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CHARACTERIZATION OF RECOMBINANT HUMAN SEROTONIN $5HT_{1A}$ RECEPTORS EXPRESSED IN CHINESE HAMSTER OVARY CELLS

[^{3}H]SPIPERONE DISCRIMINATES BETWEEN THE G-PROTEIN-COUPLED AND -UNCOUPLED FORMS

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Abstract—$5HT_{1A}$ serotonin (5-hydroxytryptamine) receptors have been characterized by ligand binding in a recombinant Chinese Hamster Ovary cell line expressing the human receptor gene. The agonist ligand [^{3}H]2-(*N,N*-dipropylamino)-8-hydroxy-1,2,3,4-tetrahydronaphthalene ([^{3}H]8-OH-DPAT) and the antagonist [^{3}H]spiperone were used. For both radioligands the binding sites labelled have the properties of $5HT_{1A}$ receptors and most antagonists show roughly equal affinities for the receptors labelled by either [^{3}H]8-OH-DPAT or [^{3}H]spiperone. Agonists, however, show higher affinities for the sites labelled by [^{3}H]8-OH-DPAT and the antagonist spiperone conversely shows a higher affinity for the sites labelled by [^{3}H]spiperone. Whereas [^{3}H]8-OH-DPAT binding is inhibited by guanosine triphosphate (GTP) the binding of [^{3}H]spiperone is increased by GTP. A model is proposed for the results whereby [^{3}H]8-OH-DPAT labels a form of the receptor coupled to a G-protein and [^{3}H]spiperone labels a form of the receptor uncoupled from G-proteins (or possibly coupled to a different G-protein).

A large family of distinct receptor subtypes has been identified for the neurotransmitter serotonin (5-hydroxytryptamine, 5HT†) using pharmacological and molecular biological techniques [1]. Of these receptor isoforms the $5HT_{1A}$ receptor is of particular interest as it is the site of action of a new class of anxiolytic drug, e.g. buspirone [2]. $5HT_{1A}$ receptors have been characterized using radioligand binding techniques with the agonist [^{3}H]2-(*N,N*-dipropylamino)-8-hydroxy-1,2,3,4-tetrahydronaphthalene ([^{3}H]8-OH-DPAT) [3]. It would, however, be desirable to use additionally an antagonist radioligand but no sufficiently selective antagonist exists to permit such a characterization in tissues such as the brain where there are other potentially cross-reacting receptor sites.

The human and rat $5HT_{1A}$ receptors have been cloned [4, 5]. In order to facilitate characterization of the $5HT_{1A}$ receptor protein we have expressed the human gene stably and at high levels in Chinese Hamster Ovary (CHO) cells [6]. The derived cell line (CHO-$5HT_{1A}$-7) expresses the receptor at high levels (~3 pmol/mg protein, ~4×10^5 receptors/cell) and the receptor is coupled to the endogenous G-proteins of the cells. CHO-$5HT_{1A}$-7 cells can be grown readily in suspension culture affording the production of large amounts of receptor-bearing membranes.

The presence in CHO-$5HT_{1A}$-7 cells of a high level of $5HT_{1A}$ receptors and the absence of other related receptors allow the use in radioligand binding assays of relatively unselective antagonist radioligands, e.g. [^{3}H]spiperone. [^{3}H]Spiperone has a moderate affinity for the $5HT_{1A}$ receptor (K_i 48 nM [7]) and would be difficult to use in brain tissue to characterize $5HT_{1A}$ receptors owing to its higher affinity for D_2 dopamine and serotonin $5HT_2$ receptors [8]. In CHO-$5HT_{1A}$-7 cells there should be no cross-reactivity with other receptors and the high level of $5HT_{1A}$ receptor expression should permit identification of $5HT_{1A}$ receptors even at the low occupancy necessitated by the relatively high K_i value.

In this report we describe the use of the agonist [^{3}H]8-OH-DPAT and the antagonist [^{3}H]spiperone to characterize $5HT_{1A}$ receptors in CHO-$5HT_{1A}$-7 cells.

