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Calmodulin Plays a Pivotal Role in Cellular Regulation

Wai Yiu Cheung

Summary. The role of calcium ions (Ca^{2+}) in cell functions is beginning to be unraveled at the molecular level as a result of recent research on calcium-binding proteins and particularly on calmodulin. These proteins interact reversibly with Ca^{2+} to form a protein · Ca^{2+} complex, whose activity is regulated by the cellular flux of Ca^{2+}. Many of the effects of Ca^{2+} appear to be exerted through calmodulin-regulated enzymes.

A living cell is the epitome of ingenious design; although highly complex, it functions with orderliness and efficiency. Its ability to coordinate a wide range of biological activities rests with an elaborate communications system directed toward a single goal—homeostasis and survival. Uncoordinated activities invariably lead to pathological conditions and, if not checked, to uncontrolled proliferation or cell death.

In a multicellular organism, communication poses an added complexity. Apart from coordinating its own activities, each cell must act in concert with neighboring cells. To meet the need for intercellular communication, each cell possesses a set of messenger molecules and cell-surface receptors that transduce the chemical messages into a recognizable signal. The signal either activates or inhibits a biochemical reaction that is controlled by a rate-limiting step, which is usually governed by a cellular regulator, defined here as a molecule that controls one or more critical processes.

The purpose of this article is to review the salient features of cellular regulators in general and Ca^{2+}-binding proteins in particular; emphasis is placed on calmodulin, a ubiquitous Ca^{2+}-binding protein that is emerging as an important mediator of Ca^{2+} functions in eukaryotes (*1*).

Cellular Regulators

Although the complexity of intercellular communication increases with the complexity of the organism, there does not appear to be a parallel increase in the number of cellular regulators. Hormones, cyclic nucleotides, and calcium ion are the three most important sets of regulators or messengers in mammalian systems, and their activities are interrelated; that is, the biochemical effect and metabolism of one invariably modify those of the others. This interrelationship is depicted schematically in Fig. 1.

The actions of hormones have long held the interest of endocrinologists. It was originally believed that the manifestation of hormonal effects required intact cells. In a series of pioneering studies on glycogenolysis, Sutherland and his

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colleagues demonstrated that the effect of glucagon, a peptide hormone, on glycogenolysis could be mimicked by a heat-stable, dialyzable factor (*2*). This led to the discovery of 3′,5′-adenosine monophosphate (cyclic AMP) and the enzyme systems involved in its metabolism: adenylate cyclase, which catalyzes the synthesis of cyclic AMP from adenosine triphosphate (ATP) (*3*), and 3′,5′-nucleotide phosphodiesterase, which catalyzes the hydrolysis of cyclic AMP to 5′-AMP (*4*). Adenylate cyclase is associated with the cell membrane; the enzyme is believed to harbor a hormone receptor site facing the extracellular space and a catalytic site facing the cytoplasmic space. Stimulation of the enzyme leads to an increase in cyclic AMP, which mediates the effect of the hormone. According to this concept, the hormone serves as a first messenger, whereas cyclic AMP serves as a second messenger (*5*). The second messenger hypothesis has provided the basis for a new understanding of hormone action and of cellular regulatory mechanisms.

In the mammalian cell, many but perhaps not all of the actions of cyclic AMP are mediated through cyclic AMP–dependent protein kinase (*6*), an enzyme consisting of two regulatory and two catalytic subunits; this holoenzyme is not active. When the regulatory subunits bind cyclic AMP, the catalytic subunits dissociate into free and active entities (*7*). Protein kinase catalyzes the phosphorylation of proteins, invariably at their serine residues (*8*). The phosphorylated proteins serve as effectors or modulators of various cellular reactions or physiological responses (*6*, *9*). The cascade of enzymes involved in glycogen metabolism furnish the most extensively studied and best understood model of how cyclic AMP works at the molecular level (*9*).

Calcium ion exerts a profound influence on many biological processes, such as cell motility, muscle contraction, axonal flow, cytoplasmic streaming, chromosome movement, neurotransmitter release, endocytosis, and exocytosis (*10–12*). Yet, because of the paucity of information about the Ca^{2+} receptors, the mechanism of Ca^{2+} action in most of these processes has remained obscure. Evidence acquired in this and other laboratories over the past few years suggests that calmodulin, a multifunctional, Ca^{2+}-dependent modulator protein, is a primary receptor of this important divalent cation.

In many instances—such as glycogen metabolism, muscle contraction, stimulus-division coupling, and stimulus-secretion coupling—the effects of Ca^{2+} and cyclic nucleotides are often intertwined; that is, one messenger may accentuate or attenuate the effect of the other [see (*11*)]. Of the several naturally occurring cyclic nucleotides, cyclic AMP is by far the best characterized. The physiologic function of cyclic guanosine monophosphate (cyclic GMP) is not well established, and very little is known about other cyclic nucleotides.

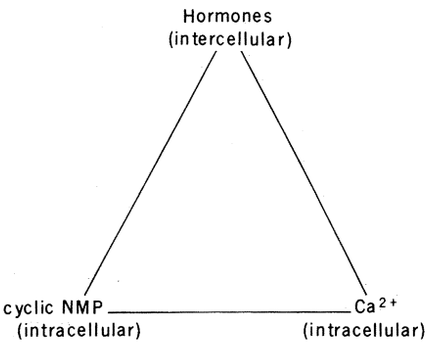

Fig. 1. The three messengers represent the major cellular regulators. Their metabolism and functions are interrelated; they have complementary roles with respect to the distance over which the signal is delivered as well as with respect to time of response and duration of action. NMP, nucleotide monophosphate.

