CHARACTERIZATION OF PERIPHERAL HUMAN CANNABINOID RECEPTOR
(hCB2) EXPRESSION AND PHARMACOLOGY USING
A NOVEL RADIOLIGAND, [35S]SCH225336*

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Studies to characterize the endogenous expression and pharmacology of peripheral human cannabinoid receptor (hCB2) have been hampered by the dearth of authentic anti-hCB2 antibodies and the lack of radioligands with CB2 selectivity. We recently described a novel CB2 inverse agonist, N-[1(S)-[4-[(4-methoxy-2-[(4methoxyphenyl)sulfonyl]phenyl)sulfonyl]phenyl]ethyl]methane-sulfonamide (Sch225336) which binds hCB2 with high affinity and excellent selectivity versus hCB1. The precursor primary amine of Sch225336 was prepared and reacted directly with 35S-mesyl chloride (synthesized from commercially obtained 35S-methane sulphonic acid) to generate [35S]Sch225336. [35S]Sch225336 has high specific activity (>1400 Ci/mmol) and affinity for hCB2 (65 pM). Using [35S]Sch225336, we assayed hemopoietic cells and cell lines to quantitate the expression and pharmacology of hCB2. Lastly, we used [35S]Sch225336 for detailed autoradiographic analysis of CB2 in lymphoid tissues. Based on these data, we conclude that [35S]Sch225336 represents a unique radioligand for the study of primate CB2 endogenously expressed in blood cells and tissues.

The endocannabinoids, anandamide and 2-arachidonyl glycerol (2-AG) are ligands for two G protein-coupled cannabinoid receptors, CB1 and CB2. It has been hypothesized that human CB2 (hCB2) and its ligands are immune regulators due to the defined expression of hCB2 mRNA in peripheral immune cells and tissues. This hypothesis was bolstered by studies showing that 2-AG, a full agonist at hCB2 (1), stimulated chemotaxis of various hemopoietic cells including differentiated monocyctic and eosinophilic cells such as HL-60, THP-1, U937, leukemia EoL-1 cells as well as human monocytes, eosinophils and dendritic cells (2, 3, 4). CB2 was implicated in this response due to its sensitivity to blockade by the CB2 inverse agonist, SR144528. However, direct measurement of hCB2 protein expression in immune cells has been hampered by the lack of radioligands with CB2 selectivity and high specific activity. Binding analyses of CB2 have used tritiated agonists such as [3H]CP55,940 or [3H]WIN55,212 which bind with low nanomolar affinity but have low specific activities (S.A. = 20-180 Ci/mmol) and little or no selectivity vs. CB1. As a result, CB2 pharmacology has been almost completely restricted to studies with recombinantly expressed receptor (for review, see 5). Recently, we described a novel CB2 inverse agonist, N-[1(S)-[4-[(4-methoxy-2-[(4methoxy-phenyl)sulfonyl]phenyl)sulfonyl]phenyl]phenyl]ethyl]methanesulfonamide (compound 4j; Sch225336) which binds hCB2 with picomolar affinity and has excellent selectivity vs. hCB1 (6). Using commercially obtained 35S-methane sulphonic acid, 35S-mesyl chloride was prepared and reacted directly with the precursor primary amine of Sch225336 to generate [35S]Sch225336, a highly potent and CB2-selective radioligand with high specific activity (>1400 Ci/mmol). The studies...
presented herein describe the use of this unique radioligand to extensively characterize CB2 expressed endogenously in blood cells, cell lines and tissue sections.

**EXPERIMENTAL PROCEDURES**

**Cells and Cell Culture** - The clonal CHO-hCB2 cell line was generated and cultured as previously described (1). Ba/F-hCB1 and –hCB2 cells were cultured as previously described (7). MonoMac6, Jurkat, U937, Hut78 and HL-60 cells were cultured in the presence or absence of the indicated differentiation factors as previously described (8, 9, 10, 11). Activated human peripheral blood lymphocytes were generated as previously described (12). Experimental cultures were used 1 to 5 days after seeding. Cell culture medium was purchased from GIBCO-BRL (Grand Island, NY).

**Cell Membrane Preparation** - Sf9 membranes exogenously expressing hCB2 + βγ2Gαi1 or βγ2Gαi1 alone and RB-hCB2 membranes were purchased from PerkinElmer Life and Analytical Sciences (Boston, MA). Other cell membrane preparations were generated as previously described (1). Briefly, adherent cells were harvested using cell dissociation buffer according to the manufacturer instructions (GIBCO-BRL, Grand Island, NY). Dissociated cells and cells grown in suspension were collected by centrifugation and used immediately or stored at -80°C. Cell pellets were resuspended and incubated on ice for 30 min in homogenization buffer (10 mM Tris-HCl, 5 mM EDTA, 3 mM EGTA, pH 7.6) containing 1 mM phenylmethylsulfonyl fluoride (PMSF) as a protease and amidase inhibitor (13, 14). Cells were then homogenized with 15-20 strokes at 900 rpm with a Dounce homogenizer using stirrer type RZR1 polytron homogenizer (Caframo, Wiarton, Ont.). Intact cells and nuclei were removed by low speed centrifugation (500g for 5 min at 4°C). Membranes in the supernatant were pelleted by centrifugation at 100,000g for 30 min at 4°C and then resuspended in gly-gly buffer (20 mM glycyglycine, 1 mM MgCl2, 250 mM sucrose, pH 7.2) and stored at -80°C. Protein determinations were performed using the Bradford method (15).

