Journal of Cellular Biochemistry 65:565–573 (1997)

Parathyroid Hormone Uses Both Adenylate Cyclase and Protein Kinase C to Regulate Acid Production in Osteoclasts

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Abstract Osteoclasts, isolated from the endosteum of 2.5- to 3-week-old chickens, were treated with acridine orange, a hydrogen ion concentration-sensitive fluorescent dye, in order to monitor changes in acid production. The adenylate cyclase inhibitor, alloxan, blocked parathyroid hormone (PTH)-stimulated acid production. Dibutyryl cyclic adenosine monophosphate, a membrane-permeant form of cyclic adenosine monophosphate, mimicked the PTH effect. Bisindolylmaleimide, a specific inhibitor of protein kinase C (PKC), blocked the initial stimulation (15, 30, and 60 min) of acid production by PTH but had no effect on long-term stimulation (120 min). Confocal microscopy of osteoclasts stained with fluorescein-conjugated bisindolylmaleimide revealed a shift in location of PKC from the cytoplasm to the plasma membrane region after treatment with parathyroid hormone. The results of these studies support the hypothesis that PTH regulation of acid production in osteoclasts involves both adenylate cyclase and PKC as effectors. J. Cell. Biochem. 65:565–573. © 1997 Wiley-Liss, Inc.

Key words: calcium-regulating hormones; bone cells; acridine orange; signal transduction

Bone remodeling requires the concerted action of both osteoblasts, the bone-forming cells, and osteoclasts, the bone-resorbing cells. Following the initial observations of Wong and Cohen [1974], many studies have shown that osteoblasts bind and respond to parathyroid hormone (PTH). These studies led to the concept that PTH acts on osteoblasts, which then secrete factors that stimulate osteoclasts to resorb bone. Studies revealing such a factor have been reported [Morris et al., 1990]. Despite substantial evidence to support the concept of the PTH–osteoblast axis, the concept is monolithic in nature and appears to be in need of being broadened. Challenging the singular nature of the PTH–osteoblast axis as the sole means of regulating osteoclasts are studies that reveal PTH binding directly to rat and avian osteoclasts [Rao et al., 1983; Teti et al., 1991; Agarwala and Gay, 1992], as well as the demonstration of mRNA for PTH receptor in murine osteoclasts [Tong et al., 1995]. Further, the acidification process in osteoclasts has been shown to be stimulated by PTH in both organ cultures [Anderson et al., 1985] and isolated cell preparations [Gay et al., 1993]. As indicated by recent reviews [Simmons and Grynpas, 1990; Partridge et al., 1994], the signaling mechanisms of PTH in osteoblasts have received considerable attention. The signaling mechanisms of PTH in osteoclasts need to be elucidated.

Bone resorption is a complex process that uses an ATP-driven proton pump for the secretion of hydrogen ions into the resorption pit as described in recent reviews [Gay, 1992; Delaissé and Vaes, 1992]. Carbonic anhydrase has been established as the enzyme that provides the protons for the proton pump, as reviewed by Gay [1996]. Both the ATP-driven proton pump [Akisaki and Gay, 1986; Baron et al., 1985; Väänänen et al., 1990], and carbonic anhydrase [Anderson et al., 1982] have been localized to the ruffled border of osteoclasts. Carbonic anhy-

Contract grant sponsor: National Institutes of Health, contract grant number DE04345 and DE09459.

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Received 12 August 1996; accepted 18 February 1997

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drase could be a potential focus of control for hormonal regulation of bone resorption.

Guanine nucleotide-binding proteins (G-proteins) are responsible for linking the receptor on the extracellular face of the plasma membrane to an effector on the intracellular face and thereby transducing the signal across the plasma membrane [Hepler and Gilman, 1992]. This laboratory has defined the profile of G-proteins in osteoclasts involved in regulating acidification [May and Gay, 1997]. Multiple effectors exist that further relay and amplify the signal as a result of the generation of second messenger molecules.

