Stimulation of creatine kinase BB activity by $1\alpha,25$ -dihydroxycholecalciferol and $24R,25$ -dihydroxycholecalciferol in rat tissues

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Vitamin D metabolites stimulate creatine kinase BB activity in organs of vitamin D-deficient rats. In epiphyses of long bones, creatine kinase BB activity increases 2.6-fold 24 h after injection of $24R,25$ -dihydroxycholecalciferol but not of $1\alpha,25$ -dihydroxycholecalciferol. Contrariwise, $1\alpha,25$ -dihydroxycholecalciferol, but not $24R,25$ -dihydroxycholecalciferol, increases creatine kinase BB activity in diaphyses and in kidney. Neither metabolite affects creatine kinase activity in duodenal mucosa.

The mechanism of action of $1\alpha,25(\text{OH})_2\text{D}_3$ has been extensively examined in chick and rat intestine (Tsai & Norman, 1973; Lawson & Wilson, 1974; Brumbaugh & Haussler, 1974; Emtage *et al.*, 1974; Zerwekh *et al.*, 1976; Fraser, 1980). These studies suggest a mode of action of $1\alpha,25(\text{OH})_2\text{D}_3$ in intestinal cells similar to that of other hormonal steroids in their responsive organs (O'Malley & Schrader, 1976; DeLuca, 1980). This mechanism involves the stimulation of the synthesis of specific proteins which take part in intestinal Ca^{2+} transport (Wasserman & Taylor, 1966, 1968; Haussler *et al.*, 1970; Wilson & Lawson, 1978).

$24,25(\text{OH})_2\text{D}_3$, another renal metabolite of cholecalciferol, is probably involved in the development of embryonic cartilage and in endochondral bone formation (Ornoy *et al.*, 1978; Endo *et al.*, 1980; Malluche *et al.*, 1980). There is increasing evidence of the presence of specific receptors for $24R,25(\text{OH})_2\text{D}_3$, particularly in chondrocytes and parathyroid glands (Corvol *et al.*, 1980; Merke & Norman, 1981; Sömjén *et al.*, 1982b). Parallel studies have shown that $24R,25(\text{OH})_2\text{D}_3$ stimulates ornithine decarboxylase and DNA polymerase activities and DNA synthesis in epiphyseal cartilage and in epiphyseal chondrocytes *in vivo* and *in vitro* (Corvol *et al.*, 1980; Sömjén *et al.*, 1982a, 1983). These studies also parallel recent

observations that $1\alpha,25(\text{OH})_2\text{D}_3$ stimulates ornithine decarboxylase activity, polyamine synthesis and DNA synthesis in the duodenal mucosa (Shinki *et al.*, 1981; Takahashi *et al.*, 1982; Sömjén *et al.*, 1983). Moreover $1\alpha,25(\text{OH})_2\text{D}_3$ stimulates the differentiation of human and mouse myeloid leukaemia cells into macrophage-like cells *in vitro* (Miyaura *et al.*, 1981; Abe *et al.*, 1981), but inhibits the proliferation of human melanoma (Colston *et al.*, 1981) and osteosarcoma cells (Majeska & Rodan, 1982). All of these findings indicate that the cholecalciferol metabolites, in addition to their role in calcium homeostasis, are trophic hormones associated with cell proliferation and differentiation.

It has been shown fairly recently (Reiss & Kaye, 1981) that the brain type (BB) isoenzyme of creatine kinase (EC 2.7.3.2) is the major component of the 'oestrogen-induced protein' (IP) of the rat uterus (Notides & Gorski, 1966). IP has been a favoured marker for studies on the mechanism of action of oestrogens in the uterus (Katzenellenbogen & Gorski, 1975; Kaye, 1983) and other tissues containing oestrogen receptors because of its early response to the hormone *in vivo* (Barnea & Gorski, 1970) and *in vitro* (Katzenellenbogen & Gorski, 1972). The finding of an enzymic activity for IP provides a sensitive and rapid assay procedure which facilitates the study of whether creatine kinase might be a more general marker for the action of steroid hormones which, like oestrogen, cause increased cell growth. Indeed, $24R,25(\text{OH})_2\text{D}_3$ stimulates creatine kinase BB

Abbreviations used: $25(\text{OH})\text{D}_3$, 25-hydroxycholecalciferol; $1\alpha,25(\text{OH})_2\text{D}_3$, $1\alpha,25$ -dihydroxycholecalciferol; $24R,25(\text{OH})_2\text{D}_3$, $24R,25$ -dihydroxycholecalciferol; IP, 'oestrogen-induced protein'.

activity in chick-embryo limb-bud chondrocyte-like cells *in vitro* (Sömjen *et al.*, 1984). In the present study we examined whether the stimulation of creatine kinase activity by vitamin D metabolites can serve as a marker for their action in mammalian as well as avian organs.

