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# Receptor density as a factor governing the efficacy of the dopamine $D_4$ receptor ligands, L-745,870 and U-101958 at human recombinant $D_{4.4}$ receptors expressed in CHO cells

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**1** The relationships between the density of dopamine $D_{4.4}$ receptors and the agonist efficacies of L-745,870 (3-(4-[4-chlorophhenyl]piperazin-1-yl)-methyl-1H-pyrrolo [2,3-b]pyridine) and U-101958 ((1-benzyl-piperidin-4-yl)-(3-isopropoxy-pyridin-2-yl)-methyl-amine) were investigated in Chinese hamster ovary (CHO) cells, after treatment with the gene expression enhancer, sodium butyrate.

**2** In CHO cells expressing $D_{4.4}$ receptors (CHO/$D_4$ cells), dopamine inhibited forskolin-stimulated cyclic AMP accumulation ($E_{max}$ 56±1% inhibition, $pEC_{50}$ 7.4±0.1, $n=10$). U-101958 behaved as a partial agonist (39±7% the efficacy of dopamine, $pEC_{50}$ 8.1±0.3, $n=4$), whereas L-745,870 had no detectable agonist effect.

**3** Receptor density, as estimated by [$^3$H]-spiperone saturation binding was 240±30 fmol mg$^{-1}$ protein ($n=8$) in CHO/$D_4$ cell homogenates. It reached 560±150 ($n=6$), 1000±190 ($n=4$) and 840±120 ($n=4$) fmol mg$^{-1}$ protein after treatment with sodium butyrate (5 mM) for 6, 18 and 48 h, respectively.

**4** The increase in receptor density was associated with a gradual enhancement of the agonist effects (increased $E_{max}$ and $pEC_{50}$ values) of dopamine. The efficacy of U-101958 (relative to dopamine) doubled and L-745,870 was turned into a partial agonist (efficacy 49% relative to dopamine, $pEC_{50}$ 8.6±0.2, $n=6$, after 48 h treatment with sodium butyrate). These agonist effects of U-101958 and L-745,870 could be antagonized by spiperone (0.1 μM) but not by raclopride (10 μM).

**5** The results show that U-101958 and L-745,870 are partial agonists at human dopamine $D_{4.4}$ receptors expressed in CHO cells. Their efficacy is governed by receptor density. Agonist effects of these two compounds *in vivo* cannot be excluded under circumstances of increased receptor levels.

**Keywords:** L-745,870; U-101958; expression system; dopamine $D_4$ receptor; cyclic AMP inhibition; [$^3$H]-spiperone binding; schizophrenia

**Abbreviations:** CHO, Chinese hamster ovary; CHO/$D_4$ cells, CHO cells expressing dopamine $D_{4.4}$ receptors; HEK, human embryonic kidney; L-745,870, (3-(4-[4-chlorophenyl]piperazin-1-yl)-methyl-1H-pyrrolo [2,3-b]pyridine); 7-OH-DPAT, 7-hydroxy-2-dipropylaminotetralin; U-101958, ((1-benzyl-piperidin-4-yl)-(3-isopropoxy-pyridin-2-yl)-methyl-amine)

## Introduction

Dopamine receptors have been originally classified into two pharmacological subtypes, namely $D_1$ and $D_2$ (Kebabian & Calne, 1979). However, recent molecular cloning studies have led to the discovery of multiple dopamine receptor genes and to their classification into $D_1$-like and $D_2$-like subfamilies (for a review, see Hartman & Civelli, 1997). The $D_1$-like subfamily comprises $D_1$ and $D_5$ receptors, both positively coupled to cyclic AMP formation, whereas the $D_2$-like subfamily consists of $D_2$, $D_3$ and $D_4$ receptors, all negatively coupled to cyclic AMP formation.

$D_2$-like receptors appear to play an important role in schizophrenia. Thus, all antipsychotics currently used to treat the symptoms of schizophrenia have the ability to antagonize $D_2$-like receptors (Lahti *et al*., 1993; Seeman & Van Tol, 1995). Within the $D_2$-like receptor family, $D_4$ receptors have received particular attention for a number of reasons. Firstly, the atypical neuroleptic, clozapine has a small degree of selectivity for the $D_4$ receptor over the $D_2$ receptor (Van Tol *et al*., 1991). Secondly, mRNA expression of the dopamine $D_4$ receptor, as well as the receptor protein itself, is found primarily in limbic and cortical regions of the brain, areas thought to be involved in emotional/affective behaviour and cognition (Meador-Woodruff *et al*., 1996; Primus *et al*., 1997). In addition, a selective increase in the density of dopamine $D_4$ binding sites has been found in *post-mortem* brain tissue from schizophrenics (Seeman *et al*., 1993; Murray *et al*., 1995), although this remains controversial (Reynolds & Mason, 1995; Reynolds, 1996). All these findings strongly suggest that the dopamine $D_4$ receptor is an interesting target for the treatment of schizophrenia and several pharmaceutical companies have therefore developed putative dopamine $D_4$ receptor antagonists (see reviews by Hartman & Civelli, 1997; Wilson *et al*., 1998).

