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Distinct function of the cytoplasmic tail in human D1-like receptor ligand binding and coupling

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Abstract To delineate the role of the cytoplasmic tail in the distinct binding and coupling properties of human dopamine D1-like receptors, chimeric receptors were generated in which the entire tail region of wild-type human D1A (or D1) and D1B (or D5) receptors was exchanged. The hD1A-D1BT, but not hD1B-D1AT, receptor expression was dramatically reduced compared with wild-type receptor expression. Swapping the cytoplasmic tail resulted in a full switch of dopamine binding affinity and constitutive activity, while dopamine potency decreased and agonist-mediated maximal activation of adenylyl cyclase increased for both chimeras. Hence, the cytoplasmic tail plays a crucial role in D1-like receptor expression, agonist binding affinity and constitutive activation but regulates in a distinct fashion the formation of D1A and D1B receptor active states upon dopamine binding.

Key words: G protein-coupled receptor; Constitutive activity; Dopamine; D1-like receptor; Binding affinity; G protein activation

1. Introduction

The broad physiological effects of dopamine are mediated by D1-like and D2-like receptors that belong to the superfamily of G protein-coupled receptors (GPCRs) [1]. In mammals, dopaminergic D1-like receptors are divided into two subtypes, D1A (or D1) and D1B (or D5) which couple to the activation of adenylyl cyclase [1]. The binding and coupling properties of the D1B receptor differ from that of the D1A receptor by a higher constitutive activity (agonist-independent activity), an increased affinity and potency for agonists, as well as a lower affinity for inverse agonists [2]. The functional characteristics of D1B receptors resemble those of constitutively active mutant GPCRs [3]. However, the structural determinants and molecular interactions underlying these differences in ligand binding and activation properties of D1A and D1B receptors are poorly understood.

Mutagenesis studies suggest that GPCRs exist in an equilibrium between two interchangeable conformational states, inactive (R) and active (R*) [3–6]. In the absence of ligand (agonist), GPCRs are predominantly maintained in an inactive R state by intramolecular constraints that prohibit interactions with G proteins. These intramolecular constraints are released upon agonist binding or by mutations. Constitutively active mutant GPCRs have a greater propensity to adopt an R* state in the absence of agonist [4,5]. For instance, mutations in the carboxyl end of the third cytoplasmic loop of GPCRs can result in mutant receptors displaying high levels of agonist-independent activity or constitutive activation [3,6]. Importantly, naturally occurring activating mutations of GPCRs have been shown to underlie numerous pathological conditions [6,7].

Previously, we have shown that a variant amino acid found in a similar region of the carboxyl end of the third cytoplasmic loop of human D1A and D1B receptor could partially explain the distinguishing features of these two receptors [8]. In a recent study, we have shown that the terminal receptor locus (TRL), a domain encompassing transmembrane regions 6 and 7 as well as the third extracellular loop (EL3) and cytoplasmic tail, is an important structural domain regulating dopamine binding, constitutive activation and coupling properties of D1-like receptors [9]. Specifically, swapping the TRL between D1A and D1B receptors resulted in a switch in the affinity of these chimeric receptors for dopamine, as well as agonist-independent activity and dopamine potency, when compared with wild-type receptors. However, the difference observed in agonist-mediated maximal activation of adenylyl cyclase elicited by D1A and D1B receptors was not altered in cells expressing these chimeric receptors. The construction of two additional chimeras, where just the EL3 region was exchanged between D1A and D1B receptors, mapped this region as the domain underlying the degree of agonist-mediated maximal activation elicited by the D1-like receptors [9]. Hence, it appears that discrete receptor domains are responsible for regulating specific GPCR activation properties (e.g. constitutive activation and agonist-mediated maximal activation of adenylyl cyclase) and these properties can be separated with specific mutations.

