Bacterial Expression and Characterization of Proteins Derived from the Chicken Calmodulin cDNA and a Calmodulin Processed Gene*

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Both normal chicken calmodulin (CaM) and a CaM-like mutant protein have been expressed in bacteria, isolated and evaluated with respect to several physical and biological properties. The mutant CaM is derived from a CaM-like gene that lacks intervening sequences and processed gene is evolved from a CaM-processed gene (Stein, J. P., Munjaal, R. P., Lagace, L., Lai, E. C., O'Malley, B. W., and Means, A. R. (1983) Proc. Natl. Acad. Sci. U. S. A. 80, 6485–6489). The mutant CaM protein contains 16 of the 19 amino acids encoded by the CaM-like gene. Normal chicken CaM produced in bacteria is identical to rat CaM by all criteria tested except that it is not trimethylated. The protein product of the CaM-like gene has been termed CaML and exhibits properties which are very similar to CaM despite the presence of 16 amino acid substitutions. CaML binds Ca\(^{2+}\) as evidenced by Ca\(^{2+}\)-dependent binding to phenothiazine- and phenyl-Sepharose affinity resins and a Ca\(^{2+}\)-dependent electrophoretic mobility shift which is similar to but distinct from CaM. CaML cross-reacts with a monospecific CaM antibody and has an immunodilution curve which is identical to bacterially synthesized CaM. Finally, CaML can maximally activate rat brain phosphodiesterase but with altered kinetic parameters as compared to CaM. These data suggest that the nucleotide substitutions in the putative CaM processed gene are not random but are selected to retain CaM-like functions in the encoded protein. Such a mechanism may exist for other processed genes.

CaM\(^{I}\) is the principal intracellular calcium receptor in all eucaryotic cells and is involved in the regulation of such diverse cellular processes as cell motility and nerve transmission (for review see Means et al., 1982 and Klee and Vanaman, 1982). To study the nucleic acids involved in the synthesis of CaM, this laboratory has isolated the cDNA molecules which encode the eel and chicken protein (Munjaal et al., 1981; Lagace et al., 1983; Putkey et al., 1983). These cDNAs were then used as probes to screen a chicken genomic library from which two distinctly different CaM genomic clones were isolated.

The first genomic clone, CL1, encodes normal chicken CaM and contains seven intervening sequences (Simmen et al., 1985). The second genomic clone, CM1, contains an open reading frame which is not interrupted by intervening sequences and encodes a protein of the same length as CaM but with 19 amino acid substitutions (Stein et al., 1983). The homology between CM1 and CL1 and the lack of intervening sequences in CM1 suggests that it has evolved from a CaM processed gene. In contrast to other known processed genes, CM1 is transcribed to yield a mRNA species which appears to be muscle specific (Stein et al., 1983). This presents the intriguing possibility that there is present in muscle a CaM-like protein encoded by a CaM processed gene.

The predicted properties of the protein encoded by CM1 should be considerably different from CaM based on the presence of 19 amino acid substitutions. In order to determine functional difference purified proteins are required. We reasoned that attempts to isolate the putative protein product of CM1 would have a low probability of success. First no direct evidence exists for the presence of such a protein in muscle. Second, even if it does exist, we know nothing of its abundance. Finally it is difficult to design an assay specific for this molecule that would not also recognize CaM. For these reasons we have constructed bacterial expression plasmids which harbor either the chicken CaM cDNA or the CM1 gene. The CM1 expression plasmid contains a hybrid CaM gene in which amino acids 12 to 148 are encoded by CM1. The protein product of the CM1 expression plasmid has been called CaML and retains 16 of the 19 amino acid substitutions encoded by CM1. The bacterially synthesized CaM is identical to rat CaM by all criteria tested except for the absence of post-translational modifications. Contrary to our expectations, CaML has properties which are very similar to CaM, including the ability to maximally activate rat brain phosphodiesterase. The similarity between CaM and CaML suggests that the putative CaM processed gene has undergone selective mutations that preserve or only modify the functional properties of the originally encoded protein.

