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Effects of Ca^{2+} and Protein Kinase C on the Chick Myoblast Differentiation

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Alteration of intracellular calcium ion concentration by adding of either calcium ionophore A23187 or EGTA in culture medium at 24 hr after cell plating resulted in remarkable changes in the progression of differentiation of chick embryo myoblast. When separated myoblast proteins using two-dimensional gel electrophoresis, synthesis patterns of several proteins changed upon the addition of either A23187 or EGTA. Treatment of A23187 and calcium-activated neutral protease at 24 hr after initial plating caused an increase in the rate of fusion compared to control culture. However, EGTA inhibited the myoblast fusion to a marked degree. A23187 treated at 24hr also increased the activity of protein kinase C during the fusion-progressed period. It seems that intracellular calcium ion plays an important role in the myoblast differentiation *in vitro* together with the protein kinase C and calcium-activated neutral protease.

KEY WORDS: Chick myoblast, Protein kinase C, Calcium-activated neutral protease.

The functional element of differentiated skeletal muscle, the nondividing multinucleate myotube, is formed by cytoplasmic fusion of mononucleated precursor cells, myoblasts. The fusion of myoblasts is a multistep process including at a minimum of the following separable components: (1) cell migration, recognition, and alignment, and (2) membrane fusion leading to cytoplasmic continuity (Nameroff and Munar, 1976).

The involvement of calcium ion in the fusion of biological membranes is an almost universal phenomenon. Although a direct role for calcium ion in the distinct process of membrane union during myogenesis has not been documented, an absolute requirement for calcium ion in membrane fusion during fertilization (Schackmann *et al.*, 1978), cellular secretion (Miller and Nelson, 1977), and virus-induced erythrocyte fusion (Volsky and Loyter, 1978) has been demonstrated.

Most animal cells so far examined contain a calcium-activated and phospholipid-dependent protein kinase (Protein kinase C) which is thought to be one such cellular regulator (Kishimoto *et al.*, 1981). This protein kinase is active in a reversible manner in the presence of both Ca^{2+} and phospholipids with a preference for phosphatidylserine. *In vivo*, the protein kinase C is thought to be activated by diacylglycerol, a second messenger generated from a signal-induced breakdown of inositol phospholipids (Kishimoto *et al.*, 1981; Berridge and Irvine, 1984). Also, a calcium-activated neutral protease is able to activated protein kinase C by limited proteolysis (Kishimoto *et al.*, 1983). The protein kinase C is present in the cytosolic and membrane fractions depending upon the physiological state and source of tissue. It has been suggested that the association of protein kinase C with membrane is required for the subsequent physiological responses.

In the present study, we have investigated the

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roles of Ca^{2+}, and protein kinase C on the chick myoblast differentiation by treating the cells with calcium ionophore A23187 and EGTA. We have also studied the effects of calcium-activated neutral protease on the chick myoblast differentiation.

Materials and Methods

Cell Culture

Cultures were prepared by a modified method of O'Neill and Stockdale (1972). Cells were obtained from 12-day old chick embryos. The cells suspended in 811 medium (MEM containing 10% horse serum, 10% embryo extract and 1% antibiotics) were plated at a concentration of 5×10^5 cells/ml. Medium was changed after 24 hr and thereafter at 2-day intervals with 8102 medium (MEM containing 10% horse serum, 2% embryo extract, and 1% antibiotics). The degree of fusion was determined by the ratio of the number of nuclei within the myotubes of three or more nuclei divided by the total number of nuclei.

Treatment of EGTA, A23187, and calcium-Activated Neutral Protease

EGTA was prepared as a 50 mM stock solution at pH 7.0, and was added at a final concentration of 2 mM. The calcium ionophore A23187 was prepared as a 10 mM stock solution in 100% dimethyl sulfoxide. This stock was diluted with vigorous vortexing to 1:1000 with MEM to prepare a 10 μM working solution immediately before use. The diluted solution was added to medium to provide a final concentration of 0.1 μ M. The calcium-activated neutral protease (Sigma, from rabbit skeletal muscle) was treated to cell culture at a final concentration of 10 μg/ml. All were treated at 24 hr after cell plating.

