The Ionic Control of 1,25-Dihydroxyvitamin D₃ Synthesis in Isolated Chick Renal Mitochondria

THE ROLE OF CALCIUM AS INFLUENCED BY INORGANIC PHOSPHATE AND HYDROGEN ION

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ABSTRACT Isolated kidney mitochondria prepared from vitamin D-deficient chicks catalyze the conversion of 25-hydroxyvitamin D₃ to 1,25 dihydroxyvitamin D₃. It was found that changes in the concentrations of Ca⁺⁺, HPO₄⁻⁻, and H⁺ altered synthesis in an interrelated fashion. Increasing the Ca⁺⁺ concentration from 10⁻⁶ to 10⁻⁴ M caused a four- to fivefold increase in 1α-hydroxylase activity when the medium pH was between 6.5 and 7.0. Increasing the [Ca⁺⁺] to 10⁻⁴ M caused no further stimulation. At higher pH values, Ca⁺⁺ had little effect upon 1α-hydroxylase activity. In the absence of calcium ([Ca⁺⁺] ≤ 10⁻⁴ M), a change in pH from 6.5 to 7.1 had no effect upon 1α-hydroxylase activity. In the presence of 10⁻⁴ M calcium, increasing the medium pH had a biphasic effect. An increase in pH from 6.5 to 6.9 caused a 1.5-fold increase in 1α-hydroxylase activity, but a further increase of the pH to 7.1 caused a profound decrease in rate of hydroxylation to approximately 20% of the peak value.

Neither 10⁻⁴ M LaCl₃ nor 10 μg/ml of oligomycin altered the effects of Ca⁺⁺ upon hydroxylase activity. However, the effect of calcium was blocked by 2.5 × 10⁻⁵ M ruthenium red, 0.83 μg/ml of antimycin A, and 500 μM dinitrophenol. The calcium ionophore, A23187, decreased but did not prevent the stimulatory effect of calcium. These data are consistent with the concept that the [Ca⁺⁺] in the mitochondrial matrix space is of importance in regulating the 1α-hydroxylase.

The presence of potassium modified the interrelated effects of calcium and phosphate in two ways: 10⁻⁴ M calcium blocked the stimulation by phosphate; and in the presence of phosphate, 10⁻⁴ M calcium resulted in less 1,25(OH)₂D₃ production than 10⁻⁴ M calcium.

The effects of calcium, phosphate, and hydrogen ion on 1,25(OH)₂D₃ production by isolated mitochondria are qualitatively similar to the effects of these ions on 1,25(OH)₂D₃ production by isolated renal tubules.

INTRODUCTION

The regulation of 25-hydroxyvitamin D₃ 1α-hydroxylase activity by calcium and phosphate has been demonstrated both in vivo (1–3) and in vitro (3–8). No simple correlation between the calcium and phosphate levels in vivo and the observed rate of 1,25 dihydroxyvitamin D₃ (1,25(OH)₂D₃) production can be made because of the other factors influenced by calcium and phosphate that may also affect 1,25(OH)₂D₃ synthesis (4). Studies with isolated renal tubules in vitro permit better control of the variables being studied (3), but changes in the 1α-hydroxylase activity resulting from changes in the extracellular ionic environment may arise from either a direct action of the ion on the 1α-hydroxylase or an indirect action via some other aspect of cellular metabolism. In a preceding report (3) we demonstrated that both calcium and phosphate, under the appropriate conditions, stimulated 1,25(OH)₂D₃ production by isolated renal tubules. An increase in hydrogen ion concentration caused a decrease in the amount of stimulation by calcium. In the present study, the effects of changes in calcium, phosphate, and hydrogen ion concentrations on 1,25(OH)₂D₃ production by chick renal mitochondria

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FIGURE 1  A. The effect of calcium on 1,25(OH)₂D₃ production by mitochondria incubated in Tris chloride buffers at four different pHs. The free calcium concentration was regulated by calcium-EGTA buffers. The pH was determined at 37°C. B. Part of the data from A replotted to indicate the effect of pH on 1,25(OH)₂D₃ production by mitochondria incubated at 0 and 10⁻⁴ M calcium.

are reported and compared to our previous results with isolated renal tubules.

