1,25-DIHYDROXYCHOLECALCIFEROL: DYNAMICS OF THE STIMULATION OF DUODENAL CALCIUM-BINDING PROTEIN, CALCIUM TRANSPORT AND BONE CALCIUM MOBILIZATION IN VITAMIN D AND CALCIUM-DEFICIENT RATS

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1. Introduction

$1,25(OH)_2D_3$ is presently known as the most potent and rapidly acting metabolite of vitamin D_3 in increasing intestinal calcium absorption and bone calcium mobilization [1]. Intestinal CaBP synthesis can be considered to be a molecular expression of the hormonal action of $1,25(OH)_2D_3$ on the enterocyte [2] and consequently a sensitive index of the intestinal response to various metabolites of vitamin D_3 [3,4].

With the aim of assessing the relation of the vitamin D-dependent CaBP to calcium absorption, the time course of intestinal changes in calcium absorption and CaBP levels in response to $1,25(OH)_2D_3$ has received considerable attention in the chick [5–7] but has not yet been investigated in mammals.

Using a highly sensitive and specific RIA, recently developed in the rat [8], we report here, in this species, the dynamic of changes in intestinal CaBP levels in response to $1,25(OH)_2D_3$. The changes in calcium transport and bone calcium mobilization were also examined.

2. Materials and methods

2.1. *Animals and diets*

Male weanling rats of the Sprague strain (Charles River, France) were raised in the dark on a vitamin D-free non-rachitogenic diet (0.50% Ca, 0.36% P) during 4–5 weeks and then on a low-calcium vitamin D-free diet (0.03% Ca, 0.36% P) for 1 week. Animals were fed ad libitum and were not fasted before sacrifice. According to their serum calcium concentrations the hypocalcemic rats were randomized into different groups. Thereafter, each rat received a single intravenous dose of 100 ng $1,25(OH)_2D_3$ in 10 μl 95% ethanol and the animals were sacrificed by exsanguination at different times to determine either intestinal CaBP and bone calcium mobilization or intestinal calcium transport. Control animals received an intravenous injection of 10 μl solvent alone.

Abbreviations: $1,25\text{-}(OH)_2D_3$, 1,25-dihydroxycholecalciferol; CaBP, calcium-binding protein; RIA, radioimmunoassay

2.2. *CaBP measurements*

The proximal 10 cm of intestine from the pyloric valve was removed. All subsequent analyses were performed at 4°C. The mucosal tissue of each rat was scraped then homogenized in Tris buffer (13.7 mM Tris–HCl, 120 mM NaCl, 3 mM KCl (pH 7.4)) centrifuged at 100 000 × g for 1 h and the S100 supernatant was stored at −30°C. Its protein content was measured by a modified Lowry procedure [9] and its cytosolic CaBP was quantitated by RIA [8] and expressed as μg/mg protein.

2.3. *Calcium transport*

The whole duodenum was taken from the pyloric valve to the angle of Treitz. Active intestinal calcium transport was measured in vitro using everted duodenal sacs [10].

2.4. *Bone calcium mobilization*

The increase in serum calcium after the injection

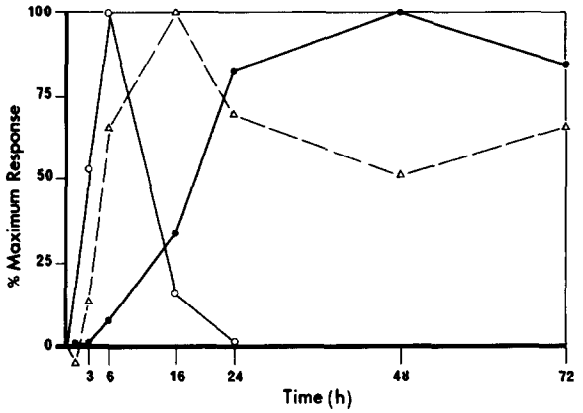

Fig.1. Time course of events occuring in the vitamin D- and calcium-deficient rat after a single intravenous injection of 100 ng $1,25(OH)_2D_3$. (○) duodenal calcium transport in vitro; (●) CaBP level in duodenal cytosol; (△) bone calcium mobilization (increment of serum calcium).

of steroid in vitamin D-deficient rats fed a low-calcium diet is regarded as resulting from the mobilization of bone calcium. Serum calcium was determined by atomic absorption spectrophotometry.

