# Calmodulin: A Protein for All Seasons

*Recent research suggests that calmodulin plays a central role in regulating a broad spectrum of cellular activities*

Calmodulin is hardly a household word, but in the world of cell biology it is one of the most exciting discoveries since recombinant DNA appeared on the horizon early in this decade. Calmodulin is a protein, apparently found in all nucleated cells, that plays a central role in regulating many cellular activities. Says an enthusiastic Thomas Vanaman of Duke University Medical Center, "It probably regulates everything in the cell from division to movement to secretion."

What calmodulin does is to act as an intracellular intermediary for calcium ions, which have long been implicated in the control of a broad spectrum of fundamental cell activities. For example, when a nerve or muscle cell is stimulated, or when an egg is fertilized, one of the first events to occur is a transient, but marked, increase in free intracellular calcium concentrations. The increase apparently signals the cells to produce the appropriate response, such as muscle contraction or release of the nerve's chemical transmitter. Similarly, many hormones trigger transient increases in the calcium concentrations of their target cells. Moreover, the element is involved in the control of cellular movements, including the sliding apart of the chromosomes during cell division and the beating of the flagella or cilia that propel sperm and many single-celled organisms.

In fact, the wide range of calcium's activities led Howard Rasmussen of Yale University Medical School to suggest in 1970 that the mineral is a "second messenger" that mediates the cell's responses to many stimuli, just as cyclic AMP (adenosine 3′,5′-monophosphate) is the second messenger for hormones such as glucagon and epinephrine.

Despite the extensive evidence for calcium's role in cellular regulation, however, researchers had few clues to how it produces its diverse effects. Discovery of calmodulin has now changed that.

The current, but still evolving, theory holds that calmodulin is an intracellular calcium receptor that binds to calcium ions when their concentration increases in response to a stimulus. This binding induces a distinct change in the shape of the calmodulin molecule, as a result of which the calmodulin-calcium complex becomes capable of binding to any of several enzymes. Consequently, the enzyme is activated, setting in motion the biochemical changes that produce the response to the stimulus. Not only can calmodulin affect cell activities in this direct manner, it may also do so indirectly by affecting the concentration of calcium itself and that of other important cellular regulators, including cyclic AMP.

The early history of calmodulin is complicated because the protein was discovered independently by several investigators, who, at the time, did not realize they were all working on the same protein. Some discovered it as an activator of a specific enzyme, others as a calcium-binding protein. This confusion is reflected in the fact that, for a time, calmodulin bore several names, among them calcium-dependent regulatory protein and activator protein. Eventually it became clear that the various proteins were identical. Wai Yiu (George) Cheung of St. Jude Children's Hospital suggested the name calmodulin because the protein modulates calcium's effects on the cell, and this is now the generally accepted designation.

The first enzyme to be linked to calmodulin was one of the phosphodiesterases (3′,5′-nucleotide phosphodiesterase), which breaks down cyclic nucleotides, including cyclic AMP and its cousin cyclic GMP (guanosine 3′,5′-monophosphate). The connection emerged gradually over a period of about 4 years. Some 10 years ago, Cheung discovered that a phosphodiesterase prepared from brain has very little activity unless another protein is present to activate it. At about the same time, Shiro Kakiuchi of Osaka University Medical School discovered a brain phosphodiesterase that would not work without calcium ions. This enzyme was also activated by a protein factor that turned out to be identical to the one discovered by Cheung. Jerry Wang of the University of Manitoba then showed that the activator was a calcium-binding protein and the calmodulin story was launched.

Investigators soon learned that the phosphodiesterase is not unique in its dependence on calmodulin. Charles Brostrom and Donald Wolff of Rutgers Medical School, and also Cheung, showed that the same was true for at least one form of adenylate cyclase, an enzyme that catalyzes the formation of cyclic AMP. As Brostrom points out, "We then knew that calmodulin was a multifunctional enzyme regulator."

By having the same factor stimulate both the enzyme that produces cyclic AMP and the one that breaks it down, the cell would appear to be spinning its wheels. But this is probably not the case. Brostrom and Wolff propose that the phosphodiesterase is more effective at breaking down cyclic GMP than cyclic AMP. If so, the simultaneous activation of the two enzymes could lead to an increase in the ratio of the concentration of cyclic AMP to that of cyclic GMP. Investigators have speculated that changes in this ratio may be more important for regulating some cellular activities, including cell division, than are the absolute concentrations of the nucleotides.

