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Characterisation of a chimeric hD_3/D_2 dopamine receptor expressed in CHO cells

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The D_2 dopamine receptor is known to be functionally coupled when expressed in CHO cells, whereas the effector systems for the D_3 dopamine receptor remain unclear. A chimeric, human D_3/D_2 receptor (hD_3/D_2) was constructed containing the third intracellular loop region of the D_2 receptor. CHO cells stably expressing the D_2, D_3, or hD_3/D_2 receptors were created and the pharmacology of the receptors was examined. The chimeric hD_3/D_2 receptor retained D_3-like affinities for dopaminergic ligands. However, in contrast to the D_2 receptor neither the D_3 receptor nor the hD_3/D_2 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 (D_1 and D_2) based upon their pharmacological properties [4]. The advent of gene cloning techniques has so far allowed the identification of five dopamine receptors designated D_1 to D_5 [5–11]. D_1 and D_5 dopamine receptors demonstrate properties similar to the pharmacologically defined D_1 dopamine receptor and are coupled to activation of adenylate cyclase [12], whereas the D_2, D_3, and D_4 dopamine receptors are more closely related to the classical D_2 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 D_3 dopamine receptors displayed similar affinities to their D_2 counterparts for dopamine antagonists [9,14,15]. However, D_3 dopamine receptors demonstrated much higher affinities for some dopamine agonists (e.g. dopamine, quinpirole) than D_2 dopamine receptors. In contrast to the human and rat D_2 receptors, which can mediate an inhibitory adenylate cyclase response and arachidonic acid release, both human and rat D_3 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 D_2 dopamine/m1 muscarinic receptor in which the third intracellular loop region of the hD_2 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 D_2 dopamine receptor itself. In this work, we have examined the pharmacology of a chimeric receptor (hD_3/D_2) in which the third intracellular loop region (i3) of the apparently uncoupled human D_3 dopamine receptor (hD_3) has been replaced with the analogous region of the functional human D_2 dopamine receptor (hD_2).

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Abbreviations: hD_2, human D_2 dopamine receptor; hD_3, human D_3 dopamine receptor; hD_3/D_2, human chimeric D_3/D_2 dopamine receptor; GTP, guanosine triphosphate; cAMP, adenosine 3′,5′-cyclic monophosphate.

2. MATERIALS AND METHODS

2.1. *Receptor clones*

DNA and a stably transfected CHO-K1 cell line for the human D_3 dopamine receptor were obtained from Sokoloff et al. (INSERM). D_2 cDNA clones were obtained using PCR techniques [20]. Based on the published sequence of the human D_{2B} receptor [17] the following

