To delineate the structural determinants involved in the constitutive activation of the D1 receptor subtypes, we have constructed chimeras between the D1A and D1B receptors. These chimeras harbored a cognate domain corresponding to transmembrane regions 6 and 7 as well as the third extracellular loop (EL3) and cytoplasmic tail, a domain referred herein to as the terminal receptor locus (TRL). A chimeric D1A receptor harboring the D1B-TRL (chimera 1) displays an increased affinity for dopamine that is indistinguishable from the wild-type D1B receptor. Likewise, a chimeric D1B receptor containing the D1A-TRL cassette (chimera 2) binds dopamine with a reduced affinity that is highly reminiscent of the dopamine affinity for the wild-type D1A receptor. Furthermore, we show that the agonist independent activity of chimera 1 is identical to the wild-type D1B receptor whereas the chimera 2 displays a low agonist independent activity that is indistinguishable from the wild-type D1A receptor. Dopamine potencies for the wild-type D1A and D1B receptor were recaptured in cells expressing the chimera 2 or chimera 1, respectively. However, the differences observed in agonist-mediated maximal activation of adenyl cyclase elicited by the D1A and D1B receptors remain unchanged in cells expressing the chimeric receptors. To gain further mechanistic insights into the structural determinants of the TRL involved in the activation properties of the D1 receptor subtypes, we have engineered two additional chimeric D1 receptors that contain the EL3 region of their respective cognate wild-type counterparts (hD1A-EL3B and hD1B-EL3A). In marked contrast to chimera 1 and 2, dopamine affinity and constitutive activation were partially modulated by the exchange of the EL3. Meanwhile, hD1A-EL3B and hD1B-EL3A mutant receptors display a full switch in the agonist-mediated maximal activation, which is reminiscent of their cognate wild-type counterparts. Overall, our studies suggest a fundamental role for the TRL in shaping the intramolecular interactions implicated in the constitutive activation and coupling properties of the dopamine D1 receptor subtypes.
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their ability to interact with antagonists. Overall, these results suggest that the carboxyl end of the third cytoplasmic loop plays a role in constraining the D1A and D1B receptor into their inactive and active allosteric states, respectively. However, the results also indicate that the molecular properties of these two D1 receptor subtypes can only be explained partially by amino acid sequences of the carboxyl end of the third cytoplasmic loop. Therefore, it is likely that other structural determinants within these receptors exist to define the intramolecular interactions responsible for the distinct features of the D1A and D1B receptors. Indeed, studies have shown that mutations occurring in transmembrane regions, extracellular loops, or the cytoplasmic tail of GPCRs can lead to a constitutive activation (2, 9, 13–16). In the present study, we use a chimeric receptor approach (Fig. 1) to delineate further the potential structural determinants that underlie the molecular properties of the human D1A and D1B receptors. We report that chimeric D1A/D1B receptors harboring the terminal receptor locus (TRL) cassette which includes the transmembrane region (TM) 6 and 7, the third extracellular loop (EL3), and the cytoplasmic tail display constitutive activity, dopamine affinity, and potency that are indistinguishable from their respective cognate wild-type receptors. Furthermore, studies with chimeric D1A/D1B receptors containing only the EL3 region suggest an important role for this region in the agonist-mediated maximal activation (intrinsinc efficacy) of the D1 receptor subtypes. The present study identifies an important structural domain regulating the activation process of the D1A and D1B receptor but demonstrates also that the molecular determinants involved in the GPCR activation properties (constitutive activation, agonist potency, and intrinsic efficacy) can be separated.

MATERIALS AND METHODS

Drugs—N-[methyl-3H]SCH23390 (84 Ci/mmol), [3H]Adenine (24 Ci/mmol), and [3H]cAMP (275 Ci/mmol) were from Amersham Pharmacia Biotech. Dopamine, deschloro-SCH23390 (SCH23982), flupentixol, and (+)-butaclamol were purchased from Research Biochemicals International. 1-Methyl-3-isobutylxanthine was obtained from Sigma.

Construction of Chimeric Human D1A and D1B Receptors—To construct the chimeric receptors, we took advantage of the high degree of nucleotide identity between the human D1A and D1B receptor (17). Using the conserved BoII restriction site located within the nucleotide sequence coding for the TM6, we constructed two chimeric D1A and D1B receptors harboring the TRL cassette of their respective cognate wild-type counterparts (Fig. 1). The TRL cassette includes sequences coding for the TM6 and TM7 as well as the EL3 and carboxyl cytoplasmic tail. Moreover, the EL3 region of the D1A and D1B receptor was exchanged to create two additional chimeric receptors. The swapping of the EL3 region was done by gene splicing using a polymerase chain reaction (PCR) approach. The products were subcloned in pBluescript SK+ (Stratagene) and the identity of the chimeras confirmed by dideoxy sequencing using Sequenase version 2.0 kit (U. S. Biochemical Corp.). Expression constructs for the wild-type and chimeric D1A and D1B receptors were engineered into the expression vector pCMV5.

