Effects of reciprocal chimeras between the C-terminal portion of third intracellular loops of the human dopamine D_2 and D_3 receptors

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Abstract The dopamine D_3 receptor is a member of the G protein-coupled superfamily of receptors. However, its coupling with intracellular events is still not well understood. We have performed chimera constructions in which amino acid residues located in a region of the receptor involved in the coupling with second messengers (the C-terminal portion of the third intracellular loop) have been exchanged between dopamine D_2 and D_3 receptors. Chimera constructions did not modify substantially the pharmacological profiles, nor G protein coupling, as compared to their respective wild-type receptors. However, the D_2 receptor chimera, containing the C-terminal portion of the third intracellular loop of the D_3 receptor, has a lower potency to inhibit cyclic AMP production. The reciprocal construction generated a D_3 receptor that is fully coupled to this second messenger pathway whereas, the native D_3 receptor is uncoupled to this pathway in our transfected cells. These results suggest that the sequence selected is important for specific coupling characteristics shown by these two dopamine receptor homologues.

Key words: G protein-coupled receptor; Dopamine receptor D_2; Dopamine receptor D_3; Cyclic AMP; GTP shift; Chimera

1. Introduction

Dopamine receptors are members of the superfamily of G protein-coupled receptors. On the basis of their genetic organization, pharmacology and intracellular signaling properties, they are divided into two subgroups, termed D_1-like (which include D_1 and D_5 subtypes) and D_2-like (including D_2, D_3 and D_4 subtypes) receptors [1]. Among these various subtypes, the D_3 receptor displays properties which suggest that it may represent a target for antipsychotic drugs [2]. The D_3 receptor has a large third intracellular loop and a short C-terminal tail, which are typical of G protein-coupled receptors that interact with a $G_o/G_{i\alpha}$ protein and mediate the inhibition of adenylate cyclase (AC) activity. However, coupling of the D_3 receptor with inhibition of cyclic AMP (cAMP) production has not been clearly demonstrated [2–9]. On the other hand, in some heterologous expression systems, D_3 receptor can couple with various second messenger systems, including inhibition of cAMP production [10–12], ion channel activity or stimulation of Na^+/H^+ exchange [6,10,13,14], or to produce intracellular responses, such as increased mitogenesis [5,10,15] or stimulation of Fos-like immunoreactivity [5]. Whether these coupling pathways of the receptor represent true natural interactions or are artifacts, because of high levels of receptors in heterologous expression systems, remains a matter of controversy.

The modulation of agonist binding to the D_3 receptor by guanosine triphosphate nucleotide (GTP), an indicator of receptor/G protein interactions, is most of the time not observed [2,9,16]. When observed, this GTP shift, as it is called, is very weak, as compared to other G_i-coupled receptors, such as the D_2 receptor [5,8,10,16]. Interestingly, a similar weak GTP shift is also observed in tissue homogenates from lobules IX and X of the rat cerebellum, where the D_3 receptors is the only representative of the D_2-like receptor subgroup [17]. Thus, it is possible that the relative absence of GTP shift is an intrinsic property of the D_3 receptor. Recently, a coupling of the D_3 receptor with the type V isoform of AC, but not type II, has been observed [18,19]. But again, activation of $G_{i\alpha}$ subunits, as measured by agonist-induced guanosine 5′[γ-thio]triphosphate-[^{35}S] ([^{35}S]GTPγS) binding, is quite low, as compared to D_2 receptor-mediated effect in similar conditions [18].

Investigations of the coupling of D_3 receptors have also been performed using D_2/D_3 receptor chimera constructions [20–22]. These studies indicate that the third intracellular loop of D_2 and D_3 receptors is important for the coupling to AC. Moreover, in D_3/D_2 receptor chimeras that fully coupled to inhibition of cAMP production, agonist binding to these chimeras is still resistant to the effect of GTP or its analogues [23]. We undertook the present study in order to identify more specific components of the third intracellular (i3) loop of human dopamine D_2 (hD_2) and D_3 (hD_3) receptor subtypes involved in the coupling of these receptors with AC. We have studied a sketch of 12 divergent amino acid residues located at the C-terminal portion of the i3 loop of these receptors (Fig. 1). Reciprocal chimera constructions between hD_2 and hD_3 dopamine receptors using this amino acid segment produce minimal effects in the pharmacological profiles of the chimera receptors, as compared to their respective wild-type receptors, but affect the coupling of these receptors with AC activity.

