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# Studies on the Differentiation of Skeletal Muscle Cells *in vitro*: The Phosphorylation and Down Regulation of Protein Kinase C in Myoblasts of Chick Embryos

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**In the short-term treatment of 12-0-tetradecanoylphorbol-13-acetate (TPA) or platelet-derived growth factor (PDGF), the TPA and PDGF induced the Protein Kinase C (PKC) activation and migration from the cytoplasm to the peripheral nulcear membrane. And the activated PKC which was directly or indirectly stimulated by TPA or PDGF phosphorylated many kinds of PKC's targeting proteins and induces various biological responses. Especially, the cytoplasmic PKC was phosphorylated within 1 hr and 10 min by TPA-and PDGF-treatment respectively.**

**In the long-term treatment of TPA or PDGF, both of them induced the down-regulation and translocation of PKC in the myoblasts. The down-regulation of PKC isozymes, the pattern of PKC I and II was similar to the PKC III isozymes in the cytoplasm. But in the nucleolus, the TPA did not induce and down-regulation or the inhibition of the immunoreactivity of PKC III antibody. This investigation indicates that each isozymes of PKC may be performed the different effects to the down-regulation of the cytoplasm or nucleolus. And down-regulated myoblasts contained low immunoreactivity of PKC antibodies.**

**KEY WORDS: Protein kinase C III, TPA, Phosphorylation, Down regulation.**

Protein Kinase C (PKC) isozymes are known to induce many physiological responses in various tissues and cells (Nishizuka, 1986). They have been identified in brain and tissue (Beh, *et al.*, 1989; Brandt *et al.*, 1987; Coussens *et al.*, 1986; Huang *et al.*, 1988; Knopf *et al.*, 1986; Niedel and Blackshear, 1986; Osada *et al.*, 1990; Schaap and Parker, 1990; Sekiguchi *et al.*, 1988). It has been observed that diacylglycerol (DAG) in the presence of $Ca^{++}$ or phospholipids can induce PKC activation (Drust and Martin, 1985; May *et al.*, 1985; Bell, 1986; Thomas *et al.*, 1988). Phospholipase C (PLC) which is activated by the stimulation of membrane receptors induces DAG and inositol 1, 4, 5-triphosphate ($IP_3$) from the hydrolysis of inositol phospholipids (Majerus *et al.*, 1986). DAG directly stimulates the activation of PKC. On the contrary, $IP_3$ indirectly activates PKC by inducing $Ca^{++}$ from the endoplasmic reticulum and the sarcoplasmic reticulum of muscle cells (Berridge and Irvine, 1984). Especially, PKC activity changes during the normal differentiation of skeletal muscle cells (Vaidya *et al.*, 1991). Our experiments with primary cultured cells have led to detection of PKC according to the syncytium of myoblasts. The syncytium which is the formation of multinucleate and the transforming into myotubes take places in the differentiation of myoblasts. In addition, $Ca^{++}$ and DAG-bound PKC is activated and translocated from the cytosol to the membrane. Therefore, PKC in the differentiation of myoblasts is important to determin-

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ing whether it can be arising of signal transduction or not. At the time, of stimulation PKC is activated and translocated from the soluble cytoplasmic fraction to the insoluble membrane fraction. Activated PKC (membrane associated form) is easily proteolysed by a neutral proteinase which is activated by $Ca^{++}$ (Kishimoto *et al.*, 1983). The 80 KD PKC is proteolysed into 35 KD and 45 KD proteins. The 35 KD protein is the regulatory domain containing the binding sequences of $Ca^{++}$, phospholipid and DAG or phorbol ester, while 45 KD protein is the protein kinase domain having the ATP-binding sequence.

In general, 12-O-tetradecanoylphorbol-13-acetate (TPA), a known tumor promoter of the phorbol ester family (Philips and Jaken, 1983) and platelet-derived growth factor (PDGF), a mitogen, can stimulate PKC. Phorbol esters and DAG share structural similarities (Chida *et al.*, 1986). The chemical functions of TPA and DAG are very similar as well. Therefore, it has been suggested that TPA may function through direct stimulation of PKC which in turn stimulates phosphorylation of many kinds of proteins. Based on previous evidence it has also been suggested that PKC activation is characterized by phosphorylation of *Mr.* 40,000 protein (Castagna *et al.*, 1982, Nishizuka, 1983).

However, the chronic treatment of PDGF or TPA also induces a feed back inhibition phenomenon (Nishizuka, 1984; Presta *et al.*, 1989). This known as down regulation of PKC. Consequently, DNA synthesis which is induced by TPA or PDGF is decreased dramatically. Addition of TPA desensitizes the cell to a second phorbol ester challenge, including TPA. As a results down regulation of receptor binding occurs. Down regulated cells exhibit little or no PKC activity (Stable *et al.*, 1987). There is evidence that PKC itself is degraded by proteolytic digestion resulting in reduced PKC activity.

