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Retinoic Acid and Its Geometrical Isomers Block Both Growth and Fusion of L6 Myoblasts by Modulating the Expression of Protein Kinase A

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All-*trans* retinoic acid (RA) and its geometrical isomers, such as 9-*cis* RA, 13-*cis* RA, and 9,13-di-*cis* RA, strongly inhibited both growth and fusion of L6 myoblasts. However, illumination of white light diminished their inhibitory activity on membrane fusion with little effect on cell growth. During myogenic differentiation, the intracellular level of cAMP decreased whereas the total activity of protein kinase A as well as the protein level of its regulatory subunit Iα (RIα) and catalytic subunit (Cα) increased. RAs raised the intracellular level of cAMP by over 3-fold, but decreased the total activity of protein kinase A. Like RAs, dibutyryl-cAMP inhibited myoblast fusion and reduced the expression of both RIα and Cα subunits. These results suggest that RAs negatively modulate the differentiation of L6 myoblasts by increasing the intracellular level of cAMP, which may in turn down-regulate the expression of protein kinase A and hence its activity.

Keywords: cAMP; L6 Myoblasts; Membrane Fusion; Myogenesis; Protein Kinase A; Retinoic Acid.

Introduction

Myogenic differentiation of skeletal muscle cells is characterized by membrane fusion of mononucleated myoblasts into multinucleated myotubes. Concurrent with the morphological differentiation, muscle-specific proteins, such as myosin heavy chain, α-actin, acetylcholine receptor, and creatine kinase, are synthesized and accumulated during the differentiation process (Olson *et al*., 1995; Park *et al*., 1992; Wakelam, 1985). This myogenic differentiation of muscle cells largely depends on their environment, such as growth factors and hormones (Florini *et al*., 1991). cAMP is one of the important intracellular second messengers that is involved in the control of cellular events in response to external signals. It has been previously reported that the intracellular level of cAMP changes during differentiation of myoblasts (Zalin, 1973) and that high concentration of cAMP impairs myogenic differentiation (Baek *et al*., 1994; Winter *et al*., 1993).

Effects of cAMP are generally mediated by a cAMP-dependent protein kinase, namely protein kinase A (PKA). During differentiation of myoblasts, the PKA activity rises with simultaneous increase in the extent of membrane fusion (Adamo *et al*., 1989; Rogers *et al*., 1985). Retinoic acid (RA), a major metabolite in the physiological pathway of retinol metabolism, has been shown to increase the activity of PKA in psoriatic fibroblasts (Raynaud *et al*., 1988) and tumoral cells (Ludwig *et al*., 1980; Plet *et al*., 1982). It is well known that RA regulates growth and differentiation of many cell types and tissues (De Luca 1991; Sporn and Roberts, 1983), although its effect is rather different depending on cell types even in related cell lines. In the myogenic differentiation of C2 and rat rhabdomyosarcoma cell lines, RA promotes growth arrest and induces differentiation (Alric *et al*., 1998; Arnold *et al*., 1992; Froeschle *et al*., 1996; Gabbert *et al*., 1988). On the other hand, all-*trans* RA inhibits not only thymidine

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Abbreviations: Cα, catalytic subunit; PKA, protein kinase A; RA, retinoic acid; RIα, regulatory subunit Iα.

incorporation but also membrane fusion of chick embryonic myoblasts (Kim *et al*., 1995). Two families of transcriptional factors mediating signals of RA are reported: retinoic acid receptors (RARs) and retinoid X receptors (RXRs). RARs are activated by all-*trans* RA and 9-*cis* RA, whereas RXRs are by 9-*cis* RA only (Allegretto *et al*., 1993; Allenby *et al*., 1993; Levin *et al*., 1992).

In an attempt to elucidate the mechanism of how RA regulates myogenic differentiation, we examined the effect of all-*trans* RA and its geometrical isomers, including 9-*cis* RA, 13-*cis* RA, and 9,13-di-*cis* RA, on growth and fusion of L6 myoblast cells. We also examined the effects of RAs on the expression of PKA as well as on the changes in its enzyme activity.

