Open Question

Vitamin D – still an unsolved problem

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The discovery of the steroid hormone 1,25-dihydroxycholecalciferol (1,25-$(OH)_2D$) has led to an explosion of publications involving vitamin D and its metabolites. These substances have attracted interest in many branches of biology, and the impression has been created that substantial progress has been made in our understanding of the manner in which vitamin D regulates calcium homeostasis. I believe this impression is more a reflection of the meagre state of previously existing knowledge and that, for biochemists, the fundamental problem of the nature of the mechanisms involved in the transport of calcium across both cells and membranes and in the action of the regulatory hormones on these processes is still unsolved.

Three hormones are involved in calcium homeostasis: parathyroid hormone (PTH), 1,25-$(OH)_2D$ and calcitonin (Fig. 1). The first two hormones act to prevent plasma calcium concentrations from falling whereas the latter hormone prevents an excessive rise. Their combined effect is to keep the plasma calcium concentration in most animals within the range 2.2–2.6 mM. Until a little over 10 years ago most research in this area concentrated on the two protein hormones PTH and calcitonin but today there is a somewhat different balance of research efforts.

Formation of 1,25-dihydroxyvitamin D

The biosynthesis of 1,25-$(OH)_2D$ begins with the action of solar ultraviolet light on 7-dehydrocholesterol to form vitamin D in the skin[1] (Fig. 2). There is very little vitamin D in other tissues or blood as it is rapidly metabolized to 25-hydroxyvitamin D (25-OHD) – the main form of vitamin D activity in the body – primarily, and probably exclusively, in the liver[2]. The control mechanism that prevents over-production of 25-OHD is not understood but it appears not to involve the 25-hydroxylase. The third and final reaction in the biosynthesis of 1,25-$(OH)_2D$, the insertion of an hydroxyl group at C-1 of 25-OHD, usually occurs only in the kidney[3]. Very recently it has been reported that placenta and embryonic bone can also form 1,25-$(OH)_2D$ from 25-OHD. Nevertheless, in non-pregnant animals, kidney appears to be the sole site of 1,25-$(OH)_2D$ synthesis since the hormone cannot be detected in anephric animals. Attention has been drawn to the similarity between this hydroxylation reaction and the well-defined adrenal hydroxylases. In addition to the 1-hydroxylase in the mitochondria of kidney cells, the reaction involves a flavoprotein reductase, a non-haem-iron protein (ferredoxin) and a cytochrome *P*-450. In reconstitution experiments the pure 1-hydroxylase and these three cofactors are sufficient for full activity. Synthesis of 1,25-$(OH)_2D$ is carefully regulated to provide appropriate stimuli to its target tissues and thereby affect plasma calcium concentrations. At least eleven factors have been considered to be involved in the regulation of 1,25-$(OH)_2D$ synthesis but although the plasma concentration of 1,25-$(OH)_2D$ (or the renal 1-hydroxylase enzyme activity) changes in response to these substances, only PTH has definitely been shown to be a regulatory factor. PTH added directly to kidney cells in culture stimulates the formation of 1,25-$(OH)_2D$ from 25-OHD. The renal 1-hydroxylase is also affected by calcium but whether this is regulatory or not is uncertain. Certainly, the enzyme's activity *in vivo* varies inversely with plasma calcium concentrations and the addition of calcium to preparations of kidney mitochondria alters the 1-hydroxylase activity. However, until calcium concentrations of less than 100 nM can be measured it will remain uncertain whether under normal physiological circumstances concentrations of calcium in the cytoplasm vary by the amount required to change the 1-hydroxylase. There is substantial evidence that 1,25-$(OH)_2D$ production varies in direct proportion to the calcium demands of the animal. During periods of increased demand for calcium (e.g. growth, lactation and egg shell formation) the concentration of 1,25-$(OH)_2D$ in plasma is raised. Furthermore, growth hormone, prolactin and oestradiol administered to chicks and/or rats increases the activity of 1-hydroxylase. Thus, experiments *in vivo* show that many hormones which increase calcium turnover are also associated with enhanced 1,25-$(OH)_2D$ formation. However, a direct effect of these hormones on 1-hydroxylation in isolated kidney tubules or cells has not been demonstrated and consequently caution must be exercised in postulating a specific role in regulating the activity of 1-hydroxylase. Expressing the regulation of the 1-hydroxylase enzyme in its simplest terms it appears that any tendency for plasma calcium concentrations to fall results in an increased release of PTH and this stimulates the formation of 1,25-$(OH)_2D$. The action of this steroid in its main target tissues (intestine, kidney and

