MECHANISMS OF CALCIUM TRANSPORT IN SMALL INTESTINE

Progress Report

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ABSTRACT

The macromolecular components of rat intestinal mucosal cytosol have been examined for their ability to bind radioactive $1,25-(OH)_2D_3$ and radioactive $25-OH-D_3$. In a variety of media the binding of these metabolites is found only to a 6S macromolecular component. No evidence for the existence of a 3.5S component which has been reported in the chick can be found in this species. The 6S component prefers $25-OH-D_3$ to $1,25-(OH)_2D_3$ and furthermore saturation experiments in vivo have shown that the 6S component does not bind $1,25-(OH)_2D_3$ in vivo but instead binds the $25-OH-D_3$. This allowed the conclusion that the 6S macromolecular component does not play a role in the $1,25-(OH)_2D_3$'s function in stimulating intestinal calcium absorption.

Work on the cytosolic macromolecular components in the chick intestinal mucosa has revealed the existence of a 3.5S macromolecular component which will bind $1,25-(OH)_2D_3$ and not $25-OH-D_3$. However, the variability of appearance of this macromolecular component has hampered further identification.

Attempts at purification of nuclei in good yields from chick and rat intestinal mucosa have been frustrated by failures. The best preparations give only a 20% yield of pure nuclei. Using this technique only 30% of the total tissue radioactivity of intestinal mucosa can be accounted for in the nucleus. This agrees with a 30% figure for isolated pure chromatin. Thus the exact subcellular location of 50% of the total mucosal radioactivity remains undetermined.

Analogues of $1,25-(OH)_2D_3$ have been chemically synthesized which provides important new information regarding the requirements of structure for stimulating intestinal calcium transport. $1\alpha-OH-D_3$ is almost as active as $1,25-(OH)_2D_3$ yet lacks the 25-hydroxyl group. The 3-deoxy- $1\alpha-OH-D_3$ compound has also been synthesized and has been shown to be active in intestinal calcium transport but only to 1/10 that observed with $1,25-(OH)_2D_3$. Nevertheless, this illustrates that the 3-hydroxyl is no longer necessary for intestinal calcium transport if a 1α -hydroxyl group is present. Side chain analogs include 1-hydroxylated vitamin D's in which the entire side chain is replaced by a hydroxyl on carbon 21 and in which the side chain is shortened by 1 carbon in $1,25-(OH)_2D_3$. The compound minus the entire side chain is totally inactive and the compound which is missing 1 carbon in the side chain has activity of about 1/1000 of that of $1,25-(OH)_2D_3$. The results, therefore, allow the conclusion that an intact side chain is required for intestinal calcium transport activity and a hydroxyl in the 1 position is also required but the hydroxyl in the 3 position is not required. To determine whether 25-hydroxylation of $1\alpha-OH-D_3$ takes place prior to its stimulating intestinal calcium transport, synthesis of $[6\alpha-^3H]-1\alpha-OHD_3$ has been completed and will be studied to determine whether such is the case.

The administration of strontium, ethane-1-hydroxy-1,1-diphosphonate and parathyroidectomy markedly reduces intestinal calcium transport which can be restored by the administration of $1,25-(OH)_2D_3$ from exogenous sources. Direct examination of the $25-OH-D_3$ - 1α -hydroxylase in isolated chick kidney mitochondria has shown that these substances or treatments result in the disappearance of 1α -hydroxylase activity. Administration of parathyroid hormone to chicks stimulates the appearance of the $25-OH-D_3$ - 1α -hydroxylase in the isolated mitochondria. These results illustrate that these treatments result in a failure of accumulation of $1,25-(OH)_2D_3$ in the intestine by inhibiting its synthesis. Phosphate deprivation

also stimulates $1,25-(\text{OH})_2\text{D}_3$ production and stimulates intestinal calcium transport. Another laboratory has reported that phosphate deprivation of the chick does not stimulate the 1α -hydroxylase of chick kidney. However, we have completed extensive studies in this area which illustrate that phosphate deprivation does stimulate the appearance of the 1α -hydroxylase in isolated chick kidney mitochondria although phosphate deprivation does not produce as marked an increase as does calcium deprivation.

