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PRESENCE OF A 1,25-DIHYDROXY-VITAMIN D_3 RECEPTOR IN CHICK SKELETAL MUSCLE MYOBLASTS

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SUMMARY: The presence of a specific receptor for 1,25-dihydroxy-vitamin D_3 was investigated in myoblasts released from chick embryo skeletal muscle by trypsin and collagenase treatment. Density gradient analysis of the cytosol obtained from these muscle cell preparations showed that 1,25-dihydroxy-vitamin D_3 binds specifically to a 3.7 S macromolecule. Scatchard analysis yielded an equilibrium dissociation constant of 2.46×10^{-10} M and a Nmax of 74 fmol/mg of cytosol protein. The data is in agreement with previous evidence which indicates that the action of the vitamin D metabolite on muscle Ca uptake is mediated by *de novo* protein and RNA synthesis, and supports the concept that muscle is a target organ for 1,25-dihydroxy-vitamin D_3.

Various lines of evidence have shown that vitamin D_3 plays a role in Ca transport by muscle membranes (1-3). Recent investigations using muscle culture preparations have, in addition, demonstrated that 1,25-dihydroxy-vitamin D_3 is the most active vitamin D metabolite in muscle cellular Ca (4,5). Moreover, it has been shown that 1,25-dihydroxy-vitamin D_3 stimulation of Ca uptake by chick embryo skeletal muscle myoblast cultures can be suppressed by inhibitors of protein and RNA synthesis. On the basis of these data it was suggested that the metabolite acts on muscle via a nuclear mechanism like in classical target organs (6). In support of this contention, the present work provides evidence which indicates that embryonic muscle cells contain a receptor specific for 1,25-dihydroxy-vitamin D_3.

MATERIAL AND METHODS:

Materials: 1,25-dihydroxy(26,27-methyl-^{3}H)vitamin D_3 (180 Ci/mmol) with a radiochemical purity of 97 % was obtained from Amersham/Buchler Co. (Braunschweig, FRG). Radioinert chromatographycally pure 1,25-dihydroxy-vitamin D_3, 25-hydroxy-vitamin D_3, 24,25-dihydroxy-vitamin D_3 and vitamin D_3 were obtained from Duphar Co. (Amsterdam, Netherlands). Bovine serum albumin, (^{14}C)-methylated (20 µCi/mg protein) and ovalbumin, (^{14}C)-methylated (20 µCi/mg protein) were provided by New England Nuclear (Boston, USA). Hydroxylapatite was supplied by Bio-Rad Laboratories (Richmond, CA, USA). Trypsin from bovine

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pancreas (10,000 BAEEU/mg) and collagenase, type II, from Clostridium histolyticum were obtained from Serva (Heidelberg, FRG) and Sigma (St. Louis, MO, USA), respectively. Other chemicals were of analytical grade. Fertilized eggs of White Leghorn chickens were obtained from Hockenberger Hatchery (Sinsheim, FRG).

Preparation of myoblasts from embryonic chick skeletal muscle: Myoblasts were isolated from breast and thigh muscles of 12- to 13-day-old chicken embryos (4,7). Approximately 20 embryos were used in each experiment. Dissection was performed at room temperature in Earle's balanced salt solution. The muscles were carefully cleaned of fat and connective tissue and minced to a size of 1-2 mm^3. The tissue was collected by centrifugation and washed twice with phosphate buffered saline solution (PBS). Incubation was then performed with 50 ml of 0.1 % trypsin-0.15 % collagenase in PBS for 40 min at 37° C and with gentle magnetic stirring. The suspension was filtered through gauze and the released cells were collected by centrifugation of the filtrate for 5 min at 500 x g. The cells were resuspended in PBS with repeated pipetting and pelleted again. This procedure was repeated twice. The final suspension was plated on gelatin-coated Petri dishes to remove contaminating fibroblasts. The unabsorbed cells were used for preparation of cytosol. These cell preparations have been shown to proliferate and differentiate into skeletal muscle multinucleated myotubes when they are cultured in medium supplemented with chick serum and chick embryo extract (4).

