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Rapid Effects of Retinoic Acid on CREB and ERK Phosphorylation in Neuronal Cells

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Retinoic acid (RA) is a potent regulator of neuronal cell differentiation. RA normally activates gene expression by binding to nuclear receptors that interact with response elements (RAREs) in regulatory regions of target genes. We show here that in PC12 cell subclones in which the retinoid causes neurite extension, RA induces a rapid and sustained phosphorylation of CREB (cyclic AMP response element binding protein), compatible with a nongenomic effect. RA also causes a rapid increase of CREB phosphorylation in primary cultures of cerebrocortical cells and of dorsal root ganglia neurons from rat embryos. RA-mediated phosphorylation of CREB leads to a direct stimulation of CREB-dependent transcriptional activity and to activation of the expression of genes such as c-*fos*, which do not contain RAREs but contain cAMP response elements (CREs) in their promoters. CREB is a major target of extracellular signal regulated kinase ERK1/2 signaling in neuronal cells, and we demonstrate here that RA induces an early stimulation of ERK1/2, which is required both for CREB phosphorylation and transcriptional activity. These results demonstrate that RA, by a nongenomic mechanism, stimulates signaling pathways that lead to phosphorylation of transcription factors, which in turn activate the transcription of genes involved in neuronal differentiation.

INTRODUCTION

PC12 cells, cloned from a rat pheochromocytoma have been widely used for studying molecular mechanisms of neuronal differentiation. On incubation with neurotrophic factors such as nerve growth factor (NGF) these cells extend long neurites and undergo biochemical changes characteristic of mature sympathetic neurons (Segal and Greenberg, 1996). The neuron-like differentiation of PC12 cells induced by NGF involves activation of the Ras/extracellular signal-regulated kinase (ERK1/2) signaling pathway. Sustained activation of ERK1/2 permits its translocation to the nucleus, where it may modulate gene expression via the phosphorylation of transcription factors or activation of other kinases (Qiu and Green, 1991; Marshall, 1995).

The nuclear transcription factor cAMP response element-binding protein (CREB) is a major downstream target of ERK1/2 signaling that contributes to neuronal differentiation in PC12 cells (Heasley *et al.*, 1991; Ginty *et al.*, 1994) and to brain neuroplasticity (Impey *et al.*, 1998b, 1999; Sgambato *et al.*, 1998; Pham *et al.*, 1999) and neuronal survival (Bonni *et al.*, 1999; Riccio *et al.*, 1999; Nakagawa *et al.*, 2002). CREB binds constitutively to the specific DNA motif, 5′-TGACGTCA-3′ known as CRE, and its activity is triggered by phosphorylation in Ser133 (González and Montminy, 1989). Phosphorylation of Ser133 recruits the CREB binding protein, CBP, to the initiator complex and thereby promotes transcription (Chrivia *et al.*, 1993; Arias *et al.*, 1994; Mayr and Montminy, 2001). CREB was initially identified as a substrate for PKA and as a mediator of cAMP-regulated gene expression, but later studies showed that CREB can be phosphorylated and activated by multiple signaling pathways including ERK1/2 (Shaywitz and Greenberg, 1999). Although CREB is not directly phosphorylated by ERK1/2, it is phosphorylated and transactivated by the ERK1/2-activated Rsk (ribosomal S6 kinase) family of protein kinases pp90rsk (Xing *et al.*, 1996, 1998; Impey *et al.*, 1998a).

Many genes are activated by CREB, including other transcription factors such as c-*fos* (Sheng *et al.*, 1988; Sheng and Greenberg, 1990), through which CREB signaling can indirectly activate an expanded range of genes. It has been demonstrated that the CRE of the c-*fos* promoter is critical for NGF activation of c-*fos* gene transcription (Ginty *et al.*, 1994; Ahn *et al.*, 1998). An NGF-inducible Ras-dependent protein kinase, which was identified as Rsk2, was found to trigger this activation (Xing *et al.*, 1996). In addition, it has been demonstrated that the stimulation of neuronal c-*fos* expression by neurotrophins involves at least two signaling pathways, the Ras-dependent pathway and a calcium/calmodulin kinase-dependent pathway (Finkbeiner *et al.*, 1997; Wu *et al.*, 2001).

All-trans-retinoic acid (RA) and other retinoids are potent regulators of morphogenesis, growth and cell differentiation. RA, which is abundant in mammalian nervous system (Maden 2001), induces neurite outgrowth and neuronal differentiation from various sources, including embryonic stem cells (Bain *et al.*, 1995; Li *et al.*, 1998), dorsal root ganglia (Corcoran and Maden, 1999), or neuroblastoma cells (Haussler *et al.*, 1983). RA-induced neuronal differentiation is characterized by the expression of tissue-specific genes, proteins, ion channels, and receptors in a developmentally controlled manner (Guan *et al.*, 2001). Although we have previously shown that RA inhibits PC12 cell proliferation and mimics the effect of neurotrophins on the expression of several genes, the retinoid does not cause neurite extension and does not increase the levels of early response genes such as

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c-*fos* in parental PC12 cells (Cosgaya *et al.*, 1996, 1997a, 1997b, 1998). In contrast, RA can stimulate the differentiation of some PC12 cell subclones. Thus, RA causes neurite extension and a rapid increase of c-*fos* mRNA levels (Scheibe *et al.*, 1991) in a mutant cell line (A126-1B2) that is deficient in cAMP-dependent protein kinase A (PKAII), and in another line (123.7) that is deficient in both cAMP-dependent protein kinase I and II (Van Buskirk *et al.*, 1985).

The actions of RA are mediated by binding to nuclear receptors (RARs), which normally act as ligand-inducible transcription factors by binding as heterodimers with the retinoid X receptors (RXRs) to RA response elements (RAREs) located in regulatory regions of target genes (Chambon, 1996). However, there is increasing evidence that nuclear receptors can mediate rapid extragenomic effects that stimulate signaling pathways by still not well-defined mechanisms, which are independent of receptor binding to DNA response elements (Valverde and Parker, 2002). The levels of both RARα and RARβ receptor isoforms are markedly higher in A126-1B2 and 123.7 cells than in the parental PC12 cells (Scheibe *et al.*, 1991). The high levels of RARβ could play an important role in the phenotypic changes induced by the retinoid, because this receptor is thought to be responsible for RA-mediated differentiation of several cell types (Liu *et al.*, 1996; Faria *et al.*, 1999; Pérez-Juste and Aranda, 1999). Moreover, it has been postulated that RARβ induction is an essential part of the neuronal differentiation process induced by NGF in neuronal cells (Corcoran and Maden, 1999; Cosgaya and Aranda; 2001; Corcoran *et al.*, 2002).

In this work we demonstrate that RA induces CREB phosphorylation and CREB-mediated transcription in the PC12 cell clones that undergo morphological differentiation upon incubation with the retinoid. The effects of RA are similar to those caused by NGF and the combination of both factors does not induce further changes, suggesting that they could use common pathways to stimulate CREB phosphorylation. Indeed, RA also causes a rapid and strong activation of ERK1/2 in these cells, and this stimulation is required for CREB phosphorylation and activation. RA also induces CREB and ERK phosphorylation in primary cultures of neural cells. Our data demonstrate that RA can stimulate signaling cascades and expression of genes involved in neuronal differentiation by a rapid nongenomic mechanism that does not appear to involve direct binding of its receptors to RAREs in target genes.

