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The Inhibitory Effect of Dopamine on Myoblast Fusion *in vitro*

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Dopamine의 배양근원세포 융합억제 작용

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적 요

근세포가 분화하는 과정에 있어서 신경전달물질의 역할을 알아보기 위해서 배양중인 근원세포에 dopamine을 처리하고, 융합지수, creatine kinase 합성률 및 dopamine에 대한 차등감수성을 조사하였다.

배양후 34시간된 근원세포에 3×10^{-5} M의 dopamine을 처리하면 그후 전 시기에 걸쳐 융합지수가 크게 감소되며, 이와 더불어 creatine kinase의 합성률도 감소하는 사실로 미루어 이들 사이에 상관관계가 있음을 알 수 있었다. 또한 dopamine의 융합억제효과는 세포주기에 따라 감수성이 달라지는 차등감수성을 관찰할 수 있었는데, 이는 근세포막에 위치할 것으로 생각되는 dopamine receptor의 배치가 세포주기에 따라 달라지는 데 연유되는 것으로 추정되었다.

INTRODUCTION

The transition from dividing myoblasts to multinucleate myotubes provides the most striking example to the terminal differentiation that takes place *in vitro.* A central interest in the myoblast differentiation, therefore, has been focussed on the nature of the control mechanism that regulates the transition of the proliferating myoblasts to the differentiating myotubes.

Myogenesis is characterized by the fusion of membrane of myoblasts to form postmitotic multinucleate myotubes and the concomitant elaboration of muscle specific proteins, such as creatine kinase (Turner *et al.*, 1974), acetylcholine receptor (Paterson and Prives, 1973), and contractile proteins (Devlin and Emerson, 1978).

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When cultured *in vitro*, myoblasts proliferate and then fuse almost synchronously to form myotubes. This synchronous nature of differentiation of myoblasts *in vitro* has been attributed to the extracellular environment which differs from the *in vivo* internal environment.

Among the reports dealt with the myoblast differentiation, a suggestion was made that myoblast differentiation might be regulated by the nervous system. For example, Filogamo *et al.* (1978) observed the contacts between the myogenic cells of somites and primitive axons growing from the neural tubes as early as 35 hr of incubation in chick embryos. Likewise, *in vitro* culture of muscle cells derived from the muscle that had been denervated by cauterization of spinal cord or by the injection of curare exhibited varying degree of differentiation in morphology according to the time of denervation (Bonner, 1978; Bonner and Adams, 1982). Furthermore, embryonic muscle cells and nerve cells grew well when cultured together (Kidocoro, 1980). Along these lines of evidence, it has been speculated that myoblast differentiation might be mediated, at least, by trophic factors originated from the nerve cells.

In addition, there has been some evidence that the differentiation of myoblasts as well as myotubes was affected by innervation. Bonner (1978) found that chick myoblast populations were changed by *in vivo* manipulation of the nervous system. Although the mechanism for the interaction between neurons and myogenic cells has not been elucidated, the above evidence suggests that myoblast differentiation may be affected by neurotransmitter. Furthermore, because the protocol of cell culture eliminates neurons from the myoblast culture, one way of obtaining some insight into the nature of synchronous differentiation of myoblasts *in vitro* involves the investigation of the effect of neurotransmitter on the myoblasts differentiation in culture.

As to the role of catecholamine in myoblast differentiation, Curtis and Zalin (1981) reported that epinephrine that raised cAMP by interaction with β-adrenergic receptors was capable of inducing precocious cell fusion. Kang *et al.* (1984) observed that dopamine and epinephrine, at higher concentration than that used by Curtis and Zalin, exhibited an inhibitory effect of myoblast fusion, and suggested that α-adrenergic receptors might be involved in the process. Furthermore, as there was some evidence that α_1-adrenergic receptors existed in a nonfusing muscle cell line (Mauger *et al.*, 1982) and β-adrenergic receptors on fusing muscle cells, we could not rule out the possibility that catecholamine might have dual effect on myoblast fusion, because epinephrine stimulated prolactin release at low concentration whereas at high concentration inhibited prolactin release (Birge *et al.*, 1970).

