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Biochemical Pharmacology 67 (2004) 2039–2045
Biochemical Pharmacology
www.elsevier.com/locate/biochempharm

Inverse agonist properties of atypical antipsychotic drugs

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Received 21 October 2003; accepted 9 February 2004

Abstract

Mechanisms of action of several atypical antipsychotic drugs have been examined at the D_2 dopamine receptor expressed in CHO cells. The drugs tested were found to exhibit inverse agonist activity at the D_2 dopamine receptor based on their effects to potentiate forskolin-stimulated cyclic AMP (cAMP) accumulation. Each of the antipsychotic drugs tested (clozapine, olanzapine, quetiapine and risperidone) increased cAMP accumulation to the same extent. The increase in cAMP was also similar to that seen with typical antipsychotic drugs. Inverse agonism at the D_2 dopamine receptor seems, therefore, to be a property common to all classes of antipsychotic drugs. The effect of sodium ions on the binding of the drugs to the receptor was also assessed. Each of the atypical antipsychotic drugs tested here bound with higher affinity in the absence of sodium ions. Previous studies have shown that some antipsychotic drugs are insensitive to sodium ions and some bind with higher affinity in the presence of sodium ions. Given that all of these antipsychotic drugs are inverse agonists, it may be concluded that this sodium ion sensitivity is unrelated to mechanisms of inverse agonism.

Keywords: D_2 dopamine receptor; Inverse agonism; Atypical antipsychotic drugs; cAMP; Sodium ion sensitivity; Mechanism

1. Introduction

The antipsychotic (neuroleptic) drugs constitute a large class of chemically diverse drugs that possess the ability to suppress the positive symptoms of schizophrenia [1–4]. Most antipsychotic drugs also cause a broad range of side effects, including motor (extrapyramidal) side effects (dystonia, akathisia, parkinsonism, tardive dyskinesia), sedation, postural hypotension, dry mouth, weight gain, amenorrhea, etc. (see [3] for more details). The antipsychotics have been divided in to two groups (typical and atypical) on the basis of their ability to precipitate the motor side effects. The typical antipsychotics do cause the motor side effects whereas the atypical drugs have a lower propensity to do this [5–7]. The mechanisms of action of the antipsychotic drugs have been much debated but it seems that actions at the D_2, D_3 and D_4 dopamine receptors are central to their activities [8]. Actions at D_2 or D_3 receptors in limbic/cortical regions may be responsible for the therapeutic effects of the drugs, whereas actions at D_2 receptors in the striatum may be responsible for the motor side effects [9].

It had been assumed that the antipsychotic drugs were acting as antagonists at dopamine receptors. It has been shown recently, however, that some antipsychotic drugs possess inverse agonist activity at D_2 and D_3 receptors. This inverse agonism has been demonstrated using a variety of techniques including stimulation of prolactin secretion from pituitary cells [10], inhibition of [^{35}S]GTPγS binding [11], inhibition of [^{3}H]thymidine incorporation [12], stimulation of cyclic AMP (cAMP) accumulation [13–15] and inhibition of phospholipase C activation in a D_2 dopamine receptor/α_{1b} adrenoceptor chimera [16]. In studies on the D_2 dopamine receptor, the inverse agonist actions of several representative antipsychotic drugs were studied in CHO cells expressing this receptor at high levels. Each of the drugs tested was able to increase forskolin-stimulated cAMP accumulation and the extent of inverse agonism was similar for each of the drugs [13]. Subsequently it was shown that a mutant D_2 receptor (T343R) provided a more sensitive means to detect this inverse agonism [15]. So far, all of the antipsychotic drugs tested have exhibited inverse agonism.

