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Palmitoylation of the luteinizing hormone/human chorionic gonadotropin receptor regulates receptor interaction with the arrestin-mediated internalization pathway

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The luteinizing hormone/human chorionic gonadotropin receptor (LH/hCGR) undergoes palmitoylation at cysteine residues 621 and 622 located in the carboxyl terminal tail of the receptor. This study examined the biological function of palmitoylation with respect to its effect on receptor internalization. Coexpression of wild-type (WT) or C621/622G mutant receptors with arrestin-2 increased receptor internalization in 293T cells. Furthermore, measurements of rate enhancement upon overexpression of arrestin indicate that the palmitoylation deficient mutant receptor is more prone to utilizing the arrestin mediated internalization pathway than the WT receptor. Coexpression of G-protein-coupled receptor kinase 4 (GRK4) with wild type receptor resulted in an increase in internalization, while coexpression with the mutant receptor did not result in further enhancement of internalization. Additionally, 293T cells expressing mutant receptor were responsive to hCG with respect to production of inositol phosphates. Taken together, these results suggest that the palmitoylation state of the receptor governs internalization by regulating the accessibility of the receptor to the arrestin-mediated internalization pathway.

Keywords: arrestin; GRK; internalization; LH/hCGR; palmitoylation.

Luteinizing hormone (LH) and its placental counterpart, human chorionic gonadotropin (hCG), bind to their receptor (LH/hCGR) in gonadal tissues, resulting in the increased production of cAMP and subsequent steroidogenesis [1]. LH/hCGR is a member of the G-protein-coupled receptor (GPCR) family. These receptors contain an N-terminal extracellular domain, seven transmembrane domains, and a C-terminal cytoplasmic tail [2,3].

LH/hCGR is palmitoylated at cysteine residues 621 and 622 [4]. While palmitoylation of GPCRs is well conserved, its biological role varies from one member of the family to another [5–8]. For example, palmitoylation deficient mutants of the β_2 adrenergic receptor (β_2AR) exhibit a decreased ability to activate adenylate cyclase [5,9] while corresponding mutants of the LH/hCGR retain the ability to mediate activation of this pathway [4,10]. Furthermore, palmitoylation has been shown to produce varying effects on receptor turnover [4,11,12].

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Abbreviations: β_2AR, β_2 adrenergic receptor; cAMP, adenosine 3′,5′-cyclic monophosphate; ER, endoplasmic reticulum; GPCR, G-protein-coupled receptor; GRK, G-protein coupled receptor kinase; hCG, human chorionic gonadotropin; LH, luteinizing hormone; PMA, 4β-phorbol 12-myristate 13-acetate; WT, wild-type.

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Our previous studies showed that mutagenesis of palmitoylation sites has no effect on the ability to stimulate cyclic AMP production, but enhanced the rate of ligand-induced receptor internalization [4,10]. The present study examines the mechanism by which palmitoylation of the receptor affects internalization of the ligand–receptor complex. Specifically, we examined the interaction of the WT and palmitoylation deficient mutant receptor with arrestin, a component of the cell's endocytotic machinery [13–16]. Additionally, the role of palmitoylation on the receptor's ability to undergo G-protein coupled receptor kinase (GRK) stimulated internalization was examined, as GRKs are generally believed to play a role in hormone-induced phosphorylation of GPCRs and subsequent internalization [13–16]. The role of palmitoylation on the receptor's ability to undergo phosphorylation was also examined, as arrestin is believed to preferentially bind the phosphorylated form of GPCRs [13,14].

In addition to stimulation of cyclic AMP production, LH is also able to stimulate phosphotidylinositol hydrolysis [17,18]. Activation of downstream kinases stimulated by this pathway could lead to receptor phosphorylation [19–21]. To further delineate the role of palmitoylation in receptor function, the activation of the phosphotidylinositol hydrolysis signaling pathway in response to hCG was measured in cells expressing WT or mutant receptor.

The results presented in this study show that a lack of palmitate at cysteine residues 621 and 622 renders the receptor more susceptible to arrestin-mediated internalization. Based on these results it is proposed that depalmitoylation might be an integral part of the mechanism by which the LH/hCG receptor is internalized.

EXPERIMENTAL PROCEDURES

Materials

Human chorionic gonadotropin (CR-127) was a gift from the Center for Population Research, NICHD, National Institutes of Health. $Na^{125}I$ was purchased from ICN. Human embryonic kidney cells (293T cells) expressing the large T antigen were a gift from G. P. Nolan, Stanford University. 293 cells were purchased from American Tissue Culture Collection. hCG for nonspecific binding was purchased from Sigma. All cell culture media was purchased from Gibco BRL. Fura-2 AM was purchased from Molecular Probes. *myo*[^{3}H] inositol, the ECL detection system, and secondary antibody to anti-GRK4 were purchased from Amersham Pharmacia. LiCl was purchased from Mallinckrodt Laboratories. AG 1-X8 formate resin and nitrocellulose membrane were purchased from Bio-Rad Laboratories. cDNAs for arrestin 2, 3, and (319–418) arrestin-2 were kindly provided by J. L. Benovic, Thomas Jefferson University. cDNAs for GRKs 2, 4 and 6 were kindly provided as a gift by R. Lefkowitz, Duke University. Primary antibody to arrestin-2 and GRK4, as well as secondary antibody to antiarrestin-2 were purchased from Santa Cruz Biotechnology, Inc. pCMV4 plasmid was a gift from D. Russell, University of Texas Southern Medical Center. All reagents used for molecular and cell biology were purchased in a suitably purified form. Mutant receptor cDNA was prepared as described previously [4,10].

