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Retinoic acid regulates selectively the expression of immediate early response genes in PC12 cells

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Abstract Nerve growth factor (NGF) induces neuronal differentiation and growth arrest in PC12 cells. One of the initial effects of NGF in these cells is the induction of the expression of immediate early genes (IEGs). In this study we have analyzed the influence of retinoic acid (RA), which exerts important effects on PC12 cell proliferation and function, on the expression of IEGs. Incubation with RA did not alter NGFI-A mRNA levels, but significantly reduced the NGFI-B and c-*fos* response to NGF and serum. The response to NGF was maximal in the presence of cycloheximide, and RA also reduced the superinduction of NGFI-B and c-*fos* mRNA levels. Sequences contained within the 5′ flanking region of the c-*fos* gene confer responsiveness to NGF and mediate the inhibitory effect of RA. The differential regulation by RA suggests that NGF induces expression of the three IEGs by different mechanisms.

Key words: Retinoic acid; Nerve growth factor; Immediate early gene

1. Introduction

The primary response to many agents that promote cell growth and differentiation is the rapid and transient induction of a set of genes called immediate early genes (IEGs) [1]. Transcription of these genes does not require de novo protein synthesis and, in fact, stimulation of cells in the presence of a protein synthesis inhibitor prolongs transcription and stabilizes the corresponding mRNAs, resulting in superinduction [2]. Many IEGs encode transcription factors including, among others, members of the Fos and Jun families and several zinc finger proteins. These transcription factors are thought to couple early signalling events with long-term alterations in gene expression.

Transcriptional stimulation of IEG expression, especially c-*fos*, has been extensively studied in PC12 cells [3]. This cell line has characteristics of precursor cells for both sympathetic neurons and chromaffin cells. Glucocorticoid hormones promote differentiation of PC12 cells along the chromaffin pathway with induction of chromaffin markers [4]. However, upon incubation with nerve growth factor (NGF), which stimulates the Ras-MAPK (mitogen-activated protein kinase) signalling pathway, PC12 cells acquire a phenotype resembling sympathetic neurons. NGF causes a rapid induction of IEGs which is followed by a later expression of 'secondary' genes responsible for the neuronal phenotype [5,6].

Retinoic acid, an endogenous metabolite of vitamin A, has pronounced effects on cellular proliferation and differentiation. The actions of retinoic acid (RA) are mediated by nuclear receptors (RARs and RXRs) which belong to the steroid/thyroid hormone receptor superfamily and act as ligand-inducible transcription factors [7,8]. We have recently shown that RA blocks proliferation of PC12 cells [9]. In addition, RA causes a transient expression of the low-affinity neurotrophin receptor $p75^{NTR}$, tyrosine hydroxylase and TGF-β1 genes similar to that observed during NGF-induced neuronal differentiation [9,10], and cooperates with NGF and Ras [11] to induce the expression of transin, a metalloprotease involved in neuronal differentiation. On the other hand, RA has been shown to induce IEG expression in embryonal carcinoma [12] and preosteoblastic cells [13], whereas in pituitary cells we have shown that this ligand decreases the c-*fos* response to different stimuli [14,15].

In this study we have compared the effect of RA on the response of three IEGs, c-*fos*, NGFI-A (also called *zif*268, *egr*-1, *krox*24, TIS8 and D2), and NGFI-B (also called N10 and *nur*77), to NGF in PC12 cells. NGFI-A encodes a member of the zinc finger transcriptional activator family [16], and NGFI-B encodes an orphan receptor member of the steroid/thyroid hormone receptor superfamily [17]. Our data show that RA has a specific effect on the expression of different IEGs in PC12 cells. RA attenuates the response of NGFI-B and c-Fos to NGF, but does not alter NGF induction of NGFI-A. This differential regulation suggests that NGF induces expression of these IEGs by different mechanisms.

2. Materials and methods

2.1. PC12 cell cultures

PC12 cells were cultured as previously described [9–11] in RPMI medium containing 10% donor horse serum (Quality Biological Inc.) and 5% fetal calf serum (Gibco).

