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Calcium/Calmodulin Signaling Controls Osteoblast Growth and Differentiation

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Abstract Ca^{2+} is a ubiquitous intracellular messenger responsible for controlling numerous cellular processes including fertilization, mitosis, neuronal transmission, contraction and relaxation of muscles, gene transcription, and cell death. At rest, the cytoplasmic Ca^{2+} concentration $[Ca^{2+}]_i$ is approximately 100 nM, but this level rises to 500–1,000 nM upon activation. In osteoblasts, the elevation of $[Ca^{2+}]_i$ is a result of an increase in the release of Ca^{2+} from endoplasmic reticulum and/or extracellular Ca^{2+} influx through voltage gated Ca^{2+} channels. Many of the cellular effects of Ca^{2+} are mediated by the Ca^{2+} binding protein, calmodulin (CaM). Upon binding up to four calcium ions, CaM undergoes a conformational change, which enables it to bind to specific proteins eliciting a specific response. Calmodulin kinase II (CaMKII) is a major target of the Ca^{2+}/CaM second messenger system. Once bound to Ca^{2+}/CaM, the multimeric CaMKII is released from its autoinhibitory status and maximally activated, which then leads to an intraholoenzyme autophosphorylation reaction. Calcineurin (Cn) is another major target protein that is activated by Ca^{2+}/CaM. Cn is a serine-threonine phosphatase that consists of a heterodimeric protein complex composed of a catalytic subunit (CnA) and a regulatory subunit (CnB). Upon activation, Cn directly binds to, and dephosphorylates nuclear factor of activated T cells (NFAT) transcription factors within the cytoplasm allowing them to translocate to the nucleus and participate in the regulation of gene expression. This review will examine the potential mechanisms by which calcium, CaM, CaMKII, and Cn/NFAT control osteoblast proliferation and differentiation. J. Cell. Biochem. 97: 56–70, 2006.

Key words: osteoblast; calmodulin; CaMKII; NFAT; calcium; Runx2; AP-1; calcineurin

EXTRACELLULAR CALCIUM IN THE BONE MICROENVIRONMENT

Bone is a dynamic tissue that is constantly in a state of remodeling throughout life. In the normal adult skeleton, bone formation by osteoblasts occurs after the initiation of bone resorption by osteoclasts. Throughout life, old bone is constantly removed and new bone is formed in its place, a process called remodeling cycle. The resorption of bone can produce local increases in the extracellular calcium concentration $[Ca^{2+}]_o$ beneath a resorbing osteoclast and reach levels as high as 40 mM [Silver et al., 1988]. It is thought that this increase in $[Ca^{2+}]_o$ during remodeling plays a critical role in regulating osteoblast proliferation and/or differentiation via either activation of the calcium-sensing receptors (CaSR) and/or increasing the Ca^{2+} influx into osteoblasts.

Calcium-Sensing Receptors (CaSR)

CaSR is a G protein coupled receptor with seven trans-membrane domains. It functions as a Ca^{2+} sensor in the parathyroid and the kidney to detect the extracellular calcium concentration and thus helps to maintain calcium homeostasis. Mutations that inactivate CaSR cause familial hypocalciuric hypercalcemia, whereas mutations that activate CaSR are the cause of autosomal dominant hypocalcemia [Brown et al., 1993; Garrett et al., 1995; Ruat et al., 1995]. In parathyroid cells, the CaSR regulates PTH synthesis and secretion in response to $[Ca^{2+}]_o$ and in the kidney it plays a role in regulating calcium reabsorption at the distal tubules [Brown et al., 1993; Pollak et al., 1993].

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CaSR functions by activating several phospholipases, C, A_2, and D in parathyroid cells [Kifor et al., 1997]. The CaSR-mediated activation of PLC appears to result from a direct G protein-mediated process, probably involving $G_{q/11}$, since this effect is not known to be blocked by pertussis toxin. The activation of PLC, formation of diacylglycerol (DAG) and inositol 1,4,5-trisphosphate (IP3), increase in Ca^{2+} influx and Ca^{2+} release from the ER appear to be an important mechanism(s) through which the CaSR exerts its biological actions [Brown and MacLeod, 2001]. Furthermore, CaSR agonists, similarly to increasing $[Ca^{2+}]_o$, also activate PLC and result in an increase in intracellular calcium $[Ca^{2+}]_i$. The activation of PLA_2 and PLD by high $[Ca^{2+}]_o$ and CaSR agonists is thought to be by indirect mechanism, utilizing CaSR-mediated, PLC-dependent activation of PKC, as inhibiting PKC has been shown to abolish this response [Kifor et al., 1997]. In addition, it has been reported that the isoform of PLA_2 activated by the CaSR is the cytosolic form and that the mechanism underlying its activation involves its phosphorylation by mitogen-activated protein kinase (MAPK) [Kifor et al., 2001].

Many mesenchymal derived cells such as fibroblasts, chondrocytes, and adipocytes have been shown to express functional CaSR [McNeil et al., 1998; Chang et al., 1999; Cifuentes et al., 2005]. However, previous studies examining the expression of CaSR in different osteoblastic cell lines, MC3T3-E1 and MG-63, produced contradictory results [Yamaguchi et al., 1998b; Pi et al., 1999]. There are several lines of evidence suggesting that an increase in $[Ca^{2+}]_o$ induces both chemotaxis and proliferation of mouse stromal cells and the MC3T3-E1 osteoblastic cell line [Yamaguchi et al., 1998a,b]. Elevated levels of $[Ca^{2+}]_o$ increased DAG and cAMP levels in these cells but did not promote the formation of IP3 that would occur with activation of PLC [Quarles et al., 1994; Hartle et al., 1996]. Although MC3T3-E1 cells were able to respond to an increase in $[Ca^{2+}]_o$ or CaSR agonists with an increase in proliferation demonstrated by an increase in DNA synthesis and serum response element transactivation, the authors were not able to detect any CaSR expression by Northern analysis, using a mouse CaSR-specific probe, or by reverse transcribed polymerase chain reaction (RT-PCR) to amplify CaSR suggesting that an extracellular cation-sensing mechanism is present in MC3T3-E1 osteoblasts that is functionally similar to but molecularly distinct from CaSR [Quarles et al., 1997]. In contrasts, other groups were able to demonstrate that MC3T3-E1 and MG-63 cells express both the CaSR transcripts and protein detected by RT-PCR, Northern analysis, and Western analysis, immunocytochemistry, respectively [Yamaguchi et al., 1998b; Kanatani et al., 1999]. This contradiction was explained by the potential differences between the cells that were used in these studies and the presumption that murine osteoblast-like cells (MC3T3-E1) are the same as human osteosarcoma cells (MG-63). Therefore, primary rat calvarial osteoblasts that are considered "bona fide" osteoblasts were used to examine the expression and function of CaSR [Chattopadhyay et al., 2004]. Chattopadhyay et al. [2004] reported that primary osteoblasts express functional CaSR that plays a role in inducing osteoblast proliferation in response to Ca^{2+} (5 mM) by inducing cyclin D expression. These data suggest that in response to an increase in $[Ca^{2+}]_o$, in the bone microenvironment, osteoblast proliferation is increased by a CaSR-dependent mechanism (Fig. 1).

