Activation of mitogen-activated protein kinase couples neurotensin receptor stimulation to induction of the primary response gene *Krox-24*

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Neurotensin (NT) is a neuropeptide that is important in a variety of biological processes such as signal transduction and cell growth. NT effects are mediated by a single class of cell-surface receptors, known as neurotensin receptors (NTRs), which exhibit structural features of the G-protein-coupled receptors superfamily. We investigated NTR signalling properties with Chinese hamster ovary (CHO) cells stably transformed with human NTR (hNTR). First, we showed that NTR stimulation by NT induced the activation of the mitogen-activated protein kinases (MAPKs) in time- and dose-dependent manners. Both p42 and p44 MAPK isoforms were retarded in gel-shift assays, which was consistent with their activation by phosphorylation. In addition we showed that NT caused a prolonged activation of MAPK as measured by in-gel kinase assay. Secondly, we demonstrated that NT induced the expression of the growth-related gene *Krox-24* at the protein level, as assessed by Western-blot analysis, and at the transcriptional level, as demonstrated in CHO cells transfected with hNTR and a reporter gene for *Krox-24*. Activation of MAPK and induction of *Krox-24* were both prevented by the NTR antagonist SR 48692, confirming the specific action on NTR. Furthermore we observed coupling of NTR to a mitogenic pathway and *Krox-24* induction in the human adenocarcinoma cell line HT29, which naturally expresses NTRs. Considering coupling pathways between NTR stimulation and MAPK activation, we observed a partial inhibition by pertussis toxin (PTX) and a complete blockade by the protein kinase C (PKC) inhibitor GF 109203X. Taken together, these results suggest that (1) stimulation of NTR activates the MAPK pathway by mechanisms involving dual coupling to both PTX-sensitive and PTX-insensitive G-proteins as well as PKC activation, and (2) these effects are associated with the induction of *Krox-24*, which might be a target of MAPK effector.

INTRODUCTION

Neurotensin (NT), discovered in bovine hypothalamus [1], is a tridecapeptide found primarily within nerves of the central and peripheral nervous systems and in endocrine cells of the pituitary as well as in the small intestine. This peptide displays a wide spectrum of biological activities, including roles in hypotension, hyperglycaemia and regulation of vascular permeability. NT is also an important hormone of the gut because it facilitates the translocation of fatty acids to the proximal small bowel and affects numerous aspects of gastrointestinal functions such as secretion and motility.

In addition to the above-mentioned biological functions, several investigators have reported that NT could stimulate cell growth. For example, NT displays trophic effects *in vivo* on rat pancreas [2] and intestinal mucosa [3]. A mitogenic effect was also observed *in vitro*, on small-cell lung cancer cells [4], on pancreatic tumour cells [5] and on the prostate cancer cell line PC-3 [6]. Furthermore it has been speculated that NT could exert autocrine functions in small-cell lung cancer cells [4] as well as in prostate cancer cells.

The various functions of NT involve its interaction with specific NT receptors (NTRs) that belong to the family of the GTP-binding protein-coupled receptors (GPCRs) [7,8]. The biochemical events resulting from NTR stimulation include protein kinase C (PKC) activation [9,10], production of cAMP [11] and cGMP [12,13] as well as the induction of phospholipid hydrolysis with subsequent Ca^{2+} mobilization.

An unresolved issue concerns the mechanism by which NT might promote cell growth. Several studies have shown that the activation of mitogen-activated protein kinases (MAPKs), also known as extracellular-signal-regulated protein kinases (ERKs), is a necessary event for mitogenesis [14]. The best understood pathways of MAPK activation are those used by tyrosine kinase receptors (RTK). However, the MAPK cascade can also be activated by certain GPCRs [15]. The 42/44 kDa serine/threonine MAPKs are key components of this activation cascade, which occurs during the pre-replicative growth (G_1) stage of the cell cycle [16]. Once activated, MAPKs translocate to the nucleus, phosphorylate and regulate nuclear proteins such as c-Myc and $p62^{TCF}$/Elk1 [16], which ultimately regulate the expression of genes essential for proliferation, such as c-*fos*, *Krox-24* and c-*jun*.

Here we demonstrate that the binding of NT to its receptor induces activation of MAPKs. This activation is mediated by PKC as an upstream regulator. Furthermore we show that NT stimulates transcription of the immediate-early gene *Krox-24*, which is a target of the MAPK cascade.

Abbreviations used: CHO, Chinese hamster ovary; CHO-hNTR, CHO cells stably transformed with human NTR; CHO-NTR-KL, CHO-hNTR cells transfected with *Krox-24*/luciferase; ERK, extracellular-signal-regulated kinase; FCS, fetal calf serum; MAPK, mitogen-activated protein kinase; MBP, myelin basic protein; MEM, minimal essential medium; NT, neurotensin; NTR, neurotensin receptor; PKC, protein kinase C; PMA, phorbol 12-myristate 13-acetate; PTX, pertussis toxin; GPCR, GTP-binding protein-coupled receptors.

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EXPERIMENTAL

Reagents

NT, bovine myelin basic protein (MBP), PMSF, sodium orthovanadate, pertussis toxin (PTX) and anti-rabbit IgG conjugated with peroxidase were purchased from Sigma Chemicals (Saint-Quentin-Fallavier, France). Phorbol 12-myristate 13-acetate (PMA) was obtained from Gibco BRL (Eragny, France). The bisindolylmaleimide (GF 109203X) and *N*-2-(methylamino)-ethyl-5-isoquinoline sulphonamide (H8) were from Calbiochem (Meudon, France). SR 48692 was synthesized at the Chemistry Department, Sanofi Recherche (Montpellier, France). [^{32}P]ATP (3000 Ci/mmol) was from Amersham (Les Ullis, France). Anti-p44 (C-16, anti-ERK-1), anti-p42 (C-14, anti-ERK-2) rabbit polyclonal antibodies and anti-Krox-24 antibody were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, U.S.A.).

