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Expression and functional characterisation of a synthetic version of the human D_4 dopamine receptor in a stable human cell line

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Abstract

A synthetic version of the human D_4 (hD4) dopamine receptor was prepared. The G/C content of the natural gene was reduced by 14% without altering the amino acid composition of the corresponding protein sequence. HEK293 cells were transfected with the synthetic hD4 gene and stable clones resistant to G418 selected. The hD4 receptor expressed from the synthetic gene had identical pharmacological characteristics to the native hD4 receptor [(1991) Nature 350, 610–619; (1992) Nature 358, 149–152]. Functional studies with cells expressing the synthetic hD4 gene indicated negative coupling of this receptor to adenylate cyclase.

Key words: D_4 dopamine receptor; Synthetic; Stable expression; Functional characterisation; HEK 293 cell

1. Introduction

Dopamine receptors are the sites of action of many of the anti-psychotic drugs currently used in the treatment of schizophrenia. Recent advances in molecular biology have dramatically expanded the number of known dopamine receptors. It is now clear that there are two families of receptors, the 'D_1-like' (D_1 and D_5) and the 'D_2-like' (D_2, D_3 and D_4) [3].

The human D_4 (hD4) dopamine receptor was cloned initially as a hybrid of genomic DNA and cDNA. This clone has been shown, by transient expression in COS-7 cells, to have a high affinity for the atypical neuroleptic, clozapine [1]. Variants of the hD4 receptor, containing a variable number of 48 bp imperfect repeat units in intracellular loop three, were later reported [3–5]. Expression of the hybrid hD4.2 (hD4 receptor with two repeat units) receptor clone was reported in baculovirus [6]. Expression was achieved by deleting the first two introns of the D_4 genomic–cDNA hybrid and replacing them with a synthetic oligonucleotide with a reduced G/C content.

The most common form of the hD4 receptor in human populations is D4.4 [5]. Stable expression of a functionally coupled hD4.4 receptor in a human cell line is therefore important for drug discovery. The hD4 gene has an unusually rich G/C content, and difficulties have been found in attempting stable expression of this receptor [7]. We decided to produce a synthetic version of the gene with an overall reduction in G/C content. The synthetic hD4.4 gene was transfected into HEK293 (human embryonic kidney) cells for stable expression. Functional expression of other G-protein coupled receptors, including the human dopamine D_2 receptor, has been demonstrated in this cell line [8–10]. The transfected HEK293 cells expressed high levels of a receptor with pharmacology similar to that described for the hD4.2 isoform [1,2,6]. The receptor expressed from the synthetic gene was also shown, in functional assays, to be negatively coupled to adenylate cyclase.

2. Materials and methods

2.1. Production of a synthetic hD4 gene

A modified version of the human D4.4 receptor was constructed by British Biotechnology Plc. Ltd. UK. the G/C content of the synthetic hD4 was reduced to 60%. Suitable restriction sites (*Eco*R1 and *Hin*dIII) were added to allow subcloning into an expression vector. Unique *Pst*1 and *Bam*H1 restriction sites were engineered into the construct. This allowed the insertion of differing numbers of 48 bp imperfect repeats into intracellular loop III.

2.2. Construction of an expression vector for the hD4.4 gene

The synthetic hD4.4 cDNA was isolated as an *Eco*R1–*Hin*III fragment by the GeneClean procedure (Bio101). The purified hD4.4 fragment was ligated into the corresponding sites in the mammalian expression vector, pCDN, using the Amersham ligation system. The ligated DNA was transformed into *Escherichia coli* Sure cells (Stratagene) and colonies were selected by ampicillin resistance. Colonies containing the hD4.4 were selected by PCR screening with primers specific for the pCDN polylinker, using Cetus Amplitaq. The DNA sequence of the hD4.4 cDNA was confirmed across the cloning sites in pCDN. One construct, pCDNHD4.4, was chosen to transfect into mammalian cells. Transfecting DNA was purified by Magic maxi columns (Promega). All procedures were done using standard methodologies [11].

