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Effects of Osteoclast Activating Factor from Human Lymphocytes on Cyclic AMP Concentrations in Isolated Mouse Bone and Bone Cells

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Summary. Osteoclast activating factor (OAF) is a lymphokine which may participate in the pathologic destruction of bone observed in a number of disorders. In the current studies, we investigated the action of OAF on cAMP accumulation by bones and isolated bone cells in culture. OAF was shown to stimulate accumulation of cAMP in mouse cranial bones at doses between 1 and 1000 ng/ml. Stimulation of bone resorption was observed in bones treated with the same doses of OAF. In order to investigate the cell types responsible for cAMP responses to OAF, we isolated bone cells and grew them in monolayer culture. The cells were isolated by a variety of techniques which separate bone cells into two types of parathyroid hormone (PTH)-responsive populations: (a) cells derived from the periosteal regions of the bone, which also respond to calcitonin with increases in cAMP; and (b) cells derived from the matrix, which do not respond to calcitonin. OAF caused elevation of cAMP levels in both the periosteum-derived cells and the matrix-derived cells. The magnitudes and time courses of OAF effects in these populations resembled the effects previously reported for PTH in the same populations. OAF stimulated adenyl cyclase in both types of cell populations, but did not produce significant changes in cAMP phosphodiesterase activity. OAF differed from PTH in that its effects on cAMP accumulation decreased sharply at supramaximal doses in both bone and isolated cells, especially in the matrix-derived populations. Bone resorption did not decrease as markedly as did cAMP accumulation at high doses of OAF, suggesting that cAMP accumulation and resorption could be dissociated under some conditions. These results indicate that OAF has effects on cAMP production in the same cell populations as PTH, and suggest that OAF could modify not only resorption but also formation of bone in vivo. OAF may exert its effects on bone by means of cAMP-dependent mechanisms, but more data will be necessary to establish this unequivocally. The observed differences between OAF and PTH may be of relevance in the mechanism and treatment of pathologic bone destruction in vivo.

Key words: Bone — Bone cells — Cyclic AMP — Osteoclast activating factor — Lymphokine.

Introduction

The lymphokine osteoclast activating factor (OAF) has been shown to stimulate destruction of bone in vitro [1]. OAF or similar factors are secreted by human peripheral lymphocytes activated by specific antigens [1] or mitogens [1, 2], by human tonsil lymphocytes activated by mitogens [3], by unstimulated primary cultures of human myeloma and lymphoma [4] cells, and by stable lymphoid cell lines derived from human myelomas and other bone-destroying lymphoid tumors [5]. The species of OAF secreted by phytohemagglutinin (PHA)-activated human tonsil lymphocytes has been purified [3], and shown to be a peptide of molecular weight approximately 9000. Bone destruction is observed frequently in association with diseases involving chronic accumulation of lymphoid cells, e.g., periodontal disease [6], or lymphoid tumors such as myelomas [4] or lymphomas [7]. This bone destruction histologically appears to be mediated by osteoclasts rather than by the inflammatory cells or tumor cells themselves [4–7]. The above observations have led to suggestions [1–5] that OAF or a similar factor secreted by lymphocytes is responsible for pathologic bone destruction in some diseases. Recently, a bone-destroying factor with the same ultrafiltration and electrophoresis characteristics as

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OAF has been demonstrated in the serum of some patients with myeloma [8].

Studies in vitro of the effects of OAF on bone metabolism have demonstrated that it stimulates osteoclastic resorption [9] and inhibits synthesis of new collagen by bone [10]. These activities of OAF are similar to those observed in vitro for the primary hormones which regulate bone metabolism, parathyroid hormone (PTH), and 1,25 dihydroxyvitamin D_3 [1,25$(OH)_2D_3$]. Although the precise mechanisms of action of PTH and 1,25$(OH)_2D_3$ still are not well understood in bone, it is clear that PTH causes accumulation of adenosine-3′5′-monophosphate (cAMP) in skeletal tissue [11] and isolated bone cells [12] whereas 1,25$(OH)_2D_3$ does not appear to have this effect [13, 14]. In order to examine the biological effects of OAF on bone, it was therefore of interest to determine whether or not this factor could influence cAMP metabolism in bone or bone cells.

In addition, it was of interest to determine which cell types in bone could respond to OAF. Several studies have shown that different populations of cells can be isolated from bone [12, 15–18]. The isolated populations can be grown in primary monolayer culture and their responses to bone-active agents can be monitored biochemically. In previous studies [12], we have shown that PTH has effects on separated bone cell populations which account for many of the effects of the hormone on intact bones. Other investigators [15–18] have also shown that separated populations of bone cells have substantially different cAMP responses to PTH and other agents. Therefore, we have examined the effects of OAF on accumulation of cAMP in isolated bone cell populations which were prepared by a variety of methods. The results suggest a possible role for cAMP in the responses of bone and bone cells to OAF, and imply that other lymphokines also may act on their target cells by means of cAMP-dependent mechanisms.

