Calpain Is Required for Normal Osteoclast Function and Is Down-regulated by Calcitonin*

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Osteoclast motility is thought to depend on rapid podosome assembly and disassembly. Both µ-calpain and m-calpain, which promote the formation and disassembly of focal adhesions, were observed in the podosome belt of osteoclasts. Calpain inhibitors disrupted the podosome belt, blocked the constitutive cleavage of the calpain substrates filamin A, talin, and Pyk2, which are enriched in the podosome belt, induced osteoclast retraction, and reduced osteoclast motility and bone resorption. The motility and resorbing activity of µ-calpain−/− osteoclast-like cells were also reduced, indicating that µ-calpain is required for normal osteoclast activity. Histomorphometric analysis of tibias from µ-calpain−/− mice revealed increased osteoclast numbers and decreased trabecular bone volume that was apparent at 10 weeks but not at 5 weeks of age. In vitro studies suggested that the increased osteoclast number in the µ-calpain−/− bones resulted from increased osteoclast survival, not increased osteoclast formation. Calcitonin disrupted the podosome ring, induced osteoclast retraction, and reduced osteoclast motility and bone resorption in a manner similar to the effects of calpain inhibitors and had no further effect on these parameters when added to osteoclasts pretreated with calpain inhibitors. Calcitonin inhibited the constitutive cleavage of a fluorescent calpain substrate and transiently blocked the constitutive cleavage of filamin A, talin, and Pyk2 by a protein kinase C-dependent mechanism, demonstrating that calcitonin induces the inhibition of calpain in osteoclasts. These results indicate that calpain activity is required for normal osteoclast activity and suggest that calcitonin inhibits osteoclast bone resorbing activity in part by down-regulating calpain activity.

Osteoclasts are large multinucleated cells of the monocyte-macrophage lineage that play a critical role in skeletal development and repair and in calcium homeostasis by resorbing mineralized cartilage and bone. They display a high degree of motility, which is required for normal bone resorbing activity. As in other highly motile cells, the integrin-based attachment complexes of osteoclasts are podosomes, which are structurally and functionally distinct from focal adhesions (1–6). Although many of the same proteins are present in both podosomes and focal adhesions, podosomes are notably more dynamic, undergoing assembly and disassembly within minutes (5–7). The rapid and cyclic podosome assembly and disassembly and their dynamic interaction with elements of the actin cytoskeleton are thought to be critical for the high motility of osteoclasts (7).

Recently, calpains have been found to play crucial roles in the regulation of motility in cells such as fibroblasts that form focal adhesions (8). Calpains are a family of cytosolic cysteine proteases, many of which are Ca2+ dependent, that catalyze the limited cleavage of specific proteins during regulatory signaling (9, 10). The most studied members of the calpain family are the ubiquitously expressed µ-calpain (calpain 1) and m-calpain (calpain 2). These calpains are heterodimers composed of a large 80-kDa catalytic subunit that is unique for each of the two enzymes and a common 30-kDa regulatory subunit, termed calpain 4 (11).

In these cells, calpains are thought to promote cell spreading and locomotion by modulating the assembly and disassembly of the focal adhesion complexes (8, 12). µ-Calpain promotes the formation of adhesion sites in the leading edge of the cell. Conversely, recent evidence suggests that m-calpain promotes the disassembly of focal adhesion structures (8), in part by cleaving talin (13). Calpains have been reported to cleave a number of attachment-related proteins that are enriched in the peripheral podosome belt of osteoclasts, including talin (14–17), vinculin (18), paxillin (15), β integrin (10, 19), Src (20), Pyk2 (21), and focal adhesion kinase (15, 22, 23). Therefore, it is likely that one or more calpains could contribute to the regulation of podosome formation and assembly and thereby help regulate osteoclast attachment and motility.

Calpains could also affect the regulation of osteoclast bone resorption by cleaving caspase 3 (24–26), thereby promoting osteoclast apoptosis. Calcitonin (CT)5 is a potent inhibitor of osteoclastic bone resorption that acts directly and rapidly on osteoclasts via the calcitonin receptor, a class B-type G protein-coupled receptor that couples to numerous signaling pathways via Gαi, Gβγ/11, and G1 (27–38). Treatment of osteoclasts with CT reduces osteoclast motility, induces cell retraction, and disrupts the peripheral belt of podosomes that forms in osteoclasts plated on glass or dentin (27, 30, 39). This suggests that CT regulates at least some of the mechanisms responsible for podosome formation and function.
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ation, but little is known of the specific mechanisms by which CT exerts its effects. The CT receptor (CTR) activates both cyclic AMP-dependent protein kinase (PKA) and protein kinase C (PKC), and it also induces transient increases in [Ca^{2+}], (29, 34, 36–38). All of these signaling mechanisms are known to modulate calpain activity, either directly or indirectly through the highly specific endogenous calpain inhibitor calpastatin (40–43), suggesting that CT could regulate calpain activity and thereby modulate the assembly/disassembly of podosomes and consequently osteoclast motility and bone resorption.

