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Evidence that antipsychotic drugs are inverse agonists at D_2 dopamine receptors

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1 The effects of a number of D_2-like dopamine receptor antagonists have been determined on forskolin-stimulated cyclic AMP accumulation in Chinese hamster ovary (CHO) cells expressing the human D_{2short} dopamine receptor (CHO-D2S cells).

2 Dopamine inhibited the effect of forskolin (as expected for a D_2 receptor). However, all of the antagonists tested, apart from UH232 and (−)-butaclamol, were able to increase cyclic AMP accumulation above the forskolin control level. (+)-Butaclamol elicited a similar stimulation of forskolin-stimulated cyclic AMP accumulation in a CHO cell line expressing human D_{2long} dopamine receptors whereas it exhibited no stimulating effect on forskolin-stimulated cyclic AMP accumulation in untransfected CHO-K1 cells.

3 There was a strong correlation between the EC_{50} values of these compounds for potentiation of cyclic AMP accumulation and their K_i values from radioligand binding experiments in CHO-D2S cells.

4 The effects of both (+)-butaclamol and dopamine in CHO-D2S cells were inhibited by pre-treatment with pertussis toxin indicating a role for Gi/Go proteins.

5 UH232 did not significantly affect forskolin-stimulated cyclic AMP accumulation but this substance was able to inhibit the effects of both dopamine and (+)-butaclamol in a concentration-dependent manner. Thus the effects of (+)-butaclamol on forskolin-stimulated cyclic AMP accumulation are mediated directly via the D_2 receptor rather than by reversal of the effects of an endogenous agonist.

6 These data suggest that the D_2 dopamine receptor antagonists tested here, many of which are used clinically as antipsychotic drugs, are in fact inverse agonists at human D_2 dopamine receptors.

Keywords: Inverse agonism; dopamine receptors; antipsychotic drugs

Introduction

Molecular biological techniques have revealed that dopamine receptors can be divided into five distinct subtypes (D_1–D_5) (for reviews see Sibley & Monsma, 1992; Civelli *et al*., 1993). The D_1 and D_5 receptors show pharmacological properties similar to those of the classically defined D_1 receptor and are therefore termed D_1-like, whilst the D_2, D_3 and D_4 receptors show properties similar to the classically defined D_2 receptor and are termed D_2-like. The D_2 receptor exists as two variants, D_{2short} (D_{2S}) and D_{2long} (D_{2L}); the D_{2S} receptor lacks a 29 amino acid insert in the third intracellular loop (Sibley & Monsma, 1992; Civelli *et al*., 1993). The presence or absence of this insert seems to affect the ability of these receptors to interact with G-proteins (Montmayer *et al*., 1993; Senogles, 1994), although the G-protein specificity of these receptor subtypes has yet to be unequivocally defined.

The D_2-like receptors are of particular interest as antagonism of these receptors is a common pharmacological property of antipsychotic (or neuroleptic) drugs. It is thought that these drugs exert their therapeutic effects by blocking the actions of dopamine at one or more of the D_2-like dopamine receptor subtypes in the brain (Strange, 1992). For example, there is a strong correlation between the free plasma concentrations achieved by antipsychotic drugs at therapeutic doses and their dissociation constants at D_2 receptors determined in radioligand binding experiments (Seeman, 1980). Also, there is a delay between the start of therapy with these drugs and the onset of their antipsychotic effects, which may indicate that an adaptive change in the dopaminergic systems is occurring in response to these drugs (Strange, 1992).

It has recently become clear from studies on other classes of G-protein-coupled receptors that a number of compounds which have previously been thought to be antagonists at these receptors in fact exhibit inverse agonist activity and are able to reduce spontaneous activity of the receptor system below its basal level (Cerione *et al*., 1984; Costa *et al*., 1990; Chidiac *et al*., 1993; Tian *et al*., 1993; Barker *et al*., 1994; Bond *et al*., 1995). Furthermore, studies on the β_2-adrenoceptor over-expressed in transgenic mice have now shown that inverse agonism is a phenomenon relevant to *in vivo* systems (Bond *et al*., 1995). In the present study we have used a Chinese hamster ovary (CHO) cell line (CHO-D2S) expressing the short isoform of the human D_2-dopamine receptor (Hayes *et al*., 1992) to study the effects of a number of D_2-like dopamine receptor antagonists on forskolin-stimulated adenosine 3′:5′-cyclic monophosphate (cyclic AMP) accumulation (D_2 dopamine receptors are known to inhibit this response (Vallar & Meldolesi, 1989)). We present evidence which suggests that these compounds, many of which are used clinically as antipsychotic drugs, are in fact inverse agonists at this receptor subtype. These results are of significance in the mechanism of action of these drugs, as prolonged therapy with an inverse agonist may alter the responsiveness of a receptor system in ways which differ from those of a simple neutral antagonist.