Using immunocytochemical technique, Choi (1990) investigated the signal transduction system in EC-4 cells (fibroblasts) treated with 12-0-tetradecanoylphorbol-13-acetate (TPA) or platelet drived growth factor (PDGF). TPA, a tumor promoting phorbol ester can stimulate PKC (Philips and Jaken, 1983). PKC itself may act as a receptor for TPA (Stabel *et al.*, 1987). Hence, TPA has shown not only it activated PKC but also phosphorylates many substrates of PKC. However, the treatment of chronic TPA induces down regulation of PKC (Nishizuka, 1984; Presta, 1989). It has been demonstrated that PKC protein is degraded by proteolytic activity of proteases. Our laboratory has shown that long-term-TPA treated myoblasts gave rise a reduction of immunoreactive PKC, which is correlated with down regulation. Presently, there are many unsolved problems concerning the down regulation mechanism of PKC in signal transduction system. Down regulation occurs in a variety of cells, therefore, it has been explored with other receptor systems. TPA-induced down regulations are unique with respect to other transmembrane cell receptors (Stable *et al.*, 1987). The PKC proteolytic degradation is responsible for the down regulation phenomenon. But the study on the down regualtion of PKC in the chick embryo myoblast is very rare. Therfore, this report deals with that PKC activation and the down regulation examine the role of PKC in the 72 hr cultured of chick embryo myoblasts.

## Materials and Methods

### Materials

Ten-day-old chick embryos purchased from Chunsung Hatchery of Kim-Hae city were used. Antibody against PKC III was supplied from the National Institutes of Health (NIH, USA).

#### 1) Cell Culture

Myoblast cells of the chick embryos were prepared according to the methods of O'neil and Stockdale (1972) with minor modification. Briefly, the skins were removed from the breast region, the breast muscles were dissected out and digested with 0.1% typsin for 30 min in incubator. Digested muscles were collected by centrifugation and suspended in minimum essential medium (MEM, Gibco) containing 10% horse serum, 10% chick embryo extracts, and 1% antibiotics (811 medium). Then in order to remove unseparated mass cells and fibroblasts, the muscle suspension was filtered through a sterile Swanny filter, and preplanted on collagen coated dishes for 10 min. At this time, most fibroblasts were attached on the collagen coated dish, but myoblast cells were not.

The myoblast cells (approximately $5 \times 10^5$ cells per ml of 811 medium) were planted on new collagen coated dishes and cultured in a humidified incubator of 6% $CO_2$ at 37°C. After 24 hrs, the medium was replaced with MEM containing 10% horse serum, 2% embryo extracts, and 1% antibiotics (8102 medium).

**2) Isolation of nuclei**

The 72 hr-precultured cells in 811 and 8102 medium were cultured in serum-free MEM for 24 hrs. They were incubated in 200 nM TPA (12-*O*-tetradecanoylphorbol-13-acetate) for 1 hr. The procedure for nuclei isolation was described in a previous paper (Choi, 1990). The TPA-treated cells were centrifuged at the 10,000 × g for 3 min. After centrifugation, pellet (cells) was suspended again. Then the suspended pellet was incubated in swollen buffer (10 mM Tris-HCl, pH 7.4, 10 mM NaCl, 1.5 mM $MgCl_2 \cdot 6H_2O$) for 20 min. The swollen cells were homogenized with Potter-Elvehjam homogenizer. And then the homogenized cells were centrifuged at 1,000 × g for 20 min. Following suspension of pellet, the pellet was washed with 0.25 M sucrose in buffer A (10 mM Tris-HCl, pH 7.4, 3 mM $CaCl_2 \cdot 2H_2O$) at 1,000 × g centrifugation for 20 min. The crude nuclear pellet and crude cytosol were obtained. The crude nuclear pellet was homogenized in 2.2 M sucrose, centrifuged at 40,000 × g for 1 hr and homogenized in 0.25 M sucrose in buffer A containing 1% Triton X-100. After centrifugation at 1,000 × g for 20 min, the nuclear pellet was washed twice with 0.25 M sucrose in buffer A without Triton X-100 to obtain the purified nuclei.

**3) Immunoprecipitation**

The 72 hr-cultured cells in 811 and 8102 medium were cultured in serum-free MEM for 24 hrs. The serum-starved cells were treated with TPA for 1 hr. And then the nuclei were isolated according to the above described methods for nuclei isolation. The purified nuclei were sonicated in homogenation buffer pH 7.3 (50 mM Tris-HCl, 5 mM NaF, 2 mM sodium vanadate, ethyleneglycolbis (b-aminoethylether)-N,N,N′,N′-tetraacetic acid (EGTA), 4 mM ethylenediaminetetraacetic acid (EDTA), 2 mM phenylmethylsulfonyl fluoride (PMSF), 0.1 mM dithiothreitol (DTT) and 0.25 mg/ml aprotinin). Next, 5 μg of antibody against PKC III (/50 μg protein) was added into the control nuclei fraction and the TPA-treated nuclei fraction. Then the fractions were shaken for 2 hrs at 4°C. 200 μl of 50% protein A agarose was added to both fraction, and shook for 1 hr at 4°C. The fractions were centrifuged at 10,000 × g for 2 min at 4°C. The supernatants were discarded. The pellets were cooked for 3 min at 90°C in 2× sample buffer (0.0625 M Tris/HCl, pH 6.8, containing 4% SDS, 10% 2-mercaptoethanol, 20% sucrose, and 0.004% Bromophenol blue) and applied to the gel electrophoresis.

