E64d, a Membrane-Permeable Cysteine Protease Inhibitor, Attenuates the Effects of Parathyroid Hormone on Osteoblasts In Vitro

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Parathyroid hormone (PTH) activates calpains I and II (calcium-activated papain-like proteases) and stimulates the synthesis and secretion of cathepsin B (a lysosomal cysteine protease) in osteoblastic cells. Anabolic doses of PTH also stimulate osteoprogenitor cell proliferation and differentiation into mature, fully functional osteoblasts capable of elaborating bone matrix, whereas catabolic doses of PTH stimulate calcium mobilization and matrix turnover. Previous investigations in other cell types have demonstrated that calcium-activated calpains play a major role in regulating proliferation and differentiation by catalyzing limited regulatory proteolysis of nuclear proteins, transcription factors, and enzymes. We tested the hypothesis that inhibition of intracellular cysteine proteases such as the calpains will ablate PTH-mediated osteoblast proliferation and differentiation, two fundamental indices of bone anabolism. A brief preincubation with the membrane-permeable, irreversible cysteine protease inhibitor E64d (10 μg/mL) before short-term PTH treatment blunted PTH-induced cell proliferation in subconfluent cultures and also attenuated proliferation and inhibited differentiation in longer-term confluent cultures. This confirms the hypothesis that cysteine proteases such as the calpains are important in mediating the proliferative and prodifferentiating or anabolic effects of PTH on MC3T3-E1 cells in culture. Immunofluorescent localization demonstrated that calpain I, calpain II, and calpastatin (the endogenous calpain inhibitor) are abundant and widely distributed within actively proliferating MC3T3-E1 preosteoblasts. Since the calpains are active and stable at neutral intracellular pH levels in osteoblasts, whereas cathepsins are not, our results support a role for these calcium-activated regulatory proteases in mediating the anabolic effects of PTH in bone.

PARATHYROID HORMONE (PTH) can have either catabolic or anabolic effects on bone, depending on the dose and frequency of administration of the hormone. High doses or a constant infusion of PTH stimulate bone catabolism, including calcium mobilization and collagen turnover.[1] In contrast, lower doses or more transient administration of PTH stimulate bone anabolism, including osteoprogenitor cell mitogenesis and differentiation, as well as enhanced synthesis of local growth factors, growth factor receptors, collagen, and noncollagenous proteins.[1] Osteoblast differentiation is a complex, multistep process marked by an initial surge in proliferation, followed by downregulation of proliferation and a reciprocal upregulation of expression of osteoblast-specific markers, elaboration of the extracellular matrix, and mineralization.[2]

PTH acts by binding to its specific, high-affinity receptors on the osteoblast and stimulating the classic cyclic nucleotide, Ca^{2+}, and phospholipid second-messenger cascades that activate target enzyme systems and mediate its biological effects in bone.[1] We have previously demonstrated that PTH stimulates calpain I and II activity in MC3T3-E1 osteoblastic cells.[3] The calpains (EC 3.4.22.17) are a ubiquitous family of Ca^{2+}-dependent cysteine endoproteases important in regulating cell proliferation and differentiation.[4] Calpain I and II (μ- and m-calpain) are synthesized as heterodimeric 80-kd/30-kd proenzymes that undergo autolytic processing in the presence of activating [Ca^{2+}], resulting in generation of the fully functional 76- to 78-kd/17-kd proteolytic isozymes that differ chiefly in Ca^{2+} requirements.[4] Once activated, calpains can undergo subcellular redistribution to the nucleus, where they can catalyze limited regulatory proteolysis of nuclear proteins, transcription factors, and kinases during normal cell proliferation and differentiation.[4,5]

