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Mechanism of ET_A-receptor stimulation-induced increases in intracellular Ca^{2+} in SK-N-MC cells

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1 The mechanism underlying endothelin-1 (ET-1)-induced increases in intracellular Ca^{2+} concentrations in the human neuroblastoma cell-line SK-N-MC was investigated.

2 ET-receptor agonists increased inositol phosphate (IP)-formation (assessed as accumulation of total [^{3}H]-IPs in [^{3}H]-myo-inositol prelabelled cells) and intracellular Ca^{2+} (assessed by the FURA-2 method) with an order of potency: ET-1 > sarafotoxin 6b (S6b) > ET-3 = S6c; the ET_A-receptor antagonist BQ-123 inhibited both responses with apparent pK_i-values of 8.3 and 8.6, respectively, while the ET_B-receptor antagonist BQ-788 did not.

3 Pretreatment of the cells with pertussis toxin (PTX, 500 ng ml^{-1} overnight) reduced ET-1-induced Ca^{2+} increases by $46 \pm 5\%$, but rather enhanced ET-1-induced IP-formation.

4 Chelation of extracellular Ca^{2+} by 5 mM EGTA did not affect ET-1-induced IP-formation. However, in the presence of 5 mM EGTA or SKF 96365, an inhibitor of receptor mediated Ca^{2+} influx ($1.0-3.0 \times 10^{-5}$ M) ET-1-induced Ca^{2+} increases were inhibited in normal, but not in PTX-treated cells.

5 [^{125}I]-ET-1 binding studies as well as mRNA expression studies (by RT–PCR) detected only ET_A-receptors whereas expression of ET_B-receptor mRNA was marginal.

6 ET-1 (10^{-8} M) inhibited isoprenaline-evoked cyclic AMP increases; this was antagonized by BQ-123, not affected by BQ-788 and abolished by PTX-treatment.

7 We conclude that SK-N-MC cells contain a homogeneous population of ET_A-receptors that couple to IP-formation and inhibition of cyclic AMP formation. Stimulation of these ET_A-receptors increases intracellular Ca^{2+} by at least two mechanisms: a PTX-insensitive IP-mediated Ca^{2+} mobilization from intracellular stores and a PTX-sensitive influx of extracellular Ca^{2+}.

Keywords: Endothelin; ET_A-receptors; inositol phosphates; pertussis toxin-sensitive G-protein; SK-N-MC cells

Introduction

The human neuroblastoma cell line SK-N-MC possesses a variety of receptor systems including cyclic AMP-linked receptors (e.g. β_1-adrenoceptors (Fishman *et al*., 1991; Michel *et al*., 1993); DA_1 dopamine receptors (Sidhu & Fishman, 1990), and receptors linked to inositol phosphate (IP)-formation such as α_1-adrenoceptors (Fisher & Landon, 1991; Esbenshade *et al*., 1993), muscarinic (possibly M_1; Fisher & Landon, 1991; Fowler *et al*., 1991; Michel *et al*., 1992), Y_1-like neuropeptide Y (Feth *et al*., 1991) and endothelin (ET) receptors (Fisher & Landon, 1991; Wilkes & Boarder, 1991; Huggins *et al*., 1994). Thus, SK-N-MC cells might be a suitable tool to study the properties and interaction of different receptor systems.

It has been recently shown that, in SK-N-MC cells, activation of α_1-adrenoceptors (Esbenshade *et al*., 1993), muscarinic (Michel *et al*., 1992), and Y_1-like neuropeptide receptors (Feth *et al*., 1991) leads to increases in intracellular Ca^{2+} whereby the mechanisms underlying these effects differed: α_1-adrenoceptor stimulation caused Ca^{2+} increases by a pertussis toxin (PTX)-insensitive IP-mediated Ca^{2+} mobilization from intracellular stores and a PTX-insensitive influx of extracellular Ca^{2+} (Ebenshade *et al*., 1993), whereas PTX inhibited Ca^{2+} influx caused by carbachol (Michel *et al*., 1992) and mobilization of Ca^{2+} from intracellular stores evoked by neuropeptide Y (Feth *et al*., 1991).

Activation of ET-receptors in SK-N-MC cells has been shown to increase intracellular Ca^{2+} (Huggins *et al*., 1994; Hiley *et al*., 1992). However, whether or not this Ca^{2+} increase involves a PTX-sensitive G-protein is not known at present. The aim of this study was, therefore, to characterize further the mechanism underlying ET-induced Ca^{2+} increases in SK-N-MC cells. For this purpose we assessed the effects of several ET receptor agonists (ET-1, ET-3, sarafotoxin 6b [S6b] and S6c) on IP-formation and increases in intracellular Ca^{2+} levels; furthermore, we studied the influence of the selective ET_A-receptor antagonist BQ-123 (Moreland, 1994) on these effects in order to subclassify the ET-receptor mediating these responses. Moreover, we pretreated SK-N-MC cells with PTX to find out whether or not a PTX-sensitive G-protein might be involved in ET-induced IP-formation and/or increases in intracellular Ca^{2+}. And finally, we tried to find out which ET-receptor subtype might be present in SK-N-MC cells; for this purpose we assessed the abundance of ET_A- and ET_B-receptors on a protein- (by [^{125}I]-ET-1 binding) and mRNA-level (by the reverse transcriptase polymerase chain reaction (RT–PCR)).

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Methods

Cell culture

SK-N-MC cells were obtained from American Type Culture Collection (Rockeville, Maryland, U.S.A.). Cells were grown at 37°C in a humidified incubator under 5% CO_2/95% air in MEM medium supplemented with foetal calf serum (10%), L-glutamine (4 mM), non-essential amino acids (1%), sodium pyruvate (1 mM) and penicillin/streptomycin (100 IU and 100 μg ml^{-1}, respectively) as previously described (Feth *et al*., 1991). Cell medium was replaced twice weekly. After attaining confluence, the cells were subcultured by trypsinization. In some experiments 500 ng ml^{-1} PTX were added to the incubation medium of subconfluent cells 24 h before cell harvesting.

Measurement of intracellular Ca^{2+} concentration

The intracellular free Ca^{2+} concentration was estimated using the fluorescent indicator dye fura-2/AM and a Hitachi F-2000 spectrofluorimeter as previously described (Feth *et al*., 1991). Briefly, aliquots of cells, cultured for 5 days, were separated by centrifugation at $200 \times g$ for 10 min. Cells were resuspended in phosphate-buffered saline (PBS) supplemented with 1 mg ml^{-1} glucose and 1 mg ml^{-1} bovine serum albumin. Then 1 μM fura-2/AM was added, and the cells were incubated for 60 min at room temperature (20–23°C). Unloaded fura-2/AM was removed by centrifugation at $200 \times g$ for 10 min. Cells were resuspended at a density of 1×10^6 ml^{-1} in PBS supplemented with 1 mg ml^{-1} glucose. After two washes the cells were resuspended at 1×10^6 cells ml^{-1} transferred to a quartz cuvette and stirrred continuously. Fluorescence emission (510 nm) was monitored with the wavelength cycling between 340 and 380 nm. At the end of each experiment, fluorescence maximum and minimum values at each excitation wavelength were obtained by first lysing the cells with 0.02% digitonin (maximum) and then adding 10 mM EGTA (minimum). Raw fluorescence data were converted to Ca^{2+} concentrations with software supplied by the manufacturer, that uses the equation given by Grynkiewicz *et al*. (1985).