2.3. Transfections into COS-1 cells (transient) and HEK293 cells (stable)

For transient transfections 1.5×10^6 COS-1 cells were seeded in 75 cm^2 flasks (Costar) in MEM medium (containing Earl's salts and L-glutamine; Gibco) supplemented with 10% FCS (Gibco) and 1% L-glutamine (Gibco). Cells were incubated at 5% CO_2, 37°C overnight. Each flask of COS-1 cells was transfected with 20 μg of DNA using the DEAE dextran methodology [12]. Cells were scraped 48 h post-transfection. The human D_2 dopamine receptor (hD2) cDNA was trans-

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fected as the positive control. This receptor was obtained from the University of Oregon.

For stable transfections 2×10^6 HEK293 cells were seeded in 100 mm petri-dishes (Costar) in MEM medium supplemented with 10% FCS (Gibco) at 5% CO_2, 37°C overnight. Each Petri-dish of cells was transfected with 20 μg DNA using the calcium phosphate protocol [13]. During transfection cells were incubated at 3% CO_2 overnight. Selection medium containing 800 μg/ml G418 (Gibco; geneticin) was added 48 h post-transfection. After 15 days G418-resistant clones were selected by ring cloning and expanded for further analysis.

2.4. Preparation of membranes and radioligand binding studies

Colony selection was carried out using in situ binding. Cells were grown to confluence in 6-well plates. The medium was removed and the cells were washed with 1 ml assay buffer (50 mM Tris containing 120 mM NaCl, 5 mM KCl, 5 mM $MgCl_2$, 1.5 mM $CaCl_2$, pH 7.4, at 22°C). Cells were then incubated with approximately 1.5 nM [^{3}H]spiperone in a final volume of 1 ml assay buffer containing 0.35% BSA for 2 h at 22°C. At the end of the incubation, separation of bound and free ligand was achieved by inversion of the plates and rapid washing of each well with 4×5 ml ice-cold assay buffer. Radioligand remaining bound to cells was removed with 10% SDS and radioactivity counted in the normal manner. Membranes for radioligand binding studies were prepared from frozen pellets of hD4.4 cells and radioligand binding carried out as described earlier [14]. The concentration of [^{3}H]spiperone used was 0.4–0.6 nM.

2.5. Functional studies

HEK293 cells expressing the hD4.4 receptor were removed from confluent 75 cm^2 flasks by replacing the medium with phosphate-buffered saline, and harvested by centrifugation. Cells (2×10^5) were pre-incubated (15 min) with isobutylmethylxanthine (500 μM) in Ultra-CHO (BioWhittaker). Samples were then incubated with the agonist and/or antagonist for 10 min at 37°C before the addition of forskolin (20 μM; Sigma). The final volume was 0.5 ml. After a further 10 min at 37°C the reaction was terminated by the addition of 3 M perchloric acid. Cyclic AMP was extracted into the aqueous phase of a 50:50 mixture of trioctylamine:trichlorotrifluoroethane and measured by cAMP radioimmunoassay (NEN DuPont).

Fig. 1. Wilbur–Lipman DNA alignment of the synthetic and native D4.4 genes. Matching residues are marked. Both genes are 86% identical and differ in their respective G/C contents by 14% without altering the amino acid sequence.

3. Results and discussion

In order to obtain successful and stable expression of the hD4.4 in a human cell line we adopted a strategy involving the construction of a synthetic version of the receptor cDNA. This strategy allowed us to reduce the gene's G/C content as well as design a receptor subtype with an appropriate number of repeats. We achieved an overall reduction in the hD4.4 G/C content of 14% (74% to 60%) without altering the amino acid sequence (Fig. 1).

The synthetic gene was subcloned into the mammalian expression vector, pCDN, producing pCDNHD4.4 (Fig. 2). This construct contains both G418 and DHFR (dihydrofolate reductase) resistance determinants and places hD4.4 under the control of a CMV promoter.

Transient expression of the synthetic gene in COS-1 cells was performed. Cells transfected with synthetic hD4.4 receptor displayed D_4-like pharmacology as assessed by [^{3}H]spiperone and clozapine binding (results not shown).

