Postreceptor Signal Transduction by Cyclic Adenosine Monophosphate and the Ca^{2+}-Calmodulin Complex

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To survive in a dynamic environment, all living cells must be able to identify and respond to variations in specific extracellular signals. Peptide hormones are one type of signal to which mammalian cells respond. Cells recognize hormones through specific receptors on the outer surface of the plasma membrane. The binding of a hormone to its receptor initiates a series of rapid events that eventually translate this external signal into a specific cellular response mediated by a selective alteration of intracellular metabolism. These metabolic responses include ion flux, enzyme activity, RNA and protein synthesis, steroid synthesis and secretion, protein secretion, cell division, cell motility, and cell-to-cell communication. The mechanism by which the extracellular event is translated into these intracellular events is not completely understood. However, it is now well accepted that virtually all of the intracellular events triggered by peptide hormones are related to changes in the concentration of two "second messengers" namely, cyclic adenosine monophosphate (cAMP)[1] and Ca^{2+}. The mechanisms of action of these intracellular messengers are in some ways similar. Nonmuscle cells possess a single receptor for each of these two messengers. Calmodulin constitutes the primary receptor for Ca^{2+} (1, 2), whereas the regulatory subunits of cAMP-dependent protein kinase are the predominant cAMP-binding proteins in eukaryotic cells (3, 4). The interaction of Ca^{2+} with calmodulin causes a conformational change in this protein so that it can interact with a variety of enzymes to produce a stimulation (5–7). Thus, calmodulin can be considered a receptor, and the enzymes regulated by the Ca^{2+}-binding protein can be considered as "acceptor" proteins. Similarly, for the cyclic nucleotide system, the binding of cyclic nucleotides to the regulatory components of protein kinase produces a conformational change that, in all cases, is accompanied by phosphorylation. These events result in the release of catalytic activity. Catalytic activity can also be regulated by the presence of low molecular weight protein kinase inhibitors. These protein inhibitors have been found in virtually all eukaryotic cells and are competitive inhibitors for the substrates of the catalytic subunit of the cAMP-dependent protein kinase (8–10). The purpose of this article is to review the contributions made by the workers in our laboratory to our knowledge of the mechanisms by which cAMP and the Ca^{2+}-calmodulin complex affect the regulation of intracellular events related to motility.

Regulation of Motility

It has been proposed that both cAMP and Ca^{2+} are involved in the regulation of the secretion of specific products from cells. In many instances the effects of these two intracellular messengers appear to be antagonistic, but additive or synergistic effects of Ca^{2+} and cAMP have also been reported (see references 1 and 4 for reviews). At least two components of the cellular cytoskeleton have also been implicated in the secretory process. Microtubules constitute a network that defines and orients the flow of secretory granules from their site of synthesis and packaging in the Golgi apparatus to the site of discharge at the plasma membrane. Microfilament bundles could provide the motive force for the fusion of the granules to the plasma membrane and/or exocytosis of the products. Calmodulin-binding proteins have been identified on secretory granules (11) and as components of the actin-binding proteins associated with the plasma membrane (12). The interaction of Ca^{2+} and cAMP in the control of motility and secretion can be appreciated by consideration of the intracellular localization of the structural and regulatory molecules involved. Calmodulin, myosin light chain kinase, and myosin light chains (LC_{20}) are localized on actin-containing microfilament bundles in interphase cells (1, 13, 14). Protein kinase inhibitor (15), the regulatory (16) and catalytic (17) components of cAMP-dependent protein kinase, and tubulin (18) are all associated with microtubules.

Calcium binds to calmodulin, allowing interaction of this complex with myosin light chain kinase. Phosphorylation of myosin light chains by this activated enzyme promotes a conformational change that allows stimulation of myosin ATPase. Hydrolysis of ATP provides the energy necessary for tension development and motility (19). Cyclic AMP binds to the regulatory component of cAMP-dependent protein kinase, releasing the active catalytic subunit (3). This enzyme phosphorylates the α- and β subunits of tubulin, resulting in the enhanced polymerization of microtubules in interphase cells (20) or flagellar motility (in spermatozoa) (21). Both

[1] *Abbreviations used in this paper:* cAMP, cyclic adenosine monophosphate; FSH, follicle-stimulating hormone.

