Published in final edited form as:

Ann N Y Acad Sci. 2010 March ; 1192(1): 358–364. doi:10.1111/j.1749-6632.2009.05242.x.

CALMODULIN – AN OFTEN IGNORED SIGNAL IN OSTEOCLASTS

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Abstract

Calcium signaling plays a key role in bone turnover, regulating both osteoblasts and osteoclasts. Despite this the role of calmodulin, the primary intracellular calcium receptor regulatory protein, has received relatively little attention. In this brief review, the function of Ca²⁺/calmodulin signaling in osteoclast development, function and apoptosis is reviewed. Considerable evidence supports an important regulatory role for Ca²⁺/calmodulin signaling in each of these processes. The overall role of Ca²⁺/calmodulin in regulating bone turnover is also supported by animal and human studies showing that calmodulin antagonists preserve bone mass.

Keywords

osteoclasts; calcium; calmodulin; calcineurin; calmodulin kinase II; apoptosis

INTRODUCTION

The era of molecular biology has yielded many extraordinary findings about the mechanisms of bone metabolism, disorders of bone metabolism and new therapeutics for bone diseases. In the process, however, the fundamental role of certain key cell signals has been de-emphasized. One such critical pathway is the Ca²⁺/calmodulin signaling pathways in bone resorption and metabolism. Studying the Ca²⁺/calmodulin signaling pathways directly often involves techniques that are not commonly used in modern molecular biology. Intracellular Ca²⁺ measurements and enzyme activity measurements, for instance, of calcineurin, calmodulin kinase II (CaMKII) and phospholipase C are seldom published despite clear evidence that downstream events (e.g. NFATc₁, translocation, substrate phosphorylation, etc.) are involved. Protein or mRNA expression levels give little or no insight into dynamic changes in enzyme activity. Signal redundancy is a built in feature of Ca²⁺ signaling. For example, although many investigators believe NFATc₁ is a master regulator of osteoclastogenesis, there is also evidence that CaMKII is critical and functions independent of NFATc₁ translocation.

Ca²⁺/calmodulin-mediated cell signaling is ubiquitous and functions at all levels of the cell life cycle including cell genesis, cell function/activity and cell death. This is also true for osteoclasts. Calcineurin and CaMKII are important for osteoclastogenesis from progenitor cells, calmodulin directly regulates bone resorption by mature osteoclasts and calmodulin interaction with the Fas death receptor modulates apoptosis of osteoclasts. Here we briefly

review evidence to support each of these and review data from the use of CaM antagonists in animal models and humans.

EFFECT OF CaM ANTAGONISTS IN HUMANS AND OVARECTOMIZED RODENTS

Two calmodulin antagonists are approved for use in humans and are well tolerated by animals. They are the anti-estrogen drug, tamoxifen, and the antipsychotic drug, trifluoperazine. Trifluoperazine has been used for years in the laboratory as a calmodulin antagonist. In contrast, the effects of tamoxifen, which is equally as potent as trifluoperazine as a calmodulin antagonist, have seldom been attributed to its ability to inhibit calmodulin interactions with target proteins. Calmodulin is normally activated by binding up to four Ca^{2+} molecules and the activated form binds (either activating or inhibiting) target proteins. Calmodulin antagonists typically bind calmodulin preventing it from interacting with target proteins.

There are no clinical studies assessing the effect of trifluoperazine on bone mineral density or related bone properties. However, trifluoperazine treatment of ovariectomized mice for five weeks reversed the effect of ovariectomy on bone mineral density. This was accompanied by a reduction in the ovariectomy-induced increase in TRAP positive osteoclasts. *In vitro* studies confirmed that TFP inhibited osteoclastogenesis through inhibiting calmodulin.(1)

The effects of tamoxifen to preserve bone in postmenopausal women have now been confirmed in many studies. For example, Powle, et al. reported that in postmenopausal women treated with tamoxifen, the annual increases in spine and hip BMD were 1.2% and 1.7%, respectively, compared to an insignificant loss in non-treated controls.(2) An independent study by Ryan et al. confirmed these results.(3) There are numerous studies in rats in which tamoxifen prevents or protects against ovariectomy-induced bone loss.(4–7) As would be expected in premenopausal women replete with estrogen these positive effects on BMD are lost because of the anti-estrogen effects of tamoxifen. *In vitro* effects of tamoxifen on osteoclast function and differentiation and calmodulin will be discussed in the following sections.