**Western blot analyses** – Western blots were performed as previously described (16). CHO-hCB2, MonoMac6, Sf9-hCB2, Ba/F-hCB1 and Ba/F–hCB2 cell membranes were solubilized in sample buffer (62.5 mM Tris-HCl, 2% sodium dodecyl sulfate, 10% 2-mercaptoethanol (v/v), 6 M urea, 20% glycerol, pH 6.8) by incubation at 60°C for 15 min. Where indicated, membrane proteins were prepurified by wheat germ agglutinin-agarose chromatography prior to solubilization in sample buffer (16). Proteins were resolved on a 7.5% sodium dodecyl sulfate-polyacrylamide gel and transferred to PVDF membrane. The membrane was then blocked for 2h with “Blotto” (10 mM NaH2PO4, 10% nonfat dry milk, 10% glycerol, 0.2% Tween 20) and incubated overnight at 4°C with the indicated antibody dilutions. Following incubation with appropriate conjugated secondary antibodies, immunoreactive proteins were detected with the ECL chemiluminescent antibody detection system (Amersham Corp.).

**Synthesis of N-(1-{4-[4-Methoxy-2-(4-methoxy-benzenesulphonyl)-benzenesulphonyl]-phenyl}-ethyl)-methane[35S]sulphonamide (35S)Sch225336** - As can be seen in the schematic shown in Fig. 2, [35S]Sch225336 (2.4) was generated using a [35S]mesyl chloride precursor (2.1). To prepare the [35S]mesyl chloride, an aqueous solution of [35S]methane sulphonic acid (2.1) (5 mCi, 40 µL, ca. 1400 Ci/m mole) was evaporated to dryness in a 10 mL V-Vial (Wheaton Scientific) and dissolved in anhydrous methylene chloride (1.5 mL). 150 µL oxaly chloride was added and the reaction was stirred for 30 minutes under Argon prior to the addition of 20 µL 10% DMF. The reaction stirred overnight at room temperature, diluted with methylene chloride (1.5 mL), cooled in an ice bath and washed successively with ice cooled 1% aqueous sodium bicarbonate (2 x 2 mL), room temperature 1% aqueous sodium bicarbonate (2 mL) and 2% aqueous sodium bisulphate (1 mL) solutions. The methylene chloride solution was dried for 1 h over anhydrous sodium sulphate and concentrated to a volume of approximately 50 µL via distillation for immediate use in the next step. In a 300 µL V-Vial (Wheaton Scientific), 1-{4-
[Methoxy-2-(4-methoxy-benzenesulphonyl)-benzenesulphonyl]-phenyl]-ethylamine (2.3) (10 mg) was dissolved in anhydrous methylene chloride (20 µL) and triethylamine (10 µL). The methylene chloride solution of [35S]mesyl chloride (2.2)(ca. 50 µL) was added, and the reaction stirred vigorously for 1 hour. The reaction was concentrated to dryness and the product purified by HPLC on a 9.4 x 250mm Zorbax Extend C18 column with a mobile phase of 0.05M pH9 triethylammonium acetate (40:60) acetonitrile at a flow rate of 5 mL/min. Detection was at 254nm. A total batch of 1.3 mCi of (2.4) at a specific activity of >1400 Ci/mmmole at a radiochemical purity of 99.5% was isolated.

Radioligand Binding and [35S]GTPγS Exchange Assays - In radioligand binding assays, cell membranes (0.1-13 µg/point, in triplicates) were incubated in 100-150 µL binding buffer (20 mM HEPES, 100 mM NaCl, 5 mM MgCl2, and 0.2% (w/v) bovine serum albumin (BSA; Factor V, lipid free), pH 7.4) in 96-well microplates. Incubations contained the indicated concentrations of cold ligands and [3H]CP55,940 (S.A.=180 Ci/mmol; NEN Boston, MA) or [35S]Sch225336 (S.A.=1400 Ci/mmol). After the indicated incubation time, the reaction was terminated by rapid filtration of the membranes through the microfiltration plates coated with 0.5% polyethylenimine (UniFilter GF/C filter plate; Packard, Meriden, CT.), using a Tomtek 96-well cell harvester (Hamden, CT.). The membranes were washed ten times with ice-cold buffer composed of 50 mM Tris, 3 mM MgCl2, 1 mM EDTA, 0.1% (w/v) BSA, pH 7. Membrane bound radioactivity was measured by liquid scintillation using a TopCount NXT Microplate Scintillation and Luminescence Counter (Packard, Meriden, CT.).

Guanosine 5’-[γ-35S]triphosphate ([35S]GTPγS) exchange was measured using a scintillation proximity assay (SPA) as previously described (17). For each assay point, membranes (2 µg/well in triplicate) were preincubated for 30 min at room temperature with 150 µg of wheat germ agglutinin-coated SPA beads (Amersham, Arlington Heights, Ill.) in SPA binding buffer (20 mM HEPES, 100 mM NaCl, 5 mM MgCl2, and 0.2% (w/v) BSA (Factor V, lipid free), pH 7.4) supplemented with 5 µM GDP.). The beads and membranes were transferred to a 96-well Isoplate (Wallac, Gaithersburg, Md.) and incubated for 60 min at room temperature with the indicated concentrations of cannabinoids. The reactions were then incubated for a further 60 min in the presence of 0.1 nM [35S]GTPγS (triethyl ammonium salt; specific activity = 1250 Ci/mmol; NEN, Boston, Mass.). Membrane-bound [35S]GTPγS was then measured using a Trilux 1450 MicroBeta counter (Wallac, Turku, Finland).

