British Journal of Pharmacology (1998) 124, 619–622
http://www.stockton-press.co.uk/bjp

SPECIAL REPORT

Evidence for inverse agonism of SR141716A at human recombinant cannabinoid CB_1 and CB_2 receptors

[1]S.J. MacLennan, P.H. Reynen, J. Kwan & D.W. Bonhaus

Department of Molecular Pharmacology, Center for Biological Research, Neurobiology Unit, Roche Bioscience, 3401 Hillview Avenue, Palo Alto, CA 94304, U.S.A.

The cannabinoid receptor antagonist SR141716A has been suggested to be an inverse agonist at CB_1 receptors in some isolated intact tissues. We found that the basal incorporation of [^{35}S]-GTPγS in Chinese hamster ovary cells expressing human recombinant CB_1 and CB_2 receptors was inhibited by SR141716A (mean pEC_{50}s 8.26 and 6.00, respectively), whereas cannabinol (10 μM) had no significant effect at hCB_1 receptors but inhibited the binding at hCB_2 receptors. As cannabinol had no effect on basal [^{35}S]-GTPγS binding at hCB_1 at a concentration 100 fold higher than its binding affinity ($K_i = 0.1$ μM), we conclude that endogenous cannabinoid receptor agonists are not a confounding factor and suggest the actions of SR141716A at the hCB_1 receptor, and the actions of SR141716A and cannabinol at the hCB_2 receptor, are due to inverse agonism.

Keywords: Cannabinoids; human recombinant receptors; CB_1 receptor; CB_2 receptor; inverse agonism; SR141716A; [^{35}S]-GTPγS

Introduction Two distinct human cannabinoid receptors (hCB_1 and hCB_2) have been identified in pharmacological and molecular biology studies whilst a splice variant of the hCB_1 receptor, described as CB_{1A}, has also been described (reviewed by Pertwee, 1996). The functional characterization of cannabinoid receptors has advanced considerably with the identification of SR141716A, a selective antagonist for the CB_1 receptor which has 100 fold selectivity over the CB_2 subtype and other receptors and ion channels (Rinaldi-Carmona *et al*., 1994). However, in several systems SR141716A has effects which are not consistent with it being a silent antagonist. Thus, in the mouse field-stimulated bladder and guinea-pig myenteric plexus-longitudinal muscle, SR141716A increases the twitch contraction amplitude (Pertwee *et al*., 1996; Pertwee & Fernando, 1996), whilst *in vivo*, it causes hyperalgesia in a rodent model of thermal pain (Richardson *et al*., 1997). These effects suggest that either SR141716A is antagonizing an endogenous agonist in these systems, or that it is an inverse agonist. To investigate further the actions of SR141716A, we have studied its effects in Chinese hamster ovary (CHO) cells expressing high levels of transfected human CB_1 and CB_2 receptors, by examining the binding of guanosine 5′-O-(γ-[^{35}S]-thio)triphosphate ([^{35}S]-GTPγS), an established technique for measuring receptor-mediated activation of G-proteins.

Methods CHO-K1 cells expressing the human CB_1 and CB_2 receptors were obtained from the National Institute of Mental Health. The cells were grown in Hams F-12 media supplemented with 10% foetal calf serum and 500 μg ml^{-1} G418 and harvested with 0.5 mM EDTA (4Na). Cells were homogenized using a Branson benchtop sonicator for 60 s, then centrifuged at 50,000 × *g* for 10 min at 4°C. The pellet was resuspended in ice-cold HEPES buffer (10 mM), supplemented with 1.0 mM $MgCl_2$, using a Polytron (High speed, 1 × 5 s). The [^{35}S]-GTPγS binding assay was carried out in 10 mM HEPES buffer supplemented with 100 mM NaCl, 32 mM $MgCl_2$, 320 μM GDP, 5 μg protein and 1.0 nM [^{35}S]-GTPγS in a final volume of 250 μl. Non-specific binding was determined using an excess of GTP (10 μM). Assay tubes were incubated at 37°C for 60 min, filtered using a Packard 96 well harvester, washed 3 times with ice cold HEPES buffer and the radioactivity counted using a Packard Top Count microplate scintillation counter.

