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D1 dopamine receptor activity is not altered by a mutation in the first intracellular loop

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Abstract

The first intracellular loop of the G protein-coupled receptors (GPCRs) is probably the domain that has been studied least. According to the limited data available, mutations of this region can increase, decrease or not affect receptor-G protein coupling, depending on the receptor. Melanocyte-stimulating hormone (MSH) receptors with a Ser69Leu mutation of the first intracellular loop phenotypically confer tobacco color to the coat of mice, and have constitutive activity and enhanced agonist stimulation of adenylyl cyclase. Since the human D1 dopamine receptor (D1DR) has a serine at the equivalent position, we were interested to see if this serine is involved in receptor-G protein coupling in a similar fashion. Our site-directed mutagenesis study showed that the replacement of this serine by leucine (Ser56Leu) in D1DR did not affect the ability of the receptors to bind ligand or couple to G protein. © 1998 Elsevier Science B.V.

Keywords: Receptor; Dopamine; Mutant; Adenylyl cyclase; Intracellular loop

1. Introduction

A variety of naturally occurring mutations of GPCRs have been found in several diseases. Some mutations cause reduction or loss of function, e.g., hormone resistance to vasopressin in nephrogenic diabetes insipidus [1]. Others cause disease states by gaining function, e.g., retinitis pigmentosa [2,3] and Jansen-type metaphyseal chondrodysplasia [4], due to constitutively active mutant receptors. Different functional roles have been determined by studying the structural variations, occurring in different regions of the receptor proteins. However, studies on the functional role of the first intracellular loop of GPCRs are lacking. Table 1 lists the mutations that have been found or made in the first intracellular loop of GPCRs (see Section 4 for details). An interesting mutation that occurs naturally in the first intracellular loop of the melanocyte-stimulating hormone (MSH) receptor, changes the pigmentation phenotype in mice [5]. The Ser69Leu mutation in mice with tobacco coat color was shown not only to have higher basal level of activity but also a greater agonist-mediated ability to stimulate adenylyl cyclase compared to the wild-type

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Table 1
Summary of the mutations in the first intracellular loop of GPCRs

Receptors	Mutations	Effects on mutant(s)	Ref.
MSHR	WT: KNRNLH^{69}S PMYY	↑Basal AC activity	[5]
	MUT: KNRNLH**L**PMYY	↑AC V_{max}	
β2AR	WT: ER^{64}L QTVT	↓Binding to CYP	[9]
	MUT: ERL(**-6-**)QTVT, ERL(**-8-**)QTVT	↓AC V_{max}	
β2AR	WT: KFER^{64}L QTVTN	↓Receptor expression	[10]
	MUT: KFER**G**QTVTN, KFE**G**L**L**TVTN, KFE**D**L**E**TVTN	↓AC V_{max}	
TSHR	WT: T^{441} SHYKLTVPR	Binding unchanged	[11]
	MUT: **AGQAQ**L**A**VP**Q**	↓AC V_{max}	
TSHR	MUT: (1) **CNRHLR**TVPR, (2) TSHYKLT**PTN**, (3) **KFERLQ**TVPR, (4) TSHYKLTV**TN**, (5) **AGQAQ**L**A**VP**Q**, (6) TSHYKL**Q**VPR	(1), (2), (5) and (6): loss of IP3 production; (3) and (4): ↓IP3 production. All: ↓AC basal activity and AC V_{max} except (5) (no change in V_{max})	[12]
Rhodopsin	WT: VQHKK^{68}L RT^{71}P L	↓Transducin activation	[13]
	MUT: VQHKKL, VQHKKLRL, VQHKKLR**AA**L		
TxA2-R	WT: QGGSHT^{60}RSSFLT	↓IP3 production	[14]
	MUT: QGGSHT**L**SSFLT		
hCRFR II	29 a.a. insert in the first intracellular loop when compared to its splicing variant hCRFR I	↓AC V_{max} and ↓IP3 production (compared To R I)	[15]
CCKBR	The first intracellular loop of CCKBR was replaced by the counterpart of CCKAR	Gained the ability to stimulate adenylyl cyclase	[16]

