Antisense oligonucleotides targeted against protein kinase c α inhibit proliferation of cultured avian myoblasts

D. A. Capiati^{*†}, G. Vazquez^{*}, M. T. Tellez Iñón[†] and R. L. Boland^{*‡}

^{*}Departamento de Biología, Bioquímica y Farmacia, Universidad Nacional del Sur, San Juan, Bahía Blanca and [†]Instituto de Investigaciones en Ingeniería Genética y Biología Molecular, CONICET, Buenos Aires, Argentina

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Abstract. Protein kinase C (PKC) has been implicated in the control of proliferation and differentiation of many cell types. There is evidence indicating that it plays a role in signal transduction mechanisms related to myogenesis, but little is known about the individual functions of PKC isoforms in muscle cell development. Data obtained in previous studies using cultured chick embryo skeletal muscle cells suggested that PKC α is linked to the regulation of myoblast proliferation. However, this causal relationship could not be definitively established as no experiments based on selective inhibition of this isoform were carried out. In the present work, specific inhibition of the expression of PKC α in cultured myoblasts by using antisense oligonucleotide technology resulted in a significant decrease of culture cell density and DNA synthesis, clearly showing that this isoenzyme is involved in signalling pathways which promote muscle cell proliferation.

INTRODUCTION

Protein kinase C (PKC) consists of a family of isoenzymes capable of modulating protein activity via serine/threonine phosphorylation (Parker *et al.* 1986; Nishizuka 1988; Nishizuka 1992). PKC has been an important subject of investigation in the last few years because of its role in the control of cell proliferation and differentiation (Clemens, Trayner & Menaya 1992).

In skeletal muscle cells, a requirement for PKC activity has been linked to signalling processes mainly related to myoblast fusion and progression to the terminally differentiated state (Adamo *et al.* 1989; David, Faser & Perrot 1990; Vaidya *et al.* 1991; Zhu, Schwartz & Crow 1991; Li *et al.* 1992). However, there is no information on the individual roles of PKC isoenzymes in muscle cell growth. Primary cultures of embryonic skeletal muscle cells, in which mononucleated myoblasts proliferate actively followed by differentiation into multinucleated myotubes expressing phenotypic characteristics of mature muscle fibres (O'Neill & Stockdale 1972), have been widely used to study the process of myogenesis and its regulation by a number of factors (Wakelam 1985; Schultz & McCormick 1994; Boland *et al.* 1995). Using this *in vitro* experimental model, we have recently obtained evidence suggesting

Correspondence: Dr Ricardo Boland, Department of Biología, Bioquímica & Farmacia, Universidad Nacional del Sur, San Juan 6708000 Bahía Blanca, Argentina. Fax: +54 2914595130; E-mail: rboland@criba.edu.ar

that PKC α may have a function in maintaining chick embryo myoblast proliferation. Thus, Western blot analysis of changes in the expression of PKC isoforms showed high levels of PKC α in the proliferating phase which markedly decreased as myoblasts differentiated; in addition, treatment of proliferative myoblasts with the phorbol ester TPA inhibited DNA synthesis and selectively down-regulated PKC α (Capiati *et al.* 1999a). Furthermore, it has been shown that stimulation of myoblast proliferation by 1,25(OH)₂-vitamin D₃ correlates to increased PKC α expression whereas decreased PKC α levels are observed during the subsequent activation of myoblast differentiation by the hormone (Capiati, Tellez-Iñón & Boland 1999b).

However, as no experiments based on specific inhibition of PKC α were carried out, no definitive conclusions could be made on the participation of this PKC isoform in embryonic muscle cell division. Various synthetic strategies have been applied to develop isoenzyme-specific inhibitors of PKC but these efforts have been hampered by the similarities in protein structure of the several PKC isoforms (Hofmann 1997). To overcome this difficulty, in the present work we have used an antisense oligodeoxynucleotide to specifically inhibit the expression of myoblast PKC α , and, through this selective experimental approach, we have obtained strong direct evidence linking PKC α and the regulation of muscle cell proliferation.