[^{35}S]Methionine Labeling and Two-Dimensional Gel Electrophoresis

After the cells were incubated in the MEM containing 10 μCi/ml of [^{35}S] methionine (Amersham, S. A. 1426 Ci/mol) for 2 hrs, the medium was removed and the cells were rinsed twice with cold Earl's balanced salt solution and harvested in microcentrifuge tubes.

Two-dimensional gel electrophoresis was carried out by the method of O'Farrell(1975) with minor modification. In the first dimension, isoelectric focusing gel containing ampholines(LKB) of a ratio of 4 parts pH range 5-7 to 1 part pH range 3-10 was used. In the second dimension, SDS/10% polyacrylamide slab gel was used.

Prepration of Lipid Vesicles

Phosphatidylserine(PS) and 1,2-diacyl-*sn*-glycerol(DAG) were dissolved separately in a minimal amount of chloroform: methanol (19:1 v/v). Lipids were mixed immediately before use. The organic solvents were evaporated under nitrogen gas. After addition of an appropriate amount of 20 mM Tris-HCl (pH 7.5), lipids were suspended by sonication for 5 min.

In Vitro Assay of Protein Kinase C

Protein kinase C activity was assayed by measuring the incorporation of ^{32}P into histone III-S (Sigma) as previously described (Honnor *et al.*, 1985; Navarro, 1987). An aliquot (40 μl) of the cell extract was added with 60 μl of the reaction mixture (20 mM Tris-HCl, pH 7.5, 1 mM $CaCl_2$, 10 mM $MgCl_2$, 2.5 nM ATP containing 1 μCi [γ-^{32}P]ATP (New England Nuclear, S. A. 3000 Ci/mM), 100 μg/ml PS, 10 μg/ml DAG, and 100 μg/ml histone III-S). Reaction was carried out for 10 min at 30°C, and terminated by addition of cold 20% TCA and 5 mM sodium pyrophosphate. The TCA-precipitates were sedimented by centrifugation for 10 min at 1,300 $\times$g at 4°C. The precipitates were dissolved in 200 μl of 100% TCA and reprecipitated with 20% TCA and 5 mM sodium pyrophosphate. The resulting precipitates were then decanted onto a glass fiber filter (Whatman, GF/C) under vacuum, that had been preweted with the washing medium containing 5% TCA and 5 mM sodium pyrophosphate. The tubes were flushed with 3 ml of the washing medium, and washed with three additional 3 ml aliquots of the medium. The filters were placed in liquid scintillation fluid for counting of their radioactivity.

Detection of ^{32}P-Labeled Phosphoproteins

Protein kinase A activity was detected by the similar method for the protein kinase C assay, except that the reaction mixture contained 0.25 nM of cAMP, but not $CaCl_2$, PS, and DAG. The reaction was stopped by addition of 1/3 reaction mixture volume of 3X SDS sample buffer. Electrophoresis was performed in the SDS/7-14% gradient polyacrylamide gel (Laemmli, 1970).

Results

Effects of the Calcium Ionophore A23187 and EGTA on Fusion

The cell fusion was initiated at about 36 hr, and almost completed by 72 hr after initial plating under the control culture condition (Figs. 1, 2). Addition of A23187 to culture resulted in an increase in the rate of fusion compared to control culture. In the A23187 treated culture, the fusion was initiated earlier. The final extent of fusion, however, in both control and A23187-treated culture was similar. A23187 shifted the time course of fusion forward about 12 hrs, without affecting general shape of the curve. On the other hand, EGTA was an effective inhibitor of myoblast fusion. The percentage of the fused cell in EGTA-treated culture reached 30% to that of control cells.