2.2. RNA extraction and hybridization

Total RNA was extracted from the cell cultures with guanidine thiocyanate. The RNA was run in 1% formaldehyde-agarose gels and transferred to nylon-nitrocellulose membranes (Nytran) for Northern blot analysis. The RNA was stained with 0.02% methylene blue, and the blots were sequentially hybridized with cDNA probes for NGFI-A [16], NGFI-B [18], and c-*fos* [19] labelled by random oligonucleotide priming. Hybridizations were carried out at 42°C with 50% formamide and the most stringent wash was at 42°C with 0.1×SSC/0.1% SDS. Quantification of mRNA levels was carried out by densitometric scan of the autoradiograms. The values obtained were always corrected for the amount of RNA applied in each lane which was determined by densitometry of the methylene blue-stained membranes.

2.3. DNA transfection

The plasmid FC4 [20] contains 404 bp upstream of the c-*fos* start of transcription linked to the chloramphenicol acetyltransferase (CAT) gene. In the construct SRE/TATA, the 20 bp dyad symmetry region located at −310 within the c-*fos* promoter was fused to minimal

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promoter sequences containing essentially the TATA box. In the plasmids p4xSRE and p4xCRE, four copies of the SRE or CRE elements, respectively, were inserted in tandem in front of the thymidine kinase (TK) promoter of pBLCAT2 [14]. For transient expression assays the cells were plated 24 h prior to transfection by the calcium phosphate method in 60 mm petri dishes. The cells were transfected with 5 μg of the reporter CAT constructs in the presence of 10 μg carrier DNA (high molecular weight calf thymus DNA, Boehringer). After overnight incubation with the calcium phosphate precipitate, the cells were incubated for 48 h with RA in the presence or absence of NGF for the last 8 h of treatment, and CAT activity was determined. Each treatment was performed in triplicate cultures which normally exhibited less than 10–15% variation in CAT activity, and each experiment was repeated twice with similar differences in regulated expression.

3. Results

In order to determine whether RA regulates the expression of IEGs in PC12 cells, the effect of incubation for 30 min with 30 ng/ml NGF was analyzed in untreated cells and in cells preincubated with 1 μM RA for 48 h. Fig. 1 shows the effect of RA on the NGF response of NGFI-A, NGFI-B and c-*fos* mRNAs. Unstimulated PC12 cells expressed undetectable levels of these mRNAs, and these levels were greatly increased by treatment with NGF for 30 min. Thereafter, the transcripts of the three IEGs decreased rapidly, being barely detectable at 2 h. Basal mRNA levels remained undetectable in PC12 cells incubated with RA. Similar results were obtained in additional experiments in which the influence of RA was analyzed at different time periods ranging between 30 min and 72 h (data not shown). The response of NGFI-A mRNA to NGF was essentially unchanged in cells that were pretreated with RA for 48 h. However, the induction of NGFI-B and c-*fos* transcripts was significantly reduced in RA-treated cells. Stimulation in the presence of cycloheximide leads to superinduction of the mRNAs for the three IEGs, and under these conditions the response to NGF reached the highest levels at 2 h. Again, RA was ineffective in regulating the superinduced levels of