Cytosol preparation: Myoblasts were collected by centrifugation and sonicated in three volumes (v/v) of 0.4 M KCl, 10 mM Tris-HCl, 1.5 mM EDTA, 1 mM dithiothreitol, 10 mM Na-molybdate, pH 7.4 (KTEDMo) using a model B12 sonifier cell disruptor (Branson Sonic Power Co.), 3 x 5-s bursts, in ice. The preparation was centrifuged at 5,000 x g for 10 min. The supernatant was used to obtain the cytosolic fraction by centrifugation at 105,000 x g for 45 min. The cytosol was kept at 4° C and rapidly used for density gradient or saturation analysis. Protein concentration was determined employing the procedure of Bradford (8).

Sucrose density gradient analysis: Aliquots (0.2 ml/1.0 mg protein) of cytosol were incubated with sterols (dissolved in 20 µl ethanol) for 3 h at 4° C. The samples were layered onto cold linear gradients of 5 to 20 % sucrose in KTEDMo buffer prepared with a self-designed gradient former. Centrifugation was then performed at 4° C in a Beckman L5-65 ultracentrifuge using a SW-60 rotor at 255,000 x g, 21 h. Fractions (7 drops) were collected and counted in Rotitainer scintillation fluid (Carl Roth Co., Karlsruhe, FRG). Sedimentation coefficients were estimated by comparison with protein markers (ovalbumin, 3.67 S; bovine serum albumin, 4.4 S). As a control of the procedure employed paralell runs were made with cytosol from chick intestinal mucosa labelled with (^{3}H)-1,25-dihydroxy-vitamin D_3.

Saturation analysis: Aliquots of cytosol (0.20 ml/1 mg protein) and various concentrations of (^{3}H)1,25-dihydroxy-vitamin D_3 (0.1-4.0 nM) dissolved in 10 µl ethanol were incubated 16 h at 4° C. Parallel incubations containing labelled sterol plus 100-fold excess of unlabelled sterol were processed similarly to estimate nonspecific binding. Separation of bound from free metabolite was performed using the hydroxylapatite procedure (9). Five hundred microliters of a 50 % slurry of Bio-gel HT hydroxylapatite in KTED buffer were added to the assay tubes. After an incubation period of 15 min at 4° C with frequent vortexing, the samples were centrifuged at 12,000 x g for 5 min. The hydroxylapatite pellets were washed three times with 1 ml TED-0.5 % Triton X-100 pH 7.4 buffer, by vortexing and centrifuging, as above. Tritiated sterols were extracted from the final washed pellet with ethanol, dried and counted in Aquasol scintillation fluid (New England Nuclear Co., Boston, USA). Binding data were analyzed by the method of Scatchard (10) using linear regression analysis to obtain the line of best fit.

RESULTS AND DISCUSSION:

Sucrose density gradient analysis of the cytosolic fraction from chick embryo skeletal muscle myoblasts incubated with (^{3}H)-1,25-dihydroxy-vitamin D_3 gave a single peak sedimenting at 3.7 S (Fig. 1A). When labelled in the presence of a 200-fold molar excess of nonradioactive 1,25-dihydroxy-vitamin D_3 most of this 3.7 S peak was displaced. However, a 200-fold molar excess of 25-hydroxy-vitamin D_3 and 24,25-dihydroxy-vitamin D_3 caused only a minor displacement (Fig. 1B and 1C, respectively). In addition, it could be observed that a 10,000-fold molar excess of vitamin D_3 exerted no competition on (^{3}H)1,25-dihydroxy-vitamin D_3 binding by muscle cell cytosol (Fig. 1D). Figure 2 shows the saturation analysis of (^{3}H)1,25-dihydroxy-vitamin D_3 binding. Incubation of myoblast cytosol at 4 °C for 16 h with increasing concentrations of (^{3}H)1,25-dihydroxy-vitamin D_3 and determination of binding by the hydroxylapatite procedure gave a plateau for specific binding at 2-4 nM. A straight line indicating a single class of

