Subtype-Specific Desensitization of Human Endothelin ET_A and ET_B Receptors Reflects Differential Receptor Phosphorylation†

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ABSTRACT: Endothelins regulate blood pressure in mammals through G protein-coupled receptors. Two receptor subtypes, ET_A and ET_B, exist which differ by their agonist profiles. Here we show subtype-specific differences in the inactivation of these endothelin receptors. Using a modified inositol phosphate accumulation assay, we found that stimulation of ET_A by endothelin-1 results in sustained activation of the subtype, retaining >30% of its initial activity even 20 min after agonist administration, whereas the ET_B rapidly deactivated after agonist stimulation, losing >80% of its initial activity within 5 min after endothelin application. The discrepancy in receptor inactivation is reflected by subtype-specific differences in receptor phosphorylation. Whereas ET_A failed to undergo ligand-induced phosphorylation, the ET_B was rapidly phosphorylated in response to agonist stimulation. By contrast, the kinetics of ligand-induced internalization were essentially identical for the receptor subtypes, suggesting endothelin receptor internalization being independent of ligand-induced receptor phosphorylation. Interestingly, a strong correlation was observed between the time course of ET_A inactivation and ET_A internalization. Therefore, our data suggest a subtype-specific inactivation of human endothelin receptors: fast receptor phosphorylation in the case of ET_B and slow receptor internalization in the case of ET_A. Subtype-specific modulation of endothelin receptors may account for the short-term hypotensive effects of endothelins via rapidly down-regulating ET_B receptors and the long-lasting hypertensive effects due to sustained ET_A activation.

Receptor desensitization in response to agonist stimulation is an important feature of G protein-coupled receptors (*1*−*3*). Desensitization is thought to prevent (i) overstimulation and potential damage of activated cells and (ii) saturation of the cells by weak stimuli (*4*). Accumulating evidence suggests that phosphorylation of G protein-coupled receptors at serine and threonine residues is involved in agonist-induced receptor desensitization (*1*−*3*). Hypothesis has been put forward that receptors in their activated state are substrates for G protein-coupled receptor kinases (GRKs) (*5*). According this model, phosphorylation promotes binding of arrestin to the tagged receptor protein. Arrestin binding prevents coupling of G proteins to receptor, thereby attenuating the capacity of the receptor to activate intracellular effector systems (*6*). Prototypical receptors governed by this mechanism are the adrenergic receptors, the muscarinic receptors, and peptide hormone receptors, e.g., for bradykinin (*7*).

Endothelins (ETs), a family of 21 amino acid peptides, exist in 3 distinct isoforms, ET-1, ET-2, and ET-3. They are implicated in a variety of physiological responses such as modulation of vascular smooth muscle cell contraction, blood pressure regulation, and renal function (*8*, *9*). The biological effects of endothelins are mediated by two distinct ET receptor subtypes A ("ET_A") and B ("ET_B"). Stimulation of ET_A or ET_B prompts the interaction of the receptors with G proteins of the $G\alpha_\theta$ family to activate PLC_β[1] with the subsequent release of IP_3 and a rise in the intracellular Ca^{2+} concentration, $[Ca^{2+}]_i$. ET_A also interacts with $G\alpha_s$ which stimulates adenylate cyclase whereas ET_B activates $G\alpha_i$ which in turn inhibits adenylate cyclase (*10*−*12*). In the rat cardiovascular system, the distinct distribution of the ET receptors allows a differential response to endothelin injection: a single dose of ET-1 results in a transient vasodilation due to the ET_B-induced NO release from endothelial cells, followed by a long-lasting vasoconstriction due to an ET_A-mediated elevation of $[Ca^{2+}]_i$ in vascular smooth muscle cells. These findings have prompted the notion that the biphasic response to a single endothelin injection might reflect the rapid desensitization of ET_B present in endothelial cells, and the persistent activity of ET_A prominent in vascular smooth muscle cells (*9*). The molecular basis of the hypothesized differential desensitization of ET_A and ET_B, respectively, has remained unclear. Premont *et al.* (*13*) reported the internalization of ET_A into caveolin-containing vesicles, hypothesizing that the activated ET_A might function for more than 2 h (*14*), whereas (*15*) reported the rapid ligand-induced desensitization of ET_A recombinantly expressed in *Xenopus* oocytes, thus pointing to the possibility that the cell system used for receptor expression might interfere with the desensitization mechanisms of ET receptors. A direct comparison of desensitization patterns of ET receptors in the same cell type is not available. Furthermore, the role of ligand-induced phosphorylation in the regulation

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[1] Abbreviations: EC_{50}, concentration at half-maximal stimulation; ELISA, enzyme-linked immunosorbent assay; IP_3, inositol triphosphate; PAGE, polyacrylamide gel electrophoresis; PBS, phosphate-buffered saline; PLC, phospholipase C; SDS, sodium dodecyl sulfate.

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of ET_A and ET_B desensitization and internalization is still obscure.

An intrinsic experimental problem in studying ET receptor desensitization is the almost irreversible binding of endothelin to ET_A and especially to ET_B at neutral pH. The ET_A/ET-1 complex is stable for at least 2 h inside CHO cells; the ET_B/ET-1 complex is so tight binding that it even withstands treatment with low concentrations of SDS (*16*). This special feature of ET receptors makes it almost impossible to dissociate receptor−ligand complexes under "physiological" conditions, a necessary prerequisite to study receptor desensitization by repeated stimulation of a given receptor population. To circumvent this problem, we have used an experimental approach where we analyze ET receptor activities by following the time course of inositol phosphate accumulation. Our findings suggest the existence of subtype-specific inactivation of endothelin receptors probably by receptor internalization in the case of ET_A and phosphorylation in the case of ET_B. Furthermore, endothelin receptor internalization seems to function independently of receptor phosphorylation.

