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Review Article

The multifunctional role of phospho-calmodulin in pathophysiological processes

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Calmodulin (CaM) is a versatile Ca^{2+}-sensor/transducer protein that modulates hundreds of enzymes, channels, transport systems, transcription factors, adaptors and other structural proteins, controlling in this manner multiple cellular functions. In addition to its capacity to regulate target proteins in a Ca^{2+}-dependent and Ca^{2+}-independent manner, the posttranslational phosphorylation of CaM by diverse Ser/Thr- and Tyr-protein kinases has been recognized as an important additional manner to regulate this protein by fine-tuning its functionality. In this review, we shall cover developments done in recent years in which phospho-CaM has been implicated in signalling pathways that are relevant for the onset and progression of diverse pathophysiological processes. These include diverse systems playing a major role in carcinogenesis and tumour development, prion-induced encephalopathies and brain hypoxia, melatonin-regulated neuroendocrine disorders, hypertension, and heavy metal-induced cell toxicity.

Introduction

Calmodulin (CaM) is a ubiquitous highly conserved Ca^{2+} sensor/transducer protein in all eukaryotic cells. The Ca^{2+}-dependent and Ca^{2+}-independent functions of CaM are exerted by interacting and controlling the activity of hundreds of enzymes, channels, transport systems, and a variety of non-enzymatic proteins that are vital for the physiology of the cell [1–6]. As a Ca^{2+} sensor, CaM transduces the oscillations of the intracellular concentration of this cation during cell activation by growth factors, hormones, neuro-transmitters, and other effectors in meaningful cellular responses via CaM-dependent proteins. In addition, diverse posttranslational modifications of CaM have been recognized as a fine-tuning mechanism by which CaM-dependent proteins are also modulated [7]. This includes phosphorylation [8], trimethylation of Lys115 [9], acetylation of the N-terminal alanine residue [10], and carboxymethylation of aspartic and glutamic acids [11]. In this context, however, the phosphorylation of CaM takes a central functional stage.

Since the first report in 1983 by Plancke and Lazarides [12] describing the presence of Ser-phosphorylated CaM in chicken brain and skeletal muscle, and the *in vitro* phosphorylation of CaM by a preparation of phosphorylase b kinase, the biological importance of CaM phosphorylated at Ser/Thr and Tyr residues has been recognized. These phosphorylated CaM forms differentially regulate, when compared with non-phosphorylated CaM, a great variety of CaM-dependent systems [8]. The different residues phosphorylated by Ser/Thr- and Tyr-protein kinases are shown in Figure 1. It is important to remark the abundance of phosphorylatable residues located at the different EF-hands (Thr26, Thr29, Thr44, Tyr99, Tyr138, Ser101, Thr117) although not all are within the sequence of the Ca^{2+}-binding sites, and in the flexible central linker (Thr79, Ser81) connecting the N- and C-lobes of the protein. This underscores the importance that these phosphorylated residues may have in modifying the Ca^{2+}-binding capacity of CaM and/or its interaction with target proteins. In recent years, new progress has been done that helps to unravel the role of different phospho-CaM species in the control of important physiological processes and its implication in different pathologies. This short review

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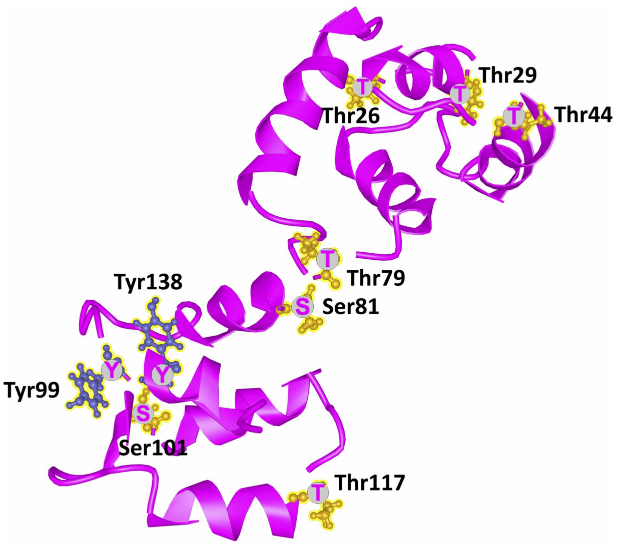

Figure 1. NMR structure of apo-CaM showing the amino acid residues known to be phosphorylated by diverse protein kinases.

The figure depicts the NMR-derived structure of Ca^{2+}-free CaM (apo-CaM) of *Xenopus laevi* showing the amino acid residues known to be phosphorylated by diverse Ser/Thr- and Tyr-protein kinases. Note the location of the phosphorylatable residues located at EF-hand I (Thr26 and Thr29), EF-hand III (Tyr99, Ser101) and EF-hand IV (Tyr138), and the flexible inter-lobe linker (Thr79 and Ser81). When phosphorylated, these residues may alter either the Ca^{2+}-binding capacity of CaM or its interaction with target CaM-binding proteins. The structure was obtained from PDB ID: 1DMO [87].

covers the advances done since our previous one in 2002 [8], but centred on the functionality of phospho-Tyr-CaM and/or phospho-Ser/Thr-CaM in tumour cell biology, during neurological disorders, the dysfunction of the vascular system particularly its role in hypertension, dysregulations of the neuroendocrine melatonin system, and other cellular processes.