Cell culture and transfection

Experiments were performed as described previously using 293 or 293T cells [4]. 293T cells were grown in DMEM containing 4500 mg·L^{-1} glucose, 50 μg·mL^{-1} gentamycin, 9 U·mL^{-1} nystatin, 10 mM Hepes, and 10% fetal bovine serum at 37 °C in a humidified atmosphere containing 5% CO_2. Cells in exponential growth were plated 5–9 h before transfection at cell densities of 5–6 × 10^6 cells per 100 mm plate for inositol phosphate studies and 2–3 × 10^6 cells per 100-mm plate for internalization studies. 293T cells were transiently transfected using the calcium phosphate coprecipitation method. Stable transfections were performed in a similar manner to transient transfections, utilizing (geneticin) G418 for selection.

^{125}I-Labelled hCG binding assay

All ^{125}I-labelled hCG binding assays were performed as described previously [4] using a saturating concentration of ^{125}I-labelled hCG. Briefly, cells were incubated with a saturating concentration (100 or 120 ng·mL^{-1}) of hCG at 4 °C or 37 °C for 30 min with or without a 1000-fold excess of cold hCG to determine nonspecific binding. The cells were then washed twice at 4 °C with Waymouth's media. The radioactivity remaining with the cell pellet was then counted in a Gamma Trac 1290 γ-counter (Tm Analytic). Specific binding was determined by subtracting nonspecific binding from total binding.

Receptor-mediated ^{125}I-hCG internalization assay

The assays were performed as previously described [4,22] with slight modification. Transiently transfected 293T cells (2–3 × 10^6) were harvested with NaCl/P_i-EDTA, washed, and resuspended in 3–8 mL of Waymouth's 752/1 medium as described in [22], the cell suspensions (0.2 mL) transferred into 12 × 75 mm tubes, and incubated with a 100-ng·mL^{-1} saturating concentration of ^{125}I-labelled hCG at 37 °C for the time periods specified. Cells were incubated in the presence of a 1000-fold excess of unlabeled hCG to measure nonspecific binding. The reaction was stopped by the addition of 1.5 mL of Waymouth MB752/1 (4 °C) medium. After vortexing, the tubes were centrifuged at 4 °C at 1000 ***g*** for 5 min. The supernatant was aspirated and the cells washed again. Following the second wash, the cells were incubated twice at 4 °C with 0.5 mL acid buffer (150 mM NaCl, 50 mM glycine, pH 3.0) for 5 min to remove the surface bound ^{125}I-labelled hCG. Previous studies have shown that greater than 90% of the surface bound hormone is removed by this procedure [4]. After each incubation, the cells were centrifuged at 4 °C at 2000 ***g*** for 5 min. The [^{125}I] in the pellets and acid washes was counted in a Gamma Trac 1290 γ counter (Tm Analytic). The radioactivity associated with the pellet represents the internalized receptor bound ligand, while radioactivity released by acid wash represents surface bound ligand.

Determination of the internalization rate constant

The first order rate constant of internalization was calculated as described previously [22]; the extent of internalization at 37 °C of ^{125}I-labelled hCG was measured after 0–30 min of ^{125}I-labelled hCG exposure. The rate constant was calculated from the equation $kt = 2.3$ log (surface bound + internalized/surface bound), where k is the first-order rate constant (min^{-1}), and t = time (min) [22].

Western blot analysis

Firstly, 2.4–2.5 × 10^6 293T cells were plated and transiently transfected in a 100 mm tissue culture dish as described above. Approximately 60 h after transfection, the cells were harvested in NaCl/P_i-EDTA and subsequently lysed in NaCl/P_i 1% Tx-100. After allowing lysis to occur for 20 min on ice, cell lysate was centrifuged for 30 min at approximately 8 °C at 100 000 ***g***. An aliquot of the supernatant was subsequently run on an SDS/PAGE gel under denaturing and reducing conditions. After separation on the gel, proteins were transferred to a nitrocellulose membrane. Antibody incubations were carried out as per the manufacturer's instructions, with a 1 : 500 dilution of goat anti-(arrestin-2) Ig and rabbit anti-GRK4 Ig, and a 1 : 2500 and 1 : 5000 dilution of horse radish peroxidase linked anti-goat and anti-rabbit Ig, respectively. Protein detection was performed using the ECL detection system (Amersham Pharmacia).

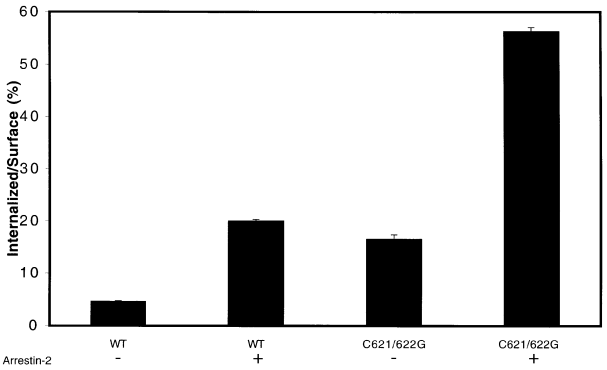

Fig. 1. Internalized ^{125}I-labelled hCG as a percentage of surface bound ^{125}I-labelled hCG in transiently transfected 293T cells expressing WT or C621/622G receptor with, or without, arrestin-2. 293T cells (2.2×10^6) were transiently transfected as described in Experimental procedures with 2.0 μg of WT cDNA, or 6.0 μg of C621/622G cDNA in the presence, or absence, of 6.0 μg of arrestin-2 cDNA. Empty pCMV4 vector was also transfected into cells to equalize the mass of DNA used in all plates. Approximately 60 h after transfection, the cells were harvested and the internalization assays were performed as described in Experimental procedures. Internalization was measured after 20 min of exposure to 100 ng·mL^{-1} ^{125}I-labelled hCG at 37°C. Data = % internalized ^{125}I-labelled hCG/surface bound ^{125}I-labelled hCG (%) ± SEM.

