5-Hydroxytryptamine 4(a) receptor expressed in Sf9 cells is palmitoylated in an agonist-dependent manner

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The mouse 5-hydroxytryptamine 4(a) receptor [5-$HT_{4(a)}$] was expressed with a baculovirus system in insect cells and analysed for acylation. [^{3}H]Palmitic acid was effectively incorporated into 5-$HT_{4(a)}$ and label was sensitive to the treatment with reducing agents indicating a thioester-type bond. Analysis of protein-bound fatty acids revealed that 5-$HT_{4(a)}$ contains predominantly palmitic acid. Treatment of infected Sf9 (*Spodoptera frugiperda*) cells with BIMU8 {(endo-N-8-methyl-8-azabicyclo[3.2.1]oct-3-yl)-2,3-dehydro-2-oxo-3-(prop-2-yl)-1H-benzimid-azole-1-carboxamide}, a 5-HT_4 receptor-selective agonist, generated a dose-dependent increase in [^{3}H]palmitate incorporation into 5-$HT_{4(a)}$ with an EC_{50} of approx. 10 nM. The change in receptor labelling after stimulation with agonist was receptor-specific and did not result from general metabolic effects. We also used both pulse labelling and pulse–chase labelling to address the dynamics of 5-$HT_{4(a)}$ palmitoylation. Incorporation studies revealed that the rate of palmitate incorporation was increased approx. 3-fold after stimulation with agonist. Results of pulse–chase experiments show that activation with BIMU8 promoted the release of radiolabel from 5-$HT_{4(a)}$, thereby reducing the levels of receptor-bound palmitate to approximately one-half. Taken together, our results demonstrate that palmitoylation of 5-$HT_{4(a)}$ is a reversible process and that stimulation of 5-$HT_{4(a)}$ with agonist increases the turnover rate for receptor-bound palmitate. This provides evidence for a regulated cycling of receptor-bound palmitate and suggests a functional role for palmitoylation/depalmitoylation in 5-hydroxytryptamine-mediated signalling.

Key words: acylation, antibodies, G-protein-coupled receptors, recombinant baculovirus, signal transduction.

INTRODUCTION

The covalent attachment of fatty acids to proteins (acylation) is a widespread modification of both cellular and viral polypeptides [1–3]. Two main modes of acylation have been described: N-myristoylation and palmitoylation (S-acylation). N-myristoylation is a co-translational modification catalysed by N-myristoyltransferase, which modifies a glycine residue within a consensus sequence at the protein N-terminus via an amide linkage [4,5]. In contrast with myristoylation, the addition of long-chain fatty acids (mainly palmitic acid) is a post-translational event that occurs through the covalent linkage of palmitate via a labile thioester bond to cysteine residues [6]. Among the cellular palmitoylated proteins, polypeptides involved in signal transduction [e.g. G-protein-coupled receptors (GPCRs), G-protein α-subunits and adenylate cyclases] are prevalent. GPCRs are often palmitoylated on cysteine residues located at the boundary between the seventh transmembrane region and the cytoplasmic tail. Although there is no clear consensus sequence for the acylation of integral membrane proteins, it has been suggested that the signals for palmitoylation comprise a complex conformational nature and are located mainly within the transmembrane domain [7,8].

With the finding that palmitoylation is a dynamic process it is now widely accepted that repeated cycles of palmitoylation and depalmitoylation could be involved in the regulation of signalling processes [9–11]. In GPCRs the functions of palmitoylation cover a wide spectrum of biological activities: from regulated endocytosis to receptor phosphorylation and desensitization [9,10,12]. For example, prevention of palmitoylation of the β-adrenergic receptor causes an increased basal phosphorylation and rapid desensitization in response to ligand binding [13]. The long-term stimulation of the β_2-adrenergic receptor with agonist, which promotes receptor phosphorylation, also increases receptor depalmitoylation, resulting in decreased signalling through the receptor [14]. A mutation of the corresponding palmitoylation site in the α_{A2}-adrenergic receptor completely abolishes the down-regulation of this receptor during prolonged exposure to agonist [15]. In addition, the activation of α-adrenergic and β-adrenergic receptors itself modulates the palmitoylation of receptor-coupled G-proteins [16–18]. Removal of the palmitoyl anchors from the rhodopsin C-terminus impairs its stimulatory activity towards all-*trans*-retinal, suggesting the involvement of palmitoylation in dark adaptation [19]. These results show that palmitoylation can modulate different biological activities of receptors, although it seems that there is no common function applicable to all GPCRs. An analysis of the functions of palmitoylation is therefore necessary for an understanding of the signalling mechanisms of individual receptors.

5-Hydroxytryptamine is a neuromodulator involved in a wide range of physiological functions via the activation of a large family of receptors. With the exception of the 5-HT_3 receptor, which is a cation channel, all other 5-hydroxytryptamine receptors are members of the superfamily of seven transmembrane-spanning GPCRs. The 5-HT_4 receptor is expressed in a wide variety of tissues, including brain, gastrointestinal tract and heart [20,21]. In the mammalian brain the 5-HT_4 receptor contributes to the control of dopamine secretion and regulates learning and long-term memory [22,23]. Furthermore, the 5-HT_4 receptor is thought to be involved in various

Abbreviations used: 5-$HT_{4(a)}$, mouse 5-hydroxytryptamine 4(a) receptor; β_2AR, β_2-adrenergic receptor; BIMU8, (endo-N-8-methyl-8-azabicyclo[3.2.1]oct-3-yl)-2,3-dehydro-2-oxo-3-(prop-2-yl)-1H-benzimid-azole-1-carboxamide; GR113808A, {1-[2-(methylsulphonylamino)ethyl]-4-piperidinyl}methyl-1-methyl-1-H-indole-3-carboxylate, maleate salt; GPCRs, G-protein-coupled receptors.