The cell proliferation was little affected by addition of A23187 or EGTA, except for slight increase of growth rate at early culture period (48 hr) in the A23187-treated culture.

Patterns of Protein Synthesis

Cells usually alter their normal pattern of protein synthesis under abnormal growth conditions. Therefore, we examined if some proteins have different synthesis patterns according to the culture conditions. Fig. 4 showed the [^{35}S] pulse-labeled polypeptides synthesized in the myoblast. Several proteins showed different synthesis patterns of protein by addition of either A23187 or EGTA in the culture (Table 1).

The 100 kDa and 80 kDa proteins were synthesized in the A23187-treated cells at 48 hr after cell plating, although these were not or little synthesized in the control culture at that time. These results are well agreed with other report (Wu *et al.*, 1981). In addition to 100 kDa and 80 kDa proteins, 170 kDa, 110 kDa, and 44 kDa proteins were synthesized more active than the con-

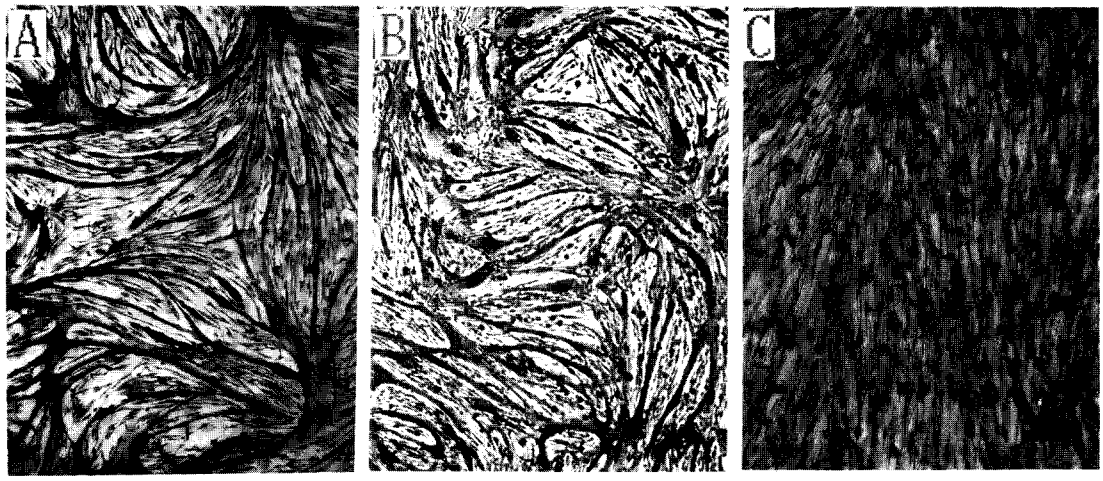

Fig. 1. The 72 hr-cultured myoblast in the presence of calcium ionophore A23187 (0.1 μM) and EGTA (2 mM). (A) control culture, (B) A23187-treated culture, and (C) EGTA-treated culture.

Table 1. ^{35}S-labeled proteins showing different patterns of synthesis as the culture cnditions.

Mr	control			A3187-treated			EGTA-treated			remarks*
(kDa)	48 hr	72 hr	96 hr	48 hr	72 hr	96 hr	48 hr	72 hr	96 hr	
170	−	−	−	+	+	−	−	−	−	6
110	−	+	+	++	++	++	+	+	+	1
100	−	++	+	++	++	++	+	++	++	2
80	+	+	+	++	++	++	+	+	+	7
68	+(−)	+	−	+	+	−	−	−	−	3
64	+(−)	+	+	+	+	+	−	+	+	4
58	−	+	+	−	−	+	+	+	+	8
44	+	+++	+	+++	++	+	++	++	+	5

*These indicate the numbers labeling in Fig. 4.

