Effect of High Extracellular Phosphate Concentration on Arachidonic Acid Production by Parathyroid Tissue In Vitro

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Abstract. Recent in vivo and in vitro studies show that high phosphate directly stimulates parathyroid hormone (PTH) secretion. However, little is known about the intracellular signaling system involved in the regulation of PTH secretion by extracellular phosphate. High extracellular calcium is coupled to the activation of phospholipase A₂ (PLA₂) and the formation of arachidonic acid (AA), a potent inhibitor of PTH release. The present study was designed to evaluate whether a high phosphate concentration has an effect on the PLA₂-AA pathway in parathyroid cells. In vitro experiments were performed in parathyroid tissue obtained from normal rats and dogs. AA production was measured in parathyroid tissue in response to 1- and 4-mM phosphate concentration and after addition of PLA₂ to the medium. To determine whether the effect of phosphate on AA production in parathyroid cells was tissue specific, separate experiments were performed to test the effect of phosphate in rat adrenal glomerulosa cells, which are known to increase AA production in response to angiotensin II. The effect of sulfate, an ion with chemical characteristics similar to phosphate, on PTH secretion was also evaluated. In parathyroid tissue, a high phosphate concentration decreased the high calcium-induced AA production. This effect of phosphate was associated with an increase in PTH secretion. The addition of AA reversed the stimulatory effect of phosphate on PTH secretion. In another type of AA-responsive tissue, the adrenal glomerulosa, a high phosphate concentration did not affect the production of AA when stimulated by angiotensin II. In a normal phosphate concentration, the addition of PLA₂ stimulated AA production and decreased the PTH secretion. However, in a 4-mM phosphate concentration, the addition of PLA₂ did not reduce PTH secretion and did not stimulate AA production. Finally, sulfate did not affect PTH secretion. In conclusion, a high phosphate concentration affects the production of AA by parathyroid tissue. This effect of phosphate may be the mechanism by which a high phosphate concentration stimulates PTH secretion.

Phosphate retention as a result of decreased renal function is an important factor in the pathogenesis of secondary hyperparathyroidism (1–3). Recent in vivo and in vitro studies show that a high phosphate concentration directly stimulates parathyroid hormone (PTH) secretion (4–10). Recently, a parathyroid cell membrane phosphate cotransporter was cloned (11); the synthesis of this protein is modulated by changes in the dietary content of phosphate, and it has been proposed that this transporter may function as a putative "phosphate sensor" for the parathyroid cell (12). However, little is known about the intracellular signaling system involved in the regulation of PTH secretion by extracellular phosphate.

By contrast, the early signal transduction mechanisms involved in the stimulation of PTH release by low extracellular calcium are increasingly understood. Extracellular calcium concentration modulates PTH secretion via a G-protein-coupled calcium-sensing receptor (13). This effector system includes the hydrolysis of membrane phospholipids by phospholipase C (PLC), phospholipase D (PLD), and phospholipase A₂ (PLA₂) to generate the appropriate intracellular signals (14). High extracellular calcium is coupled to the activation of PLA₂ and the formation of arachidonic acid (AA), a potent inhibitor of PTH release, which acts via the 12- and 15-lipoxygenase pathway (15,16).

In previous in vitro studies, we have shown that a high extracellular phosphate concentration prevents the normal inhibition of PTH secretion by calcium (4). Because AA seems to be an important mediator for the reduction of PTH release by calcium, additional experiments were performed to determine whether AA is involved in the regulation of PTH secretion by high phosphate. The results of these experiments showed that despite the presence of high phosphate concentration in the medium, the addition of exogenous AA to the medium restored the capacity of a high calcium concentration to inhibit PTH secretion. Although these results suggest that AA plays a role in the regulation of PTH secretion by phosphate, they do not show that phosphate directly affects AA production by parathyroid cells. The present study was designed to determine whether a high phosphate concentration has an effect on the PLA₂-AA pathway in parathyroid cells.
Materials and Methods

Parathyroid Tissue

The experiments were performed in intact rat parathyroid glands. The rat parathyroid glands were obtained from 170- to 200-g male Wistar rats fed a 0.6% calcium, 0.6% phosphorus diet with 100 IU/100 g vitamin D. The animals were anesthetized with pentobarbital (50 mg/kg), and blood was drained by aortic puncture; thereafter, the parathyroid glands were dissected free of thyroid under a dissecting microscope. The elapsed time from exsanguination to the completion of parathyroidectomy was approximately 2 min.

