Journal of Cellular Biochemistry 20:317–330 (1982)
Evolution of Hormone-Receptor Systems 237–250

Calmodulin as a Mediator of Hormone Action and Cell Regulation

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INTRODUCTION

In order to survive in a dynamic environment all living cells must be able to identify and respond to variation in specific extracellular signals. The action of peptide hormones on mammalian cells is a specific case in point. Target cells recognize the hormone through specific receptors on the outer surface of their plasma membrane. The binding of hormone to receptor initiates a series of rapid events which eventually translates this external signal into a specific cellular response mediated by a selective alteration of the intracellular metabolism. The mechanism by which the extracellular event is transduced to an intracellular event is still not totally understood. The effect of adrenergic agents on cAMP metabolism led Sutherland to propose that cAMP was the second messenger responsible for this tranduction through the activation of a cAMP-dependent protein kinase. While this mechanism explains many hormonal events, it does not explain the studies of Hutson et al [1] and Cherrington et al [2], who demonstrated that cAMP metabolism of activation of cAMP-dependent protein kinase was not involved in the α-adrenergic activation of glycolysis and glyconeogenesis in rat liver. In fact, Keppens et al [3] and Assimacopoulos et al [4] presented data which supported the concept that the α-adrenergic response was mediated through Ca^{++}. It is now apparent that Ca^{++} plays a major role in the regulation of cellular activity. Berridge [5] and Rassmussen et al [6] have written extensive reviews delineating the involvement of Ca^{++} in the regulation of cell metabolism. The extent to which calcium is involved in cellular processes has led Rassmussen to propose that calcium be classed with the cyclic nucleotides as a second messenger to external stimuli. Indeed, the relationship between the cyclic nucleotides and calcium has been alluded to in several reviews on the subject [5–9]. Although the involvement of Ca^{++} in the transduction of external stimuli into cellular responses has been well documented, the biochemical mechanisms mediating the Ca^{++} effects have yet to be elucidated. Kretsinger [10,11] has pointed out the importance of the cytosolic Ca^{++}-binding proteins, and has suggested that the intracellular targets for Ca^{++}, functioning as second messenger, are these proteins. Because of the multitude of enzymatic systems which are known to be Ca^{++}-regulated, Ca^{++} would have to interact with

Received July 9, 1982; accepted November 28, 1982.

either a great many different proteins, each with one specific function or one or several proteins, each with multiple functions. This latter group of proteins could be viewed as intracellular Ca^{++} receptors.

CALMODULIN AS THE Ca^{++} RECEPTOR

Several criteria must be met before a protein may be classified as a Ca^{++} receptor: (1) It must specifically bind Ca^{++} with high affinity; (2) the binding of Ca^{++} to the protein must be mandatory to its regulatory role; (3) it must regulate vital intracellular processes common to all eucaryotic cells; (4) it must be ubiquitous; and (5) because of its critical role in cell survival it must be highly conserved.

Calmodulin is a heat-stable, 17,000-M_r multifunctional Ca^{++}-binding protein which meets all the criteria set forth for a Ca^{++} receptor [12]. It contains four equivalent Ca^{++}-binding sites with a K_d of 2.4×10^{-6} M which do not bind Mg^{++} under physiological conditions [13]. Ca^{++} binding induces a more α-helical conformation of the protein which preceeds activation of the calmodulin-dependent phosphodiesterase [13–15]. In further investigating this conformational transition accompanied by Ca^{++} binding, Richman [16] has examined the microenvironment of the two tyrosine residues of the protein. In the absence of free Ca^{++}, both tyrosine residues 99 and 138 are accessible to acetylation by N-acetylimidazole, whereas in the presence of Ca^{++}, only residue 99 can be acetylated. These findings suggest that Ca^{++} binding alters the microenvironment of tyrosine residue 138 and could play a role in the biological activity of the protein.

This protein has been shown to mediate the calcium regulation of a large number of fundamental intracellular enzyme systems. These enzymes include a calcium-dependent form of phosphodiesterase [17,18], brain adenylyl cyclase [19,20], human erythrocyte membrane Ca^{++}, Mg^{++}-ATPase [21–23], myosin light chain kinase [24–26], skeletal muscle phosphorylase kinase [27], glycogen synthase kinase [28,29], human phospholipase-A_2 [30], and pea and sea urchin NAD kinase [31,32]. Calmodulin has also been reported to be the calcium-binding protein regulating calcium transport in the sarcoplasmic reticulum [33,34] and autophosphorylation of membrane proteins [35,36]. In addition, immunofluorescence studies on a variety of cultured cells have demonstrated that calmodulin is localized on the actomyosin-containing stress fibers in interphase and is a dynamic component of the mitotic apparatus [31,37–39]. In this regard, this protein has been shown to regulate the calcium-dependent assembly-disassembly of microtubules in vitro [40].

