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Nitric Oxide Regulation of cGMP Production in Osteoclasts

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Abstract Bone resorption by osteoclasts is modified by agents that affect cyclic guanosine monophosphate (cGMP), but their relative physiological roles, and what components of the process are present in osteoclasts or require accessory cells such as osteoblasts, are unclear. We studied cGMP regulation in avian osteoclasts, and in particular the roles of nitric oxide and natriuretic peptides, to clarify the mechanisms involved. C-type natriuretic peptide drives a membrane guanylate cyclase, and increased cGMP production in mixed bone cells. However, C-type natriuretic peptide did not increase cGMP in purified osteoclasts. By contrast, osteoclasts did produce cGMP in response to nitric oxide (NO) generators, sodium nitroprusside or 1-hydroxy-2-oxo-3,3-bis(3-aminoethyl)-1-triazene. These findings indicate that C-type natriuretic peptide and NO modulate cGMP in different types of bone cells. The activity of the osteoclast centers on HCl secretion that dissolves bone mineral, and both NO generators and hydrolysis-resistant cGMP analogues reduced bone degradation, while cGMP antagonists increased activity. NO synthase agonists did not affect activity, arguing against autocrine NO production. Osteoclasts express NO-activated guanylate cyclase and cGMP-dependent protein kinase (G-kinase). G-kinase reduced membrane HCl transport activity in a concentration-dependent manner, and phosphorylated a 60-kD osteoclast membrane protein, which immunoprecipitation showed is not an H^+-ATPase subunit. We conclude that cGMP is a negative regulator of osteoclast activity. cGMP is produced in response to NO made by other cells, but not in response to C-type natriuretic peptide. G-kinase modulates osteoclast membrane HCl transport via intermediate protein(s) and may mediate cGMP effects in osteoclasts. J. Cell. Biochem. 73:478–487, 1999.

Key words: guanylate cyclase; V-type ATPase; acid transport; cGMP-dependent protein kinase

The osteoclast is a multinucleated macrophage-monocyte-derived cell that is specialized to secrete HCl to degrade bone. Bone degradation is regulated by changes in osteoclast number and changes in osteoclastic activity, which depend on complex signals involving several cytokines, cell attachment, and multiple intracellular pathways [reviewed by Suda et al., 1997]. Several reports indicate that stimuli that affect cyclic guanosine monophosphate (cGMP) regulate osteoclastic activity. Kasten et al. [1994] showed that inhibition of nitric oxide-synthase (NO-synthase) potentiates osteoclastic activity. Because NO stimulates a cytosolic guanylate cyclase to produce cGMP, this suggested that cGMP is a downregulatory signal in osteoclasts. Our previous work showed that osteoclasts express a cGMP-dependent protein kinase that may function to transduce such cGMP signals [Van Epps-Fung et al., 1994].

However, other reports are not consistent with a simple model of osteoclast response to NO. MacIntyre et al. [1991] had reported NO effects on osteoclasts that appeared to be independent of cGMP. Further, studies of bone marrow explants by Holliday et al. [1995] showed that C-type natriuretic protein, which activates a membrane-receptor guanylate cyclase to synthesize cGMP, increases the number of active osteoclasts. By contrast, osteoblasts make factors that modify osteoclasts and this cell type is known to respond to C-type natriuretic factor [Nashida et al., 1996; Suda et al., 1996]. Later work (Holliday et al. 1997) indicates that low concentrations of NO reduce osteoclast forma-

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tion by a cGMP-dependent mechanism. By contrast, van't Hof and Ralston [1997] suggested that NO causes apoptosis of osteoclast precursors, reducing osteoclastic activity at the level of cellular differentiation. Mancini et al. [1997] suggested that low levels of NO increased osteoclast formation, while high levels reduced cellular activity. Thus, it is unclear to what extent osteoclastic response to pathways dependent on cGMP reflect cell formation or cell survival, and whether the primary responding cell is the osteoblast, osteoclast, or precursor cells.

In addition, interleukins-8 and -10 (IL-8, IL-10) were reported to stimulate avian osteoclasts to express inducible NO-synthase, suggesting autocrine NO-dependent regulation of osteoclasts [Sunyer et al., 1996]. However, Helfrich et al. [1997] found NO synthase in osteoblasts and macrophages, but not in osteoclasts, calling into question the physiological importance of this pathway. Mancini et al. [1998] suggested that the major effects of NO on osteoclasts involve cGMP but may also involve other pathways, including peroxynitrite-mediated osteoclast detachment. These various reports suggested that different NO or cGMP-dependent pathways affect osteoclastic activity, by autocrine or paracrine mechanisms, in potentially opposing directions.

In this work, the objective was to clarify the role of cGMP in regulating mature osteoclasts. We compared the response of mixed bone cells and purified osteoclasts with the two main types of guanylate cyclase agonists: C-type natriuretic peptide and NO. Production of cGMP was determined in the presence of phosphodiesterase inhibitors to evaluate whether significant autocrine synthesis occurred, and effects of agonists on cellular activity were correlated with effects on cGMP synthesis. In addition, previous studies indicating the presence of the cGMP-dependent protein kinase (G-kinase) in avian osteoclasts were followed up to determine whether osteoclastic acid secretion is directly modulated by the G-kinase.

