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Palmitoylation of the rat μ opioid receptor

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Abstract We examined whether the μ opioid receptor was palmitoylated and attempted to determine sites of palmitoylation. Following metabolic labeling with [^{3}H]palmitic acid and immunoaffinity purification of the μ opioid receptor, SDS-PAGE and fluorography revealed a broad labeled band with M_r of ~80 kDa in CHO cells stably expressing the rat μ receptor, but not in CHO cells transfected with the vector alone, indicating that the μ receptor is palmitoylated. Activation of the receptor with morphine did not affect the extent of palmitoylation. Hydroxylamine or dithiothreitol treatment removed most of the radioactivity, demonstrating that [^{3}H]palmitic acid is incorporated into Cys residue(s) via thioester bond(s). Surprisingly, mutations of the only two Cys residues in the C-terminal domain did not reduce [^{3}H]palmitic acid incorporation significantly. Thus, unlike many G-protein coupled receptors, the palmitoylation site(s) of the rat μ opioid receptor do(es) not reside in the C-terminal domain.

Key words: Palmitoylation; Mu opioid receptor

1. Introduction

Mu opioid receptors play important roles in many physiological functions, most notably modulation of pain perception and regulation of mood (for a review [1]). Analgesic effects of many opiates and opioids are mediated primarily by μ opioid receptors. Opiates (such as morphine and heroin) are among the most widely abused drugs, mainly because of their actions on the μ opioid receptor to induce euphoria. Development of tolerance to and dependence on opiates is mediated largely by the μ receptor. Activation of μ opioid receptors couples via pertussis toxin-sensitive G proteins to various effectors, including adenylate cyclase and K^+ and Ca^{2+} channels (for a review, [2]) and mitogen-activated kinase [3]. Following the cloning of a mouse δ opioid receptor [4,5], μ receptors were cloned [6–8]. A splice variant of the μ receptor was reported with sequence variation at the last few amino acids of the C-terminal domain [9,10]. Deduced amino acid sequences of these clones display the motif of seven transmembrane helices, which is characteristic of G-protein coupled receptors (GPCRs).

Many GPCRs have been shown to be covalently modified by palmitic acid (for a review [11]), including rhodopsin [12–14], β_2-adrenergic [15,16], α_{2A}-adrenergic [17], luteinizing hormone/human choriogonadotropin [18,19], endothelin A [20], serotonin$_{1B}$ [21], dopamine D_1 [22], dopamine D_{2L} [23], dopamine D_{2S} [24], mGluR4 metabotropic glutamate [25] and thyrotropin [26] receptors. The sites for palmitoylation were determined to be conserved cysteine residues in the proximal portion of the C-terminal domain in a few GPCRs, including rhodopsin [13,14] and β_2-adrenergic [15], α_{2A}-adrenergic [17], luteinizing hormone/human choriogonadotropin [18,19], endothelin A [20] and thyrotropin [26] receptors. Alignment of 74 GPCRs, including receptors mediating diverse signal transduction pathways, shows that ~78% have a cysteine at this position [27]. Both splice variants of the μ receptor also have a conserved cysteine at this position. Using fluorescent palmitate derivative, Moench et al. [28] demonstrated that the palmitoylation sites of rhodopsin were embedded in the membrane, thus forming a small fourth cytoplasmic loop in the C-terminal domain. Similar topology was postulated for other receptors [13,15,29]. However, palmitoylation appears to play diverse functional roles among GPCRs, such as coupling to G-proteins [15,30], or agonist-induced receptor desensitization [15,31], phosphorylation [31], internalization [18,26,32] or down-regulation [33].

In this study, we examined incorporation of [^{3}H]palmitic acid into the rat μ opioid receptor [6], the effect of morphine stimulation on palmitoylation of the μ receptor and the nature of the chemical bond formed between palmitic acid and the μ receptor. In addition, we examined palmitoylation of two mutant μ receptors, C351A and C346A/C351A, since Cys-346 and Cys-351 reside in the proximal portion of the C-terminal domain and are the only two cysteine residues in the C-terminal domain.

