3 Results

3.1 Detection of the wild-type and mutant ET_B receptors in immunoblots

The human ET_B receptor gene encodes a protein of 442 amino acids. The first 26 aa represent a cleavable signal peptide, which is removed in the ER lumen during protein synthesis. The removal of the signal peptide has been determined experimentally by N-terminal sequence analysis. In immunoblot and low-temperature SDS-PAGE analysis two isoforms of the human ET_B receptor have been described:

1) A full-length ET_B receptor lacking the signal peptide (aa 27-442).
2) An N-terminally truncated ET_B receptor lacking further 38 amino acids C-terminal of the signal peptide cleavage site (aa 65-442) (see Fig. 7).

So far no study has analyzed, which isoforms are expressed at the cell surface and how expression of the different isoforms is regulated. For a detailed analysis of the N-terminal cleavage, ET_B receptor fused to GFP and ET_B·GFP receptor mutants were used (see Fig. 7). The GFP moiety fused to the intracellular C terminus of the ET_B receptor neither alters the receptor`s pharmacological properties nor its intracellular trafficking, but allows the visualization by fluorescence microscopy and immunoblot analysis. Furthermore, several mutant ET_B receptors, with an altered amino acids sequence in the extracellular N terminus, were generated as indicated in Fig. 7. The Δ2-64 mutant corresponds to the N-terminally truncated ET_B receptor lacking the first 64 amino acids. In the R64H/S65A ET_B·GFP receptor mutant, the proteolytic cleavage site (R64↓S65) is altered. In the HIS ET_B·GFP fusion protein, the proteolytic cleavage site (R64↓S65) and the consensus site for N-linked glycosylation (N59-A60-S61) are mutated.

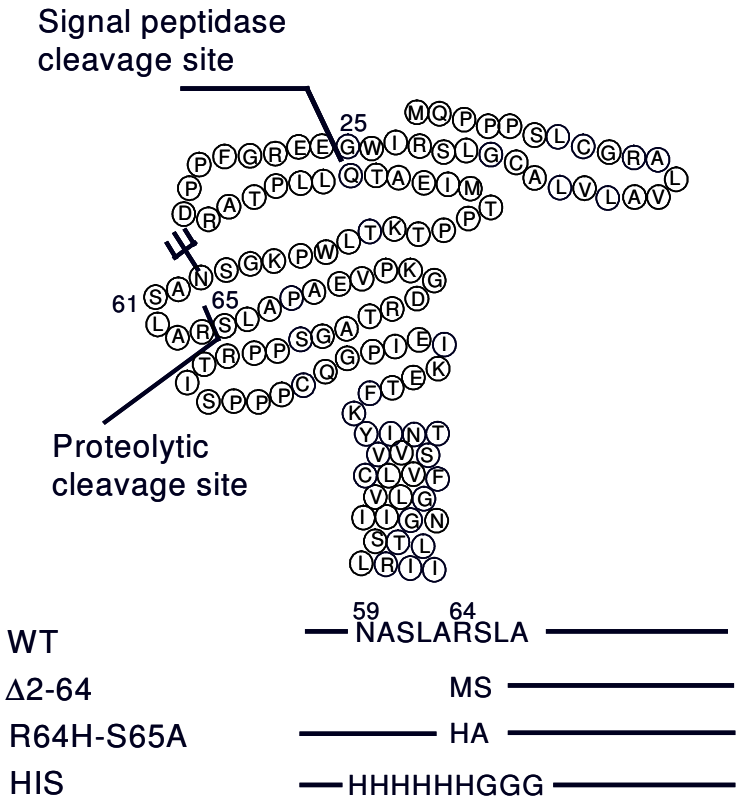

Fig. 7. Model of the ET_B receptor's N terminus. Top: Amino acid sequence of the ET_B receptor's N terminus and the first transmembrane domain. The cleavage sites for the signal peptidase (G25↓E26) and an additional proteolytical cleavage site between (R64↓S65) are indicated. A potential N-linked glycosylation at asparagine 59 is shown. Bottom: Mutant ET_B receptors investigated in this study. WT: wild-type receptor, Δ2-64: N-terminally truncated ET_B receptor lacking amino acids 2 to 64, R64H-S65A: ET_B receptor in which the proteolytic cleavage site (R64↓S65) is altered, HIS: ET_B receptor in which amino acids 59 to 67 are altered, thereby deleting the consensus site for N-linked glycosylation and altering the proteolytical cleavage site (R64↓S65).

To analyze that all mutant receptors are expressed, an immunoblot analysis with membrane preparations of HEK 293 cells stably expressing the different mutants was performed. A polyclonal anti-GFP antibody was used as primary antibody.

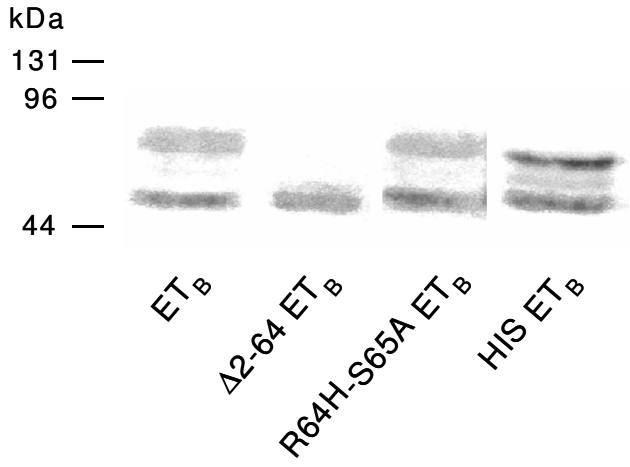

Fig. 8. Immunoblot analysis of wild-type and mutant ET_B·GFP receptor. Membrane preparations of HEK 293 cells stably expressing the wild-type or mutant ET_B·GFP receptors were separated by SDS-PAGE and blotted onto nitrocellulose filters. The fusion proteins were detected with polyclonal rabbit anti-GFP antibodies and horseradish-conjugated goat anti-rabbit antibodies as first and second antibodies, respectively. ET_B: wild-type receptor, Δ2-64 ET_B: N-terminally truncated ET_B receptor, R64H-S65A: ET_B receptor in which the proteolytic cleavage site (R64↓S65) is mutated, HIS: ET_B receptor in which amino acids 59 to 67 are mutated.

For all mutants an immunoreactive band at about 55 kDa was detected, which could represent the N-terminally cleaved receptor (65-442), as it corresponds in size to the band detected in cells expressing the Δ2-64 ET_B·GFP mutant (Fig. 8). For the wild-type ET_B·GFP and the mutant R64H/S65A ET_B·GFP a broad band at about 75 kDa was observed, which represents the complex-glycosylated full-length ET_B receptor, as it was not detected in membranes from cells expressing the Δ2-64 ET_B·GFP. In case of the HIS mutant a distinct band at 72 kDa was found. This band represents the non-glycosylated full-length receptor. The immunoblot experiments demonstrate that the wild-type, the R64H/S65A and the HIS ET_B·GFP mutants are expressed at comparable levels. However, the Δ2-64 ET_B·GFP receptor was expressed at lower levels. To compensate this, three times more protein was loaded onto the gel. In addition, mutations within the N terminus involving the cleavage site (R64↓S65) were insufficient to inhibit the N-terminal proteolysis.

3.2 The ET_B receptor is expressed as a full-length receptor at the cell surface of HEK 293 cells and vascular smooth muscle cells

From the immunoblot experiments it is not clear whether the ET_B receptor is expressed at the cell surface as a full-length or also as an N-terminally truncated (Δ2-64) receptor. To answer this question, intact HEK 293 cells stably expressing the native ET_B receptor or the ET_B·GFP fusion protein were incubated with 100 pM ^{125}I-ET-1 for 30 min at 4°C. Under these conditions cell surface receptors were labeled quantitatively (as determined in radioligand binding studies) and receptor endocytosis was inhibited. Cells were then lysed immediately in Lämmli buffer to prevent proteolytic cleavage of the receptor or incubated for up to 180 min at 37°C prior to lysis. Since ^{125}I-ET-1 forms a stable complex with the ET_B receptor that does not dissociate even under denaturing conditions, the proteins were separated by low-temperature polyacrylamide gel electrophoresis (LT-PAGE) (Takasuka et al., 1991) and receptor/ligand complexes were visualized in dried gels by autoradiography.

