Calmodulin: a prototypical calcium sensor

David Chin and Anthony R. Means

Calmodulin is the best studied and prototypical example of the E–F-hand family of Ca\(^{2+}\)-sensing proteins. Changes in intracellular Ca\(^{2+}\) concentration regulate calmodulin in three distinct ways. First, at the cellular level, by directing its subcellular distribution. Second, at the molecular level, by promoting different modes of association with many target proteins. Third, by directing a variety of conformational states in calmodulin that result in target-specific activation. The calmodulin-dependent regulation of protein kinases illustrates the potential mechanisms by which Ca\(^{2+}\)-sensing proteins can recognize and generate affinity and specificity for effectors in a Ca\(^{2+}\)-dependent manner.

Calcium (as Ca\(^{2+}\)) is an element that is crucial for numerous biological functions. In many organisms, the vast majority of Ca\(^{2+}\) is complexed with phosphates to form exo- or endoskeletons that not only serve as structural scaffolds but also buffer the levels of Ca\(^{2+}\) within extracellular fluids at ~10\(^{-7}\) M. By contrast, the resting concentrations of intracellular free Ca\(^{2+}\) (~10\(^{-10}\) M) is 10\(^4\) times lower than that outside cells, providing the potential for the ready import of Ca\(^{2+}\) into cells, where it can act as a second messenger.

Various extracellular stimuli promote the movement of Ca\(^{2+}\) either from outside the cell (via plasma-membrane Ca\(^{2+}\) channels) or from intracellular stores into the intracellular milieu (Fig. 1a). The Ca\(^{2+}\) is released in elemental aliquots called sparks, puffs or waves depending on the extent of the intracellular area covered. This free Ca\(^{2+}\) is only briefly available to act as a cellular signal, however, because Ca\(^{2+}\)-binding proteins and Ca\(^{2+}\) pumps immediately combine to sequester and transport it to intracellular storage sites or outside the cell.

The short pulses of Ca\(^{2+}\) exert specific changes in cellular function depending on their route of entry into the cell, their local sites of action and, finally, by their pattern of modulation. The particular membrane channel or intracellular receptor responsible for the release of Ca\(^{2+}\) exerts considerable influence on the eventual effects of the Ca\(^{2+}\) signal. The mode of cellular entry also influences the site of action of the Ca\(^{2+}\) signal. Hence, separate intracellular loci or organelles are potentially distinct compartments of localized Ca\(^{2+}\) signalling (Fig. 1a). Therefore, Ca\(^{2+}\) signals in the nucleus exert different effects from those generated in the cytoplasm or near the plasma membrane of the same cell. Additionally, the modulation of the amplitude or frequency of Ca\(^{2+}\) spikes (AM and FM, respectively) encodes important signalling information. This has recently been illustrated for cases in which an optimal frequency of intracellular Ca\(^{2+}\) oscillations is important for the expression of different genes.

Calcium-regulated proteins: calmodulin

How do Ca\(^{2+}\) signals produce changes in cell function? The information encoded in transient Ca\(^{2+}\) signals is deciphered by various intracellular Ca\(^{2+}\)-binding proteins that convert the signals into a wide variety of biochemical changes. Some of these proteins, such as protein kinase C, bind to Ca\(^{2+}\) and are directly regulated by a Ca\(^{2+}\)-dependent manner. Other Ca\(^{2+}\)-binding proteins, however, are intermediaries that couple the Ca\(^{2+}\) signals to biochemical and cellular changes (Fig. 1b). Among this latter group are a family of proteins that is distinguished by a structural motif known as the E–F hand. An E–F hand consists of an N-terminal helix (the E helix) immediately followed by a centrally located, Ca\(^{2+}\)-coordinating loop and a C-terminal helix (the F helix). The three-dimensional arrangement of these domains is reminiscent of the thumb, index and middle fingers of a hand, hence the name ‘E–F hand’.