The previous experiment from this laboratory showed that dopamine profoundly inhibited myoblast differentiation compared to epinephrine (Kang *et al.*, 1984). The present investigation, therefore, was undertaken to examine the effect of dopamine on myoblast fusion, creatine kinase synthesis, and changes in the sensitivity to dopamine during myogenesis.

MATERIALS AND METHODS

Cell Culture

Myoblast cultures were prepared by the method of O'Neill and Stockdale (1972) with minor modifications. Pectoral muscle was dissected out from 12-day old chick embryos. After removing connective tissues, the muscle was minced and incubated for 10 min at 37°C in 0.05% trypsin with gentle stirring. The supernatant was discarded and the tissue clumps were rinsed with RPMI 1640 medium and reincubated for 10 min at 37°C in 0.125% trypsin with gentle stirring. The supernatant was then centrifuged to collect cells. The cell pellet was resuspended in complete medium (RPMI 1640 supplemented with 10% horse serum, 4% chick embryo extract, and 1% antibiotics; designated as 8104 medium) and filtered through four layers of lens paper. The cell suspension was preplated on collagen-coated dishes for 10 min to remove fibroblasts. Cells were then counted in a hemocytometer and plated at 2×10^5 cells/ml in 35 mm plastic culture dishes for chemical treatment. The cells were grown for the first 24 hr in 8104 medium and the medium was changed at 24 hr and at 2-day intervals thereafter to 8102 medium (the same as complete medium except 2% chick embryo extract).

Dopamine dissolved in phosphate-buffered saline was added at 34 hr or at varying times after cell seeding at a final concentration of 3×10^{-5} M.

Measurement of Cell Fusion

Cell fusion was determined by direct microscopic examination of ethanol-fixed and Giemsa-stained cultures at a magnification of 400×. Cells were considered as fused only if there were clear cytoplasmic continuity and at least three nuclei present in each myotube. For each culture, randomly chosen 20 fields were counted.

DNA Estimation

DNA content was estimated by the method of Rissane and Robins (1958) with minor modifications. The samples were incubated at 60°C for 45 min with 0.1 ml of 40% (w/v) 3,5-diaminobenzoic acid that had previously been decolorized with charcoal. The reaction was stopped by the addition of 2 ml of 1 N HCl, and the fluorescence was measured in a spectrofluorometer (excitation, 405 nm and emission, 520 nm). Calf thymus DNA was used as a standard.

Creatine Kinase Assay

The chemical-treated cells stored at −70°C were rapidly thawed on the tap water and the cell lysates were scraped off in Tris-magnesium buffer (100 mM-25 mM). Creatine kinase activity was estimated by the method of Koedam (1969). Briefly, samples were centrifuged and the supernatant was used for quantitation. Reaction was started by the addition of ADP solution and stopped by adding barium hydroxide-EDTA and zinc sulfate solution. The reaction mixtures were centrifuged and the supernatant was diluted with distilled water

and then ninhydrin solution was added. The fluorescence was measured 6 min after the addition of potassium hydroxide solution. Creatine was used as a standard.

RESULTS

Effect of Dopamine on Myoblast Fusion

Treatment of cultured myoblasts with 1×10^{-5} M or 3×10^{-5} M dopamine at 34 hr after cell plating resulted in an inhibition of myoblast fusion, as shown in Fig. 1. The degree of inhibition, however, was found to be dependent on the concentration of dopamine administered. At high concentration, the myoblast fusion was drastically inhibited throughout the whole myogenesis stages, whereas at low concentration the inhibitory effect was not apparent until late stage of differentiation. The inhibitory effect of dopamine at high concentration was more than twice that of epinephrine and was not attributed to the toxic effect of dopamine on the myoblasts (data not shown).

Effect of Dopamine on Creatine Kinase Activity

Creatine kinase activity was chosen as an index of biochemical differentiation of the myoblasts, since this parameter has been known to be muscle-specific and coincides quantitatively with the progress of muscle differentiation. The changes in the creatine kinase activity of both control and dopamine-treated myoblasts are shown in Fig. 2.

