Eur. J. Biochem. **269**, 3619–3631 (2002) © FEBS 2002 doi:10.1046/j.1432-1033.2002.03038.x

REVIEW ARTICLE

Phosphorylation of calmodulin
Functional implications

Gustavo Benaim[1] and Antonio Villalobo[2]

[1]*Instituto de Biología Experimental, Facultad de Ciencias, Universidad Central de Venezuela, Caracas, Venezuela;* [2]*Instituto de Investigaciones Biomédicas, Consejo Superior de Investigaciones Científicas and Universidad Autónoma de Madrid, Spain*

Calmodulin (CaM) is phosphorylated *in vitro* and *in vivo* by multiple protein-serine/threonine and protein-tyrosine kinases. Casein kinase II and myosin light-chain kinase are two of the well established protein-serine/threonine kinases implicated in this process. On the other hand, within the protein-tyrosine kinases involved in the phosphorylation of CaM are receptors with tyrosine kinase activity, such as the insulin receptor and the epidermal growth factor receptor, and nonreceptor protein-tyrosine kinases, such as several members of the Src family kinases, Janus kinase 2, and p38Syk. The phosphorylation of CaM brings important physiological consequences for the cell as the diverse phosphocalmodulin species have differential actions as compared to nonphosphorylated CaM when acting on different CaM-dependent systems. In this review we will summarize the progress made on this topic as the first report on phosphorylation of CaM was published almost two decades ago. We will emphasize the description of the phosphorylation events mediated by the different protein kinases not only in the test tube but in intact cells, the phosphorylation-mediated changes of CaM activity, its action on CaM-dependent systems, and the functional repercussion of these phosphorylation processes in the physiology of the cell.

Keywords: calmodulin; calmodulin-dependent systems; cellular signalling; phosphocalmodulin; protein kinases; phosphoprotein phosphatases.

INTRODUCTION

Calmodulin as a Ca^{2+} sensor

The average cytosolic concentration of free Ca^{2+} in resting cells ranges from 20 to 50 nM, reaching values close to 1 μM when the cell is stimulated by a variety of physiological stimuli, while in the extracellular milieu this concentration is about 1 mM. This large concentration gradient allows intracellular Ca^{2+} to work as an useful second messenger [1,2]. The cytosolic Ca^{2+} concentration is exquisitely regulated by the operation of transport systems responsible for its increase, represented by different types of Ca^{2+} channels and $Na^+(H^+)/Ca^{2+}$ exchangers located in the plasma membrane, the endo(sarco)plasmic reticulum, and/or the mitochondria; and extrusion transport systems represented by Ca^{2+}-ATPases located in both the plasma membrane and the endo(sarco)plasmic reticulum, and the Na^+/Ca^{2+} exchanger also located within the plasma membrane [1,3]. The operation of theses transporters gives rise to oscillations in the concentration of Ca^{2+} not only in the cytosol but in the nucleus and intracellular organelles [1,4,5]. It has been possible to observe in living cells inhomogeneities in these transient changes in

Correspondence to A. Villalobo, Instituto de Investigaciones Biomédicas, Consejo Superior de Investigaciones Científicas and Universidad Autónoma de Madrid, Arturo Duperier 4, E-28029 Madrid, Spain. E-mail: antonio.villalobo@iib.uam.es

Abbreviations: AdeCycl, adenylate cyclase; CaM, calmodulin; CaM-BD, calmodulin-binding domain; CaMPK-II, calmodulin-dependent protein kinase II; CK-II, casein kinase II; CKR, cytokine receptor; dans-CaM, dansylated calmodulin; dans-CaM-BD, dansylated calmodulin-binding domain; dans-P-(Ser/Thr)-CaM, dansylated calmodulin phosphorylated at serine/threonine residues; EGF, epidermal growth factor; EGFR, epidermal growth factor receptor; InsR, insulin receptor; Jak2, Janus kinase 2; MLCK, myosin light-chain kinase; NHE1, Na^+/H^+ exchanger isoform 1; NLS, nuclear localization sequence; NOS, nitric oxide synthase; P-CaM, phosphocalmodulin; PDE1, calmodulin-dependent cyclic nucleotide phosphodiesterase; PP2B, protein-phosphatase 2B (calcineurin); P-(Ser/Thr)-CaM, calmodulin phosphorylated at serine/threonine residues; PTB domain, phosphotyrosine binding domain; P-(Thr)-CaM, calmodulin phosphorylated at threonine residues; PTK, protein-tyrosine kinase; P-(Tyr)-CaM, calmodulin phosphorylated at tyrosine residues; SH2 domain, Src noncatalytic homology 2 domain; TFP, trifluoperazine.

Enzymes: Adenylate cyclase (EC 4.6.1.1), Ca^{2+}-ATPase (EC 3.6.3.8), calcineurin (EC 3.1.3.16), calmodulin-dependent protein kinase II (EC 2.7.1.123), calmodulin(lysine) *N*-methyltransferase (EC 2.1.1.60), carboxylmethyltransferase (EC 2.1.1.8), carboxypeptidase (EC 3.4.17.20), casein kinase II (EC 2.7.1.37), cyclic nucleotide phosphodiesterase (EC 3.1.4.17), myosin light-chain kinase (EC 2.7.1.117), nitric oxide synthase (EC 1.14.13.39), phosphoprotein phosphatase (EC 3.1.3.16), phosphorylase kinase (EC 2.7.1.38), phosphotyrosine-specific phosphatase (EC 3.1.3.48), protein N-terminal acetyltransferase (EC 2.3.1.88), protein-serine/threonine kinase (EC 2.7.1.37), protein-tyrosine kinase (EC 2.7.1.112).

(Received 23 January 2002, revised 24 April 2002, accepted 10 June 2002)

Ca^{2+} concentration represented by singular events of progressively higher magnitudes which generate global changes modulated both in amplitude and frequency [6–8]. Free Ca^{2+} regulates multiple cellular functions, and the role of Ca^{2+} as an intracellular messenger depends in great extent on the existence of Ca^{2+}-binding proteins capable of decoding very small changes in its intracellular concentration [1,3].

Calmodulin is an ubiquitous highly conserved acidic Ca^{2+}-binding protein present in all eukaryotic organisms ranging from mammals to unicellular protists [9,10]. The tertiary structure of CaM has been elucidated, presenting a dumb-bell shape formed by two globular clusters at its C- and N-termini that are connected by a flexible central α helix [11] (Fig. 1). The interaction of CaM with its targets occurs due to the Ca^{2+}-induced increase in its α helix contents and the exposure of hydrophobic amino-acid residues. The function of CaM is not confined to its Ca^{2+}-bound form as it can recognize different protein targets as apocalmodulin [10,12].

Calmodulin has been implicated in a myriad of cellular processes, ranging from the synthesis and degradation of cyclic nucleotides and phosphoinositides, the phosphorylation/dephosphorylation cycle of proteins, gene transcription, and the regulation of different transport systems. This pluripotential intracellular Ca^{2+} sensor hence regulates crucial signalling pathways that are involved, among others, in the control of cellular metabolism, cytoskeletal organization, cytokinesis, muscle contraction, osmotic cell volume regulation, endo- and exocytosis, zygote fertilization, intercellular communication, cell proliferation, differentiation, and apoptosis [1,3,9,10].

Posttranslational modifications of calmodulin

As it would be expected for a protein of such a paramount importance, CaM is susceptible to undergo post-translational modifications. In addition to the acetylation of its N-terminal alanine [13], process which is catalysed by an acetyltransferase, one of the most notable post-translational modifications in mammalian CaM is the trimethylation of its Lys115 catalysed by a calmodulin (lysine) *N*-methyltransferase [13,14]. The dimethylation of Lys13 in CaM from *Paramecium* has also been described [15]. In addition, mammalian CaM is able to undergo carboxylmethylation catalysed by a carboxylmethyltransferase using *S*-adenosyl-L-methionine as donor of methyl groups [14]. Nevertheless, the function of all these methylation processes remains unclear. Another interesting post-translational modification of CaM is the proteolytic cleavage of its C-terminal lysine residue, which is mediated by a carboxypeptidase [14].

In any event, protein phosphorylation is by far the most common mechanism of protein modification aimed to regulate cellular functions. The existence of multiple well-regulated protein kinases and phosphoprotein phosphatases in all cells guarantees the occurrence of these reversible phosphorylations. Therefore, it is not surprising that CaM actions were subjected to phosphorylation-dependent modulation. In this respect, mammalian CaM possesses several putative phosphorylatable amino-acid residues, which includes four serine, 12 threonine and two tyrosine, some of which have been shown indeed to be phosphorylated. Figure 1 shows in the three-dimensional structure of human CaM the location