In the course of studies on the effects of D_2 receptors on cAMP accumulation [13,15], we identified one compound,

Abbreviations: D_2 receptor, D_2 dopamine receptor; DMEM, Dulbecco's modified Eagle's medium; (+)-UH-232, *cis*-(+)-5-methoxy-1-methyl-2-(di-*N*-propylamino)tetralin

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0006-2952/$ – see front matter © 2004 Elsevier Inc. All rights reserved.
doi:10.1016/j.bcp.2004.02.017

the aminotetralin, UH-232, which behaved as a neutral antagonist. UH-232 was originally identified as a selective presynaptic antagonist of D_2-like receptors [17] with some degree of selectivity for the D_3 dopamine receptor [18]. Other mechanistic studies using the D_2 receptor expressed in CHO cells have shown that UH-232 is a low efficacy partial agonist [19,20]. It seems that, overall, UH-232 exhibits low agonist efficacy or neutral efficacy at the D_2 dopamine receptor and is, therefore, a useful reference compound in studies on the efficacy of other drugs.

There has been some discussion about the mechanisms of inverse agonism [21–24]. For the D_2 dopamine receptor mechanisms of inverse agonism have not been unequivocally established. In the context of the extended ternary complex model [25] the drugs could be stabilising inactive R states of the receptor at the expense of R^* or R^*G states. Alternatively, the drugs could be stabilising forms of the receptor that may couple to G proteins but are inactive. One suggestion has been that the sodium ion sensitivity of the binding of some drugs reflects their ability to elicit inverse agonism [23]. For example the substituted benzamide drugs, e.g. sulpiride exhibit higher affinity binding in the presence of sodium ions [26–29] and this could reflect the ability of the drug to bind with higher affinity to an inactive (R) state of the receptor at the expense of the active states (R^*, R^*G).

The inverse agonist property of the antipsychotic drugs may be important for their antipsychotic effects. Given that the atypical antipsychotic drugs exhibit a different clinical and pharmacological profile, it is of interest to determine whether the atypical drugs are also inverse agonists. In this study, therefore, we have determined the inverse agonist profile of a range of atypical antipsychotic drugs. The sodium ion sensitivity of their binding has also been determined.

2. Materials and methods

2.1. Cell culture

The native human $D_{2(short)}$ and a T343R mutant of human $D_{2(short)}$ dopamine receptors were stably expressed in CHO-K1 cells [15]. Both cell lines (CHO-D2-native and CHO-D2-T343R cells) were cultured in DMEM containing 5% foetal bovine serum, 2 mM L-glutamine and 400 μg ml^{-1} active geneticin (to maintain an active selection pressure). For the cAMP accumulation assays the cells were seeded at 35,000–40,000 cells per well in 24-well tissue culture plates and were used 2 days later when 80–85% confluent.

2.2. Radioligand binding studies

[^{3}H]Spiperone saturation binding assays were performed to estimate the receptor expression level in different membrane preparations from CHO-D2-native and CHO-D2-T343R cells. Membranes were prepared from the two cell lines as previously described [30] and the specific binding of a range of [^{3}H]spiperone concentrations (0.001–2 nM) was determined. Assays were performed in triplicate in buffer (20 mM HEPES, 6 mM $MgCl_2$, 1 mM EDTA, 1 mM EGTA, pH 7.4 at room temperature) in a final volume of 1 ml using 20 μg membrane protein. Non-specific binding was determined by inclusion of 3 μM (+)-butaclamol. Assays were initiated by the addition of membranes and incubated for 3 h at 25 °C. The assay was terminated by rapid filtration using a Brandel cell harvester with four times 3-ml washes of ice-cold PBS (140 mM NaCl, 2.7 mM KH_2PO_4, 8 mM Na_2HPO_4, 1.5 mM KH_2PO_4, pH 7.4 at room temperature). Filters were soaked in 2 ml of Optiphase Hi-Safe 3 scintillation fluid overnight before radioactivity was determined by liquid scintillation spectrometry.

Competition binding assays with [^{3}H]spiperone (0.25 nM) and competing ligands were performed in triplicate in a final volume of 1 ml of either a buffer containing sodium ions (20 mM HEPES, 6 mM $MgCl_2$, 1 mM EDTA, 1 mM EGTA, 100 mM NaCl, pH 7.4 at room temperature) or a sodium ion-free buffer (20 mM HEPES, 6 mM $MgCl_2$, 1 mM EDTA, 1 mM EGTA, 100 mM *N*-methyl-D-glucamine, pH 7.4 at room temperature). Non-specific binding was determined by inclusion of 3 μM (+)-butaclamol. Reactions were started by the addition of 20 μg membranes followed by incubation for 3 h at 25 °C. Assays were terminated as described for the saturation binding assays.