2. Materials and methods

2.1. Materials

[^{3}H]Diprenorphine (58 Ci/mmol) and [^{3}H]palmitic acid (~50 Ci/mmol) were purchased from NEN Life Science (Boston, MA). Morphine and [^{3}H]β-funaltrexamine ([^{3}H]β-FNA) (19 Ci/mmol) were provided by the National Institute on Drug Abuse. Naloxone was a gift from DuPont/Merck Co. (Wilmington, DE). Muta-Gene kit was purchased from Bio-Rad Co. (Hercules, CA); Lipofectamine from Gibco BRL (Gaithersburg, MD); peptide-N^4-[*N*-acetyl-β-glucosaminyl]asparagine amidase (*N*-glycanase) from Genzyme Co. (Boston, MA); silica gel thin layer chromatography (TLC) plates from Analtech Inc. (Newark, DE); NP-40 from Pierce Co. (Rockford, IL); Sequenase version 2.0 from USB Biochemicals (Cleveland, OH); geneticin, protease inhibitors and other commonly used chemicals from Sigma.

2.2. Labeling with [^{3}H]palmitic acid and solubilization

CHO cells stably expressing the rat μ receptor [6] were examined for palmitoylation according to a modified procedure of O'Dowd et al. [15]. CHO cells transfected with the vector pRc/CMV served as the control. Twenty-four hours before labeling, cells were plated at ~80% density in 100 mm dishes. After washed once with serum-free medium, cells were incubated in 5 ml serum-free medium per dish with 0.2 mCi/ml [^{3}H]palmitic acid for 4 h. For examination of the effect of morphine, 2 μM morphine was added to cells 1 h before

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Abbreviations: CHO cells, Chinese hamster ovary cells; CHO-μR cells, Chinese hamster ovary cells stably expressing the rat μ opioid receptor; DAMGO, Try-D-Ala-Gly-*N*-Me-Phe-Gly-ol; TLC, thin layer chromatography; GPCR, G-protein coupled receptor; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis

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termination of the labeling. Labeled cells were then detached in Versene solution (0.67 mM EDTA, 136.9 mM NaCl, 2.7 mM KCl, 8.1 mM Na_2HPO_4, 1.5 mM KH_2PO_4 and 1.1 mM glucose, pH 7.0), collected by centrifugation and washed twice with ice-cold Versene. The cells were sonicated for 10 s in ice-cold buffer A (2% NP-40, 0.1 M Tris-HCl, pH 7.0, 0.1 M NaCl, 10 μg/ml of each of the protease inhibitors aprotinin, leupeptin, pepstatin A and soybean trypsin inhibitor, 1 mM ethylenediaminetetraacetic acid and 1 mM phenylmethylsulfonyl fluoride) and left on ice for 30–60 min to complete solubilization. The lysate was centrifuged at 40 000×*g* for 20 min at 4°C and the supernatants were filtered through a 0.22 μm filter.

2.3. Immunoaffinity purification of labeled μ opioid receptor

Immunoaffinity purification of labeled μ opioid receptor using rabbit antiserum against the C-terminal peptide 383–398 (CTNHQLENLEAETAPLP) was performed according to our published procedures [34].

2.4. TLC of [^{3}H]fatty acid associated with the immunoaffinity-purified rat μ opioid receptor

These experiments were conducted according to the method of O'Dowd et al. [15]. The immunoaffinity-purified labeled receptor was acidified with HCl to pH 1.0 and extracted three times with ethyl acetate and three times with hexane to remove free [^{3}H]palmitic acid. The aqueous phase was then treated with 1 N KOH for 12 h at 37°C, followed by acidification and extraction as above. The extract was dried under N_2 and applied to a silica gel TLC plate along with authentic [^{3}H]palmitic acid in a parallel lane. The TLC plate was developed with hexane:ethyl acetate:acetic acid (80:20:1), cut into 1 cm fractions, and the radioactivity in each fraction determined by liquid scintillation counting.

