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Adriamycin Inhibits PTH-Mediated but Not PGE_2-Mediated Stimulation of Cyclic AMP Formation in Isolated Bone Cells

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Summary. We have examined the effect of adriamycin, an anthracycline antibiotic which modifies plasma membrane functions, on the cyclic AMP response to PTH and PGE_2 in isolated osteoblastlike cells. Adriamycin blunted the increment in bone cell cyclic AMP caused by exposure to PTH. This effect appeared rapidly (within 3 min after bone cells were exposed to adriamycin) and disappeared soon after exposure of adriamycin-treated cells to adriamycin-free incubation medium. Inhibition was evident over the entire time course of PTH action, at low as well as high PTH concentrations, and was one-half maximal at 31 μM adriamycin. It could not be attributed to alterations in cyclic AMP exodus, degradation or interference with the cyclic AMP assay, nor to impaired cell viability. Adriamycin also reduced the stimulatory effect of PTH on adenylate cyclase activity in a crude plasma membrane preparation. By contrast, adriamycin failed to modify the effects of PGE_2 on cyclic AMP generation in intact bone cells, and on adenylate cyclase activity in broken cells. Moreover, concentrations of adriamycin that blunted the effect of PTH on adenylate cyclase activity did not inhibit the stimulatory effects of sodium fluoride or of GppNHp. These results suggest that adriamycin selectively alters the interaction between PTH and its receptor or impairs the transmission of information from hormone-receptor complex to adenylate cyclase (or both), perhaps by binding to specific lipid domains in the plasma membrane. Structural analogues of adriamycin, which vary in their lipophilic properties, also varied in their capacity to perturb the cyclic AMP response. One such analogue in fact inhibited the response to PGE_2, and several appeared to augment the PGE_2 effect. These substances may well be useful in probing the membrane properties required for selectivity in hormone action.

Key words: Bone cells — Parathyroid hormone — PGE_2 — Adriamycin — cAMP

A large body of evidence points to the osteoblast as a target cell for parathyroid hormone (PTH). Parathyroid hormone has been shown to modify many aspects of osteoblast metabolism *in vitro,* and to influence bone formation *in vitro* and *in vivo* [1]. Furthermore, the possibility that the resorption-stimulating effect of PTH arises indirectly, via a primary action on osteoblasts, has received experimental support [2]. Available data suggest that cyclic AMP, and perhaps ionic calcium (Ca^{2+}), are second (intracellular) messengers in the osteoblast response to PTH [3]. Accordingly, PTH is thought to interact with specific receptors on the osteoblast plasma membrane, thereby promoting both the activity of membrane-located adenylate cyclase and the entry of Ca^{2+} into the cell. Rises in cellular cyclic AMP and Ca^{2+} levels trigger a cascade of metabolic events that culminates in purposive cell behavior. There is reason to believe that prostaglandins of the "E" series (PGE) act in similar fashion to influence osteoblast function [4,5]; PGE binds specifically to osteoblastlike cells and stimulates cyclic AMP formation [6].

Clearly, plasma membrane-located events are key elements in the effects of these agents: binding, transduction of the message initiated by the hormone-receptor complex, catalysis of cyclic AMP production, and transport of Ca^{2+}. The unique

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structure of the plasma membrane, currently visualized as a fluid mosaic dominated by phospholipids and protein, provides a supportive medium for these reactions and facilitates their regulation [7]. We have undertaken a series of experiments to probe these relationships as they pertain to PTH and PGE action, using agents known to interact with and modify the function of the plasma membrane. In initial experiments, we examined the effects of adriamycin, an anthracycline antibiotic which alters multiple membrane-associated processes in mammalian cells, and which is thought to do so by binding to specific lipid components [8]. The results presented herein demonstrate that adriamycin inhibits PTH-mediated but not PGE_2-mediated enhancement of cyclic AMP formation in isolated osteoblastlike cells, and define the characteristics of that apparently selective inhibitory effect.