**4) Phosphorylation**

After cells were cultured in 811 and 8102 medium for 72 hrs, they were starved with serum-free and phosphate-free MEM for 48 hrs. They were treated with [$^{32}$P] orthophosphoric acid (200 μci/ml) for 2 hrs. The cells were divided into three groups control, TPA (200 nM/ml) treated, and PDGF (50 ng/ml) treated. The cells were treated with TPA according to the time course, 10 min, 1 hr and 6 hrs. For the cytosol and membrane phosphorylation, the treated cells were washed with PBS twice briefly and sonicated in homogenized buffer (50 mM Tris/HCl, pH 7.3, 5 mM NaF, 2 mM sodium vanadate, 5 mM EGTA, 4 mM EDTA, 2 mM PMSF, 0.25 mg/ml aprotinin, and 0.1 mM DTT). After sonication, a small amount of the crude fractions were taken out. Others were centrifuged at 100,000 × g for 1 hr. The supernatant was used for cytosol fractions, and the pellet for membrane fractions. Both fractions were sonicated in homogenized buffer containing deoxycholate. All samples were loaded in 10% polyacylamide gel electrophoresis. Dried gels were exposed on Kodak X-Omat AR film in −70°C deepfreezer. The films were developed.

**5) Immunocytochemical Study**

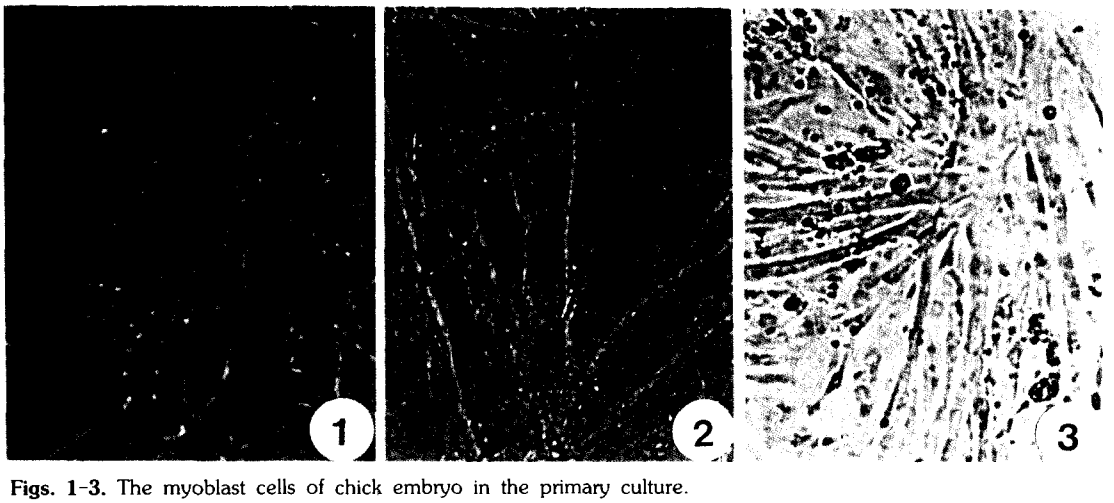
Chick embryo myoblast cells ($1 \times 10^2$) were seeded into wells (1 $cm^2$) of Lab-Tek chamber slides (Nune, Inc.) allowed to attach and grow for 24 hrs in the above 811 and 8102 culture medium. The attached cells were washed three times with serum free MEM in order to reduce the effect of serum. The cells were cultured in serum

free and phosphate-free medium for 24 hrs. The serum-and phosphate-starved cells were incubated in 200 nM TPA according to the time course (1 hr, 6 hrs, and 12 hrs). Then the cells were washed with PBS-washing buffer (containing 0.005% Tween-20, 0.014% $CaCl_2 \cdot 2H_2O$ and 0.01% $MgCl_2 \cdot 6H_2O$) three times for 5 min each. They were fixed with 2% formaldehyde in PBS for 5 min at room temperature. They were washed three times for 15 min with PBS, permeabilized with cold absolute methanol for 5 min, washed with PBS three times for 15 min and blocked with 2% bovine serum albumin (BSA) in PBS for 1 hr. The cells were incubated in antibody against PKC III for 24 hrs at 4°C. The antibody was diluted (1 : 300 dilution) in PBS containing 2% normal goat serum and 0.5% Triton X-100. Following incubation in antibody, the cells were washed with PBS four times for 20 min. After washing, the cells were reacted according to the avidin-biotin-Texas Red method (Bayer and Wilchek, 1979: Guesdon *et al.*, 1979). They were reacted with biotinylated goat anti-rabbit IgG (2.5 mg/ml, BRL) in PBS containing 1% BSA and 0.05% Tween 20 for 2 hrs at room temperature. They were washed with PBS three times for 15 min and then reacted with 0.15% streptavidin-Texas Red in PBS containing 1% BSA and 0.05% Tween 20. Then, they were washed with PBS three times for 15 min. 1% BSA in PBS was used for a final washing. The slides were mounted with 50% glycerol in PBS and photographed with p 800/1600 Kodak Ektachrome film under an Olympus BH-2 microscope at × 400.

