Necessity of inositol (1,4,5)-trisphosphate receptor 1 and μ-calpain in NO-induced osteoclast motility

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Summary

In skeletal remodeling, osteoclasts degrade bone, detach and move to new locations. Mechanical stretch and estrogen regulate osteoclast motility via nitric oxide (NO). We have found previously that NO stimulates guanylyl cyclase, activating the cGMP-dependent protein kinase 1 (PKG1), reversibly terminating osteoclast matrix degradation and attachment, and initiating motility. The PKG1 substrate vasodilator-stimulated protein (VASP), a membrane-attachment-related protein found in complexes with the integrin αvβ3 in adherent osteoclasts, was also required for motility. Here, we studied downstream mechanisms by which the NO-dependent pathway mediates osteoclast relocation. We found that NO-stimulated motility is dependent on activation of the Ca^{2+}-activated proteinase μ-calpain. RNA interference (RNAi) showed that NO-dependent activation of μ-calpain also requires PKG1 and VASP. Inhibition of Src kinases, which are involved in the regulation of adhesion complexes, also abolished NO-stimulated calpain activity. Pharmacological inhibition and RNAi showed that calpain activation in this process is mediated by the inositol (1,4,5)-trisphosphate receptor 1 [Ins(1,4,5)P_3R1] Ca^{2+} channel. We conclude that NO-induced motility in osteoclasts requires regulated Ca^{2+} release, which activates μ-calpain. This occurs via the Ins(1,4,5)P_3R1.

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Introduction

The osteoclast is a motile, multinucleated monocyte-derived cell. It degrades mineralized cartilage or bone. In air-breathing vertebrates, the skeleton is the principal support for the body and is also a reservoir of minerals for Ca^{2+} homeostasis. Skeletal weight must be minimized while retaining adequate strength to resist complex physical and metabolic stresses. As the cell that mediates bone turnover, the osteoclast is subject to regulation of activity that is sensitive to time and place. Regulation of osteoclastic motility is important to its overall function, as the cell must constantly move to new sites of active bone turnover.

Triggers of osteoclast motility include nitric oxide (NO) (Yaroslavskiy et al., 2005). Osteoclasts express inducible NO synthase (iNOS or NOS2) (Kasten et al., 1994), and a small amount of NO production occurs in osteoclast cultures (Yaroslavskiy et al., 2004). Osteoclastic NO production may be increased by upregulation of expression of iNOS (Sunyer et al., 1996). In addition to autocrine production of NO, osteoblasts and vascular endothelial cells regulate bone turnover via NO from the endothelial NOS (eNOS or NOS3). The NOS3 is, in turn, regulated by two important bone-mass-governing agents, estrogen (Armour, K. E. et al., 2001) and mechanical stretch (Nomura and Takano-Yamamoto, 2000).

NO regulates osteoclast motility via the NO-dependent guanylyl cyclase and the cGMP-dependent protein kinase 1 (PKG1) (Yaroslavskiy et al., 2004). The vasodilator-stimulated protein (VASP) is a target of PKG1 that appears to be essential for NO-induced osteoclast motility (Yaroslavskiy et al., 2005). However, the links between the NO-PKG1-VASP pathway and processes that initiate motility are unknown. A mechanism to mediate cell detachment is clearly required, and this is an uncharacterized key step in the process. Potential targets for NO effects on osteoclast movement include the activation of proteinases that are required for motility in other contexts. Members of the calpain family of proteinases are implicated in the detachment and movement of numerous cell types in response to diverse stimuli, although their involvement in NO-dependent motility has not been studied. The calpain proteinases usually require increases in intracellular Ca^{2+} for activation. Indirect evidence links osteoclast motility with increases in intracellular Ca^{2+}. Osteoclast motility involves Ca^{2+}-dependent protein kinase activity (Sanjay et al., 2001), but the source and regulation of the Ca^{2+} are not clear.

To resolve these issues, we investigated the regulation of proteinase activity in osteoclasts after motility was induced with NO or cGMP agonists. We found that μ-calpain (CAPN1) activity is a key element required for efficient NO-induced motility of osteoclasts. The μ-calpain is regulated, at least in major part, by a Ca^{2+} signal. Generation of this Ca^{2+} signal by NO or cGMP stimulation requires PKG1 and a VASP-containing protein complex. Further, this calpain activation is dependent on inositol (1,4,5)-trisphosphate receptor 1 [Ins(1,4,5)P_3R1], an endosomal Ca^{2+} channel whose

occurrence and function in the osteoclast is described for the first time here.

Results

NO and cGMP activate calpain in osteoclasts

We investigated the activity of proteinases after NO- or cGMP-stimulation of osteoclast motility. Calpain activity was measured using the calpain substrate t-butoxycarbonyl-Leu-Met-chloromethylaminocoumarin (BOC), a membrane-permeable substrate that fluoresces after calpain cleavage (Rosser et al., 1993). Assays compared activity in situ in untreated osteoclasts and in osteoclasts treated with agonists or antagonists of NO and cGMP (Fig. 1A). Addition of the NO donor sodium nitroprusside (SNP) increased calpain substrate degradation. The NO synthase inhibitor NG-monomethyl-L-arginine acetate (L-NMMA) reduced calpain activity compared with untreated controls, consistent with inhibition of the endogenous NO production, although this response was variable owing to the variability of autocrine NO activity in osteoclasts. Treatment with Rp-cGMPS, a blocking analog of cGMP, similarly inhibited calpain activity. Fig. 1B shows examples of BOC assay results, demonstrating the differences in BOC fluorescence in control and NO activated cells. In this instance L-NMMA is clearly less than control, but this did not occur in every case due to the variability of autocrine NO production (see Fig. 1B). These experiments are consistent with the activation of calpain by NO and cGMP.

Calpain activation depends on PKG1 and VASP

NO-stimulated osteoclast motility is dependent on cGMP, PKG1 and the adaptor protein VASP (Yaroslavskiy et al., 2004; Yaroslavskiy et al., 2005). We therefore studied osteoclasts using small interfering RNA (siRNA) targeting PKG1 or VASP to determine whether calpain activation is downstream from PKG1 and VASP, or whether more proximate effectors suffice for calpain activation. Compared with control-siRNA-transfected cells, calpain activation by the cGMP analog 8-pCPT-cGMP was inhibited in cells with siRNA-mediated suppression of PKG1 or VASP (Fig. 1C).

cGMP-stimulated motility is severely curtailed when calpain activity is inhibited

To test whether μ-calpain is required for NO and cGMP-stimulated osteoclast motility, cells were treated with the