Our recent study [9] raises the issue of the potential role the cytoplasmic tail plays in the TRL regulation of functional properties of the D1A and D1B receptors. The human D1A and D1B receptors share only about 30% identity between the cytoplasmic tails. In addition, the human D1B cytoplasmic tail contains more acidic amino acids than the D1A cytoplasmic tail (~20% vs. ~10%). These features suggest the presence of sequence-specific motifs within the cytoplasmic tail that regulate the binding and coupling properties of D1A and D1B receptors. Studies using goldfish D1A (a naturally truncated form of the receptor) and chimeric *Xenopus* D1A and D1B receptors suggested that the cytoplasmic tail does

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Abbreviations: GPCR, G protein-coupled receptor; EL3, third extracellular loop; TRL, terminal receptor locus; IBMX, 1-methyl-3-isobutylxanthine

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not play a major role in the dopamine binding and coupling properties of these vertebrate D1-like receptors [10,11]. However, there is a significant degree of divergence within the cytoplasmic tail between the human and *Xenopus* D1-like receptors (58–72%). Hence, the sequence-specific motifs within the human cytoplasmic tail may be responsible for some of the functionally divergent roles of D1-like receptor subtypes.

In the study described herein, the cytoplasmic tail was exchanged between the human D1A and D1B receptor to examine the potential role of this region in the unique binding and coupling properties of D1-like receptors. To our knowledge, our study provides the first evidence for an important role of the cytoplasmic tail in the regulation of the ligand binding and activation properties of the D1A and D1B receptors.

2. Materials and methods

2.1. Drugs

N-[Methyl-^{3}H]SCH23390 (83 Ci/mmol), [^{3}H]adenine (26 Ci/mmol) and [^{14}C]cAMP (275 mCi/mmol) were from Amersham Pharmacia Biotech. Dopamine, deschloro-SCH23390 (SCH23982), flupentixol and (+)-butaclamol were purchased from Research Biochemicals International. 1-Methyl-3-isobutylxanthine (IBMX) was obtained from Sigma.

2.2. Construction of chimeric human D1A and D1B receptors

The cytoplasmic tail region of the human D1A and D1B receptor was swapped by gene splicing using a PCR-based overlap extension approach. The receptor sequences were swapped at the junction between the seventh transmembrane domain and the cytoplasmic tail. This region corresponds to amino acid residue 334 in D1A and residue 362 in D1B. To facilitate the construction of these chimeric receptors, a silent mutation was introduced in each construct to create a unique restriction endonuclease site. Specifically, a *Mlu*I site was introduced at amino acid residues 331 and 332 (5′-TATGCC-3′ → 5′-TACGCG-3′) near the 3′ end of transmembrane seven of the D1A receptor, immediately upstream of the D1B receptor tail sequence. Likewise, a *Hin*dIII site was introduced at residues 367 and 368 (5′-AAGGCA-3′ → 5′-AAAGCT-3′), which is located 3′ of the junction between the seventh transmembrane domain of the D1B receptor and D1A receptor tail sequence. The resulting chimeras, referred to as hD1A-D1BT and hD1B-D1AT, were subcloned in pBluescript II SK$^+$ (Stratagene) and their identity was confirmed by dideoxy sequencing using Sequenase version 2.0 kit (US Biochemical Corp.). Sequencing of the D1B-D1A tail receptor revealed the introduction of a silent point mutation encoding amino acid residue 357 (5′-GTC-3′ → 5′-GTT-3′). Expression constructs for the wild-type and chimeric receptors were engineered into the expression vector pCMV5.

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 and gentamicin (100 μg/ml) (Life Technologies, Inc.). Cells were seeded into 100-mm dishes (2.5×10^6 cells/dish) and transiently transfected with 0.05–5 μg of DNA/dish using a modified calcium phosphate precipitation procedure as described [12].

2.4. Membrane preparation

Following an overnight incubation with the DNA–calcium phosphate precipitate, HEK293 cells were washed with phosphate-buffered saline (PBS), trypsinized, reseeded in 150-mm dishes and grown for an additional 48 h. Transfected HEK293 cells were then washed with cold PBS, scraped from the dish in ice-cold lysis buffer (10 mM Tris–HCl, pH 7.4, 5 mM EDTA) and centrifuged twice at $40\,000\times g$ for 20 min at 4°C. The crude membrane pellet was resuspended in lysis buffer using a Brinkmann Polytron (17 000 rpm for 15 s), frozen in liquid nitrogen and stored at −80°C until used.