## Results

The isolated myoblasts of chick embryo were cultured for 72 hours, in order to compare the changes of protein kinase C (PKC) according to the TPA-treated duration during differentiation of myoblast cells. The myoblast cells which attached with collagen began to grow. Under normal conditions, myoblast cells were elongated and assumed a spindle shape by 12 hr-culture (Fig. 1). At this time the developing cells were referred to as single nuclear myoblasts. After 24 hrs the myoblasts (Fig. 2) were more elongated and closely aggregated together to be a loss of cell envelope, and they were then brought to form a syncytial cells. Following this syncytium, the myoblasts had multinuclei. Also each nucleus contained from one to multinuclei. The syncytial myoblasts had grown very confluently by 72 hr (Fig. 3). They began to develop and form to the long cylindrical muscle cells.

To examine TPA-induced phosphorylation of




**Figs. 1-3.** The myoblast cells of chick embryo in the primary culture.
**Fig. 1:** The myoblast cells were cultured for 24 hrs. **Fig. 2:** The myoblast cells were cultured for 48 hrs. The cells began to differentiate to syncytial cells. **Fig. 3:** The myoblast cells were cultured for 72 hrs. The cells contained multinuclei.

myoblasts, cells were incubated with two hour-[$^{32}$P] orthophosphoric acid. Subsequently TPA treatment for one hour resulted in autophosphorylation of Mr. 80,000 PKC. At this time, any other proteins were not observed (Fig. 4). After 6 hr-TPA-treatment, some proteins in the cytosolic fraction were occurred phosphorylation: *Mrs.* 20,000 and 40,000 proteins (Fig. 5). *Mr.* 20,000 protein was significantly more phosphorylated than *Mr.* 40,000 protein.

Another common characteristic of phorobol esters including TPA is their ability to desensitize the cells to additional phorbol ester challenge. Long-term exposure to TPA reduces the cellular responsiveness to subsequent exposure of TPA. After 24 hr-TPA-treatment, most proteins could not be further phosphorylated and TPA may induce down-regulation. This may be due to a decrease in the binding capacity of PKC.

Likewise, PDGF, a mitogen, has also been treated to the myoblasts. PKC has been implicated in the proliferative action of PDGF in smooth muscle. The autophosphorylation of PKC (*Mr.* 80,000)

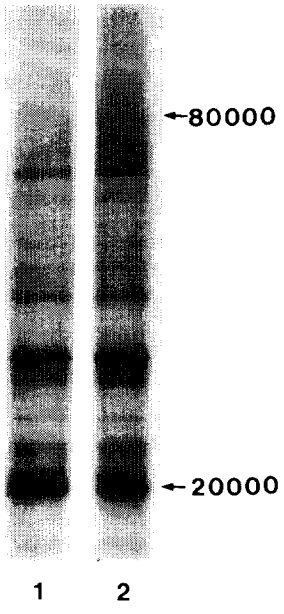



**Fig. 4.** The phosphorylation of cytosolic fraction in myoblasts exposed with [$^{32}$P] orthophosphoric acid. Lane 1: cytosolic fraction in control cells of no TPA-treatment. Lane 2: TPA-treated cytosolic fraction for 1 hr.

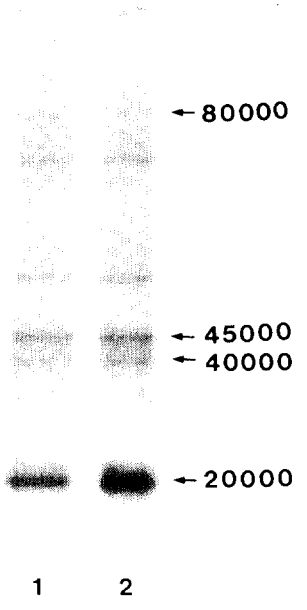



**Fig. 5.** The phosphorylation of cytosolic fractions in myoblast cells exposed with [$^{32}$P] orthophosphoric acid. Lane 1: Cytosolic fraction in control cells of no TPA-treatment. Lane 2: TPA-treated cytosolic fraction for 6 hrs.

