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Palmitoylation occurs at cysteine 347 and cysteine 351 of the dopamine D_1 receptor

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Abstract

To determine the palmitoylation sites in the human dopamine D_1 receptor, we expressed wild type and mutant receptors in which candidate cysteines in the carboxyl tail were substituted by alanines both individually (A347, A351) and together (AA). Our results showed that palmitoylation levels of A347 and A351 were reduced substantially and that AA had no detectable signal of palmitoylation. These data indicate that cysteines 347 and 351 were both palmitoylated and that they were the only sites of palmitoylation. We introduced a cAMP-dependent protein kinase site encompassing the position 351. We predicted that a functional cAMP-dependent protein kinase site would impair receptor-G protein coupling if it is not occluded by palmitoylation. Our results demonstrated that indeed, the introduction of the cAMP-dependent protein kinase site caused reduced potency of dopamine stimulation of adenylyl cyclase, and thus confirmed that when unoccluded, the cAMP-dependent protein kinase site introduced to position 351 of dopamine D_1 receptor could confer constitutive desensitization.

Keywords: G protein-coupled receptor; Dopamine; Palmitoylation; Phosphorylation; Desensitization; Adenylyl cyclase

1. Introduction

Many G protein-coupled receptors have been shown to be palmitoylated (recently reviewed by Jin et al., 1999) at cysteine residues in the cytoplasmic tail and these include rhodopsin (Ovchinnikov et al., 1988; Karnik et al., 1993), β_2-adrenoceptor (O'Dowd et al., 1989), α_2-adrenoceptor (Kennedy and Limbird, 1993; Eason et al., 1994), luteinizing hormone/chorionic gonadotropin (Kawate and Menon, 1994), endothelin ET_A (Horstmeyer et al., 1996), endothelin ET_B (Okamoto et al., 1997), and vasopressin V_2 receptors (Sadeghi et al., 1997). The 5-HT_{1A} (Butkerait et al., 1995) and 5-HT_{1B} receptors (Ng et al., 1993), dopamine D_1 (Ng et al., 1994a) and D_2 receptors (Ng et al., 1994b), and metabotropic glutamate $mGlu_4$ receptor (Alaluf et al., 1995) have also been reported to be palmitoylated, however the actual sites of palmitoylation for these receptors have not been demonstrated (Table 1), although it is assumed that palmitoylation occurs at the cysteine residues in their carboxyl tails. Recently, it was reported that a mutant μ-opioid receptor with its two cysteines in the tail replaced by alanines was still palmitoylated as in wild type (Chen et al., 1998), suggesting that palmitoylation of μ-opioid receptor occurred elsewhere.

Accumulating evidence reveals that palmitoylation serves to enhance the association of cytosolic proteins with the membrane (Bizzozero, 1997; Mumby, 1997). However, the function of G protein-coupled receptor palmitoylation remains unknown. The elimination of palmitoylation sites attenuated the receptor-G protein coupling in β_2-adrenoceptor (O'Dowd et al., 1989; Moffett et al., 1993), endothelin ET_B (Okamoto et al., 1997) and somatostatin sst_5 receptors (Hukovic et al., 1998), but not in other G protein-coupled receptors studied including rhodopsin (Karnik et al., 1993), α_2-adrenoceptor (Kennedy and Limbird, 1993; Eason et al., 1994), muscarinic M_2 receptor (Van Koppen and Nathanson, 1991), muscarinic M_3 receptor (Zeng et al., 1999), thyrotropin releasing hormone receptor

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Table 1
Palmitoylation of G protein-coupled receptors
Abbreviations: 5-HT_{1A} = 5-hydroxytryptamine$_{1A}$ receptor; 5-HT_{1B} = 5-hydroxytryptamine$_{1B}$ receptor; α_2 = α_2-adrenoceptor; β_2 = β_2-adrenoceptor; D_1 = dopamine D_1 receptor; D_2 = dopamine D_2 receptor; ET_A = endothelin ET_A receptor; ET_B = endothelin ET_B receptor; LH/CG = luteinizing hormone/chorionic gonadotropin receptor; M_2 = muscarinic m_2 receptor; M_3 = muscarinic m_3 receptor; $mGlu_4$ = metabotropic glutamate $mGlu_4$ receptor; μ = μ-opioid receptor; SST_5 = somatostatin sst_5 receptor; TRH = thyrotropin releasing hormone receptor; TSH = thyrotropin receptor; V_2 = vasopressin V_2 receptor.

