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Acute stimulation of creatine kinase activity by vitamin D metabolites in the developing cerebellum

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There is increasing evidence that vitamin D metabolites have a developmental function. We have investigated the influence of the vitamin D status on the activity of creatine kinase in the brain. Normally fed rats show an increase in the specific activity of cerebral and cerebellar creatine kinase during postnatal development. Vitamin-D-depleted rats failed to show this normal increase. Developing cerebellum, but not cerebrum, in both vitamin D-depleted rats and in normally fed animals, responded sequentially to a single injection of a vitamin D metabolite by displaying increased creatine kinase specific activity. In 5–25-day-old rats, 24*R*,25-dihydroxyvitamin D-3 significantly increased creatine kinase specific activity 24 h after injection. In contrast, 1,25-dihydroxyvitamin D-3 stimulated cerebellar creatine kinase activity from 20 days after birth. A similar pattern of sequential responsiveness to vitamin D metabolites, but at an earlier age, was shown in the cerebellum of the rabbit, which is a 'perinatal brain developer' compared to the rat, a 'postnatal brain developer'. Because of the difficulty in obtaining vitamin D-depleted rabbits, studies were carried out in normally fed animals. In these rabbits, 24*R*,25-dihydroxyvitamin D-3 stimulated cerebellar creatine kinase activity between 6 days before birth and 9 days after birth, while 1,25-dihydroxyvitamin D-3 caused an increase in cerebellar creatine kinase specific activity from 8 days after birth. These developmental differences found in creatine kinase basal activity and responsiveness are correlated with differences in cellular growth rates, both in the rabbit and in the rat, suggesting that vitamin D metabolites may be required for optimal cerebellar development.

Introduction

Vitamin D is a prohormone for the more polar metabolites, 1α,25-dihydroxyvitamin D-3 (1,25 $(OH)_2D_3$) and 24*R*,25-dihydroxyvitamin D-3 (24,25$(OH)_2D_3$). 1,25$(OH)_2D_3$ is the classical metabolite involved principally in calcium metabolism and acts on a range of adult organs containing specific receptors (reviewed in Ref. 1). The vitamin D metabolite, 24,25$(OH)_2D_3$ has not been implicated in calcium transport. However, it is probably involved in embryonic development of endochondral bone [2–5] and in growth of cartilage [6,7] including activation of enzymes in cartilage such as alkaline phosphatase [8].

Abbreviations: 1,25$(OH)_2D_3$, 1α,25-dihydroxyvitamin D-3; 24,25$(OH)_2D_3$, 24*R*,25-dihydroxyvitamin D-3.

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In addition to the classical organs responsive to vitamin D, such as bone, intestine and kidney, evidence is accumulating for interactions of vitamin D metabolites in other organs, including the brain (see review by Norman et al., Ref. 1). Receptors for $1,25(OH)_2D_3$ have been found in specific neurons and in the hippocampus [9,10] by both autoradiographic and immunocytochemical techniques. Moreover, specific localization of tritium labeled $1,25(OH)_2D_3$ was demonstrated in neurons of rat forebrain, hindbrain and spinal cord. In the pituitary, a receptor for $1,25(OH)_2D_3$ has been identified biochemically [11]. In addition to vitamin D receptors, vitamin D-dependent calcium-binding protein was also found in the brain. Its highest concentration is in the cerebellum in which the protein is localized in the Purkinje cells [10,12,13]. Since there is also autoradiographic evidence that granule cell nuclei of the cerebellum contain receptors for $1,25(OH)_2D_3$ [10], these observations suggest that the cerebellum may respond to the hormone. If this were so, it would broaden the functional significance of vitamin D to include the cerebellum and contribute to the recent extension of the action of $1,25(OH)_2D_3$ beyond its role in calcium translocation in epithelial cells [1].

