Review

The many faces of calmodulin in cell proliferation, programmed cell death, autophagy, and cancer

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ABSTRACT

Calmodulin (CaM) is a ubiquitous Ca^{2+} receptor protein mediating a large number of signaling processes in all eukaryotic cells. CaM plays a central role in regulating a myriad of cellular functions via interaction with multiple target proteins. This review focuses on the action of CaM and CaM-dependent signaling systems in the control of vertebrate cell proliferation, programmed cell death and autophagy. The significance of CaM and interconnected CaM-regulated systems for the physiology of cancer cells including tumor stem cells, and processes required for tumor progression such as growth, tumor-associated angiogenesis and metastasis are highlighted. Furthermore, the potential targeting of CaM-dependent signaling processes for therapeutic use is discussed.

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1. Introduction

Calmodulin (CaM) is considered the major regulator of Ca^{2+}-dependent signaling in all eukaryotic cells. Its ubiquitous distribution, its high conservation during evolution, its broad spectrum of functions and the fact that it is essential in all so far tested organisms underlay the importance of this EF-hand type Ca^{2+}-binding protein in cell physiology. Since its discovery as a cyclic nucleotide phosphodiesterase (PDE) activator in the early '70 of the past century [1,2], a vast amount of data has been accumulated on its important function in the control of numerous physiological processes such as e.g. cell motility, cytoskeleton architecture and function, cell proliferation, apoptosis, autophagy, metabolic homeostasis, phospholipid turnover, protein folding, phosphorylation/dephosphorylation of proteins, ions transport, osmotic control, reproductive processes, muscle contraction and gene expression among others. Much has been learned during the recent years on the structure and functionality of CaM and its target proteins, the dynamics of Ca^{2+} binding and the different Ca^{2+}-dependent as well as Ca^{2+}-independent mechanisms of interaction with its multiple targets (reviewed in Refs. [3–8]). One reason for the unprecedented diversity of CaM functions is connected with its ability to interact with several hundred different target proteins and to modulate their activity in many different ways. In this review we will describe accumulated information and recent advances on the action of CaM and different CaM-dependent systems in the control of cell proliferation, programmed cell death and autophagy, with a particular focus on the implication of these mechanisms for the physiology of tumor cells.

The transient increase in the concentration of free Ca^{2+} in the cytosol and its spread to the nucleus upon cell activation by a broad range of stimuli including mitogenic factors and other agonists has been recognized as the principal event responsible for the initiation of many signal transduction processes. The transport systems that control the concentration of Ca^{2+} in the nucleus are of upmost importance for cell

proliferation, as Ca^{2+} at this location not only binds to nuclear CaM but also controls the activity of many transcription factors (reviewed in Refs. [9,10]). The study of the complex network of Ca^{2+}-binding proteins and their interactions, denoted as 'calciomics, try to understand the multiple effects of Ca^{2+} leading to changes in cell physiology (reviewed in Ref. [11]). Besides CaM other Ca^{2+}-binding proteins including e.g. calpains (reviewed in Ref. [12]), ALG-2 (apoptosis-linked gene 2 product) (reviewed in Ref. [13]), sorcin (reviewed in Ref. [14]), S-100 proteins (reviewed in Ref. [15]), oncomodulin [16,17] and annexins (reviewed in Ref. [18]) also play important roles in signal transduction leading to the control of cell proliferation as well as cell death. However, this topic lies outside the scope of this review. The activation of Ca^{2+}-binding proteins occurs by Ca^{2+} entry into the cytosol through the temporospatial opening of Ca^{2+} channels initiated by a variety of signaling molecules. They may be derived from the metabolism of phospholipids, such as inositol-1,4,5-trisphosphate (IP_3) resulting from the hydrolysis of phosphatidylinositol 4,5-bisphosphate by phospholipase $C\gamma$ ($PLC\gamma$) (reviewed in Refs. [19–24]), and leukotriene C_4 formed from arachidonic acid released by the action of phospholipase A_2 (PLA_2) on phospholipids (reviewed in Refs. [25–27]). Additional messengers implicated in Ca^{2+} mobilization are the nucleotides cyclic ADP-ribose (cADPR) and nicotinic acid adenine dinucleotide phosphate (NAADP) derived from NAD^+ and $NADP^+$, respectively. They activate ryanodine receptors located at the endo(sarco)plasmic reticulum and two-pore channels located at endo-lysosomes, respectively (reviewed in Refs. [28–31]). Another important Ca^{2+} mobilizing agent is ADP-ribose (ADPR), synthesized by the poly(ADP-ribose) polymerase 1/2-poly(ADP-ribose) glycohydrolase (PARP1/2-PARG) pathway. ADPR acts on transient receptor potential melastatin 2 (TRPM2) Ca^{2+} channels located at the plasma membrane and lysosomes regulating autophagy and apoptosis induced by oxidative stress [32] (reviewed in Ref. [33]).

The feedback control of the opening/closing states of the above mentioned channels by different mechanisms results in Ca^{2+} influxes of distinct intensity, spatial distribution and duration, either as single transients or as oscillatory phenomena. These events are orchestrated among other factors by the assembly of clustered channels in units of different sizes and by the counter activity of Ca^{2+} transport systems involved in the sequestration of Ca^{2+} into intracellular stores and efflux to the extracellular medium. Distinct pumps, denoted sarco(endo)plasmic reticulum Ca^{2+}-ATPase (SERCA) and secretory pathway Ca^{2+}-ATPase (SPCA) (reviewed in Refs. [34,35]) mediate Ca^{2+} uptake into the endoplasmic reticulum (ER) and the Golgi secretory pathway, respectively. Ca^{2+} sequestration into the mitochondria is mediated by an electrophoretic Ca^{2+} uniporter (mCU) and its release by $Na^+(H^+)/Ca^{2+}$ exchangers (reviewed in Refs. [36–40]). Removal of Ca^{2+} from the cell is under the control of a Na^+/Ca^{2+} exchanger (reviewed in Ref. [41]) and the CaM-dependent Ca^{2+}-ATPase located at the plasma membrane (reviewed in Refs. [35,42,43]). The entry of Ca^{2+} into the nucleoplasm, in addition to the nuclear pore pathway, is mostly mediated by IP_3 and ryanodine receptors located at the nuclear envelope-ER network [10,38,44–46]. Ca^{2+} re-uptake into this compartment is mediated by a SERCA [10,44,45] and a nuclear Na^+/Ca^{2+} exchanger regulated by ganglioside M1 (monosialotetrahexosylganglioside) (GM1) [47].

Many Ca^{2+} transport systems in the cell are directly regulated by CaM (Fig. 1), counting for example the plasma membrane Ca^{2+}-ATPase (reviewed in Refs. [35,42,43]), diverse Ca^{2+} channels such as IP_3 receptors [48], ryanodine receptors [49], Orai/CRAC (calcium release-activated calcium) channels [50,51] and its regulator stromal-interacting molecule 1 (STIM1) [52], voltage-gated Ca^{2+} channels (reviewed in Ref. [53]), TRPM2 [54] and other transient receptor potential (TRP) channels (reviewed in Ref. [55]), as well as gap junction channels and hemichannels (reviewed in Ref. [56]). Furthermore, the hyperpolarizing Ca^{2+}-dependent K^+ channels, that favor Ca^{2+} entry via hyperpolarization-sensitive channels, are also CaM-dependent (reviewed in Ref. [57]).

Intracellular Ca^{2+} signals can be propagated in tissues as intercellular Ca^{2+} waves by mechanisms involving the intercellular transport of calcium-mobilizing messengers, e.g. IP_3, across gap-junction channels, each formed by six connexin molecules. Some connexins are CaM-binding proteins, e.g. connexin-32 and connexin-43 [58,59]. Alternatively, the release of ATP to the extracellular fluid across connexin hemichannels and maxi-anion channels or by vesicular release may stimulate P_2 receptors in neighboring cells where additional Ca^{2+} signals are elicited (reviewed in Ref. [60]). Moreover, the open/closed states of connexin hemichannels appear to be controlled by both the extracellular and intracellular concentrations of Ca^{2+} [61], and Ca^{2+} can itself permeate the hemichannels as demonstrated for example with reconstituted connexin 26 in liposomes [62].

The increase in intracellular Ca^{2+} triggers the formation of the Ca^{2+}/CaM complex, as monitored in intact cells [63,64], followed by modulation of the functionality of the target proteins (Fig. 2). Saucermann and Bers [65] have proposed that there are two distinct pools of CaM in the cell: one, named 'dedicated', formed by CaM molecules already tethered to their targets (e.g. Ca^{2+} channels) ready to activate them upon Ca^{2+} availability; and the other minor pool, 'named promiscuous' formed by free CaM molecules in the cytosol, which may interact and regulate CaM-binding protein upon Ca^{2+} binding (Fig. 2). Frequently, the interaction of Ca^{2+}/CaM with their targets implicate wrapping the CaM-binding region of the target by the CaM molecule collapsed by bending around its central linker α-helix and bringing the N- and C-globular regions closer to each other, as for example with CaM-dependent kinase (CaMK)-I [66] (Fig. 3), CaMK-II [67] or calcineurin (CaN) also denoted protein phosphatase 2B (PP2B) [68]. CaMK-II, when activated by Ca^{2+} and CaM converts itself into a Ca^{2+}-independent enzyme, with function in many biological processes. Table 1 summarizes a selection of the most relevant CaM-binding proteins controlling the processes to be discussed in the different chapters of this review.

Of interest for the purpose of this review is the fact that in tumor and transformed cells the mobilization of Ca^{2+} is altered, which has important implications for tumor development and progression (reviewed in Refs. [69,70]). Also important in this context is the anomalous intracellular concentration of CaM and other Ca^{2+}-binding proteins in tumor cells [71,72].

As described in earlier reviews [73–79], an influx of extracellular Ca^{2+} into the cell upon growth factors stimulation is essential for initiating cell proliferation. The Ca^{2+}/CaM complex plays a transducing role in signaling events by directly or indirectly controlling mitogenic pathways and the cell cycle machinery, most significantly acting at the G_1/S transition and at the G_2 and M phases (discussed in detail in Section 3.6). This implicates multiple molecular mechanisms responsible for the progression of the cell cycle and other relevant processes required for mitogenesis. Processes such as the activation of cyclin-dependent kinase (Cdk)/cyclin complexes, nucleotide metabolism, DNA synthesis, chromosomal reorganization and cytokinesis are dependent on the presence of CaM, which acts to a major degree through distinct CaM-dependent protein kinases and CaM-dependent protein phosphatases, most prominently CaN.

Early observations identified Ca^{2+} overload as a noxious signal that can induce cell death. This may occur by various ways including necrosis and apoptosis (discussed in detail in Section 4). More recently a prominent functional role played by Ca^{2+} and Ca^{2+}-sensor proteins in autophagy has been identified, as for example [80] (reviewed in Ref. [81] and discussed in detail in Section 5). Ca^{2+}-dependent signal transducing mechanisms during apoptosis and autophagy involve different CaM-regulated systems with important contributions by mitochondria, the endoplasmic reticulum and lysosomes, all able to mobilize Ca^{2+} (reviewed in Refs. [82–85]).

Altered CaM-dependent cell cycle regulation and proliferation has been observed in many tumor cells, and targeting CaM and/or the CaM-dependent systems has been considered useful strategies for potential therapeutic intervention in cancer (discussed in detail in Section 6.5). Inhibition of CaM function or its targets with chemical antagonists or downregulating its expression using interfering RNA technology, alone or in combination with different chemotherapeutic agents, have recently been attempted, albeit with modest success; for
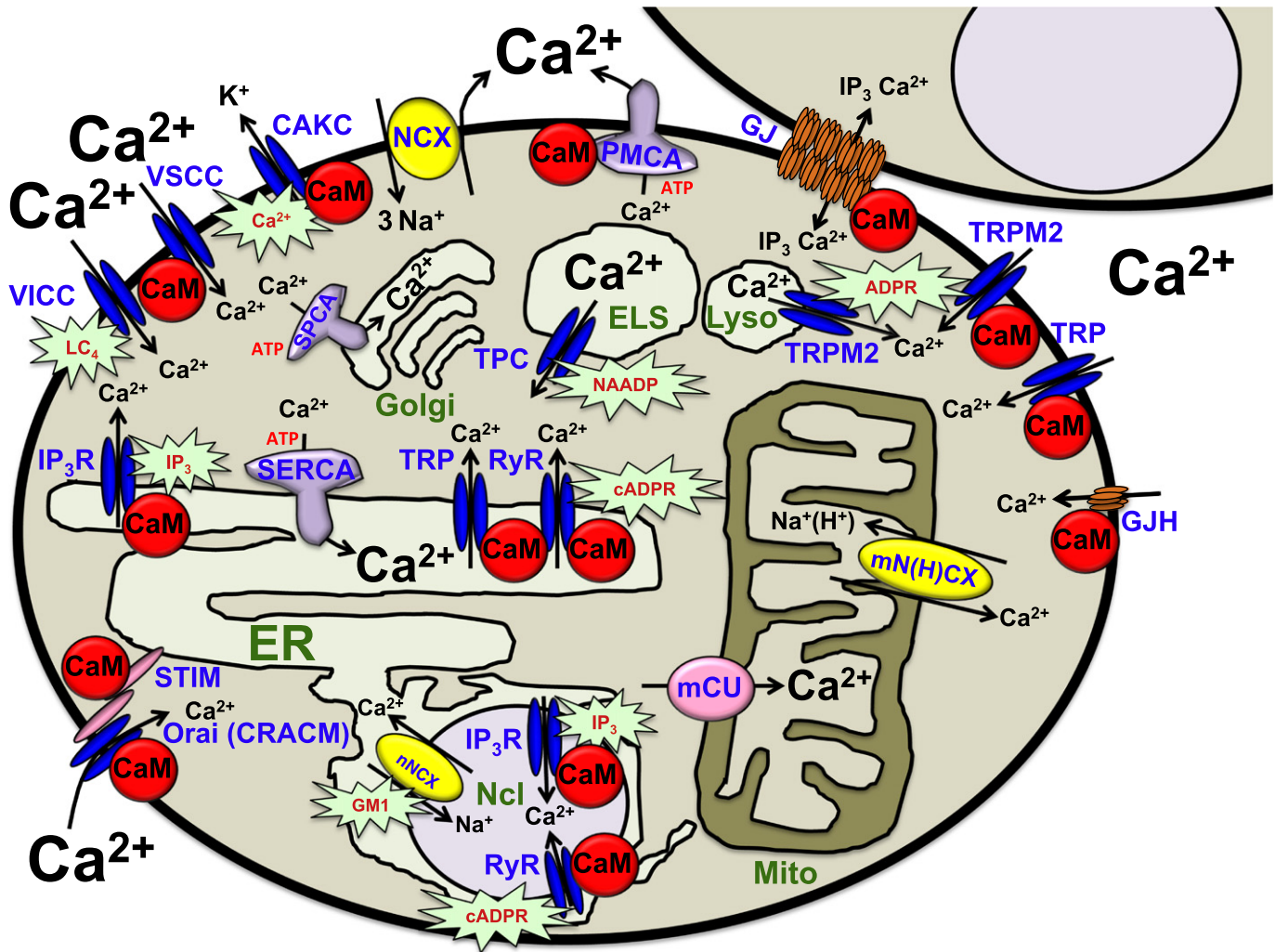
Fig. 1. The Ca^{2+} transport systems. The scheme depicts the major transport systems mobilizing Ca^{2+} at the plasma membrane, the endoplasmic reticulum (ER), mitochondria (Mito), the nucleus (Ncl), the Golgi, endolysosomes (ELS), and lysosomes (Lyso), highlighting the ones regulated by calmodulin (CaM). The effectors activating the different transport systems are also indicated. cADPR, cyclic ADP-ribose; CAKC, Ca^{2+}-activated K^+ channel; CaM, calmodulin; CRACM, calcium release-activated calcium channel molecule (Orai); GJ, gap junction; GJH, gap junction hemichannel; GM1, ganglioside M1 (monosialotetrahexosylganglioside); IP_3, inositol-1,4,5-trisphosphate; IP_3R, IP_3 receptor; LC_4, leukotriene C_4; mCU, mitochondrial Ca^{2+} uniport; mN(H)CX, mitochondrial Na^+/Ca^{2+} or H^+/Ca^{2+} exchangers; NAADP, nicotinic acid adenine dinucleotide phosphate; nNCX, nuclear Na^+/Ca^{2+} exchanger; NCX, Na^+/Ca^{2+} exchanger; PMCA, plasma membrane Ca^{2+}-ATPase; RyR, ryanodine receptor; SERCA, sarco(endo)plasmic reticulum Ca^{2+}-ATPase; SPCA, secretory pathway Ca^{2+}-ATPase; STIM, stromal-interacting molecule; TPC, two-pore channel; TRP, transient receptor potential; TRPM2, TRP melastatin 2; VICC, voltage-independent Ca^{2+} channel; VSCC, voltage-sensitive Ca^{2+} channel.

example, in a clinical trial with TFP combined with the DNA strand break inducer bleomycin [86] (reviewed in Ref. [87]).

The detailed elucidation of the CaM-dependent control of both cell proliferation and cell death during cancer development and progression is a major research goal. The complexity of CaM actions complicates the identification of particular CaM-dependent routes that could be both specific and effective targets in tumor cells for therapeutic purpose without greatly disturbing the physiology of normal cells. Understanding the molecular mechanisms underlying the CaM-dependent systems potentially involved in the enhanced proliferative capacity of tumor cells, the deficiencies in the onset of apoptosis upon DNA damage, the enhanced migration and invasiveness, and/or the angiogenesis associated with tumor growth, may lead to identifying new strategies for therapeutic intervention. In this review we will describe what is it known so far about the action of CaM and CaM-dependent systems in the control of cell proliferation, programmed cell death and autophagy of tumor and non-tumor cells.

2. Calmodulin and the proliferation of lower eukaryotic cells

Our understanding of the role of CaM in cell proliferation has been greatly aided by the important pioneer studies done in phylogenetically lower eukaryotic organisms, such as the baker's yeast *Saccharomyces cerevisiae*, the fission yeast *Schizosaccharomyces pombe* and the filamentous fungi *Aspergillus nidulans* among others (reviewed in Refs. [88–91]). Therefore, although this review will be concerned with the actions of CaM on the proliferation of vertebrate cells and their dismissal by programmed cell death and other mechanisms, it is pertinent to make some brief remarks highlighting the action of CaM on the proliferation of these microorganisms to emphasize evolutionary connections and similarities as well as differences of the implicated mechanisms.

The need for CaM during cell division was first demonstrated in a lethal mutant of *S. cerevisiae* where CaM expression was disrupted by knocking out its single gene or conditionally-repressing its expression upon galactose withdrawal [92,93]. Interestingly, expression of the N-terminal or the C-terminal halves of CaM in the CaM-null mutant was sufficient for rescuing cell viability, although in a temperature-dependent manner [94]. In addition, a temperature-sensitive *S. cerevisiae* mutant, with two point mutations in the CaM gene (I100D and E104V), when synchronized at the G_1 phase progressed through the cell cycle but retained very poor viability after the first mitosis. However, this mutant could form colonies when cultured at the permissive temperature, but lost viability when synchronized in G_2/M and shifted to the non-
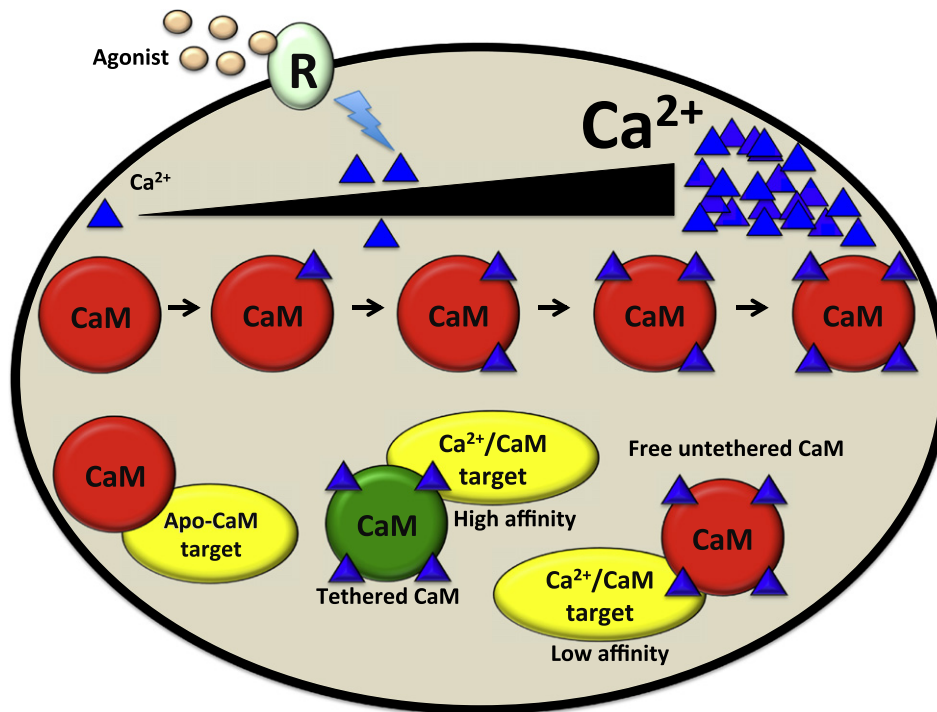
Fig. 2. Activation of calmodulin and target systems. The scheme depicts the sequential saturation of calmodulin (CaM) by increasing concentrations of cytosolic free Ca^{2+} (*blue triangles*) upon activation of a cell surface receptor (R) by an agonist; and the sequential activation of high and low affinity protein targets by the Ca^{2+}/CaM complex or the activation of tethered CaM and its target protein upon binding of Ca^{2+}. Activation of some target proteins by Ca^{2+}-free CaM (apo-CaM) is also shown.
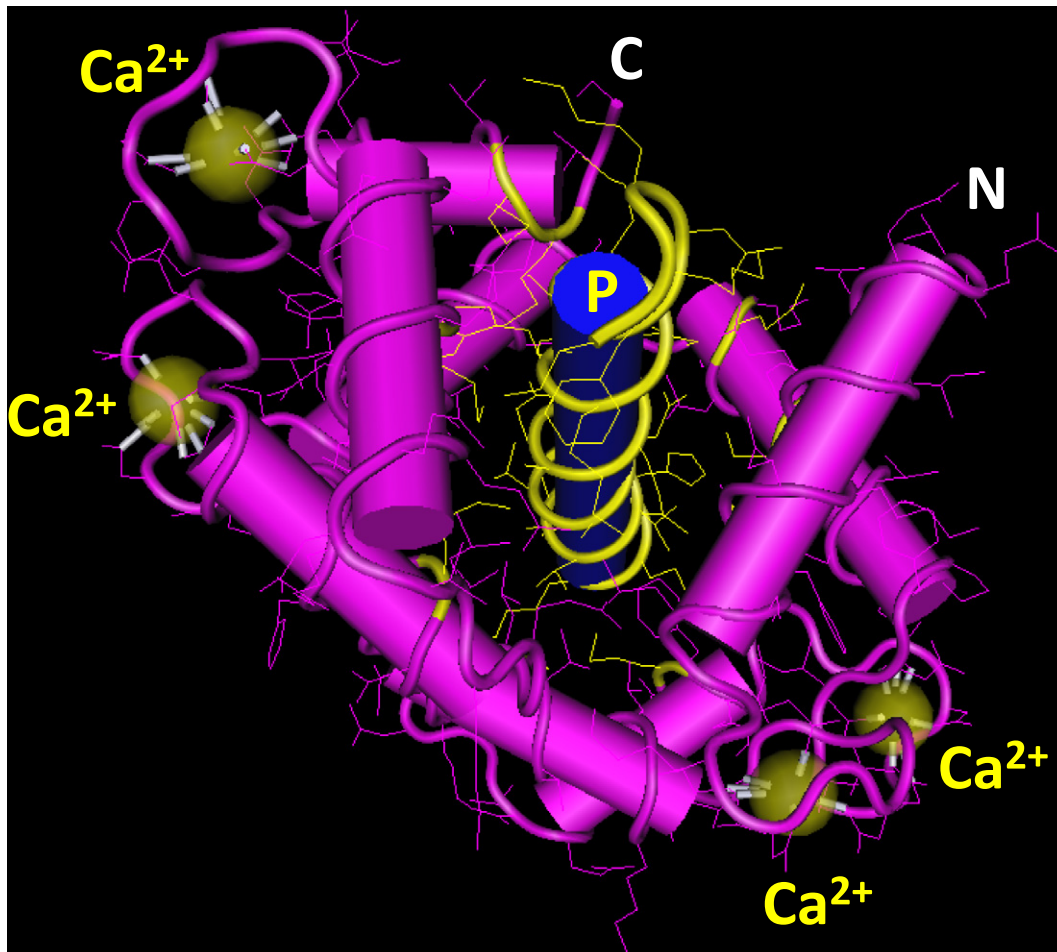
Fig. 3. Interaction of calmodulin with a CaMK-I peptide. Model of Ca^{2+}/CaM (pink) collapsed around a peptide (blue/yellow barrel labeled P) corresponding to the CaM-binding site of CaMK-I (MMDB ID: 21255 PDB ID: 1MXE). CaM methionine residues are labeled in yellow. The N- and C- termini of CaM and Ca^{2+} ions are indicated.

