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Stable expression of human D_3 dopamine receptors in GH_4C_1 pituitary cells

G.R. Seabrook, S. Patel, R. Marwood, F. Emms, M.R. Knowles, S.B. Freedman and G. McAllister

Merck Sharp & Dohme Research Laboratories, Neuroscience Research Centre, Terlings Park, Harlow, Essex, CM20 2QR, UK

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Human D_3 dopamine receptor DNA was stably transfected into GH_4C_1 pituitary cells. Displacement of iodosulpiride binding in hD3 transfected cells (K_d = 0.3 nM, B_{max} = 89 fmol/mg protein) by dopaminergic ligands was indistinguishable from that of hD3 receptors in CHO cells. Only two clonal cell lines exhibited weak GppNHp-dependent shifts in [^{3}H]N-0437 binding, and these were used for functional assays. Neither arachidonic acid metabolism, cAMP levels, inositol phosphate turnover, intracellular calcium, or potassium currents were consistently affected by dopamine (1–10 μM). The paucity of responses indicates that human D_3 receptors do not couple efficiently to these second messengers in GH_4C_1 cells.

D_3 dopamine receptor; Arachidonic acid; Potassium channel; cAMP; Schizophrenia; GH_4C_1 cell

1. INTRODUCTION

To date five structural classes of human dopamine receptors, termed D_1–D_5, have been described [1–6]. Hydrophobicity analysis indicates that all of them have 7 putative transmembrane spanning regions, and are thus homologous to previously described G-protein-linked receptors. D_3 dopamine receptors have been implicated as a target for neuroleptic drugs [7,8]. This assertion is based upon the affinity of atypical neuroleptics, including clozapine, and drugs such as (+)-AJ76 and (+)-UH232, which have been suggested to act preferentially on dopamine 'autoreceptors' [9]. The localization of rat D_3 mRNA and binding sites for the D_3 selective ligand [^{3}H]quinpirole to limbic brain structures [10,11], which have been implicated in the etiology of schizophrenia, lend further support to this view.

Dopamine, acting on D_2-like receptors, has robust inhibitory effects on neurones of the basal ganglia, including those in the ventral tegmental area and substantia nigra [12]. Although these brain regions also contain D_3 mRNA [10], no description of a physiological response which can be unambiguously attributed to D_3 receptor activation has yet been described. This is partly because many brain regions may also contain D_2 and/or D_4 receptors, but also because few dopaminergic ligands such as quinpirole have an apparent selective affinity for D_3 receptors. G-protein-linked receptors can have both low and high affinity for agonists, depending upon their G-protein coupling state. Consequently, the selectivity of ligands such as quinpirole for D_3 vs. D_2 receptors in CHO cells, in which only D_2 receptors are coupled ([7], Freedman et al. unpublished observations), may not necessarily reflect intrinsic differences in the agonist recognition sites between these receptors.

Cloned rat D_2 receptors can couple to several second messenger systems, including the inhibition of cAMP production, stimulation of arachidonic acid metabolism, and calcium mobilisation [13]. However, some of these effects are dependent upon the expression system used, presumably due to the selective expression of G-proteins in different cell lines [14]. Activation of cloned D_2 receptors may also have direct effects on ion channels, including the activation of potassium channels as in GH_4C_1 cells [15].

Although human D_3 receptors have high homology to D_2 and D_4 receptors in the predicted transmembrane domains, these receptors have only 33 and 24% identity, respectively, in the third intracellular domain. This region of G-protein-linked receptors is thought to be involved in coupling to effector mechanisms [16]. At least 20 types of G-protein alpha; subunits, belonging to 4 different structural classes, have been described [17]. Of these, GH_4C_1 cells are known to contain at least 8, some of which, including G_{i1} and G_o, are not typically found in CHO cells ([18], G. Milligan, pers. commun.). The lack of functional coupling of D_3 receptors in CHO cells may thus be due to the absence of appropriate G-proteins to which the receptor may couple. The aim of this study was to stably transfect human D_3 receptors into GH_4C_1 cells, determine the affinities of dopaminergic ligands for these receptors, and study receptor function.

