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STIMULATION OF GUANYLATE CYCLASE ACTIVITY IN CULTURED OSTEOGENIC MURINE CALVARIAL MESENCHYMAL CELLS BY PTH, CALCITONIN AND INSULIN

Marlin W. Walling*, Vicki Marvaso** and George W. Bernard**

*Research and Dental Services
VA Wadsworth Hospital Center, Los Angeles, CA 90073
and
Departments of Orthodontics and Medicine
University of California, Los Angeles, CA 90024

**Departments of Pathology, Anatomy and Oral Biology
University of California, Los Angeles, CA 90024

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Summary: These studies provide the first evidence that parathyroid hormone (PTH), calcitonin (CT), and insulin, all known effectors of bone cell metabolism, stimulate the activity of guanylate cyclase in osteogenic cells derived from fetal mouse calvarial mesenchyme. Adenylate cyclase activity was stimulated by PTH and epinephrine, but not by CT, the latter effect being consistent with an absence of osteoclast-progenitor cells in this osteogenic mesenchyme. Adenylate cyclase activity was associated entirely with the particulate fraction of the cells while guanylate cyclase, as well as acid and alkaline phosphatase, were present in both soluble and particulate material. The activation of guanylate cyclase by hormones may provide a better basis for understanding the differentiation and regulation of osteogenic cells.

INTRODUCTION

It is clear that regulation of bone cell function occurs in part via the stimulation of adenylate cyclase by hormones such as parathyroid hormone (PTH) calcitonin (CT) and epinephrine (1-4), with the resulting increases in cyclic 3',5' adenosine-monophosphate (cAMP) mediating changes in metabolism and differentiation through interactions with enzymes such as protein kinases. In addition to adenylate cyclase, bone cells also possess guanylate cyclase because cyclic 3',5' guanosine-monophosphate (cGMP) has been observed in chick long bones. (5). However, the regulation of this enzyme has not been defined.

In the present investigation, hormonal stimulation of both adenylate and guanylate cyclase was studied in embryonic mouse calvarial cells, both before

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calcification began and during the onset and continuation of osteogenesis in a tissue culture system (6).

MATERIALS AND METHODS

Thirteen day fetuses were aseptically removed from timed pregnant Swiss Webster mice anesthetised I.P. with 0.3 ml of sodium nembutal. The epithelium was peeled away from the head and the calvaria dissected away from the brain. Portions of temporal, frontal, and parietal mesenchyme were placed in 10 ml of sterile phosphate buffered saline supplemented with gentamicin (150 µgm/ml), and incubated with crude collagenase (1 mg/ml Type 1, Worthington Biochem. Corp) for 90 minutes at 37°C (7). After collagenase dispersion the cell suspension contained mesenchymal cells, endothelial cells, and blood cells (erythrocytes and leucocytes). The tissue was dissociated by trituration, the cells washed three times in Eagle's Minimum Essential Medium, and finally resuspended in BGjb medium (8) supplemented with 10 percent fetal calf serum and 50 µgm/ml gentamicin. Aliquots containing 2 x 10^6 cells were seeded onto a 60 mm petri dish. Within 24 hours mesenchymal cells adhered to the bottom of the dish in a confluent monolayer and subsequently migrated into multi-layered aggregates within which intramembraneous osteogenesis began. Endothelial cells and blood cells did not adhere and were removed during the medium changes that were made on a daily basis. Cultures were maintained and scored only if littermate control tissue, serially sectioned and stained with Von Kossa reagents, was subsequently shown to be negative for complexed calcium.

For enzyme studies, the cultures were removed from petri dishes with a rubber policeman, pelleted by low speed centrifugation at 5°C, resuspended in the phosphate buffer and stored in ice. The cells were then repelleted and resuspended in 2 ml of hypotonic buffer, pH 8.1 (25mM NaCl, 1mM Tris HCl) and disrupted by equilibrating them with N_2 at 1000 psi in a pre-cooled Kontes Mini-bomb for 30 min.