Materials and Methods

Isolation of OAF

OAF was isolated from the culture medium of human tonsil lymphocytes which had been activated with PHA [3]. Pooled culture medium was concentrated 100× by ultrafiltration using an Amicon H1P10 hollow-fiber device with a molecular weight cutoff (nominal) of 10,000. The 100× concentrate was further concentrated to approximately 1000× using an Amicon ultrafiltration cell with a UM2 membrane (nominal molecular weight cutoff 1000). The concentrate was fractionated by gel filtration using Sephacryl S-200. Active OAF fractions (K_D = 0.5–0.6) were pooled and further purified as described previously [3]. The purified material produced a single protein band in native gel and sodium dodecylsulfate-urea gel electrophoresis. It was biologically active at concentrations between 1 and 1000 ng/ml, as assessed by stimulation of calcium release from cultured mouse cranial bones [19]. OAF prepared by these procedures has been shown previously [2, 5] to be free of bone-resorbing activity from prostaglandins or vitamin D metabolites, and to contain no immunoreactive PTH.

Assay of cAMP Responses in Isolated Bone

Cranial bones were removed from 2- to 3-day-old Swiss albino mice using aseptic dissection procedures. The cranium (frontal and parietal bones) was split in half at the midline suture and halves were placed in separate wells of multiple-well culture dishes (Linbro) containing 0.5 ml Eagle's minimal essential medium (MEM) with 5 *mM* theophylline, without added serum. The bones were equilibrated 30 min at 37°C in 5% CO_2:95% air. Then an additional 0.5 ml of MEM, containing OAF or other test agents as described (also equilibrated in 5% CO_2:95% air at 37°C), was added to each well with rapid mixing. At a designated time (normally 5 min after addition of the test medium) the culture medium was rapidly aspirated. The dish, containing bones, was quickly placed in a commercial microwave oven (450 W, radiation frequency 2450 MHz) and irradiated for 2 min to stabilize tissue cAMP levels. The bone was then immersed in 0.5 ml of 0.2*M* sodium acetate buffer, pH 4.0, and left at 4°C for at least 18 h to extract soluble cAMP. If more than 24 h was to elapse between treatment and cAMP radioimmunoassay, the samples were frozen at −30°C until the day of assay. The extracted samples were acetylated [20] at room temperature by adding 20 μl of triethylamine per 0.5 ml sample with vigorous mixing, followed immediately by 10 μl of acetic anhydride, with further vigorous mixing. Aliquots of this acetylated sample were used for cAMP radioimmunoassay.

Isolation of Bone Cells

Three methods were used for preparation of bone cell populations. The most frequently used method (method A) was sequential digestion of bones with 0.1% collagenase at room temperature. This was carried out exactly as described previously [12] yielding five separate populations of cells. The cells were grown in plastic flasks in MEM containing 10% fetal bovine serum (Gibco) in an atmosphere of 5% CO_2:95% air. The monolayers reached confluence at 6–7 days after isolation. At this time cells were resuspended using 4 *mM* EDTA in Ca^{2+}- and Mg^{2+}-free Tyrode's solution, pH 7.4. The cells were then subcultured to multiple-well culture dishes at 0.5–1 × 10^5 cells per well in MEM with 10% fetal bovine serum. These cells were allowed to attach to the dish and were cultured for at least 24 h before use in assays. In other experiments an alternative bone cell isolation (method B), described by Peck et al [15], was used. Periosteal cells were separated from bone matrix by stripping the periosteal layers from bones using fine forceps under a dissecting microscope. Then periosteum and matrix were subjected separately to total digestion with 0.2% collagenase at 37° C [21]. The suspended cells were washed twice with MEM containing 10% fetal bovine serum, then cultured to confluence (5–8 days) before subculture to multiple-well dishes, as described above. A final variation of this cell isolation procedure (method C), developed by Chen and Feldman [16], was used in other experiments: instead

of manually stripping the periosteum, periosteal cells were removed by a 30-min digestion in 0.1% collagenase at 25°C. Histologic examination showed that this procedure effectively removed the periosteal layers of the bone. The remaining matrix was then subjected to total digestion using 0.2% collagenase at 37°C. Periosteal cells and matrix-associated cells were cultured separately, subcultured to multiple-well culture dishes as described above, and treated for hormone response.

Assay of cAMP Responses in Bone Cell Monolayers

After 24 h culture of bone cells in multiple-well dishes, the medium was replaced with 0.5 ml per well of fresh MEM containing 5 *mM* theophylline and no serum. The cultures were then allowed to equilibrate 30 min at 37°C in 5% CO_2:95% air. Another 0.5 ml of pre-equilibrated MEM, containing OAF or other test substance as described, was added to each well to initiate the experiment. Aspiration of medium, irradiation by microwave, extraction of cAMP, and acetylation of samples were carried out exactly as described above for cranial bones.

