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Activation of the Human Peripheral Cannabinoid Receptor Results in Inhibition of Adenylyl Cyclase

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SUMMARY

The human peripheral cannabinoid (CB_2) receptor has been cloned by reverse transcription-polymerase chain reaction from human spleen RNA and expressed, to study both ligand binding characteristics and signal transduction pathways. Receptor binding assays used the aminoalkylindole [^{3}H]Win 55212–2 and membranes from transiently transfected COS-M6 cells. Saturation analysis showed that [^{3}H]Win 55212–2 specific binding to the CB_2 receptor was of high affinity, with a K_d of 2.1 ± 0.2 nM (four experiments), and a high level of expression was attained, with a maximal number of saturable binding sites of 24.1 ± 4.4 pmol/mg of protein (four experiments). The rates of association and dissociation for [^{3}H]Win 55212–2 specific binding were both rapid when measured at 30°. [^{3}H]Win 55212–2 specific binding to the CB_2 receptor was moderately enhanced by divalent and monovalent cations but was only slightly inhibited by guanosine-5′-*O*-(3-thio)-triphosphate. Competition for [^{3}H]Win 55212–2 specific binding to the CB_2 receptor was stereoselective, with the following rank order of potency for the more active stereoisomers: HU-210 > (−)-CP-55940 ~ Win 55212–2 ≫ (−)Δ^9-THC > anandamide. The signaling pathway of the human CB_2 receptor was investigated in a CB_2-CHO-K1 stable cell line. CB_2 receptor activation by cannabinoid agonists inhibited forskolin-induced cAMP production in a concentration-dependent and stereoselective manner but did not increase either cAMP production or Ca^{2+} mobilization in fura-2/acetoxymethyl ester-loaded CB_2-CHO-K1 cells. The CB_2 receptor-mediated inhibition of forskolin-induced cAMP production was abolished by pretreatment of the cells with 10 ng/ml pertussis toxin. These results demonstrate that the CB_2 receptor is functionally coupled to inhibition of adenylyl cyclase activity via a pertussis toxin-sensitive G protein.

The cloning of the human peripheral CB_2 receptor has been recently reported (1). The CB_2 receptor shares 44% sequence identity with the previously described human central CB_1 receptor (2) and, together, the CB_2 and CB_1 receptors form a distinct class within the expanding family of G protein-coupled receptors. Cannabinoids such as Δ^9-THC (the active component of marijuana), which are known to interact with at least the CB_1 receptor, are potent psychotropic substances (3). Along with these psychoactive effects, however, the medical uses of marijuana have been documented throughout the centuries (4). Its diverse properties include antiemetic, anticonvulsive, analgesic, and anti-inflammatory effects, and therapeutic utility has been reported in the treatment of glaucoma, asthma, and muscle spasms (4, 5).

With the recent cloning of the CB_2 receptor, the opportunity has arisen to clarify further the medicinal properties of cannabinoids. For example, the CB_2 receptor has been primarily localized to the marginal zones around the periarteriolar lymphoid sheaths of the spleen and was present in splenic macrophages and monocytes but was not detected in brain (1). This distribution suggests a role for the CB_2 receptor in mediating the immunosuppressive but not the psychoactive properties attributed to cannabinoids. Several aspects of immune function have been shown to be suppressed by Δ^9-THC *in vitro* (6), including macrophage activation (7) and tumor necrosis factor-α production in both macrophages (8) and large granular lymphocytes (9). Furthermore, a cannabinoid binding site has been identified in cultured murine splenic lymphocytes. In these cells, cannabinoids stereoselectively inhibit both IgM production in response to sheep red

ABBREVIATIONS: CB receptor, cannabinoid receptor; Win 55212–2, (−)-3-(4-morpholinylmethyl)-*S*-methyl-6-(1-naphthylcarbonyl)-2,3-dihydropyrrolo[1,2,3-*de*]-1,4-benzoxazinemethanesulfonic acid; Win 55212–3, (+)-enantiomer of Win 55212–2; CP-55940, *cis*-3-[4-(1,1-dimethylheptyl)-2-hydroxyphenyl]-*trans*-4-(3-hydroxypropyl)cyclohexanol; HU-210, (−)-(6a*R*,10a*R*)-3-[(1,1-dimethyl)heptyl]-9-(hydroxymethyl)-6,6-dimethyl-6a,7,10,10a-tetrahydro-6*H*-dibenzo[*b*,*d*]pyran-1-ol; HU-211, (+)-(6a*S*,10a*S*)-3-[(1,1-dimethyl)heptyl]-9-(hydroxymethyl)-6,6-dimethyl-6a,7,10,10a-tetrahydro-6*H*-dibenzo[*b*,*d*]pyran-1-ol; Δ^9-THC, (6a*R*,10a*R*)-6,6,9-trimethyl-3-pentyl-6a,7,8,10a-tetrahydro-6*H*-dibenzo[*b*,*d*]pyran-1-ol; anandamide, eicosa-5,8,11,14-tetraenoic acid-(2-hydroxyethyl)amide; MES, 2-(*N*-morpholino)ethanesulfonic acid; Bis-Tris, bis(2-hydroxyethyl)iminotris(hydroxymethyl)methane; BES, *N*,*N*-bis(2-hydroxyethyl)-2-aminoethanesulfonic acid; Tricine, *N*-[tris(hydroxymethyl)methyl]glycine; GTPγS, guanosine-5′-*O*-(3-thio)triphosphate; ATPγS, adenosine-5′-*O*-(3-thio)triphosphate; HSA, human serum albumin; BSA, bovine serum albumin; CHO, Chinese hamster ovary; AM, acetoxymethyl ester; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; PTX, pertussis toxin; $[Ca^{2+}]_i$, intracellular calcium concentration.

blood cell antigen and proliferation in response to T cell receptor activation (10, 11). However, it is presently unclear whether these effects are CB_2 receptor mediated, because selective ligands for the peripheral receptor are not yet available.

At the present time, the CB_1 receptor has been the most thoroughly studied. Radioligand binding analyses have characterized rat and human CB_1 specific binding sites in tissues such as cerebellum and in recombinant cell lines (12–14). In addition, the signal transduction pathway of the CB_1 receptor has been determined. Agonist stimulation of both native and recombinant CB_1 receptors has been shown to inhibit adenylyl cyclase activity through coupling with the α_i class of G proteins (2, 15, 16). Cannabinoids also inhibit N-type calcium channels in neuroblastoma-glioma cells (17) and stimulate inwardly rectifying K^+ channels in CB_1 receptor-transfected AtT20 cells (18). Finally, a potential endogenous ligand for the CB_1 receptor has been identified. The arachidonic acid derivative anandamide, originally isolated from porcine brain (19), has been shown to act as an agonist at the CB_1 receptor (20, 21).

Although the nucleotide and amino acid sequences of the CB_2 receptor have been reported (1), extensive radioligand binding analysis of the CB_2 receptor has not been performed, nor has the signal transduction pathway for this receptor been elucidated. In this report, we describe the high affinity binding characteristics of the tritiated aminoalkylindole Win 55212–2 with the recombinant human CB_2 receptor expressed both in COS-M6 cells and in CHO-K1 cells. In addition, we have established that the signal transduction pathway of the CB_2 receptor expressed in CHO-K1 cells involves the inhibition of adenylyl cyclase through coupling to a PTX-sensitive G protein.