MATERIALS AND METHODS

Plasmids

The RARβ2 luciferase reporter construct containing the promoter fragment −124 to +14 with a RARE at position −37 to + 43 has been previously described (Cosgaya and Aranda, 2001). The FC4 construct contains the c-*fos* flanking region with the CRE at position −60, fused to the chloramphenicol acetyltransferase (CAT) gene (Verma and Sassone-Corsi, 1987). The ICER-CAT plasmid possesses four CREB-binding motifs, termed CAREs (Molina *et al.*, 1993) and the SOM-CAT construct contains a somatostatin promoter fragment with one CRE motif (Montminy and Bilezikjian, 1987). The c-Jun-CAT construct contains the −1600 to +170 promoter fragment of the c-*jun* rat gene, and in the ΔAP-1c-Jun-CAT plasmid the AP-1 site located at position −72 has been mutated (Angel *et al.*, 1987). CAT reporter plasmids containing 5′flanking sequences extending to −5.3 and −2.5 kb of the *Jun*B gene have been previously described (Kitabayashi *et al.*, 1993). Fusions of either wild-type CREB or CREB in which serine 133 was substituted by an alanine with the DNA-binding domain of GAL4 were a gift of S. Gütkind (NIH, Bethesda, MD). These fusions were cotransfected with a reporter plasmid containing four binding sites for GAL4 (pE1b 4xUAS-luc) to determine activity of the transcription factor. An expression vector for a dominant negative mutant of CREB (K-CREB) under control of the Rous sarcoma virus (RSV) promoter has been previously described (Walton *et al.*, 1992).

Cell Lines

The A126-1B2 cell line was selected from mutagenized PC12 cells on the basis of its resistance to the toxic effects of dibutyryl cAMP and is deficient in PKAII activity (Van Buskirk *et al.*, 1985). 123.7 cells are stably transfected with a mutant regulatory subunit of PKAI, and expression of this mutant allele reduces the level of both PKAI and PKAII activity by more than 80% (Van Buskirk *et al.*, 1985). These cells as well as parental PC12 cells were grown in RPMI medium containing 10% donor horse serum and 5% fetal bovine serum as previously described (Cosgaya and Aranda, 2001). In 123.7 cells, the medium was supplemented with 500 μg/ml G418 to maintain selection. For the experiments, cells were shifted to a medium supplemented with 10% AG1-X8 resin and charcoal-stripped newborn calf serum. RA was dissolved in ethanol and the same amount of vehicle (0.1 μl ethanol/ml medium) was used in the control cells.

Primary Cortical Cell Cultures

The cerebral cortex from fetal brains of E17 Sprague Dawley rats were dissected as described (McManus *et al.*, 1999). Cell suspensions were prepared by trituration of the dissected pieces in HBSS. Once dissociated, cells were pelleted and resuspended in serum-free DMEM containing N1 supplement (Sigma, St. Louis, MO) and 1 mM sodium pyruvate (defined medium; Bottenstein and Sato, 1979), to which bFGF (20 ng/ml) was added. Cells were seeded into poly-ornithine–coated 10-cm dishes at a density of 2–4 $\times$ 10^4 cells/cm^2 and incubated at 37°C. Medium was replaced every 2 d, and cells were passaged before reaching confluence (usually 5–6 d after plating) by treatment with 0.025% trypsin, which was inactivated by adding DMEM/10% FBS. Cells were then washed, resuspended in defined medium containing bFGF, and seeded into 35-mm dishes at a plating density of 3 $\times$ 10^4 cells/cm^2. These cells are nestin positive and present a bFGF-dependent proliferation characteristic of neural precursors/stem cells, characteristic for the neuroepithelial precursors (McManus *et al.*, 1999). Basic FGF-containing medium was replaced with bFGF-free defined medium 24 h before adding vehicle, RA 1 μM or Fk 10 μM. After 30 min of treatment, the cells were processed for Western blot.

Dorsal Root Ganglia Neuronal Cultures

Neuronal cultures were established from dorsal root ganglia (DRG) neurons obtained from Sprague Dawley rat embryos at 12 d gestation as described (Chan *et al.*, 2001). DRG neurons were dissociated and plated onto collagen-coated coverslips and were kept at 37°C in a medium consisting of 10% heat-inactivated FBS in MEM and 100 ng/ml NGF for one night. Two hours before the experiment, the cells were switched to MEM plus 0.5% heat-inactivated charcoal-treated FBS without NGF. The cells were subsequently treated with RA 1 μM for 30 or 120 min and processed for Western blot analysis.

Cell Proliferation and Viability Assays

A126-1B2 cells were seeded at 2 $\times$ 10^4 cells/well in 24-well plates and treated with RA at different concentrations. At the end of the experiment (5 d), cell density was determined by crystal violet staining. In brief, cells were fixed with 1% glutaraldehyde and stained with 0.1% crystal violet. After extensive washing, the dye was solubilized with 10% acetic acid and quantitated in a Microplate ELISA reader at 595 nm. For cell viability assays, A126-1B2 cells were seeded at 10^4 cells/well in 96-well plates in complete media. One day before the experiment, medium was changed to DMEM plus 10% charcoal/resin-treated newborn calf serum to eliminate retinoids from the medium. After treating the cells with increasing concentrations of RA for 24 h, cellular viability was determined by the MTT-based Cell Proliferation Kit I (Roche, Indianapolis, IN) as suggested by the manufacturer.

Transfections

For transient transfection assays the cells were plated 24 h before transfection in serum-containing DMEM medium. Cells were transfected in serum-free medium with 5–8 μg of reporter plasmids with a mixture of cationic liposomes in 60-mm dishes. After a 16-h incubation with the liposomes, cells were treated in medium supplemented with 10% AG1-X8 resin and charcoal-stripped newborn calf serum for the times indicated in the presence or in the absence of RA (1 μM) or NGF (50 ng/ml). All data shown are mean ± SD obtained from at least four independent transfections, and the experiments were repeated at least twice with similar relative differences in regulated expression.

