reflect a relatively poor signal transduction property of the cannabinoid CB_2 receptor.

3.3. The cannabinoid CB_1 receptor-mediated luciferase induction is blocked by H-89, a protein kinase A inhibitor

Gene transcription follows the cAMP response element-binding protein recognition of the cAMP response element motifs. The cAMP response element-binding protein itself has to be phosphorylated on Ser^{117} in order to be activated and this can be accomplished by several enzymes, including protein kinase A, protein kinase C and calmodulin kinase II (De Groot et al., 1993). However, it was reported (Himmler et al., 1993) that in a CRE-luciferase system stably expressed in CHO cells, stimulation of protein kinase C by phorbol esters or raising of calcium levels by the calcium ionophore A23187 did not induce luciferase expression. In addition, several investigations with the cannabinoid CB_1 receptor expressed in tissues (Reichman et al., 1991) or in heterologous expression systems (Felder et al., 1992, 1993) have failed to establish a functional link between the cannabinoid CB_1 receptor and the inositol phosphate/calcium pathway. From the cAMP accumulation results, it was clear that protein kinase A would be the obvious intermediary and the results in Fig. 5 support this assertion. We tested the effects on CP 55,940-induced luciferase activity of H-89, GF109203X and PD98059, inhibitors of three kinases, protein kinase A, protein kinase C and mitogen-activated protein kinase kinase 1, respectively. Only the protein kinase A inhibitor H-89 reduced the CP 55,940-induced luciferase level, a result fully compatible with a cAMP/protein kinase A/gene transcription pathway.

3.4. Use of chimeric cannabinoid CB_1 / CB_2 receptor constructs to determine the regions of the cannabinoid CB_1 receptor involved in G protein coupling

The discovery of the positive coupling of the cannabinoid CB_1 receptor to the CRE-luciferase response through the cAMP/protein kinase A pathway, contrasting with its absence for the cannabinoid CB_2 receptor, led us to use chimeric cannabinoid CB_1/CB_2 receptors as a first approach towards defining the regions of the cannabinoid CB_1 receptor implicated in this pathway. The chimeras were previously found (Shire et al., 1996a) to bind CP 55,940 with affinities similar to those of the wild type

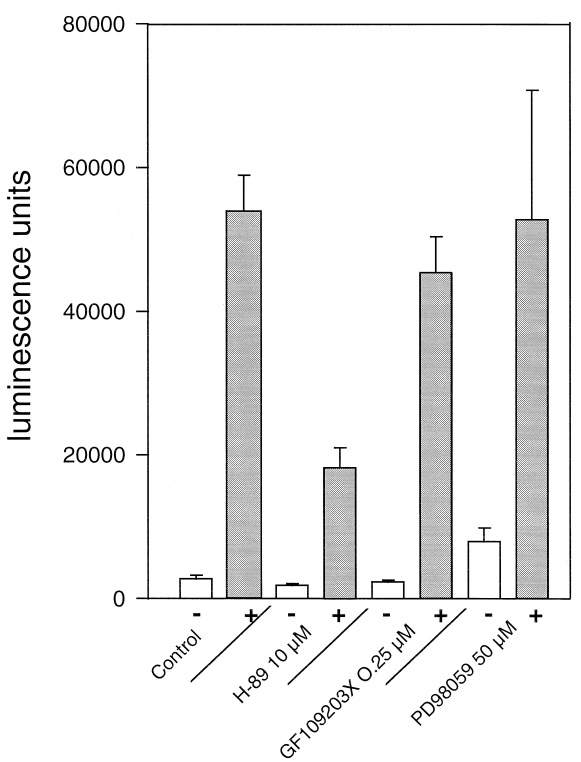

Fig. 5. Inhibition of luciferase activity induced by CP 55,940 in CHO cells transiently coexpressing CRE-luciferase and the cannabinoid CB_1 receptor. The histogram shows the effect of second messenger inhibitors on luciferase activity in arbitrary light units induced in the absence, *white*, and the presence, *gray*, of 10^{-7} M CP 55,940.