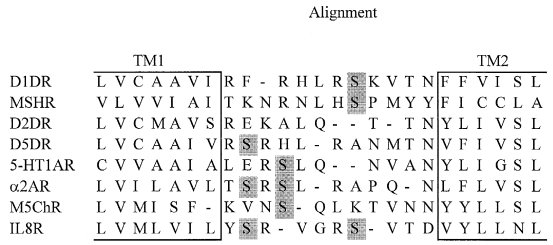

Fig. 1. An alignment of selected GPCRs. The serines in the first intracellular loop are shaded. The transmembrane domains 1 (TM1) and 2 (TM2) are indicated in brackets. D1DR has a serine at a similar position as MSHR. D1DR = D1 dopamine receptor, MSHR = melanocyte-stimulating hormone receptor, D2DR = D2 dopamine receptor, D5DR = D5 dopamine receptor, 5-HT1AR = 5-hydroxytryptamine 1A receptor, α2AR = α2 adrenergic receptor, M5AChR = M5 acetylcholine receptor, and IL8R = interleukin 8 receptor.

receptor. The D1DR has a similar serine residue at the equivalent position in the first intracellular loop (Fig. 1). We were interested to see if the substitution of leucine for serine at position 56 of D1DR by site-directed mutagenesis would also produce constitutive activity and/or increased maximal stimulation of adenylyl cyclase.

2. Materials and methods

2.1. Materials

[α-^{32}P]ATP (800 Ci/mmol), [α-^{33}P]ATP (2000 Ci/mmol), [^{3}H]SCH23390 and [^{3}H]cAMP (30

Ci/mmol) were from Du Pont/NEN. Geneticin (G418), penicillin and streptomycin were from GIBCO/BRL. Dopamine, GTP, ATP, cAMP and forskolin were from Sigma. Butaclamol was purchased from RBI (Natick, MA). Dowex Resin (100–200 mesh) was from Bio-Rad.

2.2. Cell culture

Chinese Hamster Ovarian (CHO) cells were maintained in alpha-MEM containing 10% fetal bovine serum, 100 U/ml penicillin and 100 μg/ml streptomycin. Permanent cell lines expressing the wild type and the mutant receptors were maintained as monolayer culture in alpha-MEM containing 10% fetal bovine serum, 100 U/ml penicillin, 100 μg/ml streptomycin and 1 mg/ml G418.

2.3. Site-directed mutagenesis of D1DR gene

Mutagenesis was carried out on the human D1 dopamine receptor gene using the PCR as described [6]. The PCR products were subcloned into expression vector pRC/CMV (Invitrogen) and the DNA sequences encoding the wild type and the mutant receptors were verified by double-stranded DNA sequencing using Sanger's dideoxynucleotide termination method with T7 DNA Sequencing Kit (Pharmacia).

2.4. Permanent transfection of CHO cells with D1DR genes

Monolayers of cells on Petri dishes at about 50% confluence were transfected using Ca^{2+} coprecipitation according to the instructions of the manufacturer (GIBCO/BRL). Stable cell lines expressing the receptors were obtained using selection media containing G418 and selected as described [6].

2.5. Ligand binding assays

Radioligand binding was performed essentially as described [6]. The final concentrations in binding buffer were 75 mM Tris–HCl, 5 mM EDTA, and 5 mM $MgCl_2$. Cells were collected in the binding buffer by scraping with a rubber policeman. Cell membrane suspensions were obtained by Polytron homogenization of the collected cells (6500 rpm for 20 s). Saturation experiments were carried out in triplicates with [^{3}H]SCH23390 in increasing concentrations and nonspecific binding was determined by binding that was not displaced by 1 μM (+)-butaclamol.

2.6. Adenylyl cyclase assay

Cell membranes were prepared by Polytron homogenization (6500 rpm for 20 s) in binding buffer, ultracentrifugation of the supernatant at 12 000 rpm for 15 min to collect membranes, and resuspension of the membrane pellet in buffer containing 75 mM Tris–HCl, 8 mM $MgCl_2$ and 5 mM EDTA. Adenylyl cyclase assays were conducted in duplicate as described [7] at 37°C for 20 min, in a total volume of 50 μl of the assay mix, containing 20 μg membrane protein, 12 μM ATP, 100 μM cAMP, 53 μM GTP, 2.7 mM phosphoenol-pyruvate, 0.2 units of pyruvate kinase, 1 unit of myokinase and 5 mM ascorbic acid, with 0.13 μCi of [α-^{32}P]ATP or 3–5 μl of [α-^{33}P]ATP, in the presence of indicated concentrations of dopamine. Reactions were stopped by the addition of 1 ml of an ice-cold solution containing 0.4 mM ATP, 0.3 mM cAMP and [^{3}H]cAMP (approximately 75 000 dpm). The resulting solutions were decanted directly onto the Dowex columns. The eluate was then subjected to purification on alumina columns. Protein concentrations were measured using Bio-Rad reagents.