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central and peripheral disorders, including neurodegenerative disease [24]. The 5-HT_4 receptor is positively coupled to adenylate cyclase, increasing cAMP levels and leading to the phosphorylation of a number of target proteins [25]. The broad distribution of 5-HT_4 receptors is paralleled by the existence of various 5-HT_4 splicing variants. Functional expression has been reported for five C-terminal variants and one internal splice variant in humans [26]. In addition, four 5-HT_4 receptor isoforms, 5-$HT_{4(a)}$, 5-$HT_{4(b)}$, 5-$HT_{4(e)}$ and 5-$HT_{4(f)}$, were cloned in mouse [27]. All of these variants except the internal splice product in humans share the same sequence up to Leu-358 followed by unique C-termini.

In the present study we investigated fatty acylation of the mouse 5-HT_4 receptor. After expression of the recombinant receptor in insect *Spodoptera frugiperda* (Sf9) cells and immunoprecipitation with newly developed antibodies that specifically recognize the 5-$HT_{4(a)}$ isoform, we show that this receptor contains covalently bound palmitic acid. The cleavage of fatty acids after treatment with hydroxylamine and 2-mercaptoethanol indicates that palmitate is attached in an *S*-ester-type linkage. By the use of pulse-labelling and pulse–chase labelling we show that the palmitoylation of the 5-$HT_{4(a)}$ receptor is a reversible process. We also report that the exchange of palmitate bound to 5-$HT_{4(a)}$ is accelerated after exposure to the receptor-specific agonist (endo-N-8-methyl-8-azabicyclo[3.2.1]oct-3-yl)-2,3-dehydro-2-oxo-3-(prop-2-yl)-1H-benzimid-azole-1-carboxamide (BIMU8).

EXPERIMENTAL

Materials

[9,10-^{3}H(N)]Palmitic acid (30–60 Ci/mmol) was purchased from Hartmann Analytic GmbH (Braunschweig, Germany); Tran^{35}S-label (more than 1000 Ci/mmol) was from ICN (Eschwege, Germany). Enzymes used in molecular cloning were obtained from New England Biolabs (Schwalbach, Germany). 5-Hydroxytryptamine and Protein A–Sepharose CL-4B beads were from Sigma (Deisenhofen, Germany), {1-[2-(methylsulphonylamino)-ethyl]-4-piperidinyl}methyl-1-methyl-1-H-indole-3-carboxylate, maleate salt (GR113808A) was a gift from Glaxo Wellcome (Stevenage, Herts., U.K.). BIMU8 was kindly provided by Boehringer (Ingelheim, Germany). TC-100 insect cell medium, Cellfectin® Reagent and fetal calf serum were purchased from Life Technologies (Eggenstein-Leopoldshafen, 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 Gibco BRL (Karlsruhe, Germany). A polyclonal antiserum, designated AS9459, was raised against a peptide containing amino acid residues 364–380 (CHSGHHQELEKLPIHNDP; single-letter amino acid codes) corresponding to the deduced C-terminus of 5-$HT_{4(a)}$. The peptide was purified by HPLC, coupled to keyhole-limpet haemocyanin, mixed with Freund's complete adjuvant and used for the first injection into the rabbit. Second and third injections of peptide mixed with Freund's incomplete adjuvant were performed at 4 week intervals. Serum collected 4 weeks after the third injection was used for immunoprecipitation and immunofluorescence at dilutions of 1:60 and 1:200 respectively.

Construction of recombinant baculovirus

All basic DNA procedures were as described by Sambrook et al. [28]. The gene encoding 5-$HT_{4(a)}$ was kindly provided by Dr Aline Dumuis (Montpellier, France). The cDNA for 5-$HT_{4(a)}$ was cleaved with *Xba*I and *Hin*dIII endonucleases to yield a 1.1 kb fragment containing the entire coding sequence. The fragment was ligated to the *Xba*I and *Hin*dIII sites in the multiple cloning site of the pFastBac donor plasmid (Life Technologies). The resulting plasmid was transfected into DH10Bac *Escherichia coli* cells containing bacmid and helper DNA. Recombinant bacmid DNA was then purified, checked for the presence of the gene encoding 5-$HT_{4(a)}$ by PCR with receptor-specific primers and transfected into Sf9 cells with Cellfectin reagent. Finally, the recombinant virus was purified and amplified as described previously [29].

Metabolic labelling and immunoprecipitation

Sf9 cells were grown in TC-100 medium supplemented with 10% (v/v) fetal calf serum and 1% (w/v) penicillin/streptomycin (complete TC-100). For expression, Sf9 cells (1.5×10^6) grown in 3.5 mm dishes were infected with recombinant baculovirus encoding 5-$HT_{4(a)}$ at a multiplicity of infection of 10 plaque-forming units per cell. After 48 h, Sf9 cells were labelled with Tran^{35}S-label (50 μCi/ml in TC-100 medium without methionine) or [^{3}H]palmitic acid (300 μCi/ml in TC-100 medium, 30–60 Ci/mmol) for the periods indicated in figure legends. For the pulse–chase experiments, cells were subsequently incubated with complete TC-100 medium supplemented with 100 μM unlabelled palmitate and 5 mM sodium pyruvate. In some experiments, BIMU8 and GR113808A were added to the final concentrations indicated in figure legends. To block protein synthesis, cycloheximide (50 μg/ml) was added 10 min before incubation with [^{3}H]palmitate or [^{35}S]methionine. After labelling (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% (v/v) Nonidet P40/150 mM NaCl/50 mM Tris/HCl (pH 7.9)/5 mM EDTA/10 mM iodoacetamide/1 mM PMSF]. Insoluble material was pelleted (5 min, 20000 ***g***) and antibodies AS9459 raised against the C-terminal peptide of 5-$HT_{4(a)}$ (CHSGHHQELEKLPIH-NDP) were added to the resulting supernatant at a dilution of 1:60. After agitation overnight at 4 °C, 30 μl of Protein A–Sepharose CL-4B was added, and samples were incubated with gentle rocking for 2 h. After a 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 non-reducing electrophoresis sample buffer [62.5 mM Tris/HCl, pH 6.8, containing 20% (v/v) glycerol, 6% (w/v) SDS and 0.002% Bromophenol Blue]. Radiolabelled polypeptides were analysed by SDS/PAGE [12% (w/v) gel] and detected by fluorography with Kodak X-Omat AR films. Densitometric analysis of fluorograms was performed with Gel-Pro Analyzer software, version 3.1.