The administration of exogenous $1,25-(\text{OH})_2\text{D}_3$ prevents the adaptive response of intestine to low and high dietary calcium, but does not prevent the adaptation to low dietary phosphate. These animals show a high rate of intestinal calcium transport even in the presence of exogenous $1,25-(\text{OH})_2\text{D}_3$ which suggests that another factor besides increased $1,25-(\text{OH})_2\text{D}_3$ synthesis is involved in stimulating intestinal calcium transport by phosphate deprivation.

A phosphate transport system independent of calcium is known to exist in the jejunum and ileum. This system has now been shown to respond to both 25-OH-D_3 and $1,25-(\text{OH})_2\text{D}_3$ in addition to the already reported response to vitamin D_3 . Nephrectomy prevents the response to physiologic doses of 25-OH-D_3 which suggests that $1,25-(\text{OH})_2\text{D}_3$ is the active form in phosphate transport as well.

Studies on an extract from Solanum malacoxylon has revealed a substance like $1,25-(\text{OH})_2\text{D}_3$ which has a marked ability to stimulate intestinal calcium transport in nephrectomized rats. However, unlike $1,25-(\text{OH})_2\text{D}_3$, this substance does not stimulate the mobilization of calcium from bone, does not stimulate phosphate transport or the rise in serum phosphate in rachitic animals. However, the substance has a marked effect on intestinal calcium transport which has warranted our attempts at isolation and identification. This substance has now been highly purified, it is known now to be stable to alkali and unstable to acid, it has a molecular weight of over 2,000 and is methanol-water soluble.

I. Mechanism of Intestinal Calcium Transport

A. The primary effort expended in this area during the last year has been in a study of the mechanism whereby 1,25-dihydroxyvitamin D₃ (1,25-(OH)₂D₃), the known active form of the vitamin in stimulating intestinal calcium transport, progresses to its subcellular sites of action. Previously under this AEC contract it was reported that 1,25-(OH)₂D₃ localizes in the nuclear fraction. However, the nuclear fraction from small intestine is especially heavily contaminated with all other cell fractions primarily because of the presence of excessive amounts of mucous which interferes with standard subcellular fractionation procedures. A great deal of work has been expended during the past year in an attempt to develop methods whereby pure nuclei could be isolated from small intestine of rats and chicks in high yields. This work has been frustrated by the continued persistence of mucous and other materials which interfere with fractionation. To this date this problem has not been solved, but utilizing the best procedure we can account for only 20% of the cellular radioactivity in pure nuclei. This determination is made by determining the yield of DNA on isolation of pure nuclei and determination of the radioactivity found with that DNA since it is assumed that the nuclei which are isolated are representative of the whole population of intestinal nuclei, then the amount of radioactive 1,25-(OH)₂D₃ associated with pure nuclei can be calculated. Thus the figure of 20% has been determined. The isolation of chromatin in pure form by the methods of Marushige and Bonner have also yielded identical results. That is, 20-30% of the total intestinal radioactivity resulting from an injection of radioactive 1,25-(OH)₂D₃ to vitamin D-deficient animals can be found associated with pure chromatin. It is, therefore, obvious that 50% of the cellular radioactivity which sediments in the nuclear fraction has not been accounted for.

In view of these findings and in view of reports in the literature from Haussler's laboratory and from Norman's laboratory that there exists a cytoplasmic protein in the intestines of vitamin D-deficient animals which binds 1,25-(OH)₂D₃ in a specific manner, we have carried out extensive experiments aimed at determining if there are macromolecular receptors in intestinal cytosol specific for 1,25-(OH)₂D₃. Techniques of high resolution density gradient centrifugation have been developed in this laboratory with the help of Dr. Elwood Jensen and his staff at the Ben Gay Laboratories at the University of Chicago. Dr. Joyce Knutson was sent there from my laboratory for a period of time to learn the techniques and was successful in setting up their high resolution density gradient system in our laboratory. In addition, we have used Sephadex G-200 chromatography for resolution of the binding proteins.

