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The 5-Hydroxytryptamine(4a) Receptor Is Palmitoylated at Two Different Sites, and Acylation Is Critically Involved in Regulation of Receptor Constitutive Activity*

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We have reported recently that the mouse 5-hydroxytryptamine(4a) (5-$HT_{4(a)}$) receptor undergoes dynamic palmitoylation (Ponimaskin, E. G., Schmidt, M. F., Heine, M., Bickmeyer, U., and Richter, D. W. (2001) *Biochem. J.* 353, 627–663). In the present study, conserved cysteine residues 328/329 in the carboxyl terminus of the 5-$HT_{4(a)}$ receptor were identified as potential acylation sites. In contrast to other palmitoylated G-protein-coupled receptors, the additional cysteine residue 386 positioned close to the COOH-terminal end of the receptor was also found to be palmitoylated. Using pulse and pulse-chase labeling techniques, we demonstrated that palmitoylation of individual cysteines is a reversible process and that agonist stimulation of the 5-$HT_{4(a)}$ receptor independently increases the rate of palmitate turnover for both acylation sites. Analysis of acylation-deficient mutants revealed that non-palmitoylated 5-$HT_{4(a)}$ receptors were indistinguishable from the wild type in their ability to interact with G_s, to stimulate the adenylyl cyclase activity and to activate cyclic nucleotide-sensitive cation channels after agonist stimulation. The most distinctive finding of the present study was the ability of palmitoylation to modulate the agonist-independent constitutive 5-$HT_{4(a)}$ receptor activity. We demonstrated that mutation of the proximal palmitoylation site ($Cys^{328} \rightarrow Ser/Cys^{329} \rightarrow Ser$) significantly increases the capacity of receptors to convert from the inactive (R) to the active (R*) form in the absence of agonist. In contrast, the rate of isomerization from R to R* for the $Cys^{386} \rightarrow Ser$ as well as for the triple, non-palmitoylated mutant ($Cys^{328} \rightarrow Ser/Cys^{329} \rightarrow Ser/Cys^{386} \rightarrow Ser$) was similar to that obtained for the wild type.

Covalent binding of long chain saturated fatty acids occurs within a wide variety of cellular as well as viral polypeptides (1–3). Two of the most common modifications involve acylation with myristate (*N*-myristoylation) and palmitate (*S*-acylation). Myristic acid is usually attached co-translationally to the NH_2-terminal glycine residue in an amide linkage by *N*-myristoyltransferase (4, 5). Palmitic acid is attached to cysteine residues via a labile thioester bond (6). In contrast to myristate, which generally remains attached to the polypeptides until protein degradation, palmitic acid is added post-translationally and turns over rapidly as a protein itself (7, 8).

Among the cellular palmitoylated proteins, polypeptides involved in signal transduction (*e.g.* receptors, G-protein α-subunits, and adenylyl cyclases) are prevalent. With the finding that palmitoylation states of several proteins may be dynamically regulated, it is now widely accepted that repeated cycles of palmitoylation and depalmitoylation could have important functional consequences for signaling (9–11). In G-protein-coupled receptors (GPCRs),[1] the functions of palmitoylation cover the wide spectrum of their biological activities: from coupling to G-proteins and regulated endocytosis to receptor phosphorylation and desensitization (9, 10, 12). For example, the palmitoylation state of the β_2-adrenergic receptor (β_2AR) regulates the synergistic action of cAMP-dependent protein kinase and β-adrenergic receptor kinase involved in receptor phosphorylation and desensitization in response to ligand binding (13, 14). The long term agonist stimulation of the β_2-adrenergic receptor, which promotes receptor phosphorylation, also increases receptor depalmitoylation, resulting in decreased signaling through the receptor (15). A mutation that prevents palmitoylation of the α_{A2}-adrenergic receptor has been found to strongly inhibit receptor down-regulation (16). For the bovine rhodopsin, abolition of palmitoylation by mutagenesis impairs its all-*trans*-retinal stimulatory activity, demonstrating the importance of palmitoylation for dark adaptation (17). These findings show that receptor acylation plays differing functional roles at different receptor-G-protein interfaces, suggesting that there is no common function applicable to all GPCRs. Therefore, an analysis of the functions of palmitoylation is necessary for each individual receptor to understand its signaling mechanism.

Five-hydroxytryptamine (5-HT or serotonin) mediates physiological functions in both the central nervous system and the periphery of vertebrates through a large number of receptors. With the exception of the 5-HT_3 receptor, which is a cation channel, all other 5-HT receptors belong to the GPCR family. Among the 13 known genes coding for 5-HT GPCRs, only 3 (5-HT_4, 5-HT_6, and 5-HT_7) are positively coupled to G-proteins (G_s) that stimulate adenylate cyclases (18, 19). The 5-HT_4 receptor is expressed in a wide variety of tissues, including

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[1] The abbreviations used in this paper: GPCR, G-protein-coupled receptor; 5-$HT_{4(a)}$, mouse 5-hydroxytryptamine(4a); BIMU8, (endo-*N*8-methyl-8-azabicyclo[3.2.1]oct-3-yl)-2,3-dehydro-2-oxo-3-(prop-2-yl)1H-benzimidazole-1-carboxamide; GTPγS, guanosine 5′-*O*-(3-thiotriphosphate); dFBS, dialyzed fetal bovine serum; DMEM, Dulbecco's modified Eagle's medium; FCS, fetal calf serum; PBS, phosphate-buffered saline; HvCNG, *Heliothis virescens* voltage-activated cyclic nucleotide-gated cation channel; AR, adrenergic receptor; ET, endothelin.

brain, colon, urinary bladder, gastrointestinal tract, and heart (20, 21). In the mammalian brain, the 5-HT$_4$ receptor contributes to the control of acetylcholine and dopamine secretion, facilitates cognitive performance and is also implicated in anxiety (22–24). Furthermore, the 5-HT$_4$ receptor is thought to be involved in various central and peripheral disorders, including neurodegenerative diseases and atrial fibrillation (25, 26). The wide distribution of 5-HT$_4$ receptors is paralleled by the existence of many 5-HT$_4$ splicing variants. Functional expression has been reported for five COOH-terminal and one internal splice variants in humans (27). In mouse, four 5-HT$_4$ receptor isoforms, 5-HT$_{4(a)}$, 5-HT$_{4(b)}$, 5-HT$_{4(e)}$, and 5-HT$_{4(f)}$, have been cloned (28). All these variants, except the internal splice product in humans, share the same sequence up to Leu^{358} followed by an unique COOH terminus. Because the COOH-terminal tail seems to be involved in the fine tuning of the coupling of GPCRs to G-proteins, it has been proposed that a diversity of function can be attributed to the different splicing variants of 5-HT$_4$ receptor (29, 30).

Recently we have shown that the 5-HT$_{4(a)}$ receptor undergoes palmitoylation. Our data also demonstrated that palmitoylation of the 5-HT$_{4(a)}$ is a reversible process and that agonist stimulation of the receptor increases the turnover rate for receptor-bound palmitate (31). In the present study we explored the functional role of palmitoylation/depalmitoylation in serotonin-mediated signaling. By site-directed mutagenesis, we identified Cys^{328}/Cys^{329} as potential acylation sites in the 5-HT$_{4(a)}$ receptor. In contrast to most other palmitoylated GPCRs, an additional cysteine residue Cys^{386} located in the very distal portion of the COOH-terminal domain was also identified as a palmitoylation site. Whereas the coupling with the G$_s$ protein, agonist-promoted cAMP production as well as intracellular distribution of non-palmitoylated receptor mutants were unaffected, our experimental data provide clear evidence for a functional role of palmitoylation in the modulation of constitutive activity on the 5-HT$_{4(a)}$ receptor. We demonstrate that mutation of the proximal palmitoylation site (Cys^{328} → Ser/Cys^{329} → Ser) significantly increases the capacity of the receptor to convert from the inactive (R) to the active (R*) form in the absence of a agonists. In contrast, the rate of isomerization from R to R* for the Cys^{386} → Ser as well as for the triple, non-palmitoylated mutant (Cys^{328} → Ser/Cys^{329} → Ser/Cys^{386} →Ser) was similar to that obtained for the wild type.

EXPERIMENTAL PROCEDURES

Materials—[9,10-^{3}H]Palmitic acid (30–60 Ci/mmol) was purchased from Hartmann Analytic GmbH (Braunschweig, Germany), Tran^{35}S-label (>1000 Ci/mmol) from ICN (Eschwege, Germany), [^{35}S]GTPγS (1300 Ci/mmol) from PerkinElmer Life Sciences (Köln, Germany). Enzymes used in molecular cloning were obtained from New England Biolabs (Frankfurt am Main, Germany). 5-Hydroxytryptamine (5-HT) and protein A-Sepharose CL-4B beads were from Sigma (Deisenhofen, Germany), GR113808A was a gift from GlaxoWellcome (Stevenage, United Kingdom). BIMU8 was kindly provided by Boehringer (Ingelheim, Germany). SB 207266 ((*N*-1-butyl-4-piperinylmethyl)-3,4-dihydro-2H-[1,3]-oxazino[3,2-*a*]indole-10-carboxamide, hydrochloride) was obtained from Laboratoires Fournier-Debat (Daix, France), and ML 10375 (2-(cis-3,5-dimethylpiperidino)ethyl 4-amino-5-chloro-2-methoxybenzoate) was obtained from M. Langlois (CNRS-BIOCIS, Châtenay-Malabry, France). TC-100 insect cell medium, Dulbecco's modified Eagle's medium (DMEM), trypsin versene, fetal calf serum (FCS), 2× YT medium, Cellfectin®, and Lipofectin® reagents were purchased from Invitrogen (Karlsruhe, Germany). TC-100 medium without L-methionine and L-glutamine was from PAN Biotech GmbH (Aidenbach, Germany). Cell culture dishes were purchased from Nunc (Wiesbaden, Germany). Oligonucleotide primers were synthesized by Invitrogen. AmpliTaq® DNA polymerase was from PerkinElmer Life Sciences. The polyclonal antiserum AS9459, raised against the COOH-terminal peptide of m5-HT$_{4(a)}$ receptor, has been described previously (31).

Recombinant DNA Procedures—All basic DNA procedures were performed as described by Sambrook *et al.* (32). The construction of recombinant baculovirus coding for the m5-HT$_{4(a)}$ receptor has been described previously (31). For the expression in COS-7 cells, the m5-HT$_{4(a)}$ cDNA was cleaved from pFastBac plasmid (Invitrogen) with *Xba*I and *Hin*dIII endonucleases to yield the 1.1-kb fragment containing the entire coding sequence. The fragment was treated with T4 DNA polymerase to create the blunt ends and then ligated to the *Pme*I site in the multiple cloning site of the pTracer-CMV2 donor plasmid (Invitrogen).