MATERIALS AND METHODS

Cell Cultures

Isolation of osteoclast-rich cell fractions from chicken bone is detailed elsewhere [Blair et al., 1986; Williams et al., 1996a]. Briefly, medullary bone from *Gallus domesticus*, white leghorn hens, on a Ca^{2+}-restricted diet, was harvested and cells were removed by vigorous washing and sieved through 110-μm nylon mesh; erythrocytes were lysed in hypotonic media and cells sedimented through 70% serum to recover bone cells. Adherent cells obtained are 50–80% osteoclasts (these mixed cell cultures were used only in comparisons of mixed cell cultures and pure osteoclasts). For work requiring pure osteoclasts, essentially homogeneous osteoclasts were selected by binding to 40–80 μm bone particles. Cells were maintained in Dulbecco's modified Eagle's medium (DMEM) at 37°C in humidified air with 5% CO_2. Media contained streptomycin and penicillin, 100 μg/ml, and 10% heat-inactivated serum (5% chicken, 5% newborn bovine), except during 1- to 2-h preincubation and assays for cGMP production, where 1% serum (preincubation) or no serum (assays) were used to minimize serum artifacts.

Analysis of cGMP and Cell Protein

cGMP was determined by radioimmunoassay (RIA) as described [Harper and Brooker, 1975]. Cyclic nucleotide extracts were collected from cell cultures with treatments as indicated by adding 750 μl of 0.1 M HCl in 50% methanol per 10 cm^2 well. After incubating 5 min on ice, supernatants were lyophilized and frozen at −80°C until analyzed. Proteins were determined by dye binding [Bradford, 1976] on cellular material remaining after aspiration of media for cGMP determination.

Bone Degradation

Radiolabeled substrate was used to compare degradation rates. A total of 100 μg of 40- to 80-μm bone pieces, ~60 dpm [^{3}H]proline/μg bone, were added to $\sim 3 \times 10^3$ osteoclasts in 2-cm^2 tissue culture wells, after Blair et al. [1986], but using larger bone pieces to preclude possible effects due to substrate internalization; osteoclasts attached to the substrate are demonstrated by Williams et al. [1996a]. Assays were less than 7 days to avoid the effects of pre-osteoclast differentiation [Williams et al., 1996b] in mixed cultures. Effect of macrophages in mixed cultures was assessed using 2.5×10^4 macrophages and the same substrate; results were typically indistinguishable from no cell blanks, and never more than 10% of the degradation by control osteoclasts. Untreated control osteoclasts degraded 10–20% of 100 μg total substrate during the assay periods reported. Comparisons with pit assays are shown by Carano et al. [1993]. Unless stated, results

are averages (less no cell blanks) of four determinations, and ranges are standard deviations. Substrate was L-[2,3,4,5-^{3}H]proline rat bone, labeled in vivo [Blair et al., 1986]. Substrate specific activity was determined after hydrolysis in 6 N HCl, 110°C, 18 h.

Cell Fractions and Phosphorylation Reactions

Membrane isolation was after Blair et al. [1991]. Briefly, osteoclasts were fragmented by nitrogen cavitation and membranes were recovered by sequential centrifugation. The proton pump was isolated by immunoprecipitation using monoclonal antibody to H^+-ATPase, the gift of Michael Forgac, Tufts University, Boston, MA [Mattson et al., 1994]. Polyclonal antibody to H^+ATPase was the gift of Dennis Stone, Southwestern University, Dallas, TX. Antibody to the 70-kD subunit of H^+-ATPase was provided by Jan Mattson (Astra Hassle AB, Molndal, Sweden). Phosphorylation assays contained 50 µg of membrane fraction target proteins in 10 mM $MgCl_2$ and 10 mM HEPES, pH 7.4, in 50-µl test mixtures, with [γ-^{32}P]-ATP, 2 µCi/reaction, adjusted to 100 µM with unlabeled ATP, to label products. Purified cGMP-dependent protein kinase was the gift of T. Lincoln, University of Alabama [Lincoln et al., 1988]. cGMP-dependent protein kinase was added after dilution to 10 µg/ml in 1% bovine serum albumin (BSA) at 4°C, from 1 mg/ml stock in 30% glycerol at −20°C, just before use. In phosphatase reversal reactions, phosphatase was added after indicated times, but kinase was not stopped. Phosphorylation was assessed by autoradiography after separation of proteins on 7% polyacrylamide gels in 2% sodium dodecyl sulfate (SDS) in Laemmli buffers.

