PTH Regulation by Phosphate and miRNAs

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Introduction

Beyond the steady rise in the regulating processes being discovered in the last decades for PTH, the far-known canonical function is the minute to minute regulation of extracellular calcium concentrations. Thus, the main natural canonical effector able to regulate parathyroid function, and specifically PTH secretion, is calcium itself. Hypocalcemia sensed trough plasma membrane calcium sensing receptors (CaSR) lead to an increase in PTH release which, in turn, acts on target tissues to restore normal calcium levels. Vitamin D, i.e. calcitriol, the natural active form, by acting through specific intracellular vitamin D receptors (VDR), is also able to regulate PTH synthesis. Hypocalcemia leads to an increase in vitamin D levels to enhance intestinal calcium absorption; then after the calcemia is restored, vitamin D inhibits the PTH synthesis in a safe-guard feedback manner.

This dual calcium-vitamin D model would appear robust enough to cope with the regulation of the parathyroid function. However, compelling clinical and experimental evidences pointed insistently to look at an old well-known key player of the mineral metabolism, phosphate, as a putative new direct effector of the parathyroid glands. But it has resulted highly difficult to state clearly a mechanism of action for the phosphate. In fact, despite the significant efforts made

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by researchers to find out a specific phosphate sensor it remained quite elusive. Recent data, however, may be knocking the door to come into the discovery of a receptor able to sense phosphate levels. Furthermore, along the elucidation of the effects of phosphate there was sometimes the perception of being in the footsteps of a ghost, since the observed alterations in the parathyroid function due to the increase in the phosphate burden was not always associated with an elevation in the serum phosphate levels. And here is where the fibroblast growth factor 23 (FGF23), a phosphatonin that favors renal phosphate excretion, took a preponderant role lasting from the last two decades. In part as a surrogate of high phosphate levels with the capacity to be sensed by parathyroid glands through specific receptors (a complex of the FGF23 receptor, FGFR1, with the co-receptor α-klotho), but also by targeting other organs involved in the homeostasis of the mineral metabolism. This chapter will cover the mechanisms whereby high phosphate modulates the secretion of PTH. And it will also cope with the role of miRNAs, perhaps the last guest star mechanism found to regulate PTH secretion, and which might open new therapeutic prospects.

The Direct Effect of Phosphate on PTH Secretion

From far, clinical and experimental data pointed to phosphate as a modulator of the parathyroid function. Hyperphosphatemia was consistently associated to the production of uremic hyperparathyroidism (secondary hyperparathyroidism, SHPT). Thus, both restriction of dietary phosphorus and chelation of phosphorus by binders are effective strategies in the prevention and treatment of SHP. However, it was certainly difficult to demonstrate an independent effect of phosphate on parathyroid cell function, especially in vivo. The beneficial effect of lowering the phosphate load, e.g. through a low phosphate diet, could be attributed to concomitant changes such as the stimulation of calcitriol production [1–3] and the improvement of hypocalcemia due to an increase in the calcemic response to PTH [3–5]. In an attempt to overcome these physiological constraints, Lopez-Hilker et al. showed that dietary phosphorus restriction improved renal hyperparathyroidism in dogs independent of changes in calcitriol and serum calcium levels [6]. But, in any case, the undoubted stating of a direct effect of phosphate would require in vitro studies.

Some authors had successfully used rat parathyroid gland in organ culture to test the effect of 1,25-dihydroxyvitamin D, cortisol and calcium on PTH secretion [7–9]. However, the first attempts by using parathyroid cell lines or dispersed cells from primary cultures failed to show it. The first study to report a clear direct effect of phosphate on PTH secretion was that of Almaden et al. [10] performed in whole rat parathyroid glands, which was confirmed promptly by other authors in the same model [11]. Then, Nielsen et al. [12] showed this in bovine parathyroid glands and confirmed that it was only observed in tissue with intact architecture. Interestingly, though isolated cells had been shown to be less responsive to

changes in extracellular calcium due to a progressive decrease in the expression of the CaSR along the culture time [13–15], both the bovine dispersed cells and tissue preparations responded to changes in the calcium concentration. Furthermore, though an effect of phosphate was observed in dispersed parathyroid cells [16], it was accounted to the presence of cell clusters with close cell-to-cell interaction. To date, however, there is not a clear explanation of why cell-to-cell interaction is important to observe an effect of phosphate on PTH secretion. Sun et al. [17] demonstrated that parathyroid cells in close proximity are stimulated to secrete more PTH and suggest the presence of a paracrine interaction among parathyroid cells. Intercellular communication might be also required to observe an effect of phosphate on PTH secretion. Later studies using a pseudogland model of parathyroid tissue grown in collagen [18] also showed the importance of the 3-D tissue architecture in parathyroid gland function.