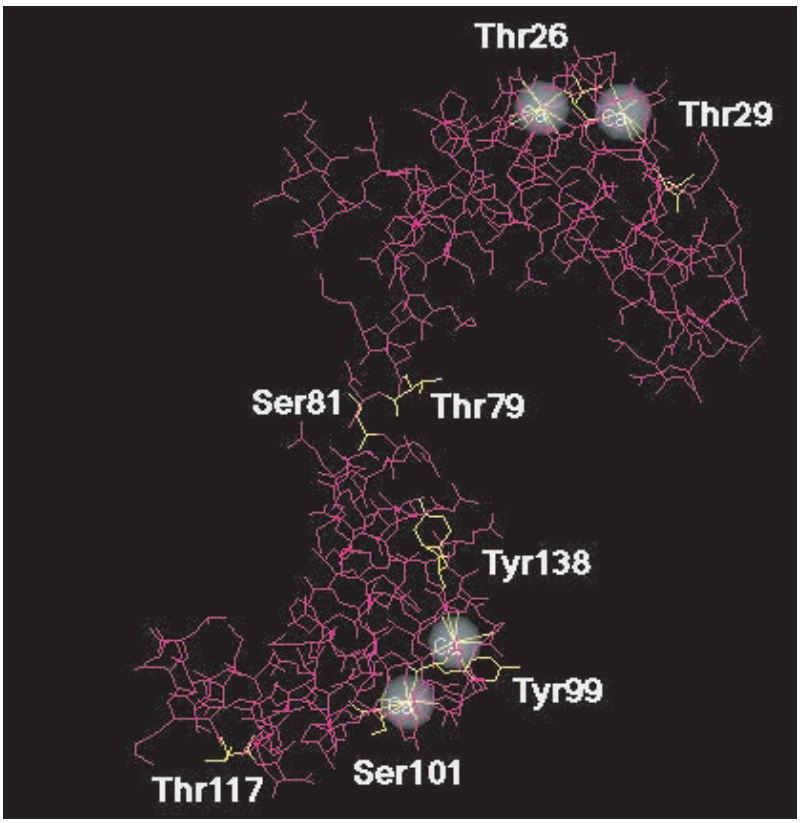

Fig. 1. Amino-acid residues of calmodulin known to be phosphorylated by different protein kinases. The amino-acid residues phosphorylated by protein-serine/threonine kinases and protein-tyrosine kinases are highlighted (*yellow colour*) in a tridimentional wire-frame model of human CaM in its Ca^{2+}-bound conformation. The figure was prepared from its reported crystallographic structure [82] using the Cn3D 3.0 program. The three-letter amino-acid code is used to label the targeted residues indicated by numbers. Notice the location of Thr79 and Ser81 in the central α-helix, the location of Thr29, Tyr99 and Ser101 in different Ca^{2+}-binding pockets, and the greater exposure of Tyr99 as compared to Tyr138.

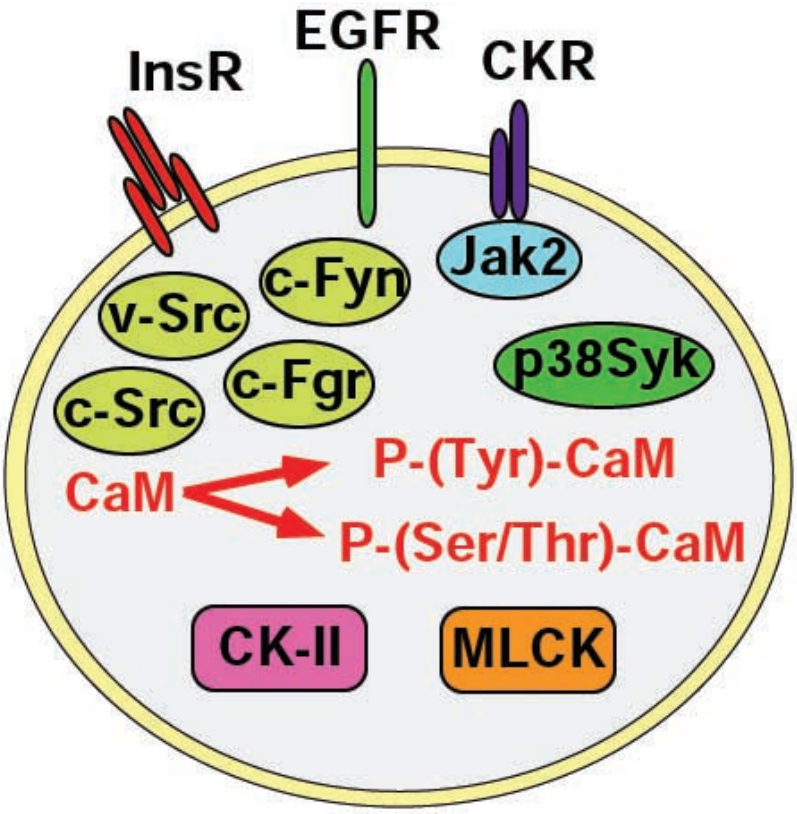

Fig. 2. Protein kinases known to phosphorylate calmodulin. The protein-tyrosine kinases and protein-serine/threonine kinases known to phosphorylate calmodulin are shown in a cartoon representing a cell. Jak2 is represented to be associated to a CKR.

Table 1. Phosphorylation sites of calmodulin. Phosphorylated residues of mammalian CaM are represented by the three-letter amino-acid code. Minority phosphorylations are indicated in parenthesis and expressed in percentage when known.

Protein kinase	Phosphorylated residues	References
Serine/threonine kinases		
CK-II	Thr79, Ser81, Ser101 (Thr117)	[23,28–31]
MLCK	Thr29 (Thr26, 10%)	[34]
Tyrosine kinases		
InsR	Tyr99 (Tyr138, 33%)	[31,39,49]
EGFR	Tyr99 (Tyr138, 15%)	[54,56,57]
c-Src	Tyr99 (Tyr138, 26%)	Unpublished[a]
PTK-III (c-Fyn + c-Fgr)	Tyr99 (Tyr138, 10%)	[23,62]

[a] Salas, V., Benaim, G. & Villalobo, A, unpublished results.

of the most relevant amino-acid residues on which phosphorylation by different protein-serine/threonine kinases and protein-tyrosine kinases have been demonstrated. Figure 2 summarizes the protein kinases involved and Table 1 shows the extent of these phosphorylations.

Early evidence for calmodulin phosphorylation

About three decades ago, it was initiated the isolation of a series of low molecular mass Ca^{2+}-binding phosphoproteins some of which, although presented properties that not always matched those of CaM [16–18], were able to activate typical CaM-dependent enzymes, such as cyclic nucleotide phosphodiesterase and adenylate cyclase [19,20]. These observations opened the debate on the possibility that CaM could be a phosphoprotein. That discussion was temporally set aside when CaM, the genuine activator of those enzymes, was purified and shown in those days to lack covalently bound phosphate. Ten years later the situation was reversed when the first strong evidence for the existence of phosphorylated CaM in chicken tissues was published by Plancke and Lazarides [21]. Since then, the accumulated evidence for CaM phosphorylation has grown and it has been firmly consolidated. The goal of the present review is to discuss the phosphorylation of CaM by different protein kinases, to discern how these phosphorylations affect its biological activity when acting on different CaM-dependent systems, and to uncover the possible functional implications of these phosphorylation processes in living cells.

PHOSPHORYLATION OF CALMODULIN BY PROTEIN-SERINE/THREONINE KINASES

The large number of protein-serine/threonine kinases present in the cell are involved in many cellular functions as they intervene in multiple signalling pathways and regulatory control systems. So far, only casein kinase II and myosin light-chain kinase have been shown to phosphorylate CaM within this group of protein kinases (Fig. 2).

Casein kinase II

The first report on phosphorylation of CaM by CK-II did not describe the need for basic polycations [22]. The requirement for these basic cofactors was subsequently found using a variety of positively charged molecules [23–25]. Thereafter, all reports dealing with CaM phosphorylation by CK-II included basic polycations in the assay systems used. The action of polycations appears to be more complex than expected, as these agents activate CK-II independently of its interaction with CaM. Moreover, the phosphorylation state of the catalytic α subunit of CK-II and the inhibitory action of its regulatory β subunit play important roles in the phosphorylation process [24–27].

Conflicting early reports were published on the phosphorylation sites of CaM by CK-II *in vitro*. Hence, some authors indicated that the major phosphorylation sites were Ser81 and Thr79 [23], while others reported phosphorylation at Ser101 and Thr79, but not at Ser81 [28]. More recently, phosphorylation of the three mentioned amino-acid residues, plus a limited phosphorylation of Thr117, has been reported [29–31] (Fig. 1). Moreover, as CaM isolated from rat liver has been shown to be phosphorylated at Thr79, Ser81 and Ser101, this gives support to the notion that CK-II may phosphorylate the three mentioned residues *in vivo* [30] (Table 1).

Thr79 and Ser81 are located in the central α helix of CaM, a region of great importance for its interaction with target CaM-dependent proteins. In contrast, Ser101 is located in its third Ca^{2+}-binding pocket (Fig. 1). Thus, phosphorylation of these residues may affect the interaction of CaM with target proteins and/or its Ca^{2+} binding capacity. Indeed, it was demonstrated that the phosphorylation of CaM by CK-II increases the binding affinity for Ca^{2+} as compared with nonphosphorylated CaM by the use of Tb^{3+} as an analogue of Ca^{2+} [32]. In contrast, a subsequent report using flow dialysis showed that phosphorylation of CaM by CK-II slightly decreases its affinity for Ca^{2+} [33]. It is interesting to mention in this context that the presence of Ca^{2+} at low concentrations stimulates the phosphorylation of CaM by CK-II [24], while higher concentrations inhibit this process, particularly affecting the phosphorylation of serine residues [23,24].

Myosin light-chain kinase

Chicken gizzard MLCK phosphorylates CaM *in vitro* in the presence of poly(L-arginine) [34]. In contrast to its phosphorylation by other protein kinases, Ca^{2+} stimulates this process in a biphasic manner, reaching a maximum at 50 μM [34]. This phosphorylation takes place predominantly at Thr29, located in the first Ca^{2+}-binding site of CaM (Fig. 1), although a limited phosphorylation of Thr26 was also observed [34] (Table 1). Using different CaM mutants, it was demonstrated that the duplication of the N-terminal EF-hand pair, or switching the C- and N-terminal EF-hand pairs, increases its phosphorylation as compared to wild type CaM. A CaM mutant with a duplicated C-terminal EF-hand pair was not phosphorylated [35]. These results suggest that the orientation of the different Ca^{2+}-binding pockets of CaM can be distinguished by MLCK [35].