Recently, we showed that an increase in activity of the brain type isozyme of creatine kinase (ATP: creatine phosphotransferase, EC 2.7.3.2) is stimulated in the rat kidney, by the two vitamin D metabolites [14,15]. They act at different stages of postnatal development, correlated with changes in the concentrations of $1,25(OH)_2D_3$ receptors or $24,25(OH)_2D_3$ binding proteins [15]. It was therefore of interest to discover whether this pattern of differential responsiveness to vitamin D metabolites could be found in the brain. We report here that normal development of creatine kinase activity does not take place in the brain of vitamin D-depleted rats. The cerebellum, but not the cerebrum, was found to be sequentially responsive to the two metabolites during different stages in development. It was particularly interesting to find similar sequential responsiveness in the cerebellum of the rat, which is a postnatal developer, and in the rabbit, which, like the human, is a 'perinatal brain developer' [16].

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Materials and Methods

Experimental animals

Rats. Vitamin D-depleted Wistar rats were raised as described previously [17]. Females were fed a vitamin D-deficient diet containing 0.75% calcium and 0.50% phosphorus (which kept them normocalcemic and normophosphatemic) and were kept in the dark. When they were 130 days old, the females were mated with normal males and maintained during gestation and lactation on the same vitamin D-deficient diet. Male and female vitamin D-depleted rats were used between the ages of 5 and 65 days as indicated. None of the vitamin D-deficient animals had any detectable circulating $25(OH)D_3$ (no more than 0.16 ng/ml compared to 15 ± 4 ng/ml in normally fed animals) or $1,25(OH)_2D_3$ (no more than 5 pg/ml compared to 95 ± 10 ng/ml), as measured by a competitive protein-binding assay, following preparative Sephadex LH-20 chromatography of the lipid extract [18,19] of their serum.

Rabbits. New Zealand white does were raised at the Tel Aviv University animal colony; fetuses or newborn animals were used between 6 days before and 15 days after birth.

Biochemicals

$24,25(OH)_2D_3$ and $1,25(OH)_2D_3$ were kindly provided by Professor S. Edelstein, Department of Biochemistry, Weizmann Institute of Science. Reagents for the assay of creatine kinase activity were purchased from Sigma, St. Louis, MO.

Hormonal treatment

Batches of vitamin D-depleted rats at different ages were randomly divided into three experimental groups and were injected intraperitoneally with 1 ng/g body weight of $1,25(OH)_2D_3$ or 3 ng/g body weight of $24,25(OH)_2D_3$ or with vehicle (20% ethanol in propylene glycol) and killed 24 h later for collection of cerebellar and cerebral samples. These doses were chosen as they were close

to the midpoint of a previously reported dose-response curve [20] for the action of 24,25$(OH)_2D_3$ on the epiphyses of vitamin D-depleted rats, and had been used for the stimulation of rat renal creatine kinase [15].

Normally fed rats and rabbits were injected with three times the dose given to vitamin D-depleted rats in order to produce a circulating level of vitamin D metabolites significantly higher than that in the untreated animals. These doses were based on previous data [15,20] and on measurements of average fetal weight from previous experiments.

In order to inject fetuses with the metabolites, a hysterotomy was performed; the uterine horns were gently lifted out, avoiding unnecessary trauma. The fetuses in each female were separated into three groups for intraperitoneal injection of the metabolite or the vehicle into the abdomen of each fetus. The uterus was gently returned to the abdominal cavity and muscle and skin each closed with a single stitch.

Creatine kinase preparation and assay

Rats or rabbits were killed with diethyl ether 24 h after injection and the cerebrum and cerebellum were removed and stored at −20 °C. The time of 24 h was chosen, since, in other systems in which the brain type isozyme of creatine kinase was increased by a single injection of a vitamin D metabolite, 24 h samples (the latest time tested) showed the highest activity [20,21].

Rabbit fetuses were removed by cesarean section 24 h after injection; the brain was excised and the separated cerebrum and cerebellum were stored at −20 °C.

Samples were homogenized in a buffer containing isotonic sucrose [20], using a teflon-glass homogenizer and centrifuged in a microcentrifuge (Eppendorf) at 12000 × *g* for 5 min. The creatine kinase activity was determined in the supernatant using a coupled spectrophotometric assay [20]. 1 unit was defined as the activity catalyzing the formation of 1 μmol of ATP per min at 30 °C, and the specific activity was defined in units per mg protein.