The findings by Almaden et al. [10] in normal rat parathyroid glands indicated that a phosphate concentration of 4 or 3 mM increased the basal rate (calcium 1.25 mM) of PTH secretion but it did not increase further the maximal rate of PTH secretion induced by low calcium. Therefore, a high phosphate concentration maintains an abnormally elevated PTH secretion rate despite a normal extracellular calcium level, but it did not further increase PTH secretion when parathyroid glands are maximally stimulated by low extracellular calcium levels. Thus, in vitro, a high phosphate level shifted the PTH-calcium curve to the right, making parathyroid cells less sensitive to inhibition by calcium. This may explain, at least in part, the increase in the set point of the PTH calcium curve observed in hyperphosphatemic patients with uremic hyperparathyroidism.

A later study by Almaden et al. [19] addressed the effect of high phosphate on the secretion of PTH in human hyperplastic parathyroid glands. These parathyroid glands have frequently areas of nodular growth and possess a decreased number of vitamin D and calcium sensor receptors [14, 20]. Thus, they are less responsive to calcium and calcitriol so that the concentration of calcium required to inhibit PTH secretion is greater than normal [21, 22]. Experiments were performed using small pieces of parathyroid glands. In diffuse hyperplastic tissue, a high concentration of phosphate in the incubation media prevented the calcium-induced inhibition of PTH secretion. This effect was more marked with 4 than 3 mM P, suggesting a dose-response effect. In nodular hyperplasia, high phosphate reduced the ability of high calcium to inhibit the PTH secretion; however, the reduction of PTH secretion by calcium was not significantly different between 3 and 1 mM phosphate. Therefore, it was demonstrated in an in vitro setting that high phosphate level stimulates PTH secretion independently of a low calcium concentration and a calcitriol deficiency, which are usually present in uremic patients.

Further demonstration of a direct effect of phosphate on PTH secretion was obtained in in vivo studies in hemodialysis patients [23] and in dogs [24]. Interestingly, these in vivo studies also demonstrated that the effect of phosphate on PTH secretion is dose-dependent; however, the degree of PTH response to phosphate is much lower than to calcium.

The direct stimulatory effect of phosphate on PTH secretion is rapid. In the in vitro setting, it was observed after 2 hours of incubation (unpublished results from our laboratory). In vivo, in uremic rats adapted to a high phosphate diet (HPD), a switch to a meal of low phosphate diet (LPD), caused a decrease in 80% of serum PTH within the 2-hour feeding period with no change in plasma calcium but a 1 mg/dl fall in plasma phosphate [25]. In contrast, HPD gavage increased PTH by 80% within 15 minutes with no change in plasma phosphate or calcium. Furthermore, duodenal and intravenous infusion of sodium phosphate increased PTH within 10 minutes, whereas infusion of sodium chloride had no effect.

Phosphate and PTH Gene Expression

Soon after the demonstration of a direct effect of phosphate on PTH secretion, the search of the underlying molecular mechanisms began. The first issue to be addressed was the possible effect of phosphate on PTH synthesis. In fact, even before the direct effect of phosphate on PTH secretion was uncovered, a number of in vivo studies with different rat models suggested that PTH synthesis was affected by dietary phosphate manipulation in early chronic renal failure [26] and hypophosphatemic rats [27]; a high serum phosphate level was associated with increased PTH mRNA It was also shown that in normal rats a high phosphate diet increased PTH mRNA independent of calcium and CTR levels [28]. Subsequent, in vitro studies with hyperplastic parathyroid tissues from hemodialysis patients showed that the stimulation of PTH secretion by high phosphate levels (4 mM) was accompanied by an increase in PTH mRNA in both diffuse and nodular hyperplasia [19].

