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Mutagenesis of Residues Adjacent to Transmembrane Prolines Alters D_1 Dopamine Receptor Binding and Signal Transduction

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SUMMARY

Proline is highly conserved in the presumed transmembrane α-helices of seven-transmembrane helix-containing, G protein-coupled receptors. Unique properties of this imino acid have led to speculations of structural and perhaps dynamic importance for seven-transmembrane helix-containing receptor function. To avoid potentially deleterious consequences of proline-directed mutagenesis, substitutions were made in the X residue of X-Pro peptide bonds (where X is the residue on the amino-terminal side of proline), which may influence static geometries and potential agonist-induced conformational changes at the X-Pro peptide bond. In the fifth helix, Ile205 was substituted with either an alanine (I205A) or a tyrosine (I205Y). Similarly, in the sixth helix, Leu286 was substituted with either an alanine (L286A) or a tyrosine (L286Y). Mutant I205A demonstrated subtle changes in D_1 pharmacology and signal transduction. The I205Y and L286Y mutations produced comparatively drastic impairments in both binding and signal transduction. Remarkably, the L286A mutation resulted in constitutive activity characterized by elevated basal signal transduction and increased agonist potencies. In addition, (*R*)-(+)-SCH23390, a classical antagonist at the wild-type D_1 receptor, behaved as a partial agonist at L286A. This is the first report of a constitutively active receptor resulting from this point mutation and the first report of a constitutively active mutant dopamine receptor. These results are discussed in terms of binding pocket geometry and potential mechanisms of signal transduction.

The cloning of several dopamine receptor subtypes (reviewed in Ref. 1) reveals that the dopamine receptor family belongs to a superfamily of structurally and functionally related proteins, the 7TM, G protein-coupled receptor superfamily. The members of this superfamily of membrane receptors are presumed to share a canonical structure characterized by seven membrane-traversing α-helical barrels connected by three extracellular and three intracellular loops. The amino terminus is extracellular, whereas the carboxyl terminus lies within the cytosol. Structure-function studies, using a variety of strategies, have focused attention upon specific regions of this complex tertiary structure that are responsible for at least two functions, i.e., ligand binding and G protein coupling.

Site-directed mutagenesis efforts have characterized the binding domain of 7TM receptors that utilize small cationic ligands. The binding sites appear to lie within a "pocket" defined by certain α-helical barrels. A highly conserved aspartate residue in transmembrane helix 3 is critical for agonist and some antagonist binding to the dopamine D_2 receptor (2) and β-adrenergic receptors (3), most likely through ionic interactions with positively charged groups on the ligands. Several helix 5 serine residues of dopamine D_1 (4) and D_2 receptors (2, 5) and β-adrenergic receptors (6) seem to be important for binding of catecholamine ligands and structurally related compounds, presumably stabilized by hydrogen bonding between the serine side groups and the hydroxyl groups of the catecholamine moiety.

In contrast to ligand binding, which occurs at the transmembrane helices, coupling with G proteins appears to involve intracellular portions of the 7TM receptor protein. Deletional mutations and construction of receptor chimeras highlight portions of the third intracellular loop and proximal portions of the carboxyl terminus as being important for signal transduction (7–10). Some studies indicate that the second intracellular loop (8, 11, 12) may also be involved. To date, most structure-function studies have investigated ligand binding domains and G protein coupling domains independently, and few studies have provided clues to the mechanisms that link these disparate receptor functions. Presumably, upon ligand binding, the receptor protein undergoes some type of conformational change or isomerization

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ABBREVIATIONS: 7TM, seven-transmembrane helix-containing; DMEM, Dulbecco's modified Eagle's medium; CMV, cytomegalovirus; FBS, fetal bovine serum; TM6, transmembrane region 6; TM5, transmembrane region 5.

that initiates subsequent signal transduction events. However, the mechanisms involved in 7TM receptor conformational shifts remain largely unknown.

Under the assumption that structurally related 7TM receptors share some common functional mechanisms of receptor activation, an analysis of aligned 7TM protein sequences may provide clues to this mechanism by emphasizing highly conserved, presumably critical, residues (for a review of aligned sequences, see Ref. 13). Such an analysis reveals several highly conserved proline residues within the presumed transmembrane α-helices. The conservation of proline in α-helical regions is interesting in light of several peculiar properties of this imino acid. The covalent linkage of the side chain to the adjacent tertiary amine produces a "kink" in the helical backbone; the severity of this helical redirection can be observed in the model by Henderson *et al.* (14), which is built upon high-resolution electron microscopic data. In addition, the cyclized structure of this "helix breaker" sterically restricts the space available to the adjacent amino-terminal residue (15). Finally, the imide nitrogen of proline lacks a hydrogen atom for participation in intrahelical hydrogen bonds. Despite these helix-destabilizing properties (reviewed in Ref. 16), intrahelical prolines are highly conserved. Thus, strategically located prolines may be involved in crucial structural motifs used by 7TM receptors.

Aside from potential structural roles, prolines may be of dynamic importance for 7TM function. The X-Pro peptide bond (where X indicates any residue on the amino-terminal side of proline) is thought to be particularly susceptible to isomerization between the *trans*- and *cis*-conformations (reviewed in Refs. 16 and 17), mainly because of the absence of hydrogen-bond stabilization between the imine group of proline and the carboxyl group provided by the adjacent helical turn. Although the drastic redirection of the peptide chain caused by a full *cis/trans*-isomerization could be alleviated to some degree by compensatory changes in the ϕ and φ angles (18), agonist-induced isomerization events in membrane-embedded regions are most likely partial and much more subtle. Computer modeling of proline-containing peptides in a low polarity environment suggests that both the full-*trans*- and full-*cis*-isomers are energetically achievable, as are several other *trans*-isomers, as a result of rotation about one torsion angle and rearrangement of hydrogen-bonding patterns (18). Conformational changes of a receptor may involve transitions between these *trans*-conformers, as well as partial transitions or "wobbles" about the full-*trans*- or -*cis*-isomers. These peculiarities of proline and the X-Pro peptide bond have led to the speculation that highly conserved prolines may be not only structurally but also dynamically important for membrane protein structure and function (19).