Materials and Methods

Cell culture L6 rat myoblasts were plated on plastic tissue culture dishes at a concentration of 1.5×10^4 cells/ml in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum (Gibco). Three days after cell seeding, the culture medium was changed to a differentiation medium, which contained 5% horse serum in place of 10% fetal bovine serum. The time of the medium change was regarded as 0 h of the culture, and RAs were added to the culture medium at this time of medium change. All-*trans* RA, 9-*cis* RA, and 13-*cis* RA were purchased from Sigma, and 9,13-di-*cis* RA was a gift from Dr. Akira Murayama (Tokyo Metropolitan Institute of Medical Science, Japan). All RAs were dissolved in absolute ethanol and kept in the dark. RA-treated cells were cultured in a dark incubator. When needed, cells were illuminated under white light (2,000 Lux) for 15 min, and cultured without shielding from light thereafter. After the culture, the cells were washed with ice-cold phosphate buffered saline (PBS) and stained with hematoxylin solution. Extents of myoblast fusion were expressed as percentages of the number of nuclei in fused cells compared to the total number of nuclei in 10 randomly chosen fields observed under a microscope. Cells containing more than three nuclei were regarded as fused cells.

cAMP assay Cells cultured with or without RAs were washed three times with PBS, harvested by centrifugation, and kept at −70°C until use. Cells were disrupted by sonication in the assay buffer containing 50 mM sodium acetate (pH 5.8), 0.02% bovine serum albumin, 0.01% preservative, and 1 mM 3-isobutyl-1-methyl-xanthine. Soluble proteins were obtained by centrifugation of the cell lysates at $15{,}000 \times g$ for 30 min. Aliquots of the samples (0.1 mg) were then subjected to cAMP assay using the cAMP EIA system (Amersham) by following the manufacturer's instructions. Proteins were assayed as described (Bradford, 1976).

Assay of the PKA activity The activity of PKA was measured according to Fujimori *et al*. (1992) with slight modifications. Cells were harvested and disrupted by sonication in 50 mM Tris·HCl (pH 7.5), 1 mM dithiothreitol, and 10 mM $MgCl_2$. After centrifugation at $15{,}000 \times g$ for 30 min, aliquots of the supernatants (50 μg protein) were added with 50 mM kemptide as a substrate in the presence or absence of 10 μM cAMP. The reaction was initiated by incubation with 1.5 μCi [γ-^{32}P]ATP (New England Nuclear) for 5 min at 36°C, and terminated by immediately transferring the tubes into ice-water. The samples (40 μl) were then spotted on phospho-cellulose papers, and washed six times with 1% phosphoric acid. The radioactivity remaining on the papers was then determined using a liquid scintillation counter.

Immunoblot analysis After appropriate culture periods, the cells were washed three times with ice-cold PBS, harvested by centrifugation, and disrupted by sonication in 50 mM Tris·HCl (pH 7.4), 5 mM $MgCl_2$, and 0.5 mM EDTA. The cell lysates were then subjected to electrophoresis on 10% polyacrylamide gels containing SDS (Laemmli, 1970). The proteins in the gel were transferred onto polyvinylidene fluoride membranes (PVDF, Millipore), and reacted with antibodies raised against RIα, RIIα, or Cα of PKA and then with an anti-rabbit IgG conjugated with horseradish peroxidase (Sigma). Antisera to the subunits of PKA were kindly provided by Dr. S. H. Hong (Seoul National University, Korea). The proteins in the membranes were then visualized using an enhanced chemiluminescence detection system (ECL, Amersham).

Data analysis Statistical analysis was performed by one-way ANOVA. The critical value for significance was determined at $P < 0.05$. All data are expressed as the mean ± standard deviation (SD).

Results

Effects of RAs on growth and fusion of L6 myoblasts We have previously shown that all-*trans* RA blocks both proliferation and membrane fusion of chick embryonic myoblasts (Kim *et al*., 1995). However, there is still a controversy regarding whether RA can inhibit or induce myogenic differentiation, depending on the cell type used. Therefore, under dark conditions, we examined the effects of RA and its geometrical isomers, 9-*cis* RA, 13-*cis* RA, and 9,13-di-*cis* RA, on the myogenic differentiation of rat L6 myoblasts. All of the RAs at 50 μM concentration strongly inhibited cell proliferation as well as membrane fusion (Figs. 1A and 1B, respectively). Among the RAs treated, the inhibitory effect of 9-*cis* RA on the fusion was the highest. Under the same experimental conditions, only about 20% of the cells treated with 9-*cis* RA fused to form myotubes, whereas more than 80% of the untreated control cells completed the fusion (Fig. 1B).

