

Characterisation of a chimeric hD₃/D₂ dopamine receptor expressed in CHO cells

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The D₂ dopamine receptor is known to be functionally coupled when expressed in CHO cells, whereas the effector systems for the D₃ dopamine receptor remain unclear. A chimeric, human D₃/D₂ receptor (hD₃/D₂) was constructed containing the third intracellular loop region of the D₂ receptor. CHO cells stably expressing the D₂, D₃, or hD₃/D₂ receptors were created and the pharmacology of the receptors was examined. The chimeric hD₃/D₂ receptor retained D₃-like affinities for dopaminergic ligands. However, in contrast to the D₂ receptor neither the D₃ receptor nor the hD₃/D₂ receptor could functionally couple to the adenylate cyclase or arachidonic acid release mechanisms

Dopamine receptor. Signal transduction; Functional coupling

1. INTRODUCTION

Dopamine has been known to be an important neurotransmitter in the central nervous system for many years. Dopamine receptors are particularly interesting because of their apparent involvement in the aetiology of Parkinson's disease and schizophrenia [1–3]. Until recently, dopamine receptors were subdivided into two classes (D₁ and D₂) based upon their pharmacological properties [4]. The advent of gene cloning techniques has so far allowed the identification of five dopamine receptors designated D₁ to D₅ [5–11]. D₁ and D₅ dopamine receptors demonstrate properties similar to the pharmacologically defined D₁ dopamine receptor and are coupled to activation of adenylate cyclase [12], whereas the D₂, D₃, and D₄ dopamine receptors are more closely related to the classical D₂ dopamine receptor [8–10]. All of these cloned dopamine receptors contain the seven putative transmembrane regions characteristic of G protein-coupled receptors [13]. When expressed in CHO (Chinese hamster ovary) cells the human and rat D₃ dopamine receptors displayed similar affinities to their D₂ counterparts for dopamine antago-

nists [9,14,15]. However, D₃ dopamine receptors demonstrated much higher affinities for some dopamine agonists (e.g. dopamine, quinpirole) than D₂ dopamine receptors. In contrast to the human and rat D₂ receptors, which can mediate an inhibitory adenylate cyclase response and arachidonic acid release, both human and rat D₃ dopamine receptors did not couple efficiently to these signal transduction mechanisms in CHO cells [9,14,15].

The putative third cytoplasmic loop of G protein-linked receptors has been implicated as being important for interacting with the G proteins required for effector coupling. In fact, a previous study examined a chimeric D₂ dopamine/m1 muscarinic receptor in which the third intracellular loop region of the hD₂ receptor had been replaced by the equivalent region of the m1 muscarinic receptor [16]. The resulting chimeric receptor mediated an increase in intracellular calcium levels in response to dopamine, an effect not found with the D₂ dopamine receptor itself. In this work, we have examined the pharmacology of a chimeric receptor (hD₃/D₂) in which the third intracellular loop region (i3) of the apparently uncoupled human D₃ dopamine receptor (hD₃) has been replaced with the analogous region of the functional human D₂ dopamine receptor (hD₂).

2. MATERIALS AND METHODS

2.1. Receptor clones

DNA and a stably transfected CHO-K1 cell line for the human D₃ dopamine receptor were obtained from Sokoloff et al. (INSERM). D₂ cDNA clones were obtained using PCR techniques [20]. Based on the published sequence of the human D_{2B} receptor [17] the following

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Abbreviations: hD₂, human D₂ dopamine receptor; hD₃, human D₃ dopamine receptor; hD₃/D₂, human chimeric D₃/D₂ dopamine receptor; GTP, guanosine triphosphate; cAMP, adenosine 3',5'-cyclic monophosphate.

