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Calcium Signaling and Tissue Calcification

Diane Proudfoot

Signalling Division, Babraham Institute, Babraham, Cambridge CB22 3AT, United Kingdom

Correspondence: diane.proudfoot@babraham.ac.uk

Calcification is a regulated physiological process occurring in bones and teeth. However, calcification is commonly found in soft tissues in association with aging and in a variety of diseases. Over the last two decades, it has emerged that calcification occurring in diseased arteries is not simply an inevitable build-up of insoluble precipitates of calcium phosphate. In some cases, it is an active process in which transcription factors drive conversion of vascular cells to an osteoblast or chondrocyte-like phenotype, with the subsequent production of mineralizing "matrix vesicles." Early studies of bone and cartilage calcification suggested roles for cellular calcium signaling in several of the processes involved in the regulation of bone calcification. Similarly, calcium signaling has recently been highlighted as an important component in the mechanisms regulating pathological calcification. The emerging hypothesis is that ectopic/pathological calcification occurs in tissues in which there is an imbalance in the regulatory mechanisms that actively prevent calcification. This review highlights the various ways that calcium signaling regulates tissue calcification, with a particular focus on pathological vascular calcification.

Calcification or mineralization are terms given to describe the crystallization of ionized calcium (Ca^{2+}) and PO_4^{3-}. This is a highly regulated physiological process occurring in bones and teeth, and a pathological process occurring in soft tissues. For calcification to initiate, a nidus for crystallization is required, along with an adequate supply of local Ca^{2+} and PO_4^{3-}. As might be expected, the systemic concentrations of Ca^{2+} and PO_4^{3-} are tightly controlled.

Approximately 99% of total body Ca^{2+} and 85% of PO_4^{3-} is found in bone, and this large mineral pool is under continuous turnover (e.g., via bone formation, resorption, and remodeling) regulated by numerous factors, including parathyroid hormone, thyroid hormone, calcitriol (1,25-hydroxyvitamin D_3), prostaglandins, aluminum, fluoride, acidification, and osteo-inductive factors (Greger 2000). The relatively minor component of Ca^{2+} and PO_4^{3-} found outside the bone is distributed between extracellular and intracellular compartments. Approximately half of the total Ca^{2+} content of blood is bound to proteins, whereas the remainder circulates free or complexed to anions. Systemic Ca^{2+} is regarded as a hormone itself, as it can modulate the function of the parathyroid gland, the thyroid gland, the kidney, and other organs and cells via the calcium-sensing receptor (CaSR). In fact, Ca^{2+} can have a more generalized effect as a hormone caused by the expression of the CaSR in numerous tissues. Systemic Ca^{2+} and PO_4^{3-} levels normally oscillate between ~1 mM and 1.5 mM (Brown 1991), although it has been proposed

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that larger extracellular Ca^{2+} changes may occur in the tiny interstitial spaces between cells, particularly at sites of inflammation (Olszak et al. 2000). Many other functions of Ca^{2+} are related to its role as a messenger within cells. Cells maintain a low, resting cytosolic Ca^{2+} concentration (~70 mM to 100 nM) through the action of calcium ATPases and exchange mechanisms that sequester the ion in intracellular stores, or extrude it across the plasma membrane (Carafoli 1991; Tsien et al. 1991; Moore et al. 1993; Sucher et al. 1996; Berridge 1997). Various stimuli, including hormones, growth factors, depolarization, and mechanical activation, trigger transient increases in cytosolic Ca^{2+} that lead to alterations in cell activity.

The main regulators of PO_4^{3-} homeostasis are parathyroid hormone, calcitriol, and peptides known as phosphatonins such as fibroblast growth factor 23 (FGF23). Interestingly, the physiological concentrations of Ca^{2+} and PO_4^{3-} in extracellular body fluids are known as metastable, which means they are sufficiently high to induce precipitation of calcium phosphate crystals. However, as discussed in the following sections, precipitation does not usually occur in extraskeletal tissues in health because of the presence of factors that actively inhibit calcification.

PHYSIOLOGICAL CALCIFICATION AND Ca^{2+} SIGNALING

Physiological mineralization is precisely controlled in terms of crystal type, morphology, and orientation, and a distinct spatial relationship exists between the mineral and organic phases. Bones contain calcified material in the form of hydroxyapatite, $(Ca_{10}(PO_4)_6(OH)_2)$, or carbonate-substituted hydroxyapatite organized within collagen fibrils. Bone is synthesized either directly by osteoblasts (via intramembranous ossification in flat bones, such as the skull), or by hypertrophic chondrocytes in the growth plate of cartilage (in long and short bones). This mineralized cartilage precursor is then later replaced by a mineralized bone matrix. In teeth, odontoblasts orchestrate the formation of dentin, a bone-like material that is found beneath the enamel. Bone turnover is regulated by osteoclasts that resorb mineralized matrix. Mineral-depleted matrix is recalcified via osteoblast-mediated bone deposition (Clarke 2008).

Osteoblasts express various Ca^{2+} channels including, stretch-activated, voltage-gated, store-operated (ORAI1), and TRP (TRPV6) channels (Jørgensen et al. 1997; Hwang et al. 2012; Lieben and Carmeliet 2012; Robinson et al. 2012; Choi et al. 2018). Voltage-gated channels are thought to be particularly important in maintaining Ca^{2+} waves between cells after mechanical stimulation (Lieben and Carmeliet 2012). Interestingly, L-type channels predominate in osteoblasts, whereas Ca^{2+} entry occurs via T-type channels in osteocytes (cells embedded within bone) (Lu et al. 2012). Little is known about osteoblast differentiation but it appears that Ca^{2+} signaling may be involved because inactivation of ORAI1 inhibits both osteoblast and osteoclast differentiation in vitro and in vivo (Robinson et al. 2012). In osteoclasts, Ca^{2+} oscillations, followed by sustained Ca^{2+} entry via TRP channels, stimulate osteoclast maturation by activating the Ca^{2+}/calmodulin signaling cascade and promoting the activity of NFATc1 (the nuclear factor of T cells c1) and CREB (cAMP response element-binding protein) (Negishi-Koga and Takayanagi 2009). Likewise, Ca^{2+} entry via TRPV4 and L-type Ca^{2+} channels controls chondrocyte differentiation, with Sox9 being the target transcription factor activated by Ca^{2+}/calmodulin signaling in chondrocytes (Lieben and Carmeliet 2012). Thus, Ca^{2+} signaling appears to be crucial in stimulating gene expression specific for the development of the cell types involved in bone formation and turnover.

