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Review Article

The role of calmodulin and calmodulin-dependent protein kinases in the pathogenesis of atherosclerosis

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Abstract

Atherosclerosis is a chronic inflammatory disease that triggers severe thrombotic cardiovascular events, such as stroke and myocardial infarction. In atherosclerotic processes, both macrophages and vascular smooth muscle cells (VSMCs) are essential cell components in atheromata formation through proinflammatory cytokine secretion, defective efferocytosis, cell migration, and proliferation, primarily controlled by Ca^{2+}-dependent signaling. Calmodulin (CaM), as a versatile Ca^{2+} sensor in diverse cell types, regulates a broad spectrum of Ca^{2+}-dependent cell functions through the actions of downstream protein kinases. Thus, this review focuses on discussing how CaM and CaM-dependent kinases (CaMKs) regulate the functions of macrophages and VSMCs in atherosclerotic plaque development based on literature from open databases. A central theme in this review is a summary of the mechanisms and consequences underlying CaMK-mediated macrophage inflammation and apoptosis, which are the key processes in necrotic core formation in atherosclerosis. Another central theme is addressing the role of CaM and CaMK-dependent pathways in phenotypic modulation, migration, and proliferation of VSMCs in atherosclerotic progression. A complete understanding of CaM and CaMK-controlled individual processes involving macrophages and VSMCs in atherogenesis might provide helpful information for developing potential therapeutic targets and strategies.

Keywords: *Atherosclerosis, Calmodulin, Calmodulin-dependent kinases, Macrophages, Vascular smooth muscle cells*

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Introduction

Atherosclerosis, a chronic inflammatory disease, occurs in the vascular walls of large and medium arteries. Atherosclerotic lesion formation in the tunica intima of the vascular wall evolves from light phase I to severe phase VI [1]. Vulnerable lesions are prone to rupture and thrombus formation, which is a major cause of thrombotic cardiovascular diseases, such as stroke, renovascular hypertension, and myocardial infarction [2,3]. The risk factors for atherosclerosis include elevated low-density lipoprotein (LDL) levels, high blood pressure, smoking, obesity, and diabetes mellitus [4]. Since the serum LDL level is the root of atherosclerotic pathogenesis, current therapy focuses on preventing lipid accumulation beneath the vascular endothelium by using statins [5]. However, these atherothrombotic vascular events continue to cause high mortality rates in industrialized society [2,4,6]. Because the progression of an atherosclerotic lesion to a rupture-prone plaque involves complex processes, research into the associated inflammatory mechanisms, characterization of the cell types involved, and cell–cell as well as cell–microenvironment interactions has increased rapidly to find an alternative treatment. In atherosclerotic plaques, macrophages and vascular smooth muscle cells (VSMCs) are two significant components, for which their role is gradually transformed with atherosclerotic lesion evolution. In both cell types, Ca^{2+}-dependent signaling is crucial in controlling the inflammation, cell death, proliferation, and migration occurring in atherosclerosis [7,8]. In this regard, calmodulin (CaM), the most critical calcium sensor in cells, and its target kinases might play a crucial role in bridging Ca^{2+} signaling and cellular reactions in response to environmental cues. Therefore, in this review, we focus on how CaM and CaM-dependent kinases (CaMKs) alter the functions of macrophages and VSMCs, and how these alterations contribute to the progression of atherosclerosis.

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The pathogenesis of atherosclerosis

