DIRECT CONTROL BY CALCIUM OF 25-HYDROXYCHOLECALCIFEROL-1-HYDROXYLASE ACTIVITY IN CHICK KIDNEY MITOCHONDRIA

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Summary: Kidney mitochondria were isolated from rachitic chicks and their activity in the metabolism of 25-OH-D_3 was studied in relation to the amount of calcium added in vitro. The addition of 0.05-0.2 mM calcium to a mitochondrial suspension caused a marked and dose-related stimulation of 1-hydroxylation. A sharp decline in the activity was induced by higher concentrations(0.3-0.5 mM) of calcium. The rate of 24-hydroxylation was not influenced by calcium. In these effects, calcium was relatively specific among various divalent cations. These data strongly suggest that calcium is directly involved in the regulation of the vitamin D activation in kidney mitochondria.

It has been generally accepted that vitamin D must be metabolically activated in the liver(1) to its 25-hydroxy derivative (25-OH-D_3), and subsequently in the kidney(2) to its 1,25-dihydroxy derivative [1,25-$(OH)_2$-D_3](3,4) before it can function in the initiation of intestinal calcium transport and in bone mineral mobilization(5). These hydroxylations were shown to take place in mitochondria of respective organs(1,2). Boyle et al first reported that the conversion in the kidney of 25-OH-D_3 to 1,25-$(OH)_2$-D_3 or 24,25-$(OH)_2$-D_3(6)(a metabolite, whose function has not been established) is regulated by serum calcium levels(7). Under conditions of hypocalcemia the kidney produces predominantly 1,25-$(OH)_2$-D_3, while under conditions of normo- and hypercalcemia the predominant metabolite synthesized by the kidney is

Abbreviations used: 25-OH-D_3, 25-hydroxycholecalciferol; 1,25-$(OH)_2$-D_3, 1,25-dihydroxycholecalciferol; 24,25-$(OH)_2$-D_3; 24,25-dihydroxycholecalciferol; PTH, Parathyroid hormone

24,25-$(OH)_2$-D_3(6,7). It was soon considered that parathyroid hormone(PTH) actually regulates the synthesis of 1,25-$(OH)_2$-D_3 in the kidney, since the secretion of PTH is stimulated in hypocalcemia. In 1972, the DeLuca's group(8) and the Rasmussen's group(9) independently reported *in vivo* and *in vitro* experiments suggesting that PTH, rather than the serum calcium level itself, triggers the synthesis of 1,25-$(OH)_2$-D_3. However, in view of the report that PTH increases the calcium influx into kidney cells(10), we considered it still possible that within the cells calcium functions as a direct regulator of the synthesis of 1,25-$(OH)_2$-D_3 in mitochondria. Evidence in favor of this concept is presented in this communication.

MATERIALS AND METHODS

Animals and Preparation of Mitochondria: One-day-old white Leghorn cockerel chicks were maintained on a vitamin D deficient purified soy protein diet(5) containing 1.2% calcium for 2 weeks and then on the same vitamin D deficient diet containing 0.1% calcium for another 2 weeks. Their serum calcium concentration dropped to 5.0-5.5 mg% at the end of feeding. The chicks were killed by decapitation, and the kidneys were removed, rinsed and homogenized in 5 volumes of 0.37 M sucrose containing 0.5 mM Tris-EDTA (pH 7.4). The homogenate was centrifuged at 700 x g for 10 min at 0°C. The supernatant fraction was centrifuged at 8000 x g for 10 min at 0°C. The resulting mitochondrial fraction was then pretreated for 5 min at room temperature in a solution, which consisted of 0.1% bovine serum albumin, 225 mM mannitol, 75 mM sucrose, 10 mM KCl, 10 mM Tris-HCl(pH 7.4), 1 mM $MgCl_2$, 3.2 mM EGTA (Glycoletherdiaminetetraacetic acid) and 0.1 mM DNP. The pretreated mitochondria were centrifuged for 10 min at 0°C, and the resulting pellet was washed once, centrifuged and

Table I. Effects of various divalent cations on the synthesis of 1,25-$(OH)_2$-D_3 by kidney mitochondria