MATERIALS AND METHODS

Membrane preparation. Recombinant CHO-$5HT_{1A}$-7 cells were grown either in adherent culture in RPMI medium supplemented with 10% dialysed foetal bovine serum or in suspension culture in the same medium, in an humidified atmosphere containing 5% CO_2 at 37° [6]. For suspension growth, 1.5 L of medium was inoculated with 1×10^7 cells taken from adherent culture. The cells grew exponentially without clumping and were harvested when the cell density reached 1×10^6 cells/mL of medium. A crude cell homogenate was obtained by centrifuging the cells (300 *g*, 10 min, 4°), resuspending the pellet in assay buffer (HEPES 20 mM, $MgSO_4$ 5 mM, pH 7.4), washing (by repeating the centrifugation/wash procedure once) and breaking the cells by Polytron homogenization at maximum

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† Abbreviations: 8-OH-DPAT, 2-(*N,N*-dipropylamino)-8-hydroxy-1,2,3,4-tetrahydronaphthalene; 5HT, serotonin (5-hydroxytryptamine); CHO, Chinese Hamster Ovary.

Table 1. Pharmacological characterization of recombinant $5HT_{1A}$ receptor expressed in CHO-$5HT_{1A}$-7 cells

	K_i (nM)	
	[^{3}H]8-OH-DPAT	[^{3}H]Spiperone
Agonists		
Buspirone	21.1 ± 3.9 (4)	212 ± 30 (3)
5-Carboxamidotryptamine	0.53 ± 0.21 (4)	17.5 ± 5.3 (3)
Ipsapirone	3.85 ± 0.75 (2)	45.2 ± 13.3 (3)
Lisuride	0.41 ± 0.18 (3)	6.90 ± 0.45 (3)
8-OH-DPAT	3.76 ± 1.10 (6)	27.1 ± 5.1 (6)
Serotonin	3.98 ± 1.13 (3)	102.3 ± 8.0 (4)
Antagonists		
(+)-Butaclamol	680 ± 75 (7)	370 ± 62 (2)
(−)-Butaclamol	2876 ± 337 (4)	>3000 (3)
Methiothepin	22.2 ± 5.6 (3)	24.6 ± 5.8 (3)
Pindolol	33.3 ± 13.9 (3)	61.1 ± 6.9 (3)
Spiperone	411 ± 50 (4)	17.2 ± 3.8 (3)

K_i values for a range of agonists and antagonists were obtained in competition experiments versus [^{3}H]8-OH-DPAT or [^{3}H]spiperone as described in Materials and Methods. All competition curves fitted well to one binding site models and the K_i values obtained using the LIGAND computer non-linear least squares curve fitting programme are given (mean ± SEM, N experiments; or mean ± range, two experiments).

speed for 10 sec. The homogenate was divided into aliquots and stored at −80°.

Radioligand binding assays. Cell homogenate (from suspension-grown cells unless stated) was incubated in a final volume of 1 mL with either [^{3}H]8-OH-DPAT (0.5 nM) or [^{3}H]spiperone (2 nM) and competing drugs at 25° for 2.5 hr in triplicate ([^{3}H]8-OH-DPAT) or 1 hr in duplicate ([^{3}H]-spiperone). Specific binding was defined as that binding inhibited by 10 μM 5HT in both cases. For [^{3}H]8-OH-DPAT experiments 50 μg homogenate protein were used and, unless otherwise stated, samples were harvested by rapid filtration (GF/B filter) and washing with 15 mL ice-cold phosphate-buffered saline (NaCl 137 mM, KCl 2.7 mM, Na_2HPO_4 8.1 mM, KH_2PO_4 1.5 mM, pH 7.4 at 4°) on a Brandel cell harvester.

For [^{3}H]spiperone binding experiments, 500 μg homogenate protein were incubated in 1.5 mL plastic microcentrifuge tubes (final assay volume 1 mL). Homogenate pellets were obtained by centrifugation at 15,000 *g* for 1 min as described in Ref. 9. The supernatant was discarded and residual free radioligand rinsed out of the tubes by twice rapidly dipping into ice-cold phosphate-buffered saline and pouring off. The tubes were then allowed to drip dry before cutting off the ends containing the pellets into scintillation vials containing scintillant and determining radioactivity.

