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PHOSPHATE TRANSPORT BY EMBRYONIC CHICK DUODENUM

STIMULATION BY VITAMIN D_3 *

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Summary

Embryonic chick duodenum maintained in organ culture is a well-suited model for the study of vitamin D effects on inorganic phosphate (P_i) absorption. The system is sensitive to as little as 6.5 nM vitamin D_3 (0.1 I.U./ml culture medium). Increased phosphate absorption is observed after 6—12 h of culture. Maximal response (133% of vitamin D-efficient control) is achieved at 24 h.

Phosphate uptake by embryonic chick duodenum involves a saturable and a non-saturable component. The former displays characteristics of an active sodium-dependent transport mechanism and is also sensitive to vitamin D_3. Presence of the sterol in culture medium raises the maximal velocity from 55 to 75 nmol P_i/min per g tissue. K_m remains unchanged (0.5 mM P_i).

Duodena cultured in presence of inhibitors of protein synthesis (actinomycin D, α-amanitin and cycloheximide) display reduced rates of phosphate absorption. This treatment also prevents vitamin D_3 action on phosphate transport. It is concluded that the sterol affects phosphate transport by modulation of synthesis of proteins which are functional in the P_i absorptive process.

Introduction

Embryonic chick duodenum maintained in organ culture has proven useful for studies of vitamin D effects at the cellular level [1—4]. Before hatching, the small intestine resembles vitamin D-deficient tissue. Although vitamin D_3 contained in yolk [5] would be a potential source for 1,25-dihydroxyvitamin D_3,

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biotransformation into this biologically active sterol [6] and uptake into intestine occurs, if at all, only in minute amounts insufficient for evoking any biological response [7,8]. However, duodenum from 20-day-old embryos cultured in presence of vitamin D_3 or of its biologically more active metabolites responds by de novo synthesis of the vitamin D-dependent calcium-binding protein [9] and by increased uptake of calcium and inorganic phosphate (P_i) [1–4,10]. Since vitamin D_3 apparently is not further metabolized in this tissue [2], the effect of the compound itself on the calcium and phosphate absorptive mechanism can be directly assessed. Of these, calcium absorption was studied in great detail [1–4]. Hitherto, little attention was paid to the phosphate transport system.

It is now well established that phosphate transport by the small intestine occurs separately from the calcium absorptive process and that vitamin D affects both systems independently [11–13]. Therefore, investigations described in this paper were aimed at characterization of the phosphate absorptive mechanism of embryonic chick duodenum and the effect of vitamin D_3 thereon to obtain more insight into the various cellular actions of the vitamin.

Methods

Embryonated eggs from a local poultry farm were incubated for 20 days at 37° C and 60% relative humidity. Duodena were cultured exactly as described by Corradino [1]. Briefly, the slit-open guts were placed mucosal side-up on rectangular grids in sterile Petri dishes. Usually, four duodena were cultured on a single grid. The serosal side was in contact with the culture medium (McCoy's 5A modified medium, 40 ml per dish).

Vitamin D_3 and actinomycin D, respectively, were dissolved in ethanol. Final ethanol concentration in culture medium did not exceed 0.1%. Cycloheximide and α-amanitin were dissolved in isotonic saline. No more than 1.0 ml was added to each Petri dish. Only vehicle was added to control groups.

Petri dishes were kept in an incubator (National Appliance Co., U.S.A.) for various time periods at 37° C in 95/5% air/CO_2 atmosphere.

Established methods were used for determination of tissue uptake of radiophosphate and radiocalcium [1]. Cultured duodena were transferred into 25 ml Erlenmeyer vials containing 3.0 ml bathing solution. The tightly sealed vials were incubated for 45 min (if not otherwise stated) at 37° C. Thereafter, the guts were quickly transferred on filter paper mounted in a Millipore 3025 Sampling Manifold and were rinsed extensively with ice-cold saline under continuous suction. Guts were then carefully blotted on tissue paper, and weighed. For determination of radioactivity, tissue samples were digested in 1.0 ml of a tissue solubilizer (TS-2, Koch-Light Laboratories, U.K.) by overnight shaking at 50° C. After addition of 10 ml of a toluene/PPO/POPOP scintillation cocktail radioactivity was determined in a Beckman LS 230 liquid scintillation counter equipped with external standardization.

