Controlling the Cell Cycle: The Role of Calcium/Calmodulin-stimulated Protein Kinases I and II

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ABSTRACT

Many studies have implicated Ca^{2+} and calmodulin (CaM) as regulators of the cell cycle. Ca^{2+}/CaM-stimulated proteins, including the family of multifunctional Ca^{2+}/CaM-stimulated protein kinases (CaMK), have also been identified as mediators of cell cycle progression. CaMKII is the best-characterized member of this family, and is regulated by multi-site phosphorylation and targeting. Using pharmacological inhibitors that were believed to be specific for CaMKII, CaMKII has been implicated in every phase of the cell cycle. However, these 'specific' inhibitors also produce effects on other CaMKs. These additional effects are usually ignored, and the effects of the inhibitors are normally attributed to CaMKII without further investigation. Using new specific molecular techniques, it has become clear that CaMKI is an important regulator of G1, whereas CaMKII is essential for regulating G2/M and the metaphase-anaphase transition. If the mechanisms controlling these events can be fully elucidated, new targets for controlling proliferative diseases may be identified.

INTRODUCTION

The generation of new cells from existing cells is a fundamental requirement for all living organisms. A cell reproduces via an orderly sequence of events, known as the cell cycle, which is classified into several distinct phases in mammalian cells (Figure 1). The first phase is *Interphase*, and is comprised of G1 (Gap/Growth Phase 1), S (DNA replication), and G2 (Gap/Growth Phase 2). The second phase is *M Phase*, which consists of Mitosis (prophase, prometaphase, metaphase, anaphase, telophase). The cell finishes dividing by undergoing Cytokinesis (separation of the daughter cell from the parent cell). Each phase of the cell cycle is tightly controlled by a network of regulatory proteins, and transition from one stage to the next is regulated by a number of events known as checkpoints. Entry and exit from these checkpoints is largely controlled by the cyclin-dependent kinases (CDK) and their associated cyclin proteins [1]. Understanding precisely how various cell cycle control mechanisms are regulated has important implications for the treatment of proliferative diseases, such as cancer.

Figure 1

This review investigates the role of a family of protein kinases - the multifunctional Serine (S)/Threonine (T) Ca^{2+}/calmodulin (CaM) stimulated protein kinases (CaMK) - in regulating the cell cycle. One member of this family, CaMKII, has been implicated as a regulator of every phase of the cell cycle (Figure 1). There is some controversy regarding its role, and the aim of this review is to identify the factors that account for this controversy and to clarify the role of CaMKII and other CaMK family members in controlling the cell cycle.

CALCIUM, CALMODULIN, AND THE CELL CYCLE

Studies over several decades have implicated Ca^{2+} as a regulator of a variety of cellular functions, including cell cycle progression, and many reviews have highlighted the important role that Ca^{2+} plays in regulating cellular proliferation, fertilisation, and early development [2-11]. Ca^{2+} plays an important role throughout the cell cycle, and has been implicated in the regulation of the G1/S and G2/M transitions, as well as the metaphase-anaphase transition [6, 8].

CaM is a small 16.7 kDa protein, and is one of the major Ca^{2+} sensors in eukaryotes. CaM has also been implicated in the regulation of the cell cycle. Progression through G1 (specifically the G1/S transition) and exit from mitosis are sensitive to changes in the concentration of CaM [12]. Upon binding with Ca^{2+}, CaM undergoes a major conformational change and the Ca^{2+}/CaM complex interacts with a variety of target proteins [13]. A range of proteins are stimulated by Ca^{2+}/CaM, including protein kinases and ion channels, but this review will focus on the multifunctional CaMK family and their role in cell cycle regulation. This family is comprised of CaMK kinase (CaMKK), CaMKI, CaMKII, and CaMKIV. This review focuses on how CaMKII regulates the cell cycle, and will attempt to resolve contradictions that currently exist in the literature.

THE MULTIFUNCTIONAL Ca^{2+}/CaM STIMULATED PROTEIN KINASES

There are numerous kinases that are stimulated by Ca^{2+}/CaM, some of which have a very specific substrate and function (for example, myosin light chain kinase (MLCK) and glycogen synthase kinase (GSK)). However, there are other Ca^{2+}/CaM-

stimulated protein kinases that can phosphorylate a broad range of substrates in many cell types, hence eliciting a wide variety of functions; these are the multi-functional CaMK family.