In addition, cathepsins L and B, two major lysosomal cysteine proteases, have recently been identified in the osteogenic stromal cell line MN7 and in normal and transformed human osteoblasts.[6,7] PTH stimulates the synthesis and secretion of cathepsin B from human osteoblastic cells.[7] The precise biochemical role of the cathepsins in bone anabolism remains unclear, since the intracellular form is not resistant to degradation at neutral pH, acidic conditions are required for proenzyme activation, and the enzyme is presumably inactive in the nonlysosomal intracellular compartments of intact osteoblasts.[7] However, extracellular cathepsin B is relatively stable and active, and it may be important in bone matrix turnover and local liberation of growth factors such as transforming growth factor-beta (TGF-β) or bone morphogenetic proteins (BMPs) that stimulate autocrine or paracrine induction of osteoblastic cell proliferation and differentiation.[7]

In the present study, we tested the hypothesis that cysteine proteases such as the calpains or cathepsins are important in PTH-regulated bone metabolism by briefly pretreating MC3T3-E1 preosteoblastic cells with the nontoxic, irreversible membrane-permeable cysteine protease inhibitor, E64d, and then culturing them in the absence or presence of PTH under conditions that favor anabolism (short-term or relatively low-dose PTH treatment). Cell number and alkaline phosphatase activity were measured as indices of proliferation and differentiation, respectively. The results suggest that cysteine protease activity is essential for proliferation and differentiation of osteoblastic cells under anabolic conditions of short-term or low-dose PTH treatment. Immunofluorescent localization demonstrated that the calpain-calpastatin system proteins (calpain I,

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Submitted October 31, 1996; accepted February 17, 1997.

Supported by the Geriatric Research, Education, and Clinical Center and the Research Service of the Department of Veterans Affairs and the National Institute of Diabetes and Digestive and Kidney Diseases.

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0026-0495/97/4609-0021$03.00/0

calpain II, and their endogenous inhibitor, calpastatin) are abundant and widely distributed within actively proliferating MC3T3-E1 preosteoblasts, suggesting that a major regulatory role for these proteases in PTH-induced bone anabolism is plausible based on their occurrence and activity at neutral intracellular pH.

MATERIALS AND METHODS

Chemicals

Rat PTH (1-34) was purchased from Bachem (Torrance, CA). E64d [(2*S*,3*S*)-*trans*-epoxylsuccinyl-L-leucylamido-3- methylbutane ethyl ester], routine biochemicals, and media were obtained from Sigma (St Louis, MO). E64d is the membrane-permeable ethyl ester derivative of E64c, an epoxysuccinyl peptide that irreversibly and selectively inhibits cysteine proteases (papain, calpains, and cathepsins) by forming a thioether with the active-site cysteine.[8] E64 derivatives have no effects on other thiol-dependent enzymes such as creatine kinase and lactic dehydrogenase.[9] Once inside the cell, E64d is cleaved by endogenous esterases to E64c, a potent irreversible nonpermeable cysteine protease inhibitor.[8] E64 (the parental compound isolated from *Aspergillus japonicans* for preventing calpain-mediated muscle cell death in dystrophic hamsters) and its derivatives are not cytotoxic,[10] and prevent or inhibit tissue loss in muscular dystrophy or following ischemic injury, myocardial infarction, or calcium ionophore treatment.[4]

Cell Culture

MC3T3-E1 mouse osteoblastic cells were routinely maintained at 37°C in a humidified atmosphere of 95% air and 5% CO_2 in Dulbecco's modified Eagle's media (DMEM) containing 10% newborn calf serum (NCS), 2 mmol/L glutamine, penicillin (100 U/mL), and streptomycin (100 μg/mL).[11]

Acute Effects of E64d and PTH on Cell Proliferation in Short-Term Cultures

MC3T3-E1 cells were plated at a density of $3.5 \times 10^3/cm^2$, cultured under routine conditions for 2 days, and switched to DMEM supplemented with 2% NCS plus vehicle or E64d (10 μg/mL) for 30 minutes. The pretreatment media were then removed and replaced with DMEM supplemented with 2% NCS and 0.0, 0.1, 1.0, or 10 nmol/L PTH, and the cells were cultured for an additional 2 days. The spent media were aspirated, and the cultures were rinsed with phosphate-buffered saline (PBS). The cells were liberated by mild trypsinization, pelleted by centrifugation at 800 × *g* for 15 minutes at 22°C, and resuspended in 2.0 mL of PBS. The cell number was determined in triplicate 250-μL aliquots of cell suspension diluted in 20 mL Isoton using a Z_1 Coulter counter (Coulter, Hialeah, FL).[12]

