2 Materials and Methods

2.1 Materials

2.1.1 Chemicals, kits, cells and antibodies

Chemicals and kits	Purchased
2-Mercaptoethanol	Carl Roth, Karlsruhe, Germany
Myo-[2-^{3}H]inositol (37 MBq/ml)	Amersham Biosciences, Freiburg, Germany
^{125}I-cAMP-tyrosylmethylester (81.4 TBq/mmol)	Biotrend, Germany
3-isobutyl-1-methylxanthine	Sigma-Aldrich, Steinheim, Germany
Acetic acid	MERCK, Darmstadt, Germany
Ammonium chloride	FLUKA, Swiss
Ammonium persulfate (APS)	Sigma-Aldrich, Steinheim, Germany
Aprotinine	MERCK, Darmstadt, Germany
Bacitracin	MERCK, Darmstadt, Germany
Batimastat	Roche Diagnostics, Pharma Research Penzberg, Germany
Benzamidine	Sigma-Aldrich, Steinheim, Germany
Bovine serum albumin	Sigma-Aldrich, Steinheim, Germany
Bromphenol blue	Carl Roth, Karlsruhe, Germany
BQ123 (ET_A receptor antagonist)	Alexis, Läufelfingen, Swiss
BQ788	Calbiochem-Novabiochem, Bad Soden, Germany
Cy3.5 monofunctional NHS ester	Amersham Biosciences, Freiburg, Germany

D-(+)-Sucrose	Carl Roth, Germany
Disodium-hydrogen phosphate	MERCK, Darmstadt, Germany
Dithiothreitol (DTT)	Sigma-Aldrich, Steinheim, Germany
Dried milk powder (low fat)	Nestlé AG, Frankfurt, Germany
Dulbecco's Modified Eagle's Medium	Sigma-Aldrich, Steinheim, Germany
DNase I, RNase free	Boehringer Mannheim, Germany
Endothelin-1	NEN, Boston, USA
^{125}I-ET-1 (2200Ci/mmol)	PerkinElmer Life Sciences, Germany
Endothelin-3	Calbiochem-Novabiochem, Bad Soden, Germany
Ethanol	J.T. Baker, Holland
Ethidium bromide	Carl Roth, Karlsruhe, Germany
Ethylendiamine-tetraacetate (EDTA)	Carl Roth, Karlsruhe, Germany
Ethylendiamine-glycol-bis-(ß-aminoethylether)-tetraacetic acid (EGTA)	Carl Roth, Germany
Fetal calf serum	Biochrom, Berlin, Germany
Formic acid	MERCK, Darmstadt, Germany
Forskolin	Sigma-Aldrich, Steinheim, Germany
HEPES	Sigma-Aldrich, Steinheim, Germany
HCl	J.T. Baker, Holland
Geneticin	Calbiochem-Novabiochem, Bad Soden, Germany
Glycerine	Sigma-Aldrich, Steinheim, Germany
Glycine	Carl Roth, Karlsruhe, Germany
Immobilized NeutrAvidin™	PIERCE, USA
IRL1038	Alexis, Läufelfingen, Swiss
IRL1620 (ET_B receptor agonist)	Alexis, Läufelfingen, Swiss
Isopropanol	J.T. Baker, Holland
Lactacystine	Sigma-Aldrich, Steinheim, Germany
LipofectAMINE	Invitrogen, Leek, Holland
Lithium chloride	Sigma-Aldrich, Steinheim, Germany