Autoradiography and Cytochemical Analysis of Frozen Sections - Adjacent 20 µm tissue sections were cut on a cryostat maintained at -20°C, mounted onto precleaned microscope glass slides (Fisher, Colorfrost/Plus) and stored at -80°C until the day of the assay. Before the experiments, slides were brought to a room temperature and then equilibrated in the assay buffer containing 50 mM HEPES, 5 mM MgCl2, 125 mM NaCl, 1 mM CaCl2, 0.0002% NaN3, 1% (w/v) bovine serum albumin (BSA; Factor V, lipid free, pH 7.4) for 5 min at 25°C. Slides then were transferred to a fresh assay buffer containing 0.1 – 0.3 nM [35S]Sch225336 (1400 Ci/mmol) or 3 nM [3H]CP55,940 in the presence or absence of cold cannabinoids and incubated for 2 hrs at 25°C. Slides were then rinsed three times for 5 min each in cold phosphate-buffered saline supplemented with 0.2% (w/v) bovine serum albumin, rinsed in deionized H2O, and gently dried under a stream of cold air. Sections were exposed to a tritium storage phosphor screen (Molecular Dynamics) for 24 hours at 25°C. The phosphor screens were then scanned and digitized using a phosphor imager Storm 860 (Molecular Dynamics). The sections were then exposed to either Kodak BioMax MS film (tritium) or Kodak BioMax MR film ([35S]Sch225336) (Eastman Kodak, Rochester, NY) for 5-72d at -80°C. In parallel, adjacent sections were either fixed in 10% phosphate-buffered paraformaldehyde and stained with hematoxylin/eosin or fixed with cold 100% acetone and used for immunohistochemistry with anti-CD79α and/or anti-CD3 (DakoCytomation, Carpinteria, CA). For immunohistochemistry, acetone fixed slides were pretreated with 3% H2O2 for 10 min before incubation with primary antibodies for 1hr at R/T. Following the incubation with biotinylated anti-mouse antibody for 30 min
the binding of the antibodies was visualized with Vectastain® Elite ABC kit plus DAB or Vectastain® ABC-AP kit plus Vector Red (Vector, Burlingame, CA). When necessary, the slides were counter stained with hematoxylin.

Data Analysis - Data are reported as mean values ±S.E.M. of at least three independent experiments, each of which were performed in triplicate. Nonlinear regression analysis of saturation data and of concentration-response data was performed using Prism 2.0b software (GraphPad Software, San Diego, CA) to calculate $K_d$, $B_{\text{max}}$, IC$_{50}$ and EC$_{50}$. IC$_{50}$ values were converted to apparent Ki values by the method of Cheng and Prusoff (18) using the $K_d$ values for [3H]CP55,940 determined from saturation experiments.

Materials - The CB2-selective bis-sulfone Sch225336 (N-[1(S)]-[4-[[4-methoxy-2-[[4-methoxyphenyl]sulfonyl]phenyl]sulfonyl]phenyl]ethyl)methanesulfonamide) and the cannabinoid inverse agonist standards SR141716A (N-(piperidin-1-yl)-5-(4-chlorophenyl)-1-(2,4 dichlorophenyl)-4-methyl-1H-pyrazole-3 carboxamide hydrochloride) and SR144528 (N-[[1(S)-endo-1,3,3-trimethylbicyclo[2.2.1]heptan-2-yl]-5-(4-chloro-3-methylphenyl)-1-(4 methyl benzyl)-pyrazole-3carboxamide were prepared by the Department of Chemical Research (Schering-Plough Research Institute). HU-210 was purchased from BioMol Research Laboratories, Plymouth Meeting, PA. All other reagents were of the best grade available and purchased from common suppliers.

RESULTS

Immunoblot analysis of hCB2 protein - Anti-hCB2 antibodies were tested in Western blots for their utility in immunodection of HA-tagged CB2 using an anti-HA antibody as a positive control. Membrane proteins from untransfected CHO-K1 cells or CHO-hCB2 cells overexpressing HA-tagged CB2 were solubilized were separated by SDS-PAGE and electrophoretically transferred to polyvinylidene difluoride membrane. The membranes were incubated with an anti-HA monoclonal antibody or polyclonal antibodies raised against peptides representing unique amino acid sequences in hCB2. The anti-HA antibody recognized a diffuse 55 kDa protein and a faint but more defined 45 kDa protein in the CB2-transfected (but not parental) CHO cells (Fig. 1A). The appearance and $M_r$ of these proteins is reminiscent of that seen in the Western blots of CHO-hCB2 membranes generated by Carayon et al (19) using their polyclonal antibody raised against a synthetic peptide derived from the predicted amino acid sequence of the carboxylic tail of hCB2. The diffuse appearance of the 55 kDa receptor protein is consistent with the migration patterns of a glycoprotein (20, 21). The fainter 45 kDa protein may be a receptor precursor similar to that has been described for other G protein-coupled receptors (20). The classification of hCB2 as a glycoprotein is verified by its successful purification with wheat germ-agglutinin chromatography prior to Western blot analysis (Fig. 1B). None of the purported anti-hCB2 antibodies that we tested (Calbiochem #209552 and #209554, ABR #PA1-744 and #PA1-746, Alexis) successfully identified the 55 kDa (or 45 kDa) receptor protein using a panel of recombinant and tumor cell lines. Representative immunoblots are shown in Fig 1C-E. None of the purported anti-CB2 antibodies immunoreacted with proteins consistent with the experiments with the HA-tagged receptor i.e. a diffuse 55 kDa glycoprotein. With all the antibodies tested, immunoreactive bands were quite sharp, inconsistent with hCB2 (Fig. 1A-B) and G protein-coupled receptors in general (16, 20, 21). Moreover, the immunoreactive protein(s) were either too small or too large and/or were equally evident in samples from cells which do express hCB2 (Fig. 1D-E). With the Calbiochem #209552 antibody (Fig. 1C), a single sharp immunoreactive band (M$_r$ ~60 kDa) was evident in the CHO-hCB2 sample but not in either the Sf9-hCB2 nor the Monomac6 samples. The 746 antibody did not immunoreact with any specific protein. Taken together, these data indicate that these antibodies are less than optimal for the immunodection of authentic hCB2. Therefore, we endeavored to develop a CB2-selective radioligand.