Bathing solution was basically Krebs-Henseleit bicarbonate buffer (1.2 mM P_i/2.5 mM Ca, pH 7.4) [14]. When necessary, P_i concentrations were adjusted by changes in the amount of KH_2PO_4 added. K^+ concentration was kept constant by appropriate addition or omission of KCl. In experiments where Na^+ was

replaced, this was done by isosmolar substitution with choline chloride and choline hydrogencarbonate.

When calcium uptake was compared with phosphate accumulation, a low sodium buffer containing mannitol was used [15] which had proven most suitable for measurement of calcium uptake [1]. This buffer contained 1.2 mM P_i and 0.25 mM Ca^{2+}. In control experiments mannitol was replaced isotonically by NaCl.

All buffers were gassed with O_2/CO_2 (95/5%) before use to adjust pH to 7.4. The vials were flushed with the same gas mixture for 30 s before incubation and then stoppered tightly. This was found sufficient to keep pH constant and, consequently, to prevent precipitation of calcium phosphate even at the highest P_i concentration used (2.4 mM).

Radioisotopes ($^{32}P_i$ obtained as $H_3{}^{32}PO_4$ in 0.02 M HCl from the Radiochemical Centre, Amersham, and $^{45}CaCl_2$ from the same source) were added to the bathing solution at a concentration of 0.5 μCi/ml.

Crystalline vitamin D_3 was purchased from Merck, G.F.R. Actinomycin D, cycloheximide and carbonyl cyanide *m*-chlorophenyl hydrazone were from Sigma (U.S.A.). α-Amanitin was a gift from Dr. Peter Swetly, Ernst Boehringer-Institut für Arzneimittelforschung, Vienna.

Student's *t*-test was used for statistical evaluations. Regression lines were calculated with a built-in program of a Compucorp 342 desk calculator.

Results

Kinetics of phosphate uptake by embryonic chick duodenum. Guts cultured in the presence of 26 μM vitamin D_3 (+D group) for 48 h accumulate significantly more P_i from the bathing solution (1.2 mM P_i) than their vitamin D_3-free controls (—D group). Fig. 1 shows that tissue accumulation of radiophos-

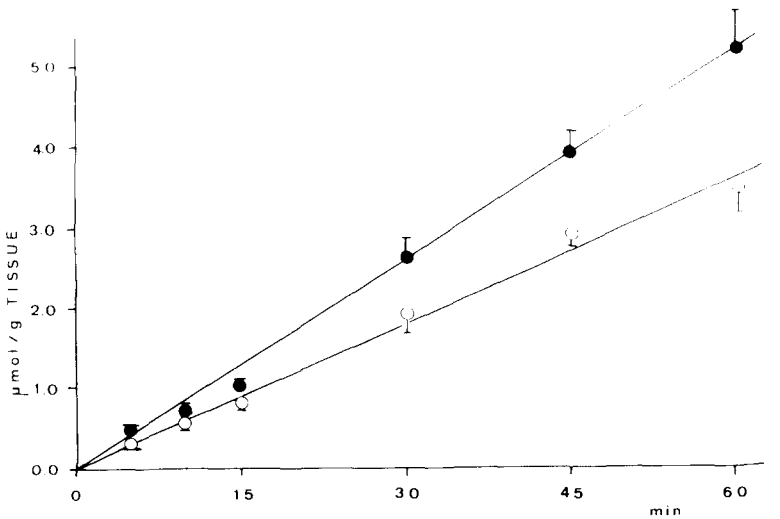

Fig. 1. Phosphate accumulation by embryonic chick duodenum as function of incubation time. Data are means (4 duodena per group) ± S.E. (vertical bars). ●, 26 μM vitamin D_3 in culture medium; ○, vitamin D_3-free culture medium. Culture period 48 h.

phate in both +D and —D duodena is a linear function ($r > 0.99$) of incubation time for periods up to 60 min. Calculated average transport rates are 80 ± 4 and 61 ± 3 nmol/min per g tissue, respectively. Thus, the vitamin D_3-related increment of phosphate absorption amounts to 31% of —D controls ($P < 0.001$).

