Journal of Cellular Biochemistry 77:200–212 (2000)

Role of Protein Kinase C in 1,25(OH)$_2$-Vitamin D$_3$ Modulation of Intracellular Calcium During Development of Skeletal Muscle Cells in Culture

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Abstract Regulation of muscle cell Ca^{2+} metabolism by 1,25-dihydroxy-vitamin D_3 [1,25(OH)$_2$D$_3$] is mediated by the classic nuclear mechanism and a fast, nongenomic mode of action that activates signal transduction pathways. The role of individual protein kinase C (PKC) isoforms in the regulation of intracellular Ca^{2+} levels ($[Ca^{2+}]_i$) by the hormone was investigated in cultured proliferating (myoblasts) and differentiated (myotubes) chick skeletal muscle cells. 1,25(OH)$_2$D$_3$ (10^{-9} M) induced a rapid (30- to 60-s) and sustained (>5-min) increase in $[Ca^{2+}]_i$ which was markedly higher in myotubes than in myoblasts. The effect was suppressed by the PKC inhibitor calphostin C. In differentiated cells, PKC activity increased in the particulate fraction and decreased in cytosol to a greater extent than in proliferating cells after 5-min treatment with 1,25(OH)$_2$D$_3$. By Western blot analysis, these changes were correlated to translocation of the PKC α isoform from cytosol to the particulate fraction, which was more pronounced in myotubes than in myoblasts. Specific inhibition of PKC α activity using antibodies against this isoform decreased the 1,25(OH)$_2$D$_3$-induced $[Ca^{2+}]_i$ sustained response associated with Ca^{2+} influx through voltage-dependent calcium channels. Neomycin, a phospholipase C (PLC) inhibitor, blocked its effects on $[Ca^{2+}]_i$, PKC activity, and translocation of PKC α. Exposure of myotubes to 1,2-dioleyl-rac-glycerol (1,2-diolein), also increased $[Ca^{2+}]_i$, PKC activity, and the amount of PKC α associated with the particulate fraction. Changes in $[Ca^{2+}]_i$ induced by diolein were inhibited by calphostin C and nifedipine. The results indicate that PKC α activation via PLC-catalyzed phosphoinositide hydrolysis is part of the mechanism by which 1,25(OH)$_2$D$_3$ regulates muscle intracellular Ca^{2+} through modulation of the Ca^{2+} influx pathway of the Ca^{2+} response to the sterol. J. Cell. Biochem. 77:200–212, 2000.

Key words: muscle cells; development; intracellular Ca^{2+}; 1,25 (OH)$_2$-vitamin D$_3$; nongenomic effects; protein kinase C; PKC isoforms

1,25-Dihydroxy-vitamin D_3 [1,25(OH)$_2$D$_3$], the hormonally active form of vitamin D_3, is an important regulator of mineral homeostasis [Norman et al., 1982; DeLuca et al., 1990]. It also plays a role in the control of cell growth and differentiation and modulation of the immune system [Tanaka et al., 1982; Walters, 1992]. This secosteroid hormone acts through at least two different mechanisms [Walters, 1992]. In addition to regulating gene transcription via its specific intracellular receptor (vitamin D receptor [VDR]) [Minghetti and Norman, 1988; Perlman et al., 1990], 1,25(OH)$_2$D$_3$ induces, like growth factors and peptide hormones, rapid, nontranscriptional responses involving activation of transmembrane signal transduction pathways [De Boland and Nemere, 1992; Nemere and Farach-Carson, 1998].

Abbreviations used: 1,25(OH)$_2$D$_3$, 1,25(OH)$_2$-vitamin D$_3$; PKC, protein kinase C; $[Ca^{2+}]_i$, intracellular Ca^{2+} concentration; DG, 1,2-dioleyl-rac-glycerol (diolein); DAG, diacylglycerol; PLC, phospholipase C; VDCC, voltage-dependent calcium channel.

Grant sponsor: Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET); Grant sponsor: Agencia Nacional de Promoción Científica y Tecnológica; Grant sponsor: Comisión de Investigaciones Científicas de la Provincia de Buenos Aires (CIC), Argentina; Grant sponsor: Volkswagen Foundation, Hannover, Germany.

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Received 11 August 1999; Accepted 12 October 1999

Clinical and experimental studies have shown that skeletal muscle is a target tissue for $1,25(OH)_2D_3$, in which the hormone regulates calcium metabolism and contractility [Boland, 1986]. Avian embryonic skeletal muscle cells (myoblasts/myotubes) have been shown to provide an appropriate model to characterize the effects of $1,25(OH)_2D_3$ on muscle, as they are endowed with the molecular machinery to respond both genomically and nongenomically to the steroid. Chick myoblasts express VDR [Boland et al., 1985] accordingly, with the long-term transcriptional effects of $1,25(OH)_2D_3$ on Ca^{2+} transport [De Boland and Boland, 1985], Ca^{2+}-binding protein synthesis [Drittanti et al., 1989a; Zanello et al., 1995] and DNA replication [Drittanti et al., 1989b] in these cells. Moreover, $1,25(OH)_2D_3$ modulates voltage-dependent Ca^{2+} channel (VDCC)-mediated Ca^{2+} influx in cultured chick muscle cells [Vazquez and De Boland, 1993] by a nongenomic pathway involving G-protein-dependent stimulation of both the adenylyl cyclase/cyclic AMP/PKA messenger system [Vazquez et al., 1995] and a phosphoinositide-specific phospholipase C (PLC) [Morelli et al., 1996]. Steroid-dependent activation of phospholipases A_2 and D has also been reported [De Boland et al., 1994, and references therein]. The rapid activation of PLC, in turn, generates diacylgycerol (DAG) and inositol-1,4,5-trisphosphate (IP_3), promoting the activation of protein kinase C (PKC) and rapid release of Ca^{2+} from endogenous stores [Vazquez et al., 1997].

PKC consists of a family of enzymes capable of modulating protein activity via serine/threonine phosphorylation. Once activated, an event that generally involves translocation of the enzyme from cytosol to membranes, PKC can phosphorylate a number of proteins and thereby regulate many short-term cellular events [Parker et al., 1986; Nishizuka, 1988]. Based on structure and biochemical characteristics, the PKC family is divided into three major groups. The conventional isoforms PKC α, β_I, β_{II}, and γ are dependent on calcium, phospholipids, and diacylglycerol (DAG) for full activation. The novel isoforms δ, μ, η, and θ do not require calcium for stimulation. A third class of PKC enzymes, the atypical isoforms ζ, λ, and ι, are dependent on phosphatidylserine but independent of DAG and calcium [Nishizuka, 1992; Olivier and Parker, 1994]. Crucial roles have been assigned to the PKC family, such as regulation of ion channels and the cell cycle [Nishizuka, 1992].

