Silencing of the Constitutive Activity of the Dopamine D1B Receptor

RECIPIROCAL MUTATIONS BETWEEN D1 RECEPTOR SUBTYPES DELINEATE RESIDUES UNDERLYING ACTIVATION PROPERTIES*†‡§©

(Received for publication, July 23, 1996, and in revised form, August 26, 1996)

Stéphane Charpentier‡, Keith R. Jarvie, Diana M. Severynse, Marc G. Caron§, and Mario Tiberi¶

From the Howard Hughes Medical Institute Laboratories, Department of Cell Biology, Duke University Medical Center, Durham, North Carolina 27710

Recently, we have shown that the dopamine D1B/D5 receptor displays binding and coupling properties that are reminiscent of those of the constitutively activated G protein-coupled receptors when compared with the related D1A/D1 receptor subtype (Tiberi, M., and Caron, M. G. (1994) J. Biol. Chem. 269, 27925–27931). The carboxyl-terminal region of the third cytoplasmic loop of several G protein-coupled receptors has been demonstrated to be important for the regulation of the equilibrium between inactive and active receptor conformations. In this cytoplasmic region, the primary structure of dopamine D1A and D1B receptors differs by only two residues: Phe264/Arg266 are present in D1A receptor compared with Ile288/Lys290 in the D1B receptor. To investigate whether these structural differences could account for the distinct binding and coupling properties of these dopamine receptor subtypes, we swapped the variant residues located in the carboxyl-terminal region by site-directed mutagenesis. The exchange of the D1A receptor residue Phe264 by the D1B receptor counterpart isoleucine led to a D1A receptor mutant exhibiting D1B-like constitutive properties. In contrast, substitution of D1B receptor Ile288 by the D1A receptor counterpart phenylalanine resulted in a loss of constitutive activation of the D1B receptor with binding and coupling properties similar to the D1A receptor. The Arg/Lys substitution had no effect on the function of either receptor. These results demonstrate that the carboxyl-terminal region, and in particular residue Ile288, is a major determinant of the constitutive activity of the dopamine D1B receptor. Moreover, these results establish that not only can agonist-independent activity of a receptor be induced, but when given the appropriate mutation, it can be reversed or silenced.

Based upon the degree of homology in their primary structure and their capacity to either stimulate or inhibit adenylyl cyclase, dopaminergic receptors can be grouped into two families referred to as D1-like (D1A, D1B) and D2-like (D2short, D2long, D3, and D4) receptors, respectively. Notwithstanding the high degree of homology in their primary structure and their similar pharmacological profile, D1-like receptors have been shown to exhibit some differences in binding and coupling properties when expressed in 293 or COS-7 cells (Grandy et al., 1991; Sunahara et al., 1991; Tiberi et al., 1991; Weishank et al., 1991; Tiberi and Caron, 1994). The affinity of agonists is generally higher for the D1B receptor, whereas antagonists bind with a greater affinity to the D1A receptor. Recently, we have shown that the D1B receptor exhibits a higher agonist-independent activity in comparison with the D1A receptor (Tiberi et al., 1994). Furthermore, agonists are more potent at stimulating adenylyl cyclase in 293 cells expressing D1B receptors than D1A receptors. Overall these properties of the D1B receptor are reminiscent of those described for constitutively active receptors (Lefkowitz et al., 1993) and suggest that the D1B receptor may represent a naturally occurring constitutively active counterpart of the D1A receptor.

It has been shown that G protein-coupled receptors for biogenic amines can be converted into constitutively active forms by engineering single point mutations in the carboxyl-terminal region of the third intracellular loop (Cotecchia et al., 1990; Ren et al., 1993; Samama et al., 1993; Högger et al., 1995). These studies have suggested that the carboxyl-terminal region of these receptors is crucial in constraining the receptor into an inactive conformation (Kjelsberg et al., 1992; Lefkowitz et al., 1993).

Based largely on these considerations, a modified “allosteric” ternary complex model has been formulated for the agonist activation of G protein-coupled receptors. The model postulates that receptors exist in an equilibrium between “inactive” (R) and “active” (R*) conformational states (Samama et al., 1993; Leff, 1995), and by virtue of a higher affinity for the active conformation, agonists bind preferentially to and stabilize R*. This interaction leads to the formation of an active complex AgR*, resulting in a displacement of the thermodynamic equilibrium R–R* to the right. The two molecular species generated, R* and AgR*, are both able to interact with G proteins and trigger signal transduction. In addition to a high agonist-independent activity, constitutively active receptors share other pharmacological properties: (i) they display an increased binding affinity for agonists, (ii) the potency of agonists in activating an effector is augmented, and (iii) partial agonists have a greater intrinsic activity. These properties support the hypothesis that the constitutively activating mutations increase the propensity of the receptor to adopt an active confor-
mation in its unliganded state (R* state).