In any case, the thorough studies performed by the group of Silver and Naveh-Many along a decade on the regulation of PTH synthesis by calcium and phosphate lead to the conclusion that the effects were post-transcriptional, as shown by nuclear transcript run-on experiments [27, 29, 30]. Thus, calcium and phosphate regulate PTH gene expression by changes in protein-PTH mRNA 3′-untranslated region (UTR) interactions, which determine PTH mRNA stability. By combining in vivo experimental models of hypocalcemic and hypophosphatemic rats with in vitro mRNA degradation assay (IVDA) this group identified both the cis-acting sequences and the trans-acting factors involved in PTH mRNA stabilization and/or decay. There was an approximately 60-fold difference in PTH mRNA levels between hypocalcemic and hypophosphatemic rats.

In a first study, these authors found that a number of parathyroid cytosolic proteins bind to a conserved *cis*-acting element in the parathyroid hormone 3′-UTR [29]; the binding being dependent upon the terminal 60 nucleotides. Parathyroid proteins from hypocalcemic rats showed increased binding and proteins from hypophosphatemic rats showed decreased binding that correlated with PTH mRNA levels. Through IVDA they showed that a PTH mRNA probe maintained intact for 180 minutes after being incubated with cytosolic proteins from hypocalcemic rats

but only for 5 minutes in the presence of hypophosphatemic proteins; conversely parathyroid proteins from control rats led the transcript to steady for 40 minutes, while a transcript lacking this region showed no degradation in the presence of hypophosphatemic proteins. Importantly, it was also shown that upon incubation with parathyroid proteins from uremic rats, the PTH mRNA was not degraded at all after 120 min and was moderately decreased at 180 min [30]. Further studies delimited the PTH mRNA-protein binding region to a minimum sequence of 26 nucleotides shown to be necessary and sufficient to confer responsiveness to calcium and phosphate through the regulation of PTH mRNA stability [31].

After the 3′-UTR regulatory region was identified, it was the turn for the specific proteins that bind it. The first was the 50 kDa AU-rich binding (AUF1) protein, which was able to bind to the PTH mRNA 3′-UTR and stabilize the PTH transcript [32]. Interestingly, calcineurin regulates AUF1 post-translationally in vitro and PTH gene expression in vivo but still allows its physiological regulation by calcium and phosphate [33]. The Upstream of N-ras (Unr) protein was shown to be another PTH mRNA 3′-UTR binding protein as part of the parathyroid RNA binding complex [34]. Furthermore, the mRNA decay promoting K-homology splicing regulator protein (KSRP) appeared as a master key in the PTH mRNA post-transcriptional regulatory complex [35]. The binding of KSPR to the 3′-UTR PTH mRNA is decreased in glands from calcium-depleted or experimental chronic kidney failure rats in which PTH mRNA is more stable, compared with parathyroid glands from control and phosphate-depleted rats in which PTH mRNA is less stable. Of note, the activity of KSRP is regulated via its interaction with the peptidyl-prolyl isomerase (PPIase), Pin1, which led to KSRP dephosphorylation and activation [36]. Pin1 activity is decreased in parathyroid protein extracts from both hypocalcemic and CKD rats.

Taken together all these pieces of information, the model whereby phosphate modulates post-transcriptionally the PTH synthesis begins to emerge. Thus, in low serum phosphate conditions active Pin 1 lead to KSRP dephosphorylation and activation, favoring its association with the PTH mRNA 3′-UTR ARE and preventing the binding of the stabilizing complex consisting of AUF1 and Unr. The result is the recruitment of the exosome leading to PTH mRNA decay and then to decreased PTH production [37, 38]. Interestingly, the same mechanism but working in an inverse sense, governs the regulation of PTH gene expression in hypocalcemia and CKD, where Pin 1 activity is reduced favoring the binding of AUF1 and Unr to inhibit PTH mRNA degradation. In any case, this picture still appears incomplete so as it is pending to connect the sensing processes with the regulation of Pin 1 activity and the possible contribution of other new players. Therefore, by contrast to the regulation of PTH synthesis at the transcriptional level defined for the vitamin D, involving the VDR and the VDRE sequences in the *p*th gene, a post-transcriptional mechanism related to cytosolic endonuclease activity, resulting in a more or less stable PTH transcript, appears to account for the effect of calcium and phosphate in the normal and uremic settings. Importantly, this post-transcriptional mechanism appears to be in accordance with the short time taken for the high phosphate levels to increase PTH release.