Dog parathyroid tissue was used when the experiments required a large amount of tissue. Dog parathyroid glands were obtained from euthanized mongrel dogs, 2 to 5 yr old and weighing 13 to 20 kg, donated by the Center for Animal Control. These dogs were healthy and ingested a standard chow diet containing 1.2% calcium, 0.9% phosphate and 1200 IU/kg vitamin D for a least 1 mo before the experiment. Euthanasia was achieved by the administration of 10 mg of intramuscular ketamine followed by 1 g of thiopental as an intravenous bolus. The time required to complete the parathyroidectomy in the dog was approximately 3 min.

Intact rat parathyroid glands or small (1 mm (3)) pieces of dog parathyroid tissue were placed in individual wells containing 2 ml of incubation media resting inside a nylon basket; the glands were maintained in a constant rocking and shaking motion (AOS-0, SBS Instruments SA, Badalona, Spain) at 37°C. Before and after each experiment, parathyroid cells were mechanically dispersed, and greater than 80% cell viability was confirmed by trypan blue exclusion.

Incubation Medium

The incubation medium was prepared using a distilled-deionized water (Elgastat UHQ PS, Elga Ltd, High Wycombe, Bucks, England) and was buffered (pH 7.4) and contained in 125 mM NaCl, 5.9 mM KCl, 0.5 mM MgCl₂, 0.4 to 2.0 mM CaCl₂, Na-pyruvate 1, glutamine 4, glucose 12, Heps 25 with human insulin 0.1 IU/ml, bovine serum albumin 0.1%, penicillin g 100 IU/ml, and streptomycin 100 mg/ml. Phosphorus was added in the form of NaH₂PO₄ and Na₂HPO₄ in 1:2 proportion to achieve the desired phosphorus concentration. The calcium concentration in the medium was modified by adding CaCl₂. The target calcium concentration was confirmed by measurement using a selective electrode (model 634; Ciba Corning, Essex, England); the ionized calcium was measured 10 min before and immediately after completion of each 1-h incubation period. The ionized calcium values did not change after incubation. All chemical products were obtained from Sigma (St. Louis, MO).

Effect of High Phosphate on AA Production by the Parathyroid Cell

In a previous report, we showed that PTH secretion was increased by a high phosphate concentration and that this effect of phosphate was completely reversed by the addition of AA (20 μM) (4). In the present experiment, the effect of phosphate on AA production by parathyroid cells was tested.

AA production was measured using gas chromatography (17). In the first experiment, the effect of high calcium concentration on AA production was evaluated. Dog parathyroid tissue was incubated for 1 h each in a low (0.6 mM) and high (1.35 mM) calcium concentration. A second experiment, in which dog parathyroid tissue was incubated in normal (1 mM) and high (4 mM) phosphate concentration, was designed to evaluate the effect of a high phosphate concentration on AA production. After completion of the incubation period, parathyroid tissue was homogenized with a glass-ball homogenizer in a solution containing 50 mM Tris-HCl (pH 7.5 at 25°C), 0.2 mM EDTA, and 0.5 mM DTT. The AA was quantified by gas chromatography as described elsewhere (17) (model 5890-A; Hewlett Packard, Avondale, PA); with the use of this method, the coefficient of variation for AA quantification was 2.5%. The protein content of the tissue sample was determined using the Bradford method (18).

To confirm that the effect of phosphate was also observed in incubations of dog parathyroid tissue, separate experiments were performed using dog parathyroid tissue in which hourly PTH production was measured in 1.35 mM calcium in the presence of a normal and high phosphate concentration. Intact PTH in the incubation media was measured using a human intact PTH IRMA kit (Nichols Institute, San Juan de Capistrano, CA). The intra- and interassay coefficients of variation were 4.3 and 4.7%, respectively. The DNA content of the tissue sample was measured by spectrophotometry after isolation using a kit (Purogene; Gentra Systems, Inc., Minneapolis, MN).