Receptor	Sites	Mutant	Coupling	Other functions	References
α_2^a	C442	C442A/S	No change	Failed to down-regulate	(Kennedy and Limbird 1993; Eason et al., 1994)
β_2^a	C341	C341G	Decreased		(O'Dowd et al., 1989)
D_1^a	C347, C351				(Ng et al., 1994a)
		C347/351G	Decreased		(Jensen et al., 1995)
		C347/351A	No change		(Jin et al., 1997; this report)
ET_A^a	C1215	C1215S, C1215A	No change		(Horstmeyer et al., 1996)
ET_B^a	C402, C403, C405	C402S; C403/405S; C402/403/405S	No change for C402S; abolished for rest		(Okamoto et al., 1997)
LH/CG[a]	C621, C622	C621/622A	No change	Increased internalization and down-regulation	(Kawate et al., 1997; Kawate and Menon 1994)
Rhodopsin[a]	C322, C323	C322S/C323S	No change		(Karnik et al., 1993)
TSH[a]	C699	C699A	No change	Slowed intracellular trafficking	(Tanaka et al., 1998)
V_2^a	C341, C342	C341/342S	No change		(Sadeghi et al., 1997)
D_2^b	C442				(Ng et al., 1994b)
5-HT_{1A}^b	C417, C420				(Butkerait et al., 1995)
5-HT_{1B}^b	C388				(Ng et al., 1993)
$mGlu_4^b$	C890				(Alaluf et al., 1995)
M_2^c	C457	C457G	No change		(Van Koppen and Nathanson, 1991)
M_3^c	C560, C562	C560/562S	No change		(Zeng et al., 1999)
SST_5^c	C320	C320A	Decreased		(Hukovic et al., 1998)
TRH[c]	C335, C337	C335/337S/G	No change	Decreased internalization	(Nussenzveig et al., 1993)
μ^d		C346/351A			(Chen et al., 1998)

[a] Receptors have been shown to be palmitoylated, and the palmitoylation sites were clearly determined by site-directed mutagenesis.
[b] Receptors have been shown to palmitoylated, but no mutagenesis study was carried out, hence sites still unknown.
[c] The putative sites (cysteine residues) were mutated, but no palmitoylation data were available. Thus the palmitoylation site was not verified.
[d] Receptors have been shown to be palmitoylated and mutagenesis of the putative sites (cysteines) was carried out. However, the elimination of cysteines in the C-tail did not eliminate palmitoylation signal. Therefore, the palmitoylation site remains undetermined.

(Nussenzveig et al., 1993), luteinizing hormone/chorionic gonadotropin (Kawate and Menon, 1994), endothelin ET_A (Horstmeyer et al., 1996), vasopressin V_2 (Sadeghi et al., 1997), dopamine D_1 (Jin et al., 1997) and thyrotropin receptors (Tanaka et al., 1998), indicating that palmitoylation is not essential for receptor-G protein coupling, although other functions such as receptor internalization and down-regulation may be affected by elimination of palmitoylation as summarized in Table 1.

The β_2-adrenoceptor contains a cAMP-dependent protein kinase consensus sequence in the close vicinity of the palmitoylation site, being separated by only one amino acid residue. It has been speculated that the elimination of palmitoylation of β_2-adrenoceptor may expose the cAMP-dependent protein kinase site and cause constitutive desensitization of this receptor (Bouvier et al., 1995). The fact that elimination of the cAMP-dependent protein kinase site from the depalmitoylated β_2-adrenoceptor restored the normal receptor-G protein coupling relationship (Moffett et al., 1996) lent further support to this hypothesis. No cAMP-dependent protein kinase consensus site at a similar position is found in the other receptors listed in Table 1.

The purpose of our study was twofold. In the first part, we wished to elucidate the actual sites of palmitoylation of dopamine D_1 receptor. We eliminated the putative palmitoylation sites by replacing cysteine 347 (Cys^{347}) and cysteine 351 (Cys^{351}) with alanine, both individually (designated as A347 and A351 hereafter) and together (designated as AA) (Jin et al., 1997), and these receptors were expressed. In this report, we showed that elimination of Cys^{347} or Cys^{351} substantially reduced the level of palmitoylation and no palmitoylation was shown in the double mutant (AA), indicating that these two cysteines are the only palmitoylation sites.