These proteins respond to Ca\(^{2+}\) in one of two ways (Fig. 1b). One group (e.g. parvalbumin and calbindin) do not undergo a significant change in conformation on binding Ca\(^{2+}\) and function as Ca\(^{2+}\) buffers or Ca\(^{2+}\) transporters. The second group, the Ca\(^{2+}\) sensors, undergo a Ca\(^{2+}\)-induced change in conformation. The most prominent examples of sensors include troponin C (a protein dedicated to regulating striated-muscle contraction), the multifunctional Ca\(^{2+}\) transducer calmodulin (CaM), the S100 family of proteins and, most recently, the neuronal myristoylated proteins such as recoverin.

The molecular and cellular mechanisms underlying the ability of a majority of the Ca\(^{2+}\)-sensor proteins to integrate Ca\(^{2+}\) signals into specific cellular responses are not clearly understood. Much of what we do know about the mechanisms that the sensor proteins use to transduce Ca\(^{2+}\) signals is based on information gained from CaM, probably the most intensively studied member of the E–F-hand family of sensors. In the remainder of this article, CaM will therefore serve as a model or prototype for other potential Ca\(^{2+}\) transducers. A review of some of the mechanisms responsible for regulating CaM at the subcellular and molecular levels might reveal valuable clues as to how Ca\(^{2+}\)-sensor proteins convert Ca\(^{2+}\) signals into cellular function.

CaM is expressed in all eukaryotic cells where it participates in signalling pathways that regulate many crucial processes such as growth, proliferation and movement. It is relatively small (vertebrate CaM...
Ka considerably higher affinity (it has additional discrimination for Ca\textsuperscript{2+} buffers by sequestering excess free Ca\textsuperscript{2+}).

Ca\textsuperscript{2+} trends in within the range of intracellular Ca\textsuperscript{2+}.

E–F hands 3 and 4 (Fig. 2).

A highly homologous C-terminal domain consisting of domain that is separated by a short flexible linker from hands combine to form a globular N-terminal and comprises four E–F hands. The first two E–F has 148 residues, evolutionarily highly conserved and comprises four E–F hands.

The two domains of CaM adopt different conformations in the absence of Ca\textsuperscript{2+}. (a) Sources of intracellular Ca\textsuperscript{2+} signals. Ca\textsuperscript{2+} enters cells via extracellular plasma-membrane receptors or from intracellular stores, producing transient local or global changes in its distribution. The Ca\textsuperscript{2+} oscillations are modulated in their amplitudes (AM) or frequencies (FM) and are therefore capable of conveying signaling information in complex ways. (b) E–F-hand Ca\textsuperscript{2+}-binding proteins are classified as buffers/transporters and sensors. The Ca\textsuperscript{2+} sensors change conformation on binding Ca\textsuperscript{2+} and transduce changes in cell function by regulating downstream effectors.

In the event of a transient rise in Ca\textsuperscript{2+}, the Ca\textsuperscript{2+}-binding event into biochemical energy that characterizes the Ca\textsuperscript{2+}-sensor proteins and is the basis of their ability to transduce Ca\textsuperscript{2+} signals.

Calmodulin: location, mobility and translocation

Calmodulin is regulated at the subcellular level, and how is this related to Ca\textsuperscript{2+} signalling? The concentration and location of CaM do appear to play an important role in regulating its biochemical activity. CaM constitutes at least 0.1% of the total protein present in cells (10\textsuperscript{-6} M - 10\textsuperscript{-5} M) and is expressed at even

![FIGURE 2](image)

