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Cholinergic differentiation of cultured sympathetic neurons induced by retinoic acid

Induction of choline acetyltransferase-mRNA and suppression of tyrosine hydroxylase-mRNA levels

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Abstract

Here we show that retinoic acid (RA) has the ability to alter the transmitter phenotype of cultured sympathetic neurons from newborn rats superior cervical ganglia (SCG). In the presence of RA, the level of choline acetyltransferase (ChAT) mRNA was increased, while the level of tyrosine hydroxylase (TH) mRNA was reduced in the cultured SCG neurons. Selective PCR amplification of different upstream regions of the ChAT-mRNA indicates that RA promotes the transcription of ChAT gene from R and M exons. The RA-induced upregulation of ChAT-mRNA level was significantly diminished by the chronic treatment with phorbol ester, suggesting that PKC has an important role in the induction of ChAT-mRNA in this system.

Key words: Transmitter phenotype; Sympathetic neuron; Choline acetyltransferase; Tyrosine hydroxylase; Retinoic acid; Polymerase chain reaction

1. Introduction

The neurotransmitter phenotype is determined at certain stages during neuronal differentiation [1–3] and it has been shown that environmental signals play an important role in this determination. For example, when grown in the presence of nonneuronal cells such as heart myocytes and ganglionic nonneuronal cells, adrenergic sympathetic neurons dissociated from the superior cervical ganglia (SCG) of newborn rats can be induced to become cholinergic [2]. Leukemia inhibitory factor (LIF) purified from the heart cell conditioned medium [4] and ciliary neurotrophic factor (CNTF) [5] also have the ability to induce cholinergic phenotype.

Another regulator of neural differentiation which may influence the transmitter phenotype is retinoic acid (RA), a natural derivative of vitamin A. For example, the localization of receptors and binding proteins for RA suggest its regulatory role in the development of neural structure in the central nervous system in vivo [6]. Complementarily, in vitro studies indicate that RA induces embryonal carcinoma cells to differentiate into muscle, glial and neuronal (including cholinergic phenotype) cells in a concentration dependent manner [7,8]. In a previous paper we showed that the exposure of clonal rat pheochromocytoma cells (PC12) to low concentrations of RA increased the activity of choline acetyltransferase (ChAT), a marker enzyme of cholinergic neurons, concomitantly with the suppression of the activity of tyrosine hydroxylase (TH), a marker enzyme of adrenergic neurons [9]. In the present study, to further investigate whether the effects of RA on the clonal cells reflect the action of RA on the transmitter choice of the developing neuron in vivo, we analyzed the effects of RA on the expression of ChAT- and TH-mRNAs in primary cultured SCG neurons. The results demonstrate that RA induces the cholinergic differentiation of SCG neurons and strengthen its possible in vivo role in the determination of transmitter phenotype.

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Abbreviations. ChAT, choline acetyltransferase; TH, tyrosine hydroxylase; RA, retinoic acid; CNTF, ciliary neurotrophic factor; LIF, leukemia inhibitory factor; TPA, phorbol 12,13-myristate acetate; PKC, protein kinase C; SCG, superior cervical ganglion; RT-PCR, reverse transcription-polymerase chain reaction.

2. Materials and methods

2.1 Cell culture

Adrenergic sympathetic neurons were obtained essentially as described by Saadat et al. [5]. Superior cervical ganglia (SCG) were dis-

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sected from postnatal day 2 rats, treated with 3 mg/ml collagenase (Sigma) in Ca^{2+}/Mg^{2+}-free PBS for 30 min and further incubated with 2 mg/ml trypsin (Difco) for 30 min. Dissociated cells were plated on poly-D-lysine/laminin coated 35 mm dishes at a density of 5 ganglia per dish. Cells were fed with Ham's F-12 medium (Sigma) containing 40 ng/ml NGF (Promega), 0.1 mg/ml BSA, antibiotics (50 U/ml penicillin and 0.1 mg/ml streptomycin), and N2 supplement (5 μg/ml insulin, 100 μg/ml transferrin, 20 nM progesterone, 30 nM selenium, 100 μM putrescine) [10]. Culture medium was changed every 3 days. The cells were treated with 10 μM Ara-C (Sigma) for 72 h from the day following plating to prevent proliferation of nonneuronal cells. All-*trans* retinoic acid (Sigma; simply abbreviated as RA in the following text) was added into the medium from one day after plating of the cells and replenished at the time of medium change. The effects of 9-*cis* RA (generous gift from Dr. H. Kagechika and Dr. K. Shudo) and 13-*cis* RA (Sigma) were also examined. These RA isomers were dissolved in ethanol, and the volume of ethanol added into the culture medium was controlled so as not to exceed 0.2%.

