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Isoforms of Protein Kinase C during the Differentiation of Chick Limb Mesenchyme

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The present studies were undertaken to examine the activitites of PKC isoforms in cultures of chick limb mesenchyme. Micromass cultures were prepared using wing buds of stage 23/24 (Hamburger and Hamilton, 1951) chick embryo. The cells were homogenized and DEAE-cellulose column chromatography was performed to get fraction containing protein kinase C (PKC) activity. PKC isoforms were resolved with hydroxyapatitie column chromatography. Profile of PKC isoforms of cultures were compared with that of rat brain. Activity of PKC-β isoform was appeared at the early stage of chondrogenesis. On 3 day of culture, activities of both PKC α and β were observed with remarkable increase but no activity of γ isoform was appeared. Treatment of phorbol-12-myristate-13-acetate (PMA) (10^{-7} M) to the culture inhibited chondrogenesis and down-regulated α and β isoforms. Staurosporine promoted chondrogenesis without any effect on PKC isioforms profile. These data indicate that PKC α and β, especially β isoform is related to chondrogenesis and the promoting effect of staurosporine on chondrogenesis is not related to PKC isoforms activities.

KEY WORDS: Chondrogenesis, Isoforms of Protein Kinase C, PMA, Staurosporine

The protein kinase C (PKC) family consists of at least nine different isoforms that can be grouped into two major groups, Ca^{2+}-dependent and Ca^{2+}-independent. Among them, PKC α, β and γ isoforms are Ca^{2+}-dependent kinases. PKC from brain tissue can be resolved into three fractions, type I, II and III, by hydroxyapatite chromatography, which correspond to the enzyme encoded by r,- β- and α-cDNA clone, respectively (Huang *et al.*, 1987; Kikkawa *et al.*, 1987; Ohno *et al.*, 1987). These PKC isoforms show tissue-specific distribution or expression (Sawamura *et al.*, 1989; Wetsel *et al.*, 1992; Disatnik *et al.*, 1994) and different differentiation profile (Connor and Clegg, 1993; Gherzi *et al.*, 1992; Sparatore *et al.*, 1993; Wada *et al.*, 1989; Wooten *et al.*, 1992).

PKC is known to be related to cartilage differentiation. PMA, an activator of PKC, inhibits expression of cartilage specific type II collagen (Sasse *et al.*, 1983) and transcription of type II collagen gene (Kulyk, 1991). PMA also suppresses synthesis of the type IV sulfated proteoglycan in chondroblasts from chick sternal cartilage. Staurosporine, an inhibitor of PKC, promotes chondrogenesis of chick limb bud (Kulyk, 1991) and facial primordia (Kulyk and Reichert, 1992) *in vitro*. Recently, PKC activity has been measured in the cultures of chick limb mesenchymal cells. PKC activity increases as chondrogenesis proceeds and PMA inhibits chondrogenesis by depressing PKC (Kang *et al.*, 1991; Sonn and Solursh, 1993). However, involvement of PKC isoforms in the differentiation process of limb bud mesenchyme is not known yet.

In order to investigate the spectrum of PKC

isoforms activity during chondrogenesis, we resolved PKC isoforms from cultures of chick limb mesenchyme by hydroxyapatite chromatography and assayed PKC activity. Also the effect of PMA and staurosporine on PKC isoform profile during chondrogenesis was examined.

Materials and Methods

Preparation of cultures

Wing buds were removed from HH stage 23/24 embryos of chicken (Hanmaeum Co., Taegu) and they were collected in Saline G. The wing bud mesoderm tissues were dissociated by treatment of 0.1% trypsin-0.1% collagenase (Ahrens *et al.*, 1977). Single cells were obtained by filtration of cell suspension through 2 layers of Nytex 20 filter.

After centrifugation, the cells were suspended in Ham's F12 medium with 10% fetal calf serum and cell density was adjusted to 2×10^7 cells/ml. The cells were applied in 10-μl drops to 35- or 100-mm Corning culture dishes. Plates were incubated for 1 hr at 37°C to permit cell attachment and then flooded with 1.5 ml of medium. PMA and staurosporine were dissolved in DMSO for stock solution and added at the beginning of culture. Medium was changed everyday and PMA and staurosporine were also renewed daily.

Histochemistry

Cells were rinsed 3 times with Saline G and fixed with Kahles' fixative for 5 min. After rinse with distilled water, cells were stained with Alcian blue at pH 1.0 (Lev and Spicer, 1964).