One thing for certain is that more studies are needed to completely characterize the expression of CaSR in osteoblasts and to elucidate its function and role in osteoblast proliferation and differentiation. Studies should include in vitro examination of osteoblasts harvested from animals where CaSR has been knocked out. Finally, although some studies report that the absence of CaSR does not have a major impact on the development of bone due to a redundancy and that other Ca^{2+} sensors could compensate its loss, the unequivocal answer will only be presented by generating an osteoblast conditional knockout of Ca^{2+} sensors and examining the bone phenotype of these animals [Pi et al., 2000].

INTRACELLULAR CALCIUM IN OSTEOBLASTS

During remodeling, osteoblasts and osteoclasts form and resorb bone, respectively. Several hormones, such as vitamin D3, IGF, and PTH, play major roles in the regulation of the remodeling process by modulating osteoblast differentiation. In addition, many factors such as cytokines and growth factors released from the bone microenvironment after bone

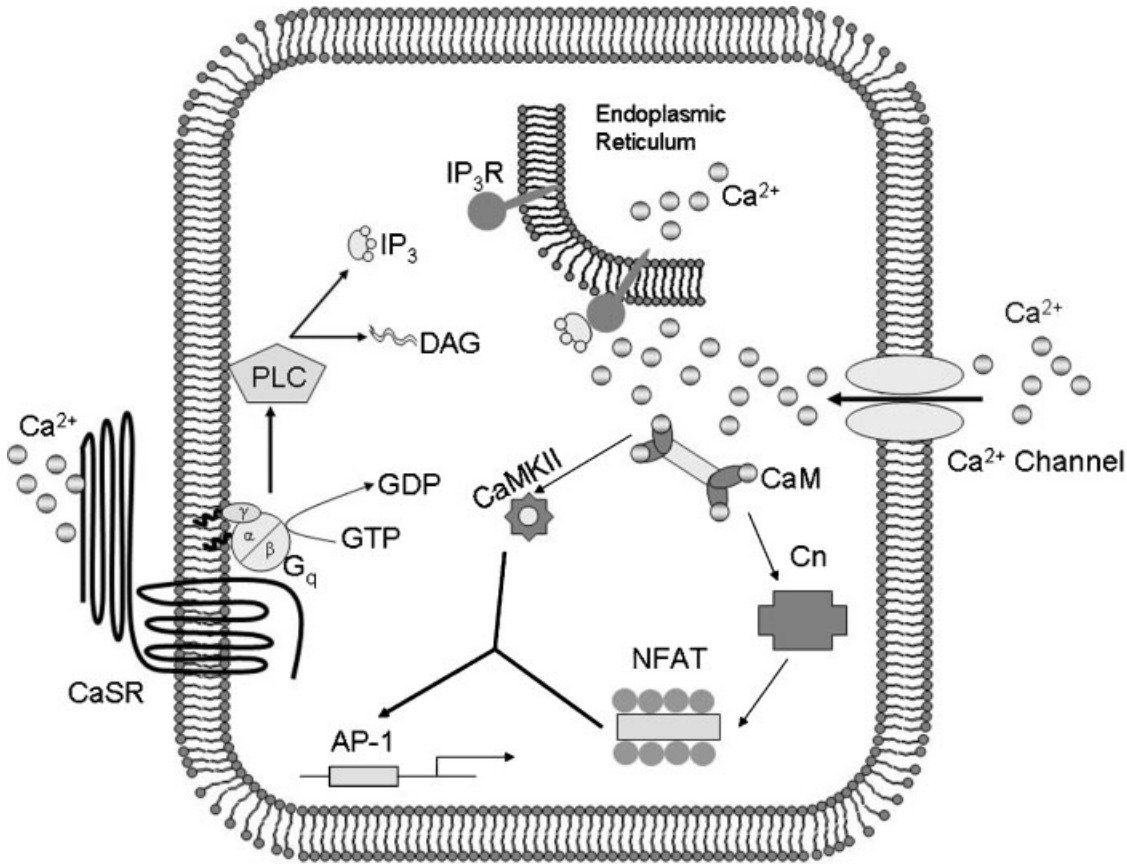

Fig. 1. Calcium/calmodulin signaling regulates gene expression in osteoblasts. Extracellular Ca^{2+} increases intracellular Ca^{2+} by a calcium-sensing receptors (CaSR)-mediated activation of PLC that leads to increasing IP3. Calcium activates calmodulin which in turn activates both CaMKII and calcineurin leading to modulation of the expression of AP-1 transcription factor family members.

resorption could play a critical role in osteoblast differentiation. Although the mechanisms of action of many of these hormones have been previously elucidated there is one thing that they all have in common, they are capable of increasing intracellular calcium by different mechanisms. Furthermore, the increase in $[Ca^{2+}]_o$ can raise $[Ca^{2+}]_i$ by increasing Ca^{2+} transport through L-type and non-L-type isoforms of voltage-gated calcium channels [Barry, 2000; Francis et al., 2002]. The pharmacologic and kinetic characteristics of L-type voltage-gated channels have already been identified in rat osteoblast-like cells, UMR-106 and ROS 17/2.8 osteosarcoma cells and in primary mouse and rat osteoblasts [Chesnoy-Marchais and Fritsch, 1988; Guggino et al., 1988; Duncan and Misler, 1989; Grygorczyk et al., 1989; Karpinski et al., 1989; Yamaguchi et al., 1989; Barry, 2000]. These channels have been shown to be activated by high and low voltage in addition to being responsive to several hormones, including PTH and vitamin D [Caffrey and Farach-Carson, 1989; Bidwell et al., 1991]. Calcium channels are thought to play a more significant role in osteoblast proliferation than in differentiation suggesting that increasing $[Ca^{2+}]_i$ activates Ca^{2+} signaling pathways that are dedicated to inducing proliferation. Several reports indicate that this pathway is a CaMK-dependent pathway that will ultimately lead to the activation of cyclin protein expression and acceleration of the rate of entry of cells into the cell cycle [Madgwick et al., 2005]. Although Ca^{2+} is one of the most important second messengers in cell biology, its cellular homeostasis, transport, signaling, and its role in gene expression in osteoblasts remains understudied. It is critical to understand the role Ca^{2+} plays in both the proliferation and differentiation of osteoblasts and to dissect the different pathways that one molecule can specifically

activate in order to further our understanding of osteoblast growth and differentiation (Fig. 1).