Involvement of a carrier-mediated transfer in P_i uptake by embryonic chick duodenum can be implicated when absorption rates are measured at different extracellular P_i concentrations (Fig. 2). In both +D and —D duodena, P_i uptake becomes saturated at higher concentrations in the bathing solution. The shape of the curves does not resemble typical Michaelis-Menten kinetics but can be converted into these if consideration is given to a non-saturable, vitamin D-independent diffusional step of P_i entry into the tissue (see below). By subtraction of a linear term the obtained data can be fitted into rectangular hyperbolas. From a linearized plot of the saturable components (see insert in Fig. 2) it can be calculated that the presence of vitamin D_3 in the culture medium (26 μM) increases the maximal velocity of P_i absorption from 55 to 75 nmol/min per g tissue, while no distinct effect on K_m is observed (—D, 0.52 mM; +D, 0.55 mM). To test whether the saturable entry of P_i into duodenal tissue takes place by facilitated diffusion or by an energy-dependent mechanism, the effect of metabolic inhibitors and of incubation at low temperature on P_i uptake was determined. Addition to the bathing solution of carbonyl cyanide *m*-chlorophenyl hydrazone (50 μM) or 2,4-dinitrophenol (100 μM) as well as incubation at 20° C caused significant reduction of P_i absorption in both the +D and —D group so that the vitamin D_3 increment was completely abolished (Table I). The similarity of residual P_i uptake in both groups under these conditions suggests that a common vitamin D-independent diffusional pathway exists in addi-

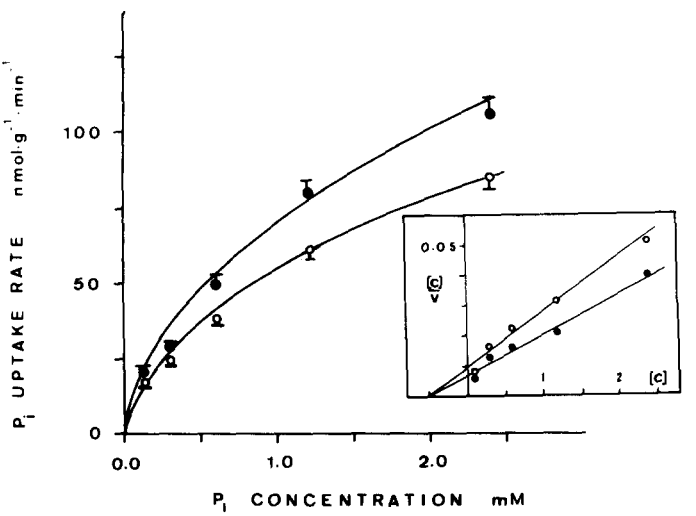

Fig. 2. Phosphate absorption rates at various phosphate concentrations in incubation buffer. Data are shown as means from 4—6 duodena per group ±S.E. (vertical bars). •, guts cultured in the presence of 26 μM vitamin D_3 for 48 h; ○, vitamin D_3-free controls. Inset: linearized plot of saturable components of P_i uptake. [*C*], P_i concentration in bathing solution; *v*, P_i uptake rate. Units and symbols are the same as in large graph.

TABLE I

INFLUENCE OF METABOLIC INHIBITION, AND OF EXTRACELLULAR Na^+ ON PHOSPHATE AND CALCIUM ABSORPTION BY EMBRYONIC CHICK DUODENUM

Data are means from 4–6 duodena per group ± S.E. For detailed composition of buffers see Methods. —D, guts cultured in vitamin D_3-free medium for 48 h; +D, 26 μM vitamin D_3 in culture medium; *m*ClCCP, carbonyl cyanide *m*-chlorophenyl hydrazone; 2,4-DNP, 2,4-dinitrophenol; n.d., not determined. Superscripts indicate statistically significant difference at least at $P < 0.05$ level: [a], +D vs. —D; [b], metabolic inhibition vs. control; [c], normal vs. low Na^+ concentration.