EXPERIMENTAL PROCEDURES

Molecular Cloning—All restriction endonucleases and DNA-modifying enzymes were used as recommended by the manufacturers. The expression plasmids were constructed as described in Fig. 2 and used to transform the JM103 strain of Escherichia coli as described by Hanahan (1983). The plasmid pDR540 was used as a source of the Tac promoter and was obtained from P-L Biochemicals. Essentially all cloning procedures were designed such that 80–90% of the resulting recombinant bacteria contained the desired plasmid. Screening under these conditions was accomplished by the rapid alkaline minilysate procedure (Birnboim and Doly, 1979). Step 1 of Fig. 2 which generated the hybrid ATG initiation codon was a less efficient cloning step. Recombinant bacteria resulting from this cloning step were initially screened by the minilysate procedure for the presence of a TaqI site and the absence of MnlI, AluI, and SalI sites.
which would be present if 1, 2, or 3 bp, respectively, were missing from the 3'-side of the blunt-end ligation junction. Two of 16 selected bacterial colonies contained plasmids which conformed to this preliminary criteria. These plasmids were sequenced around the ligation junction by the method of Maxam and Gilbert (1980); both plasmids were found to contain the hybrid ATG.

**Protein Isolation**—Bacterially synthesized CaM23 and CaML were isolated from bacterial lysates by phenyl- Sepharose chromatography. Overnight cultures were prepared from single colonies of the desired bacteria and used to inoculate 10-1 liter amounts of L broth (20 ml overnight culture/liter of L broth). The cultures were grown at 37 °C with shaking to an A600 of 0.1-0.2 at which time IPTG was added to a final concentration of 1 mM. After an additional 3-4 h the bacteria were collected by centrifugation and washed twice with 50 mM phosphate, pH 7.5, 100 mM NaCl and once with 40 mM Tris, pH 7.5. The washed bacteria were lysed by hypo-osmotic shock/lysozyme treatment (Seeburg et al., 1978; Liu and Milman, 1983). The washed bacterial pellet was resuspended in 50-100 ml of 2.4 M sucrose, 40 mM Tris, pH 8.0, 10 mM EDTA and incubated on ice for 30 min. Four volumes of 50 mM MOPS, pH 7.5, 100 mM KCl, 1 mM EDTA, 1 mM dithiothreitol, 100 μg/ml lysozyme were then added, and the suspension was placed at 0-4 °C overnight. The bacterial lysate was then centrifuged at 100,000 × g for 30 min to obtain soluble and particulate fractions. The soluble fraction was passed over an initial phenyl-Sepharose column to absorb proteins that bind in a calcium-independent manner. The nonabsorbed fraction from this first column was added to a second column. This second column was washed sequentially with 1) 50 mM Tris, pH 7.5, 1 mM CaCl2 (buffer A); 2) buffer A made 500 mM in NaCl; 3) buffer A. The bound CaM23 or CaML was then eluted with 50 mM Tris, pH 7.5, 1 mM EDTA. This procedure takes 1 day to perform and yields 30-40 mg of CaM23 or CaML from 1 liter of bacterial culture. This corresponds to a recovery of greater than 85%.

**Electrophoresis**—Electrophoresis of DNA fragments was performed in either 1% agarose or 5% polyacrylamide gels (291, acrylamide: bisacrylamide). Both the running and gel buffer contained 89 mM boric acid, 1 mM EDTA, pH 8.3. DNA fragments were isolated from either 1% low melting point agarose or polyacrylamide:bisacrylamide. Both the running and gel buffer contained 89 mM borate, 1 mM EDTA, pH 8.3. DNA fragments were isolated from either 1% low melting point agarose or polyacrylamide gels with the use of "NACS" prepacked columns according to procedures recommended by the manufacturer (Bethesda Research Laboratories).

All protein gel electrophoresis was performed on 10-20% exponential gradient gels with a Laemmli buffer system (Laemmli, 1970). The samples were solubilized in sample buffer which contained either 5 mM CaCl2 or 5 mM EDTA.