receptors, with K_d values in the range of 1.0×10^{-10} M to 6×10^{-10} M, thereby allowing us to use this agonist in the following activation studies. CHO cells cotransfected with CRE-luciferase and the chimeric receptor in which the entire carboxyl-terminal region of the cannabinoid CB_1 receptor was replaced by that of the cannabinoid CB_2 receptor, CB1/2(S^{401}/G^{304}), gave a luciferase response to CP 55,940 (Fig. 6a) similar in all respects to that of the wild type CB_1 receptor (Fig. 3). As we observed with the wild type cannabinoid CB_1 receptor, there was complete inhibition of the 10^{-8} M CP 55,940 response by 10^{-7} M SR 141716A, the same amount of SR 141716A having much less effect on CP 55,940 at 10^{-6} M. This is in line with the binding affinity of the antagonist (IC_{50} 3.4 ± 0.8 nM). Similar results were obtained with the cannabinoid CB_1 receptor construct containing the 7th transmembrane region to the carboxyl-terminal region of the cannabinoid CB_2 receptor, CB1/2(V^{364}/H^{267}) (Fig. 6b). SR 141716A

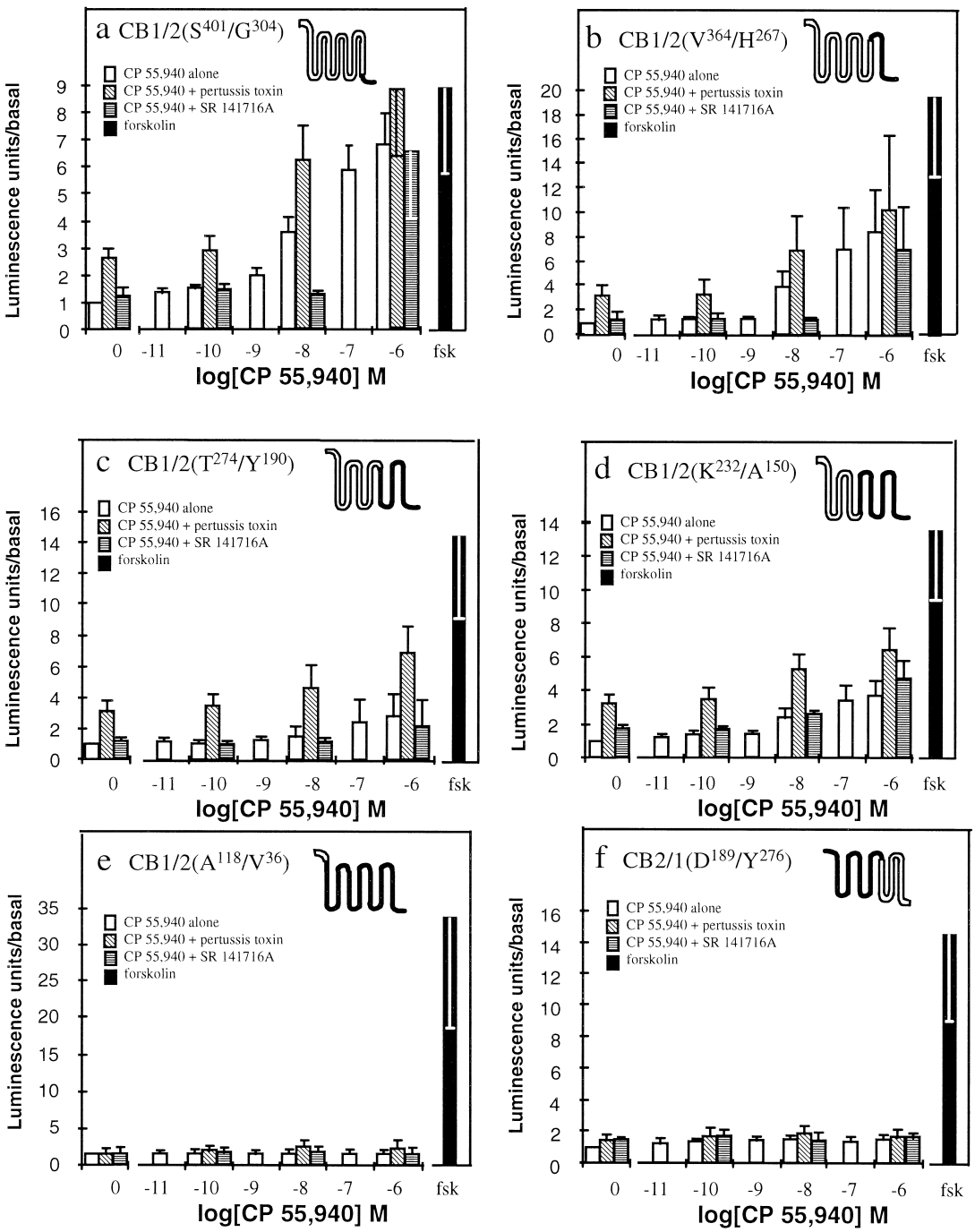

Fig. 6. Induction of luciferase activity through the mediation of chimeric cannabinoid CB_1/CB_2 receptors. The results are the means ± S.E.M. of three separate experiments carried out in triplicate.

at 10^{-7} M again inhibited CP 55,940 at 10^{-8} M, confirming that the high affinity binding site of the antagonist (IC_{50} 23.4 ± 7.7 nM) was present in the construct (Shire et al., 1996a).

Similar responses to CP 55,940 were obtained with the constructs joining the 2nd extracellular loop region of the cannabinoid CB_1 receptor to the 5th transmembrane region of the CB_2 receptor, CB1/2(T^{274}/Y^{190}) (Fig. 6c) and the 2nd intracellular loop region of the