Previous attempts to investigate the importance of conserved proline residues within the transmembrane helices of G protein-coupled catecholamine receptors were met with limited success (20). Substitution of the TM7 Pro323 of the β-adrenergic receptor appeared to result in an incorrectly or incompletely processed protein. The authors speculated that this conserved proline must be critical for proper receptor folding events. If the importance of proline residues in G protein-coupled receptor structure and function is to be understood, more subtle mutagenesis strategies must be used. Interestingly, the rates and equilibria of X-Pro isomerization appear to be influenced by the nature of the adjacent X, or amino-terminal, residue (21, 22), presumably secondary to steric interactions with the adjacent proline. The present study investigates the importance of conserved prolines in D_1 receptor function by mutating residues on the amino-terminal side of prolines within transmembrane helices. If the residue in the X position is able to influence helical geometry or the energetics of isomerization, then mutations within TM5 and TM6, which form the boundaries of the third cytosolic loop, may yield altered ligand binding and possibly altered signal transduction.

To test these ideas, site-directed mutagenesis was directed at residues on the amino-terminal side of prolines within the fifth and sixth helices of the human D_1 receptor (Fig. 1). Prolines in helices 5 and 6 were selected as targets because these barrels surround the third cytosolic loop, a region believed to critical for G protein coupling (7–10), and because helix 5 serines are believed to be important for D_1 ligand binding (4). The prolines here may be important for linking ligand binding and signal transduction. Ile205 in TM5 of the human D_1 receptor was substituted with either an alanine (I205A) or a tyrosine (I205Y), and Leu286 in TM6 was similarly substituted with either an alanine (L286A) or a tyrosine (L286Y). The wild-type residues at positions 205 and 286, isoleucine and leucine, both consist of four-carbon branched aliphatic chains. The substitutions, alanine and tyrosine, differ substantially from the wild-type amino acids, changing the characteristics of the sterically restricted space on the amino-terminal side of proline. The volume of the side chain of alanine, consisting of only one carbon atom, is smaller than than the volumes of the wild-type side chains, whereas that of tyrosine contains a large six-carbon aromatic phenyl ring. The consequences for both binding and signal transduction are discussed below. The D_1 dopamine receptor subtype was used in this study because its pharmacological and signal transduction properties are well characterized, providing a broad database against which to compare results of these mutations.

Materials and Methods

Mutagenesis. A cDNA containing the entire coding region of the human D_1 receptor was ligated into the eukaryotic expression vector pCMVneo, which contains the simian virus 40 origin of replication (pCMVhD1). Transcription of the D_1 receptor was driven by the CMV promoter. A full-length cDNA for the long isoform of the human D_1 receptor was simultaneously subcloned into pGEM as a template for mutagenesis. Site-directed mutants were created using the Clontech mutagenesis kit V. Complementary; mutant oligonucleotides of 22–25 bases were synthesized and purified twice by high performance liquid chromatography. Mutants were verified by dideoxy chain-termination DNA sequencing (Sequenase, version 3.0; United States Biochemical, Cleveland, OH). Mutated fragments were then subcloned into pCMVhD1. A β-galactosidase cDNA construct was also created in pCMVneo for transfection efficiency measurements (pCMVβ-gal).

Cell culture and transfection. COS-7 cells were maintained in DMEM, supplemented with 10% FBS, at 37° in 5% CO_2/95% O_2 and were subcultured every 3–5 days. Cells were seeded at a density of 1–1.5 $\times$ 10^6 cells/10-cm dish for transfections by the calcium phosphate precipitation protocol of Chen and Okayama (23), using 25 μg of pCMVhD1 and 5 μg of pCMVβ-gal for transfection efficiency measurements. Transfected cells were incubated at 37° in 3% CO_2 for 18–24 hr, after which the cells were washed twice with versene. For radioligand binding experiments, the versene washes were fol-

