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Palmitoylation of the 5-Hydroxytryptamine$_{4a}$ Receptor Regulates Receptor Phosphorylation, Desensitization, and β-Arrestin-Mediated Endocytosis

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ABSTRACT

The mouse 5-hydroxytryptamine$_{4a}$ (5-HT$_{4a}$) receptor is an unusual member of the G protein-coupled receptor superfamily because it possesses two separate carboxyl-terminal palmitoylation sites, which may allow the receptor to adopt different conformations in an agonist-dependent manner (*J Biol Chem* **277:**2534–2546, 2002). By targeted mutation of the proximal (Cys-328/329) or distal (Cys-386) palmitoylation sites, or a combination of both, we generated 5-HT$_{4a}$ receptor variants with distinct functional characteristics. In this study, we showed that upon 5-HT stimulation, the 5-HT$_{4a}$ receptor undergoes rapid ($t_{1/2}$ ~ 2 min) and dose-dependent (EC_{50} ~ 180 nM) phosphorylation on serine residues by a staurosporine-insensitive receptor kinase. Overexpression of GRK2 significantly reduced the receptor-promoted cAMP formation. The Cys328/329-Ser mutant, which is constitutively active in the absence of ligand, exhibited enhanced receptor phosphorylation under both basal and agonist-stimulated conditions and was more effectively desensitized and internalized via a β-arrestin-2 mediated pathway compared with the wild-type 5-HT$_{4a}$. In contrast, G protein activation, phosphorylation, desensitization, and internalization of the other palmitoylation-deficient receptor mutants were affected differently. These findings suggest that palmitoylation plays an important role in modulating 5-HT$_{4a}$ receptor functions and that G protein activation, phosphorylation, desensitization, and internalization depend on the different receptor conformations.

5-Hydroxytryptamine (5-HT or serotonin) is an important neurotransmitter and a local hormone that acts in the central nervous system as well as in various peripheral organs. It regulates cellular activities by binding to specific membrane-bound 5-HT receptors. Most of these proteins belong to the superfamily of G protein-coupled receptors (GPCR). The 5-HT$_4$ receptor was shown to mediate several important physiological effects of 5-HT, including memory facilitation and the regulation of intestinal motility (Eglen et al., 1995; Bockaert et al., 1997). Mice deficient in 5-HT$_4$ receptors displayed abnormal stress-induced feeding and locomotor behavior, which suggests that this receptor may serve as drug target in the treatment of eating disorders (Compan et al., 2004). Moreover, 5-HT$_4$ receptors have been implicated in the pathogenesis of central and peripheral disorders such as neurodegenerative diseases, irritable bowel syndrome, and atrial fibrillation (Wong et al., 1996; De and Tonini, 2001; Callahan, 2002).

Murine 5-HT$_4$ receptors are coded by a complex gene that generates four carboxyl-terminal splice variants: 5-HT$_{4a}$, 5-HT$_{4b}$, 5-HT$_{4e}$, and 5-HT$_{4f}$ (Claeysen et al., 1999). Cloning of 5-HT$_4$ homologs from human and rat tissue revealed further structural diversity of this receptor (Blondel et al., 1998). All of these receptor variants share the same sequence up to the common splicing site (Leu-358), followed by a unique C terminus. All splice variants were able to stimulate adenylyl cyclase and raise intracellular cAMP levels upon agonist

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ABBREVIATIONS: 5-HT, 5-hydroxytryptamine; GPCR, heterotrimeric GTP-binding protein-coupled receptor; PKA, cAMP-dependent protein kinase; GRK, G protein-coupled receptor kinase; PMA, phorbol 12-myristate 13-acetate; A23187, calcimycin; Ro-20-1724, 4-(3-butoxy-4-methoxybenzyl)-2-imidazolidinone; H-89, *N*-[2-((*p*-bromocinnamyl)amino)ethyl]-5-isoquinolinesulfonamide; DMEM, Dulbecco's modified Eagle's medium; PAGE, polyacrylamide gel electrophoresis; DFBS, dialyzed fetal bovine serum; GR 113808, [1-[2-(methylsulfonylamino)ethyl]-; 4-piperidinyl]methyl 1-methyl-1*H*-indole-3-carboxylate; YFP, yellow fluorescent protein; WT, wild type; 5-HT$_{4a}$, mouse 5-hydroxytryptamine$_{4a}$; HvCNG, *Heliothis virescens* voltage-activated cyclic nucleotide-gated cation channel; Sf.9, *Spodoptera frugiperda* insect cells.

stimulation (Blondel et al., 1998; Claeysen et al., 1999; Bender et al., 2000; Mialet et al., 2000a; Vilaro et al., 2002), albeit with different efficacy and potency (Mialet et al., 2000a,b). Differences may also extend to the type of G proteins to which the various splice variants are coupled. For example, we recently showed that the 5-HT$_{4a}$ receptor is coupled to both Gαs and Gα13 proteins (Ponimaskin et al., 2002b), whereas the 5-HT$_{4b}$ isoform activates Gi and Gs proteins (Pindon et al., 2002). Moreover, different 5-HT$_4$ receptor variants significantly differ in their sensitivity toward homologous agonist-induced desensitization (Mialet et al., 2003). Together, these results indicate that the carboxyl terminus of the 5-HT$_4$ receptor plays an important role in G protein coupling as well as in the initiation of counter-regulatory mechanisms.

The 5-HT$_4$ receptor contains potential sites for post-translational modifications within their cytoplasmic carboxyl-terminal domains. We showed previously that the 5-HT$_{4a}$ receptor is palmitoylated at two different sites, which involve a conserved cysteine pair at amino acid positions 328/329 and an unusual additional site (Cys-386) near its C terminus (Ponimaskin et al., 2002a). We showed that 5-HT$_{4a}$ receptor palmitoylation is dynamically regulated upon agonist stimulation of the receptor (Ponimaskin et al., 2001). Common to many GPCRs, acylation of 5-HT$_4$ receptors is believed to anchor their C-terminal tails to the plasma membrane, thereby creating additional intracellular loops.

The role of agonist-induced receptor phosphorylation by second-messenger–activated and GPCR kinases for regulating the receptor function and cell surface expression is well documented for many receptors (Ferguson, 2001). According to the paradigm, established mainly in the β2-adrenergic receptor system, phosphorylation on serine or threonine residues located preferentially in the receptor carboxyl terminus promotes β-arrestin binding, which interferes with further G protein activation. β-Arrestin also acts as an adaptor protein to couple the phosphorylated receptor with the heavy chain of clathrin, thereby initiating receptor endocytosis.

Palmitoylation has been shown to affect several aspects of GPCR function, including the efficiency and selectivity of G-protein coupling, receptor phosphorylation and desensitization, endocytosis, and transport to the plasma membrane (Qanbar and Bouvier, 2003). In the case of the β2-adrenergic receptor, it was postulated that agonist-dependent changes in receptor palmitoylation modulates access of regulatory kinases such as cAMP-dependent protein kinase (PKA) and G protein-coupled receptor kinases (GRK) to the C-terminal domain (Moffett et al., 2001). Mutation of putative palmitoylation sites was also shown to increase basal phosphorylation of the adenosine A3 receptor (Palmer and Stiles, 2000). In contrast, palmitoylation-deficient forms of the CCR5 chemokine receptor or the V1a vasopressin receptor showed significant defects in receptor phosphorylation, both at basal and agonist-stimulated levels (Hawtin et al., 2001; Kraft et al., 2001). Together, these results suggest that palmitate turnover controls access of carboxyl-terminal receptor phosphorylation sites to regulatory kinases in a differential manner.

In the present study, we demonstrate that the 5-HT$_{4a}$ receptor is phosphorylated in an agonist-dependent manner and examine the significance of palmitoylation for controlling receptor phosphorylation, desensitization and β-arrestin-mediated endocytosis.

