Phosphate Accumulation by Muscle *in vitro* and the Influence of Vitamin D_3 Metabolites

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Phosphate accumulation by muscle *in vitro* and the effects of vitamin D_3 metabolites thereupon were studied in cultures of chick embryo skeletal muscle myoblasts and intact chick soleus muscles. A significant proportion of phosphate accumulation by the cells was Na^+-dependent, saturable with respect to phosphate, energy-dependent and inhibited by ouabain and arsenate, in agreement with the operation of a Na^+-phosphate cotransport system in the muscle cell plasma membrane as has been described for intestine and kidney. This was further supported by the demonstration of substrate-saturable phosphate uptake in sarcolemma vesicles isolated from chick skeletal muscle. Preincubation of myoblast and soleus muscle cultures with physiological levels of 25-hydroxy-vitamin D_3 resulted in a significant stimulation of phosphate accumulation by cultures. 1,25-dihydroxy-vitamin D_3 had no effects on the differentiated tissue whereas it markedly increased phosphate accumulation by embryonic muscle cells. In addition, it could be shown that 25-hydroxy-vitamin D_3 affects the Na^+-linked component of cell phosphate uptake through a mechanism dependent on *de novo* protein synthesis.

Introduction

Vitamin D_3 plays an essential role in the mechanisms of phosphorus homeostasis in higher animals. The sterol stimulates the absorption of phosphate by the intestine [1–5], the mobilization of phosphate from bone [6] and enhances the reabsorption of phosphate by the renal tubule [7–9]. Phosphate transport in intestine and kidney has been studied in detail using intact cell [10, 11] and brush border vesicle membrane [12–15] preparations. 1,25-Dihydroxy-vitamin D_3 ($1,25(OH)_2D_3$) has been shown to affect this process [5, 16–19]. An stimulation of phosphate fluxes in intestine [20, 21] *in vitro* and kidney [8] *in vivo* by 25-hydroxy-vitamin D_3 ($25OHD_3$) has also been reported.

There is now increasing evidence which indicates that muscle may be another tissue which participates in the maintenance of plasma phosphate [22]. Moreover, there are data which suggest that vitamin D regulates phosphate exchange between skeletal muscle and plasma and extracellular fluid. Birge and Haddad [23] have reported that $25OHD_3$ affects *in vivo* phosphate accumulation by rat muscle. In addition, an effect *in vitro* of the metabolite on muscle ATP content was shown by these authors. However, information on the characteristics of muscle phosphate accumulation and action of vitamin D_3 metabolites on this process is lacking. The present work studies these aspects using *in vitro* models of differentiated and embryonic chick skeletal muscle.

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Material and Methods

Material

Purified samples of chemically synthetized $1,25(OH)_2D_3$ and $24,25(OH)_2D_3$ were supplied by Hoffman-La Roche (New Jersey, USA). $25OHD_3$ was supplied by the Upjohn Co. (Michigan, USA). $[^{32}P]Na_2HPO_4$ was purchased from the Atomic Energy National Commission (Argentina). Bovine pancreas trypsin, type III-S, was obtained from Sigma Chemical Co. (St. Louis, USA). Other reagents used in this study were of analytical grade.

Animals

One-day-old chicks were fed a vitamin D-deficient diet containing 1.6% Ca and 1.0% P [3] during 4–6 weeks. The animals were maintained deprived of light. At the end of the experimental period, serum levels of $25OHD_3$ were less than 4 ng/ml. The concentration of Ca in serum was 40–50% lower than in chicks repleted of vitamin D. Chicken embryos employed in the preparation of myoblast cultures

were obtained by incubation of fertile eggs at 37.8 °C under 70% humidity during 12 days.