Data were analysed using the LIGAND computer non-linear least squares computer curve fitting program.

RESULTS

Characterization of $5HT_{1A}$ receptors using [^{3}H]8-OH-*DPAT binding*

A range of agonists and antagonists were used in competition binding assays with [^{3}H]8-OH-DPAT to characterize the $5HT_{1A}$ receptor expressed in CHO-$5HT_{1A}$-7 cells and inhibition constants (K_i values) are shown in Table 1. Representative competition curves are shown in Fig. 1 and in all cases the data were best fitted by a one binding site model. The K_i values obtained were in close agreement with values reported in the literature with the exception of the K_i for (+)-butaclamol (680 nM). Raymond *et al.* [10] have reported a K_i of 6.6 nM for this ligand. Competition of (+)-butaclamol for [^{3}H]8-OH-DPAT binding to $5HT_{1A}$ receptors to bovine hippocampus gave a K_i value of 1049 nM (data not shown). We assume the values we have obtained to be accurate under the particular experimental conditions and the recombinant $5HT_{1A}$ receptor expressed in CHO-$5HT_{1A}$-7 cells is therefore displaying ligand-binding properties of a typical $5HT_{1A}$ receptor.

Characterization of $5HT_{1A}$ receptors using [^{3}H]-*spiperone binding*

Specific [^{3}H]spiperone binding to CHO-$5HT_{1A}$-7 cell membranes could be readily detected although a centrifugation assay was used owing to the anticipated affinity of the radioligand for the $5HT_{1A}$ receptor (Table 1). Specific [^{3}H]spiperone binding reached equilibrium within 60 min and this time was used for all subsequent experiments. Competition experiments were performed with a range of agonists and antagonists (Table 1, Fig. 1). For all the compounds that competed with high affinity (mostly $5HT_{1A}$ agonists and antagonists) there was full competition for specific [^{3}H]spiperone binding at high concentrations of the competitors. The competition data fitted best to a one binding site model and K_i values are given in Table 1. Competition experiments with ketanserin ($5HT_2$ selective),

