

# Retinoic Acid Stimulates the Differentiation of PC12 Cells That Are Deficient in cAMP-dependent Protein Kinase

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**Abstract.** Retinoic acid (RA) induced neuronal differentiation in A126-1B2 cells and 123.7 cells, two mutant lines of PC12 that are deficient in cAMP-dependent protein kinase, but not in the parental PC12 cell line. A single exposure to RA was sufficient to cause neurite formation and inhibit cell division for a period of >3 wk, suggesting that RA may cause a long-term, stable change in the state of these cells. In A126-1B2 cells, RA also induced the expression of other markers of differentiation including acetylcholinesterase and the mRNAs for neurofilament (NF-M) and GAP-43 as effectively as nerve growth factor (NGF). Neither NGF nor RA stimulated an increase in the expression of smg-25A in A126-1B2 cells, suggesting that the cAMP-dependent protein kinases may be required for an increase in the expression of this marker. RA also caused a rapid increase in the expression of the early response

gene, *c-fos*, but did not effect the expression of *egr-1*. RA equivalently inhibited the division of A126-1B2 cells, 123.7 cells and parental PC12 cells, so RA induced differentiation is not an indirect response to growth arrest. In contrast, the levels of retinoic acid receptors (RAR $\alpha$  and RAR $\beta$ ), and retinoic acid binding protein (CRABP) mRNA were strikingly higher in both A126-1B2 cells and 123.7 cells than in the parental PC12 cells. The deficiencies in cAMP-dependent protein kinase may increase the expression of CRABP and the RARs; and, thus, cAMP may indirectly regulate the ability of RA to control neurite formation and neural differentiation. Thus, RA appears to regulate division and differentiation of PC12 cells by a biochemical mechanism that is quite distinct from those used by peptide growth factors.

RETINOIC ACID (RA)<sup>1</sup> and retinol (vitamin A) have dramatic effects on development and differentiation of many cell types (Brockes, 1989). There is evidence that the anterior-posterior axis is regulated by a concentration gradient of RA, suggesting that RA is a morphogen (Thaller and Eichele, 1987). A cellular RA binding protein (CRABP) is found in many tissues and may be important in the mechanism of RA action (Ong and Chytil, 1978; Shubaita et al., 1987; Stoner and Gudas, 1989; Wang and Gudas, 1984). The mechanisms whereby RA act to regulate cell responses has been advanced by the discovery of a family of nuclear RA receptors (RAR $\alpha$ , RAR $\beta$ , RAR $\gamma$ ), which appear to regulate gene expression by binding to *cis*-acting sequences and activating transcription (Petkovich et al., 1987; Giguere et al., 1987; Brand et al., 1988; Krust et al., 1989; Zelent et al., 1989). RA-responsive DNA elements that regulate the expression of RA induced genes have been defined (LaRosa and Gudas, 1988; Vasios et al., 1989; de Thé et al., 1990).

RA has been implicated in a number of congenital malfor-

mations including brain deformities (Lammer et al., 1985). The RARs and CRABP are expressed in neural tissues (Benbrook et al., 1988; Zelent et al., 1989; Rees et al., 1989; Haussler et al., 1983; Maden et al., 1989), supporting a role for RA in regulating neural development. In the developing CNS, RA can transform anterior neural tissue to posterior neural tissue (Durstion et al., 1989). RA is known to stimulate neurite outgrowth, enhance the expression of neural markers, and inhibit proliferation of cultured neuroblastoma cells (Perez-Polo et al., 1982; Sidell et al., 1983, 1984, 1987; Shea et al., 1985; Sugimoto et al., 1987) as well as some teratocarcinoma cell lines (Jones-Villeneuve et al., 1983; Andrews et al., 1984; McBurney et al., 1988).

PC12 cells are a clonal line that was derived from a rat pheochromocytoma (Greene and Tischler, 1976). The PC12 cell line responds to nerve growth factor (NGF), acidic fibroblast growth factor (aFGF), and basic fibroblast growth factor (bFGF) by extending long nerve-like processes and expressing a number of markers of neural differentiation (Togari et al., 1983; Wagner and D'Amore, 1986; Rydel and Greene, 1987). The line has been a useful model system for the study of a number of the differentiated properties of nerve cells (for a review see Fujita et al., 1989). Continual exposure to these peptide growth factors is required for PC12

1. *Abbreviations used in this paper:* CRABP, cellular retinoic acid binding protein; NF-M, 150-kD neurofilament; NGF, nerve growth factor; PKA, protein kinase A; RA, retinoic acid; RAR, retinoic acid receptor.

cells to maintain a neuron-like phenotype; and, thus, neurotrophic factors act to regulate the expression of differentiated properties in this line. Mutants that have been derived from the original PC12 clone have been extremely useful in the elucidation of the signal transduction pathways used by NGF, aFGF, and bFGF in this line (Van Buskirk et al., 1985; Cremins et al., 1986; Damon et al., 1990; Brady et al., 1990). Because PC12 cells and neuroblastoma lines have already committed to differentiate along a neural lineage, the study of RA effects on these line is conceptually different from studying the effects of RA on teratocarcinoma differentiation (Jones-Villeneuve et al., 1983; Andrews et al., 1984; McBurney et al., 1988).

We have discovered that RA has long-term effects on PC12 cells. RA inhibited division in the PC12 pheochromocytoma cell line and stimulated the stable expression of differentiated markers in both a PC12 mutant cell line (A126-1B2) that is deficient in PKAII (cAMP-dependent protein kinase II; Van Buskirk et al., 1985) and another line (123.7) that is deficient in both cAMP-dependent protein kinase I and II (Ginty et al., 1991a). Thus, the cAMP-dependent protein kinases appear to modulate the responsiveness of PC12 cells to RA. RA induced *c-fos*, but not *egr-1*, demonstrating that neural differentiation can occur even if only some of the NGF-inducible early response genes are activated. Thus, PC12 cells are a useful model system to study the possible biochemical interaction between the RA and protein kinase A (PKA) signaling pathways and the molecular mechanisms of neural differentiation.

## Materials and Methods

### Materials

[ $\alpha$ -<sup>32</sup>P]dCTP (3000 Ci/mmol = 370 MBq/ml), and [ $\alpha$ -<sup>32</sup>P]CTP (300 Ci/ml = 370 MBq/ml) were obtained from New England Nuclear (Boston, MA). SP6 RNA polymerase and ribonucleotide triphosphates were from Promega Biotec (Madison, WI). DNA polymerase I was from New England BioLabs (Beverly, MA). Acetylthiocholine iodide and all-*trans*-RA were from Sigma Chemical Co. (St. Louis, MO); and NGF was purified from mouse submaxillary gland as described by Mobley et al. (1976).

