Palmitoylation of Endothelin Receptor A

Differential Modulation of Signal Transduction Activity by Post-Translational Modification*

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Post-translational modifications such as phosphorylation and palmitoylation play important roles for the function and regulation of receptors coupled to heterotrimeric guanyl nucleotide-binding proteins. Here we demonstrate that the human endothelin receptor A (ETA) incorporates [3H]palmitate. Mutation of a cluster of five cysteine residues present in the cytoplasmic tail of ETA into serine or alanine residues completely prevented palmitoylation of the receptor. The ligand binding affinity of the non-palmitoylated ETA mutants was essentially unchanged as compared to the palmitoylated wild type ETA suggesting that the replacement of the cysteine residues did not alter the overall structure of the receptor. Furthermore, the ligand-induced stimulation of adenyl cyclase by the mutant ETA was unaffected by the mutation. In contrast, the mutated non-palmitoylated receptors but not the wild type receptor failed to stimulate phosphatidylinositol hydrolysis by phospholipase C activation upon challenge by endothelin-1. Furthermore, the mutant receptors failed to stimulate the ligand-induced transient increase in the cytoplasmic calcium seen with the wild type ETA. Endothelin-1 induced mitogenic stimuli via the wild type receptors but not through the mutated receptors suggesting an important role for phospholipase C in this signal transduction pathway. The differential regulation of distinct signal transduction pathways by post-translational modification suggests that palmitoylation of the ETA provides a novel mechanism of modulating ETA receptor activity.

Post-translational modifications of receptors coupled to heterotrimeric guanyl nucleotide-binding proteins (G protein) modulate receptor function and activity (1–3). One such modification is the palmitoylation of conserved cysteine residues located in the cytoplasmic tail of many G protein-coupled receptors. It has been suggested that this acylation provides a membrane anchor that creates a fourth cytoplasmic receptor membrane anchor that creates a fourth cytoplasmic receptor functional role of palmitoylation in the modulation of signal transduction activity. Cys341 of the β-adrenergic receptor causes functional uncoupling of the receptor from the adenyl cyclase pathway (4). Furthermore, the non-palmitoylated receptor shows an increased basal phosphorylation and a rapid desensitization in response to its ligand (6). Activation of the β2-adrenergic receptor results in an enhanced palmitoylation thereby increasing the amount of functionally coupled receptor (7). Adrenergic receptor activation also increases palmitoylation of a receptor-associated stimulatory G protein (8). These findings clearly show the dynamic properties of protein/receptor palmitoylation. Unlike the β2-adrenergic receptor a mutation of the corresponding palmitoylation site in the α2a-adrenergic receptor did not influence the signal transduction activity but decreased the ligand-promoted down-regulation of the receptor (9). On the other hand, purified non-palmitoylated bovine rhodopsin shows an increased signal transduction activity compared to the palmitoylated receptor as evidenced by the increase in transducin activity (10). The reported pleiotropic effects of palmitoylation on receptor activity raise the question of how this post-translational modification will influence the signal transduction activity of other G protein-coupled receptors, especially of those that stimulate multiple signal transduction pathways such as the phospholipase C (PLC)1 and the adenyl cyclase pathways by coupling to different G proteins within the same cell.

Endothelins, a family of three closely related peptide hormones, endothelin-1, -2, and -3 (ET-1, ET-2, and ET-3) (11–13) mediate their multiple biological effects by at least two types of receptors, endothelin receptor A (ETA) (14) and endothelin receptor B (ETB) (15). The ET receptors belong to the large receptor family characterized by seven transmembrane-spanning regions which regulate distinct intracellular signaling pathways through the activation of G proteins. Stimulation of ETA expressed in CHO cells revealed a ligand-dependent increase in cAMP formation via Gαs whereas activation of ETB expressed in the same cell type inhibited forskolin-stimulated cAMP synthesis via Gαi (16, 17). Ligand-activated ETA and ETB both stimulate phosphatidylinositol hydrolysis, most likely via a pertussis toxin insensitive G protein such as Gq. In addition, ETA and ETB stimulate release of arachidonic acid and exhibit mitogenic activity. Whether these latter effects are indirectly produced by the increase in cytoplasmic Ca2+ ([Ca2+]i) and/or by the modulation of cAMP formation, or whether they are due to direct activation of effector enzymes by ETA and ETB remains unclear.

In the present study we provide experimental evidence for a functional role of palmitoylation in the modulation of signal...
transduction activity of the human ETA. We demonstrate that the mutated non-palmitoylated ET_2 receptor has lost its ability to stimulate phosphatidylinositol hydrolysis while its capacity to stimulate cAMP formation is unchanged. The activated non-palmitoylated ET_2 failed to stimulate the release of arachidonic acid and showed no mitogenic activity. The differential regulation of signal transduction pathways might indicate activation of adenyl cyclase and of phospholipase C by ETA are two independently controlled processes that could be regulated by receptor palmitoylation.

