

## What's New with Calcium?

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Calcium ions are key second messengers in eukaryotic cells. A requirement for  $\text{Ca}^{2+}$  during muscle contraction was first observed over 100 years ago, but only in the last 20 years has the role of  $\text{Ca}^{2+}$  as an important signaling molecule expanded to include nearly every eukaryotic cell. An increase in the intracellular concentration of  $\text{Ca}^{2+}$  caused by hormonal or electrical signals can trigger responses as varied as contraction, cellular proliferation, secretion, metabolic adjustments, and changes in gene expression. Considering the diverse processes regulated by  $\text{Ca}^{2+}$  and the complex mechanisms required to control this essential ion, it is not surprising that even a meeting devoted entirely to  $\text{Ca}^{2+}$  and  $\text{Ca}^{2+}$ -binding proteins can cover only the highlights of what has been learned in the last two years. At the Eighth International Symposium on Calcium-Binding Proteins and Calcium Function in Health and Disease (August 23–27, Davos, Switzerland, organized by a group led by E. Carafoli), advances were reported in understanding the initiation and propagation of the  $\text{Ca}^{2+}$  signal, the structures and functions of  $\text{Ca}^{2+}$  receptors, and the activities of the effectors. Finally, our analysis of  $\text{Ca}^{2+}$  and  $\text{Ca}^{2+}$ -binding proteins has provided insights into the molecular basis of several diseases and immunosuppression.

### The $\text{Ca}^{2+}$ Signal

The concentration of  $\text{Ca}^{2+}$  in the cytoplasm is kept low, at  $10^{-7}$  M to  $10^{-8}$  M, whereas outside cells  $[\text{Ca}^{2+}]$  is  $10^{-3}$  M. Induction, propagation, and termination of the  $\text{Ca}^{2+}$  signal requires an elaborate system of channels, pumps, and exchangers. Specific channels that open in response to electrical or hormonal stimuli allow  $\text{Ca}^{2+}$  to enter the cytoplasm either from outside the cell or from internal organelles such as the sarcoplasmic reticulum in muscle cells or the endoplasmic reticulum in nonmuscle cells. The  $\text{Ca}^{2+}$  ATPase and  $\text{Na}^{+}/\text{Ca}^{2+}$  exchanger in the plasma membrane or the  $\text{Ca}^{2+}$  ATPase in internal membranes terminate the  $\text{Ca}^{2+}$  signal by moving  $\text{Ca}^{2+}$  into the extracellular fluid or into internal stores, respectively.

Our understanding of the machinery that produces the  $\text{Ca}^{2+}$  signal has recently been bolstered by the molecular genetic analysis of the cDNAs encoding many of the components. Mutational analysis of the  $\text{Ca}^{2+}$  ATPase from the sarcoplasmic reticulum has been essential in correlating functions with structural elements (Andersen et al., 1992; N. Green, National Institute for Medical Research, London). Similar analysis is in progress on the plasma membrane  $\text{Ca}^{2+}$  ATPase (E. Carafoli, Swiss Federal Institute of Technology, Zurich) as well as the  $\text{Na}^{+}/\text{Ca}^{2+}$  exchanger from heart plasma membrane (K. Philipson, University of

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California, Los Angeles) and a  $\text{Ca}^{2+}$ ,  $\text{Na}^{+}$ , and  $\text{K}^{+}$  exchanger from retinal rod cells, which shows no sequence similarity to the heart exchanger (N. Cook, Max-Planck-Institut für Biophysik, Frankfurt).

Voltage-gated  $\text{Ca}^{2+}$  channels are one of the most extensively studied class of channels (reviewed by Tsien and Tsien, 1990). They are activated by depolarization and show a 1000-fold preference for  $\text{Ca}^{2+}$  over  $\text{K}^{+}$  and  $\text{Na}^{+}$ . They are generally classified into four groups on the basis of biophysical and pharmacological properties: L type, T type, N type, and P type. What is the relationship between these four groups and two of the major subfamilies that have been thus far revealed by analysis of cDNA clones of  $\alpha 1$  subunits? One subfamily encodes the L type channels, which are activated by strong depolarizations and are sensitive to 1,4-dihydropyridine (DHP). An elegant mutational analysis of these channels is discussed below. The other subfamily, exemplified by the brain  $\alpha 1$  clone BI, is expressed at highest levels in the cerebellum (Mori et al., 1991). Cerebellar Purkinje cells contain almost solely P type channels, which are activated by moderately high voltage and insensitive to DHP but blocked by  $\omega$ -Aga-IVA, a toxin from the funnel web spider. Thus, it was believed that BI cDNA encodes the P type channel. However, R. Tsien and coworkers (Stanford University) have shown that when BI is expressed in oocytes, these channels do not display the expected inactivation kinetics or sensitivity to  $\omega$ -Aga-IVA, although they are insensitive to DHP as expected. The discrepancies hold true regardless of which  $\beta$  subunit is coexpressed with BI. Either expression in oocytes alters the inactivation and pharmacology of the channel, or the BI cDNA does not in fact encode the P type channel. Its pharmacological properties also do not correspond to any of the previously recognized channels.

