EARLY EFFECT OF 25-HYDROXYCHOLECALCIFEROL (25-OH-D_3) AND 1,25-DIHYDROXYCHOLECALCIFEROL (1,25-$(OH)_2$-D_3) ON THE ABILITY OF PARATHYROID HORMONE (PTH) TO ELEVATE CYCLIC AMP OF INTACT BONE CELLS

Ahmed Mahgoub and Herbert Sheppard

Department of Cell Biology, Research Division
Hoffmann-La Roche Inc., Nutley, New Jersey 07110

Received December 17,1974

Summary: 25-OH-D_3 and 1,25-$(OH)_2$-D_3 had no effects by themselves on the cyclic AMP levels of isolated bone cells but enhanced the stimulation seen following an exposure with submaximal concentrations of PTH for as little as 2 minutes. Preincubation with the 25-OH-D_3 or 1,25-$(OH)_2$-D_3 resulted in a time dependent decrease in the enhancement of PTH response over a 1 hr period. It is, therefore, suggested that cyclic AMP may be involved in some aspects of the action of vitamin D_3 derivatives on bone cells.

Introduction

Vitamin D_3 (cholecalciferol) and its metabolites have been shown to act in the intestine to increase the absorption of calcium and the level of a calcium binding protein which is believed to play a role in the movement of this cation (1).

The two major metabolites of vitamin D_3, namely 25-OH-D_3 and 1,25-$(OH)_2$-D_3, have been shown to mobilize bone calcium both *in vivo* (2) and *in vitro* (3).

As with parathyroid hormone (PTH) these effects on bone take several hours to become manifest and are considered to depend on some earlier as yet unidentified biochemical action. The 1,25-$(OH)_2$-D_3 is several orders of magnitude more active than its precursor 25-OH-D_3 and is now being considered as a hormone (4).

The delayed ability of PTH to mobilize bone calcium has been associated with its very rapid action in stimulating the adenyl cyclase of calvaria (5) and isolated bone cells (6).

Vitamin D_3 administration *in vivo* has recently been demonstrated to result in an increased intestinal adenylate cyclase (7). Using cultures of chick embryo intestines, vitamin D_3 has also been shown to elevate cyclic AMP after 3 hrs and this preceded the rise in calcium absorption and calcium binding protein. Since these effects on cyclic AMP are considered late in terms of a direct hormone action at the adenylate cyclase level and since vitamin D_3 behaves mechanistically like most of the steroid hormones, an attempt was made to determine if the active metabolites of the vitamin would in some way facilitate the action of PTH in a homogeneous cell system. The results of these experiments will demonstrate that the active vitamin D_3 metabolites can within minutes, potentiate the ability of PTH to elevate the cyclic AMP levels of cultured rat fetal bone cells.

Materials and Methods

Bone cells were prepared according to the procedure of Peck *et al.* (8). Calvaria from 20-21 days old rat fetuses were digested at 37°C with 3% collagenase (Type II-Worthington) solution in 5 mM Tris buffer. Cells were separated, then inoculated in 25 ml culture flasks, or in 35 mm dishes on 22 mm^2 glass cover slips (Corning). Flasks were inoculated with 10^6 cells each and dishes with 5×10^5 cells. Cells were cultured in 20% fetal calf serum in MEM Eagle medium (Grand Island Biological Co.). Only confluent cultures were used. PTH (highly purified, Wilson) with a biological potency 970-1240 units/mg was added in 1 mM HCl. Synthetic 25-OH-D_3 and 1,25-$(OH)_2$-D_3 (Hoffmann-La Roche Inc.) were added in 10 µl of 95% ethanol. The incubation medium consisted of 2 ml of 2% bovine serum albumin, Fraction V (Armour) in MEM Eagle medium. PTH and vitamin D_3 derivatives were added to the culture and the incubations were carried out for the specified times at room temperature. The cells were then washed with Earle's salt solution. The reaction was stopped by the addition of boiling water to the culture, followed by

