25-Hydroxycholecalciferol Stimulation of Muscle Metabolism

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ABSTRACT Intact diaphragms from vitamin D-deficient rats were incubated in vitro with [^{3}H]leucine. Oral administration of 10 μg (400 U) of cholecalciferol 7 h before incubation increased leucine incorporation into diaphragm muscle protein by 136% ($P < 0.001$) of the preparation from untreated animals. Nephrectomy did not obliterate this response. ATP content of the diaphragm muscle was also enhanced 7 h after administration of the vitamin. At 4 h after administration of cholecalciferol, serum phosphorus concentration was reduced by 0.7 mg/100 ml ($P < 0.025$) and the rate of inorganic $^{32}PO_4$ accumulation by diaphragm muscle was increased by 18% ($P < 0.025$) over the untreated animals. Increasing serum phosphate concentration of the vitamin D-deficient animals by dietary supplementation with phosphate for 3 days failed to significantly enhance leucine incorporation into protein. However, supplementation of the rachitogenic, vitamin D-deficient diet with phosphorus for 3 wk stimulated the growth of the animal and muscle ATP levels. This increase in growth and muscle ATP content attributed to the addition of phosphorus to the diet was less than the increase in growth and muscle ATP levels achieved by the addition of both phosphorus and vitamin D to the diet.

To eliminate systemic effects of the vitamin, the epitrochlear muscle of the rat foreleg of vitamin D-depleted rats was maintained in tissue culture. Addition of 20 ng/ml of 25-hydroxycholecalciferol (25-OHD$_3$) to the medium enhanced ATP content of the muscle and increased leucine incorporation into protein. Vitamin D$_3$ at a concentration of 20 μg/ml and 1,25-dihydroxycholecalciferol (1,25-(OH)$_2$D$_3$) at a concentration of 500 pg/ml were without effect. Analysis of muscle cytosol in sucrose density gradients revealed a protein fraction which specifically bound 25-OHD$_3$ and which demonstrated a lesser affinity for 1,25-(OH)$_2$D$_3$. These studies suggest that 25-OHD$_3$ may influence directly the intracellular accumulation of phosphate by muscle and thereby play an important role in the maintenance of muscle metabolism and function.

INTRODUCTION

Stimulation of growth of the vitamin D-deficient patient or experimental animal has long been a recognized response to vitamin D replacement therapy (1–3). This increase in musculoskeletal growth has been attributed to the specific action of vitamin D on calcium homeostasis and specifically, the increase in intestinal absorption of calcium and secondarily an increase in bone mineralization and growth (4, 5). More recently a direct action of vitamin D on bone has been recognized which is characterized by the stimulation of osteoclastic bone resorption (6). The role of vitamin D in mineral homeostasis has more recently been extended to include the action of the vitamin in the mobilization of phosphate from the intestine (7–9) and other tissues (10). The stimulation of intestinal calcium and phosphorus transport both appear to be mediated by the 1,25-dihydroxy metabolite of vitamin D synthesized by the kidneys (11, 12) from 25-hydroxycholecalciferol (25-OHD$_3$)[1] which is derived from the hepatic hydroxylation of vitamin D$_3$ (13). The earlier reported failure of orally administered 1,25-dihydroxycholecalciferol (1,25-(OH)$_2$D$_3$) to cure the rachitic state in rats (14) has now been attributed to the more rapid clearance of 1,25-(OH)$_2$D$_3$ relative to its immediate precursor, 25-OHD$_3$

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[1] *Abbreviations used in this paper:* 1,25-(OH)$_2$D$_3$, 1,25-dihydroxycholecalciferol; 25-OHD$_3$, 25-hydroxycholecalciferol; PCA, perchloric acid.

(15), which was effective in this regard. Accordingly, the 1,25-dihydroxy metabolite of cholecalciferol is considered to be the physiologically active form of the vitamin in mediating the mobilization of calcium and phosphorus from the intestine and bone.

However, in addition to the skeletal lesion and intestinal transport defects, muscle weakness and atrophy are also prominent features of nutritional vitamin D deficiency (16–19). Vitamin D replacement therapy rapidly reverses these often incapacitating manifestations of the deficiency state. The influence of vitamin D on muscle metabolism and growth has not been specifically examined although two decades ago Steenbock and Herting (1) suggested on the basis of their nutritional studies that vitamin D may have a "widespread effect on organic tissue metabolism of which increased growth is merely one manifestation." Evidence is presented in this report of a direct action of 25-OHD_3 on muscle metabolism and muscle protein synthesis which is independent of the action of vitamin D and its metabolites on intestinal calcium and phosphorus absorption.