Phospho-Tyr-CaM in pathophysiology

Different phospho-Tyr-CaM species are implicated in signalling processes with pathophysiological implications. The kinases so far known to participate in CaM phosphorylation are the EGFR, the insulin receptor, and several non-receptor kinases such as c-Src and other Src family members, as well as Jak2 and p38Syk (see Table 1).

CaM regulates many signalling pathways and cellular functions relevant for the biology of cancerous cells [1]. It is expected, therefore, that different phospho-CaM species could be implicated in signalling routes leading to tumour cell transformation. In this section, we discuss some processes in which phospho-Tyr-CaM species are involved. There are several signalling systems very relevant for tumour cell proliferation and survival that are regulated by CaM, and where phospho-Tyr-CaM could play prominent regulatory roles. This includes the EGFR, the non-receptor tyrosine kinase c-Src, and the K-Ras/PI_3K/Akt pathway.

CaM has been shown to interact with the cytosolic juxtamembrane region of the EGFR in a Ca^{2+}-dependent manner facilitating the ligand-dependent activation of the receptor in living cells [13–16]. As CaM is also a good substrate for the EGFR [17], it was expected that phospho-Tyr-CaM could also interact with the receptor. This indeed was the case [18,19], and the site of interaction of phospho-Tyr-CaM with the receptor was stablished to be the same that the one for non-phosphorylated CaM located at the cytosolic juxtamembrane region encompassing the sequence ^{645}RRRHIVRKRTLRRLLQ^{660} [19]. The NMR-derived structure of a peptide corresponding to the transmembrane (TM) and the cytosolic juxtamembrane (JM_{cyt}) segment, where the CaM-BD is located, reconstituted in lipid bicelles shows that they form dimers and that the CaM-BD presents two helical segments divided by a flexible linker [20,21] (see Figure 2). This flexible linker is important to understand the expected bending of the JM_{cyt} to facilitate electrostatic interaction with the phosphoinositide-rich negatively charged inner leaflet of the plasma membrane to attain auto-inhibition of the EGFR in the absence of ligand

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Table 1 Pathophysiological implication of phospho-Tyr- and phospho-Ser/Thr-CaM

Phospho-CaM species	Phosphorylated residue	Action on CaM	[1]Kinase	Exp. evidence	Biological effect (Ref.) Functional and pathological implications
p-Y-CaM	DGNG**Y^{99}**ISAA	Affect EF-hand III	EGFR	*in vitro*	Phospho-Y-CaM enhances ligand-dependent EGFR activation [19]. Cancer
	GQVN**Y^{138}**EEFV	Affect EF-hand IV	c-Src	*In vivo*	The pleiotrophin/PTPRZ1 pathway enhances CaM phosphorylation in SCLC [37]. Cancer
				In silico *In vitro*	Phospho-Y99-CaM binds to the SH2 domains of the regulatory p85 subunit of PI$_3$K activating its catalytic p110 subunit and the K-RasB/PI$_3$K/Akt pathway [32,33]. Enhances proliferation, cell survival, and resistance to apoptosis. Cancer
				In vivo	Hypoxia activates EGFR and c-Src enhancing phospho-Y99-CaM [41,44]. Brain hypoxia
			[2]SFKs	*In vitro*	Unknown effect of p-Y-CaM [8]. Leukaemia and lymphomas (?)
			Jak2	*In vivo*	Jak2/phospho-Tyr-CaM/NHE3 complex activates Na$^+$ reabsorption in kidney [48]. Hypertension
			INSR	*In vitro*	Unknown effect of p-Y-CaM [92]. Diabetes (?)
			p38Syk	*In vitro*	Unknown effect of p-Y-CaM [93].
p-S/T-CaM	DGDG**T^{26}**ITTK	Affect EF-hand I	CK2	*In vitro*	PrP increases the catalytic activity of CK2 inducing CaM phosphorylation [60]. Spongiform encephalopathy
	GTIT**T^{29}**KELG	Affect EF-hand I		*In vitro*	Phospho-S^{101}-CaM inhibits eNOS [62]. Decreased NO output result in lower vasodilation. Phospho-mimetic CaM(T79D) mutant decreases the Ca^{2+} sensitivity of SK2 channel [65]. Phospho-T-CaM diminishes SK channel conductance [66]. Hypertension
	GQNP**T^{44}**EAEL	Affect EF-hand II		*In vivo*	
	KMKD**T^{79}**DSEE	Affect flexible central linker			
	KDTD**S^{81}**EEEI	Affect flexible central linker		*In vitro*	Phospho-T^{29}-CaM enhances uranium binding [75,76]. Cell toxicity
	NGYI**S^{101}**AAEL	Affect EF-hand III	CaMK-IV	*In vitro*	Phospho-T^{44}-CaM, phosphorylated by CaMK-IV, inhibits CaMK-II [68]
	GEKL**T^{117}**DEEV	Affect EF-hand IV	PKCα	*In vitro* *In vivo*	Melatonin enhances phospho-S/T-CaM levels [72]. Sleep disorders
			MLCK	*In vitro*	Unknown effect of p-T-CaM [77]. Myopathies (?)
			PhK	*In vitro* *In vivo*	Unknown effect of p-S-CaM [12] Glucogen metabolopathies (?)