Methods

Cell culture

CHO-D2S cells (Hayes *et al*., 1992) and CHO-D2L cells (a CHO cell line expressing the long isoform of the human D_2 dopamine receptor (Hayes *et al*., 1992)) were grown in RPMI 1640 medium containing 5% FBS, 2 mM L-glutamine and 200 μg ml^{-1} active geneticin (to maintain selection pressure). Untransfected CHO-K1 cells were grown in RPMI 1640 medium containing 5% FBS and 2 mM L-glutamine. For the

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cyclic AMP accumulation assays the cells were seeded at 35–40,000 per well in 24 well tissue culture plates in medium without geneticin and were used 3 days later when approximately confluent.

Measurement of cyclic AMP accumulation

Cyclic AMP accumulation was determined as previously described (Gardner *et al*., 1996). Briefly, cells were grown to confluence in 24 well plates and loaded for 2 h with [^{3}H]-adenine (3.3 μCi ml^{-1} of medium; 300 μl per well). The cells were then washed with 1 ml of HEPES-buffered (20 mM; pH 7.5) RPMI 1640 medium and incubated (37°C) in HEPES-buffered RPMI 1640 containing 1 mM IBMX (a phosphodiesterase inhibitor) for 40 min. Following this incubation forskolin (10 μM) was added and the cells were incubated for a further 10 min. The reaction was terminated by removal of the medium and addition of 0.5 ml of ice-cold perchloric acid (0.5 M) containing ~2500 d.p.m. [^{14}C]-cyclic AMP to act as a recovery standard for the subsequent chromatographic steps. The plates were left on ice for a further 40 min after which the perchloric acid extracts were subjected to sequential chromatography on dowex and alumina to separate the [^{3}H]-cyclic AMP from other radiolabelled nucleotides. ^{3}H and ^{14}C in the final eluent (1 ml) were determined by scintillation counting and the [^{3}H]-cyclic AMP content of each well was calculated based on the recovery of ^{14}C. When the effects of antipsychotic drugs were determined, these were included in the medium during the 40 min pre-incubation with IBMX. Dopamine, where used, was added simultaneously with the forskolin. In experiments with PTX the medium was replaced with medium containing PTX or vehicle 16 h before the [^{3}H]-adenine loading. In experiments with dialysed serum the cells were washed twice with serum-free medium and then grown for 16 h in medium containing either 5% FBS or 5% dialysed FBS before the [^{3}H]-adenine loading (in the appropriate medium).

Radioligand binding studies

Membranes were prepared from CHO-D2S or CHO-D2L cells as previously described (Castro & Strange, 1993). Binding assays were performed as previously described (Castro & Strange, 1993) with the following modifications: the assay buffer was supplemented with 100 mM NaCl (as the presence of Na^+ is known to affect the affinity of substituted benzamide drugs (Theodorou *et al*., 1980) and clozapine (Malmberg *et al*., 1993)) and 100 μM GTP (as the presence of GTP might be expected to affect the affinity of inverse agonist drugs (Schütz & Freissmuth, 1992)), 10 μg (CHO-D2S) or 25 μg (CHO-D2L) of membrane protein was used and the assay was incubated for 1 h (rather than 45 min) to ensure complete equilibration.

Materials

[Phenyl-4-^{3}H]-spiperone (15–30 Ci mmol^{-1}) was obtained from Amersham International. [2,8-^{3}H]-adenine (20–40 Ci mmol^{-1}) and [8-^{14}C]-cyclic AMP (40–60 mCi mmol^{-1}) were from New England Nuclear. Ascorbic acid, dimethylsulphoxide, ethylenediamine-N, N, N′, N′-tetraacetic acid (EDTA), ethylene glycol-bis(β-aminoethyl ether) N, N, N′, N′-tetraacetic acid (EGTA), foetal bovine serum (FBS), forskolin, L-glutamine, guanosine 5′-triphosphate (GTP), (N-[2-hydroxyethyl]piperazine-N′[2-ethanesulphonic acid]) (HEPES) and isobutylmethyl-xanthine (IBMX) were from Sigma. Pertussis toxin (PTX) was obtained from Sigma as a sterile solution in 50% glycerol containing 50 mM Tris, 10 mM glycine and 0.5 M NaCl, pH 7.5. Geneticin was obtained from GIBCO. Tris base was from Boehringer Mannhein. (+)-Butaclamol, (−)-butaclamol, chlorpromazine, domperidone, haloperidol and spiperone were obtained from Research Biochemicals Incorporated. UH232 (*cis*-(+)-(1**S**,2**R**)-5-methoxy-1-methyl-2-(n-dipropylamino)tetralin) was from Tocris Cookson. Clozapine, nemonapride, *cis*- and *trans*-flupenthixol and (+)- and (−)-sulpiride were generous gifts from Sandoz, Yamanouchi, Lundbeck and Ravizza, respectively. Perchloric acid and buffer salts were obtained from Fisons Scientific Equipment.