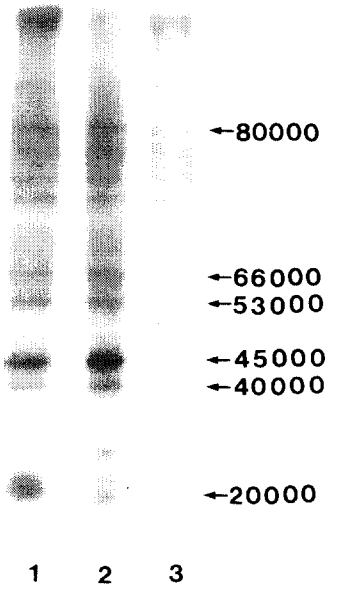



**Fig. 6.** The phosphorylation of crude extract fractions treated with PDGF in myoblast cells exposed with [$^{32}$P] orthophosphoric acid. Lane 1: Crude extract fraction in control cells of no PDGF-treatment. Lane 2: PDGF-10 min treated fraction. Lane 3: PDGF-6 hr treated fraction.

was occurred after 2 minute-TPA treatment in the cytosolic fraction (Fig. 6). Control cells of no PDGF-treatment did not increase phosphorylation of *Mrs.* 40,000 and 45,000 (Fig. 6. Lane 1). In general during short-term-PDGF-treatment phosphorylation of *Mrs.* 40,000, 45,000 and 66,000 are significantly increased and this was observed after 10 min-PDGF-treatment. However, the *Mr.* 45,000 protein was more phosphorylated than other proteins (Fig. 6, Lane 2). On the contrary, the *Mr.* 20,000 protein was dephosphorylated in 10 min-treatment with PDGF. This is interesting because *Mr.* 20,000 was not dephosphorylated during 1 hr-TPA-treatment. Furthermore, 6 hr-TPA-treatment produced a highly phosphorylated *Mr.* 20,000. PDGF may more repidly stimulate PKC than TPA. Although PDGF may not play by the same mechanisms as TPA, the signal transduction and down-regulation took place in the cytoplasm of myoblasts. Like TPA, PDGF treatment for 10 min led to the autophosphorylation of *Mr.* 80,000 PKC (Fig. 6). Generally, the same

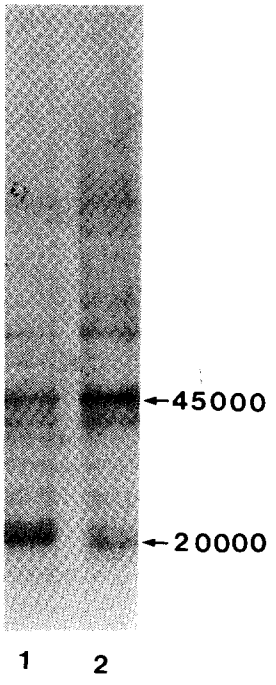
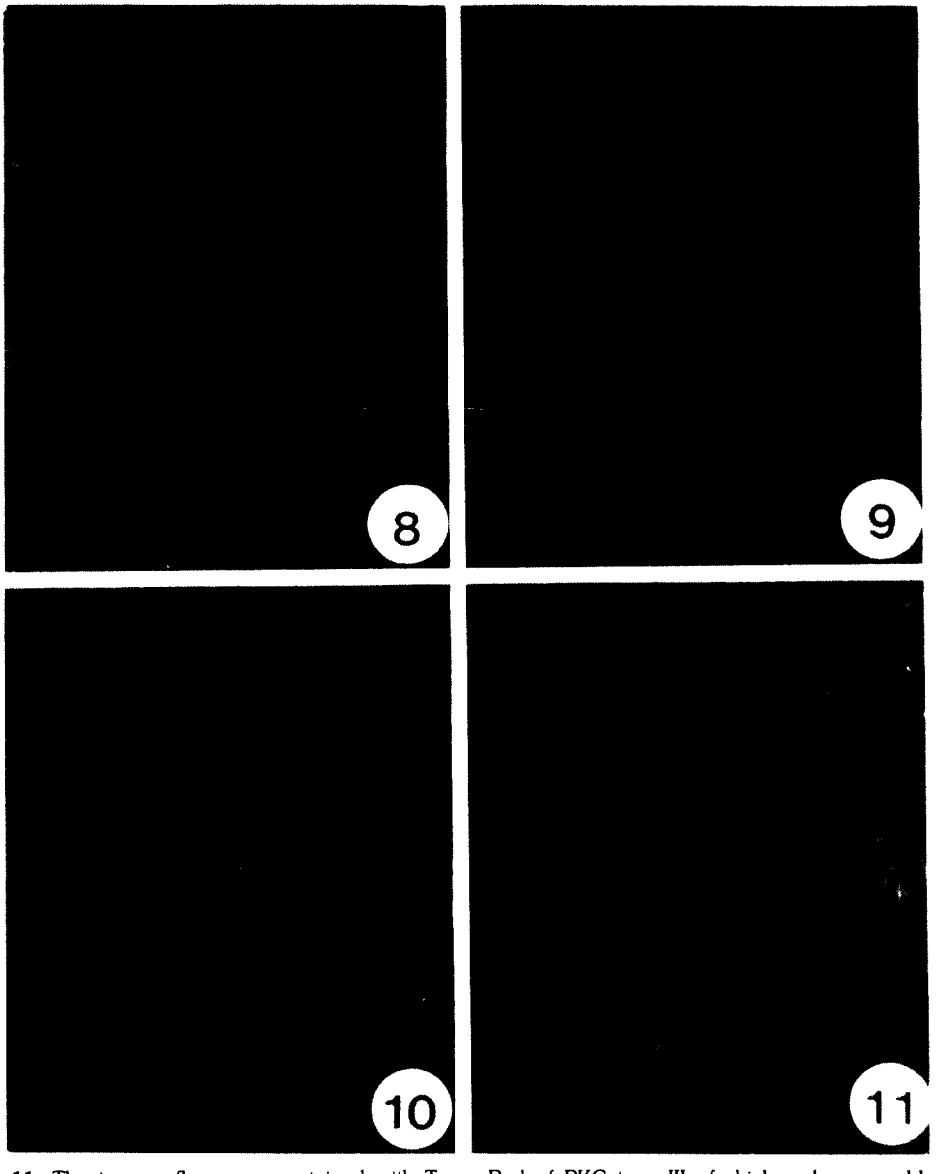