2.3. Measurement of cyclic AMP accumulation

cAMP accumulation was determined as described previously [13,15]. Briefly, cells (CHO-D2-native and CHO-D2-T343R) were grown to about 80% confluence in 24-well tissue culture plates and loaded with [^{3}H]adenine for 2 h (1.0 μCi ml^{-1} of medium; 300 μl per well). The cells were washed with 1 ml of HEPES-buffered (20 mM pH 7.5 at 37 °C) DMEM for at least 10 min. After the initial wash the cells were maintained at 37 °C in HEPES-buffered DMEM containing isobutylmethylxanthine for 40 min. Subsequently forskolin (10 μM) was added to the cells which were then incubated for a further 10 min (37 °C). Termination of the reaction was achieved by removal of the medium and addition of 0.5 ml of ice-cold perchloric acid (0.5 M) containing ~5000 d.p.m. [^{14}C]cAMP (column recovery standard). The plates were then left on ice for about 1 h to enable complete cell lysis after which the perchloric acid extracts were subjected to sequential chromatography on dowex and alumina [31]. Levels of both ^{3}H and ^{14}C in the final eluent were determined by liquid scintillation spectrometry following the addition of 3 ml Optiphase Hi-Safe 3. Total [^{3}H]cAMP content of each well was calculated on the basis of the ^{14}C

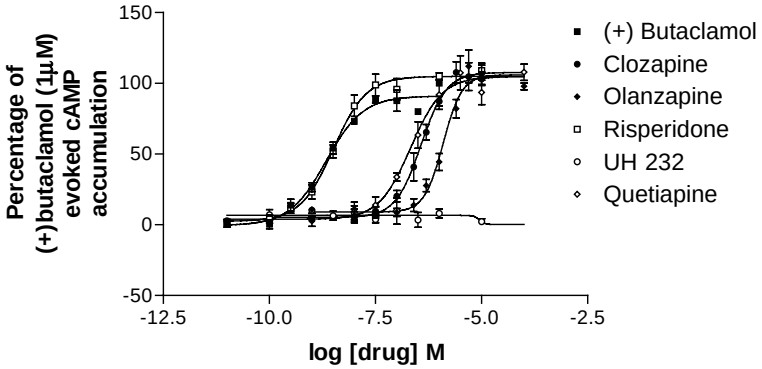

Fig. 1. Potentiation of forskolin-stimulated cAMP accumulation by antipsychotic drugs. Forskolin-stimulated cAMP accumulation was determined in CHO-D2-T343R cells in the presence of increasing concentrations of antipsychotic drugs as described in Section 2. Data were expressed as a percentage of the cAMP accumulation in the presence of 1 μM (+)-butaclamol. The data shown are the mean ± S.E.M. of data from four separate experiments.

recovery. Antipsychotic drugs were included in the medium during the 40-min pre-incubation, where appropriate.

2.4. Materials

Ascorbic acid, (+)-butaclamol, clozapine and isobutylmethylxanthine were from Sigma. Forskolin and (+)-UH-232 were supplied by Tocris Cookson. Olanzapine, risperidone and quetiapine were generous gifts from Eli Lilly, Janssen and AstraZeneca, respectively. [Phenyl-4-^{3}H]spiperone (approx. 30 Ci mmol^{-1}) and [2,8-^{3}H]adenine (approx. 40 Ci mmol^{-1}) were obtained from Amersham International and [8-^{14}C]cAMP (approx. 50 mCi mmol^{-1}) was from Dupont.

2.5. Data analysis

For cAMP experiments, concentration/response curves were fitted to a sigmoidal concentration/response equation using Prism (Graph Pad, San Diego) to generate EC_{50} values. Competition ligand binding data were analysed to provide K_i values using Prism and all data fitted well to a one-binding site model ($P < 0.05$). Statistical analyses were performed using negative logarithms of dissociation constants and either Student's *t*-test or two-way ANOVA with a Bonferroni post-test.