oligonucleotides were synthesized: DR1, 5'-TTTTGAATTCGCCCT-GATGGATCCACTG-3'; DR2, 5'-TTTTCTCGAGAAGAAGAG-GAGGCCGATC-3' and DR3, 5'-TTTTCTCGAGTGGGAAG-CAGGCTGCTCTGT-3'. cDNA was prepared from 1 µg of human brain mRNA (Clontech Inc.) and used as a template for two sequential rounds of PCR. Both rounds of PCR used the following cycle conditions: 1 min at 95°C/1 min at 56°C/2.5 min at 72°C. The first round utilized primers DR1 and DR2 and the second used DR1 and DR3. The 1,320-bp product of this PCR amplification was restriction digested with *EcoRI* and *XhoI* and subcloned into the expression vector pcDNA1neo (Invitrogen Corp.) and its identity confirmed by DNA sequencing. Chimeric hD₃/D₂ DNA was generated in three stages using PCR techniques. Cloned hD₃ DNA in pBluescript (Stratagene) was used as template to generate PCR products containing the first 5 transmembrane regions of the hD₃ clone (Primers CD1 and CD2). CD1 generated a PCR product containing part of the multiple cloning site of pBluescript (including a *HindIII* site) and CD2 contained a *BamHI* site just downstream of a *SpeI* site. This PCR product (PCR 1) was subcloned into pBluescript after digestion with *HindIII* and *BamHI*. Next, PCR 2 containing transmembrane regions six and seven of the hD₃ clone was generated using primers CD3 and CD4. CD3 contained a *BamHI* site upstream of a *NheI* site and CD4 generated a PCR product containing part of the pBluescript multiple cloning site (including a *SpeI* site). After digestion with *BamHI* and *SpeI*, PCR 2 was subcloned into the PCR 1 containing vector digested with *BamHI* and *XhoI*. This generated a plasmid containing the seven transmembrane regions of the hD₃ receptor but missing the third intracellular loop region (i3). This construct also contained *SpeI* and *NheI* restriction sites suitable for inserting DNA in the i3 region. PCR 3 was carried out using CD5 and CD6 primers and hD₂ DNA as a template and generated a product containing the i3 region of the hD₂ receptor and *SpeI* and *NheI* at the 5' and 3' ends, respectively. PCR 3 was subcloned into the vector containing PCR 1 and 2 to generate the chimeric hD₃/D₂ receptor. The construct was sequenced to confirm its structure and was then subcloned into the *HindIII/NotI* sites of pcDNA1neo (Invitrogen) for use in subsequent transfection studies. PCR reactions were carried out for 1 min at 94°C/1 min at 55°C/2 min at 72°C for 20 cycles. The primers used were as follows: CD1, 5'-ATACGACTCACTATAGGGCGAATTG-3', CD2, 5'-ATGGATC-TGGCATAGACTAGTACAGTCA-3', CD3, 5'-ATGGATCCAC-TTCGGGAGAAGAAGGCTAGCCA-3', CD4, 5'-AACCTCAC-TAAAGGGAACAAAAGC-3', CD5, 5'-CCCTTCATTGTCACC-CTACTAGTCTA-3', CD6, 5'-CGAGCATCTGGCTAGCTTTCTT-CTCCT-3'.

2.2. Cell culture and transfection

Chinese hamster ovary (CHO) cells (ATCC CCL 61) were grown at 37°C in a humidified atmosphere of 95% air/5% CO₂ in Iscove's Modified Dulbecco's Medium (Gibco) supplemented with 10% foetal bovine serum, 1% glutamine and HT media supplement. Stably transfected cell lines were obtained by transfecting the mammalian expression vector pcDNA1neo (Invitrogen) containing the appropriate dopamine receptor cDNA into CHO-K1 cells by a standard calcium phosphate method (Cullen 1987). Transfected cells were selected for their resistance to the antibiotic G418 and assayed for their ability to bind [¹²⁵I]iodosulpiride. Of 18 individual chimera clones tested, 17 demonstrated specific radioligand binding.

2.3. Radioligand binding

2.3.1. Iodosulpiride binding

Clonal cell lines stably expressing dopamine receptors were harvested in PBS and then lysed by polytroning in 10 mM Tris-HCl pH 7.4 containing 5 mM MgSO₄ for 10 s on ice. Membranes were centrifuged at 50,000 × g for 15 min at 4°C and the resulting pellet resuspended in assay buffer (20 mM HEPES Krebs buffer pH 7.4 containing 118 mM NaCl, 4.7 mM KCl, 1.2 mM MgSO₄, 5 mM NaHCO₃, 1.2 mM KH₂PO₄, 2.5 mM CaCl₂, 10 µM pargyline and 0.1% ascorbic acid) at 20 mg/ml wet weight. Scatchard analysis and displacement

studies were carried out on chimeric clone 34. Incubations were in the presence of 0.05–1 nM [¹²⁵I]iodosulpiride or 0.2 nM for displacement studies and were initiated by the addition of 100–200 µg protein to a final assay volume of 0.1 ml. The incubation was for 30 min at 30°C and was terminated by rapid filtration over GF/B filters presoaked in 0.3% PEI and washed with 10 ml ice cold 0.9% NaCl. Specific binding was defined by 1 µM haloperidol and radioactivity determined by liquid scintillation spectrometry.