3. Results

Table 1 and fig.1 show the results.

In our experimental rats, the low basal serum calcium levels give evidence of actual vitamin D deficiency. The rise in serum calcium level was significant ($P < 0.01$) as early as 6 h after the intravenous injection of $1,25(OH)_2D_3$, and remained elevated at 16 h ($P < 0.001$), 24 h ($P < 0.01$), 48 h ($P < 0.01$) and 72 h ($P < 0.05$). The maximal calcemic response was observed by 16 h.

Despite a severe vitamin D deficiency the immunoreactive CaBP level was low but was not zero (3 μg/mg protein). An increment in CaBP synthesis was first detected 6 h after dosing ($P < 0.05$), it increased to a level of 10 μg/mg at 16 h and at 24 h approached its maximal level (80%) ($P < 0.001$). Maximal quantities (22 μg/mg) were obtained by 48 h, the levels slowly declining to ~85% of the maximum by 72 h.

Duodenal sacs prepared from rats killed 3 h after dosing transported calcium at a rate significantly higher ($P < 0.05$) than that of the controls, the maximum rate being reached by sacs obtained 6 h

Table 1

Response of bone calcium mobilization, duodenal CaBP levels and duodenal calcium transport to 100 ng $1,25(OH)_2D_3$ administered intravenously to vitamin D-deficient rats fed a low-calcium diet

Time (h) after dosing	Body wt (g)	Bone calcium mobilization serum Ca (mg/dl)		CaBP (μg/mg protein)	^{45}Calcium transport (serosal/mucosal)
		Base	Sacrifice		
1 (6)	168 ± 6.2	5.3 ± 0.12	5.1 ± 0.20	3.7 ± 0.51	
3 (6)	163 ± 5.7	5.1 ± 0.15	5.4 ± 0.13	3.8 ± 0.35	2.7 ± 0.13[a]
6 (6)	174 ± 9.0	5.2 ± 0.07	6.8 ± 0.23[b]	5.1 ± 0.34[a]	4.0 ± 0.91[b]
16 (5)	174 ± 9.1	4.9 ± 0.08	7.5 ± 0.25[c]	10.1 ± 0.86[c]	1.7 ± 0.27
24 (5)	181 ± 12.0	5.2 ± 0.22	7.0 ± 0.42[b]	19.1 ± 1.27[c]	1.0 ± 0.04
48 (5)	175 ± 8.1	5.4 ± 0.15	6.6 ± 0.55[b]	22.5 ± 1.55[c]	
72 (5)	170 ± 7.1	5.6 ± 0.28	7.3 ± 0.45[a]	19.8 ± 1.66[c]	
Controls (6)	169 ± 9.2	5.3 ± 0.09	5.3 ± 0.16	3.8 ± 0.28	1.3 ± 0.07

Calcium transport was measured in groups of rats bred simultaneously according to the same protocol as those used for CaBP and bone calcium mobilization measurements. After ligature of the biliary duct each sac of duodenum everted from the pyloric valve to the angle of Treitz was filled with 0.4 ml medium containing 10 mM fructose, 125 mM NaCl, 0.25 mM $CaCl_2$, 30 mM Tris–HCl buffer (pH 7.4) and $^{45}CaCl_2$ (200 000 cpm/ml) and incubated with 2.5 ml of the same medium gassed with O_2. The incubation was performed in a 20 ml Warburg flask for 90 min at 37°C with shaking. Portions (100 μl) were then removed from the serosal and mucosal solutions (inside and outside, respectively) for radioactivity measurements. Results are the mean ± SEM. The values in brackets represent the number of rats. Analysis for statistical significance was performed by Student's *t*-test: [a] $P < 0.05$; [b] $P < 0.01$; [c] $P < 0.001$; significantly different from control rats

after dosing ($P < 0.01$). Later, the ability of the duodenal sacs to transport calcium was insignificant and represented only 15% of the maximum by 16 h and was down to zero by 24 h.