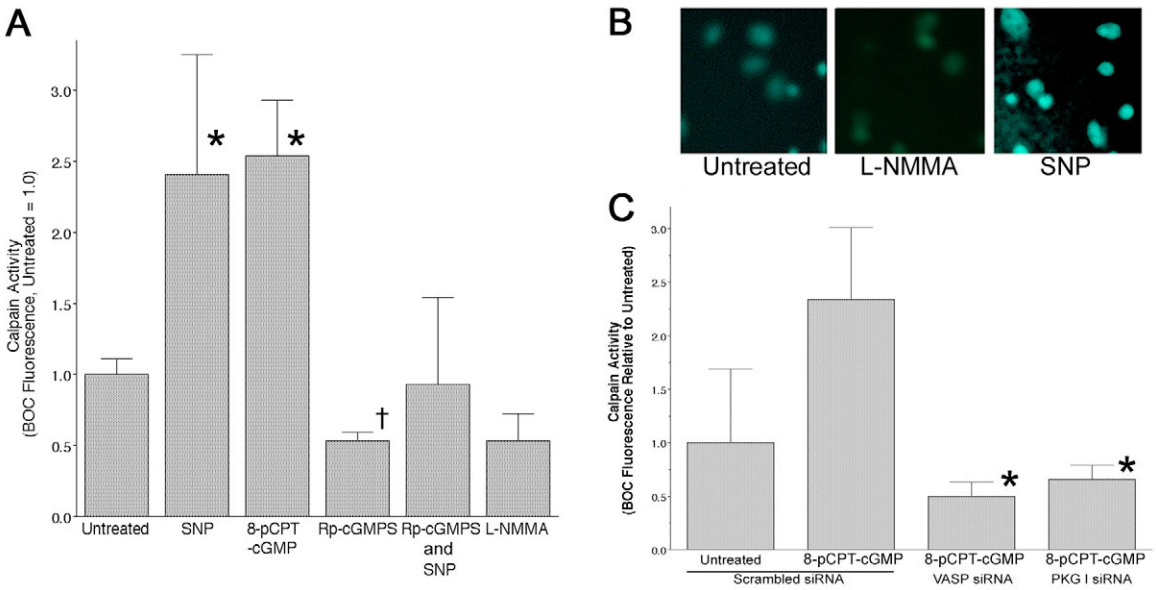

Fig. 1. NO activates calpain in osteoclasts, antagonists of NO or cGMP inactivate it. (A) Fluorescent calpain substrate assays in living NO-inhibited or NO-activated osteoclasts. Cells were incubated 20 minutes in 50 μM of the cell-permeable calpain substrate BOC. Quenching BOC is separated from the fluorescent coumarin when proteinase activity cleaves the peptide linker (Leu-Met), increasing fluorescence intensity. Untreated cells are compared with cells incubated with the NO donor SNP (100 μM), the activating cGMP analog 8-pCPT-cGMP (100 μM), the cGMP-blocking analog Rp-cGMPS (50 μM) and the inhibitor of NO synthase L-NMMA (1 μM). Results from four experiments, each measuring a minimum of 16 cells, summarized as the mean ± s.d. of each experiment, normalized to untreated controls. The NO and cGMP agonists increased calpain activity relative to untreated controls (*P<0.05), and relative to the inhibitors Rp-cGMPS and L-NMMA (P<0.01). Inhibitors reduced substrate degradation relative to untreated control, but the difference was significant only for Rp-cGMPS ($^{\dagger}P$<0.05). Note that, in the presence of Rp-cGMPS, the change in calpain activity after NO addition is small and not significant (fifth versus fourth bar). (B) Images of BOC fluorescence in cells with key treatments. SNP (right) increased fluorescence in essentially all of the cells with respect to control (left). Inhibitors such as L-NMMA (middle) reduce activity relative to untreated control, but the difference varies from experiment to experiment due to the variability in autocrine NO production in untreated cells. This is reflected also in the variability of response of osteoclasts to Rp-cGMPS and L-NMMA shown in A. (C) Calpain activity is suppressed by knockdown of VASP or PKG1. BOC fluorescence assays were carried out as in A but cells had been transfected with scrambled control siRNA or siRNAs targeting VASP and PKG1. The cells had been transfected with Cy3-labeled siRNAs 72 hours prior to the assay, and treated with 100 μM 8-pCPT-cGMP for 60 minutes prior to the assay. The assay scored BOC fluorescence over background in cells labeled with Cy3 (red). PKG1 suppression and VASP suppression reduced cGMP-stimulated calpain activity relative to the control (*P<0.05, in both cases; n=6, mean ± s.e.m.). Western blots for knockdown efficacy are shown in Fig. 6.

calpain inhibitor Calpeptin and stimulated or not with the active cGMP analog 8-pCPT-cGMP. In the presence of Calpeptin, the effect of the cGMP analog on motility over 2 hours was reduced by 60-70% relative to cells treated with 8-pCPT-cGMP alone (Fig. 2A). Inhibition of calpain activity equivalent to that in Calpeptin occurred when 50 μM calpastatin was used, but not when using 50 μM scrambled calpastatin peptide (Fig. 2B). The calpain inhibitors reduced calpain activity to below that of untreated cells, and blocked the increase due to NO or cGMP agonists by over 90%. The calpain antagonist, N-acetyl-Leu-Leu-norleucinal (ALLN; 20 μg/ml) was also tested; its effect was similar to that of calpeptin (not shown). Acute effects of the calpain inhibitor Calpeptin on cGMP-stimulated cell attachment were evaluated. Cell attachment footprint decreased over 10 minutes in response to cGMP activation, despite treatment with Calpeptin (Fig. 2C). Thus, although osteoclast motility is sensitive to calpain antagonists, changes in osteoclast attachment in response to NO and cGMP reflect also – at least in part – mechanisms that are independent of calpain.

μ-calpain is the major Ca^{2+}-dependent osteoclast calpain, and its suppression greatly reduces NO-stimulated calpain activity

Non-amplified transcriptional profiling using osteoclast mRNA prepared as described (Garcia-Palacios et al., 2005) revealed that osteoclasts express both of the calpains commonly associated with motility: μ-calpain and m-calpain (CAPN2). Four specialized calpains present in the microarray were not detected (supplementary material Table S1). We studied calpain activity in vitro in osteoclast lysates using zymography. Zymograms were developed in 30 μM or 100 μM Ca^{2+} using Ca^{2+}-EGTA buffers calibrated with a Ca^{2+} electrode. These Ca^{2+} concentrations activate μ-calpain. Mechanisms by which m-calpain is activated are not fully defined, but, when phosphorylated, m-calpain can be activated, to some extent, at micromolar Ca^{2+} (Glading et al., 2004). In zymograms of osteoclast lysates developed at either 30 μM or 100 μM Ca^{2+}, only μ-calpain activity was detected (Fig. 3A). As expected, when zymograms were performed after Ca^{2+} was reduced to low levels by excess EGTA, calpain activity was abolished (not shown). Lysates from NO or cGMP-treated osteoclasts were evaluated to determine whether these treatments altered the calpain activity at micromolar Ca^{2+}. Lysates from osteoclasts stimulated with NO or cGMP showed no consistent differences from unstimulated osteoclast lysates in zymograms at 30 μM or 100 μM Ca^{2+}: again, μ-calpain, but not m-calpain, activity was detected (not shown). Thus, it did not appear that NO or cGMP treatment resulted in post-translational modifications of m-calpain that permitted activity at micromolar Ca^{2+} concentrations.

Following NO or cGMP activation of osteoclasts, western blots of osteoclast lysates showed only minor increases in degradation fragments from the attachment-related calpain

