Differentiation of the Changes in Alkaline Phosphatase from Calcium Ion-Activated Adenosine Triphosphatase Activities Associated with Increased Calcium Absorption in Chick Intestine

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The pattern of response of the intestinal enzymes Ca^{2+} -activated adenosine triphosphatase and alkaline phosphatase in the chick to 1,25-dihydroxycholecalciferol is consistent with a role for the former but not the latter enzyme in the vitamin D-dependent absorption of calcium.

The importance of cholecalciferol in calcium absorption is well established, but the manner in which the vitamin exerts its effect at a molecular level is still unclear. The active form of cholecalciferol in the intestine is a steroid hormone 1,25-dihydroxycholecalciferol, which is known to be necessary for calcium absorption and the synthesis of the cytoplasmic calcium-binding protein (Lawson & Emtage, 1974; Wasserman *et al.*, 1974). However, comparative studies of the sequence and time scale of changes in calcium absorption and calcium-binding-protein concentrations observed in response to 1,25-dihydroxycholecalciferol have shown that calcium-binding-protein production is not a primary event in the absorption of calcium. Thus other factors in addition to this protein must be involved in this process and these factors must in some way be affected by the hormone (Spencer *et al.*, 1976). Such factors are at present unidentified, but a number of suggestions have been made. It has recently been shown that the synthesis of two membrane-bound proteins stimulated by 1,25-dihydroxycholecalciferol occurs before the onset of calcium-binding-protein production (Wilson & Lawson, 1977). One of these proteins has been identified as an actin-like protein (Wilson & Lawson, 1978) and the other, at present unidentified, has an apparent mol.wt. of 84000. In addition the activities of the membrane-bound enzymes alkaline phosphatase and Ca-ATPase* are increased by cholecalciferol and the possibility has often been considered that they are involved in the calcium absorption process (Haussler *et al.*, 1970; Holdsworth, 1970; Melancon & DeLuca, 1970; Norman *et al.*, 1970; Kowarski & Schacter, 1972).

Some confusion has arisen as to whether these two

activities are merely different manifestations of the same enzyme. Both phosphatases have been shown to be inhibited by L-phenylalanine, beryllium and zinc (Haussler *et al.*, 1970), and to have similar temperature sensitivity (Russel *et al.*, 1972).

However, a disparity between the intestinal concentrations of the phosphatases has been reported in animals subjected to a low-calcium diet, for example duodenal alkaline phosphatase activity was increased in the adapted animal, but no such increase was apparent for the Ca-ATPase (Krawitt *et al.*, 1973). Furthermore butanol extracts of intestine containing alkaline phosphatase activity do not have Ca-ATPase activity (Russel *et al.*, 1972), thus providing chemical evidence that the two phosphatases are not molecularly identical. It has been shown more recently that the increased alkaline phosphatase activity in response to 1,25-dihydroxycholecalciferol is not related to the increase in calcium absorption (Norman & Henry, 1974; Morrissey *et al.*, 1978).

It is therefore important to establish whether alkaline phosphatase and Ca-ATPase are the same enzyme, as information from other systems such as sarcoplasmic reticulum has implicated this latter enzyme as having a role in the active accumulation of calcium which can be observed with this cell fraction and it is reasonable to assume that the potent Ca-ATPase in the intestinal cell is involved in the energy-dependent pathway for calcium absorption (see for instance Norman & Henry, 1974).

The most convincing evidence to date in favour of such a role for this enzyme comes from the close correlation between changes in calcium absorption and in the concentration of the enzyme in the intestine in response to cholecalciferol. However, as shown for calcium-binding protein (Spencer *et al.*, 1976) and for alkaline phosphatase (Norman & Henry, 1974; Morrissey *et al.*, 1978), correlations observed in

* Abbreviation used: Ca-ATPase, Ca^{2+} -dependent adenosine triphosphatase.

response to cholecalciferol are not always found after a dose of the hormone. In this study we have compared the temporal changes in Ca-ATPase, alkaline phosphatase and calcium absorption which result in the intestines of vitamin D-deficient chicks after a pulse dose of 1,25-dihydroxycholecalciferol.