Alternatively, the adenylate cyclase may be turned on by lower concentrations of the calcium-calmodulin complex than those that activate the phosphodiesterase. This could cause a transient increase in cyclic AMP concentrations. Whatever the situation, calmodulin is now firmly implicated in control of the concentrations of compounds that are themselves key cellular regulators.

The effects of calcium ions and cyclic AMP overlap to a great extent. The concentrations of both are frequently altered by the same hormones and both often work on the same enzymes, with one agent either accentuating or attenuating the effects of the other. The finding that calcium, in cooperation with calmodulin, can alter cyclic AMP concentrations indicates one way in which the actions of the two regulatory agents are integrated.

In muscle, calcium is the excitation-contraction coupler—that is, the link between the stimulus and the biochemical events that produce contraction. Research from the laboratory of David


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Hartshorne at the University of Arizona suggests that calmodulin mediates this calcium effect, at least in smooth muscles such as those lining the intestines.

Contraction depends on the coming together of the two major muscle proteins, actin and myosin, to form the complex actomyosin. Activated actomyosin is an enzyme that splits adenosine triphosphate (ATP), a reaction providing the energy needed to generate the contractile force.

Although the biochemical steps producing contraction in skeletal muscle have been relatively well understood for several years, those in smooth muscle have been harder to unravel, partly because the less well-organized structure of smooth muscle cells makes them harder to study than the skeletal type. The discovery of calmodulin has cleared up some of the mystery.

According to the current picture, the interaction between actin and myosin in smooth muscle occurs following the addition of a phosphate group to certain of the protein chains comprising the myosin molecule. This phosphorylation is catalyzed by an enzyme called a myosin light chain kinase. (The kinases are enzymes that transfer a phosphate group from ATP to one or another of several potential acceptor molecules.) What Hartshorne has shown, with Renata Dabrowska of the Nencki Institute of Experimental Biology in Warsaw, Poland, is that the myosin light chain kinase is activated by calcium, but only in the presence of calmodulin. It is the calmodulin, then, that detects the calcium concentration increase caused by the stimulus and transmits the signal to the contractile machinery by turning on the kinase.

Calmodulin apparently does not play this role in skeletal muscle even though calcium is the contraction trigger there, too. Nevertheless, troponin C, the calcium-receptor protein of skeletal muscle, is structurally very similar to calmodulin. Both proteins have molecular weights of about 17,000. When Vanaman determined the sequence of amino acids in calmodulin and compared it to that in troponin C, he found that about 75 percent of the amino acids of the two proteins are either identical or chemically related. Because the amino acid sequences of calmodulin and troponin C are so similar, the two proteins probably have about the same shape when they are folded into their native three-dimensional configurations.

Calmodulin may also help to coordinate contraction with the production of the chemical energy needed by a working muscle cell. According to Philip Cohen of the University of Dundee, Scotland, the enzyme phosphorylase kinase from skeletal muscle contains four different protein chains, not just three, as was once thought. The fourth chain is none other than calmodulin. (Phosphorylase kinase is the only enzyme thus far discovered in which calmodulin appears to be a nondissociable constituent.) Phosphorylase kinase, when activated by calcium, in turn activates the enzyme phosphorylase, which breaks down glycogen. This releases glucose to be burned by the cell to produce energy for contraction in the form of ATP.

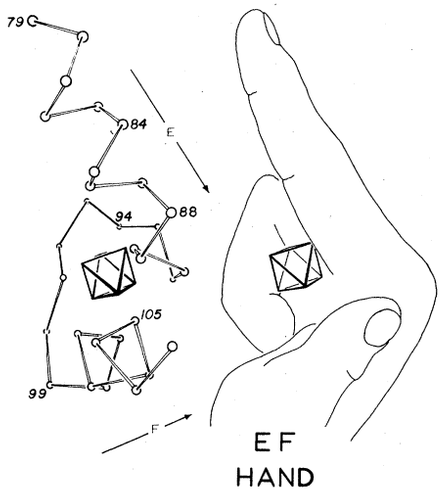


*Diagram of the "EF-hand," a basic structural unit found in many calcium-binding proteins. The F helix constitutes the extended thumb of the hand, and the E helix is the index finger. The octahedron denotes the oxygen binding site. [Source: Robert Kretsinger, University of Virginia]*