Cell lines and culture conditions

CHO wild-type cells were routinely grown as monolayers at 37 °C in a humidified atmosphere containing 5% CO_2 in alpha minimal essential medium (MEM; Gibco BRL) supplemented with 5% (v/v) fetal calf serum (FCS), 60 μg/ml Tylocine (Gibco BRL) and 20 μg/ml gentamycin. CHO cells stably transformed with human NTR (CHO-hNTR) and CHO-hNTR cells transfected with *Krox-24*/luciferase (CHO-NTR-KL) were grown in MEM supplemented with 5% (v/v) dialysed FCS, 40 μg/ml L-proline, 1 mM sodium pyruvate, 60 μg/ml Tylocine and 20 μg/ml gentamycin. The human colon adenocarcinoma HT-29 cell line was obtained from the American Type Culture Collection (Rockville, MD, U.S.A.). HT29 cells were cultured at 37 °C in Dulbecco's modified Eagle's medium containing 10% (v/v) FCS, 60 μg/ml Tylocine, 25 mM Hepes and 2.5 mM sodium pyruvate. Cells were maintained in 0.5% (v/v) FCS for 24 h before stimulation.

Generation of stable transformants

Plasmids

The cDNA coding for hNTR [8] was inserted into the expression plasmid p7055 [17] in place of the interleukin-2 cDNA. The resulting hNTR expression vector was called p1274. The pUT112/*Krox-24* expression vector was constructed by cloning the *Xba*I fragment containing the -395 to $+65$ sequence of the murine *Krox-24* promoter isolated from the pBL395 CAT vector into the *Spe*I site of pUT112 (Eurogentech, Seraing, Belgium), upstream of the firefly luciferase coding sequence.

Obtaining CHO-hNTR cells

The expression vector p1274 was used to transfect CHO dihydrofolate reductase-negative cells [18] by a modified calcium phosphate precipitation method [19]. Clones grown in selective medium were isolated 12–14 days later by Bellco glass-cylinder cloning and then screened by assaying the binding of NT on whole cells as previously described [8]. Clones stably expressing high density levels of receptors were subcloned by limiting dilution and further characterized. The established cell line 1274.20.5, expressing 421000 ± 39000 receptors per cell, was selected for further analysis.

Obtaining a double transformant for NTR and *Krox-24*/luciferase

CHO-hNTR cells were stably transformed with the pUT112/*Krox-24* expression vector by electroporation (250 μF, 450 V) with the Bio-Rad Gene pulser. Selection was achieved in MEM enriched with 20% (v/v) FCS, essential and non-essential amino acids and 1 mM sodium pyruvate and supplemented with 20 μg/ml phleomycin. Clones of transfected cells (CHO-NTR-KL) were screened 2 weeks later for the expression of NT-induced *Krox-24*/luciferase.

Luciferase assay

Cells were seeded at 10^4 cells per well in white 96-well microplates for luminescence in MEM supplemented with 5% (v/v) FCS. After 24 h the culture medium was replaced by medium containing 0.5% (v/v) FCS and cells were further incubated for 24 h. Cells were then stimulated for 5 h with various doses of NT. After stimulation, cell extracts were prepared and intracellular luciferase contents were determined by using the Luciferase Assay System (Promega, Charbonnières, France) in accordance with the supplier's instructions. Luminescence was detected by a charge-coupled device camera (MTP Reader; Hamamatsu Photonics, Hamamatsu, Japan). Quantification of light emission was made by accumulation of photon counting, and mean values from triplicates were expressed as fold induction, defined by the ratio of values in stimulated and unstimulated cells.

Immune-complex kinase assay

MAPK activity was determined as described by Frödin et al. [20]. Briefly, cells grown to 80% confluence in 24-well plates were placed in medium containing 0.5% (v/v) FCS for 24 h before stimulation. Cells were then washed twice in buffer A [50 mM Hepes (pH 7.5)/150 mM NaCl/10 mM $Na_4P_2O_7$/100 mM NaF/10 mM EDTA/20 mM glycerophosphate/1 mM EGTA/2 mM Na_3VO_4] and lysed for 15 min in buffer A containing 1% (v/v) Triton X-100, 100 units/ml aprotinin, 20 μM leupeptin and 0.2 mg/ml PMSF. Solubilized cell extracts were clarified by centrifugation at 14000 ***g*** for 15 min and then incubated for 3 h with the agarose-coupled antibodies anti-ERK1 (C-16) and anti-ERK2 (C-14). After immunoprecipitation, pellets were washed three times with solubilization buffer and twice with buffer B [50 mM Hepes/150 mM NaCl/10% (v/v) glycerol/0.1% (v/v) Triton X-100/0.2 mM Na_3VO_4], then air-dried and resuspended in 50 μl of buffer B supplemented with 100 units/ml aprotinin, 20 μM leupeptin and 0.2 mg/ml PMSF. Phosphorylation of MBP was initiated by the addition of 10 μl of a 6× mixture of 150 μg/ml MBP, 10 mM magnesium acetate, 1 mM dithiothreitol and 5 μM [γ-^{32}P]ATP (3000 Ci/mmol). The phosphorylation reaction was performed for 30 min at 30 °C (linear assay condition) and was stopped by spotting samples on Whatman P-81 filter papers, which were then dropped into a solution of 0.1% (v/v) orthophosphoric acid solution. The paper sheets were soaked in this solution, rinsed with ethanol and air-dried; the radioactivity incorporated into MBP was determined by liquid-scintillation counting.

