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Perspective Article

Hylonome

Calcium homeostasis: Solving the solubility problem

R.V. Talmage[1] and D.W. Talmage[2]

[1]Department of Orthopedics, University of North Carolina, School of Medicine, Chapel Hill, NC, USA;
[2]Department of Immunology, University of Colorado, School of Medicine, Denver, CO, USA

Abstract

This report summarizes the evidence that the control of the concentration of free calcium ions in body fluids is centered at mineralized bone surfaces. This process involves an increase in the solubility of bone mineral produced by the non-collagenous proteins existing in the bone extracellular fluid (ECF) and on the adjacent surfaces of bone. The result is a basic equilibrium level produced in the absence of parathyroid hormone (PTH), which is well above the solubility of bone mineral. The effect of PTH is to increase the solubility of bone mineral still further, but the mechanism by which the hormone acts is unknown. The lining cells of the bone contain receptors for PTH and can be observed to respond to this hormone, but the relationship between this response and the increased solubility of bone remains to be discovered. Further research in this field is strongly urged.

Keywords: Calcium Homeostasis, ECF, Extracellular Fluid, PTH, Parathyroid Hormone, PTX, Parathyroidectomized

Introduction

The control of the free calcium concentration of body fluids has been the subject of controversy over the last 75 years. Most of the controversy has concerned the action of parathyroid hormone (PTH). The first argument was whether the hormone raised plasma calcium levels by stimulating renal phosphate excretion or by acting on bone. When this was solved in favor of bone, the question was how the hormone affected bone to raise plasma calcium levels. When it was observed that PTH increased the activity of cells that break down bone (osteoclasts), it became generally accepted that PTH controlled calcium levels by stimulating osteoclasts.

However, problems with this hypothesis soon developed. There was a lack of correlation between the number of osteoclasts and the ability of PTH to increase calcium levels and evidence was obtained that showed that PTH affected the activity of the lining cell-osteocyte complex.

An important problem that is relevant to this controversy is the supersaturation of plasma and extracellular fluid (ECF) relative to bone mineral. The primary investigator in this field was William F. Neuman, a physical chemist who insisted that this solubility problem must be solved before calcium homeostasis could be understood. An important observation was that even in the absence of PTH, free calcium levels were highly supersaturated with respect to bone mineral. In 1982 Neuman and his associates reported the isolation of two bone surface proteins, which increased the solubility of the bone mineral hydroxyapatite in the absence of cells[1,2]. Unfortunately, these reports were published after his death in 1981 and did not receive much attention.

In the 20 years following Neuman's death, there was a hiatus in the study of calcium homeostasis, which resulted in continued acceptance of the osteoclast theory of parathyroid action. This acceptance of the osteoclast theory is reflected in two recent articles[3,4], which do not mention any other possibility. The primary exception to this was Michael Parfitt who stressed the necessity for including bone surface activity in solving the problem of calcium homeostasis[5,6]. At the turn of the century, however, activity in this area increased when Talmage and his associates proposed a new hypothesis for calcium control based on the activity of non-collagenous proteins on bone surfaces[7]. For a discussion of this hypothesis see[8]. In 2003 the journal, Bone, published together two opposing perspectives on calcium homeostasis by R. Heaney and M. Parfitt[3,9], which confirmed the fact that the controversy concerning calcium control was not over. In the past year a study reported by Marenzana et al.[10] demonstrated the speed and magnitude of the changes in the fluxes

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Corresponding author: Roy V. Talmage, Buckner Westminster Place, Apt. 211, 2201 Horseshoe Lane, Longview, TX 75605, USA
E-mail: rvtalmage@aol.com

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between ECF and bone mineral. Using a scanning electrode technique they found evidence for instantaneous calcium efflux from bone that was not due to resorption. This paper stressed the importance of the bone mineral surfaces in controlling the minute-by-minute concentration of free calcium in the ECF.

The purpose of our report is to review the evidence that the control of calcium homeostasis is centered at bone surfaces and that PTH controls the calcium level in the plasma and ECF by causing the bone lining cells to release substances that increase the solubility of bone mineral.

Chemistry and historical background

The solubility of the calcium phosphate in human bone is approximately 7 x 10^{-5} M at physiological pH and temperatures. Yet the concentration of free calcium in the plasma and extracellular fluid (ECF) is more than 10^{-3} M. This can be explained in part by the fact that the concentration of the PO_4^{3-} ion is very low in plasma and that most of the phosphate is in the form of the HPO_4^{2-} ion. But the problem is more complex than that because bone mineral added to plasma will remove most of its calcium.

The element calcium is abundant in nature but its concentration in aqueous solutions is limited due to the low solubility of most of its common salts, the most common of which are those combined with phosphates and carbonates.

