Mechanisms of Endothelin Receptor Subtype-specific Targeting to Distinct Intracellular Trafficking Pathways*

Joachim D. Paasche‡, Toril Attramadal‡, Cecile Sandberg, Heidi K. Johansen, and Håvard Attramadal§

From Merck Sharp & Dohme Cardiovascular Research Center and Institute of Surgical Research, University of Oslo, The National Hospital, 0027 Oslo, Norway

We recently reported that the endothelin (ET) receptor subtypes ETA and ETB are targeted to distinct intracellular destinations upon agonist stimulation (Bremnes, T., Paasche, J. D., Mehlum, A., Sandberg, C., Bremnes, B., and Attramadal, H. (2000) J. Biol. Chem. 275, 17596–17604). The ETA receptor was shown to follow the recycling route of transferrin, whereas ETB is targeted to lysosomes for degradation. In the present study we have investigated the mechanisms of ET receptor subtype-specific targeting to distinct intracellular trafficking pathways. Truncation mutants of the ETA and ETB receptors with deletions of the cytoplasmic carboxyl-terminal tail distal to the palmitoylation site were found to mediate inositol phosphate accumulation and to internalize upon agonist stimulation, although internalization occurred at a slower rate as compared with the wild-type receptors. However, the truncated ETA receptor was no longer able to undergo recycling. Rather, both truncation mutants were recognized by β-arrestin for recruitment to endocytosis and were sorted to lysosomes by a dynamin-dependent internalization pathway. Furthermore, studies of chimeric ETA and ETB receptors where the cytoplasmic tail of ETA was swapped with the corresponding domain of ETB, and vice versa, revealed that the cytoplasmic tail of ETB is required for efficient lysosomal sorting and that signals for targeting to recycling reside in the cytoplasmic tail of the ETA receptor.

The multiple physiological effects of the vasoactive peptide endothelin (ET)1 (1) are mediated by the G protein-coupled receptors (GPCRs) ETA and ETB (2). In the vasculature, ETA receptors residing on the smooth muscle cells mediate prolonged vasconstriction (3), whereas ETB receptors, which are on the plasma membrane of endothelial cells, are primarily considered to cause NO-mediated vasodilatation (4). In addition, considerable evidence now also supports a role for ETB receptors in the clearance of plasma ET-1 from the circulation (5–8). In order to elucidate the molecular mechanisms of these distinct physiological responses, we recently characterized the intracellular trafficking pathways of the ETA and ETB receptors (9). Upon agonist stimulation both receptor subtypes are rapidly internalized by mechanisms that depend on G protein-coupled receptor kinase, arrestin, clathrin, and dynamin. Interestingly, the internalized ETA and ETB receptors initially appear to share a common path into Rab5-positive early endosomes. However, the two receptor subtypes are subsequently targeted to different intracellular fates. Whereas the ETA receptor follows the recycling pathway through the pericentriolar recycling compartment and reappears at the plasma membrane, the ETB receptor is directed to lysosomes for degradation.

In terms of physiological effects, rapid recycling of the ETA receptor may provide the basis for reestablishment of the signaling response, and thus for the sustained vasoconstriction mediated through this receptor. Conversely, lysosomal targeting of the ETB receptor is consistent with a role for this receptor subtype in the clearance of ET-1 from the circulation. In this respect, it was recently also demonstrated that ET-1 is cotransported with ETB receptors to lysosomes (10). The mechanisms that allow for sorting of GPCRs to recycling versus degradation in lysosomes are unknown. However, recent evidence obtained with the substance P receptor and the protease-activated receptor-1 (PAR-1) indicates that the signals for sorting may reside in the cytoplasmic carboxyl-terminal tail (11). In the latter report analysis of chimeric substance P and PAR-1 receptors demonstrated that the cytoplasmic carboxyl-terminal domains of these receptors contained information critical for receptor-specific targeting to recycling or lysosomal degradation, respectively.

