Interactions of Endothelin Receptor Subtypes A and B with G_i, G_o, and G_q in Reconstituted Phospholipid Vesicles

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ABSTRACT: To understand the biochemical basis for the functional divergence of the human endothelin receptor subtypes A (ET_AR) and B (ET_BR), they were expressed, purified from insect Sf9 cells, and reconstituted into phospholipid vesicles with the G_o, G_q, and G_i proteins. For each G protein, a unique pattern of reactivity was observed with the different receptor subtypes. Both ET_AR and ET_BR activated G_o to a similar maximal extent, and both subtypes activated G_q with similar EC_{50} values; however, the ET_AR displayed a 2−3-fold higher maximal extent of activation. In contrast, both subtypes activated G_i to a similar maximal extent, but the ET_AR displayed a 4-fold higher EC_{50} value as compared to the ET_BR. To test whether these coupling specificities are influenced by C-terminal palmitoylation of the receptor, we mutated a cluster of cysteine residues near the end of the seventh transmembrane helix in both receptors. While the cysteine mutations in the ET_BR resulted in a partially palmitoylated receptor, the replacement of these cysteine residues in the ET_AR yielded a mostly palmitoyl-deficient receptor and had no effect on G_o activation, but caused a reduction in the extents of G_i and G_q stimulation. Together, these studies provide important insights into the specificity of G protein coupling in the endothelin receptors. The ability to discriminate between the different G proteins under various physiological conditions may be a key element in the selection of distinct signal transduction pathways by the two receptor subtypes.

The endothelins (ETs)[1] are potent vasoconstrictor peptides, and are represented by three, 21 residue long isopeptides, ET-1, ET-2, and ET-3 (*1*, *2*). A large number of studies have elucidated that ETs play many important roles in vasoconstriction/vasodilation, as well as in cardiovascular, renal, pulmonary, endocrine, gastrointestinal, and neuronal functions, mitogenesis, and so on (*3*, *4*). The ETs utilize seven-transmembrane G protein-coupled receptors to transmit various signals into the cell. In mammals, two receptor subtypes, ET_AR and ET_BR, have been identified. The ET_AR is selective to ET-1 and ET-2, while the ET_BR is nonisopeptide-selective (*5*). Both of the receptors mediate multiple signal transduction processes in various tissues and cell lines (*3*, *4*, *6*). For example, the addition of ET-1 to cells, such as vascular smooth muscle cells (*7*, *8*), atrial cells (*9*), or myocytes (*10*), causes a rapid increase of inositol 1,4,5-triphosphate accumulation. In atrial myocytes, ET_AR inhibited the accumulation of cAMP via the pertussis toxin (PTX) sensitive G protein (*11*, *12*). In the rat liver, ET_BR couples with G_q and G_s (*13*). Furthermore, the existence of ET_BR subtypes, $ET_{B1}R$ and $ET_{B2}R$, has been suggested, based on the distinct pharmacological properties (*14*, *15*), although these subtypes have not yet been identified.

In such complex signal transduction, it is of great interest to learn how each receptor subtype functions and is regulated differently. Investigations of the behaviors of ET_AR and ET_BR in cultured cell lines (*16*−*18*) or in native tissues (*7*−*13*) have revealed their interesting, cell-type-specific natures. On the other hand, studies of the interactions of ETR and G proteins in vitro should also provide biochemical information about the functional differentiation of ETRs. We and others showed previously that human ET_AR and ET_BR were reasonably well-expressed in Sf9 cells and were relatively stable after solubilization (*19*, *20*).

We have further purified ET_AR and ET_BR, modified with a histidine-tag and an epitope-tag, to near-homogeneity using affinity chromatographies, and have reconstituted them into phospholipid vesicles with the G proteins, G_i, G_o, and G_q. The reconstitution studies of purified β-adrenergic receptors, muscarinic acetylcholine receptors, and so on have reported the mechanisms and the quantitative properties of the interactions with G proteins (*21*−*23*). Using these reconstitution techniques, we present a quantitative comparison of the receptor−G protein coupling and show the similar efficiency of ET_AR and ET_BR in G_i/G_o coupling, as well as their different affinities to G_i and activities in G_q coupling. In addition, we report partial activation of the purified ET_BR via ligand-affinity chromatography. We also describe the coupling properties of the palmitoylation-deficient cysteine mutants of ET_AR in the reconstituted system. The palmi-

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[1] Abbreviations: ET, endothelin; ETR, endothelin receptor; ET_AR and ET_BR, endothelin receptor subtypes A and B; G protein, heterotrimeric guanine nucleotide binding protein; GPCR, G protein-coupled seven transmembrane receptor; GTPγS, guanosine 5′-*O*-(3-thiotriphosphate); PTX, pertussis toxin; Chaps, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate; DTT, dithiothreitol.

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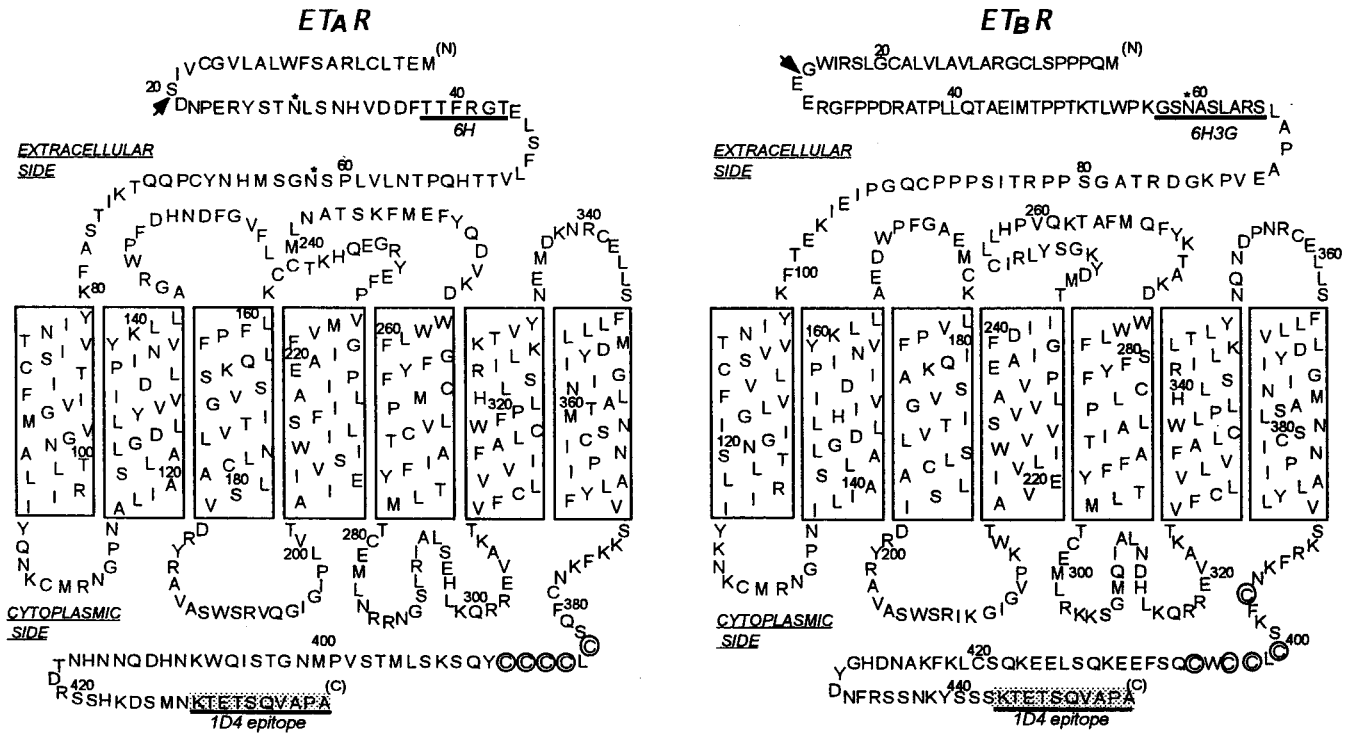
A.