Ca^{2+} /CALMODULIN REGULATION OF OSTEOCLASTOGENESIS

Although complex and not fully understood, osteoclastogenesis is regulated primarily by two factors, RANKL and macrophage colony stimulating factor (M-CSF). These factors stimulate mononuclear precursors to form multinuclear osteoclasts. M-CSF acts as a growth factor, stimulating survival and proliferation of precursors, while RANKL binds its receptor to trigger differentiation. Upon binding to RANKL, RANK recruits TNF receptor-associated factors (TRAF) 1, 2, 3, 5, and 6 to its cytoplasmic domain.(8) This leads to activation of NF- κ B, ERK, JNK and p38, as well as Akt. ERK and JNK activation lead to AP-1 induction of osteoclast-specific genes cathepsin K, calcitonin receptor, tartrate-resistant acid phosphatase, and $\alpha\text{V}\beta 3$ integrin subunits. Additionally, RANK activation leads to increased $[\text{Ca}^{2+}]_i$ and activation of the calmodulin-dependent phosphatase, calcineurin. Calcineurin dephosphorylates nuclear factor of activated T cells (NFAT), leading to NFAT translocation to the nucleus and activation of downstream gene expression. *In vitro* studies showed that the calmodulin antagonist TFP inhibits osteoclastogenesis through inhibiting calmodulin,(1) but which calmodulin signaling pathway was affected was not determined.

CaMKII is a serine/threonine kinase that functions as a large, multimeric holoenzyme and is activated by calcium and calmodulin. CaMKII isoforms include α , β , γ , and δ , which are encoded by separate genes, and multiple RNA splicing can result in as many as 30 distinct variants. There are multiple RANK signals that could either regulate, or be regulated by, CaMKII. TRAF6 interacts with c-SRC,(9) which in turn activates phospholipase C γ and leads

to endoplasmic reticulum release of calcium.(10) Upstream of MAPK is c-raf, which has been shown to be regulated by CaMKII and leads to ERK activation.(11) CaMKII has been shown to regulate c-fos expression, which regulates AP-1 activity and osteoclastic gene expression.(12) CaMKII also increases Akt activity,(13) an important mediator of osteoclast survival. Recent studies have shown that mice with reduced expression of CaMKII α have defects in osteoclast formation and have reduced bone mass, providing the most direct evidence that CaMKII is important in this process.(14)

Osteoclastogenesis is regulated by CaMKII functioning independently of NFATc1

Figure 1A shows representative photomicrographs of cells treated with RANKL, RANKL plus 1 μ M of the CaMKII inhibitor, KN93, or RANKL plus 5 μ M KN93. A quantitative summary of three experiments is shown in Fig. 1B and 1C, demonstrating that KN93 dramatically inhibits the development of osteoclasts, with maximal inhibition at 5 μ M. Although KN93 is a potent inhibitor of CaMKII, it can have non-specific effects, including inhibition of protein kinase C (PKC) and CaMKI and IV. A second CaMKII inhibitor, ANT-AIPII, which is based on the substrate sequence for CaMKII and does not inhibit PKC or CaMKIV, also reduces osteoclast formation (data not shown).