The aims of the present study were to investigate the mechanisms of ET receptor subtype-specific targeting to distinct intracellular trafficking pathways, i.e. recycling in the case of the ETA receptor and lysosomal sorting in the case of the ETB receptor. The kinetics of ET receptor internalization and recycling were investigated by monitoring [125I]-ET-1 trafficking in the absence and presence of Rab protein mutants known to interfere with the recycling process. The data provided in the present study corroborate the evidence that ETB receptors follow the recycling pathway through the pericentriolar recycling compartment via a transport route controlled by Rab5 and Rab11. To elucidate the role of the carboxyl-terminal domains of the ET receptors in intracellular trafficking, truncation mutants of the ETA and ETB receptors with deletions of the cytoplasmic tail were constructed and analyzed in transfected Chinese hamster ovary (CHO) cells. Furthermore, chimeric ET receptors where the carboxyl-terminal domains of ETA and ETB were interchanged were subjected to studies of agonist-
dependent internalization and intracellular trafficking. Strikingly, the carboxy-terminally truncated ET$_A$ receptor lost the ability to undergo recycling. Moreover, the chimeric ET$_B$ receptor with the carboxy-terminal tail of ET$_A$ was capable of recycling, whereas the chimeric ET$_B$ receptor with the carboxy-terminal domain of ET$_A$ was rapidly targeted to lysosomes. Both ET receptor subtypes with deletions of the cytoplasmic tail were targeted to lysosomal compartments in an agonist-dependent manner, although internalization occurred at a slower rate as compared with the wild-type receptors. However, over-expression of β-arrestin was able to rescue the reduced rate of internalization. Thus, although the cytoplasmic tail of ET$_B$ augments the rate of internalization, lysosomal trafficking appears to be a default pathway. Sorting to recycling, on the other hand, appears to depend on a signal residing in carboxy-terminal domain of the ET$_A$ receptor.

**EXPERIMENTAL PROCEDURES**

**Plasmid Constructs**—Subcloning of the cDNAs of the human endothelin receptors ET$_A$ and ET$_B$ into pcDNA1 has been described previously (9). The cDNAs of hemagglutinin epitope-tagged wild-type dynamin and the dynamin K44A mutant (12) in pcK5 were gifts from Dr. C. Van Koperen (University of Essen, Germany). Rat β-arrestin-1 and β-arrestin-2 were in the expression vector pCMV5 (13). Their respective mutants V53D and V54D in pcDNA1 were from Dr. M. G. Caron (Duke University, Durham, NC). The Rab11 S25N and Rab5 S79L cDNA constructs in pcDNA1 were from Dr. H. Stemmark (The Norwegian Radium Hospital, Oslo, Norway). Subcloning of the human transferrin receptor cDNA into pcDNA1 has been described previously (9).

**Construction of ET Receptor Truncation Mutants and ET Receptor Chimera**—Truncation mutants of ET$_A$ (ET$_A^T$) and ET$_B$ (ET$_B^T$) with deletion of the cytoplasmic carboxy-terminal tail were constructed in pcDNA1 by polymerase chain reaction-directed mutagenesis replacing Gln-390 and Gln-406, respectively, by stop codons and introduction of a RI-BamHI restriction site. The primers for ET$_A^T$ were 5’-ccctctgccatccagct-3’ and 5’-gctctagagcctagtaacagcagcagcagaggcatgactgg-3’ (XhoI site underlined), and the primers for ET$_B^T$ were 5’-gattcccaatagatgtgaac-3’ and 5’-gctctagagcctagtaacagcagcagcagaggcatgactgg-3’ (XhoI site underlined). The polymerase chain reaction products were digested with RI-BamHI. The polymerase chain reaction-directed mutagenesis replacing the human endothelin receptors ET$_A$ and ET$_B$ into pcDNA1 has been described previously (9).

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**RESULTS**

**Effects of Rab Mutants on ET Receptor Internalization and Recycling**—In Fig. 1A is shown the characteristic kinetics of ET$_A^T$ and ET$_B^T$ receptor internalization as determined by 125$^I$-ET$^1$-1 uptake at 37 °C in transiently transfected CHO cells. As shown, the time course of 125$^I$-ET$^1$-1 internalization is principally different in ET$_A^T$ receptor-expressing cells as compared with ET$_B^T$ receptor-expressing cells. Whereas internalization of the ET$_A^T$ receptor is characterized by a monophasic time course that asymptotically increases toward a maximum level, the ET$_B^T$ receptor displays a biphasic internalization curve. Internalization of the ET$_A^T$ receptor peaks after 10 min of agonist stimulation and subsequently declines from 10 to 60 min. As shown in Fig. 1, B and C, the sum of surface-bound and inter-
Subtype-specific Sorting of the Endothelin Receptors

