Neurotensin type 1 receptor-mediated activation of *krox24*, c-*fos* and Elk-1: preventing effect of the neurotensin antagonists SR 48692 and SR 142948

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Abstract Stimulation of neurotensin (NT) type 1 receptors (NT1-R) in transfected CHO cells is followed by the activation of mitogen-activated protein kinases and the expression of the early response gene *krox24*. By making point mutations and internal deletions in the *krox24* promoter, we show that proximal serum responsive elements (SRE) are involved in transcriptional activation by NT. In addition, we show that the related early response gene c-*fos* and the Ets protein Elk-1 are also induced by NT. The involvement of NT1-R in NT-mediated activation of *krox24*, c-*fos* and Elk-1 was demonstrated by the preventing effect of the specific antagonists SR 48692 and SR 142948. Finally, we show that the activation of *krox24* and Elk-1 on the one hand, and that of c-*fos* on the other hand, result from independent transduction pathways since the former are pertussis toxin-sensitive whereas the latter is insensitive to pertussis toxin.

Key words: Neurotensin; Neurotensin type 1 receptor; *krox24*; c-*fos*; Elk-1; cAMP; Luciferase

1. Introduction

Neurotensin (NT) is a tridecapeptide which interacts with specific G protein-coupled receptors belonging to the seven transmembrane receptor superfamily, and mediates various biological functions in the central nervous system and peripheral organs [1]. The first originally cloned receptor for NT (NT1-R) binds NT with high affinity and is insensitive to the histamine H1 receptor antagonist levocabastine [2]. The genomic structure of the human NT1-R gene was recently characterized and reveals the presence of three introns within the coding sequence, a proximal promoter sequence rich in putative transcription factor binding sites, and a tetranucleotide repeat polymorphism within the 3′-flanking region, with at least 23 alleles [3]. Rat NT1-R cDNA leads to the expression of two proteins with high affinity for NT, generated by the use of alternative translation initiation sites [4]. A second type of receptor (NT2-R) was cloned in rats [5], mice [6] and recently in humans [7], and differs from NT1-R in its low affinity for NT and its sensitivity to levocabastine. The mouse NT2-R is only 36% homologous to mouse NT1-R in global amino acid sequence, with increasing homology (60–75%) within transmembrane domains and extracellular loops [6]. Messenger RNAs for a deleted variant of NT2-R were identified in mouse brain, but were shown to encode a non-functional receptor [8]. We have characterized two potent and selective non-peptide antagonists for NT1-R, SR 48692 [9] and SR 142948 [10]. The binding domains of NT and SR 48692 on NT1-R were recently described to be distinct [11]. Unexpectedly, we have recently demonstrated that both SR 48692 and SR 142948 behave as potent agonists on NT2-R when assayed on inositol phosphate formation, calcium mobilization and arachidonic acid release [7]. In addition, NT alone does not elicit a biological response on NT2-R but completely antagonizes the effect of SR 48692 and SR 142948 [7].

Many of the central effects of NT are thought to involve interactions with dopamine systems [12]. Physiological or pharmacological neuronal stimulation activates several transcription factors, and following intrathecal administration of NT in rats, discrete sites of nuclei within the amygdala and hypothalamus have been shown to be immunoreactive with specific antibodies for c-*fos* and *krox24* [13]. We have also described that stimulation by NT of NT1-R-transfected CHO cells induces a transient activation of p42/p44 mitogen-activated protein kinases (MAPKs) and the expression of the *krox24* gene, both of which are thought to be mediated by the $G_{\beta\gamma}$ subunits of the G protein-coupled receptor [14]. In this study, we investigated in detail the signalling components from NT1-R leading to induction of *krox24* and c-*fos* genes in CHO cells.

2. Materials and methods

2.1. Reagents

Neurotensin and forskolin were purchased from Sigma Chemicals (St Quentin Fallavier, France). The non-peptide NT receptor antagonists SR 48692 [8] and SR 144528 [9] were synthesized in the Chemistry Department of Sanofi Recherche (Montpellier, France). Pertussis toxin (PTX) and GF109203X were purchased from Calbiochem (Meudon, France).

