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Involvement of Type I Protein Kinase A in the Differentiation of L6 Myoblast in Conjunction with Phosphatidylinositol 3-Kinase

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To investigate the role of protein kinase A (PKA) (EC 2.7.1.37) in myogenesis, PKA activity was closely monitored during the differentiation of L6 rat skeletal myoblasts. As the differentiation proceeded, total PKA activity increased about 2–3 fold, and the protein levels of PKA RIα and Cα subunits increased about 3–4 fold. We then looked at the effect of the specific inhibitor for PKA, *N*-[2-(*p*-bromocinnamy-lamino)-ethyl]-5-isoquinoline-sulfonamide (H89), on the differentiation of L6 myoblasts. H89 completely blocked the myotube formation and abolished the up-regulation of RIα and Cα. This inhibitory effect of H89 was dose-dependent and could be reversed upon removal of H89 from the culture medium. Furthermore, we demonstrated that specific inhibitors of phosphatidylinositol 3-kinase (PI3K), wortmannin, and LY294002 blocked the myotube formation and abolished the increase of PKA activity, which normally accompanied the differentiation of myoblasts. These results suggest that type I PKA may play a functional role(s) in the differentiation of myoblast as a putative downstream effector of the PI3K signaling pathway.

Keywords: Differentiation; L6 Myoblasts; Phosphatidylinositol 3-Kinase; Type I Protein Kinase A.

Introduction

Skeletal muscle differentiation is accomplished through a complex cascade of events, such as an exit from the cell cycle, expression of muscle-specific transcription factors, and formation of myotubes and muscle fibers. During this process, skeletal muscle-specific transcription factors (MyoD, myogenin, Myf-5, and MRF4), Pax3, MEF2, and structural proteins (α-skeletal actin, myosin heavy chain, and myosin light chains) are expressed (Braun *et al*., 1989; Lin *et al*., 1997; Tajbakhsh *et al*., 1997). In addition to these specific gene expressions, dramatic morphological changes (such as the physical fusion of plasma membrane and rearrangement of intracellular organelles) occur during this differentiation process.

Since the discovery of cyclic AMP (cAMP) as a mediator of hormonal signals, cAMP has been considered to play important roles in the regulation of cell proliferation and differentiation in a variety of cell types (Pastan *et al*., 1975). It was reported that a high concentration of cAMP inhibits myogenic differentiation (Baek *et al*., 1994; Winter *et al*., 1993), and retinoic acid can block the growth and fusion of L6 myoblasts (Shin *et al*., 2000). Once the extracellular signals (such as hormones and neurotransmitters) trigger the elevation of the intracellular cAMP concentration, cAMP binds and activates protein kinase A (PKA), a major effector protein in the cAMP signal transduction pathway.

PKA is a serine/threonine kinase that is composed of a dimer of regulatory (R) subunits 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 the R subunits [RIα (Sandberg *et al*., 1987), RIβ (Solberg *et al*., 1991)], RIIα (Oyen *et al*., 1989), RIIβ (Levy *et al*., 1988)] and three isoforms of the C subunits (Cα, Cβ, and Cγ) have been identified. It was reported that the differential expression

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Abbreviations: AKAP, A-kinase anchoring protein; cAMP, cyclic AMP; H89, *N*-[2-(*p*-bromocinnamylamino)ethyl]-5-isoquinoline-sulfonamide; PDK, phosphoinositide-dependent kinase; PI3K, phosphatidylinositol 3-kinase; PKA, protein kinase A; PMSF, phenylmethylsulfonyl fluoride; RI and RII, type I and type II regulatory subunits of protein kinase A.

of two types of PKA was related to the control of cell growth (Cho-Chung *et al*., 1990). An enhanced expression of RI/type I PKA correlates with the active cell growth and transformation; whereas, an increase of RII/type II PKA is related to growth inhibition and differentiation.