Effect of High Phosphate on AA Production by Glomerulosa Cells

To determine whether the effect of phosphate on AA production in parathyroid cells was tissue specific, separate experiments were performed in rat adrenal glomerulosa cells, which are known to increase AA production in response to angiotensin II (AII) (19). Rat adrenal tissue was sliced into 1-mm (3) pieces and incubated in the previously described medium with 1.35 mM calcium and either a normal (1 mM) or a high (4 mM) phosphate concentration. All (1 mM) or vehicle was added to the medium, and after 120 min the tissue was homogenized for the measurement of AA and protein content using the same methodology as described for parathyroid tissue.

Effect of High Phosphate on the Inhibition of PTH Secretion by Exogenous PLA₂

Intact rat parathyroid glands were incubated with PLA₂ in concentrations of 0 (vehicle), 5, 25, and 50 μM/ml in the presence of normal (1 mM) and high (4 mM) phosphate. The calcium concentration in the medium was maintained at 0.6 mM; this low calcium concentration has been associated with low endogenous PLA₂ activity (15). PTH secretion was measured in the medium after a 1-h incubation period. The intact PTH concentration in the incubation medium was measured using a rat intact PTH IRMA kit (Nichols Institute). The intra- and interassay coefficients of variation were 4.3 and 4.7%, respectively.

Effect of High Phosphate on Parathyroid Cell Production of AA Stimulated by Exogenous PLA₂

Dog parathyroid tissue was incubated in 1.35 mM and 0.6 mM calcium with 1 mM phosphate and either PLA₂ (25 μM/ml) or vehicle added to the medium. The experiments were repeated using a high (4 mM) phosphate in the medium. The production of AA and protein content was determined in homogenized tissue as described previously.

Effect of Sulfate on PTH Secretion

These experiments were performed to determine whether sulfate, an ion with chemical characteristics similar to phosphate, has an effect on PTH secretion. The effect of sulfate on PTH secretion was tested in vitro using intact rat parathyroid glands. The experiments were performed following the same methodology used in our previous study in which a direct effect of phosphate on PTH secretion was shown (4). Rat parathyroid glands (10 per well) were incubated for 9 h.
in the above described media using a calcium and phosphate concentration of 1.25 and 1 mM, respectively. The incubation was prolonged to 10 h because in previous studies (4), the maximum effect of phosphate on PTH secretion was observed between 5 and 10 h and other authors have shown that the effect of phosphate on PTH secretion increases with time (6). The concentration of sulfate in the medium was either 0.5 or 4 mM. Adequate amounts of sodium sulfate were added to the medium to obtain the desired concentration of sulfate in the medium. During the tenth hour, the calcium concentration was decreased to 0.6 mM to stimulate PTH secretion and the sulfate and phosphate concentrations were unchanged. The PTH secretion rate was measured during both the ninth and tenth hours of incubation.

Statistical Analyses
Differences between more than two means were evaluated by analysis of variance followed by the Duncan test. Paired or unpaired t tests were used to compare two means from the same or from different groups of glands, respectively. The results are expressed as mean ± SEM.

Results
The production of AA by dog parathyroid tissue after a 1-h incubation in 1.35 mM calcium was greater than in 0.6 mM calcium (0.042 ± 0.006 versus 0.015 ± 0.003 µg/µg protein, P < 0.01). As expected, the increase in AA induced by a high calcium concentration was accompanied by a decrease in PTH secretion from 119 ± 7 pg/µg DNA in 0.6 mM calcium to 61 ± 5 pg/µg DNA in 1.35 mM calcium, P < 0.02.

In separate experiments using rat parathyroid glands, the addition of AA (20 µM) to a medium with normal (1 mM) phosphate and low (0.6 mM) calcium concentration resulted in a reduction of PTH secretion from 81 ± 7 to 32 ± 4 pg/µg DNA (P < 0.01). This low level of PTH secretion induced by AA was similar to that obtained with high (1.35 mM) calcium in the medium (34 ± 3 pg/µg DNA). The inhibition of PTH secretion by calcium was not further enhanced by the addition of AA.

In a high calcium (1.35 mM) concentration, an increase in phosphate concentration from 1 to 4 mM resulted in an increase in PTH secretion (34 ± 3 versus 86 ± 7 pg/µg DNA, P < 0.01); the addition of AA (20 µM) to a high phosphate medium reduced the PTH level to that obtained with normal (1 mM) phosphate concentration (39 ± 9 pg/µg DNA).

