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Elimination of palmitoylation sites in the human dopamine D_1 receptor does not affect receptor-G protein interaction

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Abstract

We have eliminated putative palmitoylation sites in the carboxyl tail of the human dopamine D_1 receptor by replacing the two cysteine residues with alanines either separately or together. The wild type and the three mutated dopamine D_1 receptors were stably expressed in baby hamster kidney cells and characterized to detect any resulting alterations in receptor-G protein interactions. The three mutant dopamine D_1 receptors retained the same proportion of high affinity state for agonists as wild type receptors and also no difference was observed in the stimulation of adenylyl cyclase activity. Our results are in contrast to those observed with the β_2-adrenoceptor and consistent with similar studies of luteinizing hormone/human chorionic gonadotropin (LH/hCG) receptors, α_2-adrenoceptors, muscarinic M_2 receptors and thyrotropin releasing hormone (TRH) receptors. Thus, we suggest that palmitoylation appears to play a unique role in the β_2-adrenoceptors, and appears not to be essential in G protein coupling for the dopamine D_1 receptors.

Keywords: Dopamine; Dopamine D_1 receptor; Palmitoylation; Adenylyl cyclase; Desensitization

1. Introduction

Acylation by long-chain saturated fatty acids such as myristic and palmitic acids, is a common feature of membrane-associated proteins (Towler et al., 1988). Palmitoylation, a post-translational modification that initially takes place on nascent proteins in a post-endoplasmic reticulum compartment (Bonatti et al., 1989), is involved in important roles inside the cell, including anchorage of protein to the cell membrane, protein interaction with protein and determination of intracellular transportation/localization (Bizzozero et al., 1994). The alignment of the amino-acid sequences (Fig. 1) shows that the majority of members of the G-protein-coupled receptor superfamily have one or more cysteines located in the carboxyl tails near the seventh transmembrane domain. The receptors that have been shown to be palmitoylated via thioester bonds at these residues, are β_2-adrenoceptors (O'Dowd et al., 1989), rhodopsin (Ovchinnikov et al., 1988; Karnik et al., 1993), α_{2A}-adrenoceptors (Kennedy and Limbird, 1993), and the luteinizing hormone/human chorionic gonadotropin (LH/hCG) receptors (Kawate and Menon, 1994).

Following our cloning of the human dopamine D_1 and the serotonin 5-$HT_{1D\beta}$ receptors, we have demonstrated that both receptors and also the dopamine D_{2L} receptor are palmitoylated (Ng et al., 1993a,b, 1994). Furthermore we demonstrated that dopamine D_1 receptors are palmitoylated in the absence of agonist and undergo an increase in palmitoylation that is agonist-induced (Ng et al., 1993a). It was demonstrated previously that the palmitoylation at Cys^{341} in the carboxyl tail of β_2-adrenoceptors is important for receptor-G protein coupling, and that mutant β_2-adrenoceptors with Cys^{341} replaced by glycine greatly reduced their ability to form the high affinity state for agonists and their ability to stimulate adenylyl cyclase activity (O'Dowd et al., 1989; Moffett et al., 1993). Subsequent to the initial experiments with β_2-adrenoceptors, substitutions of the equivalent cysteines on other receptors, including muscarinic M_2 (Van Koppen and Glaster, 1991), rhodopsin (Karnik et al., 1993), α_{2A}-adrenergic (Kennedy and Limbird, 1993), thyrotropin-releasing hormone (TRH) (Nussenzveig et al., 1993) and LH/hCG receptors (Kawate and Menon, 1994), showed that elimination of the palmi-

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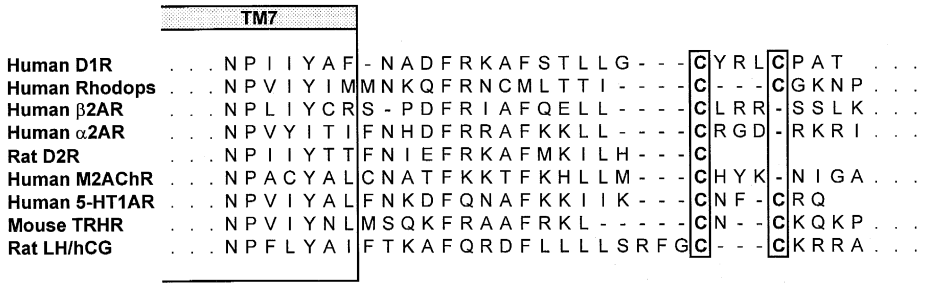