Lumi-Light Western blotting substrate	Roche Diagnostics, Germany
Magnesium chloride-6-hydrate	J.T. Backer, Holland
Magnesium sulfate	Sigma-Aldrich, Steinheim, Germany
Methanol	J.T. Baker, Holland
Minimal Essential Medium (MEM Earl`s)	Biochrom, Berlin, Germany
MMP inhibitor Ro 28-2653	Roche Diagnostics, Pharma Research Penzberg, Germany
MMP inhibitor Ro 32-7315	Roche Diagnostics, Pharma Research Penzberg, Germany
N,N,N´,N´-Tetramethylethylene diamine (TEMED)	Sigma-Aldrich, Steinheim, Germany
Nucleofection solution #4837	Amaxa, Cologne, Germany
OPTITRAN BA-S 85	Schleicher & Schuell, Germany
Ovalbumin	Sigma-Aldrich, Steinheim, Germany
Penicillin/Streptomycin	Sigma-Aldrich, Steinheim, Germany
Phenanthrolinedisulfonic acid	Sigma-Aldrich, Steinheim, Germany
Phenylmethylsulfonyl fluoride (PMSF)	Carl Roth, Germany
Phenylarsine oxide	Sigma-Aldrich, Steinheim, Germany
PD145065	Calbiochem, Bad Soden, Germany
Poly-L-Lysin	Sigma-Aldrich, Steinheim, Germany
Ponceau S	Roche Diagnostics, Germany
Potassium acetate	Carl Roth, Germany
Potassium chloride	Sigma-Aldrich, Steinheim, Germany
Potassium dihydrogen phosphate	J.T. Baker, Holland
QIAGEN Plasmid Mini Kit	QIAGEN, Germany
QIAGEN Plasmid Midi Kit	QIAGEN, Germany
QuickChange site-directed mutagenesis kit	Stratagene, Heidelberg, Germany
RES-701-1	Alexis, Läufelfingen, Swiss
Rotiphorese® Gel 30 (37,5:1), Acrylamid- and bisacrylamid solution	Carl Roth, Karlsruhe, Germany

Sarafotoxin (Sf6xc)	Alexis, Läufelfingen, Swiss
Sodium chloride	J.T. Baker, Holland
Sodium hydroxide	Carl Roth, Karlsruhe, Germany
Sodiumdodecyle sulfate (SDS)	Carl Roth, Karlsruhe, Germany
Sodium hydrogen carbonate	Sigma-Aldrich, Steinheim, Germany
Tris(hydroxymethyl)-aminomethan	MERCK, Darmstadt, Germany
Triton X-100	Sigma-Aldrich, Steinheim, Germany
Trypanbleu	Seromed, Germany
Trypsin	Sigma-Aldrich, Steinheim, Germany
Trypsin inhibitor from soybean	Sigma-Aldrich, Steinheim, Germany
Tryptose phosphate broth solution	Sigma-Aldrich, Steinheim, Germany
Tween® 20	Sigma-Aldrich, Steinheim, Germany

Cells	**Purchased**
HEK 293 (Human embryonic kidney cells, Adenovirus type 5 transformed)	DSMZ, Braunschweig, Germany
VSMC (Vascular smooth muscle cells from aorta of newborn rats)	H.P. Reusch, University of Bochum, Germany

Antibodies	**Purchased**
Polyclonal anti-phospho-ERK 1/2 antibody	Cell Signaling Technology, Beverly, USA
Polyclonal anti-ERK 1/2 antibody	Cell Signaling Technology, Beverly, USA
Polyclonal rabbit anti-GFP-antibody	Forschungsinstitut für Molekulare Pharmakologie, Germany
Cy™3-conjugated goat-anti-mouse IgG	Jackson ImmunoResearch Laboratories, Inc., USA
Horseradish peroxidase-conjugated goat	Jackson ImmunoResearch

anti-rabbit IgG antibody	Laboratories, Inc., USA
Sepharose-conjugated sheep anti-rabbit antibody	Pharmacia & Upjohn Diagnostics, Uppsala, Sweden

2.1.2 Technical equipment and software

2.1.2.1 Technical eduipment

Acryl-amid-gel cast-stand	BioRad
Acryl-amid-gel electrophoresis chamber	BioRad
BAS-SR imaging plate	Raytest Isotopenmessgeräte, Freiburg, Germany
Brandel cell harvester	Forschungsinstitut für Molekulare Pharmakologie, Germany
Cell counter	Casy, Schärfe System, Germany
Centrifuges	Beckman LE-70
	Beckman TLK-100
	Heraeus Biofuge 15
	Hereaus Biofuge *pico*
	Sorvall RC5C Plus (Dupont)
	Stratagene PicoFuge®
Clean bench	Heraeus, Hanau, Germany
Films	Ortho-Film (Agfa), X-OMAT-Röntgenfilme (Kodak)
Fuji Film BAS-3000 imaging analyzer	Raytest Isotopenmessgeräte, Freiburg, Germany
Incubator	Heraeus, Hanau, Germany
Gel dryer	BioRad Modell 583
γ-counter	FHT 111M, Contamat, Germany
Homogenisor	POTTER S, B. Braun Biotech International
Lumi-Imager F1	Boehringer Mannheim, Germany