EXPERIMENTAL PROCEDURES

Materials. Reagents were obtained from the following manufacturers: pcDNAIII, pVL-1392, and pVL-1393 vectors and Liposom-Kit from Invitrogen; Dowex AG:1−8x, hydroxide form, 200−400 mesh, from BioRad; [^{125}I]endothelin-1 ([^{125}I]ET-1) and [9,10-^{3}H]palmitic acid from Amersham; *myo*-[2-^{3}H]inositol and $H_3{}^{32}PO_4$ from ICN; endothelin-1 from Boehringer Mannheim; phorbol 12-myristate 13-acetate (PMA) and Pansorbin from Calbiochem; Pefabloc SC from Roth; and bis(indoylmaleimide) (GF109203) from Applichem. Cell culture reagents were from Life Technologies, Inc. Other reagents were obtained from standard commercial sources.

Cloning of the ET_A and ET_B Receptors. Cloning and expression of the ET_A human receptor have been described recently (*17*). The nucleotide sequence encoding the human ET_B receptor was amplified by the polymerase chain reaction using phage DNA isolated from a human placenta cDNA library. The following primers were used to specifically amplify a 1.3 kb fragment containing the complete ET_B coding sequence: *Bam*HI (5′-ATAGGATCCTATGCAGC-CGCCTCCAAGT-3′) and *Hin*dIII (5′-ATGTCGAGTA-GAACTTTCGAACCTAGGCCC-3′). The fragment was first subcloned into the Blueskript KS^+ (Bks^+) DNA vector, and then into the *Sma*I site of pVL-1392 or into the *Xho*/*Bam*HI site of pCDNAneo. Individual clones were isolated, and the nucleotide sequence of the ET_B receptor was verified by the Sanger sequencing method on both strands.

Cell Culture. CHO-K1 were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with Ham's F12 nutrient mixture (Ham's F12), 10% (v/v) fetal calf serum (FCS), and 0.5% (w/v) penicillin/streptomycin in a humidified CO_2 atmosphere at 37 °C. COS-1 cells were kept under the same conditions except that RPMI medium was used. Insect cells (Sf9) were cultured as monolayers in TC-100 medium supplemented with 10% FCS and 0.5% penicillin/streptomycin in a normal atmosphere at 27 °C (*18*).

Transfection of CHO-K1 and COS-1 Cells. CHO-K1 and COS-1 cells were transfected by the calcium phosphate method with recombinant pcDNAIII constructs together with a β-galactosidase expression plasmid for determination of transfection efficiency (*19, 20*). Transfected CHO-K1 cells were isolated after selection with 500 μg/mL G418 and analyzed for specific [^{125}I]ET-1 binding. Immunoprecipitation experiments were performed with transfected COS-1 cells incubated for 50−72 h after transfection.

Cloning of Recombinant Baculovirus and Infection of Sf9 Cells. Recombinant baculovirus was generated by cotransfection of Sf9 cells, grown in 60 mm dishes, with the pVL1392 constructs and with linearized AcMVPV by the lipofection method following the instructions of the manufacturer (Invitrogen). Two days after lipofection, fresh Sf9 cells were infected with varying dilutions (10^{-3}−10^{-6}) of harvested medium in 96-well plates to isolate positive viral clones. Subcloned recombinant viruses were identified by their ability to direct expression of the appropriate protein as revealed by immunoblotting (see below). Sf9 cells were infected with recombinant baculovirus at a multiplicity of infection of 2−5. For immunoprecipitation of the recombinant receptor, the cells were harvested 36−48 h after infection.

Kinetics of Internalization. Internalization of surface-bound ET-1 was measured as described (*21*). Briefly, clonal CHO-K1 cells were grown in 96-well plates, rinsed 3× with phosphate-buffered saline (PBS: 0.15 M NaCl, 0.1 M sodium phosphate, pH 7.4), and incubated for 2−4 h at 4 °C in 50 μL of the assay buffer (116 mM NaCl, 5.3 mM KCl, 0.81 mM $MgSO_4 \cdot 7H_2O$, 1 mM $CaCl_2$, 5 mM α-glucose, 20 mM Hepes, 1× minimum essential medium amino acids, and 0.1% bovine serum albumin, pH 7.4) containing [^{125}I]-ET-1 (50 pM). After three rapid washes with 100 μL each of ice-cold PBS, the cells were incubated at 37 °C for various periods of time (0−20 min), treated for 15 min at 4 °C with 50 mM glycine, pH 2.5 (ET_A), including 0.5 M NaCl (ET_B), and centrifuged. The remaining pellet was dissolved in 1 N NaOH containing 1% SDS. The fractions of surface-bound ET-1 present in the acid wash and of internalized ET-1 present in the dissolved pellet were determined using a γ-counter. Data points used for the calculations were corrected for unspecific binding by subtracting the amount of [^{125}I]ET-1 bound in the presence of 50 nM unlabeled ET-1.

Determination of Inositol Phosphate. Kinetics of inositol phosphate (IP_n) hydrolysis were measured by modifying the original procedure of (*22*). Briefly, clonal CHO-K1 cells were grown to confluence in 24-well plates and labeled with *myo*-[2-^{3}H]inositol at 1 μCi/mL of assay buffer (see above) for 20 h. The cells were incubated with 10 mM LiCl for 5 min at 37 °C and stimulated with 100 nM ET-1 in the presence of 10 mM LiCl for the indicated times. The reactions were stopped by aspiration of the medium followed by the addition of 1 mL of ice-cold 10 mM formic acid, pH 3. After incubation for 2 h at 4 °C, the samples were centrifuged, and the resultant supernatants were mixed into 3 mL of 50 mM ammonia solution (final pH >9). The mixtures were applied to anion exchange columns prepared from 0.3−0.5 g of Dowex AG:1−8x where the hydroxide form had been exchanged to the formate form as described by the manufacturer (BioRad). Columns were eluted with 10 mL of H_2O to remove free inositol, followed by 2× elution with 2 mL each of 2 M ammonium formate/formic acid, pH 5.2, to elute total inositol phosphates. The radioactivity present in the eluates was quantified in a liquid scintillation counter.