cannabinoid CB_1 receptor to the 4th transmembrane region of the cannabinoid CB_2 receptor, CB1/2(K^{232}/A^{150}) (Fig. 6d), but the responses were not inhibited by SR 141716A, either in the CB1/2(K^{232}/A^{150}) construct, for which the antagonist has very low affinity (IC_{50} 734 ± 134 nM), similar to that of the cannabinoid CB_2 receptor, or in CB1/2(T^{274}/Y^{190}), for which it has moderate affinity (43.7 ± 25) (Shire et al., 1996a). Significantly, pertussis toxin treatment of the cells transfected with each of these two chimeras resulted in an enhanced luciferase response, indicating that they were constitutively coupled both to $G_{i/o}$ and to a stimulatory pathway. Both CB1/2(T^{274}/Y^{190}) and CB1/2(K^{232}/A^{150}) contain the cannabinoid CB_2 receptor 3rd intracellular loop and carboxyl-terminus and the 1st and 2nd intracellular loops of the cannabinoid CB_1 receptor, which strongly suggests that the stimulatory response is associated with the latter regions. The sandwich construct CB1/2(L^{151}-G^{204}), in which the cannabinoid CB_2 receptor's 4th and 5th transmembrane regions replaced those of the cannabinoid CB_1 receptor, failed to show any CP 55,940-induced luciferase response in CHO cells, although it contained all the intracellular components of the wild type cannabinoid CB_1 receptor, but it did exhibit a marked pertussis toxin response (data not shown). This result appears surprising considering that the construct still has a high-affinity CP 55,940 binding site (Shire et al., 1996a), but since it also failed to mediate a CP 55,940-induced inhibitory effect (see below), then it is clear that although the construct is capable of ligand binding it is unable to mediate agonist-induced signals to G proteins.