Effects of Intermittent E64d Pretreatment and Short-Term PTH Administration on Cell Proliferation in Longer-Term Cultures

The cells were plated and cultured for 24 hours in DMEM containing 10% NCS. The cells were then cultured for an additional 6 days, during which time they were subjected to three 2-day cycles of pretreatment with vehicle or E64d (10 μg/mL) for 20 minutes, followed by refeeding with DMEM supplemented with 2% NCS plus 0.0, 0.1, 1.0, or 10.0 nmol/L PTH. Cells were harvested and counted as already outlined.

Alkaline Phosphatase Activity Assay

Cells remaining after the aliquots had been removed for counting were repelleted by centrifugation, resuspended in 1.0 mL 10-mmol/L Tris hydrochloride, pH 7.4, 1.0 mmol/L $MgCl_2$, 20 μmol/L $ZnCl_2$, and 0.02% (vol/vol) Triton X-100, and sonicated for 30 seconds at 4°C.[12] Alkaline phosphatase activity was determined spectrophotometrically at 410 nm in cell extracts as the release of *p*-nitrophenol from *p*-nitrophenylphosphate at 37°C in a solution containing 10 mmol/L *p*-nitrophenylphosphate, 100 mmol/L diethanolamine (pH 10.5), and 1 mmol/L $MgCl_2$.[12] The reaction was terminated by addition of 5.0 mL 0.05 mol/L NaOH. Total protein in cell extracts was determined spectrophotometrically at 592 nm by commercial dye-binding assay (Biorad, Hercules, CA) using bovine γ-globulin as the protein standard.

Immunocytochemistry

Rapidly proliferating MC3T3-E1 cells were cultured under routine conditions as previously outlined, rinsed with DMEM, and fixed in 2% paraformaldehyde for 15 minutes at room temperature. Immunolabeling of calpain-calpastatin system proteins in osteoblastic cells was conducted in a manner similar to that previously outlined for muscle sections.[13] The cells were washed in TBS with azide (50 mmol/L Tris hydrochloride, pH 7.6, 150 mmol/L NaCl, and 0.02% NaN_3) for 5 minutes and then placed in blocking buffer (TBS containing 0.5% Tween 20, 0.2% gelatin, and 0.3% nonfat dry milk) for 2 hours. The cell layer was then incubated with semipurified preimmune immunoglobulin preparations or with identically diluted, highly specific calpain system antibodies directed against the human erythrocyte calpain I large subunit, human placental calpain II large subunit, or human red blood cell calpastatin[14] overnight at 4°C, followed by biotinylated secondary antibody in TBS for 2 hours at room temperature, and then streptavidin-Texas Red in TBS for 1 hour. After each of these steps, the cells were rinsed in TBS for 10 minutes, incubated in blocking buffer again for 1 hour, and rerinsed in TBS for 10 minutes. The cells were overlaid with TBS and viewed by epifluorescence.

Statistical Analysis

All data are expressed as the mean ± SEM. Results were analyzed by ANOVA using Statview 512+ (BrainPower, Agoura Hills, CA) or InStat 2.00 software (GraphPad, San Diego, CA) on a Macintosh 6100/66 computer (Apple, Cupertino, CA).