2.5. Treatment of [^{3}H]palmitate-labeled μ receptor with hydroxylamine or dithiothreitol (DTT)

Immunoaffinity-purified [^{3}H]palmitate-labeled receptor was incubated with 1 M NH_2OH or 0.1 M DTT at pH 7.5 in 2% sodium dodecyl sulfate (SDS) at 37°C for 30 min for NH_2OH treatment [12,14,35] and for 2 h for DTT treatment [14]. The reaction mixture was acidified with 1/10 volume of 1 N HCl and extracted with an equal volume of ethyl acetate four times to remove free [^{3}H]palmitate. The aqueous phase was counted for radioactivity. For SDS-polyacrylamide gel electrophoresis (SDS-PAGE) analysis, the treated preparations were loaded onto SDS-PAGE without ethyl acetate extraction.

2.6. Oligodeoxynucleotide-directed mutagenesis

Mutations were generated on the rat μ receptor with the uracil replacement method of Kunkel et al. [36] using Muta-Gene kit. The primers used to introduce mutations were: CTTCAAGCGAGCCTTCAGAGAG (C346A); CAGAGAGTTCGCCATCCCAACC (C351A); TCAAGAGAGCCTTCAGAGAGTTCGCCATCCCAAC (C346A/C351A)

Mutations were confirmed by DNA sequence determination with the method of Sanger et al. [37].

2.7. Other methods employed

The following methods were described in our previous publications: stable expression of the μ receptor in CHO cells [38], labeling of μ opioid receptors with [^{3}H]β-FNA [38], SDS-PAGE and detection of radioactivity in gels by fluorography [39], *N*-glycanase treatment [39] and protein determinations [38,40].

3. Results and discussion

We first examined whether [^{3}H]palmitic acid was incorporated in the rat μ opioid receptor. CHO-μR cells (μ66), which stably express approximately 10 pmol/mg protein of the rat μ opioid receptor, were used. Similar numbers of CHO-μR (μ66) and CHO cells transfected with the vector pRc/CMV were labeled with [^{3}H]palmitic acid. Membranes were prepared, solubilized and the μ receptor was partially purified using immunoaffinity chromatography. SDS-PAGE of the immunoaffinity-purified material and subsequent fluorography revealed a broad and diffuse labeled protein band with M_r

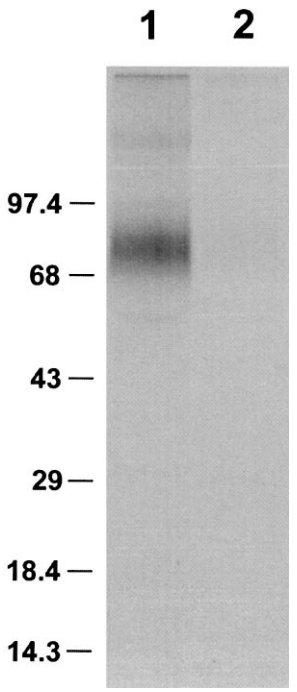

Fig. 1. Incorporation of [^{3}H]palmitic acid into the μ opioid receptor stably expressed in CHO cells. Incubation of CHO-μR cells or CHO cells transfected with the vector pRc/CMV with [^{3}H]palmitic acid and immunoaffinity chromatography were performed as described in Section 2. Lanes 1 and 2: immunoaffinity-purified materials from [^{3}H]palmitic acid-labeled CHO-μR cells (40 000 dpm, ∼5 μg protein) and CHO cells transfected with the vector pRc/CMV (20 000 dpm, ∼5 μg protein), respectively. This figure represents one of two experiments. Exposure time was 7 days.