Treatment with KN93 prevented osteoclast formation but has no effect on the NFATc1 pathway, including NFAT upregulation and translocation. Figure 2 shows a Western analysis for NFATc1 in nuclear protein extracts from RAW 264.7 cells treated with RANKL and increasing concentrations of KN93 (top panel) that demonstrates NFAT translocation is unaffected by KN93 concentrations which completely block osteoclast formation. The bottom panel of Figure 2 shows photomicrographs of cells treated as indicated and stained for NFATc1. These data indicate that NFATc1 localization moves from being primarily cytosolic in untreated cells or cells treated with RANKL for 48 hours to a compartment that overlies the nuclear stain (not shown for clarity) in cells treated with RANKL for 96 hours. Cells treated with RANKL and KN93 do not form multinucleated osteoclasts, but the pattern of NFATc1 localization is equivalent to cells treated with RANKL alone. Cells were treated with cyclosporine A to inactivate calcineurin (and thus prevent NFAT dephosphorylation) as a control. Thus CaMKII regulation of osteoclastogenesis is independent of NFAT activation and functions at a late point in the differentiation process. This has potential physiological significance, since it is possible that these developing osteoclasts are able to promote the recruitment and activation of osteoblast precursors, despite not differentiating into fully mature osteoclasts.

RANKL Activates CaMKII and CaMKII Knockdown Inhibits Osteoclastogenesis

One of the unknown mechanisms of osteoclastogenesis is the integration of short-term stimuli into a differentiation signal several days after the initial stimulus. CaMKII is an ideal molecule to integrate such signals, as it remains active long after the initial signal has abated. CaMKII activity increases six-fold within 5 minutes of RANKL addition. Activity decreases between 10–120 minutes following RANKL but remains approximately 4 fold above basal activity levels (Fig. 3A). Long-term treatment of RAW cells with RANKL shows a similar increase in CaMKII activity, doubling after 24–48 hours and peaking at 72–96 hours, coincident with differentiation into osteoclasts (Fig. 3B). The pattern of increased CaMKII activity fits very well with its proposed dual role as an early signal transducer and a late regulator of differentiation. Downregulation of CaMKII isoforms using siRNA nearly completely abolishes osteoclastogenesis, regardless of the isoform targeted (data not shown). This is consistent with the fact that CaMKII functions as a hetero-multimer and it is likely that the total activity of CaMKII is more important than the specific isoforms comprising the holoenzyme.

CA²⁺/CALMODULIN REGULATION OF OSTEOCLAST ACTIVITY

Seales et al.(15) summarized the extent of calmodulin dependent signaling in bone in 2006. Recent research has shifted from calmodulin per se, and focused on key calmodulin binding proteins including calcineurin and calmodulin dependent kinases and the downstream factors affected by these two classes of enzymes that are important in bone development/metabolism. However, one mechanism by which osteoclast activity is altered by calmodulin-dependent processes is by increased expression of calmodulin when cells are cultured on bone.(16–17) The up-regulation of calmodulin is concentrated in the specialized ruffled membrane that is responsible for acid secretion.(16) Dynamic regulation of calmodulin levels prompted investigation of the role of tamoxifen on osteoclast activity due to tamoxifen being both a calmodulin antagonist and an estrogen receptor antagonist. We showed that tamoxifen inhibited bone resorption by bone affinity purified mature osteoclasts with half maximal inhibition at approximately 10^{-6} M, while estrogen and diethylstilbestrol had no effect at concentrations ranging from 10^{-15} to 10^{-5} M. In these studies, the pure antiestrogen ICI 182780, had no effect on bone resorption between 10^{-15} and 10^{-7} M. The K_d of ICI 182780 for the estrogen receptor is 10^{-9} M. Bone resorption was inhibited up to 50% by ICI182780 concentrations of 10^{-6} to 10^{-5} M, concentrations that inhibit calmodulin dependent phosphodiesterase activity in *in vitro* assays.(18) Additional acid transport experiments with isolated ruffled membrane vesicles revealed a similar pattern of inhibition by all of the compounds indicating that tamoxifen has effects in mature osteoclasts that are independent of estrogen receptor.