The purpose of the present work was to identify and elucidate effectors and second messengers involved in the PTH stimulation of osteoclast acidification. Evidence is presented for the involvement of adenylate cyclase, cAMP, and protein kinase C (PKC) in the regulation of acid production by PTH as measured by acridine orange fluorescence. The involvement of PKC was corroborated by detection of a shift in the location of PKC from cytoplasm to the plasma membrane region upon PTH treatment of isolated osteoclasts vitally stained with fluorescein-conjugated bisindolylmaleimide.

MATERIALS AND METHODS

Materials

Synthetic salmon calcitonin, heat-inactivated calf serum (CS), heat-inactivated fetal bovine serum (FBS), minimum essential medium (MEM, Eagle's modification with nonessential amino acids and Earle's salts), L-glutamine, type 1-S hyaluronidase, type 1-A collagenase, N-α-p-Tosyl-L-lysine-chloromethylketone (TLCK), dibutyryl cyclic adenosine monophosphate (dbcAMP), alloxan, isobutylmethylxanthine (IBMX), lithium chloride, and propidium iodide were obtained from Sigma Chemical Co. (St. Louis, MO). MEM was further modified by the 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, which matches 3-week-old chicken plasma. Culture medium also contained 10 ml/L 10,000 units penicillin–10 mg streptomycin (Sigma). Phenol red was avoided in the medium due to its mild estrogenic properties. Trypsin (bovine pancreatic, 3× crystallized) was purchased from Worthington Biochemicals (Freehold, NJ), human fibronectin from Biomedical Technologies (Stoughton, MA), acridine orange from Polysciences (Warrington, PA), bisindolylmaleimide (BIM) from Calbiochem (La Jolla, CA), fim-1 diacetate from Molecular Probes (Eugene, OR), and phorbol-12,13-dibutyrate was purchased from Gibco BRL Life Technologies (Grand Island, NY). Bovine PTH (biopotency of 1300 USP U/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 [Hunter et al., 1988; Gay et al., 1993] as completely described in May and Gay [1997]. Briefly, osteoclasts were obtained from 2.5- to 3-week-old calcitonin-treated chickens (Peterson × Arbor Acre) maintained on normal chick starter diet. Tibias were split longitudinally and the bone marrow discarded. The tibias were then subjected to a mild enzymatic digestion sequence of 0.05% hyaluronidase, 0.03% trypsin, and 0.1% collagenase + 0.0027% TLCK. All digestion steps were carried out at 37°C, 5% CO_2, 95% air, and 96% humidity. Briefly, the suspension of osteoclasts derived from endosteal surfaces was filtered through 250- and 105-μm polypropylene meshes and pelleted. The pellet was resuspended in MEM + 5% FBS and plated on fibronectin-coated plates for 20 min. Loosely adherent osteoclasts that had attached to the fibronectin-coated plates were shaken free in MEM + 5% FBS by gentle swirling. Tightly adherent cells consisted mainly of fibroblasts and were discarded. The highly enriched (up to 86%) osteoclast suspensions as defined previously [May and Gay, 1997], were combined, plated, and maintained in MEM + 0.5% FBS on 12-mm glass coverslips (10^4 cells/ml) in 24-well tissue culture plates (1 ml/well) or on 100-mm culture dishes (10^5 cells/plate).

Assessment of Acid Production

Assessment of acid production in individual osteoclasts was performed as described previously [Hunter et al., 1991; Gay et al., 1993; May and Gay, 1997]. Osteoclasts were neutralized with 20 mM ammonium chloride for 15 min and allowed to recover in the presence or absence of stimulatory (10^{-8} M PTH, 10^{-4} M dbcAMP) or inhibitory substances (10^{-8} M alloxan, 10^{-4} M