Cultures

Soleus muscles were dissected intact from both legs of vitamin D-deficient chicks or animals fed a normal diet. This operation was performed in the cold room. The tissue was immediately placed in ice-cold Krebs-Ringer solution [24] until incubation was initiated. Muscles were incubated in a chemically defined medium [23] supplemented with 10% vitamin D-deficient chick serum under 95% O_2 – 5% CO_2. To evaluate the effects of vitamin D_3 metabolites, paired samples of soleus muscles obtained from both legs of the same animal were used for each treatment and the corresponding control. The viability of the tissue was evidenced by the lack of significant changes in [^{3}H]leucine incorporation into protein and of [^{32}P]phosphate and ^{45}Ca uptake between 1 and 6 h of culture. To obtain myoblast cultures, carefully dissected breast muscles from 12-day-old chicken embryos were treated with 0.1% trypsin in Earl's balanced salt solution at 37 °C during 30 min [25]. 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 (8:1:1). The suspension was dispersed by pipetting and preplated on gelatin-coated 100 mm Petri dishes to remove contaminating fibroblasts. The unabsorbed cells were cultured at concentrations of 250,000 cells/ml in medium 8:1:1 at 37 °C for 24 h in a tissue culture incubator (Forma Scientific, USA) under a constant flow of 95% air – 5% CO_2 saturated with water. The medium was then changed with Eagle's Minimum Essential Medium containing 10% vitamin D-deficient chick serum, 2% chick embryo extract, and 1% antibiotic-antimycotic solution (8:1:0.2). 25OHD_3 levels in chick serum and chick embryo extracts used to supplement the cultures were not detectable (less than 4 ng/ml); 1,25$(OH)_2D_3$ levels were 13 pg/ml and less than 2 pg/ml, respectively. The metabolites were assayed as described elsewhere [26, 27]. Treatment with vitamin D_3 metabolites was carried out after the initial adaptation period of the cells in medium 8:1:1. Viability of the cells from control and treated preparations was ensured by measurements of trypan blue exclusion.

Treatment with vitamin D_3 metabolites

Vitamin D_3 metabolites were added to intact muscle and myoblast cultures dissolved in ethanol. Ethanol alone was added to control cultures. The concentration of solvent was not greater than 0.1%. Concentration and treatment intervals with metabolites are indicated for each experiment in the Results section.

Measurement of phosphate accumulation

Chick soleus muscles were incubated in Krebs-Henseleit solution (pH 7.4) at 37 °C under 95% O_2 – 5% CO_2. After an equilibration period of 60 min, ^{32}P–Na_2HPO_4 (0.05 μCi/ml) was added. ^{32}Pi uptake was measured for various time intervals. The tissue was then washed with cold Krebs-Henseleit solution and blotted dry on filter paper. The muscles were homogenized in 1 N NaOH. Aliquots were taken for determination of protein by the Lowry procedure [28] and of radioactivity using a Beckman liquid scintillation spectrometer. A similar procedure was employed to measure phosphate accumulation by myoblast cultures except that the Krebs-Henseleit solution used contained 0.2% glucose. To terminate the uptake reaction 1 ml samples were diluted with 30 ml of cold Krebs-Henseleit solution and centrifuged 1 min at 1,200 × g to collect the myoblasts. The cells were homogenized in 0.1% SDS. Isolation and determination of phosphate uptake by sarcolemma vesicles were carried out as previously described [29, 30].

Results

Characteristics of muscle phosphate accumulation in vitro

Fig. 1 shows the time courses of phosphate accumulation by cultures of intact chick soleus muscle (A) and chick embryo myoblasts (B) at 4 °C and 37 °C. At 4 °C, the uptake of ^{32}P-phosphate by both type of preparations was considerably lower than at 37 °C. Maximum accumulation at 4 °C was already reached within the shortest time measurements (2–5 min) whereas at 37 °C it increased up to 30 min in myoblast cultures and was still augmenting after 60 min in soleus muscle preparations.

