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Investigation of the mechanism of agonist and inverse agonist action at D_2 dopamine receptors

David J Roberts, Hong Lin, Philip G Strange*

School of Animal and Microbial Sciences, University of Reading, Whiteknights, Reading, RG6 6AJ, UK

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Abstract

This study investigated, for the D_2 dopamine receptor, the relation between the ability of agonists and inverse agonists to stabilise different states of the receptor and their relative efficacies. K_i values for agonists were determined in competition versus the binding of the antagonist [^{3}H]spiperone. Competition data were fitted best by a two-binding site model (with the exception of bromocriptine, for which a one-binding site model provided the best fit) and agonist affinities for the higher (K_h) (G protein-coupled) and lower affinity (K_l) (G protein-uncoupled) sites determined. K_i values for agonists were also determined in competition versus the binding of the agonist [^{3}H]-*N*-propylnorapomorphine (NPA) to provide a second estimate of K_h. Maximal agonist effects (E_{max}) and their potencies (EC_{50}) were determined from concentration–response curves for agonist stimulation of guanosine-5′-*O*-(3-[^{32}S]thiotriphosphate) ([^{35}S]GTPγS) binding. The ability of agonists to stabilise the G protein-coupled state of the receptor (K_l/K_h determined from ligand-binding assays) did not correlate with either of two measures of relative efficacy (relative E_{max}, K_l/EC_{50}) of agonists determined in [^{35}S]GTPγS-binding assays, when the data for all of the compounds tested were analysed. For a subset of compounds, however, there was a relation between K_l/K_h and E_{max}. Competition-binding data versus [^{3}H]spiperone and [^{3}H]NPA for a range of inverse agonists were fitted best by a one-binding site model. K_i values for the inverse agonists tested were slightly lower in competition versus [^{3}H]NPA compared to [^{3}H]spiperone. These data do not provide support for the idea that inverse agonists act by binding preferentially to the ground state of the receptor.

Keywords: D_2 dopamine receptor; Ligand binding; [^{35}S]GTPγS binding; Agonists; Inverse agonists; Relative efficacy

1. Introduction

There is much current interest in understanding the mechanisms of action of agonists and inverse agonists at G protein-coupled receptors (GPCRs). This is important in terms of understanding how these receptors work, but will also be very important in drug design. A very influential model of GPCR action has been the ternary complex model and its recent extensions [1–4]. The present formulation of the model includes a ground state of the receptor (R) which can isomerise to a partially activated form (R*) which is able to couple better to the G protein to form the active (R*G) state. Agonists stabilise the AR*G form (ternary complex). In the binary (R*G) and ternary (AR*G) complexes, GDP/GTP exchange on the G protein is catalysed, leading to release of the α and βγ subunits of the G protein and alteration of effector activity. According to this model, the ability of the agonist to stabilise the ternary complex (AR*G state) relative to the ground state should determine agonist efficacy. There have been suggestions, however, that agonists are able to modulate the activity of the G protein in the ternary complex so that agonism may be determined by effects at more than one step [5,6].

The degree of stabilisation of the ternary complex relative to the ground state can be examined using ligand binding. In many cases, agonist binding to GPCRs can be described in terms of a higher affinity state (K_h), which is guanine nucleotide sensitive and, therefore, believed to correspond to receptor coupled to an empty G protein, and a lower affinity state (K_l), corresponding to uncoupled receptor and, therefore, the receptor close to the ground state (R). The ratio of the affinities for these two states (K_l/K_h) is proposed to represent how well the agonist

Abbreviations: [^{35}S]GTPγS, guanosine-5′-*O*-(3-[^{35}S]thiotriphosphate); NPA, *N*-propylnorapomorphine; 3-PPP, 3-(3-hydroxyphenyl)-*N*-propylpiperidine; (+)-UH-232, *cis*-(+)-5-methoxy-1-methyl-2-(di-*N*-propylamino)tetralin.

* Corresponding author. Tel.: +44-118-378-8015; fax: +44-118-378-6537.
E-mail address: p.g.strange@reading.ac.uk (P.G. Strange).

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stabilises the ternary complex and is predicted to correlate with agonist efficacy [1]. We have recently examined the relation between K_l/K_h and the relative efficacy of agonists in functional tests for a receptor that conforms to the ternary complex model [7]. In that study, we used two measures of relative efficacy (maximal agonist effect (E_{max}) and ratio of agonist dissociation constant to concentration of agonist giving half E_{max} (K_l/EC_{50})). We showed that the relation between K_l/K_h and E_{max} was hyperbolic but could appear linear if a limited range of E_{max} values was used. The relation between K_l/K_h and K_l/EC_{50} was linear.