Materials and Methods

Reagents. Human RNA samples were from Clontech (Palo Alto, CA). Reverse transcription-polymerase chain reaction kits were from Perkin Elmer Cetus (Norwalk, CT). The Bluescript II SK+ vector was from Stratagene (La Jolla, CA), and pcDNAIamp and pcDNAIneo were from Invitrogen (San Diego, CA). Sequenase was from United States Biochemicals (Cleveland, Ohio). Qiagen plasmid purification kits were from Qiagen (Chatsworth, CA). CHO-K1 cells (CCL61) were from the American Type Culture Collection (Rockville, MD). LipofectAMINE, Geneticin (G418), enzyme-free cell dissociation buffer, and all tissue culture media were from Gibco-BRL Canada (Burlington, Ontario, Canada). [^{3}H]Win 55212–2 (37 Ci/mmol) and Biofluor scintillation fluid were from DuPont-NEN Canada (Mississauga, Ontario, Canada). Win 55212–2, Win 55212–3, (−)-CP-55940, (+)-CP-55940, HU-210, HU-211, (+)-Δ^9-THC, (−)-Δ^9-THC, and anandamide were all synthesized by the Department of Medicinal Chemistry, Merck Frosst Centre for Therapeutic Research. HSA (fraction V, 96–99% albumin), BSA (fraction V, 96–99% albumin), and ionomycin were from Sigma Chemical Co. (St. Louis, MO). GTPγS and ATPγS were from Boehringer Mannheim Canada (Montréal, Québec, Canada). PTX, RO-201724, and forskolin were from Biomol (Plymouth Meeting, PA). ^{125}I-cAMP radioimmunoassay kits were from Amersham Canada (Oakville, Ontario, Canada). Fura-2/AM was from Molecular Probes (Eugene, OR). All other chemicals were of analytical grade.

Construction of recombinant CB_2 receptor expression vectors. The open reading frame of the CB_2 receptor was generated by reverse transcription-polymerase chain reaction amplification of human spleen poly(A)$^+$ RNA, using oligonucleotides based on the published sequence (1). The sense and antisense oligonucleotides were 5′-ATGGAGGAATGCTGGGTGACA-3′ and 5′-TCAGCAATCAGAGAGGTCTAG-3′, respectively. Poly(A)$^+$ RNA (1 μg) was first converted to single-stranded cDNA using Moloney murine leukemia virus reverse transcriptase and 2.5 μM random hexamers. Polymerase chain reactions were carried out in a buffer containing 10 mM Tris·HCl, pH 8.3, 50 mM KCl, 2.5 mM $MgCl_2$, 0.2 mM deoxynucleotide triphosphates, and 0.5 μM primers, using a cycling program of 94° for 1 min, 53° for 1 min, and 72° for 1 min (in a Perkin Elmer Cetus 9600 thermal cycler) for 36 cycles, followed by a 10-min extension at 72°. The amplified 1.1-kilobase CB_2 receptor cDNA was purified by agarose gel electrophoresis and subcloned into the *Eco*RV site of plasmid pBSK$^+$, and its DNA sequence was confirmed on both strands by dideoxy sequencing. The CB_2 receptor open reading frame was excised from pBSK$^+$ as a 1.1-kilobase *Hind*III/*Eco*RI fragment and was subcloned into the *Hind*III/*Eco*RI sites of the mammalian expression vectors pcDNAIamp and pcDNAIneo, yielding pcDNAIamp-CB_2 and pcDNAIneo-CB_2, respectively. These plasmids were prepared for transfection of mammalian cell lines using Qiagen plasmid purification kits.

pcDNAIamp-CB_2 expression in COS-M6 cells and pcDNAIneo-CB_2 expression in CHO-K1 cells. Transient expression of the CB_2 receptor was achieved by transfection of the pcDNAIamp-CB_2 plasmid into COS-M6 cells by cationic liposome-mediated transfer of DNA using LipofectAMINE reagent, according to the manufacturer's instructions. The cells were maintained in culture for 48–72 hr after transfection before being harvested by incubation with enzyme-free cell dissociation buffer (Gibco-BRL). Harvested cells were then recovered by centrifugation at 300 × *g* for 7 min at 4°, washed once with phosphate-buffered saline, pH 7.4, and recovered by centrifugation under the same conditions. The final cell pellet was resuspended at 10^7 cells/ml in 10 mM HEPES-KOH, pH 7.4, 1 mM EDTA, frozen in liquid nitrogen, and stored at −80°.

Stable expression of the CB_2 receptor was achieved by calcium phosphate-mediated transfection of the pcDNAIneo-CB_2 plasmid into CHO-K1 cells, followed by clonal selection. Cells were maintained in culture for 48 hr after transfection and then trypsinized, diluted, and replated in the presence of 500 μg/ml G418 to select for clonal cell colonies. Individual colonies were expanded and clonal CHO-K1 cell lines expressing the CB_2 receptor (CB_2-CHO-K1) were identified by [^{3}H]Win 55212–2 binding assays.

COS-M6 and CHO-K1 membrane preparation and [^{3}H]Win 55212–2 binding assays. Membranes were prepared from COS-M6 and CHO-K1 cells by differential centrifugation (1000 × *g* for 10 min and then 100,000 × *g* for 30 min, both at 4°) after lysis of the cells by nitrogen cavitation at 800 psi for 15 min on ice, in the presence of 2 mM phenylmethylsulfonyl fluoride. The 100,000 × *g* membrane fraction was resuspended at a protein concentration of 3–5 mg/ml by homogenization with a Dounce homogenizer (pestle A, 10 strokes), frozen in liquid nitrogen, and stored in aliquots at −80°. Initial [^{3}H]Win 55212–2 binding experiments showed that <5% of the CB_2 receptors were recovered in the 1000 × *g* membrane fraction, which was routinely discarded.

[^{3}H]Win 55212–2 binding assays were performed in 0.2 ml of 10 mM HEPES-KOH, pH 7.4, containing 1 mM EDTA, 0.3 mg/ml HSA, 1 mM $MgCl_2$, and 2.4 nM [^{3}H]Win 55212–2. The reaction was initiated with the addition of 3–5 μg (CB_2-COS-M6) or 12–15 μg (CB_2-CHO-K1) of protein from the 100,000 × *g* membrane fraction. Samples were incubated for 40 min at 30° before separation of the bound and free radioligand by rapid filtration through GF/B filters that had been soaked in 10 mM HEPES-KOH, pH 7.4, containing 0.01% (w/v) BSA. The individual filters were then washed with 16 ml of the HEPES/BSA soaking solution, and the residual [^{3}H]Win 55212–2 bound to the filters was determined by liquid scintillation counting after a 6-hr equilibration in 5 ml of Biofluor scintillation cocktail (efficiency for tritium of approximately 55%). Specific binding was defined as the difference between total binding and nonspecific binding, which was determined in samples containing a 420-fold excess of

Win 55212–2 (1 μM) or 10 μM Δ⁹-THC. Under these [³H]Win 55212–2 binding assay conditions, total binding represented approximately 10% of the radioligand added to the incubation mixture, with specific binding representing 80–90% of the total binding. In addition, total binding was in the linear range with respect to the protein and radioligand concentrations used in the assay.

cAMP assays in CHO-K1 cells expressing the CB_2 receptor. CB_2-CHO-K1 and control CHO-K1 cells were seeded at 10^6 cells/175-cm^2 T flask in minimal essential medium-α containing 25 mM HEPES-KOH, pH 7.4, 10% heat-inactivated fetal bovine serum, 20 units/ml penicillin G, and 20 μg/ml streptomycin sulfate, with or without 500 μg/ml G418 (for the CB_2-CHO-K1 and CHO-K1 cells, respectively). The cells were maintained in culture for 2 days, and the medium was replaced 16–24 hr before the cells were harvested. Cells were harvested at approximately 80% confluence by incubation in enzyme-free cell dissociation buffer (Gibco-BRL), washed once with HEPES-buffered Krebs-Ringer solution (1.25 mM $MgSO_4$, 1.5 mM $CaCl_2$, 5 mM KCl, 124 mM NaCl, 8 mM glucose, 1.25 mM KH_2PO_4, 25 mM HEPES-KOH, pH 7.4) containing 1 mg/ml HSA, and resuspended in the same buffer. The cAMP assay was performed in a final volume of 0.2 ml of HEPES-buffered Krebs-Ringer solution containing 1 mg/ml HSA and 100 μM levels of the phosphodiesterase type IV inhibitor RO-20–1724, to prevent hydrolysis of cAMP. Assays were also performed in the absence and presence of 5 μM forskolin, to measure both stimulation and inhibition of intracellular cAMP production. RO-20–1724, forskolin, and the compounds under evaluation were added to the incubation mixture in dimethylsulfoxide or ethanol to a final vehicle concentration of 0.35% (v/v), which was kept constant in all samples. The reaction was initiated by the addition of either 1 or 1.5 × 10^5 cells/incubation. Samples were incubated at 37° for 15 min, and the reaction was then terminated by immersion of the samples in boiling water for 3 min. Cell viability was always ≥96%, as determined by trypan blue exclusion. Measurement of cAMP was performed by radioimmunoassay using ^{125}I-cAMP.