Treatment with hydroxylamine

SDS-containing gels containing 5-$HT_{4(a)}$ labelled with [^{3}H]-palmitic acid were fixed [10% (v/v) acetic acid/10% (v/v) methanol] and then soaked in water for 30 min. They were then treated overnight, under gentle agitation, with 1 M hydroxylamine, pH 7.5, or 1 M Tris/HCl, pH 7.5. The gels were washed in water and then rocked for 30 min in DMSO to wash out cleaved fatty acids. The gels were again soaked twice in water for 30 min to remove DMSO, then processed for fluorography.

Fatty acid analysis

[^{3}H]Palmitate-labelled 5-$HT_{4(a)}$ was purified by immunoprecipitation and subjected to SDS/PAGE and fluorography.

The acyl protein band was excised from the gel, soaked in water twice for 30 min and then dried under vacuum in a desiccator. Fatty acids were cleaved by treatment of the dried gel slices with 6 M HCl for at least 16 h at 110 °C in tightly sealed ampoules. Fatty acids were then extracted three times with hexane; the upper phases were pooled and concentrated. Separation into individual fatty acid species was performed on RP-18 TLC plates (Merck) with acetonitrile/acetic acid (1:1, v/v) as the mobile phase. Radiolabelled fatty acids were detected by fluorography after the plates had been sprayed with En^{3}Hance (DuPont). For identification of fatty acid species, radiolabelled marker fatty acids ([^{3}H]myristate, [^{3}H]palmitate and [^{3}H]stearate) were run on the same plate in parallel.

Indirect immunofluorescence

At 48 h after infection with recombinant 5-HT$_{4(a)}$ baculovirus or with wild-type baculovirus, Sf9 cells grown on coverslips were fixed with paraformaldehyde [3 % (w/v) in PBS] for 15 min. The cells were washed three time with PBS and unreacted paraformaldehyde was quenched with 100 mM glycine for 15 min. Cells were permeabilized with Triton X-100 [0.1 % (v/v) in PBS] and then incubated for 1 h with the first antibody, AS9459, diluted 1:200 in PBS containing 2 % (w/v) BSA. Subsequently, the second antibody (Fluor 488; Alexa, Netherlands) [diluted 1:100 in PBS containing 2 % (w/v) BSA] was adsorbed to the cells for 1 h. Unbound antibodies were washed off after every step with PBS and coverslips were finally mounted in 90 % (v/v) glycerol. Cells were observed under a fluorescence microscope (Axioscop1; Zeiss) with a 'green' filter set (488 nm excitation; 520 nm emission). The results were recorded with a digital camera RTE/CCD-782 (Princeton Instruments) at × 630 magnification.

RESULTS

Detection of 5-HT$_{4(a)}$ by anti-peptide antibody

To examine post-translational modifications of 5-HT$_{4(a)}$, antibodies against a synthetic peptide corresponding to its C-terminus-specific sequence (His-364 to Pro-380) were generated in rabbits. High-titre baculovirus stock containing the cDNA of the 5-HT$_{4(a)}$ isoform was prepared as described in the Experimental section and used to infect Sf9 cells. To monitor the expression and intracellular distribution of the receptor, infected Sf9 cells were first subjected to immunofluorescence. The results in Figure 1(A) show that 5-HT$_{4(a)}$ was specifically detected with the newly developed antibodies and was exposed mainly on the cell surface. As seen in Figure 1(B), labelling with [^{35}S]methionine followed by immunoprecipitation and SDS/PAGE analysis revealed a single protein band with a molecular mass of approx. 42 kDa, which corresponds to the predicted molecular mass of the 5-HT$_4$ receptor. No specific bands were found in immunoprecipitates of non-infected Sf9 cells or of Sf9 cells infected with the wild-type baculovirus (Figure 1B, left panel). This emphasizes that the immunoprecipitated 42 kDa protein shown in Figure 1(B) was indeed 5-HT$_{4(a)}$. In addition, the functional expression of 5-HT$_{4(a)}$ in Sf9 cells was confirmed by several assays including coupling to co-expressed G-proteins as well as the modulation of a cyclic-nucleotide-activated and voltage-activated ion channel by receptor agonists (results not shown).

Palmitoylation of 5-HT$_{4(a)}$

To examine whether 5-HT$_{4(a)}$ is acyl-modified, Sf9 cells infected with recombinant or wild-type baculovirus were metabolically labelled with [^{3}H]palmitic acid. The resulting fluorogram (Figure 1B, right panel) demonstrates that 5-HT$_{4(a)}$ effectively incorporated [^{3}H]palmitate and that the labelled polypeptide co-migrated with the [^{35}S]methionine-labelled 5-HT$_{4(a)}$ protein.

Next we determined the chemical nature of the fatty acid bond in 5-HT$_{4(a)}$ to distinguish between amide-type and ester-type fatty acid linkages. In contrast with the amide bond linkage, the *S*-ester and hydroxyester linkages are sensitive to the presence of 2-mercaptoethanol [30]. Moreover, the *S*-ester bond can be distinguished from the hydroxyester by its sensitivity to treatment with hydroxylamine [31]. To investigate whether the fatty acid was attached to 5-HT$_{4(a)}$ by an *S*-ester bond, proteins labelled