Preincubation of myoblast cultures with 2,4-dinitrophenol to directly block the muscle cell ATP supply caused a marked reduction of phosphate accumu-

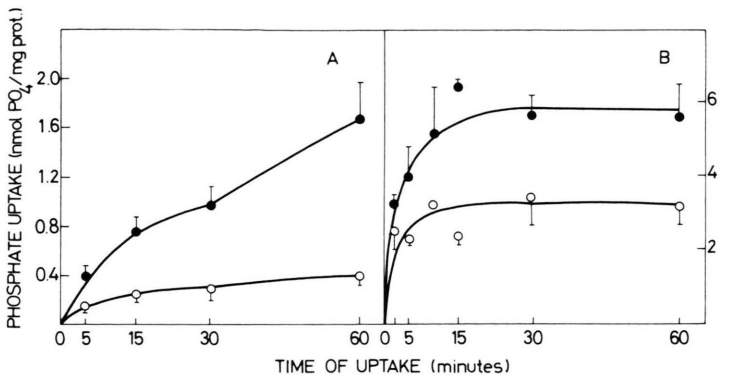

Fig. 1. Influence of temperature on the time course of phosphate uptake by chick soleus muscle (A) and chick embryo myoblast (B) cultures. Intact soleus muscles and myoblast suspensions were obtained as described in the text. Myoblasts were cultured 24 h in medium 8:1:1 followed by 8 h in medium 8:1:0.2 prior to uptake measurements. Soleus muscles were preincubated 3 h in medium [23] supplemented with 10% chick serum. The uptake of [^{32}P]phosphate was measured in Krebs-Henseleit solution containing 1 mM Na_2HPO_4 and 140 mM Na^+ at 37 °C and 4 °C under steady-state conditions for various times. Values represent means ± S.D.; n = 5 and 4 for soleus muscle and myoblast cultures, respectively.

lation. A similar decrease was observed in the absence of Na^+ (Fig. 2). These observations are in agreement with the requirement of a Na^+ gradient generated by the $Na^+ + K^+ -$ ATPase to support phosphate accumulation. In addition, arsenate (5 mM) was found to inhibit the uptake of phosphate by the cells in the same proportion as the omission of Na^+ from the medium (Fig. 2).

To characterize the Na^+-dependence of phosphate accumulation by myoblast cultures, the uptake of [^{32}P]phosphate by the cells was measured at various phosphate concentrations in the presence and absence of Na^+ (replaced by an equimolar concentration of K^+). The results are presented in Fig. 3. An appreciable inhibition of the accumulation of the anion was seen in the absence of Na^+. The extent of inhibition varied with the different external concentrations of phosphate employed. The data show, in addition, that the sodium-independent phosphate accumulation by the cells increases linearly with substrate concentration. At a concentration of 1 mM, which approaches the cell levels of the anion, phosphate uptake was reduced by 65%. A similar inhibition of phosphate uptake by cultured soleus muscle

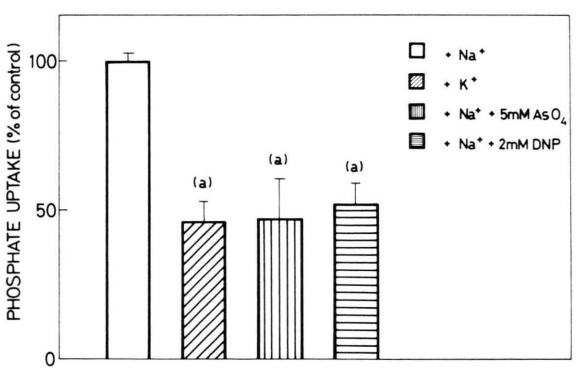

Fig. 2. Effect of inhibitors on phosphate uptake by myoblast cultures. Phosphate uptake was measured in the presence of 140 mM Na^+ ($+Na^+$) or 140 mM K^+ ($+K^+$) in the medium. 2 mM 2,4-dinitrophenol (DNP) or 5 mM arsenate were added 60 min prior to the addition of [^{32}P]Na_2HPO_4 (1 mM). The uptake period was 60 min. The values represent means ± S.D.; n = 4.