Correlations between K_l/K_h and relative efficacy have been reported previously for the β_2 adrenergic [1], CB_1 cannabinoid [8], 5-$HT_{2A/C}$ [9] and 5-HT_{1A} serotonin receptors [10]. Such correlations for the D_2 dopamine receptor have also been reported [11–13]. Other investigations for this receptor have shown no such correlation [5,14]. One possible explanation for these differences in the studies on the D_2 dopamine receptor may be due to the different methodologies used. For example, radiolabelled agonists and antagonists were used to determine values for K_h and K_l, respectively, in some of the studies reporting correlations [11,12], whereas K_h and K_l were determined from agonist/[^{3}H]spiperone competition experiments in the studies reporting no correlations [5,14]. Determinations of K_h and K_l from agonist/[^{3}H]spiperone competition curves may introduce complications. For example, there may be difficulties in analysing such curves where the separation between K_h and K_l is not great. It is also possible that determinations of K_h in competition versus an [^{3}H]agonist and versus an [^{3}H]antagonist may yield different values. This has been discussed in detail in [15,16] where, if the available G protein is limiting, the [^{3}H]agonist and [^{3}H]antagonist may label different populations of receptors.

It is commonly assumed that inverse agonist action depends upon the stabilisation of the ground state of the receptor, in preference to active states [16]. This mechanism has been probed by creating mutant receptors that may lie more towards the R^* state of the receptor than the native receptor. In some cases these mutants exhibited decreased affinity for inverse agonists, providing evidence for the mechanism [17,18]. In the case of the D_2 dopamine receptor we have reported the properties of such a mutant receptor [19]. Although the mutant had the characteristics of a receptor that adopted the activated conformation, there were no effects on the affinities of inverse agonists, a finding reported for some other receptors mutated to increase agonist-independent activation (e.g. $\alpha_{1A/B}$ adrenergic receptors [20]). An alternative method for probing the affinities of inverse agonists for the different states of the receptor is to determine their affinities in competition versus a tritiated agonist (labels R^*G) and a tritiated antagonist (labels each state with equal affinity). In principle, if the inverse agonist preferentially labels the R state, then affinities determined versus a tritiated agonist may be lower than versus a tritiated antagonist. We have used this method to determine the affinities of inverse agonists for different states of the 5-HT_{1A} serotonin receptor [21].

In the present study, therefore, we have probed these aspects of the mechanisms of agonism and inverse agonism for the D_2 dopamine receptor. The D_2 dopamine receptor is a member of the family of dopamine receptors and is an important site of action of the antipsychotic and anti-parkinsonian drugs. We have determined the affinities of a range of agonists and inverse agonists at the D_2 receptor stably expressed in CHO cells in competition-binding experiments versus the agonist, [^{3}H]*N*-propylnorapomorphine (NPA), and the antagonist [^{3}H]spiperone. [^{3}H]NPA has been shown to label with high affinity a state of the receptor that corresponds to the receptor coupled to G protein [22]. [^{3}H]Spiperone has been widely used to label D_2 receptors and appears to label both the uncoupled and G protein-coupled forms of the receptor with similar affinity in most reports (see, e.g. Ref. [23]). Competition experiments versus [^{3}H]spiperone should, therefore, provide information on affinities for the G protein-coupled and uncoupled states of the receptor. We have then compared the ligand-binding data with determinations of the relative efficacy of the agonists based on their abilities to stimulate guanosine-5′-*O*-(3-[^{32}S]thiotriphosphate) ([^{35}S]GTPγS) binding.

2. Methods

2.1. Materials

[^{35}S]GTPγS (~37 TBq mmol^{-1}) and [^{3}H]spiperone (~600 GBq mmol^{-1}) were purchased from Amersham Biosciences. [^{3}H]NPA (~1 TBq mmol^{-1}) and Optiphase HiSafe-3 scintillation fluid was purchased from Perkin-Elmer Life Sciences. Dopamine, bromocriptine and *cis*-(+)-5-methoxy-1-methyl-2-(di-*N*-propylamino)tetralin ((+)-UH-232) were purchased from TOCRIS. (+)-Butaclamol, clozapine, haloperidol, NPA, β-phenylethylamine, 3-(3-hydroxyphenyl)-*N*-propylpiperidine ((−)-3-PPP), (−)-sulpiride, *m*-tyramine and *p*-tyramine were purchased from Sigma.

2.2. Cell culture

CHO cells stably expressing human D_{2short} dopamine receptors [19] were grown in Dulbecco's modified Eagle's medium containing 5% foetal bovine serum and 400 μg ml^{-1} active geneticin (to maintain selection pressure). Cells were grown at 37 °C in an humidified atmosphere of 5% CO_2.

2.3. Membrane preparation

Membranes were prepared from CHO cells expressing D_{2short} dopamine receptors as described previously [24].

Briefly, confluent 175 cm^2 flasks of cells were washed once with 5 ml HEPES buffer (20 mM HEPES, 1 mM EGTA, 1 mM EDTA, 10 mM $MgCl_2$; pH 7.4). Cells were then removed from the surface of the flasks using 5 ml HEPES buffer and glass balls (2 mm diameter) and were then homogenised using an Ultra-Turrax homogeniser (two 5-s treatments). The homogenate was centrifuged at 1700 × *g* (10 min, 4 °C) after which the supernatant was centrifuged at 48,000 × *g* (60 min; 4 °C). The resulting pellet was resuspended in HEPES buffer at a concentration of 3–5 mg protein ml^{-1} (determined by the method of Lowry et al. [25] and stored in aliquots at −70 °C until use.