Other protein-serine/threonine kinases

The phosphorylation of endogenous and exogenous CaM by a crude preparation of skeletal muscle phosphorylase kinase has been reported [21]. However, other authors failed to detect phosphorylation of exogenously added CaM by this kinase [23]. This could be due to a very low phosphorylation stoichiometry, as its value was reported to be 0.01 mol of phosphate incorporated per mol of CaM [21], what may rest physiological significance to this observation. Additional protein-serine/threonine kinases have been tested for their capacity to phosphorylate CaM. This included protein kinase C, cyclic AMP-dependent protein kinase and casein kinase I, and it was shown that all these kinases were unable to phosphorylate CaM *in vitro* [22,23].

PHOSPHORYLATION OF CALMODULIN BY PROTEIN-TYROSINE KINASE RECEPTORS

Of the 50+ members in the superfamily of protein-tyrosine kinase receptors, only the insulin receptor and the epidermal growth factor receptor have been shown to phosphorylate CaM (Fig. 2).

Insulin receptor

The insulin-dependent phosphorylation of CaM by the InsR isolated from different sources such as adipose tissue, liver, skeletal muscle, and placenta has been described [36–42]. This phosphorylation requires the presence of diverse polycations or basic proteins to be optimal, such as poly (L-lysine), protamine or histone [40]. These cofactors have a concentration-dependent biphasic behaviour, thus presenting a stimulatory phase at low concentrations and an inhibitory effect at higher concentrations [38,42,43]. The mode of action of the polycations is heterogeneous. Thus, in experiments using poly(L-lysine), it was demonstrated that although the formation of a poly(L-lysine)/calmodulin complex appears to be an essential factor for the phosphorylation to occur [42,43], poly(L–lysine) itself directly activates the tyrosine kinase activity of the InsR by an insulin-independent mechanism [42,44].

The search for cofactors that may work in physiological conditions increasing the phosphorylation of CaM is undoubtedly a topic of great interest. In this context, it has been proposed that the C-terminal region of K-Ras, but not of N-Ras, may play this role, as a peptide corresponding to this highly basic segment of K-Ras stimulates the phosphorylation of CaM by the insulin receptor [45,46]. This observation could be related to the observed interaction of CaM with K-Ras, but not with

N-Ras or H-Ras [47]. The phosphorylation of CaM by the InsR is sensitive to Ca^{2+}, as concentrations above 1–10 μM progressively inhibit this process [37,40–42,45,48], while lower concentrations appear to have a significant stimulatory effect [37].

Earlier reports on the phosphorylation of CaM by the InsR showed very low stoichiometry, in the order of 0.01–0.15 mol of phosphate incorporated per mol of CaM [38–40]. Subsequent improvement in the assay conditions increased the observed stoichiometry to values close to 1 (mol/mol) [42,49]. The phosphorylation of CaM by isolated InsR preparations takes place at tyrosine residues, with little phosphorylation at serine, process that is mediated by contaminant protein-serine/threonine kinase(s) [36,40]. The information available on the actual tyrosine residues that are phosphorylated by this receptor is not homogeneous. Hence, some reports indicated that the phosphorylation took place only or predominantly at Tyr99 [38,43,50], while others demonstrated that this phosphorylation occurred at Tyr99 and Tyr138 [31,39,49] (Table 1 and Fig. 1). The detection of significant phosphorylation at Tyr138 was favoured by the use of more sophisticated analytical techniques, such as the assay of a CaM mutant that lacks Tyr99 or by sequence analysis of CaM phosphopeptides [31,39,49]. The typical Ca^{2+}-induced electrophoretic mobility shift of nonphosphorylated CaM was absent or of little magnitude in CaM phosphorylated by the InsR [38,50]. Interestingly, the InsR increased its ability to phosphorylate CaM that was previously phosphorylated by CK-II [29].

Epidermal growth factor receptor

An early short report showed that plasma membrane fractions from A431 tumour cells, a cell line that overexpresses the EGFR, phosphorylate CaM in an EGF-stimulated manner [51]. This phosphorylation occurred exclusively at serine residues [51], therefore excluding the EGFR as the direct actor in this process. Thereafter, it was demonstrated that the EGFR isolated from rat liver by CaM-affinity chromatography was able to phosphorylate CaM at tyrosine residues in the presence of polycations as cofactors with a stoichiometry close to 1 (mol/mol) [52–55]. The basic cofactors used, poly(L-lysine) and histone, present also biphasic effects. Thus, the observation of an optimal polycation/calmodulin molar ratio to attain maximum phosphorylation of CaM was a prominent feature of this process [54]. This phosphorylation takes place essentially at Tyr99, with only residual phosphorylation of Tyr138 if any [54,56,57] (Table 1 and Fig. 1). It is interesting to mention that in contrast to CaM phosphorylated by the InsR, CaM phosphorylated by the EGFR presents a Ca^{2+}-induced electrophoretic mobility shift identical to nonphosphorylated CaM [54], underscoring the different nature of these P-CaM species, particularly in its relative proportion of Tyr99 and Tyr138 phosphorylation (Table 1).

We have also demonstrated that exogenous CaM can be phosphorylated in an EGF-dependent manner after entering into permeabilized transfected murine fibroblasts overexpressing the human EGFR with similar characteristics than those produced by its phosphorylation with the isolated receptor from rat liver [58]. Calcium ion has a very pronounced inhibitory effect on CaM phosphorylation, exhibiting a K'_i below 1 μM [54,58]. This is particularly important, as the cytosolic Ca^{2+} rise generated by the activation of the EGFR in intact cells is of a magnitude compatible with the transient prevention of the EGF-dependent phosphorylation of CaM at this point of the receptor activation cycle [59].

PHOSPHORYLATION OF CALMODULIN BY NONRECEPTOR PROTEIN-TYROSINE KINASES

Several nonreceptor protein-tyrosine kinases has been shown to phosphorylate CaM including several members of the Src family, and other nonrelated kinases such as Jak2 and p38Syk (Fig. 2).

Src family kinases

A viral variant of Src isolated from cells transformed by the Rous sarcoma virus was the first nonreceptor protein-tyrosine kinase identified to phosphorylate CaM [60]. The phosphorylation of CaM by isolated v-Src was measured in the absence of added polycations, and this phosphorylation was inhibited by Ca^{2+} with a K'_i of 30 μM [60]. This value is significantly higher than the one described for the phosphorylation of CaM by the EGFR [54,58]. We have observed, however, that addition of poly (L-lysine) strongly stimulates the phosphorylation of CaM by purified c-Src (V. Salas, G. Benaim & A. Villalobo, unpublished results), the normal nontumorigenic form of this kinase. Moreover, this phosphorylation takes place at Tyr99, and to a lesser but significant extent at Tyr138 (Table 1 and Fig. 1). In agreement with the expected proportionally high phosphorylation of Tyr138, the P-CaM species resulting from the catalysis by v-Src has a slower electrophoretic mobility than native nonphosphorylated CaM [60].

Other protein-tyrosine kinases of the Src family have been tested for their capacity to phosphorylate CaM with mix results. Thus, PTK-III from spleen, a preparation containing c-Fyn plus c-Fgr [61], efficiently phosphorylates CaM in the presence of different polycations, preferentially at Tyr99, although phosphorylation at Tyr138 was also detected [23,62] (Table 1 and Fig. 1). In contrast, it was shown that PTK-I/c-Lyn, and PTK-IIA (also related to c-Lyn) isolated from the same source fail to phosphorylate CaM [23].

Janus kinase 2

Little information is available about the phosphorylation of calmodulin by the Janus kinase family. Thus, there is only a report describing that a purified preparation of Jak2 coupled to agarose beads and isolated from a cell line derived from inner medullar collecting ducts from kidney was able to phosphorylate CaM in a time-dependent manner in the absence of added polycations [63].

Other nonreceptor protein-tyrosine kinases

A few additional nonreceptor protein-tyrosine kinases have been tested for their ability to phosphorylate CaM. The protein-tyrosine kinase PTK-IIB/p38Syk from spleen is not

related to Src but to p72Syk. Interestingly, this kinase has been shown to phosphorylate CaM in the presence of polycations, albeit at a very low rate [23]. In contrast, c-Abl, a cellular homologue of the tumorigenic gene product from Abelson murine leukaemia virus, fails to phosphorylate CaM [23].

PHOSPHORYLATION OF CALMODULIN BY UNIDENTIFIED PROTEIN KINASES

Phosphorylation of CaM tightly bound to purified rat liver plasma membrane fractions by unidentified membrane-bound protein kinases has been described previously [64,65]. This phosphorylation was inhibited by Ca^{2+}, does not require the addition of exogenous polycations and takes place exclusively at serine residues [64,65]. In contrast, the phosphorylation of exogenous CaM by these plasma membrane preparations requires the presence of polycations presenting a biphasic behaviour toward the polycation/calmodulin (mol/mol) ratio, and takes place at serine, threonine plus tyrosine residues [65]. The phosphorylation of exogenous CaM was more sensitive to the inhibitory action of Ca^{2+} than that of endogenous CaM, presenting a K'_i of 1 μM by the former and higher than 0.1 mM by the latter [65]. The differential phosphorylation of endogenous and exogenous CaM by protein kinases associated to the plasma membrane is an interesting observation, as underscores that both P-CaM pools could have different functions in the cell. Plasma membrane fractions isolated from Ehrlich ascites tumour cells lack bound CaM although contain protein kinase(s) able to phosphorylate exogenously added CaM in the presence of polycations [65]. CaM was also phosphorylated in the presence of polycations by cytosolic preparations from the inner and outer zones of the adrenal cortex [66]. This latter phosphorylation took place with low stoichiometry, was inhibited by Ca^{2+} and occurred exclusively at threonine residue(s) [66].