MATERIALS AND METHODS

Materials

Dulbecco's Modified Eagle's Medium (DMEM), leupeptin, aprotinin, and Immobilon P (polyvinylidene difluoride, PVDF) were from Sigma Chemical Co. (St. Louis, MO, USA). Western blot chemiluminescence reagents (Renaissance) and [³H]thymidine (specific activity, 20 Ci/mmol) were provided by New England Nuclear (Chicago, IL, USA). PKC α , β , δ , ϵ and ζ antibodies, antirabbit IgG horse raddish peroxidase-conjugated antibody and Lipofectin reagent were from Gibco BRL (Gaithersburg, MD, USA). Oligodeoxynucleotides (ODNs) were synthesized by the DNA Agency (Malvern, PA, USA).

Cell culture

Myoblasts were obtained from the breast muscle of 13-day-old white leghorn chick embryos by stirring in Earle's balanced salt solution (SSBE) containing 0.1% trypsin during 30 min, essentially as previously described (Drittanti, De Boland & Boland 1989). The freed cells were collected by centrifugation and the pellet was resuspended in DMEM supplemented with 10% Foetal Bovine Serum (FBS) and antibiotic-antimycotic solution. The suspension was dispersed by pipetting, filtered through nylon mesh and plated onto glass Petri dishes to remove contaminating fibroblasts and epithelial cells. The unattached myogenic cells (100 000 cells/cm²) were plated onto plastic Petri dishes (100 mm in diameter) and incubated under humidified air with 5% CO₂. For transfection experiments, 24 h cultures were used when myoblasts are at their proliferative state. Under these culture conditions, myoblasts differentiate to myotubes within 6–7 days as revealed by morphological characterization and measurement of specific biochemical parameters, e.g. DNA synthesis, creatine kinase activity and myosin levels (Capiati *et al.* 1999a).

Cell transfection

The following oligodeoxynucleotide (ODN) sequences with phosphorothioate linkages throughout the entire ODN molecule were used: antisense-PKC α (AS): 5'-CATGGTPCCCCCAACCACC-3', P = T or C (antisense sequence against 20 nucleotides

upstream of the AUG codon); sense-PKC α (S): 5'-GGTGGTTGGGGGGRACCATG-3', R = A or G and scrambled (SC) ODN: 5'-ATCCTTAGAGTCGCGTACTT-3'. Either AS or S sequences showed no homology to any DNA in the GenBank except PKC α (human, rat, mouse and rabbit species) whereas the SC exhibited no similarity to any known DNA sequence. The Transfection with ODNs using Lipofectin was performed according to manufacturer's instructions. ODNs (5 μ M) were incubated with Lipofectin in DMEM for 15 min at room temperature. Myoblasts cultured for 24 h were washed to remove serum before addition of ODN-Lipofectin mixtures and incubation was performed for 20 h at 37°C. The ODN solution was removed, DMEM was added and the plates were placed into a metabolic incubator for an additional period of either 18, 24 or 48 h. Control treatments included DME medium or Lipofectin only. Dose-and time-response curves for Lipofectin and ODN were previously determined to optimize conditions (data not shown). Around 65–70% of the cell population was effectively transfected by using this protocol (not shown), as assayed by *in situ* staining for β -galactosidase in cells transfected with plasmid DNA containing the lacZ gene under transcriptional control of the CMV promoter (Ciccarone, Hawley-Nelson & Jessee 1993). Moreover, myoblast transfection with either S, SC or AS ODNs had no deleterious effects on metabolic and functional behaviour of the cells under both basal or stimulating conditions (Vazquez, De Boland & Boland 1999).

Cell death quantification

Myoblasts cultured in 6-multiwell plates under the conditions described above were transfected with either S, AS or SC ODNs or incubated in the presence of DME or Lipofectin alone and incubated for 20 h at 37°C. The medium was then removed, DMEM was added and cells were cultured for an additional period of either 18, 24 or 48 h. Cell death was quantified at each time by trypan blue staining (Zhuang, Lynch & Kochevar 1998). Nonadherent cells were combined with adherent cells released from the culture dish using trypsin-EDTA, resuspended in serum-containing medium and collected by centrifugation. Subsequently, 0.04% trypan blue was added and the percentage of cells exhibiting both nuclear and cytoplasmic staining (dead cells) was determined using a haemocytometer. Four hundred cells were counted for each condition at each assayed time and the number of dead cells was expressed as the percentage of the total cell population.