In certain cell types, both genomic and nongenomic effects of $1,25(OH)_2D_3$ have been shown to be mediated, at least in part, by PKC. An increase in PKC β expression underlies the sterol-induced differentiation of HL-60 cells [Simpson et al., 1998]; changes in the subcellular distribution of PKC isoforms have been observed in kidney cells exposed to the sterol [Simboli-Campbell et al., 1994]. PKC has been implicated in the $1,25(OH)_2D_3$-dependent nongenomic regulation of Ca^{2+} fluxes in chick duodenum [De Boland and Norman, 1990] and Caco-2 cells [Bissonnette et al., 1994], whereas a rapid increase in PKC activity that temporally correlates with sterol-induced polyphosphoinositide hydrolysis occurs in rat colonic epithelia [Wali et al., 1990] and chondrocytes [Boyan et al., 1994].

PKC also seems to play an important role in the mechanisms by which $1,25(OH)_2D_3$ acts on skeletal muscle cells, as sterol modulation of myoblast DNA synthesis has been shown to depend on a rapid increase in both PKC activity and Ca^{2+} influx through VDCC [Bellido et al., 1993]. Moreover, the rapid $1,25(OH)_2D_3$-stimulated L-type-mediated Ca^{2+} influx requires activation of PKC, which in turn cross-talks with the adenylyl cyclase/cyclic AMP cascade [Vazquez and De Boland, 1996].

There is no information on which PKC isoforms are specifically activated by $1,25(OH)_2D_3$ in muscle cells. In view of the fact that the expression of functionally competent VDCC increases during differentiation of myoblasts [Kano et al., 1989], and considering the link between PKC activity and L-type channel function, information regarding this point becomes relevant to understand fully the signaling pathway involved in sterol regulation of muscle intracellular Ca^{2+} levels ($[Ca^{2+}]_i$) along muscle cell development. To that end, the aims of the present work were to characterize the role of individual PKC isozymes in the rapid effects of $1,25(OH)_2D_3$ on $[Ca^{2+}]_i$ in chick proliferating myoblasts and differentiated myotubes. Furthermore, the participation of PLC in PKC-dependent $1,25(OH)_2D_3$ stimulation of intracellular Ca^{2+} was studied.

MATERIALS AND METHODS

Materials

$1,25(OH)_2D_3$ was provided by Hoffmann-La Roche (Nutley, NJ). Bovine pancreas trypsin, Dulbecco's modified Eagle's medium (DMEM), leupeptin, aprotinin, calphostin C, bisindolylmaleimide I, 1,2-dioleyl-rac-glycerol (1,2-diolein, DG), 1,3-diolein, phosphatidylserine, Immobilon P (polyvinylidene difluoride [PVDF]) membranes, and the synthetic peptide GS (PLSRTLSVAAKK) were from Sigma Chemical Co. (St. Louis, MO). Western blot chemiluminescence reagents (Renaissance) and [γ-^{32}P]-ATP (3,000 Ci/mmol) were provided by New England Nuclear (Chicago, IL). PKC α, β, δ, ϵ, and ζ antibodies and anti-rabbit IgG horseradish peroxidase (HRP)-conjugated antibody were from Gibco-BRL (Gaithersburg, MD).

Cell Culture

Myoblasts were obtained from the breast muscle of 12-day-old white leghorn chick embryos (*Gallus gallus*) by stirring in Earle's balanced salt solution (SSBE) containing 0.1% trypsin for 30 min, essentially as previously described [Vazquez and De Boland, 1993; O'Neill and Stockdale, 1972]. The freed cells were collected by centrifugation, and the pellet was resuspended in DMEM supplemented with 10% fetal 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 unadsorbed myogenic cells (100,000 cells/cm^2) were placed in plastic dishes (100-mm diameter) and incubated under humidified air with 5% CO_2 for 1–5 days. Characterization of cultures during this incubation interval by histological and scanning electron microscopy analysis initially showed the presence of mononucleated spindle-shaped cells (myoblasts) followed by the progressive appearance of cells with defined directions of alignment, fused cells (80% with more than one nucleus after 4 days of culture) and well-differentiated fibers (myotubes); no fibroblasts were observed [Bellido, 1988].

Measurement of Intracellular Calcium

Changes in intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) were monitored by using the Ca^{2+}-sensitive fluorescent dye Fura-2 as previously described [Vazquez et al., 1997]. Cell dye loading was achieved by incubating myoblasts grown on coverslips (24 × 6 mm) in buffer A containing 138 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, 5 mM glucose, 10 mM Hepes pH 7.4, 1.5 mM $CaCl_2$ plus 0.1% bovine serum albumin (BSA); 4 μM of the penta-acetoxymethylester derivative (membrane-permeable) Fura-2/AM; and 0.012% (w/v) pluronic F127 in the dark for 45 min at room temperature, in order to minimize dye compartmentalization. Unloaded dye was washed out, and cells were maintained in buffer B (i.e., buffer A without BSA, Fura-2/AM, and pluronic F127) in the dark (room temperature) for at least 40 min before use to allow for complete intracellular dye de-esterification. Coverslips containing the cells were placed into quartz cuvettes of a thermostatted (37°C) SLM Aminco 8100 spectrofluorimeter (Spectronics, Urbana, IL) sample compartment under constant controlled stirring. Fura-2 intracellular fluorescence intensity was monitored at an emission wavelength of 510 nm (8-nm bandpass) by alternating (300-Hz) the excitating wavelength between 340 and 380 nm (4-nm bandpass) with a dual excitation monochromator (DMX1100, Spectronics). Signals from short and long wavelengths were ratioed (R = 340/380); thus, the measurement was independent of variations in cellular dye content, dye leakage, or photobleaching. For calibration of the Fura-2 fluorescence signal to calculate $[Ca^{2+}]_i$ values, maximal (R_{max}) and minimal (R_{min}) intracellular dye fluorescence signals were determined by adding 5 μM ionomycin plus 3 mM Ca^{2+} and 10 mM EGTA (pH 9.0), respectively. Under these conditions of measurement, the dissociation constant (K_d) for the Ca^{2+}-Fura-2 complex was assumed to be 224 nM, and $[Ca^{2+}]_i$ derives from

$$[Ca^{2+}]_i = Kd(R - R_{min})/(R_{max} - R) \times \beta$$

where R is the ratio of Fura-2 fluorescence at the selected wavelengths, R_{max} and R_{min} represent ratios from Ca^{2+}-saturated and Ca^{2+}-free intracellular dye, respectively, and β is the ratio of the specific fluorescence of Ca^{2+}-free and Ca^{2+}-bound forms of the dye at the longer wavelength (Sf_2/Sb_2). Basal $[Ca^{2+}]_i$ values fluctuated from 25 to 68 nM, which agrees with the distribution of resting intracellular Ca^{2+} reported for other cell systems [Klin et al., 1994].