2.3.2. N-0437 binding

Cells were prepared as described above. Washed membranes were resuspended at 300 mg/ml in 20 mM HEPES buffer pH 7.4 containing 5 mM MgSO₄ and 0.1% ascorbate. Binding was determined using 1 nM [³H]N-0437 and non-specific binding determined with 1 µM haloperidol. Incubations were for times up to 60 min at 30°C and were terminated by filtration over GF/C filters presoaked in 0.3% PEI. Guanine nucleotide sensitivity was determined in the presence and absence of 100 µM GTP-γ-S (Sigma). Eight clones representing low, medium and high levels of expression were examined.

2.4. Functional coupling assays

Adenylate cyclase, arachidonic acid release and phosphatidylinositol hydrolysis measurements were carried out as described previously [18].

3. RESULTS AND DISCUSSION

3.1. Receptor expression, ligand binding and GTP shifts

Fig. 1 shows a schematic diagram of the chimeric hD₃/D₂ receptor that was constructed. The construction was carried out in three parts using PCR methodology (see section 2). Firstly, a hD₃ dopamine receptor which did not contain the third cytoplasmic loop was made. This construct was made such that it had convenient restriction sites for the insertion of the hD₂ receptor i3 region (or any other loop region with suitable restriction sites). Secondly, the hD₂ loop region was inserted and the fidelity of the construct was confirmed by DNA sequencing. The introduction of the *NheI* site at the boundary of the third cytoplasmic loop and transmembrane region VI resulted in a threonine to serine point mutation (see Fig. 1). However, this is a conservative substitution in a residue that is not conserved through all dopamine receptors (e.g. it is a methionine in D₄ receptors and a leucine in D₁) which would not be expected to change the pharmacology of the receptor. Thirdly, the chimeric construct was subcloned into the expression vector pcDNA1neo to generate the plasmid pD_{3/2}neo which was used in subsequent transfection studies. The hD₂(short) receptor cDNA was isolated using PCR techniques from human brain cDNA. Its identity was confirmed by DNA sequencing and it was subsequently cloned into pcDNA1neo and used for transfection studies. Preliminary studies demonstrated that when pD_{3/2}neo or pD₂neo was used to transiently transfect HEK 293 (human embryonic kidney) cells the resulting membranes showed considerable specific binding for the dopaminergic ligand [¹²⁵I]iodosulpiride (data not shown). We then created stable cell lines expressing the chimeric hD₃/D₂ or the hD₂ receptors by transfecting CHO-K1 cells with pD_{3/2}neo and pD₂neo, re-

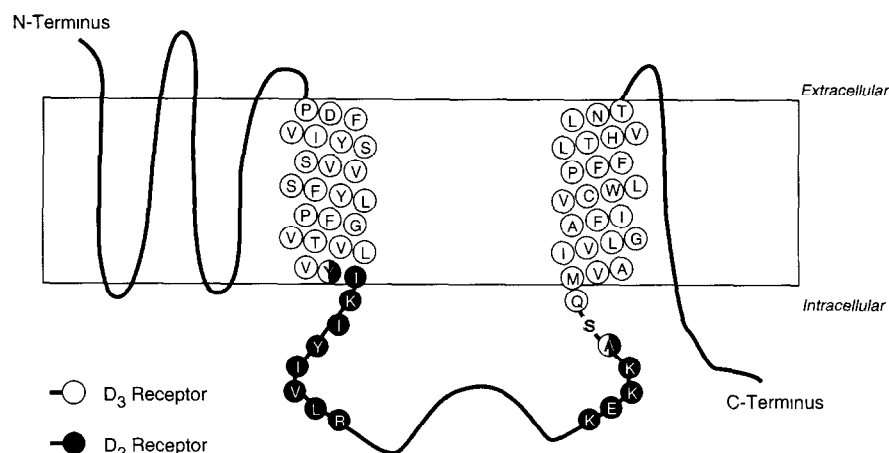


Fig. 1. A schematic diagram of the chimeric receptor. Amino acids (in the single letter code) up to and after those in black circles are derived from the hD₃ receptor. Amino acids in between are derived from the hD₂ receptor. Construction of the chimera is described in section 2. Amino acids in half black and white circles represent the junctions of the chimera and are common to the hD₂ and hD₃ receptors. The cross-hatched serine (S) residue is a point mutation introduced in the construction of the chimera to generate the convenient *Spe*I site.