Synthesis of [35S]Sch225336 - The synthesis of [35S]Sch225336 is shown schematically in Fig. 2, 35S-mesyl chloride (2.2) was prepared from
commercially obtained $^{35}$S-methane sulphonic acid (2.1) by the method of Dean et al (22) using oxalyl chloride. The resulting $^{35}$S-mesyl chloride (2.2) was then reacted directly with a large molar excess (ca. 12000) of the precursor primary amine of Sch225336 (2.3) to generate $[^{35}S]$Sch225336 (2.4) in about a 50% crude yield. After preparative reverse phase high liquid chromatography, a batch of 1.3 mCi at a radiochemical purity of 99.5% with a specific activity of > 1400 Ci/mmol was isolated.

**Equilibrium binding analyses with $[^{35}S]$Sch225336** - As can be seen in the saturation binding analysis with CHO-hCB2 membranes shown in Fig. 3A, $[^{35}S]$Sch225336 bound hCB2 in a saturable manner with a $K_d = 0.065$ nM at equilibrium. This affinity constant is slightly lower than the $K_i$ derived in competition binding analyses with $[^3H]$CP55,940 ((6); data not shown). Scatchard analysis of the saturation binding data (Fig. 3A; inset) revealed a single binding site with a Hill $\approx 1$. Saturation analyses with SF9 membranes exogenously expressing hCB2 + $\beta_1\gamma_2\alpha_i$ (Fig. 3B) showed similar high affinity, saturable $[^{35}S]$Sch225336 binding ($K_d = 0.071 \pm 0.002$ nM) with no significant $[^{35}S]$Sch225336 binding in SF9 membranes expressing only $\beta_1\gamma_2\alpha_i$.

The utility of $[^{35}S]$Sch225336 relative to $[^3H]$CP55,940 in competition binding analyses was assessed in the CHO-hCB2 membranes. Radioligand binding was also displaced in a concentration-dependent manner by unlabeled compound (Hill $\approx 1$) (Fig. 4A) though competition bindings with $[^{35}S]$Sch225336 conferred a much superior signal:noise ($B/B_0$) relative to the tritiated agonist (Fig. 4B). $[^{35}S]$Sch225336 binding was inhibited by structurally unrelated cannabinoid agonists including CP55,940 and unlabeled Sch225336 (Fig. 4C), WIN55,212-2, HU210, anandamide and 2-AG (data not shown) and the inverse agonist SR144528 (Fig. 4C). There was no measurable $[^{35}S]$Sch225336 binding in membranes expressing hCB1 (data not shown) consistent with the low affinity of Sch225336 for CB1 measured in competition binding analyses with $[^3H]$CP55,940 (6). Taken together, these data show that $[^{35}S]$Sch225336 represents a novel, high affinity CB2-specific radioligand with high specific activity.

**Analyses of hCB2 expression in peripheral blood lymphocytes and hematopoietic cell lines** - CB2 expression in hematopoietic cells has been reported, largely based on the detection of its mRNA (23, 24, 25, 26) and/or by functional responses to cannabinoids that were attenuated with CB2-selective antagonists (2, 23, 26, 27). As our efforts in CB2 immunodetection were unsuccessful (above), we assessed the utility of $[^{35}S]$Sch225336 to define and quantitate hCB2 expression in hemopoietic cells and cell lines. Saturation binding analyses were performed on membranes from human Jurkat T lymphoma cells (clone E6-1), human T lymphoma HUT78 cells, human monocyctic U937 and MonoMac6 cells, and activated human peripheral lymphocytes (PBL). All of these cells express measurable hCB2 with highest expression in MonoMac6 cell membranes while the PBL and HUT78 had the lowest expression (Fig. 5A). The effect of in vitro activation conditions on PBL expression of hCB2 is shown in Fig. 5B. Membranes were prepared from PBL isolated from two different individuals and treated with PHA in the presence of either IL-2 to stimulated a TH1-like phenotype (28, 29) or IL-4 to stimulated a TH2-like phenotype (29, 30). There was no measurable differences in $[^{35}S]$Sch225336 binding correlating with the two differentiation conditions.