Data analysis

Individual concentration-response curves from cyclic AMP accumulation assays were analysed by fitting Hill equations to the recovery corrected ^{3}H d.p.m. data to define EC_{50} values and Hill coefficients by use of the Fig P computer programme. The Hill coefficients from these fits were generally not significantly different from unity ($P>0.05$, Student's t test). When concentration-response curves were pooled the [^{3}H]-cyclic AMP in each sample was expressed as a percentage of the total perchloric acid soluble ^{3}H pool in the sample. This is referred to as the '% conversion' on the ordinate axes in the figures. The data from the radioligand binding experiments were analysed with the LIGAND programme and in all cases the data were consistent with binding to a single class of binding sites.

Results

Saturation ligand binding studies with [^{3}H]-spiperone showed that the expression level of the D_2-dopamine receptor in the

Table 1 Functional and binding parameters of dopamine receptor ligands

	Inverse agonist activity (cyclic AMP accumulation)				*Ligand binding*		
Drug	pEC_{50}[a,b]	EC_{50} (nM)	*Maximal effect*[a,c]	n[a]	pK_i[a,b]	K_i (nM)	n[a]
(+)-Butaclamol	8.41±0.08	3.9	1.00	9	8.24±0.02	5.8	3
(−)-Butaclamol	<5	>10,000	N.D.[d]	3	<5	>10,000	3
Chlorpromazine	7.89±0.11	13.0	0.88±0.08	3	8.14±0.04	7.2	3
Clozapine	6.52±0.18	297	0.86±0.11	4	6.38±0.06	418	3
Domperidone	8.75±0.05	1.8	0.95±0.05	3	8.41±0.01	3.9	3
cis-Flupenthixol	8.65±0.11	2.2	0.77±0.11	3	8.12±0.04	7.6	3
trans-Flupenthixol	6.78±0.20	163	1.10±0.10	3	6.82±0.09	148	3
Haloperidol	8.69±0.08	2.1	0.95±0.03	3	8.54±0.04	2.9	3
Nemonapride	9.34±0.01	0.46	0.92±0.04	4	9.57±0.01	0.27	3
Spiperone	9.26±0.19	0.55	1.03±0.05	4	9.44±0.04	0.36	3
(−)-Sulpiride	7.34±0.10	45.5	1.01±0.05	4	7.41±0.04	38.6	3
(+)-Sulpiride	6.01±0.13	977	1.05±0.25	3	5.89±0.01	1292	3
UH232	N.D.	N.D.	N.D.	3	7.14±0.16	72.7	3

[a] Values are the mean±s.e.mean of n determinations in CHO-D2S cells. [b] These values are compared in Figure 4. [c] Maximal effect was calculated relative to the effect of a maximally active concentration of (+)-butaclamol (1 μM) which was determined with each concentration-response curve. None of these values was significantly different from the butaclamol controls ($P<0.05$, Student's t test). [d] N.D. – values could not be determined.

CHO-D2S cells was 3.5 ± 0.4 pmol mg^{-1} protein (mean $\pm$ s.e.mean, $n = 5$). [^{3}H]-spiperone bound with a K_D of 126 pM ($pK_D = 9.90 \pm 0.10$) in these experiments. The pharmacological profile of this receptor has been characterized and the recombinant receptor exhibits properties typical of a D_2-dopamine receptor (compare the data in Table 1 and in Gardner *et al*., 1996 and Sokoloff *et al*., 1990 with Seeman, 1980). The affinity of [^{3}H]-spiperone for D_2 dopamine receptors in CHO-D2L cells was 93.2 pM ($pK_D = 10.03 \pm 0.10$) and the level of D_2 receptor expression was 1.2 ± 0.1 pmol mg^{-1} protein (mean $\pm$ s.e.mean, $n = 7$).