The interplay of the metabolism and function of cyclic nucleotides and those of Ca^{2+} is widely recognized; the realization that calmodulin functions both as a mediator of Ca^{2+} functions and as a regulator of Ca^{2+}-dependent adenylate cyclase and phosphodiesterase provides a molecular link between these two classes of regulators. As is indicated in Fig. 1, the hormones essentially play the role of intercellular regulator, whereas the cyclic nucleotides and Ca^{2+} act as intracellular regulators. In other words, hormones mediate communication between cells, while cyclic nucleotides and Ca^{2+} communicate between the intracellular organelles. In addition, these messengers have a complementary role with respect to time. The response time and the duration of hormonal action range from minutes to hours, and even to days; those of cyclic nucleotides, by contrast, range from seconds to minutes, while those of Ca^{2+} are probably in the millisecond range. Thus, the three messengers complement one another not only in the distance over which the signal is delivered but also in time of response and duration of action.

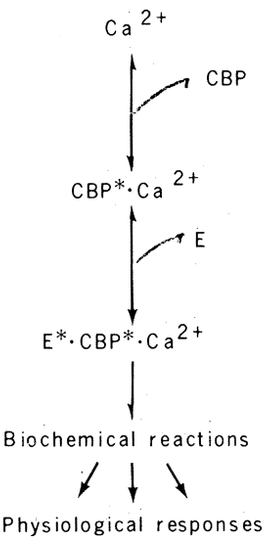

Fig. 2. Simplified scheme for the mechanism of Ca^{2+} action. CBP signifies calcium-binding proteins, E an apoenzyme or a receptor protein, and the asterisk (*) a new conformation. Stimulation of the cell increases the cellular Ca^{2+} concentration from $10^{-8}M$ or $10^{-7}M$ to $10^{-6}M$, allowing the formation of the CBP · Ca^{2+} complex, which is the active species, and regulates the activity of an appropriate enzyme. The enzyme whose activity is altered, in turn, controls some cellular processes.

Ca^{2+}-Binding Proteins as Ca^{2+} Receptors

Heilbrunn was probably the first to appreciate that Ca^{2+} serves as an important cellular regulator, when he noted some 20 years ago that the injection of a small amount of Ca^{2+} into the muscle fiber causes contraction (*13*). The regulatory function of Ca^{2+} has since been extended to a broad range of cellular reactions and processes. Although the mechanism of Ca^{2+} action in many instances is still largely unknown, the available evidence suggests that the different effects of this divalent cation are mediated through a homologous class of Ca^{2+}-binding proteins that serve as receptors for Ca^{2+} (*14*, *15*), much as the cyclic AMP– or cyclic GMP–dependent protein kinases act as receptors for their corresponding cyclic nucleotides (*9*). On the basis of evidence from numerous sources, the mode of Ca^{2+} action is outlined as in Fig. 2. The experimental basis for the scheme comes mainly from available information on calmodulin and troponin C, the Ca^{2+}-receptor subunit of the troponin system (*10*). In mammalian cells, the steady-state concentration of Ca^{2+} in cytosol ranges from 10^{-8} to $10^{-7}M$ and is the limiting factor in the above scheme. Stimulation of the cell may cause a transient increase of Ca^{2+} to $10^{-6}M$ or higher, a level sufficient to cause the Ca^{2+}-binding proteins to form an active complex with Ca^{2+}. The complex in turn combines with the target apoenzyme or effector protein to trigger a biochemical reaction, culminating in a physiological response.

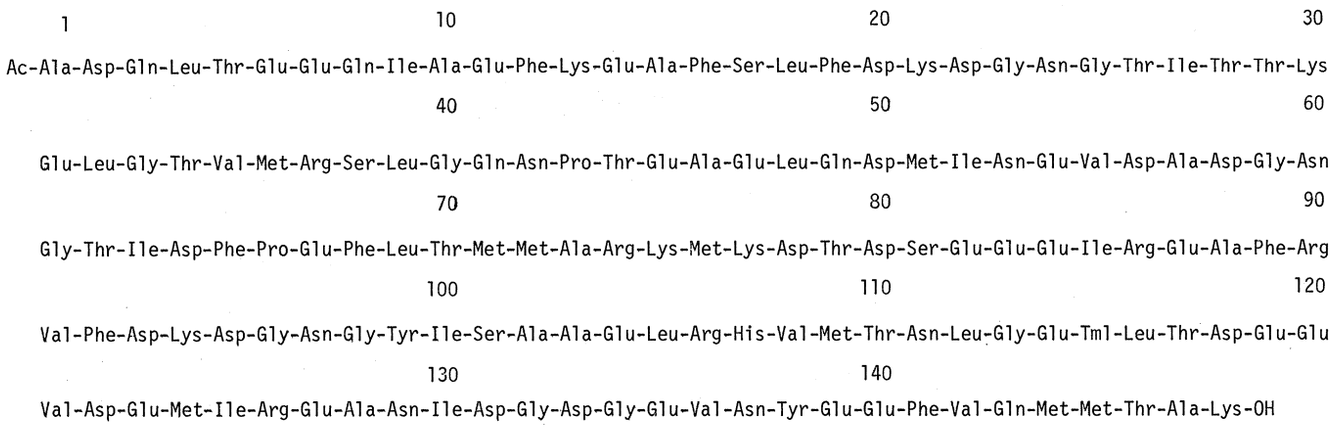

Fig. 3. The complete amino acid sequence of bovine brain calmodulin as determined by Watterson *et al.* (*26*). The protein contains many acidic amino acids, and has a trimethylated lysine (*Tml*) at position 115. However, the protein lacks cysteine, hydroxyproline, and tryptophan. The abundance of acidic amino acids furnishes carboxylate groups for the reversible binding of Ca^{2+}, while the absence of cysteine and hydroxyproline furnishes a tertiary structure highly flexible to interact with the various calmodulin-regulated proteins (*91*).

Proteins (but not nucleic acids, carbohydrates, or phospholipids) serve as receptors for Ca^{2+} because they offer carboxylate groups that bind Ca^{2+} (crystal radius, 0.099 nanometer) reversibly and selectively in the presence of millimolar Mg^{2+} (crystal radius, 0.065 nm) (*16*). Proteins have this affinity and selectivity because they can arrange six to eight oxygen atoms, each carrying partial or fully negative charge, in a helix-loop-helix structure, termed E-F band by Kretsinger, to form a cavity to harbor a cation with the radius of Ca^{2+} (*16*). Moreover, proteins afford a large potential for structural variation, a feature advantageous for transmitting allosteric information in cellular regulation. Several excellent reviews and symposiums have dealt with the roles of Ca^{2+} in cell functions (*11, 14, 15*).