The L type channels play a central role in excitation-contraction coupling in cardiac and smooth muscle and excitation-secretion coupling in endocrine cells and some neurons. In cardiac muscle, a depolarizing voltage change across the T tubules (invaginations of the plasma membrane) rapidly activates L type channels in the T tubules. Extracellular  $\text{Ca}^{2+}$  entering through these channels interacts with the ryanodine-sensitive  $\text{Ca}^{2+}$  release channel to cause a much larger release of  $\text{Ca}^{2+}$  from the sarcoplasmic reticulum. Skeletal muscle also has L type channels, but they are slowly activated and contraction does not require entry of extracellular  $\text{Ca}^{2+}$  through these channels. Instead, the voltage-induced conformational change of the L type channels in the plasma membrane apparently directly opens the intracellular  $\text{Ca}^{2+}$  release channel (Catterall, 1991).

What structural features of cardiac muscle L type channel distinguish it from skeletal muscle channels? The primary structures of the  $\alpha 1$  subunits of L type channels from both cardiac and skeletal muscle are similar and contain four repeat units, each with six predicted  $\alpha$ -helical membrane-spanning segments. K. Beam and coworkers (Colorado State University) in collaboration with the laboratory

of the late Shosaku Numa (Kyoto University) made chimeras of the skeletal and cardiac L type channels and assayed whether each chimera behaved as a skeletal or a cardiac channel. Measuring the activity of the chimeras was simplified by expressing them in dysgenic myotubes, which do not have a wild-type channel because they lack the  $\alpha 1$  subunit. The region required to induce  $\text{Ca}^{2+}$  release from the sarcoplasmic reticulum directly was limited to the cytoplasmic loop between repeats II and III. If that small region derives from skeletal muscle, induction of  $\text{Ca}^{2+}$  release does not require extracellular  $\text{Ca}^{2+}$  (Tanabe et al., 1990). Furthermore, the first repeat of putative transmembrane helices is required for fast activation of the  $\text{Ca}^{2+}$  current as is seen in cardiac muscle (Tanabe et al., 1991). In this way, the regions involved in voltage sensing and inducing  $\text{Ca}^{2+}$  release have been limited to small sections that now can be characterized by more detailed mutagenesis. Using photoactivatable derivatives of DHP, H. Glossman and coworkers (Institut für Biochemische Pharmakologie, Innsbruck) and F. Hofmann and coworkers (University of Munich) have localized the DHP-binding site to the sixth membrane-spanning segment of the third repeat.

In most cell types in which hormones or growth factors (as opposed to membrane depolarization) release  $\text{Ca}^{2+}$  from intracellular stores, the release is triggered by inositol 1,4,5-trisphosphate ( $\text{InsP}_3$ ) produced by phospholipase C-mediated breakdown of phosphatidylinositol-4,5-bisphosphate. The plasma membrane receptors that trigger this cascade belong to the seven transmembrane helix family. Although the requirement for GTP suggested that a G protein linked the receptor to phospholipase C, the G protein remained elusive until recently. Smrcka et al. (1991) isolated a new member of the heterotrimeric G protein family,  $G_q$ , that could stimulate the activity of polyphosphoinositide-specific phospholipase C. At the meeting, A. Harootyan and coworkers (University of California, San Diego) provided evidence that  $G_q$  regulates  $\text{Ca}^{2+}$  signals elicited by three growth factors. When microinjected into BALB/c3T3 or REF52 fibroblasts, inhibitory antibodies to the  $\alpha$  subunit of  $G_q$  block both release of  $\text{Ca}^{2+}$  from internal stores and  $\text{Ca}^{2+}$  influx in response to bradykinin, thrombin, or vasopressin. Two important controls showed first that the antibodies do not block the rise in  $\text{Ca}^{2+}$  elicited by platelet-derived growth factor, and thus the cells are still competent to increase  $\text{Ca}^{2+}$  levels by methods independent of  $G_q$ . Second, inhibitory antibodies against the  $\alpha$  subunit of  $G_i$  or a yeast G protein have no effect on the  $\text{Ca}^{2+}$  response to bradykinin, thrombin, or platelet-derived growth factor.

$\text{InsP}_3$  produced by phospholipase C opens the  $\text{InsP}_3$ -sensitive  $\text{Ca}^{2+}$  release channel (Mikoshiba, University of Tokyo) found in the endoplasmic reticulum, although its location is still somewhat controversial. Development of methods to measure  $\text{Ca}^{2+}$  levels in individual living cells (Tsien, 1989) revolutionized our understanding of the nature of the  $\text{Ca}^{2+}$  signal. Instead of a uniform rise in internal  $\text{Ca}^{2+}$ ,  $\text{Ca}^{2+}$  signals are highly organized in space and time. They are propagated as waves across a cell (Figure 1) and are not necessarily a single wave but repeated waves or