$[Ca^{2+}]_i$ assays in CHO-K1 cells expressing the CB_2 receptor. $[Ca^{2+}]_i$ measurements were performed with both the CB_2-CHO-K1 stable cell line and control CHO-K1 cells. The cells were harvested with enzyme-free cell dissociation buffer (Gibco-BRL), recovered by centrifugation at 300 × *g* for 7 min at 22°, and resuspended at 10^7 cells/ml in minimal essential medium-α (25 mM HEPES-KOH, pH 7.4, 10% heat-inactivated fetal bovine serum, 20 units/ml penicillin G, and 20 μg/ml streptomycin sulfate). The cells were incubated for 1 hr at 37°, in 6% CO_2, with 3 μM fura-2/AM. Unincorporated fura-2/AM was removed by three washes in Hanks' buffered saline solution (1.3 mM $CaCl_2$, 5 mM KCl, 0.3 mM KH_2PO_4, 0.9 mM $MgCl_2$, 138 mM NaCl, 4 mM $NaHCO_3$, 0.3 mM Na_2HPO_4, 5.6 mM D-glucose), followed by centrifugation at 300 × *g* for 7 min at 22°. The cells were finally resuspended at a concentration of 10^6 cells/ml in Hanks' buffered saline solution. $[Ca^{2+}]_i$ was measured at 37° after agonist challenge of 2 × 10^6 fura-2/AM-loaded cells, using a Perkin-Elmer LS-5 spectrofluorometer. The wavelengths of the spectrofluorometer were set at 340 and 380 nm for excitation and 509 nm for emission. Cell viability at the end of each individual experiment was verified by challenge with 1 μM ionomycin.

Protein assays. Protein concentrations were determined by monitoring the absorbance at 562 nm of the bicinchoninic acid-Cu^+ complex formed from the biuret reaction, using Pierce bicinchoninic acid reagent, according to the manufacturer's instructions, with BSA as the protein standard.

Results

Ligand Binding Properties of the CB_2 Receptor

[³H]Win 55212-2 specific binding in COS-M6 cells. A detailed analysis of the specific binding of the agonist [³H]Win 55212–2 to the CB_2 receptor has been conducted using membranes prepared from COS-M6 cells expressing this receptor at high levels after transient transfection with pcDNAIamp-CB_2. [³H]Win 55212–2 specific binding was not detectable using membrane fractions prepared from COS-M6 cells transfected with the pcDNAIamp plasmid alone. It has been concluded, therefore, that the results described below represent the characteristics of [³H]Win 55212–2 specific binding to the CB_2 receptor.

Saturation analysis. Saturation analysis of [³H]Win 55212–2 specific binding to the CB_2 receptor was performed as described in the legend to Fig. 1. Transformation of the deduced specific binding saturation isotherm by nonlinear

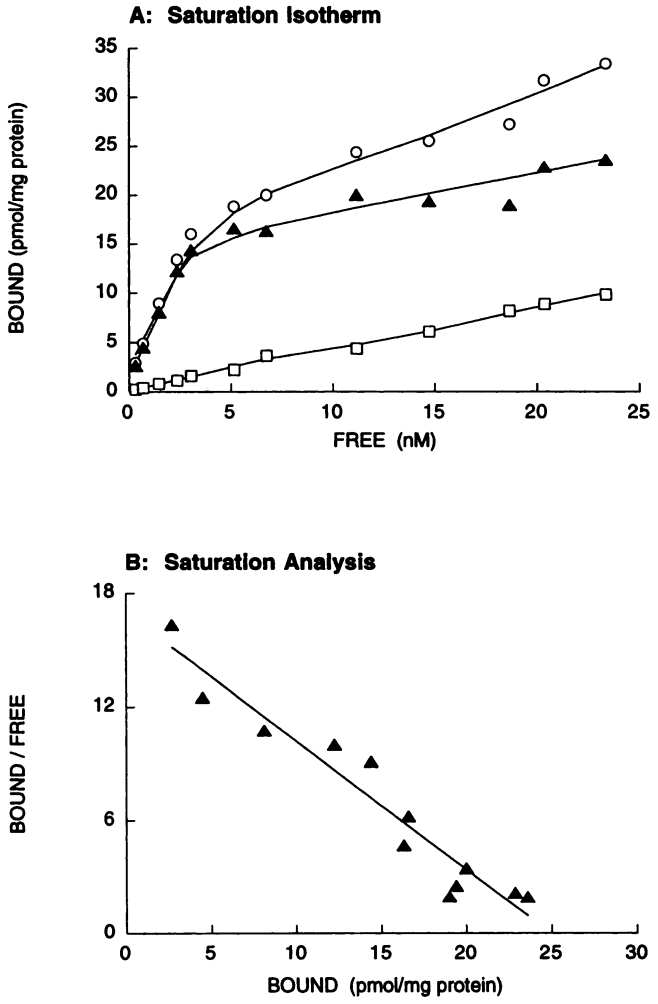

Fig. 1. Saturation analysis of [³H]Win 55212–2 binding to the CB_2 receptor expressed in membranes from COS-M6 cells. CB_2 receptor binding assays were performed over a concentration range of 1–30 nM [³H]Win 55212–2, as described in Materials and Methods. Total (○) and nonspecific (□) binding was measured in the absence and presence of a 170-fold excess of Win 55212–2 (5 μM) at each radioligand concentration. The deduced specific binding saturation isotherm (▲) was obtained by subtracting nonspecific from total binding (A). Transformation of the deduced specific binding isotherm was performed using Accufit Saturation Two-Site data analysis software (Beckman Instruments), based on a nonlinear, least-squares, regression analysis adapted from the work of Feldman (22) (B). The total radioligand bound was calculated from the equation $[(B_{max_1} \times F)/(K_{d_1} + F)] + [(B_{max_2} \times F)/(K_{d_2} + F)]$, where B_{max} is the maximal number of binding sites, K_d is the equilibrium dissociation constant, and F is the concentration of free radioligand. The nonspecific radioligand bound was determined from linear regression analysis of experimentally determined nonspecific binding values.

least-squares analysis (22) showed that [^{3}H]Win 55212–2 specific binding to the CB_2 receptor was of high affinity, with an equilibrium dissociation constant (K_d) of 2.1 ± 0.2 nM (four experiments), and conformed to a one-site model. [^{3}H]Win 55212–2 specific binding was also saturable, with a maximal number of binding sites (B_{max}) of 24.1 ± 4.4 pmol/mg of membrane protein (four experiments).