The 5-HT$_{4(a)}$ mutants with the substitution of serine for cysteines 328/329; 346; 386; 328/329 and 346; 328/329 and 386; and 346 and 386 were performed in pFastBac/5-HT$_{4(a)}$ plasmid using an oligonucleotide containing the mutation(s) corresponding to the above substitutions by standard PCR protocols, using the overlap extension technique. The recombinant baculoviruses encoding for 5-HT$_{4(a)}$ mutants were constructed, purified, and amplified as described previously (33). All mutants were verified by double-stranded dideoxy DNA sequencing at the level of the final plasmid.

Metabolic Labeling and Immunoprecipitation of Sf9 Insect Cells—*Spodoptera frugiperda* (Sf9) cells were grown in TC-100 medium supplemented with 10% FCS and 1% penicillin/streptomycin (complete TC-100). For expression, Sf9 cells (1.5×10^6) grown in 35-mm dishes were infected with recombinant baculovirus encoding for wild type or mutated 5-HT$_{4(a)}$ receptors at a multiplicity of infection (m.o.i.) of 10 plaque-forming units/cell. After 48 h, cells were labeled with Tran^{35}S-label (50 μCi/ml) in TC-100 medium without methionine or with [^{3}H]palmitic acid (300 μCi/ml) in TC-100 medium for the time periods indicated in the figure legends. For the pulse-chase experiments, cells were subsequently incubated in complete TC-100 medium supplemented with 100 μM unlabeled palmitate and 50 μM sodium pyruvate. In some experiments, BIMU8 was added to the final concentrations as indicated in figure legends. After labeling (or chase), cells were washed once with ice-cold PBS (140 mM NaCl, 3 mM KCl, 2 mM KH_2PO_4, 6 mM Na_2HPO_4, pH 7.4) and lysed in 600 μl of NTEP buffer (0.5% Nonidet P-40, 150 mM NaCl, 50 mM Tris-HCl, pH 7.9, 5 mM EDTA, 10 mM iodoacetamide, 1 mM phenylmethylsulfonyl fluoride). Insoluble material was pelleted (5 min, 20,000 × *g*), and antibodies AS9459 were added to the supernatant at a dilution of 1:60. After overnight agitation at 4 °C, 30 μl of protein A-Sepharose CL-4B was added, and samples were incubated under gentle rocking for 2 h. After brief centrifugation, the pellet was washed twice with ice-cold NTEP buffer, and the immunocomplexes were released from the beads by incubation for 30 min at 37 °C in nonreducing electrophoresis sample buffer (62,5 mM Tris-HCl, pH 6.8, containing 20% glycerol, 6% SDS, 0,002% bromphenol blue). Radiolabeled polypeptides were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) on 12% acrylamide gels and visualized by fluorography using Kodak X-Omat AR films. Quantification of fluorograms was carried out by means of a SnapScan1236 scanner and Gel-Pro Analyzer version 3.1 software. The amount of [^{3}H]palmitate incorporated was calculated relative to the expression of the various proteins as indicated by its [^{35}S]methionine/cysteine labeling.

Assay for [^{35}S]GTPγS Binding in Membranes of Sf9 Cells—Agonist-promoted binding of [^{35}S]GTPγS to different G-proteins induced by stimulation of 5-HT$_{4(a)}$ receptors was performed according to the method described previously (34). Briefly, membranes from Sf9 cells expressing the 5-HT$_{4(a)}$ receptor wild type or acylation-deficient mutants and G-protein α subunits (G_{i1}, G_{i2}, G_{i3}, G_q, G_s, and G_{12}) together with $\beta_1\gamma_2$ subunits were resuspended in 55 μl of 50 mM Tris-HCl, pH 7.4, containing 2 mM EDTA, 100 mM NaCl, 3 mM $MgCl_2$, and 1 μM GDP. After adding [^{35}S]GTPγS (1300 Ci/mmol) to a final concentration of 30 nM, samples were incubated for 5 min at 30 °C in the absence or presence of BIMU8. The reaction was terminated by adding 600 μl of 50 mM Tris-HCl, pH 7.5, containing 20 mM $MgCl_2$, 150 mM NaCl, 0,5% Nonidet P-40, 200 μg/ml aprotinin, 100 μM GDP, and 100 μM GTP for 30 min on the ice. The samples were incubated for 20 min with 150 μl of a 10% suspension of Pansorbin cells (Calbiochem) preincubated with nonimmune serum to remove nonspecific bound proteins. Samples were agitated for 1 h at 4 °C with 5–10 μl of appropriate Gα subunits-directed antiserum preincubated with 100 μl of 10% suspension of protein A-Sepharose. Immunoprecipitates were washed three times and boiled in 0.5 ml of 0.5% SDS; 4 ml of Ecolite+ was then added and radioactivity measured by scintillation spectrometry.

Electrophysiology and Data Fitting—Electrophysiological measurements were performed using the method described previously by Heine *et al.* (35). Currents were measured in the whole cell patch-clamp mode. Borosilicate glass pipettes were fire polished and had a final series

resistance of 4–8 megohms. For measurements we used the discontinuous single-electrode voltage clamp amplifier SEC-05L from NPI Electronic (Tamm, Germany), which was connected to a computer through an ITC-16 interface from Instrutech Corp. (Great Neck, NY). Data were acquired and stored by Pulse-PulseFit 8.31 software from HEKA (Lambrecht, Germany) and data analysis was performed with Igor-WaveMetrics software. The temperature of the superfusing solution was stabilized at 25 ± 0.2 °C controlled with a Peltier control unit from ESF Electronic (Göttingen, Germany). Recordings were performed in TC-100 medium without FCS. In this medium it was possible to hold cells for 4–7 h. The standard pipette solution contained 110 mM potassium gluconate, 1 mM $CaCl_2$, 2 mM $MgCl_2$, 2 mM Na_2ATP, 0.4 mM GTP, 10 mM HEPES, 10 mM EGTA, pH 7.25. The high Ca^{2+}-buffered pipette solution (15.31 nM free Ca^{2+} as calculated by Patcher's Power Tools software (Francisco Mendez, Göttingen, Germany)) allowed us to exclude side effects of Ca^{2+} ions on channel activation kinetics, as described by Lüthi and McCormick (36). The osmolarity of the pipette solution was 20 mosmol below the osmolarity of the TC-100 medium. The 5-HT$_{4(a)}$ receptor was stimulated by 0.1–10 μM serotonin (5-HT) by pressure application (PDES 2L-unit from NPI Electronic) from pipettes with a diameter of 3–5 μm.

The activation time constants (τ) were estimated by an exponential fitting. Statistical values were given as means ± S.E.

Transfection of COS-7 Cells—The cDNA encoding for the wild type or mutated 5-HT$_{4(a)}$ receptors in pTracer-CMV2 plasmid was introduced into COS-7 cells by electroporation as described previously (37). Briefly, cells were trypsinized, centrifuged, and resuspended in EP buffer (50 mM K_2HPO_4, 20 mM CH_3CO_2K, 20 mM KOH, 26.7 mM $MgSO_4$, pH 7.4) containing 25–2000 ng of receptor cDNA. The total amount of DNA was kept constant at 15 μg/transfection using pTracer vector. After 15 min of exposure at room temperature, 300 μl of cell suspension (10^7 cells) were transferred to a 0.4-ml electroporation cuvette (Bio-Rad) and pulsed using a Gene-Pulser apparatus (setting 1000 microfarads, 280 V). Afterward, cells were diluted in DMEM (10^6 cells/ml) containing 10% dialyzed fetal bovine serum (dFBS) and plated on 15-cm dishes or into 12-well clusters at the desired density.

Determination of cAMP Production in Intact COS-7 Cells—Six h after transfection, the complete medium was exchanged by DMEM without dFBS containing 2 μCi/ml [^{3}H]adenine to label the ATP pool. After overnight incubation, cAMP accumulation was measured as described previously (38).

Assay for [^{3}H]GR 113808 Binding in Membrane of COS-7 Cells—Membranes were prepared from transfected cells plated on 15-cm dishes and grown for 24 h in DMEM with 10% dFBS as described previously (39). The cells were washed twice with PBS, scraped with a rubber policeman, harvested in PBS, and centrifuged at 4 °C (200 × *g* for 4 min). The pellet was resuspended in buffer containing 10 mM HEPES, pH 7.4, 5 mM EGTA, 1 mM EDTA, and 0.32 M sucrose and homogenized 10 times with a glass-Teflon potter at 4 °C. The homogenate was centrifuged at 20,000 × *g* for 20 min, the membrane pellet was resuspended in 50 mM Hepes, pH 7.4 (5 mg of protein in 1 ml of solution), and stored at −80 °C until use.

To perform radioligand binding studies with [^{3}H]GR 113808 (specific activity: 83 Ci/mmol), 100 μl of membrane suspension prepared as described above was diluted with 50 mM HEPES, pH 7.4 (2–20 μg of protein), containing 10 mM pargyline and 0.01% ascorbic acid. Samples were incubated at 20 °C for 30 min with 100 μl of [^{3}H]GR 113808 and 50 μl of buffer or competing drugs. For saturation analysis assays, various concentrations of [^{3}H]GR 113808 (0.001–1 nM) were used. For competition binding experiments, the [^{3}H]GR 113808 concentrations were kept at 0.15 nM. The receptor densities were estimated using the specific radioligand [^{3}H]GR 113808 at a saturating concentration (0.4–0.6 nM, K_d = 0.12 nM) as described previously (38). 5-HT (0.5 μM) was used to determine nonspecific binding. Protein concentration was determined using the Bio-Rad protein assay.

Immunohistochemistry—COS-7 cells were maintained as a monolayer culture in DMEM supplemented with 10% FCS. For the expression of the 5-HT$_{4(a)}$ receptor, the cells grown on coverslips were washed once with OptiMEM without FCS and then transfected with 1 μg of plasmid DNAs using LipofectAMINE 2000. At 48 h after transfection, cells were fixed with paraformaldehyde (3% in PBS) for 15 min and washed three times with PBS, and unreacted paraformaldehyde was quenched with 50 mM glycine for 15 min. Cells were permeabilized with Triton X-100 (0.1% in PBS) and then incubated for 1 h with the first antibody AS9459 diluted 1:200 in PBS containing 2% bovine serum albumin. The second antibody (Fluor 594, (MoBiTec, Göttingen, Germany), diluted 1:100 in PBS containing 2% bovine serum albumin) was adsorbed successively to the cells for 1 h. Unbound antibodies were washed off after every step with PBS. Coverslips were finally mounted in 90% (v/v) glycerol. Cells were monitored under a confocal microscope (LSM510, Zeiss) at a magnification of 630 with appropriated filter sets.