Resolution of Protein kinase C isoforms

Fresh rat brains, chick brains or cultured cells were homogenized using an Elvehjem homogenizer or ultrasonicator. The homogenate was centrifuged at 100,000 ×g for 1 hr and the supernatant was used as a cytosolic PKC source. In case of cells *in vitro*, precipitate was dissolved in homogenation buffer containing 5% Triton X-100 and centrifuged at 100,000 ×g for 1 hr and the supernatant was used a particulate PKC source. Samples were loaded on to DEAE-cellulose columns (1 × 3 cm) equilibrated with 20 mM Tris HCl/1 mM ethylenediaminotetraacetic acid (EDTA)/20 mM NaCl/1 mM ethylene glycol-bis (β-aminoethyl ether) N, N, N′, N′-tetraacetic acid (EGTA)/50 mM 2-mercaptoethanol, pH 7.5. After washing the column with same buffer, bound proteins were eluted with same buffer containing 200 mM NaCl. The eluate was dialysed against 10 mM potassium phosphate buffer (pH 7.5) containing 10% glycerol, 0.5 mM EGTA, 0.5 mM EDTA before loading on to a column of hydroxyapatite (0.75 × 5 cm) equilibrated with the same buffer. Then 4 column volume of the same buffer was used to wash unbound protein before applying a step gradient of KPO_4, such as 10 to 25 mM, 25 to 60 mM, 60 to 120 mM and 120 to 250 mM. Fractions (1 ml) were collected and assayed promptly for PKC activity.

Assay of PKC activity

The complete assay mixture contained 20 mM Tris, pH 7.5, 20 mM $MgCl_2$, 1 mM $CaCl_2$, 20 μM [γ-^{32}P]ATP (1 – 1.5 × 10^6 cpm), and 50 μM acetylated myelin basic protein (AcMBP). Incubation was performed at 30°C. Phosphate incorporation into AcMBP was measured by spotting samples of assay mixtures on to squares of P81 paper and processing them as described by Sonn and Solursh (1993).

Results

Profile of PKC isoforms during chondrogenesis

In order to obtain a standard profile of PKC isoforms, rat brain was homogenized and PKC isoforms were resolved by a hydroxyapatite column chromatography. PKC was separated into the three fractions even though the first and second peak are not clearly separated in our system (Fig. 1A). These fraction are named Type I, II, and III according to the order of elution from the column (Huang *et al.*, 1986) and each fraction corresponds with PKC isoforms γ, β, and α, respectively. PKC from chicken brain was resolved into two major peaks upon hydroxyapatite column chromatography (Fig. 1B). When compared with elution profile of rat brain, they are identified to be

