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Participation of Type II Protein Kinase A in the Retinoic Acid-Induced Growth Inhibition of SH-SY5Y Human Neuroblastoma Cells

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To examine the role of protein kinase A (EC 2.7.1.37) isozymes in the retinoic acid-induced growth inhibition and neuronal differentiation, we investigated the changes of protein kinase A isozyme patterns in retinoic acid–treated SH-SY5Y human neuroblastoma cells. Retinoic acid induced growth inhibition and neuronal differentiation of SH-SY5Y cells in a dose- and time-dependent manner. Neuronal differentiation was evidenced by extensive neurite outgrowth, decrease of N-Myc oncoprotein, and increase of GAP-43 mRNA. Type II protein kinase A activity increased by 1.5-fold in differentiated SH-SY5Y cells by retinoic acid treatment. The increase of type II protein kinase A was due to the increase of RIIβ and Cα subunits. Since type II protein kinase A and RIIβ have been known to play important role(s) in the growth inhibition and differentiation of cancer cells, we further investigated the role of the increased type II protein kinase A by overexpressing RIIβ in SH-SY5Y cells. The growth of RIIβ-overexpressing cells was slower than that of parental cells, being comparable to that of retinoic acid-treated cells. Retinoic acid treatment further increased the RIIβ level and further inhibited the growth of RIIβ-overexpressing cells, showing strong correlation between the level of RIIβ and growth inhibition. However, RIIβ-overexpressing cells did not show any sign of neuronal differentiation and responded to retinoic acid in the same way as parental cells. These data suggest that protein kinase A participates in the retinoic acid–induced growth inhibition through the up-regulation of RIIβ/type II protein kinase A. J. Cell. Physiol. 182:421–428, 2000.

Retinoic acid (RA) has dramatic effects in the development and differentiation of many cell types (Brockes, 1989). RA works as a morphogen that is important in the regulation of anterior–posterior axis in the developmental process (Thaller and Eichele, 1987). It has been well established that the effects of RA are mediated mainly by two classes of nuclear receptors, retinoic acid receptors (RARs) (Petkovich et al., 1987; Benbrook et al., 1988; Krust et al., 1989), and retinoid X receptors (RXRs) (Hamada et al., 1989; Mangelsdorf et al., 1990, 1992; Leid et al., 1992). All-*trans* RA binds to and activates RARs, and 9-*cis* RA activates both RARs and RXRs, both of which modulate the expression of their target genes by interacting with retinoic acid response elements (RAREs) as homodimeric or heterodimeric forms (for review, see Chambon, 1996).

Protein kinase A (PKA) is a well-known effector protein in the cAMP-mediated signal transduction pathway. PKA is a serine/threonine kinase composed of a regulatory (R) subunit dimer and two catalytic (C) subunits. Two different regulatory subunits (RI and RII) bind to common catalytic subunits, distinguishing two types of PKA holoenzymes, type I and type II PKA, respectively. So far, four isoforms of R subunits, RIα

Abbreviations: AKAP, A-kinase anchoring protein; BDNF, brain-derived neurotrophic factor; cAMP, cyclic AMP; CRABP, cytoplasmic retinoic acid binding protein; CRE, cAMP-response element; dbcAMP, N^6,2′-*O*-dibutyryl cyclic AMP; DEAE-cellulose, diethylaminoethyl cellulose; NGF, nerve growth factor; PKA, protein kinase A; PMA, phorbol 12-myristate 13-acetate; PMSF, phenylmethylsulfonyl fluoride; R and C, regulatory and catalytic subunits of protein kinase A, respectively; RA, retinoic acid; RAR, retinoic acid receptor; RARE, retinoic acid response element; RI and RII, type I and type II regulatory subunits of protein kinase A, respectively; RXR, retinoid X receptor.

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(Sandberg et al., 1987), RIβ (Solberg et al., 1991), RIIα (Oyen et al., 1989), and RIIβ (Levy et al., 1988), and three isoforms of C subunits, Cα (Maldonado et al., 1988), Cβ, and Cγ (Beebe et al., 1990) have been identified. Differential expression of two types of PKA has been linked to the control of cell growth (Cho-Chung, 1990). An enhanced expression of RI/type I PKA correlates with active cell growth and transformation, whereas an increase of RII/type II PKA is related to growth inhibition and differentiation.

There are reports that RA signals change the activity and intracellular distribution of PKA in F9 teratocarcinoma cells (Plet et al., 1982, 1987). In addition, F9 cells respond to dbcAMP with appearance of neuron-like cells only when pretreated with RA (Kuff and Fewell, 1980). These results suggested that RA signals may change the responsiveness of the F9 cells to the cAMP signal. However, the nature of the changes and the mechanism by which RA signals change the responsiveness to cAMP were not elucidated.