The Ca\textsuperscript{2+}-regulated conformational change in calmodulin. The main chain structure of Ca\textsuperscript{2+}-free (apo) CaM (a) and Ca\textsuperscript{2+}-CaM (b) are shown in red with their respective E–F hands in purple to denote the location of potential hydrophobic pockets in each of the two domains. Ca\textsuperscript{2+} binding produces large changes in the helices in both domains, resulting in the exposure of several hydrophobic residues.
higher levels in rapidly growing cells, especially those undergoing cell division and differentiation. The local intracellular availability of CaM is likely to be biologically significant because various CaM-dependent effectors are regulated over a wide range of free CaM concentrations (10^{-12} - 10^{-6} M). Recent studies of CaM tagged with green-fluorescent protein (GFP) show that CaM is found throughout the cytosol and nucleus in HeLa cells, although it is concentrated around the mitotic apparatus in cells undergoing mitosis (Fig. 3), especially around the centrioles and the cytoplasmic furrow during cytokinesis. Other fluorescently labelled CaM molecules have provided information on its cellular mobility and location. Examples with serum-deprived Swiss 3T3 fibroblasts first indicated that the majority of CaM was freely diffusible, but the CaM was then immobilized in response to stimulation by serum. However, other studies on unstimulated smooth-muscle cells showed that most CaM is bound, possibly to Ca^{2+}-independent binding proteins, at resting concentrations of free Ca^{2+}.

In response to a rise in Ca^{2+}, CaM exhibits a complex pattern of cellular localization, including a significant redistribution from the cytoplasm to the nucleus. This stimulus-dependent movement of CaM to the nucleus and its activation has also been detected in neurons. CaM has also been seen to accumulate slowly in the nucleus of hormone-treated pancreatic acinar cells. The mechanism of translocation in smooth-muscle cells apparently involves the passive diffusion of CaM into the nucleus, where it might associate with targets in a Ca^{2+}-dependent manner.

The synchronization between CaM and Ca^{2+} signals is also being explored. A direct relationship between a rise in the levels of intracellular free Ca^{2+} and the Ca^{2+}-dependent activation of CaM was first observed during a response to wound healing in fibroblasts. Ca^{2+} oscillations in the secretory granules of pancreatic acinar cells have also been correlated with oscillations in the local concentration of CaM. Recent studies on sea-urchin eggs undergoing mitosis, however, indicate that the spatial patterns of Ca^{2+} are different from those of Ca^{2+}-activated CaM. Interestingly, the Ca^{2+}-dependent activation of CaM exhibits a heterogeneous distribution pattern in the cells that have been studied, indicating the presence of discrete populations of CaM. These studies emphasize the importance of temporal and spatial relationships between Ca^{2+} signals and CaM function.

### TABLE I – SOME RECENT EXAMPLES OF CALMODULIN-REGULATED PROTEINS

<table>
<thead>
<tr>
<th>Protein</th>
<th>Comments</th>
<th>Ref.</th>
</tr>
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<tbody>
<tr>
<td>Cabin1</td>
<td>Thymocyte transcriptional regulator</td>
<td>45</td>
</tr>
<tr>
<td>NAP-22</td>
<td>Neuronal substrate of protein kinase C</td>
<td>46</td>
</tr>
<tr>
<td>Striatin</td>
<td>Neuronal, associates with phosphatase 2A</td>
<td>47</td>
</tr>
<tr>
<td>CAP-19</td>
<td>Neuronal, IQ calmodulin-binding motif</td>
<td>48</td>
</tr>
<tr>
<td>EGF-receptor</td>
<td>Human, CaM binds at juxtamembrane</td>
<td>49</td>
</tr>
<tr>
<td>MLC phosphatase (targeting subunit)</td>
<td>Participant in muscle contraction/relaxation</td>
<td>50</td>
</tr>
<tr>
<td>Connexin 32</td>
<td>Located at gap junctions</td>
<td>51</td>
</tr>
<tr>
<td>CHUP</td>
<td>Located in the nucleus</td>
<td>52</td>
</tr>
<tr>
<td>High MW protein</td>
<td>Cardiac muscle phosphoprotein</td>
<td>53</td>
</tr>
<tr>
<td>Beta-2-glycoprotein</td>
<td>Membrane-associated protein in kidney</td>
<td>54</td>
</tr>
<tr>
<td>Retinal proteins</td>
<td>Involved in neuronal synaptic transmission</td>
<td>55</td>
</tr>
<tr>
<td>Exocellular proteins</td>
<td>Located in animal body fluids</td>
<td>56</td>
</tr>
<tr>
<td>Sperm proteins</td>
<td>Spermatocyte, acrosome reaction</td>
<td>57</td>
</tr>
<tr>
<td>Plant proteins</td>
<td>Plasma membrane transporter</td>
<td>58</td>
</tr>
<tr>
<td>Yeast proteins</td>
<td>Involved in cell growth and division</td>
<td>59</td>
</tr>
<tr>
<td>Phosphatidylinositol 3-kinase</td>
<td>Component in receptor signalling</td>
<td>60</td>
</tr>
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indirectly involved in protein phosphorylation. CaM also regulates the activities of the plasma-membrane Ca<sup>2+</sup>-pump, various ion channels, the ryanodine receptor and isoforms of the inositol (1,4,5)-trisphosphate receptor. The list of CaM targets is extensive and constantly growing (Table 1).

