Journal of Cellular Biochemistry 64:161–170 (1997)

Multiple G-Protein Involvement in Parathyroid Hormone Regulation of Acid Production by Osteoclasts

Lisa G. May[1] and Carol V. Gay[1,2]*

[1]Department of Biochemistry and Molecular Biology, The Pennsylvania State University, University Park, Pennsylvania 16802
[2]Department of Poultry Science, The Pennsylvania State University, University Park, Pennsylvania 16802

Abstract The involvement of multiple G-proteins in parathyroid hormone regulation of acid production was demonstrated in a highly enriched osteoclast population. Osteoclasts were isolated from the endosteum of 2.5 to 3-week-old chicken tibia using sequential enzymatic digestion. Single cell analysis of acid production was accomplished using microscope photometry and vital staining with acridine orange, a hydrogen ion concentration sensitive fluorescent dye. Lithium chloride, an uncoupler of G-proteins from their respective receptors, blocked parathyroid hormone stimulated production of acid. Cholera toxin, which permanently activates G_s-proteins, mimicked PTH stimulation. Pertussis toxin, which prevents receptor interaction with G_i- and G_o-proteins, blocked both 10^{-8} M and 10^{-11} M PTH stimulated acid production, suggesting that the pertussis toxin-sensitive G-protein is utilized at both PTH concentrations. Immunoblots of osteoclast plasma membrane proteins, using a panel of antibodies generated against specific G-protein α subunits, revealed a 48 kDa $G_s\alpha$, a 41 kDa $G_o\alpha$, a 34 kDa $G_i\alpha$-3, and a unique 68 kDa $G\alpha$ subunit, with the 41 kDa and 34 kDa bands being the most intense. Immunoblots of osteoblast plasma membrane proteins had a substantially different profile with the most intense bands being a $G_s\alpha$ (48 kDa) and a $G_o\alpha$ (36 and 38 kDa). The studies suggest the utilization of at least two different G-proteins in the parathyroid hormone regulation of acid formation by osteoclasts, a G_s and a pertussis toxin-sensitive G-protein (G_o and/or $G_i\alpha$-3). J. Cell. Biochem. 64:161–170.

Key words: calcium-regulating hormone; bone cells; acridine orange; signal transduction; GTP-binding protein

The major role of the osteoclast is to resorb bone, a process long known to be stimulated by parathyroid hormone (PTH). Following the initial observations of Wong and Cohen [1974], it has become well established that bone forming osteoblasts serve as an intermediary in the regulation of osteoclast resorptive activity. The signalling mechanism in osteoblasts utilized by PTH is quite well understood, as described in recent reviews by Simmons and Grynpas [1990] and Partridge et al. [1994].

A small body of literature indicates that osteoclasts are also directly influenced by PTH. The direct association of PTH with bone resorbing osteoclasts was first reported in an immunocytochemical study by Rao and colleagues [1983]. Subsequently, specific binding of both radiolabelled PTH and biotinylated PTH to osteoclasts has been shown [Teti et al., 1991; Agarwala and Gay, 1992]. Very recently, a burst in superoxide anion production following direct binding of PTH to osteoclasts has been reported; osteoblasts had no role in the process [Datta et al., 1996]. The present study is the first to evaluate the signalling mechanisms utilized when PTH binds to receptors on osteoclasts. We show that PTH utilizes at least two different G-proteins to stimulate acidification. One of these is a G_s-protein, whereas the other one is a pertussis toxin-sensitive G-protein and could be either a G_o-protein or a G_i-protein.

MATERIALS AND METHODS

Materials

Synthetic salmon calcitonin, heat-inactivated calf serum (CS), heat-inactivated fetal bovine

Portions of this work were presented at the Fourteenth and Fifteenth Annual Meetings of the American Society for Bone and Mineral Research in September, 1992 and 1993, and at the fifth International Conference on Avian Endocrinology in September, 1992.

Contract grant sponsor: NIH; Contract grant numbers: DE04345, DE09459.

*Correspondence to: Dr. Carol V. Gay, Professor of Cell Biology, Department of Biochemistry and Molecular Biology, 468A N. Frear Building, The Pennsylvania State University, University Park, PA 16802. E-mail: cVG1@OAS.PSU.EDU

Received 28 May 1996; Accepted 25 July 1996

serum (FBS), minimum essential medium (MEM, Eagles modification with non-essential amino acids and Earle's salts), L-glutamine, type 1-S hyaluronidase, type 1-A collagenase, Nα-p-tosyl-L-lysine-chloromethylketone (TLCK), lithium chloride, cholera toxin, pertussis toxin, Percoll, Trizma base, molecular weight markers (low range), Tyrodes buffer, and acid phosphatase staining kit were obtained from Sigma Chemical Co. (St. Louis, MO). MEM was further modified by addition of 26 mM L-glutamine, 2 mM sodium bicarbonate, and additional sodium chloride, potassium chloride, and calcium chloride (~0.8 g/L, 0.05 g/L, and 0.03 g/L, respectively) to adjust tonicity to 310 mOsm in order to match the tonicity of 3-week-old chicken plasma. The culture medium also contained 10 ml/L 10,000 U penicillin − 10 mg streptomycin (Sigma Chemical Co.). Phenol red was avoided in the medium due to its mild estrogenic properties. Acrylamide, bisacrylamide, TEMED, and ammonium persulfate were purchased from Bio-Rad (Hercules, CA). Trypsin (bovine pancreatic, 3× crystallized) was from Worthington Biochemicals (Freehold, NJ); human fibronectin from Biomedical Technologies (Stoughton, MA); acridine orange from Polysciences, Inc. (Warrington, PA). Bovine PTH (biopotency of 1300 USP units/mg) was provided by the National Hormone and Pituitary Program of the National Institute of Arthritis, Diabetes and Digestive and Kidney Diseases.