B.

Tag-modified receptors	N-terminal tail	C-terminal tail
ET_AR (wild)	-	-
6hN ET_AR epiC	38-43 : 6H	1D4-epitope attached
ET_BR (wild)	-	-
6hN ET_BR	57-65 : 6H3G	-
6hN ET_BR epiC	57-65 : 6H3G	1D4-epitope attached

Cysteine mutants	Cysteine residues mutated to alanine
1CA/ET_AR	C^{385}
5CA/ET_AR	C^{383}, C^{385}, C^{386}, C^{387}, C^{388}
1CA/ET_BR	C^{396}
4CA/ET_BR	C^{400}, C^{402}, C^{403}, C^{405}

FIGURE 1: (A) Secondary structure models of human ET_AR and ET_BR. The putative seven α helices are boxed. The tag-modified sequences are underlined. Residues 38−43 of ET_AR were replaced with six histidines, and the 1D4 epitope sequence was attached to the C-terminus of the 6hNET_ARepiC. The sequence of amino acids 57−65 of the ET_BR was changed to HHHHHHGGG in the tag-modified 6hNET_BR and 6hNET_BRepiC. The 1D4 epitope sequence was also attached to the C-terminus of the 6hNET_BRepiC. The mutated cysteines in the C-terminal tails are marked with circles. The asterisks indicate the potential N-linked glycosylation sites, and the arrows indicate the signal peptidase cleavage sites. (B) Nomenclature of the tag-modified receptors and the cysteine mutants. The cysteine mutants were constructed in the 6hNET_ARepiC and in the 6hNET_BR.

toylation of the C-terminal tails of ET_AR and ET_BR has been shown to play important roles in G protein coupling (*24*, *25*), and its roles are varied among other G protein-coupled receptors (*26*−*29*). Deficient palmitoylation led to impaired activities of the ET_AR in G_i and G_q coupling, but not in G_o coupling. The palmitoylation of the C-terminal tail provides different efficacies to the G protein coupling of the ET_AR. The observed subtle differences between the two receptors and the effects of the palmitate modification on the interactions with G proteins may form part of the biochemical basis for their functional differences in various cell types.

EXPERIMENTAL PROCEDURES

Materials. Plasmid pVL1393 was purchased from Invitrogen. [^{125}I]ET-1 (81.4 TBq/mmol) and [^{35}S]GTPγS (37 TBq/mmol) were purchased from DuPont NEN. The cDNAs encoding human ET_AR and ET_BR were kindly provided by Dr. T. Masaki (Kyoto University) and Dr. Y. Furuichi (AGENE Research Institute), respectively. The cDNAs for α_q (*30*) and γ_2 (*31*) were generously supplied by Drs. V. Mancino and M. Simon (California Institute of Technology). The pQE60-based expression plasmids for rat α_{i1} and bovine α_s (*32*, *33*) in *E. coli* strain BL21 and the recombinant virus of the rat α_{i1} for expression in Sf9 cells were generously provided by Drs. H. Itoh and Y. Kaziro (Tokyo Institute of Technology). The hybridoma producing the anti-bovine rhodopsin monoclonal antibody 1D4 was from Dr. R. Molday (University of British Columbia). The cyclic peptide antagonist for ET_BR RES701-1 (*34*) was kindly provided by Dr. M. Yoshida (Kyowa Hakko Kogyo Co., Ltd.). Digitonin was purchased from Wako.

Construction of the Tag-Modified ETR Genes in the Transfer Plasmids. The wild-type ET_BR and 6hNET_BR genes were introduced into the *Bam*HI/*Not*I sites in the pVL1393 vector, as described (*19*). In the 6hNET_BR gene, the sequence encoding amino acids 57−65 was replaced with the sequence encoding HHHHHHGGG. In the 6hNET_BRepiC gene, the sequence encoding KTETSQVAPA, the epitope for the anti-rhodopsin monoclonal antibody 1D4, was attached to the C-terminus. In the ET_AR construct (6hNET_ARepiC), the *Xba*I

Table 1: Densities and Affinities of the Wild-Type and Tag-Modified ET_AR and ET_BR Expressed in Sf9 Cells[a]

receptor	K_D (pM)	B_{max} (pmol/mg of protein)
ET_AR	55 ± 8	21 ± 1.7
$6hNET_ARepiC$	86 ± 15	31 ± 3.9
ET_BR	83 ± 11	69 ± 7.8
$6hNET_BR$	100 ± 19	77 ± 9.3

[a] Sf9 cell membranes, 140 ng of the ET_AR constructs, and 64 ng of the ET_BR constructs were used for the examination of binding parameters by a saturation isotherm of [^{125}I]ET-1 binding, as described under Experimental Procedures. Each experiment was done in duplicate and at least 2 independent times.

Table 2: Ligand Affinities for Wild-Type and Tag-Modified ETRs[a]

	agonists K_i (nM)				*antagonists* K_i (nM)		
ETR	ET-1	ET-3	S6C[c]	IRL1620	BQ123	BQ788	RES701-1
ET_AR	0.30	10	nd[d]	nd	26	nd	nd
$6hNET_ARepiC$	0.29	11	nd	nd	17	nd	nd
ET_BR	0.17	0.28	0.33	1.4	$>10^5$ [b]	7.8	44
$6hNET_BR$	0.33	0.38	0.48	1.9	$>10^5$ [b]	8.0	36

[a] Competitive binding between [^{125}I]ET-1 (50 pM) and unlabeled ligands was performed as described under Experimental Procedures, using 1.2 μg of the ET_AR-containing Sf9 membranes and 0.3 μg of the ET_BR-containing Sf9 membranes. The K_i values were calculated from the IC_{50} values determined from nonlinear regression of the competition experiments, and are the averages of two to three experiments. The maximum range of the K_i values was ±19% of the mean values. [b] Lower limits of the K_i values for BQ123 of the ET_BRs are derived from the maximum ligand concentration used. [c] S6C, sarafotoxin 6C. [d] nd, not determined.

and *Not*I sites were attached to the 5′ and 3′ termini of the gene for introduction into pVL1393, six histidines were substituted for the sequence of amino acids 38−43, and the 1D4 epitope sequence was attached to the C-terminus. The cysteine mutants were constructed in $6hNET_ARepiC$ or in $6hNET_BR$, respectively. The addition of the epitope sequence and the introduction of mutations were done by replacement with the desired gene fragment, produced by PCR.

Purification of ET_AR and ET_BR. Cell culture, expression of ET_AR and ET_BR in Sf9 cells, preparation of Sf9 cell membranes, and purification of ET_BR by ligand-affinity chromatography using biotinylated ET-1 were performed as described previously (*19*). Purification of $6hNET_ARepiC$ and $6hNET_BRepiC$ by 1D4-affinity chromatography was carried out as described (*35*) using 1D4-immobilized Sepharose 4B and the epitope peptide at 50 μM. After the ligand- or antibody-affinity chromatography, nickel affinity column chromatography was performed.