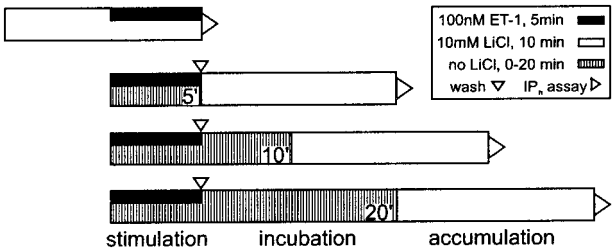

FIGURE 1: Schematic outline of measurement of endothelin receptor desensitization. Each bar represents the measurement at one time point (from top to bottom: $t = 0$, $t = 5$, $t = 10$, $t = 20$). The duration of the incubation steps is indicated. ET-1 incubation was stopped by 3 washes with PBS (wash). Following the final 10 mM LiCl incubation step, inositol phosphate accumulation was measured using the standard assay (IP_n assay).

Desensitization Assay. Transfected CHO-K1 cells were stimulated with 100 nM ET-1 for 5 min in the absence of LiCl, followed by the removal of unbound ligand by washing the cells 3× with assay buffer. The washed cells in assay buffer were incubated at 37 °C for various periods of desensitization (0−15 min). Then 10 mM LiCl was added, and the cells were incubated for another 10 min to allow accumulation of inositol phosphates. The reaction was stopped by aspiration of the medium and addition of 1 mL of 10 mM formic acid, and total inositol phosphates were quantified as above (a schematic outline of the procedure is given in Figure 1).

Gel Electrophoresis and Immunoblotting. Cells expressing human ET receptor were washed 3× with PBS and incubated in sample buffer (63 mM Tris/HCl, pH 6.8, containing 2.5% SDS, 5% glycerol, 5% β-mercaptoethanol, and 0.005% bromophenol blue) for 30 min at 42 °C. SDS−polyacrylamide electrophoresis (PAGE) was carried out in 10% acrylamide gels, and immunoblotting was done according to the manufacturer's instructions (Westran, Schleicher & Schuell). Antisera were used at a dilution of 1:2500 throughout. To visualize protein bands, anti-rabbit immunoglobulin conjugated to horseradish peroxidase and the chemiluminescence detection method were employed.

Preparation of Sf9 Membranes. Membranes were prepared as described (*23*) with the following modifications: the cells were washed 2× with PBS, resuspended in ice-cold suspension buffer (15 mM Hepes, 5 mM EDTA, and 5 mM EGTA, pH 8.0, containing 0.1 mM Pefabloc SC), and homogenized using a 20-gauge needle attached to a syringe. The membranes were collected by centrifugation, resuspended in suspension buffer, and centrifuged again. The pellet was suspended in suspension buffer to give a final protein concentration of 1−2 mg/mL, and stored at −80 °C.

Immunoprecipitation of Solubilized ET Receptors. Cells expressing ET receptors were washed twice with ice-cold PBS and suspended in ice-cold RIPA buffer (150 mM NaCl, 50 mM Tris-HCl, pH 8.0, containing 0.1% SDS, 0.5% deoxycholate, 1% Nonidet P40, 1 mM EDTA, and 0.1 mM Pefabloc SC) under rotation for 1 h at 4 °C. The cell lysates were centrifuged at 14000*g* for 10 min at 4 °C. The supernatants of the cell lysates were first incubated for 1 h at 4 °C under rotation with preimmune serum, and then with specific antibodies previously bound to staphylococcus A (Pansorbin). The reaction was terminated by centrifugation at 8000*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, and 0.002% bromophenol blue) and 20 μL of 10 M urea for 30 min at 42 °C.

^{32}P-Phosphorylation of ET Receptors in Sf9 and COS-1 Cells. Sf9 cells were harvested after 36 h of infection, and COS-1 cells 50 h following transfection. Cells were incubated for 1 h in phosphate- and serum-free IPL41 (Sf9) or DMEM (COS-1) medium followed by metabolic labeling with 0.5 mCi/mL $^{32}PO_4^{3-}$ for 4 h. The reaction was stopped by aspiration of the medium and rinsing of the cell layer with ice-cold PBS. Receptor solubilization and immunoprecipitation were carried out as given above except that phosphatase inhibitors (50 mM NaF, 25 mM sodium pyrophospate, 1 mM sodium orthovanadate) were present. The immunoprecipitated proteins were resolved by reducing SDS−PAGE in 10% acrylamide gels including 1 M urea. The gels were fixed with methanol/H_2O/acetic acid (10:77.5:12.5), dried for 2 h at 60 °C, and exposed to Fuji films for 1−3 days at −70 °C.

Phosphoamino Acid Analysis. Immunoprecipitated ^{32}P-labeled protein was subjected to reducing SDS−PAGE; the band of 45 kDa corresponding to ET_B was cut from the dried unfixed gel and incubated with 50 mM ammonium bicarbonate, pH 7.5, containing 0.1% SDS and 5% 2-mercaptoethanol for 12 h at room temperature. The extracted radiolabeled protein was precipitated with 20% trichloroacetic acid and centrifuged. The protein pellet was dried under vacuum and then hydrolyzed with 5.6 N HCl for 1 h at 110 °C and dried again. The released phosphoamino acids were resolved by two-dimensional thin-layer electrophoresis on cellulose plates at pH 1.9 (first dimension) and pH 3.5 (second dimension) as described (*24*).

^{3}H-Palmitoylation of ET Receptors in Sf9 Cells. Sf9 cells were infected with recombinant baculovirus encoding ET receptor. After 48 h, the cells were washed twice with serum-free TC100 medium and cultured for 12 h in serum-free TC100 medium. Then the cells were placed in fresh serum-free TC100 medium and metabolically labeled with 0.2 mCi/mL [9,10-^{3}H]palmitic acid for 4 h at 27 °C. Labeling was terminated by aspiration of the medium and rinsing of the cells 2× with ice-cold PBS. Receptor solubilization, immunoprecipitation, and SDS−PAGE were carried out as detailed above. The gels were fixed with methanol/H_2O/acetic acid (10:77.5:12.5), incubated in H_2O for 45 min at room temperature, washed, and incubated with 15% (w/v) sodium salicylate for another 30 min at room temperature. The gels were dried for 2 h at 60 °C and exposed to Fuji X-ray film for 1 week at −70 °C.