Inositol phosphate determination

Subconfluent cells were incubated in 175 cm^2 culture flasks for 24 h with 20 ml medium 199 supplemented with 5% foetal calf serum, 2 mM L-glutamine, and penicillin/streptomycin (100 IU and 100 μg ml^{-1}, respectively) and 100 μCi [^{3}H]-myo-inositol. Thereafter cells were peeled off by trypsin-EDTA treatment and unincorporated [^{3}H]-myo-inositol was washed out by centrifugation and resuspension in Hanks' buffered saline solution (HBSS: 140 mM NaCl, 5 mM KCl, 25 mM $NaHCO_3$, 1.2 mM $MgSO_4$, 1.2 mM KH_2PO_4, 2.5 mM $CaCl_2$, 10 mM HEPES) supplemented with 10 mM LiCl and 1% bovine serum albumin. Aliquots (970 μl) of the suspension (6×10^6 cells ml^{-1}) were then incubated with the ET-agonists in the presence or absence of the ET-antagonists for 30 min at 37°C in a final volume of 1 ml. (±)-Propranolol (10 μM) was present in all experiments. The incubation was stopped by addition of 1 ml ice-cold methanol and 2 ml chloroform. The mixture was vigorously vortexed twice, and thereafter the phases were separated by centrifugation at $820 \times g$ for 10 min at 4°C. Aliquots (1.6 ml) of the upper phase were placed on Dowex AG 1-X8 columns (200 mg/column). Free inositol was eluted twice with 5 ml H_2O and 5 ml of 60 mM ammonium formate. Total inositol phosphates were eluted by addition of 2×1 ml 1 M ammonium formate dissolved in 100 mM formic acid. Each data point was determined in quadruplicates in each experiment.

Radioligand binding sites

SK-N-MC cells were harvested using a 'rubber policeman' and washed twice by centrifugation at $400 \times g$. They were homogenized in ice-cold 10 mM Tris-HCl, 1 mM EGTA buffer pH 7.4 containing 0.25 mM phenylmethylsulphonyl-fluoride (PMSF) with a glass-Teflon homogenizer (Braun, Melsungen, Germany) with 15 strokes at 1500 rev min^{-1}. Thereafter the suspension was kept on ice for 10 min, and centrifuged at $1000 \times g \times 15$ min; the pellets were discarded, the supernatant filtered through four layers of cheesecloth and centrifuged at $50{,}000 \times g \times 20$ min. The pellets were washed twice by resuspension and recentrifugation and finally resuspended in incubation buffer (HBSS-buffer containing 0.1% bovine serum albumin and 1 mg ml^{-1} soybean trypsin inhibitor) to yield a protein concentration of 100 μg ml^{-1}. Protein was assessed by the Bradford method (Bradford, 1976), using immunoglobulin G as standard. Membranes (20–40 μg protein) were incubated with 15 different concentrations of ET-1 ranging from 10^{-13}–10^{-6} M and approximately 10,000–15,000 c.p.m. of [^{125}I]-ET-1 for 90 min at 37°C. Bound ligand was separated by vacuum filtration over Whatman GF/C filters coated with 4% bovine serum albumin followed by washing with 2×10 ml of 10 mM Tris-HCl buffer 7.4. The radioactivity of the wet filters was determined in a Gamma-Counter (Cobra Autogamma, Packard, Meriden, CT, U.S.A.) at an efficiency of 80%. Non-specific binding was defined as binding not displaced by 1 μM bosentan.

To assess the relative amount of ET_A- and ET_B-receptors in membranes from SK-N-MC cells membranes were incubated with 10,000–15,000 c.p.m. of [^{125}I]-ET-1 and various concentrations of either the selective ET_A-receptor antagonist BQ-123 (10^{-10}–10^{-5} M), the selective ET_B-receptor antagonist BQ-788 (10^{-10}–10^{-6} M (Ishikawa *et al*., 1994)), or the non-selective ET_A- and ET_B-receptor antagonist bosentan (10^{-10}–10^{-5} M (Clozel *et al*., 1994)). Specific binding was determined as described above.

RNA isolation

Total RNA from cells was isolated by guanidinium thiocyanate/cesium chloride centrifugation according to standard methods (Sambrook *et al*., 1989).

Construction of standards for competitive reverse transcriptase polymerase chain reaction

Human ET_A- and ET_B-receptor mRNA was quantified by standard calibrated RT–PCR. First, ET_A- and ET_B-specific cDNA fragments of 928 basepairs (bp) (ET_A, position 522–1449 according to Hosada *et al*., 1991) and 702 bp (ET_B, position 838–1539 according to Ogawa *et al*., 1991) were amplified by PCR using following primers: ET_A sense primer: 5′-CACTGGTTGGATGTGTAATC-3′, ET_A antisense primer: 5′-AGAGGGAACCAGCAAAGAGC-3′; and ET_B sense primer: 5′-CGAGCTGTTGCTTCTTGGAGTAG-3′, ET_B antisense primer: 5′-ACGGAAGTTGTCATATCCGT-GATC-3′, respectively.

ET_A- and ET_B-specific fragments were subsequently cloned into pCR-Script Amp SK(+) Cloning Vector (Stratagene,

Heidelberg, Germany) and their identity confirmed by DNA sequencing (ABI PRISM™ Dye Terminator Cycle Sequencing Ready Reaction Kit with AmpliTaq DNA Polymerase, FS, Perkin-Elmer Co., Foster City, CA, U.S.A.; ABI 373 DNA Sequencer) (data not shown). Second, internal deleted cRNA standards were constructed (modified after Förster, 1994). Briefly, ET_A- and ET_B-specific cDNA fragments were amplified with 30-mer linker primers and antisense primers. Linker primers included at 5′-end 10 bp of 3′-end of ET_A or ET_B sense primers, followed at 3′-end by 20 bp of ET_A- or ET_B-specific cDNA sequences, located 89 or 174 bp in 3′-direction from sense primer (ET_A linker primer: 5′-ATGTGTAATCTACCACTCATCAACCCACTA-3′, ET_B linker primer: 5′-CTTGGAGTAGGCAGTTTTACAAGA-CAGCAA-3′). The cDNA fragments subsequently isolated were reamplified with ET_A- and ET_B-specific sense and antisense primer. This method resulted in internal deleted ET_A- and ET_B-cDNA standards bound by sense and antisense primers of first PCR amplification step (length of ET_A-standard: 839 bp, and ET_B-standard: 528 bp). The identity of standards was confirmed by cloning and DNA sequencing as described (data not shown).

Third, internal deleted cDNA standards were *in vitro* transcribed into cRNA (RNA Transcription Kit, Stratagene, Heidelberg, Germany). Finally, standard cRNA was quantified spectrophotometrically (Sambrook *et al*., 1989).

Quantification of human ET_A- and ET_B-receptor mRNA by competitive reverse transcriptase polymerase chain reaction

In competitive RT–PCR experiments equal amounts of total RNA (100 ng) were incubated in separate reactions with defined amounts of ET_A- and ET_B-standard cRNA (10^7, 10^6, 10^5, 10^4 and 10^3 cRNA molecules, respectively) for 3 min at 70°C, and subsequently reverse-transcribed into cDNA using random hexamer primers and Superscript II Plus reverse transcriptase (Life Technologies, Eggenstein, Germany) for 1 h at 42°C. Afterwards, 20% of each reverse transcription reaction were amplified in separate reactions with 20 pmol ET_A or ET_B sense and antisense primers by following PCR protocols: ET_A: 38 cycles: 30 s 95°C, 30 s 58°C, 30 s 72°C; ET_B: 40 cycles: 30 s 95°C, 1 min 69°C, 1 min 72°C.