In the second part, we tested the hypothesis that the cAMP-dependent protein kinase site in β2-adrenoceptor is responsible for its difference from other G protein-coupled receptors. We introduced a cAMP-dependent protein kinase site into dopamine D_1 receptor at position 351. A functional cAMP-dependent protein kinase site would result in a similar constitutive desensitization. Indeed, we demonstrated that the addition of the cAMP-dependent protein kinase consensus site did cause a reduction in the potency of dopamine stimulation of adenylyl cyclase compared with the wild type. Thus, the introduced cAMP-dependent protein kinase site conferred a degree of constitutive desensitization when not interfered with by palmitoylation.

2. Materials and methods

2.1. Materials

Grace's supplemented medium, fetal bovine serum, gentamycin sulfate, fungizone, goat serum, rabbit serum, lipofectin, geneticin (G418), penicillin and streptomycin were purchased from Gibco/BRL (Toronto, ON). [9,10-^{3}H]palmitic acid (70–80 Ci/mmol), [α-^{32}P]ATP (800 Ci/mmol), [^{3}H]SCH23390 and [^{3}H]cAMP (30 Ci/mmol) were purchased from Du Pont/NEN (Boston, MA). Dopamine, serotonin, leupeptin, benzamidine, soybean trypsin inhibitor, GTP, ATP, cAMP and forskolin were purchased from Sigma (St. Louis, MO). Butaclamol was purchased from RBI (Natick, MA). Dowex Resin (100–200 mesh) was purchased from Bio-Rad (Richmond, CA).

2.2. Construction of dopamine D_1 receptor recombinant baculoviruses

The wild type and mutant receptor genes (Jin et al., 1997) were subcloned into Bacmid supplied with the Bac to Bac kit (Life Technologies) and subsequently expressed in Sf9 cells according to the instructions of the manufacturer.

2.3. Cell culture

Sf9 cells were grown in monolayer or suspension culture as described by Summers and Smith (1987). Chinese hamster ovary (CHO) 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 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.

2.4. Metabolic labeling with tritiated palmitate

At 24 h post viral infection, Sf9 cells expressing dopamine D_1 receptors were cultured in serum-free medium for 18 h prior to the labeling experiment. Following this period, cells were resuspended in Grace's insect medium supplemented with 1% fetal bovine serum for 1 h at 27°C. [9,10-^{3}H]palmitate was dissolved in dimethyl sulfoxide and added to the suspension culture at the concentration of 0.2 mCi/ml for 4 h. Palmitic acid labeling was terminated by centrifugation at $100 \times g$ and cells washed twice with cold PBS.

2.5. Solubilization and immunoprecipitation of dopamine D_1 receptor and SDS–polyacrylamide gel electrophoresis (SDS–PAGE)

Cells were broken by sonication twice for 20 s at the setting of 3.5 in buffer A: 5 mM Tris–HCl and 2 mM EDTA with protein inhibitors (10 μg/ml benzamidine, 5 μg/ml leupeptin and 5 μg/ml soybean trypsin inhibitor). Unbroken cells were removed by centrifugation at $100 \times g$ for 5 min. The supernatant was centrifuged at $27\,000 \times g$

for 20 min and resuspended in solubilization buffer containing 100 mM NaCl, 20 mM Tris–HCl (pH 7.4), 2% digitonin and 5 mM EDTA with protease inhibitors. The suspension was stirred at 4°C for 2 h and centrifuged at $27\,000 \times g$ for 20 min. The solubilized fraction was washed and concentrated in Centriprep 30 (Amicon) using buffer B: 100 mM NaCl and 10 mM Tris–HCl (pH 7.4) with protease inhibitors. The resultant solution was precleared with 1/20 normal rabbit serum and protein A-Sepharose beads for 2 h on ice. The solubilized receptors were immunoprecipitated with the rabbit antiserum for dopamine D_1 receptor (kindly provided by Dr. Mark Brann, University of Vermont) at a 1/40 dilution in buffer B for 2 h on ice, and agitated gently overnight at 4°C with 1/40 dilution of agarose fixed goat anti-rabbit immunoglobulin G (IgG). The immunoprecipitate was washed several times with cold buffer B, solubilized in SDS sample buffer: 50 mM Tris–HCl (pH 6.5), 10% SDS, 10% glycerol, and 0.003% bromophenol blue, sonicated and electrophoresed on SDS–PAGE. Following electrophoresis, the gel was fixed and treated with Enlightning (NEN) for 30 min, dried and exposed to Kodak X-AR film at −70°C for 6 weeks.