The chimeric construct CB1/2(A^{118}/V^{36}) was similar to the wild type cannabinoid CB_2 receptor in that no luciferase response was observed either by CP 55,940 or by pertussis toxin treatment (Fig. 6f). The same negative result was obtained with CB2/1(D^{189}/Y^{276}), a chimera containing the amino-terminal region of the cannabinoid CB_2 receptor fused after its 2nd extracellular loop to the 5th transmembrane region-carboxyl terminus of the cannabinoid CB_1 receptor (Fig. 6g). This result, as with CB1/2(T^{274}/Y^{190}) and CB1/2(K^{232}/A^{150}), again indicated that the first two intracellular loop regions were necessary for coupling to the G protein mediating the positive luciferase response. We wished to discover whether the non-stimulatory CB1/2(A^{118}/V^{36}) construct could nevertheless couple to $G_{i/o}$ to inhibit cAMP accumulation and to do this we applied a coexpression technique.

3.5. Effect of cannabimimetics on corticotrophin releasing factor stimulation of cAMP accumulation in CHO cells coexpressing corticotrophin releasing factor receptors and wild-type or mutated cannabinoid receptors

Investigations into the functional activities of $G_{i/o}$-coupled receptors generally rely on the ligand-induced inhibition of the cAMP accumulation that follows treatment of the cells by forskolin. This is acceptable for cell lines since clones can be selected that carry high numbers of receptors and the effect on forskolin cAMP accumulation can be efficiently counteracted. It is less satisfactory for transient expression systems in which the numbers of the receptors can vary considerably and the forskolin-induced cAMP accumulation often overwhelms the $G_{i/o}$-mediated inhibitory effect. To replace forskolin, we have developed a system in which an efficient G_s-coupled receptor normally absent from CHO cells is coexpressed with the cannabinoid receptors. This technique has been widely used and has been validated with, for example, the luteinizing hormone receptor (Wong et al., 1991; Chabre et al., 1994). To stimulate cAMP accumulation in our system we chose the corticotrophin releasing factor agonism of its receptor. In control experiments with CHO cells transiently coexpressing the wild type cannabinoid CB_1 receptor and the corticotrophin releasing factor receptor, CP 55,940 up to 10^{-5} M produced a basal cAMP accumulation similar to that shown in Fig. 1. Corticotrophin releasing factor induced a high amount of cAMP accumulation in a dose–response manner, which was blocked by pretreating the cells with CP 55,940 (results not shown). From these control experiments a concentration of 10^{-9} M corticotrophin releasing factor was chosen for stimulating cAMP accumulation in subsequent experiments.

Using this coexpression system, CP 55,940 treatment of the wild type cannabinoid CB_1 and cannabinoid CB_2 receptors caused blockade of cAMP accumulation with EC_{50}'s of 0.5 nM and 0.6 nM, respectively (Fig. 7, CB_2 receptor not shown). The chimeric CB2/1(D^{189}/Y^{276}) construct which had failed to transduce an agonist-induced luciferase response, was indeed functional and coupled to $G_{i/o}$, since on coexpression with the corticotrophin releasing factor receptor in CHO cells it could mediate the CP 55,940-induced inhibition of corticotrophin releasing factor-induced cAMP accumulation to an extent similar to that of both the wild type cannabinoid CB_1 receptor and the CB2/1(D^{189}/Y^{276}) construct, with a maximum inhibition of about 50% at 10^{-6} M CP 55,940 (Fig. 7). The results were adjusted for differences in expression levels by expressing them as a function of the signal obtained at 10^{-11} M CP 55,940. The CB1/2(V^{364}/H^{267}) chimera is interesting in that it does not contain the proximal carboxyl-terminal sequence that have been shown from studies with synthetic peptides to be capable of directly stimulating guanosine-5′-*O*-(3-thio)triphosphate exchange (Howlett et al., 1998), but it does still contain the amino side of