A comparison of the carboxyl-terminal region of the third cytoplasmic loop between D1A and D1B receptors reveals that these subtypes differ by only two amino acids (Phe<sup>286</sup>, Arg<sup>288</sup> in D1A and Ile<sup>288</sup>, Lys<sup>290</sup> in D1B) (Fig. 1). To investigate whether these differences play a role in the regulation of the agonist-independent activity of the D1A and D1B receptors, we substituted the two variant amino acids found in this cytoplasmic region of the D1A receptor by those found in the D1B receptor and vice versa. The ability of the various mutant receptors to bind dopaminergic agonists and antagonists, and their capacity to stimulate adenylyl cyclase in absence or presence of dopamine were assessed and compared with their cognate wild-type receptors. Using this approach, we demonstrate that a single residue in the region of the two receptors plays a crucial role in regulating the transition between active and inactive receptor conformations. Therefore, these results suggest that some of the pharmacological differences observed between D1A and D1B receptors are explained by a difference in their unliganded conformations (R and R*), which appear to be controlled at least in part by the carboxyl-terminal region of the third cytoplasmic loop.

**EXPERIMENTAL PROCEDURES**

**Construction of Mutated Receptor cDNAs—**Human dopamine D1A and D1B receptors were introduced into the expression vector pCMV5 as described previously (Dearnry et al., 1990, Tiberi et al., 1991). Point mutations were engineered using the polymerase chain reaction (PCR). To generate human D1A receptor mutants, a 224-base pair HindIII-BclI fragment was amplified using specific primers containing mutated sequences and subcloned into the wild-type D1A receptor construct. Similarly, mutations in the human D1B receptor were introduced by amplification of a BsmI-Noel fragment. PCR reactions were performed with Taq DNA polymerase (Perkin-Elmer) in a Hybaid thermocycler using the following conditions: 35 cycles (94°C for 1 min, 55°C for 2 min, 72°C for 3 min) terminated by a final extension step at 72°C for 7 min. PCR products were separated on 1.0% agarose gel and purified on Qiaex resin (Qiagen Inc.). All mutations were confirmed by dye-sequencing of the PCR amplified receptor region using a Sequenase V 2.0 kit (U. S. Biochemical Corp.).

**Cell Culture and Transfection—**Human embryonic kidney cells (293 cells; American Type Culture Collection) were grown at 37°C and 5% CO<sub>2</sub> in minimal essential medium supplemented with 10% heat-inactivated fetal bovine serum and gentamicin (100 μg/ml) (Life Technologies, Inc.). Cells were plated in 100-mm dishes (2.5 × 10<sup>5</sup> cells/dish) and transiently transfected with 5 μg of DNA/dish using a modified calcium phosphate precipitation method (Didsbury et al., 1991).

**Radioligand Binding Assay—**24 h after transfection, cells were trypsinized, reseeded in 150-mm dishes, and allowed to grow for an additional 36–40 h. To prepare membranes, 293 cells were 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 40,000 × g for 30 min at 4°C. The pellet was resuspended in lysis buffer by homogenization with a Brinkmann Polytron (15,000 rpm, 10–15 s) and stored at −80°C until used. 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<sub>2</sub>, 1.5 mM CaCl<sub>2</sub>, 1 mM EDTA) at a concentration of 5–10 μg of protein/100 μl. [<sup>35</sup>S]ICSH23982 was used as radioligand and prepared by radiiodination of the deschloro analog of SCH23390. Saturation analyses were done using [<sup>35</sup>S]ICSH23982 at concentrations ranging from 0.015 to 4.8 nM with 100 μl of membranes in a total volume of 200 μl. For competition curves, 100 μl of membranes were incubated with [<sup>125</sup>I]ICSH23982 (−0.5 nM) and increasing concentrations of competing ligand. Nonspecific binding was determined in the presence of 10 μM flupentixol (Research Biochemicals International). Binding assays were incubated for 120 min at room temperature and terminated by rapid filtration through glass fiber filters (GF/C, Whatman). Filters were washed three times with 3 ml of ice-cold wash buffer (100 mM NaCl, 50 mM Tris-HCl, pH 7.4) and monitored by γ counting. All measurements were performed in duplicate, and each experiment was repeated 2–3 times. Protein concentration was determined using the Bio-Rad assay kit with bovine serum albumin as standard. Saturation and competition binding curves were analyzed by nonlinear least square regression method (GraphPad Software, Inc.).

