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Functional Analysis of the D_{2L} Dopamine Receptor Expressed in a cAMP-Responsive Luciferase Reporter Cell Line

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ABSTRACT. A Chinese hamster ovary (CHO) cell line expressing the firefly luciferase gene under the control of six cAMP response elements (CREs) was stably transfected with the long form of the rat D_2 dopamine receptor. Saturation binding analysis using [^{3}H]spiperone showed that the receptor was expressed at low levels (B_{max} = 96.5 ± 15.8 fmol/mg), but with an affinity characteristic of the D_2 receptor (K_d = 21.5 ± 3.7 pM). Luciferase expression in this cell line was modified in a dose dependent manner with dopamine receptor agonists (*N*-propylapomorphine > apomorphine > quinpirole > dopamine) and antagonists (spiperone > (+)-butaclamol > D0710 > (−)-sulpiride > tiapride > remoxipride), according to their rank order of potency in binding and cAMP accumulation studies. Dopamine-mediated inhibition of forskolin-stimulated luciferase expression was pertussis toxin sensitive. This demonstrated the efficiency of the luciferase reporter gene assay for the functional testing of D_2 dopamine receptors, which are negatively coupled to the adenylyl cyclase signaling pathway, when heterogously expressed at low levels in CHO cells. BIOCHEM PHARMACOL **56**;1:25–30, 1998.

KEY WORDS. dopamine (D_2) receptor; adenylyl cyclase; CRE response element; reporter gene assay; luciferase; CHO cells

The dopamine receptors mediate diverse physiological actions and are important targets for the treatment of diseases such as schizophrenia and Parkinson's disease. The transduction of dopaminergic signals across cell membranes is mediated by at least five different dopamine receptor subtypes that belong to the gene superfamily of GPCRs§ [1, 2]. The D_1-like receptors (D_1 and D_5) stimulate adenylyl cyclase when expressed in mammalian cells, whereas the D_2-like receptors (D_2, D_3 and D_4) inhibit adenylyl cyclase.

At the molecular level, activation of D_2 dopamine receptors has been shown to affect a variety of cell type specific signal transduction pathways in addition to adenylyl cyclase [3] such as the stimulation of phosphatidylinositol turnover [4], potentiation of arachidonic acid release [5] or regulation of K^+- and Ca^{2+}-channel activity [4, 6]. All of these different signaling pathways are thought to involve the contribution of the G_i/G_o family of guanine nucleotide binding proteins (G-proteins).

In recent years, the molecular and pharmacological characterisation of GPCR's has been facilitated by modern cloning techniques and the development of sensitive functional assays, including reporter gene based assay systems (reviewed in [7]). Reporter genes, such as chloramphenicol acetyltransferase (CAT), β-galactosidase, secreted alkaline phosphatase (SEAP), and more recently, green fluorescent protein (GFP), can be coupled to regulatory gene sequences to produce a readily measurable phenotype upon expression [8].

In this study, CHO cells have been stably transfected with the luciferase gene from the firefly, *Photinus pyralis*, under the control of promoter sequences containing cAMP response elements and the activity of this gene was then measured using bioluminescence. There are already several reports of use of this system to monitor the functional coupling of GPCRs to the adenylyl cyclase signaling system [7]. However, there is only one report of the use of this reporter system to measure the differential coupling of receptors coupled both negatively and positively to the adenylyl cyclase signaling pathway-namely, the adenosine A1 and A2 receptors respectively [9]. In contrast, the M4 muscarinic acetylcholine receptor, which normally decreases cAMP in cells upon activation, exhibited little agonist induced inhibition of luciferase expression using such a reporter system, either at low or high levels of receptor expression [10]. In the case of the dopamine receptor, only the D_1 and D_5 subtypes, which couple

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§ *Abbreviations:* CHO, Chinese hamster ovary; CRE, cyclic AMP response element; DMEM, Dulbecco's modified Eagles medium; D_{2L}, long form of the D_2 dopamine receptor; GPCR, G-protein coupled receptors; and NPA, *N*-propyl norapomorphine.