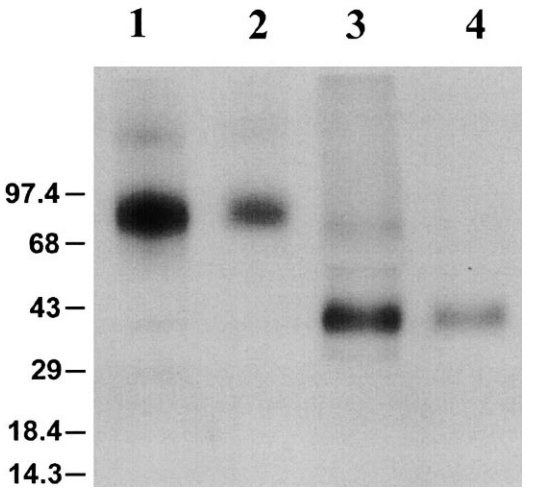

Fig. 2. Deglycosylation by *N*-glycanase of immunoaffinity-purified [^{3}H]β-FNA-labeled μ opioid receptors and [^{3}H]palmitate-labeled materials in solubilized CHO-μR membranes. [^{3}H]β-FNA-labeled μ opioid receptors and [^{3}H]palmitate-labeled materials in solubilized CHO-μR membranes were treated with or without *N*-glycanase (1 unit) at 37°C for 6 h and subjected to SDS-PAGE and fluorography. Lanes 1 and 3: [^{3}H]β-FNA-labeled μ opioid receptors, control and *N*-glycanase-treated, respectively, 37 000 dpm and ∼0.8 pmol of μ receptors each. Lanes 2 and 4: [^{3}H]palmitate-labeled materials, control and *N*-glycanase-treated, respectively, 20 000 dpm and ∼2 pmol of receptor each. This experiment was performed twice with the same results. Exposure time was 7 days.

~80 kDa present in the CHO-μR preparation, but not in the preparation of CHO cells transfected with the vector pRc/CMV alone (Fig. 1), despite the fact that the two preparations showed similar levels of total [^{3}H]palmitic acid incorporation. The broad and diffuse pattern of the labeled protein band indicates its glycoprotein nature. Following deglycosylation by *N*-glycanase, the labeled protein band became a relatively sharp band of M_r ~40 kDa (Fig. 2), which is in accordance with the deduced molecular weight of the rat μ receptor [6]. This labeled band was identical to [^{3}H]β-FNA-labeled rat μ receptor (Fig. 2) in its M_r and electrophoretic properties. These results indicate that the rat μ receptor in CHO-μR cells is palmitoylated.

Since [^{3}H]palmitic acid can be metabolized to other fatty acids, we examined whether it was [^{3}H]palmitic acid that was incorporated into the rat μ opioid receptor. The [^{3}H]fatty acid released from purified labeled material by alkali treatment had an identical migration distance to that of authentic [^{3}H]palmitic acid in silica gel TLC analysis, indicating that the receptor is acylated with [^{3}H]palmitic acid (Fig. 3).

Exposure to isoproterenol led to a specific increase in the incorporation of palmitic acid in the β_2-adrenergic receptor [41]. We determined if palmitoylation of the rat μ receptor was affected by morphine. As shown in Fig. 4, exposure to 2 μM morphine for 1 h did not affect significantly [^{3}H]palmitate incorporation into the rat μ receptor. The X-ray films of two independent experiments were scanned and the area of each band was integrated by use of NIH Image software program. The extents of palmitoylation of morphine-treated μ receptors represented 105% and 82% (mean = 93.5%)