### Cell Culture

PC12 cells, A126-1B2 cells (Van Buskirk et al., 1985), and 123.7 cells were cultured at 37°C in 10% CO<sub>2</sub> in DME supplemented with 10% FBS and 5% horse serum. Cells were plated directly on untreated tissue culture plastic (Falcon Labware, Oxnard, CA). The clone 123.7 was isolated as a stable transfectant containing the expression vector, pHL-REV B<sub>1&2</sub>neo (Clegg et al., 1987). This line expressed a mutant RI regulatory subunit of the PKAI, and had undetectable levels of PKAI and an 80% reduction in the levels of PKAII (Ginty et al., 1991a). 123.7 cells do not form neurites in response to cAMP agonists or agents that increase cAMP levels, but they do form neurites in response to NGF (Ginty et al., 1991b). For morphology studies and enzyme assays, parallel cultures were seeded at an initial density of  $1 \times 10^4$  cells per 60-mm dish, and treated with NGF (100 ng/ml) or RA as indicated in the figure legends. Neurites were defined as a process whose length exceeded one and one-half the diameter of the cell body and had a clearly defined growth cone. 300 cells per plate were scored and the percentage of cells with neurites was calculated.

### RNA Isolation and Analysis

For RNA isolation cells were plated in 150-mm dishes. The medium was changed 2 d after the cells were seeded and 100 ng/ml NGF or 1  $\mu$ M RA was added. Media and supplements were added as described in the figure legends. The cell density used for Northern (RNA) blot analysis was  $\sim 10^7$  cells per 150-cm<sup>2</sup> dish. Total cellular RNA was purified from PC12 cells,

A126-1B2 cells, or 123.7 cells according to the method of Chirgwin et al. (1974). The RNA was size fractionated on 1.2% agarose-formaldehyde gels and transferred to nitrocellulose filter. The cDNAs were labeled with [<sup>32</sup>P]dCTP using random hexamers as a primer (Feinberg and Vogelstein, 1983). The RAR $\alpha$ , RAR $\beta$ , and RAR $\gamma$  mRNAs were detected using digested cDNA probes generated as described by Hu and Gudas (1990). The RAR $\alpha$  probe hybridized to two mRNA species of 2.8 and 3.5 kb, the RAR $\beta$  probe hybridized to two mRNA species of 2.9 and 3.2 kD, and the RAR $\gamma$  probe hybridized to 2.7-kb mRNA transcript, indicating that there were no cross-hybridizations with other members of the steroid-thyroid receptor family. Filters were prehybridized at 42°C for at least 5 h in a solution containing 50% formamide, 5 $\times$  SSC (1 $\times$  SSC = 0.15 M sodium chloride/0.015 M sodium citrate, pH 7), 0.02% polyvinylpyrrolidone, 0.02% Ficoll, 0.02% BSA, 1% SDS, and 100  $\mu$ g/ml denatured calf thymus DNA. Hybridization was performed for 12 h at 42°C in the same solution containing 1-5  $\times 10^6$  cpm/ml of labeled DNA probes. Blots were washed with 0.2 $\times$  SSC and 0.5% SDS at 65°C twice, each for 30 min. Autoradiography was performed with intensifying screens at -70°C.

### Other Methods

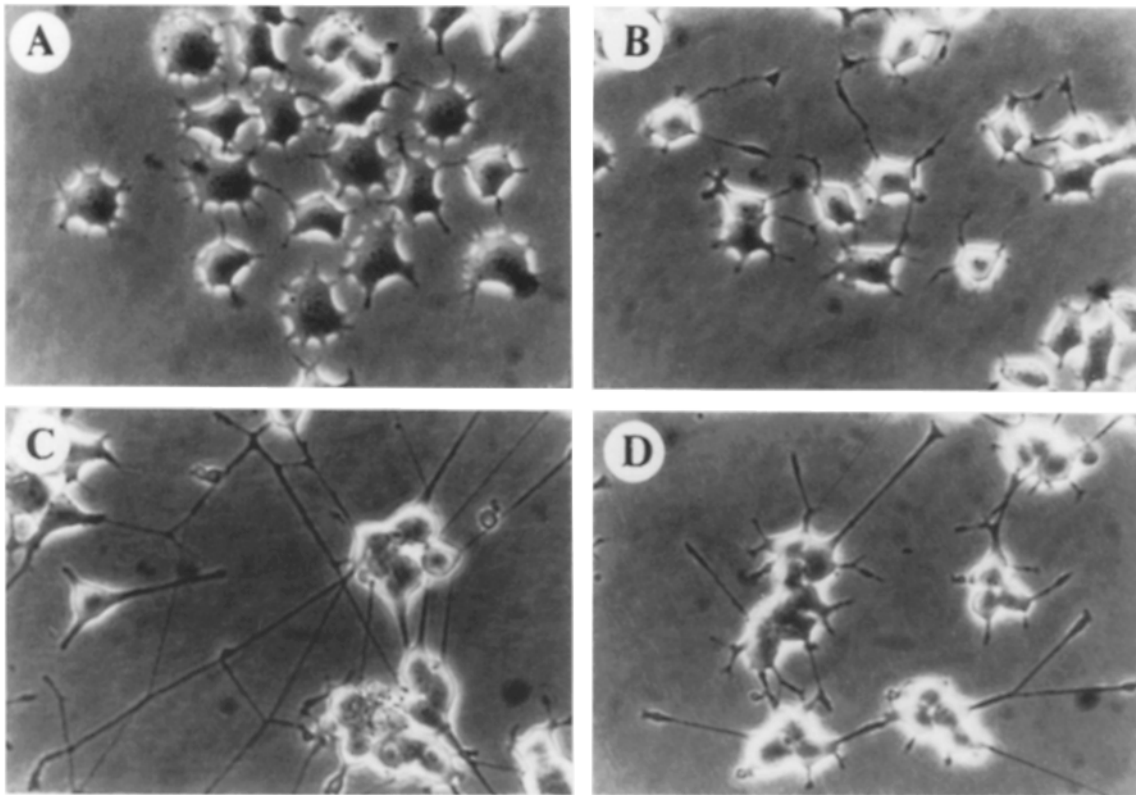
Cultures were harvested, homogenized, and assayed for acetylcholinesterase activity as described previously (Ellman, 1959). Protein concentration as determined by the method of Oyama and Eagle (1978) and DNA concentrations as previously described (Thomas and Farquhar, 1978). For all experiments triplicate cultures were assayed for each time point.

## Results

### RA Induces Neurite Formation in PC12 Mutants That Are Deficient in PKA, But Not in the Parental PC12 Cell Line

The A126-1B2 cell line was selected from mutagenized PC12 cells on the basis of its resistance to the toxic effects of dbcAMP, and is deficient in PKAII activity (Van Buskirk et al., 1985). This line retains many of the responses to NGF that are seen in PC12 cells including neurite formation, protein phosphorylation, and changes in gene expression; but it is resistant to many of the effects that are elicited by cAMP agonists or agents that act via cAMP (Glowacka and Wagner, 1990; Damon et al., 1990; Van Buskirk et al., 1985). Exposure of A126-1B2 cells to RA for 48 h stimulated the formation of long processes, which are a morphological marker of differentiation (Fig. 1, B and D). RA-induced processes are similar to those induced by NGF (compare Fig. 1, C and D) in that RA-induced processes have growth cones and are quite strait as though they are under tension. The rate of neurite production in RA-treated cells appears to be somewhat less than the rate of neurite production in NGF-treated cells (Table I). A single exposure of cells to 1  $\mu$ M RA was sufficient to cause the formation of neurites in a significant fraction of the cells by the third day, and nearly all cells had processes by 6 d. The effect was half maximal at 100 nM (Fig. 2 A). In the majority of cells (>90%), the RA induced neurites were stable for 20 d, even though RA was present only for the first 2 d and the media was changed every second day (Fig. 2 B).