Materials and Methods

Incubation media, antibiotics, and glutamine were obtained from Flow Laboratory, McLean, Virginia; theophylline from Nutritional Biochemicals, Cleveland, Ohio; bovine serum albumin (4 × crystallized) from Miles Laboratories, Kankakee, Illinois; radioactively labeled materials from New England Nuclear Corporation, Boston, Massachusetts; radioimmunoassay kits for cyclic 3′,5′-AMP from Becton-Dickinson Co., Orangeburg, New York; synthetic bovine parathyroid hormone *N*-terminal peptide (bPTH, 1-34, 3850 U/mg) from Beckman, Palo Alto, California; prostaglandin E_2 (PGE_2), adriamycin, crude bacterial collagenase, 3′-isobutyl-1-methylxanthine (IBMX) and other chemicals from Sigma Chemical Company, St. Louis, Missouri; and fetal calf serum from Sterile Systems, Inc., Logan, Utah. Various adriamycin analogs were generous gifts from Dr. N. Bachur, National Institutes of Health, and PGE_2 was also provided by Dr. Philip Needleman, Washington University.

Isolation and Culture of Bone Cells

The frontal portions of fetal rat calvaria were stripped of peripheral soft tissue and periosteum. Osteoblastlike bone cells were dispersed from the periosteum-free calvaria with crude bacterial collagenase, harvested, and cultured according to the procedure followed in this laboratory [9], except that the initial 5 min collagenase digestion solution was discarded and fresh collagenase mixture added for the final 55 min period. Freshly isolated cells were seeded at an initial density of 25,000 cells/cm^2 in Costar 12-well plates for studies of cyclic AMP formation and/or T75 flasks for subcellular component preparation. Growth medium contained modified BGJ_b [10], 10 μg/ml of human transferrin, 5% fetal calf serum, 2 mM glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin. All cultures were maintained at 37° C with 5% CO_2 for 5-6 days before being studied.

Treatment of Intact Cells and Estimation of Cyclic AMP

When cell cultures reached near-confluence, serum-containing growth medium was replaced with Ca^{2+}- and Mg^{2+}-free Earle's balanced salt solution supplemented with 0.5% bovine serum albumin, 0.8 mM $MgSO_4$ and 1.8 mM $CaCl_2$, and adriamycin or its analogs (100 μM), as warranted by individual experiments. bPTH (1-34) or PGE_2 was added 30 min later and the incubation continued for varying periods of time. The experiment was terminated with 3 × 2 ml washes of cold Earle's solution. Cyclic AMP was then extracted with cold 5% trichloroacetic acid and ether and its level estimated by radioimmunoassay as previously described [11]. All experiments were performed at room temperature. The estimation of cyclic AMP is expressed as picomoles of cyclic AMP formed per culture, there being virtually no variation in cell number among individual cultures.

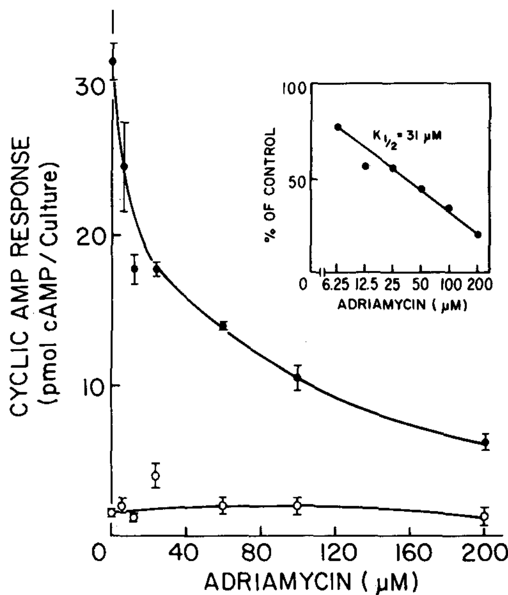
Preparation of Particulate Fraction and Measurement of Adenylate Cyclase Activity

The preparation of a particulate fraction and subsequent assay of adenylate cyclase activity were the same as previously reported [12].

