3. Results

3.1 Generation of HEK293 cell clones stably expressing ET_A and ET_B receptors

To investigate the dimerisation of the endothelin receptor subtypes HEK293 cells were stably transfected with plasmids encoding different endothelin receptor fusion proteins (see table 1) to obtain HEK293 cell clones suitable for biophysical (FRET, LSM) and biochemical (western blot, immunoprecipitation) analysis. There are certain advantages of using stable cell lines. The transfection procedure is only performed once. Variations in the expression levels due to changing transfection conditions are eliminated. The following table summarises the features of the designed plasmids encoding different endothelin receptor fusion proteins.

Table 1: ***Synopsis of expression plasmids used for stable and transient transfection of HEK293 cells.***

ET_Amyc.CFP	Encodes an N-terminally c-myc-epitope-tagged ET_A receptor with a CFP moiety at the very C terminus
ET_B.GFP	Encodes an ET_B receptor with a GFP moiety at the very C terminus
ET_B.YFP	Encodes an ET_B receptor with a YFP moiety at the very C terminus
ET_Bflag.YFP	Encodes an N-terminally FLAG-epitope-tagged ET_B receptor with a YFP moiety at the very C terminus
ET_Bflag.GFP	Encodes an N-terminally FLAG-epitope-tagged ET_B receptor with a GFP moiety at the very C terminus

Table 2: ***Synopsis of HEK 293 cell lines expressing different subtypes of endothelin receptor fusion proteins.***

ET_Amyc.CFP	HEK293 cell line stably expressing a c-myc-epitope-tagged ET_A.CFP fusion protein
ET_B.GFP	HEK293 cell line stably expressing a ET_B.GFP fusion protein
ET_B.YFP	HEK293 cell line stably expressing a ET_B.YFP fusion protein
ET_Bflag.GFP	HEK293 cell line stably expressing an ET_B receptor, C-terminally fused to the green fluorescent protein
ET_Bflag.YFP	HEK293 cell line stably expressing a FLAG-epitope-tagged ET_B.YFP fusion protein
ET_Amyc.CFP/ ET_B.YFP	HEK293 cell line stably expressing a c-myc-epitope-tagged ET_A.CFP- and a ET_B.YFP fusion protein.
ET_Amyc.CFP/ ET_Bflag.YFP	HEK293 cell line stably expressing a c-myc-epitope-tagged ET_A.CFP- and a FLAG-epitope-tagged ET_B.YFP fusion protein.

3.2 Functional analyses of fluorescent endothelin receptor fusion proteins

3.2.1 Saturation binding experiments

In our and in other laboratories it was previously shown that ET_A and ET_B receptors with a C-terminal GFP moiety show identical properties as the wild type receptors (Bremnes et al. 2000; Oksche et al. 2000; Grantcharova et al. 2002). Studies in our lab further demonstrated that c-myc- and FLAG-epitope-tagged ET_A and ET_B receptors (which were genetically inserted in the N terminus of the receptors) showed proper transport to the plasma membrane and wild-type affinity for ET-1. Here in this study at least two independently generated cell clones which express ET_Amyc.CFP, ET_B.GFP, ET_B.YFP, ET_Bflag.YFP fusion proteins individually or in combination (ET_Amyc.CFP/ET_B.YFP and ET_Amyc.CFP/ET_Bflag.YFP) were analyzed in saturation binding experiments with ^{125}I-ET-1 as radioligand to determine the binding capacity (B_{max}) and the affinity for ET-1 (K_d).

For the various receptor fusion proteins expressed in the different HEK293 cell clones very similar K_d values were obtained (**Tab. 3**). ^{125}I-PD151242 (ET_A receptor selective) and ^{125}I-ET-3 (ET_B receptor selective) were used as radioligands in saturation binding analyses to define the ratio of ET_A and ET_B receptor expression in ET_Amyc.CFP/ET_B.YFP cell clones. In the cell clones co-expressing ET_A and ET_B receptors, the ratio of ET_A and ET_B receptors varies between 70:30 and 60:40 (**Tab. 3**).

Table 3: ***Synopsis of K_d and B_{max} values of HEK293 cell clones stably expressing ET_Amyc.CFP,ET_B.YFP or ET_Amyc.CFP/ET_B.YFP.***
Saturation binding experiments with membranes of HEK293 cell clones were performed as described in "Experimental Procedures." K_d and B_{max} values were calculated from specific binding isotherms obtained with ^{125}I-ET-1, ^{125}I-ET-3 and ^{125}I-PD151242 used as radioligands. For ET_Amyc.CFP, ET_B.YFP, and ET_Amyc.CFP/ET_B.YFP two independently generated cell clones were tested. Values are means ± S.D. of three independent experiments each performed in duplicate.

HEK293 cell clones	Kd		Bmax	
	^{125}I-ET-1	^{125}I-ET-1	^{125}I-ET-3	^{125}I-PD-151242
	pM	pmol/mg protein		
ET_Amyc.CFP	26.7 ± 4	5.6 ± 0.4	ND	ND
	31.0 ± 3.9	9.0 ± 1.0	ND	ND
ET_B.YFP	10.5 ± 0.9	6.2 ± 1.6	ND	ND
	12.3 ± 2.0	7.2 ± 1.1	ND	ND
ET_Amyc.CFP/	28.0 ± 19.5	13.7 ± 0.7	4.2 ± 0.3	10 ± 2.4
ET_B.YFP	29.7 ± 7.8	15.2 ± 6.4	6.5 ± 0.6	11 ± 2.2

3.2.2 LSM analysis of HEK293 cell lines expressing fluorescent endothelin receptor fusion proteins

With the use of fluorescent proteins, such as GFP, YFP and CFP it is possible to investigate in the living cell whether a protein is localised in the cell's interior or in the plasma membrane. In this work LSM was used to determine the localisation of the endothelin receptor fusion proteins in the stably expressing HEK293 cell lines. Figure 6 shows a representative LSM image of a HEK293 cell clone expressing ET_Amyc.CFP receptors. The ET_Amyc.CFP receptors are mainly localised within the plasma membrane. Very similar results were obtained for HEK293 cell clones expressing ET_B.YFP receptors (figure 7). Both endothelin receptor subtypes were observed in the plasma membrane in HEK293 cell clones co-expressing ET_Amyc.CFP and ET_B.YFP receptors (figure 8). Taken together these results demonstrate that the cell clones expressing ET_Amyc.CFP, ET_B.YFP or co-expressing both receptor subtypes revealed a uniform and predominant expression of both receptor subtypes in the plasma membrane.

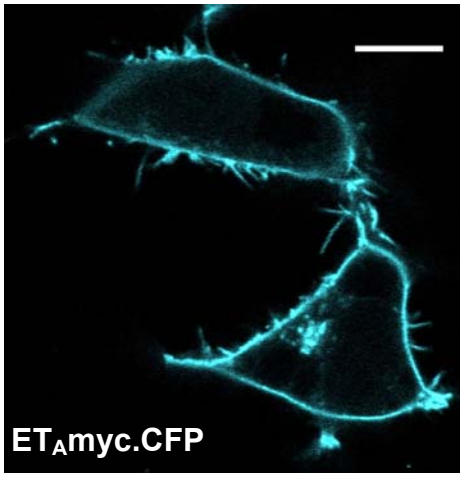

Fig. 6. **Subcellular localisation of ET_Amyc.CFP receptors.** LSM image of HEK293 cells expressing ET_Amyc.CFP. The receptors are mainly localised within the plasma membrane. Visualisation of ET_Amyc.CFP was performed as described in Experimental Procedures. *Bar*, 10 μm.

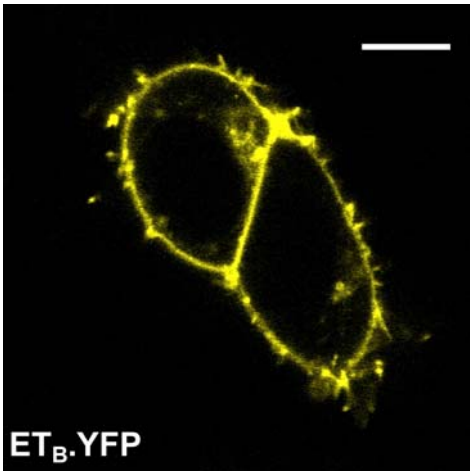

Fig. 7. **Subcellular localisation of ET_B.YFP receptors.** LSM image of HEK293 cells expressing ET_B.YFP. The receptors are mainly localised within the plasma membrane. Visualisation of ET_B.YFP was performed as described in Experimental Procedures. *Bar*, 10 μm.

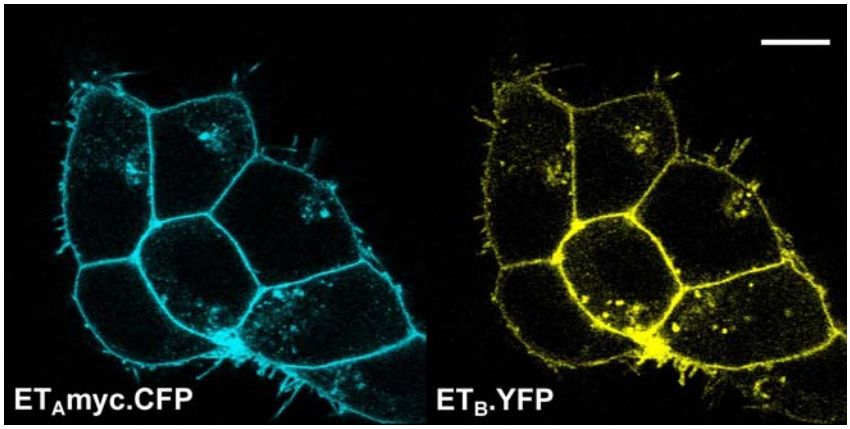

Fig. 8. **Subcellular localisation of ET_Amyc.CFP and ET_B.YFP receptors.** LSM image of HEK293 cells co-expressing ET_Amyc.CFP and ET_B.YFP. The receptors are mainly localised within the plasma membrane. Visualisation of ET_Amyc.CFP and ET_B.YFP was performed as described in Experimental Procedures. *Bar*, 10 μm.

3.2.3 Characterisation of polyclonal ET_B and ET_A receptor antibodies

Polyclonal antisera directed against the N- and C-terminal regions of the endothelin receptors were developed for Western blot analysis and co-immunoprecipitation studies. The following table summarises the generated antibodies and their antigens.

Table 4: ***Synopsis of endothelin receptor antibodies.*** Polyclonal sera were raised against synthetic peptides (right column) coupled to the carrier protein keyhole limpet hemocyanin (Callbiochem-Novabiochem).

Antibody	Peptide
anti-NT-ET_A	DNPERYSTNLSNHVDDFTTF
anti-CT-ET_A	SIQWKNHDQNNHNTDRSSHKDSMN
anti-NT-ET_B	CGLSRIWGEERGFPPDRATP
anti-CT-ET_B	CLKFKANDHGYDNFRSSNKYSSS

Anti-NT-ET_A:

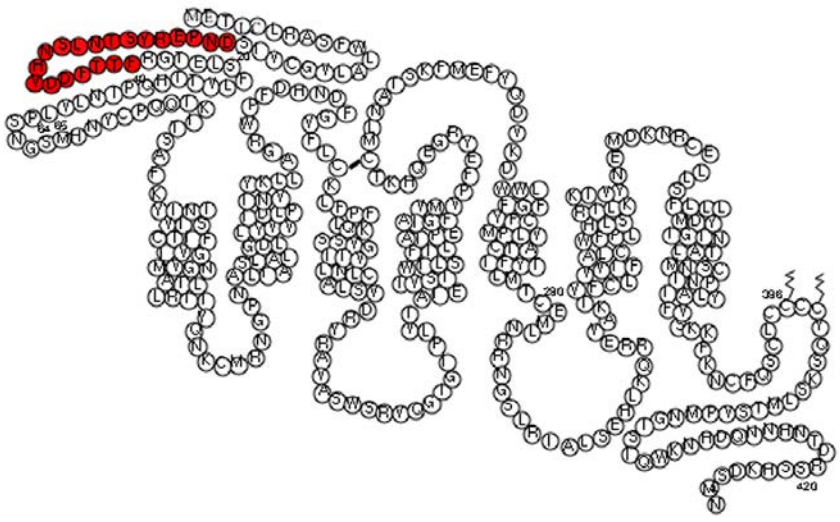

Fig. 9. **Model of the ET_A receptor.** A polyclonal anti-NT-ET_A serum was raised against a conjugate consisting of a synthetic peptide (red circles corresponding to amino acids 21-40 in the N terminus of the ET_A receptor) and the carrier protein keyhole limpet hemocyanin (Calbiochem-Novabiochem).

The anti-NT-ET_A antibody was directed against the N-terminus of the ET_A receptor as illustrated in figure 9. Efforts to detect the ET_A receptor using this antibody in western blot experiments failed. No reproducible results could be obtained (data not shown).

Anti-CT-ET_A:

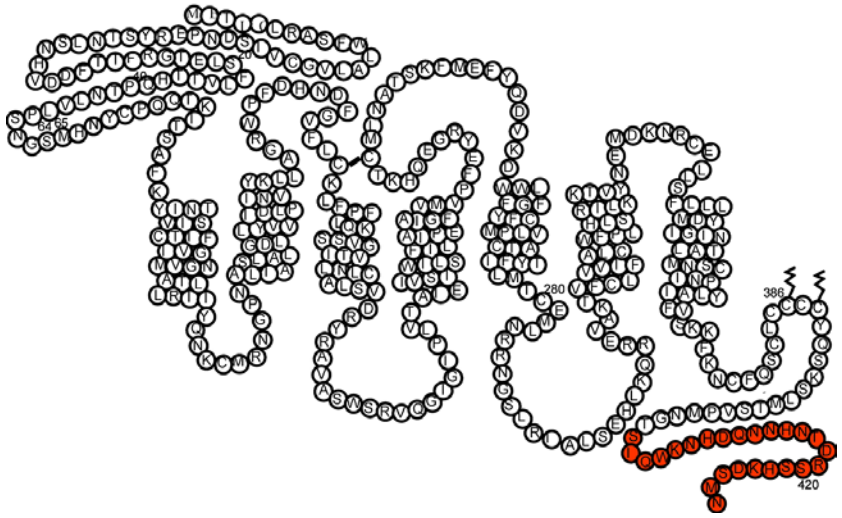

Fig. 10. **Model of the ET_A receptor.** A polyclonal anti-CT-ET_A serum was raised against a conjugate consisting of a synthetic peptide (red circles corresponding to amino acids 404-427 in the C terminus of the ET_A receptor) and the carrier protein keyhole limpet hemocyanin (Calbiochem-Novabiochem).