Atherogenesis is initiated by the subendothelial accumulation of oxidized lipoproteins, triggering the subsequent diapedesis of monocytes and macrophage transformation [Figure 1a] [9]. These phagocytes begin to clear the oxidized lipoproteins and store them as cytoplasmic droplets resulting in a foamy appearance of macrophages. During this process, inflammatory markers are clearly identified in macrophages within human and animal plaques, and inflammatory cytokines secreted by activated macrophages induce the phenotypic switching of VSMCs [2,9]. Proliferation and migration of the phenotype-switched VSMCs from the media to the intima layer of the vascular wall also contribute to increased cell mass in atherosclerotic plaques [Figure 1a]. As the lesion progresses, markers of endoplasmic reticulum (ER) stress begin to appear in macrophages and VSMCs, initiating apoptotic processes [10,11]. When apoptotic cells cannot be effectively and timely removed by the stressed phagocytes, a process termed defective efferocytosis, a necrotic core secondary to cell apoptosis, occurs. Rupture of vulnerable plaques usually occurs in sites close to necrotic cores [9]. In atherogenesis, macrophages and SMC activation is accompanied by the altered activity or expression of membrane and internal Ca^{2+} channels implicating the importance of Ca^{2+} signaling in these processes [7,12].

A brief overview of calmodulin and calmodulin-dependent kinases

Ca^{2+} ions are crucial second messengers that transmit external signals into cell responses to cope with environmental changes. There are many molecules involved in deciphering Ca^{2+} signals in cells, and CaM is the most important, owing to its ubiquitous expression in all eukaryotic cells. CaM is a 16.7 kDa protein that is extremely conserved across species [13]. With four Ca^{2+}-binding sites, CaM exerts flexibility to respond to a wide range of internal Ca^{2+} oscillations, establishing a dynamic system to trigger subsequent signaling pathways [14]. Due to the lack of kinase activity, the diverse actions of CaM are mediated through activation of a variety of targeted protein kinases. Based on substrate specificity, CaMKs can be classified into two groups, restricted and multifunctional kinases [15]. Restricted CaMKs have a limited number of substrates and are more specific to certain stimuli and pathways. Death-associated protein kinase (DAPK), phosphorylase kinase, elongation factor 2 kinase (eEF2K/CaMKIII), and myosin light-chain kinase (MLCK) belong to this category. In contrast, multifunctional kinases, mainly CaMKI, CaMII, CaMKIV, and CaMK kinase (CaMKK), are powerful controllers of other kinases [16]. Given the ubiquitous expression in distinct tissues, the multifunctional kinases regulate a broad range of cell functions involved in pathophysiological changes in macrophage- and VSMC-mediated atherosclerotic progression, including protein synthesis, apoptosis, cytoskeletal reorganization, and proliferation. Thus, we summarize

