Serotonin-Mediated Palmitoylation and Depalmitoylation of G Alpha Proteins in Rat Brain Cortical Membranes

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ABSTRACT

We investigated serotonin stimulated palmitoylation of G alpha subunits in rat brain cerebrocortical membranes. Serotonin dose dependently stimulated palmitoylation of membrane G alpha proteins. The highest [3H] palmitate incorporation observed was by G alpha-q (7-fold), followed by G alpha-o (5-fold), G alpha-i (4-fold) and G alpha-s (3-fold) and these increases in palmitoylation were blocked by methiothepin, a serotonin receptor antagonist. Isoproterenol selectively stimulated G alpha-s palmitoylation which was blocked by propranolol. Immunoprecipitates of palmitoylated G alpha subunits yielded single labeled bands on SDS-PAGE. In an attempt to define the sequence of palmitoylation/depalmitoylation that follows receptor stimulation, nonreceptor mediated palmitoylation was carried out in the presence of guanine nucleotides and receptor mediated G alpha depalmitoylation was then monitored. Receptor stimulation did not result in depalmitoylation when membranes were prelabeled with [3H] palmitic acid in the presence of the nonhydrolyzable analogue of GTP, Gpp(NH)p. However, serotonin receptor stimulation in the presence of guanine nucleotides, depalmitoylated (90%) membrane G alpha proteins when prelabeled in the presence of GTP. Immunoprecipitation experiments revealed decrease in G beta immunoreactivity associated with G alpha immunoprecipitates obtained from membranes prelabeled in presence of GTP prior to reincubation with Gpp(NH)p and serotonin. These observations suggest that receptor activation results in depalmitoylation of the trimer, followed by guanine nucleotide exchange and dissociation of the alpha subunit from beta-gamma dimer and that the activated alpha subunit is a substrate for repalmitoylation.

Heterotrimeric G proteins are central in transducting signals from cell surface receptors to appropriate membrane effectors. G proteins consist of three subunits. To date, ~25 alpha, 5 beta and 11 gamma subunits have been identified (Simon et al., 1991). Activation of G proteins by G protein coupled-receptors results in an exchange of GDP for GTP on the alpha subunit followed by dissociation of the alpha subunit from the beta-gamma subunit of the alpha-gamma dimer. Intrinsic GTPase activity of the alpha subunit subsequently hydrolyzes GTP and the reassociation of alpha-GDP with beta-gamma completes the inactivation of the G protein. The free alpha subunit and beta-gamma complex can interact with target effectors (Birnbaumer, 1992; Neer, 1994, 1995; Sternweis, 1994). The specificity of signal transduction is determined by the association of receptors with different subtypes of G proteins that are an assembly of alpha-, beta- and gamma subunits (Birnbaumer and Birnbaumer, 1995; Simon et al., 1991). Homology at the amino acid level has lead to the grouping of the alpha subunits into 4 subfamilies, G alpha-s, G alpha-i, G alpha-q and G alpha-12.

Covalent lipid modification of the G protein subunits is known to facilitate membrane association and interaction with effectors (Wedegaertner et al., 1995; Casey, 1994). The alpha subunit is known to be myristoylated as well as palmitoylated, no known modification for the beta subunit has been reported so far, while prenylation of gamma subunits and its significance in signal transduction is well documented (Casey, 1994, 1995). Myristoylation of the alpha subunit involves the co-translational linkage of a 14 carbon fatty acid via amide bond on an N-terminal glycine residue (Casey, 1994; Buss et al., 1987; Mumbly et al., 1990). High stability of the amide bond makes this modification irreversible except in a few cases. The alpha subunits of the alpha-i family (alpha-i1, alpha-12, alpha-i3, alpha-o , alpha-z and alpha-t) are substrates for myristoylation (Birnbaumer and Birnbaumer, 1995; Wedegaertner et al., 1995). The G alpha-s and G alpha-q subunits lack the N-terminal sequence required for myristoylation. Myristoylation enhances membrane association of the alpha subunit and also its affinity for the beta-

ABBREVIATIONS: HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; EGTA, ethylene glycol bis (beta-aminoethyl N, N', N''-tetraacetic acid); EDTA, ethylenediaminetetraacetic acid; PMSF, phenylmethyl sulfonyl fluoride; SDS, sodium dodecyl sulfate; TBS, Tween-20 containing phosphate-buffered saline.
Palmitoylation is a reversible posttranslational modification whereby the 16 carbon fatty acid is attached to an N-terminal cysteine residue of the alpha subunit via a thioester linkage (Wedegaertner et al., 1995; Parenti et al., 1993; Linder et al., 1991). Palmitoylations of alpha-o, alpha-s, alpha-z and alpha-q have been reported. Dual acylation of G protein alpha subunits is also known to occur (Casey, 1995; Wedegaertner et al., 1995) and in some cases myristoylation is a prerequisite for subsequent palmitoylation (Wedegaertner et al., 1995).