oligonucleotides were synthesized: DR1, 5′-TTTTGAATTCGCCCTGATGGATCCACTG-3′; DR2, 5″-TTTTCTCGAGAAGAAGAGGAGGCCGATC-3′ and DR3, 5″-TTTTCTCGAGTGGGAAGCAGGCTGCTCTGT-3′ cDNA was prepared from 1 μg of human brain mRNA (Clonetech 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 *Eco*RI and *Xho*I and subcloned into the expression vector pcDNA1neo (Invitrogen Corp.) and its identity confirmed by DNA sequencing. Chimeric hD_3/D_2 DNA was generated in three stages using PCR techniques. Cloned hD_3 DNA in pBluescript (Stratagene) was used as template to generate PCR products containing the first 5 transmembrane regions of the hD_3 clone (Primers CD1 and CD2). CD1 generated a PCR product containing part of the multiple cloning site of pBluescript (including a *Hin*dIII site) and CD2 contained a *Bam*HI site just downstream of a *Spe*I site. This PCR product (PCR 1) was subcloned into pBluescript after digestion with *Hin*dIII and *Bam*HI. Next, PCR 2 containing transmembrane regions six and seven of the hD_3 clone was generated using primers CD3 and CD4. CD3 contained a *Bam*HI site upstream of a *Nhe*I site and CD4 generated a PCR product containing part of the pBluescript multiple cloning site (including a *Spe*I site). After digestion with *Bam*HI and *Spe*I, PCR 2 was subcloned into the PCR 1 containing vector digested with *Bam*HI and *Xba*I. This generated a plasmid containing the seven transmembrane regions of the hD_3 receptor but missing the third intracellular loop region (i3). This construct also contained *Spe*I and *Nhe*I restriction sites suitable for inserting DNA in the i3 region. PCR 3 was carried out using CD5 and CD6 primers and hD_2 DNA as a template and generated a product containing the i3 region of the hD2 receptor and *Spe*I and *Nhe*I at the 5′ and 3′ ends, respectively. PCR 3 was subcloned into the vector containing PCR 1 and 2 to generate the chimeric hD_3/D_2 receptor. The construct was sequenced to confirm its structure and was then subcloned into the *Hin*dIII/*Not*I 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′-ATGGATCCTGGCATAGACTAGTACAGTCA-3′, CD3, 5′-ATGGATCCACTTCGGGAGAAGAAGGCTAGCCA-3′, CD4, 5′- AACCCTCACTAAAGGGAACAAAAGC-3′, CD5, 5′- CCCTTCATTGTCACCCTACTAGTCTA-3′, CD6, 5′-CGAGCATCTGGCTAGCTTTCTTCTCCT-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% CO_2 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 [^{125}I]iodosulpiride. Of 18 individual chimera clones tested, 17 demonstrated specific radioligand binding.

2.3. *Radioligand binding*

2.3.1. Iodosulpride 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 $MgSO_4$ 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 $MgSO_4$, 5 mM $NaHCO_3$, 1.2 mM KH_2PO_4, 2.5 mM $CaCl_2$, 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 [^{125}I]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 $MgSO_4$ and 0.1% ascorbate. Binding was determined using 1 nM [^{3}H]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 phosphatidyl inositol 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 hD_3/D_2 receptor that was constructed. The construction was carried out in three parts using PCR methodology (see section 2). Firstly, a hD_3 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 hD_2 receptor i3 region (or any other loop region with suitable restriction sites). Secondly, the hD_2 loop region was inserted and the fidelity of the construct was confirmed by DNA sequencing. The introduction of the *Nhe*I 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 D_4 receptors and a leucine in D_1) 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 $hD_{2(short)}$ 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 pD_2neo was used to transiently transfect HEK 293 (human embryonic kidney) cells the resulting membranes showed considerable specific binding for the dopaminergic ligand [^{125}I]iodosulpiride (data not shown). We then created stable cell lines expressing the chimeric hD_3/D_2 or the hD_2 receptors by transfecting CHO-K1 cells with $pD_{3/2}$neo and pD_2neo, re-