^{35}S Metabolic Labeling. Infected Sf9 cells were washed with cysteine/methionine-free IPL41 medium, and incubated in the same medium for 30 min. The cells were metabolically labeled with 50 μCi/mL each of [^{35}S]cysteine and [^{35}S]-methionine for 6 h at 27 °C, followed by receptor solubilization, immunoprecipitation, and SDS−PAGE as outlined above. Fixed and dried gels were exposed to Fuji X-ray films for 8 h at −70 °C.

RESULTS

Generation and Characterization of Antisera to the ET_B Receptor. To facilitate the biochemical characterization of the human endothelin receptor B, specific antisera were raised. Immunization of either rabbits or mice with synthetic peptides corresponding to the C-terminus (α-CLK23) and

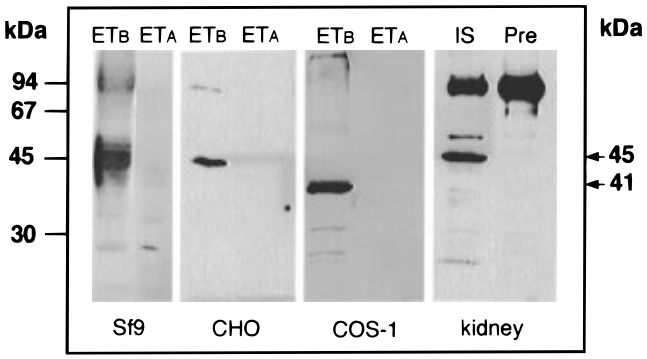

FIGURE 2: Immunoprinting of human ET_B receptor. Lysates from Sf9 cells infected with baculovirus containing the human ET_B cDNA, or from COS-1 and CHO cells transfected with ET_B cDNA, were subjected to reducing 10% SDS−PAGE, and the resolved proteins were electroblotted on poly(vinylidene fluoride) sheets. Transferred proteins were probed by a mixture of the ET_B-specific antisera α-CLK23 and α-CGL26 at a dilution of 1:2500 each, and bound antibodies were visualized by the chemiluminescence detection method. Lysates from cells expressing the human ET_A receptor were probed with ET_B-specific antisera to serve as a control for antibody specificity. For comparison, primary epithelial cells from human kidney expressing endogenous ET_B receptor were tested using the same immune sera (IS) and preimmune serum (Pre) as the control. Molecular masses (kDa) of standard proteins are given.

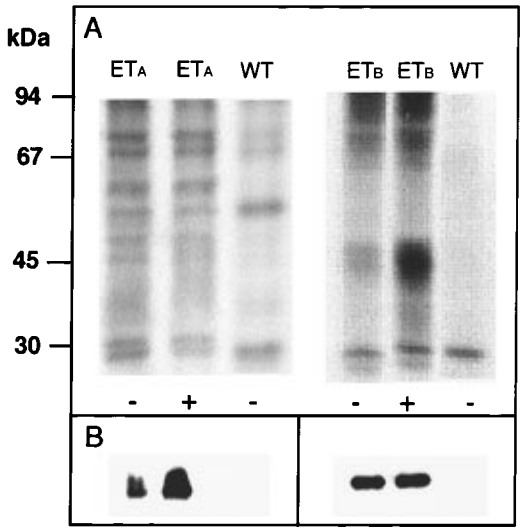

FIGURE 3: Ligand-induced phosphorylation of endothelin receptor in Sf9 cells. (A) 48 h after the infection, Sf9 cells expressing ET_A receptor or ET_B receptor were metabolically labeled for 4 h with [^{32}P]orthophosphate, washed, and incubated for 15 min in the absence (−) or presence (+) of 100 nM ET-1. Membrane fractions of the harvested cells were prepared, lysed, and immunoprecipitated with mixtures of rabbit antisera α-CTS24/α-CDN25 (ET_A) or α-CLK23/α-CGL26 (ET_B). Immunoprecipitates were analyzed by reducing 10% SDS−PAGE and autoradiography. For control, Sf9 cells infected with wild-type baculovirus were used. (B) To monitor the yield of receptor precipitation, a fraction of the immunoprecipitates was analyzed by Western blotting using mouse antisera α-CTS24/α-CDN25 (ET_A) or α-CLK23/α-CGL26 (ET_B), followed by an anti-mouse immunoglobulin and the chemiluminescence detection method.

N-terminus (α-CGL26) of the ET_B protein resulted in antisera, α-CLK23 and α-CGL26, that strongly recognized the corresponding peptides in the indirect enzyme-linked immunosorbent assay (ELISA; data not shown). A mixture of these antisera cross-reacted with the cognate antigen, i.e., human ET_B receptor, in Western blots using total cell lysates of Sf9, COS-1, or CHO cells that express high copy numbers of ET_B receptor (Figure 2), whereas no specific signal was observed in cell lysates containing the ET_A receptor (Figure 2). A specific band was also detected in primary human kidney epithelial cells known to be rich in ET_B receptors (Figure 2). The apparent molecular mass of ET_B ranged from 41 kDa (COS-1) to 45 kDa (Sf9, CHO, epithelial cells), probably reflecting differential glycosylation patterns of the receptor in the various cell types. Identical cross-reactivity profiles were obtained with the single antisera, and with corresponding anti-peptide antisera raised in mouse (not shown). Similar results were obtained when ET_B was immunoprecipitated from recombinant cells that had been metabolically labeled with ^{35}S-labeled amino acids, and using a mixture of α-CLK23 and α-CGL26, thus underlining the specificity and sensitivity of our antisera against the human ET_B receptor.