On the other hand, PI3K is involved in the signal transduction pathway that is initiated by different stimuli in eukaryotic cells. Activated PI3K generates 3-phosphoinositides, which may act as second messengers in signal transduction cascades (Toker and Cantley, 1997). The effects of PI3K activation are mediated through downstream kinases, such as phosphoinositide-dependent protein kinase 1 (PDK1) (Cohen *et al*., 1997). PDK1 phosphorylates the protein kinase B (PKB) (Stephens *et al*., 1998), p70 ribosomal protein S6 kinase (Pullen *et al*., 1998), protein kinase C (Le Good *et al*., 1998), and possibly PKA (Cheng *et al*., 1998). These protein kinases have a high degree of sequence similarity in the phosphorylation site. However, while the role of PI3K has been extensively studied in the signal transduction pathway that is initiated by growth factors [such as platelet-derived growth factor, epidermal growth factor, insulin, and insulin-like growth factors (IGF)], the physiological role of PI3K in the differentiation process is not well elucidated.

In the present study, we investigated the involvement of PKA and PI3K in the differentiation of L6 rat skeletal myoblasts. Our goal is a better understanding of the regulatory mechanism of myoblast differentiation.

Materials and Methods

Materials The cAMP, Kemptide (phosphate acceptor peptide, Leu-Arg-Arg-Ala-Ser-Leu-Gly), LY294002, and wortmannin were purchased from Sigma. Radioactive [γ-^{32}P]ATP (6,000 Ci/mmol) was purchased from DuPont-NEN. The phosphocellulose disc sheet was from Gibco-BRL. H89 was purchased from Calbiochem®.

Cell culture L6 rat skeletal myoblasts were maintained in Dulbecco's modified Eagle medium (DMEM; Gibco-BRL) that was supplemented with 10% (v/v) heat-inactivated fetal bovine serum (Gibco-BRL), 100 units/ml penicillin G (Gibco-BRL), and 100 μg/ml streptomycin (Gibco-BRL) in a humidified atmosphere of 5% CO_2 at 37°C. For the differentiation, myoblasts were plated at a density of 2×10^4 cells/35 mm culture dish on differentiation day 3. After 3 d (differentiation day 0), the cells were shifted to a differentiation medium (DMEM, Gibco-BRL) with 5% (v/v) horse serum in order to induce differentiation.

Preparation of cell extracts All of the procedures were performed at 0–4°C. The myoblasts that were cultured for the indicated times were washed three times with ice-cold phosphate-buffered saline (PBS), harvested by centrifugation, and kept frozen at –70°C until they were used. Cell pellets were suspended in 500 μl of a RIPA buffer [150 mM NaCl, 1.0% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% sodium dodecylsulfate (SDS), 50 mM Tris-Cl, pH 8.0] with a protease inhibitor cocktail [0.1 mM pepstatin, 0.1 mM antipain, 0.1 mM chymostatin, 0.2 mM leupeptin, and 1 mM phenylmethylsulfonyl fluoride (PMSF)], passed through a 20-gauge needle five times, incubated on ice for 15 min, then centrifuged at 14,000 × *g* at 4°C for 5 min. The supernatant was used for a PKA activity assay and Western analysis.

Western blot analysis Western blot analysis was performed as described previously (Kim *et al*., 1996). The polyclonal antibodies that were specific for RIα, RIIβ, and Cα were prepared as described (Kim *et al*., 2000; Lee *et al*., 1999). The monoclonal antibody against the Myosin Heavy Chain (MHC) was from Santa Cruz Biotechnology.

Evaluation of fusion index At the indicated time points, cultured myoblasts were washed with PBS three times, fixed with 3.7% paraformaldehyde at 4°C for 1 h, then permeablized in a permeablization buffer (0.3% Triton X-100, 1% bovine serum albumin in PBS) for 20 min. After staining with Hoechst dye (Polyscience) for 10 min, the myotubes that had at least 5 nuclei were counted in ten randomly chosen microscopic fields.

