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Studies on the Differentiation of Skeletal Muscle Cells *in vitro*: Protein Kinase C in the Differentiation of Skeletal Muscle Cells

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Treating 12-0-tetradecanoylphorbol 13-acetate (TPA) or platelet-derived growth factor (PDGF), the signal transduction of protein kinase C (PKC) is occurred by the phosphorylation. However the targeting proteins phosphorylated by PKC were found to be different proteins in molecular weights when TPA or PDGF was treated to the myoblast. In the TPA-treated myoblast cells, the protein of *Mr.* 20 Kd was phosphorylated. In the PDGF-treated cells, the protein of *Mr.* 40 Kd was phosphrylated, while the protein of *Mr.* 20 Kd which phosphorylated in the TPA-treatment was dephosphorylated. These results indicate that not only TPA and PDGF are different in activating the signal transduction pathways, but also they may involve in the down regulation of PKC during the long-term treatment. But PDGF gave rise more rapidly down regulation than in the case of TPA.

Using immunocytochemical approach, two distinct PKC isozymes, PKC II and PKC III, have been localized in cytoplasm and both cytoplasm and nucleolus, respectively. Therefore, the expression of two types of PKC in the myoblast suggests that the isozymes of PKC may involve in each different pathway of signal transduction or down-regulation.

KEY WORDS: Protein kinase C, Myoblast, Phosphorylation, Down-regulation, Signal transduction

Protein kinase C is widely distributed in many animals tissues (Jaken and Kiley, 1987; Nishizuka, 1986), as well as in the fibroblast cells (Fearon and Tashijan, 1985; Skoglaund *et al.*, 1985; Girard *et al.*, 1987). Protein kinase C is a mediator of the transduction for extracellular signals that induce phosphatidylinositol breakdown (Habenicht *et al.*, 1981). The activation of protein kinase C is required Ca^{++}, phospholipid and diacylglycerol. PDGF (platelet-derived growth factor) that generates diacylglycerol leads to signal translocation of cytoplasmic protein kinase C to membrane *via* PDGF-specific receptor. The translocated protein kinase C is activated in the membrane. Nishizuka (1983) demonstrated that the activation of protein kinase C is prevented when phosphatidylinositol breakdown was inhibited.

TPA (12-0-teradecanoylphorbol 13-acetate), a well-known tumor promoter, has been revealed to mimic the function of diacylglycerol to activate the protein kinase C. TPA, however, activates directly protein kinase C by-passing the agonist-mediated increase of diacylglycerol. TPA give rises the phosphorylation of protein kinase C associated with the plasma membrane.

TPA or PDGF are thought to contribute to the cell response, such as the onset of DNA replication *via* the phosphorylation of their targeting pro-

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teins by the phosphorylation of protein kinase C (Berridge, 1984). Although alterations in the phosphorylation patterns by protein kinase C were investigated in many kinds of cells and tissues, it is not quite evident in chick myoblasts. Therefore, the present study deals with the signal transduction and signal translocation of protein kinase C following treated with TPA or PDGF.

Materials and Methods

Primary Cell Culture

The 10th day chick embryos were purchased from a local hatchery in Kim-hae city. The embryos in egg shells were aseptically collected and transferred into minimum essential medium (MEM) in petri dishes and washed three times with MEM. Under the dissecting microscope, the skin and loose connective tissue of chick breast were removed and pectoralis muscles were gently isolated. The isolated muscles were minced with a sharp knife and the minced muscle tissue was incubated for 30 min at 36°C in MEM with trypsin (1%). After incubation, the muscle fragments were further loosened with repeated pipettings, washed trypsin with MEM and finally cells were precipitated by centrifugation. The myoblast cells were filtered through Swany-Filter and transferred onto the collagen treated culture flasks. After 10 min incubation, the myoblast cells which were mainly contained in the supernatant were collected and cultured in 811 medium (100 ml of MEM, 12.5 ml of horse serum, 12.5 ml of chick embryo extract, and 1.25 ml of antibiotic-antimycotic) for 1 day. And the next day, the culture medium was changed from 811 medium to 8102 medium (110 ml of MEM, 12.5 ml of horse serum, 2.5 ml of embryo extract, and 1.25 ml of antibiotic-antimycotic).

Immunocytochemical Localization of Protein Kinase C

Myoblast cells (1×10^2) were seeded into wells (1 cm^2) in collagen coated Lab-Tek chamber slides, and were allowed to attach and grow for 24 hrs in the 811 medium. Then, the myoblasts were cultured for another 48 hours in the serum-free 8102 medium. Upon the completion of culture, the serum-starved cells were washed three times with serum-free MEM, and were further incubated in MEM containing 200 nM of 12-0-tetradecanoylphorbol 13-acetate (TPA; Pharmacia P-L Biochemicals) for 10 min, 20 min, 30 min, 1 hr, 6 hrs, 12 hrs, and 24 hrs or 5 ng/ml of platelet-derived growth factor (PDGF, PDGF Inc.) for 5 min, 10 min, 1 hr, 6 hrs, 12 hrs and 24 hrs. After incubation the cells were washed three times with PBS-washing buffer for 5 min each. The cells were then fixed with 2% formaldehyde in PBS for 5 min at room temperature, were washed three times for 15 min at room temperature, were washed three times for 15 min with PBS and permeabilized with cold absolute methanol for 5 min. They were washed three times with PBS for 15 min and blocked with 2% bovine serum albumin (BSA) in PBS for 1 hr. After completion of the above preparatory steps, the fixed cells were immunoreated with the antibodies against protein kinase C (185 or 186) in PBS containing 2% normal goat serum and 0.5% Triton X-100 for 24 hrs at 4°C. Following this reaction, cells were washed four times with PBS for 20 min, and the bound antibodies were visualized following the protocols based on avidin-biotin-Texas Red complex formation (Bayer and Wilchek, 1979; Guesdon *et al.*, 1979).