Ligand-Induced Phosphorylation of Human ET Receptors. To analyze the ligand-induced phosphorylation of human ET receptors, Sf9 cells infected with baculovirus containing the cDNAs for human ET_A and ET_B, respectively, were metabolically labeled with [^{32}P]orthophosphate. The cells were stimulated with 100 nM ET-1 for 15 min and lysed, and the receptor protein was solubilized from the crude membrane preparation. Immunoprecipitation was done with a mixture of antisera to ET_A (α-CTS24/α-CDN25) and to ET_B (α-CLK23/α-CGL26), respectively. SDS−PAGE and fluorography of the immunoprecipitated proteins failed to produce a significant band of approximately 48 kDa as would be expected for a phosphorylated ET_A receptor, whereas a major band of 45 kDa indicative of a phosphorylated ET_B receptor was seen (Figure 3). In the absence of ET-1, no such phosphorylation was observed although the amounts of precipitated receptors were similar in the absence or presence of ET-1 as can be judged from the corresponding Western blots where we employed specific mouse antibodies as the probes (Figure 3B). For control, Sf9 cells infected with the wild-type baculovirus were used (Figure 3). Hence, it appears that ET_B but not the ET_A receptor is phosphorylated in response to ligand stimulation in Sf9 cells.

Phosphoamino acid analyses revealed that the majority of the ^{32}P label was present as phosphoserine together with a minor fraction of phosphothreonine. Phosphotyrosine was undetectable (Figure 4C). Moreover, the ET_B phosphorylation was dose-dependent with an apparent EC_{50} of about 50 nM, and maximum phosphorylation occurring at 100 nM ET-1 (Figure 4A). These findings are largely compatible with observations made for the ligand-induced phosphorylation of many G protein-coupled receptors [for review, see (*25*)].

Palmitoylation of Human ET Receptors in Sf9 Cells. Sf9 cells may have the capacity to phosphorylate the ET_B receptor but lack the competence to posttranslationally modify the ET_A receptor. We therefore addressed the ligand-independent modification of ET receptors by palmitoylation. Metabolic labeling of ET receptor-bearing Sf9 cells was done with [^{3}H]palmitic acid, followed by immunoprecipitation with specific antisera and resolution of the precipitated proteins by SDS−PAGE. Fluorography revealed a major radiolabeled band of 48 kDa for ET_A and a single band of 45 kDa for ET_B (Figure 5B). These bands correspond well with the size of the proteins immunoprecipitated from ^{35}S-labeled cells (Figure 5A). For control, lysates from Sf9 cells infected with the wild-type virus were used which failed to produce specific bands under the conditions of the experiment (Figure

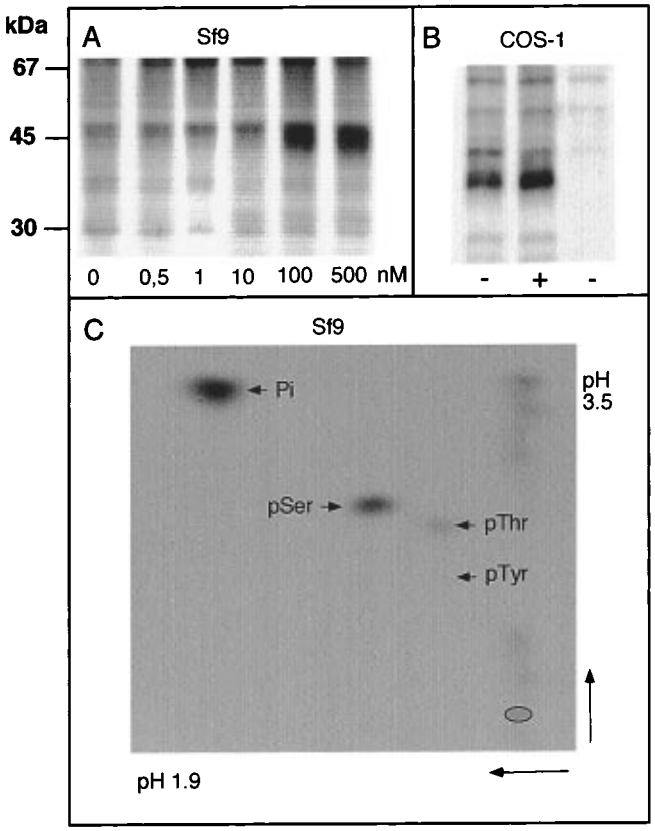

FIGURE 4: Characterization of ET_B receptor phosphorylation. (A) Sf9 cells expressing human ET_B were radiolabeled 24 h after infection with [^{32}P]orthophosphate for 4 h, and stimulated for 15 min with the indicated concentration (in nM) of ET-1. Membranes were prepared, lysed, and immunoprecipitated by α-CLK23/α-CGL26. Precipitated proteins were resolved by reducing 10% SDS−PAGE and visualized by autoradiography. (B) COS-1 cells expressing human ET_B were radiolabeled with [^{32}P]orthophosphate for 4 h and incubated in the absence (−) or presence (+) of 100 nM ET-1 for 15 min. Total cell lysates were immunoprecipitated and analyzed by reducing 10% SDS−PAGE, followed by autoradiography. For control, nontransfected COS-1 cells (right lane) were used. (C) Sf9 cells expressing ET_B were labeled with [^{32}P]-orthophosphate, immunoprecipitated, and analyzed as detailed in (A). The band corresponding to ET_B was excised from the unfixed dried gel, extracted, and hydrolyzed in 5.6 N HCl. Phosphorylated amino acids were resolved by two-dimensional thin-layer electrophoresis at pH 1.9 (first dimension) and 3.5 (second dimension), and identified by a set of standard phosphoamino acids.

5A,B). Therefore, we conclude that Sf9 cells have the capacity to express and modify both types of human ET receptors. Our finding that the ET_B but not the ET_A receptor is ligand-dependently-phosphorylated in these cells points to a difference in the intrinsic properties of these closely related receptor proteins rather than to a specific receptor kinase deficiency of Sf9 cells that prevents ET_A phosphorylation. Similar experiments in transfected COS-1 cells confirmed the agonist-induced phosphorylation of ET_B (Figure 4B) and, the lack of ET-inducible phosphorylation of the ET_A receptor (not shown); however, the low copy number prevented us from making firm conclusions as to the absence or presence of ET_A phosphorylation in COS-1 cells.

Desensitization of Endothelin Receptors. To explore the possibility that differential phosphorylation of ET_A and ET_B is reflected by differences in the desensitization or internalization of these receptors, we studied the time courses of receptor inactivation by following the accumulation of inositol phosphates after a single application of ET-1.