2.2. Plasmids

The original plasmids containing deletions of the murine *krox24* promoter as well as the mutant form of SRE_3, located upstream of chloramphenicol acetyltransferase (CAT), were previously published [15]: pBL395 contains the sequence −395 to +65 with respect to the initiation start site, pBL395Δ274 is pBL395 deleted for the portion −274/+45, pBL395Δ335 is pBL395 deleted for the portion −335/+45, and pBL395mSRE_3 is derived from pBL395 by PCR-generated point mutations in the SRE_3. Inserts were subcloned as *Xba*I fragments into the *Spe*I site of pUT112 (Eurogentech, Seraing, Belgium), upstream of the firefly luciferase coding sequence. The expression vector for hu-

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Abbreviations: CHO, Chinese hamster ovary; CRE, cAMP responsive element; NT, neurotensin; NT1-R, neurotensin type 1 receptor; NT2-R, neurotensin type 2 receptor; MAPK, mitogen-activated protein kinase; RLU, relative light unit; SRE, serum response element; TCF, ternary complex factor

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man NT1-R (p1274) as well as the corresponding empty vector (p658) were previously described [14]. pAdFosLuc was kindly provided by A.P. Czernilofsky (Vienna, Austria). The *trans* reporting system for Elk-1 (Stratagene, La Jolla, CA) consists of a plasmid containing the activation domains of Elk-1 linked to the DNA binding domain of Gal4 (Gal4/Elk-1) and a reporter plasmid containing five Gal4 DNA binding sites cloned upstream of a minimal promoter and the firefly luciferase gene (pFR-Luc).

2.3. Transient transfections and luciferase assay

CHO cells were maintained in αMEM medium (Gibco-BRL, Cergy Pontoise, France) containing ribonucleosides and deoxyribonucleosides, and supplemented with 10% fetal calf serum (FCS) and antibiotics. For transfections, exponentially growing cells were electroporated in PBS buffer together with a maximal amount of 15 μg plasmid as already described [14]. Following transfection, cells were diluted in complete αMEM medium, plated in 96-well microplates, and incubated overnight at 37°C. The next day, the medium was removed and replaced with FCS-free medium. Cells were further incubated for 24 h at 37°C before stimulation. For the analysis of *krox24*- and c-*fos*-luciferase genes, cells were stimulated for 90 min with NT. In the Elk-1 transactivation assay, cells were stimulated for 5 h with NT. When indicated, cells were treated for 5 min with SR 48692 or SR 142948 before adding NT. Following stimulation, cells were washed twice with PBS, cell extracts were prepared using the Cell Culture Lysis Reagent (Promega, Charbonnières, France), and luciferase activities were determined using the Luciferase Assay System (Promega) and a CCD camera (MTP Reader, Hamamatsu Photonics,