Radioimmunoassay of Cyclic AMP

Acetylated extracts from bone or bone cells, prepared as described above, were analyzed for cAMP using the radioimmunoassay method of Steiner et al. [22], as modified by Harper and Brooker [20]. The antisera used were prepared in this laboratory using the procedures suggested by Steiner et al. The antisera showed no cross-reactivity with ATP or with nucleotides other than cAMP at concentrations up to 10^6-fold excess over the cAMP concentrations normally encountered. The assay was sensitive, i.e., greater than 2 S.D. deflection from total binding or nonspecific binding, between 3 and 1000 fmol cAMP per sample. The within-assay coefficient of variation [23] at 30–100 fmol was less than 10%, and the between-assay coefficient of variation was 8.5% for a representative series of 20 assays. Data were processed using a logit-log transformation program for a desk-top computer. In order to validate the results obtained with the cAMP assay, we also assayed some samples in parallel experiments using the cAMP-binding protein method of Gilman [24]. Representative samples were also treated with phosphodiesterase prior to radioimmunoassay, in order to test for nonspecific, non-cAMP substances which might compete for ligand binding to antibody. In addition, some experiments contained tissue samples to which cAMP had been added in known quantities in order to monitor recovery through the extraction and acetylation steps. Recovery in these experiments was greater than 90%.

Adenyl Cyclase and Phosphodiesterase Assays

Bone cell homogenates were assayed for these activities by the methods of Rapoport and Adams [25]. Cell monolayers were suspended in Dulbecco's phosphate-buffered saline (PBS; Gibco), washed twice with PBS, and sonicated in 10 m*M* Tris-HCl, pH 7.5, 4 m*M* $MgCl_2$. Aliquots of homogenate containing 20–50 μg protein were incubated in an adenyl cyclase assay mixture containing the same buffer and 2 m*M* ATP, 1 m*M* 1-methyl-3-isobutyl xanthine, 10 m*M* creatine phosphate, 0.75 mg/ml creatine phosphokinase, 0.1% bovine serum albumin, and OAF or PTH as indicated. After 10 min incubation at 37°C, the reaction was stopped by boiling for 3 min; 0.5 ml of 50 m*M* sodium acetate buffer, pH 6.2, was added, and the mixture was centrifuged at 13,000 × *g* for 2 min. Aliquots of the supernatant were acetylated and cAMP was determined by radioimmunoassay. For phosphodiesterase assay, the cells were washed twice with PBS and sonicated in 40 m*M* Tris-HCl, pH 8.0, 5 m*M* $MgCl_2$. Aliquots containing 50μg protein were incubated in the same buffer containing 0.1 m*M* unlabeled cAMP, 0.05 μCi/ml [$_3$H]cAMP (Amersham), and OAF or PTH as indicated. After 30 min incubation at 30°C the reaction was stopped by boiling. Then 50 μg of *Ophiophagus hannah* snake venom (Sigma) was added and the mixture was incubated for another 20 min. at 30°C following which 1 ml of Dowex AG-1-X8 resin slurry in 3 m*M* acetic acid was added. The samples were shaken at room temperature for 10 min, centrifuged, and the supernatants were counted in a liquid scintillation counter. Counts of [^{3}H]adenosine remaining in the supernatant were derived from cAMP hydrolyzed by phosphodiesterase; blank values were obtained by treating some samples with homogenate which had been boiled prior to the assay. These blanks were subtracted from all hydrolysis values.

Materials

All tissue culture media and sera were obtained from Gibco. Linbro multiwell dishes were obtained from Flow Laboratories. Bovine parathyroid hormone was obtained from the Hormone Distribution Officer, NIAMDD. Dr. Anthony W. Norman kindly furnished samples of 1,25$(OH)_2D_3$. Synthetic salmon calcitonin (SCT) was supplied by Armour. All other materials were reagent grade or better, and were obtained from various commercial suppliers.

Results

Validation of cAMP Assay Procedures

Microwave irradiation has been used as a rapid and efficient means of fixing cAMP in tissues and whole animals [26]. Fixation is accomplished primarily by thermal inactivation of cAMP forming and degrading enzymes. We adopted this procedure as a means of simultaneously fixing multiple tissue culture samples without the laborious extraction and recovery procedures previously employed. Because of the low mass and physical thinness of cell monolayers and of the cranial bones used (net thickness < 0.5 mm), it was not necessary to employ the specialized high-power microwave beams used by other investigators [26] for large-scale tissue fixation. A household microwave oven (J.C. Penney model 863-5700-70-021) produced stabilization of concentrations of cAMP in bone cell monolayers within 30 s under the conditions employed for these experiments (Table 1). Recovery of known amounts of cAMP, added to the tissue before irradiation, remained near 100% for times up to 5 min of irradiation. The standard irradiation time of 2 min was chosen because this produced slightly higher recoveries of tissue cAMP than either 30 s or 1 min (Table 1). The protocol used in these experiments (2 min of