**Fig. 7.** The comparison of protein patterns treated with TPA and PDGF in myoblast exposed with [$^{32}$P] orthophosphoric acid. Lane 1: The cytosolic fraction of 1 hr-TPA treatment. Lane 2: The cytosolic fraction of 10 min-PDGF treatment.

effects of phosphorylation occurred at different times. The times of phosphorylation for TPA (Fig. 7, Lane 1) and PDGF-treatment (Fig. 7, Lane 2) were observed about 1 hr and 10 min respectively. After 6 hr-PDGF-treatment, most proteins could not be further phosphorylated (Fig. 7, Lane 3) indicating that most of the proteins did not induce continued activation but induced down regulation instead.

Immunofluorescence microscopy was used to determine whether the pattern change of antigen recognition of polyclonal antibody against PKC was occurred by the TPA treatment. Figs. 8-11 show the distribution of PKC after treatment with TPA according to the time course. As the time spans of treatment increased, PKC concentration declined in the cytoplasm. The antibody against PKC (type III) was mainly immunolabeled in the cytoplasm and nucleoli of control group (fig. 8). And after 12 hrs, immunolabeled pattern of PKC antibody was no changed in the control group, while the TPA induced the immunoreactive change. Previous studies have shown that agents capable of activating and/or translocating PKC from cytoplasmic to membrane compartments. Similarity, the PKC migrated already around the nucleus (Fig. 9) and the low level of immunolabeled enzymes were observed in the cytoplasm. And it is very interesting that PKC was also located on the nucleoli. After 24 hrs treated with TPA, the immunoreactivity of PKC gradually decreased by over 50% in the cytoplasm of myoblasts (Fig. 10), but the high immunolabeled nucleoli were unchanged with respect to immunoreactive density. But the immunoreactivity of PKC in the nucleoil as well as the 12 hr-TPA-treatment was also not decreased any further. At this time, the normal myoblasts retained a higher level of PKC than the 24 hr-TPA-treatment myoblasts and began to appear the syncytium. But no evidence found the syncytium in the myoblast treating with TPA for 24 hrs. After 72 hr-TPA-treatment (Fig. 11), the immunoreactivity of PKC in the cytoplasm was drastically lower than that of 12 hr-TPA-treatment. It has been shown that prolonged treatment with TPA caused to disappear PKC. This disappearance, the down-regulation of PKC, in the cytoplasm began to appear from at least 24 hrs later.




**Figs. 8-11.** The immunofluorescence stained with Texas-Red of PKC type III of chick embryo myoblast cells. **Fig. 8:** Immunoreactivity of control cells occurred throughout the cells. **Fig. 9:** The nucleoli and cytoplasm were immunoreacted with PKC after 1 hr-TPA treatment. **Fig. 10:** Cytoplasmic immunoreactivity gradually decreased, but immunoreactvity did not decrease after 6 hr-TPA treatment. **Fig. 11:** The immunoreaction was more decreased than earlier treatment times of TPA.

The long-term TPA-treatment in the chick embryonic myoblasts appears to be associated directly with the down regulation of PKC. Also, the immunoreactivity of PKC in the nucleoli was not decreased any further until this time. In the 72 hr-cultured myoblasts in normal medium, generally the syncytial cells were occurred over 70%. On the contrary, in the myoblasts treated with TPA for 72 hrs, the syncytial cells were not observed. Although the cytoplasmic PKC disappeared, the immunoreactivity of PKC was remained very clearly on the nucleolus which was single form.

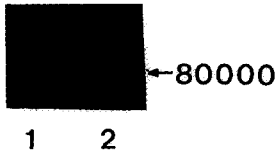



**Fig. 12.** After isolation of nuclei, nuclear PKC type was detected by immunoprecipitation techniques. Lane 1: Nuclear PKC III was observed in the control cells. Lane 2: Nuclear PKC III of 1 hr-TPA-treated cells had increased more than that of Lane 1.

The multinucleoli were not investigated because the myoblasts were blocked the transformation into the syncytium by TPA.