Rates of association and dissociation. The association rate of [^{3}H]Win 55212–2 specific binding to the CB_2 receptor was rapid, and equilibrium was reached by 20 min at 30° (Fig. 2). After equilibrium was established, there was no subsequent decrease in the level of [^{3}H]Win 55212–2 specific binding over the 120-min incubation period. This indicates that neither the radioligand nor the receptor was degraded under the binding assay conditions. The rate of dissociation of [^{3}H]Win 55212–2 specific binding from the CB_2 receptor, provoked by addition of a 400-fold excess of Win 55212–2, was also rapid, reaching nonspecific binding levels within 5 min. The addition of 100 μM GTPγS with Win 55212–2 did not affect the rate of dissociation.

Effects of divalent and monovalent cations. A substantial component of [^{3}H]Win 55212–2 specific binding to the CB_2 receptor was observed in the absence of added cations (Fig. 3A). The inclusion of divalent cations in the incubation medium, however, resulted in an enhancement of [^{3}H]Win 55212–2 specific binding to the CB_2 receptor, with the following order of potency: $Mg^{2+} \geq Mn^{2+} > Ca^{2+}$. The maximal stimulation of specific binding was obtained using 1 mM Mg^{2+} and represented an increase of 57% over the nonstimulated level, from 1052 ± 116 cpm to 1649 ± 246 cpm (three experiments), under the standard receptor binding assay conditions.

The presence of monovalent cations in the incubation medium also resulted in a stimulation of [^{3}H]Win 55212–2 specific binding to the CB_2 receptor (Fig. 3B). In this case Na^+ and K^+ were equipotent, producing a 2-fold increase in specific binding above nonstimulated levels over a cation concentration range of 3–100 mM. Monovalent cations also enhanced [^{3}H]Win 55212–2 specific binding to the CB_2 receptor in the presence of 1 mM Mg^{2+}, although the stimulation was less pronounced (Fig. 3B).

The effect of divalent and monovalent cations on [^{3}H]Win 55212–2 specific binding to the CB_2 receptor was examined in more detail by performing saturation analyses in the absence and presence of 1 mM Mg^{2+} or 100 mM Na^+. In the absence of added cation, the affinity of [^{3}H]Win 55212–2 for the CB_2 receptor was reduced (K_d of 6.7 nM and 4.1 nM, two experiments). The addition of either 1 mM Mg^{2+} or 100 mM Na^+ provoked an increase in the affinity of the radioligand for the receptor [K_d of 1.8 nM and 2.0 nM (two experiments) with 1 mM Mg^{2+} and 2.0 nM and 2.1 nM (two experiments) with 100 mM Na^+]. The presence of divalent and monovalent cations also increased the apparent number of [^{3}H]Win 55212–2 specific binding sites, up to 1.4-fold in the presence of 1 mM Mg^{2+} and up to 2-fold in the presence of 100 mM Na^+, depending upon the membrane preparation used. Divalent and monovalent cations, therefore, increase both the affinity of [^{3}H]Win 55212–2 for the CB_2 receptor and the maximal number of detectable [^{3}H]Win 55212–2 specific binding sites.

Effects of nucleotide analogues. [^{3}H]Win 55212–2 specific binding to the CB_2 receptor was only slightly inhibited by the addition of the nonhydrolyzable GTP analogue GTPγS to the incubation medium at concentrations up to 1 mM (Fig. 3C). This inhibition by GTPγS was significant at concentrations from 0.1 μM to 100 μM, compared with [^{3}H]Win 55212–2 specific binding to the CB_2 receptor in control experiments using ATPγS over the same concentration range. Saturation analyses performed in the absence and presence of 100 μM GTPγS resulted in both similar K_d values of 2.3 nM and 1.9 nM and comparable B_{max} values of 25 pmol/mg of protein and 23 pmol/mg of protein, respectively. These data are in accordance with the demonstration that [^{3}H]Win 55212–2 specific binding to the CB_2 receptor is not strongly modulated by GTPγS.

Effect of pH. [^{3}H]Win 55212–2 specific binding to the CB_2 receptor was enhanced by increasing the pH of the binding assay incubation buffer over the range of pH 5.0–7.0 (Fig. 3D). Optimal specific binding was observed between pH 7.0 and 7.5, with a slight decrease occurring at pH 8.0. All [^{3}H]Win 55212–2 binding assays were performed at pH 7.4 in 10 mM HEPES-KOH.

Effect of HSA. Traditionally, CB receptor binding assays have been performed in the presence of high concentrations (1–5 mg/ml) of BSA, to maintain hydrophobic ligands such as Δ^9-THC in solution (2, 10, 14). Saturation analyses of [^{3}H]Win 55212–2 specific binding to the CB_2 receptor were therefore performed in the absence and presence of HSA, to determine whether the inclusion of this additive in the incubation medium affected either K_d or B_{max} values. When saturation analysis was performed in the absence of HSA, ap-

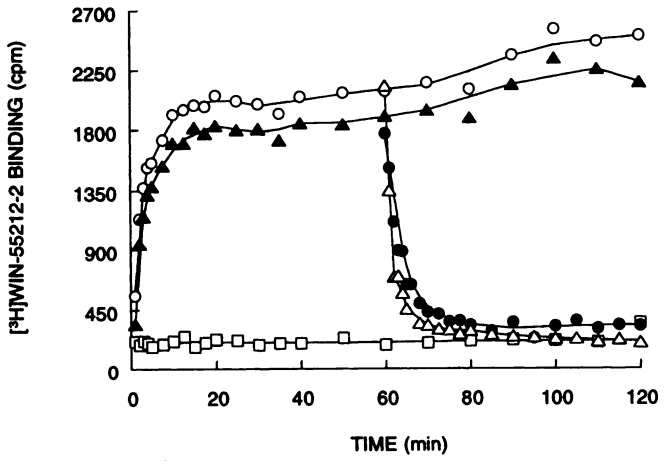

Fig. 2. Rates of association and dissociation of [^{3}H]Win 55212–2 specific binding to the CB_2 receptor expressed in membranes from COS-M6 cells. The rates of association of total (○) and nonspecific (□) [^{3}H]Win 55212–2 binding were monitored by the sequential addition of COS-M6 membranes to separate incubation tubes containing the [^{3}H]Win 55212–2 binding assay incubation mixture (preincubated to 30°). Membranes were added at the required time intervals, beginning with the final time point (120 min) and continuing to the initial time point (1 min). The incubation was conducted at 30° and the reaction was terminated by simultaneous filtering of all samples at time 0. Specific binding (▲) was deduced by subtracting nonspecific binding, measured in the presence of 1 μM Win 55212–2, from total binding. The rate of dissociation of [^{3}H]Win 55212–2 binding was monitored at 30° after sequential addition of 1 μM Win 55212–2 (●) or 1 μM Win 55212–2 with 100 μM GTPγS (△) to individual [^{3}H]Win 55212–2 binding assay incubations at equilibrium. Competing ligands were added at the required time intervals, beginning with the final time point (60 min) and continuing to the initial time point (120 min). The reaction was terminated by simultaneous filtering of all samples at 120 min. These are representative data from two experiments giving similar results.