For single-cell analysis, cells loaded with Fura-2 were mounted on the stage of an inverted

fluorescence microscope (Nikon Diaphot 200, Nikon, Melville, NY) equipped with a 40× NA objective lens (Nikon). By means of an electronically controlled chopper (5.5 Hz), the excitation wavelength was rapidly switched over 340 and 390 nm, employing a dual excitation monochromator (DMX1100) from the SLM-Aminco 8100 spectrofluorimeter connected to the epifluorescence port of the microscope through an optic fiber to allow excitation light to enter the objective and reach the sample. Emitted cellular fluorescence was reflected by a 400 nm dichroic mirror and filtered through a 510-nm bandpass filter. Ratios from short and long wavelength signals were obtained (R = 340/390) and for each single cell measured, calibration of the cytosolic Ca^{2+} signal was accomplished in situ at the end of each experiment as described.

Subcellular Fractionation

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 phenylmethylsulfonyl fluoride (PMSF), 40 μg/ml leupeptin, 40 μg/ml aprotinin (100 μl buffer/15–20 × 10^6 cells). Similar homogenization buffers containing EDTA and EGTA have been previously used to study the subcellular localization of PKC isozymes [Leli et al., 1993; Ozawa et al., 1993; Kiley and Parker, 1995]. The muscle cell lysates were then centrifuged at 200*g* for 10 min to eliminate cell debris. The supernatants were ultracentrifuged at 100,000*g* for 1 h to obtain the cytosolic (supernatant) and particulate (pellet) fractions.

PKC Assay

PKC was assayed by measuring the incorporation of ^{32}P from [γ; -^{32}P]ATP into the synthetic Glycogen synthase peptide (GS) [Gomez et al., 1988]. The reaction mixture (60 μl) contained 20 mM Tris-HCl pH 7.4, 10 mM $MgCl_2$, 25 μM GS, 50 μM [γ; -^{32}P]ATP, 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. PS and DG were added as vesicles obtained by vortexing the lipids in distilled water for 1 min. 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 concentrations. 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, dried, and assayed for radioactivity in the scintillation mixture [Roscoski, 1983]. The activity measured in the presence of EGTA was considered nonspecific and subtracted from the activities obtained when Ca^{2+}, PS, and DG were present in the incubation mixture.

Western Blot Analysis

Protein samples were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (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 antibodies (2 μg/ml) overnight at 4°C in TBST containing 5% (w/v) nonfat dry milk, followed by incubation with HRP-conjugated anti-rabbit IgG in TBST 5% nonfat dry milk (1:2,000). Bands were visualized by chemiluminescence detection. Images were obtained with a model GS-700 Imaging Densitomer (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). It is important to note that the antibodies used to detect the various PKC isoforms studied do not cross-react. They are directed against well-characterized epitopes, which are different in the PKC isoenzymes tested. Competition experiments with the synthetic peptides used to generate the antibodies did not demonstrate cross-reactivity (data not shown). Furthermore, as shown under Results, there are differences in the blot band patterns (molecular weight and number of bands).

Treatment of Muscle Cells With Anti-PKC α Antibodies by Membrane Permeabilization

Myotube monolayers were washed with buffer A containing 138 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, 1 mM Na_2HPO_4, 2 mM $NaHCO_3$, 5 mM glucose, 20 mM Hepes pH 7,4, 0.1% BSA, and 1.5 mM $CaCl_2$ and were incubated with 25 μg/ml of anti-PKC α antibodies (or normal rab-

bit serum) in permeabilization buffer containing 110 mM KCl, 20 mM NaCl, 40 mM Hepes pH 7.0–7.1, 8 mM $MgCl_2$, 1 mg/ml BSA, 2 mM EGTA, and 50 μg/ml saponin, for 5 min at room temperature. Cells were then washed with buffer A plus 25 μg/ml of anti-PKC α antibodies (or normal rabbit serum) for 30 min at room temperature. The muscle cells were then loaded with Fura-2, and spectrofluorimetric determinations of $[Ca^{2+}]_i$ by single-cell analysis were carried out. This membrane permeabilization saponin-based protocol has been recently applied for efficient introduction of antibodies against PLC isozymes into bone cells without affecting hormone responses nonspecifically [Le Mellay et al., 1997].

Statistical Evaluation

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

RESULTS

$1,25(OH)_2D_3$ regulation of intracellular Ca^{2+} levels ($[Ca^{2+}]_i$) in chick myoblasts and myotubes grown in vitro for 2 and 5 days, respectively, was studied by fluorimetric analysis of cells loaded with the fluorescence Ca^{2+} indicator Fura-2. At both stages of development, $1,25(OH)_2D_3$ (10^{-9} M) significantly increased $[Ca^{2+}]_i$ within 1 min of treatment (Fig. 1A,C). However, the response was markedly higher in