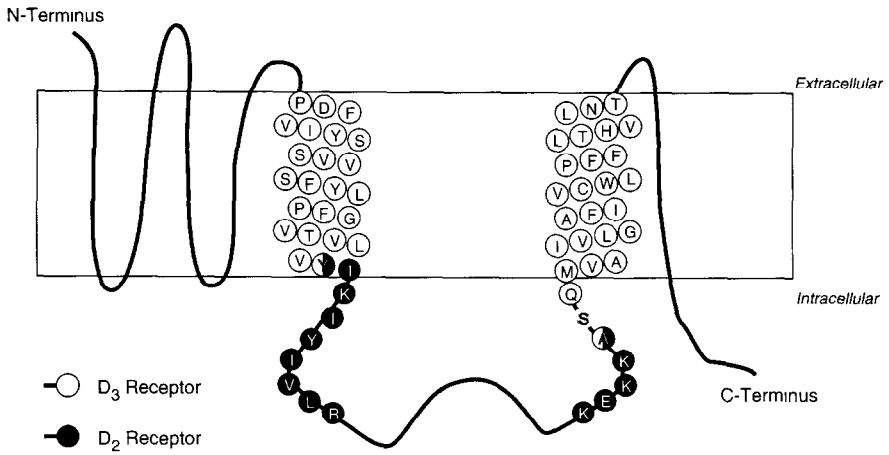

Fig. 1. A schematic diagram of the chimeric receptor Amino acids (in the single letter code) up to and after those in black circles are derived from the hD_3 receptor. Amino acids in between are derived from the hD_2 receptor. Construction of the chimera is described in section 2. Amino acids in half black and white circles represent the junctions of the chimera and are common to the hD_2 and hD_3 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 hD_2 receptor is functionally coupled in this cell line [14,15]. Of 18 individual hD_3/D_2 clonal lines examined, 17 demonstrated specific [^{125}I]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, $hD_3/D_2(34)$, expressing relatively high levels of receptor was used in radioligand studies. A typical hD_2 clone $hD_2CHO(11)$ was used in all subsequent studies. The pharmacological binding profiles of the hD_2, hD_3 and hD_3/D_2 receptors expressed in CHO cells were compared to see what effect substituting the i3 region had upon the hD_3 receptor's affinity for selected ligands. The affinity of [^{125}I]iodosulpiride was determined for membranes made from cells expressing the hD_2, hD_3 and hD_3/D_2 receptors, respectively (data not shown). The K_D for the hD_2 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 hD_3 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 hD_3/D_2 (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 [^{125}I]iodosulpiride. The results are shown in Table I. All three receptor types tested displayed D_2-like affinities for the D_2 antagonists (−)sulpiride and haloperidol and low affinity for the D_1 antagonist, SCH-23390 as was expected. Like the hD_3 receptor, the hD_3/D_2 receptor displays higher affinity for hD_2 agonists such as dopamine and quinpirole compared to the hD_2 receptor, whereas other hD_2 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 hD_3 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 [^{3}H]N-0437 to membranes prepared from the hD_2, hD_3, and hD_3/D_2 cell lines in the presence and absence of the GTP analogue GTP-γ-S (100 μM). The inhibition of [^{3}H]N-0437 binding by GTP-γ-S is consistent with the hD_2 receptor interacting with a dissociable G protein in CHO cells (Fig. 2A). In contrast, neither the hD_3 (Fig. 2B) nor the hD_3/D_2 (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 [^{3}H]N-0437. However, no significant inhibition of agonist binding by GTP-γ-S was observed in any of them. The results for clone $hD_3/D_2(34)$ are depicted in Fig. 2C. Two recent reports have demonstrated a small rightward shift in dopamine competition of [^{125}I]iodosulpiride binding at the hD_3 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 hD_3 receptor.

3.2. *Functional coupling assays*

CHO cells expressing the hD_2, hD_3, and hD_3/D_2 re-

ceptors, respectively, were also examined for functional coupling to inhibition of adenylate cyclase activity, arachidonic acid release and phosphatidyl inositol turnover. Forskolin (10 μM) produced an increase in cAMP levels in all three cell lines. However, only in the hD_2 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 μM haloperidol, and that dopamine (10 μM) could not inhibit the forskolin-stimulated increase in cAMP levels in untransfected CHO cells, confirming that this effect is mediated by the transfected hD_2 dopamine receptors [14]. In the hD_3 (Fig. 3B) and hD_3/D_2 (clone 34) (Fig. 3C) cell lines forskolin (10 μM) 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 hD_2 receptor couples via an inhibitory adenylate cyclase activity in CHO cells and that the hD_3 does not. The lack of functional coupling of the chimeric receptor to this second messenger system suggests that inserting the D_2 i3 loop into the D_3 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 hD_2 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 μM) enhanced arachidonic acid release from the hD_2 cell line in the presence of 1 μM A23187 (Fig. 4A). This increase was reversed by pretreatment with 0.1 μM haloperidol. No increases in arachidonic acid release were observed following dopamine treatment of hD_3 or hD_3/D_2 expressing cell lines (Fig. 4B and C). This again suggests that the chimeric receptor does not functionally couple like the hD_2 receptor in CHO cells.