Hormones and Intracellular Calcium

Increasing $[Ca^{2+}]_o$ results in significant changes in bone formation, such as stimulation of bone formation in bone explants, increases in proliferation and/or chemotaxis [Yamaguchi et al., 1998a,b] that are partly attributable to CaSR activation. Increasing Ca^{2+} influx through different Ca^{2+} channels has also been suggested as a potential mechanism for the cellular response to $[Ca^{2+}]_o$. It has been demonstrated that neonatal rat calvarial osteoblasts express two classes of voltage-gated Ca^{2+} channels [Chesnoy-Marchais and Fritsch, 1988]. These channels were found to be of the "low threshold" (T-type) and "high threshold" (L-type) [McCleskey et al., 1986]. It has also been shown that osteoblast-like osteosarcoma cells, ROS 17/2.8, UMR-106.01, and MG-63 also express voltage gated L-type Ca^{2+} channels [Guggino et al., 1989].

Many hormones and cytokines such as vitamin D3, PTH, and TGF have been shown to affect bone remodeling by direct action on osteoblasts [Marks and Popoff, 1988]. These agents elicit their function by modulating the expression of osteoblast-specific genes, such as collagen type I and osteocalcin [Rowe and Kream, 1982; Franceschi et al., 1988; Noda et al., 1988]. Physiological concentrations of these agents have been shown to produce short-term changes in the permeability of the cell surface membrane that triggers the activation of several intracellular signaling pathways involving second messengers such as DAG, cAMP, IP3, and $[Ca^{2+}]_i$ [Yamaguchi et al., 1987; Caffrey and Farach-Carson, 1989]. Earlier studies have shown that treating osteoblasts with PTH results in modulating $[Ca^{2+}]_i$ [Dziak and Stern, 1975a,b]. Later, when the ability to accurately measure changes in free cytosolic calcium concentration using fluorescent probes was achieved, UMR-106 osteoblast-like cells were treated with PTH and $[Ca^{2+}]_i$ was measured. These studies showed that PTH induces an increase in $[Ca^{2+}]_i$ occurring in three successive phases: a rapid increase in $[Ca^{2+}]_i$ occurring within seconds, rapid decrement in $[Ca^{2+}]_i$ to near-resting levels within 1 min, and a slow increment in $[Ca^{2+}]_i$ [Yamaguchi et al., 1987]. The first phase of increase in $[Ca^{2+}]_i$ was dependent on extracellular Ca^{2+} and was blocked by Ca^{2+} channel blockers while the third phase of increase was also dependent on extracellular Ca^{2+} but was mediated by a cAMP-dependent Ca^{2+} channel that is insensitive to PKC and to Ca^{2+} channel blockers. The second phase, decrease in $[Ca^{2+}]_i$, was due to the rapid inactivation of the phase one calcium channels. Based on these studies it was concluded that UMR-106 cells respond to PTH stimulation by the activation of a cAMP-independent Ca^{2+} channel, which is rapidly inactivated. The subsequent PTH-dependent increase in cellular cAMP is followed by activation of a cAMP-dependent Ca^{2+} channel resulting in a slow rise in $[Ca^{2+}]_i$ [Yamaguchi et al., 1987]. However, when the effect of PTH on $[Ca^{2+}]_i$ was examined in other osteoblastic cells, SaOS-2, ROS 25/1, ROS 17/2.8, and primary neonatal mouse calvarial osteoblasts there was no acute (1 s–20 min) spike in $[Ca^{2+}]_i$, in response to PTH, in any of the cells examined apart from SaOS-2. In these cells, there was a 15% increase in $[Ca^{2+}]_i$ 3 min after PTH treatment that lasted for 20 min. Furthermore, PTH was capable of increasing cAMP in both ROS and SaOS-2 osteoblastic cells. These studies concluded that the increase in $[Ca^{2+}]_i$ in osteoblasts, in response to PTH is not dependent on the activation of the PTH receptor and/or adenylate cyclase [Boland et al., 1986]. Finally, in order to verify the effect of PTH on $[Ca^{2+}]_i$, several other studies reported that PTH has a powerful capacity to mobilize Ca^{2+} into the cytosolic compartment [Donahue et al., 1988; van Leeuwen et al., 1988]. Based on these studies and the contradictory reported results, it is critical to accurately determine the precise role of Ca^{2+} in response to PTH. It is known that this hormone has two modes of action. It can act as a resorptive agent when used continuously or as an anabolic when used intermittently. It is possible that the different methods of treatment can produce different levels of $[Ca^{2+}]_i$ ultimately activating different signaling pathways that will either increase or decrease osteoblast differentiation. Further work is needed to completely elucidate the effects of PTH on $[Ca^{2+}]_i$ in osteoblasts.

Similar to PTH, vitamin D3 has also been shown to increase $[Ca^{2+}]_i$ in osteoblasts by both increasing the release of Ca^{2+} from the intracellular stores as well as activating transmembrane influx mediated by voltage-gated Ca^{2+} channels [Lieberherr, 1987; Yamaguchi et al.,

1987]. When cultured neonatal calvarial osteoblasts were treated with 1,25-$(OH)_2$, 24,25-$(OH)_2$, or 25-(OH)-vitamin D3, a rapid increase in intracellular Ca^{2+} was observed. Interestingly, the concomitant use of a Ca^{2+} channel blocker completely inhibited the effects of 1,25-$(OH)_2$ on $[Ca^{2+}]_i$ while partially inhibiting the elevation of $[Ca^{2+}]_i$ in response to 24,25-$(OH)_2$ and 25-(OH)-vitamin D3 [Lieberherr, 1987]. These results suggest that vitamin D3 metabolites have a direct action on voltage sensitive Ca^{2+} channels. The effects of vitamin D3 on Ca^{2+} influx in osteoblasts was further examined by treating ROS 17/2.8 osteosarcoma cells with 1,25-$(OH)_2$-vitamin D3 and performing ion flux studies using $^{45}Ca^{2+}$. These studies confirmed that the only voltage-gated inward current in ROS 17/2.8 osteoblastic cells is generated by "L-type" calcium channels [Caffrey and Farach-Carson, 1989]. Comprehensive studies are needed to fully elucidate the signaling pathways that are activated by increasing $[Ca^{2+}]_i$ in response to vitamin D3 treatment in osteoblasts.

Finally, not only hormones and growth factors are capable of inducing Ca^{2+} influx but also integrin-mediated cell adhesion is known to modulate $[Ca^{2+}]_i$ in a variety of cell types [Richardson and Parsons, 1995]. It has been shown in a skeletal muscle cell line, that integrin-mediated Ca^{2+} signaling requires both Ca^{2+} release from IP3-sensitive SR Ca^{2+} stores and extracellular Ca^{2+} influx through L-type Ca^{2+} channels. The interaction between α-7 integrin and laminin induced a transient elevations in $[Ca^{2+}]_i$ as a result of both IP3-dependent Ca^{2+} release from intracellular stores and extracellular Ca^{2+} influx through voltage-gated, L-type Ca^{2+} channels. Interestingly, it seems that intracellular Ca^{2+} release was a prerequisite for Ca^{2+} influx in this system.