Adenylate and guanylate cyclase activities were assayed by measuring the conversion of ^{32}P-ATP or GTP (ICN, Irvine, CA) to cAMP or cGMP respectively ([ATP] = 2mM [Mg] = 5 mM; [GTP] = 1 mM, [Mn] = 5.5mM (9,11). A creatine phosphate-creatine phosphokinase regenerating system was used and reactions were conducted at 37C for 10 min with a range of protein concentrations so that reaction rates were linear (32 to 59 mg). Purification of the cyclic nucleotides was achieved by chromatography first through Dowex 50 AGW-4 resin and then with neutral alumina columns. Acid and alkaline phosphatase were measured at pH 5.8 and 9.5 respectively using paranitrophenol phosphate as a substrate (12). The hormones used were ℓ-epinephrine (Sigma), synthetic salmon calcitonin, porcine insulin and bovine PTH (1300 units/mg, courtesy of the NIAMDD hormone distribution program).

RESULTS AND DISCUSSION

In an initial set of experiments adenylate cyclase and guanylate cyclase were assayed in the calvarial mesenchyme at the time of dissection (Day 0) and after 3, 7 and 11 days in culture. (See Figure 1 for a description of temporal changes in cell differentiation). Temporal changes in basal enzy-

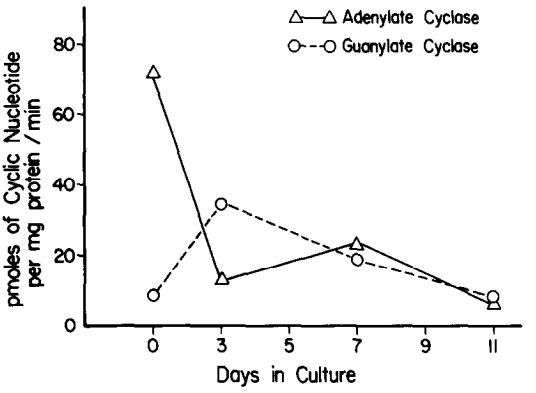

Figure 1. Temporal changes in basal nucleotide cyclase specific activity. Day 0 is freshly dissected tissue and consists of undifferentiated mesenchymal cells, erythrocytes and leukocytes. On Day 3, blood cell types are gone, and 2-3 confluent cell layers are present and undergoing differentiation. By Days 5-7, morphologic differentiation into osteoblasts and fibroblasts has occurred and initial calcification of woven bone begins. Days 9-11, woven bone growth continues and organization into lamellar bone begins (6).

matic specific activities are shown in Figure 1. Basal adenylate cyclase was maximal in the initial material, markedly diminished after 3 days in culture, increased at 7 days and decreased again at 11 days (NaF-stimulated activity followed the same pattern). Conversely, guanylate cyclase activity was low at Day 0, reached a peak at Day 3 and progressively decreased at Days 7 and 11. The changes in these specific activities may reflect alterations in non-enzyme protein synthesis; however, in a single experiment, the same pattern was observed when activity was expressed per g DNA.

The stimulation of the nucleotide cyclases by hormones was also studied as a function of the number of days in tissue culture. Interestingly, no hormonal stimulation of adenylate cyclase was observed in the initial material and no stimulation of guanylate cyclase occurred in the Day 3 material. While the explanation for the lack of response is unclear, it may be due to the fact that basal activities for the enzymes were elevated on these two days to levels higher than the maximal stimulation produced by the hormones (Figure 1, Table 1.) In any case, in agreement with studies in other experimental models (3,4), both PTH and epinephrine stimulated adenylate cyclase in cells from 3, 7 and 11 day

TABLE 1

STIMULATION OF BASAL NUCLEOTIDE CYCLASE ACTIVITIES BY HORMONES AFTER VARYING TIMES IN CULTURE

Percentage Increase above Basal Level

	Adenylate Cyclase					Guanylate Cyclase			
	DAY					DAY			
	0	3	7	11		0	3	7	11
PTH	0	22	26	42	PTH	6	0	97	104
CT	0	6	0	0	CT	0	0	116	25
Epi	0	54	29	50	Ins	42	0	102	40

Assays were conducted in duplicate or triplicate ± hormone. Day 0 is the freshly dissected material. PTH, CT and insulin were present at 10^{-7}M, Epi was 10^{-4}M. Zero percentage change means that the hormonal effect was not greater than the variation in basal values which usually averages 3% (11).

cultures while CT had no consistent effect. The lack of CT-stimulated adenylate cyclase in chick limb-bud mesenchymal cells that form bone-like osseous material in tissue culture has recently been reported (13) and was suggested to possibly indicate that the mesodermal cells that form bone do not contain osteoclast precursors. The results we obtained in Tables 1 and 2 are consistent with this hypothesis.