[1] The kinases may phosphorylate one or more of the indicated residues.
[2] Includes other SFKs (v-Src, c-Fyn, c-Fgr).
CaM, calmodulin; CaMK-II/IV, CaM-dependent kinases II/IV; c-Src, cellular sarcoma kinase; EGFR, epidermal growth factor receptor; INSR, insulin receptor; Jak2, Janus kinase 2; MLCK, myosin light-chain kinase; NHE3, Na$^+$/H$^+$ exchanger 3; PhK, phosphorylase b kinase; PrP, prion protein; PTPRZ1, protein tyrosine-phosphatase receptor Z1; SCLC, small cells lung carcinoma; SFKs, Src-family kinases.

[22]. *In vitro* studies show that phospho-Tyr-CaM enhances the ligand-dependent activation of the EGFR, and this effect was observed as well using the phospho-mimetic CaM(Y99D/Y138D) mutant [19]. This suggests that phospho-Tyr-CaM could be an intracellular co-activator of the EGFR more efficient than non-phosphorylated CaM in detaching the CaM-binding domain of the inner leaflet of the plasma membrane to which is electrostatically bound [22], most provably due to the extra negative charges of phosphate. If this

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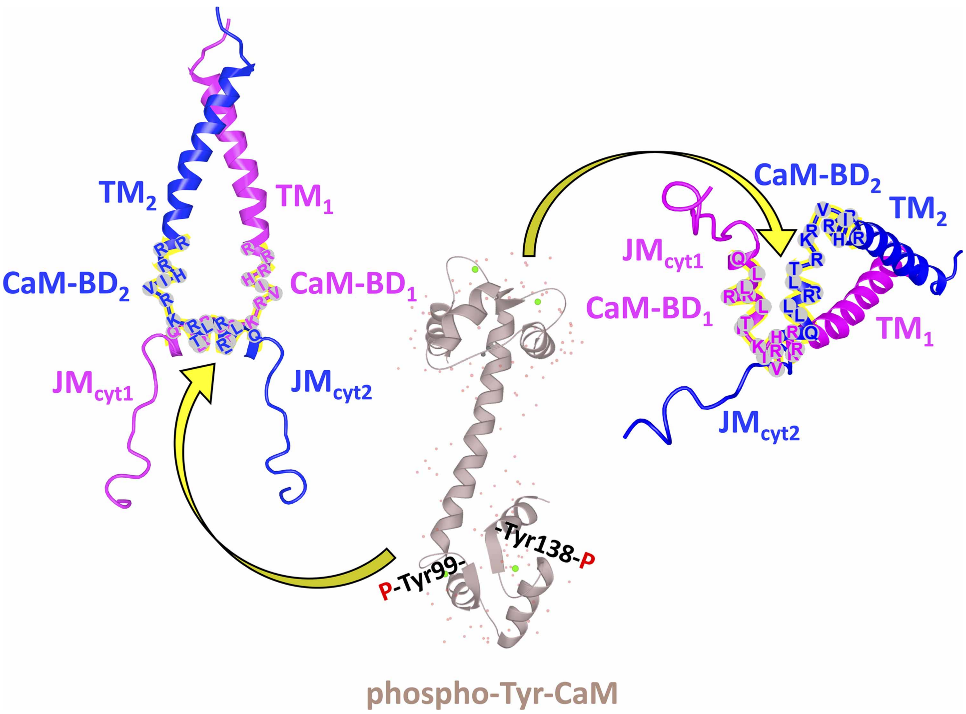

Figure 2. NMR structure of the CaM-BD of the human EGFR reconstituted in lipid bicelles.

The figure depicts the NMR-derived dimeric structure of a TM-JM$_{cyt}$ peptide of the EGFR reconstituted in lipid bicelles observed at different angles, and the interaction of phospho-Tyr99/Tyr138-CaM in the presence of Ca^{2+}. The CaM-binding domain (CaM-BD), site of interaction of non-phosphorylated CaM and phospho-Tyr-CaM, in both TM-JM$_{cyt}$ monomers is highlighted indicating the amino acid residues corresponding to the sequence ^{645}RRRHIVRKRTLRRLLQ^{660}. For clarity, the TM-JM$_{cyt}$, including the CaM-BD segments, of each monomer is indicated by numbers. The structures were obtained from PDB ID: 2M20 [20] (human EGFR TM-JM$_{cyt}$) and 1CLL (human CaM).

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assumption is correct, we may expect that phospho-Tyr-CaM could play a relevant role in EGFR-signalling, particularly in tumours overexpressing this receptor.

The binding of CaM to c-Src was first demonstrated *in vitro* and several potential CaM-binding sites in this kinase identified [23,24]. The interaction of apo-CaM, and in lesser extent Ca^{2+}/CaM, with c-Src induces a robust increase in its autophosphorylation and kinase activity towards exogenous substrates, as demonstrated *in vitro* and living cells [24,25]. In contrast with non-phosphorylated CaM, phospho-Tyr138-CaM does not co-immunoprecipitate with c-Src [18]. However, the phospho-mimetic mutants CaM (Y99D/Y138D) and CaM (Y99E/Y138E) strongly activate c-Src to the same level that non-phosphorylated CaM does, both in the absence and presence of Ca^{2+} [24]. One possibility is that phospho-Tyr99 could be required for the activation process. Nevertheless, it is not possible to infer from these experiments whether phospho-Tyr99-CaM and/or phospho-Tyr99/Tyr138-CaM interact with c-Src in living cells. This remains a possibility that deserves to be explored in the future. The identification of potential CaM-binding sites in the proximal [24] and terminal [23] regions of the SH2 domain of c-Src underscores the possibility that phospho-Tyr-CaM could interact with these sites.