METHODS

Preparation of animals. Male Wistar rats, 3 wk of age, were maintained for 4 wk on a synthetic diet described by Kenny and Munson (20) from which vitamin D, calcium, and phosphorus salts could be omitted. All animals were maintained on this diet containing 1.5% calcium, 0.1% phosphorus, and no vitamin D. To insure the availability of adequate phosphate to the tissues, the phosphorus content was increased from 0.1% to 0.4% 4 days before sacrifice unless stated otherwise. Vitamin D, 10 μg in cotton seed oil, or cotton seed oil alone was administered by oral intubation at varying time intervals before sacrifice. At a dose of 0.4 μg per animal, 25-OHD_3 was administered intravenously in 0.1 cm^3 of vitamin D-deficient rat serum. These doses of the vitamin were selected because they generated physiologic concentrations of 25-OHD_3 in serum at 7 h. Animals used for epitrochlear muscle incubations were also maintained on the synthetic diet with 1.5% calcium, 0.1% phosphorus, and without vitamin D.

Diaphragm studies. Animals were sacrificed by decapitation and the diaphragms quickly removed intact connected to the rib cage. The diaphragm was then divided through the midline connective tissue without cutting the muscular portion, then placed into stoppered 25-ml Erlenmeyer flasks gassed with 95% oxygen, 5% CO_2. The composition of the incubation medium is presented in Table I. The incubations were carried out in the presence of $Na^{32}PO_4$ (0.01 μCi/ml) or [^{3}H]leucine (0.05 μCi/ml). Cycloheximide (Sigma Chemical Co., St. Louis, Mo.) at a concentration of 50 μg/ml was used to block diaphragm muscle protein synthesis. In the examination of the incorporation of [^{3}H]leucine into protein, the diaphragms were incubated for 1 h at 37°C in the presence of the labeled amino acid. In terminating the incubation, the hemidiaphragms were removed, rinsed, and the diaphragm muscle cut away from the rib cage. The muscle was then homogenized in cold 5% perchloric acid (PCA) with a Teflon pestle tissue grinder. The precipitated protein was washed with additional 5% PCA then dissolved in 1 N KOH for radioactivity and Lowry protein measurements (21). In determining the intracellular accumulation of free amino acid, the procedure was modified so that diaphragms were incubated for 40 min in the presence of [^{3}H]leucine, and the supernatant fraction was of the PCA precipitate measured for radioactivity. Total "free" [^{3}H]leucine was corrected for extracellular [^{3}H]leucine by the ^{14}C-insulin space. Accumulation of $^{32}PO_4$ was determined after a 20-min incubation in the presence of the isotope. Both total and inorganic phosphate were measured by the procedure of Short et al. (22). The radioactivity of the various studies was measured in a Tricarb liquid scintillation spectrometer (Packard Instrument Co., Inc., Downers Grove, Ill.) and expressed as dpm per milligram Lowry protein. Total ATP content of muscle was determined by the enzymatic method of Lowry (23) on tissue removed immediately from the animal and frozen on dry ice within 2 min of sacrifice of the animal and stored at −80°C. The frozen muscle was homogenized in 3 M PCA at −10°C. The ATP content of the resulting supernate was expressed as nanomoles ATP per milligram of the PCA-precipitable protein. Serum concentration of calcium was determined by atomic absorption spectrophotometry (24) and the serum phosphorus concentration was determined colorimetrically (25). A competitive protein-binding assay (26) was used to measure serum concentrations of 25-OHD_3.