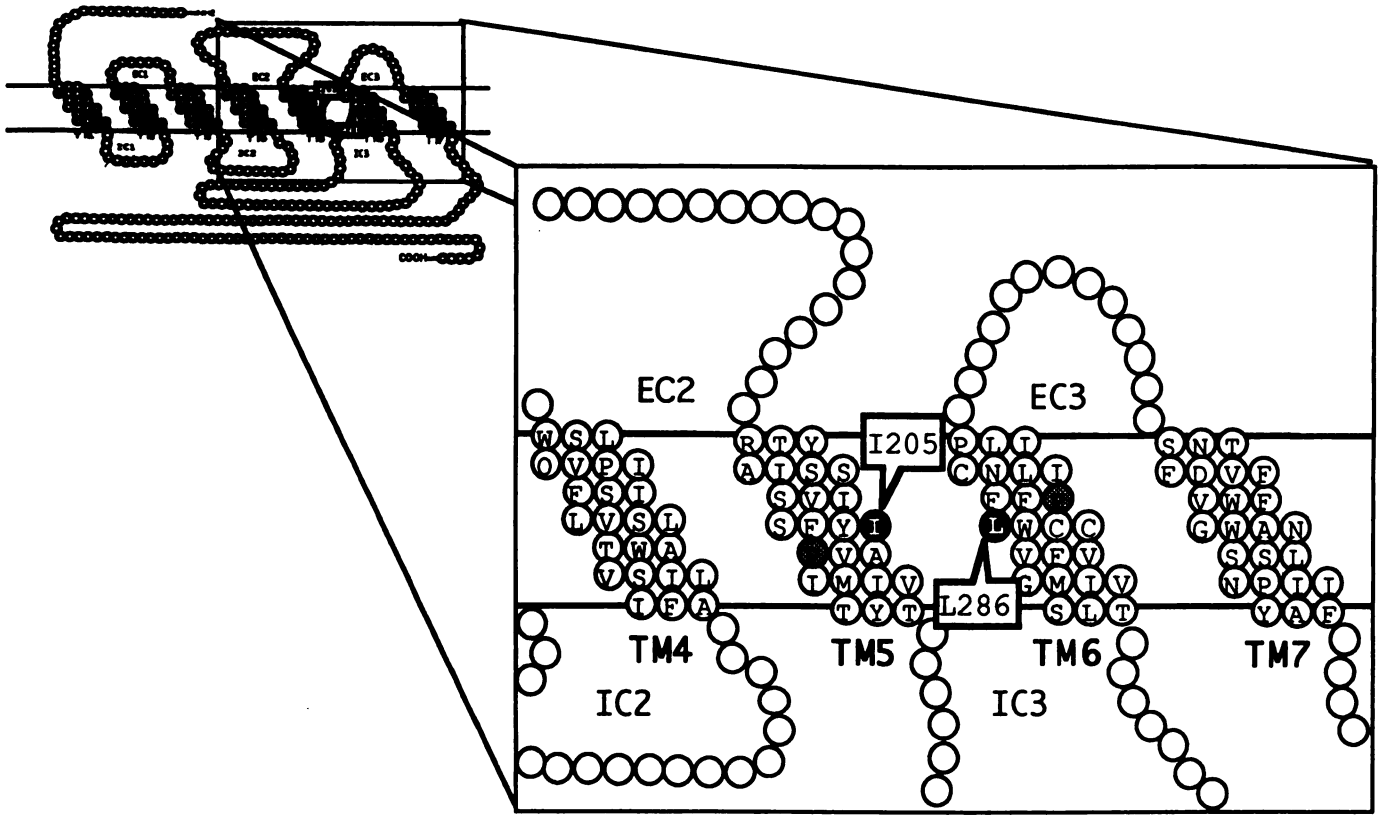

Fig. 1. Schematic representation of the D_1 dopamine receptor subtype. *Black circles*, wild-type residues that were mutated (*I205*, Ile205 in TM5; *L286*, Leu286 in TM6). *Gray circles*, highly conserved proline residues immediately adjacent to I205A and L286Y on the carboxyl-terminal side. *EC1*, first extracellular loop; *EC2*, second extracellular loop; *EC3*, third extracellular loop; *IC1*, first intracellular loop; *IC2*, second intracellular loop; *IC3*, third intracellular loop; *TM4*, transmembrane region 4; *TM7*, transmembrane region 7.

lowed by one wash with DMEM/10% FBS, and the transfected plates were then grown in 5% CO_2 at 37° for an additional 24–48 hr before cell harvest. For radioimmunological measurement of cAMP accumulation in intact cells, the versene washes were followed by mechanical trituration in versene. The cells, once lifted, were pelleted at 500 × *g* for 1 min, resuspended in DMEM/10% FBS, and plated into individual wells of 24-well plates. The replated cells were then returned to incubation at 37° in 5% CO_2 for 24–48 hr before pharmacological treatments.

Radioligand binding assays. Cells transfected by the calcium phosphate method were washed twice and harvested in binding buffer (50 mM Tris, 5 mM KCl, 5 mM $MgCl_2$, 1 mM EDTA, pH 7.4 at 22°). Harvested cells were pelleted at 500 × *g* for 5 min, resuspended in binding buffer, and homogenized (Virtishear homogenizer, 80% maximum rpm, 30 sec) (Vertishear; Virtis, Gardiner, NY). For Scatchard analysis, cellular homogenates were incubated with the D_1 antagonist (*R*)-(+)-[^{3}H]SCH23390 (specific activity, 83 Ci/mmol; Amersham, Arlington Heights, IL) at six concentrations. Nonspecific binding was determined by displacement with 10 mM unlabeled (*R*)-(+)-SCH23390 or 10 mM *cis*-(*Z*)-flupenthixol. The total volume in each incubation reaction was 250 μl/tube. Incubations were performed in duplicate for 60 min at 22°. For the generation of competition isotherms, cellular homogenates were incubated in the presence of saturating concentrations of (*R*)-(+)-[^{3}H]SCH23390 (10–100-fold greater than the K_d), displaced by increasing concentrations of the unlabeled antagonist *cis*-(*Z*)-flupenthixol or unlabeled agonists [(±)-SKF38393, (±)-SKF82958, or dopamine]. The total volume in each incubation reaction was 240 μl/tube. Incubation reactions were performed in duplicate at 22° for 60–90 min.