2.6. Site-directed mutagenesis of dopamine D_1 receptor gene

Mutagenesis was carried out on the human dopamine D1 receptor gene using polymerase chain reaction as described by Jin et al. (1997). The PCR products were subcloned, as described by Jin et al. (1997) into an expression vector pRC/CMV (Invitrogen), which was linearized by *Xba*I, 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).

2.7. Permanent transfection of CHO cells with wild-type and mutant D_1 receptor genes

Monolayers of 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 200 fmoles/mg protein) as assessed by [^{3}H]SCH23390 saturation binding.

2.8. Ligand binding assay

Ligand binding assays were carried out essentially as described by Jin et al. (1997). The final concentrations in binding buffer were 75 mM Tris–HCl (pH 7.4), 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 triplicate 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.

2.9. Adenylyl cyclase assay

Cell membranes were prepared by Polytron homogenization (6500 rpm for 20 s) in binding buffer, centrifugation at 12 000 rpm to collect membranes, and resuspension of the pellet in reaction buffer containing 75 mM Tris–HCl (pH 7.4), 8 mM $MgC1_2$ and 5 mM EDTA. Adenylyl cyclase assays were conducted in duplicates 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. Reactions were stopped by the addition of 1 ml of 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 elute was then subjected to purification on aluminum columns. Protein concentrations were measured using Bio-Rad reagents.

2.10. Data analysis

Signals from western blot and palmitoylation in SDS–PAGE were analyzed by densitometry using MCID-M4 (Imaging Research, St. Catherine, Ontario). Ligand binding and adenylyl cyclase assay data were analyzed by nonlinear least square regression using the GraphPad Prism computer programs.

3. Results

3.1. Palmitoylation occurs at both Cys^{347} and Cys^{351} of dopamine D_1 receptor

To determine the sites of palmitoylation of dopamine D_1 receptor, we replaced Cys^{347} and Cys^{351} with alanine

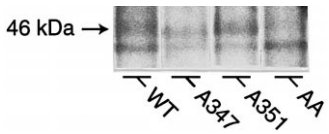

Fig. 1. Palmitoylation of dopamine D_1 receptors. Metabolic labeling with tritiated palmitic acid was performed in Sf9 cells expressing the wild type dopamine D_1 receptor and three mutant receptors as described in Materials and Methods. Cell membranes were immunoprecipitated with dopamine D_1 receptor specific antibodies. The immunoprecipitated membrane proteins were resolved on SDS–PAGE and exposed to Kodak X-AR film at −70°C for 6 weeks. The upper bands (arrow) of the first three lanes from the left represent palmitoylated dopamine D_1 receptors: WT, A347 and A351. No palmitoylation was detected in the last lane representing the double cysteine mutant AA. The size of receptors was approximately 46 kDa. The lower bands represent a nonspecific protein immunoprecipitated with the anti-dopamine D_1 receptor antibodies. This autoradiograph was a representative of two similar experiments.

Fig. 2. A sequence alignment of the wild type and three mutant dopamine D_1 receptors. A partial sequence of wild type dopamine D_1 receptor and three mutant receptors are aligned including a part of TM 7 and the adjacent carboxyl tail region. A consensus cAMP-dependent protein kinase sequence R–R–X–S that was introduced to the vicinity of position 351 in P and AAP is shown in bold. Cysteines and their alanine substitutions are underlined in WT, AA, P and AAP receptors.

separately (A347 and A351) and together (AA), and expressed the wild type, A347, A351 and AA receptors in Sf9 cells. As shown in Fig. 1, WT, A347 and A351 (upper band in lanes 1, 2 and 3) were each palmitoylated; AA was not palmitoylated (lane 4). The levels of palmitoylation were reduced in the single mutations as compared with the wild type. The level of receptor expression was corrected for by standardization with western blot signals. The A347 mutant was palmitoylated to 24% of the level of wild type, whereas the A351 was palmitoylated to 47% in comparison with the wild type (Table 2). No palmitoylation signal could be detected for the double mutant AA. The fact that the palmitoylation signal of wild type was at least twofold greater than that of the single mutants and absent in the double mutant, indicated that these two cysteines were both palmitoylated, and that they were the only sites of palmitoylation for dopamine D_1 receptor.