By the immunoprecipitation technique, the antibody against PKC III recognized *Mt.* 80,000 of the isolated nuclear PKC in the control group (Fig. 12, Lane 1). The appearance of the band in the control group clearly indicated that the nuclear PKC was already in existence before TPA treatment (Lane 1). After 1 hr-TPA-treatment, the isolated nuclear PKC was more increased than that of the control group (Lane 2). It appears that although the PKC was present in a very small amount, the antibody against PKC III was immunoreacted with the nucleolus.

## Discussion

The early stage of the differentiation of myoblasts in chick embryo is taking place that the cells appear somewhat elongated, the cells then increase in volume and round up. They have generally mononucleus and mononucleous. The next stage is that the cells are elongated, becoming almost spinder shape. Later these cells are gathered together and begin to be loss of cell membrane to make a syncytium. At this stage, the multinucleation and multinucleoleation are brought by the syncytium. We investigated a localization of Protein Kinase C (PKC) and a function of 12-0-tetradecanoylphorbol-13-acetate (TPA), as a tumor promoting phorbol eate, during the developing myoblasts.

Generally, PKC, an important mediator in signal transduction, has been characterized as a phospholipid-and $Ca^{++}$-dependent kinase (Kaibuchi *et al.*, 1981). Diacylglycerol (DAG) and inositol triphosphate are induced by the hydrolysis of phospholipids in the membrane (Nishizuka, 1984a). DAG stimulates PKC directly and inositol triphosphate induces $Ca^{++}$ mobilization from the endoplasmic reticulum and the sarcoplasmic reticulum of muscle cells. A small quantity of DAG dramatically increases the apparent affinity of PKC for $Ca^{++}$ (Castagna *et al.*, 1982; Nishizuka, 1984). DAG and $Ca^{++}$ are effected synergetically, and induce various physiological events (Nishizuka, 1984). The TPA is characterized by its ability to enhance the formation of tumors. The biological function of TPA is similar to that of DAG which stimulates PKC directly. PKC has been characterized as a phospholipid-and $Ca^{++}$-dependent kinase (Kaibuchi *et al.*, 1981). It has been suggested that PKC is the receptor of TPA. Likewise, PKC has been implicated in the proliferative action of platelet-derived growth factor (PDGF) (Kariya and Takai, 1987; Stiles, 1983). PDGF indirectly stimulates PKC through the PLC mediated hydrolysis of phosphoinositides. PDGF also induces DNA synthesis.

PKC I, II and III (Jaken and Kiley, 1987), which can be distinguished by their respective specific antibodies. Presently, at least 8 isozymes have been identified (Nishizuka, 1988; Parker *et al.*, 1986). But the translocation of PKC in myoblasts has not been studied extensively. The effects of signal transduction of PKC upon treatment with TPA were as follows: (1) all protein levels continuously kept in the control group (without TPA treatment); (2) most protein levels gradually decreased with long-term TPA-treatment. It is possible, therefore, that the down regulation of PKC is induced by chronic stimulation of long-term TPA treatment and (3) most protein levels are increased up to 24 hrs, thereafter the protein levels rapidly decreased by chronic stimulate of TPA. Understanding the mechanism of translocation of PKC will be an important step in comprehending the function of signal transduction (Nishizuka, 1984) and down regulation by TPA (Anderson and Salomon, 1985; Castagna *et al.*, 1982) and PDGF (Rozengurt, 1986; Presta *et al.*, 1989). Generally, the stimulated PKC was translocated from the cytoplasmic fraction to the membrane fraction

(Nishizuka, 1984) on treatment with TPA. A decrease in cytoplasmic PKC activity was coupled with an increase in membrane fraction PKC activity (Kuroki and Chida, 1988). The membrane associated form of PKC is easily proteolysed into a hydrophobic regulatory domain and a hydrophilic protein kinase domain (Ono and Kikkawa, 1987). The regulatory domain contains binding sites for phospholipid, DAG (or phorbol ester) and $Ca^{++}$ (Leach *et al.*, 1989). On the other hand, the protein kinase domain, the catalytic region, contains the ATP binding sequence and induces *c-myc and/or c-fos* gene expression (Kelly *et al.*, 1983; Muller *et al.*, 1984; Bravo and Muller, 1986).

It is possible that the proliferative and antiproliferative actions of PKC and governed by different subforms of this enzyme. The syncytium of myoblasts, that we do not observe by TPA treatment, could be commonly occurred in the normal condition. In the myogenesis, there is no proliferation of nuclei but that multinucleation is brought by a syncytium process in the early stages of myoblasts (Stockdale and Holtzer, 1961). It may be possibly the increased in the number of nuclei per cell may be brought about by mitosis (Alsteschul, 1946).

