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Evidence on the Participation of Protein Kinase C α in the Proliferation of Cultured Myoblasts

Daniela A. Capiati,[1,2] Fabiana Limbozzi,[1] Maria T. Téllez-Iñón,[2] and Ricardo L. Boland[1]*

[1]Departamento de Biología, Bioquímica y Farmacia, Universidad Nacional del Sur, 8000 Bahía Blanca, Argentina
[2]Instituto de Investigaciones en Ingeniería Genética y Biología Molecular, CONICET, 1428 Buenos Aires, Argentina

Abstract There is evidence involving protein kinase C (PKC) in the signal transduction pathways that regulate the differentiation of myoblasts into mature multinucleated muscle cells (myotubes). In order to obtain information on the possible role of individual PKC isozymes in myogenesis, in the present work we investigated the differential expression of PKC isoforms α, β, δ, ϵ, and ζ during muscle cell development in vitro. Chick embryo myoblasts cultured from 1 to 6 days were used as experimental model. Morphological characterization and measurement of specific biochemical parameters in cultures, e.g., DNA synthesis, creatine kinase activity, and myosin levels, revealed a typical muscle cell developmental pattern consisting of an initial proliferation of myoblasts followed by their differentiation into myotubes. PKC activity was high at the proliferative stage, decreased as myoblasts elongated and fused, and increased again in differentiated myotubes. In proliferating myoblasts, the PKC inhibitors calphostin C and bisindolylmaleimide I decreased DNA synthesis whereas in myoblasts undergoing differentiation they exerted the opposite effect, suggesting that PKC plays a role at both stages of myogenesis. Western blot analysis of changes in the expression of PKC isoforms during muscle cell development showed high levels of PKC α in the proliferating phase which markedly decreased as myoblasts differentiated. Treatment with TPA of proliferative myoblasts inhibited DNA synthesis and selectively down-regulated PKC α, suggesting that this isozyme may have an important role in maintaining myoblast proliferation. On the other hand, an increase in the expression of PKC β, δ, and ϵ was detected during myogenesis, suggesting that one or more of these isoforms may participate in the differentiation process of myoblasts. J. Cell. Biochem. 74:292–300, 1999. © 1999 Wiley-Liss, Inc.

Key words: protein kinase C; PKC isozymes; myoblasts; DNA synthesis; myogenesis

Protein kinase C (PKC) consists of a family of related enzymes capable of modulating protein activity via serine/threonine phosphorylation. Once activated, an event which generally involves translocation of the enzyme from cytosol to membrane, PKC can phosphorylate a number of proteins and thereby regulate many short-term cellular events. PKC phosphorylation also influences long-term events, such as proliferation and differentiation [Nishizuka, 1988]. The cloning and study of the molecular structure and biochemical characteristics of the various PKC isoforms have revealed that the PKC family is divided into three major groups [Nishizuka, 1992; Parker et al., 1989]. The conventional isoforms PKC α, β_I, β_{II}, and γ, are dependent on calcium, phospholipids, and diacylglycerol for full activation. The novel isoforms δ, μ, ϵ, η, and θ lack the C2 DNA sequence which codes for the region responsible for calcium sensitivity of the protein and therefore do not require calcium for activation. A third class of PKC enzymes, the atypical isoforms ζ, λ, and ι, are dependent on phosphatidylserine, but independent of diacylglycerol and calcium. Since the multiple subspecies of PKC show distinct enzymological properties, different tissue ex-

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*Correspondence to: Dr. Ricardo Boland, Dept. Biología, Bioquímica & Farmacia, Universidad Nacional del Sur, San Juan 670-8000 Bahía Blanca, Argentina. E-mail: rboland@criba.edu.ar

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pression and subcellular distribution, it is likely that the various isoforms possess individual and varied functions.

PKC has been implicated indirectly in the signal transduction cascades which repress myogenesis. In 23A2 cells, a mouse myogenic cell line, PKC activity is down-regulated when myoblasts differentiate [Vaidya et al., 1991], suggesting that it may play a role as negative modulator of myogenesis. It has been shown that treatment of avian myoblasts with the phorbol ester TPA, which activates PKC, inhibits muscle-specific gene expression [Zhu et al., 1991]. Similarly, many growth factors that inhibit myogenesis increase PKC activity [Spizz et al., 1986; Clegg et al., 1987]. Stimulation of myoblast proliferation by the secosteroid hormone 1,25$(OH)_2$-vitamin D_3 correlates to an increase in PKC activity [Bellido et al., 1993]. It has been demonstrated that activated PKC induces phosphorylation of the muscle-specific transcription factor myogenin and abolishes its ability to bind DNA and to activate muscle transcription [Li et al., 1992].