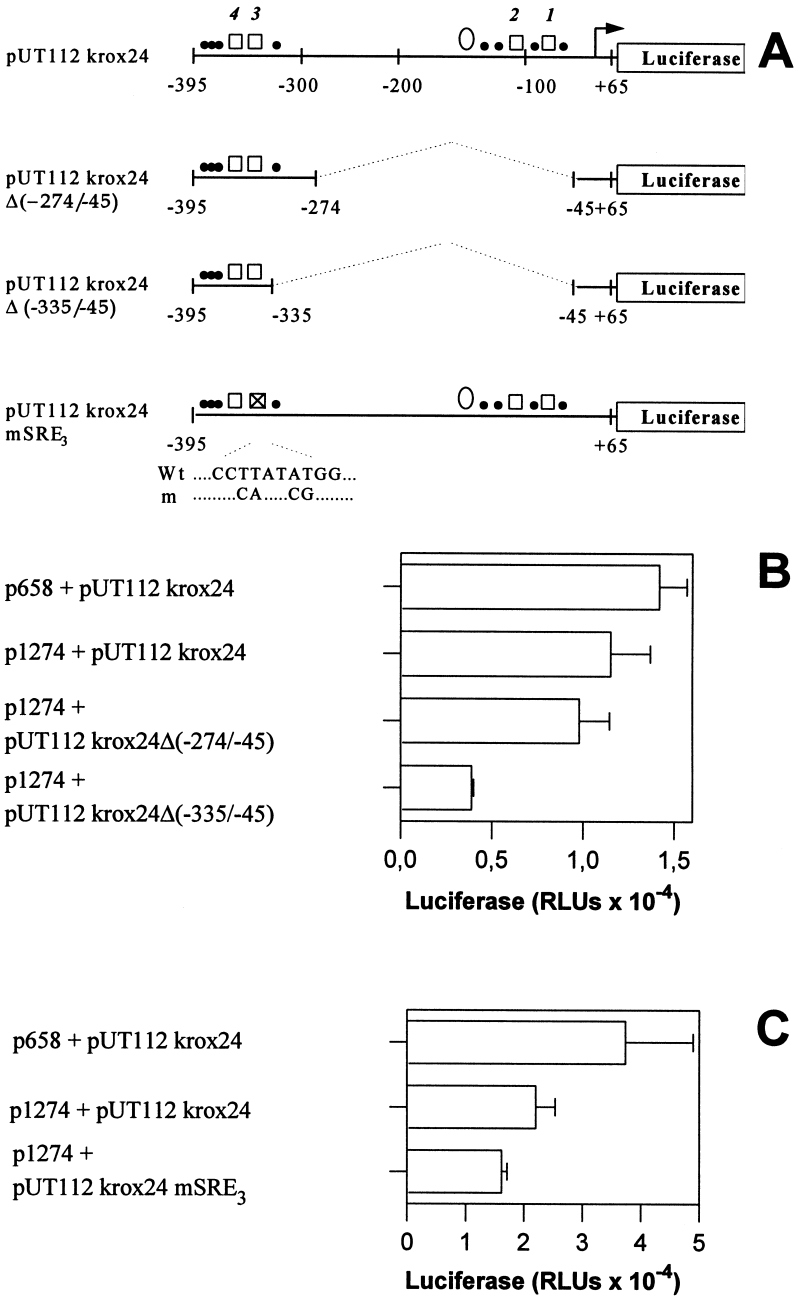

Fig. 1. *krox24* reporter constructions. A: Schematic illustration of the reporter plasmid containing the Wt murine *krox24* promoter, the plasmid deleted for the −274/−45 or the −335/−45 sequence, and the plasmid containing the mutated version of the SRE_3. The open squares represent the SREs, the open circle the CRE and the closed circles the Ets motifs. B, C: CHO Wt cells were co-transfected with 5 μg of the expression vector for hNT1-R (p1274) or the empty vector (p658) and 10 μg of the *krox24* reporter construct, as indicated. Basal luciferase activities in unstimulated cells were determined as described in Section 2. Mean results from triplicate samples are expressed in relative light units (RLUs).

Hamamatsu, Japan). Mean luciferase values from triplicate samples were expressed in relative light units (RLU) or as fold increase, defined by the ratios of RLU in stimulated versus unstimulated cells. All experiments were repeated at least three times.

2.4. Western blot analysis

CHO wild-type cells (CHO Wt) or CHO cells stably expressing hNT1-R [14] were stimulated for 90 min with NT receptor ligands before being lysed in Laemmli's buffer containing 6 M urea. When indicated, cells were treated for 5 min with the antagonists SR 48692 or SR 142948 before the addition of NT. Preparation of proteins and conditions of Western blot were previously published [14]. Briefly, cell extracts were heated for 5 min at 95°C and proteins were fractionated by SDS-PAGE on a 4–20% gradient acrylamide gel. Proteins were transferred onto nitrocellulose filters and blots were hybridized with the rabbit anti-*krox24* Ab (sc-110, Santa Cruz, CA, USA) or the rabbit anti-c-*fos* Ab (sc-52, Santa Cruz) at a concentration of 0.25 μg/ml. Immunocomplexes were revealed by a peroxidase-labeled anti-rabbit IgG conjugate associated with the enhanced chemiluminescence detection system (Amersham, Les Ulis, France).