The anti-CT-ET_A antibody was directed against the C terminus of the ET_A receptor as illustrated in figure 10. After affinity purification of the antibody cell lysates of HEK293 cell clones expressing ET_Amyc.CFP receptors were analysed in Western blot experiments. A prominent band at about 87 kDa corresponding to the ET_Amyc.CFP could be detected (Figure 11, *lane 2*). As a control for the specificity of the antibody a peptide competition experiment was performed. The anti-CT-ET_A antibody was preincubated with the peptide used for immunisation prior to labelling of the nitrocellulose. In that case the band was not observed (see figure 11, *lane 5*). Furthermore, the band was not detected when lysates of non-transfected HEK293 cells were analysed in Western blot

experiments using the anti-CT-ET_A antibody (*lane 1*). The data demonstrate that the anti-CT-ET_A antibody specifically detects the ET_Amyc.CFP receptor. To determine the glycosylation pattern of the ET_Amyc.CFP receptor lysates of HEK293 ET_Amyc.CFP cell clones were treated with endoH and PNGaseF. EndoH, which removes high manose glycans from immature, core-glycosylated proteins, did not increase the mobility of the 87 kDa band (Figure 11, *lane 3*). Treatment with PNGaseF, which removes *N*-linked glycans from both mature, complex-glycosylated and immature, core-glycosylated proteins, increased the mobility of the band, which now migrated at about 75 kDa (Figure 11, *lane 4*). Take together the data show, that the ET_Amyc.CFP receptor is mainly expressed as a complex-glycosylated protein in HEK293 cell clones.

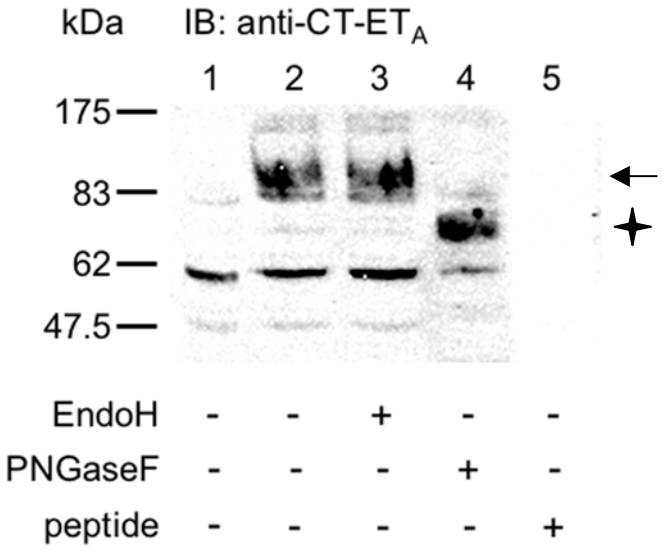

Fig. 11. **Western Blot analysis of the ET_Amyc.CFP receptor.** Lysates (30 µg per lane) from non-transfected (*lane 1*) and from a HEK293 ET_Amyc.CFP/ET_B.YFP cell clone (*lanes 2-5*) were treated with EndoH or PNGaseF as indicated, separated by SDS-PAGE (10% gels) and subjected to Western blotting. The blots were probed with anti-CT-ET_A antibody. The specificity of the antibody was verified by peptide competition with the peptide used for immunization. The antibody was incubated in a 2 µM peptide solution for 30 min at room temperature before probing the filters (*lane 5*). *Arrow*, mature, complex-glycosylated ET_Amyc.CFP receptor; *star*, deglycosylated ET_Amyc.CFP receptor.

Monoclonal c-myc (9E10) antibody:

Lysates of HEK293 cell clones expressing the endothelin A receptor as a myc-tagged fusion protein (ET_Amyc.CFP) were also analysed by Western blotting using the anti-c-myc antibody. The data were consistent with the results obtained for the polyclonal anti-CT-ET_A antibody. The ET_Amyc.CFP receptor migrated at 87 kDa (Fig. 12, *lane 2*), EndoH had no effect on the protein mobility (Fig. 12, *lane 3*), whereas treatment with PNGaseF increased the mobility, resulting in a protein migrating at about 75 kDa (Fig. 12, *lane 4*).

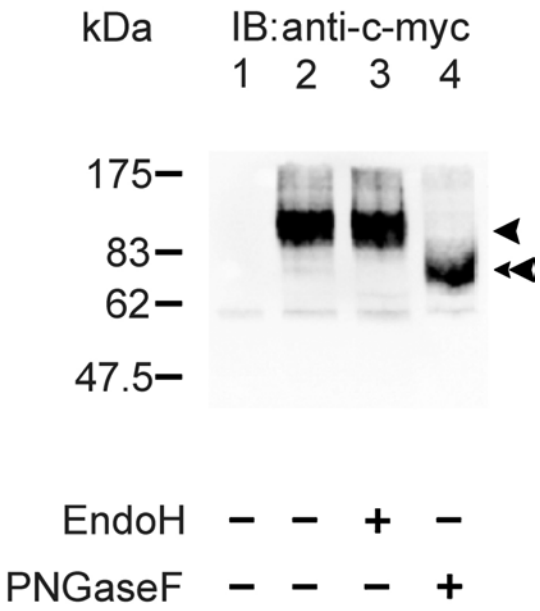

Fig. 12. **Western Blot analysis of the ET_Amyc.CFP receptor.** Lysates (30 µg per lane) from non-transfected (*lane 1*) and from an HEK293 ET_Amyc.CFP/ET_B.YFP cell clone (*lanes 2-4*) were treated with EndoH or PNGaseF as indicated, separated by SDS-PAGE (10% gels) and subjected to Western blotting. The blots were probed with the monoclonal anti-c-myc antibody. *Arrow*, mature, complex glycosylated ET_Amyc.CFP receptor; *double arrowhead*, deglycosylated ET_Amyc.CFP receptor; *IB*, immunoblot.

Anti-NT-ET_B:

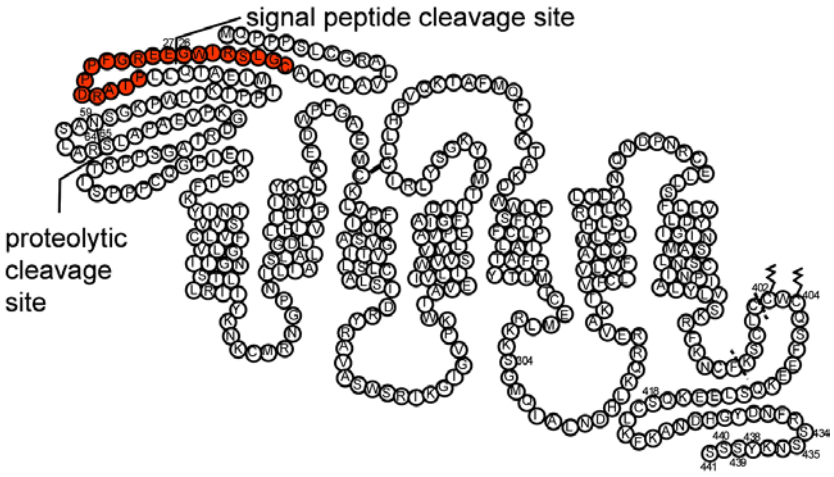

Fig. 13. **Model of the ET_B receptor.** A polyclonal NT-ET_B serum was raised against a conjugate consisting of a synthetic peptide corresponding to amino acids 19-37 (red circles) in the N terminus of the ET_B receptor and the carrier protein keyhole limpet hemocyanin (Calbiochem-Novabiochem).

The NT-ET_B antibody was directed against the N-terminus of the ET_B receptor as illustrated in figure 13. First, lysates of a HEK293 cell clone expressing ET_B.GFP were analysed in Western blot experiments. The Western blot shown in figure 14 was probed with the anti-NT-ET_B antiserum (Fig. 14, *lanes 1* and *2*) and with the affinity-purified anti-NT-ET_B antibody (Fig. 14, *lanes 3* and *4*). Both detected a prominent band at 75 kDa corresponding to the ET_B.GFP receptor. When the antiserum and the affinity-purified anti-NT-ET_B antibody were preincubated with the peptide used for immunisation, the band was not observed (Fig. 14, *lane 2* and *4*). Thus the anti-NT-ET_B antibody specifically detects the ET_B.GFP receptor.

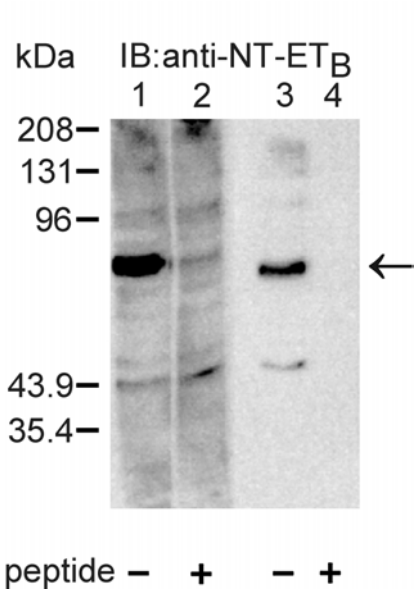

Fig. 14. **Western Blot analysis of the ET_B.GFP receptor.** Lysates (30 μg per lane) from HEK293 ET_B.GFP cell clones were separated by SDS-PAGE (10% gels) and subjected to Western blotting. The blots were probed with the anti-NT-ET_B antiserum (*lane 1* and *2*) or with affinity-purified anti-NT-ET_B antibody (*lane 3* and *4*). The specificity of the antibody was verified by peptide competition with the peptide used for immunization. The antibody was incubated in a 2 μM peptide solution for 30 min at room temperature before probing the filters (*lane 2* and *4*). *Arrow*, ET_B.GFP receptor; *IB*, immunoblot.

The same prominent band migrating at 75 kDa was detected in lysates from HEK293 ET_Amyc.CFP/ET_B.YFP cell clones (Fig. 15, *lane 2*). The glycosylation pattern of the ET_B receptor fusion protein was investigated by treatment of the lysates with EndoH and PNGaseF. No shift in protein mobility was observed when the lysates were treated with EndoH (Fig. 15, *lane 3*), whereas PNGaseF treatment increased the protein mobility, resulting in an deglycosylated ET_B.YFP receptor which migrated at about 67 kDa (Fig. 15, *lane 4*). The results demonstrate that the ET_B receptor is mainly expressed as a complex-glycosylated protein in HEK293 cell clones.

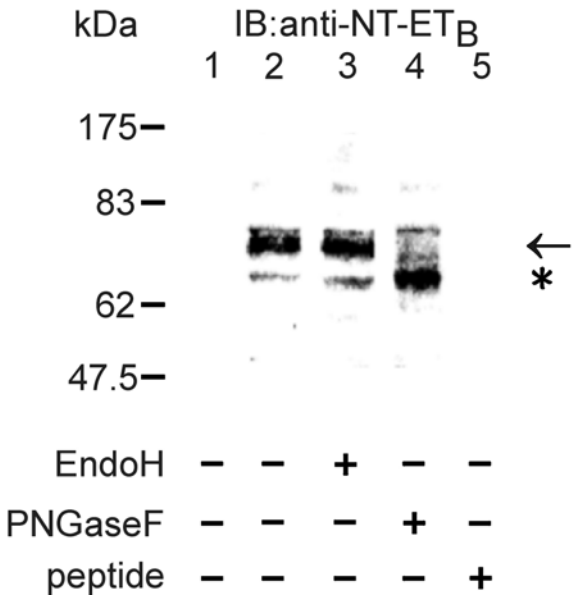

Fig. 15. **Western Blot analysis of the ET_B.YFP receptor.** Lysates (30 μg per lane) from non-transfected (*lane 1*) and from a HEK293 ET_Amyc.CFP/ET_B.YFP cell clone (*lanes 2-5*) were treated with EndoH or PNGaseF if indicated, separated by SDS-PAGE (10% gels) and subjected to western blotting. The blots were probed with the anti-NT-ET_B antibody. The specificity of the antibody was verified by peptide competition with the peptide used for immunization. The antibody was incubated in a 2 μM peptide solution for 30 min at room temperature before probing the filters (*lane 5*). *Arrow*, mature, complex glycosylated ET_B.YFP receptor; *asterisk*, deglycosylated ET_B.YFP receptor; *IB*, immunoblot.

Furthermore it was tested whether the anti-NT-ET_B antibody specifically detects the ET_B receptor in a different cell line. For this purpose Sf9 cells expressing the ET_B.GFP receptor (kindly donated from Dr. Ludwig Krabben, FMP Berlin) were used. Here the antibody detected two prominent bands. One band migrating at 75 kDa corresponding to the glycosylated ET_B.GFP receptor (Fig. 16, *lane 2*) and one band migrating at about 67 kDa corresponding to the immature, non glycosylated ET_B.GFP receptor (Fig. 16, *lane 2*). The antibody failed to detect any

band in non-transfected Sf9 cells (Fig. 16, *lane 1*). Peptide competition resulted in a loss of signal (Fig. 16, *lane 3*). Thus the anti-NT-ET_B antibody specifically detects the ET_B receptor in a Sf9 cell line.

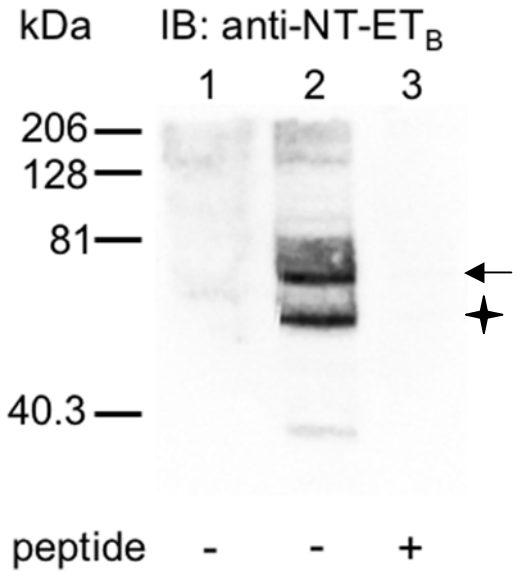

Fig. 16. **Western Blot analysis of the ET_B.GFP receptor expressed in Sf9 cells.** Lysates (30 µg per lane) from Sf9 cells expressing the ET_B.GFP receptor were separated by SDS-PAGE (10% gels) and subjected to Western blotting. The blots were probed with the affinity-purified anti-NT-ET_B antibody (*lane 1*-3). The specificity of the antibody was verified by peptide competition with the peptide used for immunization. The antibody was incubated in a 2 µM peptide solution for 30 min at room temperature before probing the filters (*lane 3*). *Arrow*, complex glycosylated ET_B.GFP receptor; *star*, de- or core glycosylated ET_B.GFP receptor; *IB*, immunoblot.