Materials and Methods

Materials. $^{32}P_i$ label was ordered from MP Biomedicals (Irvine, CA). ECL Western blotting analysis system and peroxidase-conjugated secondary antibodies were purchased from Amersham Biosciences. Enzymes used in molecular cloning were obtained from New England Biolabs (Beverly, MA). Protein A-Sepharose CL-4B beads and 5-HT were from Sigma-Aldrich (St. Louis, MO). Forskolin, PMA, A23187, Ro-20-1724, staurosporin, and H-89 were from Calbiochem (San Diego, CA). TC-100 insect cell medium, DMEM, Cellfectin, and LipofectAMINE 2000 reagents were purchased from Invitrogen (Carlsbad, CA). Cell culture dishes were from NUNC GmbH & Co. KG (Wiesbaden, Germany).

Recombinant DNA Procedures. All basic DNA procedures were performed as described by Sambrook et al. (1989). The plasmid encoding β-arrestin2/GFP has been described previously (Kraft et al., 2001). The construction of recombinant baculovirus encoding for the murine 5-HT$_{4a}$ receptor wild-type and its acylation deficient mutants with substitutions of serine for cysteine Cys328/329-Ser, Cys386-Ser, and Cys328/329/386-Ser has been reported previously (Ponimaskin et al., 2002a). All mutants were verified by double-stranded dideoxy DNA sequencing at the level of the final plasmid.

Phosphorylation Experiments. *Spodoptera frugiperda* (Sf.9) cells were grown in TC-100 medium supplemented with 10% fetal calf serum and 1% penicillin/streptomycin (complete TC-100). For expression, Sf.9 cells (1.5×10^6) grown in 35-mm dishes were infected with recombinant baculovirus encoding wild-type or mutated 5-HT$_{4a}$ receptors at a multiplicity of infection of 10 plaque-forming units per cell. Labeling experiments were started after 48 h of incubation. One hour before $^{32}P_i$-labeling, complete TC-100 was replaced by phosphate-free TC-100 medium, and cells were labeled with [$^{32}P_i$] (150 μCi/ml) in phosphate-free TC-100 medium for the time periods indicated in the figure legends. In some experiments, 5-HT, forskolin, PMA, A23187, or staurosporin were added to final concentrations, which are given in the figure legends. After labeling, cells were washed twice with ice-cold PBS and lysed in 600 μl of buffer A (0.5% Nonidet P-40, 150 mM NaCl, 50 mM Tris/HCl, pH 7.9, 5 mM EDTA, 10 mM iodoacetamide, and 1 mM phenylmethylsulfonyl fluoride) containing 10 mM sodium fluoride and 10 mM disodium pyrophosphate. Insoluble material was pelleted (5 min, 20,000*g*), and the antibody AS9459 against the C-terminal domain of the 5-HT$_{4a}$ receptor (Ponimaskin et al., 2001) was added to the supernatant together with 30 μl of protein A-Sepharose CL-4B. Samples were incubated under gentle rotation for 4 h. After brief centrifugation, the pellet was washed three times with ice-cold buffer A, 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, and 0.002% bromphenol blue). Radiolabeled polypeptides were analyzed by SDS-polyacrylamide gel electrophoresis (PAGE) on 12% acrylamide gels, visualized by autoradiography and analyzed by filmless autoradiographic analysis.

The expression of 5-HT$_{4a}$ receptors was analyzed in parallel by immunoblot analysis with antibodies AS9459 at a 1:1000 dilution. Detection was achieved with the enhanced chemiluminescence system (ECL+; Amersham Biosciences, Piscataway, NJ) and protein bands were visualized by fluorography using Kodak X-Omat AR films. The amount of [$^{32}P_i$]phosphate incorporated into each of these mutants was then calculated by autoradiographic analysis in relation to the expression level of the various proteins as assessed by densitometry of ECL films.

Phosphoamino Acid Analysis. For the determination of phosphoamino acids, $^{32}P_i$-labeled Sf.9 cells (3×10^6 cells per 60-mm dish) expressing 5-HT$_{4a}$ receptor wild-type or its acylation-deficient mutants were treated for 5 min with 10 μM 5-HT. Receptors were immunoprecipitated with the receptor-specific antibody AS9459 and resolved by SDS-PAGE on 12% polyacrylamide gel. After electrophoretic transfer to a polyvinylidene difluoride membrane (Immo-

bilon; Millipore Corporation (Bedford, MA)) and autoradiography, receptors were excised and hydrolyzed in 6 N HCl for 2 h at 110°C. Hydrolysates were vacuum-dried, resolubilized in pH 1.9 buffer [formic acid/acetic acid/H_2O, 10:100:1890 (v/v/v)] containing phosphoamino acid standards and spotted on a thin-layer cellulose plate. Phosphoamino acids were separated by electrophoresis (900 V/1.5 h) at pH 1.9 buffer, followed by a second electrophoresis (900 V/45 min) at pH 3.5 [pyridine/acetic acid/H_2O, 10:100:1890 (v/v/v) and 0.5 mM EDTA] in the orthogonal direction. After ninhydrin staining of phosphoamino acid standards, thin-layer plates were exposed to autoradiographic screens.

Electrophysiology and Data Fitting. Electrophysiological measurements were performed in Sf.9 cells using methods described previously (Heine et al., 2002). Membrane currents were measured in the whole-cell, patch-clamp mode. We used a discontinuous single electrode voltage clamp amplifier SEC-05L (NPI Electronic, Tamm, Germany), which was connected to a computer via an ITC-16 interface (Instrutech Corp., Port Washington, NY). Pipettes had a resistance of 3 to 7 MΩ. Data were acquired and stored by Pulse-PulseFit 8.31 software (HEKA, Lambrecht/Pfalz, Germany) and data analysis was performed with Igor-Wavemetrics software (Wavemetrics, Lake Oswego, OR). The temperature of the superfusing solution was stabilized at 25 ± 0.2°C controlled with a Peltier control unit (ESF-electronic, Göttingen, Germany). Recordings were performed in TC-100 medium without fetal calf serum. The used 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, and 10 mM EGTA (pH 7.25, osmolarity was 20 mOsmol below the osmolarity of the TC-100 medium). The cells were stimulated with serotonin (5-HT) by local pressure-application via pipettes with a resistance of 2 to 4 MΩ (PDES 2L-unit; NPI Electronic) for the periods as indicated in Fig. 4. Before repeated stimulation, the agonist was washed out. The activation time constants (τ) of hyperpolarization-induced currents were estimated by exponential curve fitting as described previously (Heine et al., 2002).

Transfection of COS-7 Cells. The cDNA encoding the wild-type or the mutated 5-HT_{4a} receptors in pTracer-CMV2 plasmid, as well as the cDNA coding for β-arrestin2/GFP or GRK2, were introduced into COS-7 cells by electroporation as described previously (Claeysen et al., 1996). In brief, 1×10^7 cells were trypsinized, centrifuged, resuspended in 500 μl of buffer B (50 mM K_2HPO_4, 20 mM CH_3CO_2K, 20 mM KOH, and 26.7 mM $MgSO_4$, pH 7.4) containing 2.5 to 10 μg of receptor cDNA. The total amount of DNA was kept constant at 15 μg per transfection using the pTracer vector. After a 15-min exposure at room temperature, 300 μl of cell suspension was transferred to a 0.4-ml electroporation cuvette (Bio-Rad) and pulsed using a Gene-Pulser apparatus (1000 μF, 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 Transfected COS-7 cells. Intracellular cAMP levels were determined by measuring the conversion of the [^{3}H]adenine nucleotide precursor [^{3}H]ATP to [^{3}H]cAMP. In brief, 6 h after transfection, the complete medium was exchanged for serum-free DMEM containing 2 μCi/ml [^{3}H]adenine to label the endogenous ATP pool. After overnight incubation, cAMP accumulation was measured as described previously (Dumuis et al., 1988).