Incubation of CHO-D2S cells with forskolin resulted in an increase in [^{3}H]-cyclic AMP accumulation and this was inhibited in a concentration-dependent manner by dopamine (Gardner *et al*., 1996). The antagonist (+)-butaclamol was not only able to reverse this effect of dopamine back to the forskolin control level but was also able to increase the level of [^{3}H]-cyclic AMP produced in the absence of any agonist significantly above the forskolin control. This (+)-butaclamol-induced potentiation of the effect of forskolin was concentration-dependent (Figure 1a) with a pEC_{50} value of 8.41 ± 0.08 ($n = 9$, $EC_{50} = 3.9$ nM) and was quite large amounting to $\sim$150% above the control level (Figure 1a). Similar results were obtained with the CHO-D2L cell line, even though the density of D_2 receptors in these cells was only about one third of that in CHO-D2S cells. (+)-Butaclamol induced concentration-dependent increases in forskolin-stimulated cyclic AMP accumulation in CHO-D2L cells with an EC_{50} value of 4.9 nM ($pEC_{50} = 8.31 \pm 0.11$, $n = 9$) and this increase reached $\sim$180% above the control level (Figure 1b). There was no effect of (+)-butaclamol on forskolin-stimulated cyclic AMP accumulation in the parental CHO cell line (Figure 1c).

The effects of both (+)-butaclamol and the agonist dopamine in CHO-D2S cells were abolished by pretreatment of the cells with PTX (Figure 2a), indicating that both of these effects are mediated by G-proteins of the Gi/Go family. However, the effects of these compounds were unchanged when the cells were grown overnight in medium containing dialysed FBS (to remove any small molecular weight compounds which could potentially behave as agonists at the D_2 receptor) before the experiment (Figure 2b).

A number of other commonly used D_2 receptor antagonists were tested for their ability to potentiate forskolin-stimulated cyclic AMP accumulation in the CHO-D2S cell line. Of these compounds, the majority were indeed able to increase forskolin-stimulated cyclic AMP accumulation, the maximal increases being similar to that of (+)-butaclamol (Table 1). However, in the case of UH232 no significant increase in forskolin-stimulated cyclic AMP accumulation was seen at con-

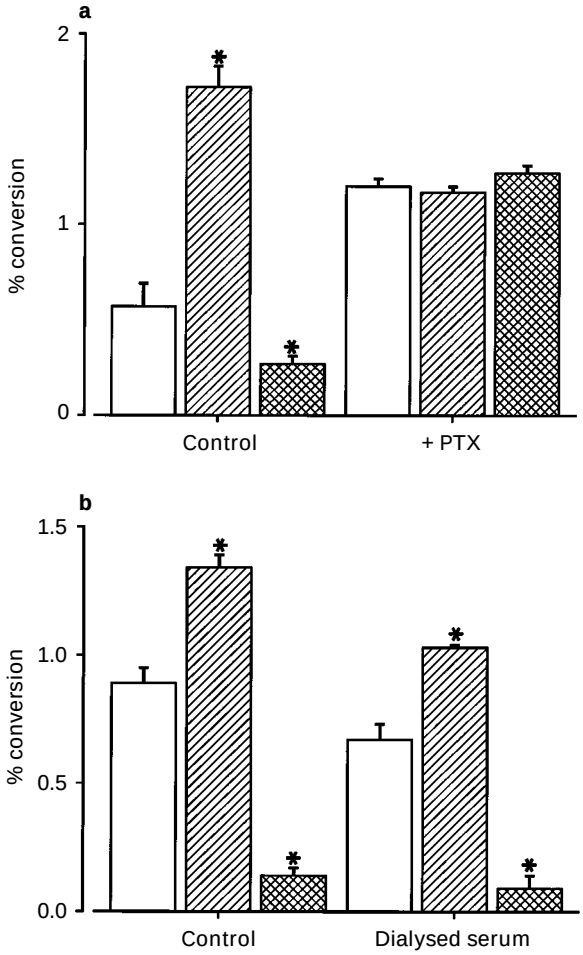

Figure 2 (a) Effect of pretreatment (16 h) of CHO-D2S cells with PTX (200 ng ml^{-1}) on forskolin (10 μM)-stimulated cyclic AMP accumulation (expressed as a % of the total perchloric acid soluble tritium pool in each well, referred to as '% conversion' on the ordinate axes) in the absence (open columns) or presence of (+)-butaclamol (1 μM, hatched columns) or dopamine (10 μM, cross-hatched columns). (b) Effect of incubation of cells overnight in medium containing dialysed serum on forskolin-stimulated cyclic AMP accumulation in the absence (open columns) or presence of (+)-butaclamol (1 μM, hatched columns) or dopamine (10 μM, cross-hatched columns). In (a) and (b) values are the the mean $\pm$ s.e.mean of 3 separate experiments in triplicate. *Significantly different from the appropriate forskolin control value, $P < 0.05$ (Student's t test).