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positively to adenylyl cyclase, have been investigated with this reporter system [11], although the differential coupling of the long and short isoforms of the D_2 dopamine receptor has been monitored using a similar CRE-cat reporter system [12], but with a limited number of ligands.

The aim of this study therefore was to assess the suitability of the reporter gene assay as a method for characterising the functional activity of heterologously expressed dopamine receptor, which was negatively coupled to adenylyl cyclase. This was achieved by stably co-expressing the D_{2L} receptor and the cAMP responsive luciferase gene in CHO cells. The responses to a wide range of agonist and antagonist ligands were investigated and the results compared to those obtained with binding and cAMP accumulation studies.

MATERIALS AND METHODS

Materials

The following ligands were purchased from Research Biochemicals International: spiperone, (+)- and (−)-butaclamol, apomorphine, NPA and quinpirole. The following substances were obtained as gifts and are gratefully acknowledged: remoxipride (Astra Pharmaceuticals), tiapride (Synthelabo), (−)-sulpiride (Ravizza Laboratories) and D0710 (Dr. A. Mann, CNRS, Strasbourg, France).

DMEM minus phenol red was purchased from Life Technologies. All other ligands and chemicals were purchased from Sigma. The reporter plasmid, pADneo2-C6-BGL, encoding the luciferase gene from the firefly *Photinus pyralis* under the control of the rabbit β-globin promoter and six cAMP response elements, was kindly donated by Dr. A. Himmler (Bender & Co., GmbH). The cDNA encoding the long form of the rat D_2 receptor was obtained from Drs. P. Vernier and J. Mallet (CNRS, Gif sur Yvette, France).

Cell Culture

Chinese hamster ovary cells deficient in the enzyme dihydrofolate reductase (CHO dhfr$^-$), were maintained in DMEM supplemented with 10% (v/v) foetal calf serum (FCS), L-glutamine (2 mm), hypoxanthine and thymidine (HT) supplement, penicillin (100 units/mL) and streptomycin (100 μg/mL). Cells were grown at 37° in a humidified atmosphere of 5% CO_2 in air.

DNA Transfection

The cAMP responsive reporter plasmid (pADneo2-C6-BGL) was transfected into CHO dhfr$^-$ cells using the calcium phosphate precipitation technique, as previously described [13]. This CHO-luc cell line was then stably cotransfected with the rat D_{2L} dopamine receptor (D_2pSVL) and a vector containing the DHFR gene (pSV2 dhfr), again using the calcium phosphate precipitation technique. Cells were grown in selective medium containing geneticin (400 μg/mL) to select for the reporter plasmid, and dialysed FCS lacking hypoxanthine and thymidine supplement to select for the DHFR gene. Following two rounds of dilution cloning, the clonal cell line exhibiting the maximum inhibition of forskolin stimulated luciferase expression in the presence of dopamine (10 μM) was selected, and is hereafter referred to as the D_2 CHO-luc cell line.

Membrane Preparation and Ligand Binding Assays

Medium was removed, and cell membranes prepared as previously described [14]. For saturation binding assays, 30 μg of membrane protein was incubated with [^{3}H]spiperone (Amersham; specific activity, 28 Ci/mmol). [^{3}H]spiperone was added over a range of concentrations up to 0.4 nM, and the reaction mixture was made up to 1 mL with Hepes I buffer [20 mM of Hepes (pH 7.4), 1 mM of EDTA, 1 mM of EGTA, and 120 mM of NaCl]. Specific binding was determined as the binding that was inhibited by 3 μM of (+)-butaclamol, and saturation analysis performed as previously described [14].