2.4. Radioligand-binding assays

Cell membranes (25 μg) were incubated with either [^{3}H]spiperone (10 pM–2 nM for saturation binding experiments; 0.35 nM for competition-binding experiments) or [^{3}H]NPA (30 pM–15 nM for saturation experiments; 0.1 nM for competition experiments) and competing drugs in HEPES buffer (20 mM HEPES, 1 mM EGTA, 1 mM EDTA, 10 mM $MgCl_2$, 100 mM *N*-methyl-D-glucamine (to maintain ionic strength in the absence of sodium ions); pH 7.4 (using HCl) containing 0.1 mM dithiothreitol) in a final volume of 1 ml for 3 h at 25 °C. The assay was terminated by rapid filtration (through Whatman GF/C filters) using a Brandel cell harvester followed by four washes with 4 ml ice-cold phosphate-buffered saline (0.14 M NaCl, 3 mM KCl, 1.5 mM KH_2PO_4, 5 mM Na_2HPO_4; pH 7.4) to remove unbound radioactivity. Filters were soaked in 2 ml of scintillation fluid for at least 5 h and bound radioactivity was determined by liquid scintillation counting. Non-specific binding of [^{3}H]spiperone was determined in the presence of 3 μM (+)-butaclamol.

2.5. [^{35}S]GTPγS-binding assays

Cell membranes (25 μg) were incubated with ligands in a volume of 0.9 ml of HEPES buffer containing 1 μM GDP and with 100 mM NaCl replacing the *N*-methyl-D-glucamine for 30 min at 30 °C. The assay was initiated by addition of 100 μl of diluted [^{35}S]GTPγS to give a final concentration of ~100 pM. The assay was incubated for a further 30 min and terminated by rapid filtration as above. For the experiments with (+)-butaclamol, GDP was omitted and the HEPES buffer with *N*-methyl-D-glucamine was used.

2.6. Data analysis

Radioligand-binding data (saturation and competition experiments) were analysed using Prism (GraphPad) and were assumed to conform to a one-binding site model unless a statistically better fit could be obtained using a two-binding site model ($P < 0.05$, *F*-test). The equilibrium dissociation constants (K_d) of the radioligands were measured by saturation binding analysis. Saturation binding data for [^{3}H]spiperone were fitted best by a one-binding site model whereas saturation data for [^{3}H]NPA were fitted best by a two-binding site model ($P < 0.05$).

In competition experiments that were fitted best by a one-binding site model, a single IC_{50} value was obtained, whereas in competition experiments that were fitted best by a two-binding site model, two IC_{50} values (for the higher and lower affinity sites) and the % higher affinity sites were obtained. The inhibition constants (K_i from the single IC_{50}, K_h, K_l from the IC_{50} values for the higher and lower affinity sites) were calculated from IC_{50} values, derived from competition-binding analyses, using the Cheng–Prusoff equation [26]. This corrects for the concentration of the radioligand and its dissociation constant at the relevant binding site. This correction makes no assumptions about mechanism and can be shown to be valid for systems where there is ligand-induced receptor isomerisation, e.g. as in the system of Leff [27] (data not shown).

Statistical significance of differences between two data sets (e.g. two sets of pK_i values) was determined using unpaired two-way ANOVA followed by a Bonferroni post-test with significance determined as $P < 0.05$. Correlations were analysed using Prism and statistical significance determined using the Pearson correlation coefficient.

3. Results

3.1. [^{3}H]Spiperone-binding data

Saturation data for [^{3}H]spiperone binding to membranes derived from cells expressing D_2 dopamine receptors were described well by a one-binding site model (pK_d 10.52 ± 0.03; K_d 0.03 nM (mean ± S.E.M., $n = 5$; Fig. 1A); B_{max} 1.15 ± 0.13 pmol mg^{-1} protein). Competition of a range of agonists versus [^{3}H]spiperone binding was carried out (Table 1). The resulting competition curves were fitted best by a two-binding site model and dissociation constants for the higher (K_h) and lower (K_l) affinity states were derived. One exception was bromocriptine, which, in agreement with previous observations, gave binding data that were fitted best by a one-binding site model (Fig. 2).

3.2. [^{3}H]NPA-binding data

Saturation analysis of [^{3}H]NPA binding produced data characterised by Hill coefficients less than one (0.66 ± 0.07, mean ± S.E.M., $n = 4$). When the data were analysed using one-binding site and two-binding site models, the data were fitted best by a two-binding site model, corresponding to the higher and lower affinity-binding sites observed in the competition experiments versus [^{3}H]spiperone (Fig. 1B). Dissociation constants were: pK_h 10.27 ± 0.08 (0.05 nM) and pK_l 9.09 ± 0.06 (0.82 nM) (mean ± S.E.M., $n = 4$) and the higher affinity sites represented 40 ± 6% of the total