Cell Culture and Transfection—Human embryonic kidney 293 (HEK293) cells were from American Type Culture Collection (Manassas, VA). HEK293 cells were cultured at 37 °C and 5% CO2 in minimal essential medium 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 transfected with 0.25–5 μg of DNA/dish using a modified calcium phosphate precipitation procedure as described (18).

Membrane Preparation—After an overnight incubation with the DNA-calcium phosphate precipitate, HEK293 cells were washed with phosphate-buffered saline, trypsinized, reseeded in 150-mm dishes and grown for an additional 36–48 h. Transfected HEK293 cells were then washed with cold phosphate-buffered saline, scraped in ice-cold lysis buffer (10 mM Tris-HCl, pH 7.4, 5 mM EDTA), and centrifuged twice at 4°C for 20 min at 4,000 g. The supernatant was resuspended in lysis buffer using a Brinkmann Polytron (17,000 r.p.m. for 15 s). The crude membranes were frozen in liquid nitrogen and stored at −80 °C until used.

Radioligand Binding Assays—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 MgCl2, 1.5 mM CaCl2, 1 mM EDTA) to achieve a concentration of 0.1 mM ascorbic acid. Binding assays were incubated for 90 min at room temperature and terminated using rapid filtration through glass fiber filters (GFC, Whatman). The filters were washed three times with 5 ml of cold washing buffer (50 mM Tris-HCl, pH 7.4, 120 mM NaCl) and the bound radioactivity was determined by liquid scintillation counting (Beckman Counter, LS1701). Protein concentration was measured using the Bio-Rad Protein assay kit with bovine serum albumin as standard. The equilibrium dissociation constant (Kd) and binding capacity (R) values, binding isotherms were analyzed using the non-linear curve-fitting program LIGAND (19).

Whole Cell cAMP Assay—Regulation of adenyl cyclase activity by wild-type and chimeric D1A and D1B receptors was assessed using a whole cell cAMP assay as described previously (8). Following overnight incubation with the DNA-calcium phosphate precipitate, HEK293 cells were reseeded in 6- or 12-well dishes. The next day, HEK293 cells were cultured in fresh minimal essential medium containing 5% (v/v) fetal bovine serum, gentamicin (100 μg/ml), and [3H]Adenine (2 μCi/ml) for 18–24 h at 37 °C and 5% CO2. The labeling medium was then removed and HEK293 cells incubated in 20 mM HEPES-buffered minimal essential medium containing 1 μM 1-methyl-3-isobutylxan-

**FIG. 1.** Schematic representation of the wild-type and chimeric D1A and D1B receptors. A, putative topology of the wild-type D1A (open circles) and D1B receptor (filled circles), chimera 1 and chimera 2 is represented. B, alignment of the primary structure corresponding to the TRL cassette derived from the human D1A (hD1A) and D1B (hD1B) receptors is shown. The TM regions are delimited with a thick line above the amino acid sequence. Identical amino acids found between the two TRL sequences are indicated with an asterisk. The number of amino acids in TRL cassettes is also shown.
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thine in the presence or absence of dopamine for 30 min at 37 °C (in the presence of 0.1 mM ascorbic acid). At the end of the incubation period, the medium was aspirated, and each well filled with 1 ml of lysis solution containing 2.5% (v/v) perchloric acid, 1% sodium citrate (0.2 M), and [3H]N-methyl-3H]-SCH23390 (2.5–5 nCi, 5,000–10,000 cpm) for 20–30 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 (1,500 rpm) at 4 °C. The amount of intracellular [3H]N-methyl-3H]-SCH23390 was determined from supernatants purified by sequential chromatography using Dowex and alumina columns as described before (20). The amount of [3H]N-methyl-3H]-SCH23390 in the cellular compartment was calculated to determine the relative adenyl cyclase activity (CA/TU). Dose-response curves to dopamine were compared by a four-parameter logistic equation using ALLFIT (21). The [3H]cAMP was determined from supernatants purified by sequential chromatography using Dowex and alumina columns as described previously (20). The amount of [3H]cAMP (CA) over the total amount of intracellular [3H]adenine (TU) was calculated to determine the relative adenyl cyclase activity (CA/TU). Dose-response curves to dopamine were analyzed by a four-parameter logistic equation using ALLFIT (21).