Palmitoylation confers greater hydrophobicity to G alpha proteins and enhances their interaction with the membrane (Wedegaertner et al., 1993; Milligan et al., 1995). The exact role of this regulated modification in the G protein cycle is not fully elucidated. Receptor-stimulated palmitoylase turnover of G alpha subunits (Wedegaertner et al., 1993) suggests that palmitoylation may play a role in transmembrane signal transduction. In fact, palmitoylation of G alpha-s and G alpha-q is required for membrane attachment and subsequent interaction of these alpha subunits with effectors (Wedegaertner et al., 1993). Wedegaertner and Bourne (1994) have suggested that depalmitoylation and palmitoylation reactions are consequences of G protein activation that determine the cytosol/membrane distribution of G alpha subunits.

Our current understanding of the role of palmitoylation/dpalmitoylation of G alpha subunits in transmembrane signal transduction is based on investigations conducted in cell lines transfected with G proteins. In the present work we attempted to examine the role of receptor-mediated palmitoylation of G alpha subunits in native biological membrane preparations obtained from rat brain cortex. We report that serotonin and beta-adrenergic receptor stimulated palmitoylations are specific with regard to receptors and G proteins. Further, our studies suggest that receptor stimulation leads to palmitoylation of G alpha proteins followed by nucleotide exchange and dissociation of alpha subunit from the beta-gamma complex; and that the activated alpha subunit is subsequently the substrate for palmitoylation.

**Methods**

**Materials.** Male Sprague Dawley rats (150–200 g) were purchased from Zivic Miller (Zillienopoles, PA) and group housed at ambient temperature of 22 ± 1°C. Animals had access to tap water and rat chow pellets (Agway pellets, 3000 Agwat, INC, Syracuse, NY). All chemicals used for buffer preparations were obtained from Sigma Chemical (St. Louis, MO). Serotonin hydrochloride (5-hydroxytryptamine hydrochloride) was purchased from Sigma Chemical. Methiothepin mesylate (1-[10,11-dihydro-8-(methylthio)dibenzo[b,f]thiepin-10-yl]-4-methylpiperazine mesylate) was a gift from Hoffman LaRoche (Nutley, NJ), Prazosin HCl from Pfizer (New York, NY), SCH23390 hemimaleate (8-chloro-2,3,4,5-tetrahydro-3-methyl-5-phenyl-1H-3-benazepin hemimaleate) from Schering Corp. (Bloomfield, NJ), l-propranolol HCl [(±)-1-(isopropylamino)-3-(1-naphthoxy)-2-propanol hydrochloride] from Ayerst Laboratories INC (New York, NY). S(-)-Sulpiride [-[S]-5-aminosulfonyl-N-[1-ethyl-2-pyrrolidinyl]methyl[2-methoxbenzamid]e] was purchased from Research Biochemicals (Natick, MA). Polyclonal antiserum to the G alpha proteins: G alpha-q (11, QL), G alpha-o (C2), G alpha-s (RM/2) and G alpha-i (A8/7) were purchased from DuPont-New England Nuclear (Boston, MA). These antiseria were previously characterized (Spiegel et al., 1990). Antiseria to G alpha-z (29319) was a generous gift from Dr. David Manning (Department of Pharmacology, University of Pennsylvania) and was characterized for specificity (Carlson et al., 1989). [9,10-3H] palmitic acid was purchased from DuPont-New England Nuclear (Boston, MA). GTP, gaunine imidazole diphosphate [Gpp(NH)p] and guanosine 5’ O (2-thiophosphate) (GDP beta-S) were purchased from Sigma Chemical. Normal rabbit serum and Pansorbin were purchased from Calbiochem (San Diego, CA).