2.2. RNA preparation and Northern blot analysis

The total RNA was prepared from SCG neurons according to the method of Chomzynski and Sacchi [11] with slight modification. For the precipitation of the total RNA from a small number of cells, a high molecular weight acrylamide compound (Ethachinmate, Nippon Gene, Toyama) was added to the phenol extracts of SCG neurons as carrier. Aliquots of the total RNA samples were glyoxylated and electrophoresed on a 1.5% agarose gel, and then transferred to a nylon membrane (Hybond-N, Amersham). The membranes were fixed by UV irradiation, prehybridized and hybridized with ^{32}P-labeled TH-cRNA probe (transcribed from a rat TH-cDNA plasmid which was a generous gift from Dr. H. Fujisawa and Dr. S. Okuno) or ChAT-cRNA probe (transcribed from a rat ChAT-cDNA plasmid) at 65°C in 50% formamide. Radioactivity bound to TH-mRNA and ChAT-mRNA was quantified with Bio-Image-Analyzer (FUJI).

2.3. RT-PCR

Although RA-dependent changes were observed on both TH- and ChAT-mRNA levels in cultured SCG neurons by Northern blot analysis, the levels of ChAT-mRNA were too low to be analyzed quantitatively. Therefore, we analyzed the levels of ChAT-mRNA quantitatively by reverse transcription-polymerase chain reaction (RT-PCR). Aliquots (0.3 μg) of the total RNA samples were treated with 0.6 U/μl DNaseI (Takara) at 37°C for 15 min. Single strand cDNA was synthesized with 0.5 μM random hexamer and 0.25 U/μl AMV-RT (Life Sciences Inc.) at 42°C for 45 min. The reaction mixture for PCR amplification was prepared using 10 × PCR buffer (Promega), and contained 0.5 μM each of ChAT primer pair, 0.2 mM each dNTP, 50 nCi/μl [α-^{32}P]dCTP and 0.02 U/μl Taq DNA Polymerase (Promega). For the amplification of the ChAT coding region, samples were subjected to total of 36–39 cycles of PCR according to the following scheme: 94°C for 50 s, 55°C for 1 min, 72°C for 1 min. After 16 cycles, 0.5 μM each of β-actin primer pair was added to the reaction mixture and the PCR cycles were further continued. The amplified PCR products were electrophoresed on a 8% polyacrylamide gel and the radioactivity incorporated into ChAT (330 bp) and β-actin (218 bp) cDNA bands was quantified with a Bio-Image-Analyzer. The primer sequences used for the amplification of the coding regions of ChAT and β-actin were as follows: ChAT CA3, 5'-CAAGGCCATCTC GCCTCCTCAGCA-3'; ChAT CA8, 5'-CTGCAGCAGCTGCAGTTTCTCAGA-3'; β-actin BA5, 5'-AAGAGAGGCATCCTGACCCT-3'; β-actin BA3, 5'-TACATGGCTGGGGTGTTGAA-3'.

For the amplification of 5'-ends of ChAT-mRNA, the total RNA samples were reverse transcribed and subjected to 45 cycles of PCR in the absence of a radio-labeled nucleotide according to the following scheme: 94°C for 40 s, 65°C for 2 min, 72°C for 3 min. After amplification, the samples were electrophoresed on 2% agarose gel and visualized by staining with ethidium bromide. The primer sequences used for the amplification of 5'-ends of ChAT-mRNA were as follows: 5'-Exon-specific upstream primers; R, 5'-TCTGTTCAGCCTGTCGCTGCAAG-3'; N, 5'-TAGTCCCGTCTTTTAGGGTCCTGGCCT-3'; M, 5'-AGTCGAGAGAGGTGTGGCTGGTTT-3'. Common downstream primer: REV, 5'-GCTCTTCCTGAACTGCTCTTCAGGTACCA-3'. The designations of the upstream primers correspond to those of 5'-exons of rat ChAT gene by Kengaku et al. [12]