Statistical treatment. A student's *t*-test was used to compare treated versus control means.

Results

Decreased creatine kinase activity in brain of vitamin D-depleted rats during postnatal development

The normal pattern of increase of creatine kinase activity in both the cerebrum and cerebellum during postnatal development of normally fed rats is a classical saturation-type curve (Fig. 1), which shows the steepest slope between 5 and 10 days and reaches a plateau in the cerebrum by 15–20 days and in the cerebellum a few days later – at approx. 25 days after birth. In contrast, vitamin D-depleted rats failed to show any increase in creatine kinase activity in either cerebrum and cerebellum between 5 and 65 days after birth (Fig. 2). The basal activity of creatine kinase showed a roughly parallel pattern of fluctuations in cerebrum and in cerebellum of vitamin D-depleted rats during this period (Fig. 2).

Age-dependence of brain creatine kinase response to vitamin D metabolites in vitamin D-depleted and normal rats

There was a striking difference between cerebrum and cerebellum in their responsiveness to vitamin D metabolites during postnatal development in vitamin D-depleted rats (Fig. 3). The

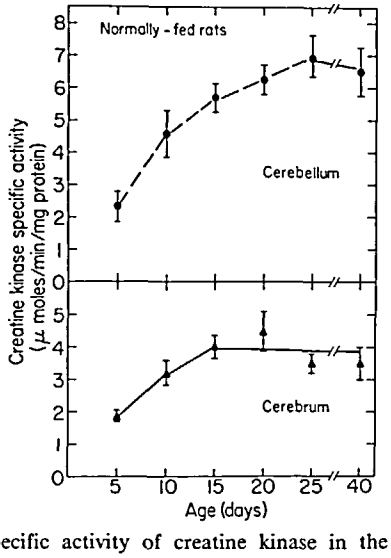

Fig. 1. Specific activity of creatine kinase in the brain of normally fed rats during postnatal development. Portions of cerebrum and the entire cerebellum were assayed as described previously [20]. The results are means ± S.E. for $n = 5–8$.

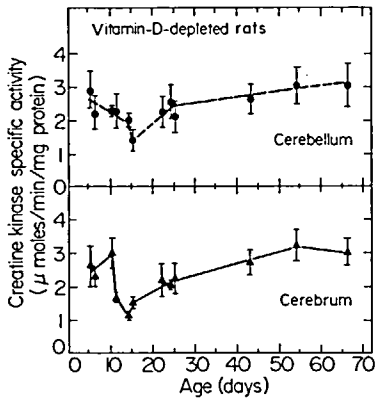

Fig. 2. Specific activity of creatine kinase in the brain of vitamin D-depleted rats during postnatal development. Portions of cerebrum and the entire cerebellum were assayed as described previously [20]. The results are means ± S.E. for $n = 6$–12.

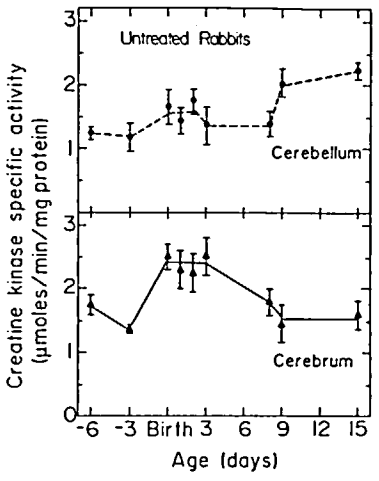

Fig. 4. Specific activity of creatine kinase in rabbit brain, during perinatal development. Portions of cerebrum and cerebellum were assayed as described previously [20]. The results are means ± S.E. for $n = 3$–9.

data in Fig. 3 (and Fig. 5 to follow) are expressed as ratios of values from experimental (vitamin D-injected) and control (vehicle-injected) animals to simplify presentation, since the values for vehicle-injected animals (Figs. 2 and 4) are not simple functions of age. For example, in vitamin D-depleted rats, 5 days after birth, the specific activity of creatine kinase in cerebellum is nearly 3 μmol/min per mg protein. Injections of $24,25(OH)_2D_3$ (3 ng/g body weight), increased the specific activity of creatine kinase 24 h later to a value of 6.8 μmol/min per mg protein, resulting in the ratio of experimental to control values (E/C) of 2.25, presented in Fig. 3.