Correspondence address: G.R. Seabrook, Merck Sharp & Dohme Research Laboratories, Neuroscience Research Centre, Terlings Park, Harlow, Essex, CM20 2QR, UK. Fax: (44) (279) 440390.

Abbreviations: cAMP adenosine 3′:5′-cyclic monophosphate, GTP guanosine triphosphate; PI, phosphatidylinositol; GppNHp, 5′-guanylylimidodiphosphate.

2. MATERIALS AND METHODS

2.1. *Transfection procedure and binding assays*

Human D_3 cDNA was subcloned into the mammalian expression vector, pcDNA1neo (Invitrogen). Stably transfected cell lines were obtained by transfecting this construct into GH_4C_1 cells by a standard calcium phosphate method [19]. Transfected cells were selected for their resistance to the antibiotic G418 and assayed for their ability to bind [^{125}I]iodosulpiride. GTP shifts were measured by examining the ability of GppNHp (100 μM) to reduce binding of the dopamine receptor agonist [^{3}H]N-0437 (0.1 nM).

2.2. *Arachidonic acid, cAMP, and phosphatidyl inositol (PI) hydrolysis*

The release of [^{3}H]arachidonic acid was measured according to the methods of Kanterman et al. [20]. Briefly, GH_4C_1 cells were incubated with [^{3}H] arachidonic acid (0.2 μCi/well) to isotopic equilibrium (18–24 h). Cells were washed twice with 25 mM HEPES DMEM supplemented with 0.5% fatty acid-free bovine serum albumin solution. Experimental agents were applied in a final volume of 1 ml and the reaction was allowed to proceed for 30 min at 37°C. The reaction was stopped by removal of 750 μl of the incubation medium, which was then centrifuged at 12,000 × g for 3 min to remove non-adherent cells. 500 μl of supernatant was removed, and released [^{3}H]arachidonic acid was measured using liquid scintillation spectrophotometry.

[^{3}H]cAMP levels were measured in cells grown to confluence using a radioimmunoassay (TRK 432 Amersham). The PI turnover assay was performed in 24-well culture plates allowed to grow to just below confluence. Cells were labelled overnight with 5 μC/ml of [^{3}H]myoinositol. The next day cells were washed twice with 2 ml of HEPES-buffered Krebs' solution, pH 7.4. The plates were subsequently incubated for 15 min at 37°C. 10 μl of LiCl (final concentration 10 mM) was added to each well and 20 min later test compounds were added in a volume of 10 μl. Assays were terminated after 30 min by aspiration of the incubation media followed by addition of 1 ml 0.1 M HCl. 15 min later 750 μl of the extract was placed into tubes and extracted with chloroform/methanol (1:1). The mixture was subsequently centrifuged at 5°C for 10 min at 2,500 rpm. 750 μl of the aqueous layer was loaded onto a 1-ml Dowex column (AG 1-X8). Columns were washed with 10 ml of 0.1 M formic acid and 5 ml of 0.025 M ammonium formate/0.1 M formic acid. The inositol monophosphate was then eluted with 0.2 M ammonium formate/0.1 M formic acid into scintillation vials. Radioactivity was then determined using liquid scintillation spectrophotometry. CHO cells expressing the human M1 muscarinic receptor were used a positive control.

2.3. *Calcium imaging*

Cells were loaded with the fluorescent indicator FURA-2 AM (2 μM; 30 min) in a saline containing (in mM): NaCl 150, KCl 3, HEPES 10, glucose 10, sucrose 20, $CaCl_2$ 2, and $MgCl_2$ 2 at pH 7.4. Essentially, methods were as previously described [21,22]. The dye fluorescence at 540 nm was measured during alternate excitations at 340 and 380 nm wavelength light (0.1–1 Hz) using an MCID imaging system (Imaging Research Inc., Canada).