3. Results and discussion

3.1. NT1-R-mediated activation of krox24

We have previously described that stimulation with NT of CHO cells stably transfected with hNT1-R led to the induction of endogenous *krox24* protein [14]. In order to examine the transcriptional activation of *krox24* gene we have also generated a stable double transfected CHO cell line expressing hNT1-R and a luciferase reporter linked to the −395/+65 sequence of the murine *krox24* promoter [14]. The specific involvement of NT1-R in both endogenous *krox24* response and reporter induction by NT was demonstrated by the preventing effect of the non-peptide antagonist SR 48692 in cells expressing hNT1-R, whereas no modulation by the ligands were observed in wild-type cells [14,16]. Regulatory elements located within the 400 bp proximal *krox24* upstream sequence are mainly composed of four serum responsive elements (SREs) and one cAMP response element (CRE) ([17] and Fig. 1A). We first investigated those preferentially activated following stimulation of NT1-R. Co-transfecting CHO cells with NT1-R (p1274) and pUT112-*krox24* (comprising the −395/+65 sequence) resulted in an increase in reporter activity of about 30-fold when stimulating with 30 nM NT, whereas co-transfecting cells with pUT112-*krox24* and the control

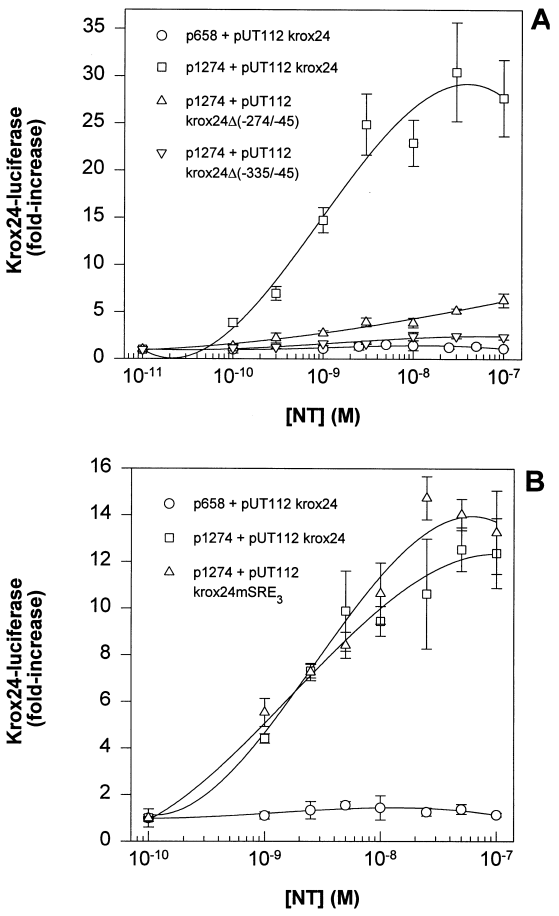

Fig. 2. *krox24* promoter analysis. A, B: CHO Wt cells were co-transfected as described in the legend to Fig. 1, and treated with increasing concentrations of NT for 90 min. The level of induction by NT is defined as the ratio of mean luciferase activities in stimulated vs. unstimulated cells and is expressed as fold increase above basal level.