In contrast, the parental PC12 line from which the A126-1B2 cells were derived did not form neurites in response to RA even if additional RA was added to the PC12 cultures at day 3 (Fig. 2 B) or if RA was replenished at day 3, 5, and 7 (data not shown). To help determine if the responsiveness of A126-1B2 cells relative to parental PC12 cells is indeed due to the mutation in PKA, we measured the morphological response of another PKA-deficient PC12 clone (123.7) to RA. The line 123.7 was constructed by transfecting PC12



**Figure 1.** RA induces neurite outgrowth in PC12 cells that are deficient in cAMP-dependent protein kinase: morphology of neurites. A126-1B2 cells, which are deficient in cAMP-dependent protein kinase type II, were plated in the absence of any additives (A), in the presence of 1  $\mu$ M RA (B and D) or 100 ng/ml NGF (C) and photographed at either 3 d (A-C) or 6 d (D). In this experiment RA and NGF were added only at the time of plating. All micrographs are shown at the same magnification.

cells with a vector that encodes a mutant regulatory subunit of PKAI (Clegg et al., 1987) that is not effectively activated by cAMP. Expression of this mutant allele reduced the level of both PKAI and PKAII activity by >80% (Ginty et al., 1991a). The 123.7 line, like the A126-1B2 line, formed neurites in response to NGF, but not in response to cAMP agonists or agents that increased cAMP levels (Ginty et al., 1991b). In response to RA, the 123.7 cell extended processes that are similar in appearance to those seen on A126-1B2 cells. A single exposure of 123.7 cells to RA elicited the formation of neurites in ~20% of the cells, whereas readdition of RA on day 3 elicited neurite formation from >60% of the

123.7 cells (Fig. 2 B). The similarity in the response of the A126-1B2 line and the 123.7 line implicates the PKA-deficiency as a significant factor in regulating the morphological response to RA. This conclusion is further supported by studies on a third PKA-deficient clone, AB.11, a PC12-derived line that expresses a mutant type 1 regulatory subunit with lesions in both cAMP binding sites (Clegg et al., 1987). The line has <20% of parental PKA activity (Ginty et al., 1991a); and, like the 123.7 line, it responded to RA by forming neurites (data not shown).

In contrast, a single addition of NGF to PC12 cells, 123.7 cells, or A126-1B2 cells induced neurites that formed significantly faster than those formed in response to RA, but these neurites began to degenerate after a week and had almost completely disappeared by day 20 (Fig. 2 B). This is because in both PC12 cells and PKA-deficient PC12 cells, NGF is required for both the initiation and the maintenance of neurites. Thus, both NGF and RA can induce neurite formation in PKA-deficient variants of PC12; however, the neurites induced by RA are more stable than those elicited by NGF. This observation suggests that RA may be causing a stable change in the differentiated state of these cells.

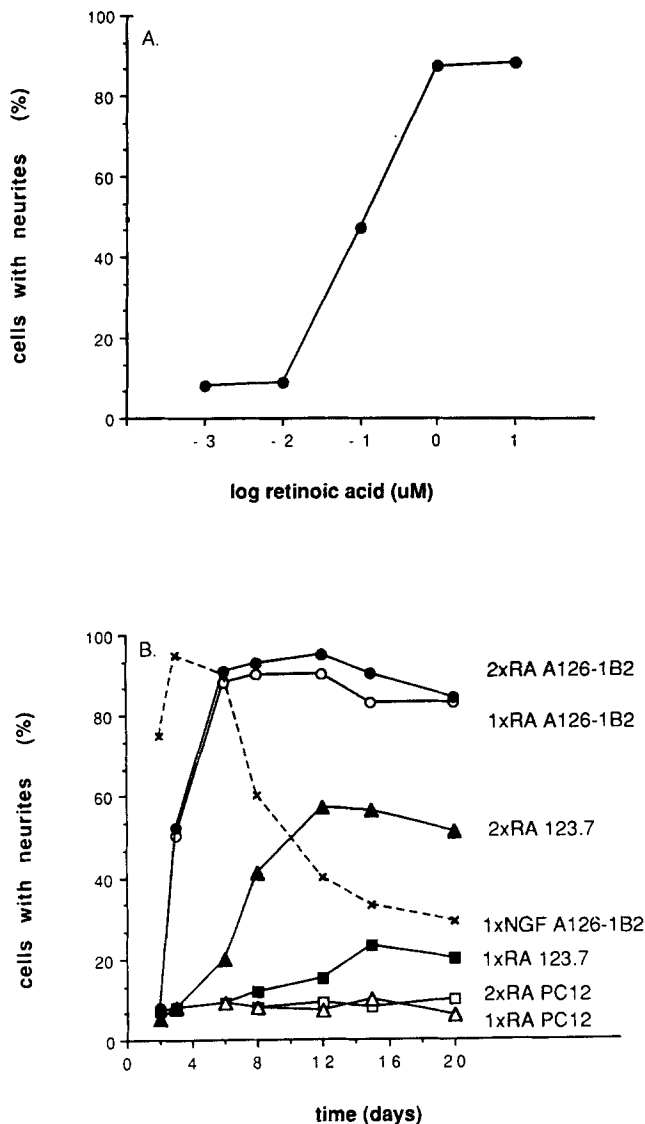
**Table I.** Rate of Neurite Production in Response to NGF and RA

Days	Length of average neurite	
	NGF	RA
2	3.0	1.6
3	6.1	2.2
4	11.4	4.2
6	15.6	6.3

A126-1B2 cells were plated in the presence of NGF (100 ng/ml; added on day 0 and day 3) or RA (1.0  $\mu$ M; added on day 0 only), the cultures were photographed at 2, 3, 4, and 6 d. The length of each neurite and the diameter of the cell body for differentiated cells were measured. The ratio of average neurite length to average cell diameter is shown above. At least 30 cells were counted for each time point.

### RA Induces Biochemical and Molecular Markers of Neural Differentiation

To determine if RA also regulated the expression of other markers of differentiation in A126-1B2, 123.7 or in PC12



**Figure 2.** RA induces neurite outgrowth in PC12 cells that are deficient in cAMP-dependent protein kinase: dose-response and time course of neurite formation. A126-1B2 cells and 123.7 cells were plated in the presence of increasing concentrations of RA as indicated and the fraction of cells with a neurite was measured after 6 d (A). PC12 cells, A126-1B2 cells, or 123.7 cells were plated in either 1  $\mu$ M RA or 100 ng/ml NGF and the fraction of cells producing neurites was measured as a function of time (B). Media was replaced every other day. NGF was added only at the time of plating. If NGF is replenished at 3-d intervals the NGF-induced neurites will continue to elongate during the course of the experiment rather than degenerate (data not shown). RA was added either only at the time of plating (1 $\times$  RA) or it was added both on the day of plating and on the day 3 after plating (2 $\times$  RA) as indicated in the figure. Neurites were defined as a process whose length exceeded one and one-half cell body diameter and had a clearly definable growth cone.

cells, we measured the change in expression of several genes in response to RA and NGF. NGF stimulates acetylcholinesterase (AChE) activity in PC12 cells (Fig. 3 A; Greene and Rukenstein, 1981), A126-1B2 cells, and 123.7 cells (Fig. 3, B and C) about twofold. In contrast, RA induced AChE expres-

sion about twofold in A126-1B2 cells and 123.7 cells (Fig. 3, B and C), but not in parental PC12 cells (Fig. 3 A). Thus the induction of AChE is correlated with the ability to induce a morphological differentiation.