The subunits of all the CaMKs, except CaMKII, have similar overall domain structures (Figure 2) [14]. All members of this family have a homologous regulatory domain containing an autoinhibitory region, which keeps the kinase inactive until Ca^{2+}/CaM binds, and a conserved CaM binding region to which Ca^{2+}/CaM binds and activates them. All family members have an N terminal catalytic domain, of varying lengths, however, only CaMKII has a C terminal association domain that enables it to assemble into dodecameric oligomers, whereas the other family members are monomeric. All members of the CaMKI and CaMKIV subfamilies are activated by phosphorylation of a conserved T in their activation loop by CaMKKα or CaMKKβ, making up a 'CaMK cascade' [15]. However, CaMKII does not possess an activation loop. Thus, while the CaMK family possess many similarities, there are some fundamental differences in the structure and regulation of these kinases (Table 1).

Figure 2

Table 1

CaMKI

CaMKI is encoded by four genes (α, β, γ, and δ), with each gene producing at least one splice variant. All members of this family are monomeric and are between 38 and 42 kDa in size, except for CaMKIγ, which is 53 kDa. The various isoforms of

CaMKI are expressed in the brain and other tissues, with CaMKIα being expressed in most mammalian cells [16]. Phosphorylation of the conserved T (T174 to T180, depending on the isoform) in the activation loop by CaMKK is required for maximal CaMKI activity [17], although this depends on the substrate [18], which suggests that CaMKI may also be regulated by targeting. Some isoforms of CaMKI have been shown to translocate once they become activated [19, 20], suggesting that molecular targeting may play a role in the regulation of CaMKI function, however, this remains to be determined. Furthermore, the effects of phosphorylation at T177 on targeting of CaMKI have not been examined. CaMKI has been implicated in a variety of cellular functions, including the control of synapsin in nerve terminals [21], growth cone motility and axon outgrowth [22], the cystic fibrosis transmembrane regulator [23], aldosterone synthase expression [24], and the cell cycle [25, 26].

CaMKIV

CaMKIV is encoded by one gene (α), which produces at least one splice variant (β). CaMKIV requires Ca^{2+}/CaM to become active, as well as phosphorylation of the conserved T in the activation loop (T200 in human CaMKIV) by CaMKK [27]. This phosphorylation generates an autonomously active kinase. All splice variants are monomeric and are between 65 and 67 kDa in size. CaMKIV is primarily expressed in the brain, but is also present in immune cells and the testis/ovary [28-31]. CaMKIV can translocate between the nucleus and cytoplasm [32], suggesting that targeting may also play a role in CaMKIV function. CaMKIV has been implicated in the regulation of cyclic AMP element binding protein (CREB) [33], neurite outgrowth [34], immune and inflammatory responses [35], and cell cycle control [25].

CaMKK

CaMKK is encoded by two genes (1 and 2) that encode CaMKKα and CaMKKβ, respectively. The CaMKK2 gene produces several splice isoforms [36]. CaMKK require Ca^{2+}/CaM for maximal activity [37], and can also undergo autophosphorylation [38]. Autophosphorylation, however, does not seem to alter kinase activity [38]. All CaMKKs are monomeric, and are between 54 and 68 kDa in size. CaMKK is primarily expressed in the brain, but is also present in the thymus, spleen, and testis [28-31]. CaMKKs phosphorylate CaMKI and CaMKIV, but they can also phoshorylate other substrates, such as AMP activated protein kinase (AMPK) [39].

CaMKII

CaMKII is encoded by four genes (α, β, γ, and δ) [40], which produce over 30 isoforms ranging in size from 50 to 60 kDa. One or more members of this family are found in virtually every tissue, and mediate diverse physiological responses triggered by increases in intracellular Ca^{2+} concentrations, activation by the binding of Ca^{2+}/CaM, and regulated by the ability to undergo autophosphorylation, and targeting. Unlike other members of the CaMK family, CaMKII is active without phosphorylation, as binding to Ca^{2+}/CaM is adequate for activation. CaMKII also does not possess an activation loop. CaMKII is expressed most abundantly in neurons, and is involved in regulating many aspects of neuronal function, including neurotransmitter synthesis and release, cellular morphology and neurite extension, long-term plasticity, learning, memory consolidation, and memory erasure following retrieval [41-46]. Non-neuronal CaMKII has been implicated in the regulation of other biological processes, such as fertilisation [4], osteogenic differentiation [47], the maintenance of vascular tone [48], and cell proliferation [49].