Using these techniques, Drs. Knutson and Reynolds examined in great detail the ability of rat intestinal cytoplasmic macromolecules to bind specifically 1,25-(OH)₂D₃. They were able to show that both 25-hydroxyvitamin D₃ (25-OH-D₃) and 1,25-(OH)₂D₃ bind to a macromolecule which sediments in the 6S region. Even when the medium used by Haussler and his associates was used, we found no binding of 1,25-(OH)₂D₃ to the 3.5S macromolecular region, which was reported in the chick by these workers. The 6S protein, which was the only 1,25-(OH)₂D₃ binding macromolecule found in rat intestinal mucosal cytosol, showed a very clear preference for 25-OH-D₃ over the 1,25-(OH)₂D₃. This was demonstrated by displacement studies and competition studies using radioactive 25-OH-D₃ or radioactive 1,25-(OH)₂D₃ and the addition of increasing amounts of non-radioactive 1,25-(OH)₂D₃ and/or 25-OH-D₃. Certainly the 25-OH-D₃ competed more favorably and displaced more readily the 1,25-(OH)₂D₃ than vice versa. When radioactive 25-OH-D₃ was administered in vivo the intestinal cytosol 6S protein was shown to bind radioactive

25-OH-D₃. However, when radioactive 1,25-(OH)₂D₃ was administered, no *in vivo* binding to the 6S protein could be observed. Of great interest is the fact that when saturating amounts of non-radioactive 1,25-(OH)₂D₃ are given to vitamin D-deficient, nephrectomized rats and then the intestinal mucosal cytosol isolated and incubated with radioactive 1,25-(OH)₂D₃, the 6S macromolecule region binds clearly the radioactive 1,25-(OH)₂D₃. On the other hand if the nephrectomized vitamin D-deficient animals are given saturating amounts of non-radioactive 25-OH-D₃ and their intestinal cytosol incubated with radioactive 1,25-(OH)₂D₃ or 25-OH-D₃, no binding to the 6S component could be demonstrated. Thus it is clear that the 6S macromolecule is not a specific receptor for 1,25-(OH)₂D₃ and this component cannot be a receptor protein which functions in intestinal calcium transport since 1,25-(OH)₂D₃ has been shown to be the metabolically active form of the vitamin in that system. Furthermore, the 6S macromolecule can be demonstrated in almost every tissue of the body except plasma.

Similar work carried out in the chick has revealed that in fact there may be a 3.5S macromolecular component which binds 1,25-(OH)₂D₃. However, there is a great deal of variability in when this 3.5S protein appears. The reason for this variability is not yet known and furthermore it is not yet known whether the 3.5S protein is a true receptor. During the next three years extensive work is planned in the area of the postulated receptor proteins for the metabolites of vitamin D.

B. As a part of our continuing work on the metabolism of vitamin D to its active and inactive forms, we have initiated an extensive program to synthesize analogs of 1,25-(OH)₂D₃ to determine what structures are necessary for its activity and to synthesize a possible antivitamin D. Previously the synthesis and biological activity of 1 α -hydroxyvitamin D₃ (1 α -OH-D₃) was described. This compound proved to be approximately one-half as active as 1,25-(OH)₂D₃ in stimulating the intestine to absorb calcium in the rat and is equally active as the natural metabolite in the chick. We recently were able to complete synthesis of the 3-deoxy-1 α -OH-D₃ to ask the question of whether the 3-hydroxyl is essential for vitamin D to stimulate intestinal calcium absorption. The 3-deoxy-1 α -OH-D₃ is active in the intestinal calcium transport system, but is approximately 1/10³ as active as 1,25-(OH)₂D₃. Work is now in progress to synthesize 3-deoxy-1 α ,25-(OH)₂D₃ since the discrimination against the 3-deoxy-1 α -OH-D₃ may be at the level of 25-hydroxylation. The synthesis of [6 α -³H]-1 α -OH-D₃ has also been completed and work is now in progress to determine whether 25-hydroxylation is essential for the 1 α -OH-D₃ to initiate intestinal calcium transport.