Is Ca^{2+} signaling involved in the process of bone crystal formation? To answer this question, we need to know exactly how calcification occurs. A proposed mechanism for crystal formation is that the initial nidus occurs in matrix vesicles. These are extracellular particles of ~100 nm in diameter that were first described as membrane-bound vesicles that bud from the plasma membrane (Anderson 1995). More recently, they have been reported to share many similarities with exosomes, which are a product

of the endosomal pathway (Shapiro et al. 2015). Matrix vesicles and exosomes are distinct from apoptotic bodies released from dying cells.

There are two different types of matrix vesicles produced by chondrocytes in the bone growth plate: one type that does not mineralize and another that specifically undergoes mineralization in the hypertrophic zone of the growth plate (Kirsch et al. 1997). Importantly, an intracellular Ca^{2+} signal is thought to alter chondrocyte gene transcription such that matrix vesicles become endowed with transport systems promoting the accumulation of Ca^{2+} and PO_4^{3-} inside the vesicle lumen (Kirsch et al. 1997). The evidence to support the notion that a Ca^{2+} signal is involved originates partly from studies in which higher intracellular Ca^{2+} levels were detected in mature chondrocytes, compared with immature cells (Iannotti and Brighton 1989; Gunter et al. 1990) and also from studies using the chelator BAPTA to block intracellular Ca^{2+} elevations (Wang and Kirsch 2002; Wang et al. 2003). The "mineralization-competent" vesicles contain enhanced levels of Ca^{2+}-binding annexins, phosphatases, Na^{+}-dependent phosphate transporters, cholesterol, phosphatidyl serine, and a reduced level of calcification-inhibitors. In addition to effects on cell differentiation and matrix vesicle calcification potential, recent studies in osteocytes suggest that Ca^{2+} oscillations stimulate matrix vesicle release from cells and lead to enhanced bone formation (Morrell et al. 2018). Thus, Ca^{2+} signaling is thought to regulate bone formation via stimulation of matrix vesicle/exosome release, and by stimulating gene expression such that matrix vesicles/exosomes are loaded with the required proteins to orchestrate formation of the initial nidus for calcium phosphate crystal formation.

Although much evidence supports the scheme involving matrix vesicles described above, alternative hypotheses for initiation of calcium phosphate crystallization in the normal skeleton have been suggested. For example, collagen, noncollagenous proteins (such as bone sialoprotein), and lipids independently nucleate calcium phosphate crystals, because of their Ca^{2+}-binding and hydroxyapatite-binding properties (Raggio et al. 1986; Goldberg et al. 1996; He and George 2004). Other evidence indicates that levels of potent inhibitors of bone mineralization, such as pyrophosphate, are selectively reduced, thereby allowing calcification to occur (Hessle et al. 2002). Several studies have suggested that amorphous (noncrystalline) calcium phosphate acts as a precursor for crystal formation. Amorphous calcium phosphate forms either transiently within the gap zones inside collagen fibrils or within cells (i.e., in vesicles or mitochondria) and is subsequently secreted into the collagen-rich environment of bone where it progresses to fully crystalline calcium phosphate (Lehninger 1970; Mahamid et al. 2011; Boonrungsiman et al. 2012). It is possible that all of these mechanisms contribute toward physiological bone formation, and their links to Ca^{2+} signaling have yet to be fully established.

PATHOLOGICAL CALCIFICATION

Pathological calcification can occur in any tissue. It occurs commonly in aging, and is often associated with blood vessels, joints, and tumors. The calcified deposits (anchored calcium phosphate crystals within extracellular matrix) are often used as a marker of disease, although the deposits themselves can be harmful by causing mechanical stress or stiffness in affected tissues and have also been associated with cell damage and inflammation. Different terms have been assigned to calcification depending on the setting for calcium phosphate crystal formation. Dystrophic calcification occurs in association with damaged cells/tissues, and metastatic calcification occurs in normal tissue in the setting of high extracellular levels of Ca^{2+} and PO_4^{3-} (e.g., hypercalcemia); and where there is evidence for differentiation of cells into a bone-like phenotype, other terms have been used such as heterotopic ossification, metaplasia, or osteo/chondrocytic conversion. Fully formed bone containing osteocytes and bone marrow has been detected in diseased blood vessels, although its occurrence is relatively rare (Deneke et al. 2001). Bone cells at these sites may be derived from preexisting cells within the blood vessel, driven by osteogenic morphogens (Boström et al. 2011), or from circulating cells such as

osteogenic monocytes (Collin et al. 2015). Unlike physiological mineralization, the mineral found in ectopic calcification is less organized, features variable crystal sizes and shapes, and consists of calcium phosphate crystals and other calcium salts. The various forms of calcium-containing crystals found at different locations in vivo are summarized in Table 1. Chemical and nuclear magnetic resonance (NMR) studies revealed that the mineral of bone and calcification in blood vessels were very similar, both containing glycosaminoglycans on the surface of hydroxyapatite crystals (Duer et al. 2008). Why calcification should occur in nonskeletal tissues has gained a great deal of interest in recent years, and various stimuli and mechanisms have been proposed (Demer and Tintut 2014). The main concepts and their links with intracellular Ca^{2+} signaling have been highlighted in the following sections, with a particular focus on blood vessel calcification.

Blood Vessel Calcification and Ca^{2+} Signaling

Calcification is found at two distinct sites in the blood vessel: in the intimal layer in association with atherosclerosis and in the medial layer in association with aging, diabetes, and renal disease. The severity of calcification in coronary arteries correlates positively with cardiovascular deaths and strokes, highlighting the need to understand the mechanisms involved (Hulten et al. 2011; Otsuka et al. 2013; Cho et al. 2015; Motoyama et al. 2015). In atherosclerosis, the calcium phosphate crystals have a spotty/speckled morphology, occurring within and around ma-