Table 1

Calmodulin-binding proteins involved in functions relevant in cell proliferation, apoptosis, autophagy and tumor progression.

CaM-binding protein	Major functions	References (on CaM binding)
14-3-3ε	Proliferation	[269]
Aurora A/B	Proliferation	[345,346]
AKAP12	Proliferation	[285][a]
	Cell migration (metastasis)	
Akt	Cell survival	[499]
	Autophagy	
ASPM	Proliferation	[364]
CaN	Proliferation	[243][a]
	Apoptosis	
	Autophagy	
CaMK-I	Proliferation	[227][a]
	Autophagy	
CaMK-II	Proliferation	[227][a]
	Apoptosis	
CaMK-III (eEF2K)	Proliferation	[227][a]
	Autophagy	
CaMK-IV	Proliferation	[227][a]
	Apoptosis	
	Autophagy	
CaMKK	Proliferation	[220][a]
	Apoptosis	
	Autophagy	
CaMKLK	Apoptosis	[391]
CAMTA1/2	Proliferation	[289][a]
C/EBP-β	Proliferation	[291]
	Autophagy	
Cdk5	Proliferation	[310]
c-FLIP	Apoptosis	[450]
Connexins (32 and 43)	Intercellular communication	[58,59]
Cyclin E1	Proliferation	[312]
DAPK1/2	Apoptosis	[373][a]
	Autophagy	
DRP-1	Apoptosis	[373][a]
	Autophagy	
EGFR (ErbB1)	Proliferation	[174]
	Differentiation	[175][a]
	Cell migration	
	Cell survival	
ErbB2	Proliferation	[176]
	Differentiation	
	Cell migration (metastasis)	
	Cell survival	
	Autophagy	
FADD	Proliferation	[286]
	Apoptosis	
	Autophagy	
Fas (CD95)	Apoptosis	[443]
FKBP38	Apoptosis	[410]
	Autophagy (?)	
gp160 (HIV envelope protein)	Apoptosis	[436]
Grb7	Cell migration (metastasis)	[279]
	Cell adhesion	
	Proliferation	
	Angiogenesis (tumor-associated)	
HRPAP20	Proliferation	[552]
	Apoptosis	
	Tumor cells invasion	
Hsp70	Proliferation	[325]
	Apoptosis	
	Autophagy	
Hsp90	Proliferation	[322]
InsR	Metabolism	[179]
	Proliferation	
IQGAP1	Proliferation	[282]
	Cell adhesion (metastasis)	
IRS1/2	Metabolism	[630]
	Proliferation	

Table 1 (continued)

CaM-binding protein	Major functions	References (on CaM binding)
Kir/Gem	Proliferation	[199]
	Cytoskeletal organization (metastasis)	
K-Ras B	Proliferation	[193]
p21[Cip1/Waf1]	Proliferation	[315]
p35 (Cdk5 neural activator)	Proliferation	[311]
p68 RNA helicase	EMT	[555]
	Proliferation	
	Cell migration (metastasis)	
Cyclic nucleotides PDE	Proliferation	[1,2]
PI₃K	Cell survival	[501]
PI₃K-III (VPS34)	Autophagy	[631]
Plk1	Proliferation	[338]
Src (v- and c-)	Proliferation	[250,251]
	Cell migration	
TrkA	Proliferation	[178]
	Cell survival	
	Differentiation	

AKAP12, A-kinase anchor protein 12; ASPM, abnormal spindle-like microcephaly-associated protein; CaN, calcineurin; CaMK-I/II/III/IV, CaM-dependent kinases I/II/III/IV; CaMKK, CaM-dependent kinase kinase; CaMKLK, CaM-dependent protein kinase-like kinase; Cdk5, cyclin-dependent kinase 5; c-FLIP, cellular FLICE-like inhibitory protein; DAPK1/2, death-associated protein kinases 1/2; DRP-1, DAPK-related protein kinase 1; EGFR (ErbB1), epidermal growth factor receptor; EMT, epithelial/mesenchymal transition; ErbB2, erythroblastic leukemia viral oncogene homologue 2; FADD, Fas-associated protein with death domain; Fas, TNF receptor superfamily member 6; FKBP38, FK506-binding protein 38; Grb7, growth factor receptor bound protein 7; HIV, human immunodeficiency virus; HRPAP20, hormone-regulated proliferation-associated protein 20; Hsp70, heat shock protein 70; Hsp90, heat shock protein 90; InsR, insulin receptor; IRS1/2, insulin receptor substrates 1/2; IQGAP1, IQ-containing Ras GTPase-activating-like protein 1; PDE, phosphodiesterase; PI₃K, phosphatidylinositol 3-kinase; PI₃K-III, phosphatidylinositol 3-kinase type III; TrkA, nerve growth factor receptor A.

[a] Review article.

permissive temperature highlighting the important role of CaM for cell cycle progression [95].

The conditional repression of the single CaM gene in the yeast *S. pombe* [96] and in a genetically manipulated strain of *A. nidulans* [97], or the insertional disruption of the CaM gene in the human pathogenic fungus *Cryptococcus neoformans* [98], blocked the proliferation of these organisms, and arrested the cell cycle at the G_2 phase in the case of *A. nidulans* [97]. Moreover, even though Ca^{2+} binding to CaM is not necessary for the growth of *S. cerevisiae* [99], mutations which block Ca^{2+} binding to CaM lead to the loss of viability when proliferation was arrested by the mating pheromone α-factor [100]. The spindle pole protein Nuf1p/Spc110p of *S. cervisiae* was demonstrated to be an essential target for CaM during mitosis, even when all the Ca^{2+}-binding sites were inactivated [101]. The CaM of *S. cerevisiae* has only three functional Ca^{2+}-binding motifs because site IV is unable to bind Ca^{2+} [102–105]. Its heterologous expression in *S. pombe*, endogenously expressing CaM with four functional Ca^{2+}-binding sites, did not support growth in contrast to the expression of vertebrate CaM in *S. pombe* [106]. This indicates a major functional difference between *S. cerevisiae* and *S. pombe* CaMs.

In *S. cerevisiae*, CaM concentrates at regions of cell growth, as for example the tip developed upon treatment with the α-factor. It is associated to actin filaments during progression of the cell cycle in a Ca^{2+}-independent manner, as similar localization was attained expressing a null Ca^{2+}-binding CaM mutant [107]. Furthermore, Ca^{2+}-independent binding of CaM to the IQ motif of myosin Myo2p was also found at the sites of polarized cell growth [108].

The implication of the CaM target calcineurin (CaN) in the growth of *S. cerevisiae* is contentious. Disruption of the genes encoding the catalytic subunit of the two isoforms of this phosphatase does not affect cell viability under normal growth conditions [109,110]. On the other hand, disrupting the regulatory subunit of CaN enhances the sensitivity

towards the mating α-factor leading to a cell cycle arrest at the G_2/M boundary [100] (reviewed in Ref. [111]). CaN controls as well the degradation of the transcription factor Yap1p, a suppressor of Ca^{2+} sensitivity in *S. cervisiae*, to ensure a delay of the cell cycle at G_2 when this organism is exposed to high concentrations of Ca^{2+} [112].

CaM kinases (CaMKs) positively contribute to cell survival when *S. cerevisiae* is in a non-proliferating state [100]. In *A. nidulans* CaMK is required for cell proliferation, particularly for the G_2/M transition [113]. However, the expression of a constitutively active form of this kinase induces the premature activation of the fungus-equivalent of the vertebrate Cdk1(cdc2)/cyclin B complex when the cells are still in G_0/G_1 and hence prevents entry into the S phase and further cell cycling [114]. CaM may participate in other growth-related processes in lower eukaryotes, as for example in the control of the number of nuclei in the slime mold *Dictyostelium* upon binding to nucleomorphin during mitosis [115,116] (reviewed in Ref. [117]).

The combined observations in yeast/fungi briefly described above underscore the fact that CaM plays a central role in growth regulation of lower eukaryotic organisms and that Ca^{2+}/CaM-dependent protein phosphorylation/dephosphorylation mechanisms are central for regulating cell proliferation.

3. Calmodulin and the proliferation of vertebrate cells

3.1. Calcium is required for cell proliferation

A variety of mitogenic factors and other effectors, including some hormones, stimulate vertebrate cell growth upon activation of their respective receptors by inducing an early and transient increase of the intracellular concentration of free Ca^{2+} [118,119]. The activation of different Ca^{2+} channels located at the plasma membrane is essential for the correct regulation of cell proliferation. Alterations of external Ca^{2+} influx has been observed in transformed and cancerous cells (reviewed in Refs. [120,121]). Store-operated Ca^{2+} entry is of vital importance for cell proliferation. Inhibition of cell cycle progression and cell growth has been demonstrated by silencing the expression of players implicated in the regulation of Ca^{2+} entry, such as the calcium sensor STIM1 and the Ca^{2+} channels Orai1 or TRP canonical 6 (TRPC6) in a variety of tumor cells including human hepatomas and cervical carcinomas [122–124]. In contrast, silencing STIM1 and Orai1 in human breast adenocarcinoma MDA-MB-231 cells did not affect their proliferation rate [125], suggesting that Ca^{2+} mobilization from other plasma membrane channels or from intracellular stores may compensate in these cells, although silencing these systems affected cell migration and the metastasic potential of a variety of tumor cells [123–125].

Ca^{2+} regulates important processes implicated in cell proliferation by the formation of a complex with CaM and other Ca^{2+}-binding proteins, targeting proteins that control cell proliferation. Some high-affinity CaM targets are readily activated at low Ca^{2+} concentrations, as they require low levels of Ca^{2+}, while the low-affinity ones are only activated at higher Ca^{2+} levels, solely attained in specific subcellular regions or during restricted time periods when global Ca^{2+} concentrations reach higher levels [64] (Fig. 2). Many transport systems, responsible for balancing the cytosolic free Ca^{2+} concentration, are under feedback control by the Ca^{2+}/CaM complex (Fig. 1). The most prominent systems regulated in this manner are the plasma membrane Ca^{2+}-ATPase (PMCA) (reviewed in Refs. [42,43]), a large number of plasma membrane Ca^{2+} channels (reviewed in Ref. [126]) and some family members of the TRP channels located at the endo(sarco)plasmic reticulum and other intracellular membranes as well as the plasma membrane (reviewed in Refs. [33,55]). Of relevance for cell proliferation, a Ca^{2+}/CaM-regulated feedback control of K^+ exit has been demonstrated, as blocking Ca^{2+}/CaM-activated K^+ channels inhibited T cells proliferation. The resulting hyperpolarization facilitated further Ca^{2+} entry through a membrane potential-driven mechanism [127].

3.2. Calmodulin is required for cell proliferation

Early reports described that in proliferation-arrested rat liver cells, which were shifted from a medium containing low to a high Ca^{2+} concentration, DNA synthesis was initiated and that this process was inhibited by CaM antagonists and an anti-CaM antibody. The effect was reverted upon addition of exogenous CaM [128,129]. This early observation underscores the positive role of CaM facilitating entry in the S phase of the cell cycle; but most intriguingly the observed effects of the CaM-antibody and exogenous CaM hinted to the possibility of an extracellular action of CaM. The signaling function of extracellular CaM in plant cells controlling among other functions cell proliferation has been well documented, for example in [130]. In *Dictyostelium*, extracellular CaM binds to a specific CaM-binding protein located within the slime sheath and negatively regulates cell proliferation [131]. The presence of CaM in different human body fluids including serum, most likely derived from platelets at least in part, has been reported [132], and extracellular CaM appeared to stimulate DNA synthesis in murine melanoma cells [133] and human leukemia cells [134,135]. In addition, the presence of a CaM-like activity was suggested to stimulate the proliferation of human umbilical vein endothelial cells by an autocrine mechanism [136]. However, these results are highly controversial, as given the acidic nature of CaM, the artefactual interaction of this protein with the ligand-binding pocket of mitogenic receptors or other non-relevant targets at the plasma membrane and/or CaM internalization via pinocytosis cannot be excluded. Therefore, these uncertainties cast serious doubts on the physiological role if any of extracellular CaM in animal tissues.

Viral vector-driven overexpression of recombinant CaM in non-transformed mammalian cells resulted in enhanced cell proliferation by shortening the length of the G_1 phase of the cell cycle and mitosis, while downregulation of CaM with antisense CaM RNAs arrested cell cycling and proliferation in mouse epithelial non-transformed C127 cells derived from a mouse breast tumor [75,137–139]. Such experiments are particularly difficult to realize with high efficiency in human and other mammalian cells, as three different CaM genes code for a single highly conserved protein sequence [140]. Nevertheless, the role of CaM in tumor cell proliferation was ascertained by using antisense RNA technology targeting the transcripts corresponding to the three CaM genes. CaM downregulation inhibited the proliferation of rat pheochromocytoma PC12 cells [141,142] and rat glioblastoma C6 cells [143]. While overexpression of CaM has a positive effect on the former cells [141] this is not the case in the latter cell type [143], perhaps reflecting the excess of CaM in C6 cells.

In human T lymphocytes [144], several human lymphoblastoid cell lines [145], and human teratoma cells [146], the mRNA level of *CALM3* was higher than those of *CALM1* and *CALM2* during cell proliferation. However, the transcripts of *CALM1* and *CALM2* appear to be more relevant for the proliferation of rat pheochromocytoma PC12 cells than the transcript of *CALM3*, as antisense-based suppression of the latter had no significant effect on the overall level of CaM expression [142]. This may suggest a differential expression of the three CaM genes among species and/or cell types. Knocking out the CaM gene II in the chicken bursa of Fabricius B lymphocyte neoplastic DT40 cell line that reduced the expression of CaM by 60% only slightly decreased cell proliferation but without altering the cell cycle profile [147] indicating that the ubiquitously expressed chicken CaM I gene is sufficient for DT40 cells survival [148]. Recently, the essential role of CaM to sustain vertebrate cell viability and proliferation was demonstrated using conditional CaM-knockout DT40 cells [149]. In addition, this cellular system allowed the direct testing of the role of Ca^{2+} binding to CaM for vertebrate cell proliferation by the expression of CaM mutants with impaired capacity to bind Ca^{2+} to one or more of its four Ca^{2+}-binding sites after suppressing endogenous wild type CaM expression. Single Ca^{2+} binding site inactivation had no measurable effect on cell proliferation

whereas mutating two Ca^{2+} binding sites decreased cell viability most dramatically when sites III and IV were inactivated [149].

3.3. Calmodulin levels during cell proliferation

During exponential growth of Chinese hamster ovary (CHO)-K1 cells the concentration of CaM was shown to progressively increase due to the enhanced transcription of the CaM genes, and reached high levels at the G_1/S transition. The CaM level remained high during the ensuing progression of the cell cycle and reached a maximum at the late G_2 or early M phases, decreasing thereafter when cells abandon the plateau phase [150–152] (Fig. 4). A similar phenomenon was evident in a hepatoma cell line *in vitro* and in the derived tumors developed *in vivo* [153], in promyelocytic leukemia cells [154], and in chemically transformed fibroblasts [155]. Moreover, CaM levels and the corresponding mRNAs also increased before cell replication during the regeneration of rat liver after partial hepatectomy [156]. Protein kinase C (PKC) appears to regulate the expression of CaM during growth of normal rat kidney cells [157].

The positive action of CaM on cell proliferation was demonstrated *in vivo* by the targeted overexpression of CaM in the heart of transgenic mice. CaM induced enhanced proliferation of cardiomyocytes with the occurrence of a high number of polyploid cells, and the subsequent development of ventricular hypertrophy, a phenomenon that was reversed by turning off the ectopic expression of CaM [158,159]. In this context, tumor necrosis factor-α (TNF-α) has been shown to induce hypertrophy in neonatal cardiomyocytes by a mechanism implicating both CaMK-II and CaN [160], possibly explaining at least in part the mechanistic action of CaM in this process, which is further described in Section 4. The changes in CaM levels during the different phases of the cell cycle appear to be evolutionarily conserved as it also occurs in protozoa, see for example [161].

In agreement with the above described observations, the action of CaM antagonists in non-tumor cells, such as the CHO-K1 cells [151,162], or in tumorigenic cells, such as avian sarcoma virus (ASV)-transformed kidney cells [163], melanoma B16 cells [164] or human breast cancer MCF-7 cells [165], is due to an arrest of the cell cycle at the G_1 phase, indicative of the prominent role that CaM plays at the G_1/S transition. However, when the CaM antagonist was added much later, during the S phase, the cell cycle was arrested at the late G_2 or early M phases [150], further pointing to the involvement of CaM in later phases of the cell cycle (Fig. 4).

3.4. Subcellular localization of calmodulin during cell proliferation

Not surprisingly, due to its multiple functions CaM is normally located in many cellular compartments including the cytosol, attached to membranes, inside the nucleus, and in different organelles, as demonstrated by sub-cellular fractionation and by expressing fluorescently-labeled CaM species in cultured living cells. Early studies performed in mammalian cells indicated a dynamic localization of CaM during cell division, moving from the cytosol to the nucleus in preparation for cell division, and attaching to the centrioles and the mitotic spindle during mitosis [166–168]. In sea urchin, it was demonstrated that CaM was progressively accumulated inside the nucleus when cells approach mitosis and this nuclear localization could be blocked by a cell-permeable CaM inhibitory peptide [169]. Similarly, in HeLa cells CaM was shown to be mostly concentrated in the cytosol during the G_1 phase and to move to the nucleus when cells entered the S phase, reaching high levels at the G_2 phase, and thereafter during mitosis concentrate at the polar regions of the mitotic spindle [170,171]. CaM was also co-localized with the astral microtubules during inter-phase, as observed in sea urchin zygotes when approaching mitosis [169]. Furthermore, CaM participated in the lysis of the nuclear membrane, distributing thereafter to the polar region of the mitotic spindle, and was shown to be involved in chromatin condensation both in sea urchin and vertebrate cells [169–171]. The targeted localization of CaM to different subcellular sites during mitosis has a dual nature, as Ca^{2+}-dependent and Ca^{2+}-independent mechanisms are operative [169]. In addition, targeted inhibition of nuclear CaM by a peptide derived from the CaM-binding domain (CaM-BD) of myosin light chain kinase (MLCK) totally prevented the entry in S phase of COS-7 cells [172]. As an example, Fig. 5 shows the dynamic distribution of green fluorescent protein (GFP)-CaM at different structures during mitosis,
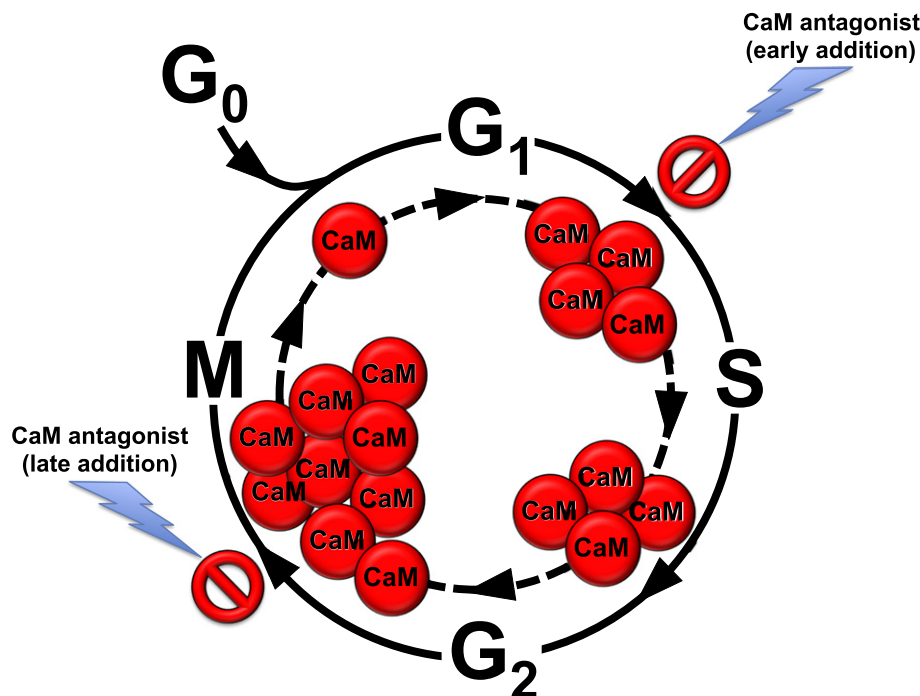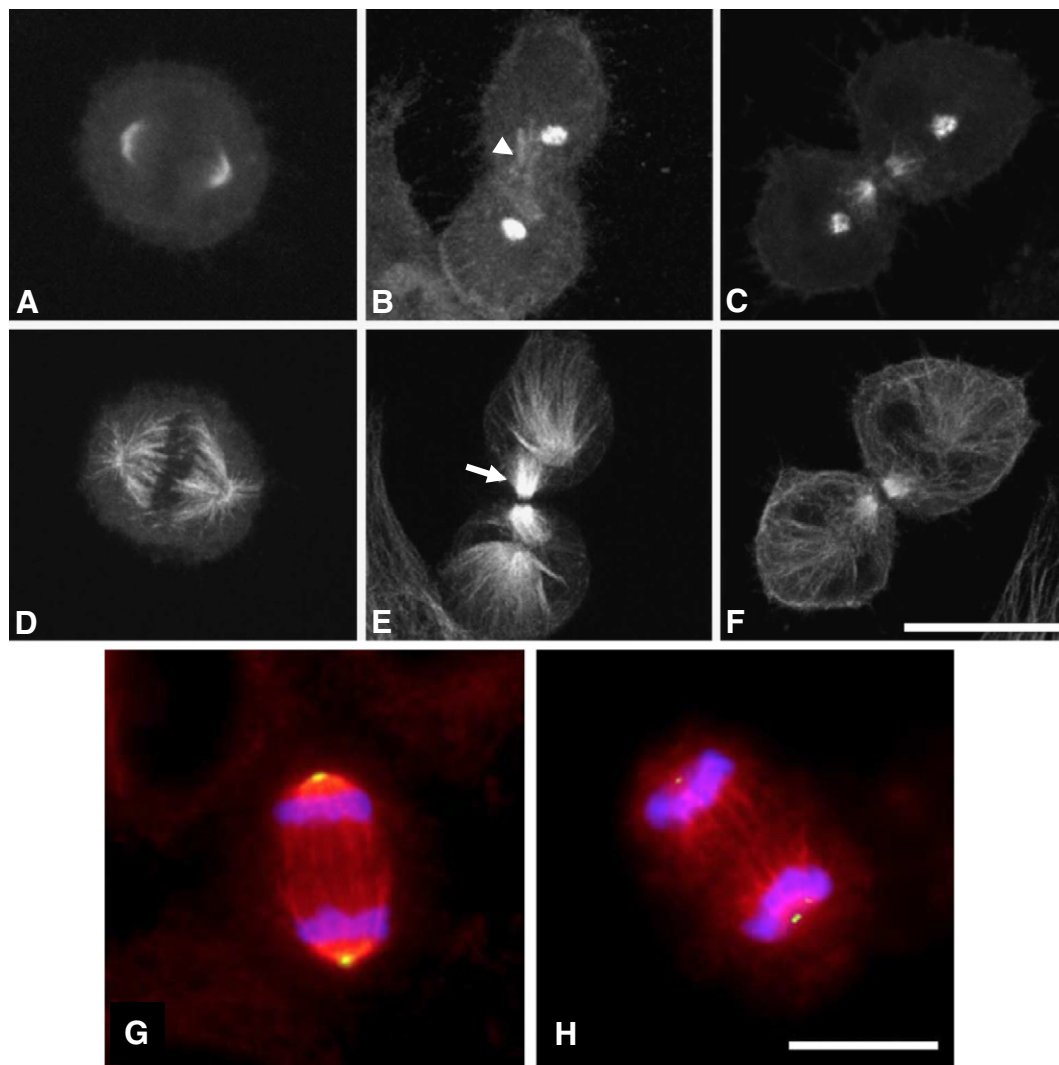

Fig. 4. Calmodulin levels during cell cycle progression. The scheme depicts the increment of calmodulin (CaM) expression at the different phases of the cell cycle; and the two major points of cell cycle arrest by CaM antagonists added at the indicated phases.