RESULTS

Chimeric Receptors Delineate a Structural Domain Underlying the Dopamine Affinity for the D1 Receptor Subtypes—The binding affinities (Kd, values) of the radioligand N-[methyl-3H]-SCH23390 for wild-type and chimeric human D1 receptors obtained using saturation studies are summarized in Table I. Results indicate that the chimeric receptors retain their ability to bind N-[methyl-3H]-SCH23390 with high affinity. In addition, no statistical differences between the binding capacities of wild-type and chimeric receptors were detected (8–10 pmol/mg of protein). These results suggest that swapping the TRL cassette between the two receptors does not alter significantly the protein folding necessary for appropriate cell surface expression.

Competition studies were performed to determine whether the TRL contains the underlying structural requirements involved in the dopamine binding to wild-type human D1A and D1B receptors. Dopamine exhibits a higher affinity for the D1B subtype in comparison with the D1A receptor (Table II) as previously reported (8, 10). The chimera 1 displays an affinity for dopamine which is highly reminiscent of the binding affinity observed for the wild-type D1B receptor (Table II). In an opposite fashion, the chimera 2 binds dopamine with an affinity very similar to the one measured for the wild-type D1A receptor (Table II). To strengthen further the dominant role the TRL cassette plays in determining the D1A and D1B receptor conformations responsible for the distinct dopamine binding affinity, we calculated the free binding energy using the relation \( \Delta G = -RT \ln (1/K_d) \) (24). As depicted in Fig. 2A, the calculated net free energy difference relative to the dopamine binding energy for the wild-type D1A receptor suggests that chimera 1 displays a reduction in the binding energy preference for dopamine. This reduction is statistically different from the wild-type D1A receptor but indistinguishable from the wild-type D1B receptor. Meanwhile, the binding energy preference of chimera 2 for dopamine is not statistically different from the wild-type D1A receptor. In addition, the binding energy preference of chimera 2 for dopamine exhibits an increase that is statistically different from the wild-type D1B and chimera 1 (Fig. 2A).

The TRL Cassette Unravels Distinct Structural Require-

ments for the Binding of Antipsychotic Drugs—Previous studies have shown that antagonists or antipsychotic drugs bind with a lower affinity to the D1B receptor in comparison with the D1A receptor (8, 10). We tested the binding affinity of flupentixol and (+)-butaclamol, two antipsychotic drugs having a distinct chemical structure and displaying inverse agonism at the D1A and D1B receptors (8, 25). As shown in Table II, both drugs have lower affinity for the wild-type D1B receptor as reported before (8). The binding affinities of flupentixol for the chimera 1 and 2 were not statistically different from the wild-type D1A and D1B receptors, respectively (Table II, Fig. 2B). Interestingly, the small fold difference in the flupentixol affinity (~1.5-fold) observed between the wild-type D1 receptors remains unchanged with the exchange of the TRL cassette.

In striking contrast to flupentixol, (+)-butaclamol displays a greater fold difference in the binding affinity (~5-fold) between the two D1 receptor subtypes (Table II). As shown in Fig. 2C, the wild-type D1B receptor has an increased binding energy preference for (+)-butaclamol in comparison with the wild-type D1A receptor. The chimera 1 binds (+)-butaclamol with an affinity which is not statistically different from the wild-type D1A receptor (Table II, Fig. 2C). However, our binding data indicate that chimera 2 has an increased affinity for (+)-butaclamol (Table II, Fig. 2C). Indeed, the net binding energy preference for (+)-butaclamol of the chimera 2 is decreased in comparison with the wild-type D1B receptor but remains statistically different from the wild-type D1A subtype.

We then studied the binding properties of the benzazepine

| Kd (nM) | R (pmol/mg prot.) |
|---------|------------------|
| 0.53±0.09 | 8.0±1.3 |
| 0.63±0.06 | 9.8±3.1 |
| 0.30±0.04 | 7.6±0.5 |
| 0.37±0.05 | 11.0±1.3 |
SCH23982 which is structurally different from both flupentixol and (-)-butaclamol. This benzazepine has been described as a classical antagonist that binds preferentially to D1-like receptors (26). In the present study, SCH23982 exhibits lower affinity for the wild-type D1B subtype (Table II) which correlates with a significant increase in the binding energy preference while contrasted to the wild-type D1A receptor (Fig. 2). Surprisingly, chimera 1 and 2 bind to SCH23982 with an increased affinity that is statistically significant in comparison with the wild-type D1A or D1B receptor (Table II, Fig. 2). This trend is also observed using the radiolabeled benzazepine analog N-[methyl-3H]SCH23390 (Table I).