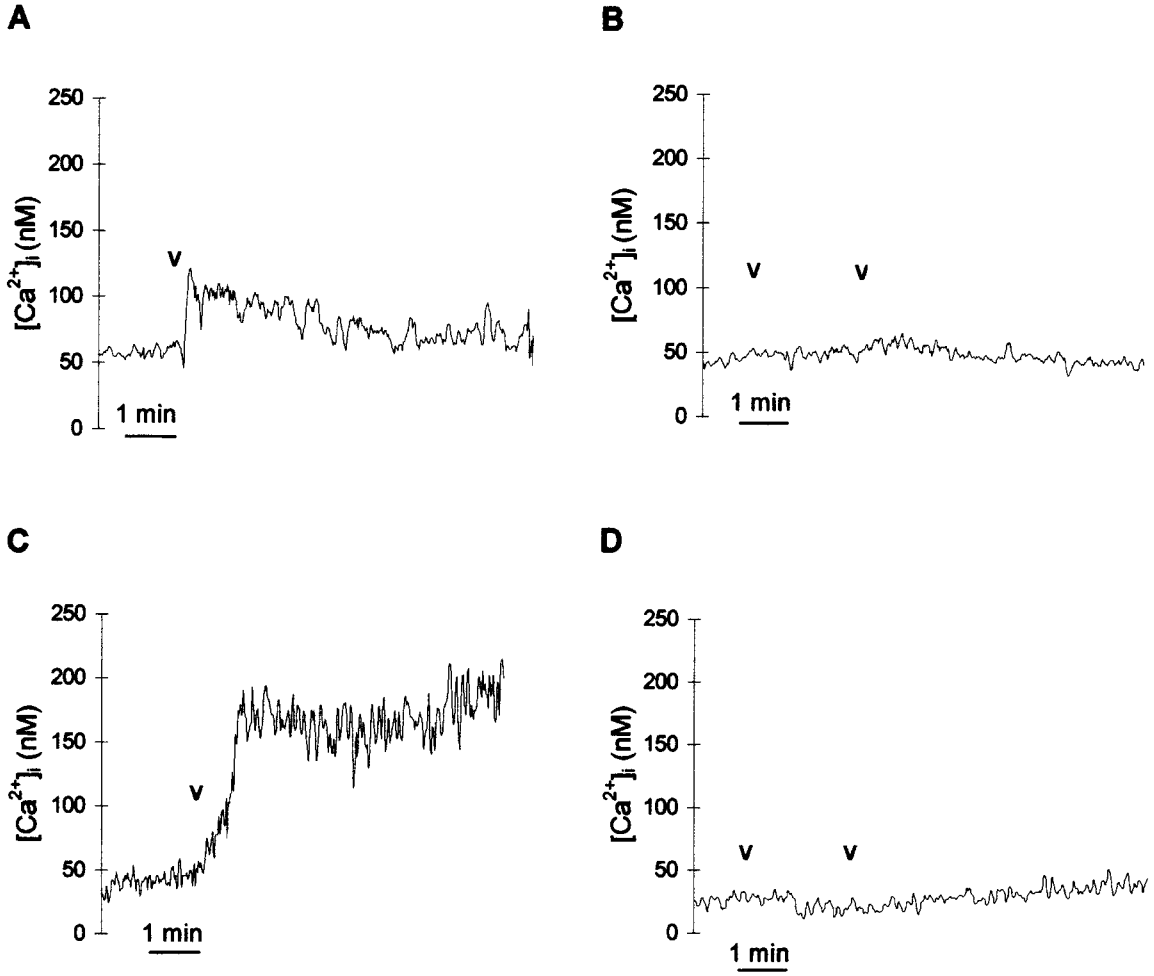

Fig. 1. Changes in intracellular Ca^{2+} levels ($[Ca^{2+}]_i$) induced by $1,25(OH)_2D_3$ in myoblasts and myotubes. Cultured chick embryo proliferating myoblasts (**A,B**) and differentiated myotubes (**C,D**) were treated with 10^{-9} M $1,25(OH)_2D_3$ (arrowhead, **A,C**, right arrowhead, **B,D**) in the absence or presence of 100 nM calphostin (left arrowhead, **B,D**), which was added 3 min before the hormone. $[Ca^{2+}]_i$ was determined fluorimetrically in cells loaded with Fura-2, as described under Materials and Methods. Shown are representative time tracings of three independent experiments.

myotubes (212.5 ± 19.9%, above control after 5 min of treatment; $P < 0.05$) than in myoblasts (45.3 ± 13.2%; $P < 0.025$). The addition of vehicle alone (ethanol, <0.1%) did not affect basal $[Ca^{2+}]_i$ values (not shown). Typical patterns of proliferation (myoblasts) and differentiation (myotubes) in these cultures have been previously established by morphological characterization and measurement of specific biochemical parameters (e.g., DNA synthesis, creatine kinase activity, and myosin levels) [Capiati et al., 1999].

In order to determine whether protein kinase C mediates the fast $1,25(OH)_2D_3$-induced increase of muscle cellular Ca^{2+} levels, measurements were made in the presence of calphostin C, a specific PKC inhibitor [Kobayashi et al., 1989]. Pretreatment of the cells with 100 nM calphostin C abolished the effect of $1,25(OH)_2D_3$ on $[Ca^{2+}]_i$ in both proliferating myoblasts (Fig. 1B) and differentiated myotubes (Fig. 1D). These results suggest that PKC plays a major role in $1,25(OH)_2D_3$ regulation of intracellular Ca^{2+} at both stages of muscle cell development.

Studies were then conducted to investigate the role of individual PKC isoforms in $1,25(OH)_2D_3$ modulation of Ca^{2+} influx through voltage-dependent calcium channels (VDCC). Activation of PKC is usually associated with its translocation from the cytoplasmic compartment to membranes [Nishizuka, 1992]. It was previously shown that $1,25(OH)_2D_3$-induced VDCC-mediated Ca^{2+} entry into chick muscle cells becomes significantly stimulated after 3 min of hormone exposure [Vazquez and De Boland, 1993], rapidly (30–60 s) reaching a plateau level. Also, it was recently demonstrated that the fast and transient (1–2 min) Ca^{2+} increase observed in these cells upon sterol stimulation in Ca^{2+}-free medium is entirely dependent on PLC activity [Morelli et al., 1996; Vazquez et al., 1997, 1998], with the subsequent activation of PKC a consequence of both cytosolic Ca^{2+} elevation and DAG formation. On these grounds, myoblasts and myotubes were exposed for 5 min to 10^{-9} M $1,25(OH)_2D_3$, followed by isolation of the cytosolic and particulate fractions and determination of PKC activity using a specific peptide substrate. The hormone treatment decreased PKC specific activity (pmol/min/mg prot.) associated with the cytosol and increased it in the particulate fraction in myoblasts (Fig. 2A) and myotubes (Fig. 2B). Once again, the effect of $1,25(OH)_2D_3$ was more pronounced in differentiated cells. These changes were also evident when expressing the data as a percentage of total PKC activity (myoblasts: 30% and 44%, myotubes: 17% and 56%; for the particulate fraction of control and $1,25(OH)_2D_3$-treated cells, respectively).

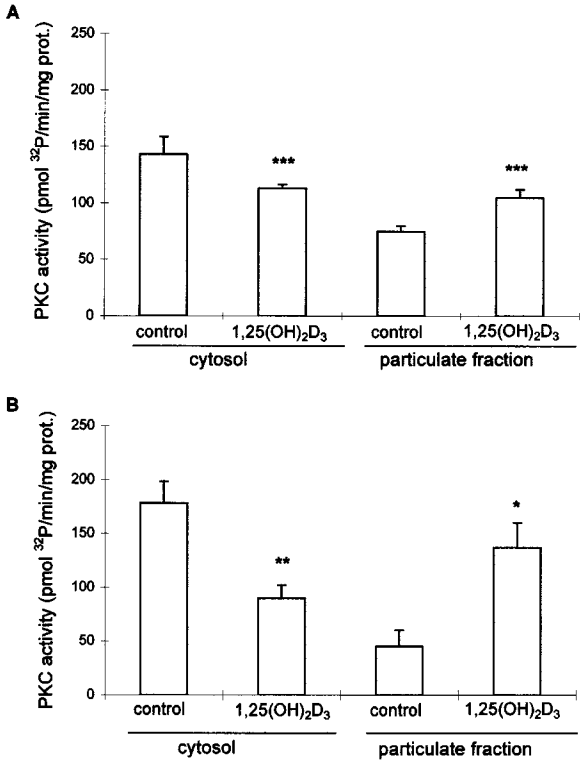

Fig. 2. Rapid effects of $1,25(OH)_2D_3$ on the subcellular distribution of PKC activity in myoblasts and myotubes. Cultured chick embryo proliferating myoblasts (**A**) and differentiated myotubes (**B**) were treated with 10^{-9} M $1,25(OH)_2D_3$ for 5 min. The cells were then homogenized and centrifuged to isolate cytosolic and particulate subcellular fractions. Determination of PKC activity was as described under Materials and Methods. Results shown are the mean ± SD of three independent experiments. *$P < 0.005$; **$P < 0.01$; ***$P < 0.05$.