**Membrane preparation.** Rats were decapitated, brains removed and cortex dissected out on ice and stored at −80°C until use. Cortical membranes were prepared as follows: tissue was homogenized in glass/glass homogenizer in HEPES buffer, pH 7.4, which consisted of 25 mM HEPES, 100 mM sucrose, 1 mM EGTA, 50 μg/ml leupeptin, 2 μg/ml soybean trypsin inhibitor, 40 μM phenylmethysulfonyl fluoride (PMSF) and 0.2% beta-mercaptoethanol. The homogenates were spun at 700 × g and the resulting supernatants were centrifuged at 41,000 × g for 20 min at 4°C. The pellets were washed once by resuspending in homogenization buffer followed by recentrifugation at 48,000 × g, for 20 min at 4°C. The final pellet was resuspended in HEPES buffer, pH 7.4, without EGTA and in which sucrose was replaced by 100 mM NaCl (suspension buffer). Protein was estimated by the method of Lowry et al. (1951).

**Palmitoylation/depalmitoylation.** The assay mixture (250 μl) which consisted of 400 μg of membrane protein in suspension buffer containing 1 mM MgCl₂, 2 μM of [3H] palmitic acid (specific activity 36–50 Ci/mmol) and 8 μM of unlabeled palmitic acid was incubated at 30°C for 10 min prior to addition of either buffer or 50 nM of GTP or the nonhydrolyzable analogue of GTP, Gpp(NH)p and incubated for an additional 5 min (basal palmitoylation). Agonist stimulated palmitoylation was monitored in the presence of varying concentrations of agonist. For the study of antagonists, after the initial 10 min incubation with [3H] palmitic acid, drugs were added and incubated for 10 min prior to addition of Gpp(NH)p with agonist. The reactions were stopped by adding ice cold suspension buffer containing 1 mM EDTA followed by centrifugation. The pellet obtained was further subjected to immunoprecipitation with antisera specific for G alpha subunits. To monitor palmitoylation, basal palmitoylation of cortical membranes was carried out in the presence of GTP or Gpp(NH)p and the pellets obtained were washed with suspension buffer containing 1–2% bovine serum albumin (BSA) to remove free [3H] palmitic acid and receptor stimulated palmitoylation was then monitored in the presence or absence of guanine nucleotides followed by immunoprecipitation with G alpha antisera.

**Immunoprecipitation.** The immunoprecipitation procedure followed was described previously (Friedman et al., 1993; Wang et al., 1993). The pellets obtained after palmitoylation were solubilized in immunoprecipitation buffer which was made up of 100 mM Tris-HCl, pH 7.4, 200 mM NaCl, 10 mM EDTA, 50 μg/ml leupeptin, 2 μg/ml soybean trypsin inhibitor and 2.5% NP-40. In addition, 0.2% sodium dodecyl sulfate (SDS) was also added. The solubilized membranes were preclarified with normal rabbit serum and the antigen-antibody complex was precipitated with Pansorbin cells. The resulting supernatant was equally divided into two tubes, and the palmitoylated G alpha subunits were precipitated by addition of normal rabbit serum or G alpha antisera followed by precipitation with Pansorbin cells. The difference in radioactivity in the pellets obtained from the reactions with normal rabbit serum and G alpha antisera (+, agonist) was used to analyze palmitoylated G alpha proteins.

**SDS-PAGE.** The palmitoylated immunoprecipitates were washed and solubilized in Laemmli (1970) sample buffer and resolved by 10% SDS-PAGE. The gels were stained with Coomassie Brilliant Blue, and specific bands were visualized by fluorography after treating the gels with enhancer (Fluro Enhancer, Research Product Corps.) according to the manufacturer’s instruction. The gels were dried and exposed to Amersham Hyperfilm Tm for 30 to 45 days at −80°C. Treatment of gels with 1 M hydroxylamine followed by fluorography was used to confirm the thiosteer linkage. For Western immunoblot studies, the immunoprecipitates were run on SDS-PAGE and transferred to nitrocellulose membrane (Towbin et al., 1979).
The membranes were incubated at 4°C overnight with 1% nonfat dry milk in 0.1% TBS, washed with 0.1% TBS, followed by 1 hr incubation with affinity purified G beta protein antibody (0.25 μg/ml, Santa Cruz Biochemicals, Santa Cruz, CA) or G alpha antisera (1:1000 dilution) in 0.1% TBS. The unbound antibody was washed with 0.1% TBS. The membranes were then incubated for 1 hr with horseradish peroxidase-conjugated anti-rabbit IgG (Amersham, Arlington Heights, IL) (1:10,000 in 1% TBS). The blots were washed in 3% TBS for 10 min, followed by three 5-min washes in 0.1% TBS and the bands were visualized using Enhanced Chemiluminescence Western blot detection system kit (Amersham/Searle, Des Plaines, IL).