Are there differences in the mechanisms by which CaM and other Ca<sup>2+</sup>-transducers regulate their targets? CaM performs a variety of roles, and CaM-binding proteins can be categorized into at least six classes based on their modes of regulation in the presence and absence of Ca<sup>2+</sup> (Fig. 4). One group of effectors, which we designate class A, binds essentially irreversibly to CaM irrespective of Ca<sup>2+</sup>-CaM. This group is thus more appropriately considered a subunit of these proteins. One example is phosphorylase kinase, an enzyme that requires denaturing conditions to dissociate CaM but is activated in the presence of Ca<sup>2+</sup> (1,4,5)-trisphosphate receptor type 1. Members of a second group of effectors (class B) bind to CaM in the absence of Ca<sup>2+</sup> (i.e. to the apo-CaM form) but dissociate reversibly in the presence of Ca<sup>2+</sup> (Ref. 20). Examples include proteins such as neuromodulin and neurogranin, which might serve as intracellular reservoirs for CaM at resting concentrations of Ca<sup>2+</sup> but liberate Ca<sup>2+</sup>-activated CaM in response to a transient Ca<sup>2+</sup> signal.

A third group of effectors (class C) includes smooth-muscle myosin-light-chain kinase (MLCK) and calcineurin. These class-C effectors form low-affinity complexes with CaM at low concentrations of Ca<sup>2+</sup>, when CaM is unoccupied or partially occupied by Ca<sup>2+</sup> (<2 (mole Ca<sup>2+</sup>) (mole CaM)<sup>-1</sup>). At high concentrations of Ca<sup>2+</sup>, these targets engage in a high-affinity complex and are activated by CaM<sup+=2</sup>. A fourth class of proteins (class D) binds to CaM in the presence of Ca<sup>2+</sup> but, in this case, CaM inhibits their function. This group includes enzymes such as select members of the G-protein-receptor kinases<sup>22</sup>, as well as the inositol (1,4,5)-trisphosphate receptor type 1. At high Ca<sup>2+</sup> concentrations (red), class D dissociates from CaM, classes E and F associate with CaM, classes A, C, E and F are activated by CaM<sup>-+</sup>, and class D is inactivated by CaM<sup>-</sup>.

![FIGURE 4](https://example.com/figure4.png)

**FIGURE 4** Ca<sup>2+</sup>-dependent functions of various classes of calmodulin-binding (CaM-binding) proteins. CaM and various classes of targets exist in free or bound states. Target classes A, B and C are associated with CaM or CaM<sup>-+</sup>-free CaM (apo-CaM) at low (resting) intracellular free Ca<sup>2+</sup> concentrations (red). When Ca<sup>2+</sup> concentrations are high (green), class B dissociates from CaM, classes D, E and F associate with CaM, classes A, C, E and F are activated by CaM<sup>-+</sup>, and class D is inactivated by CaM<sup>-</sup>.