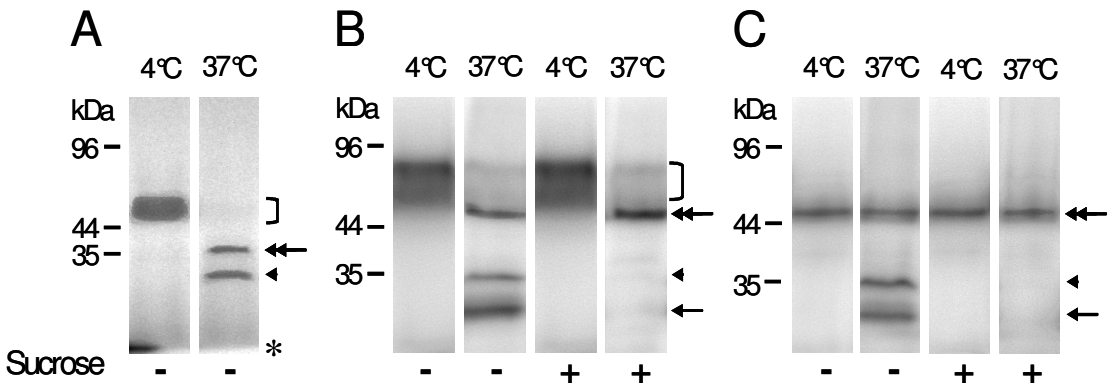

Fig. 9. N-terminal cleavage of the agonist-occupied ET_B receptor at the cell surface. HEK 293 cells stably expressing the native ET_B receptor (**A**), the ET_B·GFP receptor (**B**) or the Δ2-64 ET_B·GFP receptor (**C**) were incubated with ^{125}I-ET1 for 30 min at 4°C in the absence or presence of sucrose and then either lysed in sample buffer or incubated for further 180 min at 37°C in the absence or presence of sucrose (inhibitor of clathrin-mediated internalization) prior to lysis. Lysates were separated by LT-PAGE (see Materials and Methods). Dried gels were exposed on imaging plates and visualized with a BAS 3000 Imaging Analyzer. *Bracket*: full-length receptor; *double arrow*: N-terminally truncated receptor; *arrowhead*, *arrow*: degradation products; *star*: dye front.

In cells immediately lysed after the incubation period with ^{125}I-ET-1 at 4°C, only the full-length ET_B receptor or the ET_B·GFP receptor were expressed at the cell surface (Fig. 9A, B). The full-length receptor appeared as a broad band, which migrated at about 50-55 and 75-80 kDa in case of the native ET_B receptor and the ET_B·GFP receptor, respectively. The differences in the apparent molecular weights of the ET_B receptor and the ET_B·GFP receptor are explained by the GFP moiety, which has a molecular mass of 27 kDa.

Upon incubation at 37°C for 180 min the full-length ET_B receptor and the ET_B·GFP receptor were hardly detectable. Instead, two new distinct bands were observed for the full-length ET_B receptor (at 35 and 30 kDa) and three bands for the ET_B·GFP receptor (at about 55, 35 and 30 kDa). The bands at 35 kDa for the ET_B receptor and at 55 kDa for the ET_B·GFP (double arrows in Fig. 9A and B) represent the N-terminally truncated receptor (the latter migrates at the same position as the Δ2-64 mutant receptor, Fig. 9C, double arrow). The 35 kDa band observed for the ET_B·GFP and the Δ2-64 ET_B·GFP receptor (Fig. 9B, C, arrowhead) most likely represents the receptor without the GFP portion as the band was not detected in immunoblots using an antibody directed against the GFP moiety. In addition, this band has the same mobility as the N-terminally truncated ET_B receptor (compare Fig. 9A: double arrow with Fig. 9B, C: arrowhead). The band at 30 kDa is most likely generated by further proteolytic cleavage of the receptor (Fig. 9A: arrowhead, Fig. 9B, C: arrow). Taken together, the data show that ET_B receptor is expressed as a full-length receptor at the cell surface. Despite N- and C-terminal cleavage, ET-1 remains bound to a core-complex of the ET_B receptor.

Essentially similar results were obtained with cultured VSMCs nucleofected with the ET_B·GFP receptor (Fig. 10), indicating that the N-terminal proteolysis is not a process only observed in HEK 293 cells.

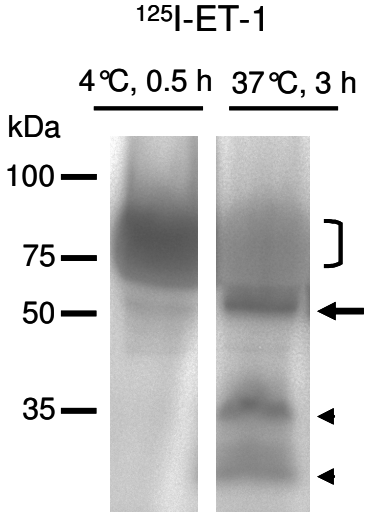

Fig. 10. Cell surface expression of the full-length ET_B receptor and ligand-induced N-terminal cleavage in VSMCs. VSMCs nucleofected with the full-length ET_B·GFP receptor were incubated with ^{125}I-ET-1 for 30 min at 4°C and either lysed in sample buffer or incubated for an additional 3 h at 37°C prior to lysis. Lysates were then separated by LT-PAGE (see Materials and Methods). Dried gels were exposed on imaging plates and visualized with a BAS 3000 Imaging Analyzer. *Bracket*-full-length receptor, *arrow*-N-terminally truncated receptor, *arrowhead*-degradation products.

3.3 The N-terminal cleavage of the ET_B receptor occurs at the plasma membrane

To analyze whether N-terminal proteolysis occurs at the plasma membrane or within an endosomal compartment, HEK 293 cells stably expressing the full-length ET_B·GFP receptor or the N-terminally truncated mutant (Δ2-64 ET_B·GFP) were incubated with ^{125}I-ET-1 in presence or absence of 450 mM sucrose (see Fig. 9B and C). It has been reported that the ET_B receptor is internalized *via* a clathrin-mediated pathway, which can be efficiently inhibited following addition of hypertonic sucrose (450 mM) (Oksche et al., 2000a). In the presence of sucrose the N-terminal cleavage was not prevented indicating that this cleavage occurs at the plasma membrane. Similarly, phenylarsine oxide, an inhibitor of clathrin-dependent and –independent endocytosis, also did not inhibit the N-terminal proteolysis. However, the C-terminal proteolysis was prevented under both

conditions, indicating that C-terminal cleavage requires sorting to endosomes. As expected, no N-terminal proteolysis was observed for the Δ2-64 ET_B·GFP mutant in the presence of sucrose: Δ2-64 ET_B receptors incubated at 37°C in the presence or absence of 450 mM sucrose did not reveal any size differences when compared to samples incubated at 4°C only. The data indicate that proteolytic cleavage of the N terminus occurs at the plasma membrane. In contrast, C-terminal cleavage requires receptor internalization, i.e. trafficking to endosomal / lysosomal compartments.

3.4 The N-terminal cleavage of the ET_B receptor is ligand-dependent

In experiments designed to detect the ET_B·GFP fusion proteins expressed on intact HEK 293 cells, we directly lysed cells kept under different conditions in sample buffer (thereby avoiding activation or release of proteases as it is the case in membrane preparations).

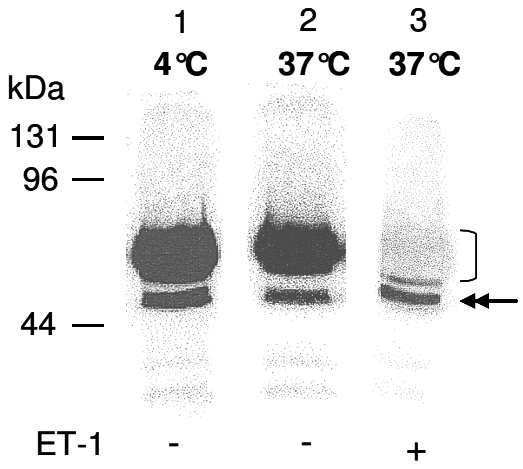

Fig. 11. Cleavage of the agonist-occupied wild-type ET_B·GFP receptor. HEK 293 cells stably expressing the wild-type ET_B·GFP receptor were incubated without (*lanes 1 and 2*) or with 100 nM ET-1 (*lane 3*) for 30 min at 4°C. The cells were either lysed immediately (*lane 1*) or incubated for further 180 min at 37°C prior to lysis (*lanes 2 and 3*). Lysates were separated by SDS-PAGE and blotted onto nitrocellulose filters. The fusion proteins were detected with polyclonal rabbit anti-GFP antibodies and horseradish-conjugated goat anti-rabbit antibodies as first and second antibodies, respectively. *Bracket*: full-length receptor; *double arrow*: N-terminally truncated receptor.

Cells incubated without ET-1 at 4°C for 30 min and lysed directly in sample buffer or incubated for a further 180 min at 37°C prior to lysis yielded identical patterns in immunoblot experiments with an anti-GFP antibody: a prominent band at 75 kDa representing the full-length receptor and a weak band at 55 kDa representing the N-terminally truncated receptor (Fig. 11, lane 1 and 2). However, when cells were incubated with ET-1 (100 nM) at 4°C for 30 min and incubated further at 37°C for 180 min prior to lysis, the 75 kDa disappeared (Fig. 11, lane 3) and only the lower band at 55 kDa (N-terminally truncated receptor) remained. A corresponding increase in band intensities, however, was not observed. As is shown above, the lack of increase is caused most probably by the removal of the GFP moiety from the C terminus of the ET_B·GFP receptor (intracellular C-terminal cleavage, see LT-PAGE experiments, see Fig. 9). As a consequence, the ET_B receptor lacking the GFP portion cannot be detected with the anti-GFP antibody. The data show that cleavage is promoted upon binding of the receptor agonist and suggest that the full-length receptor is expressed at the cell surface.