Fig. 5. Dynamic distribution of calmodulin during mitosis and the effect of W-7 in HeLa cells. (*Top*) The spatial-temporal distribution of CaM and microtubules in the identical cell during metaphase (A and D) anaphase (B and E) and telophase (C and F). The distribution pattern of the GFP-CaM fusion protein was monitored (A–C), and microtubules were immunostained in the fixed cells (D–F). The central spindle was assembled after metaphase/anaphase transition (arrow), while fiber-like distributed CaM was found in central spindle regions (arrowhead). Bar: 10 μm. (*Bottom*) Comparison of the central spindle microtubules of W-7 treated and untreated cells. The quantity of the central spindle microtubules and poles in W-7 treated cells was decreased (H) compared to the normal cells with their well-organized central spindle microtubules (G). Bar: 10 μm.
Reproduced with minor changes from Ref. [347].

and the effect of a CaM antagonist on the microtubular organization of the central spindle and poles.

3.5. Calmodulin targets involved in cell proliferation

3.5.1. Calmodulin-regulated growth factor receptors and signaling pathways

Proliferation signals are often initiated upon the ligand-dependent activation of receptors located at the cell surface. Several tyrosine kinase receptors are known to be CaM-binding proteins including the epidermal growth factor receptor (EGFR) [173–175], the related erythroblastic leukemia viral oncogene homologue 2 product (ErbB2) [176,177], the nerve growth factor receptor A (TrkA) [178], and the insulin receptor [179], all playing prominent roles in cell proliferation (Fig. 6).

It has been demonstrated that the Ca^{2+}/CaM complex binds to the cytosolic juxtamembrane region of the EGFR modulating its tyrosine kinase activity (reviewed in Ref. [175]). The functional importance of the cytosolic juxtamembrane region of the EGFR, containing the CaM-BD, during the ligand-dependent activation of the receptor following an asymmetric allosteric model has been corroborated [180–182]. Recently, by using conditional CaM-knockout DT40 cells it was demonstrated that CaM participates in the ligand-dependent activation of the

EGFR [183]. In addition, CaMK-II phosphorylates Ser1046/Ser1047 in the EGFR downregulating its tyrosine kinase activity and its internalization [184]. Interestingly, it has been shown that a peptide comprising the CaM-BD of the EGFR (Arg645–Arg662), conjugated to a human immunodeficiency virus (HIV) transactivator of transcription (Tat) sequence (HIV Tat 49–58) used to facilitate its cellular entry, inhibited the EGFR and exhibited anti-cancer activity when tested in a variety of tumor cells [185]. Similarly, a modified EGFR CaM-BD peptide (Arg651-Leu658) in which Thr654 was replaced by a cysteine to introduce PKC inhibitory properties [186], tagged with the hydrophobic signal sequence of the Kaposi fibroblast growth factor (FGF) to allow cell entry, also inhibited the EGFR and arrested cell proliferation [187]. Ca^{2+}/CaM and apo-CaM both bind to the ErbB2 receptor at nearby sites in the cytosolic juxtamembrane segment modulating receptor activity and signaling [176,177]. The high-affinity CaM-BD present in EGFR/ErbB1, ErbB2, and ErbB4 is not well conserved in ErbB3, which lacks intrinsic tyrosine kinase activity and exhibits lower CaM affinity [176,188,189].

The regulatory role of CaM on EGFR also includes its action on the transactivation of the receptor mediated by PKC [190], the intracellular traffic of the ligand-bound receptor once it is internalized [191,192], and
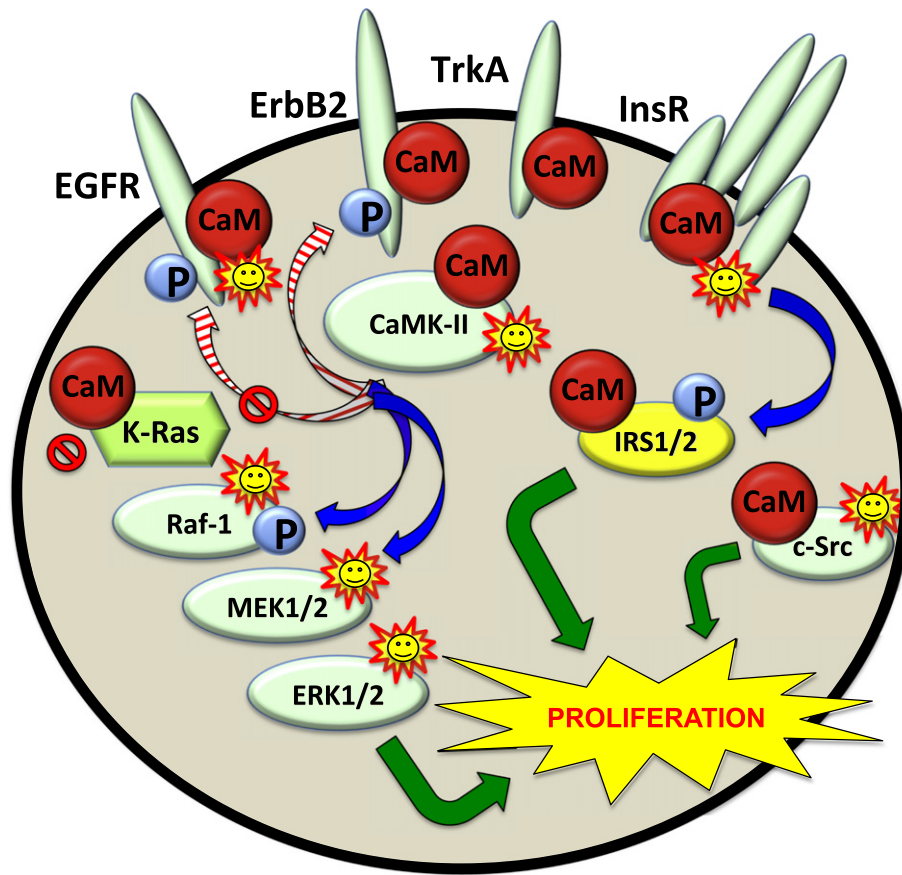
Fig. 6. Action of calmodulin on tyrosine kinase receptors and some signaling pathways implicated in cell proliferation. The scheme depicts the binding of calmodulin (CaM) to the following tyrosine kinase receptors: epidermal growth factor receptor (EGFR)/ErbB1, its related family member ErbB2, the nerve growth factor receptor A (TrkA), and the insulin receptor (InsR). The binding and activation of the non-receptor tyrosine kinase c-Src by CaM, and the binding and inhibitory action of CaM on K-Ras are also shown. The scheme also shows the stimulatory (*blue arrows*) and inhibitory (*red striped arrows*) actions of phosphorylation of diverse target systems by CaM-dependent protein kinase II (CaMK-II), and the eventual generation of cell proliferation signals (*green arrows*). ERK1/2, extracellular regulated kinases 1/2; IRS1/2, insulin receptor substrates 1/2; MEK1/2, mitogen-activated protein kinase kinases 1/2.

the modulation of downstream signaling pathways. The latter includes CaM binding to and down-regulation of K-Ras, but not of H-Ras or N-Ras, preventing its phosphorylation by PKC [193–195], and the modulation of the mitogen-activated protein kinase (MAPK) pathway at different points including the kinase Raf-1 (reviewed in Ref. [196]). Other Ras-related GTP-binding proteins, which are under the control of CaM are Kir and Gem. The latter protein has been shown to be involved in growth regulation, as its downregulation prevented the proliferation of mouse 3T3 fibroblasts [197]. A peptide corresponding to the CaM binding domain of Kir/Gem was found to bind CaM with high affinity, and the full length Kir/Gem proteins bind CaM in a Ca^{2+}-dependent fashion as analyzed by a newly developed protein overlay technique (Far-Western blotting) to visualize CaM-binding proteins [198]. In addition, CaM binding to Kir/Gem inhibited its GTP-binding activity indicating a direct regulatory function of CaM [199].

The CaMKK/CaMK-I system regulates the MAPK/extracellular regulated kinase (ERK) pathway stimulating cell proliferation; see for example [200] (reviewed in Ref. [201]). CaMK-II can be activated by different mitogenic factors, e.g. platelet-derived growth factor (PDGF) [202] or tumor/transforming growth factor-β (TGF-β) [203] which induces the proliferation of hepatic stellate cells among other cell types [204–207]. In vascular smooth muscle cells it was demonstrated that CaMK-II associates with ERK facilitating the phosphorylation of both kinases via Raf-1 and the nuclear localization of CaMK-II [207]. Mechanistically, it has been shown that CaMK-II negatively regulates the levels of p21$^{Cip1/Waf1}$ in lymphoblasts via the MAPK(ERK1/2) pathway by inducing the phosphorylation of the transcription factor Forkhead box O3a (Foxo3a). This is

followed by the exit of CaMK-II from the nucleus to the cytosol and its eventual degradation by a murine double minute protein 2 (MDM2)-mediated pathway, processes that appear to be defective in lymphoblasts from Alzheimer's disease patients diminishing entry in apoptosis (see also Section 4) [208]. Moreover, the interaction of CaM with the putative guanine exchange factor β-PIX (β-p21-activated kinase interacting exchange factor), and its association with the E3 ubiquitin ligase c-Cbl (Casitas B-cell lymphoma protein), might also regulate the degradation of ErbB receptors as well as other tyrosine kinase receptors [209].

Both CaM and Janus kinase 2 (Jak2) control the activation of the Na$^+$/H$^+$ exchanger (NHE) by the EGFR and other mitogenic receptors through a mechanism involving the phosphorylation of CaM at tyrosine residues [210,211]. The binding of CaM to NHE has been ascertained, as the crystallographic structure of its regulatory region in complex with Ca^{2+}/CaM was recently determined [212]. Interestingly, the high proliferation rate of lymphoblasts isolated from Alzheimer's disease patients, as compared to those of healthy donors, appears to be mediated by the CaM-dependent activation of NHE leading to intracellular alkalinization [213]. This might also be relevant for cancer biology, as NHE activation has been shown to play a significant role in supporting proliferation of tumor cells, e.g. bladder carcinoma cells [214].

Overall, these observations demonstrate that the regulatory role of CaM in controlling cell proliferation involves the regulation of key mitogenic receptors including the ErbB family and their downstream signaling pathways (reviewed in Ref. [175]), as well as cytosolic membrane-attached tyrosine kinases such as Src (Fig. 6). This is highly relevant for tumor cell biology, as these receptors along with non-receptor

tyrosine kinases play a key role in the development of many solid tumors either due to gene duplication, enhanced expression, the appearance of point mutations, and/or constitutively active truncated forms. Nevertheless, no mutations affecting the CaM-BD of the EGFR in human astrocytic gliomas (89 cases), where altered expression and high rate of mutations of this receptor are very common, were found so far, which highlights the functional importance of this site [215,216]. The CaM-like domain (CaM-LD) of the EGFR is an acidic region that has been suggested to interact with the CaM-BD as it has a sequence resembling CaM (reviewed in Refs. [175,188]). The CaM-LD is located in the CAIN domain, distal of the tyrosine kinase catalytic site, which is presumably involved in Ca^{2+} mobilization and receptor internalization [217]. In-frame tandem duplication of exons 18–25 of the EGFR in human gliomas has been shown to result in an aberrant receptor with two CaM-LDs [215,216].

3.5.2. Calmodulin-dependent kinases and phosphatases

Many mechanisms of CaM action in cell proliferation and the control of the cell cycle are mediated by CaM-dependent phosphorylation/ dephosphorylation events (reviewed in Refs. [78,218–220]). The best-studied kinases involved in these processes are the multifunctional CaM-dependent protein kinases (CaMKs)-I/II/IV (reviewed in Refs. [221–224]); the distinct and more substrate-restricted CaMK-III [225]; and the upstream CaM-dependent kinase kinase (CaMKK) that activate CaMK-I/IV (reviewed in Refs. [220,226,227]). The roles of these CaMKs and other relevant CaM-regulated kinases in the control of cell proliferation are presented in Table 2.

The pharmacological inhibition of CaM-dependent kinases [206,228–231], or their downregulation by small interfering RNA (siRNA) [232,233], results in the inhibition of proliferation of different cell types, particularly arresting the cell cycle at the G_1/S transition [228,229], or at the G_2/M transition increasing in this manner polyploidy [232]. In contrast, the expression of a constitutively active Ca^{2+}-independent truncated-variant of CaMK-II in murine C127 cells arrests the cell cycle at the G_2 phase [234]. The Ca^{2+}/CaM-dependent kinase cascades also controls the functionality of many transcription factors and transcriptional co-activators as well as co-repressors (reviewed in Ref. [235] and discussed in detail in Section 3.5.5), which are differentially responding to changes in amplitude and oscillating frequency of the intracellular Ca^{2+} transients (reviewed in Ref. [236]).

CaM-dependent kinases appear to be very active in tumor cells, where they contribute to accelerated cell proliferation. Examples are CaMK-II in myeloid leukemia, glioma, and endometrial and thyroid carcinoma cells [237,238]; CaMK-III and CaMK-IV in glioma cells [228,237]. The enhanced activity of CaMK-IIγ in leukemia cells as compared to normal cells appears to be controlled by the oncogenic fusion-protein *bcr-abl*, resulting in the hyper-activation of several downstream signaling routes involved in cell proliferation such as the MAPK, Jak/signal transducers and activators of transcription (Stat) and glycogen synthase kinase 3β (GSK3β)/β-catenin pathways [238]. In colon carcinoma cells CaMK-II also interacts and activates mitogen-activated protein kinase 1 (MEK1) followed by the phosphorylation and subsequent degradation of the Cdk inhibitor p27[Kip1] promoting the progression of the S phase and the G_2/M transition [239]. In addition, PNCK is upregulated in a subset of primary human breast cancers [240], and in clear-cell kidney carcinomas its overexpression is associated with poor prognosis [241].

Among phosphatases the CaM-dependent calcineurin (CaN) plays a central role in cell cycle regulation (reviewed in Refs. [242–245]), as it controls the G_0/G_1 and G_1/S transitions. Thus, inhibition of CaN with cyclosporine A inhibits DNA replication in fibroblasts stimulated with basic fibroblast growth factor [246]. The calcineurin B homologous protein 2 enhances the proliferation of HeLa tumor cells [247], and the α isoform of CaN appears to play an important role in small-cell lung cancer by promoting not only cell proliferation but also metastatic invasion [248].

Interestingly, small-cell lung carcinomas highly express the protein tyrosine phosphatase receptor Z1 (PTPRZ1). Binding of its ligand pleiotrophin inhibits PTPRZ1 activity thereby enhancing the phosphorylation levels of CaM, which is concomitant with enhanced tumor progression [249]. The direct connection between phospho-Tyr-CaM levels and tumor progression, however, is not yet established, besides the fact that both the EGFR and Src kinase family members, all playing prominent roles in cell proliferation and tumorigenesis, are known to phosphorylate CaM (reviewed in Ref. [7]). This may be facilitated because as previousy discussed, the EGFR (reviewed in Ref. [175]), v-Src [250] and c-Src [251] are CaM-binding proteins.

A significant interplay between CaM-dependent kinases and phosphatases exists as demonstrated using *in vitro* assay systems (Fig. 7). CaM-dependent protein kinase phosphatase (CaMKP) dephosphorylates the multi-functional CaMKs-I/II/IV inducing their inactivation [252–254] (reviewed in Ref. [255]), and reciprocally CaMKs-I/II phosphorylate and activate CaMKP when assayed *in vitro* [256,257]. In addition, CaMK-IV inactivates CaMKK, while its reactivation is mediated by PP2A. This phosphatase also dephosphorylates CaMK-IV inducing its inactivation (reviewed in Ref. [227]). The implication of CaMKP and its nuclear isoform CaMKP-N in cell growth and viability has been demonstrated in zebrafish embryos *in vivo*, as its downregulation brings about the initiation of apoptosis, specifically in brain and spinal cord neural cells with the subsequent occurrence of developmental abnormalities [258,259]. Of interest, the removal of the C-terminus from CaMKP-N by an ubiquitin-proteasome-mediated proteolytic pathway favors its translocation from the nucleus to the cytosol increasing its activity toward phosphorylated CaMK-I and CaMK-II thereby inactivating these kinases, while its unprocessed form inactivates nuclear CaMK-IV at a much lower rate [260].

3.5.3. Calmodulin-regulated enzymes other than kinases/phosphatases

As briefly mentioned in the Introduction, CaM was discovered as a cyclic nucleotide PDE activator. PDE activity was lost during purification

Table 2
Roles of calmodulin-regulated kinases in cell proliferation.

CaM-kinase	Selected comments	References
CaMK-I	Controls the cell cycle (G_0/G_1)	[221–224][a]
CaMK-II	Activates the MAPK pathway Controls the cell cycle (G_1/S and G_2/M)	[221–224][a]
CaMK-III	Phosphorylates eEF2 during active cell proliferation	[225][a] [632]
CaMK-IV	Controls cell proliferation phosphorylating transcription factors (Sp1, CREB, ATF1)	[221–224][a] [292,566]
CaMKK	Controls cell proliferation by phosphorylating CaMK-I/IV	[220,226,227][a]
CASK	Contains an N-terminal CaMK domain Negatively regulates the cell cycle upregulating p21[Cip1/Waf1]	[633,634]
DAPK	Activates cell growth and protein synthesis by phosphorylation of ribosomal protein S6 via the EGFR/MAPK(ERK)/DAPK pathway	[635][a]
PNCK	Closely related to CaMK-I. Downregulated upon entry in cell proliferation. Role in mammary glands development	[240,636]
PYK2	Activated during zebrafish oocyte fertilization in preparation for cell division	[637,638]
WNK1/4	WNK1 activates the MAPK/ERK5 pathway Mutation of the CaM-BD of WNK4 causes pseudohypoaldosteronism type II (Gordon's syndrome)	[639][a] [640]

ATF1, activating transcription factor 1; CaM, calmodulin; CaMK-I/IV, CaM-dependent protein kinases I/IV; CaMKK, CaM-dependent protein kinase kinase; CASK, CaM-dependent serine protein kinase; CREB, cAMP response element-binding protein; DAPK, death-associated protein kinase; ERK5, extracellular regulated kinase 5; MAPK, mitogen-activated protein kinase; PNCK, pregnancy up-regulated non-ubiquitous calmodulin-dependent kinase; PYK2, proline-rich tyrosine kinase 2; WNK1/4, with-no-lysine kinases 1/4.

[a] Review article.
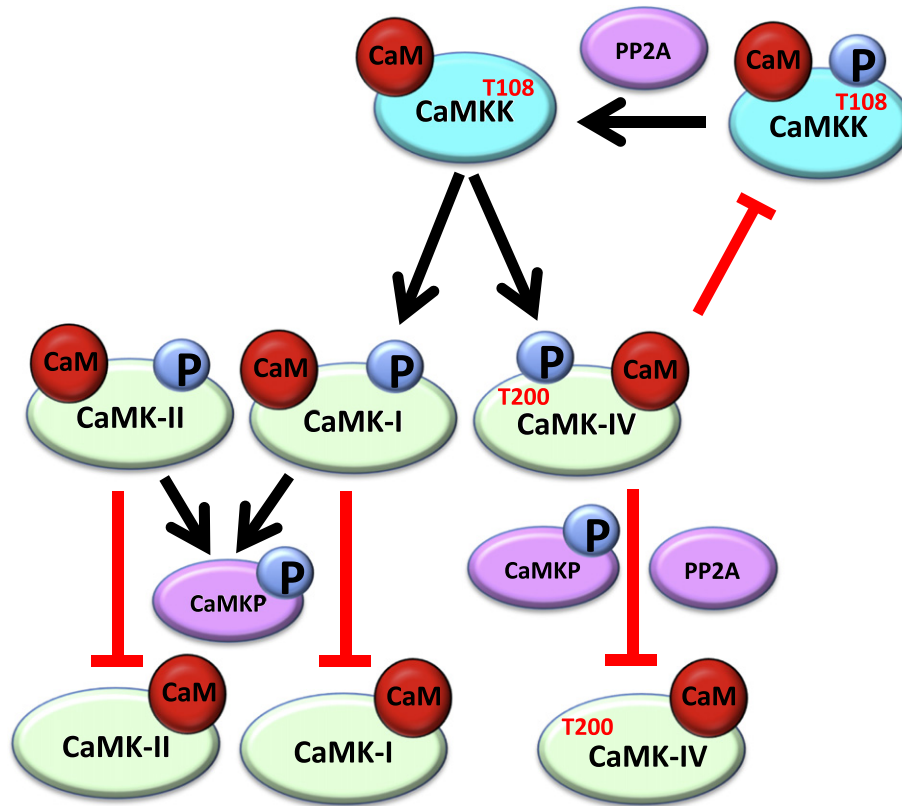
Fig. 7. Crosstalk among calmodulin-dependent kinases and phosphatases. The scheme depicts the CaM-dependent kinase kinase (CaMKK), which phosphorylates (activates) CaMK-I and CaMK-IV (at Thr200), while dephosphorylation of this residue by CaM-dependent protein kinase phosphatase (CaMKP) or protein phosphatase 2A (PP2A) induces its inactivation. Active CaMK-IV retro-phosphorylates CaMKK (at Thr108) inducing its inhibition, while dephosphorylation of this residue by PP2A induces its reactivation. Active CaMK-I and CaMK-II both phosphorylate (activate) CaMKP, and this phosphatase dephosphorylates both kinases inducing its inhibition. *Black arrows* indicate activation and *red Ts* inhibition.

but was regained after addition of a protein fraction later identified as a PDE activator and named CaM [1,2]. After the initial identification of this first CaM-binding/regulated enzyme many more followed, trailed by all sorts of proteins without recognizable enzymatic activity but of important physiological significance.

An early report suggested the implication of the CaM-dependent cyclic nucleotide PDE in cell proliferation, as its expression and activity was increased upon mitogenic stimulation of bovine peripheral lymphocytes with phytohemagglutinin (PHA) [261]. The isoform 1A of PDE preferentially hydrolyzes cGMP and plays a positive role in the proliferation of vascular smooth muscle cells upon its localization in the nucleus. Blocking its activity by chemical agents or downregulating its expression by siRNA induces an increase in intracellular cGMP. This was shown to lead to a proliferative arrest of cells at the G_1 phase and the onset of apoptosis, respectively mediated by the upregulation of p27^{Kip1} and the downregulation of cyclin D1, as well as the activation of p53 [262]. Connected with this observation, oscillating levels of CaM and cGMP may play a prominent role during the progression of the cell cycle. In accordance, changes in the activity of guanylate cyclase in the ciliate *Tetrahymena* mirror the changing levels of CaM during this process [263].

Nitric oxide is a biphasic modulator of cell proliferation inducing either activation or inhibition depending of its concentration (reviewed in Refs. [264,265]). In this context, another CaM-dependent enzyme that has been shown to participate in the control of cell proliferation is the nitric oxide synthase (NOS). Meine *et al.* [266,267] have shown that nitric oxide released from a chemical donor or by treating neuronal tumor cells with interleukin (IL)-1β at low concentrations induces their proliferation by activating the MAPK pathway. NOS inhibitors, as well as CaM and MAPK inhibitors, block the proliferation, suggesting that nitric oxide produced by NOS is implicated in this process.

3.5.4. Calmodulin-regulated adaptors and scaffolding proteins

The 14-3-3 proteins conform a family of adaptors involved in the regulation of signaling pathways in all eukaryotic cells by interacting with an extensive number of proteins by a phosphorylation-dependent manner. This includes among many others, diverse kinases as for example Raf-1, Cdks, Wee1; phosphatases such as cdc25; and regulatory Cdk inhibitory proteins such as p21$^{Cip1/Waf1}$, all of them implicated in cell proliferation; as well as proteins controlling apoptosis such as Bcl-2-associated death promoter (Bad), Bcl-2-associated X protein (Bax) and the transcription factor Foxo (reviewed in Ref. [268]). CaM has been shown to directly interact with 14-3-3ε in a Ca^{2+}-dependent manner. This mechanism represents another way by which CaM may control cell proliferation [269]. Moreover, it is known that 14-3-3 proteins play an important role in tumorigenesis [270–272]. In addition, 14-3-3 negatively regulates CaMKKα when this kinase is phosphorylated by protein kinase A (PKA) *in vitro* and in transfected HEK293 cells [273,274].