Measurement of cAMP. DMEM/10% FBS was aspirated from 24-well culture dishes containing COS-7 cells transfected with either wild-type or mutated pCMVhD1 or vector without any receptor (pCMVneo) and was replaced with DMEM. Cells were incubated in DMEM without FBS for 2 hr before drug treatments. Individual wells were treated with 0.5 mM 3-isobutyl-1-methylxanthine for 30 min at 37° before incubation with dopaminergic agonists or antagonists for 15 min at 37°; wells to be treated with agonist were also incubated with 10 μM (−)-alprenolol, to block any nonspecific stimulation of β-adrenergic receptors; mock-transfected cells were allowed to remain at 37° for a total of 45 min in the absence of dopaminergic ligands. The drug-containing medium was then quickly removed by aspiration. Each well was washed twice with ice-cold phosphate-buffered saline, and the cells were subsequently lysed in 65% ethanol for 8–12 hr at 4°. The lysate was collected, centrifuged at 2000 × *g* for 15 min, and lyophilized. The lyophilized cAMP extract was resuspended and diluted appropriately in 0.05 M sodium acetate, pH 6.2. Quantities of cAMP were assessed by equilibrium radioimmunoassay. Duplicate tubes of resuspended cAMP were incubated with anti-cAMP antiserum (Sigma Immunochemical) for 4 hr at 4°. ^{125}I-cAMP tracer was then added to each tube. Tubes were quickly vortex-mixed and incubated for an additional 18–24 hr. Antigen-antibody complexes were coprecipitated with bovine serum albumin (fraction V; Sigma) in ethanol at 2000 × *g* for 15 min at 4°. The supernatant was aspirated, and the remaining pellets were measured for undisplaced ^{125}I-cAMP using a GammaTrac 1290/TM analytical γ-radiation counting system. The amount of cAMP in each tube was determined by comparison with a standard curve of known concentrations of unlabeled cAMP. All cAMP measurements were determined in six to nine independent experiments and normalized to total protein in each well. Protein content was measured by lifting one well from each transfection by incubation with 1 mM EDTA for 15 min, followed by Bio-Rad colorimetric assays.

Data analysis. For the calculation of affinity constants, data from two to four individual binding experiments were fitted using the

TABLE 1

Summary of antagonist binding affinities for the wild-type D_1 receptor and mutants I205A, I205Y, L286A, and L286Y transiently expressed in COS-7 cells

K_d values for (*R*)-(+)-[^{3}H]SCH23390 were determined from Scatchard analysis of direct binding experiments; K_i values for *cis*-(*Z*)-flupenthixol were determined by displacement of (*R*)-(+)-[^{3}H]SCH23390. Values represent mean affinities ± standard errors derived from two or three saturation or displacement isotherms.

Receptor	(*R*)-(+)-[^{3}H]SCH23390 K_d (× 10^{-9})	*cis*-(*Z*)-Flupenthixol K_i (× 10^{-9})
	M	
Wild-type D_1	0.71 ± 0.2	0.45 ± 0.09
I205A	0.37 ± 0.1	0.79 ± 0.12
I205Y	5.2 ± 3.0	0.31 ± 0.07
L286A	1.1 ± 0.3	0.70 ± 0.17
L286Y	1.8 ± 0.6	0.90 ± 0.03

TABLE 2

Summary of agonist binding affinities for the wild-type D_1 receptor and mutants I205A, I205Y, L286A, and L286Y transiently expressed in COS-7 cells

K_i values for agonists were determined by displacement of (*R*)-(+)-[^{3}H]SCH23390. Values represent mean affinities ± standard errors derived from two to four displacement isotherms.

Receptor	K_i		
	Dopamine (× 10^{-6})	(±)-SKF82958 (× 10^{-9})	(±)-SKF38393 (× 10^{-9})
		M	
Wild-type D_1	1.9 ± 0.2	23 ± 12	530 ± 80
I205A	2.5 ± 0.2	13 ± 5	900 ± 270
I205Y	94 ± 60	500 ± 300	13,000 ± 42
L286A	1.2 ± 0.1	46 ± 14	680 ± 270
L286Y	6.5 ± 2.1	280 ± 8	4,100 ± 1,200

Ligand program for Macintosh, version 4.0 (National Institutes of Health) (24). Comparisons between mutant L286A and wild-type D_1 receptors were performed using two-tailed paired *t* tests. Basal levels of cAMP in cells transfected with either wild-type or mutant D_1 receptors, from 11 experiments, were compared by one-way analysis of variance at a 95% confidence interval, using the Scheffe *F* test for *post hoc* analysis. Agonist potencies of L286A were compared with those of the wild-type D_1 receptor by two-tailed paired *t* tests from six to eight individual experiments.

Results and Discussion

Wild-type receptor characteristics. The present studies address, indirectly, the potential role of two well conserved prolines within the transmembrane helices of the human D_1 dopamine receptor. The results support the hypothesis that residues immediately adjacent to prolines may be critical for both ligand binding and signal transduction of this receptor. In general, this work has led to three interesting conclusions. First, the nature of the residue on the amino-terminal side of membrane-embedded prolines is important for ligand binding affinity and efficacy. Second, substitution of a leucine with an alanine in TM6 confers constitutive activity to the D_1 receptor, which is characterized by enhanced basal and agonist-stimulated accumulation of cAMP. Lastly, this constitutively activating substitution induces (*R*)-(+)-SCH23390, a classical antagonist at the wild-type D_1 receptor, to behave as a partial agonist.

The wild-type D_1 receptor, when transfected into COS-7 cells, was expressed at consistently high (5–10 pmol/mg) levels. The wild-type receptor exhibited a pharmacological profile (Tables 1 and 2) similar to those in previous reports (4, 25, 26), except that in this study the antagonist *cis*-(*Z*)-flupenthixol was observed to bind with approximately 10-fold higher affinity. Curves generated from agonist displacement of (*R*)-(+)-[^{3}H]SCH23390 were best fit by a single binding site. The monophasic nature of these curves was most likely the result of high levels of receptor expression obtained in COS-7 cells, such that the proportion of high affinity sites was too small to discern. Despite the inability to discriminate a high affinity binding site, a subpopulation of transfected D_1 receptors is presumed to be coupled with G proteins, because agonist stimulation of the G_s/adenylyl cyclase pathway was observed in a dose-dependent manner. The rank order of agonist potency for cAMP stimulation, (±)-SKF82958 > (±)-SKF38393 > dopamine, paralleled the rank order observed