The fact that CaMKII is ubiquitously expressed raises the question of how a widely expressed kinase can produce such varied cell-specific responses. The answer lies in its unique regulatory mechanisms, the subtlety of which has only recently been appreciated.

Regulation of CaMKII

The biological properties of CaMKII are regulated by multi-site phosphorylation and targeting to specific subcellular locations through interactions with other proteins. These two control mechanisms can also influence one another, as the interaction between CaMKII and some binding partners can be modified by the phosphorylation state of the kinase, as well as by phosphorylation of the binding partner [49].

Phosphorylation at the well-characterized CaMKII phosphorylation sites, T286 and the paired sites T305/306 (numbered for the CaMKIIα isoform), directly alters CaMKII activity, as well as targeting. T286 phosphorylation generates autonomous activity and increases the targeting of CaMKII to various subcellular locations, including the post-synaptic density (PSD) of neurons. However, unlike the T200 site in CaMKIV, phosphorylation of T286 is not required for kinase activity. T305/306 phosphorylation prevents the binding of Ca^{2+}/CaM to CaMKII, thereby making the enzyme insensitive to changes in intracellular Ca^{2+}, and also targets CaMKII away from the PSD [50].

Emerging evidence from several laboratories shows that the behaviour of CaMKII *in vivo* cannot always be predicted from our understanding of its behaviour *in vitro*,

indicating that additional regulatory interactions occur in intact cells. Recently, a new phosphorylation site on CaMKII at T253 was identified *in vivo* [51]. T253 has previously been overlooked as a phosphorylation site of interest as it has no direct effect on the kinase activity of CaMKII *in vitro*. However, T253 phosphorylation has marked effects on CaMKII targeting [49, 51]. Furthermore, the overall stoichiometry of T253 phosphorylation is relatively low in the cell as a whole because it occurs only in a subpopulation of CaMKII molecules at particular cellular locations [51].

T253 may represent a new class of phosphorylation site that does not directly alter kinase activity but rather modifies interactions with binding proteins [49], thereby varying the cellular location of CaMKII. CaMKII located in different molecular environments can respond to stimuli differently, and become phosphorylated at different sites resulting in differential targeting and functional outcomes [50]. Variations in expression and intracellular location of binding proteins can give rise to cell specific functional responses to CaMKII activation. It is well established that the appropriate targeting of signalling molecules plays an important role in establishing the cellular responses to extracellular stimuli. With respect to CaMKII function, CaMKII must be co-localized with the correct binding protein to provide the appropriate cellular response.

INHIBITORS OF CaMKII

Interpretation of previous studies relies on the use of pharmacological inhibitors as well as expression of constitutively active or kinase dead mutants of CaMKII, or other members of the CaMK family.

The most widely used small molecular CaMKII inhibitors are 1-[N,O-bis-(5-isoquinolinesulphonyl)-N-methyl-L-tyrosyl]-4-phenylpiperazine (KN-62) and 2-[N-(2-hydroxyethyl)]-N-(4-methoxybenzensulfonyl)]amino-N-(4-chlorocinnamyl)-N-methylbenzylamine (KN-93). KN-62 and KN-93 are membrane permeable, and are thought to be competitive for CaM [52]. KN-62 and KN-93 were originally described as CaMKII specific inhibitors, as they do not affect the catalytic activity of other CaM-dependent enzymes, such as cAMP-dependent protein kinase, Ca^{2+}/phospholipid dependent protein kinase, MLCK, and Ca^{2+}-phosphodiesterase [53, 54]. Once inhibitors have been shown to produce an effect and cells have been demonstrated to express one or more isoforms of CaMKII, the effects of KN-62 and KN-93 are normally attributed to CaMKII, without any further investigation. However, subsequent studies have demonstrated that these inhibitors also affect CaMKI and CaMKIV [55, 56], and have shown that the inhibition constant (K_I) is very similar for all three kinases (Table 1). Therefore, the validity of studies that only utilize these pharmacological agents and conclude that observed effects are due to CaMKII can be questioned.