PHOSPHORYLATION OF CALMODULIN IN INTACT CELLS AND TISSUES

The first strong evidence for the existence of P-CaM *in vivo* was obtained from chicken brain and muscle, where CaM phosphorylated exclusively at serine residues was identified as a major component of these tissues [21]. Interestingly, this P-CaM species does not present the Ca^{2+}-induced electrophoretic mobility shift observed in nonphosphorylated CaM [21]. Thereafter, P-CaM was isolated from rat brain, where it appears to be more prevalent in its particulate fraction than in its soluble fraction, suggesting that it was bound to membranes [22]. The actual kinase(s) responsible for the phosphorylation of CaM in brain tissue was not identified although it was suggested that CK-II could be implicated [22]. Thereafter, it was demonstrated that the phosphorylation sites of CaM *in vivo*, as isolated from rat liver, where Thr79, Ser81 and Ser101, which correspond to three of the four sites phosphorylated by CK-II *in vitro*, suggesting that this kinase may be involved in the phosphorylation of CaM in intact tissues [30]. Likewise, it was estimated that approximately 15% of CaM present in liver was in its phosphorylated form [30].

Calmodulin phosphorylated at serine and threonine residues has been detected in normal nontransformed fibroblasts, while CaM isolated from cells transformed by the Rous sarcoma virus presented phosphorylation at serine, threonine plus tyrosine residues [60]. This suggests that v-Src was responsible for the tyrosine-directed phosphorylation of CaM in physiological conditions [60].

The first indication that physiological concentrations of insulin stimulate the phosphorylation of CaM in intact cells, presumably at tyrosine residues, was demonstrated in adipocytes [67]. This report was challenged by other authors who argued that the identified phosphoprotein was not P-CaM [68]. They indicated that the misidentified protein was phosphorylated by a protein-serine/threonine kinase and that insulin does not stimulate the phosphorylation of genuine CaM [68]. Nevertheless, subsequent reports further demonstrated both the presence of P-CaM in isolated hepatocytes and the existence of insulin-stimulated phosphorylation of CaM at serine, threonine plus tyrosine residues in intact cells with an stoichiometry close to 0.5 (mol/mol) [30,69]. Using baculovirus-transfected cells expressing the cytosolic domain of the β-subunit of the InsR it was demonstrated that this receptor was directly involved in the phosphorylation of CaM at both Tyr99 and Tyr138 [31] (Table 1 and Fig. 1). The insulin-stimulated phosphorylation of serine/threonine residues of CaM in intact cells was proposed to be due, at least in part, to the action of CK-II, an insulin-sensitive protein kinase [29,31,69].

The activation of G protein-coupled receptors, such as bradykinin receptors, generate signalling events mediated by Jak2. During this process, it was demonstrated in intact kidney cells the assembly of this protein-tyrosine kinase with CaM and NHE1. The formation of this molecular complex results in the subsequent phosphorylation of CaM at tyrosine residues and the increased alkalinization of the cytosol mediated by this antiporter [63].

DEPHOSPHORYLATION OF PHOSPHOCALMODULIN

Although P-CaM is very prone to dephosphorylation *in vivo* [21], little information is available on the actual protein phosphatases involved in the dephosphorylation of the different forms of P-CaM. Nevertheless, P-(Ser/Thr)-CaM, as phosphorylated by CK-II, has been shown to be dephosphorylated, albeit with low efficiency, by the pleiotropic phosphatases PP1γ and PP2A. No evidence for dephosphorylation of P-(Ser/Thr)-CaM by calcineurin (PP2B) was found [33]. Up to day, the phosphotyrosine-specific phosphatase(s) involved in the dephosphorylation of P-(Tyr)-CaM has not been identified.

FUNCTIONAL IMPLICATIONS FOR THE DIFFERENTIAL MODULATION OF CALMODULIN-DEPENDENT SYSTEMS BY CALMODULIN AND PHOSPHOCALMODULIN

The effects of different species of CaM phosphorylated at serine/threonine and/or tyrosine residues on the multiple CaM-dependent systems of the cell have been explored to a limited extent. Nevertheless, several enzymes, transport systems and receptors have been studied, although most of those works were performed *in vitro* (Table 2).

Table 2. Action of phosphocalmodulin on calmodulin-dependent systems. The action and kinetics parameters of different P-CaM species on diverse CaM-dependent systems are indicated. When the phosphorylated amino acids in CaM is not shown the protein kinase involved in the phosphorylation of CaM is indicated in parenthesis (Table 1). P-(Tyr99)-CaM was purified from a preparation containing other P-CaM species as phosphorylated by spleen PTK-III [62].

System	P-CaM species (kinase)	Action	References
PDE1 (brain)	P-(Ser/Thr)-CaM (CK-II)	Threefold to fivefold higher K_{act} and similar V_{max} than CaM	[33,70,71]
PDE1 (brain)	dans-P-(Ser/Thr)-CaM (CK-II)	21-Fold higher K_d than dans-CaM for CaM-BD peptide	[71]
PDE1 (brain)	P-(Tyr)-CaM (InsR)	Twofold higher K_{act} and similar V_{max} than CaM	[49]
PDE1 (brain)	P-(Tyr99)-CaM	Twofold lower K_{act} and similar V_{max} than CaM, fivefold lower K_d than CaM for a CaM-BD peptide	[62]
PDE1 (liver)	P-(Tyr)-CaM (InsR)	Similar K_{act} and V_{max} than CaM	[50]
PDE1 (heart)	P-(Tyr)-CaM (EGFR)	90% inactive	[57]
AdeCycl	P-(Tyr99)-CaM	Ninefold lower K_d than CaM for a CaM-BD peptide	[62]
Ca^{2+}-ATPase	P-(Ser/Thr)-CaM (CK-II)	Similar K_{act} and twofold lower V_{max} than CaM	[33]
Ca^{2+}-ATPase	P-(Ser/Thr)-CaM (CK-II)	Two to threefold higher K_{act} and similar V_{max} than CaM, 1.5-fold to twofold higher K_d than CaM for dans-CaM-BD peptides	[33,71,72]
Ca^{2+}-ATPase	P-(Tyr)-CaM (InsR)	Similar K_{act} and V_{max} than CaM	[72]
Ca^{2+}-ATPase	P-(Tyr99)-CaM	Threefold lower K_{act} and similar V_{max} than CaM, lower K_d than CaM for a CaM-BD peptide	[62]
CaMPK-II	P-(Ser/Thr)-CaM (CK-II)	Two to threefold higher K_{act} and 10% to 5-fold lower V_{max} than CaM, 8-fold higher K_d than CaM for a CaM-BD peptide	[71,74]
CaMPK-II	dans-P-(Ser/Thr)-CaM (CK-II)	Threefold lower K_d than dans-CaM for CaM-BD peptide	[71]
CaMPK-II	P-(Tyr)-CaM (InsR)	Similar K_{act} and 30% higher V_{max} than CaM, similar K_d than CaM for a CaM-BD peptide	[74]
CaMPK-II	P-(Tyr99)-CaM	1.5-Fold lower K_{act} and 15% lower V_{max} than CaM, 200-fold lower K_d than CaM for a CaM-BD peptide	[62]
MLCK	P-(Thr)-CaM (MLCK)	Similar K_{act} and V_{max} than CaM	[34]
MLCK	P-(Ser/Thr)-CaM (CK-II)	Inactive, 30% inhibition basal activity	[70]

Table 2. Continued.

System	P-CaM species (kinase)	Action	References
MLCK	dans-P-(Ser/Thr)-CaM (CK-II)	More than sixfold higher K_d than dans-CaM	[71]
EGFR	P-(Tyr)-CaM (EGFR)	Twofold to threefold higher tyrosine kinase activity while CaM inhibits	[52,53,55,59,75]
PP2B	P-(Ser/Thr)-CaM (CK-II)	Similar K_{act} and V_{max} than CaM	[74]
PP2B	P-(Ser/Thr)-CaM (CK-II)	Sixfold higher K_{act} and similar V_{max} than CaM, 15% lower K_d than CaM for a CaM-BD peptide	[33,71]
PP2B	P-(Tyr)-CaM (InsR)	Similar K_{act} and V_{max} than CaM	[74]
PP2B	P-(Tyr99)-CaM	Similar K_{act} and V_{max} than CaM, 18-fold lower K_d than CaM for CaM-BD peptide	[62]
NOS	P-(Ser/Thr)-CaM (CK-II)	Twofold higher K_{act} and 2.6-fold higher V_{max} than CaM	[33,71]
NOS	dans-P-(Ser/Thr)-CaM (CK-II)	Threefold higher K_d than dans-CaM for CaM-BD peptide	[71]
NOS	P-(Tyr99)-CaM	Twofold higher K_{act} and 3.4-fold higher V_{max} than CaM, fourfold lower K_d than CaM for CaM-BD peptide	[62]
NHE1	P-(Tyr)-CaM (Jak2)	Activation	[63]

Cyclic nucleotide phosphodiesterase

Calmodulin phosphorylated by CK-II has lower affinity for bovine brain PDE1 as compared with nonphosphorylated CaM, although no differences were found on the V_{max} or the affinity of the enzyme for Ca^{2+} [33,70,71]. In agreement with these observations, the affinity of dansylated P-(Ser/Thr)-CaM for a peptide corresponding to the CaM-binding site of this PDE1 was lower than its affinity for dansylated nonphosphorylated CaM [71]. Moreover, this phosphorylation decreased the stoichiometry from two peptides bound per CaM molecule to only one [71] (Table 2).