It has been reported that dopamine can stimulate phosphatidyl inositol turnover when hD_2 receptors are expressed in certain cell lines [19]. Accordingly, we examined the effect of dopamine (0.01, 1, or 100 μM) on the hD_2, hD_3, and hD_3/D_2 cell lines. Dopamine was unable to stimulate inositol monophosphatase levels in any of the cell lines (data not shown). Under the same

Table I

K_i values (nM) for dopaminergic ligands at the D_2, D_3 and hD_3/D_2 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). IC_{50} 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.

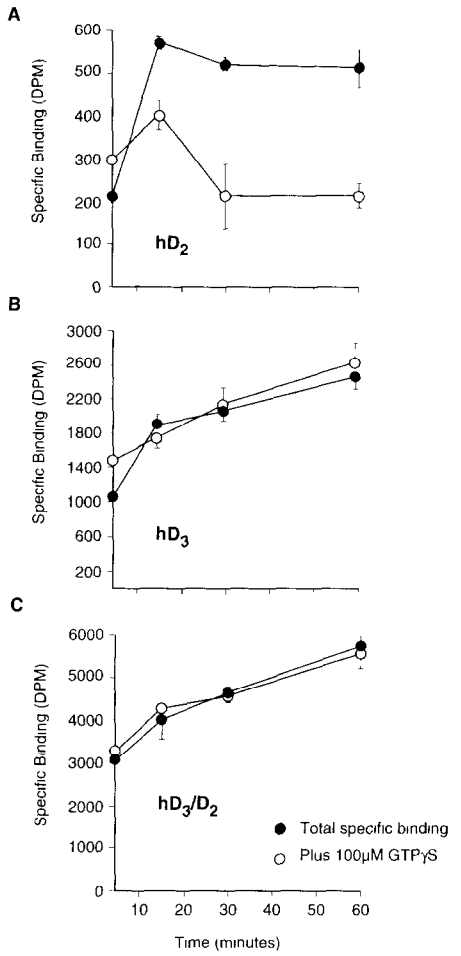

Fig. 2. The effect of guanine nucleotides on [^{3}H]N-0437 binding Specific binding was determined as described in the method section in the presence (●) or absence (○) of 100 μM GTP-γ-S at 0, 15, 30 and 60 min. (A) In CHO cells expressing hD_2 receptors specific [^{3}H]N-0437 binding was significantly decreased by the presence of GTP-γ-S, indicating coupling to a G protein. In contrast CHO cells expressing the hD_3 receptors (B) or the hD_3/D_2(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.

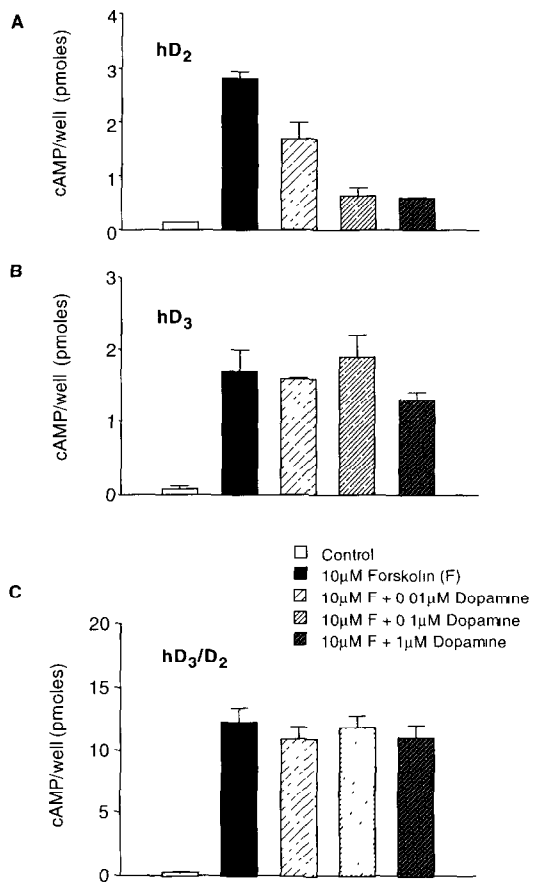

Fig. 3. Coupling of human dopamine receptors to inhibition of adenylate cyclase activity. (A) Forskolin stimulation of adenylate cyclase activity was inhibited by the activation of hD_2 receptors expressed in CHO cells in a dose-dependent manner. In contrast, CHO cells expressing the hD_3 receptors (B), or the $hD_3/D_2(34)$ 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.