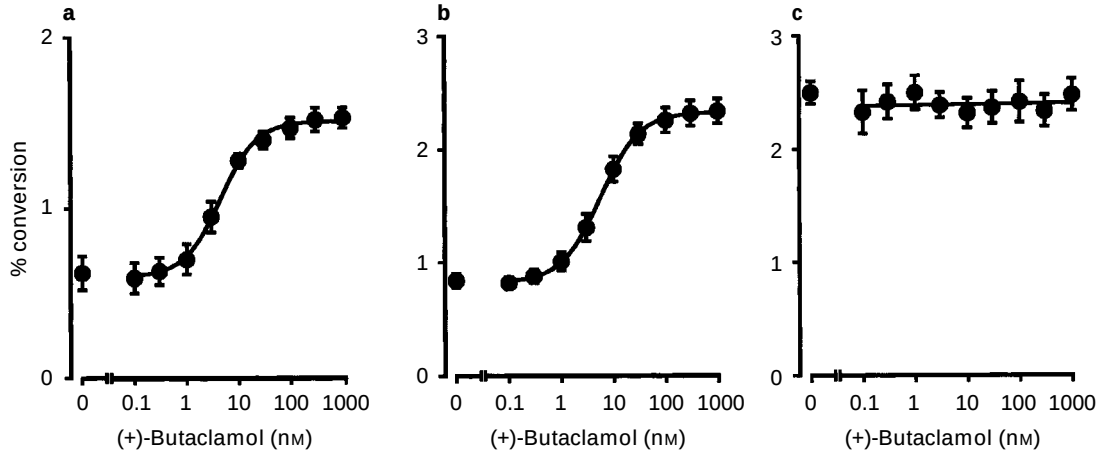

Figure 1 Inverse agonism of (+)-butaclamol. (a) Effect of (+)-butaclamol on forskolin (10 μM)-stimulated cyclic AMP accumulation in CHO-D2S cells (expressed as % of the total perchloric acid soluble tritium pool in each well, referred to as '% conversion' on the ordinate axes). (b) Effect of (+)-butaclamol on forskolin-stimulated cyclic AMP accumulation in CHO-D2L cells. (c) Effect of (+)-butaclamol on forskolin-stimulated cyclic AMP accumulation in untransfected CHO-K1 cells. Values are the mean of 9 (a and b) or 3 (c) separate experiments in duplicate; vertical lines show s.e.mean.

centrations up to 30 μM (Figure 3a). When concentration-response curves were constructed to (+)-butaclamol in the presence of various concentrations of UH232 the effect of (+)-butaclamol was inhibited in a concentration-dependent manner (Figure 3b). Schild analysis of this inhibition resulted in a pK_B value of 6.18 ± 0.05 (Figure 3b inset) indicating an apparent affinity for UH232 of 660 nM. The slope of the Schild plot was not significantly different from unity (1.13 ± 0.12). In a similar experiment with (−)-sulpiride to potentiate forskolin-stimulated cyclic AMP accumulation Schild analysis gave a pK_B value of 7.46 ± 0.09 indicating an apparent affinity of 34.7 nM for UH232. UH232 also acted as an antagonist of the dopamine-inhibition of cyclic AMP accumulation and Schild analysis of this effect gave a pK_B of 7.84 ± 0.03 indicating an apparent affinity for UH232 of 14.5 nM.

When the potencies of the drugs which were able to potentiate forskolin-stimulated cyclic AMP accumulation were compared with their affinities at D_2 dopamine receptors, obtained from radioligand binding assays in membranes prepared from CHO-D2S cells (Table 1; Figure 4), a very good correlation was observed ($r = 0.979$; $P < 0.001$, Student's t test). The slope of the regression line was not significantly different from unity (0.95 ± 0.07) and the intercept was not significantly different from zero (0.44 ± 0.53).