The dramatic upregulation of calmodulin protein levels by mature osteoclasts in response to bone attachment for bone resorption raises the question whether upregulation of calmodulin is related to the dramatic increase in CaMKII activity in differentiating preosteoclasts discussed above. In fact, the time frame and magnitude for the dramatic increase in CaMKII activity is strikingly similar to the increased bone resorption and increase in calmodulin expression levels when osteoclasts are treated with phorbol esters. Treatment of osteoclasts with phorbol esters which activates PKC is known to induce a pronounced transformation into giant cells. Therefore, studies were conducted to determine whether tamoxifen's effect on mature osteoclasts could be explained via actions of PKC since tamoxifen has been reported to inhibit PKC activity. Phorbol ester treatment of osteoclasts markedly increases calmodulin protein levels. This effect was independent of PKC since all detectable isoforms of PKC in osteoclasts were dramatically downregulated within 24 hour of phorbol ester treatment,(19) while calmodulin protein levels in whole cell lysates increased approximately 4 fold between 48 and 72 hours post treatment. Interestingly, coincubation of osteoclasts with either tamoxifen or trifluoperazine blocked the phorbol ester stimulated increase in calmodulin protein levels.

The dual nature of tamoxifen as both calmodulin and estrogen receptor antagonists is largely ignored in the literature. The complexity of the system is further enhanced by the fact that the estrogen receptor itself is a calmodulin binding protein.(20) This suggests that tamoxifen may compete for binding with estrogen to the estrogen receptor on the one hand, but also disrupts estrogen receptor signaling by preventing calmodulin binding the receptor. The predominant focus of tamoxifen being solely an estrogen receptor antagonist in the bone field has often resulted in overinterpretation of the mechanism of action of tamoxifen, a lack of understanding of the molecular regulation of estrogen receptor function and the consequent unintended oversimplification of the biological regulation of osteoclast activity.

CA²⁺/CALMODULIN REGULATION OF OSTEOCLAST APOPTOSIS

Apoptosis, programmed cell death, is a physiologic process that eliminates cells. Apoptosis of osteoclasts has received considerable attention as the elimination of these cells should, at least

in theory, decrease bone resorption. There are numerous reports demonstrating that bisphosphonates stimulate osteoclast apoptosis. Other agents associated with osteoclast apoptosis include glucocorticoids, estrogen, TGF- β_1 , $\alpha_v\beta_3$ integrins, intracellular tyrosine kinases, mitochondrial-derived H₂O₂, Bcl-2 family members and the death receptor, TRAIL. Calmodulin and Ca²⁺, the focus of this review, have been shown to play a role in Fas-death receptor-mediated apoptosis in osteoclasts as well as lymphocytes and tumor cells.

Mature osteoclasts express Fas on the surface that mediates apoptosis along classically described intracellular pathways involving release of mitochondrial cytochrome C and activation of caspases 3 and 9.(21) The *in vivo* significance of Fas mediated apoptosis of osteoclasts was characterized in two well-characterized mouse models, the Lpr and Gld mice. These mice have defects in the Fas and Fas-ligand genes, respectively, that markedly weaken the Fas apoptotic system. The Lpr mouse has decreased bone mineral density, bone volume and trabecular thickness and increased osteoclast numbers. The Gld mice also have enhanced osteoclast numbers and a slight but insignificant decrease in bone mineral density. Further *in vitro* studies characterized the regulation of Fas expression by RANKL during osteoclastogenesis and in mature osteoclasts.(22) RANKL upregulates Fas expression in osteoclast progenitors through NF- κ B but reduces levels of Fas and Fas-mediated apoptosis in mature osteoclasts, thus enhancing survival of osteoclasts.

