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Facts and conjectures on Calmodulin and its cousin proteins, parvalbumin and troponin C.

Jacques Haiech^{1*}, Marc Moreau², Catherine Leclerc² and Marie-Claude Kilhoffer¹

¹Laboratoire d'Excellence Medalis, Université de Strasbourg, BSC CNRS UMR 7242, F-67000 Strasbourg, France

²Centre de Biologie du Développement (CBD), Centre de Biologie Intégrative (CBI), Université de Toulouse, CNRS, UPS, F-31062, Toulouse, France.¹

*Corresponding author: haiech@unistra.fr

ABSTRACT

This review aims at giving a rational frame to understand the diversity of EF hand containing calcium binding proteins and their roles, with special focus on three members of this huge protein family, namely calmodulin, troponin C and parvalbumin. We propose that these proteins are members of structured macromolecular complexes, termed calcisomes, which constitute building devices allowing treatment of information within eukaryotic cells and namely calcium signals encoding and decoding, as well as control of cytosolic calcium levels in resting cells.

Calmodulin is ubiquitous, present in all eukaryotic cells, and pleiotropic. This may be explained by its prominent role in regulating calcium movement in and out of the cell, thus maintaining calcium homeostasis which is fundamental for cell survival. The protein is further involved in decoding transient calcium signals associated with calcium movements after cell stimulation. We will show that the specificity of calmodulin's actions may be more easily explained if one considers its role in the light of calcisomes.

Parvalbumin should not be considered as a simple intracellular calcium buffer. It is also a key factor for regulating calcium homeostasis in specific cells that need a rapid retrocontrol of calcium transients, such as fast muscle fibers.

Finally, we propose that troponin C, with its four calcium binding domains distributed between two lobes presenting different calcium binding kinetics, exhibits all the characteristics needed to trigger and then post modulate muscle contraction and thus appears as a typical Feed Forward Loop system.

If the present conjectures prove accurate, the way will be paved for a new pharmacology targeting the cell calcium signaling machinery.

Keywords: EF-hand calcium binding proteins; calcium signal; calcium signaling; calcium homeostasis; caldisome; Feed forward loop, toggle switch, oscillator, logical gates

Introduction

Calcium is the arch-enemy of the cell that uses phosphate-based metabolism. For life to develop, evolution has selected devices able to lower intracellular Ca^{2+} concentration and avoid calcium- phosphate precipitates. Pumps and calcium exchangers were set up to maintain a Ca^{2+} concentration gradient with a resting cytosolic Ca^{2+} concentration around 20-100 nM versus 1-2 mM in the external environment [1]. A massive increase in cytosolic Ca^{2+} is a death signal. Conversely, the possibility to modulate the Ca^{2+} concentration, both spatially and temporally, over a range from 20 nM to 1-2 μM , opens the possibility to use Ca^{2+} as an intracellular messenger. This implies the existence, within the cell, of a regulated system for Ca^{2+} entry. A set of Ca^{2+} channels, sensitive to a whole range of stimuli appeared during evolution [2]. The two sets of cellular devices that manage Ca^{2+} entry and exit can maintain a robust Ca^{2+} homeostasis provided the presence of feedback mechanisms. These mechanisms involve one or more sensors (or detector(s)) sensing the Ca^{2+} concentration and able to modulate Ca^{2+} entry and exit. To perform this role, the Ca^{2+} detector(s) must bind Ca^{2+} at concentrations ranging from 20-100 nM to 1-2 μM and interact with the proteins involved in Ca^{2+} entry and withdrawal. By modulating these three devices (Ca^{2+} entry, Ca^{2+} output and feedback to ensure a robust Ca^{2+} homeostasis), Ca^{2+} signals may be obtained with a wide variety of temporal and spatial characteristics, leading to Ca^{2+} signatures. It is thus possible to generate signals modulated in amplitude, frequency and even in phase if one considers the existence of two such coupled systems. However, a Ca^{2+} signal is of interest only if it can be decoded, i.e detected and lead to a cellular event (modification of cell or tissue function). To perform this decoding, a Ca^{2+} signaling chain comprising Ca^{2+} detectors (one or more) that specifically interact with target proteins is needed. Those interactions will start a cascade of molecular modifications ultimately triggering a cellular event.

The hunt for calcium binding proteins

The importance of Ca^{2+} in the extracellular environment was shown by Ringer at the end of the 19th century [3] but it was only in the second part of the 20th century that its role as intracellular messenger (second messenger) was unveiled [4]. During the same period, proteins able to bind Ca^{2+} with an affinity in the micromolar range were discovered. Parvalbumin was the first calcioprotein to be described in 1936 and purified in 1952 [5]. Its ability to bind Ca^{2+} was highlighted in 1971 [6] and its 3D structure, the first for calcioproteins, was published in 1972 [7]. The protein is organized in three domains, each composed of a 12 amino acid loop surrounded by two 8-9 amino acid alpha helices. In the protein structure, the alpha helices are referred to as A, B, C, D, E and F starting from the N-terminus of the protein. Among the three domains present in parvalbumin (AB, CD and EF domains), only two bind Ca^{2+} (CD and EF). The C terminal domain (EF domain) is the best defined and has been used to characterize the canonical EF-hand Ca^{2+} binding motif (Fig. 1). It was initially found in fish white muscles, but is present in a variety of cell types, including human neurons [8]. Troponin C was the second Ca^{2+} binding protein to be purified in 1967 [9] and characterized, with the description of its tertiary structure in 1985 [10]. The protein presents four EF-hand Ca^{2+} binding domains, distributed between two lobes. It is part of a protein complex that decodes muscle Ca^{2+} signals and induces muscle cell contraction by regulating the interaction between actin and myosin. Calmodulin was the third Ca^{2+} binding protein to be discovered in 1970, as an activator of cAMP phosphodiesterase [11,12]. It was purified in the 1970's [13] but its 3D structure was only unveiled in 1985 [14]. The protein also presents four EF-hand Ca^{2+} binding domains distributed between two lobes. Unlike parvalbumin and troponin C, calmodulin is ubiquitous, present in all eukaryotic cells. It interacts with numerous proteins and enzymes, thus modulating a great variety of cell functions, namely Ca^{2+} homeostasis through its activity on the Ca^{2+} entry and exit machinery in all cells of the body. It is also partner of different proteins in given cell types, organs or tissues. Since then, more than 800 proteins with at least one EF-hand domain have been characterized [15–17]. The importance of Ca^{2+} in the extracellular environment was shown by Ringer at the end of the 19th century [3] but it was only in the second part of the 20th century that its role as intracellular messenger (second messenger) was unveiled [4]. During the same period, proteins able to bind Ca^{2+} with an affinity in the micromolar range were discovered.