These findings were confirmed by pulse-chase experiments, allowing to detect newly synthesized receptor proteins (see Fig. 12). HEK cells, stably expressing ET_B·GFP were incubated with ^{35}S (pulse) for 30 min followed by a chase for up to 4 h. Cells were then lyzed and ET_B·GFP receptors were immunoprecipitated either with anti-GFP antibody or with antibody, which detects the N terminus of the receptor. The lysates were then applied onto SDS-PAGE and visualized by autoradiography.

After a 30 min pulse, two bands were observed - a 75 kDa band, corresponding to the glycosylated full-length receptor and a 72 kDa band, representing the non-glycosylated ET_B receptor. After a chase of 1 h, the 72 kDa band was hardly detectable (Fig. 12, lane 2). After a chase of 4 h, only the complex glycosylated ET_B receptor was detected. The absence of further bands in the lysates chased for 4 h strongly suggest that the ET_B receptor is synthesized as a full-length receptor with an intact N terminus.

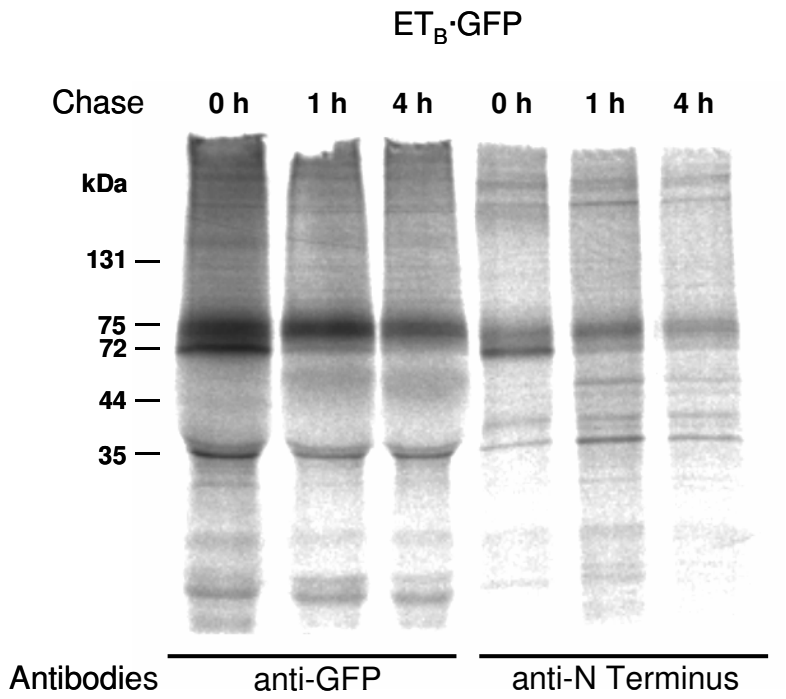

Fig. 12. The ET_B receptor is synthesized as a full-length receptor with an intact N terminus. Stably transfected HEK 293 cells expressing ET_B·GFP receptor were pulse-labeled for 30 min with 200 µCi/ml [^{35}S] methionine/cysteine and chased for the times indicated. Cell extracts were immunoprecipitated with anti-GFP or anti-N terminus antibodies and separated by SDS-PAGE. Dried gels were exposed for 2 to 4 days on a BAS-SR imaging plate and analyzed in a Fuji Film BAS-3000 Imaging Analyzer.

3.5 Time-course of N- and C-terminal proteolysis of the ET_B receptor

To analyze the process of the N- and C-terminal cleavage in more detail, intact HEK 293 cells stably expressing the ET_B·GFP receptor were incubated (after labeling with ^{125}I-ET-1) for up to 3 h in the absence or presence of 10 µM leupeptin, an inhibitor of lysosomal cysteinyl proteases. In the absence of leupeptin the N terminus of the ET_B receptor was already cleaved after 15 min and the cleavage was almost complete after 60 min, whereas the C-terminal degradation was first seen after 30 min and completed after 120 min (see Fig. 13). Further incubation for up to 180 min did not reveal any changes in the band pattern. In the presence of leupeptin the formation of C-terminal degradation products was

completely inhibited (similar to sucrose- or phenylarsine oxide-mediated inhibition of endocytosis). The N-terminal cleavage, however, was not influenced in the presence of leupeptin. The data confirm that proteolytic cleavage of the N terminus occurs at the plasma membrane and is independent of endocytosis.

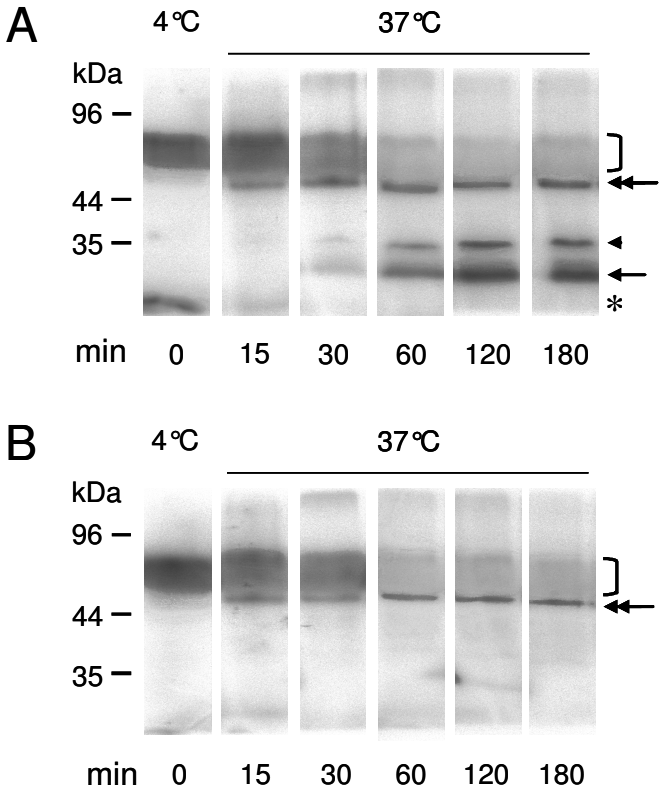

Fig. 13. Time-course of the N-terminal cleavage of the full-length ET_B·GFP receptor. HEK 293 cells expressing the ET_B·GFP receptor were preincubated with ^{125}I-ET-1 for 30 min at 4°C, followed by an incubation at 37°C for the indicated time periods in the absence (**A**) or presence (**B**) of leupeptin (10 µM). Cells were finally lysed and lysates were separated by LT-PAGE (see Experimental Procedures). *Bracket*: full-length receptor; double *arrow*: N-terminally truncated receptor; *arrowhead*, *arrow*: degradation products; *star*: dye front

3.6 The N-terminal cleavage is reduced by metal chelators but not by inhibitors of lysosomal proteases or the proteasome

To characterize which protease(s) might be involved in the N-terminal proteolysis of the ET_B receptor the effect of various protease inhibitors was analyzed. Intact HEK 293 cells stably expressing the full-length ET_B·GFP receptor were incubated with 450 mM sucrose to block the internalization, thereby preventing receptor degradation during endocytosis. Cells were then treated with different inhibitors and subsequently stimulated with ^{125}I-ET-1 at 37°C for up to 3 h.