EXPERIMENTAL PROCEDURES

Materials—Materials were obtained from the following manufacturers: pcDNAI11, pVL-1392, and pVL-1393 vectors and Liposome Kit from Invitrogen; polymerase chain reaction and mutation primers from Roth; and oligonucleotides from Life Technologies, Inc. Other reagents were obtained from standard commercial sources.

Cloning of the ETA Receptor—The nucleotide sequence encoding the ET_A receptor was amplified by the polymerase chain reaction using human placenta cDNA as a template together with the following primers: T4 (5'-GGCTACCATGTGAGTTGTT-3') and T5 (5'-ATTTTCTTCGGTGTGGAA-3'). The 1,400-base pair fragment that was specifically amplified was subcloned into the EcoRV-Smal sites of Bluescript KS⁺ (Bks⁺) and also into the Smal site of pVL-1392. Individual clones were isolated, and the nucleotide sequence of the ET_A receptor was verified by the Sanger sequencing method.

Mutagenesis of the ETA Receptor cDNA—The ET_A receptor cDNA excised from Bks⁺ was subcloned into pcDNAII using XbaI/Apal restriction sites. This construct was used as the template for the polymerase chain reaction-based site-directed mutagenesis according to standard procedures. The following primers were used: two ET_A receptor cDNA flanking primers P1 (5'-GGCTTACCATGTAAGTTGTTCCG-3') and P2 (5'-CTCTAAATGGCTCTAGTAGGAAACCTT-CCCGGAGCCATCC-3'); the ET_A-specific mutation primers Msr (CCTCCCTCCCTTACATCAGCTTCCC-3') to mutate a cluster of 5 cysteine residues to serine (ETA-1215SS and Mra (5'-CCATCGAGCGCTTTTGTAGGAGAAAGCCTTCGTAC-3') for the mutation of the histidine to alanine residue (ETA-1215H). The identity of the mutations was verified by sequence analysis. The cDNA fragments containing the mutated ET_A-1215S or ET_A-1215A gene were subcloned into the EcoRV/Xbal restriction sites of pcDNAII and in the Smal/Xbal restriction sites of pcDNAI11.

Cell Culture—CHO-K1 cells were grown in Dulbecco's modified Eagle's medium supplemented with Ham's F-12 nutrient mixture, non-essential medium amino acids, pH 7.4) for 30 min and then extracted and separated into the cAMP/mitogen-activated protein kinase signaling pathways using as described (21) with minor modifications. Briefly, cloned CHO-K1 and transfected COS7 cells were grown in 96-well plates, rinsed three times with 0.15 M NaCl, 0.1 mM CaCl₂, pH 7.4 (PBS), and incubated under gentle agitation for 4 h at 4 °C in 50 µl of the assay buffer (PBS, 0.5% bovine serum albumin, 0.26 g of α-glucose/100 ml) containing [3H]ET-1 (50 µCi, 2000 Ci/mmol) and competing peptides in the indicated concentrations. After three rapid washes with 100 µl each of ice-cold PBS, the cells were solubilized with 0.5 M NaOH, 1% SDS, and the amount of bound [3H]ET-1 was determined by γ-counting. Sf9 cells infected for 12 h were washed three times with PBS, collected by centrifugation (1,500 g for 10 min at 20 °C), resuspended in assay buffer, and incubated for 12 h at 4 °C. Bound ligand was measured as described with the addition of 0.1% SDS. The supernatants were transferred to a polypropylene tube by filtration through Whatman GF/C filters followed by three washes with PBS. Filter-bound radioactivity was measured as a γ-counter.

Determination of Whole Cell cAMP Formation—CHO-K1, COS7, and Sf9 cells expressing wild-type ETA or mutants ET_A-1215S and ET_A-1215A were cultured in 24-well plates before incubation with 300 µM of medium containing 1.5 mM CaCl₂, 30 µg/ml of theophylline, and 0.1% bovine serum albumin. The cells were stimulated for 15 min in the same medium with varying concentrations of ETA-1 as indicated. The reactions were stopped after aspiration of the medium followed by the addition of 500 µl/well of ice-cold 50 mM Tris-HCl, pH 8.0, containing 4 mM EDTA. Cells were then collected into a tube containing 500 µl of 10% TCA, centrifuged (14,000 g for 3 min at 4 °C). The supernatants were assayed for cAMP using a [3H]cAMP competition binding kit according to the manufacturer's instructions.