Searching for a Phosphate Sensor

The way the living organisms cope with phosphate sensing is highly dependent on the compartment in which the phosphate levels must be regulated and thus, two key models are distinguished. The metabolic phosphate sensing functions to maintain levels of phosphate in the intracellular compartment to support cellular metabolism, while the endocrine phosphate sensing drives the homeostatic regulation of phosphate in the extracellular compartment in multicellular organisms [39]. Phosphate sensing mechanism in bacteria and yeast is mainly based on plasma membrane proteins able to modulate phosphate uptake and the activation of signal transduction pathways. Interestingly, metabolic phosphate sensing in multicellular organism as mammals have been also shown to be related to plasma membrane transporters as the type 3 sodium- dependent phosphate transporters PIT1 and/or PIT2, ubiquitous suppliers of phosphate to the cell. However, it has been difficult to identify the endocrine phosphate sensing in mammals [40], which extends to that of parathyroid cell.

Due to the lack of a known specific phosphate sensor in the parathyroid cells that could explain the direct effect of phosphate on the parathyroid function, during the last decades a number of phosphate transporter systems, with a special role for the PIT ones, were proposed to be involved. In fact, a phosphate uptake- independent signaling function of PIT1was reported to be important for VSMC processes mediating vascular calcification [41]. Through this so-called single sensor phosphate hypothesis, the transporter would work together with a co-receptor, as found in the osteocyte to regulate PTH secretion with the participation of FGFR1upon stimulation with phosphate [39]. By the contrary, the so-called multiple sensor hypotheses imply the existence of a second independent sensor, as might be the case for the parathyroids, which has maintained elusive up to now. However, a study by Geng et al. [42] appears to shed new light regarding the phosphate sensing in the parathyroid cells. And, amazingly, it seems to operate at the very core of the regulation of the parathyroid function since it concerns to the CaSR itself. In fact, this outstanding study confirms previous views on this receptor, but also opens new paradigms.

Though extracellular calcium was initially recognized as the specific agonist of the CaSR, it was promptly found out a wide distribution and functional plasticity derived from its ability to bind to other different ligands (including various divalent and trivalent cations, polyamines and cationic polypeptides), and to activate different G proteins-downstream signaling pathways. A main point also came after the demonstration that L-amino acids were allosteric activators able to activate it provided that calcium concentration is above a threshold [43]. Now, as revealed by X-ray crystallography-derived crystal structure of the entire extracellular domain of CaSR in the resting and active conformations, Geng et al. [42] found novel binding sites for calcium, phosphate (PO_4^{3-}) and L-Trp and identified L-Trp as an agonist of the receptor, demonstrating that these ions and amino acids collectively control the function of the CaSR.

The functional CaSR is a disulfide-tethered homodimer composed of three main domains, the Venus Flytrap (VFT) module (that includes two domains, LB1 and LB2), a cysteine-rich domain and the seven-helix transmembrane region. As demonstrated by Geng et al. [42], binding of amino acids, as L-Trp, to specific binding sites facilitates extracellular domain closure of CaSR, a crucial first step during activation, by contacting both LB1 and LB2 domains of the VFT module. Thus, L-Trp directly activates CaSR-mediated intracellular calcium mobilization in the presence of extracellular calcium, which is completely abolishes by the mutation of the L-Trp-binding residues. Therefore, calcium ions are not the main activator of the receptor, since the CaSR maintains an inactive state in the absence or presence of calcium ions, and it is only after the amino acid is bound that the active state forms. Calcium binds to up to four sites with different occupancy and affinity levels resulting in the stabilization of the active conformation of the receptor by facilitating homodimer interactions between the membrane-proximal LB2 and CR domains. Importantly, the effect of amino acids and calcium depends on the each other since a level of calcium is needed for amino acids to activate the CaRS, while this increase its sensitivity toward calcium to gain in stability. Therefore, amino acids appear as orthosteric agonists of CaSR, and they act concertedly with calcium to achieve full receptor activation.