2.5. Radioligand binding assay

Frozen membranes were thawed on ice and resuspended in binding buffer (50 mM Tris–HCl, pH 7.4, 120 mM NaCl, 5 mM KCl, 4 mM $MgCl_2$, 1.5 mM $CaCl_2$, 1 mM EDTA) using a Brinkmann Polytron. Binding assays were performed with 100 μl of membrane in a total volume of 500 μl using *N*-[methyl-^{3}H]SCH23390 as radioligand. Saturation studies were done on fresh membrane using concentrations of *N*-[methyl-^{3}H]SCH23390 ranging from 0.01 to 6 nM. Non-specific binding was determined by the addition of 10 μM flupentixol. For competition studies, membranes were incubated in a constant concentration of *N*-[methyl-^{3}H]SCH23390 (∼0.6 nM) and increasing concentrations of competing ligand. Competition studies using dopamine were done in the presence of 0.1 mM ascorbic acid. Binding assays were incubated for 90–120 min at room temperature and terminated with rapid filtration through glass fiber filters (GF/C, Whatman). The filters were washed four times with 5 ml of cold washing buffer (50 mM Tris–HCl, pH 7.4, 120 mM NaCl) and bound radioactivity was determined by liquid scintillation counting (Beckman Counter, LS1701). Protein concentration was measured using the Bio-Rad assay kit with bovine serum albumin as standard. To determine the equilibrium dissociation constant (K_d) and binding capacity (R) values, binding isotherms were analyzed using the non-linear curve-fitting program LIGAND [13].

2.6. Whole cell cAMP assay

Regulation of adenylyl cyclase activity by wild-type and chimeric receptors was assessed using a whole cell cAMP assay as described previously [9]. Following overnight incubation with the DNA–calcium phosphate precipitate, HEK293 cells were reseeded in 6- or 12-well dishes. The next day, the growth medium was replaced with fresh MEM containing 5% (v/v) fetal bovine serum, gentamicin (100 μg/ml) and [^{3}H]adenine (2 μCi/ml) for 16–18 h at 37°C and 5% CO_2. The labeling medium was then removed and HEK293 cells were incubated in 20 mM HEPES-buffered MEM containing 1 mM IBMX in the presence or absence of dopamine for 30 min at 37°C. At the end of the incubation period the medium was aspirated and each well was filled with 1 ml of lysis solution containing 2.5% (v/v) perchloric acid, 1 mM cAMP and [^{14}C]cAMP (2.5–5 nCi, ∼5000–10 000 cpm) for 30 min at 4°C. The lysates were then transferred to tubes containing 0.1 ml of 4.2 M KOH (neutralizing solution) and precipitates were sedimented by a low speed centrifugation (1500 rpm) at 4°C. The amount of intracellular [^{3}H]cAMP was determined from supernatants purified by sequential chromatography using DOWEX and alumina columns as previously described [14]. The amount of [^{3}H]cAMP (CA) over the total amount of intracellular [^{3}H]adenine (TU) was calculated to determine the relative adenylyl cyclase activity (CA/TU). Dose–response curves to dopamine were analyzed by a four-parameter logistic equation using ALLFIT [15]. Receptor expression was determined using a saturating concentration (∼6 nM) of *N*-[methyl-^{3}H]SCH23390.

2.7. Statistics

Equilibrium dissociation binding constants (K_d) are expressed using the geometric mean ± S.E.M. as described [16]. All other data are reported as arithmetic means ± S.E.M. unless stated otherwise. All statistical tests used in this study have been described elsewhere [17,18]. Homoscedasticity of variances were assessed using Cochran or Bartlett tests prior to statistical analyses. One-sample *t*-test and analysis of variance (one-way ANOVA) with Newman–Keuls multiple comparison test were performed using GraphPad Prism version 3.00 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. Chimeric receptors delineate a structural domain underlying receptor expression and dopamine affinity for human D1 receptor subtypes

The binding affinities (K_d values) of the radioligand *N*-[methyl-^{3}H]SCH23390 for wild-type and chimeric human D1 receptors obtained using saturation studies are shown in Table 1. The similar K_d values indicate that the chimeric receptors retained their ability to bind *N*-[methyl-^{3}H]SCH23390 with high affinity. Compared with hD1A, hD1B and hD1B-

Table 1
Dissociation constants (K_d) and binding capacity (R) values for wild-type and chimeric receptors