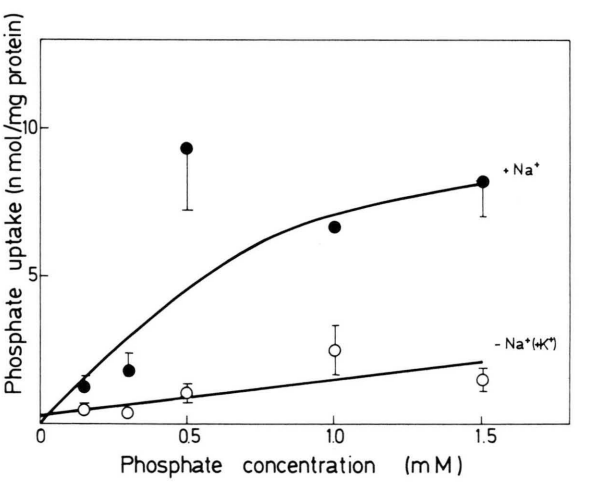

Fig. 3. Effect of external sodium on the uptake of phosphate by myoblast cultures at various phosphate concentrations. Preincubation of chick embryonic muscle cells was carried out as in Fig. 1. [^{32}P]phosphate uptake was measured using a 60 min incubation interval and a phosphate concentration range of 0.15–1.5 mM. (●----●), 140 mM Na^+; (○----○), 140 mM K^+. Values represent means ± S.D.; n = 4.

was observed at 1 mM phosphate when the medium Na^+ was replaced by K^+ (8.55 ± 1.50 *vs* 4.16 ± 0.88 nmol/mg prot for $+Na^+$ and $-Na^+$ preparations, respectively; $p < 0.0005$).

In a separate experiment, ouabain affected phosphate accumulation by myoblast cultures to the same extent as that caused by the replacement of Na^+ by K^+. This excludes possible toxic effects of high concentrations of K^+ in the preceding experiments, as the inhibitor of the sodium pump was added to the medium in the presence of 140 mM Na^+ and low levels of K^+. Ouabain exerted its effect at a concentration of 2 mM which is similar to that required to inhibit Na^+-dependent phosphate accumulation by chick intestine *in vitro* [20].

Calculation of kinetic parameters from saturation plots of phosphate uptake by cultured chick embryo myoblasts (Fig. 4A) and purified chick skeletal muscle sarcolemma vesicles (Fig. 4B) by the double-reciprocal plot corresponding to Lineweaver-Burk (insets) revealed a close agreement between the K_m values of both preparations (0.34 mmol/L and 0.24 mmol/L for cells and isolated membranes, respectively). However, V_{max} was approximately one of order of magnitude lower in vesicles than in myoblasts (0.034 nmol/mg prot $\times$ min^{-1} *vs* 0.59 nmol/mg prot $\times$ min^{-1}, respectively).

At a concentration of 0.3 mM phosphate in the external medium, which is near the K_m value of the phosphate transporter of the muscle plasma membrane, highest inhibition of phosphate accumulation due to replacement of K^+ for Na^+ was observed in cultured myoblasts (74%; Fig. 4) and soleus muscles (64%; 3.25 ± 0.53 *vs* 1.18 ± 0.13 nmol prot for $+Na^+$ and $-Na^+$ preparations, respectively; $p < 0.0005$).

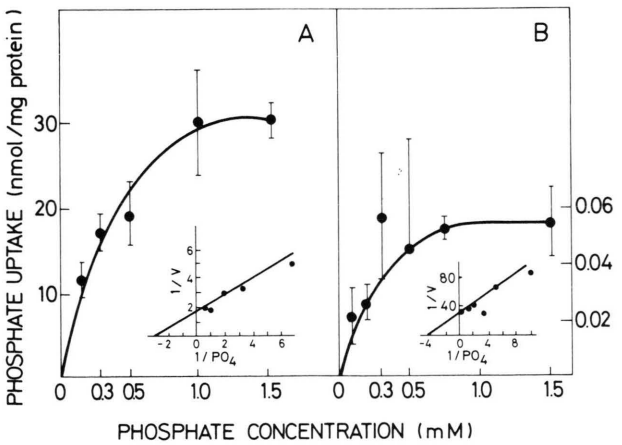

Fig. 4. Influence of phosphate concentration on the accumulation of phosphate by cultures of chick embryo skeletal muscle myoblasts (A) and sarcolemma vesicles (B). Experimental conditions for cultured preparations were similar to those described in Fig. 1. Sarcolemma vesicles were isolated from chick leg muscles by sucrose density gradient ultracentrifugation as indicated previously [29]. Phosphate uptake was measured at the various phosphate concentrations using 60 min and 15 min incubation intervals for cultures and vesicles, respectively. Insets: Lineweaver-Burk transformation of the data; 1/V and $1/PO_4$ are expressed in $(nmol/mg\ prot \times min)^{-1}$ and $(nmol/L)^{-1}$, respectively.