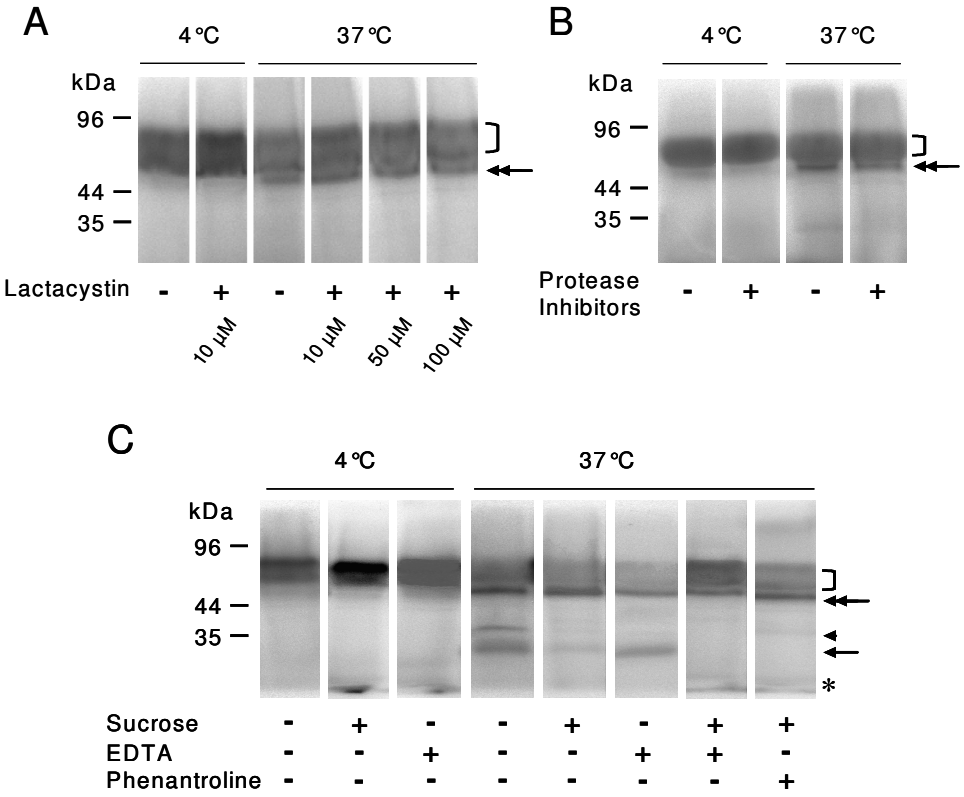

Fig. 14. N-terminal proteolysis can be reduced by metal chelators. HEK 293 cells expressing the ET_B·GFP receptor were incubated with ^{125}I-ET-1 for 30 min at 4°C and either lysed immediately or incubated for additional 180 min at 37°C. The presence of sucrose (inhibitor of clathrin-mediated internalization) and of lactacystin (10 µM) (A), protease inhibitors (PMSF, leupeptine and aprotinine) (B) or metal chelators (10 mM) (C) is indicated. *Bracket*: full-length receptor; *double arrow*: N-terminally truncated receptor; *arrowhead*, *arrow*: degradation products; *star*: dye front.

As shown on Fig. 14 lactacystin (inhibitor of the proteasome, Fig. 14A), a mixture of PMSF, leupeptin and aprotinin (inhibits various serine and cysteine proteases, Fig. 14B) did not prevent the N-terminal cleavage. Only the addition of EDTA dose-dependently reduced the N-terminal proteolysis of the ET_B receptor (Fig. 14C). An inhibition of N-terminal proteolysis was noted at concentrations at 100 µM but was maximal at 10 mM (Fig. 14C, lane 7). However, even at 10 mM EDTA the cleavage was not completely prevented. Similar to EDTA, also phenantroline (10 mM) reduced the proteolytic cleavage of the N terminus (Fig. 14C, lane 8). The data suggest that the proteolysis of the N terminus of the ET_B receptor is mediated by metalloproteases.

3.7 The N-terminal cleavage of the ET_B receptor is prevented by metalloprotease inhibitors

In order to identify which metalloprotease is involved in the N-terminal cleavage of the ET_B receptor, the effects of different metalloproteases inhibitors were investigated. LT-PAGE analyses of HEK 293 cells, stably expressing the full-length ET_B·GFP receptor were performed. To prevent receptor degradation during endocytosis, the inhibitory effect was studied in the presence of 450 mM sucrose. Both the hydroxamic acid-based metalloprotease inhibitor batimastat (Fig. 15A) and also the inhibitor of the tumor necrosis factor α-convertase (TACE) Ro 32-7315 (Fig. 15B) reduced the N-terminal cleavage in the absence of sucrose (Fig. 15A and B, lane 5) and completely inhibited it in the presence of hypertonic medium (Fig. 15A and B, lane 6). The fact that batimastat and Ro 32-7315 were more effective in the presence than in the absence of sucrose may be attributed to a decrease in the effective concentration of the inhibitors during endocytosis and endosomal trafficking. In addition, the acidic pH in the endosomal compartments may interfere with the inhibitory action of MMP inhibitors. The data provide strong evidence that the cleavage of the N terminus of the ET_B receptor is mediated by metalloproteases. However, as batimastat and the Ro 32-7315 inhibit a variety of different metalloproteases, including members of a disintegrin and metalloprotease (ADAM) family, it remains elusive which type of metalloprotease is involved in the N-terminal cleavage. Therefore the effect of a

selective metalloprotease inhibitor Ro 28-2653 (inhibits gelatinase A/MMP-2 and gelatinase B/MMP-9) was further analyzed in LT-PAGE experiments. Ro 28-2653 was not able to prevent the N-terminal proteolysis of the ET_B·GFP receptor neither in the presence nor in the absence of sucrose (Fig. 15C). The results indicate that the N terminus of the ET_B receptor is cleaved by metalloproteases other than gelatinases.

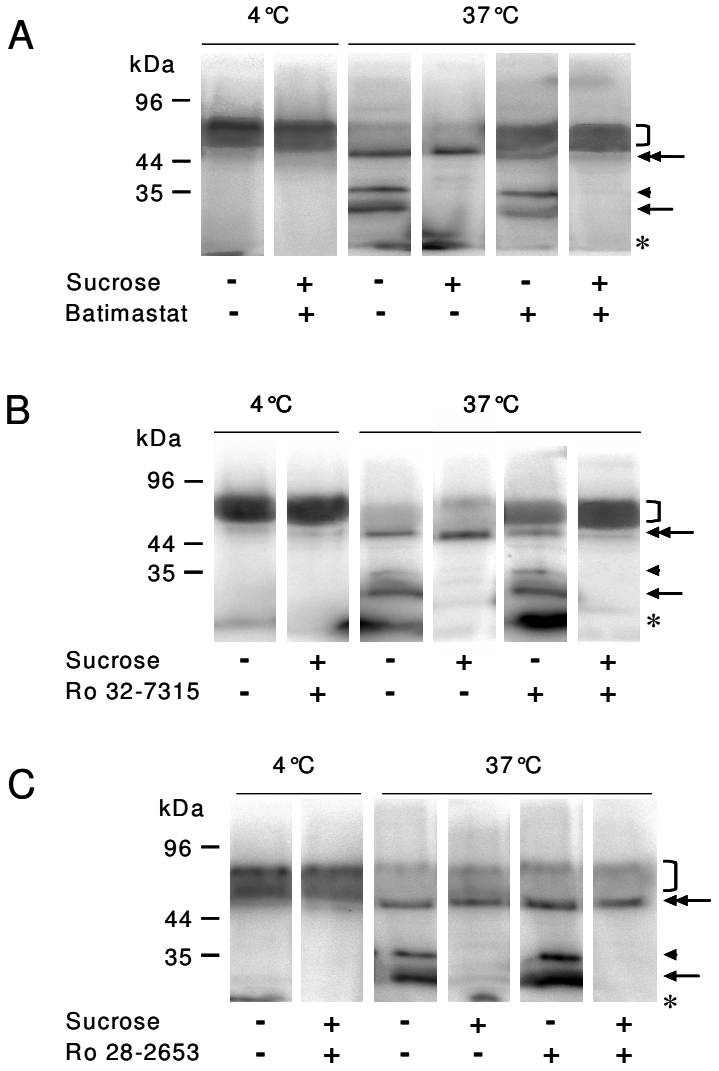

Fig. 15. Inhibitors of metalloproteases prevent N-terminal cleavage of the full-length ET_B·GFP receptor. HEK 293 cells expressing the ET_B·GFP receptor were incubated with ^{125}I-ET-1 for 30 min at 4°C and either lysed immediately or incubated for additional 180 min at 37°C. Lysates were separated by LT-PAGE (see Materials and Methods). The presence of sucrose (inhibitor of clathrin-mediated internalization) or metalloprotease inhibitors (10 µM) is indicated. *Bracket*: full-length receptor; *double arrow*: N-terminally truncated receptor; *arrowhead*, *arrow*: degradation products; *star*: dye front.

3.8 Substitutions of amino acids in the cleavage site do not prevent N-terminal proteolysis

To analyze whether the metalloproteases depend on a specific amino acid motif for the N-terminal proteolysis, two receptors with a mutated cleavage site R64H-S65A and HIS ET_B·GFP (see Fig. 7) were used. The mutant receptors were stably expressed in HEK 293 cells. The cells were then incubated with ^{125}I-ET-1 at 4°C and either lyzed immediately or further incubated at 37°C. The samples were then analyzed in LT-PAGE analysis (Fig. 16).