PCR primers compete for sample-specific and standard molecules in the amplification reaction. The PCR reactions were separated by standard agarose gel electrophoresis, stained with ethidium bromide and documented by photography using Polaroid film 665. The optical density of sample specific and standard PCR fragment was estimated by a densitometer (Personal Densitometer, Molecular Dynamics, Sunny Vale, CA, U.S.A.). Density of standard PCR fragments was normalized with a correction coefficient (ET_A: 928 bp/839 bp = 1.106; ET_B: 702 bp/528 bp = 1.329), and the logarithm of the quotient of sample specific and standard PCR fragment density was plotted graphically vs amount of standard RNA molecules. In this graph equal amounts of RNA molecules in sample and standard are present at equivalence point.

Cyclic AMP determination

Cells were suspended in phosphate buffered saline containing 10^{-4} M isobutylmethylxanthine. Aliquots of 300 μl (1×10^6 cells) were incubated for 15 min at 37°C in the presence or absence of isoprenaline (10^{-9}–10^{-5} M) in a total volume of 330 μl. In some experiments ET-1 (10^{-8} M), BQ-123 (10^{-6} M) or BQ-788 (10^{-6} M) were added. Incubation was terminated by boiling the entire reaction mixture for 4 min. After cooling, samples were centrifuged at $12{,}000 \times g$ for 5 min, and the cyclic AMP content was determined in 100 μl aliquots of the supernatant by using a radioimmunoassay (detection limit for cyclic AMP: 0.5 pmol cyclic AMP/assay).

Data analysis

Data given are means ± s.e.m. of n experiments. Experimental data were analysed by computer-supported iterative non-linear regression analysis using the InPlot program (GraphPad Software, San Diego, CA, U.S.A.). In the [^{125}I]-ET-1 binding studies, B_{max} values were calculated from the ET-1 competition curve as described by DeBlasi *et al*. (1989). For details see Becker *et al*. (1996). Data from ET-1-induced IP-formation and Ca^{2+} increases were fitted to sigmoid function; in these calculations the bottom of the curves was fixed at 0 (i.e. 0% IP-stimulation over basal or no Ca^{2+} increase) and stimulation of IP-formation or Ca^{2+} increases induced by 1 μM ET-1 were taken as maximal stimulation; the Hill-slopes were kept variable. From these curves EC_{50}-values were obtained that were not significantly different from those calculated with a Hill-slope fixed at 1.0 and/or with a non-fixed maximal stimulation induced by ET-1.

The apparent BQ-123 affinity (K_i) for inhibition of ET-1-induced IP-accumulation or Ca^{2+} increases was calculated according to the Cheng & Prusoff-equation (Cheng & Prusoff, 1973):

$$K_i = IC_{50}/([S]/EC_{50}) + 1$$

with IC_{50} being the concentration of BQ-123 yielding half-maximal inhibition of ET-1-induced IP-formation, [S] the concentration of ET-1 in the assay and EC_{50} the concentration of ET-1 causing 50% of maximal IP-formation or Ca^{2+} increases (assessed as described above).

Statistical significance of differences was analysed by unpaired two-tailed Student's t-test or, if appropriate, by repeated measures analysis of variance followed by the t-test using Bonferroni corrections for multiple comparison. A P-value < 0.05 was considered to be significant. All statistical calculations were performed with the Instat program (GraphPad Software, San Diego, CA, U.S.A.).

Chemicals

Endothelin-1, endothelin-3, sarafotoxins 6b and -6c, and BQ-123 (Cyclo[D-Asp-Pro-D-Val-Leu-D-Trp]) were purchased from Saxon Biochemicals (Hannover, Germany); BQ-788 (Dmpc-L-γ-MeLeu-D-Trp(1-CO_2CH_3)-D-Nle-OH) from Alexis Corporation (Grünberg, Germany); (±)-propranolol hydrochloride, (−)-isoprenaline bitartrate, trypsin (crude) and fura-2/AM from SIGMA (Deisenhofen, Germany); SKF 96365 hydrochloride (1-[β-[3-(4-methoxyphenyl) propoxy]-4-methoxyphenethyl]-1H-imidazole, HCl) from Calbiochem Novabiochem Corporation, La Yola, CA, U.S.A.; and [^{3}H]-myo-inositol (specific activity 80–120 Ci mmol^{-1}, prepurified with PT6-271) and a radioimmunoassay for cyclic AMP from Amersham (Braunschweig, Germany), [^{32}P]-NAD (specific activity 30 Ci mmol^{-1}) from New England Nuclear (Dreieich, Germany). Hanks' balanced salt solution, culture medium M199, and Penicillin-Streptomycin were obtained from Life Technologies (Eggenstein, Germany), Bosentan (sodium salt) was a gift from Dr M. Clozel, Hoffman-La Roche Ltd (Basel, Switzerland).

All other chemicals were of the highest purity grade commercially available.

Results

Endothelin-induced inositol phosphate formation

In SK-N-MC cells ET-1 (10^{-12}–10^{-6} M) concentration-dependently increased IP-formation; the maximal increase at 10^{-6} M was 148±39% above basal (Figure 1a); the pEC_{50} value for ET-1 was 9.8±0.3 ($n=6$). ET-1 was about 10,000 times more potent than ET-3. Among the sarafotoxins investigated S6b (10^{-8}–10^{-6} M) stimulated IP-formation (pEC_{50}-value 7.1±0.3), while S6c (up to 10^{-6} M) was without any effect.

The selective ET_A-receptor antagonist BQ-123 (10^{-10}–10^{-5} M) concentration-dependently inhibited 10^{-8} M ET-1-induced IP-formation (Figure 2); the apparent pK_i-value for BQ-123 was 8.3±0.3 ($n=5$). On the other hand, the selective ET_B-receptor antagonist BQ-788 (10^{-9}–10^{-6} M) only marginally affected 10^{-8} M ET-1-induced IP-formation: maximal inhibition at 10^{-6} M BQ-788 was only 12.9±5.5% ($n=4$).

Pretreatment of SK-N-MC cells with PTX (500 ng ml^{-1} for 24 h) completely inactivated PTX-substrates (Figure 3). In PTX-pretreated cells the ET-1-induced increase in IP-formation was not attenuated but rather enhanced: in these experiments the maximal increase in IP-formation in non-treated cells was 118±9% ($n=5$), whereas in PTX-treated cells it was 171±31% ($n=5$, Figure 4). This difference, however, did not reach statistical significance ($P=0.1392$).