Action of vitamin D_3 metabolites on muscle phosphate accumulation in vitro

Previous *in vitro* and *in vivo* studies suggested an action of vitamin D on phosphate fluxes in skeletal muscle [23, 29]. Experiments were carried out in this study to identify vitamin D metabolites active on muscle phosphate accumulation *in vitro* and to obtain information about their site of action in the muscle cell. In Table I, a comparison of the biological activity of $25OHD_3$, $1,25(OH)_2D_3$ and $24,25(OH)_2D_3$ on phosphate accumulation by cultures of soleus muscle from vitamin D-deficient chicks is shown. The preparations were preincubated with each metabolite for 5 h and [^{32}P]phosphate uptake was then measured during a 60 min interval at a phosphate concentration of 1 mM. Physiological concentrations of $25OHD_3$ significantly increased phosphate accumulation by the intact muscle cultures. $1,25(OH)_2D_3$ in the concentration range of 0.05 to 25 ng/ml (500-fold higher than the physiological level) had no effects. $24,25(OH)_2D_3$ was also inactive when employed at a concentration two-fold higher than that normally found in chick serum [31]. In a separate experiment, it could be shown that the effect of $25OHD_3$ (40 ng/ml) on soleus muscle phosphate uptake was already evident after 1 h of treatment (13% increase over controls, $p < 0.10$), maximum responses being achieved at 3 h (55% increase over control, $p < 0.0125$). Effects of $1,25(OH)_2D_3$ on intact muscle phosphate uptake at treatment intervals shorter than 5 h were not detected.

$25OHD_3$ also promoted phosphate accumulation in embryonic muscle cells. Fig. 5A shows the time course of the $25OHD_3$ phosphate uptake responses by myoblast cultures at two different sterol concentrations within the physiological range. An increase in cell [^{32}P]phosphate uptake was detected after 4 h

Table I. Effects of vitamin D_3 metabolites on phosphate accumulation by chick soleus muscle cultures[a].

Treatment	Concentration [ng/ml]	Phosphate uptake [% of control]	
Control	–	100	–
25-hydroxy-vitamin D_3	25	111 ± 3 (3)	$p < 0.025$
	40	120 ± 7 (5)	$p < 0.025$
1,25-dihydroxy-vitamin D_3	0.05	105 ± 7 (4)	$p < 0.10$
	0.10	103 ± 10 (5)	$p < 0.25$
	0.50	102 ± 2 (4)	N.S.
	25	105 ± 6 (4)	$p < 0.10$
24,25-dihydroxy-vitamin D_3	4.6	97 ± 13 (5)	$p < 0.25$

[a] Soleus muscles dissected intact from 5-week-old vitamin D-deficient chicks were cultured for 5 h in medium [23] supplemented with 10% vitamin D-deficient chick serum in the presence of various vitamin D_3 metabolites at the indicated concentrations. Paired muscle samples were used for each treatment and its control as indicated in the text. The uptake of [^{32}P]-Na_2HPO_4 (1 mM) was measured at 37 °C during 60 min. Values represent means ± S.D. (n).