FIG. 1. Time course of ETA and ETB receptor internalization in CHO cells. A, kinetics of $^{125}$I-ET-1 internalization in CHO cells transiently transfected with ETA (△) or ETB receptor (□). $^{125}$I-ET-1 was bound to the cells for 3 h at 4 °C to obtain equilibrium binding, and unbound ligand was removed by extensive washing in ice-cold phosphate-buffered saline. Internalization assay was subsequently performed by incubation at 37 °C for the indicated times. The fraction of intracellular receptor at each time point was calculated as the radioactivity still associated with the cells after low pH wash as described under “Experimental Procedures.” B and C, histograms demonstrating intracellular (closed) and surface-associated (hatched) $^{125}$I-ET-1 activity at the different time points of ETA (B) and ETB (C) receptor internalization based on the assays in A. Data are mean ± S.D. of three parallel wells and representative of at least three separate experiments.

FIG. 2. Effects of Rab mutants on the kinetics of ETA and ETB receptor internalization. A and B, kinetics of $^{125}$I-ET-1 internalization in CHO cells transiently transfected with ETA (A, △) or ETB (B, □) alone or together with either Rab5 Q79L (▲) or Rab11 S25N (●). $^{125}$I-ET-1 uptake was performed as described in the legend to Fig. 1 and under “Experimental Procedures.” C, histogram demonstrating changes in the fraction of surface-bound $^{125}$I-ET-1 (“% surface receptor”) between 10 and 30 min, based on the data in A and B, of cells transfected with ETA or ETB receptor alone (closed bars), and together with either Rab5 Q79L (hatched bars) or Rab11 S25N (open bars). The data are mean ± S.D. of three parallel wells and representative of at least three independent experiments.

Receptors increased, consistent with net externalization during this time. In cells cotransfected with Rab5 Q79L or Rab11 S25N externalization of ETB receptor in the same time span was substantially blunted or even reversed. Conversely, neither Rab5 Q79L nor Rab11 S25N altered the rate of ETB receptor internalization during the same time span. However, the initial rate of ETB receptor internalization appeared to be lower in the presence of Rab11 S25N. Taken together, these data show that when the recycling pathway is inhibited by mutant Rab proteins, the typical biphasic internalization curve of recycling ETB receptors shifts to a monophasic process characteristic of terminally internalized ETB receptors. Hence, the distinct intracellular pathways of the ETA and the ETB receptors appear to reflect on the internalization kinetics of these receptors.

Internalization and Intracellular Trafficking of ET Receptors with Deletion of the Cytoplasmic Carboxy-terminal Tail—To investigate the role of the cytoplasmic carboxy-terminal tail of the ET receptors in internalization and intracellular trafficking, we constructed deletion mutants of ETA (ETAΔT) and ETB (ETBΔT) by introducing stop codons at positions Gln-390 and Gln-406, respectively, as illustrated in Table I. Thus, both mutants retained the clusters of cysteines representing the palmitoylation sites (17, 18). As shown in Table II, the ligand binding characteristics of these truncated receptors did not differ significantly from those of wild-type ETA and ETB receptors. Furthermore, deletion of the cytoplasmic carboxy-terminal tail did not appear to alter the capacity of the ET receptors to activate phospholipase C. As shown in Fig. 3, C and D, ET-1-stimulated accumulation of inositol phosphates was nearly identical for the truncated receptors and their wild-type counterparts. However, agonist-stimulated internalization-
tion of ET<sub>A</sub>T and ET<sub>B</sub>T was substantially impaired as compared with wild-type ET<sub>A</sub> and ET<sub>B</sub>, respectively (Fig. 3, A and B). The biphasic internalization curve characteristic for the recycling ET<sub>A</sub> wild-type receptor was abolished in agonist-stimulated internalization of ET<sub>T</sub>. Thus, the monophasic internalization of ET<sub>A</sub>T provides evidence that deletion of the carboxyl-terminal tail of ET<sub>A</sub> prevents recycling of this receptor subtype. Deletion of the cytoplasmic tail of ET<sub>B</sub> reduced the rate and extent of agonist-stimulated internalization of this receptor subtype, indicating that the mechanism for fast sorting of ET<sub>B</sub> may reside in its carboxyl-terminal tail.