3. Results and discussion

3.1. Quantitation of ChAT-mRNA expressed in cultured SCG neurons

In order to quantify low levels of ChAT-mRNA expression in cultured SCG neurons, we employed quantitative RT-PCR. When a range of amplification cycles between 35 and 40 cycles were tried, logarithmic amplifications of both ChAT-cDNA and β-actin-cDNA (as an endogenous control) were detected with the total RNA samples prepared from SCG neurons cultured in the absence or presence of 10^{-10} M RA for 6 days (Fig. 1). Although the levels of ChAT-cDNA amplification increased by the RA-treatment, the levels of β-actin-cDNA amplification were not changed significantly by the RA-treatment, indicating that the amplifications of ChAT- and β-actin-cDNAs did not interfere with each other (Fig. 1, see also Fig. 2C). However, the levels of β-actin expression decreased significantly over the culture period concomitantly with the differentiation of SCG neurons, as revealed by Northern blot analysis (data not shown). Therefore, in the following experiments, the levels of ChAT-mRNA expression were first calculated as the ratios of radioactivity of the ChAT PCR products to that of the β-actin PCR products, then normalized with the relative levels of β-actin expression measured by Northern blot analysis and finally expressed as relative values per μg of total RNA.

3.2. Effects of RA on ChAT- and TH-mRNA levels

The treatment of dissociated SCG neurons with RA up to 10^{-6} M for 6 days brought about an elevation of the ChAT-mRNA level and a parallel suppression of the TH-mRNA level in a concentration dependent manner (Fig. 2A,B). Significant effects of RA on the levels of

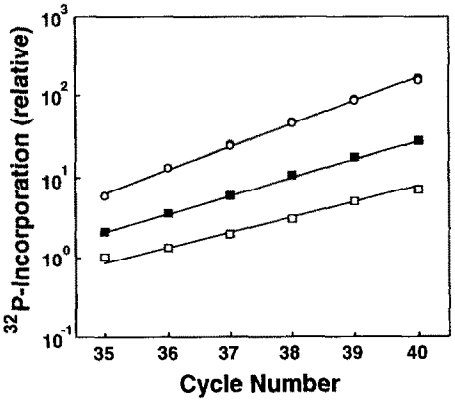

Fig. 1. Quantitation of ChAT-mRNA in the cultured SCG neurons. RT-PCR was carried out on 0.3 μg of the total RNA from SCG neurons cultured in the absence (open symbols) or presence (closed symbols) of 10^{-10} M RA for 6 days with ChAT and β-actin primers with inclusion of ^{32}P-labelled dCTP. Samples were taken out after the indicated number of cycles and the level of radioactivity of electrophoretic ChAT-cDNA (square) and β-actin-cDNA (circle) bands were quantified with Bio-Image-Analyzer.