Human promyelocytic leukemia HL-60 cells have been reported to express functional hCB2 (2, 19, 26). Saturation analysis in membranes from HL-60 cells differentiated towards a more neutrophil-like phenotype by prolonged incubation in the presence of DMSO or db-cAMP reveal saturable, high affinity $[^{35}S]$Sch225336 binding (Fig. 5C). $[^{35}S]$Sch225336 binding in membranes from monocytic (PMA-treated) HL-60 cells (Fig. 5D) is considerably less than that measured in DMSO-treated HL-membranes or in the monocyte-like MonoMac6 cells (Fig. 5A). There was no measurable $[^{35}S]$Sch225336 binding in membranes prepared from either retinoic acid-pretreated HL-60 cells which stimulates monocytic differentiation (10) (data not shown) nor in human monocyte THP-1 cell membranes (Fig. 5D). Taken together, these data demonstrate that
[35S]Sch225336 has excellent utility for detection of CB2 at physiological expression levels.

The effect of hCB2 coupling state on [35S]Sch225336 pharmacology - We have previously established that Sch225336 is an inverse agonist (7) based on its ability to inhibit constitutive CB2 signaling and its higher affinity for uncoupled hCB2 conformation(s). To further define the pharmacology of Sch225336, we measured the effect of the nonhydrolysable GTP analog, GppNHp, on [35S]Sch225336 binding in CB2 transfectants (RB-hCB2, Sf9-hCB2) and hemopoietic cell lines (HL-60(DMSO) and U937). GppNHp increased [35S]Sch225336 binding in a concentration-responsive manner although the extent of the increase varied between lines (Fig. 6A and 6B). In RB-hCB2 membranes, which have the most [35S]Sch225336 binding sites, binding increased only slightly with uncoupling, much less than the increase measured either in HL-60(DMSO) membranes or in Sf9-hCB2 membranes which overexpress both hCB2 and its G proteins (1). GppNHp had the largest relative effect on [35S]Sch225336 binding in U937 membranes (Fig. 6B) suggesting that although CB2 expression is relatively low, a greater proportion of the receptor in these membranes is coupled to G proteins. As would be expected, CB2 uncoupling with GppNHp decreased the binding of the agonist [3H]CP55,940 in the Sf9-hCB2 and U937 membranes while [35S]Sch225336 binding increased (Fig. 6C). Note that [3H]CP55,940 binding in U937 membranes is barely measurable. To better assess the impact of receptor coupling in the U937 membranes, we performed saturation analyses with [35S]Sch225336 in these membranes in the presence or absence of another nonhydrolysable GTP analogue, GTPγS (Fig. 6D). In the absence of the uncoupling agent, [35S]Sch225336 bound with a Kd = 0.13 ± 0.04 nM. Consistent with the effect of GppNHp (above), co-incubation with GTPγS increased the affinity of [35S]Sch225336 (Kd = 0.066 ± 0.022 nM) in the U937 membranes without affecting the Bmax. The increased affinity associated with CB2 uncoupling is readily apparent in the Scatchard analyses of the data (Fig. 6D: inset).

Autoradiographic analyses of CB2 expression in spleen and lymphoid tissues - We assessed the utility of [35S]Sch225336 or [3H]CP55,940 for autoradiographic analysis of CB2 expression in human spleen (Figs. 7A and 7B) or human peripheral lymph node (Fig. 7C). Incubation of the spleen slices with either radioligand results in a distinct, punctuate labeling of areas representing splenic white pulp based on lymphocyte surface marker staining (Fig. 8). The images generated with [35S]Sch225336 are much more detailed than those generated with the tritiated material and require relatively short film exposure times (5-8 days) versus that required with [3H]CP55,940 (72 days). Based on superior signal:noise, [35S]Sch225336 was used for analysis of human lymph node sections. As can be seen in Fig 7C, [35S]Sch225336 binding relative to nonspecific binding is apparent, although the displaceable binding is too small to be conclusive. We can conclude, however, that CB2 expression in the lymph node section is significantly lower than in the spleen.

In order to identify the cell types accounting for [35S]Sch225336 binding in the spleen (Figs 8A and B), adjacent sections were stained with B and T cell surface markers, CD79α and CD3 (Figs. 8C and 8D). The pattern of [35S]Sch225336 binding corresponds predominantly to B lymphocytes in the primary lymphoid structures of the spleen (Fig. 8C). [35S]Sch225336 binding to T lymphocytes appears fairly low compared to the B cells (Fig. 8D), although we cannot conclusively rule out T cell binding. Interestingly, there are CD79α-positive B cells scattered in the red pulp (arrows in Fig. 8D) although no significant [35S]Sch225336 binding localized with these cells.

DISCUSSION

Based on initial in situ hybridization studies, it was apparent that hCB2 has a rather defined expression in peripheral immune cells and multiple lymphoid organs (31, 32). CB2 mRNA is was found in spleen, tonsils, bone marrow, mast cells, peripheral blood leukocytes and a variety of hemopoietic cell lines including the myeloid cell line U937 and HL-60 cells (33, 34, 35, 36, 37, 38, 39). Based on these data, it has been suggested that hCB2 may have a direct role in immune
function and/or mediate the immunosuppressive effects of cannabinoids. Subsequently, it was shown that 2-AG, a full agonist at hCB2 (1), stimulated chemotaxis of hemopoietic cells (monocytic HL60, THP-1, U937, human monocytes, leukemia Eo-L1 cells and human peripheral blood eosinophils) via an endogenous receptor (2, 3) The authors reasonably credited this response to CB2 due to its sensitivity to blockade by the CB2 inverse agonist, SR144528 and immunoblot data suggesting that CB2 protein was expressed in eosinophils. Indeed, direct demonstration of CB2 expression has been difficult due to the scarcity of commercially available antibodies with substantial and/or authentic CB2 immunoreactivity. Also, in contrast to the relatively high expression of CB1 in the brain (≥ 1 pmol/mg; (40), CB2 expression in immune cells/tissues is more moderate (10-300 fmol/mg; Fig 5). Lastly, there are no CB2-selective radioligands with high specific activity. Not surprisingly, this has resulted in the almost exclusive use of recombinant expression systems for CB2 binding studies (5). Nonselective cannabinoid radioligands such as [3H]CP55,940 or [3H]WIN55,212 have certainly shown utility in the study of cannabinoid receptor pharmacology. Although these ligands bind with low nanomolar affinity, they have poor specific activities (50-180 Ci/mmol) inherent to tritiated radioligands. As a result, binding studies using [3H]CP55,940 could not quantitate (or even convincingly establish) hCB2 expression in the hemopoietic cells tested in this study (Fig. 6C and data not shown). [35S]Sch225336 is relatively easily synthesized using commercially obtained 35S-methane sulfonic acid. The specific activity achieved in this synthesis (>1400 Ci/mmol) verges on that achieved with carrier-free 125I-labeled ligands. Hence, [35S]Sch225336 represents a first-in-class radioligand for the study of recombinantly or (more importantly) endogenously expressed hCB2.