Statistical analysis. All values are presented as mean ± S.E.M. of at least 3–4 experiments. Statistical significance was determined by the nonpaired Student’s t test or by analysis of variance followed by either one way or two way Duncan’s test as appropriate.

Results

Initial studies were carried out to establish optimal conditions for incorporation of [3H] palmitic acid by G alpha subunits in cortical membrane preparations. Palmitoylation was found to be maximal at a concentration of 50 nM Gpp(NH)p and the time required for optimal incorporation of [3H] palmitic acid was 5–10 min in the presence or absence of agonist. No incorporation of [3H] palmitic acid by the G alpha subunits under basal conditions was observed in the absence of magnesium. In the presence of 1 mM magnesium, palmitoylation of membrane G alpha subunits occurred in the absence of either guanine nucleotides or agonist. Concentrations of Mg²⁺ greater than 2 mM suppressed palmitoylation. Basal palmitoylation increased several-fold in the presence of Gpp(NH)p plus serotonin, however levels of incorporated [3H] palmitic acid were lower when the reaction was carried out in the presence of GTP (Table 1). The lower response achieved in the presence of GTP is probably the result of GTP hydrolysis. Table 1 also shows that agonist-stimulated palmitoylation is unaffected by washing and resuspension of the membranes suggesting that this protocol may be used in our experiments.

Serotonin, dose dependently increased the incorporation of [3H] palmitic acid by the G alpha subunits. Maximal palmitoylation of G alpha-q, G alpha-o and G alpha-i were achieved at 1 μM serotonin, while maximal palmitoylation of G alpha-s was observed at 100 nM serotonin (fig. 1a). The neurotransmitter increased the palmitoylation of G alpha-q, G alpha-i, G alpha-o and G alpha-s, by 7, 5, 4 and 3-fold, respectively. The EC₅₀ values for palmitoylation of the various G alpha subunits by serotonin are as follows: G alpha-q, 22 nM; G alpha-o, 460 nM; G alpha-i, 18 nM and G alpha-s, 8 nM. Serotonin-stimulated [3H] palmitic acid incorporation by G alpha subunits was also determined in precipitates after chloroform:methanol extractions. While net counts were reduced similar receptor-enhanced palmitoylations of G alpha subunits were obtained when compared to immunoprecipitates which were counted directly. Serotonin did not increase palmitoylation of G alpha-s at concentrations of up to 5 μM (data not shown). The beta-adrenergic receptor agonist, isoproterenol elicited a dose dependent increase in G

![Fig. 1. Agonist-stimulated incorporation of [3H] palmitic acid by cerebrocortical membrane G alpha subunits. Cortical membranes were preincubated for 10 min with [3H] palmitic acid followed by Gpp(NH)p plus varying concentrations of a) serotonin or b) isoproterenol for additional 5 min at 30°C. Palmitoylated G alpha subunits were immunoprecipitated with G alpha-antisera. The data is expressed as the-fold increase in [3H] palmitate incorporated in presence of agonist as compared to basal palmitate incorporation. The values are the mean ± S.E.M. of 3–6 experiments. Basal palmitate incorporation’s were for G alpha-q 4072 ± 332, G alpha-o 3520 ± 313, G alpha-i 5784 ± 250 and G alpha-s 5497 ± 469 dpm.](https://example.com/fig1.png)

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<sup>a</sup> P < .01 and <sup>b</sup> P < .05 compared with values obtained when serotonin-stimulated palmitoylation was carried out in presence of Gpp(NH)p as analyzed by Student’s t test.
alpha-s palmitoylation (fig. 1b) but did not increase the palmitoylation of other G alpha subunits. A maximal 3-fold increase in [3H] palmitic acid incorporation by G alpha-s was observed at 100 nM isoproterenol. The EC_{50} for isoproterenol induced palmitoylation of G alpha-s was calculated to be 41 nM.