Radioligand binding studies were conducted essentially as described by Felder *et al*. (1995). Briefly, confluent cells were washed with phosphate-buffered saline, harvested and homogenized in ice-cold buffer (50 mM Tris, 5 mM $MgCl_2$, 2.5 mM EDTA, pH 7.4). The homogenate was centrifuged at 2,000 × *g* for 15 min at 4°C. The supernatant was collected and centrifuged at 43,000 × *g* for 30 min at 4°C. The membranes were resuspended in buffer and stored at −80°C until used in binding assays. Competition binding studies were conducted by incubating membranes and competing ligands with 1.0 nM [^{3}H]-CP-55,940 in buffer containing 0.5% fatty acid-free bovine serum albumin, at 30°C for 60 min. Ten different concentrations of competing ligand were used to generate competition binding curves. Saturation binding studies were conducted by incubating membranes in buffer and 0.5% fatty acid-free bovine serum albumin with twelve concentrations of radioligand. Nonspecific binding was determined in the presence of 5 μM non-radioactive CP-55,940.

The ligand-induced change in the incorporation of [^{35}S]-GTPγS was expressed as a percentage of the basal incorportion and the data fitted to the Hill equation to determine the maximum effect and concentration producing the half-maximal effect (EC_{50}). For competition radioligand binding assays, IC_{50} values were obtained by fitting data to the Hill equation. K_i values were then calculated using the Cheng-Prusoff (1973) equation. The iterative curve fitting programmes of Prism (GraphPad Software, CA, U.S.A.) were utilized for these procedures. Saturation binding isotherms were similarly analysed yielding K_D and B_{max} values. Hams F-12, G-418 and HEPES buffer were purchased from Life Technologies (MD, U.S.A.), foetal calf serum from Summit (CO, U.S.A.). [^{35}S]-GTPγS (1250 Ci mmol^{-1}) and [^{3}H]-CP-55,940 (165 Ci mmol^{-1}) were purchased from NEN Life

[1] Author for correspondence.

Sciences (MA, U.S.A.). All other chemicals were obtained from Sigma (MO, U.S.A.). The drugs used were from the following sources; CP-55,940 ((−)*cis*-3-[2-hydroxy-4-(1,1-dimethylheptyl)phenyl]-trans-4-(3-hydroxypropyl)cyclohexanol) from the Department of Chemistry (Neurobiology Unit, Roche Bioscience); SR141716A (N-(piperidin-1-yl)-5-(4-chlorophenyl)-1-(2,4-dichlorophenyl)-4-methyl-1H-pyrazole-3-carboxamide) from Tocris Cookson (U.K.); WIN55,212-2 (R)(+)-[2,3-dihydro-5-methyl-3-[(morpholinyl) methyl]pyrrolo[1,2,3-de]-1,4-benzoxazin-yl]-(1-naphthalenyl)methanone mesylate) from RBI (MA, U.S.A.) and cannabinol from Sigma (MO, U.S.A.).

Statistical analyses of data were undertaken using two-sample *t* tests, with $P<0.05$ regarded as statistically significant.

Results The affinity of [^{3}H]-CP55,940 at hCB_1 and hCB_2 receptors was similar (K_D values of 2.8 ± 0.8 and 4.7 ± 1.8 nM, respectively, $n=5$). Each receptor type was highly expressed. However, the density of CB_1 receptors was approximately 10 fold less than that of CB_2 receptors (Figure 1; B_{max} values were 6.2 ± 1.9 and 78.2 ± 8.5 pmol mg^{-1} protein, respectively, $n=5$). The cannabinoid receptor ligands CP55,940, WIN55,212-2, cannabinol and SR141716A displaced [^{3}H]-CP55,940 at each receptor subtype with Hill slopes not significantly different from one (Figure 2), with affinities appropriate for the labeling of CB_1 and CB_2 receptors (Table 1; see Felder *et al*., 1995).