Anti-CT-ET_B:

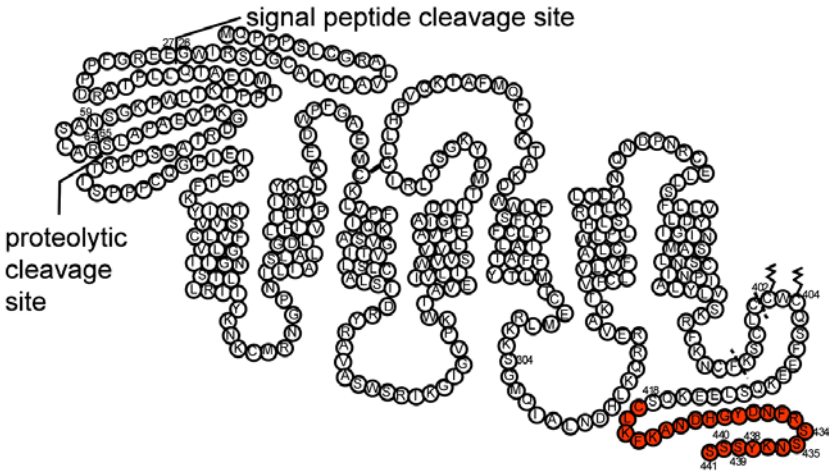

Fig. 17. **Model of the ET_B receptor.** A polyclonal CT-ET_B serum was raised against a conjugate consisting of a synthetic peptide corresponding to amino acids 419-441 (red circles) in the C-terminus of the ET_B receptor and the carrier protein keyhole limpet hemocyanin (Calbiochem-Novabiochem).

The anti-CT-ET_B antibody was directed against the C terminus of the ET_B receptor as illustrated in figure 17. First, lysates of a HEK293 cell clone expressing the ET_B.GFP receptor were analysed in Western blot experiments. The Western blot shown in figure 18 was probed with an anti-CT-ET_B antiserum (Fig. 18, *lanes 2* and *3*) and with the affinity-purified anti-CT-ET_B antibody (Fig. 18, *lanes 4* and *5*). Both detected a prominent band at 75 kDa corresponding to the ET_B.GFP receptor and a further band at 64 kDa. When the antiserum and the affinity-purified anti-CT-ET_B antibody were preincubated with the peptide used for immunisation, the 75 kDa band was not observed (Fig. 18, *lane 3* and *5*), however the 64 kDa band remained, suggesting the presence of non-specific immunoreactivity. Further evidence that the 75 kDa band represents the ET_B.GFP receptor was provided by detection of the receptor

with a polyclonal GFP antibody (GFP 08, Dr. Alexander Oksche, FMP Berlin), which also recognized the 75 kDa band (Fig. 18, *lane 1*). Thus the anti-CT-ET_B antibody specifically detects the ET_B.GFP receptor.

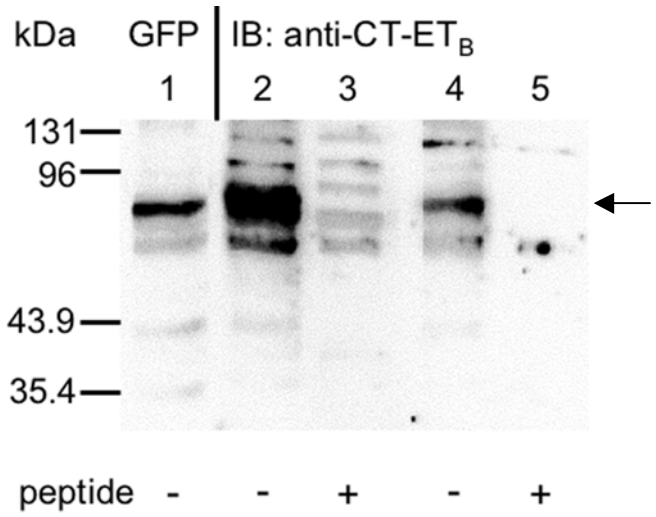

Fig. 18. **Western Blot analysis of the ET_B.GFP receptor.** Lysates (30 μg per lane) from HEK293 ET_B.GFP cell clones were separated by SDS-PAGE (10% gels) and subjected to Western blotting. The blots were probed with GFP 08 (*lane 1*) the anti-CT-ET_B antiserum (*lane 2* and *3*) or with affinity-purified anti-CT-ET_B antibody (*lane 4* and *5*). The specificity of the antibody was verified by peptide competition with the peptide used for immunization. The antibody was incubated in a 2 μM peptide solution for 30 min at room temperature before probing the filters (*lane 2* and *4*). *Arrow*, ET_B.GFP receptor; *IB*, immunoblot.

The same prominent band migrating at 75 kDa was detected in lysates from HEK293 ET_Amyc.CFP/ET_B.YFP cell clones (Fig. 19, *lane 2*). The glycosylation pattern of the ET_B receptor fusion protein was investigated by treatment of the lysates with EndoH and PNGaseF. No shift in protein mobility was observed when the lysates were treated with EndoH (Fig. 19, *lane 2*), whereas PNGaseF treatment increased the protein mobility, resulting in a deglycosylated ET_B.YFP

receptor that migrated at about 67 kDa (Fig. 19, *lane 3*). After peptide competition an unspecific band was still detected (Fig. 19, *lane 4*).

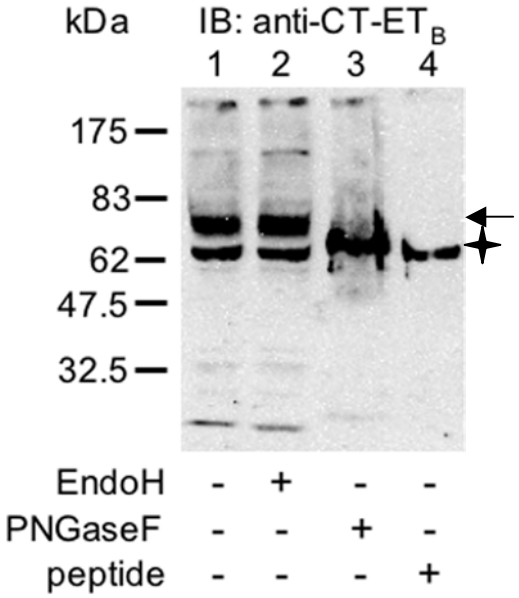

Fig. 19. **Western Blot analysis of the ET$_B$.YFP receptor.** Lysates (30 µg per lane) from a HEK293 ET$_A$myc.CFP/ET$_B$.YFP cell clone (*lanes 1-4*) were treated with EndoH or PNGaseF as indicated, separated by SDS-PAGE (10% gels) and subjected to Western blotting. The blots were probed with the anti-CT-ET$_B$ antibody. The specificity of the antibody was verified by peptide competition with the peptide used for immunization. The antibody was incubated in a 2 µM peptide solution for 30 min at room temperature before probing the filters (*lane 5*). *Arrow*, mature, complex glycosylated ET$_B$.YFP receptor; *star*, deglycosylated ET$_B$.YFP receptor.

Figure 20 shows two Western blots of lysates derived from a HEK 293 cell clone co-expressing ET_Amyc.CFP and ET_B.YFP receptors probed with anti-NT-ET_B and anti-CT-ET_A. Prior to lysis cells were treated for 5 min with ET-1 in order to prove whether the ligand-occupied endothelin A and endothelin B receptors are more resistant to proteolytic degradation after cell disruption. ET-1 binding did not improve the detection of the ET_A and ET_B receptors in Western blot analysis. For the untreated, the EndoH and PNGaseF treated lysates similar results as for the non-ET-1 pre-treated cells were found.

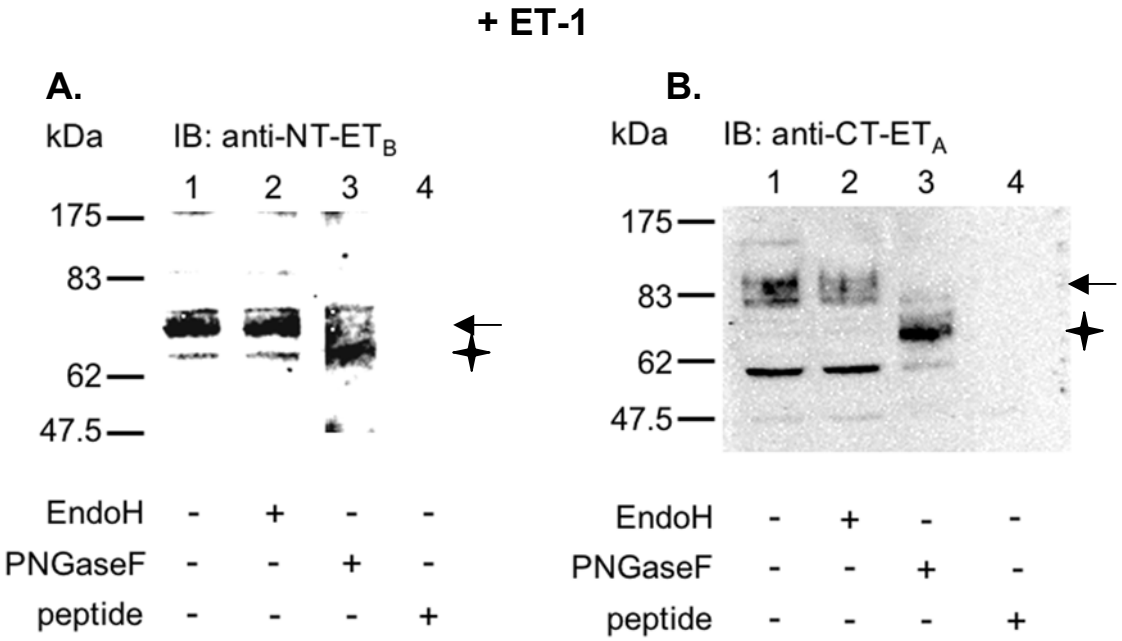

Fig. 20. **Western Blot analysis of the ligand occupied ET_B.YFP and ET_Amyc.CFP receptors.** HEK293 ET_Amyc.CFP/ET_B.YFP cell clones were treated with ET-1 for 5 min prior to preparation of the lysates. Lysates (30 μg per lane, *lanes 1-4* , A and B) were treated with EndoH or PNGaseF as indicated, separated by SDS-PAGE (10% gels) and subjected to Western blotting. The blots were probed with the anti-NT-ET_B (A) or the anti-CT-ET_A antibodies. The specificity of the antibodies was verified by peptide competition with the peptide used for immunization. The antibodies were incubated in a 2 μM peptide solution for 30 min at room temperature before probing the filters (*lane 4*, A and B). A, *Arrow*, mature, complex glycosylated ET_B.YFP receptor; *star*, deglycosylated ET_B.YFP receptor. B, *Arrow*, mature, complex glycosylated ET_Amyc.CFP receptor; *star*, deglycosylated ET_Amyc.CFP receptor.

3.3 Immunoprecipitation

3.3.1 Antibodies for immunoprecipitation studies

After characterisation of the polyclonal endothelin receptor antibodies in Western blot experiments, the antibodies were tested whether they were suitable for immunoprecipitation studies. The ET_B.GFP receptor could only be precipitated with the anti-NT-ET_B antibody (Fig. 22, *lane 3*). Several attempts to precipitate the ET_Amyc.CFP receptor with the polyclonal anti-CT-ET_A or the monoclonal c-myc antibody failed (data not shown). Therefore the immunoprecipitation experiments were performed with the anti-NT-ET_B antibody.

3.3.2 Selection of HEK293 cell clones for immunoprecipitation studies

Different HEK293 cell clones co-expressing ET_Amyc.CFP and ET_B.YFP receptors were analysed for their suitability for immunoprecipitation studies. Therefore, lysates of the various cell clones were separated by SDS-PAGE and analysed by probing with the anti-NT-ET_B and the monoclonal anti-c-myc antibody. Figure 21 shows two Western blots of 11 different cell clones co-expressing ET_Amyc.CFP and ET_B.YFP receptors. The blots were probed with the anti-NT-ET_B (A) or with the monoclonal anti-c-my antibody (B). Clones 2, 6 and 11 were selected for further immunoprecipitation studies because of a strong signal for both the ET_Amyc.CFP and the ET_B.YFP receptor.

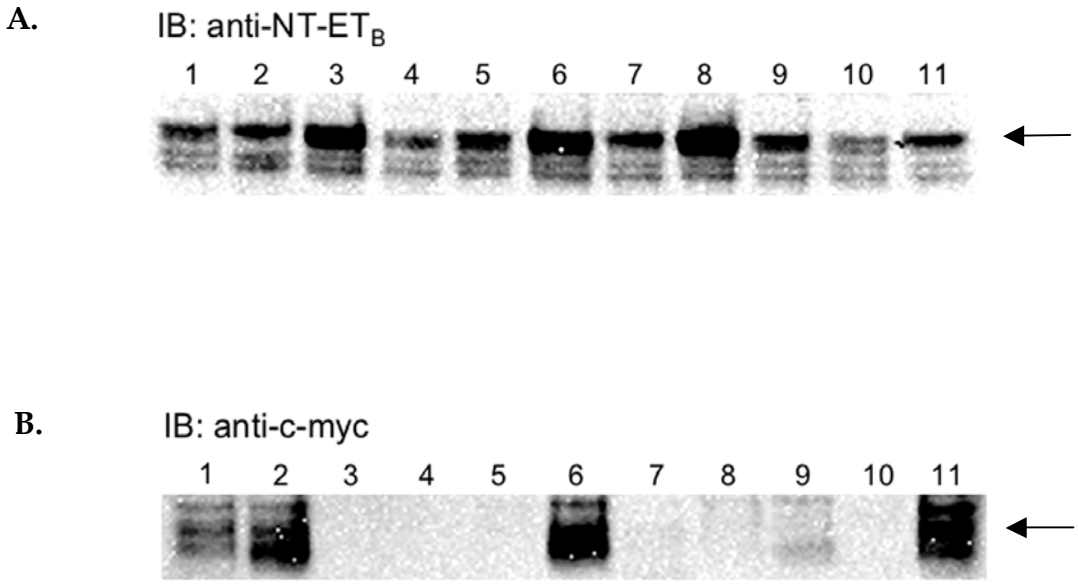

Fig. 21. **Western blot analysis of HEK293 ET$_A$myc.CFP/ET$_B$.YFP cell clones.** Lysates (30 µg per lane) from HEK293 ET$_A$myc.CFP/ET$_B$.GFP cell clones were separated by SDS-PAGE (10% gels) and subjected to Western blotting. A. The blot was probed with affinity-purified anti-NT-ET$_B$ antibody (*lane1*-11). *Arrow*, ET$_B$.YFP receptor; B. The blot was probed with anti-c-myc antibody (*lane1*-11). *Arrow*, ET$_A$myc.CFP. *IB*, immunoblot.