Determination of Inositol Phosphate—Inositol trisphosphate hydrolysis was measured as described recently (22). Briefly, cloned CHO-K1 cells were grown to confluence in 24-well plates and labeled with [32P]inositol (1 µCi/ml) for 12 h in serum-free medium containing 116 mM NaCl, 5 mM KCl, 0.81 mM MgCl₂, 7.1 mM H₃PO₄, 5 mM CaCl₂, 5 mM α-glucose, 20 mM Hepes, 1 × minimal essential medium amino acids, pH 7.4). Following incubation with 10 µM LiCl for 15 min the cells were directly stimulated at 37 °C in this medium with varying concentrations of ET-1 as indicated. The reactions were stopped after 10 min by aspiration of the medium and addition of 1 ml of ice-cold formic acid (10 µM, pH 3). After incubation for 30 min at 4 °C these supernatants were mixed into 3 ml of 50 mM ammonium formate (final pH 9–9), and these extracts were applied to anion exchange columns prepared with 0.3 to 0.5 g of Dowex AG-1–8x where the hydroxide form had been exchanged to the formate form as described by the manufacturer (Bio-Rad). Columns were eluted with 10 ml of H₂O to remove free inositol, followed by 7 ml of 2 x ammonium formate/formic acid, pH 5.2, to elute total inositol phosphates. The eluates were quantitated by liquid scintillation counting.

[Ca²⁺]i Measurement by Fura-2/AM—Intracellular Ca²⁺ concentrations were determined by the fluorescent Ca²⁺ chelating agent, fura-2/AM. Clonal CHO-K1 cells confluent cultured on glass coverslips, and aliquots of Sf9 cells suspension were inoculated with lo} incubation (116 mM NaCl, 5.3 mM KCl, 0.81 mM MgSO₄ × 7 H₂O, 1 mM CaCl₂, 20 mM Hepes, minimal essential medium amino acids, pH 7.4) for 30 min at 37 °C (CHO) or for 1.5 h at room temperature (Sf9). The incubation was followed by two washes with loading solution containing 5 mM α-glucose. Loaded cells were stimulated with different concentrations of ET-1 in indicated, and the fluorescent emission using a HITACHI spectrophotometer. To test for the PLC activity of donor CHO-K1 cells previously challenged with ET-1, the cells were stimulated with 4 mM ATP. To investigate the involvement of PLC in the transient increase in [Ca²⁺]i, Sf9 cells were incubated with 4 µM U73122, a specific PLC inhibitor, or with 10 µM LaCl₃, an inhibitor of Ca²⁺ channel opening, prior to stimulation with 100 mM ET-1. Fluorescence monitoring, calibration procedures, and all other experimental details were as described (23, 24).

Determination of [3H]Arachidonic Acid Release—To measure the receptor-mediated arachidonic acid release the cloned CHO-K1 cells were cultured in 24-well plates until confluent, and labeled by incubation for 2.5 h with serum-free Ham's containing [3H]arachidonic acid (1 µCi/ml) to label the phospholipase A₂ activity. After carefully washing three times with PBS the cells were incubated with serum-free Ham's for 30 min to equilibrate. The cells were stimulated for 15 min at 37 °C in 500 µl of fresh serum-free medium containing ET-1 at the concentrations indicated. The amount of [3H]arachidonic acid released into the supernatant (450 µl) was determined by liquid scintillation counting (25, 26).
for 1 week at m
for 30 min at 4°C, followed by another wash with ice-cold PBS. Acid-
glycerol, 5%

receptor solubilization and immunoprecipitation were carried out as
developed above. Reducing SDS-PAGE was performed with the immu-

incubation with 15% (w/v) sodium salicylate for 30 min at room tem-
mperature. Following incubation in the same medium for 30 min the cells

acid-tol 0M uretic acid for 30 min at 42°C.

RESULTS

TABLE I

<table>
<thead>
<tr>
<th>Competition binding IC_{50}a</th>
<th>Bosentan</th>
<th>cAMP EC_{50}b</th>
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<tr>
<td>ET-1</td>
<td>BQ123</td>
<td>Bosentan</td>
</tr>
<tr>
<td>CHO cell</td>
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<tr>
<td>wt-ET_{A}</td>
<td>11.2 ± 0.32</td>
<td>1.47 ± 0.82</td>
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<tr>
<td>ETA-S1,-S2,-S3; Sf9 cells</td>
<td>7.01 ± 2.36</td>
<td>0.75 ± 0.13</td>
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<tr>
<td>ETA-S2,-S3</td>
<td>9.96 ± 0.04</td>
<td>1.61 ± 0.33</td>
</tr>
<tr>
<td>ETA-S1,-S2,-S3; Sf9 cells</td>
<td>9.96 ± 0.04</td>
<td>1.61 ± 0.33</td>
</tr>
<tr>
<td>ETA-1215S</td>
<td>20.3 ± 0.18</td>
<td>1.96 ± 0.03</td>
</tr>
<tr>
<td>ETA-1215A</td>
<td>9.96 ± 0.11</td>
<td>2.40 ± 0.40</td>
</tr>
<tr>
<td>ETA-S1,-S2,-S3; Sf9 cells</td>
<td>5.95 ± 0.09</td>
<td>2.95 ± 0.25</td>
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</table>

aIC_{50} values were obtained in competition binding experiments measuring binding of [125I]endothelin-1 competed with endothelin-1 (ET-1), BQ123, and Bosentan. Data are presented as mean ± S.D. of at least three independent experiments performed in triplicate.

bEC_{50} values of ET-1 induced cAMP formation. Cell lines used: CHO cells expressing wild type ET_{A} (wt-ET_{A}) or mutant ET_{A}-1215S (clones ETA-S1,-S2,-S3); Sf9 cells expressing wild type ET_{A} (wt-ET_{A}) or ET_{A} mutants, ET_{A}-1215S and ET_{A}-1215A.