Protein kinase A activity assay PKA activity was estimated as described previously (Kim *et al*., 2000). For a determination of total cellular PKA activity, the cell extract was prepared as described previously and used directly for a PKA activity assay. Ten micrograms of cell extracts were mixed with 50 μl of a kinase assay buffer (50 mM Tris-Cl, pH 7.5, 1 mM DTT, 10 mM $MgCl_2$, 30 μM Kemptide, 5 μM [γ-^{32}P]ATP) either in the presence or absence of 5 μM cAMP, and incubated at 37°C for 5 min. The reaction mixture were spotted onto phosphocellulose filters and washed three times with 1% phosphoric acid. Filters were air-dried and radioactivity was measured with a liquid scintillation counter (Pharmacia).

Results

Changes in the level of PKA subunits and PKA activity In order to understand the role of PKA in the myoblast differentiation, levels of the PKA subunits and PKA activity were examined during the differentiation of rat L6 skeletal myoblasts. During the first 24 to 48 h of differentiation, the myoblast became elongated and aligned with the adjacent cells. Multinucleated myotubes began to form on differentiation day 3. Figure 1A shows the time-course of the myotube formation during the myoblast differentiation process. The myotube formation began on differentiation day 3 and increased dramatically thereafter. About 70–80% of the myoblasts were fused to form myotubes on differentiation day 5. The intracellular levels of various PKA subunits during this differentiation pro-

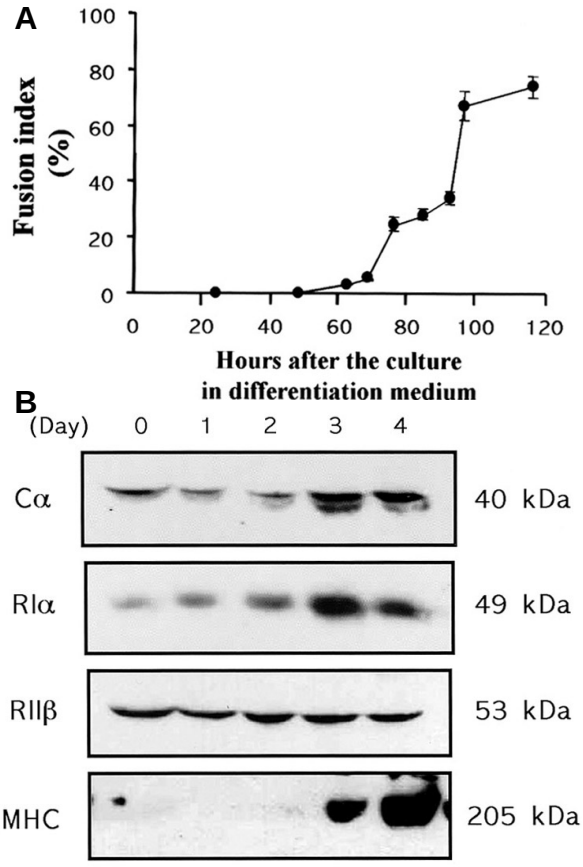

Fig. 1. Time courses of the myotube formation and protein levels of PKA subunits and MHC during the differentiation of L6 myoblast. **A.** Myotube formation during the differentiation of myoblasts. L6 rat skeletal myoblasts were grown in a proliferation medium, then allowed to differentiate in a differentiation medium that contained 5% horse serum. Under the ten randomly selected microscopic fields, the myotubes that had at least 5 nuclei were counted at the indicated time points. The degree of myoblast fusion was expressed as the number of nuclei within the fused cells in the total number of nuclei (as a percentage). Each data point represents the average of the number of myotubes in three independent experiments. Error bar, S.D. **B.** Protein levels of Cα and RIα subunits. Cells were allowed to differentiate for indicated time, and cell extracts were prepared. One hundred micrograms of cell extracts were subjected to SDS-PAGE and examined by Western analyses using anti-RIα, RIIβ, Cα, and MHC antibodies.

cess were examined by a Western blot analysis (Fig. 1B). The level of catalytic subunit of PKA, Cα of about 40 kDa, increased about 2–3 fold on differentiation day 3. The RIIβ regulatory subunit level (53 kDa) was not significantly changed during this entire differentiation process. In contrast to the RIIβ level, the RIα subunit level (49 kDa) started to increase after 24 h and reached its maximum of 5–6 fold on differentiation day 3. The Cα and RIα levels showed similar patterns of time course.