In this study, we investigated whether calpain might play a role in the regulation of osteoclast retraction, motility, and bone resorbing activity and whether the inhibitory effects of CT upon osteoclast motility and bone resorption might involve the modulation of calpain activity. We found that reducing calpain activity with chemical inhibitors or by deleting the μ-calpain gene impaired osteoclast spreading, motility, and bone resorption in vitro and that CT transiently inhibited calpain activity. At the same time, reducing calpain activity prolonged osteoclast survival in a manner similar to the effect of CT. The in vivo effect of deleting the μ-calpain gene was a marked increase in the numbers of osteoclasts with low intrinsic bone resorbing activity, resulting in late onset osteopetrosis.

EXPERIMENTAL PROCEDURES

Reagents and Antibodies—Salmon calcitonin was purchased from Peninsula Laboratories, Inc. (Belmont, CA). Calphostin C, prostaglandin E2, minimum essential medium, α modification (α-MEM), Igepal CA-630, and fetal bovine serum (FBS) were from Sigma. Calpeptin, MDL28170, PD151746, (R)-isomer of 8-bromo-adenosine 3′,5′-cyclic monophosphorothioate ([R]),-8-Br-cAMPS), H-89, and anti-m-calpain antibody were purchased from Calbiochem. Anti-μ-calpain antibody was purchased from Biomol (Plymouth Meeting, PA). Anti-talin antibodies were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Anti-filamin antibody was purchased from Chemicon International (Temecula, CA). Anti-Pyk2/CAK antibody was purchased from BD Biosciences. Anti-cleaved caspase-3 antibody was purchased from New England Biolabs, Inc. (Beverly, MA). Both anti-mouse and anti-rabbit horseradish peroxidase-conjugated secondary antibodies were obtained from Fisher. Boc-Leu-Met-7-amino-4-chloromethylcoumarin (Boc-LM-CMAC) was purchased from Molecular Probes, Inc. (Eugene, OR).

Osteoclasts and Osteoclast-like Cells—Rabbit osteoclasts and osteoclast-like cells (OCLs) were generated as described elsewhere (44) with modifications. Briefly, the long bones and scapulae of neonatal New Zealand White rabbits (60–90 g) were dissected free of soft tissue and minced in α-MEM with 0.55 mg/liter sodium bicarbonate and 10 mM HEPES (pH 7.10). The bone fragments were then allowed to settle, the mesenchymal cells at 37 °C. The OCLs were lysed in 1% Igepal CA-630, 150 mM NaCl, 50 mM Tris-HCl (pH 8.0), 5 mM EGTA, 1 mM phenylmethylsulfonyl fluoride, 10 mM NaF, 0.4 mM Na2VO4, and 10 μg/ml leupeptin and aprotinin. The lysates were processed for immunoprecipitation and incubated with specific antibodies and protein A- or protein G-Sepharose, and the immune complexes were washed with 150 mM NaCl, 50 mM Tris-HCl (pH 8.0), 5 mM EGTA, 10 mM NaF, 0.4 mM Na2VO4, and 10 μg/ml each aprotinin and leupeptin. The samples were then processed for immunoblotting, as described elsewhere (46).

Calpain Activity Assay—Authentic osteoclasts were plated on glass coverslips for 24 h. They were then loaded with 30 μM Boc-LM-CMAC for 1 h at 37 °C. After loading, images of the cells were obtained before and after CT addition. Calpain-catalyzed cleavage of Boc-LM-CMAC creates a fluorescent product; fluorescence correlates with calpain activity (47–49).

Immunofluorescence Analysis—Authentic osteoclasts on coverslips were fixed in phosphate-buffered saline (PBS) containing 3.7% formaldehyde for 10 min at room temperature and washed in PBS. Coverslips for actin labeling were extracted in ice-cold acetone for 3–5 min and returned to PBS. All other coverslips were permeabilized in 0.05% saponin for 30 min. The coverslips were blocked in 5% normal goat serum for 30 min and then incubated in the appropriate primary antibody for 2 h, washed in PBS, incubated for 1 h in the appropriate fluorescent- or rhodamine-conjugated secondary antibody, and washed. Those coverslips used for actin labeling were incubated in a 1:40 dilution (in PBS) of rhodamine phalloidin (Molecular Probes) for 20 min and washed. All coverslips were mounted in FluorSave. Cells were examined using a scanning laser confocal imaging system (MRC-600; Bio-Rad). Images were recorded, composite images were compiled, and image enhancements were performed using Adobe Photoshop 6.0.

Cell Retraction Assay—Authentic rabbit osteoclasts were plated on glass or dentine slices for 24 h and then incubated for 30 min with vehicle or calpain inhibitors as described under “Results.” Cells were then fixed (inhibitor only) or treated with CT for 20 min before fixing (CT only, inhibitor plus CT). The fixed cells were stained with toluidine blue or TRAP-stained (50, 51), and the area of at least 70 osteoclasts/group was measured with a computerized system for histomorphometry (Osteometrics, Atlanta, GA).