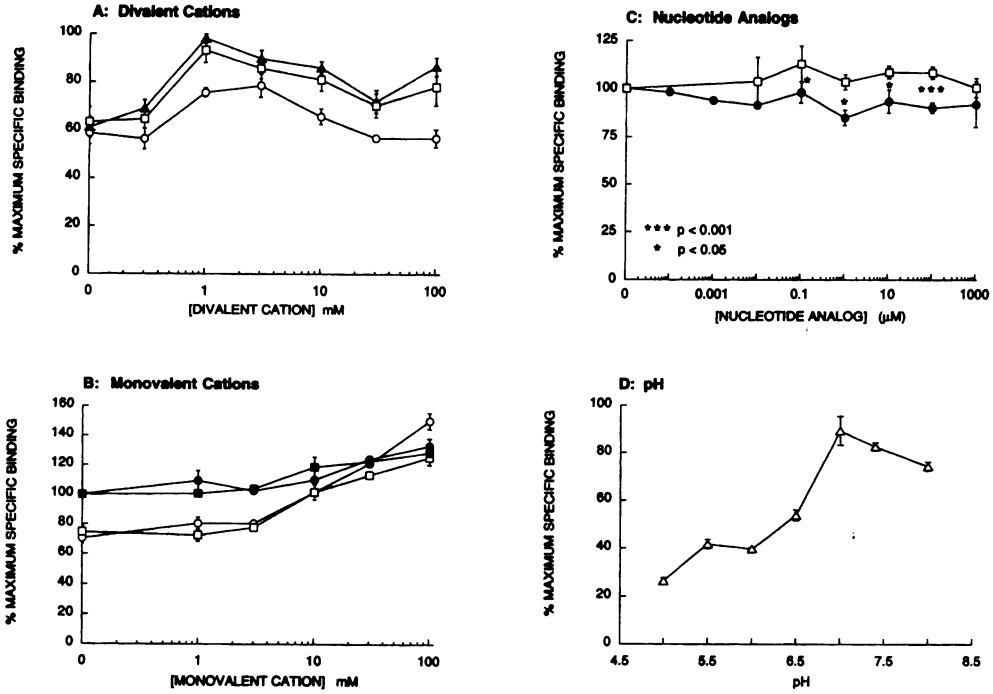

Fig. 3. Effects of divalent and monovalent cations, nucleotide analogues, and pH on [^{3}H]Win 55212–2 specific binding to the CB_2 receptor expressed in membranes from COS-M6 cells. [^{3}H]Win 55212–2 binding assays were performed, as described in Materials and Methods, in the presence of 0–100 mM $MgCl_2$ (▲), $CaCl_2$ (□), or $MnCl_2$ (○) (A), 0–100 mM NaCl (●, ○) or KCl (■, □) in the absence of (○, □) or presence (●, ■) of 1 mM $MgCl_2$ (B), or 0–1 mM GTPγS (●) or ATPγS (□) (C) and over the range of pH 5.0–8.0 with 10 mM concentrations of the following buffers: sodium acetate, pH 5.0; sodium acetate, pH 5.5; MES-KOH, pH 6.0; Bis-Tris-HCl, pH 6.5; BES-KOH, pH 7.0; HEPES-KOH, pH 7.5; and Tricine-KOH, pH 8.0 (D). The specific binding obtained at each cation concentration has been calculated as a percentage of the specific binding obtained in the presence of 1 mM $MgCl_2$ and expressed as a function of cation concentration. The specific binding obtained at each nucleotide analogue concentration has been calculated as a percentage of the specific binding obtained in the absence of nucleotide analogue and expressed as a function of nucleotide analogue concentration. The specific binding obtained at each pH has been calculated as a percentage of the maximal specific binding obtained in each individual experiment and expressed as a function of pH. Data points are the mean ± standard error from six experimental values. Significance was calculated by *t* distribution.

proximately 75% of the radioligand was lost during the experimental manipulations and saturation of the receptor binding sites was not achieved, precluding the calculation of K_d and B_{max} values (data not shown). This loss was completely prevented by the inclusion of 0.3 mg/ml HSA in the incubation medium, which resulted in a K_d of 2.1 ± 0.2 nM (four experiments) and a B_{max} of 24.1 ± 4.4 pmol/mg of protein (four experiments). Increasing the HSA concentration in the binding assay mixture to either 1.0 or 3.0 mg/ml did not significantly change the affinity of Win 55212–2 for the CB_2 receptor, with K_d values of 1.7 nM with both HSA concentrations. The maximal number of detectable specific binding sites was also unaltered, remaining within the standard error of the B_{max} determined with 0.3 mg/ml HSA (B_{max} = 26 pmol/mg of protein with 1 mg/ml HSA and B_{max} = 19 pmol/mg of protein with 3 mg/ml HSA). As a result of these data, 0.3 mg/ml HSA was routinely included in [^{3}H]Win 55212–2 binding assays.

Equilibrium competition assays. CB receptor agonists were evaluated for their ability to compete stereoselectively for [^{3}H]Win 55212–2 specific binding to the CB_2 receptor (Table 1). The classical cannabinoid (−)-Δ^9-THC had an equilibrium inhibition constant (K_i) value of 24.7 ± 2.3 nM (three experiments) in competition for [^{3}H]Win 55212–2 specific binding to the CB_2 receptor. The corresponding enantiomer, (+)-Δ^9-THC, was approximately 20-fold less active, with a K_i value of 537 ± 160 nM (three experiments). In comparison, the (−)-Δ^9-THC analogue HU-210 (23) displayed the highest affinity for the CB_2 receptor of all the competing ligands tested, with a K_i of 0.36 ± 0.05 nM (four experiments). Again, the corresponding enantiomer HU-211 was approximately 1100-fold less active than HU-210, with a K_i value of 385 nM (two experiments).

TABLE 1

Competition for [^{3}H]Win 55212–2 specific binding to the CB_2 receptor expressed in membranes from COS-M6 cells

[^{3}H]Win 55212–2 equilibrium binding assays were performed as described in Materials and Methods. Competing ligands were added to the incubation medium in dimethylsulfoxide to a final vehicle concentration of 1% (v/v), which was kept constant in all samples. Competition experiments were analyzed and the IC_{50} values were determined from the four-parameter logistic fit using the equation $y = (m1 - m2)/[1 + (m0/m3)e^{m4}] + m2$, where *m1* is the maximum and *m2* is the minimum of the curve, *m3* represents the inflection point, *m4* is the slope of the line at the inflection, *m0* is the concentration of the competing ligand, and *y* is the percentage specific binding. The K_i values were calculated from the equation $K_i = IC_{50}/1 + ([radioligand]/K_d)$ and are expressed as the mean ± standard error from three to seven individual experiments performed in duplicate.

Ligand	K_i
	nM
(−)-Δ^9-THC	24.7 ± 2.3
(+)-Δ^9-THC	537 ± 160
HU-210	0.36 ± 0.05
HU-211	184, 585
(−)-CP-55940	1.0 ± 0.2
(+)-CP-55940	28.0 ± 4.5
Win 55212–2	1.3 ± 0.2
Win 55212–3	>1000
Anandamide	90.3 ± 5.1

Two structurally distinct, synthetic, CB receptor agonists were also evaluated, i.e., the (−)-Δ^9-THC analogue (−)-CP-55940 (24) and the indole Win 55212–2 (25). These ligands were equipotent in competition for [^{3}H]Win 55212–2 specific binding to the CB_2 receptor, with K_i values of 1.0 ± 0.2 nM (three experiments) and 1.3 ± 0.2 nM (seven experiments), respectively (Table 1). As expected, the corresponding stereoisomers displayed reduced affinities for the CB_2 receptor, with a K_i value of 28.0 ± 4.5 nM (three experiments) for (+)-CP-55940, whereas Win 55212–3 was inactive at concentrations up to 10 μM (three experiments). Anandamide, which is a putative endogenous ligand for the central CB_1 receptor (19), was also evaluated and had a K_i value of 90.3 ± 5.1 nM (four experiments) in competition for [^{3}H]Win 55212–2 specific binding to the CB_2 receptor. The K_i value for anandamide was not altered by the presence of 50 μM phenylmethylsulfonyl fluoride, which has been reported to prevent the metabolism of anandamide by amidases present in some membrane and tissue preparations (data not shown) (26).

[^{3}H]Win 55212–2 specific binding to the CB_2 receptor expressed in CHO-K1 cells. [^{3}H]Win 55212–2 specific binding to the CB_2 receptor was also investigated in the CHO-K1 stable cell line used in the signal transduction studies described below. Saturation analysis demonstrated saturable [^{3}H]Win 55212–2 specific binding of high affinity, with a K_d of 3.8 nM and a B_{max} of 4 pmol/mg of protein. In addition, [^{3}H]Win 55212–2 specific binding to CB_2 receptors in CHO-K1 cell membranes was moderately stimulated by divalent and monovalent cations but was unaffected by GTPγS, in a manner analogous to that of the CB_2 receptor in COS-M6 cell membranes (data not shown).