Effect of High Phosphate on AA Production by Parathyroid Cells
The time effect of normal (1 mM) and high (4 mM) phosphate concentration on AA production by parathyroid tissue is shown in Figure 1. In a medium with normal phosphate concentration (1 mM), a calcium of 1.35 mM resulted in a three-fold and significant increase in AA production; the values subsequently decreased to basal levels at 60 min. By contrast, in 4 mM phosphate and the same high calcium concentration, the production of AA remained unchanged during the 1-h observation period.

The secretion of PTH by dog parathyroid tissue was determined during 3 h of incubation in 1.35 mM calcium with 1 or 4 mM phosphate. In 1 mM phosphate, PTH secretion remained unchanged (64 ± 5, 60 ± 6, and 61 ± 4 pg/µg DNA at 1, 2, and 3 h, respectively); by contrast, in 4 mM phosphate, PTH secretion increased progressively (76 ± 7, 82 ± 4, and 87 ± 5 pg/µg DNA at 1, 2, and 3 h, respectively). Values at 2 and 3 h were significantly increased (P < 0.05) in 4 mM phosphate as compared with 1 mM phosphate.
Effect of High Phosphate on AA Production by Adrenal Glomerulosa Cells

To evaluate whether the effect of phosphate on AA production was specific for parathyroid cells, separate experiments were performed in rat adrenal glomerulosa tissue, which are known to increase AA production in response to AII. The addition of AII (1 nM) to the medium containing 1 mM phosphate induced a threefold increase in AA production (P < 0.05). With high phosphate (4 mM) in the medium, the basal and AII-induced AA production were not significantly different from those obtained with 1 mM phosphate in the medium (Figure 2). Thus, in glomerulosa cells, basal and AII-stimulated AA production were unaffected by the extracellular phosphate concentration.

Effect of High Phosphate on the Inhibition of PTH Secretion by Exogenous PLA₂

The effect of phosphate on the inhibition of PTH secretion in the presence of exogenous PLA₂ is shown in Figure 3. With normal phosphate (1 mM) in the medium and a low calcium (0.6 mM) concentration, the addition of increasing concentrations of PLA₂ inhibited PTH secretion in a dose-dependent manner; the maximum inhibition of PTH secretion was achieved with 25 mU/ml PLA₂. By contrast, in 4 mM phosphate, the same concentration of PLA₂ did not reduce PTH secretion. Thus, exogenous PLA₂ in concentrations that induced maximum inhibition of PTH secretion did not reduce the increased PTH secretion induced by high phosphate.

Effect of PLA₂ on AA Production by Parathyroid Cells with High Extracellular Phosphate

In a medium containing 1.35 mM calcium, the addition of PLA₂ did not further increase production of AA. An elevation of phosphate in the medium from 1 to 4 mM resulted in a significant decrease in AA production (Figure 4A), and the addition of PLA₂ (25 mU/ml) to the medium containing high phosphate did not increase AA production (Figure 4A).

Figure 3. Inhibition of parathyroid hormone (PTH) secretion by phospholipase A₂ (PLA₂). The effect of a high phosphate concentration in the medium. Intact rat parathyroid glands were incubated in low calcium (0.6 mM) with high (4 mM) and normal (1 mM) phosphate concentration in the medium. Increasing concentrations of PLA₂ were added to the medium, resulting in a dose-dependent inhibition of PTH secretion in glands incubated in 1 mM phosphate. By contrast, in 4 mM phosphate, the addition of PLA₂ did not reduce PTH secretion. *, P < 0.01 versus P = 1 mM.