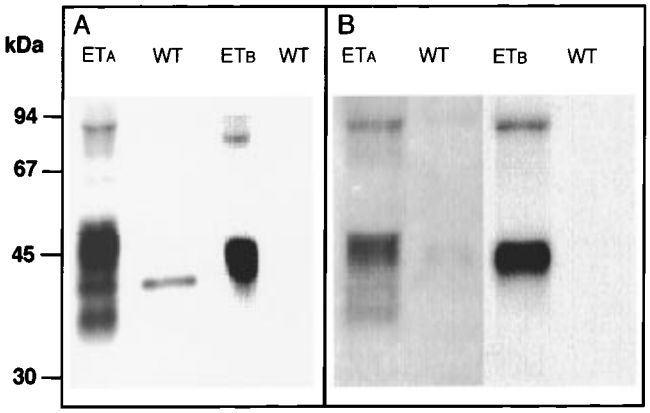

FIGURE 5: Immunoprecipitation of ^{35}S- and ^{3}H-labeled endothelin receptors. Sf9 cells expressing recombinant ET_A or ET_B were metabolically labeled for 4 h with a mixture of [^{35}S]cysteine/[^{35}S]-methionine (A) or [^{3}H]palmitic acid (B). ET receptor was immunoprecipitated from total cell lysates of Sf9 cells using mixtures of α-CLK23/α-CGL26 (ET_A) or α-CTS24/α-CDN25 (ET_B). Immune complexes were resolved by reducing 10% SDS−PAGE, followed by fluorography (^{35}S) or autoradiography (^{3}H). For control, cells infected with wild-type baculovirus (WT) were used. Molecular masses of standard proteins are given in kDa.

Because in Sf9 cells measurement of receptor inactivation was hampered by insufficient accumulation of inositol phosphates (probably due to an inefficient labeling of Sf9 cells with *myo*-[2-^{3}H]inositol), we employed CHO cells bearing recombinant ET_A ("CHO-ET_A") or ET_B ("CHO-ET_B") receptors. First we studied the time course of receptor activity of CHO-ET_A and CHO-ET_B cells that had been pretreated with 10 mM LiCl for 10 min and were subsequently challenged by 100 nM ET-1. Accumulation of total inositol phosphates for CHO-ET_A was almost linear with time over a period of 5 min, whereas in the case of ET_B the inositol phosphate accumulation slowed down after 3 min and seemed to level off after 5 min (Figure 6A). The observed differences in the time course of inositol phosphate accumulation between the ET receptors were even more obvious when CHO cells were stimulated with 100 nM ET-1 for 5 min, washed extensively to remove unbound ligand, and then incubated in a LiCl-free medium for 0−15 min to allow the ET receptors to desensitize (for a detailed outline of the experimental setting, see Figure 1). After the indicated time periods, a single pulse of 10 mM LiCl was applied for 10 min, and thereafter total inositol phosphates were measured. At $t = 0$ min, i.e., when LiCl was added immediately after ET-1 stimulation, inositol phosphate accumulation was almost identical for ET_A and ET_B receptors (=100%). When LiCl was added after a lag phase of 5 min, ET_A was still operating with maximum activity (98%) whereas the ET_B activity, measured as total inositol phosphate production, was reduced to about 15% of the maximum (Figure 6B). This dramatic difference was even more pronounced after 10 min of incubation in the absence of LiCl, where ET_A had a relative activity of 68% whereas the ET_B activity was undetectable (<1%). After 20 min in the absence of LiCl, the ET_A activity was still 28%, and the ET_B remained at <1% (Figure 6B). Both experimental modes demonstrate that the ET_A receptor activity declines slowly after receptor stimulation whereas the ET_B receptor shuts off quickly after ligand activation.

Role of Protein Kinase C in Endothelin Receptor Desensitization. It has been speculated that protein kinase C (PKC) is involved in desensitization of G protein-coupled receptors