[^{32}P] Labeling of transfected cells used for receptor purification

Approximately 60 h after transfection of $2–3 \times 10^6$ 293T cells, the DMEM medium was removed and replaced with P_i-free medium containing 100 μCi·mL^{-1} of [^{32}P]orthophosphate. The cells were then incubated at 37 °C for 3 h; 500 nM 4β-phorbol 12-myristate 13-acetate (PMA) was then added to the experimental plates and incubation extended for another hour.

Purification of LH/hCG receptor using affi-gel chromatography

LH/hCG receptor was purified as described previously by hCG affi-gel chromatography [4]. After incubation with [^{32}P]orthophosphate, cultures were placed on ice and washed with phosphate buffered saline (NaCl/P_i). The cells were harvested and washed with buffer A (NaCl/P_i) supplemented with protease inhibitors (5 mM EDTA, 1 mM phenylmethanesulfonyl fluoride, 1 mM benzamidine, 1 mM *N*-ethylmaleamide, 2 μM leupeptin, and 2 μM *trans*-epoxysuccinyl-L-leucylamido-(4-guanidino)butane (E64), and phosphatase inhibitors (100 nM okadaic acid, 200 nM microcystin-LR, 100 μM orthovanadate, and 10 mM NaF). The cell pellets were solubilized with buffer B (0.15 M NaCl and 20 mM Hepes, pH 7.4) containing 0.5% Nonidet P-40, 20% glycerol, and the protease and phosphatase inhibitors present in buffer A. The supernatants were applied to affi-Gel covalently linked to highly purified hCG, and the suspension mixed end-over-end at 4 °C for 16 h. The gel was washed several times and the receptor eluted with sample buffer C (2% SDS, 10% glycerol, 20 mM EGTA, 0.5 mg·mL^{-1} bromphenol blue and 62.5 mM Tris, pH 6.8) containing the protease and phosphatase inhibitors contained in buffer A.

SDS/PAGE autoradiography

The purified receptor was subjected to SDS/PAGE on a 7.5% acrylamide gel under reducing conditions (0.05 M dithiothreitol, 5% 2-mercaptoethanol). The gels were then dried at 80 °C and exposed to Kodak X-Omat AR film for approximately 17 h.

Ca^{2+} measurements

Assays were performed using a slight modification of the procedure described by Liao *et al*. [23]. Stably transfected 293 cells were labeled with 20 μM Fura-2 AM at 37 °C for 30 min. After three washes with assay buffer (140 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, 1.8 mM $CaCl_2$, 10 mM dextrose, 0.1% BSA, and 15 mM Hepes, pH 7.4), the cells were resuspended at a concentration of 1×10^6 cells·mL^{-1}, the cells placed in a SLM 8000 spectrophotometer (SLM-Aminco) and maintained at a temperature of 37 °C. Fluorescence was monitored with an excitation wavelength of 340 nm and an emission wavelength of 510 nm in the absence and presence of 1 μg·mL^{-1} CR-127 hCG. EDTA was added to a final concentration of 13 mM prior to hormone exposure.

Inositol phosphate assays

Assays were performed using a slight modification of the procedure described by Berridge *et al*. [24]. Briefly, transiently transfected 293T cells (5×10^6) were labeled at 37 °C for 48 h with 75 μCi [^{3}H]*myo*-inositol. The cells were then harvested in NaCl/P_i-EDTA (1 mM), and resuspended in assay buffer (142 mM NaCl, 30 mM Hepes, 5.6 mM KCl, 3.6 mM $NaHCO_3$, 2.2 mM $CaCl_2$, 1.0 mM $MgCl_2$, 1 mg·mL^{-1} D-glucose, 15 mM LiCl adjusted to pH 7.4). The cells were incubated in this buffer at 37 °C for at least 15 min prior to stimulation with hormone in a total volume of 0.5 mL per tube. After exposure of cells to varying concentrations of hormone at 37 °C for 40 min, the cells were immediately placed on ice, and 0.5 mL of 20% trichloroacetic acid was added. The cells were then vortexed and placed on ice for an additional 15 min. Following centrifugation at 2000 ***g***, the supernatant was transferred to new tubes and washed five times with 2 mL of dH_2O saturated ether. The pH was adjusted to > 6.0 using 1 M $NaHCO_3$, and 1.7 mL of H_2O was added to each tube. Subsequently, 0.5 mL of an AG 1-X8 formate resin slurry was added to each tube. After vortexing, the samples were placed at 4 °C overnight. The following day, the samples were vortexed again, the supernatant was aspirated, and the resin was washed five times with 2.5 mL of 5 mM myo-inositol. The resin was then washed with 1 mL of an elution buffer (0.1 M formic acid, 1.2 M ammonium formate), vortexed, and incubated for 10 min at room temperature, and centrifuged. An aliquot of the supernatant was examined for [^{3}H]inositol phosphates using a Beckman liquid scintillation counter.