Calmodulin represents the major intracellular calcium receptor in all non- and smooth muscle cells [12,41]. It constitutes between 0.1% and 1.0% of the total protein in all cells from the lowest- to the highest-order plants and animals. As might be expected for a protein so important and widely distributed, calmodulin is remarkedly conserved phylogenetically. The molecule has been sequenced from representatives of mammalian, fish, coelenterate, protozoan, and plant phyla [42–48]. Of the 148 amino acids, no more than seven have been shown to be altered between species. In all cases, amino acid substitutions are of a conservative nature and could be accounted for by a single base point mutation in the genes. In addition to the conservation of the primary amino acid sequence, calmodulins appear to be immunologically identical. A radioimmunoassay developed utilizing an antibody prepared against unmodified mammalian protein has revealed identical competition curves generated by calmodulin

present in extracts of tissue prepared from a wide variety of species from ameoba to man [49].

RECENT STUDIES ON THE CALMODULIN GENE

The calmodulin antibody has recently been used in an immunoprecipitation assay to monitor the purification of calmodulin mRNA [49]. Total poly(A)$^+$ RNA was isolated from the electroplax tissue of the electric eel and fractionated by sucrose gradient centrifugation [50]. One fraction was demonstrated to contain approximately 39% calmodulin mRNA by in vitro translation and hybridization analysis. This partially purified mRNA was transcribed into double-stranded cDNA by reverse transcriptase and cloned in the Pst I site of pBR-322 using Escherichia coli RRI as the host. A structural gene probe (pCM109) was identified and shown by sequence analysis to contain the nucleotides coding for amino acids 93–148 of calmodulin as well as 180 nucleotides from the 3′-nontranslated region including a AAUAAA polyadenylation signal.

pCM109 was shown to hybridize to DNA isolated from diverse animal and plant species suggesting that the calmodulin gene may also be highly conserved during evolution. Analysis of the DNA hybridization data revealed that the calmodulin gene was represented less than three times in both eel and chicken. The eel cDNA was also utilized to select and characterize a full-length cDNA complementary to electroplax calmodulin mRNA. DNA sequence of this recombinant molecule, pCM116, has shown it to contain 26 nucleotides of 5′-nontranslated sequence, the initiator codon AUG, the entire coding region, the terminator codon UGA, 409 nucleotides of 3′-nontranslated DNA and a poly(a) tail. Translation of the coding region revealed only a single conservative amino acid substitution compared to the published calmodulin sequence from bovine and human material. Hybridization of ^{32}P-labeled pCM116 to poly(A)$^+$ mRNA from electroplax distributed on a polyacrylamide gel and then transferred to nitrocellulose paper yielded three bands of approximately equal intensity at 820,1100 and 2,000, respectively. These data were obtained whether the RNA was isolated from total cell, cytoplasm, or polyribosomes. However, when nuclear RNA was similarly analyzed an additional RNA species 5,500 nucleotides in length was also observed. pCM116 was found to contain an AAUAAA sequence at 580 nucleotides and another at 873 nucleotides. The sequence of the 3′-nontranslated region of pCM109 was identical to the same portion of pCM116 up to the first polyadenylation site. A DNA fragment isolated 3′ from the first AAUAAA of pCM116 hybridized to the 5,500 nuclear RNA species as well as to the 2,000 and 1,100 nucleotide cytoplasmic mRNA but not to the 820 nucleotide RNA. Together these data suggest that the three cytoplasmic mRNAs differ only in the 3′-nontranslated region and could be derived from the 5,500 nuclear molecule by differential addition of poly(A). It also appears that pCM109 may have been generated from the 820 nucleotide mRNA, whereas the 1100 nucleotide species gave rise to pCM116. Multiple species of calmodulin mRNA have been identified in poly(A)$^+$ RNA from rat, cow, chicken, baboon, and human arguing for a common mechanism, for processing of calmodulin mRNA.