Experimental

Animals

Female Wistar rats (4 weeks old) were raised on a vitamin D-deficient diet containing 0.75% calcium, 0.5% phosphorus and soya-bean protein instead of casein (Lawson *et al.*, 1969), and kept in darkness. At 130 days of age, the females were mated with normal male rats and maintained during gestation and lactation on the same vitamin D-deficient diet (Sömjen *et al.*, 1982a). The weanling pups were fed with the same diet for 2 additional weeks. Vitamin D deficiency was verified by plasma analysis of 25(OH)D₃ and 1 α ,25(OH)₂D₃ (Eisman *et al.*, 1976; Weisman *et al.*, 1977). All animals had no detectable 25(OH)D₃ (<1.6 ng/ml) or 1 α ,25(OH)₂D₃ (<5 pg/ml). Vitamin D metabolites in 0.2 ml of 20% ethanol were injected intraperitoneally.

Tissue preparation

After injection of the hormones, rats were killed with diethyl ether at the specified times. The epiphyses and diaphyses of the tibiae, the duodenal mucosa and the kidneys were quickly removed, washed thoroughly with cold 0.9% NaCl and homogenized with an Ultra-Turrax homogenizer [homogenizing buffer: 50 mM-Tris/HCl (pH 6.8)/5 mM-Mg₂SO₄ / 0.4 mM-EDTA / 2.75 mM-dithiothreitol/250 mM-sucrose]. The homogenate was centrifuged at 38000g for 10 min, and the resulting supernatant was used for the enzyme assay; its protein content was determined by the Coomassie Brilliant Blue dye-binding method (Bradford, 1976), with bovine serum albumin as standard.

Assay of creatine kinase activity and separation of isoenzymes

Creatine kinase activity was measured in a Gilford 250 automatic recording spectrophotometer at 340 nm, by using a coupled assay of a 0.5 ml incubation mixture containing 50 mM-imidazole/acetate buffer, pH 6.7, 25 mM-creatine phosphate, 2 mM-ADP, 10 mM-magnesium acetate, 20 mM-D-glucose, 2 mM-NAD, 5 mM-EDTA, 5 μ g of bovine serum albumin, 50 μ M-diadenosine pentaphosphate (myokinase inhibitor), 20 mM-N-acetylcysteine, 2 mM-dithiothreitol, 1.2 units of glucose-6-phosphate dehydrogenase and 0.8 unit of hexokinase. A unit of enzyme activity was defined as the amount yielding 1 μ mol of product/min at 30°C.

The separation of creatine kinase isoenzymes was performed as described previously (Kaye *et al.*, 1981). Preparations in 20 mM-NaCl/100 mM-Tris/HCl (pH 7.9)/5 mM-magnesium acetate/0.4 mM-EDTA were applied to a DEAE-cellulose column (volume 1 ml). The MM (muscle type) isoenzyme of creatine kinase was not adsorbed; the MB (hybrid) isoenzyme was eluted with 40 mM-NaCl/100 mM-Tris/HCl (pH 6.4)/5 mM-magnesium acetate/0.4 mM-EDTA and the BB (brain type) isoenzyme was eluted with 250 mM-NaCl in the same buffer.

Biochemicals

24R,25(OH)₂D₃ and 1 α ,25(OH)₂D₃ were kindly provided by Professor S. Edelstein, Department of Biochemistry, Weizmann Institute of Science. All biochemicals for enzyme assays were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.).

Results

Administration of 24R,25(OH)₂D₃, but not of 1 α ,25(OH)₂D₃, to vitamin D-depleted rats caused, 24 h later, a 2.6-fold increase in creatine kinase activity in the epiphyses of tibiae

Table 1. Stimulation of creatine kinase activity by cholecalciferol metabolites in organs from vitamin D-depleted rats. Methods are described in the Experimental section. Results are means $\pm$ S.E.M. for at least six determinations. The ratios of experimental to control values are given in parentheses. Statistical significance of difference between treated and control groups by Student's *t* test: **P* < 0.01; ***P* < 0.005; insignificant differences are unmarked.