Table 1. Occurrence of different types of calcium salts

Calcium salt	Formula	Occurrence
Hydroxyapatite	$Ca_{10}(PO_4)_6(OH)_2$	Enamel, bone, atherosclerosis, medial calcification, kidney stones (Schmid et al. 1980; LeGeros 2001; Barrere et al. 2006) Myocardium (Pillai et al. 2017) Breast tumors (Scimeca et al. 2014)
Octacalcium phosphate (OCP)	$Ca_8\ H_2(PO_4)_6\ 5(OH)_2$	Dental and urinary caliculi, joints (Cheung et al. 1997; Tomazic 2001; Whelan et al. 2005; Barrere et al. 2006)
Carbonate-apatite	$Ca_5(PO_4,CO_3)_3(OH)$	Enamel, bone, dental caliculi, atherosclerosis, medial calcification, urinary stones, soft-tissues, joints (Schmid et al. 1980; LeGeros 2001; Whelan et al. 2005)
Amorphous calcium phosphate	$Ca_9(PO_4)_6$	Microcalcification in coronary arteries (Roijers et al. 2008) Aortic valve (Kim and Trump 1975) Bone (Boonrungsiman et al. 2012)
Calcium carbonate	$CaCO_3$	Atherosclerosis, apoptotic bodies (in vitro) (Proudfoot et al. 2000)
Calcium pyrophosphate dehydrate (CPPD)	$Ca_2P_2O_7\ 2H_2O$	Pseudogout (Cheung et al. 1997)
Tricalcium phosphate	$(Ca)_3\ (PO_4)_2$	Joints (Cheung et al. 1997; Whelan et al. 2005; Barrere et al. 2006)
Mg-substituted tricalcium phosphate	$(Mg, Ca)_3\ (PO_4)_2$	Arthritic cartilage, dental caliculi, salivary stones, soft tissue deposits (LeGeros 2001) Aorta, breast cancer (Scimeca et al. 2014)
Dicalcium phosphate dihydrate (DCPD) (brushite)	$CaHPO_4\ 2H_2O$	Dental caliculi, chondrocalcinosis (LeGeros 2001; Barrere et al. 2006)
Calcium oxalate	CaC_2O_4	Kidney stones, breast cancer (Scimeca et al. 2014)

Mg and Na can substitute Ca. Cl_2 and F can substitute OH.

trix vesicles and apoptotic bodies, with crystals appearing as aggregates of nanoparticles or as needle-like sheets located near fatty deposits, inflammatory cells, and vascular smooth muscle cells (VSMCs) (Kim 1976; Shanahan et al. 1999). Calcification has been detected either within VSMCs (in mitochondria), or in matrix vesicles closely aligned with elastin fibrils that surround VSMCs (Kim 1976).

In healthy vessels, VSMCs reside in the medial layer of blood vessels and have a contractile phenotype. VSMCs have a large intracellular Ca^{2+} store (the sarcoplasmic reticulum [SR]) that is used to trigger contraction. However, VSMCs are not terminally differentiated and, in diseased blood vessels, they lose their contractile properties and adopt a "repair" or "synthetic" phenotype, accompanied by conversion to a cell type that expresses various bone-regulatory proteins (Tyson et al. 2003). Whether VSMCs require intracellular Ca^{2+} signals to trigger this phenotypic conversion is not yet known, but it coincides with a loss of expression of voltage-activated L-type Ca^{2+} channels and an increased expression of T-type Ca^{2+} channels. Ca^{2+} influx via voltage-gated Ca^{2+} channels stimulates expression of VSMC-specific contractile proteins (smooth muscle myosin heavy chain and α-smooth muscle actin) via RhoA/Rho kinase and myocardin (Wamhoff et al. 2004). Expression of ryanodine receptors and SR ATPase isoform 2a are reduced in synthetic VSMCs, compared with contractile cells (House et al. 2008). Additionally, purinergic receptor expression is reduced on VSMC phenotypic change, leading to a lack of response to purinergic agonists, which would normally stimulate Ca^{2+} signaling in contractile VSMCs (Erlinge 1998). Thus, conversion of VSMCs to a disease phenotype leads to changes in several Ca^{2+} homeostatic/signaling mechanisms, which may influence their potential in regulating calcification.

VSMC Ca^{2+} Entry and Efflux in Relation to Calcification

Does VSMC Ca^{2+} signaling, or changes in Ca^{2+} homeostasis, influence the calcification occurring in diseased blood vessels? Clinical trials and experimental models have revealed that the calcium channel antagonists verapamil and nifedipine can reduce vascular calcification (Fleckenstein-Grün et al. 1994; Motro and Shemesh 2001; Motro et al. 2001; Chen et al. 2010). This implies that calcification may be directly inhibited by blocking Ca^{2+} entry into VSMCs. However, Ca^{2+} entry leads to a pleiotropic cellular response with many different downstream effects that may influence calcification (Orth et al. 1996). For example, effects on intracellular Ca^{2+} mobilization (Marche and Stepien 2000), cell differentiation, down-regulation of alkaline phosphatase (an enzyme that generates phosphate to encourage calcification), and direct effects on matrix vesicle membrane phospholipids (Chen et al. 2010). Therefore, blocking Ca^{2+} entry into VSMCs via voltage-gated channels may be beneficial in preventing calcification but further studies are required to establish the exact mechanisms responsible for reducing calcification.

Another group of proteins to consider are those involved in Ca^{2+} efflux from VSMCs; NCX1 (Na^+/Ca^{2+} exchanger), PMCA1 (plasma membrane Ca^{2+}ATPase), and NCKX4 (K^+-dependent Na^+/Ca^{2+} exchanger), which have all be implicated in VSMC calcification (Gui et al. 2012). The evidence for their involvement is that messenger RNA (mRNA) and protein levels for these Ca^{2+} efflux channels were reduced in the aortic media of a murine model of calcification (Klotho null mice) and an in vitro VSMC calcification model. MicroRNAs (miRNAs) that target expression of these proteins were induced in these calcification models: miR-135a*, miR-762, miR714, and miR-712*, and inhibitors directed against these miRNAs reduced calcification. Thus, perturbation of Ca^{2+} efflux mechanisms altered cytosolic Ca^{2+} levels within VSMCs and this appeared to influence the development of calcification.

Loss of Calcification Inhibitors and Presence of Calcification Triggers

Studies in mouse knockout models have shown that vascular calcification of the medial layer of blood vessels is under the control of several inhibitors. These include matrix Gla protein

(MGP) (Luo et al. 1997); osteoprotegerin, a member of the tumor necrosis factor (TNF) superfamily that regulates osteoclast differentiation (Bucay et al. 1998); FGF23, a circulating factor released from bone in response to high PO_4^{3-} levels to provide feedback control of PO_4^{3-} concentration (Shimada et al. 2004); Klotho, a protein associated with aging that acts as a cofactor for FGF23 signaling and has a clear link with expression of Ca^{2+} efflux proteins, as detailed above (Kuro-o et al. 1997; Gui et al. 2012); and fetuin-A, a circulating protein that binds hydroxyapatite (Jahnen-Dechent et al. 1997).