Expression and Purification of G Proteins. The rat α_{i1} subunit was expressed in *E. coli* strain BL21 or in Sf9 cells, and was prepared as described (*36*, *37*). In the beginning, the α_{i1} subunit prepared from Sf9 cells was used for the ETR-stimulated GTPγS binding assays. Since the α_{i1} subunit prepared from *E. coli* turned out to have activity similar to that of the Sf9-derived α_{i1} subunit in the assays, most of the experiments with G_i were performed with the *E. coli*-derived α_{i1} subunit. The bovine α_o subunit was purified from bovine brain (*38*). The $\beta_1\gamma_2$ subunits and the $\alpha_q\beta_1\gamma_2$ subunits were coexpressed in Sf9 cells and were purified as heterodimers or heterotrimers (*39*). The α_o, $\beta_1\gamma_2$, and $\alpha_q\beta_1\gamma_2$ subunits were stored in a 0.6% Chaps solution [20 mM Tris-HCl, 1 mM EDTA, 1 mM DTT, 120 mM potassium phosphate (pH 8.0), and 0.6% Chaps].

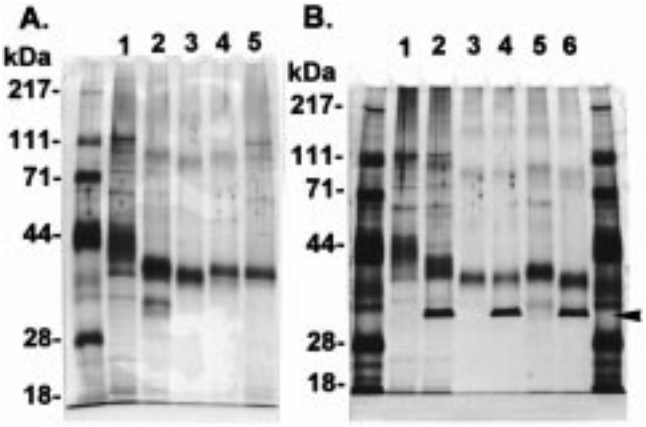


FIGURE 2: Purified and reconstituted ETRs (A) and the deglycosylated ETRs (B) were analyzed by SDS−12.5%PAGE followed by silver staining. (A) Lane 1, the $6hNET_ARepiC$; lane 2, the ET_BR; lane 3, the $6hNET_BR$; lane 4, the $6hNET_BRepiC$ purified by ligand-affinity and nickel-affinity chromatographies; lane 5, the $6hNET_B$-RepiC purified by antibody-affinity and nickel-affinity chromatographies. (B) The purified ETRs were incubated at 37 °C for 20 h with (lanes 2, 4, and 6) or without (lanes 1, 3, and 5) glycopeptidase F. Lanes 1 and 2, the $6hNET_ARepiC$; lanes 3 and 4, the $6hNET_BR$; lanes 5 and 6, the ET_BR. The bands around 35 kDa, indicated by an arrowhead, correspond to the glycopeptidase F. Repeated digestions of $6hNET_ARepiC$ still failed to complete the deglycosylation.

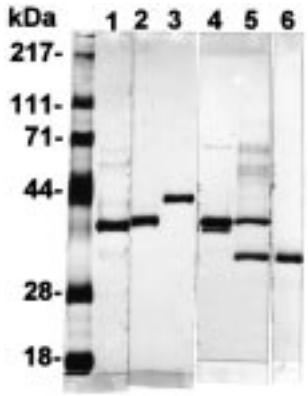


FIGURE 3: Purified G proteins were analyzed by SDS−12.5%PAGE visualized by silver staining. The proteins were alkylated by *N*-ethylmaleimide before loading onto the gel for better resolution (*46*). Lane 1, α_o purified from bovine brain; lane 2, α_i expressed in *E. coli*; lane 3, α_s expressed in *E. coli*; lane 4, α_i expressed in Sf9 cells; lane 5, $\alpha_q\beta\gamma$ expressed in Sf9 cells; lane 6, $\beta\gamma$ expressed in Sf9 cells. The γ subunit migrated with the dye front and is not visible.

Reconstitution of Receptors and G Proteins. Purified receptors (100−1000 pmol), in 100−300 μL of 20 mM Tris-HCl (pH 7.5), 0.1 M NaCl, and 0.1% digitonin, were mixed with 250−300 μL of lipid solution at 4 °C, containing 1-palmitoyl-2-oleoyl-phosphatidylethanolamine (Avanti) at 0.6 mg/mL, bovine brain phosphatidylserine (Avanti) at 0.4 mg/mL, cholesteryl hemisuccinate (Sigma) at 28 μg/mL, and 0.7% Chaps in HENM solution [20 mM Hepes (pH 8.0), 1 mM EDTA, 0.1 M NaCl, and 2 mM $MgCl_2$] (*40*). The mixtures were subjected to gel filtration using columns (7 × 230 mm) of Ultrogel AcA34. The void fractions without detergent were collected, divided into aliquots, and stored at −70 °C. The receptor recoveries were 3−10%, and vesicle suspensions containing receptor proteins at 10−40 nM were routinely obtained. For coreconstitution with G proteins, the