The differential roles of PKC isozymes in myogenesis remain unknown. In the present work, individual changes in the expression of PKC isozymes along myogenesis are studied and evidence is provided indicating that PKC α may be involved in the proliferation of myoblasts at early times of culture, and its expression decreases to allow differentiation.

MATERIALS AND METHODS

Materials

Bovine pancreas trypsin, Dulbecco's modified Eagle medium (DMEM), leupeptin, aprotinin, calphostin C, bisindolylmaleimide I, TPA (phorbol 12-myristate 13-acetate), 1,2 dioleyl-rac-glycerol, phosphatidylserine, Immobilon P (polyvinylidene difluoride, PVDF) membranes, BCIP/NBT (5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium), anti-myosin antibodies, and the synthetic peptide GS (PLSRTLSVAAKK) were from Sigma Chemical Co. (St. Louis, MO). Western blot chemiluminescense reagents (Renaissence), [γ-^{32}P]ATP and [^{3}H]thymidine were provided by New England Nuclear (Chicago, IL). PKC α, β, δ, ϵ, and ζ antibodies, anti-rabbit IgG alkaline phosphatase-conjugated antibody and histone H1 were from GIBCO BRL (Gaithesburg, MD). Monotest CK NAC kit for creatine kinase activity determinations was from Boehringer-Mannheim (Germany).

Cell Culture

Myoblasts were obtained from 12-day-old chick embryo breast muscles by stirring in Earl's balanced salt solution (SSBE) containing 0.1% trypsin during 30 min as previously described [Drittanti et al., 1987; De Boland and Boland, 1985]. The freed cells were collected by centrifugation and the pellet was resuspended in DMEM suplemented with 10% Foetal Bovine Serum (FBS) and antibiotic-antimycotic solution. The suspension was dispersed by pipetting, filtered through nylon mesh, "preplated" on gelatin coated Petri dishes, and incubated for 1 h at 37°C to remove contaminant fibroblasts. The unabsorbed cells were placed in 10 cm dishes (10^7 cells/dish) and cultured at 37°C under humidified air with 5% CO_2. Histological analysis of cultures during 1–6 days have shown progressive fusion of spindle-shaped cells ($>$ 80% with more than one nucleus after 4 days of culture) and the presence of multinucleated well-differentiated fibers at the end of this culture interval, in agreement with the morphological characteristics described in Figure 1; moreover, ultrastructural analysis has revealed subcellular morphology characteristic of skeletal muscle cells; no fibroblasts could be detected (T. Bellido and R. Boland, unpublished results). Whole cell lysates were obtained by scraping the cells from the dishes, followed by sonication in 20 mM Tris/HCl pH 7.4, 0.33 M sucrose, 1 mM EDTA, 0.5 mM EGTA, 1 mM dithiothreitol, 1 mM phenylmethanesulphonyl fluoride (PMSF), 40 μg/ml leupeptin, 40 μg/ml aprotinin.

Thymidine Incorporation

The rate of thymidine incorporation into DNA was determined by adding 0.5 (μCi [^{3}H]thymidine/ml of DMEM to myoblast monolayers, incubating for 1 h at 37°C, and washing three times with Krebs-Henseleit-0.2% glucose solution. DNA and proteins were precipitated with ice cold 12% trichloroacetic acid, resuspended in 1 N NaOH and the radioactivity was counted in a liquid scintillation counter.

Creatine Kinase Assay

Creatine kinase activiy was measured using the Monotest CK NAC kit (Boehringer-Mann-