Desensitization Analysis. COS-7 cells expressing wild-type or acylation-deficient 5-HT_{4a} receptors were grown in serum-free DMEM and incubated with [^{3}H]adenine for 24 h. To analyze receptor desensitization, cells were preincubated with 5-HT (1 μM) for the time periods indicated in figure legends. After removal of the agonist, cells were washed three times with DMEM, and cAMP accumulation was initiated by addition of medium containing 5-HT (10 μM) and phosphodiesterase inhibitor RO-20-1724 (0.1 mM). The cAMP accumulation within a 10-min stimulation period was determined. To analyze the role of GRK2 in the agonist-promoted desensitization of the 5-HT_{4a} receptor, COS-7 cells transiently expressing either the 5-HT_{4a} WT receptor or acylation-deficient mutants were cotransfected with or without the vector encoding GRK2 (500 ng/10^7 cells). The 5-HT (10 μM) induced cAMP production was then directly measured during a 10-min incubation period.

Assay for [^{3}H]GR 113808 Binding in Intact COS-7 Cells. To measure radioligand binding in intact cells, transiently transfected COS-7 cells were plated into 12-well dishes (6×10^5 cells/ml), grown for 6 h in DMEM containing 10% dFBS and then for another 20 h in serum-free medium. Experiments were performed in HEPES-buffered saline (20 mM HEPES, 150 mM NaCl, 4.2 mM KCl, 0.9 mM $CaCl_2$, 0.5 mM $MgCl_2$, 0.1% glucose, and 0.1% bovine serum albumin). The reaction mixture (250 μl) containing saturating concentrations of [^{3}H]GR 113808 (0.4 to 0.6 nM, K_d = 0.12 nM; specific activity, 83 Ci/mmol) was added to the cells and incubated for 2 h at 4°C. Incubation was terminated by aspiration of the incubating buffer followed by washing the cells with ice-cold HEPES-buffered saline. Cells were lysed in 0.1 N NaOH, then 8 ml of scintillation liquid (PCS scintillation cocktail; Amersham Biosciences) was added and radioactivity was measured by scintillation counting. The receptor densities were estimated as described previously (Claeysen et al., 1999). An excess of 5-HT (0.5 μM) was added to determine nonspecific binding. Protein concentration was determined using a Bio-Rad protein assay.

Fluorescent Microscopy. To analyze the receptor internalization, COS-7 cells growing on coverslips were cotransfected with the plasmids encoding for the YFP-tagged 5-HT_{4a} receptor and GFP-tagged β-arrestin2. At 14 to 16 h after transfection, cells were washed and starved for 24 to 26 h in serum-free DMEM. Then, cells were treated either with vehicle (H_2O) or with 5-HT (1 μM) for 1 h and fixed with paraformaldehyde (3% in phosphate-buffered saline) for 15 min. The cells were washed three times with phosphate-buffered saline, and coverslips were mounted in 90% (v/v) glycerol. Finally, cells were monitored under a confocal laser-scan microscope LSM510-Meta (Zeiss, Welwyn Garden City, UK) at 63× magnification by using a λ mode setting that allows for spectral unmixing using individual reference spectrums.

Results

Phosphorylation of the 5-HT_{4a} Receptor. G protein-coupled receptors often undergo agonist-induced phosphorylation, which is functionally linked to receptor desensitization. We therefore studied whether the 5-HT_{4a} receptor is phosphorylated upon agonist stimulation. Sf.9 insect cells expressing recombinant 5-HT_{4a} receptors were incubated with $^{32}P_i$ to label their ATP pool and then exposed to the agonist 5-HT (10 μM, 10 min). After immunoprecipitation and SDS-PAGE, the receptor was analyzed by autoradiography as described under *Materials and Methods*. As shown in Fig. 1A, exposure of the receptor to 5-HT promoted the phosphorylation of a single protein band with a molecular mass of approximately 42 kDa corresponding to the predicted molecular mass of the 5-HT_4 receptor. This band comigrated with the 5-HT_{4a} protein band detected by the Western blot and was absent in noninfected, $^{32}P_i$-labeled control cells. To examine the dose dependence of $^{32}P_i$ incorporation, Sf.9 cells expressing 5-HT_{4a} receptor were incubated in the presence of varying concentrations of 5-HT. Levels of radiolabel incorporation were then quantified by autoradiographic analysis after immunoprecipitation of 5-HT_{4a} receptors and SDS-PAGE. Figure 1B demonstrates that 5-HT induced a dose-dependent increase in the phosphorylation intensity of the receptor with an EC_{50} of 180 nM. Kinetic analysis revealed a rapid 5-HT-induced increase in $^{32}P_i$ incorporation into the

5-HT$_{4a}$ receptor ($t_{1/2}$ ~ 2 min), and maximal phosphorylation level was reached after 5 min (Fig. 1C).

Phosphorylation of GPCR may be controlled by multiple second messenger-activated protein kinases, including protein kinase C, PKA, and calcium/calmodulin-dependent kinase. We investigated whether these protein kinases may participate in phosphorylation of the 5-HT$_{4a}$ receptor. Treatment of $^{32}P_i$-labeled cells with forskolin, PMA, or with the Ca^{2+} ionophore A23187 to stimulate different kinase activities, however, did not induce increased receptor phosphorylation (Fig. 1A). Moreover, pretreatment of the receptor-expressing cells with the protein kinase inhibitor staurosporin at 1 μM concentration did not reduce the agonist-promoted increase in 5-HT$_{4a}$ receptor phosphorylation (Fig. 1D). These results demonstrate that the second messenger-dependent kinases tested do not mediate 5-HT$_{4a}$ receptor phosphorylation.