Finally, calmodulin may even facilitate the removal of calcium ions from the cytoplasm of muscle cells, thus enabling them to relax. There are indications that the protein stimulates the "pump" (actually an enzyme) that transports calcium into the sarcoplasmic reticulum (SR). The SR, which consists of an intracellular network of membranous sacs and tubes, stores calcium and is the source of the calcium ions that flow into the cytoplasm when a muscle cell is stimulated. The calcium pump of the SR removes the ions from the cytoplasm to terminate contraction. If calmodulin does stimulate this pump, then the same regulatory protein that acts to initiate contraction may help turn it off.

Studying membrane transport in simple cells, such as red blood cells, is much easier than studying it in the more complicated muscle cells. Frank Vincenzi and Thomas Hinds of the University of Washington School of Medicine and John Penniston of the Mayo Clinic have shown that calmodulin activates the calcium pump in the outer membranes of red blood cells. This pump may or may not be similar to that of the SR, but all cells are thought to have some sort of calcium pump to maintain their internal calcium concentrations at very low levels, possibly to prevent intracellular precipitation of the highly insoluble salt calcium phosphate. Although more work is needed to confirm the hypothesis, calmodulin, by activating assorted pumps, may be a major regulator of cellular calcium concentrations.

Just as muscle stimulation causes a short-lived increase of calcium ions in the cytoplasm, so does stimulation of nerve cells. This increase may mediate the release of the stimulated neuron's neurotransmitter, another phenomenon that may depend on calmodulin.

Neurotransmitter release is one of the processes in which there is overlap between the actions of calcium and of cyclic AMP. Increases in the concentration of the nucleotide in response to nerve stimulation also appear to mediate release of the nerve chemicals. According to Paul Greengard of Yale University, cyclic AMP produces many and perhaps all of its effects, in nerve and other cells, by stimulating the phosphorylation of certain cell proteins. Investigators in his laboratory, for example, have found that it stimulates the phosphorylation of two specific proteins in the membranes of nerve terminals, the site of neurotransmitter release. It does this by activating a protein kinase.

The Greengard group has now found that calcium ions stimulate the phosphorylation of the same two proteins affected by cyclic AMP. The calcium-stimulated phosphorylation requires the presence of calmodulin and appears to be another example of calmodulin activation of a kinase. "This is exciting," says Greengard, "because there is a common focus for the two regulatory systems." The discovery suggests another way in which the two regulatory agents may interact.

According to Robert DeLorenzo, also of Yale, work in his laboratory has produced direct evidence that calcium ions stimulate the release of the neurotransmitter norepinephrine from a test-tube preparation of nerve terminals, an event accompanied by the phosphorylation of terminal membrane proteins. The phosphorylation and the norepinephrine release again require calmodulin.

In addition, DeLorenzo finds that phenytoin, an anticonvulsant drug used to treat epilepsy, blocks both phosphorylation of the proteins and norepinephrine release. Although more work will be

needed to confirm the hypothesis, this finding suggests that phenytoin works by inhibiting the activation of protein phosphorylation by the calcium-calmodulin complex. Other investigators, including Benjamin Weiss of the Medical College of Pennsylvania, have evidence that some drugs used to treat mental illness interfere with the binding of calcium-calmodulin complexes to their target enzymes. As DeLorenzo points out, "A better understanding of calcium's effects means we can look for new mechanisms of drug action."

Egg fertilization is still another fundamental biological event that is accompanied by a marked increase in internal calcium ion concentrations, triggered in this case by the penetration of the first sperm. Among the consequences of this increase is the turning on of an enzyme that helps to provide energy for the synthesis of the many cell constituents needed by a rapidly dividing young embryo. Cheung and David Epel of the Hopkins Marine Station of Stanford University have found that calmodulin activates a kinase that transfers a phosphate group to NAD (nicotinamide adenine dinucleotide) to form NADP. The latter compound is an essential participant in many synthetic biochemical pathways. James Anderson and Milton Cormier of the University of Georgia had previously shown that calmodulin activates an NAD kinase in plants.

One of the outstanding puzzles of cell biology concerns cell division and how it is regulated. The discovery of calmodulin has by no means solved the puzzle, but it has provided some new leads.