In non-transfected CHO-K1 cells, the basal incorporation of [^{35}S]-GTPγS was 68 ± 5 fmol mg^{-1} protein ($n=5$) and was not significantly ($P>0.05$) affected by either CP55,940 (10 μM) or SR141716A (10 μM). In cells transfected with the hCB_1 or the hCB_2 receptor, the basal incorporation of [^{35}S]-GTPγS was significantly increased to 175 ± 29 and 281 ± 45 fmol mg^{-1} protein (hCB_1 and hCB_2, respectively, $n=5$). PMSF (100 μM), an irreversible inhibitor of anandamide synthase, did not significantly ($P>0.05$) affect the basal incorporation of [^{35}S]-GTPγS at either receptor subtype (180 ± 19 and 303 ± 50 fmol mg^{-1} protein at hCB_1 and hCB_2, respectively, $n=5$). At each receptor type, the incorporation of [^{35}S]-GTPγS was concentration-dependently increased by WIN55,212-2 and CP55,940 (Figure 3, Table 1). However, for both agonists the maximum incorporation was much greater in cells expressing the CB_1 receptor (increase over basal of approximately 100%) than the CB_2 receptor (approximately 40% and 20%, respectively). SR141716A inhibited basal [^{35}S]-GTPγS binding

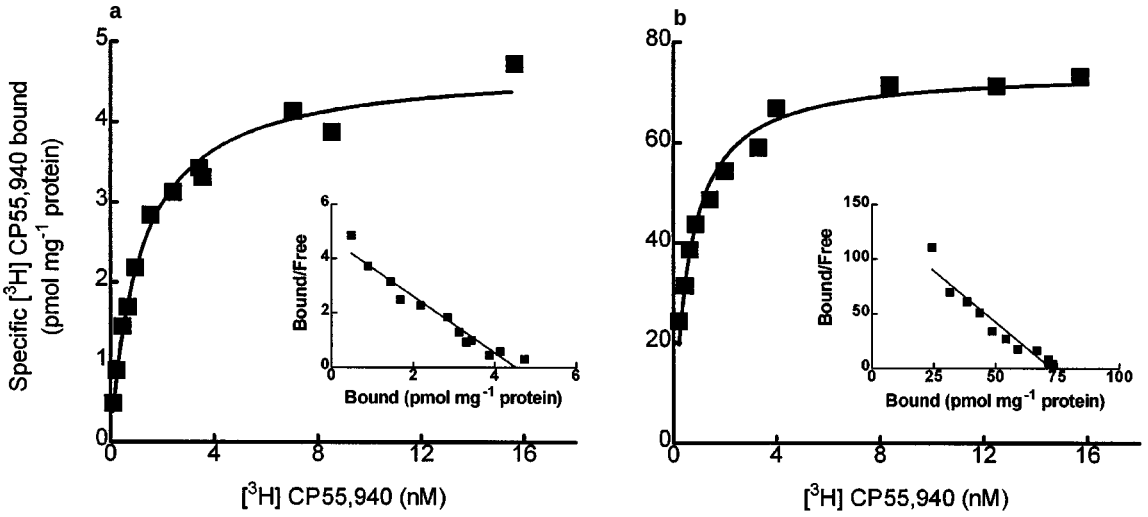

Figure 1 Radioligand ([^{3}H]-CP55940) binding isotherms at the human recombinant CB_1 (a) and CB_2 (b) receptors. For each receptor the data shown are representative of 5 separate experiments which were conducted. The inserts are Scatchard plots.

