Phosphate Accumulation by Muscle in vitro and the Influence of Vitamin D₃ Metabolites

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Phosphate accumulation by muscle in vitro and the effects of vitamin D₃ 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 D₃ resulted in a significant stimulation of phosphate accumulation by cultures. 1,25-dihydroxy-vitamin D₃ 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 D₃ affects the Na⁺-linked component of cell phosphate uptake through a mechanism dependent on de novo protein synthesis.

Introduction

Vitamin D₃ 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 D₃ (1,25(OH)₂D₃) 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 D₃ (25(OH)D₃) 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 25(OH)D₃ 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 D₃ metabolites on this process is lacking. The present work studies these aspects using in vitro models of differentiated and embryonic chick skeletal muscle.

Material and Methods

Material

Purified samples of chemically synthesized 1,25(OH)₂D₃ and 24,25(OH)₂D₃ were supplied by Hoffman-La Roche (New Jersey, USA). 25(OH)D₃ was supplied by the Upjohn Co. (Michigan, USA). [³²P]Na₂HPO₄ 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 25(OH)D₃ 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

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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% O₂—5% CO₂. To evaluate the effects of vitamin D₃ 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 [³H]leucine incorporation into protein and of [³²P]phosphate and ⁴⁵Ca 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% CO₂ 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). 25OHD₃ levels in chick serum and chick embryo extracts used to supplement the cultures were not detectable (less than 4 ng/ml); 1.25(OH)₂D₃ levels were 13 pg/ml and less than 2 pg/ml, respectively. The metabolites were assayed as described elsewhere [26, 27]. Treatment with vitamin D₃ 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.

**Measurement of phosphate accumulation**

Chick soleus muscles were incubated in Krebs-Henseleit solution (pH 7.4) at 37 °C under 95% O₂—5% CO₂. After an equilibration period of 60 min, ³²P—Na₂HPO₄ (0.05 μCi/ml) was added. ³²P 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 sarcotroma 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 ³²P-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$_2$HPO$_4$ 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.

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$_2$HPO$_4$ (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 *Kₘ* values of both preparations (0.34 mmol/L and 0.24 mmol/L for cells and isolated membranes, respectively). However, *Vₘₐₓ* was approximately one of order of magnitude lower in vesicles than in myoblasts (0.034 nmol/mg prot x min⁻¹ vs 0.59 nmol/mg prot x min⁻¹, respectively).

At a concentration of 0.3 mM phosphate in the external medium, which is near the *Kₘ* 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*).

**Action of vitamin D₃ 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 25(OH)D₃, 1,25(OH)₂D₃ and 24,25(OH)₂D₃ 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 [³²P]phosphate uptake was then measured during a 60 min interval at a phosphate concentration of 1 mM. Physiological concentrations of 25(OH)D₃ significantly increased phosphate accumulation by the intact muscle cultures. 1,25(OH)₂D₃ in the concentration range of 0.05 to 25 ng/ml (500-fold higher than the physiological level) had no effects. 24,25(OH)₂D₃ 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 25(OH)D₃ (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)₂D₃ on intact muscle phosphate uptake at treatment intervals shorter than 5 h were not detected.

25(OH)D₃ also promoted phosphate accumulation in embryonic muscle cells. Fig. 5A shows the time course of the 25(OH)D₃ phosphate uptake responses by myoblast cultures at two different sterol concentrations within the physiological range. An increase in cell [³²P]phosphate uptake was detected after 4 h
Table I. Effects of vitamin D$_3$ metabolites on phosphate accumulation by chick soleus muscle cultures.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Concentration [ng/ml]</th>
<th>Phosphate uptake [% of control]</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>-</td>
<td>100</td>
</tr>
<tr>
<td>25-hydroxy-vitamin D$_3$</td>
<td>25</td>
<td>111 ± 3 (3) p &lt; 0.025</td>
</tr>
<tr>
<td></td>
<td>40</td>
<td>120 ± 7 (5) p &lt; 0.025</td>
</tr>
<tr>
<td>1,25-dihydroxy-vitamin D$_3$</td>
<td>0.05</td>
<td>105 ± 7 (4) p &lt; 0.10</td>
</tr>
<tr>
<td></td>
<td>0.10</td>
<td>103 ± 10 (5) p &lt; 0.25</td>
</tr>
<tr>
<td></td>
<td>0.50</td>
<td>102 ± 2 (4) N.S.</td>
</tr>
<tr>
<td>24,25-dihydroxy-vitamin D$_3$</td>
<td>25</td>
<td>105 ± 6 (4) p &lt; 0.10</td>
</tr>
<tr>
<td></td>
<td>4.6</td>
<td>97 ± 13 (5) p &lt; 0.25</td>
</tr>
</tbody>
</table>

* 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 [32P]-Na$_2$HPO$_4$ (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 [32P]-Na$_2$HPO$_4$ (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.

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 25OH D₃ on myoblast phosphate accumulation in the presence and absence of external Na⁺. Chick embryo skeletal muscle myoblast cultures were treated 8 h with 25OH D₃ (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 D₃-mediated increase in phosphate accumulation by cultured myoblasts. Cultures were treated 8 h with 25OH D₃. Cycloheximide (50 μM) was added 1 h before addition of the metabolite. After treatment with the sterol, [³²P]-Na₂HPO₄ (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 describes, 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 [³²P]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 Kₘ 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 Vₘₐₓ 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 D₃ metabolites are involved in the regulation of phosphate fluxes across muscle membranes. 25OHD₃, in physiological concentrations, stimulated the accumulation of phosphate by vitamin D-deficient chick soleus muscle cultures. The presence of 1,25(OH)₂D₃, even in large excess, or 24,25(OH)₂D₃ 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 25OHD₃ to vitamin D-deficient rats caused a fall in serum P and an increase in in vitro [³²P]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)₂D₃ was not required.

An effect of 1,25(OH)₂D₃ on muscle Ca transport has been clearly demonstrated [32–34]. The lack of effects of 1,25(OH)₂D₃ 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, 25OHD₃ 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 25OHD₃ 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 25OHD₃ on phosphate transport in the kidney by a mechanism independent of its conversion into 1,25(OH)₂D₃ [8].

Embryonic muscle cells also showed increased phosphate accumulation upon treatment with 25OHD₃. 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 25OHD₃ 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)₂D₃. The present studies were not addressed to further characterize these effects. However, it may be speculated that 1,25(OH)₂D₃ 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 25OHD₃ affects the sodium-linked entry of phosphate into the muscle cells. A less pronounced increment of [³²P]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 D₃ 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 D₃ 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 25OHD3 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 25OHD3 (Fig. 5). Vitamin D3 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.


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