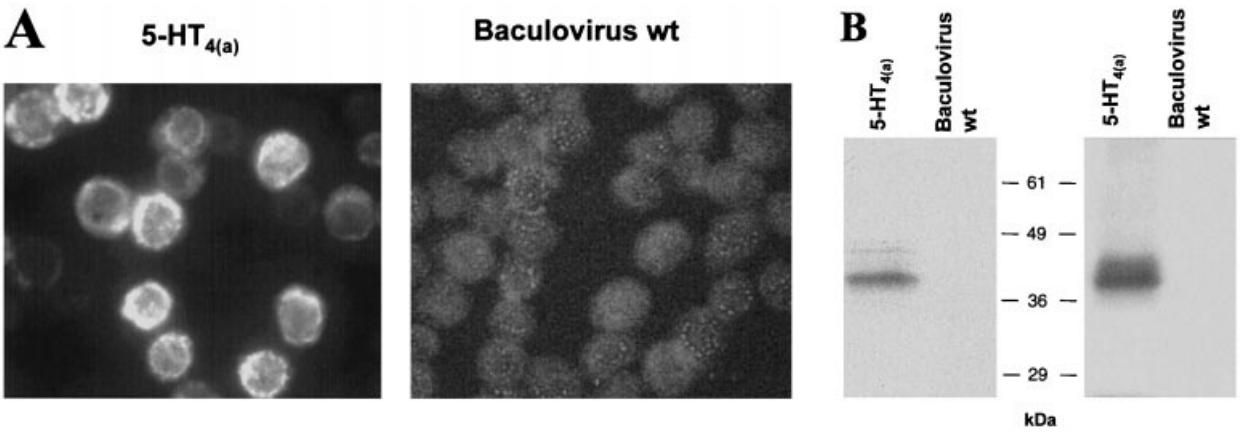

Figure 1 Expression and palmitoylation of 5-HT$_{4(a)}$

(**A**) Sf9 cells infected with recombinant or wild-type (wt) baculovirus were fixed with paraformaldehyde at 48 h after infection and then subjected to immunofluorescence staining with an antibody (AS9459) raised against the C-terminus of 5-HT$_{4(a)}$. (**B**) 5-HT$_{4(a)}$ was expressed in Sf9 cells, labelled either with [^{35}S]methionine (left panel) or [^{3}H]palmitic acid (right panel) and subjected to immunoprecipitation with antibody AS9459 followed by SDS/PAGE and fluorography. The exposure time was 1 day for labelling with [^{35}S]methionine and 2 days for labelling with [^{3}H]palmitate. Sf9 cells infected with wild-type baculovirus served as control. The positions of molecular mass markers are indicated between the panels.

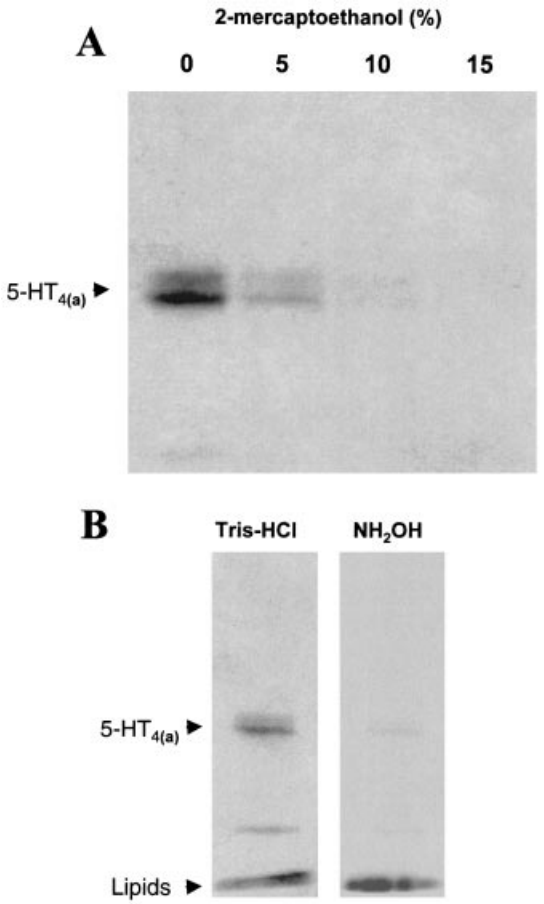

Figure 2 Sensitivity of the fatty acyl bond to hydroxylamine and 2-mercaptoethanol

(**A**) [^{3}H]Palmitate-labelled 5-HT$_{4(a)}$ was immunoprecipitated and treated with non-reducing gel-loading buffer or with the same buffer containing 5%, 10% or 15% (v/v) 2-mercaptoethanol for 30 min at 37 °C before SDS/PAGE and fluorography. Representative of three independent experiments. (**B**) Sf9 cells expressing 5-HT$_{4(a)}$ were labelled with [^{3}H]palmitate, immunoprecipitated and subjected to SDS/PAGE. The gel was treated with 1 M Tris/HCl (left panel) or 1 M hydroxylamine (right panel) before fluorography. The fluorogram shown is representative of two independent experiments.

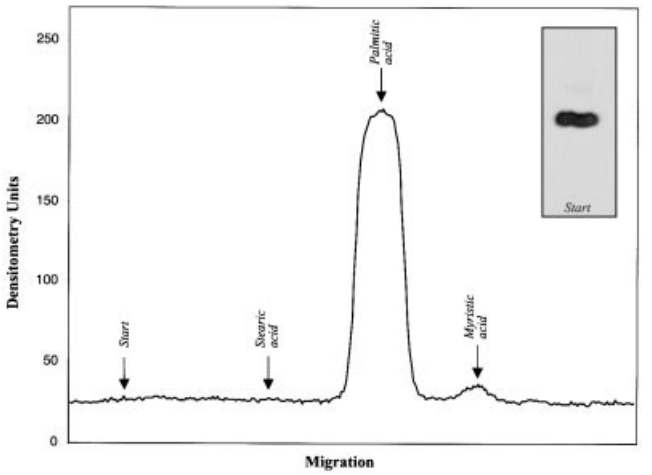

Figure 3 Chromatographic identification of receptor-bound fatty acids

[^{3}H]Palmitate-labelled 5-HT$_{4(a)}$ was purified by immunoprecipitation, then subjected to SDS/PAGE and fluorography. Receptor-bound fatty acids were hydrolysed, extracted and separated by TLC. The fluorogram obtained from the TLC plate after 2 days of exposure (inset) was analysed with Gel-Pro Analyzer software, version 3.1.