Currently, the specific functions of each PKA isozyme, such as specific modulation of intracellular signaling and cell growth control, are being discovered (Cho-Chung, 1990; Skalhegg et al., 1994; Tortora et al., 1997). The importance of PKA isozymes and each regulatory subunit in the neural functions was also exaggerated by the findings from knock-out mice of PKA regulatory subunits (Brandon et al., 1995, 1998; Huang et al., 1995; Adams et al., 1997; Malmberg et al., 1997; Hensch et al., 1998). Thus, it became necessary to consider the isozyme-specific roles of PKA in analyzing the cellular events started by RA.

In the present study, we employed SH-SY5Y human neuroblastoma cells as a model system to study the role of PKA isozymes in the RA-induced growth inhibition and neuronal differentiation. We found that RIIβ and type II PKA activity specifically increase along with RA-induced growth inhibition and neuronal differentiation of SH-SY5Y cells. Through the experiments with RIIβ-overexpressing cells, we showed that the increased RIIβ/type II PKA is critically involved in the RA-induced growth inhibition but not in the neuronal differentiation.

MATERIALS AND METHODS

Cell culture

SH-SY5Y human neuroblastoma cells were maintained in Eagle's minimum essential medium (GIBCO-BRL, Gaithersburg, MD) supplemented with 10% heat-inactivated fetal bovine serum (GIBCO-BRL), 100 units/ml penicillin-G, and 100 μg/ml streptomycin in a humidified atmosphere of 5% CO_2 at 37°C. For all the experiments, all-*trans* RA was treated on the day after cell seeding from 1,000X stock in ethanol.

Generation of RIIβ-overexpressing cells

RIIβ expression construct was prepared by inserting human RIIβ cDNA into *Eco*RI cloning site of pLXSN plasmid (Miller et al., 1989). SH-SY5Y cells were transfected with empty pLXSN plasmid and RIIβ expression construct by calcium phosphate/DNA coprecipitation method. Stable transfectants were selected against 500 μg/ml G418 (GIBCO-BRL). G418-resistant vector-transfected cells were pooled and used as vector control cells. Clonal cells transfected with RIIβ expression constructs were obtained by dilution cloning and the levels of RIIβ were examined by Western blot. Three clones of two- to threefold increased RIIβ level were selected and pooled for further characterization.

Monolayer growth assay

Cells were seeded at a density of 2×10^5 cells/35-mm culture dish at Day −1. All-*trans* RA was treated at indicated concentrations at Day 0 when necessary. Cell numbers were determined at indicated time points with Coulter's counter.

Western blot analysis

Monolayer cells were washed with phosphate-buffered saline and then harvested by scraping and centrifugation. Cell pellet was suspended in an extraction buffer (20 mM Tris–Cl, pH 7.4; 100 mM NaCl; 1% NP-40; 0.5% sodium deoxycholate; 5 mM $MgCl_2$; 0.1 mM PMSF; 0.1 mM pepstatin A; 0.1 mM antipain; 0.1 mM chymostatin; 0.2 mM leupeptin; 0.5 μg/ml aprotinin; 0.5 mg/ml soybean trypsin inhibitor; 1 mM benzamidine), incubated on ice for 15 min and then centrifuged. Resulting supernatant was used as cell extract. Western blot analysis was performed as described previously (Kim et al., 1996). Polyclonal antibodies against PKA regulatory subunits were generated in rabbit using recombinant proteins (Lee et al., 1999). Polyclonal antibody against Cα subunit was also generated in rabbit using recombinant Cα protein (amino acids 19–350). Monoclonal antibody for N-Myc was purchased from Santa Cruz Biotechnology (Santa Cruz, CA).

Northern blot analysis

Total cellular RNA was isolated by the method described by Chomczynski and Sacchi (1987). For Northern blot analysis, 20 μg of total RNA were resolved in formaldehyde-denaturing agarose gel, transferred onto nitrocellulose filter, and hybridized with ^{32}P-labeled probe. Radioactive probes for Northern blot analyses were prepared by unidirectional polymerase chain reaction or random primer-labeling method. For probing the blots, 451-bp *Hind*III fragment of RIIβ (nucleotides 354–804), 521-bp *Rsa*I-*Eco*RI fragment of RIα (nucleotides 223–743), 1.1 kb of total Cα cDNA, *Bam*HI–*Eco*RI fragment (5B fragment) of mouse 18S rRNA gene (Bowman et al., 1981), and 604-bp fragment (242–845) of human GAP-43 cDNA were used.