To investigate the intracellular trafficking pathways of the truncation mutants ET<sub>A</sub>T and ET<sub>B</sub>T, we constructed GFP fusion proteins of these receptor mutants for fluorescence microscopy studies. ET<sub>A</sub>-GFP and ET<sub>B</sub>-GFP were transiently transfected into CHO cells and subjected to analysis of agonist-stimulated internalization. The GFP-tagged wild-type receptors ET<sub>A</sub> and ET<sub>B</sub> were able to recycle efficiently, and no colocalization with LDL in the lysosomal compartments of ETA-GFP- or ETAT-GFP-transfected cells was observed (Fig. 4, D). ETAT-GFP was not found to enter the recycling compartment (Fig. 4, G—I), and the same was the case for the truncated ET<sub>A</sub>-GFP (Fig. 4, J—L). Rather, both ET<sub>B</sub>-GFP (Fig. 5, G—I) and ETB-T-GFP (Fig. 5, J—L) were found to accu-

### Table I

| Receptor | K<sub>d</sub> (pM) | B<sub>max</sub> (pmol/mg) |
|----------|----------------|------------------------|
| ET<sub>A</sub> wt | 99 ± 6 | 5.89 ± 0.11 |
| ET<sub>A</sub>T | 87 ± 8 | 1.66 ± 0.55 |
| ET<sub>A</sub>B | 241 ± 26 | 0.78 ± 0.04 |
| ET<sub>A</sub>T-GFP | 62 ± 5 | 2.71 ± 0.07 |
| ET<sub>B</sub>T-GFP | 111 ± 13 | 1.78 ± 0.08 |
| ET<sub>B</sub>B-GFP | 169 ± 33 | 3.43 ± 0.29 |
| ET<sub>B</sub> wt | 31 ± 3 | 1.15 ± 0.03 |
| ET<sub>B</sub>T | 40 ± 5 | 0.46 ± 0.02 |
| ET<sub>B</sub>B | 72 ± 10 | 4.15 ± 0.17 |
| ET<sub>B</sub>T-GFP | 24 ± 2 | 3.52 ± 0.08 |
| ET<sub>B</sub>B-GFP | 34 ± 3 | 5.76 ± 0.14 |
| ET<sub>B</sub>B-GFP | 169 ± 33 | 3.43 ± 0.28 |

# Subtype-specific Sorting of the Endothelin Receptors

The properties of ET-1 binding, including the number of binding sites, were determined in CHO cells transiently transfected with wild-type ET receptors, the carboxyl-terminal truncation mutants ET<sub>T</sub>, or ET<sub>T</sub>T, or the ET receptor chimera ET<sub>A</sub> wt or ET<sub>A</sub>B with or without GFP tags. Binding of <sup>125</sup>I-ET-1 was performed on membranes as described under "Experimental Procedures" to determine the equilibrium dissociation constants (K<sub>d</sub>) and the maximal binding (B<sub>max</sub>) for the different receptors. All data represent mean of three parallels, presented with 95% confidence intervals (GraphPad Prism), and are representative of at least three independent experiments.

### Table II

Radioligand binding characteristics of wild-type and mutant ET receptors

The properties of ET-1 binding, including the number of binding sites, were determined in CHO cells transiently transfected with wild-type ET receptors, the carboxyl-terminal truncation mutants ET<sub>T</sub>, or ET<sub>T</sub>T, or the ET receptor chimera ET<sub>A</sub> wt or ET<sub>A</sub>B with or without GFP tags. Binding of <sup>125</sup>I-ET-1 was performed on membranes as described under "Experimental Procedures" to determine the equilibrium dissociation constants (K<sub>d</sub>) and the maximal binding (B<sub>max</sub>) for the different receptors. All data represent mean of three parallels, presented with 95% confidence intervals (GraphPad Prism), and are representative of at least three independent experiments.
mulate in LDL-positive lysosomes after 60 min of internalization. Thus, deletion of the cytoplasmic tail of ETB did not seem to alter the intracellular trafficking route of this subtype. Taken together, our data suggest that the cytoplasmic tail of ETA contains information for receptor recycling.