2. Materials and methods

2.1. Materials

[^{3}H]Spiperone was purchased from Amersham Life Science (Oakville, Ont., Canada). 5′-Guanylylimidodiphosphate (Gpp(NH)p), forskolin and isobutylmethylxanthine (IBMX) are from Sigma-Aldrich (Oakville, Ont., Canada). Antagonists (+)butaclamol, clozapine, haloperidol, raclopride and risperidone, as well as agonists quinpirole, *R*(+)-7-hydroxy-*N,N*-di-*n*-propyl-2-aminotetralin (R(+)7-OH-DPAT), bromocriptine, apomorphine and pergolide were from Research Bio-

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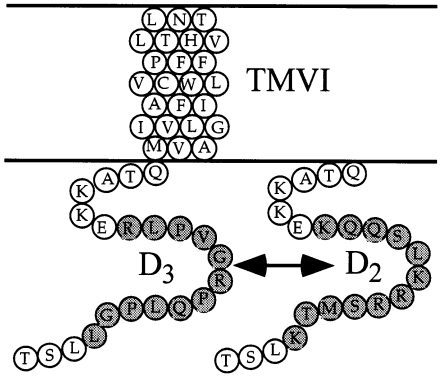

Fig. 1. Schematic representation of the dopamine hD_2 and hD_3 sequences (shaded circles) that were used for chimera constructions. The diagram illustrates the C-terminal portion of the third intracellular loop of the hD_3 receptor with the transmembrane domain VI (TMVI). The one letter code for amino acid residues is used. Reciprocal chimera constructions were performed (see Section 2) where the represented sequence of the hD_3 receptor was replaced by the hD_2 receptor sequence and vice et versa (chimeras are named hD_3/hD_{2i3} and hD_2/hD_{3i3}, respectively).

chemicals International (RBI, Natick, MA, USA). Cyclic AMP levels were measured using the Rianen [^{125}I]cAMP radioimmunoassay kit purchased from DuPont NEN (Guelph, Ont., Canada). Cell culture media Dulbecco's modified Eagle medium (DMEM) and F12, as well as certified fetal bovine serum were obtained from Gibco BRL (Life Sciences Technologies, Burlington, Ont., Canada).

2.2. Cell lines and chimera constructions

The construction and stable transfection of hD_2 [24], hD_3 receptor [25] and receptor chimeras were performed in Chinese hamster ovary (CHO) cells. Cell lines were maintained in Ham's F12 medium supplemented with L-glutamine (0.3 mg/ml), 10% heat-inactivated fetal bovine serum, 100 U/ml penicillin and 100 μg/ml streptomycin at 37°C and 5% CO_2. DNA transfections into CHO cells were performed with the calcium/phosphate method in standard conditions. Chimeras were constructed using polymerase chain reaction (PCR) technology. Subcloning of the hD_3 receptor into the mammalian expression vector pRC/CMV containing a resistance gene for neomycin (G418, Gibco BRL) was previously described [5]. To introduce the hD_2 receptor peptide, corresponding to amino acid 327– 338 (KTMSRRKLSQQK) of the C-terminal portion of the i3 loop (Fig. 1), into the hD_3 receptor, two PCR reactions were combined. One set of primers containing the initiation ATG codon of the hD_3 receptor (with a *Hin*dIII site) and overlapping sequences of hD_2 and hD_3 receptors were synthesized (sense: 5′-ACGAAGCTTATGGCATCTCTGAGTCAGCTGA-3′ and antisense: 5′-CTTACGTCGACTCATGGTCTTCAAAGATGTCGATAA-3′). A second set of primers were synthesized in order to obtain the remaining portion of the hD_3 receptor sequence (sense: 5′-CATGAGTCGACGTAAGCTCTCCCAGCAGAAGGAGAAGAAGGCAACCCAAATGGTG-3′ and antisense 5′-TGCAGATCTTCAGCAAGACAGGATCTTGAGG-3′, containing the stop codon and a *Xba*I site). The degenerate nature of trinucleotide codons for amino acid residues serine and arginine was exploited to generate a *Sal*I restriction site (nucleotide sequences underlined) in the middle of the sequence corresponding to the segment of the hD_2 receptor to be introduced in the hD_3 receptor. Amplified PCR fragments were then digested with corresponding sets of endonuclease restriction enzymes (*Hin*dIII/*Sal*I and *Sal*I/*Xba*I), ligated and introduced into the polylinker of a pRC/CMV plasmid (*Hin*dIII/*Xba*I) to form the hD_3 receptor chimera with the C-terminal portion of the i3 loop of the hD_2 receptor (named hD_3/hD_{2i3}). Similar experiments were performed to construct the reciprocal hD_2 chimera, where a hD_3 receptor peptide sequence, corresponding to amino acid 312–323 (LGPLQPRGVPLR) of the C-terminal portion of the i3 loop of the receptor is introduced into the reading frame of the hD_2 receptor (Fig. 1). This chimera is named hD_2/hD_{3i3}. For this construction, we started with a hD_2 (short isoform) subcloned into a pCMV5 plasmid (a gift from Dr. Pierre Falardeau). The first set of primers included a hD_2 ATG primer with an *Eco*RI site (5′-TGCGAATTCGCCACCATGGATCCACTGAAT-3′) and the antisense primer in the third intracellular loop containing a *Ksp*I (*Sac*II, underlined) site (5′-ACTCCCCGCGGTTGCAGGGGCCCCAGGGAGGTCCGGGTTTTGCCATTGGG-3′), the second set of primer consisted to a sense primer containing a *Ksp*I site (5′-CCTGCAACCGCGGGGAGTGCCACTTCGGGAGAAGAAAGCCACTCAGATGCTT-3′) and antisense primer containing a *Kpn*I site and a stop codon (5′-CATGGTACCTCAGCAGTGGAGGAT-3′). Again, the *Ksp*I site was introduced by using the degenerative nature of trinucleotide codons for the amino acid proline and arginine. After digestion with appropriate restriction enzymes, the resulting hD_2 receptor chimera containing a segment of the i3 loop of the hD_3 receptor was subcloned into the pRC/CMV expression vector. All the constructions were sequenced using Sequenase Version 2.0 DNA sequencing kit (USB, Cleveland, OH, USA) to verify the sequences.