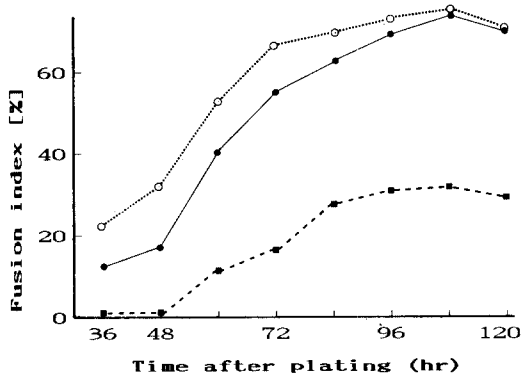

Fig. 2. Myoblast fusion in the presence of calcium ionophore A23187 (0.1 μM) and EGTA (2 mM). • —— •, control culture; ○ ····· ○, A23187-treated culture; ■ -- ■, EGTA-treated culture.

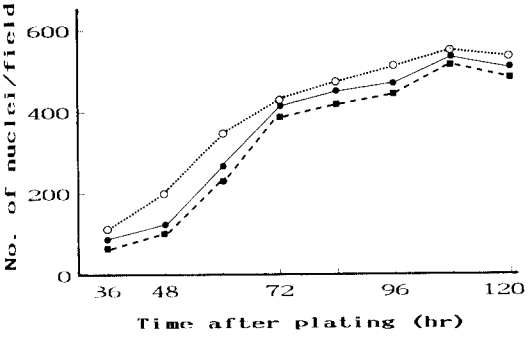

Fig. 3. Effects of calcium ionophore A23187 and EGTA on the myoblast proliferation. Cell growth was determined by the number of total nuclei per field. • —— •, control culture; ○ ····· ○, A23187-treated culture; ■ -- ■, EGTA-treated culture.

trol culture at 48 hr after cell plating, The 170 kDa, 110 kDa, and 80 kDa proteins were continuously synthesized in the A23187-treated cells at higher rates than control cells at 72 hr after cell plating. In the contrary, 58 kDa proteins are synthesized in the control culture more actively than the A23187-treated culture at 72 hr after cell plating. The patterns of protein synthesis were similar to both control and A23187-treated culture at fusion-completed point, which is about 96 hr after cell plating. On the other hand, the 110 kDa and 100 kDa were synthesized more actively in the EGTA-treated cells than the control culture, but less actively than the A23187-treated culture at 48 hr after cell plating. The 58 kDa proteins were synthesized only under the EGTA-treated condition at 48 hr after cell plating.

Roles of Calcium-Activated Neutral Protease

The addition of calcium-activated neutral protease resulted in a slight increase in the rate of fusion compared to the control culture (Fig. 5, A). Fusion under the protease-treated condition was initiated earlier than the control culture, but not earlier than A23187-treated culture. The final extent of fusion in the protease-treated culture was similar to the control culture. The cell proliferation was not affected by the protease (Fig. 5, B).

Activities of Protein kinase C

Protein kinase C activity presented in the cultured myoblast was assayed by measuring of the incorporation of [^{32}P] derived from [γ ^{32}P]ATP