Discovery of Calmodulin

The first evidence for an activator protein of 3′,5′-nucleotide phosphodiesterase was obtained serendipitously in our laboratory in the late 1960's during studies to characterize phosphodiesterase from bovine brain (*17*). At that time, I was interested in the regulatory aspects of cyclic AMP metabolism and had chosen brain as the experimental tissue because it is readily available in bulk and contains high levels of cyclic AMP metabolic enzymes. Before phosphodiesterase could be characterized in detail, a purified enzyme was needed. During the course of enzyme purification, I noted that the activity of phosphodiesterase decreased precipitously after passage of the enzyme in an anion-exchange column (*18*). The decrease could not be attributed to enzyme inactivation, as the activities of both the crude and the partially purified enzyme were quite stable. In fact, the activity of the purified enzyme could be restored to its original level if the assay was conducted with a snake venom present in the incubation mixture. Snake venom was used as a source of 5′-nucleotidase, an ancillary enzyme added to the reaction mixture to convert the product of the phosphodiesterase reaction, 5′-AMP, to adenosine and inorganic phosphate, which was measured to monitor enzyme activity (*4*). A loss of phosphodiesterase activity was observed if the assay was done with a two-stage procedure in which snake venom was added during the second stage of incubation, that is, after the phosphodiesterase activity had been terminated in the first stage. No such loss of activity was noted if the assay was done with a one-stage procedure in which snake venom was added before the enzyme assay was begun. In addition, the activity of phosphodiesterase, either in a crude homogenate or in the preparation applied to the anion-exchange column, was not affected by the type of assay performed, whether the one-stage or the two-stage procedure. Other investigators also noted a loss of phosphodiesterase activity during the course of enzyme purification (*4, 19*). The stimulatory effect of snake venom was later traced to its proteolytic activities (*17*).

The apparent loss of phosphodiesterase activity could be explained in several different ways. My experiments suggested that a cofactor required for phosphodiesterase was dissociated from the enzyme at the step of the anion-exchange column, since mixing experiments indicated that an activator, now termed calmodulin, had indeed been removed from the enzyme during the course of purification (*18*). A more detailed account of this aspect of the work has been described (*20*). The existence of calmodulin as an activator of phosphodiesterase was soon confirmed by others (*21*). Thus, the stimulatory effect of snake venom on phosphodiesterase, an observation that appears to have no immediate biological significance, provided a crucial lead to the discovery of an important biological regulator.

Biological and Biochemical Properties of Calmodulin

To characterize calmodulin and elucidate its mode of action, two experimental approaches were initiated. In one we investigated the tissue, cellular and subcellular localization of the protein as well as its developmental changes (*22*); in the other we purified the protein to homogeneity to permit physical and chemical characterization (*23*). Calmodulin was present in greater concentrations than phosphodiesterase and appeared in all tissues examined, even in those without calmodulin-dependent phosphodiesterase activity (*24*). Further, the activity of calmodulin did not parallel that of phosphodiesterase during ontogenetic development, suggesting separate genetic regulation of the two proteins (*22*). Others extended this study and demonstrated that calmodulin was present in all eukaryotes examined (*25*). Collectively, these observations strongly suggest that, besides the regulation of phosphodiesterase activity, calmodulin is involved in some basic cellular functions.

A prominent feature of calmodulin is its thermal stability and acidic nature (*18*), which have been exploited in the development of a simple procedure for

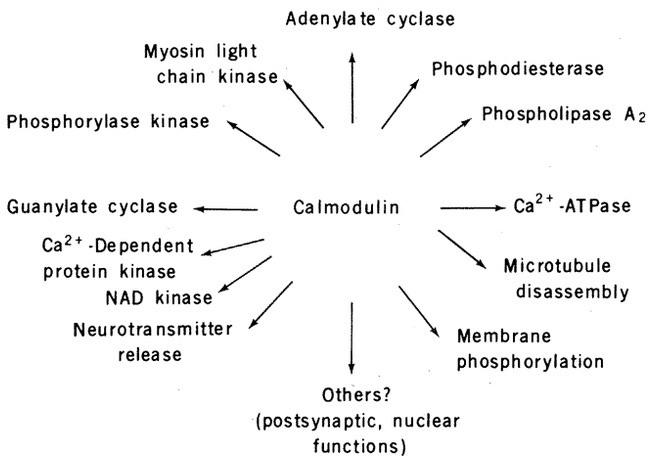

Fig. 4. Calmodulin-regulated enzymes or cellular processes. The roles of calmodulin on some of the enzymes or cellular processes listed above have not been established beyond doubt. The evidence indicating the effect of calmodulin on phopholipase A_2, guanylate cyclase, Ca^{2+}-dependent protein kinase, microtubule disassembly, and neurotransmitter release appears preliminary. The postulated roles of calmodulin in postsynaptic, nuclear, and other functions are speculative.

purifying the protein from bovine brain to apparent homogeneity (*23*). Calmodulin is a single polypeptide with a molecular weight of 16,700. Some 30 percent of its amino acids consist of aspartate and glutamate, accounting for the *p*I of 4.3. The protein contains no cysteine, hydroxyproline, or tryptophan, but it does contain a trimethylated lysine at position 115 (*26*) (Fig. 3). The lack of cysteine and hydroxyproline would allow calmodulin to assume a tertiary structure highly flexible to interact with its receptor proteins, a property not to be overlooked in view of its multifunctions. Because of its high ratio of phenylalanine (eight residues) to tyrosine (two residues), calmodulin displays a distinctive ultraviolet absorption pattern, with five peaks at 253, 259, 365, 269, and 277 nm, and a shoulder at 282 nm, a spectrum characteristic of the fine structure of phenylalanine.