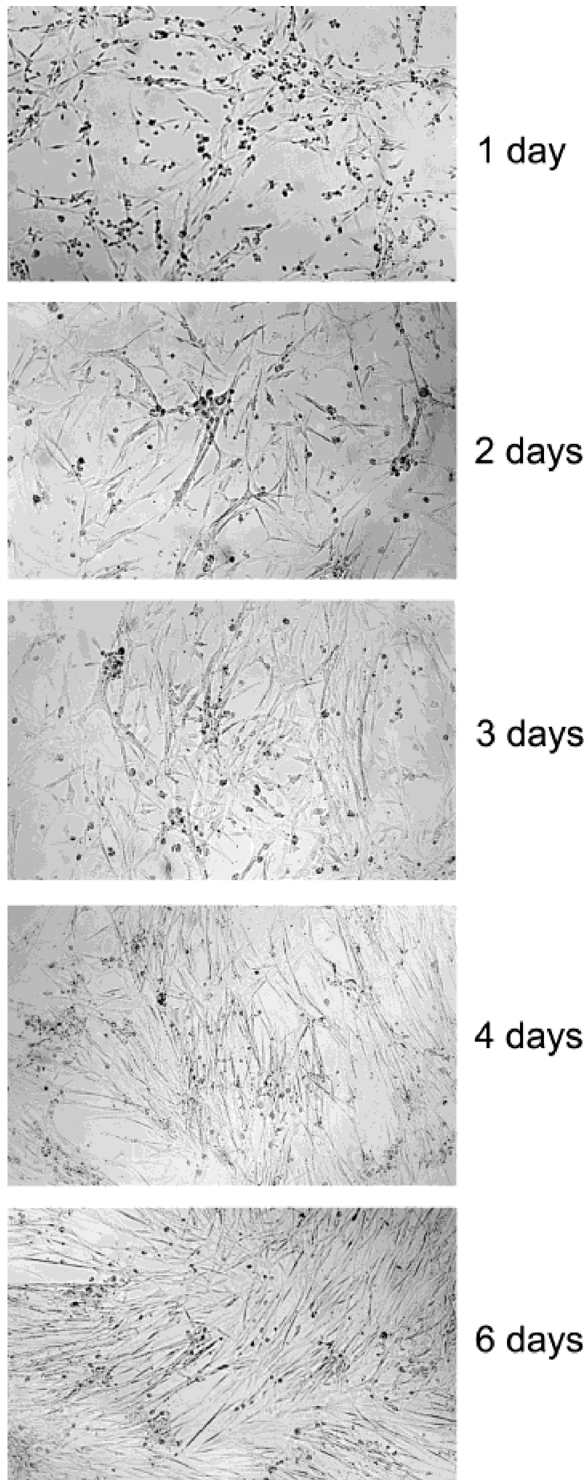

Fig. 1. Morphological characteristics of myoblasts during culture in vitro. Myoblasts were isolated from chick embryos and cultured in DMEM for 1–6 days as described under Materials and Methods. Micrographs of representative cultures obtained with a 10× objective are shown.

heim) by coupling the hexokinase and glucose-6-phosphate dehydrogenase reactions. One unit of activity represents 1 mol of NADPH generated/min per mg protein.

Protein Kinase C Assay

Protein kinase C was assayed by measuring the incorporation of ^{32}P from [γ-^{32}P]ATP into the synthetic Glycogen Synthase peptide (GS) or histone H1 as described before [Gomez et al., 1988]. The reaction mixture (60 μl) contained 20 mM Tris/HCl, pH 7.4; 10 mM $MgCl_2$; 25 μM GS (or 0.25 mg/ml of histone H1); 50 μM [γ-^{32}P]ATP (specific activity: 100 cpm/pmol) and the sample to be assayed in the presence of 1 mM $CaCl_2$, 60 μg/ml phosphatidylserine (PS) and 3 μg/ml 1,2 dioleyl-rac-glycerol (DG), or 1 mM EGTA. Reactions were initiated by the addition of [γ-^{32}P]ATP and incubated for up to 10 min at 30°C. Assay conditions were selected so that phosphorylation had a linear dependence on incubation time and enzyme concentration. The reactions were stopped by pipetting 40 μl of the reaction mixture onto Whatman P-81 phosphocellulose papers which were immediately soaked in 75 mM phosphoric acid and washed three times for 10 min in the same solution, then dried and radioactivity was counted in scintillation mixture [Roskoski, 1983]. The activity measured in the presence of EGTA was considered unspecific and substracted from the activities obtained when Ca^{2+}, PS, and DG were present in the incubation mixture.

Western Blot Analysis

Protein samples were subjected to SDS-PAGE according to the method of Laemmli [1970]. The separated proteins were electrophoretically transferred to PVDF membranes using a Bio-Rad Semidry Transfer Cell. Nonspecific sites were blocked by 5% nonfat dry milk in TBST (50 mM Tris/HCl, pH 7.5, 0.15 M NaCl, 0.05% Tween-20) for 1 h at room temperature. Membranes were incubated with anti-PKC (2 μg/ml) antibodies overnight at 4°C in TBST containing 5% nonfat dry milk followed by incubation with a 1:6,000 dilution of alkaline phosphatase- or with a 1:2,000 dilution of horseradish peroxidase-conjugated anti-rabbit IgG in TBST 5% nonfat dry milk. For myosin detection the membranes were incubated with myosin antibody (1:100 dilution) followed by incubation with horseradish peroxidase-conjugated