There is now considerable evidence supporting Ca²⁺ and calmodulin as regulators of Fas-mediated apoptosis. In the ovariectomized mouse, the calmodulin antagonist, trifluoperazine, rescues bone loss with a concomitant decrease in osteoclasts.(1) Treatment of mouse osteoclasts with either trifluoperazine or tamoxifen induces osteoclast apoptosis. Calmodulin binds directly to Fas and this binding is decreased by trifluoperazine and, as in Jurkat cells, increased when Fas is stimulated by Fas-activating antibody.(23) Consistent with this, Fas with a point mutation (V254N) which is analogous to the Lpr mouse mutation that inactivates the Fas pathway has reduced CaM binding. In recent related studies in cholangiocarcinoma cells, calmodulin regulates Fas-mediated apoptosis in part by binding to FLICE-like inhibitory protein (FLIP) which is a physiological inhibitory protein in the death-inducing signaling complex.(24) Studies in cholangiocarcinoma and Jurkat cells show that of all the death receptor family members, calmodulin, binds only to Fas, and Fas and FLIP are the only two calmodulin binding proteins in the death inducing signaling complex which is formed during Fas activation. Calmodulin thus appears to be a regulatory docking protein in the Fas-induced signaling complex and calmodulin inhibitors act, at least in part, by affecting binding of calmodulin to Fas and/or FLIP.

Although clearly there are multiple pathways for osteoclasts to undergo apoptosis, data, from both animal studies and *in vitro* isolated cell studies, indicate that Ca²⁺ and calmodulin are relevant regulators of the Fas-mediated pathway in osteoclasts. In addition, Fas expression is regulated by RANKL lending more credence to its relevance in determining the longevity of osteoclasts.

SUMMARY

Ca²⁺ and calmodulin signaling pathways play an important role in regulating osteoclastogenesis, osteoclast activity and osteoclast apoptosis and both animal and human studies show that calmodulin antagonists preserve bone. Ca²⁺/calmodulin targets, calcineurin and CaMKII are important rate limiting pathways for osteoclastogenesis. Ca²⁺/calmodulin has a dynamic and direct effect on the activity of mature osteoclasts, and calmodulin regulates the Fas-apoptotic pathway in osteoclasts. How these pathways are integrated with each other and with osteoblasts in the overall regulation of bone turnover remains to be determined.

Acknowledgments

The author's work was supported by a grant from the National Institutes of Health: P30 AR046031 (JMM).

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Fig. 1. Inhibition of osteoclastogenesis by KN93

RAW 264.7 cells (A and B) or mouse primary macrophages (C) were treated with RANKL (20ng/ml) for 96 hours in the presence of the indicated concentrations of KN93, then TRAcP stained. (A) Photomicrographs show a representative example from three independent experiments. (B and C) Osteoclasts were counted at 100 $\times$ magnification (three fields per sample, averaged) and the mean $\pm$ SD from three independent experiments performed in triplicate is shown. (*) $p < 0.05$ compare to RANKL alone treated samples.

Fig. 2. Inhibition of CaMKII by KN93 does not affect NFATc1 translocation

(top) Untreated RAW 264.7 cells or cells treated with RANKL (20ng/ml) were incubated for 72 hours in the presence of the indicated concentrations of KN93, then nuclear proteins were extracted and Western blotted for NFATc1. Shown is a representative blot from two independent experiments. (Bottom) RAW 264.7 cells were treated as indicated and immunostained for NFATc1 (red). Shown are representative micrographs from three independent experiments.

Fig. 3. RANKL increases CAMKII activity after short-term and long-term treatments
 RAW264.7 cells were treated with RANKL for the times indicated, then lysed. CaMKII activity was determined in lysates treated with RANKL for 0–120 minutes (A) or from 0–96 hours (B). Activity assay (kit was purchased from Millipore and performed according to manufacturer's protocol) in the presence (upper red lines) or absence (lower blue lines) of calcium, and activity is expressed as pmol phosphate incorporated per minute per mg of total lysate protein. Data is expressed as the mean of two separate experiments performed in triplicate $\pm$ SD.