Other striking features of calmodulin are its lack of tissue or species specificity and the similarity of its physicochemical properties to those of troponin C (*27*). Indeed, bovine brain calmodulin substituted effectively for troponin C in activating muscle actomyosin adenosine triphosphatase (ATPase) (*28*); moreover, about 70 percent of the amino acid sequence in calmodulin appears homologous with that of troponin C (*26*, *29*).

Teo and Wang (*30*) demonstrated that calmodulin is a calcium-binding protein. That calmodulin is a Ca^{2+}-binding protein was suggested indirectly in earlier studies on the metal requirements of phosphodiesterase. Mammalian phosphodiesterase was known to require divalent cations to express full activity (*4*). Kakiuchi and co-workers (*31*) observed that calmodulin increased the sensitivity of phosphodiesterase to Ca^{2+}. Further, when purified to a stage at which sufficient calmodulin was still retained for full activity, bovine brain phosphodiesterase contained 1 mole of Ca^{2+} per 250,000 grams of enzyme (*32*). The question therefore arose as to whether the metal was associated with the enzyme or with calmodulin, or both. The availability of pure calmodulin permitted experiments to resolve at least part of this question.

Measurements with atomic absorption spectrophotometry showed that calmodulin as purified under our conditions contained 1 mole of Ca^{2+} (*23*). This would account for the Ca^{2+} found in the partially purified phosphodiesterase (*32*). Measurement with equilibrium dialysis indicated that calmodulin contains four Ca^{2+}-binding sites, with dissociation constants ranging from 4 to 18 μM (*23*). Calmodulin from several different tissues was also found to have four Ca^{2+}-binding sites, although the dissociation constants showed considerable variation (*30*, *33*), probably because of different experimental conditions.

Molecular Mechanism of Action of Calmodulin

The mode of action of calmodulin was first established with the phosphodiesterase system (*23*, *30*, *34*). Calmodulin itself is not active; its active form is the calmodulin Ca^{2+} complex (*23*, *30*). Once bound to Ca^{2+}, calmodulin assumes a more helical conformation to become the active species (*33*, *35*), which binds reversibly to the apoenzyme of phosphodiesterase, resulting in the formation of an active holoenzyme (*36*). The sequence of events associated with the stimulation of phosphodiesterase by calmodulin may be depicted as follows:

$$(\text{calmodulin})_{\text{inactive}} + Ca^{2+} \rightleftharpoons (\text{calmodulin}^{*}\cdot Ca^{2+})_{\text{active}} \quad (1)$$

$$(E)_{\text{less active}} + (\text{calmodulin}^{*}\cdot Ca^{2+})_{\text{active}} \rightleftharpoons (E^{*}\cdot\text{calmodulin}^{*}\cdot Ca^{2+})_{\text{active}} \quad (2)$$

where E stands for the apoenzyme and the asterisk (*) for a new conformation. According to this scheme, the cellular flux of Ca^{2+} plays a crucial role in regulating phosphodiesterase activity. Subsequently, brain adenylate cyclase (*37*), erythrocyte Ca^{2+}-ATPase (*38*), myosin light chain kinase (*39*), and nicotinamide adenine dinucleotide (NAD) kinase (*40*) were found to be stimulated by calmodulin, apparently by a similar mechanism. As is shown in Fig. 4, calmodulin also regulates the activities of phospholipase A_2 (*41*), synaptosomal Ca^{2+}-ATPase (*42*), phosphorylase kinase (*43*), phosphorylation of membranes (*44*), neurotransmitter release (*45*), Ca^{2+} transport in erythrocytes and sarcoplasmic reticulum (*46*), and disassembly of microtubules (*47*). How calmodulin exerts its effect on these systems has not been determined. In the case of phosphorylase kinase, calmodulin appears to be tightly bound to the holoenzyme throughout the course of enzyme purification. Exogenous calmodulin further stimulates phosphorylase kinase activity (*43*).

Structure-Function Relation of Calmodulin

The primary structure of calmodulin appears to have been well conserved throughout the phylogenetic scale. Calmodulin from different eukaryotes shows biological cross-reactivity; moreover, an antibody directed against calmodulin from bovine brain recognizes calmodulin from cotton seed (*48*). Similarly, antibody against rat testis calmodulin cross-reacts with that from coelenterates (*Remilla reniformis*) (*49*). Comparison of the amino acid sequence of calmodulin from bovine brain with the partially completed sequence of calmodulin from rat testis (*29*) and bovine uterus (*50*) indicates that the primary structures of the three proteins are virtually identical.

Not only is the primary structure of calmodulin from phylogenically distant species highly conserved, but the protein shares a large homology with troponin C,

as well as with parvalbumin, a Ca^{2+}-binding protein found in the skeletal muscle of vertebrates (*26*). Like calmodulin, troponin C has four Ca^{2+}-binding sites. One notable feature of the primary structure of calmodulin is its internal homology. As pointed out by Vanaman and co-workers (*26*), calmodulin could be subdivided into four domains having a homologous sequence. The homology is greatest when the first domain (residues 8 to 40) is aligned with the third (residues 81 to 113), and the second domain (residues 44 to 76) is aligned with the fourth (residues 117 to 148) (Fig. 5). In the first and second domains, 18 residues are identical and 6 are conservative replacements; and in the second and fourth domains, 14 residues are identical and 11 are conservative replacements. This level of internal homology appears to be greater than that observed within the muscle troponin C.

On the basis of the crystal structure of parvalbumin and its amino acid sequence homology to troponin C, Kretsinger and Barry (*51*) have predicted the three-dimensional structure of rabbit skeletal muscle troponin C and have identified in each domain the putative calcium-binding residues. The corresponding calcium-binding residues in calmodulin are believed to be those indicated by the asterisks in Fig. 5. The regions of sequence immediately adjacent to these putative calcium-binding residues contain the appropriate hydrophobic group necessary for the helices that have been predicted to be essential for calcium-binding.