METHODS

Animals. White Leghorn cockerels (Moyers Chickens, Quakertown, Pa.) were raised on a vitamin D-deficient diet containing 0.43% calcium and 0.3% P as inorganic phosphate for 21-25 days.

Materials. [26,27-³H]1²5(OH)₂D₃, specific activity 1 mCi/mmol, was obtained from Amersham/Searle Corp. (Arlington Heights, Ill.). Whatman SG81 chromatography paper was obtained from H. Reeve Angel & Co., Inc. (Clifton, N. J.). All chemicals were reagent grade and obtained from commercial suppliers.

Mitochondrial preparation and incubation. The kidneys were perfused in situ with ice-cold 50 mM Tris acetate buffer, pH 7.4 (pH adjusted at room temperature), containing 250 mM sucrose and 2 mM EGTA (buffer A). The kidneys were removed, rinsed, and passed through a tissue press. Six pairs of kidneys were homogenized in 20 ml of buffer A with a Potter-Elvehiem apparatus, the homogenate was then centrifuged at 600 g for 10 min, and the supernate was removed and saved. The pellet was re-suspended in 20 ml of buffer A, and the above was repeated. The combined supernates were centrifuged at 7,500 g for 10 min. The pellet was washed twice in 20 ml of ice-cold Tris chloride, pH 6.8 (pH adjusted at 37°C), containing 300 mM sucrose and 10 mM EGTA (buffer B). The third wash was performed in 20 mM Tris chloride, 300 mM sucrose buffer with pH adjusted to the pH of the incubation mixture (6.8 in most experiments), and without EGTA (buffer C). The washed pellet was suspended in 6 ml of buffer C to give a mitochondrial suspension of 10-12 mg protein/ml. The final incubation occupied a volume of 1 ml and contained 5-6 mg mitochondrial protein, 16 mM Tris chloride, 150 mM sucrose, 10 mM sodium malate, 2.1 mM magnesium sulfate, and 10 mM EGTA with sufficient calcium to give the desired free calcium concentration for the desired pH employed (9). The pH for the incubation medium was determined in all cases at 37°C. Phosphate was added as the sodium salt adjusted to the appropriate pH. The mitochondria were preincubated for 12 min at 37°C under an atmosphere of O₂ before the addition of 25 pmol of 250HD₃₀ specific activity 1 mCi/μmol. After 12 min more of incubation the reaction was terminated with CHCl₃: MeOH, 1:2, and the CHCl₃ extract was prepared for Whatman SG81 chromatography as described previously (10). The amount of 1,25(OH)₂D₃ produced was calculated from the percent of total radioactivity appearing in the 1,25(OH)₂D₃ peak on the chromatograms. The rate of 1,25(OH)₂D₃ production was linear over this time period for the amount of mitochondrial protein employed.

Mitochondrial ion concentrations. Perchloric acid extracts of the mitochondria were made by the method of Kimmich and Rasmussen (11). Calcium, phosphate, and protein determinations were performed as previously described (3).

RESULTS

Calcium concentrations between 10⁻⁴ and 10⁻⁴ M stimulated the production of 1,25(OH)₂D₃ four- to fivefold by mitochondria incubated at a pH below 7.0 (Fig. 1A). No inhibition of 1,25(OH)₂D₃ production was noted even at calcium concentrations as high as 10⁻⁴ M. Between pH 6.45 and 6.90, hydrogen ion decreased the extent of stimulation by optimal levels of calcium but had no apparent effect on the 1α-hydroxylase activity at calcium concentrations below 10⁻⁴ M (Fig. 1B). However, above pH 7.0, little stimulation by calcium was observed until 10⁻⁴ M concentrations were used (Fig. 1A and 1B). A separate experiment with mitochondria incubated at pH 7.0 showed a response intermediate to that found at pH 6.9 and 7.1, with some stimulation by calcium noted at 10⁻⁴ M, but still not to the extent seen at a lower pH. This marked sensitivity of the calcium effect to extramitochondrial pH was reproduced not only in Tris chloride buffers but in NaHCO₃-CO₂ buffers as well. Little difference in mitochondrial content of calcium was seen between mitochondria washed at pH 7.1 and 6.8 (Table I). However, washing mitochondria at the higher pH resulted in a decreased amount of phosphate.