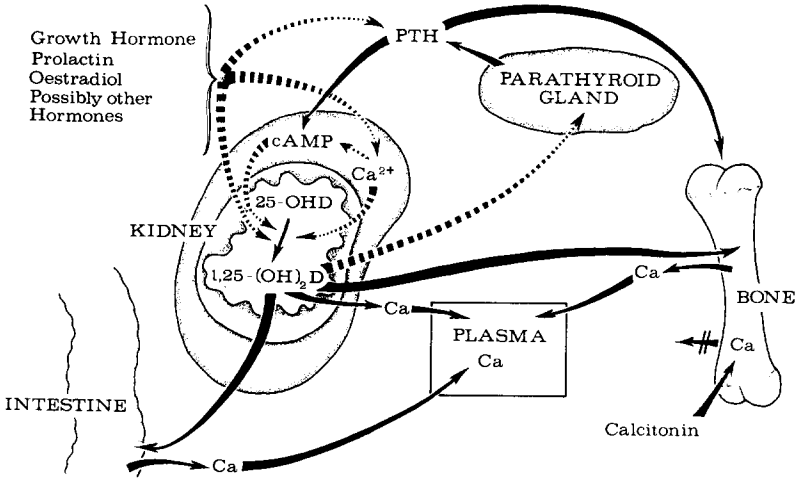

Fig. 1. Inter-relationship of parathyroid hormone (PTH), 1,25-dihydroxyvitamin D (1,25-$(OH)_2D$) and calcitonin in regulating plasma calcium concentrations. Solid lines indicate processes known to occur. Broken lines indicate possible processes; where there is more than one effect indicated they are not mutually exclusive.

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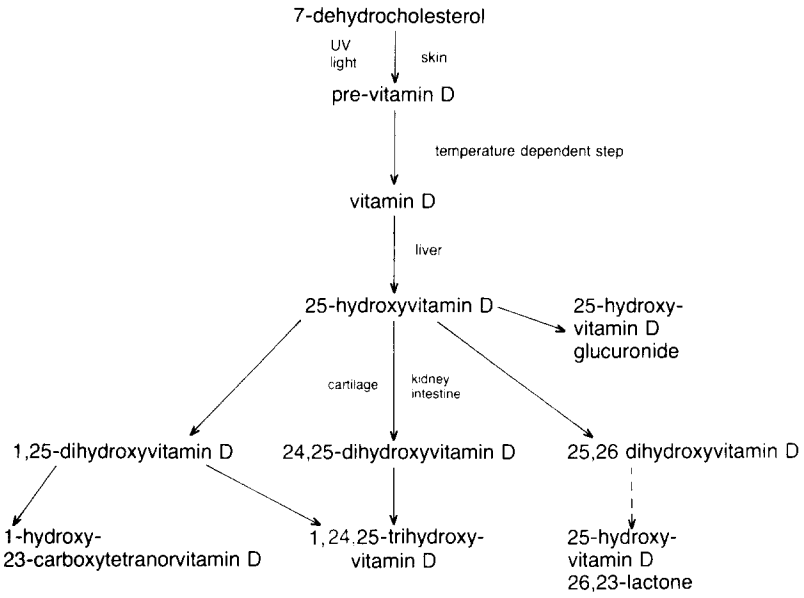

Fig. 2. Conversion of 7-dehydrocholesterol to the vitamin D series of metabolites. Broken line indicates possible route by which this metabolite could be formed.

bone) is to raise plasma calcium concentrations and thereby switch off future formation of 1,25-$(OH)_2$D. The control of the renal 1-hydroxylase by PTH probably occurs through a 'second messenger' and it may be in this way that calcium plays a regulatory role.

Calcium absorption and 1,25-$(OH)_2$D

The well-established physiological functions of vitamin D involve permissive roles in the following processes:
(a) absorption of calcium and phosphorous in the intestine; (b) renal re-absorption of Ca^{2+}; (c) mobilization of calcium and phosphorous from bone; (d) mineralization of bone. Most studies on the function of vitamin D have been concerned with its stimulation of intestinal calcium absorption, an action readily reproduced in a variety of well-established systems used to study the processes of absorption. This effect of vitamin D is thought to be due to 1,25-$(OH)_2$D since only vitamin D derivatives with a hydroxyl group at C-1 are active when injected in physiological amounts into anephric animals. Furthermore, 1,25-$(OH)_2$D is the most potent vitamin D metabolite known to stimulate Ca^{2+} absorption, it is the most rapidly acting metabolite and it is accumulated in the intestine where it constitutes the majority of vitamin D steroids. Specific, high-affinity, low-capacity binding proteins for 1,25-$(OH)_2$D have been identified in the cytoplasm and nucleus of mucosal cells[4]. The only evidence still required to establish that 1,25-$(OH)_2$D is the active form of vitamin D is to show that mature mucosal cells in culture can respond to 1,25-$(OH)_2$D without further metabolism of this compound. That 1,25-$(OH)_2$D is the active metabolite in the other systems is not so clear, but only in the case of bone mineralization[5,6] and possibly in the chick embryo[7,8] is there any indication that another metabolite, 24-25-$(OH)_2$D, may be an active form. Before this latter conclusion is accepted, further consideration must be given to discovering the optimum dose and mode of administration of 1,25-$(OH)_2$D. It is possible that the failure to obtain a complete response to 1,25-$(OH)_2$D in processes occurring over an extended period as with mineralization may be a consequence of injecting the hormone as a single bolus which is then rapidly metabolized so as to disappear almost completely before the next dose. Under physiological conditions 1,25-$(OH)_2$D concentrations are probably maintained within fixed limits and 1,25-$(OH)_2$D-dependent processes kept continuously stimulated. If this situation could be maintained experimentally, lower 1,25-$(OH)_2$D concentrations than currently used might be effective, in which case this would indicate that the apparent ineffectiveness of 1,25-$(OH)_2$D may be a response to excessive amounts of the hormone.