THE JOURNAL OF CELL BIOLOGY · VOLUME 99 NO. 1 PT. 2 JULY 1984 226s–231s

contractility in response to Ca^{2+} and motility in response to cAMP are positive reactions resulting from stimulation of microfilaments or microtubules, respectively. On the other hand, the Ca^{2+}-calmodulin complex can cause a depolymerization of microtubules. This effect has been observed in vitro, (22) in vivo after the microinjection of calmodulin into living cells (23), and in situ in a lysed cell system (20). Ca^{2+}-calmodulin also attenuates the stimulatory effect of cAMP on sperm motility. Calmodulin can be found associated with cytoplasmic microtubules (24) as well as microfilaments (5). These associations are Ca^{2+} independent, whereas the regulation of enzyme activities on these organelles requires Ca^{2+} bound to calmodulin. Thus, calmodulin can serve a structural role by binding to other proteins in the absence of Ca^{2+} and yet still respond to alterations in Ca^{2+} concentration by undergoing a conformational change and activating specific enzymes. Finally, phosphorylation of the myosin light chain kinase by cAMP-dependent protein kinase markedly decreases the affinity of this enzyme for Ca^{2+}-calmodulin (25). This reaction could be one mechanism by which cAMP antagonizes at least one positive effect of Ca^{2+}. Together, these observations suggest that cell motility, as exemplified by exocytosis, is regulated by the ratio of cAMP to Ca^{2+}-calmodulin in the cytoplasm.

Regulation of Energy Production

It is generally accepted that protein phosphorylation is the major common mechanism by which intracellular events in eukaryotic cells are controlled by external physiologic stimuli. Whereas calcium and cAMP enjoy widespread acceptance as the two primary mediators of ligands that bind to cell surface receptors, it is only recently that the idea has been considered that widely divergent cellular processes are regulated by common phosphorylation-dephosphorylation reactions (4). We have offered one explanation of how the common mechanisms apply to the regulation of motility and contractility. One point still to be considered is how nonmuscle cells supply the energy required for motile responses mediated by calcium and cAMP. Glycogen is the primary source of energy for muscle contraction. Synthesis and degradation of this complex carbohydrate are regulated by the hormones epinephrine and insulin as well as by the contractile state of the tissue. Regulation is through changes in the phosphorylation state of glycogen phosphorylase and glycogen synthase. These changes are subserved by a discrete number of protein kinases, protein phosphatases, and regulatory components. In every case, the regulatory components are modulated by cAMP and calcium.

Evidence is now emerging that the energy required for motility in nonmuscle cells is generated in a manner analogous to energy generation in muscle. The rat testis Sertoli cell contains glycogen, glycogen synthetase, phosphorylase kinase, and phosphorylase (26). The peptide hormone follicle-stimulating hormone (FSH) markedly alters the phosphorylase activity ratio, leading to glycogen breakdown and energy production. The phosphorylase in the Sertoli cell is activated by an increase in the intracellular concentration of either cAMP or calcium. The mechanism includes conversion of inactive phosphorylase *b* to active phosphorylase *a* by phosphorylation. The converting enzyme phosphorylase kinase also is activated by phosphorylation. In the Sertoli cell (as in muscle) this phosphorylation occurs via the catalytic subunit of cAMP-dependent protein kinase released in response to FSH-induced cAMP accumulation. In addition, increases in intracellular calcium also stimulate phosphorylase kinase in a manner mediated by interaction with a constitutive calmodulin subunit (4).

Glycogen-multienzyme complexes found in muscle consist not only of the polymer and the enzymes directly involved in glycogen synthesis and degradation but also of the enzymes necessary to degrade glucose to lactate (27). These complexes have been found by electron microscopy to be localized in muscle at the I band of the sarcomere and are thus in close proximity to the contractile elements. FSH not only stimulates glycogen breakdown in the Sertoli cell but also stimulates conversion of glucose to lactate and pyruvate, which are the preferred substrates for the surrounding germ cells (28, 29). Therefore, ths glycolytic enzymes are also present in the Sertoli cell and are regulated by changes in cAMP and calcium. The structure analogous to the muscle sarcomere in nonmuscle cells is the microfilament bundle. These structures contain actin, myosin, and all of the regulatory proteins required to control contractility. Phosphorylase has recently been shown by indirect immunofluorescence microscopy to be associated with the stress fibers in Sertoli cells (26). In addition, this localization was abolished by incubation of the cells with α-amylase, which suggests the association of phosphorylase with glycogen particles.