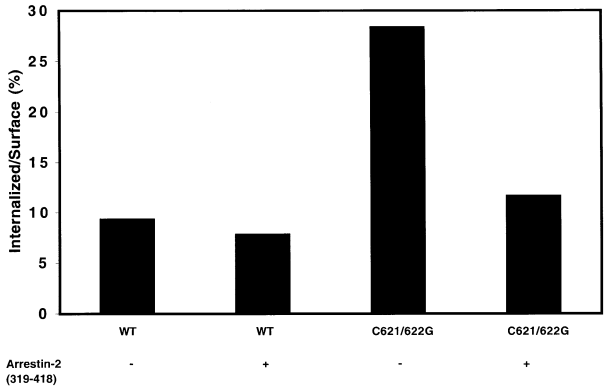

Fig. 2. Internalized ^{125}I-labelled hCG as a percentage of surface bound ^{125}I-labelled hCG in transiently transfected 293T cells expressing WT or C621/622G receptor with or without (319–418) arrestin-2. 293T cells (2.2×10^6) were transiently transfected as described in Experimental procedures with 1.68 μg of WT cDNA, or 4.40 μg of C621/622G cDNA, with, or without, 6.71 μg of (319–418) arrestin-2 cDNA. Empty pCMV4 vector was also transfected into cells to equalize the mass of DNA used in all plates. Approximately 60 h after transfection, the cells were harvested and the internalization assays performed as described in Experimental procedures. Internalization was measured after 20 min of exposure to 100 ng·mL^{-1} ^{125}I-labelled hCG at 37°C. The data shown are the results of one of two experiments conducted and show internalized ^{125}I-labelled hCG/surface bound ^{125}I-labelled hCG (%).

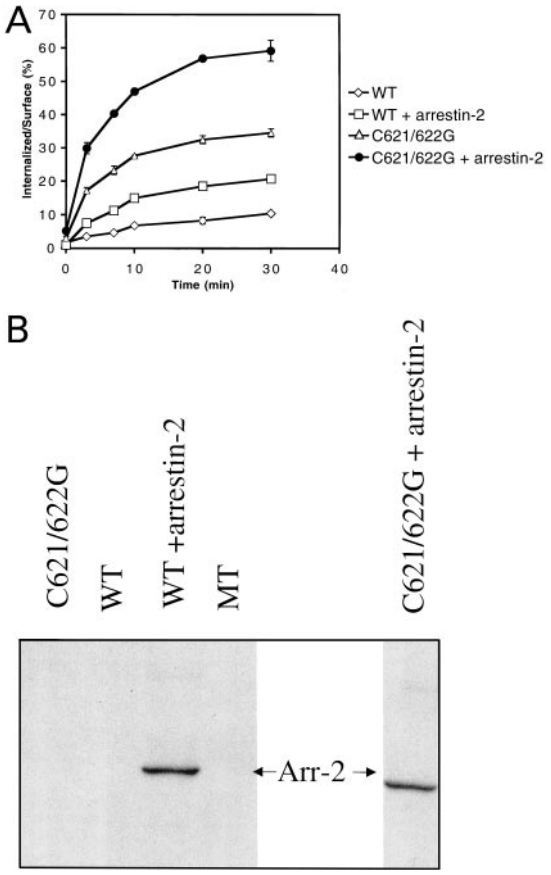

Fig. 3. Time course and rate of ^{125}I-labelled hCG internalization in cells expressing WT or C621/622G receptor with or without arrestin-2. 293T cells ($2.4–2.5 \times 10^6$) were transiently transfected as described in Experimental procedures with 2.0 μg of WT cDNA, or 6.0 μg of C621/622G cDNA with or without 6.0 μg of arrestin-2 cDNA. Empty pCMV4 vector was also transfected into cells to equalize the mass of DNA used in all plates. Approximately 60 h after transfection, cells were harvested and internalization assays performed as described in Experimental procedures. Internalized ^{125}I-labelled hCG was measured, as described in Experimental procedures, from 0 to 30 min of exposure to 100 ng·mL^{-1} ^{125}I-labelled hCG at 37°C and is expressed as a percentage of surface-bound ^{125}I-labelled hCG in cells expressing WT or mutant receptor with or without (A) arrestin-2. The data are representative of four independent experiments ± SEM. (B) Western blot showing relative expression of arrestin-2 in cells transfected with WT or C621/622G receptor cDNA with or without cDNA expressing arrestin-2 as described for (A) loaded with approximately 11.5 μg of total protein per lane, except for MT (mock transfect) lane, where approximately 7.5 μg of total protein was loaded.

RESULTS

Effect of arrestin-2 on internalization of palmitoylation deficient mutant receptor

We determined whether the rate of internalization of the palmitoylation deficient mutant receptor [4,10] is affected by overexpression with arrestin. To test this, 293T cells were transiently transfected with WT or mutant receptor cDNA in the presence, or absence, of a second vector encoding arrestin-2. Cells were harvested 60 h after transfection and internalization was measured at 37 °C after 20 min of exposure to ^{125}I-labelled hCG. The internalization of WT and C621/622G LH/hCGR increased upon coexpression with arrestin-2 (Fig. 1), suggesting that the palmitoylation deficient mutant receptor can be internalized by an arrestin-mediated pathway.

To further substantiate that the palmitoylation deficient receptor is internalized via an arrestin-mediated mechanism, 293T cells were transiently transfected with cDNA encoding WT or mutant receptor and a truncated, dominant negative form of arrestin-2 (amino-acid residues 319–418) that retains the ability to bind clathrin, but is unable to bind the phosphorylated receptor [16]. Internalization of the C621/622G mutant receptor was inhibited when coexpressed with the dominant negative form of arrestin-2 (Fig. 2). A 62.5 ± 3.5% (SEM of two independent experiments) decrease in internalization of the mutant receptor was seen when coexpressed with (319–418) arrestin-2 compared to a 20.5 ± 4.5% (SEM of two independent experiments) decrease for the WT control. The inhibition of mutant receptor internalization by the dominant negative arrestin-2 further shows that the palmitoylation deficient mutant receptor internalization occurs in large part through an arrestin-mediated pathway.