Recently, it has been reported that the composition of RA and its geometrical isomers rapidly change by photoisomerization reaction under natural light (Murayama *et al*., 1997). Therefore, we examined whether illumination might influence the inhibitory effects of RAs on the growth and fusion of L6 myoblasts. Without treatment with RAs, light showed little or no effect on growth and fusion of the cells (Fig. 1). In addition, the inhibitory effects of RAs on

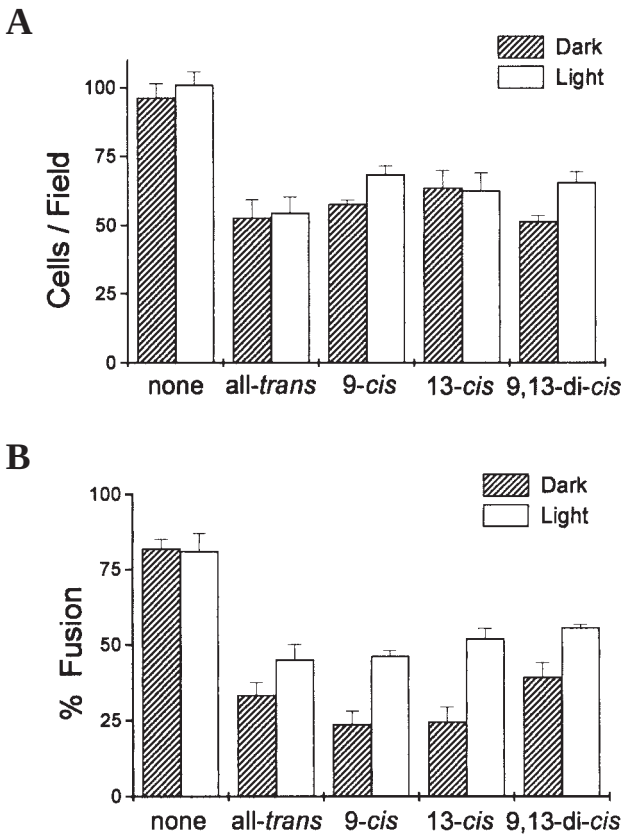

Fig. 1. Effects of RA and its geometrical isomers on growth (**A**) and fusion (**B**) of L6 myoblasts. At the time of the medium change, cells were treated with 50 μM of RA or its geometrical isomers, and cultured for 72 h in the absence or presence of light as described in Materials and Methods. After the culture, cells were stained with hematoxylin solution and observed under a microscope (× 200). Number of nuclei in randomly chosen 10 fields were counted, and their averages were expressed as growth index (**A**). Fusion index was defined as the percentages of nuclei in myotubes compared to total nuclei in the same field (**B**). The values shown are averages (mean ± SD) of one representative experiment ($P < 0.001$ by ANOVA). The same work was repeated three times.

cell growth were not influenced by illumination (Fig. 1A). On the other hand, illumination significantly diminished the inhibitory effects of RAs on membrane fusion, although it could not completely reverse their effects. It has been shown that illumination generates equilibrium mixtures of various isoforms of RAs (Murayama *et al.*, 1997). Accordingly, the inhibitory effects of RAs became approximately the same upon illumination. Thus, it appears that the RA receptors in myoblasts show different affinity to RA isomers at least for the signaling pathway linked to the fusion process. These results clearly show that RAs negatively modulate membrane fusion of rat L6 myoblasts.

Changes in the PKA activity and cAMP level during myogenesis To examine whether the level of cAMP may change during differentiation of L6 myoblasts, cells were harvested and assayed for cAMP level at every 24 h after medium change with differentiation medium. As shown in Fig. 2A, the level of cAMP dramatically decreased during the period of myoblast differentiation. Surprisingly, however, a slight increase in the total activity of PKA was observed during the same culture periods, whether or not exogenous cAMP was treated (Fig. 2B). In an attempt to clarify the discrepancy in the correlation between the cAMP level and PKA activity, we examined the changes in the protein level of PKA subunits during the same differentiation period upon immunoblot analysis using antibodies raised against the PKA subunits. Figure 2C shows that the levels of RIα and Cα markedly increased with myoblast fusion (after 48 h), whereas the RIIα level gradually decreased to an insignificant extent during the entire course of culture period. These results raised the possibility that cAMP may be involved in down-regulation of the expression of RIα and Cα but with relatively little effect on the expression of RIIα.