Epitrochlear muscle studies. The epitrochlear muscle of the foreleg was excised at its tendinous insertions from the exposed limb with care taken not to injure the muscle. Control and vitamin-treated incubations were carried out on paired muscles from the same animals. The conditions of the incubation were the same as those for the diaphragm muscle studies with the exception of the addition of 10% vitamin D-deficient rat serum. The latter served as a vehicle for the addition of vitamin D and its metabolites to the incubation. The procedure for the preparation of the muscle for protein and radioactivity measurements was the same as that described for diaphragm muscle. Incorporation of [^{3}H]leucine into PCA-precipitable protein was measured as a function of time and vitamin D preparation. The data has been expressed as dpm of [^{3}H]leucine per milligram Lowry protein and treated statistically by analysis of the paired data. The 1,25-$(OH)_2D_3$ was prepared from the incubation of 25-OHD_3 of known specific radio-

TABLE I
Incubation Medium

	Addition/liter
Dextrose	1.0 g
NaCl	6.8 g
KCl	0.4 g
$MgSO_4$	0.1 g
$MgCl_2$	0.1 g
$NaHCO_3$	2.2 g
$CaCl_2$	0.155 g
HEPES buffer	1.4 g
Glutamine	0.25 g
0.2 M Na_2HPO_4-NaH_2PO_4, pH 7.4	2.5 ml
Penicillin, 10,000 U-streptomycin, 10 mg/ml	10 ml
Insulin	40 U
MEM amino acid mixture (50×)	5 ml

HEPES buffer, *N*-2-hydroxyethylpiperazine-*N'*-2-ethane sulfonic acid; MEM, minimal essential medium.

TABLE II

Influence of Vitamin D_3 on Muscle

Time	Treatment*	[^{3}H]Leucine in protein			ATP content			$^{32}P_i$ accumulation		
		Mean±SEM	*n*	*P* value	Mean±SEM	*n*	*P* value	Mean±SEM	*n*	*P* value
h		*dpm/mg protein*			*nmol/mg protein*			*cpm/mg protein*		
4	−D	2,018±167	16	NS	42±1	12	NS	453±23	15	<0.025
	+D	2,381±190	16		44±2	12		536±27	15	
7	−D	2,212±110	19	<0.001	43±1	16	<0.025	462±33	12	NS
	+D	2,942±203	20		51±2	16		494±39	12	

* At 4 or 7 h after oral administration of 10 μg of vitamin D_3 (+D) or the cotton seed oil vehicle (−D), the animals were killed and the intact diaphragm was incubated with either [^{3}H]leucine for 1 h or $^{32}PO_4$ for 20 min or frozen immediately for ATP determination.

activity with chick kidney homogenates as described by Boyle et al. (27). The product was extracted and purified by silicic acid and Sephadex LH-20 (Pharmacia Fine Chemicals, Inc., Piscataway, N. J.) chromatography. The biologic potency was also established in a chick intestinal organ culture system sensitive to 50 pg/ml of the dihydroxy metabolite of vitamin D.

Cytosol receptor protein. Skeletal muscle from the lower limbs of vitamin D-depleted rats was minced in cold isotonic saline and homogenized in a VirTis homogenizer (VirTis Co., Inc., Gardiner, N. Y.). The homogenate was centrifuged at 500 *g* at 4°C for 15 min and the pellet discarded. The supernate was then centrifuged at 105,000 *g* in an SW50.1 rotor in a Beckman L-65B ultracentrifuge (Beckman Instruments, Inc., Spinco Div., Palo Alto, Calif.) for 1 h. This high-speed supernate (cytosol) was then directly used in binding studies. [26,27-^{3}H]25-OHD_3 (Amersham/Searle Corp., Arlington Heights, Ill.; sp act 1.1–6.9 Ci/mmol) was purified on columns of Sephadex LH-20 in chloroform: *n*-hexane (65:35, vol/vol) as previously described (28). Saturation analyses were carried out by using the coated-charcoal technique of removing free sterol (26). Identical aliquots of diluted muscle cytosol were incubated with increasing amounts of radioactive 25-OHD_3 for 1 h at 4°C. Total binding (supernatant dpm in absence of unlabeled 25-OHD_3) minus nonspecific binding (supernatant dpm in the presence of 1 μg of unlabeled 25-OHD_3) yielded specific binding. Specific binding was analyzed by the method of Scatchard (29) and related to the protein content (21) of the cytosol.

TABLE III

Influence of Vitamin D on Free [^{3}H]Leucine Accumulation by Diaphragm Muscle

Treatment*	Time	*n*	[^{3}H]Leucine (±SEM)	
			Free	Bound
	h		*dpm/mg protein*	
−D −cyclo.	7	16	2,784±86	1,869±83
+D −cyclo.	7	16	2,823±102	2,326±86
−D +cyclo.	7	16	2,618±98	202±23
+D +cyclo.	7	16	2,551±106	187±30

* The animals were killed 7 h after treatment as described previously. The intact, hemidiaphragm was incubated either in the presence (+cyclo.) or absence (−cyclo.) of 50 μg/ml of cycloheximide for 20 min before transfer to [^{3}H]leucine-containing buffer.