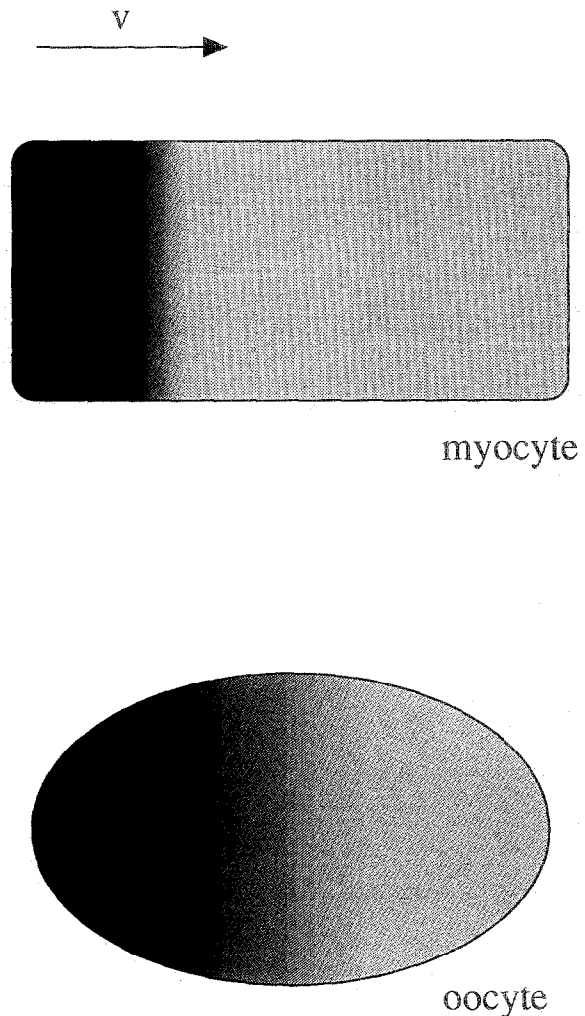


Figure 1. Schematic Representation of Calcium Waves in Myocytes and Oocytes

A high concentration of calcium is represented in black; the resting level of  $\text{Ca}^{2+}$  is light grey. The calcium gradient at the wave front is much steeper in myocytes than oocytes. (Adapted from Meyer [1991] by E. Muller and T. Davis).

oscillations. One of the controversies in the field is how the waves are propagated. Two models have been proposed, and both suggest a positive feedback loop. The models differ in the mechanism of positive feedback. One model (M. Berridge, University of Cambridge) proposes that the wave is propagated by  $\text{Ca}^{2+}$  diffusion and then amplified by  $\text{Ca}^{2+}$ -induced  $\text{Ca}^{2+}$  release from internal stores as is seen in cardiac cells. In nonmuscle cells, the initial pulse of  $\text{Ca}^{2+}$  comes from  $\text{InsP}_3$ -sensitive stores, whereas subsequent  $\text{Ca}^{2+}$  is released from  $\text{Ca}^{2+}$ -sensitive,  $\text{InsP}_3$ -insensitive stores (Berridge, 1990). In the latest version of the model, Berridge has argued that the  $\text{InsP}_3$ -sensitive stores may also be capable of propagating calcium waves through a process of  $\text{Ca}^{2+}$ -induced  $\text{Ca}^{2+}$  release. Therefore, the  $\text{Ca}^{2+}$  wave represents the diffusion of  $\text{Ca}^{2+}$  across the cell, releasing new stores of  $\text{Ca}^{2+}$  as it diffuses. The other model (T. Meyer, Duke University) suggests that the wave is propagated by  $\text{InsP}_3$  diffusion. The initial pulse of

$\text{Ca}^{2+}$  from  $\text{InsP}_3$ -sensitive stores locally activates phospholipase C  $\beta$ , which is activated both by  $\text{Ca}^{2+}$  and  $G_q$  in vitro (Srncka et al., 1991). Phospholipase C produces more  $\text{InsP}_3$ , which then diffuses across the cell to release more  $\text{Ca}^{2+}$  from  $\text{InsP}_3$ -sensitive stores (Meyer and Stryer, 1991). Thus, the two models differ in that  $\text{Ca}^{2+}$  is the propagating messenger in the first, whereas  $\text{InsP}_3$  is the propagating messenger in the second. Using experimental values of the velocity of the wave and the length of the wavefront, Meyer (1991) calculated that the diffusion coefficient of the messenger in oocytes, hepatocytes, and astrocytes is about  $300 \mu\text{m}^2/\text{s}$ . Diffusion measurements of  $^{45}\text{Ca}^{2+}$  and radiolabeled  $\text{InsP}_3$  in *Xenopus* cytoplasm were presented at the meeting (L. Stryer and coworkers, Stanford University).  $\text{InsP}_3$  has a diffusion coefficient of  $260 \mu\text{m}^2/\text{s}$ , while the high buffering of  $\text{Ca}^{2+}$  slows its diffusion to less than  $30 \mu\text{m}^2/\text{s}$ . Thus,  $\text{InsP}_3$  has the properties required for propagation of a wave of  $\text{Ca}^{2+}$  in oocytes, hepatocytes, and astrocytes, and  $\text{Ca}^{2+}$  does not. Furthermore, the time required for wave development (1 s to 10 s) in these cells is consistent with a mechanism requiring  $\text{Ca}^{2+}$ -dependent production of  $\text{InsP}_3$  and much slower than the time required for direct activation of a channel by  $\text{Ca}^{2+}$ . The two models are not mutually exclusive. Both forms of amplification might coexist in some cells with  $\text{InsP}_3$ -mediated long-range  $\text{Ca}^{2+}$  signals locally amplified to higher peak  $\text{Ca}^{2+}$  levels by the faster feedback of direct activation of a  $\text{Ca}^{2+}$ -sensitive channel.