Divalent Cations added		Amounts of 1,25-$(OH)_2$-D_3* produced		Percentage of Control
Control	mM	4.3	4.4**	100
(EGTA	0.5)	4.4		
Ca^{++}	0.1	20.7	19.7**	448
		18.6		
Sr^{++}	0.1	12.2	12.8**	291
		13.4		
Ba^{++}	0.1	12.5	12.0**	273
		11.5		
Mn^{++}	0.1	5.1	4.7**	107
		4.2		
Cd^{++}	0.1	0.0	0.0**	0
		0.0		

* The amounts of 1,25-$(OH)_2$-D_3 produced in vitro was expressed as ng/mg mitochondrial protein/20 min.
** mean of two experiments

resuspended in 0.37 M sucrose. An aliquot of the suspension was taken for protein determination by the method of Gornall et al(11), and for calcium determination by means of a Perkin Elmer Model 403 atomic absorption spectrometer.

Incubation and Extraction of Samples: In a 25 ml Erlenmeyer flask, mitochondria (7 mg protein) were preincubated in air for 5 min at 30°C in 1 ml of a solution of Gray et al(12)(15 mM Tris-acetate, pH 7.4, 2 mM $MgCl_2$, 5 mM succinate, 0.4 mM NADP and 0.22 M sucrose). Various concentrations of other divalent cations (as chloride salts) or EGTA were added as indicated in the text. The reaction was started with the addition of 770 ng (0.14 μCi) of [26,27-^{3}H]-25-OH-D_3 (Amersham, Buckingshire, England), and stopped 20 min thereafter by the addition of 10 ml of 2:1 methanol:chloroform. Extraction was performed as reported by Gray et al(12).

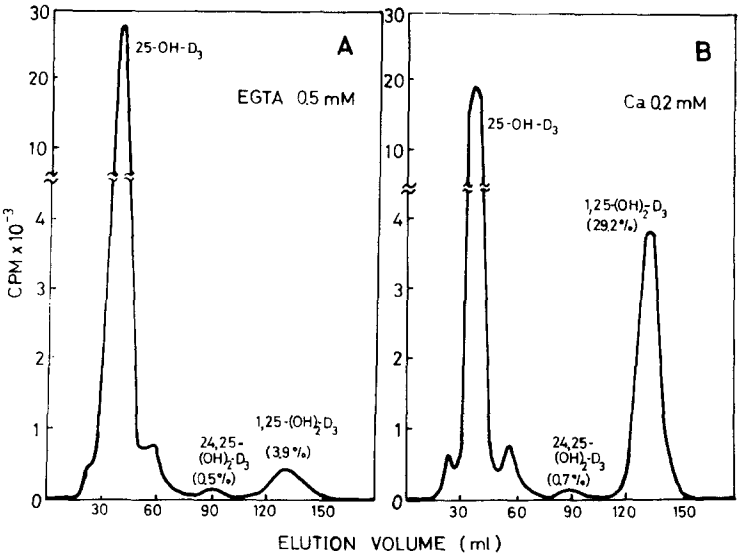

Fig 1. Sephadex LH-20 chromatographic profiles of chloroform extracts of mitochondrial suspension incubated with 770 ng of [^{3}H]-25-OH-D_3 in the absence of calcium (in the presence of 0.5 mM EGTA)(A) and in the presence of 0.2 mM calcium (B). Columns were eluted using a solvent of 65% chloroform-35% hexane. Three ml fractions were collected automatically into liquid scintillation counting vials and evaporated to dryness under a stream of air. The radioactivity was determined using a toluene counting solution(13) and a Packard Model 3320 liquid scintillation spectrometer. The position of elution of each of vitamin D metabolites was determined with standards of radioactive 25-OH-D_3, 24,25-$(OH)_2$-D_3 and 1,25-$(OH)_2$-D_3 as previously described(3,6).