RESULTS

Oral administration of vitamin D to vitamin D-deficient rats alters a number of parameters of diaphragm muscle metabolism (Table II). These changes can be appreciated as early as 4 h after administration of the vitamin and are characterized by an increase in the rate of accumulation of inorganic phosphate by the tissue. At 7 h after administration of the vitamin, ATP content of the muscle and [^{3}H]leucine incorporation into 5% PCA-precipitable protein was also greater than in muscle preparations from untreated animals. Accumulation of "free" intracellular [^{3}H]leucine was not increased by vitamin D. Under conditions in which protein synthesis was inhibited by 90%, vitamin D did not enhance the accumulation of "free" intracellular [^{3}H]-leucine (Table III). Thus the increased incorporation of [^{3}H]leucine into protein cannot be attributed to an increased accumulation of the isotope within the cell.

In addition to changes in certain parameters of muscle metabolism, unexpected changes in serum phosphorus were observed (Table IV). At 7 h after administration of vitamin D, serum phosphorus of vitamin-treated animals was significantly greater ($P < 0.01$) than untreated control animals, consistent with the observations of Chen et al. (10). At 7 h, circulating concentrations of 25-OHD_3 were 16±3 ng/ml for this metabolite of vitamin D. This compares to a 25-OHD_3 level of 11.4±1.7 (SD) ng/ml in normal rats on Purina rat chow. At 4 h after administration of 10 μg of vitamin D, circulating concentrations of 25-OHD_3 were 5±1 ng/ml, and the concentration of the serum phosphorus was now significantly reduced ($P < 0.025$) in the vitamin-treated animals. These data suggest that the first demonstrable

TABLE IV

Influence of Vitamin D_3 on Serum Calcium and Phosphorus

Time	Treatment	Calcium		Phosphorus		25-OHD
		Mean±SEM	*P* value	Mean±SEM	*P* value	(±SEM)
h		*mg/100 ml*		*mg/100 ml*		*ng/ml*
2	−D	10.3±0.3	NS	6.9±0.3	NS	<1
	+D	10.7±0.2		6.8±0.3		<1
4	−D	10.2±0.2	NS	6.9±0.2	<0.025	<1
	+D	9.9±0.2		6.2±0.3		5.2±0.6
7	−D	10.2±0.2	NS	7.0±0.5	<0.01	<1
	+D	10.3±0.2		8.4±0.3		15.9±3.6

response to vitamin D is an accelerated accumulation of inorganic phosphate by muscle which is reflected by a reduction in serum phosphate concentrations.

The question then raised was whether vitamin D or a metabolite of the vitamin was responsible for the observed metabolic effects. Administration of 0.4 μg of 25-OHD_3 intravenously generated serum levels of this metabolite at 4 h (13±3 ng/ml) which were comparable to those generated by vitamin D given orally at 7 h. Significant stimulation of [^{3}H]leucine incorporation into protein was also apparent at 4 h after 25-OHD_3 administration, 3 h before the same response was observed in animals given the parent vitamin (Table V). Removal of both kidneys (and therefore the mechanism for the conversion of 25-OHD_3 to 1,25-$(OH)_2D_3$) did not obliterate the response of the muscle to vitamin D administration. From these data it was concluded that at least 25-OHD_3 had the potential of altering muscle amino acid metabolism.

The second question raised by the observed changes in serum and muscle was whether these changes reflected a direct action of the vitamin or its metabolites on muscle or whether the changes in muscle metabolism were secondary to a systemic effect of the vitamin, as for example, an alteration in the serum Ca and P as a result of the influence of the vitamin on the intestinal transport of these ions. In Fig. 1, it is apparent that protein synthesis in the vitamin-deficient diaphragm, as reflected in the rate of incorporation of [^{3}H]leucine into protein, is relatively refractory to acute changes in extracellular phosphate concentration. Although by increasing the phosphate concentration of the incubation medium from 0.5 to 1.5 mM, [^{3}H]leucine incorporation into protein was increased, no significant change was observed by increasing the phosphate concentration from 1.5 to 3.0 mM. Since the in vitro incubations provide only a brief exposure to the different extracellular phosphate concentrations, more prolonged exposure to increased phosphate was achieved by increasing the serum phosphate of the vitamin D-deficient animals (Table VI). Neutral sodium phosphate (35 mmol/liter) was added to the drinking water over a