The primary advantage of [35S]Sch225336 over other cannabinoid radioligands may be its utility for autoradiography. This application is important as the commercially available anti-CB2 antibodies that we tested were ineffective in detecting authentic receptor protein in immunoblot analyses of samples prepared from cells which express hCB2 at high levels either recombinantly (~7-20 pmol/mg) or endogenously in Monomac6 cells (~0.3 pmol/mg hCB2). Using a cell line expressing HA-tagged hCB2, we showed that the receptor migrates as a diffuse 55 kDa protein which is absent in parental CHO cells. The diffuse nature of the band and its absorption to wheat germ-agglutinin-agarose is consistent with the expression of hCB2 as a glycoprotein. The appearance of the receptor in our Western blots is very reminiscent of that seen in immunoblots of CHO-hCB2 membranes by Carayon (19) using their polyclonal antibody raised against a peptide sequence within the carboxylic tail of the receptor. The authors performed very nice studies on endogenous CB2 expression with this antibody, demonstrating expression of the receptor on CD4/CD8 T cells, natural killer cells and B cells. Unfortunately, this reagent is not commercially available.

As an alternative (or additional) approach to immunodetection of cannabinoid receptors, tritiated radioligands have been used successfully for autoradiographic studies of CB1 expression in the central nervous system wherein CB1 is highly expressed (41, 42). The non-selective nature of [3H]CP55,940 or [3H]WIN55,212 for CB1 can be addressed indirectly in these studies through competition with the CB1 and CB2 antagonists (SR141716A and SR144528, respectively) or directly with [3H]SR141716A autoradiography (43). [35S]Sch225336 is the pharmacological counterpart of [3H]SR141716A although its affinity for CB2 (0.065 nM; Figs. 3-6) is higher than the affinity of [3H]SR141716A for CB1 (~ 2 nM) (44). [3H]CP55,940 has been used with some success for autoradiography studies to detect CB2 (presumably) in immune tissues from rat (31, 45) and human (Fig. 7B). However, in our studies, the image quality arising from studies with [35S]Sch225336 are much superior to that generated with the tritiated agonist and the necessity of using emulsions or specialized film to detect the lower energy emissions from tritium is avoided. Indeed, due to its higher specific activity relative to tritiated cannabinoids (> 1400 vs. ~ 20 Ci/mmol), experiments with [35S]Sch225336 required much shorter exposure times to film (5-8d versus 72d for tritium). Based on IHC in parallel sections, CB2 appears to be expressed...
predominantly on B cells in the white pulp with little expression in the red pulp or T cells. This observation is consistent with the results published by Rayman et al. (46). These authors used two specific anti-CB2 antibodies in immunohistochemistry and showed that CB2 receptor was predominantly expressed by follicular B cells in the primary follicles of human spleen.

In conclusion, $^{35}$S]Sch225336 is a CB2-selective radioligand with unparalled specific activity (> 1400 Ci/mmol) and binding affinity. As such, $^{35}$S]Sch225336 is uniquely suited for the study of endogenously expressed CB2 both by equilibrium binding analyses and autoradiography.

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FOOTNOTES

* We would like to acknowledge Madeline Hipkin for her critical reading of this manuscript.
The abbreviations used are: CB1, central cannabinoid receptor; CB2, peripheral cannabinoid receptor; CHO, Chinese hamster ovary; 2-AG, 2-arachidonyl glycerol; FBS, fetal bovine serum; PMSF, phenylmethylsulfonyl fluoride; IBMX, isobutyl methylxanthine; Sch225336, N-[1(S)-4-[4-(methoxy-2-[4(methoxymethyl)sulfonyl]phenyl)sulfonyl]phenyl]ethyl)methane-sulfonamide; SR141716A, N-(piperidin-1-yl)-5-(4-chlorophenyl)-1-(2,4-dichlorophenyl)-4-methyl-1H-pyrazole-3-carboxamide hydrochloride; SR144528, N-[(1S)-endo-1,3,3-trimethyl bicyclo[2.2.1]heptan-2-yl]-5-(4-chloro-3-methylphenyl)-1-(4-methylbenzyl)-pyrazole-3-carboxamide; CP55940, (1R,3R,4R)-3-[2-hydroxy-4-(1,1-dimethylheptyl)phenyl]-4-(3-hydroxypropyl)cyclohexan-1-ol; PMA, phorbol 12-myristate 13-acetate; HU-210, 6aR,10aR analog of 11-hydroxy-Δ8-tetrahy-drocannabinol; GppNHp, guanosine 5'-β,γ-imido-triphosphate; DMSO, dimethylsulphoxide; GTPγS, guanosine 5'-γ-S-triphosphate; WIN55,212, (R)-(+)-[2,3-dihydro-5-methyl-3-(4-morpholinylmethyl)pyrrolo-[1,2,3-de]-1,4 benzoxazin-6-yl]-1-naphthalenyl-methanonesemelate.