The proportion of dietary calcium absorbed by adult man ranges from 15–35% depending upon a number of factors, particularly the amount of calcium in the diet. This adaptive response to decreasing amounts of calcium in the diet depends on vitamin D since a fall in the intake causes the animals to absorb an increasing proportion of calcium by an active process. It is this energy-dependent process which requires vitamin D and it is the pathway which is quantitatively the most important for calcium absorption in young animals. In older animals, with bone growth completed, there is less demand for calcium and now the main pathway for absorption is a passive (energy-independent) process. It is not clear whether this second route requires vitamin D. During absorption calcium must pass across two external membranes and the cytoplasm of the intestinal cell. Although a paracellular pathway for the absorption of Ca^{2+} through the tight junction between the mucosal cells has been considered, the available evidence does not support this possibility[9]. Experiments on the uptake and transport of calcium across the mucosal cell, together with the effects on these two processes of various inhibitors and of calcium ionophores, suggests that movement of calcium across the brush-border membrane occurs by facilitated diffusion and that movement across the basal-lateral membrane is an energy-dependent step that presumably involves a Ca-ATPase. Vitamin D in the form of 1,25-$(OH)_2$D almost certainly has an effect on the movement of calcium across the mucosal cell brush-border and may have an effect on transfer across the cytoplasm. It is not clear at present whether vitamin D has any direct effect on a basal-lateral membrane event involved in calcium absorption[10].

Biochemical responses of the intestine to 1,25-dihydroxyvitamin D

A number of biochemical responses to 1,25-$(OH)_2$D have been observed in intestine (Fig. 3). A major effect is the stimula-

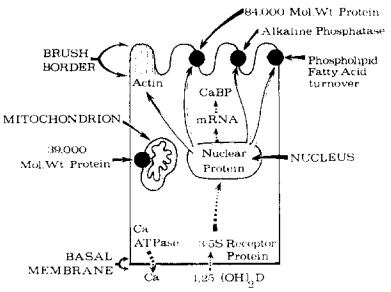

Fig. 3. Intestinal mucosal cell components known to be affected by 1,25-$(OH)_2$D. Solid lines indicate reactions known to occur. Broken lines indicate that the mechanism by which these components are affected is unknown. Ca-ATPase in basal membrane may respond directly to changing cytoplasmic calcium concentrations or be affected by 1,25-$(OH)_2$D.

tion of synthesis of vitamin D-dependent calcium-binding protein (CaBP) by increasing the synthesis of its mRNA. The other evidence implicating CaBP in calcium absorption includes (a) its high concentration in tissues which are very active in transporting calcium, such as intestine, kidney and hen's shell gland; (b) an increase in CaBP concentration as well as calcium absorption in animals on a diet low in calcium; and (c) comparison of the relative potencies of divalent cations to compete with calcium in absorption and for the binding sites in CaBP[11]. However, the changes in calcium absorption in chicks and rats in response to 1,25-$(OH)_2$D do not follow exactly the changes in CaBP concentrations. When the effect of the hormone on absorption has decreased almost to its original value, substantial amounts of the protein are still present in the intestine and, in addition, the mRNA for CaBP is only detected after absorption first begins to increase[1]. Nevertheless, the synthesis of this protein after 1,25-$(OH)_2$D dosing is typical of that shown by other hormone-responsive proteins in that it is increased three or fourfold by a second dose of hormone. It is also now accepted that the majority of the CaBP in the cell is located in the cytoplasm and that there is none, or at least very little, in the intestinal brush border. CaBP's role is still unknown but as calcium can change the conformation[12] of bovine intestinal CaBP it could act as a regulator.