Table 1. Effect of fixation method on parameters of cAMP radioimmunoassay

Fixation method	Nonspecific binding (cpm)	Specific binding (cpm)	Measured cAMP (pmol/10^5 cells)		
			Control	PTH	PDE-sensitive
TCA precipitation	2808 ± 124	6935 ± 64	4.3 ± 0.3	32.4 ± 2.8	84%
Ethanol-HCl extraction	2247 ± 148	7088 ± 113	3.9 ± 0.2	28.4 ± 3.3	88%
Microwave irradiation (min)					
0	1315 ± 93	3842 ± 102	6.0 ± 0.8	9.5 ± 1.4	95%
0.5	1014 ± 136	6344 ± 215	4.4 ± 0.3	27.4 ± 2.5	93%
1	1135 ± 2.02	7418 ± 125	3.8 ± 0.4	31.4 ± 4.0	96%
2	1047 ± 78	8814 ± 113	4.2 ± 0.4	33.2 ± 2.8	95%
5	1788 ± 102	7542 ± 83	1.6 ± 0.3	16.4 ± 4.1	81%

Bone cell monolayers were prepared from population 4 cells produced using digestion method A (see Methods). These cells were incubated for 5 min at 37°C in the presence or absence of 300 ng/ml PTH. The medium was removed and the monolayers were then fixed and the cAMP extracted using 3 methods. (a) TCA precipitation: 0.5 ml of ice-cold 10% trichloroacetic acid (TCA) was added directly to the cell layer and the cells were homogenized with a rubber policeman. TCA was removed by 5 extractions of the aqueous phase using 3 vol of ether, the samples were lyophilized and then reconstituted with 0.5 ml of 0.2 *M* sodium acetate buffer, pH 4.0. (b) Ethanol-HCl extraction: 0.5 ml of 95% ethanol-0.02 *N* HCl at −20°C was added to the cell layers, and the dishes were kept at −20°C overnight. The cells were then homogenized with a rubber policeman, and the ethanol-HCl solution was removed by boiling to dryness. The residue was redissolved in 0.5 ml of pH 4.0 buffer as above. (c) Microwave irradiation: cell monolayers were irradiated for the designated period of time, then 0.5 ml of pH 4.0 buffer was added and the samples were allowed to stand overnight at 4°C

Some samples from all 3 fixation procedures were treated with cAMP phosphodiesterase (PDE). Then all samples were acetylated as described in Methods, and analyzed for cAMP by radioimmunoassay. *Nonspecific binding* represents the cpm of ^{125}I-succinyl cAMP tyrosine methyl ester which were associated with antibody when a high cAMP concentration (> 100 pmoles/sample) was added to the sample before acetylation. *Specific binding* represents the amount of ^{125}I-derivative bound to antibody in phosphodiesterase-treated samples, less the nonspecific binding (i.e., nonspecific binding + specific binding = total binding). A total of 20,000 cpm of ^{125}I derivative was added per assay tube; thus total binding was 40%–50% at zero cAMP concentration. Values in the "PDE-sensitive column denote the percentage decrease in measured cAMP which was induced by phosphodiesterase treatment of identical samples. All values are means ± SEM of 4 samples.

microwave fixation, 18 h of sodium acetate extraction) produced higher recoveries and less variability in recovery of cAMP than extractions with either trichloroacetic acid [22] or ethanol-HCl [12]. In addition, this method of fixation and extraction of samples resulted in significantly lower values for nonspecific binding and for phosphodiesterase-insensitive displacement of ligand in the cAMP radioimmunoassay (Table 1).

Stimulation of cAMP Accumulation in Bones by OAF and PTH

Addition of OAF to the culture medium produced a rapid stimulation of accumulation of cAMP in mouse cranial bones (Fig. 1). The peak in cAMP concentration occurred approximately 5 min after addition of OAF, and was followed by a more gradual decrease in concentration, reaching baseline (i.e, prestimulation) levels after approximately 20 min. These results are not significantly different from the results obtained for PTH in this system (Fig. 1). The cAMP response to OAF was markedly dose dependent (Fig. 2), with minimum significant stimulation of cAMP accumulation occurring below 1 ng/ml purified OAF, and maximum effect at about 10 ng/ml. At low doses, the effects of OAF on cAMP accumulation were correlated with increases in calcium release from mouse cranial bones treated with the same doses of OAF for 72 h in organ culture (Fig. 2). At doses of OAF above 10 ng/ml, stimulation of both bone resorption and cAMP accumulation decreased progressively (Fig. 2). No significant decrease in either bone resorption or cAMP accumulation was observed when supramaximal doses of PTH were used in this system (data not shown).