spectively. Transfected cells were selected for G418 resistance. CHO-K1 cells were chosen because they do not express endogenous dopamine receptors and because the hD₂ receptor is functionally coupled in this cell line [14,15]. Of 18 individual hD₃/D₂ clonal lines examined, 17 demonstrated specific [¹²⁵I]iodosulpiride binding. Eight of these positive clones representing low, medium and high levels of expression were studied for GTP sensitivity of agonist binding (see below). A chimeric clone, hD₃/D₂(34), expressing relatively high levels of receptor was used in radioligand studies. A typical hD₂ clone hD₂CHO(11) was used in all subsequent studies. The pharmacological binding profiles of the hD₂, hD₃ and hD₃/D₂ receptors expressed in CHO cells were compared to see what effect substituting the i3 region had upon the hD₃ receptor's affinity for selected ligands. The affinity of [¹²⁵I]iodosulpiride was determined for membranes made from cells expressing the hD₂, hD₃ and hD₃/D₂ receptors, respectively (data not shown). The K_D for the hD₂ receptor was 0.26 nM (0.22, 0.33) (mean, range, $n = 3$) with a B_{max} of 700 ± 180 fmol/mg of protein, while for the hD₃ receptor the affinity was 0.42 nM (0.38, 0.47) (mean, range, $n = 3$) with a B_{max} of 290 ± 33 fmol/mg of protein. These values are in good agreement with reported affinities. The hD₃/D₂ (clone 34) chimeric receptor displayed a slightly lower affinity of 0.7 nM (0.57, 0.88) with a B_{max} of 370 fmol/mg of protein. The fidelity of the expressed receptors was further examined by testing the binding affinities of seven dopaminergic ligands in a competition binding assay with 0.2 nM [¹²⁵I]iodosulpiride. The results are shown in Table I. All three receptor types tested displayed D₂-like affinities for the D₂ antagonists (-)sulpiride and haloperidol and low affinity for the D₁ antagonist, SCH-23390 as was expected. Like the hD₃ receptor, the hD₃/D₂ receptor displays higher affinity for hD₂ agonists

such as dopamine and quinpirole compared to the hD₂ receptor, whereas other hD₂ agonists such as apomorphine and bromocryptine did not show this selectivity. We conclude from this data that the chimeric receptor has maintained the pharmacological binding profile of the hD₃ receptor and we therefore proceeded to examine the functional coupling, if any, of these receptors.

A characteristic of G protein coupled receptors is that the affinity of agonist binding for the receptors is reduced by the presence of guanine nucleotides. We examined (Fig. 2) the time course of binding of the dopamine agonist [³H]N-0437 to membranes prepared from the hD₂, hD₃, and hD₃/D₂ cell lines in the presence and absence of the GTP analogue GTP- γ -S (100 μ M). The inhibition of [³H]N-0437 binding by GTP- γ -S is consistent with the hD₂ receptor interacting with a dissociable G protein in CHO cells (Fig. 2A). In contrast, neither the hD₃ (Fig. 2B) nor the hD₃/D₂ (Fig. 2C) receptors demonstrate any inhibition of agonist binding in the presence of GTP- γ -S. Eight chimeric clones representing low, medium and high levels of expression were tested, all of which specifically bound [³H]N-0437. However, no significant inhibition of agonist binding by GTP- γ -S was observed in any of them. The results for clone hD₃/D₂(34) are depicted in Fig. 2C. Two recent reports have demonstrated a small rightward shift in dopamine competition of [¹²⁵I]iodosulpiride binding at the hD₃ receptor suggesting some level of interaction with a G protein in CHO cells [15,23]. The assay used in this study measures radiolabelled agonist binding directly and is a more sensitive indicator of receptor-G protein interactions. None of these studies demonstrated functional coupling of the hD₃ receptor.

