24*R*,25-Dihydroxyvitamin D stimulates creatine kinase BB activity in chick cartilage cells in culture

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In chick limb-bud cartilage cell cultures 24*R*,25-dihydroxycholecalciferol (24*R*,25$(OH)_2D_3$), but not 24*S*,25$(OH)_2D_3$, 1α,25$(OH)_2D_3$ or 25$(OH)D_3$, stimulates the activity of the brain type (BB) isozyme of creatine kinase (EC 2.7.3.2), the 'estrogen-induced protein' first identified in rat uterus. Cultures treated with bromodeoxyuridine, in which cartilage formation is inhibited, show no stimulation of creatine kinase BB by 24*R*,25$(OH)_2D_3$.

24R,25-Dihydroxycholecalciferol *Creatine kinase BB* *Chicken cartilage* *Limb bud* *Estrogen-induced protein*

1. INTRODUCTION

1α,25$(OH)_2D_3$ is the active metabolite of vitamin D involved in calcium homeostasis which acts on a range of organs in which specific receptors have been demonstrated [1]. It stimulates RNA polymerase [2] and ornithine decarboxylase (ODC) activity [3,4], and the synthesis of specific proteins [5], and DNA [4,6].

The more recently recognized vitamin D metabolite 24*R*,25$(OH)_2D_3$, which is not implicated in calcium transport, is involved in embryonic development of endochondral bone [7–10]. It is also active in embryonic cartilage [11–13] in which it has specific receptors [4,11,12] and where it stimulates DNA and protein synthesis and increases ODC activity [4]. An advantageous system for the study of 24*R*,25$(OH)_2D_3$ is micromass cell cultures prepared by trypsin dispersion from chick limb-bud mesenchymal cells, obtained from stage 24 embryos. These cultures develop into nodules of cartilage cells, surrounded by mesenchymal cells, within 4 days. Their differentiation and calcification can be regulated by manipulation of the growth medium [14,15]. The cells contain specific receptors in their cytosol for 24*R*,25$(OH)D_3$ [12] which stimulates increases in the rate of protein and DNA synthesis, and in ODC activity [13,16].

An increase in the rate of synthesis of a specific protein [17], within 1 h after treatment [18], identified as the brain type isozyme of creatine kinase (CK-BB) [19], is one of the earliest changes induced by estrogen in rat uterus, preceding increased synthesis of protein [17] and cell growth and division [20]. Estrogen also stimulates CK-BB activity in other organs containing estrogen receptors [21,22]. CK regulates the intracellular concentration of ATP via its ATP 'buffering action' [23], regenerating ATP from ADP and creatine phosphate following ATPase activity [24]. The finding of an enzymic activity for the estrogen-induced protein makes available a sensitive and reproducible assay which facilitates the study of whether creatine kinase might be a more general marker for

Abbreviations: D_3, cholecalciferol, in all metabolites (e.g., 24*R*,25$(OH)_2D_3$ = 24*R*,25$(OH)_2$ cholecalciferol); ODC, ornithine decarboxylase; BUdr, bromodeoxyuridine

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the action of steroid hormones such as vitamin D metabolites which, like estrogen, stimulate cell growth.

2. MATERIALS AND METHODS

2.1. *Biochemicals*

25(OH)D_3, 1α,25(OH)$_2D_3$ and 24*R*,25(OH)$_2D_3$ were kindly provided by Dr S. Edelstein, Department of Biochemistry, the Weizmann Institute of Science and 24*S*,25(OH)$_2D_3$ by Teva Pharmaceutical Industries Ltd, Jerusalem. All other biochemicals were purchased from Sigma, St Louis, MO.

2.2. *Cell cultures*

Preparation of micro-mass cell cultures derived from embryonic chick limb-bud mesenchyme was described in [14]. The mesenchymal cells undergo chondrogenesis within 4 days.

2.3. *CK activity*

After incubation cells were washed and sonicated in buffer consisting of 50 mM Tris–HCl (pH 6.8), 5 mM $MgSO_4$, 0.4 mM EDTA, 2.75 mM dithiothreitol and 250 mM sucrose. Supernatant solutions were obtained by centrifugation at 4°C at 38000 × *g* for 15 min and their protein concentrations determined as in [25] using bovine serum albumin as the standard. The activity of CK in the supernatant solutions was assayed as in [21] in a Gilford 250 automatic recording spectrophotometer at 30°C. Unit activity was defined as the amount yielding 1 μmol ATP/min.

