A direct effect in vitro of phosphate on PTH release from bovine parathyroid tissue slices but not from dispersed parathyroid cells

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Abstract

**Background.** Phosphate retention has long been considered to be of importance for the pathogenesis of secondary hyperparathyroidism in chronic renal failure. Hyperphosphatemia in vivo is associated with alterations of calcium and vitamin D levels, both of which are known to alter the parathyroid hormone (PTH) release independently.

**Materials and methods.** We have investigated the direct effect of phosphate on PTH release in vitro using two different preparations of bovine parathyroid tissue: Acutely dispersed bovine parathyroid cells and tissue slices of 0.5 x 0.5 mm were prepared from bovine parathyroid glands. Parathyroid dispersed cells and tissue slices were incubated for 4 h in media containing normal phosphate (1.0 mM) or high phosphate (3.5 mM).

**Results.** High phosphate induced a significant (P<0.01) increase in PTH release in the preparation of tissue slices, but not in preparations of dispersed cells. The 4 h incubation in high phosphate medium did not change the set-point for calcium. Bovine parathyroid tissue slices incubated in increasing phosphate concentrations from 1.0 to 3.5 mM and with a fixed calcium concentration of either 0.8, 1.2 or 1.8 mM responded with a dose dependent stimulation of PTH release. The degree of stimulation of PTH release by high phosphate (3.5 mM), was significantly (P<0.05) higher at low calcium levels (0.8 mM), 172% above baseline values (1.0 mM phosphate) as compared to high calcium levels (1.8 mM), 139% above baseline values.

**Conclusions.** This study shows that phosphate directly stimulates the PTH release in bovine parathyroid glands, and that this effect is only seen in preparations of parathyroid tissue slices and not in preparations of dispersed cells. This indicates that maintenance of near normal architecture of the parathyroid glands is essential in order to elicit the effect of high phosphate on the PTH release.

Key words: parathyroid cells; cell culture; phosphate; PTH

Introduction

Renal insufficiency is characterized by several alterations in the mineral homeostasis. Thus secondary hyperparathyroidism is present even in early stages of renal insufficiency [1,2]. The two major pathogenetic mechanisms responsible for the development of the secondary hyperparathyroidism in advanced renal insufficiency are phosphate retention and low levels of 1,25(OH)2D3. As early as the 1970s Slatopolsky and Bricker showed in dogs with experimental uremia that dietary phosphate restriction was effective in preventing the development of secondary hyperparathyroidism [2]. Clinical studies have since demonstrated that phosphate restriction in patients with uremia prevented the increase in serum PTH levels [3-7]. The mechanism of this effect has not been clear, but is in part considered to be due to changes in serum 1,25(OH)2D3 concentrations. Both in vitro [8,9] and in vivo [3] phosphate directly regulates the renal production of 1,25(OH)2D3. Besides, hyperphosphatemia leads to a decrease in serum calcium by several mechanisms, including inhibition of renal 1α-hydroxylase activity, [8], and secondly the activity of low levels of 1,25(OH)2D3 which lead to a decrease in intestinal calcium absorption, and to altered PTH synthesis and PTH secretion [10].

Several investigators have shown, that phosphate restriction in vivo prevents secondary hyperparathyroidism. Lopez-Hilker showed in uremic dogs that phosphate restriction reversed hyperparathyroidism independently of changes in calcium and 1,25(OH)2D3 [11] and Kilav et al. showed in rats that hypophosphatemia without alterations in ionized calcium and circulating 1,25(OH)2D3 levels down regulated the PTH gene transcription [12].

Until recently in vitro studies have failed to demonstrate a direct effect of phosphate on PTH release. However, Almadén et al. [13] have just demonstrated in a rat model, using whole parathyroid glands in...
Culture, that high phosphate stimulated PTH release and that this stimulatory effect of phosphate on PTH release in vitro also could be found in pathological parathyroid tissue slices from humans [14].

In the present study, we have examined the effect of phosphate on PTH secretion in dispersed bovine parathyroid cells as compared to that of tissue slices of bovine parathyroid glands. Our results provide further evidence of the direct in vitro effect of phosphate on the parathyroid glands, an effect which is only mediated in parathyroid tissue with intact structure and not in dispersed cells.