The TRL Cassette Is the Underlying Structural Domain of D1 Receptor Constitutive Activation—Previous studies have shown that the D1B receptor shares the functional features of constitutively activated mutant GPCRs (8, 10). The role of the TRL cassette in the agonist-independent activation of adenylyl cyclase by wild-type and chimeric receptors was assessed using a whole cell cAMP assay. The results are summarized in Fig. 3. In brief, the D1B receptor has a 3.5-fold higher agonist independent activity than the D1A receptor as shown previously (8, 10). Interestingly, chimera 1 shows an increase in its constitutive activation that is statistically different from the wild-type D1A but indistinguishable from the wild-type D1B receptor (Fig. 3). In striking contrast, chimera 2 exhibits a significant decrease of its agonist independent activity when compared with the wild-type D1B receptor (Fig. 3). In fact, the constitutive activation level of chimera 2 is highly reminiscent of the one measured for the wild-type D1A receptor.

### Table II

| Dissociation constants (K_d), nM |
|---------------------------------|
| **DA** | **FLU** | **BUTA** | **SCH** |
|-------|--------|---------|--------|
| 8792  | 13     | 8.5     | 0.52   |
| ±     | ±      | ±       | ±      |
| 384   | 2.0    | 0.8     | 0.02   |
|-------|--------|---------|--------|
| 817   | 21     | 42      | 0.73   |
| ±     | ±      | ±       | ±      |
| 48    | 2.5    | 1.1     | 0.02   |
|-------|--------|---------|--------|
| 1094  | 14     | 12      | 0.33   |
| ±     | ±      | ±       | ±      |
| 64    | 1.8    | 0.9     | 0.01   |
|-------|--------|---------|--------|
| 6012  | 20     | 21      | 0.34   |
| ±     | ±      | ±       | ±      |
| 281   | 2.7    | 1.5     | 0.01   |

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**FIG. 2.** Free energy of binding of dopaminergic ligands to wild-type and chimeric D1 receptors. For each drug tested the net free energy differences relative to hD1A was calculated using K_d values from competition studies shown in Table II. Data are expressed as arithmetic mean ± S.E. of seven to 10 experiments done in duplicate determinations. *, p < 0.05 when compared with hD1A; †, p < 0.05 when compared with hD1B. DA, dopamine; FLU, flupentixol; BUTA, (-)-butaclamol; SCH, SCH23982.

**FIG. 3.** Constitutive activity of wild-type and chimeric D1 receptors expressed in HEK293 cells. Basal levels of adenylyl cyclase activity were determined in single wells of a six-well dish using whole cell cAMP assays and calculated relative to hD1A receptor. Data are expressed as arithmetic mean ± S.E. of seven experiments done in triplicate determinations. The receptor expression in picomole/mg of membrane protein (expressed as the arithmetic mean ± S.E.) was 8.4 ± 1.9 (hD1A), 11.6 ± 1.8 (hD1B), 8.0 ± 1.1 (chimera 1) and 10.8 ± 1.8 (chimera 2). *, p < 0.05 when compared with hD1A; †, p < 0.05 when compared with chimera 2.

The TRL Cassette Is Involved in the D1A and D1B Receptor Coupling Properties—Differences in the agonist-mediated coupling properties of the D1A and D1B receptors have been described previously (8). To test whether the TRL cassette delineates the structural requirements for the dopamine potency and intrinsic efficacy, dose-response curves were done in HEK293 cells transfected with the wild-type and chimeric re-
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As depicted in Fig. 4A, the dopamine potency is about 10-fold superior at the wild-type D1B receptor in comparison with the wild-type D1A, a value in agreement with previous studies (8, 10). Chimera 1 exhibits an increase in dopamine potency as compared with its wild-type D1A counterpart (Fig. 4A). The potency of dopamine at the chimera 1 is not statistically different from the wild-type D1B receptor. Alternatively, chimera 2 displays a loss of dopamine potency that is significantly different from the wild-type D1A and D1B receptor (Fig. 4A).