RESULTS

Subconfluent MC3T3-E1 preosteoblastic cells cultured under conditions that favor PTH-induced bone anabolism (short-term administration of a single relatively low dose of PTH) proliferated to a greater extent than cells that received no PTH (Table 1). Pretreating these short-term preconfluent cultures with E64d (a nontoxic, membrane-permeable irreversible cysteine protease inhibitor) for 30 minutes before refeeding completely ablated the PTH-induced increase in cell number observed under anabolic culture conditions (Table 1) but had no effect on

Table 1. Acute Effects of E64d and PTH on Cell Proliferation in Short-Term Cultures

PTH Dose (nmol/L)†	Cell No./Culture*	
	0 μg E64d/mL	10 μg E64d/mL
0.0	158,123 ± 8,358	155,791 ± 6,491
0.1	178,323 ± 8,389	139,910 ± 2,535
1.0	198,668 ± 7,651	142,398 ± 10,215
10.0	202,649 ± 12,017	139,391 ± 7,316

NOTE. MC3T3-E1 mouse osteoblastic cells were plated in DMEM supplemented with 10% NCS for 2 days, switched to DMEM supplemented with 2% NCS plus or minus 0 or 10 μg E64d/mL for 30 minutes, and then cultured in the absence or presence of PTH for an additional 2 days. Data are expressed as the mean ± SEM (n = 3).

*By 2-factor ANOVA, the effect of E64d was significant at $P = .0001$.

†In the absence of E64d, the effect of PTH was significant at $P = .032$ by 1-factor ANOVA (F test).

cells that received no PTH, suggesting that cysteine protease activity is essential for PTH-induced proliferation of subconfluent preosteoblasts.

MC3T3-E1 preosteoblastic cells cultured for a prolonged period under anabolic conditions (intermittent short-term treatment with low 0.1- and 1.0-nmol/L doses of PTH) achieved confluence at a relatively stable cell number (Table 2) and expressed high levels of alkaline phosphatase, an osteoblast-specific marker (Table 3). Brief, 20-minute preincubations in E64d before refeeding reduced both the rate of cellular proliferation (Table 2) and the level of alkaline phosphatase activity (Table 3) in cultures maintained to postconfluence with 0.0, 0.1, and 1.0 nmol/L PTH.

MC3T3-E1 preosteoblastic cells cultured for prolonged periods at the highest dose of PTH (10.0 nmol/L) did not exhibit cell contact–dependent inhibition of proliferation, and instead became overgrown (Table 2) and exhibited low levels of alkaline phosphatase activity (Table 3). In all cases, the cell number per culture increased with the length of time in culture, and all cultures appeared fully confluent at the end of the longer-term experiment.

The occurrence and distribution of calpain-calpastatin system proteins (calpain I, calpain II, and calpastatin) was determined by immunofluorescence (Fig 1). All of the calpain-calpastatin system components, including calpain I (Fig 1B), calpain II (Fig 1C), and calpastatin (Fig 1D), are abundant and widely distributed within actively proliferating MC3T3-E1 preosteoblastic cells.

DISCUSSION

Differentiation of osteoblastic cells in response to anabolic stimuli is a highly organized process that requires an initial surge in the rate of proliferation of preosteoblasts, followed by a density-dependent downregulation of proliferation, elaboration of the extracellular matrix, and mineralization.[2] As expected, PTH-induced differentiation of preconfluent MC3T3-E1 preosteoblastic cells was associated with enhanced proliferation (Table 1), followed by proliferation to confluence (Table 2) and induction of alkaline phosphatase activity (Table 3) in longer-term cultures. Brief pretreatment with E64d, the membrane-permeable cysteine protease inhibitor, prevented PTH-induced cell proliferation (Tables 1 and 2) and induction of the differentiated phenotype (Table 3). In conclusion, it appears that intracellular cysteine protease activity is required for PTH-induced osteoblastic cell proliferation in subconfluent cultures, as well as full expression of the differentiated phenotype once the rate of proliferation declines and osteoprogenitor cells undergo progressive development of the osteoblastic phenotype required for formation of the mature extracellular bone matrix.[2]

Table 2. Effects of Intermittent E64d Pretreatment and PTH on Cell Proliferation in Longer-Term Cultures