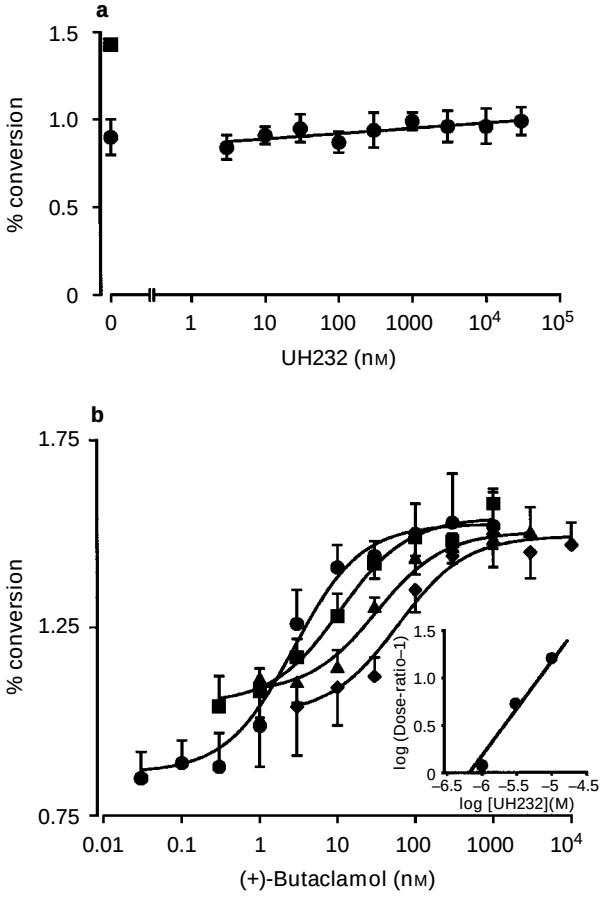

Figure 3 Efficacy of UH232. (a) Effect of UH232 (●) on forskolin (10 μM)-stimulated cyclic AMP accumulation in CHO-D2S cells (expressed as a % of the total perchloric acid soluble tritium pool in each well, referred to as '% conversion' on the ordinate axes). The effect of 1 μM (+)-butaclamol (■) in these experiments is shown for comparison. (b) Effect of (+)-butaclamol on forskolin-stimulated cyclic AMP accumulation in CHO-D2S cells in the absence (●) or presence of UH232 at 1 μM (■), 3 μM (▲) or 10 μM (◆). Values are the mean of 3 separate experiments in duplicate; vertical lines show s.e.mean. The Hill coefficients of the individual and pooled concentration-response curves were not significantly different from unity (the 95% confidence interval of the fitted value overlapped unity), therefore, when curves were fitted to the pooled concentration-response curves for Schild analysis the Hill coefficients were constrained to unity. Inset, Schild plot of the data presented in (b). As UH232 appeared to be a partial inverse agonist in these experiments dose-ratios were calculated from the concentrations of (+)-butaclamol causing 1.3% conversion from each of the pooled concentration-response curves.

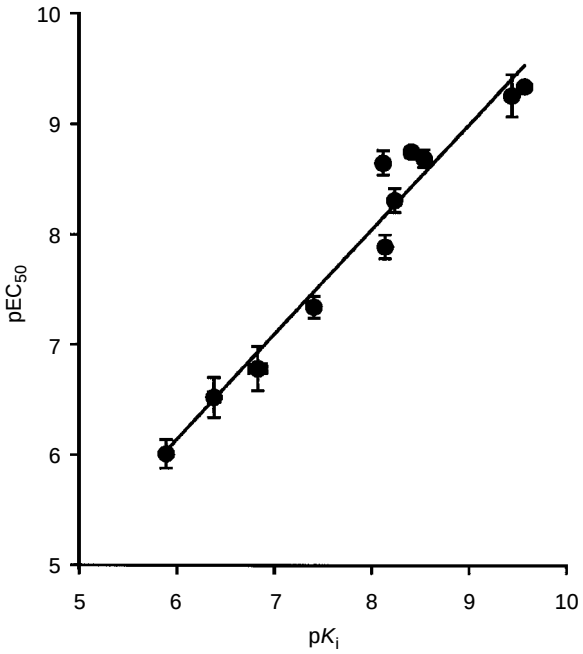

Figure 4 Comparison of the EC_{50} values of inverse agonists at the D_2 receptor for potentiation of forskolin-stimulated cyclic AMP accumulation with their apparent affinities (K_i) at D_2 dopamine receptors in competition with [^{3}H]-spiperone. Values are the mean of at least 3 separate determinations in duplicate; vertical lines show s.e.mean.

Discussion

This study shows that a number of drugs that are commonly considered to be antagonists at the D_2 dopamine receptor in fact possess inverse agonist activity. These observations are of additional significance owing to the widespread clinical use of these drugs as antipsychotics.