In cardiac cells,  $\text{Ca}^{2+}$ -induced  $\text{Ca}^{2+}$  release from the sarcoplasmic reticulum is the sole mechanism of wave propagation, and  $\text{InsP}_3$  is not involved. The rapid rate at which the signal is amplified in cardiac cells ( $<0.16$  s) is consistent with the direct activation of a channel by  $\text{Ca}^{2+}$  and inconsistent with the slower feedback loop involving  $\text{Ca}^{2+}$ -dependent production of  $\text{InsP}_3$ . As predicted for a wave produced by a slowly diffusing messenger, the length of the wavefront is very narrow, although it is difficult to measure accurately (see Meyer, 1991). Furthermore, the T tubules of cardiac cells permit  $\text{Ca}^{2+}$  to travel very short distances before it induces  $\text{Ca}^{2+}$  release.

### Structures of the Intracellular $\text{Ca}^{2+}$ Receptors

Transmission of the  $\text{Ca}^{2+}$  signal requires intracellular  $\text{Ca}^{2+}$  receptors to trigger the cellular response. Except for the annexins (see below), intracellular receptors share a common  $\text{Ca}^{2+}$ -binding motif—a 29 residue helix-loop-helix structure called an EF hand, first described by Kretsinger in 1973 (reviewed by Strynadka and James, 1989). The residues that bind  $\text{Ca}^{2+}$  are in a contiguous 12 residue sequence that spans the loop and the beginning of the second  $\alpha$  helix. The crystal structures of 35 EF hands are published, and the similarity is remarkable (C. Bugg, University of Alabama). All show 7-fold coordination of the calcium ion in a pentagonal bipyramid. With the exception of the EF hands in a sarcoplasmic  $\text{Ca}^{2+}$ -binding protein (Vijay-Kumar and Cook, 1992), the two helices are approximately perpendicular in the  $\text{Ca}^{2+}$ -bound form, and the residues that form ligands to  $\text{Ca}^{2+}$  are highly conserved. Nevertheless, the EF hands bind  $\text{Ca}^{2+}$  with  $K_d$ s ranging over

four orders of magnitude from  $10^{-9}$  M in parvalbumin to  $10^{-5}$  M in the low affinity sites of calmodulin. One of the future challenges is to identify the features of the EF hand that determine affinity; at present, it is not possible to predict the affinity of a site even when the three-dimensional structure is known.

Upon binding  $\text{Ca}^{2+}$ , the intracellular receptors undergo conformational changes, which allow activation of target proteins. To understand the activation mechanism, the structure of all three forms of the receptor—with  $\text{Ca}^{2+}$ , without  $\text{Ca}^{2+}$ , and bound to the target—must be solved. Substantial progress has been made in solving these structures, and each has yielded surprises. When the structure of troponin C, a receptor specific to muscle, was analyzed and first presented, the response was virtual disbelief. The two globular domains each containing a pair of EF hands were not unexpected, but the domains were connected by a long central helix, a structure that seemed unlikely to exist in solution (Herzberg and James, 1985; Sundaralingam et al., 1985). The crystal structure of calmodulin, a ubiquitous intracellular  $\text{Ca}^{2+}$  receptor, demonstrated that it also has two globular domains connected by a central helix (Figure 2). Analysis of calmodulin by multidimensional nuclear magnetic resonance (NMR) shows that the globular domains are folded in a very similar fashion in solution as in the crystal structure, but the region of the helix from residues 77–81 is flexible (Ikura et al., 1991), and the tumbling of the N-terminal and C-terminal domains (as measured by  $^{15}\text{N}$  relaxation) is effectively independent (Barbato et al., 1992). The results of small angle X-ray-scattering experiments are consistent with calmodulin existing in solution in a range of structures, from extended, as in the crystal structure, to more compacted forms (J. Head, Boston University School of Medicine). The compact forms are probably molecules in which the helix is bent and the two domains are brought together (R. Kretsinger, University of Virginia).

The solution structure of a complex of  $\text{Ca}^{2+}$ -calmodulin and a small peptide encompassing the calmodulin-binding site of skeletal muscle myosin light chain kinase (MLCK) has been solved using advanced methods of multidimensional NMR (A. Bax, National Institutes of Health; Ikura et al., 1992). Although both small angle X-ray-scattering (Heidorn et al., 1989) and modeling studies (Persechini and Kretsinger, 1988) had predicted a compact structure, few were prepared for the transformation that occurs when calmodulin binds to this target peptide. The two globular domains retain their overall shape, but they are collapsed around the peptide with an approximate 2-fold symmetry (Figure 2). The central helix adopts an alternative conformation of two short helices connected by a flexible loop that spans from residues 74–82 (Ikura et al., 1992). The crystal structure of a complex of  $\text{Ca}^{2+}$ -calmodulin and the calmodulin-binding peptide of smooth muscle MLCK has also been analyzed (Meador et al., 1992). Overall, the crystal structure is very similar to the solution structure, but in the crystal, the bend in the central helix is well defined and occurs only from residues 73–77. The differences between the crystal and solution structures could be due to the differences in the target peptides (from skeletal muscle