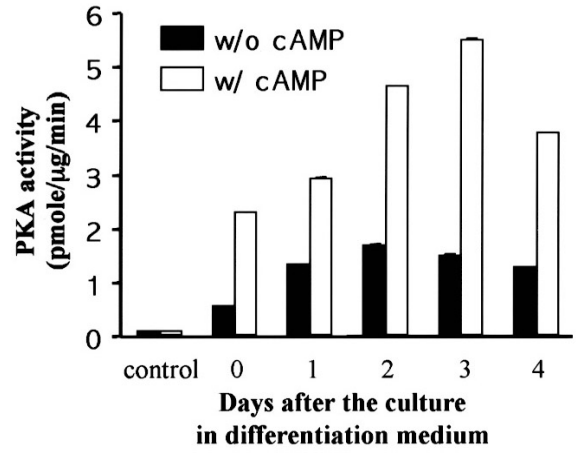

Fig. 2. Increase of total PKA activity. The myoblasts were differentiated under the same condition as in Fig. 1. Each day cell extracts were prepared and PKA activity was determined either in the presence or absence of 5 μM cAMP. The graph shows a representative of three separate experiments. Open bar indicates PKA activity in the presence of cAMP, and closed bar shows PKA activity in the absence of cAMP. Error bars, S.D.

The myosin heavy chain level (MHC), which was considered one of the marker genes for myogenesis (Medford *et al.*, 1983), was also checked. The intracellular MHC level began to increase on differentiation day 3 (Fig. 1B).

Since the level of RIα and Cα subunits increased, it could be assumed that the PKA activity increased during that process. In order to examine this possibility, PKA activity was measured during the differentiation of L6 rat skeletal myoblasts. Both the free catalytic subunit activity (in the absence of cAMP) and the total PKA activity (in the presence of cAMP) were measured. As shown in Fig. 2, total PKA activity gradually increased, then reached its maximum of 2 fold on differentiation day 3. The free catalytic subunit activity showed a similar pattern. Together with the time course of myotube formation (Fig. 1A) and PKA activities (Fig. 2) during the differentiation process, these results indicate that there might be a correlation between the change of type I PKA activity and myoblast differentiation, especially at the point of membrane fusion (i.e., differentiation day 3).

Inhibition of the myoblast differentiation by H89 Next, the effect of the specific PKA inhibitor, H89 {*N*-[2-(*p*-bromocinnamylamino)ethyl]-5-isoquinoline-sulfonamide}, was examined. H89 has a potent and selective inhibitory action against PKA (Chijiwa *et al.*, 1990). On differentiation day 1 (Fig. 3B), the myoblasts were treated with H89. The numbers of myotubes were counted on differentiation day 4. As shown in Fig. 3A, H89 inhibited the myotube formation in a dose-dependent manner, whereas control myoblasts were fused to form the myotubes (Fig. 3C). A half-inhibition with H89 was observed at 5 μM. The myo-