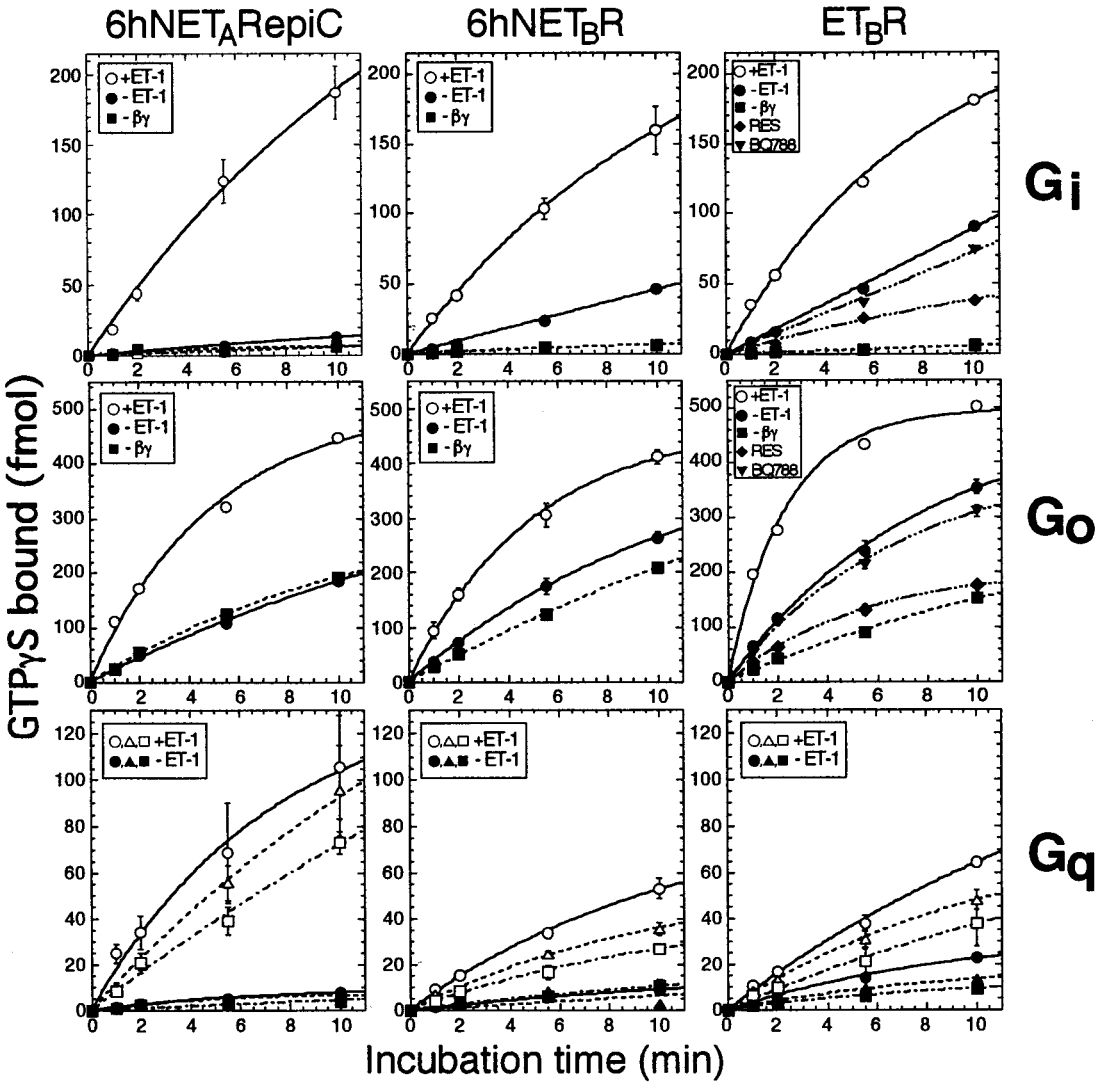


FIGURE 4: ET-1-dependent stimulation of [^{35}S]GTPγS binding to G_i, G_o, and G_q by the 6hNET$_A$RepiC, the 6hNET$_B$R, and the ET$_B$R. The 6hNET$_B$R and the ET$_B$R were purified by ligand-affinity chromatography accompanied by nickel-affinity chromatography. The receptors and the G proteins in the reconstituted vesicles, prepared as described under Experimental Procedures, were incubated with 5 nM [^{35}S]-GTPγS at 30 °C for G_i and G_q, and at 28 °C for G_o, in the presence of 1.0 μM ET-1 (open symbols) or in the absence of ET-1 (closed symbols). The α_{i1} protein expressed and purified from Sf9 cells was used. GDP, at a concentration of 1.0 μM, was added to the assays for G_i and G_o. The amounts of receptors in the isolated phospholipid vesicles were measured by [^{125}I]ET-1 binding, and the amounts of G_α proteins added to the coreconstitution mixtures were measured by [^{35}S]GTPγS binding as described (*38*). The following amounts of receptors in the vesicles and G proteins were contained in the 20 μL aliquots of the respective coreconstitution mixtures: 6hNET$_A$RepiC/G_i, 28 fmol of receptor (1.4 nM) and 1066 fmol of α_{i1} (53 nM); 6hNET$_B$R/G_i, 28 fmol of receptor and 1066 fmol of α_{i1}; ET$_B$R/G_i, 46 fmol of receptor (2.3 nM) and 1066 fmol of α_{i1}; 6hNET$_A$RepiC/G_o, 28 fmol of receptor and 1724 fmol of α_o (86.2 nM); 6hNET$_B$R/G_o, 28 fmol of receptor and 1724 fmol of α_o; and ET$_B$R/G_o, 46 fmol of receptor and 1724 fmol of α_o. Together with each α subunit, 1600 fmol of $\beta\gamma$ subunits was added. The results without $\beta\gamma$ subunits (■) are also shown. In the panels of the ET$_B$R/G_i and the ET$_B$R/G_o, the results in the presence of the antagonists RES701-1(◆) and BQ788 (▼) at 10 μM are shown. In the assays with G_q, different receptor concentrations were compared, and the amount of G_q ($\alpha_q\beta_1\gamma_2$) was fixed at 1145 fmol (57 nM) in 20 μL. The amounts of receptor in the 20 μL aliquots were as follows: 6hNET$_A$RepiC/G_q, 90 fmol (○,●), 28 fmol (△,▲), and 14 fmol (□,■); 6hNET$_B$R/G_q, 90 fmol (○,●), 28 fmol (△,▲), and 14 fmol (□,■); ET$_B$R/G_q, 102 fmol (○,●), 46 fmol (△,▲), and 24 fmol (□,■). Data are the means of duplicate or triplicate determinations.

vesicles were incubated at 4 °C for 1 h with a 20−100-fold molar excess of G proteins in 100 μL of HENM solutions containing 1 mM DTT and 0.06−0.1% Chaps. Then the mixtures were diluted with HENM to 220 μL (*41*) and were used for ligand-dependent [γ-^{35}S]GTPγS binding assays (*39, 19*). The [γ-^{35}S]GTPγS binding rates in the presence of ET-1 were analyzed according to the apparent first-order kinetics.

Radioligand Binding Assays. Saturation binding assays, competition binding assays, and determination of the dissociation constant (K_D), the maximum ligand binding sites (B_{max}), and the K_i were performed according to the methods described (*42*), using the LIGAND program (Biosoft). The amounts of ETRs contained in the reconstitution mixtures of [γ-^{35}S]GTPγS binding assays were measured by the [^{125}I]-ET-1 binding assays as described (*19*).

Analysis of Metabolically-Labeled Products. Metabolic labeling of the virus-infected Sf9 cells was performed as described (*43*). The harvested cells were solubilized in 0.5 mL of a 1% digitonin solution for 1 h at 4 °C, and the supernatants, resulting from centrifugation at 100000*g* for 30 min, were used for purification with 50 μL of the antibody-immobilized resin. The 1D4-resin and the 2A5-resin, containing the anti-ET$_B$R monoclonal antibody (unpublished work), were used. The procedures were as de-

Table 3: GTPγS Binding of G Proteins Stimulated by the ETRs[a]

G proteins	6hN ET_ARepiC	6hN ET_BR	ET_BR
G_i	0.88 ± 0.22	0.81 ± 0.065	0.65 ± 0.056
G_o	2.3 ± 0.18	2.2 ± 0.08	3.3 ± 0.12
G_q	0.59 ± 0.07	0.27 ± 0.05	0.19 ± 0.09

[a] The ETR-stimulated GTPγS binding values presented in Figure 4 are expressed as moles of GTPγS bound per minute per mole of receptor. The values at 1 min were calculated by nonlinear least-squares fitting to the first-order rate equation, and were normalized by dividing by the receptor concentrations. For the G_o coupling only, the values obtained in the absence of the $\beta\gamma$ subunits were subtracted as backgrounds from the values obtained in the presence of ET-1 and the $\alpha_o\beta\gamma$ subunits. For G_q coupling, the results obtained at the lowest receptor concentration (Figure 4) were used.

scribed above. The purified samples were analyzed by SDS−12.5% PAGE, followed by either fluorography or immunoblotting.

RESULTS

Ligand Binding Properties of the Wild-Type and the Tag-Modified ETRs. To facilitate the purification of the ETRs, the six-histidine tag and the epitope tag were introduced into the N- and the C-termini, respectively. The modifications of the ETRs are shown in Figure 1. The membranes, prepared from Sf9 cells expressing either the wild-type or the modified receptors, were examined for their ligand binding profiles. The Scatchard analysis of the specific binding to [^{125}I]ET-1, from saturation binding experiments, indicated the presence of a single class of high-affinity binding sites. The apparent dissociation constants (K_D) and the maximum binding (B_{max}) are summarized in Table 1. The binding site of ET_BR was usually expressed 3−5 times better than that of the ET_AR in Sf9 cells. Table 2 summarizes the apparent K_i values of each ligand to the wild-type or the tag-modified ETRs. The results indicate that the modification of the ETRs with the tag sequences at the N- or the C-terminus did not change their ligand binding profiles, and that the Sf9-expressed ETRs exhibited pharmacological properties similar to those of the ETRs expressed in mammalian cells (*44*, *45*, *25*).