Data Analysis—The dose-response curves were fitted by the equation $y = ((y_{max} - y_{min})/1 + (x/EC_{50})n_H + y_{min})$, where EC_{50} is the concentration of agonist producing a response equal to 50% of the maximum, y_{max} and y_{min} correspond to the maximal and minimal values, and n_H to the Hill coefficient, by using Kaleidagraph software. Competition and saturation experiments were analyzed by nonlinear regression using LIGAND software. Saturation experiments were also analyzed according to Scatchard. Statistical significant differences were determined with the StatView Student program (Abacus Concepts, Berkeley, CA) with Student's *t* test.

RESULTS

Identification of Potential Acylation Site(s) on 5-HT$_{4(a)}$—Given that the 5-HT$_{4(a)}$ receptor contains covalently bound palmitic acid (31), we sought to identify potential acylation site(s) by construction of a series of mutant receptors where serine was substituted for cysteine residues at the receptor carboxyl terminus (Fig. 1*A*). All substitution mutants along with the wild type receptor were expressed in Sf9 insect cells by the baculovirus system and labeled with either Tran^{35}S-label or [^{3}H]palmitic acid followed by immunoprecipitation, SDS-PAGE, and fluorography. Results of the [^{35}S]Met labeling demonstrated that all mutants were expressed at the levels comparable with those of the 5-HT$_{4(a)}$ wild type control (Fig. 1*B*), which allows for a quantitative comparison of palmitoylation levels in the different products. The amount of [^{3}H]palmitate incorporated into each of these mutants was calculated by densitometry of fluorograms in relation to the expression level of the various proteins as indicated by its [^{35}S]methionine/cysteine labeling (40).

Of the four cysteine residues at the COOH-terminal cytoplasmic domain of the receptor, Cys^{328}/Cys^{329} are highly conserved among GPCRs and also correspond to the site that has been shown to be palmitoylated. However, replacement of these cysteine residues with serine did not completely abolish palmitoylation. Incorporation of [^{3}H]palmitate into Cys^{328} → Ser/Cys^{329} → Ser mutant was 43.7 ± 4.8% (n = 4). Replacement of Cys^{346} alone or in combination with Cys^{328}/Cys^{329} did not significantly affect the relative palmitoylation efficiency of the resulting mutants as compared with the wild type (100% for the wt and 104 ± 6.3% for the Cys^{346} → Ser) and Cys^{328} → Ser/Cys^{329} → Ser construct (43.7 ± 4.8% for the Cys^{328} → Ser/Cys^{329} → Ser and 38.1 ± 3.9% for the Cys^{328} → Ser/Cys^{329} → Ser/Cys^{346} → Ser), respectively (Fig. 1*A*). A single mutation of Cys^{386} located very close to the COOH-terminal end, however, resulted in a decreased palmitoylation as compared with the wild type. The relative palmitoylation value demonstrated that incorporation of [^{3}H]palmitate into the Cys^{386} → Ser mutant was 61.5 ± 5% (n = 4). When Cys^{386} was mutated together with Cys^{346}, relative palmitoylation efficiency (57.4 ± 5.6%) was similar to the Cys^{386} → Ser receptor (61.5 ± 5%). In contrast, simultaneous substitution of Cys^{328}/Cys^{329} and Cys^{386} led to a complete elimination of any detectable [^{3}H]palmitic acid incorporation (Fig. 1*B*). Absence of palmitoylation on the Cys^{328} → Ser/Cys^{329} → Ser/Cys^{386} → Ser mutant was also confirmed after prolonged (up to 6 weeks) gel exposure. Thus, we conclude that Cys^{328}/Cys^{329} and Cys^{386}, but not Cys^{346}, are the potential palmitoylation sites of the 5-HT$_{4(a)}$ receptor.

Palmitoylation of Both Acylation Sites (Cys^{328}/Cys^{329} and Cys^{386}) Is Agonist-promoted—We have demonstrated previously that palmitoylation of the 5-HT$_{4(a)}$ receptor is a dynamic process and that receptor stimulation by agonists increases the rate of palmitate turnover (31). Because palmitoylation of the 5-HT$_{4(a)}$ receptor occurs on two different sites (Cys^{328}/Cys^{329} and Cys^{386}), we next studied the time course of agonist-induced incorporation of [^{3}H]palmitic acid into individual mutants. As shown in Fig. 2*A* (control), the intensity of radiolabel incorpo-

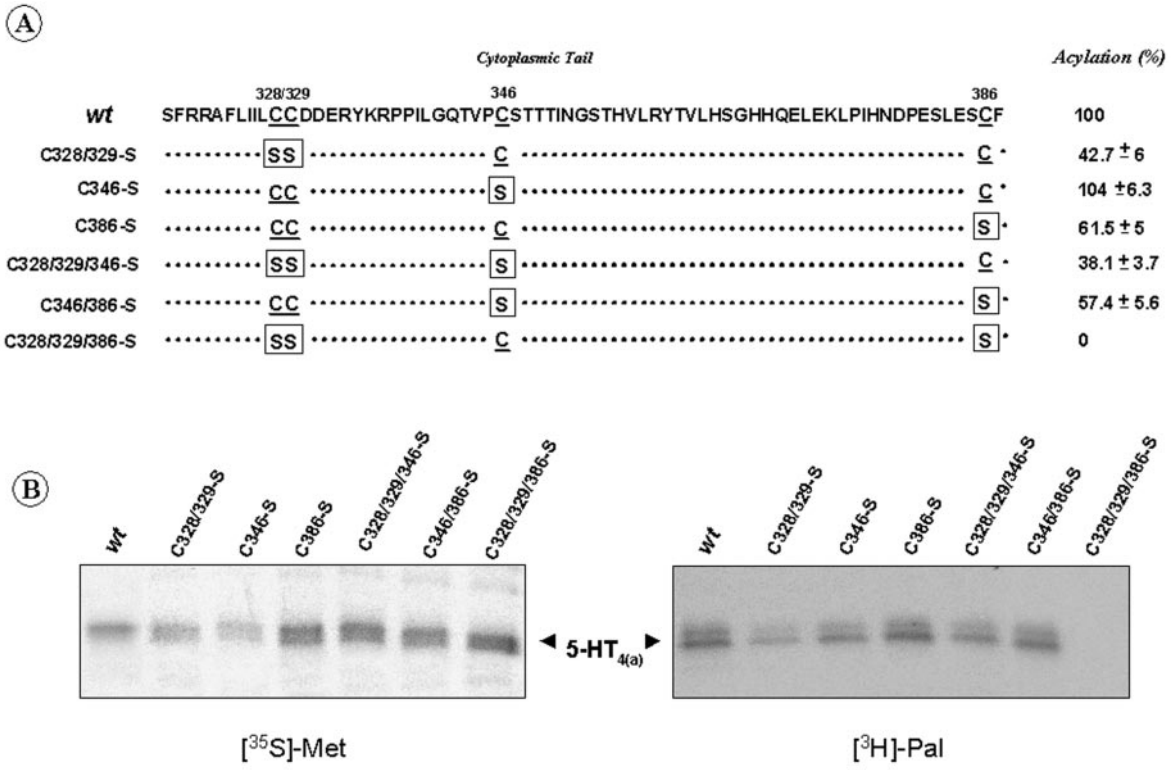

FIG. 1. **5-HT$_{4(a)}$ receptor is palmitoylated on two different sites (Cys^{328}/Cys^{329} and Cys^{386}).** *A*, schematic view of the 5-HT$_{4(a)}$ mutants. The cytoplasmic, carboxyl-terminal sequences of the 5-HT$_{4(a)}$ receptor, and six substitution mutants are given in a *single-letter code*. The amino acid numbers for four cysteine residues are indicated. *Boxed* are the serine residues substituted for the corresponding cysteine residues. *Numbers* on the *right*, mean ± S.E. acylation efficiencies determined by densitometric analysis of fluorograms in relation to the expression level of the various proteins as indicated by [^{35}S]methionine/cysteine labeling ($n = 4$). 5-HT$_{4(a)}$ wild type was used as control and the values obtained for 5-HT$_{4(a)}$ wild type were set to 100%. *B*, 5-HT$_{4(a)}$ receptor wild type and different substitution mutants were expressed in Sf9 cells, labeled either with [^{35}S]methionine/cysteine (*left panel*) or [^{3}H]palmitic acid (*right panel*) and subjected to immunoprecipitation, SDS-PAGE and fluorography. Exposure time is 1 day for labeling with [^{35}S]-methionine/cysteine and 1 week for labeling with [^{3}H]palmitate. A representative fluorogram is shown.

ration into both mutants increased steadily, reflecting basal changes in palmitoylation. Next, the kinetics of [^{3}H]palmitate incorporation were studied in the presence of BIMU8, a 5-HT$_4$ receptor-selective agonist (21). The results shown in Fig. 2*A* reveal that exposure to BIMU8 significantly increased radiolabel incorporation into each individual mutant over the whole labeling period. In both cases, BIMU8 induced an approximate 2-fold increase in labeling compared with controls (Fig. 2*B*). Labeling with [^{35}S]methionine done in parallel demonstrated that exposure to the agonist did not influence the expression level of these mutants (data not shown).

To determine whether the agonist-promoted increase in palmitoylation results from an elevation in the stoichiometry of acylation or reflects a faster exchange between labeled and unlabeled palmitate, pulse-chase labeling experiments in the presence or in the absence of BIMU8 were performed with individual mutants. As seen in Fig. 2*C*, in the absence of agonist (control), [^{3}H]palmitate was released from mutated receptors over time, representing basal depalmitoylation. BIMU8 apparently promoted the release of radiolabel from both mutants at all time points, reducing the amount of receptor-bound palmitate by ~50–55% of control. We interestingly obtained quite similar rates of depalmitoylation for the 5-HT$_{4(a)}$ receptor wild type (31). The effect of BIMU8 was receptor-specific because the increased release of [^{3}H]palmitate was effectively blocked by GR113808, high affinity 5-HT$_{4(a)}$ receptor antagonist (data not shown). Parallel labeling with [^{35}S]methionine demonstrated that the rate of turnover for mutants itself appeared to be unaffected by the treatment with agonists. Taken together, these results (Fig. 2) suggest that both acylation sites (Cys^{328}/Cys^{329} and Cys^{386}) are dynamically palmitoylated and that agonist stimulation increases the rate of palmitate turnover on each of them.