Gel-shift assay

Phosphorylation of p42 (ERK-2) and p44 (ERK-1) MAPKs was determined by electrophoretic mobility shift assay as described [21,22]. Briefly, quiescent cells were incubated in six-well plates at 37 °C for various durations, then washed once in ice-cold buffer containing 50 mM Hepes (pH 7.4) and 0.2 mM sodium orthovanadate before being lysed in Laemmli's loading buffer containing 6 M urea. Samples were heated for 10 min at 95 °C and proteins were separated by SDS/PAGE on 11% (w/v) acrylamide gels. Proteins were then transferred to nitrocellulose filters

in 25 mM Tris, 0.19 M glycine and 20 % (v/v) methanol. Membranes were blotted in TN buffer (20 mM Tris/120 mM NaCl, pH 7.5) containing 10 % (w/v) dried milk powder. The blots were then incubated with the anti-p42 and anti-p44 antibodies at a concentration of 0.25 μg/ml in blotting solution for 3 h at room temperature. After extensive washes in TN buffer containing 0.1 % (v/v) Tween-20, the second antibody, a peroxidase-labelled anti-rabbit IgG antibody, was added for 45 min at room temperature. After additional washes, immunostained MAPKs were revealed with the enhanced chemiluminescence detection (ECL) system in accordance with the instructions of the supplier (Amersham).

Western-blot analysis

After stimulation, cells were washed and lysed. Proteins were extracted, subjected to SDS/PAGE and blotted on nitrocellulose filters as described above. Blots were incubated with the anti-Krox-24 antibody for 3 h at room temperature. After washes, Krox-24 proteins were detected by the peroxidase conjugate of anti-rabbit IgG and the enhanced chemiluminescence system.

Gel kinase assay

Quiescent CHO-hNTR cells were stimulated with 10 nM NT for various periods, lysed in the Laemmli buffer and analysed for MAPK activity with an in-gel kinase assay as previously described [23]. Samples were subjected to SDS/PAGE [10 % (w/v) gel] containing 0.2 mg/ml MBP. Gels were incubated once in buffer I [50 mM Tris/HCl (pH 8)/5 mM 2-mercaptoethanol] containing 20 % (v/v) methanol for 20 min, once in buffer I for 30 min, twice in buffer I containing 6 M guanidinium chloride for 30 min, twice in buffer I containing 0.04 % (v/v) Tween-20 at 4 °C for 16 h, once in buffer I containing 100 μM Na_3VO_4 and 2 mM $MgCl_2$ for 30 min, and once in buffer I containing 100 μM Na_3VO_4, 2 mM $MgCl_2$, 50 μM ATP, and 50 μCi of [γ-^{32}P]ATP for 2 h at 30 °C. The reaction was stopped by extensive washes of the gels in 5 % (v/v) trichloroacetic acid. Finally gels were dried and subjected to autoradiography.

RESULTS

MAPK activation after NT stimulation

To study the signal transduction induced by NT on growth-related activities, we derived a CHO cell line stably expressing hNTR. A Scatchard plot analysis revealed a dissociation constant (K_d) of 0.72 ± 0.12 nM and a maximal binding (B_{max}) of $421\,000 \pm 39\,000$ sites per cell. We investigated whether stimulation through NTR could result in the activation of MAPK. Growth-arrested CHO-hNTR cells were exposed to NT for various periods of time, proteins were extracted and MAPKs were immunoprecipitated with anti-ERK-2 (anti-p42) and anti-ERK-1 (anti-p44) antibodies. The immune complexes were assayed for kinase activity with MBP as the substrate. Quiescent cells displayed a slight constitutive MAPK activity, which was markedly enhanced by treatment with agonist (Figure 1A). This effect was measurable after 2 min of stimulation, reached a maximum between 6 and 10 min, then slowly declined. MAPK activity returned to the basal level only after 1 h (results not shown). Activation was also determined by the appearance of more slowly migrating forms that resulted from phosphorylation on specific threonine and tyrosine residues [21]. Treatment of cells with NT stimulated both 42 and 44 kDa proteins as judged by the upward mobility shift (Figure 1B). The appearance of the more slowly migrating forms was time-dependent and fitted with

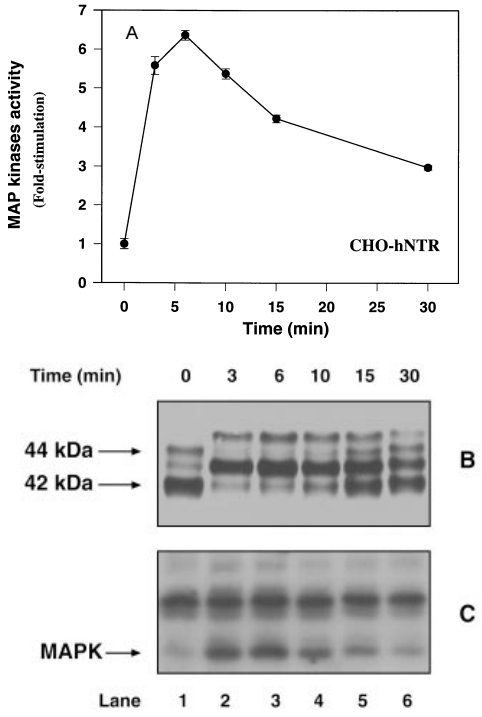

Figure 1 Time course of NT stimulation of MAPK activity in CHO-hNTR cells

(**A**) MAPK activity in CHO-hNTR cells after stimulation with NT. Quiescent CHO-hNTR cells were stimulated with 10 nM NT for the indicated periods of time. MAPK activity was determined as described in the Experimental section. Each data point is the mean $\pm$ S.E.M. for triplicate samples and experiments were repeated twice. (**B**) Western blot analysis of MAPK in growth-arrested CHO-hNTR cells treated with 10 nM NT for the indicated periods. Total protein (7 μg) was loaded in each lane. (**C**) Detection of MAPK activity. CHO-hNTR cells were stimulated as indicated in (**B**) and 30 μg of protein extracts were subjected to SDS/PAGE in the presence of MBP. Kinase actitity was determined after protein renaturation in the gel.

the activation pattern depicted in Figure 1A. An additional confirmation of the observed phosphorylation of MBP by MAPKs was obtained by the in-gel kinase assay performed with proteins extracted from NT-treated cells and subjected to SDS/PAGE: as illustrated in Figure 1(C), intact MBP protein, trapped in the polyacrylamide gel, was efficiently phosphorylated by 42–44 kDa MAPKs, and the kinetics of stimulation was similar to the kinetics of kinase activation observed in whole extracts with the MBP peptide as the substrate.