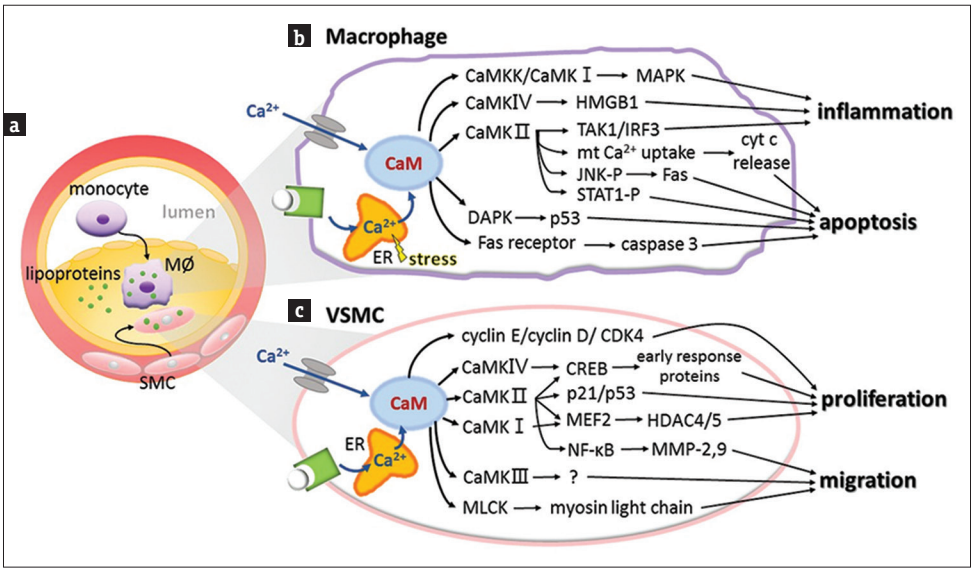

Figure 1: Overview of the mechanisms underlying CaM- and CaMK-mediated regulation of macrophage and VSMC functions in atherogenesis. (a) Schematic diagram depicting the involvement of macrophages and VSMCs in atherosclerotic plaque formation. Accumulation of lipoproteins beneath the endothelium of a blood vessel induces myeloid monocyte infiltration and the movement of local VSMCs. The recruited monocytes subsequently differentiate into macrophages in lesions, and VSMCs undergo phenotypic modulation through the activation of specific transcription factors and signaling molecules. (b) Schematic illustrating CaM- and CaMK-dependent signaling pathways contributing to macrophage inflammation and apoptosis involved in atherosclerosis. (c) CaM- and CaMK-dependent signaling pathways in a number of processes occurring in VSMCs during the progression of atherosclerosis. CaM: Calmodulin, CaMK: Ca^{2+}/calmodulin-dependent kinase, CDK4: Cyclin-dependent kinase 4, CREB: cAMP response element binding protein, cyt c: Cytochrome c, DAPK: Death-associated protein kinase, ER: Endoplasmic reticulum, HDAC4/5: Histone deacetylases 4 and 5, HMGB1: High mobility group box 1, IRF3: Interferon regulatory factor 3, JNK-P: Phosphorylated c-Jun N-terminal kinase, MAPK: Mitogen-activated protein kinase, MEF2: Myocyte enhancing factor 2, MLCK: Myosin light chain kinase, MMP: Matrix metalloproteinase, MØ: Macrophage, mt: Mitochondria, NF-κB: Nuclear factor-κB, STAT1-P: Phosphorylated signal transducer and activator of transcription 1, TAK1: Transforming growth factor-β-activated kinase 1, VSMC: Vascular smooth muscle cell

the impact of alterations in CaM and CaMK activity on macrophage and VSMC functions during atherosclerosis in the following sections.

Role of calmodulin and calmodulin-dependent kinases in atherosclerotic macrophages

Immunohistological examinations reveal that plaques from symptomatic patients are populated with abundant macrophages [17]. Macrophages are traditionally classified into two major subtypes, M1 and M2, with a proinflammatory role for the former and an opposite role for the latter [18,19]. Numerous studies have attempted to define the macrophage subtype in plaques using macrophage markers; however, due to multiple stimuli gradients in the plaque and the flexible transition between macrophage subtypes, recognizing separate macrophage subsets in early, late, or ruptured plaques is difficult [20]. Therefore, compared to characterizing macrophage subtypes, it might be more effective to clarify the inflammatory mechanisms in macrophages at every atherogenic stage. In the early- to mid-stage, invaded macrophages predominantly form foam cells that secret many proinflammatory cytokines or growth factors causing chronic inflammation in the intima. In the advanced stage, overwhelmed inflammatory reactions cause lesions in macrophages and massive cell death, a critical factor for necrotic core formation. Suppression of CaM or CaMK activity by inhibitors or small-interfering (si) RNA ameliorates inflammation and reduces cell death [21,22], indicating the importance of CaM and CaMKs in macrophage-mediated atherogenesis.

Calmodulin and calmodulin-dependent kinases regulate the inflammatory processes of macrophages

Emerging evidence shows that CaM and multiple CaMKs are associated with macrophage-specific inflammatory responses by modulating the activity of transcription factors and signaling kinases. CaMKII promotes Toll-like receptor (TLR)-mediated proinflammatory cytokine production by activating transforming growth factor-β-activated kinase 1 and interferon regulatory factor 3 signaling in macrophages [22]. CaMKK/CaMKIα triggers interleukin-10 and high mobility group box 1 (HMGB1) release through mechanisms involving mitogen-activated protein kinase (MAPK) activation in lipopolysaccharide-induced macrophages [23]. A previous study showed that CaMKKβ-null macrophages exhibit low migratory activity and cytokine production after TLR4 activation by uncoupling the TLR4 cascade from the downstream kinase [24]. Furthermore, CaMKIV stimulates nucleocytoplasmic shuttling and HMGB1 release through CaMKIV-dependent serine phosphorylation of HMGB1 in TLR4-activated macrophages [25]. As illustrated in Figure 1b, these studies show that CaMKs are required for sustained macrophage activation, which intensifies inflammation and subsequently modulates other cell types in the vascular wall. Overwhelmed inflammatory responses accelerate lesion progression and massive macrophage death causing a deficit in the clearance of dead cells (or defective efferocytosis) that facilitates the necrotic core formation, a key factor in atherosclerosis-induced severe vascular accidents [9].