DEAE-cellulose chromatography

Cells were washed with ice-cold phosphate-buffered saline and harvested by scraping and centrifugation. Cell pellet was suspended in 10 ml of column buffer (10 mM Tris–Cl, pH 7.1; 1 mM EDTA; 1 mM DTT; 0.5 mM PMSF; 0.1 mM pepstatin A; 0.1 mM antipain; 0.1 mM chymostatin; 0.2 mM leupeptin; 0.5 μg/ml aprotinin; 0.5 mg/ml soybean trypsin inhibitor; 1 mM benzamidine) and kept on ice for 20 min. Cells were broken up with a Dounce homogenizer (50 strokes with A pestle) and centrifuged at 10,000*g* for 20 min. The resulting supernatant was used as cell extract for DEAE-cellulose chromatography. Two milligrams of cell extract were loaded onto the DEAE-cellulose column (10 × 1 cm, Bio-Rad, Richmond, CA) preequilibrated with column buffer, and the column was washed with 30 ml of column buffer. The column was developed with 100 ml of NaCl gradient (0–350 mM) in column buffer at a flow

rate of 0.5 ml/min. Two-milliliter fractions were collected and used for PKA activity assay.

Protein kinase A activity assay

For the determination of total cellular PKA activity, cell extract was prepared as in DEAE-cellulose chromatography and used directly for PKA activity assay. Ten microliters of the column fractions or 10 μg of total cell extract in 10 μl were mixed with 50 μl of kinase assay buffer (50 mM Tris–Cl, pH 7.5; 1 mM DTT; 10 mM $MgCl_2$; 30 μM Kemptide [Leu-Arg-Arg-Ala-Ser-Leu-Gly], 5 μM [γ-^{32}P]ATP) in the presence or absence of 5 μM cAMP, and incubated at 37°C for 5 min. Fifty microliters of the reaction mixture were spotted onto phosphocellulose filters and washed three times with 1% phosphoric acid. Filters were air-dried and radioactivities were measured with liquid scintillation counter.

RESULTS

Growth inhibition and neuronal differentiation of SH-SY5Y cells by retinoic acid

Induction of growth inhibition and differentiation of undifferentiated cells are the most well known effects of RA. Neuroblastoma cells have been known to differentiate in response to RA treatment (Pahlman et al., 1984). To examine the response of SH-SY5Y cells toward RA treatment, the changes in the growth rate and differentiation status were tested. When SH-SY5Y cells were treated with all-*trans* RA of various concentrations, the cell proliferation was inhibited in a dose-dependent manner (Fig. 1A). The growth-inhibiting effect of RA was evident as early as Day 2 of RA treatment (Fig. 1B).

Differentiation of SH-SY5Y cells was examined by cellular morphology and changes in the differentiation markers. Cells responded to RA treatment by extensive neurite outgrowth (Fig. 2A), decreased level of N-Myc (Fig. 2B), and increased mRNA level of growth cone–associated protein 43 (GAP-43) (Fig. 2C). Decrease of N-Myc level was found to be a prerequisite for the neuronal differentiation of neuroblastoma cells (Thiele et al., 1985). GAP-43 has been considered as a marker of neuronal differentiation and its level correlates with neurite outgrowth (for review, see Gispen et al., 1991). The decrease in N-Myc level and the increase of GAP-43 mRNA were manifested within 6 h of RA treatment (Fig. 2B and 2C), showing that the biochemical changes related to neuronal differentiation initiate within 6 h of RA treatment. The level of GAP-43 mRNA showed two peaks at 6 and 72 h of RA treatment, suggesting biphasic effect of RA on the GAP-43 mRNA level.

Increase of type II PKA activity during retinoic acid–induced growth inhibition and differentiation

Since PKA plays important roles in the control of cell growth and differentiation, we examined the changes in PKA activity during the RA-induced growth inhibition and neuronal differentiation of SH-SY5Y cells. In RA-treated cells, total PKA activity gradually increased up to twofold at Day 6, with only a slight increase of basal PKA activity (Fig. 3A). The significant increase of total PKA activity was observed from Day 3. These results suggest that both the catalytic and regulatory subunits of PKA increased and formed holoenzyme in RA-treated cells.

