1,25-Dihydroxyvitamin D_3 Affects the Synthesis, Phosphorylation and *in vitro* Calmodulin Binding of Myoblast Cytoskeletal Proteins

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Incubation of chick embryo myoblasts with 1,25-dihydroxyvitamin D_3 [1,25$(OH)_2D_3$] (10^{-10} M, 24 h), markedly stimulated the incorporation of [^{3}H]leucine into total cytoskeletal proteins and this effect was abolished when the sterol treatment was performed in the presence of cycloheximide or actinomycin D. 1,25$(OH)_2D_3$ selectively stimulated the *de novo* synthesis of several proteins with apparent molecular masses (isoelectric points) of 220 kDa (6.1 and 9.7), 150 kDa (7.5), 110 kDa (7.2), 68 kDa (9.5, 7.5 and 4.5), 50 kDa (8.5), 44 kDa (6.3), 27 kDa (7.8) and 15 kDa (5.5). Labelling of proteins with [^{125}I]calmodulin after their separation on SDS-polyacrylamide gels showed that 1,25$(OH)_2D_3$-dependent protein of 110 kDa is the major calmodulin-binding component of myoblasts cytoskeleton.

In addition, the sterol increased the phosphorylation of several cytoskeletal proteins including that of 110 and 15 kDa whose synthesis potentiates.

Introduction

Various lines of evidence have involved 1,25$(OH)_2D_3$ in the regulation of skeletal muscle function [1]. In animal model and myoblast cell culture studies it has been shown that the sterol plays a role in the regulation of intracellular Ca^{2+} in skeletal muscle [2–5]. In addition, 1,25$(OH)_2D_3$ has been involved in the stimulation of cell proliferation and subsequently fusion of myoblasts into myotubes [4, 6, 7]. The sterol acts, at least in part, *via* a nuclear mechanism. RNA and protein synthesis-dependent changes in myoblasts Ca^{2+} uptake and phospholipid metabolism have been shown to occur in response to 1,25$(OH)_2D_3$ [8, 9]. In agreement with these observations, a receptor specific for 1,25$(OH)_2D_3$ has been detected in myoblasts and myotubes [6, 10, 11]. Partial information on proteins synthesized in response to 1,25$(OH)_2D_3$ in cultured muscle cells has recently been obtained [12]. Since the cell cytoskeleton play a key role, among others, in cell morphogenesis, membrane and cell surface properties and functions of growth control and hormone action [13], these work was performed to determine wheather 1,25$(OH)_2D_3$ alters cytoskeletal protein synthesis and properties, *e.g.* phosphorylation and calmodulin binding, in cultured myoblasts.

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Materials and Methods

Chemicals

1,25$(OH)_2D_3$ was kindly donated by Dr. M. Uskokovic (Hoffmann-La Roche Co., N.J., U.S.A.). L-[4,5-^{3}H]leucine, 1-[U^{14}C]leucine, Na[^{125}I] and ^{32}P were purchased from New England Nuclear (MA, U.S.A.). Ampholines (Servalit AG 3–10) for isoelectric focusing were obtained from Serva Feinbiochemical (Heidelberg, F.R.G.). Cycloheximide and actinomycin D were provided by Sigma Chemical Co. (MO., U.S.A.). All other reagents were of analytical grade.

Cell cultures

A myoblasts suspension was prepared by controlled trypsin digestion of breast muscle from 12 day-old chick embryos essentially as described previously [14]. The freed cells were collected by centrifugation and resuspended in Eagle's minimum essential medium containing 10% serum from vitamin-D deficient chicks, 10% chick embryo extract and 1% antibiotic-antimycotic solution [4]. The suspension was dispersed by pipetting, filtered through nylon mesh and "preplated" on gelatin-coated petri dishes to remove contaminating fibroblasts. The unadsorbed cells were brought to a concentration of 2.5×10^{-5} cells/ml. Cells were incubated at 37 °C with mild shaking under constant flow of 95% air: 5% CO_2 saturated with water. The medium was replaced after 24 h

incubation, lowering the concentration of chick embryo extract to 2%. $1,25(OH)_2D_3$ (10^{-10} M) was added after medium replacement and the cells were cultured for an additional 24 h period. Cycloheximide and actinomycin D were added to the cultures 1 h before the addition of the sterol.

Viability of the cells from control and treated preparations was ensured by trypan blue exclusion.