HEK293 cells were transfected with the hD4 construct, pCDNHD4.4, and selected with G418. Resistant clones (212) were tested for receptor expression by [^{3}H]spiperone binding in 6-well plates. 26 positive clones were expanded. K_d and B_{max} were determined in saturation experiments. One of the clones, expressing 2 pmol/mg protein, was selected for radioligand binding and functional studies.

Radioligand binding studies were carried out under the same conditions as we have used previously for hD2 and hD3 receptors cloned in CHO cells [14]. These conditions are different to those employed by others in previous hD4.2 studies [1,2,6,16]. However, neither the change in assay buffer nor the change in temperature had any effect on radioligand binding (data not shown). Fig. 3 shows the results of [^{3}H]spiperone binding studies with membranes obtained from hD4.4/293 cells. Data was analysed according to a single site fit [15], using the K_d for [^{3}H]spiperone obtained in the saturation studies (0.14 nM).

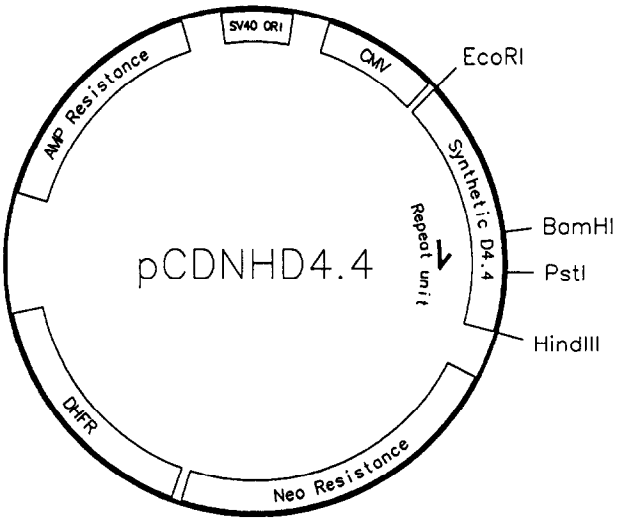

Fig. 2. Plasmid pCDNHD4.4 including the synthetic hD4.4 gene. The position of the hD4.4, 48 bp repeat cassette is marked by an arrow.

The K_i's obtained are shown in Table 1. Dopamine, spiperone and clozapine showed affinities at the hD4.4 receptor expressed in HEK293 clones consistent with the values obtained by others in different cell lines [1,2,6,16]. The binding characteristics of the product of our synthetic gene are therefore similar to those described by others for this receptor.

Stimulation by forskolin (20 μM) produced a 25-fold stimulation of cellular cAMP over basal levels (approxi-

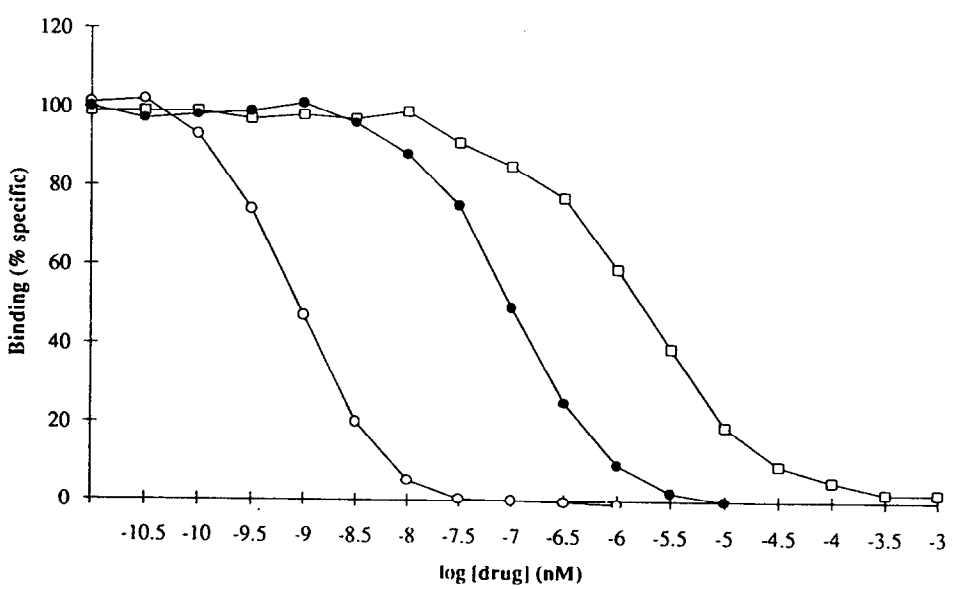

Fig. 3. Inhibition of [^{3}H]spiperone (0.4–0.6 nM) binding by spiperone (○), clozapine (●) or dopamine (□). Each point is the mean of 6 separate experiments.