$CaHPO_4$ under physiological conditions has a solubility of approximately 2 x 10^{-3} M or 10 mg of calcium per 100 ml. All living organisms must adapt to this low solubility of calcium salts to survive. The concentration of calcium ions inside most animal cells is very low (1 X 10^{-7} M) and therefore cellular calcium is not a solubility problem. But this also means that it would be very difficult, if not impossible, to rapidly transport calcium through cells. Calcium homeostasis is a problem of controlling the free calcium level in the ECF and plasma. The level in both compartments must be maintained at approximately 10^{-3} M or 5 mg of free calcium ions per 100 ml of fluid.

One of the unique characteristics of calcium is its ability to complex with organic compounds, particularly proteins. The amount of calcium that a protein can bind depends upon the composition and conformation of the protein. The binding of calcium to proteins is reversible and is therefore in a state of equilibrium. Because of the low concentration of calcium in the ECF, most proteins in contact with the ECF are not saturated with calcium. In plasma approximately one-half of the calcium is bound to protein (mostly albumin), which makes the total plasma calcium approximately 2 x 10^{-3} M or 10 mg per 100 ml.

Proteins are major constituents of cell membranes and most of these proteins are in contact with the ECF. It follows that the amount of calcium bound to membrane proteins is dependent upon the concentration of free calcium in the ECF. Bound calcium on membrane proteins influences the strength and function of membrane proteins. This is why it is necessary to control the concentration of free calcium in the ECF.

Vertebrates appeared around 500 million years ago and were the first to have an internal living skeleton. The extremely insoluble salt used was the crystallized calcium phosphate, hydroxyapatite. This skeletal compound was inside the animal but outside the cells. Accordingly it was in direct contact with the ECF. This arrangement produced a major chemical problem. Although the ECF is under saturated with respect to $CaHPO_4$ it is supersaturated with respect to bone. This condition can easily be demonstrated by adding a small amount of hydroxyapatite or bone mineral to a sample of ECF or plasma. After the sample is shaken and centrifuged the supernatant quickly loses most of its calcium. This means that if the ECF comes in contact with bone mineral, the calcium in the fluid should be rapidly removed. The animal would cease to exist. Obviously vertebrates would not have appeared on the planet if this problem had not been solved millions of years ago.

The role of parathyroid hormone (PTH)

The fact that land vertebrates and those descended from them are the only animals to possess parathyroid glands, suggests that the hormone came into being as a supplement to an existing process. This is also indicated by the observation that after parathyroidectomy (PTX) land vertebrates still can maintain a free calcium concentration in the ECF that is at least 10 times the solubility of bone mineral, a drop of approximately one-third from that seen in intact animals. Examples of this can be seen in the human, frog, rat and dog[7,11-13].

One of the earliest observed effects of PTH injection in animals or the effect of elevated hormone activity in humans was an increase in the number and activity of cells identified as osteoclasts. The clinical observation was that the activity of osteoclasts and the elevation of plasma calcium occurred simultaneously. It was not surprising that it was concluded that one process caused the other, and that the calcium released by osteoclasts following the action of PTH supplied the calcium that caused the increase in plasma calcium levels. This unconfirmed assumption is still widely held in clinical circles today[3,4].

However, it has been adequately demonstrated that plasma calcium remains normal even when the number of osteoclasts is greatly reduced or inactive as in osteopetrosis, or when their number is significantly increased as in Paget's disease. Plutonium destruction of osteoclasts did not cause a drop in blood calcium. These studies were carried out both in dogs[14] and in rats[15]. Also, an increase in the activity of osteoclasts by prostaglandin E_2 did not raise the blood calcium in PTX rats[16]. This study also showed that the calcium released by increased osteoclast activity was too small to have a significant effect on free calcium concentration. Finally recent studies[17,18] in both humans and other mammals have shown that antibodies to RANK-Ligand (RANKL), a key regulator of osteoclastic activity, were able to achieve prolonged near total blockage of osteoclast-driv-

en markers of bone resorption. Nonetheless, blood calcium levels were still maintained within the range of normal values. However, hypocalcemia can be produced in osteopetrosis by deprivation of the calcium in the diet[19] and there is no doubt that in cases of severe hyperparathyroidism the increased osteoclast activity does major damage to bone. All these effects of osteoclast activity require time. Therefore, it must be concluded that, while PTH does directly affect osteoclast function, this activity is separate from the action of the hormone on minute-by-minute calcium homeostasis. The possibility that PTH had two distinct actions in bone was first suggested by Doty et al. in 1965[15] and further elaborated by Talmage[20].