The calmodulin cDNA probes have also been employed to screen a chicken DNA library. Two calmodulin (or calmodulinlike) genes have been isolated and characterized. The first gene was isolated as a 10.6 kilobase (kb) fragment and the

calmodulin DNA was found to be localized to a 1.4-kb fragment at the extreme 5′-end. DNA sequence analysis has shown this fragment to contain all but 33 nucleotides complementary to the coding region of calmodulin mRNA (410 nucleotides) plus 481 nucleotides of 3′-nontranslated DNA, a polyadenylation site (AAUAAA), and a stretch of polyT residues. Derivation of the amino acids coded for by the structural segment illustrates that this genomic DNA begins with animo acid 12 (phe). Sixteen differences exist between this amino acid sequence and that derived from the eel cDNA (pCM116). Nine of the substitutions are nonconservative with two of the most striking being the presence of cysteine at positions 26 and 130. When this DNA was used as a hybridization probe in Northern analysis, only skeletal muscle demonstrated a positive signal (at 800 nucleotides) out of seven chicken tissues examined. Together these data suggest the possibility that this DNA represents either a processed calmodulin gene or a pseudogene.

The second chicken DNA was cloned as a 13.5-kb fragment. Eco RI cleaved this DNA into a 5′ 7.0-kb and a 3′ 6.5-kb fragment. Sequence analysis of the 6.5-kb fragment (5′→3′) revealed that this DNA begins with nucleotide 34 of the coding region of the calmodulin gene. Nucleotides 34–168 are identical to those of pCM116. However, restriction hybridization analysis has shown the calmodulin DNA to encompass approximately 4-kb and to be interrupted by at least three introns in the structural sequence region. Whether additional introns exist in the 5′-nontranslated region remains to be established. A mRNA was isolated from chicken brain poly(A)$^+$ RNA using pCM116 as a hybridization probe. This mRNA has been cloned and sequenced. It is now apparent that this mRNA is encoded by the calmodulin gene containing intervening sequences. Indeed, the sequence of the chick brain cDNA is identical to pCM116 within the entire coding segment (444 nucleotides). Poly(A)$^+$ RNA from all eight chicken tissues examined (including skeletal muscle) contain species complementary to the second chicken gene. In all cases, the predominant mRNA is approximately 1,600 nucleotides. Finally, Southern analyses under stringent hybridization conditions suggest that this calmodulin gene is unique.

CALMODULIN AND HORMONE ACTION

Since calmodulin (CaM) is a component of virtually every intracellular compartment as well as the plasma membrane [51], efforts have been made to determine whether cell surface acting agents promote an alteration in the distribution of CaM. Distinct anatomical regions of the central nervous system such as the corpus striatum contain dopamine receptors which seem to be coupled to adenylyl cyclase [52]. Calmodulin has been suggested to mediate dopamine action since phosphorylation of membrane proteins promotes the apparent release of CaM from membrane-bound to soluble form [53]. Since a soluble CaM-dependent phosphodiesterase (PDE) exists, it has been proposed that long-term stimulation of dopamine receptors is associated with an increase in the soluble CaM content thereby activating PDE and decreasing receptor responsiveness. Similar data suggest interneuronal pathways also exist where opiates increase soluble CaM via a release of dopamine and thus act as indirect dopamine agonists [54]. Smoake and Solomon [55] have reported altered CaM distribution in liver cells from rats with streptozotocin-induced diabetes. These authors conclude that such changes might play a role in the alteration of cAMP metabolism known to exist in such pathological states.

The difficulties with interpretation of most CaM distribution studies is that the protein is assayed by its ability to stimulated a CaM-dependent enzyme. Since all such assays are Ca^{++}-dependent and other CaM-binding proteins are likely to be present in each subcellular fraction, it is difficult to obtain quantitative values for CaM. This difficulty is circumvented when a radioimmunoassay is employed since the assay can be performed in the presence of EGTA and is therefore Ca^{++}-independent [49]. The radioimmunoassay has been utilized to determine the quantity and subcellular distribution of CaM in the rat pituitary gonadotrope before and during Gonadotropin Releasing Hormone (GnRH)-induced luteinizing hormone (LH) release [56]. Indeed, the distribution of CaM does change in response to GnRH. There is an initial rise in the percentage of CaM associated with the plasma membrane which appears concomitantly with the depletion of cytoplasmic CaM. These changes occur temporally in concert with secretion of LH. As the CaM begins to be cleared from the plasma membrane, its level increases first in the secretory granule and microsomal fractions before finally replenishing the cytoplasm. The magnitude of the changes that occur between plasma membrane and cytoplasmic content of CaM is related to the dose of GnRH. Calmodulin redistribution is also hormone specific since analogs such as des^{1} GnRH (2–10) which has no efficacy in promoting LH secretion did not alter intracellular changes in CaM. Finally, a budget of CaM content in all subcellular fractions revealed that GnRH did not increase total CaM and greater than 95% of the cellular CaM was recovered.