The Ca<sup>2+</sup>-controlled exposure of hydrophobic groups in the two domains of CaM releases a considerable amount of biochemical energy, which is transduced into two separable effects: a change in the affinity of CaM for the effector and an alteration in the effector’s function. Studies focusing on one group of CaM-regulated enzymes in particular, the CaM-dependent protein kinases, have provided important insights into some of the mechanisms underlying these phenomena. A short peptide of ~20 residues that is responsible for binding Ca<sup>2+</sup>–CaM, designated a CaM-binding domain, has been identified in many CaM-regulated proteins (Fig. 5a) and in other types of CaM-binding proteins.<sup>23</sup> The crystal structure of CaM kinase I reveals that the CaM-binding domain directly interacts with and sterically obstructs the putative substrate-binding sites of the inactive enzyme<sup>23</sup>. Furthermore, the N-terminal part of the CaM-binding sequence loops away from the enzyme, exposing the hydrophobic side chain of Trp303 to solvent and providing potential access for Ca<sup>2+</sup>–CaM to bind (Fig. 5b). This proposal is supported by experiments showing that the mutation of Trp303 to Ser in CaM kinase I significantly lowers the apparent affinity of CaM kinase I for Ca<sup>2+</sup>–CaM.<sup>23</sup>
A homologous hydrophobic residue is conserved in other CaM kinases (Fig. 5a). In the absence of detailed information on complexes between CaM and its intact effectors, spectroscopic and crystallographic studies of Ca\textsuperscript{2+}–CaM complexed with peptides corresponding to the CaM-binding domains of four CaM kinases including CaM kinase I show in each case that this conserved hydrophobic residue interacts exclusively with the methionine-rich hydrophobic pocket in the C-terminal domain of Ca\textsuperscript{2+}–CaM\textsuperscript{29–32} (Fig. 5c). Recently determined three-dimensional structures of Ca\textsuperscript{2+}–CaM bound to peptides from the plasma membrane Ca\textsuperscript{2+}-pump and a CaM-kinase kinase also reveal additional modes of interaction between CaM and these other CaM-binding peptides\textsuperscript{33,34}. These peptide studies indicate that the C-terminal domain of Ca\textsuperscript{2+}–CaM might confer binding energy on the intact enzymes. Indeed, this appears to be the case because complementary mutagenesis experiments on the Met residues of CaM showed that an evolutionarily invariant Met124 in the C-terminal domain of Ca\textsuperscript{2+}–CaM that contacts the conserved hydrophobic residues in several CaM-binding peptides (Fig. 5c) is necessary for high-affinity binding and activation of CaM kinase I as well as for three other CaM-dependent protein kinases\textsuperscript{35,36}.

In contrast to the C-terminal domain of Ca\textsuperscript{2+}–CaM, residues in the hydrophobic pocket of the N-terminal domain of Ca\textsuperscript{2+}–CaM perform varying functions with different CaM-dependent kinases. The results from the crystallographic studies show that hydrophobic residues in the N-terminal domain of Ca\textsuperscript{2+}–CaM mainly interact with the C-terminal part of the CaM-binding peptides of smooth-muscle MLCK and CaM kinase II, respectively (Fig. 5a). Progressive C-terminal deletions and chimeric substitutions in the CaM-binding domain of smooth-muscle MLCK showed that the C-terminal half of the CaM-binding domains of these enzymes...
are required for Ca\(^{2+}\)-CaM-dependent activation\(^6,10,11\). Furthermore, deletion studies of CaM kinase I show that the C-terminal portion of its CaM-binding sequence confers high affinity for Ca\(^{2+}\)-CaM, as well as CaM-dependent activity\(^12\). These results complement those from experiments on CaM showing that the hydrophobic pocket in its N-terminal domain generates a high-affinity complex with CaM kinase II, activates smooth-muscle MLCK and combines the functions of high-affinity binding and activation of CaM kinase I\(^13,14\).