Neither $1,25(OH)_2D_3$ nor $24,25(OH)_2D_3$ stimulated creatine kinase activity in the vitamin D-depleted rat cerebrum (Fig. 3). However, in the cerebellum of the vitamin D-depleted rat, $24,25(OH)_2D_3$ significantly increased creatine kinase activity from 5 to 25 days after birth while $1,25(OH)_2D_3$ stimulated creatine kinase activity from day 21 (Fig. 3).

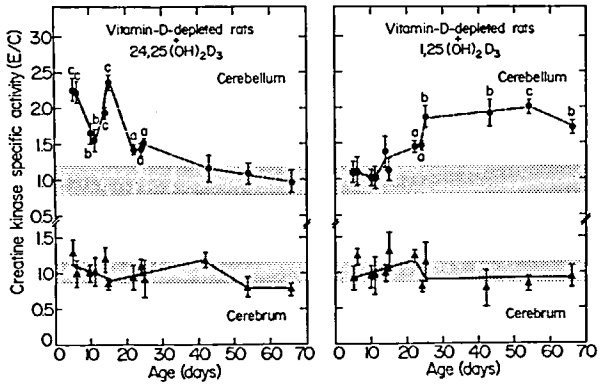

Fig. 3. Creatine kinase specific activity in the brain of vitamin D-depleted rats, during postnatal development, 24 h after intraperitoneal injection of vitamin D metabolites. Rats were treated as described in Materials and Methods and creatine kinase was assayed as described previously [20]. The results (E/C) are means ± S.E. (for $n = 6$–12) of experimental divided by control (vehicle-injected) values. The broken lines indicate the average S.E. of the mean control values taken from data presented in Fig. 2. Significance of experimental vs. control values was calculated by a Student's t-test; *a*, $P < 0.05$; *b*, $P < 0.01$; c, $P < 0.001$.

A preliminary test of responsiveness to vitamin D metabolites in normally fed rats showed that stimulation of brain creatine kinase occurred also in these animals. As in the vitamin D-depleted rats, only cerebellar and not cerebral creatine kinase was stimulated. Cerebellar creatine kinase activity, was stimulated by 3.2-fold at 5 days (compared to the normal values shown in Fig. 1), 1.8-fold at 10 days and 1.3-fold at 20 days by $24,25(OH)_2D_3$, while $1,25(OH)_2D_3$ stimulated cerebellar creatine kinase activity 1.4-fold at 20 days and 1.7-fold at 40 days. These results indicated that experiments could be made using normally fed animals.

Creatine kinase activity in perinatal rabbit brain

It was particularly interesting to study the response of the rabbit, since like the human and unlike the rat, it is a 'perinatal brain developer' [16]. Because of the difficulty in obtaining vitamin D-deficient rabbits, normally fed rabbits were used in these studies.

We chose to study creatine kinase activity from 6 days prenatally until 15 days postnatally. This is within the time period of the brain growth spurt, which is normally from 10 days before birth to 30 days after birth [16]. During the period studied, the basal activity of creatine kinase shows a different developmental pattern in the rabbit cerebrum and cerebellum (Fig. 4). The basal creatine kinase activity in embryonic cerebrum at 6 days before birth is higher than the value in the cerebellum at this age (Fig. 4). In both cerebrum and cerebellum, creatine kinase activity rises between 3 days before birth and birth. The cerebrum maintains the high creatine kinase activity reached at birth until and including day 3; then there is a decline by day 8 to the level of activity measured at 3 days before birth. This value for cerebral creatine kinase activity remains the same up to day 15 after birth. Cerebellar creatine kinase, also higher at birth than at 3 days before birth, declines by day 3 after birth then shows a second rise by day 9, which is maintained to reach peak activity at day 15, the latest day tested (Fig. 4).