Osteoclast Isolation

Osteoclasts were isolated from the endosteal surface of chicken tibias using a combination of earlier methods [Gay et al., 1983; Hunter et al., 1988; Gay et al., 1993]. Osteoclasts were obtained from the tibias of 18 2.5- to 3-week-old male chickens (Peterson × Arbor Acre) maintained on normal chick starter diet. The chickens were injected subcutaneously with salmon calcitonin (30 mU/100 g body weight), in order to enhance release of osteoclasts from the endosteum. Chickens were decapitated 30 min after calcitonin injection. Tibias were aseptically removed, freed of attached tendon and muscle with sterile gauze, split longitudinally, and the bone marrow was lifted out with forceps. The bones were then placed in MEM + 10% CS, flushed with vigorous pipetting, and subjected to a mild enzymatic digestion sequence of (a) 0.05% hyaluronidase in MEM + 10% CS for 10 min and rinsed in MEM; (b) 0.03% trypsin in MEM for 20 min and rinsed in MEM + 10% CS; and (c) 0.1% collagenase + 0.0027% TLCK, an inhibitor of trypsin and papain, in MEM + 10% CS for 75 min and rinsed in MEM + 10% CS. All digestion steps were carried out at 37°C, 5% CO_2, 95% air, 96% humidity. The endosteal surfaces were gently scraped with a rubber policeman into MEM + 10% CS. The cell suspension was filtered through 250 and 105 μm polypropylene meshes (Small Parts, Inc., Miami Lakes, FL), centrifuged at 625*g* in a swinging bucket rotor for 10 min and the pellet was resuspended in 16 ml MEM + 5% FBS. The resulting cell suspension was plated (8 ml/plate) on fibronectin coated plates (described below), incubated at 37°C, 5% CO_2, 95% air for 20 min. Nonadherent cells were transferred to a second set of fibronectin coated plates and incubated for 25 min at 37°C, 5% CO_2, 95% air. Osteoclasts that had attached to the fibronectin coated plates were shaken free in 8 ml MEM + 5% FBS by gentle swirling. Tightly adherent cells consisted mainly of fibroblasts and were discarded. The cell suspensions were combined, then diluted and plated at a concentration of 10^4 cells/ml on glass coverslips (12 mm diameter) in 24 well tissue culture plates (1 ml/well) for microscopy. Cells were incubated at 37°C, 5% CO_2, 95% air for 24 h followed by a rinse in MEM to remove nonadherent cells and transferred to MEM + 0.5% FBS. Half of the medium was replaced every 2 to 3 days.

For coating culture plates with fibronectin, 2 mg human fibronectin was dissolved in 2 ml of 2 M urea in 0.05 M Tris-HCl (pH 7.5) for 1 h and added to 100 ml sterile Tyrodes buffer under sterile conditions. Tissue culture plates were treated with reconstituted fibronectin (3 ml/100 mm plate, 2 h). The solution was transferred to a second set of tissue culture plates and again held at room temperature for 2 h. The remaining solution was discarded and the plates were air dried and stored at room temperature. The plates were rinsed once with sterile MEM prior to use.

Assessment of Purity of the Cell Preparation

Cells maintained on coverslips for 4–6 days were fixed with 3.7% formaldehyde-1% calcium chloride for 10 min, rinsed with distilled water, then stained for acid phosphatase in the presence of 6.7 mM tartrate to determine the proportion of tartrate resistant acid phosphatase (TRAP), a characteristic marker for osteoclasts. The coverslips were air dried, mounted, and examined by light microscopic image analysis

(Bioquant System IV, R & M Biometrics, Nashville, TN). Nine coverslips were assessed at 25× magnification (20 fields of view per coverslip) to determine the number of both TRAP positive and TRAP negative cells. For estimating the number of TRAP stained cells after Percoll purification, subsequently described, cells in suspension were smeared onto slides, air dried at 4°C, fixed, and stained as described above. The average purity was determined to be 89% (experiment performed in triplicate).

Assessment of Acid Production

Acid production in individual osteoclasts was monitored as described previously [Hunter et al., 1991; Gay et al., 1993]. Briefly, coverslips with adherent cells were cultured for 4–6 days and neutralized with 20 mM ammonium chloride in MEM for 15 min, then rinsed three times, 30 s each in MEM. The cells were allowed to recover in the presence or absence of stimulatory or inhibitory substances for 15, 30, 60, and 120 min, followed by 10 min in MEM containing the stimulatory or inhibitory agent and 10 μM acridine orange. Extracellular acridine orange was then removed by rinsing in MEM which contained the stimulatory or inhibitory agent for 1 min. Neutralization and acridine orange staining was accomplished at 37°C, 5% CO_2, 95% air. The coverslips were then inverted over a live cell observation chamber along with MEM and the stimulatory or inhibitory agent. The coverslips were scanned using phase optics to identify single multinucleate cells that were separated from other cells by a distance of several cell diameters. The fluorescent intensity of individual cells was then determined using a Leitz microscope MPV photometer with an H_2 filter cube plus a GG455 cutoff filter (excitation range 455–490 nm, emission 510 nm, suppression at 515 nm). A neutral density filter (N4, 25% transmittance) was used to reduce light intensity and fading. Twenty osteoclasts on each coverslip were measured per time period per treatment. A background level of fluorescence was obtained by exposing one coverslip per experiment to 20 mM ammonium chloride throughout neutralization and staining with acridine orange. One coverslip per time point per experiment was neutralized and exposed to vehicle without stimulators or inhibitors to provide normal levels of acidity in osteoclasts. Experiments were performed a minimum of three times.

Preparation of Osteoclast Plasma Membrane Samples

Plasma membranes were prepared from osteoclasts using a modification of the method of Bekker and Gay [1990]. Osteoclasts were released from bone surfaces as described above, filtered through 250 and 105 μm polypropylene meshes, and centrifuged (900*g*, 10 min, 4°C) in a swinging bucket rotor. The resulting pellet was resuspended in 10 ml 35% Percoll solution in 1.5× Tyrodes buffer (pH 7.2–7.4, 4°C). The suspension of osteoclasts was carefully overlaid with 1 ml cold Tyrodes buffer (pH 7.2–7.4) to preserve a sharp interface and centrifuged in a swinging bucket rotor (700*g*, 20 min, 4°C). The cloudy layer at the interface was recovered by aspiration, placed in 20 ml cold Tyrodes buffer, and centrifuged (900*g*, 10 min, 4°C). The resulting white pellets were suspended in 9.6 ml homogenization buffer, which consisted of 0.3 mg/ml EDTA, 5 mM HEPES (pH 8.0, 37 mOsm) and protease inhibitors (0.5 μg/ml leupeptin, 8.6 μg/ml phenylmethylsulphonyl fluoride). The partially lysed suspension was maintained on ice for 10 min, then aspirated into a syringe using a 22 gauge needle. Lysis was enhanced by passing the suspension through a 30 gauge needle three times at 4°C and completed by sonicating three times, 1 min each, with intermittent periods of 1 min each, on ice. Examination by light microscopy revealed that all osteoclasts were ruptured. The homogenate was then centrifuged (700*g*, 10 min, 4°C) to remove nuclei, followed by centrifugation at 11,000*g* for 10 min. The resulting supernatant (9.6 ml) was restored to isotonicity by adding 0.79 ml 2.5 M sucrose; 2.34 ml Percoll, and 0.26 ml 2.5 M sucrose was then added to make a final Percoll concentration of 20%. Centrifugation at 47,800*g* for 30 min at 4°C, created a gradient, the top 2.5 ml of which contained the plasma membrane fraction. The purity of the plasma membrane preparation was monitored by assaying 5′-nucleotidase, the plasma membrane marker; succinic dehydrogenase, the mitochondrial marker; NADH dehydrogenase, the endoplasmic reticulum marker; and N-acetyl-glucosaminidase, the lysosomal marker, as described in Bekker and Gay [1990]. The plasma membrane marker was found to be enriched 28-, 4.7-, and 3.1-fold more than the markers for mitochondria, endoplasmic reticulum, and lysosomes, respectively.