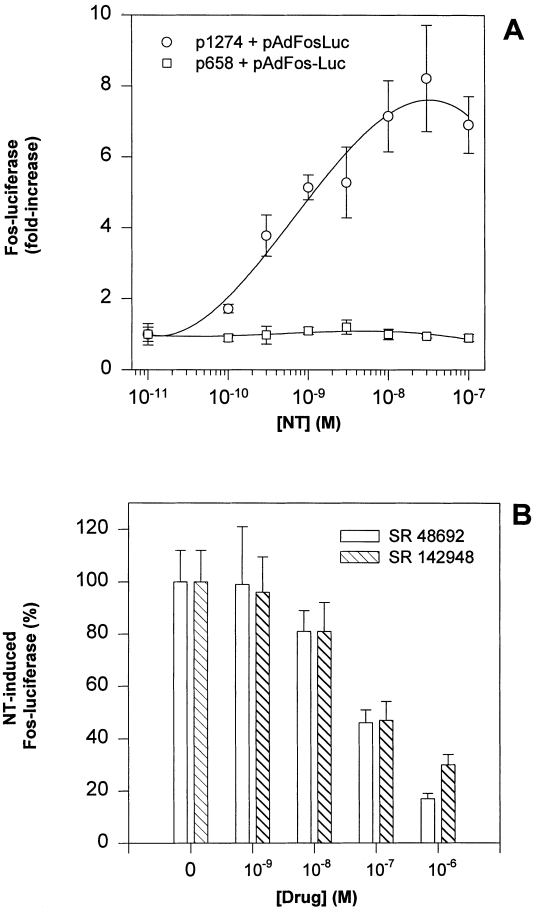

Fig. 3. Effect of SR 48692 and SR 142948 on c-*fos* gene induction by NT. A: Dose-response effect of NT on the expression of c-*fos*-luciferase. CHO Wt cells were co-transfected with 10 μg pAdNeoFos-Luc and either 5 μg hNT1-R (p1274) or the empty vector (p658). Cells treated as described in the legend to Fig. 1 were stimulated for 90 min with increasing concentrations of NT and mean luciferase activities in cell extracts from triplicate samples were determined. B: Effects of NT1-R antagonists on c-*fos* gene induction. Cells treated as described in the legend to Fig. 1 were preincubated for 5 min with increasing concentrations of the antagonists SR 48692 or SR 142948 prior to the addition of 10^{-8} M NT. Results are expressed as a percentage of luciferase values in cells pretreated with the vehicle only before the addition of NT.

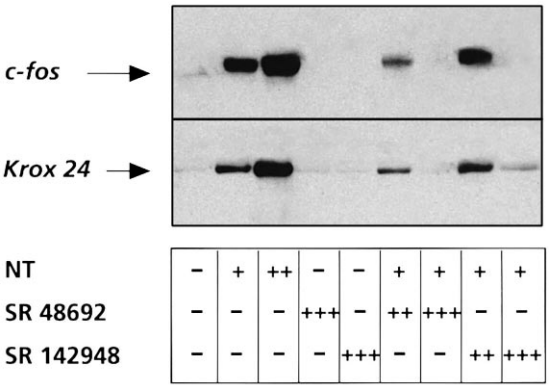

Fig. 4. Effects of SR 48692 and SR 142948 on the expression of *krox24* and c-*fos* proteins by NT. CHO cells expressing hNT1-R (CHO-hNT1-R) were treated for 90 min with NT, SR 48692, SR 142948, or pretreated for 5 min with the antagonists before the addition of NT. Protein extracts were prepared, run on SDS-PAGE, and blotted onto nitrocellulose filters as described in Section 2. Filters were hybrized with anti-c-*fos* or anti-*krox24* Ab. Concentrations of the ligands: −: untreated; +: 10^{-8} M; ++: 10^{-7} M; +++: 10^{-6} M.