Figure 4. Stimulation of AA production by PLA₂. The effect of extracellular calcium and phosphate. (A) In a high calcium concentration (1.35 mM), the production of AA was increased as compared with a low calcium concentration (0.6 mM) (B), and it was not further increased by the addition of PLA₂ (25 mU/ml). The addition of phosphate (4 mM) to the medium reduced the production of AA, which was not affected by the addition of PLA₂. *, P < 0.01 versus P = 1 mM without PLA₂. (B) In a low calcium (0.6 mM) and a normal (1 mM) phosphate concentration, the production of AA was low and the addition of PLA₂ (25 mU/ml) significantly increased the production of AA. With the same low calcium (0.6 mM) and a high phosphate (4 mM) concentration, AA production was reduced, but the addition of PLA₂ (25 mU/ml) did not increase the AA production. *, P < 0.01 versus P = 1 mM without PLA₂ and P = 4 mM with PLA₂ added.
With a low calcium concentration in the medium (0.6 mM), the AA production was low; however, in the presence of normal phosphate, the addition of PLA₂ produced a significant increase in AA production. With low calcium and a high phosphate concentration in the medium, the AA production was low and was not stimulated by the addition of PLA₂ to the medium (Figure 4B).

**Effect of Sulfate on PTH Secretion**

The modification of sulfate concentration in the medium did not significantly change PTH secretion (Figure 5). With low calcium, the PTH secretion increased significantly and the presence of a high sulfate concentration did not affect the stimulation of PTH secretion by low calcium. Thus, *in vitro*, PTH secretion was not affected by high sulfate concentration in the medium.

**Discussion**

The results obtained in the present study show that a high concentration of extracellular phosphate stimulated PTH secretion by parathyroid tissue *in vitro*, and this effect was associated with a decrease in AA production. While in the presence of a normal phosphate concentration exogenous PLA₂ decreased PTH secretion, a high phosphate concentration prevented the inhibition of PTH secretion by exogenous PLA₂. A high phosphate concentration also prevents the stimulation of AA production by PLA₂. The inhibitory effect of phosphate on AA production was not observed in other AA-producing tissue such as rat adrenal glomerulosa tissue. These results suggest that high extracellular phosphate regulates PTH secretion through the PLA₂-AA pathway. Sulfate, another divalent anion similar to phosphate, did not have an effect on PTH secretion by parathyroid tissue *in vitro*.

Our experiments show that in parathyroid cells, AA production is stimulated by high extracellular calcium and in the presence of a low calcium concentration, the addition of AA inhibits the PTH secretion. Similar results have been reported by others (14–16). The high calcium-induced AA production was observed after a 30- to 45-min incubation, and at 60 min the AA values had returned to baseline. This temporal effect of a high calcium concentration–induced AA production was also reported by Bourdeau et al. (15), but the peak production of AA was observed after 5 min. Such differences in the time effect were probably because these experiments were performed in dispersed parathyroid cells, whereas in our experiments, slices of parathyroid tissue were used. Kifor et al. (14) found that a high calcium (3 mM) concentration induced a progressive increase in AA until 30 min, but there was no further follow-up, so it was not possible to determine a peak effect. Nevertheless, these authors reported that AA production was increased in parathyroid cells exposed to high calcium concentration in the medium. Thus, the activation of PLA₂-AA pathway likely functioned to mediate the inhibition of PTH secretion by calcium. The addition of high phosphate concentration to the medium produced a remarkable decrease in the AA production induced by high calcium (Figure 1), and the supplementation of AA prevented the stimulation of PTH secretion by high phosphate (4). These data suggest that high phosphate stimulates PTH secretion by inhibiting AA production.

To investigate whether the phosphate effect on PTH secretion was tissue specific, we performed additional experiments to evaluate the effect of high phosphate in adrenal glomerulosa tissue. Aldosterone production by adrenal glomerulosa cells is stimulated by AII, and this effect is mediated by activation of PLA₂, which results in high AA production (19). Our results confirmed that AII stimulates AA production by adrenal glomerulosa cells; in fact, we observed a threefold increase in AA production, which is comparable to the twofold elevation in AA levels reported by Kojima et al. (19). A high phosphate concentration in the medium did not modify the basal AA or the AII-stimulated AA levels. These results suggest that the effect of phosphate is specific for parathyroid tissue. It has been proposed that the recently cloned PiT-1 Na/P cotransporter expressed in parathyroid cells may act as phosphate sensor. To our knowledge, this cotransporter is not expressed in adrenal glomerulosa cells and this may explain the lack of an effect of phosphate in these cells.