As a result of these findings, attempts have been made to recognise other vitamin D-dependent intestinal components. The incorporation of radioactive amino acids into at least three chick intestinal proteins is increased by 1,25-$(OH)_2$D. One of these proteins, actin, is present in the core of the brush-border. A second (mol. wt 39,000) is found in the mitochondrial and microsomal fractions but not apparently in the intestinal brush borders. Finally, there is a protein (mol. wt 84,000) in the brush-border membrane. This protein can be phosphorylated by a reaction that does not require Mg^{2+} but which is stimulated by Ca^{2+}. Considerable effort has also been expended in trying to show that phospholipids are affected by vitamin D. Recently it has been reported that 1,25-$(OH)_2$D increases the turnover of the fatty acids in phospholipid side chains[13]. There is no evidence that 1,25-$(OH)_2$D is in the intestinal brush-border and consequently this action must be indirect. The activities of alkaline phosphatase and Ca^{2+}/Mg^{2+}-ATPase in the intestine are increased by 1,25-$(OH)_2$D but it is still not completely clear that these are separate enzymes. Although changes in adenyl cylase activity and in concentrations of cAMP in response to vitamin D have been reported it has proved difficult to relate them to calcium absorption. One general conclusion emerging from these studies is that 1,25-$(OH)_2$D acts by increasing the total amount of the intestinal component which is sensitive to it, rather than simply causing them to be more effective (i.e. an increase in V_{max} rather than K_m). Interestingly this conclusion was confirmed by analysis of the effect of vitamin D on calcium absorption or uptake by chick intestinal membrane vesicles. In the latter case there is also substantial vitamin D-dependent calcium binding by the membranes and a vitamin D-dependent calcium-binding protein (mol. wt about 20,000) has been isolated from rat intestine[14].

With our present knowledge of the intestinal action of vitamin D we can define the nature of the information required for a more complete understanding of one aspect of calcium homeostasis – calcium absorption. Which components of the brush-border membrane are involved in calcium absorption, taking into consideration that the process is one of facilitated diffusion? Since nuclei are the only organelles of the intestine to contain 1,25-$(OH)_2$D how does any effect elicited here become apparent in the brush-border of these cells? Is there a mechanism for protecting the internal mucosal cell membranes from high concentrations of calcium during absorption? What is the nature of the calcium pump in the basal-lateral membrane and does it respond directly to 1,25-$(OH)_2$D?

In conclusion, although vitamin D is known to be merely the precursor of a steroid hormone, many of the most important problems associated with this substance still remain unsolved. These include the regulation of 25-OHD synthesis when vitamin D is synthesized from its physiological source, sunlight; the factor(s) controlling 1-hydroxylation; the number of tissues or cell types responsive to 1,25-$(OH)_2$D and the sequence of biochemical changes occurring within them and, finally, whether 1,25-$(OH)_2$D is the only active form of vitamin D.

References

1 Lawson, D. E. M. and Davie, M. (1979) *Vitamins and Hormones*, 37, 1–67
2 DeLuca, H. F. (1977) *Adv. Clin. Chem.* 19, 125–174
3 Fraser, D. R. (1980) *Physiol. Rev.* 60, 551–613
4 Norman, A. W. and Henry, H. L. (1979) *Trends Biochem. Sci.* 4, 14–18
5 Bordier, P., Rasmussen, H., Marie, P., Miravet, L., Gueris, J. and Ryewoert, A. (1979) *J. Clin. Endocrinol. Metab.* 46, 284–290
6 Ornoy, A., Goodwin, D., Noff, D. and Edelstein, S. (1978) *Nature (London)* 276, 517–519
7 Henry, H. L. and Norman, A. W. (1978) *Science* 2001, 835–837
8 Sunde, M. L., Turk, C. M. and DeLuca, H. F. (1978) *Science* 200, 1067–1069
9 Nellans, H. N. and Kimberg, D. V. (1978) *Am. J. Physiol.* 235, E726–E737
10 Wasserman, R. H. (1968) *Calc. Tiss. Res.* 2, 301–313
11 Wasserman, R. H., Fullmer, C. S. and Taylor, A. N. (1978) in *Vitamin D* (D. E. M. Lawson, ed.) p. 133, Academic Press Inc. London
12 Birdsall, W. J., Levine, B. A., Williams, R. J. P., Fullmer, C. S. and Wasserman, R. H. (1979) *Biochem. Soc. Trans.* 7, 702–703
13 Max, E. E., Goodman, D. B. P. and Rasmussen, H. (1978) *Biochim. Biophys. Acta* 511, 224–233
14 Kowarski, S. and Schachter, D. (1980) *J. Biol. Chem.* 255, 10834–10840