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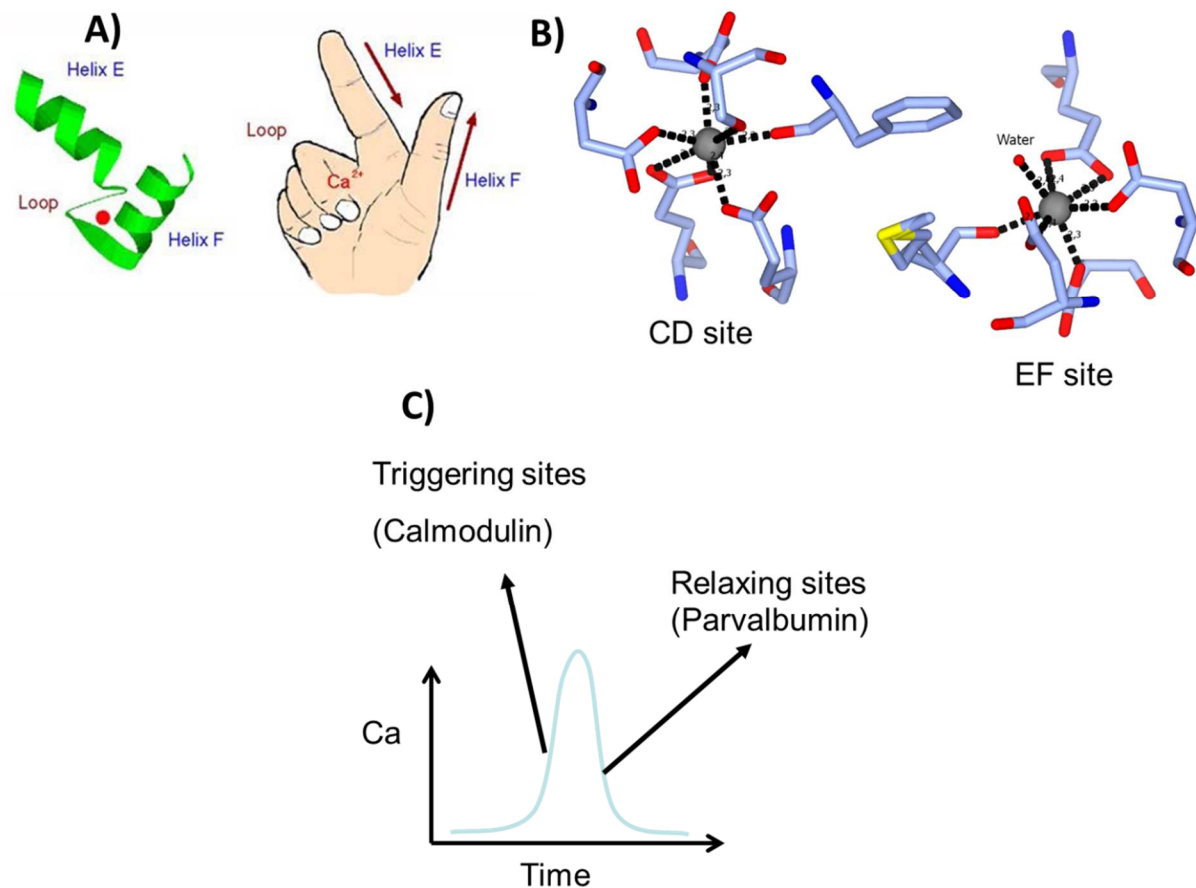


Figure 1: Ca^{2+} binding to and Ca^{2+} signaling via EF-hand domains. Representation of a 29 amino acids EF-hand domain comprising two alpha-helices and a 12 amino acid loop involved in Ca^{2+} ligandation (panel A). Panel B shows Ca^{2+} coordination in the two Ca^{2+} binding sites of pike parvalbumin. In the CD site, all the ligands are provided by the protein (oxygen atoms shown in red of carbonyl or carboxyl groups). In the EF site, one of the ligands is a water molecule (red sphere annotated "water») [15]. The Ca^{2+} coordination structure forms a bipyramid with a pentagonal basis. These domains usually work as couple and are involved either in Ca^{2+} signal onset (triggering sites) as found in calmodulin or offset (relaxing sites) as found in parvalbumin (panel C).

Characteristics of the Ca^{2+} binding sites

In the free state, Ca^{2+} is surrounded by seven or eight water molecules forming its first hydration layer. In the Ca^{2+} binding domains, Ca^{2+} is complexed through seven ligands in a pentagonal bipyramid structure. The seven ligands are provided by the oxygen of the protein

carbonyl or carboxyl groups or water molecules (Fig. 1B). In biological systems and whatever the Ca^{2+} binding protein, this ligation geometry is respected. In one of the Ca^{2+} binding sites of parvalbumin (CD site), the seven ligands are provided by the protein. There is no residual water molecule. In the EF site of parvalbumin, one of the ligands is a water molecule. In calmodulin, the four Ca^{2+} are complexed via six ligands from the protein and one water molecule, as in the EF site of parvalbumin. The affinity of a calprotein for Ca^{2+} depends on the number of water molecules remaining after ligation [18] but the geometry of the liganded complex is the same whether or not there is a water molecule.