The activation of MAPK by NT was dose-dependent, with noticeable effects on CHO-hNTR cells at 0.1 nM and maximal effects at 10 nM, whereas no effect was observed on CHO wild-type cells (Figure 2A). The EC_{50} was in the nanomolar range, which was in accordance with the K_d for NT. The specific involvement of NTR was further demonstrated by experiments where CHO-hNTR cells were exposed to NT and SR 48692 together: SR 48692, which by itself had no effect, potently antagonized NT action (Figure 2B). Taken together, these results provide strong evidence that NT-induced activation of p42/p44 MAPKs is specifically mediated by NTRs.

MAPK activation in HT29 cells

It is not unusual for cloned receptors expressed in large amounts by foreign cell lines to couple to transduction systems that might not be relevant to receptors endogenously expressed. We there-

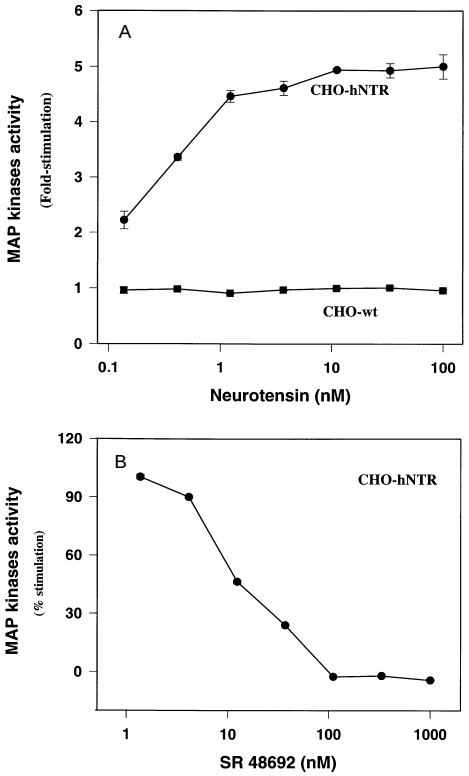

Figure 2 Specificity and dose-dependent effects of NT on MAPKs

(**A**) Growth-arrested CHO-hNTR (●) and wild-type CHO (■) cells were treated for 6 min with the indicated concentrations of NT. MAPK activity was determined as described in the Experimental section. (**B**) Effect of the antagonist SR 48692 on NT-induced MAPK activity in CHO-hNTR. Quiescent cells were treated for 6 min with 1 nM NT and the indicated concentrations of SR 48692. MAPK activity was determined as described in the Experimental section. Results are the means of triplicate determinations in three independent experiments.

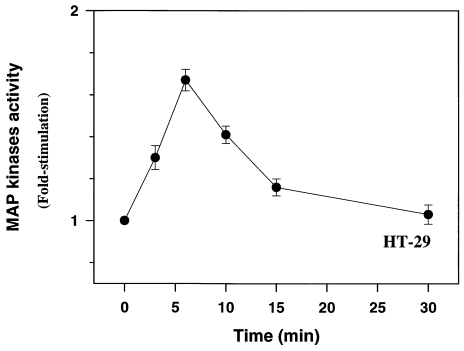

Figure 3 Effect of NT on MAPK activation in HT-29 cells

Quiescent HT-29 cells were treated with 10 nM NT for the indicated periods and MAPK activity was measured as described in the Experimental section. Each data point is the mean ± S.E.M. for triplicate samples and experiments were repeated twice.

fore examined whether the above results obtained by using an overexpressed receptor system could be extended to a human cell line such as the human colon carcinoma cell line HT29, which expresses NTRs as do cells from normal intestinal mucosa [24]. As shown in Figure 3, stimulation of NTR in HT29 cells also induced a rapid and marked increase in MAPK activity, although in a lower range than in CHO-hNTR cells. HT29 cells exhibit

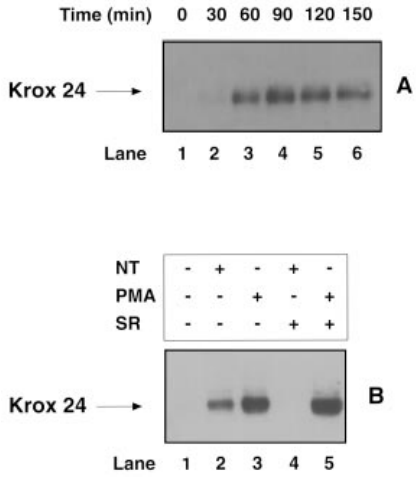

Figure 4 Induction by NT of *Krox-24* expression in CHO-hNTR cells

(**A**) Kinetics of induction of *Krox-24* in CHO-hNTR cells. Quiescent CHO-hNTR cells were stimulated with 10 nM NT for the indicated periods. Proteins were extracted and 7 μg per lane was fractionated by SDS/PAGE. Western blots were incubated with the anti-Krox-24 antibody. (**B**) Effects of the antagonist SR 48692 (SR) on NT-induced *Krox-24* expression in CHO-hNTR cells. Quiescent cells were treated as indicated for 90 min, proteins were extracted and treated as above: lane 1, untreated cells; lane 2, 1 nM NT; lane 3, 20 ng/ml PMA; lane 4, 1 nM NT + 1 μM SR 48692; lane 5, 20 ng/ml PMA + 1 μM SR 48692.