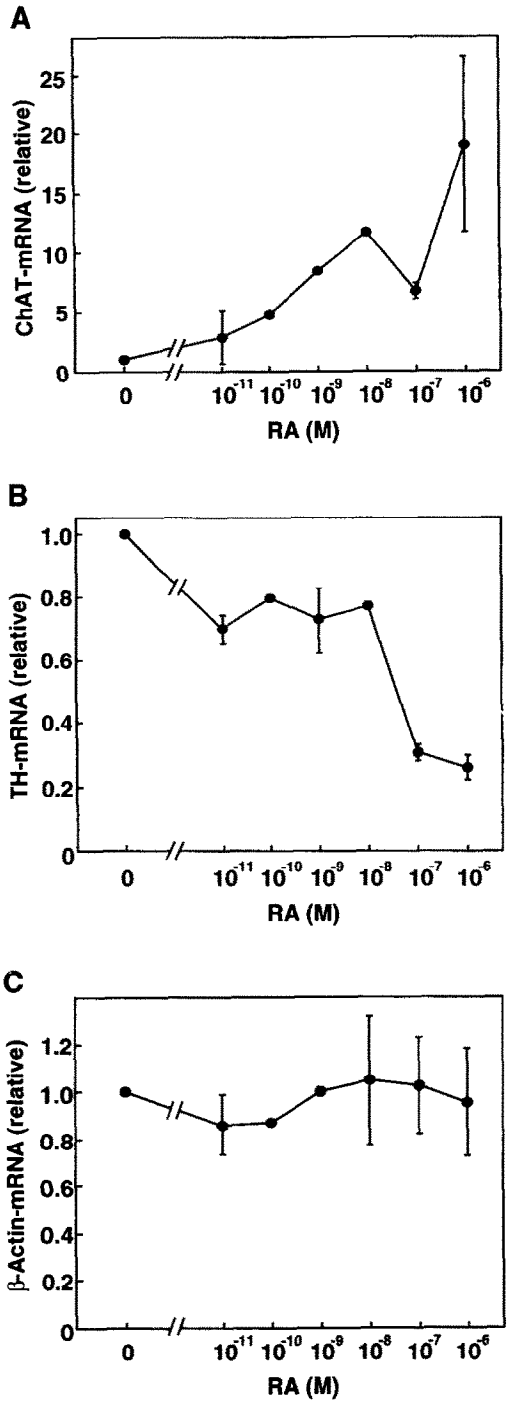

Fig. 2. Effects of RA on ChAT-, TH- and β-actin-mRNA levels in the cultured SCG neurons. SCG neurons were cultured for 6 days in the presence of indicated concentrations of RA. Then, the total RNA was prepared and subjected to quantitation of ChAT-mRNA (A: RT-PCR) and TH- and β-actin-mRNA (B and C: Northern blot analysis) levels. Points represent the mean and range of two independent cultures.

ChAT- and TH-mRNAs of cultured SCG neurons were detectable with a RA concentration as low as 10^{-11} M. The treatment of SCG neurons with 10^{-6} M RA increased the ChAT-mRNA level 18-fold but suppressed the TH-mRNA level to 25% of that of the control culture. In contrast, the level of β-actin-mRNA was not significantly changed by RA-treatment up to 10^{-6} M, suggesting specific actions of RA on the ChAT and the TH gene expressions (Fig. 2C). Cell viability and amount of the total RNA extractable from the cultured SCG neurons was also not changed significantly by treatment with RA up to 10^{-6} M. However, neither a plateau of ChAT-mRNA induction nor complete suppression of TH-mRNA by RA was obtained, since RA above 10^{-6} M exerted significant toxicity on the culture of SCG neurons.

The effects over time of RA on the expressions of ChAT- and TH-mRNAs in the cultured SCG neurons were assessed in Fig. 3. In the absence of RA, the TH-mRNA level increased 2.5-fold between day 1 and day 13, but the ChAT-mRNA level did not change significantly during this period. When 10^{-7} M RA was added to the culture of SCG neurons at day 1, TH-mRNA level began to decrease and reached 50% of the original level (20% of that of the control culture) at day 13. On the other hand, ChAT mRNA level in the RA-treated culture continued to increase over the two-week culture period. The induction of ChAT-mRNA and the suppression of TH-mRNA were detectable with RA treatment at 10^{-7} M after as little as 3 days.

RA added at the initial stage of culture (day 1) might exert its effect primarily on the nonneuronal cells and then subsequently affect the ChAT- and TH-mRNA levels in neuronal cells, since there existed a number of contaminating nonneuronal cells in the initial stage of culture. Therefore, we examined the effects of RA added on day 7 when virtually all the nonneuronal cells were eliminated by Ara-C treatment. As shown in Fig. 3, 10^{-7} M RA added on day 7 increased and suppressed the ChAT- and TH-mRNA levels, respectively, to extents similar to the effects of RA added on day 1, suggesting that RA acts directly on neurons to alter their transmitter phenotype. It is also suggested that SCG neurons do not lose their plasticity of transmitter phenotype during the culture period of at least 1 week although their adrenergic phenotype was further strengthened during this period.

Our results of the RA action on the cholinergic gene regulation of the cultured SCG neurons are consistent with our previous observation on PC12 cells [9] and with a recent report that RA increases the specific activity of ChAT and suppresses the specific activity of TH in the cultured SCG neurons [13]. Selective action of RA on enhancement of the cholinergic properties has been also reported with spinal cord neurons in primary culture [14]. Considering these findings, our observation suggests that RA can promote cholinergic differentiation in certain in vivo situations during neural development.