The unit of creatine kinase activity was expressed in terms of nmoles of creatine produced per μg of DNA. In the control culture, the level of creatine kinase activity began to increase after about 60 hr, which corresponded to the onset of myoblast fusion. In contrast, the myoblasts treated with dopamine at 34 hr started to increase the level of

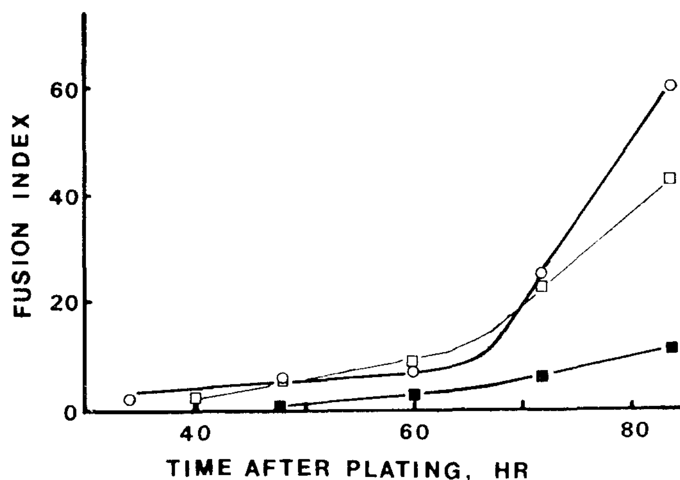

Fig. 1. The effect of dopamine administration on the fusion index of myoblasts at 32 hr after plating and scored at designated times. ○-○, control culture; □-□, 1×10^{-5} M dopamine; ■-■, 3×10^{-5} M dopamine.

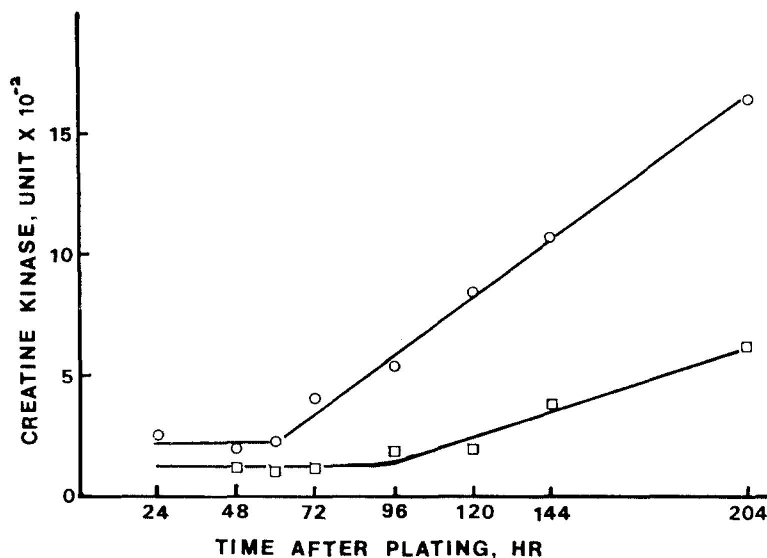

Fig. 2. The effect of dopamine treatment on the creatine kinase activity of myoblasts. The creatine kinase activity is expressed in terms of nmoles of creatine produced per μg of DNA. ○-○, control culture; □-□, 3×10^{-5} M dopamine.

creatine kinase activity at about 96 hr with milder slope than that of the control. Thus, the delay in the synthesis of creatine kinase of 36 hr and the decreased rate of synthesis were apparent, indicating that dopamine somehow inhibits the synthesis of muscle-specific protein.

Differential Action of Dopamine with the Stages of Myoblasts

The fusion index of myoblasts treated with dopamine at varying times from 34 through 69 hr and scored at 96 hr is shown in Fig. 3, and a representative morphology is shown in Fig. 4. As is evident from Fig. 3, there appeared to be a cyclic fashion of dopamine inhibition, being low in fusion index at 34, 41~45 and 58~61 hr and high at 37, 49~53, and 65~69 hr. The cyclic nature of dopamine inhibition might be interpreted as the differential sensitivity of myoblasts to dopamine as the differentiation proceeded. The differential sensitivity to dopamine is likely to be attributed to the difference in dopamine receptor density on the myoblast membrane.