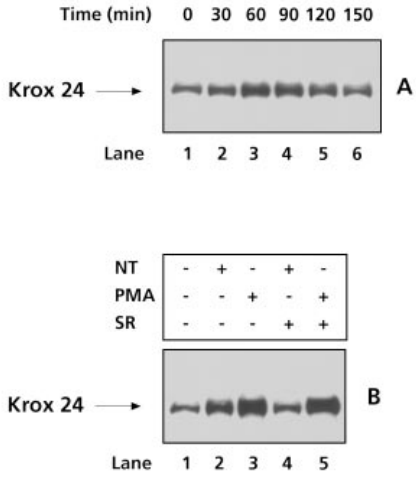

Figure 5 Induction by NT of *Krox-24* expression in HT29 cells

(**A**) Kinetics of induction of *Krox-24* in HT29 cells. Quiescent HT29 cells were stimulated with 10 nM NT for the indicated periods. Proteins (14 μg) were fractionated by SDS/PAGE, and Western blots were incubated with the anti-Krox-24 antibody. (**B**) Effects of the antagonist SR 48692 (SR) on NT-induced *Krox-24* expression in HT29 cells. Quiescent cells were treated as indicated for 90 min, proteins were extracted and treated as above: lane 1, untreated cells; lane 2, 1 nM NT; lane 3, 20 ng/ml PMA; lane 4, 1 nM NT + 1 μM SR 48692; lane 5, 20 ng/ml PMA + 1 μM SR 48692.

approx. 2×10^4 NTR sites per cell, as determined by binding experiments (results not shown), and this lower level of expression could explain the differences observed between the two cell types.

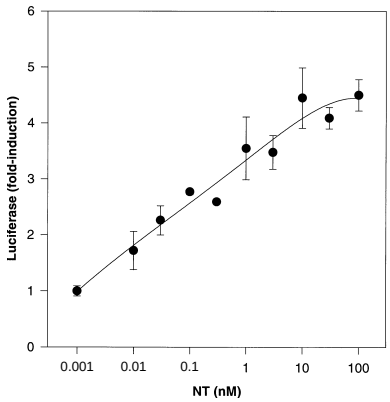

Figure 6 Dose response of *Krox-24* reporter induction by NT

CHO-hNTR cells stably expressing the *Krox-24*/luciferase gene were cultured in medium containing 0.5% (v/v) FCS 24 h before stimulation with increasing concentrations of NT; 5 h after stimulation, cell extracts were prepared. Luciferase activities were measured and means ± S.E.M. for triplicates were calculated. Results were expressed as fold induction, defined as the ratio of values from stimulated and unstimulated cells. The experiment was repeated three times with similar results.

Krox-24 activation in response to NTR stimulation

MAPKs play a role in the control of gene transcription by phosphorylating transcription factors, which in turn modulate the expression of target genes. One of these genes encodes a zinc-finger transcription factor, the early growth response gene 1 (*egr-1*) also called *Krox-24*, *NGFI-A* or *zif/268* [25]. We first examined whether the activation of NTR was associated with induction of Krox-24 protein in CHO-hNTR cells by Western blot analysis with anti-Krox-24 antibodies. NT treatment induced the expression of Krox-24 protein in a time-dependent manner, with maximum levels observed after 90 min (Figure 4A). The NT antagonist SR 48692 completely prevented this increase, whereas it had no effect on PMA-induced Krox-24 expression (Figure 4B). A similar enhancement of Krox-24 protein was also observed in HT29 cells after stimulation with NT and reversed by the NT antagonist (Figures 5A and 5B).

Secondly, we examined whether the increase in Krox-24 protein level reflected an increase in the transcriptional activity of the *Krox-24* gene. For this purpose, CHO-hNTR cells were stably transformed with an expression vector (pUT112/*Krox-24*) containing the −395 to +65 sequence of the *Krox-24* promoter located upstream of the luciferase reporter gene. As shown in Figure 6, when CHO-NTR-KL cells were treated with NT, a dose-dependent increase in luciferase activity was observed.

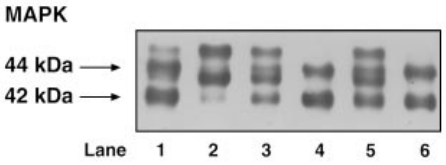

Figure 7 Effects of PTX and GF 109203X on NT-induced MAPK activity in CHO-hNTR cells

CHO-hNTR cells were treated as described in Table 1 and MAPK phosphorylation was analysed by electrophoretic mobility shift assay as described in the Experimental section. Lane 1, untreated; lane 2, 10 nM NT; lane 3, 10 nM NT + 100 ng/ml PTX; lane 4, 100 ng/ml PTX; lane 5, 10 nM NT + 5 μM GFX; lane 6, 5 μM GFX. Each lane was loaded with 7 μg of total protein.

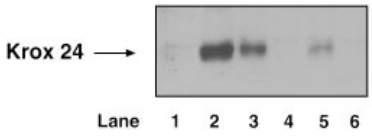

Figure 8 Effects of PTX and GF 109203X on NT-induced *Krox-24* expression

CHO-hNTR cells were treated as indicated for 90 min. Protein were extracted and 7 μg per lane was submitted to electrophoretic mobility shift assay. Lane 1, untreated; lane 2, 10 nM NT; lane 3, 10 nM NT + 100 ng/ml PTX; lane 4, 100 ng/ml PTX; lane 5, 10 nM NT + 5 μM GFX; lane 6, 5 μM GFX.