	Cell No. (millions)/Culture	
PTH Dose (nmol/L)*	0 μg E64d/mL	10 μg E64d/mL
0.0	1.513 ± 0.023	1.365 ± 0.027
0.1	1.527 ± 0.007	1.367 ± 0.032
1.0	1.510 ± 0.029	1.343 ± 0.029
10.0	1.777 ± 0.018	1.703 ± 0.037

NOTE. Mouse MC3T3-E1 osteoblastic cells were plated in DMEM supplemented with 10% NCS for 1 day, and then cultured for an additional 6 days in DMEM plus 2% NCS. At the beginning of days 2, 4, and 6 in culture, the cells were pretreated with either vehicle or E64d for 20 minutes and refed with DMEM supplemented with 2% NCS plus or minus PTH to achieve E64d inhibition of intracellular cysteine protease activity immediately before refeeding in half of the cultured cells. Data are expressed as the mean ± SEM (n = 3).

*By 2-factor ANOVA, the effect of E64d is significant at $P = .0046$ and the effect of PTH is significant at $P = .0001$.

Table 3. Effects of Intermittent E64d Pretreatment and PTH on Alkaline Phosphatase Activity in Longer-Term Cultures

	Alkaline Phosphatase Activity (nmol *p*-NP/mg protein · 60 min)	
PTH Dose (nmol/L)*	0 μg E64d/mL	10 μg E64d/mL
0.0	281.3 ± 8.8	123.7 ± 10.2
0.1	387.3 ± 12.6	203.3 ± 15.6
1.0	337.7 ± 4.4	185.3 ± 20.3
10.0	130.3 ± 2.2	127.0 ± 3.0

NOTE. Mouse MC3T3-E1 osteoblastic cells were cultured as detailed in Table 2. All values are expressed as the mean ± SEM (n = 3).

*By 2-factor ANOVA, the effects of PTH and E64d and the interaction between PTH and E64d are all significant at $P = .0001$.

Treatment with the highest dose of PTH (10 nmol/L) for the longest period (6 days) was associated with cellular overgrowth (Table 2) and relatively low levels of alkaline phosphatase activity (Table 3). The concentration of intact PTH in control human serum ranges from about 1 to 5 pmol/L,[15] compared with 0, 100, 1,000, and 10,000 pmol/L used in this in vitro experiment; therefore, it is likely that the biphasic effects of PTH observed in postconfluent MC3T3-E1 cells (Tables 2 and 3) represent a pharmacologic effect of the hormone at the highest concentration. For the experiments described here, we attempted to work within the range of PTH concentrations that are associated with optimally high levels of second-messenger generation and receptor occupancy and measurable biological effects in a variety of osteoblastic cell types in vitro.[16-19] In addition, the cells were cultured in DMEM containing only 2% serum to minimize the potentially confounding effects of high concentrations of other hormones. Hence, the total amount of proliferation in the absence or presence of PTH was relatively modest. However, the effects of PTH on proliferation and differentiation in DMEM supplemented with 2% serum were highly reproducible and statistically significant. The full anabolic effects of PTH may not be observed upon addition of PTH alone, but appear to require the presence of other hormones such as growth hormone for the maximum effect to be observed.[20]

E64d (the epoxysuccinyl peptide that irreversibly inhibits cysteine proteases by alkylating the active-site cysteine) is not sufficiently specific to permit exact identification of the protease(s) responsible for PTH-induced osteoblastic cell proliferation and differentiation. It irreversibly inhibits both of the major families of cysteine proteases (calpains and cathepsins) present in osteoblastic cells.[3,21,22] Abundant amounts of the neutral proteases calpain I and II are present in MC3T3-E1 cells (Fig 1). Unlike the cathepsins, which require acidic conditions for activation of the proenzymes, the calpains are optimally active