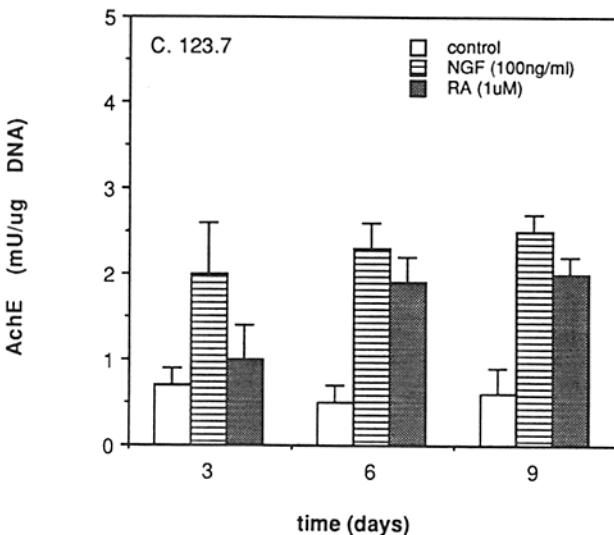
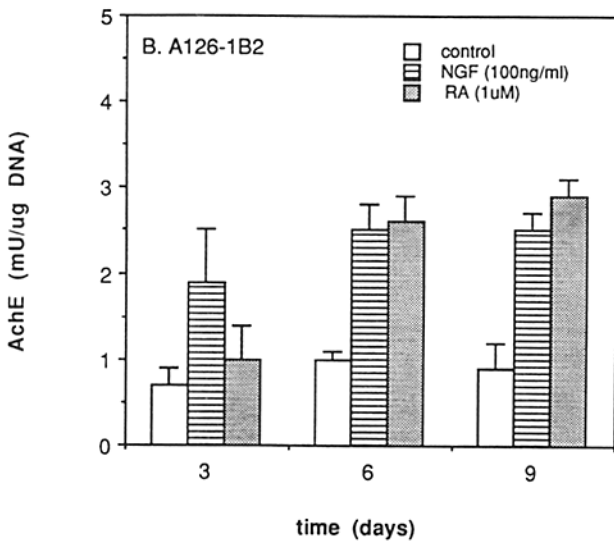
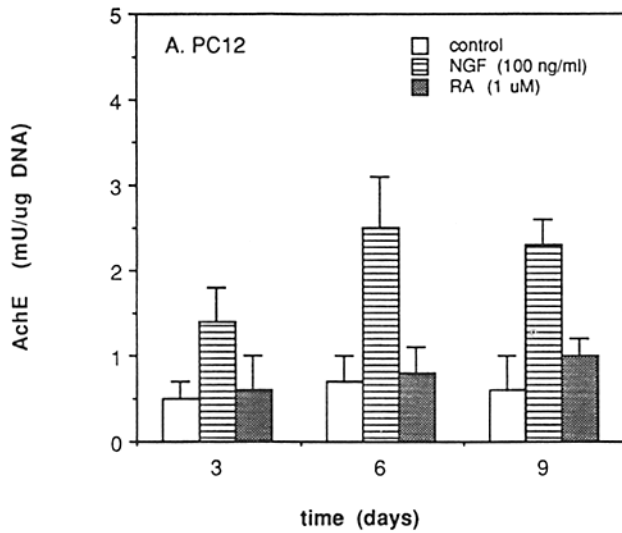
Morphological differentiation of PC12 cells in response to NGF is correlated with an increase in the expression of the middle molecular weight neurofilament gene (NF-M) (Lindenbaum et al., 1988; Fig. 4). At several days after exposure to NGF, this induction is thought to occur at both the transcriptional and posttranscriptional level (Doherty et al., 1987). RA induces the expression of NF-M mRNA in A126-1B2 cells as effectively as NGF, but RA does not induce NF-M mRNA in the parental PC12 cells. There was a higher level of NF-M expression in the both control and NGF treated A126-1B2 cells than in the parental line, possibly because PKA, which is present at higher levels in the parental line may decrease the expression of NF-M (Doherty et al., 1987). (On the basis of sequence analysis [data not shown], we have now demonstrated that the d4 gene, which is rapidly induced by NGF at a posttranscriptional level [Cho et al., 1989], encodes the NF-M [Napolitano et al., 1987; unpublished data]. We conclude that NGF can rapidly induce the expression of the neurofilament gene, but at the posttranscriptional level.)

GAP-43 is one of a number of growth-associated proteins that are found in increased concentrations in developing and regenerating nerves (Skene, 1989). GAP-43 expression is induced by NGF in PC12 cells at a posttranscriptional level (Fig. 4 B; Karns et al., 1987; Federoff et al., 1988). The mRNA for GAP-43 is induced by both NGF and RA in A126-1B2 cells, whereas it is induced only by NGF in the parental line (Fig. 4). Thus, in addition to neurite formation, RA induces the expression of at least three markers of differentiation (i.e., AChE, NF-M, and GAP-43) in A126-1B2 cells.

The gene *smg-25A*, which encodes an NGF-inducible G protein (Fig. 4; Sano et al., 1989) is induced by NGF, but not RA in PC12 cells. This gene is not induced in A126-1B2 cells by either RA or NGF (Fig. 4). Our observation suggests the possibility that active cAMP-dependent protein kinase is required for the transcriptional regulation of this gene by both NGF and RA.

### Induction of Early Response Genes by RA

One of the first steps in the response of PC12 cells to NGF is the rapid induction of a number of genes (e.g., *egr-1*/NGF1A, *c-fos*, NGF1B, et cetera) whose expression is considered by many investigators to be important for subsequent steps in differentiation (see Sheng and Greenberg [1990], for review). To determine if the expression of these early response genes may be important for RA-induced differentiation, we measured the expression of *egr-1* mRNA (Sukhatme et al., 1987; Christy et al., 1988; Milbrandt, 1987; Cho et al., 1989; Lemaire et al., 1988) and *c-fos* mRNA (Sheng and Greenberg, 1990). In A126-1B2 cells, RA induces the expression of *c-fos*, but not *egr-1* within 1 h of the exposure of the cells to RA (Fig. 5). In PC12 cells, neither gene was induced by RA. As expected, both *egr-1* and *c-fos* mRNAs were induced by NGF in both A126-1B2 and PC12 (Fig. 5). We conclude that a rapid increase in the expression of *egr-1* is not essential for differentiation in response to RA, but that RA is capable of rapidly inducing the expression of *c-fos*.