In osteoblasts, the effects of integrin-mediated cell adhesion on $[Ca^{2+}]_i$ are not well understood and it was thought that they were mediated via transforming growth factor-beta 1 (TGF-β1). TGF-β1 is a growth factor implicated in the control of proliferation, migration, differentiation, and survival of many cells, including osteoblasts [Janssens et al., 2005]. It was previously reported that the TGF-β1 mediated $[Ca^{2+}]_i$ signaling is required for increasing human osteoblast adhesion to extracellular matrix substrate independent of Smad2 activation [Nesti et al., 2002]. The same group then examined whether the TGF-β1 stimulated Ca^{2+} signaling pathway is involved in enhancing the interaction between the extracellular matrix protein fibronectin, and $\alpha_5\beta1$ integrin on the osteoblast surface. These studies demonstrate that the TGF-β1 induced intracellular Ca^{2+} signal is partly responsible for stimulating the gene and protein expression of α_5 integrin in primary human osteoblasts. This effect was inhibited by nifedipine, a selective L-type Ca^{2+} channel blocker. This suggests TGF-β1-induced intracellular Ca^{2+} signaling in human osteoblasts is an important mechanistic step in the regulation of α_5 integrin expression and ultimately, cell adhesion [Nesti et al., 2002]. It is well known that osteoblast interaction by $\alpha_2\beta1$ integrin to collagen type I in the extracellular matrix is critical for osteoblast differentiation [Meyers et al., 2004]. It is therefore possible that the interaction of collagen I and $\alpha_2\beta1$ integrin leads to an increase in $[Ca^{2+}]_i$ by increasing Ca^{2+} influx through the L-type Ca^{2+} channel. This could ultimately lead to the activation of Ras and the extracellular signal-related protein kinase (ERK) signaling pathway that is critical for Runx2 activation and osteoblast differentiation.

THE ROLE OF CALMODULIN IN OSTEOBLAST DIFFERENTIATION

Many of the cellular effects of Ca^{2+} are mediated by the Ca^{2+} binding protein, calmodulin (CaM). The functions of CaM are not confined to its Ca^{2+}-bound form, as Ca^{2+}-free CaM can also recognize different target proteins. CaM is a ubiquitous, calcium-binding protein expressed in all eukaryotes and it can bind to and regulate different protein targets, resulting in multiple and different cellular functions. Structurally, CaM is a dumb-bell shape formed by two globular domains at its C- and N-termini that are connected by a flexible helical linker region. Each globular end contains a pair of Ca^{2+}-binding motifs, and the binding of Ca^{2+} to CaM exposes a hydrophobic surface which is responsible for binding to various target proteins. CaM plays a critical role in the regulation of many biological processes, such as inflammation, metabolism, apoptosis, muscle contraction, intracellular transport, short-term and long-term memory, growth cone elongation, gene expression, ion channel function, and the immune response [O'Day, 2003]. CaM is expressed in many cell types and localizes to different subcellular

compartments such as the cytoplasm, within organelles, or associated with the plasma or organelle membranes. Many of the downstream targets that CaM binds to and activates are unable to bind calcium themselves; therefore they use CaM as a calcium sensor and signal transducer. This gives CaM the ability to be involved in almost all aspects of cellular function via the diversity of its target proteins, which include adenylyl cyclases and phosphodiesterases, many protein kinases (CaMKII), and protein phosphatases (calcineurin), nitric oxide synthase, Ca^{2+} pumps, and many ion channels [Tran et al., 2003]. CaM is activated by intracellular calcium that increases by a different processes involving membrane potential-dependent Ca^{2+} entry through L-type calcium channels, release from Ca^{2+} stores in the endoplasmic and the sarcoplasmic reticulum. Upon binding up to four calcium ions, CaM undergoes a conformational change, which enables it to bind to specific proteins eliciting a specific response. In addition, CaM undergoes post-translational modifications, such as phosphorylation, acetylation, methylation, and proteolytic cleavage, each of which can potentially modulate its actions [O'Day, 2003].

CaM-binding proteins are diverse and their interactions with CaM are divided into calcium-dependent and independent binding and modes of regulation. Ca^{2+}/CaM complexes alter the function of target proteins upon binding and activate a specific calcium signal transduction pathway while the calcium-free apocalmodulin (ApoCaM) binds to different targets, eliciting different cellular responses [Jurado et al., 1999; O'Day, 2003] (Table I). Several mechanisms have been proposed to explain CaM interactions with target proteins. CaM is thought to activate myosin light chain kinase (MLCK) and CaM kinase II (CaMKII) by displacement of their auto-inhibitory domains. Other proposed mechanisms of activation include binding of the CaM C-terminal alone, binding of CaM in an extended conformation, and dimer formation with its target. This mechanistic diversity means that there is no single conserved amino acid sequence for CaM binding, however several different binding motifs have been recognized based on the positions of conserved hydrophobic residues [O'Day, 2003].

CaM not only mediates the effects of changes in $[Ca^{2+}]_i$, but also $[Ca^{2+}]_i$ itself by regulating the activity of Ca^{2+} pumps and channels,

TABLE I. A List of Some Calmodulin and Apocalmodulin Binding Proteins

Class	Name	Function
Ca^{2+} Calmodulin-binding proteins		
Kinases	CASK	Scaffolding and regulation of cell growth?
	CaMKI	Multifunctional
	CaMKII	Multifunctional
	CaMKIII	Multifunctional
	CaMKIV	Multifunctional
	Myosin light chain kinase	Regulates the concentrations of smooth muscles
	Phosphorylase B kinase	Regulates glycogen metabolism
Phosphatase	Calcineurin 2b	Multifunctional
	CaMKPase	Regulates CaMKII
Second messenger	Adenylate cyclases, type 1	Produces cAMP
	Ca^{2+} transporter-ATPase	Decreases $[Ca^{2+}]_i$
	cAMP phosphodiesterase	Degrades cAMP
	G-protein coupled receptor	Multifunctional
	Inositol 3-kinase A	Produces IP4
	NOS	Produces nitric oxide
Cytoskeletal and muscle	α-1 syntrophin	Inhibits dystrophin binding
	Caldesmon	Inhibits F-actin binding
	Dystrophin	Inhibits F-actin binding
	MARCKS	Inhibits F-actin binding
	Spectrin α-chain	Inhibits F-actin binding
Apocalmodulin-binding proteins		
Neuroprotein	Glutamate decarboxylase	Glutamate decarboxylation
	Neuromodulin	Multifunctional
	Neurogranin	Unidentified
	PEP-19	Unidentified
Second messenger	Inositol 1,4,5 triphosphate receptor	Inositol 1,4,5 trisphosphate binding
	cGMP-dependent protein kinase	cGMP and cAMP-dependent phosphorylation
	SR Ca^{2+} release channel	Increase Ca^{2+} release from SR
	iNOS	Produces nitric oxide
Cytoskeletal and muscle	IQGAP1	Unidentified
	Myosin I	Multifunctional
	P190	Actin-activated ATPase