plasmid empty of hNT1-R cDNA (p658) showed no modulation of the reporter, confirming that NT effects are mediated by NT1-R (Fig. 1B). Deleting the sequence −274/−45 of the *krox24* promoter, comprising the two proximal SREs and the CRE sequence led to a dramatic decrease of reporter inducibility (six-fold activation with 30 nM NT, 80% decrease, Fig. 2A). The deletion −335/−45 further contributed to reduce activation from six-fold to two-fold. In addition, the deletion −274/−45 did not significantly affect basal reporter activity whereas the deletion −335/−45 led to a dramatic decrease (more than 60%) of basal luciferase (Fig. 1B). Our results indicate that elements comprised in the proximal −275/−45 sequence are necessary for maximal activation by NT. Similar results were described for nerve growth factor (NGF) response in PC12 cells and the authors demonstrated that mutating either SRE_1 or SRE_2 reduced *krox24* gene inducibility by NGF [18]. Further deleting the sequence −274/−335 nearly completely abolished the NT effect (Fig. 2A). To analyze the potential involvement of the Ets binding sequence located in this region (around −315), we next investigated whether introducing point mutations in the adjacent SRE_3 altered reporter inducibility NT. Results of co-transfections of hNT1-R with either the wild-type −395/+65 *krox24* sequence or the mutated version of SRE_3 (*krox24* $mSRE_3$) indicated that neither basal expression (Fig. 1C) nor the level of induction by NT (Fig. 2B) was significantly modified by the mutations, suggesting that there is no cooperative effect between SRE_3 and the adjacent Ets sequence for maximal transcriptional activity.

3.2. NT1-mediated activation of c-fos and Elk-1

Since the c-*fos* promoter contains similar regulatory elements as the *krox24* promoter [19], we next investigated whether the c-*fos* gene could be activated by hNT1-R. In a transient transfection assay of CHO cells with hNT1-R and pAdFosLuc, a plasmid comprising the c-*fos* regulatory sequences linked to luciferase, we observed a dose-dependent increase in luciferase activity following stimulation with NT (Fig. 3A). The specific involvement of hNT1-R in this activation was demonstrated by the absence of stimulation by NT in co-transfection experiments with pAdFosLuc and the control vector lacking NT1-R (Fig. 3A) and by the observation that the antagonists SR 48692 and SR 142948 dose-dependently prevented NT response in NT1-R-transfected cells (Fig. 3B).

We next asked whether the endogenous c-*fos* protein was similarly regulated by NT receptor ligands in CHO cells stably expressing hNT1-R, and we compared c-*fos* and *krox24* induction. Western blot analysis show that NT strongly induced the amount of both proteins in CHO-hNT1-R cells (Fig. 4), whereas NT has no effect in CHO Wt cells (data not shown). Maximal induction was obtained with concentrations of NT ranging from 10^{-8} M to 10^{-7} M and was similar for both proteins, which is consistent with reporter assays. The antagonists SR 48692 and SR 142948 alone had no effect on basal expression of either c-*fos* or *krox24* in CHO-hNT1-R cells (Fig. 4) or CHO Wt cells (data not shown). When CHO-hNT1-R cells were treated with SR 48692 or SR 142948, a dose-dependent inhibition of c-*fos* and *krox24* expression