Despite the homology between calmodulin and troponin C, one significant difference exists. Calmodulin contains at position 115 a trimethylated lysine that may have a role in the multiregulatory functions. Comparative structure and function studies on bovine brain calmodulin and rabbit skeletal muscle troponin C as inhibitors of kinase-mediated phosphorylation of troponin I suggest that the third domain in calmodulin may

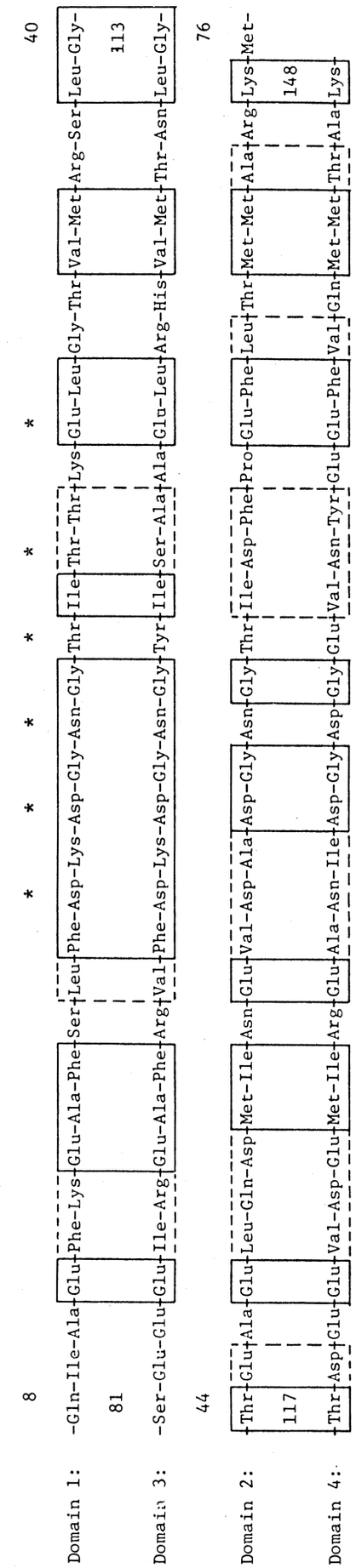

Fig. 5. Internal sequence homology in calmodulin. According to Watterson *et al.* (*26*), the amino acid sequence of bovine brain calmodulin can be divided into four homologous domains as shown. All four domains are related in sequence; the level of homology is greatest when domain 1 is aligned with domain 3 and domain 2 is aligned with domain 4. The numerals refer to positions in the calmodulin sequence. Solid line boxes indicate positions for which the two domains are identical. Dashed line boxes indicate positions for which the two domains are related by functionally conservative replacements. Asterisks indicate putative calcium-binding residues predicted by the Kretsinger model (*14*).

interact specifically with troponin I (*26*). Whether this is also the region involved in binding to other calmodulin-regulated proteins is not known.

Attempts have been made to relate the structure and function of calmodulin. One approach was chemical modification of certain selective groups. Carboxylation of the only histidine, iodination of one tyrosine, nitration of the two tyrosines, or modification of four out of six arginine residues did not affect the ability of calmodulin to stimulate phosphodiesterase. Nevertheless, modification of one or more methionine or lysine residues or blockade of several carboxyl groups resulted in partial or complete loss of activity (*52*).

A second approach was controlled tryptic digestion of calmodulin to yield peptides of various sizes. Peptide 1 (residues 1 through 77) and peptide 2 (residues 78 through 148) showed no biological activity, although peptide 2 retained the ability to undergo conformational change and to interact with troponin I in the presence of Ca^{2+}. Peptide 3 (residues 1 through 106) is 1/200th as active as the native protein. The removal of a 16-residue fragment (residues 91 through 106) from peptide 3 abolishes the remaining activity (*53*). Thus, it appears that the biological activity of calmodulin depends heavily on the integrity of the entire polypeptide.

Calmodulins isolated from widely divergent species appear to have similar, if not identical amino acid sequence, and they share in common several basic characteristics. They contain a trimethylated lysine and four Ca^{2+}-binding sites with high affinities. In the presence of Ca^{2+}, the protein undergoes a conformation change, assuming a more helical, compact structure, which is stable during electrophoresis in an alkaline polyacrylamide gel containing 8*M* urea. Further, calmodulin forms a complex with troponin I, and the complex is also stable under similar conditions (*28*). In addition, all calmodulins stimulate Ca^{2+}-dependent phosphodiesterase, and the stimulation is blocked by trifluoperazine, a phenothiazine derivative often used as an antipsychotic agent. Calmodulin binds 2 moles of trifluoperazine with a dissociation constant of $10^{-6}M$; upon binding trifluoperazine, calmodulin becomes biologically inactive (*54*).

Conceivably, the role of calmodulin in cellular function is of such fundamental importance that a mutation resulting in a gross change of its primary sequence would be lethal to the cell. Relevant to this point are the histones, the most high-

ly conserved proteins (*55*), which coat and condense the DNA of eukaryotes and are believed to regulate transcriptions (*56*).

Calmodulin and Cellular Regulation

Calmodulin-regulated enzymes. Several years after the discovery of calmodulin as an activator of phosphodiesterase, brain adenylate cyclase was shown to be regulated by this protein (*37*), as were numerous other enzymes and cellular processes summarized in Fig. 4. The enzymes listed in Fig. 4 control not only important steps in cellular metabolism, but also the metabolism of certain key regulators. For example, calmodulin governs both the synthesis and degradation of cyclic AMP, at least in the brain (*1*) and the adrenal gland (*57*). The regulation of both adenylate cyclase and phosphodiesterase by the influx of Ca^{2+} through the plasma membrane or the release of membrane-bound Ca^{2+} in response to stimuli may allow a sequential stimulation of the synthesis and subsequent degradation of cyclic AMP, resulting in a transient accumulation of cyclic AMP. However, the cytoplasmic phosphodiesterase also catalyzes the hydrolysis of cyclic GMP; in fact, at a physiological concentration of substrates, the rate of cyclic GMP hydrolysis is greater than that of cyclic AMP. The influx of Ca^{2+} could therefore result in an increase of cyclic AMP and a concomitant decrease of cyclic BMP (*1, 37*).