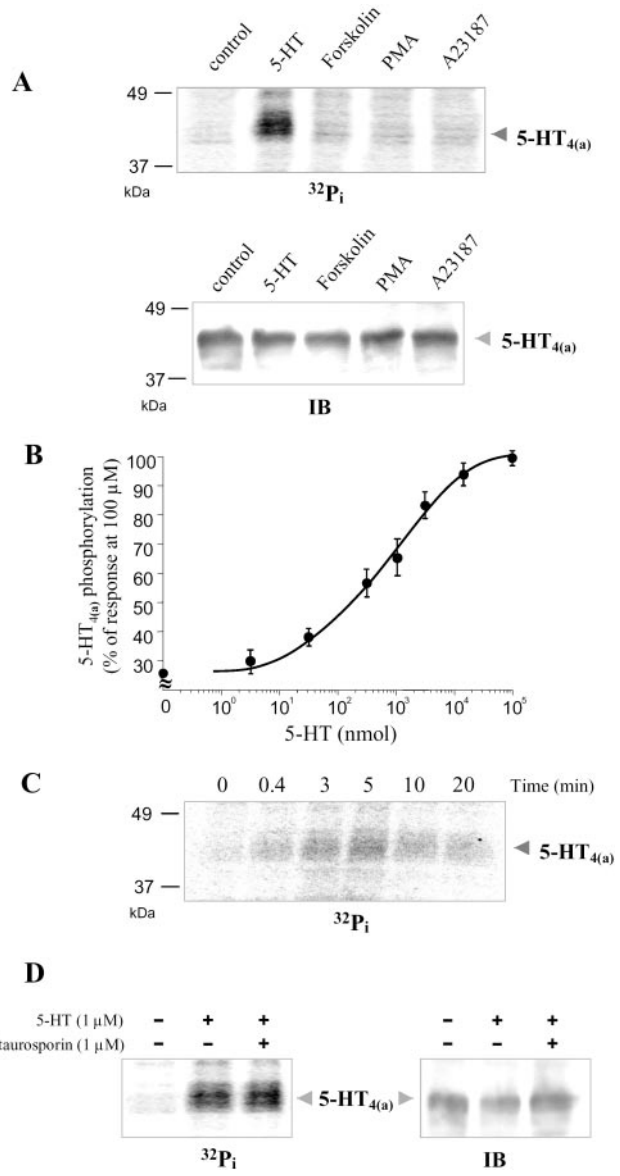

Fig. 1. Phosphorylation of the 5-HT$_{4a}$ receptor. A, the 5-HT$_{4a}$ receptor was expressed in Sf.9 insect cells, labeled with [$^{32}P_i$]phosphate (top), and treated with vehicle (H_2O, control), 10 μM 5-HT, 50 μM forskolin, 200 nM PMA, or 10 μM A23187 for 10 min. After immunoprecipitation with the antibody AS9459 directed against C-terminal domain of the 5-HT$_{4a}$ receptor, samples were subjected to SDS-PAGE and autoradiography. Expression of the 5-HT$_{4a}$ receptor was documented in parallel by immunoblot (IB; bottom). Representative fluorograms are shown. B, [$^{32}P_i$]phosphate-labeled Sf.9 cells were treated with increasing concentrations of 5-HT for 10 min. Receptors were immunoprecipitated, and $^{32}P_i$-incorporation was calculated after SDS-PAGE and autoradiography. The values represent the means ± S.E.M. from three independent experiments performed in duplicate. C, phosphorylation kinetics were analyzed in Sf.9 cells expressing the 5-HT$_{4a}$ receptor. After labeling with [$^{32}P_i$]phosphate, cells were treated with 10 μM 5-HT for the time interval shown and then subjected to immunoprecipitation, SDS-PAGE, and autoradiography. D, insect Sf.9 cells expressing recombinant 5-HT$_{4a}$ receptor were labeled with [$^{32}P_i$]phosphate and incubated with 1 μm of staurosporin for 20 min followed by stimulation with 10 μM 5-HT for 10 min. Expression of the 5-HT$_{4a}$ receptor was determined in parallel by IB. In all cases, representative images are shown.

Phosphorylation of Palmitoylation-Deficient 5-HT$_{4a}$ Receptor Mutants. We have shown recently that the 5-HT$_{4a}$ receptor undergoes agonist-dependent palmitoylation and also localized the palmitoylation sites by site-directed mutagenesis (Ponimaskin et al., 2001, 2002a). Because the 5-HT$_{4a}$ receptor undergoes agonist-induced phosphorylation, we next analyzed a possible interplay between these two post-translational receptor modifications. Sf.9 insect cells expressing either the wild-type 5-HT$_{4a}$ receptor or its palmitoylation-deficient mutants were loaded with $^{32}P_i$, stimulated with 5-HT, and then subjected to immunoprecipitation, SDS-PAGE, and autoradiography.

Immunoblot analysis revealed that all mutants were expressed at levels comparable with those of the 5-HT$_{4a}$ wild-type control (Fig. 2A), which allows for a quantitative comparison of phosphorylation levels in the different products. The amount of [$^{32}P_i$]phosphate incorporated into each of these mutants was calculated by densitometry of fluorograms and normalized for expression levels of the various proteins as determined by immunoblotting. Analysis of the results obtained in $^{32}P_i$ labeling experiments demonstrated that both basal and agonist-mediated phosphorylation efficiency for all acylation-deficient mutants was significantly elevated up to 2.7-fold compared with the wild-type receptor (Fig. 2, A and B). The increase in the phosphorylation efficiency over the basal level was particularly pronounced in the case of Cys328/329 and Cys386-Ser receptor mutants.

Phosphoamino Acid Analysis. The C-terminal domain of the 5-HT$_{4a}$ receptor contains several serine and threonine residues that represent potential phosphate acceptor sites for GRK-mediated receptor phosphorylation. Next, we defined which amino acids within the 5-HT$_{4a}$ receptor are modified by ligand-induced phosphorylation. We also analyzed whether a different phosphorylation site(s) may be used in the acylation-deficient mutants. Phosphoamino acid analysis revealed that agonist-mediated phosphorylation of the wild-type receptor as well as all acylation-deficient mutants occurs exclusively at serine residues (Fig. 3).

Desensitization of the 5-HT$_{4a}$ Receptor in Sf.9 Cells as Assessed by an Electrophysiological Assay. A possible functional implication of the palmitoylation for the 5-HT$_{4a}$ receptor desensitization was analyzed in the insect Sf.9 cells by using the patch-clamp technique, as described previously (Heine et al., 2002). To this end, the hyperpolarization- and cyclic nucleotide-sensitive cation-channel from *Heliothis virescens* (HvCNG) was coexpressed in Sf.9 cells together with the heterotrimeric Gs protein (Gαs, β1 and γ2 subunits) and with the 5-HT$_{4a}$ receptor wild-type or its acylation-deficient mutants. The recorded activation currents

during a hyperpolarization to −100 mV were fitted by a single exponential function. The resulting channel activation time constant τ was used as a parameter for the analysis of the channel activity.

As shown in Fig. 4, treatment of the wild-type and mutated 5-HT_{4a} receptors with 5-HT resulted in a fast and significant decrease of the activation time constant τ, which reflects an increase in the intracellular cAMP levels, leading to a faster activation of the HvCNG-channel (Fig. 4). The stimulatory effect was reversible and the currents returned nearly to control values after washout of the agonist. To analyze the receptor desensitization, the agonist was applied repeatedly, and changes in the activation time constant τ were measured over the time. After every receptor stimulation, the agonist was thoroughly washed out from the bath before the new stimulation. Figure 4 demonstrates that repetitive application of the agonist to the 5-HT_{4a} receptor wild-type resulted in similar activation kinetics for the channel for the following stimulation after washout. It is noteworthy that repetitive agonist stimulation of the Cys328/329-Ser mutant resulted in a reduction of the channel activation reflected in an increase of the activation time constant (Fig. 4). Analysis of the ascending slope for the minimal τ values (maximal channel activation during agonist application) obtained after repeated agonist applications revealed that the slope value was increased from 3.7 (n = 4) for the wild-type to 13.5 (n = 4) in

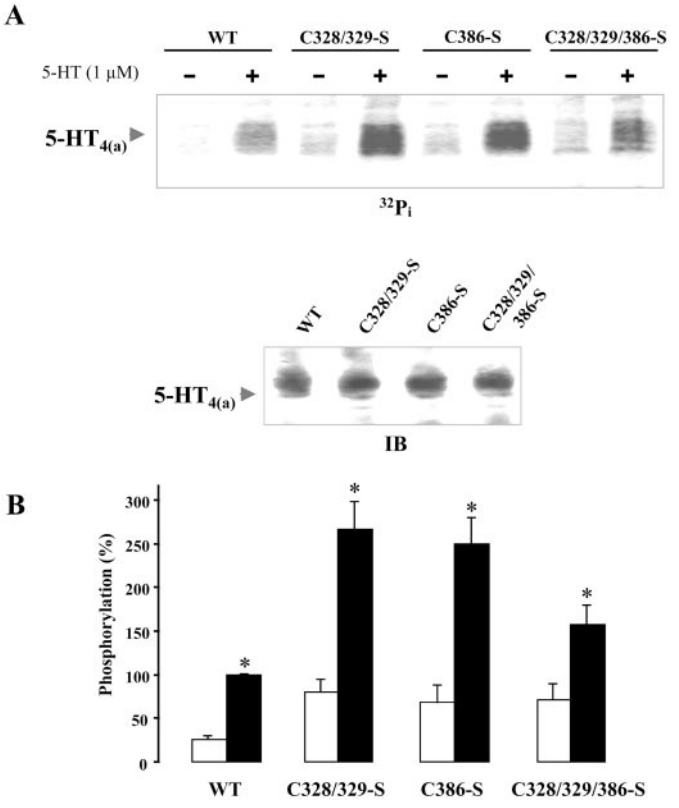

Fig. 2. Phosphorylation of palmitoylation-deficient receptor mutants. A, Sf.9 cells expressing the 5-HT_{4a} receptor wild-type or its acylation-deficient mutants were incubated with either [$^{32}P_i$]phosphate (top) in the presence of either vehicle (H_2O, control) or 10 μM 5-HT for 10 min. Receptors were immunoprecipitated, resolved by SDS-PAGE, and analyzed by autoradiography. Expression of the 5-HT_{4a} receptor was analyzed in parallel by immunoblot (IB; bottom). Representative fluorograms are shown. B, changes in receptor phosphorylation after 5-HT stimulation versus vehicle were determined by autoradiographic analysis in relation to receptor expression levels as determined by immunoblot. Results are shown as mean percentage ± S.E.M. compared with cells expressing stimulated wild-type receptors (n = 4) for the wild-type and palmitoylation-deficient mutants. The value obtained for the receptor wild-type after 5-HT stimulation was set at 100%. A statistically significant difference between agonist-stimulated and nonstimulated phosphorylation is indicated (*, $p < 0.01$).