2.3. Binding assay

Confluent cells were trypsinized, resuspended in F12 medium and rapidly centrifuged at $2000\times g$ for 5 min. They were homogenized using a polytron (at moderate velocity) and resuspended in a Tris-HCl buffer (15 mM) containing 5 mM $MgCl_2$, pH 7.4, then recentrifuged at $50\,000\times g$ for 15 min. Incubation buffer contained 50 mM Tris-HCl, 120 mM NaCl and 5 mM $MgCl_2$, pH 7.4. [^{3}H]Spiperone (specific activity: 94–107 Ci/mmol) binding was performed. For isotherm saturation experiments increasing concentrations (0.01–3 nM) of [^{3}H]spiperone were added to the incubation buffer. The reaction was started by addition of cell homogenates (20–50 μg of protein determine by the method of Lowry [26]) in a final volume of 1 ml. (+)Butaclamol (1 μM) or raclopride (1 μM) was used to determine non-specific binding for hD_2 or hD_3 receptors or respective receptor chimeras constructions. Incubations lasted for 1 h at room temperature and were stopped by rapid filtration through GF/C filters (Whatman) with ice-cold Tris-NaCl buffer. 10 ml of scintillation cocktail was added to the filters and radioactivity was counted at 30% efficiency. Competitions of [^{3}H]spiperone binding (0.8–1.0 nM) with agonists and antagonists were performed in similar conditions. For GTP-induced shift of dopamine affinities, Gpp(NH)p (100 μM) was added. Each concentration points were performed in triplicate. Computer assisted non-linear regression curve analysis were performed using the GraphPad software (Prism 2.0, GraphPad Software Inc.)

Table 1
Pharmacological profile of dopamine receptor chimera constructions compared to the human D_2 and D_3 wild-type receptor subtypes

	hD_2	hD_2/hD_{3i3}	hD_3	hD_3/hD_{2i3}
Agonists				
Quinpirole	1708 ± 961	1124 ± 473	7.9 ± 3.0	14.9 ± 3.9
Pergolide	24 ± 6	37 ± 11	0.5 ± 0.1	1.7 ± 0.3
Apomorphine	83 ± 34	123 ± 33	24 ± 4	33 ± 3
R(+)7-OH-DPAT	313 ± 88	270 ± 25	0.6 ± 0.1	1.3 ± 0.2
Bromocriptine	10.4 ± 4.7	7.9 ± 1.4	2.5 ± 0.3	4.9 ± 0.9
Antagonists				
Haloperidol	1.0 ± 0.2	0.6 ± 0.1	1.0 ± 0.3	1.0 ± 0.4
Raclopride	2.3 ± 0.6	2.0 ± 0.5	1.5 ± 0.4	1.4 ± 0.1
Clozapine	477 ± 114	437 ± 66	371 ± 123	380 ± 53
Risperidone	4.1 ± 0.6	3.6 ± 0.9	11.4 ± 2.2	10.3 ± 0.6