We recently reported that two such compounds, L-745,870 (3-(4-[4-chlorophenyl]piperazin-1-yl)-methyl-1H-pyrrolo [2,3-b]pyridine; Patel *et al*., 1997) and U-101958 ((1-benzyl-piperidin-4-yl)-(3-isopropoxy-pyridin-2-yl)-methyl-amine; Schlachter *et al*., 1997) were in fact agonists at human recombinant dopamine $D_{4.4}$ receptors stably expressed in human embryonic kidney (HEK)293 cells (Gazi *et al*., 1998). In these cells, L-745,870 and U-101958 mimicked dopamine at inhibiting forskolin-stimulated cyclic AMP accumulation, in

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contrast to spiperone and clozapine which were silent. Furthermore, the effects of L-745,870 and U-101958 could be antagonized by spiperone and clozapine but not by raclopride, consistent with a dopamine $D_4$ receptor-mediated effect. In the absence of recognized functional model for native dopamine $D_4$ receptors, these intriguing results prompted us to investigate the activities of L-745,870 and U-101958 in another expression system, namely Chinese hamster ovary (CHO) cells, using the same functional readout as previously (inhibition of cyclic AMP formation). We paid particular attention to the relationships between receptor density and ligand efficacy. Receptor density is known to be a factor strongly influencing ligand efficacy in both tissues and recombinant systems (Kenakin, 1997). Using sodium butyrate as an enhancer of receptor gene expression, we now show that the putative antipsychotics, L-745,870 and U-101958 are not silent antagonists at dopamine $D_{4.4}$ receptors expressed in CHO cells. Their efficacy is a function of receptor density.

## Methods

### *Cell line and culture*

A mammalian expression vector containing a human dopamine $D_{4.4}$ receptor cDNA (Gazi *et al*., 1998) was cotransfected with the pRSVNeo plasmid (in a 9 : 1 ratio) into CHO-K1 cells by the calcium phosphate precipitation method of Chen & Okayama (1987). Selection for stable integration was performed by adding 0.8 mg $ml^{-1}$ of geneticin to the culture and transfectants were then tested for a cyclic AMP inhibitory response to dopamine. The best line (hereafter referred to as $CHO/D_4$ cells) was propagated in Dulbecco's modified Eagle medium supplemented with 10% foetal calf serum, 100 iu $ml^{-1}$ penicillin, 100 $\mu$g $ml^{-1}$ streptomycin, non-essential aminoacids and 1 mg $ml^{-1}$ geneticin. Cells were grown in a humidified atmosphere at 37°C in the presence of 5% $CO_2$. They were split twice a week using a trypsin/EDTA solution. For radioligand binding experiments, cells were grown in 24.5 cm squared ('bio-assay') dishes. For cyclic AMP measurements, cells were seeded at a density of $2-3\times10^5$ cells/well in 24-well plates.

### *Radioligand binding assay*

$CHO/D_4$ cells grown to confluence in bio-assay dishes were collected by scraping in 50 mM HEPES buffer, pH 7.4, containing 1 mM $MgCl_2$, 2.5 mM $CaCl_2$, 0.1% (w $v^{-1}$) bovine serum albumin, 0.025% (w $v^{-1}$) bacitracin and 0.025% (w $v^{-1}$) sodium azide. They were then centrifuged at 1200 r.p.m. for 10 min at 4°C. After the removal of the supernatant, the cells were frozen at −70°C until the day of the experiment. For each binding assay experiment, the cells were resuspended in 50 mM Tris-HCl buffer (pH 7.4) containing (mM): NaCl 120, EDTA 5, $MgCl_2$ 1.5 and KCl 5, using a Polytron tissue homogenizer at setting 3–4 for 15 s. One hundred and 50 $\mu$l of the cell suspension (corresponding to ~250,000 cells/assay) were added to 96-well microtitre plates containing 50 $\mu$l drug and 50 $\mu$l (~40,000 c.p.m./assay) of [$^3$H]-spiperone (110 Ci $mmol^{-1}$, Amersham, Rahn AG, Zürich, Switzerland). The plates were incubated at room temperature for 120 min, rapidly filtered through Packard Unifilter-96, GF/C plates and washed three times with 300 $\mu$l ice-cold 10 mM Tris-HCl buffer containing 154 mM NaCl, pH 7.5. Filter-bound radioactivity was counted in 40 $\mu$l Microscint 40 in a Packard TopCount scintillation counter. Non specific binding was defined in the presence of 1 $\mu$M U-101958. Saturation experiments were performed using eight concentrations of the radioligand, ranging from approximately 0.4 to 25 nM. Assays were performed in triplicate and the determinations were replicated at least three times. The protein content was determined according to Bradford (1976).

**Table 1** Radioligand binding and cyclic AMP data in $CHO/D_4$ cells treated or not with 5 mM sodium butyrate (NaB) for the indicated periods of time

| | | *Untreated* | *NaB 6 h* | *NaB 18 h* | *NaB 48 h* |
|---|---|---|---|---|---|
| *[$^3$H]-spiperone binding* | | | | | |
| $B_{max}$ | fmol $mg^{-1}$ protein | 240±30 | 560±150 | 1000±190 | 840±120 |
| $pK_D$ | | 9.1±0.1 | 9.0±0.2 | 8.9±0.1 | 8.7±0.2 |
| | *n* | 8 | 6 | 4 | 4 |
| *Cyclic AMP* | | | | | |
| Dopamine | $E_{max}$ (% inhibition) | 56±1 | 78±1 | 83±2 | 77±1 |
| | $pEC_{50}$ | 7.4±0.1 | 7.7±0.1 | 8.4±0.2 | 8.6±0.1 |
| | relative efficacy | 100 | 100 | 100 | 100 |
| | *n* | 10 | 4 | 6 | 12 |
| Quinpirole | $E_{max}$ (% inhibition) | 53±6 | 75±1 | 79±4 | 78±3 |
| | $pEC_{50}$ | 7.7±0.2 | 8.3±0.1 | 8.4±0.3 | 9.1±0.2 |
| | relative efficacy | 99±11 | 97±1 | 97±1 | 98±2 |
| | *n* | 3 | 4 | 3 | 4 |
| 7-OH-DPAT | $E_{max}$ (% inhibition) | 49±1 | | | 72±3 |
| | $pEC_{50}$ | 7.0±0.1 | | | 7.8±0.2 |
| | relative efficacy | 94±2 | | | 103±5 |
| | *n* | 3 | | | 3 |
| U-101958 | $E_{max}$ (% inhibition) | 23±4 | 29±5 | 53±6 | 49±5 |
| | $pEC_{50}$ | 8.1±0.3 | 9.3±0.4 | 8.8±0.2 | 8.8±0.1 |
| | relative efficacy | 39±7 | 37±7 | 64±6 | 64±5 |
| | *n* | 4 | 4 | 6 | 6 |
| L-745,870 | $E_{max}$ (% inhibition) | 0 | 26±5 | 42±4 | 39±3 |
| | $pEC_{50}$ | | NC | 8.8±0.3 | 8.6±0.2 |
| | relative efficacy | 0 | 34±6 | 48±5 | 49±3 |
| | *n* | 3 | 4 | 4 | 6 |