The localization of phosphorylase on Sertoli cell stress fibers could be a mechanism by which the two mediators of postreceptor signal transduction, cAMP and calcium, regulate the production of ATP required for myosin ATPase. The localization of a metabolic complex at the site of energy utilization provides an efficient mechanism for coupling an energy-requiring motile structure with an energy-producing system. Even though glycogen may provide a small proportion of the total energy requirements for nonmuscle cells such as the Sertoli cell, its localization on stress fibers might permit these cells to reserve this energy for specific functions associated with cytoskeletal movement. In this way the Sertoli cell could rapidly respond to changes in cAMP or Ca^{2+} by microfilament-induced shape changes and protein secretion.

Involvement of Calmodulin in the Regulation of Cell Proliferation

The information summarized in the preceding sections suggests that changes in the intracellular levels of calmodulin also are important in the regulation of cell function by Ca^{2+}. The first series of observations in this regard was somewhat disappointing, because the expression of calmodulin levels, even in target cells for both peptide and steroid hormones, appears to be constitutive (1, 2, 30). However, calmodulin levels are 2–10 times higher in several transformed cells (1, 2, 30, 31). This increase is regulated at the level of synthesis and not degradation and occurs regardless of the nature of the transforming agent (31). Thus, elevated levels of calmodulin, determined by radioimmunoassay, have been reported in cells transformed by RNA and DNA oncogenic viruses, chemical carcinogens, or hormones (31). Calmodulin has also been shown to be synthesized in a cell cycle-dependent manner. Chafouleas et al. (32) found that calmodulin is increased by a factor of two at the G_1/S boundary of Chinese hamster ovary cells. Moreover, treatment of these cells in exponential growth with the anticalmodulin drug W13 results in the selective but reversible inhibition of the progression through

the S phase. Further experiments revealed that this inhibition of DNA synthesis was due to a single W13-induced block at the G_1/S boundary. The drug had no effect on progression through G_1, G_2, or M.

The calmodulin concentration also seems to be important for the reentry of quiescent (plateau or G_0) cells into the cell cycle. Chafouleas et al. (33) have presented data indicating that calmodulin plays a role in at least two distinct phases during reentry of cells from the plateau phase into the cell cycle. First, radioimmunoassay has shown that release from plateau is associated with a decrease in calmodulin content within the first hour. This rapidly occurring decrease results in a calmodulin content similar to that found in G_1 cells after cytokinesis and appears to be linked to the calmodulin mRNA concentration in that an identical decrease in the calmodulin mRNA also occurs upon release. The early decrease in intracellular calmodulin is intrinsic to reentry into the cell cycle, because it is observed when the cells are released by two different methods. Indeed, these changes appear to be highly correlated with reentry into the cell cycle, because the magnitude of the change in calmodulin appears to be proportional to the degree of synchrony of the released cultures. On the other hand, analysis of β-actin mRNA has revealed no decrease in content upon release, regardless of the method employed. Thus, the decrease in mRNA appears to be selective for calmodulin. The involvement of calmodulin early in quiescent cell release into the cell cycle has also been substantiated by the observation that cells released directly into medium containing W13 are markedly inhibited in their ability to progress into S phase. However, this block is reversible and cells begin to enter S phase 8–10 h after removal of the drug. This lag period is exactly the same length for the nontreated (control) cultures upon plateau release, which is again consistent with a W13-induced block at a very early stage in plateau release.

The mechanism by which calmodulin levels are rapidly reduced during plateau reentry is unknown, but it may be secondary to the rate of transcription of the single calmodulin gene. It is known that mRNA degradation is coupled with rate of transcription in the case of the histones (34). Unfortunately, this is not readily apparent when mRNA degradation is coupled to protein degradation in such an acute fashion.