Western Blot analysis

Cells were scraped from the dishes and homogenized 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 phenylmethylsulphonyl fluoride (PMSF), 40 μ g/mL leupeptin, 40 μ g/mL aprotinin. Protein samples were subjected to SDS-PAGE according to the method of Laemmli (Laemmli 1970) and electrophoretically transferred to PVDF membranes using a Bio-Rad Semidry Transfer Cell. Non-specific sites were blocked by 1% (w/v) bovine seroalbumin (BSA) in TBST (50 mM Tris-HCl pH 7.5, 0.15 M NaCl, 0.05% Tween-20) for 1 h at room temperature. The equivalence of transferred proteins from each gel lane was evaluated by staining membrane-bound proteins with 0.1% Coomassie blue R250. Membranes were incubated with anti-PKC antibodies (2 mg/ml) overnight at 4°C, followed by incubation with horse radish peroxidase-conjugated antirabbit IgG. Bands were visualized by chemiluminescence detection. Images were obtained with a model GS-700 Imaging Densitomer (Bio-Rad, Hercules, CA, USA) by scanning at 1200 dpi and printing at the same resolution.

Figure 1. Decreased expression of PKC α in myoblasts transfected with an antisense oligodeoxynucleotide (ODN). Twenty-four hour-cultured chick embryo myoblasts were transfected with sense (S), antisense (AS), or scrambled (SC) ODNs against PKC α . Treatments with Lipofectin (LPF) or DME medium (DME) alone were carried out as controls. Forty-eight hours after transfection whole cell lysates were obtained and then subjected to SDS-PAGE followed by immunoblotting using an specific anti PKC α antibody. No changes in the expression of PKC β , δ , ε and ζ were detected by Western blot analysis performed in parallel (not shown).

[3 H]Thymidine incorporation

DNA synthesis by the [3 H]-thymidine incorporation technique was performed essentially as previously described (Drittanti *et al.* 1989). Myoblast monolayers were washed with DMEM and incubated with DMEM containing 0.5 μ Ci/mL [3 H]thymidine for 1 h in a metabolic incubator. The cells were then washed 3 times with KrebsHenseleit (116 mM NaCl, 50 mM KCl, 1 mM CaCl_2 , 1 mM MgSO_4 , 24 mM NaHCO_3 , pH 7.4)-0.2% glucose solution. Myoblast proteins were precipitated with 12% trichloroacetic acid and dissolved in 1 N NaOH. Incorporated radioactivity was measured in a liquid scintillation counter.

Statistical evaluation

The significance of the results was evaluated by Student's *t*-test, where $P < 0.05$ was considered significant (Snedecor & Cochran 1967).

RESULTS AND DISCUSSION

Various lines of evidence previously described have suggested that PKC α is involved in the regulation of proliferation of chick myoblasts (see Introduction; Capiati *et al.* 1999a, 1999b). Given the fact that PKC inhibitors available are not isoenzyme-specific, we applied antisense oligonucleotide technology to further demonstrate the participation of this PKC isoform in embryonic skeletal muscle cell division. Antisense oligonucleotides can be targeted to mRNA sequences which are unique to a given PKC isoenzyme, leading to the selective inhibition in expression of that protein (Dean *et al.* 1996; McGraw *et al.* 1997).

To specifically characterize the role of PKC α in chick myoblast proliferation we used an antisense oligodeoxynucleotide (ODN) complementary to a 20-mer nucleotide sequence of the PKC α mRNA, targeting an untranslated region upstream of the AUG starting codon. Oligos aimed at the 5 or 3 untranslated regions have shown to be the most effective for suppression of PKC α in other cell systems (Mochly-Rosen & Kauvar 1998). As the base sequence of the chick (*Gallus gallus*) mRNA is not known for PKC α , we chose this sequence which is highly conserved among all the cDNA sequences reported for human, rat, mouse and rabbit PKC α . Myoblasts transfected with the antisense sequence showed a significant decrease in PKC α 48-h after transfection, as revealed by Western blot analysis (Fig. 1) whereas the levels of PKC isoforms β , δ , ε and ζ , also expressed in chick muscle cells (Capiati *et al.* 1999a, 1999b), were not affected (data not shown). A significant reduction in cell density (–63%) and DNA synthesis (–46%) was observed in myoblasts transfected with the antisense sequence as compared to sense and scrambled ODNs (Figs 2 and 3, respectively).