Effect of Cysteine(s) Replacement on the Receptor-G-protein Coupling as Assessed by GTPγS Binding—Co-expression of receptor and G-protein in insect cells followed by measurement of agonist-promoted binding of [^{35}S]GTPγS to the Gα subunit provides a useful experimental approach for assessing the selectivity of receptor-G-protein coupling [41]. Using this system we analyzed communication of the 5-HT$_{4(a)}$ receptor with G-proteins belonging to the different families. Fig. 3 represents a set of experiments in which Sf9 cell membranes containing G_{i2}, G_{i3}, G_q, G_s, or G_{12} (in all cases the appropriate α-subunit was co-expressed with $\beta_1\gamma_2$ subunits) and the 5-HT$_{4(a)}$ receptor were incubated with [^{35}S]GTPγS in the presence or in the absence of BIMU8. Gα subunits were subsequently immunoprecipitated with the appropriate antibody and bound [^{35}S]GTPγS was counted directly. As shown in Fig. 3*A*, no binding occurred with α_{i2}, α_{i3}, α_q, and α_{12} subunits. In contrast, co-expression of the 5-HT$_{4(a)}$ receptor with G_s resulted in the increase of [^{35}S]GTPγS binding, even in the absence of agonist. Activation of the receptor with agonist (BIMU8) elicited an ~4-fold increase in binding when compared with control, demonstrating that 5-HT$_{4(a)}$ receptor couples to G-proteins of the G_s family. Omitting the receptor from the assay demonstrated that G_s alone did not bind [^{35}S]GTPγS (data not shown).

Having demonstrated that the 5-HT$_{4(a)}$ receptor communicates with G_s, we next assessed the potential of G_s for coupling to different acylation-deficient mutants of 5-HT$_{4(a)}$. As shown in Fig. 3*B*, activation of mutated receptors with BIMU8 re-

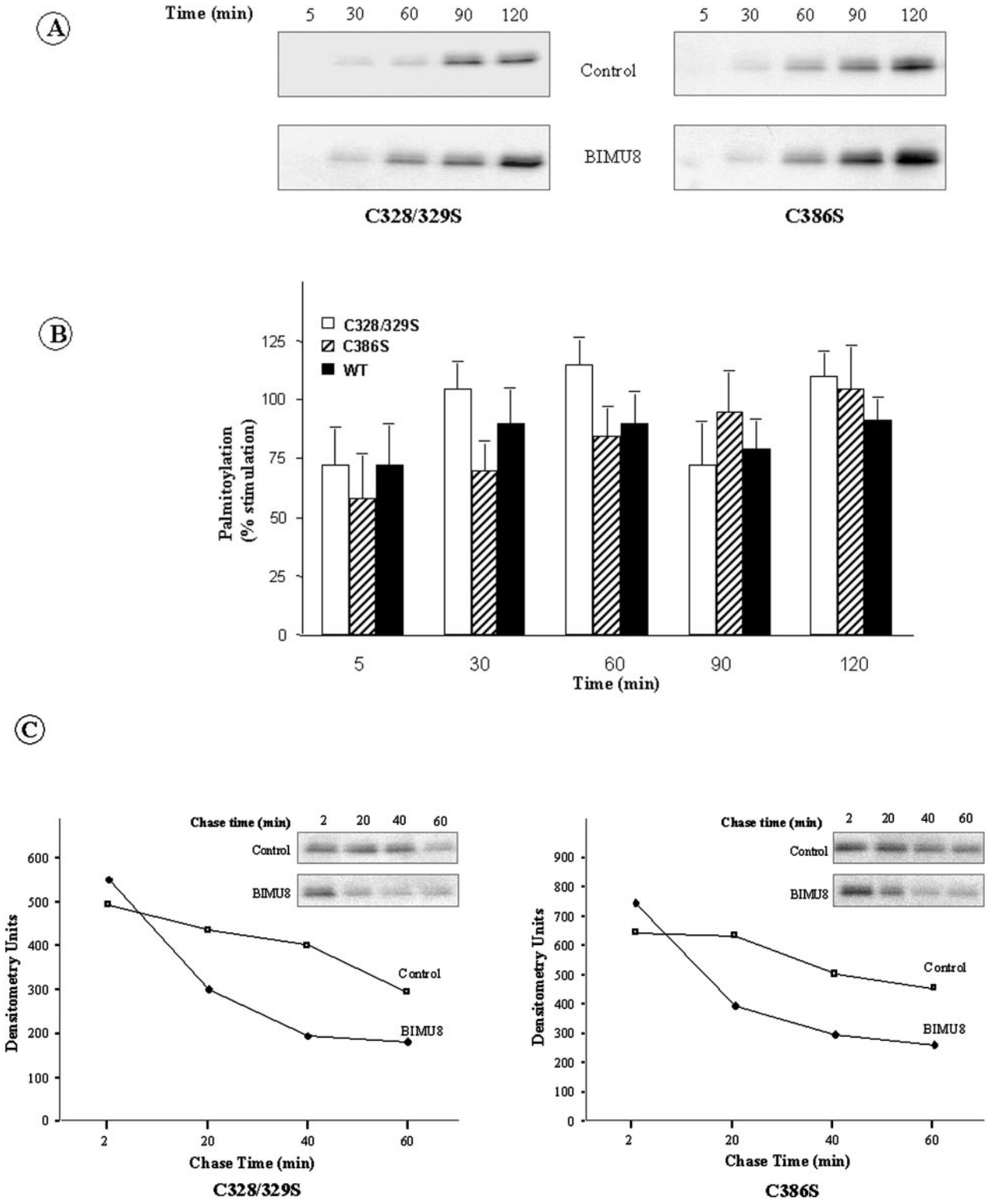

FIG. 2. **Palmitoylation of both acylation sites (*C328/329S* and *C386S*) is a dynamic process.** *A*, Sf9 cells expressing the 5-HT$_{4(a)}$ receptor mutants were incubated with [^{3}H]palmitate in the presence of either vehicle (H_2O, control) or 100 nM BIMU8 for the time periods indicated. Receptors were immunoprecipitated, resolved by SDS-PAGE, and visualized by fluorography. A representative fluorogram is shown. A percentage change of palmitoylation after BIMU8 stimulation *versus* vehicle is shown in *B* as an average ± S.E. ($n = 4$) for the wild type and individual mutants. *C*, insect cells expressing the individual mutants were labeled with [^{3}H]palmitate for 1 h and chased with medium containing nonlabeled palmitate for the time periods indicated. During the chase time, cells were treated with vehicle (H_2O, control) or BIMU8. Incorporation of the radiolabel was assessed by receptor immunoprecipitation followed by SDS-PAGE and densitometry. One representative experiment ($n = 3$) is shown.

sulted in an increase of [^{35}S]GTPγS binding, which was quite similar to that obtained for the wild type. Next, the basal constitutive receptor activity was analyzed for the single mutants. As seen in Fig. 3*B*, the level of agonist-independent binding of [^{35}S]GTPγS to G_s was significantly increased for the $Cys^{328} \rightarrow Ser/Cys^{329} \rightarrow Ser$ mutant compared with the receptor

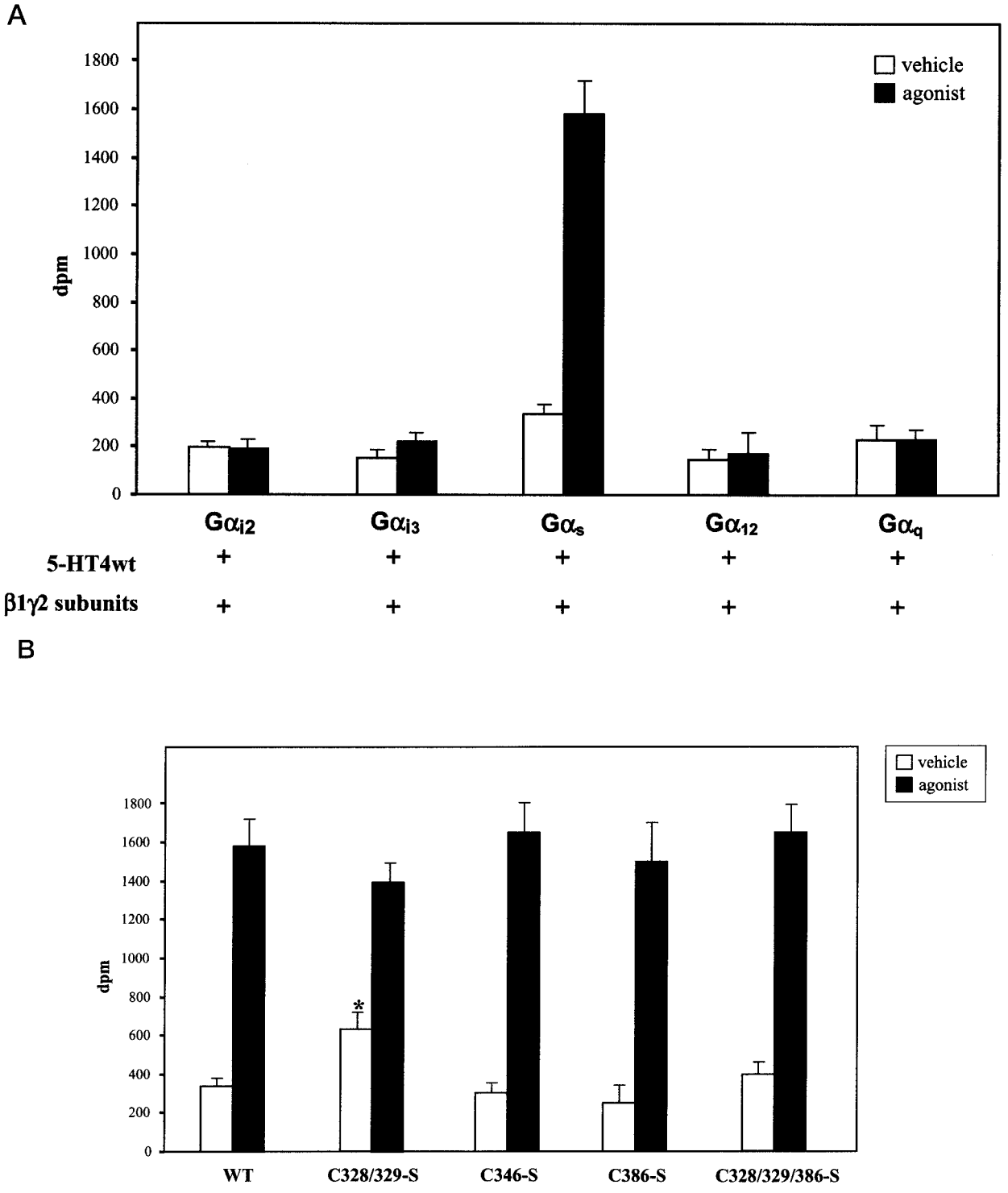

FIG. 3. **Communication of the 5-HT$_{4(a)}$ receptor with different G-proteins: effect of palmitoylation.** *A*, membranes were prepared from Sf9 cells expressing recombinant proteins as indicated and then incubated with [^{35}S]GTPγS in the presence of either vehicle (H_2O) or 100 nM BIMU8. Immunoprecipitations were performed with appropriate antibodies directed against indicated Gα subunits. In *B*, 5-HT$_{4(a)}$ receptor wild type or different mutants were expressed beside the G_s protein. Data points represent the means ± S.E. from at least four independent experiments. A statistically significant increase from values obtained without agonist for Cys^{328} → Ser/Cys^{329} → Ser mutant as compared with the wild type basal activity is noted (*, $p < 0.05$).