KN-62 and KN-93 prevent the activation of CaMKII by Ca^{2+}/CaM but do not inhibit autonomously active CaMKII [55], therefore, CaMKII that is autonomously active due to T286 phosphorylation or interaction with a specific binding protein will be unaffected by these inhibitors. The effects on autonomously active CaMKIV have not been examined, but could be hypothesized to be similar. Furthermore, KN-62 and KN-93 can inhibit molecules unrelated to the CaMK family, such as ion channels [57-59]. CaMK-specific effects of these inhibitors can only be determined by comparing the effects of KN-62 and KN-93 with their inactive analogues, KN-04 and KN-92,

respectively, but these inhibitors do not distinguish between the multifunctional CaMK family members. Whilst KN-92 is widely used as a control for KN-93 treatment, KN-04 is infrequently used as a negative compound for KN-62.

The autocamtide-2-related autoinhibitory peptide (AIP; KKALRRQEAVDAL) [52] is a novel peptide that competes with substrates at the active site of CaMKII, thereby inhibiting all enzyme activity irrespective of how CaMKII was activated. AIP is a nonphosphorylatable analog of autocamtide-2, which is a peptide based on the autoinhibitory domain of CaMKII, and AIP is competitive for autocamtide-2, but not syntide-2 [60]. AIP-II (KKKLRRQEAFDAY) is a more potent peptide analogue of AIP, where Alanine 3 (A3) and Valine 10 (V1) are replaced with a Lysine (K) and Phenylalanine (F) [61]. This modification reduces the half maximal inhibitory concentration (IC_{50}) of AIP for CaMKII from 40nM to 4nM. Autocamide-3 derived peptide inhibitor (AC3-1; KKALHRQEAVDCL) is also an analogue of AIP, however, its IC_{50} for CaMKII is 150nM [62], meaning that it is not quite as potent with CaMKII as the parent analog.

CaMK REGULATION OF THE CELL CYCLE

Studies in many cell types using a variety of approaches [49, 63-87] have established that the CaMK family are important regulators of the cell cycle and that they are involved in every phase of the cell cycle (Table 2). Many of these studies have concluded that CaMKII controls the observed effects based on the action of the 'CaMKII specific inhibitors' KN-62 and KN-93 (Figure 1). However, due to the reasons outlined above, these conclusions need further examination.

Table 2

G1 Phase

Treatment of various cell lines with KN-62 or KN-93 blocks cells in G1 [72, 77, 78, 83, 84, 87, 88]. A variety of molecular mechanisms have been proposed to account for these effects including a decreased expression of cyclin D1 [72, 78], decreased phosphorylation of the Retinoblastoma protein (Rb) [72, 78, 87], prevention of cdk4 activation [69], and increased $p27^{Kip1}$ association with cdk2 [72]. Many of these studies attributed the observed effects of treatment with KN-62 or KN-93 to inhibition of CaMKII activity [72, 77, 84], although at the doses utilized (5 - 20μM), CaMKI would also be inhibited (Table 1). However, selectively inhibiting the activity of CaMKI by downregulating CaMKI expression using short interfering RNA (siRNA) [78], or overexpressing a kinase dead mutant of CaMKI [69], elicited the same effects as KN-62 and KN-93 treatment on cell cycle progression, Rb phosphorylation, cyclin D1 expression and cdk4 activation. This implies that the G1 phase specific effects that previously have been attributed to CaMKII are actually mediated by CaMKI. Furthermore, overexpression of a CaMKII kinase dead mutant did not alter cdk4 activation [69], suggesting that CaMKII does not control G1 progression.

S Phase

Of all the cell cycle phases in which CaMKII has been implicated, the evidence supporting a role in S phase progression is the weakest. Relatively few studies have demonstrated that inhibition of CaMKII, either through the use of pharmacological or endogenous inhibitors, can cause a block in the S phase of the cell cycle [71, 86, 89]. Both KN-62 and AIP treatment either blocked [71] or slowed [86] the progression of cells

through S phase, depending on the method used to examine cell proliferation. Both of these experiments used cell lines that expressed CaMKI, CaMKII, and CaMKIV, and used KN-62 at a concentration that would inhibit all of these kinases (10μM), making the results difficult to interpret.