Growth factor receptor bound protein 7 (Grb7), together with Grb10 and Grb14, belongs to a family of mammalian adaptor proteins phylogenetically related to the *Caenorhabditis elegans* protein Mig10 implicated in embryonic neuronal cell migration [275,276]. These adaptors transmit signals from receptor and non-receptor tyrosine kinases and other tyrosine-phosphorylated proteins after binding to phospho-tyrosine residues via the SH2 domain located at their C-terminus [277,278]. Grb7 is a Ca^{2+}-dependent CaM-binding protein containing a CaM-BD site in the proximal region of its PH domain [279]. Grb7 is involved in cell migration processes [277,278], but also plays a major role in cell proliferation [187,280,281], tumor growth and tumor-associated angiogenesis [279,281].

Another scaffold protein that binds CaM is the IQ-containing Ras GTPase-activating-like protein 1 (IQGAP1) [282,283], which is involved

in many signaling pathways playing important roles for example in the control of cell proliferation, cell–cell adhesion, β-catenin-mediated transcriptional events, tumorigenesis and metastasis (reviewed in Ref. [284]). The interaction of CaM with IQGAP1 occurs in a Ca^{2+}-dependent and -independent manner at their four IQ motifs located in tandem in its central region [282], and in addition interacts in a Ca^{2+}-dependent manner at its calponin homology domain located in its N-terminus [283]. A-kinase anchor protein 12 (AKAP12) is a scaffolding protein that appears to suppress metastasis. Its downregulation, frequently occurring in many human cancers, results in tumor progression. AKAP12 interacts with some G protein-coupled receptors, several kinases (PKC, PKA, Src), other enzymes such as PDE, and structural as well as signaling proteins including CaM. In this manner AKAP12 participates in mitogenic signaling and the remodeling of the cytoskeleton during cell migration, processes associated to oncogenic progression (reviewed in Ref. [285]). The adaptor protein denoted Fas-associated protein with death domain (FADD), involved in apoptosis by connecting death receptors to the initiating caspases-8/10 forming the death-inducing signaling complex (DISC) (discussed further in Section 4), has been found to bind CaM in a Ca^{2+}-dependent manner. FADD is also implicated in autophagic cell death, proliferation and cell cycle control, among other functions [286].

3.5.5. Calmodulin-regulated transcription factors

Cell proliferation requires the coordinated activation or silencing of multiple genes at specific time points. CaM directly regulates these processes by binding to transcriptional regulators and most importantly through CaM-dependent phosphorylation/dephosphorylation events mediated by CaMKs cascades and CaN, thus modulating the activity of transcriptional factors that are expressed in a cell cycle specific fashion (reviewed in Refs. [244,287,288]). This has been observed for example with the expression of CaM-binding transcription activators 1/2 (CAMTA1/2) during cardiac growth and tumor suppression (reviewed in Ref. [289]). CAMTA1 controls the S and M phases of the cell cycle in neuroblastoma SK-N-SH cells [290]. CaM also binds to two different isoforms of the transcription factor C/EBP-β (CCAAT/enhancer binding protein-β), denoted C/EBP-β-LAP (liver activator protein) and C/EBP-β-LIP (liver inhibitor protein), preventing the growth promoting activity of the latter. C/EBP-β-LIP controls the transcription of genes required for the S phase by preventing the repressing action of the retinoblastoma protein (pRb) on the E2F promoter and disrupting the E2F1–pRb complex [291]. The pRb-regulated transcription factor Sp1 is also under the control of CaM due to its phosphorylation by both CaMK-II and CaMK-IV [292]. Furthermore, inhibition of CaM in human fibrosarcoma cells enhances the expression of early growth response protein 1 (Egr-1), repressing cell proliferation by a mechanism involving the control of the Ras/MAPK/Elk-1 (E twenty six-like transcription factor 1) pathway exerted by CaMK-II and CaN [293].

Activation of CaN by the Ca^{2+}/CaM complex results in the dephosphorylation of the transcription factor denoted nuclear factor of activated T cells (NFAT) and its translocation to the nucleus. In cooperation with AP-1 (Fos/Jun) NFAT positively regulates the transcription of multiple genes or induces gene silencing by interacting with histone deacetylases (reviewed in Refs. [294,295]). The CaN/NFAT pathway has been shown to control the growth of a variety of cells including cardiomyocytes [296], pancreatic β cells [297], vascular smooth muscle cells [298,299] and hepatocellular carcinoma cells [300] among others. Deregulation of this signaling route plays an important role in tumorigenesis (reviewed in Refs. [301–303]). The CaN/NFAT pathway seems to have diverse and cell type specific effects. In T lymphocytes and Jurkat cells CaN/NFAT negatively controls the expression of Cdk4 [304], required for the G_1/S transition. However, in vascular smooth muscle cells CaN/NFAT induces the expression of cyclin A [305], required for the progression of the S and G_2/M phases. Furthermore, the reduced growth rate of leukemic HL-60 cells induced by retinoic acid-mediated differentiation appears to be due to increased CaN expression and

activity [306]. Transcriptional processes regulated by CaM through phosphorylation and dephosphorylation events catalyzed, respectively, by CaMK-II and CaN, have been described as well in normal tissue growth and development, as for example in skeletal muscle (reviewed in Ref. [307]). In addition, CaM regulates the nuclear entry of the transcription factors sex-determining region on the Y chromosome (SRY) and Sry-related high-mobility-box (SOX) family by binding to one of its nuclear localization sequences. This is an important event for gonadal cells terminal differentiation (reviewed in Ref. [308]).

3.5.6. Calmodulin-regulated cell cycle proteins

In addition to the CaM-regulated kinases and phosphatases controlling the cell cycle, there are other proteins participating in the cell cycle machinery that directly bind CaM. One example is Cdk5, an atypical Cdk family member negatively controlling the cell cycle arresting the cells at G_o by stabilizing the Cdk2/cyclins E/A inhibitor $p27^{Kip1}$ [309]. Cdk5 has been shown to be a CaM-binding protein in *Dictyostelium* [310], however information on Cdk5/CaM interaction in vertebrate cells is not yet available to our knowledge. The neural Cdk5 activator p35 is a CaM-binding protein [311]. Cyclin E1, a Cdk2 partner, is another CaM-binding protein that controls the G_1/S transition [312,313]. The CaM interacting 14-3-3 proteins e.g. 14-3-3ε [269], described in Section 3.5.4, control the G_1/S and G_2/M transitions. They sequester key cell cycle components such as the Cdk2/cyclin E and Cdk1(cdc2)/cyclin B complexes and the phosphatases cdc25A/B/C in the cytosol, preventing progression of the cell cycle, or sequestering phospho-$p27^{Kip1}$ facilitating its progression [314]. The C-terminal domain of the Cdk4/cyclin D inhibitor $p21^{Cip1/Waf1}$ directly interacts with CaM in a Ca^{2+}-dependent manner [315,316], facilitating its nuclear entry by preventing its phosphorylation by PKC [315,317], and therefore controlling the G_1/S transition.

3.6. Calmodulin and the control of specific cell cycle phases

3.6.1. The G_0/G_1 transition

Several CaM-dependent systems are involed in the regulation of the G_0/G_1 transition (Fig. 8). CaN via NFAT appears to favor the expression of cyclin D at early G_1 but paradoxically repressing the Cdk4 promoter and inactivating this kinase by dephosphorylation, hence regulating the cyclin D/Cdk4 complex in mammalian cells (reviewed in Refs. [78,318]). CaMK-I more likely than CaMK-II may participate in G_1 progression (reviewed in Ref. [78]). The Ca^{2+}-independent activity of CaMK-II and the Ca^{2+}-dependent activity of CaN both increase when vascular smooth muscle cells transit from G_0 to G_1/S [319].

3.6.2. The G_1/S transition

The G_1/S transition is one of the most critical checkpoints of the cell cycle where CaM controls a variety of systems (Fig. 8). The Ca^{2+}/CaM complex regulates the phosphorylation of pRb at an early stage of the cell cycle. CaM antagonists added at an early time after stimulation with growth factors inhibited this process in a variety of cells. This did not happen when the inhibitors are added at a later time point [320,321] (see also Fig. 4). Mechanistically, CaM antagonist-induced arrest of the cell cycle in normal rat kidney cells at the G_1/S transition appears to be due to the inhibition of Cdk4 and Cdk2, as this results in pRb hypophosphorylation [321]. The association of CaM with Cdk4/cyclin D1 was proposed to be mediated by heat shock protein 90 (Hsp90) [321], which was earlier shown to interact with CaM [322]. In WI-38 fibroblasts CaN induced the expression of cyclin D1 at the early G_1 phase [323]. On the other hand, it inhibits Cdk4, but not Cdk6 or Cdk2 in Jurkat cells, by removing the phosphate introduced by cyclin activating kinase (CAK), that is the activating kinase of Cdk4, and therefore negatively controls the G_1/S transition [304,324]. The heat shock protein 70 (Hsp70) has a CaM-binding motif [325]. In the nucleus of mammalian cells the interaction of Hsp70 with CaM during the S phase participates in the arrest of cell cycle at this point inducing apoptosis [326].
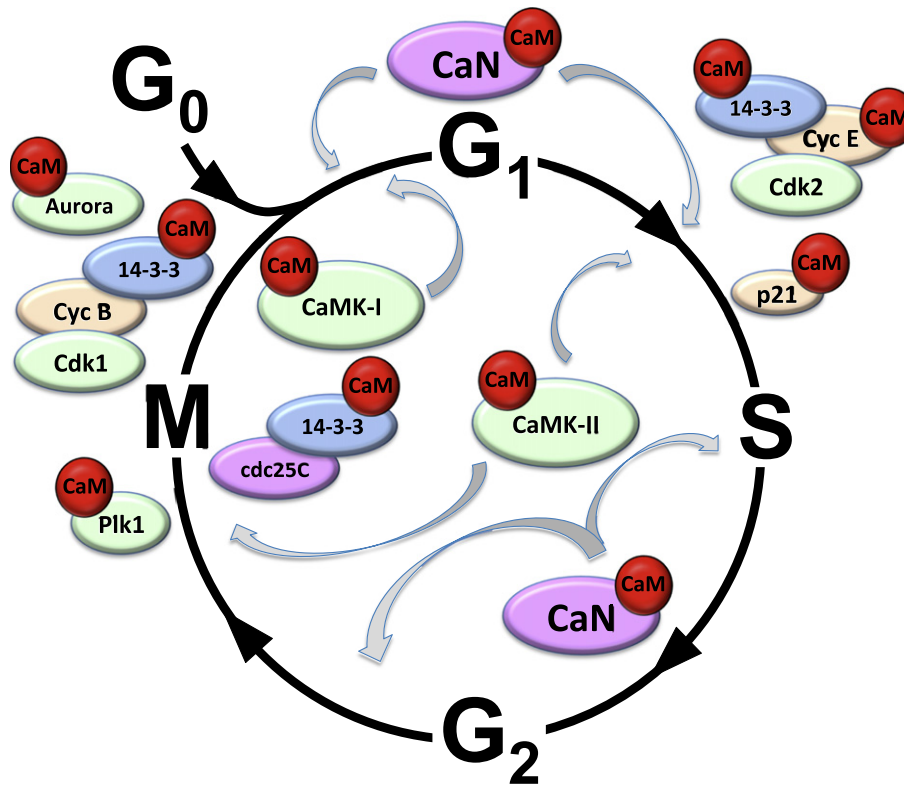
Fig. 8. Calmodulin-controlled systems in cell cycle progression. The scheme depicts major calmodulin-dependent systems controlling the different phases of the cell cycle. CaM, calmodulin; CaMK-I/II, CaM-dependent protein kinases I/II; CaN, calcineurin; Cdk1/2, cyclin-dependent kinases 1 and 2; Cyc B/E, cyclins B and E; Plk1, Polo-like kinase 1.

Another key cell cycle regulatory point with CaM involvement at the G_1/S transition is its interaction with cyclin E1 leading to activation of the Cdk2/cyclin E complex in a Ca^{2+}-dependent manner [312,313]. In this context, an inhibitory peptide based on the CaM-BD of cyclin E disrupted the interaction of this cyclin with Cdk2 and arrested the cell cycle at the G_1/S transition in primary mouse aortic smooth muscle cells [327]. Moreover, the CaM-binding adaptor protein 14-3-3 inhibits the G_1/S transition by sequestering the Cdk2/cyclin E complex in the cytoplasm (reviewed in Ref. [314]).

CaMK-I positively controls cell proliferation, as its downregulation with siRNA inhibited cell growth and diminished the expression of c-Fos [233]. CaMK-I also appears to participate in Cdk4 activation facilitating the Cdk4/cyclin D assembly. The pharmacological inhibition of this kinase with KN-93 or the overexpression of a dominant negative kinase-null mutant induced the association of p21[Cip/Waf1] and p27[Kip1] with the Cdk4/cyclin D complex arresting the cell cycle at G_1 in human WI-38 fibroblasts [328]. On the other hand, direct binding of CaM to p21[Cip/Waf1] in a variety of cells (NRK, NIH3T3, COS7) facilitated the entry of this Cdk inhibitor into the nucleus by preventing its phosphorylation by PKC, that otherwise will block its nuclear localization as shown in NRK cells [315,317] (reviewed in Ref. [313]). In breast carcinoma MCF-7 cells, the inhibition of the CaMKK/CaMK-I pathway also resulted in the arrest of the cell cycle at the G_1 phase, accompanied by the downregulation of cyclin D1 and pRb hypophosphorylation [329]. In normal and tumor lung epithelial cells, the ubiquitin E3 ligase element F-box protein 12 (Fbxl12) participates in G_1 arrest by inducing the degradation of CaMK-I [330].

The activation of CaMK-II and its downstream MAPK pathway can result from signals arriving from the tyrosine kinase receptor RET, hyperactive B-Raf or Ras mutated forms, as demonstrated in thyroid carcinoma cells [331]. In colon cancer cells CaM inhibition by different curcumin derivatives induced a sustained phosphorylation of ERK1/2, but most significantly an upregulation of p21[Cip1/Waf1], and hence arresting the cell cycle [332,333]. A similar effect on ERK2 activation

and upregulation of p21[Cip1/Waf1] upon inhibition of CaM with W-13 was observed in NIH3T3 fibroblasts [334]. This is in agreement with studies done using CaMK-II$\delta^{-/-}$ transgenic mice, and their derived vascular smooth muscle cells, where decreased expression of cyclins E and D1, lower Cdk2 and Cdk4 activity, and upregulation of p21[Cip1/Waf1] were detected, while the opposite was found in control wild type cells [335]. It was also found that the elevated expression of p21[Cip1/Waf1] in these cells was due to the upregulation of the p53 pathway [335]. Nevertheless, CaMK-II may also negatively regulate the G_1/S transition by phosphorylating the large subunit of the replication factor C thus preventing DNA replication [336]. Overall, this emphasizes the occurrence of multiple and apparently conflicting CaM-regulated control mechanisms acting at the G_1/S transition, which may operate in a cell-specific fashion.

3.6.3. The G_2/M transition

CaM controls the G_2/M transition via CaMK-II (Fig. 8). Inhibition of CaM induces the downregulation of cyclin A and Cdk1(cdc2) [321]. The Cdk1(cdc2)/cyclin B complex is able to phosphorylate and activate the phosphatase cdc25C by a positive auto-activation loop, further releasing the inhibitory Wee1-mediated phosphorylation of Thr14/Tyr15 in remaining Cdk1(cdc2) molecules. The activating phosphorylation of cdc25C can initially be catalyzed by CaMK-II, hence facilitating the further activation of the Cdk1(cdc2)/cyclin B complex during the G_2/M transition as demonstrated in HeLa cells [337]. Polo-like kinase 1 (Plk1) is also an important regulator of the G_2/M transition. Recently, this kinase was shown to bind and to be activated by CaM when cells are transiting from G_2 to M phase stimulating thereby the phosphorylation of cdc25C [338]. When human erythroid leukemia cells were subjected to ionizing radiation arresting the cell cycle at G_2/M, the observed activation of CaMK-II was preceded by enhanced Ca^{2+} entry controlled by voltage-gated K^+ channels (Fig. 1), followed by the inactivation of both cdc25B and Cdk1(cdc2) [339]. The sequestration of the phosphatases cdc25B and the Cdk1(cdc2)/cyclin B complex in the

cytosol by 14-3-3 proteins also results in cell cycle arrest (reviewed in Ref. [314]).

3.6.4. The M phase and cytokinesis

CaM plays important roles during mitosis and cytokinesis, shifting subcellular location during mitotic progression and acting on a series of important targets, which regulate these processes. The potential role of CaM during mitosis can be studied by visualizing the localization of fluorescent CaM species, such as by expressing the chimera GFP-CaM (see for example Refs. [170,171] and Fig. 5). Alternatively, CaM tagged with a fluorescent probe, such as RITC-CaM, TA-CaM or FL-CaM, can be microinjected to study its functionality in living cells [63,166,168], or localized in fixed cells by electron microscopy and immunocytochemistry using an antibody against performic acid-treated CaM [167]. The fluorescence emitted by TA-CaM represents active Ca^{2+}/CaM bound to its targets while the fluorescence emitted by FL-CaM is insensitive to Ca^{2+} binding [63]. By measuring the differential signals of both fluorescent CaM species it was demonstrated that active CaM is mostly located in the nucleus in preparation for mitosis (interphase at G_2 and prophase). CaM was also shown to be associated with the aster at interphase; and during metaphase active CaM associated to the spindle, the spindle poles, the astral microtubules and centrosomes, remaining at this location during early anaphase but fading away thereafter [63] (Fig. 9). A role of CaM in the mitotic spindle appears to be mediated by CaMKK, which is, together with liver kinase B1 (LKB1), an upstream kinase for the phosphorylation of AMP-activated protein kinase (AMPK). AMPK in turn phosphorylates the downstream substrate myosin regulatory light chain (MRLC) to achieve proper mitotic spindle orientation [340]. The participation of CaMKKβ as the leading upstream kinase of AMPK in LKB1-deficient tumor cells was demonstrated by activating this pathway by baicalin, a natural flavonoid that prevents lipid accumulation in the liver [341]. MLCK, a CaM-binding enzyme that may have a species-specific role in these processes, was shown to be localized in the cleavage furrow and may be phosphorylated by the kinase Aurora B in mammalian cells during cytokinesis (reviewed in Ref. [342]).

It has been suggested that CaM could participate in the shortening of the interpolar microtubules and those associated to the kinetochores, participating as well in the assembly of chromosomes at the metaphase plate, but CaM does not appear to participate in the shortening of the astral microtubules. Microinjection of Ca^{2+}/CaM during early prometaphase prolonged the time that the cells take to reach anaphase and therefore retarded mitosis, an effect that was also observed with Ca^{2+} only, although with lower efficiency [343]. On the other hand, antagonizing CaM with W-7, calmidazolium or a phenothiazine-CaM adduct denoted CAPP$_1$-CaM inhibited metaphase progression [344]. An additional proposed mechanism explaining the implication of CaM in the control of mitosis is through binding to the centrosome-located Ca^{2+}/CaM-activated kinase Aurora A, thereby facilitating mitotic progression and cytokinesis. Inhibition of CaM on the other hand was shown to reduce the interaction of this kinase with its activator neural precursor cell expressed developmentally down-regulated protein 9 (Nedd9) preventing these processes [345]. Aurora B was also shown to interact with CaM, protecting this kinase from ubiquitination by competing with the E3 ligase subunit F-box and leucine-rich repeat protein 2 (Fbxl2) and thereby preventing destabilization of chromosome segregation, the occurrence of multi-spindle formation and tetraploidy [346].

CaM participates in cytokinesis by associating to the central spindle, controlling the migration of the centrioles and mediating the formation of the cleavage furrow, processes that can be prevented by W-7 treatment, not only in vertebrate cells [347,348], but also in protozoa [349]. Along this line, down-regulation of CaM by antisense RNA technology in *Dictyostelium* prevented completion of cytokinesis [350]. In *Tetrahymena*, CaM was shown to act during cytokinesis by its Ca^{2+}-dependent interaction with an 85 kDa protein that participates in the recruitment of G-actin to the cell division plane for the formation of

the contractile ring responsible for the generation of the cleavage furrow [349,351]. In early embryos of *C. elegans* downregulation of CaM using RNA interference did not alter cytokinesis, even though it induced minor defects in chromosomes segregation [352]. However, it cannot be excluded that the residual ($\approx 4\%$) CaM remaining after RNA interference could be responsible for the apparent absence of major effects of CaM during cytokinesis in this organism.

Several CaM-binding proteins with functions in cell cycle regulation are also involved in cell death programs, one of them being FADD (discussed in Section 4). Interestingly, when FADD regulates non-apoptotic functions in HeLa cells controlling the cell cycle, the interaction of CaM with this protein induced the co-localization of CaM together with casein kinase I at the mitotic spindle during metaphase and anaphase [286].

3.7. Cell cycle progression during egg fertilization

Fertilization of vertebrate eggs induces a rise in the cytosolic Ca^{2+} concentration mediated by a set of Ca^{2+} oscillations and propagating waves, processes that are required to resume the proliferation of unfertilized eggs previously arrested at meiotic metaphase II. Ca^{2+} release from the endoplasmic reticulum and Ca^{2+} entry by the STIM1/Orai1 CaM-binding system play important roles in mammalian egg fertilization (reviewed in Ref. [353]).

In *Xenopus* eggs the proteolytic degradation of cyclin B by the ubiquitin-dependent pathway during exit from meiotic metaphase II occurs after inactivation of the cytostatic factor c-mos and the M phase-promoting factor by CaMK-II-mediated phosphorylation. This was demonstrated either by inhibiting this kinase with specific inhibitors or sequestering CaM by an inhibitory Ca^{2+}/CaM-binding peptide corresponding to the CaM-BD of MLCK, and conversely by activating the process expressing a constitutively active form of CaMK-II [354–356].

The translational control of cyclin B1 during cell cycle progression is mediated by a complex formed by the protein denoted Maskin with cytoplasmic polyadenylation element-binding protein (CPEB) and eukaryotic initiation factor 4E (eIF4E) among other factors [357]. In this context, phosphorylation of Maskin by Cdk1(cdc2) allowed the translation of cyclin B1 mRNA, which is required for progression to the M phase, while dephosphorylation of Maskin by CaN prevented cyclin B1 translation [358]. Upon a surge of Ca^{2+} during fertilization of *Xenopus* eggs the activation of CaMK-II by Ca^{2+}/CaM was shown to lead to phosphorylation of the phosphatase cdc25C at Ser287 inhibiting its activity thereby delaying reentry into the M phase [359]. A particular form of CaMK-II denoted as cyk has been identified as a cyclin B2 kinase, although phosphorylation of this cyclin does not appear to affect its functionality [360]. CaMK-II also plays a role in the resumption of the cell cycle in metaphase II-arrested mouse eggs [361]. This effect was shown to be mediated by the single CaMK-II isoform γ3 in mouse eggs [362]. In addition, phosphorylation of myosin-V by CaMK-II prevented organelle transport along the actin microfilaments, such as melanophores, during mitosis in *Xenopus* eggs, as it released the molecular motor from the organelle [363]. Furthermore, CaM was shown to interact with the microtubule-associated protein ASPM (abnormal spindle-like microcephaly-associated protein) during mitosis I in mouse oocytes, thereby participating in the assembly of the spindle during meiosis [364].