Effects of RAs on the PKA activity and protein level of its subunits To determine whether RAs had influence on the PKA activity during differentiation of the L6 myoblasts, we first examined the effects of RAs at the intracellular level of cAMP. At the time of the medium change with differentiation medium, all-*trans* RA or 9-*cis* RA (10 μM) was added to the culture medium and cultured for 72 h in the presence or absence of light. As shown in Fig. 3A, the cAMP level increased over 3-fold in the cells treated with RAs compared to that in the untreated control cells. The cAMP level also increased in the presence of light but to extents less than those seen under dark conditions. These results confirm that the increase in the intracellular cAMP level, which is mediated by RA-treatment, inhibits myoblast fusion.

We then examined whether the increased level of cAMP might be related with the activity of PKA and the protein level of its subunits. As shown in Fig. 3B, the PKA activity in the RA-treated cells was instead reduced to approximately one-half of that seen in the control cells regardless of whether light was provided. Furthermore, treatment with RAs also resulted in a decrease in the protein level of both RIα and Cα with little effect on that of RIIα (Fig. 3C). These results suggest that the decrease in the PKA activity upon RA treatment is due to down-regulation of the expression of the PKA subunits and this effect may be mediated by the increase in the intracellular level of cAMP.

Effect of cAMP on expression of PKA subunits In order to determine the effect of cAMP on expression of PKA subunits, cells were treated with 100 μM dibutyryl-

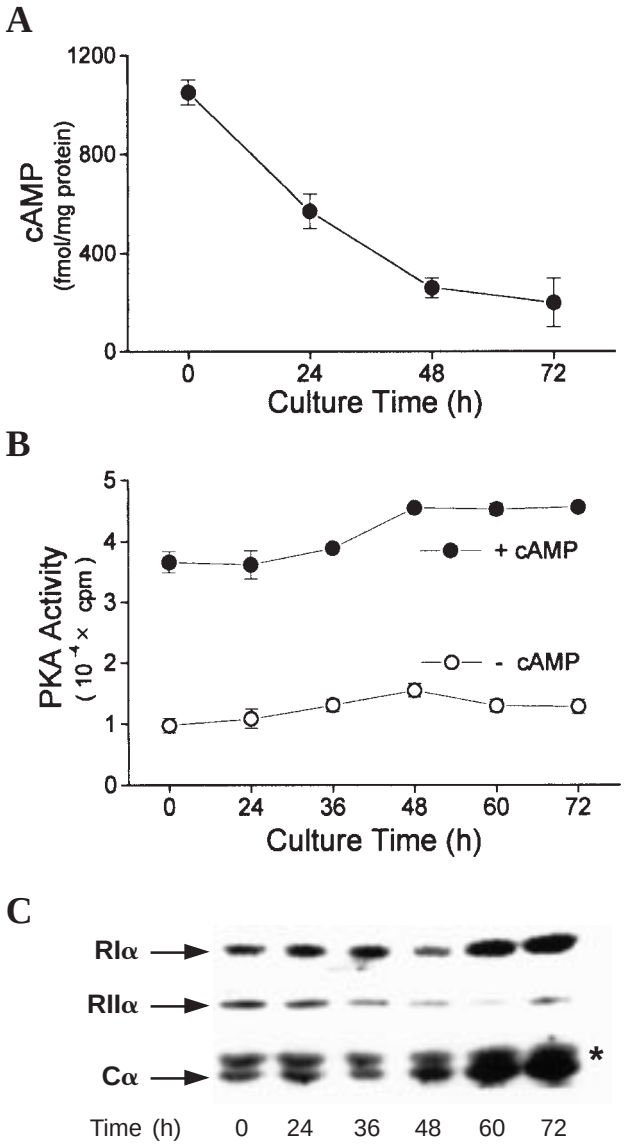

Fig. 2. Changes in the level of cAMP (**A**) and PKA activity (**B**) and the expression of PKA subunits (**C**) during myogenesis. At the indicated culture times, cells were harvested and assayed for the cAMP level using the cAMP assay kit (Amersham). The PKA activity was assayed using kemptide as a substrate in the presence or absence of 10 μM cAMP. The expression of RIα, RIIα, and Cα subunits of PKA were determined by immunoblot analysis using the antibodies raised against each subunit. The band just above Cα (indicated by an asterisk) is a protein that interacts nonspecifically with an anti-Cα antibody. The values shown are averages (mean ± SD) of one representative experiment ($P < 0.001$ by ANOVA). The same work was repeated three times in **A**, **B** and twice in **C**.