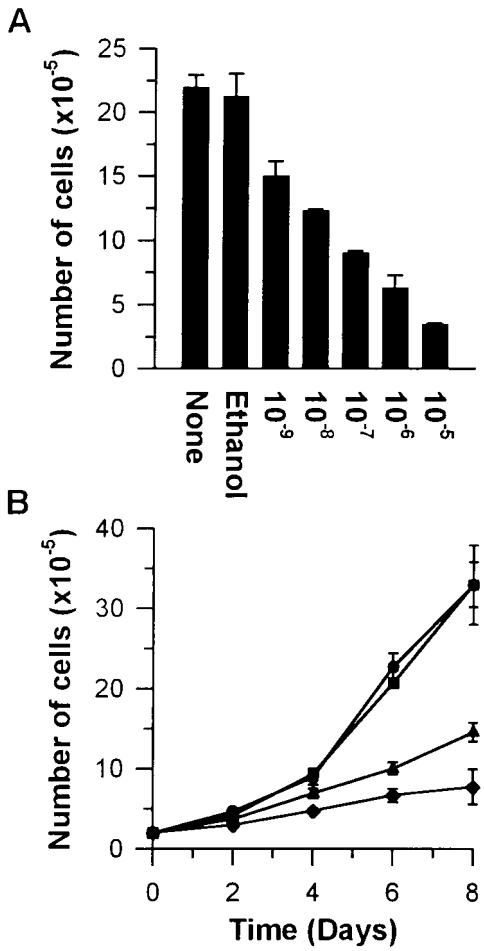

Fig. 1. Inhibition of cell growth by retinoic acid. **A:** Effects of various concentrations of all-*trans* RA on the cell growth. Cells were seeded at a density of 2×10^5 per 35-mm tissue-culture dish at Day −1. All-*trans* RA was treated at Day 0, and cell number was determined at Day 6. Designations are: None, nontreated; Ethanol, ethanol-treated (0.1%); numbers are concentrations of treated all-*trans* RA in molarity. **B:** Time-course experiment on the effect of all-*trans* RA on the cell growth. Cells were grown at the same condition as in panel A. Cell number was determined at days 0, 2, 4, 6, and 8. Symbols are: ●, nontreatment; ■, 0.1% ethanol; ▲, 10^{-7} M all-*trans* RA; ◆, 10^{-6} M all-*trans* RA. The cell numbers of both 10^{-7} and 10^{-6} M all-*trans* RA-treated cells were significantly different from Day 2 ($P < 0.05$, two-sided *t*-test). Data show a representative from three separate experiments of triplicate determinations. Error bar: SD.

PKA holoenzymes are divided into type I and type II PKA, which have opposite functions in the cell-growth regulation; therefore, we decided to look at the nature of the increase of total PKA activity. To determine the activities of type I and type II PKA separately, cell extracts were fractionated by DEAE-cellulose chromatography and their kinase activities were examined. Nontreated SH-SY5Y cells showed almost undetectable type I PKA activity and prominent type II PKA activity. The 10^{-6} M RA-treated cells showed 1.5-fold increased type II PKA activity without any change in type I PKA activity (Fig. 3B). These data indicate that type II PKA increases specifically during the process of RA-induced growth inhibition and differentiation.