Role of Arrestin and Dynamin in Regulation of ETA and ETB Receptors with Carboxyl-terminal Truncations—Since the carboxyl-terminally truncated receptors ETA,T and ETB,T were found to both internalize and follow a typical intracellular trafficking route to lysosomes, we pursued their mechanisms of internalization. As shown in the overlays to the right, ETA-GFP demonstrated the most prominent colocalization with Tf in the recycling compartment (C). Only minor colocalization with Tf was seen for ETAT-GFP (F), and no colocalization with Tf was seen for ETAT-GFP (I) and ETBT-GFP (L).

In Fig. 5, subcellular localization of the GFP-tagged truncation mutants ETAT-GFP and ETBT-GFP compared with ETAA-GFP and ETAB-GFP after ET-induced internalization; probing for receptor colocalization with LDL in lysosomes. CHO cells were transiently transfected with ETAA-GFP (A-C), ETAT-GFP (D-F), ETBT-GFP (G-I) or ETBT-GFP (J-L) and transferrin receptor. After binding of ET-1 (0.2 μM) at 4 °C, agonist-stimulated internalization of the ET receptors was analyzed after 15 min at 37 °C. Red fluorescent Tf was added during the 15-min period for labeling of the recycling compartment. As shown in the overlays to the right, ETA-GFP demonstrated the most prominent colocalization with Tf in the recycling compartment (C). Only minor colocalization with Tf was seen for ETAT-GFP (F), and no colocalization with Tf was seen for ETAT-GFP (I) and ETBT-GFP (L).

FIG. 4. Subcellular localization of the GFP-tagged truncation mutants ETAA-GFP and ETAB-GFP compared with ETA-GFP and ETB-GFP after ET-induced internalization; probing for receptor colocalization with transferrin in the recycling compartment. CHO cells were transiently transfected with ETAA-GFP (A–C), ETAT-GFP (D–F), ETBT-GFP (G–I), or ETBT-GFP (J–L) and transferrin receptor. After binding of ET-1 (0.2 μM) at 4 °C, agonist-stimulated internalization of the ET receptors was analyzed after 15 min at 37 °C. Red fluorescent Tf was added during the 15-min period for labeling of the recycling compartment. As shown in the overlays to the right, ETA-GFP demonstrated the most prominent colocalization with Tf in the recycling compartment (C). Only minor colocalization with Tf was seen for ETAT-GFP (F), and no colocalization with Tf was seen for ETAT-GFP (I) and ETBT-GFP (L).

FIG. 5. Subcellular localization of the GFP-tagged truncation mutants ETAT-GFP and ETBT-GFP compared with ETA-GFP and ETB-GFP after ET-induced internalization; probing for receptor colocalization with LDL in lysosomes. CHO cells were transiently transfected with ETAA-GFP (A–C), ETAT-GFP (D–F), ETBT-GFP (G–I), or ETBT-GFP (J–L). Agonist-induced receptor internalization was performed for 60 min together with DiI-LDL to probe for lysosomal sorting as described under “Experimental Procedures.” As shown in the overlays to the right, ETAT-GFP (F), ETBT-GFP (I), and ETBT-GFP (L) colocalized with LDL in lysosomes. Note that ETAA-GFP could not be detected in lysosomes. However, ETAT-GFP demonstrated principally plasma membrane staining after 60 min of internalization, consistent with recycling.

that increasing DNA levels during transfection resulted in proportional elevations in protein levels (Fig. 7E). As shown in Fig. 7, A–D, cotransfection with increasing amounts of β-arrestin-1 V53D or β-arrestin-2 V54D resulted in a concentration-dependent decrease in the internalization of the wild-type receptors (Fig. 7, A and C), whereas less pronounced effects were seen for the carboxyl-terminally truncated receptors ETA,T and ETB,T (Fig. 7, B and D). Conversely, cotransfection of wild-type β-arrestin-1 or β-arrestin-2 significantly augmented the internalization rates of ETA,T and ETB,T (Fig. 6, C and F). Thus, the β-arrestins appear to be able to rescue the internalization defect imposed by deletion of the carboxyl-terminal cytoplasmic tail, indicating that the β-arrestins are able to bind and recruit the carboxyl-terminally truncated ET receptors to endocytosis, albeit with lower affinities than the wild-type receptors.

In Fig. 8, A–D, are shown the effects of dynamin and the dominant negative mutant dynamin K44A on the intracellular trafficking route of wild-type and truncated ET receptors. The internalization rates of ETA and ETB are dramatically reduced upon overexpression of dynamin K44A. Dynamin K44A also reduced the rate of ETA,T and ETB,T internalization (Fig. 8, C–D), indicating that the residual capacity of these receptor mutants to enter endocytosis is through a dynamin-dependent mechanism similar to the wild-type ETA and ETB receptors.