Preparation of Osteoblast Plasma Membrane and Heart Samples

Osteoblasts were isolated as previously described [Gay et al., 1994] and cultured in Dulbecco's Modified Eagle's medium + 10% FBS for 8 days to confluence. Briefly, osteoblasts were obtained from the periosteal surface of 2–3-week-old chicken tibias after mild sequential enzymatic digestion with 0.05% hyaluronidase, 0.03% trypsin, and 0.1% collagenase followed by gentle scraping. Plasma membrane vesicles were prepared as described by Gay and Lloyd [1995], a method similar to the osteoclast vesicle preparation method.

Cardiomyocyte homogenate from chicken heart for immunoblotting of G-protein α subunits was prepared according to Puceat et al. [1995]. Briefly, this procedure involved homogenization of a 3 week chicken heart in 3 ml homogenization buffer (50 mM Tris, 2 mM EDTA, 10 mM EGTA, 5 mM dithiothreitol, 250 mM sucrose, 0.5% Triton), followed by centrifugation (15,000*g*, 15 min, 4°C). The supernatant was collected, stabilized with 1.2 ml glycerol and stored at −20°C until use.

Sodium Dodecyl Sulfate (SDS) Polyacrylamide Gel Electrophoresis

Plasma membranes from approximately 10^6 osteoclasts were concentrated using acetone precipitation [Hames, 1981] prior to SDS-polyacrylamide gel electrophoresis (SDS-PAGE) according to Laemmli [1970]. Five volumes of ice cold acetone were added to the membrane samples, which were mixed and maintained for 3 h at −20°C, then centrifuged (9000*g*, 10 min). The pellet was air dried and resuspended in 300 µl loading buffer (62.5 mM Tris-HCl, pH 6.8, 2% SDS, 10% [vol/vol] glycerol, 0.1 M dithiothreitol, and 0.01% bromophenol blue).

The acetone precipitated proteins in loading buffer were heated (60°C, 20 min), loaded into wells (25 µl/well), and separated by SDS-PAGE using a 12% gel in a Mini-Protean II Electrophoresis Cell apparatus (Bio-Rad, Hercules, CA). SDS-PAGE gels were typically run 45 min at 200 volts in buffer (25 mM Tris, 0.2 M glycine, 0.1% SDS).

Immunoblot Analysis of G-Protein α Subunits

The proteins separated by SDS-PAGE were electroblotted onto nitrocellulose (0.45 µm, Schleicher and Schuell, Keene, NH) in a Bio-Rad Mini Trans-Blot Cell. Transfer was performed for 1 h at 100 volts in 25 mM Tris, pH 8.3, 192 mM glycine, and 20% (vol/vol) methanol. The blots were rinsed (2×, 5 min) in Tris buffered saline (TBS, 25 mM Tris, pH 8, 137 mM sodium chloride, and 2.7 mM potassium chloride). After blocking with 5% nonfat dry milk (Carnation) in TBS + 0.1% Tween 20 (TBST) for 1 h, the blots were rinsed thoroughly in TBST. The blots were then cut into strips the width of the gel lanes, placed in disposable incubation trays (Schleicher and Schuell, Keene, NH), and incubated in primary antibody in TBST (1:1000) for 1 h. The primary antibodies used were polyclonal and recognized: (A) residues 159–168 of the $G_i\alpha$-1 subunit (antibody A); (B) residues 345–354 which are common to both $G_i\alpha$-1 and $G_i\alpha$-2 (antibody B); (C) residues 345–354 which are common to $G_i\alpha$-3 and $G_o\alpha$ (antibody C); (D) residues 345–354 of the $G_i\alpha$-3 subunit (antibody D); (E) residues 385–394 of the $G_s\alpha$ subunit (antibody E); (F) residues 127–139 of the G-protein β subunit (antibody F); (G) another preparation of $G_s\alpha$ (antibody G); and (H) residues 40–54 common to multiple forms of Gα subunits [$G_s\alpha$, $G_i\alpha$-1, $G_i\alpha$-2, $G_i\alpha$-3, $G_o\alpha$, $G_z\alpha$, and $G_t\alpha$] (antibody H). Antibodies A to F and antibody H were obtained from Calbiochem, La Jolla, CA; antibody G was from Dupont, Boston, MA. Antibody G was obtained since antibody E did not react in our system. Following application of primary antibody, the blots were thoroughly rinsed in TBST. The blots were transferred to secondary antibody in TBST (1:5000, rabbit IgG, horseradish peroxidase-linked whole antibody, Amersham, Arlington Heights, IL) for 1 h, then rinsed thoroughly. An enhanced chemiluminescent procedure was employed to detect reaction product. Equal volumes of detection reagent 1 and detection reagent 2 (Enhanced Chemiluminescence Kit, Amersham, Arlington Heights, IL) were mixed and applied to the nitrocellulose blots. After incubation for 1 min, excess reagent was removed, the blot sandwiched between plastic sheets (overhead transparency, 3M Corporation, Austin, TX), and exposed to Hyperfilm-ECL (Amersham, Arlington Heights, IL) for 1, 5, or 15 min, depending on band intensity. Each blot used for assessing molecular weight contained duplicate samples and molecular weight markers that were electrophoresed and electroblotted simultaneously with those used for immunoblotting followed by staining with India Ink.

RESULTS

Assessment of Acid Production

Image analysis of cells grown on coverslips and stained for TRAP revealed that the purity of the preparation ranged from 40–86%, with the average being 60%. Impurity in the cell preparation was due to contaminating cells which had an elongated shape and appeared fibroblast-like. Only cells that were separated from all other cells by 50 µm or more were selected for analysis.