including the plasma membrane Ca^{2+}-pumps, ryanodine receptors, IP3-receptors, and Ca^{2+}-channels. One of the best examples of the diverse functions of CaM is in its regulation of Ca^{2+} channels. CaM exerts two opposing effects on P/Q-type calcium channels, first promoting, then inhibiting channel opening. Both of these responses require Ca^{2+} and CaM to bind to the C-terminal tail of the channel. The different effects depend upon which CaM domain binds the calcium: Ca^{2+}-binding to the C-terminal CaM domain promotes channel opening, while Ca^{2+}-binding to the N-terminal CaM domain promotes channel closure. The modulation of Ca^{2+} influx via the control of calcium channels has important consequences. For instance, Ca^{2+} is essential for cardiac excitation–contraction coupling and the propulsion of blood: Ca^{2+} activates myofilaments during cardiac systole causing contraction; the release of Ca^{2+} from the myofilaments during cardiac diastole then causes relaxation.

One of the earliest reports that examined the role of CaM in bone reported a decrease in calmodulin levels in the weight-bearing cartilage of animals treated with steroids and concluded that the characterization of calmodulin activity is warranted in the study of osteopenic states at the subcellular level [Lehman et al., 1984]. Several studies have been performed since then confirming that CaM indeed plays a critical role in osteoblast differentiation and that CaM also is critical in the osteoblast response to a variety of stimuli. Due to the ability of PTH and vitamin D3 to increase $[Ca^{2+}]_i$, as discussed above, the role of these hormones in activating CaM has also been studied. It has been shown that CaM plays an important role in the osteoblast response to both PTH and to vitamin D3 [Wong, 1983; Rao et al., 1989]. In addition, it has been demonstrated the CaM also plays a role in regulating bone morphogenetic protein-2 (BMP-2) signaling. BMP-2 and TGF-β are important growth factors that modulate osteoblast differentiation by activating Smad1 and Smad2, respectively. It was found that CaM physically interacts and binds to two distinct and conserved regions of both Smad1 and 2. This interaction inhibits the subsequent ERK-dependent phosphorylation of the Smads [Scherer and Graff, 2000]. Furthermore, it was shown that CaM also interacts with and binds to the estrogen receptor. This interaction was found to increase the transcription activity of the estrogen receptor [Li et al., 2003]. Finally, we have recently demonstrated that the pharmacologic inhibition of CaM by trifluoperazine (TFP) decreases bone formation in vivo and osteoblast differentiation in vitro [Zayzafoon et al., 2005]. This effect was mediated by the ability of CaM to activate CaMKII and ultimately to regulate the expression of the *c-fos* transcription factor. Despite all of these studies, many questions remain unanswered such as if CaM expression is altered during osteoblast differentiation, the localization and the function of CaM in osteoblasts and most importantly what would be the skeletal phenotype of an osteoblast conditional CaM knockout animal. When such an animal is generated, it will be very exciting to examine the effects of many hormones such as PTH, vitamin D3, BMP-2, TGF-β, and estrogen on bone formation.

CALMODULIN KINASE AND OSTEOBLAST DIFFERENTIATION

CaMKII is a multifunctional kinase that is highly expressed in neuronal cells and is known to be critical for decoding Ca^{2+} signal transduction. The multifunctionality of CaMKII is evident by the important role it plays in the regulation of synaptic transmission [Pettit et al., 1994], gene expression [Enslen and Soderling, 1994; Dolmetsch et al., 1997], cell growth [Lorca et al., 1994], mRNA translation [Qin et al., 2003], cytoskeletal organization [Shen et al., 1998], growth of synaptic arbor [Zou and Cline, 1999], learning [Braun and Schulman, 1995], and memory [De Koninck and Schulman, 1998] all of which are closely dictated by its multimeric structure, autoregulation, isozymic type, and subcellular localization [Hudmon and Schulman, 2002a]. CaMKII is a serine/threonine kinase that is activated in response to Ca^{2+} signals and phosphorylates several downstream proteins [Hook and Means, 2001]. The frequency of the CaMKII response has been shown to be modulated by both the amplitude and duration of individual Ca^{2+} spikes that are translated into distinct levels of CaMKII activity, thus the designation Ca^{2+} decoder [De Koninck and Schulman, 1998].

There are now over 30 identified isoforms of CaMKII, with molecular masses ranging from 52 to 83 kDa, resulting from alternative messenger RNA splicing of four CaMKII genes (α, β,

γ, δ) [Braun and Schulman, 1995; Fink and Meyer, 2002]. The CaMKII isoforms α and β are highly homologous, with the exception of an ~30-amino acid sequence in the β isoform that serves as an actin-targeting domain [Fink and Meyer, 2002]. These two isoforms were once thought to be expressed only in the brain [Erondu and Kennedy, 1985], but it is becoming increasingly evident that the α isoform is expressed in other cells such as thyroid, hepatic, and smooth muscle cells [Muthalif et al., 1996; Illario et al., 2003]. In contrast, the γ and δ isoforms are known to be expressed in various extraneural cells but predominantly in embryonic cells and lymphocytes, respectively [Caran et al., 2001]. All CaMKII isoforms are capable of homo- and hetero-multimerization (6–12 kinase subunits) via their C-terminal domain to form a holoenzymes with a wheel-like structure (Fig. 2). Two wheels may also interact and form a double-wheel structure (Fig. 2) [Hudmon and Schulman, 2002a,b; Colbran, 2004]. Upon activation, in response to an increase in Ca^{2+} and the activation of CaM, Ca^{2+}/CaM displaces the auto-inhibitory domain on CaMKII by wrapping around it and triggering its phosphorylation. The kinase can then remain in an activated state by promoting autophosphorylation of the Thr-286 on the auto-inhibitory domain of an adjacent CaMKII monomer [Maier and Bers, 2002]. This autophosphorylation property of CaMKII is critical to the ability of this enzyme to remain active after the decline in $[Ca^{2+}]_i$. Auto-phosphorylation, although not essential for CaMKII activation, plays a critical role in increasing the affinity of CaM for CaMKII by decreasing the rate of CaM dissociation, a process known as "calmodulin trapping." CaM is trapped by autophosphorylation so that even when there is a decrease in $[Ca^{2+}]_i$ the kinase remains fully active until CaM dissociates (several hundreds of seconds) [Hook and Means, 2001]. This mechanism is believed to be responsible for the increased sensitivity of CaMKII to changes in $[Ca^{2+}]_i$.