3.3. Mechanism of cholinergic differentiation by RA

It has been shown that there are at least five different mRNA species for ChAT, each differing in their 5′-non-

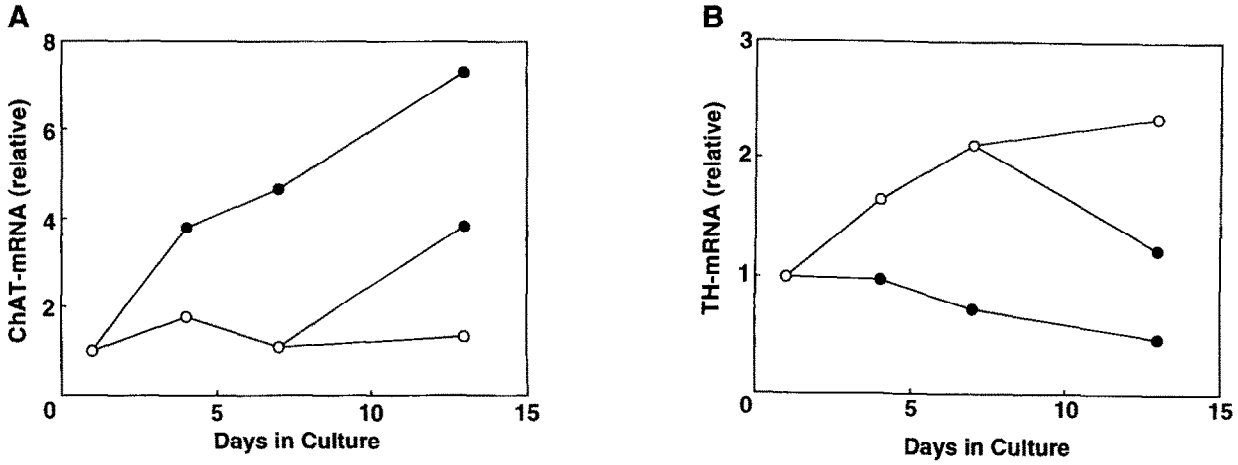

Fig. 3. Time course of the effects of RA on ChAT- (A) and TH-mRNA (B) levels. RA (10^{-7} M) was added to SCG neurons at 1 or 7 days after plating. Then, neurons were cultured for the indicated periods (closed symbols). ChAT- and TH-mRNA levels in the control culture without RA are shown with open symbols.

coding sequences, in rat spinal cord [12]. The multiple ChAT-mRNA species are thought to be generated by the combination of the use of different promoter regions and alternative splicing. We attempted to identify species of ChAT-mRNA specifically induced by RA in the cultured SCG neurons. Therefore, we synthesized oligonucleotide primers specific to different 5′-noncoding exons and performed RT-PCR with the total RNA samples from the cultured SCG neurons (Fig. 4). In the control culture, without the RA-treatment, only the 328 bp band corresponding to N2-type ChAT-mRNA was detected. In the culture of SCG neurons treated with 10^{-8} M RA for 6 days, the 262 and 406 bp bands corresponding to R2- and M-type mRNA, respectively, were detected. This result indicates that the RA-treatment enhances the ChAT gene transcription from the two different promoter regions around R and M exons. Although the presence of a responsive element for nuclear retinoid receptors is not known, the presence of a number of other transcriptional regulatory sites has been reported upstream of R exon and downstream of M exon [15,16]. Future study on the analysis of the sequence elements around R and M exons mediating the RA-action would identify factors involved in the RA-induction of ChAT-mRNA.