Subjects and methods

Parathyroid cell and tissue preparations

Fresh bovine parathyroid glands were obtained within 10 min of slaughter at a local abattoir and transported to the laboratory in ice-cold magnesium and calcium deleted Hank’s balanced salt solution (Gibco BRL, Life Technologies, Roskilde, Denmark), with a pH of 7.4, containing penicillin 10 IU/ml and streptomycin 10 μg/ml. Using sterile conditions, the glands were trimmed free of excess fat and connective tissue and in the same buffer finely minced into 0.5 mm fragments. After the tissue was minced it could be used either at the same day or stored for the subsequent day at 4°C in Coon’s modification of Ham’s F12 containing CaCl2 0.5 mM and MgCl2 0.5 mM, Insulin 100 IU/l (Novo Nordisk, Bagsvaerd, Denmark), non essential amino acids and L-glutamin 0.3 mg/ml (Gibco BRL, Life Technologies, Roskilde, Denmark) with 5% heat inactivated fetal calf serum (Biological Industries, Beth Haemek, Israel).

Preparation of dispersed parathyroid cells

Dispersed cells were prepared with minor modifications as described previously by Brown [15] from the stock of minced parathyroid tissue, by digestion in Hank’s balanced salt solution, with dispase grade II 1 IU/ml (Boehringer-Manheim GmbH, Germany) and collagenase 0.5 mg/ml (Sigma, St Louis, MO) for 45–60 min in an incubator at 37°C, 5% CO2, with vigorous pipetting every 15 min by a 60 cc syringe. The solution was then filtered through two layers of gauze and the cells were sedimented at 1800 r.p.m. (600 x g) for 5 min at 20°C, washed three times in Coons modification of Ham’s F12. Cell viability was more than 95% by trypan blue exclusion. At least 90% of the acutely dispersed cells were parathyroid cells by morphological criteria. Cells prepared by digestion were used immediately after preparation as acutely dispersed cells at a concentration of 1 × 10⁶ cells/ml.

Preparation of tissue slices

Tissue slices were prepared from the same stock of minced parathyroid tissue as used for the preparation of dispersed cells. Tissue slices were trimmed free of fat and connective tissue and placed in 10 cc polycrylonite tubes containing 5 ml of culture medium.

Incubation took place in an incubator (Forma Scientific, OH) at 37°C, 5% CO2, with the 10 cc polycrylonite tubes placed on a shaker. Each experiment was performed in triplicate or quadruplicate. When comparing tissue slices and dispersed cells the parathyroid tissue for preparing dispersed cells as well as tissue slices were taken from the same stock of minced tissue, and incubated in parallel.

The incubation medium consisted of Ham’s modified F12 with Insulin 100 IU/l (Novo Nordisk, Bagsvaerd, Denmark) non essential amino acids and L-glutamin 0.3 mg/ml (Gibco GBL, Life Technologies, Roskilde, Denmark) with 5% heat inactivated fetal calf serum (Biological Industries, Beth Haemek, Israel). The potassium concentration was adjusted to 5.8 mM in all media. The osmolality was 298 mosM.

The supernatants for subsequent measurements of PTH were obtained after centrifugation for 5 min at 600 x g at the end of the incubation time. All supernatants were removed and STM buffer was immediately added to the dispersed cells and then frozen at −20°C for subsequent measurements of DNA concentration. Tissue slices were immediately frozen in liquid nitrogen and stored at −20°C for subsequent measurements of DNA concentration.

Experiments

I: Effects of calcium on PTH release in dispersed cells and in tissue slices

To examine the effect of calcium on PTH release, dispersed cells and tissue slices were incubated for 4 h in medium with increasing calcium concentrations (0.5–2.3 mM). Ionized calcium was carefully titrated to the concentration wanted with an accepted tolerance of <0.03 mM and at a fixed phosphate concentration of 1.0 mM. The inverse sigmoidal relationship between PTH secretion and ionized calcium is illustrated by the four parameter model using the equation Y = ((A−D)/(1 + (X/C)^B)+D), where Y is the secretory rate for PTH and X is ionized calcium concentration. The four parameters are as follows: A, maximal secretory rate; B, slope of the curve at its midpoint; C, midpoint or set-point (Ca** concentration producing half maximal change in PTH release); and D, minimal secretory rate, as described by Brown [16].

II: Stability of the medium containing phosphate and calcium

High concentrations of phosphate in calcium and magnesium containing solutions are known to precipitate at room temperature, therefore, we examined the stability of the medium regarding ionized calcium and ionized magnesium prior to the experiments involving cell and tissue preparations.