PTH (10^{-8} M) treatment of osteoclasts for 15, 30, 60, and 120 min resulted in stimulation of acid production, as indicated by increased levels of fluorescence relative to osteoclasts exposed to vehicle alone (Fig. 1). When osteoclasts were pretreated (30 min) with 10^{-4} M lithium chloride, an agent which uncouples G-proteins from the receptors with which they interact [Avissar et al., 1988], and then treated simultaneously with lithium chloride and PTH, the PTH stimulated increase in acidity was prevented (Fig. 1). Lithium chloride alone had no consistent effect on acidity (Fig. 1). These results indicate the involvement of a G-protein in the signaling pathway of PTH stimulated acidification.

Cholera toxin, a substance which permanently activates G-proteins of the G_s type, was utilized to further characterize the identity of the G-protein indicated by the lithium effect. Treatment of osteoclasts with cholera toxin (0.01 µg/ml) elicited an effect similar to that of PTH on acid production (Fig. 1). Exposure of osteoclasts to PTH and cholera toxin simultaneously did not further stimulate acid production beyond levels that were generated by cholera toxin alone. Taken together, these findings indicate that a stimulatory G-protein is involved in the PTH mediated increase in acid production.

Pertussis toxin, a substance which prevents receptor interaction with G_i- and G_o-proteins, was used to assess the presence of additional G-proteins with which the PTH receptor could interact. When osteoclasts were pretreated (3 h) with pertussis toxin (0.01 µg/ml) and subsequently treated with PTH (10^{-8} M), a decrease in levels of acid production was observed that was dramatically different from levels in PTH stimulated osteoclasts (Fig. 2). Pertussis toxin alone had no consistent effect on acid production. These results suggest the involvement of another G-protein, potentially a G_i- or G_o-protein, in addition to the G_s-protein implicated by cholera toxin. To determine if the two G-proteins present in osteoclasts were linked to different receptor subtypes, with one of the subtypes being functional at lower concentrations of PTH, osteoclasts were exposed to 10^{-11} M PTH and simultaneously treated with pertussis toxin. Pertussis toxin was also capable of blocking 10^{-11} M PTH stimulation of acid production (Fig. 2). No differences were observed between the two concentrations of PTH, suggesting that the pertussis toxin sensitive G-

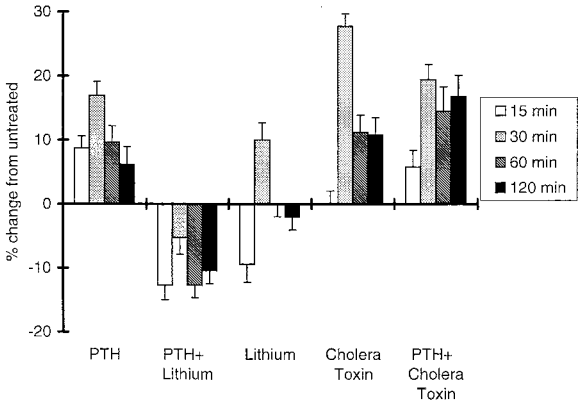

Fig. 1. Effect of lithium chloride and cholera toxin on PTH stimulated acid production by osteoclasts. Osteoclasts, neutralized with 20 mM ammonium chloride followed by staining with 10 µM acridine orange to monitor acid production, were treated with 10^{-8} M PTH, 10^{-4} M lithium chloride, 0.01 µg/ml cholera toxin, or the combinations indicated for the times shown. Results from four (lithium chloride) and three (cholera toxin) experiments are expressed as percentages of acid production in treated cells relative to acid production in untreated cells ± SEM.

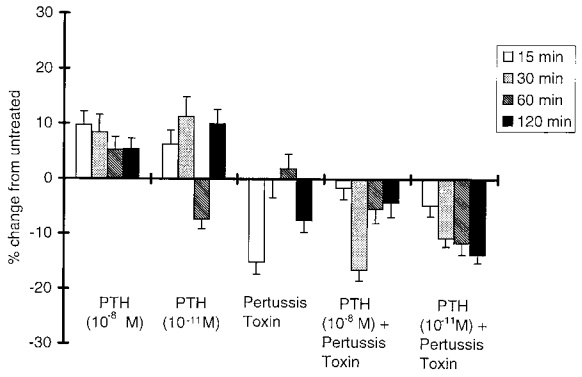

Fig. 2. Effect of pertussis toxin on PTH stimulated acid production by osteoclasts. Osteoclasts, neutralized with 20 mM ammonium chloride and stained with 10 µM acridine orange to monitor acid production, were treated with 10^{-8} M PTH, 10^{-11} M PTH, 0.01 µg/ml pertussis toxin, or the combinations indicated for the times shown. Results from three (10^{-8} M PTH) and four (10^{-11} M PTH) experiments are expressed as percentages of acid production in treated cells relative to acid production in untreated cells ± SEM.

protein is functional at both nanomolar and picomolar concentrations of PTH in the osteoclast.

Immunoblot Analysis of G-Protein α Subunits

To further identify the G-proteins implicated by the use of lithium chloride, cholera toxin, and pertussis toxin, immunoblot analysis of osteoclast plasma membranes was performed using antibodies against several G-protein α-subunits. Initial experiments were performed with antibody H, which recognizes multiple forms of the G-protein α-subunit in order to establish relative abundance of the proteins. Two bands in osteoclast plasma membranes with relative molecular weights of approximately 39 and 53 kDa were evident (data not shown).

Specific antibodies (A to F) were used to define the types of G-protein α-subunits present (Fig. 3). Comparison of lanes stained with either antibody A (lane A) or antibody B (lane B) revealed two bands, one at 56 kDa due to nonspecific binding of the secondary antibody (see lane X) and the other at 34 kDa. This band is likely to be $G_i\alpha$-2. Similarly, detection with antibody C (lane C) and antibody D (lane D) was used to determine if $G_i\alpha$-3 or $G_o\alpha$ was present. These two α subunits have slightly different molecular weights and amino acid sequences; however, they share the same four C-terminal amino acids. Antibody C reacted strongly with a 41 kDa protein which is therefore considered to be $G_o\alpha$. The band that reacted with antibody D had a relative molecular weight of 34 kDa and is identified as $G_i\alpha$-3.