Values represent means ± S.E.M. of inhibition constants (K_i) expressed in nM ($n = 3$–5).

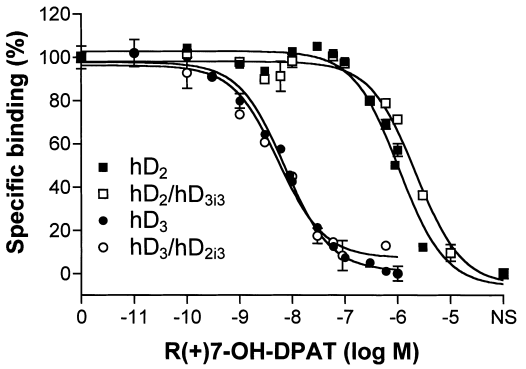

Fig. 2. Representative examples of displacement curves obtained from the competition of [^{3}H]spiperone binding (0.8–1.0 nM) with various agonists and antagonists (competition with R(+)-7-OH-DPAT is presented) with wild-types and chimera constructions. Inhibitory constants (K_i) were calculated from half-maximal inhibitory concentration values (IC_{50}) according to the Cheng and Prusoff correction: $K_i = IC_{50}/(1+[L]/K_d)$, where L and K_d are the concentration and the dissociation constant of the radiolabeled ligand used, respectively [38].

and each set of data were successively fitted according to a one- or a two-site binding model. Then the fits were statistically compared and a two-site model was retained only if it was statistically better ($P < 0.05$) than the one-site analysis.

2.4. Cyclic AMP assay

Cells were plated in 96-well plates at a density of 40 000 cells/well. After 24 h culture, cells were washed twice for 15 min with 0.2 ml of DMEM without serum at 37°C and 5% CO_2. Cells were then incubated in five replicates for 15 min at 37°C in 5% CO_2 with increasing concentration of dopamine (0.1 nM–10 μM) in the presence of 100 μM IBMX and 10 μM forskolin in a final volume of 0.15 ml of DMEM (forskolin and dopamine were omitted to determine basal cAMP levels). Then, the medium containing the drugs was rapidly removed and 50 μl/well of 0.1 N HCl was added, and the plates were placed on ice. Cells were sonicated for 5 s and the extract was neutralized with 0.4 ml of a mixture of 2.4 M sodium acetate, pH 6.0, and 2 N NaOH. Cyclic AMP levels were measured using the Rianen [^{125}I]cAMP radioimmunoassay kit from DuPont NEN (Guelph, Ont., Canada) in the conditions recommended by the manufacturer.

2.5. Statistical analysis

The data were compared using an analysis of variance (one-way ANOVA), followed by a Fisher probability of least significant difference (PLSD) test.

3. Results

Among all the positive clones selected in our transfection experiments we only kept a series of clones that expressed similar levels of dopamine hD_2, hD_3, hD_2/hD_{3i3} and hD_3/hD_{2i3} receptors (maximal receptor capacities (B_{max}) were: hD_2, 364 ± 81; hD_3, 438 ± 84; hD_2/hD_{3i3}, 370 ± 13 and hD_3/hD_{2i3}, 366 ± 42 fmol/mg of protein, $n = 5$). The hD_2 and hD_3 receptor chimeras have similar equilibrium dissociation constant (K_d), as compared to their respective wild-type homologues (K_d were: hD_2, 80 ± 20; hD_3, 107 ± 17; hD_2/hD_{3i3}, 93 ± 30 and hD_3/hD_{2i3}, 99 ± 9 pM, $n = 5$). Comparisons of inhibitory constants (K_i) for dopamine agonists and antagonists indicate that the pharmacological profiles shown by hD_2/hD_{3i3} and hD_3/hD_{2i3} receptor chimeras are quite similar to their respective wild-type receptors (Table 1). Overall, binding of agonists is not affected in the hD_2/hD_{3i3} chimera, as compared to the hD_2 receptor, whereas binding affinities of agonists are slightly (2-fold) decreased in the hD_3/hD_{2i3} chimera, as compared to the hD_3 receptor (Table 1). However, compounds which display selectivity for a receptor subtype, such as R(+)7-OH-DPAT for the D_3 receptor, maintained their selectivity (Table 1 and Fig. 2). Binding of antagonists is not affected in any cases (Table 1).