	Dissociation constants (K_d), nM					R
	[^{3}H]-SCH	DA	FLU	BUTA	SCH	(pmol/mg prot.)
hD1A	0.35 ± 0.02	7244 ± 411	3.15 ± 0.22 (#)	1.98 ± 0.02 (Ψ#)	0.39 ± 0.01	11.8 ± 1.57
hD1B	0.44 ± 0.02	642 ± 20 (*#)	5.35 ± 0.31	16.9 ± 0.43	0.48 ± 0.02	15.2 ± 1.62
hD1A-D1BT	0.16 ± 0.01 (*Ψ#)	584 ± 47 (*#)	2.61 ± 0.08 (#Ψ)	1.28 ± 0.04 (*Ψ#)	0.26 ± 0.02 (Ψ)	0.73 ± 0.06 (*Ψ#)
hD1B-D1AT	0.41 ± 0.04	5113 ± 119	4.45 ± 0.27	13.8 ± 0.25	0.38 ± 0.02	16.5 ± 2.79

K_d and R values are expressed as geometric and arithmetic means ± S.E.M., respectively. Means are from four to five experiments done in duplicate determinations. [^{3}H]-SCH, *N*-[methyl-^{3}H]-SCH23390; DA, dopamine; FLU, flupentixol; BUTA, (+)-butaclamol; SCH, SCH23982.
*$P < 0.05$ when compared with hD1A; $^{\Psi}P < 0.05$ when compared with hD1B; $^{\#}P < 0.05$ when compared with hD1B-D1A tail receptor.

D1AT receptors, hD1A-D1BT receptors displayed a significant increase in binding affinity for *N*-[methyl-^{3}H]SCH23390 (Table 1). In addition, there was a significant reduction (~15-fold) in the expression of the hD1A-D1BT receptor, compared with both parent wild-type receptors and the hD1B-D1AT receptor (Table 1). Increasing the amount of transfected DNA to 10 μg did not augment significantly the hD1A-D1BT receptor expression (data not shown).

Competition studies were performed to determine whether the cytoplasmic tail contains the underlying structural requirements to influence dopamine binding to wild-type human D1A and D1B receptors. As previously reported [2,8,9], dopamine exhibited a higher affinity (~11-fold) for the D1B subtype in comparison with the D1A receptor (Table 1). Importantly, the hD1A-D1BT receptor displayed an increased affinity for dopamine that was indistinguishable from the binding affinity observed for the wild-type D1B receptor (Table 1). Reciprocally, the hD1B-D1AT receptor bound dopamine with a lower affinity that was similar to that observed for the wild-type D1A receptor (Table 1).

3.2. The cytoplasmic tail does not govern the binding affinity of antipsychotics

Previous studies have shown that antagonists bind to the D1B receptor with lower affinity in comparison with the D1A receptor [2,8,9]. We tested the binding affinity of flupentixol and (+)-butaclamol, two antipsychotic drugs that display inverse agonism at both D1 receptor subtypes [2,19]. Both drugs have lower affinity for the wild-type D1B versus the D1A receptor (Table 1). The binding affinity of both chimeras for flupentixol was not statistically different from their respective parent receptors (Table 1). A similar trend applies for (+)-butaclamol, except that the hD1A-D1BT receptor displayed an increased affinity for this drug compared with wild-type D1A receptor (Table 1).

Next, we studied the binding properties of the benzazepine SCH23982, a high affinity antagonist that binds selectively to D1-like receptors [20] but behaves as a partial agonist toward D1 receptors when expressed in HEK293 cells [2]. In the present study, SCH23982 exhibited a lower affinity for the wild-type D1B subtype compared to the D1A receptor. Surprisingly, both chimeras bound SCH23982 with an increased affinity in comparison with their parent receptor (Table 1). A similar finding was observed with the swapping of the TRL cassette [9].