In the MGP knockout mouse, large elastic arteries display extensive calcification, leading to premature death from arterial rupture. MGP contains five γ-carboxyglutamic acid (hence the name "Gla protein") residues, formed by a vitamin K-dependent modification of specific glutamic acid residues. When the Gla residue formation is inhibited with the vitamin K antagonist warfarin, vascular calcification is induced both in vivo and in vitro (Price et al. 1998; Schurgers et al. 2008). The Gla residues allow Ca^{2+} binding and hydroxyapatite binding, which may explain their inhibitory effects on calcification. The arteries of MGP knockout mice contained chondrocytes rather than VSMCs, which suggests that MGP also maintains the VSMC phenotype. This effect may be via its binding to and inactivation of bone morphogenetic protein 2 (BMP-2), a potent inducer of calcification and bone formation (Zebboudj et al. 2003). In the rare cartilage calcification disorder, Keutel syndrome, in which DNA mutations cause truncated forms of MGP, pulmonary artery calcification occurred prematurely in affected individuals (Munroe et al. 1999; Meier et al. 2001). In addition, rare monogenic disorders caused by mutations in *ENPP1* in generalized arterial calcification of infancy, *ABCC6* in pseudoxanthoma elasticum, and *NT5E* in arterial calcification and distal joint calcification clearly indicate a genetic component to the regulation of inorganic PO_4^{3-} metabolism and adenosine signaling in arterial calcification (Rutsch et al. 2011).

In the intimal layer of blood vessels, loss of calcification inhibitor activity may contribute to the development of calcified deposits in atherosclerosis (Morony et al 2008; Nitschke et al 2011; Schurgers et al 2012). However, it is clear that there are many other triggers of calcification such as oxidized lipids, reactive oxygen species, inflammatory cytokines, uremic toxins, and high levels of extracellular Ca^{2+}. The resulting calcification is thought to be caused by an imbalance of inhibitors and triggers of calcification (summarized in Fig. 1).

The Ca^{2+}-Sensing Receptor (CaSR) and Calcification

The CaSR is a G-protein-coupled receptor that is not only involved in systemic Ca^{2+} homeostasis, but is also expressed by VSMCs where it has a role in regulating myogenic tone and proliferation (Ohanian et al. 2005; Li et al. 2011). In an in vitro model using VSMCs, blocking CaSR function increased vascular calcification, and calcimimetic drugs (CaSR agonists other than Ca^{2+}) that increase the sensitivity of the CaSR to Ca^{2+} reduced calcification (Alam et al. 2009). The potent calcification-inhibitor, MGP, is thought to be expressed at higher levels in tissues where extracellular Ca^{2+} is high and appears to be regulated by the CaSR. Raised extracellular Ca^{2+} has been reported to increase MGP transcription in vitro and in vivo, possibly a critical response to prevent calcification (Farzaneh-Far et al. 2000; Mendoza et al. 2011). MGP is thought to undergo a conformational change on Ca^{2+} binding, increasing its affinity for hydroxyapatite. Interestingly, PO_4^{3-} and Mg^{2+} reduced MGPs affinity for hydroxyapatite. One study suggests that although Mg^{2+} is an inhibitor of calcification, Mg^{2+} inhibited a Ca^{2+}-induced increase in MGP expression in a chondrocyte cell line (Nakatani et al. 2006). The role of Mg^{2+} in calcification is still not fully understood but there is some evidence that it has a stimulatory effect on MGP expression, involving the TRPM7 channel (Montezano et al. 2010). Thus, the expression and activity of MGP may be influenced by the CaSR, TRPM7 channels, and the local ionic environment. Some of the observations regarding Mg^{2+} and MGP expression are conflicting and

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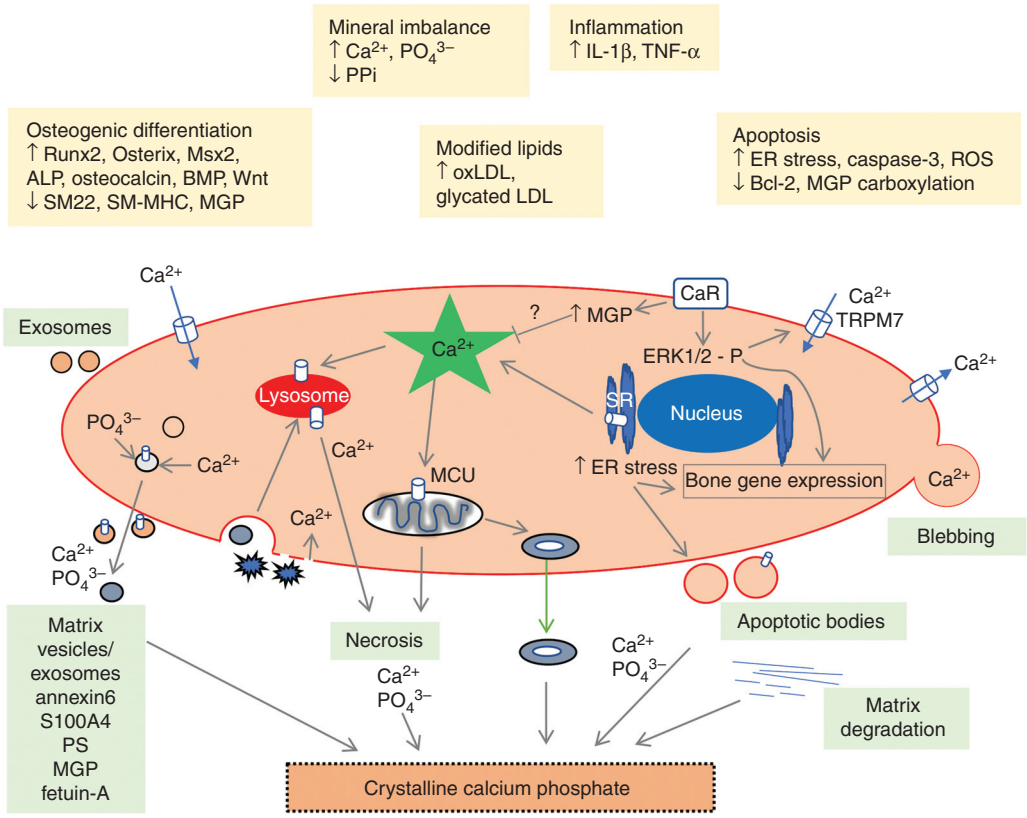