The data presented above suggest that CaM may be important in the regulation of protein secretion, but provide little information concerning the mechanism. At this juncture it is impossible to predict whether CaM redistribution is a cause or consequence of the secretory process. In the red blood cell [57], pancreatic islet [58], and adipocyte [59], CaM-activated ATPases are found in the plasma membrane, and, at least in the adipocyte, the enzyme appears to be hormonally regulated. Plasma membranes from islet cells also have been reported to contain a CaM-stimulated adenylyl cyclase activity [60]. Calmodulin is also a major component of postsynaptic membranes [51,61,62], has been proposed to mediate the Ca^{++} effects on synaptic transmission [52,53,63] and, accordingly, may play a role in neurotransmitter release [64]. Finally trifluoperazine and naphthalenesulfonamides are drugs that bind to CaM and inhibit many of its actions. These drugs also inhibit the receptor-mediated secretory process in a variety of systems.

Receptor-mediated endocytosis is also a Ca^{++}-dependent process and also involves clathrin-coated vesicles [65,66]. Although internalization of GnRH does not appear to be required for the LH release process, the gonadotrophe response to this releasing hormone does include the pattern of patching, capping, and internalization observed for many cell surface-mediated ligand systems [56]. This receptor redistribution pattern in the gonadotrophe is mimicked by changes found in CaM associated with the plasma membrane when assessed by indirect immunofluoresence microscopy. Recruitment of clathrin-coated vesicles to the plasma membrane of human lymphoblastoid cells occurs following stimulation with multivalent anti-IgM antibodies [66]. This recruitment is inhibited by the presence of anticalmodulin drugs, and CaM is a component of such vesicles. Thus, the appearance of CaM at the plasma membrane may be associated with the accumulation of coated pits involved in the receptor internalization process. Insulin, which also is internalized following cap formation, promotes the translocation of glucose transport activity from the microsomal or Golgi fractions to the plasma membrane [67,68]. Actin and myosin have also

been reported to co-cap with several cell surface receptors [69,70] and actin-containing matrices have been isolated from Dictyostelium discoideum [71], murine tumor cells and lymphocyte plasma membranes [72] associated with various receptors. Thus, the phenomenon of redistribution of new activities to the plasma membrane may be a generalized occurrance for plasma membrane receptor-mediated events. This redistribution suggests a mechanism by which CaM-regulated events could be affected without the requirement for new protein synthesis. It is likely that CaM redistribution is secondary to alterations in the net flux or distribution of Ca^{++} within the cell.

CALMODULIN AND MICROFILAMENTS

Antibodies developed against native rat testis calmodulin have been employed to localize the protein in a variety of tissue culture cells by indirect immunofluorescence microscopy. In cells in interphase, calmodulin predominantly decorates the actin-containing stress fibers [43], whereas in skeletal muscle, the protein is preferentially associated with the I-bands. The logic of these distributions becomes readily apparent when considering the biochemical reactions involved in contractility. In all types of cells, an enzyme termed myosin light chain kinase (MLCK) is present that phosphorylates one of the myosin light chains (LC_{20}) [see 73 for review]. This enzyme was found to be calmodulin-dependent [24,25]. Myosin light chain kinase has been purified from smooth [25,74,75], skeletal [76–78], and cardiac muscle [79,80]. The reported molecular weights of the enzymes from these tissues range from 80,000 to 130,000 depending on both the tissue and procedure used for purification. It has been suggested that either there are multiple forms of MLCK in these tissues [73,81] or the smaller molecular weight proteins are proteolytic fragments of the native enzyme [80]. A recent study by Guerriero et al [75] addressed this question by producing an antibody against chicken gizzard MLCK. Using this probe and the protein transfer technique of Towbin et al [82], the molecular weight of the enzyme in a variety of chicken tissues was found to be 130,000. These data suggest that some proteolysis of the native enzyme may occur during purification.

In non- and smooth muscle cells LC_{20} phosphorylation is positively correlated with tension development and contractility [73]. Thus, Ca^{++} binds to CaM, which promotes the association of this complex with an inactive myosin light chain kinase (MLCK). Activation of MLCK results in LC_{20} phosphorylation. This modification promotes a conformational change in myosin that allows actin binding and consequent stimulation of the myosin ATPase. Hydrolysis of ATP provides the energy required for contraction. Indeed myosin, tropomyosin and myosin light chain kinase have also been revealed to be components of the stress fibers in nonmuscle cells [75,83]. Contractility of skeletal muscle is primarily controlled by the troponin system and LC_{20} phosphorylation does nothing to enhance the primary contractile response. Rather, this CaM-dependent alteration seems to be involved in posttetanic potentiation of the action potential. In order for this to occur, actin, MLCK, and CaM should all be present on the I-band (which they are). Only following contraction would the I-band be in register with myosin to allow LC_{20} phosphorylation and the posttetanic response. Enzymes that dephosphorylate the regulatory light chain of myosin have been purified from skeletal [84] and smooth muscle [85]. Dephosphorylation of smooth muscle myosin results in a form of myosin ATPase that cannot be activated

by actin. The removal of the phosphate from the regulatory light chain, then, leads to smooth muscle relaxation.