lithium chloride, 5 nM BIM) for 15, 30, 60, and 120 min. The cells were incubated for an additional 10 min in MEM containing 10 μM acridine orange, as well as the stimulatory or inhibitory agent. A 1-min rinse in MEM to remove external acridine orange followed. Osteoclasts on coverslips were then mounted in a live cell observation chamber on a microscope stage along with MEM and the stimulatory or inhibitory agent. Single multinucleate cells that were not in close proximity to other cells were located using phase optics. 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 continuous exposure of one coverslip per experiment to the neutralizing agent, 20 mM ammonium chloride, throughout the entire protocol. One coverslip per time point per experiment was neutralized and exposed to vehicle alone to determine levels of acidity in untreated osteoclasts. Experiments were performed three or four times and, because there was less than 10% variation between experiments, the data for each set of experiments were averaged and expressed as % change from untreated.

Localization of Protein Kinase C

Osteoclasts cultured for 4–6 days on coverslips were rinsed in MEM (5 min, 37°C, 5% CO_2, 95% air) to remove potential stimulatory agents in serum. Coverslips were then incubated with 200 nM fim-1 diacetate in MEM (30 min, 37°C, 5% CO_2, 95% air). PTH (10^{-8} M) was included before and/or during incubation with fim-1 diacetate to provide treatment times of 15, 30, 60, or 120 min. Cells still attached to coverslips were rinsed in MEM, then mounted in a live cell observation chamber containing MEM or PTH (10^{-8} M) and observed using a BioRad MRC-600 laser scanning confocal microscope configured to excite at 488 nm and collect fluorescent images through the BHS filter. Propidium iodide (2 μg/ml) was present to permit detection of dead or membrane-impaired cells. Images were obtained by making projections of individual optical z-sections (i.e., sections parallel to the coverslip) taken at 1-μm intervals through the cell. For each experiment, the background level of fluorescence was assessed in an osteoclast not exposed to fim-1 diacetate. For a positive control, osteoclasts on coverslips were stained with fim-1 diacetate and mounted in phorbol-12,13-dibutyrate (1 nM), a known activator of PKC at short times of treatment [Castagna et al., 1982]. A total of 121 cells were analyzed representing 16 different isolations.

RESULTS

Assessment of Acid Production

The involvement of PTH and its effector, adenylate cyclase, in osteoclast acidification are shown in Figure 1. PTH 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 treated with vehicle alone. Osteoclasts treated with PTH in the presence of alloxan, an inhibitor of adenylate cyclase, had consistently decreased levels of acid production compared to osteoclasts treated with PTH alone. Treatment of osteoclasts with alloxan alone resulted in low levels of acid production, similar to osteoclasts treated with vehicle alone (data not shown). Also, as shown in Figure 1, dibutyryl cAMP, a membrane-permeant form of cAMP, mimicked the PTH effect. Osteoclasts simultaneously

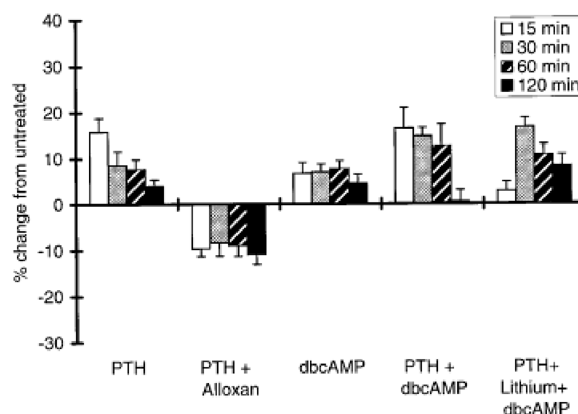

Fig. 1. Effect of alloxan, dbcAMP, and lithium chloride on PTH stimulation of acid production. Osteoclasts were neutralized with 20 mM ammonium chloride, then treated with 10^{-8} M PTH, 10^{-8} M alloxan, 10^{-4} M dbcAMP, 10^{-4} M lithium chloride, or the combinations shown for the times indicated, followed by staining with 10 μM acridine orange to monitor acid production. Results were obtained from three experiments in all cases except the last (PTH + lithium + dbcAMP) which was repeated four times. Results are expressed as percentages of acid production in treated cells relative to acid production in untreated cells ±SEM.

treated with PTH and dbcAMP were similar to PTH alone. Lithium chloride, which uncouples G-proteins from their receptors and has previously been shown to prevent PTH stimulation of acid production [May and Gay, 1997], was ineffective in the presence of dbcAMP.