To examine more closely the influence of the palmitoylation state of the receptor on interaction with the arrestin-mediated internalization pathway, the time course of WT or mutant receptor internalization in the absence or presence of coexpressed arrestin-2 was examined. Cells (293T) were transiently transfected with the appropriate cDNAs, harvested 60 h later, and the time course of internalization was determined. The percent internalization of WT and mutant

Table 1. Effects of arrestin-2 overexpression on WT and mutant receptor internalization. The data presented are the average of four independent experiments.

Receptor constructs	First order rate constant of ^{125}I-labelled hCG internalization $\times 10^{-3}$ (min^{-1}) ± SEM	Rate enhancement[a]
WT	2.01 ± 0.30	–
WT + arrestin-2	4.49 ± 0.66	2.2
C621/622G	5.18 ± 1.23	–
C621/622G + arrestin-2	8.86 ± 1.52	1.7

[a] Rate enhancement is the factor by which the rate constant increases in the presence of coexpressed arrestin-2 for each receptor construct.

receptor in the absence or presence of coexpressed arrestin-2 increased with time (Fig. 3A).

The first order rate constants of internalization were calculated from the data presented in Fig. 3A and are summarized in Table 1. The results show that the rate constants of internalization were higher upon coexpression of arrestin-2 for both the WT and the mutant receptor. Interestingly, coexpression of arrestin-2 along with the WT receptor resulted in a greater rate enhancement compared to that seen for the mutant receptor. This suggests that a lack of palmitoylation of the receptor renders it more susceptible to internalization via endogenous arrestin, such that overexpression of exogenous arrestin can not stimulate mutant receptor internalization as much as WT receptor internalization. LH/hCGR cell surface expression was measured by incubating cells with ^{125}I-labelled hCG for 30 min at 4 °C. Results indicated comparable cell surface receptor expression between WT and mutant receptor expressing cells (data not shown). Furthermore, arrestin-2 overexpression in cells expressing mutant receptor was slightly lower than in cells expressing WT receptor (Fig. 3B). Densitometric analysis showed that arrestin-2 overexpression in cells expressing the C621/622G receptor was approximately 80% of that in cells expressing the WT receptor (data not shown). However, in both cases, arrestin-2 was overexpressed at a high level, and therefore any differences in arrestin-2 overexpression are unlikely to contribute to the differences in rate enhancement.

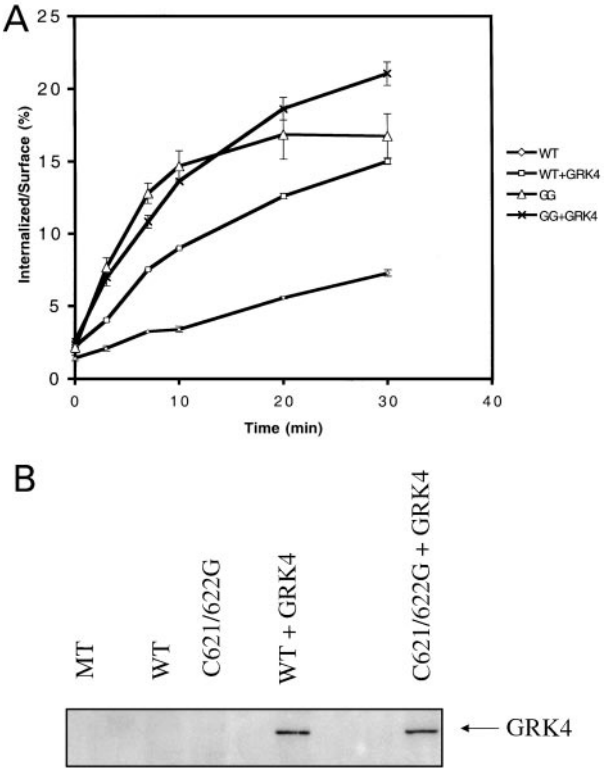

Fig. 4. Time course and rate of ^{125}I-labelled hCG internalization in cells expressing WT or C621/622G receptor with or without GRK4. (A) 293T cells ($2.4–2.5 \times 10^6$) were transiently transfected as described in Experimental procedures with 2.0 μg of WT cDNA, or 6.0 μg of C621/622G cDNA in the presence, or absence, of 6.0 μg GRK4 cDNA. Empty pCMV4 vector was also transfected into cells to equalize the mass of DNA used in all plates. Approximately 60 h after transfection, the cells were harvested and internalization assays performed as described in Experimental procedures. Internalized ^{125}I-labelled hCG was measured as described in Experimental procedures at 37°C after exposure from 0 to 30 min to 100 $ng \cdot mL^{-1}$ ^{125}I-labelled hCG and expressed as a percentage of surface-bound ^{125}I-labelled hCG in cells expressing WT or mutant receptor with, or without, GRK4. The data are representative of five independent experiments ± SEM. (B) Western blot showing relative expression of GRK4 in cells transfected with WT or C621/622G receptor cDNA with or without cDNA expressing GRK4, as described in (A). Approximately 7.5 μg of total protein was loaded in the WT and C621/622G lanes, 5 μg of total protein in the MT (mock transfect) lane, and approximately 5.5 μg of total protein in the WT + GRK4, and C621/622G+GRK4 lanes, respectively.