Effects of OAF on Bone Cell cAMP

In order to compare the effects of OAF with those reported in other studies using isolated bone cells, we prepared bone cells by three techniques previously shown to produce separate populations of cells with different metabolic characteristics. These cell populations were cultivated in vitro and the effects of OAF on cAMP accumulation were assessed (Tables 2 and 3; Figs. 3 and 4). The digestion technique of Wong (method A in the current study), which produces five separate populations of cells

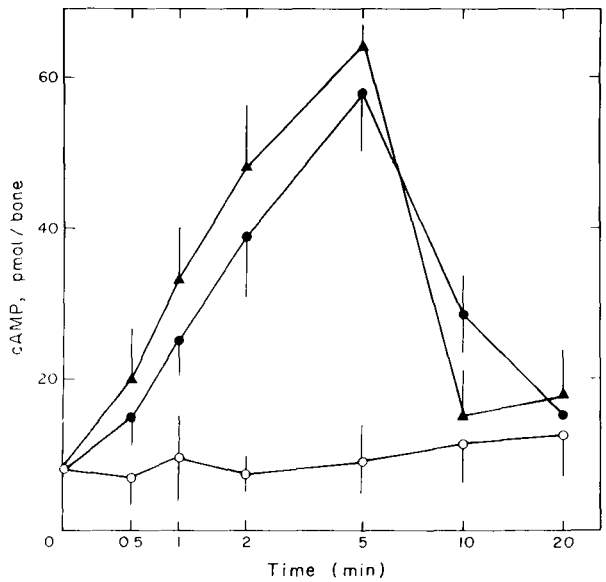

Fig. 1. Time course of cAMP accumulation in mouse cranial bones treated for the indicated times with 300 ng/ml PTH (▲—▲), 10 ng/ml OAF (●—●), or no addition (○—○). All values are mean ± SEM for 4 bones per point

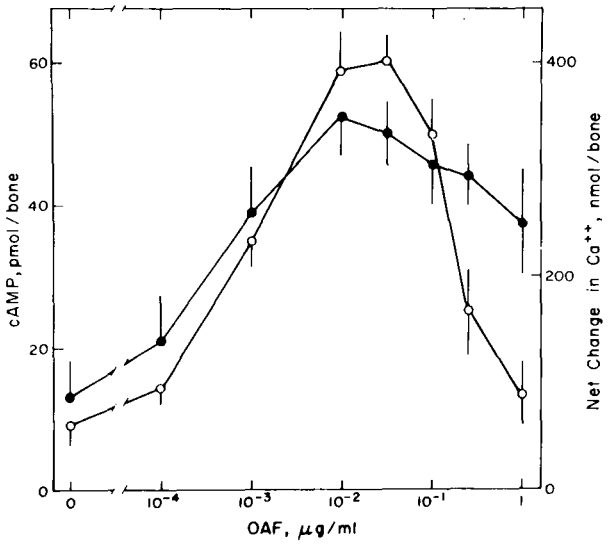

Fig. 2. Dose-response relationships in bone for OAF on cAMP accumulation (○—○), measured at 5 min of treatment, and on calcium release (●—●), measured after 72 h treatment in organ culture. Calcium release was measured by atomic absorption spectrometry, and is expressed relative to bones killed by freezing and thawing prior to culture. Values are mean ± SEM for 3-4 bones per point

[17], was the primary preparation procedure used for these studies. Using this system, it was shown (Table 2) that there were two distinct populations in which OAF stimulated cAMP accumulation. One of these was population 2 (i.e., those cells derived from the second 20-min digestion with collagenase). The stimulation of cAMP accumulation observed was relatively low, 1.5- to 3-fold, even with maximal doses of OAF. Similar magnitudes of cAMP accumulation were observed using PTH or SCT in the same population (Table 2), but another stimulator of bone resorption, 1,25-dihydroxyvitamin D_3, did not affect cAMP concentrations in these cells (data not shown). OAF also caused increases in cAMP in populations 4 and 5 of the cells prepared by method A. In these populations, the stimulation was much higher, approximately tenfold (Table 2). These data also were comparable with those obtained for PTH in the same populations, although PTH caused somewhat higher peak cAMP concentrations, approximately 15-fold higher than baseline concentration. Again 1,25-dihydroxyvitamin D_3 failed to cause elevation of cAMP concentrations in the population 4 and 5 cells. Likewise, SCT failed to stimulate cAMP accumulation in these populations, as reported previously [12].

Reproducibility of Effects

The basal levels of cAMP in bone cell populations varied between 0.8 and 2.5 pmol/10^5 cells for population 2 cells and between 1.8 and 4.2 pmol/10^5 cells for population 4 cells over a series of experiments, probably because of minor differences in the animal populations and bone digestion technique from experiment to experiment. The maximal levels of stimulation induced by PTH or OAF varied in proportion to the basal levels of cAMP, with the result that the ratios between treated and control cultures were consistent: e.g., 1.5- to 3-fold maximum stimulation in population 2 and 10- to 15-fold maximum stimulation in population 4, for both OAF and PTH. All major findings reported here were reproduced in 4 to 10 experiments within these variations.