Treatment of osteoclasts with bisindolylmaleimide (BIM), a specific inhibitor of PKC [Toullec et al., 1991], was used to determine whether PKC was an effector for PTH (Fig. 2). BIM alone had no consistent effect on basal levels of activity. PTH stimulation of acid production was blocked by BIM during treatment times of 15, 30, and 60 min but was ineffective at 120 min of treatment. To determine whether the inability of BIM to inhibit PTH stimulation of acid production at 120 min of treatment was due to degradation of the inhibitor, the experiments were repeated with BIM being completely replaced every 15 min. Similar results were obtained, with BIM having no effect on PTH-stimulated acid production at 120 min of treatment (data not shown).

Localization of Protein Kinase C

Vital staining of osteoclasts with fim-1 diacetate, a fluorescein-conjugated derivative of a PKC-specific inhibitor, revealed that PKC is distributed throughout the cytoplasm, but not in nuclei, the clear zone, or plasma membrane of untreated osteoclasts (Fig. 3a,b).

Plasma membrane staining was observed in osteoclasts stimulated with PTH for 15 min (Fig. 3c,d), indicating PKC migration to the plasma membrane, a prerequisite of PKC activation. Osteoclasts treated with PTH for 30 min exhibited minimal staining (Fig. 3e,f), suggesting downregulation of PKC. Exposure of osteoclasts to PTH for 60 min resulted in a pattern of staining similar to that of untreated osteoclasts, i.e. mainly cytoplasmic staining (Fig. 3g,h). Osteoclasts stimulated with PTH for 120 min exhibited strong dense staining throughout the cytoplasm and nuclei whereas the plasma membrane was not stained (Fig. 3i,j). Superimposing the fluorescent image over the phase-contrast image revealed that some staining was diffuse, whereas other staining was within the vesicles.

Osteoclasts treated with 1 μM phorbol-12,13-dibutyrate, a known PKC activator, served as a control to verify that fim-1 diacetate could be used to monitor PKC activation (Fig. 4). Staining intensity increased progressively in some regions of the plasma membrane of osteoclasts exposed to phorbol-12,13-dibutyrate, peaking at around 12.5 min of treatment. No staining was evident after osteoclasts were exposed to phorbol-12,13-dibutyrate for 24 hr, suggesting complete downregulation of PKC (micrograph not shown). Of the cells analyzed, 82% of 72 cells responded to PTH, and 77% of 18 cells responded to phorbol-12,13-dibutyrate treatment. Some nonresponders lacked staining to

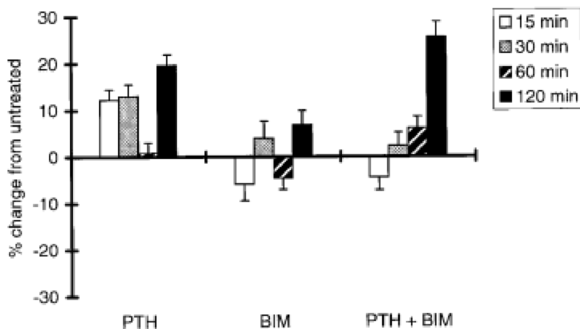

Fig. 2. Effect of bisindolylmaleimide (BIM) on PTH stimulation of acid production. Osteoclasts were neutralized with 20 mM ammonium chloride, pretreated for 15 min with 5 nM BIM or vehicle, then treated with 10^{-8} M PTH, or 10^{-8} M PTH and 5 nM BIM, for the times shown and stained with 10 μM acridine orange to monitor acid production. Results were obtained from four experiments and expressed as percentages of acid production in treated cells relative to acid production in untreated cells ±SEM.