The second phase of plateau release in which calmodulin appears to be involved occurs approximately 5–8 h after release. During this period there is a rapid increase in calmodulin levels associated with the entry into S phase. This pattern is similar to that observed during normal cell-cycle progression in Chinese hamster ovary cells (32) and occurs at a rate compatible with the synthetic rate constants determined for the protein by Chafouleas et al. (31). This change in protein level is preceded by an increase in calmodulin mRNA. The relative importance of calmodulin in this phase of reentry was also revealed by the studies with W13. Cultures appear to be susceptible to W13 treatment for at least the first 5 h of plateau release. However, in contrast to treatment at 0 time, an increasing number of cells enter S phase when treatments are initiated further from release. Although cells enter S phase, no progression through this phase can occur in the presence of the drug. Analysis by flow cytometry have revealed that this inhibition of S phase progression is due to a block at early S phase and not to a change in the transit time through S phase. This effect of W13 on progression into and through S phase in the plateau-released cells is similar to that observed for cells cycling normally (32). It would appear that this event is similar to the normal G_1/S transition.

The involvement of calmodulin in two distinct areas in plateau release is further supported by W13 reversal studies. When cultures are treated with W13 5 h after release, some of the cells enter but do not progress through S phase. Removal of the drug appears to reinstate progression in two distinct populations of cells. First, the cells that are blocked early in S phase progress through the S phase. This is then followed by the synchronous entry into S phase of a second population of cells initially blocked in G_0/G_1. This subsequent progression into S phase occurs 8–10 h after removal of the drug and is identical to that observed for the cultures treated at 0 h. Removal of the drug 5 h later only shifts this labeling index profile to the right by exactly 5 h. These data are consistent with reports of the existence of either two (35) or three (36) steps in the G_0–G_1–S transition. In addition, recent studies (37–40) performed on serum-deprived 3T3 cells have demonstrated that reentry into the cell cycle can be broken down into at least two distinct steps. The first is induction of competence. Cells that have been rendered competent by such mitogenic agents as platelet-derived growth factor are primed for the next step, progression into S phase. Progression is mediated by other growth factors and can only occur in competent cells. Calmodulin may be involved in both of these steps.

Recent experimental results obtained in our laboratory (40a) can be interpreted as suggesting that the increase in calmodulin at G_1/S is important for optimal DNA repair before replicative DNA synthesis. Bleomycin is known to cause DNA damage by defined pathways. Concentrations of this drug that result in potentially lethal damage to tissue culture cells can be readily determined. Under such conditions, ~90% of the cells are killed. The remaining 10%, however, can recover from the drug by repairing DNA and eventually repopulate the culture dish. If cells are selected for potentially lethal damage and released from bleomycin into media containing W13 (but not W12), all the cells are killed within 3 h. The most obvious explanation of these results is that calmodulin is required for DNA repair. When calmodulin is neutralized with W13, cells cannot repair the DNA damage caused by bleomycin. This results in cell death during the subsequent replicative phase. However, the possibility existed that the effect of W13 was not mediated via calmodulin. Therefore, we evaluated the effects of this drug on growth and DNA repair in bacteria. No effect of W13 was noted on the growth of *Escherichia coli*, even at concentrations of up to 200 μg/ml. Moreover, when bleomycin was used to induce DNA damage, W13 had no potentiating effect. Because prokaryotes do not contain calmodulin, this suggests that the effects of W13 seen in Chinese hamster ovary cells were due to inhibition of calmodulin activities.

A more direct approach to the postulate that calmodulin is involved in DNA repair was to examine the effects of W13 on recovery from bleomycin-induced damage by the nucleoid sedimentation assay described by Durkacz et al. (41). Nucleoids are defined as nuclei treated with high salt to remove the histones. Dispersion of the chromatin results in a characteristic sedimentation when it is centrifuged through a 15–30% sucrose gradient. Damage to DNA results in a more dispersed chromatin and, consequently, the nucleoids sediment more slowly. The difference in the distance migrated between nucleoids from control and experimentally damaged

cells is a measure of the degree of DNA breakage. The rate and extent of recovery from such damage is related to the ability of the cells to repair the DNA. W13 completely prevents recovery from bleomycin-induced DNA damage, which is further evidence that calmodulin is involved in the repair process.