3. Results

CHO cell lines expressing similar levels of the native and T343R mutant D_2 dopamine receptors (CHO-D2-native, CHO-D2-T343R, respectively) were used for this work. Expression levels were determined using [^{3}H]spiperone saturation binding analyses. Saturation data all fitted well to one-binding site models and the binding parameters were: CHO-D2-native B_{max} 1.75 ± 0.04 pmol mg^{-1}, pK_d 10.52 ± 0.07, K_d 30 pM; CHO-D2-T343R B_{max} 1.22 ± 0.01 pmol mg^{-1}, pK_d 10.41 ± 0.03, K_d 39 pM (mean ± S.E.M., four experiments).

3.1. Effects of drugs on forskolin-stimulated cAMP accumulation

Forskolin-stimulated cAMP accumulation was determined in the two cell lines. Dopamine inhibited forskolin-stimulated cAMP accumulation to a similar extent (~90%) in the two cell lines but with a significantly higher potency in CHO-D2-T343R cells (pEC_{50} 8.85 ± 0.06 (1.4 nM)) than in CHO-D2-native cells (pEC_{50} 8.30 ± 0.05 (5 nM)) (mean ± S.E.M., five experiments, $P < 0.05$, *t*-test).

Four atypical antipsychotic drugs were tested and each was found to potentiate forskolin-stimulated cAMP accumulation in CHO-D2-T343R cells but not in CHO-D2-native cells. The extent of the potentiation (~threefold) was similar for the different drugs and similar to that seen with (+)-butaclamol (Fig. 1). EC_{50} values are given in Table 1. One compound, (+)-UH-232, was found to be without effect in these assays in either cell line.

Table 1
Effect of antipsychotic drugs on forskolin-stimulated cAMP accumulation in CHO-D2-T343R cells

	pEC_{50} ± S.E.M. (EC_{50}, nM)	*n*
(+)-Butaclamol	8.66 ± 0.03 (2.2)	3
Clozapine	6.41 ± 0.14 (389)	7
Olanzapine	6.21 ± 0.07 (616)	8
Quetiapine	6.62 ± 0.13 (240)	4
Risperidone	8.43 ± 0.14 (3.7)	5
UH-232	NP	4

Forskolin-stimulated cAMP accumulation was determined as described in Section 2 in the presence of increasing concentrations of drugs. Data for potentiation of forskolin-stimulated cAMP accumulation were analysed using Prism with a sigmoidal concentration/response model and EC_{50} values determined. Data are mean ± S.E.M. (*n*) from separate experiments performed in duplicate. NP—no potentiation of forskolin-stimulated cAMP accumulation was observed. The drugs were also tested on CHO-D2-native cells and no potentiation of forskolin-stimulated cAMP accumulation was seen (data not shown).