Treatment (24h)	Organ ...	Creatine kinase activity (units/mg of protein)			
		Epiphyses	Diaphyses	Kidney	Intestine
Control [0.2 ml of 20% (v/v) ethanol in propylene glycol]		4.4 $\pm$ 0.2	4.5 $\pm$ 0.1	6.8 $\pm$ 1.0	5.3 $\pm$ 1.1
1 α ,25(OH) ₂ D ₃ (60 ng/rat)		4.9 $\pm$ 0.1 (1.10)	7.0 $\pm$ 0.6* (1.51)	16.8 $\pm$ 2.0** (2.47)	6.3 $\pm$ 1.4 (1.19)
24R,25(OH) ₂ D ₃ (300 ng/rat)		11.4 $\pm$ 1.2** (2.6)	4.8 $\pm$ 0.3 (1.0)	5.1 $\pm$ 1.0 (0.8)	4.8 $\pm$ 1.5 (0.9)

Fig. 1. Time course of creatine kinase response in organs of vitamin D-depleted rats. Changes in (a) renal, (b) duodenal, (c) diaphysal and (d) epiphyseal creatine kinase activity were measured after intraperitoneal injection of 60 ng of $1\alpha,25(\text{OH})_2\text{D}_3$ (●), 300 ng of $24\text{R},25(\text{OH})_2\text{D}_3$ (■), or vehicle (○). Methods are described in the Experimental section. Results are means $\pm$ S.E.M. for five determinations.

(Table 1). Contrariwise, $1\alpha,25(\text{OH})_2\text{D}_3$ but not $24\text{R},25(\text{OH})_2\text{D}_3$, caused a 1.5-fold increase in the enzyme activity in the diaphyses and a 2.5-fold increase in the kidney. No change in creatine kinase activity was found in the intestinal mucosa after the administration of either $1\alpha,25(\text{OH})_2\text{D}_3$ or $24\text{R},25(\text{OH})_2\text{D}_3$ (Table 1). The time-dependence of these increases in epiphyseal, diaphysal and renal creatine kinase activity after administration of $1\alpha,25(\text{OH})_2\text{D}_3$ or

Fig. 2. Dose-dependence of the stimulation of creatine kinase activity by $24\text{R},25(\text{OH})_2\text{D}_3$ in the epiphyses of vitamin D-depleted rats. $24\text{R},25(\text{OH})_2\text{D}_3$ was injected intraperitoneally, and the animals were killed 24 h later. Methods are described in the Experimental section. Results are means $\pm$ S.E.M. for four determinations.

$24\text{R},25(\text{OH})_2\text{D}_3$ is shown in Fig. 1. The enzyme activity in the kidney increased significantly at 1 and 4 h and was still increasing 24 h after the injection of $1\alpha,25(\text{OH})_2\text{D}_3$, the longest time interval tested. Creatine kinase activity in the diaphysis increased significantly 4 and 24 h after the injection of $1\alpha,25(\text{OH})_2\text{D}_3$. In the epiphyses of long bones, the activity of creatine kinase increased 1 h after $24\text{R},25(\text{OH})_2\text{D}_3$ treatment and then decreased to close to control values at 4 h. At 24 h, the longest interval tested, the enzyme activity attained its highest value. The activity of epiphyseal creatine kinase, 24 h after injection of $24\text{R},25(\text{OH})_2\text{D}_3$, rose steeply with dose, then began to reach a plateau between 300 and 3000 ng of $24\text{R},25(\text{OH})_2\text{D}_3$ (Fig. 2). The stimulation of creatine kinase activity in long bones and kidney, after the injection of cholecalciferol metabolites, was due to an increase in the BB isoenzyme of creatine kinase, as shown by DEAE-cellulose column chromatography (Fig. 3).