ND, not determined.

Determination of [3H]Thymidine Incorporation—Growth promoting activity of ET-1 was measured by [3H]thymidine incorporation as described (25, 27). Clonal CHO-K1 cells were grown in 24-well plates for 48 h. At a confluence of 70% the cell cultures were synchronized for 24 h in serum-free Ham’s, then stimulated for 18 h with varying concentrations of ET-1 in serum-free medium as indicated, followed by an incubation for 4 h in fresh serum-free medium containing [3H]thymidine (1 μCi/ml) and different concentrations of ET-1. After washing three times with ice-cold PBS the cells were treated with 10% trichloroacetic acid for 30 min at 4°C, followed by another wash with ice-cold PBS. Acid-insoluble fractions were solubilized with 0.1 N NaOH, 1% SDS for 30 min at 37°C, and the associated radioactivity was quantified by liquid scintillation counting.

Gel Electrophoresis and Immunoblotting—After 48–72 h of infection the SF9 cells were washed three times with PBS and resuspended in sample buffer (63 mM Tris-HCl, pH 6.8, containing 2.5% SDS, 0.5% mercaptoethanol, 0.005% bromphenol blue) and incubated for 30 min at 42°C. SDS-polyacrylamide electrophoresis (SDS-PAGE) was carried out in 10% acrylamide gels. Gels were processed for immunoblotting as described in the manufacturer’s instructions (“Westrans,” Schleicher & Schuell). All antisera were used at a 1:2,500 dilution. Anti-rabbit IgG conjugated to horseradish peroxidase and the chemiluminescence detection method were employed for visualization of the protein bands.

Immunoprecipitation of ET_{A}—SF9 cells that had been infected for 60 h were washed twice with ice-cold PBS and resuspended in ice-cold RIPA buffer (350 mM NaCl, 50 mM Tris-HCl, pH 8.0, containing 0.1% SDS, 0.5% deoxycholat, 1% Nonidet P-40) with rotation for 1 h at 4°C. The cell lysates were centrifuged at 14,000 × g for 10 min at 4°C and the supernatants incubated under rotation for 1 h at 4°C with specific antibodies previously coupled to staphylococcus A. The reactions were terminated by centrifugation (8,000 × g for 2 min at 4°C). The pellets were washed twice with ice-cold RIPA buffer and resuspended in 30 μl of sample buffer (250 mM Tris-HCl, pH 6.8, containing 4% SDS, 20% glycerol, 10% β-mercaptoethanol, 0.002% bromphenol blue) and incubated with 20 μl of 10 μM urotic acid for 30 min at 42°C.

Palmitoylation of Human ET_{A} Receptor—Infected SF9 cells were washed twice with serum-free TC100 medium and cultured in serum-free TC100 medium for 12 h. Following this period the cells were plated in fresh serum-free TC100 medium and metabolically labeled with [9,10-3H]palmitic acid (0.2 mCi/ml) for 4 h at 27°C. The reaction was stopped by washing the cells twice with ice-cold PBS. Receptor solubilization and immunoprecipitation were carried out as described above. Reducing SDS-PAGE was performed with the immunoprecipitated samples using 10% acrylamide gels including 1% urea. The gels were fixed with methanol/H_{2}O/acetic acid (10:77.5:12.5), dried for 2 h at 60°C, and exposed to a Fuji x-ray film for 8 h at −70°C.

Detection of the ET_{A} by Anti-peptide Antibodies—To examine post-translational modifications of the human ET_{A} receptor, antibodies against five synthetic peptides from the extracellular and intracellular domains of the receptor were generated in rabbits (Table II). The titers of specific antibodies were similar for the various peptides as judged by indirect enzyme-linked immunosorbent assay using titer plates coated with the cognate peptides. Only antisera directed against the amino-terminal (α-CDN25) or the carboxyl-terminal (α-CTS24) region of the receptor cross-reacted with the ET_{A} protein in Western blotting and immunoprecipitations. Identical cross-reactivity profiles were obtained with anti-peptide antisera raised in mice (not shown).