The effects of various inhibitors on the calcium stimulation of 1α-hydroxylase activity are shown in Fig. 2. Neither 10 μg/ml oligomycin nor 10⁻⁴ M LaCl₃ prevented the calcium stimulation of the 1α-hydroxylase activity. However, 500 μM dinitrophenol, 0.83 μg/ml anti-

| Table I |
| Mitochondrial Content of Calcium and Phosphate |
|         | pH   | Calcium | Phosphate |
|         |      |         |          |
| Prepara-| 6.8  | ±0.1    | 47.6±0.4 |
| tion    | 7.1  | ±0.1    | 27.4±0.5 |
mycin, and 2.5 × 10^{-4} M ruthenium red each prevented the stimulatory effect of calcium on 1,25(OH)\textsubscript{2}D\textsubscript{3} production. The calcium ionophore A23187, at 10^{-4} M, decreased but did not totally block the effect of calcium. At these concentrations antimycin A blocked state 4 oxygen consumption by the mitochondria, whereas ruthenium red, LaCl\textsubscript{3}, and oligomycin did not (Table II).

Phosphate, added as the sodium salt, exerted a biphasic effect on the 1α-hydroxylase activity. Increasing the phosphate concentration over the range of 0–1 mM led to increased mitochondrial synthesis of 1,25(OH)\textsubscript{2}D\textsubscript{3} at all calcium concentrations tested (Fig. 3). Further increases of phosphate above 1 mM resulted in slightly diminished 1α-hydroxylase activity (Fig. 3). The stimulatory effects of calcium and phosphate on 1,25(OH)\textsubscript{2}D\textsubscript{3} production were synergistic, at least at suboptimal concentrations for each. In the absence of phosphate little stimulation by 10^{-4} M calcium was seen in this experiment. However, in the presence of 1 mM phosphate, 10^{-4} M calcium more than doubled the rate of synthesis of 1,25(OH)\textsubscript{2}D\textsubscript{3}. Likewise, only a 20% stimulation by 0.3 mM phosphate was seen in the absence of calcium, but in the presence of 10^{-4} M calcium, 0.3 mM phosphate nearly doubled the rate of 1,25(OH)\textsubscript{2}D\textsubscript{3} production.

If the phosphate was added as the potassium salt so that 10 mM potassium was present in the incubation medium (Fig. 4), the effects of calcium and phosphate on 1α-hydroxylase activity were modified in the following three ways: (a) potassium stimulated the activity of the 1α-hydroxylase in the absence of calcium and phosphate; (b) the calcium effect was biphasic in the presence of phosphate, so that 10^{-4} M calcium resulted in less 1,25(OH)\textsubscript{2}D\textsubscript{3} production than 10^{-4} M calcium, and this difference was more marked at 3 mM phosphate than at 0.3 mM phosphate, and (c) the stimulatory effect by

**Table II**

<table>
<thead>
<tr>
<th>O\textsubscript{2}</th>
<th>Control</th>
<th>nato/min/mg</th>
<th>%</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>7.01</td>
<td>100</td>
<td></td>
</tr>
<tr>
<td>2.5 × 10^{-4} M ruthenium red</td>
<td>7.58</td>
<td>108</td>
<td></td>
</tr>
<tr>
<td>10^{-5} M LaCl\textsubscript{3}</td>
<td>8.16</td>
<td>116</td>
<td></td>
</tr>
<tr>
<td>10 μg/ml antimycin A</td>
<td>7.05</td>
<td>100</td>
<td></td>
</tr>
<tr>
<td>0.83 μg/ml antimycin A</td>
<td>1.77</td>
<td>25</td>
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Preparation and incubation conditions of the mitochondria were as described in the Methods except the reaction was performed at 22°C with a mitochondrial protein concentration of 1.67 mg/ml in 3 ml total vol and in the absence of 25OHD\textsubscript{3}. The inhibitors were added to the incubation mixture before the addition of the mitochondria.