Addition of the Ca^{2+} chelating agent EGTA (5 mM) to the medium led to a significant increase in basal IP-formation (1461±248 c.p.m. ($n=6$) vs 4446±738 c.p.m. ($n=6$), $P<0.05$); in the presence of EGTA 10^{-8} M ET-1-induced IP-formation was less than that in the absence of EGTA if the data were expressed in per cent above basal (without EGTA 116.3±37%, $n=6$; with EGTA 38.9±8%, $n=6$, $P=0.0681$); however, absolute increases in IP-formation were not different between cells with or without EGTA (without EGTA 1342±225 c.p.m.; with EGTA 1702±450 c.p.m., $n=6$). Thus, chelation of extracellular Ca^{2+} appears not to affect ET-1-

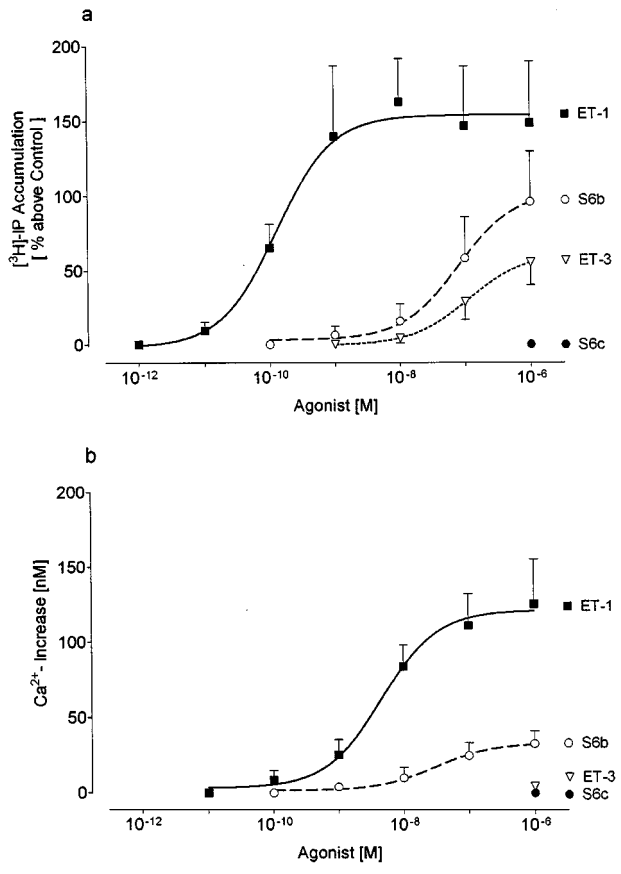

Figure 1 (a) Effects of endothelin-1 (ET-1), sarafotoxin 6b (S6b), ET-3 and S6c on inositol phosphate (IP)-formation in SK-N-MC cells. Ordinate: [^{3}H]-IP-formation in per cent above basal; Abscissa: molar concentrations of the ET-agonists. Values are means±s.e.m. of six experiments. Basal [^{3}H]-IP-formation was about 1–2% of the incorporated radioactivity and amounted to 1437±222 c.p.m. ($n=21$). (b) Effects of ET-1, ET-3, S6b and S6c on intracellular free Ca^{2+} in SK-N-MC cells. Ordinate: Increase in intracellular Ca^{2+} in nM; Abscissa: molar concentrations of the ET-agonists. Values are means±s.e.m. of six experiments.

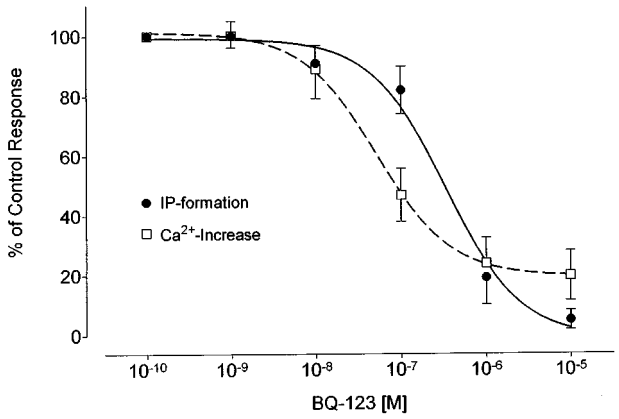

Figure 2 Inhibition of 10^{-8} M endothelin-1 (ET-1)-induced inositol phosphate (IP)-formation and of 10^{-7} M ET-1-induced increases in intracellular Ca^{2+} by BQ-123 in SK-N-MC cells. Ordinates: ET-1-induced IP-formation and increase in intracellular Ca^{2+} in % of control (i.e. in the absence of BQ-123 = 100%); Abscissa: molar concentrations of BQ-123. Means±s.e.m. of five experiments each.

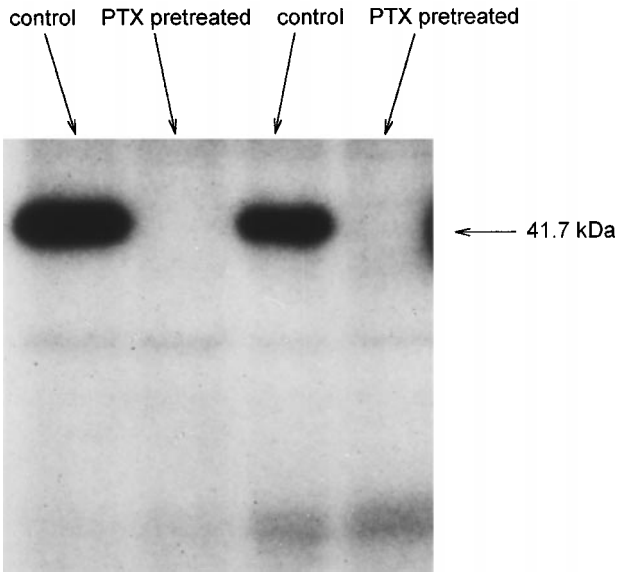

Figure 3 Effect of pertussis toxin (PTX) treatment of SK-N-MC cells on PTX-catalyzed ADP-ribosylation of membranes derived from these SK-N-MC cells. Cells were treated with 500 ng PTX ml^{-1} culture medium for 20 h at 37°C. Following washout membranes were prepared and incubated with fresh PTX and [^{32}P]-NAD for 1 h at 30°C. The figure shows an autoradiogram of the SDS–polyacrylamide gel analysis of the reaction products. Lanes were loaded with membranes vehicle-treated cells (control) or from PTX-treated cells.

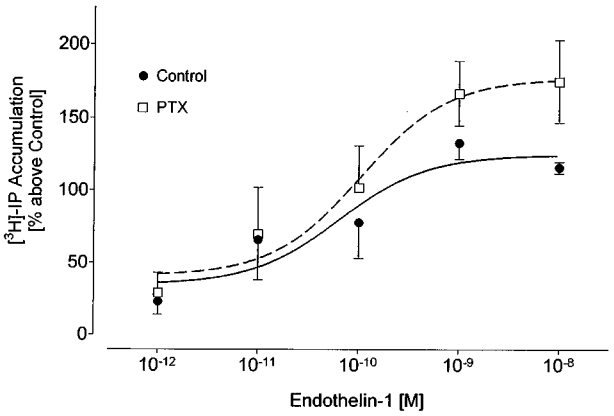

Figure 4 Effect of pertussis toxin (PTX, 500 ng ml^{-1}) treatment overnight on endothelin-1 (ET-1)-induced inositol phosphate (IP)-formation in SK-N-MC cells. For details see legend to Figure 1. Basal [^{3}H]-IP-formation was in control cells 1846±489 c.p.m. ($n=5$), in PTX-treated cells 1549±335 c.p.m. ($n=5$). Means±s.e.m. of five experiments.

induced increases in IP-formation. Similar findings have been also obtained in rat renal slices where EGTA increased basal IP-formation but did not affect absolute increases in IP-formation induced by noradrenaline (Büscher *et al.*, 1994).