CALMODULIN AND MICROTUBULES

In mitotic cells, CaM was determined to be localized in the half-spindle [37,38,42]. During most of mitosis, CaM is found between the chromosomes and spindle poles in apparent association with the spindle fibers. However, CaM and the spindle microtubules network do not share identical locales since microtubules are found throughout the mitotic apparatus. CaM also seemed to be present in those regions of the mitotic apparatus characterized by the microtubule shortening that accompanies anaphase chromosome movements and cytokinesis.

It has long been known that Ca^{++} promotes diassembly of the mitotic apparatus as well as of microtubules both in vivo and in vitro. Marcum et al [40] evaluated whether the Ca^{++} regulation of microtubule assembly/disassembly might be mediated by CaM. Microtubules were isolated from bovine brain in the absence of glycerol as described by Borisy et al [86]. Microtubule (MT) polymerization was monitored in a recording spectrophotometer by evaluating the change in A_{320} with time. Ingredients were equilibrated to 37°C and the reaction was initiated by the addition of GTP. In all experiments, Ca^{++}/EGTA buffers were utilized in order to calculate the free Ca^{++} concentration. The Ca^{++} concentration in the normal polymerization solution was 0.3 μM, and under these conditions, microtubule assembly proceeded without a timelag and reached a plateau within 10 min. Increasing Ca^{++} to 11 μM resulted in a slight 10% decrease in the plateau value. Addition of CaM to the mixture prior to addition of GTP was without effect on either rate or extent of MT assembly. However, if 11 μM Ca^{++} was added at the plateau, a rapid and biphasic disassembly occurred that was complete within 45 min. Addition of 11 μM Ca^{++} and CaM at the beginning of the assay completely prevented MT polymerization.

The effect of Ca^{++}-CaM on MT assembly was not all-or-none. Ca^{++} alone caused complete disassembly of polymerized MTs, but the concentration required was 10^{-3} M. This concentration is far beyond that occurring in a cell under any circumstances. Addition of CaM resulted in a 2 log shift in the sensitivity to Ca^{++}. Thus, complete disassembly was achieved at 10^{-5} M Ca^{++}, a level that can occur in the cell during mitosis [11]. These data suggest that if CaM alters MT assembly in the cell, then there should be an inverse relationship between CaM concentration and degree of MT polymerization. Such a relationship does occur in the mitotic spindle. CaM concentration decreases from pole to chromosome during metaphase. Brinkley and Cartwright [87] have shown that no MTs are present at the centrioles but increase in number between the poles and the chromosomes. In very late anaphase, CaM is transiently found on both sides of the chromosomes, but is absent from the portion of the cell which corresponds to the developing cleavage furrow. On the other hand, MTs are present only in this latter location. Thus, an inverse relationship does seem to occur between CaM concentration and the number of MT profiles present in the mitotic cell [88].

Salmon and Segall [89] have succeeded in isolating mitotic apparati from sea urchin eggs that demonstrate μM sensitivity to Ca^{++}. The CaM radioimmunoassay was employed to confirm the presence of CaM in such structures. Evaluation of the localization of MTs by tubulin indirect immunofluorescence revealed their presence

throughout the spindle and in the asters surrounding the poles. CaM was only demonstrable as a band surrounding the centrioles. Therefore, if the theory held, cross-sections of the spindle should fail to reveal MTs in any section containing portions of centrioles. This prediction proved to be true, thereby strengthening the argument that CaM might regulate the extent of MT polymerization in the cell [88].

REGULATION OF CALMODULIN

The prediction from the observations discussed above is that alteration of the intracellular concentration of CaM should result in profound effects on the architecture and functions of the cell. We evaluated 13 hormone-regulated systems in order to determine whether CaM levels were selectively altered [90]. The systems included one representative of each of the five classes of steroid hormones and eight peptide hormone-regulated cell types. In every case, the target cell was known to respond to the hormone via increases in the synthesis of at least one specific protein. Calmodulin levels were found to be constitutively regulated in target cells for both steroid and peptide hormones. Included in the systems evaluated was the chick oviduct that responds to estrogen and progesterone by the induction of a large number of specific proteins. Even in this model of steroid hormone-mediated tissue differentiation, CaM levels per cell were unchanged regardless of the hormonal status of the animal.