Cells expressing wild type and mutant receptors were treated with 10 μM dopamine for 20 min. We did not observe any increase in the level of palmitoylation in the wild type or single mutants (Table 2). Again, no signal was detected in the double cysteine mutant.

3.2. Introduction of cAMP-dependent protein kinase site in dopamine D_1 receptor

In β_2-adrenoceptor, elimination of the palmitoylation site attenuated receptor-G protein coupling (O'Dowd et al., 1989). It has been demonstrated (Moffett et al., 1996) that a cAMP-dependent protein kinase site in β_2-adrenoceptor was responsible for the attenuated receptor-G protein coupling in the Gly341β_2-adrenoceptor mutant. We introduced a cAMP-dependent protein kinase site into the wild type dopamine D_1 receptor and the AA double mutant. In order to create a consensus cAMP-dependent protein kinase sequence R–R–X–S, we replaced the wild type sequence R–L–C351–P with R–R–C351–S (designated as P) and replaced the R–L–A351–P in the double cysteine mutant with R–R–A351–S (designated as AAP) (Fig. 2). We subsequently expressed the WT, AA, P and AAP receptors in CHO cells. Stable cell lines expressing similar receptor densities were selected for further study. As shown in Table 3, the stable cell lines expressed WT, AA, P, and AAP at comparable levels. The dissociation constant K_d values for SCH23390 for the four receptors were also similar (Table 3), indicating that the introduction of the

Table 2
Palmitoylation level of dopamine D_1 receptor
Immunoprecipitated membrane proteins obtained from Sf9 cells expressing the WT, A347, A351 and AA receptors in the absence and presence of dopamine (DA) treatment (10 μM for 20 min) were electrophoresed on SDS–PAGE. Palmitoylation signal intensity was standardized by dividing the absorbence of the palmitoylation signal read from the densitometer by the absorbance of Western blot signal. The percentage in the parentheses indicates the ratio of the mutant signal compared with the WT.

Dopamine D_1 receptor	−DA	+DA
WT	0.26 (100%)	0.26
A347	0.06 (24%)	0.04
A351	0.12 (47%)	0.07
AA	0.01 (4%)	0.00

Table 3
The binding characteristics of wild type and mutant dopamine D_1 receptors
The B_{max} and K_d values for the antagonist SCH23390 were measured in triplicates and shown as mean ± S.E.M. The numbers of independent experiments are indicated in the parentheses.

Dopamine D_1 receptor	B_{max} (fmol/mg)	K_d (nM)
WT	155.5 ± 15.1 (3)	311.6 ± 34.8 (3)
AA	151.4 ± 19.3 (3)	323.6 ± 51.7 (3)
P	236.7 ± 77.0 (4)	282.4 ± 28.4 (4)
AAP	218.8 ± 42.2 (3)	198.3 ± 53.1 (3)

cAMP-dependent protein kinase site did not alter receptor affinity for the antagonist SCH23390.

3.3. The effect of the introduced cAMP-dependent protein kinase site on the receptor-G protein coupling

We had predicted that the cAMP-dependent protein kinase site introduced into the wild type receptor should not produce any constitutive desensitization because it might be occluded by palmitoylation. However, phosphorylation of the cAMP-dependent protein kinase site in the AAP was expected to confer some degree of constitutive desensitization, since the elimination of two palmitoylation sites might have promoted cAMP-dependent protein kinase-mediated phosphorylation. Therefore, we expected that WT, AA, and P should have the same level of basal activity and maximum dopamine stimulation of adenylyl cyclase, whereas the AAP should have lower potency and/or efficacy of agonist stimulation of adenylyl cyclase activity.

As shown in Fig. 3 and summarized in Table 4, the basal adenylyl cyclase activities of WT, AA, P, and AAP were similar. However, the EC_{50} value for AAP was shifted to the right and was at least twofold higher than the other three receptors. The V_{max} of AAP was not decreased but was higher.