Calmodulin-binding Proteins Assessed by Gel Overlay

If calmodulin is involved in DNA repair, then some of the enzymes involved in purine and/or pyrimidine metabolism (or that of other proteins involved in DNA repair) must be regulated by calmodulin. It is known that such enzymes are induced at the G_1/S boundary, as is the case for calmodulin. The correlate of this hypothesis is that specific calmodulin-binding proteins also are induced before the G_1/S boundary in both normally cycling cells and in G_0 cells stimulated to reenter the cell cycle. We have evaluated such changes in calmodulin-binding protein during plateau reentry. The calmodulin-binding proteins were detected by the binding of ^{125}I-calmodulin to cellular proteins distributed on polyacrylamide gels (42, 43). By the inclusion of M_r standards it was revealed that calmodulin could bind directly to glycogen phosphorylase assessed by the gel overlay technique. Because of the results of earlier studies on the Ca^{2+} regulation of phosphorylase (4) and the fact that it colocalizes with calmodulin (as determined by immunofluorescence microscopy) (26) this fortuitous observation was further investigated.

The amount of calmodulin that bound to purified phosphorylase was compared with that that interacted with purified myosin light chain kinase. Conditions were manipulated in such a way that the amount of ^{125}I-calmodulin bound to each enzyme was directly proportional to the amount of protein applied to the gel. Quantitation of the data obtained revealed that phosphorylase and myosin kinase bound equal amounts of calmodulin on a molar basis. In both cases the binding was Ca^{2+} dependent. These similarities suggested the possibility that calmodulin directly interacts with phosphorylase in a Ca^{2+}-dependent manner in vitro, as is the case in the regulation of the activity of myosin kinase (14, 25).

Indeed, calmodulin activates both phosphorylase *a* and *b* at a limiting concentration of either of the substrates glycogen or glucose-1-phosphate. However, the affinity for calmodulin is 1,000 times lower than that of myosin light chain kinase, and activation is not Ca^{2+} dependent. The activation of phosphorylase is also not protein specific and can be mimicked by other molecules, as diverse as troponin C and bovine serum albumin, which exhibit similar binding constants. These experiments illustrate the limitation of a correlation of the characteristics of ^{125}I-calmodulin binding to a protein as determined by the gel overlay technique with those assessed by an independent assay, such as in vitro enzyme activation. The properties predicted from the observation of calmodulin interaction with proteins distributed on polyacrylamide gels may, therefore, be quite different from those found when binding is assessed by other independent techniques. Caution should be exercised in interpreting the results of gel overlay determinations if physiological relevance is the goal.

On the other hand, the gel overlay technique is a convenient method by which to analyze a subset of protein in a complex protein composition such as that in a total cell extract. Application of this procedure to Chinese hamster ovary cells in plateau phase revealed approximately 20 proteins. Some of these proteins bound ^{125}I-calmodulin only in the presence of Ca^{2+}, some bound in a qualitatively similar manner, regardless of the Ca^{2+} concentration, and some were detected only in the presence of EGTA. No demonstrable quantitative changes occurred within the first 6 h after plateau release. Subsequently, however, proteins of four types were observed. Two of these exhibited Ca^{2+}-dependent and two Ca^{2+}-independent ^{125}I-calmodulin binding. The changes can be illustrated by the following examples: (*a*) a 58,000-M_r Ca^{2+}-dependent protein that exhibited changes similar to those of calmodulin (33); (*b*) a 51,000-M_r Ca^{2+}-dependent species that remained unchanged for 18 h after release; (*c*) a 47,000-M_r protein whose binding was independent of Ca^{2+} (this protein showed a continuous increase during reentry, reaching a level 10 times that present in quiescent cells 18 h after release); and (*d*) a 28,000-M_r Ca^{2+}-independent protein that began to decrease in amount until by 12 h after the mitogenic signal it was no longer detectable. The identity and functional role of these proteins are unknown. However, it is obvious that dynamic changes in the protein composition of cells occur during plateau reentry. Because the changes in the amount of the 58,000-M_r protein were identical to those observed for calmodulin, this molecule likely is a calmodulin-dependent protein whose synthesis also is regulated at the G_1/S boundary. Future studies will be designed to evaluate this interesting possibility.

