Role of the fourth intracellular loop of D1-like dopaminergic receptors in conferring subtype-specific signaling properties

Katerina Tumova, Dongling Zhang, Mario Tiberi*

Ottawa Health Research Institute, Neuroscience Program, and Departments of Medicine/Cellular and Molecular Medicine/Psychiatry, University of Ottawa, Ottawa, ON, Canada

Received 20 July 2004; revised 17 September 2004; accepted 20 September 2004

Available online 6 October 2004

Edited by Sandro Sonnino

Abstract **We investigate whether the fourth intracellular loop (IL4) of D1 and D5 dopaminergic receptors (D1R, D5R) confers D1-like subtype-specific signaling properties. Using chimeric receptors (D1R-IL4B and D5R-IL4A), we show that swapping of IL4 leads to a switch in dopamine affinity and constitutive activity of D1R and D5R. Dopamine potency was reduced for both chimeras in comparison with wild-type receptors. Moreover, dopamine-mediated maximal activation was drastically increased in cells expressing D1R-IL4B when compared with those harboring D5R-IL4A or wild-type receptors. In conclusion, IL4 plays a pivotal role in imparting subtype-specific ligand binding and activation properties to highly homologous seven-transmembrane receptors.**

Keywords: Dopamine; Seven-transmembrane receptor; Helix-8; Ligand binding; Constitutive activation; G protein

1. Introduction

Dopamine (DA) mediates a variety of physiological effects through the activation of seven-transmembrane proteins belonging to the superfamily of G protein-coupled receptors (GPCRs). Five distinct genes encoding the DA receptors have been isolated and classified into D1-like and D2-like receptors based on their ability to activate or inhibit adenylyl cyclase (AC) activity, respectively. In mammals, two genes encoding D1-like dopaminergic receptors are known and referred to as D1 and D5 subtypes (also called D1A and D1B, respectively) [1]. Functionally, the D5 receptor (D5R) differentiates itself from the D1 receptor (D1R) by a higher constitutive activity (agonist-independent activity), increased affinity and potency for agonists, decreased affinity for antagonists/inverse agonists, and a lower agonist-mediated maximal activation [2].

The cytoplasmic tail (CT) plays a crucial role in conferring the subtype-specific DA binding affinities and G protein coupling properties displayed by the D1-like receptors [3,4]. However, the precise CT structural determinants imparting specific functional signatures to the D1-like receptors remain to be identified. Recently, we have observed that the D1R truncated at Cys^{351}, and thus harboring only the N-terminal segment of the CT, displayed an increased DA affinity and constitutive activity suggesting a key role of this CT region in regulating the equilibrium between the inactive and active states of D1R [5]. Interestingly, Cys^{351} of D1R belongs to a group of conserved Cys residues of CT shown to undergo palmitoylation [6]. As Cys palmitoylation may anchor the CT to the membrane, the encompassed N-terminal segment of CT has been proposed to form a fourth intracellular loop (IL4) and implicated in the proper G protein coupling of rhodopsin and β2-adrenergic receptors (β2-AR) [7–10]. Moreover, its unique α-helical conformation, deduced from a crystal structure of rhodopsin, has been postulated to govern the formation of IL4 [11]. As a result, the IL4 segment has been referred to as cytoplasmic α-helix 8 region (H8), whose involvement in GPCR function is gaining increasing support [12–15]. To date, the contribution of the putative IL4 to the function of D1-like dopaminergic receptors has not been explored. Most importantly, the primary structure of D1R and D5R extending from the seventh transmembrane region to two conserved Cys residues displays striking differences that may underlie CT-conferred D1-like receptor-specific functional features [3].

The main focus of the present paper is to investigate the potential role of IL4 in the CT-conferred ligand binding and G protein coupling properties of the D1-like receptors [3,4]. Our results strongly suggest that IL4 is a major structural determinant underlying the CT-conferred D1-like subtype-specific functional signatures.

2. Materials and methods

2.1. Drugs

N-[methyl-^{3}H]-SCH23390 (82 Ci/mmol), [^{3}H]-adenine (27 Ci/mmol), and [^{14}C]-cAMP (252 mCi/mmol) were from Amersham Pharmacia Biotech (Baie d'Urfé, Québec, Canada). DA, *cis*-flupentixol (+)-butaclamol, SCH23390 (SCH) and 1-methyl-3-isobutylxanthine were from Sigma/Research Biochemicals International (Oakville, Ontario, Canada).

* Corresponding author. Address: Ottawa Health Research Institute, Moses and Rose Loeb Research Centre, 725 Parkdale Avenue, Ottawa, ON, Canada K1Y 4E9. Fax: +1-613-761-5365.
E-mail address: mtiberi@ohri.ca (M. Tiberi).

Abbreviations: AC, adenylyl cyclase; AR, adrenergic receptor; B_{max}, maximal binding capacity; CT, cytoplasmic tail; DA, dopamine; GPCR, G protein-coupled receptor; H8, α-helix 8 region; HEK293, human embryonic kidney 293; IL4, fourth intracellular loop; MEM, minimal essential medium; PBS, phosphate-buffered saline; PCR, polymerase chain reaction; TM7, transmembrane region 7