Incorporation of labeled palmitic acid by specific G alpha subunits was visualized after the separation of immunoprecipitated G alpha subunits on SDS-PAGE followed by fluorography (fig. 2a). Single labeled bands indicated specific incorporation of palmitic acid by the respective G alpha subunits. The incorporation of [3H] palmitic acid in the respective G alpha subunits was increased by serotonin (fig. 2a, lanes 2, 4, 6). Moreover, palmitoylated G alpha proteins were sensitive to hydroxylamine treatment as indicated by the disappearance of the signal from gels that were treated with 1 M hydroxylamine under alkaline conditions (data not shown). Aliquots of the same immunoprecipitates were also used for Western immunoblotting (fig. 2b). These revealed single immunoreactive bands with G alpha-q, G alpha-o and G alpha-i antisera respectively, the intensity of which was not altered by incubation of the membranes with serotonin (fig. 2b, lanes 1–6).

To evaluate whether agonist-stimulated G alpha palmitoylation is mediated by specific activation of cell surface receptors, the effect of receptor antagonists was examined. The serotonin receptor antagonist, methiothepin, dose dependently decreased serotonin-induced palmitoylation of G alpha-q and G alpha-o (fig. 3a). Maximal inhibition (80%) of palmitoylation was observed at 100 nM methiothepin. Serotonin stimulated palmitoylation of G alpha subunits was not affected by antagonists of beta-adrenergic (propranolol), alpha-1 adrenergic (prazosin) or dopamine D_1 (SCH) and D_2 (sulpiride) receptors (fig. 3b). Isoproterenol (100 nM) stimulated palmitoylation of G alpha-s was dose dependently inhibited by the beta-adrenergic receptor antagonist, propranolol (fig. 3c). Maximal inhibition (70%) was observed at 0.1 μM propranolol. Basal palmitoylation of G alpha-q, G alpha-i and G alpha-s was unchanged in the presence of the antagonists alone (data not shown).

To determine the sequence of palmitoylation/depalmitoylation which follows receptor stimulation, we first palmitoylated G alpha subunits in the presence of guanine nucleotides and then examined the effect of receptor activation on G alpha depalmitoylation. The palmitoylation reaction was carried out in the presence of either GTP or the nonhydrolyzable analogue of GTP, Gpp(NH)p, the reaction was stopped and free [3H] palmitic acid was removed by washing the membrane pellets with buffer containing bovine serum albumin. The effect of serotonin receptor stimulation on incorporated [3H] palmitic acid was then monitored in the presence or absence of guanine nucleotides by quantitating [3H] palmitoylated G alpha-q and G alpha-o in the respective immunoprecipitates of solubilized membranes. When palmitoylation was carried out in the presence of Gpp(NH)p, [3H] palmitic acid incorporated by membrane G alpha-q or G alpha-o was unaffected upon reincubation with guanine nucleotides or serotonin alone or by guanine nucleotides plus serotonin (fig. 4a). On the other hand, when the palmitoylation reaction was carried out in the presence of GTP, depalmitoylation of G alpha-q or G alpha-o was unaffected upon reincubation with guanine nucleotides alone. However, incubation with serotonin resulted in a 50–60% loss in palmitoylated G alpha subunits (fig. 4b); serotonin plus guanine nucleotides elicited almost a complete (90%) depalmitoylation of G alpha-q or G alpha-o subunits (Fig. 4b). The effects of Gpp(NH)p plus serotonin shown in figure 4, a and b, were duplicated when GTP was substituted for Gpp(NH)p in the reincubation phase of the experiment (data not shown). Moreover, the guanine nucleotide analogue GDPbeta-S did not facilitate the serotonin-induced depalmitoylation (60%) of the G alpha subunits.

In order to monitor the changes in the state of the trimer during palmitoylation/depalmitoylation in the G protein cycle, the G beta subunit associated with the immunoprecipi-
G protein was assessed by Western blotting. Using an antibody to the common beta subunit of G proteins, immunoblots revealed similar immunoreactivities associated with precipitates wherein basal palmitoylation was carried out in presence of Gpp(NH)p without serotonin (fig. 5, a and b, lane 1) and precipitates in which the membranes were further incubated with guanine nucleotide plus serotonin (fig. 5, a and b, lane 2). However, the intensity of the immunoreactive G beta bands were reduced by about 50% of controls (lanes 4 in a and b) in precipitates in which palmitylation was carried out in the presence of GTP and the membranes were further incubated with Gpp(NH)p and serotonin (fig. 5). This set of experiments demonstrate that dissociation of the heterotrimeric G protein and depalmitoylation of the G alpha subunit require receptor stimulation.