with [^{3}H]palmitic acid were subjected to treatment with 2-mercaptoethanol. The results in Figure 2(A) show that [^{3}H]-palmitate-derived radioactivity bound to the protein was sensitive to heating with buffer supplemented with increasing concentrations of 2-mercaptoethanol. This suggests that 5-HT$_{4(a)}$ contains exclusively *S*-ester-linked acyl groups and no fatty acids linked by an amide bond, which would have been resistant to such treatment. Furthermore, after treatment of gels containing fatty-acid-labelled 5-HT$_{4(a)}$ protein with neutral hydroxylamine, hydroxylamine cleaved the [^{3}H]palmitate-derived label from the receptor, whereas labelled lipids remained unaffected (Figure 2B). This sensitivity to neutral hydroxylamine and reducing agent indicates that fatty acid is bound to 5-HT$_{4(a)}$ via an *S*-ester-type linkage.

To prove the identity of the actual protein-bound fatty acids in 5-HT$_{4(a)}$ protein after labelling with [^{3}H]palmitic acid, receptors were subjected to fatty acid analysis. Fatty acids were hydrolysed from gel-purified 5-HT$_{4(a)}$ and separated into the individual fatty acid species by TLC. Radiochromatographic scanning of the TLC plates (Figure 3) revealed that 5-HT$_{4(a)}$ contained predominantly palmitic acid, with traces (less than 1%) of myristic acid.

Activation of 5-HT$_{4(a)}$ induces changes in its palmitoylation

To test whether the incorporation of [^{3}H]palmitate into 5-HT$_{4(a)}$ is promoted by agonist, we performed experiments with Sf9 cells expressing 5-HT$_{4(a)}$ protein. Cells were incubated for 60 min in the absence or presence of increasing concentrations of BIMU8, a 5-HT$_4$ receptor-selective agonist [21]. Levels of radiolabel incorporated were evaluated from fluorograms of SDS-containing gels after the immunoprecipitation of 5-HT$_{4(a)}$. As shown in Figure 4, BIMU8 generated a dose-dependent increase in the labelling intensity, with an EC$_{50}$ of approx. 10 nM. The change in receptor labelling after stimulation with agonist was not due to any general metabolic effects because the incorporation of [^{3}H]palmitate into total membrane proteins was not affected by treatment with agonist (Figure 4C). It should also be noted that stimulation with agonist did not increase the amount of newly synthesized receptor. Indeed, as seen in the inset to Figure 4(A) and in Figure 4(C), the incorporation of [^{35}S]methionine into 5-HT$_{4(a)}$ as well as into total membrane proteins was even decreased slightly after the application of agonist. In general we obtained a 5–15% decrease in protein synthesis after stimulation with agonist, depending on the experiment. Thus the absolute increase in the rate of palmitate incorporation was approx. 3-fold after stimulation with 100 nM agonist. In all subsequent experiments the BIMU8 concentration was kept at 100 nM.

To exclude further the influence of possible changes in the rate of receptor synthesis on palmitoylation, receptor-expressing cells were labelled with [^{3}H]palmitate or [^{35}S]methionine in the absence or presence of cycloheximide. The resulting fluorogram (Figure 5) shows that despite the block of protein synthesis, 5-HT$_{4(a)}$ still incorporated palmitic acid and the increase in its radiolabelling was promoted by agonist. These results demonstrate that fluctuations in receptor synthesis cannot account for the above observations. In addition, these results emphasize the post-translational nature of palmitoylation and indicate that