wild type. In contrast, agonist-independent binding of [^{35}S]GTPγS to G_s obtained for the Cys^{386} → Ser mutant was quite similar to the wild type. Surprisingly, the substitution of all palmitoylated cysteine residues with serine (Cys^{328} → Ser/Cys^{329} → Ser/Cys^{386} → Ser) reduced the high constitutive activity obtained for the Cys^{328} → Ser/Cys^{329} → Ser mutant to the wild type level. Fig. 3*B* also illustrates that the high basal activity obtained for the Cys^{328} → Ser/Cys^{329} → Ser mutant leads to a decrease in relative efficacy of agonist to stimulate over basal constitutive activity. For the 5-HT$_{4(a)}$ receptor wild type, we found a 5-fold increase in the binding of [^{35}S]GTPγS in response to the agonist, whereas, for the Cys^{328} → Ser/Cys^{329} → Ser mutant, agonist stimulation resulted only in 2.2-fold increase in [^{35}S]GTPγS binding. It is noteworthy that these

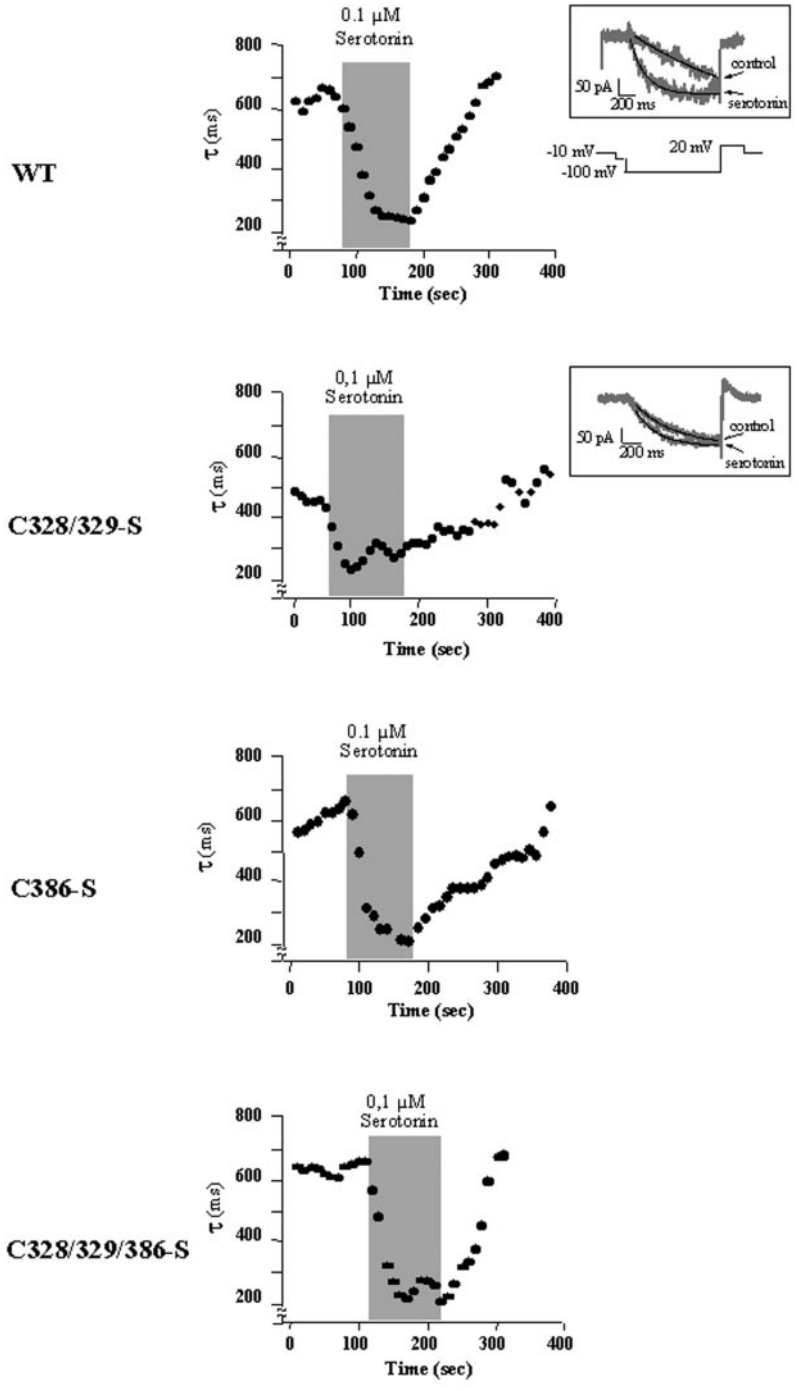

FIG. 4. **Activation of the HvCNG cation channel mediated by the 5-HT$_{4(a)}$ receptor wild type or by acylation-deficient mutants.** HvCNG currents from Sf9-cells expressing the 5-HT$_{4(a)}$ receptor (wild type or mutants) together with the G-protein subunits α_s, β_1, γ_2 and the HvCNG channel were recorded in the whole-cell patch-clamp mode. Channel's activation time constants (τ) were obtained from exponential fits of currents evoked via a voltage step to −100 mV at a temperature of +25 °C, and each point represents the activation time constant of a 1-s-long pulse. Stimulation by 100 nM serotonin (*5-HT*) induced a pronounced decrease of the activation constants (τ) in all cases. *Insets*, voltage protocol and one representative measurement of currents from at least four independent experiments for the wild type and Cys^{328} → Ser/Cys^{329} → Ser mutant in the absence (control) or in the presence of 5-HT are shown.

values did not change significantly, when binding of [^{35}S]GTPγS was analyzed as function of time after 5, 20, 40, and 60 min of incubation (data not shown). These results indicate that proximal (Cys^{328}/Cys^{329}) but not the distal (Cys^{386}) acylation site is selectively involved in modulation of agonist-independent receptor activity.

Effect of Cysteine(s) Replacement on the Receptor-G-protein Coupling as Assessed by Electrophysiological Assay—To analyze the effect of palmitoylation on the activation of downstream signal cascades, we established a new functional assay using the patch-clamp technique (35). For this purpose, the hyperpolarization- and cyclic nucleotide-sensitive cation channel from *Heliothis virescens* (HvCNG) (42) was co-expressed in Sf9 cells in addition to the 5-HT$_{4(a)}$ receptor and G$_s$. The choice of the HvCNG channel had several reasons. (i) The channel is not activated at the resting potential of the Sf9 cells, which ranges between −10 and −40 mV. (ii) The channel is highly sensitive to cAMP. (iii) Use of the HvCNG activation kinetics was independent of the channel expression level. The recorded activation currents during a hyperpolarization to −100 mV were fitted by a single exponential function, and the resulting activation time constant for the channel (τ) was used as parameter for the approximation of the intracellular cAMP concentration.

As shown in Fig. 4, stimulation of wild type and mutated 5-HT$_{4(a)}$ receptors with 0.1 μM serotonin resulted in a faster activation of the current and in a significant decrease of the channel's activation time constant τ, demonstrating that activation of receptors is able to activate HvCNG via stimulation of G$_s$ and endogenous adenylyl cyclase(s). The activation kinetics

obtained after agonist stimulation was similar for the mutants and for the wild type. The stimulatory effect was reversible and the current returned to the control values after wash-out of agonist (Fig. 4). When the receptor or G_s were excluded from the assay, channel activation by serotonin was not seen (35). Repetitive application of the agonist to the 5-HT$_{4(a)}$ wild type resulted in significantly declined responses, and even a 10-fold elevation of the serotonin concentration (1 μM) only marginally decreased τ during a second application. In contrast, repetitive agonist stimulation of acylation-deficient mutants resulted in the efficient channel activation (data not shown), suggesting only low rates of agonist-promoted receptor desensitization for the acylation-deficient mutants of the 5-HT$_{4(a)}$ receptor.

It was also of particular interest to check whether the electrophysiological assay is suitable for measuring basal constitutive activity of the receptor. To test for that, we compared the first currents recorded after the establishing whole cell configuration, but before stimulation with serotonin in the cell expressing the HvCNG channel alone or with co-expressed 5-HT$_{4(a)}$ and G_s. For the Cys^{386} → Ser mutant as well as for the acylation-deficient receptor (Cys^{328} → Ser/Cys^{329} → Ser/Cys^{386} → Ser), we obtained no significant differences in the basal, agonist-independent channel stimulation as compared with the receptor wild type (Fig. 4). In contrast, the mutant Cys^{328} → Ser/Cys^{329} → Ser considerably increased the level of agonist-independent channel stimulation (Fig. 4, *C328/329-S*). The activation constant τ calculated for the wild type was decreased from 665 ± 114 ms (control) to 272 ± 47 ms (5-HT stimulation; n = 4). However, in the case of the Cys^{328} → Ser/Cys^{329} → Ser mutant, τ value was changed from 423 ± 100 ms (control) to 243 ± 40 ms (5-HT stimulation, n = 3).

Taken together, these data demonstrate that using the sensitive HvCNG ion channel as a cAMP sensor provides a new functional method to monitor receptor-effector interactions. In addition, electrophysiological measurement of agonist-independent channel activation confirmed the involvement of a proximal acylation site (Cys^{328}/Cys^{329}) of the 5-HT$_{4(a)}$ receptor in the regulation of constitutive receptor activity.