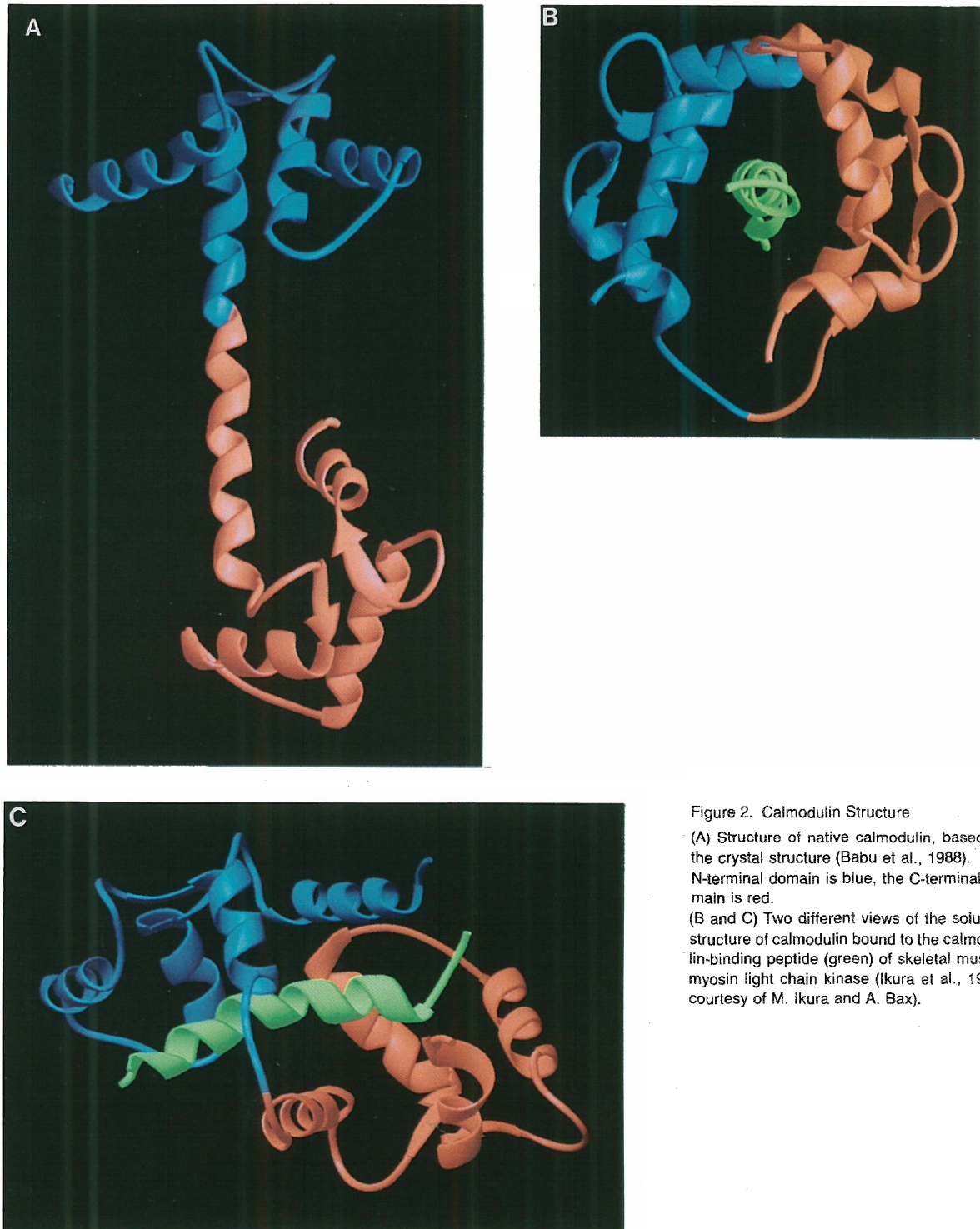


Figure 2. Calmodulin Structure

(A) Structure of native calmodulin, based on the crystal structure (Babu et al., 1988). The N-terminal domain is blue, the C-terminal domain is red.

(B and C) Two different views of the solution structure of calmodulin bound to the calmodulin-binding peptide (green) of skeletal muscle myosin light chain kinase (Ikura et al., 1992; courtesy of M. Ikura and A. Bax).

MLCK or smooth muscle MLCK), the calmodulins (*Drosophila* or mammalian), or the methods used to obtain the structures. Substantial evidence indicates that different targets interact differently with calmodulin (Klee, 1988). Thus, these structures represent one method of interaction, which will be interesting to compare with structures

of calmodulin bound to other targets or the structure of troponin C bound to troponin I.

The structures of apo-calmodulin and apo-troponin C remain to be solved. In both the crystal structure and the solution structure of calmodulin, all four  $\text{Ca}^{2+}$ -binding sites are filled. Obtaining crystals of apo-calmodulin or solving

the solution structure by NMR will be extremely difficult because the molecule is quite flexible. In the troponin C structures, only the two C-terminal EF hands are filled; the N-terminal sites are devoid of metal ions. A model for the  $\text{Ca}^{2+}$ -induced conformational change was proposed based on the hypothesis that upon binding  $\text{Ca}^{2+}$ , the N-terminal domain adopts a conformation similar to the  $\text{Ca}^{2+}$ -bound C-terminal domain (Herzberg et al., 1986). Characterization of the biochemical effects of mutations in the N-terminal domain support the hypothesis (Fujimori et al., 1990; Grabarek et al., 1990). Many of the interactions predicted by a similar model of the C-terminal domain of calmodulin can be detected by NMR (S. Forsén, University of Lund, Sweden).