Relative efficacy values are $E_{max}$ values of the agonists expressed as percentage of the $E_{max}$ value of dopamine in each individual experiment. NC, not calculable.

### *Measurement of cyclic AMP accumulation*

Cells grown to confluence in 24-well plates were washed with 1 ml of HEPES-buffered salt solution (in mM): NaCl 130, KCl 5.4, $CaCl_2$ 1.8, $MgSO_4$ 0.8, $NaH_2PO_4$ 0.9, glucose 25, HEPES 20, pH 7.4, containing phenol red 5 mg $l^{-1}$, and incubated with 6 $\mu$Ci $ml^{-1}$ of [8-$^3$H]-adenine (23 Ci $mmol^{-1}$, Anawa Trading SA, Wangen, Switzerland) at 37°C for 2 h in 0.5 ml of the same buffer. They were then washed twice with 1 ml of the buffer solution supplemented with 1 mM isobutylmethylxanthine. The cells were incubated in 1 ml of the same solution at 37°C, in the presence and absence of forskolin (10 $\mu$M) and of test compounds at the indicated concentrations. Experiments were conducted in duplicate. After 15 min, the medium was removed and replaced by 1 ml of 5% trichloroacetic acid solution containing cyclic AMP and ATP (both 0.1 mM). After 30 min at 4°C, the trichloroacetic acid extracts were directly subjected to sequential chromatography on Dowex AG 50W-X4 and alumina columns (Salomon, 1991). Cyclic AMP accumulation was calculated as the ratio [$^3$H]-cyclic AMP/([$^3$H]-cyclic AMP + [$^3$H]-ATP). The recovery of cyclic AMP was 76%.

### *Analysis of data*

Cyclic AMP data were expressed as percentage of forskolin-stimulated cyclic AMP accumulation. Radioligand binding saturation and inhibition curves, as well as concentration-response curves were fitted to the non linear logistic function of the Microcal Origin software package. Values of $B_{max}$, $K_D$, $K_i$, $E_{max}$ (maximal effect) and $EC_{50}$ (concentration producing half the maximal effect) were derived from this fit. The apparent $pK_B$ values of antagonists were calculated according to the formula: $pK_B = \log[B] - \log(CR-1)$ where [B] is the concentration of the antagonist used and CR (concentration-ratio) is the ratio of agonist $EC_{50}$ measured in the presence of antagonist over that measured in the absence of antagonist. Results are given as mean ± s.e.mean of the indicated $n$ values.