Fig. 1. Alignment of the amino-terminal regions of carboxyl tails of selected G protein-coupled receptors. The bracket indicates the seventh transmembrane domains (TM7) and the cysteine residues for palmitoylation are highlighted.

toylation site(s) did not perturb receptor-G protein coupling. However, palmitoylation has been implicated as being involved in sequestration and down-regulation of the α_{2A}-adrenergic (Eason et al., 1994), muscarinic M_2 (Van Koppen and Glaster, 1991), TRHR (Nussenzveig et al., 1993) and LH/hCG receptors (Kawate and Menon, 1994). These studies showed that prevention of palmitoylation had variable effects on G protein-coupled receptors.

The human dopamine D_1 receptor has an overall structure similar to the β_2-adrenoceptor and contains two putative palmitoylation sites (Cys^{347} and Cys^{351}) in the carboxyl tail (Fig. 2) (Sunahara et al., 1990). To clarify the role of palmitoylation in dopamine D_1 receptors, we eliminated these putative palmitoylation sites, both individually and together, using site-directed mutagenesis. We have found that substitution of either cysteine or both together did not affect ligand binding, receptor-G protein coupling, receptor desensitization, or agonist-induced stimulation of adenylyl cyclase activity.

2. Materials and methods

2.1. Materials

[α-^{32}P]ATP (800 Ci/mmol), [^{3}H]7-chloro-8-hydroxy-1-phenyl-2,3,4,5-tetrahydro-1*H*-3-benzazepine ([^{3}H]SCH23390) and [^{3}H]cAMP (30 Ci/mmol) were purchased from Du Pont/NEN. Lipofectin, geneticin (G418), penicillin and streptomycin were purchased from

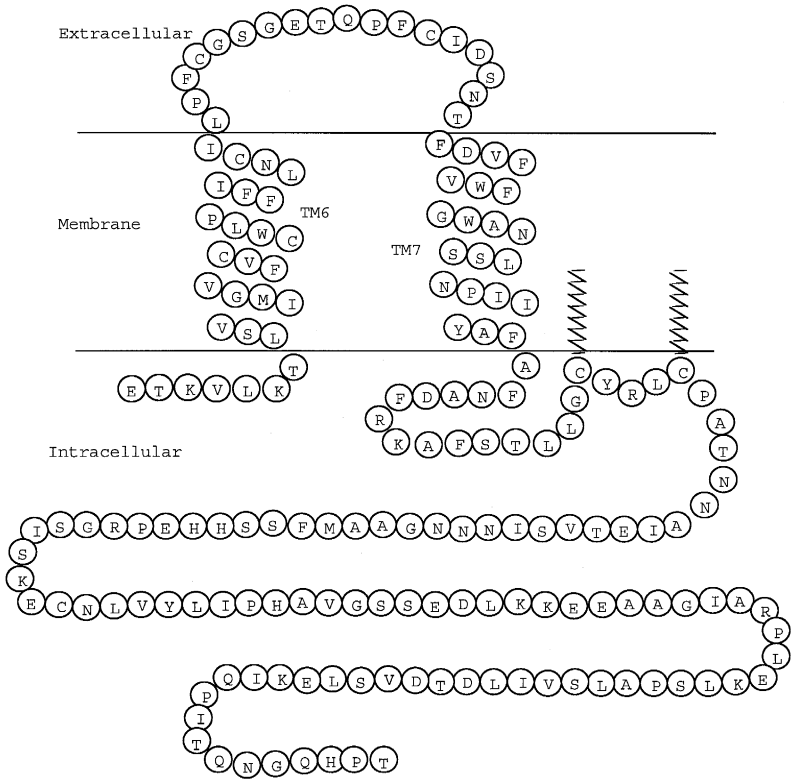

Fig. 2. A topographic model of the TM6 to carboxyl tail of the human D_1 dopamine receptor. The cysteine residues which are postulated to be post-translationally modified by palmitic acids are indicated. These cysteine residues were substituted by alanine either separately (Cys^{347}-Ala and Cys^{351}-Ala) or together (Cys^{347}-Ala;Cys^{351}-Ala) by site-directed mutagenesis.