Western Blot

Cells were lysed in buffer containing 0.5% SDS, 50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1.25 μg/μl pepstatin, leupeptin, and aprotinin, 1 mM PMSF, 1.25 mM sodium orthovanadate, 1.25 mM sodium fluoride, and 30 mM pyrophosphate. Cell proteins (30–40 μg) were run in a 12% SDS-polyacrylamide gel and transferred to permeabilized nylon membranes (Immobilon P, Millipore, Bedford, MA). Blots were incubated overnight at 4°C with antibodies recognizing specifically CREB phosphorylated in serine 133 (P-CREB), total CREB,

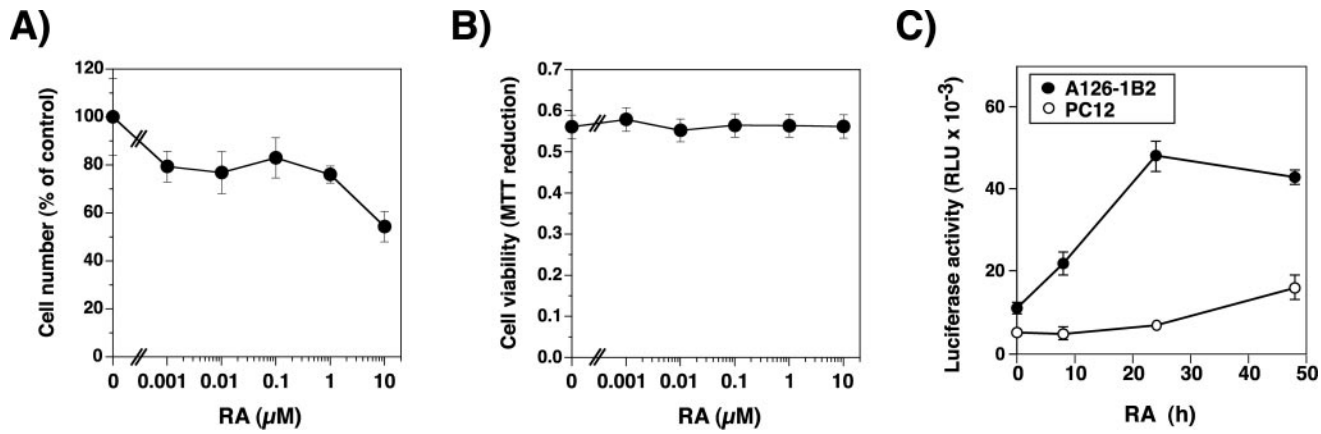

Figure 1. Expression of functional RA receptors and influence of RA on A126-1B2 cell proliferation and viability. (A) A126-1B2 cells were treated with increasing concentrations of RA for 5 d and the total cell number was determined. (B) Cellular viability was determined with MTT after 24-h treatment with increasing concentrations of RA. (C) Cells were transiently transfected with the RARβ2 reporter plasmid that contains a RARE, and luciferase activity was determined in cells treated with 1 μM RA for the indicated time periods.

ERK1/2 phosphorylated in threonine 202 and tyrosine 204 (P-ERK), or total ERK2, followed by a second incubation with a peroxidase-conjugated secondary antibody. All primary antibodies were obtained from New England Biolabs (Beverly, MA) and were used at a 1:1000 dilution. Blots were revealed by chemiluminescence (ECL; Amersham Pharmacia Biotech, Piscataway, NJ). Blots were quantified and the ratio of the phosphorylated vs. total protein calculated. The data presented in the figures show a representative experiment obtained from duplicate cultures with variation <20–30%, and similar qualitative results were obtained in at least two additional experiments. Results are expressed as fold-induction over the values obtained in control cells treated with vehicle alone. RARβ levels were determined by Western blot with 20 μg of total protein and the antibody SC-552 (Santa Cruz Biotechnology, Santa Cruz, CA) at a 1:1000 dilution.

Northern Blot

Total RNA was extracted from the cell cultures with guanidine thiocyanate and hybridized with a 1.5 kb *Eco*RI cDNA fragment from *jun*B, as previously described (Cosgaya *et al.,* 1996, 1998). Membranes were stained with 0.02% methylene blue to detect rRNA as a control for loading. The mRNA levels were quantified by densitometric scanning of the autoradiograms, and the values obtained were corrected by the amount of the rRNA applied.

RESULTS

Effect of RA on Cell Differentiation, Proliferation, and Viability

It has been previously reported that PKA-deficient PC12 cells differentiate upon incubation with RA (Scheibe *et al.,* 1991). Thus, we have observed that after 4 d of incubation of A126-1B2 cells with 1 μM RA, more than 40% of the cells extended neurites with more than two cell body sizes in length. No further neurite extension was elicited by the combination of RA plus NGF, and morphology was identical to that obtained with either factor alone (unpublished data). As shown in Figure 1A, RA caused a decrease in cell proliferation. The reduction in cell number obtained at 1 μM RA was similar to that obtained with lower concentrations and is not due to a toxic effect, because MTT assays (Figure 1B) showed that the great majority of the cells were viable even after incubation with a higher RA concentration (10 μM).

It has been previously shown (Scheibe *et al.,* 1991) that the levels of RARβ and RARα are markedly higher (5- and 15-fold, respectively) in A126-1B2 cells than in parental PC12 cells that do not extend neurites in response to RA. To confirm that functional RA receptors are increased in the mutant cells, a reporter plasmid containing the RARβ2 promoter was transiently transfected into PC12 and A126-1B2 cells. This promoter contains a strong RARE that binds RXR/RAR heterodimers. As illustrated in Figure 1C, RA-dependent transactivation was observed earlier and was stronger in A126-1B2 than in wild-type PC12 cells.

RA Induces CREB Phosphorylation in A126-1B2 and 123.7 Cells but Not in Parental PC12 Cells

Because CREB phosphorylation appears to play an important role in neurotrophic responses, we analyzed whether this modification also occurs during RA-induced morphological differentiation of PKA-deficient PC12 cells. For this purpose, the amount of phosphorylated CREB (P-CREB) was assessed by Western blot using an antibody specific for the Ser133-phosphorylated form of the transcription factor. As shown in Figure 2A, incubation with 1 μM RA resulted in a rapid stimulation of CREB phosphorylation, with a similar increase being observed between 10 and 60 min of incubation. In contrast, the retinoid did not affect CREB phosphorylation after 3 min of treatment (unpublished data). The amount of total CREB remained unaltered upon incubation with RA, as analyzed with an antibody recognizing total CREB. The effect of RA on CREB phosphorylation was quantitatively similar to that caused by 30 min. incubation with NGF (Figure 2B). In addition, as also illustrated in Figure 2B, the combined effect of RA and NGF on the levels of P-CREB was not additive. Induction of CREB phosphorylation by RA was mimicked by 1 μM of the 9-*cis*-RA isomer and also by the same concentration of the RAR-specific ligand TTNPB (unpublished data), indicating that most likely RARs mediate stimulation by RA.

Stimulation of CREB phosphorylation by RA was also found in 123.7 cells (Figure 2C), which also differentiate in response to this compound (Scheibe *et al.,* 1991). In these cells, as well as in A126-1B2 cells, activation was also very sustained, with P-CREB levels remaining elevated for at least 8 h. In contrast, in parental PC12 cells, which do not extend neurites in response to RA, stimulation of CREB phosphorylation by RA was never found (Figure 2C).