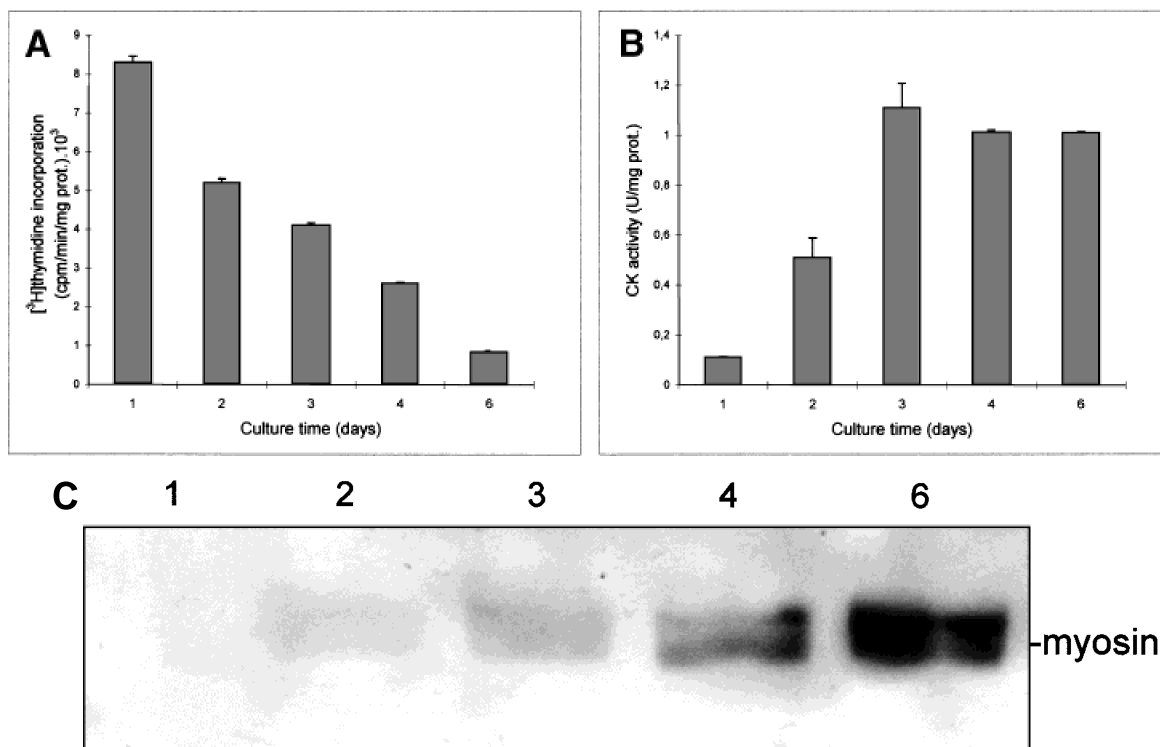

Fig. 2. Changes in biochemical parameters during myogenesis in vitro. DNA synthesis **(A)**, creatine kinase activity **(B)**, and myosin expression **(C)** were determined in myoblasts cultured for 1–6 days as described in Materials and Methods. The results are the means ±S.D. of determinations from three independent experiments; a representative Western blot for myosin levels along the culture interval is shown (numbers indicate days of culture).

anti-mouse IgG as for PKC isoform detection. Proteins were visualized using enhanced chemiluminescence system for horseradish peroxidase-conjugated IgG or BCIP/NBT as substrate for alkaline phosphatase. Competition assays were carried out employing peptides corresponding to the same regions used to generate the antibodies. Images were obtained with a model GS-700 Imaging Densitomer from Bio-Rad (Hercules, CA) by scanning at 600 dpi and printing at the same resolution. Bands were quantified using the Molecular Analyst program (Bio-Rad).

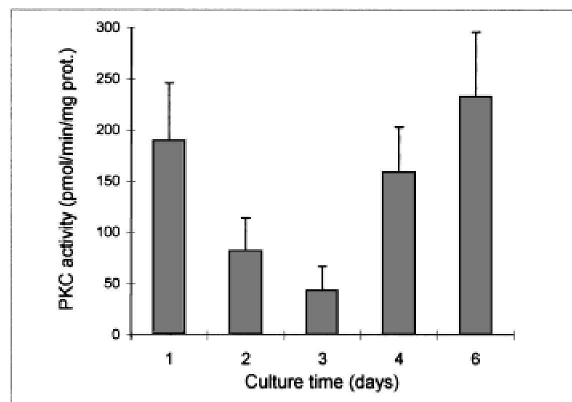

Fig. 3. Modifications in PKC activity during myogenesis in vitro. PKC activity levels in whole cell lysates from myoblasts cultured for 1–6 days were measured as described under Materials and Methods using GS as substrate. The results are the means ±S.D. of three independent experiments. $P < 0.025$ for 1 day vs. 3 days and 3 days vs. 6 days; $P < 0.05$ for 3 days vs. 4 days.