Cell Migration Assay—Cell migration was analyzed as described elsewhere (52) with modifications. Authentic murine osteoclasts or OCLs were plated on semipermeable membranes coated with vitronectin in Boyden chambers (CHEMICON) and incubated for 18 h with and without calpain inhibitors as described under “Results” and then fixed and stained for TRAP. The TRAP-positive cells on the bottom side of the membrane were counted. Data values are expressed as a ratio relative to the values obtained from the untreated control chambers.

In Vitro Bone Resorption—Authentic murine or rabbit osteoclasts or murine OCLs were plated on dentine slices and cultured for 24 h. They were then incubated with or without CT or calpain inhibitors for an additional 16 h and then fixed and stained with toluidine blue. The pit resonance assays as described below. All animal protocols were approved by the Yale University Institutional Animal Care and Use Committee.
were fixed in 4% formalin and then embedded in methylmethacrylate as described (52). 5-μm sections were cut and stained either with toluidine blue or by the Von Kossa method for staining calcified tissues (52). 10-μm sections were cut from the same samples and coverslipped unstained for dynamic measurements. Histomorphometric analysis was carried out with an Osteomasure system (Osteometrics, Inc.,) using standard procedures (53) and in blind fashion. Tibial sections were measured in the proximal metaphysis beginning 340 μm below the chondro-osseous junction. Tibial cortical thickness and periosteal mineral appositional rates were measured beginning 680 μm from the chondro-osseous junction on the anterofibular side. Serum levels of pyridinoline cross-links (collagen type I fragments resulting from proteolytic digestion), a marker of osteoclastic bone resorption, were measured using an ELISA kit from Nordic Biosciences (Copenhagen, Denmark). At least six animals per group were examined. Statistical analysis was performed using analysis of variance, with p values less than 0.05 accepted as significant; error bars represent ± S.D.

Osteoclast Survival—OCL survival was measured as reported previously (45). OCLs were generated by coculturing bone marrow cells from μ-calpain−/− mice and genetically matched wild type mice with calvarial osteoblasts from neonatal CD-1 mice. Once the OCLs were formed, the calvarial cell layer was removed by collagenase/disase treatment. Some plates were immediately stained for TRAP. The remaining cells were cultured in α-MEM and 10% FBS with vehicle, calpain inhibitors, or CT for various times up to 24 h. The OCLs were then fixed and TRAP-stained. The numbers of TRAP-positive multinucleated cells were determined and expressed as a percentage of the TRAP-positive multinucleated cells present at the time when the calvarial cell layer was removed.

RESULTS

Both μ-Calpain and m-calpain Are Expressed in Osteoclasts and Localize in Podosomes—Calpain is known to modulate spreading (54) and motility (8, 55) of cells that form focal adhesions by cleaving several focal adhesion proteins, cytoskeletal proteins, and downstream effectors, including talin, focal adhesion kinase, Pyk2, Src, Rac1, and RhoA, and thereby regulating focal complex assembly/disassembly (8, 56). We hypothesized that calpains might therefore regulate the turnover of the podosomes and consequently osteoclast motility and bone resorption. Western blotting of lysates of OCLs generated in vitro revealed the presence of both μ-calpain and m-calpain (Fig. 1, A and B). In addition, immunofluorescence analysis showed that high levels of both μ-calpain (Fig. 1, C and D) and m-calpain (Fig. 1, E and F) were associated with the F-actin-rich belt of podosomes in authentic osteoclasts from both rabbit (Fig. 1, C and E) and mouse (Fig. 1, D and F). Calpain was enriched throughout the region between the individual podosomes (Fig. 2C).

Talin, Filamin A, and Pyk2 Are Present in the Podosome Belt and Are Constitutively Cleaved by Calpain in Osteoclasts—The calpain substrates talin and filamin A are actin-binding proteins that associate with focal adhesion structures (14, 57–59), and talin has been shown to localize to podosomes in osteoclasts and other highly motile cells (60, 61). Immunofluorescence confirmed that talin is associated with podosomes (Fig. 2A) and showed that filamin A is also expressed in the osteoclasts and localized in and around podosomes (Fig. 2B) in a manner similar to calpain (Fig. 2C). Pyk2, another calpain substrate protein (21), is a nonreceptor tyrosine kinase related to focal adhesion kinase that is highly enriched in the podosomes of osteoclasts (39, 62) and plays a key role in the Src-dependent regulation of osteoclast adhesion and motility following the activation of α5β1 integrin (46, 63, 64). Since podosomes form and disappear at a much higher rate than focal adhesions (5–7), we reasoned that if calpain-catalyzed proteolysis of these or other podosome-associated proteins contributes to the regulation of podosome turnover, and thus to the regulation of osteoclast attachment, motility, and spreading, it might be possible to detect calpain-generated cleavage products from these proteins in osteoclasts. Western blotting of OCL lysates with antibodies against talin (Fig. 3A), filamin A (Fig. 3B), or Pyk2 (Fig. 3C) detected all three proteins as well as less intense bands below the parent proteins of sizes consistent with reported cleavage products (15, 21, 65). Pretreatment of the OCLs with the calpain inhibitors calpeptin or MDL28170, which inhibit calpains by binding to the catalytic site (66), or PD151746, which inhibits calpains by binding to the calcium-binding site (67), reduced the amount of the lower bands in all three blots, strongly suggesting that one or more calpains are cleaving podosome-associated proteins in osteoclasts.