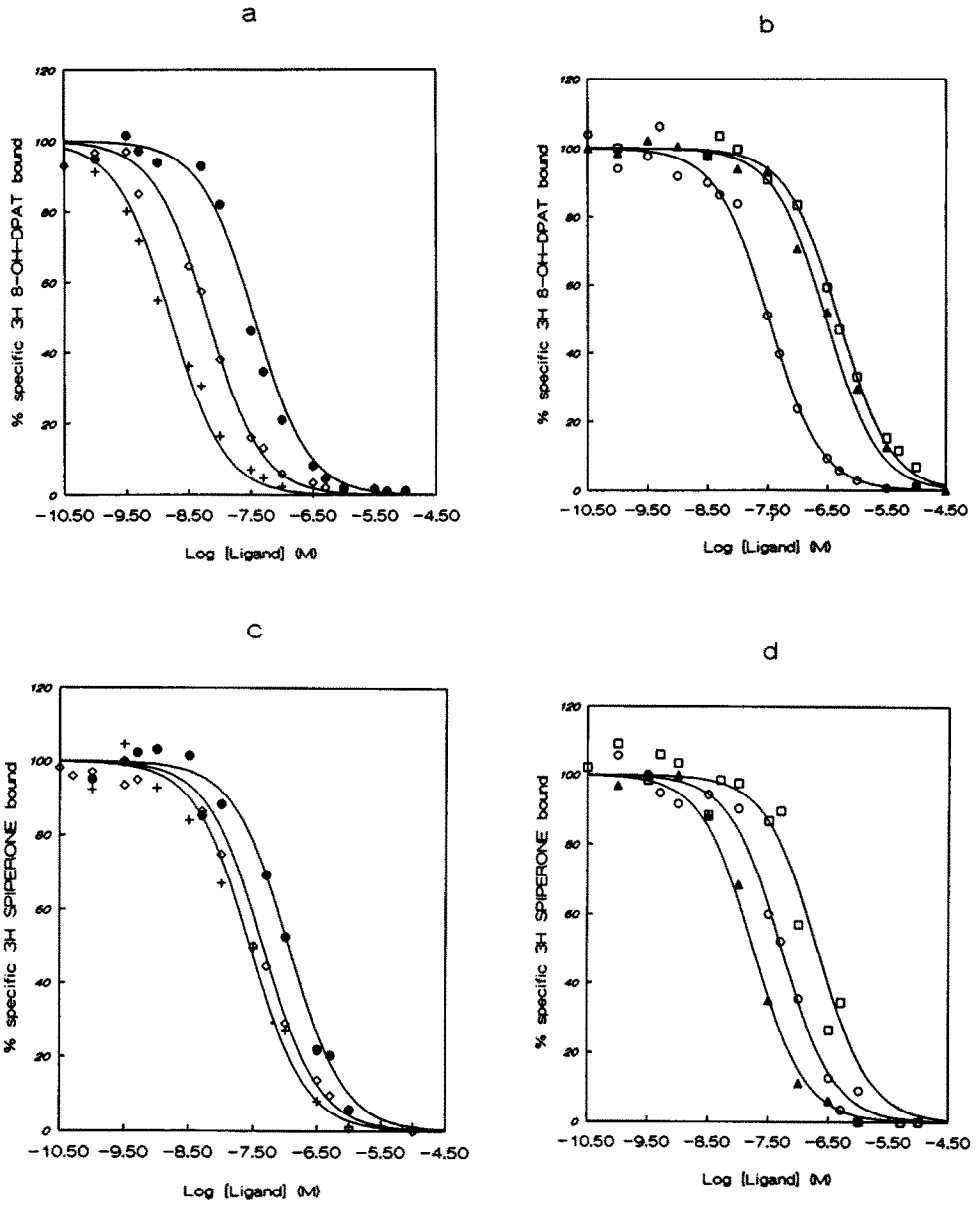

Fig. 1. Pharmacological characterization of recombinant $5HT_{1A}$ receptors expressed in CHO-$5HT_{1A}$-7 cells. A range of agonists (panel a and c) and antagonists (panel b and d) was competed with [^{3}H]8-OH-DPAT (panel a and b) or [^{3}H]spiperone (panel c and d) binding to $5HT_{1A}$ receptors in CHO-$5HT_{1A}$ cells as described. The curves shown are the best fits to one binding site models, the data points are the means of triplicate determinations and the experiments have been replicated as in Table 1. (+) 8-OH-DPAT, (●) buspirone, (◇) ipsapirone, (○) pindolol, (▲) spiperone, (□) (+)-butaclamol.

domperidone (D_2 selective), prazosin (α_1 selective) and R5573 (spirodecanone selective) gave IC_{50} values greater than 1 μM (N = 3). These potential [^{3}H]-spiperone binding sites are therefore not present in the experiments with CHO-$5HT_{1A}$-7 cells. This was supported by experiments with the parent untransfected CHO cell line from which CHO-$5HT_{1A}$-7 cells were derived. The untransfected CHO cells showed no specific [^{3}H]spiperone binding. Thus, it seems that in CHO-$5HT_{1A}$-7 cells it is possible to assay $5HT_{1A}$ receptors with the antagonist [^{3}H]-spiperone and specific [^{3}H]spiperone binding is to a single population of $5HT_{1A}$ receptors.