Mutations of Palmitoylated Cysteine Residues Resulted in Different Constitutive Activities of the 5-HT$_{4(a)}$ Receptor after Expression in COS-7 Cells—The experiments with insect cells demonstrated the possible involvement of palmitoylation in regulation of basal constitutive activity (Figs. 3 and 4). Therefore, we next tested agonist-independent activation of acylation-deficient 5-HT$_{4(a)}$ mutants in the mammalian cell system. The cDNA encoding for wild type and mutant receptors was cloned in a pTracer plasmid (Invitrogen), and the ability of these proteins to modulate an intracellular level of cAMP was examined in transfected COS-7 cells as the function of receptor density. As shown in Fig. 5*A* and as reported previously (28, 29), the native 5-HT$_{4(a)}$ receptor expressed in these cells possess a high basal constitutive activity as compared with mock-transfected cells. The constitutive activity of the wild type 5-HT$_{4(a)}$ receptor was almost linearly proportional to the receptor density (Fig. 5*A*). When the basal activity of the Cys^{328} → Ser/Cys^{329} → Ser mutant was analyzed, we found more than a 2.5-fold increase in the agonist-independent cAMP production as compared with the wild type receptor. In contrast, agonist-independent cAMP production for the Cys^{386} → Ser as well as for the Cys^{328} → Ser/Cys^{329} → Ser/Cys^{386} → Ser mutants expressed at the same receptor density was quite similar to the wild type (Fig. 5*A*). The *inset* in Fig. 5*A* also illustrates that the high basal activity obtained for the Cys^{328} → Ser/Cys^{329} → Ser mutant leads to a decrease in relative efficacy of 5-HT (10^{-6} M) to stimulate over basal constitutive activity. Also notable is the fact that the 5-HT-mediated maximal response was slightly reduced in acylation-deficient mutants. Based on the two-state model (29), we also calculated the constant J, *i.e.* the equilibrium constant denoting the ratio of the receptor in the inactive *versus* active state (J = [R]/[R*]) for the wild type and mutants. We found that [R*] was 16 ± 4.2, 31.5 ± 3.7, 13.1 ± 2.8, and 17.6 ± 3.1% for the wild type, Cys^{328} → Ser/Cys^{329} → Ser, Cys^{386} → Ser, and Cys^{328} → Ser/Cys^{329} → Ser/Cys^{386} → Ser, respectively.

Although our experiments were performed with dialyzed serum, it was important to check whether some contamination by 5-HT could be responsible for the observed intrinsic activity. Therefore, we used highly selective 5-HT$_{4(a)}$ receptor agonists/antagonists. Whereas the neutral antagonist ML 10375 did not have any effect on the 5-HT-induced constitutive receptor activity, the inverse agonist the SB 207266 reduced the basal constitutive activity of all receptors (Fig. 5*B*). Both substances used in Fig. 5*B* were highly potent in inhibiting the 5-HT-induced cAMP production (data not shown). When these antagonists were used together, the neutral antagonist reversed the effect of the inverse agonist, indicating that both drugs acted on the same 5-HT$_4$ receptor site (data not shown). Thus, these experiments indicated that the observed constitutive activity was not because of 5-HT contamination.

Pharmacological Characterization of Wild type and Mutated 5-HT$_{4(a)}$ Receptors—Next we compared the pharmacological profiles of wild type and mutated 5-HT$_{4(a)}$ receptors. The extended ternary complex (43) as well as cubic ternary complex (44) introduced for the modeling of the GPCR isomerization from R to R* and for their interaction with G-proteins, indicated that the observed affinity of the agonist increased as a function of the isomerization allosteric constant J (J = [R]/[R*]). Indeed, we observed that the affinity of 5-HT for the mutated receptor (Cys^{328} → Ser/Cys^{329} → Ser) increased by almost a factor of 10 when compared with the wild type and other mutants expressed at similar densities (Fig. 6, Table I). 5-HT binding was measured by competition with the high affinity antagonist [^{3}H]GR 113808 in the membrane preparation of transfected COS-7 cells. All displacement curves were monophasic. As shown in Table I, the affinity of [^{3}H]GR 113808 for wild type 5-HT$_{4(a)}$ receptors (K_D = 0.12 nM), was similar to that obtained for the Cys^{386} → Ser (K_D = 0.13 nM) and Cys^{328} → Ser/Cys^{329} → Ser/Cys^{386} → Ser (K_D = 0.13 nM) mutants. However, it was different from that of the Cys^{328} → Ser/Cys^{329} → Ser (K_D = 0.25 nM) mutant. Competition binding analysis also demonstrated that the K_i value for 5-HT obtained with the Cys^{328} → Ser/Cys^{329} → Ser mutant was ~10-fold lower when compared with the wild type receptor and other mutants (Table I). We also found that the EC_{50} of 5-HT to stimulate cAMP was about 5 times lower in mutant Cys^{328} → Ser/Cys^{329} → Ser than in other mutants or in the wild type (Fig. 6*B*, Table I).

Intracellular Distribution of Wild type and Mutant Receptors Expressed in COS-7 Cells—To examine the intracellular localization of the wild type and mutated 5-HT$_{4(a)}$ receptors, the genes encoding for the appropriate proteins were cloned in a pTracer plasmid and expressed in COS-7 cells. Because this vector also contains the green fluorescent protein gene under control of separate promotor, the cells expressing recombinant receptors may be visualized easily by green fluorescence. To monitor expression and intracellular distribution of receptors, transfected COS-7 cells were subjected to immunofluorescence. As seen in Fig. 7, there were no apparent differences in the immunostaining between wild type and mutated receptors. This suggests that palmitoylation did not critically contribute to the intracellular distribution of the recombinant 5-HT$_{4(a)}$ receptors.

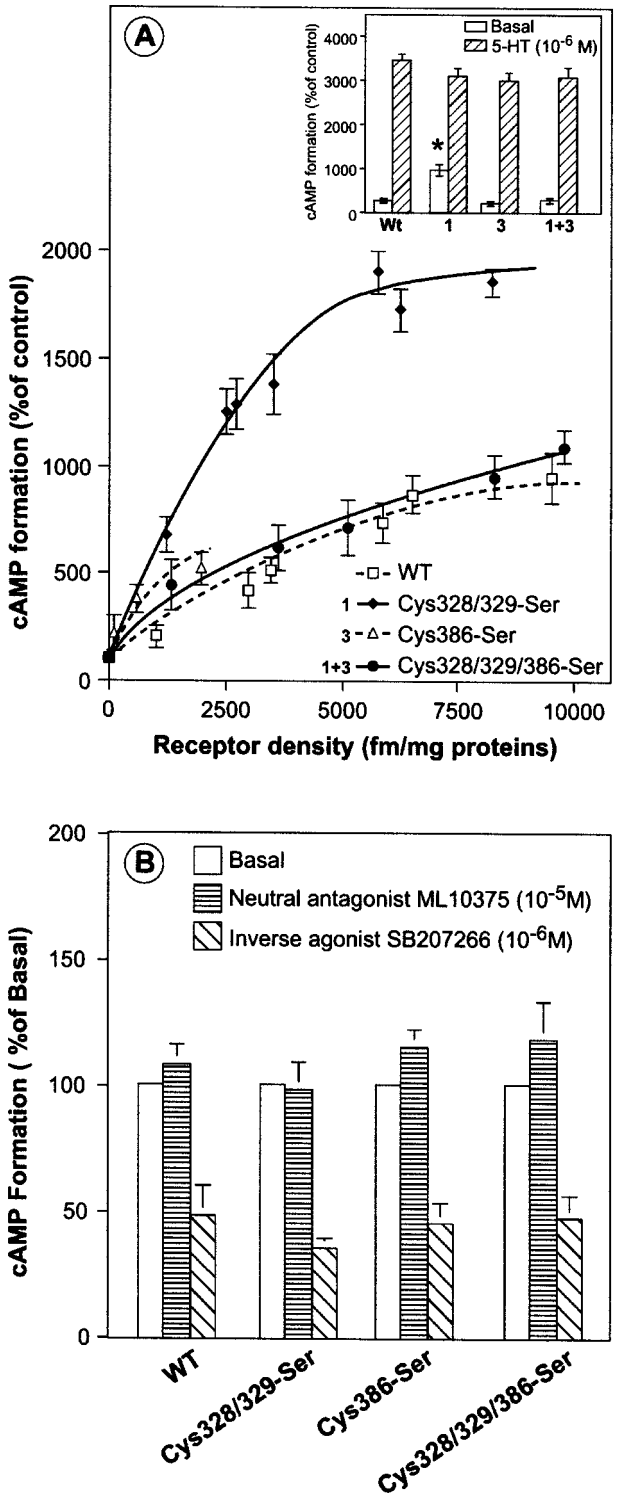

FIG. 5. **Mutations of palmitoylated cysteine residues of the 5-HT$_{4(a)}$ receptor differently affect the agonist-independent cAMP production.** *A*, COS-7 cells expressing different receptor amounts (from 200 to 10,000 fmol/mg protein) were assayed for basal cAMP production. The percentage of conversion of [^{3}H]ATP to [^{3}H]cAMP in mock-transfected COS-7 cells was 0.105 ± 0.014 (control). Levels of cAMP accumulation were measured after 15 min of incubation and shown as a percentage of the control. Each *point* represents the means ± S.E. from four independent experiments performed in triplicate. *Inset*, basal and maximal cAMP accumulation in response to 10^{-6} M 5-HT measured in COS-7 cells expressing between 1527 and 1780 fmol of receptor/mg of protein. The percentage conversion of [^{3}H]ATP to [^{3}H]cAMP in mock-transfected COS-7 cells was 0.128 ± 0.011 (control). Each value represents the means ± S.E. from four independent experiments performed in triplicate. A statistically significant increase from values obtained without agonist with Cys^{328} → Ser/Cys^{329} → Ser mutant is noted (*, $p < 0.05$). *B*, effect of two potent 5-HT$_{4(a)}$ receptor drugs: an inverse 5-HT$_4$ receptor agonist SB 207266 and a neutral antagonist ML10375 on agonist-independent cAMP production in COS-7 cells expressing either wild type or mutated 5-HT$_{4(a)}$ receptors. The receptor surface density was between 1800 and 2300 fmol/mg of protein. The percentage conversion of [^{3}H]ATP to [^{3}H]cAMP in mock-transfected cells was 0.14 ± 0.018 (control). The basal cAMP formations in wild type and mutants Cys^{328} → Ser/Cys^{329} → Ser, Cys^{386} → Ser, and Cys^{328} → Ser/Cys^{329} → Ser/Cys^{386} → Ser were 700 ± 67, 1890 ± 59, 780 ± 65, and 930 ± 80% of control, respectively. Each value is the means ± S.E. from three independent experiments performed in triplicate.