Gibco/BRL. Dopamine, serotonin, leupeptin, benzamidine, soybean trypsin inhibitor, 5′-guanylylimidodiphosphate (Gpp(NH)p), GTP, ATP, cAMP and forskolin were purchased from Sigma. 1-Phenyl-2,3,4,5-tetrahydro-(1*H*)-3-benzazepine-7,8-diol (SKF38393) and (+)-butaclamol were purchased from Research Biochemical International (Natick, MA, USA) and the Dowex Resin (100–200 mesh) was purchased from Bio-Rad (Hercules, CA, USA).

2.2. Cell culture

Baby hamster kidney-3 (BHK3) cells were maintained in Dulbecco's modified essential medium (DMEM) (Gibco/BRL) containing 10% fetal bovine serum, 100 U/ml penicillin and 100 μg/ml streptomycin. Permanent cell lines expressing the wild type and the three mutant receptors were maintained as monolayer culture in DMEM containing 10% fetal bovine serum, 100 U/ml penicillin, 100 μg/ml streptomycin and 1 mg/ml G418.

2.3. Site-directed mutagenesis of dopamine D_1 receptor gene

Mutagenesis was carried out on the human dopamine D_1 receptor gene using the polymerase chain reaction (PCR) as described (Higuchi et al., 1988). The PCR products were subcloned into expression vector pRC/CMV (Invitrogen), which was linearized by XbaI, blunt-ended with Klenow fragment and dephosphorylated by calf alkaline phosphatase (Pharmacia). The orientation of inserts was confirmed by restriction mapping, 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 cells with wild type and mutant dopamine D_1 receptor genes

Monolayers of BHK3 cells on Petri dishes at about 50% confluence were transfected using Lipofectin according to the instructions of the manufacturer. Cells were trypsinized at 48 h and diluted 1:30 in DMEM supplemented with 10% fetal bovine serum, 100 U/ml penicillin, 100 μg/ml streptomycin and 1 mg/ml G418 and incubated at 37°C in 5% CO_2. After 2 weeks, G418 resistant clones were isolated in small Petri dishes, expanded in 75 cm^2 flasks, and screened using dopamine D_1 receptor antagonist, SCH23390, with saturation isotherm curves to determine the expression level of the receptors. One cell line was selected for each type of receptor with a similar expression level (approximately 5 pmol/mg protein for the wild type D_1, D_1(Cys^{347}-Ala) and D_1(Cys^{347}-Ala;Cys^{351}-Ala)) as assessed by B_{max} of [^{3}H]SCH23390 saturation curves (see below). The D_1(Cys^{351}-Ala) mutant was expressed at a higher level (15 pmol/mg protein).

2.5. Ligand binding assays

Radioligand binding was performed essentially as described (Sunahara et al., 1990). The final concentrations of binding buffer were 50 mM Tris-HCl, 5 mM EDTA, 1.5 mM $CaCl_2$, 5 mM $MgCl_2$ (and 120 mM NaCl for antagonists) with protease inhibitors (10 μg/ml benzamidine, 5 μg/ml leupeptin, and 5 μg/ml soybean trypsin inhibitor). 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 duplicate with [^{3}H]SCH23390 in increasing concentrations (20–5000 pM) and non-specific binding was determined by binding that was not displaced by 1 μM (+)-butaclamol. The concentration of [^{3}H]SCH23390 in competition assays was approximately equal to 600 pM. In competition curves using dopamine, a final concentration of 5 mM ascorbic acid was added to prevent dopamine oxidation.

2.6. Desensitization study

Prior to harvesting, cells were pretreated with 10 μM dopamine for 15 min (with 5 mM ascorbic acid), and competition binding assays of dopamine (10^{-10}–10^{-4} M) against [^{3}H]SCH23390 (600 pM) were performed as described above.