**RESULTS**

**FIG. 1.** Representation of the putative topology of a 7-transmembrane G protein-coupled receptor and sequence alignment of the carboxyl-terminal region of the third cytoplasmic loop of the human dopamine D1A and D1B receptors. Variant amino acid residues in the carboxyl-terminal region used as mutagenesis sites are shown in black circles (Phe<sup>286</sup> and Arg<sup>288</sup> in D1A, and Ile<sup>288</sup> and Lys<sup>290</sup> in D1B).

Dopamine receptors are explained by a difference in their unliganded conformations (R and R*), which appear to be controlled at least in part by the carboxyl-terminal region of the third cytoplasmic loop.

**Structural Basis of Dopamine D1A and D1B Receptor Activation**

**Saturation Binding Studies**

Binding studies were performed on membrane homogenates of transfected cells to determine the potential role that variant amino acids residing in the carboxyl-terminal region of the third cytoplasmic loop play in D1A and D1B receptor affinity for dopaminergic agonists and antagonists. Saturation binding experiments showed that [<sup>125</sup>I]ICSH23982 binds to D1A, D1B receptors, and their mutants with a similar affinity constant (K<sub>d</sub> = 0.6 nM). Receptors were expressed at similar levels (B<sub>max</sub> = 1–2 pmol/mg of proteins), suggesting that the mutant receptors are appropriately processed at the cell surface.
Substitution of Phe264 by Ile in the Third Cytoplasmic Loop of D1A Receptor Increases Agonist Affinity without Affecting Antagonist Binding—Substitution of Phe264 and Arg286 in the D1A receptor by the reciprocal Ile and Lys residues found in the D1B receptor leads to a mutant D1A receptor that exhibits an increased affinity for the endogenous agonist dopamine. This higher dopamine affinity for the double mutant is manifested by a shift of the competition curve toward the D1B receptor isotherm (Fig. 2A). To delineate the underlying residues responsible for this increased affinity, we tested the ability of the two single point mutants (F264I-D1A and R266K-D1A) to bind dopamine. Competition binding experiments performed with these mutants indicate that the increased affinity observed for the double point mutant is solely explained by the F264I substitution. Indeed, the F264I-D1A receptor displays a leftward shift in the affinity for dopamine while the R266K substitution has no significant effect. We then examined the ability of these mutant D1A receptors to bind butaclamol (the most selective antagonist). In striking contrast with the dopamine binding, the different substitutions had no significant effect on the binding affinity of butaclamol for mutant D1A receptors (Fig. 2B).

Substitution of Ile288 by Phe in the Third Cytoplasmic Loop of D1B Receptor Reduces Agonist Affinity without Affecting Antagonist Binding—In a similar manner as described above, we substituted the residues Ile288 and Lys290 in the carboxy-terminal region of the third cytoplasmic loop of the D1B receptor by their D1A residue counterparts (Phe and Arg). The double substitution (I288F/K290R) in the D1B receptor results in a loss of affinity for dopamine (Fig. 3A). In fact, the mutant receptor displays a dopamine binding profile similar to that of the D1A receptor (Fig. 3A). To further explore which of these two residues potentially modulate dopamine binding, we engineered single point mutations. The single substitution, I288F, results in a mutant receptor that mimics the effect of the double mutation while the conservative mutation, K290R, does not alter the dopamine binding affinity. Therefore, the loss of affinity for dopamine obtained with the double point mutant can be explained by I288F substitution. None of these substitutions affect the binding affinity of the antagonist butaclamol (Fig. 3B).

Basal cAMP Production

The basal adenyl cyclase activity was determined by measuring the intracellular content of [3H]cAMP formed in 293 cells transfected with the various wild-type and mutant D1 receptor
subtypes. For these studies, transfection conditions were chosen to give similar levels of receptor expression to allow a meaningful comparison of the agonist-independent activity obtained in cells transfected with the wild-type and mutant receptors. As we previously demonstrated, cells transfected with the dopamine D1B receptor have a 3–4-fold higher basal adenyl cyclase activity than those transfected with the D1A receptor (Fig. 4).