Effect of GRK4 on receptor internalization

As arrestin is known to preferentially bind the phosphorylated form of GPCRs [13,14], the time course of WT and palmitoylation deficient mutant receptor internalization in the absence, or presence, of coexpressed GRK4, which can promote agonist induced phosphorylation of the receptor [19], was examined. The internalization of the WT and the mutant receptors increased over time when coexpressed with GRK4 (Fig. 4A). The first order rate constants of internalization were calculated from the data presented in Fig. 4A and are summarized in Table 2. While the rate constant of internalization of the mutant receptor was not increased by GRK4 coexpression, the rate enhancement seen for the WT receptor was substantial. This suggests that a lack of palmitoylation renders the receptor less susceptible to stimulation of internalization by exogenous GRKs. LH/hCGR cell surface expression was measured by incubating cells with ^{125}I-labelled hCG for 30 min at 4 °C. Results indicated comparable receptor expression between WT and mutant receptor expressing cells (data not shown). Furthermore, GRK4 overexpression was comparable in cells expressing WT or mutant receptor (Fig. 4B). Densitometric analysis showed that GRK4 overexpression in cells expressing the C621/622G mutant receptor was approximately 90% of that in cells expressing the WT receptor (data not shown). This difference is too small to account for the large difference

Table 2. Effects of GRK4 overexpression on WT and mutant receptor internalization. The data presented are the average of five independent experiments.

Receptor constructs	First order rate constant of ^{125}I-labelled hCG internalization $\times 10^{-3}$ (min^{-1}) ± SEM	Rate enhancement[a]
WT	1.79 ±0.22	–
WT + GRK 4	3.50 ±0.22	2.0
C621/622G	4.46 ±0.92	–
C621/622G + GRK 4	4.97 ±0.62	1.1

[a] Rate enhancement is the factor by which the rate constant increases in the presence of coexpressed GRK4 for each receptor construct.

in rate enhancement seen upon GRK4 overexpression between cells expressing the WT and mutant forms of the receptor.

Effect of PMA on receptor phosphorylation

As the C-terminal portion of the receptor contains consensus phosphorylation sites for protein kinase C and protein kinase A [25], the ability of WT and palmitoylation deficient mutant receptors to undergo phosphorylation in

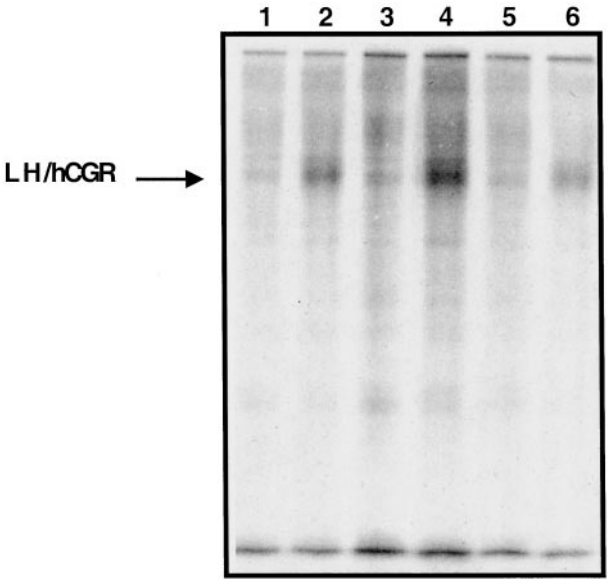

Fig. 5. PMA-stimulated phosphorylation of WT and palmitoylation deficient mutant receptors. Sixty hours after transient transfection of 2–3 × 10^6 293T with cDNA expressing WT, C621/622G, or C621/622S cDNA, the cells were labeled with 100 μCi·mL^{-1} [^{32}P]orthophosphate for 3 h as described in Experimental procedures. The cells were then incubated with, or without, 500 nM PMA for 1 h. The cells were harvested, and the LH/hCGR purified as described in Experimental procedures. The purified receptors were subjected to SDS/PAGE under reducing conditions, and the labeled phosphorylated receptor was visualized by autoradiography. An equivalent amount of eluate from the purification procedure was loaded into each lane. Lanes 1 and 2, C621/622G; lanes 3 and 4, C621/622S; Lanes 5 and 6, WT receptor; Lanes 1, 3, 5, without PMA; lanes 2,4, and 6 with PMA.