TABLE V

Role of 25-OHD_3 in Stimulating [^{3}H]Leucine Incorporation into Rat Diaphragm Muscle

Animal	Treatment	*n* +D/−D	Time	[^{3}H]Leucine		Serum 25-OHD_3 (±SEM)
				% control*	*P* value	
			h			*ng/ml*
Intact	10 μg D_3, p.o.	16/16	4	112	<0.1	5±1
Intact	10 μg D_3, p.o.	20/19	7	133	<0.001	16±3
Intact	0.4 μg 25-OHD_3, i.v.	10/10	4	126	<0.05	13±3
Ureteral ligation	10 μg D_3, p.o.	14/14	7	129	<0.005	11±2
Bilateral nephrectomy	10 μg D_3, p.o.	12/13	7	123	<0.01	13±3

* The incorporation of [^{3}H]leucine (dpm per milligram protein) into muscle of the vitamin-treated rats during 1 h incubation with label has been expressed as a percent of the incorporation by an identically prepared control group of rats. Student's *t* test was used to analyze the statistical difference between the response of the vitamin-treated and their respective controls for each preparation of animals.

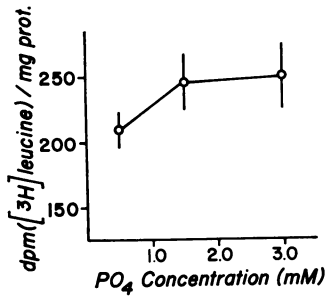

FIGURE 1 Incorporation of [^{3}H]leucine into diaphragm muscle protein incubated in vitro for 1 h as a function of increasing concentrations of phosphate in the incubation medium. The vertical bars indicate the SEM of 12 diaphragms at each phosphate concentration.

3-day period before sacrifice. All three experimental groups were otherwise maintained on the vitamin D-deficient synthetic diet containing 0.1% phosphorus. Dietary calcium of those receiving additional phosphate was increased from 1.5 to 2.25%. Again, increase in extracellular phosphate was not effective in significantly stimulating protein synthesis.

However, the importance of phosphate in maintaining normal growth of the animal is demonstrated by the data in Table VII. In these studies the dietary intake of calcium was adjusted to provide a similar serum concentration of calcium in an effort to minimize the influence on growth of this cation. The four groups of animals were pair-fed their respective diets for 3 wk. Over the 3-wk period the addition of phosphate to the diet accelerated growth both in the presence and absence of vitamin D, although maximal growth was achieved with the addition of both vitamin D and phosphorus to the diets. The rate of growth was correlated

TABLE VI

The In Vivo Influence of Vitamin D and PO_4 on [^{3}H]Leucine Incorporation into Diaphragm Muscle Protein

Treatment*	n	P_s	Ca_s	[^{3}H]Leucine % control	[^{3}H]Leucine P value
		mg/100 ml			
A. Control	35	3.4	11.1	—	—
B. $+PO_4$	24	4.2	11.1	106	NS
C. +D	16	4.3	10.9	129	<0.01

* All animals were maintained on the synthetic vitamin D-deficient diet containing 0.1% phosphorus. Group B rats were given 35 mmol/liter of phosphate in the drinking water. Group A rats were given 56 mmol/liter of NaCl in the drinking water. Group C rats were given 56 mmol/liter of NaCl in the drinking water plus 10 μg vitamin D_3 orally 7 h before removal of the diaphragm.

TABLE VII

Influence of Dietary Phosphorus and Vitamin D_3 on Growth and Muscle ATP Content

Vitamin D	Diet P	Diet Ca	Serum P(±SEM)	Serum Ca(±SEM)	Wt gain* (±SEM)	ATP (±SEM)
	%		*mg/100 ml*		*g*	*nmol/mg protein*
−	0.1	1.0	5.0±0.2	12.3±0.2	42±3	33.5±2.2
−	0.4	1.5	8.3±0.4	12.1±0.3	71±5	41.1±2.1
+	0.1	0.5	6.4±0.3	12.2±0.2	60±2	48.0±3.8
+	0.4	0.5	9.1±0.3	11.6±0.3	89±4	53.1±5.2

* Total average weight gain for the 16 animals in each group over the 3-wk study period.

with the concentration of serum phosphate but not the level of ATP in muscle.