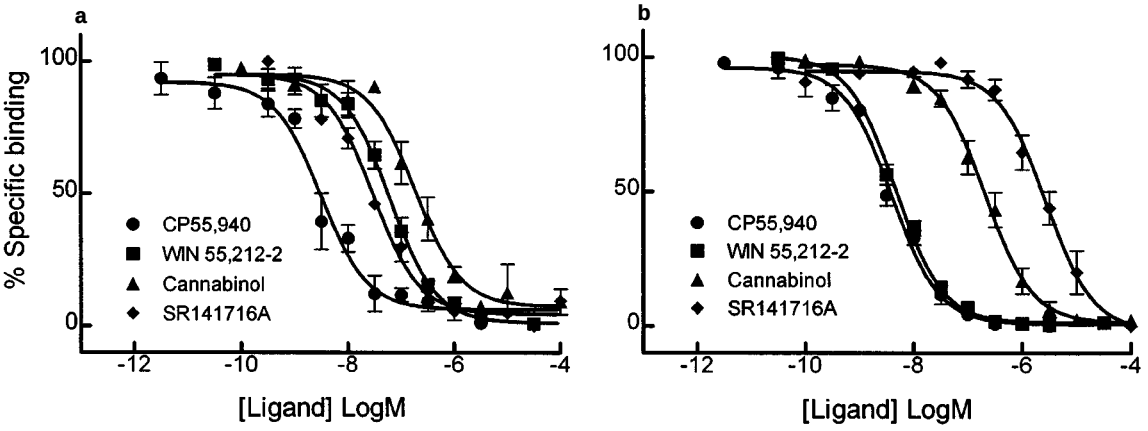

Figure 2 Inhibition by cannabinoid receptor ligands of specific binding of [^{3}H]-CP55940 to membranes of CHO cells expressing human recombinant CB_1 (a) and CB_2 (b) receptors. Data are the mean from 5 separate experiments; vertical lines show s.e.mean.

Table 1 Affinity and efficacy of ligands at human recombinant cannabinoid receptors

		hCB1			*hCB2*	
	pK_i	*pEC50*	*Maximum effect*	*pK_i*	*pEC50*	*Maximum effect*
CP55,940	8.67±0.09	7.73±0.07	103±14	8.74±0.02	8.56±0.09	42±3
WIN55,212−2	7.38±0.07	6.21±0.08	92±12	8.71±0.06	8.65±0.33[a]	27±7[a]
SR141716A	7.72±0.07	8.26±0.06	−47±6	5.52±0.13	6.00±0.08	−65±6
Cannabinol	6.92±0.14	ND	−5±11[b]	7.00±0.10	6.24±0.04	−53±4

pK_i values are from competition radioligand binding. pEC_{50} and maximum effects of ligands are from measuring the effects on [^{35}S]-GTPγS binding. All values are the mean±s.e.mean of 5 determinations with the exception of some data for WIN55,212−2 at hCB_2 ([a]) which is the mean of 3 determinations. ND, not able to be determined as cannabinol had no effect upon [^{35}S]-GTPγS binding to hCB_1; [b]maximum effect of cannabinol at 10 μM.

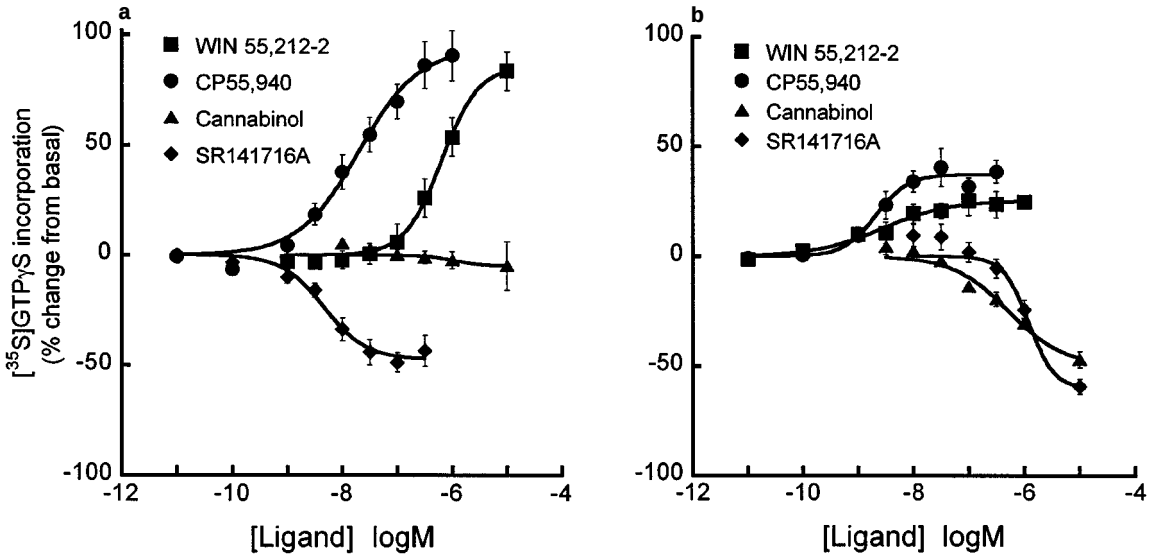

Figure 3 Effects of cannabinoid receptor ligands on the binding of [^{35}S]-GTPγS to human recombinant CB_1 (a) and CB_2 (b) receptors. Data are expressed as the % change in the basal binding of [^{35}S]-GTPγS and are the mean from 5 separate experiments with the exception of data for WIN55,212-2 at hCB_2 which is from 3 experiments; vertical lines show s.e.mean.