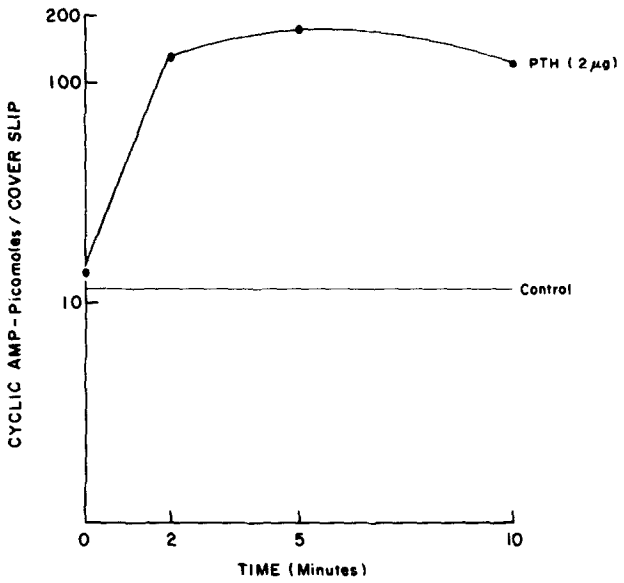

Fig. 1. Time response curve for PTH (2 μg). The hormone was added to the confluent cells attached to the cover slips for the specified time. Each point is the average of a triplicate. Control: 2 min.

heating for 10 min in a boiling water bath to extract cyclic AMP. Cyclic AMP was determined by the protein binding assay (9). In each experiment, random flasks or dishes were selected and protein determined by a modified Lowery method (10). Experiments were run in triplicate. The t-Student test was used to test for statistical significance.

Results

Protein determinations showed a difference of protein content of less than 5% between randomly selected flasks or cover slips, and the results were, therefore, expressed as picomoles cyclic AMP/flask or cover slip.

PTH caused a marked stimulation of cyclic AMP content of bone cells and the effect was maximum at 2-5 minutes (Fig. 1). A dose response curve is shown in Fig. 2. PTH stimulation of cyclic AMP was linear up to a concentration of 2 μg per 2 ml of incubation medium. Control cyclic AMP levels were usually very low or undectectable.

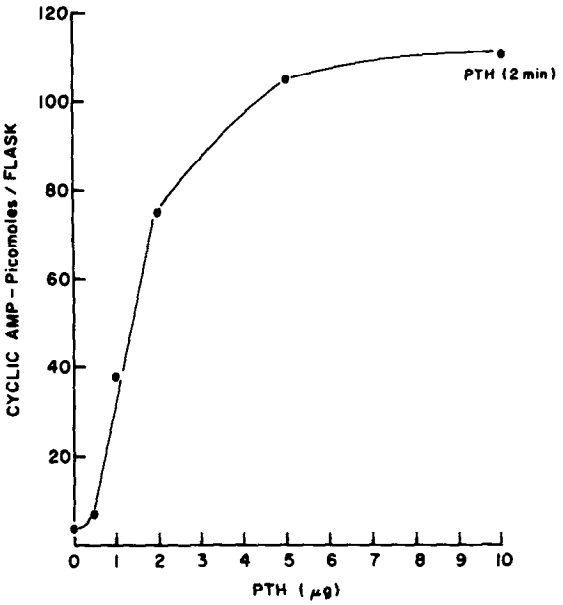

Fig. 2. Dose response curve. PTH added in one volume for 2 minutes to culture flasks. Experiment run in triplicates.

The addition of 5 and 10 μg of 25-OH-D_3 had no effect on the basal cyclic AMP levels of bone cells (Table 1). The significant increase in cyclic AMP levels obtained with 1 μg of PTH for 5 minutes was increased further by the addition of 25-OH-D_3 (5 and 10 μg). The failure to obtain enhancement of the response to 2 μg of PTH has been observed again in a subsequent experiment. Both levels of 25-OH-D_3 enhanced the stimulation of cyclic AMP obtained with 1 μg of PTH for only 2 minutes. 25-OH-D_3 was ineffective at a dose of 1 μg.

As little as 0.01 μg of 1,25-$(OH)_2$-D_3 had no effect by itself on the basal cyclic AMP levels of bone cells (Fig. 3), but was able to cause a marked increase in the stimulation produced by 2 μg of PTH for 2 minutes. Pretreatment of bone cells with 0.01 μg of 1,25-$(OH)_2D_3$ for 15 and 30 minutes before the addition of PTH, caused a reduced but still significant potentiation of the PTH effect. However, no enhancement of the PTH effect was found when 1,25-$(OH)_2$-D_3 was added for 60 minutes.