Discussion

The present study demonstrates that creatine kinase BB activity is stimulated by other steroid hormones in addition to oestrogens (Reiss & Kaye, 1981), in this case, the dihydroxylated metabolites of cholecalciferol. The finding of a protein stimulated by $24\text{R},25(\text{OH})_2\text{D}_3$ and $1\alpha,25(\text{OH})_2\text{D}_3$, and which shows creatine kinase activity, provides a convenient and sensitive marker for the study of the mechanism of action of cholecalciferol metabo-

Fig. 3. Creatine kinase isoenzyme distribution in organs of vitamin D-depleted rats

$24R,25(OH)_2D_3$ (300 ng), $1\alpha,25(OH)_2D_3$ (60 ng) or vehicle were injected intraperitoneally, and the rats were killed 24 h later. Isoenzymes of creatine kinase were separated and fractions assayed as described in the Experimental section. ■, MM isoenzyme; □, MB isoenzyme; ▤, BB isoenzyme. (a) kidney, untreated; (b) kidney, $1\alpha,25(OH)_2D_3$ -treated; (c) diaphyses, untreated; (d) diaphyses, $1\alpha,25(OH)_2D_3$ -treated; (e) epiphyses, untreated; (f) epiphyses, $24R,25(OH)_2D_3$ -treated.

lites in their responsive organs and for the determination of vitamin D responsiveness in both basic and clinical situations.

What is the physiological role of increased creatine kinase activity? Creatine kinase is necessary for the process of ATP regeneration. Excess ATP formed during glycolysis or fatty acid catabolism is converted into creatine phosphate, which in turn, may be used for ATP-consuming processes such as transport and cell growth and proliferation (Kenyon & Reed, 1983). Cholecalciferol-stimulated creatine kinase activity could be involved in the two major ATP-consuming functions of vitamin D: one, in transcellular Ca^{2+} transport, which is crucial for Ca^{2+} homeostasis,

and the other, in events leading to cell growth and division. Our observations that creatine kinase is stimulated in the epiphyseal cartilage by $24R,25(OH)_2D_3$, whereas $1\alpha,25(OH)_2D_3$ stimulates its activity in the diaphyses, are parallel with the results of previous studies showing a corresponding localization of specific receptors for vitamin D metabolites (Sömjen *et al.*, 1982b). In turn, receptor localization is correlated with the effects of $24R,25(OH)_2D_3$ and $1\alpha,25(OH)_2D_3$ on cell growth and division in the epiphysis and diaphysis of long bones and in chondrocytes from chick-embryo limb-bud cultures (Sömjen *et al.*, 1983; Binderman & Sömjen, 1984). These parallels support the suggestion that each of these metabolites has its specific site of action in the developing bone. Apparently, the hydroxy group at C-24 is necessary for the observed biological action on the epiphyseal cartilage, since $1\alpha,25(OH)_2D_3$ has no effect on creatine kinase activity in this tissue. It is surprising that $1\alpha,25(OH)_2D_3$ has no effect on creatine kinase activity in the duodenal mucosa, in contrast with its stimulation of ornithine decarboxylase activity and [3H]thymidine incorporation into DNA (Shinki *et al.*, 1981; Sömjen *et al.*, 1983). On the other hand, in the kidney, which contains receptors for $1\alpha,25(OH)_2D_3$ (Colston & Feldman, 1979), the stimulation of creatine kinase activity appears to be the first-reported response to a vitamin D metabolite.

The presence of cholecalciferol-stimulated creatine kinase BB activity in developing skeletal tissues is not confined to the rat, since we have found that $24R,25(OH)_2D_3$, but not other vitamin D metabolites, stimulates creatine kinase BB synthesis and activity in chondrocyte-like cells from chick-embryo limb-bud cell cultures (Sömjen *et al.*, 1984). These observations *in vivo* and *in vitro* suggest that cholecalciferol metabolites may act in a wide range of systems to increase the rate of synthesis of creatine kinase BB, possibly by an increase in mRNA for creatine kinase BB as in the oestrogen-stimulated rat uterus (Walker & Kaye, 1981). The tissue and metabolite specificity of the effect also suggests that the rapid stimulation of creatine kinase activity by cholecalciferol metabolites *in vivo* is due to their direct action and is not secondary to an increase in the concentration of serum Ca^{2+} or other intermediate. However, it is not ruled out that changes in the intracellular concentration of Ca^{2+} may lead to increased creatine kinase activity, since it has been shown that cholecalciferol metabolites affect Ca^{2+} transport in primary cultures of bone cells (Eilam *et al.*, 1980). The creatine kinase BB response may therefore be one of many secondary changes resulting from a primary effect on Ca^{2+} transport. Changes in the cytoplasmic concentration of Ca^{2+} modify

myriad biochemical reactions including those affecting growth and differentiation (Wasserman & Corradino, 1973; Boyce & Ham, 1983). However, aside from considerations of the mode of action of vitamin D metabolites, the present work provides further evidence that different cell types respond to different vitamin D metabolites.

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