Interaction of G protein-coupled receptors with heterotrimeric G proteins can be indirectly visualized by the GTP-induced shift of the high affinity state into the low affinity state for the endogenous ligand. As expected, we observed a high and low affinity states for the hD_2 receptor. Addition of Gpp(NH)p, a non-hydrolyzable analogue of GTP, induced a complete conversion of the high affinity into the low affinity state of the hD_2 receptor for dopamine (Table 2). For the hD_3 receptor, in most experiments, we were not able to observe high and low affinity states for dopamine in our transfected cells (Table 2). However, in three experiments out of eight, we did see a better fit with the two-site model (see Fig. 3). When observed, the high affinity state of the hD_3 receptor was never converted into a low affinity state in the same conditions (neither with higher Gpp(NH)p concentrations, data not shown). Although the high affinity state of the hD_3 is similar to the value of the hD_2 receptor, the fraction of the total hD_3 receptor number in this state is more important than the hD_2 receptor (Table 2). Moreover, the low affinity state of the hD_3 receptor is only 10-fold lower than the high affinity state of the receptor, whereas it is about 1000-fold lower for the hD_2 receptor (Table 2). The hD_2/hD_{3i3} receptor chimera also displayed a complete conversion of the high affinity state for dopamine into a low affinity state, but the high affinity state for dopamine was lower, whereas the proportion of the total receptor population in the high affinity state is higher in this receptor chimera, as compared to the wild-type hD_2 receptor (Table 2 and Fig. 3). All the hD_3/hD_{2i3} receptor chimera binding parameters were identical to the wild-type hD_3 receptor (Table 2). However, in three GTP shift experiments out of

Table 2
GTP-induced affinity shifts of dopamine for receptor chimera constructions compared to the human D_2 and D_3 wild-type receptor subtypes

		CHO cell types expressing human dopamine receptors			
		hD_2	hD_2/hD_{3i3}	hD_3	hD_3/hD_{2i3}
Dopamine	K_h (nM)	5.5 ± 2.5	17.2 ± 5.5*	4.1 ± 1.3	3.9 ± 1.3
	K_L (nM)	3800 ± 1200	2510 ± 670	(74 ± 6)	(54 ± 28)
	R_h (%)	29.6 ± 5.3	47.1 ± 13.7	(66.4 ± 1.4)*	(84.6 ± 6.2)
Dopamine+Gpp(NH)p	K_h (nM)	–	–	–	–
	K_L (nM)	2460 ± 640	2400 ± 700	7.7 ± 1.6	6.5 ± 1.4

Values represent means ± S.E.M. ($n = 6$–8).
*$P < 0.05$ vs. hD_2 receptor. Values presented in parentheses were obtained in three out of eight experiments with hD_3 and hD_3/hD_{2i3} receptors. See Fig. 3 for additional information.