Microscopes	Zeiss Axiovert 13S,
	Laser Scanning Microscope-Zeiss LSM 510
	META; Carl Zeiss, Jena, Germany
Nucleofector	Amaxa, Cologne, Germany
Photometer	Pharmacia UV-visible Spectrophotometer
PCR machines	Perking Elmer Thermocycler 9700
	Biometra UNO-Thermoblock™
	Biometra Trio-Thermoblock™
pH-meter	Hanna Instruments HI 9321, Germany
Pipettes	Eppendorf
Power supply	BioRad
Transilluminator	Herolab UVT-28MP
Rotation vacuum concentrator	Alpha-RVC, Christ, Germany
Scales	Scaltel SBA52
	Melter Toledo AG245
Sequencer ABI 373 A	Perkin Elmer, USA
Sonicator	Sonopuls UW 2040, Bandelin Electronics
Thermomixer	Eppendorf-Thermomixer 5436
Videocamera	Herolab E.A.S.Y. 429K
Western blot chamber	BioRad

2.1.2.2 Software

Axio Vision	Zeiss
Excel 2003	Microsoft
GraphPad Prism 3.02	GraphPad Software Inc.
Photoshop 6.0	Adobe
PowerPoint 2003	Microsoft
RADLIG 4.0	Cambridge, UK
Word 2003	Microsoft

2.2 Methods

2.2.1 Cell culture, transient transfection and nucleofection

2.2.1.1 Cell culture of HEK 293 cells and VSMCs

Media and solutions

Cell culture medium for HEK 293 cells	DMEM (pH 7.4)	90% (v/v)
	Fetal calf serum	10% (v/v)
	Antibiotics	100 U/ml Penicillin G
		100 µg/ml streptomycin
Cell culture medium for VSMCs	MEM Earl´s (pH 7.4)	90% (v/v)
	Fetal calf serum	10% (v/v)
	Tryptose phosphate broth	2% (v/v)
	Antibiotics	100 U/ml Penicillin G
		100 µg/ml streptomycin
PBS	NaCl	80 g/l
	KCl	0.2 g/l
	$Na_2HPO_4 \cdot 2H_2O$	1.44 g/l
	KH_2PO_4	0.2 g/l →pH 7.4
Trypsin/EDTA solution	Trypsin	0.05% (w/v)
	EDTA	0.02% (w/v)
	PBS	ad 100 ml

HEK 293 cells and VSMCs were grown in DMEM or MEM Earl's, respectively, (see above) at 37°C in a humidified atmosphere of 95% air / 5% CO_2. For sub-culturing, confluent cell monolayers were rinsed once with prewarmed PBS (without Ca^{2+} and Mg^{2+}) and incubated with trypsin/EDTA solution for 10 min at 37°C to detach cells from the cell culture surface. Confluent cell monolayers were passaged every 4-5 days and split in ratio

of 1:3-1:6. For HEK 293 cells, the seeding densities were 2 x 10^6 cells/25 cm^2 flask; 2 x 10^6 cells/100-mm culture dish; 1 x 10^5 cells/35-mm culture dish); 0.5 x 10^5 cells per well of a 24-well plate.

2.2.1.2 Transient transfection of HEK 293 cells

For transfection of HEK 293 cells, the lipid-mediated DNA-transfection procedure with LipofectAMINE was used. The cationic lipids (= LipofectAMINE) bind to the negatively charged DNA and form a liposome-DNA complex, which associates with the plasma membrane. Thus DNA is introduced into cells for expression of protein.