The inverse agonist nature of these drugs is seen in the present study from their ability to potentiate forskolin-stimulated cyclic AMP accumulation in CHO cells expressing human D_2 dopamine receptors. These effects are due to an interaction with the D_2 dopamine receptors in the cells as the response shows the specificity expected for a D_2 dopamine receptor and similar effects were not seen in the parent untransfected CHO cells. Both the inverse agonist ((+)-butaclamol) and agonist (dopamine) effects were eliminated by pretreatment of the cells with PTX, indicating that the effects are mediated via a G-protein of the Gi/Go family and that the increase in cyclic AMP accumulation due to (+)-butaclamol is not due to a Gs protein stimulating adenylyl cyclase. The inverse agonist effects are not due to the antagonism of an endogenous catecholamine as the effects of both dopamine and (+)-butaclamol were unaffected after the CHO-D2S cells were cultured overnight in dialysed serum. CHO-K1 cells are not of a neuronal phenotype and are therefore unlikely to secrete dopamine.

Several compounds were tested for inverse agonist activity at the D_2-dopamine receptor and most of these gave maximal activities similar to that of (+)-butaclamol (Table 1). The pharmacological profile indicated by these studies was characteristic of the D_2-dopamine receptor (Seeman, 1980), with strong stereoselectivity being seen for the stereoisomers of the drugs butaclamol, flupenthixol and sulpiride and an ex-

cellent correlation between the potencies of the compounds as inverse agonists and their affinities at the D_2-dopamine receptor in ligand binding assays. However, in the case of UH232, no significant increase in forskolin-stimulated cyclic AMP accumulation was seen at concentrations up to 30 μM, suggesting that this compound may be a neutral antagonist. UH232 has been shown to be an antagonist at D_2 dopamine receptors in behavioural and neurochemical studies (Johansson *et al*., 1985) and, in the present study, we confirmed that UH232 antagonizes competitively the effects of dopamine to inhibit cyclic AMP accumulation with an apparent affinity (14.5 nM) in agreement with its affinity seen in ligand binding studies. UH232 also antagonizes competitively the effects of the inverse agonists, (+)-butaclamol and (−)-sulpiride, and Schild analysis of the data gave apparent affinities for UH232 of 660 nM and 34.7 nM. Whereas the affinity for UH232 derived in competition with (−)-sulpiride is in good agreement with data derived from ligand-binding, the affinity obtained versus (+)-butaclamol is lower than expected. This is likely to reflect the lack of equilibration of UH232 with the higher affinity, slower dissociating, (+)-butaclamol whereas with the lower affinity (−)-sulpiride (and dopamine) equilibrium is reached quickly during the incubation time of the experiment.

It can also be seen from the concentration-response curves in Figure 3 that there is a small but consistent potentiation of the forskolin-stimulated level of cyclic AMP accumulation in the presence of UH232 alone suggesting that this compound is in fact a weak partial inverse agonist rather than a truly neutral antagonist. Indeed, from the UH232 concentration-response curve there is a trend towards an increase in cyclic AMP accumulation at the higher concentrations although this never reached significance at the 5% level. These observations also provide stronger evidence that the effect of (+)-butaclamol and the other neuroleptic drugs tested are indeed due to inverse agonism rather than to the reversal of the effects of an endogenous agonist, as if the latter were the case, all of the antagonists tested would have been expected to produce the same maximal effect.

The D_2 receptors in the CHO-D2S cell line, therefore, appear to be constitutively active, leading to a tonic inhibition of adenylyl cyclase. This basal receptor activity is reversed by a number of dopamine receptor ligands which have previously been classified as antagonists, suggesting that these compounds are in fact inverse agonists at this receptor. (+)-Butaclamol was also able to potentiate cyclic AMP accumulation in CHO-D2L cells which are, by their nature, a distinct CHO cell isolate expressing a distinct isoform of the receptor. Also, other studies have shown effects of some of these compounds in other cell systems which are consistent with inverse agonist activity (see below). Therefore the inverse agonism of these drugs seems to be a general observation. The mechanism of this inverse agonist effect is as yet unclear but there are a number of possibilities. For example, inverse agonists could bind selectively to the free form of the receptor, thereby reducing its ability to interact with and therefore activate G-proteins (this type of selectivity for receptor states has been shown for the 5-hydroxytryptamine $(5\text{-HT})_{1A}$ receptor (Sundaram *et al*., 1995)) or an inverse agonist may induce a conformation of the receptor which is able to bind to G-protein but which is unable to activate it.