Calmodulin- and calmodulin-dependent kinases-mediated regulation of macrophage death

Macrophage apoptosis can be extrinsically elicited through death receptor activation by cytokines, such as Fas and TNF-related apoptosis-inducing ligand [26] or intrinsically through signals induced by stressed organelles, such as mitochondria and the ER [21]. As demonstrated by different groups, CaM and CaMKs regulate both extrinsic and intrinsic apoptotic pathways [Figure 1b]. Fas is a cell-surface receptor that receives external signals to regulate internal apoptotic signaling via its cytoplasmic domain [27]. Direct interaction between the cytosolic domain of the Fas death receptor and CaM has been reported. CaM binding to Fas recruits downstream pro-apoptotic factors that initiate caspase-3-mediated cell apoptosis [28]. A mutation in the Ca^{2+}/CaM-binding domain of Fas reduces Fas-dependent apoptosis, further supporting the Fas/CaM interaction and its role in macrophage death. Notably, studies on human vulnerable plaques show that signals from ER stress activated through the unfolded protein response caused by lipoprotein clearance are closely correlated with macrophage apoptosis in atherosclerosis [21]. Prolonged ER stress triggers Ca^{2+} release from internal stores, with this subsequently activating CaM. Thereafter, multiple CaM-targeted molecules, including CaMKII, CaMKIV, CaMKKβ, and DAPK, are stimulated and activate the following signaling pathways in macrophages. CaMKII is a crucial integrator of ER-stress-induced apoptosis in atherosclerosis [21]. CaMKIIγ is the major subtype that controls ER-stress-induced macrophage apoptosis by activating Janus N-terminal kinase (JNK) and signal transducer and activator of transcription 1 to facilitate mitochondrial Ca^{2+} uptake, trigger mitochondrial cytochrome c release, and induce a loss of mitochondrial membrane potential [21]. These detrimental effects are ameliorated by *CaMKIIγ* knockout or pharmacological inhibition, further supporting the role of CaMKIIγ in ER-stress-induced apoptosis [29]. CaMKIV is another multifunctional CaMK highly expressed in unstable atherosclerotic plaques and activated by an upstream regulator (CaMKK) [30,31]. The functions of the CaMKK/CaMKIV complex are best understood in the forebrain, cerebellum, testis, thymus, and tumors, where it controls anti-apoptosis, cell cycle arrest, and autophagy processes [32]. Moreover, CaMKIV activation inhibits β cell apoptosis [33] and CaMKII autophosphorylation and activation [34]; however, it remains unclear whether elevated CaMKIV expression within unstable plaques functions as a survival signal to counteract the apoptotic effect induced by CaMKII in macrophages. Therefore, additional investigation in this area is needed. DAPK belongs to the class of restricted CaM-dependent kinases that function as pro-apoptotic factors [35]; however, the mechanism associated with DAPK-regulated apoptosis remains unknown. It might act as an integrator in the p53-mediated apoptotic pathway [36]. DAPK1-deficiency also attenuates ER-stress- and Fas-ligand-induced cell death in cultured macrophages [37]. As demonstrated previously, the CaMK expression pattern in vulnerable plaques differs from that in stable plaques and

determines the fate of atherosclerotic plaques [37]; therefore, CaMKs might represent a potential biomarker and therapeutic target for atherosclerosis.