Briefly, primary antibody treated cells 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 then washed three times with PBS three times for 15 min and reacted with 0.15% streptoavidin-Texas Red in PBS containing 1% BSA and 0.05% Tween-20, and washed twice with PBS for 15 min, then washed finally with in PBS containing 1% BSA. After the completion of immunostaining, slides were mounted with 50% glycerol in PBS, and viewed and photographed with P800/1600 Kodak Ektachrome film under an Olympus BH-2 microscope at × 400.

Autoradiography for Phosphorylation Study

The myoblast cells were initially cultured in

MEM containing horse serum for 24 hrs. And w/ or w/o serum-treated cells were cultured for another 48 hrs in phosphate-free MEM. Upon the completion of culture, cell layer was washed with serum- and phosphate-free (SPF) MEM and scraped off the dish to harvest the cells. After brief centrifugation, the pellet of cells was recovered and incubated in SPF-MEM containing [^{32}P] orthophosphoric acid (200 μCi/ml) for 2 hrs. The ^{32}P-exposed cells were treated either with TPA (200 nM) for 30 min, 1 hr, 6 hrs, 12 hrs, 24 hrs, and 48 hrs or with PDGF (50 ng/ml) for 10 min, 6 hrs, 12 hrs, 24 hrs, and 48 hrs. After treatment cells were breifly washed in PBS and homogenized with Potter-Elvehjam homogenizer. The homogenate was then centrifuged at 2,000 × g for 20 min, and the nuclear pellet was discarded. The recovered supernatant was sonicated in homogenation buffer containing 50 mM Tris/HCl (pH adjusted to 8.0), 5 mM NaF, 2 mM sodium vanadate, 2 mM phenylmethylsulfonyl fluoride (PMSF), 0.25 mg/ml aprotinin, 5 mM ethyleneglycol bis (β-aminoethyl ether)-N, N, N′, N′-tetraacetic acid (EGTA), 0.1 mM Dithiothreitol (DTT), and 4 mM ethylenediaminotetraacetic acid (EDTA). The part of sonicated homogenates were taken as crude samples. And then the other homogenates were centrifuged at 100,000 × g for 1 hr. The supernatants and pellets were taken as cytosol and membrane fractions, respectively. The membrane fractions were resonicated in homogenation buffer containing 0.5% sodium deoxycholate. After sonication, all samples were subjected to SDS/polyacrylamide gel electrophoresis. After the run, gels were stained with coomassie brilliant blue R 250, destained, dried and finally exposed to Kodak X-Omat AR film for 24 to 48 hrs. Molecular weight estimations were made using the following markers: lysozyme (*Mr.* 14,300), β-lactoglobulin (*Mr.* 18,400), trypsinogen (*Mr.* 24,000), bovine albumin (*Mr.* 66,000), β-galactosidase (*Mr.* 116,000).

Isolation of Nuclei

To determine the localization of PKC in the nucleus, the myoblast cells were treated with TPA (200 nM) for 1 hr and then centrifuged at 10,000 × g for 3 min. The pellet was suspended and stayed in swollen buffer (10 mM Tris/HCl, pH 7.4 containing 10 mM NaCl, and 1.5 mM $MgCl_2 \cdot 6H_2O$) for 20 min. The swollen cells were homogenized in the swollen buffer with Potter-Elvehjam homogenizer. And homogenates were centrifuged at 1,000 × g for 20 min. The pellet were suspended and washed in 0.25 M sucrose in buffer A (10 mM Tris/HCl, pH 7.4 containing 3 mM $CaCl_2 \cdot 2H_2O$) and centrifuged at 10,000 × g for 10 min. The crude nuclear and cytosol were obtained. And the curde 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 rinsed twice with 0.25 M sucrose in buffer A without Triton X-100. Then the pellet was used as purified nuclei.

Immunoblotting

The primary cultured, then serum starved-myoblasts were treated with TPA (200 nM) for 1 hr. The nucleus and cytosol fractions were isolated as samples according to the above mentioned methods. The samples were electrophoresed on the polyacrylamide slab gel, and transferred to nitrocellulose paper in the electrode buffer (20 mM Tris-HCl, 150 mM glycine and 20% methanol). After transferring the nitrocellulose paper was saturated with 2% BSA and was incubated overnight at 4°C with primary antibody (15 μg/ml). The paper was washed in TBS (0.05 M Tris/HCl, pH 7.5, 0.2 M NaCl, 0.5% bovine serum albumin (BSA), and 0.1% Tween-20) and incubated with biotinylated secondary antibody (biotinylated goat-anti-rabbit IgG) for 4 hrs at 4°C. The incubated paper was washed three times for 15 min with TBS. Finally, it was incubated with streptavidin conjugate horseradish peroxidase, followed by 4-chloro-1-napthol and hydrogen peroxide treatment. The nitrocellulose paper was washed, dried and photographed.

Results

To determine the effects of the PKC and other proteins in the cellular signalling system, the antibodies against PKC were immunoreacted onto the myoblasts after TPA and PDGF treatment. In this experiment, we used two kinds of antibodies: the antibody (185) against PKC type II and the antibody (186) against PKC type III.