A recently identified endogenous CaMKII inhibitor protein, CaMKIIN, has been implicated in the control of progression of cells through S phase. CaMKIIN was first identified in rat brain by yeast two hybrid assay [90], and there are two known rat isoforms - CaMKIINα [90] and CaMKIINβ [91]. Human CaMKIIN (hCaMKIIN) has also been identified in human dendritic cells [92]. CaMKIIN only interacts with activated CaMKII [90]. Importantly, CaMKIIN does not inhibit other kinases, such as protein kinase C (PKC), protein kinase A (PKA), CaMKI, CaMKIV, or CaMKK [90]. It is therefore the most specific CaMKII inhibitor currently known. Overexpression of hCaMKIINα in human colon adenocarcinoma cells caused an accumulation of cells in S phase, whereas silencing hCaMKIINα increased cell proliferation rates [89]. In addition, hCaMKIINβ overexpression decreased ovarian cancer cell growth and tumorigenicity [93]. These findings provide the strongest evidence supporting a role of CaMKII in the control of S phase progression. However, whether the functions controlled by CaMKII in S phase are regulated by the endogenous CaMKII inhibitor, CaMKIIN, or by another regulatory mechanism remains to be seen.

G2 Phase and Mitosis

Mitotic Entry

The strongest evidence indicating that CaMKII plays a role in cell cycle regulation is the wealth of data in a variety of cell lines using numerous methods that demonstrate

that CaMKII function is essential for progression through the G2 and M phases of the cell cycle [64, 66, 68, 70, 73-76, 80, 82, 9495]. Pharmacological inhibition with KN-93 or AC3-1 (a peptide inhibitor) [73, 74, 80, 82], down-regulation of CaMKII using siRNA [68], or overexpression of a kinase dead [68] mutant of CaMKII caused cells to accumulate in the G2 or M phases. Consistent with these results, overexpression of CaMKII increased the rate of cell division [49].

By contrast, Planas-Silva and Means [75] showed that overexpression of a constitutively active truncated mutant blocked cells in the G2 phase of the cell cycle. This is the only study that appears to contradict the wealth of evidence demonstrating that CaMKII function is essential for progression through the G2 and M phases of the cell cycle and recent advances in our understanding of the importance of molecular targeting in the regulation of CaMKII may explain the unexpected results in this study. The constitutively active form of CaMKIIα used by Planas-Silva and Means [75] was truncated and monomeric, lacking the entire association domain and part of the regulatory domain of the enzyme which are the regions of CaMKII that contain most of the known binding sites for targeting proteins [50]. Consequently, this constitutively active, truncated, monomeric enzyme would be predicted to exhibit aberrant targeting in cells and therefore may produce functional responses that are different from those normally produced by CaMKII action. Furthermore, multimeric CaMKII can act as an adaptor to assemble multimeric protein complexes [50] and, if this adaptor activity is involved in cell cycle regulation, monomeric CaMKII would be expected to be deficient in adaptor activity. Therefore, the interpretation of the results of Planas-Silva and Means [75] is problematic.

Recent results from our laboratory [49] show that overexpression of a full length constitutively active T286 phospho-mimic mutant of CaMKIIα stimulated proliferation. While these experiments were performed in different cell lines to those used by Planas-Silva and Means [75], we observed the same effects on cell proliferation in more than one cell type. This is consistent with the results from many laboratories on the effects on the cell cycle of reducing CaMKII activity and supports the interpretation that the unexpected results of Planas-Silva and Means [75] are due to aberrant targeting of the transfected CaMKII. The importance of correct CaMKII targeting in regulating the cell cycle was further illustrated by our results. We showed that overexpression of a full length T253 phospho-mimic mutant of CaMKII, which has normal activity but altered targeting, resulted in a strong inhibition of cell proliferation [49] and transient overexpression of this T253 phospho-mimic caused cells to accumulate in G2 or M phases of the cell cycle (data not shown). Taken together, these results strongly suggest that a combination of CaMKII activity and its selective targeting to specific cellular locations is a major regulator of the G2 or M phases of the cell cycle.