When the K_i values for competing ligands versus [^{3}H]spiperone are compared with similar data obtained versus [^{3}H]8-OH-DPAT, for most antagonists very similar values are seen. An exception is spiperone which shows an approximately 20-fold lower affinity when competing with [^{3}H]8-OH-DPAT. Conversely all agonists show much higher

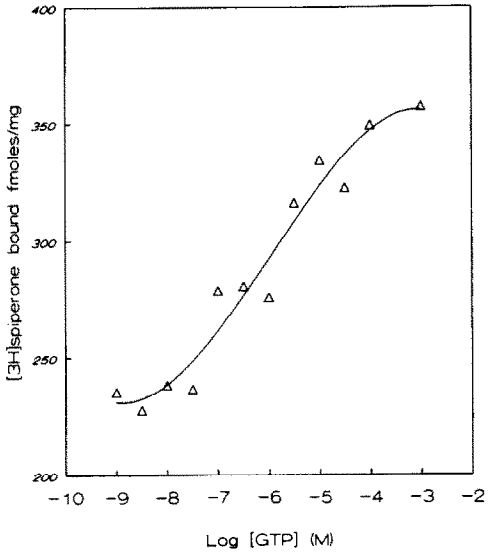

Fig. 2. Effect of GTP on [^{3}H]spiperone binding to $5HT_{1A}$ receptors in homogenates of CHO-$5HT_{1A}$-7 cells. The specific binding of [^{3}H]spiperone (2 nM) to $5HT_{1A}$ receptors was determined in the presence of different concentrations of GTP. The data points are the means of duplicate determinations and the experiment has been replicated four times (mean EC_{50} for GTP 3.82 ± 1.11 μM).

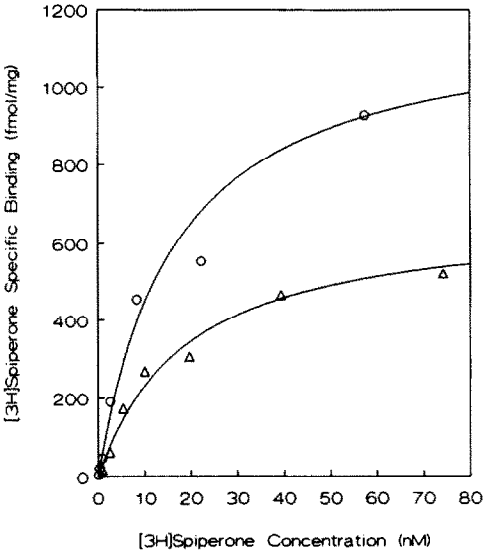

Fig. 3. Effect of GTP on [^{3}H]spiperone binding to $5HT_{1A}$ receptors in homogenates of CHO-$5HT_{1A}$-7 cells. Saturation analysis of specific [^{3}H]spiperone (1–70 nM) binding was carried out in the presence (○) and absence (△) of GTP (100 μM). The curves shown are the best fit curves to one binding site models. The data points are the means of duplicate determinations and the experiment has been replicated as described in the text.

affinities when competing with [^{3}H]8-OH-DPAT. The discrepancy in affinity estimates for spiperone was not due to differences in growth conditions or assay conditions. Spiperone competing with [^{3}H]-spiperone showed a K_i value of 25.3 ± 5.2 nM (mean ± SE, N = 3) when cells grown in adherent culture were used. This is very similar to the value reported for cells grown in suspension culture (Table 1). Also, when spiperone was competed against [^{3}H]8-OH-DPAT using a centrifugation assay a K_i value of 400 ± 130 nM (mean ± SE, N = 3) was obtained. This is very similar to the value obtained using a filtration assay (Table 1).

Effect of GTP on [^{3}H]spiperone binding to $5HT_{1A}$ receptors

The binding of [^{3}H]spiperone (2 nM) to $5HT_{1A}$ receptors in CHO-$5HT_{1A}$-7 cells was increased in a dose-dependent manner by increasing concentrations of GTP (Fig. 2). The maximum increase was about 45% at high concentrations of GTP (100 μM) and a half maximal increase was obtained with 3.8 ± 1.1 μM GTP (mean ± SE, N = 4).