Luciferase Assays

Reporter cells were seeded at a density of 2×10^5 cells/well in a 48-well culture plate 18–20 hr prior to assay. Cells were washed twice with serum-free DMEM before the addition of drugs in 100 μL of serum-free DMEM. Agonists, antagonists, and forskolin were added simultaneously and the cells incubated at 37° for 4 hr as previously described [11, 13, 24] as preincubations were found to be unnecessary (for toxin studies, the cells were pretreated with pertussis toxin (100 ng/mL) for 20 hr before incubating with ligands). After incubation, 100 μL of Packard Lucite reagent was added to the cells which were shaken for 5 min at room temperature to ensure complete lysis before being transferred to a white (opaque) 96-well plate. Luminescence was measured on a Top Count 228 Microplate Scintillation and Luminescence Counter (Packard 9912V) in single photon counting mode for 0.02 min/well, following 10-min adaptation in the dark.

Data Analysis

Concentration-response data generated using the luciferase assay, following incubation of cells with receptor ligands, were analysed by subtracting basal levels (i.e. with diluent alone), then normalising values as a percentage of controls (see figure legends for details). These data were analysed by nonlinear regression with variable slope, using the computer package Graphpad Inplot 4. If slopes were found to be not significantly different from unity at the 5% confidence level using the Student's *t*-test, then slopes were constrained to 1 for the purpose of estimating EC_{50} or IC_{50} values. These are expressed as the mean ± SEM for at least

3 independent experiments (N = 3), unless otherwise stated in the figure legend. Estimate of the antagonist-receptor equilibrium dissociation constant K_b, were made from IC_{50} values derived from antagonist inhibition curves according to the Gaddum equation [13, 15].

When NPA dose response curves were carried out in the presence of different concentrations of butaclamol, the pK_b was estimated as the intercept of a plot of log (dr-1) against log [antagonist] analysed by linear regression (Schild plot). The dose ratio (dr) was calculated as the EC_{50} of the agonist in the presence of antagonist divided by the EC_{50} of the agonist in the absence of antagonist, where EC_{50} was determined by nonlinear regression as described above. To assess the correlation between luciferase and cAMP accumulation data, linear regression analysis was carried out using Graphpad Inplot 4 and the regression value (r) determined.

RESULTS

Expression of the D_{2L} Receptor in the CHO-luc Reporter Cell Line

The reporter cell line, CHO-luc, containing the luciferase reporter gene under the control of six CRE regulatory regions, has previously been shown to be insensitive to dopamine, suggesting the lack of endogenous receptors of this class in this cell line [11, 13]. This reporter cell line was transfected with the long form of the D_2 dopamine receptor (D_{2L}), and stable cell lines were selected by measuring the inhibition of forskolin stimulated luciferase expression after incubation with dopamine (10 μM). The cell line isolated, D_2 CHO-luc, demonstrated about 20-fold stimulation of luciferase in the presence of forskolin (0.5 μM) over the background luciferase level (200 counts per second), and this was inhibited by 70–80% in the presence of dopamine (10 μM). Saturation analysis of [^{3}H]spiperone binding to these cells (between passage 10–13) gave a K_d of 21.5 ± 3.7 pm and a B_{max} value of 96.5 ± 15.8 fmol/mg. There was no specific binding of [^{3}H]spiperone to untransfected CHO-luc cells.

Characterisation of Changes in Luciferase Expression Mediated by the Dopamine Receptor

Toxin studies. In order to confirm that the effect of dopamine on luciferase expression was mediated by a G-protein, the effect of pertussis toxin (PTX) on the response was investigated (Fig. 1). In the absence of toxin (control), 0.5 μM of forskolin gave a mean increase in luciferase expression of 19.3 ± 2.2-fold over basal, and coincubation with 10 μm of dopamine produced a 68.5 ± 3.0% inhibition of this forskolin stimulated level. Pre-incubation with pertussis toxin slightly decreased both basal and forskolin stimulated levels of luciferase expression to 84.4 ± 2.2, and 87.9 ± 2.8% of basal and forskolin stimulated levels respectively in the absence of toxin. However, the mean induction with 0.5-μm forskolin was not significantly altered at 19.6 ± 1.1-fold over basal. Pre-incubation with the toxin abolished dopamine mediated inhibition of the forskolin stimulated expression, with expression in the presence of 10 μM of dopamine being 99.4 ± 1.9% of the forskolin control.