Na^+ in incubation buffer (mM)	Na^+ replaced by	Inhibition by	Phosphate absorption		Calcium absorption	
			—D	+D	—D	+D
			(nmol/min per g tissue)			
143	—	—	53 ± 5	85 ± 4 [a]	n.d.	n.d.
143	—	50 μM *m*ClCCP	21 ± 1 [b]	25 ± 2 [b]	n.d.	n.d.
143	—	100 μM 2,4-DNP	24 ± 1 [b]	24 ± 2 [b]	n.d.	n.d.
143	—	20 °C	17 ± 1 [b]	18 ± 1 [b]	n.d.	n.d.
0	Choline	—	16 ± 2 [c]	16 ± 2 [c]	n.d.	n.d.
125	—	—	34 ± 3	39 ± 3	4.7 ± 0.4	6.2 ± 0.3 [a]
25	Mannitol	—	23 ± 1 [c]	27 ± 1 [a,c]	11.1 ± 0.6 [c]	15.4 ± 0.6 [a,c]

tion to a saturable energy-dependent mechanism sensitive to vitamin D_3. From P_i accumulation under metabolic inhibition and at low temperature the average rate of diffusional P_i entry at 1.2 mM P_i was calculated (data from +D and —D groups were combined, Table I). The obtained value was found appropriate for calculation of the linear term used for transformation of overall uptake kinetics into rectangular hyperbolas (see above).

Influence of extracellular Na^+ on P_i and Ca^{2+} absorption by embryonic chick duodenum. Isosmolar substitution of Na^+ by choline in the incubation buffer reduces radiophosphate absorption to equal levels in the vitamin D_3-treated group and in vitamin D_3-deficient controls (Table I). The transport rates observed under this condition are similar to those under metabolic inhibition and probably reflect sodium- and vitamin D_3-independent entry of phosphate into duodenal tissue by a diffusional pathway. Apparently, the active P_i absorptive mechanism is completely inhibited by incubation in Na^+-free buffer.

In contrast to inorganic phosphate, calcium absorption by embryonic duodenum is stimulated by low extracellular sodium. Absorption of both ions was measured in a low sodium buffer containing mannitol [15], which is most suitable for measurements of calcium uptake [1], and compared to uptake at normal sodium concentration. The tissue accumulates significantly more calcium from the bathing solution at 25 mM than at 125 mM Na^+. Obviously, the increment due to vitamin D_3 is also higher at the low sodium level (Table I). Phosphate uptake, however, is inversely related to extracellular sodium concentration. A heavily reduced accumulation rate and a small vitamin D effect is observed with the mannitol buffer. Adjustment of the Na^+ concentration to normal stimulates uptake in both +D and —D groups (Table I).

Time- course and dose-response-relationship of vitamin D_3 effect on phos-

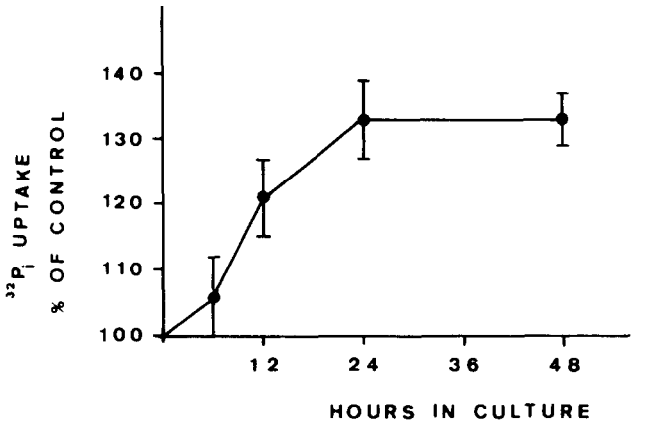

Fig. 3. Time-course of vitamin D_3 effect on phosphate transport. Data were converted to 'percentage of zero vitamin D_3 control'. Values are means ± S.E. from 4—8 guts per group.

phate absorption. Stimulation of tissue accumulation of P_i was studied at the 26 μM vitamin D_3 dose level (Fig. 3). Phosphate absorption tends to increase after 6 h of culture. A statistically significant rise above the —D level was observed at 12 h ($P < 0.05$). Full expression of the stimulatory effect occurs as early as 18 h and is maintained up to 48 h. Longer culture periods were not studied.