To further establish the mechanism of vitamin D stimulation of muscle metabolism, the epitrochlear muscle of the forelimb of the rachitic rat was studied in tissue culture. Thus muscle is flat with a uniform thickness of approximately 500 μm and can easily be removed without damage to the body of the muscle. These properties facilitate the maintenance of the tissue in culture. Linear rates of [^{3}H]leucine incorporation into protein were observed for 5 h in culture. Addition of 25-OHD_3 at a concentration of 20 ng/ml, the approximate physiologic concentration of this metabolite of vitamin D in man and rat, stimulated the incorporation of [^{3}H]leucine into protein and increased the ATP content of the muscle (Table VIII). The parent vitamin, cholecalciferol, at 1,000 times this concentration was without effect. Finally, 1,25-$(OH)_2D_3$, at a concentration of 0.5 ng/ml and therefore at a concentration considerably greater than the estimated concentration of this biologically active metabolite in the circulation, also failed to stimulate protein synthesis and the accumulation of ATP in the muscle preparation. The biologic potency of the 1,25-$(OH)_2D_3$ used in these studies was established by demonstrating the ability of this preparation of 1,25-$(OH)_2D_3$ at a concentration of 50 pg/ml to stimulate the incorporation of ^{45}Ca into chick intestinal explants maintained in culture.

In contrast to the 4.1S sedimentation of ^{3}H on sucrose gradients when radioactive 25-OHD_3 is incubated with rat serum, we have previously demonstrated that muscle cytosol binding of [^{3}H]25-OHD_3 is ligand specific at 6.3S (30). The binding at 37°C is of high affinity ($K_{eq} = 5 \times 10^{-10}$ M) and 12 pmol of 25-OHD_3 is specifically bound by 1 mg of cytosol protein. Of the several steroids tested for their ability to compete for these sites, none have been found by either sucrose gradient analyses or by the coated-charcoal technique to bind significantly to the 25-OHD_3-binding protein in

muscle (Table IX). Since rat serum 25-OHD_3 concentrations are normally 2–4 × 10^{-8} M, the muscle is normally perfused by a concentration of 25-OHD_3 which is in excess of the concentration at which half of the muscle cytosol sites are occupied.

DISCUSSION

The influence of vitamin D on intestinal transport of calcium and phosphorus and the influence of the vitamin on the maintenance of normal skeletal growth have been well established (1-6, 8-10). Although muscle wasting and weakness are prominent features of nutritional rickets (16-19), a direct action of the vitamin on muscle metabolism and growth has heretofore not been appreciated. The generalized growth failure characteristic of vitamin D deficiency has previously been attributed to the failure of skeletal growth and the disordered calcium and phosphorus homeostasis attributed to the action of the vitamin on intestinal transport of these ions (4, 5). The observations reported in these investigations demonstrate that vitamin D repletion alters muscle metabolism, resulting in increased ATP concentrations and accelerated incorporation of an amino acid into muscle protein. This presumed increase in ATP and in protein synthesis could also be induced in skeletal muscle maintained in an organ culture system by the addition of physiologic concentrations of 25-OHD_3 to the medium. Thus, one must conclude that the vitamin is acting directly on muscle and that the increases in protein synthesis and skeletal muscle growth of the animal may, at least in part, be independent of the action of vitamin D on intestinal transport of calcium and phosphorus and the resulting increases in circulating levels of these ions.

TABLE VIII

*Influence of Vitamin D and Metabolites In Vitro on Epitrochlear Muscle**

Incubation time	n	Control‡	Treated‡	P value
h		*dpm [³H]leucine/mg protein*		
[³H]Leucine incorportion into protein				
25-OHD_3, 20 ng/ml				
1	8	115±8	105±5	NS
2.5	10	240±9	253±9	<0.1
5.0	32	476±4	522±5	<0.001
1,25$(OH)_2D_3$, 0.5 ng/ml				
5.0	12	490±12	465±15	NS
Vitamin D_3, 20 μg ml				
5.0	12	488±11	472±12	NS
		nmol ATP/mg protein		
Muscle ATP content				
25-OHD_3, 20 ng/ml				
2.5	13	45.9±1.7	53.2±1.7	<0.001
5.0	13	41.5±1.3	46.1±1.5	<0.025

* Epitrochlear muscles from rachitic rats were divided in pairs between control incubations and vitamin-supplemented incubations. The data was treated statistically by the application of Student's t test to the paired data.
‡ ±SEM.