Ionized calcium was measured in a stock of medium with increasing amounts of calcium and containing 1.0 mM phosphate. We added an additional amount of phosphate as KH2PO4 to these solutions reaching a final concentration of 3.5 mM. Potassium chloride was added to the low phosphate medium, reaching a potassium concentration of 5.8 mM in all solutions, pH was adjusted to 7.45 by 1 M NaOH. The medium was incubated at 37°C, 5% CO2 and ionized calcium was measured during the following 10 days. A small additional amount of calcium chloride was added to the high phosphate (3.5 mM) medium to match the ionized calcium levels of the low phosphate (1.0 mM) medium. In each experiment ionized calcium and magnesium was measured just prior to and at the end of each incubation. Osmolality was measured for the different media.

III: Effects of phosphate on PTH release and on the set-point for calcium

In order to examine the effect of high phosphate on PTH release from dispersed cells versus tissue slices at different calcium levels and in order to investigate whether high phosphate had an influence on the set-point for calcium, we incubated dispersed cells and tissue slices at phosphate concentrations of 1.0 and of 3.5 mM phosphate and with increasing calcium concentrations from 0.5 to 2.3 mM.
In order to examine further the effect of increasing phosphate concentrations from 1.0 to 3.5 mM on PTH release from dispersed cells and tissue slices, incubation at a fixed calcium concentration of 1.0 mM and increasing phosphate concentrations from 1.0 to 3.5 mM were performed. In order to examine whether the effect of phosphate on PTH release in preparations of tissue slices was dependent upon the secretory state of the parathyroids, tissue slices were incubated at low calcium (0.8 mM), normal calcium (1.2 mM) and at high calcium (1.8 mM) concentrations.

**Bovine PTH measurements**

Bovine PTH was measured in the supernatants by a highly sensitive two site IRMA assay (Allegro, Nichols, San Juan Capistrano, CA), which is specific for intact human 1-84 PTH. This assay also recognizes intact bovine PTH, but does not recognize hormonal fragments [17,18]. A standard curve using intact (1-84) bovine PTH (Bachem, CA) was parallel to the human standard curve throughout the range of 7.5-1700 pg/ml, with a recovery of ~20%. The detection limit in our laboratory was 1.5 pg/ml, and the inter-assay variation was for low levels (40 pg/ml) 7.9% and for intermediate levels (250 pg/ml) 5.8%, respectively (n = 6). The intra-assay variation at the same levels were 3.5% and 2.1%, respectively (n = 6).

**Electrolyte measurements**

Ca$^{2+}$, Mg$^{2+}$, K$^+$, Na$^+$ and pH were measured in the medium just prior to and just after the incubation using ICA2 and KNa analyzers (Radiometer, Copenhagen, Denmark) and NOVA 8 (Nova Biomedical, Waltham, MA) for measurements of ionized magnesium. Total calcium, total magnesium and phosphate were measured in the supernatant after each experiment using Kodak Ectachem 250 Analyzer (Eastman Kodak Company, Rochester, NY).

**DNA measurements**

Total DNA measurements were performed in order to express the PTH secretion as a function of the DNA content. Thus, in the dispersed parathyroid cells DNA was measured in each individual sample using a colorimetric method as described by Burton [19], and modified by Giles and Myers [20] for measurements of DNA in preparation of dispersed cells.

DNA measurements in the tissue slices were performed using a DNA extraction Kit (Stratagene, La Jolla, CA), which is a modification of a procedure based on separating contaminating proteins from DNA by salt precipitation [21]. The tissue slices were homogenized by a polytron in the extraction solution and the cellular proteins digested by pronase for 18 h at 37°C. Subsequent to the removal of the proteins by 'salting out' using sodium chloride and enzymatic degradation of RNA by RNase, determination of DNA yield was performed on 250 µl of the supernatant and by use of the colorimetric method, as described above. The DNA yield in parathyroid tissue was 74.5 ± 2.6 µg DNA/100 mg tissue. In five samples (93–305 mg) the coefficient of variance was 3.5%.

In our assay the detection limit was 1 µg DNA/ml, the intra-assay variation at the 30 µg DNA/ml level was 1.6% (n = 5) and the inter-assay variation at 30 µg and 50 µg DNA/ml levels was 8.7% and 5.2%, respectively (n = 6).

**Results**

**Experiments**

I: Effects of calcium on PTH release in dispersed cells and in tissue slices. Initially it was examined whether the inverse sigmoidal relationship between ionized calcium and PTH release was identical in dispersed bovine parathyroid cells and tissue slices of bovine parathyroid glands. Figure 1 demonstrates that the suppressibility of PTH release at 2.3 mM calcium compared to that of 0.5 mM calcium was 64% and 54% for dispersed cells and tissue slices, respectively.