NT induces MAPK activation and *Krox-24* expression via a PKC-dependent transduction pathway

To investigate which receptor-linked effector pathway might be responsible for MAPK activation, we first examined the effect of PTX. PTX treatment partly inhibited the NT-induced MAPK activation measured either by the MAPK activity (Table 1) or by Western blotting (Figure 7). This indicated that both a PTX-sensitive component and a PTX-insensitive component were involved in MAPK activation through NTR stimulation.

As NTRs were shown to activate PKC [9,10], which is known to represent an upstream regulator of the MAPK cascade [26], we explored this pathway by using the non-toxic PKC-specific inhibitor, bisindolylmaleimide (GF 109203X) [27]. Table 1 and Figure 7 show that treatment of CHO-hNTR cells with GF 109203X markedly decreased NT-stimulated MAPK activity, suggesting the involvement of PKC in MAPK phosphorylation.

The effects of PTX and GF 109203X were also analysed on Krox-24 protein expression levels by Western blotting (Figure 8).

Table 1 Effects of PTX and GF 109203X on NT-induced MAPK activity in CHO-hNTR cells

Cells were treated for 18 h with PTX or for 3 h with GFX before the addition of NT for 6 min. Fold increase is the ratio of values in stimulated and non-stimulated cells. Results are expressed as means ± S.E.M.; the experiment was repeated twice with similar results.
Abbreviation: n.d., not determined.

	Control		GF 109203X (5 μM)		PTX (100 ng/ml)	
Stimulation	c.p.m.	Fold increase	c.p.m.	Fold increase	c.p.m.	Fold increase
None	7703 ± 284	1 ± 0.037	5315 ± 168	1 ± 0.032	5539 ± 37	1 ± 0.0067
NT (10 nM)	42212 ± 1496	5.48 ± 0.194	10424 ± 302	1.96 ± 0.057	20918 ± 284	3.78 ± 0.051
PMA (20 ng/ml)	25400 ± 1095	3.3 ± 0.14	5119 ± 95	0.96 ± 0.018	n.d.	n.d.

Results show a partial but significant decrease in production of Krox-24 after treatment with PTX and a more marked effect of GF 109203X, which is in accordance with the results obtained for MAPK assays.

DISCUSSION

In the present study, using CHO cells stably transformed with human NTR, we have characterized novel aspects of biochemical signals induced by NT.

NTR stimulation induces MAPK activation

In this study we first provide several lines of evidence for MAPK activation by NT: (1) NT induced a time- and dose-dependent activation of MAPKs towards the MBP peptide as substrate; (2) Western blotting analysis revealed that p42/p44 MAPK proteins exhibited an upward mobility shift, suggesting the phosphorylation of MAPKs, which is part of their mechanisms of activation [28] after NT stimulation; and (3) p42/p44 were shown to be responsible for the increased MBP peptide kinase activity measured in NT-stimulated cells, as assessed by a kinase renaturation assay.

It has previously been shown that receptors expressed in foreign hosts can couple to different signal transduction systems depending on the cell type [29]. This was shown for example for dopamine D2 receptors, which have different effects on adenylate cyclase activity, intracellular Ca^{2+} concentrations and inositol phosphates production, when expressed in different hosts [30]. With the human colon carcinoma cell line HT29, which naturally expresses NTRs, we showed that MAPKs were also activated after NT stimulation, although at lower levels than in CHO-hNTR cells. To our knowledge these results are the first to demonstrate that NT mediates MAPK activation in transfected but also in cells naturally expressing NTR.

Transduction pathway between NTR and MAPKs

Although the molecular mechanisms located downstream of the G-protein and leading to MAPK activation by NT still remain unclear, some elements can be offered by the present study. We showed that PTX partly blocked MAPK activation. PTX is known to induce ADP-ribosylation of G_i/G_o-type G-proteins, thus preventing the dissociation of their α and β/γ subunits [31]. Our findings are consistent with two independent pathways mediated by GTP-binding proteins, one G_i/G_o-dependent and one G_i/G_o-independent, both leading to the activation of the MAPK cascade. Maximal responsiveness is obtained when both pathways are used. This situation is similar to that reported in mouse neuroblastoma N1E115 cells, where NT effects on inositol phosphate production were partly inhibited by PTX [32].

It was shown that stimulation of CHO-hNTR cells with NT induces a dose-dependent increase in the level of cAMP [33]. We indeed found that NT increased cAMP levels in CHO-hNTR cells (results not shown). Because hydrolysis-resistant cAMP analogues such as dibutyryl cAMP or 8-Br-cAMP, which maintain high cellular levels of cAMP, are able either to prevent or to increase the MAPK level, depending on the cell type [34], we examined whether MAPK activation could be related to cAMP metabolism. In CHO-hNTR cells, cAMP analogues alone did not significantly affect this activity. Moreover, the protein kinase A inhibitor H8 did not affect the NT-stimulated MAPKs in CHO-hNTR cells (results not shown). Together these results argue against the possibility that MAPK activation is secondary to a G-protein-mediated stimulation of cAMP levels, and suggest that NT stimulates cAMP production and MAPK cascade through independent pathways.

NTRs are coupled to phospholipase C through G-proteins. Activation of phospholipase C by NT is followed by the hydrolysis of PtdIns(4,5)P_2, generating two second messengers, Ins(1,4,5)P_3 and diacylglycerol. Subsequently, Ins(1,4,5)P_3 mobilizes the intracellular Ca^{2+} whereas diacylglycerol activates PKC [7,9,10]. Many types of receptor signalling involve PKC as effectors of MAPKs [35,36]. We showed, by using the protein kinase inhibitor GF 109203X, that a PKC lies on the route between G-protein and MAPKs. Among the molecular mechanisms that could be suggested, an interesting possibility would be that PKC activates the Ras/Raf/MAPK cascade through the activation of $p21^{ras}$ [37]. However, in HT29 cells we observed that MAPK activation by NT was not affected by GF 109203X, suggesting that in these cells NT does not operate through PKC (results not shown).