Plasma membranes from osteoblasts were used as a positive control to verify that the antibodies employed cross reacted with the avian species and to demonstrate differences between the osteoclast and osteoblast G-protein profiles. Plasma membrane samples from osteoblasts reacted only with antibody C, which recognizes $G_i\alpha$-3 and $G_o\alpha$ (Fig. 4). Since antibody D, which is specific for $G_i\alpha$-3, was not reactive, it is apparent that the intense band in lane C is the $G_o\alpha$ subunit.

Avian heart samples were used to determine if antibody E was capable of recognizing the $G_s\alpha$ subunit. Rat hearts have been shown by immunoblotting to contain multiple forms of $G_s\alpha$, $G_i\alpha$, and $G_o\alpha$ [Wichelhaus et al., 1994; Johnson et al., 1995]. Heart samples contained bands that were identified as $G_i\alpha$-2, $G_i\alpha$-3, and $G_o\alpha$. However, no bands were detected for $G_s\alpha$ (data not shown). Consequently, reactivity by

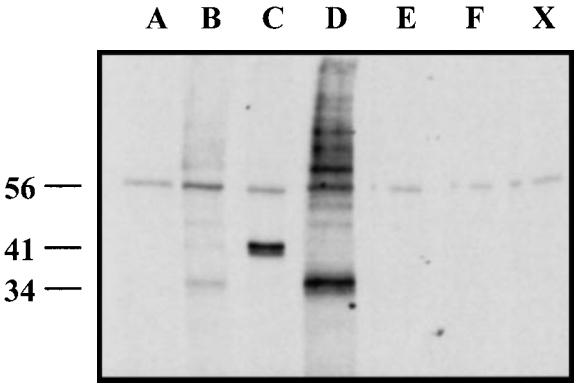

Fig. 3. Immunoblot analysis of osteoclast plasma membrane using antibodies against specific G-protein α-subunits. Each lane contained 25 µl of the proteins precipitated from the osteoclast plasma membrane fraction. Comparison of Lane A (anti-$G_i\alpha$-1) and Lane B (anti-$G_i\alpha$-1 and $G_i\alpha$-2) indicates the presence of a 34 kDa $G_i\alpha$-2 subunit in Lane B. Comparison of Lane C (anti-$G_i\alpha$-3 and $G_o\alpha$) and Lane D (anti-$G_i\alpha$-3) reveals a 41 kDa $G_o\alpha$ in Lane C as well as a 34 kDa $G_i\alpha$-3 subunit and several unidentified bands in Lane D. Lane E (anti-$G_s\alpha$) and Lane F (anti-G-protein β subunit) had no detectable bands other than that observed in Lane X, which was reactive with secondary antibody only. Cross reactivity by another anti-$G_s\alpha$ is shown in Figure 5. The nonspecific band due to binding by the secondary antibody was present at 56 kDa and appeared in all lanes. The immunoblot was exposed to Hyperfilm-ECL for 5 min. Identical results were obtained from a second cell isolation. The results are representative of two experiments.

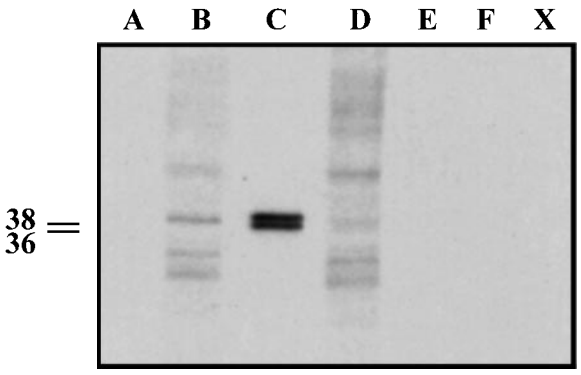

Fig. 4. Immunoblot analysis of osteoblast plasma membrane using the same antibodies against specific G-protein α-subunits as used in Figure 3. Lane order is the same. By comparing Lanes A and B, $G_i\alpha$-1 (37 kDa) is faintly evident in Lane B; comparing Lanes C and D reveals $G_o\alpha$ as an intense doublet at 36 and 38 kDa in Lane C and a faintly staining $G_i\alpha$-3 (37 kDa) in Lane D. Several unidentified bands are also present in Lanes B and D. There was no reactivity against $G_s\alpha$ (Lane E) or Gβ (Lane F) proteins. There was no nonspecific staining by the secondary antibody (Lane X). The immunoblot was exposed to Hyperfilm-ECL for 1 min. These results are representative of two experiments.

another $G_s\alpha$ antibody (antibody G) was explored.

Immunoblots of the heart, osteoclast, and osteoblast preparations were probed with antibody G purchased from a different supplier but generated against the same C-terminal sequence of amino acids 385–394. This antibody reacted with two bands of relative molecular weights 66 and 48 kDa in heart and osteoblast preparations and 68 and 48 kDa in osteoclast preparations (Fig. 5), indicating that, as expected, the $G_s\alpha$ protein is present in all three tissues.

DISCUSSION

Although specific binding of PTH to osteoclasts has been demonstrated by several investigators [Rao et al., 1983; Teti et al., 1991; Agarwala and Gay, 1992], few studies of the direct action of PTH on osteoclasts have been reported. PTH has been shown to enhance the amount of acid in secretory vacuoles in isolated osteoclasts in a dose dependent manner [Hunter et al., 1988] and to stimulate carbonic anhydrase activity in isolated osteoclasts [Silverton et al., 1987]. The PTH enhancement of osteoclast acidity in isolated cells is blocked by carbonic anhydrase inhibition [Gay, 1991]. The relevance of the two latter points is that carbonic anhydrase in osteoclasts has been established as providing hydrogen ions to an ATP-driven hydrogen ion translocation pump in the ruffled border [for reviews see Delaissé and Vaes, 1992; Gay, 1988, 1992, 1996]. PTH enhanced acidification is also reduced by treatment of isolated cells with 17-β estradiol [Gay et al., 1993]. Tong and colleagues [1995] have reported that micro-isolated osteoclasts express mRNA for the PTH receptor. Recently, Datta et al. [1996] have reported that PTH stimulates a burst of superoxide anion production by osteoclasts, an effect that did not depend on the presence of osteoblasts. The purpose of the present study was to identify signalling mechanisms utilized by occupied PTH-receptors in osteoclasts.