CK isozymes were separated by DEAE–cellulose chromatography [26].

3. RESULTS AND DISCUSSION

The presence of 12 nM 24*R*,25(OH)$_2D_3$, but not 25(OH)D_3 or 1α,25(OH)$_2D_3$, in 6-day-old micromass chick embryo limb-bud mesenchyme cell cultures caused, within 4 h, a 2.5-fold increase in CK activity (fig.1). 24*S*,25(OH)$_2D_3$, the inactive isomer, has a much smaller effect on CK activity (which can be attributed to contamination with 24*R*,25(OH)$_2D_3$ in this preparation [fig.1]). Triamcinolone acetonide, which promotes calcification of cartilage, does not affect CK activity (fig.1).

The stimulation of CK activity by

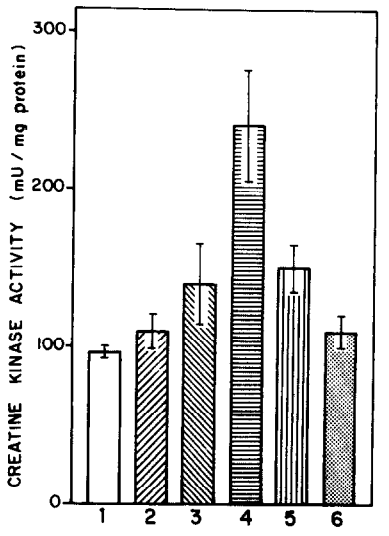

Fig.1. Stimulation of creatine kinase activity in micromass chick embryo limb-bud cell cultures. Embryonic chick limb bud mesenchymal cells (4×10^5 in a 20 μl drop) were placed in a 35 mm diameter tissue culture dish and allowed to attach before adding the medium (Eagle's minimum essential medium [MEM] supplemented with 10% fetal calf serum). After 4 h incubation with the test hormone, cells were extracted and assayed as described in section 2. The assay mixture contained in 1 ml: 50 mM imidazole acetate (pH 6.7), 25 mM creatine phosphate, 2 mM ADP, 10 mM Mg acetate, 20 mM D-glucose, 2 mM NAD, 5 mM EDTA, 50 μM diadenosine pentaphosphate (myokinase inhibitor), 20 mM *N*-acetylcysteine, 2 mM DTT, 10 μg/ml bovine serum albumin, 2.4 units glucose-6-phosphate dehydrogenase and 1.6 units hexokinase. Results are means ± SE for $n \geqslant 5$. (1) Control; (2) 25(OH)D_3, 12 nM; (3) 1α,25(OH)$_2D_3$, 12 nM; (4) 24*R*,25(OH)$_2D_3$, 12 nM; (5) 24*S*,25(OH)$_2D_3$, 12 nM; (6) triamcinolone acetonide, 1.0 nM.

24*R*,25(OH)$_2D_3$ was rapid (table 1), showing a hint of a biphasic response, similar to uterine CK-BB [21], with an increase in CK activity after 1 h exposure to the hormone, and a greater than 2-fold increase in activity after 4 and after 24 h. The increase in CK activity shows a dependence on the concentration of 24*R*,25(OH)$_2D_3$ but not 1α,25(OH)$_2D_3$ (table 2), similar to the induction of DNA synthesis [13,16]. The increase was shown to be due, at least in part, to an increase in the rate of synthesis of CK (cf. [19,22]) and not to enzyme activation. The increase in synthetic rate was demonstrated by polyacrylamide gel elec-

Table 1

Time-dependent stimulation of creatine kinase activity by 24*R*,25(OH)$_2$D$_3$ in micro-mass chick embryo limb-bud cell cultures

Time (h)	CK activity	
	(nmol·min^{-1}·mg protein^{-1})	Activity relative to control
0	130 ± 30	(1.0 ± 0.3)
1	240 ± 40	(1.9 ± 0.2)
2	180 ± 30	(1.4 ± 0.2)
4	310 ± 30	(2.4 ± 0.1)
24	350 ± 90	(2.7 ± 0.3)

Cells were incubated in 12 nM 24*R*,25(OH)$_2$D$_3$. Enzyme assays were performed as described in section 2 and fig.1. Results are means ± SE for $n \geqslant 3$

trophoresis and fluorography (not shown) of [^{35}S]methionine-labelled cytosols prepared from cultures, 1 h after exposure to 24*R*,25(OH)$_2$D$_3$ or to vehicle.