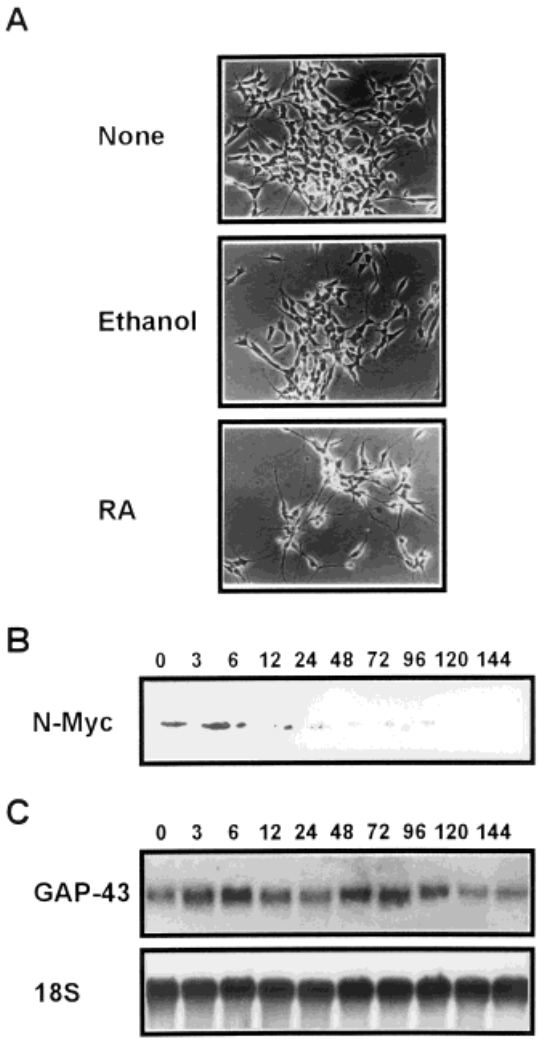

Fig. 2. Neuronal differentiation of SH-SY5Y cells by retinoic acid. **A:** Morphological assessment of neuronal differentiation. Nontreated cells, and cells treated with ethanol (0.1%) and 10^{-6} M all-*trans* RA were maintained for four days. In contrast to nontreated or ethanol-treated cells, extensive neurite outgrowth and formation of connections between cells were visualized in RA-treated cells. Photographs were taken at the same magnifications (×300). **B:** Decrease of N-Myc protein by RA treatment. Cells were treated with 10^{-6} M all-*trans* RA for indicated time (h). Level of N-Myc protein was examined by Western blot analysis. **C:** Change of GAP-43 mRNA by RA treatment. Total cellular RNA was extracted from cells treated with 10^{-6} M all-*trans* RA as in panel B, and analyzed by Northern blot analysis. 18S rRNA was used as an internal control.

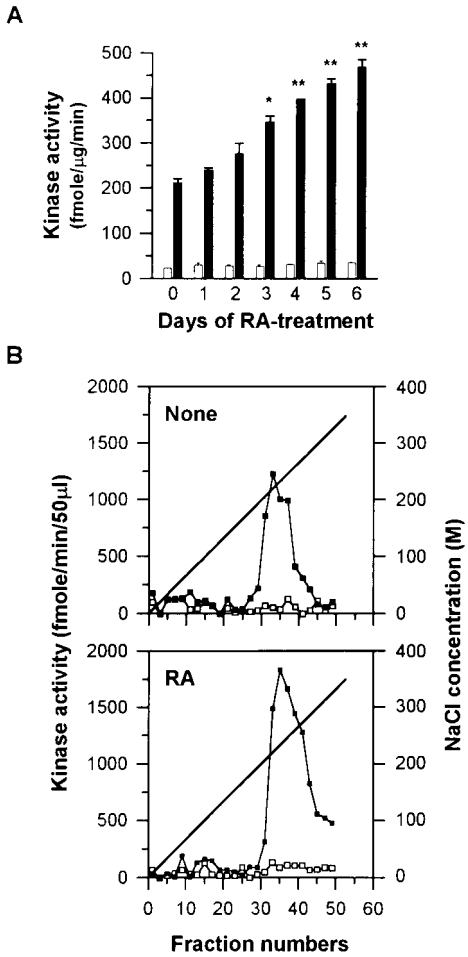

Fig. 3. Effect of retinoic acid on the activities of protein kinase A isozymes. **A:** Changes in total PKA activity. Cells were grown in the presence of 10^{-6} M all-*trans* RA and the PKA activity in cell extract was examined at indicated time points. Open bar indicates PKA activity assayed without cAMP and closed bar shows PKA activity measured in the presence of 5 μM cAMP. Data show means of three separate experiments with SD. Asterisks show significance difference in PKA activities between Day 0 and others (*, $P < 0.05$; **, $P < 0.01$, two-sided *t*-test). **B:** Increase of type II PKA activity by RA. Cells were grown in the absence (None) or presence (RA) of 10^{-6} M all-*trans* RA for 6 days, and 2 μg of cell extracts were subjected to DEAE-cellulose chromatography. The kinase activity around fraction number 11 shows type I PKA activity. The other peak around fraction number 35 represents type II PKA activity. Open square shows kinase activity measured without cAMP and closed square designates kinase activity measured in the presence of 5 μM cAMP. Graph shows a representative from three separate experiments with similar results.

Changes in mRNA and protein levels of PKA subunits

Because type II PKA activity was increased in RA-treated cells, it was assumed that RII subunits of PKA should be increased. To examine whether RII subunits of PKA are really increased by RA treatment, Northern blot and Western blot analyses on PKA subunits were carried out.

In RA-treated cells, RIIβ mRNA increased up to twofold at 24 h of treatment, which was maintained up to Day 6 (Fig. 4). The mRNA levels of RIα and RIIα were not changed by the treatment of 10^{-6} M RA (Fig. 4 and data not shown). Cα mRNA showed only statistically insignificant increase at 3 h of treatment and no considerable change (Fig. 4). RIIβ protein level also increased up to two- to threefold at Day 6 (Fig. 5). In contrast to the mRNA level, the Cα protein level also increased with the similar time course to RIIβ (Fig. 5), suggesting the increase of Cα protein is due to the stabilization of Cα protein by the formation of type II PKA holoenzyme. RIα and RIIα proteins did not show remarkable change (Fig. 5). Thus, the increase of type II PKA activity is due to the increase of RIIβ and Cα protein and the formation of type II PKA holoenzyme.