DISCUSSION

Contradictory or conflicting findings have been reported for the role of catecholamine in the myogenesis. Curtis and Zalin (1981) reported that catecholamine which raised cAMP by interaction with β-adrenergic receptors induced precocious cell fusion. In accord with this finding was the observation that on the cell surface of rat myoblast cell lines were β-adrenergic receptors (Atlas *et al.*, 1977) and that adenylate cyclase was activated by

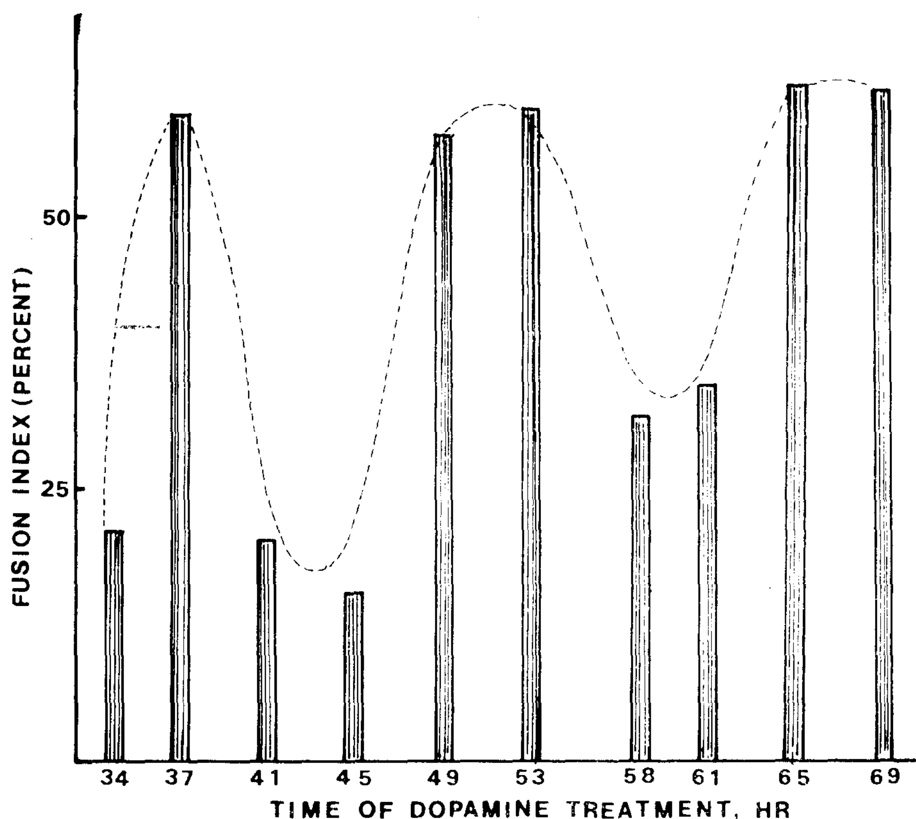

Fig. 3. The effect of time of dopamine treatment on the fusion index of myoblasts treated at designated times and scored at 96 hr after plating. The cyclic nature of dopamine sensitivity is apparent.

catecholamine through β-adrenergic receptors (Hirata *et al.*, 1979).

In contrast, β-adrenergic receptors were not detected on quail myoblasts before differentiation (Parent *et al.*, 1980) and the release of melanocyte stimulating hormone (MSH) was completely inhibited in the presence of epinephrine or dopamine (Bower *et al.*, 1974). Furthermore, Bower suggested that the inhibitory effect of catecholamine on MSH release *in vitro* might be controled through α-adrenergic receptors, because the release was inhibited by a specific α-adrenergic agonist and not by a β-adrenergic agonist.