In pancreatic tumour cells, it was shown that the interaction of CaM with the terminal region of the SH2 domain of c-Src facilitates its recruitment to the Fas death receptor signalling complex in a FADD-independent manner activating the c-Src/ERK pathway which promotes proliferation, cell survival, and resistant to apoptosis [23]. In this context, a series of compounds that disrupt the interaction of CaM with the SH2 domain of c-Src have been identified [26]. These compounds could be useful to prevent any potential action of CaM, and possibly phospho-Tyr-CaM, on c-Src signalling pathways. The possible action of phospho-Tyr-CaM on v-Src and other Src-family kinases relevant in diverse lymphoblastic tumours is an interesting topic pending to be

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investigated, particularly since there is an intense cross-talk between the Ca^{2+} signal and many Src-family kinases [25,27].

The K-Ras/PI$_3$K/Akt pathway participates in cell proliferation and cell survival processes. K-RasB, but not H-Ras or N-Ras, binds to and is down-regulated by CaM [28]. Also, PI$_3$K is regulated by CaM upon binding to its 110 kDa catalytic subunit (p110) [29] and the 85 kDa regulatory subunit (p85) [30]. Phospho-Tyr99-CaM, probably produced by a Src-family kinase, also interacts with the two SH2 domains of the regulatory p85 subunit of PI$_3$K, activating in this manner its catalytic p110 subunit [31]. It has been shown that an extended form of phospho-Tyr99-CaM binds to the proximal SH2 domain (nSH2) of p85, while its distal SH2 domain (cSH2) interacts with a collapsed conformation of phospho-Tyr99-CaM which carries in addition K-Ras4B bound as determined *in vitro* by molecular simulation (see Figure 3). This tripartite interaction activates PI$_3$K, since K-Ras4B also allosterically acts on its catalytic subunit. The transformation of PIP$_2$ to PIP$_3$ in the cell membrane upon activation of PI$_3$K induces Akt binding to the membrane and activation, transmitting downstream proliferative signals that are relevant for K-Ras-driven tumours [32,33].

Using an engineered chicken B-lymphocyte tumour DT-40 cell line with the two functional CaM genes deleted, and one allele replaced by a CaM transgene repressible by tetracycline, it was shown that the expression of the non-phosphorylatable CaM mutants, CaM (Y99F), CaM (Y138F), or CaM (Y99F/Y138F) in the absence of endogenous wild-type CaM did not affect the growth and survival of the tumour cells. This suggests that phosphorylation of CaM at tyrosine residues was not required for the initiation and progression of these

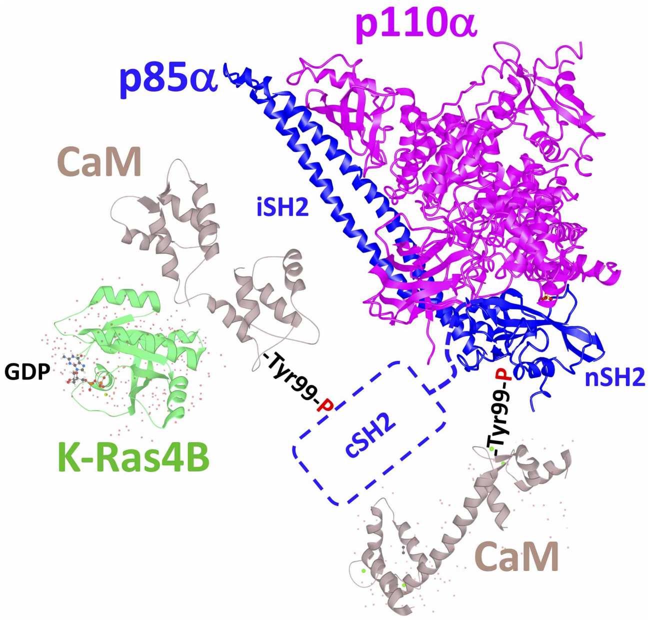

Figure 3. Tripartite PI$_3$K/phospho-Tyr99-CaM/K-Ras4B complex.
The figure depicts a composite complex formed by phosphatidylinositol-4,5-bisphosphate-3-kinase (PI$_3$K) interacting with phopho-Tyr99-CaM (phospho-Tyr99-CaM) and K-Ras4B. To attain crystallization, the full-length p110α catalytic subunit (*pink*) was fussed to a segment of the regulatory p85α subunit (*blue*) of PI$_3$K comprising the proximal SH2 domain (nSH2) and the inter-SH2 segment (iSH2) [88,89]. The distal SH2 domain (cSH2) is absent from the construct used for crystallization and it is shown as a dashed box. Phospho-Tyr99-CaM (*brown*) in an extended conformation interacts with the proximal SH2 domain (nSH2) of the regulatory p85α subunit of PI$_3$K (*blue*), while a collapsed conformation of phospho-Tyr99-CaM (*brown*), which is bound to K-Ras4B (*green*), interacts with its distal SH2 domain (cSH2) which is shown as a dashed box. K-Ras4B is represented in its GDP-bound conformation, and phospho-Tyr99 (-Tyr-P) CaM is highlighted. This theoretical model is based on reference [30], but it does not depict the actual interaction sites of the different proteins in the complex. The structures were obtained from PDB ID: 4L1B (human PI$_3$K, full-length p110α and 318–615 amino acid segment of p85α, at 2.6 Å X-ray resolution [88,89]), 1CLL and 1CFC (human and *Xenopus* CaM at 1.7 Å X-ray resolution and NMR-derived structure, respectively [87,90]) and 4LDJ (human K-Ras4B at 1.15 Å X-ray resolution [91]).