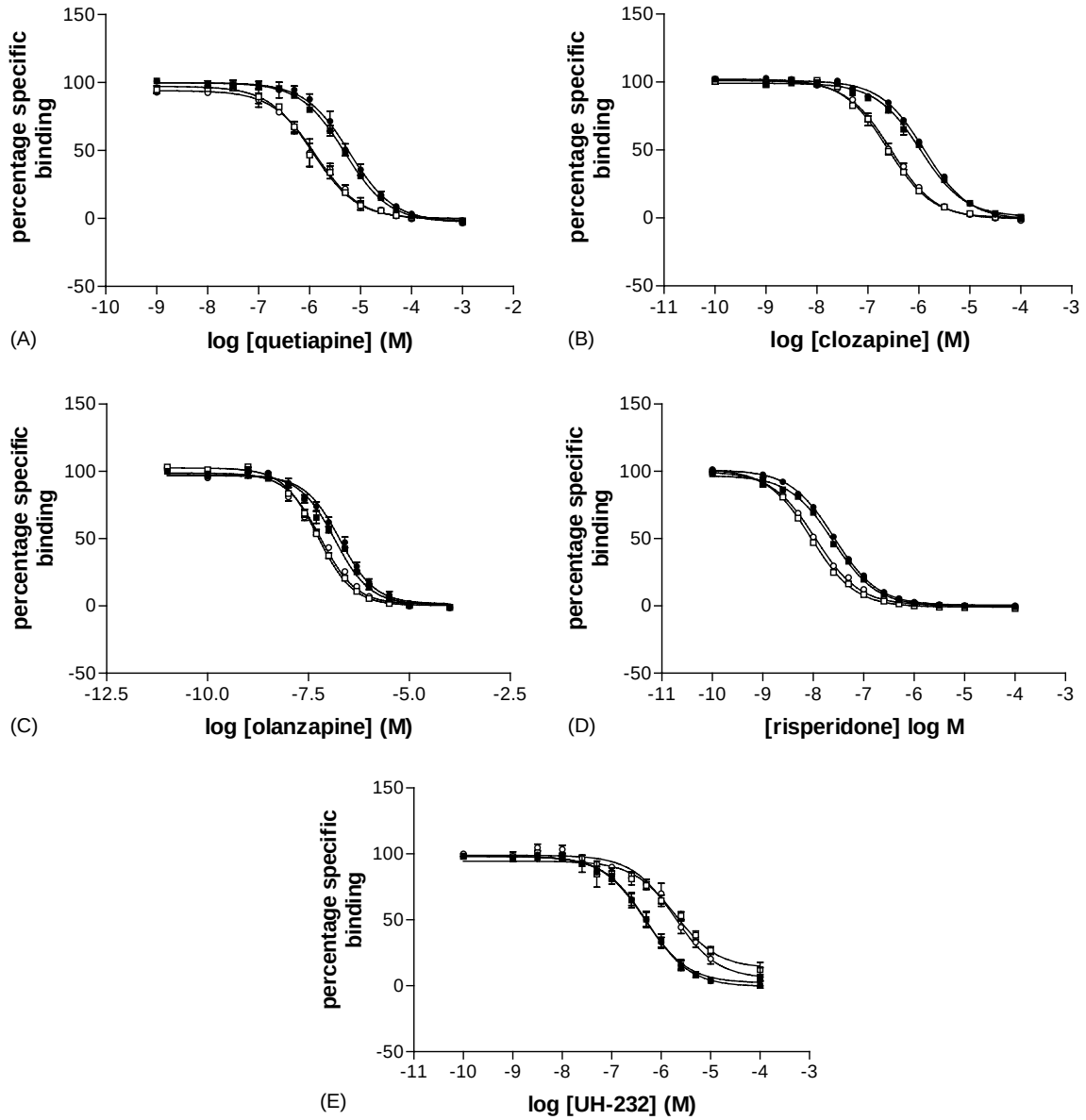

Fig. 2. Binding of antipsychotic drugs to D_2 dopamine receptors (native and T343R mutant): effects of sodium ions. Drug/[^{3}H]spiperone competition binding experiments were performed as described in Section 2 for quetiapine (A), clozapine (B), olanzapine (C), risperidone (D) and (+)-UH-232 (E). Data are given for the native receptor with Na^+ ions (■) and without Na^+ ions (□) and for the T343R mutant receptor with Na^+ ions (●) and without Na^+ ions (○). All competition data fitted well to one-binding site models and representative experiments are shown in the figure. Numbers of replicate experiments performed are given in Table 2.

3.2. Sodium ion sensitivity of drug binding to D_2 dopamine receptors

Each of the drugs tested above was examined in competition ligand binding assays versus [^{3}H]spiperone in membranes from the two cell lines. Competition experiments were performed in buffer containing sodium ions (100 mM) or in a buffer containing *N*-methyl-D-glucamine (100 mM) to act as a cation substitute to maintain ionic strength. In all cases, the drugs gave competition data that fitted best to a one-binding site model (Fig. 2) and the K_i values are given in Table 2.