4. Calmodulin in programmed cell death

4.1. General comments and main players in programmed cell death related to calmodulin signaling

Higher eukaryotes have developed a number of essential processes leading finally to cell death. Failure of these processes often leads to diseases including e.g. cancer. Understanding the molecular mechanisms
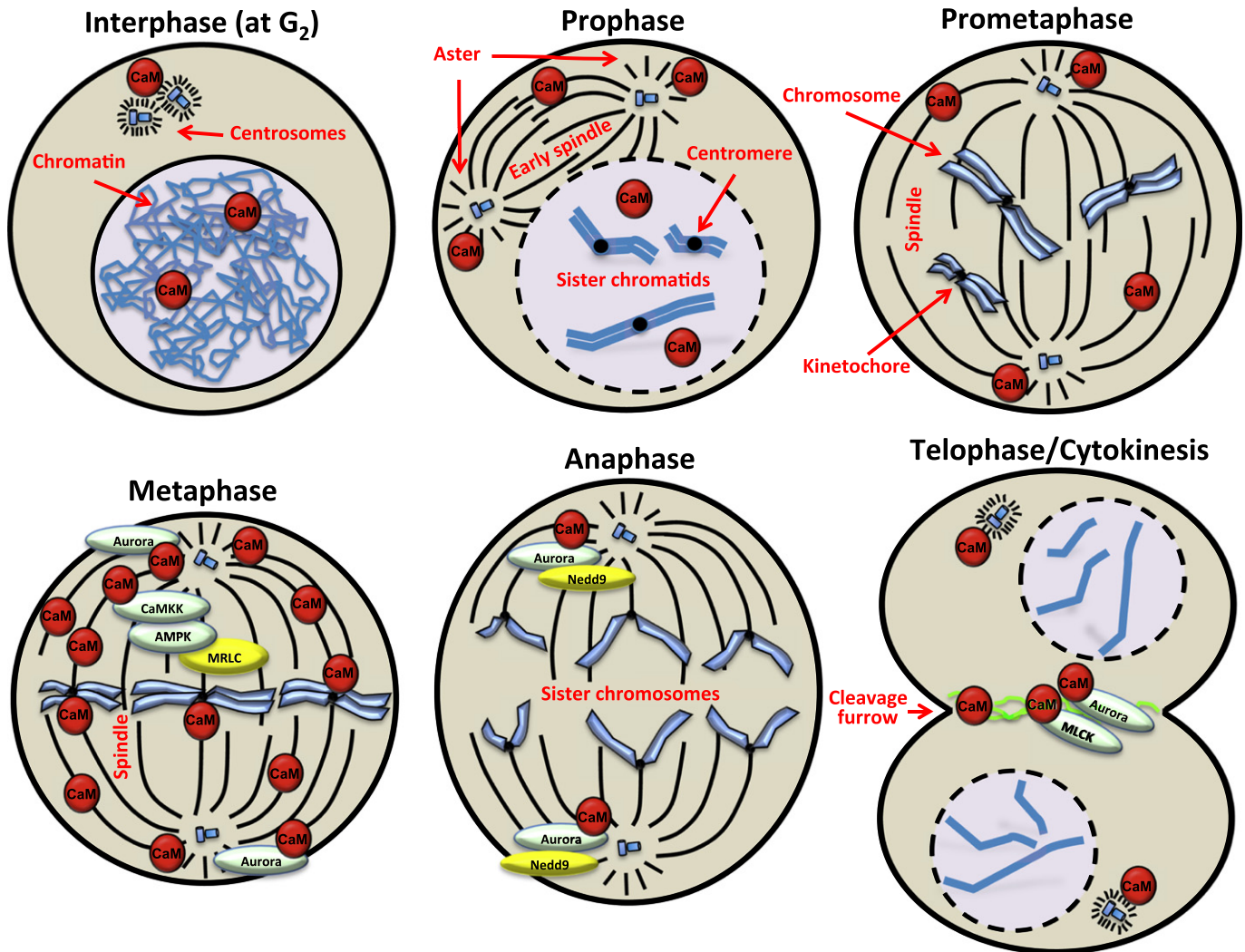
Fig. 9. Location of calmodulin during mitosis and cytokinesis. The scheme depicts the major points of localization of calmodulin (CaM) during the different phases of mitosis and cytokinesis. The scheme also shows the location of Aurora A, and its activator neural precursor cell expressed developmentally down-regulated protein 9 (Nedd9), at the spindle poles during metaphase and anaphase; and at the cleavage furrow, together with myosin light chain kinase (MLCK), during cytokinesis. The CaMKK/AMPK/MRLC cascade acting to attain proper spindle orientation is also shown in metaphase. AMPK, AMP-activated protein kinase; CaMKK, CaM-dependent protein kinase kinase; MRLC, myosin regulatory light chain.

involving cell death is therefore of highest interest in basic biology and has huge implications for the advances of novel therapies. Even though distinct molecular pathways were delineated in the recent years, these mechanisms are highly interconnected and players in the cell death mechanisms often have other functions as well. Because of misconceptions and confusions in the vocabulary of scientific reports dealing with cell death the Nomenclature Committee on Cell Death (NCCD) has proposed a classification of death processes, which is mostly based on morphological characteristics and recommended methods to describe cell death pathways [365]. The basic categories of cell death are described as apoptosis, autophagy, cornification and necrosis. All these processes may occur in a programmed fashion. Later, based on the rapidly growing knowledge on molecular and functional features of cell death, the NCCD published the molecular definitions of cell death [366]. This functional classification includes extrinsic apoptosis involving cell death receptors such as Fas (TNF receptor superfamily member 6) and related receptors, caspase-dependent or caspase-independent intrinsic apoptosis involving mitochondria, regulated necrosis, autophagic cell death and mitotic catastrophy. Of note, autophagy also plays an important prosurvival role in the cell and this is why it will be discussed separately in Section 5.

Transient elevation of Ca^{2+} provoked by the action of e.g. hormones, chemical inhibitors such as thapsigargin, which blocks the endoplasmic reticulum Ca^{2+} uptake pump, and also cancer drugs has been shown to induce apoptosis. On the other hand, Ca^{2+} signals appearing later during the apoptotic process also seem to be important for the execution of programmed cell death, and uptake of Ca^{2+} by mitochondria may lead to permeability change and release of proapoptotic proteins (reviewed in Ref. [85]). The Ca^{2+}-activated protease calpain has been shown to be a proapoptotic factor by its ability to cleave and activate various proteins implicated in the apoptotic process including Bax and Bid (BH3 interacting-domain death agonist) (reviewed in Ref. [367]).

As CaM is the main intracellular Ca^{2+} signal mediator protein and an important factor of cell viability and proliferation it is not surprising that its interaction with target proteins and their modulation has a major impact on cell death. In addition, because elevated intracellular Ca^{2+} can have both growth promoting and also cell death-inducing consequences, CaM regulates apoptotic processes both positively and negatively (Fig. 10). CaM is involved in various ways in different types of apoptotic processes including the activation of transcriptional programs as shown e.g. in IL-2 deprivation-induced T-cell death that functionally involves the CaM-binding protein neurogranin, also named RC3 [368]. Neurogranin, which is transcriptionally induced by IL-2, increases the intracellular Ca^{2+} concentration leading to cell death by sequestering CaM and stabilizing its Ca^{2+}-free form thereby increasing intracellular Ca^{2+}.
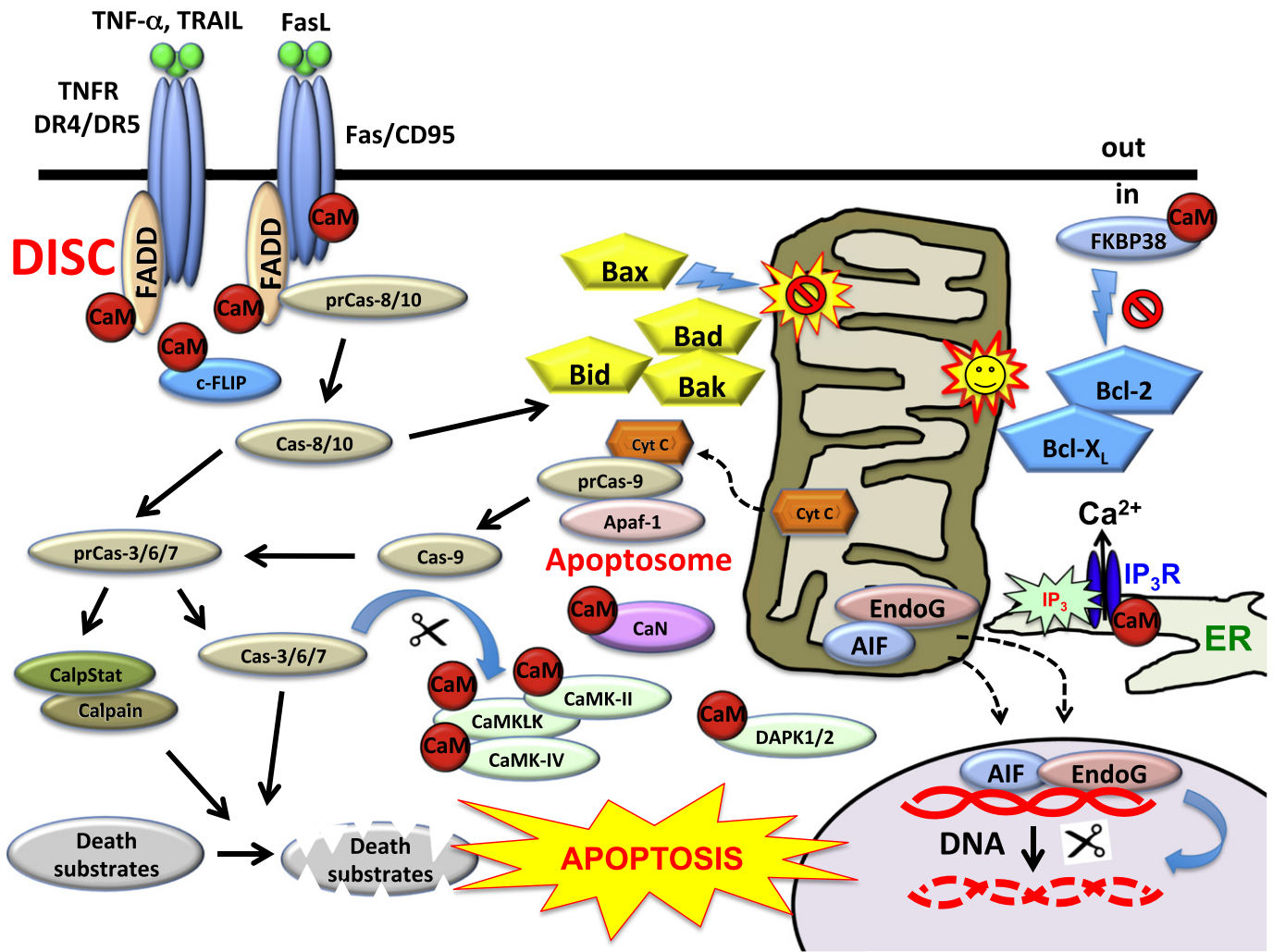
Fig. 10. Calmodulin action on systems controlling apoptosis. The scheme depicts the major components participating in the extrinsic and intrinsic apoptotic pathways and the different calmodulin (CaM)-dependent systems controlling apoptosis. Connections between the extrinsic and intrinsic pathways are also shown. AIF, apoptosis-inducing factor; Apaf-1, apoptotic protease activating factor 1; Bad, Bcl-2-associated death promoter; Bak, Bcl-2 homologous antagonist; Bax, Bcl-2–associated X protein; Bcl-2, B-cell lymphoma 2 protein; Bcl-X$_L$, B-cell lymphoma-extra large protein; Bid, BH3 interacting-domain death agonist; CalpStat, calpastatin; CaMK-II/IV, CaM-dependent protein kinases II/IV; CaMKLK, CaM-dependent protein kinase-like kinase; CaN, calcineurin; Cas3/6/7/8/9/10, caspases 3/6/7/8/9/10; c-FLIP, cellular FLICE-like inhibitory protein; cyt c, cytochrome c; DAPK1/2, death-associated protein kinases 1/2; DISC, death-inducing signaling complex; DR4/5, death receptors 4/5; EndoG, endonuclease G; ER, endoplasmic reticulum; FADD, Fas-associated protein with death domain; Fas (CD95), TNF receptor superfamily member 6; FasL, Fas ligand; FKBP38, FK506-binding protein 38; IP$_3$, inositol-1,4,5-trisphosphate; IP$_3$R, IP$_3$ receptor; prCas3/6/7/8/9/10, pro-caspases 3/6/7/8/9/10; TNFR, tumor necrosis factor-α receptor; TRAIL, TNF-related apoptosis-inducing ligand.

The most prominent targets of CaM for its function in apoptosis are CaM-dependent kinases and phosphatases. One CaM-dependent serine/threonine protein kinase discovered in apoptosis research by Kimchi and collaborators [369] is the CaM-dependent death-associated protein kinase (DAPK), and its related but shorter family member DRP-1 (reviewed in Refs. [370–374] A shorter spliced-variant of DRP-1 denoted DRP-1β lacks, however, the CaM-binding motif [375]. The extensive literature on the function of DAPK and related proteins will not be discussed in this chapter, as comprehensive review articles exist (reviewed in Refs. [373,374,376]). CaMK-II has as well been intensively studied in the context of cell death regulation during the recent years, particularly in connection with death of normal and transformed neuronal, heart and pancreatic cells, as well as a number of cancerous cells. An intriguing study by Olofsson et al. [377] who screened 40 known apoptosis-inducing chemicals for their site of action in connection with four important apoptotic mediators including p53, activator protein 1 (AP1), NFAT and nuclear factor κ-light-chain-enhancer of activated B cells (NFκB), showed that Ca^{2+} was involved in most of the pathways in a late stage and that CaM and CaMK-II play major roles in several Ca^{2+}/CaMK-dependent apoptosis-inducing pathways. The authors proposed that Ca^{2+}/CaM signaling might be involved in apoptotic pathways by

sustained activation of c-Jun N-terminal kinase (JNK) through the function of CaMK-II [378]. The importance of CaM in apoptotic pathways is highlighted also by the evolutionary conservation of CaM-dependent cell death pathways. One example is its participation in an apoptosis pathway in Drosophila, where Reaper, which has similarity to vertebrate Fas and the TNF-α receptor, has been shown to activate a Ca^{2+}/CaM-dependent signaling pathway finally leading to caspase activation and death of cultured Drosophila S2 cells inducibly transfected with Reaper [379]. Based on a complete inhibition of apoptosis by the CaM inhibitor W-7 the authors suggested that CaM plays an essential role in Reaper-induced apoptosis. However, a specific CaM-dependent pathway could not be identified in this study.

Using the monocytic cell line U937 Wright and collaborators [380] reported on a proapoptotic function of CaMK-II in signaling leading to apoptosis induced by TNF-α and ultraviolet (UV) light. Both of these apoptosis-inducing stimuli markedly increased the CaM-independent activity of CaMK-II. Blocking CaMK-II activity by inhibitors of TNF-α- and UV-induced apoptosis could also be shown in a variety of lymphoma cell lines. However, these inhibitors could not block Fas-induced apoptosis indicating that CaMK-II is not generally necessary for apoptosis induction. The 24 kDa apoptotic serine protease (AP24), which induces

DNA fragmentation by activating L-DNAase II, was found to depend of the activity of CaMK-II in the used experimental system. CaMK-II activation was shown to be mediated through the activity of caspases indicating that it acts downstream of caspases and upstream of the apoptotic protease AP24.

CaMK-II seems to be important for endoplasmic reticulum (ER) stress-induced apoptosis. ER stress is connected with increased cytosolic Ca^{2+}, which triggers a variety of processes. Importantly, increased Ca^{2+} uptake into mitochondria may lead to apoptosis. Timmins *et al.* [381] found that increased cytosolic Ca^{2+} as a consequence of ER stress induces the expression of the Fas receptor (CD95) through the activity of CaMK-IIγ and JNK. Mitochondria-mediated apoptosis was also dependent on CaMK-IIγ as shown in the same article by loss of the mitochondrial membrane potential and cytochrome *c* release in a variety of cells. Besides, CaMK-IIγ deficient mice showed reduced mitochondrial dysfunction and apoptosis induced by ER stress as well as other improvements of pathophysiological processes. This led the authors to propose that CaMK-IIγ inhibitors may be used as drugs to prevent ER-mediated apoptosis.

CaN is known to dephosphorylate the transcription factor NFAT, which then translocates to the nucleus and activates gene transcription (discussed in detail in Section 3.5.5). Overexpression of CaN was shown to lead to apoptosis in growth factor-deprived baby hamster kidney BHK-21 fibroblasts [382]. Well documented is the function of CaN in dephophorylating Bad allowing its translocation to the mitochondria and heterodimerization with the B-cell lymphoma-extra large protein (Bcl-X$_L$) [383]. In addition, CaN has been shown to be involved in p53-induced cell death in cancer cells downstream of proline oxidase, which produces proline-dependent reactive oxygen species (ROS) followed by Ca^{2+} mobilization from intracellular pools and activation of CaN [384].

4.2. Calmodulin in neuronal cell death

Extensive information exists on the function of CaMK-II in neuronal tissue. This kinase is a central enzyme playing important roles in pre- and post-synaptic functions, including neurotransmitter release, as well as in the process of long-term potentiation. Early work indicated that ischemia- or hyperthermia-induced neuronal damage leads to inhibition of Ca^{2+}-dependent phosphorylation mediated by CaMK-II, suggesting that impaired CaMK-II activity may be important for the development of ischemia-induced cell death in gerbils [385]. A more recent study by Uno and co-workers [386] indicated that CaMK-II activity is connected with neuroprotection in the gerbil hippocampus where CaMK-II is especially abundant. This study showed that under neuroprotective conditions such as hypothermia the decrease in cytosolic CaMK activity seen after ischemia is markedly reduced. Supporting this finding Xu *et al.* [387] discovered that treatment of CA1 pyramidal cells with subtoxic concentrations of *N*-methyl-D-aspartate (NMDA), known to enhance neuronal cell survival *in vivo* and *in vitro*, involves the phosphatidylinositol 3-kinase (PI$_3$K)/Akt pathway. They showed that binding of CaM to the 85 kDa subunit of PI$_3$K was enhanced by this treatment, which leads to the activation of Akt. This indicated a direct participation of CaM in this process.

An antiapoptotic function of CaMKs was found by McGinnis and colleagues who reported that CaMK-IV was proteolytically cleaved by caspase-3 in thapsigargin-induced apoptosis in human neuroblastoma SH-SY5Y cells [388], and that inhibiting CaMK-II using KN-93 enhanced Ca^{2+}-induced apoptosis in the same cell line [389]. CaMK-IV also seems to be an important player in neuronal cell survival as it phosphorylated cAMP response element-binding protein (CREB) at Ser133 known to be important for neuronal viability and overexpression of CaMK-IV attenuated neuronal apoptosis in cerebellar granule neurons [390]. CaM-dependent protein kinase-like kinase (CaMKLK), an enzyme with structural similarity to CaMK-IV, it was shown to be cleaved by caspase activity, which accelerated apoptosis in NG108 cells [391].

Overexpression of a non-cleavable mutant of CaMKLK protected from apoptotic cell death indicating an active role of this kinase in the apoptotic process. CaMK-IV also seems to protect from amyloid precursor protein-induced neuronal cell death, which involves CaN and calpain but is independent of caspase-3 and nucleosomal cleavage [392].

CaM may prevent ischemic neuronal death through CaMKK, which is an upstream regulator of CaMK-IV (Fig. 7) known to inhibit apoptosis by phosphorylation of CREB as described above (reviewed in Ref. [393]) and the prosurvival kinase Akt, suggesting that both CaMK-IV and Akt may be important players in the induction of ischemic tolerance mediated by the upstream CaMKK activity.

Studies indicate that CaMK-II may also have a proapoptotic function. Inhibiting CaMK-II using the inhibitor KN-62 may protect from neuronal cell death induced by NMDA or hypoxia in fetal rat cortical cultures [394]. This is explained by insults leading to NMDA-mediated neuronal death initiated by imbalanced Ca^{2+} and Na^+ influx into cells, which could activate a number of downstream systems including CaMK-II-mediated phosphorylation. Pretreatment with the CaMK-II inhibitor KN-62 reduced Ca^{2+} transients induced by NMDA and shortened the time when Ca^{2+} reached the basic level after stimulation. These results indicated that CaMK-II is involved in phosphorylation of the NMDA channel potentiating its activity. Indeed it has been shown that the NMDA channel regulatory subunit contains a CaMK-II phosphorylation site (reviewed in Ref. [224]). On the other hand, this may not be the only way CaM-dependent kinases trigger neuronal cell death. Depolarization of neuronal cells is another way that leads to brain cell death. Takano *et al.* [395] found that inhibiting CaM and CaMK-II by a variety of inhibitors prevented death of cortical neurons induced by the alkaloid veratridine, a voltage-sensitive Na^+ channel activator. A possible explanation for this observation is that increased Ca^{2+} in the cell leads to CaMK-II activation followed by phosphorylation and activation of L-type Ca^{2+} channels. This is supported by the observation described in the same article that the L-type Ca^{2+} channel blocker nifedipine contributed to cell viability. Based on these results the authors suggest CaM and CaMK-II as targets for anti-stroke therapies. Supporting the view of a pro-apoptotic function of CaM and CaMK-II it has been shown that the CaM inhibitor DY-9760e protects against neuronal death [396]. In contrast to *in vitro* studies, work by Hashiguchi *et al.* [397] indicates that DY-9760e when applied post-ischemically mainly inhibits Ca^{2+}/CaM-dependent neuronal nitric oxide synthase (nNOS) and not CaMK-II and CaN in the model of CaM antagonist-mediated delayed neuronal death in gerbil hippocampus. This indicated that inhibition of nitric oxide formation by nNOS, known to cause nitrotyrosine-mediated protein dysfunction, might correlate with the neuroprotective effect of the CaM antagonist.

Shirasaki *et al.* [398] showed that expressing CaM with inactivated Ca^{2+}-binding sites renders cortical rat neurons resistant to a variety of neuronal death-inducing insults, including depolarization by KCl. Overexpression of PEP-19, a brain protein thought to sequester CaM in a Ca^{2+}-independent manner, similar to neurogranin as mentioned above, prevents glutamate-induced cell death, and PEP-19 was markedly reduced after glutamate treatment resulting from calpain-mediated degradation [399]. These investigations suggest that CaM activation may naturally be controlled by PEP-19 and when PEP-19 is degraded CaM may activate target proteins involved in proapoptotic functions.

A proapoptotic function of CaMK-II was also found in the embryonic stem cell line P19 stably transfected with the microtubuli modulating protein τ (tau), a cell model system to study processes relevant for Alzheimer's disease (AD). Neurofibrillar tangles composed of the hyperphosphorylated τ protein are AD hallmarks. Apoptotic cell death during neuronal differentiation of these cells induced by retinoic acid was evident in contrast to cells not expressing the τ protein [400]. As CaM and CaMK-II inhibitors protected tau/P19 cells and as CaMK-II phosphorylates τ protein at tubulin interaction sites the authors suggest that the τ protein may dissociate from microtubules and may translocate into the nucleus where it activates apoptosis-mimicking neurodegenerative processes in

AD. On the other hand, a CaM-dependent pathway preventing apoptosis was found in lymphoblasts of AD patients [401]. These cells escape from undergoing serum deprivation-induced apoptosis as compared to control cells. ERK1/2 seems to be involved in this process, as ERK1/2 activity is lower in these cells and ERK1/2 inhibition in normal cells prevents apoptosis. CaM seems to be involved in ERK1/2 downregulation and preventing cell death of AD lymphoblasts as CaM inhibitors lead to increased ERK1/2 activity and accelerated apoptosis due to serum deprivation.

Lewy bodies, a hallmark of Parkinson's disease (PD) contain large amounts of α-synuclein, which may negatively regulate the protein phosphatase A2 (PPA2), known to have a neuroprotective function. Yang et al. [251] overexpressed α-synuclein in neuronal cells and found that PPA2 was phosphorylated at a known Src phosphorylation site (Y307) decreasing its activity and levels of phospho-activated Src were enhanced compared to control cells. Importantly, CaM/Src complex formation was also higher after α-synuclein expression. The CaM-dependent activation of Src was earlier demonstrated by Fedida-Metula et al. [402]. The cytotoxic effect of α-synuclein could be ameliorated through activating PPA2 by ceramide C_2 or by buffering Ca^{2+}. These findings lead to the hypothesis that neurodegeneration in PD induced by α-syuclein is mediated by Src/CaM-dependent inactivation of PPA2.

Retinopathy involving death of retinal ganglion cells (RGCs) leading to dysfunction of the vision is a major complication in diabetes. CaMK-II is known to be involved in RGC death. Diabetic mice exhibit massively induced CaMK-II and active phosphorylated CaMK-II levels in RGCs. Inhibition of CaMK-II activity by downregulation of the enzyme using siRNA or specific inhibitors protected from diabetes induced RGC apoptotic death [403]. In addition, the plant-derived natural polyphenol resveratrol known to have neuroprotective properties could be shown to prevent diabetic retinopathy induced by CaMK-II activation indicating its potential therapeutic value. As suggested by the authors, one way CaMK-II could be transcriptionally regulated is by the action of voltage-gated Ca^{2+} channels known to regulate Ca^{2+}-dependent gene expression and to contribute to RGCs apoptotic death (reviewed in Ref. [403]). These results were later supported and extended by a study of Li et al. [404] using the macaque choroid-retinal endothelial cell line RF/6A. They found that hyperglycemia-induced apoptotic cell death is mediated by extracellular ATP-triggered Ca^{2+} entry and dependent on CaMK-II activity. Both the Fas-dependent and mitochondria-dependent apoptotic pathways were affected by inhibitors blocking Ca^{2+} entry and CaMK-II function in these cells.