Calpain Is Required for Normal Osteoclast Spreading and Motility—Attachment, spreading, and motility of cells that form focal adhesions require the binding of attachment proteins, typically integrins, to the extracellular matrix and the subsequent engagement of the cytoskeleton by the integrin intracellular domain. The tension generated by these interactions leads to cell locomotion (68, 69). Since the calpain-catalyzed cleavage of cytoskeletal and attachment-related proteins regulates the assembly and disassembly of adhesion complexes (54) and cell motility (17, 55, 70, 71), we examined the effects of calpain inhibitors on osteoclast spreading and motility.

To determine if calpain promotes osteoclast spreading, authentic osteoclasts were isolated from rabbits or mice, cultured for 24 h, and then treated with calpain inhibitors or with CT as a positive control and fixed as described under "Experimental Procedures." Both CT and the calpain inhibitors induced a prompt cellular retraction of rabbit (Fig. 4A) and mouse (not shown) osteoclasts plated on either glass (not shown) or dentine (Fig. 4A) to 30–50% of the area of untreated cells, suggesting that calpain activity is required for the normal spreading of osteoclasts. Similar results were obtained with three different calpain inhibitors (calpeptin, MDL28170, and PD151746). The addition of both CT and a calpain inhibitor together induced no more retraction than either agent alone.

The effect of calpain inhibitors on osteoclast spreading suggested that calpain might also play a role in osteoclast motility. To address this question, osteoclasts were plated on vitronectin-coated semipermeable membranes in Boyden chambers, incubated with or without CT (positive control) or calpain inhibitors for 16 h, and analyzed for the amount of migration across the membrane (Fig. 4B) as described under "Experimental Procedures." Data were normalized to untreated controls. Treatment with calpain inhibitors caused a reduction of cell motility that was comparable with that induced by CT and, as was true of cell spreading, there was no additive reduction of motility when CT and a calpain inhibitor were both present.

To examine the specific role of μ-calpain in osteoclast migration, we analyzed the migration of OCLs generated from precursors obtained from μ-calpain−/− mice (72) and genetically matched wild type animals. The μ-calpain−/− OCLs exhibited less motility than the wild type controls but more motility than the CT-treated wild type OCLs (Fig. 4C). However, in contrast to the inability of CT to further reduce the motility of osteoclasts treated with calpain inhibitors (Fig. 4B), treatment with CT further reduced the motility of the μ-calpain−/− OCLs.
These results, together with the more complete inhibition obtained with different calpain inhibitors, suggest that osteoclast locomotion is also promoted by calpains other than \( \mu \)-calpain, most likely m-calpain, and that CT down-regulates all of the calpains that are blocked by the chemical inhibitors.

**Calpain Is Required for Normal Osteoclast Bone Resorbing Activity**

Bone resorbing activity is dependent on osteoclast motility (73). Thus, reducing motility by inhibiting calpain might reduce bone resorption. To test this, authentic rabbit osteoclasts were isolated as described under “Experimental Procedures,” plated on dentine slices, and incubated for 16–18 h in the presence or absence of calpain inhibitors or CT as a positive control, as indicated (Fig. 5A). The calpain inhibitors reduced pit formation by 80–90%, at least as much as the CT-induced inhibition. There was no additive effect when the osteoclasts were treated with combinations of CT and calpain inhibitors. To examine the specific role of \( \mu \)-calpain, we analyzed the resorbing activity of \( \mu \)-calpain \(^{-/-}\) OCLs plated on dentine slices for 16–18 h (Fig. 5B). Consistent with the results obtained with the rabbit osteoclasts, CT reduced the bone resorbing activity of the wild type murine OCLs by about 75%. The resorption by the \( \mu \)-calpain \(^{-/-}\) OCLs was about half of the resorption by the wild type OCLs, a significant reduction from the wild type activity but not as great a reduction as that achieved by treating the wild type OCLs with CT or by treating the rabbit osteoclasts with either CT or the calpain inhibitors. In contrast to the lack of an additional effect of CT on the bone resorbing activity of rabbit osteoclasts treated with calpain inhibitors, however, treatment of the \( \mu \)-calpain \(^{-/-}\) OCLs with CT induced a further reduction in the bone resorbing activity, suggesting again that at least one other effector that is inhibited by the calpain inhibitors and CT, most likely m-calpain, also contributes to osteoclast bone resorbing activity.