In the normal chick myoblasts, PKC type II was mainly detected in the cytoplasm (Fig. 1), while PKC type III was localized in both cytoplasm and nucleolus (Fig. 2). Also the multinucleoli were clearly immunoreacted with the antibody 186.

In the TPA-treated myoblast for 1 hr (Fig. 3), any phenomenon of the translocation of protein kinase C, type II or type III, was not clearly observed. The labeling of antibody 186 (type III) was also found on the nucleoli within the nucleus comparable to control. Especially, the high-level immunoreactivity appeared in the nucleoli. However, the protein kinase C did not migrate into the nucleus from the cytoplasm.

In the TPA-treated cells for 12 hrs, the immunoreactivity of antibody (186) in the cytoplasm was lower than that of 1 hr-TPA treated-cells (Fig. 3) because the cytoplasmic protein kinase C level began to decrease (Fig. 4). Also, the level of type II protein kinase C, in the cytoplasm began to decrease as well as type III. Those results suggest that TPA treatment causes the down-regulation of protein kinase C between 1 hr and 12 hrs. On the contrary, the immunolabeling of nucleoli, was even more distinct and the pattern maintained continuously. In the TPA treated-cells for 24 hrs (Fig. 5), the antibody 186 was weakly labeled cytoplasmic protein kinase C. The immunolabeling was not changed to decrease or increase in the nucleoli. In the TPA treated-cells for 48 hrs (Fig. 6), the protein kinase C was severely decreased in the cytoplasm. The cytoplasmic protein kinase C in this time had maintained the lowest level in the immunoreaction than that in other treated times. But no nucleolar changes in the immunoreactivity were observed.

In order to clarify in which kinds of proteins show the phosphorylation or dephosphorylation according to the time course, myoblast cells were labeled with ^{32}P. In the 20 min-TPA-treatment (Fig. 7), only the protein kinase C, 80,000 dalton, was phosphorylated although most other proteins were not phosphorylated at this time.

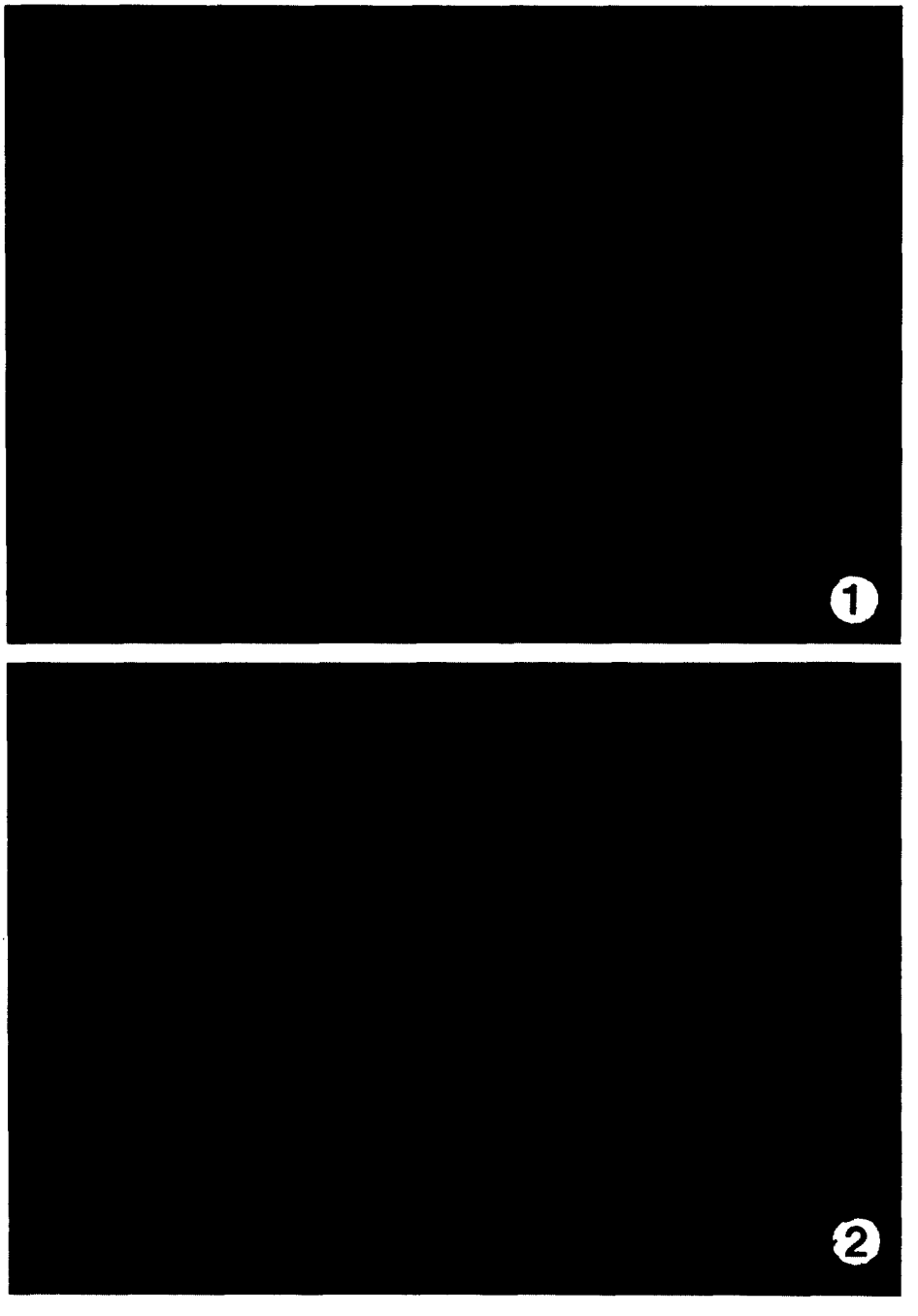
In the 6 hrs-TPA-treated cells, the protein of 20,000 dalton was phosphorylated as shown in Fig. 8.