We observed in embryonic myoblasts may be the phosphorylation of some protein by the TPA-treatment for short-term. The activated PKC by TPA may induce phosphorylation of many kinds of PKC targeted proteins and as a result induces many cellular responses. It is well known that PKC phosphorylates many kinds of target proteins in DAG, $Ca^{++}$ and phospholipids (Collins and Rozengurt, 1982; Coussens *et al.*, 1986; Feuerstein *et al.*, 1984; Naka *et al.*, 1983). Our result confirm this study. We have shown that a *Mr.* 20,000 protein phosphorylated by PKC is a myosin light chain and could be related to the calmodulin-dependent protein in TPA-treatment. It has been established that the addition of phorbol esters induces phosphorylation of a myosin light chain kinase (Lukas *et al.*, 1988). And *Mr.* 40,000 protein phosphorylation was observed after treatment with TPA and PDGF. The phosphorylation of *Mr.* 40,000 protein was associated with an increase in serotonin concentration (Lyons *et al.*, 1975; Haslan and Lynhan, 1977) which is often coupled with PKC activation. Furthermore, autophosphorylation of PKC *Mr.* 80,000 also occurred after TPA-or PDGF-treatment for 1 hr and 10 min respectively. However, the PKC (type III) was initially depleted in the cytoplasm of myoblasts, and induced then down-regulation upon long-term treatment with TPA or PDGF. And chronic TPA-treatment also induces a PKC depletion or loss of phosphorylation of some proteins. Cooper and coworkers (1989) have shown in BC3H-1 myocytes that chronic TPA-treatment induces the loss of histone phosphorylation and PKC depletion. The loss of protein phosphorylation, dephosphorylation, is investigated in many targeting proteins of the PKC. Although the mechanism of down regulation of PKC and other targeting proteins of PKC is still not clear demonstrated in myoblasts, Stabel and coworkers (1987) proposed that phorbol esters may effect PKC synthesis, degradation or both.

The major finding of the present report concerns the relationship between the immunoreactivities of the cytoplasmic PKC and of nucleolar PKC after TPA treatment. In untreated control cells, high immunoreactivity of cytoplasmic PKC was observed throughout the cytoplasm. In the 1 hr-TPA-treatment, data from the cytoplasm to membrane translocation studies of PKC produced interesting results. PKC I and II exhibited different translocation patterns compared to PKC III. As a result, the translocation of PKC from cytoplasm to the peripheral nuclear membrane of myoblasts was investigated in the all of PKC isozymes. The antibody against PKC II was not immunoreacted in the nucleoli not only in the TPA-untreated group, but also in the TPA-treated cells. But the immunolabeled PKC did appear in the cytoplasm. On the contrary, the translocation of PKC III in myoblasts from cytoplasm to peripheral nuclear membrane took place very intensively. And the PKC III was also distributed on nucleolus in the both of control group and TPA-treated cells. Therefore, it is believed that PKC III may naturally exist in the nucleolus. This nucleolar PKC may be precursor PKC later distributed in the cytoplasm. And long-term TPA treatment, PKC in cytoplasm may take place a down-regulation in general. It may indicate that the binding of phorbol esters to their receptors reduces the affinity of the latter (PKC) for phorbol esters on subsequent exposure

(Kuroki and Chida, 1988) and that PKC down regulation is a result of proteolytic degradations of PKC (Chida *et al.*, 1986). But the immunoreactivity of the PKC antibody bound with the nucleoulus is neither decreased nor increased. The PKC antibody (type III) could be bound very strong with the nucleolar components. And no evidence was found the down-regulation of the nucleolar PKC although long-term treatment of TPA was performed to the myoblasts. The PKC (type III) binding with nucleolus may not be due to inhibition of receptor-ligand complex internalization.

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## 근세포 분화에 관한 연구 : 계배의 Myoblasts에 있어서 Protein Kinase C (PKC)의 인산화작용과 Down Regulation

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12-0-tetradecanoylphorbol 13-acetate(TPA)나 platelet-derived growth fator의 단기간 처리시에는 Protein Kinase C(PKC)를 활성시킴과 더불어 세포질로부터 핵막주변으로 이동을 유도하였다. 그리고 그 PKC는 TPA에 의해 직접적으로 또는 PDGF에 의해 간접적으로 활성화 되어 여러종류의 PKC의 표적단백질을 인산화 시키며 많은 생물학적인 반응을 일으킨다. 특히 세포질에 있는 PKC는 TPA에 의해서는 1시간내에, PDGF에서는 10분내에 각각 인산화작용이 일어났다.

TPA나 PDGF의 장시간 처리시에는 양자가 다 myoblast의 세포질에 있는 PKC의 down-regulation이나 translocation을 일으키게 유도하였다. PKC 동위효소의 translocation에 있어서, 세포질에 있는 PKC I과 II의 양상은 PKC III 동위효소와 매우 비슷하였다. 그러나 인에서는 TPA가 PKC III antibody의 면역반응성은 억제나 어떤 down-regulation을 유도하지 않았다. 이러한 관찰은 PKC의 동위효소들은 각각 다른 영향을 인이나 세포질에 미치는 것을 나타낸다. 그리고 down-regulation된 myoblasts는 PKC antibody의 낮은 면역반응성을 나타낸다.