2.7. Adenylyl cyclase assay

Cell membranes were prepared by Polytron homogenization (6500 rpm for 20 s) in 5 mM Tris-HCl (pH 7.4) and 2 mM EDTA (pH 8.0), centrifugation at $800 \times g$ for 10 min to remove unbroken cells, ultracentrifugation of the supernatant at 15 000 rpm for 30 min to collect membranes, and resuspension of the membrane pellet in the reaction buffer containing 75 mM Tris-HCl, 12.5 mM $MgCl_2$ and 2 mM EDTA. Adenylyl cyclase assays were conducted in duplicate as described (Johnson and Salomon, 1991) at 28°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, in the presence of indicated concentrations of dopamine or 100 μM forskolin. 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 25 000 cpm). The resulting solutions were decanted directly onto the Dowex columns. The eluate was then subjected to either purification on alumina columns or $ZnSO_4$/$Ba(OH)_2$ precipitation. Both methods produced comparable, low blank values (Johnson and Salomon, 1991). Protein concentrations were measured using Bio-Rad reagents.

Table 1
SCH23390 affinity for wild type and mutant dopamine D_1 receptors

Cell Line	Wild type	Cys^{347}-Ala	Cys^{351}-Ala	Cys^{347}-Ala; Cys^{351}-Ala
K_d (pM)	284.2 ± 27.5	369.6 ± 38.6	363.0 ± 30.6	318.1 ± 32.2
n	4	4	3	3

[^{3}H]SCH23390 at six concentrations of 10^{-10} to 10^{-4} M was used in the saturation binding isotherm to determine the dissociation constants (K_d) of the wild type and mutant receptors (see Section 2). Non-specific binding was the binding not displaced by the presence of 1 μM (+)-butaclamol. The data shown (mean ± S.E.M.) were obtained from the indicated number of experiments performed in duplicates.

2.8. Data analysis

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

3. Results

3.1. Radioligand binding profiles for wild type and mutant dopamine D_1 receptors

There are two cysteines in the carboxyl tail of the dopamine D_1 receptor corresponding to those that are shown to be palmitoylated in other receptors. Cell lines were established, each expressing one of the following four receptors, namely, wild type dopamine D_1, D_1(Cys^{347}-Ala), D_1(Cys^{351}-Ala) and D_1(Cys^{347}-Ala;Cys^{351}-Ala) receptors. To determine whether the substitution of these cysteines affected their ligand binding profiles, the dissociation constants (K_d values) for [^{3}H]SCH23390 and the rank orders of potency for agonists and antagonists were determined.

The K_d values (Table 1) for each of the receptors closely approximate that of dopamine D_1 receptor in human caudate tissue (Seeman and Niznik, 1988). Catecholamine agonists and some dopaminergic antagonists were selected to compete for [^{3}H]SCH23390 binding. Agonist displacement was observed to be the same for each of the four receptors, with the following rank order of potency: dopamine > SKF38393 > serotonin (Table 2). Similarly, antagonist competition exhibited the same rank order for both the wild type and the three mutant receptors: SCH23390 > (+)-butaclamol > haloperidol (Table 3). The

Table 3
Antagonist rank order for dopamine D_1 receptors

Antagonist	Receptor	K_i (nM)	n
SCH23390	Wild type	3.93 ± 1.44	2
	Cys^{347}-Ala	3.13 ± 1.52	2
	Cys^{351}-Ala	5.91 ± 3.05	2
	Cys^{347}-Ala;Cys^{351}-Ala	3.37 ± 1.17	2
Butaclamol	Wild type	31.75 ± 16.71	2
	Cys^{347}-Ala	26.34 ± 10.75	2
	Cys^{351}-Ala	42.52 ± 4.815	2
	Cys^{347}-Ala;Cys^{351}-Ala	19.11 ± 1.575	
Haloperidol	Wild type	177.40 ± 24.00	2
	Cys^{347}-Ala	159.89 ± 61.02	2
	Cys^{351}-Ala	223.75 ± 36.65	2
	Cys^{347}-Ala;Cys^{351}-Ala	183.00 ± 27.80	2

Competition binding assays were performed to determine the rank order of potency for dopamine D_1 receptor antagonists (see Section 2). The K_i values (mean ± S.E.M.) were determined from experiments carried out in duplicates using InPlot and InStat computer programs.