Since hormones did not selectively alter CaM levels, we searched for a system where the MT network was purportedly altered. One such system is the nature of the cytoplasmic microtubule complex (CMTC) in some fibroblastic tissue culture cells in relation to that in virally transformed counterparts. Swiss mouse 3T3 cells exhibit an extensive array of cytoplasmic MTs. However, in 3T3 cells transformed by the DNA oncogenic virus SV40 (SV-3T3), the complex has been reported to be diminished as viewed by immunofluoresnce [91] or electron microscopic procedures [92]. This finding has become somewhat controversial since several groups have reported that the tubulin content of 3T3 and SV-3T3 cells is identical [90,93,94]. However, SV-3T3 have been demonstrated by radioimmunoassay to exhibit a 2–3-fold increase in CaM compared to 3T3 cells [90,94]. Indeed, this elevation of CaM levels seems to be a general characteristic of such cells whether transformation was achieved by oncogenic viruses, chemical carcinogens, or hormones. In all cases, the increase in CaM levels are due to an enhanced rate of synthesis and not degradation. Moreover, this effect is selective since neither tubulin nor actin content is altered. Thus, the SV-3T3 cell exhibits a CaM:tubulin ratio two times greater than the 3T3 cell.

Such changes in the CaM:tubulin ratio may explain why immunofluorescent techniques apparently show a diminished cytoplasmic microtubule complex in transformed cells, whereas biochemical procedures reveal no change in tubulin content. Three pieces of evidence support this hypothesis. First, Marcum et al [40] found that CaM mediates the Ca^{++}-dependent assembly-disassembly of microtubules in vitro. Second, using a cell system made permeable by detergent to study polymerization of microtubules from 6S tubulin, Brinkley et al [95] showed that SV40-transformed 3T3 cells have an attenuated ability to nucleate and elongate microtubules; this difference is twofold. Tash et al reported that the SV40-3T3 cell can be restored to normal by preincubation with anti-CaM. Third, Rubin and Warren [92] used quantitative electron microscopy to evaluate the number of polymerized microtubules in Normal Rat Kidney (NRK) and Rous sarcoma virus transformed (RSV)-NRK cells. They found

that the transformed cells contain only 50% as many polymerized microtubules as NRK cells. Together these studies suggest that the degree of polymerization of tubulin into microtubules in vivo may depend on the concentration of CaM within the cell relative to that of tubulin. The elevated CaM levels in transformed cells could destabilize microtubules and signifcantly reduce the number and length of these cytoskeletal components compared with those in nontransformed cells.

Cell transformation is usually accompanied by alterations in structure, cyclic nucleotide metabolism, metabolic rate, and intracellular Ca^{++} levels. Transformed cells also lose the requirements for anchorage to substrate and Ca^{++} for cell proliferation. All these processes could depend on CaM, based on the enzymes known to be regulated by this ubiquitous Ca^{++}-binding protein. Certainly one major Ca^{++}-CaM regulated system, the cytoplasmic microtubule network, seems to be altered in virus-transformed cells. If CaM does afffect the intracellular regulation of tubulin assembly, it could also indirectly alter intracellular tubulin metabolism. Ben Ze'er et al [96] and Cleveland et al [97] have shown that an increase in the ratio of unpolymerized to polymerized 6S tubulin increases the rate of degradation of these proteins by affecting the turnover of tubulin mRNA. These observations predict that an increase in unpolymerized tubulin in the transformed cell should result in an increase in the rate of tubulin degradation relative to what occurs in the nontransformed state. Chafouleas et al [90,94] have shown such a situation to exist in cells transformed by both RNA and DNA oncogenic viruses. Thus, CaM may alter the cytoskeleton not only through a direct effect on microtubule assembly, but also indirectly through regulation of the intracellular levels of tubulin.