NTR stimulation induces *Krox-24* gene expression

MAPKs phosphorylate a variety of intracellular substrates including transcription factors, which in turn modulate the expression of target genes implicated in cell growth [16,25]. To investigate further the involvement of MAPK in NT-induced cell growth, we explored whether the activation of NTR was associated with such gene induction. We have chosen the induction of the *Krox-24* gene as an endpoint because the activation of MAPK has been correlated with an increase in its transcription [38] and also because its induction is a ubiquitous correlate of mitogenic stimulation [39]. *Krox-24* expression is independent of protein synthesis and appears within minutes of mitogenic stimulation [40,41]. Our results showed a massive accumulation of Krox-24 protein on stimulation with NT in both CHO-hNTR and HT29 cells. The NT antagonist SR 48692 completely blocked this effect, which unequivocally demonstrated that induction of *Krox-24* expression is mediated by NTRs. Furthermore, by generating double stable transformants for hNTR and a reporter gene for *Krox-24*, we showed a dose-dependent stimulation of *Krox-24* expression by NT, which indicates that the regulation is imposed at the transcriptional level.

Importantly, we showed that stimulation of *Krox-24* by NT was parallel to MAPK stimulation in several aspects: (1) both stimulations were selectively blocked by the SR 48692 antagonist; (2) the responses were similar in their sensitivities towards PTX; (3) the inhibition of MAPK activity by a PKC inhibitor was associated with a concomitant decrease in *Krox-24* expression, indicating that the two responses were PKC-linked; and (4) both pathways were PKA-independent (results not shown). Thus it seems that MAPK might participate in NTR stimulation of the early response gene *Krox-24*.

The pivotal link between MAPK and *Krox-24* could be p62TCF/Elk1, a member of the Ets family that has been shown to be phosphorylated by MAPK [42,43] and binds with high affinity to specific ETS motifs of which three copies are located in the *Krox-24* promoter, two of them being necessary for transcriptional activity [38,44].

We propose that the action of NT involves the coupling of NTR to *Krox-24* induction through the MAPK cascade. This can be supported by the following points: (1) studies with dominant-negative MAPK have provided evidence that this pathway can lead to the stimulation of DNA synthesis [45], (2) *Krox-24* induction is an early response event to all cell division signals examined so far [46–49], and (3) studies with anti-sense oligonucleotides have demonstrated that the induction of *Krox-24* in lymphocytes is necessary for cell proliferation to occur

[49,50]. This is presumably accomplished by the induction of a second wave of function-related genes that are controlled by *Krox-24*. Indeed, Krox-24 is a member of the Krox family, which contain three zinc-finger DNA-binding domains and act as transcriptional factors. Gene activation by Krox-24 is observed after high-affinity binding to the consensus regulatory element GCG(G/T)GGGCG in a zinc-dependent manner. Although only few target genes of *Krox-24* have been characterized so far, the thymidine kinase gene seems to represent a physiologically relevant target for *Krox-24* in mitogenesis, because it is involved in cell cycle progression from the G_1 to the S phase [51].

Concluding remarks

These findings have several implications. First, the increase in *Krox-24* gene expression can be used as a marker for studying the molecular events associated with NTR stimulation. Secondly, we recently published results demonstrating the involvement of the GPC cannabinoid receptors in *Krox-24* activation mediated by MAPK [52]. The results reported here provide an additional example of a GPCR that can activate *Krox-24*. Thirdly, these results strengthen the notion that NT could be important in the regulation of cell growth.