The action of P-(Tyr)-CaM on PDE1 appears to vary depending on the number of phosphorylated tyrosine residues and the origin of the enzyme. Moreover, it should be considered the variable proportion of P-CaM and native CaM present in the different preparations used, as not always P-CaM was free of its nonphosphorylated form. CaM phosphorylated at Tyr99 plus Tyr138 by the InsR has lower affinity for bovine brain PDE1 than its nonphosphorylated counterpart [49]. In contrast, purified P-(Tyr99)-CaM has comparatively higher affinity for this enzyme than nonphosphorylated CaM, and a peptide corresponding to the CaM-binding site of PDE1 appears to have higher affinity for P-(Tyr99)-CaM that for its nonphosphorylated form [62]. In both instances, no changes on the V_{max} of the enzyme were observed when nonphosphorylated CaM was compared with the two P-(Tyr)-CaM species mentioned above [49,62]. CaM phosphorylated by the InsR does not show any differential effect as compared to nonphosphorylated CaM when acting on a PDE1 isolated from rat hepatocytes [50], while a preparation of P-(Tyr)-CaM, as phosphorylated by the EGFR, and totally free of nonphosphorylated CaM, was unable to significantly activate bovine heart PDE1 [57] (Table 2).

Another interesting observation is that the phosphorylation of CaM by the InsR alters its interaction with different CaM inhibitors [49,50]. Hence, by monitoring the PDE1 activity it was shown that P-(Tyr)-CaM was inhibited by TFP and W7 less efficiently than CaM [50], while mastoparan, another CaM inhibitor, increased its inhibitory potential toward P-(Tyr)-CaM as compared to nonphosphorylated CaM [49].

Overall, the above mentioned observations suggest that the action of the diverse P-(Ser/Thr)-CaM and P-(Tyr)-CaM species, as produced by different kinases, could be highly distinct in the different tissues, as it could either increase or decrease the amount of cAMP and cGMP available in the cell depending on the sensitivity of the PDE1 isoform to P-CaM.

Adenylate cyclase

The only information available on the possible effect of different P-CaM species on adenylate cyclase is that purified P-(Tyr99)-CaM has significant higher affinity than nonphosphorylated CaM for a peptide corresponding to the CaM-binding domain of this enzyme [62] (Table 2). As the action of this phosphorylated form of CaM on the activity of the enzyme has not been tested, it is not possible to predict its function.

Plasma membrane Ca^{2+}-ATPase

Calmodulin phosphorylated by CK-II has a diminished ability to activate the plasma membrane Ca^{2+}-ATPase. In a first report it was shown that the V_{max} of the enzyme decreased in the presence of P-(Ser/Thr)-CaM, while only a marginal effect on its affinity was detected [30]. Thereafter, it was reported that P-(Ser/Thr)-CaM had lower affinity than nonphosphorylated CaM for the Ca^{2+}-ATPase, and that the V_{max} of the enzyme was not significantly affected [30,72]. The discrepancy between those reports could be due to differences in the phosphorylation extent of serine and threonine residues. Moreover, the binding of CaM phosphorylated by CK-II to two synthetic peptides corresponding to the CaM-binding domain of the Ca^{2+}-ATPase occurs with slightly lower affinity than that of nonphosphorylated CaM [71], supporting the results on the impaired activation of the Ca^{2+}-ATPase activity described above (Table 2).

Concerning the effect of CaM phosphorylated by different protein-tyrosine kinases, there are also conflicting evidences. While some workers did not found any differences in the V_{max} or the K_{act} of the Ca^{2+}-ATPase, or in the affinity of the enzyme for Ca^{2+}, when CaM and P-(Tyr)-CaM where compared [72], similar V_{max} and an increase in the affinity of P-(Tyr99)-CaM for the Ca^{2+}-ATPase, when compared with native CaM, was observed by other group [62]. An apparent difference among those experiments were the species of P-CaM tested. While some investigators used CaM phosphorylated by the cytosolic domain of the β subunit of the insulin receptor with not further fractionation [72], that it was shown to be phosphorylated at both Tyr99 and Tyr138 [31,39,49]; others used PTK-III (a mixture of c-Fyn and c-Fgr) from spleen to phosphorylate CaM and purified the CaM species exclusively phosphorylated at Tyr99 for its assay [62]. The binding of P-(Tyr99)-CaM to a synthetic peptide corresponding to the CaM-binding domain of the Ca^{2+}-ATPase showed higher affinity than that of nonphosphorylated CaM [62]. However, the binding of P-(Tyr99)-CaM to the same dansylated peptide showed lower affinity than native CaM [62] (Table 2).

Using site-directed mutagenesis it was demonstrated that CaM with a Tyr138Phe substitution has lower affinity for the Ca^{2+}-ATPase than native CaM [72]. The phosphorylation at Tyr99 of this mutant CaM increased its affinity for the Ca^{2+}-ATPase, approaching to values closer to those of nonphosphorylated wild type CaM [72]. The Ca^{2+}-ATPase presented identical V_{max} and affinity for phosphorylated and nonphosphorylated wheat germ CaM, which contains a single tyrosine residue at position 139, albeit the K_{act} for plant CaM was fourfold higher than for nonphosphorylated mammalian CaM [72].

The kinetics parameters for Ca^{2+} transport by the plasma membrane Ca^{2+}-ATPase have not yet been studied in the presence of CaM phosphorylated at serine/threonine or tyrosine residues, so it remains to be elucidated if these phosphorylations are able to modify the efficiency of Ca^{2+} removal from the cytosol under physiological conditions. In this context, it was demonstrated that physiological concentrations of insulin inhibited the Ca^{2+}-ATPase activity from isolated adipocyte plasma membranes [73], suggesting that phosphorylation of CaM could be responsible for this effect [37]. It is interesting to mention that the insulin-sensitive CK-II could be involved in the process, as P-(Ser/Thr)-CaM is known to affect the kinetics parameters of the Ca^{2+}-ATPase more dramatically than P-(Tyr)-CaM [30,33,71,72] (Table 2).

Calmodulin-dependent protein kinase II

Calmodulin phosphorylated by CK-II has a decreased ability to activate CaMPK-II because a diminished affinity for this enzyme. It also slightly decreased its V_{max} and its affinity for Ca^{2+} [74]. On the contrary, CaM phosphorylated by the InsR increased the V_{max} of CaMPK-II without affecting other kinetics parameters [74], while purified P-(Tyr99)-CaM slightly decreased the V_{max} of this enzyme [62] (Table 2). Therefore, it is expected than this kinase could work less efficiently in the presence of P-(Ser/Thr)-CaM, and with higher efficiency in the presence of P-(Tyr)-CaM, as phosphorylated by the insulin receptor.

Surface plasmon resonance studies demonstrated that phosphorylation of CaM by CK-II, but not by the InsR, produced a decreased affinity for a peptide corresponding to the CaM-binding domain of CaMPK-II [74]. Other binding studies using different techniques demonstrated that a peptide corresponding to the CaM-binding site of CaMPK-II has several order of magnitude higher affinity for purified P-(Tyr99)-CaM than for nonphosphorylated CaM [62]. Also, dansylated P-(Ser/Thr)-CaM, as phosphorylated by CK-II, has higher affinity than dansylated nonphosphorylated CaM for a peptide corresponding to the CaM-binding domain of CaMPK-II [71] (Table 2).

Myosin light-chain kinase

Calmodulin phosphorylated by MLCK does not have a differential effect as compared to nonphosphorylated CaM on the activity of this kinase [34]. In contrast, CaM phosphorylated by CK-II not only fails to activate MLCK but also slightly inhibits its basal activity as measured in the absence of CaM, as compared to assays with nonphosphorylated CaM [70] (Table 2). This kinase maintains the same affinity for Ca^{2+} when assayed with phosphorylated and nonphosphorylated CaM [70]. These experiments demonstrate that the site of phosphorylation of CaM is a critical factor that affects the activity of MLCK, as CK-II and MLCK itself target different residues in CaM (Table 1). Interestingly, titration experiments show that the interaction of dansylated CaM with a peptide corresponding to the CaM-binding site of MLCK has a sigmoidal behaviour allowing for two peptides bound per each dansylated CaM molecule [71]. In contrast, the interaction of this peptide with dansylated

P-CaM, as phosphorylated by CK-II, has a lower affinity and an hyperbolic behaviour, suggesting the existence of a single binding site in this instance [71] (Table 2). CaM phosphorylated by CK-II was equally sensitive to TFP than nonphosphorylated CaM when assayed on the MLCK activity [34].

Epidermal growth factor receptor

The effect of CaM phosphorylated by the EGFR on the tyrosine kinase activity of this receptor has been studied in a preliminary manner. The results indicate that this P-(Tyr)-CaM species could be an activator of the tyrosine kinase activity of the receptor both in the presence and absence of Ca^{2+} [53,59,75], while nonphosphorylated CaM has an inhibitory effect only in the presence of Ca^{2+} [52,55] (Table 2). The space-temporal phases on which CaM and P-(Tyr)-CaM interact with the EGFR in intact cells have not yet been identified. Therefore, it is not possible at present to give a detailed description of the putative physiological functions of this process. Nevertheless, we have proposed that nonphosphorylated CaM could play a role on receptor internalization and intracellular processing [76]. This has received experimental support in intact cells, as permeant calmodulin inhibitors favour the accumulation of the EGFR in endosomes preventing both its migration to lysosomes and its recycling back to the cell surface [77].