For immunoprecipitations human ET_{A} was expressed in SF9 cells that had been metabolically labeled with [35S]cysteine/methionine. The total cell lysates were immunoprecipitated with the combined antisera α-CDN25 and α-CTS24 and subjected to SDS-PAGE. Two distinct bands, a major band of approximately 48 kDa and a minor band of 38 kDa were immunoprecipitated by the antisera (Fig. 3A). In addition a band of 96 kDa was detected that might represent a dimerized receptor. No specific bands were found in immunoprecipitates of
Sf9 cells expressing the human ET<sub>B</sub> receptor or of control Sf9 cells infected with the wild type baculovirus (Fig. 3A). Western blotting of lysates from Sf9 cells expressing the wild type ET<sub>A</sub> revealed a similar pattern of protein bands as that observed with the immunoprecipitation experiments (data not shown).

Palmitoylation of ET<sub>A</sub>—To investigate post-translational modifications of the human ET<sub>A</sub> receptor by acylation we incubated Sf9 cells expressing ET<sub>A</sub> with [3H]palmitic acid. ET<sub>A</sub> was immunoprecipitated by the combined antisera α-CDN25 and α-CTS24 and subjected to SDS-PAGE, followed by fluorography and autoradiography. Incorporation of [3H]palmitate into the ET<sub>A</sub> protein was detected in three radiolabeled bands of approximately 38, 48, and 96 kDa (Fig. 3B and C). Stimulation of wt-ET<sub>A</sub> with 100 nM ET-1 did not influence incorporation of [3H]palmitate into the ET<sub>A</sub> protein (data not shown). The labeled proteins co-migrated with the 35S-labeled ET<sub>A</sub> protein (Fig. 3A). The incorporated [3H]palmitate was released upon incubation of the immunoprecipitated receptor protein with 1 M hydroxylamine, pH 7.0, prior to SDS-PAGE whereas treatment with 1 M Tris-HCl, pH 7.0, showed no effect (Fig. 3C). It can therefore be concluded that the incorporated radiolabel is [3H]palmitate covalently bound to ET<sub>A</sub> via a thioester linkage, most probably to cysteine residue(s).

Mutation of Cysteine Residues Eliminates [3H]Palmitic Acid Incorporation—Given that ETA incorporates [3H]palmitate we sought to identify the receptor region containing cysteine residue(s) covalently linked to [3H]palmitate. Unlike the α<sub>2</sub>- or β<sub>2</sub>-adrenergic receptors where palmitoylation occurs at a single conserved cysteine residue at the receptor carboxyl terminus, the ET<sub>A</sub> contains a cluster of five cysteine residues (Cys<sup>383</sup>, Cys<sup>385</sup>, Cys<sup>386</sup>, Cys<sup>387</sup>, and Cys<sup>388</sup>) in the corresponding region (Fig. 4). Replacement of this cluster by five serine residues using polymerase chain reaction-based site-directed mutagenesis generated a mutated ET<sub>A</sub> receptor, ET<sub>A</sub>-1215S (Fig. 4). The exchange of the cysteine residues resulted in the elimination of a detectable incorporation of [3H]palmitate into the mutant ET<sub>A</sub> protein as judged from an immunoprecipitation of [3H]palmitate- labeled Sf9 cells expressing either wild type ET<sub>A</sub> or ET<sub>A</sub>-1215S protein (Fig. 3B). While the replacement of the cysteine residues abolished the [3H]palmitate incorporation into ET<sub>A</sub>-1215S the expression levels of the wild type and mutant ET<sub>A</sub> receptor protein were similar: immunoprecipitation of 35S-labeled wild type ET<sub>A</sub> or ET<sub>A</sub>-1215S protein demonstrated similar expression levels of the two receptor variants (Fig. 3D). Replacement of the five cysteine residues with alanine (“ET<sub>A</sub>-1215A”) gave comparable results, i.e. lack of palmitoylation.


Table II

<table>
<thead>
<tr>
<th>Sequencea</th>
<th>Locationb</th>
<th>Positionsc</th>
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<tr>
<td>CDNPERYSTLNSHVDDFTTFRGTE</td>
<td>ED1</td>
<td>21-44</td>
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<td>ED7</td>
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<td>ED3</td>
<td>140-159</td>
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<td>ED4</td>
<td>329-342</td>
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<td>CTSIQWKNHQWNNTHDRSHKDS</td>
<td>ID4</td>
<td>403-425</td>
<td>CTS 24</td>
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</table>

*a* The single letter amino acid code is used.
*b* Orientation with respect to the cytoplasmic membrane: ED, extracellular domain; ID, intracellular domain.
*c* Positions in the sequence of the human ETA receptor (accession number; P25101).
*d* Peptide names are composed by the first three amino terminal residues using the one letter code, followed by the total number of residues of the peptide.

Fig. 3. Incorporation of [3H]palmitic acid into immunoprecipitated wild type ETA or ETA-1215SS protein.