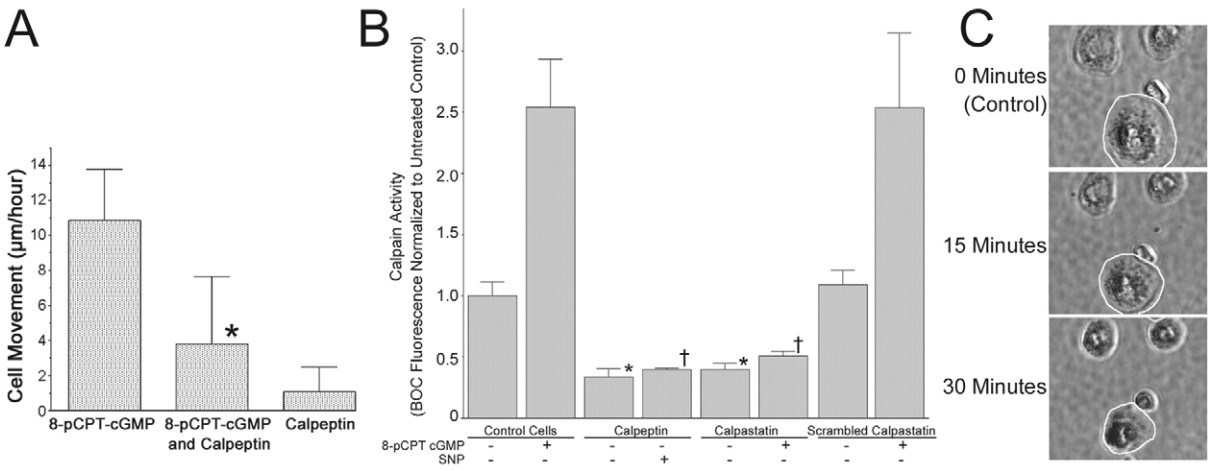

Fig. 2. Effects of calpain antagonists on on cGMP-induced motility and calpain activity. (A) Motility of NO-activated cells with and without 10 μM of the calpain inhibitor Calpeptin. There was very little cell movement of Calpeptin-treated osteoclasts relative to cGMP-activated cells (8-pCPT-cGMP). In cGMP-activated cells with Calpeptin added (middle bar), motility was reduced relative to cGMP-activated cells by 60%, with considerable variation between cells. However, the effect relative to 8-pCPT-cGMP-only treatment is significant (*P<0.05), averaging measurements over 20 cells ± s.d. (B) Effect of Calpeptin, calpastatin and calpastatin scrambled peptide on calpain activity in osteoclasts. Calpain activity was determined using the BOC assay as described for Fig. 1, in osteoclasts without stimulus or treated with the cGMP agonist 8-pCPT-cGMP or the NO donor SNP. Note that the scrambled calpastatin gives results essentially congruent with the controls (right two versus left two bars), whereas Calpeptin and calpastatin inhibited activity to below control cells and prevented calpain activation after cGMP or NO stimulation by more than 90%. Average of four experiments ± s.d. (left two bars) and of two experiments ± s.d. (right six bars) with each experiment measuring fluorescence in ~40 cells. *P<0.05 relative to control unstimulated cells (first bar). $^{\dagger}P$<0.01 relative to control stimulated cells (second bar). (C) Cell diameter decreased after cGMP was activated in Calpeptin-treated cells that did not show significant linear translocation. (Top panel) Group of three osteoclasts and a single smaller cell, probably an un-fused CD14 derived macrophage. Calpeptin and 8-pCPT-cGMP were added added at the time the top phase photograph was taken. (Middle and bottom panels) No linear translocation but the footprint of the cells shrank (compare encircled cell). Cell diameter decreased 15.7±5.1% at 15 minutes and 18.1±1.4% at 30 minutes (n=3, mean ± s.d.). After 30 minutes the mean diameter did not change significantly (not shown). Cell diameter also decreased with cGMP activation only (Yaroslavskiy et al., 2005), but not with Calpeptin treatment alone (not shown). Thus, NO-induced rearrangement of the cell attachment does not solely depend on calpain.

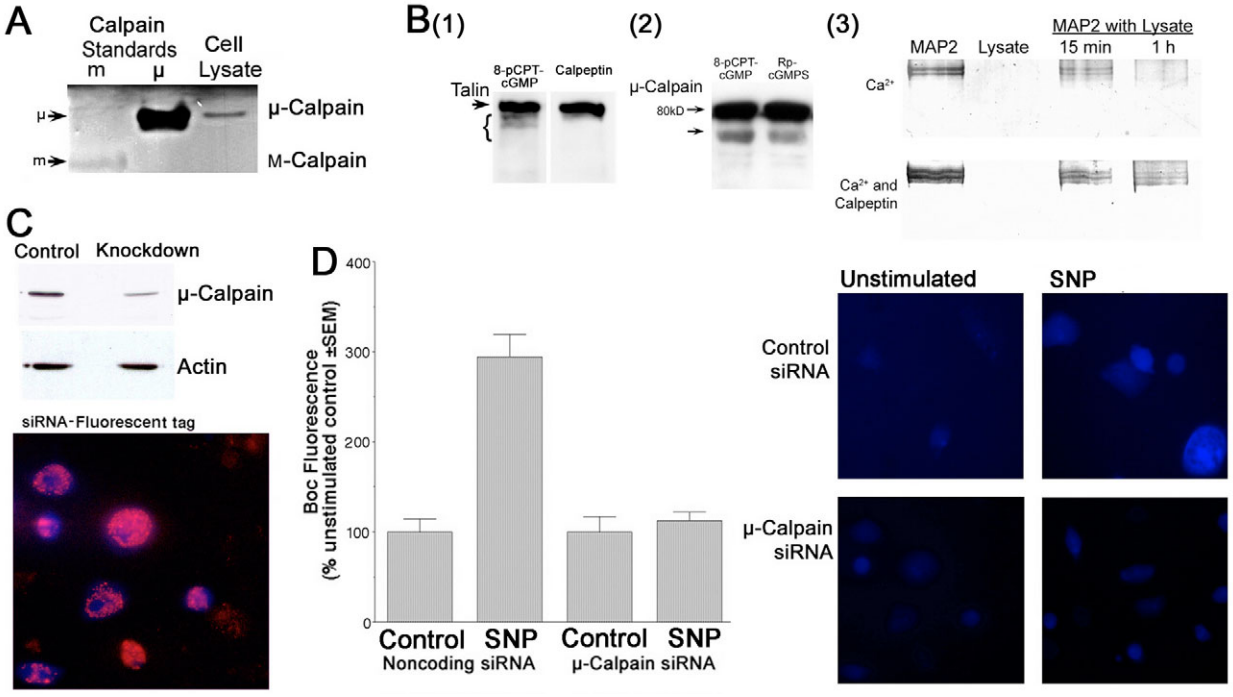

Fig. 3. Calpain activity in osteoclast lysates and intact osteoclasts, and the effect of suppressing μ-calpain expression on NO-induced calpain activity in situ in osteoclasts. (A) The main active calpain in osteoclasts is μ-calpain. A zymogram is shown as a negative image (degradation is dark) with recombinant μ-calpain and m-calpain standards, and osteoclast lysate (10 μg). The zymogram was developed in Ca^{2+}-EGTA buffers with 30 μM Ca^{2+} activity. Additional zymograms (not shown) were performed under the same conditions (30 μM Ca^{2+}) or at 100 μM Ca^{2+} with lysates from osteoclasts treated with NO or cGMP. These studies showed no consistent differences between lysates from stimulated and unstimulated cells. m-calpain activity was not detected even when zymograms were developed at 100 μM Ca^{2+}. (B) Only small amounts of processed μ-calpain or of talin (a calpain substrate) accumulate in cGMP-activated osteoclasts, although osteoclast lysates degrade calpain substrates at constantly elevated Ca^{2+} levels. (1) Western blot of talin (10% SDS-PAGE). A minor amount of degradation fragments accumulated in osteoclasts activated by 8-pCPT-cGMP for 1 hour, compared with cells treated with the calpain inhibitor Calpeptin (another lane from the same gel). Several other conditions were tested; the difference shown is the largest seen. Talin links actin to the integrin complex and is an established target for partial proteolysis by calpain. (2) Western blot of μ-calpain (10% SDS-PAGE). Osteoclast lysates showed small amounts of partially cleaved enzyme after cGMP activation. The large subunit of μ-calpain (~80 kD) did not vary measurably after treating cells with several NO or cGMP activators and inhibitors (not shown), although partially cleaved enzyme (~70 kD) was increased slightly at treatment with NO donor or cGMP activating analog (compare lower bands). Osteoclast lysates (30 μg) were prepared from osteoclasts treated 1 hour with 100 μM of the cGMP activator 8-pCPT-cGMP or 50 μM of the inhibitor Rp-cGMPS. (3) MAP2 degradation by osteoclast calpain in vitro (6% SDS-PAGE; silver stain). MAP2 is a structural protein (280 kDa) that is not expressed in osteoclasts and is a sensitive and well-studied calpain substrate. (Top panel) Comparisons show 2 μg of untreated MAP2, 3 μg of osteoclast lysate (Lysate), and MAP2 incubated for 15 minutes and 1 hour in buffer containing 100 μM Ca^{2+} with 3 μg of osteoclast lysate. Lysate was made from osteoclasts pre-treated 30 minutes with 8-pCPT-cGMP. (Bottom panel) Results with lysates from osteoclasts pre-treated with 10 μg Calpeptin. (C) siRNA inhibition of μ-calpain. Five days after transfection of siRNA targeting μ-calpain, the protein was reduced 85-90% as determined by western blotting. Micrograph shows efficiency of siRNA uptake was >95% (fluorescence microscopy of Cy3-labeled siRNA). Nuclei were labeled with Hoechst dye (blue) to demonstrate that very few cells were without siRNA uptake. (D) Specificity of intra-osteoclastic proteolysis for μ-calpain. The graph shows average BOC fluorescence of 20 cells (mean ± s.e.m.) normalized to unstimulated controls, for osteoclasts transfected with either non-coding siRNA or siRNA targeting μ-calpain, and treated or not with 200 μM of the NO-donor SNP for 10 minutes. Note that the NO donor fails to stimulate significant degradation of the fluorescent calpain substrate in cells transfected with the siRNA targeting μ-calpain. The reduction in NO-dependent calpain activity was comparable to the reduction in μ-calpain protein. Photomicrographs show sample fields of cells transfected with non-coding siRNA (top panels) or μ-calpain-targeting siRNA (bottom panels) without (left) or with (right) treatment with SNP. Note that the NO donor causes significant degradation of the fluorescent calpain substrate only in the control cells (top right panel).

target talin (Fig. 3B1). Western blots also showed only minor changes in μ-calpain, suggesting that μ-calpain degradation fragments do not accumulate in significant quantities in vivo (Fig. 3B2). However, osteoclast lysates completely degraded an exogenous μ-calpain substrate in vitro during a similar period (Fig. 3B3). The difference between calpain activity in vitro and in vivo probably reflects tight regulation of calpain access to substrates in the intact cell, and also that calpain activation is intermittent in vivo. Removal of degradation products and regeneration of the attachment site apparently is sufficient to prevent accumulation of damaged proteins.