For determination of the dose-response relationship, duodena were cultured for 48 h in the presence of different vitamin D_3 concentrations. The lowest dose level effective in raising absorption significantly ($P < 0.05$) above control values was 6.5 nM vitamin D_3 corresponding to a concentration of 0.1 I.U./ml culture medium. Maximal response was observed at 0.65 μM vitamin D_3 (Fig. 4). Higher vitamin D_3 concentrations had no additional effect on P_i absorption.

Effect of inhibitors of protein synthesis on vitamin D_3 stimulation of phosphate transport. Since the active metabolite of vitamin D_3, 1,25-dihydroxy-

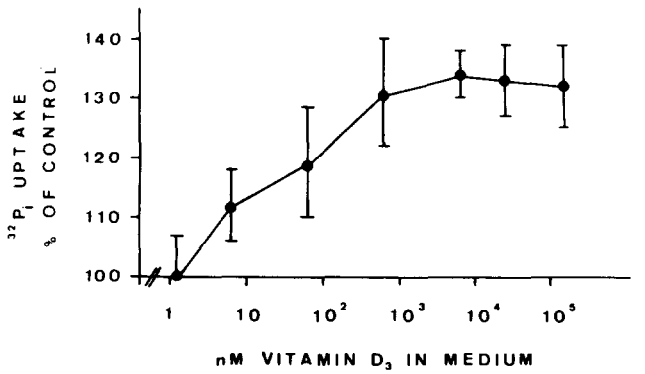

Fig. 4. Dose-response relationship of vitamin D_3 stimulation of phosphate absorption. Duodena were cultured for 48 h in presence or absence of vitamin D_3. For presentation data were converted to 'percentage of zero vitamin D_3 control'. Values are the mean ± S.E. (vertical bars) from 4 duodena per group.

TABLE II

EFFECT OF INHIBITORS OF PROTEIN SYNTHESIS ON PHOSPHATE ABSORPTION BY EMBRYONIC CHICK DUODENUM

Duodena were cultured for 24 h. Inhibitors were added 6 h after begin of culture. —D, no vitamin D_3 present in culture medium; +D, 26 μM vitamin D_3 in culture medium. Data are means (4—6 duodena per group) and S.E. Significance of difference between treated groups and their respective controls (no inhibitor present) at least at $P < 0.05$ level. No significant differences between +D and —D groups when inhibitors were added to culture medium.

Inhibitor in culture medium	Phosphate uptake (nmol/min per g tissue)	
	—D	+D
None	77 ± 5	94 ± 8
Actinomycin D (5.5 μg/ml)	56 ± 2	56 ± 8
α-Amanitin (0.5 μg/ml)	51 ± 7	63 ± 9
Cycloheximide (125 μg/ml)	52 ± 4	45 ± 2

vitamin D_3, is known to exert its effect on intestinal calcium absorption via alteration of the genetic expression of the intestinal cell [16] leading to the synthesis of a specific calcium-binding protein [17], the effect of transcriptional and translational inhibitors on phosphate transport was tested. Actinomycin D [18], α-amanitin [19] and cycloheximide [20], when present in the culture medium, not only reduced basal phosphate absorption in vitamin D_3-free controls but also abolished any vitamin D stimulation (Table II). This proves that P_i transport by embryonic chick duodenum depends on intact protein synthesis. Interaction of the sterol with a transcriptional step and subsequent modulation of protein synthesis necessarily preceeds stimulation of phosphate absorption in cultured embryonic duodenum.

Discussion

The results obtained prove embryonic chick duodenum maintained in organ culture a useful model for the study of cellular vitamin D effects with respect to absorption of inorganic phosphate. Dose range and time course of the vitamin D effect are both comparable to those pertinent to induction of calcium-binding protein and increase in calcium absorption observed in this system [1].

The present study reveals several features of the phosphate absorptive mechanism of embryonic chick duodenum. Tissue uptake involves a saturable step which depends on metabolic energy and displays characteristics of a sodium-linked transfer [22]. Vitamin D_3 stimulates this pathway by increasing its maximal velocity. An alteration of affinity of the carrier complex to P_i cannot be deduced from the experiments since the apparent Michaelis-Menten constant remains virtually unchanged. Thus, with respect to its basic characteristics, vitamin D_3-induced phosphate absorption cannot be distinguished from the unstimulated process.