Role of calmodulin and calmodulin-dependent kinases in phenotypic regulation of vascular smooth muscle cell during atherosclerosis

In addition to macrophages, VSMCs are another primary cell type that participates in atheroma formation. Molecular and histological examinations of human and animal plaques show that VSMCs express macrophage markers, whereas specific VSMC markers are silenced [38]. The origin of VSMCs within atherosclerotic lesions was controversial for a long time. Recent studies using SMC lineage tracing approaches in mouse models and Y chromosome lineage tracing techniques demonstrated that a considerable proportion of SMC-like cells and cells expressing macrophage markers within the plaques do not have a myeloid origin [38-40]. Furthermore, studies using cross-gender-tracing and arterial transplantation concluded that most SMC-like cells within lesions originate from the local vascular wall [41,42]. These observations indicate that contractile VSMCs can be reversed to an undifferentiated state through a series of complex structural and functional changes, a process termed phenotype switching/modulation, upon mechanical or chemical stimulation and migrate into lesions. Compelling evidence indicates that Ca^{2+}-mediated CaM and CaMK activation is involved in regulating phenotype modulation of VSMCs. Here, we summarize the recent evidence regarding the role of CaM and CaMKs in structural and functional changes in VSMCs during the atherosclerotic process.

From contractile vascular smooth muscle cells to synthetic vascular smooth muscle cells: Ca^{2+}/CaM-dependent transcriptional control

Gene expression is implicated in the control of numerous cell functions. Therefore, transcriptional regulation is a critical event to define the cell phenotypic state. Emerging evidence indicates that CaM and CaMKs are involved in the phenotypic switch of VSMCs by controlling the activity of cAMP response element-binding protein (CREB), myocyte enhancing factor 2 (MEF2), and nuclear factor kappa B (NF-κB). CREB activation is associated with proliferation-associated gene transcription, and both CaMKII and CaMKIV activate CREB via phosphorylation at Ser133 under elevated Ca^{2+} concentrations [43-45]. CREB activation promotes the expression of early-response genes, such as *c-fos* and *egr-1*, which induce VSMC proliferation and migration [46,47]. Whereas CaMKIV only phosphorylates Ser133 for CREB activation, Ser142 represents a second phosphorylation site targeted by CaMKIIδ to inhibit CREB activity in cultured VSMCs [48]. The interaction between CaMKII and CaMKIV in CREB activation and inhibition remains unclear. According to a previous report, CaMKII expression and activity are decreased in unstable carotid plaques, whereas CaMKIV expression is significantly upregulated in human carotid plaques [37]. Predominant expression of CaMKIV might cause CREB activation promoting VSMC phenotypic transformation.

MEF2 is a member of the MADS-box family of transcription factors that is required for upregulating the transcriptional activity in synthetic VSMCs in injured arteries and cultured VSMCs upon angiotensin II stimulation [49,50]. The increase in MEF2-induced transcription is regulated by CaMKI, CaMKII, and CaMKIV through indirect mechanisms involving Class II histone deacetylase (HDAC) 4, HDAC5, and the 14-3-3 chaperon protein [51-53]. During the resting state, MEF2-dependent gene transcription is repressed by forming an inactive complex with HDAC4 and HDAC5 in the nucleus [52,54]. The phosphorylation of HDAC4/HDAC5 by CaMKIIδ triggers nucleocytoplasmic translocation of the phosphorylated HDACs that subsequently derepresses MEF2 activity. This causes altered transcription of multiple genes that regulate phenotype switching in VSMCs [52]. Conversely, a study by Ellis *et al*. indicated that CaMKI and CaMKIV promote the cytoplasmic sequestration of HDAC4 by phosphorylating 14-3-3 resulting in increased MEF2-dependent transcription of smooth muscle-specific genes [51]. This evidence shows that the balance of CaMK activity is important in determining MEF2 activation in synthetic VSMCs, especially in terms of positive or negative regulation.