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processes, although endogenous phospho-Tyr-CaM was detected in the conditional CaM-knockout cell line before CaM down-regulation [34]. It is important to mention that these tyrosine-defective mutants were previously characterized and were found to be biologically active [35].

Pleiotrophin is a ligand of the tyrosine-phosphatase receptor PTPRZ1 that upon binding to its extracellular domain induces dimerization. This phosphatase receptor is characterized by having two phosphatase sites, although the distal one is generally inactive. The distal phosphatase site of one PTPRZ1 monomer blocks the proximal active site of the apposed monomer, suppressing in this manner its activity [36]. As a consequence, pleiotrophin enhances the protein tyrosine phosphorylation level in the cell, and consequently it was shown that the pleiotrophin/PTPRZ1 pathway enhances CaM phosphorylation in SCLC cells [37]. Whether or not PTPRZ1-mediated tumour progression directly correlates with phospho-Tyr-CaM levels remains unknown.

Phospho-Tyr-CaM also participates in signalling processes relevant in the regulation of the vascular tone. In agreement with the stimulatory action of phospho-Tyr-CaM on nNOS, phospho-Tyr-CaM and the phospho-mimetic mutant CaM (Y99E/Y138E) also enhanced, albeit in minor degree, the catalytic activity of the eNOS isoform when assayed *in vitro*, when compared with wild-type non-phosphorylated CaM [38]. In contrast, phospho-Tyr-CaM and the phospho-mimetic mutant CaM (Y99D/Y138D) slightly inhibited bovine brain PDE1 when compared with wild-type non-phosphorylated CaM [38]. However, bovine hearth PDE was inhibited *in vitro* by phospho-Tyr-CaM in a larger extent, as was reported earlier [39]. Moreover, the phospho-mimetic CaM(Y99E) mutant significantly inhibited eNOS *in vitro*, while no significant effect was found on the activity of nNOS or iNOS [40]. Overall, these observations suggest that the action of phospho-Tyr-CaM on NOS depends on the enzyme isoform, its tissular origin, and provably the phospho-Tyr residue of CaM involved. This underscores the existence of a great variety in regulatory mechanisms controlling CaM-dependent NO output implicated in vascular relaxation.

During experimental hypoxia of the brain, activation of the EGFR and c-Src [41,42] was observed. This leads to enhanced CaM phosphorylation at Tyr99 in the cerebral cortex and brain nuclei, particularly mediated by c-Src [43]. Phospho-Tyr99-CaM activates nNOS increasing the production of NO, and this potentially accelerates neuronal cell death. Puzzlingly, addition of an nNOS inhibitor before the onset of hypoxia prevents phospho-Tyr-CaM accumulation, suggesting that NO regulates EGFR and/or c-Src activation and hence the CaM phosphorylation process [41,44]. This is consistent with the biphasic regulation that NO exerts on EGFR activation [45,46].

Essential hypertension is a widespread heterogeneous illness in which a major pathophysiological factor is the inability of the kidney to excrete sodium at normal blood pressure. This results in altered fluid balance and increased arterial resistance that affect the correct function of many organs [47]. The Na^+/H^+-exchanger (NHE) plays a central role in Na^+ reabsorption in the kidney proximal tubular system. It was demonstrated in cultured podocytes that NHE1 is regulated by CaM, which is Tyr-phosphorylated by Jak2, a process in which the EGFR also plays an important role [48]. In normal physiological conditions, Ang-II (angiotensin-II) activates this exchanger promoting Na^+ retention and increasing the arterial blood pressure. Moreover, oxidative stress up-regulates the Ang-II receptor AT_1R, inducing enhanced Jak2-induced CaM phosphorylation and formation of the Jak2/phospho-Tyr-CaM/NHE3 complex, exacerbating Na^+ retention leading to hypertension [49].

Phospho-Ser/Thr-CaM in pathophysiology

The best-studied Ser/Thr-kinase participating in CaM phosphorylation is CK2 (casein kinase 2), although other kinases, such as PhK, CaMK-IV, PKCα, and MLCK, also phosphorylate this protein. Phospho-Ser/Thr-CaM also participates in signalling pathways relevant in several diseases (see Table 1). In this section, some of these processes are discussed.