Purified and Reconstituted ETRs in Phospholipid Vesicles. The tag-modified 6hNET_ARepiC and 6hNET_BRepiC proteins were purified by antibody-affinity and subsequent nickel-affinity column chromatographies. The wild-type ET_BR was purified by the ligand-affinity column. The 6hNET_BR and 6hNET_BRepiC proteins were purified by ligand-affinity and nickel-affinity column chromatographies. The purified receptors were reconstituted into phospholipid vesicles, as described under Experimental Procedures. Figures 2A and 3 show the silver-stained SDS−PAGE analysis of the isolated vesicles and the purified G proteins used for reconstitution with the ETRs. The ET_BRs were purified to near-homogeneity. The 6hNET_ARepiC protein was purified to the major species, and displayed smeared multiple bands around 44 kDa, due to glycosylation. The specific activity of the purified 6hNET_ARepiC was approximately 2.2 nmol/mg of protein, while those of the wild-type and modified ET_BRs were 17−22 nmol/mg of protein. After deglycosylation by glycopeptidase F, the bands for the 6hNET_ARepiC and the wild-type ET_BR were shifted to around 40 kDa (Figure 2B), while the 6hNET_BR, which lacks the N-glycosylation site, did not change its mobility in SDS−PAGE.

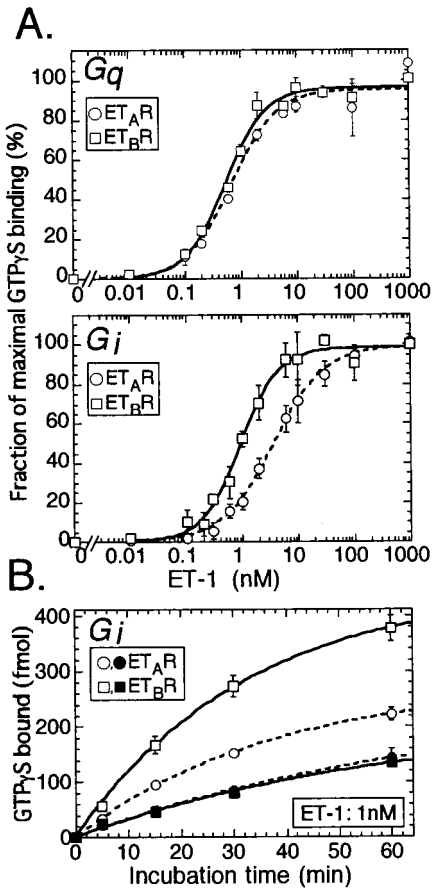


FIGURE 5: Effect of ET-1 concentration on the [^{35}S]GTPγS binding of G_q and G_i. (A) Each sample was incubated for 10 min at 30 °C. Data are the means of triplicate determinations. Each 20 μL aliquot contained 1600 fmol of G_q, and either 20 fmol of 6hNET_ARepiC (○) or 20 fmol of ET_BR (□) in the upper panel; and 1600 fmol of α_{i1}, 1600 fmol of $\beta\gamma$ subunits, and either 20 fmol of 6hNET_ARepiC (○) or 20 fmol of ET_BR (□) in the lower panel. (B) 1600 fmol of α_{i1}, $\beta\gamma$ subunits, and either 32 fmol of the 6hNET_ARepiC (○,●) or 30 fmol of the ET_BR (□,■) in 20 μL aliquots were incubated with (○,■) or without (●,■) 1.0 nM ET-1, and were assayed as described in Figure 4. The ET_BR purified by the 2A5-affinity column was used for comparison with the 6hNET_ARepiC at similar backgrounds to each other. The α_{i1} used was prepared from *E. coli*.

Reconstitution of ETRs and G_i, G_o, and G_q Proteins. The ETRs (6hNET_ARepiC, 6hNET_BR, and ET_BR) in the phospholipid vesicles were further reconstituted with G proteins to test the coupling efficiency. Similar receptor concentrations (1−2 nM) were chosen, and identical amounts of each G protein (50−80 nM) were coreconstituted into the respective ETR vesicles. Figure 4 shows the stimulation of [^{35}S]GTPγS binding of G_i, G_o, and G_q by the ETRs. The ETRs stimulated [^{35}S]GTPγS binding by G_i ($\alpha_{i1}\beta_1\gamma_2$) and G_o ($\alpha_o\beta_1\gamma_2$) similarly in the presence of ET-1 (Table 3). The stimulation of G_i and G_o binding observed in the absence of ET-1 by the ET_BRs was due to the purification method, and is described below (*Artificial Activation of the ET_BRs*). This stimulation was inhibited by a cyclic peptide antagonist, RES701-1, to some extent. Therefore, the actual background binding was represented by the binding observed in the absence of the $\beta\gamma$ subunits. In contrast to G_i/G_o, the [^{35}S]GTPγS binding by G_q was stimulated 2−3-fold more efficiently by the 6hNET_ARepiC than by the ET_BRs (Table 3). This occurred in the receptor concentration range from 0.7 to 5.1 nM. Slight stimulation by the ET_BRs without ET-1 was also observed.

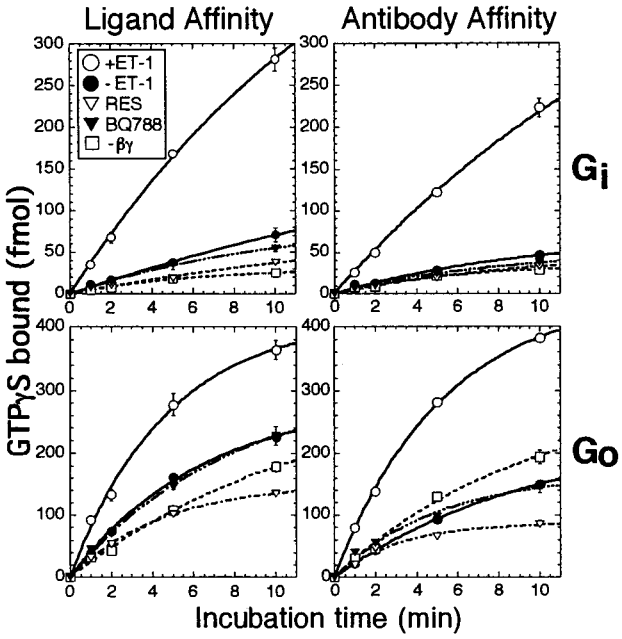


FIGURE 6: Ligand-purified (left panels) and antibody-purified (right panels) 6hNET$_B$RepiCs were compared in the stimulation of [^{35}S]-GTPγS binding by G_i and G_o. The α_{i1} used was derived from *E. coli*. Each 20 μL aliquot contained 30 fmol of the receptor, either 1825 fmol of α_{i1} or 1520 fmol of α_o, and 1600 fmol of $\beta\gamma$ subunits. The reactions were done at 30 °C for G_i and at 25 °C for G_o in the presence (○) or the absence (●) of 1.0 μM ET-1, or in the presence of either 10 μM RES701-1 (▽), 10 μM BQ788 (▼), or without $\beta\gamma$ subunits (□).