**Calcitonin Modulates Calpain Activity in Osteoclasts**

The similar effects of CT and calpain inhibitors on osteoclast retraction, motility, and bone resorbing activity, together with the lack of any additive effect of the two agents on these functions, suggested that CT might act at least in part by inhibiting calpain. Calpain activity can be inhibited by either PKC or PKA (43), both of which are activated downstream of the CTR (29, 34, 36, 37). Thus, CT could inhibit calpain activity in osteoclasts and thereby modulate osteoclast attachment, motility, and bone resorbing activity. To determine whether CT affected calpain activity in osteoclasts, rabbit osteoclasts were incubated in the presence of 30 \( \mu \)M Boc-LM-CMAC, a membrane-permeable fluorogenic calpain substrate, for 1 h, during which time the fluorescence increased in the...
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osteoclasts and contaminating cells as a consequence of constitutive calpain activity. Following the administration of CT, the fluorescence in the osteoclasts decreased, in contrast to the continued increased fluorescence in contaminating cells, particularly erythrocytes, that do not express CT receptors (Fig. 6A), consistent with the hypothesized down-regulation of osteoclast calpain activity by CT.

Since calpain inhibitors blocked the constitutive fragmentation of filamin, talin, and Pyk2 in osteoclasts (Fig. 3), we asked whether CT also affected the proteolysis of talin and filamin in a similar manner. To address the question, rabbit OCLs were treated with 10^{-8} M CT for various times, and the lysates were blotted with antibodies against filamin and talin (Fig. 6B). As noted before, both the full-length proteins and high MW cleavage products were detected in untreated OCLs. CT treatment transiently reduced the amounts of the fragments, which were largely absent at 5 min after the addition of CT and present again by 10–20 min, providing further evidence that CT inhibits calpain activity in osteoclasts.

PKC Mediates the CT-induced Inhibition of Calpain—The CTR signals through several effectors that are known to inhibit calpain in other cell systems, including PKA and PKC (29, 34, 36, 37). In order to determine whether either PKA or PKC mediate the CT-induced decrease in calpain activity, we examined the effect of PKC and PKA inhibitors on the CT-induced inhibition of the constitutive cleavage of talin. Mouse OCLs were treated with 10^{-8} M CT for 5 min in the absence and in the presence of calphostin C (25, 50, or 100 nM), H89 (50 or 100 nM) or Rho-8-Br-cAMPS (1 or 5 μM) and then lysed and processed for Western blotting with anti-talin antibody. Three high molecular weight proteins obtained from μ-calpain−/− mice and genetically matched wild type animals, as described under “Experimental Procedures,” in the presence or absence of 10^{-8} M CT. *p < 0.01 relative to untreated controls (C), the migration assay was performed with OCLs generated from bone marrow cells obtained from mouse and genetically matched wild type animals, as described under “Experimental Procedures,” and the TRAP-positive cells on the bottom surface of the membranes were counted. **p < 0.01 relative to untreated WT. #, p < 0.01 relative to the untreated μ-calpain−/− OCLs.
appearance of the talin cleavage product (Fig. 7A), whereas the PKA inhibitors had little effect (Fig. 7B), suggesting that CT acts via PKC but not PKA to inhibit calpain activity.

The Absence of μ-Calpain Causes Bone Loss in Mice—Based on the ability of calpain inhibitors to reduce osteoclast spreading, motility, and bone resorbing activity in vitro, the decreased motility and bone resorbing activity of μ-calpain−/− OCLs, and the CT–induced inhibition of calpain, we hypothesized that the μ-calpain−/− mice would have increased bone mass due to decreased bone resorption. Contrary to our prediction, however, Von Kossa staining of sections of tibias from 10 week-old μ-calpain−/− mice and genetically matched wild type mice revealed a marked decrease in the trabecular bone volume of the secondary spongiosa of the μ-calpain−/− bones (Fig. 8A). Histomorphometric analysis of 5- and 10-week-old μ-calpain−/− and wild type mice confirmed this observation. Trabecular bone volume (Fig. 8B) was markedly decreased at 10 weeks (but not at 5 weeks) in the μ-calpain−/− mice due to diminished trabecular number (Fig. 8, C and D). No difference was observed in the cortical bone thickness at either age (Fig. 8E).

Both osteoclast numbers and the amount of bone surface covered by osteoclasts were strongly increased by 30–60% in μ-calpain−/− mice relative to wild type controls at both 5 and 10 weeks (Fig. 8, F and G), whereas no difference was observed in either osteoblast surface or number at either age (Fig. 8, H and I).

Bone formation was assessed in the same mice by examining fluorochrome labels incorporated during mineralization. There were no significant differences in either mineralizing surface or mineral apposition rate at either 5 or 10 weeks (Fig. 8, J and K). As a result, bone formation rate was also unchanged (data not shown).