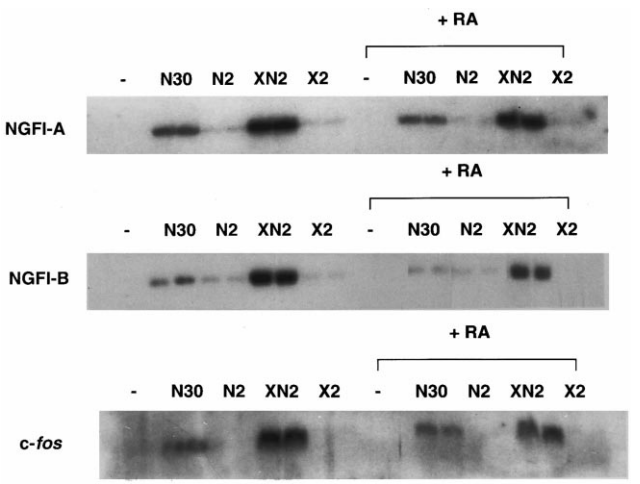

Fig. 1. Influence of RA on the induction of IEG mRNAs in PC12 cells. The cells were incubated in the absence or presence of 1 μM RA for 48 h, and treated with 50 ng/ml NGF (N) and/or 5 μg/ml cycloheximide (X) for the last 30 min or 2 h as indicated. −: control; N30: NGF 30 min; N2: NGF 2 h; XN2: cycloheximide+NGF 2 h; X2: cycloheximide 2 h. Northern blot analysis was carried out with 30 μg total RNA and labeled NGFI-A, NGFI-B and c-*fos* cDNA probes. Representative blots obtained from duplicate cultures for each treatment are shown.

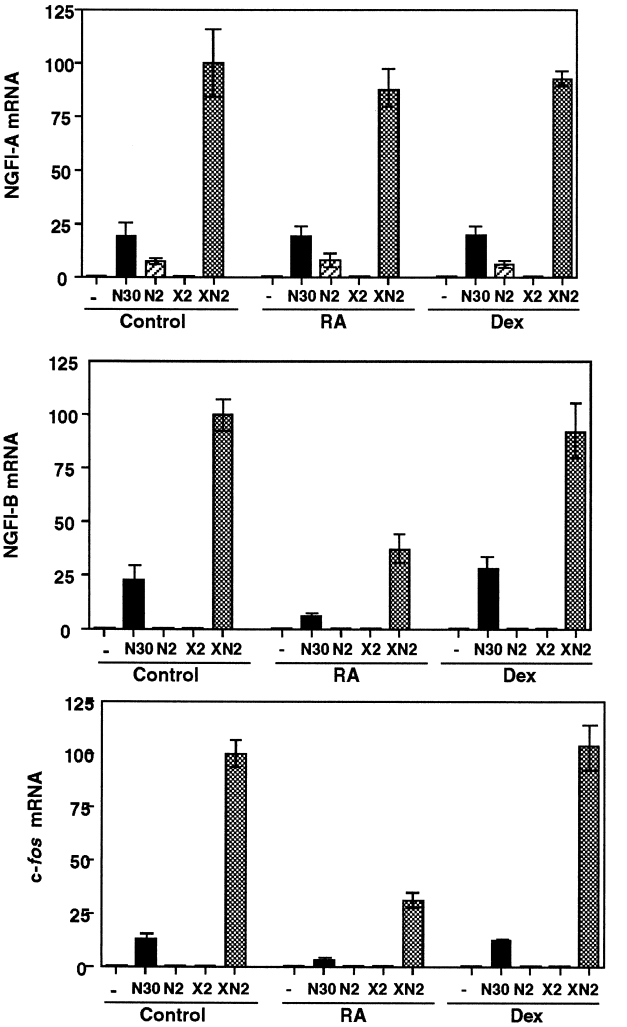

Fig. 2. Comparison of the effects of RA and dexamethasone on IEG expression in PC12 cells. The cells were treated with medium alone (Control), 1 μM RA or 100 nM dexamethasone (Dex) for 48 h. The same treatments as in Fig. 1 were administred for the last 30 min or 2 h: N30: NGF 30 min; N2: NGF 2 h; X2: cycloheximide 2 h; XN2: cycloheximide+NGF 2 h. Northern blot analysis was carried out with total RNA and labeled NGFI-A, NGFI-B and c-*fos* cDNA probes. The blots were quantitated by densitometry, and the values obtained were corrected for the amount of RNA applied to each lane. The data for each mRNA are expressed as the percent of the maximal superinduced levels (obtained in cells incubated with NGF and cycloheximide), which were arbitrarily set to 100%. The data represent the mean ± S.D. values obtained from three independent experiments performed in duplicate.

NGFI-A mRNA, but significantly decreased the superinduction of NGFI-B and c-*fos* transcripts found in cells treated with cycloheximide and NGF for 2 h. The repressive effect of RA on both induction and superinduction of these mRNAs was not a rapid effect, since 2 or 8 h of treatment with RA were not sufficient to alter this response to NGF or to the combination of NGF plus cycloheximide (data not shown).