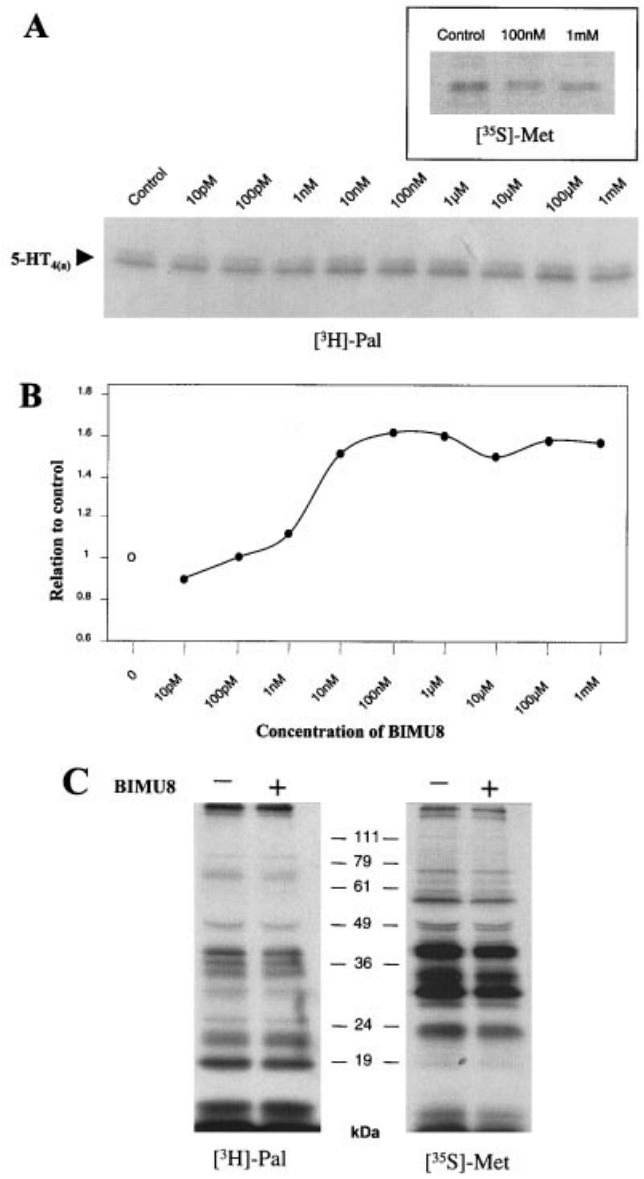

Figure 4 Agonist-promoted incorporation of [^{3}H]palmitate into 5-HT$_{4(a)}$

(**A**) Sf9 cells expressing 5-HT$_{4(a)}$ were labelled with [^{3}H]palmitate ([^{3}H]-Pal) for 60 min in the absence [open circle in (**B**)] or in the presence of increasing concentrations of BIMU8 [filled circles in (**B**)]. The receptor was immunoprecipitated, separated by SDS/PAGE [12% (w/v) gel] and subjected to fluorography. The inset shows the effect of BIMU8 on 5-HT$_{4(a)}$ synthesis as assessed by labelling with [^{35}S]methionine ([^{35}S]-Met). The results shown are representative of three independent experiments. (**B**) Results of densitometric analysis of fluorograph shown in (**A**). (**C**) Incorporation of [^{3}H]palmitate and [^{35}S]methionine into total membrane protein. Sf9 cells infected with recombinant virus were labelled with the respective isotopes for 1 h in the absence (−) or presence (+) of 100 nM BIMU8 and then subjected to SDS/PAGE and fluorography. Fluorograms after exposure for 3 days ([^{35}S]methionine) and 14 days ([^{3}H]palmitate) are shown. The positions of molecular mass markers are indicated between the panels.

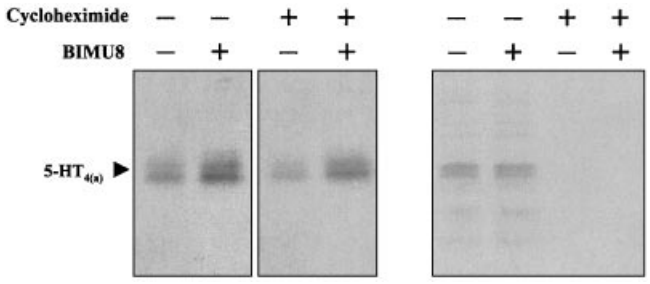

Figure 5 Palmitoylation of 5-HT$_{4(a)}$ does not require protein synthesis

Insect cells expressing 5-HT$_{4(a)}$ were labelled for 60 min with [^{3}H]palmitate (left panel) or [^{35}S]methionine (right panel) in the absence (−) or presence (+) of cycloheximide (50 μg/ml). In parallel, 100 nM BIMU8 or vehicle (water) was added. The incorporation of radiolabel was detected by immunoprecipitation followed by SDS/PAGE and fluorography. The fluorograms shown are representative of three independent experiments.

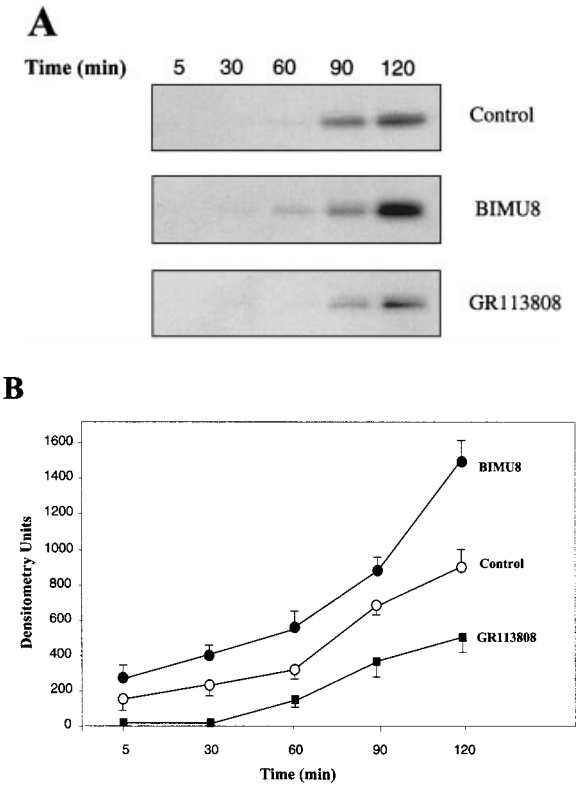

Figure 6 Time course of agonist or antagonist effects on the incorporation of [^{3}H]palmitate into 5-HT$_{4(a)}$

Sf9 cells expressing 5-HT$_{4(a)}$ were incubated with [^{3}H]palmitate in the presence of vehicle (water, control), 100 nM BIMU8 or 10 μM GR113808 for the periods indicated. Receptor was immunoprecipitated, resolved by SDS/PAGE and detected by fluorography. (**A**) A representative fluorogram of three independent experiments. (**B**) The intensity of labelling was assessed by densitometry with Gel-Pro Analyzer software, version 3.1; results are means ± S.D.

previously synthesized 5-HT$_{4(a)}$ must have been made available for further rounds of palmitoylation by deacylation.

To obtain information on the dynamics of palmitoylation, we studied the incorporation of [^{3}H]palmitic acid into 5-HT$_{4(a)}$ as a function of time. Sf9 cells infected with recombinant virus were incubated for 5, 30, 60, 90 and 120 min with [^{3}H]palmitate before immunoprecipitation and SDS/PAGE. As shown in Figure 6 (control), the intensity of radiolabelling of the receptor increased steadily, reflecting a basal turnover of palmitate. To determine whether the activation of receptor influenced its palmitoylation state, the kinetics of [^{3}H]palmitate incorporation was studied in