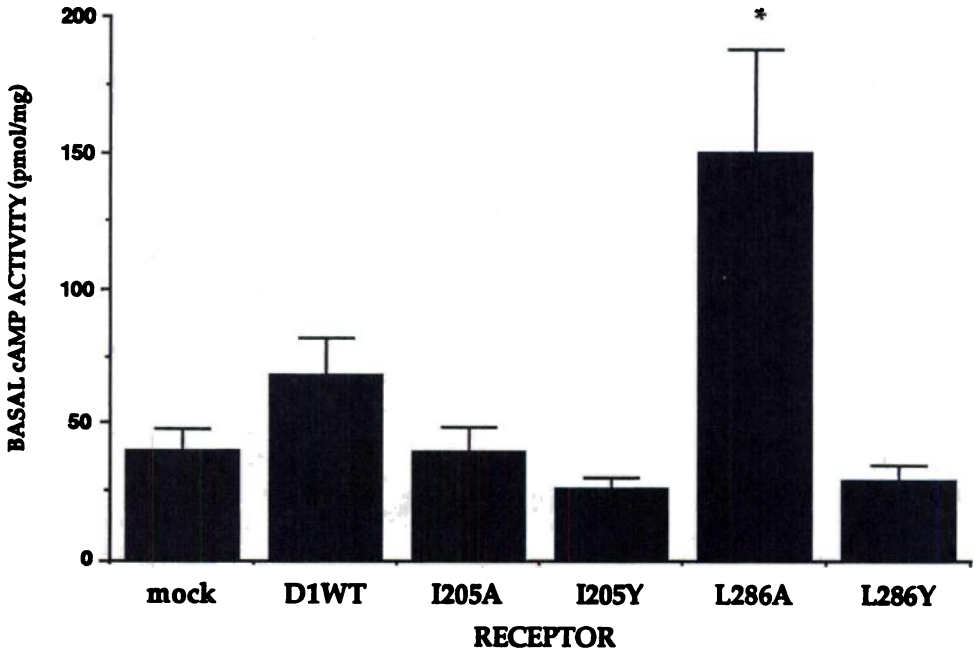

Fig. 2. Basal accumulation of cAMP in COS-7 cells that were mock-transfected or transfected with either the wild-type D_1 receptor (*D1WT*) or the mutants I205A, I205Y, L286A, and L286Y. Values represent means ± standard errors from 12 independent experiments. *, *post hoc* significance of difference from the wild-type and mock-transfected cells at 95% by one-way analysis of variance (overall *p* value < 0.0001).

in binding assays. Fig. 2 represents basal levels of cAMP accumulation in cells transfected with either the wild-type receptor, the individual mutants, or vector alone (mock transfection). Compared with mock-transfected cells, cells expressing the wild-type D_1 receptor demonstrated a modest elevation in basal levels of cAMP; however, this basal elevation failed to reach significance when analyzed by one-way analysis of variance at a 95% confidence limit.

Constitutive activity produced by substitution of Leu286 with alanine. Mutant L286A, which incorporates an alanine residue instead of a leucine at position 286 of TM6, appeared to exhibit binding affinity changes for both antagonists (Table 1) and agonists (Table 2) within 2-fold of wild-type values. The absence of drastic affinity changes, in conjunction with high expression levels (5–10 pmol/mg of protein), suggests that processing events and the tertiary structure of this mutated receptor were not significantly altered. Notably, the affinity for dopamine was improved ($p = 0.03$) by the L286A mutation. Perhaps the influences of two TM6 phenylalanine residues (at positions 288 and 289) that are predicted to stabilize ligand binding for several 7TM receptor subtypes (10, 13, 27, 28) may have been altered by this TM6 mutation.

More interesting than the binding changes characterizing L286A were the changes in signal transduction exhibited by this mutant. Substitution of the TM6 leucine with an alanine enhanced both basal and agonist-induced activities. The basal activity of L286A (Fig. 2), in serum-free medium, was 2-fold greater than that of the wild-type D_1 receptor and 3.5-fold greater than mock-transfected cells (overall analysis of variance, $p < 0.0001$). The significantly increased basal activity of L286A, compared with the wild-type D_1 receptor, was not the result of higher levels of expression, because binding assays and cAMP measurements from the same transfection demonstrated nearly identical levels of expression for L286A and the wild-type receptor (between 5 and 10 pmol/mg). This elevation in basal cAMP level represents an essential characteristic of constitutively active mutant receptors (reviewed in Ref. 29). Consistent with other reports of constitutive activity (30–33), L286A demonstrated increased potency of agonist-induced second messenger stimulation. This mutation produced a leftward shift, or increased potency, in the dose-response curve for dopamine, from an EC_{50} value of 4.3×10^{-6} (log $EC_{50} = -5.4 \pm 0.3$) for the wild-type receptor to 1.6×10^{-6} M (log $EC_{50} = -5.8 \pm 0.3$) ($p = 0.01$) (Fig. 3), and a similar increase in potency for (±)-SKF82958, from 4.8×10^{-7} (log $EC_{50} = -6.3 \pm 0.2$) to 1.2×10^{-7} M (log $EC_{50} = -6.9 \pm 0.1$) ($p = 0.01$) (Fig. 4). It must be noted that, for the agonist (±)-SKF82958, the potency was increased, whereas the binding affinity was not. The partial agonist (±)-SKF38393 also displayed a trend toward increased potency.