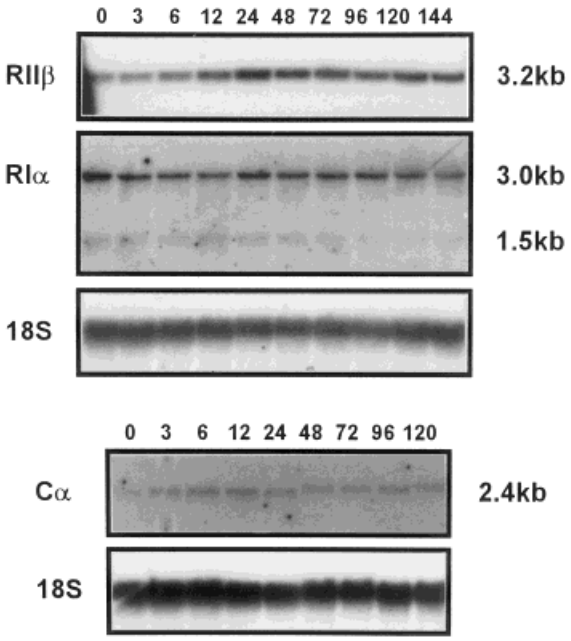

Fig. 4. Effects of retinoic acid on the mRNA levels of protein kinase A subunits. Messenger RNA levels of PKA subunits were examined by Northern blot analyses in 10^{-6} M all-*trans* RA treated cells. Total cellular RNA was extracted at the indicated time points (h) after treatment of all-*trans* RA. The same blot was used to probe RIIβ and RIα mRNA. 18S rRNA is shown as an internal control. The sizes of each band are shown at the right side of the autoradiogram.

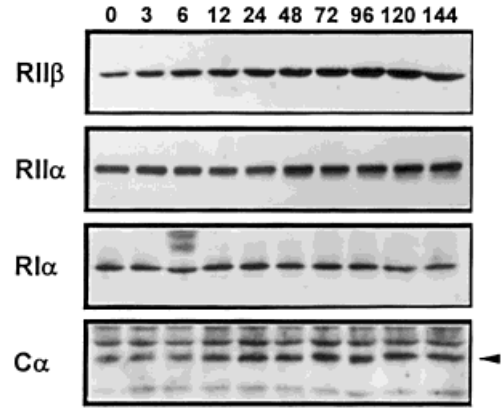

Fig. 5. Changes in protein levels of protein kinase A subunits by retinoic acid. Cells were treated with 10^{-6} M all-*trans* RA for indicated time (h) and total cell extract was prepared. Fifty micrograms of cell extracts were used for Western blot analyses. The bands for Cα were marked with arrowhead. The approximate sizes of each protein are 53 kDa for RIIβ, 52 kDa for RIIα, 49 kDa for RIα, and 40 kDa for Cα.

Effect of retinoic acid in RIIβ-overexpressing cells

RIIβ and Type II PKA is known to induce the growth inhibition and differentiation in cancer cells (Cho-Chung, 1990). In addition, overexpression of RIIβ in SK-N-SH neuroblastoma cells induced the inhibition of cell growth (Kim et al., 1996, 1997). Thus, we examined the role of the increase of RIIβ and type II PKA in the RA-induced growth inhibition and differentiation. To this end, we introduced the expression construct for RIIβ into SH-SY5Y cells and obtained clonal cells with high RIIβ levels. Through clonal selection, three clonal lines of two- to threefold higher levels of RIIβ compared to parental cells were selected and pooled for further characterization. The level of RIIβ protein in RIIβ-overexpressing cells was threefold higher than that in parental cells and was further increased by RA treatment (Fig. 6A). The growth of RIIβ-overexpressing cells was slower than that of parental cells and further decreased by RA treatment, showing the strong correlation of growth inhibition with the level of RIIβ (Fig. 6B). These data indicate that the up-regulation of RIIβ participate in the RA-induced growth inhibition.

However, RIIβ-overexpressing cells did not show neurite outgrowth that was seen in RA-treated cells (Fig. 6C). When RA was treated, RIIβ-overexpressing cells showed the same extent and time course of neurite outgrowth as parental cells (Fig. 6C). In addition, RIIβ-overexpressing cells were not different in the level of N-Myc and GAP-43 mRNA from the parental cells (data not shown). Thus, it is likely that increase of RIIβ/type II PKA is not sufficient to induce neuronal differentiation.

DISCUSSION

The role of PKA in the action of RA has been investigated previously in other systems. One of the most important studies was performed with PC12 cell lines. It was shown that RA could induce neuronal differentiation in mutant PC12 cells that are deficient in PKA but not in wild-type PC12 cells (Scheibe et al., 1991). Although it was not extensively examined, this inhibitory effect of PKA on RA-induced neuronal differentiation was ascribed to the suppression of RARs and cytoplasmic RA binding protein (CRABP) by PKA. The negative regulation of mRNA levels of RARs by the activation of PKA was also demonstrated in B16 melanoma cells (Xiao et al., 1996). These reports suggested that the catalytic activity of PKA may be inhibitory to RA-induced neuronal differentiation in relation to the suppression of RARs. However, a growing body of evidence shows that PKA isozymes play different roles in the modulation of intracellular signals and the regulation of cell proliferation and differentiation. Thus, we thought that the role of PKA in the action of RA should not be confined to the simple catalytic activity but might be extended to the differential functions of PKA isozymes.