Phospholipase A_2, whose activity is governed by Ca^{2+}, catalyzes the deacylation of phosphoglycerides at the 2 position to give a lysophosphatide and a free fatty acid such as arachidonic acid, a precursor of prostaglandin (*58*). The steady-state level of arachidonic acid is generally low and appears to be the limiting step in prostaglandin synthesis (*59*). In human platelets, calmodulin stimulates the activity of phospholipase A_2 and thus may control the metabolism of endoperoxides and thromboxane A_2, which are important modulators of cellular function not only in platelets but also in other tissues (*60*). Phospholipase A_2 activity in all tissues requires Ca^{2+} for maximum activity (*61*); the effect of calmodulin on this important enzyme may be applicable to other tissues. In the platelet, cyclic AMP inhibits the synthesis of thromboxane A_2, presumably by acting on phospholipase A_2 (*62*). In controlling the metabolism of cyclic nucleotides and prostaglandins, calmodulin serves as a molecular link between these two classes of regulators.

Plasma membrane Ca^{2+}-ATPase is generally believed to be the Ca^{2+} pump responsible for extruding Ca^{2+} from the cytoplasmic compartment. An increase of intracellular Ca^{2+} activates the Ca^{2+}-ATPase, and increases the Ca^{2+} efflux; this action would constitute a self-regulating device for maintaining a low steady-state level of intracellular Ca^{2+}. Thus, Ca^{2+} modulates not only the activity of the Ca^{2+}-ATPase, but also its own cellular concentration (*38, 46*).

Myosin light chain kinase, which appears unique among calmodulin-regulated proteins in that its activity, like that of phosphorylase kinase, is absolutely dependent on calmodulin and catalyzes the phosphorylation of a 20,000-dalton myosin light chain. Phosphorylation of the light chain activates the actomyosin ATPase, leading to the hydrolysis of ATP and contraction of the myosin in smooth muscle or nonmuscle cells. The role of calmodulin in regulating the actomyosin ATPase of smooth muscle appears comparable to that of troponin C in regulating the actomyosin ATPase of skeletal and cardiac muscles; both calmodulin and troponin C serve as the Ca^{2+}-sensor for their respective contractile systems. Cardiac muscle also contains low activities of a calmodulin-dependent myosin light chain kinase (*63*) although its role in this tissue is not clear. Recently, calmodulin has been shown to regulate the activity of human platelet myosin light chain kinase (*39*).

Phosphorylase kinase and glycogen synthase control the degradation and synthesis of glycogen (*64*). Phosphorylase kinase consists of 16 subunits with the molecular structure of $(\alpha\beta\gamma\delta)_4$. Calmodulin has been shown to be the δ subunit, and appears to be tightly bound to the holoenzyme (*43*). This contrasts with other calmodulin-regulated enzymes, in which calmodulin is easily dissociated in the presence of EGTA, a specific chelator for Ca^{2+}. Phosphorylase kinase also acts on glycogen synthase (*65*). Phosphorylation of phosphorylase b causes an increase in activity, while that of glycogen synthase causes a decrease (*66*). In skeletal or cardiac muscle, as the concentration of Ca^{2+} increases in response to stimuli, calmodulin stimulates phosphorylase kinase while inhibiting glycogen synthase. This results in the degradation of glycogen with the simultaneous inhibition of its synthesis, an excellent example of a coordinated metabolic regulation. In the liver, the increase of cyclic AMP in response to stimulation by glucagon follows a similar concerted mechanism in the regulation of glycogen metabolism. Stimulation of cyclic AMP–dependent kinase by cyclic AMP activates phosphorylase kinase while inhibiting glycogen synthase (*66*), setting the stage for the onset of glycogenolysis and the concomitant shutoff of glycogen synthesis.

Preliminary evidence suggests that a Ca^{2+}-dependent protein kinase is present in many tissues. In the synaptosome, the enzyme catalyzes the phosphorylation of certain plasma membrane components that may be involved in the release of neurotransmitters (*45*). In other cellular systems, the enzyme may be involved in different reactions; for example, a calmodulin-dependent protein kinase stimulates a tryptophan 5-monooxygenase (*67*).

NAD kinase catalyzes the conversion of NAD to NADP (NAD phosphate); the ratio of NAD to NADP usually determines the activities of certain metabolic reactions (*68*). Calmodulin regulates NAD kinase activity in plant and sea urchin eggs (*40*), and an increase of NAD kinase activity is one of the early events following fertilization in the eggs (*69*).

Recently, Nagao *et al.* (*70*) and Suzuki *et al.* (*71*) demonstrated that calmodulin activates guanylate cyclase in a protozoan (*Tetrahymena pyriformis*). Mammalian guanylate cyclase requires Ca^{2+} for maximum activity (*72*); it remains to be shown whether the effect of Ca^{2+} on the mammalian enzyme is mediated through calmodulin.

Subcellular localization of calmodulin. Welsh *et al.* (*73*) and Anderson *et al.* (*74*), using indirect immunofluorescence, found that the antibody directed against calmodulin labels the mitotic spindle during division of certain cultured cells, indicating a possible role of calmodulin in chromosomal movement. Marcum *et al.* (*47*) observed that calmodulin regulates the assembly-disassembly of microtubules in vitro. In this study, the amount of calmodulin used in relation to tubulin appeared to be high. Wood *et al.* (*75*) noted that, in mouse basal ganglia, antibody to calmodulin labeled the postsynaptic densities and the microtubules associated with postsynaptic dendrites, suggesting that calmodulin influences postsynaptic function. This notion is supported by the finding that calmodulin is a component of postsynaptic densities isolated from rat brain (*76*).