The fact that both A126-1B2 and 123.7 cells are PKA deficient, suggests that this pathway may block RA signaling. To address this issue, the influence of RA on CREB phosphorylation was analyzed in parental PC12 cells incu-

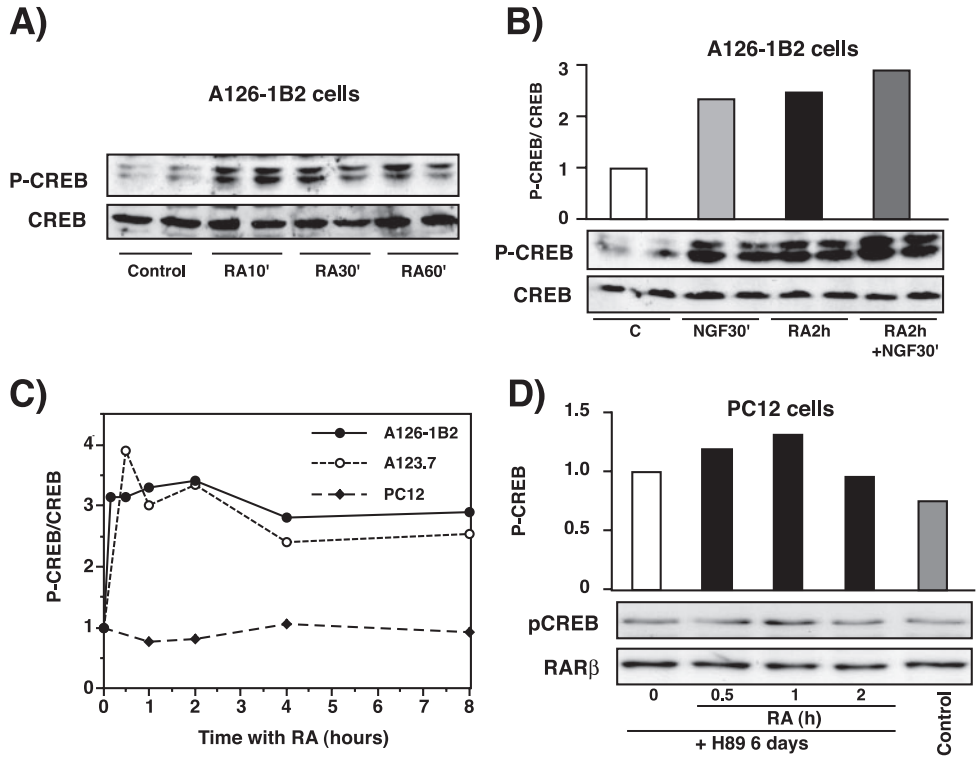

Figure 2. RA induces CREB phosphorylation in A126-1B2 cells. (A) Western blot using antibodies against phosphorylated CREB (P-CREB) and total CREB of proteins from cells treated with 1 μM RA during the time periods indicated. In B, A126-1B2 cells were incubated for 60 and 120 min with RA in the presence and absence of NGF for the last 30 min. (C) The quantification of the ratio P-CREB/CREB obtained in A126-1B2, 123.7, and parental PC12 cells treated with RA for the times indicated shown as the fold induction above the levels obtained in untreated cells. (D) PC12 cells were incubated in the presence and absence of 20 μM H89 for 8 d. Medium and inhibitor were replaced every 24 h. CREB phosphorylation and RARβ levels were analyzed in control PC12 cells and in H89-treated cells incubated with RA for the time periods indicated.

bated with the PKA inhibitor H89. As shown in Figure 2D, incubation with RA did not elicit a significant increase in CREB phosphorylation. In addition, RA did not cause differentiation of PC12 cells treated with H89 (unpublished data). The different behavior of the PKA-deficient stable mutant cell lines and the H89-treated PC12 cells could be due to the fact that whereas the levels of RARs are very high in A126-1B2 and 123.7 cells (Scheibe *et al.*, 1991), RARβ levels did not increase after treatment of PC12 cells with the inhibitor (Figure 2D).

RA Stimulates CRE-mediated Transcription and CREB Transcriptional Activity

Although phosphorylation of CREB at Ser133 is required for the induction of CREB-dependent transcription, there are instances in which phosphorylation of CREB at Ser133 is not sufficient for target gene activation (Bonni *et al.*, 1995). To examine whether RA can stimulate CRE-mediated transcriptional responses, transient transfection assays were carried out in A126-1B2 cells with CRE-containing reporter plasmids. It is important to note that these plasmids do not contain RAREs to which RA receptors could bind to activate transcription. One of the best known targets of activated CREB in PC12 cells is the c-*fos* gene (Bonni *et al.*, 1995, Ahn *et al.*, 1998). As illustrated in Figure 3A, in A126-1B2 cells activity of the c-*fos* promoter was stimulated by RA and NGF to a similar extent (left panel). This promoter is not stimulated by RA in parental PC12 cells (Cosgaya *et al.*, 1998). To determine whether binding of CREB or CREB-related transcription factors to the CRE was required for RA-induced c-*fos* transcription, a dominant negative CREB mutant, KCREB (Walton *et al.*, 1992), was cotransfected with the c-*fos* promoter construct into A126-1B2 cells. Figure 3A (right panel) shows that RA failed to activate the promoter in cells cotransfected with KCREB, whereas a significant activation was found with the same amount of the empty vector. These results demonstrate that CREB, or a closely related family member, is required for RA-induced c-*fos* dependent transcription.

Other promoter activated by NGF in PC12 cells as a consequence of CREB phosphorylation is the intrinsic P2 promoter of the transcription factor CREM (cAMP response element modulator) that gives rise to the inhibitory isoform ICER (inducible cAMP early repressor; Monaco and Sassone-Corsi, 1997). The P2 promoter presents two pairs of CREB binding sites named CAREs (cAMP autoregulatory elements; Molina *et al.*, 1993), and as illustrated in Figure 3B, the activity of this promoter was also significantly stimulated by RA as well as by NGF. Finally, the activity of the somatostatin promoter, which contains a well-characterized CRE (Montminy and Bilezikjian 1987), was increased in A126-1B2 cells by RA and NGF (Figure 3C). As occurred with CREB phosphorylation, the effects of both agents were not additive, suggesting the existence of common mechanisms for their actions.

As an additional strategy to detect direct effects of RA on CREB-mediated transcription and to test whether phosphorylation in Ser133 is necessary for this response, GAL-CREB expression plasmids were cotransfected into A126-1B2 cells with a luciferase reporter plasmid containing binding motifs for GAL4. GAL-CREB consists of the DBD of yeast GAL4 fused to full-length CREB. Transfected cells were then treated with RA for different time periods. As shown in Figure 3D, treatment with RA resulted in a marked increased in GAL-CREB–dependent gene expression, indicating that RA activates CREB-mediated transcription in these cells. Similar experiments were performed with a mutant GAL-CREB hybrid in which Ser133 was replaced by an alanine. This single point mutation abolished induction by RA, showing that the increase in CREB phosphorylation in this residue shown in Figure 2 is responsible for the induction of CREB-transcriptional activity by the retinoid.