in cells expressing both hCB_1 and hCB_2 receptors, whereas cannabinol inhibited basal incorporation of [^{35}S]-GTPγS in hCB_2 only (Figure 3, Table 1). At hCB_1 receptors cannabinol did not affect the basal incorporation of [^{35}S]-GTPγS at concentrations up to 10 μM.

Discussion These results suggest that SR141716A is an inverse agonist at the human recombinant CB_1 and CB_2 receptors. At each subtype, the basal incorporation of [^{35}S]-GTPγS was reduced by SR141716A. In the absence of other evidence this could be explained by either inverse agonism or by antagonism of an endogenous cannabinoid agonist. However, the finding that 10 μM cannabinol did not have any effect at hCB_1 receptors, at concentrations 100 fold higher than its binding affinity (K_i=0.1 μM) implies that endogenous agonist(s) are not a confounding factor. A caveat to this conclusion would be if both the potency and efficacy of the endogenous agonist at hCB_1 was equal to the potency and inverse efficacy of cannabinol, in which case their effects would self-cancel. This scenario seems unlikely. Further evidence against a role for endogenous agonists such as anandamide is provided by the observation that PMSF (100 μM), an irreversible inhibitor of anandamide synthase (Deutsch *et al*., 1997), had no effect on the basal incorporation of [^{35}S]-GTPγS in either hCB_1 or hCB_2. As both hCB_1 and hCB_2 receptors were expressed in the same cell line and maintained under identical conditions, the results imply that the actions of both SR141716A and cannabinol at hCB_2 receptors are due to inverse agonism also. However, the effects of SR141716A and cannabinol at hCB_2 were observed in a system overexpressing the receptor protein (78.2 pmol mg^{-1} protein). Clearly, it will be interesting to determine whether these observations have physiological relevance, for example at CB_2 receptors on mouse spleen cells, which have a relatively low expression level of approximately 1000 receptors per cell (Kaminski *et al*., 1992).

Our data confirm and extend recent observations with SR141716A at hCB_1 receptors. Landsman *et al*. (1997) and Bouaboula *et al*. (1997) found that SR141716A inhibited the incorporation of [^{35}S]-GTPγS in CHO cells expressing the hCB_1 receptor. The former study did not address endogenous agonist(s) as a factor to explain the effects of SR141716A, while the latter study showed an effect of SR141716A at a single concentration only, but did provide evidence, if somewhat indirect, to suggest that constitutive activity of the receptor could not be attributed to endogenous agonist.

Although we have provided evidence that SR141716A behaves as an inverse agonist at recombinant receptors in a heterologous expression system, this may not explain its

actions in isolated, intact tissue preparations. Thus, in the field stimulated mouse bladder and guinea-pig myenteric plexus-longitudinal muscle preparations, SR141716A caused a small but significant increase in twitch contraction amplitude (Pertwee *et al*., 1996; Pertwee & Fernando, 1996). The constitutive activity of CB_1 receptors in these tissues, suggested by the actions of SR141716A, could not be attributed unequivocally to either displacement of endocannabinoids or to inverse agonist actions of the drug. It remains to be determined whether *in vitro*, these systems are able to synthesize endogenous cannabinoids, for example, anandamide, which has been isolated principally from brain tisuse (Felder *et al*., 1996). The search for an endogenous cannabinoid in these tissues, if such exists, may not be straightforward as anandamide represents only one of perhaps several cannabinoids (e.g. sn-2 arachidonylglycerol; Stella *et al*., 1997) which are endogenous ligands for cannabinoid receptors.

The effects of SR141716A *in vivo* are not simple to explain. In the absence of data regarding the presence of endocannabinoids, the constitutive activation of cannabinoid receptors, suggested by the hyperalgesic effects of SR141716A (Richardson *et al*., 1997), cannot be explained unequivocally by the inverse agonist actions of this compound.