Reagent quantities for different sized culture dishes

Culture plate diameter (mm)	DNA amount (µg/µl)	LipofectAMINE
60	2.5 µg in 250 µl medium	18.75 µl in 250µl medium
35	1 µg in 100 µl medium	7.5 µl in 100 µl medium
24-well plate (per well)	0.25 µg in 25 µl medium	2 µl in 25 µl medium

HEK 293 cells were grown to 80% confluence before transient transfection with plasmid DNA. The LipofectAMINE and DNA were diluted separately in serum- and antibiotic-free medium. Both solutions were mixed and incubated for 20 min at room temperature to allow DNA-liposome complexes to form. Then serum-free medium was added (2 ml / 60-mm culture dish; 800 µl / 35-mm culture dish; 250 µl per well of a 24-well plate). Cells were washed once with serum-free medium and overlaid with the DNA-liposome mixture. After 5 h incubation at 37°C in a humidified atmosphere of 95% air / 5% CO_2, the serum-free medium was replaced with complete growth medium. Cells were then further incubated for additional 48 h at 37°C in a CO_2 incubator.

2.2.1.3 Nucleofection of VSMCs

Nucleofection of VSMCs was performed using the Nucleofector^{TM} technology (Amaxa GmbH, Cologne, Germany). By this non-viral transfection method which is based on a

combination of electroporation and cell-type specific solution, the DNA is transported rapidly into the cell nucleus.

Confluent VSMCs were washed once with PBS and harvested with trypsin / EDTA. After determination of the cell density, 2×10^6 cells were centrifuged at 1000 rpm for 5 min. The cell pellet was mixed with 10 µg DNA diluted in 100 µl nucleofection solution # 4837 (Amaxa) and transferred to an Amaxa certified cuvette. Cells were nucleofected using program T-28 and seeded at a density of 600 cells/mm^2. Cells were grown in the presence of 10% fetal calf serum for 48 h before the experiments.

2.2.2 Protein analysis

2.2.2.1 Preparation of membranes for ligand binding and immunoblot experiments

Reagents and solutions

PBS	(see 2.2.1.1)	
1 N HCl solution	HCl	1 N
Tris-ME	Tris HCl	50 mM
	EGTA	2 mM
	$MgCl_2$	10 mM → pH 7.2
Bacitracin stock solution	Bacitracin	2.13 g
	H_2O	ad 10 ml
		(1 h at 70°C)
Aprotinine stock solution	Aprotinine	0.15 g
	H_2O	ad 10 ml
Tris-BAME buffer	Tris-ME	500 ml
	Bacitracin stock solution	500 µl
	Aprotinine stock solution	500 µl
PMSF stock solution	PMSF	40 mM (in Ethanol)

Protease inhibitors mix	Benzamidine	100 mM
	Trypsin inhibitor	2 µg/ml
	Aprotinine	1 µg/ml
PBSI	PBS	10 ml
	PMSF stock solution	125 µl
	Protease inhibitors mix	80 µl

All procedures were performed at 4°C. HEK 293 cells expressing ET_B·GFP fusion proteins were grown on 100-mm Petri dishes, washed once with 5 ml ice cold PBS, harvested with a rubber policeman and centrifuged at 400 x g for 10 min at 4°C. For ligand binding experiments, the pellet was resuspended in Tris-BAME buffer and homogenized with a glass/teflon homogenizer with 10 strokes at 800 rpm. The homogenate was centrifuged at 26,000 x g for 30 min at 4°C. The pellet was resuspended in Tris-BAME, and stored at -80°C until use. For immunoblot experiments, the initial pellet (see above) was resuspended in 1 ml PBSI. The samples were briefly sonicated and centrifuged at 100,000 x g for 60 min at 4°C. The pellet was resuspended in PBSI, and aliquots were stored at -80°C until use. Protein concentrations of samples were determined in duplicates by the method of Bradford (see 2.2.2.3).

2.2.2.2 Preparation of total cell lysates

Reagents and solutions

PBS	(see 2.2.1.1)	
3 x Lysis buffer	Glycerin	30% (v/v)
	SDS	6% (w/v)
	Tris-HCl (pH 6.8)	190 mM
	Dithiothreitol (DTT)	150 mM
	Brome-phenol blue	0.3% (w/v)

HEK 293 cells were grown on 35-mm Petri dishes in DMEM/10% fetal calf serum for 48 h. For the detection of phospho-ERK1/2 and ERK1/2, cells were maintained in serum-free medium for additional 18 h. Cells were then incubated with medium alone (control) or with medium and ET-1 (100 nM) for up to 3 h at 37°C. After washing with ice cold PBS, cells were lysed directly in lysis buffer (300 µl per dish; see above). Extracts were transferred to microcentrifuge tubes and were stored at -20°C until use.