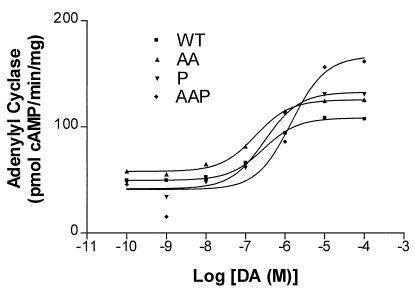

Fig. 3. Dopamine stimulation of adenylyl cyclase activity in dopamine D_1 receptors. Adenylyl cyclase assays were conducted on WT, AA, P and AAP as described in the Materials and Methods. The curves are representatives of at least three independent experiments that were carried out in duplicate. EC_{50} and V_{max} values are summarized in Table 4.

4. Discussion

We have shown previously that dopamine D_1 receptor is palmitoylated (Ng et al., 1994a,b); however, the palmitoylation site(s) was not determined. The two cysteines (Cys^{347} and Cys^{351}) most proximal to the membrane were assumed to be the palmitoylation sites. Within the cytoplasmic domains, three other cysteine residues exist in dopamine D_1 receptor, two in the third intracellular loop (Cys^{297} and Cys^{306}) and one in the carboxyl tail (Cys^{385}). In this report, we have demonstrated the following: (1) wild type dopamine D_1 receptors were palmitoylated at both Cys^{347} and Cys^{351}; (2) these two cysteines were palmitoylated independently, consistent with luteinizing hormone/chorionic gonadotropin (Kawate and Menon, 1994) and vasopressin V_2 receptors (Sadeghi et al., 1997) but in contrast to rhodopsin, in which the substitution of the first cysteine prevented the palmitoylation of the second cysteine (Karnik et al., 1993); (3) palmitoylation occurred only at Cys^{347} and Cys^{351}, consistent with most studies listed in Table 1 with the exception of μ-opoid receptor, where elimination of the two cysteines in the carboxyl tail did not affect palmitoylation of the mutant receptor; and (4) the other three cysteines in the cytoplasmic regions were not palmitoylated.

The study of receptors listed in Table 1 can roughly be classified into the following three categories.

(1) No palmitoylation data were available and only cysteine mutagenesis was carried out. These receptors include muscarinic M_2 receptor (Van Koppen and Nathanson, 1991), muscarinic M_3 receptor (Zeng et al., 1999), somatostatin SST_5 (Hukovic et al., 1998), and thyrotropin-releasing hormone (Nussenzveig et al., 1993).

(2) Receptors were shown to be palmitoylated but no mutagenesis studies were carried out; therefore, the actual sites of palmitoylation were not determined. In these receptors, it has always been assumed that palmitoylation occurs at the cysteine residues of their carboxyl terminals close to the membrane. These receptors include 5-HT_{1A} (Butkerait

Table 4
The effect of PKA site in dopamine D_1 receptor
The dopamine stimulation of adenylyl cyclase activity on the wild type and mutant receptors was carried out in duplicate as described in Materials and Methods. The concentration of forskolin was 10 μM. The values are shown as mean ± S.E.M. The number in parentheses indicates the number of replicated experiments.

Dopamine D_1 receptor	Basal (pmol cAMP/min/mg)	V_{max} (pmol cAMP/min/mg)	EC_{50} (μM)	Forskolin (pmol cAMP/min/mg)
WT	42.2 ± 4.3 (3)	90.3 ± 9.1 (3)	0.56 ± 0.15 (3)	300.5 ± 54.5 (5)
AA	47.2 ± 8.4 (4)	116.7 ± 16.7 (4)	0.21 ± 0.03 (4)	294.5 ± 49.0 (5)
P	37.2 ± 3.9 (5)	127.7 ± 12.1 (5)	0.41 ± 0.06 (5)	443.9 ± 23.4 (5)
AAP	37.6 ± 4.9 (3)	152.6 ± 15.3 (3)	1.02 ± 0.32 (3)	387.6 ± 56.7 (3)

et al., 1995), 5-HT_{1B} (Ng et al., 1993), dopamine D_2 (Ng et al., 1994b), and metabotropic glutamate $mGlu_4$ receptors (Alaluf et al., 1995).