The effect of the T343R mutation on the K_i values of the drugs tested was small (<two-fold) and was not significant ($P > 0.05$, ANOVA). The binding of all of the drugs with the exception of (+)-butaclamol was sensitive to removal of sodium ions and for some drugs (clozapine, olanzapine, risperidone and quetiapine), K_i values were higher (two- to four-fold, $P < 0.05$, ANOVA) in the presence of sodium ions and for (+)-UH-232, the K_i value was lower (~three-fold, $P < 0.05$, ANOVA).

4. Discussion

In this report, we have examined some of the properties of a group of atypical antipsychotic drugs. The data show that each of the drugs tested possesses inverse agonist

Table 2
Binding of antipsychotic drugs to native and T343R mutant D_2 dopamine receptors: effects of sodium ions

	+Na, pK_i values ± S.E.M. (K_i, nM)		−Na, pK_i values ± S.E.M. (K_i, nM)	
	D2-native	D2-T343R	D2-native	D2-T343R
(+)-Butaclamol	9.81 ± 0.10 (0.16)	9.59 ± 0.09 (0.26)	9.98 ± 0.10 (0.11)	9.78 ± 0.10 (0.17)
Clozapine	7.67 ± 0.06 (22)	7.48 ± 0.05 (33)	8.26 ± 0.03* (5)	8.08 ± 0.04* (8)
Olanzapine	8.50 ± 0.04 (3)	8.26 ± 0.08 (5)	8.89 ± 0.04* (1.3)	8.72 ± 0.04* (2)
Quetiapine	7.06 ± 0.04 (87)	6.84 ± 0.04 (145)	7.65 ± 0.04* (23)	7.45 ± 0.04* (35)
Risperidone	9.14 ± 0.07 (0.72)	9.02 ± 0.07 (0.95)	9.60 ± 0.06* (0.25)	9.41 ± 0.05* (0.39)
UH-232	7.96 ± 0.07 (11)	7.83 ± 0.05 (15)	7.42 ± 0.07* (38)	7.33 ± 0.09* (47)

The binding of [^{3}H]spiperone to membranes from CHO-D2-native or CHO-D2-T343R cells was determined in the presence of increasing concentrations of competing drug as described in Section 2. Competition data were analysed using Prism and in each case a one-binding site model provided the best fit to the data ($P < 0.05$). K_i values were derived and data are given as mean ± S.E.M. for four experiments. Statistical analyses of the effect of Na^+ ($^*P < 0.05$) and the T343R mutation ($P > 0.05$) were performed using ANOVA on pK_i values.

activity at the D_2 dopamine receptor so that this may be a common feature of the antipsychotic drugs.

In the first part of this study, we examined inverse agonism in the drugs based on their ability to potentiate forskolin-stimulated cAMP accumulation. We have previously demonstrated that (+)-butaclamol is an inverse agonist at the D_2 receptor [13] and in the present study (+)-butaclamol was able to potentiate forskolin-stimulated cAMP accumulation in CHO cells expressing the T343R mutant D_2 receptor but not in cells expressing the native receptor at a similar level. This agrees with our previous findings on this mutant receptor, which appears to lie more towards the active state in the absence of agonist and is more sensitive to the effects of inverse agonists [15]. We tested four atypical drugs (clozapine, olanzapine, risperidone and quetiapine) and each of these was able to potentiate forskolin-stimulated cAMP accumulation in CHO-D2-T343R cells but not in CHO-D2-native cells. Each of these drugs, therefore, possesses inverse agonist activity. The extent of the inverse agonism was similar for each of the drugs and similar to that for (+)-butaclamol. Previously we have shown that drugs from several chemical classes (butyrophenones, phenothiazines, substituted benzamides, dibenzodiazepines) exhibit inverse agonism at the D_2 dopamine receptor [13,15]. Based on these studies and the present study, it seems that inverse agonism at the D_2 dopamine receptors is an attribute that is common to all of the antipsychotic drugs tested so far from different chemical and therapeutic classes.