Discussion

In the present communication we have demonstrated receptor mediated palmitoylation of specific G alpha subunits in biological membranes obtained from rat brain. Serotonin stimulated the palmitoylation of G alpha-q, G alpha-o, G alpha-i and G alpha-s proteins in cortical membranes, while isoproterenol increased the palmitoylation of G alpha-s protein in the same tissue. Palmitoylation of multiple G alpha proteins by serotonin and of G alpha-s by the beta-adrenergic receptor agonist probably reflect the fact that the multiple serotonin receptor subtypes in the cortex are linked to different G proteins while the beta-adrenergic receptors found in the cortex are coupled solely to G alpha-s protein. Previous results from our laboratory obtained by examining receptor-mediated increases in GTP binding to G alpha proteins in rat brain cortical membranes have shown coupling of the 5-HT1A and 5-HT1B receptors to G alpha-i and G alpha-o, the 5-HT2A receptor to G alpha-q, G alpha-o and G alpha-i, the 5-HT2C receptor to G alpha-q and the 5-HT3, 5-HT4 and 5-HT7 receptors to G alpha-s (Friedman and Wang, 1990). Incorporation of [3H] palmitic acid by membrane G alpha subunits, both under basal as well as under receptor stimulated conditions, was found to be completely magnesium dependent but not absolutely dependent on the presence of guanine nucleotides. Consistent with the nature of the thioester bond.
in palmitoylated G alpha subunits, the product was sensitive to hydroxylamine treatment.

Serotonin-stimulated incorporation of [3H] palmitic acid by the G alpha subunits was highest for G alpha-q, followed by G alpha-o and G alpha-i and least for the G alpha-s subunit. This order is clearly not related to the concentration of membrane G alpha subunits, but may reflect the abundance of the particular receptor subtypes which are linked to respective G proteins in the cortex. The effect of serotonin was dose-dependent and sensitive to blockade by the serotonin receptor antagonist, methiothepin, suggesting that palmitoylation is receptor mediated. The specificity of this effect is further confirmed by the observation that antagonists of other receptors failed to block serotonin stimulated palmitoylation.

Furthermore serotonin receptor stimulation did not result in [3H] palmitic acid incorporation by G alpha-z, suggesting that cortical serotonin receptors are not coupled to G alpha-z protein (data not shown). The dose-dependent effect of isoproterenol on the incorporation of [3H] palmitate by G alpha-s protein noted in the present work, is similar to earlier observations made in S49 and COS cells by previous workers (Degtyarev et al., 1993a). However, the concentration of isoproterenol required for maximal palmitoylation of G alpha-s observed in our study is at least 20 times lower than that reported in S49 cells (Degtyarev et al., 1993a), suggesting that receptor-stimulated palmitoylation of G alpha-s may be more efficient in native cerebrocortical membranes. The selective incorporation of palmitate by G alpha-s is also consistent with our earlier observation that beta-adrenergic receptor stimulation selectively enhanced GTP binding by G alpha-s in human cerebrocortical membranes (Friedman and Wang, 1996).

The sequence of receptor mediated palmitoylation/depalmitoylation is not fully elucidated and the enzymes involved in these reactions are not yet fully characterized, although it has been suggested that palmitoylated G alpha subunits are substrates for thioesterase purified from bovine brain (Camp and Hofman, 1993). Previous studies conducted in cell lines or in cells transfected with G proteins have indicated that activation of G protein-coupled receptors results in enhanced palmitoylation of G alpha subunits (Mumby et al., 1994; Wedegaertner and Bourne, 1994; Degtyarev et al., 1993a). Wedegaertner and Bourne (1994) have furthermore suggested that receptor-mediated activation of G proteins results in depalmitoylation which is followed by palmitoylation. Understanding the sequence of these reactions under physiological conditions is vital in the elucidation of the role of G protein palmitoylation in receptor mediated signal transduction. In the present study we examined the effect of receptor stimulation on palmitoylation. In these experiments palmitoylation of membrane G alpha proteins was first carried out in the presence of either Gpp(NH)p or GTP and subsequently depalmitoylation of G alpha proteins was monitored after receptor stimulation in the presence or absence of 50 nM of the guanine nucleotides and residual [3H] palmitic acid (10 μM) and Gpp(NH)p (lanes 1 and 2) or GTP (lanes 3 and 4). The reactions were stopped with ice-cold HEPES buffer, pH 7.4, containing 1 mM EDTA, centrifuged and pellets were reincubated with Gpp(NH)p plus serotonin (lane 2 and 4) followed by immunoprecipitation with antisera against G alpha-o (a) or G alpha-q (b) subunits. The immunoprecipitates were resolved on 10% SDS-PAGE, transferred to nitrocellulose membranes and immunoblotted with antisera against common G beta subunit. The immunocomplexes were detected using antirabbit IgG and enhanced chemiluminescence. The relative optical densities (±S.E.M.) for immunoreactive bands in lanes 1, 2, 3 and 4 are 3251 ± 284, 2617 ± 228, 5792 ± 371 and 3251 ± 218 (a) and 1807 ± 138, 1952 ± 196, 5745 ± 212 and 1872 ± 172 (b). G betai- immunoreactivity in lanes 4 (a and b) are significantly lower (P < .0001) when compared to their respective control (lanes 3) as analyzed by two way ANOVA followed by Dun- cans test.  