Another class of intracellular  $\text{Ca}^{2+}$ -binding proteins, the annexins, lack the EF hand  $\text{Ca}^{2+}$ -binding motif. They were first described as phospholipid and  $\text{Ca}^{2+}$ -binding proteins and have since been ascribed many interesting properties, but their physiological role is in dispute. Annexin V can act as a voltage-gated  $\text{Ca}^{2+}$  channel (Rojas et al., 1990). The crystal structure suggests a unique mechanism for  $\text{Ca}^{2+}$  conductance (Huber et al., 1992). Mutants in annexin V display altered channel activities, so the channel is dependent on the annexin (A. Burger, D. Vöges, and R. Berendes, Max-Planck-Institut für Biochemie, Munich). However, the significance of this observation is controversial, largely because it is not yet resolved whether the channel activity represents most of the molecules or only a small fraction. Also, proteins unrelated to transport have been observed to form channels in lipid bilayers. An alternative model is that annexins bind to the cytoplasmic face of membranes to mediate a variety of unknown functions (M. Crumpton, Imperial Cancer Research Fund, London).

### Functions of the Intracellular $\text{Ca}^{2+}$ Receptors and the Enzymes They Regulate

With each meeting, the number of  $\text{Ca}^{2+}$ -binding proteins increases and  $\text{Ca}^{2+}$  signals are detected in more cells. However, specific intracellular  $\text{Ca}^{2+}$  receptors have been correlated with responses to specific signals in only a few systems. In skeletal muscle,  $\text{Ca}^{2+}$  binding to troponin C induces a conformational change in the troponin complex, which triggers contraction. In smooth muscle,  $\text{Ca}^{2+}$ -calmodulin activates MLCK, which induces contraction by phosphorylation of myosin light chain. At the meeting, another connection was made in visual signal transduction. In darkness, cationic channels in the plasma membrane of rod outer segments are kept open by bound cyclic GMP. Light activates an enzymatic cascade that stimulates cyclic GMP hydrolysis leading to channel closure. In the dark the  $[\text{Ca}^{2+}]$  inside retinal rod cells is 400 nM, whereas light-induced closure of the channel lowers the  $[\text{Ca}^{2+}]$  to <50 nM. Restoration of the dark state requires resynthesis of cyclic GMP by guanylate cyclase to reopen the cationic channels in the plasma membrane. Although it was known that guanylate cyclase activity increases when  $\text{Ca}^{2+}$  is lowered to less than 100 nM, the protein that senses the change in  $\text{Ca}^{2+}$  and contributes to the activation of guanylate cyclase was only identified last year as a 23 kD  $\text{Ca}^{2+}$ -

binding protein called recoverin (Dizhoor et al., 1991). Recoverin and its close relatives visinin (Yamagata et al., 1990) and frequenin (J. Lindemeier, University of Hamburg) have several unique features. The recoverin crystal structure reveals four EF hand  $\text{Ca}^{2+}$ -binding motifs, although only two have a high affinity for  $\text{Ca}^{2+}$  (L. Stryer). Like calmodulin and troponin C, recoverin has two globular domains, each with two EF hands. However, there is no long central helix; instead, the two domains make a narrow cleft at their interface, an arrangement not previously seen in EF hand proteins. Recoverin is unusual in that it is myristoylated (14:1) on the N-terminus. A similar myristoyl (14:0) modification is found on another  $\text{Ca}^{2+}$  receptor, the regulatory subunit of the phosphatase calcineurin, where it is proposed to be involved in the interaction between regulatory and catalytic subunits (C. Klee, National Institutes of Health). Finally, recoverin and its near relatives are the only known  $\text{Ca}^{2+}$  receptors for which a decrease in  $\text{Ca}^{2+}$  levels triggers target activation. Understanding the role of the myristoyl group and the nature of the interaction between recoverin and its target in the absence of  $\text{Ca}^{2+}$  will provide new insights into the range of activities adopted by  $\text{Ca}^{2+}$  receptors.

Multifunctional  $\text{Ca}^{2+}$ -calmodulin-dependent protein kinase (CaM kinase) is an important factor in neuronal signal transduction. Numerous studies implicate CaM kinase in modulation of both neurotransmitter release and neurotransmitter synthesis (reviewed by Hanson and Schulman, 1992). Mice homozygous for a deletion of the  $\alpha$  form of CaM kinase show specific defects both in induction of long-term potentiation in the hippocampus (Silva et al., 1992b) and in spatial learning (Silva et al., 1992a). Mutational analyses of CaM kinase have revealed a complex system of regulation. Recombinant CaM kinase made entirely of  $\alpha$  subunits can be produced by expression in mammalian cells and retains the same characteristics as the enzyme purified from brain, which is a mixture of  $\alpha$  and  $\beta$  subunits.  $\alpha$ -CaM kinase is a multimeric enzyme: the  $\alpha$  subunit is a polypeptide of 478 residues with a catalytic domain, an autoinhibitory domain, and an association domain required to form the holoenzyme.  $\text{Ca}^{2+}$ -calmodulin binding to the autoinhibitory domain activates CaM kinase 100- to 1000-fold. One of the best-studied substrates of CaM kinase is CaM kinase itself. The enzyme autophosphorylates at numerous sites, but autophosphorylation at Thr-286 in the autoinhibitory domain is necessary and sufficient to convert CaM kinase to a  $\text{Ca}^{2+}$ -independent form. Autophosphorylation of truncated molecules that cannot associate is concentration dependent, and thus phosphorylation is an intersubunit reaction (H. Schulman, Stanford University). In addition, a subunit can phosphorylate an adjacent subunit even if the adjacent subunit is inactive owing to a mutation in the active site, but not if the adjacent subunit is free of calmodulin.