Membrane Acid Transport

Mg^{2+}-ATP-dependent HCl transport was determined using membranes isolated from purified osteoclast by nitrogen cavitation and differential centrifugation [Blair et al., 1991]. Membrane vesicles for transport studies were produced by allowing membrane fragments from cavitation to reconstitute into vesicles in 120 mM KCl, 20 mM NaCl, 10 mM HEPES, pH 7.4, at 1.0 mg/ml protein by incubation 30 min at 4°C. These vesicles are randomly assorted inside-out and outside-out; the ATPase assay only measures the inside-out fraction, the remaining vesicles being inert. Acid transport in vesicles was determined by acridine orange (3 µM) uptake by fluorescence spectrophotometry; averaged emission was recorded at 540 nm at 5-s intervals with 468-nm excitation. Reactions used 50-µg vesicle protein in 2.5 ml of 1 mM ATP, 3 µM acridine orange, in reconstitution buffer, at 37°C; transport was initiated with 2 mM $MgCl_2$. Washout with 1 mM NH_4Cl at the end of each reaction was used to control for nonspecific effects.

RESULTS

Production of cGMP in Marrow Cells and Osteoclasts in Response to Natriuretic Peptides

Osteoclast cGMP production was determined by RIA. Effect of C-type natriuretic peptide was expected, while atrial natriuretic peptide was run as a control. Surprisingly, in mixed avian bone cells, there was a response to both atrial natriuretic peptide (Fig. 1A) and C-type natriuretic peptide; measurable cGMP in either case required the phosphodiesterase inhibitor isobutylmethylxanthine. Response was near-maximal at 10 min; 20 min was used in further analysis. Response to C-type natriuretic peptide required ~0.1 µM peptide (Fig. 1B), higher by 10-fold the expected concentration; at >1 µM, no additional effect was seen (Fig. 1C). We hypothesized that the relative insensitivity of concentration response was attributable to the mixed cell preparation, with a minority of the cells reacting to the peptide, so that the observed effect might reflect secondary response by receptor-negative cells. When cGMP production by bone affinity-purified osteoclasts was compared (Fig. 1D), there was essentially no accumulation of cGMP in osteoclasts in response to A- or C-type natriuretic peptide. This finding suggested that osteoclasts do not respond directly to these peptides, and lack of signal in the presence of 300 µM isobutylmethylxanthine suggested that basal osteoclast cGMP syntheses is quite low.

Production of cGMP in Osteoclasts in Response to NO

The other major pathway for cGMP response is an NO-activated soluble guanylate cyclase. Whether this pathway regulated osteoclastic cGMP was studied by adding NO generators, sodium nitroprusside (100 µM), or 1-hydroxy-2-oxo-3,3-bis(3-aminoethyl)-1-triazene (100 µM), to purified osteoclast cultures and measuring cGMP (Fig. 2A, B). cGMP response to the NO

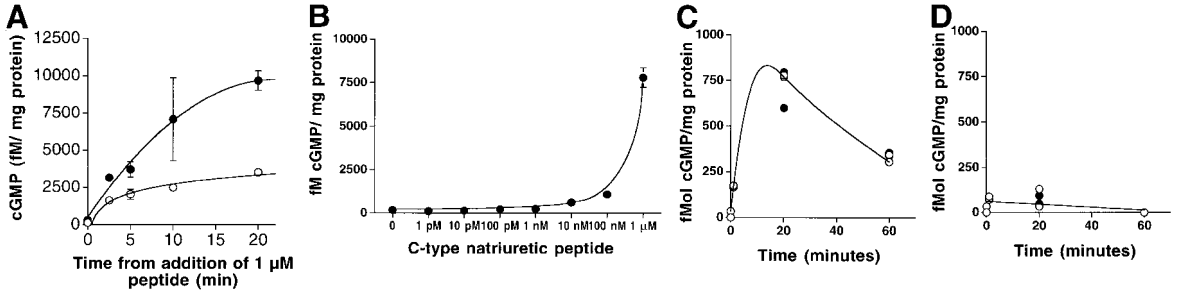


Fig. 1. cGMP synthesis by bone cells (50–80% osteoclasts) and essentially homogeneous osteoclasts in response to A- and C-natriuretic peptides. All determinations included the phosphodiesterase inhibitor isobutylmethylxanthine, 300 μM. **A:** Response of avian bone cells, 50–80% osteoclasts, to saturating (10 μM) atrial (open symbols, lower trace) and C-type (closed symbols, upper trace) natriuretic peptides. Responses plateaued beyond 10 min. **B:** Avian bone cell (50–80% osteoclasts) concentration response for C-type natriuretic peptide. Maximal response occurred at 0.1–1 μM. **C:** Time course of cGMP response of bone cells (50–80% osteoclasts) to C-type natriuretic peptide at high concentrations (10 μM, closed symbols; 1 μM, open symbols). The concentrations give the same effect, showing that these are saturating concentrations; decline in response was observed at periods of >20 min. **D:** cGMP was not detected in purified mature avian osteoclasts (essentially homogeneous, purified by bone-affinity) in response to 10 μM C-type natriuretic peptide (10 μM, closed symbols; 1 μM, open symbols). A–B, duplicate determinations with range; C–D, averages of duplicate determinations.

generators that peaked in ~15 min; levels from 15 min to 2 h remained relatively constant with 1-hydroxy-2-oxo-3,3-bis(3-aminoethyl)-1-triazene, which releases NO with a half-life of ~2 h. Adding 0.5–10 mM L-arginine, which would provide excess substrate for endogenous NO synthase, had no effect on cGMP synthesis at 1 h, or on bone degradation at 3 days, suggesting that the NO system does not have a major autocrine component under conditions tested (not illustrated). That the NO, but not natriuretic peptide, response co-isolated with osteoclasts was confirmed by assaying the cGMP response to 1-hydroxy-2-oxo-3,3-bis(3-aminoethyl)-1-triazene (100 μM) and C-type natriuretic peptide (1 μM) both before and after osteoclast separation. The peptide drove high cGMP accumulation only in the mixed cells, while purified osteoclasts retained a large portion of the response to NO (Fig. 2C).