In the previous study, we demonstrated that the induction of cholinergic phenotype in neuronal cell lines was a specific action of RA among retinoid derivatives including retinol and retinal [9]. In order to analyze the mechanism of the alteration of transmitter phenotype by RA, it is important to identify the primary target of RA action. It is now generally accepted that RA exerts its effects on gene expression through two types of nuclear retinoid receptors, i.e. RARs and RXRs. RARs recognize all-*trans* RA (RA) and other isomers of RA such as 9-*cis* RA and 13-*cis* RA with similar affinities, while RXRs are activated selectively by 9-*cis* RA [17,18]. Although the expression of RARs or RXRs in rat SCG is not known, temporal expression of RXRβ in the neural crest of chick embryo has been reported [19]. The concentration of RA required to change ChAT- and TH-mRNA levels in SCG neurons (Fig. 2) roughly coincides with that required for activation of nuclear retinoid receptors [17,18]. Therefore, we attempted to elucidate the type of nuclear retinoid receptor which regulates the transmitter phenotype in the cultured SCG neurons by comparing the actions of RA isomers at the concentrations exerting submaximal effects. Thus, 9-*cis* RA and 13-*cis* RA added at 10^{-10} M and 10^{-8} M increased ChAT-mRNA and suppressed TH-mRNA to extents similar to

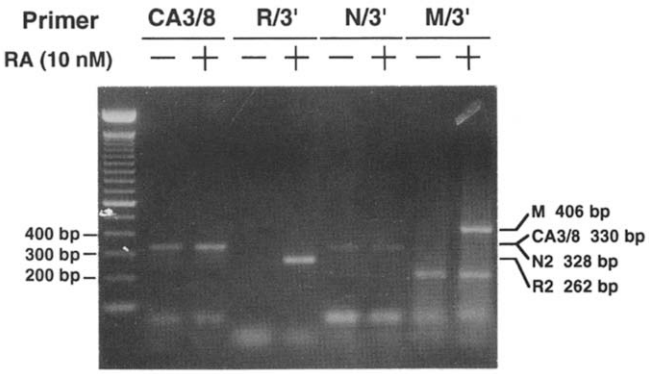

Fig. 4. PCR amplification of 5′-regions of ChAT-mRNA from the cultured SCG neurons. SCG neurons were cultured in the absence or presence of 10^{-8} M RA for 6 days. Then, the total RNA was prepared and 5′-regions of ChAT-mRNA were amplified by RT-PCR using the primer pairs each consisting of a common 3′-primer (REV) and a different 5′-primer corresponding to 5′-exons (R, N, and M) of rat ChAT gene. Coding region of ChAT-mRNA (330 bp) was also amplified with CA3 and CA8 primers. After separation on a 2% agarose gel, the amplification products were stained with ethidium bromide. Expected positions of various types of ChAT-mRNA species are indicated on the right side of the gel; M (406 bp), N2 (328 bp), R2 (262 bp).

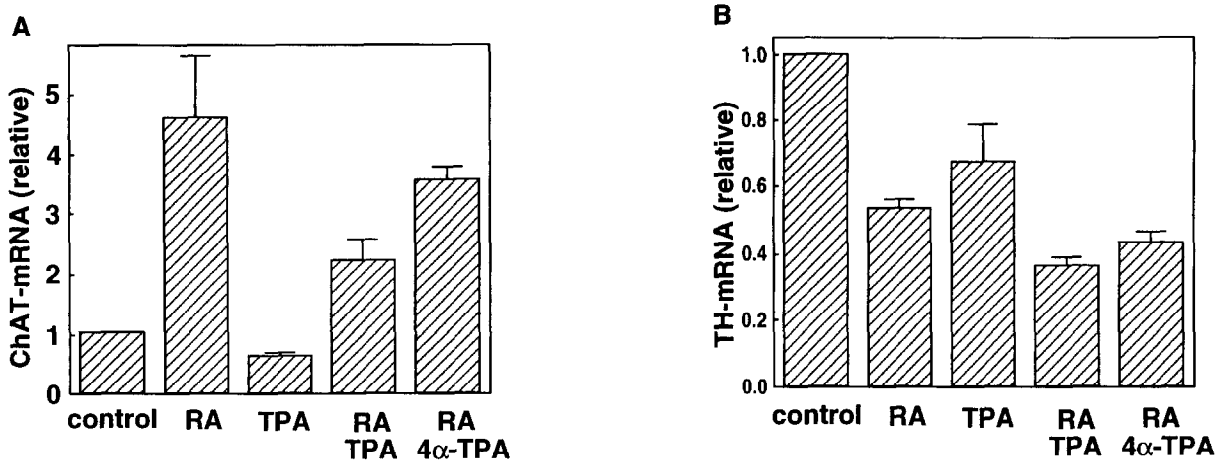

Fig. 5. Effects of chronic treatment of the SCG neurons with phorbol esters on the ChAT- and TH-mRNA levels. SCG neurons were cultured for 6 days in the presence of the indicated combinations of 10^{-7} M RA, 5 nM TPA and 5 nM 4α-TPA. Neurons were then harvested and subjected to the quantitation of ChAT- (A) and TH-mRNA (B) levels. Data represent the mean and the range of the two independent cultures.