(3) Receptors were shown to be palmitoylated and the actual palmitoylation sites were determined by peptide sequencing and mass spectrometry in rhodopsin (Ovchinnikov et al., 1988) or by site-directed mutagenesis studies in the majority of other receptors as listed below. These receptors include the following: α_2-adrenoceptor (Kennedy and Limbird, 1993; Eason et al., 1994), β_2-adrenoceptor (O'Dowd et al., 1989; Moffett et al., 1993), endothelin ET_A (Horstmeyer et al., 1996), endothelin ET_B (Okamoto et al., 1997), luteinizing hormone/chorionic gonadotropin (Kawate and Menon, 1994; Zhu et al., 1995; Kawate et al., 1997), rhodopsin (Karnik et al., 1993), thyroid-stimulating hormone (Tanaka et al., 1998) and vasopressin V_2 receptors (Sadeghi et al., 1997). Dopamine D_1 receptor (Ng et al., 1994a; Jin et al., 1997; and this report) also falls into this category.

Of the receptors in which palmitoylation has been shown and the sites identified, endothelin ET_A and ET_B, luteinizing hormone/chorionic gonadotropin, rhodopsin and vasopressin V_2 receptors contain more than one cysteine residue in the carboxyl terminal. In endothelin ET_B (Okamoto et al., 1997), luteinizing hormone/chorionic gonadotropin (Kawate and Menon, 1994), rhodopsin (Karnik et al., 1993) and vasopressin V_2 (Sadeghi et al., 1997), multiple cysteines of the palmitoylation sites were mutated both individually and together. In endothelin ET_B, luteinizing hormone/chorionic gonadotropin, and vasopressin V_2 receptors, these cysteines were palmitoylated individually and palmitoylation did not require the presence of the other cysteines. However, in rhodopsin, the mutation of the first of the adjacent cysteine residues prevented the palmitoylation of the next cysteine, indicating that the first cysteine is the primary palmitoylation site (Karnik et al., 1993). The five cysteines for palmitoylation in endothelin ET_A (Horstmeyer et al., 1996) were not mutated separately. Our results that in dopamine D_1 receptor Cys^{347} and Cys^{351} were independent palmitoylation sites were consistent with the results of the endothelin ET_B, luteinizing hormone/chorionic gonadotropin and vasopressin V_2 receptors.

Recently, it was reported that palmitoylation of μ-opioid receptor occurred at sites other than the two cysteines of the carboxyl tail (Chen et al., 1998). The reason for this discrepancy is not clear. Since there is another cysteine in the second intracellular loop of μ-opioid receptor, palmitoylation might occur at that cysteine. Our data in this report demonstrated that palmitoylation occurs only at Cys^{347} and Cys^{351} in the carboxyl tail of dopamine D_1 receptor.

In the second part of this report, we introduced a cAMP-dependent protein kinase site in the vicinity of position 351 in the wild type and AA. Elimination of palmitoylation of β_2-adrenoceptor has been suggested to constantly expose the adjacent cAMP-dependent protein kinase site to cAMP-dependent protein kinase leading to constitutive desensitization and impaired receptor-G protein coupling (Moffett et al., 1996). A functional cAMP-dependent protein kinase site introduced in the AAP was expected to result in some constitutive desensitization. Indeed, EC_{50} of dopamine stimulation of adenylyl cyclase in AAP was shifted to the right. The decreased potency of dopamine stimulation suggested that the cAMP-dependent protein kinase site introduced into the carboxyl tail was functional, and that it conferred a degree of constitutive desensitization to this mutant receptor. The reason why this desensitization affected only potency but not efficacy is unknown. This phenomenon of desensitization affecting only potency (EC_{50}) and not efficacy (V_{max}), has been observed in other mutated receptors as well, such as a β_2-adrenoceptor mutant with its β_2-adrenoceptor kinase sites removed from the carboxyl tail (Hausdorff et al., 1989), a chimeric β_3-adrenoceptor with a β_2-adrenoceptor third intracellular loop (Jockers et al., 1996), and a chimeric adenosine A_1 receptor containing the A_3 receptor tail (Palmer et al., 1996).

In summary, our results have shown for the first time that palmitoylation occurs exclusively at Cys^{347} and Cys^{351} in dopamine D_1 receptor. Introduction of cAMP-dependent protein kinase site in the carboxyl tail induces a constitutively desensitized state of the dopamine D_1 receptor, which clarifies the mechanism whereby palmitoylation of dopamine D_1 receptor differs from β_2-adrenoceptor.

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