In the myoblast cells treating with PDGF for 10 min (Fig. 9), there were two kinds of phosphorylated protein patterns, *Mrs.* 80,000 and 40,000 in the cytosol fraction. The protein pattern of *Mr.* 20,000 showed dephosphorylation.

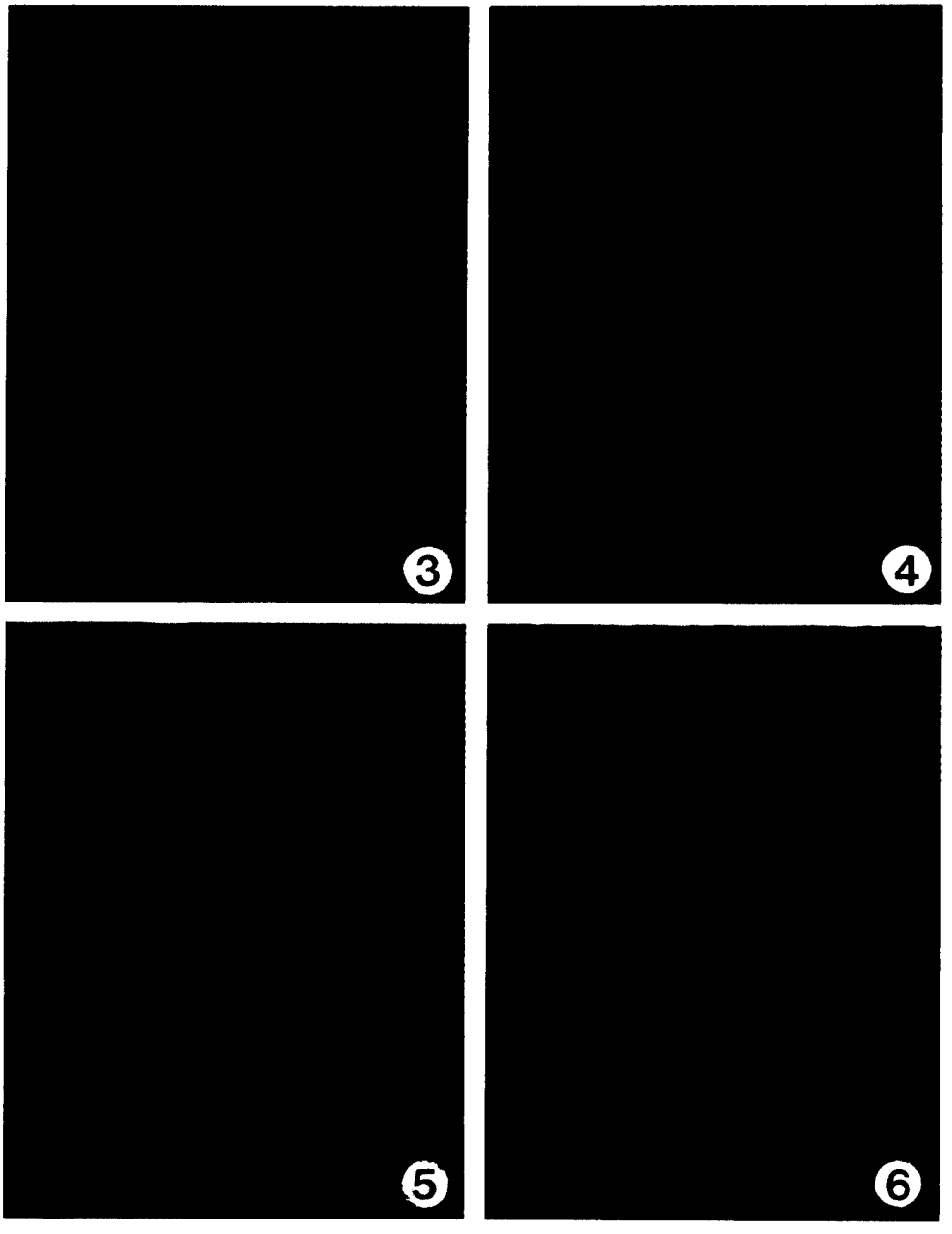
In the PDGF-treatment cells for 6 hrs (Fig. 10), the dephosphorylation was revealed not only in the protein kinase C of *Mr.* 80,000, but also in the proteins of *Mrs.* 35,000 and 40,000 which showed the phosphorylation in the 10 min treated-cells. The most proteins were degraded. This phenomenon of degradation may be the down-regulation.

Fig. 11 shows the bands of protein kinase C, type III, by immunoblotting. The band of the enzyme was occurred in the cytoplasm (Lane 1), and in the nucleus fraction of control group (Lane 2). The enzyme of protein kinase C in the nucleus was revealed smaller quantity than that of cytoplasm. In the TPA-treated cells for 1 hr, the band of cytoplasmic enzyme (Lane 3) was similar to that shown in control group. At this time, the band of the nuclear enzyme was more increased than that of the nucleus of control group.