2.7. Data analysis

All data were analyzed by nonlinear least squares regression using the InStat and InPlot GraphPad computer programs.

3. Results

CHO cell lines stably expressing similar levels of wild type and mutant D1DR were developed and selected (Table 2) to determine whether the Ser56Leu substitution in the first intracellular loop of D1DR would elicit the same effect as the MSH receptors,

Table 2
SCH23390 binding parameters for wild type and mutant D1DR in CHO cells

Receptor type	B_{max} (fmol/mg)	K_d (pM)
Wild type	159.8 ± 21.7 (11)	489.9 ± 75.8 (11)
Mutant	214.8 ± 21.4 (3)	212.6 ± 50.8 (3)

The values shown in the table are the means ± SEM of saturation binding. The numbers in parentheses indicate the number of independent experiments that were carried out, each in triplicate. The data of the wild-type receptor was the summation of eleven experiments carried out in four cell lines expressing similar levels of wild-type receptors.

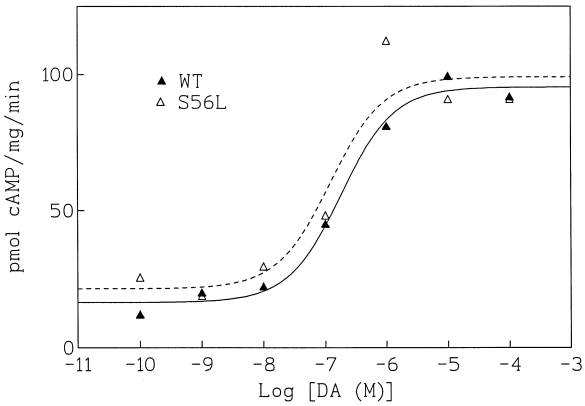

Fig. 2. Adenylyl cyclase activation by dopamine in the wild type and mutant receptors. The curves shown in this figure are representatives of 15 cyclase assays performed in four cell lines for wild-type receptor, and of 5 assays in a cell line expressing mutant receptors at a similar level. The basal level, V_{max} and EC_{50} values are listed in Table 3.

namely an increase in both basal activity and maximal adenylyl cyclase activity (V_{max}) upon agonist stimulation. As shown in Table 2, the cell lines expressed receptors at about 200 fmol/mg protein, which is comparable to the physiological receptor concentration, and demonstrated similar binding affinity for the D1 selective antagonist [^{3}H]SCH23390, with similar dissociation constant (K_d) values. Therefore, the introduction of the Ser56Leu mutation did not alter the antagonist binding capability of the D1DR. The observation that binding of the receptor to ligand was not affected by the mutation in the first intracellular loop of D1DR is consistent with previous studies that ligand binding domains reside on the transmembrane domains for small molecule ligands and on the extracellular regions of the receptors for large molecules such as peptides [8].

We studied the effect of the Ser56Leu mutation on the effect of basal activity of adenylyl cyclase in these cells. As shown in Table 3 and Fig. 2, the basal activity of the cell line expressing the mutant receptors was not increased when compared with the wild-type receptor cell line. Therefore, no constitutive activity could be demonstrated in the mutant receptors as shown in the analogous MSH receptors.

Adenylyl cyclase activity upon maximal stimulation by dopamine was identical in mutant compared to the wild type D1DR, unlike the observations with the MSH mutant receptors. The Vmax values of the wild type and the mutant D1 receptors were almost identical (approximately 100 pmol cAMP mg^{-1} min^{-1}) (Table 3 and Fig. 2). EC_{50} values were also similar for the wild type and mutant receptors (Table 3).

In summary, we did not observe any constitutive activity nor any enhanced stimulation of adenylyl cyclase of the mutant D1 receptor as in the case of MSH receptor. Receptor binding characteristics were expectedly unaltered as well by the engineered mutation.

Table 3
Basal and dopamine-stimulated adenylyl cyclase activities of wild type and mutant D1DR

Receptors	Basal (pmol cAMP mg^{-1} min^{-1})	V_{max} (pmol cAMP mg^{-1} min^{-1})	EC_{50} (pM)
Wild type	32.3 ± 3.6 (15)	88.0 ± 4.9 (15)	434.5 ± 80.7 (15)
Mutant	24.5 ± 3.7 (5)	104.3 ± 15.8 (5)	284.2 ± 56.0 (5)

Basal activities and maximal activation of adenylyl cyclase in the cell lines expressing similar receptor levels were determined as described in the Section 2. The values listed in the table are mean ± SEM of the number of experiments indicated in parentheses carried out in duplicates. The data for the wild-type receptor were the summation of fifteen adenylyl cyclase assays carried out in four cell lines with approximately the same receptor expression level.