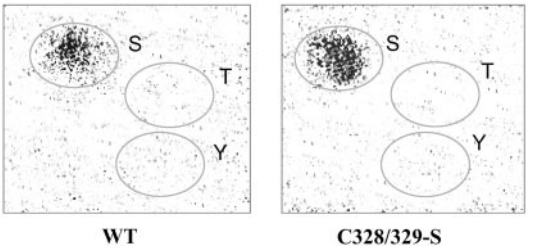

Fig. 3. Two-dimensional phosphoamino acid analysis of the 5-HT_{4a} receptor. Sf.9 insect cells expressing the 5-HT_{4a} receptor wild type or Cys328/329-Ser mutant were labeled with [$^{32}P_i$]phosphate in the presence of 10 μM 5-HT. Phosphoamino acid analysis of the immunoprecipitated receptors was performed as described under *Materials and Methods*. Positions of phosphoserine (S), phosphothreonine (T), and phosphotyrosine (Y) were determined by ninhydrin staining of phosphoamino acid standards, which were separated together with radioactive samples. A representative fluorogram from three independent experiments is shown.

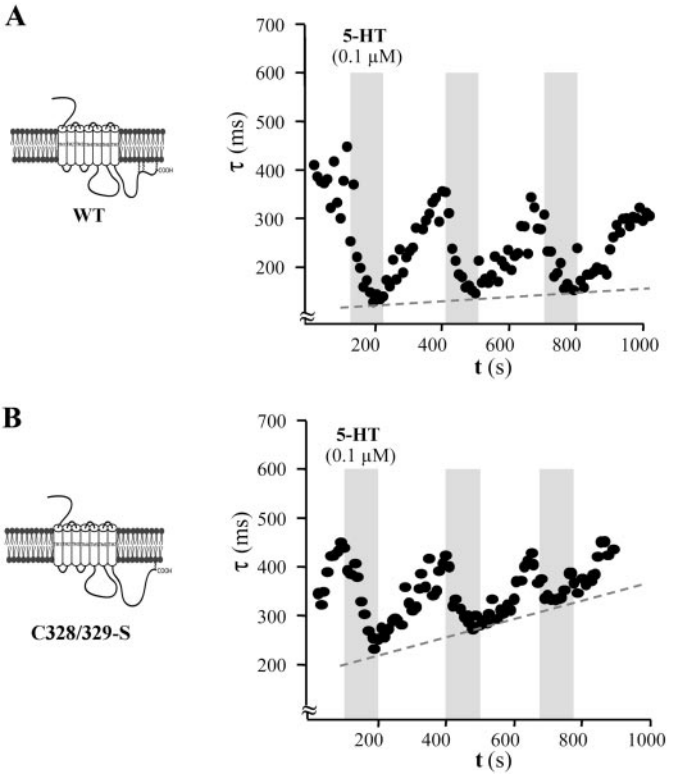

Fig. 4. Activation of the HvCNG cation-channel is mediated by repeated stimulation of the 5-HT_{4a} receptor. Currents from Sf.9 cells expressing the HvCNG-channel, the 5-HT_{4a} receptor wild-type (A) or Cys328/329-Ser mutant (B) together with the G protein-subunits $G\alpha_s$, $\beta 1$, and $\gamma 2$ were recorded in the whole-cell patch-clamp mode. Channel 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 pulse. Stimulation by 0.1 μM serotonin induced a pronounced decrease of the activation constants τ. This effect was reversible after washout of agonist. The repeated application of agonist results in a decrease of the 5-HT evoked response. The rate of desensitization for the wild-type and mutant was compared by analysis of the ascending slope for the τ values obtained after repeated 5-HT applications. Schematic representations of the 5-HT_{4a} receptor wild-type and Cys328/329-Ser mutant are shown on the left.

the case of Cys328/329-Ser mutant (Fig. 4). This demonstrates that the channel activation after repeated stimulation of the Cys328/329-Ser mutant was reduced compared with the wild-type, reflecting a reduced number of mutated receptor that induce intracellular cAMP changes. It is noteworthy that the agonist-induced receptor desensitization did not significantly differ from that of the wild-type receptor when the Cys386-Ser and Cys328/329/386-Ser mutants were analyzed (data not shown). These data suggest that the palmitoylation state of a proximal acylation site (Cys328/329), which was previously shown to selectively modulate constitutive receptor activity (Ponimaskin et al., 2002a), may also affect 5-HT$_{4a}$ receptor desensitization.

Desensitization of the 5-HT$_{4a}$ Receptor Wild-Type and Its Acylation-Deficient Mutants Expressed in COS-7 Cells. To further assess the role of palmitoylation for the agonist-induced 5-HT$_{4a}$ receptor desensitization in a different cellular context, the effects of sustained agonist treatment on the signaling efficacy of the WT and acylation-deficient mutants were compared in COS-7 cells. Receptor activities were measured in transfected cells that were pretreated with or without 5-HT for different periods. Desensitization was then defined as the reduction in the efficiency of agonist to stimulate adenylyl cyclase activity after pretreatment. As illustrated in Fig. 5, the onset of agonist-promoted desensitization was accelerated for all acylation-deficient mutants and particularly for the Cys328/329-Ser construct, compared with wild-type. However, the rate of desensitization was relatively low; after 1 h preincubation, we found that the residual response to the agonist was 80 ± 8%, 72 ± 6%, 68 ± 9%, and 62 ± 7% for the WT, Cys386-Ser, Cys328/329/386-Ser, and Cys328/329-Ser, respectively. Even at the longest desensitization time studied (18 h), we could still detect residual adenylyl cyclase activity of 62 ± 6%, 50 ± 5%, 45 ± 8% and 33 ± 7% for the WT, Cys386-Ser, Cys328/329/386-Ser, and Cys328/329-Ser, respectively (Fig. 5B). This is in contrast to our previous observations in rat esophagus and colliculus neurons of mouse, where exposure of cells to selective 5-HT$_4$ receptor agonists was accompanied by a very potent and rapid desensitization of the receptor-stimulated adenylyl cyclase response (Ansanay et al., 1992; Ronde et al., 1995). Such divergent findings may either indicate cell type-specific effects based on a differential expression of regulatory proteins or may result from overexpression of receptors in transfected cell lines.

Taken together, our results indicate that the acylation-deficient mutants were reproducibly more sensitive to agonist pre-exposure than the receptor wild-type. The rate of agonist-promoted receptor desensitization was greatest for the proximal acylation mutant Cys328/329-Ser. This suggests that, in agreement with our findings with receptor phosphorylation, desensitization of the 5-HT$_{4a}$ receptor may also be regulated by its palmitoylation state.

Role of GRK in Desensitization of the 5-HT$_{4a}$ Receptor. Phosphorylation of the 5-HT$_{4a}$ receptor is not affected by stimulation of several different second messenger-activated protein kinases (Fig. 1, A and D). In addition, we had previously demonstrated that the functional desensitization of the 5-HT$_4$ receptors in mouse colliculus neurons is cAMP-independent and agonist-selective (Ansanay et al., 1992; Ronde et al., 1995). This suggests a possible role of GRKs in the desensitization process. To test this hypothesis, we coexpressed GRK2 with the different 5-HT$_{4a}$ receptor mutants and determined the amount of cAMP accumulation over a 10-min incubation period. In this experimental setting, "desensitization" is interpreted as the cumulative effect of GRK overexpression on 5-HT-induced cAMP generation compared with cells with endogenous GRK levels. As shown in Fig. 6, the basal activity of cells coexpressing the different receptors together with GRK2 was not significantly changed. In contrast, the agonist-induced cyclase response was significantly ($p < 0.01$) impaired in cells overexpressing GRK2 compared with the control (Fig. 6). After a 10-min stimulation period with 5-HT, the maximal stimulation of adenylate cyclase in cells overexpressing GRK2 was reduced to 28 ± 8%, 35 ± 5%, 55 ± 11%, and 51 ± 9% of control cells (without coexpression of GRK2) for the wild-type, Cys328/329-Ser, Cys386-Ser, and Cys328/329/386-Ser, respectively. A 60 min-agonist pre-exposure performed before the period of cAMP accumulation did not significantly decrease the 5-HT response (data not shown). Taken together, these results strongly suggest a role for GRK2 (or a related receptor kinase) in homologous desensitization of the 5-HT$_{4a}$ receptor.