Wild type ETA or ETA-1215SS protein was immunoprecipitated from total Sf9 cell lysates using α-CDN25/α-CTS24 antisera. Lysates were prepared from Sf9 cells expressing either wild type or mutant ETA. Cells were metabolically labeled for 4 h with either [3H]lysine/methionine (A and D) or [3H]palmitic acid (B and C). Immune complexes were resolved in SDS-PAGE, followed by fluorography and autoradiography. Panels A, B, and D, lysates of Sf9 cells infected with recombinant ETA-virus (ETA), ETA-1215S virus (ETA-1215S), ETA-1215A virus (ETA-1215A), or non-recombinant AcNPV-virus (wt); panel C, lysates of Sf9 cells infected with ETA-virus treated with 1 M Tris, pH 7.0 (ETA), or 1 M hydroxylamine, pH 7.0 (ETA + hydroxylamine).

Palmitoylation of Human ETA Receptor

Effect of Cysteine Replacement on the Regulation of [Ca^{2+}]i and Phosphatidylinositol Hydrolysis by ETA-1215AXNext we compared the signal transduction properties of ETA-1215S to the wild type ETA. In CHO cells the dose-response curve of the ET-1-stimulated increase of inositol phosphates revealed an EC_{50} of 10.8 ± 5.1 nM ET-1 for the wild type ETA, whereas three independent CHO clones expressing ETA-1215S failed to show a ligand-dependent increase in phosphatidylinositol hydrolysis (Fig. 5). This lack of stimulation of phosphatidylinositol hydrolysis by ETA-1215S indicates that the mutant ETA-1215S receptor has lost the ability to stimulate PLC. This hypothesis is supported by the finding that ETA-1215S also failed to increase [Ca^{2+}], upon challenge with ET-1 in Sf9 cells: using fura-2/AM to analyze [Ca^{2+}], changes we were able to show a ligand-dependent transient in [Ca^{2+}], only for the wild type ETA (Fig. 1A) but not for ETA-1215S (Fig. 1C) or ETA-1215A (Fig. 1D). Similar results were obtained with three independent CHO cell clones stably expressing the ETA-1215S receptor (Fig. 6). Because the specific PLC inhibitor, U73122, completely blocked the ET-1-induced [Ca^{2+}] increase mediated by the wild type ETA (Fig. 1B), we conclude that the non-palmitoylated ETA mutants have lost their ability to stimulate PLC and consequently to increase [Ca^{2+}] in CHO and Sf9 cells.

Effect of Cysteine Replacement on the Stimulation of cAMP Synthesis by ETA—The failure of ETA-1215S/A to stimulate phosphatidylinositol hydrolysis and to increase [Ca^{2+}], might suggest that the replacement of the cysteine cluster had generated a functionally deficient mutant of ETA. However, CHO cells stably expressing wild type or mutant ETA showed no apparent differences in the ET-1-induced increase of cAMP synthesis. The EC_{50} values calculated from the dose-response curves of cAMP stimulation were 12.9 nM ET-1 for the wild type ETA (Fig. 2A) and 6.3 nM ET-1 (Fig. 2C) for the most active
The maximum stimulation of cAMP synthesis was about 10-fold for the wild type ETA (Fig. 2A) and 2.5- to 7-fold for the various ETA-1215S clones (Fig. 2C). The capacity of ETA-1215S to stimulate cAMP formation was confirmed with Sf9 cells (Fig. 2D) where EC50 values of 3.4 nM ETA-1215S and 4.1 nM ETA were found (Table I). In addition, the efficacy values were similar for the wild type and the mutated receptors: maximum stimulation of cAMP formation was 2-fold in the case of the wild type ETA and 2.3-fold for ETA-1215S (Fig. 2, B and D). We conclude that the non-palmitoylated ETA has selectively lost its capacity to stimulate the phosphohialase C pathway but retained its capacity to activate the adenylyl cyclase pathway.

Effect of Cysteine Replacement on [3H]Arachidonic Acid Release and Mitogenic Activity of ETA—ETA mutants unable to stimulate the PLC pathway provide an ideal means to study cellular effects of endothelins that are thought to be regulated via PLC activation. For example, stimulation of arachidonic acid release by ETA is believed to be indirectly regulated via the [Ca2+]i transient following PLC stimulation. To address this hypothesis we used clonal CHO cells expressing the wild type ETA or ETA-1215S, three different clones, ETA-S1, ETA-S2, and ETA-S3 were incubated in the absence (control) or presence of 100 nM ET-1 for 15 min. Data were calculated as the fold increase of inositol phosphates accumulation over control. Data are means ± S.D. representative of three separate experiments done in triplicate.