3.3.3 Immunoprecipitation analysis reveals ET$_A$/ET$_B$ heterodimers

Immunoprecipitation studies were performed using detergent extracts of HEK293 cell clones expressing the ET$_B$.GFP or co-expressing the ET$_A$myc.CFP and ET$_B$.YFP receptor. Prior to immunoprecipitation it was tested whether the detergent extracts contain sufficient levels of ET$_A$ and ET$_B$ receptors. In figure 22 a Western blot of detergent extracts of HEK293 cell clones co-expressing ET$_A$myc.CFP and ET$_B$.YFP receptors is shown (Fig. 22, *lane 1* and 2). A prominent band migrating at 75 kDa corresponding to the ET$_B$.YFP receptor

was detected with the anti-NT-ET_B antibody while with the anti-c-myc antibody a 87 kDa was detected representing the ET_Amyc.CFP receptor.

Immunoprecipitation with the anti-NT-ET_B antibody was then tested using detergent extracts of a cell clone expressing only the ET_B.GFP receptor. In the immunoblots with a GFP antibody a 75 kDa band, corresponding to the ET_B.GFP receptor was observed (Fig. 22, *lane 3*). In immunoprecipitation experiments using detergent extracts from HEK293 cell clones which co-express the ET_Amyc.CFP and the ET_B.YFP receptors with the anti-NT-ET_B antibody two bands were detected after probing the immunoblots with a monoclonal anti-GFP antibody: A 75- and a 87 kDa band, corresponding to ET_B.YFP and ET_Amyc.CFP receptors, respectively (Fig. 22, *lane 4*). The presence of the ET_Amyc.CFP receptor in the immunoprecipitate was verified by using a c-myc antibody, which detected a single band at 87 kDa (Fig.22, *lane 5*). Thus co-immunoprecipitation of the ET_Amyc.CFP receptor was observed, when the ET_B.YFP receptor was immunoprecipitated with the anti-NT-ET_B antibody from detergent extracts of HEK293 cell clones co-expressing ET_Amyc.CFP and ET_B.YFP receptors.

When a mixture of detergent extracts derived from HEK293 cell clones individually expressing ET_Amyc.CFP and ET_B.YFP receptors was subjected to immunoprecipitation with the anti-NT-ET_B antibody, no co-immunoprecipitation of the ET_Amyc.CFP receptor was observed. Here, only the ET_B.YFP receptor was detected (Fig. 22, *lane 6*) but not the ET_Amyc.CFP receptor (Fig. 22, *lane 7*). The results demonstrate that heterodimerisation of the endothelin receptor fusion proteins is not due to secondary aggregation of the fusion proteins after cell disruption and preparation of the detergent extracts.

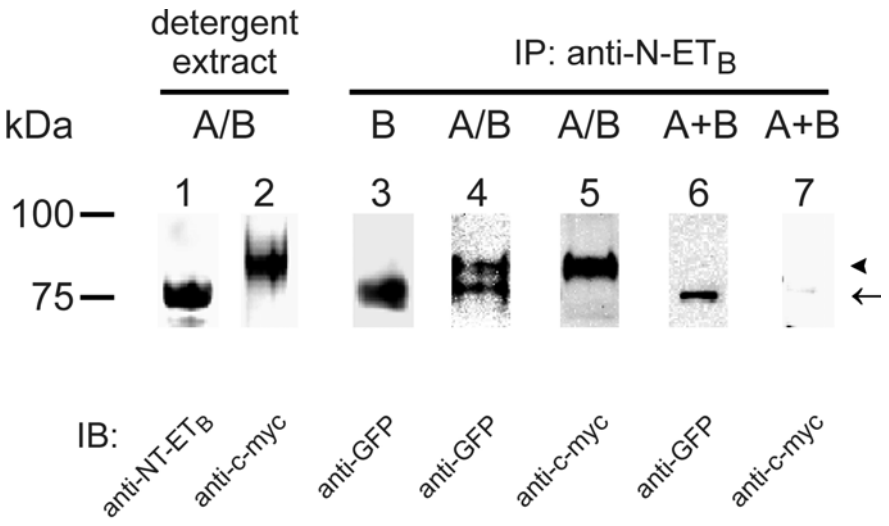

Fig. 22. **Co-immunoprecipitation of endothelin A and endothelin B receptors.** Detergent extracts from ET_B.GFP (*lane 3*), ET_B.YFP (*lane 6* and *7*), ET_Amyc.CFP (*lanes 6* and *7*), ET_Amyc.CFP/ET_B.YFP cell clones (*lane 1*, *2*, *4*, and *5*) were either left untreated (*lanes 1* and *2*) or subjected to immunoprecipitation with the affinity-purified polyclonal anti-NT-ET_B antibody (*lane 3* to *7*). Detergent extracts and immunoprecipitated proteins were analysed by Western blotting, using the indicated antibodies. *Arrowhead*, ET_Amyc.CFP receptor; *arrow*, ET_B.GFP or ET_B.YFP receptor; A/B, ET_Amyc.CFP/ ET_B.YFP cell clone; A+B, mixture of ET_Amyc.CFP and ET_B.GFP cell clones; B, ET_B.GFP cell clone

To exclude that heterodimerisation of ET_Amyc.CFP and ET_B.YFP receptors is mediated by the CFP and YFP moieties, HEK293 cells transiently expressing ET_Amyc and ET_Bflag receptors without CFP/YFP moieties were analysed. Immunoprecipitation of detergent extracts from these cells was performed with the anti-NT-ET_B antibody. Subsequent immunoblotting with FLAG and c-myc antibodies demonstrated the presence of both ET_B (Fig. 23, *lane2*, arrow) and ET_A receptors (Fig. 23, *lane 3*, arrowhead). The c-myc antibody detected an unspecific band, when immunoprecipitation with the anti-NT-ET_B antibody from detergent extracts of non-transfected HEK293 cells was performed (Fig. 23, *lane 1*, asterisk). In summary, the data prove that ET_A and ET_B receptors

form heterodimers and that heterodimerisation is not mediated by the CFP and YFP moieties fused to the C termini of the receptor subtypes.

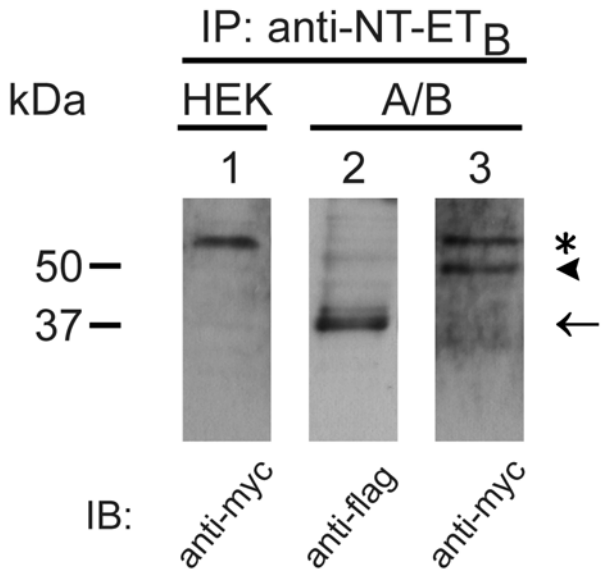

Fig. 23. **Co-immunoprecipitation of ET_Amyc and ET_Bflag receptors.** Immunoprecipitation with the anti-NT-ET_B antibody was performed with detergent extracts from mock-transfected HEK293 cells (*lane 1*) or HEK 293 cells transiently co-transfected with ET_Amyc and ET_Bflag cDNAs (*lanes 2* and *3*). Precipitates were analysed by Western blotting using the indicated antibodies. *Arrow*, ET_Bflag; *arrowhead*, ET_Amyc; *asterisks*, unspecific band; *IB*, immunoblot; *IP*, immunoprecipitation.

3.4 Fluorescence resonance energy transfer

3.4.1 The principle of FRET

FRET is a quantum mechanical process and is widely used as a biophysical tool to study protein-protein interactions. Excitation of a donor fluorophore can give rise to non-radiative transfer of the absorbed energy to an adjacent acceptor fluorophore provided that the emission spectrum of the donor overlaps sufficiently with the excitation spectrum of the acceptor. Direct energy transfer depends on the coupling of the respective dipole moments. Since the extent of energy transfer is proportional to orientation and inversely proportional to the sixth power of the distance ($1/R^6$) between the donor and the acceptor, FRET can be used to deduce the respective positional or rotational contexts of the two molecules. The positional constraints limit efficient FRET to distances of less than 10 nm and therefore sufficiently close to infer interaction. FRET between two fluorophores therefore provides evidence for a direct protein-protein interaction.

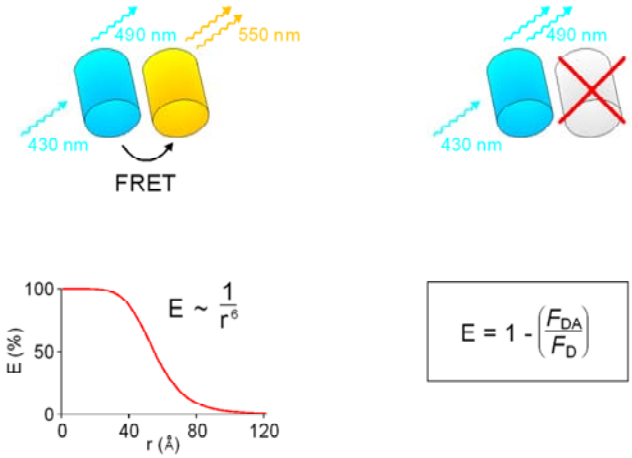

Fig. 24. **The principle of FRET.** FRET between two fluorophores is restricted to distances of less than 10 nm and can be assessed as recovery of the donor (e.g. CFP) fluorescence during acceptor (e.g. YFP) bleach. The FRET efficiency can be expressed according to the above formula, where F_{DA} and F_D are intensities of the donor fluorescence in the presence and absence of the FRET acceptor, respectively.

3.4.2 FRET analysis demonstrate hetero- and homodimerisation of endothelin receptor subtypes in living HEK293 cells

FRET was measured in HEK293 cells transiently co-expressing ET_Amyc.CFP and ET_B.YFP receptors or in HEK293 cells co-expressing ET_Amyc.CFP and ET_Amyc.YFP or ET_B.CFP and ET_B.YFP receptors, respectively. FRET efficiencies of 12-18% were obtained when ET_Amyc.YFP or ET_B.YFP receptors (acceptors) were expressed at a higher level (>1.5) than the ET_Amyc.CFP or ET_B.CFP receptors (donor). The molar ratio of ET_Amyc.CFP or ET_B.CFP receptor to ET_Amyc.YFP or ET_B.YFP receptor was assessed by quantitative analysis of CFP and YFP fluorescence intensities (Lenz et al. 2002). In the stably transfected HEK293 cell clones co-expressing ET_Amyc.CFP and ET_B.YFP receptors, the ET_B.YFP receptor was expressed at a lower level than the ET_Amyc.CFP receptor. Thus, these cell clones were not suitable for the FRET experiments. Therefore, FRET experiments were performed with transiently transfected HEK293 cells, in which an appropriate ratio of acceptor and donor expression (>1.5) was achieved when plasmids encoding ET_Amyc.CFP or ET_B.CFP and ET_Amyc.YFP or ET_B.YFP receptors were transfected in a 1 : 1.5 ratio. Figures 25-27 show three representative FRET experiments of HEK293 cells transiently co-expressing ET_Amyc.CFP and ET_B.YFP receptors (Fig. 25), ET_Amyc.CFP and ET_Amyc.YFP (Fig. 26) or ET_B.CFP and ET_B.YFP (Fig. 27) receptors, with a steep decrease in the YFP emission during selective photobleaching of YFP **(a)** and a coincident increase in CFP emission **(b)**. FRET efficiency **(c)** was determined by plotting CFP fluorescence during acceptor bleach against the remaining YFP fluorescence according to the formula described under *Experimental Procedures*. Consistently FRET efficiencies between 12 and 18% were obtained.

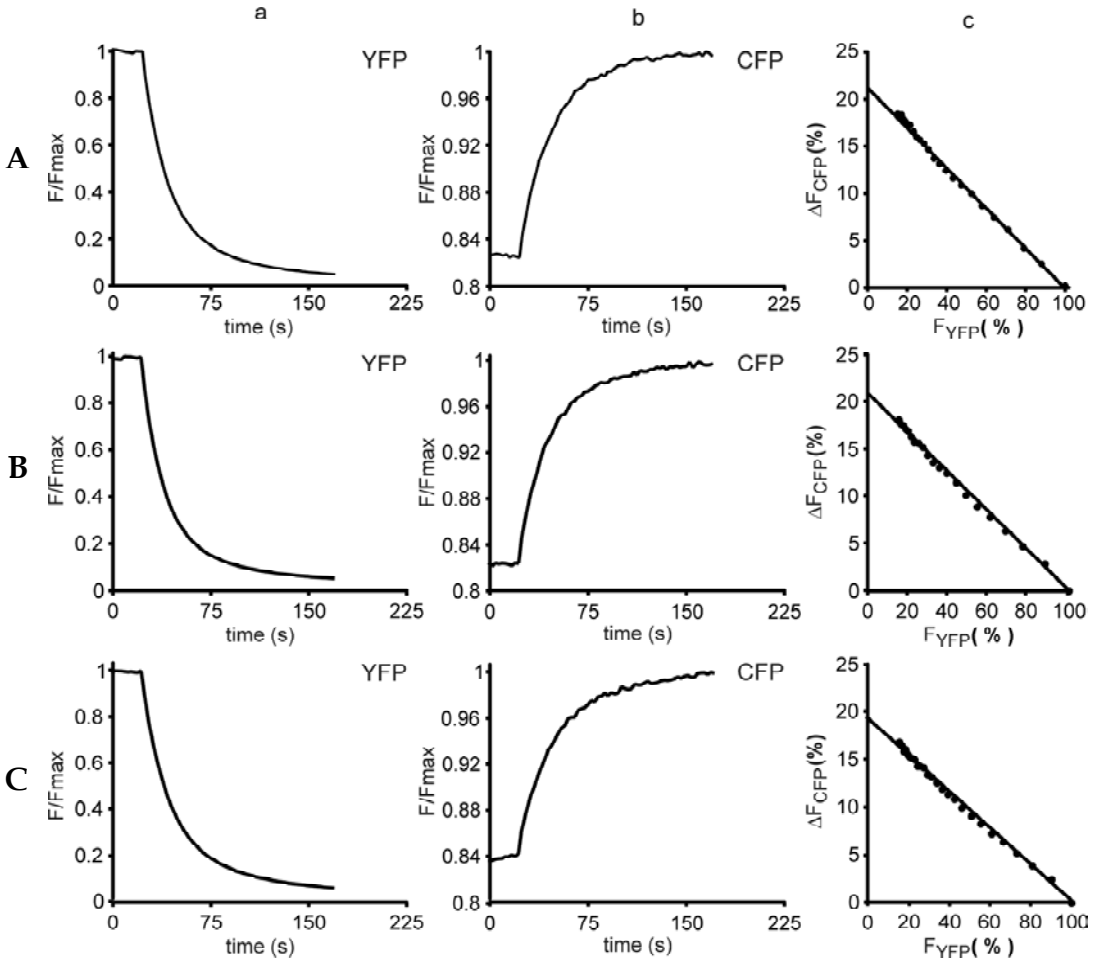

Fig. 25. **FRET analysis of HEK293 cells transiently co-transfected with ET_Amyc.CFP and ET_B.YFP receptor cDNAs.** HEK293 cells were co-transfected with plasmids encoding ET_Amyc.CFP and ET_B.YFP receptors. Following a baseline recording of CFP and YFP emission for 20 cycles, YFP was selectively photobleached at 512 nm, resulting in a decrease in YFP emission (a), which is accompanied by an increase in CFP emission (b). The increase in CFP fluorescence during YFP bleach is indicative of FRET. FRET efficiencies were calculated from the relative increase in CFP emission and the decrease in YFP emission by linear regression (see *Experimental Procedures)* (c). The calculated molar ratio of YFP : CFP was 1.8 (A), 1.5 (B), 1.6 (C); FRET efficiencies were 18% (A), 17.4% (B), 16% (C).