Table 2
Agonist rank order for wild type and mutant dopamine D_1 receptors

Receptors	K_i high (nM)	K_i low (nM)	% High	*n*
Dopamine				
Wild type	5.5 ± 1.8	6 410 ± 2 335	11.1 ± 2.2	2
Cys^{347}-Ala	9.1 ± 3.8	1 165 ± 15.5	33.6 ± 5.0	2
Cys^{351}-Ala	2.9 ± 0.3	3 081 ± 601	15.3 ± 6.1	2
Cys^{347}-Ala;Cys^{351}-Ala	5.4 ± 2.1	804.6 ± 212.4	18.0 ± 2.4	4
SKF38393				
Wild type	12.3 ± 7.3	787.1 ± 426.2	13.9 ± 5.1	2
Cys^{347}-Ala	19.7 ± 14.1	1 784 ± 1 362	17.0 ± 3.9	3
Cys^{351}-Ala	25.6 ± 3.7	641.7 ± 355.2	17.9 ± 3.1	3
Cys^{347}-Ala;Cys^{351}-Ala	19.4 ± 4.8	1 458 ± 1 279	17.2 ± 6.9	2
5-HT				
Wild type		28 007 ± 20 093		2
Cys^{347}-Ala		36 037 ± 6 847.9		3
Cys^{351}-Ala		53 920 ± 14 610		2
Cys^{347}-Ala;Cys^{351}-Ala		26 240 ± 410.0		2

The rank orders of the dopamine D_1 receptor agonists shown above were determined by competition curves against [^{3}H]SCH23390 (600 pM) with a series of concentrations (10^{-10} to 10^{-4} M) of the agonists listed in the table (see Section 2). The values shown (mean ± S.E.M.) were obtained from at least two experiments in duplicates.

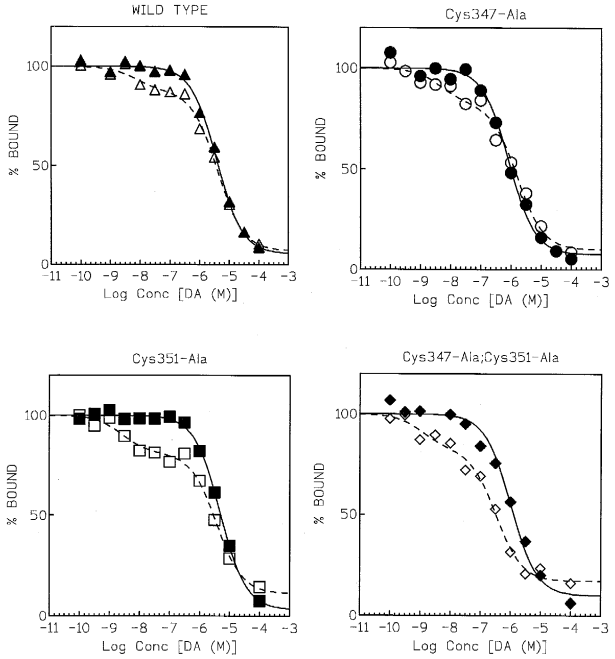

Fig. 3. Desensitization of D_1 receptors induced by dopamine pretreatment. Cells expressing the wild type and mutant receptors were pretreated with 1 μM dopamine for 15 min. Dopamine (10^{-10} to 10^{-4} M) was used as the cold agonist to compete against 600 pM [^{3}H]SCH23390. The data shown are representatives of two to four independent experiments done in duplicates. Controls and pretreated samples are indicated by open and closed symbols, respectively.