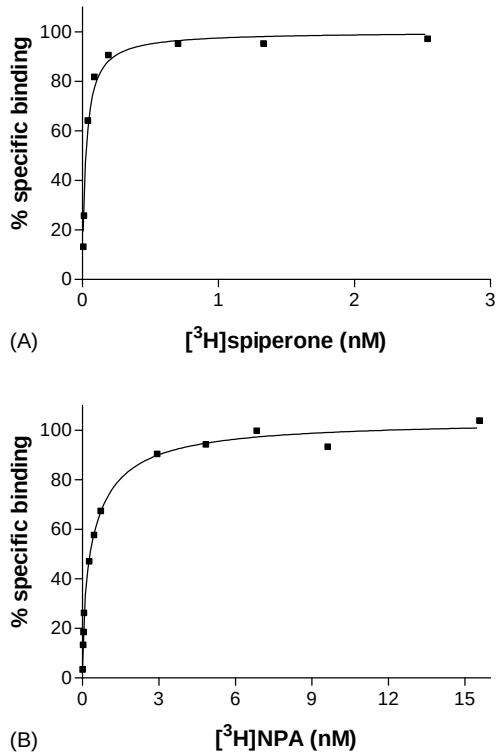

Fig. 1. Saturation analysis of [^{3}H]spiperone binding (A) and [^{3}H]NPA binding (B) to D_2 dopamine receptors expressed in CHO cells. Saturation binding analyses were performed as described in Section 2 and the data are from representative experiments, performed in triplicate, and replicated as outlined in the text. [^{3}H]Spiperone binding was fitted best by a one-binding site model whereas [^{3}H]NPA binding was fitted best by a two-binding site model ($P < 0.05$).

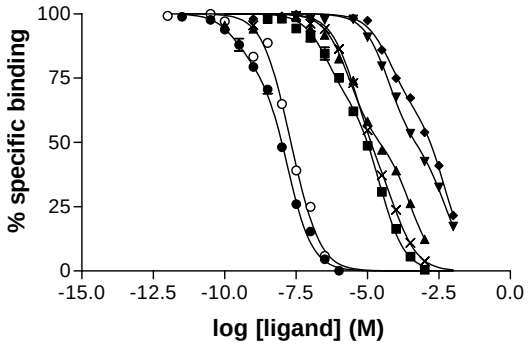

Fig. 2. Binding of agonists to D_2 dopamine receptors expressed in CHO cells. The binding of dopamine (■), *m*-tyramine (▲), β-phenylethylamine (▼), *p*-tyramine (◆), NPA (●), (−)-3-PPP (×) and bromocriptine (○) was determined in competition vs. [^{3}H]spiperone to membranes of CHO cells expressing the D_2 receptor as described in Section 2. Data are from representative experiments replicated as in Table 1. All curves were fitted best by a two-binding site model, except that for bromocriptine, which was fitted best by a one-binding site model ($P < 0.05$).

population. The B_{max} value for total [^{3}H]NPA binding was 0.96 ± 0.06 pmol mg^{-1} and this was not significantly different from the value for the B_{max} of [^{3}H]spiperone binding measured on the same preparation ($P > 0.05$). Binding of the agonists was tested in competition versus [^{3}H]NPA binding. The concentration of [^{3}H]NPA used was ~0.1 nM in order to restrict binding to the higher affinity population of sites. Under these conditions binding of [^{3}H]NPA to lower affinity sites was ~20% of the total specific [^{3}H]NPA binding. Competition data were all characterised by Hill coefficients close to one (dopamine 0.92 ± 0.09; *m*-tyramine 0.99 ± 0.13; *p*-tyramine 1.12 ± 0.09; β-phenylethylamine 0.88 ± 0.14; NPA 1.00 ± 0.07; bromocriptine 0.98 ± 0.08; (−)-3-PPP 1.08 ± 0.11). When analysed using one- and two-binding site models, competition data fitted best to a one-binding site model and the derived K_i values are given in Table 1. No significant difference was found between the K_h determined versus [^{3}H]spiperone and the K_i determined versus [^{3}H]NPA ($P > 0.05$).

3.3. Agonist stimulation of [^{35}S]GTPγS binding

Agonist stimulation of [^{35}S]GTPγS binding was used as a measure of relative efficacy of the agonists tested (Table 2). The compounds exhibited a range of relative efficacies, with (−)-3-PPP showing the lowest efficacy (27% that of dopamine) and NPA the highest (106% that

Table 1
The binding of agonists to human D_2 dopamine receptors expressed in CHO cells

Ligand	[^{3}H]Spiperone					[^{3}H]NPA
	pK_h (K_h, nM)	pK_l (K_l, nM)	%R_h	K_l/K_h	*n*	pK_i (*n*) (K_i, nM)
Dopamine	7.24 ± 0.11 (58)	5.49 ± 0.09 (3240)	53 ± 6	62	6	7.59 ± 0.14 (8) (26)
m-Tyramine	6.56 ± 0.05 (275)	4.69 ± 0.04 (20400)	47 ± 3	68	5	6.85 ± 0.13 (8) (140)
β-Phenylethylamine	5.39 ± 0.05 (4070)	3.63 ± 0.04 (234000)	43 ± 3	50	7	5.61 ± 0.11 (4) (2460)
p-Tyramine	5.22 ± 0.05 (6030)	3.59 ± 0.12 (257000)	33 ± 5	55	4	5.28 ± 0.07 (4) (5250)
NPA	10.53 ± 0.04 (0.03)	8.67 ± 0.03 (2)	37 ± 4	67	5	10.05 ± 0.25 (4) (0.09)
Bromocriptine	8.76 ± 0.04 (2)	–	–	1	7	9.12 ± 0.20 (5) (0.8)
(−)-3-PPP	6.86 ± 0.09 (140)	5.32 ± 0.04 (4790)	48 ± 7	27	4	6.74 ± 0.18 (4) (180)