The set-point, calculated as half maximal inhibition of the secretion was 1.04 mM in the tissue slices and 1.03 mM in the dispersed cells. The overall shape of the curves were identical. Results are the means ± SEM of six independent experiments performed in triplicate.

Statistical analysis was performed using the Student's unpaired t-test. For analysis of multiple comparisons ANOVA was used. The null hypothesis was rejected when P<0.05 was obtained. Statistical calculations were based on an n equal to the number of separate experiments.
1.10 mM in dispersed cells (p; ns). Further, the overall shape of the curves was identical for the two preparations, but the amount of PTH released per µg DNA was 3-fold higher in the tissue slices than that determined in the preparation of dispersed cells.

II: Stability of the medium containing phosphate and calcium. Calcium phosphate precipitation by time is shown in Figure 2. The medium containing 3.5 mM phosphate and increasing amount of calcium remained stable throughout the first 6 h of incubation. With time the amount of calcium phosphate precipitation increased at high calcium and high phosphate concentrations, and as a consequence of these findings the medium was freshly made just prior to each experiment.

We found no change in ionized calcium, potassium, sodium, pH levels or osmolality during our short term incubation of 4 h (Table 1).

III: Effects of phosphate on PTH release and on the set-point for calcium. Dispersed bovine parathyroid cells and tissue slices of bovine parathyroid glands were prepared as described from the same stock, and incubated in Ham's F12 with increasing concentrations of calcium (0.5–2.5 mM), in either 1.0 or 3.5 mM phosphate and incubated for 4 h. In dispersed cells no difference between the Ca²⁺/PTH curves were seen (P = 0.28) whether incubated in high or normal phosphate medium (Figure 3). No difference in the set-point for calcium was demonstrated (1.07 mM).

However, in the tissue slices a significant (P < 0.05) increase of PTH release was seen at low calcium concentrations of 0.8 mM: 672 ± 43 pg PTH/µgDNA/ at 3.5 mM phosphate versus 563 ± 19 pg PTH/µgDNA at 1.0 mM phosphate. Similar results were found at a high calcium concentration of 1.8 mM: 421 ± 17 pg PTH/µgDNA versus 351 pg PTH/µgDNA at high and normal phosphate, respectively. The PTH release was increased after 4 h of incubation throughout the range of calcium investigated (P = 0.0017) (Figure 4), while there was no difference in the set-point for calcium (1.04 mM).

IV: Effect of increasing concentrations of phosphate on PTH release. Tissue slices incubated for 5 h at a fixed calcium level of 1.0 mM and increasing concentrations of phosphate (1.0 mM–3.5 mM) resulted in a significant increase in PTH release of 180% above basal PTH levels (P < 0.05). Such a dose dependency was not found in dispersed cells incubated under the same conditions. These results are based on four independent experiments in quadruplicate with tissue slices and dispersed cells from the same stock of minced parathyroid glands. The incubation was initiated at the same time after the tissue was harvested and the parathyroid tissue slices were kept on ice-cold Ham’s F12 until the incubation was initiated (Figure 5).

Incubating tissue slices at different calcium concentrations of 0.8, 1.2 or 1.8 mM, respectively, showed a significant, stimulation of PTH release by 172%, 159% and 139%, respectively, in seven independent experiments in triplicate, indicating a more pronounced (P < 0.05) stimulation of PTH release at low calcium levels (Figure 6) and clearly showing the stimulatory effect of high phosphate concentration on the PTH release at all calcium levels.

Discussion

The present in vitro study has clearly demonstrated that parathyroid tissue prepared either as dispersed cells or tissue slices resulted in different responses to incubation with high phosphate concentration. Thus, tissue slices responded to increasing phosphate concentrations by stimulation of PTH release in a dose dependent manner, while such a stimulatory effect was not found in the same bovine parathyroid glands when prepared as dispersed cells.

Recently, in vitro studies [13,14,22], culturing normal rat parathyroid glands and pathological human parathyroid glands in a high phosphate medium at a fixed calcium level of 1.25 mM produced a significant stimulation of PTH release after 5–6 h of incubation. In these studies the entire parathyroid glands from rats were used and they were the first to show a direct stimulatory effect of phosphate on PTH release in vitro.