CALMODULIN AND THE CELL CYCLE

During the growth cycle of mammalian cells in tissue culture, dramatic changes in cell shape and function occur. Nontransformed cells require rather large amounts of Ca^{++} in the media, and Ca^{++} seems to be necessry both for progression from G_1 into S as well as for mitosis. On the other hand, transformed cells appear to require much less Ca^{++} for cell growth. For these reasons, Chafouleas et al [98] evaluated changes in CaM that occur during the growth cycle of Chinese hamster ovary (CHO)-K_1 cells. These Chinese hamster ovary cells were chosen because they exhibit a relatively short cell cycle (12–16 hr) and, consequently, can be synchronized by mitotic shake [99] without the need for the addition of drugs such as thymidine or colchicine. The population of cells utilized for the initial experiments were shown to exhibit at 16-hr cell cycle distributed as follows: $M=1$, $G_1=5$, $S=8$, and $G_2=2$. Mitotic cells were released into the cycle by increasing the temperature from 4°C to 37°C. During the subsequent 16 hr, the percentage of cells in S was evaluated by 10-min pulses with ^{3}H-thymidine (TdR) followed by radioautography to quantitate the number of cells with labeled nuclei. Calmodulin content was monitored by radioimmunoassay and the percentage mitotic cells was also scored as a measure of cell synchrony.

At mitosis, the calmodulin content was 150 ng/10^6 cells. This value fell abruptly by 50% coincident with separation of the daughter cells. Between 4 and 6 hr after initiation of cell synchrony, CaM values again increased to 150 ng/10^6 cells and remained at this value for at least 12 hr. The increase in CaM content slightly preceded the ^{3}H-TdR-labeled cells, suggesting that synthesis of this protein occurred at the G_1/

S boundary. This postulate was tested by repeating the experiments with a population of cells that exhibited a 2-hr G_1 period. Again, CaM values halved as cells were released from mitotic synchrony and increased again preceding DNA synthesis. Four such experiments with cells that revealed both 2- and 5-hr periods were carried out. The data were graphed as CaM content (ng/10^6 cells) as a function of the percentage of cells labeled with ^{3}TdR. Linear regression analysis was performed and these parameters were found to show a correlation coefficient of 0.966 (slope = 1.07). Thus, CaM synthesis is coupled to the G_1/S boundary—a fact which raises the question as to whether the doubling in CaM content may play a role in the G_1/S transition.

If the elevation in CaM concentration is important in the progression of cells from G_1 into S, then anticalmodulin drugs might be expected to arrest the cells at this growth cycle boundary. The primary question was which drugs to choose for these experiments. The most widely used anti-CaM compounds are members of the phenothiazine family. These molecules, characterized by trifluoperazine (TFP) and chlorpromazine, bind to CaM in a Ca^{++}-dependent manner [100] and have been linked to inert supports to form affinity columns [101,102]. These columns have been used to isolate CaM from a wide range of cells types. Whereas the in vitro specificity of these drugs is considerable ($K_d = 10^{-6}$ M), the specificity in vivo is questionable. Trifluoperazine is highly lipophilic and as such will bind nonspecifically to cell membranes [103]. Moreover, TFP has been shown to interact with the dopamine receptor [53] as well as receptors for α-adrenergic agonists [104]. Control compounds such as TFP-sulfoxide bind much less avidly to CaM, but also possess a much different hydrophobicity index (measured by the octanol:H_2O partition coefficient). These problems are compounded because CaM undergoes a conformational change upon binding Ca^{++}. This interaction exposes a highly lipophilic surface and it is this surface that reportedly binds to both CaM-dependent enzymes as well as to the phenothiazines [105,106]. The importance of these considerations can be appreciated when considering the fact that phosphodiesterase and MLCK [106] can be fully activated in vitro by phospholipids [107,108]. This type of activation precludes the need for CaM as a regulatory component. Therefore, in designing drugs to be used as anti-CaM compounds, it is necessary to separate the lipophilic nature of the drugs from their ability to bind CaM. The ideal control compounds would be those that bind with less affinity to CaM, but exhibit similar hydrophobicity indices to their CaM-binding counterparts.

The naphthalenesulfonamides may represent a class of drugs as described above. These "W-compounds" have been synthesized and evaluated by Hidaka and colleagues [109,110]. The highest-affinity W-compounds (W-7 and W-13) have a Cl^- attached to C-1 of the A-ring. These drugs bind to CaM in a Ca^{++}-dependent manner and exhibit similar affinities to TFP ($K_d = 10^{-6}$ M). Removal of the Cl^- decreases CaM binding by five- to ten-fold, but alters the hydrophobicity index by only 10–15%. For these reasons we decided to evaluate the anti-CaM compound W-13 and its dechlorinated control compound W-12 for effects on the growth cycle of (CHO)-K_1 cells. The first series of experiments were designed to assess the fraction of cells that survived following culture in the presence of increasing concentrations of W-13 or W-12. Addition of W-12 up to 80 μg/ml did not affect survival. Similarly 30 μg/ml of W-13 (10^{-4} M) caused no lethality but did alter the shape of the cells. Increasing the W-13 concentration to 35μg/ml resulted in 40% cell death and only 0.01% of the cells survived at 60μg/ml.