RESULTS

Morphological and Biochemical Characterization of Skeletal Muscle Cells Undergoing Differentiation In Vitro

Myoblasts are mononucleated cells which proliferate actively in culture followed by differentiation into multinucleated myotubes expressing phenotypic characteristics of mature muscle fibers [O'Neill and Stockdale, 1972; Wakelam, 1985]. Different parameters were determined in order to characterize the in vitro myogenic process. As shown in Figure 1, the morphological profiles of myoblasts cultured for 1–6 days indicate that undifferentiated muscle cells elongate, become aligned and fuse to form differentiated myotubes as the culture period

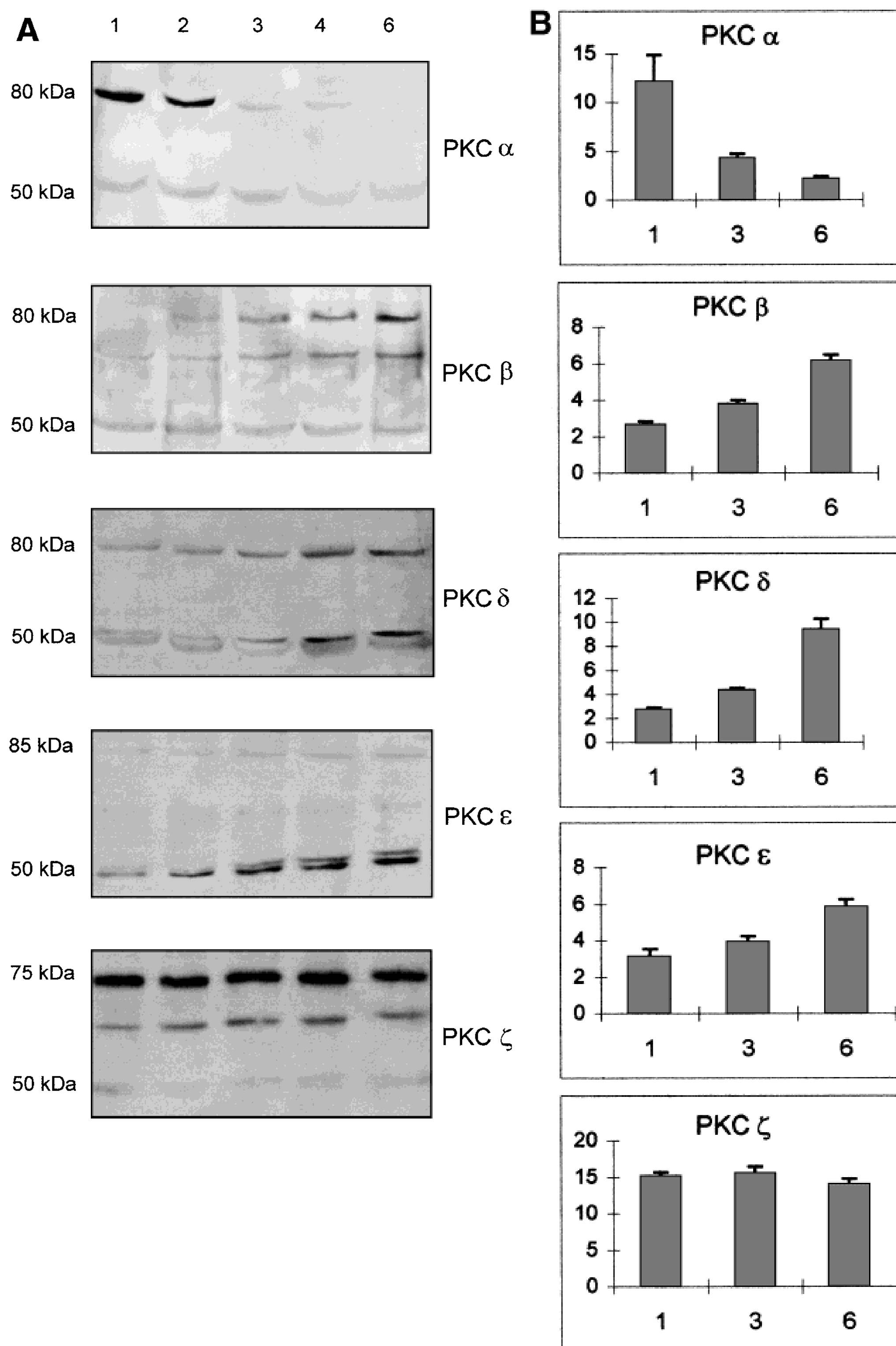

Fig. 4. Differential expression of PKC isoforms during myogenesis. Whole cell lysates of myoblasts cultured for 1–6 days were subjected to SDS-PAGE and transferred to PDVF membranes. Immunoblotting was performed using specific antibodies against PKC α, β, δ, ϵ, and ζ isoforms. The numbers indicate days of culture. **A**: Representative immunoblots. **B**: Quantitative analysis of blots; results are expressed as density values [(counts/mm^2)×$\cdot 10^3$] and are the average ± S.D. of four independent experiments. Numbers in the × scale represent days of culture. $P < 0.001$ for PKC α, 1 day vs. 3 days; PKC β, 1 day vs. 3 days and 3 days vs. 6 days; and PKC δ, 1 day vs. 3 days and 3 days vs. 6 days. $P < 0.005$ for PKC α, 3 days vs. 6 days and PKC ϵ, 3 days vs. 6 days. $P < 0.05$ for PKC ϵ, 1 day vs. 3 days.

progresses. Accordingly, in parallel to these changes the rate of DNA synthesis decreased 10-fold during the 1–6 day interval (Fig. 2A) and creatine kinase actvity increased 10–11-fold (Fig. 2B). In addition, myosin levels markedly increased from 2 to 6 days of culture during myogenesis (Fig. 2C). These results indicate that chick myoblasts cultured under the conditions described in Materials and Methods differentiate into myotubes within 6–7 days.

Changes in PKC Activity and Isoform Expression During Myogenesis

PKC activity was determined in myoblasts at different culture times along myogenesis. A decrease (−77%) in enzyme activity was observed as myoblasts elongated. In differentiated myotubes, PKC activity increased reaching the initial values. A similar biphasic profile of PKC activity was observed using histone H1 as substrate (data not shown).