Harper *et al.* (*77*) observed that antibody for calmodulin bound to the nucleus of rat liver and adrenal cortical cells;

and the amount of nuclear labeling in the adrenal cells varied as a function of hormonal activity. These findings suggest that calmodulin may have a role in nuclear function. Cellular and subcellular localization of calmodulin may help to clarify its other biological functions.

Other calmodulin-binding proteins. Bovine brain contains several calmodulin-binding proteins whose functions have not been determined (*78*). Two of these proteins have been purified to homogeneity. One has a molecular weight of 80,000, is heat labile, and consists of two subunits, one of 61,000 and another of 18,500. The protein (termed CaM-BP_{80}) is present in high concentrations in brain, especially in the putamen and caudate nucleus (*79*). Antibody directed against CaM-BP_{80} labels the postsynaptic densities and the dendritic microtubules in the basal ganglia, in exact parallel with the subcellular localization of calmodulin (*75*). Thus, CaM-BP_{80} may be another calmodulin-regulated protein with undetermined biological functions. The other calmodulin-binding protein, termed CaM-BP_{70}, possesses a molecular weight of 70,000 and is heat stable. Its concentration in the brain is much lower than that of CaM-BP_{80} (*78*).

CaM-BP_{80} and CaM-BP_{70} have been referred to as inhibitor protein and modulator-binding protein, respectively (*1*, *78*). The abbreviated designation of the two proteins appears advantageous in that it affords a systematic terminology with which to catalog calmodulin-binding proteins with unidentified functions. Other calmodulin-binding proteins have also been detected in the brain (*80*). Identification of these and other calmodulin-binding proteins will be important in clarifying further the functional activities of calmodulin.

Criteria for calmodulin-regulated reactions. In view of the mounting evidence that calmodulin regulates many cellular reactions, it is important to define a set of criteria to ascertain whether a given reaction is so governed. First, the tissue or cell should possess sufficient calmodulin in the appropriate locale. Second, depletion of endogenous calmodulin by appropriate means should alter the activity of the reaction in question. A corollary of this criterion is that the experimental system depleted of endogenous calmodulin should respond to exogenous calmodulin. Third, since calmodulin requires Ca^{2+} for activity, sequestering Ca^{2+} in the reaction system by an appropriate chelater, such as EGTA, should return the calmodulin-induced activity to the basal level. Fourth, calmodulin in the presence of Ca^{2+} avidly binds trifluoperazine (an antidepressant drug) (dissociation constant $K_d = 10^{-6}M$); upon binding trifluoperazine, calmodulin becomes biologically inactive. The addition of this compound should return the calmodulin-dependent activity to the steady-state level. The use of trifluoperazine may be more specific than that of EGTA to chelate Ca^{2+}, which may be required in the test reaction other than to activate calmodulin. However, a cautionary note should be added. Trifluoperazine contains a tricyclic structure, and is hydrophobic. It is important to ascertain whether its effect on the test system is due to its interaction with hydrophobic environments or to its specific action on calmodulin. Finally, the effect of calmodulin should be reversed, at least in theory, by its antibody, provided that the calmodulin in the test system is accessible to the antibody, and that the affinity of the antigen-antibody complex (approximately $K_d = 10^{-6}M$) is comparable to or greater than that of calmodulin for its receptor. In practice, however, this criterion may not be met satisfactorily. The dissociation constants of calmodulin for phosphodiesterase and for CaM-BP_{80} are in the range of 10^{-8} to $10^{-9}M$ (*78*); and antibody to calmodulin may not effectively reverse the action of calmodulin on receptor proteins with such high affinities. The use of these criteria should be judicious, as they may be subject to refinement or enlargement when more information on the properties of calmodulin becomes available.

Other Ca^{2+}-Binding Proteins

Heilbrunn and Wiercinski (*13*) first demonstrated that injection of very small quantities of Ca^{2+} into the muscle fiber produced contraction. The significance of this observation was not at once fully appreciated since the control of the actin myosin interaction did not fit the prevailing conceptual framework based on the known properties of myofibrillar proteins. Later, Weber and Ebashi and their colleagues demonstrated convincingly that the interaction of actin and myosin depended on a low concentration of Ca^{2+} and a newly discovered troponin (*81*), which consists of three subunits: troponin T (TnT), troponin I (TnI), and troponin C (TnC). Troponin T binds to tropomyosin, troponin I inhibits actomyosin ATPase, and troponin C confers Ca^{2+}-sensitivity to the contractile system. Upon binding Ca^{2+}, troponin C assumes a more helical structure; the conformational change in troponin C alters the position of tropomyosin so that actin can interact with the myosin head, resulting in ATP hydrolysis and muscle contraction, which persists until Ca^{2+} is removed. Thus, Ca^{2+} controls skeletal and cardiac muscle contraction by an allosteric mechanism in which the signal is transmitted sequentially from Ca^{2+} to troponin, tropomyosin, actin, and then myosin (*82*).

Although Ca^{2+} also regulates the contractile system in both smooth muscle and nonmuscle cells, the mechanism of regulation appears different from that in the skeletal muscle. Troponin is prob-