0014-5793/$22.00
doi:10.1016/j.febslet.2004.09.059

2.2. Construction of chimeric human D1 and D5 receptors

To construct the IL4 chimeric receptors (Fig. 1), we took advantage of existing chimeras (D1R-CTB and D5R-CTA) in which the entire CT was swapped between the human D1R and D5R [3]. In these newly generated chimeras, all residues downstream of the second Cys of CT (Cys^{351} in D1 and Cys^{379} in D5) were restored to wild-type primary structure, leaving only IL4 in a mutated form (Fig. 1). In case of the chimeric D1R-IL4B receptor, the CT of D5R was terminated at Cys^{379}, followed by D1R residues to complete the CT. In case of the D5R-IL4A chimera, the CT of the D1R was terminated at Cys^{351}, followed by D5R residues for completion of the CT. The swap of the IL4 between the D1R and D5R was achieved by gene splicing using a PCR-based overlap extension method [16]. With respect to the D1R-IL4B chimera, the first round of PCR generated two fragments: the first fragment encoding the C-terminal portion of third intracellular loop, transmembrane region 6, third extracellular loop and transmembrane region 7 (TM7) of D1R and IL4 of D5R (using the D1R-CTB chimera as template), and the second fragment coding for CT residues of D1R located downstream of IL4 (using the D5R-CTA chimera as template). The first fragment was amplified using primers 5′-CACCACAGGTAATGGAAA-3′ (forward primer) and 5′-GCAGAAGTGGCTGCACCCCAG-3′ (reverse primer). The second fragment was amplified using primers 5′-TGCAGCCACTTCTGCCC-TGCGACGAATAAT-3′ (forward primer) and 5′-TGCAACTTA-ATTTTATTA-3′ (reverse primer). Likewise, with respect to the construction of D5R-IL4A chimera, the first round of PCR generated two fragments: the first coding the N-terminus of the D5R up to IL4 of D1R (using the D5R-CTA chimera as template) and the second fragment coding the CT residues of the D5R located downstream of Cys^{379} (using the D1R-CTB chimera as template). The first fragment was amplified using primers 5′-TACGGTGGGAGG-3′ (forward primer) and 5′-GCAAAGTCTGTAGCATCCTAAGAGGGT-3′ (reverse primer). The second fragment was amplified using primers 5′-TGCTACAGACTTTGCTCCCGCACGCCGGTG-3′ (forward primer) and 5′-TGCAACTTAATTTTATTA-3′ (reverse primer). To facilitate the construction and identification of the chimeric receptors, a silent mutation was introduced in each construct to create a unique restriction endonuclease site. For D1R-IL4B chimera, a *Mlu*I site was introduced at nucleotide sequence corresponding to amino acid residues 331 and 332 near the 3′ end of TM7 of D1R, immediately upstream of the IL4 sequence of D5R (5′-TA**T**GC**C**-3′ → 5′-TA**C**GC**G**-3′: modified nucleotides are bold and underlined). For D5R-IL4A chimera, a *Hin*dIII restriction site was introduced at nucleotide sequence corresponding to residues 367 and 368 located 3′ of the junction between the TM7 of D5R and IL4 sequence of D1R (5′-AA**G**GC**A**-3′ → 5′-AA**A**GC**T**-3′: modified nucleotides are bold and underlined). Amplified fragments were separated on a 1% agarose gel and appropriate bands were excised and purified by QIAEX II gel extraction method (Qiagen, Valencia, CA). Diluted aliquots of the paired fragments were combined and subjected to overlap PCR using appropriate 5′ and 3′ flanking primers. The resulting PCR products were introduced into the expression vector pCMV5. The D1R-IL4B fragment was subcloned into D1R-CTB/pCMV5 expression construct using restriction enzymes *Mlu*I and *Xba*I. The D5R-IL4A fragment (*Hin*dIII) was subcloned into linearized pCMV5 (*Kpn*I–*Hin*dIII) together with the N-terminal portion of D5R-CTA (*Kpn*I–*Hin*dIII) in a 3-piece ligation process. The nucleotide sequence of PCR products and cloning sites was confirmed by dideoxy sequencing using Sequenase version 2.0 from Amersham Pharmacia Biotech (Baie d'Urfé, Québec, Canada).

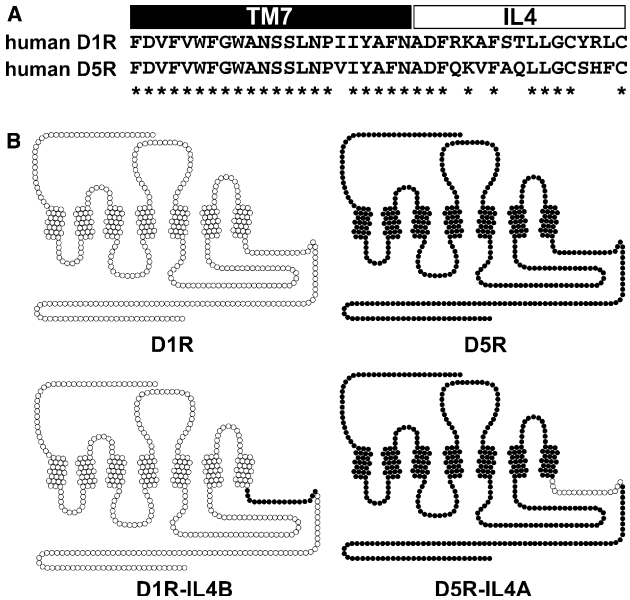


Fig. 1. Primary structure of the putative fourth intracellular loop (IL4) of the human D1-like subtypes and schematic representation of wild-type and chimeric receptors. (A) Alignment of the primary structure of transmembrane region 7 (TM7) and IL4 between human D1R and D5R. Identical residues found between the two primary structures are indicated with an *asterisk*. The TM7 and IL4 are delimited by black and white rectangles. (B) Putative topology of the wild-type D1R (*open circles*) and D5R (*filled circles*), D1R-IL4B and D5R-IL4B chimeras is depicted.