The functional role of μ-calpain in osteoclasts after cGMP activation was confirmed by suppressing μ-calpain using siRNA (Fig. 3C,D). Three-day incubation with siRNA gave poor suppression of μ-calpain (~50%; data not shown), in keeping with the reported high stability of the enzyme. However, 5 days after transfection of siRNA targeting μ-calpain the protein was reduced 85-90% (Fig. 3C, upper blot); efficiency of siRNA

uptake was >95%, as shown using Cy3-labeled siRNA (Fig. 3C, photomicrograph). NO-stimulated calpain activity in osteoclasts was then determined using the BOC assay. When μ-calpain expression is inhibited by RNA interference (RNAi), NO-dependent calpain activity was reduced ~90%, in keeping with the level of protein expression (Fig. 3D).

Ca^{2+} increases during NO- or cGMP-induced cell movement

Because μ-calpain is Ca^{2+} dependent, NO and cGMP-dependent activation of μ-calpain suggested that NO initiates intermittent Ca^{2+} fluxes in osteoclasts. Whereas Ca^{2+} and calmodulin are necessary for osteoclast activity (Radding et al., 1994), we found no previous reports of data on NO- or cGMP-activated Ca^{2+} currents in osteoclasts. We studied Ca^{2+} in osteoclasts stimulated by NO or cGMP agonists using Ca^{2+} imaging. Cells were loaded with Ca^{2+}-sensitive fluorophores, Fluo3 for single wavelength measurements, or Oregon Green 488 BAPTA with Fura Red for ratio imaging. The Ca^{2+} activity was measured as fluorescence intensity during 200- to 250-msecond periods, at intervals of 2-5 minutes, for 30-60 minutes. Motility of cGMP-treated cells corresponded to increases in Ca^{2+}, involving variable areas of the cell during movement (Fig. 4A). Ca^{2+} images (in Fig. 4A, Fig. 5, and in Movies 1-3 in supplementary material) were processed to display relative Ca^{2+} signals as false color (Materials and Methods). Ca^{2+} activity was prominent in moving cells. Inhibiting versus activating analogs of cGMP produced 75% fewer motile cells with elevated Ca^{2+} (Fig. 4B). Ratio imaging was used to estimate the average Ca^{2+} activity in cells before and after addition of SNP, which increased the Ca^{2+} level in moving cells to ~100 times that of controls (Fig. 4C). Ca^{2+} measurements after stimulation varied greatly. Thus, the average value of ~5 μM may underestimate peak Ca^{2+} activity, which might be highly localized and subject to rapid fluctuation (Fig. 4A). However, Ca^{2+} peaks in the low micromolar range are consistent with previous reports (Radding et al., 1999).

Additional Ca^{2+}-motility studies are summarized in Fig. 5. These are based on Movies 1, 2 and 3 in supplementary material. Ca^{2+} images of osteoclasts on glass, using single wavelength imaging (Fig. 5A,B) and dual-wavelength-ratio imaging (Fig. 5C) are shown. Cells were stimulated with cGMP-activating analogs and NO donors or PKG1 was blocked using cGMP-inactivating analogs. Subtraction of images that were taken 30 minutes apart shows that the cells with elevated Ca^{2+} levels comprise the major population of moving cells (Fig. 5A-C, right image of each pair). NO donors and cGMP activating analogs produced similar effects (compare Fig. 5A with C). In the presence of the cGMP antagonist Rp-cGMPs, there was much less motility. On the one hand, some moving cells showing elevated Ca^{2+} levels were seen when using cGMP antagonists (Fig. 5B), which demonstrates that cGMP-independent motility mechanisms also occur in the cells studied. On the other hand, the few motile cells that were seen when using Rp-cGMPs were small and atypical, which may represent, in part, non-osteoclastic monocytes that contaminate osteoclasts produced by in vitro differentiation.

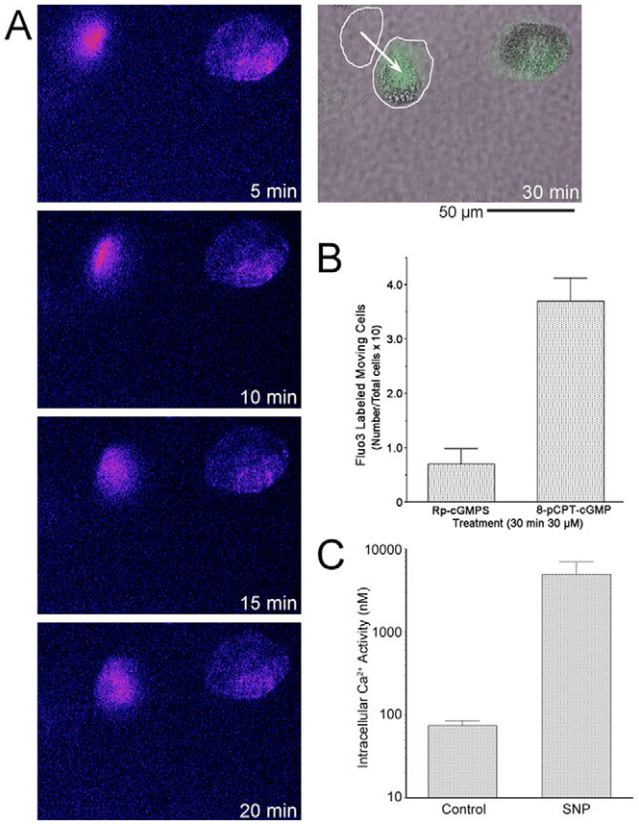

Fig. 4. Ca^{2+} signals during movement of cGMP- or NO-activated osteoclasts. (A) Elevated Ca^{2+} in a moving osteoclast after cGMP activation. Four false-color Ca^{2+} images at 5-minute intervals are shown. Each frame depicts an average Fluo 3 signal from a confocal image exposed for 200 mseconds, showing Ca^{2+} activity as fluorescence at 525 nm. Sixty minutes before imaging, cells were treated with 100 μM 8-pCPT-cGMP, which induces motility in a large fraction of cells. Two cells are shown. One shows minimal changes in Ca^{2+} and did not move (right cell in each frame). The other (left cell in each frame) moved during the period shown. Movement occurred with large, localized, changes in Ca^{2+} (first and second frames) and was largely complete after 20 minutes. False color scale: red ~1 μM > violet ~100 nM > blue; black, no signal. (B) Large Ca^{2+} transients and motility were much less common in cells when the the cGMP pathway was inhibited. The number of moving cells and a Fluo3 signal two-fold above background was determined in four experiments, at 3-minute intervals over 1 hour in each experiment. Ca^{2+} fluxes with motility were uncommon in cells treated with the cGMP antagonist Rp-cGMPS (50 μM; left bar, n=43 cells) but frequent in cells treated with the cGMP analog 8-pCPT-cGMP (100 μM; right bar, n=77 cells) 30 minutes prior to analysis. (C) Mean intracellular Ca^{2+} activity from ratio imaging of osteoclasts with Oregon-Green-BAPTA and Fura Red. The average intracellular Ca^{2+} levels in unactivated cells was 74 nM; 15-30 minutes after treatment with 100 μM SNP the average intracellular Ca^{2+} level increased to 5 μM, although some measured values were indistinguishable from the maximum G:R ratio so, probably, some cells with higher intracellular Ca^{2+} levels occur (mean ± s.e.m.; n=10).

Osteoclast calpain activation is dependent on Src

PKG1- or VASP-knockdown cells did not show calpain activity after cGMP activation (Fig. 2A), which suggests an attachment complex that includes VASP is required for calpain activity and, hence, a membrane protein complex is regulating Ca^{2+} release. Membrane receptors, such as the αvβ3 integrin, CSF-1 receptor and RANK, regulate osteoclast motility or cell spreading in other contexts. All of these can increase Src and

phosphatidylinositol-3-kinase (PI 3-kinase) activity. PI 3-kinase is associated with osteoclast spreading (Grey et al., 2000), and many attachment-related Ca^{2+}-release mechanisms depend on Src. The PI 3-kinase phosphorylates Ins(4,5)P_3 to Ins(3,4,5)P_3. Its downstream activity is related to Ca^{2+} currents (Faccio et al., 2003; Golden and Insogna, 2004; Komarova et al., 2005), but the functional role for an Ins(3,4,5)P_3-sensitive Ca^{2+} channel in the osteoclast (Hsu et al., 2000) was unknown. Thus, we studied Src dependency of calpain activity following activation of cGMP, and phosphorylation of Akt, downstream of the PI 3-kinase pathway. Calpain activation does depend on Src because the Src inhibitor PP2 abolished calpain activity after cGMP activation (Fig. 6A). Thus, either Src or Src family kinases sensitive to PP2 are elements of the pathway. Knockdown of PKG1 or VASP impaired cGMP-stimulated Src phosphorylation (Fig. 6C), confirming the importance of PKG1 and the intermediate protein VASP in Src activation downstream of cGMP activation in osteoclasts. However, cGMP activation did not increase phospho-Akt (Fig. 6B). Thus, Ca^{2+} release after NO activation is unlikely to depend on PI 3-kinase activity.