Isolation and Characterization of Nucleic Acids Encoding Calmodulin

The experiments summarized in the preceding sections yielded data suggesting that cellular calmodulin levels could be altered either by transformation or during the cell cycle at the G_1/S boundary. Because the transformed cell types used in our study had a shorter cell cycle time than their nontransformed counterparts (31), it is possible that the primary intracellular regulation of calmodulin is cell cycle dependent. To continue our search for the mechanism controlling cell calmodulin level, we turned our attention to the cloning of calmodulin cDNAs and genes. Because of the high concentration of calmodulin in the electroplax of the electric eel, we used this tissue as a source of calmodulin mRNA. The first cloned cDNA encoded the C-terminal 35% of calmodulin (amino acids 93–148) and 182 nucleotides of the 3′ noncoding region (44). This recombinant molecule (pCM109) was then used to select a clone shown by DNA sequencing to contain 26 nucleotides of the 5′ nontranslated region, the entire coding region, and 408 nucleotides of the 3′ noncoding region (45). The noncoding region contained two polyadenylation signals 280 nucleotides apart that both gave rise to functional mRNAs. We concluded that a single 5,500-nucleotide primary transcript was used to produce three functional mRNA molecules 2,000, 1,100, and 820 nucleotides in length.

The full-length eel clone pCM116 was used to select calmodulin sequences from a cDNA library prepared from chicken brain mRNA and from a chicken genomic library constructed in λ phage. Two overlapping cDNA clones were sequenced and found to contain the entire coding region of chicken calmodulin, together with 94 nucleotides of the 5′ nontranslated and 857 nucleotides of the 3′ nontranslated regions (46). The chicken cDNA clones were used to isolate calmodulin gene sequences from the phage library. We now

have sequenced the entire gene from the ATG through the transcription termination site. The gene is single copy and contains at least six intervening sequences. The first of these introns separates the ATG from the first nucleotide of the codon, which is translated into the N-terminal amino acid of calmodulin (ala). The remaining introns are positioned as follows: II between aa 10 and 11; III between aa 56 and 57; IV between aa 82 and 83; V between aa 100 and 101; and VI between aa 139 and 140. Intervening sequences III, V, and VI interrupt Ca^{2+}-binding sites I, II, and IV, respectively, whereas intron IV occurs in the linker that serves to divide the two homologous halves of the protein. We have used a 70-nucleotide end-labeled fragment of the chicken cDNA containing exclusively the 5′ noncoding sequence to screen a genomic cosmid library. We have isolated an additional recombinant containing 44 Kb of DNA, with 7.2 Kb being 5′ to the 5′ terminus of our original λ clone (CL-1). This additional λ clone (CL-2) is currently being evaluated for the presence of the remaining 5′ nontranslated region of the chicken calmodulin gene.

A second calmodulin-related DNA sequence was obtained by screening the chicken genomic library with the partial eel clone pCM109 under relaxed conditions (60°C; 6x SSC). This molecule, CM-1, is a single-copy gene that encodes a 148-amino acid protein that bears an 87% sequence homology with chicken calmodulin (47). This gene does not contain intervening sequences but does possess a TATA box, an ATG initiation codon, is in open reading frame, and has a single polyadenylation signal 486 nucleotides from the TGA termination codon. This gene is flanked both 5′ and 3′ by a nine-base pair direct repeat. These properties are similar to those attributed to so-called processed genes. If this is true, CM-1 is a reverse transcribed copy of the CL-1 processed transcript. This copy, lacking introns, would be inserted back into the genome. Hybridization analysis of poly A^+ RNA reveals the presence of an mRNA in several chicken tissues that is complementary to CM-1 (47). Such data are compatible with the tissue specific expression of a processed calmodulin gene and predict the presence of a calmodulin-like protein that differs in 19 of the 148 amino acids from calmodulin.

Construction of Prokaryotic Expression Vectors

If the calmodulin-like gene CM-1 is expressed in a tissue-selective manner, the encoded protein would be the first calmodulin-like molecule to be demonstrated in a vertebrate species. As a prelude to finding out whether this is true, we decided to develop recombinant DNA molecules capable of synthesizing the protein encoded by CM-1. Methods developed to purify the protein from bacterial extracts could then be applied directly to chicken tissues. In addition, an antibody could be produced that would selectively recognize CM-1 protein (but not authentic calmodulin) and could be used to localize this molecule in muscle as well as in other tissues and cells. Even if CM-1 protein is not expressed in vivo, the bacterially produced protein would be useful in determining which domains of calmodulin are involved in which of its many proposed functions. The protein encoded by CM-1 would have amino acid substitutions in Ca^{2+} binding domains I, II, and IV (47). By taking advantage of common restriction endonuclease sites in the three naturally occurring nucleic acids available to us (45–47), we can engineer three additional calmodulin-like proteins with specific changes introduced into (*a*) domain I; (*b*) domain II; and (*c*) domains II and IV. Finally, bacterial expression vectors will allow us to metabolically label calmodulin or the calmodulin-like proteins with any isotope or radioactive amino acid desired. In this manner, direct binding studies of unmodified proteins can be carried out in vivo, in vitro, or using the gel overlay procedure.