These findings are consistent with the current view on intestinal phosphate transport and the effect of vitamin D thereon. Previous investigations on unidirectional P_i fluxes in everted chick jejunum furnished evidence for an active

vitamin D-dependent pathway located on the mucosal side of the epithelial cell layer [13,21]. P_i uptake from the serosal side is not influenced by vitamin D and, in addition, occurs at a rate far below that of P_i entry across the mucosal surface [13,21]. If this holds true also for embryonic chick duodenum, vitamin D-dependent tissue accumulation reflects mainly mucosal P_i influx, although uptake of radiophosphate as measured in the present study also includes P_i influx from the serosal side.

The current study also yields some information on the mechanism by which vitamin D_3 might stimulate phosphate absorption. Stimulation of a passive transfer step can be excluded from the observations indicating a lack of any vitamin D_3 increment during metabolic inhibition or in the sodium-free state. The demonstration of increase in maximal velocity of the Na^+-dependent active component of P_i transport due to vitamin D_3 favors the assumption that the sterol increases the number of available carrier sites. This could be achieved either by exposure of pre-existing latent carrier complexes or by increased synthesis of carrier proteins. The blocking of vitamin D action by inhibitors of protein synthesis is in keeping with the second possibility.

The different sensitivity of calcium and phosphate uptake by embryonic chick duodenum towards extracellular Na^+ suggests that Ca^{2+} and P_i enter the tissue by separate cellular pathways. Consequently, this implies an independent effect of vitamin D_3 on the respective absorptive mechanisms.

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References

1 Corradino, R.A. (1973) J. Cell Biol. 58, 64–78
2 Corradino, R.A. (1973) Science 179, 402—405
3 Corradino, R.A. (1974) Endocrinology 94, 1607—1614
4 Corradino, R.A. (1975) in Calcium-Regulating Hormones (Talmage, R.V., Owen, M. and Parsons, J.A., eds.), pp. 346—361, Excerpta Medica, Amsterdam
5 Fraser, D.R. and Emtage, J.S. (1976) Biochem. J. 160, 671—682
6 Lawson, D.E.M., Fraser, D.R., Kodicek, E., Morris, H.R. and Williams, D.H. (1971) Nature 230, 228—230
7 Moriuchi, S. and DeLuca, H.F. (1974) Arch. Biochem. Biophys. 164, 165—171
8 Bishop, J.E. and Norman, A.W. (1975) Arch. Biochem. Biophys. 167, 769—773
9 Wasserman, R.H., Corradino, R.A. and Taylor, A.N. (1968) J. Biol. Chem. 243, 3978—3986
10 Corradino, R.A. and Wasserman, R.H. (1971) Science 172, 731—733
11 Hurwitz, S. and Bar, A. (1972) Am. J. Physiol. 222, 761—767
12 Taylor, A.N. (1974) J. Nutr. 104, 489—494
13 Peterlik, M. and Wasserman, R.H. (1978) Am. J. Physiol. 234, E 379—388
14 Cohen, P.P. (1957) in Manometric Techniques (Umbreit, W.W., Burris, R.H. and Stauffer, J.F., eds.), pp. 147—150, Burgess Publishing Co., Minneapolis
15 Hurwitz, S., Harrison, H.C. and Harrison, H.E. (1967) J. Nutr. 91, 319—323
16 Zerwekh, J.E., Lindel, T.J. and Haussler, M.R. (1976) J. Biol. Chem. 251, 2388—2394
17 Emtage, J.S., Lawson, D.E.M. and Kodicek, E. (1973) Nature 246, 100—101
18 Reich, E., Franklin, R.M., Shatkin, A.J. and Tatum, E.L. (1962) Proc. Natl. Acad. Sci. U.S. 48, 1238—1245
19 Lindell, T.J., Weinberg, F., Morris, P.W., Roeder, R.G. and Rutter, W.J. (1970) Science 170, 447—449
20 McKeehan, W. and Hardesty, B. (1969) Biochem. Biophys. Res. Comm. 36, 625—630
21 Peterlik, M. and Wasserman, R.H. (1977) in Phosphate Metabolism (Massry, S.G. and Ritz, E., eds.), pp. 323—332, Plenum Press, New York
22 Schultz, S.G. and Curran, P.F. (1970) Physiol. Rev. 50, 637—718