4. Discussion

Characterizations of the functional role of the first intracellular loop of GPCRs are generally lacking. However, in a limited number of studies, outlined in Table 1, this domain has been shown to play a critical role in receptor expression and receptor-G protein coupling in at least some receptors. One of the mutant receptors that is of particular interest has been the MSH receptor. A naturally occurring mutant receptor (Ser69Leu in the first intracellular loop) found in tobacco-coat mice showed constitutive activity and greater activation of its effector, adenylyl cyclase, than the wild type when expressed in human 293 cells [5].

In β2-adrenergic receptors, Kobilka et al. [9] showed that insertion of 4–8 amino acid residues into the middle of the first intra-cellular loop led to a marked reduction in binding of the antagonist cyanopindolol and in the maximal agonist activation of adenylyl cyclase, although activation occurred with the same EC_{50} as in the wild-type receptor. It is probable that normal folding and conformation of the receptor protein was disturbed by these insertions. In another study of the first intracellular loop of β2-adrenergic receptors, O'Dowd et al. [10] found that the single substitution of Leu64 by Gly resulted in a receptor with a 10-fold increased binding affinity for agonist isoproterenol, but normal binding for cyanopindolol. This mutant receptor had an appreciably lower level of expression, accounting for its reduced potency and efficacy of cyclase stimulation. In the same study, substitutions of the amino acids flanking position 64 had similar but more deleterious effects on receptor expression, thus preventing meaningful adenylyl cyclase activation parameters to be obtained.

In the thyrotropin (TSH) receptor, the results are controversial. Chazenbalk et al. [11] reported that a nonconserved substitution mutant involving the first intracellular loop caused the total loss of the TSH-induced cAMP response. However, Kosugi and Mori [12] reported that the first cytoplasmic loop is important for phosphoinositide signaling but not for agonist-induced adenylyl cyclase activation when replaced with the corresponding homologous amino acids from α1- and β2-ARs.

In the characterization of mutant rhodopsins responsible for autosomal dominant retinitis pigmentosa, Min et al. [13] discovered that deletion and replacement of two amino acids in the first intracellular loop resulted in a complete loss and a reduction of light-dependent transducin activation, respectively.

A mutation in the first cytoplasmic loop of the thromboxane A2 (TXA2) receptor was demonstrated by Hirata et al. [14] in affected members of two unrelated families with a mild bleeding disorder characterized by defective platelet response to TXA2. In this receptor the replacement of Arg60 by Leu, a highly conserved basic residue among GPCRs, caused impaired ability to induce agonist-mediated PI hydrolysis.

Nabhan et al. [15] reported that corticotropin-releasing factor (CRF) receptor has two alternatively spliced forms. The two splice variants are identical except for a 29-amino-acid insert present in the first intracellular loop of the type II receptor. When expressed in COS cells, type II CRF receptor had low binding affinity, a weak cAMP response and low inositol phosphate generation, suggesting that this receptor is weakly coupled to G protein, as a result of the change in the first intracellular loop.

Wu et al. [16] constructed a series of chimeric receptors containing different parts of cholecystokinin-A and -B receptors (CCK-AR and CCK-BR, respectively). One of the chimera, CCK-BA_{ICL-1}, which consisted mainly of CCK-BR sequence with only the first intracellular loop being replaced by the corresponding CCK-AR amino acid residues, gained the function to couple to the Gs as with CCK-AR, although to a less extent.

In summary, mutations in the first intracellular loop, whether occurring naturally or engineered by in vitro mutagenesis, may have variable effects on the function of different receptors, causing increased (e.g., MSH and CCKBR), decreased (e.g., β2AR, some TSHR mutants, rhodopsin and hCRFRII) or no change (e.g., some TSHR mutants) in receptor-G protein coupling. Our study showed that Ser56 in the first intracellular loop of the D1DR does not influence receptor–effector coupling. The differences that these various receptors manifest may relate to the relative position of residues within the intracellular loop, determined by the overall three dimensional architecture of the receptor, and may not therefore be directly

comparable with each other. These findings emphasize the importance of studying structure–function determinants of GPCRs individually rather than to generalize from one receptor to another based on the common topography of GPCRs. Our efforts to elucidate the final determinants of the relationship between the structure and function of G protein-coupled receptors are still at a preliminary stage, until the structures of these receptors are definitively revealed by crystallography.

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