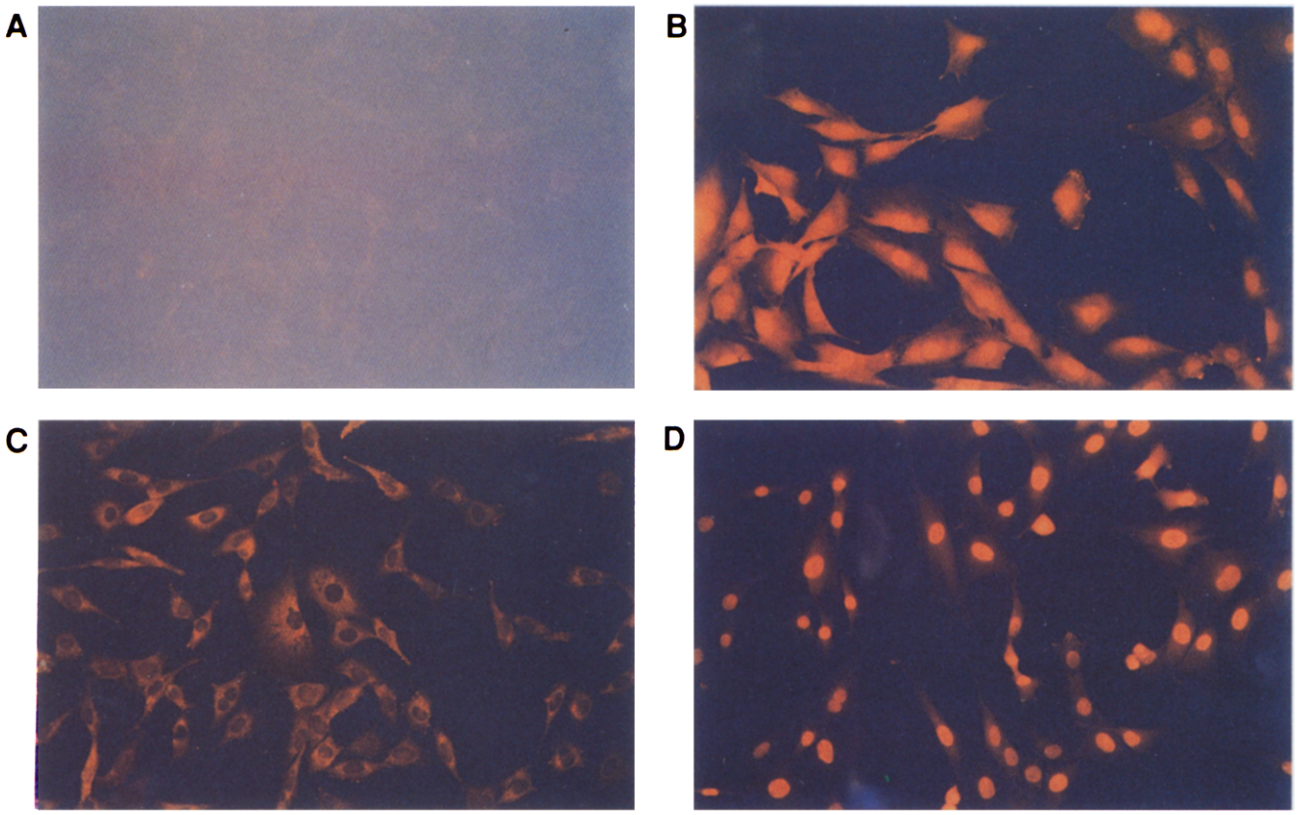

Fig 1. Immunolocalization of calpain system components in murine MC3T3-E1 cells. Cells were immunostained with preimmune rabbit serum diluted 1:100 (A), mouse antibody directed against the human erythrocyte calpain I large subunit (1:100) (B), rabbit antibody directed against the human placental calpain II large subunit (1:100) (C), and mouse antibody directed against human erythrocyte calpastatin (1:1,000) (D). All calpain system components are sufficiently abundant to be easily detected. Control cells immunostained with preimmune mouse serum had results similar to those observed in A (data not shown) (original magnification ×200).