The effects of GTP were then examined in saturation analyses of [^{3}H]spiperone binding (Fig. 3). [^{3}H]Spiperone saturation curves fitted well to one binding site models and there was little or no effect of GTP on the K_i value (control: 13.5 ± 3.4 nM; + 100 μM GTP: 18.1 ± 3.6 nM; mean ± SE, N = 4). There was however a large (70% approximately) increase in B_{max} (control: 830 ± 60 fmol/mg; +GTP: 1390 ± 70 fmol/mg). Saturation experiments with [^{3}H]8-OH-DPAT performed on the same batch of cell homogenate as was used for [^{3}H]spiperone

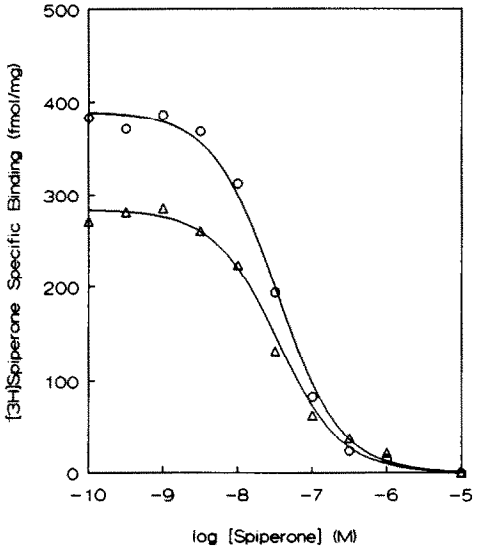

Fig. 4. Effect of GTP on spiperone competition for [^{3}H]-spiperone binding to $5HT_{1A}$ receptors in homogenates of CHO-$5HT_{1A}$-7 cells. Spiperone/[^{3}H]spiperone competition experiments were performed as described. (△) Control; (○) +GTP (100 μM). The data points are the means of duplicate determinations, the curves shown are the best fit curves to one binding site models and the experiment has been replicated as described in the text.

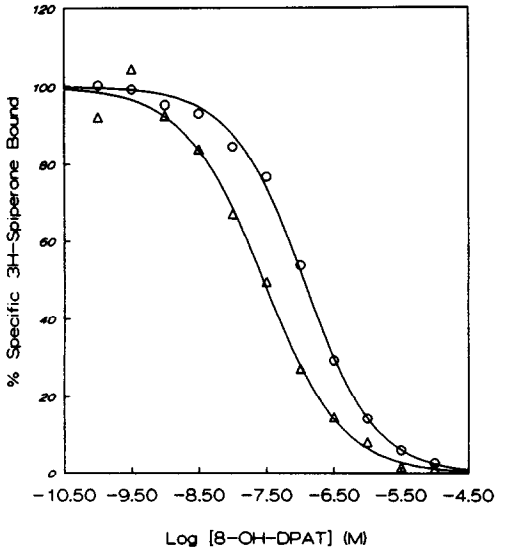

Fig. 5. Effect of GTP on 8-OH-DPAT competition for [^{3}H]spiperone binding to $5HT_{1A}$ receptors in homogenates of CHO-$5HT_{1A}$-7 cells. 8-OH-DPAT/[^{3}H]spiperone competition experiments were performed as described. (△) Control; (○) +GTP (100 μM). The data points are the means of duplicate determinations, the curves shown are the best fit curves to one binding site models and the experiment has been replicated as described in the text.

binding gave a similar B_{max} value in the absence of GTP (749 ± 99 fmol/mg, mean ± SE, N = 3, K_d 2.4 ± 0.2 nM).

When [^{3}H]spiperone was competed with non-radioactive spiperone (Fig. 4), the effect of GTP (100 μM) was to increase the maximal specific [^{3}H]-spiperone binding observed (35.8 ± 6.9% increase, mean ± SE, N = 6) with little or no change in K_i (control: 14.9 ± 2.2 nM; +GTP: 16.4 ± 1.4 nM; mean ± SE, N = 4). When the agonist 8-OH-DPAT was competed against [^{3}H]spiperone (Fig. 5), GTP increased the specific [^{3}H]spiperone binding as above and also affected the K_i value (control: 30.0 ± 7.6 nM, mean ± SE, N = 4; +GTP (100 μM): 65.7 ± 12.0 nM, mean ± SE, N = 3). The data are presented in Fig. 5 as the percentage of the maximum specific [^{3}H]spiperone binding in order to illustrate the change in K_i.