Results

In initial experiments, we examined the effects of adriamycin, added at various concentrations, on the increment in bone cell cyclic AMP formation (hereafter referred to as the cyclic AMP response) caused by PTH. Brief (3 min) incubation of the cells with adriamycin prior to PTH addition caused a substantial inhibition of the cyclic AMP response, without affecting the cyclic AMP levels in PTH-untreated cells. As little as 6.25 μM adriamycin (the lowest concentration tested) yielded a small inhibitory effect and one-half maximal inhibition appeared at 31 μM (Fig. 1). Maximal inhibition, though marked, did not totally eliminate the cyclic AMP response, even at concentrations ranging from 200–500 μM (data not shown).

Exposure of bone cells to PTH elicits a rapid increment in cyclic AMP which peaks in 2.5–5 min and then gradually declines [11], reflecting an initial burst of adenylate cyclase activity, the subsequent phosphodiesterase-mediated metabolism of newly generated cyclic AMP, and an emerging refractoriness (desensitization) to the stimulatory action of the hormone [12]. Consequently, it was of interest to determine the impact of adriamycin on the time course of the cyclic AMP response to PTH and its effect in the presence of a cyclic AMP phosphodiesterase inhibitor. Adriamycin was inhibitory during the ascending phase of PTH stimulation (Fig.



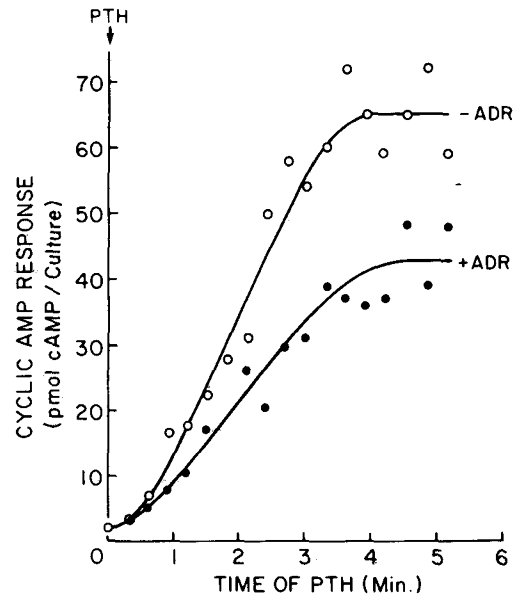


Fig. 1. Bone cells were incubated for 30 min in the presence of various concentrations of adriamycin. PTH (100 ng/ml) was added to stimulate cAMP formation for 3 min before the reaction was terminated with TCA and content of cAMP was assayed. Each point is a mean ± SE of triplicate determinations. Inset: the same data were presented as a percentage of control (no adriamycin in the incubation) on the ordinate and logarithm of adriamycin concentration was plotted on the abscissa. **Fig. 2.** Bone cells were preincubated in the presence or the absence of 100 μM adriamycin for 30 min. PTH (100 ng/ml) was added to the culture at zero time and the reaction was terminated as specified with TCA, and cAMP content was determined. Each point represents mean ± SE of triplicate determinations.

2) and during the descending phase as well (data not shown). Moreover, it was equally effective in the presence or absence of the potent phosphodiesterase inhibitor IBMX (data not shown). This finding, together with the effect of adriamycin on the early phase of the cyclic AMP response, suggests that augmentation of phosphodiesterase activity was not the mechanism of adriamycin action. Adriamycin did not modify the exodus of cyclic AMP from PTH-treated bone cells, nor did it influence cell viability, as judged by (1) trypan blue exclusion and (2) the incorporation of radioactively labeled amino acids into protein. Adriamycin (1-500 μM) failed to perturb the radioimmunoassay for cyclic AMP employed in these experiments.

The osteoblast cyclic AMP response is seen at submicrogram (near-physiological) concentrations of PTH, and increases in linear fashion over a wide dose range. To determine the effect of adriamycin on this dose-response relationship, we treated bone cells with a fixed concentration of adriamycin, and exposed them to concentrations of PTH ranging from 10–100 ng/ml. The fractional inhibition of the cyclic AMP response to PTH by adriamycin was virtually independent of the hormone concentration (Fig. 3). If anything, there was greater inhibition at lower than at higher PTH levels.