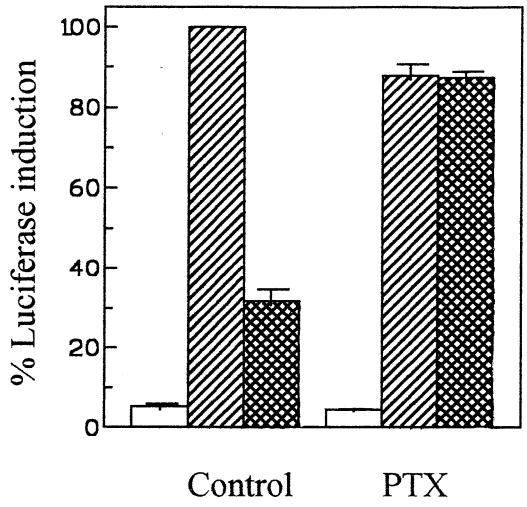

FIG. 1. The effect of PTX on dopamine-mediated inhibition of forskolin-stimulated luciferase expression in D_2CHO-luc cells. Cells were incubated with 1% DMSO (open bars), 0.5 μM of forskolin (diagonal stripe) or 0.5 μM of forskolin plus 10 μM of dopamine (criss-cross) for 4 hrs in the presence (+PTX) or absence (control) of pertussis toxin. Luciferase activity was determined as described in Methods. Values were calculated as % luciferase expression with forskolin in the absence of toxin. Bars represent the mean ± SEM of three independent experiments carried out in triplicate.

Agonists. D_2 dopamine receptor agonists gave rise to inhibition of forskolin stimulated luciferase expression in D_2 CHO-luc cells in a concentration dependent manner (Fig. 2), with a rank order of potency of NPA > apomorphine > quinpirole > dopamine. The concentration response data could be fitted to sigmoid curves, with slopes which were not significantly different to 1 ($P > 0.05$). All four ligands gave approximately the same maximum % inhibition of forskolin stimulated luciferase expression (see Table 1).

Antagonists. To further characterize the interactions between the D_2 receptor and the luciferase reporter plasmid, the activities of seven antagonists, selected to cover a range of expected affinities for the receptor and to represent different classes of drug (i.e. the substituted benzamides and classic antagonists), were investigated. These antagonists were able to reverse dopamine mediated inhibition of forskolin stimulated luciferase expression in a concentration dependent manner, with a rank order of potency as follows: spiperone > (+)-butaclamol > DO710 > (−)-sulpiride > tiapride > remoxipride (Fig. 3). The concentration response data could all be fitted to sigmoid curves, and the Hill slopes did not differ significantly from unity ($P > 0.05$) (Table 2). The Hill slope for remoxipride,