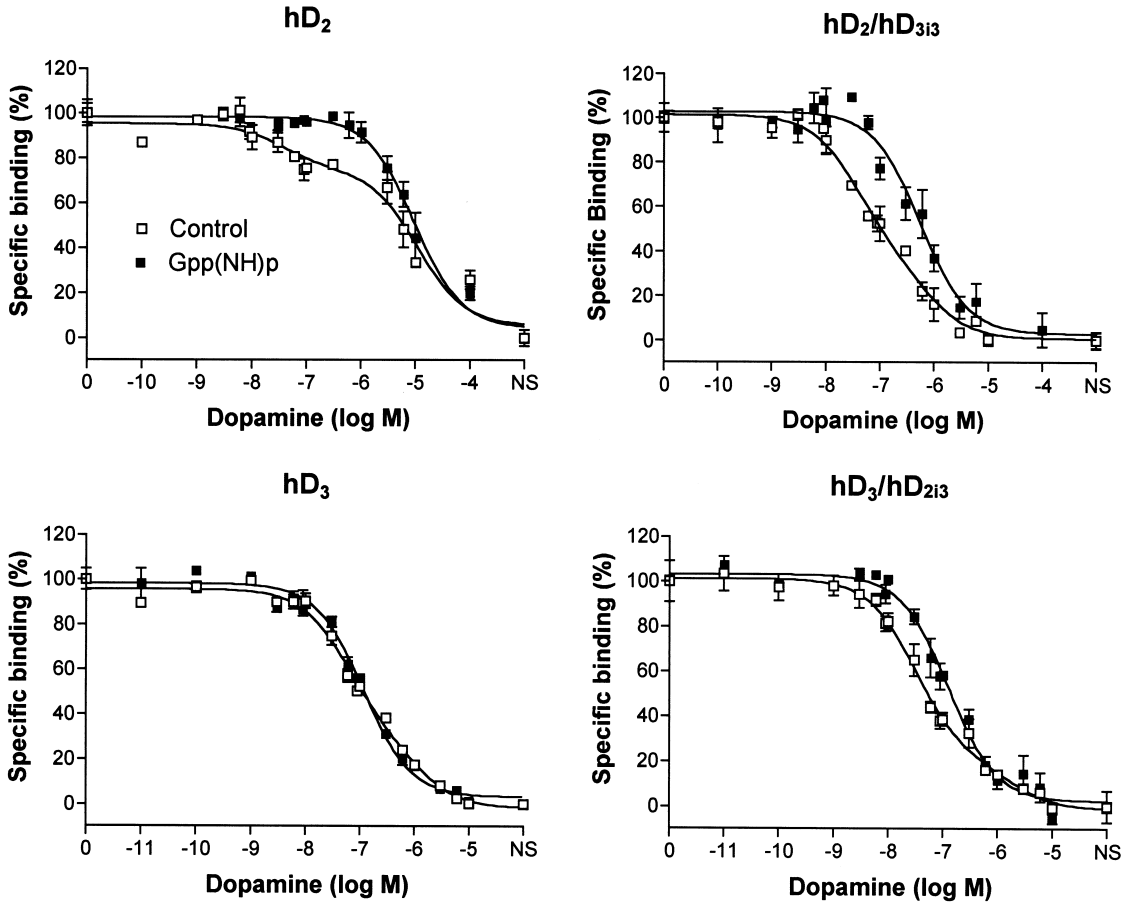

Fig. 3. Representative examples of the competition of [^{3}H]spiperone binding by dopamine in the absence or presence of Gpp(NH)p (100 μM) with dopamine hD_2 and hD_3 wild-type receptors and hD_2/hD_{3i3} and hD_3/hD_{2i3} receptor chimera constructions. Values are: hD_2 control, $K_h = 3.3$ nM, $K_L = 998$ nM, $R_h = 22.6\%$; hD_2 Gpp(NH)p, $K_L = 860$ nM; hD_3 control, $K_h = 5.2$ nM, $K_L = 88$ nM, $R_h = 34.4\%$; hD_3 Gpp(NH)p, $K_L = 11$ nM; hD_2/hD_{3i3} control, $K_h = 3.4$ nM, K_L=59 nM, $R_h = 61.6\%$; hD_2/hD_{3i3} Gpp(NH)p, $K_L = 50$ nM; hD_3/hD_{2i3} control, $K_h = 2.8$ nM, $K_L = 158$ nM, $R_h = 78.5\%$; hD_3/hD_{2i3} Gpp(NH)p, $K_L = 12$ nM. For the hD_3/hD_{2i3} chimera, the example illustrated here is taken from an experiment where a high and low affinity states (two-site model), as well as a significant GTP shift were observed (see Table 2).

seven, we observed a small but significant GTP shift with the hD_3/hD_{2i3} receptor chimera (see Fig. 3). The reasons for the variability of GTP effects in hD_3 receptor constructions is still not clear for the moment.