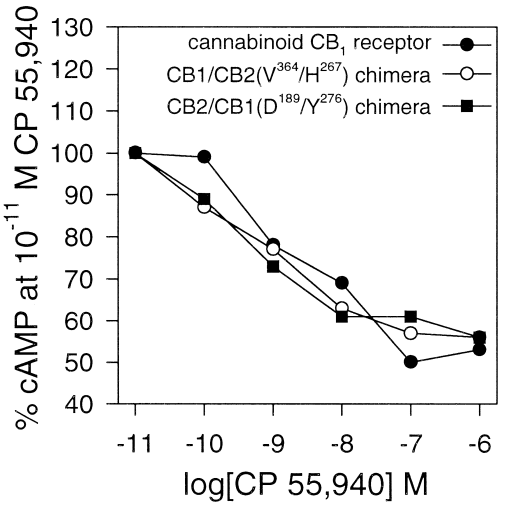

Fig. 7. CP 55,940-mediated inhibition of cAMP accumulation in CHO cells transiently coexpressing the cannabinoid CB_1 receptor and the corticotrophin releasing factor receptor. Cyclic AMP accumulation was measured by radioimmunoassay after induction with 10^{-9} M corticotrophin releasing factor in the presence of various amounts of CP 55,940 and is expressed as a percentage of the quantity obtained using 10^{-11} M CP 55,940.

the 3rd intracellular loop residues of the cannabinoid CB_1 receptor shown in the same study to be coupled to $G_{i/o}$, albeit less efficiently. At the present time we have no knowledge of the cannabinoid CB_2 receptor regions that couple to $G_{i/o}$ and therefore cannot evaluate the contribution of the cannabinoid CB_2 receptor components to the inhibitory effect.

4. Discussion

Although there were early reports prior to cloning of the cannabinoid receptors that cannabinoids could stimulate cAMP accumulation (reviewed by Pertwee, 1988), it is only recently that some indications have appeared to suggest that the cannabinoid CB_1 receptor can undergo dual G protein coupling (Maneuf and Brotchie, 1997; Bonhaus et al., 1998). In particular, it was reported (Maneuf and Brotchie, 1997) that the cannabimimetic WIN 55,212-2, in the absence of a G_s stimulant, could enhance basal cAMP accumulation in a slice preparation of globus pallidus. Here, using a heterologous in vitro expression system we provide firm experimental evidence that the cannabinoid CB_1 receptor can indeed mediate cAMP accumulation, with concomitant downstream effects on gene transcription. We have shown that wild type cannabinoid CB_1 expressed in CHO cells could mediate cAMP accumulation upon binding the cannabinoid CP 55,940, the effect being strictly dependent on the concentration of the agonist, reaching the level produced by forskolin treatment at 10^{-6} M CP 55,940, with an EC_{50} of 5×10^{-8} M. The effect produced by the agonist at 10^{-8} M was counteracted by the cannabinoid CB_1-specific antagonist SR 141716A with an IC_{50} of 5×10^{-8} M, showing that the accumulation was cannabinoid CB_1 receptor-dependent. The stimulation was enhanced by pretreatment of the cells with pertussis toxin, which, by inactivating $G_{i/o}$, blocked the natural basal constitutive activity of the cannabinoid CB_1 receptor that normally manifests itself through an inhibition of adenylyl cyclase activity. The cAMP accumulation following the pertussis toxin treatment, and that produced by the agonism of CP 55,940, were additive. Through a classical metabolic cascade the accumulated cAMP activated protein kinase A, which presumably phosphorylated the cAMP-response element binding protein, which in turn initiated gene transcription by binding to CRE motifs in a synthetic promoter. We have followed this process using a luciferase-based reporter gene system and have shown that it is indeed PKA-dependent in that it could be blocked by H-89, a protein kinase A-specific inhibitor.