The affinity of a Ca^{2+} binding domain depends also on its structuration and its ability to be well positioned around the ion. Conformational adjustment can be obtained by interaction between domains. Hence, a single EF-hand domain has approximately 100 to 10000 less affinity than this same domain coupled to another one [19]. An exhaustive analysis of EF-hand domain proteins shows that the domains are not isolated, but work in pairs, two domains forming a lobe [16]. Hence, as revealed in the proteins' 3D structure, calmodulin and troponin C have two lobes, each with two Ca^{2+} binding domains.

Finally, Ca^{2+} affinity for a domain depends on the electrostatic environment of the domain in the protein. Thus, a domain in an electronegative environment will show higher Ca^{2+} affinity than the same domain in an electropositive environment. This stems from an improvement in the interaction kinetics between Ca^{2+} carrying two positive charges and the binding domain in an electronegative environment. Electric fields not only act on Ca^{2+} affinity, but also on ion binding specificity. Indeed, Ca^{2+} binding domains also bind Mg^{2+} although with a ~ 1000 -fold lower affinity [20]. Mg^{2+} complexation involves six ligands in a square base bipyramid geometry. A recent study showed that the binding specificity between Ca^{2+} and Mg^{2+} can be modified by playing around with the domain environment [21]. Comparison of calmodulin Ca^{2+} binding constants has illustrated this point [22].

Parvalbumin binds Ca^{2+} and Mg^{2+} with affinities in the nanomolar and micromolar range, respectively [20]. In the cytosol of resting cells, parvalbumin's Ca^{2+} binding sites are occupied by Mg^{2+} and the apparent affinity for Ca^{2+} is in the micromolar range. Calmodulin has an affinity for Ca^{2+} in the micromolar range and for Mg^{2+} above millimolar. In a resting cell, calmodulin's Ca^{2+} binding sites, in either of the two lobes, are free. In troponin C, the two Ca^{2+} sites located in the N-terminal lobe behave similarly to those in calmodulin, whereas

the two sites in the C-terminal lobe are more akin to those in parvalbumin. Hence, at resting Ca^{2+} levels, the two sites in the N-terminal lobe are free, whereas the two sites in the C-terminal lobe are occupied by Mg^{2+} . Upon a Ca^{2+} signal that follows cell stimulation, the empty sites of those calcioproteins will be able to bind Ca^{2+} according to a kinetic limited by the ion diffusion rate and the capacity of the Ca^{2+} binding domain to dehydrate the ion, whereas the sites occupied by Mg^{2+} can only bind Ca^{2+} after a delay depending on the kinetics of exchange between Mg^{2+} and Ca^{2+} [23].

From a kinetic and functional point of view, we propose that the EF-hand calcium binding sites belong to two main classes (Fig. 1C):

1) Triggering sites involved in the first part of a calcium signal (Fig. 1C). These sites are free of Ca^{2+} in resting cells and can readily bind Ca^{2+} when its concentration increases upon cell stimulation. The four calmodulin Ca^{2+} binding sites and the two sites located in the N-terminal lobe of troponin C belong to this group.

2) Relaxing sites involved in the second part of the calcium signal (Fig. 1C). These sites are occupied by Mg^{2+} at resting Ca^{2+} concentrations. Following cytosolic Ca^{2+} concentration increase, Ca^{2+} must exchange with Mg^{2+} to occupy such sites, inducing a kinetic delay. These sites are present in parvalbumin and in the C-terminal lobe of troponin C.

It is now widely accepted that Ca^{2+} binding sites in calcioproteins are not independent [24]. Ca^{2+} binding to a given site will modify the protein conformation as well as its electrostatic potential [25]. These modifications directly affect Ca^{2+} binding properties of other sites. Moreover, any posttranslational modification of calcioproteins, as well as their participation in a macromolecular architecture, will modify the binding properties of each Ca^{2+} binding site, in terms of affinity and specificity. One may thus envision the ability of these proteins to adopt a large variety of conformations upon Ca^{2+} binding enabling plural Ca^{2+} signal decoding possibilities depending on the calcioprotein and its environment. These assumptions need experimentation able to catch snapshots and dissect the events as a function of time. Up to now, most biophysical studies of calmodulin looked at either direct Ca^{2+} binding to the protein, or conformational modifications induced by Ca^{2+} binding, allowing to get a macroscopic view of the system, i.e. the state of calmodulin with 0 to 4 Ca^{2+} bound. To get a microscopic view of a system, meaning the possibility to discriminate between the different

molecular conformations of the protein in a given state, it is necessary, although often not sufficient, to study single molecules. Part of the complexity of calmodulin has been unveiled in a series of publications using either optical forceps or atomic force microscopy (AFM) to analyze the effects of Ca^{2+} , at different concentrations, on a single calmodulin molecule [26–30]. These two techniques which allow analysis of the conformational landscape of single molecules can be used to precisely dissect the ensemble of modifications occurring when Ca^{2+} binds to calmodulin and when calmodulin interacts with its partners. These approaches are however limited by the fact that the experimental conditions used and thus the environment of the system do not mirror the cellular context.