We next investigated whether selective translocation of individual PKC isozymes was involved in the $1,25(OH)_2D_3$ rapid increase in muscle cell membrane-associated PKC activity. To that end, Western blot analysis of subcellular fractions were performed using antibodies against PKC isoforms α, β, δ, ϵ, and ζ, which recognize sequences mapping on the catalytic domain of the protein. As shown in Figure 3, two bands were detected for each isozyme, one corresponding to the whole enzyme, of ~80 kDa for PKC α, β, and δ, 85 kDa for PKC ϵ, and 75 kDa for PKC ζ, and the other

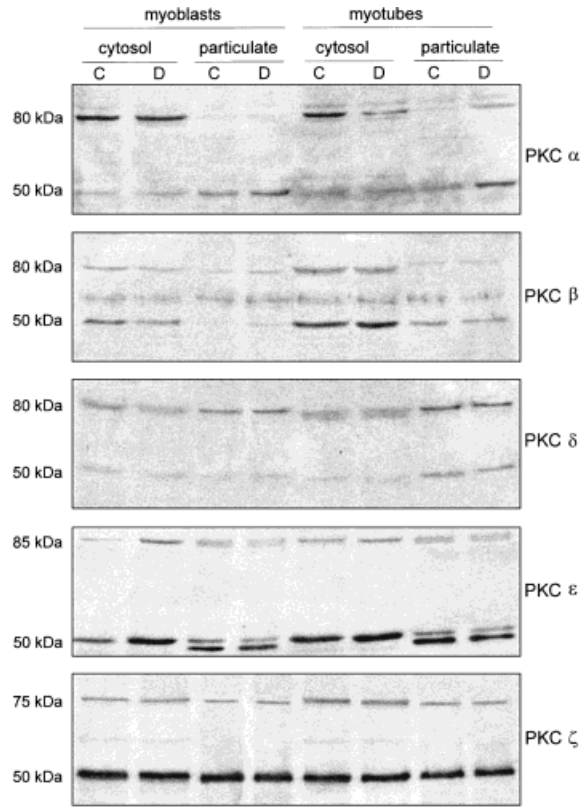

Fig. 3. Fast changes in the subcellular distribution of PKC isoenzymes in myoblasts and myotubes in response to $1,25(OH)_2D_3$. Cultured chick embryo proliferating myoblasts and differentiated myotubes were treated with vehicle (ethanol <0.1%, C) or 10^{-9} M $1,25(OH)_2D_3$ (D) for 5 min. After cell homogenization, the cytosolic and particulate fractions were isolated by differential centrifugation followed by Western blot analysis of PKC isoenzymes, as described under Materials and Methods. Representative blots from three independent experiments are shown. Quantitative data and statistical analysis are given in Table I.

(~50 kDa), corresponding to the catalytic fragment, which has been reported to be of physiological relevance by several investigators [Cressman et al., 1995; Gallicano et al., 1995; Sato et al., 1997]. An intermediate band was detected for the β and ζ isoforms; these were shown to be specific, as they totally disappeared in competitive experiments where anti-PKC β and anti-PKC ζ antibodies and the antigen peptides they were raised against were used [Capiati et al., 1999]. As shown in Figure 3 and Table I, $1,25(OH)_2D_3$ induced translocation of PKC α from cytosol to the particulate fraction in both proliferating and differentiated muscle cells, indicating that this isoform could mediate the hormonal effects on Ca^{2+} influx. Consistently, this change was greater in myotubes than in myoblasts. In the two cellular developmental stages analyzed, PKC ϵ moved from the particulate fraction to the cytosol in response to $1,25(OH)_2D_3$. No significant modifications were observed in the subcellular distribution of PKC β, δ, and ζ after hormone treatment.

To further corroborate the above observations involving PKC α in $1,25(OH)_2D_3$ modulation of $[Ca^{2+}]_i$, myotubes permeabilized with saponin were treated in vivo with anti-PKC α antibodies (25 μg/ml). As shown in Figure 4, specific inhibition of PKC α activity decreased $1,25(OH)_2D_3$-induced Ca^{2+} influx (60–70%), not affecting the fast, transient PLC-dependent increase in $[Ca^{2+}]_i$. It is unlikely that the saponin permeabilization procedure used may have affected per se PKC activity/distribution. Different lines of evidence support this contention. Using the same permeabilizing conditions, signaling proteins other than PKC (e.g., Ca^{2+} channels, heterotrimeric G proteins, phospholipases) are well preserved with regard to either functionality or subcellular localization [Hirata et al., 1984; Le Mellay et al., 1997; Vazquez et al., 1995]. Of importance, similar permeabilizing approaches do not alter neither the activity nor the subcellular distribution of PKC isoenzymes in other cell types [Cheng et al., 1999; Byung-Chang et al., 1998]. Moreover, typical Ca^{2+} responses to either $1,25(OH)_2D_3$, thapsigargin, or membrane-depolarizing agents are seen under the permeabilizing conditions described in our work (G. Vazquez, A.R. De Boland, and R. Boland, unpublished observations).