Signal Transduction Pathway of the CB_2 Receptor

Inhibition of forskolin-stimulated adenylyl cyclase. The CB_2 receptor-expressing CHO-K1 cell line was used to examine the signal transduction pathway of the agonist-stimulated CB_2 receptor. Activation of the adenylyl cyclase pathway was examined using the potent CB receptor agonist HU-210 in both CB_2-CHO-K1 cells and control CHO-K1 cells (Fig. 4). Basal cAMP levels were 0.130 ± 0.004 and 0.106 ± 0.002 pmol/10^5 cells (five experiments) in the CB_2 receptor-expressing and control cell lines, respectively, when measured in the presence of 100 μM RO-20–1724. Addition of 100 nM HU-210 did not increase intracellular cAMP production over basal levels, with values of 0.118 ± 0.003 and 0.096 ± 0.008 pmol/10^5 cells (three experiments) for the CB_2-CHO-K1 and CHO-K1 cell lines, respectively. These results show that agonist activation of the CB_2 receptor does not result in stimulation of adenylyl cyclase.

To investigate whether agonist activation of the CB_2 receptor resulted in a decrease in intracellular cAMP production, cells were challenged with 5 μM forskolin to stimulate adenylyl cyclase, both in the absence and in the presence of 100 nM HU-210 (Fig. 4). Challenge of CB_2-CHO-K1 cells with 5 μM forskolin stimulated cAMP production to 10.5 ± 0.4 pmol/10^5 cells (five experiments). The presence of 100 nM HU-210 inhibited 75% of the forskolin-stimulated cAMP production in CB_2-CHO-K1 cells, reducing the level to 2.8 ± 0.3 pmol/10^5 cells (three experiments). In contrast, challenge of control CHO-K1 cells with 5 μM forskolin alone raised cAMP levels to 21.5 ± 0.5 pmol/10^5 cells (five experiments), 2-fold

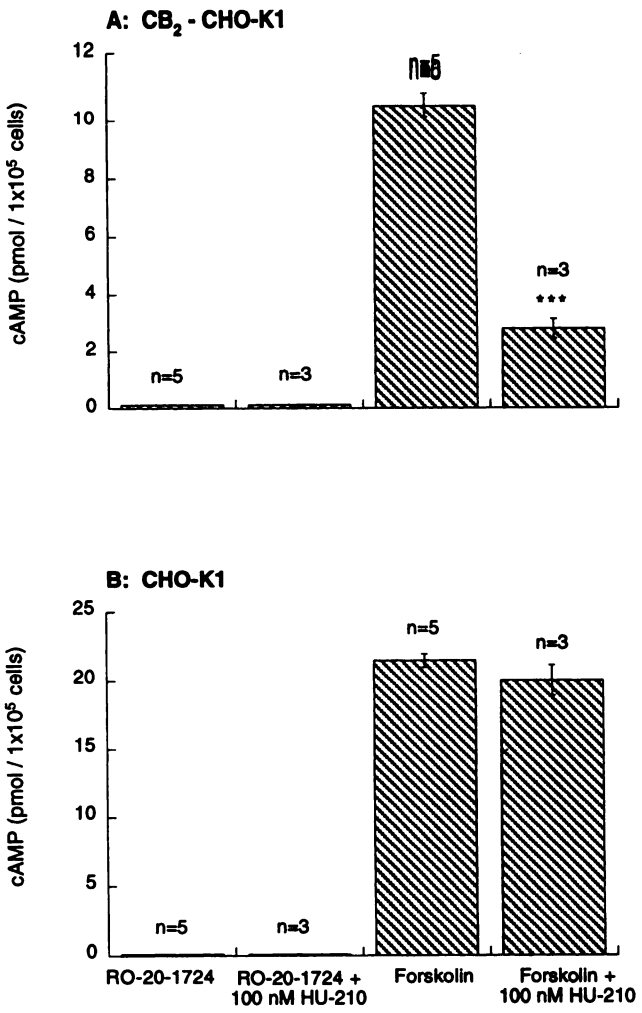

Fig. 4. Inhibition of forskolin-stimulated cAMP production by the CB receptor agonist HU-210 in CHO-K1 cells expressing the CB_2 receptor. CB_2-CHO-K1 (A) and control CHO-K1 (B) cells were incubated in the absence and presence of both 5 μM forskolin and 100 nM HU-210, in HEPES-buffered Krebs-Ringer solution containing 1 mg/ml HSA and 100 μM RO-20–1724, as described in Materials and Methods. The reaction was initiated by addition of 10^5 cells, and the samples were incubated at 37° for 15 min before termination of the reaction by immersion of the samples in boiling water for 3 min. Measurement of cAMP in the samples was performed by radioimmunoassay. Results have been expressed as the cAMP produced (in pmol/10^5 cells). Data points are the mean ± standard error from three to five experimental values. ***, Significance was $p < 0.001$, calculated by t distribution.

higher than observed in the CB_2-CHO-K1 cell line. Agonist challenge with 100 nM HU-210, however, did not significantly inhibit cAMP production in control CHO-K1 cells, which remained constant at 20.0 ± 1.1 pmol/10^5 cells (three experiments). These results demonstrate that agonist activation of the CB_2 receptor expressed in CHO-K1 cells inhibits adenylyl cyclase and that this inhibition is indeed mediated through the CB_2 receptor, because it does not occur in control CHO-K1 cells.

Stereoselective inhibition of forskolin-induced adenylyl cyclase activity. Known cannabimimetics that are agonists at the CB_1 receptor (16, 20) and that display high affinity for the CB_2 receptor (Table 1) (1) were examined for their ability to stereoselectively inhibit forskolin-stimulated cAMP production in the CB_2-CHO-K1 cell line (Fig. 5). HU-210 was the most potent compound to inhibit adenylyl cy-

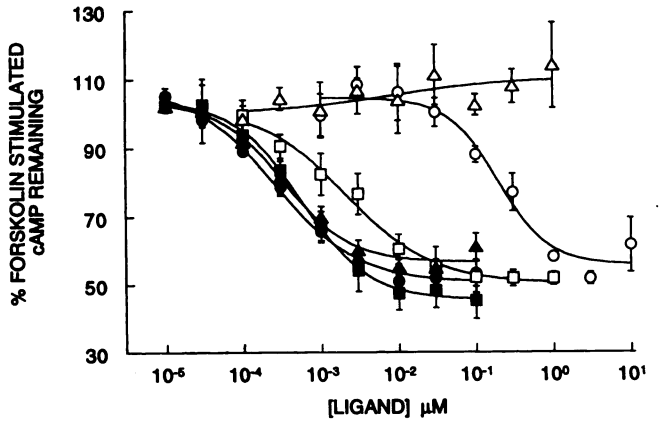

Fig. 5. Stereoselective inhibition of forskolin-stimulated cAMP production by cannabimimetics in CHO-K1 cells expressing the CB_2 receptor. CB_2-CHO-K1 cells were incubated in HEPES-buffered Krebs-Ringer solution containing 1 mg/ml HSA, 5 μM forskolin, and 100 μM RO-201724, as described in Materials and Methods. The incubation also contained HU-210 (●), HU-211 (○), (−)-CP-55940 (■), (+)-CP-55940 (□), Win 55212–2 (▲), or Win 55212–3 (△) over a concentration range up to 1 μM. The reaction was initiated by addition of 1.5×10^5 cells, and the samples were incubated at 37° for 15 min before termination of the reaction by immersion of the samples in boiling water for 3 min. Measurement of cAMP in the samples was performed by radioimmunoassay. Results have been expressed as the percentage of cAMP production obtained in the presence of 5 μM forskolin remaining at each ligand concentration. Data points are the mean ± standard error of three to five experimental values measured in duplicate.