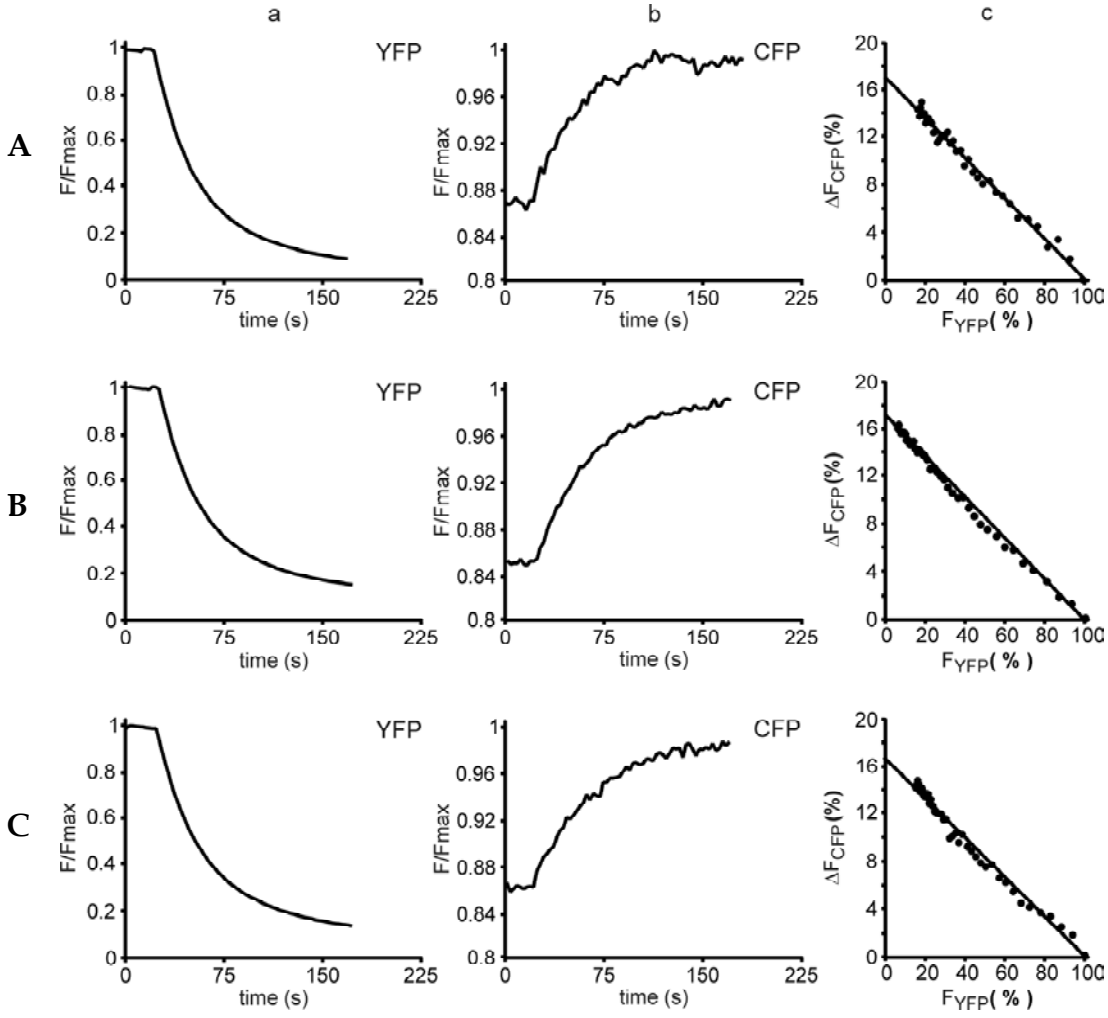

Fig. 26. **FRET analysis of HEK293 cells transiently co-transfected with ET$_A$myc.CFP and ET$_A$myc.YFP receptor cDNAs.** HEK293 cells were co-transfected with plasmids encoding ET$_A$myc.CFP and ET$_A$myc.YFP receptors. Following a baseline recording of CFP and YFP emission for 20 cycles, YFP was selectively photobleached at 512 nm, resulting in a decrease in YFP emission (a), which is accompanied by an increase in CFP emission (b). The increase in CFP fluorescence during YFP bleach is indicative of FRET. FRET efficiencies were calculated from the relative increase in CFP emission and the decrease in YFP emission by linear regression (see *Experimental Procedures)* (c). The calculated molar ratio of YFP : CFP was 2.2 (A), 2.4 (B), 2.3 (C); FRET efficiencies were 14.5% (A), 14.8% (B), 14.2% (C).

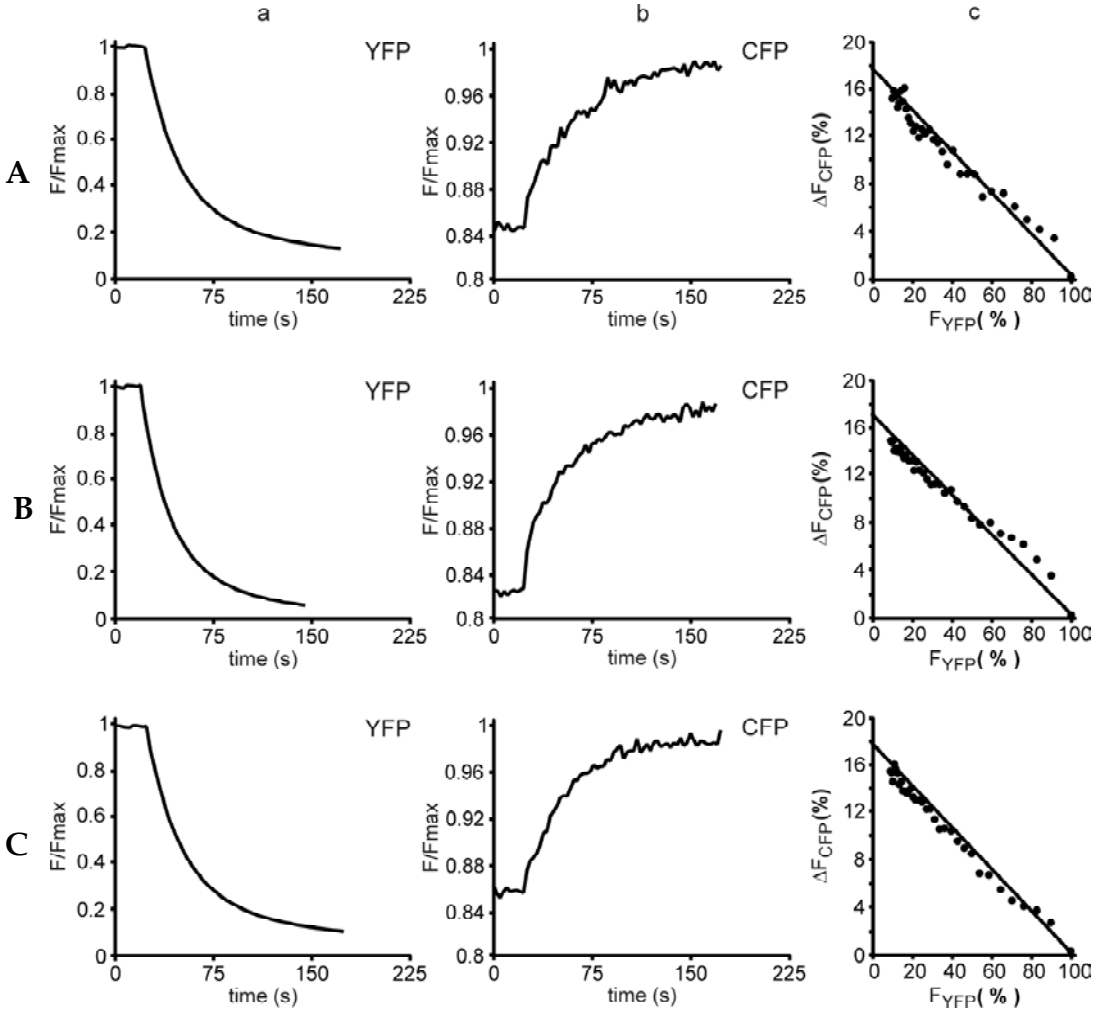

Fig. 27. **FRET analysis of HEK293 cells transiently co-transfected with ET_B.CFP and ET_B.YFP receptor cDNAs.** HEK293 cells were co-transfected with plasmids encoding ET_B.CFP and ET_B.YFP receptors. Following a baseline recording of CFP and YFP emission for 20 cycles, YFP was selectively photobleached at 512 nm, resulting in a decrease in YFP emission (a), which is accompanied by an increase in CFP emission (b). The increase in CFP fluorescence during YFP bleach is indicative of FRET. FRET efficiencies were calculated from the relative increase in CFP emission and the decrease in YFP emission by linear regression (see *Experimental Procedures)* (c). The calculated molar ratio of YFP : CFP was 3.5 (A), 2.7 (B), 3.2 (C); FRET efficiencies were 15.1% (A), 14.5% (B), 15.3% (C).

3.4.3 Specificity of ET_A and ET_B receptor heterodimerisation

To prove that heterodimerisation of ET_Amyc.CFP and ET_B.YFP receptors was not due to dimerisation of the CFP/YFP moieties, co-transfection of plasmids encoding ET_Amyc.CFP and ET_B.YFP receptors with plasmids encoding native ET_A or ET_B receptors lacking the CFP/YFP moieties (at a 1:1.5:2.5 ratio) was performed. In the case that heterodimerisation is mediated by the receptor moieties CFP and YFP tagged endothelin receptors should associate in a statistical manner with non-tagged ET_A and ET_B receptors, resulting in a decrease in FRET efficiency. Indeed, the FRET efficiency was significantly diminished to 6-8% (Fig. 28). By quantitative analysis of CFP and YFP fluorescence intensities, it was ensured that additional expression of non-tagged ET_A or ET_B receptors did not alter the molar ratio of ET_Amyc.CFP and ET_B.YFP. The acceptor to donor ratio (>1.5) was similar to that of HEK293 cells only co-expressing ET_Amyc.CFP and ET_B.YFP receptors (Fig. 28). Co-expression of ET_Amyc.CFP and ET_B.YFP receptors with the native epidermal growth factor receptor (without a CFP or YFP moiety) did not result in a significant reduction in FRET efficiencies (Fig. 28). The presented data show that FRET arises from the interaction between the receptor moieties of the fusion proteins, and hence confirms the existence of the ET_A/ET_B receptor heterodimers.

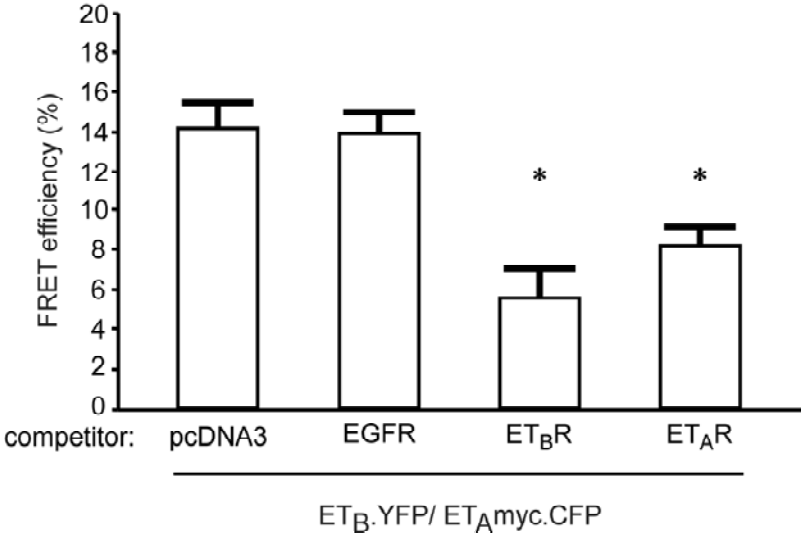

Fig. 28. **FRET analysis to prove the specificity of ET_A/ET_B dimers.** HEK293 cells were co-transfected with plasmids encoding ET_Amyc.CFP and ET_B.YFP receptors, and either vector DNA (pcDNA3) or plasmids encoding the epidermal growth factor receptor, the ET_B receptor or the ET_A receptor without CFP/YFP moieties (competitors). FRET efficiencies of cells were determined, in which the molar ratio of YFP:CFP was γ 1.5. Only for cells co-expressing ET_Amyc.CFP/ET_B.YFP receptors and ET_A or ET_B receptors a significant reduction in FRET efficiencies was observed. Values are means ± S.E. of at least three independent experiments performed in triplicate. $^*p < 0.05$ by analysis of variance and Dunett`s test.