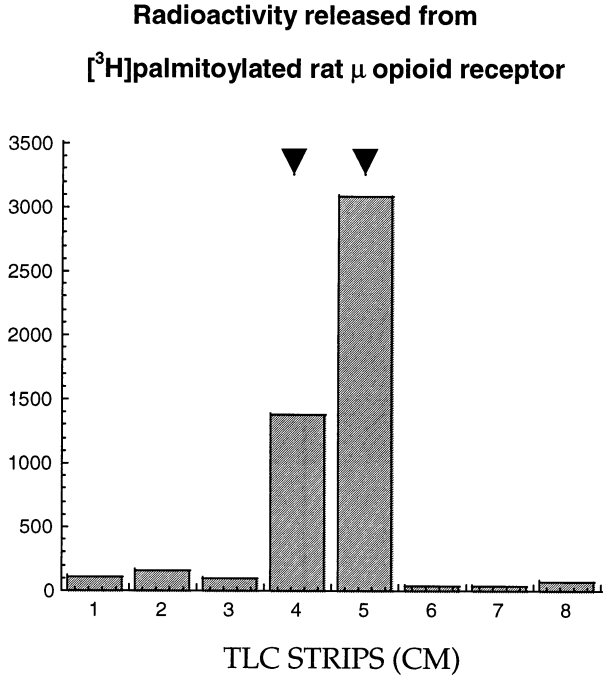

Fig. 3. Thin-layer chromatography of [^{3}H]fatty acid associated with the immunoaffinity-purified rat μ opioid receptor. Immunoaffinity-purified [^{3}H]labeled rat μ opioid receptor was subjected to alkali treatment and the released radioactivity was analyzed on silica gel TLC along with authentic [^{3}H]palmitic acid as described in Section 2. The figure shows the migration distance of radioactivity released from affinity-purified labeled rat μ opioid receptor, with the arrows indicating that of authentic [^{3}H]palmitic acid (about 1/3 at 4 cm and 2/3 at 5 cm).

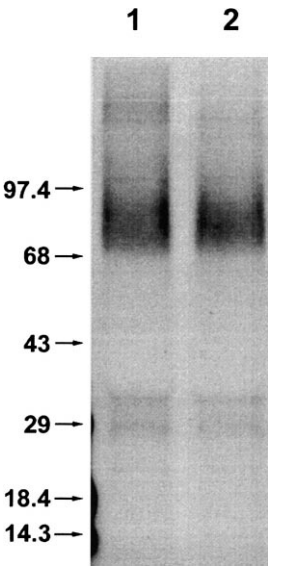

Fig. 4. Effect of morphine stimulation on palmitoylation of the rat μ opioid receptor. Similar numbers of CHO-μR cells were labeled with [^{3}H]palmitic acid with or without morphine treatment and labeled receptors partially purified and subjected to SDS-PAGE and fluorography. Lane 1: [^{3}H]palmitic acid-labeled CHO-μR cells (~2 pmol receptors, 15000 dpm); lane 2: [^{3}H]palmitic acid-labeled CHO-μR cells with 2 μM morphine added 1 h before termination of the labeling (~2 pmol receptors, 16000 dpm). Exposure time was 20 days.

of the control levels. Thus, palmitoylation of the rat μ receptor appears not to be affected by morphine.

Hydroxylamine and DTT treatments of [^{3}H]palmitate-labeled μ opioid receptor were performed to determine whether the chemical bond(s) formed between [^{3}H]palmitic acid and the μ receptor were thioester in nature or involved thiol group. Hydroxylamine (1 M) or DTT (0.1 M) removed [^{3}H]palmitate from labeled μ opioid receptor by 75% and 85%, respectively (Fig. 5A). SDS-PAGE and fluorography showed that labeling of the μ receptor was greatly reduced by hydroxylamine or DTT (Fig. 5B). Since the chemicals and the conditions used specifically cleaved thioester bonds, these results demonstrated that [^{3}H]palmitic acid was incorporated into one or multiple cysteine residues.

There are two cysteines, C346 and C351, present in the proximal portion of the C-terminal domain in the rat μ opioid receptor. One or two highly conserved cysteines in this region have been shown to be palmitoylated in a few GPCRs [13–15,17–20,26]. We replaced the two cysteines with alanines, individually and in combination, and expressed the mutant receptors stably in CHO cells. The expression levels of C346A, C351A and C345A/C351A mutants were determined by [^{3}H]diprenorphine binding and the clones with highest expression levels contained 0.2, 4.5 and 1.5 pmol/mg protein, respectively. K_d values of [^{3}H]diprenorphine binding to the mutants were not significantly different from that of the wildtype. The low expression levels of C346A made it impossible to examine its palmitoylation. Palmitoylation of C351A and C346A/C351A mutant receptors was investigated and compared to that of the wildtype. For the C351A mutant, μ66 cells were used. For the C346A/C351A mutant, another CHO-μR cell line (μ1), which expresses ~2.5 pmol/mg protein of the wildtype receptor, was used as the control to avoid complication in data interpretation due to the vast difference in