Comparison of Digestion Procedures

Two other procedures were used to separate populations of bone cells. Both of these techniques produce only two populations, previously characterized [14, 16] as periosteal cells and matrix-associated cells. An additional feature characterizing these populations is the ability of periosteal cells to respond to SCT with increased cAMP levels, whereas the matrix-derived cells fail to respond to SCT [12, 14, 16]. The procedure of Peck et al. [15] corresponds to method B in the current study. Using this procedure, we obtained modest elevations of cAMP, only about 50% over base line in cultures derived from periosteal cells (Table 2). This result

Table 2. Effect of separation technique on cAMP Responses of bone cell populations

Separation used	cAMP (pmol/10^5 cells)			
	Control	OAF (10 ng/ml)	PTH (300 ng/ml)	SCT (50 ng/ml)
Method A				
Population 1	2.1 ± 0.3	2.6 ± 0.2	2.9 ± 0.4	2.6 ± 0.3
Population 2	3.2 ± 0.4	6.9 ± 0.5[a]	7.1 ± 0.6[a]	5.7 ± 0.3[a]
Population 3	3.6 ± 0.4	4.2 ± 0.4	4.4 ± 0.5	3.5 ± 0.2
Population 4	3.9 ± 0.4	43.2 ± 1.8[a]	59.3 ± 2.8[a]	4.3 ± 0.5
Population 5	4.2 ± 0.5	22.7 ± 2.1[a]	18.4 ± 1.9[a]	4.8 ± 0.6
Method B				
Periosteum-derived	2.9 ± 0.3	4.6 ± 0.5[a]	4.8 ± 0.4[a]	5.1 ± 0.4[a]
Matrix-derived	3.6 ± 0.4	28.3 ± 3.1[a]	30.4 ± 3.6[a]	3.9 ± 0.5
Method C				
Periosteum-derived	3.2 ± 0.4	7.3 ± 0.6[a]	6.9 ± 0.5[a]	6.8 ± 0.8[a]
Matrix-derived	4.5 ± 0.5	21.2 ± 3.1[a]	19.7 ± 2.2[a]	3.9 ± 0.6

Cell monolayers prepared by the indicated procedure (see Methods for details of cell isolation procedures) were treated for 5 min with each agent, then assayed for cAMP. Values are mean ± SEM for 4 samples

[a]Significantly different from control, $P < 0.05$ (Dunnett multiple comparison test, ref. 27)

Table 3. Effects of OAF and PTH on adenyl cyclase and phosphodiesterase activities of bone cell populations

Cell population and treatment	Adenyl cyclase pmol/min/mg protein	Phosphodiesterase nmol/min/mg protein
Population 2		
Control	21.6 ± 1.9	2.6 ± 0.3
PTH (300 ng/ml)	63.2 ± 5.4[a]	2.1 ± 0.2
OAF (10 ng/ml)	58.5 ± 4.3[a]	3.1 ± 0.4
Population 5		
Control	23.5 ± 1.4	2.7 ± 0.1
PTH (300 ng/ml)	87.4 ± 6.2[a]	3.1 ± 0.4
OAF (10 ng/ml)	91.3 ± 8.6[a]	3.3 ± 0.3

Cell monolayers from the indicated populations were prepared and assayed for adenyl cyclase and phosphodiesterase as described in Methods. Homogenates were treated with OAF or PTH for 10 min for adenyl cyclase studies and 30 min for phosphodiesterase studies. Values are mean ± SEM for at least 4 samples

[a]Significantly different from corresponding control, $P < 0.05$ (Dunnett multiple comparison test, ref. 27)

was found for OAF, PTH, and SCT at maximal doses. In the cultures derived from matrix-associated cells, approximately eightfold increases over baseline values were observed for OAF and PTH (Table 2), although no response to SCT was observed.

In the cell isolation procedure of Chen and Feldman an initial collagenase digestion step was used to remove periosteal cells, followed by total digestion of the matrix. This technique (method C) was found [16] to produce periosteal cells which maintained substantial cAMP responses to PTH in culture. In our hands, periosteal cells isolated by this technique exhibited higher levels of cAMP accumulation than those cells islated by method B (Table 2), demonstrating responses essentially equal to those produced by PTH, OAF, or SCT in the cells isolated by method A. However, the responses of matrix-derived cells were lower than those derived from method B, and considerably lower than responses observed in population 4 of the cells isolated by method A. Because of the higher responses, and therefore apparently more efficient enrichment of responsive cells in populations 2 and 4, method A was used for cell isolation in the remainder of the studies reported here.

Time Courses and Dose Dependency of Responses to OAF

Periosteal cells and matrix-associated cells (i.e., population 2 and population 4 cells, respectively) showed different time courses of cAMP response to OAF (Fig. 3). Accumulation of cAMP in both cell types reached maximum levels at approximately 5 min after addition of OAF. The maximum cAMP concentration was sustained for several minutes in population 2 cells, returning to baseline levels only after at least 30 min. In contrast, cAMP concentrations decreased sharply after reaching peak levels in population 4 and had returned to baseline levels by approximately 20 min after treatment with OAF was begun. When the responses to OAF were measured at 5 min (the time of peak cAMP elevations in each cell type), similar dose-response curves were obtained for the two populations (Fig. 4). Although the magnitudes of response in the two populations differed substantially, the ascending slopes of the