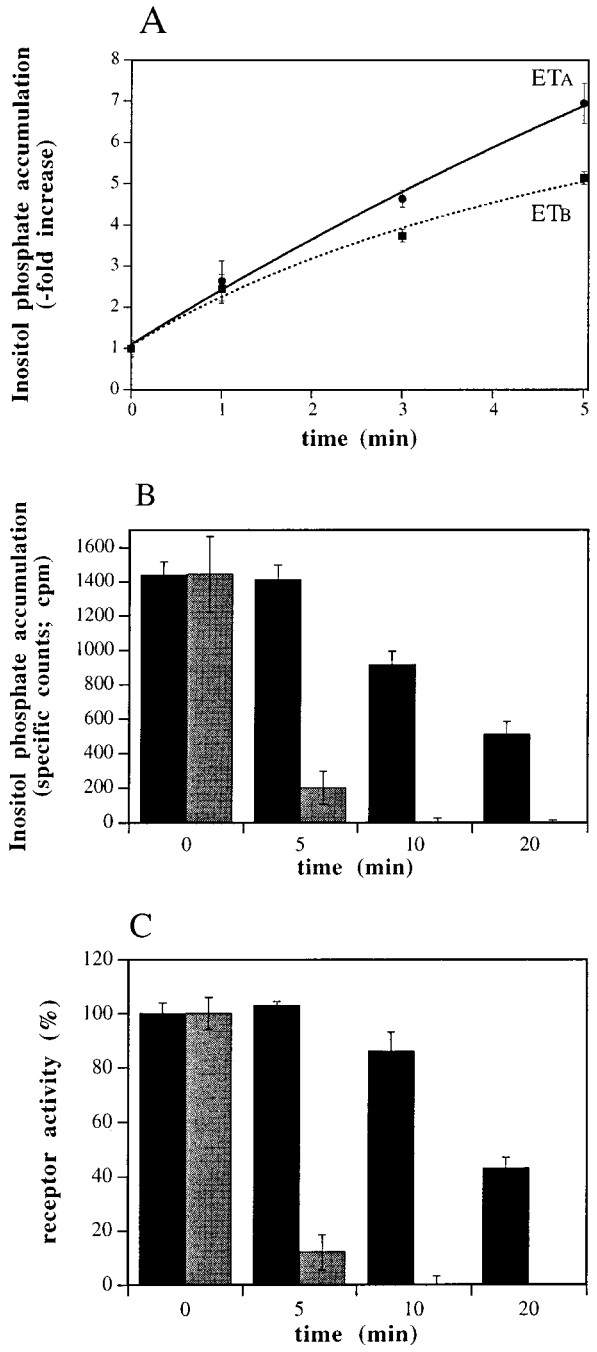

FIGURE 6: Kinetics of receptor-mediated inositol phosphate accumulation. (A) CHO cells expressing ET_A (●) or ET_B (■) were metabolically labeled with [^{3}H]inositol for 24 h. The cells were treated with 10 mM LiCl for 5 min, and incubated in the absence (control) or presence of 100 nM ET-1 for the indicated time periods. Data were calculated as the fold increase of inositol phosphate accumulation over control and represent means ± SD of two separate experiments done in triplicate. (B) [^{3}H]Inositol-labeled CHO cells expressing ET_A (black bars) or ET_B (gray bars) were treated with 10 mM LiCl for 5 min, washed, and incubated for 5 min in the absence or presence of 100 nM ET-1 in a LiCl-free buffer. Then 10 mM LiCl was added for 10 min, and total inositol phosphate was measured ($t = 0$ min). To follow receptor inactivation, the incubation period in the absence of LiCl was extended as indicated ($t = 5$, 10, or 20 min). Data are means ± SD representative of three separate experiments done in triplicate each. (C) CHO cells expressing ET_A (black bars) or ET_B (gray bars) were treated with 2 μM bis(indoylmaleimide). The time course of receptor inactivation was followed using the same protocol as in (B). Data are given as the percentage of control at $t = 0$ min. Means ± SD representative of two separate experiments done in duplicate each are presented.

(*25*). We wondered whether a potent PKC inhibitor such as bis(indoylmaleimide) might alter the desensitization kinetics of the ET receptors. To this end, we preincubated CHO-ET_A and CHO-ET_B cells, respectively, for 10 min with 2 μM bis(indoylmaleimide), followed by application of 10 mM LiCl for another 5 min, and the stimulation of the cells by 100 nM ET-1. The desensitization patterns of ET_A and ET_B were almost unchanged in the presence of the PKC inhibitor (Figure 6C).

Internalization of Endothelin Receptors. Next we addressed the possibility that the observed differences in the desensitization patterns of ET_A and ET_B are due to variances in their internalization kinetics. To this end, we analyzed the receptor-mediated uptake of a radioligand, [^{125}I]ET-1, into CHO-ET_A or CHO-ET_B cells. The apparent receptor number on the cell surface was determined after 5, 10, and 20 min following agonist exposure (Figure 7). Both CHO-ET_A and CHO-ET_B showed a rapid loss of ET receptors from the cell surface: 50% of the receptors were lost after 6 min (ET_A) and 7 min (ET_B), respectively. After 20 min of receptor stimulation, less than 20% of the initial copy number of ET_A and ET_B was present on the cell surface.

DISCUSSION

The maintenance of homeostasis in the human body requires a delicate balance between activating and inactivating mechanisms. Following stimulation by their cognate ligands, G protein-coupled receptors undergo multiple mechanisms of desensitization including receptor phosphorylation, sequestration, internalization, uncoupling of the ligand, and/or proteolytic breakdown. The molecular mechanisms underlying desensitization are well understood for the rhodopsin receptor (*26*) and the adrenergic receptor (*27*). Peptides hormones such as angiotensin, vasopressin, bradykinin, and endothelin bind to various receptor subtypes that have differential, sometimes even opposing, biological effects. Therefore, the elucidation of subtype-specific desensitization mechanisms of these receptors is of general interest. In this report, we have studied the correlation of phosphorylation, desensitization, and internalization of human ET receptor subtypes ET_A and ET_B. ET receptors have been subclassified into ET_{A1}/ET_{A2} (28) and ET_{B1}/ET_{B2} (*29*) in several species using pharmacological criteria. ET_{B1} is supposed to function on endothelial cells inducing vasodilation whereas ET_{B2} expressed in smooth muscle cells causes vasoconstriction. On the other hand, the spatial and functional divergence of ET_B receptor subtypes has been questioned (*30*). To the best of our knowledge, no data are available to show that recombinant expression of ET receptors can mimic the pharmacological profiles of the BQ123-insensitive ET_{A2} or the ET_{B1} showing "super-high" affinity sites. Therefore, our results are not conclusive whether our data also reflect the properties of ET_{A2} and ET_{B1}. Additional studies are necessary performing similar experiments as described using endothelial and smooth muscle cells.

The combined results obtained from Sf9, CHO, and COS cells expressing ET receptors indicate that the ET_A subtype (most likely ET_{A1}) is not phosphorylated in response to ligand stimulation and desensitizes slowly, whereas the ET_B subtype (most likely ET_{B2}) becomes phosphorylated on serine and threonine residues immediately after ligand stimulation and desensitizes swiftly. The internalization kinetics for ET_A and ET_B are almost identical, thus ruling out the possibility that