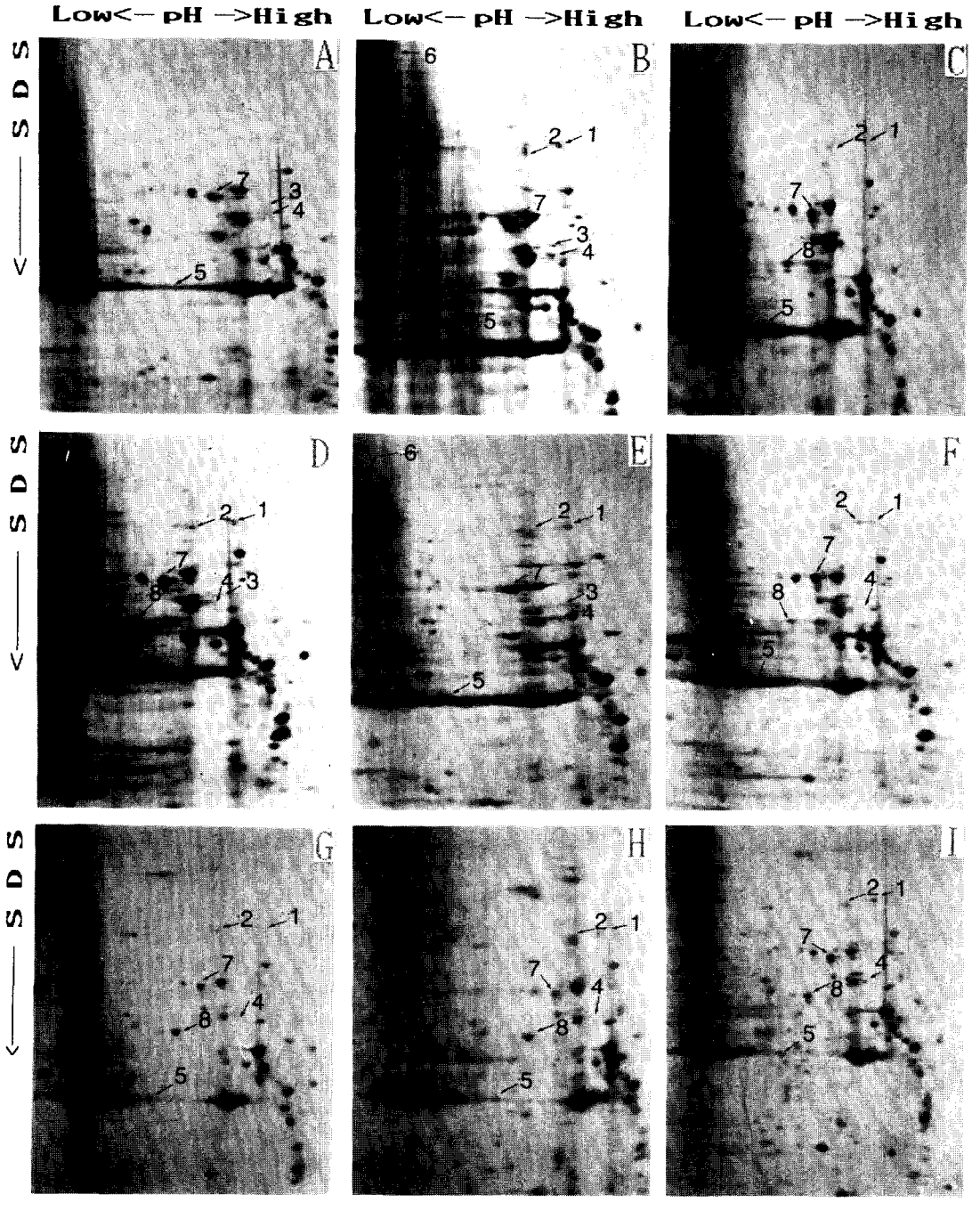

Fig. 4. ^{35}S-labeled proteins synthesized in the myoblast. The myoblast was labeled with 10μCi/ml of [^{35}S]methionine for 2 hrs, then harvested. The proteins were separated by O'Farrell's two-dimensional gel electrophoresis. The numbered spots in the fluorograms are proteins showing different patterns of synthesis as culture conditions (see Table 1). A, control 48 hr culture; B, A23187-treated 48 hr culture, C, EGTA-treated 48 hr culture; D, control 72 hr culture; E, A23187-treated 72 hr culture; F, EGTA-treated 72 hr culture; G, control 96 hr culture; H, A23187-treated 96 hr culture; I EGTA-treated 96 hr culture.