Table 4
EC_{50}, basal and V_{max} values for dopamine stimulation of adenylyl cyclase in wild type and mutant dopamine D_1 receptors

Cell lines	Wild type	Cys^{347}-Ala	Cys^{351}-Ala	Cys^{347}-Ala; Cys^{351}-Ala
EC_{50}	59.7 ± 14.7	46.9 ± 23.6	22.8 ± 16.4	19.3 ± 15.1
Basal	28.1 ± 1.86	38.5 ± 7.71	53.9 ± 10.6	43.8 ± 14.5
V_{max}	114.4 ± 20.5	119.1 ± 24.1	126.9 ± 30.0	126.6 ± 36.1
n	3	4	3	3

Adenylyl cyclase assays were carried out to determine the EC_{50} (nM) and the basal and maximal activities (pmol cAMP/min/mg protein) of the wild type and the mutant dopamine D_1 receptors (see Section 2). The values shown are mean ± S.E.M. from at least three separate experiments in duplicates.

parameters (K_d and K_i values) were similar among the wild type and the three mutant receptors, and were consistent with the published literature (Seeman and Niznik, 1988). These data indicated that the replacement of either one or both of the cysteines with alanine(s) in the carboxyl tail did not affect the antagonist binding profile of dopamine D_1 receptors. These results were predictable, as ligand binding mainly involves amino-acid residues in the transmembrane domains of the G protein-coupled receptors (Dohlman et al., 1991).

Agonist binding studies using dopamine or SKF38393 revealed the presence of two affinity states of the receptor

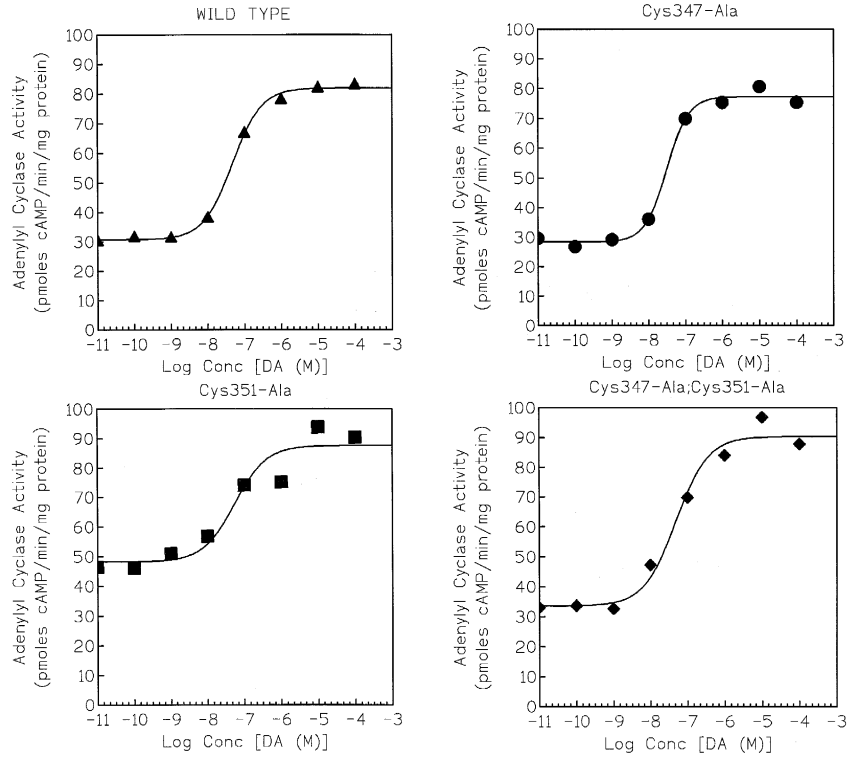

Fig. 4. Adenylyl cyclase activities stimulated by dopamine in cells expressing the wild type and mutant dopamine D_1 receptors. Dopamine (10^{-11} to 10^{-4} M) was used to stimulate adenylyl cyclase in the four cell lines expressing the wild type and mutant dopamine D_1 receptors. The results shown are representatives of at least three separate experiments done in duplicates. The parameter estimates (EC_{50} and V_{max}) are shown in Table 4.

detected by both dopamine D_1 receptor agonists in all the four stable cell lines (see Table 2). The proportion of the high affinity state was very similar for the wild type and mutant dopamine D_1 receptors, indicating the same extent of receptor-G protein coupling (De Lean et al., 1980). Thus residues Cys^{347} and Cys^{351} of the dopamine D_1 receptor appear not to be involved in high affinity agonist or antagonist binding.