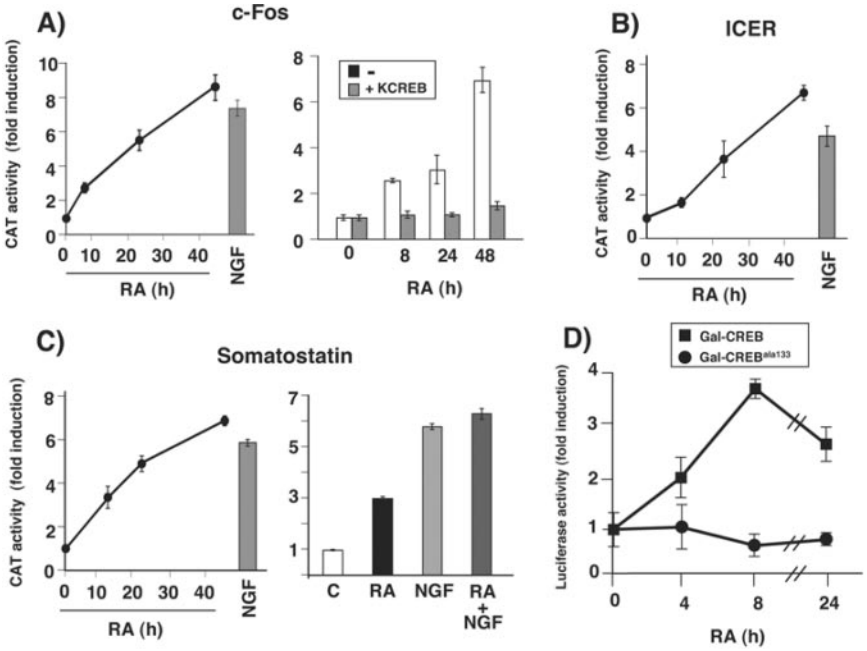

Figure 3. RA increases the activity of CRE-containing promoters in A126-1B2 cells. (A) Transient transfection assays were performed with a CAT reporter plasmid (FC4) containing the c-Fos promoter (7 μg). In the left panel CAT activity was determined in cells incubated for increasing time periods with RA or with NGF for 48 h. In the right panel the reporter construct was cotransfected with 12 μg of an expression vector for the dominant negative CREB mutant (KCREB) or with the same amount of the corresponding empty noncoding vector. CAT activity was determined after incubation with RA for the time periods indicated. (B) Cells were transfected with 7 μg of an ICER-CAT construct, and CAT activity was determined in cells treated with RA or with NGF for 24 h. In C, cells were transfected with 8 μg of a plasmid containing the somatostatin promoter. The left panel shows CAT activities determined after incubation with RA for the indicated time periods and with NGF for 24 h. In the right panel the cells were incubated with NGF alone or in combination with RA for 16 h. In all cases CAT activities are expressed as the fold induction over the values obtained in the untreated cells. (D) RA induces CREB transcriptional activity in A126-1B2 cells. Cells were transfected with 7 μg of the Gal-DBD fused to either native CREB (Gal-CREB) or CREB mutated in serine 133 (Gal-CREB^{ala133}). These constructs were cotransfected with 5 μg of a luciferase reporter plasmid containing four binding sites for Gal-DBD, and luciferase activity was determined in cells treated with RA for increasing time periods. Reporter activity is expressed as the fold induction over the values obtained in the untreated cells.

RA Induces ERK1/2 Phosphorylation in A126-1B2 Cells

Having defined CREB as a mediator of RA-regulated gene expression in A126-1B2 cells and because the Ras/ERK pathway appears to play a key role in neurotrophin-mediated CREB phosphorylation in PC12 cells (Xing *et al.,* 1996, Vaudry *et al.,* 2002), we sought next to elucidate whether ERK1/2 could be activated by RA. For this purpose, the amount of phosphorylated ERK was assessed by Western blot using an antibody specific for the phosphorylated form of ERK1/2 in cells incubated with RA. As illustrated in Figure 4A, RA caused a strong and rapid increase in the amount of phosphorylated ERK1/2. This increase was found as soon as 3 min after incubation with the retinoid and was already maximal at 10 min, decreasing thereafter. In contrast, total levels of ERK2, as determined with an antibody recognizing both phosphorylated and unphosphorylated protein, remained unaltered after RA treatment. The kinetics of induction of ERK and CREB phosphorylation by RA were different. Although ERK phosphorylation returned to basal levels after 1 h of incubation and was not increased at time periods ranging between 1 and 8 h (unpublished data), CREB phosphorylation was very sustained and was elevated during the whole period (see Figure 2).

ERK1/2 Activation Is Required for RA-induced CREB Phosphorylation and Transcriptional Activity

Having shown that RA phosphorylates ERK, we tested whether ERK activation was necessary for RA responses by interfering signaling with the MEK inhibitor U0126. Figure 4B shows that although treatment of A126-1B2 cells for 3 h with RA caused the expected increase in CREB phosphorylation, RA was unable to induce CREB phosphorylation in cells preincubated with the kinase inhibitor. Furthermore, as shown in Figure 4C, RA-induced CREB-mediated transcription, as assessed with the GAL-CREB construct, was abolished in the presence of U0126. These results indicate that ERK is a major mediator of CREB-dependent gene expression activated by RA.

RA Activates Transcription of Several Early Genes in A126-1B2 Cells

Neurotrophins induce differentiation of PC12 cells partly by initiating a cascade of early genes, some of which are transcription factors of the Fos/Jun family (Segal and Greenberg, 1996). Furthermore, expression and activation of genes of the *jun* family are involved in differentiation of PC12 cells (Lëppa *et al.,* 1998). Because ERK and CREB activation mediates in many instances expression of these genes, we tested the possibility that, in addition to c-*fos,* RA could stimulate expression of genes of the *jun* family in A126-1B2 cells. Figure 5A shows that the activity of the c-*jun* promoter increased significantly in these cells upon incubation with RA, reaching at 48 h levels similar to those obtained in NGF-treated cells. It has been described that an AP-1 site located in the proximal promoter, which it is more efficiently recognized by c-Jun/ATF-2 heterodimers than by conventional AP-1 complexes, appears to be important for c-*jun* transcriptional activity (Karin, 1995). The right panel in Figure 5A shows that mutation of the AP-1 site blocks activation of the c-*jun* promoter by RA, demonstrating that this motif is also required for induction by the retinoid. Therefore, as in the case of the c-*fos* gene, stimulation of c-*jun* promoter activity by RA does not appear to involve a classical mechanism of receptor binding to a RARE. The influence of RA on the activity of the promoter of other early gene, *junB*, which is induced by NGF in PC12 cells, was also examined. As shown in the left panel of Figure 5B, RA increased *junB* promoter activity in A126-1B2 cells to the same extent as NGF. In this case stimulation by the retinoid