The ubiquitous kinase CK2 plays many functional roles phosphorylating a variety of protein substrates, and is implicated in cell viability and progression of the cell cycle [50]. The phosphorylation of CaM by CK2 occurs by the isolated catalytic α- and α′-subunits as demonstrated *in vitro*, while the N-terminal region of its regulatory β-subunit exerts an inhibitory effect [51,52]. CaM directly interacts with the β-subunit of CK2, and the C-terminal of CaM is implicated in the inhibitory effect of this subunit, as well as in the activation mediated by polybasic peptides [53]. It is expected, therefore, that the holoenzyme is unable to phosphorylate CaM. This was consistent with a report showing that the construct $CK2\alpha_2^{1-335}\,\beta_2$ does not phosphorylate CaM, although the CK2α′-derived holoenzymes phosphorylated CaM when poly-Lys was present, while only weak phosphorylation was observed in its absence [54]. The absolute requirement of a basic polypeptide, including poly-Lys, for the phosphorylation of CaM by CK2 was earlier reported [55]. In fibroblasts, serum induces an

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unbalanced overexpression of the CK2 α′-subunit without the corresponding expression of its β-subunit. It has been stablished that the former subunit acts as an oncogene, and in concert with H-Ras has transforming capacity. This is accompanied by increased cell proliferation rate and CaM phosphorylation in cultured cells due to the unabated excess of CK2 α′-subunit [56]. This suggests that phospho-CaM could play a role in the transformation process. Nevertheless, it cannot be excluded that other CK2 α′-subunit-substrates are also involved in the transformation process. Interestingly, the SH3 domain of the protein HS1 directly interacts with the CK2 α-subunit *in vitro* inhibiting CaM phosphorylation and expected H-Ras-mediated transformation [57]. This could open the possibility of using peptides derived from the SH3 domain of HS1 as potential therapeutics to inhibit tumour transformation.

Transmissible spongiform encephalopathies from animals and human are produced by infecting agents denoted prions. These agents are misfolded conformers of the host-coded glycoprotein PrP, which is able to interact and to induce non-reversible conformational changes in normal counterpart protein molecules generating the pathological accumulation of the so-called disease-associated protein PrP^d in the brain. This process produces neural vacuolation, astrocytosis, and neurodegeneration leading to serious neurological dysfunctions and patient death. Apparently, mutation of PrP can also favour or induce disease in the absence of an external infective agent [58,59]. CK2 is able to phosphorylate PrP at Ser154 *in vitro* [60], and conversely recombinant bovine PrP binds with high affinity to the catalytic α/α′-subunits of CK2. This interaction increases the catalytic activity of CK2 inducing CaM phosphorylation, and counteracts the inhibitory role that exerts its β-subunit [61]. As CK2 and CaM are both abundant in the brain, this opens the possibility that increased activity of this enzyme in spongiform encephalopathies and accumulation of phospho-CaM could play a role in the disease.

CK2 has been shown to phosphorylate several CaM residues, primarily affecting Thr79, Ser81, Ser101, and Thr117, although phosphorylation of Ser81 prevents subsequent phosphorylation of Thr79 [8,53]. CK2-phosphorylated CaM inhibits eNOS activity as mentioned earlier, and the phospho-Ser101 residue is likely to participate in the inhibition of eNOS [62]. The down-regulation of eNOS in endothelial cells by CK2-phosphorylated CaM could have profound consequences in NO output and hence its vasodilatory capacity leading to higher arterial blood pressure. It would be interesting to investigate whether this system is altered in hypertensive individuals.

CaM is constitutively bound to the C-terminal of the subunits forming homo-tetrameric small-conductance Ca^{2+}-activated K^+ (SK) channels [63] (see Figure 4), transducing in this manner the Ca^{2+} signal and opening the channel pore [64]. The CaM/SK channel also forms a multimeric complex with CK2 and PP2A reversibly regulating CaM phosphorylation levels. CK2 phosphorylates CaM only when the channel is closed [65]. Moreover, co-expression of SK2 channels with the phospho-mimetic CaM(T79D) mutant decreases the Ca^{2+} sensitivity of the channel, suggesting that phosphorylation of Thr79 by CK2 controls this process [65]. To clarify matters, in the latter work Thr79 was mislabelled as Thr80. This is the position corresponding to full-length CaM counting the N-terminal Met residue, which is not present in the mature protein. It has been shown that up-regulated CK2 in the hypothalamic paraventricular nucleus of spontaneously hypertensive rats increases the level of CaM phosphorylation, and phospho-CaM diminishes SK channel conductance altering the excitability of presympathetic neurons [66]. PIP_2 plays an important role mediating SK channels activation by CaM, as its binding site is located in the interphase of CaM/SK interaction. Moreover, phosphorylation of Thr79 by CK2 reduces the affinity for PIP_2 inducing greater inhibition of the channel [67]. These findings suggest that CK2-phosphorylated CaM, upon regulating SK channels, could intervene in the neural control of dysregulated vessels implicated in essential hypertension.

Brain hypoxia also activates CaMK-IV via c-Src, and the former activates the transcription factor CREB phosphorylating Ser133 enhancing the expression of pro-apoptotic proteins which initiate hypoxic neural cell death [42,43]. In contrast with the case of CaMK-II that poorly phosphorylates CaM, CaMK-IV is able to efficiently phosphorylate CaM at Thr44, which is located in the proximal region of the second EF-hand Ca^{2+}-binding site. Moreover, the authors of this report also demonstrated that CaM phosphorylated by CaMK-IV inhibits CaMK-II activity, when compared with non-phosphorylated CaM [68]. However, the action of phospho-Thr44-CaM on CaMK-IV itself was not reported.