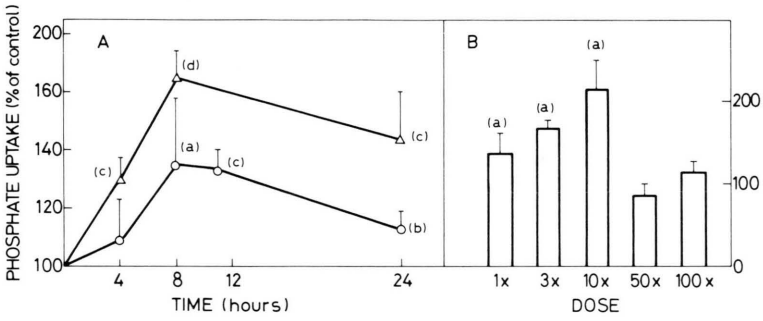

Fig. 5. Effects of 25-hydroxy-vitamin D_3 on phosphate accumulation by myoblast cultures. Myoblasts were cultured 24 h in medium 8:1:1. The cells were transferred to medium 8:1:0.2 and treated with $25OHD_3$ at the indicated concentrations and time intervals. Phosphate uptake was measured after the addition of [^{32}P]-Na_2PO_4 (1 mM) during 60 min. (A) Time-course of $25OHD_3$ effect on phosphate accumulation; (○----○), 20 ng 25 OHD_3 ml; (△----△), 40 ng $25OHD_3$/ml. (B) Dose-response relationship of $25OHD_3$ effect on phosphate accumulation; x = 20 ng/ml. (a) $p < 0.025$, (b) $p < 0.005$, (c) $p < 0.0005$; $n = 4$.

of treatment with the metabolite ($p < 0.0025$, for 40 ng/ml of $25OHD_3$). Maximal effects were observed at 8 h. The dose-dependent response of myoblast cultures to $25OHD_3$ increased up to 10-fold the physiological levels of the metabolite. At higher levels, however, the sterol exerted an inhibitory effect (Fig. 5B).

Differently as for soleus muscle, treatment of myoblast cultures with $1,25(OH)_2D_3$ (0.10 ng/ml) resulted in a significant stimulation of phosphate accumulation by the cells. The metabolite elicited maximum responses after 4 h treatment of cultures (207% increase over controls, $p < 0.0025$), its effect decreasing sharply at 8 h and 24 h.

As shown in Fig. 6, replacement of K^+ for Na^+ markedly reduced the stimulatory effect of $25OHD_3$ on phosphate accumulation by myoblasts, indicating that the sterol affects mainly the Na^+-dependent component of the cell uptake system.

The effect of cycloheximide, a ribosomal protein synthesis inhibitor, on $25OHD_3$-dependent myoblast phosphate accumulation was evaluated. The addition of the antibiotic abolished the response of increased phosphate uptake by the cultures exposed to the metabolite without significantly affecting control cultures (Fig. 7).

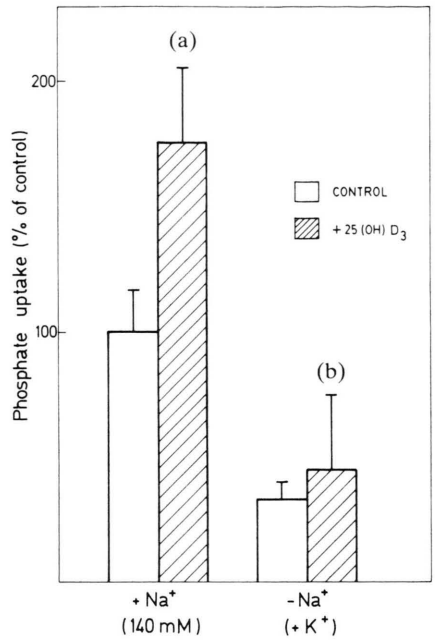

Fig. 6. Effect of $25OHD_3$ on myoblast phosphate accumulation in the presence and absence of external Na^+. Chick embryo skeletal muscle myoblast cultures were treated 8 h with $25OHD_3$ (25 ng/ml). Phosphate uptake was measured in the presence of 140 mM Na^+ ($+Na^+$) or 140 mM K^+ ($-Na^+$) using a 60 min incubation interval and a phosphate concentration of 1 mM. Values represent means ± S.D.; $n = 4$. (a) $p < 0.005$, (b) $p < 0.05$.