Changes in the amounts of α, β, δ, ϵ, and ζ PKC isoform levels along myogenesis were determined by Western blot analysis. As shown in Figure 4A, two bands were detected for each isoform, one corresponding to the holoenzyme (approximately 80 kDa for PKC α, β, and δ, 85 kDa for PKC ϵ and 75 kDa for PKC ζ), and the other to the catalytic fragment (approximately 50 kDa) already described by several authors [Cressman and Shea, 1995; Murray et al., 1987]. In some cases an intermediate band was detected for the β and ζ isoforms. This band was also specific, as indicated by the ability of the corresponding peptides to compete with each isoform (data not shown). Quantification of the blots (Fig. 4B*)* showed that PKC α levels were high in proliferating myoblasts (1 day) and decreased dramatically as the cells differentiated (2.7- and 4.8-fold at 3 and 6 days, respectively). PKC β, δ, and ϵ were poorly expressed in undifferentiated myoblasts and their levels increased during myogenesis (PKC β: 1.4- and 2.3-fold, PKC δ: 1.7- and 3.8-fold, PKC ϵ: 1.3- and 1.9-fold, at 3 and 6 days, respectively) whereas PKC ζ did not change during this process. The results of Westen blot analysis suggest that there is a correlation between the expression of specific PKC isoforms and the biphasic profile of modifications of PKC activity during myogenesis. The high levels of enzymatic activity present in undifferentiated myoblasts may be accounted for by PKC α; then PKC activity decreases in parallel to the levels of this isoform. The late increase in PKC activity in differentiated myotubes may be due to PKC β, δ, and ϵ.

Effects of Calphostin C and Bisindolylmaleimide I Treatment on DNA Synthesis at Different Stages of Myogenesis

In order to obtain additional information about the role of protein kinase C in myogenesis, myoblasts were treated for 1 h at 37°C with the specific PKC inhibitor calphostin C (100 nM) at different times of culture followed by determination of [^{3}H]thymidine incorporation into DNA. The results are shown in Table 1. In proliferative myoblasts (24 h of culture), calphostin treatment decreased [^{3}H]thymidine uptake (−49%). This effect is reversed as myoblasts differentiate. In myoblasts cultured for 72 h, calphostin induced an increase in the rate of DNA synthesis (+49%). In agreement with these results, treatment with the selective PKC inhibitor bisindolylmaleimide I decreased [^{3}H]thymidine incorporation (−85%) in myoblasts cultured for 24 h and increased it (+34%) in more differentiated cells (72 h of culture).