The inverse agonist effects described here have been obtained in CHO-D2S cells that were stimulated by forskolin to increase adenylyl cyclase activity. In these cells the EC_{50} for forskolin stimulation of cyclic AMP accumulation was 14.5 μM (pEC_{50} 4.84 ± 0.20, mean ± range, 2 experiments) (data not shown) so that adenylyl cyclase was not maximally stimulated by the concentration of forskolin (10 μM) used in the present experiments. There was no significant effect of the inverse agonists on cyclic AMP accumulation in the absence of forskolin, but since cyclic AMP accumulation is very low under these conditions it is impossible to determine whether inverse agonists are effective only in cells activated by another stimulus. In the experiments described here the D_2 receptors appear to be constitutively active in the absence of agonist leading to basal inhibition of adenylyl cyclase. This is seen in the experiments with pertussis toxin where cyclic AMP accumulation is increased after toxin treatment. Also in the parent untransfected cells cyclic AMP accumulation is greater than in the cells expressing D_2 receptors and this probably reflects the lack of basal inhibition of adenylyl cyclase in the untransfected cells.

It is often suggested that inverse agonism is a property of systems in which receptors are over-expressed and indeed the receptor densities present in the cell lines in this study (3.5 ± 0.1 and 1.2 ± 0.1 pmol mg^{-1}) are somewhat greater than those found in striatal membrane preparations (~ 200 fmol mg^{-1} protein in man (Owen *et al*., 1978)). However, a clonal cell line is a homogeneous cell population and thus all of the cells present should express dopamine receptors at a similar level, whereas brain tissue is a mixed cell population containing a large proportion of glial cells, which are unlikely to express significant levels of dopamine receptors, as well as the neuronal cells, only some of which will. Thus the presence of glial cell derived membranes in a preparation from striatum or other brain regions will dilute the apparent density of D_2 receptors present. Also, as only about 50% of the neurones in the striatum express D_2 receptors (Gerfen, 1992), their apparent density will be further reduced. If one then allows for clustering of the receptors at the synapses, say, rather than their being uniformly distrubuted around the entire surface of the neurone then local receptor densities may be further underestimated. Thus, although receptor densities in the pmol mg^{-1} range may appear relatively high compared to the levels usually found in brain, they may not in fact be unrepresentative of the true receptor density when the lack of homogeneity of receptor distribution and cell types in brain tissue are taken into account.

Many of the compounds shown here to be inverse agonists are used therapeutically as antipsychotic drugs. The results described here are therefore of importance as they may shed new light on the therapeutic actions and possibly the side effects of these drugs. It is no longer possible simply to think of them as passive antagonists, their therapeutic actions may be due to their ability to have actions over and above simple blockade of the effects of dopamine. For example, it has been shown that haloperidol has effects on acetylcholine accumulation in mouse brain after treatment with 6-hydroxydopamine (a treatment which results in the destruction of dopaminergic neurones and therefore the absence of dopaminergic signalling), suggesting that this compound can have effects in the brain even when there is no dopamine present to stimulate the dopamine receptors (Fibiger & Grewall, 1974). This may be an effect of the inverse agonism of haloperidol in this system, although reversal of the effects of small amounts of residual dopamine or effects at other neurotransmitter receptors cannot be ruled out in this case. Similarly, haloperidol has been shown to increase (although only very weakly) prolactin release from GH4C1 cells transfected with D_2 receptors but not wild-type GH4C1 cells (Nilsson & Eriksson, 1993), which could again be interpreted as in inverse agonist effect of this drug. It is also interesting to note that the drugs (+)-butaclamol and flupenthixol have been shown to behave as inverse agonists at D_1 and D_5 receptors after transient transfection into HEK 293 cells (Tiberi & Caron, 1994). However, this observation may not be relevant to their therapeutic action in schizophrenia, as blockade of D_1-like receptors is not usually considered to be important for antipsychotic activity (Sedvall & Farde, 1995).

The inverse agonist effects of these drugs at D_2 receptors may be particularly important during long term treatment, where there may be changes in the number and responsiveness of the receptors triggered specifically by their inverse agonist nature. Indeed, treatment of cells expressing D_2 receptors with sulpiride has been shown to increase receptor density under conditions where blockade of the effects of endogenous ago-

nists is also unlikely to be a satisfactory explanation (Filtz *et al*., 1994). Therefore these findings are of considerable importance in understanding both the short and long term effects of antipsychotic drugs. It will be of some interest to determine whether inverse agonist activity is a prerequisite for antipsychotic activity, as this may help to elucidate some of the mechanisms involved in the treatment and possibly the pathogenesis of schizophrenia.

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