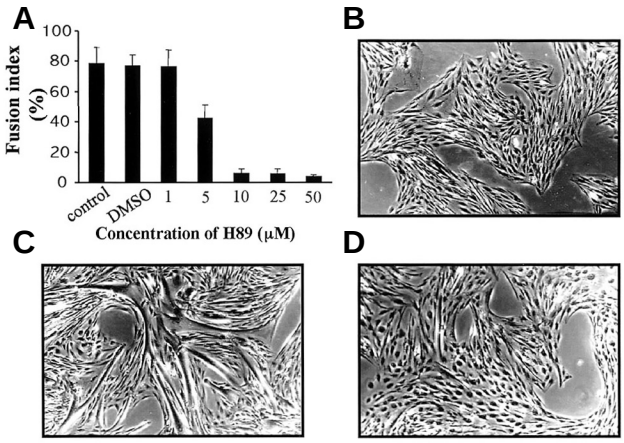

Fig. 3. Effects of H89 treatment on the myotube formation. **A.** Effect of H89 on the myotube formation. L6 rat skeletal myoblasts were grown in a proliferation medium that contained 10% FBS, then allowed to differentiate in a differentiation medium that contained 5% horse serum. H89 was treated on differentiation day 1 and myotubes that had at least 5 nuclei were counted on differentiation day 4 under the ten randomly selected microscopic fields. The degree of cell fusion was determined as described in the legend to Fig. 1. Data show means of three separate experiments with S.D. **B–D.** L6 myoblasts (differentiation day 0, panel B) were grown, then allowed to differentiate in the absence (C) or presence (D) of 10 μM H89. Cells were photographed on differentiation day 4. Magnification, ×40.

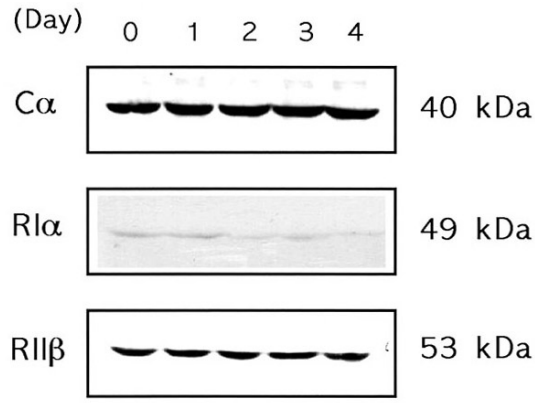

Fig. 4. Effects of H89 on the protein levels of PKA subunits. Protein levels of PKA subunits and MHC were examined by Western analyses. Cells differentiated in the presence of 10 μM H89 for the indicated times, and the cell extract was prepared. One hundred micrograms of cell extracts were used for Western analyses that used anti-RIα, RIIβ, Cα, and MHC antibodies.

tube formation was not observed in the presence of 10 μM H89 (Fig. 3D), which indicates that the inhibitory effect was evident at a 10 μM concentration, although the myoblasts became elongated and aligned. One thing that needs to be noted is that the myoblast's fusion was resumed after H89 was removed from the medium (data not shown). These results clearly show that the specific inhibitor of PKA reversibly inhibits the formation of myotubes. We then analyzed the effect of H89 on the protein levels of PKA subunits during the differentiation period. As shown in Fig. 4, H89 blocked the increase of Cα and RIα levels, which were normally observed during the differentiation period (Fig. 1B). H89 also inhibited the expression of the MHC protein. However, the RIIβ level was not influenced by the H89 treatment. These results indicate that H89 inhibits the up-regulation of the Cα and RIα subunits, MHC expression, and differentiation of myoblasts.

Inhibition of the increase of PKA activities by wortmannin and LY294002 It was reported that inhibitors of PI3K blocked the differentiation of skeletal myoblasts (Kaliman *et al.*, 1996; Sarbassov *et al.*, 1998). These results prompted us to examine whether or not the PI3K-dependent signaling pathway is connected with the PKA signal transduction in the differentiation of myoblast. To test this idea, we next analyzed the effect of the PI3K inhibitor on the increase of PKA activity, which was normally observed during the differentiation period (Fig. 2). Myoblasts were allowed to differentiate in the presence of a PI3K inhibitor, wortmannin, or LY294002. Every 12 h the culture medium was replaced with a fresh medium that contained PI3K inhibitors, as it was reported that wortmannin is highly unstable in an aqueous solution (Ui *et al.*, 1995). During the first 24 h in the differentiation medium, the control myoblasts were aligned with each other and fused to form myotubes on differentiation day 4. However, LY294002 blocked the myotube formation completely (Fig. 5A). These inhibitory effects were also similarly observed in the wortmannin-treated groups (data not shown).