[In search of the roles of these calcioproteins](#)

Calmodulin interacts with proteins involved in Ca^{2+} influx and efflux. Calmodulin exhibits all the characteristics to retro-control Ca^{2+} movements and allow robust Ca^{2+} homeostasis in all eukaryotic cells. This prominent role of calmodulin is also consistent with its strong evolutionary relationship with the ancestral protein of the EF-hand calcioprotein family [16]. But calmodulin also interacts with enzymes and structural proteins to trigger cellular events. Its role is dual, being involved both in the detection and decoding of Ca^{2+} signaling. It maintains cellular Ca^{2+} homeostasis and is a member of cell-specific Ca^{2+} pathways activated under given physiological or differentiation states. Calmodulin in the human genome is encoded by three different genes. This redundancy may be explained by the main role in calcium homeostasis. It may also have regulatory roles and be an advantage to co-regulate the expression of proteins interacting with calmodulin in a given calcium signal pathway [31,32].

Troponin C exists in two isoforms, a skeletal troponin C specific for fast muscle fibers and a cardiac troponin C found only in cardiomyocytes and in slow muscle fibers. Unlike calmodulin, expression of troponin C is tissue-specific and the protein is specifically involved in the regulation of muscle contraction. The macromolecular complex composed of troponin, tropomyosin, actin and myosin is a stereotypical example of an integrated macromolecular complex akin to an information treating device.

More generally, considering the calcioproteins of the EF-hand family, we suggest that two functional groups may be highlighted:

- 1) The proteins involved in Ca^{2+} homeostasis. Calmodulin is at the heart of this category. It can be assumed that beyond calmodulin, there is a finer regulation of Ca^{2+} homeostasis by other calcioproteins and that this regulation is adapted to a given cell type. For example, these proteins could modulate Ca^{2+} entry or exit either at the cell membrane or at the level of organelles. Parvalbumin also belongs to this group. As a soluble cytoplasmic relaxing factor, it may kinetically replace Ca^{2+} pumps to induce, when present, a more rapid decrease in cytosolic Ca^{2+} . It participates in a form of temporal regulation of Ca^{2+} homeostasis.
- 2) The proteins involved in the Ca^{2+} signal decoding. Troponin C is a typical example of this group. Calmodulin appears as a Janus protein being involved in decoding while also playing a key role in Ca^{2+} homeostasis.

Sensing and decoding the Ca^{2+} signal: calcioproteins as part of information treatment devices

Within cells, Ca^{2+} signals must be detected, transduced into cellular functions and the various signals received currently or in the past must be integrated to generate the bona fide response. This information treatment may be described using signal processing concepts from digital electronics, as illustrated below with a few examples.

Hence, troponin C with its two different lobes each containing two paired EF-hand domains, may be assimilated to a feed forward loop (FFL), a circuit composed of three elements A, B and C (Fig. 2A). Activation of A induces activation or inhibition of B and C. Activation of B triggers activation or inhibition of C. Such a device has been extensively studied in regulatory genetic networks but not in Ca^{2+} signaling pathways. In the case of troponin C, element A would correspond to the triggering sites, B to the relaxing sites and C to the cellular event (Fig. 2B). During a Ca^{2+} signal, Ca^{2+} binds to the triggering sites in the N-terminal lobe of troponin C. This allows the interaction between actin and myosin (corresponding to the cellular event, i.e. element C in the FFL scheme). After a kinetic delay due to the exchange between Ca^{2+} and Mg^{2+} , Ca^{2+} binds to the domains in the C-terminal lobe. This binding will modify the conformation of troponin C and may modulate the interaction between actin and

myosin initially triggered by Ca^{2+} occupancy of the sites in the N-terminal lobe of the protein (Fig. 2B). This system triggers a cellular event which lasts for some time depending of the kinetic delay between the activation of the triggering sites and the relaxing sites. This kinetic delay may be itself modulated by post-translational modifications.