To further assess whether bone resorption was increased in the absence of μ-calpain, we assayed the serum level of pyridinoline cross-links produced from collagen type I digestion, a well-established serum marker of bone resorption, in 6-week-old μ-calpain−/− mice and found that this parameter was moderately increased (Fig. 8L). Given the marked elevation in the number of osteoclasts, the moderate increase in cross-links is consistent with the reduced bone resorbing activity of individual μ-calpain−/− OCLs that we observed in vitro. The results suggest that the reduced activity of the individual osteoclasts partly compensates for the greater number of osteoclasts, resulting in the slowly progressing osteopenia that was seen at 10 weeks but not at 5 weeks.
Inhibition or Disruption of Calpain Prolongs the Survival of OCLs—
The increased number of osteoclasts in the spongiosa of μ-calpain−/− mice could be the consequence of either increased osteoclast differentiation or increased osteoclast survival. We therefore examined the effect of deleting μ-calpain on the formation of TRAP-positive multinucleated OCLs in the in vitro coculture system. Similar numbers of OCLs were formed in the cocultures of bone marrow cells from wild type and μ-calpain−/− mice (Fig. 9A), indicating that μ-calpain is not required for normal osteoclast differentiation and suggesting that the increased numbers of osteoclasts observed in the μ-calpain−/− mice was not due to increased recruitment. On the other hand, both the absence of μ-calpain and the pharmacological inhibition of calpain prolonged the survival of the OCLs following the removal of the supporting calvarial stromal cells (Fig. 9, B and C). The increased survival of the μ-calpain−/− OCLs may explain the increased number of osteoclasts observed in the μ-calpain−/− mice. CT prolonged the survival of the OCLs in the absence of supporting stromal cells to the same degree as the presence of calpain inhibitors. Interestingly, CT and the calpain inhibitors were more effective at prolonging the survival of the OCLs than the absence of the μ-calpain gene was, suggesting again that these agents are affecting more than μ-calpain in osteoclasts.

Calpains are reported to trigger apoptosis in various cell types by cleaving and activating caspase-3 (24–26), and our laboratory has shown that caspase-3 is cleaved during OCL apoptosis (74). We therefore examined the effect of CT on the presence of cleaved caspase-3 in wild type OCLs cultured in the absence of supporting stromal cells (Fig. 9D) and found that CT inhibited the production of activated caspase-3. We also examined the rate of appearance of cleaved caspase-3 in wild type and in μ-calpain−/− OCLs (Fig. 9E). Cleaved caspase-3 appeared in both the wild type and the μ-calpain−/− OCLs after the supporting stromal cell layer was removed, but the level of cleaved caspase-3 was higher in the wild type OCLs at 12 and 18 h, which would be predicted to promote the more rapid apoptosis of the wild type osteoclasts.

DISCUSSION
Calpains are a family of cytosolic cysteine proteases that catalyze the limited cleavage of specific proteins during regulatory signaling (9, 10). The most studied members of the calpain family are the ubiquitously expressed μ-calpain and m-calpain, which are heterodimeric proteins composed of large 80-kDa catalytic subunits that are unique for the two enzymes and a common 30-kDa regulatory subunit, calpain 4 (11). The proteolytic activities of both μ-calpain and m-calpain are influenced by Ca2+ concentration. The two enzymes are also regulated by calpastatin, a highly specific endogenous peptide inhibitor of calpain, and by various signaling events, including PKA- and PKC-dependent mechanisms (43).
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FIGURE 9. Reducing calpain activity enhances the survival of OCLs. A, OCLs were generated from bone marrow obtained from μ-calpain−/− (CT−) and genetically matched wild type mice. After removal of the supporting stromal cell layer, the cells were fixed and stained for TRAP, and the TRAP-positive multinucleated cells were counted. The numbers in the WT and μ-calpain−/− cultures were similar at the time when the stromal cells layers were removed. B, OCLs were incubated in α-MEM, 10% FBS for 12 h after removing the supporting stromal cell layer and then stained for TRAP and counted. The number of viable OCLs remaining in the WT and μ-calpain−/− cultures after 12 h is shown as a percentage of the number of cells when the stromal cell layer was removed (T0). *, p < 0.01 relative to the WT cells. C, wild type murine OCLs were generated in coculture. The supporting stromal cell layers were removed, and the OCLs were further incubated in the presence or absence of CT (10−6 M) or calpain inhibitors (40 μg/ml CP; 50 μM MDL) or dimethyl sulfoxide alone (D; 1 μM/ml) for 12 h and then TRAP-stained and counted. *, p = 0.05 relative to untreated controls. D, Western blotting of cleaved caspase-3 in WT OCLs treated with 10−6 M CT and untreated controls. E, wild type (±) and μ-calpain−/− (−) OCLs were cultured for the indicated times in the absence of the supporting stromal cell layer. The time-dependent change in cleaved caspase-3 was detected by Western blotting with anti-cleaved caspase-3 antibody. The membrane was stripped and reprobed for actin to show relative sample loading.