The acute effect of adriamycin on the cyclic AMP response to PTH did not persist when adriamycin-treated cells were refed with adriamycin-free incubation medium (Table 1). The effect of PTH was fully restored 30 min after terminating the exposure of bone cells to adriamycin-containing medium.

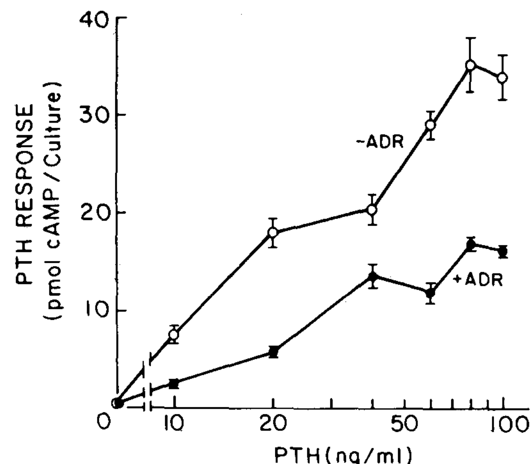


Fig. 3. Bone cells were preincubated in the presence or the absence of 100 μM adriamycin for 30 min. PTH, concentration as specified in the graph, was added to the culture for 3 min before the reaction was terminated with TCA and cAMP content determined. Each point represents mean ± SE of triplicate determinations.

PGE_2, like PTH, promotes cyclic AMP formation in oteoblastlike cells harvested by the method described herein. To define the specificity of adriamycin action, we examined its effect on the cyclic

Table 1. Adriamycin-inhibited PTH response is reversible[a]

Preincubation adriamycin (30 min)	Interval in adriamycin-free medium (min)	Response to PTH pmol cAMP/culture
+	−2	6.2 ± 0.3
+	0	6.3 ± 0.7
+	2	12.6 ± 1.7
+	10	16.7 ± 3.7
+	30	24.5 ± 4.5
−	0	25.7 ± 0.6
−	30	24.6 ± 1.8

[a] Cultures were preincubated in medium with or without 100 μM adriamycin for 30 min; adriamycin-containing medium was then removed and the cultures were washed twice and fed with fresh medium without adriamycin. PTH (100 ng/ml was added to the cultures and the reaction was terminated 3 min later. Each result is expressed as mean ± SE in triplicate samples

Table 2. Cyclic AMP response to PTH and PGE_2 in the presence and the absence of adriamycin[a]

	pmol cAMP/culture		
	Control	PTH	PGE_2
A. In a typical experiment[b]			
Control	2.8 ± 0.3	113.0 ± 10.6	44.3 ± 0.7
Adriamycin	7.5 ± 1.6	43.7 ± 2.7	42.7 ± 1.6
B. In all trials[c]			
Control		100	100
Adriamycin		46	96

[a] Cultures were treated with 100 μM adriamycin for 30 min and 100 ng/ml 1–34 bPTH or 10 μM PGE_2 was added to stimulate cAMP formation for 3 min before cAMP was extracted and assayed
[b] Absolute level of cAMP formed in a typical experiment (triplicate determinations) is expressed as mean ± SE
[c] PTH and PGE_2 controls were set at 100. Adriamycin-treated samples are expressed as percentage of the controls (n = 9)

AMP response to PGE_2. Concentrations of adriamycin which markedly inhibited the effect of PTH did not alter the influence of PGE_2, an observation that was highly reproducible (Table 2).

The results described above suggest that adriamycin-mediated inhibition is exerted at the level of PTH-target cell interaction, transduction of the hormone-receptor initiated signal, and/or adenylate cyclase catalysis. To discriminate between these possible modes of action, we examined the effects of adriamycin on adenylate cyclase activity in PTH-treated, PGE_2-treated, fluoride-treated, GppNHp (Guanylyl imidodiphosphate)-treated and untreated subcellular fractions (Table 3). Though a high concentration was required (concentrations less than 500 μM were not effective), adriamycin virtually nullified the stimulatory effect of PTH. By contrast, it failed to blunt the effect of PGE_2 and only the highest concentration tested reduced "basal" adenylate cyclase activity (in some experiments) as well as the stimulatory effect of sodium fluoride. This same high concentration of adriamycin did not impair GppNHp-mediated stimulation. These results indicate that adriamycin influences the binding of PTH to its receptor and/or the transduction of the subsequently generated message but not the activity of the catalytic or regulatory component of adenylate cyclase.