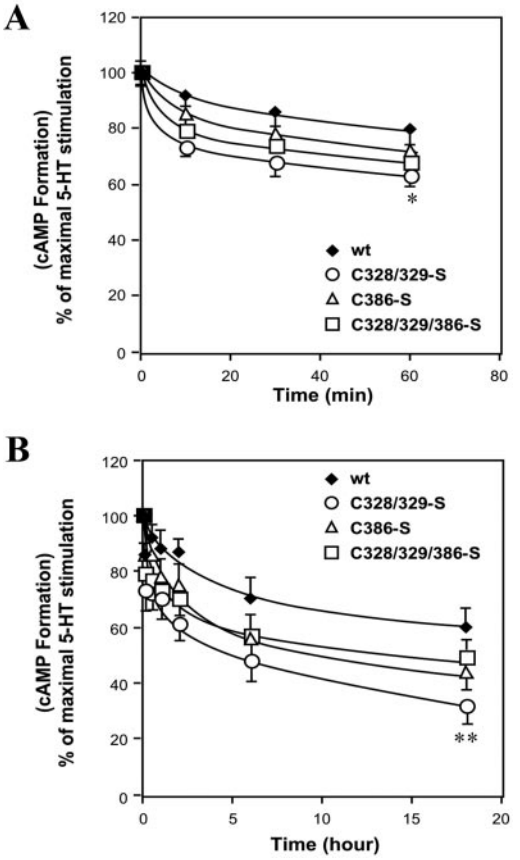

Fig. 5. Time course of desensitization of the 5-HT$_{4a}$ receptor wild-type and palmitoylation-deficient mutants. COS-7 cells expressing either the wild-type (wt) or acylation-deficient mutants were preincubated with 1 μM 5-HT for a short (0–60 min) (A) or for a long (0–18 h) (B) period of time. After extensive washes with the medium, cAMP accumulation was measured as described under *Materials and Methods*. Results are expressed as a percentage of residual stimulation relative to the maximal stimulation obtained for appropriated receptors. Each value represents the means ± S.E.M. from at least three independent experiments performed in triplicate. Statistically significant differences between receptor wild-type and Cys328/329-Ser mutant after 1 h and 18 h are indicated (**, $p < 0.01$; *, $p < 0.05$).

Role of Palmitoylation in Agonist-Induced Internalization of the 5-HT$_{4a}$ Receptor. In many GPCRs, GRK-induced receptor phosphorylation plays an important role in facilitating β-arrestin-mediated receptor internalization. To analyze whether the 5-HT$_{4a}$ receptor undergoes agonist-dependent internalization, the COS-7 cells were transiently cotransfected with YFP-tagged 5-HT$_{4a}$ receptor and GFP-tagged β-arrestin2. Functionality of receptor-YFP construct was confirmed by ligand binding, 5-HT induced adenylate cyclase activity, and serum response element assay. Distribution of the receptor and β-arrestin2 was then analyzed by confocal microscopy on LSM510-Meta microscope. Without agonist stimulation, the 5-HT$_{4a}$ receptor was mainly localized at the plasma membrane, whereas β-arrestin2 showed homogeneous cytoplasmic staining (Fig. 7A). Treatment of transfected cells with 1 μM 5-HT for 60 min induced clear receptor internalization with punctate, clustered 5-HT$_{4a}$ receptor-YFP structures that were partially colocalized with β-arrestin2 (Fig. 7A).

Having established that phosphorylation efficiency of the 5-HT$_{4a}$ receptor is modulated by receptor palmitoylation, we next compared the rate of agonist-mediated internalization for palmitoylation-deficient mutants. In addition, we analyzed whether arrestin might modulate this process. COS-7 cells were transiently transfected with plasmids encoding the wild-type or mutated receptors in the presence or absence of β-arrestin2 over-expression. Binding of the receptor-specific antagonist [^{3}H]GR 113808 on intact cells was used to quantify the number of receptors at the cell surface. This assay revealed that the wild-type and all palmitoylation-deficient mutants showed similar expression levels at the cell surface under basal conditions (Table 1). Cells were then treated with the agonist, and internalization was measured up to 60 min after agonist exposure. As shown in the Fig. 7B, in the absence of recombinant β-arrestin2 overexpression, the wild type and all acylation-deficient mutants underwent slow agonist-mediated internalization. We found that after 60 min of incubation with 5-HT, only 25% of the wild-type, Cys386-Ser, and Cys328/329/386-Ser mutants were internalized, whereas internalization of the proximal mutant Cys328/329-Ser was more efficient; approximately 35% of this mutant was removed from the cell surface (Fig. 7, Table 1).

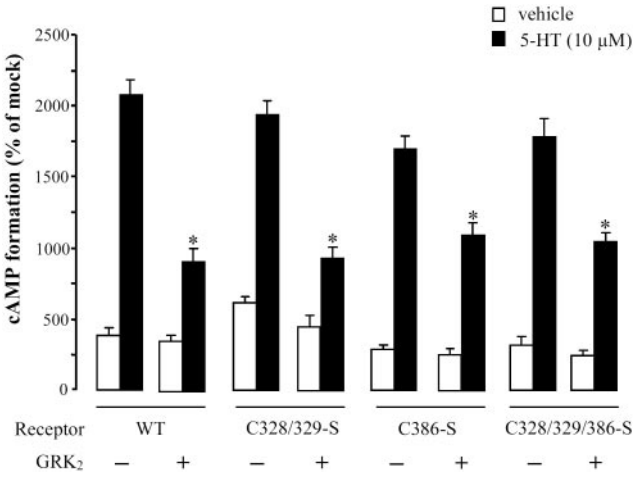

Fig. 6. Desensitization of the 5-HT$_{4a}$ receptors after overexpression of GRK2. COS-7 cells expressing either the 5-HT$_{4a}$ WT receptor or acylation-deficient mutants were cotransfected with or without the vector encoding GRK2 (500 ng/10^7 cells). The 5-HT (10 μM) induced cAMP production was directly measured during 10 min. Results are expressed as a percentage of the cAMP production in mock-transfected cells. In mock-transfected cells, 0.15 ± 0.02% of [^{3}H]ATP was converted into [^{3}H]cAMP. The values are plotted as the percentage conversion of [^{3}H]ATP into [^{3}H]cAMP. Each value represents the means ± S.E.M. from at least four independent experiments performed in triplicate. A statistically significant difference between the values is indicated (*, $p < 0.01$).

Coexpression of the 5-HT$_{4a}$ receptor wild-type with β-arrestin2 resulted only in moderate enhancement of internalization up to 30% (Fig. 7C). In the case of the Cys386-Ser and Cys328/329/386-Ser mutants, the rate of agonist-induced internalization upon coexpression with β-arrestin2 was increased up to 45% (Fig. 7C). The effect of β-arrestin2 overexpression was strongest with the Cys328/329-Ser mutant, where 77% of ligand binding sites were removed from the cell surface after 60-min incubation with 5-HT (Fig. 7D, Table 1). The different effects of β-arrestin2 overexpression on internalization of the receptor variants were found not to be caused by different β-arrestin2 expression levels in the various transfections.