Calcineurin

In a preliminary report it was shown that CaM phosphorylated by CK-II does not modify the kinetics parameters of calcineurin (PP2B) [74]. Thereafter, it was reported that CaM phosphorylated by this kinase has less affinity for calcineurin than nonphosphorylated CaM, although this enzyme has the same V_{max} for both CaM species [33,71]. Nevertheless, this phosphorylation slightly increases the affinity of CaM for a peptide corresponding to the CaM-binding site of calcineurin [71] (Table 2).

Phosphorylation of CaM by the InsR or PTK-III does not significantly affect the kinetics parameters of calcineurin [62,74], although the affinity of a peptide corresponding to the CaM-binding site of this phosphatase was significantly higher for purified P-(Tyr99)-CaM than for nonphosphorylated CaM [62] (Table 2).

Nitric oxide synthase

The effect of P-CaM on NOS isolated from brain has been investigated, and it was shown that both P-(Ser/Thr)-CaM and P-(Tyr)-CaM species stimulated the enzyme to a larger extent than nonphosphorylated CaM, and the affinity of both P-CaM species decreased with respect to its nonphosphorylated form [33,62,71]. It is interesting to mention that the magnitude of the increase in the activity of NOS mediated by phosphorylated CaM was far larger than the effects observed on other CaM-dependent enzymes (Table 2).

Displacement experiments of a dansylated synthetic peptide corresponding to the CaM-binding domain of the plasma membrane Ca^{2+}-ATPase bound to nonphosphorylated CaM or P-(Tyr99)-CaM by a peptide corresponding to the CaM-binding domains of NOS were performed in order to study the change in affinity due to this particular phosphorylation [62]. The results showed that the affinity of P-(Tyr99)-CaM for the CaM-binding site of NOS increased with respect to nonphosphorylated CaM [62], in agreement with the observed increase in activity. However, titration of dansylated CaM and dansylated P-(Ser/Thr)-CaM, as phosphorylated by CK-II, with a peptide corresponding to the CaM-binding domain of NOS, showed that this peptide has lower affinity for the phosphorylated form of CaM than for its nonphosphorylated counterpart [71] (Table 2).

Na^+/H^+ exchanger

Calmodulin binds to NHE1 in a Ca^{2+}-dependent manner, inducing at least in part its activation and the subsequent alkalinization of the cytosol [78]. It has been proposed that CaM phosphorylation by Jak2 is enhanced by the ensuing calcium signal generated by the activation of bradykinin B_2 receptors, and that P-(Tyr)-CaM could act as a potent activator of NHE1 by increasing its interaction with either of its two CaM-binding domains [63] (Table 2). As the EGFR also phosphorylates CaM (see above), this work raises an interesting possibility; as the mitogenic activation of cells via the EGFR and other growth factor receptors also results in the activation of the Na^+/H^+ exchanger. Hence, it is possible that P-(Tyr)-CaM generated during the activation of the EGFR could contribute to the production of the transient cytosolic alkalinization associated to the mitogenic activation of cells by EGF.

CONCLUDING REMARKS AND PROSPECTS

As we have learned from the different sections of this review article, the phosphorylation of CaM appears to be a widespread process leading to the regulation of diverse cellular functions. Calmodulin most frequently modulates its activity by the availability of Ca^{2+}, decoding in this manner signals generated by extracellular and intracellular stimuli. It is frequently noticed that Ca^{2+}/CaM-dependent enzymes, which play opposite physiological roles, exist in the cell. Among these, protein kinases and phosphatases such as CaMPK-II and calcineurin, and enzymes involved in the synthesis and degradation of cyclic nucleotides such as adenylate cyclase and PDE1, are relevant examples. It is apparent that effective mechanisms must be in place to prevent that these enzymes are equally activated upon arrival of a suitable Ca^{2+} signal. Certainly, the subcellular compartmentation of the conflicting enzymes, and/or the existence of kinetics control able to differentially discern minute changes in Ca^{2+} concentration, and therefore the frequency and amplitude of its oscillations, could avoid the operation of these futile regulatory cycles. Nevertheless, the efficiency of this process is expected to be tighter if additional regulatory systems were operative in the cell. CaM phosphorylation could play this role as an additional mechanism entrusted in the physiology of this modulator to achieve the fine-tuning of the multiple CaM-dependent systems of the cell independently of the generated Ca^{2+} signal.

The combinatorial phosphorylation of CaM at serine, threonine and/or tyrosine residues opens the possibility that

this Ca^{2+} sensor could have a variety of phosphorylated species with distinct properties in intact cells. This area of research is particularly interesting, as the assorted phosphorylation of CaM, that undoubtedly occurs in physiological conditions, could contribute to explain the plasticity and pleiotropicity shown by this modulator when acting on the multiple CaM-dependent systems of the cell. It is relevant to mention, that the information on the action of P-CaM on their different targets is still very limited (Table 2). Therefore, it is expected that additional CaM-dependent proteins would be added to the list of systems that are differentially modulated by CaM and P-CaM. Likewise, P-CaM is also expected to play additional, but yet unknown, roles on CaM-dependent systems on which the interaction between modulator and target occurs in the absence of Ca^{2+}. Therefore, future work in this unexplored area may help to unravel new physiological functions of apocalmodulin. It would also be important to have an outlook on whether P-(Tyr)-CaM is a distinct signalling molecule able to target proteins containing SH2 and/or PTB domains thereby affecting their functions.

The great majority of reported experiments have shown that it is required the presence of positively charged cofactors for the efficient phosphorylation of CaM. This is so for virtually all protein kinases tested, and it is possible that unnoticed basic proteins or other cationic contaminants could be present in the few examples where CaM phosphorylation was observed in the absence of added polycations. Thus, another interesting point that requires special attention is to establish the nature of the physiological ligand(s) that acts as cofactor(s) for CaM phosphorylation in intact cells, as this topic is largely neglected. Most workers have used in their experiments a series of synthetic polycations or basic proteins, such as histones, that may not have relevance for the phosphorylation of CaM in a physiological setting. Recently, a striking and disconcerting observations have been performed on the location of the EGFR and ErbB4 receptor in the nucleus and their putative role as transcription factors [79,80]. As the much needed confirmation of these unexpected observations unfolds, the possibility that CaM could be phosphorylated at the nucleus by these receptors or other protein kinases known to have nuclear location using histones as physiological cofactors is open to debate. The case for the EGFR is particularly interesting, as the NLS proposed to be involved in the translocation process [79], has been previously shown by our group to be the Ca^{2+}-dependent CaM-binding domain of this receptor located at its cytosolic juxtamembrane region [76]. A similar consensus NLS has been proposed to exist, among others, in the cytosolic juxtamembrane region of the ErbB4 receptor [81].

An additional problem which requires to be addressed in order to understand the role of the different P-CaM species in the cell is the identification of the whole set of protein kinases and phophoprotein phosphatases involved in the phosphorylation/dephosphorylation cycle of CaM in different tissues and under diverse developmental and physiological conditions. The panoply of enzymes involved in these processes in distinct organisms is expected to be large. Hence, it is expected that the progress of proteomic projects in a variety of living species, including human, may help to uncover new enzymes involved in physiologically relevant phosphorylation and dephosphorylation events of CaM in intact cells.

Finally, a significant effort must be made to determine all relevant physiological functions that the diverse sets of P-CaM species present in the cell may have at a given time. To this end, the use of transfectants that could express in an inducible manner dominant-negative CaM mutants unable to be phosphorylated by specific protein kinases could be of help.

ACKNOWLEDGEMENTS

The work in the authors laboratories was financed by grants from the Comisión Interministerial de Ciencia y Tecnología (SAF99-0052), the Agencia Española de Cooperación Internacional (2002 CN0013) and Consejería de Educación y Cultura de la Comunidad de Madrid (08.1/0027/2001–1) to AV; grants from the Fondo Nacional de Ciencia, Tecnología e Innovación del Ministerio de Ciencia y Tecnología de Venezuela (S1-99000058 and G-2001000637) and Consejo de Desarrollo Científico y Humanístico de la Universidad Central de Venezuela (C-03-10-33-4798/00) to GB; and a grant from the Spanish Ministry of Education (Programa de Cooperación con Iberoamérica) to A. V. and G. B.

REFERENCES

1. Carafoli, E. (1987) Intracellular calcium homeostasis. *Ann. Rev. Biochem.* **56**, 395–433.
2. Barritt, G.J. (1999) Receptor-activated Ca^{2+} inflow in animal cells: a variety of pathways tailored to meet different intracellular Ca^{2+} signalling requirements. *Biochem. J.* **337**, 153–169.
3. Carafoli, E., Santella, L., Branca, D. & Brini, M. (2001) Generation, control, and processing of cellular calcium signals. *Crit. Rev. Biochem. Mol. Biol.* **36**, 107–260.
4. Pozzan, T., Rizzuto, R., Volpe, P. & Meldolesi, J. (1994) Molecular and cellular physiology of intracellular calcium stores. *Physiol. Rev.* **74**, 595–636.
5. Bootman, M.D., Thomas, D., Tovey, S.C., Berridge, M.J. & Lipp, P. (2000) Nuclear calcium signalling. *Cell. Mol. Life Sci.* **57**, 371–378.
6. Berridge, M.J. (1990) Calcium oscillations. *J. Biol. Chem.* **265**, 9583–9586.
7. Berridge, M.J. (1995) Capacitative calcium entry. *Biochem. J.* **312**, 1–11.
8. Berridge, M.J. (1997) The AM and FM of calcium signalling. *Nature* **386**, 759–760.
9. Klee, C.B. & Vanaman, T.C. (1982) Calmodulin. *Adv. Prot. Chem.* **35**, 213–321.
10. Chin, D. & Means, A.R. (2000) Calmodulin: a prototypical calcium sensor. *Trends Cell Biol.* **10**, 322–328.
11. Babu, Y.S., Sack, J.S., Greenhough, T.J., Buge, C.E., Means, A.R. & Cook, W.J. (1985) Three-dimensional structure of calmodulin. *Nature* **315**, 37–40.
12. Jurado, L.A., Chockalingam, P.S. & Jarrett, H.W. (1999) Apocalmodulin. *Physiol. Rev.* **79**, 661–682.
13. Watterson, D.M., Sharief, F. & Vanaman, T.C. (1980) The complete amino acid sequence of the Ca^{2+}-dependent modulator protein (calmodulin) of bovine brain. *J. Biol. Chem.* **255**, 962–975.
14. Murtaugh, T.J., Wright, L.S. & Siegel, F.L. (1986) Posttranslational modifications of calmodulin in rat brain and pituitary. *J. Neurochem.* **47**, 164–172.
15. Schaefer, W.H., Lukas, T.J., Blair, I.A., Schultz, J.E. & Watterson, D.M. (1987) Amino acid sequence of a novel calmodulin from *Paramecium tetraurelia* that contains dimethyllysine in the first domain. *J. Biol. Chem.* **262**, 1025–1029.