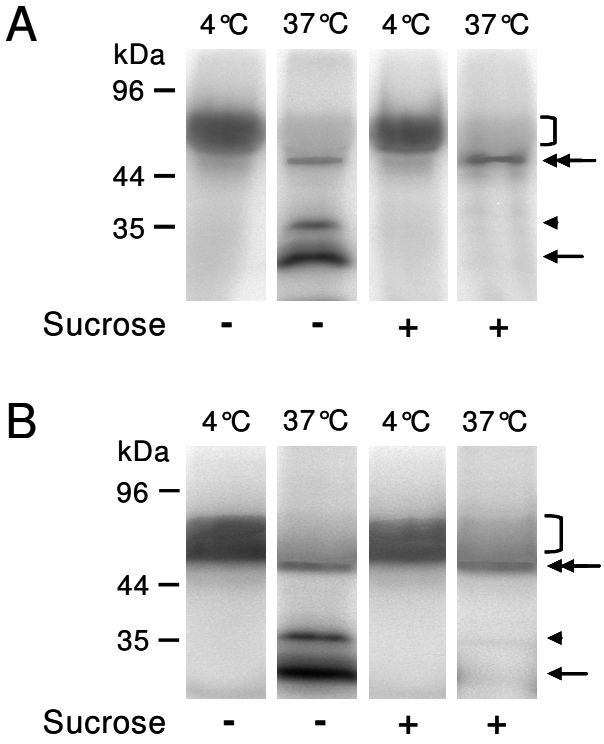

Fig. 16. Mutations of the cleavage site do not prevent N-terminal proteolysis of the full-length ET_B·GFP receptor. HEK 293 cells stably expressing the R64H-S65A (**A**) or the HIS ET_B·GFP receptor (**B**), were incubated with ^{125}I-ET-1 for 30 min at 4°C and for further 180 min at 37°C in the absence or presence of sucrose (inhibitor of clathrin-mediated internalization) and finally lysed. Lysates were separated by LT-PAGE (see Materials and Methods). The two mutants yielded patterns indistinguishable to the wild-type pattern under all experimental conditions. *Bracket*: full-length receptor; *double arrow*: N-terminally truncated receptor; *arrowhead*, *arrow*: degradation products.

Lysates of cells obtained after the incubation period at 4°C, displayed a broad band, migrating at 75-85 kDa, as found for the wild-type ET_B·GFP receptor (see Fig. 9 and Fig.

16). Lysates which were obtained after an additional incubation at 37°C for 180 min with ^{125}I-ET-1 in the presence or absence of sucrose showed an identical pattern of N- and C-terminal proteolysis as found for the wild-type ET_B·GFP receptor. The data demonstrate that modification of the amino acid sequence within the extracellular N terminus is insufficient to prevent N-terminal cleavage.

3.9 The N-terminally truncated Δ2-64 ET_B receptor shows a dramatically reduced cell surface expression when compared to the wild-type

To prove whether N-terminal cleavage alters the affinity of the remaining ET_B receptor for ET-1, saturation binding experiments were performed. Intact cells or membrane preparations derived from cells expressing the full-length or the Δ2-64 ET_B·GFP receptor mutant were incubated with the radioligand ^{125}I-ET-1 in the absence or presence of unlabeled ET-1 (10 µM) for 2 h and total and non-specifically bound radioactivity was determined. K_D and B_{max} values were calculated with the help of the Prism 3.0 Software. The K_D values for the full-length and the Δ2-64 ET_B·GFP receptor were almost identical. In contrast, the B_{max} values obtained for the Δ2-64 ET_B were about 15-fold lower than those for the full-length ET_B·GFP receptor (Tab. 2). The HIS ET_B·GFP mutant showed similar expression levels as the full-length receptor.

In displacement experiments with membrane preparations of the wild-type and Δ2-64 mutant ET_B receptor a variety of different ligands were tested. As competing ligands the natural agonists ET-1, endothelin-3 (ET-3) and sarafotoxin 6c (Sfx6c), the synthetic ET_B agonist IRL1620, the ET_A/ ET_B receptor antagonist PD 145065 and the ET_B receptor selective antagonists IRL1038 and RES-701-1 were tested (Tab. 2). The wild-type and mutant ET_B receptor Δ2-64 revealed almost identical K_i values for all of the ligands studied with the exception of IRL1620. The Δ2-64 ET_B receptor revealed significantly lower K_i values for IRL1620 than the wild-type receptor (Tab. 2). For the ET_B receptor-selective antagonists IRL1038 and RES-701-1, however, no displacement of the radioligand was found (Tab. 2). The data suggest that the N-terminal proteolysis does not alter the ligand

binding properties with the exception of an increased affinity of the Δ2-64 ET_B receptor for IRL1620.

	K_d [pM]	B_{max} [fmol/mg prot.]
ET_B/GFP	16 ± 9	3500 ± 326
Δ2-64 ET_B/GFP	10 ± 5	242 ± 140

K_i [pM]		
	ET_B/GFP	Δ2-64/GFP
ET1	44 ± 33	52 ± 3
ET3	41 ± 31	43 ± 21
Sf6xc	527 ± 38	328 ± 40
IRL1620	425 ± 35	107 ± 55
PD145065	5200 ± 424	4950 ± 71
IRL1038	>10000	>10000
RES-701-1	>10000	>10000

Tab. 2. Synopsis of K_D, B_{max} and K_i values of the full-length ET_B·GFP and the Δ2-64 ET_B·GFP receptors. Top: Saturation binding experiments with membranes of transiently transfected HEK 293 cells were performed as described in Materials and Methods. K_D and B_{max} values were calculated from specific binding isotherms. Values are means ± SD of three independent experiments each performed in duplicates. Bottom: Displacement binding experiments with membranes of stably transfected HEK 293 cells were performed as described in Materials and Methods. Membranes were incubated for 2 h with ^{125}I-ET-1 (50 pM) and increasing concentrations of unlabeled ligands. Values are means ± SD of three independent experiments each performed in duplicates.

3.10 The Δ2-64 ET_B receptor retains its ability to stimulate inositol phosphate formation and to inhibit forskolin-induced cAMP formation

The N-terminal proteolysis of the ET_B receptor yields a receptor with preserved binding-properties. However, N-terminal proteolysis could lead to a change in the activated G proteins. Alternatively, the N-terminal cleavage may be involved in receptor activation either by the generation of a tethered ligand similar to the protease activated receptors or by the release of the N-terminal peptide consisting of 38 amino acids. It has been reported that the ET_B receptor stimulates the formation of inositol phosphates *via* G proteins of the $G_{q/11}$ family (Cramer et al., 2001) and inhibits formation of cAMP *via* G_i proteins (Eguchi et al., 1993b). Therefore the ability of the full-length and the N-terminally truncated receptor to stimulate inositol phosphate formation or inhibit forskolin-induced cAMP production were studied. HEK293 cell clones expressing the full-length or the Δ2-64 ET_B receptor displayed almost identical basal values of inositol phosphates indicating that no significant constitutive activity of the Δ2-64 ET_B receptor exists. Upon stimulation with ET-1 the wild-type ET_B receptor was found to increase inositol phosphate formation to a much higher extent than the Δ2-64 mutant receptor (6-fold in the wild-type, 2-fold in the mutant; Fig. 17A). This difference, however, could result from the reduced cell surface expression of the Δ2-64 mutant receptor. In saturation binding analysis we could demonstrate that the Δ2-64 mutant receptor is expressed at much lower levels than the wild-type ET_B receptor (see Tab. 2 and 3.9.). To achieve similar numbers of cell surface receptors for the wild-type and the Δ2-64 mutant receptor, experiments with transiently transfected HEK 293 cells were performed. As the expression of the Δ2-64 ET_B receptor could not be increased by varying concentrations of plasmid DNA or by altering the plasmid/lipofectamine ratio, the amount of plasmid DNA encoding the wild-type receptor was reduced. The plasmid amount used for the wild-type receptor was lowered to $1/8^{th}$ of that used for the Δ2-64 mutant receptor. By this approach comparable receptor numbers at the cell surface were achieved (as determined in binding analysis). Under these conditions both receptors showed an almost identical potency and efficacy to stimulate inositol phosphate formation (EC_{50} and E_{max} values were 0.2 nM and 2-fold for the full-length ET_B receptor and 0.3 nM and 2-fold for the Δ2-64 ET_B receptor mutant).