FRET efficiencies and the molar ratio of ET_Amyc.CFP receptor to ET_B.YFP receptor were analysed in single cells. Single cell analysis allows dissecting the correlation between the molar ratio of donor to acceptor expression and the FRET efficiency. Reliable FRET efficiencies are depending on an appropriate ratio of donor and acceptor expression. FRET efficiencies of more than 20% were measured when the ET_B.YFP receptor (acceptor) was expressed at a higher level (>1.0) than the ET_Amyc.CFP receptor (Fig. 29). In figure 29 the molar ratio of ET_Amyc.CFP receptor to ET_B.YFP receptor was plotted against the FRET efficiency. Each black dot represents a single cell. In column B cells with a

donor-acceptor ratio < 1 were excluded from the analysis. When in FRET experiments the level of the acceptor expression was lower then for the donor (<1) the FRET values were consistently lower (Tab. 5). Mean FRET efficiencies in correlation to different molar ratios of donor and acceptor expression are shown in table 5. HEK293 cells were also co-transfected not only with plasmids encoding ET_Amyc.CFP and ET_B.YFP receptors, but also with plasmids encoding native ET_A or ET_B receptors lacking the CFP/YFP moieties (at a 1:1.5:2.5 ratio). Here the mean FRET efficiency diminished significantly when plasmids encoding the native ET_A or ET_B receptors were co-transfected (Fig. 29 and Tab. 5). Taken together the FRET analysis in single cells indicate a dependency of FRET efficiency and molar ratio within an YFP/CFP ratio of 0 - 1.5. No dependency of FRET efficiency and molar ratio was observed when the YFP/CFP ratio was higher the 1.5. Here consistent FRET efficiency for ET_Amyc.CFP and ET_B.YFP receptors was obtained.

Table 5: ***Synopsis of FRET efficiencies in single cell FRET analysis of HEK293 cells transiently co-transfected with ET_Amyc.CFP and ET_B.YFP and native ET_A or ET_B receptor cDNAs.***
HEK293 cells were co-transfected with plasmids encoding ET_Amyc.CFP and ET_B.YFP receptors or with plasmids encoding ET_Amyc.CFP and ET_B.YFP and ET_A or ET_B receptor cDNAs. FRET efficiency and YFP/CFP ratio were determined as described in figure legend 25. *, $p < 0.05$ by analysis of variance and Dunett's test.

donor/acceptor ratio	**Co-transfection of HEK293 cells with plasmids encoding**		
	ET_Amyc.CFP/ET_B.YFP	ET_Amyc.CFP/ET_B.YFP/ **ET_A**	ET_Amyc.CFP/ET_B.YFP/ **ET_B**
	FRET efficiency (%)		
total	16.93 ± 3.82	9.04 ± 2.57	6.98 ± 2.35
> 1	18.06 ± 3.95	9.98 ± 2.55	8.35 ± 2.46
< 1	12.12 ± 2.4*	5.27 ± 1.26*	4.52 ± 1.44*

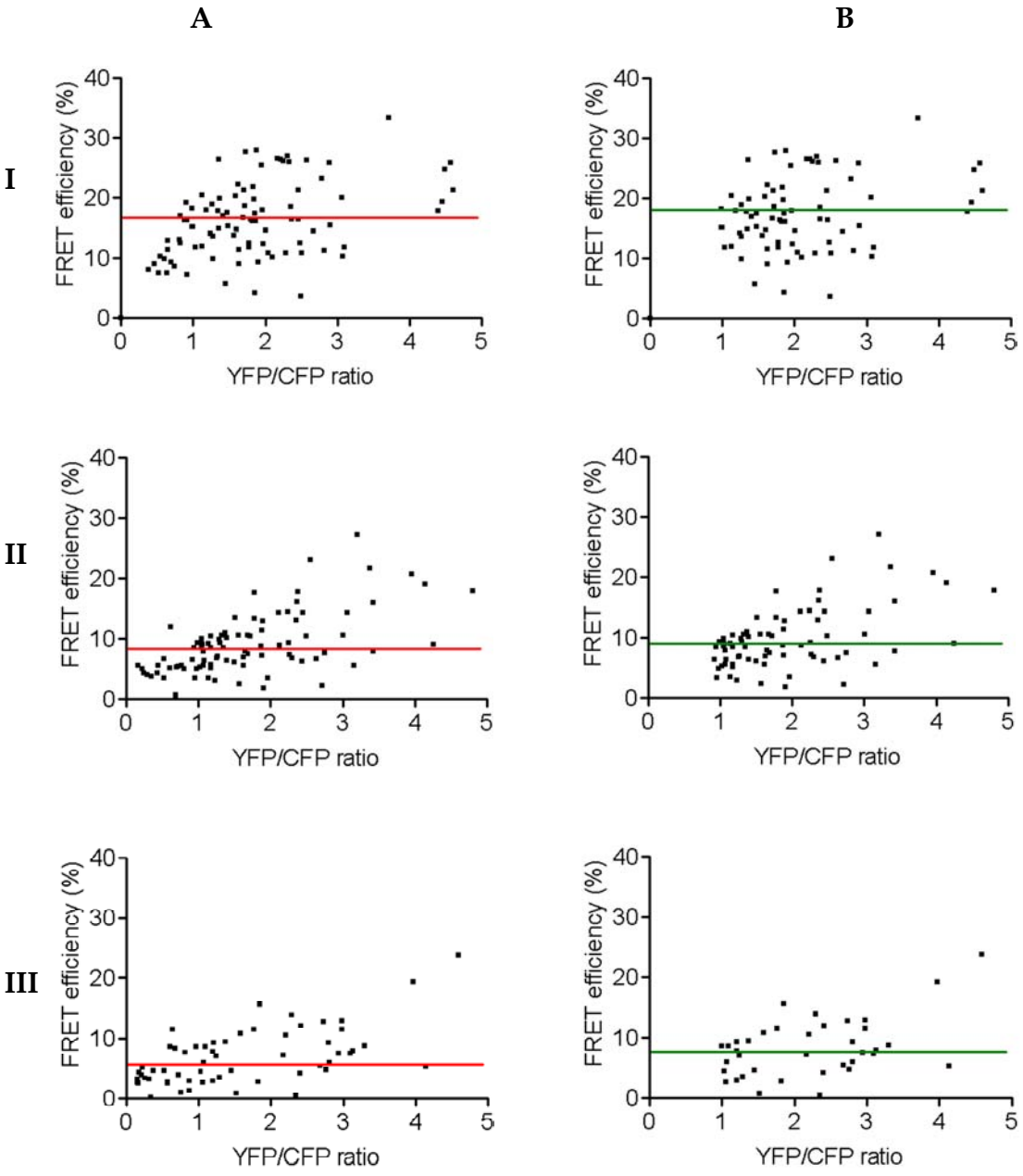

Fig. 29. **Single cell FRET analysis of HEK293 cells transiently co-transfected with ET_Amyc.CFP and ET_B.YFP and native ET_A or ET_B receptor cDNAs.** HEK293 cells were co-transfected with plasmids encoding ET_Amyc.CFP and ET_B.YFP receptors (I, A and B) or with plasmids encoding ET_Amyc.CFP and ET_B.YFP and ET_A (II, A and B) or ET_B receptor cDNAs (III, A and B). FRET efficiency and YFP/CFP ratio were determined as described in figure legend 25. Black dots represent single cells, red and green bars display the mean FRET efficiencies. Column A includes cells with a YFP/CFP ratio <1, column B cells with a YFP/CFP ratio > 1. Mean FRET efficiencies were 16.93 ± 3.82% (I, A), 18.06 ± 3.95% (I, B), 9.04 ± 2.57% (II, A), 9.98 ± 2.55% (II, B), 6.98 ± 2.35% (III, A), 8.35 ± 2.46% (III, B).

3.5 Endothelin receptor subtypes display similar ligand-binding affinities when expressed individually or in combination

To determine the K_i values of the endothelin receptor fusion proteins expressed in HEK293 cells membranes from ET_Amyc.CFP, ET_B.YFP or ET_Amyc.CFP/ET_B.YFP cell clones were prepared. The membranes were pre-incubated with 50 pM ^{125}I-ET-1 in the absence or presence of increasing amounts of non-radioactive mixed agonist ET-1, the ET_B receptor-selective agonists ET-3 or sarafotoxin 6c, or the ET_A receptor-selective antagonist BQ123 (Fig. 30). The K_i values for ET-1 did not significantly differ between ET_Amyc.CFP and ET_B.YFP receptors expressed individually or in combination (see Tab. 6). For the ET_B.YFP receptor, the K_i value for ET-3 was similar to that for ET-1 (high affinity binding site for ET-3), whereas for the ET_Amyc.CFP receptor, the K_i value for ET-3 was about 100-fold lower than that for ET-1 (low affinity binding site for ET-3; see Tab. 6 and Fig. 30**, A** and **B**). In membrane preparations of HEK293 cells co-expressing ET_Amyc.CFP/ET_B.YFP receptors two different K_i values were obtained for ET-3 (see biphasic curve in Fig. 30**, C** and Tab. 6). The lower K_i value (K_{i1}) corresponds to that of the ET_B.YFP receptor (high affinity), the higher (K_{i2}) to that of the ET_Amyc.CFP receptor (low affinity). Interestingly, the K_i value of the ET_Amyc.CFP receptor for ET-3 was significantly lower when co-expressed with the ET_B.YFP receptor than that obtained for the ET_Amyc.CFP receptor expressed alone (Tab. 6). Similarly, the K_i values for sarafotoxin 6c and BQ123 were significantly lower for co-expressed ET_Amyc.CFP and ET_B.YFP receptors than for ET_B.YFP or ET_Amyc.CFP receptors expressed alone (Tab. 6). Thus, it appeared that co-expression of the two receptor subtypes decreased the affinities of ET_A and ET_B receptor fusion proteins to receptor-selective ligands. However, when membranes of cell clones expressing either ET_Amyc.CFP or ET_B.YFP receptors were mixed prior to the binding experiments, K_i values for ET-3 (K_{i1} and K_{i2}) , BQ123, and sarafotoxin 6c were obtained similar to those for membranes of cell clones co-expressing ET_Amyc.CFP/ET_B.YFP receptors (see Tab. 6 and Fig. 30 **B**). Because binding

analysis were performed with non-saturating amounts of ^{125}I-ET-1 (50 pM), the selective ligands BQ123, sarafotoxin 6c, or ET3 may displaced the radioligand from one receptor subtype. As the selective ligands did not inhibit the binding of the displaced radioligand to the other, ligand-free receptor subtype, displacement of the radioligand could only be observed when the second receptor subtype was saturated. As a consequence, the displacement curve shifted to the right, when compared with that obtained with membrane preparations of cells expressing only one of the two receptor subtypes. In summary it appears that dimerisation does not alter the affinities of ET_A and ET_B receptors to ET-1, ET-3 and BQ123 in HEK293 cell clones.

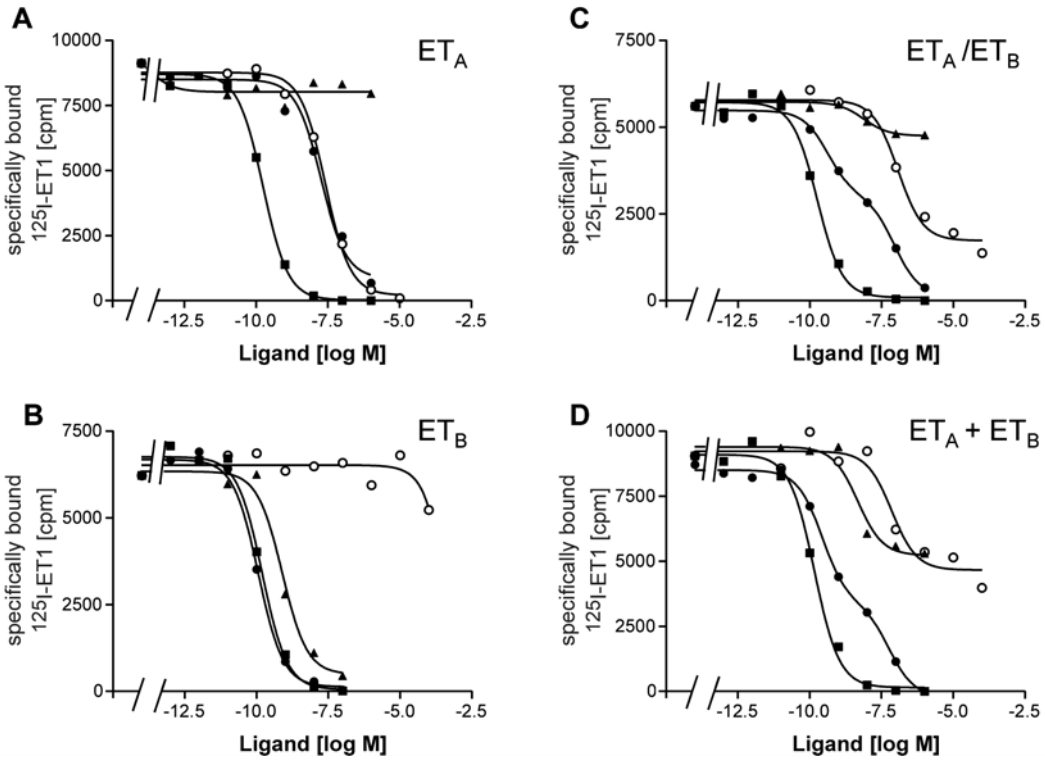

Fig. 30. **Displacement binding experiments.** Membranes from ET_Amyc.CFP **(A)**, ET_B.GFP **(B)**, ET_Amyc.CFP/ET_B.YFP cell clones **(C)** were incubated for 3 h with ^{125}I-ET-1 (50 pM), and increasing concentrations of the mixed agonist ET-1, the ET_B receptor-selective agonists ET-3 and Sarafotoxin 6c and the ET_A receptor-selective antagonist BQ123. **(D)** Membranes of ET_Amyc.CFP and ET_B.YFP cell clones were mixed prior to addition of ligands. Data represent mean values of at least three independent experiments performed in duplicate. *Triangle*, Sarafotoxin 6c; *open circle*, BQ123; *closed square*, ET-1; *closed circle*, ET-3.