2.2.2.3 Determination of protein concentrations by the method of Bradford

Reagents and solutions

Coomassie Brilliant Blue Protein	CBB	100 mg/l
Reagent (CBB PR)	Ethanol 95 Vol.%	4.25% (v/v)
	H_3PO_4 85%	8.5% (v/v)

Samples of unknown protein concentration were run in parallel with samples of a protein standard (ovalbumin in different concentrations: from 0.1 mg/ml to 1 mg/ml). Probes were run in duplicates. Proteins (5 µl) were diluted with water (45 µl) and denatured with 2 N NaOH (50 µl) for 10 min at 60°C. After addition of CBB PR (1000 µl), absorption at 595 nm was measured with a spectrophotometer. The standard curve (calibration curve) was calculated using wiacalc (Wallac and Os, 1989). The program then calculates the protein concentration of samples using the calibration curve. Protein concentrations were used only, when in the range of the calibration curve.

2.2.2.4 SDS-Polyacrylamide Gel Electrophoresis

SDS-Polyacrylamide gel electrophoresis (PAGE) was performed accordingly to Laemmli.

Reagents and solutions

4 x Laemmli-sample buffer	Glycerin	10% (v/v)
	SDS	2% (w/v)
	Tris-HCl (pH 6.8)	60 mM
	β-mercapto-ethanol	5% (v/v)
	Brome-phenol blue	0.01% (w/v)
Running buffer	Tris	50 mM
	Glycine	150 mM
	SDS	0.1% (w/v)
		pH 8.3-8.4

Separation gel 10 %	30% Acrylamide (w/v)	
	0.8% Bis-acrylamide (w/v)	3.3 ml
	1.5 M Tris-HCl, pH 8.8	2.5 ml
	20% SDS	50 µl
	H_2O	4.05 ml
	APS 10% (w/v)	50 µl
	TEMED	5 µl

Stacking gel 4 %	30% Acrylamide (w/v)	
	0.8% Bis-acrylamide (w/v)	0.66 ml
	0.5 M Tris-HCl, pH 6.8	1.3 ml
	20% SDS	50 µl
	H_2O	2.935 ml
	APS 10% (w/v)	50 µl
	TEMED	5 µl

The separating gel solution for 10% SDS-PAGE mini-gel (1 mm thickness) was prepared by adding the reagents shown above to each other. The solution was mixed and used immediately. Using BioRad gel cast chambers, separation gels were cast first, covered with 70% isopropanol and incubated for 30 min to allow polymerization. Subsequently, isopropanol was discarded and stacking gels were cast. Membrane preparations from HEK 293 cells (see 2.2.2.1) were briefly sonicated; total cell lysates (for the detection of

pERK1/2 or ERK1/2; see 2.2.2.2) were boiled for 5 min at 95°C. Samples (80 µg protein per lane of the membranes or 10 µl lysate and the appropriate molecular weight standarts) were loaded onto the gel and electrophoresis was performed for 1 h at 20 mA per gel.

2.2.2.5 Western Blot

Reagents and solutions

Blotting buffer	Tris	2.4 g
	Glycine	11.26 g
	Methanol	200 ml
	20% SDS solution	750 µl
	H_2O	ad 1 l → pH 8.3

Size-separated proteins were electrophoretically transferred to nitrocellulose membranes using a Tank Blot apparatus. Protein transfer and equal loading was monitored by Ponceau-red staining. Briefly, membranes cut in the dimensions of the minigel, were equilibrated for 5 min in blotting buffer. Subsequently, the membrane was placed on a top of a Whatman paper soaked in blotting buffer and covered with the minigel. Another wet Whatman paper was put on the top of the gel and eventually trapped air bubbles were displaced by rolling a glass rod over the blotting stack. Electrophoretic transfer of proteins was performed at 100 mA per gel for 1.5 h.