3.2. Functional coupling assays

CHO cells expressing the hD₂, hD₃, and hD₃/D₂ re-

ceptors, respectively, were also examined for functional coupling to inhibition of adenylate cyclase activity, arachidonic acid release and phosphatidyl inositol turnover. Forskolin (10 μ M) produced an increase in cAMP levels in all three cell lines. However, only in the hD₂ cell line (Fig. 3A) could this increase (from 0.19 ± 0.01 to 13.9 ± 1.1 pmol per well) be dose-dependently inhibited by dopamine. We have previously demonstrated that this inhibition was reversed by pretreatment with 1 μ M haloperidol, and that dopamine (10 μ M) could not inhibit the forskolin-stimulated increase in cAMP levels in untransfected CHO cells, confirming that this effect is mediated by the transfected hD₂ dopamine receptors [14]. In the hD₃ (Fig. 3B) and hD₃/D₂ (clone 34) (Fig. 3C) cell lines forskolin (10 μ M) also produced a stimulation in cAMP levels, however, this was not significantly altered by the presence of dopamine. Dopamine treatment alone did not modify the basal levels of cAMP in any of the cell lines (data not shown). Five individual cell lines expressing varying levels of the chimeric receptor were examined. A typical result is shown in Fig. 3C. These data confirm that the hD₂ receptor couples via an inhibitory adenylate cyclase activity in CHO cells and that the hD₃ does not. The lack of functional coupling of the chimeric receptor to this second messenger system suggests that inserting the D₂ i3 loop into the D₃ receptor is not sufficient to allow efficient coupling mediated by an appropriate G protein.

Dopamine has been shown to stimulate the release of arachidonic acid from hD₂ transfected cells in the presence of either ATP or the ionophore A23187 [14]. This effector system involves a different coupling mechanism, so the same five chimeric clones used in the adenylate cyclase experiments were tested for their ability to stimulate arachidonic acid release. Our results showed that dopamine (1 μ M) enhanced arachidonic

Table I

K_i values (nM) for dopaminergic ligands at the D₂, D₃ and hD₃/D₂ receptors

Compound	D ₂	D ₃	hD ₃ /D ₂
Agonists			
Dopamine	700 (600; 810)	25 (19; 34)	6.2 (2.7; 14)
Quinpirole	580 (490; 690)	16 (14; 19)	7.9 (5.0; 12)
Apomorphine	29 (24; 35)	14 (9.5; 21)	18 (13; 26)
Bromocryptine	0.46 (0.27; 0.80)	2.2 (1.1; 4.2)	0.65 (0.37; 1.1)
Antagonists			
(-)-Sulpiride	4.5 (3.6; 5.6)	8.0 (7.3; 8.8)	19 (12; 30)
Haloperidol	2.3 (1.5; 3.4)	1.4 (1.0; 1.9)	1.8 (1.0; 3.3)
SCH-23390	560 (530; 590)	370 (330; 410)	270 (180; 410)

Results are expressed as the geometric mean of the apparent inhibition constant (K_i). IC₅₀ values were determined in 3–8 independent experiments and converted using the Cheng–Prussoff equation [22]. Curves were generated from 8 points, each determined in duplicate. Numbers in parentheses refer to the low and high error range.