Experiments were then aimed to determine the pathway of PKC stimulation by $1,25(OH)_2D_3$. Differentiated cells were chosen for these studies because of their greater responsiveness to the hormone than proliferating cells. The involvement of PLC-catalyzed hydrolysis of inositol phospholipids in the activation of PKC and the increase in $[Ca^{2+}]_i$ by $1,25(OH)_2D_3$ was studied using the PLC inhibitor neomycin. This compound interferes the interaction of PLC with its phosphoinositide substrate [Prentki et al., 1986]. Myotubes were incubated with 500 μM neomycin for 3 min before treatment with 10^{-9} M $1,25(OH)_2D_3$. As shown in Figure 5A, the PLC inhibitor completely blocked the effect of the hormone on $[Ca^{2+}]_i$. The PLC inhibitor U73122, which impairs cou-

TABLE I. Effects of $1,25(OH)_2D_3$ on PKC Isoenzyme Subcellular Distribution in Myoblasts and Myotubes†

Subcellular distribution	PKC α	PKC β	PKC δ	PKC ϵ	PKC ζ
Myoblasts					
Cytosol					
Control	71.8 ± 1.8	56.7 ± 5.5	49.3 ± 1.4	58.8 ± 1.7	116.6 ± 2.6
$1,25(OH)_2D_3$	54.6 ± 1.0*	48.4 ± 4.0	41.8 ± 1.0	114.5 ± 3.2*	122.4 ± 6.0
Particulate					
Control	23.4 ± 1.4	9.1 ± 1.1	3.32 ± 1.6	107.4 ± 5.5	102.5 ± 1.7
$1,25(OH)_2D_3$	33.1 ± 2.5**	10.5 ± 1.0	3.98 ± 1.7	61.5 ± 3.2*	99.1 ± 5.0
Myotubes					
Cytosol					
Control	65.3 ± 2.7	93.2 ± 2.7	46.5 ± 0.8	78.5 ± 2.0	129.5 ± 2.3
$1,25(OH)_2D_3$	34.6 ± 3.1*	97.5 ± 2.9	46.4 ± 0.9	104.7 ± 7.0**	130.3 ± 2.4
Particulate					
Control	19.5 ± 1.0	36.5 ± 4.5	63.1 ± 1.7	119.4 ± 2.1	83.0 ± 6.7
$1,25(OH)_2D_3$	46.4 ± 1.0*	31.1 ± 3.2	61.8 ± 1.8	81.6 ± 1.7*	85.3 ± 2.9

†Conditions of cell treatment and processing for immunoblots of PKC isoenzymes were as in Figure 3. Films were scanned and all PKC-immunoreactive bands were quantified as described under Materials and Methods. Density is expressed in arbitrary units. Results are the mean ±SD of three independent experiments: *$P < 0.001$; **$P < 0.05$.

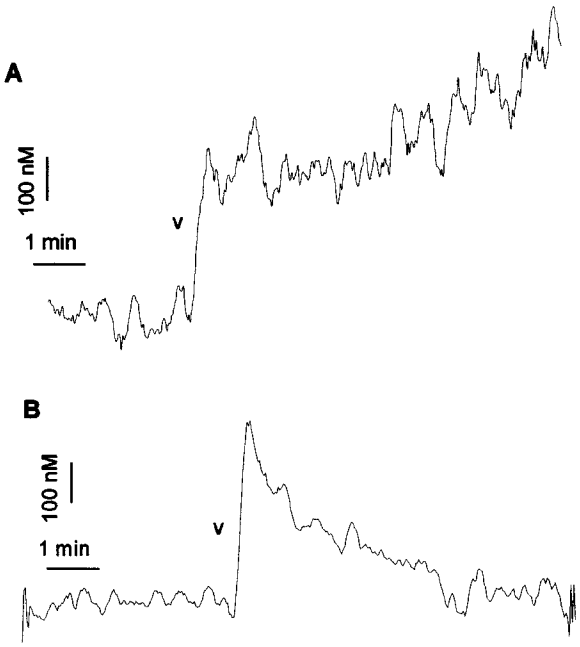

Fig. 4. Inhibition of $1,25(OH)_2D_3$-induced Ca^{2+} influx by anti-PKC α treatment. Myotubes were treated with normal rabbit serum (**A**) or 25 μg/ml of anti-PKC α antibodies (**B**), as described under Materials and Methods, loaded with Fura-2 and $[Ca^{2+}]_i$, and $[Ca^{2+}]_i$ was determined fluorimetrically after treatment with $1,25(OH)_2D_3$ 10^{-8} M by single-cell analysis. The time tracings shown are representative of three independent experiments.

pling of Gq/11 with PLC, has been previously shown to exert effects similar to those of neomycin [Vazquez et al., 1997, 1998]. In addition, preincubation of myotubes with neomycin, followed by treatment with $1,25(OH)_2D_3$ at the same concentration, suppressed the hormone-induced increase in PKC specific activity in the particulate fraction, as well as the decrease in cytosol (Fig. 5B). Similar results were obtained when the data were expressed as percentage of total PKC activity (not shown). Western blot analysis of subcellular fractions from these experiments with anti-PKC α antibody showed that neomycin also blocked $1,25(OH)_2D_3$-induced PKC α translocation from cytosol to membranes (Fig. 5C and Table II).

The synthetic diacylglycerol 1,2-dioleyl-rac-glycerol (1,2-diolein; DG), a good activator of PKC in vitro, has also been shown to stimulate the enzyme in intact cells [Mullin and McGinn, 1988]. In view of the observed correlation between $1,25(OH)_2D_3$-induced changes in intracellular Ca^{2+}, PKC activity and α isoform translocation, we tested whether 1,2-diolein mimicked the hormone effects on these parameters in myotubes. As shown in Figure 6A, 50 μM 1,2-diolein increased $[Ca^{2+}]_i$ significantly, although the response was delayed in comparison with $1,25(OH)_2D_3$ (Fig. 1A). The effect of 1,2-diolein was abolished by pretreating cells with 100 nM calphostin C (Fig. 6B) or 2 μM nifedipine (Fig. 6C) and when measurements were carried out in Ca^{2+}-free medium (29 ± 7 vs 32 ± 4 nM Ca^{2+}; basal versus 50 μM diolein, respectively). Treatment with 1,3-diolein, an