Fig. 4. Effect of guanine nucleotides present during the palmitoylation reaction on subsequent receptor-stimulated depalmitoylation of G alpha proteins. Palmitoylation of membrane G alpha proteins was carried out in presence of 50 nM of either Gpp(NH)p (a) or GTP (b) and the membranes were washed with buffer containing 1% BSA. Membranes were subsequently reincubated with 1 μM serotonin either in presence or absence of 50 nM of the guanine nucleotides and residual [3H] palmitic acid incorporated by the G alpha proteins was measured in immunoprecipitates of specific antibodies to the indicated G alpha-q and G alpha-o proteins. The values are means ± S.E.M. (n = 3–6), expressed as percent of basal incorporated [3H] palmitate. * P < .0001 compared to basal palmitoylation, ** P < .0001 compared to values obtained wherein basal palmitoylation was followed by reincubation of pellets with serotonin alone as analyzed by two way ANOVA followed by Duncans test.

Fig. 5. Western immunoblots of G beta subunit in G alpha-o and G alpha-q immunoprecipitates. Membranes were palmitoylated with unlabeled palmitic acid (10 μM) and Gpp(NH)p (lanes 1 and 2) or GTP (lanes 3 and 4). The reactions were stopped with ice-cold HEPES buffer, pH 7.4, containing 1 mM EDTA, centrifuged and pellets were reincubated with Gpp(NH)p plus serotonin (lane 2 and 4) followed by immunoprecipitation with antisera against G alpha-o (a) or G alpha-q (b) subunits. The immunoprecipitates were resolved on 10% SDS-PAGE, transferred to nitrocellulose membranes and immunoblotted with antisera against common G beta subunit. The immunocomplexes were detected using antirabbit IgG and enhanced chemiluminescence. The relative optical densities (±S.E.M.) for immunoreactive bands in lanes 1, 2, 3 and 4 are 84, 53.2, 34.9 and 5.3 (a) and 1.2, 3.4 (b). G beta- immunoreactivity in lanes 4 (a and b) are significantly lower (P < .0001) when compared to their respective control (lanes 3) as analyzed by two way ANOVA followed by Dun- cans test.
absence of guanine nucleotides. Activation of G protein in the presence of Gpp(NH)p results in the formation of free alpha-Gpp(NH)p, while activation in the presence of GTP results in the formation of alpha-GTP. However, the rapid hydrolysis of GTP followed by the reassociation of alpha-GDP with the beta-gamma dimer would be expected to yield the G protein trimer, alpha-GDPbeta-gamma. Indeed formation of the trimer was in fact suggested by higher G beta- immunoreactivity detected in association with immunoprecipitated G alpha proteins when palmitoylation is carried in the presence of GTP as compared to Gpp(NH)p (fig. 5). Thus the palmitoylated alpha subunit in the trimer, i.e. G alpha-GDPbeta-gamma, is almost completely depalmitoylated (80%) when serotonin plus Gpp(NH)p were added to membranes which were prelabeled with [3H] palmitic acid in the presence of GTP, as opposed to that observed when serotonin or serotonin plus GDPbeta-S (50–60%) were used to stimulate depalmitoylation. However, serotonin, in the presence or absence of guanine nucleotides did not result in depalmitoylation of the dissociated and palmitoylated alpha subunit that is found in membranes which were palmitoylated in the presence of Gpp(NH)p (alpha-Gpp(NH)p). The data therefore, suggest that receptor stimulation elicits depalmitoylation of the alpha subunit. Exchange of nucleotides does not seem to be the factor governing depalmitoylation, but rather receptor occupation by agonist followed by its interaction with the trimeric G protein results in depalmitoylation. This is consistent with the observed loss in basal acylated G alpha subunits upon incubation of membranes with serotonin plus GDPbeta-S. Similarly, agonist induced loss of palmitoylated G alpha-s subunit was previously reported in HA and S49 cells (Wedegaertner and Bourne,1994). Depalmitoylation appears to be followed by exchange of guanine nucleotides, leading to the dissociation of the activated G alpha protein from the beta-gamma complex. This is suggested by the decrease in G beta immunoreactivity that is found associated with the G alpha subunits in immunoprecipitates obtained from membranes which had been previously palmitoylated in the presence of GTP and then stimulated by serotonin plus guanine nucleotides. The depalmitoylated and activated G alpha subunit (alpha-GTP) appears to be the substrate for palmitoylation as serotonin stimulated palmitoylation of membrane G alpha subunits was undetectable when palmitoylation was performed in the presence of GDPbeta-S which is presumed to yield the undissociated trimer (alpha-GDPbeta-Sbeta-gamma). This formulation is further supported by observations obtained in the beta-adrenergic receptor system. In lysates of cells transfected with the alpha-s mutant (alpha-s-G 226A) which cannot mediate beta-adrenergic receptor stimulated formation of cAMP and is unable to dissociate from beta-gamma complex, isoproterenol did not stimulate palmitoylation and in fact a small degree of depalmitoylation was reported (Wedegaertner and Bourne,1994). Similarly, no increase in palmitoylation by beta-adrenergic receptor stimulation was noted in the unc mutant of S49 cells in which the G alpha-s is unable to couple to beta- receptors or in the H21a mutant in which the G proteins couple to beta- adrenergic receptors promoting exchange of guanine nucleotides but are unable to dissociate from the beta-gamma dimers (Degtyarev et al., 1993b). These observations strongly support our conclusion that palmitoylation of the alpha subunit occurs only after the G protein has been activated and the G alpha subunit has dissociated from the beta-gamma dimer.