We then looked at the changes in PKA activity throughout the differentiation period. Compared with the control group, in LY294002-treated cells, the total PKA activity did not increase (Fig. 5B). These inhibitory effects of LY294002 on PKA activity were also observed in the wortmannin-treated cells (data not shown). These results demonstrate that treatment with the inhibitors of PI3K results in the down-regulation of PKA activity during the differentiation period. Furthermore, treatment with LY294002 and wortmannin blocks the increase of Cα level (data not shown), which is observed in the differentiation of myoblast (Fig. 1B). These results suggest that PI3K activity is involved in the selective up-regulation of PKA activity during the differentiation of myoblasts.

Discussion

In the present study, we examined the specific roles of

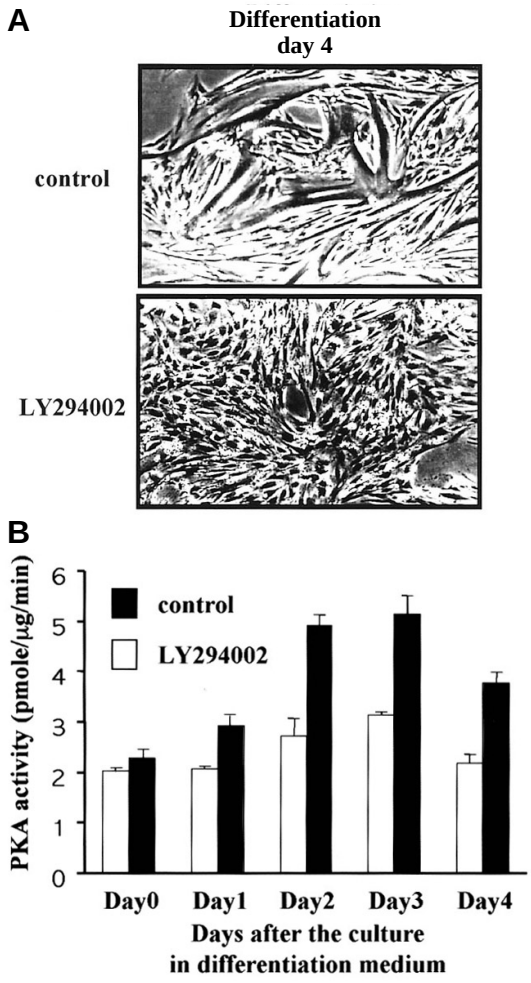

Fig. 5. Effects of specific inhibitor of PI3K on the myoblast differentiation. **A.** Inhibition of the myotube formation by LY294002. The myoblasts were differentiated either in the presence or absence of 10 μM LY294002 for 4 d. Photographs were taken on differentiation day 4. Magnification, ×40. **B.** Effect of LY294002 on the PKA activity during the differentiation of L6 myoblasts. The myoblasts were differentiated either in the presence or absence of 10 μM LY294002 for the indicated times. The PKA activity was examined in the presence of 5 μM cAMP. Open bar indicates PKA activities of the myoblasts that were differentiated in the presence of 10 μM LY294002, and closed bar shows PKA activities of the myoblasts that were differentiated in the absence of LY294002. Data show a representative of three experiments with S.D.

PKA in the control of myogenic differentiation. Many reports have suggested that each PKA isozyme plays a specific role through the action of its specific regulatory subunit. For example, the RIα subunit can reportedly bind to Grb2 and the T-cell receptor complex (Skalhagg *et al*., 1994; Tortora *et al*., 1997), and RII subunits can bind to the cAMP response element (Srivastava *et al*., 1998). In addition, studies with the knock-out mice of PKA regulatory subunits suggest the specific role of PKA isozymes in the nervous system (Huang *et al*., 1995).

We have shown that the total PKA activity increased significantly during the differentiation of L6 myoblasts, probably through an increase in the Cα and RIα levels (Fig. 1). These results suggest that the activity of type I PKA may be required for the differentiation of myoblast. The changes of PKA activities and myotube formation (Figs. 1 and 2) indicate that the increases of PKA activity preceded the myotube formation. This suggests that the increase of type I PKA activity is a prerequisite to the myotube formation. Moreover, the data indicate that the multinucleated myotube began to form abruptly on differentiation day 3 (Fig. 1), and PKA activity reached its maximum on differentiation day 3, compared with the decreased activity of PKA on day 4. This strongly indicates that type I PKA activity may be necessary to evoke the myogenic differentiation on day 3. Accordingly, the inactivation of PKA activity with H89 results in the complete inhibition of the myotube formation (Fig. 3).