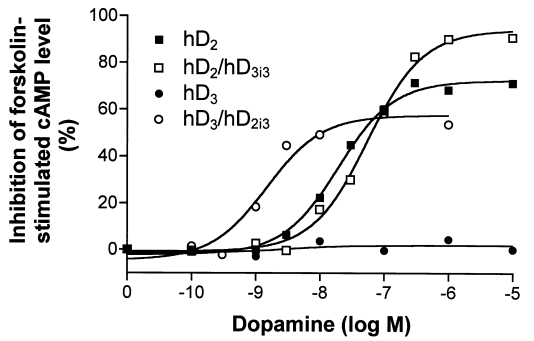

Fig. 4. Representative examples of the inhibition of forskolin-stimulated (10 μM) cAMP levels by increasing concentration of dopamine with hD_2, hD_3, hD_2/hD_{3i3} and hD_3/hD_{2i3} receptors stably transfected in CHO cells. Half-maximal inhibitory concentrations (IC_{50}) and maximal responses (MR) values for this exemple are: hD_2, $IC_{50} = 19$ nM, MR = 72.2%; hD_2/hD_{3i3}, $IC_{50} = 55$ nM, MR = 93.3%; hD_3, not coupled; hD_3/hD_{2i3}, $IC_{50} = 1.5$ nM, MR = 57.0%. Means ± S.E.M are presented in Fig. 5.

Despite the fact that apparent coupling with G proteins (as observed with the GTP shift) is not greatly affected in the chimeras, coupling to AC was dramatically modified (Figs. 4 and 5). The hD_2/hD_{3i3} receptor chimera showed a 3-fold decrease in the IC_{50} of dopamine for inhibition of forskolin-stimulated cAMP levels (Figs. 4 and 5A), as compared to the hD_2 receptor, whereas its maximal response was increased (Figs. 4 and 5B). Although the native hD_3 receptor is not coupled to cAMP pathway in our transfected CHO cell lines, the hD_3/hD_{2i3} receptor chimera construction was fully coupled with AC (even with a higher potency than the hD_2 receptor) (Figs. 4 and 5A). However, the maximal capacity for dopamine to inhibit forskolin-stimulated cAMP levels with the hD_3/hD_{2i3} receptor chimera is lower than that of the hD_2 or hD_2/hD_{3i3} receptors (Figs. 4 and 5B). Basal levels of cAMP were similar for all hD_2 and hD_3 wild-type and mutant receptors (hD_2, 1.2 ± 0.3; hD_3, 2.3 ± 1.1; hD_2/hD_{3i3}, 1.7 ± 0.5 and hD_3/hD_{2i3}, 2.1 ± 0.4 pmol/ml). However, forskolin-stimulated cAMP level (at 10 μM) was significantly higher in the hD_3/hD_{2i3} chimera, as compared to the other constructions (hD_2, 123 ± 11; hD_3, 110 ± 22; hD_2/hD_{3i3}, 110 ± 13 and hD_3/hD_{2i3}, 192 ± 23 pmol/ml, $P < 0.01$). A dose-response curve of forskolin for stimulation of cAMP production was performed. No difference in the EC_{50}(s) of forskolin to stimulate cAMP

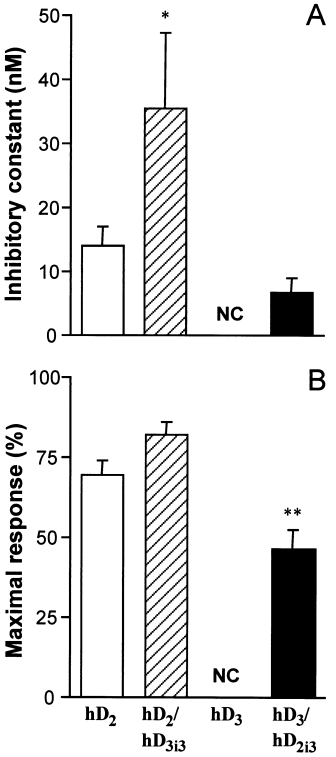

Fig. 5. Effect of receptor chimera constructions on the potency (A) and maximal response (B) for inhibition of forskolin-stimulated (10 μM) cAMP levels by dopamine. The results on potency represent half-maximal inhibitory concentration values (IC_{50} expressed in nM) and maximal inhibitory responses (expressed in % of forskolin-stimulated levels) are means ± S.E.M. obtained from 6–8 independent determinations. $*P < 0.05$ and $**P < 0.01$ vs. hD_2 receptor. NC = not coupled.

production was observed between dopamine wild-types and chimera receptor clones (data not shown).