The present experiment showed that dopamine was found to exert an inhibitory effect on both fusion index and creatine kinase activity. It was also ascertained that the level of cAMP was not altered by the addition of dopamine (Kang *et al.*, 1984). These findings led us to postulate a possibility that the inhibitory effect of dopamine on myoblast fusion might be regulated through α-adrenergic receptors. Depamine receptors were divided into two general categories (designated D-1 and D-2) on the basis of biochemical and pharmacological criteria (Kebabian and Calne, 1979). The D-1 receptor in various tissues

Fig. 4. A representative pattern of myoblast differentiation treated with dopamine at varying times and scored at 96 hr after seeding. Dopamine was treated at A, 34 hr; B, 37 hr; C, 41 hr; D, 45 hr; E, 49 hr; F, 53 hr; G, 58 hr; H, 61 hr; and I, 65 hr.

regulates a specific dopamine sensitive adenylate cyclase, and therefore increases the level of cAMP, whereas the D-2 receptor neither enhances adenylate cyclase activity nor increases the level of cAMP (Brown *et al.*, 1977). Thus, it is plausible that the interaction of dopamine or epinephrine with the D-2 receptor which may exist in myoblasts results in the inhibition of myoblast fusion. In addition, it is also probable that the α-adrenergic receptors might be involved in this inhibitory effect, but it remains to be elucidated.

How does dopamine or epinephrine inhibit myoblast fusion is uncertain. There is, however, some evidence that catecholamine affects Ca^{2+}-influx. Horn and McAffe (1980) proposed that activation of an α-adrenoreceptor inhibits a voltage-sensitive Ca^{2+} conductance, thereby reducing the inward Ca^{2+} current. Douglas and Taraskevich (1978) reported that dopamine decreased the frequency of spontaneous action potentials. Calcium normally enters a cell through a relatively specific calcium channel that is independent of membrane potential, or through a potential-dependent calcium channel (Rasmussen and Goodman, 1977). Both channels may be activated by hormonal interaction with the plasma membrane. The fusion of myoblast is also known to be dependent on Ca^{2+} (Bischoff, 1978). Little is known about the molecular action of Ca^{2+} at present, but David *et al.* (1981) have observed that a measurable increase in net calcium influx occurred just prior to fusion. This finding indicates that Ca^{2+} may play a role in myoblast differentiation. In the light of all these observations, it is likely that dopamine or epinephrine interacts with their recepters and their interaction blocks Ca^{2+} influx which is responsible for the myoblast differentiation and/or the membrane fusion of myoblasts.

Another interesting finding to be noted in the present experiment was that the inhibitory effect of dopamine appeared to vary in cyclic manner which seemed to rely on the stages of cell cycle. The inhibition appeared at about every 12 hr interval which corresponds to one cell cycle of myoblasts. Furthermore, the inhibition seemed to occur around G_1 phase of the cell cycle, as was ascertained by ^3H-thymidine uptake experiment. This stage-specific inhibition of dopamine is extremely interesting, when compared with the finding that two peaks of binding maxima for prostaglandin E_1 exist during myoblast differentiation (Hausman and Velleman, 1981). The match in the time for dopamine inhibition and that for prostaglandin E_1 binding peak would surely tell us something important, but the meaning for this match remains to be elucidated. The only suggestion we can give at present is that dopamine act specifically at G_1 stage of cell cycle as prostaglandin E_1 does, and that neurons would play some inhibitory role in myoblast differentiation *in vivo*. If it turn out to be the case, it could explain at least, in part, the nature of synchronous differentiation of myoblast cultured *in vitro*.

ABSTRACT

In order to elucidate the effect of neurotransmitter on the differention of myoblasts *in*

vitro, dopamine was administered to the myoblasts at varying stages of myogenesis, and the fusion index, the rate of creatine kinase (CK) synthesis, and the sensitivity to dopamine were determined.

When dopamine (3×10^{-5} M) was administered at 34 hr after myoblast seeding, a significant decrease in the fusion index as well as CK synthesis was observed, indicating a good correlation exists between these two parameters. In other experiment, dopamine was administered at varying stages of myogenesis and the inhibitory effect of dopamine as scored by fusion index at 96 hr was found to be cyclic in nature. This finding raised a possibility that arrangement of dopamine receptors occurs according to the cell cycle stages in myogenesis.

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