But perhaps the most exciting finding described by Geng et al. [42] concerns to the role of anions on the CaSR function. Contrary to the effect of calcium, phosphate (PO_4^{3-}) reinforce the inactive conformation. They identified a total of four anion-binding sites in the inactive and active structures; sites 1–3 located above the interdomain cleft in the LB1 domain, and site 4 as part of LB2 domain. In the inactive structure, anions bound at sites 1–3 but in the active structure, only sites 2 and 4 are occupied. Binding of phosphate to the active form at site 2 and 4 leads to a negative modulatory effect on the CaSR activity and the concomitant decrease in CaSR-mediated IP accumulation.

All these novel findings depict the functional activity of the CaSR as the result of complex relationships with at least three key ligands to modulate the inactive and active states. In the resting state, L-amino acids induce VFT closure favoring the formation of homodimer interface between subunits, while calcium binding stabilize the active state by enhancing homodimer interactions to fully activate the receptor. By contrast, phosphate binding prevent activation by promoting an inactive configuration. Importantly, these mechanistic findings meet some previous questions as the requirement of a threshold of calcium to observe the effect of phosphate on PTH secretion and previous observations that phosphate makes parathyroid cells less sensitive to inhibition by calcium. And also provide a putative mechanism for the role of polycations as polyamines, which might favor the dissociation of phosphate from the relatively weak anion-binding sites and thus prevent its inhibitory effect. Finally, since the effect of phosphate on the parathyroid cells appears mediated by a cell membrane protein, it may be affected by the tissue digestion required for cell dispersion, which would be the reason of the failure to demonstrate an effect of phosphate on PTH secretion in isolated parathyroid cells.

Regulation of PTH Secretion by miRNAs

MicroRNAs (miRNAs) are small non-coding RNAs involving in post-transcriptional regulation of gene expression. Through interfering RNAs, miRNAs exert a fine-tuning of gene expression and thus contribute as important regulators of numerous physiological and pathological mechanisms. Identification of miRNA functions can indicate novel targets for biological processes and may have significant value as biomarkers of disease etiology and progression [44–46].

The first approximations to study the involvement of miRNAs in the parathyroid cell function were performed in the setting of abnormal parathyroid growth as carcinomas and adenomas. As compared to normal or adenoma tissues, carcinoma tissues showed a specific pattern of *miRNoma* consisting of a set of down-regulated miRNAs as miR-26b, miR-30b, miR-139, miR-126–5p, and miR-296 [47], while others as miR-222, miR-503 and miR-517c appeared up-regulated [48, 49]. As expected, after looking for the putative target genes of these miRNAs, a number of genes related to the regulation of cell growth and malignancy were evidenced. Though undoubtedly this can be related to the regulation of PTH secretion through a role in the development of parathyroid hyperplasia, in the last recent years, two studies by the Naveh-Many's group addressed and uncovered a more direct involvement of miRNAs on PTH secretion [50, 51].

In the first study, Shilo et al. [50] used parathyroid-Dicer$^{-/-}$ mice (unable to produce mature miRNAs in the parathyroid glands) that showed no alterations in the key mineral metabolism parameters since they had normal calcium, phosphate and PTH. When they were subjected to an experimental acute hypocalcemia (by EGTA administration), they failed to show a full increase in PTH as the controls did. Of note, it was also replicated in vitro by incubating the thyroparathyroid glands in a calcium-depleted medium. These authors also demonstrated that a chronic challenge of hypocalcemia, by feeding the parathyroid-Dicer$^{-/-}$ mice with a calcium-depleted diet, resulted in a much-reduced increase in PTH, one third of the response observed in the control mice; and, importantly, there was no increase in PTH mRNA. Also, of special interest is that, by contrast to what it happened in the controls, there was no stimulation of parathyroid proliferation in the defective animals. Finally, Shilo et al. [50] evaluated these events in the setting of a rat model of uremia and SHP induced by an adenine high phosphate diet. In spite of having similar levels of uremia, again the uremic counterparts failed to increase PTH to the same extent as in the control mice (in a two to four-fold relationship), as well as the parathyroid proliferation. However, it was extremely interesting that when they assessed the response of the parathyroid-Dicer$^{-/-}$ mice to a hypercalcemic challenge (by an i.v. injection of calcium gluconate), they found a normal inhibition of PTH secretion, similar to that of controls. And it was correspondingly replicated after treatment with calcimimetic. These comprehensive results showed that the normal response of parathyroid glands to both hypocalcemia (acute and chronic) and uremia (involving induction of PTH secretion and proliferation) is dependent on the dicer activity and, then of specific miRNAs. The conserved