2.3. Cell culture and transfection

Human embryonic kidney 293 (HEK293) cells (American Type Culture Collection, Manassas, VA, USA) were cultured at 37 °C and 5% CO_2 in minimal essential medium (MEM) supplemented with 10% heat-inactivated fetal bovine serum (FBS) and gentamicin (10 μg/ml) (Invitrogen, Burlington, Ontario, Canada). A modified calcium phosphate-mediated transfection of cells (2.5×10^6 cells/dish) was carried out as described [17]. Briefly, 100 μl of 2.5 M $CaCl_2$ was added to a plastic tube containing 10 μg of DNA in 900 μl of sterile milli-Q-water and mixed. Then, 1 ml of 2× HEPES-buffered saline (HBS), pH 7.1 (0.28 M NaCl, 0.05 M HEPES, pH 7.0, and 1.5 mM Na_3PO_4, pH 7.1), was added dropwise to the DNA-calcium solution, gently mixed by flicking the tube and used to transfect two 100-mm dishes at a time (5 μg/dish). Typically, 1 ml of transfection mixture was added dropwise to a 100 mm-dish of cells containing 10 ml of complete MEM. When less than 5 μg receptor DNA per dish was used in transfections, empty pCMV5 vector was added to normalize the total amount of DNA. Cells were incubated with the DNA-calcium phosphate precipitate overnight prior to reseeding. All experiments were performed with cells from 38 to 52 passages.

2.4. Membrane preparation

Transfected HEK293 cells were washed with phosphate-buffered saline (PBS), trypsinized, reseeded in 150-mm dishes and grown for an additional 48 h. Crude membrane preparations expressing wild-type or chimeric receptors were prepared as previously described [18]. Membrane preparations were either used immediately (saturation studies) or frozen in liquid nitrogen and stored at −80 °C until needed (competition studies).

2.5. Receptor binding assays

Membranes were resuspended in binding buffer (final concentrations in the binding assay: 50 mM Tris–HCl, pH 7.4, 120 mM NaCl, 5 mM KCl, 4 mM $MgCl_2$, 1.5 mM $CaCl_2$, and 1 mM EDTA) using a Brinkmann Polytron. Binding assays were carried out with 100 μl of membranes in a total volume of 500 μl using [^{3}H]-SCH23390 as radioligand. Saturation studies were performed with fresh membranes and increasing concentrations of [^{3}H]-SCH23390 (0.01–6 nM). Non-specific binding was determined by the addition of 10 μM *cis*-flupentixol (dissolved in milli-Q-water). Competition studies were done using frozen membranes thawed on ice. Membrane preparations were incubated with a fixed concentration of [^{3}H]-SCH23390 (∼0.6 nM) and increasing concentrations of competing ligand. Competition studies using DA were done in the presence of 0.1 mM ascorbic acid (dissolved in milli-Q-water). Membranes were incubated for 90 min at room temperature and binding assays stopped using rapid filtration through glass fiber filters (GF/C, Whatman) as described before [18]. Radioactivity bound to filters was measured using liquid scintillation counting (Beckman Counter, LS 6500). Protein concentration was assessed using the Bio-Rad assay kit with bovine serum albumin as standard.

2.6. Whole cell cAMP assays

Transfected HEK293 cells were reseeded in 6- or 12-well dishes and whole cell cAMP assays performed in the presence of 1-methyl-3-isobutylxanthine to assess the regulation of AC activity by wild-type and chimeric receptors as described previously [2,18]. Intracellular [^{3}H]-cAMP was purified by sequential chromatography using Dowex (AG 50W-X4) and alumina columns as described previously [19]. The amount of intracellular [^{3}H]-cAMP (CA) over the total amount of intracellular [^{3}H]-adenine (TU) was computed to determine the relative AC activity (CA/TU$\times$1000). The maximal binding capacity (B_{max}) values were determined using a saturating concentration of [^{3}H]-SCH23390 (~6 nM).

2.7. Statistics

Saturation and competition binding isotherms were analyzed using the non-linear curve-fitting program LIGAND to determine the equilibrium dissociation constant or K_d (expressed as the reciprocal transformation of affinity constants) and B_{max} values [20]. A simultaneous fitting of dose-response curves was performed with ALLFIT [21] using unconstrained and constrained (shared) parameters (bottom or basal activity, top or maximal stimulation, effective concentration that elicits 50% of maximal response or EC_{50}, slope factor). This was done to establish whether differences observed between best-fitted values were statistically different, i.e., whether constraining a specific curve parameter to share a common value worsens the goodness of fit. K_d values are reported using the geometric mean $\pm$ standard error (S.E.). The S.E. of geometric means were computed using the value of geometric average multiplied by the S.E. of the averaged logarithmic transform [22]. All other data are expressed as arithmetic means $\pm$ S.E. unless stated otherwise. Homogeneity of variances was determined using F_{max} or Bartlett tests prior to statistical analyses [23]. One sample *t*-test and analysis of variance (one-way ANOVA) with Newman–Keuls multiple comparison test were performed using GraphPad Prism version 4.0 for Windows, GraphPad Software, San Diego, CA, USA, www.graphpad.com. The level of significance was established at $P < 0.05$.

3. Results

3.1. IL4 modulates ligand binding properties and receptor expression of D1-like subtypes

Our results suggest that substitution of IL4 does not impair the ability of the chimeric receptors (Fig. 1) to bind with high affinity the benzazepine SCH23390 (Table 1), a selective D1-like ligand behaving as a partial agonist in heterologous cells [2,24]. Binding affinities of SCH23390 at both chimeras were significantly increased in comparison with their respective parental receptors while retaining the wild-type pharmacological profile, i.e., a lower SCH23390 affinity constant for the D5R (Table 1). A similar trend was observed with competition studies (Table 2).