16. Wolff, D.J. & Siegel, F.L. (1972) Purification of a calcium-binding phosphoprotein from pig brain. *J. Biol. Chem.* **247**, 4180–4185.
17. Brooks, J.C. & Siegel, F.L. (1973) Calcium-binding phosphoproteins: the principal acidic protein of mammalian sperm. *Biochem. Biophys. Res. Commun.* **55**, 710–716.
18. Brooks, J.C. & Siegel, F.L. (1973) Purification of a calcium-binding phosphoprotein from beef adrenal medulla. Identity with one of two calcium-binding proteins of brain. *J. Biol. Chem.* **248**, 4189–4193.
19. Wolff, D.J. & Brostrom, C.O. (1974) Calcium-binding phosphoprotein from pig brain: identification as a calcium-dependent regulator of brain cyclic nucleotide phosphodiesterase. *Arch. Biochem. Biophys.* **163**, 349–358.
20. Brostrom, C.O., Huang, Y.-C., Breckenridge, B.M. & Wolff, D.J. (1975) Identification of a calcium-binding protein as a calcium-dependent regulator of brain adenylate cyclase. *Proc. Natl Acad. Sci. USA* **72**, 64–68.
21. Plancke, Y.D. & Lazarides, E. (1983) Evidence for a phosphorylated form of calmodulin in chicken brain and muscle. *Mol. Cell. Biol.* **3**, 1412–1420.
22. Nakajo, S., Hayashi, K., Daimatsu, T., Tanaka, M., Nakaya, K. & Nakamura, Y. (1986) Phosphorylation of rat brain calmodulin *in vivo* and *in vitro*. *Biochem. Int.* **13**, 687–693.
23. Meggio, F., Brunati, A.M. & Pinna, L.A. (1987) Polycation-dependent, Ca^{2+}-antagonized phosphorylation of calmodulin by casein kinase-2 and spleen tyrosine protein kinase. *FEBS Lett.* **215**, 241–246.
24. Sacks, D.B. & McDonald, J.M. (1992) Effects of cationic polypeptides on the activity, substrate interaction, and autophosphorylation of casein kinase II: a study with calmodulin. *Arch. Biochem. Biophys.* **299**, 275–280.
25. Song, Q., Combest, W.L. & Gilbert, L.I. (1994) Spermine and polylysine enhanced phosphorylation of calmodulin and tubulin in an insect endocrine gland. *Mol. Cell. Endocrinol.* **99**, 1–10.
26. Meggio, F., Bolddyreff, B., Marin, O., Marchiori, F., Perich, J.W., Issinger, O.-G. & Pinna, L.A. (1992) The effect of polylysine on casein-kinase-2 activity is influenced by both the structure of the protein/peptide substrates and the subunit composition of the enzyme. *Eur. J. Biochem.* **205**, 939–945.
27. Sarno, S., Marin, O., Meggio, F. & Pinna, L.A. (1993) Polyamines as negative regulators of casein kinase-2: the phosphorylation of calmodulin triggered by polylysine and by the α[66–86] peptide is prevented by spermine. *Biochem. Biophys. Res. Commun.* **194**, 83–90.
28. Nakajo, S., Masuda, Y., Nakaya, K. & Nakamura, Y. (1988) Determination of the phosphorylation sites of calmodulin catalyzed by casein kinase 2. *J. Biochem.* **104**, 946–951.
29. Sacks, D.B., Davis, H.W., Crimmins, D.L. & McDonald, J.M. (1992) Insulin-stimulated phosphorylation of calmodulin. *Biochem. J.* **286**, 211–216.
30. Quadroni, M., James, P. & Carafoli, E. (1994) Isolation of phosphorylated calmodulin from rat liver and identification of the *in vivo* phosphorylation sites. *J. Biol. Chem.* **269**, 16116–16122.
31. Joyal, J.L., Crimmins, D.L., Thoma, R.S. & Sacks, D.B. (1996) Identification of insulin-stimulated phosphorylation sites on calmodulin. *Biochemistry* **35**, 6267–6275.
32. Aiuchi, T., Hagiwara, T., Omata, K., Nakaya, K. & Nakamura, Y. (1991) Effect of phosphorylation of calmodulin on calcium binding affinity as estimated by terbium fluorescence. *Biochem. Int.* **23**, 145–149.
33. Quadroni, M., L'Hostis, E.L., Corti, C., Myagkikh, I., Durussel, I., Cox, J., James, P. & Carafoli, E. (1998) Phosphorylation of calmodulin alters its potency as an activator of target enzymes. *Biochemistry* **37**, 6523–6532.
34. Davis, H.W., Crimmins, D.L., Thoma, R.S. & Garcia, J.G.N. (1996) Phosphorylation of calmodulin in the first calcium-binding pocket by myosin light chain kinase. *Arch. Biochem. Biophys.* **332**, 101–109.
35. Davis, H.W. (1997) Phosphorylation of calmodulin by myosin light chain kinase is altered by exchange or duplication of EF-hand pairs. *Biochem. Biophys. Res. Commun.* **236**, 702–705.
36. Haring, H.U., White, M.F., Kahn, C.R., Ahmad, Z., DePaoli-Roach, A.A. & Roach, P.J. (1985) Interaction of the insulin receptor kinase with serine/threonine kinases *in vitro*. *J. Cell Biochem.* **28**, 171–182.
37. Graves, C.B., Gale, R.D., Laurino, J.P. & McDonald, J.M. (1986) The insulin receptor and calmodulin: calmodulin enhances insulin-mediated receptor kinase activity and insulin stimulates phosphorylation of calmodulin. *J. Biol. Chem.* **261**, 10429–10438.
38. Laurino, J.P., Colca, J.R., Pearson, J.D., DeWald, D.B. & McDonald, J.M. (1988) The *in vitro* phosphorylation of calmodulin by the insulin receptor tyrosine kinase. *Arch. Biochem. Biophys.* **265**, 8–21.
39. Wong, E.C.C., Sacks, D.B., Laurino, J.P. & McDonald, J.M. (1988) Characteristics of calmodulin phosphorylated by the insulin receptor kinase. *Endocrinology* **123**, 1830–1836.
40. Sacks, D.B. & McDonald, J.M. (1988) Insulin-stimulated phosphorylation of calmodulin by rat liver insulin receptor preparations. *J. Biol. Chem.* **263**, 2377–2383.
41. Sacks, D.B. & McDonald, J.M. (1989) Calmodulin as substrate for insulin-receptor kinase: phosphorylation by receptors from rat skeletal muscle. *Diabetes* **38**, 84–90.
42. Sacks, D.B., Fujita-Yamaguchi, Y., Gale, R.D. & McDonald, J.M. (1989) Tyrosine-specific phosphorylation of calmodulin by the insulin receptor kinase purified from human placenta. *Biochem. J.* **263**, 803–812.
43. Saville, M.K. & Houslay, M.D. (1993) The role of polybasic compounds in determining the tyrosyl phosphorylation of calmodulin by the human receptor. *Cell. Signal.* **5**, 709–725.
44. Fujita-Yamaguchi, Y., Sacks, D.B., McDonald, J.M., Sahal, D. & Kathuria, S. (1989) Effect of basic polycations and proteins on purified insulin receptor: insulin-independent activation of the receptor tyrosine-specific protein kinase by poly(L-lysine). *Biochem. J.* **263**, 813–822.
45. Sacks, D.B., Glenn, K.C. & McDonald, J.M. (1989) The carboxyl terminal segment of the c-Ki-*ras* 2 gene product mediates insulin-stimulated phosphorylation of calmodulin and stimulates insulin-independent autophosphorylation of the insulin receptor. *Biochem. Biophys. Res. Commun.* **161**, 399–405.
46. Fujita-Yamaguchi, Y., Kathuria, S., Xu, Q.-Y., McDonald, J.M., Nakano, H. & Kamata, T. (1989) *In vitro* tyrosine phosphorylation studies on RAS proteins and calmodulin suggest that polylysine-like basic peptides or domains may be involved in interactions between insulin receptor kinase and its substrate. *Proc. Natl Acad. Sci. USA* **86**, 7306–7310.
47. Villalonga, P., López-Alcalá, C., Bosch, M., Chiloeches, A., Rocamora, N., Gil, J., Marais, R., Marshall, C.J., Bachs, O. & Agell, N. (2001) Calmodulin binds to K-Ras, but not to H- or N-Ras, and modulates its downstream signaling. *Mol. Cell. Biol.* **21**, 7345–7354.
48. Sacks, D.B. (1994) Alteration of calmodulin–protein interactions by a monoclonal antibody to calmodulin. *Biochim. Biophys. Acta* **1206**, 120–128.
49. Williams, J.P., Jo, H., Sacks, D.B., Crimmins, D.L., Thoma, R.S., Hunnicutt, R.E., Radding, W., Sharma, R.K. & McDonald, J.M. (1994) Tyrosine-phosphorylated calmodulin has reduced biological activity. *Arch. Biochem. Biophys.* **315**, 119–126.
50. Saville, M.K. & Houslay, M.D. (1994) Phosphorylation of calmodulin on Tyr99 selectively attenuates the action of calmodulin antagonists on type-I cyclic nucleotide phosphodiesterase activity. *Biochem. J.* **299**, 863–868.