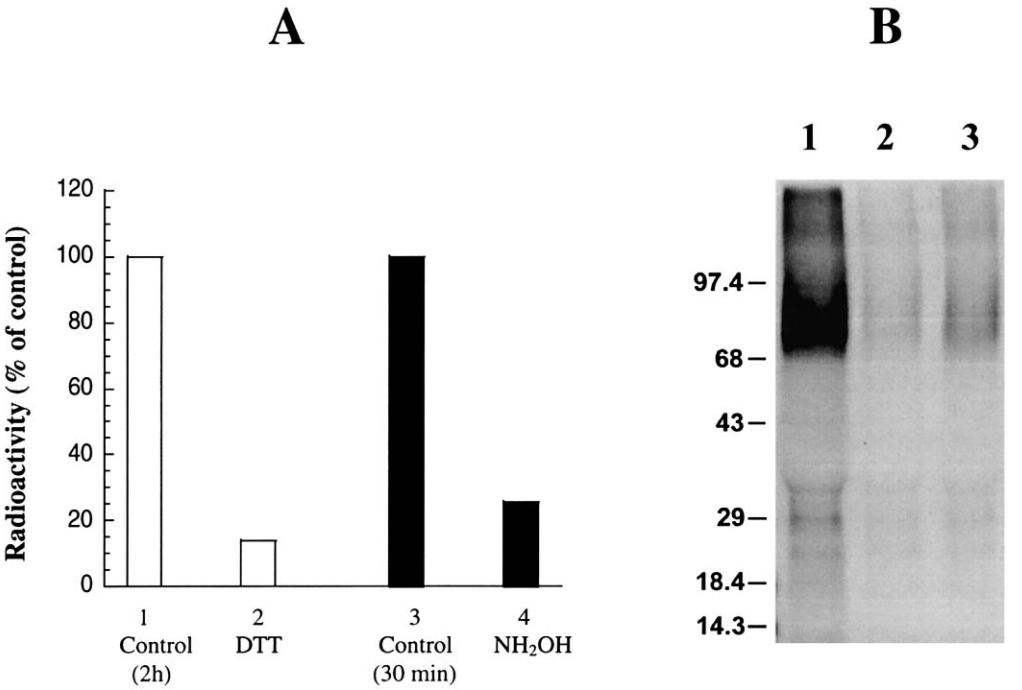

Fig. 5. Depalmitoylation of [^{3}H]palmitic acid-labeled μ receptors with DTT or hydroxylamine. A: Immunoaffinity-purified [^{3}H]palmitic acid-labeled μ receptors were incubated with or without DTT for 2 h or hydroxylamine for 30 min, extracted with ethyl acetate and the aqueous phase counted for radioactivity as described in Section 2. Data are expressed as % of the control. This experiment was repeated three times with similar results. B: SDS-PAGE (7%) and fluorography. Approximately 10 pmol each of immunoaffinity-purified labeled receptors were treated under the same conditions as in A and loaded onto the gel. Lane 1: control. Lane 2: 0.1 M DTT. Lane 3: 1 M hydroxylamine. This figure represents one of the two experiments performed with similar results. Exposure time was 7 days.

expression level. To our surprise, both C351A and C346A/C351A mutant receptors were palmitoylated to similar extents as the wildtype receptor (Fig. 6). We confirmed the presence of desired mutations and the absence of unwanted mutations in C351A and C346A/C351A mutants stably transfected into CHO cells by performing reverse transcription-polymerase chain reaction on poly(A)$^+$ RNA and DNA sequence determination of the product. Our results indicate that neither of the only two Cys residues in the C-terminal domain is the palmitoylation site. Thus, palmitoylation may occur at cysteine residues outside of the C-terminal domain. This finding is at odds with the observations that palmitoylation sites of