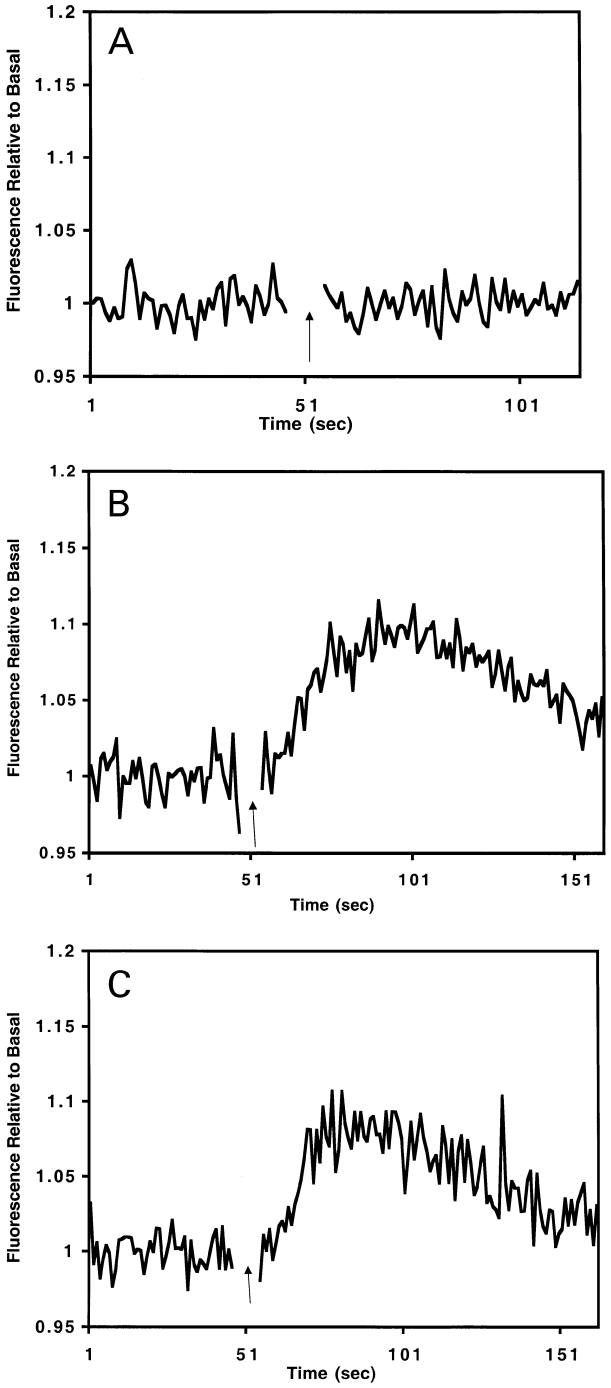

Fig. 6. Calcium release in stably transfected 293 cells expressing WT or palmitoylation deficient LH/hCG receptor. 293 cells stably transfected with pCMV4 expressing WT, or C621/622S mutant receptor were harvested and labeled with Fura 2-AM, as described in Experimental procedures; 2 × 10^6 cells were used per sample. Each plot shows the fold increase in fluorescence emission over baseline (fluorescence relative to basal) at 510 nm. [fluorescence relative to basal vs. time (s) upon addition of 1 μg·mL^{-1} hCG (arrow)] for cells expressing (A) no receptor, (B) WT receptor or (C) C621/622S receptor. Data are representative of two independent experiments.

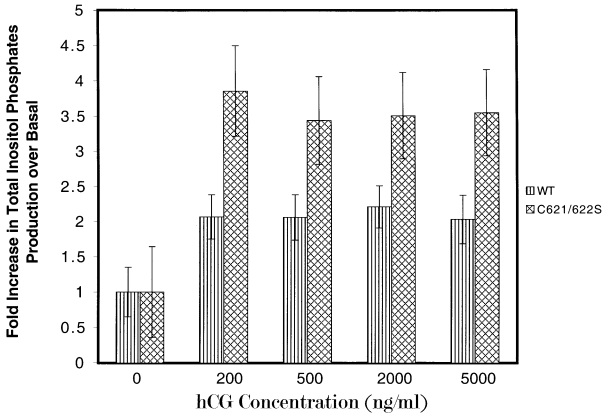

Fig. 7. Increase in total inositol phosphates production in transiently transfected 293T cells expressing WT or palmitoylation deficient LH/hCG Receptor. 293T cells (5×10^6) were transiently transfected with 3μg of WT cDNA or 12 μg C621/622S mutant cDNA to give approximately equivalent receptor expression. Twelve hours later, the cells were labelled with 75 μCi of [^{3}H]*myo*-inositol. Forty-eight hours after labeling, the cells were harvested and stimulated with 0 to 5000 ng·mL^{-1} hCG at 37°C for 40 min. Total inositol phosphates were assayed as described in Experimental procedures. The increase in inositol phosphates production was calculated as the quotient of c.p.m. of [^{3}H]inositol phosphates produced at a given hCG concentration/ c.p.m. of [^{3}H]inositol phosphates produced at [hCG] = 0 for each cell type.

response to PMA or 8-bromo cAMP was examined. The transfected cells were labeled with [^{32}P]orthophosphate for 3 h. Subsequently, cells expressing WT, C621/622G, or C621/622S palmitoylation deficient mutant receptors were treated with 500 nM PMA for 1 h. Controls were incubated in the absence of PMA. Following the incubation, the cells were harvested and solubilized. The LH/hCGR was purified by hCG-affigel chromatography, subjected to SDS/PAGE under reducing conditions, and the phosphorylated receptor was visualized by autoradiography, as described in Experimental procedures. The results show (Fig. 5) an increase in phosphorylation of both the WT and the mutant receptors upon PMA stimulation. Furthermore, both forms of the mutant receptor are more susceptible to PMA stimulated phosphorylation compared to the WT receptor. Densitometric analysis showed that PMA stimulated a 3.5-fold increase in C621/622G phosphorylation, 3.3-fold increase in C621/622S phosphorylation, and 1.8-fold increase of WT receptor phosphorylation (data not shown). 8-Bromo cAMP did not stimulate phosphorylation of the WT receptor to any appreciable extent (data not shown). These results suggest that the palmitoylation state may regulate the phosphorylation state of the LH/hCG receptor, with the unpalmitoylated form of the receptor being more susceptible to phosphorylation.

Effect of hCG on Ca^{2+} release

To further examine the biological role of receptor palmitoylation, we examined the effect of palmitoylation on receptor signaling through the phospholipase C pathway. Specifically, the ability of the palmitoylation deficient mutant to mediate the hCG-induced production of inositol phosphates and Ca^{2+} flux from the ER to the cytoplasm was measured.