Fig. 3. Protein kinase C localization in osteoclasts using fim-1 diacetate as a stain. Fluorescent (**a**) and phase-contrast (**b**) images of an unstimulated osteoclast stained for 30 min and treated simultaneously with vehicle. Protein kinase C is distributed throughout the cytoplasm but not in the nuclei (*) or the clear zone (cz) of untreated osteoclasts. The fluorescent micrographs are merged optical sections. Evaluation of individual sections permitted the distinction between cytoplasmic and membrane domains. **c–j:** Protein kinase C localization in PTH-stimulated osteoclasts. Fluorescent (**c**,**e**,**g**,**i**) and phase-contrast (**d**,**f**,**h**,**j**) images of osteoclasts stained for 30 min and simultaneously treated with 10^{-8} M PTH for 15 min (**c**,**d**); 30 min (**e**,**f**); 60 min (**g**,**h**); 120 min (**i**,**j**). Staining in the region of the plasma membrane was observed at 15 min (e.g., between *arrows*); cytoplasmic staining was reduced. At 30 min, minimal membrane and cytoplasmic staining was detected. By 60 min, the pattern of staining was similar to that of unstimulated osteoclasts, being mostly cytoplasmic. At 120 min, strong dense staining was observed throughout the cytoplasm and nuclei. In all views, the thicker central region of the osteoclast was discerned by altering the focal plane of phase-contrast images or by creating optical sections in the fluorescent images. *Arrows,* region of the plasma membrane that projected above the flattened clear zone (cz) region, the region to which fim-1 migrated after PTH treatment. *Arrowheads,* edge of the thin cytoplasmic skirt of the cell. ×640.