Metaphase-Anaphase Transition

Further compelling evidence supporting the role of CaMKII in mitotic progression has come from elegant studies examining egg activation. Mammalian eggs are arrested in metaphase of meiosis II. Fertilisation is characterised by a series of Ca^{2+} spikes [2], which activate CaMKII. Importantly, studies using pharmacological inhibitors [66, 82, 96, 97], morpholino-induced downregulation [98], or gene knockout [99] of CaMKII have shown that egg activation is prevented by CaMKII inhibition, as oocytes fail to resume meiosis. Oocytes remained arrested at metaphase II, which

indicates that CaMKII is a necessary requirement for the metaphase-anaphase transition. These results were confirmed in two somatic cell lines, as low concentrations of KN-93 (0.5 - 5μM), which would specifically inhibit CaMKI and CaMKII, transiently blocked cells at metaphase, with higher concentrations (10 - 100μM) causing permanent cell cycle arrest [74]. Furthermore, overexpression of a constitutively active CaMKII mutant in oocytes resulted in the resumption of meiosis [70, 96]. This data strongly indicates that CaMKII is essential for progression through mitosis, and specifically, the metaphase-anaphase transition.

CaMKII Regulation of the Mitosis Promoting Factor (MPF)

The key mediator of the G2/M transition in eukaryotic cells is thought to be the mitosis promoting factor (MPF), a complex formed by Cdk1 (also known as Cdc2) and cyclin B [100]. During interphase, the MPF is inhibited by Cdk1 phosphorylation at two sites, T14 and Tyrosine (Y) 15. The kinases Myt1 and Wee1 are believed to be responsible for this inhibitory phosphorylation. At the G2/M transition, these phosphorylations are reversed by the Cdc25C checkpoint phosphatase, leading to MPF activation and mitosis [101]. During interphase, Cdc25C is inhibited by S216 phosphorylation, and CaMKII is one of a number of kinases that has been reported to phosphorylate this site [102]. Therefore, CaMKII would act as an inhibitor of mitosis. Cdc25C, however, can also be phosphorylated at a number of activating sites [103, 104]. CaMKII also phosphorylates these activating sites [73], hence acting as a positive regulator of mitosis. This raises the question of how such opposing effects of CaMKII can be regulated *in vivo*. One possibility is differential molecular targeting. If CaMKII is phosphorylated at different sites throughout the cell cycle, this would alter the proteins with which it interacts, thereby targeting it to different molecular

complexes and allowing it to both positively and negatively regulate mitosis. Indeed, that full length CaMKII promotes cell proliferation [49], whilst truncated [75] or T253 phospho-mimic [49] mutants inhibit proliferation, strongly support this hypothesis.

Role of CaMKII in Regulating Microtubule Dynamics

Microtubules are composed of α- and β-tubulin heterodimers which assemble with microtubule-associated proteins (MAPs) to form polymeric filaments [105]. Microtubules are dynamic structures that are constantly growing and shortening, and their ends have the ability to switch stochastically between these states, in a phenomenon termed *dynamic instability* [106]. Microtubule growth can be interrupted by a random transition in depolymerisation, called a *catastrophe*, a process that is essential for the metaphase-anaphase transition [107]. The dynamic instability of microtubules is crucial for many microtubule-dependent processes, but it is most important in mitosis.

CaMKII is a dynamic component of the mitotic apparatus, and is essential for initial centrosome duplication [108]. During interphase, CaMKII is localized diffusely in the cytoplasm and nucleus. At metaphase, CaMKII is associated on the spindle poles, and at the metaphase-anaphase transition it is localized to the centrosomes and between the spindle poles. After anaphase, CaMKII translocates to the area between separating chromosomes [66, 109]. Recent work has demonstrated that CaMKII regulates microtubule catastrophe during anaphase, as overexpression of a constitutively active CaMKII mutant promotes microtubule destabilization [110]. Furthermore, down-regulation of CaMKIIγ in a variety of cell lines by siRNA resulted in an accumulation of prometaphase/metaphase cells, as well as cells with multipolar spindles [67], which

indicates that CaMKII is required for normal bipolar spindle formation during mitosis.