Methods

Animals

Chicks (1 day old: Rhode Island Red $\times$ Light Sussex) obtained from the National Institute for Research in Dairying (Shinfield, Reading, Berks., U.K.) were fed on a vitamin D-free diet containing 1% calcium and 0.7% phosphate (Lawson & Wilson, 1974) and used after 3–4 weeks. 1,25-Dihydroxycholecalciferol was dissolved in 10% ethanol in propylene glycol and administered intracardially in 125 ng doses to the birds. Most groups consisted of three birds at each time interval.

Preparation of intestinal homogenates

At indicated times the birds were killed, and the intestines washed initially with 0.9% NaCl, 5% EDTA and finally 0.9% NaCl. Mucosa obtained by splitting the intestine longitudinally and scraping with a glass slide was homogenized in 100 mM-Tris/HCl, pH 7.4. Protein was determined by the method of Lowry *et al.* (1951) with bovine serum albumin as

standard. For controls, homogenates were prepared at zero time and at other times during a 24 h period so that any observed effects of 1,25-dihydroxycholecalciferol could be differentiated from diurnal changes in the enzyme activities.

Enzyme activities

Both enzymes were determined in duplicate on the same day as the birds were killed.

Ca-ATPase. This enzyme was assayed as described by Melancon & DeLuca (1970) in which 0.2 ml of the homogenate (200 μ g of protein) in 40 mM-Tris/HCl, pH 7.4, was incubated at 37°C with either 2.5 mM-MgCl₂ or 5 mM-MgCl₂ and 10 mM-CaCl₂ to measure the increase in enzyme activity due to Ca²⁺. The reaction was started by addition of ATP (5 mM) and stopped after a further 10 min by adding 5% trichloroacetic acid. P_i was estimated by the method of Allen (1940).

Alkaline phosphatase. To a cuvette containing 1 ml of 0.2 M-glycine buffer, pH 9.0, 4 mM-MgCl₂, 2 mM-zinc acetate, 1 mM-CoSO₄ and 0.4 ml of water was added 0.2 ml of the homogenate (1.5–2 μ g of protein). After incubation at 37°C for 5 min 0.5 ml of the substrate *p*-nitrophenyl phosphate (20 mM) was added to initiate the reaction. The rate of appearance of the product *p*-nitrophenolate anion was determined by using a molar absorption coefficient at 410 nm of 15300 M⁻¹·cm⁻¹ (Norman *et al.*, 1970).

Fig. 1. Changes in intestinal activities of (a) alkaline phosphatase, (b) Ca-ATPase in vitamin D-deficient chicks in response to 1,25-dihydroxycholecalciferol (125 ng/bird)

The chicks were killed at time intervals indicated after single intracardial injection of the hormone or after a second injection given 24 h after the first. The enzymes were measured as described in the text. Enzyme activities for alkaline phosphatase are expressed as nmol of *p*-nitrophenol/min per μ g of protein and for Ca-ATPase they are expressed as μ mol of phosphate/10 min per mg of protein. ▲, ■, Enzyme activity after a single dose of hormone; △, □, activity produced after a second dose of the hormone given at the time indicated (↑). Bars indicate s.e.m. and are included whenever the variation from the mean is greater than 5%.

Calcium absorption

This was measured using an everted intestinal-sac procedure (Schacter & Rosen, 1959). The results were calculated as the ratio of ^{45}Ca in serosal fluid to that in mucosal fluid. The ratio at each time interval was then related in percentage terms to the maximum ratio found, in these experiments, at 8 h after a dose of the hormone.

Results and Discussion

Fig. 1(a) shows the increase in intestinal alkaline phosphatase activity observed in rachitic chicks after an intracardial injection of 1,25-dihydroxycholecalciferol. No significant difference in activity was seen at 5 h, but by 9 h after the administration of the hormone the enzyme activity was significantly increased in comparison with that observed in deficient controls killed at the same time. The enzyme activity continued to increase, reaching a plateau value at about 24 h, which was then maintained throughout the rest of the experiment. A second injection given 24 h after the initial dose elicited no further increase in activity of this enzyme.

