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Parvalbumin increases in the caudate putamen of rats with vitamin D hypervitaminosis

(vitamin D/calcium-binding proteins/brain)

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ABSTRACT **The influence of chronic vitamin D_3 application on the concentration of the four calcium-binding proteins parvalbumin, the 28-kDa calbindin-D, calmodulin, and S-100 was studied in various brain regions and in the kidney. Young rats were administered daily 20,000 international units of vitamin D_3 per kg (body weight) over a period of 4 months. This chronic treatment resulted in a clinically mild hypervitaminosis that did not affect the content of calmodulin, the 28-kDa calbindin-D, and S-100. Also the concentration of parvalbumin in the cerebral cortex, hippocampus, and kidney remained unchanged. On the other hand, parvalbumin was increased about 50% in the caudate putamen of hypervitaminotic animals as compared to controls. Our results indicate that the metabolism of parvalbumin in the caudate putamen can be influenced by variations of the blood level of this steroid hormone.**

Calcium metabolism is of critical importance in brain function as it appears to have a role in multiple areas of cellular physiology (1). An emerging area of neurochemistry, as it relates to neural function, concerns the family of calcium-binding proteins that are being identified in the brain. Certain of these are rather ubiquitous (calmodulin and S-100) whereas others appear to be distributed to specific neuronal cell types or systems (the 28-kDa calbindin-D and parvalbumin) (2). Such distributions suggest that these calcium-binding proteins may have functions related to the specific physiological characteristics or requirements of the cells in which they are localized. However, to date there have been scant direct correlations between the function or expression of these proteins and a controlled experimental condition. Therefore, we investigated the modulation of the synthesis of calcium-binding proteins by treating rats with a greater than physiological dose of vitamin D over a long period of time. Receptors for metabolites of vitamin D have been detected in many peripheral tissues (3) and in various brain regions (4). This steroid hormone has an enormous variety of effects on different cell types (5), including the induction of the synthesis of the 28-kDa calbindin-D in gut (6), kidney (7), and the peripheral nervous system (8). In the brain a vitamin D dependency in the synthesis of the 28-kDa calbindin-D after acute repletion of vitamin D-deficient animals is uncertain (9–11). We determined the concentration of 28-kDa calbindin-D and of other calcium-binding proteins, parvalbumin, calmodulin, and S-100, in discrete brain regions of animals with vitamin D hypervitaminosis. Unexpectedly, only parvalbumin in the caudate putamen showed an increase under chronic vitamin D treatment, whereas the concentration of all other calcium-binding proteins remained constant in the studied brain regions.

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METHODS

Animals and Treatments. Twenty-six female albino rats [Zur:SIV f (SPF), Institute for Laboratory Animal Sciences, University of Zürich], 24 days of age, were caged individually in screen-bottom suspended cages (39 × 23 × 15 cm) and allowed to adapt for 7 days to standardized constant-temperature and constant-humidity air-conditioned quarters with the light switched on in the day time for 12 hr. At the age of 32 days and weighing ≈135 g, the animals were randomly assigned to two groups and placed on different treatments. Fourteen animals were treated with 20,000 international units (500 μg) of crystallized vitamin D_3 (a generous gift of H. Weiser; Hoffman–La Roche) per kg (body weight), dissolved in 0.1 ml of peanut oil, and administered by gastric cannulation daily from day 8 to day 121. Controls (12 animals) received 40 international units of vitamin D_3 per animal per day throughout the same period. All rats had free access to water and food [standard diet with regard to carbohydrates, fat, protein, minerals, and vitamins, but free of vitamin D and with a calcium content of 0.9% and a phosphorus content of 0.67% (RM 2017, Klingentalmühle Kaiseraugst/Basle)]. Individual food and water consumption as well as weight gains were measured weekly and the vitamin D doses of the treated animals were adapted according to their increase in weight. Activity tests included open-field activity (12) as well as latency and "presession" activity in the "shuttle box" (13) and were performed on day 80 and then sequentially on days 119, 120, and 121. At the end of the treatment (day 122) the animals were sacrificed.