CONCLUSIONS

Understanding how the cell cycle is regulated is one of the fundamental pursuits of cell biology. At least one member of the CaMK family has been implicated in each phase of the cell cycle, however, exactly how they regulate these phases is currently unknown. In this review, we have clarified the role of CaMKII in regulating the cell cycle and microtubule dynamics. CaMKI, and not CaMKII, is involved in the progression of cells through G1, whereas CaMKII regulates G2/M and the metaphase-anaphase transition. Furthermore, CaMKII may be involved in S phase progression, however, the exact role remains unknown. Future investigations will need to focus on identifying the mechanisms regulating CaMKII in these processes, as well as the downstream effector molecules involved, as this will potentially uncover new pathways for manipulating the cell cycle, and hence, controlling proliferative diseases, such as cancer.

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ABBREVIATIONS

A	Alanine
AIP	Autocamtide-2-related autoinhibitory peptide
AMPK	AMP activated protein kinase
CaM	Calmodulin
CaMK	Calcium/calmodulin-stimulated protein kinase
CaMKK	Calcium/calmodulin-stimulated protein kinase kinase
CDK	Cyclin-dependent kinases
CREB	Cyclic AMP response element binding protein
F	Phenylalanine
GSK	Glycogen synthase kinase
IC_{50}	Half maximal inhibitory concentration
kDa	Kilodalton
K	Lysine
K_I	Inhibition constant
MAP	Microtubule-associated protein
MLCK	Myosin light chain kinase
MPF	Mitosis promoting factor
PKA	Protein kinase A
PKC	Protein kinase C
PSD	Post-synaptic density

Rb	Retinoblastoma protein
S	Serine
siRNA	Short interfering RNA
T	Threonine
V	Valine
Y	Tyrosine

Figure 1. Schematic representation of the effects of CaMK inhibitors on cell cycle progression. A cell reproduces by an orderly sequence of events, called the cell cycle. The cell cycle is classified into two distinct phases: *Interphase* (Gap/Growth Phase 1 [G1], DNA replication [S], Gap/Growth Phase 2 [G2]) and *M phase* (prophase, prometaphase, metaphase, anaphase, telophase). Specific inhibitor studies have implicated Ca^{2+}/calmodulin-stimulated protein kinase I (CaMKI; green arrow) in regulating G1, and CaMKII (red arrows) in regulating the G2/M, metaphase-anaphase transition, and potentially the S phase of the cell cycle.

Figure 2. Schematic diagram of the domain structure of CaMKI and CaMKII. All of the Ca^{2+}/calmodulin stimulated protein kinases (CaMK), except CaMKII, have similar overall domain structures. CaMKI, CaMKII, CaMKIV, and CaMKK possess two domains: an N-terminal catalytic domain (red), and a regulatory domain (purple), which is comprised of an autoinhibitory and a calmodulin binding domain. CaMKII has an additional C-terminal association domain (blue). In each instance, phosphorylation sites are numbered according to the α isoform.

Table 1. Properties of the CaMK Family

	CaMKI	CaMKII	CaMKIV	CaMKK
Number of genes	4 (α, β, γ, δ)	4 (α, β, γ, δ)	1	2 (α, β)
Number of splice variants	>5	>30	2	Unknown
Enzyme structure	Monomeric	Multimer (dodecamer) of dimers	Monomeric	Monomeric
Molecular weight	38 – 53 kDa	500 – 600 kDa (holoenzyme); 50 – 60 kDa (subunit)	65 – 67 kDa	54 – 68 kDa
Tissue distribution	Ubiquitous	CaMKIIγ and CaMKIIδ are ubiquitous, CaMKIIα and CaMKIIβ are neuronal	Neuronal, immune cells, testis	Neuronal, thymus, spleen, testis
Requirement for activation	Ca^{2+}/CaM binding, phosphorylation	Ca^{2+}/CaM binding	Ca^{2+}/CaM binding, phosphorylation	Ca^{2+}/CaM binding
Regulation	Phosphorylated (activated) by CaMKK, targeting (?)	Autophosphorylation (autonomous and inhibitory), targeting	Phosphorylated (autonomous activation) by CaMKK, targeting (?)	Phosphorylation (inhibitory)
Number of phosphorylation sites	1 (T 174 – 180, depending on isoform)	Multiple	1 (T196 - 200, depending on isoform)	Multiple
Capable of autonomous activity?	No	Yes	Yes	Yes
Inhibition by KN-62 and KN-93 (K_I)	0.8μM [111, 112]	0.8μM [111, 112]	3μM [111, 112]	No effect
Inhibition by AIP (IC_{50})	Not determined	40nM [52]	>10μM [52]	Not determined
Inhibition by AIP-II	Not determined	4nM [61]	>10μM [61]	Not determined