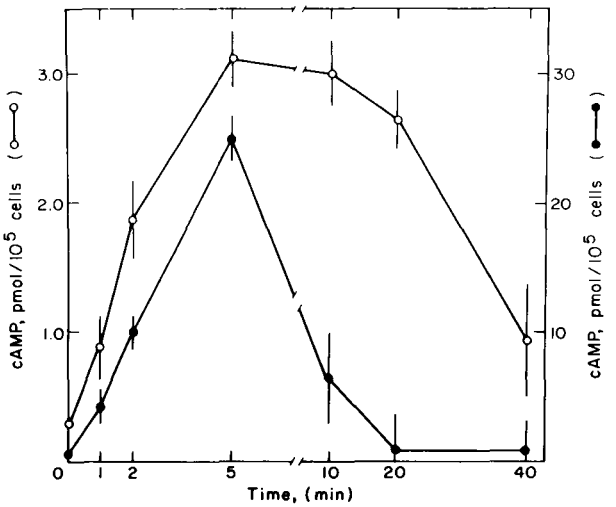

Fig. 3. Time course of cAMP response to a maximal dose of OAF (10 ng/ml) in isolated bone cells from population 2 (○—○) and population 4 (●—●). Values are mean ± SEM for 4 samples per point

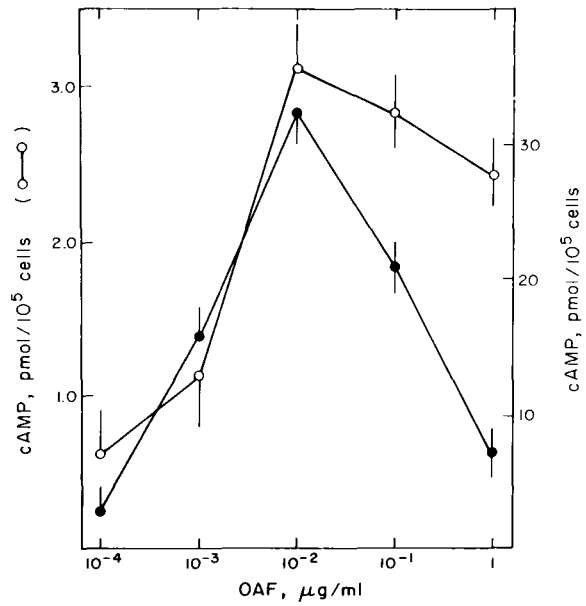

Fig. 4. Dose dependency of cAMP response to OAF in bone cells from population 2 (○—○) and population 4 (●—●). Values are mean ± SEM for 4 samples per point

dose-response curves, when compared to maximum effect, were indistinguishable. However, at supramaximal doses of OAF the cAMP response in matrix-derived cells fell off sharply while the response in periosteal cells remained relatively constant up to a dose approximately 10 times the lowest maximal dose of OAF. At even higher doses, the response of periosteal cells decreased, but more gradually than the decrease observed in matrix-derived cell populations.

Effects of OAF on Adenyl Cyclase and Phosphodiesterase Activities

OAF and PTH caused significant stimulation of adenyl cyclase activity in cell membranes from both periosteal and matrix-derived cells (Table 3). Conversely, no significant effects on overall levels of cAMP phosphodiesterase were observed in cell homogenates treated with either OAF or PTH (Table 3).

Discussion

Since the finding by Chase and Aurbach [11] that PTH caused increased accumulation of cAMP in isolated bones, there has been a general assumption that at least some of the effects of PTH on bone in vivo are mediated by cAMP. This assumption is not universally agreed on, however; other possible routes for mediation of PTH action have been proposed, notably uptake of calcium by bone cells [34, 36]. Likewise, there is little agreement about the mechanisms by which pathologic factors might mediate bone resorption, although prostaglandins also may act via a cAMP-dependent mechanism [30]. It was therefore of interest to determine whether the lymphokine OAF could modulate cAMP metabolism in skeletal tissue.

The data shown in Figures 1 and 2 demonstrate that at submaximal and maximal doses OAF stimulated cAMP accumulation in bone, with kinetics and dose dependency resembling the effects of PTH. Moreover, the doses of OAF which produced stimulation of cAMP accumulation were closely correlated with the doses found to cause stimulation of calcium release from cultured bone (Fig. 2). In order to clarify the metabolic importance of the observations in intact bone, we wished to determine which cell types in bone were responsible for the observed elevation of cAMP concentrations. As shown by numerous workers, different populations of cells in bone can differ radically in their metabolic responses to various agents. The techniques used by various workers typically result in two general types of bone cell populations: (a) those cells derived from the periosteal layers of the bone, cultures of which contain relatively high levels of lysosomal enzymes and respond to SCT as well as PTH; and (b) those cells derived from the bone matrix after prior removal of the periosteum, cultures of which contain relatively high levels of alkaline phosphatase and collagen-synthesizing enzymes, and do not respond to SCT. The former populations have been referred to as osteoclast-like cells,

whereas the latter populations are regarded as osteoblast-like [12]. We separated these populations using a variety of techniques, and found (Tables 2 and 3; Figs. 3 and 4) that both periosteal and matrix-derived cells responded to OAF with elevated cAMP levels. The cAMP responses in the cells resembled the responses of the same cells to PTH [12], with the exception that OAF produced a sharply biphasic dose-response curve in matrix-derived cells (Fig. 4), just as it did in cultured bones (Fig. 1). In contrast, the cAMP response fell off only slightly in periosteal cells at supramaximal doses of OAF. Since osteoblasts far outnumber resorptive cells in the intact bone [28], and since the osteoblast-like populations respond with about ten times as much cAMP per cell as the periosteal cells, it is reasonable to hypothesize that the biphasic cAMP response of intact bone to OAF is largely due to osteoblasts.