Tissue Extraction. The animals were anesthetized by intraperitoneal injection of pentobarbital and ketamine. Blood samples were taken from all animals by cardiac punction and centrifuged at 1000 × *g*, and the plasma was stored at −20°C for subsequent analysis of blood parameters and vitamin D metabolites. Intrasplenic injection of 1 ml of 0.01% sodium nitrite and heparin (1000 international units) in physiological saline was followed by transcardial perfusion with 100 ml of cold (10°C) 0.15 M NaCl, containing 0.0002% sodium nitrite and 500 international units of heparin. Immediately after perfusion the animals were transferred to a cold room (4°C) for dissection. Selected samples of heart, kidney, and brain were collected for morphological studies and fixed in 4% (wt/vol) paraformaldehyde/0.1 M sodium phosphate, pH 7.3 for immunohistochemistry or in 70% ethanol, followed by paraplast embedding for the Von Kossa reaction (18). Hind limbs were used for x-ray analysis of the tibial epiphyseal plate. The kidneys were removed, decapsulated, and frozen. The brain was cut in six coronal slices and distinct regions were isolated with the aid of a dissecting microscope. For this study we used only cerebral cortex, hippocampus, and caudate putamen. The tissue was frozen and stored in liquid nitrogen. All extraction steps of tissues for biochemical

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analysis were carried out at 0–4°C. Ultrasonic homogenization of the brain regions was performed in 4 mM EDTA/0.028% aprotinin. Homogenates were centrifuged for 30 min at 11,000 × *g*. After centrifugation the supernatant was decanted, and the pellet was resuspended, sonified, and recentrifuged at the same speed. This procedure was performed three times in a total volume of 2 ml. The supernatants were then pooled, vortex mixed, and centrifuged at 80,000 × *g* prior to protein determination and freezing in liquid nitrogen for subsequent radioimmunoassays (RIAs). The kidney extraction was performed in a total volume of 3 ml with addition of 0.05 M 6-aminohexanoic acid, 0.005 M benzamidine hydrochloride, and 0.001 M phenylmethylsulfonyl fluoride to the above sonication solution.

Biochemical Measurements and Histological Studies. Immunoreactive calmodulin in brain and kidney was measured with a commercial RIA kit (Amersham, IM 150). Parvalbumin content of the same tissues was determined by RIA using either monoclonal antibodies (14) or a polyclonal serum, 4064, directed against muscle parvalbumin (kind gift of U. Kaegi, University of Zürich). The 50% binding of the RIA using the antiserum occurred at a 1:1150 dilution. The sensitivity was 0.2–20 ng of parvalbumin assay, and the affinity constant was 1.13×10^{-13} M^{-1}. Purified rat muscle parvalbumin (kind gift of C. W. Heizmann, University of Zürich) was iodinated with the Bolton–Hunter reagent to a specific activity of 36.5 mCi/mg (Anawa Laboratories, Wangen/Zürich; 1 Ci = 37 GBq). The 28-kDa calbindin-D RIA for brain tissue was performed as described (15). The 28-kDa calbindin-D RIA for kidney tissue was performed with a monoclonal antibody specific for this protein as determined by two-dimensional SDS gel electrophoresis (unpublished data). The quantitative measurement of S-100 was according to ref. 16.

Protein concentration was determined by using the protein assay from Bio-Rad, with bovine serum albumin as standard. Immunohistochemical studies were performed as described (17). Paraplast sections were stained as described by Von Kossa (18) for microscopic study of metastatic calcification. Immunoblots of SDS/polyacrylamide gels were performed according to ref. 14. The blood parameters were determined in specialized clinical laboratories (for vitamin D metabolites, Institute of Pathophysiology, University of Berne; for chemical analysis, Institute of Medical Chemistry, University of Zürich). All data were analyzed by Student's *t* test; significance was defined with $P < 0.0001$. Chemicals were commercially available and of analytical or pharmaceutical grade.

RESULTS

Chronic treatment of rats with greater than physiological doses of vitamin D_3 resulted in a mild clinical hypervitaminosis. Weight increase was constant, but lagged behind that of controls. A mild anorexia accompanied by a pronounced polydipsia were induced. Macroscopic demineralization of bone was limited to a moderate rarefaction of the tibial epiphyseal growth plate and to a few metastatic calcifications in the kidneys and heart (data not shown). No calcification was observed in the nervous system. Laboratory data confirmed the hypervitaminosis (153.0 versus 33.9 pmol of 1,25-dihydroxyvitamin D_3 per liter of plasma), the hypercalcemia (3.59 versus 2.71 mmol of calcium per liter of plasma and the hyperphosphatemia (2.21 versus 1.72 mmol of phosphate per liter of plasma) of the animals treated with a greater than physiological dose of vitamin D_3 (Table 1). Neither perturbation in laboratory parameters nor calcification were observed in the controls.