It has been recently reported (Glass and Felder, 1997) that the cannabinoid CB_2 receptor, unlike the cannabinoid CB_1 receptor, was incapable of stimulating cAMP accumulation even after pertussis toxin treatment. We confirm these results in the present investigation and, benefiting from the total absence of cannabinoid CB_2 receptor coupling to cAMP stimulation, we have been able to investigate the signaling properties of chimeric cannabinoid CB_1/CB_2 receptor mutants to begin to discover the intracellular regions of the cannabinoid CB_1 receptor implicated in cAMP stimulation. The systematic replacement of the cannabinoid CB_1 receptor domains by the cognate cannabinoid CB_2 receptor domains led to constructs that maintained a high affinity CP 55,940 binding site (Shire et al., 1996a). We have used this fact to investigate the functionality of the chimeras and have found that the cAMP stimulatory property of the cannabinoid CB_1 receptor was conserved in all the chimeras that contained the first two intracellular loops of this receptor. In the present investigation we were unable to delineate the role of each loop more precisely, since the chimeric constructs containing cannabinoid CB_1/CB_2 receptor connections in the 2nd and 3rd transmembrane regions were not translocated into the plasma membrane (Shire et al., 1996a). The chimeric construct with the cannabinoid CB_2 receptor at the amino half of the construct connected to the carboxyl half of the cannabinoid CB_1 receptor was unable to stimulate cAMP, since it lacked the first two intracellular loops of the cannabinoid CB_1 receptor, but was still capable of exerting an inhibitory effect on the cAMP accumulation generated through the activation of the coexpressed corticotrophin releasing factor receptor. Interestingly, the sandwich construct consisting of the 4th and 5th transmembrane regions of the cannabinoid CB_2 receptor replacing the cognate cannabinoid CB_1 receptor region possessed the dual activity of the cannabinoid CB_1 receptor, but was incapable of transducing an agonist-mediated signal, although the high

affinity CP 55,940 binding site was present (Shire et al., 1996a). Possible motifs contained in the first two intracellular loops of the cannabinoid CB_1 receptor responsible for the positive cAMP response are under investigation.

The locations of the intracellular regions of G protein-coupled receptors associated with G_i and G_s activation vary considerably. It was found (Okamoto and Nishimoto, 1992) that synthetic peptides representing the 2nd intracellular loop of the m4 muscarinic acetylcholine receptor could stimulate G proteins, but that 30- to 100-fold higher concentrations were needed to stimulate G_s than G_i. Implication of the 1st intracellular loop of the gonadotropin-releasing hormone receptor in G_s coupling has recently been reported (Arora et al., 1998). The G_s coupling domain of the α_2-adrenergic receptor α_{2A} sub-type has been determined to be in the amino terminus of the 3rd intracellular loop, whereas the $G_{i/o}$-coupling domain appears to be elsewhere in the receptor (Eason and Liggett, 1995). From the mutation of a single arginine residue in the 7th transmembrane region, evidence was obtained (Negishi et al., 1995) that the prostaglandin EP3D receptor had two distinct active conformations, causing the receptor to couple to either G_s or G_i, but were unable to conclude whether it was the ligand or the G protein that determined each conformation. In studies of synthetic peptides representing different domains of the CB_1 receptor, it was discovered (Howlett et al., 1998) that the amino-side of the 3rd intracellular loop and the proximal carboxyl terminus were capable of interacting with the $G_{i/o}$ class of proteins. This study did not include peptides corresponding to the first two intracellular loops and it would be interesting to see whether such peptides interact with G_s.