The effects of OAF on cAMP accumulation were apparently mediated by stimulation of adenyl cyclase in both periosteal and matrix-derived populations (Table 3), as were the effects of PTH. No significant changes in overall cAMP phosphodiesterase activity were observed in cell homogenates treated with OAF or PTH (Table 3). However, this clearly does not exclude the possibility that phosphodiesterases of different kinetic or substrate specificities could be influenced in the intact cells or in specific intracellular compartments [42]. More comprehensive studies of the effects of OAF on a number of enzymes will be necessary before we can elucidate the mechanisms by which OAF changes intracellular cAMP concentrations. In addition, it will be necessary to clarify the relationships between intracellular free cAMP and the role of this cAMP in mediation of the cellular effects of OAF. For example, at the highest doses used, OAF continued to stimulate bone resorption (albeit at a reduced level), even though cAMP levels in bone were not significantly elevated. This failure to observe stimulation of cAMP accumulation was not due to changes in the time of peak cAMP levels in bones, since the time course of cAMP response at higher doses of OAF was not significantly different from that at lower doses (unpublished data). Other workers have shown that both bone resorption and cAMP accumulation can exhibit sharply biphasic responses to effectors such as dibutyryl cyclic AMP [32, 33], theophylline [33], or the ionophore A23187 [34]. In contrast, several studies show that both bone resorption and cAMP concentrations remain elevated at doses of PTH up to two orders of magnitude above those required for maximum response [35]. The sharply biphasic dose relationships of responses to OAF, and the apparent dissociation of bone resorption from cAMP accumulation at high OAF doses, may constitute major metabolic differences between OAF and PTH. These differences could be of importance in understanding the in vivo actions of OAF, particularly in determining why pathologic bone destruction apparently fails to respond to the normal homeostatic controls over bone turnover.

Thus it seems clear that OAF can cause increased accumulation of cAMP in bone and bone cells. An important question is whether or not these data indicate that increased accumulation of cAMP is *necessary* for the effects of OAF on bone. It has been shown previously [34] that the ionophore A23187 can mimic many of the effects of PTH in bone and in isolated bone cells. In that study [34], A23187 also stimulated cAMP accumulation, although it did not do so at all doses which caused bone resorption. In fact, at high doses of A23187 there was a dissociation between cAMP accumulation and bone resorption, similar to that observed for OAF in this study. Incubation of bone cells in medium containing high calcium concentrations also has been reported to cause PTH-like effects without increased cAMP accumulation [36]. It is therefore conceivable that OAF could be acting, by mechanisms similar to A23187 and/or high calcium concentrations, to cause bone resorption independent of its ability to cause increases in cAMP accumulation. The close correlation between cAMP elevation in periosteal cells and resorption in intact bones (Fig. 1) tends to argue against this concept, but this argument can hardly be called conclusive. The resolution of this and other questions will require more extensive studies of the intracellular processes which trigger resorptive events, both for OAF and for other agents which modify bone resorption.

Lymphokines bring about a large number of responses in a wide variety of target cells. For lymphokines other than OAF, the relationship between cAMP and effects on target cells may vary considerably. For example, although cAMP itself and agents known to elevate intracellular cAMP can mimic the effects of migration inhibition factor (MIF) on macrophages, MIF itself apparently does not produce elevated cAMP levels in the same cells [37]. Lymphotoxin (LT) likewise is not known to elevate cAMP in target cells, but seems instead to stimulate calcium uptake as a trigger for the intracellular processes mediating its cytotoxic effects [38]. This could conceivably be of significance in the case of OAF, bearing in mind the similarity of the dose-response curves for OAF and the ionophore A23187 on both bone resorption and cAMP accumulation. However, it is not at all clear that all of the effects of A23187 on bone cells are due to

increased calcium uptake [34]. Moreover, other immunologic mediators such as the inhibitor of DNA synthesis (IDS) and lymphocyte activating factor (LAF) do activate adenyl cyclase in their target cells [39, 40], and it is likely that this is an integral part of the biological activities of these factors [41]. A major problem which has confused interpretations of most previous studies of the mechanisms of action of lymphokines is that either the lymphokine or the target cell preparations (and often both) have been poorly characterized. Use of purified lymphokines such as the OAF used in this study, along with target cell populations whose responses to other effectors are characterized, should improve the precision of future studies. In turn, more precise knowledge of the intracellular mechanisms which mediate lymphokine action should improve understanding of the role of these factors in both normal and pathologic processes in vivo.

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