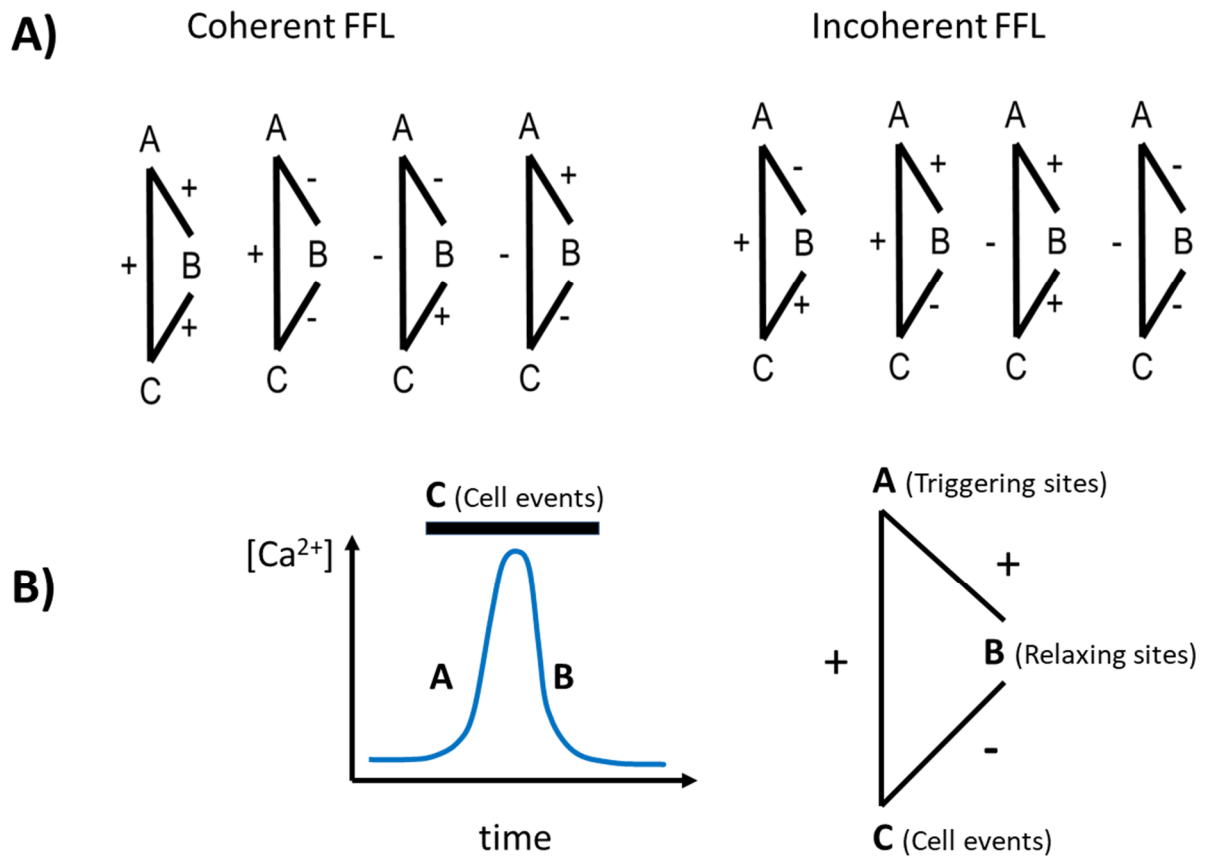


Figure 2: Troponin C as an incoherent Feed Forward Loop (FFL). Panel A: FFL is a device built from three elements A, B and C. A activates or inhibits C directly or via B. If, whatever the pathway, A is activating or inhibiting C, the FFL is coherent. If not, the FFL is incoherent. The eight putative FFL are illustrated. Panel B proposes a scheme for realizing a FFL device using the two types of EF-hand Ca^{2+} binding sites of troponin C, i.e. the triggering and relaxing sites presented in Fig. 1. When a Ca^{2+} concentration transient is triggered by an external stimulus, the triggering sites bind Ca^{2+} and activate the cell event. After a kinetic delay, the relaxing sites are occupied and may modulate the cell event in a reverse way. An incoherent FFL is thus obtained.

Troponin C is a calprotein with a dedicated function, involved in the regulation of muscle contraction. Calmodulin, on the other hand, with its multiple interactions and pleiotropic effects is at the heart of the Ca^{2+} signaling network. It is a major player in supramolecular protein complexes that possess all the properties needed to behave as digital information processing devices, such as Feed Forward Loops, Toggle switches (or bistables), logic gates or oscillators.

Ca^{2+} /Calmodulin (Ca^{2+} /CaM)-dependent protein kinase II (CaM Kinase II) may be presented as an example of such a supramolecular complex functioning as a toggle switch, which is a bi-stable device able, when triggered, to alternate between two states ON and OFF and maintaining a persistent state after triggering comparable to an ON/OFF switch with memory (similar to the caps lock key on a computer). It is a typical device that allows information storage. It can switch from one steady state to the other by increasing stimulation or inhibition or by changing other regulatory mechanisms. CaM Kinase II plays a prominent role in learning and memory mechanisms, including Long-Term Potentiation (LTP) and Long-Term Depression (LTD) [33–35]. The dodecameric protein exists either under an unphosphorylated inactive form (OFF state) or a phosphorylated form (ON state). CaM kinase II is activated by Ca^{2+} -calmodulin, which induces cooperative autophosphorylation on a threonine residue in each monomer. Once phosphorylated, the enzyme remains active, even in the absence of Ca^{2+} - Calmodulin [36]. The active enzyme can phosphorylate protein phosphatase PP2A, rendering it inactive. PP2A can also be phosphorylated by cAMP dependent protein kinase (PKA). This phosphorylation activates it and triggers dephosphorylation of CaM Kinase II [37]. This functioning can be represented by a toggle switch in which Ca^{2+} acts as the Set input and cAMP as the Reset input (Fig. 3). The basic circuit represented in Fig. 3 is one of the many necessary to describe the whole network of interactions occurring in resting and stimulated cells. Through this mechanism of toggle switch, CaM Kinase II has been involved in memory storage [35,36]. CaM Kinase II also appears to be involved in Long Term Depression through its capacity to decipher frequency coded Ca^{2+} signal [34].