The fact that the highest rate of receptor endocytosis was obtained for the mutant Cys328/329 suggests that the lack of palmitoylation at these sites renders 5-HT$_{4a}$ receptors more susceptible to β-arrestin2-mediated internalization, and this effect becomes more apparent under experimental conditions of β-arrestin2 overexpression.

Discussion

The results presented here demonstrate that the 5-HT$_{4a}$ receptor is a substrate for phosphorylation by endogenous cell kinase(s) upon exposure to agonist. A prominent role for GRKs in the regulation of this receptor can be assumed because of following observations: 1) The 5-HT$_{4a}$ receptor is rapidly ($t_{1/2} \sim 2$ min) phosphorylated upon agonist stimulation, and the concentration dependence of receptor phosphorylation ($EC_{50} \sim 180$ nM 5-HT) strictly followed ligand occupancy. 2) Neither activation nor inhibition of PKA, the kinase activated downstream of the 5-HT$_{4a}$ receptor, or other second messenger-activated protein kinases, such as protein kinase C or calmodulin kinase, affected 5-HT$_{4a}$ receptor phosphorylation in a noticeable manner. This finding is unexpected, because the receptor's third intracellular loop as well as the carboxyl terminus contains consensus sites for phosphorylation by protein kinase C and calcium/calmodulin-dependent kinase. A close correlation between dose-dependent receptor phosphorylation and ligand binding together with the apparent lack of any effect of second messenger-activated protein kinases strongly suggests the involvement of one or more G protein-coupled receptor kinases, because they specifically phosphorylate only agonist-occupied receptors (Bünemann and Hosey, 1999). GRKs from insects and mammals are evolutionarily conserved, which may explain why these kinases are capable of phosphorylating a wide range of receptor substrates (Cassill et al., 1991).

Examination of the 5-HT$_{4a}$ receptor C terminus reveals the existence of several potential serine or threonine phosphorylation sites. Some of these sites (e.g., Ser382 or Ser385) are highly conserved among human, rat, and murine 5-HT$_{4a}$ receptors. These serine residues are flanked by negatively charged amino acids, which previously were found to promote GRK-mediated phosphorylation (Onorato et al., 1991). Moreover, phosphoamino acid analysis revealed that ligand-

induced phosphorylation of the 5-HT$_{4a}$ receptor occurs exclusively on serine residues. It remains to be determined which of the six C-terminal serine residues, or possibly other serines located on cytoplasmic loops of the receptor, constitute phospho-acceptor sites for receptor kinases.

It is well documented that many GPCRs can be modified by both palmitoylation and phosphorylation and that these two post-translational modifications are interrelated. The findings of the present study are reminiscent of earlier work on the β2-adrenergic receptor in which a Cys341-Gly palmitoylation mutant found to be hyperphosphorylated at basal levels could not be enhanced by further stimulation (Moffett et al., 1993). It was concluded that lack of palmitoylated cysteine exposes a PKA phosphorylation site juxtaposed to the palmitoylation site. A marked increase in basal phosphorylation as a result of mutating two putative cysteine palmitoylation sites was also observed in the A3 adenosine receptor (Palmer and Stiles, 2000). However, GPCRs in their depalmitoylated states are not always better targets for receptor kinases. For example, a palmitoylation-deficient variant of the CCR5 chemokine receptor is not efficiently phosphorylated and reveals a profound defect in receptor desensitization and internalization (Kraft et al., 2001). Similar defects were observed for the vasopressin V1a receptor (Hawtin et al., 2001), whereas elimination of the two palmitoylated cysteine residues in the related V2 vasopressin receptor did not change basal or

TABLE 1

Cell surface expression of the 5-HT$_{4a}$ receptor

Comparison of the cell surface expression of the 5-HT$_{4a}$ receptor WT or acylation-deficient mutants in intact COS-7 cells before and after a 60-min pre-exposure to agonist in the absence or in the presence of transiently expressed β-arrestin2. Each value represents the mean ± S.E.M. from at least three independent experiments performed in triplicate. A statistically significant difference between the value obtained with β-arrestin2 expression after a 60-min pre-exposure to 5-HT for the C328/329-S mutant and the WT values is indicated (**, $p < 0.001$).

5-TH$_4$ R	5-TH$_4$R			
	Without β-Arrestin2		With β-Arrestin2	
	Before 5-HT Exposure	After 60-min 5-HT Exposure	Before 5-HT Exposure	After 60-min 5-HT Exposure
	fmol / 10^6 cells			
Wild-type	290 ± 23	217 ± 11	280 ± 12	190 ± 21
Cys328/329-Ser	310 ± 28	201 ± 14	275 ± 16	55 ± 9**
Cys386-Ser	298 ± 12	208 ± 21	305 ± 15	168 ± 11
Cys328/329/386-Ser	310 ± 18	229 ± 18	270 ± 24	148 ± 17

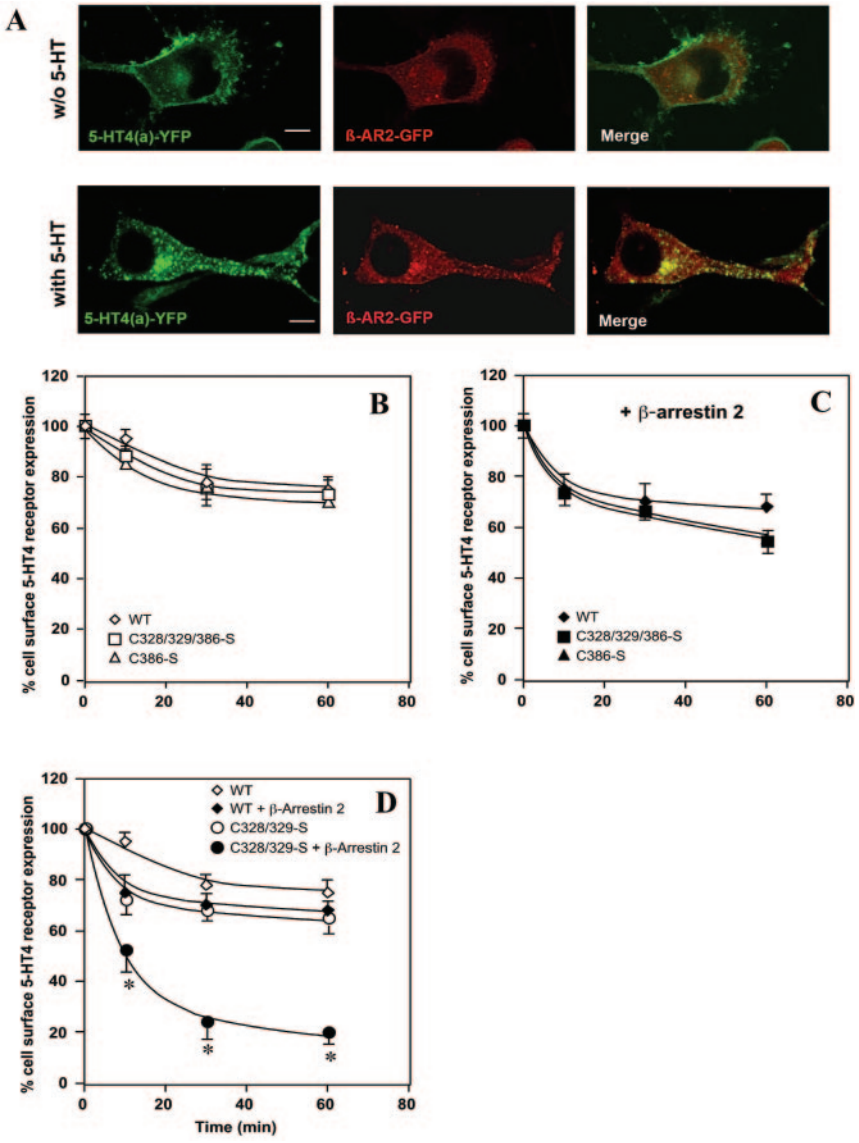

Fig. 7. Internalization of the 5-HT$_{4a}$ receptor and effect of β-arrestin2 overexpression. A, COS-7 cells expressing YFP-tagged 5-HT$_{4a}$ receptor and GFP-tagged β-arrestin2 (β-AR2-GFP) were incubated for 1 h with or without 1 μM 5-HT followed by fixation and microscopic analysis. Representative confocal images obtained with LSM510-Meta microscope at 63× magnification are shown. Scale bar, 10 μM. B–D, COS-7 cells were transiently transfected with vectors encoding WT or acylation-deficient mutants as indicated. The cells were stimulated with 1 μM 5-HT at 37°C and extensively washed; then, sequestration of antagonist [^{3}H]GR 113808 from the cell surface was measured as described under *Materials and Methods*. Kinetics of internalization for the WT and indicated acylation-deficient mutants in the absence (B) or presence (C) of β-arrestin2 overexpression are shown. D, comparison of internalization kinetics for the WT and Cys328/329-Ser mutant in the presence and absence of β-arrestin2 overexpression. Data represent the means ± S.E.M. from five independent experiments performed in duplicate. A statistically significant difference between the values for the WT and Cys328/329-Ser mutant in the presence of β-arrestin2 overexpression is indicated (*, $p < 0.01$).