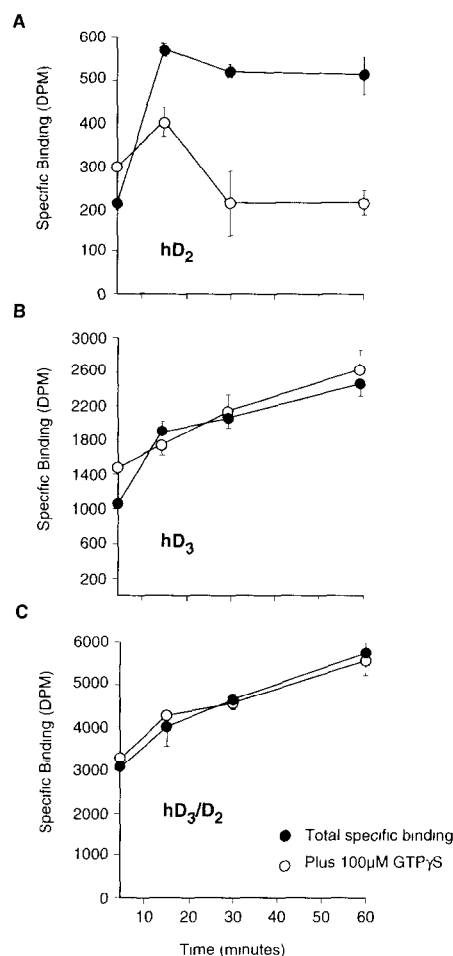


Fig. 2. The effect of guanine nucleotides on [³H]N-O437 binding. Specific binding was determined as described in the method section in the presence (●) or absence (○) of 100 μ M GTP- γ -S at 0, 15, 30 and 60 min. (A) In CHO cells expressing hD₂ receptors specific [³H]N-O437 binding was significantly decreased by the presence of GTP- γ -S, indicating coupling to a G protein. In contrast CHO cells expressing the hD₃ receptors (B) or the hD₃/D₂ (34) receptor (C) did not demonstrate any decrease of agonist binding. Each experiment was repeated at least three times and the results shown are from a typical experiment performed in quadruplicate.

acid release from the hD₂ cell line in the presence of 1 μ M A23187 (Fig. 4A). This increase was reversed by pretreatment with 0.1 μ M haloperidol. No increases in arachidonic acid release were observed following dopamine treatment of hD₃ or hD₃/D₂ expressing cell lines (Fig. 4B and C). This again suggests that the chimeric receptor does not functionally couple like the hD₂ receptor in CHO cells.

It has been reported that dopamine can stimulate phosphatidyl inositol turnover when hD₂ receptors are expressed in certain cell lines [19]. Accordingly, we examined the effect of dopamine (0.01, 1, or 100 μ M) on the hD₂, hD₃, and hD₃/D₂ cell lines. Dopamine was unable to stimulate inositol monophosphatase levels in any of the cell lines (data not shown). Under the same

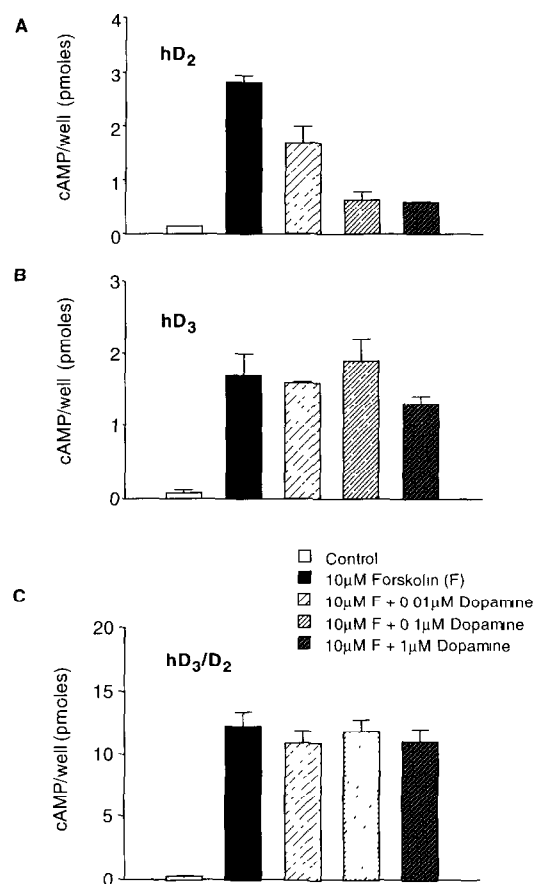


Fig. 3. Coupling of human dopamine receptors to inhibition of adenylate cyclase activity. (A) Forskolin stimulation of adenylate cyclase activity was inhibited by the activation of hD₂ receptors expressed in CHO cells in a dose-dependent manner. In contrast, CHO cells expressing the hD₃ receptors (B), or the hD₃/D₂(34) receptor (C), did not demonstrate any inhibition of the response. Results are from a typical experiment performed in quadruplicate. Each experiment was repeated on at least three separate occasions. Values are expressed as cAMP levels per well in pmol.

conditions carbachol (1 mM) produced a 6- to 7-fold stimulation in a CHO cell line stably expressing the human m1 muscarinic receptor [21]. We conclude that neither the hD₂ nor the hD₃ receptors functionally couple via phosphatidyl inositol turnover in CHO cells.