Effects of TPA Treatment on DNA Synthesis, PKC Activity and PKC Isoform Levels

The phorbol ester TPA was used to down-regulate PKC. Myoblasts were treated with 100 nM TPA since plating. After 24 h, [^{3}H]thymidine incorporation into DNA, PKC activity, and isoforms levels were determined (Fig. 5). Both

TABLE I. Effects of Calphostin C and Bisindolylmaleimide I on DNA Synthesis at Different Times of Myoblast Culture[a]

Culture time (days)	Thymidine incorporation (% of control)	
	Calphostin	Bisindolylmaleimide
1	−48.7 ± 19.1*	−85.0 ± 4.2**
2	−15.3 ± 4.0	ND
3	49.1 ± 8.2**	33.5 ± 5.0***
4	28.0 ± 7.0**	ND

[a]Myoblasts cultured for 1, 2, 3, and 4 days were treated with 100 nM calphostin C or 30 nM bisindolylmaleimide I for 1 h. After treatment, the rate of [^{3}H]thymidine incorporation into DNA was determined as described in Methods. Results are expressed as percent variation with respect to the control. Data are the means ± S.D. of three independent experiments. ND, not determined.
*$P < 0.01$.
**$P < 0.005$.
***$P < 0.001$.

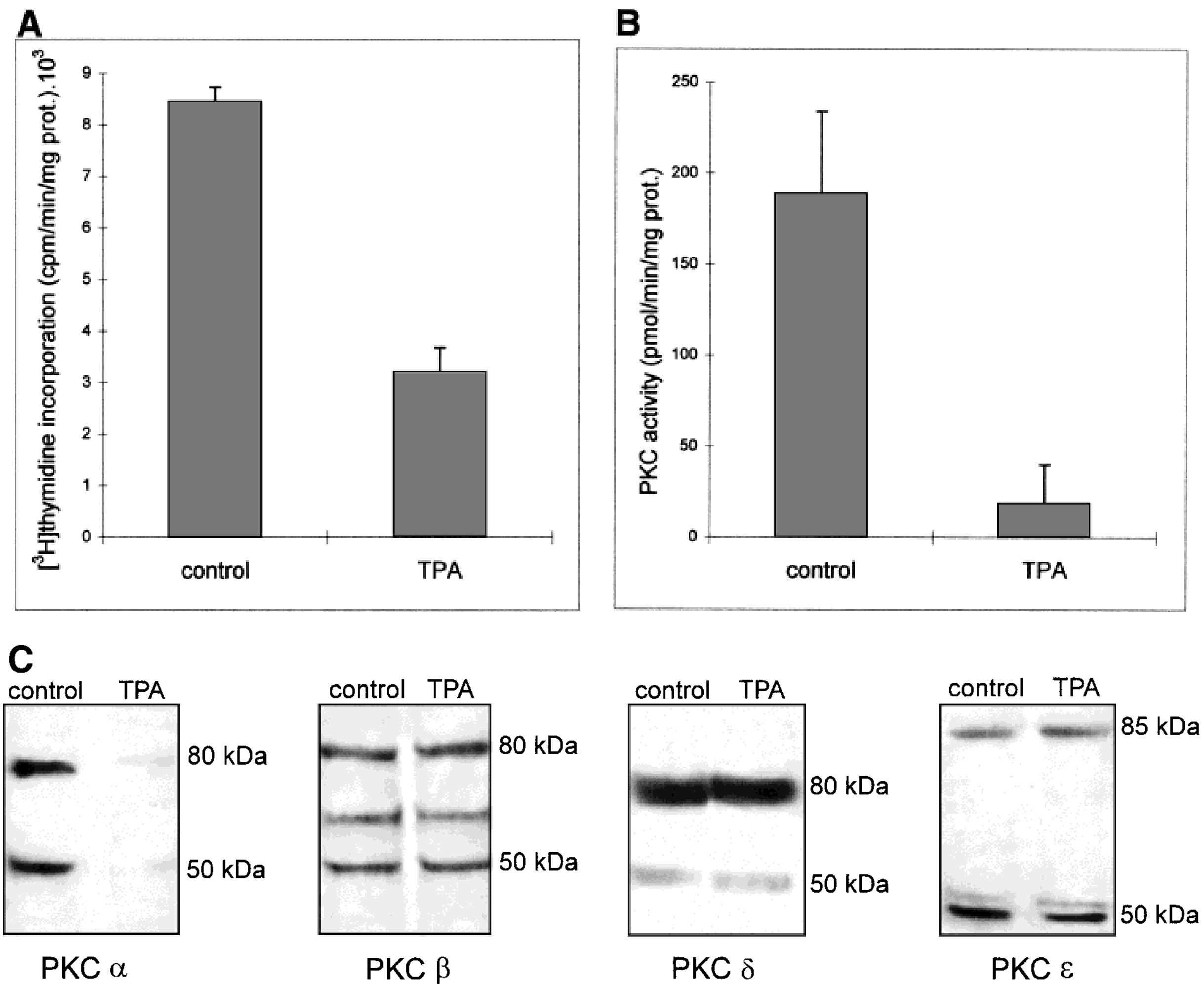

Fig. 5. Effects of TPA on DNA synthesis, PKC activity and isozyme expression. Myoblasts were cultured either in the absence or presence of 100 nM TPA for 24 h. **A**: [^{3}H]thymidine incorporation into DNA and **(B)** PKC activity were determined as described under Materials and Methods. Results are the average ± S.D. of three independent experiments. $P<0.001$ for [^{3}H]thymidine incorporation and $P<0.01$ for PKC activity. **C**: PKC α, β, δ, and ϵ isoforms were detected by immunoblot analysis of whole cell lysates of control and treated myoblasts as described under Materials and Methods. Representative blots are shown; quantitative data and statistical analysis are given in the text (Results).

DNA synthesis and PKC activity dramatically decreased in myoblasts exposed to TPA (−63% and −90%, respectively). Western blot analysis showed that PKC α isoform levels decreased in treated cells (−94.4 ± 6.9%, n = 3, $P< 0.001$), while no significant changes were observed in β, δ, and ϵ, the other TPA-sensitive PKC isoforms studied.

DISCUSSION

In the last few years PKC has become an important research subject because it has been shown to be involved in the control of proliferation and differentiation of many cell types. In skeletal muscle cells, it has been implicated indirectly in the signal transduction cascades that repress myogenesis [Vaidya et al., 1991; Zhu et al., 1991; Spizz et al., 1986; Clegg et al., 1987; Li et al., 1992], but the individual functions of PKC isozymes in muscle cell development remain unknown. The aim of the present study was to provide evidence on the distinct role of PKC isoforms in myogenesis.

Primary cultures of chick myoblasts were chosen as a model. The first step was to characterize the differentiation process of these cells by studying changes in typical morphological and biochemical parameters along myogenesis in vitro. When myoblasts are cultured, they proliferate before undergoing differentiation. In the proliferative state, they are round-shaped, then they elongate, become aligned and