3.3. Swapping the tail caused a full switch in agonist-independent activity

The role of the cytoplasmic tail in agonist-independent activation of adenylyl cyclase by wild-type and chimeric receptors was assessed using a whole cell cAMP assay. In initial experiments (Fig. 1A), HEK293 cells were transfected with 5 μg of DNA for each of the four receptors. As previously reported [2,8,9], the D1B receptor displayed a significant increase in agonist-independent activity compared to the D1A receptor (Fig. 1A,B). The D1A tail of the hD1B-D1AT receptor dramatically reduced the constitutive activity of the D1B receptor to a significantly lower level than was measured for the wild-type D1A receptor (Fig. 1A,B). To our surprise, however, this effect was not reciprocal. In fact, the agonist-independent activity of the hD1A-D1BT receptor exhibited a significant reduction compared with the D1A receptor (Fig. 1A,B). Importantly, the average receptor expression of D1A, D1B, D1A-D1BT and D1B-D1AT receptor in these experiments was 13.9 ± 4.07, 14.4 ± 4.37, 1.17 ± 0.35 and 13.8 ± 3.11 pmol/mg protein, respectively. This issue is of importance since a linear relationship exists between receptor density and constitutive activity [2,5,21,22].

To determine whether this unexpected decrease in constitutive activity of the hD1A-D1BT receptor was a function of its significantly reduced expression (see above; Table 1), we measured the constitutive activity of each receptor following transfection with increasing amounts of DNA to obtain different levels of receptor expression (Fig. 1C). Comparison of the slopes obtained from the linear regression of the curves revealed that swapping the tail between D1A and D1B receptors does in fact cause a reciprocal switch in the slope and hence level of constitutive activity. The reciprocity of this switch is evident when the slope factor from each linear regression in Fig. 1C is expressed relative to the D1A receptor (Fig. 1D). Expression of the D1B tail in the context of the D1A receptor caused an increase (~5-fold) in agonist-independent activity to levels comparable with the D1B receptor. In contrast, the hD1B-D1AT receptor displayed a dramatic reduction in constitutive activity to levels indistinguishable from the D1A receptor (Fig. 1D).

3.4. Coupling properties of D1A and D1B receptor chimeras

Differences in agonist-mediated coupling properties of D1A and D1B receptors have been reported previously [2,8,9]. To test whether the cytoplasmic tail delineates the structural requirements for dopamine potency (EC_{50}) and agonist-mediated maximal activation, dose–response curves were done in HEK293 cells expressing similar levels of wild-type and chi-