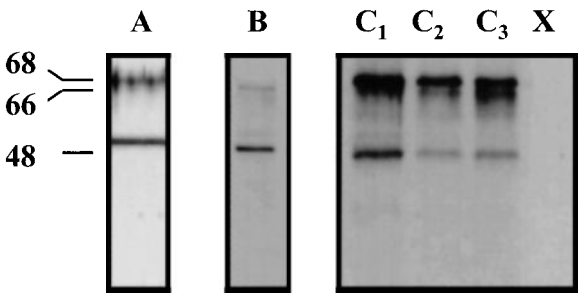

Fig. 5. Immunoblot analysis using antibody G against the $G_s\alpha$ subunit. Lane A: Heart homogenate; Lane B: osteoblast plasma membrane protein; Lanes C_1–C_3: osteoclast plasma membrane protein from three different cell isolations. Lane X contains the same material as Lane C_3 but primary antibody was omitted. The immunoblot was exposed to Hyperfilm-ECL for 5 s. A 48 kDa band ($G_s\alpha$) was present in all samples. A 66 or 68 kDa band was also present intensely in heart and osteoclast samples, but only weakly in osteoblasts. These results are representative of two experiments.

As a consequence of the pronounced effect PTH has on osteoblasts, assessing the amount of osteoblasts present in osteoclast cultures is critical. In the present study, the purity of the cell preparation was estimated on slides stained for tartrate resistant acid phosphatase (TRAP), an enzyme characteristic for osteoclasts [Andersson et al., 1992]. An average of 60% of the cells were positive for TRAP. Cells not staining for TRAP typically were elongated and appeared fibroblast-like. In previous evaluations, we have found that the fibroblast-like cells did not stain for alkaline phosphatase, the classic marker of osteoblasts. We have also determined the activity levels of alkaline phosphatase in our osteoclast preparations and found it to be 37.93 nanomoles p-nitrophenylphosphate released per min mg protein, which is approximately 1/7 that found in a pure preparation of isolated osteoblasts [May and Gay, 1996]. In similarly isolated populations, the total area occupied by cells was 77.5% osteoclasts [Gay et al., 1983]. Because the osteoclast preparation was not completely pure, careful experimental technique was employed in the single cell analysis of acid production. Individual osteoclasts that were several cell diameters (at least 50 μm) away from all other cells were analyzed.

For the immunoblot studies, an additional purification step, which utilized a 35% Percoll gradient, was also employed. Cell purity was assessed as before by histochemical staining for TRAP and found to be 89%, a value similar to that reported by Bekker and Gay [1990]. Contaminating cells, making up approximately 11% of the preparation, varied from small mononuclear cells, possibly erythrocytes, to large multinucleated cells, resembling osteoclasts but not displaying the presence of TRAP. The higher purity of this procedure results in low viability [Bekker and Gay, 1990].

The osteoclasts used in this study were derived in the same manner as in previous studies in which the ability of the cells to form

resorption pits on bone slices or bivalve shell has been shown [Hunter et al., 1991; Guillemin et al., 1995; May and Gay, 1996]. In addition, cells cultured with bone particles release Ca^{++} into the media [May and Gay, 1996]. While we have not monitored the influence of PTH on pit formation or Ca^{++} release in this study, previous studies in this laboratory have revealed that carbonic anhydrase inhibition blocked PTH-stimulated acidification [Gay, 1991] as well as pit formation [Hunter et al., 1991].

In the present study, both the use of indirect (toxin administration) and direct (immunoblotting) techniques were used to classify the G-proteins present in osteoclasts. Lithium chloride was used as an initial screen to discern whether the PTH signalling pathway in osteoclasts involved G-proteins. This was found to be the case. The lack of a consistent effect of lithium chloride on osteoclast acidification is not completely understood. Although the 15 and 30 min lithium treatments are divergent, there was no effect at the 60 and 120 min exposures. The divergent effects of lithium at the early time points could be an attempt by the osteoclast to stabilize acid production levels which result in overshooting the basal level.

Cholera toxin was found to mimic the effects of PTH on acid production, indicating that a G_s-protein is present. This observation is in agreement with studies of other investigators who have shown the presence of a cholera toxin-sensitive G-protein in osteoclasts [Ransjö et al., 1988; Moonga et al., 1990; Zaidi et al., 1990; Moonga et al., 1993].

Pertussis toxin was found to block PTH stimulated acid production, indicating the involvement of the G_i (inhibitory) family of G-proteins. This demonstration of a second type of G-protein in osteoclasts indicates the involvement of dual G-proteins in the signaling mechanism of PTH. Other investigators have demonstrated that multiple G-proteins mediate the effects of calcitonin, both in a pig kidney cell line [Chakraborty et al., 1991] and in osteoclasts [Zaidi et al., 1990].

Some pertussis toxin-sensitive G-proteins are known to be coupled to phospholipase C (PLC). One branch of the PLC pathway involves protein kinase C (PKC) activation. Jouishomme and colleagues [1992] have shown in osteoblast-like cells derived from osteosarcomas, that picomolar concentrations of PTH stimulate PKC but not adenylate cyclase; whereas, nanomolar concentrations of PTH stimulate both PKC and adenylate cyclase. Reshkin and Murer [1991] found similar results in opossum kidney cells, with both pathways being utilized at nanomolar concentrations of PTH but only the PKC pathway being used at picomolar concentrations of PTH. We found that pertussis toxin was effective in blocking PTH stimulated acid production at both nanomolar and picomolar concentrations of the hormone.

Exposure of osteoclasts to pertussis toxin alone resulted in inconsistent effects on acid production at 15 min. The possibility exists that the 15 min time point includes a population of cells that is more sensitive to pertussis toxin and are in the process of dying. At later times, that population of sensitive cells may no longer exist.

At least four G-protein α subunits are present in osteoclast plasma membranes in high abundance as shown by the use of antibodies generated against specific G-protein α subunits (Table I). The profile of G-proteins in osteoclasts is substantially different from that of osteoblasts. For osteoclasts, two intense bands with relative molecular weights of 34 and 41 kDa, were identified as $G_i\alpha$-3 and $G_o\alpha$, respectively. A less intense band with a relative molecular weight of 34 kDa was identified as $G_i\alpha$-2. One or all three of these G-proteins could be the pertussis toxin-sensitive G-protein that was identified as a component of the PTH signaling pathway. The molecular weights of the α subunits from the G_i- and G_o-class of G-proteins range from 39–41 kDa [Hepler and Gilman, 1992]. The molecular weights that we determined approximate previously reported values.