A major reason for blindness is ischemic injury of the retina. CaN activity is increased during transiently induced ischemia leading to cell death [405]. CaN directly interact with and dephosphorylates the prosurvival kinase Akt. In addition, as mentioned above, the proapoptotic protein Bad is dephosphorylated by CaN, constitutively activated by cleavage, and translocates to the mitochondria where it interacts with the antiapoptotic proteins B-cell lymphoma 2 protein (Bcl-2) and Bcl-X_L leading to mitochondria failure followed by release of proapoptotic proteins and activation of caspases, which finally leads to cell death. The CaN inhibitor FK506 (tracolimus) reduces Akt and Bad dephosphorylation so protecting from ischemia-induced retinal cell death. Huang et al. [406] showed that intraocular pressure leads to cleavage and activation of CaN in experimental glaucoma followed by induction of a retinal ganglion cell death pathway involving the same players as in the above described ischemia model, a process which can be blocked by CaN inhibitors. The same group showed later [407] that activation of CaN in normal retinal ganglia by adenovirus-mediated gene transfer caused neuronal degeneration. Based on these combined results on chronic and acute retinal ganglion cells degeneration by a specific CaN-dependent pathway new possibilities for therapeutic interventions have been proposed [407].

Preserving astrocyte viability during spinal cord injury is considered a main issue for successful recovery. Hypoxic/ischemic stress in spinal astrocytes targets primarily the ER and mitochondria. As mentioned above, CaMK-II induces apoptosis through the JNK pathway [378] and

CaMK-IIγ is involved in linking the ER and mitochondrial apoptotic pathways [381]. Liu et al. [408] using an in vitro oxygen/glucose depriving model found that hypoxia/reoxygeneration conditions lead to ROS accumulation and CaMK-II activation needed for the direct phosphorylation and activation of apoptosis signal-regulating kinase 1 (ASK1). This kinase then induces JNK-mediated death of spinal astrocytes. Downregulation of CaMK-IIγ reduced ASK1, JNK and 38 kDa mitogen-activated protein kinase (p38MAPK) activation and apoptotic cell death indicating that CaMK-IIγ plays an important role in the death of spinal cord astrocytes after injury. In support of these findings Lu et al. [409] recently found that CaMK-II is stimulated in rat hippocampal slices exposed to oxygen glucose deprivation mimicking hypoxia–ischemia. Several events including increased activity of p38MAPK leading to hypoxia induced neuronal cell death could be blocked by addition of the CaMK-II inhibitor KN-93 strongly indicating an involvement of this enzyme in hypoxia-induced neuronal cell death.

CaM could be involved in neuronal apoptosis by its direct interaction with FK506-binding protein 38 (FKBP38), an enzyme that suppresses the antiapoptotic protein Bcl-2 through its prolyl cis/trans-isomerase activity. Ca^{2+}/CaM activation is required for the interaction of FKBP38 with Bcl-2 and its regulatory function [410]. Interestingly, CaM/FKBP38 interaction occurs in a novel dual intramolecular way where the C-terminal lobe of CaM interacts with the C-terminus of FKBP38 enabling the N-terminal lobe of CaM to interact Ca^{2+}-independently with the catalytic domain of FKBP38. This interaction leads to the activation of this enzyme and thereby suppresses the antiapoptotic function of Bcl-2 [411]. Active site-directed inhibition of FKBP38 has been shown to increase the survival of neuroblastoma cells challenged with apoptotic stimuli and to provide neuroprotection and neuroregenerative effects in a rat ischemia model [412].

4.3. Calmodulin function in cardiomyocytes death

CaMK-II has been demonstrated to have proapoptotic function in cardiomyocytes. Zhu et al. [413] showed that inhibition of CaMK-II protects from β1-adrenergic receptor stimulation-induced apoptosis in cultured ventricular myocytes and overexpression of the cardiac CaMK-IIδC enhanced the effect of β1-adrenergic stimulation. The proapoptotic activity of CaMK-II was also demonstrated in vivo using mice genetically engineered to express a specific CaMK-II inhibitory peptide [414]. Persistent β-adrenergic stimulation in the failing heart leads to cardiomyocyte apoptosis mediated by PKA-dependent activation of CaMK-II. Using a transgenic mouse model and cultured feline ventricular myoctes with expression of a PKA specific inhibitor Zhang et al. [415] showed that β-adrenergic signaling leads to PKA-dependent cardiomyocyte death and PKA-independent cardioprotection. PKA inhibition prevented cytosolic and sarcoplasmic reticulum Ca^{2+} overload, and CaMK-II activation exerted a cardioprotective function mediated through a cAMP/Rap1/Rac/ERK-dependent pathway. These results indicated that inhibition of PKA could be an effective treatment of heart failure induced by β-adrenergic stimulation of CaMK-II.

Cardiac hypertrophy is characterized by a loss of myocytes due to apoptosis. Circulating angiotensin II (Ang-II), which levels are increased in heart failure, may contribute to the death of myocytes. Ang-II induces apoptosis through increased CaMK-II activity mediated by ROS and followed by p38MAPK activation finally leading to cell death involving the intrinsic apoptotic pathway [416]. Interestingly, Ang-II or ROS can activate CaMK-II at very low Ca^{2+} concentrations and in the presence of the CaM inhibitor W-7 in vitro resetting the Ca^{2+}/CaM-dependence of CaMK-II. CaMK-II plays as well a proapoptotic role in irreversible ischemia–reperfusion (I/R) injury in the heart as the CaMK-II inhibitor KN-93 and a CaMK-II inhibitory peptide enhanced the contractile recovery in parallel with a decreased extent of infarction and the decreased presence of apoptotic markers [417].

Cell death by I/R is induced by elevated mitochondrial Ca^{2+} uptake through the mitochondrial permeability transition pore (mPTP) and

production of ROS. Both Ca^{2+} and ROS activate CaMK-II, which leads to both apoptotic and necrotic cell death. Necrotic cell death then leads to a severe inflammatory response damaging the tissue. It is well known that the latter response is mediated through NFκB, which is released from its inhibitor IκBα, that undergoes phosphorylation followed by proteosomal degradation, and NFκB translocates to the nucleus where it positively regulates the transcription of a number of proinflammatory cytokines and other factors. A study by Ling et al. [418] indicated that CaMK-IIδ is involved in inflammation, infarct and ventricular malfunction induced by myocardial I/R and by NFκB. CaMK-IIδ knock-out mice were protected from I/R damage and inhibited I/R-induced inflammation and NFκB-mediated gene upregulation. These combined results are not in line with studies showing that CaMK-II may be beneficial in reversible I/R injury where phosphorylation of phospholamban at Thr17 by CaMK-II seems to be important in the stunned heart [419].

As described above CaMK-II is involved in mediating ER stress to the mitochondria-dependent apoptotic signaling pathway. Roe and Ren [420] found that oxidative activation of CaMK-II plays a major role in ER stress-induced apoptosis of cardiomyocytes. The effect of tunicamycin, an ER stress inducer, which leads to cardiomyocyte abnormalities and apoptosis could be inhibited by the CaMK-II inhibitor KN-93 in a similar way as achieved with cardiac specific overexpression of the antioxidant catalase. Recent literature shows that the effect of CaMK-II on mPTP is mediated through increasing the mitochondrial Ca^{2+} uniporter (mCU) current and reducing its Ca^{2+} tolerance. A cardiac and mitochrondria targeted CaMK-II inhibitor prevented mPTP opening, mitochondrial disruption and protected from I/R injury-mediated apoptotic cell death in vivo similar to known mPTP inhibitors such as cyclosporine A [421]. The same study indicates that CaMK-II may directly regulate the function of the mCU by phorphorylating it at serines 57 and 92.

Another CaM-dependent apoptotic pathway was described by Li et al. [422]. HEK293 cells transformed with the T-large antigen showed increased expression of the potassium channel Kv4.3 transcriptionally induced by the activity of the Sp1 transcription factor, which itself is controlled by the large T-antigen. The Kv4.3 K^+ channels are associated with a variety of diseases including heart failure and diabetes. One example is hypertrophic heart where Kv4.3 channel expression is gradually decreased. Inactivation of Kv4.3 leads to CaMK-II-mediated apoptosis and necrosis that can be reversed by the CaMK-II inhibitor KN-93 [422]. The authors suggest that downregulation of this channel may lead to the dissociation of CaMK-II from the channel followed by enzymatic activation of CaMK-II and initiation of a cell death inducing process in hypertrophic hearts.

Protein dephosphorylation by CaN may counteract the activity of proapoptotic kinases in the heart. Guo et al. [423] found that pretreatment of rat cardiomyocytes with the regulatory subunit of CaN (CNB) activates CaN, which reduces cell death in response to hypoxia/reoxygenation injury that resembles the I/R injury model. This confirmed earlier observations (reviewed in Ref. [423]), indicating an important beneficial role of CaN in hearts physiology. Guo et al. also found [423] that pretreatment of cardiomyocytes with CNB, prior to injury causing insults enhanced expression of Bcl-2 and Bcl-X_L indicating a function of these antiapoptotic proteins in the protective role of CaN in the heart [423]. However, it is not known at the present time, which are the critical direct targets of CaN for its antiapoptotic function in the heart. Not surprisingly, also proapoptotic functions of CaN in the heart induced by the β-adrenergic pathway have been described earlier [424] making it difficult to judge whether increasing CaN activity in the heart would be of general benefit for protecting patients from heart failure.

A role of microRNA in the regulation of cardiac Ca^{2+}-mediated apoptosis was recently found by Cha and collaborators [425]. They showed that micro-RNA-145 repressed the expression of CaMK-IIδ, reduced ROS-induced Ca^{2+} overload and suppressed apoptosis by directly targeting CaMK-IIδ.

4.4. Calmodulin functions in the death of platelets, hepatocytes and pancreatic cells

The non-nucleated platelet cells have important functions in the integrity of endothelium and homeostasis. CaM is attached to various cell surface receptors in platelets, and CaM inhibitors affect the receptor-mediated function of platelets. Wang et al. [426] found that the CaM inhibitors W-7, tamoxifen and trifluoperazine (TFP) all induced platelet apoptosis through the mitochondrial pathway. CaM antagonists did not impair platelet activation but affected adhesion and aggregation. As CaM antagonists increase intracellular Ca^{2+} levels it is suggested that mitochondrial inner membrane potential loss may be caused by mitochondrial Ca^{2+} overload. Thus, patients treated with CaM antagonists may suffer from thrombocytopenia due to the proapoptotic effect of these drugs on platelets.

It is well known that cytotoxic effects increase intracellular Ca^{2+}, a process that may be mediated by CaM. As an example, Tsutsui et al. [427] showed that D-galactosamine (GalN), a hepatotoxic compound that induces apoptosis, involves Ca^{2+} and CaM activity. Interestingly, increase of GalN-induced apoptosis seems to be associated with an initial increase in a perinuclear Ca^{2+} signal followed by its diffusion into the cytoplasm, whereas the necrotic effect of GalN is Ca^{2+} independent indicating that GalN operates through different mechanisms leading to cell death.

An early study by Yu et al. [428] indicated that CaM overexpression in pancreatic β-cells might lead to a rise in blood glucose, which is preceded by apoptotic β-cell death. As inhibition of nNOS prevented the CaM-induced hyperglycemia and β-cell death the authors speculated that CaM overexpression might sensitize β-cells to Ca^{2+}-dependent nNOS activation triggering cell death.

4.5. Calmodulin functions in the immune system and HIV-induced cell death

Early work indicated an active role of Ca^{2+} and CaM in glucocorticoid-induced apoptosis in immature T-cells. Buffering sustained elevation of Ca^{2+} concentration induced by glucocorticoid with Quin-2 or treatment of cells with the CaM inhibitor calmidazolium prevented DNA fragmentation and apoptotic death [429]. Furthermore, Dowd et al. [430] found an up to 10-fold increase of CaM mRNA level in WEHI7.2 lymphocytes after glucocorticoid treatment indicating steroid hormone-regulated expression of CaM. Elevated CaM expression may be important for the execution of the apoptotic program supported by the observation that treatment with the CaM inhibitor calmidazolium protected lymphocytes from apoptosis.

A major effect of HIV infection is the apoptotic death of T cells known to be frequently mediated by Ca^{2+} signaling. It was found that CaM may be involved in HIV-triggered and Fas-dependent T-cell apoptosis [431] as transfection of cells with vectors expressing the wild type gp160 HIV envelope protein, in contrast to a truncated version lacking the CaM binding domain, enhanced apoptosis. Moreover, the CaM antagonists TFP and tamoxifen inhibited enhancement of Fas-triggered apoptosis by gp160. It has also been found that an increase in CaM concentration is needed for gp160-dependent enhanced Fas-mediated apoptosis, where caspase-3 activity is involved in the apoptotic process in Jurkat T cells with inducible pg160 expression [432]. This process, which requires CaM binding to gp160, can be blocked by a single point mutation in the CaM-binding site of gp160 [433]. No effect of this mutation and several other point mutations in the CaM binding sequence of gp160, which all abolished the apoptotic effect of gp160, was found on viral replication and envelope expression supporting the specific effect of CaM on apoptosis [433]. In addition, CD4+ T-cells from acquired immunodeficiency syndrome (AIDS) patients showed enhanced spontaneous T-cell apoptosis compared to healthy controls. In the presence of the CaM inhibitors mentioned above the rate of apoptosis was significantly inhibited in vitro [434]. Confirming these data it

was found that expressing gp160, but not the gp160 cleavage product gp120, in human CD4$^+$ cells induced apoptosis mediated by Ca^{2+}/CaM-dependent intracellular Ca^{2+} release [435]. The use of the CaM antagonist W-7 and the cell permeable Ca^{2+} chelator BAPTA-AM in the gp160 expressing UE160 cells could prevent nuclear fragmentation, a typical apoptotic hallmark. The same group also showed later that the C-terminus of gp160 is responsible for direct complex formation with CaM and that this complex is essential for the initiation of cell death of the monocytoid cell line U937 that expresses high levels of CD4 [436].

Contini et al. [437] found that HLA class I-mediated apoptosis of Epstein–Barr virus specific CD8 cytotoxic T cells through Fas ligand (FasL)/Fas was dependent among other factors on the CaMK-II signaling pathway. This is followed by nuclear translocation of the transcription factor together with NFκB, was responsible for inducing FasL expression followed by T-cell apoptosis. In addition, apoptosis induced upon activation of the B-cell receptor (BCR) in B-lymphocytes appears to involve the participation of CaM. This was shown by decreasing the levels of cellular CaM to 40% of its original value by knocking down the CaMII gene in DT40 cells, which partially protected from BCR-induced apoptosis [147].

4.6. Calmodulin-mediated mechanisms of apoptosis in tumor cells

CaM antagonists have been shown to contribute to the induction of apoptosis in cancer cells. An early study by Frankfurt et al. [438] demonstrated that blocking CaM function using the CaM antagonists TFP and W-7 in combination with the estrogen receptor (ER) antagonist tamoxifen induced apoptosis in breast cancer cell lines and also in cells freshly isolated from ER-positive and ER-negative breast cancer cells in a synergistic manner. The apoptotic effect of tamoxifen was attributed to its inhibitory effect on CaM function independent of its effect on the ER [439], as it has been shown earlier that this agent binds to and inhibits CaM [440].

Lack of Fas expression in the aggressive human cholangiocarcinoma may explain the resistance of this cancer type to generally used cancer treatments. A study by Pan et al. [441] showed that TFP and tamoxifen act in a Fas-dependent fashion to induce apoptosis in cholangiocarcinoma cells. Fas/APO1 (CD95) is a major cell death receptor belonging to the TNF-α superfamily. TFP and tamoxifen induced apoptosis only in cholangiocarcinoma cells that expressed Fas, indicating its involvement in the apoptotic cell death mechanism. Cells with functional Fas expression were not tumorigenic in nude mice in contrast to cells that lost Fas, underlining the importance of the Fas signaling system to prevent uncontrolled cell proliferation. Furthermore, McDonalds' group also observed that interferon-γ increased the susceptibility towards the CaM antagonists to induce caspase-dependent and caspase-independent apoptosis in cholangiocarcinoma cells weakly expressing Fas [442]. In addition, they found that CaN and CaMK-II are not likely to be involved in the CaM-mediated cell death mechanism, as inhibitors of these proteins were not effective in inhibiting apoptosis. A possible mechanism of CaM-triggered Fas-dependent apoptosis was indicated by the discovery of a direct interaction between CaM and the cytosolic death domain (DD) of Fas in a 2:1 (mol/mol) CaM/Fas ratio with dissociation constant of 2 μM and with both the N- and C-lobes [443–445]. Binding was shown to be Ca^{2+}-dependent and specific to Fas, as other death receptors (DR) such as the TNF-α family receptors DR4 and DR5 did not interact with CaM. A mutation in the CaM-binding sequence of Fas decreased CaM-binding as well as Fas-mediated apoptosis in Jurkat T-cells. Upon Fas activation an initial increased interaction between Fas and CaM was observed after 30 min followed by a decrease to 50% after 2 h compared to controls. Direct CaM/Fas interaction and induction of Fas-mediated apoptosis by CaM antagonists were also detected in osteoclasts indicating a general significance of these findings [446].

Interestingly, knockdown of the Fas adaptor protein FADD in pancreatic cancer cells led to resistance towards Fas-mediated signaling that otherwise provokes cell death [447]. Instead, Fas initiated a survival pathway in the absence of FADD that involved ERK activation and could be inhibited by CaM antagonists. It was also found that FADD knockdown leads to a recruitment of CaM, Src and phosphorylated Src to the Fas-activated signaling complex. CaM directly interacted with the SH2 domain of Src as describe above, leading to its phosphorylation suggesting a crucial role of CaM in Fas-mediated FADD-independent cell survival. This pathway seems to be also independent of a c-FLIP (an enzymatically inactive caspase-8 homologue)-mediated signaling mechanism working through NFκB which is a known cell viability supporting process also found to operate in cancer cells. Rapold and collaborators [448] found that Fas induced a Ca^{2+}/CaMK-II-dependent pathway with involvement of ERK1/2 phosphorylation leading to lipolysis in 3T3-L adipocytes confirming the existence of a Fas-driven non-apoptotic pathway that involves CaM signaling.

TNF-related apoptosis-inducing ligand (TRAIL) signals through the TNF-α receptor family members DR4 and DR5 to induce extrinsic apoptosis in a variety of cancer cells but is not generally toxic to most normal cells making it a promising anticancer candidate drug. On the other hand, TRAIL is only effective in combination with other drugs in TRAIL-resistant tumors. Hwang and collaborators [449] found that co-treatment of lung tumor cells with TRAIL and the CaM antagonist fluphenazine-N2-chloroethan or the use of CaM siRNA sensitized the cells to TRAIL-induced apoptosis. The authors proposed that this CaM antagonist might inhibit the TRAIL-induced interaction of the apoptosis inhibitory protein c-FLIP, earlier shown to directly bind to CaM [450]. In addition, Chen et al. [451] showed that the Fas-mediated DISC contains CaM and that Fas enhanced the recruitment of CaM to the DISC in cholangiocarcinoma cells. A second possibility of CaM antagonist function in the TRAIL-mediated pathway is through inhibiting the prosurvival Akt-dependent pathway. Indeed, the CaM antagonist inhibited Akt phosphorylation leading to decreased expression of death inhibiting molecules such as c-FLIP and inhibitor of apoptosis (IAP) family members [449]. Fujikawa et al. [452] showed that CaMK-II through Akt, likely involving direct phosphorylation, might regulate TRAIL-induced caspase-8-dependent apoptosis in fibroblast-like synovial cells as inhibitors to both kinases augmented apoptosis. A recent study by Kaminskyy et al. [453] supports an antiapoptotic activity of CaM in TRAIL signaling based on the observation that Ca^{2+}/CaM suppressed the survival of non-small cells lung carcinoma (NSCLC) cells with TRAIL-mediated upregulation of the short c-FLIP form. The latter protein is induced in cells, which are resistant to undergo TRAIL, DR4 and caspase 8-mediated apoptosis, which normally occurs in most NSCLC cells.

In prostatic cancer cells a CaM/CaN-dependent signaling pathway leading to apoptotic cell death and induced by the SERCA inhibitor thapsigargin was discovered by Tombal et al. [454]. In contrast to the most frequently used anti-cancer drugs, which are only effective in rapidly proliferating cells, thapsigargin induces cell death in malignant androgen-independent prostate cancer cells proliferating at a low rate. Typical consequences of the activated CaM/CaN signaling pathway leading to cell death such as translocation of NFAT to the nucleus and Bad to the mitochondria after dephosphorylation of both proteins as well as release of cytochrome c from the mitochondria to the cytosol was observed.

Inhibition of CaMK activity has been demonstrated to induce apoptosis in human promyelocytic leukemia cells. The CaMK inhibitor KN-62 and the CaM antagonist TFP at nontoxic concentrations were shown to sensitize cells to etoposide (VP-16) cytotoxicity [455]. This effect was attributed to enhancement of the topoisomerase II (TOPO-II)-induced DNA cleavage complex formation followed by S phase-specific apoptosis of the cells. The used inhibitors enhanced phosphorylation of TOPO-II, which may explain their sensitizing effect on the cells resistant to VP-16 to undergo TOPO-II-mediated DNA cleavage. Newer results from the same group using VP-16 sensitive and insensitive HL-60 cells indicated that the CaN inhibitor KN-62 modulates the effect of VP-16 only in the S phase of the cell cycle in VP-16 sensitive cells whereas in VP-16 resistant cells nontoxic concentrations of KN-62 had

a modulatory effect in a cell cycle independent manner [456]. A possible explanation of these results is that KN-62 both stabilizes TOPO-II α and β form-induced DNA cleavage as well as protein levels and phosphorylation of the β, form which is not cell cycle regulated in contrast to the α form. In a more recent article, the effect of the CaMK selective inhibitor KN-93 on choriocarcinoma cells was tested [231]. Among other effects KN-93 induced apoptosis in these cells as demonstrated by exposure of phosphatidylserine monitored by annexin-V staining, DNA fragmentation and loss of the mitochondrial membrane potential.

The clinically widely used drug 5-fluorouracil (Adrucil™) (5-FU) for treatment of a variety of cancers is known to lead to genome impairment and inducing apoptosis through mechanisms involving p53. Recently, Can *et al.* [457] discovered that that 5-FU treatment lead to influx of Ca^{2+} from the extracellular space and that CaM-dependent phosphorylation of p53 at three positions (Ser15/33/37) was necessary for its function to activate an apoptotic pathway in colon carcinoma cells. However, it is not known whether this is a direct phosphorylation by CaMK(s) or indirect by other upstream kinase(s). Following p53 activation caspase-8 was activated through the DR5/DISC pathway finally leading to activation of downstream executer caspases and apoptotic cell death.