Figure 1. Summary of proposed mechanisms inducing vascular smooth muscle cell (VSMC) calcification. This figure depicts various triggers that stimulate changes in VSMC signaling, resulting in the generation of calcium phosphate crystals. VSMCs release matrix vesicles/exosomes and depending on their protein content and extracellular environment, they will nucleate calcium phosphate crystals. VSMCs undergoing apoptosis produce apoptotic bodies that can calcify in an environment that encourages calcification. Calcified mitochondria may also contribute to the generation of vesicle-derived extracellular calcium phosphate crystals. Engulfment of nanocrystals encourages cell death and inflammation, which facilitates further crystal growth. The presence of Ca^{2+}-binding proteins in the extracellular matrix, together with matrix degradation encourages calcification of the extracellular matrix. IL, Interleukin; TNF, tumor necrosis factor; BMP, bone morphogenetic protein; MGP, matrix Gla protein; oxLDL, oxidized low-density lipoprotein; ER, endoplasmic reticulum.

this may be the result of the different models used in each study.

Role for Apoptosis and Necrosis

A link between cell death and vascular calcification has been observed in various settings: pathological studies (Kim 1995; Kockx et al. 1998); in arteries from dialysis patients (Shroff et al. 2008); in a mouse model (Clarke et al. 2010); and in human VSMCs in culture, in which apoptotic bodies were found to initiate calcification (Proudfoot et al. 2000). Apoptotic bodies are normally efficiently removed by phagocytosis. However, in diseased blood vessels, the presence of oxidative stress, modified lipids, and the anti-phagocytic molecule CD47 are thought to reduce apoptotic body clearance and can promote calcification (Anderson et al. 2002; Proudfoot et al. 2002; Kojima et al. 2016). A lack of clearance of apoptotic cells would also lead to secondary necrosis, in which Ca^{2+} and PO_4^{3-} released from necrotic cells creates an environment favoring calcium phosphate crystal formation.

It is well established that perturbations in intracellular Ca^{2+} store levels or Ca^{2+} overload can trigger apoptosis or necrosis (Orrenius et al. 2003). Therefore, it is likely that the large variety of factors that can cause changes in VSMC Ca^{2+} homeostasis in diseased blood vessels could induce calcification via apoptosis, necrosis, and prevention of phagocytosis. It is worth noting here that engulfment of apoptotic bodies by phagocytic cells requires oscillatory Ca^{2+} signals (Melendez and Tay 2008), and these signals have been observed in human VSMCs engulfing apoptotic cells (Proudfoot and Dautova 2013). This raises the notion that in addition to professional phagocytic clearance of apoptotic cells, VSMC phagocytic capabilities may also be important in maintaining healthy, debris-free, and calcification-free blood vessels.

One of the triggers for activating apoptosis and necrosis in VSMCs is calcium phosphate crystals themselves. Studies using human VSMCs have found that crystals extracted from human calcified arteries and chemically synthesized calcium phosphate nanoparticles induced VSMC death in vitro, involving both apoptosis and necrosis (Ewence et al. 2008). Interestingly, these calcified particles stimulated a series of transient, reversible elevations in cytosolic Ca^{2+} in fura-2-loaded VSMCs, with each individual cell having a different pattern of Ca^{2+} elevations. Electron microscopy analysis revealed evidence of uptake of crystals into cells via endocytosis and also areas of plasma membrane damage soon after exposure to calcium phosphate crystals. Some cells displayed recovery to baseline cytosolic Ca^{2+} levels, suggesting that homeostatic mechanisms were activated. However, in cells that died, a large unrecoverable Ca^{2+} elevation occurred, accompanied by cell blebbing and loss of plasma membrane integrity (necrosis) (Ewence et al. 2008; Dautova et al. 2014). Evidence for repair of plasma membrane damage is that before death, blebs containing the cell-impermeant dye propidium iodide were extruded from viable cells. Ca^{2+} entry into cells via plasma membrane damage has been shown to activate repair via annexins and subsets of lysosomes (Reddy et al. 2001; Draeger et al. 2011). Membrane damage is therefore one way that Ca^{2+} can enter VSMCs leading to cytosolic Ca^{2+} elevations. Some of the Ca^{2+} increases that occurred following addition of crystals to VSMCs may have been derived from intracellular Ca^{2+} sources such as lysosomes, because bafilomycin A reduced the size of Ca^{2+} elevations and inhibited cell death (Ewence et al. 2008). Other Ca^{2+}-storing organelles may also be involved, particularly in the generation of Ca^{2+} oscillations, although this possibility is yet to be fully investigated.

Fetuin-A, a calcification-inhibitor known to bind hydroxyapatite reduced Ca^{2+} elevations in calcium phosphate crystal-treated VSMC (Dautova et al. 2014). Fetuin-A also delayed plasma membrane damage and delayed cell death, indicating that the damaging effects of calcium phosphate crystals can potentially be reduced or blocked via a variety of mechanisms. It is also interesting to note that fetuin-A levels are lower in patients with chronic kidney disease (CKD), and these patients can have particularly severe vascular calcification. Furthermore, patients with CKD have a high vascular calcium load, deposited as nanocrystals in the extracellular matrix (Shroff et al. 2008). In a study using ex vivo human blood vessels treated with high Ca^{2+} and PO_4^{3-}, similar to levels in CKD patients, VSMCs underwent apoptosis coinciding with formation of the first calcium phosphate crystals (Shroff et al. 2010). However, more recent studies using human VSMCs suggest that if the cells survive initial calcium phosphate crystal exposure, their longer-term effects can result in stimulation of proinflammatory pathways and chemokine release (Dautova et al. 2018). Thus, calcium phosphate crystals can induce cell death and promote inflammation on exposure to human VSMCs, and cytosolic Ca^{2+} elevations occur before these events. Further studies are required to determine the exact nature of Ca^{2+} elevations induced by calcium phosphate crystals and how they are involved in triggering damaging effects in vascular cells.