A rise in Ca-ATPase activity can be detected at short time intervals after 1,25-dihydroxycholecalciferol (Fig. 1b). Thus by 2 h after the initial injection the activity ($0.618 \pm 0.04 \mu\text{mol}$ of phosphate/10 min per mg of protein) was significantly different from control values ($0.213 \mu\text{mol}$ of phosphate/10 min per mg of protein; $P < 0.001$). The maximum enzyme activity ($2.77 \mu\text{mol}$ of phosphate/10 min per mg of protein) was observed at 8 h after dosing, but the enzyme activity fell quite rapidly so that it was markedly decreased by 15 h although still significantly greater than that observed in deficient controls killed at the same time. The same pattern of response in the enzyme activity was observed after a second injection of the hormone given to the birds 24 h after the first (Fig. 1b). No diurnal variation in the activity of either of these enzymes was found.

An assessment of calcium absorption in birds dosed with 1,25-dihydroxycholecalciferol was made by using a system of everted intestinal sacs *in vitro*. The results were very similar to those reported previously (Spencer *et al.*, 1976). Sacs prepared from birds killed 2 h after dosing transported calcium at a rate significantly higher than that of the controls ($P < 0.001$; $n = 16$), with the maximum rate being reached in sacs prepared from birds 8 h after dosing (Fig. 2). Calcium absorption had decreased in birds 24 h after the dose of the hormone, but was still significantly greater than that in untreated birds ($P < 0.001$; $n = 16$). As with the Ca-ATPase activities the calcium absorption response of birds to a repeated dose of the hormone was the same as that seen after a single dose. Thus by 5 h after a pulse dose of 1,25-dihydroxy-

Fig. 2. Temporal changes occurring in the activities of the two membrane-bound enzymes and in calcium absorption in the intestine of vitamin D-deficient chicks after a pulse dose of 1,25-dihydroxycholecalciferol

Results are expressed as a percentage of the maximum value obtained for each of the three parameters irrespective of the time interval at which this was reached. Enzyme data are taken from Fig. 1. Calcium absorption was measured in eight birds at each time interval. ○, Ca absorption; ■, Ca-ATPase; ▲, alkaline phosphatase.

cholecalciferol to rachitic chicks calcium absorption had been increased to about 50% of its maximum measured rate, as was the Ca-ATPase activity, whereas alkaline phosphatase activity was unchanged at this time. By 24 h when both the Ca-ATPase activity and calcium transport had decreased markedly the intestinal alkaline phosphatase activity was nearly maximal. It is of interest that the changes in the synthesis of the 84000-mol.wt. protein observed previously (Wilson & Lawson, 1977) correspond not only to the changes in calcium absorption, but also to those in Ca-ATPase activity. However, we have not found ATPase activity associated with this protein.

The conclusions from these results are as follows. Firstly, Ca-ATPase and alkaline phosphatase activities are due to two different enzymes or two different moieties of a multicomponent complex (Wasserman & Corradino, 1973) and are not merely different manifestations of the same molecule. Secondly, intestinal alkaline phosphatase is not involved in the primary events in the calcium absorption process. Thirdly, the close parallel between the changes in calcium absorption and Ca-ATPase activity in vitamin D-deficient birds in response to single or double injections of 1,25-dihydroxycholecalciferol is consistent with a role for this enzyme in the process of calcium absorption. Physiologically intracellular calcium concentrations are kept lower

than extracellular concentrations by a plasma-membrane pump. Consequently when calcium is in the process of being absorbed it might be expected that the Ca-ATPase activity would be increased. Evidence is now required as to whether the changes in Ca-ATPase reported here are brought about by a direct effect of the hormone or whether they are solely in response to changes in calcium concentrations. Such evidence will include comparisons of the extent to which it is possible to alter calcium absorption rates and Ca-ATPase activity independently. For example, the calcium ionophore filipin transfers calcium ions across the intestinal cell of vitamin D-deficient chicks (Norman & Henry, 1974) and at the same time Ca-ATPase activity is increased (S. M. Lane & D. E. M. Lawson, unpublished work). Such a result is consistent with the view that 1,25-dihydroxycholecalciferol does not stimulate Ca-ATPase directly, but more experiments of this type and with Ca-ATPase inhibitors are required before a definite answer to this problem can be given.

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