Substitution of Phe264 by Ile in the Third Cytoplasmic Loop of D1A Receptor Induces Constitutive Activation—As illustrated in Fig. 4A, exchanging the Phe264 and Arg266 residues of the D1A receptor by Ile and Lys (D1B receptor residues), respectively, leads to a mutant D1A receptor (F264I/R266K-D1A) with a higher agonist-independent activity. The significant gain of agonist-independent activity of the mutant D1A receptor (~40%) is explained by the Ile residue. Indeed, the F264I-D1A mutant displays the same degree of constitutive activation as the F264I/R266K-D1A receptor. However, the mutation R266K does not significantly modify the basal adenyl cyclase activity of the D1A receptor (Fig. 4A).

Substitution of Ile288 by Phe in a Diminution of the D1B Receptor Constitutive Activity—In contrast to the D1A mutants, the double substitution (I288F/K290R) in the D1B receptor leads to an ~50% loss of constitutive activity (Fig. 4B). However, similar to that of the D1A mutants, the effect on agonist-independent activity is attributed to the I288F substitution while the K290R substitution has no significant effect of the D1B receptor constitutive activity. Moreover, the I288F substitution is able to reproduce a similar decrease of basal adenyl cyclase activity observed with the double substitution.

Agonist-induced cAMP Production

Dose-response curves were performed in 293 cells transfected with wild-type and mutant receptors to study the effect of mutations on the dopamine potency. Fig. 5 shows that dopamine is about 10-fold more potent at the human D1B dopamine receptor as compared with the human D1A receptor, as was reported previously (Tiberi et al., 1994).

F264I Swapping in D1A Receptor Increases the Potency of Dopamine—Dopamine has a significant increased potency for the F264I/R266K-D1A receptor as compared with the wild-type D1A dopamine receptor (Fig. 5A). This increased potency of dopamine agrees with its increased binding affinity and the higher basal activity, confirming the constitutively active nature of the F264I/R266K-D1A mutant. Furthermore, this effect is explained by F264I substitution. Indeed, dopamine potency displays a 3-fold increase for the F264I-D1A receptor as compared with the wild type, whereas the R266K mutant exhibits a slight decrease of dopamine potency (Fig. 5A). None of the mutations have significantly altered the level of maximal activation of adenyl cyclase as compared with the wild-type D1A receptor (data not shown).

Eschewing Ile288 by Phe in D1B Receptor Decreases Dopamine Potency—We analyzed the D1B mutant receptors upon dopamine stimulation. Dopamine displays a decreased potency for I288F/K290R-D1B mutant toward the potency measured at the wild-type D1A receptor (Fig. 5B). Indeed, this is reflected by a 3-fold decrease in EC50 of dopamine for stimulation of cAMP as compared with the wild-type D1B receptor. This effect can be mimicked by the single substitution I288F, whereas the K290R-D1B receptor mutant is indistinguishable from the wild-type D1B receptor. No significant change in the level of adenyl cyclase maximal stimulation was observed for all the mutants as compared with the wild-type D1B receptor (data not shown).

DISCUSSION

In the present study, we have delineated some of the underlying structural determinants for the binding and coupling properties of the D1 receptor subtypes by systematically interchanging the two variant amino acid residues in the carboxy-terminal domain of their third intracellular loops. This region has been previously identified as a preferential domain for activating mutations in G protein-coupled receptors (Lefkowitz et al., 1993). Our data demonstrate that residue Ile288 is one of the amino acid residues underlying the D1B receptor constitutive activity properties. To support this assertion, substitution of Phe264 in the D1A receptor by an isoleucine residue results in a mutant D1A receptor with features of a constitutively activated receptor. Conversely, the substitution I288F in the D1B receptor results in a partial silencing of constitutive activity and a mutant D1B receptor with properties reminiscent of the wild-type D1A receptor. Interestingly, substitutions of other variant residues in the carboxy-terminal region (R266K for D1A and K290R for D1B) do not significantly alter any of the pharmacological properties observed with the respective wild-type D1 receptor subtypes. These results suggest that within the context of these two receptor subtypes, the aliphatic side chain of the isoleucine residue plays an important role in the formation of an active receptor conformation, whereas the aromatic moiety of the phenylalanine residue may help to maintain the receptor in an inactive conformation.