This differential expression of RI/type I PKA in the myogenic differentiation presents a new insight about the role of PKA isozymes. It was reported that type II PKA may be related to the growth inhibition and differentiation, and type I PKA to the cell growth and transformation (Cho-Chung, 1990; Kim *et al*., 2000). However, in this report, it was shown that the enhanced expression of type I PKA may be involved in the myogenic differentiation. This differential expression of RI/type I PKA in the myogenic differentiation may indicate the physiological differences of myogenic differentiation compared with other differentiation processes. In contrast to other differentiation processes, the myogenic differentiation is comprised of complex events, such as membrane fusions between adjacent myoblasts, as well as the assembly and rearrangement of intracellular organelles. In a sense, these events are similar to some aspects of the mitotic events in which the intracellular organelles are disintegrated, then reintegrated. These characteristics of morphological changes may explain the differential expressions of type I PKA in the differentiation of myoblasts.

On the other hand, it was reported that PKA represses myogenic differentiation and the activity of Myf5 and MyoD *in vitro* (Winter *et al*., 1993). These reports suggest the potential of PKA as an inhibitor in myogenesis. If PKA plays an inhibitory role in the differentiation of myoblast as described by Winter *et al*. (1993), then the differentiation of myoblast could be stimulated by H89, a specific PKA inhibitor. However, this was not the case. Instead of stimulating myogenesis, H89 completely blocked the myoblast fusion (Fig. 3B) and inhibited the up-regulation of the RIα and Cα levels and MHC during the differentiation of L6 myoblast (Fig. 4). Therefore, these results strongly suggest that type I PKA may play a positive role(s) in myoblast differentiation.

Furthermore, the positive role of PKA in the differentiation of myoblasts was strongly supported by experiments using inhibitors of PI3K, wortmannin, and LY294002. It was reported that PI3K inhibitors abolished the induction of muscle regulatory factors, such as myf5 and myoD, induction of p21cdk inhibitor, and down-regulation of the dominant negative bHLH Id. These events finally lead to the failure of the myotube formation (Kaliman *et al*., 1996). In the present study, we showed that wortmannin and LY294002 not only blocked the myotube formation (Fig. 5A), but also inhibited the increase of PKA activity (Fig. 5B). It was also demonstrated that these PI3K inhibitors (data not known) suppressed the increase in the protein levels of Cα and RIα , which were normally observed in the differentiation of myoblast (Figs. 1 and 2). These results support the positive role of PKA. Also, PI3K activity may participate in the up-regulation of PKA during the differentiation of myoblast.

It was reported that PI3K signaling is mediated by PDK1 (Kobayashi and Cohen, 1999; Paradis *et al*., 1999). On the other hand, it was also reported that PDK1 could phosphorylate and activate the catalytic subunit of PKA (Cheng *et al*., 1998). These reports strongly support our results that PI3K can act upstream of PKA through PDK signaling.

Therefore, the following has been shown: 1) The protein levels and activity of type I PKA increased along with the differentiation of L6 rat skeletal myoblasts. 2) Inactivation of PKA activity completely abolished the myotube formation. 3) Inhibition of PI3K blocked the myotube formation and increased type I PKA activity.

These results suggest that PKA activity is absolutely required for the myogenic differentiation through the cross talk with the PI3K signaling pathway. Further investigations are necessary to elucidate the positive role(s) of the type I PKA in the myogenic differentiation, such as in the regulation of proteins that are involved in the physical membrane fusion, or in the transcriptional control of specific genes. Also, it is hoped that this study provides clues in order to define the interconnections between the PI3K and PKA/cAMP signaling pathways in eukaryotic cells.

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