FIGURE LEGENDS

FIGURE 1. Immunoblot analyses of hCB2. Immunoreactive proteins in membranes prepared from (A) CHO-K1, (A, B, C) CHO-hCB2 (both 25 µg/lane), (C) SF9-hCB2 (10 µg/lane), (D, E) Ba/F-hCB1 or Ba/F-hCB1 cells (both 10 µg/lane) are shown. Membrane proteins were solubilized in SDS-PAGE sample buffer. In some experiments (B, D, E), both total membrane proteins (+) and wheat germ agglutinin-agarose purified proteins (+) were tested. Proteins were separated by SDS-PAGE, transferred to PVDF membrane and incubated with anti-HA antibody (A, B; 1 µg/ml), Calbiochem #209552 (C; 1:500), Calbiochem #209554 (D; 1:500), ABR #PA1-744 or ABR #PA1-746 (E; 1:250) (as described in the Experimental Procedures). Molecular size markers are shown on the left of each panel.

FIGURE 2. Synthesis of [35S]Sch225336. The radioligand was synthesized using commercially obtained 35S-methane sulphonic acid and the precursor primary amine of Sch225336 (as described in the Experimental Procedures).

FIGURE 3. Saturation binding analyses with [35S]Sch225336. Membranes (0.1 µg/well) from (A) CHO-hCB2, (B) SF9 or SF9-hCB2 cells were incubated in binding buffer (as described in the Experimental Procedures) with the indicated concentrations of [35S]Sch225336 in the absence (total binding) or presence of 3 µM unlabelled CP55,940 (nonspecific binding). Bound radioligand was measured by liquid scintillation. (A) Total, nonspecific and specific binding (total – nonspecific binding) is shown. Scatchard analysis of specific binding (A: inset). (B) Specific binding is shown. Data represent the mean ± range of triplicate determinations from a representative experiment (n=2-3).

FIGURE 4. Competition binding analyses with [35S]Sch225336 and [3H]CP55,940. Membranes (1-13 µg/well) were incubated with (A, C) 0.05 nM [35S]Sch225336 or (B) 1.0 nM [3H]CP55,940 and the indicated concentrations of Sch225336, (C) CP55,940 or SR144528. Following filtration, the membrane-associated radioactivity was measured by liquid scintillation. Data are presented as (A, C) total bound cpm ± range or (B) expressed as fold of nonspecific binding ± range of triplicate determinations from a representative experiment (n=2).

FIGURE 5. Binding analyses with [35S]Sch225336 in hemopoietic cells and cell lines. Cell membranes (1.5-10 µg/well) from (A) Monomac6 (□), Jurkat (■), U937 (○), Hut78 (◇) and activated human peripheral blood lymphocytes (▲); or (B) peripheral blood lymphocytes (from two individuals) activated in vitro for 7 days with PHA/IL-2 (□, ■) or IL-4 (○, ●); or (C) from HL-60 cells pretreated with DMSO (□) or db-cAMP (■) were incubated in binding buffer (as described in the Experimental Procedures)
with the indicated concentrations of $[^{35}\text{S}]$Sch225336 in the absence or presence of 3 $\mu$M unlabelled CP55,940. (D) Membranes (4 $\mu$g/well) from differentiated HL-60 (DMSO, ○; PMA, ●) or THP-1 cells (□) were incubated in binding buffer with 0.06 nM $[^{35}\text{S}]$Sch225336 the indicated concentrations unlabelled Sch225336. Following filtration, the membrane-associated radioactivity was measured by liquid scintillation. Data represent the mean binding ± range of triplicate determinations from a representative experiment (n =2-3).

FIGURE 6. The effect of CB2 uncoupling on the affinity of $[^{35}\text{S}]$Sch225336 in recombinant and hemopoietic cell membranes. (A) Membranes (4, 1.5, 0.5, 0.1 $\mu$g/well, respectively) from U937 (●), HL-60(DMSO) (○), RB-hCB2 (■) and SF9-hCB2 cells (□) were incubated in binding buffer (as described in the Experimental Procedures) with 0.05 nM $[^{35}\text{S}]$Sch225336 the indicated concentrations GppNHp. Data represent the mean cpm ± range of triplicate determinations from a representative experiment (n =2). (B) The same data is shown as a fold of basal binding (B/B0). (C) Membranes (10 and 4 and 1.0 and 0.1 $\mu$g/well, respectively) from U937 (●, ○) and SF9-hCB2 cells (■, □) were incubated in binding buffer (as described in the Experimental Procedures) with 1.0 nM $[^{3}\text{H}]$CP55,940 (●, □) or 0.05 nM $[^{35}\text{S}]$Sch225336 (●, ■) the indicated concentrations GppNHp. Data represent the mean cpm ± range of triplicate determinations from a representative experiment (n =2). (D) Membranes (4 $\mu$g/well) from U937 cells were incubated in binding buffer with the indicated concentrations of $[^{35}\text{S}]$Sch225336 ± excess unlabelled ligand in the absence (■) or presence (□) of 10 $\mu$M GTPγS. Bound radioligand measured by liquid scintillation. Data represent the mean specific binding ± range of triplicate determinations from a representative experiment (n =2). Scatchard analysis of specific binding is shown (D: inset).