No differences between controls and treated animals were found in various motor tasks including open field activity and the latency or "presession" activity in the "shuttle box" test. Additional behavioral tests performed on these animals are described elsewhere (19). Amounts of 28-kDa calbindin-D, parvalbumin, calmodulin, and S-100 were determined in the total cerebral cortex, total hippocampus, basal ganglia, and kidney in both groups. No significant differences were found in the content of the 28-kDa calbindin-D, calmodulin, and S-100 in the three investigated brain regions (Table 2). Similarly, the parvalbumin concentration in the cerebral cortex, hippocampus, and kidney was unchanged (Table 2).

In contrast to the brain regions mentioned, the concentration of parvalbumin in the basal ganglia was increased by 50% in animals with vitamin D hypervitaminosis [0.069 ± 0.006 versus 0.046 ± 0.002 μg/mg of soluble protein (mean ± SEM)] (Table 2 and Fig. 1). This difference is highly significant ($P < 0.0001$; 5.7406 by Student's *t* test; degrees of freedom = 20). The total number of parvalbumin-containing cells in the basal ganglia, as counted in one-half of the brain from two animals in each group, remained unchanged. In the kidney, however, the 28-kDa calbindin-D level was increased by 4-fold (Table 2). A confirmation of the RIA results for parvalbumin in the basal ganglia by immunoblot analysis failed because of the insensitivity of the immunoblot.

DISCUSSION

This paper shows that the chronic administration of large doses of vitamin D_3 for 4 months to young female rats results

Table 1. Plasma parameters and clinical signs in control rats and rats with chronic vitamin D hypervitaminosis

Parameter or clinical sign	Control rats	Rats with vitamin D hypervitaminosis
1,25-$(OH)_2$-D_3, pmol/liter of plasma	33.9 ± 9.0	153.0 ± 12.6
25-(OH)-D_3, nmol/liter	40.6 ± 2.4	699 ± 27
Calcium, mmol/liter	2.71 ± 0.03	3.59 ± 0.08
Phosphate, mmol/liter	1.72 ± 0.07	2.21 ± 0.12
Creatinine, μmol/liter	69 ± 3	71 ± 3
Urea, mmol/liter	7.2 ± 0.6	6.8 ± 0.3
Alkaline phosphatase, international units/liter	113 ± 12	107 ± 6
Final weight, g	271 ± 6	239 ± 7
Final food intake, g/day	16 ± 0.3	13 ± 1.0
Final water intake, ml/day	36 ± 1.5	51 ± 1.9

Each value represents the mean ± SEM. Twelve rats were used as controls; 14 rats with hypervitaminosis were used. Hypervitaminosis, hypercalcemia, and hyperphosphatemia as well as reduced weight development, anorexia, and polydipsia are evident in the treated animals. Creatinine, urea, and alkaline phosphatase levels remained unchanged. 1,25-$(OH)_2$-D_3, 1,25-dihydroxyvitamin D_3; 25-(OH)-D_3, 25-hydroxyvitamin D_3.

Table 2. Quantitative determination of the four calcium-binding proteins in the brain

Calcium-binding protein	Tissue	Control rats	Rats with hypervitaminosis
Calmodulin, μg/mg of soluble protein	Kidney	205 ± 11‡	240 ± 16*
	Cerebral cortex	14.8 ± 0.9	13.1 ± 0.6‡
	Hippocampus	19.5 ± 0.7	19.0 ± 0.6
	Basal ganglia	18.7 ± 0.9	16.7 ± 0.6
Parvalbumin, μg/mg of soluble protein	Kidney	0.122 ± 0.022‡	0.088 ± 0.028*
	Cerebral cortex	0.396 ± 0.009	0.385 ± 0.009*
	Hippocampus	0.457 ± 0.016	0.464 ± 0.017
	Basal ganglia	0.046 ± 0.002	0.069 ± 0.006
Calbindin-D, μg/mg of soluble protein	Kidney	17.61 ± 1.55‡	72.03 ± 4.8*
	Cerebral cortex	3.88 ± 0.42†	3.86 ± 0.35‡
	Hippocampus	1.13 ± 0.04	0.86 ± 0.03‡
	Basal ganglia	2.73 ± 0.16	2.68 ± 0.14
S-100, μg/mg of soluble protein	Cerebral cortex	2.12 ± 0.07	2.15 ± 0.03‡
	Hippocampus	10.96 ± 0.31	11.31 ± 0.38
	Basal ganglia	13.02 ± 0.70	10.86 ± 0.40

Results are expressed as mean ± SEM. The concentration of parvalbumin in the basal ganglia was increased by 50% in animals with vitamin D hypervitaminosis. This increment is highly significant ($P < 0.0001$). Calbindin-D showed an obvious increase in the kidney ($P = 0.0001$) and slight decrease in the hippocampus. The other tissues showed for each assay highly identical mean values with nonsignificant differences elsewhere. In both controls and treated animals, only the kidneys showed considerable individual variations in the parvalbumin concentration. Ten control rats and 12 rats with vitamin D hypervitaminosis were used unless otherwise indicated. *Seven animals were tested. †Nine animals were tested. ‡Eleven animals were tested.

in an increase in the concentration of parvalbumin in the caudate putamen, whereas the concentration of other calcium-binding proteins in the brain remains unchanged.