3.2. Agonist-induced desensitization of the wild type and mutant dopamine D_1 receptors

To assess the ability of the wild type and the mutant dopamine D_1 receptors to desensitize, cells expressing receptors were pretreated with 10 μM dopamine for 15 min. As shown in Fig. 3, all four types of the dopamine D_1 receptors were detected by dopamine competition against [^{3}H]SCH23390 to have two affinity sites, with similar receptor fractions in the high affinity state (approximately 20%) in the absence of agonist pretreatment. The high affinity state of the wild type and the three mutant receptors was converted almost completely to the low affinity state after exposure to dopamine, suggesting a similar pattern of agonist-induced desensitization.

In addition, in the presence of 100 μM Gpp(NH)p, a nonhydrolyzable GTP analogue, the high affinity states of both the wild type and each of the mutant receptors were decreased to undetectable levels (data not shown), indicating that the high affinity states of these receptors were equally sensitive to guanine nucleotide (De Lean et al., 1980).

Therefore, elimination of putative palmitoylation site(s) did not affect receptor-G protein coupling or agonist-induced receptor desensitization for dopamine D_1 receptors.

3.3. The effect of mutation(s) on adenylyl cyclase activity

To further characterize the effect on receptor-G protein coupling of the dopamine D_1 receptor after substitution of one or both of the palmitoylation sites, we assessed stimulation of adenylyl cyclase activity mediated by the wild type and the three mutant receptors. Representative curves are shown in Fig. 4, and EC_{50} and V_{max} values are summarized in Table 4. Dopamine D_1(Cys^{347}-Ala), D_1(Cys^{351}-Ala) and D_1(Cys^{347}-Ala;Cys^{351}-Ala) receptors had the same stimulation pattern as the wild type. The EC_{50} values for the adenylyl cyclase stimulation with dopamine were very similar if not identical to each other (Table 4). In all cases, dopamine caused the full stimulation of adenylyl cyclase activity comparable to that obtained by forskolin (approximately 90–100 pmol cAMP/min per mg protein). These effects differ from the results obtained with the β_2-adrenoceptor, although both receptors are coupled to the stimulatory GTP-binding proteins, and are more consistent with those of similar mutagenesis studies carried out on other G protein-coupled receptors as discussed below.

4. Discussion

Our results demonstrated that substitution of the two putative palmitoylation sites in the carboxyl tail of the human dopamine D_1 receptors did not perturb the ligand binding profile, high affinity agonist binding state, receptor desensitization, and the ability to stimulate adenylyl cyclase activity, i.e., no difference was observed between the wild type and the three mutant forms of these dopamine D_1 receptors in receptor-G protein interaction.

It is noteworthy that the basal level of adenylyl cyclase activity in the cell line expressing the dopamine D_1(Cys^{351}-Ala) receptors was relatively higher than the cell lines expressing the other three receptors. This is perhaps because the level of receptor expression was higher than the other cell lines. Therefore, more copies of the active form of the receptors were available stochastically at basal state (Milligan et al., 1995; Kenakin, 1995a,b). On the other hand, the maximum activity of adenylyl cyclase stimulation of the D_1(Cys^{351}-Ala) receptor cell line was not further elevated, perhaps because adenylyl cyclase activity is maximally stimulated at a receptor density of 5 pmol/mg protein. This is consistent with a quantitative relationship between the variable receptor expression levels and V_{max} of adenylyl cyclase activity (Whaley et al., 1993), which showed that although V_{max} initially increased roughly proportionally to increasing receptor density, the values reached a plateau at higher receptor densities.