The cAMP-modifying characteristics of mutant L286A were further described in the presence of the antagonists (*R*)-(+)-SCH23390 and *cis*-(Z)-flupenthixol. (*R*)-(+)-SCH23390, between 1×10^{-11} and 1×10^{-5} M, did not reverse the enhanced basal activity of L286A; thus, the elevated basal activity of L286A cannot simply be explained by residual catecholamines that were not completely washed out during the cAMP assay. Instead, within this concentration range, (*R*)-(+)-SCH23390 further increased intracellular levels of cAMP in cells transfected with L286A, with an

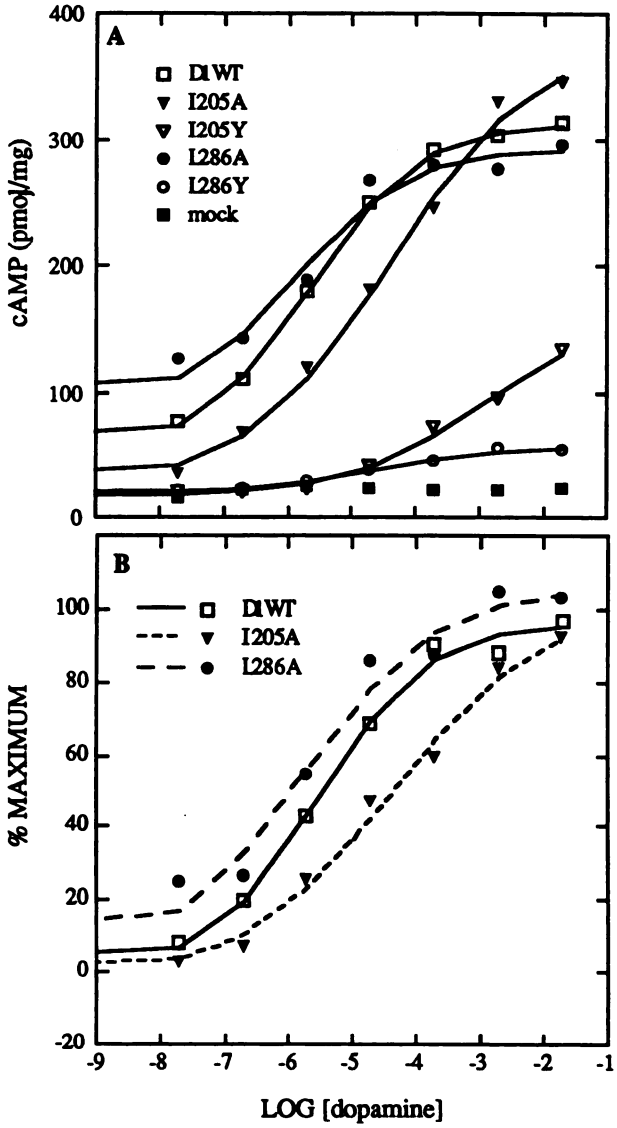

Fig. 3. Dopamine-stimulated cAMP accumulation, expressed as raw values (A) and percentages of maximal stimulation (B), in COS-7 cells that were mock-transfected or transfected with either the wild-type D_1 receptor (*D1WT*) or the mutants I205A, I205Y, L286A, and L286Y. Values are representative of means from eight experiments. Maximal stimulation by the wild-type D_1 receptor, I205A, and L286A was between 200 and 350 pmol/mg. Maximal stimulation by I205Y and L286Y was approximately 100 and 33 pmol/mg, respectively.

efficacy achieving 40% of the maximal stimulation attained with the full D_1 agonist (±)-SKF82958 (Fig. 5). Within this same concentration range, (*R*)-(+)-SCH23390 failed to change intracellular cAMP levels at the wild-type D_1 (data not shown). The antagonist *cis*-(*Z*)-flupenthixol failed to alter cAMP levels at L286A and at the wild-type D_1 receptor between 1×10^{-12} and 1×10^{-6} M (data not shown). From these data, it appears that (*R*)-(+)-SCH23390, which behaves as a "classical" antagonist at the wild-type receptor, demonstrates partial agonist activity at the L286A mutant.

Although mutant L286A is characterized by enhanced basal activity and increased agonist potencies, it also demonstrates some interesting features that have not generally been observed with other constitutively active mutant receptors. First, L286A failed to exhibit a global improvement in

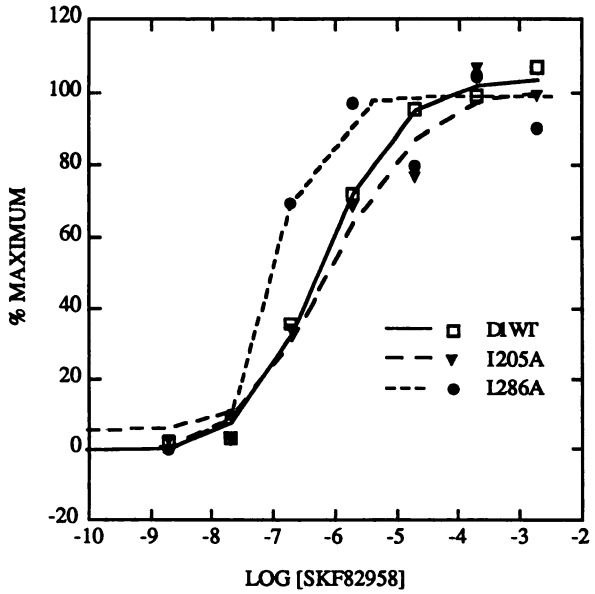

Fig. 4. (±)-SKF82958-stimulated cAMP accumulation in COS-7 cells that were transfected with either the wild-type D_1 receptor (*D1WT*) or the mutants I205A and L286A. Values are representative of means from eight experiments. Maximal stimulation by the wild-type D_1 receptor, I205A, and L286A was between 300 and 500 pmol/mg.