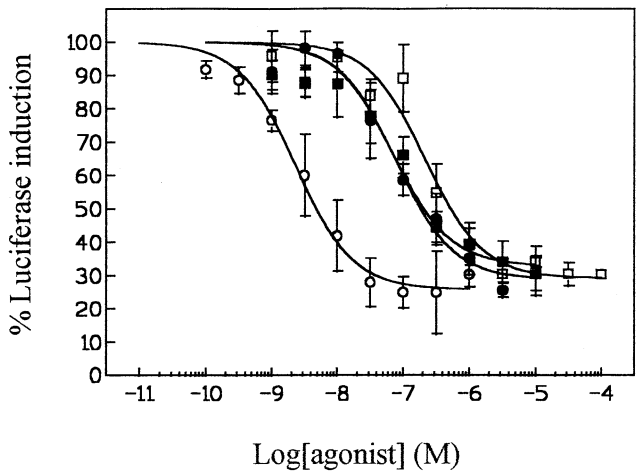

FIG. 2. The effects of dopamine-receptor agonists on forskolin-stimulated luciferase expression in D_2CHO-luc cells. Cells were incubated with 0.5 μM of forskolin in the presence of NPA (open circles), quinpirole (closed circles), dopamine (open squares), or apomorphine (closed squares). Values were calculated as % of luciferase expression in the presence of 0.5 μM of forskolin and the absence of agonist. The curves were constrained to a maximum of 100% and slopes did not differ significantly from unity ($P > 0.05$), so were constrained to 1. Data points represent the mean ± SEM of at least three independent experiments carried out in duplicate.

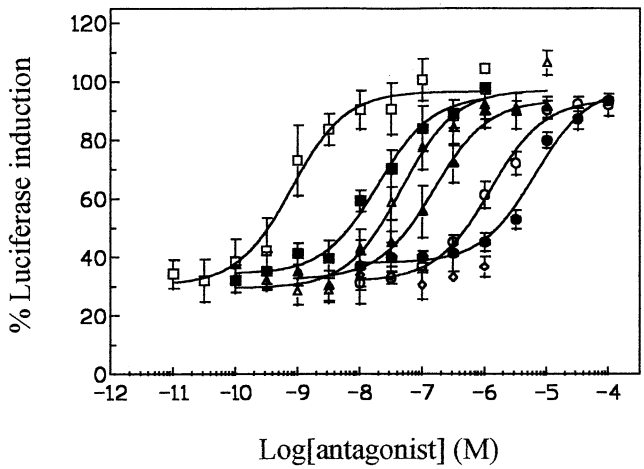

FIG. 3. The effects of dopamine-receptor antagonists on forskolin-stimulated luciferase expression in D_2CHO-luc cells. Cells were incubated with 0.5 μM of forskolin and 10 μM of dopamine in the presence of spiperone (open squares), (+)-butaclamol (closed squares), DO710 (open triangles), (-)-sulpiride (closed triangles), tiapride (open circles), remoxipride (closed circles) or (-)-butaclamol (open diamonds). Values were calculated as % of luciferase expression in the presence of 0.5 μM of forskolin and the absence of both dopamine and antagonist. The Hill slopes did not differ significantly to form unity ($P > 0.05$) and so curves were fitted with the Hill slopes constrained to 1. Data represent the mean ± SEM of at least three independent experiments carried out in duplicate.

however, significantly differed from 1 at the 10% significance level. The (−)-stereoisomer of butaclamol was unable to reverse dopamine mediated inhibition at concentrations at which the (+)-isomer was effective (Fig. 3).

The effect of increasing concentrations of (+)-butaclamol on the concentration response curve for NPA mediated inhibition of forskolin stimulated expression, was investigated. Increasing concentrations of (+)-butaclamol were found to shift the concentration response curve for NPA to the right (Fig. 4). Schild analysis gave a mean slope of 1.52 ± 0.13 and a mean pK_b estimate of 7.16 ± 1.02. This slope was not significantly different to unity according to the Student's *t*-test.

DISCUSSION

A reporter cell line, which co-expressed the dopamine D_{2L} receptor and a cAMP responsive luciferase gene, was established in CHO dhfr$^-$ cells. Before functional responses mediated by the dopamine receptor could be assessed using the luciferase assay, it was necessary to confirm the authenticity of the receptor in terms of its ability to bind dopaminergic ligands and interact with G_i-like proteins. The lack of specific binding of [^{3}H]spiperone confirmed that there was no endogenous D_2 receptor in the CHO-luc cells, and was consistent with the previous observation that dopamine was unable to inhibit forskolin-stimulated luciferase expression in this cell line [11, 13]. However, the CHO-luc D_2 cell line showed specific binding of [^{3}H]spiperone of approximately 100 fmol/mg with a K_d within the range expected for the D_2 dopamine receptor. Dopamine gave rise to 70–80% inhibition of forskolin-stimulated