The protein kinase C, type III, was not clearly detected the migration from cytoplasm to nucleus by using immunofluorescent method although the protein kinase C was immunolabeled in the nucleolus in control group. In the immunoblotting, however, the protein kinase C in the nucleus fraction was slightly increased when myoblast cells were treated with TPA for 1 hr although the increasing of nucleolar PKC was apart from the reason.



Figs. 1 and 2. Immunofluorescent photomicrographs. Fig. 1. Normal myoblasts reacted with antibody against PKC type II. The enzyme is located in cytoplasm. Fig. 2. antibody against PKC type III was reacted in normal myoblasts. The enzyme is detected both in the nucleoli and cytoplasm.



Figs. 3-6. Immunofluorescent photomicrographs of the TPA-treated myoblasts. Fig. 3. TPA-treated cells for 1 hr. These is no evidence that the type III enzyme is not migrated into the nucleus. But the immunolabeling is investigated only in the nucleolus. Fig. 4. TPA-treatment for 12 hrs. Note the clearly labeled nuclei with antibody 186. Fig. 5. TPA-treated cells for 24 hrs. The immunoreaction is distinctively present in the nucleolus, but the enzyme is degraded in the cytoplasm. Fig. 6. TPA-treated cells for 48 hrs. The enzyme was almostly degraded in the cytoplasm, but the immunoreactivity of nucleoli existed.

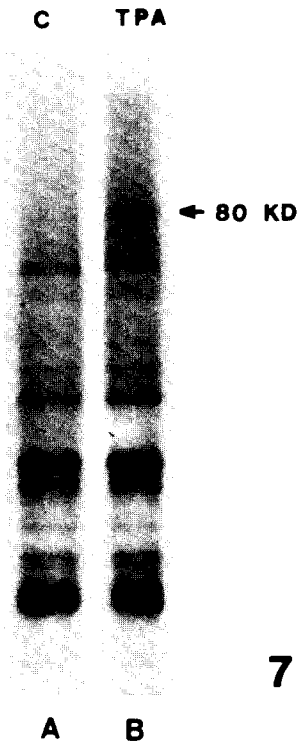


Fig. 7. The cytosolic fractions of the myoblast cells were treated with and without TPA for 20 min. A is the control group without TPA treatment. B is TPA-treated group for 20 min. The protein kinase C of *Mr.* 80,000 took place phosphorylation, but there was not occurred any phosphorylation of other proteins in B lane.

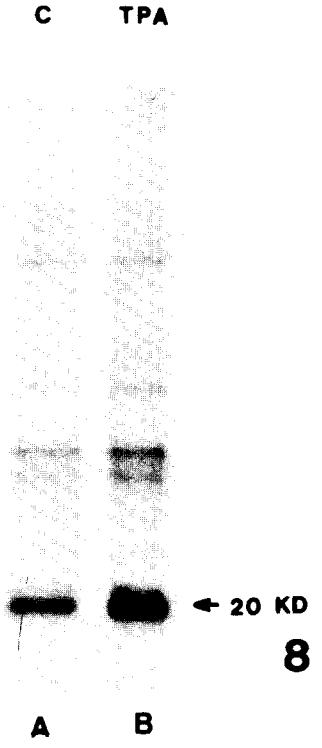


Fig. 8. The cytosolic fractions of the myoblast cells treated with and without TPA for 6 hrs. A is the control group, and B is the TPA-treatment group. The protein of *Mr.* 20,000 was phosphorylated only.

Discussion

The involvement of PDGF as agonist on the PDGF-specific receptor induces the phospholipase C (PLC) that catalyses the hydrolysis of phosphatidylinositol 4,5-bisphosphate (PIP_2)(Berridge, 1984) to form the potential second messengers inositol 1,4,5-triphosphate (IP_3) and diacylglycerol (DAG)(Berridge, 1987; Rozengurt, 1986). Blumberg and co-workers (1984) have shown that DAGs, natural activators of protein kinase C, competitively inhibit phorbol ester binding, which is consistent with their being the postulated endogenous phorbol ester analogs. The phorbol esters are tumor promotors (Anderson *et al.*, 1985; Frantz *et al.*, 1979; Sivak and Van Duuren, 1967). The hydrolysis of polyphosphoinositides, Ca^{++} mobilization and the increase of DAG induced the activation of PKC. Kishimoto and co-workers (1980) have postulated that PKC is a mediator of signal transmission.

The signal transduction system of *raf* in which effects of TPA or PDGF can be observed in NIH3T3 cells has been previousely demonstrated by us from the cytoplasm to nucleus in the *raf*-transformed NIH3T3 fibroblast (Rapp *et al.*, 1988). The protein kinase C has been shown a serine/

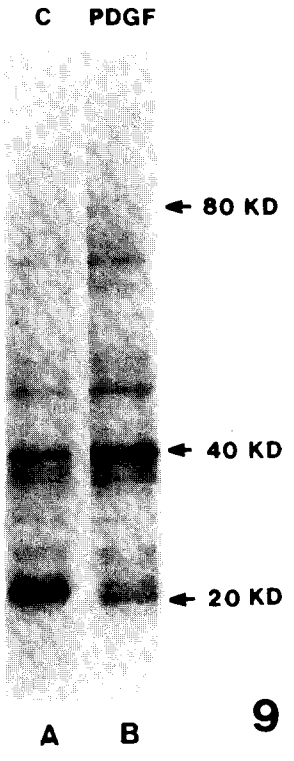


Fig. 9. The cytosolic fractions of the myoblast cells treated with and without PDGF for 10 min. A is the control group without PDGF treatment. B is the PDGF-treated group for 10 min. The proteins of *Mrs.* 40,000 and 80,000 daltons were phosphorylated, but the protein of *Mr.* 20,000 was dephosphorylated in the B lane.