Fig. 4B shows that the maximal stimulation elicited by the wild-type D1A receptor is significantly higher than the wild-type D1B receptors as described before (8). Interestingly, chimera 1 and 2 elicited a maximal activation of adenylyl cyclase that is identical to their respective wild-type receptor counterparts (Fig. 4B). On one hand, these results suggest that differences within the primary sequence of the TRL cassette does not account for the difference observed between the dopamine intrinsic efficacy of the wild-type D1A and D1B receptors. On the other hand, it is possible that the structural determinants underlying the D1A and D1B-mediated maximal activation are located within the TRL but remains unraveled using our chimeric receptors. To address this issue, we have engineered two additional chimeric D1A and D1B receptors that carry a more discrete domain of the TRL cassette, namely the EL3 region. We reasoned that the low degree of identity found between the EL3 of the D1A and D1B receptor, as shown in Fig. 1, may suggest an important structural role for this region in the agonist-independent and -dependent activation of the D1 receptor subtypes. The results are summarized below.

The EL3 Region Modulates the Dopamine Affinity and Constitutive Activity of the D1 Receptor Subtypes—Chimeric D1A and D1B receptors carrying the EL3 region of their cognate wild-type counterpart were constructed using a polymerase chain reaction-based overlap extension method (Fig. 1, Table III). In HEK293 cells, these chimeric receptors display high levels of expression (≥6 pmol/mg of protein). Radioligand binding studies indicate that dopamine affinity for the hD1A-EL3B is increased when compared with the wild-type D1A receptor (Table III). In contrast, a chimeric D1B receptor carrying the EL3 region of the D1A receptor (hD1B-EL3A) exhibits a reduction of dopamine affinity. These effects were also reflected in the binding energy preference for dopamine (data not shown). The binding affinity of N-[methyl-3H]SCH23390 remains unchanged.

The agonist independent activity of the hD1A-EL3B and hD1B-EL3A mutant receptors was studied in intact HEK293 cells expressing similar levels of receptors. The hD1B-EL3A mutant receptor displays a loss of agonist independent activity in comparison with the wild-type D1B (Fig. 5). In contrast, the hD1A-EL3B chimeric receptor exhibits a gain in the extent of constitutive activation as compared with the wild-type D1A receptor.

The EL3 Region of the D1A and D1B Receptor Underlies the Differential Dopamine-mediated Maximal Stimulation of Adenylyl Cyclase Activity—HEK293 cells expressing similar receptor levels of wild-type or chimeric receptors were stimulated with 10 μM dopamine, a concentration that produces a maximal activation of adenylyl cyclase. As described above, the wild-type D1A receptor elicits a significantly higher maximal activation of adenylyl cyclase than the wild-type D1B receptor (Fig. 6). Stimulation of HEK293 cells expressing the hD1A-EL3B chimeric receptor with 10 μM dopamine leads to a maximal activation of adenylyl cyclase that is indistinguishable from the maximal activation elicited by the wild-type D1B receptor (Fig. 6). Meanwhile, a full stimulation of the hD1B-EL3A mutant receptor or wild-type D1A receptor yields to a similar maximal activation of adenylyl cyclase (Fig. 6).

**FIG. 4.** 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. of five experiments done in triplicate determinations using single wells from a 12-well dish. For the determination of EC50 values and maximal stimulation, each point was first expressed as fold relative to hD1A basal activity and curves were then analyzed using ALLFIT. For the graphical representation, curve points are depicted as percentage of maximal response obtained with the respective wild-type or chimeric receptor after subtracting the basal value. The EC50 values are as follows (in nM): 9.3 ± 1.6 (hD1A), 1.1 ± 0.3 (hD1B), 2.5 ± 0.4 (chimera 1), and 41 ± 9.5 (chimera 2). The receptor expression was determined using picochem radioligand binding studies. The EC50 values were as follows (in nM): 3.4 ± 0.9 (hD1A); 2.7 ± 0.7 (hD1B), 2.2 ± 0.5 (chimera 1), and 1.8 ± 0.5 (chimera 2). B, maximal activation of adenylyl cyclase in HEK293 transfected with wild-type and chimeric D1 receptors. The maximal activation values were determined using ALLFIT as described in A. *p < 0.05 when compared with hD1A; †p < 0.05 when compared with chimera 1.

**DISCUSSION**

In the present study, we have used a chimeric approach to explore the structural determinants involved in the activation
properties of the D1 receptor subtypes. Previously, this approach has been useful in helping to delineate specific residues or domains underlying the binding and coupling functions of GPCRs (27–29). We have excluded the possibility that a low degree of identity between the various TRL cassettes may underlie nonspecific perturbations in the global receptor conformation of the chimeras. In effect, analysis of the primary structure reveals a degree of identity of 48% between the D1A-TRL and D1B-TRL cassette (Fig. 1). Despite this low degree of identity, the various chimeric receptors retain their ability to express at high levels of expression in HEK293 cells. These results suggest that the primary sequence of the TRL does not generate structural incompatibilities hindering the functional receptor protein folding.