FIGURE 7. Analysis of CB2 expression in human spleen and lymphoid tissues by autoradiography with $[^{35}\text{S}]$Sch225336 and $[^{3}\text{H}]$CP55,940. (A-B) Frozen human spleen and (C) lymphoid sections were incubated in autoradiography binding buffer containing 0.3 nM $[^{35}\text{S}]$Sch225336 or 3 nM $[^{3}\text{H}]$CP55,940 (as described in the Experimental Procedures) in the absence (Total) or presence (NSB) of 10 $\mu$M SR144528 for 2 hrs at 25°C. Slides were then rinsed in cold phosphate-buffered saline supplemented with 0.2% (w/v) bovine serum albumin, rinsed in deionized H2O, gently dried under a stream of cold air and then exposed to a imaging film (Kodak Biomax) for 5-72 days. The imaging film was scanned and digitized using a computer scanner (Epson Perfection 1200S).

FIGURE 8. Analysis of splenic cell types that express CB2 by $[^{35}\text{S}]$Sch225336 binding and immunohistochemistry. (A) Autoradiograph of $[^{35}\text{S}]$Sch225336 binding to human spleen showing intense binding to the primary lymphoid follicles in the white pulp. (B) The area within the red box in (A) is shown at a higher magnification. (C-D) Immunostaining of anti-CD79α (C -dark brown; D -brown). Counter staining with hematoxylin to demonstrate the T lymphocyte region (dark blue, see circles) of the white pulp (C). Its identity as T lymphocyte region was confirmed with anti-CD3 immunostaining on an adjacent serial section (data not shown). Circles indicate regions of the splenic lymphoid follicles that are filled with mostly T cells. CD79α-positive B cells in the red pulp were indicated by arrows.
Fig 2

CH$_3$SO$_2$OH
(1)

$\xrightarrow{(COCl)_2 / CH_2Cl_2}$
Cat. DMF

CH$_3$SO$_2$Cl
(2)

MeO

MeO

MeO

MeO

S

S

S

S

NH$_2$

(3)

CH$_3$SO$_2$Cl

Et$_3$N / CH$_2$Cl$_2$

MeO

NH$_2$

(4)

MeO

MeO

MeO

MeO
Fig 3A

![Graph showing the binding of [35S]Sch225336](image)
Fig 3B

The figure shows a graph with the x-axis labeled as "Free [35S]Sch225336 (nM)" and the y-axis labeled as "[35S]Sch225336 (fmol/mg)". The graph includes two data sets:
- Sf9-β1γ2Gαi1, indicated by open squares
- Sf9-hCB2–β1γ2Gαi1, indicated by solid squares

The x-axis ranges from 0.0 to 1.0, and the y-axis ranges from 0 to 7000.
Fig 4

A

Radioligand bound (cpm)

$^{[35]S}$Sch225336

$[^3]H$CP55,940

Radioligand bound (B/B₀)

Sch225336 Log (M)

B

Ki = 0.052 nM

Ki = 0.39 nM
Fig 4C

Cannabinoid Log (M)

$[^{35}S]$Sch225336 (cpm)

CP55940
SR144528
Sch225336

Cannabinoid Log (M)
Fig 6

A

\[ \text{Radioligand bound (cpm)} \]

\[ \begin{array}{c}
\text{Sf9-hCB2} \quad \text{HL-60 +DMSO} \\
\text{RB-hCB2} \quad \text{U937}
\end{array} \]

Gpp[NH]p Log (M)

B

\[ \text{Free [}^{35}\text{S}\text{]Sch225336 (nM)} \]

\[ \begin{array}{c}
\text{Control Kd = 0.083 nM} \\
\text{GTP\gamma S Kd = 0.044 nM}
\end{array} \]

Gpp[NH]p Log (M)

C

\[ \text{Free [}^{35}\text{S}\text{]Sch225336 (nM)} \]

\[ \begin{array}{c}
\text{Sf9-hCB2} \quad \text{[}^{3}\text{H}\text{]CP} \\
\text{U937} \quad \text{[}^{3}\text{H}\text{]CP} \\
\text{Sf9-hCB2} \quad \text{[}^{35}\text{S}\text{]Sch} \\
\text{U937} \quad \text{[}^{35}\text{S}\text{]Sch}
\end{array} \]

Gpp[NH]p Log (M)

D

\[ \text{Bound (fmol/mg)} \]

\[ \begin{array}{c}
\text{Control Kd = 0.083 nM} \\
\text{GTP\gamma S Kd = 0.044 nM}
\end{array} \]

Free [}^{35}\text{S}\text{]Sch225336 (nM)}
Fig 7

A \(^{[35S]}\text{Sch225336}\)

B \(^{[3H]}\text{CP55,940}\)

C
Characterization of peripheral human cannabinoid receptor (hCB2) expression and pharmacology using a novel radioligand, [\(^{35}\)S]SCH225336Waldemar Gonsiorek, David Hesk, David Kinsley, Shu-Cheng Chen, Jay S. Fine, James V. Jackson, Loretta A. Bober, Gregory Deno, Charles A. Lunn, Joseph A. Kozlowski, Brian Lavey, John Piwinski, Satwant K. Narula, Daniel J. Lundell and R. William Hipkin

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