Glucocorticoid hormones and NGF have been described to have an opposite effect on regulation of several genes in PC12 cells [4]. However, as shown in Fig. 2, treatment with 100 nM dexamethasone did not significantly influence the NGFI-A, NGFI-B and c-*fos* responses to NGF. Superinduction in the

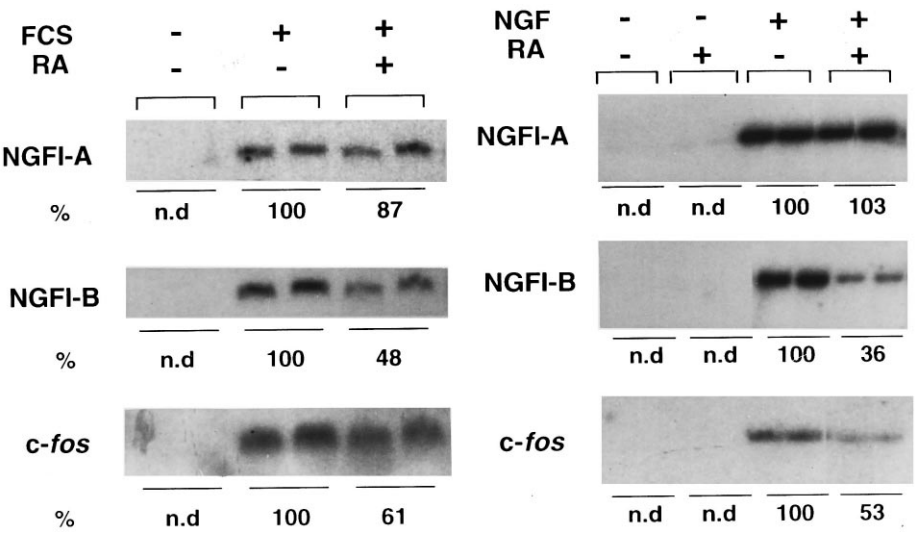

Fig. 3. Retinoic acid modulates the response of NGFI-B and c-*fos* to serum. Northern blot analysis was carried out with 30 μg of RNA obtained from PC12 cells treated with 1 μM RA for 48 h and with 20% fetal calf serum (FCS) (left panel) or with 50 ng/ml NGF (right panel) for the last 30 min, as indicated. The blots were quantitated and the data are shown as the percent of the values obtained in the cells treated with either FCS or NGF. n.d: not detectable.

presence of cycloheximide was not affected either by dexamethasone in PC12 cells. In contrast, RA decreased by 2–3-fold the NGFI-B and c-*fos* response to a 30 min incubation with NGF or NGF plus cycloheximide. The lack of effect of dexamethasone suggests that the repressive effect of RA was specific for this ligand. This was confirmed with other ligands of members of the steroid/thyroid hormone receptor superfamily, since the inductions of NGFI-B or c-*fos* were not altered by incubation of PC12 cells with 5 nM triiodothyronine (T3) or 100 nM 1,25$(OH)_2$-vitamin D3 (data not shown).

In order to analyze whether the reduced induction of NGFI-B and c-*fos* transcripts was specific for NGF, or whether it extended to other stimulators of the expression of these genes, the influence of RA on the response to serum was also examined in PC12 cells. As shown in Fig. 3, incubation with 20% fetal calf serum for 30 min increased the mRNA levels of the three IEGs to levels similar to those induced by the same time of incubation with NGF. RA again had a specific effect on NGFI-B and c-*fos* transcripts, reducing their response to serum (left panel) and NGF (right panel). The induction of NGFI-A mRNA by serum was not altered in cells treated with RA.