Incubation of asynchronously growing cells with 30 μg/ml W-13 resulted, within 24 hr, in a 50% reduction in the number of cells compared to cells grown in a similar concentration of W-12 or the usual culture medium [98]. This growth cycle arrest was completely reversible. Following removal of W-13, the cells remained quiescent for approximately 8 hr before beginning to increase in number. The cell number doubled by 30 hr, and after recovery, this population of cells continued to exhibit a cell cycle of 12.5 hr. These experiments confirmed that the anti-CaM drug W-13 delayed progression of cells through the cell cycle in a specific and reversible manner. The next studies were designed to question the location of cell cycle arrest.

Cells were synchronized by mitotic shake and 1 hr after release into the cell cycle W-13, W-12 or normal medium was added. W-13 was found to cause a delay in G_1 progression into S as monitored by the increase in the number of cells labeled with ^{3}H-TdR. W-12 caused no effect and these cells responded precisely as did cells incubated in normal medium. Whereas 92% of the cell nuclei became labeled by 8 hr when cultured in medium or medium containing W-12, only 60% became labeled in the presence of W-13. Thus, although W-13 appeared to exert an effect at the G_1/S boundary, the arrest was incomplete. This population of (CHO)/K_1 cells exhibited a G_1 of only 2 hr. Subsequent experiments with the 5-hr G_1 population demonstrated that a complete delay in G_1 to S progression could be effected by W-13. During these experiments, CaM content was monitored by radioimmunoassay (RIA). The data revealed that W-13 did not prevent the twofold increase in CaM that occurred at the G_1/S boundary. It is likely, therefore, that the W-13 was preventing an action(s) of CaM required for progression of the cells into S.

Not only does W-13 cause a delay in the progression of synchronized cells (at mitosis) from G_1 to S, but the drug also prevents progression through S phase in cells synchronized at the G_1/S by a double TdR block. On the other hand, W-13 does not affect progression through either G_2 or M. Taken together, the data suggest that the elevation in CaM concentration that occurs during the cell cycle is important for the progression of cells through replicative DNA synthesis (S phase) and that W-13 must prevent one or more actions of CaM that are permissive for this important cellular event.

The calmodulin concentration also seems to be important for the re-entry of plateau cells (G_0) into the cell cycle. Calmodulin levels increase by 50% as cells leave G_1 and enter plateau and remain at this concentration for the duration of the G_0 phase. Upon release of the cells into the growth cycle, calmodulin decreases by 50% within the first hr and remains at this concentration for 4–5 hr. By 6 hr, the intracellular levels have again doubled and remain at this level as cells pass through S, G_2 and M. This concentration is that normally achieved after the increase in calmodulin at the G_1/S boundary [98]. Addition of W-13 at the time of treatment with fresh medium prevented entry into S phase, but the changes in the intracellular concentration of calmodulin were unaltered. When cells were treated with W-13 at various times following release from plateau, a direct correlation was observed between the percentage of cells entering S phase and the time of drug addition ($r=.99$). The labeling index increased as the interval between drug treatment and G_0 release increased. However, although some cells entered S phase, no progression through this period was observed when W-13 was added as late as 5 hr following addition of fresh medium. Removal of the drug resulted, after a 5-hr lag period, in progression of all cells through S phase in a synchronous fashion. These data strengthen the contention

that calmodulin is important for the progression of cells through DNA synthesis.

Recent experimental results can be interpreted to suggest that the increase in calmodulin at G_1/S is important for optimal DNA repair prior to replicative DNA synthesis. Bleomycin is a drug known to cause DNA damage by strand scission. Concentrations of this agent can readily be found that result in potentially lethal damage to tissue culture cells. Under these conditions, approximately 90% of the cells are killed. The remaining 10%, however, can recover from the drug by repairing DNA and will eventually repopulate the culture dish. If cells are selected for potentially lethal damage and released from Bleomycin into the presence of media containing W-13, all the cells are killed within 3 hr. The most obvious explanation of these results is that calmodulin is required for DNA repair. When calmodulin is neutralized with W-13, then the cells cannot repair the DNA damage which resulted from Bleomycin. This results in cell death during the subsequent replicative phase. If such a scenario is the case, then some of the enzymes 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. Studies are currently underway to determine whether the DNA repair enzymes are calmodulin-binding proteins and whether the increased calmodulin synthesis at G_1/S is transcriptionally or posttranscriptionally regulated.

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