The $G_s\alpha$-specific antibody (antibody G) recognized a band with a relative molecular weight of 48 kDa in osteoclast plasma membranes, osteoblast plasma membranes, and heart. Two forms of the $G_s\alpha$ subunit have been identified

TABLE I. Major G-Proteins in Osteoclast Plasma Membrane Identified by Immunoblot Analysis

Subunit type	Molecular weight (kDa)	Figure number
$G_i\alpha$-3	34	3
$G_o\alpha$*	36, 38	4
$G_o\alpha$	41	3
$G_s\alpha$*	48	5
$G_h\alpha$	66, 68	5

*Also found in abundance in osteoblast plasma membranes.

with relative molecular weights of 45 and 52 kDa [Hepler and Gilman, 1992]. Since PTH has been shown to stimulate adenylate cyclase in osteoblasts, which would suggest that it does so via a stimulatory G-protein [Civitelli et al., 1990; Jouishomme et al., 1992; Kano et al., 1993], and heart has been demonstrated to express both the 45 and 52 kDa forms of $G_s\alpha$ [Wichelhaus et al., 1994; Johnson et al., 1995], the bands recognized by antibody G are believed to be a $G_s\alpha$ subunit. The second band that was recognized by antibody G had a relative molecular weight of 66 and 68 kDa. Most molecular weight values of G-protein α subunits range from 39 to 52 kDa, although a 74 kDa α subunit was discovered to couple to α_1-adrenergic receptors in rat liver membranes and has been named G_h [Im and Graham, 1990]. Other investigators have identified G_h-proteins in rat heart and liver, dog heart, bovine heart and liver, and human heart with relative molecular weights ranging from 74 to 80 kDa [Baek et al., 1993]. The 66 and 68 kDa band recognized by the $G_s\alpha$ antibody could be a G_h-protein or an undiscovered G-protein α subunit. As many as 20 α, 5 β, and 6 γ subunits of G-proteins have been identified over a wide range of cell types [Neer, 1995].

To summarize, the presence and involvement of at least two, possibly three, G-proteins in osteoclasts utilized by PTH to produce acid has been demonstrated. A stimulatory G-protein, G_s-protein, was identified by both cholera toxin and a polyclonal antibody. Two other G-proteins, $G_o\alpha$, and $G_i\alpha$-3, which were identified by polyclonal antibodies, are both capable of being ADP-ribosylated by pertussis toxin. There is also the possibility of $G_i\alpha$-2 involvement since immunoreactivity was demonstrated, although to a lesser extent than that of $G_o\alpha$, and $G_i\alpha$-3. The most intense bands were $G_s\alpha$, $G_i\alpha$-3, $G_o\alpha$, and $G_h\alpha$, as codified in Table I. Although the results from both techniques agree, the immunoblot findings are the most accurate because specific antibodies for the direct identification of G-protein α subunits were used and also the purity of the cell preparations used for immunoblotting were increased. The G-protein profile in osteoblasts was found to be considerably different from that of osteoclasts, in that in osteoblasts the 36 and 38 kDa bands ($G_o\alpha$) were more intense and G_i-3 was absent. A higher molecular weight G-protein, potentially a G_h-protein, was found in both osteoclasts and osteoblasts. In osteoclasts, the PTH signal appears to be transduced through a G_s-protein, a G_i-3 protein, and a G_o-protein. On the basis of toxin analysis, at least two of the three G-proteins appear to be utilized by PTH to stimulate acid production in the osteoclast. In the studies described here, a specific link between the immunoblot findings and the proposed involvement of G-proteins on PTH stimulation of acid production cannot be made. However, it seems highly probable that the cholera toxin-sensitive G-protein is the G_s protein identified by immunoblotting. Likewise, since both G_i- and G_o-proteins are capable of being ADP-ribosylated by pertussis toxin, it is likely that one or both of the G_i- or G_o-proteins identified in the immunoblots is the pertussis toxin-sensitive G-protein involved in PTH regulation of osteoclasts.

ACKNOWLEDGMENTS

Portions of this work were presented at the Fourteenth and Fifteenth Annual Meetings of the American Society for Bone and Mineral Research in September, 1992 and 1993, and at the Fifth International Conference on Avian Endocrinology in September, 1992. The assistance of Virginia R. Gilman with photography and editing, Qin P. Lloyd with the isolation of osteoblasts, Joseph A. Stains with the cardiomyocyte isolation, and Kristen D. Brubaker for stimulating discussions is gratefully acknowledged. This work was supported by NIH grants DE04345 and DE09459.

REFERENCES

Agarwala N, Gay CV (1992): Specific binding of parathyroid hormone to living osteoclasts. J Bone Miner Res 7:531–539.

Andersson G, Ek-Rylander B, Minkin C (1992): Acid phosphatases. In Rifkin BR, Gay CV (eds): "Biology and Physiology of the Osteoclast." Boca Raton: CRC Press, pp 55–80.

Avissar S, Schreiber G, Danon A, Belmaker RH (1988): Lithium inhibits adrenergic and cholinergic increases in GTP binding in rat cortex. Nature 331:440–442.

Baek KJ, Das T, Gray C, Antar S, Murugesan G, Im MJ (1993): Evidence that the G_h protein is a signal mediator from alpha 1-adrenoceptor to a phospholipase C. I. Identification of alpha 1-adrenoceptor-coupled G_h family and purification of G_h7 from bovine heart. J Biol Chem 268: 27390–27397.

Bekker PJ, Gay CV (1990): Characterization of a Ca^{+2}-ATPase in osteoclast plasma membrane. J Bone Miner Res 5:557–567.

Chakraborty M, Chatterjee D, Kellokumpu S, Rasmussen H, Baron R (1991): Cell-cycle dependent coupling of the calcitonin receptor to different G-proteins. Science 251: 1078–1082.

Civitelli R, Hruska KA, Shen V, Avioli LV (1990): Cyclic-AMP dependent and calcium dependent signals in parathyroid hormone function. Exp Gerontol 25:223–231.

Datta HK, Rathod H, Manning P, Turnbull Y, McNeil CJ (1996): Parathyroid hormone induces superoxide anion burst in the osteoclast: Evidence for the direct instantaneous activity of the osteoclast by the hormone. J Endocrinol 149:269–275.

Delaissé J-M, Vaes G (1992): Mechanism of mineral solubilization and matrix degradation in osteoclastic bone resorption. In Rifkin BR, Gay CV (eds): "Biology and Physiology of the Osteoclast." Boca Raton: CRC Press, pp 289–314.

Gay CV (1988): Avian bone resorption at the cellular level. CRC Crit Rev Poultry Biol 1:197–210.