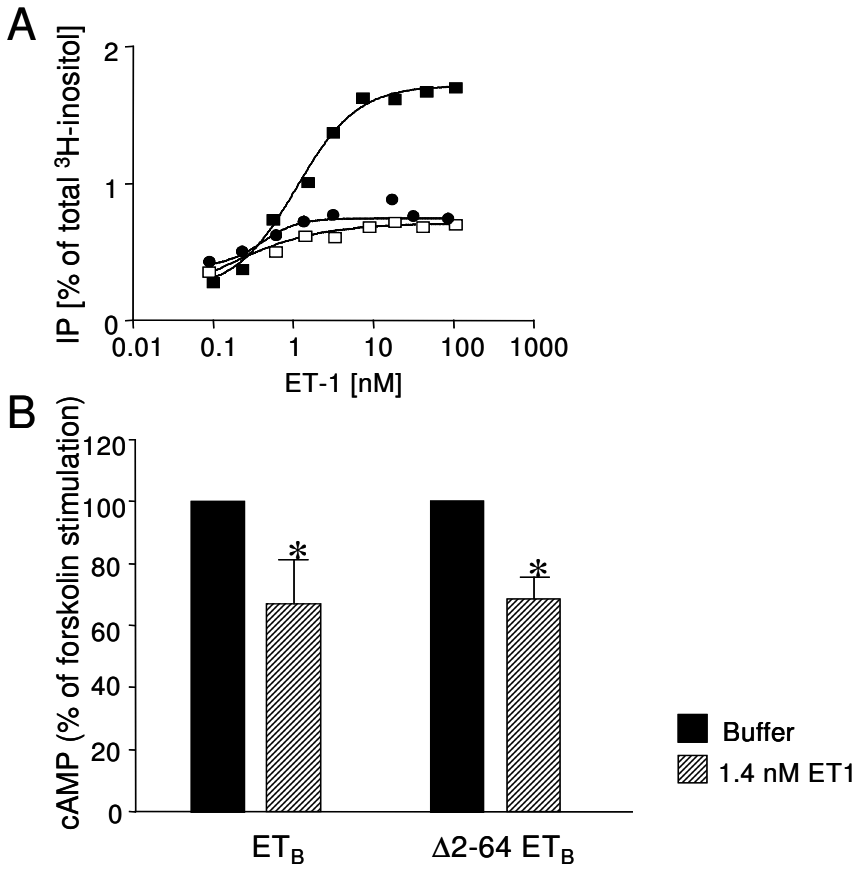

Fig. 17. Functional properties of the full-length ET_B·GFP and Δ2-64 ET_B·GFP receptors. A: HEK 293 cells were transiently transfected with plasmid DNA encoding the full-length or the Δ2-64 ET_B·GFP fusion protein. In standard transfection experiments the full-length ET_B·GFP receptor revealed about 15-fold higher expression levels than the Δ2-64 mutant (see Tab. 2). To achieve similar numbers of cell surface receptors for full-length ET_B·GFP receptor and the Δ2-64 ET_B·GFP receptor, we transfected less plasmid DNA encoding the full-length ET_B·GFP receptor (1/8 of the standard DNA amount). After labeling with [^{3}H]myo-inositol, cells were incubated with increasing concentrations of ET-1 for 30 min in the presence of 10 mM LiCl. Formed [^{3}H]inositol phosphates were determined as described in Experimental Procedures. Values are means of duplicates which differed by less than 5%. The curves are representative for three independent experiments. Solid squares: full-length ET_B·GFP fusion protein (250 ng DNA/well), open squares: full-length ET_B·GFP receptor (31.25 ng DNA/well), solid circles: Δ2-64 ET_B·GFP receptor (250 ng DNA/well) B: HEK 293 cells stably expressing the wild-type or the Δ2-64 mutant ET_B·GFP receptor were incubated with 10 µM forskolin in the absence or presence of 1.4 nM ET-1 for 30 min at 37°C. Cells were lysed, and cAMP was determined by RIA as described in Materials and Methods. Values are means ± SD of 4 independent experiments each performed in duplicates. *: $p < 0.05$ by ANOVA and Dunnett's test.

The data show that the N-terminally truncated receptor (Δ2-64 ET_B·GFP) has the ability to stimulate the formation of inositol phosphates similar to that of the wild-type receptor and that its signaling *via* G proteins of the $G_{q/11}$ family is not altered.
In cAMP analysis similar results were obtained. Upon stimulation with ET-1 both the full-length and the Δ2-64 ET_B receptor inhibited the forskolin induced formation of cAMP by about 30 % (Fig. 17B). Thus, the full-length and the Δ2-64 ET_B·GFP receptor do not differ in their ability to activate G proteins of the G_i family.

3.11 Wild-type and N-terminally truncated ET_B receptors show differences in ERK1/2 activation

We further analyzed the full-length ET_B·GFP receptor and an N-terminally cleaved receptor for their ability to mediate ERK1/2 activation. For this purpose, HEK 293 cells stably expressing the full-length ET_B·GFP receptor or the truncated Δ2-64 ET_B·GFP receptor mutant were serum-starved for 12 h and subsequently stimulated with ET-1 for up to 3 h. Cells were then lyzed directly and lysates were subjected to SDS-PAGE. Immunoblotting analysis with phosphospecific antibodies (for details see "Materials and Methods") was performed to detect the phosphorylated and non-phosphorylated form of ERK1/2. Upon ET-1 stimulation the ET_B·GFP receptor stably expressed in HEK 293 cells a biphasic ERK1/2 activation was found (Fig. 18A), while Δ2-64 ET_B·GFP showed only monophasic and transient increase in ERK1/2 phosphorylation (Fig. 18B). For both receptors the early phase of ERK1/2 activation reached a peak at 5-10 min and slowly declined thereafter toward basal levels at 30-60 min of treatment. Only the stimulation of the full-length ET_B·GFP receptor induced additionally a delayed second phase of ERK1/2 phosphorylation. The second phase was observed after 80 min of agonist application and persisted for up to 1 h (Fig. 18A). A similar biphasic ERK1/2 formation was also observed in VSMCs. In VSMCs nucleofected with ET_B·GFP (Fig. 18B) or Δ2-64 ET_B·GFP, the kinetics of ERK1/2 phosphorylation had the same pattern as those in HEK 293 cells. Because VSMCs express endogenous ET_A receptor together with ET_B, IRL 1620 (a selective ET_B receptor agonist) was used instead of ET-1 to avoid ET_A receptor activation.

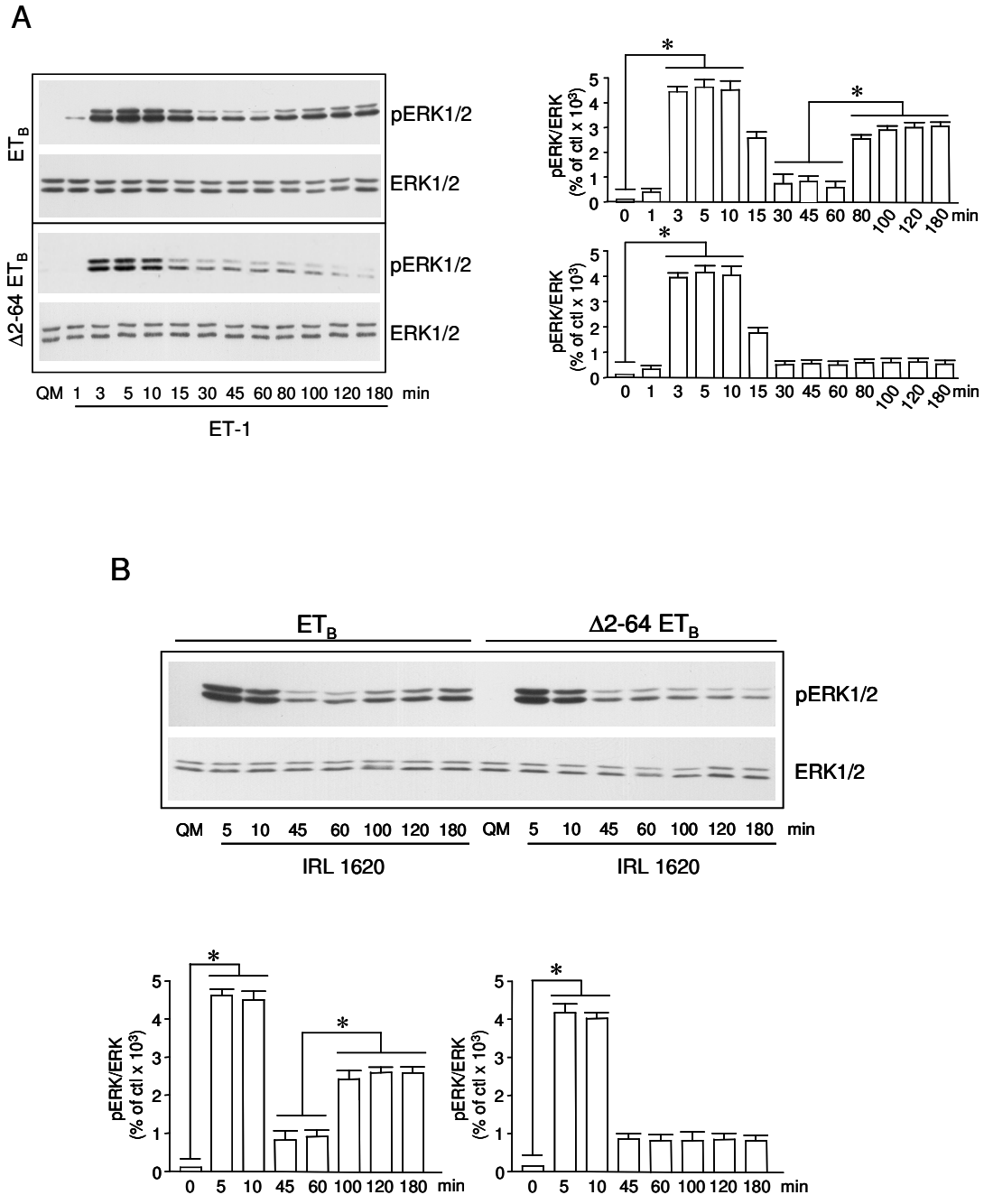

Fig. 18. The extracellular N terminus of the ET_B receptor is required for the biphasic ERK1/2 activation. HEK 293 cell clones (A) or nucleofected VSMCs (B,) expressing either the full-length ET_B·GFP or the Δ2-64 ET_B·GFP receptor were analyzed with regard to ET-1 (HEK 293 cells) or IRL1620-induced (VSMCs; both at 100 nM) activation of ERK1/2. Lysates were then subjected to immunoblotting using pERK1/2 and ERK1/2 antibodies. Graphs summarize the results of 5 to 10 different experiments. *QM*, quiescent medium, $p<0.05$.