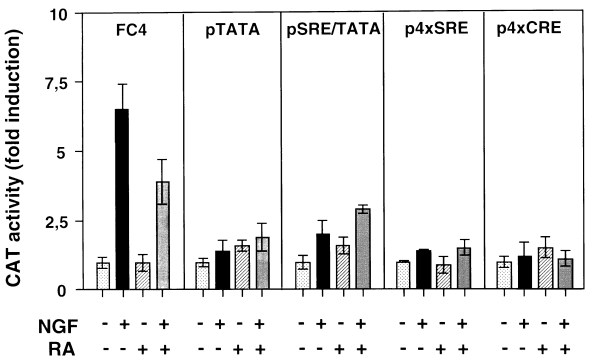

Fig. 4. Influence of NGF and RA on c-*fos* promoter activity. PC12 cells were transfected with 5 μg of the CAT construct FC4 which contains the c-*fos* 5′ flanking region linked to CAT, or with CAT constructs containing elements present in the c-*fos* promoter. pTATA contains minimal promoter sequences. In pSRE/TATA, the serum response element has been added to this construct. The plasmid p4xSRE contains four copies of the serum response element fused in tandem to the thymidine kinase (TK) promoter, and p4xCRE is similar but contains four copies of the cyclic AMP response element. After transfection the cells were incubated with 1 μM RA or medium alone for 48 h and, when indicated, with 50 ng/ml NGF for the last 8 h of treatment. The data represent the mean CAT activity obtained in two separate experiments performed with duplicate cultures and are expressed as the increase found over the corresponding levels obtained for each construct in the untreated cells.

To examine whether the decreased NGF induction following RA treatment of PC12 cells is exerted at the transcriptional level, we performed transient transfection assays using the fos-CAT fusion construct FC4 [20]. The region of the c-*fos* promoter contained within this construct includes all of the previously characterized c-*fos* regulatory elements. Fig. 4 shows the effect of a 48 h incubation with RA on basal c-*fos* promoter activity and on the promoter response to NGF. The neurotrophin increased by about 6-fold c-*fos* promoter activity. RA did not affect basal CAT levels, but decreased to less than 4-fold the response to NGF. Since phorbol esters are potent stimulators of c-*fos* gene expression, the influence of RA on the response to 100 nM TPA was also examined. TPA induced a 5-fold increase in CAT levels, and RA reduced this induction to 2.1-fold (not illustrated). A minimal promoter construct (pTATA) was affected by neither NGF nor RA. It has been described that the serum response element (SRE) of the c-*fos* promoter is critical for NGF activation of c-*fos* gene transcription [21], although more recent data also demonstrate the important role of a cyclic AMP response element (CRE) for this regulation [22]. Thus, we also analyzed the influence of NGF and RA on the activity of constructs containing isolated SRE or CRE elements. Fig. 4C shows that the construct pSRE/TATA, which contains the SRE ligated to the proximal promoter sequences of c-*fos*, was weakly stimulated by NGF, and that RA did not decrease this stimulation. Similar results were obtained with a construct in which four copies of the SRE were ligated to a heterologous (TK) promoter. In addition, the CRE alone was incapable of confer-

ring responsiveness to NGF, as shown in the right panel of Fig. 4, in which the cells were transfected with a TK-CAT plasmid containing four copies of the CRE. RA was also ineffective in regulating this construct. Therefore, although the c-*fos* promoter is responsive to both NGF and RA, cooperation among different promoter elements appears to be required for either induction by NGF or repression by RA.

4. Discussion

Incubation of PC12 cells with NGF and other growth factors causes a rapid and transient increase in the expression of different IEGs, which becomes superinduced in the presence of an inhibitor of protein synthesis. In this study we have demonstrated that RA, which affects proliferation and differentiation of PC12 cells, can modulate the response of the IEGs NGFI-B and c-*fos* to NGF.

Deletion analysis within the 5′ regulatory region of the c-*fos* gene has demonstrated that the SRE is involved in the induction of c-*fos* transcription by NGF and other growth factors [21]. In addition, more recent data have shown that this induction also requires the CRE present in the c-*fos* promoter. A NGF-inducible, Ras-dependent protein kinase, which was identified as RSK2, and catalyzes the phosphorylation of CREB (the cyclic AMP response element binding protein), was found to trigger this activation [22,23]. Immediate early transcription of NGFI-A and -B is also regulated by sequences that are similar to, but distinct from, those that regulate c-*fos* expression. In the case of NGFI-B, two AP-1-like sites which could also act as CREs, as well as a GC-rich region are essential for its induction by NGF in PC12 cells [24]. NGF appears to induce NGFI-A expression through complex regulatory elements that are also sensitive to serum and TPA, and at least two SREs and an AP-1-like element contribute to NGF induction of NGFI-A [25].