Age-dependence of the brain creatine kinase response to vitamin D metabolites in normal rabbits

The same striking difference between the cerebrum and cerebellum in their responsiveness

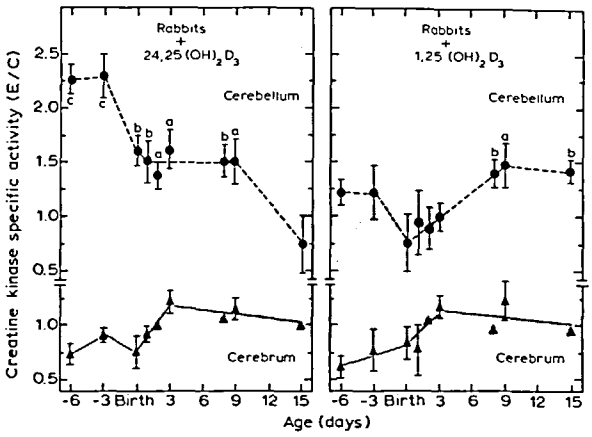

Fig. 5. Creatine kinase specific activity in the brain of the perinatal rabbit, 24 h after intraperitoneal injection of vitamin D metabolites. Rabbits were treated as described in the text and creatine kinase was assayed as described previously [20]. The results (E/C) are means ± S.E. (for $n = 6$–12) of experimental divided by control (vehicle injected) values. For $n \leq 3$ only the means and no error bars are given. The broken lines indicate the average S.E. of the mean control values taken from data presented in Fig. 4. Significance of experimental vs. control values was calculated by a Student's t-test. a, $P < 0.05$; b, $P < 0.01$; c, $P < 0.001$.

to $1,25(OH)_2D_3$ and $24,25(OH)_2D_3$ during the postnatal development of the rat was seen in the rabbit during the perinatal period. Neither $1,25(OH)_2D_3$ nor $24,25(OH)_2D_3$ induced creatine kinase activity in the rabbit cerebrum (Fig. 5). However, in the rabbit cerebellum, $24,25(OH)_2D_3$ significantly increased creatine kinase activity from 6 days before birth to 9 days after birth, while $1,25(OH)_2D_3$ stimulated creatine kinase activity only from day 8 after birth (Fig. 5). At 6 and 3 days before birth, $24,25(OH)_2D_3$ increased creatine kinase activity by 2.25-fold, and from the day of birth to 9 days after birth, by 1.5 fold. $1,25(OH)_2D_3$ increased creatine kinase activity from day 8 to day 15 after birth by the same 1.5-fold (Fig. 5).

Discussion

The results of this investigation reveal that the specific activity of creatine kinase, in the cerebellum (but not in the cerebrum) of developing rats and rabbits, can be modulated by the availability of vitamin D metabolites. Furthermore, cerebellar

creatine kinase activity is susceptible to stimulation by $24,25(OH)_2D_3$ at an early stage of development (prenatal in the rabbit, immediately postnatal in the rat). This stage lasts in the rat for 2–3 weeks and is followed by a stage in prepubertal development in which the responsiveness to $24,25(OH)_2D_3$ is lost, but is replaced by responsiveness to $1,25(OH)_2D_3$ which then persists into adult life.

The differences in creatine kinase activity and responsiveness to vitamin D metabolites found in the cerebrum and cerebellum may be a consequence of differences in their developmental patterns. During the perinatal period in the rabbit, up to 3 weeks of age [16], and postnatally in the rat, the cerebellum develops relatively late in comparison with other parts of the brain, and is thus largely a postnatal acquisition [22–24]. At this stage, growth in the cerebellum is characterized predominantly by cell proliferation, while in the cerebrum increase in cell size [16,24] is the main characteristic.