NF-κB is a critical regulator of the inflammatory responses of atherosclerotic macrophages but also regulates the phenotypic modulation of VSMCs. Pharmacological inhibition of NF-κB signaling attenuates the expression of matrix metalloproteinases (MMPs) in VSMCs isolated from symptomatic plaques and prevents VSMC proliferation and migration in culture, indicating a role for NF-κB in VSMC phenotypic modulation [55-57]. A key event for NF-κB activation is ubiquitin-mediated IκB degradation triggered by IκB kinases (IKKs) [58]. Biochemical studies reveal that CaMKIIδ directly interacts with IKKβ causing IKKβ activation and IκB degradation [59]. NF-κB activation increases the production of MMPs (mainly MMP-2 and MMP-9) and promotes the subsequent phenotypic switching to synthetic VSMCs [55,60,61]. Collectively, these findings show that the transcription factors CREB, MEF2, and NF-κB play crucial roles in VSMC fate, especially during atherosclerosis progression [Figure 1c]. Different transcription factors can separately trigger the expression of specific proteins responsible for VSMC plasticity. Because these transcription factors regulate both VSMC physiology and pathology, they might serve as excellent therapeutic targets for treating atherosclerosis. Therefore, understanding their modulation by CaMKs might provide insight for the development of novel treatment strategies.

Calmodulin and calmodulin-dependent kinases-mediated regulation of vascular smooth muscle cell migration

Medial VSMCs are normally embedded in a highly organized extracellular matrix (ECM) comprising collagen-I, collagen-III, fibronectin, elastin, and proteoglycans [62]. These matrix molecules interact with VSMC surface receptors to maintain the structure and function of smooth

muscle in a native artery. Multiple pathways are activated to enable the migration of phenotype-modulated VSMCs, and CaM and CaMKs are involved in the regulation of these pathways [Figure 1c]. ECM degradation is the first step to liberate cells from environmental confinement. ECM component turnover is associated with the upregulation of MMPs [63]. MMP-2 and MMP-9 are specifically upregulated in VSMCs during intima formation caused by vascular lesions in animal models [64-66]. Scott *et al*. [67] found that MMP-9 protein and mRNA levels are significantly lower in CaMKIIδ-deficient VSMCs in cell culture or injured carotid arteries of mice, demonstrating the importance of CaMKII in the regulation of MMP-9 expression. Additionally, *MMP-2* transcription is upregulated in cultured VSMCs treated with different growth factors related to atherosclerosis through the phosphoinositide 3-kinase–protein kinase B (Akt) signaling cascade, which is shown to be activated by CaMKKβ, CaMKII, and CaMKIV in cancer cells, T cells, and endothelial cells [68-71]. However, additional studies are required to understand CaMK-mediated Akt-activation in VSMC migration during atherosclerosis.

Concomitant to ECM degradation, the actomyosin apparatus in VSMCs needs to undergo reorganization for cell movement during migration. VSMC movement is accomplished by a cyclical process that includes leading edge extension, focal adhesion formation, and retraction of the cell rear, each of which involves interactions between actin and myosin [72]. Actomyosin contraction is triggered by multiple signaling pathways that converge on the critical controller, Ca^{2+}/CaM-dependent MLCK. These pathways include excitatory and inhibitory signals from Ca^{2+}-, myotonic dystrophy kinase-related Cdc42-binding kinase-, extracellular-regulated kinases (ERK)-, and Rho-kinase-dependent cascades [72]. Interaction between actin and MLCK-phosphorylated myosin II allows VSMCs to slide along the MMP-degraded ECM [73]. In addition to the essential role of MLCK in cell migration, CaMKII is also a critical controller of chemoattractant- or integrin-induced VSMC migration [74,75]. CaMKII positioned at the leading edge triggers actomyosin motor motion via ERK1/2, protein tyrosine kinase 2, and Rac activation, with energy provided by CaMKII-stimulated mitochondria [76-78]. CaMKII activation by gene transfer or Ca^{2+}/CaM abolishes the inhibition of VSMC migration caused by blocking the chemoattractant receptors or shedding integrin by an antibody [75,79]. In addition, a previous study found that CaMKIII (eEF2K) activation and the CaMKII–JNK axis are required for both cancer cell migration and invasiveness [80,81]. With the common migratory mechanisms shared by different cell types, CaMKIII and the CaMKII–JNK axis might also participate in VSMC migration during plaque formation.