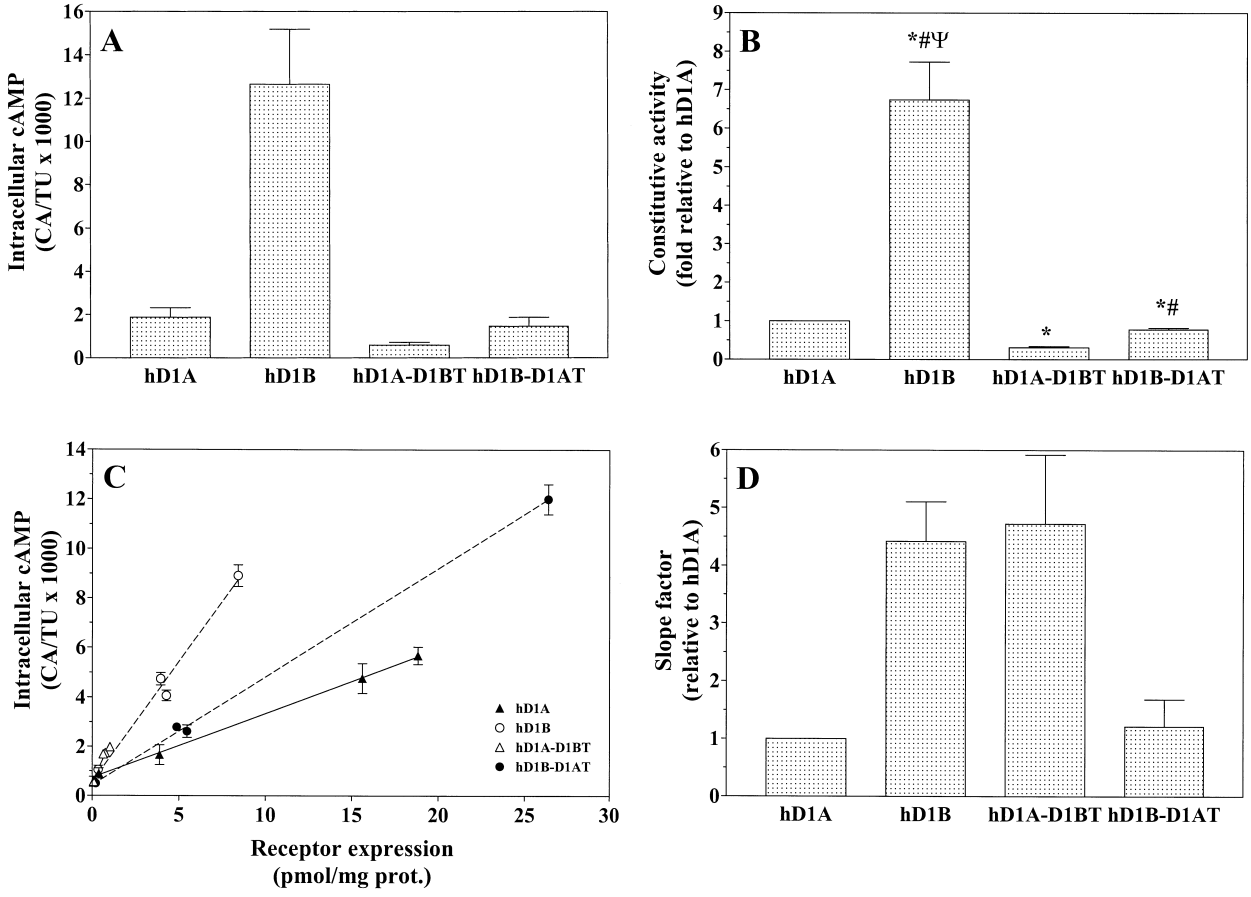

Fig. 1. Constitutive activity of wild-type and chimeric D1 receptors expressed in HEK293 cells. Basal levels of intracellular cAMP were determined in single wells of a six-well dish using whole cell cAMP assays (A) and calculated relative to hD1A receptor (B). Data are expressed as geometric mean ± S.E.M. of three experiments done in triplicate determinations. C: A representative example ($n=2$) of basal intracellular levels of cAMP versus receptor expression (pmol/mg protein) following transfection of HEK293 cells with wild-type and chimeric receptors using four different amounts of DNA (0.005, 0.05, 0.5 and 5 μg). D: The slope factor for each line plotted in C is expressed relative to hD1A receptor. $^{*}P<0.05$ when compared to hD1A; $^{\#}P<0.05$ when compared with hD1A-D1BT; $^{\Psi}P<0.05$ when compared with hD1B-D1AT. CA/TU, [^{3}H]cAMP formed divided by the total uptake.

meric receptors. As shown in Fig. 2A, dopamine potency was ~10-fold greater at wild-type D1B receptor compared with wild-type D1A receptor. In comparison with their parent receptors, hD1A-D1BT and hD1B-D1AT chimeras displayed a significant 5- and 15-fold decrease, respectively, in dopamine potency (Fig. 2A).

As previously described [2,9], agonist-mediated maximal stimulation elicited by the wild-type D1A receptor is significantly greater than the wild-type D1B receptor (Fig. 2B). Interestingly, there was a significant increase in the maximal activation of adenylyl cyclase following agonist stimulation of both D1A-D1BT and D1B-D1AT receptors, compared with their parent receptors (Fig. 2B).

4. Discussion

In the present study, we show for the first time that the cytoplasmic tail is a crucial structural determinant regulating the distinct binding and activation properties of the human D1A and D1B receptors. Several studies have implicated the cytoplasmic tail of GCPRs in G protein coupling and second messenger activation, partial agonist activity, constitutive activation, and receptor desensitization and/or internalization [10,23–29]. Recently, $GABA_A$-ligand-gated channels have been shown to complex selectively with D1B receptors through the direct binding of the D1B cytoplasmic tail with the second intracellular loop of the $GABA_A$ γ2(short) receptor subunit, resulting in a co-directional decrease in $GABA_A$ and D1B receptor function [30]. Importantly, our present study may provide insights into the molecular basis for the physical interaction between D1B and $GABA_A$ receptors.