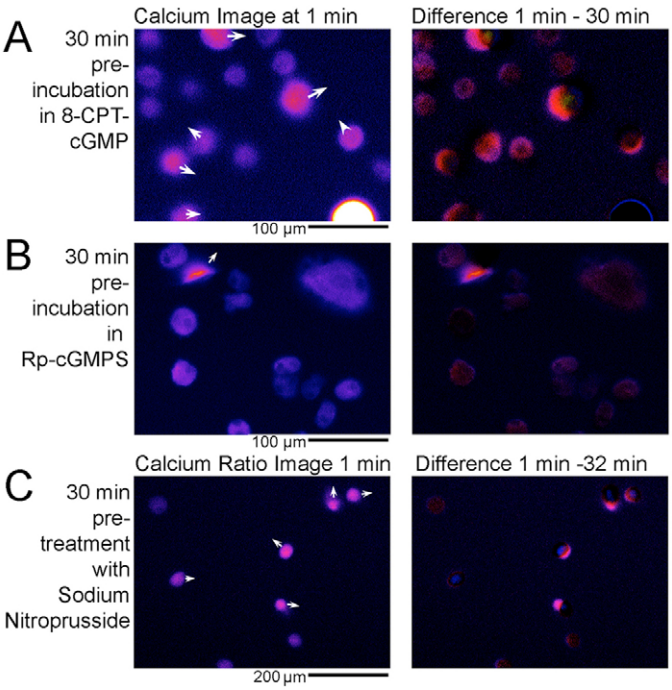

Fig. 5. Increased intracellular Ca^{2+} activity and increased motility in cells treated with cGMP agonists or NO donors compared with cGMP inhibitors, see Movies 1-3 in supplementary material for more detailed information. The false color scale is the same as that described in Fig. 4. (A) Increased motility and Ca^{2+} levels after preincubation with a cGMP agonist. After a 30-minute incubation with 100 μM 8-pCPT-cGMP, many of the cells show elevated Ca^{2+} levels using Fluo3 (left). These cells are moving, as shown by subtraction of a 30-minute image from a image taken at 0 minutes (right). (B) Low motility and low Ca^{2+} levels after preincubation with a cGMP antagonist. After a 30-minute preincubation with 50 μM 8-pCPT-cGMP only one cell with high Ca^{2+} levels is seen (left). This cell is moving, as shown by subtraction of a 30-minute image from a image taken at 0 minutes (right). The moving cell is an atypical, fusiform cell and might represent a macrophage derivative rather than an osteoclast (see text). (C) Increased motility and Ca^{2+} after preincubation with the NO donor SNP. After a 30-minute exposure to 100 μM SNP, many cells show elevated Ca^{2+} levels by ratio imaging (left) and are moving, as shown by subtraction of a 32-minute image from a image taken at 0 minutes (right).

The Ins(1,4,5)P_3R1 is required for calpain activation

The source of the Ca^{2+} pulses during NO- or cGMP-activated osteoclast movement remains unknown. Known osteoclast Ca^{2+} channels include the ryanodine receptor (RyR), a ryanodine-sensitive calmodulin-activated channel (Moonga et al., 2002), a potential target for NO activation (Xu et al., 1998). Also, the Ins(1,4,5)P_3Rs, a new class of high-molecular-weight receptor Ca^{2+} channels activated by Ins(1,4,5)P_3 (Ferris et al., 1992), are possibly involved. To determine whether these channels are required for cGMP-mediated activity, BOC assays were performed using the RyR antagonist tetracaine (50 μM) and the Ins(1,4,5)P_3R antagonist 2-aminoethoxydiphenylborane (2-APB; 100 μM). Ca^{2+}-activated calpain activity was greatly reduced when the Ins(1,4,5)P_3R was blocked, but the RyR antagonist had no effect (Fig. 7A). These results suggested that Ca^{2+} release requires Ins(1,4,5)P_3Rs. To assess the role of Ins(1,4,5)P_3Rs further, we used RNA interference (RNAi). There are three Ins(1,4,5)P_3R isoforms, but only Ins(1,4,5)P_3R1 was found on non-amplified gene screening in osteoclasts (Table S1 in supplementary material). Knock-down by using transfection of three siRNAs targeting Ins(1,4,5)P_3R1 suppressed protein synthesis of Ins(1,4,5)P_3R effectively (Fig. 7B). Ins(1,4,5)P_3R1 knockdown cells had severely reduced response to cGMP activating analogs or NO donors (Fig. 7C). Control and cGMP-activated osteoclasts are also illustrated to show labeled siRNA and corresponding BOC fluorescence after cGMP for key conditions (Fig. 7D). In the control several times more calpain activity after cGMP or NO donor activation. In Ins(1,4,5)P_3R1-knockdown cells, calpain activity after activation was significantly lower than in control cells. The Ins(1,4,5)P_3R1 knockdown cells had, on average, a greater attachment area, in keeping with very low motility in these cells.

Discussion

NO is an important regulator of Ca^{2+} homeostasis. Skeletal flexion is a primary stimulus that maintains bone mass, and stretched osteoblasts produce NO (Zaman et al., 1999). NO synthesis is also stimulated by inflammatory cytokines, such as TNFα (Ueno et al., 1998). The eNOS knockout revealed that this NO synthase also regulates osteoblast activity and that eNOS-knockout animals have a blunted response to estrogen (Armour, K. J. et al., 2001). Other work also shows that NO is an important mediator of estrogen response in bone-forming osteoblasts (O'Shaughnessy et al., 2000), and the eNOS-knockout animal lacks an estrogen anabolic response (Armour, K. E. et al., 2001). Thus, NO is a central regulator of bone mass that coordinates important signaling systems. We previously showed that NO regulates osteoclast motility via the NO-dependent guanylyl cyclase and PKG1 (Yaroslavskiy et al., 2004), and demonstrated that VASP is an essential target of PKG1 in osteoclasts (Yaroslavskiy et al., 2005). Our findings are consistent with podosomal rearrangement of osteoclasts in response to NO or cGMP activation (Yaroslavskiy et al., 2005),