REFERENCES

1 Carraway, R. E. and Leeman, S. E. (1973) J. Biol. Chem. **248**, 6854–6861
2 Feurle, G. E., Muller, B. and Rix, E. (1987) Gut **28** (suppl. 1), 19–23
3 Wood, J. G., Hoang, H. D., Bussjaeger, L. J. and Solomon, T. E. (1988) Am. J. Physiol. **255**, G813–G817
4 Davis, T. P., Burgess, H. S. and Crowell, S. (1989) Eur. J. Pharmacol. **161**, 283–285
5 Ishizuka, J., Townsend, C. M. and Thompson, J. C. (1993) Annals Surg. **217**, 439–446
6 Sehgal, I., Powers, S., Huntley, B., Powis, G., Pittlekow, M. and Maihle, N. J. (1994) Proc. Natl. Acad. Sci. U.S.A. **91**, 4673–4677
7 Tanaka, K., Masu, M. and Nakanishi, S. (1990) Neuron **4**, 847–854
8 Vita, N., Laurent, P., Lefort, S., Chalon, P., Dumont, X., Kaghad, M., Gully, D., Le Fur, G., Ferrara, P. and Caput, D. (1993) FEBS Lett. **317**, 139–142
9 Goedert, M., Pinnock, R. D., Downes, C. P., Mantyh, P. W. and Emson, P. C. (1984) Brain Res. **323**, 193–197
10 Snider, R. M., Forray, C., Pfenning, M. and Richelson, E. (1986) J. Neurochem. **47**, 1214–1218
11 Bozou, J. C., Amar, S., Vincent, J. P. and Kitabgi, P. (1986) Mol. Pharmacol. **29**, 489–496
12 Gilbert, J. A. and Richelson, E. (1984) Eur. J. Pharmacol. **99**, 245–246
13 Oury-Donat, F., Thurneyssen, O., Gonalons, N., Forgez, P., Gully, D., Le Fur, G. and Soubrie, P. (1995) Br. J. Pharmacol. **116**, 1899–1905
14 Blenis, J. (1993) Proc. Natl. Acad. Sci. U.S.A. **90**, 5889–5892
15 Howe, L. R. and Marshall, C. J. (1993) J. Biol. Chem. **268**, 20717–20720
16 Davis, R. J. (1993) J. Biol. Chem. **268**, 14553–14556
17 Miloux, B. and Lupker, J. H. (1994) Gene **149**, 341–344
18 Urlaub, G. and Chasin, L. A. (1980) Proc. Natl. Acad. Sci. U.S.A. **77**, 4216–4220
19 Graham, F. L. and Van der Eb, A. J. (1973) Virology **54**, 536–539
20 Frödin, M., Prealdi, P. and Van Obberghen, E. (1994) J. Biol. Chem. **269**, 6207–6214
21 Leevers, S. J. and Marshall, C. J. (1992) EMBO J. **11**, 569–574
22 De Vries-Smith, A. M., Burgering, B. M., Leevers, S. J., Marshall, C. J. and Bos, J. L. (1992) Nature (London) **357**, 602–604
23 Gotoh, Y., Nishida, E., Yamashita, T., Hoshi, M., Kawakami, M. and Sakaï, H. (1990) Eur. J. Biochem. **193**, 661–669
24 Amar, S., Kitabgi, P. and Vincent, J. P. (1986) FEBS Lett. **201**, 31–36
25 Seth, A., Alvarez, E., Gupta, S. and Davis, R. J. (1991) J. Biol. Chem. **266**, 23521–23524
26 Marshall, C. J. (1994) Curr. Opin. Genet. Dev. **4**, 82–89
27 Toullec, D., Pianetti, P., Coste, H., Belleverge, P., Grand-Perret, T., Ajanake, M., Baudet, V., Boissin, P., Boursier, E., Loriolle, F., Duhanel, D., Charon, D. and Kirilovski, J. (1991) J. Biol. Chem. **266**, 15771–15781
28 Nishida, E. and Gotoh, Y. (1993) Int. Rev. Cytol. **13**, 6124–6136
29 Richards, M. H. (1991) Biochem. Pharmacol. **42**, 1645–1651
30 Vallar, L., Muca, C., Magni, M., Albert, P., Bunzow, J., Mendolesi, J. and Civelli, O. (1990) J. Biol. Cell. **265**, 10320–10326
31 Casey, P. and Gilman, A. G. J. (1988) J. Biol. Chem. **263**, 2577–2580
32 Amar, S., Kitabgi, P. and Vincent, J. P. (1987) J. Neurochem. **49**, 999–1006
33 Yamada, M., Yamada, M., Watson, M. A. and Richelson, E. (1992) Eur. J. Pharmacol. **244**, 99–101
34 Sevetson, B. R., Kong, X. and Lawrence, J. C. (1993) Proc. Natl. Acad. Sci. U.S.A. **90**, 10305–10309
35 L'Allemain, G., Sturgill, T. W. and Weber, M. J. (1991) Mol. Cell. Biol. **11**, 1002–1008
36 Kazlauskas, A. and Cooper, J. A. (1988) J. Cell. Biol. **106**, 1395–1402
37 Burgering, B. M. T. and Bos, J. L. (1995) Trends Biochem. Sci. **20**, 18–22
38 MacMahon, S. B. and Monroe, J. G. (1995) J. Exp. Med. **181**, 417–422
39 Nguyen, H. Q., Hoffman-Liebermann, B. and Liebermann, D. A. (1993) Cell **72**, 197–209
40 Sukhatme, V. P., Kartha, S., Toback, F. G., Taub, R., Hoover, R. G. and Tsai-Morris, C. H. (1987) Oncogene Res. **1**, 343–355
41 Seyfert, V. L., Mc Mahon, S. B., Glenn, W. D., Yellen, A. J., Sukhatme, V. P., Cao, X. and Monroe, J. G. (1990) Science **250**, 797–800
42 Treisman, R. (1994) Curr. Opin. Genet. Dev. **4**, 96–101
43 Janknecht, R., Ernst, W. Q., Pingoud, V. and Nordheim, A. (1993) EMBO J. **12**, 5097–5104
44 Tsai-Morris, C. H., Cao, X. M. and Sukhatme, V. P. (1988) Nucleic Acids Res. **16**, 8835–8846
45 Mansour, S. J., Matten, W. T., Herman, A. S., Cnadia, J. M., Rong, S., Fukasawa, K., Woude, F. V. and Ahan, N. G. (1994) Science **265**, 966–970
46 Sukhatme, V. P. (1990) J. Am. Soc. Nephrol. **1**, 859–866
47 Lemaire, P., Vesque, C., Schmidt, J., Stunnenberg, H., Frank, R. and Charnay, P. (1990) Mol. Cell. Biol. **10**, 3456–3467
48 Seyfert, V. L., Sukhatme, V. P. and Monroe, J. G. (1989) Mol. Cel. Biol. **9**, 2083–2088
49 Perez-Castillo, A., Pipaon, C., Garcia, I. and Alemany, S. (1993) J. Biol. Chem. **268**, 19445–19450
50 Monroe, J. G., Yellen-Shaw, A. J. and Seyfert, V. L. (1993) Adv. Mol. Cell. Immunol. **1B**, 1–32
51 Molnar, G., Crozat, A. and Pardee, A. B. (1994) Mol. Cell. Biol. **14**, 5242–5248
52 Bouaboula, M., Poinot-Chazel, C., Bourrie, B., Canat, X., Calandra, B., Le Fur, G. and Casellas, P. (1995) Biochem. J. **312**, 637–641

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