Effects of NO on Osteoclastic Activity

To determine how the activity of osteoclasts was affected by NO and the reversibility of effects, isolated osteoclasts were exposed to NO generators, which decompose in water to release NO. Degradation of bone was measured and recovery on washout determined. Experiments using mixed bone cell preparations gave variable results (not illustrated; see discussion). However, the NO generators (±)-S-nitroso-N-acetylpenacillamine (Fig. 3A) or 1-hydroxy-2-oxo-3,3-bis(3-aminoethyl)-1-triazene (Fig. 3B) reduced the activity of purified osteoclast preparations with half-maximal effects at 10 and 0.1 μM, respectively, in keeping with the half-lives of these NO donors. At half-inhibitory concentrations, measurable NO effects occurred by 3 days. On washout, partially inhibited cells showed activity exceeding controls, but this difference was not statistically meaningful (Fig. 3C). At high NO donor concentrations, bone degradation was negligible after 2 days, washout showed reduced recovery, and numbers of osteoclasts were reduced, suggesting that high NO causes cell death (see Discussion).

Replication of NO Effects by cGMP, and Activity of NO Synthase in Osteoclasts

cGMP analogues and NO-synthase inhibitors were used to clarify the mechanisms mediating the effects of NO. The long-acting cGMP analogue 8-(4-chlorophenylthio)guanosine 3′:5′-cyclic monophosphate reduced osteoclastic activity with ~50% effect at 100 μM (Fig. 4A). By contrast, when the inhibitory cGMP analog (Rp)-8-bromoguanosine-3′:5′-cyclic monophosphorothioate was substituted, osteoclastic activity was increased ~60% relative to controls (Fig. 4B). This effect was seen at the lowest concentration tested, 10 μM; effects were significant at times >3 days. Whether an autocrine NO-generating pathway contributes to regulation of bone-attached isolated osteoclasts was tested by adding the NO synthase inhibitors N-methyl-(L)-arginine, 1 mM, or 0.1–10 mM aminoguanidine. There were no viable cells after treatment with N-methylarginine, so effects were re-

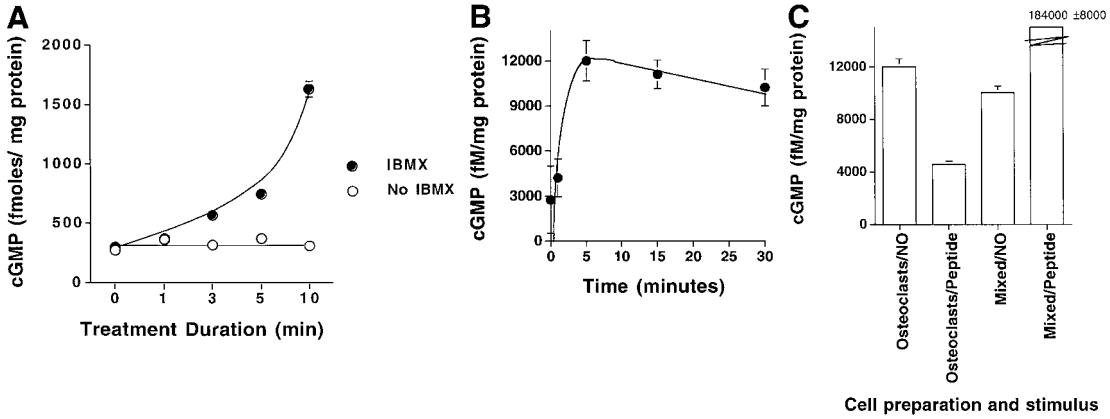


Fig. 2. Rate of cGMP production, by essentially homogeneous avian osteoclasts prepared by affinity to bone particles, in response to different NO donors. cGMP was measured by radioimmunoassay in the presence of 300 µM isobutylmethylxanthine except as indicated. NO donors were added at t = 0. **A:** NO stimulates rapid cGMP production in purified osteoclasts, but at levels detectable only when phosphodiesterase activity is inhibited. Sodium nitroprusside, 100 µM, was added at time 0. The upper trace is the assay in isobutylmethylxanthine; the lower trace is the assay omitting isobutylmethylxanthine. **B:** cGMP production peaks within ~10 min but is maintained. Purified osteoclasts were treated with 100 µM 1-hydroxy-2-oxo-3,3-bis(3-aminoethyl)-1-triazene at time 0. cGMP response peaked rapidly and was then maintained. Measurements at 1 and 2 h were similar to 30 min but are excluded to show detail of the rapid rise. **C:** The NO-response segregates with purified osteoclasts. In preparations from a single animal, responses to 100 µM 1-hydroxy-2-oxo-3,3-bis(3-aminoethyl)-1-triazene and 1-µM C-type natriuretic peptide at 5-min incubation were compared. The natriuretic peptide produced very high cGMP levels, but only in the mixed marrow cells (right two bars). The NO response is similar in mixed marrow cells and purified osteoclasts (first and third bars), but only minor peptide stimulus is present in purified osteoclasts (second bar). A–C: n = 2, mean ± range.