Calmodulin- and calmodulin-dependent kinases-mediated regulation of vascular smooth muscle cell proliferation

In a normal blood vessel, the turnover rate of medial VSMCs is very low, whereas the cell proliferation rate is drastically increased during atherogenesis. Most of these proliferating VSMCs acquire markers and functions related to macrophages, thereby contributing to an increased number of foam cells in plaques and aggravated inflammation *in situ* [82]. Cell proliferation is accompanied by the activation or upregulation of different molecules, including kinases, ion channels, and transporters, which enable the transition of orderly signaling events in a cell cycle. Among these molecules, Ca^{2+}/CaM and CaMKs are required to control the proliferative processes in the cell cycle. In the G_1 phase, CaM expression progressively increases, resulting in nuclear factor of activated T cells-dependent expression of cyclin D and cyclin D/cyclin D-dependent kinase 4 (CDK4) assembly [83]. G_1 phase progression is also positively regulated by CaMKI. Selective inhibition of CaMKI by siRNA or overexpression of an inactive variant suppresses cyclin D expression and its assembly with CDK4 [84]. The G_1/S transition is a critical checkpoint in the cell cycle and highly sensitive to changes in Ca^{2+} concentrations [85]. Cyclin E is a main controller of G_1/S transition and the S phase. Deleting the highly conserved CaM-binding motif from cyclin E or pharmacologically inhibiting CaM activity abolishes the Ca^{2+} sensitivity of cyclin E and causes G_1 arrest in cultured VSMCs [86]. Moreover, cyclin E expression is positively regulated by CaMKIIδ, a particular isoform expressed in phenotype-switched VSMCs. CaMKIIδ stimulates the G_1/S phase transition by downregulating the expression of P21 proteins either through Mdm-2-mediated P53 degradation or through HDAC4 activation [49,52]. In G_2/M transition, CaMKII is especially important in bridging Ca^{2+}/CaM signaling to cell cycle indices. Suppression of CaMKIIδ activity by inhibitors, siRNA, or gene silencing induces increased proportions of multinucleated cells in the G_2 phase, abrogates VSMC proliferation in cell culture, and prevents neointima formation *in vivo* [49,87,88]. Furthermore, throughout the M phase, Ca^{2+}/CaM is essential for maintaining well-organized spindle microtubules and the association between p85 and G-actin in cytokinesis [89,90]. Collectively, CaM plays a regulatory role in VSMC proliferation by regulating the activity of cyclin-associated proteins, CaMKI, CaMKII, and CaMKIV in atherosclerosis [Figure 1c]. Suppressing CaM and CaMK activity delays or stops cell cycle progression. Furthermore, the CaMKII subtype is specifically switched to the δ isoform in phenotype-modulated VSMCs, which might serve as a biomarker to develop therapeutic strategies.

Potential therapeutic interventions for atherosclerosis treatment by targeting calmodulin-dependent kinases

Studies on cell and animal models described previously herein demonstrate the contribution of CaM and CaMKs to atherosclerosis and the therapeutic potential, since suppressing CaM or CaMKs with inhibitors or genetic manipulation alleviates atherosclerotic severity. Because CaMKII is of particular important in both macrophage- and VSMC-mediated atherogenic processes, it has attracted pharmaceutical interest to develop CaMKII inhibitors as a therapeutic agent. The types and basic characteristics of CaMKII inhibitors are summarized in Table 1. KN-93 and KN-62 are the most

Table 1: Biochemical and pharmacological properties of calcium/calmodulin-dependent protein kinase II inhibitors