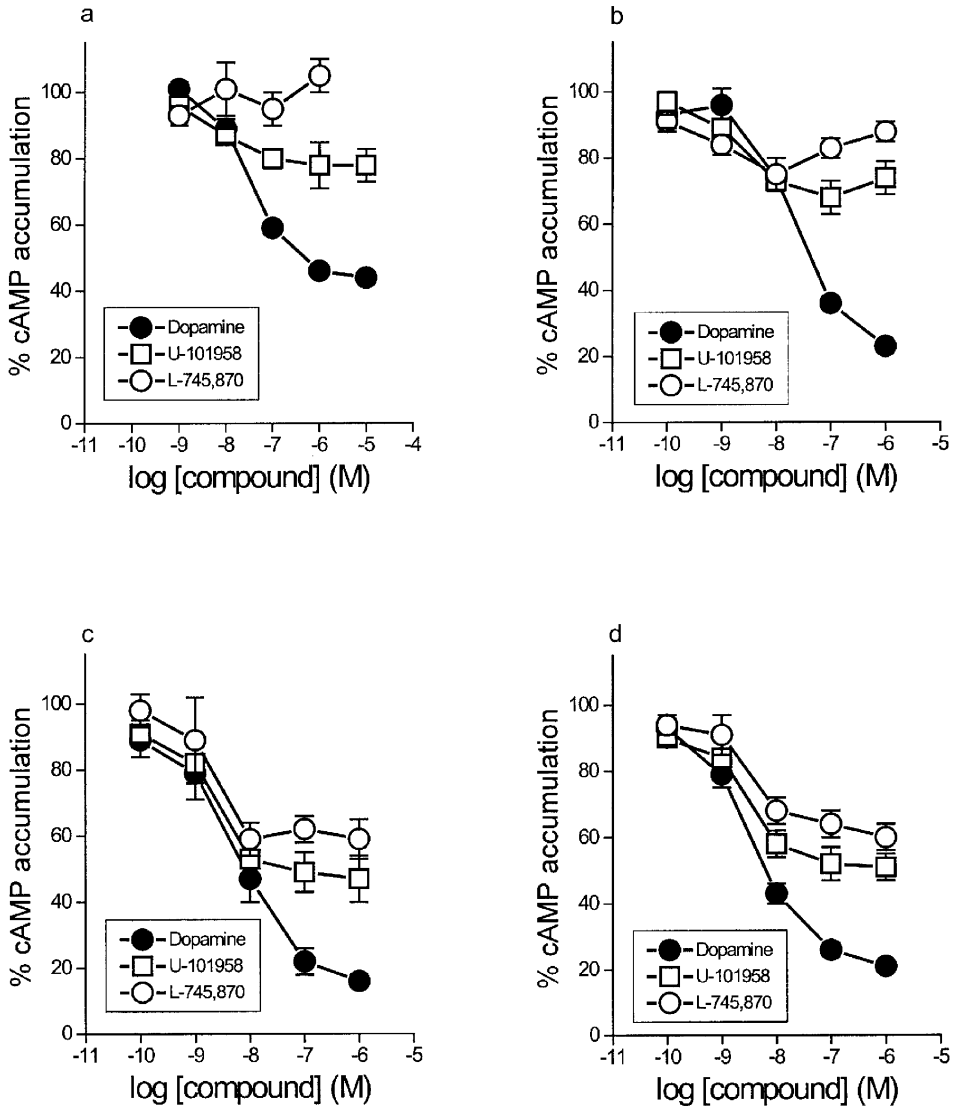


**Figure 1** Concentration-response curves of dopamine, U-101958 and L-745,870 for inhibition of forskolin-stimulated cyclic AMP accumulation in (a) untreated CHO/$D_4$ cells and CHO/$D_4$ cells treated with 5 mM sodium butyrate for (b) 6 h, (c) 18 h and (d) 48 h. Data are means and vertical lines show s.e.mean from the number ($n$) of experiments indicated in Table 1.

*Drugs and biochemicals*

The substances were obtained from the following sources: forskolin, isobutylmethylxanthine, dopamine and sodium butyrate (n-butyric acid sodium salt, Sigma, Fluka, Buchs, Switzerland); quinpirole hydrochloride (Research Biochemicals International, Rahn, Zürich, Switzerland); 7-hydroxy-2-dipropylaminotetralin hydrobromide and L-745,870 (3-[[4-(4-chlorophenyl)piperazin - 1 - yl] - methyl] - 1*H*-pyrrolo[2,3-*b*]pyridine trihydrochloride) (Tocris, Bristol, U.K.); raclopride tartrate (a gift from Astra, Sweden). U-101958 ((1-benzyl-piperidin-4-yl)-(3-isopropoxy-pyridin-2-yl)-methyl-amine; Dr R. Swoboda), spiperone and clozapine were from Novartis Pharma AG, Basel, Switzerland. The subtances were prepared daily at 40 mM, either in distilled water or in a mixture of 1-methyl-2-pyrrolidone:ethanol (1 : 1) containing