ER Stress and Calcification

Disturbances in cytosolic Ca^{2+} homeostasis can induce endoplasmic reticulum (ER) stress

(Krebs et al. 2015) and there are several reports that link ER stress with the development of vascular and aortic valve calcification (Cai et al. 2013; Shanahan and Furmanik 2017; Panda et al. 2018). In valvular interstitial cells (VICs) loaded with fluo-3-AM, oxidized low-density lipoprotein (oxLDL) was reported to increase intracellular Ca^{2+} (Cai et al. 2013). Both oxLDL and the Ca^{2+} ionophore, A23187 induced ER stress, whereas BAPTA-AM protected VICs from ER stress induced by A23187 or oxLDL, suggesting that ER stress was dependent on raised cytosolic Ca^{2+} levels. ER stress is normally relieved by the unfolded protein response (UPR), which restores ER homeostasis by reducing the aggregation and build-up of unfolded proteins. Proper functioning of the UPR is required in physiological processes, such as cartilage and bone development (Murakami et al. 2009; Saito et al. 2009), and failure of the UPR can lead to proapoptotic signaling (Szegezdi et al. 2006; Duan et al. 2009). Apoptosis may therefore be the link between raised cytosolic Ca^{2+}, ER stress, and calcification.

Mitochondrial Ca^{2+}, Mitophagy, and Calcification

Maintaining mitochondrial function to provide cellular energy and regulate cytosolic Ca^{2+} is essential for cellular physiology. Mitochondrial Ca^{2+} accumulation (Ca^{2+} overload) can impair mitochondrial function and has been described in hypertension, hypercalcemia, and CKD (Fleckenstein-Grun et al. 1992; Shroff et al. 2008). Noncrystalline forms of calcium phosphate have been found within mitochondria of bone cells as a physiological precursor to crystal formation, and also in pathological calcification, where calcified mitochondria have been detected within VSMCs. In situations in which cells are exposed to stimuli that cause elevations in cytosolic Ca^{2+}, such as with calcium phosphate crystals, homeostatic mechanisms rapidly return Ca^{2+} to resting levels and this is achieved partly by uptake into mitochondria. A sustained increase in intracellular Ca^{2+} is known to collapse the proton-motive potential gradient across the inner mitochondrial membrane (IMM) (Malis and Bonventre 1986), which may account for the cell death observed in VSMCs in response to calcium phosphate crystals.

Mitochondrial Ca^{2+} uptake occurs first by Ca^{2+} passing through the outer mitochondrial membrane (OMM) via a voltage-dependent anion-selective channel (VDAC). This channel behaves as a diffusion pore that is permeable to ions and small hydrophilic metabolites (Shoshan-Barmatz et al. 2018). Ca^{2+} ions pass into the mitochondrial matrix through a channel known as the mitochondrial Ca^{2+} uniporter (MCU). The MCU is located within the IMM, and is highly selective for Ca^{2+} (Baughman et al. 2011; De Stefani et al. 2011). MCU oligomers form the selective pore, but they are part of a multiprotein complex composed of a large number of regulatory proteins, such as MICU1 (Mallilankaraman et al. 2012).

In an interesting study of blood vessels from healthy individuals, calcified mitochondria were observed after long-term treatment with raised extracellular levels of Ca^{2+} and PO_4^{3-}, whereas in blood vessels from patients with CKD, extensive calcification was observed extracellularly but not in mitochondria (Shroff et al. 2010). An explanation for this discrepancy is that VSMCs in CKD are phenotypically modified and release huge numbers of Ca^{2+}-loaded vesicles, thus preventing Ca^{2+} overload and avoiding mitochondrial damage. If this is indeed the case, the phenotype of a cell may determine whether exposure to potentially toxic cytosolic Ca^{2+} levels results in mitochondrial Ca^{2+} uptake or rapid removal of Ca^{2+} via vesicle release. It is also possible that crystal formation within mitochondria may be a cell survival mechanism, otherwise large increases in mitochondrial Ca^{2+} may result in cell death. Very little is known about the mechanisms generating either amorphous or crystalline forms of calcium phosphate within mitochondria and whether this process is indeed preceded by large accumulations of mitochondrial Ca^{2+}. It is tempting to speculate that cells with dysfunctional mitochondria, such as in aging and disease (e.g., atherosclerosis) are more sensitive to intramitochondrial Ca^{2+} accumulation, resulting in progression of amorphous to crystalline calcium phosphate.

Damaged and dysfunctional mitochondria are normally removed and recycled within cells via mitophagy—a process regulated by cytosolic and mitochondrial Ca^{2+} levels (East and Campanella 2013; Rimessi et al. 2013). It has recently been suggested that mitophagy is part of the physiological process of normal bone formation being involved in the transport of intramitochondrial amorphous calcium phosphate for delivery to the extracellular matrix (Pei et al. 2018). Thus, mitophagy appears to be crucial in the transportation of calcium phosphate crystal precursors for extracellular release in healthy physiological bone formation. However, in aging or pathological conditions, mitophagy can be disturbed leading to an altered clearance of damaged mitochondria (Safiulina et al. 2019), and studies suggest that disturbed mitophagy accelerates VSMC apoptosis and calcification (Swiader et al. 2016; Zhu et al. 2019).

Role for Ca^{2+} in Matrix Vesicle/Exosome Release and Calcification

As mentioned earlier, matrix vesicles/exosomes derived both from VSMCs and macrophages are one of the main mechanisms for initiation of physiological and pathological calcification (Anderson 1995; Reynolds et al. 2004; New et al. 2013). Limiting matrix vesicle/exosome levels, either via inhibition of autophagy or inhibiting exosome release has been shown to reduce calcification (Dai et al. 2013; Kapustin et al. 2015). Inhibition of exosome release has also been shown to reduce inflammatory mediator release from VSMCs (Dautova et al. 2018). As introduced earlier, it was suggested that high cytosolic Ca^{2+} levels can cause increased loading of Ca^{2+} in extracellular vesicles or blebs, as a cellular defense mechanism (Lemasters et al. 1987; Kim et al. 1999). Other more recent studies in hemopoietic cell lines and cancer cells support this concept, in which intracellular Ca^{2+} elevations stimulated exosome release (Savina et al. 2003; Messenger et al. 2018). Therefore, rapid Ca^{2+} accumulation in matrix vesicle/exosomes and their release from the cell may be a protective mechanism that reduces intracellular Ca^{2+} overload. We can therefore speculate that exosome/matrix vesicle release may be an additional way that cells can avoid calcium overload by excreting Ca^{2+}-loaded exosomes to the extracellular environment. Depending on the combination of proteins contained within the secreted vesicles, and on the extracellular environment, this will determine whether Ca^{2+}-loaded exosomes will nucleate and support calcium phosphate crystal proliferation.