Endothelin-induced increases in intracellular Ca^{2+}

ET-1 rapidly induced transient increases in intracellular Ca^{2+} in SK-N-MC cells. Figure 5 shows an original tracing. ET-1 ($10^{-11}-10^{-6}$ M) concentration-dependently increased Ca^{2+}; the maximal increase at 10^{-6} M was 125±23 nM (Figure 1b); the pEC_{50} value for ET-1 was 8.3±0.4 ($n=6$). S6b ($10^{-9}-10^{-6}$ M) was only a weak agonist in this system; the maximal increase at 10^{-6} M was only 37±7 nM. However, it should be mentioned that at a concentration of 10^{-6} M the S6b effect appeared not to be maximal (Figure 1b). On the other hand, S6c and ET-3 (up to 10^{-6} M) were without any effects.

The selective ET_A-receptor antagonist BQ-123 ($10^{-9}-10^{-5}$ M) concentration-dependently inhibited Ca^{2+} increases induced by 10^{-7} M ET-1 (Figure 2); the apparent pK_i-value for BQ-123 was 8.6±0.5 ($n=5$). The selective ET_B-receptor antagonist BQ-788 ($10^{-9}-10^{-6}$ M), on the other hand, did not significantly inhibit Ca^{2+} increases induced by 10^{-7} M ET-1; the maximal inhibition at 10^{-6} M BQ-788 was 9.2±9.1% ($n=3$).

Chelation of extracellular Ca^{2+} by 5 mM EGTA significantly attenuated ET-1-induced Ca^{2+} increases by 59±7% ($n=4$, Figure 6); nevertheless, in the absence of extracellular Ca^{2+}, ET-1 still induced an increase in intracellular Ca^{2+} by 48±6 nM (Figure 6).

In PTX-treated SK-N-MC cells maximal increase in intracellular Ca^{2+} induced by 10^{-6} M ET-1 was reduced by 46±5% ($n=5$, Figure 7a); on the other hand, PTX-pretreatment did not affect ET-1-induced increase in intracellular Ca^{2+} in cells, in which extracellular Ca^{2+} had been removed through chelation by 5 mM EGTA (Figure 7b).

To further characterize the role of PTX-sensitive G-protein in ET-1-induced Ca^{2+} increases we studied the effects of SKF 96365, a receptor mediated Ca^{2+} entry inhibitor that has been shown to block agonist-activated Ca^{2+} influx in a variety of cells without affecting Ca^{2+} release from internal stores (Merritt *et al.*, 1990), on 10^{-7} M ET-1-induced Ca^{2+} increases in normal and PTX-treated cells. SKF 96365 (1.5×10^{-5} M)

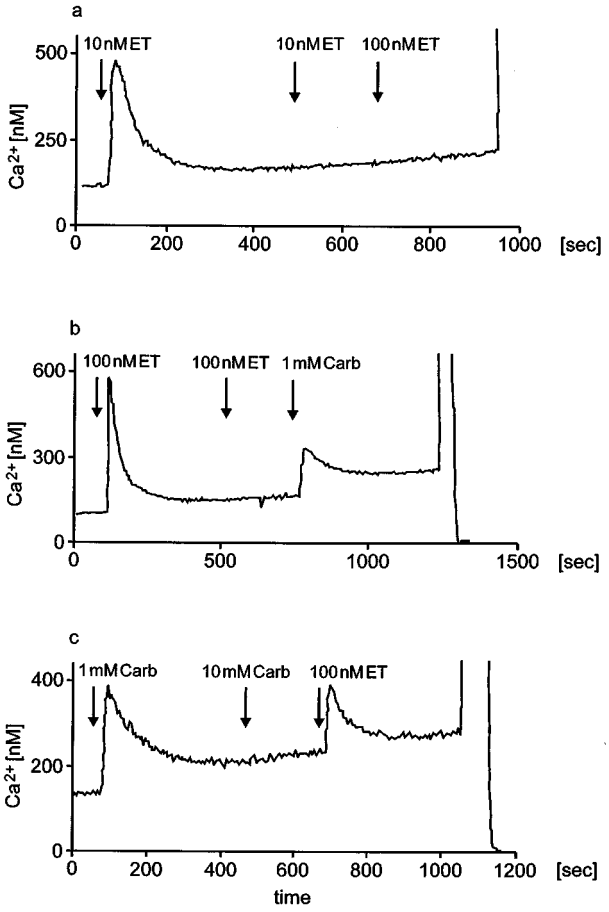

Figure 5 (a) Representative traces showing Ca^{2+} mobilization by endothelin-1 (ET) in SK-N-MC cells; (b) also shown is cross-desensitization of ET-1 and carbachol (Carb)-induced Ca^{2+} mobilization. (c) Representative traces showing Ca^{2+} mobilization by carbachol (Carb) in SK-N-MC cells. Also shown is cross-desensitization of carbachol and endothelin-1 (ET)-induced Ca^{2+} mobilization.

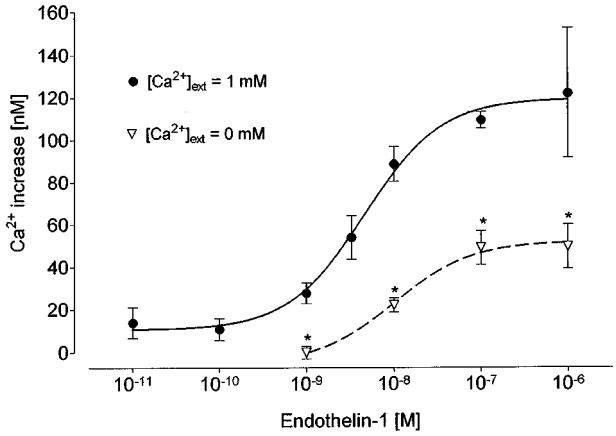

Figure 6 Effect of chelation of extracellular Ca^{2+} by 5 mM EGTA on endothelin-1 (ET-1)-induced increase in intracellular Ca^{2+} in SK-N-MC cells. Ordinate: Increase in intracellular Ca^{2+} in nM; Abscissa: molar concentrations of ET-1. Means±s.e.m. of 3–4 experiments. *$P<0.05$ vs the corresponding value at 1 mM extracellular Ca^{2+}. Basal Ca^{2+}-content was in the absence of EGTA 139.2±24.9 nM, in the presence of EGTA 38.2±14.7 nM.

significantly inhibited ET-1-induced Ca^{2+} increases in non-treated cells, but not in PTX-treated cells; similarly, at 3.0×10^{-5} M SKF 96365 inhibited ET-1-induced Ca^{2+} increases in non-treated cells more markedly than in PTX-treated cells, although the difference did not reach statistical significance (Table 1). Higher concentrations of SKF 96365, however, inhibited ET-1-induced Ca^{2+} increases in non-treated cells as well as in PTX-treated cells to a similar extent (Table 1).