2.4. *Whole-cell patch-clamp recording*

Whole-cell patch-clamp recordings were made using 2 Mohm glass microelectrodes which had been filled with (in mM); KCl 20, potassium aspartate 120, $MgCl_2$ 1, Mg-ATP 2, cyclic AMP 1, EGTA 10, HEPES 10, sodium creatine phosphate 5, creatine phosphokinase 20 U/ml, Na-GTP 1, adjusted to pH 7.4. Cells were perfused with an extracellular saline which contained (in mM); NaCl 135, glucose 10, HEPES 10, KCl 5, $MgCl_2$ 4, $CaCl_2$ 1, and tetrodotoxin 1 μM, pH 7.4 at 22°C. Cells were voltage-clamped at −80 mV, and following 1 s conditioning pulses to −100 or −120 mV, potassium currents were elicited by 100 ms voltage steps from −60 to +100 mV (0.1 Hz). In the majority of experiments the series resistance was compensated for by 90–98%, and all signals were leak subtracted. Whole-cell currents were captured on line, via a CED 1401 computer interface which was connected to an Axopatch 200 patch clamp amplifier (Axon Instruments), and were subsequently analyzed using CED voltage-clamp software (Cambridge Electronic Design). Activation and inactivation curves were fitted to Boltzman functions by least-squares analysis of variance using an iterative procedure on a VAX computer with Research System 1 software (BBN Software Products Corporation). Data are expressed as the mean ± S.E.M.

Table I

Comparative affinities of various dopamine receptor ligands for the human D3 receptor expressed in GH_4C_1 cells vs. that of the D3 and D2 receptor expressed in CHO cells (Freedman et al. unpublished observations)

Ligand	hD3-GH_4C_1	hD3-CHO	hD2-CHO
Apomorphine	28 (22,34)	14	29
Clozapine	55 (25,120)	74	33
Dopamine	49 (26,91)	25	700
Haloperidol	2.4 (1.4,4.0)	1.4	2.3
(−)-Sulpiride	5.5 (2.5,12.0)	8	5
Quinpirole	9.1 (7.0,12)	16	580

Data were fitted to a single-site model, and are expressed as K_i values (nM) ± 95% confidence limits from 4–7 separate determinations.

3. RESULTS AND DISCUSSION

3.1. *D_3 receptor expression, ligand binding, and GTP shifts*

The affinity of [^{125}I]iodosulpiride was determined to be 0.34 nM (0.28–0.44), with a B_{max} of 89 fmol/mg protein (n = 3); minimal specific binding was detected in the control cell line. Guanine nucleotide sensitivity of agonist binding was tested using [^{3}H]N-0437 binding. Only two clonal cell lines exhibited weak GppNHp-dependent shifts in agonist binding (ca. 10%). One of these clones, clone 18, was used for the functional assays. The affinities of dopaminergic ligands for the receptors expressed in these GH_4C_1 cells was indistin-

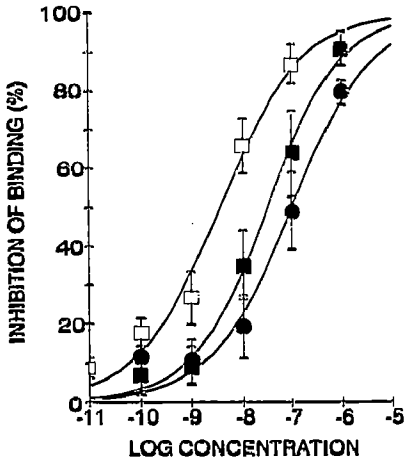

Fig. 1. Displacement of 0.2 nM [^{125}I]iodosulpiride binding by haloperidol (□), quinpirole (■) and dopamine (○) in GH_4C_1 cells transfected with human D_3 receptors. Each data point represents the mean ± S.E.M. of >4 seperate determinations.