### RA Inhibits Cell Division

In addition to inducing neurite formation, NGF inhibits the division of PC12 cells and A126-1B2 cells (Burstein and Greene, 1982). RA also causes a dose-dependent inhibition of the division of both A126-1B2 and PC12 cells with a half-maximal effect at 100 nM (Fig. 6, *A* and *B*). Between 1 and 3 wk after exposure to RA, there was no further increase in cell number (Fig. 6, *C* and *D*). DNA replication could have continued, even in the absence of cell division, so the amount of DNA in the cultures was quantified as a function of time (Fig. 6, *E* and *F*), demonstrating that RA inhibited DNA replication and cell division equivalently. Thus, in A126-1B2 cells, inhibition of cell division was temporally correlated with neurite outgrowth (compare Fig. 2 *B* with Fig. 6 *C*). RA inhibited the division of the PKA-deficient PC12 mutant, 123.7, with kinetics almost identical to those seen with the A126-1B2 cells (data not shown). In the parental PC12 cells, RA effectively inhibited cell division, but failed to elicit neurite formation. PC12 cells, unlike some neuroblastoma cell lines, do not differentiate in response to inhibition of cell division. Likewise, A126-1B2 cells do not differentiate when growth is inhibited by low serum concentration (data not shown).

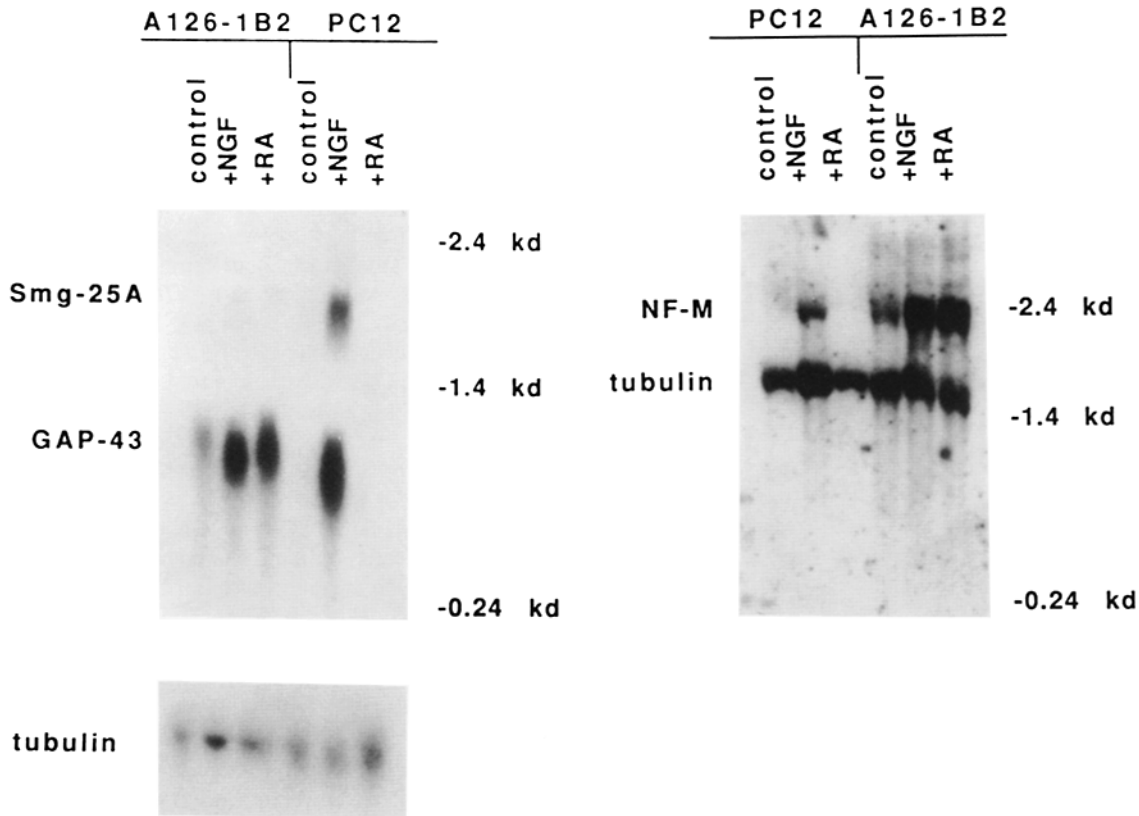
### Expression of RA Receptors and the RA Binding Proteins in PC12 Cells and in PKA-deficient Cell Lines

The relative level of the mRNA encoding the three nuclear RA receptors ( $RAR\alpha$ ,  $RAR\beta$ , and  $RAR\gamma$ ) as well as the cytoplasmic RA binding protein (CRABP) were measured in parental PC12 cells and in the two PKA-deficient clones, A126-1B2 and 123.7. In both PKA-deficient lines, the level of mRNA for both  $RAR\alpha$  and  $RAR\beta$  was increased markedly (15-fold, 5-fold) relative to the parental cells (Fig. 7). None of the three lines expressed measurable levels of  $RAR\gamma$  (Fig. 7). Both PKA-deficient lines (A126-1B2 and 123.7) also expressed a significantly higher levels (10-fold) of the CRABP mRNA than the parental line (Fig. 8). The increased levels of  $RAR\alpha$ ,  $RAR\beta$ , and CRABP may be partially responsible for the ability of A126-1B2 and 123.7 cells to respond to RA.

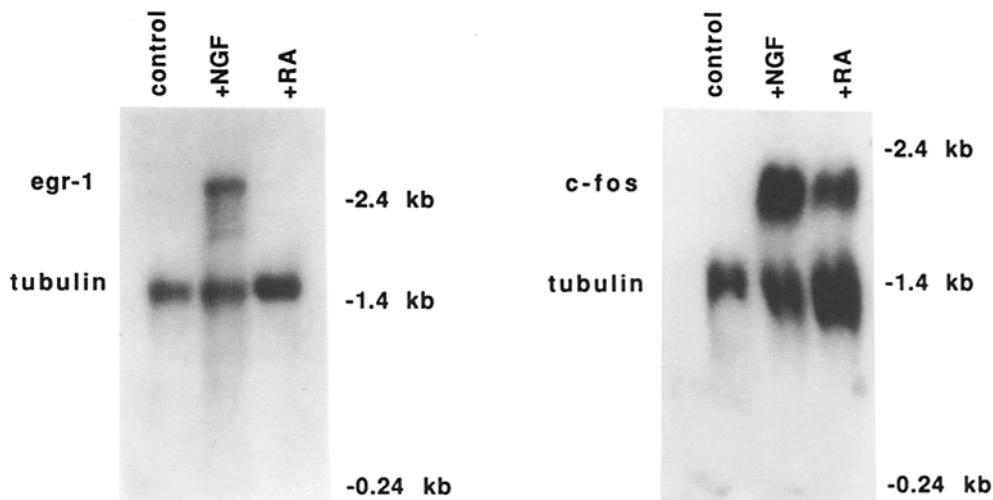
### Discussion

In PKA-deficient PC12 cells, both RA and NGF stimulate neurite outgrowth and the expression of differentiated characteristics, but there is clearly a difference in the ways these cells respond to these dissimilar signaling molecules. NGF regulates the expression of differentiated characteristics by PC12 cells and PKA-deficient PC12 mutants; and continued presence of NGF is required to maintain neurite

*Figure 3.* Induction of AchE activity by RA and NGF. PC12 cells (*A*), A126-1B2 cells (*B*), or 123.7 cells (*C*) were plated in the presence of 1  $\mu$ M RA (at the time of plating only) or 100 ng/ml NGF (added at the time of plating and at day 3) or in the absence of any additives, and the specific activity of AchE (average of six assays  $\pm$  SE) was determined at day 3, 6, and 9 as described (Ellman, 1959). One unit of esterase activity is defined as the hydrolysis of 1  $\mu$ M of acetylcholine per minute.