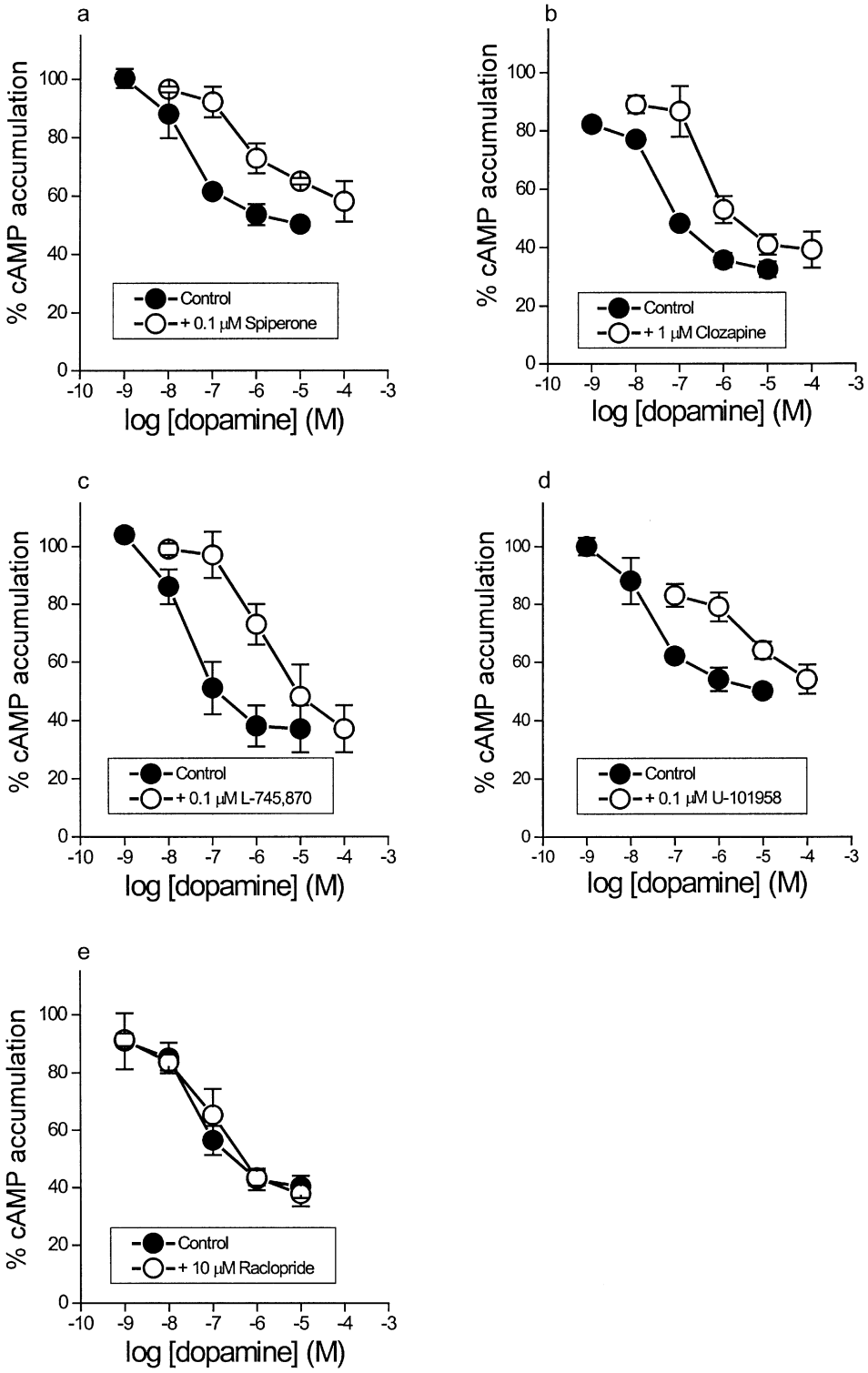


**Figure 2** Concentration-response curves of dopamine for inhibition of forskolin-stimulated cyclic AMP accumulation in CHO/$D_4$ cells, in the absence and in the presence of (a) spiperone (0.1 $\mu$M), (b) clozapine (1 $\mu$M), (c) L-745,870 (0.1 $\mu$M), (d) U-101958 (0.1 $\mu$M) and (e) raclopride (10 $\mu$M). Data are means and vertical lines show s.e.mean from three experiments.

20 mg $ml^{-1}$ ascorbic acid, and further diluted with water. Exceptions were forskolin (10 mM stock solution in ethanol) and sodium butyrate (directly dissolved at 5 mM in culture medium).

**Table 2** Antagonist parameters in $CHO/D_4$ cells

| *Antagonist* | $pK_B$ *(cyclic AMP)* | n | $pK_i$ *(binding)* | n |
|---|---|---|---|---|
| L-745,870 | 8.6±0.2 | 3 | 7.9±0.2 | 3 |
| U-101958 | 8.9±0.8 | 3 | 8.9±0.2 | 3 |
| Spiperone | 8.2±0.3 | 3 | 9.9±0.2 | 3 |
| Clozapine | 7.8±0.8 | 3 | 7.2±0.2 | 4 |
| Raclopride | <5 | 3 | 5.3±0.1 | 3 |

Values of $pK_B$ were derived from the experiments illustrated in Figure 2. Raclopride was ineffective at 10 μM in the cyclic AMP functional assay.

## Results

### *Characterization of dopamine $D_4$ receptor-mediated responses in $CHO/D_4$ cells*

Forskolin (10 μM) induced an average 15 fold stimulation of cyclic AMP accumulation in $CHO/D_4$ cells. This effect was inhibited in a concentration-dependent fashion by dopamine ($E_{max}$ 56±1% inhibition of forskolin-stimulated levels, $pEC_{50}$ 7.4±0.1, $n=10$; Figure 1a) and mimicked by the dopamine $D_2$-like receptor agonists, quinpirole and 7-hydroxy-2-dipropylaminotetralin (7-OH-DPAT; see Table 1). U-101958 also inhibited forskolin-stimulated cyclic AMP accumulation in a concentration-dependent manner ($E_{max}$ 23±4% inhibition of forskolin-stimulated levels, $pEC_{50}$ 8.1±0.3, $n=4$; Figure 1a). The $E_{max}$ of U-101958 represented 39% of the $E_{max}$ of dopamine (see Table 1). In contrast, L-745,870 did not inhibit forskolin-stimulated cyclic AMP accumulation (Figure 1a). At a concentration of 10 μM, there was even a trend for L-745,870 to enhance forskolin effects (not shown). Spiperone, clozapine