Upon the reciprocal exchange of the IL4 region, the D1R-IL4B chimera displayed an increased affinity for DA that was reminiscent of the affinity for wild-type D5R. On the other hand, the D5R-IL4B chimera displayed a partially reduced DA affinity in comparison with the wild-type D5R (Table 2). The exchange of the IL4 segment between D1R and D5R did not switch the subtype-specific inverse agonist affinities (Table 2). Although slightly increased, the affinities of both chimeras for *cis*-flupentixol were not statistically different from their respective parent receptors. Similar trend was also observed with ((+)-butaclamol) affinity, where the increase in affinity was greater and statistically significant for both chimeras compared with their respective parent receptors.

Moreover, our saturation studies revealed a drastic decrease (~20- to 30-fold) in the B_{max} of [^{3}H]-SCH23390 in cells expressing D1R-IL4B (Table 1). In contrast, the B_{max} of [^{3}H]-SCH23390 in cells expressing D5R-IL4A chimera remained unchanged in comparison with its wild-type D5R counterpart.

3.2. IL4 underlies the CT-conferred constitutive activity phenotype of D1-like receptors

The role of IL4 in CT-conferred D1-like subtype-specific agonist-independent and dependent activation of AC properties was assessed in intact transfected HEK293 cells under low and high receptor expression. Using cells expressing low and comparable receptor levels (~1–2 pmol/mg protein), dose- response curves were performed to determine DA potency. Additional inferences regarding the basal and maximal activation of AC under the low receptor expression condition were drawn from ALLFIT best-fitted values using unconstrained and constrained simultaneous curve fitting (Fig. 2A and E). In contrast, the high receptor expression condition (maximum) was achieved following a transfection with 5 μg DNA to evaluate basal activity and DA-mediated maximal activation of AC was measured under the high expression condition (Fig. 2C and D).

The D5R displayed higher agonist-independent activity in comparison with the D1R (Fig. 2B and C) as reported previously [2,3,25]. Substitution of the variant IL4 residues produced a decrease of the agonist-independent activation of D5R-IL4A chimera in comparison with the wild-type D5R and beyond the levels measured for the D1R (Fig. 2B and C). Furthermore, under comparable low but not high B_{max} values, we have shown that D1R-IL4B exhibited a significant increase in constitutive activity when compared with the wild-type D1R (Fig. 2B and C). These findings are consistent with the lower B_{max} value of D1R-IL4B obtained under conditions leading to high receptor expression and the linear relationship existing between receptor density and constitutive activity. Importantly, the constitutive activity of D1R-IL4B chimera was indistinguishable from the wild-type D5R when assessed at a similar low receptor expression (Fig. 2B and C).

3.3. IL4 modulates dopamine-mediated activation of AC by D1-like receptors

We next examined the DA-mediated maximal stimulation of AC in cells expressing comparable levels (low expression) of wild-type and chimeric receptors using the best-fitted maximal

Table 1
Dissociation constants (K_d) and maximal binding capacity (B_{max}) values of N-[methyl-^{3}H]-SCH23390 for wild-type and chimeric D1-like receptors

Receptor	K_d (nM)	B_{max} (pmol/mg prot.)
D1R	0.44 ± 0.02	11.8 ± 2.4
	(0.35–0.55)	(100)
D5R	$0.81 \pm 0.02^{*}$	16.8 ± 2.1
	(0.70–0.94)	(160 ± 20)
D1R-IL4B	$0.14 \pm 0.01^{*,\#}$	$0.59 \pm 0.14^{*,\#}$
	(0.09–0.24)	(5.0 ± 0.7)
D5R-IL4A	$0.64 \pm 0.03^{*,\psi}$	$16.0 \pm 1.0^{\psi}$
	(0.49–0.83)	(166 ± 32)

K_d and B_{max} values are expressed as geometric and arithmetic means $\pm$ S.E., respectively. B_{max} values are also expressed as percent of D1R. Lower and upper 95% of confidence intervals of geometric means are shown in brackets. Means are from six experiments done in duplicate determinations.

$^{\psi}P < 0.05$ when compared with D1R-IL4B.

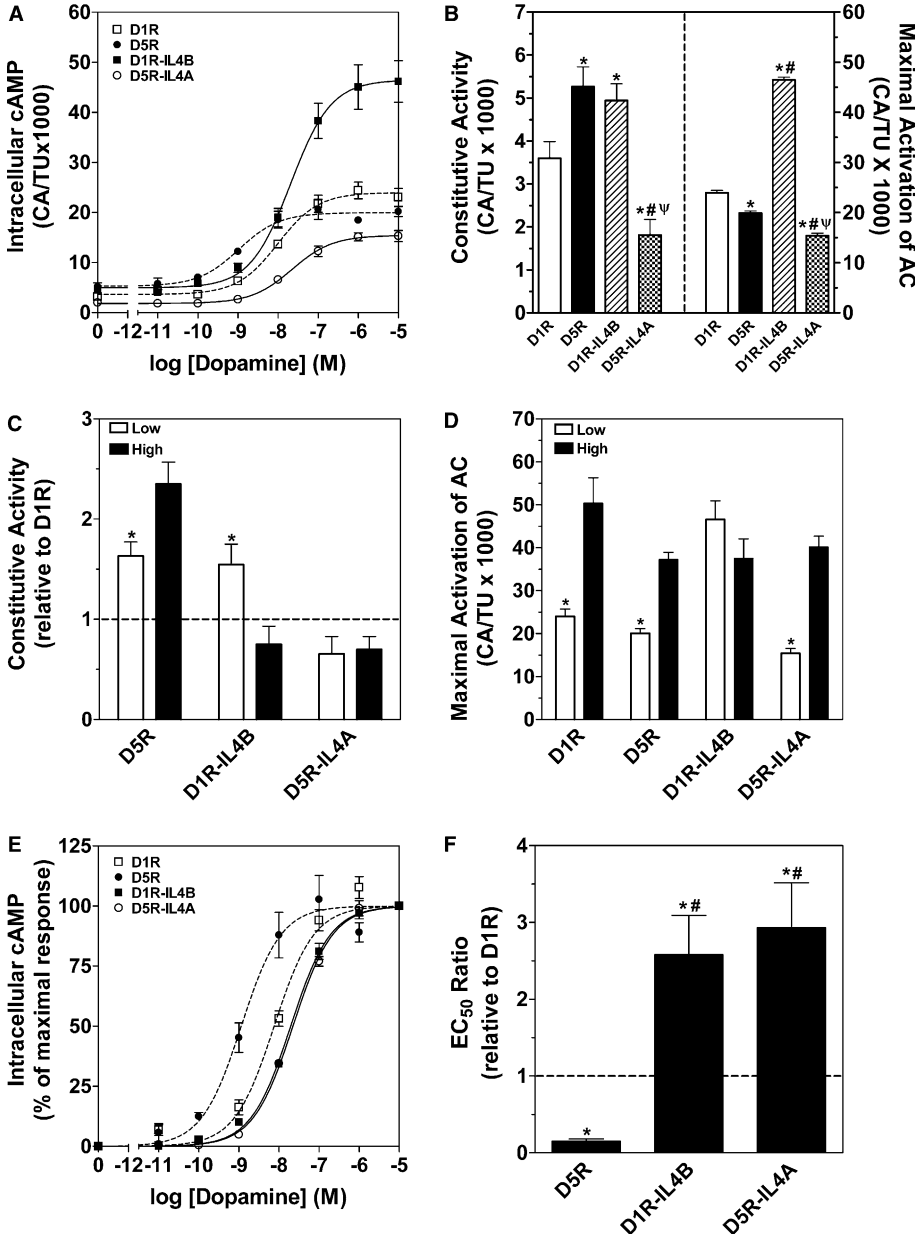
$^{\#}P < 0.05$ when compared with D5R.