Figure 5 shows the effect of the ET-1 concentration on the [^{35}S]GTPγS binding of G_i and G_q reconstituted with the ETRs. Many GPCRs display low- and high-affinity states for the agonists depending upon the interactions with G proteins (*23, 47, 48*). In the case of the ETRs, both ET$_A$R and the ET$_B$R display high affinities for ET-1, similar to each other in the plasma membranes of many tissues and cells (*4, 14*). The K_D values of the purified ET$_A$R and the ET$_B$R in the phospholipid vesicles are also similar (220 ± 27 pM, 170 ± 19 pM, respectively). In the reconstitution mixtures with G_i or G_q, they displayed similar affinities for ET-1, regardless of the presence or absence of GTPγS at 10^{-4} M, as measured by competition experiments (data not shown). Therefore, the effective ET-1 concentrations for the [^{35}S]-GTPγS binding of the receptors reflect the affinities of the ligand-bound receptors to the G protein. For G_q stimulation, the 6hNET$_A$RepiC and the ET$_B$R displayed similar EC_{50} values, 0.69 ± 0.12 nM and 0.54 ± 0.06 nM, respectively (Figure 5A). On the other hand, in G_i coupling, the 6hNET$_A$-RepiC exhibited a 4-fold higher EC_{50} value (3.7 ± 0.2 nM) than that of the ET$_B$R (1.0 ± 0.09 nM). Figure 5B shows typical ET-1-dependent [^{35}S]GTPγS binding assays, but with 1.0 nM ET-1 instead of 1.0 μM, indicating that the stimulation efficiencies of G_i by ET$_A$R and ET$_B$R are different from each other at lower concentrations of the ligand-bound receptors. Thus, the ET-1-activated ET$_A$R and ET$_B$R have similar affinities for G_q, but different affinities for G_i.

Artificial Activation of the ET$_B$Rs. In Figure 4, the ligand affinity-purified ET$_B$Rs showed slight activation of G proteins in the absence of ET-1, as compared to the 6hNET$_A$RepiC. This property is particularly significant in G_i and G_o coupling. To investigate the artificial effects caused by purification,

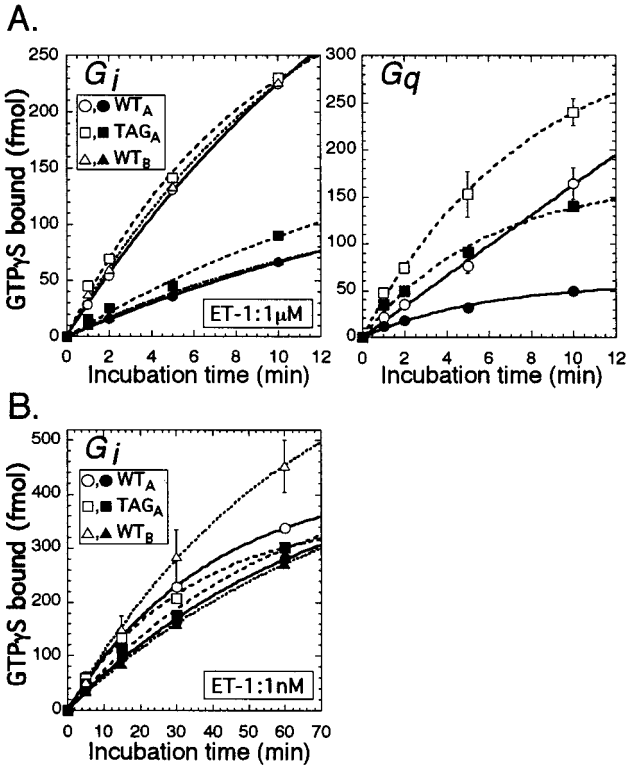


FIGURE 7: Activities of Sf9 membranes containing the wild-type ET$_A$R and the 6hNET$_A$RepiC in the stimulation of G_i and G_q. (A) The Sf9 membranes containing the wild-type ET$_A$R (WT$_A$, ○,●), the 6hNET$_A$RepiC (TAG$_A$, □,■), and the wild-type ET$_B$R (WT$_B$, △,▲) were reconstituted with G proteins, as described for the phospholipid vesicles, and were assayed for [^{35}S]GTPγS binding by G proteins with (○,□,△) or without (●,■,▲) 1.0 μM ET-1, as described for Figures 4 and 6. In coupling with G_q, only the results of wild-type ET$_A$R (WT$_A$, ○,●) and 6hNET$_A$RepiC (TAG$_A$, □,■) are shown. The amounts contained in the 20 μL aliquots were 20 fmol of each receptor, and either 1600 fmol of α_{i1}, $\beta\gamma$ subunits, or 1600 fmol of G_q. The membrane proteins contained in the 20 μL aliquots were 1.3 μg of the wild-type ET$_A$R, 1.9 μg of the 6hNET$_A$-RepiC, and 0.3 μg of the wild-type ET$_B$R. (B) The stimulation of [^{35}S]GTPγS binding by G_i was assayed with or without 1.0 nM ET-1. The amounts of respective membranes and G_i used and the symbols are as described in (A).

we expressed the 6hNET$_B$RepiC protein, in which the 1D4 epitope sequence was attached to the C-terminus of the 6hNET$_B$R. The 6hNET$_B$RepiC protein was purified either by ligand-affinity column chromatography followed by nickel-affinity chromatography or by 1D4-affinity column chromatography followed by nickel-affinity chromatography (Figure 2A). It was then reconstituted into the vesicles and examined for G protein coupling. As shown in Figure 6, while the ligand affinity-purified 6hNET$_B$RepiC showed slight activation of the [^{35}S]GTPγS binding of G_i/G_o in the absence of ET-1, the antibody affinity-purified 6hNET$_B$-RepiC displayed basically the same activity as in the absence of $\beta\gamma$ subunits. We assume that the chaotropic reagent, NaSCN, used to dissociate the ET$_B$R protein from the biotinyl-ET-1-bound avidin column, denatured the ET$_B$R to some extent, which led to partial activation of the receptor. In addition, the artificially activated ET$_B$R could be antagonized effectively by RES 701-1, but not by BQ788 (Figures 4 and 6).

Activities of the Wild-Type and Tagged ETRs. The wild-type ET$_B$R and the glycosylation-deficient 6hNET$_B$R were