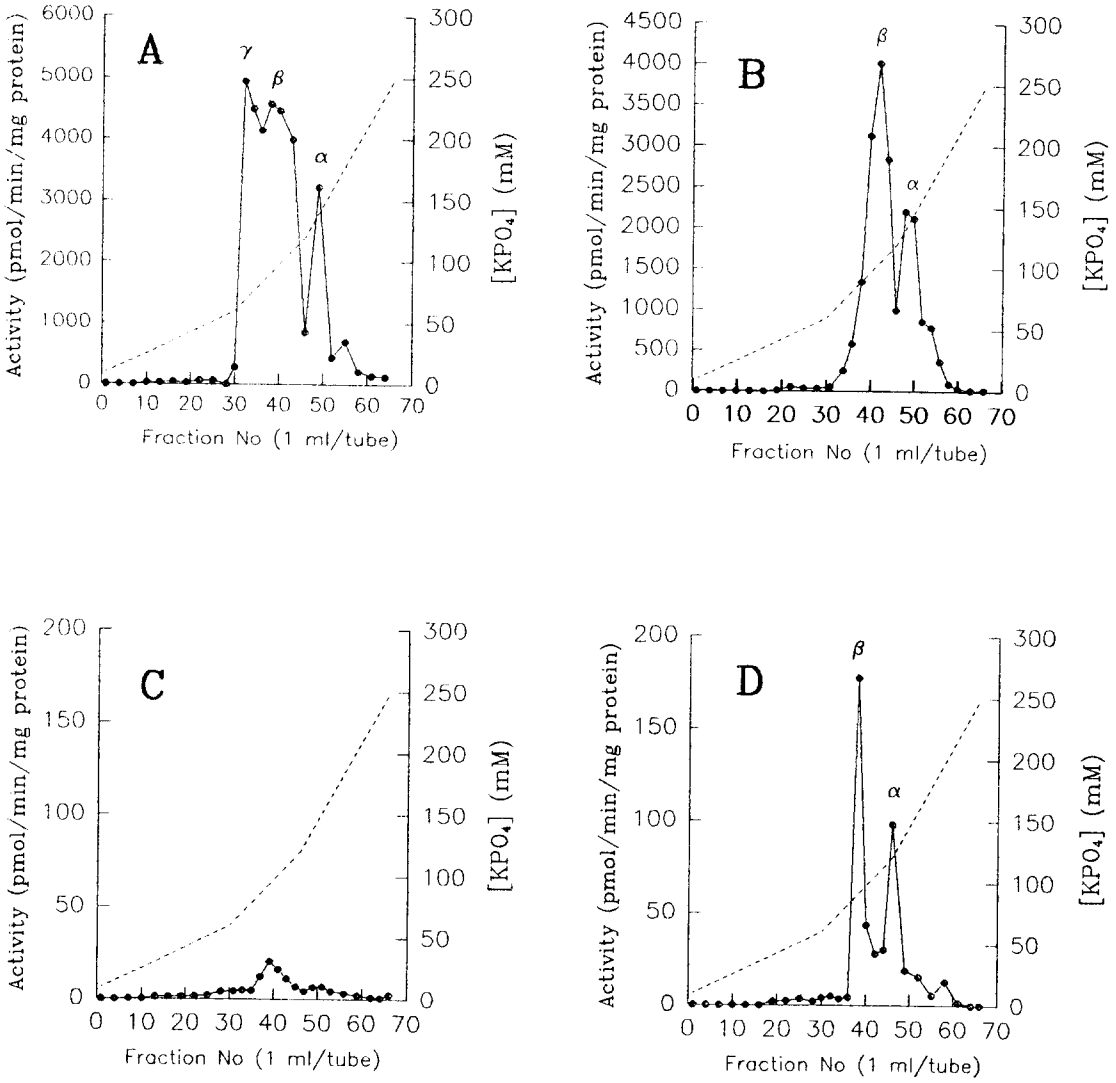

Fig. 1. Hydroxyapatite column chromatography of PKC from rat brain, chicken brain and cultures of chick wing bud mesenchyme. The PKC activity of the samples was resolved by hydroxyapatite column chromatography and enzymatic activity was assayed as described in Materials and Methods. A: rat brain, B: chicken brain, C: 1 day cultured cells, D: 3 day cultured cells.

type II and III PKC isoforms. To assay the changes of PKC isoforms profile during chondrogenesis, cells were homogenized and cytosolic and particulate fractions were obtained. In one day cultured cells of chick limb mesenchyme, there is small PKC β activity and neither activity of PKC α nor γ was detected in cytosolic fraction (Fig. 1C). Similar pattern was observed in particulate fraction (data not shown). On day 3 of culture, when chondrogenesis is accomplished and apparent Alcian blue-stained nodules are seen (Fig. 2B), activity of PKC β isoform was greatly increased and similar activity of PKC α isoform was also observed in cytosolic fraction (Fig. 1D). In particulate fraction, similar pattern of PKC α and β isoforms were observed but their activities were much smaller than in cytosolic fraction (data not shown).