It is too soon to say if the cannabinoid CB_1 receptor is simultaneously coupled to $G_{i/o}$ and G_s or whether there are two populations of receptors. Since pertussis toxin treatment reveals an inherent adenylyl cyclase stimulatory activity, a dual activity based on a switching process resulting from phosphorylation, as observed with the β_2-adrenoceptor (Daaka et al., 1997), is unlikely. Adenylyl cyclase function depends on the class of enzyme present in the tissue or cell being studies. Nine distinct isoforms have been cloned from mammalian tissues and have been divided into six subfamilies based on sequence and functional similarities (Premont et al., 1996). The isoforms present in CHO cells are unknown. Cyclase activity is enhanced by $G_s\alpha$ binding and reduced by $G_i\alpha$ binding, but several isoforms can also be regulated by $\beta\gamma$-subunits. Types 2 and 4 were reported (Tang and Gilman, 1998) to be stimulated by $\beta\gamma$-subunits, but only in the presence of $G_s\alpha$ activation. Recently, it was found (Rhee et al., 1998) that the activities of types 1, 5, 6 and 8 were inhibited by cannabinoid agonists with both the cannabinoid CB_1 and cannabinoid CB_2 receptors expressed in monkey kidney COS-7 cells, whereas those of types 2, 4 and 7 were enhanced, probably through the release of $\beta\gamma$-subunits. Since we found that pertussis toxin treatment enhanced cAMP accumulation in the absence of agonist in the case of the cannabinoid CB_1 receptor in CHO cells, it is most unlikely that autoactivated cannabinoid CB_1 receptor stimulates cAMP production through $\beta\gamma$-subunits. The stimulatory effect of the cannabinoid CB_1 receptor is probably mediated through $G_s\alpha$ binding to adenylyl cyclase, but at present we have no direct evidence that this is the case.

A dual coupling to a stimulatory and an inhibitory effect on adenylyl cyclase appears paradoxical, but there are several reports of receptors that are coupled to both $G_{i/o}$ and G_s, such as the angiotensin II AT_1 receptor (Bharatula et al., 1998), the m4 muscarinic acetylcholine receptor (Dittman et al., 1994), the dog thyrotropin receptor (Allgeier et al., 1997) and the bovine prostaglandin receptor EP3D subtype (Negishi et al., 1995). However, the best studied are the α_2-adrenoceptors (Fraser et al., 1989; Eason et al., 1992; Okamoto and Nishimoto, 1992; Chabre et al., 1994; Eason and Liggett, 1995) and, in particular, solid evidence for the physical coupling of the α_{2C10}-adrenoceptor with both $G_{i/o}$ and G_s was provided from immunoprecipitation experiments (Eason et al., 1992). From transient coexpression of α_{2A}-adrenoceptors and G proteins in the human embryonic kidney HEK293 cell line, it was concluded (Chabre et al., 1994) that the receptor preferentially coupled to G_i with an EC_{50} of 0.09 nM rather than to G_s with the much lower EC_{50} of 70 nM. In contrast to what we observe with the cannabinoid CB_1 receptor in CHO cells, it has been reported (Chabre et al., 1994) that coupling of the α_{2C10}-adrenoceptor to G_s could only be observed when the latter was expressed from an exogenous vector. In addition, the cAMP response they observed was low, about two-fold at the highest agonist concentration, a level similar to the accumulation we measured here with 10^{-6} M CP 55,940.

The physiological significance of a dual response to cannabinoid CB_1 receptor activation remains to be determined. Although the $G_{i/o}$ coupling is undoubtedly the major occurrence, there may be certain situations where the alternative coupling predominates, either through a natural imbalance in the G protein components of a particular cell type or through the sequestration of the $G_{i/o}$ proteins by colocalized receptors. The efficacy of coupling to the G proteins may also be different. Such differences may explain the anomalies observed in the relative levels of receptor density compared to receptor-activated G proteins in various brain regions (Sim et al., 1995) and the failure of cannabinoids to inhibit cAMP production by rat hippocampal membranes (Childers et al., 1994). In addition, since differential sodium effects were observed in two rat brain areas, it was speculated that different G proteins could be coupled to the receptors in the different regions (Pacheco et al., 1994). Finally, dual coupling may help explain the observation (Fan et al., 1996) that repeated injections of CP 55,940 led to a 50% decrease in receptor number in the cerebellum of treated mice, with quite different effects on the respective degrees of motor hy-

poactivity, hypothermia and immobility, without producing tolerance to the inhibitory effect of CP 55,940 on cAMP production.

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