Agonist binding was determined in competition vs. [^{3}H]spiperone (~0.4 nM) or [^{3}H]NPA (~0.1 nM) as described in Section 2. Competition curves were analysed by non-linear regression and, with the exception of bromocriptine, data were fitted best by a two-binding site model using [^{3}H]spiperone and a one-binding site model using [^{3}H]NPA ($P < 0.05$). Data for bromocriptine were fitted best by a one-binding site model in all cases ($P < 0.05$). Values are given for the dissociation constants for the different states as well as the percentage existing in high affinity (%R_h) where appropriate. Data are expressed as mean ± S.E.M. for the indicated number of experiments with data points determined in triplicate. $P > 0.05$ for comparison of K_h vs. [^{3}H]spiperone with K_i vs. [^{3}H]NPA (two-way ANOVA).

Table 2
Agonist stimulation of [^{35}S]GTPγS binding by D_2 receptors expressed in CHO cells

Ligand	pEC_{50} (EC_{50}, nM)	Relative efficacy	n
Dopamine	6.37 ± 0.02 (430)	100	13
m-Tyramine	5.46 ± 0.06 (3470)	71 ± 4	8
β-Phenylethylamine	4.84 ± 0.07 (14450)	48 ± 6	10
p-Tyramine	4.38 ± 0.08 (41700)	46 ± 2	5
NPA	8.84 ± 0.22 (1.5)	106 ± 11	10
Bromocriptine	8.88 ± 0.22 (1.3)	66 ± 5	9
(−)-3-PPP	7.14 ± 0.22 (72)	27 ± 4	7

Agonist stimulation of [^{35}S]GTPγS binding was performed using a range of agonist concentrations as described in Section 2. Data were fitted best by sigmoidal concentration–response relationships with Hill coefficients of one. pEC_{50} values were determined and maximal effects expressed as a percentage of the maximal dopamine response to give the relative efficacy for each agonist. Data are expressed as mean ± S.E.M. for the indicated number of experiments with data points determined in triplicate.

of the dopamine response). The potency values (EC_{50}) of these compounds were related to their affinities in that they lay between the K_l and K_h values derived from ligand binding. This relationship is emphasised by the properties of bromocriptine. This compound displays only one affinity in competition against [^{3}H]spiperone, which is very similar to its potency in [^{35}S]GTPγS-binding assays (Tables 1 and 2).

3.4. Inverse agonist-binding data

Inverse agonists are proposed to bind preferentially to the R state of the receptor uncoupled from G protein [17]. This was investigated by determining the dissociation constants of a number of inverse agonists, and UH-232 (previously demonstrated to be a neutral antagonist [19]), in competition versus the binding of both [^{3}H]spiperone and [^{3}H]NPA. A low concentration of [^{3}H]NPA (0.1 nM) was used in order to restrict binding to the higher affinity population of [^{3}H]NPA-binding sites. Competition data were all fitted best by a one-binding site model. A significantly higher affinity was observed for all ligands in competition versus [^{3}H]NPA compared to [^{3}H]spiperone (Table 3). The affinity difference was approximately twofold for the inverse agonists and approximately sevenfold for UH-232. These data suggest that inverse agonists do not preferentially bind to the R state of the receptor.

3.5. Inverse agonist effects of (+)-butaclamol in [^{35}S]GTPγS-binding assays

The inverse agonist effects of (+)-butaclamol were assessed in [^{35}S]GTPγS-binding assays in the absence of GDP and Na^+ ions in order to maximise basal G protein activation (Fig. 3). (+)-Butaclamol inhibited up to ∼20% of the basal [^{35}S]GTPγS binding in a concentration-dependent manner with a pIC_{50} of 9.28 ± 0.16 (0.5 nM) (mean ± S.E.M., $n = 11$).

Table 3
Dissociation constants of inverse agonists at D_2 dopamine receptors expressed in CHO cells

Ligand	[^{3}H]Spiperone	[^{3}H]NPA
	pK_i (n) (K_i, nM)	pK_i (n) (K_i, nM)
(+)-Butaclamol	9.42 ± 0.05 (4) (0.38)	9.80 ± 0.08 (3)** (0.16)
(−)-Sulpiride	6.82 ± 0.04 (4) (151)	7.12 ± 0.02 (3)* (76)
Clozapine	7.83 ± 0.02 (3) (15)	8.23 ± 0.01 (3)* (6)
Haloperidol	9.60 ± 0.04 (4) (0.25)	9.87 ± 0.09 (3) (0.13)
(+)-UH-232	6.39 ± 0.03 (3) (410)	7.24 ± 0.10 (3)*** (58)

The binding of inverse agonists and an antagonist was determined in competition vs. [^{3}H]spiperone (∼0.4 nM) and [^{3}H]NPA (∼0.1 nM) as described in Section 2. The competition curves were analysed by non-linear regression analysis to derive dissociation constants (K_i) and all data were fitted best by a one-binding site model ($P < 0.05$).