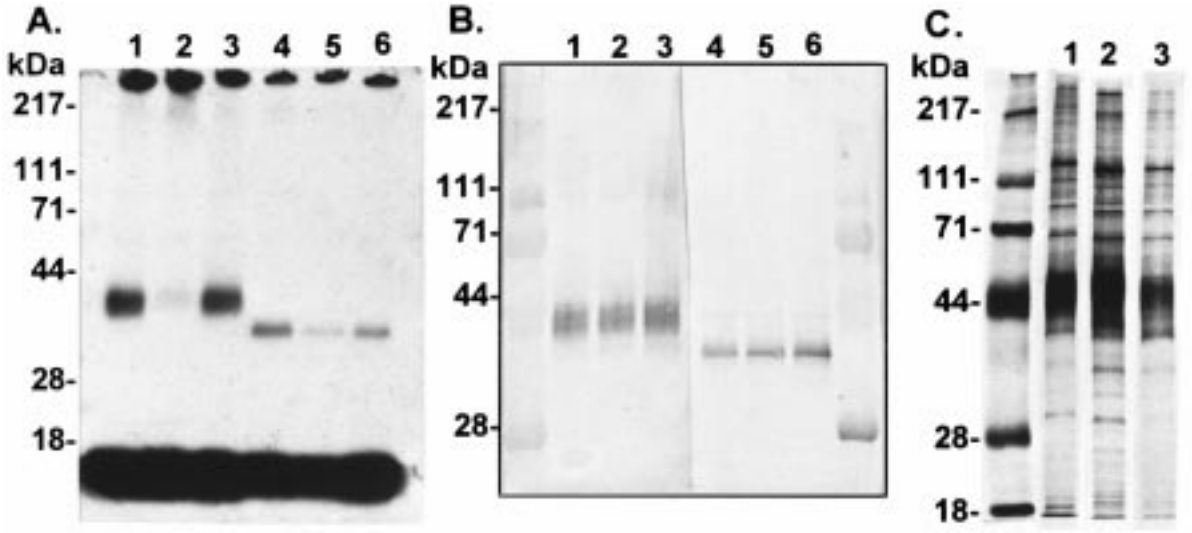


FIGURE 8: Palmitoylation of the ETRs in Sf9 cells. The Sf9 cells expressing the 6hNET$_A$RepiC (lane1), the 5CA/ET$_A$R (lane 2), the 1CA/ET$_A$R (lane 3), the 6hNET$_B$R (lane 4), the 4CA/ET$_B$R (lane 5), and the 1CA/ET$_B$R (lane 6) were metabolically labeled with [^{3}H]-palmitic acid, solubilized, and purified by antibody-affinity chromatography. The samples were analyzed by SDS−12.5% PAGE followed by (A) fluorography or (B) immunoblotting using the 1D4 mAb (lanes 1−3) and the 2A5 mAb (lanes 4−6). Prestained size markers were run next to lanes 1 and 6, whose molecular masses are indicated at the left side. (C) The purified cysteine mutants were analyzed by SDS−12.5% PAGE accompanied by silver staining. Lane 1, the wild-type 6hNET$_A$RepiC; lane 2, the 5CA/ET$_A$R; lane 3, the 1CA/ET$_A$R.

basically identical in their coupling efficiencies with G_i, G_o, and G_q (Figure 4). In the case of the ET$_A$R, only the tag-modified 6hNET$_A$RepiC was purified. To examine the effects caused by the introduced tags in the interactions with G proteins, Sf9 membranes containing the wild-type ET$_A$R and the tagged 6hNET$_A$RepiC were reconstituted with the G proteins, as described for the vesicles. Figure 7 shows the stimulation of [^{35}S]GTPγS binding by G proteins in the presence of the ET$_A$Rs. Since different amounts of membrane proteins were contained in the reconstitution mixtures to make the receptor concentrations similar, the background levels without the addition of ET-1 were different (particularly in G_q coupling). However, the extents of stimulation of [^{35}S]GTPγS binding by ET-1 were similar in the wild-type and the tagged ET$_A$Rs. In addition, the lower extents of G_i stimulation by 1.0 nM ET-1 were also observed in the wild-type and tagged ET$_A$Rs membranes, as compared to the ET$_B$R membrane (Figure 7B). Although we could not compare G_o coupling in the crude Sf9 membranes because of the high backgrounds, the wild-type ET$_B$R, the 6hNET$_B$-RepiC, and the 6hNET$_A$RepiC showed similar activities in G_o coupling in the reconstituted vesicles (Figures 4 and 6). Therefore, we conclude that the 6hNET$_A$RepiC is comparable to the wild-type ET$_A$R in the G protein interaction properties that we examined.

Palmitoylation-Deficient ETRs in G Protein Coupling. To examine whether palmitoylation in the C-terminal tail affects the properties of G protein coupling by the ETRs, a cluster of cysteine residues in the C-terminal tail was mutated, as listed in Figure 1B. These mutant receptors were expressed in Sf9 cells. The ligand binding properties of the mutant receptors were similar to those of the wild-type receptors (data not shown). The mutant receptors expressed in Sf9 cells were metabolically labeled with [^{3}H]palmitic acid, purified by antibody-affinity column chromatography, and analyzed by fluorography and immunoblotting (Figure 8A,B). While the incorporation of [^{3}H]palmitate into the 5CA/ET$_A$R was less than 5% of that of the 6hNET$_A$RepiC, the 4CA/ET$_B$R incorporated approximately 20−30% of that of the 6hNET$_B$R in Sf9 cells, probably via the residues C396 and C420 in the C-terminal tail (Figure 8A). The conserved palmitoylation sites in the C-terminal tail of GPCR in mammalian cells do not appear to be strictly recognized in Sf9 cells.

Since the 5CA/ET$_A$R lacked most of the palmitate modification, it was further purified (Figure 8C), reconstituted into phospholipid vesicles, and assayed for stimulation of [^{35}S]GTPγS binding by G proteins (Figure 9). The 5CA/ET$_A$R showed 50% less stimulation of G_i and G_q, but displayed G_o activation similar to that of the wild-type. Regarding the EC_{50} values, the 5CA/ET$_A$R exhibited a 2−3-fold higher value (1.8 ± 0.18 nM) in G_q coupling, but a similar value (2.9 ± 0.6 nM) in G_i coupling, as compared to those of the wild-type (0.69 ± 0.12 nM and 3.1 ± 0.5 nM, respectively) (Figure 9B). Thus, palmitoylation in the C-terminal tail affects the properties of G protein coupling by the ET$_A$R.

DISCUSSION

Human ET$_A$R and ET$_B$R share high homology (~55%), particularly in the transmembrane domain and the cytoplasmic domain, except for the C-terminal 35 residues (~75%). Nevertheless, they transmit different signals, depending upon the cell or tissue type. The purposes of this study were to evaluate the interactions of human ET$_A$R and ET$_B$R with the G proteins, G_i, G_o, and G_q, in phospholipid vesicles reconstituted with known concentrations of receptors and G proteins, and to obtain insight into how the differences in their properties give rise to divergent functions.

The high expression level in Sf9 cells and the stability after solubilization of human ET$_A$R and ET$_B$R (*19, 20*) facilitated their purification and reconstitution into phospholipid vesicles with G proteins. However, the specific activities after immunoaffinity purification were 5−10-fold different from each other, which suggested that the properly folded ET$_B$R was synthesized more efficiently than the ET$_A$R in Sf9 cells.