TABLE 1. The pEC_{50} and % maximal inhibition of agonist concentration response curves

Agonist	pEC_{50} ± SEM	Maximum% inhibition ± SEM
NPA	8.55 ± 0.13	79.7 ± 1.5
Apomorphine	7.28 ± 0.22	78.6 ± 10.0
Quinpirole	7.11 ± 0.20	72.7 ± 5.3
Dopamine	6.84 ± 0.18	74.1 ± 3.4

Curves were fitted using Graphpad Inplot (see Methods) with the maximum constrained to 100%. Hill slopes did not differ significantly from unity so were constrained to 1 in order to estimate the pEC_{50} values. The maximum % inhibition was taken from the minima of the fitted curves. Values are the mean ± SEM of the values from curves fitted to at least three independent experiments.

TABLE 2. The pIC_{50} and estimated K_b values from antagonist concentration response curves

Antagonist	pIC_{50} ± SEM	K_b (nM) ± SEM
Spiperone	9.11 ± 0.16	0.030 ± 0.021
(+)-Butaclamol	7.72 ± 0.17	0.49 ± 0.15
DO710	7.31 ± 0.21	2.0 ± 1.1
(−)-Sulpride	7.18 ± 0.29	6.6 ± 3.3
Tiapride	5.79 ± 0.12	34 ± 7.5
Remoxipride	5.18 ± 0.01	110 ± 22
(−)-Butaclamol	<5	>100

Curves were fitted using Graphpad Inplot 4 and Hill slopes, which were found to be not significantly different from unity were constrained to 1 in order to estimate the pIC_{50} values. Data are the mean ± SEM of values from curves fitted to at least three independent experiments. K_b values were calculated from the mean pIC_{50} as described (see Methods).

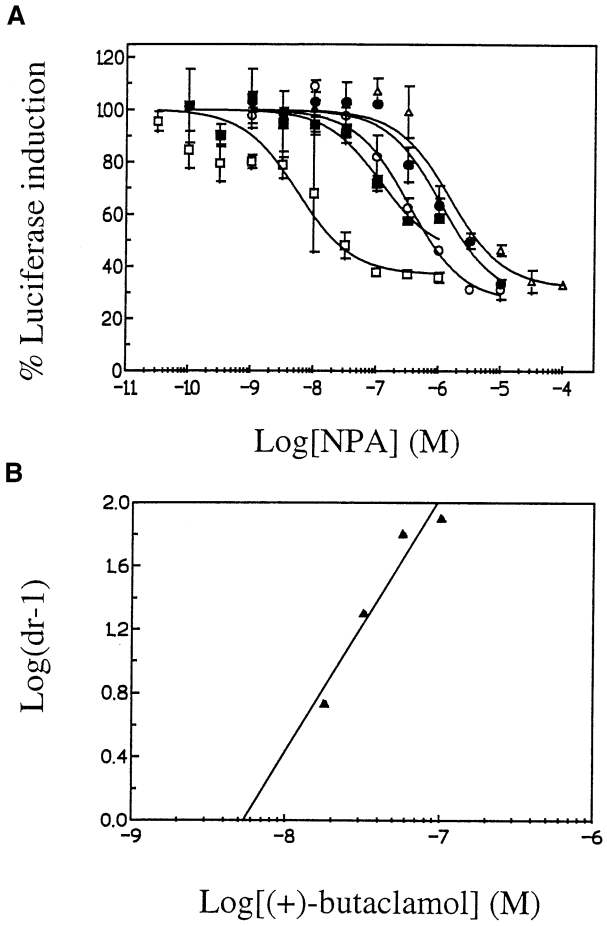

FIG. 4. The effect of (+)-butaclamol on the NPA concentration response in D_2CHO-luc cells. (A) Cells were incubated with various concentrations of NPA in the presence of 0.5 μM of forskolin and the following concentrations of (+)-butaclamol: 0 nM (open squares), 3 nM (closed squares), 10 nM (open circles), 30 nM (closed circles), or 100 nM (open triangles). Values were calculated as % of the forskolin control in the absence of NPA and (+)-butaclamol. Slopes were found to be not significantly different to unity ($P > 0.05$) so were constrained to 1. Data points represent the mean ± SEM of duplicate determinations from a single experiment representative of four carried out. (B) Shows Schild regression analysis of data presented in (A).