at physiologic pH.[4] We have previously demonstrated that calpain I and II activity can be detected in MC3T3-E1 cells, that PTH stimulates these activities, and that E64c completely inhibits PTH-stimulated calpain I and II activity in MC3T3-E1 cell extracts.[3] In addition, preincubating MC3T3-E1 cells with E64d ablates 50% of the PTH-induced retraction of intact cells.[23] In conclusion, PTH stimulates the existing activity of calpain I and II,[3] and the same concentrations of the irreversible inhibitors (E64c and E64d) that inhibit calpain activity[3] and PTH-induced osteoblastic retraction[23] also ablate PTH-induced MC3T3-E1 cell proliferation and differentiation. Therefore, these observations lend support to the hypothesis that the calpains are among the cysteine proteases involved in PTH-induced osteoblastic cell proliferation and differentiation.

Cathepsin B, another cysteine protease strongly inhibited by E64d, is present in normal human osteoblastic cells and the osteosarcoma cell line MG-63, where 94% to 99% of the activity appears to be intracellular.[7] Cathepsin B has also been detected immunohistochemically in osteoclasts and on the adjacent surfaces of bone undergoing osteoclastic resorption.[24,25] However, no positive immunostaining for cathepsins has been reported on nonresorbing bone surfaces or within the trabeculae of normal bone, indicating that cathepsins are not present in the matrix surrounding osteoblasts under normal conditions.[24] MN7 is a stromal cell line that can be induced to undergo osteoblastic differentiation.[6] Under basal conditions, MN7 cells secrete cathepsins B and L, whereas the related nonosteogenic stromal cell lines MM1 and MV1 do not.[6] MN7 cells also secrete type I collagen, tissue inhibitor of metalloproteinase-2, and an osteopontin fragment, but not osteocalcin, a marker of bone matrix synthesis.[6] Previous investigators have reported that procathepsin L and procathepsin L–like proteins that exhibit no enzymatic activity at physiological pH are present in bone homogenates.[25] No cathepsin L activity is observed in whole-bone homogenates, and cathepsin L activity cannot be detected in bone explant culture fluid even after PTH treatment.[25] Although a potential role of intracellular cathepsins B and L in PTH-induced osteoblast proliferation and differentiation cannot be excluded, these enzymes appear to be unstable in the intracellular environment and inactive at physiologic pH in bone homogenates, culture medium, or nonresorbing bone surfaces that lack active osteoclasts with ruffled borders. Therefore, in the absence of osteoclast-mediated extracellular fluid acidification and procathepsin activation, it is unlikely that the cathepsins play a major role in local bone matrix turnover and the liberation of BMPs or TGF-β following administration of PTH.

The observation that the calpains appear to play a role in mediating PTH-induced anabolism is consistent with the role of these regulatory proteases in proliferation and differentiation in muscle cells, neurons, and erythroid and epithelial cells.[4,5] Following transient elevations in intracellular [Ca^{2+}], the

calpains can undergo a specific, energy-dependent subcellular relocalization from the cytosol to the nucleus,[26] where they are capable of proteolyzing transcription factors and nuclear proteins, including AP1 (c-*Fos*/c-*Jun*), AP2, AP3, Pit-1, Oct-1, CP1a and b, c-*Myc*, ATF/CREB, the basic helix-loop-helix zipper transcription factor USF, histone H1 kinase, and nuclear proteins of 60, 120, 130, and 200 kd at physiologic [Ca^{+2}] in the presence of DNA.[5,27] Other substrates include pp60src (a tyrosine kinase),[28] the proto-oncogene c-*mos* and cyclin,[29] and protein phosphotyrosine phosphatase 1B.[30] Additional research will be required to identify the endogenous substrates of the calpains in the osteoblast, to elucidate the functions of calpain I, calpain II, and calpastatin, and to clarify the roles, if any, for other cysteine proteases in PTH-mediated bone anabolism.

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