By constructing chimeric receptors, we have clearly shown that the cytoplasmic tail of human dopamine D1-like receptors, which is unlikely to play a direct role in the docking of extracellular agonists and antagonists, regulates the binding affinity of the natural ligand, dopamine. In contrast, exchanging the tail region had little effect on the binding affinity of inverse agonists for the chimeric receptors, compared with wild-type receptors. This suggests that inverse agonist binding is most likely independent of tail-induced conformational changes. Alternatively, this binding may be modulated by residues conserved in the cytoplasmic tail of both D1-like receptors and rely also upon interactions with different residues outside of the tail region.

In addition, our results permit us to narrow down the structural region(s) responsible for dopamine binding affinity and constitutive activity, which we previously attributed to the TRL cassette, a large domain encompassing transmembrane

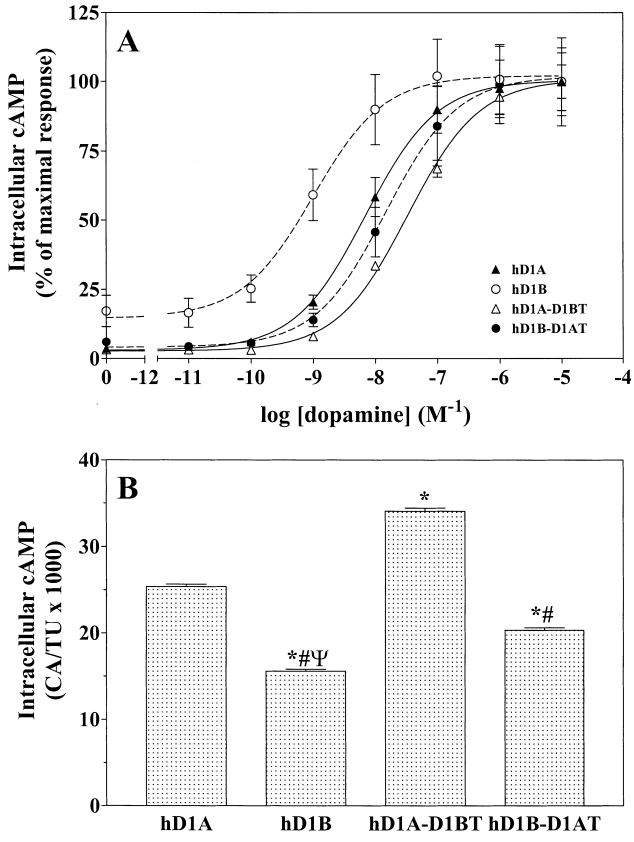

Fig. 2. Dopamine-mediated stimulation of adenylyl cyclase activity by wild-type and chimeric D1 receptors expressed in HEK293 cells. A: Dose–response curve of dopamine for adenylyl cyclase stimulation by wild-type and chimeric D1 receptors. Each point is the arithmetic mean ± S.E.M. of four to five experiments done in triplicate determinations using single wells from a 12-well dish. For the determination of EC_{50} values and maximal stimulation, each point was first expressed as percentage of maximal response obtained with the respective wild-type or chimeric receptor and curves were then analyzed by simultaneous curve-fitting using ALLFIT. The EC_{50} values are as follows (in nM): 7.0 ± 0.6 (hD1A), 1.0 ± 0.2 (hD1B), 33.3 ± 0.2 (hD1A-D1BT), and 15.0 ± 0.2 (hD1B-D1AT). The receptor expression in pmol/mg of membrane protein (expressed as the arithmetic mean ± S.E.M.) was 3.69 ± 0.24 (hD1A), 2.55 ± 0.51 (hD1B), 0.77 ± 0.08 (hD1A-D1BT), and 2.61 ± 0.69 (hD1B-D1AT). B: Maximal activation of adenylyl cyclase in HEK293 cells transfected with wild-type and chimeric D1 receptors. The maximal activation values were determined using ALLFIT as described in A. Statistical significance was determined following unconstrained and constrained curve fitting. $^{*}P < 0.05$ when compared to hD1A; $^{\#}P < 0.05$ when compared with hD1A-D1BT; $^{\Psi}P < 0.05$ when compared with hD1B-D1AT.