TABLE 1. Effect of 25-OH-D_3 on PTH-stimulation of cyclic AMP of isolated bone cells

Treatment	Dose (μg)	Cyclic AMP picomoles/cover slip
1 mM HCl	-	3.69
95% ethanol	-	6.27
25-OH-D_3	5	1.90
25-OH-D_3	10	4.05
PTH	1.0	68.80
PTH	2.0	128.00
PTH 1 μg + 25-OH-D_3	5	131.40*
PTH 1 μg + 25-OH-D_3	10	217.10**
PTH 2 μg + 25-OH-D_3	5	120.70
PTH 2 μg + 25-OH-D_3	10	142.90

PTH added for 5 minutes.

*$p < .05$, ** $p < .001$ from PTH alone.

N = 3 cover slips.

The same-time-dependent decrease in enhancement of PTH response was obtained with 25-OH-D_3 (5 μg).

Since PTH was added in 1 mM HCl and the vitamin D_3 derivatives in ethanol, a combination of the two solvents was tested and it was found to have no effect on cyclic AMP stimulation. Likewise the addition of ethanol to PTH or 1 mM HCl to the vitamin D_3 derivatives did not alter the cyclic AMP responses.

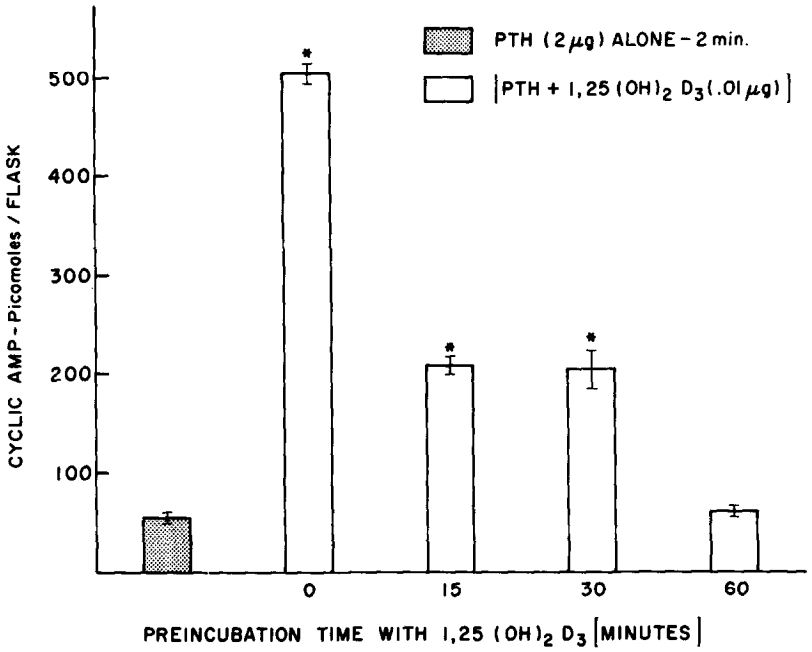

Fig. 3. Confluent cells in flasks were treated for the specified time with 1,25-$(OH)_2$-D_3, then PTH (2 μg) was added for 2 minutes (values ± S.E.M.). The solvent for this experiment was a combination of 1 mM HCl and ethanol. Control cyclic AMP levels were undetectable.

Discussion

The vitamin D_3 derivatives, 25-OH-D_3 and 1,25-$(OH)_2$-D_3 were found to have no effect by themselves on isolated bone cells in primary culture but were capable of potentiating the effects of submaximal concentrations of PTH within 2 and 5 min. Pretreatment with these vitamin D_3 derivatives, however, reduced the PTH-stimulation in a time-dependent fashion.

The first association of vitamin D_3 with cyclic AMP was seen in an elevated intestinal adenylate cyclase 3 hrs after its administration to D-deficient chicks (11). A more recent report using embryonic chick intestines *in vitro* (7) demonstrated an elevated level of cyclic AMP 3 hrs after an incubation with vitamin D_3 and this too was followed by a fall to basal level before a subsequent rise to higher levels.

It is difficult to determine from these results whether one is dealing with a direct effect since earlier cyclic AMP levels were not reported and the tissues were composed of many cell types.

The results discussed here represents the earliest report of an effect of the active metabolites of vitamin D_3 and clearly implicate them as modulators of the action of PTH on the cyclic AMP system of bone cells in culture. It remains to be determined whether the action of these vitamin D_3 metabolites is at the level of the membrane or the cyclic AMP phosphodiesterase.

The nature of the reduced enhancement of the PTH effect following a preincubation with these vitamin D_3 metabolites was surprising but may in some way be related to hormone desensitization which has been reported to follow a preincubation with a hormone in many systems. Other possible explanations remain but it is clear that a more detailed study of this inhibition is required.

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