FIGURE 2 The effect of different inhibitors on the ability of calcium to stimulate 25(OH)\textsubscript{2}D\textsubscript{3}-1α-hydroxylase activity. Oligomycin, 10 μg/ml; lanthanum chloride, 10^{-9} M; antimycin A, 0.83 μg/ml; ruthenium red, 2.5 × 10^{-4} M; dintrophenol, 5 × 10^{-4} M; and A23187, 10^{-8} M.

FIGURE 3 A. The effect of calcium on 1,25(OH)\textsubscript{2}D\textsubscript{3} production by mitochondria incubated in the response of four different phosphate concentrations. B. The data in A replotted to illustrate the effect of phosphate on 1,25(OH)\textsubscript{2}D\textsubscript{3} production by mitochondria incubated in the presence of five different calcium concentrations. Phosphate was added as the sodium salt so that 10 mM sodium was present in all incubation media. Chloride was the balancing counter-ion.

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phosphate of the 1α-hydroxylase activity was blocked by 10⁻⁴ M calcium.

**DISCUSSION**

This study has evaluated the effects of physiologic concentrations of calcium, phosphate, and hydrogen ion on 1,25(OH)₂D₃ production by vitamin D-deficient chick renal mitochondria. Care was taken to minimize the exposure of the mitochondria to extracellular concentrations of calcium and phosphate during the preparative procedures. The mitochondria were partially depleted of their calcium by washing with EGTA. The morphology of the mitochondria prepared in this fashion as judged by electron microscopy was comparable to that in the intact cell. The amount of calcium in the mitochondria (4.5 x 10⁻⁷ mol/mg protein) was less than 50% of the amount detected in vitamin D-deficient rat kidney mitochondria not washed in EGTA (11) and only 15% of the amount in normal rat kidney mitochondria (11). The incubations of the mitochondria were performed in the absence of a permeant anion (except when the effects of phosphate were studied) to avoid the potentially disruptive swelling caused by high concentrations of calcium in the presence of a permeant anion like acetate. The free calcium concentration was controlled during the incubation with an EGTA-calcium buffer system (9). Even in the presence of phosphate (up to 10 mM), this buffer system maintained the free calcium concentration within ½ pCa as calculated from the extramitochondrial calcium remaining after the incubation.

Calcium in a concentration range of 10⁻⁷ to 10⁻⁴ M stimulated the production of 1,25(OH)₂D₃ four- to fivefold. Such a calcium range overlaps both the level taken to be the cytosol concentration of calcium (12) and the Kᵣ for calcium of the mitochondrial calcium transport system (13). The effects of hydrogen ion were biphasic. Below pH 6.9, hydrogen ion decreased the ability of calcium to stimulate 1α-hydroxylase activity. It is of interest that decreased calcium accumulation by mitochondria is observed when the pH of the medium is decreased (14). However, above pH 7.0 the ability of less than 10⁻⁴ M calcium to stimulate the 1α-hydroxylase activity was blocked. This is true in both Tris chloride and NaHCO₃-CO₂ buffer systems. Whether the diminished endogenous phosphate content of the mitochondria at the higher pH (Table I) can account in some way for the decreased response of the 1α-hydroxylase activity to calcium remains to be seen. The rate of 1,25(OH)₂D₃ production by isolated renal tubules did not show this loss of calcium stimulation at an alkaline pH (3). Gray, Omdahl, Ghazarian, and De Luca (8) also observed a biphasic effect of pH on 1α-hydroxylase activity in mitochondria. However, the pH optimum observed by them was 7.4. It is not clear whether the pH's of their buffers were determined at the 37°C incubation temperature employed or at a lower temperature. If the buffers were adjusted at 22°C, the actual optimum pH observed by Gray et al. would be similar to that observed in this laboratory.