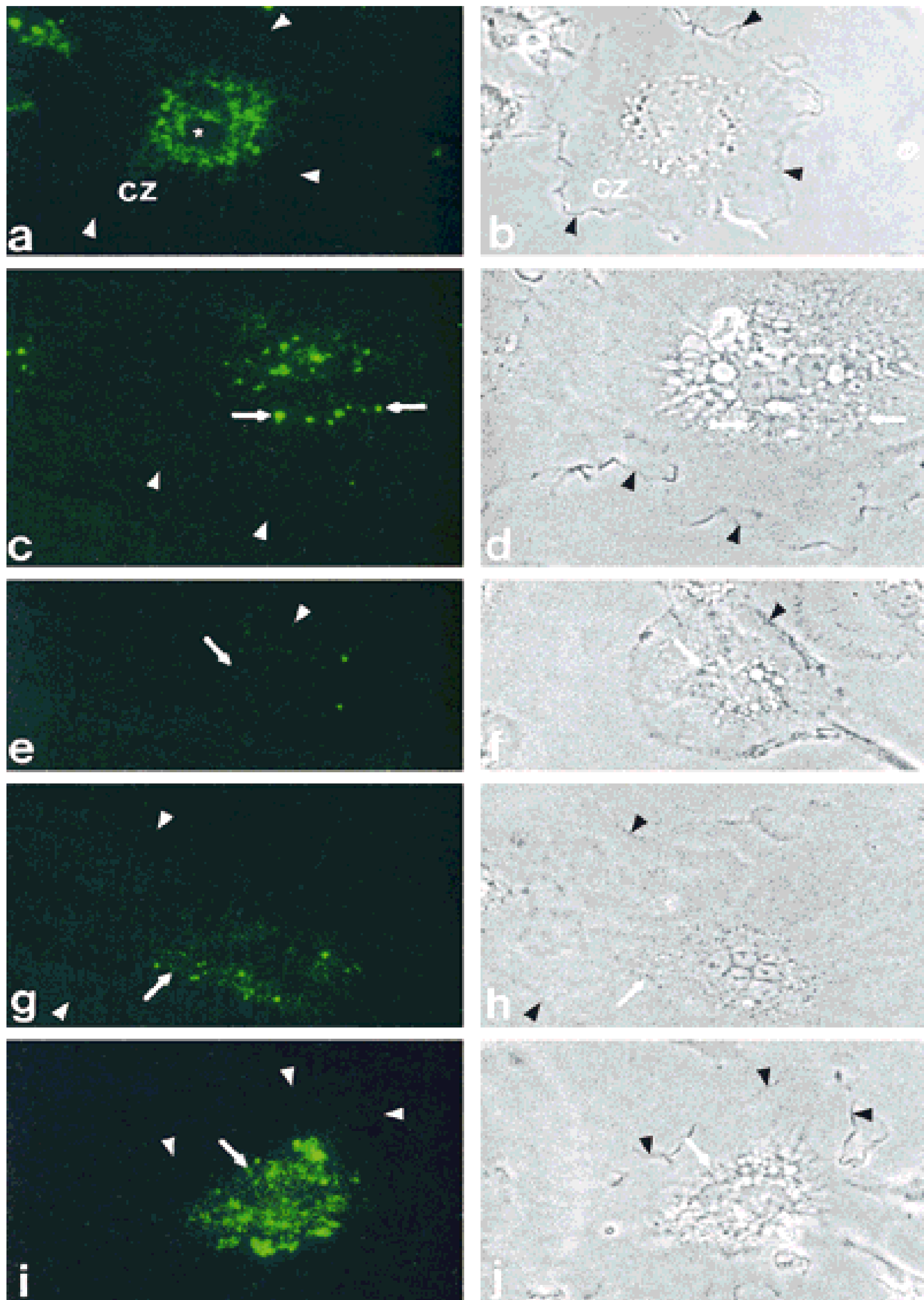

Figure 3.

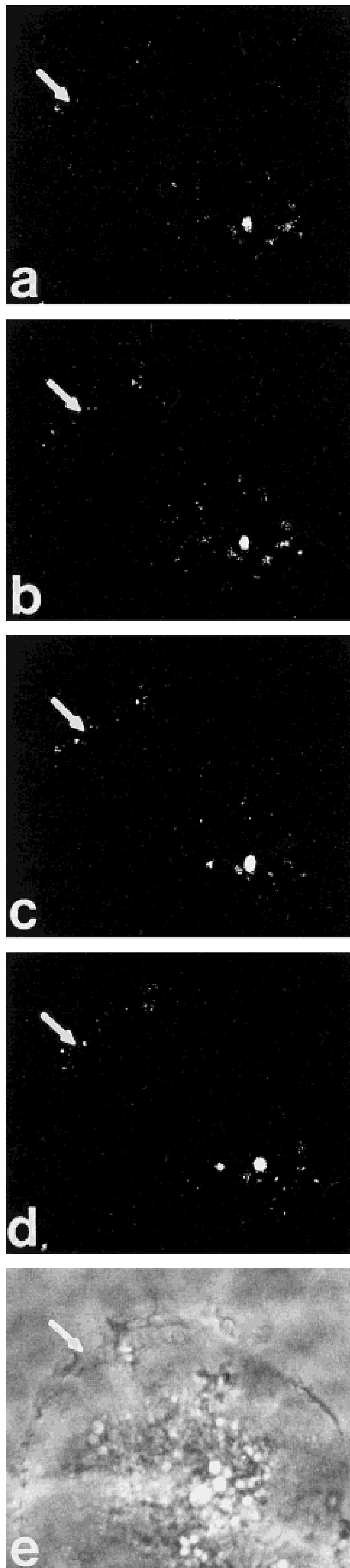

Fig. 4. Protein kinase C localization in phorbol-12,13-dibutyrate-treated osteoclasts. Shown is a representative osteoclast stained with fim-1 diacetate for 30 min and treated with phorbol-12,13-dibutyrate for (a) 0.5 min, (b) 3.0 min, (c) 12.5 min, and (d) 28.5 min. e: Phase-contrast image of the same osteoclast. *Arrows,* regions of the plasma membrane in which clusters of fluorescence increased following PTH treatment. Fluorescence in the central region of the cell (lower right) did not change with time. ×640.

begin with; in other nonresponders, the location of fim-1-diacetate remained in the cytosol. No shift in staining occurred in 31 untreated control cells.