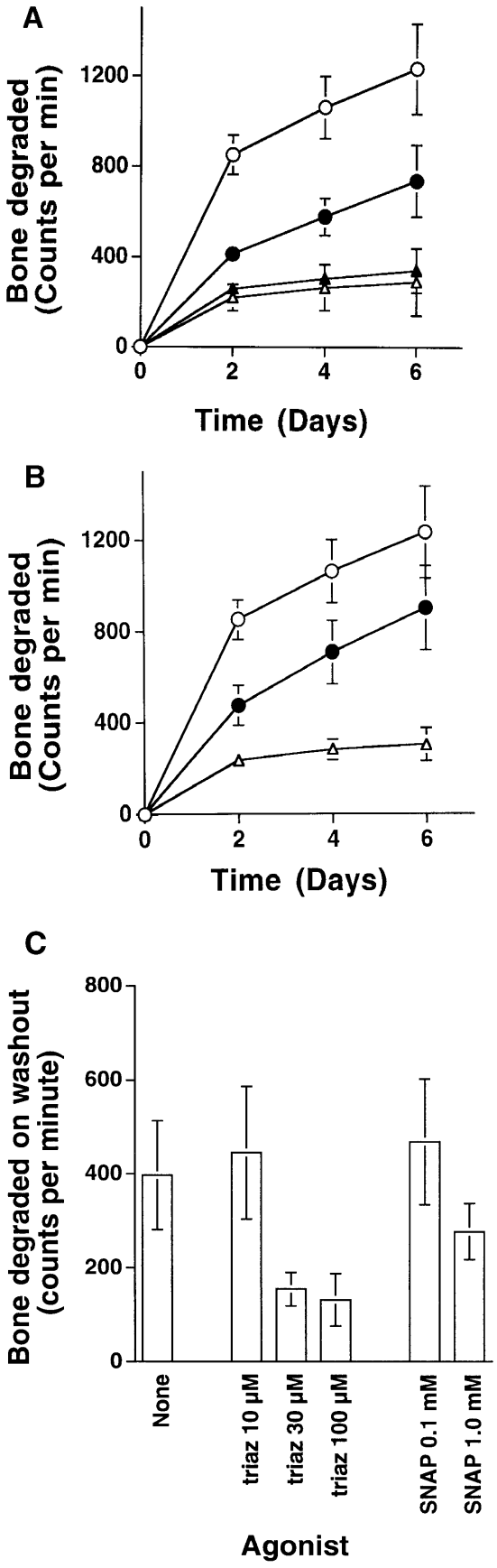
garded as uninterpretable because of toxicity (not illustrated). Aminoguanidine did not affect bone resorption at 3 days at concentrations to 10 mM (Fig. 4C). These findings indicate that NO acts on osteoclasts mainly as a paracrine factor with a cGMP intermediate.

Concentration-Dependent Inhibition of Acid Secretion by cGMP-Dependent Protein Kinase

cGMP effects are often mediated by cGMP-dependent protein kinase (G-kinase), which is present in avian osteoclasts and localizes to the osteoclast-bone attachment site [Van Epps-Fung et al., 1994]. Membrane preparations were unaffected by cGMP in the absence of added kinase, indicating that kinase is not bound to membrane structures. However, the cGMP-dependent protein kinase inhibited acid transport in isolated membrane vesicles in a kinase activity-dependent manner with 50% inhibition by 2 nM G-kinase in 1 µM cGMP (Fig. 5A). Different preparations of vesicles and enzyme gave similar patterns of concentration dependence, although half-maximal response varied from ~1–3 nM in different preparations, presumably reflecting specific activity of membranes and enzyme. Washout data from bone resorption assays suggested that cGMP-dependent osteoclast inhibition is reversible (Fig. 3C), so G-kinase effects were expected to be reversible. Surprisingly, alkaline phosphatase (ALP) did not reverse the G-kinase effect, however (see Discussion).