In some studies, extracellular exosomes themselves have been shown to promote Ca^{2+} signaling. Exosomes/matrix vesicles isolated from VSMCs treated with high levels of extracellular PO_4^{3-} induced an increase in intracellular Ca^{2+}, partially via Ca^{2+} release from intracellular stores (Chen et al. 2018). Furthermore, in a separate study, it was reported that exosomes specifically bound to autotaxin, a lysophospholipase, and through a series of signaling steps induced the release of Ca^{2+} from intracellular stores (Jethwa et al. 2016). These studies suggest that exosomes with a particular proteome signature can stimulate changes in intracellular Ca^{2+} signaling.

Ca^{2+} in Aging, Senescence, and Calcification

Calcification and aging are well recognized as risk factors for cardiovascular disease and mortality. Aging is thought to involve several different degenerative processes, including changes in Ca^{2+} homeostasis, oxidative stress, DNA damage, mitochondrial dysfunction, alterations in autophagy and mitophagy, and induction of an inflammatory, secretory-associated phenotype (SASP). Recent insights into aging disorders have aided our understanding of the mechanisms involved in aging. For example, specific mutations in the *LNMA* gene encoding the nuclear envelope protein lamin A cause premature aging in Hutchinson–Gifford progeria syndrome (HGPS), in which VSMC degeneration and calcification occur in premature atherosclerosis, leading to vascular stiffness, and heart attack or stroke usually before 20 years of age (Gonzalo et al. 2017). Prelamin A appears to accumulate in diseased and aged VSMCs and it has been suggested that lamin A can serve as a biomarker for vascular aging and disease (Rag-

nauth et al. 2010; Liu et al. 2013; Warren et al. 2015). As mentioned earlier, Klotho null mice develop premature aging and vascular calcification, and it has recently been recognized that vitamin D is linked with all of the above potential mechanisms associated with aging (Berridge 2017). For example, vitamin D can improve the nuclear morphology, DNA repair, and senescence phenotype in HGPS patient-derived cells (Kreienkamp et al. 2016) and it also controls the expression of Klotho and Nrf2 (Forster et al. 2011). Indeed, it has been suggested that vitamin D works together with Klotho and Nrf2 to maintain intracellular Ca^{2+} and redox signaling (Berridge 2017). In the vascular calcification literature, there has been some debate about whether vitamin D is beneficial, because of the observations in some studies that vitamin D–induced calcification (Fleckenstein-Grun et al. 1992), whereas in others it reduced calcification, or altered expression of calcification-regulatory proteins (Farzaneh-Far et al. 2001). The differences may simply be the result of dose-related or context-dependent effects (Shanahan et al. 2011).

Aging is often associated with diminished autophagy, and autophagy is thought to protect against vascular calcification. The role of Ca^{2+} signaling in autophagy is complex, as several studies have shown that Ca^{2+} signals can trigger autophagy; however, Ca^{2+} signals can also have anti-autophagic effects (Bootman et al. 2018). It is therefore interesting to note that calcium phosphate precipitates have been reported to induce autophagy in several cell types in vitro, and this is thought to be caused by exogenously introduced Ca^{2+} (Gao et al. 2008, 2010; Sarkar et al. 2009; Chen et al. 2012, 2014). Whether autophagy is stimulated by exposure to calcium phosphate crystals in a physiological or pathological setting in vivo is yet to be established. Studies using calcium phosphate nanoparticles as a vehicle to deliver biomolecules into cells in vivo would suggest that the calcium phosphate component of the complexed particles is relatively inert (Frede et al. 2017). This concept is supported by studies of endogenous calcium phosphate nanoparticles occurring in the gastrointestinal tract, where these particles are thought to be rapidly cleared by cells in a "silent" manner (Pele et al. 2017). However, as described earlier, studies in vascular cells and in blood vessels suggest that calcium phosphate crystals are damaging to surrounding cells (Vengrenyuk et al. 2006; Ewence et al. 2008; Dautova et al. 2014; Hutcheson et al. 2014). The impact of nano- and microparticulate crystals on cells is therefore likely to depend on the type of biomolecules bound to the crystals, the target cell phenotype, and the microenvironment. If calcium phosphate crystals do indeed stimulate autophagy in vivo, this would be expected to have a favorable effect in preventing soft tissue calcification. Evidence suggests that autophagy is a protective homeostatic mechanism in normal cartilage and bone, and its reduced function in aging is associated with cell death and osteoarthritis (Settembre et al. 2008; Caramés et al. 2010; Hocking et al. 2012). In vascular cells exposed to high extracellular PO_4^{3-} levels, autophagy was reported to be protective against calcification, and this effect was thought to be partly mediated via reduced matrix vesicle release (Dai et al. 2013). The link between autophagy and exosome biogenesis has been described in various cell types (Fader and Colombo 2006; Xu et al. 2018).

Matrix vesicle/exosome generation depends on both extracellular and intracellular Ca^{2+} levels (Kapustin et al. 2011, 2015). A recent report suggests that vesicles released by senescent endothelial cells or vesicles isolated from the plasma of elderly individuals contained higher amounts of Ca^{2+} and were able to induce VSMC calcification in vitro (Alique et al. 2017). These studies suggest that aging and triggers of cellular senescence alter cellular Ca^{2+} homeostasis such that matrix vesicles/exosomes are particularly prone to calcify and promote further calcification.

Calcification in Other Tissues

Heart valve calcification is associated with an increased mortality risk and shares similar mechanisms with arterial calcification. Its development involves osteo/chondrocytic conversion of valve interstitial cells, inflammation, cell death, and mineralized matrix vesicles (Lerman

et al. 2015). Calcification of the heart muscle (myocardium) is often associated with sites of damaged, infarcted tissue, and was thought to be driven by dystrophic mechanisms alone. However, recently a role for cardiac fibroblasts has been implicated in driving calcification via adoption of an osteoblast-like phenotype (Pillai et al. 2017).