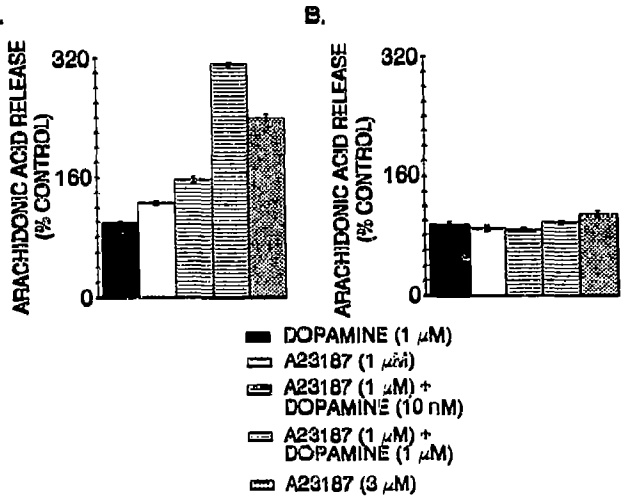

Fig. 2. Coupling of human dopamine receptors to the release of arachidonic acid. (A) Arachidonic acid release was potentiated by the activation of human D_2 receptors expressed in CHO cells following pretreatment with the calcium ionophore, A23187. (B) In contrast to the hD2 cell line, neither A23187 nor dopamine (10–1000 nM) affected the release of arachidonic acid from GH_4C_1 cells which had been transfected with the human D_3 receptor.

guishable from the hD_3-CHO expression system (Table I, Fig. 1). As with the human D_3 CHO cell line (Freedman et al. unpublished observations) clozapine did not discriminate between D_2 and D_3 receptors. Those ligands which were selective for the D_3 receptor included the highly efficacious D_2 receptor agonists, dopamine and quinpirole.

3.2. *Lack of coupling to arachidonic acid, cAMP, PI turnover, or intracellular calcium*

Forskolin (10 μM) produced an increase in cAMP levels in CHO cells transfected with human D_2(short) receptors, from 0.19 ± 0.01 to 13.9 ± 1.1 pmol per well. This was significantly inhibited by 1 and 10 μM, but not 0.01 μM, dopamine (Fig. 2a). This inhibition was reversed by pretreatment with haloperidol (1 μM, data not shown). In the hD3-GH_4C_1 cell line, forskolin also produced an increase in cAMP levels, from 0.08 ± 0.03 to 8.9 ± 0.61 pmol per well. In contrast to the hD2-CHO cell line, dopamine (0.01–10 μM) failed to reverse the forskolin stimulation (Fig. 2b). In a second series of experiments, dopamine (0.1 μM) failed to modify basal levels of cAMP in the hD3-GH_4C_1 cell line.

Dopamine has been shown to stimulate the release of arachidonic acid from rat D2-CHO cells in the presence of either ATP or the calcium ionophore, A23187 [23]. Similarly, in the present study dopamine (0.01 to 1 μM) enhanced arachidinic acid release from hD2-transfected CHO cells in the presence of either A23187 (1 μM) or ATP (1 μM, data not shown). In contrast, in the hD3-GH_4C_1 cells, dopamine (0.01–10 μM) did not enhance the release of arachidonic acid in the presence of either ATP or A23187. In a separate series of experiments, dopamine (0.01–10 μM) did not stimulate inositol monophosphate levels in the same cell line.

Intracellular calcium concentrations in hD3-GH_4C_1 cells (25 ± 1 nM, n = 10 cells) were likewise unaffected by perfusion with dopamine (10 μM; 24 ± 1 nM). Thyrotropin releasing hormone (TRH) has previously been shown to increase cytosolic calcium levels in pituitary