Melatonin (5-methoxy-*N*-acetyltryptamine) is a neurohormone mainly synthesized and secreted by the pineal gland, also named epiphysis, located in the epithalamus between the two brain hemispheres, but extra pineal tissues also synthesize melatonin. This hormone controls the cyclic 24-h circadian rhythm, and plays many physiological functions in the organism, modulating, for example, the immune, neural, and cardiovascular systems, and exerting antioxidant actions among many other functions [69]. The melatonin receptor MT_2 is

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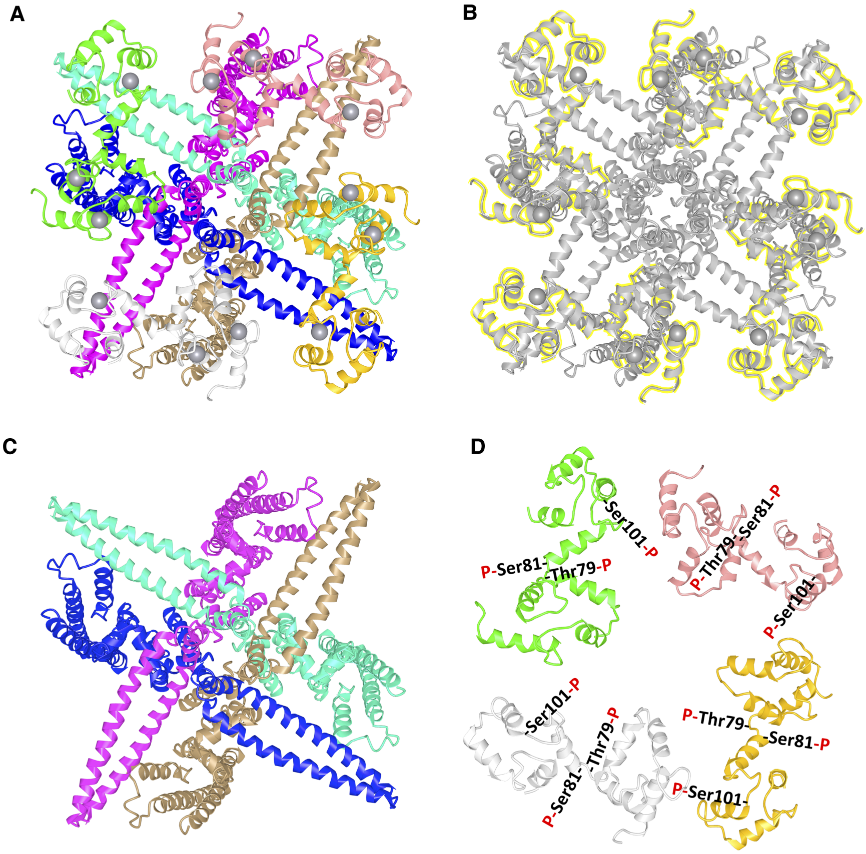

Figure 4. Cryo-EM structure of the human SK4/CaM complex.

The figure depicts the cryo-electron microscope-derived structure at 4.7 Å resolution of the human SK4/CaM complex in the presence of Ca^{2+} (**A**). The four CaM molecules are highlighted in yellow (**B**). The four subunits of the SK4 channel (**C**) and the four CaM molecules indicating the phosphorylation of Thr79, Ser81, and Ser101 by casein kinase 2 in the central flexible linker and the third EF-hand Ca^{2+}-binding site, respectively, (**D**) are shown in isolation in different colours. The structure was obtained from PDB ID: 6CNO [63].

a GPCR that is involved in many sleep and neuropsychiatric disorders, including insomnia, anxiety, and depression. Therefore, melatonin and other agonists of this receptor have been used to treat these disorders [70,71]. Melatonin has been shown to stimulate, at very low concentration, the phosphorylation of CaM mediated by PKCα in the presence of the phorbol ester PMA in an *in vitro* reconstituted system as well as in cultured cells [72]. In this process is implicated the downstream MAPK pathway, as determined using specific inhibitors blocking melatonin-induced CaM-phosphorylation. The authors of this work suggested that high serum melatonin levels during the dark phase of the circadian cycle may increase phospho-Ser/Thr-CaM concentration, down-regulating in this manner key CaM-dependent target enzymes sensitive to this phosphorylated modulator as previously described [8]. An intriguing possibility of interest to be studied in the future is the occurrence of alterations of melatonin-induced CaM-phosphorylation in sleep disorders and other neuropsychiatric illness.

Heavy metals are very toxic, and some of their actions are due to its interaction with CaM by altering its functionality [73,74]. Uranium chemical toxicity in living organisms is due, in part, to the interaction of the uranyl ion with cellular proteins. The first EF-hand Ca^{2+}-binding motif of CaM has about thousand times more affinity for uranyl than for Ca^{2+}, and *in vitro* studies demonstrated that phosphorylation of *Arabidopsis thaliana* CaM by CK2 at a threonine in the first Ca^{2+}-binding loop, increases the uranyl affinity at physiological pH [75,76]. Another kinase known to phosphorylate the first EF-hand Ca^{2+}-binding site of vertebrate CaM at Thr29, and in lesser extent Thr26, is MLCK [77]. It would be interesting to determine whether this kinase may

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also contribute to this process. Uranium has an enormous toxic effect in cells of organisms contaminated with this radioactive element. This may be due, in part, to its disrupting effect on Ca^{2+}-binding to CaM preventing in this manner its normal functionality, in addition to the lasting noxious effects exerted by this radioactive element.