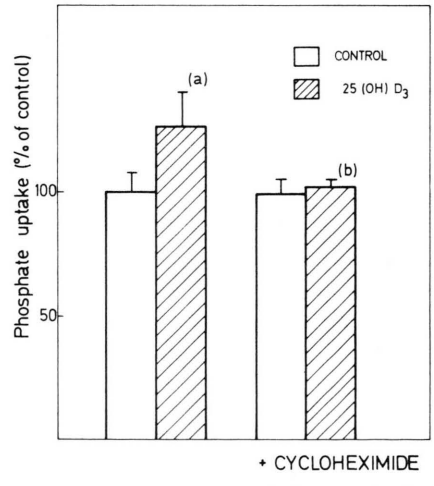

Fig. 7. Effect of cycloheximide on the 25-hydroxy-vitamin D_3-mediated increase in phosphate accumulation by cultured myoblasts. Cultures were treated 8 h with $25OHD_3$. Cycloheximide (50 μM) was added 1 h before addition of the metabolite. After treatment with the sterol, [^{32}P]-Na_2HPO_4 (1 mM) was added and phosphate uptake was measured during 60 min. Values represent means ± S.D.; $n = 4$. (a) $p < 0.025$, (b) $p < 0.25$.

Discussion

The data presented in this report firstly describe, to our knowledge, the properties of phosphate accumulation by muscle *in vitro*. Although, under the conditions employed this accumulation does not exclude the contribution of cell phosphate organification, *e.g.* phosphate incorporation into cell glycolytic intermediates, phospholipids, phosphoproteins and nucleic acids, the results are consistent with the operation in embryonic and differentiated chick skeletal muscle of a phosphate transport system whose characteristics resemble those reported for phosphate transport in intestine and kidney [10–16, 20]. The uptake of [^{32}P]phosphate by myoblasts was saturable with respect to phosphate, indicating the participation of a carrier-mediated process. This mechanism is probably located in the plasma membrane of the cells as substrate-saturable phosphate uptake could also be demonstrated in sarcolemma vesicles isolated from chick muscle (Fig. 4A and 4B). Moreover, the apparent K_m value for phosphate uptake by vesicles was similar to that estimated for cultured muscle cells and is also in reasonable agreement with values reported for kidney cells [11] and intestinal and kidney plasma membranes [12, 15]. However, the V_{max} value for phosphate uptake by sarcolemma vesicles was significantly lower than that determined in myoblasts. This may be associated to a decrease in the number of active phosphate transport sites of membrane preparations due to extensive manipulation during their isolation. Alternatively, it may reflect intrinsic differences between differentiated and embryonic muscle. Lack of a procedure adequate for the isolation of sarcolemmal membranes in sufficient quantity and purity from myoblast cultures precluded the study of this aspect.

Additional data supporting the localization of a phosphate transport system in the plasma membrane of muscle cells were obtained. A significant proportion of the phosphate taken up by both myoblast (Fig. 3) and soleus muscle cultures was Na^+-dependent. Moreover, the addition of ouabain, a specific inhibitor of the $Na^+ + K^+$-ATPase, diminished phosphate uptake by myoblast cultures to the same extent as the substitution of Na^+ by K^+ in the medium. The Na^+ pump is responsible for the maintenance of the Na^+ gradient across the plasma membrane. Using intact cell and plasma membranes isolated from intestine and kidney ample evidence has been obtained

which indicates that this gradient provides the energy for the accumulation of phosphate through a secondary active transport mechanism which involves the cotransport of phosphate and Na^+ [10–15]. The inhibition of phosphate uptake observed in the presence of 2,4-dinitrophenol (Fig. 2) or by lowering the temperature (Fig. 1) is consistent with the energy requirement of the pump, although, in the case of the metabolic inhibitor, the contribution of decreased phosphate organification cannot be discarded. In addition, it could be shown that the Na^+-dependent uptake of phosphate by myoblasts is specific as it was totally inhibited by arsenate, a structural analog of phosphate (Fig. 2).

The present work confirms and further expands previous investigations which had suggested that vitamin D_3 metabolites are involved in the regulation of phosphate fluxes across muscle membranes. $25OHD_3$, in physiological concentrations, stimulated the accumulation of phosphate by vitamin D-deficient chick soleus muscle cultures. The presence of $1,25(OH)_2D_3$, even in large excess, or $24,25(OH)_2D_3$ in the culture medium caused no changes in phosphate accumulation by the tissue (Table I). These results are in agreement with previous work of Birge and Haddad [22, 23] who reported that administration of $25OHD_3$ to vitamin D-deficient rats caused a fall in serum P and an increase in *in vitro* [^{32}P]phosphate accumulation by muscle followed by an increase in muscle ATP and protein synthesis. Nephrectomy did not obliterate these responses indicating that further conversion of the sterol to $1,25(OH)_2D_3$ was not required.