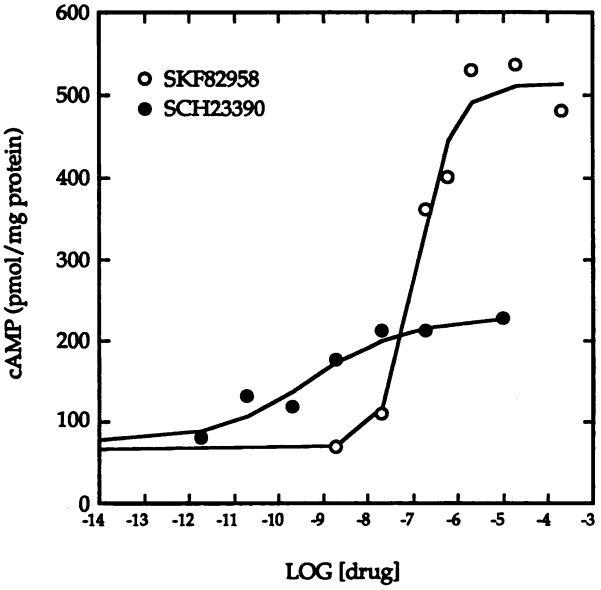

Fig. 5. (*R*)-(+)-SCH23390-mediated stimulation of cAMP accumulation through L286A. COS-7 cells were transfected with the mutant L286A. ●, Stimulation by (*R*)-(+)-SCH23390; ○, stimulation by the full D_1 agonist (±)-SKF82958. Values are representative of means from two experiments, each directly comparing stimulation by (*R*)-(+)-SCH23390 and (±)-SKF82958.

agonist (but not antagonist) affinities in the absence of G proteins. Second, the compound (*R*)-(+)-SCH23390, traditionally considered an antagonist at the D_1 receptor, was surprisingly able to stimulate the mutant L286A but not the wild-type receptor. The observation that L286A did not display indiscriminate increases in agonist potencies suggest that L286A did not faithfully mimic the activated receptor state described in the "extended" ternary complex model of Lefkowitz and colleagues (29, 32). According to this model, the receptor (R) is capable of either spontaneous or ligand-facilitated isomerization to an activated state (R*), which is then capable of coupling with G proteins. It is hypothesized that adoption of this activated conformation is favored by certain mutations that display not only increases in basal activity but also increases in agonist affinity, proportional to the intrinsic activity of the agonist. The observation that (*R*)-(+)-SCH23390 stimulates L286A may be partially explained within this extended ternary complex model; the data suggest that this ligand, which is traditionally considered an antagonist at the D_1 receptor, may actually possess very weak partial agonist activity at the wild-type receptor, which is augmented by the facilitating L286A mutation. However, like (±)-SKF82958, (*R*)-(+)-SCH23390 failed to demonstrate increased affinity at the mutated receptor, and thus L286A does not completely meet the predictions of the extended ternary complex model. The generalizations described by Lefkowitz and colleagues may hold for constitutively activating mutations that structurally resemble the activated state (R*) throughout the entire tertiary conformation of the receptor. Mutant L286A may mimic R* only in G protein-coupling topography and not in ligand-binding topography; the constitutively activating mutation at Leu286 is within the ligand-binding transmembrane core, whereas the mutations described by Lefkowitz and colleagues were located in the carboxyl-terminal region of the third cytoplasmic loop. The different mutational substrates may very well result in different local effects on binding and G protein-coupling geometries. It is conceivable that structural alterations near the binding pocket may have modified the nature of ligand/receptor interactions altogether, such that some agonists bind with decreased affinity and some antagonists are capable of stabilizing the R* conformation at the new binding interface.

It is clear from the characteristics of the dopamine receptor mutant L286A and mutants in various other receptor systems that constitutive basal activity may be independent of other pharmacological traits. A pathological mutation of the 7TM human luteinizing hormone receptor was found to confer constitutive activation as a result of a single TM6 aspartate to glycine change, without increased agonist affinity and without changes in the EC_{50} value of human chorionic gonadotropin (34). Additionally, a constitutively active thyrotropin receptor with an alanine to isoleucine mutation in the carboxyl region of the third cytosolic loop did not have increased agonist potency (35), unlike the homologous α_{1B}-adrenergic receptor mutant (30, 31). Furthermore, two naturally occurring constitutively active mutants of the melanocyte-stimulating hormone receptor were reported to display a range of responses, from no additional stimulation by agonists to a maximal response that exceeded that of the wild-type receptor (36). The potency of α-melanocyte-stimulating hormone for the latter mutant was slightly decreased. Because different constitutively activating mutations yield different pharmacological properties, the mechanisms that produce these changes are also most likely different. The L286A dopamine receptor mutant reported here may have been affected both by local conformational changes and by changes in putative dynamic events at the X-Pro peptide bond of TM6. Mutation at this position may have facilitated "hinging" or "wobbling" to favor the activated R* receptor conformation. Interestingly, a Leu387 to alanine substitution in the human D_2 receptor long isoform, homologous to the

L286A change in the D_1 receptor, produced a mutant receptor that was unable to demonstrate agonist-mediated inhibition of adenylyl cyclase, despite the ability to bind agonists with nearly wild-type affinities (28). Together, these data suggest that G_s- and G_i-coupled receptors may use this TM6 leucine-proline peptide bond differently.