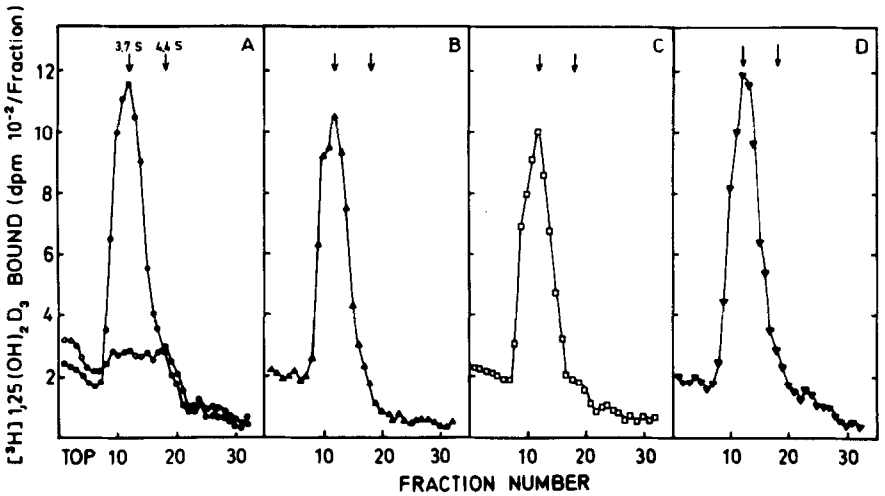

Fig. 1. Sucrose density gradient analysis of (^{3}H)1,25-dihydroxy-vitamin D_3 binding by cytosol from chick embryo skeletal muscle myoblasts. Cytosol (1.0 mg protein) was incubated with vitamin D_3 sterols for 3 h at 4 C and centrifuged in linear 5-20 % sucrose density gradients for 21 h at 255,000 x g. Fractions were collected from the bottom of the tube. The position of external markers is indicated by the arrows: 3.7 S, ovalbumin and the chick intestinal receptor for 1,25-dihydroxy-vitamin D_3, 4.4 S, bovine serum albumin. A, binding of 1 nM (^{3}H)1,25-dihydroxy-vitamin D_3 in the absence (●) and presence (○) of 200-fold molar excess of 1,25-dihydroxy-vitamin D_3. B, 1 nM (^{3}H)1,25-dihydroxy-vitamin D_3 in the presence of 200-fold molar excess of 25-hydroxy-vitamin D_3. C, 1 nM (^{3}H)1,25-dihydroxy-vitamin D_3 in the presence of 200-fold molar excess 24,25-dihydroxy-vitamin D_3. D, 1 nM (^{3}H)1,25-dihydroxy-vitamin D_3 in the presence of 10,000-fold molar excess of vitamin D_3.

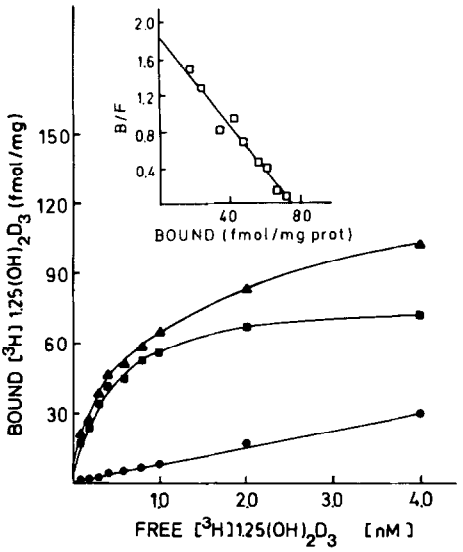

Fig. 2. Saturation analysis of (^{3}H)1,25-dihydroxy-vitamin D_3 binding to cytosol from embryonic chick skeletal muscle myoblasts. Cytosol (1.0 mg protein) was incubated with increasing concentrations (0.1-4 nM) of (^{3}H)1,25-dihydroxy-vitamin D_3 in the presence or absence of 100-fold molar excess of radioinert 1,25-dihydroxy-vitamin D_3 at 4 C for 16 h. Bound and free 1,25-dihydroxy-vitamin D_3 was separated with hydroxylapatite as indicated in Material and Methods. Saturation plot of total (▲), specific (■) and nonspecific (●) binding. The inset shows the Scatchard analysis of specific binding. The equilibrium dissociation constant (K_D) determined from the slope of the regression line was 2.46×10^{-10} M. The concentration of binding sites estimated from the abscissa intercept was 74 fmol/mg protein. Values are the average of triplicate determinations.

binding sites was obtained by Scatchard analysis of specific binding (Fig. 2, inset). From the slope of the line an equilibrium dissociation constant of 2.46×10^{-10} M was obtained. Extrapolation of the line to the abscissa yielded a maximum binding of 74 fmol/mg protein.