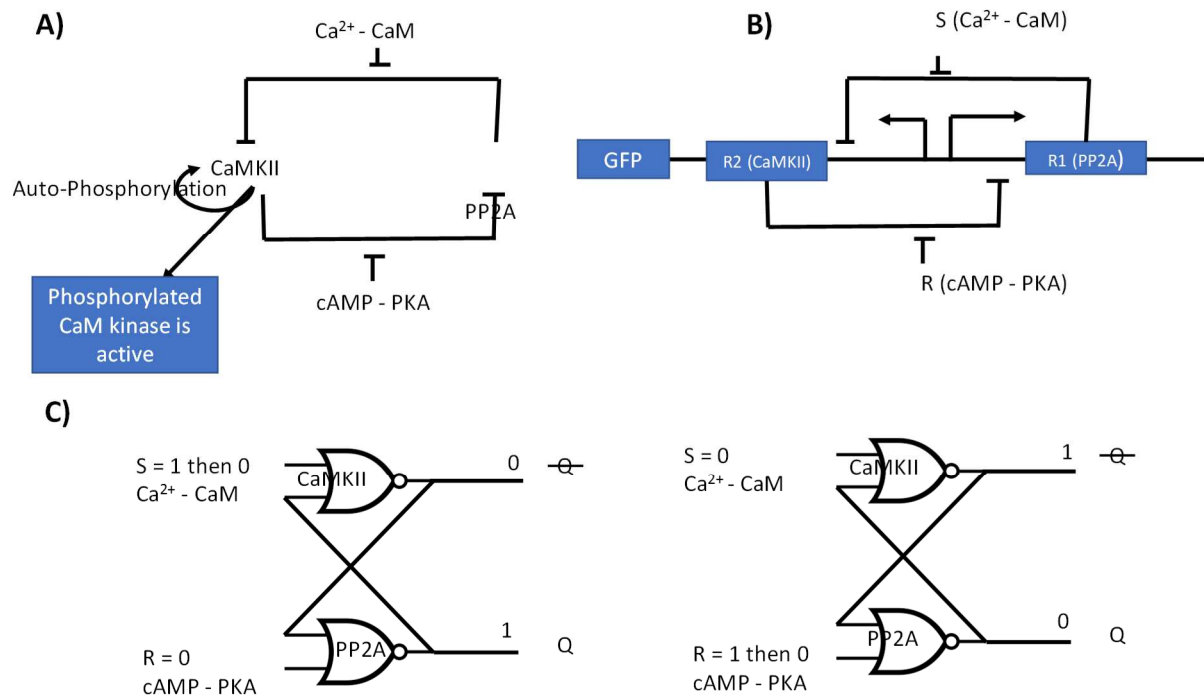


Figure 3: CaM Kinase II regarded as a memory toggle switch system with Ca^{2+} and cAMP as Set and Reset inputs. Panel A: PP2A stands for protein phosphatase 2A, CaMKII for calmodulin Kinase II, PKA for protein kinase A. CaM kinase II can switch between an unphosphorylated state where it is inactive and a phosphorylated state where it is active. Ca^{2+} - Calmodulin triggers CaM Kinase II phosphorylation and thus passage from the inactive to the active state. Once activated, CaM Kinase II remains active unless unphosphorylated by e.g. PP2A which is activated by cAMP - PKA. PP2A is itself inhibited by active CaM Kinase II. This activity can be described using genetic networks (panel B) or digital microelectronic concepts and namely a S-R latch which represents a flip flop basic memory circuit (Panel C). Panel B illustrates a Set - Reset latch built with two repressors R1 and R2. R1 represses the expression of the gene R2 and R2 represses the expression of R1. The input S inhibits the activity of R1 (respectively R inhibits the activity of R2). The output Q corresponds to the expression of R2 and GFP (respectively \bar{Q} to the expression of R1). In panel C, S and R correspond to the inputs and stand for Set and Reset, respectively. The output of the S NOR gate is one of the inputs of the R NOR gate and the output of the R NOR gate is one of the inputs of the S NOR gate. The modeling of such a S - R latch has been extensively done in electronics and for genetic regulatory networks. Due to the analogy of the CaM Kinase II circuit with these other circuits, it is possible to use the theoretical work done in electronics and genetic regulatory networks to model the CaM Kinase II toggle switch.

Several models of CaM Kinase II have been presented in the literature. Some of them presented CaM Kinase II as involved in a toggle switch, whereas a recent publication presents CaM Kinase II as a Ca^{2+} frequency signal decoder [34].

With two inputs, sixteen different logical gates can be built. The NOR gate is one among the sixteen and it is a universal gate. Each universal gate by itself is able to resume the full set of logical gates [38]. Such logical gates have been built using genetic networks. Indeed, a gene regulated by an activator and a repressor constitutes the most basic logical gate that we may built with one gene.

Other logical gates may easily be built from the Ca^{2+} signaling toolkit [39]. For example, a OR gate can be obtained by taking two G-protein coupled receptors (GPCRs) sensitive to two different inputs, with each activated receptor able to trigger a Ca^{2+} transient.

Besides FFL, toggle switches and logic gates, oscillators constitute another type of digital information processing device. Stable Ca^{2+} oscillations, amplitude or frequency modulated, can be obtained taking devices involved in Ca^{2+} homeostasis regulation coupled to an attendant IP_3 stimulus [40].