In this study, we asked whether the differential modulation of PKA isozyme is involved in RA-induced growth inhibition and neuronal differentiation. SH-SY5Y cells responded to all-*trans* RA treatment by growth inhibition (Fig. 1) and neuronal differentiation (Fig. 2). Examination of neuronal differentiation markers revealed that the response to RA occurs as early as within 6 h of RA treatment (Fig. 2B and 2C). DEAE-cellulose chromatography and PKA assay showed that type II PKA is specifically increased in RA-treated cells (Fig. 3B). The increase of type II PKA is due to the increase of RIIβ and Cα (Fig. 5). 9-*cis* RA also showed a similar effect to all-*trans* RA on the cell growth, neuronal differentiation, and the induction of RIIβ (data not shown). These results show that RA-induced growth inhibition and neuronal differentiation occur with the increase of RIIβ/type II PKA.

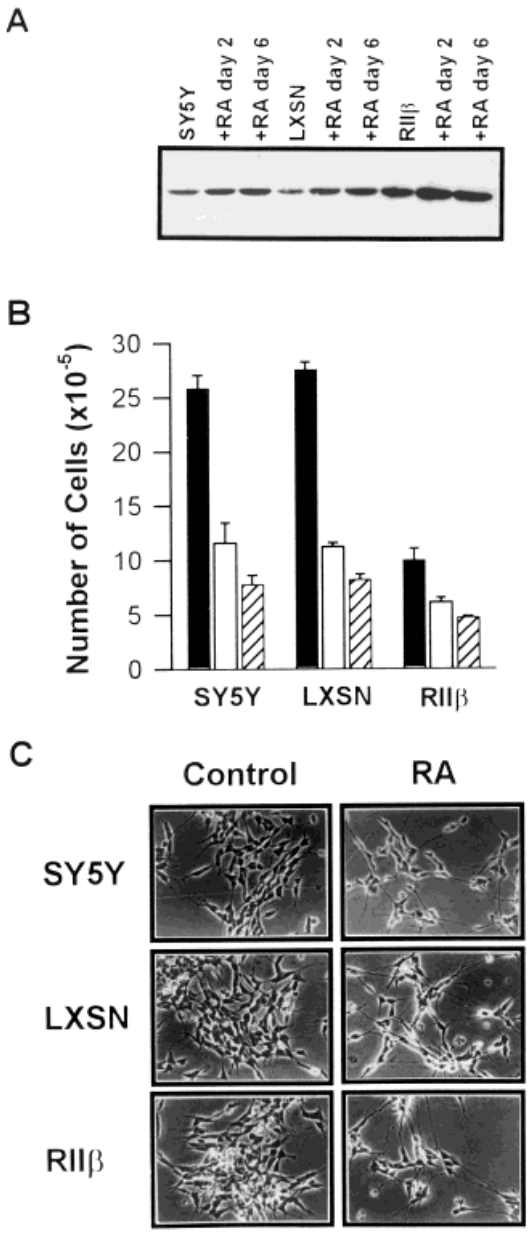

Fig. 6. Effects of retinoic acid in RIIβ-overexpressing cells. **A:** Protein levels of RIIβ in RIIβ-overexpressing cells. Parental (SY5Y), vector control (LXSN), and RIIβ-overexpressing cells (RIIβ) were treated with 10^{-6} M all-*trans* RA and cell extracts were obtained at days 0, 2, and 6. RIIβ protein levels were examined by Western blot analysis. **B:** Effect of RA on the growth of RIIβ-overexpressing cells. Cells were seeded at a density of 2×10^5 cells per 35-mm tissue-culture dish at Day −1. All-*trans* RA was treated at Day 0 and cell number was determined at Day 6. Closed bar, open bar, and hatched bar indicate nontreated cells, cell treated with 10^{-7} M and 10^{-6} M all-*trans* RA, respectively. Data show means of three separate experiments with SD. **C:** Comparison of cellular morphology of RIIβ-overexpressing cells with parental cells. Cells were cultured in the absence or presence of 10^{-6} M all-*trans* RA for 4 days. At both conditions, RIIβ-overexpressing cells did not show any morphological difference from parental or vector-introduced cells. Photographs were taken at the same magnifications (×300).