Another consideration in PTH action is that the hormone has been shown to affect the anatomy and metabolism of both osteocytes and the lining cells on the surfaces of bone mineral. Matthews and Talmage[21] reported this effect of PTH with electron micrographs of non-decalcified bone and a diagrammatic representation of the changes in the lining cells. This has been confirmed by later work[22,23], which identified specific receptor sites for PTH on lining cells and their underlying osteocytes.

A third point is that in the absence of PTH, calcium is maintained in an equilibrated state in the ECF. This equilibrium is set locally on bone surfaces and any process which elevates plasma calcium must increase the level of equilibrium at these surfaces. In support of this surface action of PTH, studies have shown that after a PTH injection the last calcium that entered bone was the first calcium to be returned to the ECF[24-25].

Early work by William Neuman

The problem of calcium ion supersaturation was a topic of considerable research during the middle of the last century. One of the pioneers studying this problem was William Neuman, a physical chemist at the University of Rochester. He spent most of his professional career studying this problem. Of particular interest were his early reports that were all related to the problems of blood bone disequilibrium[26-29]. In these studies he asked three important questions: (1) Was there a separate bone fluid compartment? (2) Was the pH of the fluid at the surfaces of bone lowered to permit increased solubility of bone mineral? and (3) Were there other more soluble calcium phosphate salts in bone which would allow for the release of calcium into the ECF? The answer to the first two questions was in the negative. The development of the use of the electron microscope by biologists showed that the lining cells on the surfaces of bone were connected by gap junctions, not tight junctions[21]. Therefore, fluid and calcium could move between cells to the surfaces of bone.

Despite these findings the membrane model still persists and was preferred by Rubinacci's group in their recent paper[10]. In this model the bone ECF is separated from the other ECF by a membrane containing the lining cells and calcium is pumped across the membrane. The problem with the membrane model is that it cannot explain the speed with which large amounts of calcium are transported or the source of energy for this transport. In 1992 Bronner and Stein[30] reported that this concept of an intracellular calcium pump was energetically implausible. Nor can the pump concept explain Neuman's finding of non-collagenous proteins that increase the solubility of bone mineral in the absence of cells. The gradients in ion concentration on which the membrane model is based (a six-fold increase in potassium and three-fold reduction in calcium) indicate an obvious contamination of the bone ECF sample with cellular content. The finding that calcium efflux can be blocked by cyanide is difficult to interpret because cyanide may have other chemical effects than the destruction of cells.

For all these reasons it remains unclear exactly what the role of the lining cell is in the control of calcium homeostasis. This is an obviously important area for future research.

Neuman's third question could be answered in the affirmative since there are calcium phosphate salts that are more soluble than hydroxyapatite, but their location is primarily in areas of bone formation.

Early physiological studies bearing on the solubility problem

During the early period of Neuman's work, three groups of investigators reported several relevant studies[12,13,32]. All three groups applied a low calcium stress to either rats or dogs, and recorded the return of plasma calcium values to their previous levels. Both control and parathyroidectomized (PTX) animals were used. The plasma calcium values of control animals returned to the previous control levels and that of PTX animals returned to their previous levels, which were 2 to 3 mg/100 ml lower than controls. The studies of Talmage and his associates[31,32] utilized a procedure of peritoneal lavage in nephrectomized rats. They showed that continuing the peritoneal lavage procedure for twelve or more hours did not diminish the rate of removal of calcium from bone. They also determined the quantity of calcium removed. If the results were extrapolated to humans on the basis of relative weight, they indicated a removal of calcium from bone of approximately 6 gm per hour even in PTX animals. The same type of control exists in other land vertebrates, including humans[6] and amphibians[11].

A few years later, Cooper et al.[33] reported a study in which the shafts of rat femurs were incubated in plasma with temperature and pH controlled. The shaft is cortical bone, which in the rat contains few, if any, osteoclasts. If the starting value for calcium in the plasma was normal, this value dropped during incubation to the level observed in PTX rats. If calcium was removed from the plasma before incubation, the concentration of calcium rose to that same level. They also reported a similar result if the shaft was subjected to a sequence of freezing and thawing in dry ice. This procedure killed the cells with minimal loss of organic material.

In 1993, Parfitt[6] published A. Dhem's ^{45}Ca radioauto-

graphs which demonstrated that injected calcium is rapidly adsorbed to all the bone surfaces accessible to the circulation. This explained why the exchangeable pool of injected calcium is much greater than the size of the ECF pool as measured by tagged sodium.

The sum total of these studies demonstrated that bone surfaces were able to continuously supply calcium to the ECF far above the solubility of hydroxyapatite, or to remove it from plasma as required to maintain normal plasma calcium levels. At that time no explanation could be given for these results.