An effect of $1,25(OH)_2D_3$ on muscle Ca transport has been clearly demonstrated [32–34]. The lack of effects of $1,25(OH)_2D_3$ on phosphate uptake by differentiated skeletal muscle reveals that the metabolite influences muscle mineral metabolism differently as in intestine, kidney and bone, where it stimulates both phosphate and Ca transport.

However, $25OHD_3$ may play a general role in cell phosphate transport. There is evidence showing an action of the metabolite on phosphate fluxes in tissues other than muscle. *In vitro* treatment of chick ileum explants with physiological levels of $25OHD_3$ resulted in an stimulation of phosphate accumulation by the explants [20]. Birge and Avioli [21] have shown that the sterol at a concentration of 100 ng/ml increases phosphate uptake by intestinal epithelial cells derived from vitamin D-deficient chicks. In addition, *in vivo* evidence has suggested an effect of $25OHD_3$ on phosphate transport in the kidney by a mechanism independent of its conversion into $1,25(OH)_2D_3$ [8].

Embryonic muscle cells also showed increased phosphate accumulation upon treatment with $25OHD_3$. The response to the metabolite was dose-dependent in the range 20–200 ng/ml. The changes caused by the sterol in cell cultures were evident after longer treatment intervals than in soleus muscle cultures (Fig. 5). Treatment intervals required to induce maximum responses in phosphate uptake by both preparations were similar to those at which the metabolite has been previously shown to cause maximal stimulation in Ca uptake [34]. On the basis of Ca efflux kinetic data it has been suggested that the predominant effect of $25OHD_3$ on muscle Ca metabolism is to promote mitochondrial Ca uptake [33, 34]. The possibility is then raised that this change is the consequence of the stimulation of muscle phosphate uptake caused by the sterol, as the anion potentiates the influx of Ca into mitochondria.

A marked stimulation of phosphate uptake by cultured myoblasts could be observed upon the addition of $1,25(OH)_2D_3$. The present studies were not addressed to further characterize these effects. However, it may be speculated that $1,25(OH)_2D_3$ acted through a modification of the metabolic state of the cells as it has been shown that the metabolite promotes the differentiation of myoblasts *in vitro* [35].

The data obtained also suggest that $25OHD_3$ affects the sodium-linked entry of phosphate into the muscle cells. A less pronounced increment of [^{32}P]phosphate uptake was observed in myoblasts pretreated with the metabolite when the uptake measurements were carried out in a sodium-free medium (Fig. 6). This result may be connected to previous studies *in vivo* which showed an effect of vitamin D_3 on phosphate uptake by skeletal muscle at the plasma membrane level. In these investigations it could be observed that administration of the sterol to vitamin D-deficient chicks resulted in a marked stimulation of phosphate transport by subsequently isolated sarcolemma vesicles. The effect of the sterol on phosphate uptake into the vesicles was dependent on the presence of an external Na^+ gradient [29]. The data are also in general agreement with the fact that in intestine and kidney vitamin D_3 metabolites affect the sodium-dependent phosphate

transport mechanism located in the plasma membrane [18, 36, 37].

Cycloheximide suppressed the stimulation of phosphate accumulation induced by $25OHD_3$ in cultured myoblasts (Fig. 7), indicating that the effects of the metabolite are mediated by *de novo* protein synthesis. This may explain the delay in observing a significant increase in myoblast phosphate uptake after treating the cultures with $25OHD_3$ (Fig. 5). Vitamin D_3 or derived metabolites have also been shown to stimulate phosphate uptake in intestine *in vivo* and *in vitro* [16, 38] and in cultured kidney cells [39] by a mechanism involving the synthesis of new protein(s). However, until the present time no information is available on the identity of the protein(s) induced by the secosterols which directly or indirectly affect phosphate uptake.

Acknowledgements

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