DISCUSSION

Since investigators demonstrated PTH receptors on osteoblasts [Silve et al., 1982; Barling and Bibby, 1985; Rouleau et al., 1986], it has become widely accepted that PTH acts directly on osteoblasts to stimulate osteoclasts indirectly. A few studies have shown that PTH also binds specifically to osteoclasts [Rao et al., 1983; Teti et al., 1991; Agarwala and Gay, 1992], although studies of the direct action of PTH on osteoclasts are few and data are difficult to obtain. As a consequence of the pronounced effect of PTH on osteoblasts, assessing the amount of osteoblasts present in the osteoclast cultures is critical. In the present study, the purity of the cell preparation was assessed by histochemical staining for tartrate-resistant acid phosphatase (TRAP), as described in a parallel study [May and Gay, 1997], and was found to be as high as 86%. Cells not staining for TRAP were mainly elongated in shape, appeared fibroblast-like and did not stain for alkaline phosphatase, the classic marker of osteoblasts. To determine the degree of contamination in our cell preparation attributable to osteoblasts, activity levels of alkaline phosphatase were determined in another parallel study [May and Gay, 1996]. The cell preparation displayed an alkaline phosphatase activity substantially lower than the activity level for a pure preparation of isolated cultured osteoblasts, approximately one-seventh that of a preparation of pure osteoblasts. In similarly isolated preparations, the area of coverslips occupied by osteoclasts was 77.5% osteoclasts and 1–2% osteoblasts, with the remainder consisting of other cells and debris [Gay et al., 1983]. Because the osteoclast preparation was not 100% pure, careful experimental technique in single-cell analy-

sis involved the measurement of individual osteoclasts that were not in close proximity to other cell types. This maneuver was performed to minimize influences by other cells. In support of the studies reported here, Tong et al. [1995] demonstrated that osteoclasts on dentine slices respond to PTH by an increase in resorption under conditions that are relatively deficient in osteoblasts. These investigators also demonstrated that microisolated osteoclasts express mRNA for the PTH receptor.

The acridine orange assay system we used to measure acid production in osteoclasts is unique in that individual living cells are monitored for their ability to produce acid in response to appropriate treatments [Hunter et al., 1988, 1991; Gay et al., 1993; Brubaker and Gay, 1994; Guillemin et al., 1995]. Using the acridine orange assay system, the involvement of adenylate cyclase in PTH stimulation of acid production in the present study was found to corroborate previous studies [Gay et al., 1993; May et al., 1993]. Additionally, alloxan, an inhibitor of adenylate cyclase, was found to block PTH stimulation of acid production, whereas alloxan by itself did not significantly inhibit or stimulate acid production. Adenylate cyclase involvement was further strengthened by demonstrating that dibutyryl cAMP, a membrane-permeant form of cAMP, was capable of mimicking PTH-stimulated acid production. These studies indicate that PTH activates adenylate cyclase, which then generates the second messenger cAMP.

PKC was also implicated in the signaling mechanism used by PTH to stimulate acid production by using bisindolylmaleimide (BIM), a specific inhibitor of PKC. It is interesting to note that the ability of PTH to stimulate acid production was only blocked at short periods of treatment. Longer exposures of osteoclasts to PTH resulted in acid production that was not blocked by BIM. This loss of inhibition was not due to degradation of BIM because similar results were obtained when BIM was completely replaced every 15 min. The loss of inhibition by BIM may be due to compensation by another pathway used by PTH, such as the adenylate cyclase/cAMP pathway.