Gay CV (1991): The role of carbonic anhydrase in bone resorbing cells. In Botré F, Gros G, Storey BT (eds): "Carbonic Anhydrase: From Biochemistry and Genetics to Physiology and Medicine." Weinheim: VHC, pp 419–422.

Gay CV (1992): Osteoclast ultrastructure and enzyme histochemistry: Functional implications. In Rifkin BR, Gay CV (eds): "Biology and Physiology of the Osteoclast." Boca Raton: CRC Press, pp 129–150.

Gay CV (1996): Role of microscopy in elucidating the mechanism and regulation of the osteoclast resorptive apparatus. Microsc Res Tech 33:165–170.

Gay CV, Ito MB, Schraer H (1983): Carbonic anhydrase activity in isolated osteoclasts. Metab Bone Dis Rel Res 5:33–39.

Gay CV, Kief NL, Bekker PJ (1993): Effect of estrogen on acidification in osteoclasts. Biochem Biophys Res Comm 192:1251–1259.

Gay CV, Lloyd Q, Gilman V (1994): Characteristics and culture of osteoblasts derived from avian long bone. In Vitro Cell Dev Biol 30A:379–383.

Gay CV, Lloyd Q (1995): Characterization of calcium efflux by osteoblasts derived from long bone periosteum. Comp Biochem Physiol A 111:257–261.

Guillemin G, Hunter SJ, Gay CV (1995): Resorption of natural calcium carbonate by avian osteoclasts in vitro. Cells Materials (Scanning Microscopy International) 5:157–165.

Hames BD (1981): An introduction to polyacrylamide gel electrophoresis. In Hames BD, Rickwood D (eds): "Gel Electrophoresis of Proteins, a Practical Approach." Oxford: IRA Press, pp 1–86.

Hepler JR, Gilman AG (1992): G-proteins. Trends Biochem Sci 17:383–387.

Hunter SJ, Schraer H, Gay CV (1988): Characterization of isolated and cultured chick osteoclasts: The effects of acetazolamide, calcitonin, and parathyroid hormone on acid production. J Bone Miner Res 3:297–303.

Hunter SJ, Rosen CJ, Gay CV (1991): In vitro resorptive activity of isolated chick osteoclasts: effects of carbonic anhydrase inhibition. J Bone Miner Res 6:61–66.

Im MJ, Graham RM (1990): A novel guanine nucleotide-binding protein coupled to the alpha 1-adrenergic receptor. I. Identification by photolabeling of membrane and ternary complex preparations. J Biol Chem 265:18944–18951.

Johnson MD, Zhou Y, Friedman E, Roberts J (1995): Expression of G-protein alpha subunits in the aging cardiovascular system. J Gerontol 50A:314–319.

Jouishomme H, Whitfield JF, Chakravarthy B, Durkin JP, Gagnon L, Isaacs RJ (1992): The protein kinase C activation domain of the parathyroid hormone. Endocrinology 130:53–60.

Kano J, Sugimoto T, Fukase M, Chihara K (1993): Crosstalk of dual signal transduction systems in the regulation of DNA synthesis by parathyroid hormone in osteoblast osteosarcoma cells. J Bone Miner Res 8:323–329.

Laemmli UK (1970): Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227:680–685.

May LG, Gay CV (1996): Development of a new method for obtaining osteoclasts from endosteal surfaces. In Vitro Cell Dev Biol—Animal 32:269–278.

Moonga BS, Moss DW, Patchell A, Zaidi M (1990): Intracellular regulation of enzyme secretion from rat osteoclasts and evidence for a functional role in bone resorption. J Physiol 429:29–45.

Moonga BS, Pazianas M, Alam T, Shankar VS, Huang C, Zaidi M (1993): Stimulation of a G_s-like G-protein in the osteoclast inhibits bone resorption but enhances tartrate-resistant acid phosphatase secretion. Biochem Biophys Res Comm 190:496–501.

Neer EJ (1995): Heterotrimeric G-proteins: Organizers of transmembrane signals. Cell 80:249–257.

Partridge NC, Bloch SR, Pearman AT (1994): Signal transduction pathways mediating parathyroid hormone regulation of osteoblastic gene expression. J Cell Biochem 55:321–327.

Puceat M, Korichneva I, Cassoly R, Vassort G (1995): Identification of band 3-like proteins and Cl^-/HCO_3^- exchange in isolated cardiomyocytes. J Biol Chem 270:1315–1322.

Rao LG, Murray TM, Heersche JNM (1983): Immunohistochemical demonstration of parathyroid hormone binding to specific cell types in fixed rat bone tissue. Endocrinology 113:805–810.

Ransjö M, Lerner UH, Heersche JNM (1988): Calcitonin-like effects of forskolin and cholera toxin on surface area and motility of isolated rabbit osteoclasts. J Bone Miner Res 3:611–619.

Reshkin SJ, Murer H (1991): Parathyroid hormone regulation in OK cells: In situ protein phosphorylation reactions involving PKA, PKC, and GTP-binding protein. Cell Physiol Biochem 1:143–159.

Silverton SF, Dodgson SF, Fallon MD, Forster RE (1987): Carbonic anhydrase activity of chick osteoclasts is increased by parathyroid hormone. Am J Physiol 253:E670–E674.

Simmons SM, Grynpas MD (1990): Mechanisms of bone formation in vivo. In Hall BK (ed): "The Osteoblast and Osteocyte." New Jersey: The Telford Press, pp 193–302.

Teti A, Rizzoli R, Zambonin Zallone A (1991): Parathyroid hormone binding to cultured avian osteoclasts. Biochem Biophys Res Comm 174:1217–1222.

Tong H, Lin H, Wang H, Sakai D, Minkin C (1995): Osteoclasts respond to parathyroid hormone and express mRNA for its receptor. J Bone Miner Res 10:M278.

Wichelhaus A, Russ M, Peterson S, Eckel J (1994): G-protein expression and adenylate cyclase regulation in ventricular cardiomyocytes from STZ-diabetic rats. Am J Physiol 267:H548–H555.

Wong G, Cohn DV (1974): Separation of parathyroid hormone and calcitonin-sensitive cells from non-responsive bone cells. Nature 252:713–715.

Zaidi M, Datta HK, Moonga BS, MacIntyre I (1990): Evidence that the action of calcitonin on rat osteoclasts is mediated by two G-proteins acting via separate post receptor pathways. J Endocrinol 126:473–481.