Side-chain analogs of 1,25-(OH)₂D₃ have also been synthesized. 22-27-Pentanol-1,25-(OH)₂D₃ has been prepared and shown to be totally inactive in stimulating intestinal calcium absorption. We have prepared 24-nor-1,25-(OH)₂D₃ in which the side chain is shortened by one carbon and have found that it is only 1/1000 as active as 1,25-(OH)₂D₃. It is already clear then that an intact side chain and a 1-hydroxyl are essential for intestinal calcium transport activity. Furthermore, the 3-hydroxyl is not necessary once the 1-hydroxyl has been placed on the molecule. Work will continue in this area to define the structural requirements for the intestinal calcium transport system and to provide compounds which will help in searching for the receptors for the active form of vitamin D in this system.

II. Regulation of Intestinal Calcium Transport by Agents Which Regulate $1,25-(OH)_2D_3$ Synthesis

Previously it had been shown that dietary strontium, ethane-1-hydroxy-1,1-diphosphonate, high dietary calcium and parathyroidectomy inhibit intestinal calcium transport by blocking the synthesis of $1,25-(OH)_2D_3$. The inhibition of intestinal calcium transport by these agents can be easily overcome by the administration of exogenous $1,25-(OH)_2D_3$. To establish on a more firm basis these phenomenon, experiments have been carried out in the chick in which α -hydroxylase can be measured in vitro. The administration of strontium, ethane-1-hydroxy-1,1-diphosphonate, high dietary calcium or parathyroidectomy in chicks will cause a fall in the activity of the $25-OH-D_3$ - α -hydroxylase in incubated mitochondria. The in vivo administration of parathyroid hormone to chicks will stimulate $1,25-(OH)_2D_3$ synthesis by isolated kidney mitochondria as has been determined in our laboratory and that of Kodicek and his colleagues. It is, therefore, clear that in response to the need for calcium, the parathyroid glands secrete parathyroid hormone which in some unknown way stimulates the level of $25-OH-D_3$ - α -hydroxylase of kidney.

The mechanism whereby strontium inhibits $1,25-(OH)_2D_3$ synthesis has not yet been determined, although work in other laboratories has demonstrated that strontium markedly represses parathyroid hormone secretion.

Ethane-1-hydroxy-1,1-diphosphonate directly inhibits the enzyme added in vitro but large amounts are required in the order of 10^{-3} to 10^{-4} M. Recently, however, systems have been devised in which the diphosphonate at much lower concentrations will inhibit the mitochondrial $25-OH-D_3$ - α -hydroxylase. When the mitochondria are swollen in 10 mM calcium and external NADPH is supplied, very small amounts of diphosphonate will inhibit the α -hydroxylase, making more feasible the idea that the diphosphonate inhibits directly the α -hydroxylase. The α -hydroxylase has now been solubilized under work carried out under my NIH grant and three components have been identified, a flavoprotein, an iron-sulfur protein and a cytochrome P-450. The P-450 has been highly purified and combined with beef adrenodoxin and beef adrenal flavoprotein together with NADPH and this system will hydroxylate $25-OH-D_3$ to the $1,25-(OH)_2D_3$. Work will now progress to demonstrate that ethane-1-hydroxy-1,1-diphosphonate given in vivo in a radioactive form will bind to the P-450 and be retained throughout the purification procedure being devised for that protein. This will constitute final proof that the diphosphonate inhibits this enzyme in vivo by interacting directly with it rather than effecting some change in a physiological regulation system for the α -hydroxylation.