DISCUSSION

In this report we have characterized the recombinant human serotonin $5HT_{1A}$ receptor expressed in CHO-$5HT_{1A}$-7 cells using the binding of agonist ([^{3}H]8-OH-DPAT) and antagonist ([^{3}H]-spiperone) radioligands. Using [^{3}H]8-OH-DPAT binding in competition assays with a range of agonists and antagonists the recombinant receptor exhibited pharmacological properties similar to those described by others in brain tissue [6]. Thus, the $5HT_{1A}$ receptor expressed in CHO-$5HT_{1A}$-7 cells is authentic and provides a good model system for the further characterization of the receptor. [^{3}H]8-OH-DPAT binding sites in CHO-$5HT_{1A}$-7 cells appear to form a homogenous population of sites. [^{3}H]8-OH-DPAT binding in CHO-$5HT_{1A}$-7 cells is eliminated by the addition of GTP [6]. Therefore, [^{3}H]8-OH-DPAT is labelling a single population of $5HT_{1A}$ receptors all coupled to G-proteins in CHO-$5HT_{1A}$-7 cells.

The use of the antagonist radioligand [^{3}H]-spiperone in this report for characterizing $5HT_{1A}$ receptors is novel. [^{3}H]Spiperone would not normally be considered suitable for ligand-binding studies on $5HT_{1A}$ receptors. The high level of expression and lack of other cross-reacting receptor sites in CHO-$5HT_{1A}$-7 cells, however, enables [^{3}H]spiperone to be used for this purpose. [^{3}H]Spiperone labels, in CHO-$5HT_{1A}$-7 cells, a single population of $5HT_{1A}$ receptors. Affinities for most antagonists are similar whether derived from competition versus [^{3}H]-spiperone or [^{3}H]8-OH-DPAT. Spiperone itself, however, has a much higher affinity when competing with [^{3}H]spiperone and the converse is true for all agonists. This suggested that the two radioligands might be labelling different states of the same receptor. It was already known that the receptors labelled by [^{3}H]8-OH-DPAT were in a state coupled to a G-protein from the effects of GTP. In the present study GTP was found to increase [^{3}H]-spiperone binding with an effect on the B_{max} but not the affinity of the radioligand. Saturation analyses showed that approximately equal numbers of receptor sites were labelled by either [^{3}H]8-OH-DPAT or [^{3}H]spiperone in the absence of GTP. Whereas GTP eliminates [^{3}H]8-OH-DPAT binding it increases [^{3}H]spiperone binding by about 70%. The number of receptor sites (labelled by [^{3}H]8-OH-DPAT) lost upon addition of GTP is roughly the same as the number (labelled by [^{3}H]spiperone) gained upon addition of GTP.

These findings may be explained in the following model. $5HT_{1A}$ receptors in CHO-$5HT_{1A}$-7 cells can exist in a form coupled to a G-protein (R–G) or uncoupled (R). GTP destabilizes R–G converting it to R. Agonists show a higher affinity for R–G than for R, whereas most antagonists show roughly equal affinities for the two states. Spiperone is an exception showing a higher affinity for R than for R–G. The differences in affinity are such that under the experimental conditions used [^{3}H]8-OH-DPAT will only label R–G and [^{3}H]spiperone will only label R (although this conclusion will be modified below). The data obtained show that normally in the cells there are roughly equal amounts of R–G and R, hence the similar B_{max} values for [^{3}H]8-OH-DPAT and [^{3}H]spiperone in control cells. Addition of GTP converts R–G to R so that [^{3}H]8-OH-DPAT binding is eliminated and [^{3}H]spiperone binding approximately doubled. The number of G-proteins in the cell membrane presumably limits the amounts of R than can form the R–G state and the equilibrium between R and R–G lies in favour of the latter in the absence of GTP or ligands.