Phosphate augmented the stimulatory effect of calcium on the rate of 1,25(OH)₂D₃ synthesis both by decreasing the threshold concentration of calcium at which stimulation was observed and by increasing the amount of stimulation seen (Fig. 3). Phosphate in the absence of calcium exerted a biphasic effect on 1α-hydroxylase activity, with maximum stimulation occurring at 1 mM phosphate. Calcium at physiologic concentrations enhanced the phosphate effect without altering its biphasic nature. Phosphate increases the entry of calcium into mitochondria (12-14). Perhaps this underlies part of the phosphate effect on 1α-hydroxylase activity. However, it is necessary to conclude that phosphate has a role independent of calcium to explain the biphasic nature of phosphate control of the 1α-hydroxylase activity observed even in the absence of calcium (Fig. 3).

The probable importance of calcium accumulation by the mitochondria as a necessary step in the calcium stimulation of 1α-hydroxylase activity is underscored by the observation that ruthenium red, at a concentration that does not inhibit state 4 oxygen consumption under the conditions used in this study (Table II) or oxidative phosphorylation in rat liver mitochondria (15), blocked the calcium stimulation of 1α-hydroxylase activity (Fig. 2). Ruthenium red blocks the calcium binding and transport processes of mitochondria (15). Furthermore, A23187, a divalent ionophore that limits the ability of mitochondria to accumulate calcium (16), decreased the effect of extramitochondrial calcium on 1α-hydroxylase activity.

**Figure 4** The same conditions as in Fig. 3, except that phosphate was added as the potassium salt so that 10 mM potassium was present in all incubation media; chloride was the balancing counterion.
activity. Dinitrophenol and antimycin A also block mitochondrial calcium transport (17) and blocked the calcium stimulation of 1,25(OH)2D3 production (Fig. 2). These effects could be attributed to a more general action on mitochondrial metabolism. Lanthanum chloride, thought to be a specific inhibitor of calcium transport by mitochondria (18), did not block the calcium stimulation of 1α-hydroxylase activity. However, Lehninger and Carafoli (19) noted that even in the presence of 10-4 M La++, calcium accumulation by mitochondria continued, although at a reduced rate, and net accumulation was equal to that by control mitochondria after 10 min. The preincubation period used in our experiments was 12 min. Oligomycin, which does not block calcium transport (17), did not block the calcium stimulation of 1,25(OH)2D3 production.

The stimulation of 1α-hydroxylase activity by calcium, demonstrated in this report, was not found by Colston, Evans, Galante, Mac Intyre, and Moss (5) or Fraser and Kodicek (4). In fact, they reported an inhibition of enzyme activity by calcium. These groups used homogenates prepared without an apparent effort to control the amount of extracellular calcium contaminating the preparation, and with no attempt either to deplete the mitochondria of calcium or to control the free calcium concentration in the homogenate with EGTA-calcium buffers. The amounts of calcium they added to achieve an inhibition of the 1α-hydroxylase activity were greater than 10-4 M and may have had a general disruptive effect on mitochondrial function, especially if permeant anions were present in the homogenate. In contrast, Suda et al. (6) found that calcium stimulated the production of 1,25(OH)2D3 by mitochondria if the mitochondria were first depleted of calcium. However, Suda et al. made no attempt to regulate free calcium concentration, so their results are not comparable to our own. They observed maximal stimulation by 2·10-4 M added calcium, a dose that resulted in 50% inhibition in the hands of Colston et al. (4). Higher concentrations of added calcium led to less 1,25(OH)2D3 production, but their incubation medium contained 15 mM acetate.

Our results are not in complete accord with those of Gray, et al. (8). These authors have reported that oligomycin inhibits, by approximately 40%, the mitochondrial 1α-hydroxylase. It is difficult to reconcile this difference. However, these authors did not control the Ca2+ content of the mitochondria during preparation, did not measure rates but reported only a single time point, and used Tris acetate as buffer. Thus, their mitochondria were likely to contain considerable amounts of Ca2+ and swell with the acetate, so that the presence of ATP might well have been required to maintain mitochondrial integrity.

The results obtained with our mitochondrial prepara-
REFERENCES