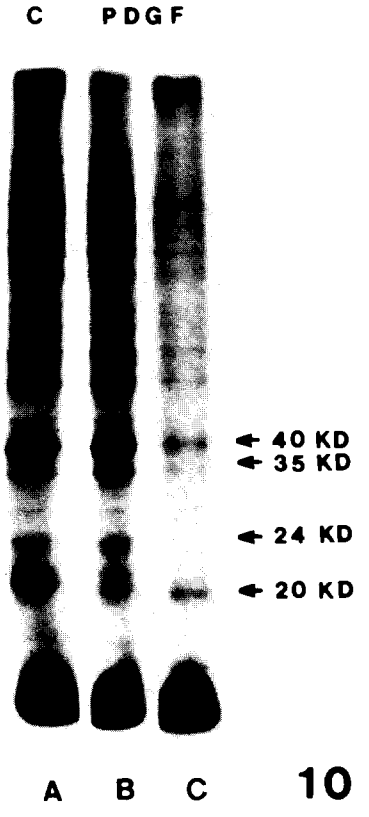


Fig. 10. The crude fractions of the myoblast cells treated with and without PDGF. A is the control group without PDGF treatment with for 10 min. B is the PDGF-treated group for 10 min, and C is PDGF-treated group for 6 hrs. After 10 min-PDGF-treatment, the proteins of *Mrs.* 35,000 and 40,000 were mainly phosphorylated in the B lane, while most proteins were dephosphorylated in the C lane atter 6 hr-PDGF-treatment.

threonine-specific protein kinase (Gilmore and Martin, 1983; Schwantke *et al.*, 1985) and contained a characteristic cystein-rich sequence in regulatory domain similar to those of *raf* protein kinase (Bonner *et al.*, 1986; Parker *et al.*, 1986; Tahira *et al.*, 1987).

During the differentiation of the myoblast of chick embryo, PKC is generally distributed in the cytoplasm. But in the results of this study, a kind of the isozymes of the protein kinase C was distributed both in the cytoplasm and the nucleolus: the immunoreactivity of the antibody 186 was occurred in the nucleolus and the cytoplasm, while the antibody 185 did not immunoreact with the nucleolus. The antibody 185 was occurred only the cytoplasm of the myoblast. During the differentiation of myoblast, the phenomenon of translocation of the PKC type II from cytoplasm into nucleus was very difficult to detect by treating with TPA or PDGF by using immunofluorescent method. It seems to indicate that the PKC type II may not be involved in the translocation of itself into the nucleus by TPA or PDGF. However, the

Fig. 11. Immunoblotting of PKC type III in the myoblasts treated w/ and w/o TPA for 1 hr. Lane 1 is the cytosolic fraction of the control group. The immunolabeled enzyme is occurred in this cytosolic fraction. Lane 2 is the nucleus fraction of the control group. The immunolabeled enzyme is also revealed, but the quantity of the enzyme is less than that of the cytosolic fraction. Lane 3 is the cytosolic fraction of TPA-treatment for 1 hr. Lane 4 is the nucleus fraction of TPA-treatment for 1 hr. The immunoreacted enzyme in the nucleus fraction existed much more than that in the nucleus fraction of the control group.

antibody 186 against the PKC type III was very intensely immunolabeled by PDGF-treatment in a time-dependent manner. It may suggest that a same region of amino acids sequences of PKC type III may locate in the nucleolar protein, or precursors of the enzyme may be produced from nucleolus.

In ^{32}P-labeled myoblast cells of chick embryo, PKC of *Mr.* 80,000 is constitutively autophosphorylated in TPA-treated for 20 min. The PKC type III requires much more quantity of Ca^{++} ions than other types of PKC for the activation. Subsequently, PKC give rises phosphorylation to the targeting protein. Such being the case, the phosphorylation of the targeting protein (*Mr.* 20,000) which was induced by the influence of the phosphorylation of PKC taken place at least 6 hrs later in TPA-treatment. In the PDGF treated-myoblast, the targeting proteins (*Mrs.* 35,000 and 40,000) were already phosphorylate at 10 min. The reaction pathway of PDGF runs complicately *via* receptor of PLC to produce IP_3 and DAG (Nishizuka, 1986; Smith and Stiles, 1981). The response of the phosphorylation by PDGF-treatment was more rapidly proceeded than that of TPA-treatment although TPA reacts directly to the PKC (Castagna *et al.*, 1982; Nishizuka, 1984; Takai *et al.*, 1985). It may suggest that in PDGF-treatment, IP_3 makes the relasing of Ca^{++} from endoplasmic reticulum, and Ca^{++} then may directly effect to PKC. While TPA may not directly and rapidly stimulate to produce Ca^{++} in order to activate PKC. But in the normal condition of cell, the Ca^{++} may be contained enough quantity in the cytoplasm for the activation of PKC.