**Figure 4.** Induction of gene expression by RA. mRNA (30  $\mu$ g) was isolated from both PC12 and A126-1B2 cells 8 d after treatment with 100 ng/ml NGF (added at day 0, 3, and 6) or 1  $\mu$ M RA (added only at the time of plating), fractionated on a 1.2% formaldehyde agarose gel, transferred to a nitrocellulose membrane, and hybridized to  $^{32}$ P-labeled NF-M cDNA ( $1 \times 10^6$  cpm/ml), GAP-43 cDNA ( $1 \times 10^6$  cpm/ml), smg-25A cDNA ( $2 \times 10^6$  cpm/ml), or tubulin cDNA ( $1 \times 10^6$  cpm/ml). The exposure times were 12 h (*tubulin*), 24 h (*GAP-43*, *NF-M*), or 48 h (*GTP-binding protein*, *smg-25A*). When normalized to the hybridization signal from  $\alpha$ -tubulin, NFM is induced  $\sim$ 30-fold by NGF and  $\sim$ 35-fold by RA. Likewise, GAP43 is induced  $\sim$ 9-fold by NGF and 13-fold by RA. Molecular size markers are ethidium bromide stained RNA components derived from bacteriophage T7, yeast, and bacteriophage  $\lambda$  DNA.



**Figure 5.** Induction of early response genes by RA. RNA (20  $\mu$ g) was isolated from A126-1B2 cells, fractionated on a 1.2% formaldehyde agarose gel, transferred to nitrocellulose, and hybridized with  $^{32}$ P-labeled *egr-1* cDNA ( $1 \times 10^6$  cpm/ml) or *c-fos* cDNA ( $1 \times 10^6$  cpm/ml) and tubulin cDNA ( $1 \times 10^6$  cpm/ml). The gel was exposed for 24 h (*egr-1*) or 3 d (*c-fos*). Cells were exposed to NGF (100 ng/ml) or RA (1  $\mu$ M) for 1 h as indicated. The absolute induction of *c-fos* is impossible to quantitate because the basal level of *c-fos* was not measurable; but, relative to  $\alpha$ -tubulin, RA is about fivefold less effective than NGF at inducing *c-fos* message. Molecular size markers are ethidium bromide stained RNA components derived from bacteriophage T7, yeast, and bacteriophage  $\lambda$  DNA.