3.12 The second phase of ERK1/2 activation depends on βγ subunits released from G_i proteins

For the ET_B receptor it has been demonstrated that the ERK1/2 activation (up to 40 min) depends on G proteins of the $G_{q/11}$ family (Arai et al., 2003; Cramer et al., 2001). However, it is not known whether $G_{q/11}$ proteins also mediate the second phase of ERK1/2 activation. To address this question, serum-starved HEK 293 cells stably expressing the full-length ET_B·GFP receptor were pretreated with pertussis-toxin (PTX, 200 ng/ml for 18 h) prior to stimulation with ET-1. PTX is known to block interactions between receptor and G_i proteins by ADP-rybosylating the C terminus of $G_{i\alpha}$ which leads to inactivation. Cells were then lyzed and probes were analyzed by immunoblotting. As seen from Fig. 19A, treatment of cells with PTX completely inhibited the second phase of ERK1/2 phosphorylation, while the first phase was not affected. Similar results were obtained when HEK293 cells were transiently co-transfected with plasmids encoding the ET_B·GFP receptor and the C-terminal fragment of the β-adrenergic receptor kinase 1 (βARK1-CT), which sequesters free βγ subunit of G_i proteins (Fig. 19B). These data suggest that the full-length ET_B receptor stimulates the early and the late phase of ERK1/2 activation *via* two different signaling cascades, e.g. a PTX-insensitive and a PTX-sensitive signaling cascade, respectively.

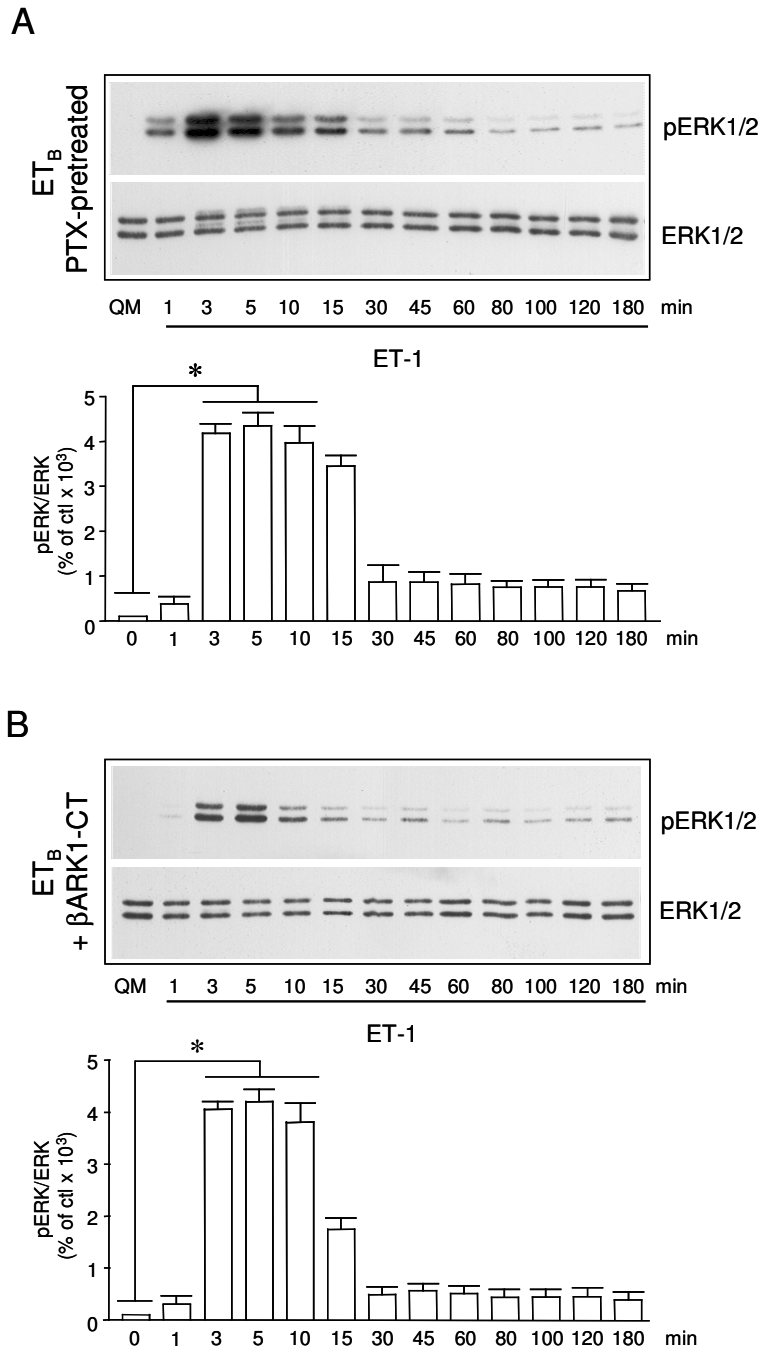

Fig. 19. βγ subunits of G_i proteins are required for the late phase of ET-1-induced ERK1/2 phosphorylation. A, HEK 293 cell clones expressing ET_B·GFP receptors were pretreated with PTX (200 ng/ml for 18 h) and then stimulated with 100 nM ET-1. Lysates of cells were immunoblotted and probed with pERK1/2 and ERK1/2 antibodies. **B**, Transiently transfected HEK293 cells, co-expressing the full-length ET_B·GFP fusion protein and the C-terminal fragment of the β-adrenergic receptor kinase 1 (βARK1-CT) were analyzed for ET-1-mediated ERK1/2 activation in immunoblots. Graphs summarize the results of at least 3 different experiments. *QM*, quiescent medium, $p<0.05$.

3.13 Matrix metalloproteases are involved in the second phase of ERK1/2 activation

As seen from Fig. 15A and B, batimastat and Ro32-7315 block the agonist-induced N-terminal shedding of the full-length ET_B·GFP receptor. To test whether these inhibitors also abolish the late phase of ERK1/2 activation, HEK 293 ET_B·GFP cell clones were treated with batimastat and Ro32-7315 for 30 min prior to agonist application. The selective MMP-2 and MMP-9-inhibitor Ro28-2653, which does not inhibit the agonist-induced N-terminal shedding of the ET_B receptor, was included as a control. As shown in Fig. 20, both batimastat and Ro32-7315 abolished the second phase of ERK1/2 activation. However, the MMP-2 / -9-selective inhibitor Ro28-2653 also abolished the late phase, pointing to a role of MMP-2 / -9 in ERK1/2 activation. Thus, matrix metalloproteases are not only required for the N-terminal cleavage of the ET_B receptor, but mediate also signaling steps involved in ERK1/2 activation.

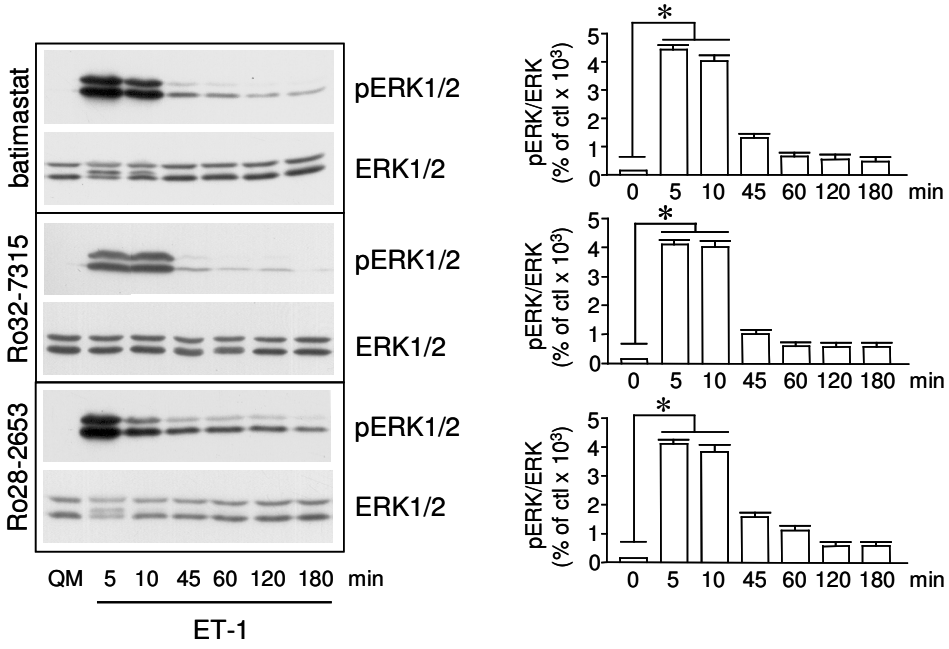

Fig. 20. The second phase of the ERK1/2 activation is abolished by metalloprotease inhibitors. HEK 293 cell clones expressing ET_B·GFP receptors were treated for 30 min with the metalloprotease inhibitors batimastat, Ro32-7315 or Ro28-2653 (all at 10 µM) prior to stimulation with ET-1 (100 nM) as indicated. Cell lysates were immunoblotted and probed with pERK1/2 and ERK1/2 antibodies. Graphs summarize the results of at least 3 different experiments.