51. Lin, P.H., Selinfreund, R. & Wharton, W. (1986) Epidermal growth factor sensitive phosphorylation of calmodulin in A431 cell membrane. *Fed. Proc.* **45**, 1693.
52. San José, E., Benguría, A., Geller, P. & Villalobo, A. (1992) Calmodulin inhibits the epidermal growth factor receptor tyrosine kinase. *J. Biol. Chem.* **267**, 15237–15245.
53. Benguría, A. & Villalobo, A. (1993) Calmodulin and the epidermal growth factor receptor: a reciprocal regulation? *Bio-Reguladores* **2**, 74–85.
54. Benguría, A., Hernández-Perera, O., Martínez-Pastor, M.T., Sacks, D.B. & Villalobo, A. (1994) Phosphorylation of calmodulin by the epidermal-growth-factor-receptor tyrosine kinase. *Eur. J. Biochem.* **224**, 909–916.
55. Benguría, A., Martín-Nieto, J., Benaim, G. & Villalobo, A. (1995) Regulatory interaction between calmodulin and the epidermal growth factor receptor. *Ann. NY Acad. Sci.* **766**, 472–476.
56. Benaim, G., Cervino, V. & Villalobo, A. (1998) Comparative phosphorylation of calmodulin from trypanosomatids and bovine brain by calmodulin-binding protein kinases. *Comp. Biochem. Physiol. Part C* **120**, 57–65.
57. Palomo-Jiménez, P.I., Hernández-Hernando, S., García-Nieto, R.M. & Villalobo, A. (1999) A method for the purification of phospho(Tyr)calmodulin free of non-phosphorylated calmodulin. *Prot. Exp. Purif.* **16**, 388–395.
58. De Frutos, T., Martín-Nieto, J. & Villalobo, A. (1997) Phosphorylation of calmodulin by permeabilized fibroblasts overexpressing the human epidermal growth factor receptor. *Biol. Chem.* **378**, 31–38.
59. Villalobo, A., Ruano, M.J., Palomo-Jiménez, P.I., Li, H. & Martín-Nieto, J. (2000) The epidermal growth factor receptor and the calcium signal. In Calcium: the Molecular Basis of Calcium Action in Biology and Medicine (Pochet, R., Donato, R., Haiech, J., Heizmann, C. & Gerke, V., eds), pp. 287–303. Kluwer Academic Publishers, Boston, MA, USA.
60. Fukami, Y., Nakamura, T., Nakayama, A. & Kanehisa, T. (1986) Phosphorylation of tyrosine residues of calmodulin in Rous sarcoma virus-transformed cells. *Proc. Natl Acad. Sci. USA* **83**, 4190–4193.
61. Brunati, A.M., James, P., Donella-Deana, A., Matoskova, B., Robbins, K.C. & Pinna, L.A. (1993) Isolation and identification of two proto-oncogene products related to c-fgr and fyn in a tyrosine-protein-kinase fraction of rat spleen. *Eur. J. Biochem.* **216**, 323–327.
62. Corti, C., LeClerc-L'Hostis, E., Quadroni, M., Schmid, H., Durusel, I., Cox, J., Hatt, P.D., James, P. & Carafoli, E. (1999) Tyrosine phosphorylation modulates the interaction of calmodulin with its target proteins. *Eur. J. Biochem.* **262**, 790–802.
63. Mukhin, Y.V., Vlasova, T., Jaffa, A.A., Collinsworth, G., Bell, J.L., Tholanikunnel, B.G., Pettus, T., Fitzgibbon, W., Ploth, D.W., Raymond, J.R. & Garnovskaya, M.N. (2001) Bradykinin B_2 receptors activate Na^+/H^+ exchange in mIMCD-3 cells via Janus kinase 2 and Ca^{2+}/calmodulin. *J. Biol. Chem.* **276**, 17339–17346.
64. Ghosh, S., Church, J.G., Roufogalis, B.D. & Villalobo, A. (1988) Phosphorylation of liver plasma membrane-bound calmodulin. *Biochem. Cell Biol.* **66**, 922–927.
65. Benguría, A., Soriano, M., Joyal, J., Sacks, D.B. & Villalobo, A. (1995) Phosphorylation of calmodulin by plasma-membrane-associated protein kinase(s). *Eur. J. Biochem.* **234**, 50–58.
66. Kubo, M. & Strott, C.A. (1988) Phosphorylation of calmodulin on threonine residue (s) by cytosol prepared from adrenal cortex. *Biochem. Biophys. Res. Commun.* **156**, 1333–1339.
67. Colca, J.R., DeWald, D.B., Pearson, J.D., Palazuk, B.J., Laurino, J.P. & McDonald, J.M. (1987) Insulin stimulates the phosphorylation of calmodulin in intact adipocytes. *J. Biol. Chem.* **262**, 11399–11402.
68. Blackshear, P.J. & McNeill-Haupt, D. (1989) Evidence against insulin-stimulated phosphorylation of calmodulin in 3T3-L1 adipocytes. *J. Biol. Chem.* **264**, 3854–3858.
69. Joyal, J.L. & Sacks, D.B. (1994) Insulin-dependent phosphorylation of calmodulin in rat hepatocytes. *J. Biol. Chem.* **269**, 30039–30048.
70. Sacks, D.B., Davis, H.W., Williams, J.P., Sheehan, E.L., Garcia, J.G.N. & McDonald, J.M. (1992) Phosphorylation by casein kinase II alters the biological activity of calmodulin. *Biochem. J.* **283**, 21–24.
71. Leclerc, E., Corti, C., Schmid, H., Vetter, S., James, P. & Carafoli, E. (1999) Serine/threonine phosphorylation of calmodulin modulates its interaction with the binding domains of target enzymes. *Biochem. J.* **344**, 403–411.
72. Sacks, D.B., Lopez, M.M., Li, Z. & Kosk-Kosicka, D. (1996) Analysis of phosphorylation and mutation of tyrosine residues of calmodulin on its activation of the erythrocyte Ca^{2+}-transporting ATPase. *Eur. J. Biochem.* **239**, 98–104.
73. Pershadsingh, H.A. & McDonald, J.M. (1979) Direct addition of insulin inhibits a high affinity Ca^{2+}-ATPase in isolated adipocyte plasma membranes. *Nature* **281**, 495–497.
74. Sacks, D.B., Mazus, B. & Joyal, J.L. (1995) The activity of calmodulin is altered by phosphorylation: modulation of calmodulin function by the site of phosphate incorporation. *Biochem. J.* **312**, 197–204.
75. Villalobo, A., Suju, M., Benaim, G., Palomo-Jiménez, P.I., Salerno, M., Martín-Nieto, J. & Benguría, A. (1997) Phosphocalmodulin activates the epidermal growth factor receptor. Proceedings of the Tenth International Symposium Calcium-Binding Proteins and Calcium Function in Health and Disease. p. 78. Lund University, Sweden.
76. Martín-Nieto, J. & Villalobo, A. (1998) The human epidermal growth factor receptor contains a juxtamembrane calmodulin-binding site. *Biochemistry* **37**, 227–236.
77. Tebar, F., Villalonga, P., Sorkina, T., Agell, N., Sorkin, A. & Enrich, C. (2002) Calmodulin regulates intracellular trafficking of epidermal growth factor receptor and the MAPK signaling pathway. *Mol. Biol. Cell* **13**, 2057–2068.
78. Bertrand, B., Wakabayashi, S., Ikeda, T., Pouyssegur, J. & Shigekawa, M. (1994) The Na^+/H^+ exchanger isoform 1 (NHE1) is a novel member of the calmodulin-binding proteins: identification and characterization of calmodulin-binding sites. *J. Biol. Chem.* **269**, 13703–13709.
79. Lin, S.-Y., Makino, K., Xia, W., Matin, A., Wen, Y., Kwong, K.Y., Bourguignon, L. & Hung, M.-C. (2001) Nuclear localization of EGF receptor and its potential new role as a transcription factor. *Natl Cell Biol.* **3**, 802–808.
80. Ni, C.-Y., Murphy, M.P., Golde, T.E. & Carpenter, G. (2001) γ-Secretase cleavage and nuclear localization of ErbB-4 receptor tyrosine kinase. *Science* **294**, 2179–2181.
81. Srinivasan, R., Gillett, C.E., Barnes, D.M. & Gullick, W.J. (2000) Nuclear expression of the c-erbB-4/HER-4 growth factor receptor in invasive breast cancers. *Cancer Res.* **60**, 1483–1487.
82. Chattaopadhyaya, R. & Quiocho, F.A. (1992) Calmodulin (vertebrate). *NCBI PubMed.,* PDB Id: 1CLL.