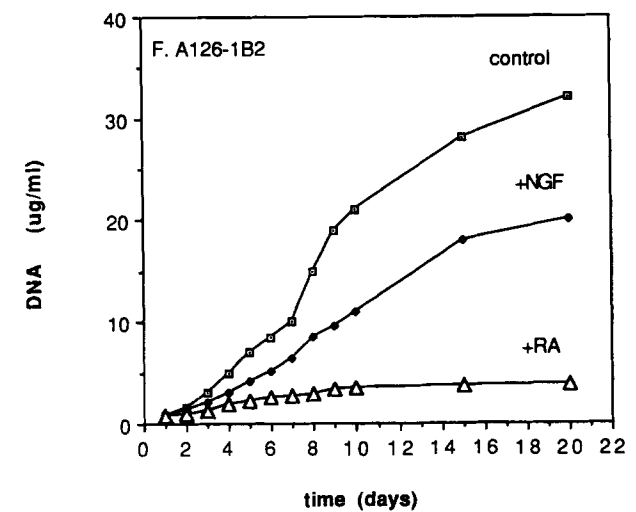
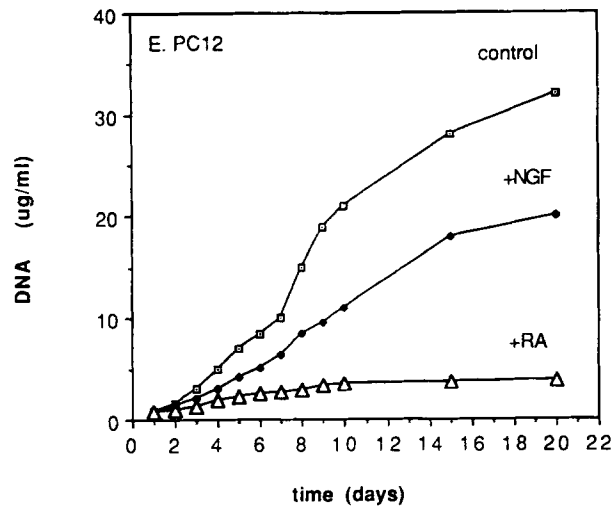
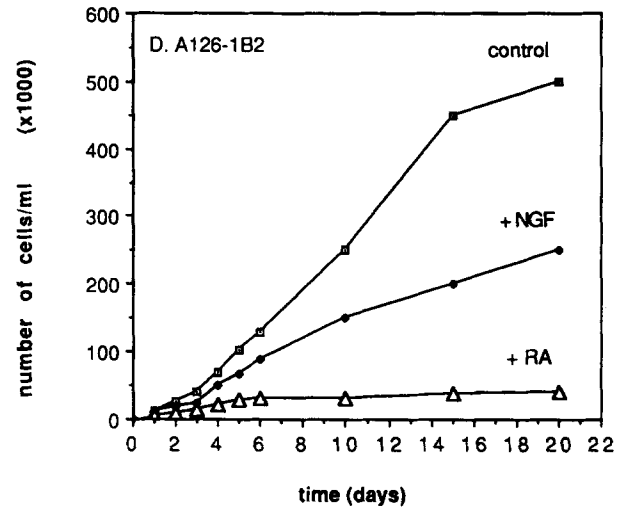
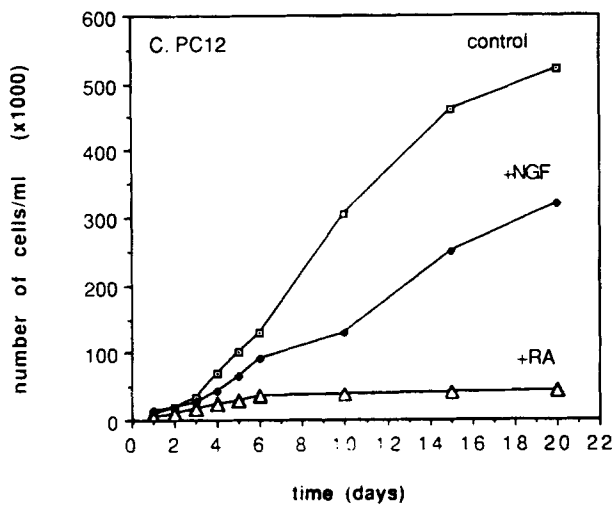
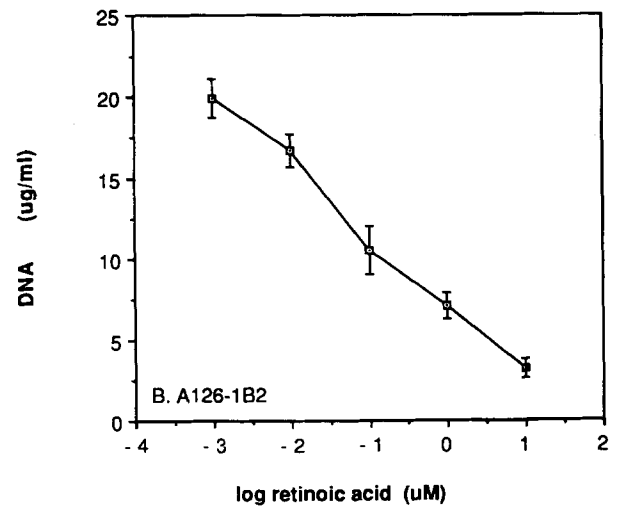
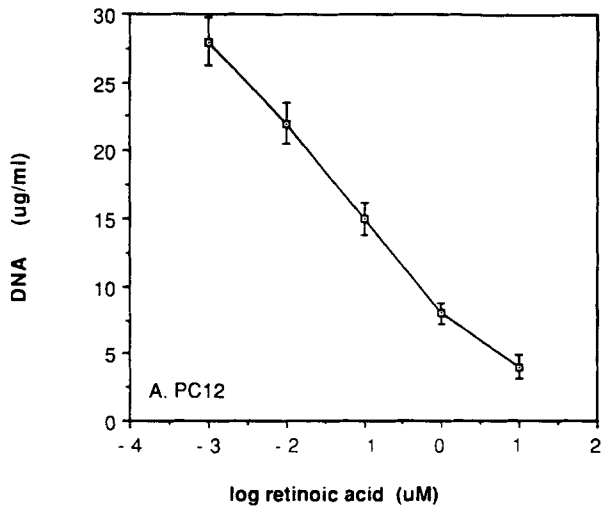
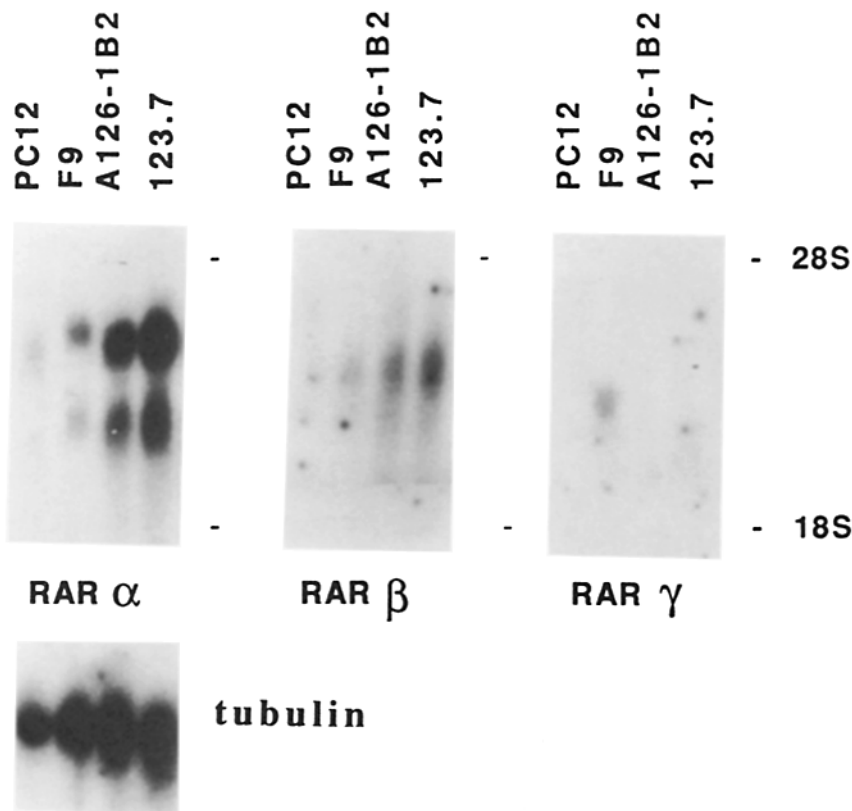


Figure 6. RA inhibits division of PC12 cells. Both PC12 (A) and A126-1B2 (B) cells were plated at an initial density of  $1 \times 10^4$  in the presence of increasing concentrations of RA and the amount of DNA per culture was measured after 6 d. In another experiment, PC12 cells (C and E) and A126-1B2 cells (D and F) were plated in the presence of either 100 ng/ml NGF (added at the time of plating and every third day) or  $1 \mu\text{M}$  RA (added only at the time of plating). The cell number (C and D) and the DNA content (E and F) of each culture was determined as a function of time.



**Figure 7.** Expression of RAR $\alpha$ , RAR $\beta$ , and RAR $\gamma$ . RNA (30  $\mu$ g) was isolated from PC12, A126-1B2, and 123.7 cells, or F9 cells fractionated on an 1.2% formaldehyde agarose gel, transferred to nitrocellulose, and hybridized with  $^{32}$ P-labeled cDNA probe prepared from a 0.6-kb RAR $\alpha$  fragment ( $5 \times 10^6$  cpm/ml), 0.6-kb RAR $\beta$  fragment ( $5 \times 10^6$  cpm/ml), 0.3-kb RAR $\gamma$  fragment ( $5 \times 10^6$  cpm/ml) or  $\alpha$ -tubulin ( $1 \times 10^6$  cpm/ml) as indicated. The RAR probes were prepared as described (Hu and Gudas, 1990). The exposure times were 24 h (RAR $\alpha$ ), 24 h (RAR $\beta$ ), 48 h (RAR $\gamma$ ), and 12 h (tubulin). The position of the 28S and 18S rRNA as detected by ethidium bromide staining is indicated.