In summary our results suggest that the occupied receptor interacts with the trimeric G protein and triggers the depalmitoylation of the G alpha subunit (fig. 6). This is in accord with the reported lower affinity of the nonacylated versus the acylated G alpha-o subunit for the beta-gamma complex (Linder et al., 1993; Iiri et al., 1996). Our data do not allow us to conclude the exact sequence of depalmitoylation and guanine nucleotide exchange. Notwithstanding this sequence of events, our data do indicate that depalmitoylation and nucleotide exchange are followed by the dissociation of the trimer which precedes palmitoylation of the active alpha subunit (alpha-GTP) (fig. 6). The present data suggest that depalmitoylation of G alpha protein results in its conformational change which results in a change in its ability to interact with G beta-gamma complex. Depalmitoylation of the G alpha subunit may result in a partial unfolding of its N-terminus, the site of palmitoylation (Linder et al., 1991; Parenti et al., 1993; Wedegaertner et al., 1993), facilitating nucleotide exchange on G alpha protein and its subsequent dissociation from the trimer. The previous estimation of the dissociation rate of GDP from the trimer (G alpha-GDPbeta-gamma) was found to be 100 times less than that from free alpha-GDP (Higashijima et al., 1987; Bourne et al., 1991) supporting the suggested model (fig. 6). The present data, thus suggest that depalmitoylation favors activation of the G protein which is followed by palmitoylation of G alpha and the activation of effector. The trimer is then reformed after hydrolyses of GTP that is bound to G alpha. It is this cycle of delipidation/lipidation of G protein that may determine the movement of the protein in plasma membrane facilitating interactions with the receptor and the effector molecules.

![Fig. 6. Schematic representation of the palmitoylation/depalmitoylation of G proteins. Receptor (R) occupation by agonist (A) leads to the interaction of agonist occupied receptor (AR) with its associated palmitoylated trimeric G protein (alpha*-GTP beta-gamma) and results in its depalmitoylation, followed by the exchange of GTP for GDP and the dissociation of activated alpha-GTP from the beta-gamma dimer. If palmitoylation does not occur, the activated alpha-GTP may be released into the cytoplasm. However palmitoylation at this point facilitates interaction of the activated alpha subunit with its effector. Subsequent GTPase activity results in the formation of palmitoylated alpha-GTP, which then reassociates with the beta-gamma complex to form the palmitoylated trimeric G protein that is ready for activation by agonist occupied receptors.](image-url)
References


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