Fig. 6. ET-1-induced increase of [Ca2+]i in CHO-K1 clones expressing wild type or mutant ETA. ET-1-induced changes of [Ca2+]i were measured as changes in the ratio of fluorescence measured at 340/380 nm in fura-2AM preloaded CHO-K1 lines expressing either wild type ETA (A) or ETA-1215S clones, ETA-S1 (B), ETA-S2 (C), ETA-S3 (D). To control for functional PLC, CHO-K1 clones expressing ETA-1215S (B, C, and D) were stimulated with 4 mM ATP. Effectors were added at the time point indicated by an arrow. The recordings are representative of three independent experiments.
Endothelins have powerful mitogenic effects through the $\mathrm{ET}_A$ receptor. These mitogenic effects are thought to be due to $\mathrm{ET}_A$-mediated PLC activation giving rise to diacylglycerol and the inositol triphosphate formation, with subsequent activation of protein kinase C (PKC) by diacylglycerol and the inositol triphosphate-triggered increase of $[\mathrm{Ca}^{2+}]_{\text{cyt}}$. To test this hypothesis we measured ET-1-induced $[^{3}H]$thymidine incorporation in CHO cells transfected with mutant $\mathrm{ET}_A$-1215S cDNA using CHO cells bearing the wild type $\mathrm{ET}_A$ receptor as a control. In the absence of FCS, ET-1 stimulated $[^{3}H]$thymidine incorporation in CHO cells expressing wild type $\mathrm{ET}_A$ in a dose dependent manner to levels over 2-fold above baseline, whereas three independent CHO clones expressing $\mathrm{ET}_A$-1215S showed no significant $[^{3}H]$thymidine incorporation (Fig. 8). Therefore the ligand-induced activation of PLC seems to be necessary for the $\mathrm{ET}_A$-mediated mitogenic stimuli, and cAMP formation alone is not sufficient to promote these effects. Hence abrogation of palmitoylation due to the mutation of a critical cysteine cluster renders the $\mathrm{ET}_A$ receptor non-mitogenic.

DISCUSSION

We have demonstrated that the human $\mathrm{ET}_A$ is covalently modified by thioesterification of palmitic acid. Replacement of cysteine residue cluster in the carboxyl terminus by serine or alanine residues results in a non-palmitoylated $\mathrm{ET}_A$ that is still able to stimulate adenylyl cyclase but has lost its ability to stimulate PLC, to increase $[\mathrm{Ca}^{2+}]_{\text{cyt}}$, to release arachidonic acid, and to mediate ET-1-induced mitogenic effects. Thus, palmitoylation appears to differentially modulate the $\mathrm{ET}_A$ signal transduction activity.

The similar expression levels of wild type $\mathrm{ET}_A$ and $\mathrm{ET}_A$-1215S/A suggest that post-translational modification of the receptor by palmitoylation is not essential for the expression and processing of $\mathrm{ET}_A$. High affinity binding of ET-1 by the mutant receptors is a good indication that the overall structure of the receptor has not been changed by the amino acid replacement. These data are in line with the findings of unchanged binding properties for the non-palmitoylated $\alpha_2$-adrenergic receptor (28) and the rat lutropin/choriogonadotropin receptor (29). The available data do not strictly prove that loss of palmitoylation and the functional impairment of $\mathrm{ET}_A$ are causally related. It is still possible that the replacement of the cysteine residue cluster alone causes the loss of function in the $\mathrm{ET}_A$ mutants, and that lack of palmitoylation is only an epiphenomenon. It is also conceivable that the mutations impair the palmitoylation of ectopic cysteine residues. For example, Hepler et al. (30) have shown by enzymatic deacetylation of critical cysteine residues that palmitoylation of $\mathrm{G}_{\text{a}q}$ is not required for activation of PLC$\beta_1$, $\mathrm{G}_{\text{a}q}$ and $m_1$-muscarinic receptor, rather that the cysteine residues are functionally important per se, and their palmitoylation might even have an inhibitory effect on G protein function. However, other reports where the corresponding cysteine residues of $\mathrm{G}_{\text{a}q}$ had been mutated to serine residues came to the conclusion that palmitoylation is essential for $\mathrm{G}_{\text{a}q}$ function (31, 32). Few such data are available for G protein-coupled receptors: depalmitoylation of purified rhodopsin results in an enhanced light-dependent GTPase activity (33). Therefore, the comparing study of the functional properties of palmitoylated and non-palmitoylated cysteine residues in the terminal domain of $\mathrm{ET}_A$ will await the purification of the wild type $\mathrm{ET}_A$ and its chemical or enzymatic deacetylation.