Treatment with Lipofectin alone caused a slight reduction in cell density (–13% with respect to DME medium) with no effects on the rate of DNA synthesis, the former change probably due to effects of the transfection reagent on the plasma membrane causing cell lysis. However, as noted the magnitude of this variation is negligible compared with that caused by antisense treatment. The possibility that the drastic reduction in cell density observed in antisense-treated cultures (Fig. 2a, b) could be due to increased cell death rather than to a strong restriction of cell proliferation was ruled out, as no differences were observed in trypan blue uptake between AS-transfected cells and control cultures (Fig. 2c). Moreover, a decrease in PKC α was already observed within 24 h after transfection, i.e. before a significant reduction in cell density could be detected (not shown). These results thus indicate that inhibition of PKC α expression leads to a significant decrease in myoblast proliferation rate. This is in line with recent observations indicating that PKC α participates in the regulation of proliferation of several cell types (Wooten *et al.* 1993; Battaini *et al.* 1994; Perletti *et al.* 1994; Kato *et al.* 1999). We recently observed (Capiati *et al.* 1999a) that total PKC activity decreases (up to –80%) in the course of myoblast elongation (2–3 days in culture) but then suddenly augments reaching initial values when the terminally differentiated (myotube) state is achieved. The down turning phase in PKC activity strongly correlates with a drastic reduction in PKC α levels, whereas a concomitant up-regulation in the expression of PKC β , δ and ϵ may account for the late increase in PKC activity exhibited by the myotubes. Similar changes in total PKC activity have been previously reported for chick embryo- (Adamo *et al.* 1989) and mouse-myogenic cells (Vaidya *et al.* 1991) but the role of individual PKC isoforms has not been addressed in these reports. As derived from our results (see also Capiati *et al.* 1999a,b) the differential expression of PKC isoenzymes suggests that they may play distinctive roles in proliferation and/or differentiation of myoblasts. Although a decrease in PKC activity levels has been postulated to be critical in achieving and maintaining a terminally differentiated phenotype (Vaidya *et al.* 1991) it must be kept in mind that changes in total PKC activity should not necessarily match the changes in expression of PKC isoenzymes.

In conclusion, these results clearly demonstrate that PKC α is involved in cell signalling mechanisms which promote proliferation of myoblasts, representing the first specific evidence on the role of an individual PKC isoform in myogenesis. In view of recent studies performed with other cell types, the mitogenic effects of PKC α on muscle cells may be mediated through stimulation of members of the MAP kinase cascade and/or independent signal transduction pathways involved in proto-oncogene regulation (Fung *et al.* 1997; Chiloehes *et al.* 1999; McKay *et al.* 1999; Soh *et al.* 1999).

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Figure 2. Effect of inhibition of PKC α expression on myoblast cellular density. Twenty-four hour-cultured chick embryo myoblasts were transfected with sense (S), antisense (AS) or scrambled (SC) ODNs against PKC α . Treatments with Lipofectin (LPF) or DME medium (DME) alone were carried out as controls. Forty-eight hours after transfection the monolayers were photographed with a 10 X objective lens (a), stained with May Grunwald–Giemsa solution and the nuclei present in 40 $\times$ fields were counted (b). Results are the means $\pm$ SD of three independent experiments, counting 10 fields in each of them; $P < 0.001$ for AS vs. S and AS vs. SC. Twenty-four hour-cultured chick embryo myoblasts were subjected to the conditions described in panel A; after the indicated times cells were harvested by trypsinization and the number of dead vs. living cells was determined by trypan blue exclusion as described in Materials and Methods. Bars represent the mean $\pm$ SD of two independent experiments. Four hundred cells were counted for each condition at each assayed time (c).

Figure 3. Effect of inhibition of PKC α expression on DNA synthesis. Twenty-four hour-cultured myoblasts were transfected with sense (S), antisense (AS) or scrambled (SC) ODNs against PKC α . Treatments with Lipofectin (LPF) or DME medium (DME) alone were carried out as controls. Forty-eight hours after transfection [³H] thymidine incorporation into DNA was determined as described in Materials and Methods. Results are the means $\pm$ SD of three independent determinations; $P < 0.005$ for AS vs. S, $P < 0.001$ for AS vs. SC.

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