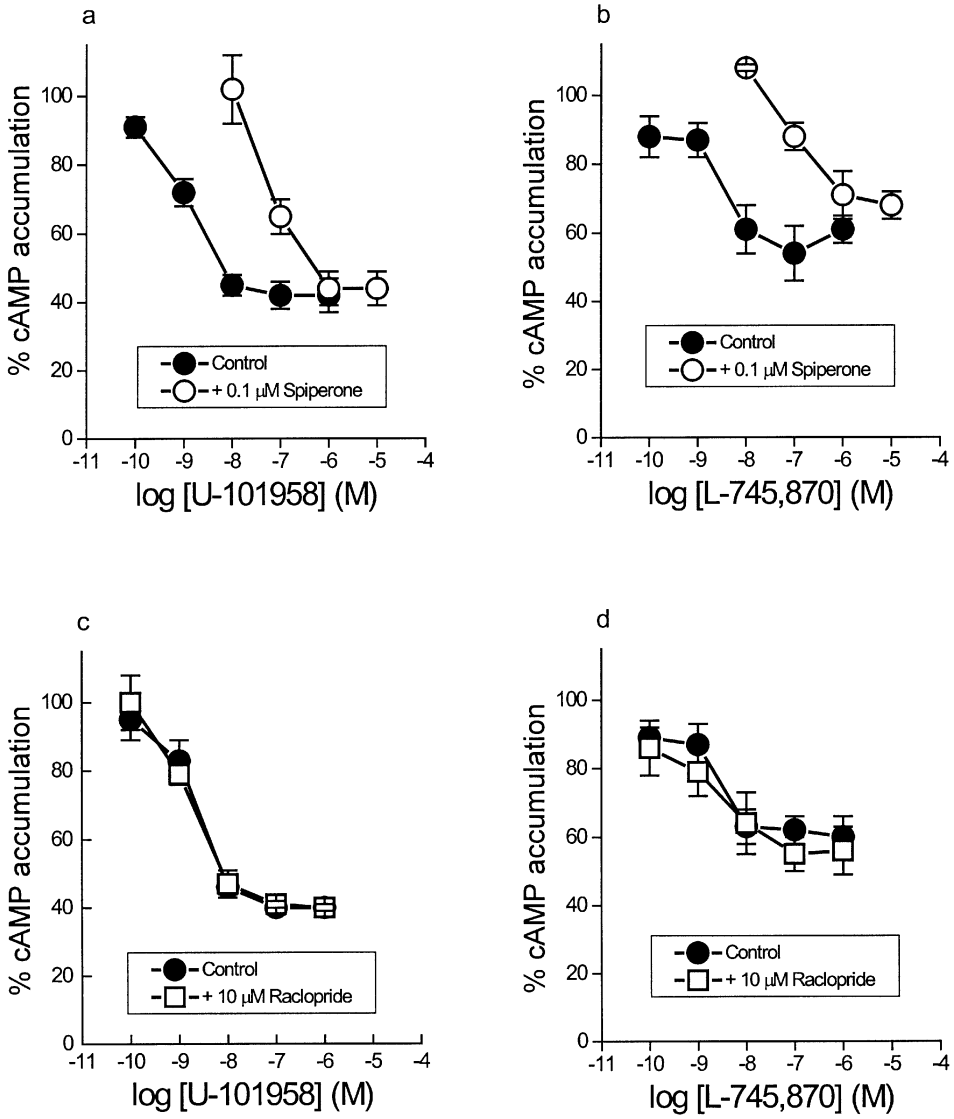


**Figure 3** Concentration-response curves of (a,c) U-101958 and (b,d) L-745,870 for inhibition of forskolin-stimulated cyclic AMP accumulation in the absence and in the presence of (a,b) spiperone (0.1 μM) and (c,d) raclopride (10 μM) in $CHO/D_4$ cells treated with 5 mM sodium butyrate for 48 h. Data are means and vertical lines show s.e.mean from three or four experiments.

and raclopride were silent in these cells up to 10 $\mu$M ($n \geqslant 3$ each).

Dopamine, quinpirole, 7-OH-DPAT, U-101958 and L-745,870 (1 nM – 10 $\mu$M) did not inhibit forskolin-stimulated cyclic AMP accumulation in control, non-transfected CHO-K1 cells ($n \geqslant 3$ each).

As illustrated in Figure 2, the concentration-response curve of dopamine was shifted to the right in a parallel manner in the presence of spiperone (0.1 $\mu$M), clozapine (1 $\mu$M), L-745,870 (0.1 $\mu$M) and the partial agonist U-101958 (0.1 $\mu$M) but not in the presence of raclopride (10 $\mu$M). Derived $pK_B$ values are to be found in Table 2.

### *[$^3$H]-spiperone binding*

In saturation binding experiments, [$^3$H]-spiperone was found to label a homogeneous and saturable population of specific binding sites in CHO/$D_4$ cell membrane homogenates. The $B_{max}$ values amounted to $240 \pm 30$ fmol mg$^{-1}$ protein and the $pK_D$ value was $9.1 \pm 0.1$ ($n = 8$). The specific binding of [$^3$H]-spiperone was inhibited in a monophasic manner by L-745,870, U-101958, spiperone and clozapine with high affinity, but much less so by raclopride (see Table 2). After treatment with sodium butyrate (5 mM) for 6, 18 and 48 h, the $B_{max}$ value reached $560 \pm 150$, $1000 \pm 190$ and $840 \pm 120$ fml mg$^{-1}$ protein, respectively, without major changes in the $pK_D$ value (see Table 1).

### *Influence of sodium butyrate treatment on the efficacy and potency of $D_4$ receptor ligands*

Figure 1b, c and d shows the effects of dopamine, U-101958 and L-745,870 on CHO/$D_4$ cells treated with 5 mM sodium butyrate for 6, 18 and 48 h, respectively. These results are summarized in Table 1, along with data on quinpirole and 7-OH-DPAT. There were no noticeable changes in basal and forskolin-stimulated cyclic AMP levels after sodium butyrate treatment. A 6 h treatment with sodium butyrate resulted in an increased $E_{max}$ of dopamine (from $56 \pm 1\%$ to $78 \pm 1\%$ inhibition of forskolin-stimulated cyclic AMP levels), with a minimal increase in the $pEC_{50}$ value (from $7.4 \pm 0.1$ to $7.7 \pm 0.1$). Prolonged treatment up to 48 h did not increase the $E_{max}$ value further, whereas the $pEC_{50}$ value increased to $8.4 \pm 0.2$ and $8.6 \pm 0.1$ after 18 and 48 h treatment, respectively (Table 1). The same trend was observed with quinpirole and 7-OH-DPAT (Table 1). Under the same conditions, the $E_{max}$ of U-101958 approximately doubled (from $23 \pm 4\%$ in untreated cells to $53 \pm 6\%$ and $49 \pm 5\%$ after 18 and 48 h treatment), along with a slight increase in the $pEC_{50}$ value (from $8.1 \pm 0.3$ to $8.8 \pm 0.1$). The relative efficacy of U-101958 (compared to dopamine) thus changed from 39% in untreated cells to 64% in sodium butyrate-treated cells. In addition, treatment with sodium butyrate revealed an agonist effect of L-745,870. After 6 h treatment, this effect reached $26 \pm 5\%$ inhibition, but the concentration-response curve of L-745,870 tended to be biphasic, preventing any accurate determination of the $EC_{50}$ value (Figure 1b). In cells treated for 18 and 48 h with sodium butyrate, L-745,870 induced concentration-dependent inhibitions of forskolin-stimulated cyclic AMP accumulation, with $E_{max}$ values of $42 \pm 4\%$ and $39 \pm 3\%$ (48 and 49% efficacy relative to dopamine) and $pEC_{50}$ values of $8.8 \pm 0.3$ and $8.6 \pm 0.2$. No such effects were observed with spiperone and clozapine after 48 h treatment with sodium butyrate ($n = 4$ each, not shown).