DISCUSSION

From analysis of the primary structure of acylated GPCRs, it is known that palmitoylation occurs exclusively on cysteine residues located at the COOH-terminal juxta-membrane portion of the receptors (40, 45). The 5-HT$_{4(a)}$ receptor possesses four cysteine residues within its COOH-terminal cytoplasmic domain, Cys^{328}/Cys^{329}, Cys^{346}, and Cys^{386}. In the present study we identify cysteine residues 328/329 and cysteine 386 as potential palmitoylation sites of the 5-HT$_{4(a)}$ receptor. Our finding that conserved cysteine residues 328/329 are modified with palmitic acid, whereas cysteine 346 located further away from the membrane is not acylated, is consistent with a general view of the location of GPCR's palmitoylation sites. Quite contrary, the existence of an additional palmitoylated cysteine 386 positioned 70 amino acids away from the plasma membrane face and close to the COOH-terminal end of the 5-HT$_{4(a)}$ receptor (Fig. 1) was surprising. Several palmitoylated GPCRs (*e.g.* vasopressin V2, endothelin B, luteinizing hormone/human chorionic gonadotropin, and β_2-adrenergic receptors) also possess multiple cysteine residues within their cytoplasmic COOH-terminal domains. In these receptors, however, only cysteine residues in positions up to 14 amino acids from the membrane border toward the cytoplasmic tail have been shown to serve as acylation sites (16, 46–48). No experimental evidence has been provided so far for the fatty acid transfer to the cysteine residues located more distantly. Thus, the 5-HT$_{4(a)}$ receptor represents the first case of palmitoylated receptors with the novel acylation site located close to its COOH terminus.

This finding could have additional implications in the context of the great number of 5-HT$_4$ receptor splicing variants differing only in their C termini sequences after residue Leu^{358} (27, 28). Among these different isoforms, only the 5-HT$_{4(a)}$ receptor possesses a Cys^{386} both localized close to the COOH terminus and susceptible to palmitoylation. To date, nothing has clearly been reported on the specific role or localization of the different receptor splicing variants, except that isoforms of 5-HT$_4$ receptor differ in their basal constitutive activity (28, 29). The present study, therefore, points out for the first time the structural divergence between different 5-HT$_4$ receptor isoforms, which could posses some functional importance, like differential regulation of basal and agonist-promoted receptor activities.

For a number of peripheral membrane proteins involved in signal transduction (*e.g.* p21^{ras}, α subunits of heterotrimeric G-protein, and endothelial nitric-oxide synthase), palmitoylation has been reported to be a regulated process (reviewed in Ref. 10). In addition, several GPCRs, including β_2-adrenergic and dopamine D1 receptors, undergo a regulated turnover of palmitate upon receptor stimulation (13, 15, 49). For the 5-HT$_{4(a)}$ receptor, we have also demonstrated that agonist stimulation enhanced the palmitate exchange on this polypeptide (31). Given that the 5-HT$_{4(a)}$ receptor is palmitoylated at two different sites within its COOH-terminal cytoplasmic domain (Fig. 1), it could be speculated that acylation of both sites may be modulated by the agonist. The results of the present

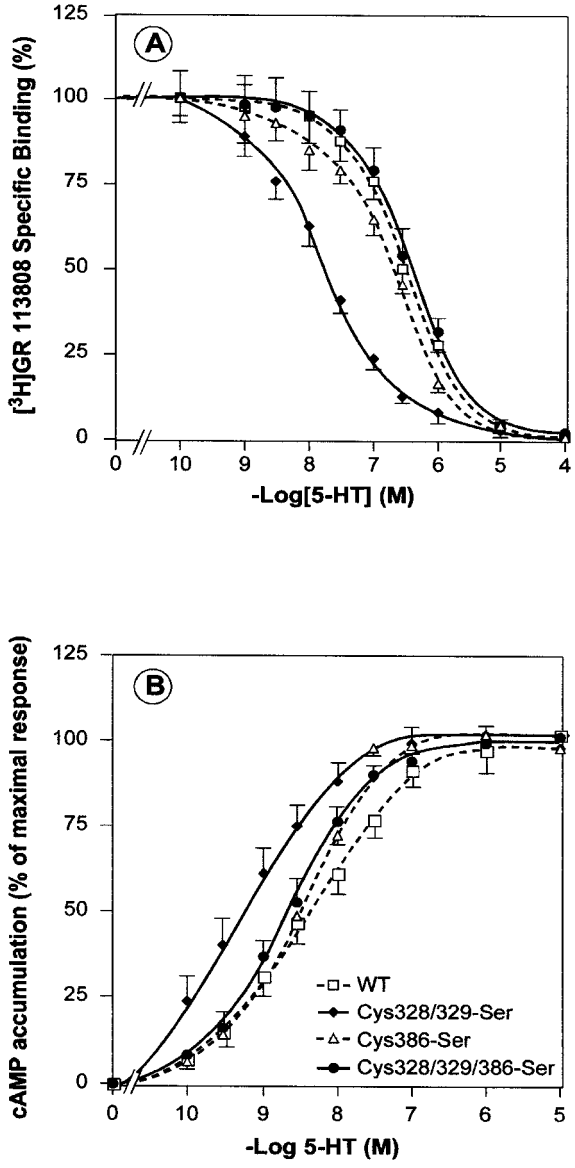

FIG. 6. **Competition binding and 5-HT-induced cAMP formation in COS-7 cells expressing 5-HT$_{4(a)}$ receptor wt or acylation-deficient mutants.** *A*, competition binding of 5-HT for [^{3}H]GR 113808 in membranes derived from COS-7 cells expressing comparable amounts of wild type and mutated receptors (receptor surface densities between 827 and 1180 fmol/mg). Data points represent the means ± S.E. from at least four independent experiments. *B*, intracellular cAMP level was measured at increasing concentrations of 5-HT in transfected COS-7 cells. The maximal increase in the ligand-dependent activity differs between receptors. The basal and 5-HT maximal adenylyl cyclase stimulation values are shown as percentage of control. Data points represent the means ± S.E. from four independent experiments performed in triplicate.

experiments support this view, demonstrating that palmitoylation of the each individual acylation site is a dynamic process (Fig. 2). Although the detailed mechanism involved in the regulation of palmitoylation/depalmitoylation cycles of individual cysteines on the 5-HT$_{4(a)}$ receptor is still unknown, our results indicate that the biological activation of the 5-HT$_{4(a)}$ receptor enhances the palmitate exchange on both acylation sites (Cys^{328}/Cys^{329} and Cys^{386}).

Functional analyses of mutant GPCRs lacking the acylation site have failed to reveal a common functional role for receptor palmitoylation. Moreover, mutagenesis of the palmitoylated cysteine residues is often associated with different, sometimes opposite effects on the functional receptor activities. Therefore, by using acylation-deficient mutants of the 5-HT$_{4(a)}$ receptor, we analyzed the possible role of dynamic palmitoylation in different receptor properties, including coupling with heterotrimeric G-proteins, ligand-binding efficiency, intracellular distribution, modulation of downstream signal cascades, as well as modulation of basal constitutive receptor activity. Evaluation of agonist-promoted binding of [^{35}S]GTPγS with G-proteins belonging to different families revealed that the recombinant 5-HT$_{4(a)}$ receptor communicates with Gα_s but not with Gα_i, Gα_q, or Gα_{12} subunits (Fig. 3). Additional assays, including agonist-promoted cAMP production (Fig. 6) and activation of cAMP-gated ion channels (Fig. 4), also confirmed that the 5-HT$_{4(a)}$ receptor operates through G$_s$. This observation is in line with the current notion that native as well as heterologously expressed 5-HT$_4$ receptors couple positively to adenylate cyclase catalyzing cAMP production (37, 50).

Analysis of acylation-deficient 5-HT$_{4(a)}$ mutants revealed that non-palmitoylated receptors are indistinguishable from the wild type in their ability to interact with G$_s$ (Fig. 3), to activate cyclic nucleotide-sensitive cation-channels (Fig. 4), and to stimulate adenylyl cyclase activity (Fig. 6) after agonist stimulation. The lack of any effect of palmitoylation on the coupling of the 5-HT$_{4(a)}$ receptor with G-proteins and on the downstream effectors, parallels recent reports on palmitoylation of the α_2-adrenergic receptor (α_2AR). The α_2AR couples to both G$_s$ as well as to G$_i$ (51), and mutation of cysteine 442, resulting in non-palmitoylated receptor, has no effect on coupling with either class of G-proteins (52). This differs from the features reported for rhodopsin, β_2AR, as well as for endothelin types A and B (ET$_A$ and ET$_B$) receptors. Recent works on rhodopsin indicate that chemical depalmitoylation enhances light-dependent GTPase activity of G$_t$ and strongly decreases the light-independent activity of opsin-*atr* (17, 53). Similarly, functional characterization of non-palmitoylated β_2AR and the ET$_B$ receptor revealed that palmitoylation is essential for agonist-stimulated coupling to G$_s$ and to both G$_q$ and G$_i$ proteins, respectively (45, 54). Analysis of the non-palmitoylated ET$_A$ receptor mutant demonstrated that ligand-induced stimulation of G$_s$ was unaffected by the lack of palmitoylation, whereas signaling through G$_q$ was abolished (55). These opposing findings suggest that palmitoylated cysteine residues may play differing roles at different receptor-G-protein interfaces. This may be because of the different subunit composition of G$_s$, G$_q$, G$_t$, and G$_{i/o}$ or, alternatively, to the differing receptor structures presented to the appropriate G-proteins.

Immunofluorescence analysis of transfected COS-7 cells (Fig. 7) suggested that palmitoylation was also not critically involved in intracellular distribution of the wild type 5-HT$_{4(a)}$ receptor. The immunofluorescence assay used, however, is not quantitative, and the intracellular traffic of the receptor molecule as well as fine tuning of receptor localization could not be assessed. Therefore, it is still an open question whether the intracellular trafficking of the 5-HT$_{4(a)}$ receptor to the "correct" site within the plasma membrane may be actively regulated by the receptor palmitoylation. In a search for the possible role of the 5-HT$_{4(a)}$ receptor palmitoylation in a defined subcellular distribution, careful analysis of intracellular transport of acylation-deficient mutants should be done.

The most distinct finding of the present study was the observation that palmitoylation modulates the agonist-independent constitutive 5-HT$_{4(a)}$ receptor activity. Spontaneous or constitutive GPCR activity has been described convincingly for 10 years in the pioneering work of Costa and Lefkowitz (56, 57). Now it is well established that GPCRs can reach their active state even in the absence of agonist, as a result of a natural shift in the equilibrium between their inactive and active con-

TABLE I

Results of binding experiments and cAMP production for the wild-type and acylation-deficient 5-HT$_{4(a)}$ receptors

Wild type (WT) and mutant receptors were transiently transfected in COS-7 cells and used for saturation (K_D values) and competition (K_i values) binding analyses. [^{3}H]GR 113808 was used as a selective antagonist. EC_{50} values refer to the agonist concentrations yielding 50% of the maximal activation. Data are expressed as means ± S.E. of at least four independent experiments. Significantly different values obtained for the Cys^{328} → Ser/Cys^{329} → Ser mutant as compared with the wt and mutant receptors Cys^{386} → Ser and Cys^{328} → Ser/Cys^{329} → Ser/Cys^{386} → Ser are noted (*, $p < 0.01$).