Calpains have previously been shown to influence spreading and motility of cells that form focal adhesions and to regulate focal complex assembly/disassembly (8). Calpains cleave several focal adhesion proteins and downstream effectors, although the identities of the specific proteins whose cleavage is most critical for calpain’s effects on the formation of focal adhesions and on cell detachment have not been firmly established (75). In addition, the effects of calpain on cell migration and cell spreading are greatly influenced by cellular context and can be quite different in different cell types (75). Finally, the regulatory mechanisms and the dynamics of assembly/disassembly of focal adhesion structures and podosomes are significantly different, and a role for calpain in podosome regulation has yet to be reported. Since high motility and the dynamic turnover of podosomes are required for efficient osteoclastic bone resorption (7, 76), we sought to test the hypothesis that calpains regulate osteoclast adhesion and motility and thereby possibly contribute to normal bone homeostasis.

Our results support this hypothesis. Pharmacological inhibition of calpain activity in authentic osteoclasts and OCLs using agents that inhibit calpains by two independent mechanisms (modifying a critical cysteine residue in the catalytic site or blocking the calcium-binding site) caused pronounced cell retraction and a sharp reduction in osteoclast motility and bone resorption. The inhibitors also reduced or prevented the constitutive cleavage of three known calpain substrates that are associated with podosomes, talin, filamin, and Pyk2. To test the physiological relevance of the results obtained with the calpain inhibitors, we examined OCLs derived from μ-calpain−/− mice. The μ-calpain−/− OCLs consistently displayed both reduced migration and reduced bone resorption compared with genetically matched wild type cells, providing further evidence that calpain activity is required for normal osteoclast function. Based on the results of these in vitro experiments, we hypothesized that the trabecular bone volume of the μ-calpain−/− mice would be increased. Contrary to our hypothesis, however, we found that bone volume was decreased in these mice at 10 weeks of age, and there was a large increase in the number of osteoclasts at both 5 and 10 weeks. The serum level of pyridinoline cross-links, a marker of bone resorption, was only moderately increased in the μ-calpain−/− mice, however, consistent with the reduced in vitro bone resorbing activity of the μ-calpain−/− osteoclasts. The opposing effects of the increased numbers of osteoclasts in the μ-calpain−/− bones and the reduced resorbing activity of the μ-calpain−/− osteoclasts may explain the relatively slow onset of detectable osteopenia. It should be noted that calpain activity is also required for the normal differentiation of osteoblast cell lines (77), and we cannot rule out the possibility that a change in osteoblast function contributes in some way to the μ-calpain−/− bone phenotype. However, the fact that the histomorphometric analysis revealed no difference in osteoblast number, osteoblast surface, mineralizing surface, or mineral apposition rate indicates that a contribution by the osteoblasts, if any, is extremely subtle. Conversely, the significantly increased values of osteoclast number, osteoclast surface, and pyridinoline cross-links provide strong evidence that bone resorption is increased in the μ-calpain−/− animals.

The increased number of osteoclasts in the bones of the μ-calpain−/− mice appears to be due to increased survival of the osteoclasts rather than to increased production of the cells (Fig. 9). Consistent with reports in the literature (43), the inhibition or absence of μ-calpain prolonged cell survival and reduced the production of caspase-3 when OCLs were cultured in the absence of supporting stromal cells. It is likely that the reduced production of caspase-3 is at least partly responsible for the increased survival, since the cleavage and activation of caspase-3 by calpain (24) is known to trigger apoptosis in other cell types (25, 26). Our finding that the bone marrow cells from μ-calpain−/− mice form osteoclasts as efficiently as those from wild type is contrary to a recently published report that μ-calpain is essential for RANK ligand-induced differentiation of RAW 264.7 cells (78). The opposite findings of the two studies regarding a role for μ-calpain in osteoclast differentiation may be due to unappreciated differences between authentic osteoclast precursors and the RAW 264.7 cell line.

We also found that CT, a potent inhibitor of bone resorption, inhibited osteoclast calpain activity, assessed both by monitoring intracellular calpain-catalyzed cleavage of a fluorogenic substrate and by immunoblotting of the calpain substrates talin and filamin. The effect of CT was rapid, occurring in less than 5 min. The rapid onset indicates that the CT-induced reduction in calpain activity is a consequence of signaling events downstream of the CTR, not changes in calpain gene transcription, a conclusion that is supported by our failure to detect a change in the amounts of μ-calpain and m-calpain in CT-treated OCLs (data not shown). CT and the calpain inhibitors induced similar cell retraction and reductions in osteoclast motility and bone resorption. In all three assays, CT had no further effect on wild type osteoclasts pretreated with calpain inhibitors. However, CT did induce additional cell retraction and a further reduction of motility and bone resorbing activity in the μ-calpain−/− OCLs, in contrast to the lack of additive effects of CT and calpain inhibitors in wild type osteoclasts and OCLs. These results suggest that the calpain inhibitors and CT act on both μ-calpain...
and one or more other targets, probably including m-calpain, which was also identified in osteoclasts.