Inhibitor	Type	Action site	IC_{50} (μM)	Limitation	References
KN-93, KN-62	Chemical compound	Allosteric binding	0.5, 1	Poor selectivity	[91,92]
AC3-I, AIP	Peptide	Substrate-binding domain	0.8, 0.04	Lack of an extensive screening of targets; delivery technique	[91,94]
CaMKIIN	Peptide	Substrate-binding domain, regulatory domain	0.05	Lack of an extensive screening of targets; delivery technique	[98]
AS105, GS-680, RA306	Chemical compound	ATP-binding domain	0.008, 0.002, 0.03	Lack of specificity for Ser/Thr kinases	[95,96]
siRNA/miRNA	RNA	mRNA	-	Difficulty in in vivo delivery	[22,97]

CaMKII: Calcium/calmodulin-dependent protein kinase II, AC3-I: Autocamtide-3-derived inhibitor peptide, AIP: Autocamtide-2-related inhibitory peptide, ATP: Adenosine triphosphate, RNA: Ribonucleic acid, siRNA: Small interfering RNA, miRNA: Micro RNA

widely used pharmacologic CaMKII inhibitors with high membrane-permeability [91]. Owing to their off-target inhibitory effects on CaMKI, CaMKIV, and voltage-gated cation channels [92,93], optimization in their selectivity and potency is needed for therapeutic use. Autocamtide-3-derived inhibitor peptide (AC3-I), autocamtide-2-related inhibitory peptide (AIP), and CaMKIIN are three substrate-competitive peptide inhibitors [91,94]. Although the three peptide inhibitors are very potent in inhibiting CaMKII, extensive screening for their off-target effects has not been conducted, and thus, their pharmacological profile remains unclear. Limited bioavailability of short peptides and delivery using viral vectors or nanoparticles are also challenges to conquer for application in humans. AS105, GS-680, and RA306 are recently developed pyrimidine-based small molecules targeting the ATP-binding site of CaMKII [95,96]. However, due to the similar structure of ATP-binding sites shared by many Ser/Thr kinases, specificity becomes a problem for this type of inhibitor. Another therapeutic approach lies in the development of RNA drugs, which directly target specific *CaMK* genes. Short hairpin RNAs, microRNAs, and small-interfering RNA have been used for decades to identify the role of a critical gene in a disease and to find cures. A recent report demonstrated that CaMKIIγ-targeting siRNA nanoparticles ameliorate the lesions in macrophages and stabilize the atherosclerotic plaque in LDL receptor-knockout mice fed with a Western diet [97]. Although techniques for the selective targeting of a specific tissue, bioavailability, and delivery are still in development, the flexibility and specificity exerted by RNA drugs shows their potential for future therapeutic use.

Conclusions and future directions

Macrophages and VSMCs are two predominant components of atherosclerotic plaques that participate in unresolved inflammation, defective efferocytosis, and massive cell death, all key factors for plaque rupture. Understanding the mechanisms underlying the regulation of their states, functions, and phenotypic switching in response to complex microenvironmental cues is essential to find new treatment strategies for atherosclerosis. The type of atherosclerotic plaque is determined by CaM and CaMK expression patterns in human and laboratory animals, indicating the potential of CaM and CaMKs as biomarkers for atherosclerosis. In addition, CaMKs and their downstream molecules contribute to the induction of massive cell death and necrotic core formation in atherosclerosis by activating inflammatory reactions and apoptotic processes in macrophages and controlling phenotypic modulation, cell migration, and proliferation in VSMCs. The signaling pathways discussed in this review are summarized in Figure 1. Furthermore, inhibition of CaM or CaMKs through pharmacological or genetic interventions successfully prevents macrophage-associated inflammation and the phenotypic modulation of VSMCs in cultures and animal models. Therefore, targeting CaM or CaMKs might be beneficial to decelerate atherogenic progress. However, human atherosclerosis mechanisms are more complicated and might not be entirely reflected by the data from cultured cells or animal models. A critical challenge for future studies will be finding ways to apply laboratory information to clinical use. Moreover, a clear definition for the fundamental functions of CaM and CaMKs in the human atherosclerotic progression is still in need to help develop an effective therapeutic intervention.

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Conflicts of interest

There are no conflicts of interest.

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