G-Kinase Phosphorylation of Membrane Proteins

Results of acid transport assays suggested that the cGMP-dependent protein kinase targets membrane proteins. This was studied by [γ-^{32}P]-ATP labeling membrane proteins with and without added G-kinase. There was endogenous phosphorylation of several proteins, but a major protein at M_r ~60 kD was specifically labeled by the G-kinase (Fig. 6A). Phosphorylation was not affected by 0.5 µM okadaic acid, a phosphatase I–IIa inhibitor, in keeping with the persistence of the cGMP-dependent protein kinase effect on these membrane fractions. Heating membrane fractions 10 min at 60°C abolished endogenous kinase activity, and simplified the labeling pattern. In some assays, proteins of ~20, 30, 120, and 200 kD also labeled weakly with the G-kinase. However, it was clear that G-kinase reaction with membrane proteins involved few proteins, and possibly one main protein. An important ~60-kD membrane protein is the B-subunit of the H^+-



ATPase. However, precipitation of the V-type H^+-ATPase after G-kinase phosphorylation showed that ATPase subunits are not phosphorylated (Fig. 6B). Thus, while membrane acid transport is inhibited by the G-kinase, the acid-secreting membrane H^+-ATPase is not a direct target of the G-kinase.

DISCUSSION

Several studies have indicated that bone degradation is modified by NO or cGMP. However, a variety of effects are reported that are difficult to fit into a logical sequence. Our purpose was to resolve questions regarding which NO and cGMP-dependent pathways function in osteoclasts. MacIntyre et al. [1991] reported that osteoclast activity, as determined by spread area and bone resorption, is downregulated by NO donors, which commonly stimulate a guanylate cyclase, but that cGMP was probably not the mediator, based on lack of response to the hydrolysis-resistant agonist 8-bromoguanosine cyclic-3′:5′-monophosphate. The observation of NO inhibition was confirmed by Kasten et al. [1994], who found that inhibiting NO synthase activity increased osteoclastic bone resorption. Our observations are in keeping with these reports, in that we find NO donors to inhibit osteoclastic activity. However, our results all indicate that this response is mediated by cGMP. We measured cGMP directly in response NO donors, and compatible effects on cellular activity were produced by hydrolysis-resistant cGMP analogues (Figs. 2, 4).

Fig. 3. Effect of NO generators on osteoclastic activity. **A:** Effect of the NO generator (±)-S-nitroso-N-acetylpenacillamine (SNAP), added at 0, 2, and 4 days. Vehicle control is the top trace (open circles); closed circles, open triangles, and closed triangles included 10, 30, and 100 µM of the test substance, respectively. **B:** Effect of the NO generator 1-hydroxy-2-oxo-3,3-bis(3-aminoethyl)-1-triazene. Vehicle control is the top trace (open circles); closed circles and open triangles included 0.1 and 1.0 µM of the NO donor, respectively. **C:** Recovery of activity after washout of (±)-S-nitroso-N-acetylpenacillamine (SNAP) or 1-hydroxy-2-oxo-3,3-bis(3-aminoethyl)-1-triazene (triaz) with plain medium at 6 days, assayed on day 10. After washout of half-inhibitory concentrations, activity exceeded controls but differences were not statistically meaningful; recovery was not seen at high NO-donor concentrations; these cultures had reduced numbers of bone-attached osteoclasts at day 10 (essentially none in 30–100 µM 1-hydroxy-2-oxo-3,3-bis(3-aminoethyl)-1-triazene), indicating that the high NO-donor concentrations caused cell death. A–C: Mean ±SD, n = 4; activity of substrate was ~60 cpm/µg.

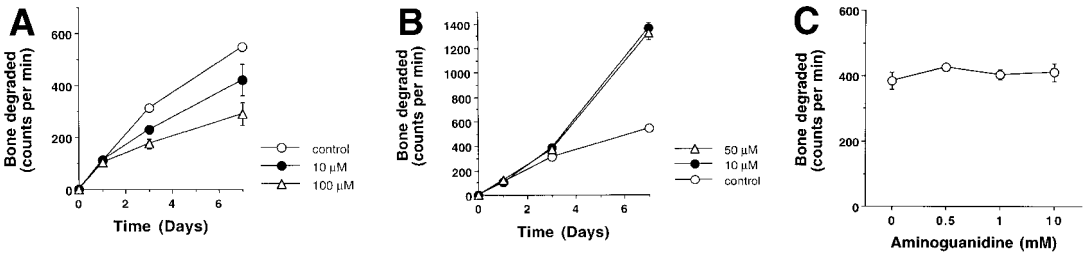


Fig. 4. Effect of cGMP analogues and NO-synthase inhibitors on osteoclastic activity. **A:** Effect of the hydrolysis-resistant cGMP agonist 8-(4-chlorophenylthio)guanosine-3′:5′-cyclic monophosphate. Controls, open circles (top trace); 10 and 100 μM compound, closed circles and triangles. **B:** Effect of the cGMP-dependent protein kinase antagonist (Rp)-8-bromoguanosine-3′:5′-cyclic monophosphorothioate. Controls, open circles (bottom trace); 10- and 50-μM compound, closed circles and triangles. **C:** Effect of aminoguanidine, an NO-synthesis inhibitor, measured over 5 days. Differences are not significant. A–C: Mean ±SEM, n = 4.