4. Discussion

The present study clearly shows the different changes in the increase in bone calcium mobilization, duodenal CaBP level and duodenal calcium transport as functions of time in response to a single intravenous injection of 100 ng of $1,25(OH)_2D_3$ to vitamin D-deficient rats fed a low-calcium diet.

The rapid onset as well as the duration of the calcemic response has been discussed elsewhere [4]. Such findings in the rat are in agreement with those reported in the vitamin D-deficient chick [5,7].

The early response (3 h) and the short duration (<24 h) of the ability of rat intestine to absorb Ca^{2+} have been tested in vitro. Using this everted gut sac technique [10] a similar kinetic was reported [6] in response to 125 ng $1,25(OH)_2D_3$ administration to the vitamin D-deficient chick, i.e., an early stimulation (2 h) with a maximal response at 8 h and a decline to almost zero at 21 h. Using the in situ ligated loop technique a significant increase in calcium transport only 3 h after dosing was observed [4].

The development of a specific rabbit antiserum and the utilization of a RIA for rat intestinal CaBP [8] for quantitative CaBP measurements allowed us to study the dynamic of the changes in the amount of duodenal CaBP in the rat. Our study confirms that, after a 5 or 6 week-vitamin D deficiency from weaning, the immunoreactive CaBP is always detectable in the rat intestine [4]. By contrast, intestinal CaBP level in day 1 cockerel fed for 2–4 weeks on a vitamin D-deficient diet was either very low (<0.1 μg/mg) by using a RIA for chick intestinal CaBP [11] or undetectable by using immunoelectrophoresis [6]. In our opinion the low level of intestinal CaBP in vitamin D-deficient rat is more probably due to an incomplete vitamin D-deficiency than to a residual vitamin D-independent CaBP level. After a single injection of $1,25(OH)_2D_3$ the increase in CaBP synthesis was first detectable at 6 h. This delay is consistent with the time-lag required for de novo CaBP synthesis reported in the chick after $1,25(OH)_2D_3$ administration to vitamin D-deficient birds [2,6]. However in similar experimental conditions described [7] traces of chick CaBP as early as 2 h after dosing were detected. As described in the chick [6,7], CaBP levels in the rat plateaued from 48–72 h. This suggests that CaBP is stable, having a turnover time very similar to that of the intestinal cells (34–40 h).

Thus it is noteworthy that, in the rat, the increase in CaBP synthesis appears later than the rise in calcium transport. Indeed by 3 h, $1,25(OH)_2D_3$-stimulated calcium transport was occuring at >50% of the maximum measured rate, whereas an increase in CaBP synthesis could not be detected. At 16 h, when calcium transport had declined markedly, the intestinal CaBP level was beginning to increase. At 24 h, when duodenal calcium transport was down to zero, the duodenal CaBP level was approaching its maximum. This lack of correlation between the rise in calcium transport and the increase in the quantity of CaBP induced by a single injection of $1,25(OH)_2D_3$ at both short and longer time intervals is consistent with early work [12] that failed to show a temporal relationship between the calcium-binding protein and the initiation of calcium absorption in response to vitamin D in the rat. At early time periods our data are in agreement with those reported in the chick [5,6]. However CaBP was detected [7] 2 h after $1,25(OH)_2D_3$ injection to vitamin D-deficient chick and significant increase in calcium transport only after 3 h. The apparent lack of correlation between CaBP level and calcium transport at the later time periods agrees perfectly well with all the studies undertaken in vitamin D-deficient chicks [5–7].

Such dissociations and others reported [13] between calcium absorption and CaBP concentrations question the possible role this protein might have in the calcium absorption process. After the usual homogenization procedure and centrifugation at 100 000 × g for 1 h, the greater part of the amount of the CaBP is cytosolic. This agrees well with the supranuclear localization of CaBP observed in the absorptive cells of rat duodenum [14] and with a 'buffer' instead of a 'carrier' role proposed for CaBP [15]. CaBP might be needed for the later stages of the complex calcium transport process whereas in the very early steps alkaline phosphatase [5,7] as well as other proteins [16] might be involved in vitamin D-dependent calcium absorption process.

This absence of temporal relationship and the inverse correlation order, between the increase in calcium transport and in CaBP synthesis at both short and longer time intervals suggest that the production of CaBP might be an event occuring consequent upon the hormonal stimulation of calcium transport.

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