Measurements of in vitro Production of 1,25-$(OH)_2$-D_3 and 24,25-$(OH)_2$-D_3 from 25-OH-D_3: Chromatography of the extracts was carried out on a 1 x 30 cm column of 10 gm of Sephadex LH-20 using a solvent of 65% chloroform-35% hexane according to Holick and DeLuca(13). Amounts of each of vitamin D metabolites produced in vitro were calculated from radioactivities of respective metabolites and the specific activity of the isotope.

RESULTS

The kidney mitochondrial suspension added with 0.5 mM EGTA did not metabolize the substrate, 25-OH-D_3, so actively as that fortified with 0.2 mM calcium (Fig 1). In the former case, more than 95% of the radioactivity was recovered as unchanged

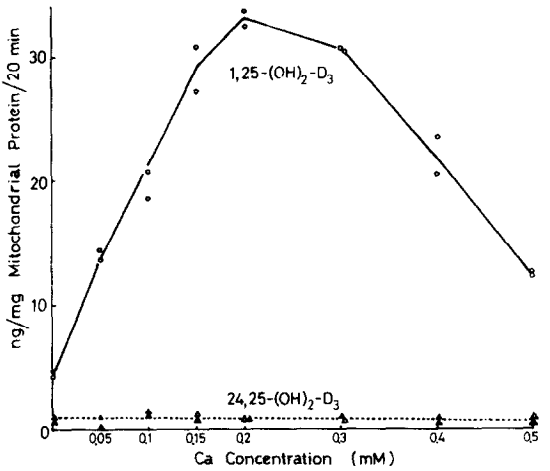

Fig 2. Relationship between calcium concentration and rate of in vitro synthesis of 1,25-$(OH)_2$-D_3 and 24,25-$(OH)_2$-D_3 in kidney mitochondria. Note that the rate of 1,25-$(OH)_2$-D_3 production was maximum at the calcium concentration of 0.2 mM. This tendency was highly reproducible in three independent repetition experiments.

25-OH-D_3 (Fig 1,A), and only 4.4 ng of 1,25-$(OH)_2$-D_3 per 20 min per mg of mitochondrial protein was produced (Table I). The addition of 0.05-0.2 mM calcium to the mitochondrial suspension caused a significant and dose-related stimulation of 1-hydroxylation. Then, in the presence of 0.2 mM calcium, as much as 33.0 ng of 1,25-$(OH)_2$-D_3 per 20 min per mg protein was produced (Fig 1,B and 2). This value is higher than that reported by Gray et al(12). The addition of higher concentrations(0.3-0.5 mM) of calcium caused a sharp decline in the production of 1,25-$(OH)_2$-D_3. On the contrary, the rate of the production of 24,25-$(OH)_2$-D_3 was very low and did not change in response to the addition of 0.05-0.5 mM calcium (Fig 1 and 2). In Table I, the effects of various divalent cations on the stimulation of 1-hydroxylation of 25-OH-D_3 are compared. Calcium showed the highest stimulation in this activity, followed by strontium and barium The addition of manganese(0.1 mM) did not stimulate this activity, and that of cadmium(0.1 mM) inhibited it completely.

DISCUSSION

The idea that intracellular calcium might directly regulate the 1-hydroxylation of 25-OH-D_3 in kidney mitochondria was suggested by many workers(14,15). However, no clearcut evidence for this has been reported. That they have failed to demonstrate the calcium effect may be explained from heavy contamination of calcium in their preparations(12,15). Our mitochondria contained about 7 nmoles of calcium per mg protein, corresponding to the calcium content estimated for *in situ* mitochondria(16). When 50 nmoles of calcium was added to 1 ml of the mitochondrial suspension containing 7 mg protein, the production of 1,25-$(OH)_2$-D_3 was significantly enhanced. This calcium level seems not very far from that attained physiologically in the cell. The dose-related rise in the 1-hydroxylation and the sharp decline by the larger amounts of calcium are most interesting, because previous reports have indicated that physiological amounts of PTH stimulate and higher doses of PTH inhibit the production of 1,25-$(OH)_2$-D_3 *in vivo* and *in vitro*(8,9,14 and 15). These data, therefore, strongly suggest that intracellular calcium is directly involved in the regulation of the vitamin D activation in kidney mitochondria.

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