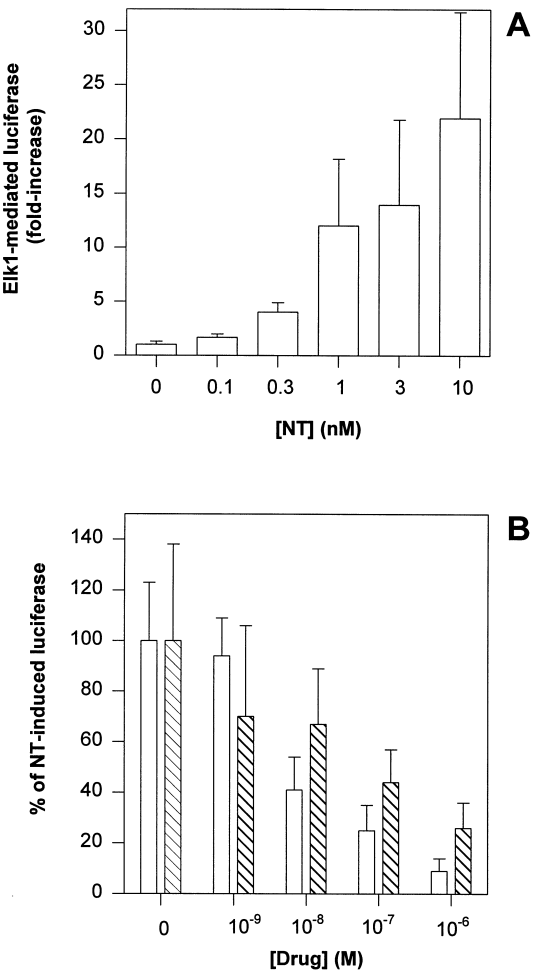

Fig. 5. NT1-mediated activation of Elk-1. A: Dose-response effect of NT. CHO cells were co-transfected with 2.5 μg hNT1-R (p1274)+500 ng Gal4-Elk-1+7.5 μg Gal4-Luc and treated as described in the legend to Fig. 1. Mean luciferase activities in cell extracts from triplicate samples were determined following 5 h of stimulation with NT. B: Effects of NT1-R antagonists. Cells transfected as above were preincubated for 5 min with increasing concentrations of SR 48692 or SR 142948 prior to the addition of 10^{-8} M NT. Results are expressed as a percentage of luciferase values in cells pretreated with the vehicle only before the addition of NT.

Table 1
NT1-R-mediated activation of *krox24*, c-*fos* and Elk-1

Stimuli	Luciferase activity (fold increase ± S.E.M.)		
	krox24 reporter	c-*fos* reporter	Elk-1 reporter
Untreated	1.00 ± 0.16	1.00 ± 0.49	–
NT	8.53 ± 0.53	5.38 ± 0.65	–
Forskolin	2.98 ± 0.35	1.33 ± 0.5	–
Forskolin+NT	10.1 ± 0.8	7.32 ± 1.2	–
Untreated	1.00 ± 0.06	1.00 ± 0.14	1.00 ± 0.52
NT	20.4 ± 1.24	38.74 ± 5.84	27.11 ± 4.44
PTX	0.96 ± 0.13	1.00 ± 0.12	0.89 ± 0.59
PTX+NT	4.06 ± 0.36	49.57 ± 5.36	6.33 ± 3.81
Untreated	1.00 ± 0.08	1.00 ± 0.13	1.00 ± 0.58
NT	19.01 ± 2.73	28.88 ± 1.37	33.16 ± 7.23
GFX	0.66 ± 0.11	0.59 ± 0.14	0.38 ± 0.27
GFX+NT	6.96 ± 1.23	8.67 ± 0.54	5.39 ± 1.97

CHO cells co-transfected with NT1-R and the indicated reporter construct were treated for 5 min with forskolin (1 μM) before the addition of NT (10^{-8} M), or for 18 h with PTX (10 ng/ml), or for 3 h with GF109203X (GFX, 1 μM) before the addition of NT (10^{-7} M). Cells were further stimulated for 1.5 h for the *krox24* and c-*fos* reporters and for 5 h for the Elk-1 reporter. As a control, for each type of stimulation, cells were pretreated with the vehicle. Results are expressed as fold increase in luciferase level above the level in untreated cells ± S.E.M.

mediated by NT was observed. Collectively, these results indicate that SR 142948 and SR 48692 potently prevent *krox24* and c-*fos* induction by NT, both at the protein level and at the transcriptional level, demonstrating that the two early genes are specifically activated through NT1-R.

We have previously shown that NT stimulation of CHO cells stably expressing NT1-R is followed by the transient activation of MAPKs [14] and similar results were also recently reported for NT2-R signalling in transfected CHO cells [7]. The coordinate biological response mediated by the various MAPK pathways was shown to converge at the SRE of either c-*fos* [20–22] or *krox24* [23] through ternary complex factors (TCFs) including Elk-1 and *SAP-1a*. We thus investigated whether Elk-1 could be involved in the activation of the early genes by NT1-R through the MAPK pathway. The activity of TCFs, which are ubiquitously expressed in the cell, is regulated by phosphorylation on serine/threonine residues on the COOH terminal end. Using a transactivation reporter assay for Elk-1, we observed a dose-dependent increase in luciferase activity following stimulation with NT in CHO cells co-transfected with NT1-R (Fig. 5A) but not in cells co-transfected with the empty vector p658 (data not shown). In addition, treatment with the antagonists SR 48692 or SR 142948 prevented the NT-induced response in NT1-R expressing cells (Fig. 5B). The antagonist potentials of both SR compounds in the Elk-1 transactivation assay are in agreement with previous ones in *krox24* or c-*fos* reporter assays.