2.2.2.6 Immunoblots for the detection of ET_B·GFP fusion proteins

Reagents and solutions

PBS	(see 2.2.1.1)	
Blocking buffer	Tween® 20	0.05% in PBS (w/v)
	Low fat milk powder	5% (w/v)

	Fetal calf serum	5% (v/v)
Washing solution	IGPal C630	1% in PBS (w/v)
	SDS	0.1% (w/v)
	Fetal calf serum	5% (v/v)

Nitrocellulose membranes were blocked for one hour at room temperature in blocking buffer and incubated over night at 4°C with polyclonal rabbit anti-GFP antibodies diluted 1:1000 in PBS / 5% low fat milk powder. Blots were washed once with washing solution, once with PBS and finally with washing solution each for 15 min. The membranes were then incubated for 1 h at room temperature with peroxidase-conjugated anti-rabbit IgG antibodies (Jackson Immunoresearch Laboratories, USA) diluted 1:2000 in PBS / 5% low fat milk powder. After a wash with washing solution and two washes with PBS (each for 15 min) the blots were incubated for 3 min in Lumi-Light Western Blotting Substrate (Roche Diagnostics Corporation, USA). Chemiluminescence was visualized and band densities were quantified using a Lumi-Imager F1 (Roche Diagnostics, Mannheim, Germany).

2.2.2.7 Immunoblots for the detection of pERK1/2 and ERK1/2

Reagents and solutions

TBS	Tris	10 mM
	NaCl	150 mM
		pH adjusted to 7.4
TBST	Tween® 20	0.1% in TBS (w/v)
Blocking buffer	Low fat milk powder	5% (w/v)
	TBST	ad 100 ml

Membranes were blocked in blocking buffer as described above and incubated with affinity-purified polyclonal anti-phospho-ERK1/2 or anti-ERK1/2 antibodies (both diluted 1:1000 in TBST with 5 % low fat milk powder) overnight at 4°C. Subsequently, blots were

washed in TBST (3 x 10 min) and primary antibodies were detected with a horseradish peroxidase-coupled goat anti-rabbit secondary antibody (diluted 1:2000 in TBST with 5 % low fat dried milk). After washing with TBST (3 x 10 min) membranes were incubated for 3 min in Lumi-Light Western Blotting Substrate and immunoreactive bands were visualized by Lumi-Imager.

2.2.2.8 Low temperature polyacrylamide gel electrophoresis (LT-PAGE)

Reagents and solutions

Buffer A	DMEM	
	Hepes	10 mM
	BSA	0.5%
		pH adjusted to 7.2
Buffer B	DMEM	
	Hepes	10 mM
	BSA	0.5%
		pH adjusted to 7.2
	^{125}I-ET-1	100 pM
Sample buffer	Tris	0.25 M
	SDS	2% (v/v)
	β-mercaptoethanol	5% (v/v)
	Glycerol	20% (w/v)
	Bromphenol blue	0.02%(w/v)
		pH adjusted to 6.8

Stably transfected HEK 293 cells or nucleofected VSMCs were grown on 35-mm Petri dishes. After 48 h cells were washed with buffer A at 4°C and incubated in buffer B for 30 min at 4°C. Subsequently, the cells were washed once with buffer A at 4°C to remove unbound ligand and either lysed directly (controls) in 100 µl sample buffer or incubated with prewarmed buffer A at 37°C for different time periods ranging from 30 to 180 min

prior to lysis. To prevent clathrin-mediated endocytosis buffers A and B were supplemented with 0.45 M sucrose (final osmolality of 750 mosm/l) or phenylarsine oxide (final concentration 50 µM). The samples were briefly sonicated and separated in 10% SDS-polyacrylamide gels in the presence of 0.1% SDS at 4°C. Prestained molecular weight markers (BIORAD, München, FRG) were run in parallel. Gels were dried, exposed for 2 to 4 days on a BAS–SR imaging plate and analyzed in a Fuji Film BAS-3000 Imaging Analyzer (Raytest Isotopenmeßgeräte, Freiburg, FRG).