The agonist effects of U-101958 and L-745,870 in cells treated with sodium butyrate for 48 h could be antagonized by spiperone (0.1 $\mu$M, Figure 3a, b), but not by raclopride (10 $\mu$M; Figure 3c, d). Spiperone $pK_B$ values were $8.8 \pm 0.1$ and $9.1 \pm 0.1$ ($n = 3$ each) against U-101958 and L-745,870, respectively.

Dopamine, quinpirole, U-101958 and L-745,870 did not inhibit forskolin-stimulated cyclic AMP accumulation in non-transfected CHO-K1 cells treated with sodium butyrate for 6, 18 or 48 h (not shown).

## Discussion

U-101958 and L-745,870 have originally been introduced as dopamine $D_4$ receptor antagonists (Patel *et al*., 1997; Schlachter *et al*., 1997). However, we recently found them to be agonists at human recombinant dopamine $D_{4.4}$ receptors expressed in HEK293 cells (Gazi *et al*., 1998). In the latter cells, U-101958 acted as a quasi-full agonist, compared to dopamine, and L-745,870 had a substantial efficacy (71% relative to dopamine). Since most of the previous *in vitro* information on these two compounds had been obtained in CHO cells, we transfected this type of cells with the same cDNA as was used for HEK293 cells (Gazi *et al*., 1998). Inhibition of forskolin-stimulated cyclic AMP accumulation was used as a functional readout. The resulting pharmacological profile was confirmed as being dopaminergic $D_4$ in nature, since: (1) dopamine, quinpirole and 7-OH-DPAT induced concentration-dependent inhibitions of forskolin-stimulated cyclic AMP accumulation in CHO/$D_4$ cells but not in non-transfected CHO-K1 cells; and (2) the effect of dopamine could be potently antagonized by spiperone and clozapine, but not by raclopride. In addition, radioligand binding studies confirmed the presence of sites with the same profile. Nevertheless, the present data notably differ from our previous results obtained with HEK293 cells in that, in CHO/$D_4$ cells U-101958 behaved as a partial agonist whereas L-745,870 was a pure antagonist. It is also noteworthy that dopamine and the full agonists, quinpirole and 7-OH-DPAT were less potent in CHO/$D_4$ cells than in human embryonic kidney 293 cells by more than one order of magnitude (see Gazi *et al*., 1998). As a whole, these findings hint toward a higher receptor density and/or a more efficient receptor-effector coupling (for some reason) in the latter cells than in CHO/$D_4$ cells. It is well known that functional responses mediated by recombinant receptors may be affected by such factors (see Kenakin, 1997).

In the present study, we addressed the possible influence of receptor density on functional responses in CHO/$D_4$ cells by treating the cells with sodium butyrate. Sodium butyrate is an enhancer of gene expression, acting by inducing mammalian promoters like the cytomegalovirus promoter (Cockett *et al*., 1990) which controlled the expression of human dopamine $D_{4.4}$ receptors in our cells (see Gazi *et al*., 1998). For instance, this agent has been successfully used for increasing the expression of 5-$HT_{1B}$ and 5-$HT_{1D}$ receptors in C6 glioma cells and HEK293 cells (Lesage *et al*., 1998). In CHO/$D_4$ cells, the $B_{max}$ value of dopamine $D_4$ receptors, as estimated by [$^3$H]-spiperone saturation binding, was 240 fmol mg$^{-1}$ protein. This value was multiplied by a factor 2 after 6 h and by a factor 4 after 18 h of treatment with sodium butyrate. This level of expression was not increased further after 48 h of treatment.

These changes in receptor density were accompanied by an enhancement of the agonist activities of dopamine, quinpirole and 7-OH-DPAT (both in terms of maximal inhibition of forskolin-stimulated cyclic AMP accumulation

and of $pEC_{50}$ values). More specifically, after 6 h treatment with sodium butyrate (when receptor density had doubled), the $E_{max}$ of dopamine increased in a substantial manner (from $56\pm1\%$ to $78\pm1\%$ inhibition of forskolin-stimulated cyclic AMP levels). The $pEC_{50}$ value was slightly changed from $7.4\pm0.1$ to $7.7\pm0.1$ under these conditions. A much more dramatic increase in the potency of dopamine was observed after 18 h of treatment with butyrate ($pEC_{50}$ $8.4\pm0.2$), i.e. when receptor density had been four times higher than in untreated cells. By contrast, the $E_{max}$ of dopamine was not altered in a significant manner between 6 and 18 h of treatment ($83\pm2\%$ versus $78\pm1\%$). Such data are consistent with classical receptor theory which predicts that increasing the receptor number will first result in an increase of the maximal responsiveness to agonists, until a ceiling is reached (i.e., the maximal achievable cell or tissue responses). Further increases in receptor number should then lead to higher agonist potencies, with no more changes in the maximal effect (Kenakin, 1997). Classical receptor theory also predicts that variations in receptor number will have a larger impact on the intrinsic activity of partial agonists than for full agonists. Examples that this is really the case in practice have been reported (e.g., Hermans *et al*., 1999). In the present study, marked increases in the efficacies of U-101958 and L-745,870 were found after 18 h of treatment with sodium butyrate (i.e., concomittantly with receptor density being four times higher than in untreated cells). The $E_{max}$ of U-101958 more than doubled (from $23\pm4\%$ to $53\pm6\%$ inhibition of forskolin-stimulated cyclic AMP accumulation) and L-745,870 was turned into an agonist under these conditions. In antagonist studies using spiperone and raclopride, the effects of both U-101958 and L-745,870 were characterized as being mediated by dopamine $D_4$ receptors. Thus, both compounds were partial agonists at dopamine $D_{4.4}$ receptors expressed in CHO cells. In particular, for L-745,870, a relatively high threshold level of receptor number appears to be required for significant agonism to be manifest. This level must lie between 560 and 1000 fmol $mg^{-1}$ protein, a range in which the agonist effects of L-745,870 became apparent in CHO/$D_4$ cells. This may provide an explanation as to why Patel *et al*. (1997) did not observe agonist effects of L-745,870. Their CHO cells expressed 710 fmol $mg^{-1}$ protein, which might have been insufficient to see clearcut agonist effects. As far as U-101958 is concerned, there was no mention of receptor number in the study by Schlachter *et al*. (1997), who reported it to be a dopamine $D_4$ receptor antagonist, using a mitogenesis assay in CHO cells.