Protein labelling

Control and treated cells were collected by centrifugation, washed and resuspended in Krebs-Henseleit-0.2% glucose solution containing [^{3}H]leucine (3 μCi/ml) or [^{14}C]leucine (1 μCi/ml) for treated and control preparations, respectively. The suspension was incubated for 2 h at 37 °C under 95% O_2:5% CO_2 with mild shaking. After centrifugation and washing the cells with Krebs-Henseleit, myoblasts cytoskeleton was isolated according to a previously described method [15]. Proteins were analyzed by SDS-PAGE according to Laemmli [16] using a 5–15% acrylamide gradient. For double labelling experiments, [^{3}H]leucine (treated) and [^{14}C]leucine (control) labelled samples were mixed in equal amounts, and electrophoresed as above. The gels were sliced in 2 mm fractions and the slices dissolved by heating at 70 °C in 100% H_2O_2 and the ^{3}H and ^{14}C radioactivity measured by scintillation spectrometry.

Isoelectric focusing

It was performed on 7.7% polyacrylamide gels equilibrated at 10 °C. The gels contained ampholines of pH 3–10 and were run at 200 V until constant amperage was attained. The voltage was then kept at 290 V for 30 min. The gels were sliced in 2 mm fractions and the slices were incubated in 2 ml H_2O_2 at 30–40 °C for 30 min with shaking. The pH was then measured. To measure radioactivity, gel slices from parallel runs were dissolved and counted as above for SDS-PAGE.

Calmodulin binding

Calmodulin was radiolabelled with ^{125}I-labelled Bolton-Hunter reagent and its specific binding to cytoskeletal proteins was assessed by the gel-overlay method [17, 18, 24].

Protein phosphorylation

Control and $1,25(OH)_2D_3$-treated (24 h) myoblasts were labelled with 250 μCi/ml of carrier-free ^{32}P at 37 °C for 60 min with mild shaking and under 95% O_2:5% CO_2. After cytoskeleton isolation, phosphoproteins were separated by SDS-PAGE, visualized by autoradiography and then excised from the gel and quantified by liquid scintillation.

Results and Discussion

We have previously shown that exposure of chick myoblasts to physiological concentrations of $1,25(OH)_2D_3$ for 24 h resulted in a marked increase in the incorporation of [^{3}H]leucine into total cell proteins [20]. In agreement with this observations, a stimulation by $1,25(OH)_2D_3$ of the incorporation of [^{3}H]leucine into myoblast cytoskeleton (insoluble matrix) was detected. Fig. 1 shows that

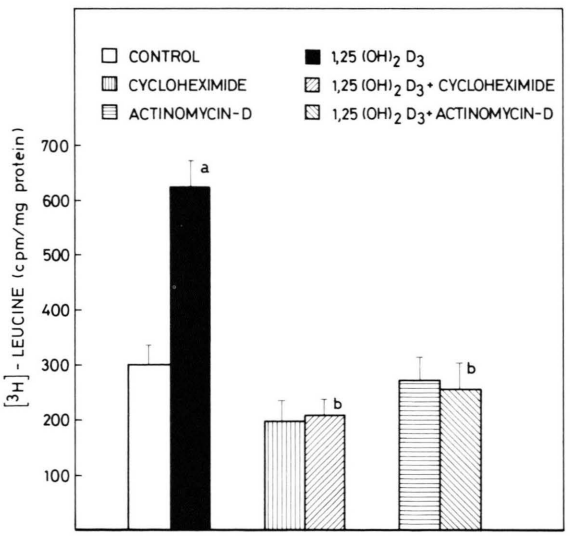

Fig. 1. Incorporation of [^{3}H]leucine *in vitro* into cytoskeletal proteins of control and $1,25(OH)_2D_3$-treated myoblasts: Effect of protein and RNA synthesis inhibitors. Primary cultured myoblasts (24 h) were further cultured for 24 h in the presence or absence of $1,25(OH)_2D_3$ (10^{-10} M) or cycloheximide (50 μM) or actinomycin D (1.6 μM). The cells were then carefully washed and incubated in Krebs-Henseleit-0.2% glucose medium containing [^{3}H]leucine for 2 h at 37 °C. Isolation of labelled cytoskeleton and determination of radioactivity incorporated into protein were carried out as indicated in Materials and Methods. Values are the mean of three experiments + S.D. a, $p < 0.005$; b, NS, with respect to the corresponding control.