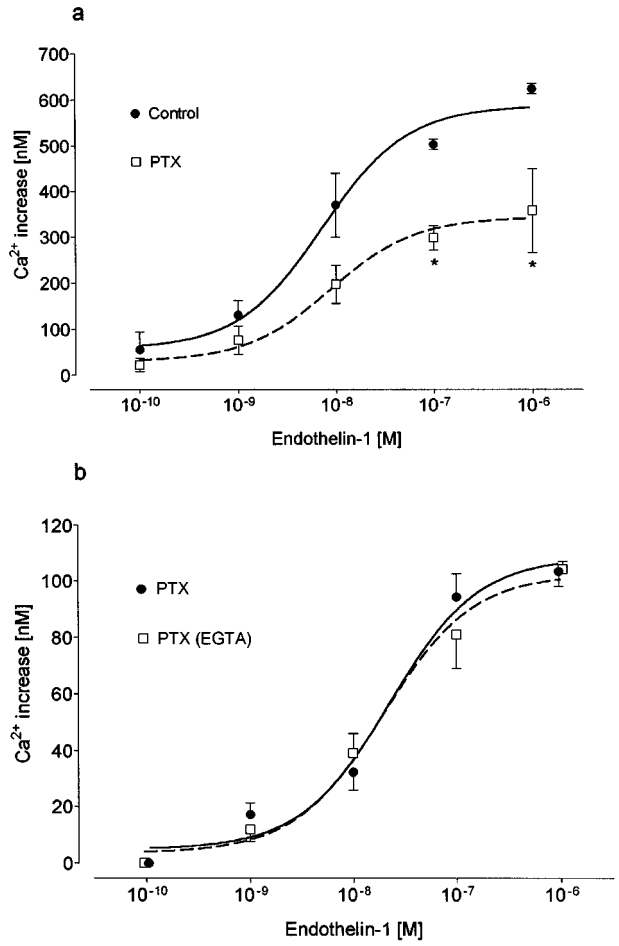

Figure 7. (a) Effect of pertussis toxin (PTX, 500 ng ml^{-1}) treatment overnight on endothelin-1 (ET-1)-induced increase in intracellular Ca^{2+} in SK-N-MC cells. Ordinate: Increase in intracellular Ca^{2+} in nM; Abscissa: molar concentrations of ET-1. Means ± s.e.m. of five experiments. $^*P<0.05$ vs the corresponding value on control cells. Basal Ca^{2+}-content was in control cells 184.5 ± 19.9 nM, in PTX-treated cells 232.7 ± 32.2 nM. (b) Effect of pertussis toxin (PTX, 500 ng ml^{-1}) treatment overnight and chelation of extracellular Ca^{2+} by 5 mM EGTA on endothelin-1 (ET-1)-induced increase in intracellular Ca^{2+} in SK-N-MC cells. Ordinate: Increase in intracellular Ca^{2+} in nM; Abscissa: molar concentrations of ET-1. Means ± s.e.m. of three experiments. Basal Ca^{2+}-content was in the absence of EGTA in PTX-treated cells 197.7 ± 43.1 nM, in the presence of EGTA in PTX-treated cells 60.9 ± 15.8 nM.

Investigation of the time-course of the ET-1-induced increase in Ca^{2+} revealed that the agonist-induced Ca^{2+} increases were only transient, they peaked within 20–30 s and thereafter declined to a small but sustained plateau although agonist was present throughout the experiment (Figure 5a). A second addition of the same or even a higher concentration of ET-1 caused virtually no response. This desensitized state was maintained for at least 30 min following washout of ET-1.

It has been recently shown that SK-N-MC cells contain muscarinic receptors that couple to increases in intracellular Ca^{2+} (Michel *et al*., 1992). We, therefore, investigated whether the Ca^{2+} response to carbachol might undergo similar rapid desensitization. As shown in Figure 5c, 1 mM carbachol induced a rapid, transient increase in Ca^{2+} that peaked within 20–30 s and then declined to a small but sustained plateau; a second addition of 1 mM carbachol did increase intracellular Ca^{2+} only by 4 ± 4% of the initial response. However, in carbachol desensitized SK-N-MC cells ET-1 was still capable of increasing Ca^{2+} (Figure 5c) as was carbachol capable of increasing Ca^{2+} in ET-1 desensitized SK-N-MC cells (Figure 5b) indicating that, in these cells, carbachol and ET-1 desensitize preferentially their own receptors.

Receptor binding and mRNA-studies

In receptor binding studies ET-1 displaced [^{125}I]-ET-1 binding with a monophasic steep competition curve and a pseudo-Hill coefficient of 0.98 ± 0.01 ($n=3$). From these curves an apparent K_D-value of 88 ± 12 pM ($n=3$) and a B_{max} of 922 ± 155 fmol mg^{-1} protein ($n=3$) was calculated. Among the ET-receptor antagonists BQ-123 (10^{-10}–10^{-5} M) and bosentan (10^{-10}–10^{-5} M) inhibited [^{125}I]-ET-1 binding with monophasic competition curves and Hill slopes not significantly different from unity; the apparent pK_i value for BQ-123 was 8.3 ± 0.3, ($n=3$); for bosentan it was 8.0 ± 0.3 ($n=3$). On the other hand, the selective ET_B-receptor antagonist BQ-788 caused, at the highest tested concentration (10^{-6} M), only a 36% inhibition of [^{125}I]-ET-1 binding.

Expression of ET_A- and ET_B-receptor mRNA in SK-N-MC cells was determined by standard calibrated competitive RT–PCR. SK-N-MC cells expressed predominantly ET_A-receptor mRNA (Figure 8A). The ET_A-receptor concentration in SK-N-MC cells was estimated to be $2.04 \pm 0.37 \times 10^6$ mRNA molecules μg^{-1} RNA.

In contrast, SK-N-MC cells expressed only very little, if any, ET_B-receptor mRNA ($<10^4$ molecules μg^{-1} RNA (Figure

Table 1 Effects of SKF 96365 (SK) on ET-1 (10^{-7} nM)-induced Ca^{2+} increases in PTX-treated and non-treated SK-N-MC cells

	Non-treated cells (%)	*PTX-treated cells (%)*
ET-1 (10^{-7} M)	100	100
ET-1 (10^{-7} M) + 1.0×10^{-5} M SK	95.8 ± 4.2 (7)	118.6 ± 4.4 (3)
ET-1 (10^{-7} M) + 1.5×10^{-5} M SK	48.0 ± 4.8 (5)	81.9 ± 9.4 (3)[a]
ET-1 (10^{-7} M) + 3.0×10^{-5} M SK	38.5 ± 7.8 (7)	69.4 ± 18.1 (3)
ET-1 (10^{-7} M) + 6.0×10^{-5} M SK	16.4 ± 2.7 (7)	24.7 ± 6.3 (3)

Data are given in % of the ET-1 (10^{-7} M)-induced Ca^{2+} increases (= 100%). Ca^{2+} increases were in non-treated cells: 605.6 ± 10.6 nM ($n=7$), in PTX-treated cells: 421.2 ± 24.4 nM ($n=3$). Means ± s.e.m.; number of experiments in parentheses. [a]$P<0.05$ vs the corresponding value in non-treated cells.