We have recently demonstrated that α and γ CaMKII isoforms are expressed in osteoblasts and that α-CaMKII regulates CRE and SRE transactivation and *c-fos* expression. We have

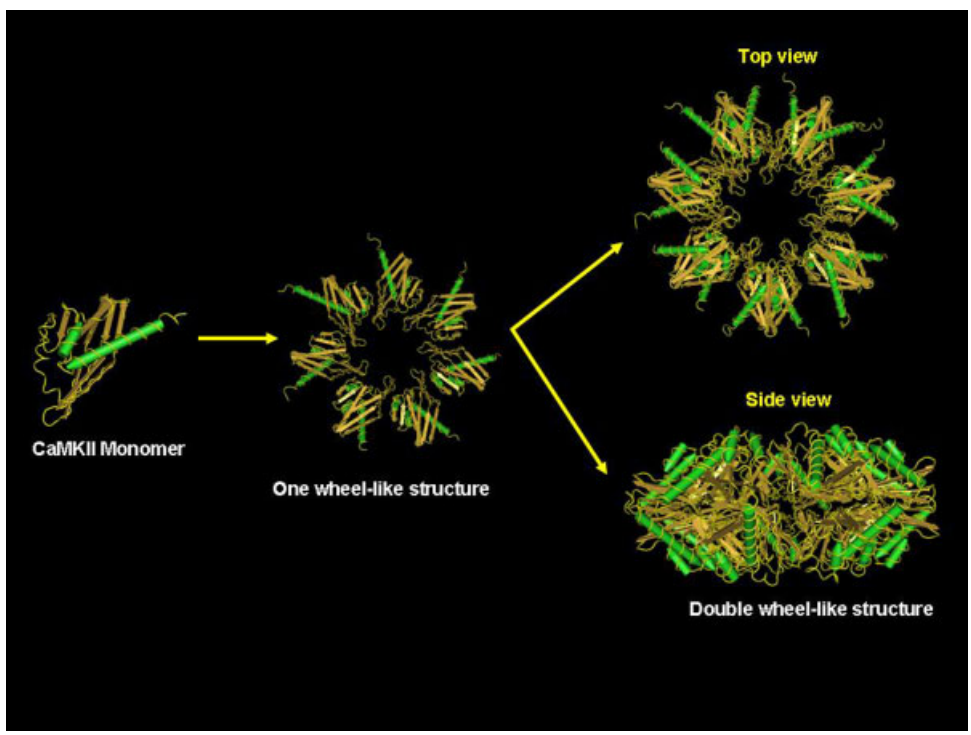

Fig. 2. The three-dimensional structure of the calmodulin kinase II (CaMKII) holoenzyme. One CaMKII monomer multimerize with other CaMKII subunits (6–12 kinase subunits) via their C-terminal domain to form a wheel-like structure. Two wheels may also interact to form a double-wheel structure. This image was created using Cn3D software provided by NCBI (http://www.ncbi.nlm.nih.gov/). [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

also shown that pharmacologic and specific genetic inhibition of α-CaMKII inhibits the activity of the CRE and SRE, leading to a decrease in AP-1 transactivation and DNA binding activity. Ultimately, this leads to a decrease in osteoblast differentiation, both in vitro and in vivo [Zayzafoon et al., 2005]. CaMKII has also been implicated, by others, in the function of osteoblasts, as a regulator of the action of PTH and vitamin D. PTH has been previously shown to increase $[Ca^{2+}]_i$ in the UMR 106-01 osteoblastic cell line [Yamaguchi et al., 1987] suggesting that CaMKII could be a downstream PTH target. It is known that PTH induces the expression of several genes, including collagenase-3 (MMP-13) [Selvamurugan et al., 2000]. Not surprisingly, it has been shown that the inhibition of CaMKII in UMR cells significantly decreases the expression of MMP-13 in response to PTH suggesting that some of the osteoblast response to PTH is mediated by CaMKII [Quinn et al., 2000]. Another osteogenic agent that has been thought to play a role in activating CaMKII in osteoblasts is vitamin D3 [Farach-Carson et al., 2004]. At physiologic levels, vitamin D3 has been shown to be a potent modulator of calcium channel function in the ROS 17/2.8 osteoblastic cell line [Caffrey and Farach-Carson, 1989]. Vitamin D3 activates both membrane-initiated rapid Ca^{2+} responses linked to influx through voltage sensitive Ca^{2+} channels and longer term nuclear receptor-mediated changes in gene expression [Caffrey and Farach-Carson, 1989]. As a result, the role of CaMK was examined in the vitamin D3 response in MC3T3-E1 osteoblasts [Farach-Carson et al., 2004]. All CaMK isoforms were detected in osteoblasts (CaMKI, II, and IV) and it was demonstrated that the increase in Ca^{2+} in response to vitamin D3 results in an activation of CaM/CaMK that leads to an increase in phosphorylation of CREB and the activation of CRE and ultimately regulation of OPG expression [Farach-Carson et al., 2004]. Consistent with this report, our results also demonstrate that CaMKII and more specifically, α-CaMKII, regulates the CREB/ATF and ERK/SRE signaling pathways and AP-1 transcription factor activity in resting osteoblasts [Zayzafoon et al., 2005].

Surprisingly, despite the importance of Ca^{2+} in osteoblast differentiation and the role of CaMKII as a decoder of Ca^{2+} signaling, the role of CaMKII in osteoblast proliferation and differentiation and the effects of many hormones, that increase $[Ca^{2+}]_i$ in osteoblasts, on CaMKII remain understudied. It is critical to elucidate the functions of CaMKII in osteoblasts based on this enzyme's mode of action in other systems. For example, in many cells including osteoblasts, integrin receptor activation after binding to extracellular matrix proteins is critical not only for cell adhesion but also for cell motility, proliferation, differentiation, and survival. Furthermore, integrin activation has also been shown to increase $[Ca^{2+}]_i$ in skeletal muscle cells as a result of an increase in the release of Ca^{2+} from sarcoplasmic/endoplasmic reticulum and extracellular Ca^{2+} influx through voltage-gated, L-type plasma membrane Ca^{2+} channels [Kwon et al., 2000]. Based on this, it was hypothesized and later shown that integrin binding to fibronectin activates the Ca^{2+}–CaM/CaMKII pathway leading to an increase in the phosphorylation of ERK through regulation of Raf-1 activation [Illario et al., 2003]. It is possible that a similar mechanism exists in osteoblasts, the binding of the integrin receptors to collagen I could activate CaMKII leading to an increase in the phosphorylation of ERK and subsequently activation of Runx2 and osteoblast differentiation.