The phosphorylation of the protein of the *Mr.* 20,000 was decreased 10 min later in the PDGF-treatment. It is indicated that protein phosphatase may give to dephosphorylation of *Mr.* 20,000 because the activation of protein phosphatase was induced by Ca^{++} ions (Burnham *et al.*, 1986). But the most proteins are also dephosphorylated 6 hours later. The dephosphorylation has been shown a down-regulation. In general, down-regulation (Young *et al.*, 1987) is caused by long-term over-expressed cell condition of effectors (TPA or PDGF). The protein kinase C which existed in the cytoplasm is bound with phosphatidylserine of the membrane, Ca^{++}, DAG, and then activated (Fearon and Tashjian, 1985; Hirota *et al.*, 1985; Kraft and Anderson, 1983). At this time, the regulatory- and catalytic-domains are divided from the PKC by a specific protease that preferentially proteolyses the membrane-bound enzyme (Kishimoto *et al.*, 1980) which was activated by Ca^{++}. The antibody recognizing the regulatory-domain fragment of PKC was not observed in the cytoplasm except the membrane, while the catalytic domain fragments were detected in the cytoplasm (Parker *et al.*, 1986). It may happen that the activated catalytic domain goes to the cytoplasm and may give a certain signal into the nucleus *via* a mechanism of other enzyme path-

way or activates a certain targeting protein for cell responses. For this above reason, the intact protein kinase C in the cytoplasm showed the degradation phenomenon. Thus, the down-regulation is effected through the degradation of the cytoplasmic intact protein kinase C. It appears then that the presence of TPA as tumor promotor or PDGF as growth factor leads to an increased rate of degradation of the intact PKC in the immunofluorescent experiment. Thus, down regulation of PKC may be due to increase degradation of the polypeptide.

In conclusion, the results of the present study suggest that the proteins which were phosphorylated under the influence of the active PKC may inhibit or block the over-expressed role of a certain mechanism in order to turn to the normal condition of cell, that TPA and PDGF activate largely separated phosphorylation pathway in this myoblast. Also, in this result the PKC may be not directly migrate into the nucleus, but the signal transduction of PKC may take place through other complicated pathways from the cytoplasm to the nucleus.

References

Anderson, W. B., A. Estival, H. Tapiovaara, and R. Gopalakrishna, 1985. Altered subcellular distribution of protein kinase C (a phorbol ester receptor). Possible role in tumor promotion and the regulation of cell growth: relationship to changes in adenylate cyclase activity. *Adv. Cyclic Nucleotide Res.* **19:** 287-306.

Bayer, E. A., and M. Wilchek, 1979. The biotin transport system in yeast. *Methods in Biochem. Anal.* **26:** 1-45.

Berridge, M. J., 1984. Inositol triphosphate and diacylglycerol as second messengers. *Biochem. J.* **220:** 345-360.

Berridge, M. J., 1987. Inositol triphosphate and diacylglycerol: two interacting second messengers. *Ann. Rev. Biochem.* **56:** 159-193.

Blumberg, P. M., S. Jaken, B. Konig, N. A. Sharkey, K. L. Leach, A. Y. Jeng, and E. Yeh, 1984. Mechanism of action of the phorbol ester tumor promoters: specific receptors for lipophilic ligands. *Biochem. Pharmacol.* **33:** 933-940.

Bonner, T. I., H. Oppermann, P. Seeberg, S. B. Kerby, M. A. Gunnell, A. C. Young, and U. R. Rapp, 1986. The complete coding sequence of the human *raf* oncogene and the corresponding structure of the c-*raf*-1 gene. *Nucleic Acids Res.* **14:** 1009-1015.

Burnham, D. B., P. Munowitz, S. R. Hootman, and J. A. Williams, 1986. Regulation of protein phosphorylation in pancreatic acini. Distinct effects of Ca^{++} inophore A23187 and 12-0-tetradecanoylphorbol 13-acetate. *Biochem. J.* **235:** 125-131.

Castagna, M., Y. Takai, K. Kaibuchi, K. Sano, U. Kikkawa, and Y. Nishizuka, 1982. Direct activation of calcium-activated, phospholipid-dependent protein kinase by tumor-promoting phorbol esters. *J. Biol. Chem.* **257:** 7847-7851.

Fearon, C. W., and A. H. Tashjian Jr., 1985. Thyrotropin-releasing hormone induces redistribution of protein kinase C in GH_4C_1 rat pituitary cells. *J. Biol. Chem.* **260:** 8366-8371.

Frantz, C. N., C. D. Stiles, and C. D. Scher, 1979. The tumor prototer 12-0-tetradecanoylphorbol 13-acetate enhances the proliferative response of Balb/c-3T3 cells to hormonal growth factors. *J. Cell Physiol.* **100:** 413-424.

Gilmore, T., and S. Martin, 1983. Phorbol ester and diacylglycerol induce protein phosphorylation at tyrosine. *Nature* **306:** 487-490.

Girard, P. R., V. L. Stevens, P. J. Blackshear, A. H. Merrill, Jr., J. G. Wood, and J. F. Kuo, 1987. Immunocytochemical evidence for phorbol ester-induced directional translocations of protein kinase C in HL60, K562, CHO, and E7SKS cells: possible role in differentiation. *Cancer Res.* **47:** 2892-2898.

Guesdon, J. L., T. Termynck, and S. Averemeas, 1979. The use of avidin-biotin interaction in immunoenzymatic techniques. *J. Histochem. Cytochem.* **27:** 1131-1138.