the sterol increased more than 100% the labelling of total cytoskeletal proteins. The increment was abolished when pretreatment with $1,25(OH)_2D_3$ was performed in the presence of 50 μM cycloheximide or 1.6 μM actinomycin D. Double labelling experiments were then performed to ascertain whether the sterol would exert a selective action on the synthesis of myoblast cytoskeletal proteins. To that end, $1,25(OH)_2D_3$-treated myoblast cultures were incubated for 2 h with [^{3}H]leucine and control cultures with [^{14}C]leucine. After cytoskeleton isolation, ^{3}H-labelled proteins were mixed with ^{14}C-labelled proteins and separated by SDS-PAGE.

Fig. 2A shows that $1,25(OH)_2D_3$ increased the labelling of proteins of relative molecular masses of 220, 150, 110, 68, 50, 44, 27 and 15 kDa, whereas the synthesis of several other proteins was not affected by the sterol. The effects of $1,25(OH)_2D_3$ could be more clearly seen by plotting $^3H/^{14}C$ ratios of each gel fraction (Fig. 2B). The changes in $^3H/^{14}C$ ratios were absent in gels obtained from control cytoskeleton separately labelled with [^{3}H]leucine and [^{14}C]leucine and processed as described above (data not shown), excluding therefore, the possibility that differential labelling of individual protein fractions in response to $1,25(OH)_2D_3$ was artifactual. In accordance with these results, it has been shown that the $1,25(OH)_2D_3$ alters the cytoskeletal structure in tissues other than muscle [21]. The sterol stimulates the synthesis of an actin-like protein in chick intestinal brush borders and in mice macrophages [22, 23].

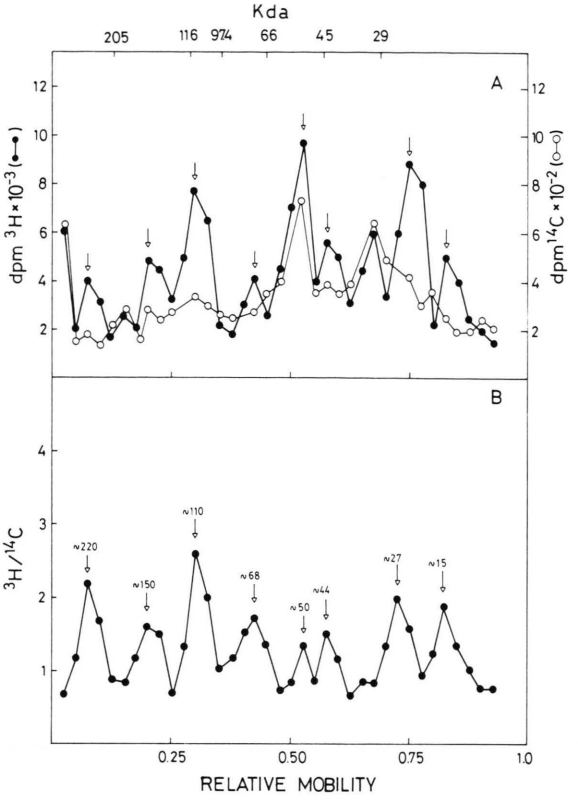

Fig. 2. (A) SDS-polyacrylamide gel electrophoresis labelling patterns of cytoskeletal proteins of myoblasts incubated with radioactive amino acids. Control and $1,25(OH)_2D_3$-treated (10^{-10} M; 24 h) chick embryo myoblast cultures were incubated in Krebs-Henseleit-0.2% glucose solution containing [^{14}C]leucine and [^{3}H]leucine, respectively, for 2 h at 37 °C. After cytoskeleton isolation, ^{3}H- and ^{14}C-labelled proteins were mixed in equal amounts for electrophoretic analysis. Radioactivity (dpm) was determined in 2 mm gel slices. (●—●) $1,25(OH)_2D_3$; (○—○) control. (B) Plot of $^3H/^{14}C$ ratios. Values are the mean of three independent experiments.