Tab. 6: ***Synopsis of K_i values obtained from membranes of HEK293 cells expressing ET_Amyc.CFP and ET_B.YFP receptors either individually or in combination.***

Displacement binding experiments with membranes of stably transfected HEK293 cell clones were performed as described under *Experimental Procedures* (bottom). Membranes were incubated for 2 h with ^{125}I-ETI (50 pM) and increasing concentrations of non-radioactive ligands. Values are means ± S.D. of three independent experiments each performed in duplicate. For ET_Amyc.CFP and ET_B.YFP receptors (either co-expressed or mixed prior to binding experiments), two different Ki values were obtained with ET-3, corresponding to the high and low affinity binding sites of ET_B.YFP and ET_Amyc.CFP receptors, respectively. Ki values of co-expressed or mixed ET_Amyc.CFP and ET_B.YFP receptors for ET-3, BQ123, and Sfx6c were significant different from those of ET_Amyc.CFP and ET_B.YFP receptors expressed alone.

* *, $p < 0.05$ by analysis of variance and Dunett's test.

	ET_Amyc.CFP	ET_B.YFP	ET_Amyc.CFP/ ET_B.YFP	ET_Amyc.CFP + ET_B.YFP
		K_i (*nM*)		
ET-1	0.029 ± 0.006	0.063 ± 0.026	0.064 ± 0.013	0.056 ± 0.004
ET-3	3.6 ± 0.4	0.045 ± 0.007	0.101 ± 0.035 (K_{i1}) 32 ± 12 (K_{i2})*	0.049 ± 0.030 (Ki1) 22 ± 10 (K_{i2})*
BQ123	7.2 ± 3.6		31 ± 16*	30 ± 7*
Sfx6c		0.397 ± 0.232	2.1 ± 0.97*	1.25 ± 0.486*

3.6 Heterodimerisation results in a decreased rate of ET-1-mediated ET_B receptor sequestration

When expressed individually, the ET_A and ET_B receptors differ in their ligand-induced internalization and intracellular trafficking. The ET_A receptor internalizes either in clathrin-dependent or –independent manner and recycles to the plasma membrane (Okamoto et al. 2000). In contrast, the ET_B receptor internalizes via a clathrin-dependent pathway and does not recycle, but is targeted to late endosomes/lysosomes leading to down-regulation (Bremnes et al. 2000; Oksche et al. 2000). To analyze the agonist-mediated sequestration of heterodimers, ELISA experiments with cell clones expressing the ET_Bflag.YFP receptor either individually or in combination, with the ET_Amyc.CFP receptor were performed. Prior to ELISA experiments in real-time imaging the association of two different fluorescent ligands (Cy3-ET-1 and Cy3-BQ3020) to the endothelin receptor subtypes was analysed. Cells expressing both endothelin receptor subtypes showed that the receptors are occupied quantitative approximately 60 seconds after the treatment with 50 nM Cy3-ET-1 (Fig. 31) or 50 nM Cy3-BQ3020 (Fig. 32). Similar results were obtained with cells expressing only the ET_B receptor. To induce receptor sequestration, cells were challenged with saturating concentrations of the non-selective agonist ET-1 (50 nM) or the ET_B receptor-selective agonist BQ3020 (50 nM) for up to 120 min. In ET_Bflag.YFP cell clones treated with ET-1 for 15, 30 and 60 min, 22%, 43% and 56% of ET_Bflag.YFP receptors were sequestered, respectively (Fig. 33, **A,** *diamonds*). However, when ET_Amyc.CFP/ET_Bflag.YFP cells were challenged with ET-1, sequestration of only 5, 21 and 30% of ET_Bflag.YFP receptors was observed after 15, 30 and 60 min, respectively (Fig. 33, **A,** *squares*). No differences in ET-1 or BQ3020-mediated sequestration of ET_Bflag.YFP receptors were observed, when cell clones were used expressing ET_Bflag.YFP receptors alone (Fig. 33, **B**). Thus, sequestration of the ET_Bflag.YFP receptor is markedly retarded in cells co-expressing both ET_Amyc.CFP and ET_Bflag.YFP receptors.

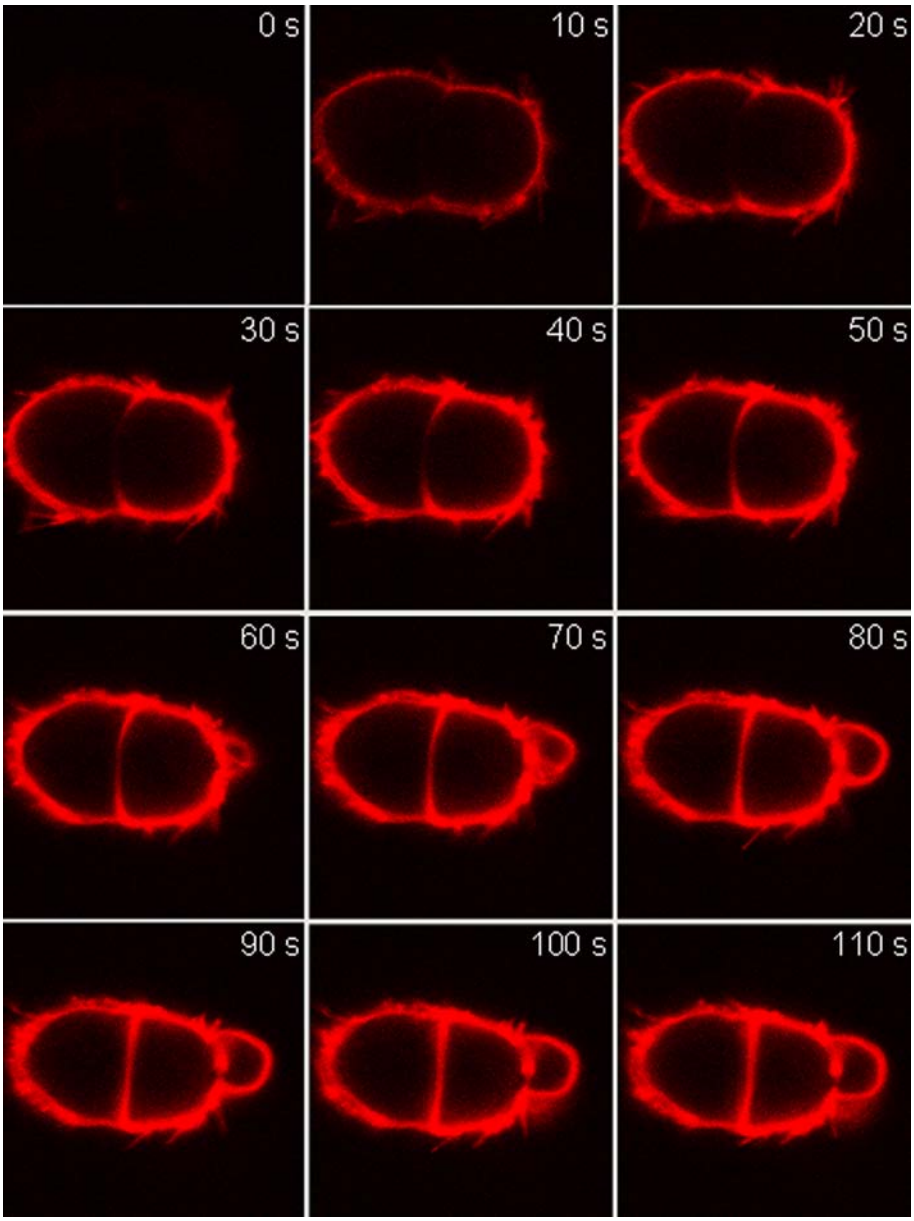

Fig. 31. **Cy3-ET-1 binding to ET_Amyc.CFP and ET_Bflag.YFP receptors in the plasma membrane of HEK293 ET_Amyc.CFP/ET_Bflag.YFP cell clones.**
ET_Amyc.CFP/ET_Bflag.YFP cell clones were grown for 48 h on glass coverslips and incubated for up to 110 seconds in the presence of Cy3-ET-1 (mixed agonist). Immediately after the application, Cy3-ET-1 bound the endothelin receptor in the plasma membrane. After approximately 60 s the intensity of the fluorescence signal was maximal indicating quantitative occupation of the receptors with the ligand.

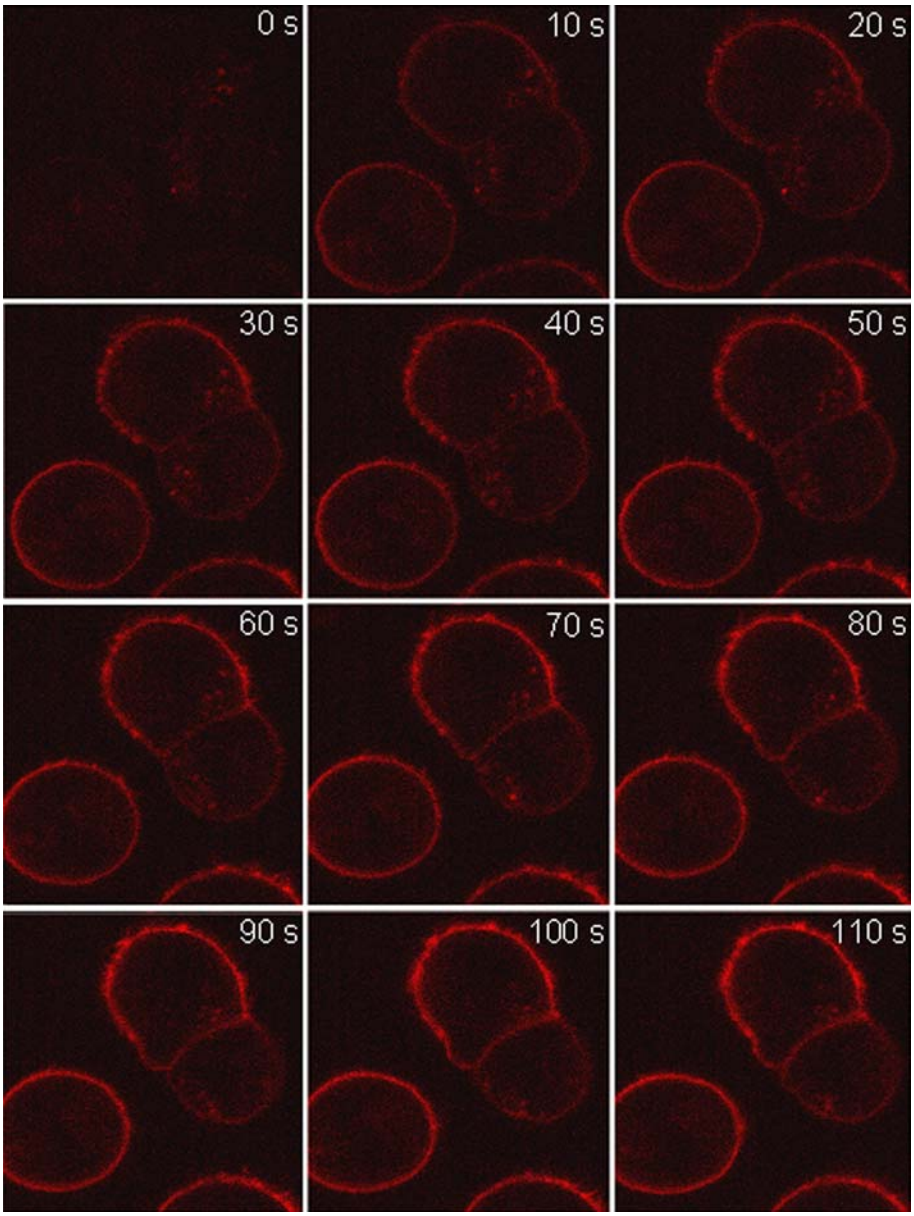

Fig. 32. **Cy3-BQ3020 binding to ET_Amyc.CFP and ET_Bflag.YFP receptors in the plasma membrane of HEK293 ET_Amyc.CFP/ET_Bflag.YFP cell clones**
ET_Amyc.CFP/ET_Bflag.YFP cell clones were grown for 48 h on glass coverslips and incubated for up to 110 seconds in the presence of Cy3-BQ3020 (ET_B receptor-selective agonist). Immediately after the application, Cy3-BQ3020 bound the endothelin receptor in the plasma membrane. After approximately 60 s the intensity of the fluorescence signal was maximal indicating quantitative occupation of the receptors with the ligand.

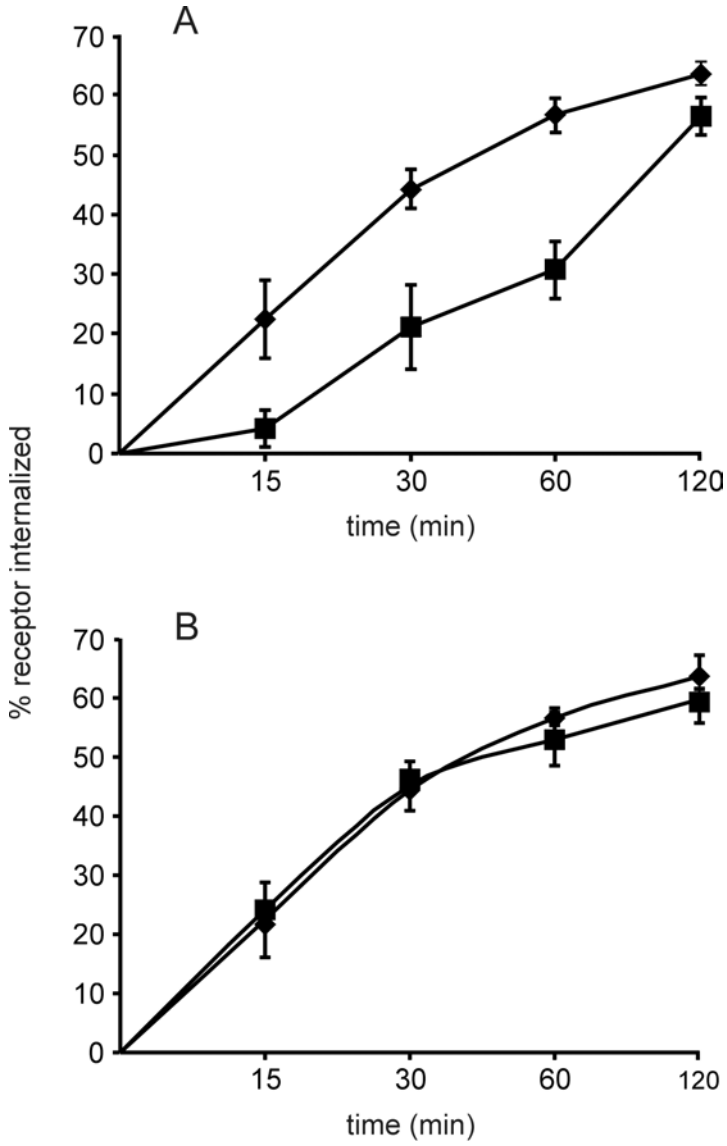

Fig. 33. **Time course of ET_B receptor sequestration.** ET_Bflag.GFP or ET_Amyc.CFP/ET_Bflag.YFP cell clones were stimulated with 50 nM ET-1 (A) or BQ3020 (B) for up to 120 min at 37°C. Values are means ± S.E. of at least three different experiments performed in triplicate. *Diamond*, ET_Bflag.GFP receptor; *square*, ET_Amyc.CFP/ET_Bflag.YFP receptors.