$\text{Ca}^{2+}$ -calmodulin-dependent autophosphorylation of CaM kinase not only converts the enzyme to a  $\text{Ca}^{2+}$ -independent form but also traps the calmodulin. Autophosphorylation at Thr-286 decreases the rate of dissociation of calmodulin by 100-fold (Meyer et al., 1992). After a  $\text{Ca}^{2+}$  spike, calmodulin does not appreciably dissociate from the

autophosphorylated kinase unless the  $\text{Ca}^{2+}$  concentration drops back to basal levels for at least 10 s. The combination of the high activity as long as calmodulin is trapped and the lower but still substantial  $\text{Ca}^{2+}$ -independent activity that persists even after calmodulin dissociates provides a mechanism of molecular memory between spikes.

Compared with our understanding of muscle, liver, and neuronal tissues, much less is known about the role of calmodulin in proliferating cells. In fungal and mammalian cells, calmodulin is required for cell growth and division. Relatives of many target enzymes found in brain and muscle are found in proliferating tissues, but little is known about how calmodulin and its targets work together during proliferation. In mammalian cells, focus has turned towards the nucleus, where calmodulin and calmodulin-binding proteins such as MLCK, CaM kinase, and calcineurin have been identified (O. Bachs, University of Barcelona). However, it is not clear that  $\text{Ca}^{2+}$  in the nucleus is regulated independently of cytoplasmic  $\text{Ca}^{2+}$ .

$\text{Ca}^{2+}$  and CaM kinase are required for entry into mitosis in sea urchin eggs. Blocking the increase in cytoplasmic  $\text{Ca}^{2+}$  that occurs at mitosis blocks entry into mitosis, and prematurely raising intracellular  $\text{Ca}^{2+}$  advances the onset of mitosis. Inhibition of sea urchin CaM kinase also blocks entry into mitosis (Baitinger et al., 1990). In a search for the target of calmodulin action, M. Whitaker and coworkers (University College, London) have shown that CaM kinase can activate the human Cdc25 protein approximately 5-fold by phosphorylation. However, CaM kinase is a multi-substrate enzyme, so it will be important to show that such phosphorylation occurs *in vivo* and has physiological effects.

A. Means and coworkers (Duke University) have studied the role of calmodulin in cell cycle progression in the filamentous fungus *Aspergillus nidulans* (Lu et al., 1992) using a strain that is conditional for calmodulin expression and carries a temperature-sensitive mutation, *nimT23*, in the *cdc25* homolog *nimT*. Progression into mitosis requires dephosphorylation of  $\text{p34}^{\text{cdc2}}$  by the protein phosphatase encoded by *nimT*. When cells carrying the *nimT23* mutation are incubated at the restrictive temperature, they accumulate at the G2/M boundary. Spores are germinated under conditions that prevent expression of calmodulin and then arrested in the first cell cycle at G2 by a shift to high temperature. When released from the G2 block (by lowering the temperature), the calmodulin-deficient cells cannot enter mitosis. If extracellular  $\text{Ca}^{2+}$  is lowered at the time the cells are shifted back to the permissive temperature, they cannot enter mitosis upon release from the G2 block even if calmodulin is present in the cells. These results argue that  $\text{Ca}^{2+}$  and calmodulin must perform some function during the cell cycle for cells to proceed from G2 to M. Exactly when calmodulin is required is not addressed because the cells are without calmodulin for 3 to 4 hr while they accumulate at the *nimT* block. However, the requirement for  $\text{Ca}^{2+}$  must exist for events that occur after the *nimT* block.

Calmodulin localizes to the mitotic spindle in plant and animal cells (Welsh and Sweet, 1989), and several lines of evidence suggest that it is required during mitosis. Expres-

sion of antisense calmodulin RNA in mouse C127 cells causes a transient cell cycle arrest in G1, and mitosis (Rasmussen and Means, 1989). Under nonpermissive conditions, temperature-sensitive calmodulin mutants in the budding yeast *Saccharomyces cerevisiae* have severe defects in the mitotic spindle (Y. Anraku, University of Tokyo; Davis, 1992). Analysis of synchronized cultures indicates that calmodulin is specifically required during chromosome segregation and not during formation of the spindle. When calmodulin is inactive during mitosis, the DNA becomes dispersed along the spindle (T. Davis). Calmodulin is also involved in polarized growth. Calmodulin localizes at sites of cell growth in *S. cerevisiae* (Brockhoff and Davis, 1992; Sun et al., 1992), and temperature-sensitive calmodulin mutants show partial defects in polarized growth (Davis, 1992).

Despite its well-established role as a  $\text{Ca}^{2+}$  receptor, calmodulin may be required in a different capacity during cellular proliferation. In *S. cerevisiae*, mutant calmodulins in which the  $\text{Ca}^{2+}$ -binding sites have been inactivated can support growth (Geiser et al., 1991) but are completely defective in activation of  $\text{Ca}^{2+}$ -calmodulin-dependent protein kinase. Further mutational analysis revealed that the features of calmodulin required for proliferation are substantially different from those required to act as a  $\text{Ca}^{2+}$  receptor (T. Davis). In animal cells, calmodulins altered so that they no longer activate the  $\text{Ca}^{2+}$ -calmodulin-dependent cyclic nucleotide phosphodiesterase nevertheless bind to the mitotic spindle (Sweet et al., 1988). Increased understanding of calmodulin function during proliferation will require investigation into  $\text{Ca}^{2+}$ -independent functions.