2.2.2.9 Pulse-Chase

Reagents and solutions

Buffer A	Tris-HCl (pH 8.0)	50 mM
	NaCl	150 mM
	EDTA (pH 8.0)	1 mM
	Triton X-100	1% (w/v)
	SDS	0.1% (w/v)
	PMSF	40 mM
	Protease inhibitors mix	(see 2.2.2.1)
Washing buffer I	Tris-HCl (pH 8.0)	50 mM
	NaCl	500 mM
	EDTA (pH 8.0)	1 mM
	Triton X-100	0.5% (w/v)
	SDS	0.1% (w/v)
Washing buffer II	Tris-HCl (pH 7.4)	50 mM
	NaCl	500 mM
	EDTA (pH 8.0)	1 mM
	Triton X-100	0.5% (w/v)
	SDS	0.1% (w/v)
Fixing solution	Methanol	50% (v/v)
	Acetic acid	10% (v/v)
	H_2O	

HEK 293 cells stably expressing ET_B·GFP were grown on 100-mm Petri dishes to 90% confluence and harvested with trypsin/EDTA. Cells were starved for 45 min in methionine/cysteine-free DMEM supplemented with 10% FCS. Then cells were pulse-labeled with [^{35}S] methionine/cysteine (150-200 µCi/ml) for 30 min at 37°C and chased for 4 h in complete DMEM supplemented with Met/Cys. The probes were centrifuged at 1000 x g for 5 min at 4°C and the pellets were resuspended with 500 µl buffer A. The lysates were centrifuged again (13,000 x g for 5 min at 4°C) to separate the insoluble and soluble fractions. For immunoprecipitations, after an overnight incubation at 4°C with anti-GFP 01 antibodies, the clear soluble fraction was further incubated with Protein A Sepharose for 1h at 4°C. After agitation the beads were washed first with washing buffer I and then with washing buffer II and the attached antigen subjected to SDS-PAGE. After the electrophoresis, gels were incubated in fixing solution for 30 min at 4°C, dried and exposed for 2 to 4 days on a BAS–SR imaging plate and analyzed in a Fuji Film BAS-3000 Imaging Analyzer.

2.2.3 Saturation and displacement binding experiments with ^{125}I-ET-1

Reagents and solutions

PBS	(see 2.2.1.1)	
Tris-BAME buffer	(see 2.2.2.1)	
Polyethylenimine buffer	Polyethylenimine	0.1% (w/v)
	H_2O	

Membrane preparations of HEK 293 cells stably expressing ET_B·GFP fusion proteins (see 2.2.2.1) were diluted with Tris-Bame buffer to a final concentration of 0.5 µg protein/µl. For saturation binding experiments, membranes (see above; 5 µg per sample) were incubated in a final volume of 200 µl Tris/BAME buffer with increasing amounts of ^{125}I-ET-1 (7.5-500 pM) in the absence (total binding) or presence of 1 µM unlabeled ET-1 (non-specific binding) for 2 h at 25°C. In case of displacement binding experiments, membranes (5 µg per sample) were incubated with 50 pM ^{125}I-ET-1 without or with

unlabeled ligand (1 x 10^{-12} to 1 x 10^{-6} M) for 2 h at 25°C. The samples were then transferred onto GF/C filters (Whatman International, Maidstone, UK) pretreated with 0.1 % (w/v) polyethylenimine and washed rapidly twice with PBS using a Brandell cell harvester. Filters were finally transferred into 5 ml vials and filter-bound radioactivity was determined in a γ-counter. Data were analyzed with the RadLig Software 4.0 (Biosoft, Cambridge, UK).

2.2.4 Inositol phosphate assay

Reagents and solutions

Buffer A	DMEM	
	Hepes	10 mM
	LiCl	10 mM
	BSA	0.5%
		pH adjusted to 7.2
Buffer B (dilution buffer)	Sodium tetraborate	5 mM
	EDTA	0.5 mM
Formic acid	0.2 M	
NaOH	0.1 N	
Regeneration buffer	Formic acid	0.1 M
	Ammonium formate	3 M
Washing buffer	Sodium tetraborate	5 mM
	Sodium formate	60 mM
Elution buffer	Formic acid	0.1 M
	Ammonium formate	0.4 M