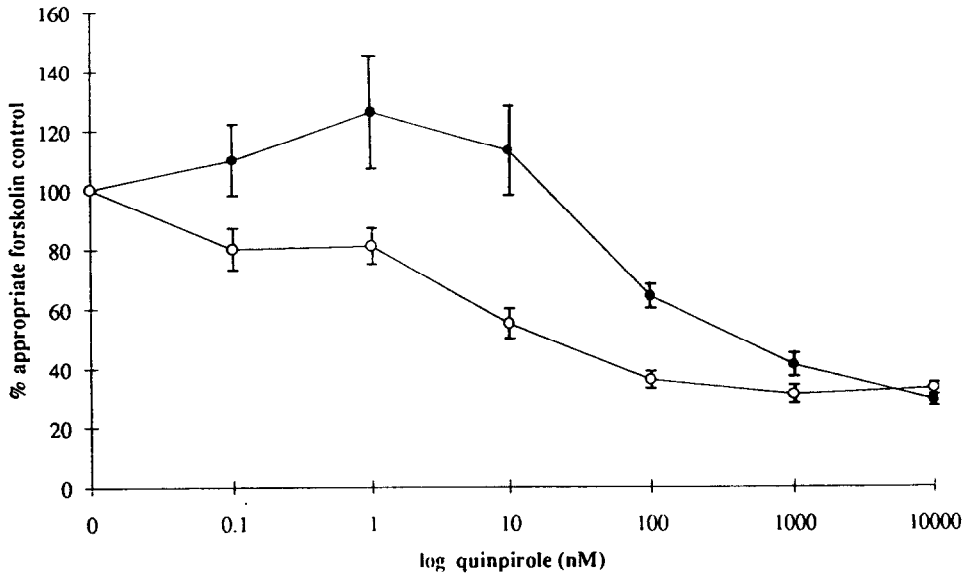

Fig. 4. Inhibition of forskolin (20 μM)-induced stimulation of adenylyl cyclase by quinpirole in the absence (○) or presence (●) of 1 nM spiperone. Each point is the mean ± S.E.M. of five experiments assayed in triplicate.

mately 2 pmol/10^5 cells), as measured by cAMP radioimmunoassay. Forskolin-stimulated cAMP was inhibited by the dopamine receptor agonist, quinpirole, in a concentration-related manner (Fig. 4), producing > 60% inhibition at 10 μM. The quinpirole inhibition curve was shifted to the right by spiperone (1 nM), which did not itself have any effect on cAMP levels. These results suggest that the hD4.4 receptor is negatively coupled to adenylate cyclase in these cells, similar to the effect reported in mouse retinas [17]. However, to the best of our knowledge, this is the first report of the hD4.4 receptor coupling in a human cell line.

In conclusion, HEK 293, a human kidney cell line, expressing high levels of hD4.4 receptor, was obtained. Negative coupling to adenylate cyclase was demonstrated in this system. The stable expression of the hD4.4 receptor in a human cell line will aid our understanding of the biochemistry and pharmacology of this receptor.

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Table 1
Affinities of dopamine, spiperone and clozapine for human D4.4 receptor expressed in HEK293 cells: comparison with literature data

Drug	HEK293	Van Tol et al. [1,2] (COS)	Mills et al. [6] (Sf9)	Lahti et al. [13] (COS)
Dopamine	394 ± 76.1	450,–	–	–
Spiperone	0.188 ± 0.016	0.08,0.10	0.3	–
Clozapine	29.6 ± 1.84	9,25	53	29

Data are expressed as K_i values (nM) ± S.E.M. from 6 separate experiments.

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