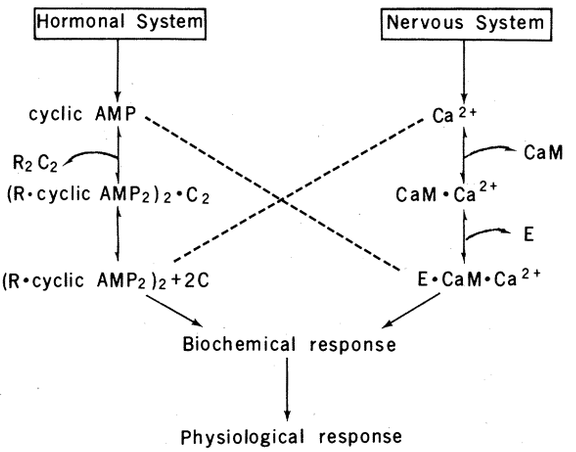

Fig. 6. Interrelations between the endocrine and nervous systems. Stimulation of adenylate cyclase by peptide hormones and certain prostaglandin increases the intracellular concentration of cyclic AMP. Upon binding cyclic AMP, the regulatory subunit (R) of protein kinase (R_2C_2) is dissociated from the catalytic subunit (C), which becomes active, and catalyzes the phosphorylation of a protein, usually at the serine residue. Phosphorylation of a protein either stimulates or inhibits its biological activity; the phosphorylated protein serves as an effector or a modulator of a cellular process. Nervous stimulation either increases the influx of Ca^{2+} or the release of Ca^{2+} from the cell membrane, or sarcoplasmic reticulum. The increase of free Ca^{2+} activates calmodulin, which forms a complex with the receptor protein to initiate a biochemical or physiological response. $E \cdot CaM \cdot Ca^{2+}$ may stand for Ca^{2+}-dependent adenylate cyclase, phosphodiesterase, or any of the calmodulin-regulated enzymes listed in Fig. 4. Calmodulin directly regulates the metabolism of cyclic AMP, which in turn could affect the availability of Ca^{2+} (*90*). Thus, one system can act independently of, in concert with, or in opposition to the effect of the other. For details, see the text.

ably not present in either smooth muscle or nonmuscle cells; the purported finding of troponin C in these tissues may be explained on the basis of the similar characteristics of troponin C to those of calmodulin. Recent studies of smooth muscle or nonmuscle cells suggest that contraction is regulated by a light chain kinase, which is activated by calmodulin in the presence of Ca^{2+}. Activation of the kinase enhances the activity of actomyosin ATPase and the contraction of the myosin system (*39*).

Parvalbumin, a sarcoplasmic protein found in the skeletal muscle of lower vertebrates and of mammals, is another Ca^{2+}-binding protein with potential importance as a cellular regulator. The protein is acidic, and has a molecular weight of 12,000. It is present in tissue in the millimolar range, which is probably adequate to bind all the Ca^{2+} in the sarcoplasm. Although parvalbumin is thought to be important in regulating muscular activity, direct evidence to support this notion is lacking (*83*).

Other Ca^{2+}-binding proteins, such as those dependent on vitamin D and vitamin K, are likewise potential regulators. Vitamin D-dependent Ca^{2+}-binding protein is important in Ca^{2+} translocation in the intestine (*84*); vitamin K-dependent Ca^{2+}-binding protein is required for a posttranslational conversion of precursors of prothrombin in liver microsomes to the plasma form which binds Ca^{2+} (*84*). Some other Ca^{2+}-binding proteins are aequorin (*85*), spasmin (*86*), S-100 (*87*), and calsequestrin (*88*).

Of the Ca^{2+}-binding proteins so far studied, calmodulin is the most widely distributed. The highly conserved structure observed in all the calmodulins that have been isolated from phylogenetically diverse species suggests that the molecule is probably the most ancient in the family of Ca^{2+}-binding proteins.

Integration of Cellular Regulation by Messengers

It has been pointed out that the effect of cyclic AMP and that of Ca^{2+} are interrelated. As Fig. 6 shows, the two systems can now be integrated on a molecular basis. Most, if not all, of the effects of cyclic AMP in the mammalian system are mediated through cyclic AMP-dependent protein kinases. The functioning of the nervous system, on the other hand, is closely related with the metabolism of Ca^{2+}, whose activity is mediated through calcium-binding proteins. As I have indicated in this article, many of these functions are mediated through calmodulin. Perhaps one of the least studied calmodulin-regulated enzymes is the Ca^{2+}-dependent protein kinase, which may govern a wide variety of cellular reactions. Numerous investigations have implied that the function and metabolism of cyclic AMP, prostaglandins, and Ca^{2+} are related. The three systems may function independently, or they may modulate or antagonize the effect of any of the others (*11, 59, 89*). In serving as a Ca^{2+} receptor, calmodulin integrates the major communications systems in the eukaryotes.

Conclusions

The many effects of Ca^{2+} in cell function have been appreciated and extensively studied in the past 30 years, but the mechanism of action is only beginning to be unraveled, thanks to the research on calmodulin and other Ca^{2+}-binding proteins, which have provided a framework for understanding the action at the molecular level. In itself Ca^{2+} is not the active species; upon interacting with a receptor protein, Ca^{2+} interacts with the protein to form an active complex. Thus Ca^{2+}-binding proteins are all potential receptors of Ca^{2+}, mediating its effect in cellular reactions.

With respect to the regulatory aspects of cellular functions, many problems remain to be solved. For example: (i) the functions of the unidentified calmodulin-binding proteins; (ii) the identification of other Ca^{2+} receptors and their functions; (iii) the factors affecting the level of cellular Ca^{2+}; (iv) whether calmodulin, present in high levels in some tissues, regulates the free Ca^{2+} by binding it; (v) the factors affecting the release of bound Ca^{2+} from calmodulin and those determining the specificity of Ca^{2+} effects; (vi) the molecular interrelation between Ca^{2+}, the cyclic nucleotides, and the hormones including the prostaglandins; (vii) whether the different enzymes, regulated by calmodulin contain a common domain at which they interact with calmodulin; and (viii) whether calmodulin also assumes a common tertiary structure in interacting with its various receptor proteins. As important cellular regulators, calcium-binding proteins should continue to be an active area of research in biology. Of the calcium-binding proteins known to act as cellular regulators, calmodulin, which is the most widely distributed, emerges with a pivotal role in mediating the Ca^{2+} functions in eukaryotes.

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91. Abbreviations for the amino acid residues are as follows: Ala, alanine; Arg, arginine; Asp, aspartic acid; Asn, asparagine; C, cysteine; Glu, glutamic acid; Gln, glutamine; Gly, glycine; His, histidine, Ile, isoleucine; Leu, leucine; Lys, lysine; Met, methionine; Phe, phenylalanine; Pro, proline; Ser, serine; Thr, threonine; Trp, tryptophan; Tyr, tyrosine; Val, valine.
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