$^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$ for comparison between K_i vs. [^{3}H]spiperone and K_i vs. [^{3}H]NPA (two-way ANOVA).

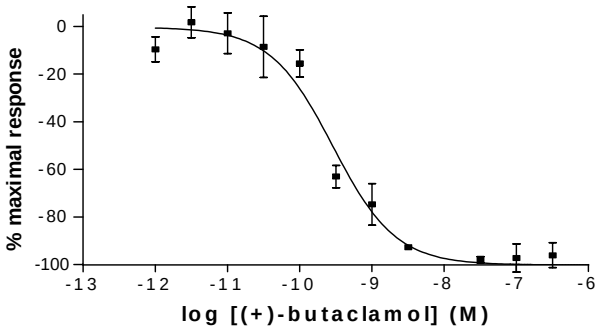

Fig. 3. Inhibition of basal [^{35}S]GTPγS binding by (+)-butaclamol via D_2 dopamine receptors expressed in CHO cells. [^{35}S]GTPγS binding was determined in the presence of different concentrations of (+)-butaclamol as described in Section 2. The data were fitted using a sigmoidal dose–response curve with a Hill coefficient of one and expressed as a percentage of the maximal (+)-butaclamol response (∼20% inhibition of basal [^{35}S]GTPγS binding). The data shown represent a single experiment, replicated as in the text. Data points are mean ± S.E.M.

4. Discussion

In this study, we have examined several aspects of the mechanism of action of agonists and inverse agonists at the D_2 dopamine receptor. In particular, we sought to validate the prediction from ternary complex models [1–4] that the degree of stabilisation of the ternary complex of agonist/receptor/G protein, relative to the ground state measured in ligand-binding studies, is a measure of agonist efficacy. In addition, we examined the hypothesis that inverse agonism is achieved by stabilisation of the ground state of the receptor at the expense of activated states.

Ligand-binding studies have been used to probe the mechanism of agonist efficacy, notably by determining the ability of agonists to bind to higher affinity states (K_h), associated with coupling to G proteins (AR^*G), and lower affinity states (K_l), in the absence of G protein coupling (R/R^*). In principle, there should be a relationship between the K_l/K_h ratio and the relative efficacy of agonists (E_{max}).

Simulations of the ternary complex model have shown that in fact E_{max} shows a hyperbolic dependence on K_l/K_h which can appear linear for limited data sets [7]. In addition, a second measure of relative efficacy (K_l/EC_{50}) should be highly correlated with K_l/K_h [7]. In experimental studies of this relationship, correlations have been reported in some cases between the K_l/K_h ratio and measures of agonist efficacy [1,8–10]. For the D_2 dopamine receptor, correlations have also been reported ([11–13], some compounds in Ref. [28]) although other studies have failed to find a correlation between the stabilisation of the ternary complex and agonist efficacy [5,14]; some compounds in Ref. [28]). In our own studies (see, e.g. Ref. [14]) we have determined the K_l/K_h ratio from agonist/[^{3}H]spiperone competition curves. Other authors [11,12] have used separate competition experiments versus a tritiated agonist and a tritiated antagonist (in the presence of GTP) to define K_h and K_l, respectively. These differences in analysis could account for the different conclusions reached and, in the present report, we have investigated this issue.

In the present study, therefore, we determined values for K_h and K_l using these two methods for the D_2 dopamine receptor. Agonist-binding curves obtained in competition versus [^{3}H]spiperone exhibited the characteristic higher and lower affinity states from which values for K_h and K_l could be obtained (Table 1). Bromocriptine, in agreement with many other observations, showed only a single affinity state, i.e. it has a K_l/K_h ratio of one. Values for K_h were also obtained in competition versus the agonist [^{3}H]NPA. A low concentration (~0.1 nM) of [^{3}H]NPA was used in these experiments in order to restrict binding to the higher affinity G protein-coupled population of sites. These data were in excellent agreement with values for K_h determined in competition versus [^{3}H]spiperone. This shows that the two methods for determining K_h provided equivalent data. The differences in the occurrence of correlations between K_l/K_h and efficacy, therefore, are not a result of the different methods used for determining K_h. We have also shown previously that values for K_l determined from the lower affinity site of the competition curve versus [^{3}H]spiperone and from the single site in the presence of GTP/Na^+ ions are equivalent [5]. Determinations of K_l and K_h from the agonist/[^{3}H]spiperone competition curve, therefore, do provide valid estimates of these parameters.