An alternative explanation for the data that must be considered is that a proportion of the available receptors are occupied by serotonin from the growth medium. These occupied receptors would be unavailable for binding [^{3}H]spiperone with high affinity unless GTP was added when the reduction

in agonist affinity would allow the serotonin to dissociate from the receptors and an apparent increase in receptor number would be seen for [^{3}H]-spiperone binding. This alternative model predicts that the affinities for antagonists would be similar whether derived from competition versus [^{3}H]-spiperone or [^{3}H]8-OH-DPAT but this is not observed for spiperone in the present experiments. In addition, experiments with the radioligand [^{3}H]-lisuride (H. Sundaram, J. D. Turner and P. G. Strange, manuscript in preparation) have shown that in CHO-5HT$_{1A}$-7 cells this radioligand can label with high affinity both the R and R–G states (labelled in the present experiments with [^{3}H]spiperone and [^{3}H]-8-OH-DPAT, respectively). This shows clearly that there is not a population of receptors occluded by serotonin.

The model outlined above conforms broadly to that which has been proposed for many G-protein-linked receptors with agonists having a higher affinity for R–G states and antagonists having equal affinities for R–G and R states. It differs significantly, however, in that spiperone, although an antagonist, has different affinities for the two states showing higher affinity for the uncoupled (R) form. There are, in fact, a number of precedents for these findings in other receptor systems. Antagonist binding has been reported to be increased by GTP at muscarinic acetylcholine [11], A1 adenosine [12] and σ-opiate [13] receptors. Spiperone itself has been reported to show different affinities for the R–G and R states of D_2 dopamine and 5HT$_2$ serotonin receptors in some studies [14, 15]. Therefore, it seems that the simple model of R–G and R states showing different affinities for agonists but similar affinities for antagonists may not always hold. In the present system, the 5HT$_{1A}$ receptor, it is only spiperone that shows the differential affinities. Since spiperone has also been found to show differential affinities for R–G and R at other receptors [14, 15] it may be that this compound binds to these receptors in a different manner than most antagonists and is therefore sensitive to the conformational change relating R–G and R. [^{3}H]Spiperone binding is more sensitive to GTP ($E_{50} \sim 4\ \mu M$) than [^{3}H]8-OH-DPAT binding ($IC_{50} \sim 15\ \mu M$) [6]. This may reflect the occupancy with high affinity of the R–G state by the agonist whereas when the antagonist is used the R–G state is unoccupied.

The models discussed above may, however, be oversimplifications in the present system. In competition studies versus [^{3}H]spiperone, the affinity of the agonist 8-OH-DPAT was reduced by the addition of GTP. If [^{3}H]spiperone is labelling only the R state of the receptor, for which agonists have a low affinity, addition of GTP should not affect the competition of 8-OH-DPAT. Therefore, it is possible that in the absence of GTP in the cells, the states thought to be uncoupled from G-proteins (R) are actually coupled to a G-protein (R–G′) and there is no free R state. The R–G′ state would be different from the R–G state, perhaps owing to a different G-protein or a different conformational state of the R–G complex:

$$R\text{–}G \rightleftharpoons R\text{–}G' \rightleftharpoons R$$

Spiperone then has equally high affinities for R–G′ and R and a lower affinity for R–G. Agonists have a high affinity for R–G and much lower but not equal affinities for R–G′ and R. Addition of GTP converts all R–G and R–G′ to R.

The nature of the G-proteins involved here is unclear. It is known that the 5HT$_{1A}$ receptors in CHO-5HT$_{1A}$-7 cells inhibit adenylyl cyclase and that this is sensitive to pertussis toxin implicating a member of the G_i/G_o family [6]. The E_{50} values for agonists and the K_i value for spiperone for the adenylyl cyclase response correspond more to the K_i values determined versus [^{3}H]spiperone binding in the present study. Therefore, at least some of the G-proteins involved may be members of the pertussis toxin-sensitive family. This system should therefore provide a useful model for understanding the different interactions of 5HT$_{1A}$ receptors, G-proteins and signalling systems.

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