3.3. Conclusions

There are several possible explanations of the apparent lack of functional coupling of the hD₃ dopamine receptor when expressed in CHO cells. One possibility is that there is no appropriate G protein present to mediate signal transduction in this cell line. We have reported elsewhere the effect of expressing the hD₃ receptor in a variety of cell lines [14,18]. A second possibility is that the hD₃ receptor couples via an as yet undescribed mechanism, although this seems unlikely given the hD₃ receptor's extensive homology with classical G protein-coupled receptors. A third possibility is that the hD₃ receptor is very tightly bound to an inappropriate

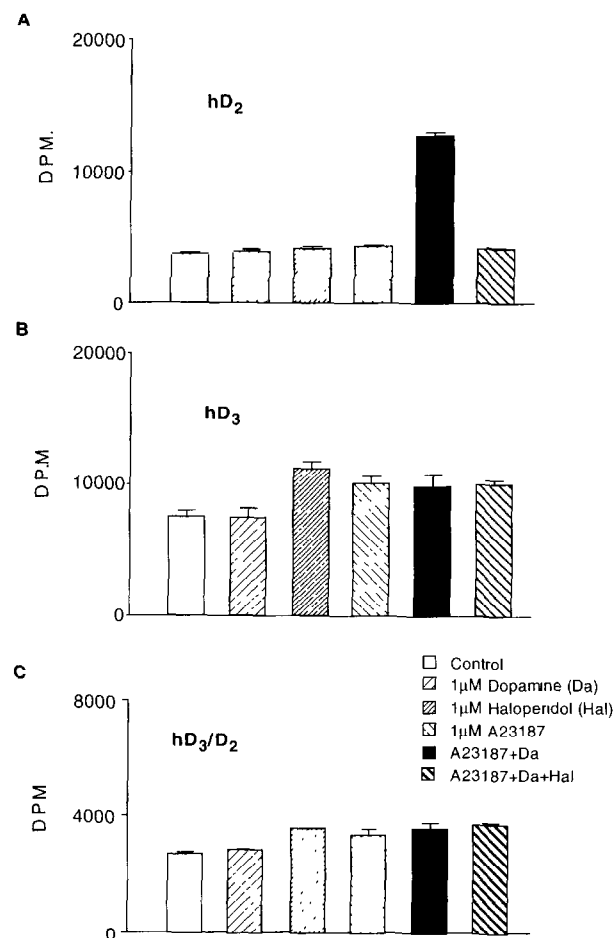


Fig. 4. Coupling of human dopamine receptors to arachidonic acid release. (A) Arachidonic acid release was potentiated by the activation of hD₂ receptors expressed in CHO cells after pretreatment with the calcium ionophore, A23187. This potentiation was blocked by pretreatment with haloperidol. In contrast CHO cells expressing the human D₃ receptors (B) or the hD₃/D₂(34) receptors (C) did not demonstrate this potentiation.

G protein such that the receptor-G protein complex cannot dissociate. This latter explanation would account for the apparent selectivity of the hD₃ receptor for certain agonists relative to the hD₂ receptor and would explain the lack of inhibition of agonist binding in the presence of guanine nucleotides. This study addresses the first and last of these possibilities. It is clear that the hD₂ receptor can functionally couple to the adenylate cyclase and arachidonic acid release systems in CHO cells [14,15], and that agonist binding to the hD₂ receptor in this cell line is modulated by the presence of guanine nucleotides. It is also well established that the i3 loop of G protein-coupled receptors is very important in determining G protein binding (e.g. [16]). Therefore, a chimeric hD₃ receptor containing the hD₂ i3 loop region may circumvent any problems associated with G protein specificity (the chimera should be able to bind to the same G protein that the hD₂ receptor utilizes). Similarly, such a chimera would be expected to mini-

mise the chances of a G protein being 'stuck' tightly to the receptor. Our initial binding data confirms that the chimeric receptor is expressed correctly in CHO cells and is able to bind dopaminergic ligands with an appropriate pharmacology. The binding profile of the chimera is very similar to that of the hD₃ receptor. However, the chimera does not behave as predicted in functional studies. There is no guanine nucleotide sensitivity of agonist binding and no evidence of functional coupling to conventional second messenger systems was detected. Thus, either other regions of the hD₃ receptor may influence G protein binding, or replacing only the third intracellular loop region is insufficient to allow interaction with the G protein(s) which couple the hD₂ receptor. Alternatively, and perhaps most interestingly, it may be that the hD₃ receptor does couple in an as yet undescribed way. Whatever the explanation, the suggestion that the hD₃ receptor constitutes an important target for antipsychotic drugs awaits a convincing demonstration that the D₃ receptor is the functional correlate of a central nervous system dopamine receptor.

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