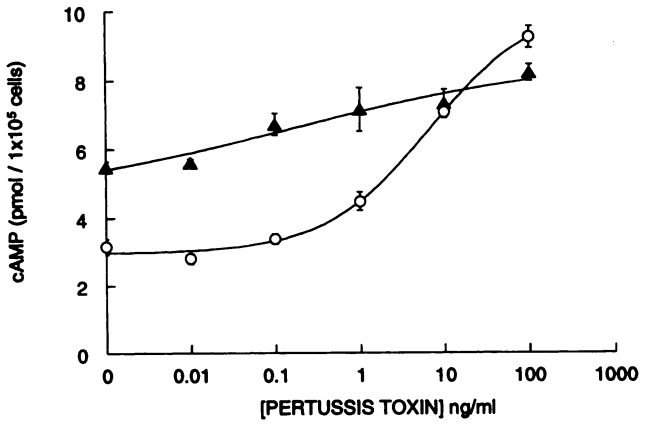

Fig. 6. Sensitivity to PTX treatment of agonist-induced inhibition of forskolin-stimulated cAMP production in CHO-K1 cells expressing the CB_2 receptor. CB_2-CHO-K1 cells were grown in culture for 12 hr in the presence of 0–100 ng/ml PTX. CB_2-CHO-K1 cells were then harvested and incubated in HEPES-buffered Krebs-Ringer solution containing 1 mg/ml HSA, 100 μM RO-201724, and 5 μM forskolin, in the absence (▲) and presence (○) of 100 nM HU-210, as described in Materials and Methods. The reaction was initiated by addition of 10^5 cells, and the samples were incubated at 37° for 15 min before termination of the reaction by immersion of the samples in boiling water for 3 min. Measurement of cAMP in the samples was performed by radioimmunoassay. Results have been expressed as the cAMP produced (in pmol/10^5 cells) as a function of PTX concentration. Data points are the means ± standard errors from three to five experimental values.

clase activity, with an EC_{50} value of 0.37 ± 0.07 nM (five experiments). The HU-210 enantiomer HU-211 was 800-fold less active, with an EC_{50} value of 286 ± 110 nM (three experiments). (−)-CP-55940 and Win 55212–2 were equipotent, with EC_{50} values of 0.72 ± 0.24 nM (three experiments) and 0.63 ± 0.09 nM (four experiments), respectively. (+)-CP-55940, the corresponding enantiomer of (−)-CP-55940, was 9-fold less active, with an EC_{50} value of 6.8 ± 1.6 nM (three experiments), whereas the Win 55212–2 enantiomer Win 55212–3 was inactive at concentrations up to 1 μM (three experiments). In control experiments, these CB receptor agonists did not stimulate adenylyl cyclase in CB_2-CHO-K1 cells, nor did they inhibit or stimulate adenylyl cyclase activity in control CHO-K1 cells when tested at concentrations shown to produce maximal inhibition of adenylyl cyclase in CB_2-CHO-K1 cells (100 nM to 10 μM) (data not shown).

In contrast, (−)-Δ^9-THC, its enantiomer (+)-Δ^9-THC, and anandamide all increased cAMP production in the presence of 5 μM forskolin, in a concentration-dependent manner, when incubated with either CB_2 receptor-expressing or control CHO-K1 cells. These compounds, however, did not increase cAMP production in the CB_2-CHO-K1 and CHO-K1 cells in the absence of forskolin.

Inhibition of forskolin-stimulated adenylyl cyclase activity and sensitivity to PTX. CB_2 receptor-expressing CHO-K1 cells were maintained in culture and pretreated with 0–100 ng/ml PTX for 12 hr. The cells were then harvested and tested for their ability to inhibit forskolin-stimulated cAMP production in the absence and presence of 100 nM HU-210. PTX blocked the agonist-induced inhibition of forskolin-stimulated cAMP production in a concentration-dependent manner, with maximal inhibition occurring at 10 ng/ml PTX (Fig. 6).

$[Ca^{2+}]_i$ measurements with fura-2/AM. Receptor-mediated increases in $[Ca^{2+}]_i$ were also examined in CB_2-CHO-K1 and control CHO-K1 cell lines after loading of the cells with the cell-permeable fluorescent probe fura-2/AM. There were no significant increases in $[Ca^{2+}]_i$ observed upon agonist challenge of either CB_2-CHO-K1 or control CHO-K1 cells, using concentrations of HU-210 up to 1 μM. Cells were viable at the end of each experiment, as monitored by the large increase in $[Ca^{2+}]_i$ observed upon challenge with 1 μM levels of the calcium ionophore ionomycin, followed by quenching of the intracellular Ca^{2+} release by addition of 1 mM EDTA (data not shown).

Discussion

The nucleotide and amino acid sequences for the CB_2 receptor have been reported (1). In this study, the cloned human CB_2 receptor has been characterized by detailed radioligand binding studies with the aminoalkylindole agonist [^{3}H]Win 55212–2. The level of expression of the CB_2 receptor achieved by transient transfection in this cell line was extremely high, as shown by the maximal number of detectable specific binding sites, which was 24.1 ± 4.4 pmol/mg of protein under the standard binding assay conditions. [^{3}H]Win 55212–2 specific binding to the CB_2 receptor was also of reasonably high affinity, with a K_d of 2.1 ± 0.2 nM (four experiments), approximately 2-fold lower than the previously reported value (1).

Radioligand binding studies with many different G protein-coupled receptors have established criteria that generally describe agonist binding to this family of receptors. For example, agonist specific binding has been shown to be regulated by both divalent and monovalent cations. Divalent cations, in particular Mg^{2+}, increase the affinity of an agonist for its receptor, which leads to stimulation of agonist

specific binding (27). In addition, high affinity Mg^{2+} binding to the G protein α subunit in the presence of agonist-stimulated receptor is required for the association of GTP and, thereafter, αGTP-$\beta\gamma$ subunit dissociation (28, 29). Monovalent cations, in particular Na^+, also allosterically regulate agonist interaction with many G protein-coupled receptors, leading to an inhibition of specific binding (30). A mechanism for this regulation was first proposed for the α_2-adrenergic receptor, whereby Na^+ interaction with aspartate-79 promoted dissociation of the agonist from the receptor (31). Because this second transmembrane domain aspartate residue is conserved among all G protein-coupled receptors, this was proposed as a generally applicable mechanism. In agreement with this idea, mutagenesis of this conserved aspartate to an asparagine residue also abolished Na^+ regulation of agonist specific binding to the somatostatin receptor subtype 2 (32) and the δ-opioid receptor (33). It seems, however, that Na^+ can exert pleiotropic effects. For example, Na^+ did not modulate agonist specific binding to the angiotensin II type 1a receptor, and subsequent mutagenesis of aspartate-74 did not alter agonist affinity, either in the absence or in the presence of Na^+ (34). In addition, Na^+ increased the affinity of agonist binding to the cloned rat D_2 dopaminergic receptor expressed in GH_4ZR_7 cells (35) but had no effect on agonist binding to the bovine pituitary D_2 dopaminergic receptor (36).