![Figure 4](image-url)
Conformational modifications induced by the Ile-Phe substitution can be rationalized by the allosteric ternary complex model that postulates an isomerization step between an inactive R state and an active R* state of the receptor (Samama et al., 1993). The activating mutation F264I in the D1A receptor leads to the formation of the R* state in the absence of agonist while the reciprocal I288F mutation in the D1B receptor preferentially constrains the receptor conformation into its R state. Consistent with the observation that agonists bind preferentially to R* (Lefkowitz et al., 1993; Samama et al., 1993), our binding data confirm the increased affinity of F264I-D1A mutant for dopamine. In addition, our results are in agreement with an expected loss of affinity of the mutant I288F-D1B receptor for dopamine. In contrast, antagonists appear to bind with similar affinities to R and R*. Indeed, no differences in butaclamol binding affinities were measured for either wild-type or constitutively active D1 receptor subtypes. As it was suggested for the constitutively activated adrenergic receptors, the Phe264 residue in the D1A receptor is likely to constrain the receptor in an inactive conformation (Kjelsberg et al., 1992). This constraint is released upon agonist binding or partially released by the isoleucine substitution that allows the D1A receptor to “relax” into an active conformation. Moreover, the allosteric ternary complex model helps to explain the coupling differences observed between wild-type D1A and D1B receptors. Indeed, the unoccupied wild-type D1A receptor exists predominantly in the inactive form R that can less favorably couple to G proteins, whereas the D1B receptor has a higher propensity to exist in the R* state that allows it to couple more readily to G protein and activate adenylyl cyclase in the absence of agonist. The conversion of D1A into a D1B-like receptor and, reciprocally, the D1B into a D1A-like receptor by manipulating the carboxyl-terminal domain of the third intracellular loop demonstrates the important role this region plays in the transition of inactive to active receptor.

While the mutations reciprocally introduced into the two receptor subtypes recapitulated in part some of the activated or silent properties of the parent receptor, they failed to completely reproduce the phenotype, suggesting that other structural determinants of the receptors exist that constrain them in an inactive or active conformation. Indeed, it is worth mentioning that in addition to the carboxyl-terminal region of the third cytoplasmic loop (Cotecchia et al., 1990; Parma et al., 1993; Ren et al., 1993; Samama et al., 1993; Högger et al., 1995; Latronico et al., 1995; Burstein et al., 1996), numerous studies have now identified activating mutations (naturally occurring or artificially introduced) in extracellular (Robbins et al., 1993, Nanivez et al., 1996), transmembrane (Robinson et al., 1992, Robbins et al., 1993, Rao et al., 1994, Duprez et al., 1994, Perez et al., 1996, Shenker et al., 1993, Kremer et al., 1993, Dryja et al., 1993, Spalding et al., 1995), or intracellular domains (Robbins et al., 1993, Schipani et al., 1995, Matus-Leibovitch et al., 1995) of G protein-coupled receptors. Several of these mutations have been found to underlie various pathological conditions. Commonly, a 2–10-fold increase in receptor basal activity has been associated with these mutations, suggesting that the organism can detect small changes in the properties of these receptors. These examples highlight the probable physiological relevance of the pharmacological differences between D1A and D1B receptors. However, the properties observed in heterologous ex-
pression systems will eventually have to be verified in the whole animal. Indeed, the use of transgenic mice expressing these mutants in specific neurons known to synthesize the mRNA for the D1 receptor subtypes may unravel their physiological functions (Milano et al., 1994; Chen et al., 1995). Interestingly, the physiological consequences of such activating mutations may not solely depend on the enhanced constitutive signaling activity as such mutations enhance the phosphorylated state of these receptors (Ren et al., 1993; Pei et al., 1994).

Finally, more studies will be required to unequivocally establish whether introduction of other residues than those found in the wild-type receptors further enhance or silence the constitutive activity when introduced in the carboxyl-terminal region of the third intracellular loop of D1 receptor subtypes, as reported previously for the α1B-adrenergic receptor (Kjelsberg et al., 1992).

In conclusion, our results demonstrate that the residue Ile288 is one of the structural determinants underlying the constitutive activation of the dopamine D1B receptor. Reversal of the effect elicited by reciprocal amino acid substitutions suggests that some of the pharmacological differences observed between D1A and D1B receptors are likely explained by their unliganded conformation states. The findings that mutations in the carboxyl-terminal part of the third cytoplasmic loop can not only induce but reverse, at least partially, agonist-independent receptor activity implies that not all receptors have evolved to display the most silent phenotype possible and also reinforces the notion that this region is of importance in the transition between the inactive and active receptor conformations.

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