The identification of a lethal mutation in the single *Drosophila* calmodulin gene allows the role of calmodulin during development to be analyzed (K. Beckingham, Rice University, Houston). An interesting foray into the function of calmodulin in the plant world was made by J. Braam (Rice University). A number of relatively mild stresses (e.g., touch, wind, and rain) stimulate the expression of genes encoding calmodulin-like proteins in *Arabidopsis*.  $\text{Ca}^{2+}$  and calmodulin may therefore be involved in the signal transduction pathways used by plants to respond to diverse stimuli.

## **$\text{Ca}^{2+}$ and Disease**

Three examples were presented of the important interplay between research aimed at understanding cellular processes and research directly guided toward analysis of mammalian disease. First, the analysis of  $\text{Ca}^{2+}$  and  $\text{Ca}^{2+}$ -binding proteins has aided our understanding of the mechanism of action of immunosuppressants that have revolutionized the field of organ transplantation. Cyclosporin A and FK506 are structurally distinct inhibitors of T cell activation (reviewed by Schreiber, 1992). Both drugs bind to and inhibit the activity of cis-trans-peptidylprolyl isomerases, termed immunophilins. However, the abilities of the immunosuppressants and their analogs to inhibit the isomerases do not correlate with their ability to cause immunosuppression. A major breakthrough in solving this puzzle was the discovery that calcineurin, the  $\text{Ca}^{2+}$ -

calmodulin-dependent protein phosphatase, binds specifically to immunophilin–ligand complexes (Liu et al., 1991). Furthermore, the ability of immunophilin–ligand complexes to inhibit calcineurin correlates well with their ability to inhibit early steps in T cell activation (C. Klee; Liu et al., 1992). Therefore, calcineurin represents the common step in the activation pathway of T cells targeted by the immunosuppressants.

In humans with malignant hyperthermia, anesthesia can induce skeletal muscle rigidity, which if not immediately reversed can lead to death (D. MacLennan, University of Toronto; MacLennan and Phillips, 1992). The corresponding disease in swine leads to stress-induced death but is not without advantages since it increases the muscle mass and the leanness of the meat. The defect has been traced to a mutation in the ryanodine-sensitive  $\text{Ca}^{2+}$  release channel in the sarcoplasmic reticulum of skeletal muscle. The single missense mutation leads to inappropriate release of  $\text{Ca}^{2+}$ , and thus even when sedentary, the pigs are exercising their muscles. A series of different mutations in the  $\text{Ca}^{2+}$ -release channel are responsible for many cases of malignant hyperthermia in humans. Identification of these mutations will further aid our understanding of the channel and allow us to screen for individuals that might be predisposed to malignant hyperthermia. Unfortunately for future American gladiators, it probably will not be useful for developing new methods of bodybuilding.

Finally, an unparalleled example of the scientific progression from human disease to identification of the genetic lesions to a molecular analysis of a protein was presented by M. Hughes (Baylor College of Medicine, Houston) and J. O'Riordan (Middlesex Hospital, London). Hereditary vitamin D-resistant rickets is an autosomal recessive disorder that results from an inability to regulate  $\text{Ca}^{2+}$  properly in the body. The symptoms are severe bone demineralization, total body loss of hair, and elevated levels of 1,25-dihydroxyvitamin D (the active form of vitamin D). The disease is caused by inherited mutations in the vitamin D receptor, a member of the steroid/thyroid family of hormone-sensitive transcription factors. The eight identified mutations fall into three classes: missense mutations in the zinc finger domain required for DNA binding; nonsense mutations that truncate the protein in or before the hormone-binding region; and one missense mutation in the hormone-binding region.

## Conclusion

Enormous progress has been made in understanding the functions of  $\text{Ca}^{2+}$  and  $\text{Ca}^{2+}$ -binding proteins. Nevertheless, we are faced with an impressive array of unanswered questions. How do the cloned  $\text{Ca}^{2+}$  channels relate to the channels defined by biophysical and pharmacological criteria? Complicated patterns of  $\text{Ca}^{2+}$  oscillations are observed: what is their significance? We have solved the structure of several intracellular  $\text{Ca}^{2+}$  receptors and have detailed structural information about the interaction between calmodulin and two target peptides. Substantial evidence suggests other targets interact differently with their receptors. How will their structures surprise us? We must

not only examine the properties of the purified proteins but also analyze them in the context of the whole cell and the whole organism. Calmodulin is very concentrated in the brain, but CaM kinase and calcineurin are present at even higher concentrations. Is there only 1 free molecule of calmodulin for every 1000 bound, as calculations suggest? What are the implications if both calmodulin and  $\text{Ca}^{2+}$  are highly buffered? The functions of  $\text{Ca}^{2+}$  receptors in muscle, liver, retina, and brain are established, but calmodulin is found in all eukaryotic cells: what is its role in proliferating cells, in developing embryos, in plants? Can calmodulin function without binding  $\text{Ca}^{2+}$ ? Analyses in genetically tractable organisms such as fungi, *Drosophila*, *Arabidopsis*, and mice will continue to be important for addressing these questions. In addition to the intracellular  $\text{Ca}^{2+}$  receptors, extracellular  $\text{Ca}^{2+}$ -binding proteins exist: what is the role of  $\text{Ca}^{2+}$  in these proteins? Is it required for signaling, for stability? The Ninth International Symposium will be held in Seattle in 1994: what additional pieces to the  $\text{Ca}^{2+}$  puzzle will be added by then?

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