The absence of an additive effect of CT and calpain inhibitors on wild type osteoclast retraction, resorption, and motility suggests that the CT-induced inhibition of calpain contributes to the inhibitory effect of CT on osteoclast activity. This is not to suggest that the calpain inhibition is entirely responsible for the inhibitory effect of CT upon bone resorption. CT also modulates a number of important downstream signaling effectors in osteoclasts, including Erk1/Erk2, mitochondrial cytochrome C oxidase, and kinases and phosphatases that regulate tyrosine phosphorylation of a number of target proteins, including components of adhesion complexes (39, 74). The total inhibitory activity of CT depends on these activities as well. The transient effect of CT on the constitutive proteolysis of talin, filamin, and Pyk2 suggests that the inhibition by CT of calpain-catalyzed cleavage of cytoskeletal elements and components of attachment complexes may contribute particularly to the rapid onset of osteoclast retraction and decreased motility.

Our results confirm an earlier report that CT promotes osteoclast survival in vitro (79). The demonstration that CT inhibits both calpain activity and the generation of caspase-3 identifies a new component in the mechanism(s) by which CT affects osteoclast survival. Our laboratory has shown that CT induces the activation of Erk1/Erk2 in osteoclasts (39), which has been shown to promote osteoclast survival (45). Determining if and how CT-induced inhibition of calpain and activation of Erk combine to protect osteoclasts from apoptosis will be an important subject of future studies, particularly since studies in other cell types suggest that Erk activation promotes calpain activity (49, 80 – 82).

The calpain-dependent mechanisms that promote osteoclast attachment, motility, and bone resorption and the mechanisms by which CT inhibits calpain activity in osteoclasts remain to be characterized. We found evidence that at least three podosome-associated proteins, talin, filamin A, and Pyk2, are constitutively cleaved by calpain in osteoclasts, and it is likely that other podosome-associated calpain substrate proteins are also cleaved. Based on the reported roles of $\mu$-calpain and m-calpain in regulating focal adhesion-based cell attachment and motility, we suggest that calpain-catalyzed cleavage of podosome-associated proteins is required for the rapid assembly and disassembly of podosomes, which in turn is required for normal cell attachment and the high motility that characterized osteoclasts. In fact, two of the cleaved proteins that we identified (talin and Pyk2) have been implicated in the regulation of podosome structure and function (6, 46, 64). Whereas the high molecular weight cleavage products that we observed represent only a small fraction of the amount of the parent proteins, particularly in the cases of talin and filamin A, this is not unexpected if the proteolytic products are generated during a single step in a dynamic and cyclic process, such as podosome assembly and disassembly. First, it is unlikely that more than a small fraction of the parent proteins will be undergoing cleavage at any given time, and second, it will be important to clear the resulting fragments as quickly as possible so that they do not accumulate and interfere with the incorporation of intact parent proteins in newly assembling complexes. In fact, at least in the cases of talin and filamin, the proteolytic fragments are rapidly cleared, as shown by their disappearance within 5 min from rabbit OCLs treated with CT (Fig. 6B). Whereas our results suggest an important role for calpain in osteoclasts, significant additional research will be required to define how calpain-catalyzed cleavage of one or more podosome-associated proteins affect podosome dynamics and osteoclast function.

Several signaling mechanisms that are activated downstream of the CTR have been reported to reduce calpain activity. Both PKA and PKC, which are activated downstream of the CT receptor (29, 34, 36, 37), phosphorylate the two calpains (42, 43, 83) and the endogenous calpain inhibitor calpastatin (84 – 86), whose specificity and inhibitory efficiency are modulated by both PKA and PKC (40, 41). In addition, calcitomin is activated by lower, more physiological cytosolic Ca$^{2+}$ concentrations (87 – 89), suggesting that the increase in [Ca$^{2+}$], induced by CT (38) might contribute to calpain inhibition in osteoclasts. Although this study provides only preliminary evidence about the mechanisms responsible for CT-induced regulation of calpain activity, the experiments with the kinase inhibitors suggest that the mechanism involves PKC but not PKA. A role for PKC and not PKA is consistent with our earlier report that calcitonin-induced osteoclast retraction and inhibition of bone resorption is mediated by a PKC-dependent signaling pathway (30).

In conclusion, the present study provides evidence that calpain regulates osteoclast function in at least two somewhat antagonistic ways: first, by inducing cytoskeletal rearrangements and modulating cell attachment, thereby promoting osteoclast spreading, motility, and bone resorbing activity; and second, by limiting the life span of the osteoclast. Both of these activities contribute to determining the rate of overall bone resorption and thereby the balance point of skeletal homeostasis. Furthermore, the present study also suggests that calcitonin exerts its antiresorptive effect and promotes osteoclast survival in part by down-regulating calpain activity, possibly via the PKC signaling pathway.

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