The increase in creatine kinase activity during the stage of most rapid cell division in the cerebellum of untreated animals and its stimulation by $24,25(OH)_2D_3$ in the cerebellum of prenatal and early postnatal animals, is reminiscent of other rat and avian systems in which creatine kinase activity parallels growth and division [25–27]. The use of stimulation of creatine kinase activity as a marker for hormone action in this case, parallels its use in chick [21], rat [20] and human [25] systems for vitamin D metabolites, and for an extremely wide range of other steroid and peptide hormones and growth factors [25–29]. Particularly striking is the parallel sequential responsiveness to vitamin D metabolites in the cerebellum, reported in this paper, and in the kidney, as reported previously [15]. In kidney cells in culture [14] we found that $24,25(OH)_2D_3$ but not $1,25(OH)_2D_3$ could accelerate the maturation of responsiveness to $1,25(OH)_2D_3$, perhaps via induction of receptors for $1,25(OH)_2D_3$ [31].

Reports of interactions between vitamin D metabolites and the brain, have, until now, dealt with the description of vitamin D binding [9] accompanied by the presence of calcium binding proteins [10,12,13] similar to those induced by $1,25(OH)_2D_3$ in the intestine [32]. An increase in immunoreactive calcium-binding protein was found in chick cerebellum following chronic, but not acute, administration of vitamin D to severely vitamin D-deficient chicks [33]. Specific rat brain nuclei, which contain receptors for $1,25(OH)_2D_3$ and/or calcium binding protein, showed an increase in choline acetyltransferase activity after 1 week of treatment with $1,25(OH)_2D_3$ [34].

To the best of our knowledge, the present report is the first to demonstrate an acute effect of vitamin D metabolites on the brain. Moreover, the pattern of creatine kinase activity as a function of age (Fig. 2) in vitamin D-deficient rats, in both cerebellum and cerebrum, is strikingly different from the normal saturation-type growth curve of creatine kinase activity in the cerebrum and cerebellum (Fig. 1) or in whole brain of normal rats [28], which shows the most rapid rate of increase between 5 and 15 days after birth. The discrepancy in availability of creatine kinase activity between normally fed and vitamin D-deficient rats suggests that vitamin D depletion may indeed have a deleterious effect on brain development. The higher specific activity of creatine kinase in cerebellum and cerebrum of vitamin D-depleted rats immediately after birth, compared to 15 days later, may be due to the incomplete vitamin D depletion in the fetus. The capacity for carrying out the synthesis of both $24,25(OH)_2D_3$ and $1,25(OH)_2D_3$ has been found in human decidua and placenta [30] which may be capable of utilizing lower circulating concentrations of precursor molecules than the kidney of the mother or the newborn. Such a hypothesis could explain the drop in creatine kinase activity after birth when the pups receive vitamin D-depleted milk.

In considering the mechanism of stimulation of creatine kinase in the cerebellum by vitamin D metabolites, a comparison with the stimulation of creatine kinase activity by estrogen in the rat uterus is a useful starting point. Despite the fact that $1,25(OH)_2D_3$ or $24,25(OH)_2D_3$ transport through the rat blood-brain barrier [35] is more restricted than the transport of 17β-estradiol [36], autoradiographic studies [9] and the results presented in this paper demonstrate that sufficient amounts of $1,25(OH)_2D_3$ and probably also $24,25(OH)_2D_3$, reach cell nuclei in the brain and presumably act there analogously to steroid

hormones such as estrogen, although an indirect effect is also possible. Since accumulation of mRNA for the brain type isozyme of creatine kinase is stimulated by estrogen in rat uterus [37], and actinomycin D prevents the stimulation of creatine kinase activity by parathyroid hormone in bone cells [26], it is possible that vitamin D metabolites stimulate cerebellar creatine kinase via increased steady-state concentrations of mRNA for the brain type isozyme of creatine kinase. This possibility is supported by our first molecular hybridization experiments showing that mRNA for CKB increases 4-fold 2 h after injection of $24,25(OH)_2D_3$ into 5 day-old vitamin D depleted rats [38]. Thus, in addition to providing a marker capable of revealing previously undetected hormonal responsiveness, the rapid stimulation of the gene for the brain type isozyme of creatine kinase may provide a favorable system for the investigation of how diverse hormones can stimulate the same gene.

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