Intracellular Trafficking of Wild-type and Chimeric ET Receptors—To investigate whether the characteristic properties of recycling and lysosomal sorting are controlled by the cytoplasmic tail of ETA and ETB, respectively, we constructed chi-
meric ET receptors where the carboxyl-terminal domains of the ET_A receptor and the ET_B receptor were interchanged between the two subtypes, i.e. ET_A receptor bearing the carboxyl-terminal tail of ET_B (ET_A/B) and ET_B receptor bearing the carboxyl-terminal tail of ET_A (ET_B/A) (see “Experimental Procedures” and Table I). To study the intracellular trafficking routes of ET_A/B and ET_B/A, GFP fusion proteins of these chimeric receptors were made, resulting in ET_A/B-GFP and ET_B/A-GFP. The
ligand binding characteristics of the chimeric receptors and the corresponding GFP-tagged variants did not differ significantly from those of the wild-type ET receptors (Table II). For characterization of the intracellular trafficking pathways of ET<sub>A/B</sub>-GFP and ET<sub>B/A</sub>-GFP after agonist-stimulated internalization, we performed simultaneous uptake of red fluorescent transferrin (Tf) or red fluorescent LDL as described under “Experimental Procedures.” As shown in Fig. 9, A–C, ET<sub>A/B</sub>-GFP did not colocalize with Tf in the pericentriolar recycling compartment, as opposed to ET<sub>A</sub>-GFP. Strikingly, ET<sub>A/B</sub> was found to colocalize with red fluorescent LDL in lysosomes, as shown in Fig. 10, A–C. Conversely, ET<sub>B/A</sub> reflect the characteristics of the ET<sub>A</sub> receptor and was found to colocalize with Tf in the recycling compartment (Fig. 9, D–F). Although the carboxy-terminal tail of ET<sub>A</sub> conferred capacity of ET<sub>B/A</sub> to be sorted to the pericentriolar recycling compartment, some degree of lysosomal sorting could also be detected in a minor fraction of the cells (Fig. 10, D–F). Taken together, however, the results obtained with the carboxy-terminally swapped receptors demonstrate that the cytoplasmic tails of the ET<sub>A</sub> and ET<sub>B</sub> receptors play decisive roles in determining the intracellular trafficking pathways of these receptors.

**Agonist-stimulated Internalization and Phosphoinositide Hydrolysis of the Chimeric ET Receptors**—The assay of ligand-induced internalization demonstrated that ET<sub>B/A</sub> internalized more efficiently than wild-type ET<sub>A</sub> (Fig. 11A). Furthermore, the carboxy-terminal tail of ET<sub>B</sub> conferred a monophasic time course of ET-1-stimulated internalization of ET<sub>A/B</sub> as opposed to the biphasic course of internalization of wild-type ET<sub>A</sub>. Conversely, the ET<sub>A/B</sub> chimera internalized at a lower rate and extent than ET<sub>B</sub> (Fig. 11B), and the carboxy-terminal tail of ET<sub>A</sub> switched the internalization kinetics to a biphasic course similar to that of ET<sub>B</sub>. Interestingly, analysis of the capacity of the chimeric receptors ET<sub>A/B</sub> and ET<sub>B/A</sub> to mediate ET-1-induced inositol phosphate accumulation showed that the carboxy-terminal tails conferred the characteristics of their respective wild-type counterparts. Thus, ET<sub>A/B</sub> the ET<sub>A</sub> chimera bearing the cytoplasmic tail of ET<sub>B</sub> was not able to stimulate accumulation of inositol phosphate to the same extent as the ET<sub>A</sub> wild-type receptor. Conversely, ET<sub>B/A</sub>, the ET<sub>B</sub> chimera bearing the cytoplasmic tail of ET<sub>A</sub>, was able to stimulate higher levels of inositol phosphate accumulation than the ET<sub>B</sub> wild-type receptor. These data demonstrate that ET-1-stimulated phospholipase C activity reflect on the intracellular trafficking pathways of the ET receptor subtypes.