The release of Ca^{2+} from the ER into the cytosol upon hCG binding was detected by an increase in fluorescence emission at 510 nm in 293 cells stably transfected with WT or C621/622S mutant receptor cDNA that were loaded with the fluorescent calcium chelator, Fura-2 AM. The results presented in Fig. 6A–C show the release of calcium from the ER into the cytosol in untransfected cells, cells transfected with WT, or C621/622S cDNA, respectively, upon addition of 1 μg·mL^{-1} hCG. 293T cells transiently transfected with C621/622G receptor cDNA also displayed Ca^{2+} release from the ER into the cytosol upon addition of 1 μg·mL^{-1} hCG (data not shown). These results show that the palmitoylation deficient mutant receptor retains the ability to mediate Ca^{2+} release from the ER into the cytosol.

WT and palmitoylation deficient mutant receptor mediated activation of inositol phosphates production

To further confirm that the WT and palmitoylation deficient mutant receptors mediate activation of the phospholipase C pathway, we measured the production of total inositol phosphates as a function of hCG concentration in 293T cells transiently expressing equal numbers of WT or C621/622S mutant receptor. The results presented in Fig. 7 show that both the WT and the mutant receptor have the ability to mediate the activation of inositol phosphate production upon hCG binding.

DISCUSSION

As a continuation of earlier studies on LH/hCG receptor palmitoylation [4,10], the present study examined the effect of overexpression of various components of the cell's endocytotic machinery on WT and palmitoylation deficient mutant receptor internalization. Our results show that a lack of palmitoylation results in a higher rate of internalization, which is largely arrestin-2 dependent, while the internalization of the WT receptor appears to be largely resistant to dominant negative arrestin-2 inhibition. Additionally, coexpression of GRK4 increases WT receptor internalization, but has no effect on the internalization of the C621/622G receptor. Finally, we show that abrogation of palmitoylation does not eliminate the ability of the mutant receptor to activate the phospholipase C signaling pathway.

The basal rate of internalization of the mutant receptor was higher than the WT, but the WT receptor showed a greater rate of enhancement of internalization than the mutant upon overexpression of arrestin-2. This suggests that the mutant receptor preferentially uses endogenous arrestin for internalization compared to the WT receptor. The minimal effect of dominant negative arrestin-2 on WT receptor internalization may be due to hindrance of the receptor–arrestin interaction by the two palmitate residues. This may result in the WT receptor using alternative pathways of internalization, such as the AP-2 mediated internalization pathway [26]. Taken together, these data suggest that a lack of palmitoylation confers on the receptor a greater ability to undergo arrestin-mediated internalization, while palmitoylation interferes with receptor–arrestin

interaction and results in the use of alternative internalization pathways. As coexpression of arrestin-3 was able to promote a greater rate enhancement of the mutant receptor than arrestin-2 (data not shown), it is unlikely that the lower rate enhancement of the mutant receptor in the presence of arrestin-2 was due to the limiting nature of other cellular components required for internalization, such as clathrin and dynamin.

The lower rate enhancement of the mutant compared to the WT was also not due to differences in transfection efficiency (data not shown). It is expected that 293T cells endogenously express arrestin-2, as coexpression with (319–418) arrestin-2 inhibited mutant receptor internalization. Furthermore, it has been previously shown that arrestin-2 is expressed in 293 cells and may be ubiquitously expressed in tissues [27–30]. Along these lines, it was found that arrestin-2 was overexpressed in cells expressing the C621/622G mutant receptor at levels comparable to that seen in cells expressing the WT receptor (Fig. 3B). Although there is a slight decrease in the level of overexpression of arrestin-2 in the mutant receptor expressing cells, this decrease is not expected to have an effect on the rate of internalization. Furthermore, the rate of internalization of the WT receptor is comparable to the rate of the C621/622G mutant internalization only when arrestin-2 is overexpressed with the WT receptor (Table 1). Thus, the palmitoylation deficient receptor is more susceptible to interaction with the arrestin-mediated internalization pathway than the WT receptor.

The GRK family of kinases may play a role in hormone-induced phosphorylation of GPCRs [13–16]. Overexpression of GRK4 resulted in an increase in the rate of internalization of the WT receptor, while there was no increase for the mutant receptor. Differences in GRK4 overexpression are unlikely to play a role in rate enhancement (Fig. 4B). Furthermore, the rate of internalization of the WT receptor is comparable to that of the C621/622G mutant receptor only when GRK4 was overexpressed in the presence of the WT receptor (Table 2). This suggests that a lack of palmitoylation facilitates internalization through promoting receptor phosphorylation. As GRKs probably stimulate internalization through phosphorylation of the C-terminus of the receptor [13–16,19], it is possible that either the C-terminus is hyperphosphorylated in the unpalmitoylated mutant, and consequently GRK4 overexpression has little effect on internalization, or that a lack of palmitoylation inhibits GRK4 stimulated internalization through an unknown mechanism. The hypothesis that the unpalmitoylated receptor might be hyperphosphorylated is supported by the data presented in Fig. 5, which show that both unpalmitoylated forms of the receptor have a greater ability to undergo PMA-stimulated phosphorylation than the WT receptor.

Previous studies on the desensitization of the β2AR have shown that it proceeds through a depalmitoylated, hyperphosphorylated state [9,28,31–34]. Our finding that GRK4 overexpression had no effect on mutant receptor internalization would support the notion that the mutant receptor might exist in an hyperphosphorylated state that is more prone to arrestin-mediated internalization. In summary, the present study suggests that LH/hCG receptor internalization might proceed through a depalmitoylated/hyperphosphorylated intermediate during prolonged exposure to hormone.

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