Although the three IEGs have similar expression kinetics following NGF stimulation, and despite the extensive similarity between the promoter elements involved, their induction must have different components since their regulation by RA is different. Whereas NGFI-A inducibility by NGF is not affected by RA, this retinoid significantly represses NGF responsiveness of the NGFI-B and c-*fos* genes. RA also attenuates serum inducibility of NGFI-B and c-*fos* without altering the response of NGFI-A to serum. This inhibitory effect of the retinoid is not in disagreement with the reported increase in the NGF sensitivity in PC12 cells due to induction of low-affinity NGF receptors [26], because neither the selectivity of the RA inhibitory effect towards some early genes, nor the RA attenuation of the serum inducibility of such genes, can be easily explained by a modulation of the levels of NGF receptors.

At least in the case of the c-*fos* gene the repressive effect of RA is exerted at the transcriptional level, since RA decreases the activation of the c-*fos* promoter by NGF in transient transfection assays. The activation by NGF appears to require the cooperation between different promoter elements, since isolated SRE or CRE elements are not sufficient for a full NGF responsiveness ([22] and Fig. 4). The SRE of the c-*fos* gene gives some response to NGF and this response is not affected by RA, suggesting that other *cis*-acting elements are involved in its inhibitory action. An anti AP-1 activity of the RA receptors is well known, and we have previously demonstrated the participation of AP-1 sites in the regulation of c-*fos* by RA in pituitary cells [14]. That these elements are likely regulated by RA in PC12 cells is also suggested by the finding that the response of the c-*fos* promoter to TPA is also repressed by RA. However, the only presence of AP-1 sites in the promoter cannot account for the RA-mediated inhibition of the c-*fos* or NGFI-B response to NGF, since the NGFI-A promoter also contains an AP-1 element that participates in the induction by NGF [25], and this response is not altered by RA.

The data obtained in the present study show that RA has effects on the expression of c-*fos* and NGFI-B that are antagonistic to those exerted by NGF. Since the important role of c-*fos* in cell proliferation, differentiation and function has been widely recognized, the regulation of the expression of this gene by RA may play a role in these processes. However, RA does not alter the morphological response to NGF [9]. In addition to inducing neurite extension, NGF inhibits division of PC12 cells, and we have previously shown that RA also markedly arrests PC12 cell growth, and that the combination of NGF plus RA does not cause a further inhibition of cell proliferation [9].

The NGFI-B-encoded protein is an 'orphan' member of the nuclear hormone receptor superfamily, and has been shown to act as a potent transcriptional activator [27]. This receptor binds as a monomer to DNA but it can also heterodimerize with RXR and, therefore, modulate the RA signal transduction pathway [28]. The regulation of NGFI-B expression by RA observed here in PC12 cells shows the existence of a complex cross-talk between the signalling pathways of NGFI-B and the retinoid receptors which could also have important consequences in PC12 cell function.

It has been previously shown that RA rapidly and transiently increases the expression of NGFI-A in embryonal carcinoma cells [12] and in an osteoblastic cell line [13], where the retinoid induces differentiation. The RA-mediated transcriptional increase in NGFI-A gene transcription appears to be mediated by interaction of RA receptors with a RA response element (RARE) in the 5′ flanking regulatory region of the gene. Although we have previously shown that RA is able to induce transcription of a reporter gene containing a RARE in PC12 cells [9], we did not observe an induction of NGFI-A gene expression by RA in these cells. A similar lack of induction by RA has been observed in mature osteoblastic cells. In the latter case, the suppression of RA-stimulated gene expression has been attributed to a 29 bp sequence located downstream of the RA-responsive region, which is bound by nuclear proteins from several cell types. It is possible that these sequences responsible for cell-type-specific suppression might be involved in the lack of NGFI-A responsiveness to RA in PC12 cells.

Since the IEGs examined appear to play an important role in cell proliferation, differentiation and function, their differential modulation by RA could help to elucidate the molecular mechanisms by which the neurotrophins and other growth factors elicit their programs of gene expression in PC12 cells.

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