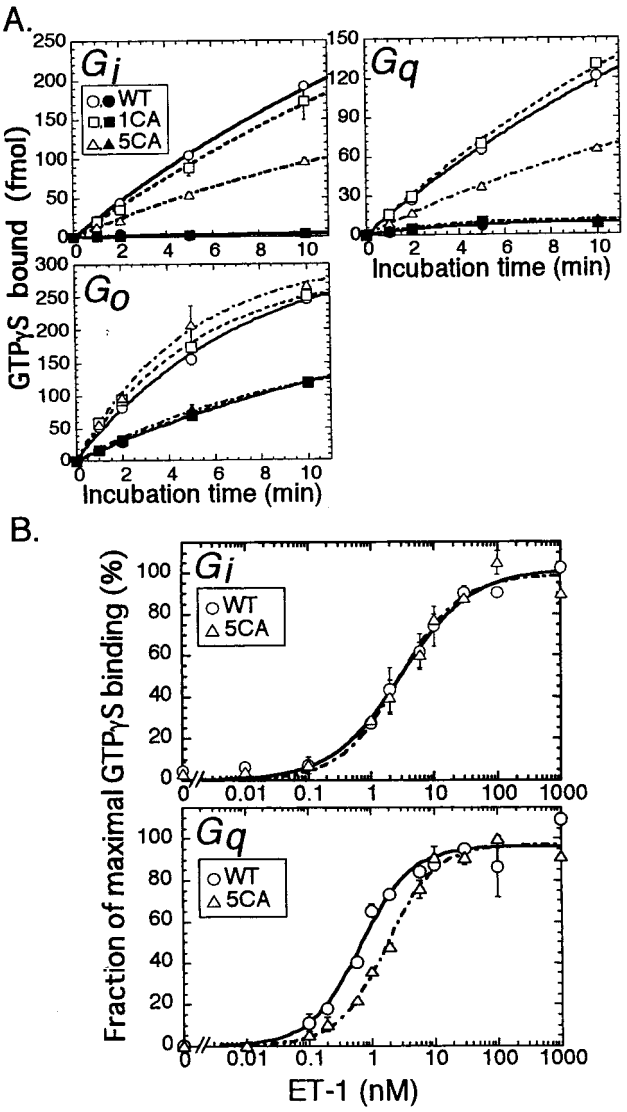


FIGURE 9: Stimulation of [^{35}S]GTPγS binding to G_i, G_q, and G_o by the cysteine mutants. (A) The purified mutant receptors were reconstituted into phospholipid vesicles with G proteins and were assayed in the presence of ET-1 at 1.0 μM (○,□,△) or in the absence of ET-1 (●,■,▲) as described in the legends of Figures 4 and 6. The 6hNET$_A$RepiC (○,●) and its mutants, the 1CA/ET$_A$R (□,■) and the 5CA/ET$_A$R (△,▲) are compared. Each 20 μL aliquot contained 16 fmol of the receptors, and either 1825 fmol of α_{i1} and 1600 fmol of $\beta\gamma$ subunits, or 1520 fmol of α_o and 1600 fmol of $\beta\gamma$ subunits, or 1600 fmol of G_q. (B) The effective ET-1 concentrations for [^{35}S]GTPγS binding of G_q and G_i stimulated by the 6hNET$_A$RepiC (○) and the 5CA/ET$_A$R (△) are compared. The assays were carried out as described in the legend of Figure 5A. The amounts contained in each 20 μL aliquot are the same as above.

The use of a cotransfection system in COS cells, with the cDNAs encoding the ETRs and the G proteins, has shown that the ETRs coupled G_s, G_i, and G_q, and that the ET$_B$R was less potent in G_q coupling, as measured by IP_3 production (*17*). This observation agrees well with the 2−3-fold higher stimulation of G_q by the ET$_A$R than by the ET$_B$R with similar receptor concentrations in our reconstituted system. However, the affinities of the ligand-bound ETRs for G_q are similar. Therefore, the different activities may originate from the catalytic rates of the ETRs. Substitution or deletion of the C-terminal region (296−305) of the third intracellular loop of ET$_A$R has been shown to impair calcium mobilization (*49*). Since the substituted region is well-conserved between the ET$_A$R and the ET$_B$R, some other region may also participate in the interaction with G_q. Unlike most other G_α subunits, the $G_{q\alpha}$ and $G_{11\alpha}$ subunits have relatively poor affinity for GTPγS in the absence of an appropriate agonist-bound receptor (*50*). Conversely, this unusual feature of the $G_{q\alpha}$ and $G_{11\alpha}$ subunits might allow each agonist-bound receptor to activate them differently, which could lead to variable and regulatory signal transduction.

In contrast, the ETRs display differences in the affinities for G_i under our conditions. Although the difference in the EC_{50} values of GTPγS binding was only 4-fold, it was observed only for G_i, and not for G_q. In cultured CHO cells, the ET$_A$R has been shown to couple to G_s, but not to G_i, whereas the ET$_B$R coupled to G_i, but not to G_s (*16*, *18*). The lower affinity of the ET$_A$R for G_i might result in its uncoupling from G_i in CHO cells. The use of chimeric receptors of human ET$_A$R and ET$_B$R in CHO cells has revealed that the replacement of the transmembrane helix 5 to 6, including the third intracellular loop of the ET$_A$R, with that of the ET$_B$R makes the chimeric receptor couple to G_i (*18*). Considering the fact that the replaced regions are highly homologous to each other, the difference in the allosteric changes in the indicated region, caused by binding ET-1, might give rise to the distinct affinity for G_i. Using $G_s\alpha$ expressed in either Sf9 cells or *E. coli*, we were unable to detect ET-1-dependent stimulation by the ETRs in the reconstituted system. Further studies on the interactions of ETRs with G_s would provide clearer insights into the regulated signal transduction of ETRs. In addition, there might be differences among α_{i1}, α_{i2}, and α_{i3} in their interactions with the ETRs, as observed with the dopamine receptor, subtype D_{2S} (*51*).

Concerning tissue- or cell-type-specific signal transduction by GPCR, the efficiency of stimulation, the affinities of the receptors for G proteins, the concentration of ligands, and the subcellular localizations of the receptors and the G proteins all could influence the G protein specificities, and lead to refined mechanisms of signal transduction. Although the ET$_B$R exhibits low G_q stimulation, many signal transduction pathways used by the ET$_B$R via the PTX-insensitive G protein have been identified (*52*−*54*, *13*). Likewise, the ET$_A$R utilizes G_i to transmit important signals (*55*, *11*, *12*, *56*). Recent extensive studies on GPCR-mediated mitogenic signaling have elucidated the heterogeneous pathways among various cell types, for example, via G_i in Rat-1 fibroblasts (*57*), via $G_{q/11}$ in CHO cells (*58*), and via G_i and $G_{q/11}$ in PC12 cells (*59*). The ability to couple to multiple G proteins and the observed subtle differences of the ETRs may contribute to the complexity of signal transduction in various cell types. Additionally, as reported for the PACAP receptor, which differentially couples to two transduction pathways through distinct ligands (*60*), the ET-3-bound ET$_B$R might exhibit different properties from those of the ET-1-bound ET$_B$R in G protein coupling.

The effective inhibition of the artificially activated ET$_B$R by a cyclic peptide antagonist, RES701-1, suggests its ability to conduct the ET$_B$R to a conformation uncoupled from G proteins, particularly G_o. It behaves like an inverse agonist, which stabilizes the inactive GPCR. Determination of the RES701-1 binding sites on the ET$_B$R should provide information on the conformational changes of ET$_B$R.

The 6hNET$_B$R, which lacks a glycosylation site at N^{59}, is as functional as the wild-type ET$_B$R in its pharmacological

properties and G protein coupling. Therefore, the lack of glycosylation did not cause any significant changes in the ET_BR. The $6hNET_BR$, which can be highly purified in milligram quantities, is well-suited for further biochemical studies.

The abrogated palmitoylation of the ET_AR and the ET_BR, by mutation of the cysteine cluster in the C-terminal tail, resulted in no detectable stimulation of phosphatidylinositol hydrolysis and no increase of the cytoplasmic calcium concentration induced by ET-1 in CHO cells (*25*) and CHO/Sf9 cells (*24*). In the purified reconstituted system, the lack of palmitoylation resulted in reduced stimulation of G_q and G_i, but not of G_o, and reduced affinity for G_q in the ET_AR, suggesting the role of palmitate in modulating the interactions with G proteins. Thus, the important posttranslational modification by palmitate may confer diversity on signal transduction by the ETRs.

In summary, we have reported the subtle differences and similarities of human ET_AR and ET_BR in a purified reconstitution system with G proteins. These properties may be significant for the elaborate signal transduction by the ETRs, which respond to various physiological conditions. Further studies of the properties and interactions of the ETRs with other proteins should lead to a better understanding of their complex signal transduction mechanisms.

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