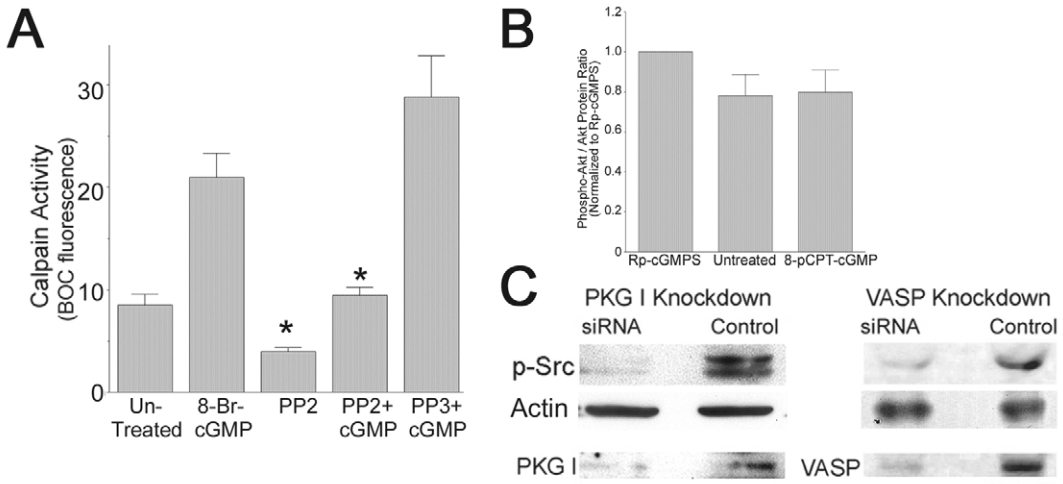

Fig. 6. Src activity is required for calpain activation by cGMP, but levels of phosphorylated Akt are not increased. (A) Src is required for cGMP-mediated activation of calpain in osteoclasts. These assays used the BOC fluorescent calpain substrate as described in Fig. 1. In the absence of cGMP (untreated) there is weak basal activity, attributed to autocrine NO production. Following activation with 8-Br-cGMP (50 μM, 1 hour) calpain is strongly activated. The Src inhibitor PP2 reduced activity of calpain to below that of the control (PP2) and reduced 8-Br-cGMP (cGMP) activation by 85% (PP2+8-Br-cGMP). An inactive control, PP3, had no effect on 8-Br-cGMP (cGMP) activation (PP3+8-Br-cGMP). *P<0.01 relative to 8-Br-cGMP; n=25-60, mean ± s.e.m. (B) Activation of cGMP did not increase phosphorylation of Akt. Phosphorylated Akt (p-Akt) was identified in osteoclast lysates by antibody against Akt phosphorylated at Ser473. Lysates of cultures after treatment with the hydrolysis-resistant cGMP analog 8-pCPT-cGMP (100 μM) or the cGMP-blocking analog Rp-cGMPS (50 μM) for 30 minutes were compared with untreated cells. Density of antibody labeling was measured and expressed as a fraction of total Akt by stripping membranes and re-labeling for total Akt protein. Whereas no differences were significant, the largest p-AKT signal was in the control containing the cGMP-blocking analog (left bar). Since PI 3-kinase activates Akt phosphorylation, this suggests that PI 3-kinase is not a direct mediator of cGMP signaling in osteoclasts; n=3, mean ± s.e.m. (C) PKG1 and VASP are required for cGMP-dependent Src phosphorylation. Phosphorylation of Src (top) at Y416 after treatment with 100 μM 8-pCPT-cGMP was reduced by knockdown of PKG1 (left) or VASP (right). Protein loading controls (middle) and knockdown controls (bottom) are also shown. In either case, p-Src was reduced by 70-90%. siRNAs used for knockdown experiments have been described previously (Yaroslavskiy et al., 2005).

which might be analogous to the motility of dendritic cells of related lineage. Dendritic cells and osteoclasts respond similarly to calpain inhibitors (Calle et al., 2006): in dendritic cells the mechanism depends on WASP and WASP shares a homology domain with VASP. Our study shows that μ-calpain is crucial for NO-induced osteoclast motility and that μ-calpain is regulated by a Ca^{2+} signal that requires PKG1, Src and VASP. Our findings are consistent with results from previous studies showing calpain activity in osteoclasts (Lee et al., 2005; Hayashi et al., 2005; Marzia et al., 2006). Earlier research suggested calpain involvement in osteoclast differentiation and function (Lee et al., 2005; Marzia et al., 2006), but here we describe for the first time μ-calpain activation and Ca^{2+} signaling in NO/cGMP-induced osteoclast motility. We also show that Ins(1,4,5)P_3R1 is responsible, at least in part, for Ca^{2+} signaling following stimulation with NO. This is the first demonstration of Ins(1,4,5)P_3R-dependent cell motility.

Ins(1,4,5)P_3, an inositol metabolite unrelated to PI 3-kinase activity, is known to cause Ca^{2+} signaling in bone cells (Falsafi et al., 1991) but the physiological role of the Ca^{2+} signal was unknown. There are no precedents for Ca^{2+} currents downstream of NO, cGMP, PKG1 or VASP in osteoclasts, although the regulation of Ca^{2+} by NO and cGMP is well established in other contexts. In hepatocytes, cGMP stimulates Ca^{2+} release that is dependent on Ins(1,4,5)P_3 (Rooney et al., 1996; Guihard et al., 1996). Furthermore, regulation of vascular permeability by NO involves Ins(1,4,5)P_3R1 (Tiruppathi et al., 2002). These are precedents for a signaling pathway starting with NO or cGMP via Ins(1,4,5)P_3 to Ca^{2+}, but there were no precedents for the involvement of Ins(1,4,5)P_3 in osteoclast motility or for a link between Ins(1,4,5)P_3R1 and PKG1 or Ins(1,4,5)P_3R1 and VASP. Other receptors involved in Ca^{2+}-release mechanisms, such as the cADP-ribose receptor and the RyR, also function in osteoclast activation (Sun et al., 2003). Moreover, RyRs can be activated by NO (Xu et al., 1998). These Ca^{2+}-release mechanisms did not appear to be of importance for motility, but they undoubtedly function under other conditions. Ca^{2+} signals also often correlate with PI 3-kinase activity. Akt phosphorylation, which is typically dependent on PI 3-kinase activation, was not increased after NO stimulation of osteoclasts (Fig. 6). This suggests that, in contrast to the essential role of Ins(1,4,5)P_3R1 in NO-stimulated Ca^{2+} signaling (Fig. 7), PI 3-kinase activity is not linked to NO in osteoclasts. These findings help explain why NO and cGMP have major effects on motility and attachment without the profound effects on cell survival that would be expected with PI 3-kinase activation. There are precedents for a relationship of PI 3-kinase activity to voltage-independent Ca^{2+}-channel activity in several cell types (Marcantoni et al., 2006; Tian et al., 2004), and our work does not exclude such a relationship in osteoclasts in contexts other than in NO/cGMP regulation.

Calpeptin reduced NO- and cGMP-induced osteoclast motility as well as calpain activity in situ (Figs 1, 2). Calpeptin is cell-permeable inhibitor of calpain, and was identified as an inhibitor of Ca^{2+}-activated actin rearrangements (Potter et al.,