We have in the present study shown that the stimula-
Table 1. Electrolyte concentration in medium spiked with phosphate

<table>
<thead>
<tr>
<th>Phosphate added to the medium</th>
<th>Ca²⁺ (mM)</th>
<th>Na⁺ (mM)</th>
<th>K⁺ (mM)</th>
<th>pH</th>
<th>Phosphate (mM)</th>
<th>Osmolality (mosM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.00 mM</td>
<td>1.00 ± 0.01</td>
<td>134 ± 0.3</td>
<td>5.8 ± 0.06</td>
<td>7.47 ± 0.14</td>
<td>1.00 ± 0.01</td>
<td>298 ± 0.8</td>
</tr>
<tr>
<td>2.00 mM</td>
<td>0.99 ± 0.01</td>
<td>134 ± 0.3</td>
<td>5.9 ± 0.07</td>
<td>7.50 ± 0.17</td>
<td>1.99 ± 0.02</td>
<td>297 ± 1.0</td>
</tr>
<tr>
<td>2.75 mM</td>
<td>1.00 ± 0.09</td>
<td>134 ± 0.3</td>
<td>5.8 ± 0.06</td>
<td>7.45 ± 0.13</td>
<td>2.76 ± 0.05</td>
<td>299 ± 0.7</td>
</tr>
<tr>
<td>3.50 mM</td>
<td>0.99 ± 0.01</td>
<td>134 ± 0.4</td>
<td>5.8 ± 0.03</td>
<td>7.38 ± 0.12</td>
<td>3.50 ± 0.11</td>
<td>298 ± 0.6</td>
</tr>
</tbody>
</table>

Electrolytes were measured in the medium at an ionized calcium level of 1.00 mM, with increasing addition of phosphate to the medium. Results are means ± SEM of four independent experiments, samples taken at the end of each experiment. Addition of phosphate did not alter the concentration of the other electrolytes. Results for other calcium levels were identical and stable throughout the experiments.

Fig. 3. Effect of high phosphate on PTH release in dispersed cells. PTH release from acutely dispersed cells cultured for 4 h in suspension in either 1.0 or 3.5 mM phosphate showed that the overall shape of the curves was identical with no significant difference (P = 0.28) in the PTH release. High phosphate did not change the set-point for calcium. Results represent means ± SEM of seven independent (n = 7) experiments performed in triplicate.

Fig. 4. Effect of high phosphate on PTH release in tissue slices. Parathyroid glands were finely minced in 0.5 × 0.5 mm pieces and incubated (4–5 pieces in each tube) in suspension with either 1.0 or 3.5 mM phosphate. Results represent mean of six independent (n = 6) experiments in triplicate. Tissue slices incubated in high phosphate increased the PTH release significantly (P < 0.01) by ~20% after 4 h of incubation with no change in the calcium set-point.

The mechanism of calcium sensing in parathyroids is mediated at least in part through a calcium sensing receptor located in the plasma membrane [25], but at present there is no evidence of the existence of a phosphate sensing receptor similar to the calcium sensing receptor. However, the long time needed to see any effect of phosphate makes the existence of a membrane receptor mediated mechanism less likely. In

BoPCaR gene transcription [24]. Whether this dedifferentiation and loss of calcium sensitivity of the bovine parathyroid cells in culture partly may be responsible for the lack of effect of high phosphate on preparations of dispersed cells is unknown.
the present study we could not exclude that the rather harsh treatment of the parathyroids by the digestion, which is necessary in order to prepare dispersed cells, could potentially destroy membrane associated phosphate transporters or receptors that might be essential for mediating the signal of high extracellular phosphate.

Kilav et al. studied in rats the effect of hypophosphatemia on PTH gene expression in vivo and showed that PTH mRNA levels were markedly decreased in second generation vitamin D-deficient rats fed on low phosphate and low calcium diet despite normal serum 1,25(OH)2D3 and normal serum calcium. These studies have provided a model to study the effect of hypophosphatemia on PTH release and PTH gene expression without changes in calcium or vitamin D levels, but it has not been possible to illustrate effects of high phosphate on PTH release in an in vivo model without changes in either calcium or vitamin D levels, both of which are known to modulate the PTH gene expression [12].

The calcium set-point [26] might be altered in severe secondary hyperparathyroidism, with a shift to the right. In the present study high phosphate concentration did not change the calcium set-point of the parathyroids during a 4 h incubation.

In summary, the present study shows that phosphate directly stimulates PTH release in bovine parathyroid glands, and that this effect is only seen in preparations of parathyroid tissue slices and not in preparations of dispersed cells. This indicates, that maintenance of near normal architecture of the parathyroid gland is essential in order to elicit the effect of high phosphate on the PTH release, but further investigations are needed in order to elucidate the pathway for the action of phosphate on parathyroid glands.

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