Non-collagenous proteins on bone mineral surfaces

The presence of proteins on the surfaces of all bone mineral is now well documented. However, until recently, the emphasis has been on the relationship of these proteins to the process of bone formation[34]. There have been at least eight different proteins identified in metaphyseal bone, many of which are known to contain considerable amounts of calcium and sodium[35,36]. However, Neuman and his associates reported in 1982 that they had isolated two proteins from cortical bone[2]. And within the last ten years it has been demonstrated by electron microscopy that these proteins do indeed cover bone mineral surfaces[36]. We believe that these proteins provide the solution to the problem of the supersaturation of bone ECF in its relationship to bone mineral. For without these proteins there is no chemical reaction known that would allow the continued existence of this supersaturation.

The solution to the problem of fluid-bone supersaturation

The material given in the preceding sections provides evidence that calcium ion concentrations in the bone ECF are elevated above the solubility of hydroxyapatite because of the presence of non-collagenous proteins in the bone ECF and on the bone surfaces. Neuman's group in 1982[1,2] first proposed that this process might be the key to the control of calcium homeostasis. They extracted two non-collagenous proteins, osteocalcin and osteonectin from compact bone taken from the shaft of the rat femur. When these proteins were added to a buffered solution containing bone mineral the calcium and phosphate content of the solution immediately rose. They also reported a reduced adsorption of tagged calcium and phosphate ions into the bone mineral. From these studies they concluded that the proteins increased the solubility of hydroxyapatite. Neuman's group isolated only two of the many non-collagenous proteins now known to exist on bone surfaces. It is unfortunate that Neuman was not able to finish his study and receive an appropriate credit for solving the problem of calcium homeostasis.

Solubility is a reflection of an equilibrium between the fluid and solid phases of a compound and indicates the maximum concentration of the compound that can exist in the fluid phase. In the reaction between soluble calcium and phosphate and insoluble bone the equilibrium is produced by the equality of the rates of movement of ions off and on the bone. If a compound such as calcium phosphate undergoes ionization the solubility product is the maximum product of the concentrations of the electrolytes, which at one temperature can continue in equilibrium with the solid phase. Neuman's proposal of an increased solubility implies that the protein changed the solubility of bone mineral by shifting the equilibrium between the soluble electrolytes and the solid phase.

Recently, R. Talmage and his associates[7] modified and extended the hypothesis that calcium homeostasis was achieved by the action of bone surface proteins setting a new, elevated equilibrium for the calcium ion in bone ECF. According to this new concept, equilibrium is achieved by a transfer of calcium from bone mineral to protein, and from the protein to the bone fluid. They suggested that this rapid two-step flux is balanced by a rapid movement of calcium back into bone caused by the excessive supersaturation of the fluid with respect to bone. Also in line with this concept are the results of Neuman's group that one of the active proteins, osteonectin, is phosphorylated and thus provides a possible source of energy for the accelerated elution of calcium and phosphate from the bone. In addition, Neuman's finding of a reduced adsorption of labeled ions suggests that, in addition to the acceleration of the elution, one of the proteins may reduce the rate of adsorption. Both of these actions would combine to shift the equilibrium toward higher solubility.

One of the remarkable aspects of the Neuman study was the speed of the reaction between calcium and bone. This has been supported by studies showing the speed by which tagged calcium is removed from plasma. The rapidity of these fluxes (bone vs. ECF and ECF vs. plasma) insures that any change in the plasma calcium level must reflect a change in the equilibrium set at the surfaces of bone.

Grubb et al. have demonstrated that PTH acts first by returning to plasma the last calcium entering bone mineral[25]. This rapid removal of just adsorbed ions might simulate the effect of a reduced rate of adsorption. This question could be answered by careful analysis of adsorption and elution curves.

Summary

This report summarizes the evidence that the control of the concentration of free calcium ions in body fluids is centered at bone mineral surfaces. This process involves changes in the solubility of hydroxyapatite produced by the noncollagenous proteins existing in the bone ECF and on the adjacent surfaces of bone. There is a basic equilibrium level produced in the absence of PTH, which is well above the solubility of bone mineral. It appears that the action of PTH involves an increase in this solubility altering process, but the mechanism by which this is accomplished remains to be discovered.

In conclusion, we hope that this report will stimulate

research into the mechanisms of calcium solubility. This should include experiments to determine the function of the two proteins isolated by Neuman and determine if the phosphorylated protein, osteonectin, provides the energy source that is needed to explain the rapid elution of calcium postulated by Talmage and his associates[7]. We hope there will be other experiments to determine the relationship of the stimulation of lining cells by PTH to the increased solubility of bone. Another area of needed research is the role of PO_4^{3-} and HPO_4^{2-} ions in the solubility problem and the relationship of these ions to PTH function.

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