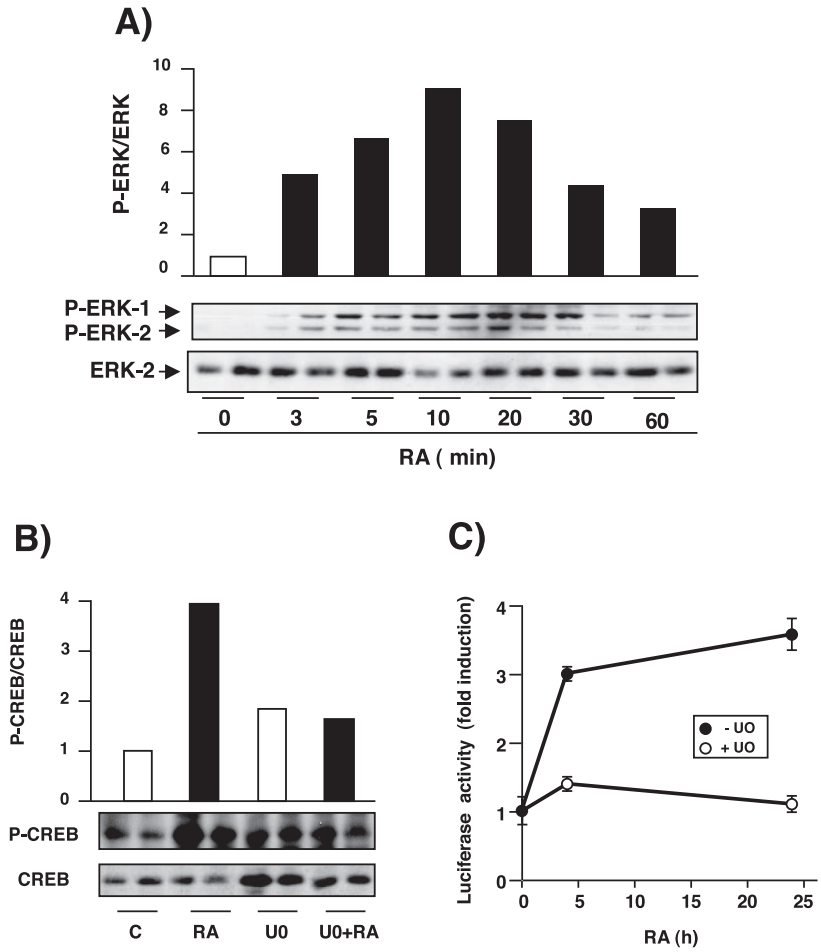

Figure 4. ERK1/2 phosphorylation is required for CREB phosphorylation and transcriptional activation by RA. (A) Western blot analysis performed with proteins from cells incubated with RA for the indicated time periods and antibodies recognizing phosphorylated forms of ERK1/2 (P-ERK-1 and -2) and total ERK-2. The top panel represents the ratio P-ERK/ERK as fold induction over the values obtained in control untreated cells. (B) CREB phosphorylation was determined in cells pretreated for 20 min with 10 μM U0126 and then incubated for 3 h in the presence and absence of RA. Quantitation of the P-CREB/CREB ratio is shown in the top panel, and Western blots with antibodies against P-CREB and CREB are shown in the bottom panels. (C) Transient transfection assays with the Gal-CREB plasmid were performed as in Figure 3D. Luciferase activity was determined in cells preincubated with U0126 for 30 min and then with RA for 4 and 24 h.

mapped to a distal promoter region, because deletion of sequences located between −5.3 and −2.5 kb abolished induction (right panel in Figure 5B). Analysis of this region, which contains two serum response elements (SRE), did not reveal the existence of a RARE, suggesting again a nonconventional mechanism for stimulation of *junB* transcription by RA.

Transcription of immediate early genes by direct inducers does not require de novo protein synthesis and inhibitors such as cycloheximide prolong transcription and stabilize the mRNA leading to superinduction. We then analyzed the effect of RA in the presence and absence of the cycloheximide on the levels of *junB* mRNA in A126-1B2. As shown in Figure 6, incubation with RA increased *junB* transcripts, and these levels were superinduced in the presence of cycloheximide. This result shows that induction by RA did not require de novo protein synthesis and proves that the effect of the retinoid on early gene expression is direct and not a secondary response to a gene induced by this compound.

RA Increases CREB and ERK Phosphorylation in Primary Neuronal Cells

To analyze whether induction of CREB phosphorylation by RA is a general property of neuronal cells or whether it was specific for the mutant PC12 cells, we conducted experiments with primary neuronal cells. Figure 7A shows that a 30-min incubation of primary cortical precursor cell cultures from fetal rats with RA caused an increase in CREB phosphorylation similar to that obtained after incubation with forskolin. This occurred without changes in the total amount of CREB. Furthermore, RA also induced a significant increase in the amount of phosphorylated ERK, which was not affected by forskolin. That RA can stimulate CREB phosphorylation in neuronal cells was confirmed in cultures from embryonic dorsal root ganglia (DRG), in which incubation with the retinoid for 30 and 120 min also increased the levels of phosphorylated CREB (Figure 7B).

DISCUSSION

CREB is a transcription factor activated by a diverse array of extracellular signals (Shaywitz and Greenberg, 1999) and a major mediator of neuronal neurotrophin responses (Finkbeiner e*t al*., 1997). In PC12 cells CREB activity is stimulated in response to NGF and other agents that induce neuronal differentiation (Ginty *et al.*, 1994). After observing that RA is able to reproduce in A126-1B2 cells many of the effects of NGF, we demonstrate in this study that CREB phosphorylation is also induced in these cells during RA-mediated differentiation. The effects of RA and NGF on CREB phosphorylation and morphological differentiation are not additive, suggesting that both agents could use common pathways to influence these processes. RA also causes a rapid and sustained stimulation of CREB phosphorylation at Ser133 in the PC12 clone A123.7, which extends neurites upon incubation with the retinoid (Scheibe *et al.*, 1991), whereas CREB phosphorylation is not affected in parental

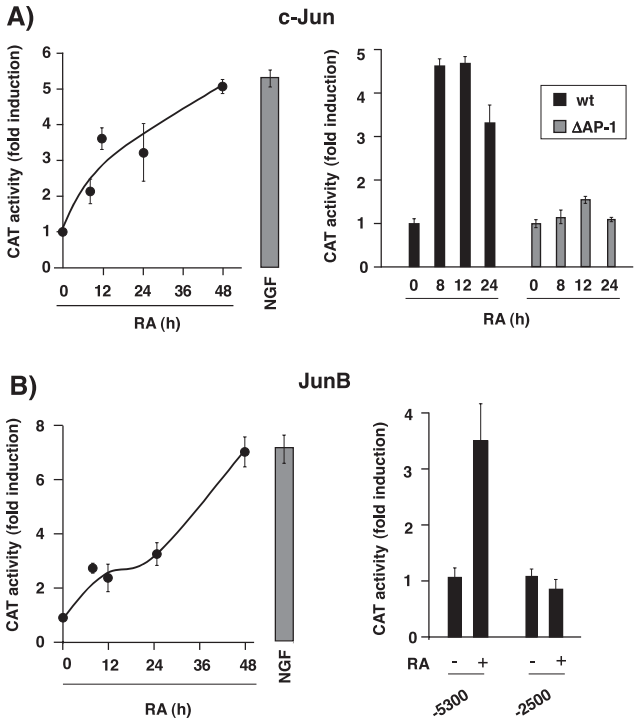

Figure 5. RA activates the c-*Jun* and *Jun*B promoters in A126.1B2 cells. (A) In the left panel cells were transfected with 7 μg of a reporter plasmid containing the 5′-flanking region (from −1600 to +170) of the c-*jun* gene and CAT activity was determined in cells treated for increasing time periods with RA and with NGF for 48 h. In the right panel cells were transfected with 8 μg of the native promoter construct (wt) or with the same amount of a plasmid in which the AP-1 site located around position −72 has been mutated (ΔAP-1). CAT activity was determined in cells treated with RA between 0 and 24 h. (B) In the left panel, cells were transfected with 8 μg of a CAT plasmid containing 5300 base pairs of the *Jun*B promoter, and CAT activity was determined in cells treated with RA for the indicated time periods and with NGF for 48 h. In the right panel, cells were transfected with 8 μg of plasmids extending to either −5300 or −2500 base pairs of the *Jun*B promoter, and CAT activity was determined in cells incubated for 48 h in the presence and absence of RA.