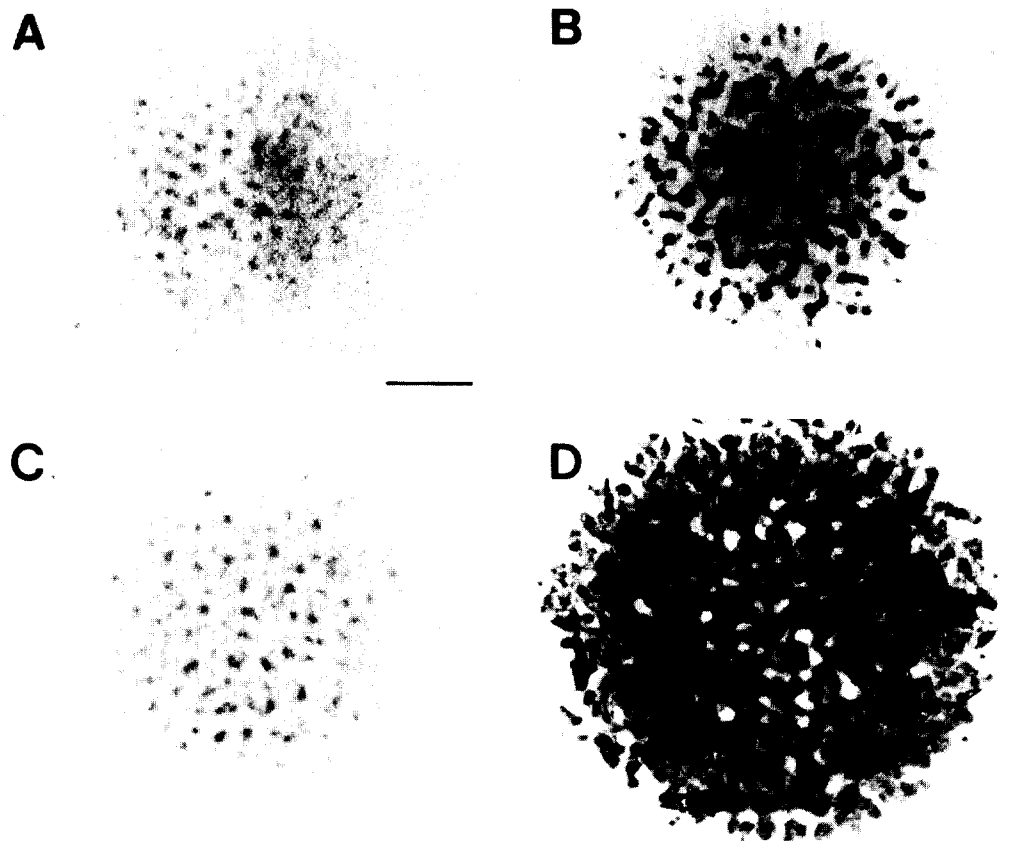

Fig. 2. Photomicrograph of cultures of stage 23/24 chick limb mesenchymal cells. Cells were grown in control media for 1 day (A), 3 days (B) or were treated with PMA (C) or staurosporine (D) for 3 days. Cells were fixed with Kahles' fixative and stained with Alcian blue at pH 1.0. Bar, 1 mm.

Effect of modulation of chondrogenesis on PKC isoforms profile

Treatment of PMA (10^{-7} M) to the cultures for three days inhibited chondrogenesis (Fig. 2C). The PKC isoforms profiles in cytosolic and particulate fractions were assayed. In the PMA-treated cultures, there is only one small peak of PKC β isoform in both cytosolic and particulate fracitons (Fig. 3A, B). These patterns of PKC isoforms are similar to those of 1 day cultured cells. As shown in Fig. 2D and described (Sonn and Solursh, 1993), staurosporine enhances the cartilage differentiation of chick wing bud. The hydroxyapatite column elution patterns of PKC isoforms in cytosolic fraction of staurosporine-treated cultures were similar to those in 3 day - control cultures. There are two peaks of PKC α and β isoforms with similar activities to those of 3 day-control cultures. In particulate fraction, only small PKC β isoform was observed. Table 1 summarizes peak activities of PKC isoforms in different states of differentiation. Again, it is clear that only small activity of PKC β isoform is present in undifferentiated cells while both PKC α and β isoforms are present with similar activity in differentiated cells.