A strategy was devised to construct an expression vector for calmodulin using the chicken cDNA (46), a pUC8 vector (48), and a Tac promoter (49) as the DNA components. The recombinant molecule was engineered so that the ATG was seven nucleotides from the ribosome binding site of the lac operon component of Tac. After introduction into a bacterial host a colony was selected that produces 3% of its total protein as calmodulin. The bacterially produced calmodulin appears to be identical to rat testis calmodulin by the following criteria: (*a*) amino acid composition; (*b*) heat stability; (*c*) migration on one- and two-dimensional denaturing gels, both in the absence and presence of Ca^{2+}; (*d*) cross-reactivity in a specific calmodulin radioimmunoassay (50); and (*e*) immunoprecipitation with a polyclonal affinity purified calmodulin IgG.

The CM-1 gene was inserted into the calmodulin expression vector in such a way that the ATG and first 10 amino acids were encoded by chicken calmodulin, whereas the remainder of the protein was encoded by CM-1 sequences. A clone was selected in which, again, the level of expression was 3% of the total protein. The CM-1 protein was characterized with respect to rat testis and bacterially synthesized calmodulin. The CM-1 protein is similar to calmodulin in that it is heat stable, can be purified on phenothiazine-Sepharose, cross-reacts in a calmodulin radioimmunoassay, and, in the presence of EGTA, migrates identically to calmodulin on one-dimensional SDS gels. CM-1 protein can be distinguished from calmodulin in that it undergoes an aberrant Ca^{2+}-dependent mobility shift on SDS gels and is more basic than calmodulin upon analysis by two-dimensional gel electrophoresis. Finally, CM-1 protein can be separated from calmodulin by chromatography on thiol-Sepharose because it contains two cys residues and calmodulin does not. We now are using these properties to determine whether the CM-1 protein exists in chicken tissues. If the answer is yes, we will have the first evidence for (*a*) expression of an apparently processed gene; (*b*) presence of more than one functional calmodulin or calmodulin-like gene in any species; (*c*) existence of a novel calmodulin-like protein in a vertebrate; and (*d*) tissue-selective (or specific) expression of a calmodulin-like protein.

Future Perspectives

Even if the CM-1 gene is not expressed at the protein level, we have capitalized on the fortuitous observation of the presence of a second calmodulin-like gene by using this DNA to produce "mutant" calmodulin proteins in bacteria. These proteins can be used to examine how calmodulin exerts a variety of effects on a large number of specific metabolic processes. As discussed above, calmodulin may play multiple roles in maintaining the structure and function of the cellular cytomatrix. Calmodulin antibodies have been used to localize this protein on cytoplasmic (24) and mitotic spindle (18, 51, 52) microtubules, cytoplasmic microfilament bundles (13), and cytoplasmic intermediate filaments (53). Calmodulin promotes the Ca^{2+}-dependent disassembly of microtubules in vitro (22) and when microinjected into living cells in culture (23). However, the effects of calmodulin in vitro can be mimicked by skeletal muscle troponin C (22) and are not inhibited by phenothiazine or naphthalene sulfonamide drugs

either in vitro or in vivo (1, 24). These observations suggest that the portions of the calmodulin molecule involved in microtubule binding and depolymerization are different from those involved in the activation of enzymes such as cyclic nucleotide phosphodiesterase (54) or myosin light chain kinase (55). The bacterially produced calmodulin "mutant" proteins can be used to directly examine whether this is so.

The work reported in this manuscript was supported, in part, by the following grants to A. R. Means: National Institutes of Health (NIH) HD-07503, American Cancer Society BC-326, and Robert A. Welch Foundation Q-611. G. R. Slaughter was the recipient of postdoctoral fellowship grant HD-06250 from the NIH, and J. A. Putkey was supported by cancer-directed fellowship DRG-582 of the Damon Runyon-Walter Winchell Cancer Fund.

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