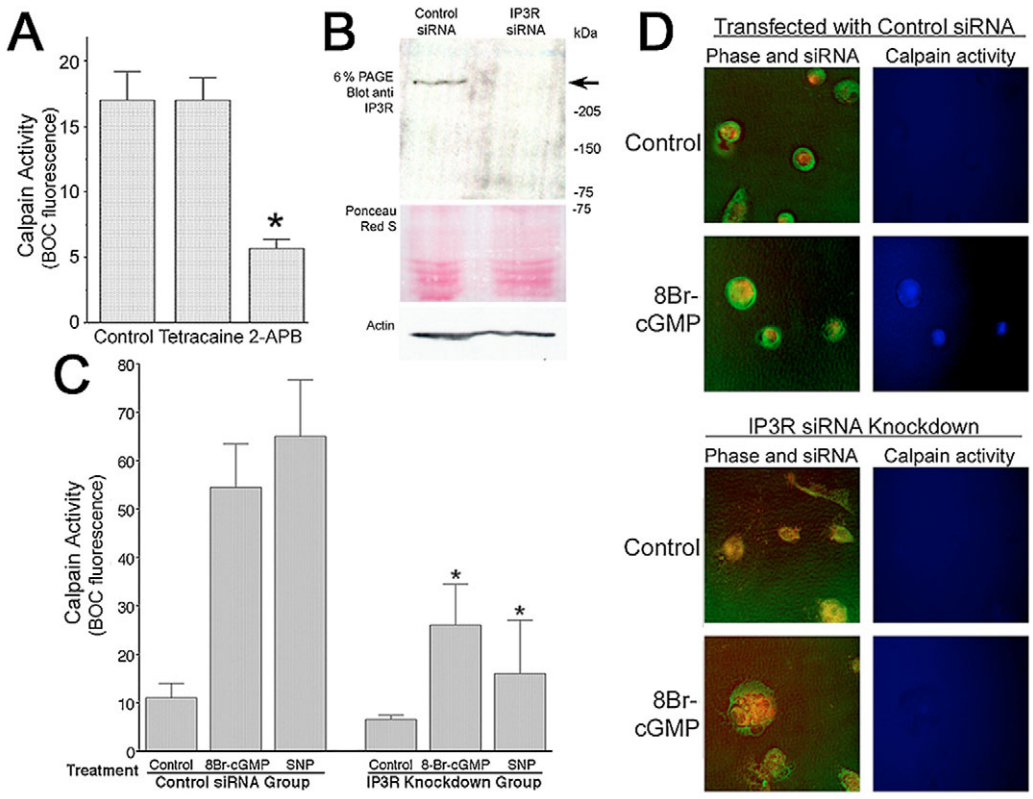

Fig. 7. Ins(1,4,5)P_3R1 is required for normal osteoclast calpain activity. (A) Effect of Ins(1,4,5)P_3R and RyR antagonists on calpain activity. BOC fluorescent calpain substrate assays as described in Fig. 1. Each culture was treated with 8-Br-cGMP. This produced strong control activity (Control). The RyR antagonist tetracaine (50 μM) did not affect activity. The Ins(1,4,5)P_3R antagonist 2-APB (100 μM) reduced activity; *P<0.01, n=30, mean ± s.e.m. (B) Knockdown of Ins(1,4,5)P_3R by using siRNA (IP3R siRNA). The protein (~240 kDa) was visualized by western blotting after separation of 35-μg aliquots of cell lysates on 6% SDS-PAGE (top). The membrane was re-blotted for actin, which runs at the front on 6% PAGE. Because of this, the membrane was also stained for total protein using Ponceau Red S (bottom image). (C) Ins(1,4,5)P_3R1 (IP3R) knockdown reduces calpain activity after cGMP analog or NO donor activation. BOC activity in transfected cells only, shown by Cy3-red labeling of siRNA, as shown in D. In cells shown on the left BOC-labeled cells were measured after control siRNA transfection, without treatment or with 8-Br-cGMP (100 μM) 40 minutes before BOC was added, or with SNP (100 μM) 10 minutes before BOC activity was assayed. The IP3R Knockdown Group was transfected with siRNA targeting Ins(1,4,5)P_3R1 (IP3R1) but was otherwise the same. The activated knockdown cells are different from matched controls, *P<0. 05, n=20, mean ± s.d. (D) Appearance of transfected cells with and without cGMP activation. Top panels show cells transfected with control siRNA; bottom panels show cells transfected with siRNA targeting Ins(1,4,5)P_3R1 (IP3R siRNA Knockdown). The left panel of each group shows phase-contrast (green) and Cy3-labeled siRNA (red). The Ins(1,4,5)P_3R1-knockdown cells show a greater average diameter, although there is significant stochastic variation in cell size within both groups. Ins(1,4,5)P_3R-knockdown cells have an average diameter 1.38±0.43 times that of control cells, n=10. The spread cells showed essentially no movement (not illustrated). The right panel of each group show BOC fluorescence (blue). Fluorescent BOC labeling was seen only in the control cells treated with nonsense siRNA after cGMP activation. Each image is 110 μm^2.

1998; Dedieu et al., 2004). Calpeptin also inhibits μ-calpain activity in osteoclast lysates (Fig. 3), consistent with work by others (Marzia et al., 2006). At the concentrations used in our studies, Calpeptin is essentially specific for calpains, although at higher concentrations it may also inhibit papain-family cysteine proteinases. The specific involvement of calpains is further indicated by the inhibitory effects of a calpastatin-derived peptide that is highly specific for calpain (Fig. 2B). RNAi to target μ-calpain expression in osteoclasts greatly diminished NO-dependent calpain activity: residual activity in siRNA-treated cells was indistinguishable from that of unstimulated cells (Fig. 3). Thus, μ-calpain appears to be the main proteinase activated by NO, although the possibility of a less important role for m-calpain cannot be entirely excluded by these studies.

Calpains can be regulated by phosphorylation as well as by Ca^{2+} (Glading et al., 2004); however, this mechanism appears to be largely restricted to m-calpain, whereas μ-calpain responds mainly to Ca^{2+} signals (Satish et al., 2005). The Ca^{2+}-dependence of NO-stimulated calpain activity in osteoclasts is thus fully consistent with the notion that NO and cGMP trigger μ-calpain activity. However, lack of μ-calpain expression is not lethal for μ-calpain knockout mice (Marzia et al., 2006), and μ-calpain does not appear to be absolutely required for osteoclast function in vivo. This might reflect adaptation, with limited activity of alternative calpains, such as m-calpain, in osteoclasts that lack μ-calpain. In this scenario, m-calpain might be activated by post-translational modification.

Ca^{2+} signals were clearly triggered by NO and cGMP (Figs 4, 5). There was variation in cellular response (Fig. 2 and Movies 1-3 in supplementary material), and Ca^{2+} levels in activated cells varied widely. This variation could have several

reasons, such as variable degrees of cell maturation. However, mainly the variability is related to cyclic activity of NO. Osteoclasts normally attach and resorb bone for several hours up to days between movement cycles. During movement, bone resorption stops, because it depends on acid secretion at cell attachment sites. Additional Ca^{2+}-activated mechanisms are expected to control the timespan of NO and cGMP effects. These probably involve counter-regulation through calmodulin. Calmodulin is a major Ca^{2+}-activated protein in the osteoclast (Radding et al., 1994). Proteins activated by calmodulin include calcineurin, which can modify osteoclast activity (Sun et al., 2003). There is also a calmodulin-activated phosphodiesterase that degrades cGMP (Mayer et al., 1993); and calmodulin activates the Ca^{2+}-ATPase that pumps cytoplasmic Ca^{2+} out of osteoclasts (Bekker and Gay, 1990). However, specific studies will be required to determine in more detail how cGMP action is terminated in the osteoclast. Osteoclasts are also regulated by Ca^{2+}-responsive proteins (Sanjay et al., 2001). The Ca^{2+}-release mechanisms involved in these pathways are, for the most part, uncharacterized. Calpain itself is also required for normal osteoclast maturation, and RANK signaling is modified by μ-calpain (Lee et al., 2005; Marzia et al., 2006).

The requirement of Ins(1,4,5)P_3 for Ca^{2+} signaling in NO-stimulated osteoclast motility undoubtedly implies additional regulatory proteins. Production of Ins(1,4,5)P_3 depends on phospholipase C (PLC) isoforms, typically PLCβ or PLCγ (Balla, 2006). These are regulated by both G-protein coupled receptors and tyrosine kinase-receptors, which have no clear relationship to NO signaling. PLCγ is expressed by osteoclasts, is implicated in cell spreading (Nakamura et al., 2002) and essential to osteoclast function in pathways independent of RANK (Koga et al., 2004). However, a link between PLCγ and the NO pathway is at this point hypothetical. In smooth muscle, inhibition of Ins(1,4,5)P_3-Ins(1,4,5)P_3R1 activity might involve additional intermediate proteins (Fritsch et al., 2004). The activity of the Ins(1,4,5)P_3R1 can be counteracted by PKG1 (Murthy and Zhou, 2003), possibly providing a feedback mechanism to limit NO-dependent Ca^{2+} release. Reports of cGMP and PKG1 potentiating Ins(1,4,5)P_3 activity in hepatocytes suggest a regulation of Ins(1,4,5)P_3R1 activity by cGMP and PKG1, because the Ins(1,4,5)P_3 signal remained unchanged (Guihard et al., 1996). It is likely that Ins(1,4,5)P_3R1 activity in the osteoclast is similarly regulated. The signal for the Ins(1,4,5)P_3R1 is, at a basal level, due to membrane-related integrin or tyrosine kinase signals, and receptor activity increases in the presence of cGMP. In osteoclasts, the Ca^{2+} activation pathway requires Src family tyrosine kinase activity in addition to PKG1. This is in keeping with the possible dependency of the Ca^{2+} signal on cell membrane integrin or tyrosine kinase signals. However, it is also possible that Src directly modifies Ins(1,4,5)P_3R1. Direct regulation of the Ins(1,4,5)P_3R1 by Src-family kinase activity has been demonstrated in lymphocytes (Cui et al., 2004).

In conclusion, we show that μ-calpain is crucial for NO-induced osteoclast motility; μ-calpain is regulated by Ca^{2+} signaling that requires PKG1, Src and VASP, through a mechanism involving Ins(1,4,5)P_3R1. Our results show for the first time Ins(1,4,5)P_3R-dependent cell motility. Our work is in keeping with studies showing calpain involvement in osteoclast differentiation and function (Lee et al., 2005; Hayashi et al., 2005; Marzia et al., 2006), but our results identify a new link between activation of μ-calpain and NO-induced motility.

Materials and Methods

Osteoclasts

Human $CD14^+$ cells were isolated from citrate-anticoagulated blood. Cells were used for osteoclast differentiation in vitro using recombinant human CSF-1 and RANKL (Yaroslavskiy et al., 2005). Procedures were approved by the institutional review board.