Treatment of chick embryo limb-bud mesenchyme cells with BUdr, which causes their differentiation into fibroblasts and inhibits the growth of cartilage cells [14], causes a parallel loss (not shown) in the ability of 24*R*,25(OH)$_2$D$_3$ to stimulate CK activity although the basal CK activity doubles, suggesting a specific biological role of 24*R*,25(OH)$_2$D$_3$ in chondrocytes. The loss of responsiveness is correlated with the loss of specific binding of 24*R*,25(OH)$_2$D$_3$ in the cultures [12].

Table 2

Concentration-dependent stimulation of creatine kinase activity by 24*R*,25(OH)$_2$D$_3$ in micro-mass chick embryo limb-bud cell cultures

Dose (nM)	CK activity (nmol·min^{-1}·mg protein^{-1})	
	24*R*,25(OH)$_2$D$_3$	1α,25(OH)$_2$D$_3$
0	60 ± 20	60 ± 20
1.2	80 ± 30	58 ± 10
12.0	110 ± 10	65 ± 10
120.0	160 ± 10	60 ± 10

Hormonal treatment was for 4 h. Enzyme assays were performed as described in section 2 and fig.1. Results are means ± SE for $n \geqslant 3$

DEAE–cellulose chromatography (fig.2) revealed that stimulation of CK specific activity by 24*R*,25(OH)$_2$D$_3$ was due mainly to an increase in CK-BB (the brain type isozyme); small quantities of CK-MM (muscle type isozyme) were also detected. This is similar to the estrogen-responsive CK activity described in the uterus and other normal as well as neoplastic tissues [19,21,22]. The stimulation of CK-BB in chick embryo limb-bud mesenchymal cultures by 24*R*,25(OH)$_2$D$_3$ is specific to the chondrocytes in these cultures, as shown by the use of BUdr, described above.

This first observation of the existence of a specific '24*R*,25(OH)$_2$D$_3$ induced protein' in chick embryo limb-bud chondrocytes, which appears to be identical to the estrogen induced protein (IP) of rat uterus [17,26], the BB isozyme of creatine kinase [19], reveals that the stimulation of CK-BB is not limited to estrogens but is a more general property of trophic hormones, and may, like the stimulation of ODC activity [27,28], serve as a more general marker of hormone action. Indeed,

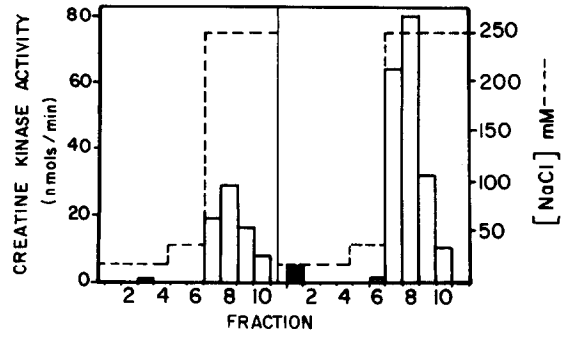

Fig.2. Creatine kinase isozyme distribution in micro-mass chick embryo limb-bud cell cultures. Extracts were prepared from either untreated (control) cells (left panel) or cells treated with 12 nM 24*R*,25(OH)$_2$D$_3$ for 4 h (right panel). Supernatant fractions of extracts were applied to a DEAE–cellulose column (1 ml) in 20 mM NaCl, 100 mM Tris–HCl (pH 7.9), 5 mM Mg acetate and 0.4 mM EDTA. The MM (muscle type) isozyme of CK (black bars) was not absorbed to the column, the MB (hybrid) isozyme (lined bars) was eluted with 40 mM NaCl in the same buffer and the BB (brain type) isozyme (open bars) with 250 mM NaCl in the same buffer. Fractions were assayed as described in section 2 and fig.1.

in vitamin D-depleted rats, in which we have demonstrated the presence of specific receptors for 24*R*,25(OH)$_2$D$_3$ in the epiphyses and for 1α,25(OH)$_2$D$_3$ in the diaphysis of long bones [16] (correlated with induction of DNA synthesis and stimulation of ODC activity [16] by the hormone specific for each region), we have shown (in preparation) that 24*R*,25(OH)$_2$D$_3$ stimulates CK activity in the epiphysis and 1α,25(OH)$_2$D$_3$ in the diaphysis of long bones.

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