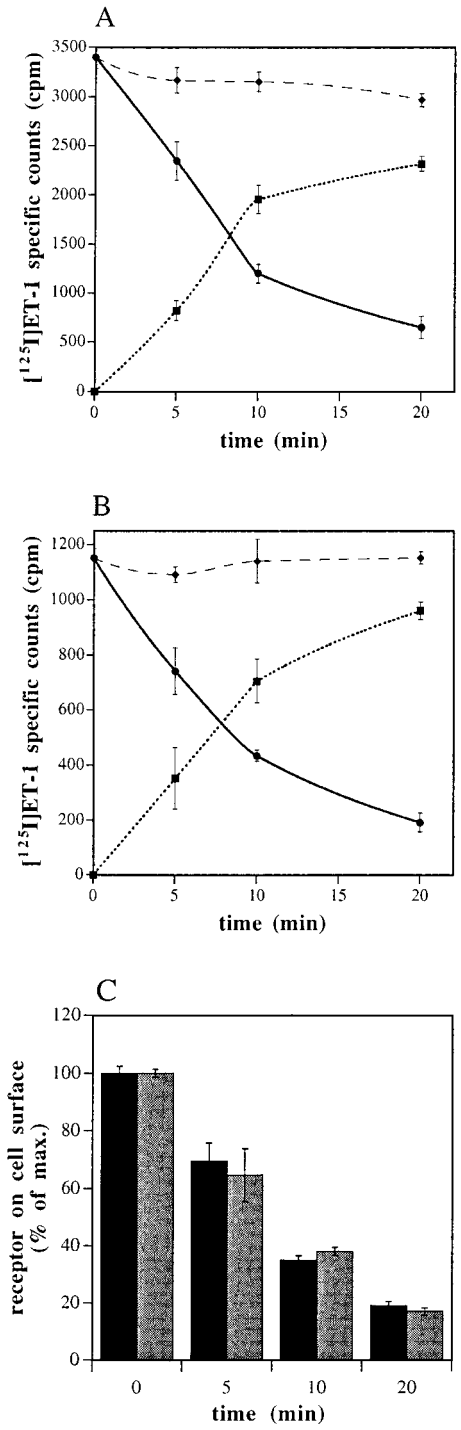

FIGURE 7: Internalization of endothelin receptors by CHO cells. CHO cells transfected with ET_A (panel A) or ET_B (panel B) were incubated with 50 pM [^{125}I]ET-1 for 2−4 h at 4 °C. Then the cells were incubated at 37 °C for the indicated time periods and analyzed. The fraction of the radioligand associated with the cell surface was measured as the acid-soluble fraction of [^{125}I]ET-1. The fraction of internalized [^{125}I]ET-1 (■) was calculated as the difference of the acid-insoluble fractions of [^{125}I]ET-1 (●) at a given time point and at $t = 0$. Total bound ligand (◆) was calculated as the sum of surface-bound and internalized fractions. The unspecific binding of [^{125}I]ET-1 was determined in the presence of 100 nM unlabeled ET-1. Means of duplicate measurements of three independent experiments are presented. (C) Time course of receptor internalization for CHO-ET_A (black bars) and CHO-ET_B (gray bars). Surface-bound [^{125}I]ET-1 is given as the percentage of the control at $t = 0$. Data are means ± SD representative of three separate experiments each done in triplicate.

the observed differences in receptor phosphorylation and desensitization are caused by differential cellular redistribution of the stimulated receptors. These findings imply a subtype-specific down-regulation of ET receptors where ET_A receptors mediate long-lasting effects of endothelins whereas ET_B receptors contribute to their short-term effects. We have not addressed the question whether ET_B agonists like IRL-1620, BQ-3020, and sarafotoxin S6c would alter the phosphorylation rate and the desensitization behavior of ET_B because no reports are available showing a ligand-specific regulation of ET_B signal transduction properties or changes in the signal duration by these ligands. On the other hand, subtle differences in the phosphorylation and desensitization pattern of ET_B cannot be ruled out and should be the subject of follow-up studies.

The differential phosphorylation patterns of ET receptor subtypes may be explained by the intrinsic properties of ET_A vs ET_B or by the endogenous properties of the recombinant cell line (Sf9) used for these experiments. Three experimental lines support the former notion: (i) both subtypes are subject to another posttranslational modification, i.e., palmitoylation in Sf9 cells; (ii) in a chimeric receptor where the carboxy-terminal tail of ET_B has been replaced by the corresponding sequence of ET_A, almost all predicted phosphorylation sites for receptor kinases are ligand-dependently-phosphorylated in Sf9 cells (data not shown); (iii) ET_B is phosphorylated in COS cells in a ligand-dependent manner. Unfortunately, we failed to assess phosphorylation of ET_A receptors in COS-1 and CHO cells because we were unable to immunoprecipitate sufficient ET_A receptor protein. Hence, we cannot entirely rule out the possibility that differential receptor phosphorylation reflects the lack of a competent ET_A receptor kinase in Sf9 cells. However, agonist-induced phosphorylation of G protein-coupled receptors has been demonstrated for muscarinic (*31*), serotonergic (*32*), and dopaminergic receptors (*33*), suggesting that Sf9 cells are endowed with kinases for a wide variety of receptors. One of these reports describes the subtype-specific receptor phosphorylation of the m2 but not of the m1 subtype of the muscarinic receptor (*31*), suggesting that differential phosphorylation might be a common mechanism shared by several receptor populations.

In the present study, we have not addressed the type of protein kinase(s) involved in ligand-dependent ET receptor phosphorylation. ET-1 stimulation induces the release of IP_3 and diacylglycerol via $G\alpha_q$ and PLC, and subsequently a rise of $[Ca^{2+}]_i$ in Sf9, CHO, and COS cells [(*17*) and data not shown]. Hence, calcium/calmodulin-dependent and/or diacylglycerol-activated protein kinases might be candidates for the putative ET_B receptor kinase(s). Preincubation of CHO-ET_B cells with the PLC inhibitor U73122 completely abolished the IP_3 and $[Ca^{2+}]_i$ signals without inhibiting ET_B phosphorylation, thus making the involvement of a calcium/calmodulin-dependent kinase unlikely (data not shown). Furthermore, inhibition of PKC was without effect on the ligand-dependent ET_B inactivation kinetics in CHO cells (this study) or on ET receptor down-regulation in C6 cells (*34*), thus ruling out the possibility that PKC serves in the ligand-dependent homologous ET_B inactivation. In contrast, a positive role of PKC for the heterologous desensitization of ET receptors was observed. PMA stimulation of PKC reduced the initial ET_B but not the ET_A activity in CHO cells (data not shown). Therefore, ET_B receptor function in CHO cells can be regulated by endothelin-independent processes that regulate PKC activity.

In summary, the presented data suggest a subtype-specific desensitization of human ET receptors which likely correlates with their differential ligand-induced phosphorylation. It will be intriguing to identify the structural receptor elements that are responsible for subtype-specific regulation of endothelin receptor activity and to evaluate the impact of the differential receptor regulation on the performance of the endothelin system.

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