3.3. Role of cAMP regulatory elements in NT-mediated transcriptional activation of krox24 and c-fos genes

In NT1-R-transfected CHO cells, it is well documented that NT raises cAMP levels [24,25], an effect which is prevented by the antagonist SR 48692 [26]. Since both *krox24* and c-*fos* promoters contain a CRE element, and since we demonstrated above that the region −274/−45 of the *krox24* promoter, including the CRE, was mainly involved in NT-mediated activation, we next asked whether cAMP could directly modulate the expression of the early genes. The cAMP-inducing agent forskolin, at a concentration of 1 μM, only slightly stimulated luciferase activity from either *krox24* (three-fold) or c-*fos* (1.3-fold) reporter plasmids, as compared to the stimulation obtained with NT (Table 1). Increasing the forskolin concentration to 10^{-5} M did not further enhance luciferase activities (data not shown). Furthermore, the effect of forskolin was additive with that of NT. Thus our results indicate that if cAMP potentially contributes to transcriptional activation of *krox24* or c-*fos* by NT through the CRE, it is not sufficient to drive optimal gene induction.

3.4. Krox24 and c-fos genes are induced through alternative signalling pathways

We have previously shown that the NT1-R-mediated activation of MAPK was partially prevented by PTX, which ADP ribosylates G proteins of the G_o/G_i type [14]. As shown in Table 1, pretreatment for 18 h with 10 ng/ml PTX also partially prevented the induction by NT of *krox24* (80%) and Elk-1 (74%) but not c-*fos*. This result clearly indicates that Elk-1-mediated gene activation may be restricted to *krox24*. The coupling of NT1-R to G_q proteins, leading to phospholipase C activation and subsequent inositol phosphate increase and Ca^{2+} mobilization, is well documented [26]. The direct involvement of G_q in c-*fos* activation through the increase in Ca^{2+} is a possible explanation since the CRE binding element is phosphorylated either by the cAMP-dependent protein kinase or the Ca^{2+}/calmodulin-dependent kinase at the same site, serine-133 [27]. It has been also demonstrated that members of the Rho family GTPases can link Gα12 to the transcriptional activation of the c-*fos* SRE, by a pathway that is independent of TCF [28,29].

NT-mediated activation of MAPK and induction of *krox24* protein expression also partially involve protein kinase C [14]. Using the protein kinase C inhibitor GF109203X we observed that it equally but partially prevented the induction by NT of reporter for *krox24* (64%), Elk-1 (58%) and c-*fos* (51%) (Table 1). Thus, although the signalling pathways leading to activation of the three proteins may differ from the type of G protein coupling, they share common elements, including a protein kinase C.

4. Concluding remarks

In this paper, we have explored in detail the signalling

components associated with activation of NT1-R in transfected CHO cells, and leading to the modulation of the early genes *krox24* and c-*fos*. We conclude that the transduction pathways are not related to cAMP metabolism, and we provide new evidence for the cascade G_i/G_o-coupled NT1 receptor/p42/p44 MAPKs/Elk-1/*krox24*, whereas signalling to c-*fos* does not involve coupling to G_i/G_o proteins. Since stimulation of both NT1-R and NT2-R is followed by the transient activation of MAPKs, and since NT and SR compounds exhibit opposite pharmacological effects (agonist or antagonist) on the two types of receptor, it may be of particular interest to investigate their functions in cells naturally co-expressing both receptors.

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