those affected by all-*trans* RA (RA) (data not shown). This result suggests that the dominant involvement of RXRs over RARs in controlling transmitter phenotype in SCG neurons by RA is unlikely and rather suggests that RARs take part in the regulation of the transmitter related genes in this system. For further identification of the responsible receptor type in the action of RA, one has to examine the expression of a particular type of RARs in SCG neurons. Detection of the expression of RARs/RXRs in SCG in any particular developmental stage would also establish the in vivo role of RA in the development of sympathetic neurons.

Besides RA, several peptide factors such as CNTF and LIF are known to induce cholinergic differentiation in the cultured SCG neurons [4,5]. Although RA, which binds to the nuclear retinoid receptor, and CNTF/LIF, which binds to specific receptors associated with common receptor subunit, gp130, on the plasma membrane, seem to have different upstream mechanisms of their actions, there still remains the possibility that they also share a common downstream mechanism of inducing cholinergic properties. Recently it was reported that the induction of cholinergic properties in SCG neurons by CNTF and LIF is mediated by the activation of protein kinase C (PKC) [20]. Therefore, we also examined the effect of chronic treatment of SCG neurons with phorbol 12,13-myristate acetate (TPA), which was shown to deplete PKC activity in the cultured SCG neurons [20], on the ChAT- and TH-mRNA levels affected by RA. As shown in Fig. 5A, The treatment of SCG neurons with 5 nM TPA alone for 6 days did not change the ChAT-mRNA level significantly. But, TPA added in combination with 10^{-7} M RA inhibited the RA-induced ChAT-mRNA level, while 4α-TPA, which does not act on PKC, was less effective than TPA in suppressing the RA-induced ChAT-mRNA level. These results suggest that the induction of ChAT-mRNA in the cultured SCG neurons is also mediated by a PKC-dependent process and is consistent with our previous result that H-7, an inhibitor of PKC, abolished the RA-induced ChAT activity in PC12 cells [21]. However, the chronic treatment with TPA alone slightly suppressed the TH-mRNA level. The combination of TPA and RA suppressed the TH-mRNA level in an additive manner suggesting that the activation of PKC is not necessary for TH-mRNA suppression by RA.

In the present study, we showed that RA regulates the expressions of ChAT and TH genes in a mutually exclusive manner. Direct activation of transcription of the ChAT gene is less likely, since a typical retinoid receptor responsive element has not been found on the identified promoter/enhancer regions of ChAT gene. It is rather tempting to assume that, in response to RA, the nuclear retinoid receptor activates the expression of its target gene whose product functions as a transcription factor for ChAT gene. Although activation of a nuclear retinoid receptor by PKC is rather unlikely, it is plausible that the putative target gene of this receptor is activated by PKC. Such a putative target gene of RA action may also serve as the target gene for the actions of CNTF and LIF [20] and thus, may have key roles in cholinergic differentiation.

On the other hand, recent observations have indicated that RA downregulates the expression of target genes by blocking transcriptional activation at an AP-1 site on their promote [22–24]. These observations raise a possibility that a RA-retinoid receptor complex blocks the activity of the AP-1 site on the TH-promoter which has been shown to be involved in the PKC-mediated activation of TH gene expression [25]. Direct binding of a RA-retinoid receptor complex to an AP-1 site is rather controversial [22–24]. Alternatively, a RA-retinoid re-

ceptor complex would activate the transcription of the target gene whose product functions as an negative regulator of TH gene transcription. It is tempting to speculate that a positive transcription factor for ChAT gene induced by RA also functions as the negative regulator for TH gene, which would explain the mutually exclusive expression of these two genes in neuronal cells. Our current efforts are concentrated on identifying such a target molecule of the RA-action in the cholinergic differentiation of neuronal cells [26].

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