Habenicht, A. J. R., J. A. Glomset, W. C. King, C. Nist, C. D. Mitchell, and R. Ross, 1981. Early changes in phosphatidylinositol and arachidonic acid metabolism in quiescent Swiss 3T3 cells stimulated to divide by platelet-derived growth factor. *J. Biol. Chem.* **256:** 12329-12335.

Hirota, K., T. Hirota, G. Aguilera, and K. J. Catt, 1985. Hormone-induced redistribution of calcium-activated phospholipid-dependent protein kinase in pituitary gonadotrophs. *J. Biol. Chem.* **260:** 3243-3246.

Jaken, S. and S. C. Kiley, 1987. Protein kinase C purification and characterization of three types of protein kinase C from rabbit brain cytosol. *Proc. Natl. Acad. Sci.* **84:** 4418-4422.

Kishimoto, A., Y. Takai, T. Mori, U. Kikkawa, and Y. Nishizuka, 1980. Activation of calcium and phospholi-

pid-dependent protein kinase by diacylglycerol: its possible relation to phosphatidylinositol turnover. *J. Biol. Chem.* **255:** 2273-2276.

Kraft, A. S., and W. B. Anderson, 1983. Phorbol esters increase the amount of Ca^{2+}, phospholipid-dependent protein kinase associated with plasma membrane. *Nature* **301:** 621-623.

Nishizuka, Y., 1983. Calcium, phospholipid turnover and transmembrane signalling. *Phil. Trans. R. Soc. Lond.* **B302:** 101-112.

Nishizuka, Y., 1984. The role of protein kinase C in cell surface signal transduction and tumor promotion. *Nature* **308:** 693-698.

Nishizuka, Y., 1986. Studies and perspective of protein kinase C. *Science* **233:** 305-312.

Parker, P. J., J. Coussens, N. Totty, L. Rhee, S. Young, E. Chen, S. Stabel, M. D. Waterfield, and A. Ullrich, 1986. The complete primary structure of protein kinase C-the major phorbol ester receptor. *Science* **233:** 853-859.

Rapp, U. R., G. Heidecker, M. Huleihel, J. L. Cleveland, W. C. Choi, T. Pawson, J. N. Ihle, and W. B. Anderson, 1988. *raf* Family serine/threonine protein kinases in the mitogen signal transduction. *Cold Spring Harbor Symposia on Quantitative Biology* **LIII:** 173-183.

Rozengurt, E., 1986. Early signals in the mitogenic response. *Science* **234:** 161-166.

Schwantke, N., F. Le Bouffant, M. Doree, and C. J. Le Peuch, 1985. Protein kinase C: properties and possible role in cellular division and differentiation. Biochim. **67:** 1103-1110.

Sivak, A. and B. L. Van Durren, 1967. Phenotypic expression of transformation: induction of cell culture by a phorbol ester. *Science* **157:** 1443-1444.

Skoglaund, G., A. Hansson, and M. Ingelman-Sunberg, 1985. Rapid effects of phorbol eaters on isolated rat adipocytes. Relationship to the action of protein kinase C. *Eur. J. Biochem.* **148:** 407-412.

Smith, J. C. and C. D. Stilles, 1981. Cytoplasmic transfer of the mitogenic response to platelet-derived growth factor. *Proc. Natl. Acad. Sci.* **78:** 4363-4369.

Tahira, T., M. Ochiai, K. Hayashi, M. Nagao, and T. Sugimura, 1987. Acitvation of human c-*raf*-1 by replacing the n-terminal region with different sequences. *Nucleic Acid Res.* **15:** 4809-4820.

Takai, Y., K. Kaibuchi, T. Tsuda, and M. Hoshijima, 1985. Role of protein kinase C in transmembrane signalling. *J. Cell. Biochem.* **29:** 4809-4820.

Takai, Y., K. Kaibuchi, T. Tsuda, and M. Hoshijima, 1985. Role of protein kinase C in transmembrane signalling. *J. Cell. Biochem.* **29:** 143-155.

Young, S., P. J. Parker, A. Ullrich, and S. Stabel, 1987. Down-regulation of protein kinase C is due to an increased rate of degradation. *Biochem. J.* **244:** 775-779.

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근세포 분화에 관한 연구 : 근세포 분화에 있어서 Protein Kinase C

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TPA나 PDGF를 처리로 인한 Protein Kinase C의 신호전달은 인산화에 의해 일어난다. 그렇지만, PKC에 의해 인산화 되어지는 targeting protein은 TPA나 PDGF 처리시에는 분자량이 서로 다른 단백질들이 인산화가 되어졌다. TPA 처리한 myoblast에서 분자량 20,000의 단백질이 인산화되었다. PDGF 처리한 세포에서는 분자량 40,000의 단백질이 인산화된 반면에 TPA 처리로 인산화 되었던 분자량 20,000의 단백질은 탈인산화 되었다. 이러한 결과들은 TPA와 PDGF가 신호전달체의 활성에 있어서 다를 뿐만 아니라 그들은 장시간의 처리 동안에 PKC의 down regulation에 관계되어 짐을 암시한다. 그러나 PDGF는 TPA의 경우에서 보다 빠른 down regulation을 유도하였다.

면역세포화학적인 연구에서 PKC의 동위효소인 PKC II는 세포질에, PKC III는 세포질과 막에 각각 분포하고 있었다. Myoblast에 있어서 PKC의 두가지 형태의 동위효소의 발현은 이들 동위효소들이 signal transduction이나 down regulation의 각기 다른 경로에 개입되어 진다는 것을 암시한다.