The studies which utilized the fluorescein-conjugate of BIM (fim-1 diacetate) support this latter explanation. The translocation of a portion of PKC from cytosol to the region of the plasma membrane following a 15-min exposure to PTH suggests that PKC became activated. Discerning the location of the plasma membrane required varying the focal plane of the phase-contrast images and making optical sections of the fluorescent images. The particulate nature of the staining is not understood. However, some particulate staining coincided with vesicles seen in the phase-contrast images; PKC could potentially be translocated to the plasma membrane in association with vesicles. PKC appears to be downregulated after 30-min exposure to PTH, since staining was absent at that time. Diminished staining was also observed in osteoclasts exposed for 30 min to phorbol-12,13-dibutyrate, a substance that has been shown to downregulate PKC [Rodriguez-Pena and Rozengurt, 1984]. PKC was localized within the cell cytosol, but not at the plasma membrane after osteoclasts were exposed to PTH for 60 min. After 120-min exposure to PTH, PKC was intensely localized within the cytosol and nuclei of living osteoclasts. The early shift in staining of PKC from cytosol to the plasma membrane suggests that PKC is activated during short times of PTH exposure. These results agree with the BIM studies using the acridine orange assay, in which BIM was only effective at inhibiting PTH-stimulated acid production during the short exposure times. PKC downregulation through a degradation process could explain the lack of PKC inhibition by BIM at exposures of greater than 30 min. The presence of PKC in nuclei of living osteoclasts after exposure to PTH for 120 min (cf. Fig. 3a,i) suggests that a role of PKC at longer treatment times might occur at the level of the DNA to promote the long-term effects of PTH. Other investigators have shown that PKC in the nucleus appears to promote the development of multidrug resistance in MCF-7 cells [Lee et al., 1992], as well as enhance the induction of immunosuppression [Zorn et al., 1995]. Neri et al. [1994] demonstrated that PKC was translocated to the nucleus of Swiss 3T3 cells after a 45-min exposure to insulin-like growth factor.

An alternate means of distinguishing between adenylate cyclase and PKC pathways, which was not explored in this study, would be to evaluate the effects of PTH fragments. The cAMP/PKA system requires the presence of the first two amino acid residues of the 1–34 PTH fragment [Goltzmann et al., 1975], whereas the phospholipase C/PKC system is dependent on a

longer domain of the N-terminus for activation [Jouishomme et al., 1992; Fujimori et al., 1992].

In the present study, both adenylate cyclase and PKC signaling pathways for PTH stimulation of osteoclasts was determined. These findings are in good agreement with the role of the three G-proteins, G_s, G_i, and G_o, identified in the PTH signaling pathway [May and Gay, 1997]. Stimulatory G-proteins (G_s) are typically linked to adenylate cyclase, as identified in the present study. Pertussis toxin-sensitive G-proteins are members of the G_i-family and have been shown to use phospholipase C as an effector molecule to activate PKC.

The complete mechanism by which PTH stimulates acid production in osteoclasts is not fully understood. It has been demonstrated that PTH treatment of osteoclasts activates carbonic anhydrase [Silverton et al., 1987; Dietsch, 1987] and that this activation appears to be due to phosphorylation through protein kinase A [Dietsch, 1987]. The activation of carbonic anhydrase results in an increased supply of hydrogen ions, which would then be translocated across the plasma membrane by the proton pump [reviewed by Gay, 1992; Delaissé and Vaes, 1992]. The gastric proton pump has been shown to be activated upon phosphorylation by a cAMP-dependent protein kinase in parietal cells [Sack, 1993] and by PKC in the yeast, *S. cerevisiae* [Brandao et al., 1994]. It is possible that the increases in cAMP proceed to activate protein kinase A, which phosphorylates carbonic anhydrase, and that PKC activates the proton pump, with the net result being increased acid secretion by osteoclasts.

ACKNOWLEDGMENTS

Portions of this work were presented at the Fourteenth Annual Meeting of the American Society for Bone and Mineral Research in September 1992 and at the Seventeenth Annual Meeting of the American Society for Bone and Mineral Research in September 1995. The authors acknowledge the assistance of Virginia Gilman with photography.

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