In parathyroid tissue incubated in a normal phosphate and low calcium concentration, the addition of PLA₂ produced a decrease in PTH secretion. This effect of PLA₂ was dose dependent, similar to the observation reported by Bourdeau et al. (15). The reduction of PTH secretion by PLA₂ was associated with an increase in AA production. The addition of PLA₂ to a medium with high calcium did not further increase the already elevated production of AA, suggesting that AA production was maximally stimulated by the high calcium. A high phosphate concentration in the medium prevented both effects.
of PLA₂, the inhibition of PTH secretion and the increase in AA production by parathyroid tissue. The lack of response to PLA₂ may suggest inability of phospholipids to release AA under the action of PLA₂ in the presence of a high phosphate concentration.

The sensing mechanism for phosphate is unknown, but the newly described phosphate cotransporter is being proposed as a possible phosphate sensor (11,12). Although there is not information about the intracellular events that mediate the regulation of PTH secretion by phosphate, it has been shown that three phospholipases, PLC, PLD, and PLA₂, participate in parathyroid cell signaling (14). The results of the present study suggest that a high phosphate concentration regulates PTH secretion through the PLA₂-AA signaling system. A role for PLC or PLD cannot be excluded, although our preliminary experiments indicate that inhibition of PLC-dependent protein kinase C activity does not modify the stimulatory effect of phosphate on PTH secretion. In rat parathyroid glands cultured in a low (0.6 mM) calcium and a normal phosphate (1 mM) concentration, the addition of H7 (10 μM), a protein kinase C inhibitor, reduced the PTH secretion from 75 ± 6 to 38 ± 7 pg/μg DNA, P < 0.01; however, with a high phosphate (4 mM) concentration, the addition of H7 did not decrease the PTH secretion significantly (89 ± 8 versus 87 ± 10 pg/μg DNA). These results suggest that intracellular signaling system for low calcium- and high phosphate-induced PTH stimulation are not the same.

Different groups have shown that the effect of phosphate on PTH secretion is observed only in tissue preparations rather than in dispersed parathyroid cells in culture. Nielsen et al. (7) performed studies to test whether the effect of phosphate on PTH secretion was influenced by the type of in vitro culture: dispersed bovine parathyroid cells versus slices of bovine parathyroid tissue. They showed that the stimulation of PTH secretion by phosphate was observed only in parathyroid tissue, but both dispersed cells and tissue preparations responded to changes in the calcium concentration. Slatopolsky et al. (20) presented data indicating that phosphate did not stimulate PTH secretion in dispersed bovine parathyroid cells. However, the same group of investigators, using intact rat parathyroid glands in culture, were able to show a direct effect of phosphate on PTH secretion (6). In a recent report by Rousson et al. (21), an adequate PTH response to calcium and persistence of the calcium-sensing receptor was observed in a long-term human parathyroid cell culture. In these cells, a high phosphate concentration significantly increased PTH secretion. According to the authors, the observation of an effect of phosphate may have been possible as a result of the presence of clusters with close cell-to-cell interaction. Therefore, there is experimental evidence that shows that the effect of phosphate on parathyroid function is observed in intact tissue rather than dispersed cells in culture. 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. (22) demonstrated that parathyroid cells in close proximity are stimulated to secrete more PTH and suggest the presence of a paracrine interaction among parathyroid cells. Following this same line of reason-

ing, intercellular communication may be required to observe an effect of phosphate on PTH secretion. The present work shows that a high phosphate concentration affects the production of AA by parathyroid cells. Thus, as in other systems (23), it is possible that eicosanoid products produced by a cell may affect neighbor cells. This may be the reason whereby intact parathyroid tissue architecture or at least a close cell-to-cell interaction is required to see an effect of phosphate.

As in our previous study (9), the results of the present study were obtained using normal parathyroid tissue. Hyperplastic parathyroid glands from humans or from uremic animals might not show the same response as normal parathyroid glands.

The rationale to evaluate a possible effect of sulfate on PTH secretion was simply that sulfate, like phosphate, is another divalent anion circulating in relatively high concentrations in the blood. A sulfate concentration as high as 4 mM did not have an effect on PTH secretion. This indicates that the effect of phosphate is not only tissue specific (parathyroid gland) but also specific for the phosphate ion.

In conclusion, a high phosphate concentration affects the production of AA by parathyroid tissue. This effect of phosphate may be the mechanism by which a high phosphate stimulates PTH secretion.

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