The present findings show that increases in the efficacy of the dopamine $D_4$ receptor ligands, U-101958 and L-745,870 are associated with increases in receptor density in CHO cells treated with sodium butyrate for various periods of time. The parallelism between these changes as well as the fact that similar findings have been described for other receptors (Esbenshade *et al*., 1995; Hermans *et al*., 1999) strongly suggest that receptor density is a factor governing the efficacy of dopamine $D_4$ receptor agonists. Nevertheless, other effects of the sodium butyrate treatment cannot be totally excluded. Because sodium butyrate has been incubated for varying periods of time in the present study, it might have had a different impact on cell proliferation and functioning in these various situations. However, we did not observe any change in the morphology of the cells during the treatment with sodium butyrate. In addition, in separate, strictly time-controlled experiments, the yield of protein was not altered by sodium butyrate treatment. Another possible consequence of this treatment, likely to impinge on the efficiency of stimulus-response coupling, might have been an alteration of the density of the G-proteins involved in the transduction of dopamine $D_4$ receptor-mediated effects. With respect to this possibility, we measured the number of dopamine-activated G-proteins in CHO cells treated or not with sodium butyrate for 18 h, using [$^{35}$S]-GTP$\gamma$S isotopic dilution saturation binding (according to Newman-Tancredi *et al*., 1997). The number of G-proteins activated per receptor tended to be reduced after the treatment with sodium butyrate (3 versus 5 in untreated cells), making it unlikely that changes at the level of G-proteins could account for the observed increases in efficacy.

Even though receptor density is a determinant of the efficacy of dopamine $D_4$ receptor ligands, it is probably not the sole one. Comparing the present data in CHO cells with those obtained previously in HEK293 cells (Gazi *et al*., 1998) also indicates an influence of the expression system. At similar receptor densities, 560 fmol $mg^{-1}$ protein in CHO cells treated with sodium butyrate for 6 h (this study) and 505 fmol $mg^{-1}$ protein in HEK293 cells (Gazi *et al*., 1998), dopamine was more potent by one order of magnitude in the latter cells ($pEC_{50}$ 8.7 versus 7.7). In addition, the efficacies of U-101958 and L-745,870 were much more marked in HEK293 cells (93 and 71% relative to dopamine; Gazi *et al*., 1998). Even under conditions of high receptor densities (1000 and 840 fmol $mg^{-1}$ protein after 18 and 48 h treatment with sodium butyrate), and even though the $pEC_{50}$ value of dopamine approached that in human embryonic kidney cells, U-101958 and L-745,870 remained less efficacious in CHO cells (64 and 49% relative to dopamine). Such differences suggest a more efficient receptor-effector coupling in HEK293 than in CHO cells. Tentative explanations are different repertoires of G protein subunits, of adenylate cyclase subtypes and the receptor/G protein/effector stoichiometry. For instance, a greater number of G proteins may be activated per receptor unit in HEK293 than in CHO cells.

As a whole, the present data confirm our previous view that U-101958 and L-745,870 are not silent antagonists but can behave as agonists at dopamine $D_4$ receptors. In the absence of any recognized model of native dopamine $D_4$ receptor activation, the relevance of these findings to the *in vivo* situation is difficult to ascertain. It is worth noting, nevertheless, that U-101958 exhibited dopaminergic agonist activity at inhibiting potassium currents in rat posterior pituitary (Wilke *et al*., 1998) and at inducing phospholipid methylation in human neuroblastoma SK-N-MC cells (Sharma *et al*., 1999). L-745,870 was found to behave as a partial agonist at dopamine $D_2$-like receptors mediating inhibition of serotonin N-acetyltransferase in chick retina (Zawilska & Nowak, 1997). That putative dopamine $D_4$ receptor antagonists may have agonist properties under some circumstances may have clinical implications. These compounds have been conceived as potential antipsychotics, in an effort to mimic the blocking effect of clozapine at dopamine $D_4$ receptors. Whereas U-101958 has not been developed further because of poor metabolic stability and bioavailability (Schlachter *et al*., 1997), clinical studies with L-745,870 have been completed (Kramer *et al*., 1997). The compound was found to be ineffective in schizophrenic patients and intriguingly, some signs of aggravation of the illness were noticed. If the density of dopamine $D_4$ binding sites is really increased by several fold in the brain of schizophrenics as has been reported (Seeman *et al*., 1993, 1995; Murray *et al*., 1995; Sumiyoshi *et al*., 1995; Marzella *et al*., 1997), L-745,870 might have acted as an agonist in these patients and this might possibly provide an explanation to the negative outcome of these trials.

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