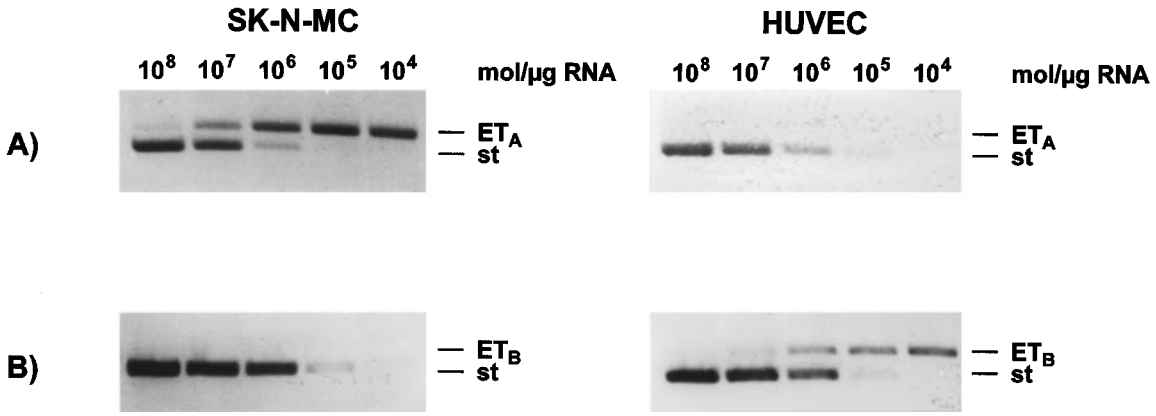

Figure 8 Expression of ET_A- and ET_B-receptor mRNA in SK-N-MC cells. ET_A- and ET_B-receptor mRNA was quantified by standard calibrated competitive RT–PCR. Equal amounts of RNA from human SK-N-MC cells and umbilical vein endothelial cells (HUVEC) were reverse transcribed with indicated amounts of internal deleted ET_A- and ET_B-cRNA molecules. Reverse transcribed ET_A- (A) and ET_B- (B) cDNA molecules were subsequently amplified by PCR, separated by agarose gel electrophoresis and their concentration determined densitometrically as described in Methods. The optical density of ET receptor-specific fragments indicate the amount of ET receptor mRNA expression in the corresponding cell type, compared to internal deleted standards (St). The gel shown is representative for three independent experiments. SK-N-MC cells express nearly exclusively ET_A- and HUVEC mainly ET_B-mRNA.

8B). In order to ascertain that our method is capable of detecting ET_B-receptor mRNA, we assessed the amount of ET_B-receptors in human endothelial cells known to possess ET_B-receptors (Ogawa *et al*., 1991). As shown in Figure 8B ET_B-receptor mRNA expression could be clearly demonstrated in the human endothelial cells: the ET_B-receptor concentration was $1.91 \pm 0.25 \times 10^5$ mRNA molecules μg^{-1} RNA.

Effects of endothelin on isoprenaline-induced elevation of cyclic AMP

Finally, in order to characterize further the functional importance of the PTX-sensitive G-protein present in SK-N-MC cells we assessed the effects of ET-1 on isoprenaline-induced cyclic AMP-increases. Isoprenaline (10^{-9}–10^{-5} M) concentration-dependently increased cyclic AMP-content in the SK-N-MC cells (Table 2a); EC_{50}-value was 21 ± 4 nM ($n = 7$). ET-1 (10^{-8} M) significantly inhibited 10^{-5} M isoprenaline-induced cyclic AMP increase (Table 2b); this inhibitory effect of ET-1 was completely suppressed by 10^{-6} M BQ-123, but nearly not affected by 10^{-6} M BQ-788. In PTX-treated cells, however, ET-1 failed to inhibit isoprenaline-induced cyclic AMP increase (Table 2b).

Table 2 (a) Effects of isoprenaline on cyclic AMP-content in SK-N-MC cells

	cyclic AMP content (pmol/10^6 *cells*)
Basal	128 ± 51
Isoprenaline (10^{-9} M)	152 ± 51
Isoprenaline (10^{-8} M)	430 ± 99
Isoprenaline (10^{-7} M)	900 ± 189
Isoprenaline (10^{-6} M)	1019 ± 211
Isoprenaline (10^{-5} M)	1065 ± 207

Means ± s.e.m. of six experiments

Table 2 (b) Effects of ET-1 (10^{-8} M) on isoprenaline (10^{-5} M)-induced cyclic AMP-increases in PTX-treated and non-treated SK-N-MC cells

	(%)
Non-treated cells	
Isoprenaline (10^{-5} M)	100
Isoprenaline (10^{-5} M) + ET-1 (10^{-8} M)	57.4 ± 5.5 (7)
Isoprenaline (10^{-5} M) + ET-1 (10^{-8} M) + BQ-123 (10^{-6} M)	91.1 ± 8.6 (3)[a]
Isoprenaline (10^{-5} M) + ET-1 (10^{-8} M) + BQ-788 (10^{-6} M)	68.1 ± 2.6 (3)
PTX-treated cells	
Isoprenaline (10^{-5}M) + ET-1 (10^{-8}M)	87.3 ± 2.3 (4)[a]

Data are given in % of the isoprenaline (10^{-5} M)-induced cyclic AMP-increase (= 100%). Means ± s.e.m.; number of experiments in parentheses. [a]$P < 0.05$ vs isoprenaline (10^{-5} M) + ET-1 (10^{-8} M).

Discussion

In the present study ET-receptor agonists stimulated IP-formation in the human neuroblastoma cell line SK-N-MC with an order of potency of ET-1 > S6b > > ET-3 = S6c indicating that this response is mediated by an ET_A-receptor (Masaki *et al*., 1994). The same order of potency was obtained for ET receptor agonists induced increases in intracellular Ca^{2+} which is in favour of the idea that both IP-formation and increases in intracellular Ca^{2+} involve the same ET receptor subtype. This view is strongly supported by the fact that the selective ET_A-receptor antagonist BQ-123 inhibited ET-1-induced IP-formation and increases in intracellular Ca^{2+} with high affinity and nearly identical apparent pK_i values (8.3 and 8.6, respectively); on the other hand, the selective ET_B-receptor antagonist BQ-788 was only a very weak inhibitor of the ET-1 responses. The high affinity of BQ-123 and the low affinity of BQ-788 for the ET receptor involved in these responses is compatible with the view that SK-N-MC cells contain a homogeneous class of ET_A-receptors that couple to IP-formation and increases in intracellular Ca^{2+}. Our radioligand binding, as well as the mRNA expression studies, support this view. In binding studies [^{125}I]-ET-1 binding was completely

inhibited by BQ-123 with an apparent pK_i value (8.3) that was nearly identical with that obtained in the IP and Ca^{2+} experiments whereas the ET_B-receptor antagonist BQ-788, even at the high concentration of 10^{-6} M only incompletely inhibited [^{125}I]-ET-1 binding. Moreover, our RT–PCR experiments clearly detected expression of mRNA for the ET_A-receptor but there was only marginal expression of mRNA of ET_B-receptor, if any at all.

Thus, the present results confirm and extend the previous literature on the existence of ET_A-receptors in SK-N-MC cells (Fisher & Landon, 1991; Wilkes & Boarder, 1991; Huggins *et al*., 1994). In radioligand binding studies ET-receptor agonists inhibited binding of [^{125}I]-ET-1 with an order of potency of ET-1 > S6b > ET-3 (Wilkes & Boarder, 1991; Huggins *et al*., 1994) an order of potency that is almost identical with that obtained in the present study. Moreover, BQ-123 inhibited [^{125}I]-ET-1 binding with a monophasic competition curve and a pseudo Hill coefficient of 0.9 (Huggins *et al*., 1994); its apparent K_i-value (0.87 nM) was in a similar range as the apparent K_i-values obtained in the present study (2.5 and 5 nM).