The finding that $\mathrm{ET}_A$-1215S stimulates adenylyl cyclase without activating PLC cannot simply be explained by differences in the receptor efficacy: even at ET-1 concentrations that were 1000-fold above those necessary for the wild type $\mathrm{ET}_A$ the mutant $\mathrm{ET}_A$-1215S/A receptor failed to activate PLC. This data suggests that the $\mathrm{ET}_A$-1215 mutant has essentially lost its capability to stimulate PLC. On the other hand the minor differences seen in the stimulation of cAMP synthesis of randomly picked CHO clones expressing $\mathrm{ET}_A$-1215S are likely to reflect variable efficacy. In Sf9 cells where donor variation does not occur the same mutant receptor had an $E_{\text{C}_{50}}$ for ET-1-stimulated cAMP synthesis which matched that of the wild type $\mathrm{ET}_A$. Furthermore, our $\mathrm{ET}_A$-1215 mutants inhibit $[^{3}H]$thymidine incorporation induced by FCS or insulin to about 30% (data not shown), thereby reproducing the recently reported inhibition of growth factor-induced $[^{3}H]$thymidine incorporation by the wild type $\mathrm{ET}_A$ (34). This latter finding provides another argument for the "partial wild type activity" of $\mathrm{ET}_A$-1215S/A, i.e. the non-palmitoylated $\mathrm{ET}_A$ selectively lost the capacity to stimulate PLC while other receptor functions are fully retained.
The selective impairment of signal transduction properties has been previously reported for thyrotropin receptor (35) and for the neurotensin receptor (36) where mutations were introduced into the third cytoplasmic loop (ED4) without affecting post-translational modifications of these receptors. These findings are in agreement with our observation that distinct structural elements of G protein-coupled receptors appear to be involved in the coupling to the various signal transduction pathways. The novelty of the present study lies in the discovery of a differential modulation of signal transduction by a post-translational modification, and in the identification of a cysteine residue cluster located in the tail domain, ED4, of the receptor. Thus, these studies reveal that the receptor that we tested is not a post-translational modification, and in the identification of a cysteine residue cluster located in the tail domain, ED4, of the receptor.

The specific role of ED4 in signal transduction is highlighted by a recent report that carboxyl-terminal truncations of the ETA protein abolished its capacity to modulate [Ca^{2+}]; (17), however, no information was provided regarding whether the mutant receptors stimulate phosphatidylinositol hydrolysis or activate CAMP synthesis.

The differential coupling of ETA-1215S provides a novel mechanism of receptor modulation by post-translational modification. Whereas the dynamic turnover of palmitate has been shown for several proteins including G protein-coupled receptors (8, 37, 38), we could not demonstrate such a ligand-dependent modulation of ETA palmitoylation in Sf9 cells. agonist-stimulated increase in receptor palmitoylation has been shown for the β2-adrenergic receptor (2) where a 2-fold up-regulation of palmitoylation was seen under certain experimental conditions. We have been unable to find such a ligand-dependent modulation for the ETA receptor. Therefore, we conclude that ETA palmitoylation is not regulated by the cognate agonist, however, it may vary depending on the cell type and/or the differentiation status of a given cell.

An obvious explanation for the failure of ETA-1215S/A to stimulate PLC might be its inability to bind Gαq protein; in our experiments, we have not directly addressed this possibility (see below). However, in the case of the homologous β2-adrenergic receptor the lower affinity of the non-palmitoylated receptor compared to the wild type receptor suggests that Gαq binding is impaired in the mutant receptor (4). Because ETA does not seem to couple to a G protein prior to ET-1 binding (39) we can only speculate that the non-palmitoylated ETA is unable to bind Gαq. Alternatively, the ligand-activated ETA receptor mutants could bind to Gαq, but their ability to stimulate the conformational change of Gαq necessary to induce the GTP/GDP exchange might be impaired (40). The latter possibility would then suggest that two different active states of ligand-activated ETA are necessary for Gαq versus Gαq activation. At present, we cannot discriminate between these possibilities.

The ET(A) receptor allowed us to clarify the importance of PLC activation for ET(A)-mediated cellular effects of endothelins such as arachidonic acid release and mitogenic activity. The failure of ETA-1215S/A to induce release of arachidonic acid in CHO cells strongly suggests that this signal transduction pathway requires phosphatidylinositol hydrolysis and/or a transient increase in [Ca^{2+}]; for cPLA2 activation to occur (41) whereas stimulation of adenylyl cyclase alone is insufficient to produce the arachidonic acid release. The discrepancies between our present data in CHO cells frequently used to analyze the signal transduction pathways of G protein-coupled receptors (10, 42), and those of earlier reports stating that endothelin-mediated arachidonic acid release is independent of phosphatidylinositol hydrolysis and [Ca^{2+}]; transients in smooth muscle cells (16, 17) might reflect cell-type specific differences in signal transduction pathways. In Swiss 3T3 and artery smooth muscle cells, ET-1 stimulates adenylyl cyclase and PLC and acts as a pow-
Palmitoylation of Human \( \text{ET}_A \) Receptor

Palmitoylation of Endothelin Receptor A: DIFFERENTIAL MODULATION OF SIGNAL TRANSDUCTION ACTIVITY BY POST-TRANSLATIONAL MODIFICATION


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