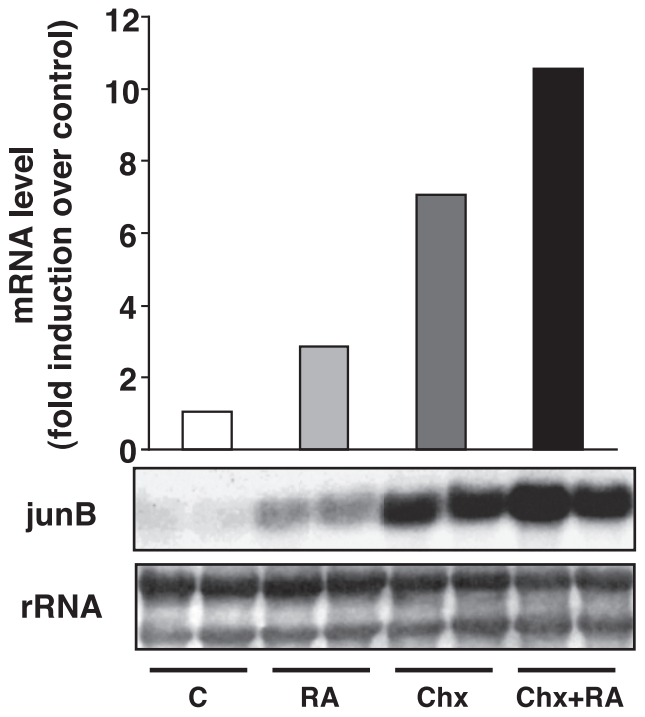

Figure 6. Regulation of *junB* mRNA by RA in A126-1B2 cells. Cells were treated with RA 1 μM alone or in combination with cycloheximide (Chx) 5 μg/ml for 4 h. *junB* mRNA content was analyzed by Northern blot with 30 μg of total RNA. The bottom panel is the control loading and shows the rRNAs after Methylene blue staining of the blot. Quantitation of the normalized *junB* mRNA levels is shown in the top panel.

PC12 cells, which do not differentiate in response to RA. Lack of induction in wild-type PC12 cells do not appear to be a direct consequence of block of RA signaling by the PKA pathway in wild-type PC12 cells. This is supported by the finding that RA neither caused differentiation nor induced CREB phosphorylation in PC12 cells treated with the PKA inhibitor H89. The different behavior of the PKA-deficient stable mutant cells and the wild-type and H89-treated PC12 cells could be due to the fact that although A126-1B2 and 123.7 cells express high functional RAR levels (Scheibe *et al.*, 1991, and Figure 1C), the levels of the RARβ isoform, which appears to play an important role on RA-mediated cell differentiation, did not increase after treatment of PC12 cells with the inhibitor.

It is important to remark that the action of RA on CREB activation is not restricted to the mutant PC12 cells and could be a general property of neuronal cells, because RA also caused rapid increases of CREB phosphorylation in primary cultures of cortical precursor cells and dorsal root ganglia neurons from rat embryos. Dorsal root ganglia cells express different isoforms of RA receptors (Corcoran and Maden, 1999; Corcoran *et al.*, 2002) and respond to the retinoid by extending neurites (Corcoran *et al.*, 2000). Furthermore, we have observed that the cerebrocortical cell cultures undergo important morphological changes upon incubation with RA (unpublished observations).

Induction of CREB phosphorylation by RA is very rapid and the magnitude of induction is similar to that obtained with NGF. However, the kinetics of stimulation are different because, as demonstrated by Monaco and Sassone-Corsi (1997), CREB phosphorylation returns to basal levels after 2 h of incubation with NGF, whereas we observe a more sustained stimulation that is still noticeable after 8 h of incubation with RA. Many actions of RA are mediated by binding of RAR-RXR heterodimers to DNA response elements. However, the early response of CREB phosphorylation to RA is not compatible with a transcriptional effect of the retinoid and shows that RA can have rapid "nongenomic" effects in neuronal cells. The identity of receptors that mediate rapid nongenomic actions of ligands of nuclear receptors is controversial. They could be mediated by a subpopulation of classical receptors localized at or near the cell membrane or by an unrelated receptor. At this respect it has been shown that although RARs are predominantly nuclear, cytoplasmic localization of some receptor isoforms can be detected (Sommer *et al.*, 1999). Although we have not unequivocally identified the receptors involved, RARs are the most likely candidates to mediate the effects of RA on CREB phosphorylation, because the RAR-selective ligand TTNPB, which is widely used to define RAR-specific effects, also causes this effect. Furthermore, the fact that both the RAR ligand all-*trans*-RA, as well as 9-*cis*-RA, which binds with high affinity to both RAR and RXR (Allenby *et al.*, 1993),

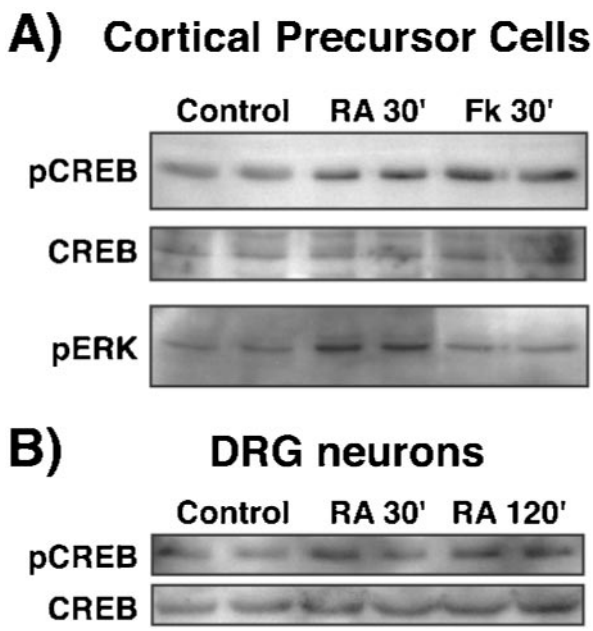

Figure 7. RA activates CREB phosphorylation in primary cultures of neuronal cells. (A) Cortical precursor cells were treated with 1 μM RA or 10 μM forskolin (Fk) for 30 min, and CREB and ERK phosphorylation as well as total CREB levels were determined by Western blot. (B) Rat DRG neurons were treated with RA 1 μM for 0, 30, or 120 min and the degree of CREB phosphorylation was determined by Western blot.

stimulate CREB phosphorylation in A127-1B2 cells reinforces this hypothesis. However, we cannot either dismiss the possibility that RXR could mediate a similar action or that a RAR/RXR heterodimer could participate in the induction of CREB phosphorylation by RA.

CRE-mediated transcription is a convergence point for multiple pathways initiated by differentiating compounds in PC12 cells, through both CREB and CBP (Vaudry *et al.*, 2002). Accordingly, we have observed that RA increases CRE-mediated transcription in A126-1B2 cells. Thus, this compound stimulates the activity of promoters that contain this motif (but do not contain a RARE) and that are also stimulated by NGF. On the other hand, the combined treatment with NGF and RA confirms again a possible redundancy in the signaling pathways used by both compounds to activate transcription.