agonist-stimulated phosphorylation (Sadeghi et al., 1997). Moreover, a mass spectrometric study of post-translational modifications of the bradykinin B_2 receptor revealed that palmitoylation at Cys356 and phosphorylation at Tyr352 were mutually exclusive (Soskic et al., 1999). Taken together, these studies clearly show that in GPCRs, palmitoylation and phosphorylation are often interrelated, albeit in different ways.

Agonist-induced 5-HT_{4a} receptor phosphorylation established in the present study seems to be functionally significant. We reported previously that 5-HT_4 receptors expressed in colliculus neurons undergo homologous receptor desensitization (Ansanay et al., 1992). Neither activation nor inhibition of cAMP-dependent protein kinase affected receptor desensitization, whereas inhibitors of G protein-coupled receptor kinases, such as heparin or Zn^{2+}, caused significant decrease in 5-HT-induced receptor desensitization. Similar to the present findings on 5-HT_{4a} receptor regulation in a heterologous expression system, the 5-HT_4 receptor desensitization in native tissues also proceeded in a biphasic manner and possessed comparable kinetics of the rapid phase of receptor desensitization. Further support for a role for GRKs in 5-HT_{4a} receptor regulation derives from the observation that overexpression of GRK2 significantly enhanced 5-HT_{4a} receptor desensitization.

A role for PKA in 5-HT_4 receptor regulation was also suggested recently based on the observation that receptor-independent activation of PKA could mimic 5-HT induced desensitization of the 5-HT_{4d} receptor isoform (Mialet et al., 2003). However, mutation of the four PKA consensus sites on this receptor did not impair PKA-induced 5-HT_{4d} receptor desensitization. In combination with our finding that, on its own, PKA activation did not result in 5-HT_{4a} receptor phosphorylation, this implies that PKA may phosphorylate another protein substrate downstream of this receptor. Adenylyl cyclase, which is inhibited by PKA phosphorylation (Iwami et al., 1995), is a potential substrate for such regulation.

The 5-HT_{4a} receptor is atypical among GPCRs because of the presence of two separate C-terminal palmitoylation sites operating as potential membrane anchorage points at positions Cys-328/329 and Cys-386. Together with the observation that the palmitoylation state of the receptor is agonist-sensitive (Ponimaskin et al., 2001), this dual lipidation suggests that in a native cellular environment, the 5-HT_{4a} receptor C terminus exists in several different conformational states that can be modulated by external stimuli. In the present work, we tested the hypothesis that regulatory cytoplasmic proteins, which bind to or phosphorylate the C-terminal domain, may interact with these receptor conformers in a differential manner. Earlier studies in which the same receptor variants were used, revealed that mutation of the proximal Cys-328/329 couplet resulted in elevated constitutive activity of this receptor (Ponimaskin et al., 2002a). In contrast, mutations of the distal Cys-386 or of all C-terminal palmitoylation sites showed no increase in the basal activity. We concluded from these studies that defective palmitoylation at the proximal membrane anchorage site induces a conformation of the 5-HT_{4a} C terminus to facilitate receptor binding and/or activation of heterotrimeric G proteins (Ponimaskin et al., 2002a). Our present finding of elevated basal phosphorylation levels of the proximal Cys328/329-Ser mutant may be caused by its higher constitutive activity, as was reported before in other receptor systems (Pei et al., 1994). This interpretation, however, does not explain why the Cys386-Ser mutant and the completely palmitoylation-deficient receptor are also better substrates for receptor kinases. Conformational changes occurring at the C terminus during basal or agonist-stimulated depalmitoylation of the receptor possibly lead to an exposure of one or several protein kinase binding site(s). Such receptor structure seems not to be identical with the conformation that is optimal for G protein activation. Several putative serine phosphorylation sites are located near the palmitoylated C-terminal cysteine residues, which supports the assumption that palmitoylation may restrain access of these sites to the receptor kinase. Enhanced receptor phosphorylation in case of the palmitoylation-deficient mutants could also be caused by the presence of additional phospho-acceptor sites that may have been introduced by the conservative replacement of cysteine by serine residues. However, this seems unlikely, because the triple mutant Cys328/329/386-Ser was phosphorylated even less than the single or double mutants.

Among the different receptor variants, the Cys328/329-Ser mutant shows high agonist-independent receptor activity. It also seems to be a preferred substrate for receptor kinases and is obviously most susceptible to ligand-induced desensitization and β-arrestin-mediated internalization. These findings demonstrate that the palmitoylation status of the 5-HT_{4a} receptor modifies its ability to interact with other downstream regulatory proteins. The receptor mutants were affected in their capacity to undergo agonist-independent receptor activation, phosphorylation, desensitization, and β-arrestin-mediated internalization with different rank orders, which suggests that interactions of regulatory proteins with the palmitoylated receptor have different structural requirements. Although all receptor mutants were hyperphosphorylated, only the Cys328/329-Ser mutant was rapidly and efficiently internalized in β-arrestin-overexpressing cells. Thus, it is unlikely that GRK-mediated hyperphosphorylation alone is responsible for the enhanced endocytosis. Instead, our data suggest that the Cys328/329-Ser receptor mutant, having only a single palmitoylation site distal to the membrane, represents a specific receptor conformation facilitating β-arrestin-mediated endocytosis. According to the sequential multisite model of β-arrestin interaction with ligand-activated receptors, high-affinity binding of arrestin to GPCR involves two separate phosphorylation-recognition and activation-recognition sites that reside within different parts of the molecule (Han et al., 2001). We did not directly assess β-arrestin binding to palmitoylation-deficient receptor mutants, as was reported previously for the V2 vasopressin receptor (Charest and Bouvier, 2003). In contrast to 5-HT_{4a} receptors, nonpalmitoylated V2 vasopressin receptor showed no difference in basal or agonist-induced phosphorylation but is much more slowly internalized than wild-type receptors (Sadeghi et al., 1997; Charest and Bouvier, 2003). Mutation of the palmitoylation site also decreased the rate and the extent of β-arrestin recruitment to this receptor. Likewise, receptor palmitoylation was also found to be required for ligand-induced β-arrestin recruitment to the cell membrane and endocytosis of the thyrotropin-releasing hormone receptor (Groarke et al., 2001). On the contrary, mutation of the membrane-proximal palmitoylation site facilitates β-arrestin-mediated internalization of 5-HT_{4a} receptors.

In summary, our study shows that 5-HT$_{4a}$ receptor palmitoylation is functionally significant, because disruption of C-terminal palmitoylation sites resulted in enhanced receptor phosphorylation, desensitization, and β-arrestin-mediated endocytosis. Because both the phosphorylation and the palmitoylation status of the 5-HT$_{4a}$ receptor are dynamically regulated by agonist stimulation, concerted interactions of these two post-translational modifications seem to play an essential role in modulating the function of 5-HT$_{4a}$ receptors.

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