These observations on the estimates of K_l derived in competition versus [^{3}H]agonist and [^{3}H]antagonist radioligands are consistent with the system being G protein limited [15]. When the [^{3}H]antagonist is used, agonists compete with higher and lower affinities. The higher affinity states correspond to binding to the RG complex whereas the lower affinity states are for binding to the uncoupled (R) state resulting from a limited availability of G protein. When the [^{3}H]agonist is used, only the RG complex is labelled with high affinity and the affinity derived agrees with that seen for the higher affinity state determined using the [^{3}H]antagonist.

It is also important also to be clear about what the two measures of agonist affinity reflect in terms of different states of the receptor. The analysis presented in this report assumes that K_l for agonists (determined versus [^{3}H]spiperone) reflects labelling of the R state of the receptor uncoupled from G protein. If the extended ternary complex model applies, however, there could be a component of the affinity of R^* included in these estimates (see discussion in Ref. [29]). This possibility may be addressed by considering the properties of a mutant D_2 receptor (T343R [19]). This receptor exhibited many of the properties of a constitutively active receptor. When the binding of dopamine to the T343R mutant receptor was analysed, there was a decrease (higher affinity) in K_l compared to the native receptor and K_l became sensitive to Na^+ with Na^+ increasing K_l. Given that the T343R mutant seems to lie towards R^*, this sensitivity to Na^+ seems to be a reflection of the R^*/R transition, with Na^+ promoting the R^* to R transition. For the native receptor K_l is largely insensitive to Na^+ ions, so we may conclude that, for the native receptor, K_l is a good measure of the ground state affinity, i.e. the R state. The single affinity obtained in the presence of GTP for agonists (K_{iGTP}) in competition with [^{3}H]spiperone agrees with estimates of K_l so that K_l and K_{iGTP} are good measures of the affinity of the R state and effects of R^* do not interfere to any great extent.

We are also assuming here that higher affinity [^{3}H]NPA binding is a measure of the G protein-coupled state of the receptor (R^*G). Evidence supporting this idea comes from the observation that high affinity NPA binding (both in saturation analyses with [^{3}H]NPA and in competition experiments [5]; D. Roberts and P.G. Strange, unpublished data) is fully sensitive to GTP and there is a complete conversion to the lower affinity state.

The relative efficacy of the agonists used here was evaluated using the stimulation of [^{35}S]GTPγS binding [30] determining the EC_{50} (concentration of agonist giving half-maximal effect) and E_{max} (maximal effect) values for each agonist in this assay. Dopamine and NPA displayed full agonism while the other agonists showed a range of partial agonism (*m*-tyramine > bromocriptine > β-phenylethylamine = *p*-tyramine > (−)-3-*PPP*). Two measures of agonist relative efficacy, the E_{max} and the K_l/EC_{50} ratio (see above and Ref. [7]) have been used here in relation to the K_l/K_h ratio.

When data for all the agonists tested are considered, there is no relation between K_l/K_h and either measure of relative efficacy (Fig. 4). Within the set of agonists tested there are, however, clear differences between compounds. Bromocriptine, for example, has a K_l/K_h value of one but it is a good partial agonist, whereas all the other compounds tested have K_l/K_h values greater than 20 with varying degrees of relative efficacy. Bromocriptine may, therefore, activate the receptor in a different manner. We have suggested that for this compound, binding to the receptor in the absence of G protein coupling may strongly stabilise

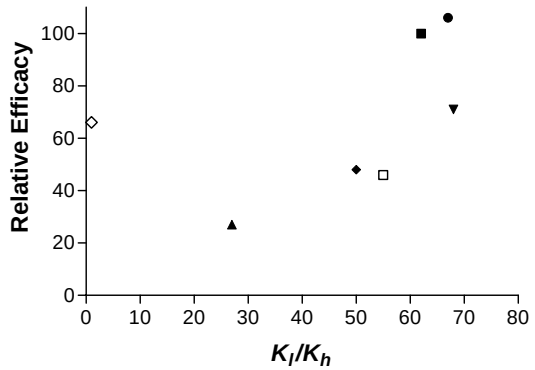

Fig. 4. Relationship between agonist relative efficacy (E_{max}) and the K_l/K_h ratio for D_2 dopamine receptors expressed in CHO cells. For each of the agonists (dopamine (■), *m*-tyramine (▼), β-phenylethylamine (◆), *p*-tyramine (□), NPA (●), (−)-3-PPP (▲), bromocriptine (◇)) the K_l/K_h ratio was determined (shown in Table 1). Agonist relative efficacy (E_{max}) was obtained from the maximal effect in the [^{35}S]GTPγS-binding assays as shown in Table 2. See text for discussion of correlations.

the activated form of the receptor (AR^*) and there may be little energy gained in ternary complex (AR^*G) formation, i.e. AR^* is close in structure to AR^*G [14]. This would give agonism but there would be no difference between binding affinities in the absence and presence of G protein coupling. For this compound the binding energy between receptor and ligand is fully expressed upon binding to the free receptor. It may, therefore, be legitimate to analyse the data excluding bromocriptine. If the data are analysed excluding bromocriptine, then there appears to be a relationship between E_{max} and K_l/K_h although this does not quite reach significance if analysed using linear regression ($P = 0.0506$). No correlation was seen, however, between K_l/EC_{50} and K_l/K_h ($P > 0.05$). There may, therefore, be some relation between the ability of an agonist to stabilise the ternary complex, as determined by K_l/K_h, and the relative efficacy for this subset of compounds, but the relationship is incomplete.