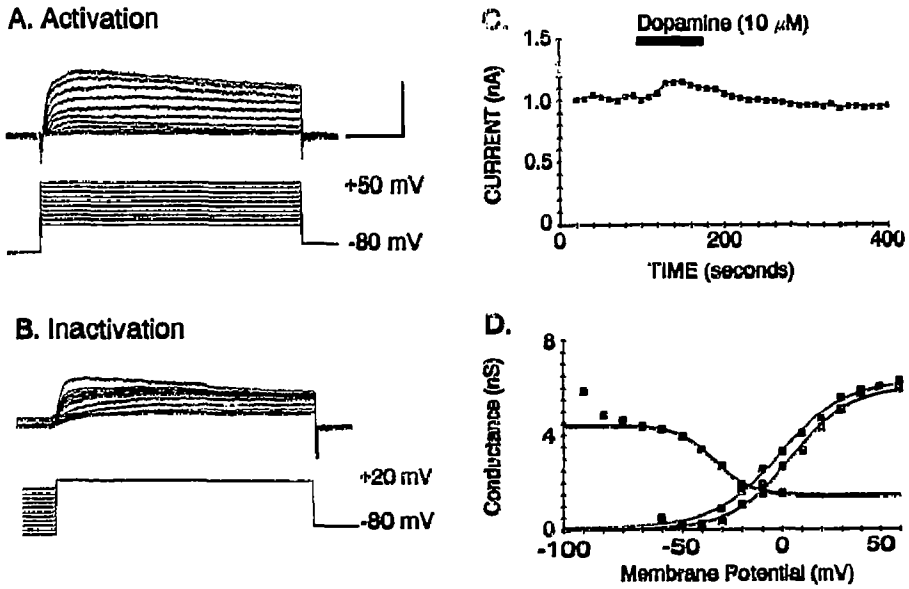

Fig. 3. Whole-cell patch-clamp recording of outward potassium currents in GH_4C_1 cells. (A) Family of currents elicited by test depolarisations of increasing magnitude. (B) Inactivation of outward currents by 1 s depolarisations prior to the test depolarisation to +20 mV. Bar = 20 ms, 1 nA. (C) In this cell dopamine caused a <10% increase in the outward current which was associated (D) with a 6 mV negative shift in the potential for half-maximal activation. The voltage-dependence of inactivation of the potassium conductance was unaffected. Stippled squares, control; filled squares, dopamine (10 μM).

cells [24] causing an increase in prolactin release. This TRH-induced prolactin release can be readily antagonized by activation of transfected D_2 receptors [13]. Following exposure of the hD3-transfected GH_4C_1 cells to TRH (1 μM for 2 min) a mean 43 ± 14% (n = 10) rise in intracellular free calcium was observed, but this was also unaffected by dopamine (10 μM).

3.3. *Characterization of voltage-dependent potassium currents and effects of dopamine*

Whole-cell patch-clamp recordings were made from 44 cells in control and transfected cell lines. GH_4C_1 cells exhibited a mean maximal potassium conductance (11 ± 1 nS), which was depressed by 44 ± 3% with 10 mM tetraethylammonium ions (n = 3). These currents were half-maximally activated at a membrane potential of +2 ± 2 mV (14 cells) and half-maximally inactivated with 1 s predepolarising pulses to −39 ± 3 mV (9 cells; Fig. 2). 36 ± 5% of the current was resistant to inactivation. This observation, coupled to the biphasic inactivation curves seen in some cells, suggests that the macroscopic current arose via activation of more than one type of voltage-dependent K^+ channel. No effects of dopamine (1–10 μM) were observed in untransfected cells. Initial experiments on clone 18 revealed that dopamine (10 μM, 2 min) caused a reversible increase in the outward current in 2 cells (Fig. 3A). In a third cell this was associated with a 6 mV hyperpolarising shift in the activation kinetics of this current (Fig. 3B). No effects of dopamine were seen in two other cells from this batch or in 25 other cells examined in subsequent experiments. Thus, unlike rat D_2 receptors [15], human D_3 receptors do not couple efficiently to voltage-dependent potassium currents in this expression system.

3.4. *Conclusions*

This study describes the stable transfection of GH_4C_1 cells with human D_3 receptors. The paucity of responses to dopamine in these transfected cell lines indicates that human D_3 receptors do not couple efficiently, or at all, to second messenger systems which are known to be modulated following activation of D_1 or D_2 dopamine receptor subtypes. Furthermore, these data suggest that either the GH_4C_1 cell line lacks appropriate G-proteins to which the receptor may couple, or that the transduction mechanism involved is different from that of previously described dopamine receptors.

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