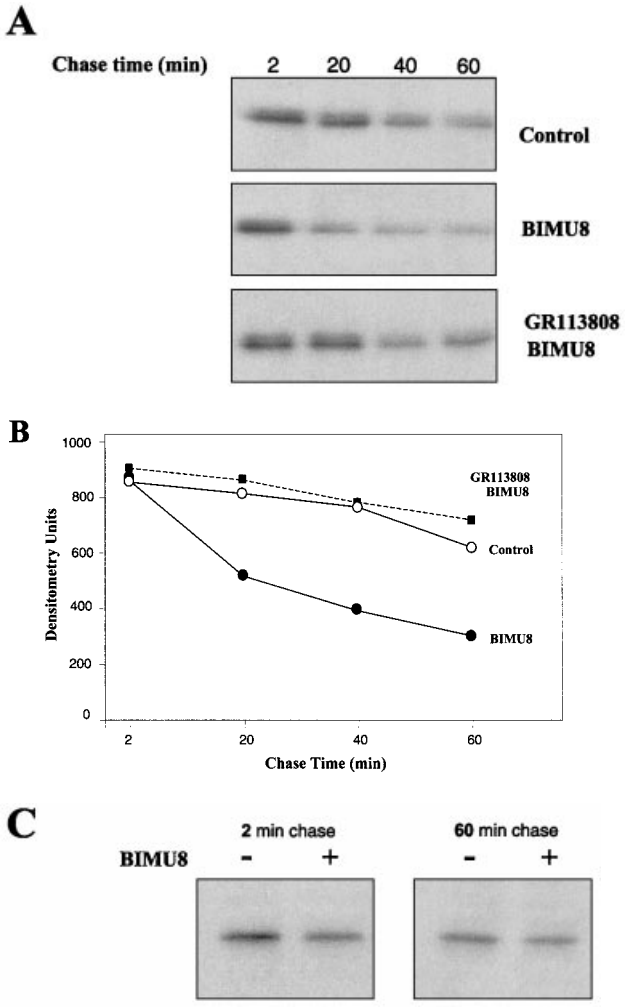

Figure 7 Effect of agonist and antagonist on the turnover rate of 5-HT$_{4(a)}$-bound palmitate

Sf9 cells expressing 5-HT$_{4(a)}$ were labelled with [^{3}H]palmitate (**A**) or [^{35}S]methionine (**C**) for 1 h and chased with medium containing unlabelled palmitate or methionine for the periods indicated. During the chase, cells were treated with vehicle (control), BIMU8 or BIMU8 plus GR113808. Labelled receptors were detected by fluorography after receptor immunoprecipitation and SDS/PAGE. One representative experiment ($n = 3$) is shown in (**A**) and (**C**). (**B**) Graph derived from a densitometric analysis of fluorogram in (**A**).

the presence of BIMU8. The results shown in Figure 6 reveal that exposure to BIMU8 significantly increased radiolabel incorporation into the receptor over the whole labelling period of 120 min. On average, BIMU8 elicited an approx. 2.5-fold increase in labelling in comparison with controls. Labelling with [^{35}S]-methionine showed that exposure to agonist slightly decreased the amount of 5-HT$_{4(a)}$ protein (results not shown), demonstrating that the real increase in the rate of palmitate incorporation into receptor was even more pronounced, as seen in Figure 6(B).

To establish the specificity of agonist-promoted palmitoylation of 5-HT$_{4(a)}$, infected Sf9 cells were labelled with [^{3}H]palmitate for the same durations in the presence of GR113808, a high-affinity 5-HT$_{4(a)}$ antagonist [32]. Treatment with GR113808 alone significantly decreased the incorporation of radiolabel at all periods in comparison with agonist-treated or control cells (Figure 6). Interestingly, the quantity of receptors was unaffected, as tested by labelling with [^{35}S]methionine over the period studied (results not shown). Because it has been reported that unstimulated 5-HT$_{4(a)}$ possesses a high constitutive activity [27], the decrease in palmitoylation obtained might have resulted from an inhibition of basal agonist activity by antagonists. When cells were treated with GR113808 and BIMU8 during the period of labelling, the time course of palmitate incorporation was similar to that obtained in the control (results not shown). Taken together, these results demonstrate that an agonist-dependent increase in palmitate incorporation into 5-HT$_{4(a)}$ is due to receptor activation rather than reflecting changes in the rate of receptor synthesis.

To determine whether the agonist-promoted increase in receptor palmitoylation resulted from an elevation in the stochiometry of acylation or reflected a faster exchange between labelled and unlabelled palmitate, pulse–chase labelling experiments were performed. Infected Sf9 cells were incubated with [^{3}H]palmitate for 60 min followed by incubation with 100 μM unlabelled palmitate and 5 mM sodium pyruvate in the presence of either BIMU8 or BIMU8 plus GR113808 for various durations. As seen in Figure 7, in the absence of agonist (control), [^{3}H]palmitate was released from 5-HT$_{4(a)}$ over time, indicative of basal depalmitoylation. BIMU8 apparently promoted the release of radiolabel from 5-HT$_{4(a)}$ at all time points, decreasing the amount of receptor-bound palmitate by approx. 52 % of control. Even after a long exposure (up to 180 min) to agonist during the chase, [^{3}H]palmitate label was not completely released from 5-HT$_{4(a)}$, suggesting the existence of a minimal acylation state (results not shown). The effect of BIMU8 was receptor-specific because the increased release of [^{3}H]palmitate was effectively blocked by GR113808. Parallel labelling with [^{35}S]methionine demonstrated only minimal changes in the amount of receptors during the chase and the rate of turnover for receptor itself seemed unaffected by the treatment with agonists (Figure 7C). Taken together with the results of the incorporation experiments (Figure 6), the results of pulse–chase experiments suggest that stimulation of 5-HT$_{4(a)}$ by agonists increases the rate of its palmitate turnover.

DISCUSSION

In this study we present evidence that 5-HT$_{4(a)}$ expressed by the baculovirus system is covalently modified by palmitic acid. The fact that the label is sensitive to reducing agents, as shown by treatment with 2-mercaptoethanol and hydroxylamine (Figure 2), indicates that fatty acids are attached through an *S*-ester linkage to the free thiol group on one or more cysteine residues.