4. Discussion

Results presented clearly indicate that the amino acid sketch selected for the construction of the chimeras is extremely important in the coupling characteristics showed by dopamine hD_2 and hD_3 receptors. Reciprocal replacements of the selected C-terminal amino acid segment of the i3 loop of these receptors induced minimal modifications of pharmacological profiles of the chimeras with only subtle effects on G protein coupling. However, the potency of the coupling with AC activity of the hD_2/hD_{3i3} receptor chimera, where the 12 amino acid sequence (see Fig. 1) of the hD_2 receptor is replaced by the equivalent sequence of the hD_3 receptor, was reduced (decreased dopamine IC_{50} for inhibition of forskolin-stimulated cAMP levels), whereas the reciprocal chimera (hD_3/hD_{2i3}) shows an inhibition of AC activity with a high potency, but with a lower maximal capacity (the hD_3 wild-type was not coupled to AC in our CHO transfected cells).

The main characteristic of the amino acid sketch selected for construction of the chimeras is that many (9 out of 12 amino acids) amino acid residues carrying charged or polar groups in the hD_2 sequence are replaced at equivalent position by aliphatic or hydrophobic groups in the hD_3 receptor (see Fig. 1). This sequence has been chosen because we were looking for divergent sequences susceptible to explain the difference in coupling properties between these two dopamine receptor homologues. Moreover, the flanking sequence EKKATQ (closed to TMVI) and the N-terminal TSL flanking residues of this segment are conserved in both receptors (Fig. 1). The K(R)EKKATQ residues have been shown to form a regulatory region (including the threonine residue and the BBXB motif, where B is a basic residue) for interaction with G protein and were shown to have conformational effects in the production of constitutive activation of the receptors [27]. The alanine residue (underlined) present in the flanking region of the segment chosen is equivalent to the alanine residue which induces constitutive activity of adrenergic receptors, when mutated [28]. Unsurprisingly, we did not see any evidence of an increase in constitutive activity in our receptor chimeras. On the contrary, the higher capacity of forskolin to stimulated cAMP production in the hD_3/hD_{2i3} chimera and the increase of the maximal response to inhibit forskolin-stimulated cAMP with the hD_2/hD_{3i3} may suggest that we in fact have relieved some pre-existing constitutive activity in both native receptors with the chimera constructions. Results from other sources also suggest that both dopamine D_2 and D_3 receptors may indeed display constitutive activity. For example, in heterologous expression systems, inverse agonist activities of some antagonists have been observed for both receptors [29,30].

The C-terminal portion of the i3 loop of G protein-coupled receptors has been shown to play a critical role in the coupling to second messenger systems of various receptor subtypes [27,31–36], including the D_2 receptor [37]. Our data indicate that this portion in the hD_3 receptor sequence may be, on the contrary, responsible for the absence of coupling with cAMP in CHO cells, because when replaced with the equivalent hD_2 sequence, it induced a coupling with AC activity. We cannot exclude the possibility that the appropriate G protein, which normally couples to the hD_3 receptor, is not expressed in this cell line. But, the similarity of GTP shifts observed with D_3 transfected cell homogenates and brain tissue homogenates containing the D_3 receptor is striking [17]. Introduction of the hD_3 sequence into the hD_2 receptor decrease the affinity of the high affinity state of the hD_2 receptor. This is consistent with the lower potency of this chimera to inhibit cAMP production. Other regions in the hD_2 receptor sequence are possibly involved in the coupling with G_i protein, because we only see a partial reduction in the potency of the receptor to inhibit cAMP production, as compared to the lack of effect of the hD_3 receptor. The hD_3/hD_{2i3} receptor chimera is coupled to inhibition of AC activity with a high potency, but with a lower maximal capacity, as compared to the hD_2 receptor. The potency and maximal response for inhibition of AC activity observed with the hD_3/hD_{2i3} chimera are similar to what is reported for the hD_3 receptor, when a coupling to AC activity is observed, in other heterologous expression systems [10,12].

It has been suggested that the C-terminal portion of the i3 loop located at the junction of TMVI of G protein-coupled receptor form a short α-helix that is extremely important for the interaction with heterotrimeric G proteins [33,36]. It is possible that proline residues (especially the proline residue at position 321) present in this portion of the i3 loop of the hD_3 receptor disrupt the α-helix structure important for interactions with heterotrimeric G proteins. Thus, despite its

general structural organization that suggests a coupling with G_i proteins, which leads to inhibition of AC activity, the hD_3 receptor may not be coupled with this pathway in physiological conditions.

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