fuse to form multinucleated myotubes. During this process creatine kinase activity and myosin expression increase [O'Neill and Stockdale, 1972; Wakelam, 1985]. The results obtained in this work indicate that cultured chick myoblasts differentiate to myotubes within 6–7 days following the morphological and biochemical patterns previously described.

PKC activity and isoform levels were determined in cultured avian myoblasts at different times during the 6-day proliferation and differentiation interval. PKC activity is high in undifferentiated cells, then decreases as myoblasts elongate and fuse, and increases again in differentiated myotubes. The results obtained by Western blot analysis of PKC α, β, δ, ϵ, and ζ, isoforms which have been found in mammalian skeletal muscle [Hong et al., 1995; Schmitz-Peiffer et al., 1996], may explain the PKC activity profile. According to the immunochemical data the high levels of enzymatic activity at early times of culture may be due to PKC α, and the late increase could correspond to the β, δ, and ϵ isoforms. The differential expression of PKC isozymes suggests that they may play distinct roles in proliferation and differentiation of myoblasts.

Treatment with PKC inhibitors calphostin C and bisindolylmaleimide I exerts opposite effects on DNA synthesis depending on culture time. In undifferentiated myoblasts, inhibition of PKC decreases myoblast proliferation. At this stage of myogenesis PKC α shows its highest levels of expression, and it is possible that the effect observed on DNA synthesis may be due to the inhibition of this isozyme. By contrast, in myoblasts undergoing differentiation (72 h of culture) calphostin treatment leads to an increase in cell proliferation. At this stage of myogenesis, PKC β, δ, and ϵ show high levels of expression compared to the proliferative stage and the stimulation of [^{3}H]thymidine incorporation into DNA may be due to the inhibition of one or more of these isoforms. Of relevance, TPA treatment of proliferative myoblasts inhibits DNA synthesis in parallel to a selective down-regulation of PKC α.

Our observations with myoblasts are in agreement with recent reports indicating that PKC α mediates proliferative events in different cell systems [Battaini et al., 1994; Wooten et al., 1992; Perletti et al., 1994]. In addition, there is also evidence involving the β, δ, and ϵ isozymes of PKC in the differentiation of various cell types other than muscle [O'Driscoll et al., 1995; Beckman et al., 1990; Doi et al., 1994].

In conclusion, the present study provides evidence indicating that PKC α may be involved in the proliferation of myoblasts. It is also possible that β, δ, and/or ϵ PKC isozymes may contribute to the differentiation process or, alternatively, play a role in maintaining the differentiated state by inhibiting muscle cell proliferation.

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