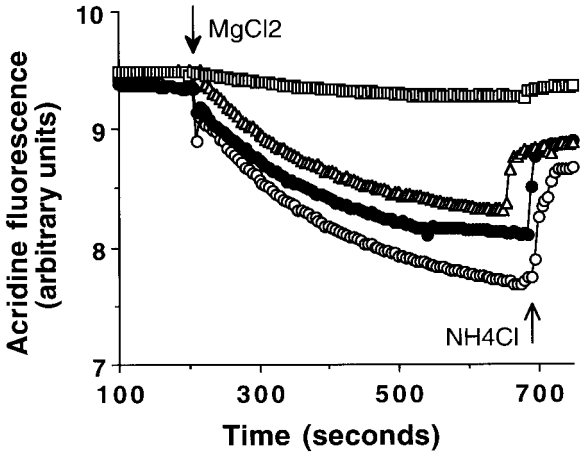


Fig. 5. Effect of cGMP-dependent protein kinase (G-kinase) on osteoclast membrane acid transport. ATP-dependent H^+-transport was measured by acridine uptake in reconstituted membrane vesicles, made from purified osteoclasts by nitrogen cavitation and differential centrifugation (see Materials and Methods). Open circles indicate control activity (bottom trace); closed circles, triangles, and squares (top trace) indicate activity after incubation with 600 pM, 2.4 nM, and 7.5 nM G-kinase, respectively. Transport was initiated by adding $MgCl_2$ (left arrow) and reversed by addition of 1 mM NH_4Cl (right arrow). Representative results from several experiments are shown.

By contrast, Holliday et al. [1995] showed that C-type natriuretic peptide, which activates a membrane guanylate cyclase, increases bone resorption in mouse marrow explants. We confirmed an impressive response to C-type natriuretic peptide in chicken marrow cell isolates, but found that this response is due to other cells than osteoclasts (Figs. 1, 2C). These results are consistent with recent work on osteoblast C-type natriuretic response [Nashida et al., 1996; Suda et al., 1996]. Other than the reliable production of cGMP in response to C-type natriuretic peptide in mixed bone cells, we found that mixed cell preparations gave variable responses to cGMP analogues. This probably reflects that multiple cell types including osteoblasts and osteoclasts respond to increases in cGMP and are therefore likely interact in unpredictable ways in uncontrolled mixed cultures.

There is direct evidence for production of NO by stimulated osteoblasts [Hukkanen et al., 1995; Sunyer et al., 1996]. Brandi et al. [1995] also reported NO-synthase in osteoclasts. However, in isolated cells we were unable to affect cells with high doses of the NO-synthase inhibitor aminoguanidine (Fig. 4C). This finding is in contrast to the data reported by Kasten et al. [1994]. Our data on the influence of cell purity (Fig. 2C) suggest that the difference is related to the degree of homogeneity of the cell preparations used. In keeping with this, Helfrich et al. [1997] reported that NO-synthase is present in osteoblasts and macrophages but not osteoclasts. Thus, while it is possible that inflammatory factor-mediated autocrine NO production occurs in avian osteoclasts [Sunyer et al., 1996], this pathway is probably not important physiologically under typical conditions. On the other hand, in osteomyelitis or other inflammatory conditions in which the factors required to upregulate inducible NO-synthase would be present, this could become an important mechanism that limits regional bone loss. In later work, Sunyer et al. [1997] found that in mature osteoclasts NO did not appear to be produced by cytokine action, although a calcium response was noted. The osteoclasts on bone used in the present study have very stable cytoplasmic calcium (data not shown), and this possibility is not addressed in the present work. van't Hof and Ralston [1997] proposed a model of NO-dependent apoptosis of osteoclast precursors. Our study did not address this issue directly, although our results are consistent with a bipha-