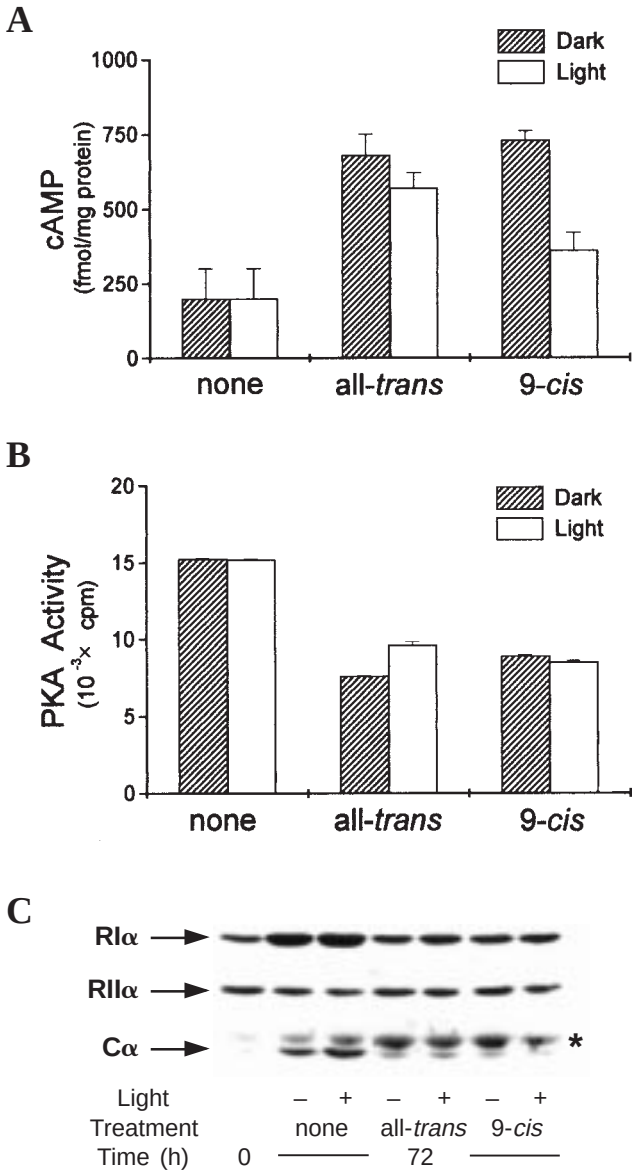

Fig. 3. Effects of RAs on the cAMP level (**A**), PKA activity (**B**), and expression of PKA subunits (**C**). At the time of medium change, cells were treated with all-*trans* RA or 9-*cis* RA (10 μM) and cultured for 72 h with and without illumination as in Fig. 1. They were then harvested and assayed for their cAMP level, PKA activity, and expression of PKA subunits as described under Fig. 2. The values shown are averages (mean ± SD) of one representative experiment ($P < 0.05$ by ANOVA). The same work was repeated three times in **A**, **B** and twice in **C**.

cAMP or 10 μM all-*trans* RA at the time of the medium change. They were then cultured for 72 h and assayed for the protein level of PKA subunits upon immunoblot analysis. Figure 4 shows that the protein levels of both RIα and Cα in the cells treated with the cAMP analog are reduced to similar extents as seen in the cells treated with all-*trans* RA. On the other hand, little or no effect was observed on the protein level of RIIα (Fig. 4). These results strongly suggest that cAMP is involved in selective down-regulation of the expression of PKA subunits and that the same negative effect of RA on the expression of PKA subunits is mediated by the increase in the intracellular cAMP level. Thus, it appears that RA-mediated inhibition of myoblast fusion is due to the increase in the intracellular level of cAMP level, which may induce down-regulation of the expression of RIα and Cα subunits and hence the decrease in the total PKA activity.

Discussion

In this report, we have demonstrated that all of the RAs used inhibit the growth and fusion of L6 rat skeletal myoblast cells. The growth arrest is a prerequisite for the terminal differentiation of muscle cells. It has been reported that RA promotes growth arrest but induces myogenic differentiation of C2 and rat rhabdomyosarcoma cell lines (Alric *et al*., 1998; Arnold *et al*., 1992; Froeschle *et al*., 1996; Gabbert *et al*., 1988). In contrast, RA has been shown to inhibit not only cell growth but also membrane fusion of chick embryonic myoblasts (Kim *et al*., 1995). The inhibitory effects of RAs on L6 myoblast fusion demonstrated in the present studies were not due to their toxic effect since membrane fusion could be fully recovered upon removal of the drugs from the medium (data not shown). Muscle differentiation is largely dependent on myogenic regulatory proteins, such as MyoD, myogenin, Myf5, and MRF4 (Lassar and Munsterberg, 1994; Weintraub *et al*., 1991). These proteins are differentially expressed in muscle cells, for example, L6 cells express primarily myogenin but not MyoD (Braun *et al*., 1989). It has been suggested that RA induces growth arrest and differentiation only in MyoD-expressing muscle cells (Froeschle *et al*., 1998). Thus, it appears clear that RAs block membrane fusion of L6 myoblasts, in contrast to their effects on MyoD-expressing cell lines.