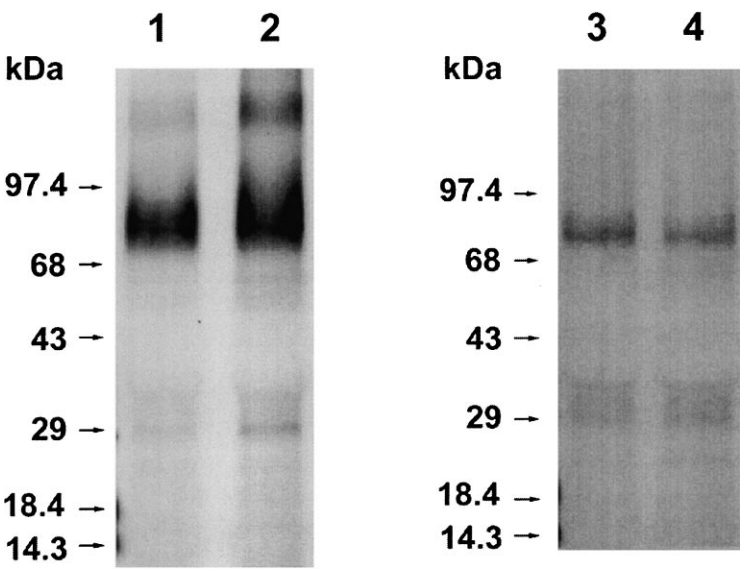

Fig. 6. Effects of (A) C351A and (B) C346A/C351A mutations on [^{3}H]palmitic acid incorporation into the μ receptor. CHO-μR cells, CHO-C351A cells and CHO-C346A/C351A cells were labeled with [^{3}H]palmitic acid, solubilized and labeled receptors purified with immunoaffinity chromatography. Eluates were subjected to SDS-PAGE and fluorography. A: Lane 1: [^{3}H]palmitic acid-labeled CHO-μR cells (μ66 cells) (~6 pmol receptors, 54 000 dpm); lane 2: [^{3}H]palmitic acid-labeled CHO-C351A cells (~7 pmol receptors, 62 000 dpm). B: Lane 3: [^{3}H]palmitic acid-labeled CHO-μR cells (μ1 cells) (~2 pmol receptors, 18 000 dpm); lane 4: [^{3}H]palmitic acid-labeled CHO-C346A/C351A cells (~2.5 pmol receptors, 23 000 dpm). This figure represents one of two (A) or three (B) experiments with similar results. Exposure time was 7 days (A) or 20 days (B).

rhodopsin [13,14] and β_2-adrenergic [15], α_{2A}-adrenergic [17], luteinizing hormone/human choriogonadotropin [18,19], endothelin A [20] and thyrotropin [26] receptors are cysteine residues in the proximal portion of the C-terminal domain. However, our results are in accord with recent findings on serotonin$_{1B}$ and serotonin$_{1D}$ receptors by O'Dowd et al. [42]. The serotonin$_{1D}$ receptor was shown to be palmitoylated despite of its lack of cysteine residues in the C-terminal domain. The serotonin$_{1B}$ receptor was palmitoylated; however, a mutant in which the only cysteine residue in the C-terminal domain was substituted with alanine was still palmitoylated.

Cys-170 in the second intracellular loop is the only other cysteine residue in the intracellular domains of the rat μ receptor. C170A and C170A/C346A/C351A mutants were generated. However, both mutants exhibited very low level of [^{3}H]diprenorphine binding and immunohistochemical staining of the μ opioid receptor (not shown). Therefore, we did not examine palmitoylation of these mutants.

In our experience, an expression level of approximately 1.5 pmol receptor/mg protein is the detection limit for [^{3}H]palmitic acid incorporation. This limit is imposed by the specific activity of [^{3}H]palmitic acid, which is lowered further by the endogenous palmitate. Many previous palmitoylation studies on GPCRs were conducted on mammalian cells with high receptor expression levels [14,15,18,20,25,43]. In more recent years, the baculovirus-Sf9 cell expression system, which usually gives a high level of expression, was used in some palmitoylation studies [21–23,41]. We opted to use CHO cells instead of the baculovirus-Sf9 cell expression system for two reasons. First, mammalian cells are more physiologically relevant. Second, the human or mouse μ opioid receptor has been expressed using the baculovirus-Sf9 cell or 'High Five' cell system and the expression level is not higher than what we observed in CHO cells [44,45].

In conclusion, the rat μ opioid receptor expressed in CHO cells undergoes palmitoylation and morphine treatment did not affect the level of palmitoylation. [^{3}H]Palmitic acid is incorporated via thioester bond(s) into Cys residue(s); however, it appears that neither Cys-346 nor Cys-351, the only two Cys residues in the C-terminal domain, is the site of palmitic acid incorporation.

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