The effects of both divalent and monovalent cations on [^{3}H]Win 55212–2 specific binding to the CB_2 receptor were examined. A significant number of relatively high affinity CB_2 receptor specific binding sites were detectable in the absence of added divalent cations. The introduction of an optimal concentration of Mg^{2+} did not strongly stimulate [^{3}H]Win 55212–2 specific binding to the CB_2 receptor, resulting primarily in a 2–3-fold increase in affinity. Surprisingly, monovalent cations did not inhibit but instead potentiated [^{3}H]Win 55212–2 specific binding to the CB_2 receptor, with 100 mM Na^+ resulting in a 2–3-fold increase in affinity and also a 1.3–2-fold increase in the number of specific binding sites. Although these results are consistent with a role for Mg^{2+} in stabilizing the high affinity Win 55212–2-CB_2 receptor-G protein complex, they are incompatible with the idea that Na^+ provokes dissociation in this system. The effects of Na^+ suggest a more general role for monovalent cations, possibly by changing the conformation of the receptor through interaction with membrane components such as phospholipids (37).

Another characteristic of agonist specific binding to G protein-coupled receptors is that it is strongly inhibited by nonhydrolyzable GTP analogues such as GTPγS. Models of G protein activation of effector enzymes involve high affinity ligand binding to the receptor, which then binds the heterotrimeric α-GDP-$\beta\gamma$ complex and initiates the concomitant replacement of GDP with GTP. The hydrolysis of GTP to GDP terminates effector stimulation (38). Nonhydrolyzable GTP analogues block the "off" GTPase reaction (39), and the subsequent shift to low affinity binding can be observed experimentally by a reduction in specific binding (40). GTPγS, however, only slightly inhibits [^{3}H]Win 55212–2 specific binding to the CB_2 receptor, both in the highly CB_2 receptor-expressing transfected COS-M6 cells and in the functionally coupled CB_2-CHO-K1 cell line. These results suggest either that there is little difference in the affinity of the CB_2 receptor for [^{3}H]Win 55212–2 between the coupled and uncoupled states or that the major population of CB_2 receptors in both the transiently and stably CB_2 receptor-expressing cell lines is uncoupled, due to the high level of expression of the CB_2 receptor found in these cell lines, and that G protein-mediated signal transduction occurs through a small population of coupled receptors that is not detectable by saturation analysis. If the latter hypothesis is true, it is possible that the small potentiation of [^{3}H]Win 55212–2 specific binding to CB_2 receptors elicited by Mg^{2+} also occurs because the divalent cation modulates only this limited population of CB_2 receptors that can couple with the available G proteins.

In comparison, radioligand binding characterization of the rat CB_1 receptor with either of the CB receptor agonists (−)-[^{3}H]CP-55940 (12) or [^{3}H]Win 55212–2 (14) showed potentiation of specific binding by divalent cations, particularly Mg^{2+} and Ca^{2+}, and inhibition of specific binding by both Na^+ and the nonhydrolyzable guanine nucleotide analogues GTPγS and guanosine-5′-(β,γ-imido)triphosphate. In contrast, the receptor binding data described in this report demonstrate that [^{3}H]Win 55212–2 specific binding to the CB_2 receptor does not display the characteristics that are typical for most agonist interactions with G protein-coupled receptors. It is possible that these data arise as a result of the heterologous expression of cloned receptors in non-native cell lines.

Competition for [^{3}H]Win 55212–2 specific binding to the CB_2 receptor by cannabinoid ligands was stereoselective. The active stereoisomers displayed a rank order of affinities such that HU-210 > (−)-CP-55940 ~ Win 55212–2 > Δ^9-THC > anandamide. With the exception of Win 55212–2 and anandamide, these results are similar to published data for (−)-[^{3}H]CP-55940 specific binding to the human CB_1 receptor (13) and [^{3}H]Win 55212–2 specific binding to the rat CB_1 receptor (14). Win 55212–2 appears to be less selective for the human CB_1 receptor. However, ligands such as Δ^9-THC and (−)-CP-55940 do not appear to be selective for either the human CB_2 or CB_1 receptors. The K_i values reported here are all lower than the K_i values reported by Munro *et al.* (1) for Δ^9-THC, (−)-CP-55940, and anandamide, which may be due to the differences in binding assay methodologies. Both studies, however, show that anandamide has 4-fold lower affinity than does (−)-Δ^9-THC at the CB_2 receptor and is not expected to be a candidate endogenous ligand for this receptor.

The CB_2 receptor signal transduction pathway was established by examining agonist activation of the recombinant receptor expressed in CHO-K1 cells. CB receptor agonist challenge of the CB_2-CHO-K1 cells inhibited forskolin-stimulated cAMP production but did not increase intracellular cAMP levels in the absence of forskolin. The inhibition of adenylyl cyclase activity was mediated by the CB_2 receptor, as it was concentration dependent and stereoselective and did not occur in the nontransfected cell line. The rank order of potency of the active stereoisomers at the CB_2 receptor was HU-210 > (−)-CP-55940 ~ Win 55212–2. The finding that Win 55212–2 is equipotent with (−)-CP-55940 differs from their relative order of potencies at the cloned rat CB_1 receptor, where Win 55212–2 is 13-fold less potent than (−)-CP-55940 (13). In addition, whereas (−)-CP-55940 displays comparable potency in evoking CB_1 and CB_2 receptor-mediated inhibition of adenylyl cyclase activity, the related enantiomer

(+)-CP-55940 is 39- and 11-fold more active in inhibiting cAMP production through the CB_2 receptor than via the recombinant human and rat CB_1 receptors, respectively (2, 16).

In contrast, (−)-Δ^9-THC, its enantiomer (+)-Δ^9-THC, and anandamide all increased cAMP production in both CB_2-CHO-K1 and control CHO-K1 cells in the presence, but not the absence, of forskolin. The increase in cAMP production obtained in the presence of these compounds, therefore, is not receptor mediated and, unfortunately, precludes determination of whether (−)-Δ^9-THC and anandamide are also agonists at the CB_2 receptor. A recent report has, however, suggested that anandamide behaves as an antagonist at the CB_2 receptor (41). That study demonstrated that the rat basophilic leukemia RBL-2H3 cell line expresses only the peripheral and not the central CB receptor. Cannabimimetics and, interestingly, palmitoylethanolamide, but not anandamide, inhibited antigen-stimulated RBL-2H3 cell degranulation, albeit at micromolar concentrations, suggesting a role for the CB_2 receptor in the negative regulation of mast cell activation. In that system, anandamide was shown to antagonize these cannabinoid-mediated effects.

The ability of the CB receptor agonist HU-210 to stimulate calcium mobilization in CB_2-CHO-K1 and control CHO-K1 cells was also examined. It was found that HU-210, at concentrations up to 1 μM, did not increase $[Ca^{2+}]_i$ in either CB_2 receptor-expressing or control CHO-K1 cells. These results are in contrast to previous findings, where HU-210, at concentrations of 1 μM and 10 μM, was able to stimulate increases in $[Ca^{2+}]_i$. This occurred through a non-receptor-mediated mechanism, however, because these responses occurred both in the rat CB_1 receptor-expressing and control CHO cells (13).

The inhibition of forskolin-stimulated cAMP production in the CB_2-CHO-K1 stable cell line by the CB receptor agonist HU-210 was completely blocked by pretreatment of the cells with 10 ng/ml PTX. There is a significant molecular diversity of substrates for the ADP-ribosylation by PTX, ranging through the α_i, α_o, α_{gust}, and α_t classes of G proteins (38). Of this PTX-sensitive class of α subunits, α_i is thought to be the major regulatory protein involved in the hormonal inhibition of adenylyl cyclase (38). Therefore, these results demonstrate the involvement of a PTX-sensitive class of G proteins, probably $G_{\alpha i}$, in the inhibition of adenylyl cyclase activity after agonist stimulation at the CB_2 receptor.

In conclusion, a comprehensive characterization of the CB_2 receptor by radioligand binding analysis using [^{3}H]Win 55212–2 has been completed. These results have established K_i values for a series of known cannabimimetics. In addition, we have shown that the signal transduction pathway for the recombinant CB_2 receptor in CHO-K1 cells involves inhibition of intracellular cAMP production.

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