Anthony Means and John Dedman of Baylor College of Medicine have linked the protein to the regulation of cell shape and mitosis. They have been using radioimmunoassay to study the distribution of calmodulin at various stages of the cell cycle. Between rounds of cell division they find the protein mainly in the cytoplasm and in filaments, composed of actin, that stretch across the cell.

Even nonmuscle cells contain actin and myosin, which are thought to be involved in such movements as changes in cell shape. The finding of calmodulin in the actin fibers is consistent with suggestions that it regulates the interaction between actin and myosin in nonmuscle cells as well as in smooth muscle.

During mitosis, the distribution of calmodulin changes as the protein becomes associated with another kind of cellular fiber, the microtubular fibers that apparently pull the duplicated chromosomes apart before the cell divides. According to Means and Dedman, calmodulin may promote the shortening of these fibers, which, many investigators think, serves to separate the chromosomes. Exactly how the calmodulin does this is as yet unclear.

A question many investigators would like to answer is, How can one protein do as many different things as calmodulin apparently does? Another aspect of calmodulin's versatility is discrimination. How does the protein "know" what it is to do at a given time?

Part of calmodulin's discriminatory ability may be simply explained by the fact that cells do not all contain the same complement of enzymes; muscle cells do not make neurotransmitters, for example. In addition, some discriminatory capacity may be built into the structure of calmodulin itself.

> "It has almost been a sort of cultural shock for me to get sucked into the calmodulin abyss."

All investigators agree that the protein has four binding sites for calcium and that the binding induces significant changes in its conformation. According to Claude Klee of the National Cancer Institute, the four sites bind calcium ions in a specific ordered sequence, with each addition producing its own conformational change. She suggests, "You may get different conformations depending on how many calciums are bound; different enzymes may then recognize various conformations." In this way, calmodulin could translate the quantitative information of varying calcium concentrations into diverse responses. Verifying Klee's hypothesis will take a detailed analysis of the complexes formed between the regulatory protein and its targets. Another possibility is that the target enzymes, some of which may be more readily activated by calmodulin than others, contribute to the discrimination.

One measure of calmodulin's regulatory importance is its ubiquitous distribution. It has been found thus far in all the nucleated cells examined, from single-celled organisms such as *Tetrahymena pyriformis* to plant cells and human brain cells. It has not yet been detected in the simpler, nonnucleated bacterial cells, but investigators think that these cells may have a counterpart.

Examination of the amino acid sequences of calmodulins obtained from various sources shows that the structure has been highly conserved during the course of evolution. Says Vanaman, who has done many of the structural comparisons, "At least throughout the entire animal kingdom, from the lowest invertebrates to the human brain, there were very few changes." He suggests that calmodulin's structure has been so carefully conserved because the protein has to be able to interact with many different enzymes and other proteins, the activities of which are essential to life.

One of the more striking features of calmodulin's structure is the presence of four internal amino acid sequences (called domains), each of which resembles the others. About 5 years ago, Robert Kretsinger of the University of Virginia determined the three-dimensional structure of another calcium-binding protein called parvalbumin. He found that this protein contained three domains, all having similar spatial arrangements of their atoms. Each domain consisted of two $\alpha$-helices, both containing about ten amino acid residues, separated by a nonhelical loop that contains the actual calcium binding site. Because the helices are arranged like the extended index finger and thumb of a hand, Kretsinger called the structure the "EF-hand." (The E and F refer to the helices of one of the parualbumin domains.) He also predicted that it would prove to be the basic structural unit of calcium-binding proteins in general, a prediction now being borne out. The four domains of calmodulin, one for each calcium bound, are EF-hands, according to Vanaman. Other calcium-binding proteins, including troponin C, also consist of domains of EF-hands.

In any event calmodulin is only one member, even if the preeminent one, of a family of related calcium-binding proteins now undergoing intensive investigation. Cell biologists are finding new roles for calmodulin and its relatives almost daily and the end is not in sight. Referring to the difficulty of keeping up with the rapid pace of developments in many different cellular systems, Vincenzi says, "It has been a sort of cultural shock for me to get sucked into the calmodulin abyss." The calcium-binding proteins are turning out to be the warp on which the complex tapestry of cell regulation is woven.—JEAN L. MARX