During the course of this work, Jensen et al. (1995) reported, in a series of transient expressions in COS cells, the effect of the carboxyl tail cysteine residues of dopamine D_1 receptor and they suggested that Cys^{347} played a role in antagonist binding, activation of adenylyl cyclase and agonist-induced desensitization. However, in the adenylyl cyclase assays, their EC_{50} values for dopamine were several hundred times higher than our estimates, and in their desensitization study, EC_{50} values were very similar to those of the controls suggesting no receptor desensitization. Moreover, although they noticed that absence of Cys^{347} caused a substantially diminished receptor expression level in the membrane fraction of the cell, the adenylyl cyclase data were not interpreted accordingly. Since the Cys^{347} mutant receptors were expressed at less than half the level of the wild type and other mutants, perhaps not surprisingly, the documented V_{max} values were also about half that for the other receptors. We believe that the adenylyl cyclase activity for their wild type and mutant receptors would have been stimulated to a similar maximum level, if the receptor expression levels had been comparable, and that their data, therefore, should actually support our observations and conclusion.

Substitution of the palmitoylation site (Cys^{341}-Gly) of β_2-adrenoceptors has been shown to result in a dramatic

loss of receptor-G protein coupling (O'Dowd et al., 1989; Moffett et al., 1993). In contrast, studies on other G protein-coupled receptors, including this study, have demonstrated that palmitoylation is not a requirement for G protein coupling (Van Koppen and Glaster, 1991; Karnik et al., 1993; Kennedy and Limbird, 1993; Nussenzveig et al., 1993; Kawate and Menon, 1994). Thus, so far, the β_2-adrenoceptor is the only G protein-coupled receptor in which palmitoylation played a critical role in its interaction with G proteins.

However, the effects on receptor sequestration and down-regulation after elimination of the carboxyl cysteine sites have been diverse. The lack of palmitoylation blocked the rapid agonist-induced internalization of TRH receptors (Nussenzveig et al., 1993). With the α_{2A}-adrenoceptor (Kennedy and Limbird, 1993), down-regulation after prolonged agonist exposure was completely abolished in these mutants, although the mutation did not alter the extent or rate of agonist promoted sequestration or the recovery from sequestration. With the LH/hCG receptors (Kawate and Menon, 1994), abolition of palmitoylation in the carboxyl tail increased agonist-induced internalization of the receptor. Thus, receptor palmitoylation appears to have different roles among various members of this family of G protein-coupled receptors in sequestration and down-regulation, and the difference between the dopamine D_1 receptors and β_2-adrenoceptors in G protein coupling does not exclude the possibility that palmitoylation may be involved in other dopamine D_1 receptor functions in a pattern as outlined above.

The β_2-adrenoceptor contains a cAMP-dependent protein kinase site almost immediately adjacent to the palmitoylation site (separated by only one amino-acid residue). Moffett et al. (1993) demonstrated that the basal level of phosphorylation is about 4 times higher in the β_2(Cys^{341}-Gly)-adrenoceptor than in the wild type and that agonist treatment cannot promote further phosphorylation of the mutant receptor. In addition, Bouvier et al. (1995) reported that elimination of the cAMP-dependent protein kinase site from β_2(Cys^{341}-Gly)-adrenoceptor restored both the phosphorylation and the effector activation to levels observed with wild type β_2-adrenoceptors. Thus, they suggested that the conformation of wild type β_2-adrenoceptors mediated by palmitoylation may prevent free access to the adjacent phosphorylation site by cAMP-dependent protein kinase and, in contrast, the β_2(Cys^{341}-Gly)-adrenoceptor constantly exposes the kinase site, thereby becoming constitutively phosphorylated even in the absence of agonist treatment. Interestingly, no such phosphorylation site is found in the carboxyl tails of rhodopsin, α_{2A}-adrenoceptors, muscarinic M_2 receptors or TRH receptors. A consensus sequence for cAMP-dependent protein kinase is present in LH/hCG receptor in its carboxyl tail and is 9 amino-acid residues downstream from the closest palmitoylation site, and a similar site in the human dopamine D_1 receptor carboxyl tail (-RKAFS-) is 5 amino acids upstream from the equivalent cysteine. Therefore, those protein kinase sites on LH/hCG and dopamine D_1 receptors are more distant from their palmitoylation sites and perhaps are not affected by the presence of palmitoylation at the cysteine residues. We suggest that the discrepancy between the results obtained for the β_2-adrenoceptors and other G protein-coupled receptors may be attributed to this different structural feature of their carboxyl tails.

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