This study differs from others performed to demonstrate a vitamin D effect on the concentration of calcium-binding proteins in the periphery (6, 7, 20) and brain (8, 9, 21). In fact, the mild chronic vitamin D treatment that we used did not severely interfere with the overall health of the animals and allowed a prolonged course of the experiment. In spite of the profound alteration of the parvalbumin content of the basal ganglia, no overt modifications of motor behavior were observed in the animals with hypervitaminosis. This, however, does not exclude functional consequences of the increased parvalbumin concentration that could be unmasked by more sophisticated tests of motor function (19).

It was surprising that the effect was restricted to the caudate putamen, where parvalbumin occurs only in a subpopulation of aspiny interneurons (22). The physiological role of these neurons is uncertain, but they are fast firing neurons using γ-aminobutyric acid as neurotransmitter (23). In the basal ganglia the presence of 1,25-dihydroxyvitamin D_3 receptors was reported by Stumpf and O'Brien (24) on "large, solitary neurons." It may be a coincidence, but their shape and localization is consistent with their being parvalbumin-positive.

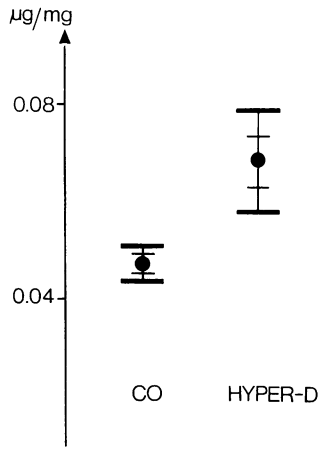

FIG. 1. Parvalbumin in the basal ganglia of control animals (CO) and animals with vitamin D hypervitaminosis (hyper-D) (mean ± 2 SEM).

The apparent lack of response of parvalbumin in cortical and hippocampal cells to vitamin D administration suggests opposite sensitivities of different neural subpopulations. In fact, whereas only one neural type in the caudate putamen is parvalbumin-positive, the cerebral cortex and the hippocampus display a parcellation and stratification of a myriad of distinct interneuronal populations. This by no means implies that the concentration of parvalbumin in these areas is not susceptible to changes under certain conditions. Baimbridge (25) described an increase of the parvalbumin concentration in the cerebral cortex of the epileptic mouse (25).

Vitamin D hypervitaminosis also produced hypercalcemia and probably triggered adaptive endocrine changes that could be responsible for the observed increase of parvalbumin in the basal ganglia. Notwithstanding, hypercalcemia alone is reported not to influence the Ca^{2+} concentration in the striatum (26) and calcitonin binding sites are absent from the basal ganglia (27).

The clear cut increase in the concentration of 28-kDa calbindin-D in the kidney but its constancy in the brain implies different regulation of the kidney and brain 28-kDa calbindin-D genes by vitamin D (28). Alternatively, it is conceivable that, if vitamin D is required, then the rate of production of 28-kDa calbindin-D in neurons that contain this protein is maximal and will not be further increased by the steroid hormone. The absence of changes in the expression of the 28-kDa calbindin-D, calmodulin, and S-100 genes to vitamin D application could also derive from an inadequacy of the used steroid or from an inaccessibility of the target cells. The first possibility influenced our choice of the "precursor" form vitamin D_3 over the more specific metabolite 1,25-dihydroxyvitamin D_3 for the induction of hypervitaminosis. The second possibility is unlikely, since vitamin D metabolites cross the blood–brain barrier, are measurable in the cerebrospinal fluid (29–31), and may directly affect nerve and glial cells (4). Therefore, we assume that neither vitamin D nor Ca^{2+} affect the expression of the 28-kDa calbindin-D in the brain, although a modulation is possible under certain experimental conditions (32).

In conclusion, this report suggests the possibility of influencing the concentration of parvalbumin in selected nerve

cells by peripheral application of the steroid hormone vitamin D.

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