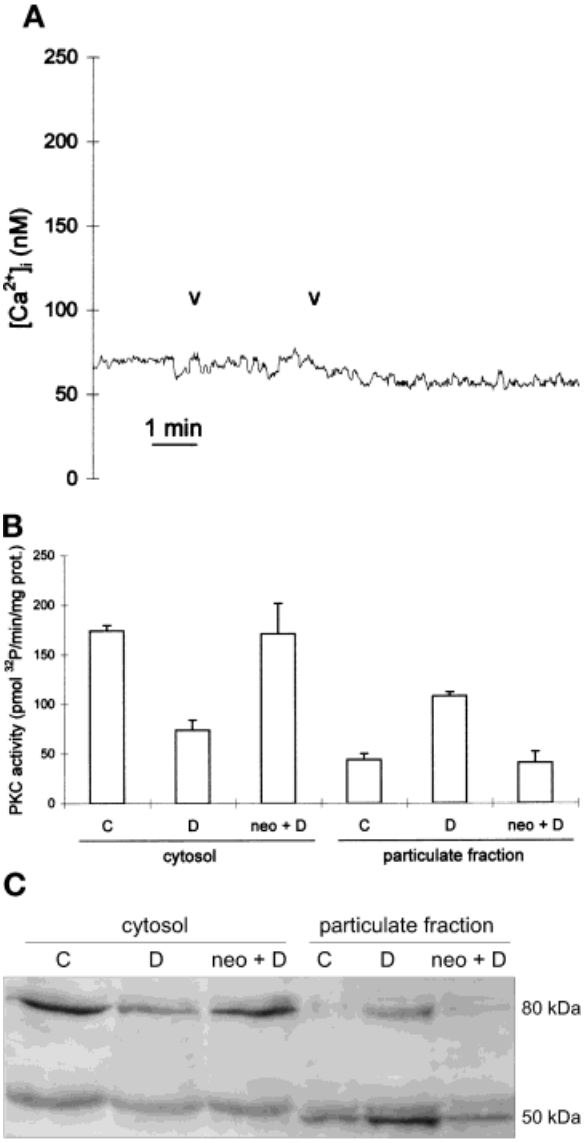

Fig. 5. Neomycin inhibition of $1,25(OH)_2D_3$ effects on intracellular Ca^{2+}, PKC activity, and PKC α translocation in cultured myotubes. Myotubes loaded with Fura-2 were treated with 500 μM neomycin (left arrowhead) for 3 min before the addition of 10^{-9} M $1,25(OH)_2D_3$ (right arrowhead). A representative time trace from three independent experiments is shown (**A**). For PKC studies, myotubes were treated for 5 min with 10^{-9} M $1,25(OH)_2D_3$ in the absence and presence of 500 μM neomycin, which was added 3 min before hormone treatment. The cells were then homogenized, followed by isolation of subcellular fractions for measurement of PKC activity (**B**) and Western blot analysis of PKC α (**C**), as described under Materials and Methods. **B**: Data are mean ± SD of three independent experiments; $P < 0.001$ for C versus D, in both cytosol and particulate fractions; $P < 0.005$ and $P < 0.01$ for D versus neo + D in particulate and cytosol fractions, respectively. **C:** Representative immunoblot of three independent experiments is shown; quantitative data and statistical analysis are given in Table II. C, control; D, $1,25(OH)_2D_3$; neo, neomycin.

TABLE II. Effect of PLC Inhibition on the Action of $1,25(OH)_2D_3$ on PKC α Translocation†

Subcellular distribution	PKC α
Cytosol	
Control	63.0 ± 3.2
$1,25(OH)_2D_3$	32.4 ± 0.8**[a]
Neo + $1,25(OH)_2D_3$	52.7 ± 4.1***[b]
Particulate	
Control	14.7 ± 1.5
$1,25(OH)_2D_3$	34.9 ± 8.9*[a]
Neo + $1,25(OH)_2D_3$	14.9 ± 0.7*[b]

†Myotubes were treated for 5 min with or without (control) 10^{-9} M $1,25(OH)_2D_3$ in the presence or absence of 500 μM neomycin (neo) as in Figure 5. Immunodetection and quantitation of 80- and 50-kDa PKC α-immunoreactive bands were as described under Materials and Methods. Density is expressed in arbitrary units. Results are the mean ±SD of three independent experiments: *$P < 0.001$; **$P < 0.005$; ***$P < 0.025$.

[a]$1,25(OH)_2D_3$ vs control.

[b]$1,25(OH)_2D_3$ + neo vs $1,25(OH)_2D_3$.

inactive diolein derivative, had no effect on $[Ca^{2+}]_i$ (not shown), indicating that the increase in $[Ca^{2+}]_i$ induced by 1,2-diolein is not the consequence of a nonspecific membrane effect. PKC specific activity decreased in cytosol and increased in the particulate fraction after short exposure of myotubes to DG (Fig. 6D). Similar results were obtained when the data were expressed as a percentage of total PKC activity (not shown). In addition, Western blot analysis showed that PKC α translocated to the particulate fraction in response to DG (Fig. 6E and Table III).

DISCUSSION

The present work provides information on the signal transduction pathway involved in $1,25(OH)_2D_3$ regulation of cytoplasmic Ca^{2+} levels ($[Ca^{2+}]_i$) during the differentiation process of skeletal muscle cells. Using cultured chick embryo proliferating myoblasts and differentiated myotubes, it was demonstrated that PKC plays an important role in nongenomic modulation of muscle $[Ca^{2+}]_i$ by $1,25(OH)_2D_3$ and that this mechanism is affected by the developmental stage of muscle cells. The hormone-induced rapid elevation in $[Ca^{2+}]_i$ was significantly higher in myotubes than in myoblasts, an observation that was correlated with greater translocation of PKC activity from the cytosol to the particulate frac-