**DISCUSSION**

In the present study we have investigated the mechanisms of ET receptor subtype-specific targeting to distinct intracellular trafficking pathways. Recycling is a characteristic feature of the ET<sub>A</sub> receptor and a plausible mechanism by which a long lasting signaling response can be achieved upon persistent agonist activation of this subtype. ET<sub>A</sub> receptors are transported to the pericentriolar recycling compartment upon stimulation with ET, and recycling back to the cell surface appears to occur within 1 h (9). A significant proportion of<sup>125</sup>I-ET-1 has been shown to remain associated with ET<sub>A</sub> receptors for up to 2 h after endocytosis (19), and ET-1 is neither degraded nor returned to the medium during the first 60 min after its binding to the ET<sub>A</sub> receptor (20). Thus, the biphasic internalization of<sup>125</sup>I-ET-1 in ET<sub>A</sub> receptor-transfected cells observed in the present study provides a means of quantitative assessment of the recycling process. As demonstrated, coexpression of ET<sub>A</sub> receptor and Rab5 Q79L altered the characteristic biphasic pattern of ET<sub>A</sub> receptor internalization, consistent with the ability of Rab5 Q79L to retard the recycling process (16) and to cause entrapment of ET<sub>A</sub> receptors in early endosomes (9). The dominant negative mutant Rab11 S25N is considered to be a more potent inhibitor of the recycling process, as transport from early endosomes to the pericentriolar recycling compartment is blocked upon its overexpression (15). As expected, coexpression of Rab11 S25N with Rab5 Q79L resulted in a more prominent change in the time course of ET<sub>A</sub> receptor internalization, altering the biphasic internalization curve to a monophasic event in which externalization of the receptor was blunted. Thus, the internalized receptor accumulated intracellularly and asymptotically reached maximal levels after 30 min of agonist-stimulated internalization. These data not only show that ET receptor trafficking is regulated by Rab proteins along the endocytic pathway but also provide quantitative evidence for recycling of the ET<sub>A</sub> receptors. As to the latter, the characteristic patterns of ET-1 internalization appeared to reflect the intracellular fate of the ET receptors. Interestingly, when correlating the internalization curves of<sup>125</sup>I-ET-1 in cells transfected with mutant ET receptors to the intracellular trafficking pathways of the corresponding GFP-tagged receptors, we found those with lysosomal sorting, i.e., ET<sub>B</sub>T, ET<sub>A</sub>T, and ET<sub>A/B</sub>, to display internalization curves characteristic of the ET<sub>B</sub> wild-type receptor. Conversely, with the ET<sub>B/A</sub> receptor chimera, a biphasic internalization event similar to that of the wild-type ET<sub>A</sub> receptor was observed, which correlates well with the recycling kinetics of these receptors as analyzed by fluorescence microscopy. It could be argued that inhibition of internalization and endocytic traffic of ET<sub>A</sub> by coexpression of the Rab mutants or by truncation of the carboxy-terminal tail of ET<sub>A</sub> may shift the internalization from a biphasic to a monophasic event by slowing the initial rate of internalization. However, Rab11 S25N blocks recycling, and carboxy-terminal truncation of ET<sub>A</sub> redirects the receptor to the lysosomal pathway. These alterations in intracellular transport of ET<sub>A</sub> are likely to be the primary causes of the shift of the internalization from a biphasic to a monophasic event. Furthermore, in the case of the ET receptor chimeras, the monophasic and biphasic internalization curves correlated with lysosomal transport and recycling, respectively, and not with the initial rates of receptor
internalization. In this respect, lysosomal transport of ET\textsubscript{A/B} was associated with monophasic internalization at higher initial rates than that of the wild-type ET\textsubscript{A} receptor. Conversely, the recycling ET\textsubscript{B/A} chimera displayed a biphasic internalization curve despite lower initial rates of internalization as compared with ET\textsubscript{B} wild type.

In the present work we have demonstrated that the cytoplasmic tail of the ET\textsubscript{A} receptor is responsible for efficient targeting of this subtype to the recycling pathway. Thus, the carboxyl-terminal tail of ET\textsubscript{A} contains a signal, which we have also shown to be phosphorylated by G protein-coupled receptor kinases (21) than that of the wild-type ET\textsubscript{A} receptor. Thus, the cytoplasmic tail of ET\textsubscript{B} may confer higher affinities of \textsuperscript{32}P-arrestin binding. Although this remains to be demonstrated, Cramer et al. (22) have reported that the ET\textsubscript{B} receptor subtype is more efficiently phosphorylated than the ET\textsubscript{A} receptor subtype after agonist stimulation in transfected CHO cells. Furthermore, this finding was found to correlate with the rapid inactivation of the inositol phosphate response through the ET\textsubscript{B} receptors as compared with the more sustained activity observed through agonist-activated ET\textsubscript{A} receptors in these cells.