5. Calmodulin in regulation of autophagy

5.1. General comments on the functional roles of autophagy

Autophagy is a physiological mechanism by which cells remove in a controlled fashion long-lived cellular proteins or protein aggregates, defective intracellular organelles and/or pathogens, by engulfing these components into intracellular multimembrane vesicles denoted autophagosomes and delivering them for their disposal to lysosomes (reviewed in Ref. [458]) (Fig. 11). Autophagy plays an important pro-survival role especially under starving conditions accompanying many physiological functions but also plays a pro-death role under certain conditions. Autophagy participates in the immune response, including the clearance of bacteria and viruses (reviewed in Refs. [459–463]); metabolic homeostasis (reviewed in Ref. [464]); organismal development (reviewed in Refs. [465,466]); and normal physiological aging (reviewed in Ref. [467]). Autophagy also participates in the pathogenesis of various diseases including cancer (reviewed in Refs. [468–471]). The carcinogenic transformation of cells is frequently associated with the suppression of autophagy, suggesting that its induction with some drugs could open venues for new anti-cancer therapeutic strategies (reviewed in Ref. [472]).
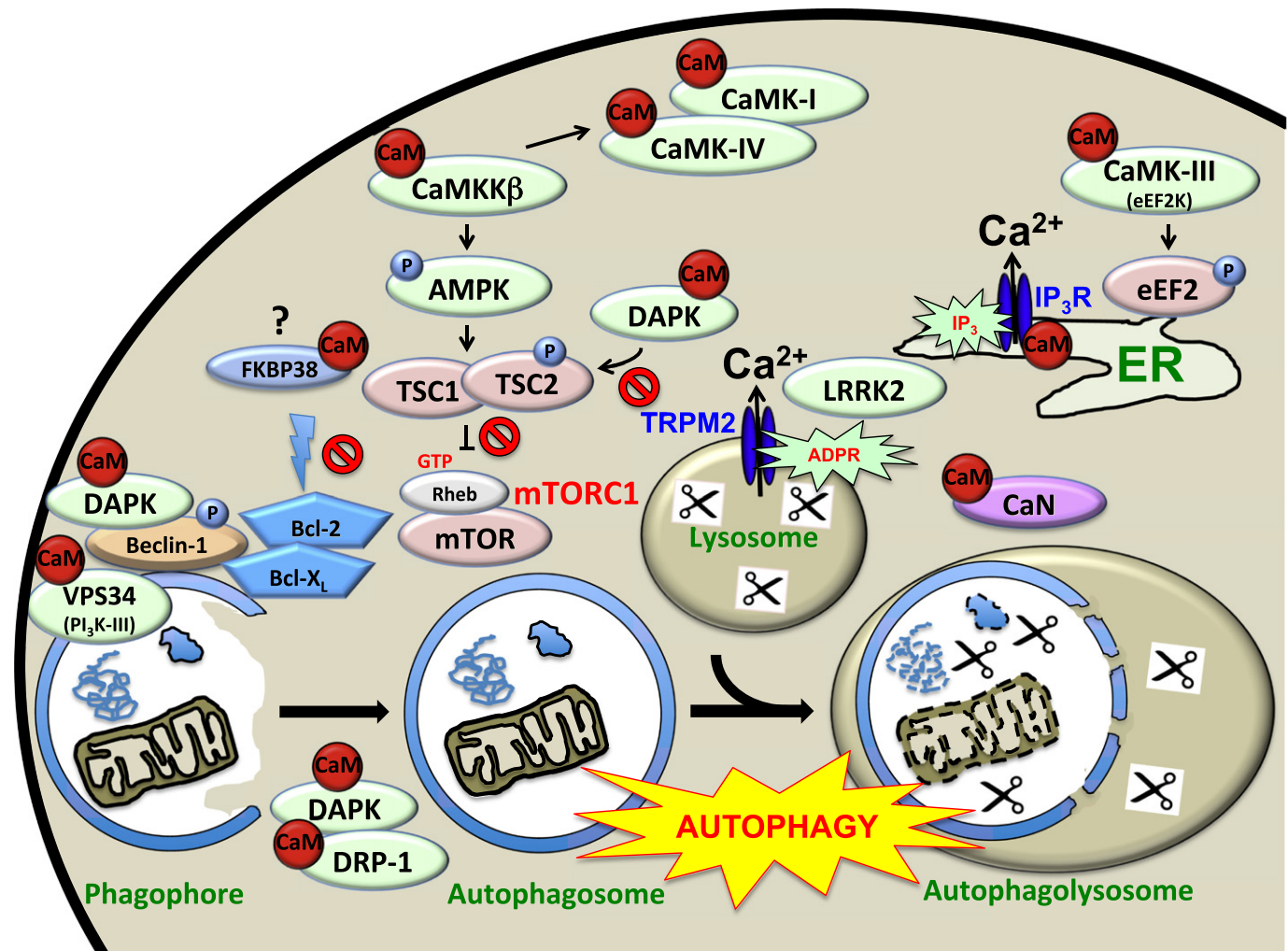
Fig. 11. Calmodulin action on systems controlling autophagy. The scheme depicts the major components participating in autophagy and the different calmodulin (CaM)-dependent systems controlling this process. ADPR, ADP-ribose; AMPK, AMP-activated protein kinase; Bcl-2, B-cell lymphoma 2 protein; Bcl-X_L, B-cell lymphoma-extra large protein; CaMK-I/III/IV, CaM-dependent protein kinases I/III/IV; CaMKKβ, CaM-dependent protein kinase β; CaN, calcineurin; DAPK, death-associated protein kinase; DRP-1, DAPK-related protein kinase 1; eEF2, eukaryotic elongation factor 2; eEF2K, eEF2 kinase (CaMK-III); ER, endoplasmic reticulum; FKBP38, FK506-binding protein 38; IP$_3$, inositol-1,4,5-trisphosphate; IP$_3$R, IP$_3$, receptor; LRRK2, leucin-rich repeat kinase 2; mTOR, mammalian target of *rapamycin*; mTORC1, mTOR complex C1; Rheb, Ras homolog-enriched in brain G protein; TRPM2, transient receptor potential melastatin 2; TSC1/2, tuberous sclerosis proteins 1 and 2; VPS34 (PI$_3$K-III), phosphatidylinositol 3-kinase type III.

The best studied type of autophagy is macroautophagy, where whole organelles, cytoplasmic components and/or pathogens are degraded in vesicular structures denoted autophagosomes after fusion with lysosomes (reviewed in Refs. [473,474]) (Fig. 11). Another type is microautophagy, in which cytoplasmic cargo, including piecemeal organelle fragments, are directly engulfed into lysosomes/vacuoles by the formation of an autophagic tube from which the vesicles are escised (reviewed in Ref. [475]). A third type is chaperone-mediated autophagy, in which proteins bound for degradations form complex with chaperones that facilitate their direct delivery to the lysosomal lumen (reviewed in Ref. [476]). Although autophagy and apoptosis are mechanistically different cellular processes, there are some common regulatory proteins that intervene in both of them, such as the anti-apoptotic/anti-autophagy regulators Bcl-2 and Bcl-X$_L$, and the all-important serine/threonine kinase DAPK among others, thus these systems establish a vivid crosstalk controlling the fate of the cells by undertaking one of these pathways to either achieve cell restoration or cell death (reviewed in Refs. [373,477]). The enzymes PARP1/2 appears to play a pivotal decision making role in directing the cells to authophagy or apoptosis by Ca^{2+}-mediated mechanisms after receiving an environmental insult such as oxidative stress [32]. Also, the transient permeabilization of the mitochondrial membrane occurring during intrinsic apoptosis triggers autophagy of the damaged mitochondrium (reviewed in Ref. [478]), further linking both phenomena. In addition, the dual-role played by DAPK as a decision maker in pro-survival or pro-death signaling appears to be determined by the activation level of the implicated mechanisms, an ancient phenomenon that is phylogenetically conserved even in the worm *C. elegans* (reviewed in Ref. [479]).

5.2. The calcium signal and the implication of calmodulin in autophagy

An important signaling component in autophagy is the mobilization of calcium (reviewed in Ref. [85]), leading to the formation of the Ca^{2+}/CaM complex and the activation of an array of CaM-dependent target systems that play prominent roles during this process (Fig. 11). Ca^{2+} mobilization during autophagy has been demonstrated to occur via IP$_3$ receptors/Ca^{2+}-release channels that might have a dual role: either a suppressing-function of autophagy, when Ca^{2+} uptake by the mitochondria enhances oxidative phosphorylation and ATP levels decreasing the activity of AMPK; or an enhancing function by different signaling routes where CaM is implicated by activating CaMKKβ and CaMK-I among other pathways (reviewed in Refs. [480–483]), inducing the phosphorylation of AMPK as demonstrated in various tissues, see for example [484]. Calcium mobilization during ER-stress induced for example by 2-deoxyglucose is also a potent signal to activate the CaMKKβ/AMPK pathway [485]. An upstream kinase denoted leucin-rich repeat kinase 2 (LRRK2) appears to activate the CaMKKβ/AMPK pathway, an effect that can be mimicked by NAADP, a lysosomal Ca^{2+}-mobilizing agent [486] (Fig. 1). This demonstrates that the autophagy-initiating Ca^{2+} signal could have different origin. The Ca^{2+} signal activating the CaMKKβ/AMPK route leads to the phosphorylation of the tuberous sclerosis proteins 1/2 (TSC1/TSC2) complex and the downstream repression of mammalian target of *rapamycin* (mTOR) with the subsequent induction of autophagy [482,487–489], a process that is inhibited by Bcl-2 ectopically located in the endoplasmic reticulum [488]. This mechanism was commented by Swerdlow and Distelhorst [490] stressing the common implication of Bcl-2 both in apoptosis and autophagy. Although the intracellular mobilization of Ca^{2+} plays an important role in setting autophagy induced by mTOR inhibition [80], in *S. cerevisiae* a Ca^{2+}-independent role of CaM has been observed in the process of microautophagy, where cytosolic components are directly delivered into the vesicular degradation system by a specialized membrane invagination [491].

Interestingly, CaMKKβ can be by-passed in some circumstances, as for example in TRAIL-induced autophagy where transforming growth factor-β-activating kinase 1 (TAK1) acts as an activator of AMPK without the involvement of the former [492]. And furthermore, AMPK can also be bypassed in the Ca^{2+}-mediated induction of autophagy as demonstrated using embryonic fibroblasts from AMPK-knockout mice [493]. In addition, Guo and collaborators [494] described a CaMK-Iα/AMPK-dependent but mTOR complex C1 (mTORC1)-independent autophagic mechanism acting during acute neutrophilic inflammation of the lung induced by lipopolysaccharides. Overall these observations underline the existence of additional compensatory mechanisms unrelated to canonical autophagic pathways.

CaMK-III, also denoted as eEF2-kinase, plays an important role controlling autophagy as its downregulation inhibits autophagy and conversely its overexpression enhances this process [495,496]. This also suggests the existence of a CaM-mediated control of protein synthesis during the autophagic process. Besides, a protective effect of CaMK-III (eEF2-kinase)-mediated autophagy in cancer cells subjected to metabolic stress has been described [497], and inhibition of CaMK-III suppressed autophagy but promotes apoptosis when tumor cells were subjected to pharmacological inhibition of Akt [498].

In accordance with the implication of mTOR inhibition in setting autophagy, it is well known that the PI$_3$K/Akt/mTOR pathway is involved in cell survival, and as previously mentioned, activation of this axis leads to inhibition of autophagy. As both PI$_3$K and Akt activities are regulated by CaM [499–501], this could represent another route by which CaM exerts a repressive action on autophagy. This issue appears to be quite complex, as suppression of autophagy by activating the PI$_3$K/Akt/mTOR pathway also appears to promote necrotic cell death, at least in cell models where autophagy plays a pro-survival function [502].

CaM-dependent serine/threonine kinases such as DAPK and other members of this kinase family play important roles in autophagy by facilitating the formation of autophagosomes and stimulating vesicular traffic (reviewed in Refs. [373,374]). Thus, it was shown that both DAPK and its related kinase DRP-1 participate in membrane bebbling and the formation of autophagic vesicles [503]. One mechanism is by the interaction of DAPK with the microtubular-associated protein 1B (MAP1B) and the autophagosomal protein Atg8 [504,505]. Moreover, DAPK also interacts with and phosphorylates TSC2, thus suppressing the activity of the TSC1/TSC2 complex. This results in the activation of mTORC1 promoting cell survival and repressing autophagy under normal but not hyperactive growth factor-stimulated MAPK signaling conditions [506]. CaM-mediated activation of DAPK also induces the phosphorylation of beclin-1 and its dissociation from their inhibitory regulators Bcl-X$_L$ and Bcl-2, followed by the induction of the autophagic process [507,508]. In a recent study, Han *et al.* [509] have shown that beclin-1 also interacts with the ErbB2 receptor increasing its phosphorylation and activating the Akt pathway. Furthermore, in breast carcinoma cells, which are resistant to the ErbB2 tyrosine kinase inhibitor lapatinib, this agent disrupts the ErbB2/beclin-1 complex increasing beclin-1 cytosolic concentration and thereby facilitating the autophagic response. The IFN-γ-regulated transcription factors ATF6 (activating transcription factor 6) and C/EBP-β are essential for the expression of DAPK1 and the subsequent induction of autophagy [510], while the degradation of DAPK, counterbalancing IFN-γ action, is controlled either by its degradation in the proteasome after polyubiquitination [511], or by lysosomal degradation after interaction with TSC2 via its death domain [512].

The lack of supply of essential amino acids is a strong signal to induce autophagy via the CaMKKβ/AMPK pathway resulting in the activation of the autophagy-initiating UNC-51-like kinase (ULK1) and inhibition of the negative regulator of autophagy mTORC1 [513]. The formation of autophagosomes upon mTOR inhibition is concomitant with the synthesis of phosphatidylinositol 3-phosphate (PI$_3$P), and this phosphoinoside interacts with the so-called WD-repeat protein interacting with phosphoinositides (WIPI) proteins located in the autophagosomal membranes. In this context, it has been demonstrated that a CaMKK/CaMK-I pathway contributes to the stimulation of WIPI-1 during Ca^{2+}-

Table 3

Effects of distinct CaM antagonists on tumor cells.

CaM antagonist	Tumor/cell/tissue	Origin	Effect	References
B859-35	Neuroendocrine lung tumors	Hamster	Inhibits tumor growth *in vivo*	[579]
	Lung carcinoid NCI-H727, adenocarcinoma NCI-H322 and NCI-H358 cells	Human	Inhibits cell proliferation (at 0.001 pM–100 nM)	[580]
Berberine[a]	Hepatocellular carcinoma Bel7402 cells	Human	Arrest the cell cycle at G_1 W-7 and TFP pontentiate its action. Inhibits CaMK-II and MEK1, and p27 degradation (CaM-unrelated effects observed)	[585]
Calmidazolium (R24571)	Breast adenocarcinoma MCF-7, T47D, ZR-75-1 cells	Human	Inhibits cell proliferation at the early to mid G_1 phase of the cell cycle. Induces apoptosis-like cell death potentiated by antiestrogen drugs	[165,439,588]
	Serous cystadenocarcinoma ovary cells	Human	Inhibits cell proliferation and prevents EGF binding to its receptor	[583]
	Pituitary tumor GH_3 cells (ER-positive)	Rat	Induces apoptosis-like cell death potentiated by antiestrogen drugs	[588]
	ASV-transformed cells	Rat	Inhibits cell cycle at late G_1	[163]
	Ehrlich ascites tumor cells	Mouse	Inhibits protein synthesis	[605]
	Astrocytoma C6 cells	Rat	Inhibits cell proliferation	[589]
Chlorpromazine	Leukemia cells	Human and mouse	Inhibits cell growth and clonogenicit	[593]
	Astrocytoma C6 cells	Rat	Inhibits cell proliferation	[589]
Compounds 1, 2 and 3[b]	Breast adenocarcinoma T-47D, MCF-7B, MCF-7T and MDA-MB-231 cells	Human	Inhibits cell proliferation	[578]
Compound 48/80[c]	Promyelocytic leukemia HL-60 cells	Human	Inhibits cell proliferation	[154]
Dequalinium	Astrocytoma C6 cells	Rat	Inhibits cell proliferation	[87][d]
Fluphenazine mustard	Astrocytoma C6 cells	Rat	Inhibits cell proliferation	[87][d]
FPCE	Lung cancer H1299 cells	Human	Sensitizes cells to TRAIL-induced apoptosis	[449]
HBC	Colon cancer HCT15 cells	Human	Inhibits cell proliferation (upregulates p21$^{Cip1/Waf1}$)	[333]
HBCP	Colon cancer HCT15 cells	Human	Inhibits cell proliferation (upregulates p21$^{Cip1/Waf1}$)	[332]
J-8	Breast adenocarcinoma T-47D, MCF-7B, MCF-7T and MDA-MB-231 cells	Human	Inhibits cell proliferation	[578]
	Melanoma A375-SM cells	Human	Reduces cell invasiveness	[641]
KS-501 and KS-502[e]	Leukemic L1210 lymphocytes	Mouse	Inhibits cell proliferation (less effective in MDR variants)	[594]
Melittin	Serous cystadenocarcinoma ovary cells	Human	Inhibits cell proliferation and prevents EGF binding to its receptor	[583]
	Leukemia cells	Human and mouse	Inhibits cell growth and clonogenicity	[593]
	Astrocytoma C6 cells	Rat	Inhibits cell proliferation	[589]
Phenothiazine[f] (thiodiphenylamine)	Colon adenocarcinoma WiDr cells	Human	Inhibits cell proliferation	[581]
Phenothiazines[g]	Glioma C6 cells	Rat	Inhibit cell proliferation	[591]
Prochlorperazine	Melanoma B16 cells	Mouse	Inhibits cell proliferation (DNA synthesis)	[133]
Promethazine	Colon adenocarcinoma WiDr cells	Human	Inhibits cell proliferation	[581]
Rhodamine-123	Astrocytoma C6 cells	Rat	Inhibits cell proliferation	[87][d]
Tamoxifen	Melanoma A375-SM cells and uveal melanoma cells	Human	Reduces cell invasiveness (effect unrelated to ER antagonism but related to CaM inhibition)	[641]
Trifluoperazine	Breast adenocarcinoma MDA-MB-231 and MCF-7 cells	Human	Inhibits cell proliferation and colony formation	[439,642]
	Fibrosarcoma HT1080 cells	Human	Enhances Egr-1 expression inhibiting cell growth	[293]
	Glioma C6 cells	Rat	Inhibits cell proliferation	[589,591,597]
	Promyelocytic leukemia HL-60 cells	Human	Inhibits cell proliferation	[154,592]
	Leukemia cells	Human and mouse	Inhibits cell growth and clonogenicity	[593]
	Ehrlich ascites tumor cells	Mouse	Inhibits protein synthesis	[605]
W-5	Serous cystadenocarcinoma ovary cells	Human	Inhibits cell proliferation and prevents EGF binding to its receptor	[583]
	Breast adenocarcinoma MCF-7 cells	Human	Inhibits cell proliferation (very weakly)	[439]
W-7	Skin tumors	Mouse	Inhibits tumor promotion by DMBA plus teleocidin	[595]
	Melanoma B16 cells	Mouse	Inhibits cell proliferation (DNA synthesis) and down-regulates metastasis-associated genes	[133,164,548]
	Serous cystadenocarcinoma ovary cells	Human	Inhibits cell proliferation and prevents EGF binding to its receptor	[583]
	Breast adenocarcinoma MDA-MB-231 and MCF-7 cells (ER-positive and ER-negative variants)	Human	Inhibits cell proliferation and colony formation	[439,578,642]
	Lewis lung carcinoma	Mouse	Inhibits metastasis development	[549]
	Colon adenocarcinoma WiDr cells	Human	Inhibits cell proliferation	[581]
	Thyroid papillary adenocarcinoma NIM 1 cells	Human	Inhibits IL-1 -induced cell proliferation (DNA synthesis)	[586]
	Hepatocellular carcinoma VX_2-L cells	Rabbit	Inhibits cell proliferation (DNA synthesis)	[584]
	ASV transformed cells	Rat	Inhibits cell cycle at late G_1	[163]
	Solid sarcoma 180	Mouse	Inhibits tumor growth *in vivo*	[587]
	Chemically-induced glioma GA-1 cells	Rat	Inhibits tumor growth *in vitro* and *in vivo*	[590]
	Glioma C6 cells	Rat	Inhibits cell proliferation	[597]
	Ehrlich ascites tumor cells	Mouse	Inhibits protein synthesis	[605]
	Promyelocytic leukemia HL-60 cells	Human	Inhibits cell proliferation and phagocytosis	[592]
W-13	Breast adenocarcinoma MCF-7, ZR-75-1B and T47D (ER-positive); LY2 and RR (ER-positive/TamoxR); and MDA-MB-231 and MDA-MB-435 (ER-negative) cells	Human	Inhibits cell proliferation and colony formation	[576,577,642]
	Promyelocytic leukemia HL-60 cells	Human	Inhibits cell proliferation and phagocytosis	[592]

mediated autophagy, a process that appears to be independent of AMPK action, as it also occurs in AMPK-deficient fibroblasts [514]. In addition, CaMK-IV plays a protective role during ischemia–reperfusion (I/R) injury of the liver by activating the prosurvival autophagic pathway in hepatocytes, as demonstrated in CaMK-IV knockout mice, where I/R produces more extensive organ damage compared to controls and a reduction of this damage by rapamycin, an agent which allosterically inhibits mTOR [515]. An interesting example of the implication of CaMKKβ during autophagy induced by virus infection is the activation of the CaMKKβ/ AMPK signaling pathway by viroporin, a rotavirus pore-forming protein that releases Ca^{2+} from the endoplasmic reticulum [516,517].

Phosphatases counterbalancing the different kinases implicated in autophagy are also important regulators of the overall process, as early demonstrated by the inhibitory action of okadaic acid and other phosphatase inhibitors on autophagy in hepatocytes, and the protective effect that CaM antagonists and CaMK-II inhibitors have on okadaic acid action [518–520]. The assayed inhibitors mostly targeted protein phosphatase type 2A (PP2A), possibly by dephosphorylating an inhibitory autophosphorylation site in DAPK resulting in the activation of this kinase during ER stress-induced autophagy [521]. Nevertheless CaM is also implicated in the dephosphorylation process, as CaN may also play a protective role in autophagy. This was shown by the occurrence of enhanced autophagy in CaN-defective strains of the worm *C. elegans* [522]. Furthermore, the ubiquitin/proteasome and autophagy–lysosome pathways both degrade the CaN inhibitory protein denoted regulator of calcineurin 1 (RCAN1) in two mammalian cell lines, possibly enhancing CaN/NFAT signaling during chaperone-mediated autophagy [523].

6. Calmodulin and tumor progression

The CaM-dependent signaling mechanisms regulating either cell proliferation, programmed cell death or autophagy described above, are all of great importance for tumor cell biology. In the following sections the implication of CaM-dependent systems in major physiological processes relevant in tumorigenesis and tumor progression will be discussed.

6.1. Calmodulin and tumor growth

The level of CaM in many tumor cells appears to be higher as compared to cells from normal tissues. This was demonstrated for example in chicken fibroblasts transformed with the Rous sarcoma virus [524], in kidney epithelial cells infected with ASV [163], in different rat hepatoma cell lines [525–528], in rat fibroblasts transfected with various PKC constructs and oncogenes [529], and in human primary lung cancer cells [530]. A positive correlation between the rate of cell growth and the degree of tumor malignancy with the level of cellular CaM has been postulated [525,527,528,530,531]. Increased CaM levels, mainly in the cytosol and a concomitant decrease of membrane-bound CaM, has been noticed in hepatoma cells compared to normal liver cells [525,527]. The increased CaM expression in tumor cell lines was shown to correlate with high intracellular Ca^{2+} and increased activity of the CaM-dependent cyclic nucleotide PDE [525–528].

The levels of CaM are higher during active growth of normal tissues, as it occurs during the postnatal development of testis and spermatogenesis [532]. Moreover, cytosolic but not membrane-bound CaM increased in normal regenerating liver during partial hepatectomy concomitant with increased DNA synthesis [526]. These observations point to higher CaM requirements in fast growing cells. In a study by Chirigan *et al.* [533] it was shown that tumor cells growing independently of serum have lower nuclear CaM levels, and CaM antagonists more efficiently inhibit their proliferation as compared to serum-dependent clones. Therefore, it was concluded that nuclear but not cytosolic CaM plays a role in autonomic cell proliferation.

6.2. Calmodulin and tumor-associated angiogenesis

Tumor growth requires an efficient supply of oxygen, nutrients and systemic and/or locally-produced endocrine/regulatory factors, and the removal of waste products. To attain sizes larger than 2 mm, were simple diffusion starts to be inefficient, tumors require a capable blood supply to perform those tasks. This is bestowed by what is called tumor-associated angiogenesis. This process involves the development of new blood vessels by either the trans-differentiation of tumor stem cells into vascular cells [534] and/or the sprouting and growth of already formed peripheral vessels. Overall, this yields a competent neo-formed tumor-associated vascular system that is highly regulated and that has been targeted for anti-tumor therapeutic purpose (reviewed in Ref. [535]). Hypoxia is a major driver of tumor-associated angiogenesis, as hypoxic stresses occurring in poorly irrigated tumor microenvironments increases the cytosolic concentration of Ca^{2+} and hence the formation of the Ca^{2+}/CaM complex. This leads to an enhancement of the transcriptional activity of hypoxia-inducible factor 1 (HIF-1) increasing the expression of vascular endothelial growth factor (VEGF) and hence angiogenesis, a process that can be inhibited by CaM antagonists [536–539].