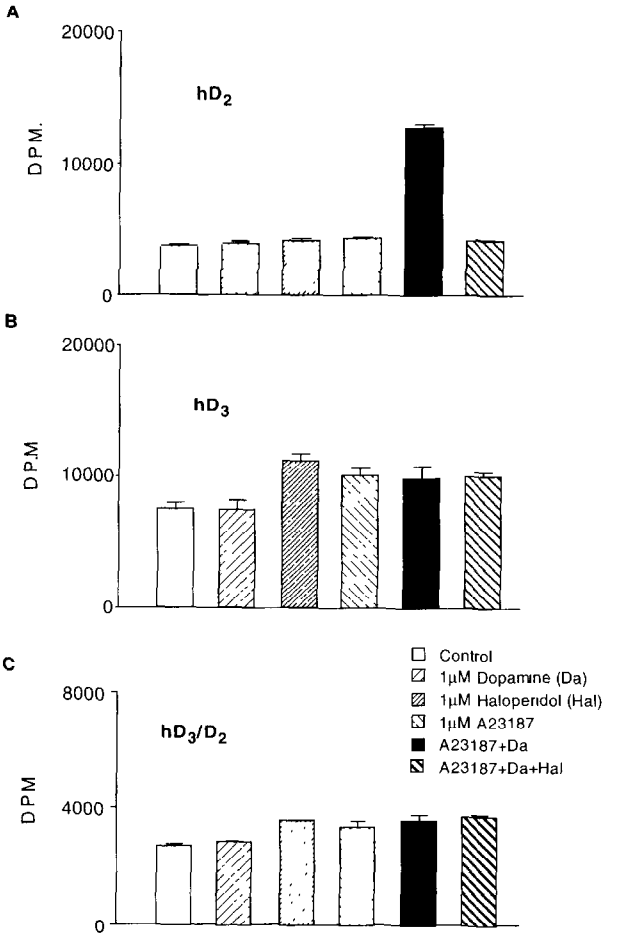

Fig. 4. Coupling of human dopamine receptors to arachidonic acid release (A) Arachidonic acid release was potentiated by the activation of hD_2 receptors expressed in CHO cells after pretreatment with the calcium ionophore, A23187. This potentiation was blocked by pretreatment with haloperidol. In contrast CHO cells expressing the human D_3 receptors (B) or the $hD_3/D_2(34)$ receptors (C) did not demonstrate this potentiation

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 hD_2 nor the hD_3 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 hD_3 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 hD_3 receptor in a variety of cell lines [14,18]. A second possibility is that the hD_3 receptor couples via an as yet undescribed mechanism, although this seems unlikely given the hD_3 receptor's extensive homology with classical G protein-coupled receptors. A third possibility is that the hD_3 receptor is very tightly bound to an inappropriate G protein such that the receptor–G protein complex cannot dissociate. This latter explanation would account for the apparent selectivity of the hD_3 receptor for certain agonists relative to the hD_2 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 hD_2 receptor can functionally couple to the adenylate cyclase and arachidonic acid release systems in CHO cells [14,15], and that agonist binding to the hD_2 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 hD_3 receptor containing the hD_2 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 hD_2 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 hD_3 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 hD_3 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 hD_2 receptor. Alternatively, and perhaps most interestingly, it may be that the hD_3 receptor does couple in an as yet undescribed way. Whatever the explanation, the suggestion that the hD_3 receptor constitutes an important target for antipsychotic drugs awaits a convincing demonstration that the D_3 receptor is the functional correlate of a central nervous system dopamine receptor.

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