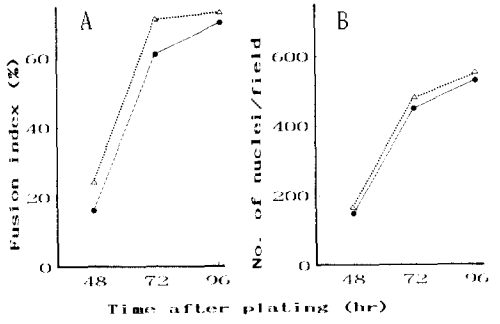

Fig. 5. Effects of calcium-activated neutral protease on the myoblast. The Protease was added myoblast culture at 24 hr after cell plating (10 μg/ml). (A) Fusion index, (B) growth rate: • —— •, control culture; △·····△, protease-treated culture.

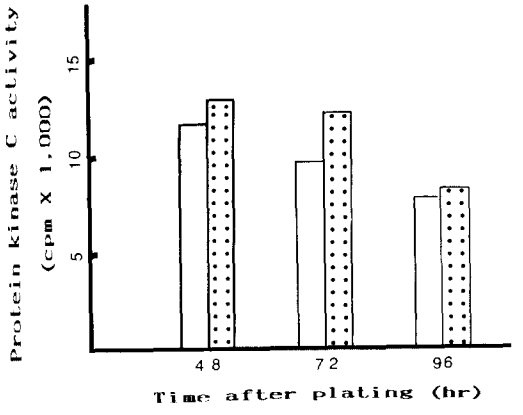

Fig. 6. The activities of intracellular protein kinase C. The cells were periodically withdrawn from culture, then assayed using the cell-free assay system in the presence of PS, DAG, and calcium ion. □, control culture; ■, A23187-treated culture.

into histone III-S. The protein kinase C activity in the control culture was slightly decreased as fusion progressed (Fig. 6). The protein kinase C showed the highest activity at the fusion-initiated point, 48 hr after cell plating, and lowest at the fusion-completed point 96 hr after cell plating.

The treatment of calcium ionophore A23187 resulted in a slight increase in the protein kinase C activity However, A23187 did not effect on the

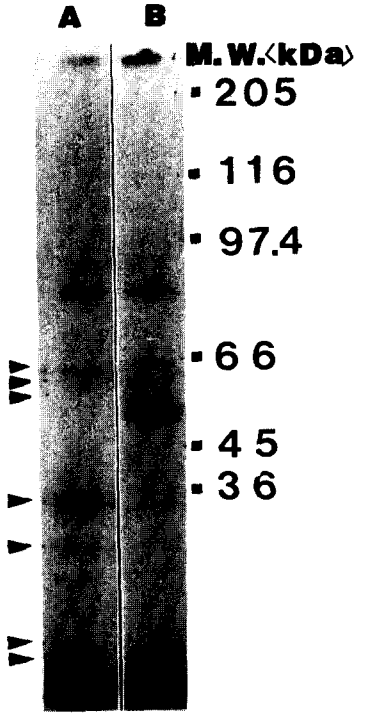

Fig. 7. Autoradiograms of ^{32}P-labeled Proteins. The protein kinase A activity was assayed with similar method of protein kinase C assay, except that the reaction mixture contained 0.25 nM of cAMP, but no Ca^{2+}, PS, and DAG. The proteins were separated in a SDS/7-14% gradient polyacrylamide gel. A, proteins phosphorylated by protein kinase A; B, proteins phosphlated by protein kinase C; ▶, proteins showing different phosphorylated patterns by either kind of protein kinase.

protein kinase C activity at the fusion-completed point. It was interesting that elevation of the protein kinase C activity and increase of the myoblast fusion caused by A23187 treatment occurred at the same period (Figs. 2, 6)

We compared the kinds of phosphorylated proteins catalyzed by protein kinase A and C. As shown in Fig. 7, a greater part of the ^{32}P-labeled proteins were phosphorylated by either protein kinase A or B.

Discussion

We observed that many proteins were synthe-

sized cultured-time dependently. Synthesis patterns of several proteins appear to change upon the alteration of the culture condition. Wu *et al.* (1981) proposed that the effect of A23187 upon the synthesis of the 80 kDa Protein is mediated through an increase in cytoplasmic calcium ion concentration.