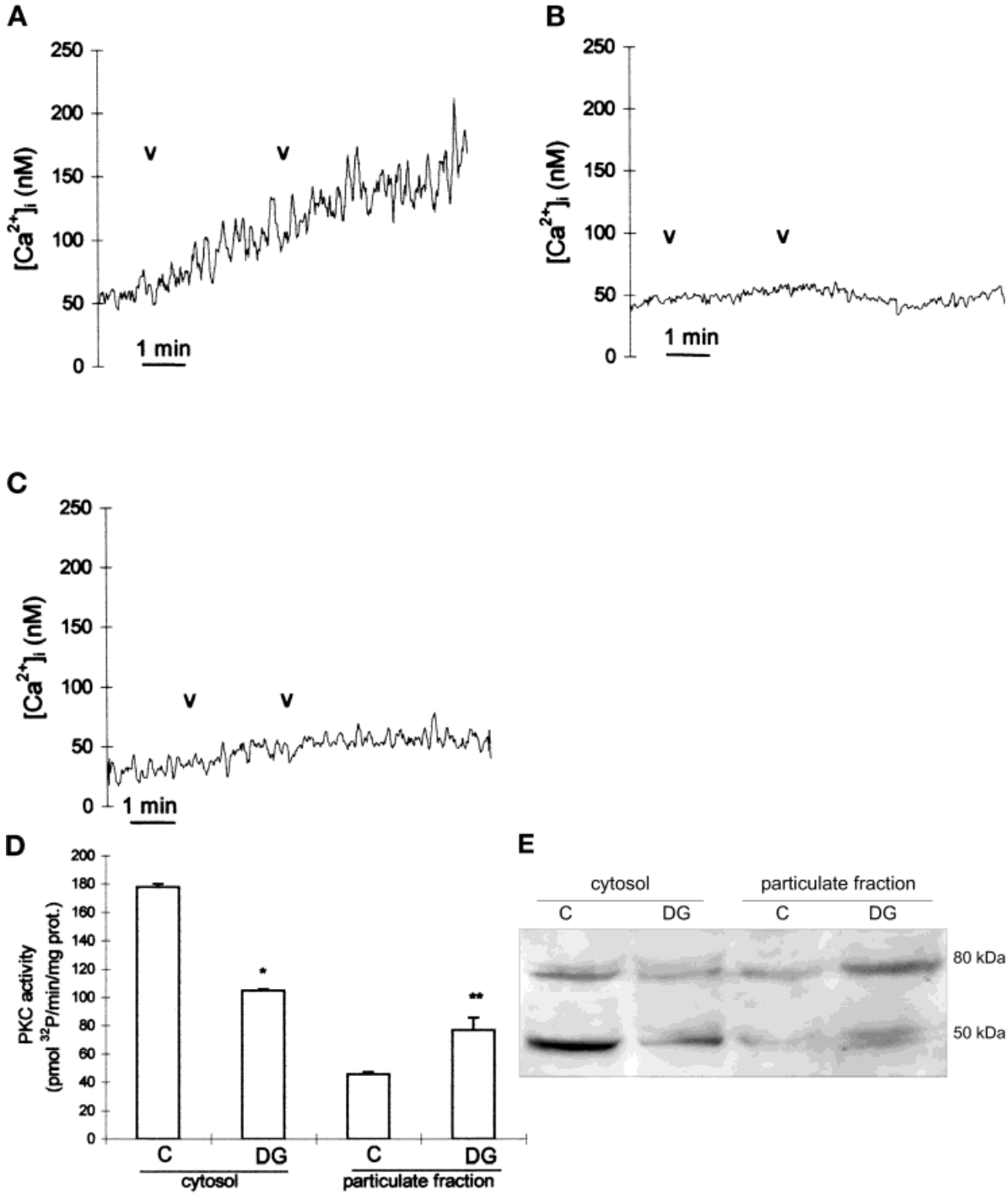

Fig. 6. Effects of diolein on intracellular Ca^{2+}, PKC activity, and translocation of PKC α in myotubes. **A:** Cultured chick myotubes loaded with Fura-2 were treated successively with 50 μM (left arrowhead) and 100 μM (right arrowhead) diolein. Alternatively, 50 μM diolein was added (right arrowhead, **B**,**C**) after preincubation with 100 nM calphostin (left arrowhead, **B**) or 2 μM nifedipine (left arrowhead, **C**). $[Ca^{2+}]_i$ was determined fluorimetrically, as described under Materials and Methods. Shown are representative time tracings of three independent experiments. For PKC studies, myotubes incubated with 50 μM 1,2-diolein for 5 min, were homogenized and processed for PKC activity (**D**) or Western blot analysis of PKC α (**E**), as described under Materials and Methods. **D:** Data are the mean ±SD of three independent experiments; $*P < 0.001$; $**P < 0.01$. **E:** Representative immunoblot of three independent experiments is shown; quantitative data and statistical analysis are given in Table III. C, control; DG, 1,2-diolein.

TABLE III. Effect of Diolein on PKC α Translocation in Myotubes†

Subcellular distribution	PKC α
Cytosol	
Control	59.2 ± 2.9
DG	26.6 ± 4.0*
Particulate	
Control	14.6 ± 2.2
DG	51.4 ± 2.1*

†Myotubes were incubated with or without (control) 50 μM diolein (DG) for 5 min and processed for immunodetection and quantitation of 80- and 50-kDa PKC α-immunoreactive bands, as described under Materials and Methods. Density is expressed in arbitrary units. Data are the mean ±SD of three independent experiments; *$P < 0.001$.

tion in differentiated muscle cells. In further support of the participation of PKC in $1,25(OH)_2D_3$ rapid effects, the PKC inhibitor calphostin C blocked the hormone-dependent changes in $[Ca^{2+}]_i$ both in myoblasts and in myotubes.

Evidence was obtained indicating that PKC α mediates the fast action of $1,25(OH)_2D_3$ in muscle cells, as Western blot analysis showed that the hormone preferentially translocates this isoform from cytosol to the particulate fraction, accompanying the changes in subcellular localization of PKC activity. In line with the above interpretation, this response was more evident in differentiated cells. Furthermore, treatment of myotubes with anti-PKC α antibodies inhibits $1,25(OH)_2D_3$-stimulated Ca^{2+} influx. PKC ε moved from the particulate to the cytosolic fraction after $1,25(OH)_2D_3$ treatment both in myoblasts and in myotubes. A similar reverse translocation has also been observed in differentiated rat skeletal muscle in response to the hormone [Facchinetti and De Boland, 1998]. As the modifications in subcellular distribution of PKC ε are opposite to those observed in PKC activity and, in addition, the substrate (GS) used in the enzyme assays is phosphorylated more efficiently by PKC α than by PKC ε, as well as by the other isoforms [Kazanietz et al., 1993], it may be concluded that translocation of PKC α mainly accounts for the changes induced by $1,25(OH)_2D_3$ in muscle cell PKC activity, leading to an increase in $[Ca^{2+}]_i$. Nevertheless, it remains to be established whether PKC ε participates in nongenomic actions of the hormone other than Ca^{2+} channel modulation.

The mechanism of $1,25(OH)_2D_3$ stimulation of protein kinase C in muscle cells was also addressed in this study. The fact that the $1,25(OH)_2D_3$-induced increase in membrane-associated PKC activity/PKC α amounts and the connected changes in $[Ca^{2+}]_i$ could be blocked by the PLC inhibitor neomycin supports the concept that phosphoinositide breakdown is the likely mechanism by which PKC is activated and in turn elevates cytosolic Ca^{2+} levels in intact muscle cells [Bellido et al., 1993]. In agreement with this contention is the observation that 1,2-dioleyl-rac-glycerol (1,2-diolein), like $1,25(OH)_2D_3$, increased $[Ca^{2+}]_i$, PKC activity and PKC α translocation in myotubes, indicating that the synthetic compound acted as substitute for hormone-released DAG. Moreover, the fact that the $[Ca^{2+}]_i$ response induced by 1,2-diolein could be suppressed by nifedipine, as has been shown for the sterol [Bellido et al., 1993] and had no effect in Ca^{2+}-free medium, further suggests that PKC α participates in $1,25(OH)_2D_3$ regulation of muscle cell $[Ca^{2+}]_i$ through modulation of voltage-dependent Ca^{2+} channels. In fact, there is evidence indicating that PKC-mediated protein phosphorylation is involved in modulation of VDCC [McDonald et al., 1994, and references therein]. However, additional studies are required to further substantiate our interpretation.

Finally, the observation that the interaction $1,25(OH)_2D_3$-PKC to regulate intracellular Ca^{2+} homeostasis was more pronounced in myotubes than in myoblasts suggests that this mechanism may be of physiological significance in differentiated skeletal muscle, in which the hormone plays an important role in the regulation of contractility [Boland, 1986].

ACKNOWLEDGMENTS

Expert technical assistance from Ms. Fabiana Limbozzi is gratefully acknowledged.

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