3.14 N–linked glycosylation is essential for the late phase of ERK1/2 activation

To elucidate, whether the shedded N-terminal peptide functions as a soluble ligand or co-activator in ET-1-induced ERK1/2 activation, HEK 293 cell clones expressing the Δ2-64 ET_B·GFP receptor were incubated with a synthetic peptide (NT26–64, 100 µM; Fig. 21A).

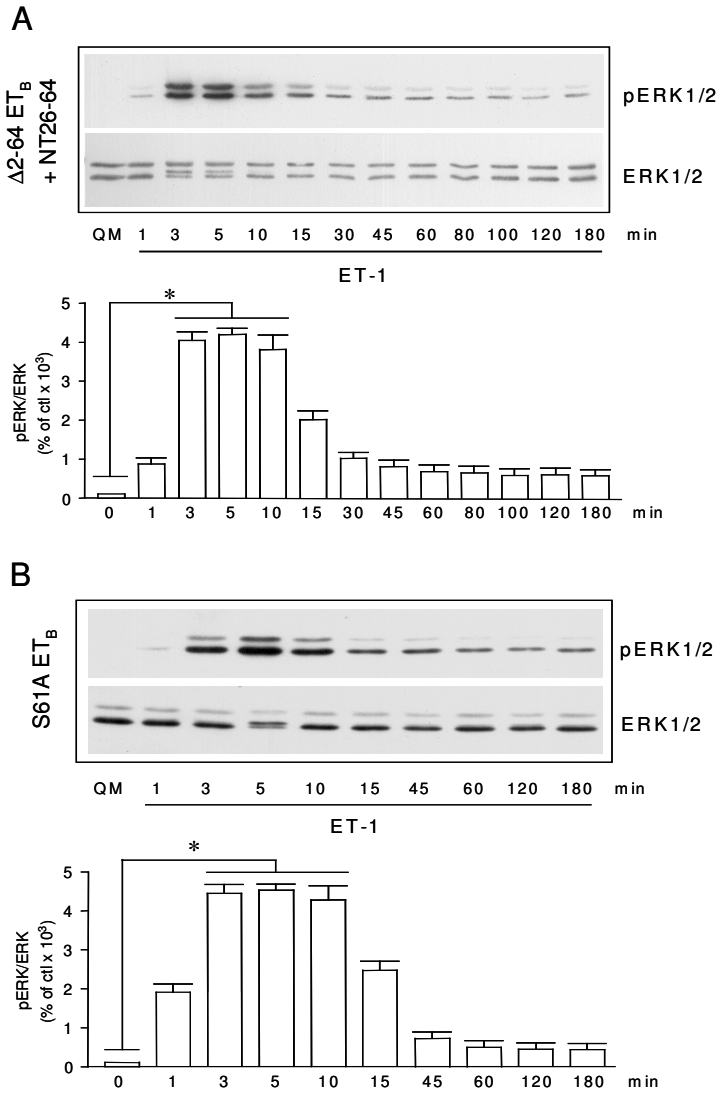

Fig. 21. The glycosylation-deficient ET_B receptor mediates only a monophasic ERK1/2 activation. A, HEK 293 cell clones expressing the Δ2-64 ET_B·GFP receptor were incubated with a synthetic, non-glycosylated N-terminal peptide (NT26–64, 100 µM) for 30 min prior to the stimulation of cells with ET-1 (100 nM) as indicated. **B**, Transiently transfected HEK 293 cells expressing the glycosylation-deficient S61A ET_B·GFP receptor were stimulated with ET-1 (100 nM) as indicated. Lysates of cells (**A**, **B**) were immunoblotted and probed with pERK1/2 and ERK1/2 antibodies. Graphs summarize the results of at least 3 different experiments. *QM*, quiescent medium, $p<0.05$

This peptide corresponds to the shedded N-terminal fragment of the ET_B receptor, with the exception that it lacks an N-linked glycosylation at asparagine 59. However, the peptide did not restore the late phase of ERK1/2 activation (Fig. 21A). To elucidate the role of the N-linked glycan, a control experiment with HEK 293 cells expressing a glycosylation-deficient S61A ET_B·GFP receptor (this mutant also undergoes agonist-dependent N-terminal cleavage, resulting in the release of an unglycosylated N-terminal peptide) was performed. Upon stimulation of cells with ET-1, monophasic ERK1/2 activation was observed (Fig. 21B). The results suggest that not only the presence of the ET_B receptor's N terminus, but also the N-linked glycosylation is necessary for the late phase of ERK1/2 activation.

3.15 The Δ2-64 ET_B receptor shows normal internalization upon ET-1 stimulation

To elucidate whether the Δ2-64 ET_B·GFP receptor has preserved its ability to internalize upon ET-1 stimulation, HEK 293 cells stably expressing the full-length ET_B·GFP or the Δ2-64 ET_B·GFP receptors were compared by using laser scanning microscopy (LSM). LSM of unstimulated cells, revealed that both receptors were expressed at the plasma membrane (Fig. 22, A and D). In the case of the Δ2-64 ET_B·GFP, receptors were also found in a perinuclear region, possibly representing transport intermediates in endosomal compartments or the Golgi apparatus (Fig. 22D). Upon stimulation with a fluorescent ET-1 analogue (Cy3.5-ET-1), both receptors internalized and were sorted into larger endosomal structures. After 35 min, the ligand/receptor complexes remained tightly associated, as ligand and receptor showed the same intracellular distribution (Fig. 22, B and C; E and F). The data demonstrate that ET-1-mediated internalization is well preserved in the Δ2-64 ET_B·GFP receptor.

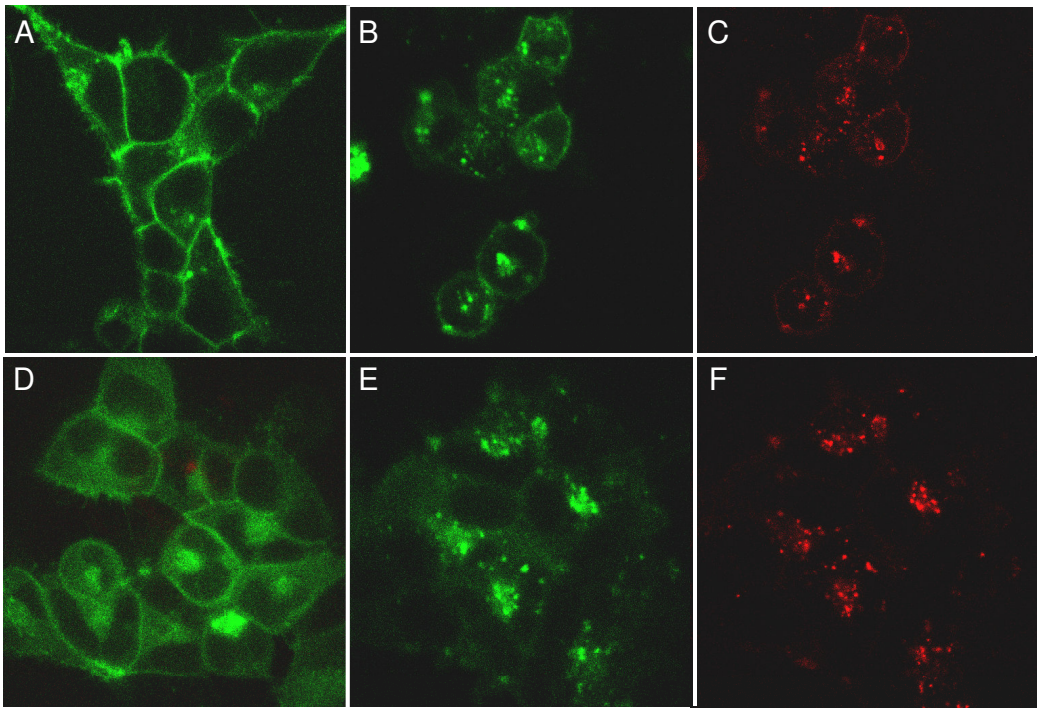

Fig. 22. Internalization of full-length ET_B·GFP and Δ2-64 ET_B·GFP receptors. HEK 293 cells stably expressing the full-length or the Δ2-64 ET_B·GFP receptor were incubated with buffer (A, D) or with Cy3.5-labeled ET-1 (20 nM) for 30 min. In unstimulated cells, the full-length and the Δ2-64 ET_B·GFP fusion protein were mainly observed in the plasma membrane. In case of the Δ2-64 mutant, also more intracellular signals were detected representing transport intermediates in endosomal compartments or in the Golgi apparatus. Upon stimulation with Cy3.5-ET-1 for 35 min, both fusion proteins were mainly found in inside the cell (B, E). Cy3.5-ET-1 (B, D) was found in the same endosomal/lysosomal structures as the two internalized fusion proteins (compare B with C and D with E).