One other report demonstrates that signals for sorting to a specific pathway may reside in the cytoplasmic carboxyl-terminal tail of GPCRs. By analyzing chimeras of the substance P receptor and the PAR-1 receptor where the cytoplasmic carboxyl-terminal domains of the two receptors were interchanged, Trejo and Coughlin (11) demonstrated that the carboxyl-terminal tails of these GPCRs contain information critical for receptor-specific targeting to recycling or lysosomal degradation, respectively. Furthermore, maximal levels of agonist-stimulated phosphoinositide hydrolysis were higher for PAR-1 bearing the carboxyl terminus of the substance P receptor, and lower for the chimeric substance P receptor bearing the carboxyl terminus of PAR-1, as compared with their wild-type counterparts. Consistently, our analyses also showed higher levels of agonist-stimulated inositol phosphate accumulation.
tion, truncation of the carboxyl terminus abolished the capacity of β-arrestin to interdict G protein coupling (29). The interaction between β-arrestin and carboxyl-terminally truncated receptors is probably relatively weak, and recognition of phosphorylated residues in the carboxyl-terminal tail might be crucial for efficient desensitization to occur. Thus, our data indicate that the ability of ET_A and ET_B to stimulate inositol phosphate accumulation to the same levels as their wild-type counterparts could be the sum of less efficient desensitization and a slower rate of internalization.

Differential regulation of GPCRs might also be due to the existence of specificity in the interaction between arrestin family members and distinct receptors. In a recent report, Oakley et al. (30) identified two classes of GPCRs as follows: class A receptors, including the ET_A receptor and the β2-adrenergic receptor; and class B receptors, including the V2 vasopressin receptor and the substance P receptor. Class A receptors displayed higher affinities for β-arrestin-2 than for β-arrestin-1 and visual arrestin, whereas class B receptors were found to bind with similar affinities to all three arrestins. Furthermore, class B receptors generally bound with higher affinities to arrestins due to the presence of a cluster of serines in their cytoplasmic tail, which is not present in class A receptors (31, 32). Interestingly, the ET_A receptor contains a cluster of serines in its extreme carboxyl terminus, which is not present in the ET_A receptor. Although the affinities of arrestin binding to the ET receptors have not been determined, it is tempting to hypothesize that the ET_A receptor may conform to the class B receptor characteristics of high affinity binding to β-arrestin. Thus, a more stable binding to β-arrestin caused by the phosphorylated carboxyl terminus of ET_B could account for the more efficient desensitization as well as the faster internalization rates of ET_B and ET_A/B. Conversely, less stable association with β-arrestin due to the less phosphorylated ET_A tail could serve to explain both the higher levels of inositol phosphate accumulation and the lower rates of internalization of ET_A and ET_B.

Based on the data of the present study, we conclude that the cytoplasmic tails of the ET receptors play decisive roles in the intracellular trafficking of these receptors. The future challenge will be to elucidate what specific signals residing in the carboxyl-terminal tail of the ET_A receptor direct this receptor subtype to recycling. Arrestin evidently plays a key role in modulating signaling responses through GPCRs. Not only receptor desensitization and internalization but also the rate of receptor resensitization seems to be determined by arrestin, the latter correlating with the ability of arrestin to dissociate from the receptor (31). In addition, emerging evidence implicates β-arrestin as a scaffolding protein for GPCR-orchestrated stimulation of mitogen-activated protein kinase (33, 34). However, the putative role of arrestin in targeting receptors to distinct intracellular trafficking pathways remains to be investigated. We believe our model system is of general interest in the field of GPCRs, as we deal with two receptors that are activated by the same ligand but that follow different intracellular routes and show distinct signaling properties.

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Mechanisms of Endothelin Receptor Subtype-specific Targeting to Distinct Intracellular Trafficking Pathways

Joachim D. Paasche, Toril Attramadal, Cecilie Sandberg, Heidi K. Johansen and Håvard Attramadal

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