In conclusion, this study has provided evidence that SR141716A is an inverse agonist at human recombinant CB_1 and CB_2 receptors. Whether this mechanism explains its effects in isolated tissues, or *in vivo*, remains to be determined.

References

BOUABOULA, M., PERRACHON, S., MILLIGAN, L., CANAT, X., RINALDI-CARMONA, M., PORTIER, M., BARTH, F., CALANDRA, B., PECCEU, F., LUPKER, J., MAFFRAND, J-P., LE FUR, G. & CASELLAS, P. (1997). A selective inverse agonist for central cannabinoid receptor inhibits mitogen-activated protein kinase activation stimulated by insulin or insulin-like growth factor 1. *J. Biol. Chem.,* **272,** 22330–22339.

DEUTSCH, D.G., LIN, S., HILL, W.A.G., MORSE, K.L., SALEHANI, D., ARREAZA, G., OMEIR, R.L. & MAKRIYANNIS, A. (1997). Fatty acid sulfonyl fluorides inhibit anandamide metabolism and bind to the cannabinoid receptor. *Biochem. Biophys. Res. Commun.,* **231,** 217–221.

FELDER, C.C., JOYCE, K.E., BRILEY, E.M., MANSOURI, J., MACKIE, K., BLOND, O., LAI, Y., MA, A. & MITCHELL, R.L. (1995). Comparison of the pharmacology and signal transduction of the human cannabinoid CB_1 and CB_2 receptors. *Mol. Pharmacol.,* **48,** 443–450.

FELDER, C.C., NIELSEN, A., BRILEY, E.M., PALKOVITS, M., PRILLER, J., AXELROD, J., NGUYEN, D.N., RICHARDSON, J.M., RIGGIN, R.M., KOPPEL, G.A., PAUL, S.M. & BECKER, G.W. (1996). Isolation and measurement of the endogenous cannabinoid receptor agonist, anandamide, in brain and peripheral tissues of human and rat. *FEBS Lett.,* **393,** 231–235.

KAMINSKI, N.E., ABOOD, M.E., KESSLER, F.K., MARTIN, B.R. & SCHATZ, A.R. (1992). Identification of a functionally relevant cannabinoid receptor on mouse spleen cells that is involved in cannabinoid-mediated immune modulation. *Mol. Pharmacol.,* **42,** 736–742.

LANDSMAN, R.S., BURKEY, T.H., CONSROE, P., ROESKE, W.R. & YAMAMURA, H.I. (1997). SR141716A is an inverse agonist at the human cannabinoid CB_1 receptor. *Eur. J. Pharmacol.,* **334,** R1–R2.

PERTWEE, R.G. (1996). Cannabinoid receptor ligands: clinical and neuropharmacological considerations relevant to future drug discovery and development. *Exp. Opin. Invest. Drugs,* **5,** 1245–1253.

PERTWEE, R.G. & FERNANDO, S.R. (1996). Evidence for the presence of cannabinoid CB_1 receptors in mouse urinary bladder. *Br. J. Pharmacol.,* **118,** 2053–2058.

PERTWEE, R.G., FERNANDO, S.R., NASH, J.E. & COUTTS, A.A. (1996). Further evidence of cannabinoid CB_1 receptors in guinea-pig small intestine. *Br. J. Pharmacol.,* **118,** 2199–2205.

RICHARDSON, J.D., AANONSEN, L. & HARGREAVES, K.M. (1997). SR141716A, a cannabinoid receptor antagonist, produces hyperalgesia in untreated mice. *Eur. J. Pharmacol.,* **319,** R3–R4.

RINALDI-CARMONA, M., BARTH, F., HEAULME, M., SHIRE, D., CALANDRA, B., CONGY, C., MARTINEZ, S., MARUANI, J., NELIAT, G., CAPUT, D., FERRARA, P., SOUBRIE, P., BRELIERE, J.C. & LE FUR, G. (1994). SR141716A, a potent and selective antagonist of the brain cannabinoid receptor. *FEBS Lett.,* **350,** 240–244.

STELLA, N., SCHWEITZER, P. & PIOMELLI, D. (1997). A second endogenous cannabinoid that modulates long-term potentiation. *Nature,* **388,** 773–778.

(Received February 6, 1998
Revised March 20, 1998
Accepted March 30, 1998)