HEK 293 cells were seeded onto 24-well plates (100,000 cells/well) and grown in DMEM supplemented with 10% fetal calf serum. After 48 h cells were incubated with 74 kBq/ml myo-[2-^{3}H] inositol (specific activity 37 MBq/ml; Amersham Biosciences, Freiburg, Germany) for further 20 h at 37°C. Cells were then washed with 1 ml of Buffer A and

stimulated with Buffer A alone (control) or with increasing concentrations of ET-1 (100 pM to 100 nM) for 60 min at 37°C. The incubation was terminated by aspiration of the medium and lysis of the cells with 0.1 N NaOH (150 µl/well). By subsequent addition of 0.2 M formic acid (50 µl/well) and dilution buffer (500 µl/well) the appropriate conditions (pH, ionic strength) were adjusted. The lysates were centrifuged at 23,000 x g for 15 min at 4°C and supernatants were transferred to anion exchange chromatography on AG 1-X8 columns (200-400 mesh; 0.8 x 4 cm; Bio-Rad Laboratories). Shortly before applying the samples the anion exchange resins were treated with 10 ml 0.5 N NaOH, 10 ml H_2O, 10 ml regeneration buffer, 10 ml H_2O, 10 ml regeneration buffer and 20 ml H_2O by subsequent passing over. After washing with 10 ml distilled water, 10 ml washing buffer and 10 ml distilled water, inositol-mono-di- and triphosphate were eluted with 4 ml elution buffer and mixed with 16 ml liquid scintillator (Aquasafe 300 plus, Zinsser, Germany). Radioactivity was determined in a liquid scintillation counter.

2.2.5 Determination of cAMP content of intact HEK 293 cells by radioimmunoassay

Reagents and solutions

Stimulation medium	DMEM	
	Hepes	10 mM
	3-isobutyl-l-methylxanthine	0.25 mM
	BSA	0.5%
		pH adjusted to 7.2
Lysis buffer	Trifluoroacetic acid	0.1%
	Triton X-100	0.005%
RIA buffer	Sodium acetate	100 mM
	BSA	0.1%
	Triton X-100	0.1%
		pH adjusted to 6.0

HEK 293 cells were seeded onto 24 well plates (100,000 cells/well) and grown in DMEM supplemented with 10% fetal calf serum. After 48 h the cells were washed with 1 ml stimulation medium and incubated for 30 min at 37°C with buffer or 10 µM forskolin in the absence or presence of 1.4 nM ET-1. The stimulation was terminated by aspiration of the medium and lysis of the cells with 750 µl lysis buffer for 30 min at 4°C. The lysates were boiled for 10 min at 95°C, dried overnight in a rotation vacuum concentrator (Alpha-RVC, Christ, Germany) and finally stored at -20°C until use. For RIA (Radioimmunoassay) measurements, the dried pellets were resuspended in RIA buffer, incubated for 20 min at 4° C and finally centrifuged at 23,000 x g for 15 min at 4°C. After appropriate dilutions the concentration of cAMP in the supernatant was determined by . To improve the sensitivity samples and standards (samples of defined cAMP concentrations) were acetylated (30 µl sample in 470 µl RIA buffer) with a mixture of acetic anhydride and triethylamine (1 volume/2 volumes) under vigorous shaking at room temperature. The cAMP content was determined using ^{125}I-cAMP-tyrosylmethylester (10,000 CPM, specific activity 81.4 TBq/mmol, Biotrend, Germany) and a polyclonal rabbit anti-cAMP-antibody (final dilution 1:160,000). After an overnight incubation at 4°C the antibody-bound fraction was precipitated using 250 µl of Sepharose-conjugated sheep anti-rabbit antibody (Pharmacia decanting suspension, Pharmacia & Upjohn Diagnostics, Uppsala, Sweden). The radioactivity of the precipitate was determined in a γ-counter. Concentrations of cAMP in a range between 4 and 100 fmoles/incubate could be determined with an interassay coefficient of variation less or equal to 10%.

2.2.6 Laser Scanning Microscopy

HEK 293 cells stably expressing ET_B·GFP or Δ2-64 ET_B·GFP receptor were grown on coverslips to 80 % confluence. Then the cells were washed twice with prewarmed PBS and transferred immediately into a self-made chamber. Cells were covered with 1 ml PBS (control) with or without Cy3.5-labeled ET-1 (20 nM) for 30 min at 37°C. The GFP and Cy3.5 fluorescence were visualized on a Zeiss 410 invert laser scanning microscope (Argon/Krypton and Argon-Ion laser). Excitation and emission wave lengths were λ_{exc} = 488 nm and λ_{em} > 515 nm for GFP and λ_{exc} = 543 nm and λ_{em} > 570 nm for Cy3.5-ET-1.