TABLE IX

Potency of Various Steroids in the Displacement of [³H]25-OHD_3 from Muscle Cytosol Binding-Protein

Steroid	Displacement potency*
25-OHD_3	1.0
1α-25-$(OH)_2D_3$	0.035
5,6 trans 25-OHD_3	0.032
Vitamin D_3	<0.02
1α-Vitamin D_3	<0.01
Cholesterol	<0.01
25-OH cholesterol	<0.01
17β-Estradiol	<0.001

* In vitro incubations of steroids with muscle cytosol were carried out as described in Methods. The molar concentrations of unlabeled 25-OHD_3 resulting in 50% decrease of [³H]25-OHD_3 bound by cytosol protein was divided by the molar concentration of test steroid resulting in a 50% decrease of [³H]25-OHD_3 bound by cytosol protein to yield the displacement potency of the test steroids.

The mechanism of action of vitamin D or, more specifically, 25-OHD_3 on muscle has not been established. Recently the action of vitamin D on intestinal phosphate transport has received attention (7-10). DeLuca and co-workers (10) suggest that the stimulation of intestinal absorption of phosphate occurs simultaneously with, if not before, the effect of the vitamin on intestinal calcium absorption and therefore may be a primary action of the vitamin on the intestine. The increase in serum phosphorus of our animals 7 h after administration of the vitamin is consistent with these observations. However, the rise in serum phosphorus in our animals was preceded by a decrease in the serum phosphorus concentration of the vitamin-treated animals 4 h after administration of the vitamin. At this time, the rate of inorganic phosphate accumulation by muscle was accelerated by the vitamin. This increase in inorganic phosphate accumulation was the earliest alteration of muscle metabolism demonstrated and therefore may be the primary effect of 25-OHD_3 on muscle. The increase in ATP levels and protein synthesis would reasonably follow from this action of the vitamin on muscle if intracellular inorganic phosphate concentrations were indeed rate limiting in ATP synthesis. Intracellular ATP concentration was in fact found to be reduced in the vitamin D-deficient animal. In pair-fed vitamin-repleted animals, intracellular concentrations of

ATP were maintained even under conditions of phosphate deprivation. One may then postulate that ATP concentrations within the cell of the vitamin-deficient animal are reduced to levels which are rate limiting in the synthesis of protein. The importance of phosphate in growth is exemplified by the demonstration of excellent correlation between serum phosphate concentration and the rate of growth. However acute and brief increases in the phosphate concentration of serum or diaphragm incubation medium were without demonstrable effects on protein synthesis. Again these observations are consistent with the role of vitamin D in facilitating the entry of phosphate into the cell and restoring one or more intracellular ATP pools required for protein synthesis. Similarly one can relate the muscle weakness of vitamin D deficiency to inadequate muscle ATP concentration.

The apparent specificity of the muscle response to 25-OHD_3 and the failure of the muscle to respond to physiologic concentrations of 1,25-$(OH)_2D_3$ is of obvious interest. This biologic specificity for 25-OHD_3 is supported by the finding of a muscle cytosol protein fraction which specifically binds 25-OHD_3. The K_{eq} of this binding to the cytosol protein fraction (5×10^{-10} M) is significantly less than the concentration of 25-OHD_3 in the circulation ($2\text{-}4 \times 10^{-8}$ M) to which the muscle would be exposed. Furthermore 1,25-$(OH)_2D_3$ demonstrated a significantly lesser affinity for this protein, again consistent with the failure of physiologic concentrations of 1,25-$(OH)_2D_3$ to alter muscle protein synthetic processes. These observations do not exclude the presence of a specific receptor protein for 1,25-$(OH)_2D_3$ and a biologic response of muscle other than the parameters measured. Previously we reported that a wide variety of tissues demonstrated similar binding characteristics for 25-OHD_3 (31). The question is then raised as to whether this metabolite of vitamin D plays a similar role in the metabolism of these tissues and that therefore the primary action of this metabolite of vitamin D may be the generalized maintenance of cell growth and function.

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