In the present study ET-1 induced, after chelation of extracellular Ca^{2+} by 5 mM EGTA, increases in Ca^{2+} that were significantly reduced but not completely abolished. These results demonstrate that the ET-1-induced increase in Ca^{2+} is partly derived from an influx of extracellular Ca^{2+} and partly from mobilization from intracellular stores. Similar findings have been made by other investigators in SK-N-MC cells who observed that the early Ca^{2+} increase evoked by ET-1 is mainly due to mobilization while the later (plateau) phase mainly comes from influx (Hiley *et al*., 1992).

Pretreatment of the cells with PTX did not decrease ET-1-induced IP-formation suggesting that ET_A-receptor mediated IP-formation does not involve a PTX-sensitive G-protein. On the other hand, PTX treatment significantly reduced ET-1-induced increases in intracellular Ca^{2+} when extracellular Ca^{2+} was normal; after chelation of extracellular Ca^{2+} by EGTA, however, PTX did not affect the ET-1-induced increases in intracellular Ca^{2+} indicating that part of the ET-1-induced Ca^{2+} increase is caused by a PTX-sensitive influx. Experiments with SKF 96365, an inhibitor of receptor mediated Ca^{2+} influx (Merritt *et al*., 1990; Cabello & Schilling, 1993), supported this view: SKF 96365, in the concentration range from $1.0-3.0\times10^{-5}$ M, concentration-dependently inhibited ET-1 induced Ca^{2+} increases in the SK-N-MC cells; on the other hand, after pretreatment of the cells with PTX (thus inhibiting ET-1-induced Ca^{2+} influx), SKF 96365 exhibited only marginal inhibitory effects (*c.f.* Table 1). At higher concentrations, however, SKF 96365 appeared to be an unselective inhibitor of Ca^{2+} increases since it suppressed ET-1-induced Ca^{2+} increases in both PTX-treated as well as non-treated cells to a similar extent (*c.f.* Table 1). Taken together, these results are in favour of the view that, in the human neuroblastoma cell line SK-N-MC, ET_A-receptors couple to PTX-insensitive mobilization and to PTX-sensitive influx of Ca^{2+}. Since IPs are well established to be mediators of mobilization of Ca^{2+} from intracellular stores (Berridge & Irvine, 1989), and since our data clearly show that ETs increase IP-formation in a PTX-insensitive manner, it is tempting to speculate that the PTX-insensitive part of ET-1-induced increases in intracellular Ca^{2+} is triggered by increases in IPs. This idea is further supported by the finding that chelation of extracellular Ca^{2+} did not affect ET-1-induced IP-formation. Accordingly, the mechanism of ET_A-receptor mediated Ca^{2+} increases in SK-N-MC cells resembles that of muscarinic receptors: previous studies have shown that carbachol increases intracellular Ca^{2+} *via* a PTX-sensitive Ca^{2+} influx and a PTX-insensitive, IPs-mediated mobilization from intracellular stores (Michel *et al*., 1992).

In SK-N-MC cells ET_A-receptors obviously couple to two different G-proteins, a PTX-insensitive (presumably $G_{q/11}$, the G-protein coupling ET_A-receptors to phospholipase C (PLC); Takigawa *et al*., 1995) and a PTX-sensitive G-protein. The PTX-sensitive G-protein is not only involved in ET_A-receptor mediated Ca^{2+} influx but also in inhibition of cyclic AMP-formation: ET-1 (10^{-8} M) significantly inhibited increases in intracellular cyclic AMP content induced by isoprenaline *via* β_1-adrenoceptor stimulation (Michel *et al*., 1993) in normal cells, but not in PTX-treated cells (*c.f.* Table 2). The inhibitory effect of ET-1 was completely suppressed by the selective ET_A-receptor antagonist BQ-123 but not all affected by the selective ET_B-receptor antagonist BQ-788. This strongly supports the view that also the adenylyl cyclase inhibiting effect of ET-1 is mediated by ET_A-receptors. Similar coupling to two different signal-transduction mechanisms, involving two different G-proteins, has been described for ET_A-receptors in rat adult cardiomyocytes (Hilal-Dandan *et al*., 1994), brain capillary endothelial cells (Ladoux & Frelin, 1991) and human right atrium (Vogelsang *et al*., 1994; Pönicke *et al*., 1998), where stimulation of ET_A-receptors caused increases in IP-formation *via* a PTX-insensitive G-protein and inhibition of adenylyl cyclase *via* a PTX-sensitive G-protein. SK-N-MC cells exhibiting the same pattern of coupling to different G-proteins could be, therefore, a very suitable cell culture system to study possible interactions between the two pathways.

In the present study, the maximal increase in IP-formation induced by ET-1 was in PTX-treated cells higher than in non-treated cells (*c.f.* Figure 4) although the difference did not reach statistical significance. This phenomenon of enhancing effects of PTX treatment on ET-1-induced IP-formation has been also observed in rat cerebellar slices (Sokolovsky, 1993a), rat neonatal cardiomyocytes (Sokolovsky, 1993b; Pönicke *et al*., 1997), and in isolated rat renal cells (Becker *et al*., 1996); similar findings of an enhanced PLC response to stimulatory hormones have also been obtained in cultured F9 mouse teratocarcinoma stem cells and ROS 17/2.8 cells in which G_i expression was abolished by antisense RNA (Watkins *et al*., 1994). This might be taken as an indication that, in certain tissues and/or cells, PLC-activity is under tonic inhibitory control of G_i.

In the present study Ca^{2+} increases induced by ET-1 were only transient and returned to levels slightly higher than baseline within a few minutes, in accordance with published data from the literature (Hiley *et al*., 1992). This rapid decline in intracellular Ca^{2+} concentration in the presence of ET-1 was obviously not due to removal or inactivation of the agonist but rather due to desensitization since a second addition of ET-1 in the same or even in higher concentrations did not produce a considerable effect. Similarly, carbachol-induced increases in intracellular Ca^{2+} that are mediated by activation of muscarinic receptors (Michel *et al*., 1992) rapidly desensitized. On the other hand, carbachol did not desensitize the ET-1 effect and vice versa indicating that in SK-N-MC cells muscarinic and ET_A-receptors undergo rapid homologous desensitization but no, or only weak, heterologous desensitization. A similar very rapid desensitization has been recently observed for α_{2A}-adrenoceptors and Y_1-like neuropeptide Y receptors when mediating Ca^{2+} increases in HEL cells (Michel, 1994); however, in contrast to the SK-N-MC cells, in these cells desensitization involved both homologous and heterologous components. The mechanism underlying this rapid desensitization of agonist-induced Ca^{2+} increases, however, remains to be elucidated.

In conclusion: the present results show that the human neuroblastoma cell line SK-N-MC contains a homogeneous class of ET_A-receptors that couple to both IP-formation and increases in intracellular Ca^{2+}. The increase in intracellular Ca^{2+} is brought about by two different mechanisms: a PTX-insensitive, IP-mediated, Ca^{2+} mobilization from intracellular stores, and a PTX-sensitive influx of extracellular Ca^{2+}. Thus, in SK-N-MC cells ET_A-receptors couple to two different G-proteins, a PTX-insensitive and a PTX-sensitive G-protein. The ET_A-receptors undergo rapid homologous desensitization following short-term activation with ET-1.

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