The specific increase of RIIβ/type II PKA with the growth inhibition and neuronal differentiation was interesting in that: (1) up-regulation of RIIβ induced growth inhibition in other cell lines including neuroblastoma cells (Tortora et al., 1994; Kim et al., 1996; Nesterova et al., 1996; Lee et al., 1999), (2) RIIβ is essential in the cAMP-induced differentiation of HL60 human leukemia cells (Tortora et al., 1990b), and (3) RIIβ knock-out mice showed abnormal motor behavior and neural functions, suggesting the importance of RIIβ in the neuron-specific function (Adams et al., 1997; Brandon et al., 1998). In addition, RIIβ was not induced in a variant of SK-N-SH, which is unresponsive to RA (data not shown). With this background, we further investigated the role of increased RIIβ in RA-induced growth inhibition and neuronal differentiation by overexpressing RIIβ in SH-SY5Y cells. We hypothesized that if the increase of RIIβ is sufficient to cause the RA-induced growth inhibition and neuronal differentiation, overexpression of RIIβ could mimic the effect of RA treatment.

The growth of RIIβ-overexpressing cells was much slower than that of parental cells and was comparable to that of RA-treated cells. In addition, the growth of RIIβ-overexpressing cells was further inhibited by RA treatment, which well correlates with the increase of RIIβ (Fig. 6A and 6B). Thus, the increase of RIIβ is, at least in part, accountable for the RA-induced growth inhibition. However, time-course experiments showed that the changes of differentiation markers precede or coincide with the increase of RIIβ (compare Fig. 2 with Figs. 4 and 5), suggesting the increase of RIIβ is not prerequisite to RA-induced neuronal differentiation. In accordance with this assumption, RIIβ-overexpressing cells did not show any sign of neuronal differentiation in cellular morphology (Fig. 6C) and the levels of neuronal differentiation markers (data not shown). In addition, parental and RIIβ-overexpressing cells showed similar response of neuronal differentiation to RA treatment, suggesting increase of RIIβ is not directly involved in the RA-induced neuronal differentiation.

It has been reported that RA induces the expression of TrkB in some neuroblastoma cells and enables BDNF-dependent neuronal differentiation (Kaplan et al., 1993). However, BDNF does not affect the growth of TrkB-expressing neuroblastoma cells and RA inhibits neuroblastoma cell growth even in the absence of functional TrkB (Kaplan et al., 1993; Matsumoto et al., 1995). Thus, it is likely that the neuronal differentiation and the growth inhibition induced by RA are regulated by separate mechanisms. Based on our present results, we propose that the increase of RIIβ and type II PKA is one of the main causes of RA-induced growth inhibition in neuroblastoma cells.

There are reports providing the explanations about the isozyme-specific functions of PKA. Type II regulatory subunits bind to a family of anchoring proteins (AKAPs) and specifically localize PKA holoenzyme near potential substrates (for review, see Lester and Scott, 1997), conferring specific functions on specific PKA isozymes. Type I regulatory subunits also bind to signal-transducing molecules, such as T-cell receptor complex and Grb2 (Skalhegg et al., 1994; Tortora et al., 1997). Thus, PKA isozymes may control different cellular processes through differential binding with cellular proteins. The specific role of RIIβ in the transcriptional control has been suggested such that the introduction of RIIβ, but not RIα, could restore CRE (cAMP-response element)-dependent transcription in

cAMP-unresponsive PC12 cell line (Tortora et al., 1990a). RIIβ protein may also act as a transcription factor acting to CRE, suggesting alternative function of PKA (Srivastava et al., 1998). Considering these previous reports, it is likely that each PKA isozyme plays a separate role through the action of its specific regulatory subunits, RI and RII.

In the present study, we have shown that RIIβ and type II PKA increase with RA-induced growth inhibition and neuronal differentiation of SH-SY5Y cells. We found that the RA-induced growth inhibition well correlates with the level of RIIβ, and overexpression of RIIβ could mimic the RA-induced growth inhibition. However, increased RIIβ is not likely to be directly involved in the RA-induced neuronal differentiation. Although the role of increased RIIβ/type II PKA such as in the transcriptional control of specific genes could be inferred, the identification of the specific biochemical events induced by the change of PKA isozyme pattern awaits further investigation. Taken together, our results show that PKA participates in the RA-induced intracellular signaling leading to cell-growth inhibition through RIIβ/type II PKA isozyme. These results also point to the possibility that the signals mediated by PKA isozymes may function in the transduction and the modulation of other intracellular signals. In this light, it would be interesting to test whether the growth inhibition and neuronal differentiation by other differentiation inducers, including NGF and PMA, is also associated with the function of type II PKA.

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