$^{*}P < 0.05$ when compared with D1R.

Table 2
Dissociation constants (K_d) for binding of dopaminergic ligands to wild-type and chimeric D1-like receptors

Receptor	SCH22390 (nM)	Dopamine (nM)	*cis*-Flupentixol (nM)	(+)-Butaclamol (nM)
D1R	0.43 ± 0.03	7953 ± 422	2.51 ± 0.32	3.97 ± 0.29
	(0.20–0.94)	(4704–13447)	(0.71–8.81)	(1.91–8.23)
D5R	$0.86 \pm 0.09^{*}$	$602 \pm 7^{*}$	$6.76 \pm 0.4^{*}$	$40.9 \pm 3.82^{*}$
	(0.30–2.43)	(538–675)	(3.78–12.1)	(16.2–103)
D1R-IL4B	$0.18 \pm 0.001^{*,\#}$	$559 \pm 56^{*}$	$1.38 \pm 0.08^{\#}$	$1.56 \pm 0.08^{*,\#}$
	(0.16–0.20)	(207–1513)	(0.76–2.52)	(0.37–6.61)
D5R-IL4A	$0.59 \pm 0.02^{\psi}$	$2488 \pm 170^{*,\#,\psi}$	$5.11 \pm 0.29^{*,\psi}$	$23.8 \pm 0.85^{*,\#,\psi}$
	(0.45–0.76)	(1265–4895)	(2.94–8.88)	(16.7–34.0)

K_d are expressed as geometric means ± S.E. Means are from two to three experiments done in duplicate determinations. Lower and upper 95% of confidence intervals of geometric means are shown in brackets.
$^{\psi}P < 0.05$ when compared with D1R-IL4B.
$^{\#}P < 0.05$ when compared with D5R.
$^{*}P < 0.05$ when compared with D1R.



response values obtained from dose-response curves analyzed by ALLFIT. As shown in Fig. 2B, DA-mediated maximal stimulation of AC was significantly greater in cells expressing the D1R than those expressing the D5R. Interestingly, upon exchange of the IL4 region, the D1R-IL4B chimera exhibited a significant increase in DA-mediated maximal activation of AC when compared with the wild-type D1R (Fig. 2B). Conversely, D5R-IL4A chimera displayed a significant decrease of the DA-mediated maximal activation of AC when compared with the wild-type D5R (Fig. 2B). An investigation of DA-mediated maximal stimulation of AC by the wild-type and chimeric D1-like receptors under the maximal (high) expression condition was carried out to assess further the role of IL4 in regulating DA efficacy. Statistically significant differences in DA-mediated maximal activation of AC were observed between low and high expression conditions for wild-type receptors and D5R-IL4A (Fig. 2D). Under the high expression condition, D1R-IL4B chimera displayed a DA-mediated maximal activation of AC akin to that of D5R and D5R-IL4A, despite an approximately 20-fold decrease in B_{max} (Fig. 2D). These results suggest that the coupling efficiency of D1R-IL4B to G_s/AC pathways is significantly increased in comparison with D1R, D5R and D5R-IL4A.

The functional role of IL4 in the CT-induced changes in DA potency (as indexed using EC_{50} values) was also tested. DA potency was significantly greater in HEK293 cells expressing D5R than D1R (Fig. 2E and F) as previously reported [2,3,25]. Substitution of the variant IL4 residues produced a significant loss of DA potency for both receptors (Fig. 2E and F). More specifically, in comparison with their respective parent receptors, D1R-IL4B and D5R-IL4A displayed a significant 2.5- and 19-fold decrease in DA potency, respectively (Fig. 2E and F).