The availability of multiple structural analogs of adriamycin, the effects of which have been tested in other systems, afforded us an opportunity to study structure-activity relationships (Table 4). None of the six analogs studied modified "basal" cyclic AMP levels. Two analogs, alcacinomycin and rubidazone, were at least as effective as adriamycin in inhibiting the cyclic AMP response to PTH. Al-

Table 3. Adriamycin inhibits PTH but not PGE_2 or NaF-stimulated adenylate cyclase activity[a]

	Adenylate cyclase activity pmol cAMP formed/mg protein/10 min			
		Adriamycin		
	Control	100 μM	500 μM	1 mM
Control	411 ± 18	412 ± 11	390 ± 9	316 ± 3
PTH	697 ± 8	687 ± 20	478 ± 20[b]	255 ± 60[b]
Control	212 ± 22	201 ± 3	271 ± 7	290 ± 8
PGE_2	417 ± 20	435 ± 11	630 ± 24	506 ± 44
Control	169 ± 13	196 ± 21	167 ± 18	139 ± 10
NaF	1001 ± 38	1045 ± 63	1076 ± 51	754 ± 2
Control	259 ± 4	296 ± 1		180 ± 20
GppNHp	823 ± 20	797 ± 37		821 ± 13

[a] Adenylate cyclase activity in the crude membrane preparation was measured in the presence of 1 μg/ml PTH, 100 μM PGE_2, 10 mM NaF or 10 μM GppNHp, with or without adriamycin at concentration listed. Results are expressed as mean ± SE
[b] Differs significantly from the control on the same row

Table 4. Effect of different adriamycin analogs on cAMP formation stimulated by PTH and PGE_2

	Modification	cAMP[a]	
		PTH	PGE_2
Control		100	100
Adriamycin		53[b]	101
Alcacinomycin	Long-chain sugar	32[b]	34[b]
AD-32	Long-chain sugar	153[b]	159[b]
7-omen	Long-chain sugar	97	130
4,epi-adriamycin	C-4 epimer	78	115
Anthracenediene	Anthracenediene group	80	150[b]
Rubidazone	Hydrazine	46[b]	144[b]

[a] Results are expressed as a percentage of the control from 2 separate triplicate determinations
[b] Differs significantly from the control ($P \leq 0.05$)

cacinomycin, but not rubidazone, inhibited the effect of PGE_2. Indeed, one analog appeared to augment the effect of both agents, and several to augment the PGE_2 effect.

Discussion

Our experimental results indicate that adriamycin is a potent inhibitor of the cyclic AMP response to PTH in isolated osteoblastlike cells. Adriamycin appears to act by modifying one or more plasma membrane-located step(s): the initial interaction between PTH and the osteoblast receptor, the transduction of the hormone-receptor signal, and the catalytic generation of cyclic AMP. Three lines of evidence support this proposition. First, the effect of adriamycin is not attributable to alterations in cyclic AMP fluxes or degradation, nor to interference with the cyclic AMP assay. Second, adriamycin-mediated inhibition appeared rapidly, and also disappeared quickly when exposure was terminated, implying inhibition exerted at a superficial cell site. Third, adriamycin blocked the effect of PTH on adenylate cyclase activity in a broken bone cell preparation, in which substrate (ATP) levels were not limiting and cyclic AMP degradation was blocked. Although higher concentrations of adriamycin were required to inhibit adenylate cyclase activity in the crude membrane preparation than the cyclic AMP response in the intact cell, the mechanism of adriamycin action was likely the same, since adriamycin inhibited the effect of PTH but not of PGE_2 in both systems.