The data obtained indicate that chick embryonic skeletal muscle cells contain a receptor-like macromolecule which specifically binds 1,25-dihydroxy-vitamin D_3 with high affinity and low capacity, as has been demonstrated in target cells for the metabolite (11,12). This finding supports previous clinical and experimental evidence which suggested that skeletal muscle is a target organ for 1,25-dihydroxy-vitamin D_3. Muscle weakness is a prominent feature in nutritional vitamin D deficiency and in chronic renal failure where synthesis of the hormone is impaired (13,14). This myopathy responds to the treatment with small amounts of 1,25-dihydroxy-vitamin D_3 (15,16). Electrophysiological cha-

racterization of muscle weakness in animals nutritionally depleted of vitamin D has shown altered kinetics of contraction and relaxation (2,17). These changes may be related to alterations of the mechanisms by which intracellular Ca^{2+} levels are regulated in the muscle cell as sarcoplasmic Ca^{2+} plays a key role in muscle contraction and relaxation (18). Several data are in agreement with this idea. Administration of vitamin D_3 to vitamin D-deficient animals stimulates Ca transport systems located in sarcoplasmic reticulum and sarcolemmal membranes (1,3). In addition, the ability of mitochondria to store Ca *in vivo* is affected by the sterol (2). 1,25-dihydroxy-vitamin D_3 may be the principal vitamin D metabolite responsible for the effects of vitamin D_3 on muscle Ca transport. It has been shown that experimental maneuvers which suppress *in vivo* 1,25-dihydroxy-vitamin D_3 synthesis affect Ca transport by sarcoplasmic reticulum. Thus, nephrectomy or strontium feeding impair Ca uptake and phosphorylation of Ca-ATPase in this membrane system. These changes are reversed by the administration of 1,25-dihydroxy-vitamin D_3 (19-21). Studies employing cultures of chick soleus muscle and chick embryo myoblasts have indicated a direct action of 1,25-dihydroxy-vitamin D_3 in muscle Ca metabolism. At physiological levels the metabolite caused a significant stimulation of ^{45}Ca uptake by these *in vitro* preparations. Moreover, 1,25-dihydroxy-vitamin D_3 was markedly more effective than 25-hydroxy-vitamin D_3 to increase Ca uptake. In addition, kinetic characterization of Ca efflux in cultures prelabelled with ^{45}Ca suggested, in agreement with the above mentioned *in vivo* evidence, an stimulation by 1,25-dihydroxy-vitamin D_3 of Ca fluxes across sarcolemma and the membranes of an intracellular compartment representing mitochondria and/or sarcoplasmic reticulum (4,5). These investigations indicated that embryonic muscle cells may represent an useful model to characterize the mode of action of 1,25-dihydroxy-vitamin D_3 on muscle Ca transport as the sterol induced similar changes in Ca fluxes as in the fully differentiated skeletal muscle culture preparations. In more recent work it was possible to demonstrate that the increase in Ca uptake induced by 1,25-dihydroxy-vitamin D_3 in soleus muscle and myoblast cultures can be suppressed by puromycin and cycloheximide, thereby, indicating

that the effects of the metabolite on muscle Ca transport are mediated by de novo protein synthesis. In addition, it could be shown that 1,25-dihydroxy-vitamin D_3-dependent Ca uptake by the cultures is effectively blocked by actinomycin D, suggesting that the sterol acts via a nuclear mechanism (6). The present data further suggests that the nuclear action of 1,25-dihydroxy-vitamin D_3 in muscle, like in classical targe organs, may involve binding to a specific receptor prior to genome activation. Earlier reports in which a 1,25-dihydroxy-vitamin D_3 receptor in skeletal muscle was not detected (22,23) may be explained by the presence in the muscle cytosol preparations employed of a 6.0 S binder which binds 1,25-dihydroxy-vitamin D_3 with low affinity. This binding protein represents a complex between serum vitamin D transport protein and actin (24). The 6.0 S binder may be present in high concentrations in cytosol fractions obtained from tissue homogenates prepared by conventional procedures due to contamination with serum. In agreement with this interpretation, initial attempts to detect a 1,25-dihydroxy-vitamin D_3 receptor in cytosol directly prepared from chick embryo muscle tissue yielded sucrose density gradient binding profiles in which a 6.0 S peak was observed in addition to the 3.7 S peak (data not shown). Finally, the results of the present study raise a question about the possible gene products, e.g. troponin c, actin, mitochondrial membrane proteins (25-27), expressed in the muscle cell through the receptor-mediated genomic action of 1,25-dihydroxy-vitamin D_3.

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