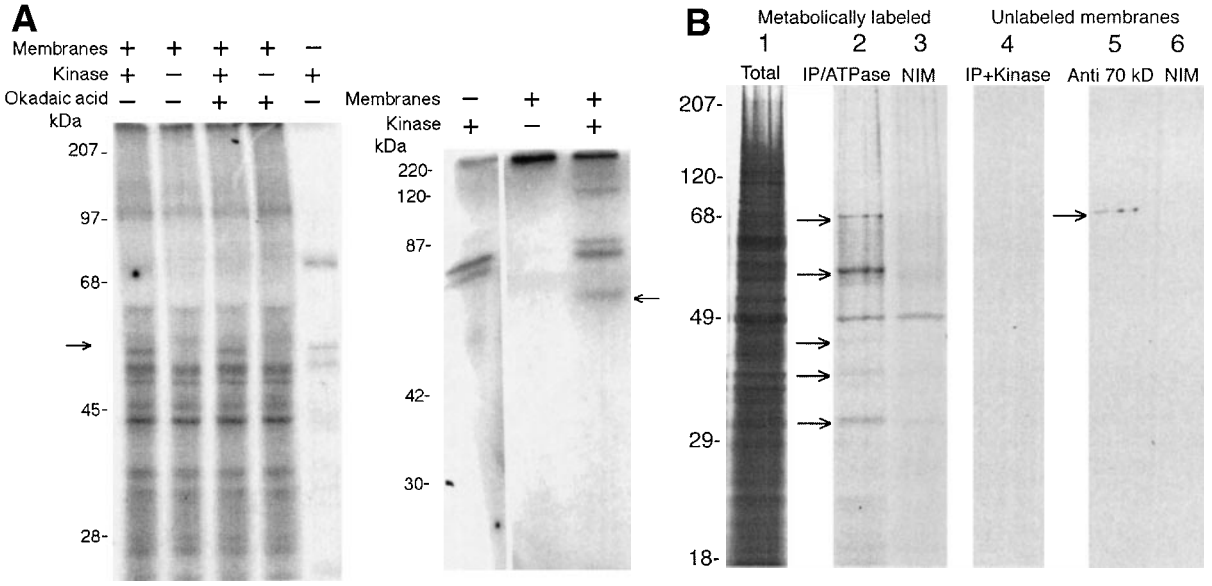


Fig. 6. Osteoclast membrane phosphorylation by G-kinase. **A:** Membrane protein phosphorylation. Left gel shows phosphorylation of G-kinase and membrane vesicles with and without added kinase and okadaic acid, 0.5 μM. Membranes were incubated with purified G-kinase, 2 nM, 0.1 mM [γ-^{32}P]-ATP, and 10 μM cGMP for 2 min at 4°C. An ~60-kD protein is labeled by the kinase; a number of autophosphorylated proteins and the G-kinase itself also label. Right gel shows similar reactions using membranes pretreated by heating 10 min to 60°C, to eliminate effects of endogenous kinases. A protein at ~60 kD is still the main labeled product. Autophosphorylated G-kinase is seen, but membrane autophosphorylation is effectively eliminated. **B:** H^+ ATPase from osteoclast membranes was not phosphorylated by G-kinase. Osteoclasts were labeled with ^{35}S methionine and cell membranes isolated (**lane 1,** total cell membrane label). Then the H^+-ATPase was isolated by immunoprecipitation (**lanes 2–3,** NIM, nonimmune control). Precipitated proteins include the major catalytic and regulatory A and B subunits, ~60 and ~70 kD, as well as 30- to 50-kD subunits (arrows). Similar but nonmetabolically labeled membrane fractions were phosphorylated with G-kinase and [γ-^{32}P]-ATP and immunoprecipitated; however, H^+-ATPase subunits are not phosphorylated (**lane 4**). Multiple determinations including very long exposures gave the same result. Western blot analysis for the 70-kD subunit (arrow, **lane 5; lane 6,** the nonimmune control, NIM) confirmed that the immunoprecipitate contained ATPase subunits.

sic response to NO. NO donors reversibly reduced osteoclastic activity at moderate concentrations, but cell death occurred at high NO levels (Fig. 3C).

Our observations indicate that short-term downregulation of osteoclastic activity occurs by an NO/cGMP pathway. These findings are in keeping with the results reported by Kasten et al. [1994]. Kinases are the common mediators of cGMP activity, via 78-kD (type I) and 86-kD (type II) cGMP-dependent protein kinases. The type I kinase is found in many tissues, including osteoclasts [Van Epps-Fung et al., 1994]. Thus, cGMP effects are probably mediated, at least in part, by cGMP-dependent protein kinase. The 78-kD type I cGMP-dependent protein kinase functions as a dimer, interacting with many target proteins including membrane channels [Lincoln et al., 1995]. Membrane acid-transport assays showed that G-kinase does directly modify cell membrane acid secretion (Fig. 5). After inactivation of membrane vesicles by G-kinase, treatment with ALP did not recover activity, although ALP does increase membrane acid transport under some conditions [Schlesinger et al., 1994]. This is in keeping with a relatively complex mechanism, with the G-kinase modifying an intermediate protein in a manner, such as dissociation of a regulatory complex, where simple dephosphorylation would not reverse the reaction. In keeping with a complex regulatory mechanism, precipitation of the H^+-ATPase complex after G-kinase labeling showed that the hydrogen pump is not a direct target of the kinase. After partial G-kinase inhibition, some increase in activity occurred when membranes were treated with the calmodulin-dependent phosphatase calcineurin (data not shown). However, calmodulin is required for acid secretion [Radding et al., 1994] and, because the nonspecific ALP did not affect G-kinase-treated vesicles, it is likely that the calmodulin-dependent phosphatase modified vesicle transport by an unrelated mechanism.

We conclude that there is no direct action of natriuretic peptides on osteoclasts. By contrast, nitric oxide causes cGMP synthesis in osteoclasts and inhibits bone degradation. NO effects were replicated by cGMP analogues. Stimuli of NO-synthase had no effect, suggesting that autocrine NO synthesis does not function in normal avian osteoclasts, although it is possible that NO-synthase may be induced by additional stimuli. The type I cGMP-dependent protein kinase is expressed in osteoclasts and regulates a membrane protein that inactivates membrane H^+-ATPase activity in vitro. These findings are consistent with an intermediate role of this kinase in the NO/cGMP-dependent regulation of acid secretion, although the kinase effects on acid secretion are indirect; hence, other unidentified downstream elements must also be involved.

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