Several studies have examined the relation between the stabilisation of the G protein-coupled state of the receptor by agonists (K_l/K_h) and relative efficacy (see earlier references). In some studies a relationship is seen between the two sets of parameters and in other studies such a relationship is not seen. It should be noted, however, that in most studies only one efficacy parameter (E_{max}) is considered. The present study shows that differences in the methods for determination of K_l and K_h do not underlie the occurrence of these relationships. Another difference between the different studies concerns the expression systems used which could affect the G protein that the D_2 receptor activates, the receptor/G protein ratio and the efficiency of receptor/G protein coupling, which in turn could affect the relationship between K_l/K_h and relative efficacy. Importantly, however, a major influence on the occurrence of such relationships is likely to be the group of agonists used. We have shown that if a structurally similar group of agonists (aminotetralins) is examined, then a correlation can be observed between K_l/K_h and both measures of relative efficacy used here [28], whereas for another group of compounds (phenylethylamines) a correlation is not seen. This suggests that correlations between K_l/K_h and relative efficacy can be seen for some compounds and not for others, as in the present study. Where correlations are not seen, the degree of stabilisation of the ternary complex by an agonist may not be the only factor that determines relative efficacy. It may be that agonists are able to regulate the activity of the ternary complex to different extents, including rates of GTP/GDP exchange [31] and ternary complex breakdown [5,14,32].

We also examined the potential mechanisms of inverse agonism. It has been proposed that inverse agonists stabilise the ground state (R) form of the receptor and thereby prevent agonist-independent activity [2]. This may be achieved by preferential binding to R over R^* or R over R^*G or combinations of these two mechanisms. In either case it is predicted that inverse agonists will show a higher affinity for the R state of the receptor than the R^*G state [21]. Therefore, we determined the affinities of a range of inverse agonists and one neutral antagonist in competition with the binding of the agonist [^{3}H]NPA and the antagonist [^{3}H]spiperone. The inverse agonists did not exhibit lower affinities versus the agonist radioligand, indeed these tended to be higher than affinities obtained versus [^{3}H]spiperone. Nevertheless, from these data it might be concluded that inverse agonist action is not achieved by stabilisation of the ground state of the receptor. It should be noted, however, that a difference in affinity for an inverse agonist when measured in competition versus an [^{3}H]agonist and versus an [^{3}H]antagonist will only be seen if the receptor couples strongly to G proteins in the absence of agonist, and there is an excess of R over G. In this case the receptor exists in two semi-independent pools (R and RG) which may be labelled by the [^{3}H]antagonist and [^{3}H]agonist, respectively [16]. We have employed this method for the 5-HT_{1A} serotonin receptor and shown that some inverse agonists do exhibit differences in affinity for the coupled and uncoupled forms of the receptor [33]. For the D_2 dopamine receptor expressed in CHO cells, there appears to be agonist-independent coupling of R and G based on the ability of (+)-butaclamol to inhibit basal [^{35}S]GTPγS binding (Fig. 3) and to potentiate forskolin-stimulated cAMP accumulation [34]. The present data do not, therefore, provide evidence in favour of the hypothesis that inverse agonists act by stabilising the ground state (R) of the D_2 receptor. Inverse agonism may, therefore, be achieved by compounds converting the receptor to a state that is unable to activate G proteins.

It should be noted, however, that the antagonist, spiperone, has been shown to distinguish higher and lower affinity-binding sites of the D_2 receptor in a guanine nucleotide-sensitive manner in membranes from the anterior pituitary gland [35,36]. Differences between these studies and the present report may result from differences

in the two cell systems, for example, different G proteins or different accessory proteins. It is interesting that, whereas the physiological relevance of inverse agonism remains unresolved, the only clear evidence for the mechanism of action of inverse agonists at dopamine D_2 receptors has been obtained from studies on a native tissue.

In the foregoing analysis, we have used the compound UH-232, which we have previously reported as a neutral antagonist [19,34]. It is notable that when this compound was analysed for K_h and K_l, there was a difference between the two parameters such that K_l/K_h for this compound was ~7. This value would be more consistent with agonism and indeed several studies have reported partial agonist effects of UH-232 [37,38]. It may be that the relative efficacy of this compound is very system dependent.

In conclusion, we have found no relation between agonist stabilisation of the ternary complex, as determined by the K_l/K_h ratio and relative efficacy, when data for all of the compounds tested here are analysed. For a subset of compounds some relation between the two parameters was seen. No significant difference was found between K_h values determined in competition against [^{3}H]spiperone or [^{3}H]NPA, ruling this out as a reason for the lack of correlation. Comparison of inverse agonist affinities using [^{3}H]spiperone and [^{3}H]NPA competition showed slightly higher affinity estimates when determined in competition with [^{3}H]NPA binding. This study, therefore, provides no evidence for the idea that stabilisation of the ground receptor state by the inverse agonist is the mechanism of action of these inverse agonists.

Acknowledgments

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