The characterization of our chimeric D1A and D1B receptors has unveiled the molecular complexity underlying antagonist/inverse agonist binding to the D1 receptor subtypes. For instance, the binding affinity of flupentixol (inverse agonist) to the various chimeric receptors remains unchanged in comparison with their cognate wild-type receptors. As reported previously, flupentixol displays a lower affinity for the D1B receptor in comparison with the D1A receptor (8). These findings may suggest that flupentixol binding requires residues that are not found within the TRL or is independent of the TRL-induced conformational changes. Alternatively, the binding of flupentixol to D1A and D1B receptor may require conserved residues located in the TRL of both D1 receptor subtypes and rely also upon interactions with different residues existing outside the TRL boundaries. Meanwhile, we show that binding of (+)-butaclamol (inverse agonist) to the D1 receptor subtypes is explained, at least partially, by TRL-induced intramolecular interactions. Indeed, results obtained with chimera 2 support a role for the D1A-TRL in conferring to the wild-type D1A receptor its ability to bind (+)-butaclamol with a higher affinity. Moreover, consonant with a partial effect of the D1A-TRL on the (+)-butaclamol affinity of chimera 2, our study suggests

|           | K_d (nM) | R (pmol/mg prot.) |
|-----------|----------|------------------|
| DA        | [N-methyl-^3H]-SCH23390 | 6897 ± 448 | 14.0 ± 2.1 |
| hD1A      |          | 668 ± 32 | 18.4 ± 1.5 |
| hD1B      |          | 4315 ± 91 | 49.6 ± 5.4 |
| hD1A-EL3B |          | 1147 ± 99 | 6.7 ± 1.8 |
| hD1B-EL3A |          |          |          |
that the structural determinants located outside the TRL may also play an important role in coordinating the conformation(s) that underlie the binding of (+)-butaclamol. In striking contrast, a similar exchange performed in the D1A receptor using the D1B-TRL (chimera 1), resulted in the lack of a significant effect on the (+)-butaclamol affinity constant, suggesting that the lower affinity of the D1B receptor for (+)-butaclamol is independent of spatial relationships controlled by the D1B-TRL. These observations underscore the complex molecular pharmacology of D1 inverse agonists. In fact, the pharmacological differences observed between the two inverse agonists may suggest that different molecular mechanisms exist to induce the same active conformational state or that the receptor can adopt multiple active conformations. Overall, our study supports the notion that binding of inverse agonists to the D1A and D1B receptor requires distinct structural determinants.

Results obtained with the benzazepine SCH23982 emphasize the role of the TRL in regulating the global receptor conformation of the D1-like receptor subtypes. Originally, this benzazepine analog has been described as a high affinity antagonist that binds selectively to D1-like receptors (26). However, recent studies have demonstrated that benzazepine analogs behave as a partial agonist toward the D1 receptors, a phenomenon not only observed in HEK293 cells (8) but also in COS-7 and Sf9 cells (30–32). This contention is supported by in vivo studies showing that benzazepine-like compounds can induce behavioral responses in a D1-like agonist fashion (33, 34). Both chimeras exhibit a statistically significant decrease in the free binding energy when compared with their wild-type receptor counterparts (Fig. 2D). The reduced binding energies translate into a higher affinity of chimera 1 and 2 for SCH23982 (Table II). It is also worth mentioning that the partial agonism property of SCH23982 remains in both chimeric receptors (data not shown). We believe that our results indicate that the TRL-dependent conformational constraints of the chimeras regulate the receptor affinity for SCH23982 by modifying the position of specific residues responsible for the direct docking interactions with the benzazepine. In fact, our results may suggest a role for TRLs in regulating specific interactions with other receptor regions to constrain the D1A and D1B receptor into a suboptimal binding state for benzazepines. Similarly, these observations may imply the existence of a complementation between residues found in the TRL and other receptor regions (for instance with the intracellular loops) that maintain the two D1 receptor subtypes into a suboptimal binding conformation for benzazepines.