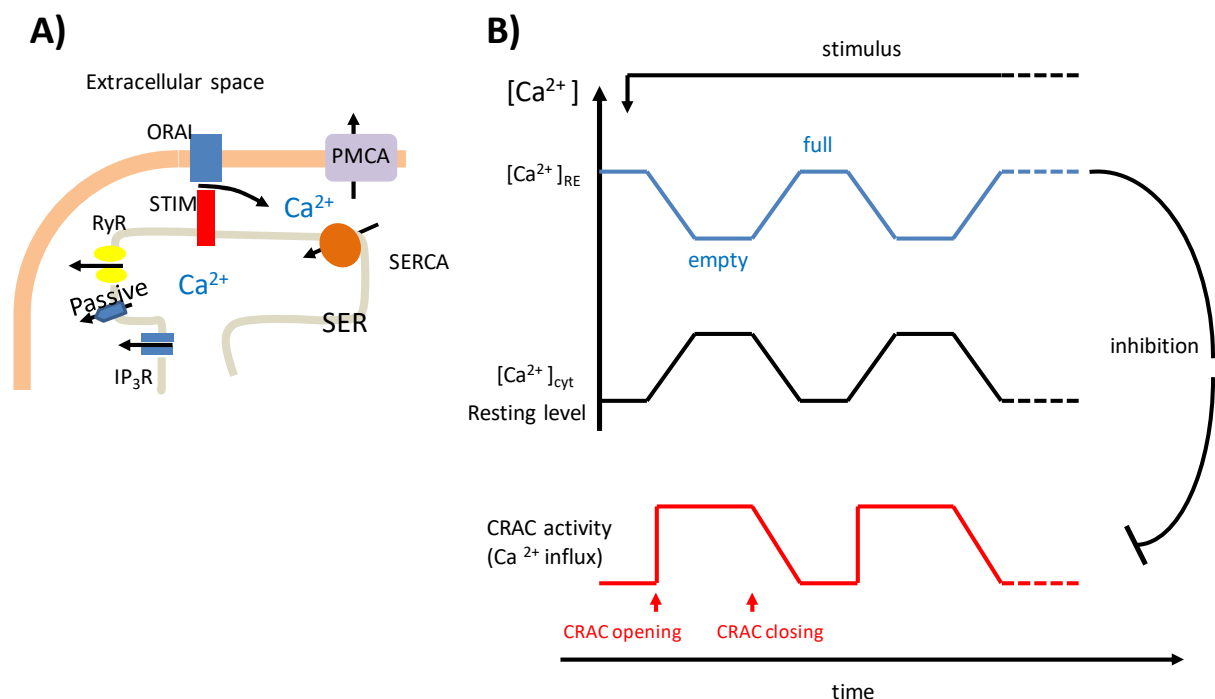


Figure 4. Illustration of a Ca^{2+} oscillator generated with three pools of Ca^{2+} . The three pools of Ca^{2+} are: extracellular Ca^{2+} , cytosolic Ca^{2+} and Ca^{2+} in the endoplasmic reticulum. Panel A illustrates the overall device. The external Ca^{2+} concentration is clamped. PMCA (plasma membrane Ca^{2+} pump) and SERCA (sarco/endoplasmic reticulum Ca^{2+} transport ATPase) are pumps involved in the maintenance of resting cytoplasmic Ca^{2+} level. The STIM/ORAI complex represents the CRAC (calcium released activated channel). This channel opens when the ER (endoplasmic reticulum) Ca^{2+} concentration is low. There is Ca^{2+} flux either passive or due to the activation of IP3 or ryanodine receptor between the ER and the cytosol. Panel B is an illustration of the of Ca^{2+} concentration variations in two of the three pools (cytosolic and endoplasmic reticulum) as a function of time. The Ca^{2+} concentration in the third pool is considered to be stable.

A classification of oscillating devices built from genetic regulatory networks has been published [41,42]. Fig. 4 gives an example illustrating the possibility to build a biochemical oscillator on the basis of three cellular Ca^{2+} pools. This oscillator relies on the SOCE system (store operated Ca^{2+} entry). The involvement of the ORAI/STIM complex in this system has been shown in different biological systems [43,44]

Hence, the calcium toolbox possesses all the elements needed to build the whole set of devices that are required to construct any information decoding pathways [45].

Calcisomes and Ca^{2+} signal spatialization

To get an integrated view of how cells manage Ca^{2+} signaling, we proposed, in 1981, the concept of calcisomes [46] defined as a "specific assembly of calcium sensors, target enzymes and inhibitory proteins able to respond specifically to a transient and localized rise in Ca^{2+} concentration". Association to or localization in vicinity of Ca^{2+} channels and Ca^{2+} pumps was proposed. Myofibrils and glycogen particles were given as examples of calcisomes.

In the calcisome concept, some or all the Ca^{2+} signaling players would be brought together in supramolecular architectures.

At the cellular level, the calcisomes need to exhibit four levels of regulation [46]:

- 1) Genetic regulation that implies control of the expression (at the genomic and translational level) of the genes coding for calcisome proteins or related to those
- 2) Spatial regulation defined by the location of the calcisome in the cell
- 3) Kinetic regulation depending on the kinetics of calcisome assembly
- 4) A memory regulation that takes into account the history of the cell and involves post-translational modifications of the calcisome partners.

At the genomic level, two gene toolboxes (set of genes) coding for the different calcisome protagonists can be considered, namely i) the toolbox involved in the management of Ca^{2+} homeostasis and generation of Ca^{2+} signatures and ii) the toolbox for detecting Ca^{2+} signals and inducing cellular events. In this second toolbox, it is interesting to pinpoint Ca^{2+} dependent transcription factors such as the KCNIP protein family. Such proteins contain four EF-hand domains and are modulating the expression of a large number of genes [47,48].

Additionally, one needs a set of genes coding for receptor proteins involved in the detection of external or internal stimuli and a set of genes coding for proteins working as actuators involved in triggering and performing the cellular event related to a specific Ca^{2+} signal. Altogether, the genome has a toolkit as defined by Michael Berridge with the two previous calcium toolboxes and the two sets of genes involved in the Ca^{2+} dependent sensor/actuator pathways (Fig. 5) [39].

Handling a Ca^{2+} signaling pathway by a cell may be described using a sensor - actuator scheme comprising (Fig. 5):

- A set of sensors which catch information about the cell context (external sensors) and the cell state (internal sensors)
- Devices able to integrate and to treat information coded by the Ca^{2+} signal and provided by the sensors (Input). Calcisomes would be these devices
- A set of actuators corresponding to effector pathways activated by signals generated by the previous devices and triggering a cellular event (transcription, metabolism, mobility, cell fate, morphological changes).

Following our rationale, calcisomes appear as supramolecular protein complexes organized around one or more Ca^{2+} binding proteins that can perform at least one of the four basic digital operations mentioned above, namely feed forward loop, toggle switch, logical gate, oscillator and to integrate them into a signal treatment device. To perform more complex digital operation, different calcisomes present in a specific cell need to be connected. Thus, calcisomes appear as the nodes of a network linked through Ca^{2+} diffusion. Such a model of calcisome networks has started to be proposed in astrocytes to simulate the different Ca^{2+} signatures in brain tissue [42].