Finally, not only osteoblast differentiation but also proliferation could be controlled by CaMKII. We have previously shown that inhibition of CaMKII significantly decreases *c-fos* expression and AP-1 activation in osteoblasts [Zayzafoon et al., 2005]. It is known that c-Fos is critical for osteoblast growth and proliferation. Based on this, it is probable that the activation of CaMKII, in response to an increase in $[Ca^{2+}]_i$, is a key regulator of osteoblast proliferation. This increase in $[Ca^{2+}]_i$ could result from either an increase in the release of Ca^{2+} from endoplasmic reticulum and/or extracellular Ca^{2+} influx through voltage-gated Ca^{2+} channels from the bone-microenvironment where the osteoclast is resorbing bone and releasing relatively large concentrations of Ca^{2+}. A significant amount of work is needed in order to understand and characterize the precise function/s of CaMKII in osteoblasts.

CALCINEURIN AND OSTEOBLAST DIFFERENTIATION

Cn is a heterodimeric protein complex consisting of a catalytic subunit (CnA) and a

regulatory subunit (CnB). The CnA subunit contains the phosphatase domain, a CnB binding domain, a calmodulin binding domain, and an autoinhibitory loop. In resting cells, the autoinhibitory domain obscures the phosphatase domain and is displaced upon binding of CnB and Ca^{2+}/CaM to CnA resulting in the full activation of Cn. Cn directly binds to and dephosphorylates the NFAT1-4 transcription factors within the cytoplasm allowing them to translocate to the nucleus and participate in the regulation of gene expression [Crabtree and Olson, 2002; Bassel-Duby and Olson, 2003; Parsons et al., 2004].

The NFAT family of transcription factors was initially identified as transcription factors that mediate Ca^{2+}/CaM and Cn-dependent transcription of many cytokines, including IL-2, as part of T-cell activation [Rao et al., 1997]. This family is composed of five proteins related to the Rel/NFκB family (NFATc1-c4 and NFAT5) [Hogan et al., 2003]. The characteristic feature of NFAT is its regulation by Ca^{2+} and the Ca^{2+}/CaM-dependent serine-threonine phosphatase, calcineurin (Cn). In resting cells, NFAT proteins are highly phosphorylated and reside in the cytoplasm. Upon activation by increases in $[Ca^{2+}]_i$, and activation of CaM they are dephosphorylated by Cn, translocate to the nucleus, and become transcriptionally active [Hogan et al., 2003]. The Cn/NFAT signaling pathway is inhibited both in vivo and in vitro by several compounds such as cyclosporine A (CsA) and FK506.

CsA and FK506 are potent immunosuppressive agents that are used clinically to prevent rejection after transplantion by inhibiting Cn activation and NFAT dephosphorylation leading to a decrease in T-cell activation and cytokine expression [Rao et al., 1997]. These drugs exhibit high levels of toxicity resulting from their ability to inhibit Cn/NFAT signaling in cells outside the immune system [Dumont et al., 1992] most importantly, in the skeletal system. It has been documented that bone disease, in the form of osteopenia and osteoporosis, is a serious complication of organ transplantation in humans [Katz and Epstein, 1992; Shane et al., 1993, 1997]. This phenomenon is not only due to the underlying disease and to the organ transplantation but also was a side effect of the drugs that are administered pre- and post-transplantation such as steroids, methotrexate, anticoagulants, and immunosuppressive agents, such as CsA [Mazanec and Grisanti, 1989; Rich et al., 1992; Katz and Epstein, 1992; Lukert and Raisz, 1994]. The inhibition of the Cn/NFAT signaling pathway by the immunosuppressive drugs, CsA and FK506, has been associated with an increased incidence of bone fractures [McDonald et al., 1991]. The co-administration of CsA with other agents in humans makes it difficult to determine specific effects of CsA monotherapy on bone. Nevertheless, CsA monotherapy in kidney transplantation patients has been shown to increase serum levels of alkaline phosphatase, even though bone mass was reduced by increasing osteoclast activation [Loertscher et al., 1983] suggesting that inhibition of Cn/NFAT has some positive effect on osteoblasts. In animals, the effects of CsA treatment on bone are contradictory. It has been reported that the administration of CsA to rats produces severe osteopenia with either a significant increase or decrease in bone remodeling and turnover [Movsowitz et al., 1988; Movsowitz et al., 1989; Fu et al., 2001; Igarashi et al., 2004]. It is unclear why CsA treatment would produce a high turnover bone loss (increase in osteoblasts and osteoclasts) in one model and a low turn over bone loss in another (decrease in osteoblasts and osteoclasts). It is possible that these contradictions are due to the use of different species, genders, ages, drug concentrations, and duration of drug administration.

The specific roles of Cn and NFAT in osteoblast differentiation are not well understood and in some instances are even contradictory. Some studies have reported that the use of CsA and FK506 enhances osteoblastic differentiation in vivo and in vitro [Erben et al., 1998; Tang et al., 2002] while other studies report that both CsA and FK506 decrease osteoblast differentiation by various mechanisms such as CsA affecting extracellular matrix synthesis and degradation or FK506 affecting the transcriptional activity of Osterix [Fornoni et al., 2001; Koga et al., 2005]. Surprisingly, many of the studies that use Cn/NFAT inhibitors use such high concentrations, more than 1,000-fold, required to inhibit NFAT signaling. In addition, few studies demonstrate that treatment with Cn inhibitors was successful and specific in inhibiting the intended target, NFAT. We have recently examined the role of NFAT signaling in osteoblasts and have shown that several NFAT family members are expressed in the MC3T3-E1 osteoblastic cell line [Yeo et al.,