We also included in the present study the drug (+)-UH-232, which we have shown previously to be a neutral antagonist in similar assays [13,15]. UH-232 was included as a control for the measurements of inverse agonism on the other drugs. In the present experiments, UH-232 was unable to stimulate or inhibit cAMP accumulation in agreement with the previous observations. (+)-UH-232 has been tested as an antipsychotic drug in a limited trial [32] and did not exhibit any antipsychotic activity. This could be taken to imply that inverse agonism of the antipsychotic drugs is important for their antipsychotic effect. UH-232 does, however, have some activity at 5-HT_2 receptors [32] which could have confounded the trial. More recently, the drug aripiprazole has been introduced and shown to be an effective antipsychotic [33]. Aripiprazole is a potent low efficacy partial agonist at D_2 dopamine receptors [33]. It seems, therefore, that antipsychotic activity is possible in the absence of inverse agonism so that the inverse agonism exhibited by the majority of presently used antipsychotic drugs may be associated with their side effect profile rather than their therapeutic effects.

The binding properties of these drugs were tested in competition binding experiments versus [^{3}H]spiperone in membranes derived from cells expressing the native and T343R mutant D_2 receptors. There were slight differences in drug affinity between the native and T343R mutant receptors for binding some of the drugs but these were not significant. When the K_i values for the binding of drugs to the D_2 receptor were compared with their potencies as inverse agonists, it was found that for several drugs the inverse agonist potency was less than the binding affinity.

We also evaluated the sodium ion sensitivity of the binding of the drugs tested in this report. It should be noted that the radioligand ([^{3}H]spiperone) used is insensitive to changes in sodium ion concentration [29]. In these competition experiments, (+)-butaclamol was found to be insensitive to changes in sodium ion concentration. This is important as (+)-butaclamol is used to define specific [^{3}H]spiperone binding in these experiments. The other drugs tested here were found to be sensitive to the presence of sodium ions in assay buffers. The four atypical antipsychotic drugs each bound with lower affinity in the presence of sodium ions. The neutral antagonist (+)-UH-232 bound with higher affinity in the presence of sodium ions. It seems that, for the D_2 dopamine receptor, drugs exhibit a spectrum of sensitivity to sodium ions in their binding and three classes of drug may be discerned based on this property:

1. Drugs that bind with higher affinity in the presence of sodium ions, e.g. substituted benzamides such as sulpiride (see for example [26–29]), aminotetralins such as (+)-UH-232 (present study);

2. Drugs that bind with lower affinity in the presence of sodium ions, e.g. clozapine, olanzapine, quetiapine and risperidone (present study);
3. Drugs whose binding is insensitive to the presence of sodium ions, e.g. butaclamol (present study), spiperone [29].

It has been suggested that the sodium ion sensitivity of the binding of drugs to the D_2 dopamine receptor may reflect their mechanism of inverse agonism [23]. In this theory, the R/R^* transition is sodium ion-sensitive with the R state being favoured in the presence of sodium ions (see for example [15]). Drugs that bind with higher affinity to the R state of the receptor than the R^* state would then exhibit sodium ion sensitivity with increased affinity in the presence of sodium ions. If the mechanism of inverse agonism for these drugs depends on stabilisation of the R state of the receptor then the sodium ion sensitivity is linked to the inverse agonism. It seems, however, that a range of drugs, e.g. sulpiride, clozapine, spiperone, etc., all of which exhibit inverse agonism, show different sodium ion sensitivity. It seems, therefore, that the sodium ion sensitivity of the binding of drugs is not a universal reflection of inverse agonist mechanisms.

Additionally, (+)-UH-232, which we included in this study as a reference compound, also exhibited sodium ion-sensitive binding. UH-232 has been shown to be either a neutral antagonist [13,15] or a low efficacy partial agonist [19,20]. The binding of UH-232 was found, in the present study, to be of higher affinity in the presence of sodium ions. This was surprising given that agonists usually exhibit lower binding affinities in the presence of sodium ions (see for example [15,28]) and further emphasises that the sodium ion sensitivity of binding is not a guide to the efficacy of the compound.

Acknowledgments

We thank Eli Lilly for financial support.

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