The most novel and potentially important observations in these studies are the activation of guanylate cyclase by PTH, CT and insulin (Table 1). While only insulin increased enzyme activities in the initial material, all three hormones were effective on material from 7 and 11 day cultures (Table 1). The data in Tables 2 and 3 show the reproducibility of the PTH and CT stimulation of guanylate cyclase together with the PTH, but not a CT effect on adenylate cyclase.

Finally, we examined the sub-cellular distribution of these enzymes and found that adenylate cyclase was entirely associated with particulate material

TABLE 2

HORMONAL STIMULATION OF ADENYLATE CYCLASE

		cAMP produced/mg prot/min					
Exp.	Basal	PTH 10^{-7}M	ΔPTH	CT 10^{-7}	ΔCT	NaF 10^{-2}M	NaF Δ
1	17.8	22.4	+4.6	16.5	-1.3	107.7	+ 89.9
2	19.2	24.6	+5.4	20.5	+1.3	123.9	+104.7
3	23.6	29.7	+6.1	23.0	-0.6	108.8	+ 85.2
			+5.4		-0.2		+ 93.3
			±0.4SE		±0.8SE		± 5.9SE
			$p < 0.01$		Not Different		$p < 0.005$

Each value is the average of duplicate or triplicate assays. The cells used were from three different groups of calvarial mesenchyme that had been in culture for 6 to 7 days.

while guanylate cyclase was present in considerable amounts in the soluble fraction as well as in the particulate phase (Table 4). The distribution of the bone cell enzymes, alkaline and acid phosphatase, was examined as well, and both enzymes were present in the soluble and particulate fractions, with acid phosphatase showing a tendency toward a predominantly soluble localization (Table 4).

The significance of the temporal differences in hormonal responsivity of these two enzymes will require further study but, in general, effects seem to be greatest in cultures that have developed osteogenic potential (Figure 1, Tables 2 and 3). Because we are studying a population of mixed cell types (primarily fibroblasts and osteoblasts), we cannot specify the cellular distribution of hormonal receptors involved in regulating these enzymes and the presence of unaffected cell types probably accounts for the low magnitude of some of the hormonal stimulations (e.g. Table 2). However, studies have been initiated (in collaboration with Dr. R. A. Luben) on bone cells biochemically defined as osteoclast- and osteoblast-like (14), that should help to alleviate

TABLE 3

HORMONAL STIMULATION OF GUANYLATE CYCLASE

cGMP produced/mg prot/min

Exp.	Basal	PTH 10^{-7}M	ΔPTH	CT 10^{-7}M	ΔCT	Triton 1%	ΔTriton
1	5.9	13.1	+7.2	9.3	+3.4	151.0	145.1
2	4.7	14.1	+9.7	8.6	+3.9	148.0	143.3
3	6.9	12.2	+5.2	9.5	+2.6	154.0	147.1
			7.3±1.2SE		3.3±0.4SE		145.2 ±1.1SE
			$p < 0.05$		$p < 0.02$		$P < 0.001$

Assays were conducted as described in Table 2. Cells used were from 10-11 day cultures.

TABLE 4

SOLUBLE VS. PARTICULATE DISTRIBUTION OF BONE CELL ENZYMES

	Percentage Distribution of Enzyme Activity	
	Soluble †	Particulate †
Protein	52.8	47.2
Adenylate Cyclase	0	100
Guanylate Cyclase	37.1	62.9
Alkaline phosphatase	44.3	55.7
Acid phosphatase	70.0	30.0

† Soluble is defined as the material remaining in the supernatant following one hour of centrifugation at 100,000 xg while particulate activity was measured on the pellet. 100% was defined as the sum of the total protein or enzyme activity in the supernatant and pellet. Cells used were from 11 day cultures.

the present interpretational problems. In any event, the fact that hormones such as PTH, CT and insulin that are known to alter bone cell function, effect

the activity of guanylate cyclase and presumably cellular cGMP levels, poses exciting possibilities for defining the mechanisms of hormonal regulation of bone cell differentiation and function.

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