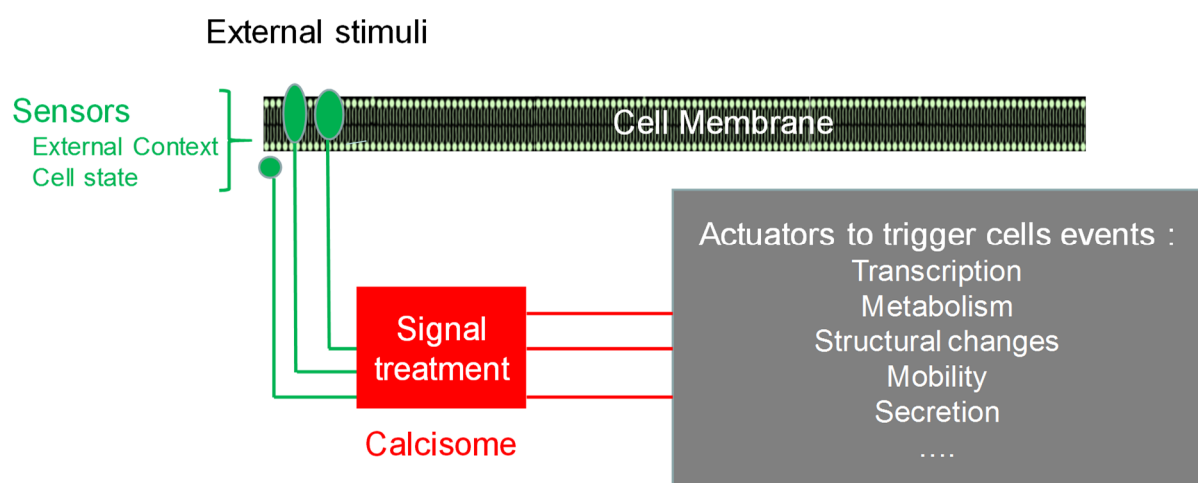


Figure 5: Illustration of a cellular Ca^{2+} signaling pathway using a sensor/actuator scheme. The system has, at one side, a set of external and internal sensors and on the other side, a set of actuators able to trigger a cellular event. In the middle, a device able to treat the Ca^{2+} signal and to send information to the actuator. We propose that this device is a multiprotein complex involving one or more Ca^{2+} binding proteins and name it calcisome.

Concluding remarks

Although parvalbumin, troponin C and calmodulin have been discovered about 60 years ago, their specific roles in the cell are not completely elucidated.

The logical frame that could be drawn from the analysis of knowledge evolution relative to those proteins, led us to the following conclusions:

- 1) Calmodulin, with its function as Ca^{2+} dependent modulator of Ca^{2+} movement in and out of the cell cytosol, is a key and ubiquitous player to maintain Ca^{2+} homeostasis in all eukaryotic cells. Parvalbumin appears to be involved in accelerating the return to basal cytosolic Ca^{2+} concentrations in specific cells. It participates to a retro-control of the Ca^{2+} signal and therefore to cellular Ca^{2+} homeostasis but under very specific physiological situations. Whereas calmodulin is a general modulator of Ca^{2+} homeostasis, evolution may have selected other Ca^{2+} -binding proteins to fine tune Ca^{2+} homeostasis depending on the cellular context and physiology.
- 2) Two types of Ca^{2+} binding domains often twinned as lobes in the protein structure exist: i) a main class with Ca^{2+} affinity around $1\ \mu\text{M}$, not occupied by Mg^{2+} at resting Ca^{2+} levels and termed triggering sites, ii) a second class, termed relaxing sites, with high Ca^{2+} affinity and occupied by Mg^{2+} at resting Ca^{2+} levels. Following a Ca^{2+} signal, those high affinity sites exhibit a kinetic delay before they complex Ca^{2+} .
- 3) Calmodulin is also a key player in deciphering Ca^{2+} signals in a given differentiated cell under a specific physiological state. This specificity may be related to the fact that it is a member of calcisomes, corresponding to a set of proteins spatially and genetically regulated. This suggests that calmodulin and calmodulin binding proteins are not homogeneously distributed inside the cell. For the time being an exhaustive analysis of such calcisomes in the cell has not been performed. Future analysis will probably unveil that most calcisomes contain calmodulin. In addition to general modulation by calmodulin, fine tuning may be performed by specific Ca^{2+} binding proteins. Moreover, in certain systems, calmodulin may be replaced by calmodulin like proteins such as troponin C, that have evolved to perform a more specific modulation in a given cellular context. We propose to classify the EF-hand calcioproteins as calmodulin partners when they colocalize with calmodulin or as specific Ca^{2+} modulators when they are the main Ca^{2+} modulators in a given calcisome, ie for instance troponin C in the muscle fibers. To experimentally approach this question, it would be important to exhaustively analyze the proteomes

of calmodulin interacting proteins from a given cell under different physiological status.

- 4) Because of this dual role of calmodulin, as a general key player in cellular Ca^{2+} homeostasis and a specific Ca^{2+} deciphering hub depending of its protein partners in a specific calcisome, the pharmacology of calmodulin is complex. The perturbation of the different calmodulin dependent calcisomes remains an open research field. One may anticipate that such knowledge would open a completely new therapeutic field if manipulation of the different calmodulin dependent calcium pathways would become possible.
- 5) The Troponin C family does not exist in plants, but a high number of calmodulin-like proteins has been selected during evolution. This suggests that the EF-hand domain proteins may adapt to different biological contexts. In addition, the ancestral calcium toolbox in prokaryotes remains an open question, as the EF hand domain proteins present in prokaryotic cells have been acquired by horizontal transfer from eukaryotic cells [16].

Although Ca^{2+} binding proteins and Ca^{2+} signaling constitute an old scientific field, we are convinced that with the emerging imaging technologies which allow to follow calcium signals with spatial and temporal resolution at the cellular level and with the new -omics technologies (single cell RNA seq and high sensitivity proteomics), the field can be approached with a new, more quantitative and dynamic impetus. It is a prerequisite to the development of a " Ca^{2+} signal pharmacology" which will open the way to new therapeutic approaches. A recent paper illustrates this new approach to treat cancer[49].

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