Substitution of TM5 Ile205 with alanine. Although the nature of this mutation is similar to the substitution of the TM6 Leu286 with an alanine, the I205A mutant displayed markedly different characteristics (Tables 1 and 2). Because the I205A mutation was located in TM5, the helix that contains binding-critical serines, the replacement of the bulky β-branched Ile205 side chain with the compact alanine side chain may have relieved steric clashes with the adjacent proline, resulting in slight reorientation of this binding-critical helix. The functional characteristics of this mutant were minimally different from those of the wild-type receptor. Although levels of expression of I205A were indistinguishable from those of the wild-type D_1 and mutant L286A, I205A demonstrated basal activity that was less than that of the TM6 mutant L286A and the wild-type receptor (Fig. 2). Consistent with this aspect of diminished functional activity, the agonists dopamine, (±)-SKF82958 (Fig. 4), and (±)-SKF38393 displayed trends toward decreased potencies. The efficacies of the full agonists dopamine and (±)-SKF82958 and the partial agonist (±)-SKF38393 at I205A, however, were similar to those at the wild-type D_1 receptor. Although the rightward shift in potency for dopamine may suggest altered dynamics for this mutant, it is difficult to determine whether this potency shift is simply the result of altered binding affinity.

Disruption of both binding and signal transduction by substitution of TM5 Ile205 or TM6 Leu286 with tyrosine. Substitution of either of the X-Pro residues, Ile205 in TM5 or Leu286 in TM6, with a tyrosine resulted in substantially diminished levels of protein expression (approximately 0.5 pmol/mg). In addition, these substitutions with tyrosines adversely affected both ligand binding (Tables 1 and 2) and D_1-mediated stimulation of intracellular cAMP (Figs. 2–4). Fig. 3 also depicts dramatically blunted efficacies for maximal cAMP stimulation. The most conservative interpretation would explain decreased efficacy as a function of decreased expression levels of the transfected receptor. However, less efficient signal transduction between receptors and G proteins cannot be ruled out. In general, the nearly universal disruption in ligand binding and decreased protein expression levels suggests significant conformational changes in mutants I205Y and L286Y, probably to a degree that interferes with protein folding and processing. Because of the restricted space available to the residue on the amino-terminal side of proline, the bulk and aromaticity of tyrosine may have significantly altered the direction of the helical backbone and, as a result, the shape of the binding pocket.

Although the hypothesis that conformational changes in 7TM receptors involve isomerization at X-Pro peptide bonds is extremely intriguing, there is little direct evidence for this mechanism. Until X-ray structures prove the existence of multiple 7TM receptor isomers, this hypothesis must be tested by less direct measures. Several dynamic spectroscopic studies of the 7TM protein bacteriorhodopsin provide evidence for conformational shifts at one or more proline residues during the photocycle of bacteriorhodopsin (37, 38). However, a few mutagenesis studies suggest nonessential roles for some bacteriorhodopsin intramembranous prolines, because individual mutations did not severely alter function (39). Single proline changes in these mutagenesis experiments, though, may not be sufficient to deleteriously affect the function of this protein. It also must be noted that, although bacteriorhodopsin contains seven transmembrane helices, it is not coupled with G proteins. In the m3 muscarinic acetylcholine receptor, single proline substitutions within helices 5, 6, and 7, homologous to the conserved prolines in helices 5, 6, and 7 of the D_1 dopamine receptor, support the structural importance of these residues (40). Their substitutions with alanines resulted in marked (35–100-fold) decreases in receptor protein levels. The substitution in helix 7 produced substantially impaired functional activity; however, substitutions in helices 5 and 6 retained wild-type agonist binding characteristics and full functional capacities. Site-directed mutagenesis of catecholamine receptor prolines has been only moderately successful. Substitution of a highly conserved proline residue within the second intracellular loop of the β_2-adrenergic receptor resulted in wild-type agonist binding characteristics but decreased potency and efficacy of cAMP stimulation by isoproterenol (8). These data are consistent with an hypothesis of X-Pro dynamics. Because this mutation was created in an intracellular domain, the disrupted mechanism may represent a final step in the transduction of binding-induced changes in transmembrane helix geometry to changes in G protein-coupling domains. The only attempt at mutation of an intramembranous catecholamine receptor proline, as previously mentioned, resulted in an incompletely processed β-adrenergic receptor (20). From these data, it appears that the potential role of dynamics at X-Pro peptide bonds in bacteriorhodopsin, muscarinic receptors, and catecholamine receptors may differ. Even within the dopamine receptor family, the D_1 and D_2 receptors may utilize homologous X-Pro peptide bonds differently (28). Such a conclusion would not be particularly surprising, because these 7TM receptor types are coupled with different G proteins (or not coupled with G proteins at all) and different second messenger systems.

In summary, the results presented here suggest the importance of structural influences of the X-Pro peptide bond upon the D_1 dopamine receptor, because mutation of either TM5 Ile205 or TM6 Leu286 to alanine resulted in subtle antagonist and agonist binding changes, whereas substitutions with tyrosine produced more drastic effects. The results do not provide direct evidence for full or partial isomerizations involving these conserved proline residues but do provide data consistent with this hypothesis. The disrupted signal transduction capacities of the TM5 and TM6 tyrosine substitutions and the substitution of TM5 Ile205 with an alanine are difficult to interpret within this hypothesis, because reduced functional characteristics were accompanied by reduced binding affinities. However, substitution of the TM6 Leu286 with an alanine produced a significant degree of agonist-independent cAMP stimulation and increased agonist potencies that were not necessarily accompanied by increased affinities. Also, this TM6 leucine to alanine substitution conferred partial agonist activity upon (*R*)-(+)-SCH23390, traditionally considered a D_1 antagonist. This is the first report of constitutive activity as a result of this point mutation. Characterization of other X-Pro mutations, possibly in

conjunction with spectroscopic studies, may demonstrate further functional significance of proline-containing 7TM receptor segments.

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