In comparison with unbound Ca\(^{2+}\)-CaM, the Ca\(^{2+}\)-CaM-peptide complexes exhibit a dramatic contraction enabling CaM to wrap around and sequester the helical CaM-binding peptides (Fig. Sc). Experiments on hydrophobic residues of CaM show that charged and polar residues are also required to activate the smooth-muscle MLCK by promoting the accessibility of substrate to this particular enzyme. Surprisingly, the hydrophobic residues on CaM responsible for this effect are originally separated from each other on both domains of free Ca\(^{2+}\)-CaM by more than 5 Å apart in the Ca\(^{2+}\)-CaM-peptide complex\(^15\). These polar groups do not contact the CaM-binding peptide directly, so they might exert their effects on the intact protein kinase by interacting with an area distinct from its CaM-binding domain. Indeed neutron- and X-ray-scattering studies on the kinase domain of skeletal-muscle MLCK indicate that the CaM-binding domain is displaced to one side of the enzyme by the binding of Ca\(^{2+}\)-CaM\(^16\). This event could expose the substrate-binding site of the enzyme and indicates how CaM might remove an inhibitory CaM-binding domain away from the kinase domain, thus leading to enzyme activation.

The preceding mechanistic studies show that the regulation of enzymes by Ca\(^{2+}\)-CaM is a highly ordered, cooperative and complementary process that contributes to both the affinity and specificity for targets. Another surprising outcome is the discovery that the structures of Ca\(^{2+}\)-CaM-peptide complexes are relevant to their corresponding enzymes. However, in addition to the obvious limitation in the use of peptides to study mechanisms of enzyme activation, there are mounting indications that peptides might not be entirely suitable for studying other functions of the full-length target protein. For example, Ca\(^{2+}\) has a considerably higher affinity for the CaM-MLCK-peptide complex than for the corresponding CaM-MLCK enzyme complex\(^17\). Also, mutations in the CaM-binding domain of smooth-muscle MLCK have a significantly greater effect on CaM binding and activation than the same changes in muscle MLCK have a significantly greater effect on CaM-binding domain of smooth-muscle MLCK and the intact enzyme have also been observed by small-angle scattering\(^18\). One explanation for the adaptability and the higher affinity exhibited by the shorter CaM-binding domains is the conformational flexibility inherent in isolated peptides. By contrast, the relatively fixed conformation of the intact, folded enzymes restricts their ability to adapt similarly to structural changes within their CaM-binding domains. It is helpful to bear these caveats in mind when peptides are used to model effector function.

Perspective and conclusion

The extensive characterization of CaM provides a useful precedent for less-well-understood Ca\(^{2+}\) sensors. At the subcellular level, the spatial and temporal coordination between Ca\(^{2+}\), CaM and its effectors are important for channeling all three components into a productive signalling pathway. The ability of CaM to integrate Ca\(^{2+}\) signals into different cellular contexts by migrating between various compartments further underscores this point. At the intermolecular level, CaM uses different modes of Ca\(^{2+}\)-dependent interaction to promote high affinity as well as specificity for targets. At the submolecular level, the Ca\(^{2+}\)-triggered exposure of energy-donating groups on CaM is coupled to energy-accepting groups on its targets, leading to changes in Ca\(^{2+}\) binding by CaM as well as in the function of its effectors.

Finally, how are these levels of regulating CaM related to each other? It is likely that the mobility of separate pools of CaM derives from the different interactions between CaM and its targets. Therefore, some classes of proteins might anchor CaM to specific cellular locations, depending on the stability of a particular CaM-effector complex in the absence or presence of a Ca\(^{2+}\) signal. The affinity of such complexes is likely to be due to complementary interactions between sites on the target proteins and sites on CaM that change conformation in response to Ca\(^{2+}\). The Ca\(^{2+}\)-dependent interactions not only affect the affinity of the complex but also regulate the activity of effectors. This apparent ability of a CaM-effector complex to decode Ca\(^{2+}\) signals has been exemplified in a recent study showing that CaM participates in converting Ca\(^{2+}\) oscillations into changes in the autonomous enzymatic activity of at least one target, CaM kinase II\(^19\). Additional studies on CaM will lead to a more-complete integration of its levels of regulation. Meanwhile, it will be interesting to see whether any of the mechanisms exhibited by CaM will be relevant to other Ca\(^{2+}\) sensors.

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