CaSR sensitivity to hypercalcemia of PT-Dicer1$^{-/-}$ cells indicates that the parathyroid functions related to the gene expression/activation of the CaSR appears not to be under the regulation by miRNAs.

In a second significant study, Shilo et al. [51] first profiled miRNAs in normal mouse, rat and human parathyroid glands by small-RNA sequencing. They found conservation of expression of miRNAs among species; among the 50 most abundant sequence families in human parathyroid, 37 were also top 50 in mouse and 39 in rat. let-7 members were the most highly expressed ranging 23−32% and then the miR-30 (8.9–14%) and miR-141/200 members (4.5–8.5%). These similar profiles of abundant miRNAs, suggest an evolutionarily conserved regulation of functions in parathyroid physiology. Then, these authors studied the function of some of these specific miRNAs in parathyroid glands from patients receiving dialysis and from experimental uremic models of SHP as a short-term uremia (induced by 1-week adenine diet with high-phosphate content), an intermediate/long-term uremia (6–8 weeks of the diet), and a hypocalcemia (induced by a 3-week feeding with a low-calcium content diet). As compared to the normal glands, mayor *miRNome* alterations were found. Of the six most abundant miRNAs families in rat parathyroid, four families (miR-30, miR-148, miR-141, and miR-21) were significantly up-regulated in SHP, whereas two (let-7 and miR-375) were no significantly down-regulated. Interestingly, while some miRNAs alterations were shared by all SPH models, other specific patterns were dependent on the cause of SHP. And, of note, in a dose-response manner, the more severe the experimental SHP was, the more outlying the miRNAs profiles were. Thus, miRNAs families were gradually up-regulated or down-regulated following a progressive trend from the early CKD to the hypocalcemia (as an intermediate CKD) and then to the late CKD.

Then, Shilo et al. [51] studied the effect of inhibiting specific miRNAs, as the abundant let-7 family, by injecting antagonizing oligonucleotides twice weekly for 4 weeks. Treatment with anti–let-7 oligonucleotides increased serum PTH in both normal and CKD rats. In accordance, let-7 anti-miRNAs added to the growth medium of mouse thyro-parathyroid organ cultures increased PTH secretion. Thus, the let-7 family members were suggested to regulate PTH by restraining PTH production or secretion. By contrast, administration of anti–miR-148 led to significant decrease in serum PTH in the CKD rats, which also was corroborated in vitro in parathyroids from CKD mice; thus, indicating that the miR-148 members promote PTH secretion.

Undoubtedly, these pioneer studies ascertaining the contribution of specific miRNAs in PTH secretion throughout the SHP pathogenesis are just opening a hopeful future in that they can be manipulated to potentially manage the disease. In fact, it is already being extending to other related branches of the disease. Thus, as compared to healthy controls, in patients with CKD stage 4 and 5 there was a downregulation of miR-223-3p and miR-93-5p, which were associated with CKD stages, parameters of vascular calcification, inflammation and kidney function [52]. Interestingly, a trend towards an association with PTH was also seen. Furthermore, this down-regulation disappeared after kidney transplantation even

when lower glomerular filtration rates persisted. In another study, vascular smooth muscle-specific miR-143 and miR-145 expressions were decreased while that of miR-126 was markedly increased, all of them regulating the expression of protein targets involved in vascular alteration associated with CKD [44]. Therefore, miRNAs appear as new players in the CKD-MBD field [45] that might be of help to assess changes and to prevent or treat complications of CKD as the vascular risk in these patients.

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