The finding that adriamycin blunted the various responses to PTH but not to PGE_2 is of considerable interest. One possible explanation for this highly reproducible observation is that the cultures contained multiple cell populations, one of which was PGE_2-responsive and adriamycin-resistant. Indeed, the cell cultures used in the present study do not contain a homogeneous population, but osteoblastlike cells are in the majority, as indicated by a variety of biochemical criteria. Osteoblasts possess receptors for prostaglandins of the E series, and respond to prostaglandins with increases in cyclic AMP formation [4–6]. It is reasonable to suggest, therefore, that the cyclic AMP response in our experimental system stems mainly from osteoblasts (or osteoblastlike cells). Hence, differential sensitivity to adriamycin, which may exist in a heterogeneous cell population, is an unlikely interpretation of the data. It is more likely that adriamycin affects an event that is unique to the action of PTH. Since bone cells probably possess separate receptors for PTH and PGE_2, and perhaps separate mechanisms for transduction, adriamycin may inhibit the binding of PTH or the transduction of the PTH message (or both). Although bone cells could contain separate adenylate cyclase regulatory and catalytic units for both agents, the failure of adriamycin to inhibit GppNHp stimulation of adenylate cyclase and fluoride-augmented catalysis at concentrations that inhibited PTH stimulation argues against these units as the possible loci of adriamycin action.

The concept that adriamycin interferes with PTH binding or action at the level of the plasma membrane is consistent with the results of studies in other systems. Adriamycin influences multiple cell membrane functions, including lectin-induced cell agglutination [13], ion transport [14], lipid organization [15], and membrane fluidity [16]. It inhibits the exchange of Ca^{2+} in isolated atria [17], reduces $(Na^+ + K^+)$-ATPase activity in cardiac cells [14], and enhances the net transport of Na^+ in frog skin epithelial cells [18]. At low concentrations, adriamycin increases the inward slow $(Ca^{2+} - Na^+)$ current in chick hearts, whereas high concentrations inhibit action potentials [17]. Adriamycin was also found to be a specific inhibitor of Na^+/Ca^{2+} exchange in heart vesicles [19]. A low concentration (10 μg/ml) has been shown to cause a noncompetitive inhibition of uridine transport in sarcoma 180 cells within 30 min [20]. These effects appear to stem from a direct interaction between adriamycin and phospholipid components of the plasma membrane. In fact, the binding of adriamycin to cardiolipin has been shown to correlate positively with its *in vitro* toxicity [8].

Efforts to demonstrate effects of adriamycin on cyclic nucleotide metabolism in other systems have yielded mixed results. Adriamycin (30 μg/ml) was reported to reduce cyclic AMP levels in line 10 guinea pig hepatoma cells [21]. By contrast, administration of adriamycin to humans failed to modify cyclic AMP levels in endomyocardial biopsy specimens [22], though there was a prominent decrease in tissue cyclic GMP content. Finally, adriamycin (50 μg/ml) rapidly (within 15 min) increased cyclic AMP in isolated, perfused chick hearts, which may underlie its positive inotropic effect, its activation of slow current, and enhancement of Ca^{2+} influx [17].

The skeletal responses to adriamycin have not been studied extensively. Long-term, low-dose administration to young, growing rabbits induced thinning of the epiphyses, a decline in the number of osteogenic cells, a depression of spongiosa trabeculae, reduced bone density, and retardation of bone

mineralization [23]. It has been suggested that adriamycin interacts with bone mineral, much like representatives of the chemically similar tetracycline group of antibiotics. Consequently, bone cells closely applied to mineralized surfaces might be exposed to particularly high concentrations of this toxic agent.

Our studies with the adriamycin analogs indicate that the various effects they exert on plasma membrane function may reflect the extent, locus, and nature of their specific interactions with membrane components. Hence, one compound (alcacinomycin) did inhibit the effect of PGE_2, in addition to that of PTH, whereas others actually stimulated the cyclic AMP response to one or both agents. These agents may emerge as probes of membrane structure function that could be useful in exploring the mechanisms of hormone action.

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