(IC_{50})				
Inhibition by AC3-1 (IC_{50})	>10μM [62]	150nM [62]	Not determined	>10μM [62]

Table 2. Effects of CaMK Inhibitors on Cell Cycle Progression

Cell Cycle Phase	Inhibitor	Cell Type	Effect Observed	Ref
G1/S				
	KN-93 KN-92	HeLa (human cervical cancer)	KN-93 arrested cells in G1/S. KN-92 had no effect.	77
	KN-93 KN-92	NIH-3T3 (human fibroblasts)	KN-93 arrested cells in G1. KN-92 had no effect.	84
	KN-93 KN-92	NIH-3T3 (human fibroblasts)	KN-93 blocked cells in G1, decreased cyclin D expression, increased $p27^{kip1}$ associated with cdk2/cyclin E, decreased phosphorylation of pRb, and decreased activity of cdk2 and cdk4. KN-92 had no effect.	72
	KN-93 KN-92	WI-38 (human diploid fibroblasts)	KN-93 blocked cells in G1, and prevented cdk4 activation. KN-92 had no effect. Overexpression of kinase dead mutants of CaMKI or CaMKII showed effects due to CaMKI.	69
	KN-93 KN-92	MCF-7 (human breast cancer)	KN-93 blocked cells in G1, decreased cyclin D and pRb expression. KN-92 had no effect. Inhibition of CaMKI by siRNA produced similar effects.	78
	KN-93 KN-92	MG-63, 143B (human osteosarcoma)	KN-93 induced cell cycle arrest in G1, decreased pRB and E2F activation, and increased $p21^{CIPKIP}$. KN-92 had no effect.	87
	KN-93	Human endometrial cancer cell lines	KN-93 caused cells to accumulate in G1.	83
S				
	KN-62	K562 (human leukaemia)	KN-62 blocked cells in S phase.	71
	AIP KN-62	Small cell lung carcinoma cell lines, SK-N-SH (human neuroblastoma), K562 (human leukaemia)	KN-62 and AIP slowed progression through S phase.	86
G2 and M phases				
	Peptide (aa273-302)	Sea urchin eggs	The peptide corresponding to aa273-302 inhibited nuclear envelope breakdown following injection into sea urchin eggs.	94
	KN-93 KN-92 AC3-1 AC3-C	HeLa (human cervical cancer)	KN-93 or AC3-1 blocked cells in G2. KN-92 or AC3-C had no effect.	73
	KN-93 KN-92	HeLa S3 (human cervical cancer), ECV304 (human endothelial)	Lower concentrations (0.5 - 5μM) of KN-93 blocked cells transiently at metaphase. Higher concentrations (10 - 100μM) blocked cells permanently at metaphase. KN-92 had no effect.	74
	KN-93 AIP	Mouse oocytes	KN-93 and myr-AIP blocked the metaphase-anaphase transition. KN-92 had no effect.	82

KN-93 KN-92 AIP-II	Pig oocytes	KN-93 and AIP-II treatment prevented meiotic resumption, accumulation of cyclin B and phosphorylation of MAP and p90rsk. KN-92 had no effect.	66
siRNA-CaMKIIγ	K562 (human leukaemia)	Inhibiting CaMKIIγ disorganized multipolar spindles and caused a block in M.	67
siRNA-CaMKIIδ	Vascular smooth muscle cells	siRNA inhibition of CaMKIIδ caused cells to accumulate in G2 or M.	68
KN-93 KN-92	LX-2 (hepatic stellate cells)	KN-93 blocked cells in G2 or M. KN-92 had no effect.	80

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