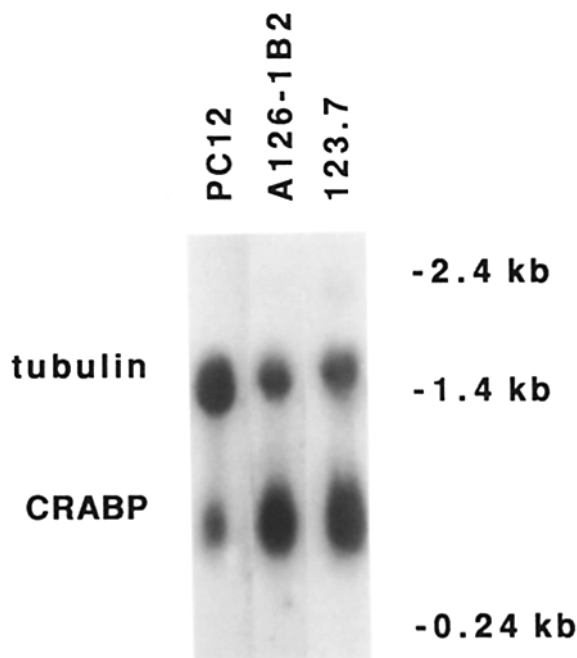
outgrowth and to inhibit cell division (Greene and Tischler, 1976). In contrast, a single exposure to RA appears to have a long term (several weeks) effect on PC12 cells and PKA-deficient PC12 cell mutants. The neurites that are formed in A126-1B2 cells in response to RA are stable, and the small decrease in the fraction of cells that have neurites at late times (Fig. 2) appears to be due to a proliferation of a subpopulation of cells that did not fully respond to RA. We conclude that RA causes a long-term stable change in the growth and differentiation state of the cells, suggesting that RA causes an apparently irreversible determination event. The RA-induced state of the cells is characterized by the entrance of the cells into a nondividing ( $G_0$ ) state, by the formation of nerve-like processes, and by the expression of other markers of neural differentiation (AChE, NF-M, and GAP-43).

Although it is often suggested that the induction of early response genes is an essential step in the cascade of events that leads to neural differentiation, it has been difficult to demonstrate the requirement for any of the early response genes in the morphological differentiation of PC12 cells (Sheng and Greenberg, 1990). Differentiation of A126-1B2 cells in response to RA occurs in absence of the induction of *egr-1*. Thus, a rapid transcriptional induction of *egr-1*, as detected in PC12 cells after NGF treatment (Milbrandt, 1987) is unlikely to be an essential trigger that is required to induce neurite outgrowth. This is consistent with the observation that NGF can cause neurite formation when the acute induction of *egr-1* is compromised by the downregulation of protein kinase C in a PKA-deficient line (Damon et al., 1990). RA does cause a rapid induction of *c-fos* mRNA. Thus, *egr-1* and *c-fos* are not coordinately regulated in response to RA in A126-1B2 cells, as they are in response to NGF (Sukhatme et al., 1988).

In PKA-deficient PC12 cells, RA induces a program of differentiation that is similar to that induced by NGF. AChE, GAP-43, and NF-M are induced in response to both agents; but neither agent induces the GTP binding protein, smg-25A. In parental PC12 cells, NGF, but not RA, induces the expression of all these molecular markers as well as neurite outgrowth. This raises the question of why smg-25A is not induced by NGF in A126-1B2 cells, when it is induced by NGF in parental PC12 cells. We suggest that the expression of smg-25A may depend on PKA activity and that the lack of PKA in the A126-1B2 cells prevents the induction of this marker. Alternatively, smg-25A may not be regulated by RA under any circumstances. The differentiated cell type that is formed in response to RA may be different from the differentiated cell type that is formed in response to NGF.

RA action is presumably mediated by the RARs and the CRABP, or by related proteins. The RARs are thought to activate gene expression by binding to specific sequences in target genes, and it is the activation of these genes that presumably leads to neural differentiation. The RA-dependent pathway that leads to neural differentiation is likely to be very different from the signal transduction pathways that are used by NGF and FGF, which are initiated by the occupation of a cell surface receptor. Occupation of these cell surface receptors initiates a cascade of events that also leads to the activation of some of the same genes that are activated by RA. Thus, genes that are involved in neural differentiation are apparently regulated by two very different signaling pathways. Furthermore, there appears to be an interaction between the RA-dependent pathway and the cAMP-dependent pathway. The higher levels of the mRNAs for the RARs and CRABP in A126-1B2 cells probably results from the deficiency in PKAII activity since the line 123.7, which also ex-





**Figure 8.** Expression of CRABP. RNA was isolated from PC12, A126-1B2, and 123.7 cells, fractionated on a 1.2% formaldehyde agarose gel, transferred to nitrocellulose, and hybridized with  $^{32}\text{P}$ -labeled cDNA probe to CRABP or tubulin as indicated. The gel was exposed for 12 h for both CRABP and tubulin. These data are representative of three independent experiments. When normalized to  $\alpha$ -tubulin, CRABP message is overexpressed  $\sim 18$ -fold in both the A126-1B2 cells and the 123.7 cells, relative to PC12 cells. Molecular size markers are ethidium bromide stained RNA components derived from bacteriophage T7, yeast, and bacteriophage  $\lambda$  DNA.

presses reduced levels of this kinase, has increased levels of the RARs and CRABP. Thus, the level of activated PKA in a cell, which is regulated by hormones and local mediators may indirectly regulate the responsiveness of a cell to RA. This idea is consistent with the observations that activation of PKA by dbcAMP causes a decrease in the expression of the RAR $\alpha$  and RAR $\beta$  in F9 cells (Hu and Gudas, 1990).

Our data show that neither the 123.7 cells nor the AB.11 cells were as sensitive to RA as the A126-1B2 cells. The processes formed by 123.7 cells or AB.11 cells in response to RA did not elongate as much as those from the A126-1B2 cells. Furthermore, although a single exposure to RA elicited neurites from nearly all A126-1B2 cells, it took several treatments with RA to elicit neurite formation from a majority of 123.7 cells (Fig. 1 B) or AB.11 cells (data not shown). The reason for these differences in the sensitivity to RA is not currently understood. The A126-1B2 cells are deficient in PKAII, but have essentially normal levels of PKAI, while both 123.7 cells and AB.11 cells are almost completely deficient in PKAI, and have reduced levels of PKAII (Ginty et al., 1991b). It is a reasonable speculation that this difference in kinase profile may account for the difference in RA responsiveness of the lines. Alternatively, the A126-1B2 cells may be carrying a mutation that is unlinked to their PKA deficiency and this mutation may be responsible for their greater responsiveness to RA. Nevertheless, the fact that all three PKA-deficient cell lines formed

neurites in response to RA is persuasive evidence that there is an interaction between the RA and cAMP signaling pathways.

RA is becoming recognized as an important signaling molecule that provides patterning information during morphogenesis (Thaller and Eichele, 1987). The CRABP and the RARs have been detected in the neural crest (Osumi-Yamashita et al., 1990) and other developing neural tissue (Maden et al., 1989; Giguere et al., 1987; Benbrook et al., 1988). RA may also play a role in controlling the pattern of innervation, and innervation may have an influence on other morphogenic processes. Our experiments establish that the PC12 cell line is a useful model to study the effect of RA on neural differentiation and the interaction between the RA and PKA signal transduction pathway. We have provided evidence that PKA may play an essential role in modulating RA-dependent neural differentiation. Although RA must act by a mechanism that is fundamentally different from that used by the peptide growth factors, RA and the peptide growth factors appear to be able to regulate an overlapping, but nonidentical set of differentiated properties. Finally, the RA signaling pathway may be influenced and modulated by cAMP.

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