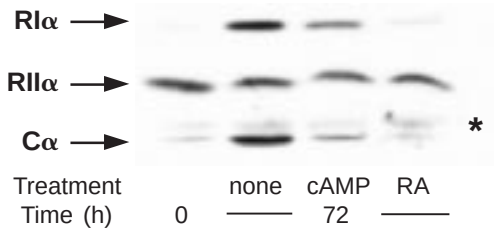

Fig. 4. Effects of cAMP and all-*trans* RA on the expression of PKA subunits. At the time of medium change, cells were treated with 100 μM dibutyryl-cAMP or 10 μM all-*trans* RA and cultured for 72 h. They were then harvested and subjected to immunoblot analysis using the antibodies raised against each of the PKA subunits.

Of interest was the finding that different geometric isoforms of RA inhibited myoblast fusion to different extents, 9-*cis* RA being the most effective. On the other hand, all of the RA isomers reduced cell growth to a similar extent. It has been shown that 9-*cis* RA specifically interacts with RXR, while all-*trans* RA preferentially binds to RAR (Allegretto *et al*., 1993; Allenby *et al*., 1993; Levin *et al*., 1992). Therefore, it is tempting to speculate that interaction of 9-*cis* RA with RXR may be primarily responsible for the RA-mediated fusion inhibition. However, given the fact that other geometric isomers also inhibit the cell fusion, although less significantly than 9-*cis* RA, it appears more likely that the differential inhibitory effect of RAs is due to their different affinity to RA receptors. However, it is at least clear that the inhibitory effects of RAs on growth and fusion of L6 myoblasts are differently regulated.

As an attempt to determine the action mechanism of RA in preventing membrane fusion of L6 myoblasts, we first examined the effects of RAs on the changes in intracellular level of cAMP. It has previously been reported that the cAMP level transiently (i.e. within a period of a few hours) increases in fusion competent cells and subsequently falls to basal levels as differentiation proceeds (Ball and Sanwal, 1980; Curtis and Zalin, 1981). Although cAMP peak was not detected in this study because it was assayed at every 24 h-interval, the overall cAMP level was found to dramatically decrease as myogenic differentiation proceeds. How do RAs raise the intracellular cAMP level is not clear in this study, but RAs increased it about 3-fold more than untreated cells (Fig. 3A). It has well been known that high level of cAMP prevents membrane fusion of myoblasts from various sources, such as primary cultures of chick embryonic myoblasts and mouse C2 and rat L6 cell lines (Baek *et al*., 1994; Winter *et al*., 1993). Thus, it is clear that cAMP negatively modulates myoblast fusion. Under the same conditions, however, the total activity of PKA increased slightly, despite the fact that the most well known action mechanism of cAMP is mediated by direct activation of PKA through binding to its regulatory subunit. Therefore, we suggest that cAMP is somehow involved in down-regulation of the expression of PKA.

Evidence presented in the present studies supports our proposal. First, RAs, which inhibit myoblast fusion, not only raised the intracellular level of cAMP but also decreased the total PKA activity. Second, RAs negatively

modulate the expression of PKA subunits and this effect is specific to the Cα subunit in addition to RIα. Third, exogenous treatment of cAMP (i.e. dibutyryl-cAMP) reduced the expression of both Cα and RIα to the similar levels seen upon treatment with RAs. Thus, it appears likely that RA-mediated inhibition of myoblast fusion is mediated by the increase in the intracellular level of cAMP level, which may in turn induce down-regulation of the expression of RIα and Cα subunits and hence in the decrease in the total PKA activity. The mechanism by which cAMP selectively down-regulated RIα and Cα subunits of protein kinase A remains unknown. There is, however, a possibility that cAMP could regulate gene expression of eukaryotes. It has been previously reported (Winter *et al*., 1993) that cAMP inhibits the expression of myogenin, which is a transcription factor and an early marker of muscle differentiation. To answer this question, further studies are needed.

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