4. Discussion

In the study reported here, we have narrowed down the CT structural determinant responsible for D1-like subtype-specific functional properties to the N-terminal segment of CT (Fig. 1), a region also known as IL4 or H8 in rhodopsin [11,13]. Our results suggest that variant residues found within IL4 region of D1-like receptors contribute to the more and less constrained DA binding conformation displayed by D1R and D5R, respectively. However, our DA binding data with D5R-IL4A fall short of the results obtained with a chimeric D5R harboring the entire CT of D1R, which exhibited a full switch in DA affinity [3,4]. Thus, the spatial relationships induced by IL4 on D1R and D5R are not reciprocally conferred to the chimeric receptors, suggesting that IL4 sequence of D1R is not the sole CT structural determinant involved in regulating DA affinity at the D1R. There are several lines of evidence supporting this assertion. Previously, we have reported that a truncated form of the D1R ending with the IL4 region displayed an increased DA affinity, suggesting that sequences downstream of the IL4 partake in the formation of a more constrained D1R [5]. Likewise, the third extracellular loop of D1R can impart on its own a more constrained conformation to the D5R, an effect that is exacerbated by swapping the entire CT sequences with those coding for the D1R [18,25]. Alternatively, the CT residues of D5R left in the D5R-IL4A chimera may interfere with the constraining effects exerted by IL4 structural determinants of D1R.

Another aspect of our study is the lack of an affinity switch of inverse agonists upon IL4 exchange. These results mirror findings obtained upon the swap of the entire CT and support the notion that the subtype-specific inverse agonist binding

Fig. 2. DA-independent and dependent G protein coupling to AC activation of wild-type and chimeric D1-like receptors expressed in HEK293 cells. (A) HEK293 cells were transfected with wild-type or chimeric D1-like receptors using the following amounts of DNA per dish: 0.02 μg of D1R, and 0.02 μg of D5R, 5.0 μg of D1R-IL4B, and 0.02 μg of D5R-IL4A. The B_{max} values in picomole/mg of membrane protein (expressed as the arithmetic mean ± S.E.) were 2.8 ± 0.3 (D1R), 2.6 ± 0.4 (D5R), 0.6 ± 0.1 (D1R-IL4B), and 1.3 ± 0.2 (D5R-IL4A). Intracellular cAMP levels were measured in single wells of a 12-well dish in the absence or presence of increasing concentrations of DA as described under Section 2 and plotted as a function of log of DA concentrations. Each point is the arithmetic mean ± S.E. of four experiments done in triplicate determinations and expressed as [^{3}H]-cAMP formed (CA) over the total amount of [^{3}H]-adenine uptake (TU) × 1000. (B) Averaged curves (shown in A) were analyzed by simultaneous curve-fitting using ALLFIT. Statistical significance was determined using unconstrained and constrained (shared) parameters. The results for constitutive activity (best-fitted parameters for minimal response) and DA-mediated maximal activation of AC (best-fitted parameters for maximal response) of wild-type and chimeric D1-like receptors are shown in *left* and *right panels*, respectively. Results are expressed as values ± approximate S.E. as obtained by ALLFIT. $^*P < 0.05$ when compared with D1R; $^{\#}P < 0.05$ when compared with D5R; $^{\psi}P < 0.05$ when compared with D1R-IL4B. (C) Constitutive activity of D5R, D1R-IL4B and D5R-IL4A relative to D1R (set as 1, broken line) was determined under transfection conditions leading to low receptor expression (as described in A) and high (maximal) receptor expression (using 5 μg of receptor DNA construct). For the low receptor expression condition, intracellular cAMP levels measured in single wells of a 12-well dish in the absence or presence of increasing concentrations of DA were expressed relative to D1R basal and individual dose-response curves analyzed using ALLFIT to obtain the best-fitted value for relative constitutive activity of D5R and chimeric receptors. For the high receptor condition, constitutive and maximal activation of AC activity were determined in single wells of a 6-well dish using whole cell cAMP assays in the absence of DA. Results are expressed as arithmetic means ± S.E. of three (high) to four (low) experiments done in triplicate determinations. Statistical analysis was done using one-way ANOVA with Newman–Keuls post test. For the low receptor expression, the B_{max} values were as stated in A. For the high receptor expression, the B_{max} values in picomole/mg of membrane proteins (expressed as the arithmetic mean ± S.E.) were 12.6 ± 2.1 (D1R), 13.6 ± 0.3 (D5R), 0.6 ± 0.2 (D1R-IL4B), and 10.1 ± 1.2 (D5R-IL4A). $^*P < 0.05$ when compared with high receptor expression. (D) DA-mediated maximal activation of wild-type and chimeric D1-like receptors was assessed under low and high receptor expression. For the low receptor expression condition, DA-mediated maximal activation was obtained from dose-response curves analyzed individually using ALLFIT. For the high receptor expression, maximal activation of AC was assessed using single wells of a 6-well dish in the presence of 10 μM DA. Results are expressed as arithmetic means ± S.E. of three (high) to four (low) experiments done in triplicate determinations. Statistical analysis was performed using one-way ANOVA with Newman–Keuls post test. The B_{max} values for the low and high receptor expression were as stated in A and C. $^*P < 0.05$ when compared with high receptor expression. (E) The percentage of DA-mediated maximal activation obtained with respective wild-type or chimeric receptor after subtracting the basal activity was calculated to normalize dose-response curves shown in A. Normalized curves were analyzed by simultaneous curve-fitting using ALLFIT as described in Section 2. Statistical significance was determined using unconstrained and constrained (shared) parameters. The EC_{50} values ± approximate S.E. as obtained by ALLFIT are as follows (in nM): 7.6 ± 1.1 (D1R), 1.2 ± 0.2 (D5R), 19.6 ± 2.8 (D1R-IL4B), and 22.3 ± 3.2 (D5R-IL4A). (F) EC_{50} ratios of D5R and chimeric receptors relative to the D1R (broken line). $^*P < 0.05$ when compared with D1R; $^{\#}P < 0.05$ when compared with D5R.

profile is most likely independent of CT-induced conformational changes [3]. Meanwhile, the increased inverse agonist affinity observed at both chimeric receptors suggests a potential role of IL4 region in regulating high affinity binding of this pharmacological class of dopaminergic ligands.