Isoelectric focusing of double-labelled cytoskeletal proteins was performed to investigate the possibility that the double-label peaks detected by SDS-PAGE would comprise more than one $1,25(OH)_2D_3$-dependent protein and to obtain information about their isoelectric points. To correlate molecular masses and isoelectric points of induced proteins, in separate experiments double-labelled protein mixtures were first separated by SDS-PAGE and then each gel fraction corresponding to a double-label peak was subjected to isoelectric focusing. Isoelectric point values of 7.5, 7.2, 8.5, 6.3, 7.8 and 5.5 were obtained, respectively, for the 150, 110, 50, 44, 27 and 15 kDa $1,25(OH)_2D_3$-dependent proteins. The 220 kDa double peak could be further resolved in two proteins of pI 6.1 and 9.7 and the 68 kDa in three proteins of pI 9.5, 7.5 and 4.5.

Since the structure and function of some protein components of the cytoskeleton are affected by their interaction with calmodulin, the calmodulin-binding properties of cytoskeletal proteins induced by $1,25(OH)_2D_3$ were investigated by means of the gel-overlay technique. A representative autoradiogram is shown in Fig. 3. The major calmodulin target has an apparent molecular mass of 110 kDa. In addition minor calmodulin-binding proteins of 150 and 68 kDa were barely detected in the gels.

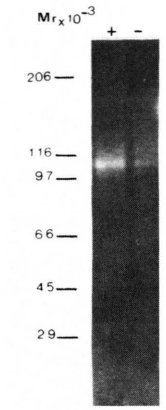

Fig. 3. *In vitro* calmodulin binding by myoblast cytoskeletal proteins. The figure shows the autoradiogram of cytoskeletal proteins (75 µg) isolated from myoblasts incubated in the presence or absence of $1,25(OH)_2D_3$ (10^{-10} M, 24 h). After isolation, the proteins were separated by SDS-PAGE and then incubated with [^{125}I]calmodulin. The position of molecular weight markers are shown on the left line.

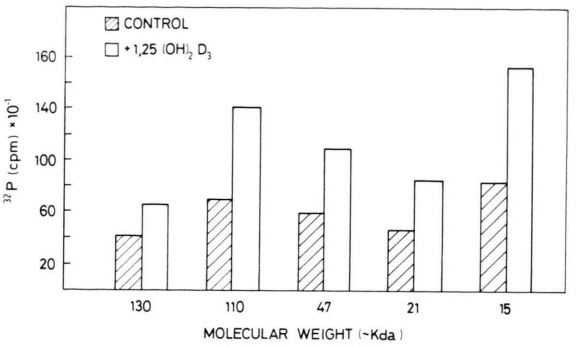

Fig. 4. Effects of $1,25(OH)_2D_3$ on the phosphorylation of myoblast cytoskeletal proteins. Myoblasts were cultured in the presence or absence of $1,25(OH)_2D_3$ (10^{-10} M, 24 h) and then phosphorylated with [^{32}P]Pi for 1 h. Cytoskeletal proteins were isolated and subjected to SDS-PAGE followed by autoradiography. Proteins from radioactive bands were excised from the gel and the radioactivity measured as described in Materials and Methods. Results are the average of three separate experiments.

The presence of flufenazine (0.1 mM), a calmodulin antagonist, prevented the binding of [^{125}I]calmodulin to these proteins, indicating that they bind calmodulin specifically (data not shown).

To quantitate the effects of the sterol on calmodulin binding by the 110 kDa macromolecule, the labelled protein was removed from the gel and their ^{125}I content was determined using gamma scintillation spectroscopy. $1,25(OH)_2D_3$ increased approx. 1-fold the amount of radioactivity associated with the 110 kDa protein, probably reflecting the stimulation of its synthesis.

Since the organization and the interaction of the cytoskeleton with other cell components is regulated in part by phosphorylation of microtubule associated proteins [25], we have also investigated the effect of $1,25(OH)_2D_3$ on the phosphorylation of myoblast cytoskeletal proteins. Differences between endogenous phosphorylation of control and $1,25(OH)_2D_3$-treated (24 h) myoblasts were shown by the higher phosphate incorporation into cytoskeletal proteins of 130, 110, 47, 21 and 15 kDa (Fig. 4). These results may be either explained by increased amount of proteins (110 and 15 kDa) synthesized in response to the sterol or alternatively they may be associated to modifications in kinase/phosphatase activities in treated myoblasts. The present investigations indicate that $1,25(OH)_2D_3$ may affect the formation and function of cytoskeletal proteins in embryonic muscle. Future studies are necessary to further characterize these $1,25(OH)_2D_3$-dependent proteins, establish their identity and role in the regulation of myoblasts function and differentiation.

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