Perspectives

The diversity of phosphorylatable residues in CaM suggests that each one may have specific functionalities (see Figure 1 and Table 1). As examples to be investigated in the future, it would be relevant to stablish whether phosphorylation of Thr79 and Ser81 in the flexible linker of CaM could play distinct or redundant roles in modifying its flexibility and therefore its capacity to rotate their N- and C-lobes, as this may be critical for its interaction with target proteins. A CaM deletion mutant lacking Thr79 and Asp80 induces the loss of a partial turn of the central α-helix resulting in the rotation of the C-lobe 220° with respect to the N-lobe positioning both lobes in a *cis* orientation, when compared with the *trans* orientation in wild-type CaM [78]. This was interpreted as to why this CaM mutant has decreased capacity to activate some CaM-binding enzymes, while has little or no effect on others. Although this is a very likely possibility when performing *in vitro* assays, it is not negligible to take in consideration that the absence of the phosphorylatable Thr79 residue in this mutant could be relevant when considering its lack of phosphorylation in living cells. To stablish whether phospho-Tyr99 and phospho-Tyr138 play a distinct role in modifying the Ca^{2+}-binding capacity of CaM and/or to determine its interaction with target proteins containing SH2 and/or PTB domains are points of interest. Likewise, whether phosphorylation of both tyrosine residues in CaM is required or not to interact with some target proteins is also a relevant point.

Another topic deserving special attention is whether, in addition to the case of PI_3K, where the binding of phospho-Tyr-CaM to the two SH2 domains of its p85 regulatory subunit has been stablished [32], there are other proteins containing SH2 or PTB domains that may interact with phospho-Tyr-CaM regulating in this manner their activities as suggested earlier [8]. This includes the SH2 domain of c-Src, where potential CaM-binding sites have been identified [23,24]. This could be of particular importance, as this interaction may explain how phospho-Tyr-CaM differentially regulates diverse signalling pathways when compared with non-phosphorylated CaM. Furthermore, as CaM has the capacity to cluster different or identical subunits of a variety of CaM-binding proteins and to interact with separate segments of the same polypeptide chain [2], it is possible to visualize that phospho-Tyr-CaM could do the same, clustering two distinct SH2/PTB-containing proteins or protein subunits if each one simultaneously bind to the phospho-Tyr99 and the phospho-Tyr138 residues.

A series of natural occurring CaM mutants, impairing Ca^{2+}-binding, affect CaM-regulated ion channels in the heart causing some arrhythmias, including the bradycardic long QT syndrome and idiopathic ventricular fibrillation [79–84]. One possibility worth to be explored in the future is whether mutations affecting phosphorylatable CaM residues may also dysregulate ion channels in the heart and/or other organs. In this context, it has been shown in *Paramecium tetraurelia* that changing Ser101 by a non-phosphorylatable residue in CaM affects the ion channel-mediated motile response of the protozoa by acting on the low conductance Ca^{2+}-dependent K^+ channel [85]. As CK2 phosphorylates CaM at different residues, including Ser101, it is tempting to speculate whether the observed effect on this channel induced by the disabled CaM mutant could primarily be due to the absence of phospho-Ser101-CaM. This is supported by the fact that *Tetrahymena pyriformis* CaM, which has a phosphorylatable Thr instead of a Ser at position 101, restores the activity of dysfunctional Ca^{2+}-dependent K^+ channels [85]. Curiously, two additional mutations simultaneously occurring in the CaM (Ser101Phe) mutant that revert the phenotype are Asp80Tyr and Arg106Lys [86]. It would be interesting to investigate whether phosphorylation of Tyr80 by a Src-family kinase occurs in this mutant, and if positive, whether this phosphorylation is responsible for the suppressor activity of this triple mutant, as phospho-Tyr80 could mimic the effect of phospho-Ser101.

Abbreviations

Ang-II, angiotensin-II; AT_1R, Ang-II type 1 receptor; CaM, calmodulin; CaMK-II/IV, CaM-dependent kinases II/IV; CK2, casein kinase 2; CREB, cAMP response element-binding protein; c-Src, cellular sarcoma kinase; EGFR, epidermal growth factor receptor; EM, electron microscope; eNOS, endothelial nitric oxide synthase; ERK, extracellular-regulated kinase; FADD, Fas-associated protein with death domain; Fas, tumour-necrosis factor receptor superfamily member 6; GPCR, G protein-coupled receptor; HS1, hematopoietic lineage cell-specific

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protein; iNOS, inducible nitric oxide synthase; Jak2, Janus kinase 2; MAPK, mitogen-activated protein kinase; MLCK, myosin light-chain kinase; NHE1/3, Na^+/H^+-exchangers 1/3; NMR, nuclear magnetic resonance; nNOS, neuronal nitric oxide synthase; PDB ID, protein data bank identification number; PDE1, 3′,5′-cyclic nucleotide phosphodiesterase 1; PhK, phosphorylase b kinase; PI_3K, phosphatidylinositol-4,5-bisphosphate-3-kinase; PIP_2, phosphatidylinositol 4,5-bisphosphate; PIP_3, phosphatidylinositol 3,4,5-trisphosphate; PKCα, protein kinase Cα; PMA, 12-*O*-tetradecanoylphorbol-13-acetate; PP2A, protein phosphatase 2A; PrP, prion protein; PrP^d, disease-associated PrP; PTB, phospho-tyrosine binding; PTPRZ1, protein tyrosine-phosphatase receptor Z1; SCLC, small cells lung carcinoma; SH2, Src homology domain 2; SH3, Src homology domain 3; $TM\text{-}JM_{cyt}$, transmembrane-cytosolic juxtamembrane segment.

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Competing Interests

The Author declares that there are no competing interests associated with this manuscript.

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