Reagents

Recombinant m-calpain and purified μ-calpain, the calpain inhibitor calpastatin, and scrambled calpastatin peptide were from CalBiochem (San Diego, CA). The Ca^{2+} indicators Fluo3, Oregon Green-488 BAPTA-1, Fura Red, and the calpain substrate t-butoxycarbonyl-Leu-Met-chloromethylaminocoumarin (BOC) were from Molecular Probes (Carlsbad, CA). Calpain inhibitors N-Acetyl-Leu-Leu-norleucinal (ALLN) and N-benzyloxycarbonyl-L-leucylnorleucinal (Calpeptin) and the NO synthase antagonist NG-monomethyl-L-arginine acetate (L-NMMA) were from Biomol (Plymouth Meeting, PA). The Src inhibitor 4-amino-5-(4-chlorophenyl)-7-(t-butyl)pyrazolo[3,4-d]pyrimidine (PP2) and its inactive congener 4-amino-7-phenylpyrazol[3,4-d]pyrimidine (PP3) were from Calbiochem. The NO donor sodium nitroprusside (SNP) was from Sigma (St Louis, MO). Hydrolysis-resistant cGMP activators 8-(4-chlorophenylthio)guanosine-3′,5′-cyclic monophosphate (8-pCPT-cGMP), 8-Br-guanosine-3′,5′-cyclic monophosphorothioate (8-Br-cGMP), and an antagonist 8-(Rp-4-chlorophenylthio)guanosine-3′,5′-cyclic monophosphorothioate (Rp-cGMPS) were from Biolog (Bremen, Germany). Monoclonal anti-phosphotyrosine and phospho-Src (Tyr416) were from Cell Signaling Technology (Beverly, MA). Polyclonal anti-Src and anti-talin were from Santa Cruz (Santa Cruz, Ca). Polyclonal anti-PKG1 was from Stressgen (Victoria, BC, Canada). Anti-β-actin was from Sigma. MAP2 was from Cytoskeleton (Denver, CO).

Western analysis and cell lysate enzyme activity assays

For western blots cells were lysed in 0.3% SDS, 50 mM Tris pH 7, with proteinase and phosphatase inhibitors (Williams et al., 1996). Proteins were separated on SDS-PAGE and transferred to polyvinylidine membranes for immune labeling with alkaline-phosphatase-coupled secondary antibodies and enhanced chemiluminescence detection (ECL plus, Amersham, Piscataway, NJ) (Yaroslavskiy et al., 2004). Zymography used cell lysates in 25 mM HEPES, 30 mM imidazole, 1 mM vanadate, 1% Triton X-100, and 10% glycerol, pH 7.0. Lysates were mixed with equal volumes of 250 mM Tris, 25 mM EDTA, 50% glycerol 1 mM β-mercaptoethanol, pH 6.8, and separated on 15%-non-denaturing acrylamide gels with 0.2% casein and 1 mM EGTA (Glading et al., 2004). Gels were washed and placed in 20 mM MOPS pH 7.2, with Ca^{2+} activity adjusted using a Ca^{2+} electrode by titrating EGTA with $CaCl_2$. Gels were developed 16 hours at 20°C and stained with 0.4% Coomassie Blue. Calpain degradation of the microtubule-associated protein 2 (MAP2) (Baki et al., 1996) was assessed using lysates as in zymography. Lysate protein (10 μg) was incubated with 1 mg of MAP2 in buffer with 1 mM $CaCl_2$. Reactions were stopped with SDS. Proteins were separated on 6% SDS-PAGE and visualized by silver staining.

RNAi

Cells were transfected with siRNA targeting two PKG1 sequences, one μ-calpain sequence, three Ins(1,4,5)P_3R1 sequences, or four VASP sequences. Controls used transfection with nonsense siRNA. Sequences were screened for homology to other proteins using BLAST (www.ncbi.nlm.nih.gov/BLAST). siRNA targeting Ins(1,4,5)P_3R1 was from Santa Cruz (Santa Cruz, CA) and included siRNAs from GenBank NM_002222 nucleotides 7944-7962 (5′-GAGACAAGTTTGACAA-CAA-3′), nucleotides 8946-8964 (5′-CCAAGTCTATGAACTGTTA-3′) and nucleotides 9360-9378 (5′-CCACAGACATGTTATTCTT-3′). Silencing of μ-calpain was carried out using siRNA from Integrated DNA Technologies (Coralville, IA) as RNA duplexes of 18 RNA bases and two chimeric DNA bases: 5′-GUUCUCGUCAAUCUCCUCTT-3′ and GAGGAGAUUGACGAGAACTT-3′. Additional siRNAs, made for this work, used PKG1 sequences from GenBank Z92867, +109-129 from the start codon (5′-AAGAGGAAACTCCACAAATGC-3′) and 124-46 (5′-AAATGCCAGCGGTGCTCCCAGT-3′). For VASP target sequences (GenBank Z46389) were +121-42 from the start codon (5′-AACCCCACGGCCAATTCCTTT-3′), +274-95, 5′-AACTTCGGCAGCAAGG-AGGAT-3′), 700-720 (5′-AAACTCAGGAAAGTCAGCAAG-3′) and +847-867 (5′-AAAACCCCCAAGGATGAATCT-3′). From these sequences, siRNA sense and antisense oligonucleotides were manufactured (Integrated DNA Technologies, Coralville, IA) by adding a 8 bp leader sequence complementary to T7 promoter primer. Templates were hybridized to T7 promoter primers and extended with Klenow DNA polymerase. The double-stranded template was transcribed by T7 RNA polymerase and hybridized to create dsRNA. RNA was digested to remove the single-strand leaders, resulting in ds-siRNA. Transfection used mixtures of siRNAs with 100 nM total siRNA. To visualize transfection, Cy3 was covalently

attached to the duplex siRNA (Silencer siRNA labeling kit, Ambion, Austin, TX). Cells were transfected with siRNA using siPORT Amine transfection reagent (Ambion), a blend of polyamines.

Microscopy, fluorescent Ca^{2+} and calpain assays in living cells

A Nikon TE2000 inverted phase-fluorescence microscope with a 12 bit 1600×1200 pixel CCD detector (Spot, Diagnostic Instruments, Sterling Heights, MI) was used to acquire images. Phase photographs used a NA 0.95 long working distance 40× objective. Intracellular Ca^{2+} was studied using Ca^{2+}-sensitive fluors. For single wavelength measurements, cells were incubated 20 minutes at 37°C in 10 mM of the membrane-permeant acetoxymethyl ester (AM) of Fluo3. Following this, fluorescence images were acquired using a 40× oil immersion lens with epifluorescence at excitation 450-490 nm, 510 nm dichroic mirror, 520 nm barrier filter. Dual wavelength Ca^{2+} measurements were obtained after preincubation with Oregon Green 488 BAPTA-AM-1 (1.25 mM) and Fura Red AM (1 mM) for 20 minutes (Yap et al., 2000). Fluorescent images were made with excitation at 450-490 nm, a 510 nm dichroic mirror, recording Fura Red emission with a 600-710 nm filter and Oregon Green with a 500-570 nm filter. Maximum and minimum ratios were determined by adding the Ca^{2+} ionophore A23187 followed by an excess of EDTA. False color Ca^{2+} images were made from 12 bit Fluo3 CCD images by compressing the images to 8 bit tiff-format files, converting these to false color using NIH Image (http://rsb.info.nih.gov/nih-image) by applying the fire2 look-up table, to produce black background with blue, violet, red, orange, yellow and white indicating increasing signal. These files were converted, using Photoshop 7 (Adobe Systems, San Jose, CA), to 24 bit color to allow jpeg compression for display of sequential files as movies (iMovie, Apple Computer, Cupertino CA). The Fluo3 single-wavelength Ca^{2+} images cannot be calibrated accurately; differences in signal are approximately proportional to relative Ca^{2+} activity. For ratio imaging, images were processed by making the inverse of the 12 bit red image and multiplying this by the green image. To reduce noise, 4:1 binning was applied before ratio calculation. The resultant ratio images were converted to false color as for the Fluo3 images. Intracellular calpain activity was determined using BOC (Glading et al., 2000). For 20 minutes, 50 μM BOC was added to osteoclasts in cover glass chambers. Fluorescence intensity was determined by imaging the activated substrate within cells, as in Fluo3 images but using excitation at 380-425 nm, a 430 nm dichroic filter and a 450 nm barrier filter. In BOC assays, false color is displayed at the approximate emission maximum of the fluorophore, except in red-green-blue comparisons (in Fig. 7) where BOC is shown in blue. Measurement of BOC fluorescence also used NIH image software, and is expressed in arbitrary units or as percent of control-cell fluorescence as indicated in the figure legends.

Statistics

Student's *t*-test was used for comparisons of groups.

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