The functional characterization of our chimeras suggests that agonist-independent and dependent G protein coupling properties of the D1-like receptors are differentially regulated by IL4. Our studies demonstrated a reversal of agonist-independent activation of AC upon a swap of IL4 between D1R and D5R implying a role of this cytoplasmic region in the regulation of the thermodynamic equilibrium between active and inactive conformations of D1-like receptors. A recent study has implicated IL4 (referred therein as H8) in regulating the conformational change of leukotriene B4 receptor to the low affinity state following G protein activation [26]. Furthermore, critical conformational changes of IL4 regulating potentially the transition between inactive and active states of rhodopsin have recently been demonstrated [27]. In fact, IL4 has been proposed to function as a conformational switch alternating between a helical conformation (inactive state) and a looplike structure (active state) in response to changes in membrane environment [27]. Likewise, a similar concept of a helix to coil transition has been put forward as a possible molecular mechanism underlying G protein coupling of angiotensin II AT_{1A} receptors [28]. Whether a similar role is performed by the IL4 of D1R and D5R remains to be investigated further.

Additionally, IL4 appears to exert a distinct influence on the formation of D1-like receptor active states upon DA binding. In agreement with our previous study highlighting the chimeric substitution of the full CT, the exchange of IL4 between D1R and D5R produced a loss of DA potency in both chimeras. The decrease in DA potency is particularly intriguing for D1R-IL4B, in light of its increased DA binding affinity. However, we have recently shown that the third extracellular loop is required for the CT-induced full switch in DA potency [3,5,18]. Likewise, the third extracellular loop may also act in concert specifically with IL4 to impart the subtype-specific DA potency to D1-like receptors. Notwithstanding the potential molecular interplay between the third extracellular loop and IL4, similar uncoupling of DA affinity and potency has been observed in our earlier studies and suggests the formation of multiple active D1-like conformational states [3,5,18].

New evidence from rhodopsin studies [29,30] also suggests that differences in the primary structure of IL4 of D1R and D5R may underlie some of the subtype-specific G protein coupling properties of D1-like receptors. In rhodopsin, IL4 has been implicated in binding of G protein α and γ subunits [29,30], and thus D1-like receptors may use IL4 to bind to distinct G protein subunits. In agreement with this view, a differential dependence of D1R and D5R on the G protein γ subunit for activation of AC has been shown [31]. Alternatively, post-translational modifications may also play an important role in controlling the responsiveness of wild-type and chimeric D1-like receptors under study. Indeed, palmitoylation of Cys and PKA phosphorylation taking place in or around the H8 region have been shown to regulate GPCR desensitization [6,32–34]. Moreover, IL4-dependent mechanisms may also be important in regulating B_{max} values, thus influencing receptor responsiveness. In our study, D1R-IL4B displays a drastically reduced B_{max}, a finding in line with a similar observation in cells expressing a chimeric D1R harboring the entire CT of D5R [3,18]. Our preliminary studies show that D1R and D5R are exclusively expressed at the plasma membrane, while D1R-IL4B is detected at the cell surface and intracellularly (Zhang and Tiberi, data not shown). Intracellular localization of D1R-IL4B may point toward an increased ER retention and/or sorting to degradation pathways leading to B_{max} reduction. Additionally, increased agonist-independent activity of D1R-IL4B may promote its structural instability, constitutive desensitization and internalization as seen with other GPCRs [35–38]. Since our G protein coupling studies were done at similar B_{max} values for wild-type and chimeric receptors, it remains to be clearly established if under our experimental conditions these cellular processes contribute significantly to D1R-IL4B signaling properties (e.g., we may underestimate the extent of G protein coupling in the absence of agonist).

Taken together, the results of our study suggest an important contribution of IL4 in conferring the distinct DA binding and G protein coupling properties to D1-like receptors. Although initial reports regarding the role of IL4 in GPCR function were contradictory [7,39–41], several studies now suggest that this cytoplasmic region is important for GPCR binding to and activation of heterotrimeric G proteins [9,10,26,29,30,42–44]. Our study now shows for the first time that differences in the primary structure of IL4 may underlie the structural basis for subtype-specific ligand binding and G protein-coupling properties of highly homologous members belonging to a seven-transmembrane receptor subfamily.

Acknowledgements: This work was supported by an operating grant (#203694) from the Natural Sciences and Engineering Research Council (NSERC) of Canada to MT. KT was the recipient of an Ontario Graduate Scholarship in Science and Technology (OGSST) from the Government of Ontario and Ottawa Health Research Institute. We thank Marie-France Nantel, Amy Slater and Kimberly Threader for technical assistance with cell culture and preparation of reagents. We thank members of Tiberi's lab for critically reading the manuscript.

References

[1] Missale, C., Nash, S.R., Robinson, S.W., Jaber, M. and Caron, M.G. (1998) Physiol. Rev. 78, 189–225.
[2] Tiberi, M. and Caron, M.G. (1994) J. Biol. Chem. 269, 27925–27931.
[3] Jackson, A., Iwasiow, R.M. and Tiberi, M. (2000) FEBS Lett. 470, 183–188.
[4] Demchyshyn, L.L., McConkey, F. and Niznik, H.B. (2000) J. Biol. Chem. 275, 23446–23455.
[5] Chaar, Z.Y., Jackson, A. and Tiberi, M. (2001) J. Neurochem. 79, 1047–1058.
[6] Jin, H., Xie, Z., George, S.R. and O'Dowd, B.F. (1999) Eur. J. Pharmacol. 386, 305–312.
[7] Konig, B., Arendt, A., McDowell, J.H., Kahlert, M., Hargrave, P.A. and Hofmann, K.P. (1989) Proc. Natl. Acad. Sci. USA 86, 6878–6882.
[8] O'Dowd, B.F., Hnatowich, M., Caron, M.G., Lefkowitz, R.J. and Bouvier, M. (1989) J. Biol. Chem. 264, 7564–7569.
[9] O'Dowd, B.F., Hnatowich, M., Regan, J.W., Leader, W.M., Caron, M.G. and Lefkowitz, R.J. (1988) J. Biol. Chem. 263, 15985–15992.
[10] Liggett, S.B., Caron, M.G., Lefkowitz, R.J. and Hnatowich, M. (1991) J. Biol. Chem. 266, 4816–4821.
[11] Palczewski, K. et al. (2000) Science 289, 739–745.
[12] Ballesteros, J.A., Shi, L. and Javitch, J.A. (2001) Mol. Pharmacol. 60, 1–19.