In osteoarthritic joints, two different types of calcium phosphate crystals have been described: (1) basic calcium phosphate (BCP), which is a mixture of hydroxyapatite and octacalcium phosphate (OCP), and (2) calcium pyrophosphate dihydrate (CPPD). The formation of CPPD and BCP crystals in articular cartilage is not completely understood, but is thought to be caused by aging, injury, or genetic predisposition. Chondrocyte matrix vesicles generated either CPPD or BCP in vitro, suggesting that matrix vesicles may initiate crystal formation in joints (Derfus 1992). Other potential triggers of CPPD formation are excess quantities of extracellular ATP that could arise via cell injury or cell death (Ryan et al. 1992), or the ATP metabolite pyrophosphate (PPi, the anionic component of CPPD crystals [Ryan et al. 1981]). PPi is generated from exogenous ATP by a variety of ectoenzymes, and complexes with local Ca^{2+} to form CPPD crystals (Costello et al. 2011). When phosphate, rather than PPi, predominates, BCP mineral is generated. Abnormalities of the extracellular matrix, characterized by increased levels of osteopontin and high activities of the protein cross-linking *trans*-glutaminase enzymes also contribute to CPPD crystal formation. Thus, in joints, it appears that calcium deposits can arise via phenotypically altered cells producing calcifying matrix vesicles and also by altered levels of local ions and proteins.

Calcium oxalate is the main type of crystal found in kidney stones and is thought to form as a result of supersaturation of soluble salts. In the kidney, cell injury is thought to cause mitochondrial Ca^{2+} overload and calcification (Ganote et al. 1975), whereas extracellular renal calcification occurs in cytoplasmic buds from renal epithelial cells known as ovoid bodies. Calcium phosphate crystal deposits are also common in tumors, and interestingly bone gene expression by cancer cells has been reported (Castronovo and Bellahcene 1998; Scimeca et al. 2014). In the brain, some studies suggest mutations in *PDGFB*, *SLC20A2* (encoding the Na^+-dependent phosphate transporter, Pit2), and *CaSR* genes are responsible for initiation and progression of calcification (Wang et al. 2012; DeMeo et al. 2018). Finally, fibrodysplasia ossificans progressiva (FOP) is a rare and debilitating condition in which calcification occurs in skeletal muscle, tendons, and ligaments exposed to injury, and is caused by mutations in the BMP type 1 receptor family member ACVR1, which leads to activation and enhancement of BMP receptor signaling (Kaplan et al. 2009).

CONCLUDING REMARKS

The events leading to the initial nidus for calcium crystal formation at the various sites of ectopic calcification are not completely understood and are likely to involve more than one mechanism, such as alteration of cell phenotype and production of calcification-stimulating factors, cell injury, and death via apoptosis or necrosis, initiation within cells (particularly mitochondria), and initiation in matrix vesicles and apoptotic bodies, where Ca^{2+}, PO_4^{3-}, and other ion concentrations are raised above physiological levels and where calcification-inhibitors are inactive, absent, or limiting. Some of these mechanisms of calcium phosphate crystal formation in disease are shared with physiological bone formation. Changes in cytosolic Ca^{2+} levels within cells appear to be key in orchestrating signaling pathways that lead to physiological and pathological calcification (overview in Fig. 2). However, we still know very little of the exact nature, timing, and factors regulating Ca^{2+} signals during the calcification process. From currently available evidence, it appears that homeostatic mechanisms controlling normal intracellular Ca^{2+} signals within cells are required to maintain healthy, calcification-free tissues and that specific intracellular Ca^{2+} signals are a prerequisite for calcium deposition in association with disease. Although calcification was regarded as an inevitable, degenerative part of aging, it is promising that we are beginning to

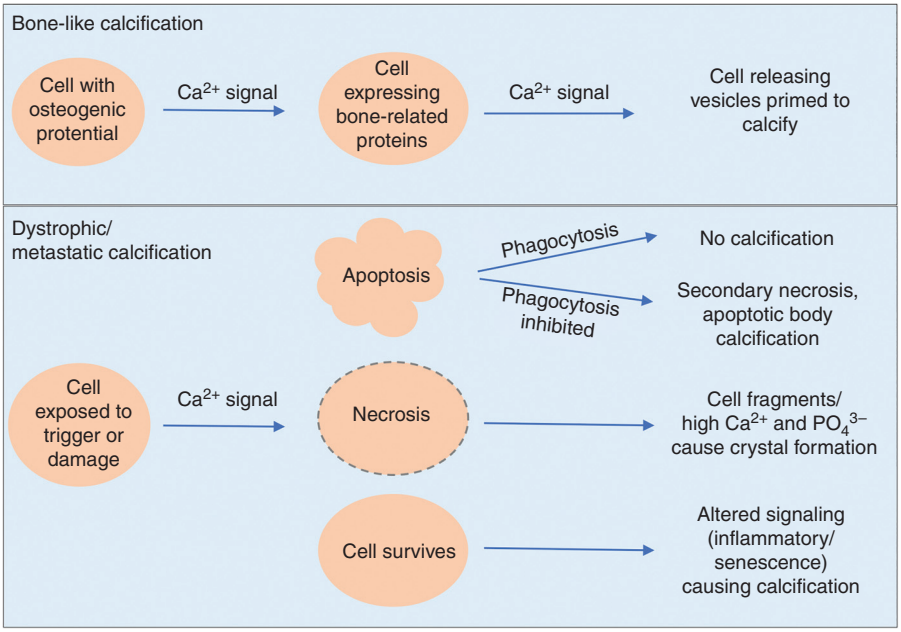

Figure 2. Schematic displaying the roles of cytosolic Ca^{2+} in calcification. In bone, Ca^{2+} signals are required for differentiation of precursor cells into specialized bone-regulatory cells. Further signals are required for release of calcification-competent matrix vesicles/exosomes. In dystrophic or metastatic calcification, cytosolic Ca^{2+} elevations occur before apoptosis, necrosis, and altered inflammatory/senescence signaling. Depending on the local environment, if phagocytosis is allowed to progress efficiently, apoptosis has no impact on calcification. Whereas impairment of phagocytosis allows apoptotic bodies to calcify and secondary necrosis to occur, generating increased local Ca^{2+} and PO_4^{3-} and nidi for calcification.

understand the various ways that the initiation and progression of calcification are regulated. Hopefully, a fuller picture of how intracellular Ca^{2+} changes control tissue calcification will emerge in future studies.

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