Nitric oxide (NO) has been shown to be an important regulator of angiogenesis, apparently acting in a biphasic fashion depending of the amount produced by the CaM-dependent enzyme eNOS in endothelial cells. NO has been demonstrated to control the upregulation of VEGF during angiogenesis by increasing the transcriptional activity of HIF-1 (reviewed in Ref. [540]). Nevertheless, it has been demonstrated that addition of VEGF to endothelial cells induces the activation of the CaMKKβ/ AMPK pathway leading to angiogenesis without the concourse of NOS [541]. Furthermore, embryonic angiogenesis mediated by activation of the heterotrimeric Gα13 protein is due to the activation of the transcription factor MEF2 (myocyte enhancer factor 2) with the participation of CaMK-IV and histone deacetylase 5, a substrate of this kinase [542]. In addition to this pathway, protein kinase D1 PKD1 (PKC-μ), newly classified as belonging to the CaMK group based on characteristics of its kinase domain (discussed in Ref. [543]), has been shown to participate in VEGF-mediated angiogenesis. This is accomplished by phosphorylation of histone deacetylases 5 and 7 and thus releasing the repression of MEF2-regulated transcription (reviewed in Ref. [543]). The formation of new capillary tubes *in vitro* also results in the expression of the *CEC5* gene in endothelial cells. This gene may encode a new CaMK with certain similarities but distinct from CaMK-II and CASK [544].

Notes to Table 3:

ASV, avian sarcoma virus; B859-35, (−) enantiomer of dihydropyrine 3-methyl-5-3-(4,4-diphenyl-1-piperidinyl)-propyl-1,4-dihydro-2,6-dimethyl-4-(3-nitrophenyl)-piridine-3,5-dicarboxylate-hydrochloride (*niguldipine*); CaMK-II, CaM-dependent protein kinase II; EGF, epidermal growth factor; Egr-1, early growth response protein 1; ER, estrogen receptor; FPCE, fluphenazine-*N*-2-chloroethane; IL-1α, interleukin-1α; MDR, multidrug resistant; MEK1, mitogen-activated protein kinase kinase 1; TFP, trifluoperazine; TRAIL, TNF-related apoptosis-inducing ligand.

[a] An isoquinoline alkaloid.
[b] Compounds 1–3 are variants of the W series of naphthalenesulfonamides such as W-7 and the related compound J-18.
[c] Condensation product of N-methyl-p-methoxyphenethylamine with formaldehyde.
[d] Review article.
[e] Compounds isolated from the fungus *Sporothrix* sp. KAC-1985.
[f] Basic compound from which a family of related neuroleptic antipsychotic drugs are derived.
[g] Assay of several phenothiazines.

Interestingly, media conditioned by HEK293T cells transiently expressing Grb7 or Grb7V deletion mutants lacking the CaM-BD presented strong anti-angiogenic activity when assayed *in vitro*, as compared to media conditioned by cells expressing wild type Grb7 or Grb7V [279]. This suggested the participation of Grb7 in angiogenic processes with CaM involvement. In this context and of relevance for tumor-associated angiogenesis, the stable expression of EYFP-Grb7 deletion mutant in rat glioma C6 cells stereotaxic implanted in the brain produced tumors with lower angiogenic capacity and of smaller size than those generated by cells expressing EYFP-Grb7 or EYFP [281].

6.3. Calmodulin and tumor metastasis

Multiple genes control the metastatic invasiveness of tumor cells. Among these are the *MTS1* gene, encoding the Ca^{2+}-binding protein metastasin 1 (also denoted S100A4), that is highly expressed in tumor cells with high metastatic potential (review in Ref. [545]); the metastasis suppressor gene denoted *NM23* (reviewed in Ref. [546]); and the *GRB7* gene encoding the adaptor protein Grb7 (reviewed in Refs. [278,547]). Interestingly, in highly metastatic murine melanoma cells the CaM inhibitor W-7 downregulated the expression of the *MTS1* and *NM23* genes [548]. Moreover, this CaM antagonist also inhibited the development of metastasis of primary Lewis lung carcinoma in a mouse model [549]. Deletion of the CaM-BD of Grb7 impaired cell attachment to the extracellular matrix and cell migration [281,550], which may be important in metastasis. This deletion also prevented the entry of this protein into the nucleus [551]. Overexpression of the CaM-binding hormone-regulated proliferation-associated protein 20 (HRPAP20) in breast tumor cells appears to be associated with their enhanced invasiveness by increased secretion of the matrix metalloproteinase 9 (MMP-9) [552]. This process may be CaM-dependent as tumor cells expressing a mutant HRPAP20 protein lacking the capacity to bind CaM fail to increase MMP-9 secretion and subsequent invasion.

In prostate cancer it has been shown that tumor cell lines derived from bone metastasis express Notch-1 and the α, β, γ and δ isoforms of CaMK-II. This is in contrast to prostate tumor cell lines not derived from bone metastasis, which lack the Notch-1 receptor and some CaMK-II isoforms, underscoring the implication of these kinases in Notch signaling and metastasis. CaMK-II does not appear to regulate the activity of the γ-secretase, responsible for the cleavage of Notch-1 necessary for its translocation to the nucleus [553]. The epithelial–mesenchymal transition (EMT) is an important phenomenon associated with metastatic invasion. In this context, Bergamaschi *et al.* [554] demonstrated amplification of the gene encoding CaMK-I (isoform D) in samples of triple-negative (estrogen, progesterone and ErbB2 negative) basal breast cancer patients, and found that engineered overexpression of this kinase in non-tumorigenic breast epithelial cells resulted in the loss of cell–cell adhesion and increased cell migration as well as invasiveness associated to EMT phenotypic changes. In addition, Wang *et al.* [555] have recently shown that the interaction of Ca^{2+}/CaM with the p68 RNA helicase promotes tumor cell migration and metastasis, as these processes were inhibited by a peptide that spans and blocks the IQ motif of p68 in two different animal models.

6.4. Calmodulin in tumor stem cells

Cancer stem cells form a small population of self-renewing cells within tumors that have the ability of unlimited proliferation in contrast to the majority of more differentiated cancer cells, which form the bulk mass of the tumor. These stem cells are responsible for tumor growth and the relapse of patients undergoing currently standard anti-tumor therapy, as they are more resistant to chemotherapeutic agents and ionizing radiation. Specifically targeting these cells is expected to open the path for more effective therapies (reviewed in Refs. [556–559]). Knowledge of the differentiation mechanisms of normal stem cells is of upmost importance to understand the physiological role of this cell subpopulation. Various CaM-dependent systems are known to play prominent roles in the differentiation of a variety of cell types. These include neurons (reviewed in Ref. [560]), where for example the expression of CaM and actin follows a similar spatio-temporal pattern during neurite outgrowth [561], and the δ isoform of CaMK-II, which participates in the outgrowth of neurites in embryonal carcinoma cells [562]. CaM also plays a role in the differentiation of bone cells involving both osteoblasts and osteoclasts (reviewed in Refs. [563,564]). As an additional example, CaMK-IV plays an essential role in hematopoietic stem cells differentiation, as demonstrated in *Camk4* knockout cells [565]. In neuroblastoma cells, used as a model of neural stem/progenitor cells, the CaMKK/CaMK-IV system is one of the decision-making centers for either inducing proliferation, by phosphorylation and activation of the transcription factors CREB and ATF1, or differentiation, after repressing CaMK-IV activity for example with retinoic acid [566].

CaM plays important roles in the growth of myeloid and erythroid progenitor cells, as different CaM antagonists inhibited cytokine-stimulated colony formation by these cells [567], suggesting that CaM may play a similar role in leukemia-stem cells. Hematopoietic stem cells and progenitor cells are subjected to the control of different cytokines including the stem cell factor (SCF), which is the ligand of the tyrosine kinase receptor c-Kit (reviewed in Ref. [568]). Interestingly, SCF has been shown to have a synergistic effect with other cytokines such as IL-3, IL-6 or granulocyte colony-stimulating factor (G-CSF) in the induction of a 68 kDa CaM-binding protein coinciding with an increase in thymidine kinase activity, implicated in the regulation of the G_1/S transition in myeloblastic progenitor cells [569,570]. Finding similarities and specific differences in the CaM-mediated mechanisms controlling the functionality of normal stem cells and their tumor counterparts could be of great importance to uncover new potential therapeutic targets.

It has been demonstrated that some pathways responsible for drug resistance in cancer stem cells are controlled by CaM-dependent systems such as different CaMKs, which are potential target for therapeutic intervention (reviewed in Ref. [571]). Hypoxia also plays a major role in tumor progression as it controls tumor stem cell division, and the major regulator of hypoxia-induced gene transcription HIF is known to be under the control of CaM [536–539]. In glioblastoma stem cells for example HIF-2α induces the expression of different genes, some of which are modulated by CaM, as for example NFAT-2, which participates in the CaN pathway [572]. In leukemic blastic cells, however, the expression of CaN and other phosphatases are rather low when they are in an early stage to become pluripotent stem cells [573]. It has been shown that the expression of the CaM-dependent transcription factor CAMTA1 reduces the formation of neurospheres and the rate of glioblastoma growth by a mechanism implicating miRNAs, suggesting that this transcriptional activator might also play an important role in glioblastoma stem cells [574]. Highly interesting is the fact that CaMK-IV has been shown to mediate the epigenetic control of embryonic carcinoma stem cell differentiation. This kinase phosphorylates histone deacetylases inhibiting their activity during the experimental differentiation of these cells into cardiomyocytes [575].

6.5. Calmodulin-directed pharmacological targeting of tumor cells

Multiple CaM inhibitors with distinct chemical structures have been used to inhibit tumor cell growth of different origins and species either in culture or *in vivo*, as for example: ER-positive and ER-negative breast adenocarcinomas [439,528,576–578], lung carcinomas [579,580], colon carcinomas [332,333,581], ovary cystadenocarcinoma [582,583], liver hepatocellular carcinoma [584,585], thyroid papillary adenocarcinoma [586], melanomas [133,164], sarcomas [587], pituitary tumors [588], gliomas [87,589–591], and leukemia cells [87,154,592–594] (Table 3). In addition, the CaM antagonist W-7 has been shown to prevent chemical-induced carcinogenesis in a classical skin tumor model [595], and therefore implicating the Ca^{2+}/CaM complex in tumor promotion. There are many natural compounds isolated from plant or

animal sources, such as alkaloids and different peptides among many others, which are potent CaM inhibitors (reviewed in Ref. [596]). These compounds may be of therapeutic interest but are so far mostly unexplored.

It is important to point out as mentioned in the previous sections that CaM antagonists may also inhibit mechanisms responsible for the proliferation of normal cells, as demonstrated in a variety of cell types (see for example among others Refs. [151,152,162,164,172,320,321,567,597–602]) (Table 4). Moreover, in some instances CaM inhibitors appear to be less effective in transformed than in normal cells (reviewed in Ref. [77]), as tumor cells appear to be defective in a variety of CaM-dependent mechanisms responsible for the correct and ordered function of cell proliferation (reviewed in Refs. [77,78]). This casts doubts on the efficacy of these compounds as potential therapeutic agents against cancer.

Notwithstanding the positive role exerted by CaM in the ordered control of the cell cycle and subsequent cell proliferation events, CaM also plays a relevant role inducing cell cycle arrest upon DNA damage mediated by genotoxic stress, such as ionizing radiation [603]. Therefore, the use of anti-CaM agents and radiation therapy might not be a wise strategy to be jointly followed for cancer treatment. In addition, and related with this, as different actions of CaM are mediated by CaMK-II, the activation of this kinase via ionizing radiation-induced Ca^{2+} entry through transient receptor potential vanilloid 5/6 (TRPV5/6)-like nonselective cation channels results in cell cycle arrest at G_2/M, thus facilitating the survival of leukemia cells due to a greater chance of DNA repair [604]. The mechanisms of action of CaM antagonists when arresting tumor cell growth could also be due to some general non-specific toxic effect, such as inhibiting protein synthesis as described in Ehrlich ascites tumor cells [605], or by other specialized mechanisms such as inducing apoptotic cell death for example

in breast and pituitary tumor cells [588] (see Section 4.6 for additional examples).

The inhibition of proliferation induced by CaM antagonists in general is directly correlated with their affinity for CaM [589,591,593]. However, some of the actions of CaM antagonists might be unspecific. In ovarian cancer cells for example W-5 was surprisingly reported to be more potent than W-7 [582,583], even though W-5 has a lower affinity for CaM than W-7 [606], since the absence of a chlorine atom in W-5 (and in W-12) might hinder efficient entry of these compounds into the cell. Nevertheless, and in agreement with its lower affinity for CaM, W-5 was shown to be mostly ineffective or having weaker effect than W-7 inhibiting the growth of murine sarcoma in vivo [587], cells neoplastically transformed by ASV [163], chemically-induced rat glioblastoma cells [590], or human leukemia cells [592], among many other examples. This suggests that the effects of these CaM antagonists could depend to some extent on the physiological context existing in ovarian cancer cells as compared to cells from other origins. W-5 was also shown to activate DNA synthesis at low concentrations in fetal glioblasts, similar to W-7 and trifluoperazine, suggesting a biphasic and non-specific effects of these compounds [597]. Another example of ambiguous effects of potential CaM antagonists is the vinblastine-derivative KAR-2, which, although able to interact with CaM in the absence and presence of Ca^{2+}, appears to lack anti-CaM activity because it does not prevent CaM interacting with most tested targets, thus explaining its low toxicity in vivo [607,608].

CaM antagonists have also been tested in combination with clinically used anti-tumor drugs because they enhance their anti-proliferative actions (Table 5). For example, combining the CaM antagonist clomipramine and the alkaloid vincristine, a mitotic inhibitor that prevents

Table 4
Effects of distinct CaM antagonists on non-tumor cells.

CaM antagonist	Cell/tissue/organ	Origin	Effect	References
Calmidazolium (R24571)	NRK cells (kidney)	Rat	Inhibits nuclear localization of Cdk4/cyclin D1	[321]
	PtK2 cells (kidney)	Rat-kangaroo (Potorous tridactylis)	Inhibits mitosis (metaphase)	[344]
	Lymphocytes (from AD patients)	Human	Dephosphorylates pRb	[643]
Chlorpromazine	T lymphocytes	Human	Inhibits PHA-induced mitogenesis	[598]
Chlorpromazine sulfoxide	T lymphocytes	Human	Inhibits PHA-induced mitogenesis	[598]
D-hexapeptides[a]	NRK cells (kidney)	Rat	Inhibits DNA synthesis	[602]
	Liver cells	Rat	Inhibits in vivo liver regeneration (after partial hepatectomy)	[602]
Fendiline	Keratinocytes (skin) (primary)	Human	Inhibits DNA synthesis	[599]
Inhibitory peptide[b]	COS-7 cells (kidney)	Green monkey (Cercopithecus aethiops)	Inhibits DNA synthesis	[172]
Iodo-C_8-W-7	Keratinocytes (epidermis) (primary)	Human	Inhibits cell proliferation (more potently than W-7)	[164]
J-8	NRK cells (kidney)	Rat	Inhibits nuclear localization of Cdk4/cyclin D1	[321]
Tamoxifen	Platelet	Human	Iduces apoptosis and subsequent thrombocytopenia	[426]
Trifluoperazine	Myeloid and erythroid progenitor cells	Human	Inhibits colony formation	[567]
	Fetal glioblasts	Rat	Inhibits GMF-induced mitogenesis	[597]
	T lymphocytes	Human	Inhibits PHA-induced mitogenesis	[598,600]
	Platelet	Human	Iduces apoptosis and subsequent thrombocytopenia	[426]
W-13	CHO-K1 cells (ovary)	Hamster	Inhibits cell proliferation Cells arrested at early G_0/G_1 and G_1/S, inhibiting entry and progression through S. No effect on G_2 progression or from M to next G_1	[151,152]
	NRK cells (kidney)	Rat	Inhibits DNA synthesis pRb phosphorylation, Cdk2/Cdk4 activation and nuclear localization of Cdk4/cyclin D1	[321]
	Myeloid and erythroid progenitor cells	Human	Inhibits colony formation	[567]
W-7	CHO-K1 cells (ovary)	Hamster	Inhibits cell proliferation Cells arrested at G_1/S	[162]
	Fibroblasts (foreskin dermis) (primary)	Human	Inhibits cell proliferation	[164]
	Keratinocytes (epidermis) (primary)	Human	Inhibits cell proliferation Cells arrested at G_1/S	[164]
	Gastric mucosal cells	Rabbit	Inhibits cell proliferation	[601]
	NRK cells (kidney)	Rat	Inhibits nuclear localization of Cdk4/cyclin D1	[321]
	PtK2 cells (kidney)	Rat-kangaroo (Potorous tridactylis)	Inhibits mitosis (metaphase)	[344]
	Vascular endothelial cells (umbilical vein)	Human	Inhibits DNA synthesis and pRb phosphorylation	[320]
	Myeloid and erythroid progenitor cells	Human	Inhibits colony formation	[567]
	Platelet	Human	Iduces apoptosis and subsequent thrombocytopenia	[426]
	Fetal glioblasts	Rat	Inhibits GMF-induced mitogenesis	[597]

AD, Alzheimer's disease; CHO, Chinese ovary hamster; GMF, glia maturation factor; NLS, nuclear localization sequence; NRK, normal rat kidney; PHA, phytohemagglutinin.
  [a] Synthetic combinatorial library D-hexapeptides isolated as inhibitors of PDE.
  [b] CaM inhibitor peptide based on tandem repeats of the CaM-BD of MLCK creating a NLS to target the nucleus.

Table 5
Synergistic effects of CaM antagonists and chemotherapeutic agents.

CaM antagonist	Tumor/cell/tissue	Origin	Effect	References
Acridone derivatives	KBChR-8-5 cells (HeLa cell variant)	Human	Reversal of MDR (vinblastine)	[618] [620]
Chlorpromazine	Lymphocytic leukemia P388 cells	Mouse	Enhances cellular retention and cytotoxicity of Adriamycin	[612]
Clomipramine	Leukemia (lymphoblastic T and B cells, and myelogenous cells)	Mouse	Circumvents resistance to vincristine *in vivo*	[609,610] [611][a]
E2	Leukemia K562 cells	Human	Reversal of MDR (doxorubicin)	[619]
EBB	Breast adenocarcinoma MCF-7/ADR cells	Human	Reversal of MDR (doxorubicin)	[621]
Phenothiazine (thiodiphenylamine)	Colon adenocarcinoma WiDr cells	Human	Potentiates the action of methotrexate and 5-FdUrd (possible reduced DNA repair)	[581]
Phenothiazines[b]	Glioma C6 cells	Rat	Potentiates the action of bleomycin	[591]
Promethazine	Colon adenocarcinoma WiDr cells	Human	Potentiates the action of methotrexate and 5-FdUrd (possible reduced DNA repair)	[581]
Trifluoperazine	EMT6/Ca/VJAC tumor cells	Mouse	In synergy with heat (44 °C) strongly enhances bleomycin cytotoxicity	[616] [611][a]
	Cancer patients	Human	Synergistic improved response (4/19 cases) with bleomycin in a clinical trial	[86,87][a]
	Breast cancer ER-positive MCF-7 and ER-negative MDA-MB-468 cells, and primary ER-positive cells from cancer patients	Human	Synergy with tamoxifen inducing apoptosis	[438]
	Lymphocytic leukemia P388 cells	Mouse	Reversal of MDR (anthrapyrazoles and anthracyclines) Enhances cellular retention and cytotoxicity of Adriamycin	[617] [612]
	Promyelocytic leukemia HL-60/ADR0.05 cells (Adriamycin-resistant)	Human	Enhances of cytotoxicity etoposide (VP-16) by inducing TOPO-II-mediated DNA damage	[455]
W-5	Cystadenocarcinoma ovary cells	Human	Potentiates the action of doxorubicin (Adriamycin) and 5-fluorouracil	[582,615]
W-7	Cystadenocarcinoma ovary cells	Human	Potentiates the action of doxorubicin (Adriamycin) and 5-fluorouracil	[582,615]
	Colon adenocarcinoma WiDr cells	Human	Potentiates the action of methotrexate and 5-FdUrd (possible reduced DNA repair)	[581]
	Breast cancer ER-positive MCF-7 and ER-negative MDA-MB-468 cells, and primary ER-positive cells from cancer patients	Human	Synergy with tamoxifen inducing apoptosis	[438]

5-FdUrd, 5-fluorodeoxyuridine; ER, estrogen receptor; MDR, multidrug resistant; TOPO-II, topoisomerase II.
[a] Review article.
[b] Assay of a family of related neuroleptic antipsychotic drugs.

tubulin polymerization, facilitated the uptake of this chemotherapeutic drug particularly in leukemia cells resistant to this latter agent [609,610] (reviewed in Ref. [611]). In human ovarian serous cystadecarcinoma cells the combination of the CaM antagonists W-5 or W-7 with the DNA-intercalating drug doxorubicin (Adriamycin™), or the DNA-crosslinking drug cisplatin, also potentiates the action of these drugs [582]. Furthermore, chlorpromazine and trifluoperazine both enhanced the retention of Adriamycin™ and its cytotoxicity in mouse lymphocytic leukemia cells, particularly strong in a resistant clone as compared to its sensitive counterpart [612]. As an additional example, combining different CaM antagonists with the DNA-damaging agents methotrexate or 5-fluorodeoxyuridine (5-FdUrd) enhanced the cytotoxic effects of these drugs in human colon adenocarcinoma cells [581]. Furthermore, downregulating the expression of CaM using siRNA against the transcript of the CaM gene *CALM1* in HeLa cells potentiated the action of the chemotherapeutic agent doxorubicin and the mitotic inhibitor paclitaxel (Taxol), but not the inhibitory action of cisplatin [613].

The effect of W-5 and W-7 increasing the uptake of the thymidylate synthase inhibitor 5-fluorouracil or the uptake of cisplatin in cells resistant to this agent, and hence synergistically arresting cell proliferation, has been reported in ovarian tumor cells [614,615]. Moreover, a synergistic effect of several phenothiazines, including TFP, in combination with heat treatment, enhancing the action of the chemotherapeutic DNA-breaking agent bleomycin has been observed [591,616] (reviewed in Ref. [611]). The action of many CaM antagonists reverting tumor cells resistance to different chemotherapeutic agents has been further studied in numerous tumors [617–621]. This underscores the direct control that the Ca^{2+}/CaM complex may exert on the P-glycoprotein, a transporter mostly responsible for multidrug-resistant in tumor cells [622].

The pharmacological inhibition of CaM-dependent enzymes in tumor cells has been attempted as well, most prominently targeting

the different CaMKs isoforms (reviewed in Ref. [623]). In this context and as an example, the CaMK-II inhibitor KN-93 has been proposed as a therapeutic agent for treating choriocarcinomas [231]. In addition, it has been demonstrated that berbamine, a compound isolated from the traditional Chinese herbal medicine *Berberis amurensis*, inhibits the proliferation of human chronic myeloid leukemia cells and liver cancer cells by blocking the catalytic site of CaMK-II inhibiting its activity [624,625]. Inhibiting the upstream CaMKKs has also been considered for therapeutic purpose. CaMKK2 has been proposed as a therapeutic target in androgen-dependent prostate cancer, as this kinase is transcriptionally regulated by the androgen receptor, contributing to enhanced proliferation of the tumor cells and subsequent tumor progression [626]. On the contrary, the targeted inhibition of CaN generally used for immunosuppression might have instead some tumor-promoting effects. Particularly, skin squamous-cell carcinomas may be induce because of the enhancement of the carcinogenic potential of keratinocyte tumor stem cells, as it happens in patients undergoing immunosuppressive therapy (reviewed in Refs. [627,628]). Along this line, it has been reported that substituting CaN inhibitors by alternative immunosuppressive agents, such as for example mTOR inhibitors, resulted in a drastic and consolidated reduction in the number of new skin tumors [629].

7. Concluding remarks and future perspectives

Because of their potential toxicity, the systemic use of CaM antagonists in the clinic may be barred or drastically restricted, perhaps for local tumor-targeted administration only. An alternative to be explored could be the targeted-inhibition of the binding site(s) for CaM in specific CaM-binding proteins, particularly if the activity of these proteins is subverted in tumor cells. Additionally, searching mutations affecting the CaM-dependent mechanisms in tumor cells could be useful to understand

some pathophysiological processes in cancer, and helpful for diagnostic/prognostic purpose and/or for predicting survival expectancy. To study how essential CaM-dependent mechanisms work in normal and tumor stem cells could also help to understand basic tumorigenic processes. Studies in this area could be important to develop methods to target CaM-dependent systems in restricted tumor cell populations and/or in certain phases of tumor development, thus opening specific therapeutic windows, as with DNA-damaging agents, where implementation of this therapy may be useful in personalized medicine if CaM-dependent pathways are known to be out of control in a subset of cancer patients.

Acknowledgments

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