The present study highlights a structural domain (referred herein to as TRL) that is fundamental in the dopamine-independent and -dependent activation process of the human D1A and D1B receptor. We clearly demonstrate that the TRL is involved in regulating the intramolecular interactions that underlie the distinct binding and coupling properties of the D1 receptor subtypes. Our ligand binding and G protein coupling data obtained with chimera 2 suggest that the intramolecular interactions induced by the D1A-TRL maintain predominantly the chimeric receptor in a constrained conformation (R state) as indexed by a decreased binding affinity and potency for dopamine, and a lower agonist-independent activity. Interestingly, the molecular properties of chimera 2 are mostly indistinguishable from those of the wild-type D1A receptor. In striking contrast, the intramolecular interactions induced by the D1B-TRL enable chimera 1 to adopt a more “relaxed” conformation (R* state) as measured by an increased binding affinity and potency for dopamine, and a higher agonist-independent activity. In fact, chimera 1 exhibits constitutive activation properties that are indistinguishable from the wild-type D1B receptor. Most importantly, the D1B-TRL contains the structural determinants that confer the functional features of constitutively active GPCRs (2, 8). As we reported previously (10), the features of a constitutively active GPCR can be reversed or silenced with the appropriate mutation. In contrast to partial effects seen with mutations introduced in the third cytoplasmic loop (10), a chimeric D1B receptor containing the D1A-TRL (chimera 2) displays the functional properties of a fully silenced receptor when compared with the wild-type D1B receptor. Overall, our results suggest that the TRL cassette may underlie the spatial relationships specific to the D1A and D1B receptors, notably those underlying the molecular properties of constitutive activation and agonist potency but not those involved in the maximal activation of adenylyl cyclase.

Indeed, in HEK293 cells and at a comparable expression level, dopamine-mediated stimulation of the D1B receptor or chimera 2 elicits a lower maximal activation of adenylyl cyclase activity in comparison with cells expressing the D1A receptor or chimera 1. Therefore, cells expressing the D1B receptor subtype exhibit a lower intrinsic efficacy for dopamine; a receptor property that eludes the spatial relationships induced by the different TRL cassettes suggesting the involvement of other structural determinants. However, it is possible that the swapping of large receptor domains such as our TRL cassettes obstruct the delineation of specific regions which are important in the regulation of the activation properties of the D1 receptor subtypes. A close examination of the primary structure of the TRL cassettes indicates that a low degree of identity (38%) is found within the EL3 region (Fig. 1). Therefore, we speculated that the EL3 region could be involved in the activation process of the D1A and D1B receptor. In support of this contention, a recent study has shown that the EL3 region is functionally important for the regulation of agonist binding and agonist-independent activity of the β2-adrenergic receptor (35).

Our results indicate that the dopamine affinity obtained with hD1A-EL3B and hD1B-EL3A mutant receptors reproduced partially the dopamine binding phenotype of their cognate wild-type receptor counterparts. The partial changes observed in dopamine affinity were also reflected in the agonist independent activation of hD1A-EL3B and hD1B-EL3A. Similar to previous work using single point mutations in the carboxyl-terminal region of the third intracellular loop (10), we show that both dopamine affinity and agonist-independent activity of the wild-type D1A and D1B receptors could be partially recapitulated with the EL3 loop exchange. However, and most importantly, we demonstrate that a swap of the EL3 region leads to a complete reversal of the dopamine intrinsic efficacy (maximal activation of adenylyl cyclase). This is in marked contrast with our results obtained using chimera 1 and 2. Therefore, EL3 plays an important role in regulating the intramolecular interactions involved in the maximal activation of adenylyl cyclase by the D1A and D1B receptor, but to a lesser extent in agonist binding and constitutive activation. The effects of EL3 may be explained by differences found in the number of proline and glycine residues (Fig. 1). In fact, the proline and glycine content may contribute to different degrees of rigidity of the agonist-dependent conformational states of the D1A and D1B receptor.

The discrimination between constitutive activation and dopamine-mediated maximal activation (intrinsic efficacy) using our chimeric receptors is very intriguing. In addition to the existence of intrinsic differences in the intramolecular interactions between these two D1 subtypes, we cannot rule out differences in receptor/Gαi protein interaction. Potentially, the interaction of the D1A and D1B receptor with different Gαi splicing variants (short and long isoforms) may contribute to
the distinct activation properties observed between these two subtypes. Recently, Seifer et al. (36) have reported that the β2-adrenergic receptor fused to the long isoform of Gαs has the hallmark of constitutively activated GPCR. The dissociation between constitutive activation and dopamine intrinsic efficacy (maximal stimulation of adenylyl cyclase) observed in our study may be explained by potential mechanistic differences in the Gαs activation by the R* state and agonist-R* complex of these two D1 subtypes. Although speculative, recent genetic and mutagenesis studies on Gαs may support this hypothesis (37–40). Mutations in the switch 3 region of Gαs (R258W or R258A) lead to a reduced ability to stimulate adenylyl cyclase upon receptor activation (37). The defective function of these Gαs mutants is associated with an increased rate of GDP release, decreased binding of GDP in the inactive state, and increased intrinsic GTPase activity (37, 38). Cleator et al. (39) have shown that a mutant form of Gαs (S54N) exhibits a phenotype that suppresses the hormone-mediated stimulation of adenylyl cyclase, mediated by a β-adrenergic receptor or thyroid stimulating hormone receptor, without inhibiting the basal levels of intracellular cAMP (39). In fact, the thyroid stimulating hormone receptor displays an increased basal activity when co-expressed with the Gαs-S54N (39). These effects could be associated with an increased preference of the Gαs-S54N mutant for GDP, notably in the presence of agonist stimulation (40). Overall, these studies may provide support to the contention that different active states of a GPCR (R* or agonist-R*) could potentially induce or relieve structural constraints underlying the regulation of Gαs functional properties.