Discussion

The protein kinase C enzyme family is involved

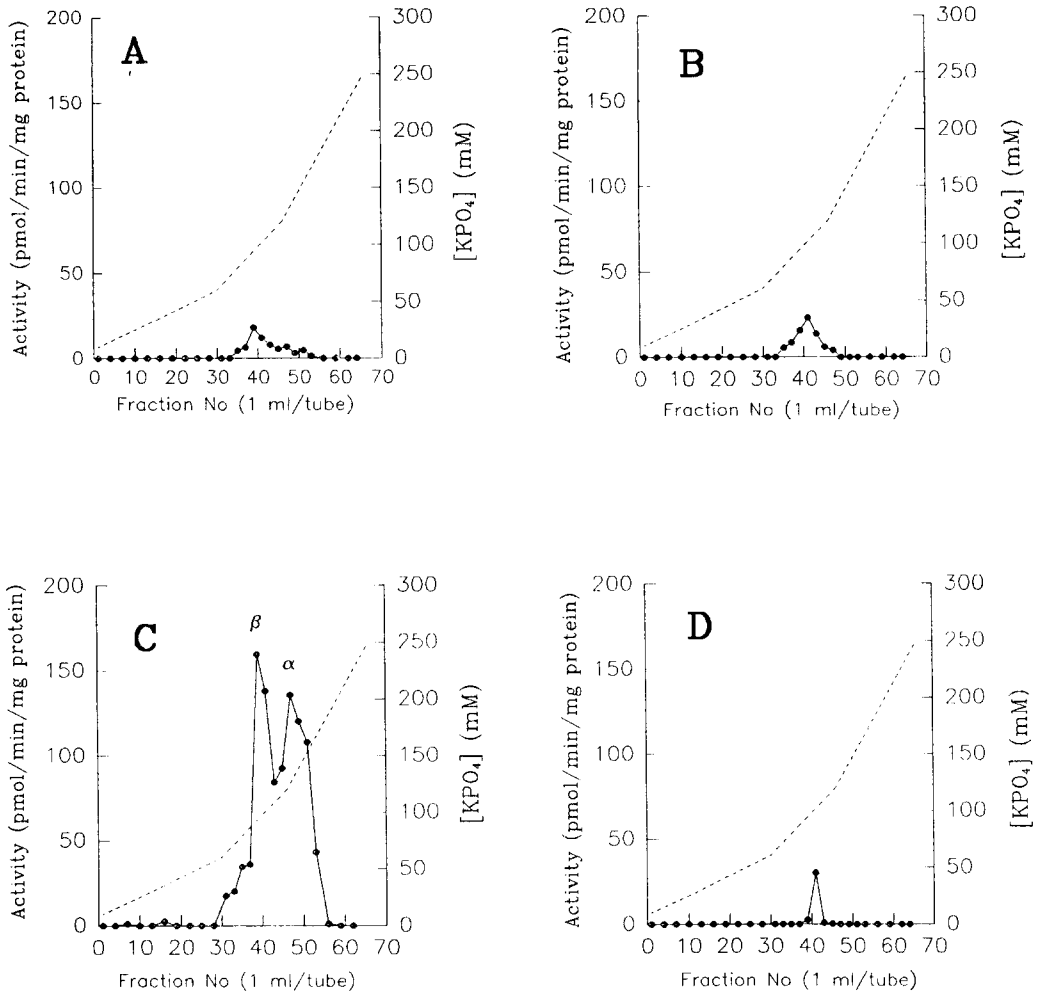

Fig. 3. Hydroxyapatite column chromatography of PKC from cultures of chick wing bud mesenchyme modulated by PMA or staurosporine. Cells were cultured in the presence of PMA (10^{-7} M) or staurosporine (5×10^{-9} M) for 3 days. Cells were homogenized and centrifuged at 100,000 x g for 1 hr and supernatant was used as cytosolic fraction (A, C). The precipitate was dissolved with extraction buffer containing 0.1% Triton X-100 and centrifuged at 100,000 x g for 1 hr. The supernatant was used as particulate fraction (B, D). The PKC activity of the cells was separated by hydroxyapatite column chromatography and enzymatic activity was assayed as described in Materials and Methods. A, B: PMA-treated cultures, C, D: staurosporine-treated cultures.

in the transductin of external growth and differentiation signals (Nishizuka, 1986). Molecular cloning and biochemical studies indicate that the members of the PKC family exhibit differential patterns of expression in a variety of cell types and suggest that different isotypes play different roles in the cellular responses to external stimuli (Nishizuka, 1988). Previously, we have demonstrated that PKC activity increases as chondrogenesis proceeds and PKC is closely related to chondrogenesis (Sonn and Solursh, 1993). However, the role of PKC isoforms in chondrogenesis is not known yet. This study shows the changes of PKC isoforms during chondrogenesis.

As far as our knowledge extend, there are no

Table 1. Peak activities of PKC isoforms in different states of chondrogenesis†.

Culture conditions	Activities		Chondrogenesis
	PKC α	PKC β	
[1]Con 1d	0	35.3	-
[2]Con 3d	99.7	170.3	+
[3]PMA 3d	0	31.4	-
[4]St 3d	131.3	150.2	++

†Enzyme activities are from cytosolic fraction and expressed as pmol/min/mg protein. [1]One day cultured cells [2]Three day cultured cells [3]Cells treated with PMA for 3 days [4]Cells treated with staurosporine for 3 days