Palmitoylation of GPCRs seems to be a general feature of these signalling molecules; approx. 80 % of all known receptors contain one or more potentially palmitoylable cysteine residues downstream of their seventh transmembrane domain [32]. The roles of receptor palmitoylation have been investigated mainly by functional approaches involving acylation-deficient mutants [33]. However, the regulation of receptor palmitoylation in response to receptor stimulation has been addressed sporadically but has not yet been reviewed critically. The results of experiments presented here demonstrate that the palmitoylation of 5-HT$_{4(a)}$ is a dynamic process that can be modulated by agonists. The use of both incorporation and pulse–chase approaches allowed us to distinguish between two possible reasons for this modulation: (1) an increased proportion of palmitoylated receptors or (2) an enhanced exchange of receptor-bound palmitate. The results of incorporation experiments demonstrate that the increase in palmitoylation most probably does not result solely from the acylation of newly synthesized receptors because the rate of agonist-promoted palmitoylation was approx. 3-fold higher than

that of untreated controls (Figures 4 and 6). More important is the observation that this increase in palmitoylation is paralleled by a slight decrease in the rate of receptor synthesis (compare with Figure 4). These effects of treatment with agonist are receptor-specific and did not result from general metabolic effects, because BIMU8 influenced neither total protein synthesis nor palmitate incorporation under any of the labelling conditions applied (Figure 4C). In addition, the results of pulse–chase experiments show that the half-life of receptor-bound palmitate is shorter than that for the receptor itself and that stimulation of the receptor increases the rate of palmitate turnover (Figure 7). Although the detailed mechanism involved in the regulation of palmitoylation/depalmitoylation cycles of 5-$HT_{4(a)}$ is still unknown, our results indicate that the exchange of unlabelled and ^{3}H-labelled palmitate is accelerated after treatment with agonist. These observations do not preclude a simultaneous change in stoichiometry but, taken together with the results obtained from the cycloheximide experiments (compare with Figure 5), the above results strongly suggest that the biological activation of 5-$HT_{4(a)}$ enhances the exchange of palmitate on this polypeptide. Palmitate turnover implies that at any given point palmitoylated and non-palmitoylated forms of 5-$HT_{4(a)}$ are present in the cell and are available for repeated cycles of palmitoylation/depalmitoylation.

The regulated turnover of protein palmitoylation was first reported for the peripheral membrane protein $p21^{ras}$ [34]. Similar results have been reported for other proteins involved in signal transduction, including α-subunits of heterotrimeric G-protein (reviewed in [10]) and endothelial nitric oxide synthase [34]. For example, a stimulation of palmitate incorporation has been observed for $G_q\alpha$, $G_s\alpha$, $G_i\alpha$ and $G_o\alpha$ after activation through 5-hydroxytryptamine receptors [16,35]. Stimulation of the β_2-adrenergic receptor also results in an increased turnover of palmitate on $G_s\alpha$ [17,18]. Although quite common, regulated palmitate turnover does not seem to be a general feature because palmitoylation of $G_z\alpha$ and $G\alpha_{12}$ is not affected by the stimulation of D2 dopamine and PAR1 thrombin receptors respectively [36,37].

The dynamic nature of palmitoylation obtained here for 5-$HT_{4(a)}$ parallels previous observations on the increased turnover of palmitate in β_2-adrenergic (β_2AR) and D1 dopamine receptors after receptor stimulation [13,14,38]. Interestingly, the effect of stimulation with agonist on the palmitoylation of β_2AR was biphasic. During the early phase the stimulation of the receptor increased [^{3}H]palmitate incorporation, whereas during the later phase (after 120 min) the incorporation of label was decreased. It has been proposed that after stimulation with agonist, palmitate exchange is accelerated, leading to an initial increase in [^{3}H]-palmitate incorporation. However, after prolonged stimulation the receptor becomes phosphorylated at its C-terminus and this seems to inhibit repalmitoylation. In contrast, we found that for 5-$HT_{4(a)}$ the increase in [^{3}H]palmitate incorporation proceeds even after a long exposure (up to 180 min) to agonist. Structurally, these differences could be explained by a lack of potential sites for phosphorylation at the C-terminus of 5-$HT_{4(a)}$. From a functional point of view it should be mentioned that the role of palmitoylation of 5-$HT_{4(a)}$ could be different from that proposed for β_2AR. For the latter it has been suggested that a dynamic interaction between palmitoylation and phosphorylation is important for the regulation of β_2AR desensitization. In contrast, for humane α_{2A}-adrenergic receptor, which is also palmitoylated but not phosphorylated, it has been reported that palmitoylation is specifically involved in an agonist-promoted receptor down-regulation, which is another common adaptive response during signalling [15]. In addition, it has been reported for the endothelin A receptor and for the A1 adenosine receptor that palmitoylation might also be involved in coupling with G-proteins and in receptor proteolysis respectively [39,40]. In the search for the specific biological functions of dynamic palmitoylation of 5-$HT_{4(a)}$, the generation and analysis of its acylation-deficient mutants should provide a useful tool.

In conclusion, we have shown that 5-$HT_{4(a)}$ expressed in Sf9 cells undergoes palmitoylation. In addition, we used both palmitate incorporation and pulse–chase techniques to address the dynamic nature of 5-$HT_{4(a)}$ palmitoylation. Our combined results show a dose-dependent and time-dependent change in [^{3}H]-palmitate incorporation into the receptor after stimulation with agonist. The effects were receptor-specific and did not represent general metabolic effects of stimulation with agonist. Although the picture is still obscure with regard to the precise molecular mechanisms involved, the conformational changes induced by the stimulation of 5-$HT_{4(a)}$ with agonist could modulate the turnover rate of receptor-bound palmitate through accelerated palmitoylation/depalmitoylation. Only the use of acylation-deficient mutants will allow us to gain better insights into the molecular mechanics of the 5-HT_4 signalling system. To address these questions, identification of the actual palmitoylation site(s) in 5-$HT_{4(a)}$ will be necessary in follow-up studies employing molecular and enzymic methods to detect the modification of individual cysteine residues.

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