[13] Sakmar, T.P. (2002) Curr. Opin. Cell Biol. 14, 189–195.
[14] Choi, G., Landin, J. and Xie, X.Q. (2002) J. Pept. Res. 60, 169–177.
[15] Katragadda, M., Maciejewski, M.W. and Yeagle, P.L. (2004) Biochim. Biophys. Acta 1663, 74–81.
[16] Horton, R.M., Cai, Z.L., Ho, S.N. and Pease, L.R. (1990) Biotechniques 8, 528–535.
[17] Gorman, C (1985) (Glover, D.M., Ed.). IRL, Washington, pp. 143–165.
[18] Tumova, K., Iwasiow, R.M. and Tiberi, M. (2003) J. Biol. Chem. 278, 8146–8153.
[19] Johnson, R.A. and Salomon, Y. (1991) Methods Enzymol. 195, 3–21.
[20] Munson, P.J. and Rodbard, D. (1980) Anal. Biochem. 107, 220–239.
[21] De Léan, A., Munson, P.J. and Rodbard, D. (1978) Am. J. Physiol. 235, E97–E102.
[22] De Léan, A., Hancock, A.A. and Lefkowitz, R.J. (1982) Mol. Pharmacol. 21, 5–16.
[23] Sokal, R. R., Rohlf, F.J. (1981) W.H. Freeman and Co., New York.
[24] Sugamori, K.S., Scheideler, M.A., Vernier, P. and Niznik, H.B. (1998) J. Neurochem. 71, 2593–2599.
[25] Iwasiow, R.M., Nantel, M.F. and Tiberi, M. (1999) J. Biol. Chem. 274, 31882–31890.
[26] Okuno, T., Ago, H., Terawaki, K., Miyano, M., Shimizu, T. and Yokomizo, T. (2003) J. Biol. Chem. 278, 41500–41509.
[27] Krishna, A.G., Menon, S.T., Terry, T.J. and Sakmar, T.P. (2002) Biochemistry 41, 8298–8309.
[28] Franzoni, L., Nicastro, G., Pertinhez, T.A., Tato, M., Nakaie, C.R., Paiva, A.C., Schreier, S. and Spisni, A. (1997) J. Biol. Chem. 272, 9734–9741.
[29] Marin, E.P., Krishna, A.G., Zvyaga, T.A., Isele, J., Siebert, F. and Sakmar, T.P. (2000) J. Biol. Chem. 275, 1930–1936.
[30] Ernst, O.P., Meyer, C.K., Marin, E.P., Henklein, P., Fu, W.Y., Sakmar, T.P. and Hofmann, K.P. (2000) J. Biol. Chem. 275, 1937–1943.
[31] Wang, Q., Jolly, J.P., Surmeier, J.D., Mullah, B.M., Lidow, M.S., Bergson, C.M. and Robishaw, J.D. (2001) J. Biol. Chem. 276, 39386–39393.
[32] Moffett, S., Adam, L., Bonin, H., Loisel, T.P., Bouvier, M. and Mouillac, B. (1996) J. Biol. Chem. 271, 21490–21497.
[33] Hukovic, N., Panetta, R., Kumar, U., Rocheville, M. and Patel, Y.C. (1998) J. Biol. Chem. 273, 21416–21422.
[34] Moffett, S., Rousseau, G., Lagacé, M. and Bouvier, M. (2001) J. Neurochem. 76, 269–279.
[35] Lee, T.W., Cotecchia, S. and Milligan, G. (1997) Biochem. J. 325, 733–739.
[36] Gether, U., Ballesteros, J.A., Seifert, R., Sanders-Bush, E., Weinstein, H. and Kobilka, B.K. (1997) J. Biol. Chem. 272, 2587–2590.
[37] Barak, L.S., Oakley, R.H., Laporte, S.A. and Caron, M.G. (2001) Proc. Natl. Acad. Sci. USA 98, 93–98.
[38] Wilbanks, A.M., Laporte, S.A., Bohn, L.M., Barak, L.S. and Caron, M.G. (2002) Biochemistry 41, 11981–11989.
[39] Takemoto, D.J., Morrison, D., Davis, L.C. and Takemoto, L.J. (1986) Biochem. J. 235, 309–312.
[40] Osawa, S. and Weiss, E.R. (1994) Mol. Pharmacol. 46, 1036–1040.
[41] Weiss, E.R., Osawa, S., Shi, W. and Dickerson, C.D. (1994) Biochemistry 33, 7587–7593.
[42] Zhong, M., Navratil, A.M., Clay, C. and Sanborn, B.M. (2004) Biochemistry 43, 3490–3498.
[43] Tetsuka, M., Saito, Y., Imai, K., Doi, H. and Maruyama, K. (2004) Endocrinology 145, 3712–3723.
[44] Natochin, M., Gasimov, K.G., Moussaif, M. and Artemyev, N.O. (2003) J. Biol. Chem. 278, 37574–37581.