regions 6 and 7 as well as EL3 and the cytoplasmic tail [9]. While it was determined that the EL3 region was responsible for partial modulation of dopamine binding affinity and agonist-independent activity [9], we have shown that swapping the cytoplasmic tail region caused a full switch in both properties. Hence, there is some overlap or complementation in the contribution of these distinct structural domains to the conformation of D1-like receptors. Interestingly, the major determinants of α2- and β2-adrenergic receptor agonist and antagonist ligand binding specificity are contained within the seventh membrane-spanning domain and several of the first five hydrophobic domains may contribute to agonist binding specificity [31]. Perhaps the cytoplasmic tail of D1A and D1B receptors differentially modulates the three-dimensional arrangement of the transmembrane domains that provide the binding pocket for dopamine [32,33], resulting in distinct agonist binding affinities for these receptor subtypes. Similarly, amino acid sequences of the intracellular loops mediate the interaction of GPCRs with G protein [33,34]. Our findings suggest that the cytoplasmic tail interacts directly or indirectly with this region to modulate G protein coupling and second messenger activation, as measured by changes in agonist-independent adenylyl cyclase activity. Hence, in the process of making our chimeric receptors, the direct or indirect 'native' interactions of amino acids in the tail with those in the intracellular loops and/or transmembrane domains are switched and now reflect those of the cognate wild-type receptor counterpart.

The tail region of human D1-like receptors, in particular the D1B tail, also appears to play an important role in determining the level of functional receptor expression in the plasma membrane of human (HEK293) cells. This was evident by a 15-fold decrease in the binding capacity of hD1A-D1BT receptor compared with hD1A, hD1B and hD1B-D1AT receptors. Perhaps the D1B tail, expressed in the context of a D1A receptor, prevents the chimera from being efficiently routed to the plasma membrane or may reduce the stability of the receptor in the membrane causing it to be quickly recycled from the surface. For example, the D1B tail in a D1A receptor 'environment' may not bind as efficiently to cytoskeletal elements or other membrane proteins that anchor the receptor in the plasma membrane, therefore increasing lateral movement to coated pits. This lateral movement is presumably a prerequisite for ligand-induced endocytosis and subsequent down-regulation [35,36]. Our finding that the D1B tail causes the D1A receptor to 'behave' more like a D1B receptor, as measured by an increase in constitutive activity, suggests that this chimeric receptor is efficiently routed to the plasma membrane and coupled to G_s protein, but is quickly recycled. In fact, studies have shown that activating mutations may lead to mutant GPCRs that are structurally unstable [37,38]. In addition, Parker and Ross [39] have shown that truncation of the cytoplasmic tail of the turkey β-adrenergic receptor increases its susceptibility to amino-terminal proteolysis.

The present study describes an important function of the cytoplasmic tail in regulating the intramolecular interactions underlying some of the distinct binding and coupling properties of the D1 receptor subtypes. The majority of our ligand binding and G protein coupling data suggest that the intramolecular interactions induced by the D1A tail in the hD1B-D1AT chimera maintain this receptor in a predominantly 'constrained' conformation, as indexed by a decreased binding affinity and potency for dopamine, and a lower agonist-independent activity. While these findings might lead one to conclude that the efficacy of coupling to G_s of hD1B-D1AT receptor is reduced and now resembles the D1A receptor, the observed increase in maximal activation of adenylyl cyclase is not consistent with such a general conclusion. Similarly, initial findings that the D1B tail enabled the D1A-D1BT receptor to increase its binding affinity for dopamine and constitutive activity suggested that this region induces intramolecular interactions enabling the chimera to adopt a more 'relaxed' conformation. However, the observed decrease in dopamine potency of the D1A-D1BT receptor compared with wild-type

D1A receptor illustrates that even a full switch in dopamine binding affinity does not necessarily allow one to predict that there will be similar changes in all coupling properties, i.e. these phenomena can be separated. In fact, this statement is further supported by our finding of an increase, instead of a decrease, in agonist-mediated maximal activation of adenylyl cyclase in cells expressing hD1A-D1BT receptors. Overall, these findings suggest that the D1A and D1B receptor can undergo multiple active receptor conformations. Studies in our laboratory are under way to define further the specific residues of the cytoplasmic tail responsible for these activation properties.

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