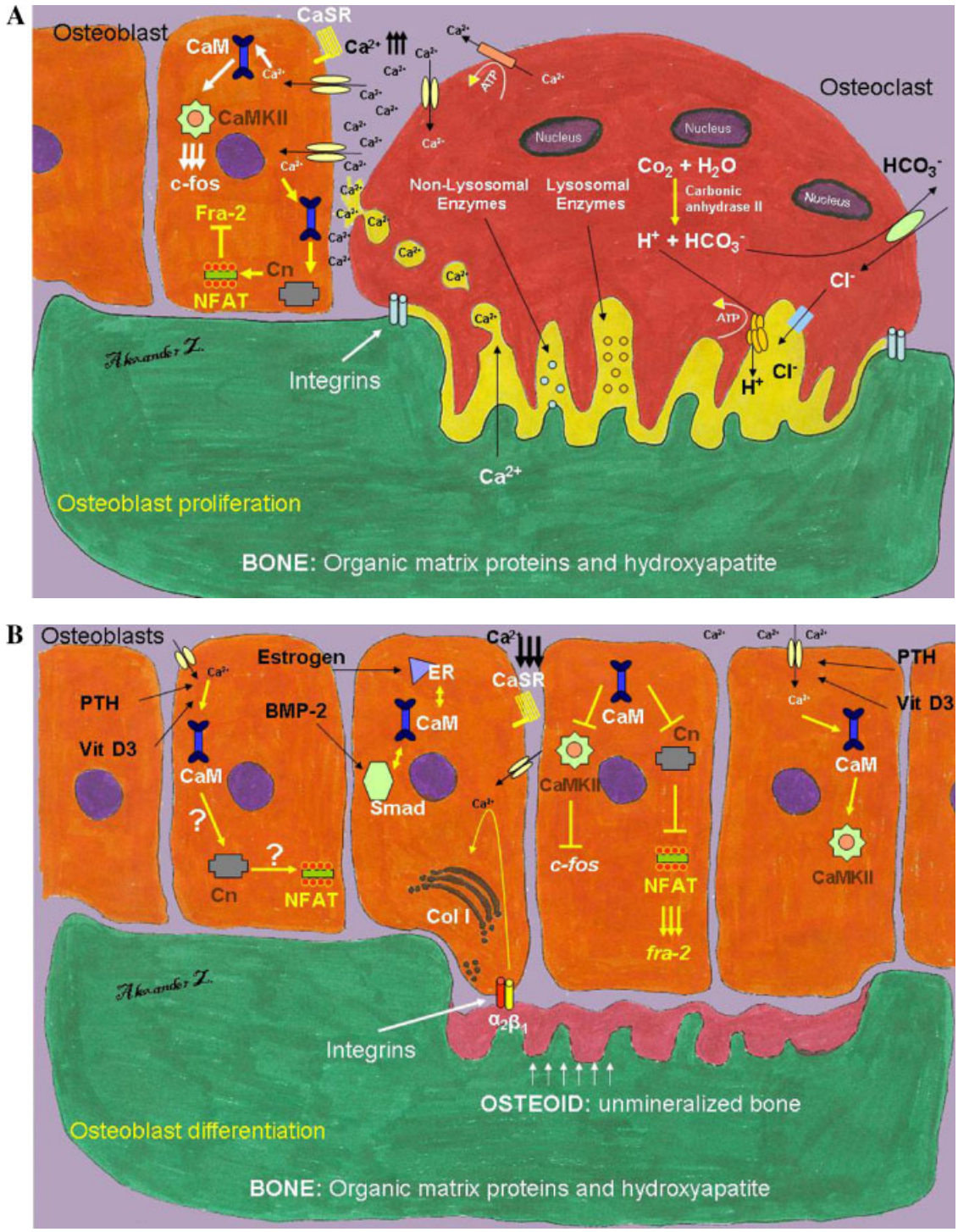

Fig. 3. The potential role of Ca^{2+}/calmodulin in regulating osteoblast proliferation and differentiation. **A**: During the resorption phase there is a dramatic increase in $[Ca^{2+}]_i$ resulting in the activation of Ca^{2+}/CaM. This signal will induce osteoblast proliferation by activation of CaMKII and *c-fos* expression as well as inhibiting osteoblast differentiation by stimulating Cn and decreasing *fra-2* expression. **B**: When $[Ca^{2+}]_o$ subsides, calmodulin activation is decreased causing a decrease in the activity of both CaMKII and Cn. This will diminish the proliferation of osteoblasts by reducing *c-fos* expression and stimulate osteoblast differentiation by inducing the expression of *fra-2*. Other factors that could potentially regulate Ca^{2+}/CaM signaling are PTH and vitamin D3 that modulate Ca^{2+} influx, thus CaM activation. Finally ER and Smad that interact and bind to CaM and regulate the function of estrogen and BMP-2 signaling, respectively. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

2005]. Our results demonstrate that inhibition of NFAT by the immunosuppressive drugs, CsA and FK506, increases osteoblastic differentiation while activation of NFATc1 by overexpressing a constitutively active NFATc1 in MC3T3-E1 osteoblasts, using a retroviral system, inhibits it [Yeo et al., 2005]. Our work is consistent with and supportive of clinical observations that patients treated with CsA have increased bone formation markers despite the observed decrease in bone density [Loertscher et al., 1983; Katz and Epstein, 1992]. Based on our work and on the work of others we believe that inhibition of the calcineurin/NFAT signaling pathway increases osteoblast differentiation and the contradiction in results stems from the wide variation in the concentrations of calcineurin inhibitors used, ranging between 1 nM and 25 μM.

Mechanistically, we have shown that NFATc1 negatively regulates the expression of *fra-2*, an AP-1 family member [Yeo et al., 2005]. Expression of the AP-1 family members has been shown to vary depending on the stage of osteoblast differentiation [McCabe et al., 1996]. During osteoblast proliferation, all AP-1 members are known to be expressed, while during mineralization, Fra-2 and to a lesser extent, JunD are known to be the major AP-1 members expressed, suggesting that Fra-2 expression is critical in osteoblast differentiation and mineralization [McCabe et al., 1996]. This was confirmed using a Fra-2 antisense construct in osteoblasts and demonstrating inhibition of differentiation and mineralization [McCabe et al., 1996]. Furthermore, it has been shown that hormones, such as PTH, increase osteoblast differentiation by increasing Fra-2 expression [McCauley et al., 2001]. Our work suggests that NFAT, and more specifically NFATc1, plays a critical role in regulating osteoblast differentiation. NFATc1 acts by negatively regulating *fra-2* expression, possibly by binding to an NFAT consensus sequence downstream of the transcriptional start site. Studies examining the role of Cn/NFAT in osteoblast differentiation are limited and more work is definitely needed in order to complete our understanding of this important signaling pathway in osteoblasts.

CONCLUSIONS AND FUTURE PROSPECTS

Osteoporosis, the major metabolic bone disease in the elderly, is characterized by decreases in the quality and quantity of bone ultimately leading to increased fragility. Almost all approved therapies for osteoporosis target the osteoclasts and the only anabolic treatment currently available is PTH. Calcium signaling is central to many aspects of osteoblast proliferation and differentiation. It is possible that the increase in $[Ca^{2+}]_o$ during the resorption phase together with the appropriate hormonal stimulation affect Ca^{2+} channels causing a dramatic increase in $[Ca^{2+}]_i$ resulting in the activation of Ca^{2+}/CaM. This signal induces osteoblast proliferation by activation of CaMKII and *c-fos* expression as well as inhibiting osteoblast differentiation by the activation of Cn and the inhibition of *fra-2* expression. When $[Ca^{2+}]_o$ subsides and again with the appropriate hormonal stimulation, the reverse will occur, CaMKII and Cn will be inhibited, *c-fos* expression will decline and *fra-2* expression will increase (Fig. 3). Hopefully, further investigation will shed more light on the role of Ca^{2+} and CaM in regulating these downstream targets and gene expression in osteoblasts. Further research will identify other molecular mechanisms by which CaMKII and/or Cn affect osteoblast differentiation and hopefully this will lead to the development of improved anabolic therapy for treating osteoporosis.

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