WT and mutated 5-HT$_{4(a)}$ receptors	5-HT$_{4(a)}$ receptor binding sites		5-HT$_{4(a)}$ receptor coupling to adenylyl cyclase
	[^{3}H]GR 113808 K_D ± S.E.	5-HT; K_i ± S.E.	Agonist: 5-HT; EC_{50} ± S.E.
	nM	*nM*	*nM*
WT	0.12 ± 0.03	86.7 ± 8.8	3 ± 0.42
Cys^{328} → Ser/Cys^{329} → Ser	0.25* ± 0.07	7.8* ± 0.2	0.6* ± 0.15
Cys^{386} → Ser	0.13 ± 0.04	64 ± 7.6	3 ± 0.28
Cys^{328} → Ser/Cys^{329} → Ser/Cys^{386} → Ser	0.13 ± 0.02	102 ± 9	3 ± 0.35

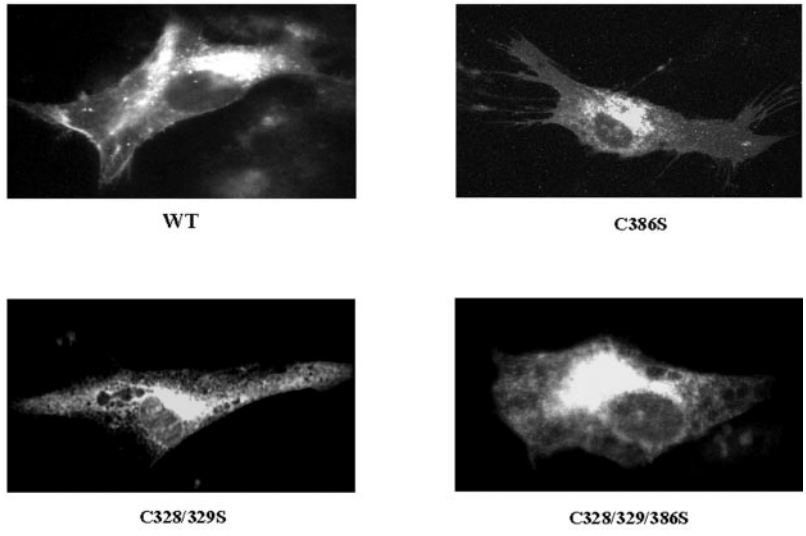

FIG. 7. **Role of palmitoylation for the intracellular 5-HT$_{4(a)}$ receptor distribution.** COS-7 cells were transfected either with wild type or mutant 5-HT$_{4(a)}$ receptor cDNAs. 48 h after transfection, cells were fixed, permeabilized, and then subjected to immunofluorescence analysis with an antibody AS9459 raised against the COOH terminus of 5-HT$_{4(a)}$ receptor. After incubation with the fluorescent second antibodies, cells were subjected to the confocal microscopy with appropriated filters set at magnification of 630.

formations (58). The capacity of a native receptor to spontaneously isomerize from an inactive form (R) to an active form (R*) is certainly a very important pharmacological characteristic because this may explain part of its physiological and possible pathological behavior, as well as the effect of drugs classified as inverse agonists (able to reverse the isomerization because of their higher affinity for R than for R*) on the receptor. Although the agonist-independent signaling has been observed for a wide variety of GPCRs (in particular after their overexpression in heterologous cells), molecular constrains involved in the regulation of receptor constitutive activity remain poorly understood. There are some data to indicate that specific sequences within the third intracellular loop *i*3, as well as composition of the COOH-terminal tail of GPCRs, may be essential for the isomerization of receptors from the R to the R* form (58).

In the present study, we demonstrate that mutation of conserved cysteine residues 328/329 located at the COOH-terminal tail of the 5-HT$_{4(a)}$ receptor elevates the receptor basal constitutive activity. Binding of [^{35}S]GTPγ S to G_s as well as the basal activation of the HvCNG channel were significantly increased after mutation of these cysteine residues (Figs. 3 and 4). Expression of the Cys^{328} → Ser/Cys^{329} → Ser mutant in COS-7 cells resulted in an ~2.5-fold higher basal level of cAMP formation when compared with the wild type receptor (Fig. 5). In addition, this mutant possesses a significantly lowered ligand-binding affinity (Table I) and its EC_{50} value was shifted from 3 nM (wild type and other mutants) to 0.6 nM 5-HT (Table I, Fig. 6). In contrast, mutation of Cys^{386}, which is also subjected to palmitoylation (Figs. 1 and 2) had no effect on the agonist-independent receptor activation. It is interesting to note that simultaneous substitution of Cys^{328}/Cys^{329} and

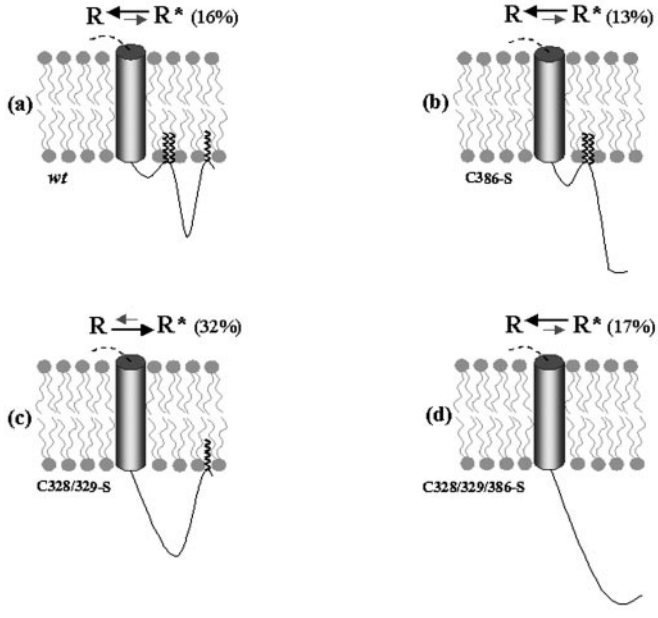

FIG. 8. **Proposed mechanism for the modulation of 5-HT$_{4(a)}$ receptor constitutive activity by palmitoylation.** The seventh transmembrane domain as well as carboxyl-terminal cytoplasmic tail of the 5-HT$_{4(a)}$ receptor are schematically shown. Depending on the number of cysteine residues modified, palmitoylation could result in the formation of two (*a*), one small (*b*), one large (*c*), or no (*d*) intracellular loops. In the present model, every conformation could be changed to one of the remaining three forms by basal or agonist-promoted palmitate turnover. Percentage of receptor population in the R* form was calculated based on the two-state model as described by Claeysen *et al.* (29).

Cys^{386}, which completely abolishes palmitoylation of the 5-HT$_{4(a)}$ receptor, reverses the high constitutive activity obtained for the Cys^{328} → Ser/Cys^{329} → Ser mutant to a level obtained for the wild type.

What could be a possible scenario in which the different palmitoylation states of the 5-HT$_{4(a)}$ receptor differently modulate its constitutive activity? It has been proposed that palmitoylation of GPCRs may provide a lipophilic membrane anchor to create an additional fourth intracellular loop in the carboxyl-terminal region of the receptor (9, 59). More recently, direct evidence for this has been obtained for rhodopsin (60, 61). The 5-HT$_{4(a)}$ receptor possesses two different acylation sites at its COOH-terminal domain (Fig. 1). Therefore, "complete" palmitoylation of the 5-HT$_{4(a)}$ receptor may result in the formation of two additional intracellular loops *i4* and *i5* (Fig. 8*a*). Functionally, this conformation could be critical for determining the basal level of agonist-independent receptor activation. When cysteine residues 328/329 become depalmitoylated (by basal or agonist-promoted palmitate turnover), a single large intracellular loop could be generated instead of *i4* and *i5* loops (Fig. 8*c*). From a functional point of view, such a change in the conformation could lead to the significant increase of the receptor's constitutive activity by shifting R → R* equilibrium toward the R* state. Based on the two-state model, we calculated that about 32% of the total Cys^{328} → Ser/Cys^{329} → Ser mutant receptor population was in the activated R* state. For the wild type 5-HT$_{4(a)}$, this value was only 16% (29). Subsequent depalmitoylation of the cysteine residue 386 destroys the large activating loop and therefore reduces the receptor activity to the wild type level (Fig. 8*d*). Generally, our model proposes that palmitoylation of the 5-HT$_{4(a)}$ receptor is directly involved in isomerization of the receptor from R to R* by dictating conformation of its flexible cytoplasmic loops involved either in the receptor/G-protein recognition process or in G-protein binding and/or receptor-mediated G-protein activation. Taken together with the fact that palmitoylation of the 5-HT$_{4(a)}$ receptor is a dynamic process (Ref. 31, Fig. 2), this model could also explain the higher constitutive activity reported for the native 5-HT$_{4(a)}$ receptor as compared with other GPCRs (28, 30). Indeed, existence of palmitate turnover implies that at any given point different populations of the palmitoylated and non-palmitoylated 5-HT$_{4(a)}$ receptors, including the form with a large activating loop (Fig. 8*c*), are present in the cell and therefore actively contribute to the agonist-independent receptor activity. Moreover, it can be proposed that agonist stimulation, by promoting depalmitoylation of the receptor, regulates membrane attachment of the carboxyl tail, providing a formation of the large activating loop. The detailed mechanism involved in the regulation of different palmitoylation states of the 5-HT$_{4(a)}$ receptor is still unclear. One possibility is that palmitoylation and/or depalmitoylation enzymes may directly regulate production of different acylation states of the receptor. Alternatively, other intracellular proteins, like the Homer protein for the metabotropic glutamate receptors (62), could be involved in the controlling process.

In conclusion, we have demonstrated that the mouse 5-HT$_{4(a)}$ receptor contains two potential palmitoylation sites (Cys^{328}/Cys^{329} and Cys^{386}) located in the carboxyl-terminal domain of the receptor. These two sites are palmitoylated independently of each other in an agonist-dependent manner. Palmitoylation does not contribute to the coupling with G$_s$ nor to the intracellular distribution. We also revealed the critical role of palmitoylation in the modulation of the receptor's constitutive activity and propose that dynamic palmitoylation of two different acylation sites within the COOH-terminal tail of the 5-HT$_{4(a)}$ receptor represents a novel mechanism regulating both agonist-promoted as well as constitutive receptor activities.

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