Notwithstanding the aspect of receptor/G protein interface, our present study raises two issues. The first issue pertains to the fact that our results support the existence of different active D1-like receptor conformations evoked in the presence or absence of dopamine. The second issue refers to the necessity of other structural determinants (presumably located in the TRL region) that act in concert with EL3 to define the spatial relationships underlying the dopamine affinity and constitutive activation of the D1A and D1B receptors. More specifically, the latter issue relates to the potential “antagonistic” or “counteracting” effect of these structural determinants (found either within or outside the TRL region) on EL3 function in the formation of active and inactive states of D1A and D1B receptors. The identification of these residues are of importance since they may underlie the molecular basis for the differential dopamine-mediated maximal activation of adenylyl cyclase observed upon stimulation of the D1A and D1B receptor.

Potentially, these residues could be located within TM6 and TM7. The highest degree of identity between D1A-TRL and D1B-TRL is found within the TM6 and TM7 (>90%). Indeed, in the TM6 and TM7, the primary structure differs only by two and one amino acids, respectively. Previous studies have shown that the TM7 of adrenergic receptors is an important structural determinant of both agonist and antagonist binding specificity, whereas TM6 influences mainly the coupling to G proteins (27). Recently, the TM6 has been implicated in discriminating between subtype-selective agonists for the human B1 and B2 bradykinin receptors and modifying antagonist affinity (29).

A role for the TM6 and TM7 in discriminating between the D1A and D1B receptor function remains to be clearly established. However, studies using chimeric receptor constructed from D1A and D2, or D1A and D3 implicate TM6 and TM7 as structural determinants that define some of the functional properties of D1-like and D2-like receptors (41–44). Mutation of the conserved tryptophan residue (at position 321) in TM7 of the D1A receptor leads to a 3-fold decrease in the SCH23982 affinity without any modification of the dopamine affinity (45).

Overall, these studies support the notion of the presence of docking sites for agonists and antagonists within these TM regions. In addition to providing docking sites for ligands, mutagenesis and genetic studies have implicated the TM6 and TM7 in the regulation of active and inactive conformational states of GPCRs (46–48). Constitutively activating mutations occurring naturally in the TM6 or TM7 of various types of GPCRs have been linked to the development of male precocious puberty, thyroid adenomas, and a form of retinitis pigmentosa (49–51). Interestingly, recent studies have shown that activation of the β2-adrenergic receptor modifies the orientation of TM6 (47, 52). Potentially, the small differences found in the primary structure of the TM6 and TM7 of D1 receptors may alter the orientation of these TM and underlie the molecular basis for their ligand-dependent and -independent receptor conformations.

Alternatively, residues present in the cytoplasmic tail may also play an important role in the regulation of active and inactive conformations of the D1A and D1B receptors. In fact, the lowest degree of identity between D1A-TRL and D1B-TRL is found in the cytoplasmic tail (31%). Because of its intracellular localization, it is unlikely that the cytoplasmic tail plays a role in the direct docking of agonists and antagonists. Meanwhile, studies have demonstrated that the cytoplasmic tail can regulate the formation of active and inactive receptor states (9, 13). However, a recent study has shown that chimeric Xenopus D1 receptors harboring cytoplasmic tail sequences exhibit no modification in the dopamine affinity and potency, and constitutive activation (32).

In conclusion, our study has delineated an important receptor domain containing the structural determinants involved in regulating the activation process of the human D1-like receptor subtypes. Studies in our laboratory are underway to define further the molecular basis of the constitutive activation and G protein coupling of the D1A and D1B receptor. To our knowledge, the present study represents the first example of mutations introduced in GPCRs that can dissociate agonist affinity, constitutive activation, and intrinsic efficacy (maximal activation of adenylyl cyclase).

Acknowledgments—We thank Simon Ginsberg for technical assistance with the cell culture. We express our sincere gratitude to Drs. Stéphane Charpentier and Adele Jackson for insightful comments and critically reading the manuscript.

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