Stimulation of CRE-mediated transcription by RA is not due to binding of RARs (either as homodimers or heterodimers with RXR) to CRE motifs, as analyzed in gel retardation assays with a CRE oligonucleotide and recombinant RA receptors that, however, bind strongly to a RARE. In addition, treatment with RA does not increase the levels of total CREB, and the amount of CRE-binding proteins present in RA-treated A126-1B2 cells remains unaltered (Cañón and Aranda, unpublished observations). Therefore, our results strongly suggest that stimulation of CRE-mediated transcription was a direct effect of an increase in CREB transcriptional activity secondary to the observed increase in CREB phosphorylation at Ser133. This was confirmed in assays with GAL-CREB chimeras that unequivocally demonstrated that RA induces CREB transcriptional activity and that this induction requires residue Ser133.

Participation of PKA, one of the main CREB kinases, in RA-mediated CREB phosphorylation is excluded, because A126-1B2 cells are deficient in PKA activity (Scheibe *et al.*, 1991), and we have verified that incubation with forskolin does not induce CREB phosphorylation. However, our data show that RA induces ERK1/2 phosphorylation and that ERK activity is required for the actions of RA on CREB phosphorylation and CREB-dependent transactivation. Moreover, activation of ERK by RA was also observed in cortical precursor cells from rat brain, suggesting that this extragenomic action of the retinoid could play a role in normal neuronal development. A key role of ERKs on CREB phosphorylation by a variety of agents in PC12 cells has been previously established. Although, as stated above, ERKs do not phosphorylate CREB, they modulate this process through activation of other kinases such as Rsks (Ginty *et al.*, 1994, Xing *et al.*, 1996), which directly phosphorylate CREB at Ser133. In A126-1B2 cells the time course of ERK1/2 activation by RA is not in line with the sustained CREB phosphorylation This suggests that stimulation of ERK1/2 could be responsible for initial CREB activation and that RA could stimulate additional and still unidentified kinases and/or repress activity of phosphatases that would contribute to maintain elevated levels of CREB phosphorylation long after ERK activity has returned to normal.

The mechanism by which RA as well as other ligands of nuclear receptors can cause ERK activation is still unknown. Rapid increase of ERKs phosphorylation by estrogen or progestins in other cell types has been previously described. In this case it has been proposed that the steroid receptors activate ERK by direct interaction with upstream components of the signaling pathway such as the Src protein (Migliaccio *et al.*, 1998; Castoria *et al.*, 1999, 2001).

Expression of early genes appears to be important for the process of PC12 cell neuronal differentiation (Segal and Greenberg, 1996), and in the PC12 clone A126-1B2, RA-induced differentiation is accompanied by a rapid induction of c-*fos* transcripts (Scheibe *et al.*, 1991). In our work we show that this induction occurs at a transcriptional level and that it is mediated by regulatory sequences located at the 5′-flanking region of the c-*fos* gene. Furthermore, our results demonstrate that CREB plays a key role in the response to RA, because a dominant negative CREB mutant blocks c-*fos* promoter stimulation by RA.

Transcription of CRE-dependent genes in PC12 cells, which is required for differentiation, may also depend on additional *cis* elements in CREB-responsive genes besides the CRE itself. For example, NGF increases expression of the c-*fos* gene through both a CRE and an SRE (Ginty *et al.*, 1994; Bonni *et al.*, 1995). Similarly, other elements could contribute to c-*fos* induction by RA. For instance, SRE-mediated transcription depends on phosphorylation of transcription factors of the Ets family that are direct targets of ERKs (Sharrocks, 2001). Because RA causes strong ERK1/2 activation, it is likely that genes containing these motifs or other motifs stimulated by ERK signaling also could be induced by the retinoid. Although we have not yet fully explored this possibility, we have observed induction of c-*jun* and *jun*B promoter activity by RA in A126-1B2 cells. These genes do not contain sequences resembling RAREs in their regulatory regions and, at least in the case of c-*jun*, induction by RA requires a promoter AP-1 motif. Interestingly, this element differs from other consensus AP-1 sites that bind Fos/Jun heterodimers, and rather binds complexes of c-Jun with ATF-2, other b-zip transcription factor of the CREB family (Karin, 1995). Induction of c-Jun by RA is not restricted to A126-1B2 cells. Thus, activation of c-*jun* transcription is observed during RA-induced differentiation of embryonic carcinoma cells (Yang-Yen *et al.*, 1990; Kitabayashi *et al.*, 1992).

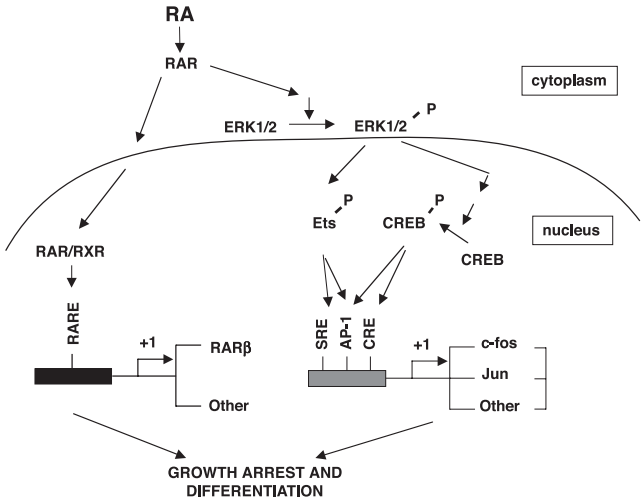

Figure 8. Model for RA-mediated differentiation of neuronal cells. In cells expressing high levels of RARs, RA can either regulate expression of genes containing RAREs in their promoters or by a rapid nongenomic mechanism activate ERK1/2. Translocation of ERK1/2 to the nuclei can lead to direct phosphorylation of transcription factors and to an indirect phoshorylation of CREB. Activation of these factors can stimulate promoters of early genes containing CRE, AP-1, or SRE motifs that then activate transcription of late genes involved in growth arrest and differentiation.

Also in this case RA appears to induce transcription by a RARE-independent mechanism through complexes that include ATF-2 and the coactivator CBP/p300 (Kawasaki *et al.*, 1998).

In summary, our results suggest the model depicted in Figure 8 for RA-mediated differentiation of neuronal precursors and PKA-deficient PC12 cells. On one hand, RA could regulate expression of genes involved in growth arrest and neurite extension by a conventional mechanism that involves binding of RXR/RAR heterodimers to RAREs. This would be the case for the RARβ gene that appears to play an important role in differentiation (Corcoran and Maden, 1999; Cosgaya and Aranda, 2001). On the other hand, RA causes ERK phosphorylation by a rapid nongenomic mechanism. ERKs could directly phosphorylate and activate transcription factors of the Ets family and can also stimulate downstream kinases that then phosphorylate members of the b-zip family of transcription factors such as CREB. This would lead to transcription of early genes, among them transcription factors of the Fos/Jun family, and to the subsequent expression of late genes involved in differentiation. The specific contribution of genomic (RARE-dependent) and nongenomic (CREB- and ERK-dependent) actions to neurite outgrowth and neuronal differentiation remains to be determined.

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