The feeding of low phosphate diets has been clearly shown by the work of Carlsson and then by the work of Wasserman to stimulate intestinal calcium absorption. In this laboratory it has been demonstrated that phosphate deprivation stimulates $1,25-(OH)_2D_3$ synthesis. This has been challenged by experiments carried out in the chick in which reportedly low phosphate diets have been fed resulting in no stimulation of the α -hydroxylase. However, we have carried out extensive work in this area and have now been able to show clearly that phosphate deprivation in the chick as well as in the rat will stimulate the activity of the $25-OH-D_3$ - α -hydroxylase in isolated mitochondria. Thus phosphate deprivation at least in part functions to stimulate intestinal calcium transport by increased synthesis of $1,25-(OH)_2D_3$. However, the stimulation by phosphate deprivation is much less than the stimulation seen by calcium deprivation in the chick.

To test more completely the idea that calcium deprivation and phosphate deprivation stimulate intestinal calcium transport by stimulating $1,25-(OH)_2D_3$ synthesis, rats were given an exogenous source of $1,25-(OH)_2D_3$ and then fed low calcium, normal calcium, low phosphorus and normal phosphorus diets. Rats on low calcium and high calcium diets as well as those on normal phosphorus diets show a high efficiency of intestinal calcium absorption quite independent of dietary calcium levels. However, the phosphate depleted animals show a much higher intestinal calcium absorption than all the others, suggesting that low phosphate diets and hence phosphate deprivation must have some additional mechanism of stimulating intestinal calcium transport besides stimulating $1,25-(OH)_2D_3$ production. This question is now being pursued more actively to determine whether the metabolism of $1,25-(OH)_2D_3$ itself is affected by phosphate depletion.

III. Phosphate Absorption or Transport

In order to separate phosphate absorption from calcium absorption, work has continued in the area of studying intestinal phosphate absorption in response to the vitamin D metabolites. In the jejunum and ileum, a phosphate transport system has been located through the work of Harrison et al., Schachter et al., and Wasserman and Taylor, as well as in our own laboratory. This system operates in the absence of calcium and bears no relationship to calcium transport. This system requires glucose and sodium ions but the sodium ions are required for the uptake of phosphate rather than the extrusion into the serosal fluid. It must be recalled that previous work in this laboratory has shown that sodium functions in the calcium transport system by causing extrusion of calcium into the serosal fluid. This intestinal phosphate transport system is stimulated by both $25-OH-D_3$ and $1,25-(OH)_2D_3$. However, the response to $25-OH-D_3$ is eliminated by nephrectomy. Work is continuing on the mechanism of the phosphate transport system and whether it is stimulated by phosphate deprivation and calcium deprivation. In addition, a strain of mice which has a genetic defect in phosphate metabolism has been obtained from the Bar Harbor Laboratories. These mice which are hypophosphatemic by an x-linked dominant trait are now being studied with regard to intestinal phosphate transport, vitamin D metabolism, and the human disease, x-linked familial hypophosphatemic vitamin D-resistant rickets.

IV. Calcium Transport Substance from Solanum malacoxylon

As discussed in the previous report, work has been carried out on a plant which in certain seasons in South America will cause death in grazing cattle. Death is caused by markedly elevated intestinal calcium absorption and soft tissue calcification. This plant, which is known as Solanum malacoxylon, stimulates the intestine to absorb calcium even in a nephrectomized state and in work carried out in Wasserman's laboratory, stimulates the appearance of calcium binding protein just as does vitamin D. The fact that it functions in nephrectomized animals suggests that the active compound is a $1,25-(OH)_2D_3$ like substance. Work in this laboratory has shown that the extract from Solanum malacoxylon, although able to stimulate intestinal calcium absorption like $1,25-(OH)_2D_3$, it will not stimulate the mobilization of calcium from bone nor will it stimulate phosphate transport nor will it cause mineralization of bone. However, work is now being carried out on the isolation of this material for its identification. The substance has been purified several hundred fold and is soluble in methanol and methanol-water solutions. It is very stable to alkaline treatment but is unstable to acid. It has a molecular weight of something over 2,000 but so far the compound which is responsible for the activity has not been obtained in homogeneous form. Work will continue on the isolation and identification of this component which may provide interesting information regarding intestinal calcium transport.

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