3.7 Intracellular trafficking of the ET_B receptor in HEK293 ET_Bflag.YFP/ET_Amyc.CFP cell clones

The ligand-dependent differences in ET_Bflag.YFP receptor sequestration were further examined in HEK293 ET_Bflag.YFP and ET_Bflag.YFP/ET_Amyc.CFP cell clones by laser scanning microscopy. When ET_Amyc.CFP/ET_Bflag.YFP cell clones were incubated for 30 min with Cy3-ET-1, ET_Amyc.CFP and ET_Bflag.YFP receptors were mainly found co-localised with Cy3-ET-1 at the plasma membrane and to a minor extent within endosomal structures. Even after 60 min, ET_Amyc.CFP and ET_Bflag.YFP receptors were still found at the plasma membrane (Fig. 34). When ET_Amyc.CFP/ET_Bflag.YFP cell clones were incubated with Cy3-BQ3020 for 30 or 60 min, Cy3-BQ3020-ET_Bflag.YFP complexes were mainly found in the perinuclear region, and only few ET_Bflag.YFP receptors remained at the cell surface (Fig. 34). In contrast, the ET_Amyc.CFP receptor was predominantly found in the plasma membrane. In agreement with the ELISA experiments (see above), the results demonstrate that the ET-1-mediated sequestration of the ET_Bflag.YFP receptor in ET_Amyc.CFP/ET_Bflag.YFP cell clones is significantly slower than that mediated by BQ3020.

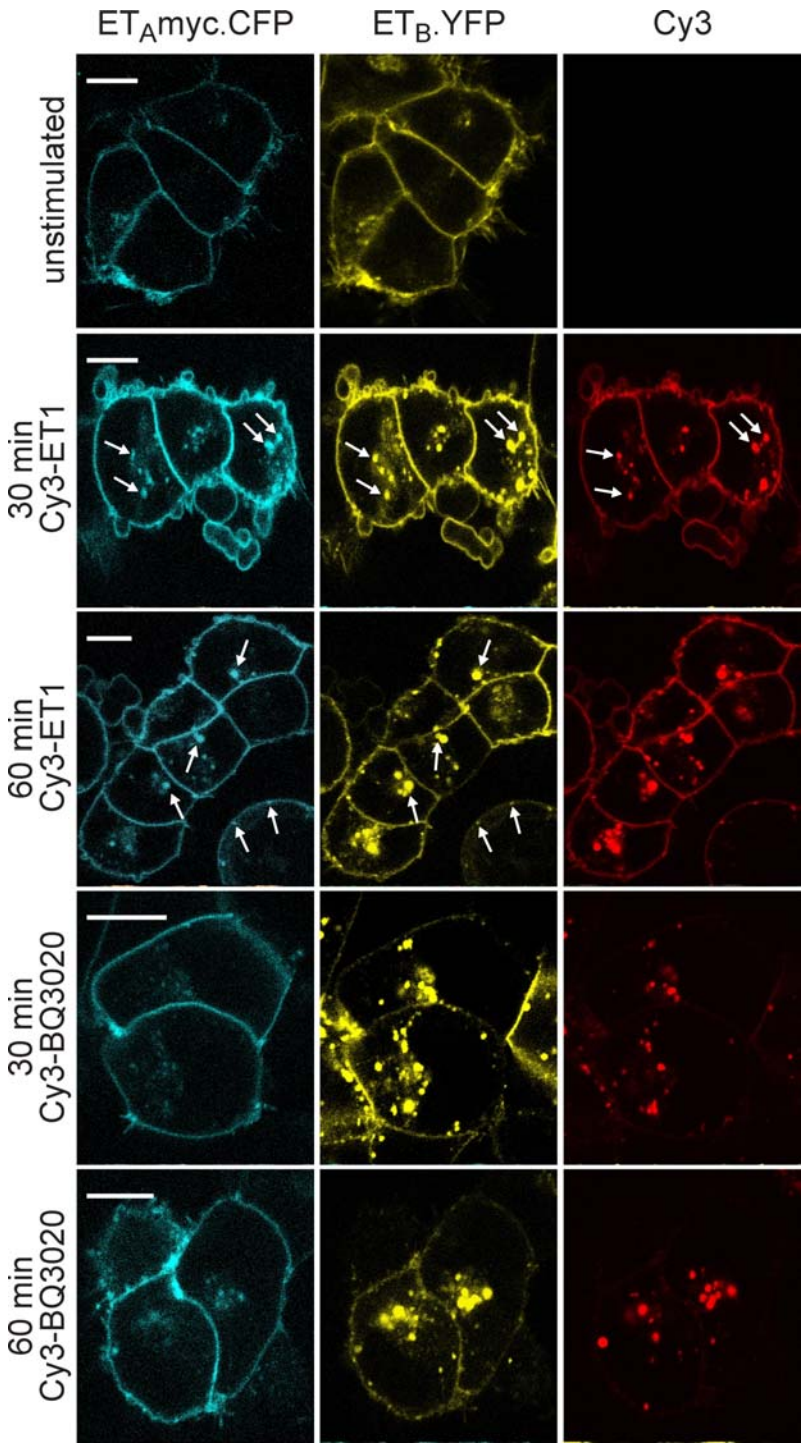

Fig. 34. **Cy3-ET-1- and Cy3-BQ3020-mediated internalisation and intracellular trafficking of ET_Amyc.CFP and ET_B.YFP receptors in HEK ET_Amyc.CFP/ET_B.YFP cell clones.** ET_Amyc.CFP/ET_Bflag.YFP cell clones were grown for 48 h on glass coverslips and incubated at 37°C for up to 60 min in the presence of Cy3-ET-1 (mixed agonist; second and third panel) or Cy3-BQ3020 (ET_B receptor-selective agonist; two lower panels). In unstimulated cells (top

panel) ET_Amyc.CFP and ET_B.YFP are predominantly located in the plasma membrane. In cells stimulated for 30 and 60 min with Cy3-ET-1, ET_Amyc.CFP and ET_B.YFP receptors were mainly found co-localised with Cy3-ET-1 at the plasma membrane and to a minor extent in endosomal compartments (see *arrows, indicating co-localisation*). In cells stimulated for 30 and 60 min with Cy3-BQ3020, ET_Bflag.YFP is predominantly localised intracellular in a complex with Cy3-BQ3020. In contrast, ET_Amyc.CFP is predominantly detected at the plasma membrane. *Bars*, 10 µm.

3.8 Influence of receptor ligands on ET_A/ET_B heterodimers

To investigate the influence of ligands on ET_A/ET_B receptor heterodimers, FRET analysis of transiently transfected HEK293 cells expressing ET_Amyc.CFP and ET_B.YFP receptors were performed. A short application (5 min) of the mixed agonist ET-1, the ET_B receptor-selective agonist BQ3020, the ET_B receptor-selective antagonist BQ788 or the ET_A receptor-selective antagonist BQ123 had no significant influence on FRET efficiencies (Fig. 35). Similarly, addition of ET-1 for 30 min, the ET_A receptor-selective antagonist BQ123 or the ET_B receptor-selective antagonist BQ788 for 30 min had no effect on FRET efficiency and thus no effect on the level of endothelin receptor dimers. In contrast, stimulation with BQ3020 (30 min) decreased FRET efficiency to about 50% of the initial value. In the presence of sucrose (450 mM), which inhibits clathrin-mediated internalisation of the ET_B receptor (Oksche et al. 2000), BQ3020 no longer decreased the FRET efficiency (Fig. 35**)**, which indicated that the dissociation of the heterodimers did not occur on the plasma membrane.

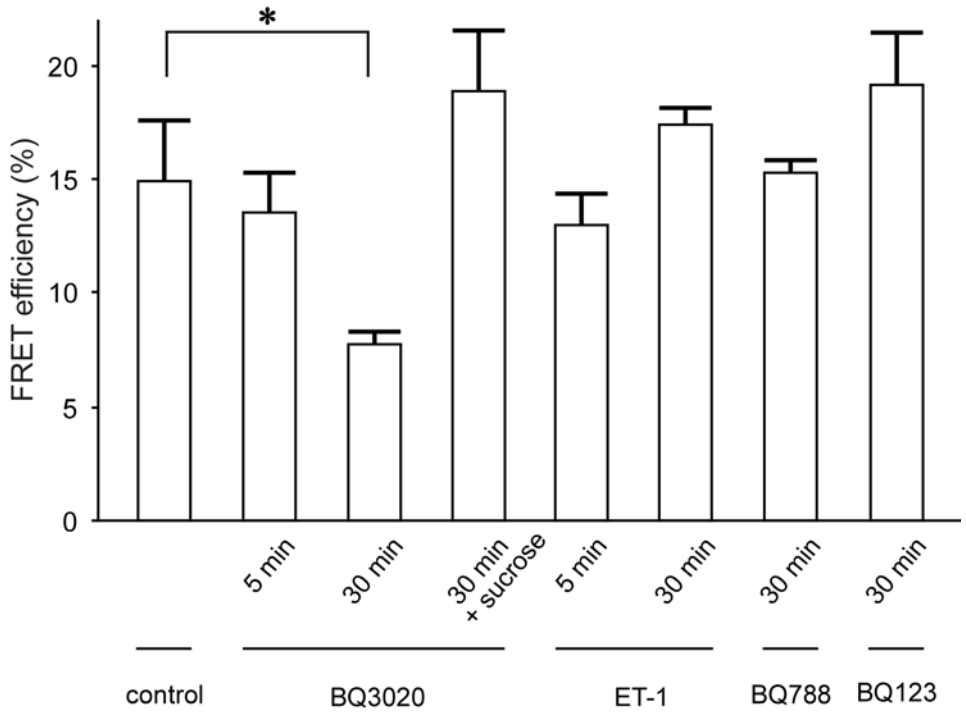

Fig. 35. **Influence of ligands on ET_A/ET_B receptor heterodimers.** Prior to FRET measurements, HEK293 cells transiently co-expressing ET_Amyc.CFP and ET_B.YFP receptors were incubated without (*control*) or with various ligands for 5 or 30 min at 37°C. Values are means ± S.E. of at least three different experiments each performed in triplicate. Clathrin-mediated internalization of the ET_Amyc.CFP/ET_B.YFP receptors was inhibited in the presence of 450 mM sucrose. *, $p < 0.05$ by analysis of variance and Dunett`s test.

3.9 Dissociation of ET_A/ET_B heterodimers occurs along the endocytic pathway

It is not clear whether the dissociation of the ET_A and ET_B receptor fusion proteins occurs immediately after the addition of the ligand at the plasma membrane, or during endocytosis and subsequent intracellular trafficking steps. To address this question, FRET analysis of transiently transfected HEK293 cells expressing ET_Amyc.CFP and ET_B.YFP receptors were performed. No decrease in FRET efficiency was detected when a dominant-negative mutant of dynamin I (K44A.dynamin I), (Damke et al. 1994), was co-transfected together with ET_Amyc.CFP and ET_B.YFP receptors (Fig. 36). To exclude the possibility that the decrease in FRET efficiencies is caused by a loss of YFP emission because of lower pH values in the endosomal compartments, the intracellular pH was clamped with the H^+-ionophores monensin and nigericin at pH 7.5 (pH of the incubation buffer). Under these conditions, the decrease in FRET efficiency of 50% was still observed (Fig. 36). However, when monensin and nigericin treated cells were incubated with an acidic buffer (pH 5), YFP emission was completely abolished and no FRET could be measured. The data confirm that ET_A and ET_B receptors exist as constitutive heterodimers in the plasma membrane. In the presence of the mixed agonist ET-1, the ET_B receptor-selective antagonist BQ788 and the ET_A receptor-selective antagonist BQ123 the extent of heterodimerisation is not altered. However, prolonged stimulation of the heterodimer with the ET_B receptor-selective agonist BQ3020 promoted the dissociation of the heterodimer. The data further demonstrate that BQ3020-mediated dissociation and separation of ET_A and ET_B receptors does not occur at the plasma membrane following binding of the agonists, but depends on endocytosis.

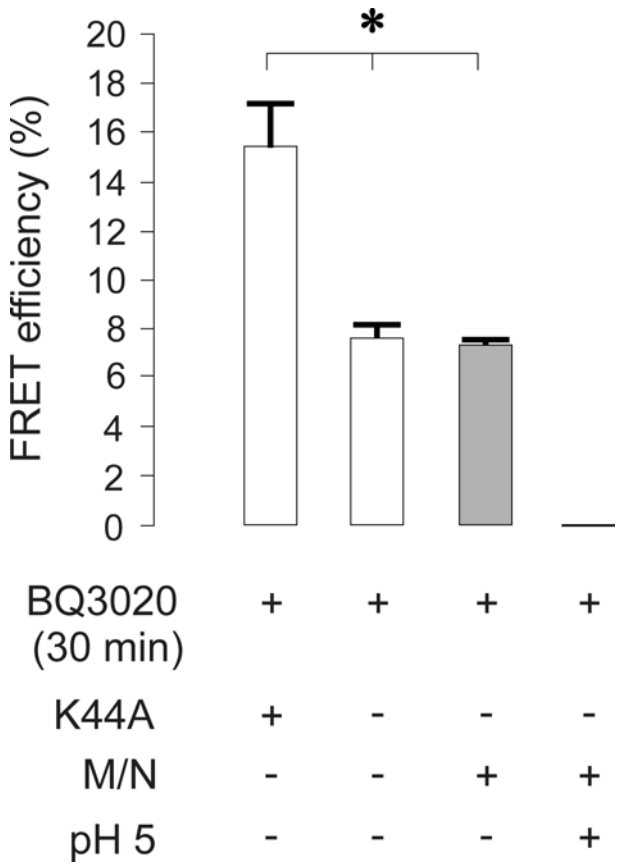

Fig. 36. **Influence of BQ3020 in the presence of K44A.dynamin I.** Prior to FRET measurements, HEK293 cells transiently co-transfected with ET_Amyc.CFP and ET_B.YFP receptors were treated for 30 min with BQ3020 at 37°C. To prevent internalization in the presence of BQ3020, cells were co-transfected with K44A.dynamin I. Monensin and nigericin were added, when pH was to adjusted to neutral (pH 7.5) or acidic (pH 5) values of the incubation buffer. *, $p < 0.05$ by analysis of variance and Dunett`s test.