It has well been documented that extracellular calcium is required for normal progression through the initial recognition-alignment phase of myogenesis (David *et al.*, 1981). We observed that the myoblast fusion was promoted by calcium ionophore A23187, but inhibited by EGTA. These results are consistent with the requirement of extracellular calcium for the normal progression of the myoblast fusion. The divalent cation ionophore has been shown to increase the permeability of a number of neutral membranes to calcium and magnesium ions.

Protein kinase C is a calcium- and phospholipid-dependent protein kinase (Kishimoto *et al.*, 1983; Ballester and Rosen, 1985; Balazovich *et al.*, 1987). As shown in Fig. 7, its catalytic specificity was strictly distinguished from cAMP-dependent protein kinase A. The calcium ionophore A23187 slightly increased the total intracellular protein kinase C activity. Activation of protein kinase C by addition of A23187 was observed not during the overall period of myoblast culture, but only during the fusion-progressed period (48 hr and 72 hr).

Also, we observed that the addition of calcium-activated neutral protease resulted in a slight increase in the rate of fusion compared to control culture. The kinetics of the myoblast fusion under the protease-treated culture was similar to A23187-treated culture (Figs. 2, 5).

It appears that calcium ion plays an important role in the myoblast differentiation together with the protein kinase C and the calcium-activated neutral protease. This suggestion is based on the following evidence. (1) Calcium ionophore A23187, EGTA, calcium-activated neutral protease, and protein kinase C affect intracellular Ca^{2+} concentration. (2) There is a measurable increase or decrease in the myoblast fusion by addition of A23178 or EGTA. (3) Calcium-activated neutral protease increases the rate of myoblast fusion as the same manner of the A23187. (4) Activation of protein kinase C by A23187 was observed only during the fusion-progressed period. (5) The increase of myoblast fusion by A23187 or calcium-activated neutral protease, and elevation of protein kinase C activity by the A23187 were all simultaneously observed at the same cultured period, which is fusion-progressed period (48-72 hr). In spite of the proposal that A23187, protein kinase C, and calcium-activated neutral protease control the myoblast differentiation with an interrelationship, the precise control mechanism still remains unclear.

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Ca^{2+} 및 Protein Kinase C가 배양한 계배근원세포의 분화에 미치는 영향

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계배 근원세포의 배양 배지에 calcium ionophore A23187이나 EGTA를 배양 24시간에 첨가함으로서 초래된 세포내 칼슘 농도의 변화는 근원세포의 분화과정에 상당한 영향을 미쳤다. 배양 24시간에 A23187이나 EGTA를 첨가한 후 배양 48시간, 72시간, 및 96시간에 각각 세포를 [^{35}S]methionine으로 1시간 표지시킨 후 수확하여 2차원 전기영동법으로 단백질을 분리시켰을 때, 일부 단백질은 배양 조건에 따라 합성 양상을 달리함을 보였다. 배양 24시간에 처리한 A23187과 calcium-activated neutral protease는 대조군에 비해 세포융합을 촉진시켰으나 동일 시기에 처리된 EGTA는 세포융합을 현저히 감소시켰다. protein kinase C의 활성도를 ^{32}P로 표지된 phosphoprotein을 정량함으로써 조사하였을 때, A23187이 배양 초기에는 대조군에 비해 약간 이 효소의 활성도를 높이는 효과를 보였으나 세포융합이 완성된 시기인 96시간에는 대조군에 비해 활성도의 차이를 나타내지 않았다. A23187 및 calcium-activated neutral protease에 의한 세포융합의 촉진, 그리고 A23187에 의한 protein kinase C 활성도의 증가가 모두 근원세포의 융합이 활발히 진행되는 시기인 배양 48-72 시간에 관찰됨을 볼 때, 세포내 칼슘의 농도는 protein kinase C 및 calcium-activated neutral protease와 상호연관을 가지면서 세포분화에 관여하는 것으로 사료된다.