luciferase expression. This inhibition was abolished by pre-incubation with PTX indicating that the response was mediated by a PTX sensitive G-protein, as expected for the D_2 dopamine receptor [10, 16]. Inhibition by dopamine was reversed by (+)-butaclamol, with the expected stereoselectivity for the D_2 receptor [17]. These results indicated that the D_2 receptor had been successfully expressed, and that it was able to alter luciferase expression through an interaction with the appropriate class of G-proteins in a ligand dependent manner.

All of the agonists tested were able to inhibit forskolin-stimulated luciferase expression via the dopamine D_2 receptor. Although the published EC_{50} values for these agonists using adenylyl cyclase assays vary considerably between studies, perhaps due to differences in receptor expression levels and/or cellular environments, the EC_{50} values obtained from the luciferase assay fall within the published range for each agonist at this receptor. The rank order of potency of the agonists from luciferase assays and adenylyl cyclase activity studies carried out on cells endogenously expressing D_2-like receptors [16, 18, 19, 20] was preserved, as previously observed with the endogenously expressed serotonin and calcitonin in receptors [13]. Moreover, the reporter gene assay has several advantages over classic cAMP assays [21]. Although the reporter gene assay initially requires a longer incubation period with drugs (typically 2–4 hr), the assay is readily automated in microtitre format, and requires minimal sample manipulation, with both the lysis and luciferase reaction being performed in a single reagent. The amplification of the intracellular signal, combined with the broad linear range and sensitivity of the luciferase assay, means that fewer cells are required to produce a measurable response, which can be determined immediately after the assay is performed. It therefore provides a highly sensitive, alternative assay to either ligand binding or second messenger assays, for the pharmacological characterisation of receptors coupled to adenylyl cyclase.

Similarly, the rank order of potency of antagonists at the D_{2L} receptor is preserved in this reporter gene assay. There is a good correlation ($r > 0.999$) between published K_i values for the same antagonists determined from competition analysis of the same receptor expressed in CHO [22] and COS-7 [14] cells, and the estimated K_b values from individual competition curves in this study. Although the affinity values estimated for the antagonists are lower (approximately three-fold) than those determined in ligand binding assays [14, 22], it is likely that this is due to differences in experimental conditions between the two assays, and in the latter instance, the cell line used. However, determination of antagonist dissociation constants for (+)-butaclamol using Schild analysis (pK_b = 7.16 ± 1.02) gave a better correlation to published K_i values determined from ligand binding studies.

In summary, a cell line which expressed the dopamine D_2 receptor and the cAMP responsive luciferase reporter gene was established in CHO dhfr$^-$ cells. The D_2 receptor was able to mediate agonist dependent inhibition of forskolin-stimulated luciferase expression through an interaction with a PTX-sensitive G-protein. D_2 receptor antagonists were able to reverse agonist dependent inhibition of forskolin-stimulated luciferase expression with a rank order of potency and stereoselectivity characteristic of the D_2 receptor. These results confirm the value of the luciferase reporter gene assay as a tool for the pharmacological characterisation of heterologously expressed dopamine receptors, which are negatively, as well as positively [11], coupled to the adenylyl cyclase signaling pathway. Moreover, it allows for the detection of subtle, but physiologically relevant, changes in cAMP levels mediated by the activation of receptors expressed at low levels in mammalian cell lines.

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