antibodies to chicken PKC isotypes so far and no interaction between mammal antibodies to PKC isoforms and chicken PKC isoforms. Therefore, we compared the hydroxyapatite elution pattern of PKC isoforms from chicken brain or cultures of chick limb mesenchyme with that of rat brain. While three isoforms are resolved in rat brain tissues, only activities of two PKC isoforms, PKC α and β were observed in cultures of chick limb mesenchyme, while PKC γ isoform is absent. In some cells, PKC β isoforms is related to the cell function or cell differentiation. Immunoblot study of PC12 cells revealed a large increase in the PKC β_{II} during induced differentiation by treatment with nerve growth factor (Wooten *et al.*, 1992). A protein kinase C agonist, selective for the βI isoform, induces expression of adhesion molecules on human umbilical vein endothelial cells (Deisher *et al.*, 1993). In other cells, such as promonocytic cell line, U1, PKC β_{I} isoform is not important in induction of latent HIC-1 in U1 cells (Kim *et al.*, 1994). In this study, the early appearance of PKC β isoforms and large increase in the β isoform activity during the differentiation of chick mesenchyme suggest that PKC β might play an important role in chondrogenesis. PMA inhibits differentiation of chick limb mesenchyme (Sasse *et al.*, 1983, Fig. 2) and down regulates PKC. The resolution of PKC isoforms by hydroxyapatite column chromatography in cultures of chick limb mesenchyme treated with PMA shows small activity of β isoform. Again, this result suggests that PKC β isoform is involved in chondrogenesis. However, the possibility remains that PKC α is somehow related to chondrogenesis because PKC α isoform is completely down-regulated by PMA treatment. We are currently exploring the relationship between α and β isoforms during chondrogenesis using PKC isoforms specific activators. PKC isoforms can be divided into two categories based on Ca^{2+} requirement for activation; Ca^{2+}-dependent (conventional PKC (cPKC)) and Ca^{2+}-independent (novel PKC (nPKC)) and nPKCs have some roles in cell function and cell differentiation as cPKCs do. PKC δ plays a negative role in the early stages of murine erytholeukemia cell differentiation (Sparatore *et al.*, 1993). Additional studies on nPKCs during chondrogenesis are needed to understand complete mechanism of PKC isoforms in this process.

Staurosporine, a microbial alkaloid, is now widely known to be an inhibitor of several kinases, including PKC, cAMP-dependent protein kinase (Nakano, *et al.*, 1987), cGMP- dependent protein kinase (Niggli and Keller, 1991), Ca^{2+}/calmodulin-dependent kinase II (Yanagihara *et al.*, 1991). It promotes differentiation of chick limb mesenchyme without changes of PKC activity (Sonn and Solursh, 1993). We observed no changes of PKC isoforms profile on hydroxyapatite column chromatography in cytosolic fraction of staurosporine-treated cultures (Fig. 2) and this result supports the suggestion that influence of staurosporine on differentiation is not related to PKC (Sonn and Solursh, 1993). The presence of small activity of PKC β isoform in particulate fraction implies that β isoform is in inactivated state by treatment of staurosporine. However, the activity of β isoform is very small compared with that of cytosolic fraction and more precise assay of PKC isoforms is needed.

In summary, the activity of PKC β isoform is present in the early stage of chondrogenesis and shows large increase during chondrogenesis indicating that it is involved in differentiation process. PKC α isoform is present with similar activity in differentiated cells. PMA inhibits chondrogenesis by down-regulation of both PKC α and β isoforms. Meanwhile, staurosporine promotes chondrogenesis without any effect on

PKC isoforms profile as judged by hydroxyapatite column chromatography.

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계배 간충직세포 분화과정에서의 Protein Kinase C Isoform들의 변화

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본 연구는 계배 limb 간충직세포의 연골분화과정에서 PKC isoform들의 활성변화를 조사하였다. HH-stage 23/24의 계배 wing bud로 micromass 배양을 실시하고 세포를 균질화 한 후 DEAE-cellulose 크로마토그래피로 PKC활성을 갖는 분획을 얻었다. 이 분획으로 hydroxyapatite 크로마토그래피를 실시하여 PKC isoform들을 분리하였다. 분리된 PKC isoform들의 양상을 흰쥐 뇌 조직으로부터 얻은 PKC isoforms들의 양상과 비교하여 PKC isoform들을 확인하였다. 배양 초기에 약한 PKC β isoform의 활성이 있었고 배양 3일째에 PKC α와 β의 활성이 크게 나타났으며 PKC γ isoform의 활성은 나타나지 않았다. 배양세포에 PMA(10^{-7} M)를 처리하였을 때 연골세포의 분화가 억제되고 PKC α와 β의 활성이 억제되었다. Staurosporine의 연골세포의 분화 촉진효과는 PKC isoform들의 양상에 영향을 주지 않고 일어났다. 이러한 결과는 PKC α와 β isoform, 특히 β isoform이 연골세포의 분화와 밀접한 관계가 있고 staurosporine의 연골세포 분화촉진 효과는 PKC isoforms 양상과는 무관하게 일어남을 보여준다.