**Preparation of Phosphodiesterase**—Brains were excised from 10 adult male rats, homogenized in 3 volumes of 10 mM Tris, pH 7.5, 10 mM mercaptoethanol, 5 mM EDTA, 1 mM MgCl2 (buffer A), and centrifuged at 100,000 × g for 1 h. The supernatant was filtered through glass wool, applied to a DE-52 cellulose column equilibrated in 10 mM Tris, pH 7.5, 16 mM mercaptoethanol, 1 mM EGTA, 1 mM MgCl2, buffer B), and washed with 200 ml of buffer B. Phosphodiesterase was eluted from the column by a 400-ml 0-0.4 M (NH4)2SO4 gradient in buffer B to separate the CaM from the enzyme (Chafouleas and Means, 1983). Fractions were assayed ± 10 μg of rat testis CaM. The CaM-stimulated fractions were pooled, and glycerol was added to a final concentration of 50%. The fractions were stable when they were stored at either -20 or -180 °C.

**Phosphodiesterase Assays**—Phosphodiesterase activity was determined in an assay containing 10 mM MOPS, pH 7.0, 3 mM Mg acetate, 5 mM mercaptoethanol, 0.2 mM ammonium chloride, and 1 mM [3H]cAMP and CaCl2 as recommended by Klee et al. (1979). The CaCl2 concentration was adjusted to give 500 μM free Ca2+ in excess of the EGTA in the enzyme preparation, except in assays to test the Ca2+-dependence of the CaM activation. For those experiments EGTA was added to a final concentration of 2 mM, and the amount of orion CaCl2 standard needed to produce the desired free Ca2+ concentration was calculated by a computer program developed by Goldstein (1979). CaM preparations were diluted into 10 mM Tris, 0.1% bovine serum albumin, pH 7.6. Phosphodiesterase buffer, CaM, and CaCl2 were preincubated for 15 min at 30 °C; then the reaction was initiated by adding [3H]cAMP. After 30 min the reaction was terminated by adding 10 mM cAMP, 20 mM EDTA. Snake venom phosphodiesterase (12.5 μg) was added and the reaction continued for 15 min. Adenosine was added and the unreacted [3H]cAMP absorbed to Bio-Rad AG5-X8 resin (Chafouleas and Means, 1983). An aliquot of the supernatant was counted in 10% BBS-3 Spectrofluor. All assays were performed in triplicate. Data are expressed as per cent maximum stimulation within an experiment. Saturating amounts of calmodulin and Ca2+ stimulated hydrolysis 6-fold over the basal activity.

**RESULTS**

The goals defined in this project required the construction of expression plasmids which produce unfused normal CaM and CaM-like proteins. The amino acid sequences of these protein products are shown in Fig. 1. The primary amino acid sequence of chicken CaM is identical to that of both bovine brain and human CaM (Watson et al., 1980; Sasagawa et al., 1982). The putative protein product of the CM1 gene has the same number of amino acids as CaM but with 19 substitutions. Fig. 1 indicates these substitutions and relates them to the proposed calcium-binding domains. In this paper we describe an expression plasmid which encodes a CaM protein containing 16 of the 19 amino acid substitutions shown in Fig. 1. The CaM-like gene in this plasmid is derived from nucleic acids which encode the first 11 amino acids of chicken CaM and amino acids 12-148 of the putative CM1 protein product. The two gene fragments are spliced together via a common EcoRI site.

A strategy was devised to construct a bacterial expression vector for CaM using the chicken cDNA and a Tac promoter (Russell and Bennett, 1982) cloned into the plasmid pUC8 (Vieira and Messing, 1982). Rather than placing the promoter at random sites 5' from the CaM gene and then screening for
CaM expression, the strategy was instead directed toward the construction of a plasmid in which there is a defined distance between the Shine-Delgarno box of the bacterial promoter and the ATG initiation codon of the CaM gene. This strategy is outlined in Fig. 2.

The key feature of the constructions listed in Fig. 2 centers on the nucleotide sequence generated in pCaM4 and shown for pCaM16. This sequence contains a hybrid initiation codon in which the A is donated by the blunt-ended SalI site of pUC8 while the TG residues are donated from the BglII site of pCB12. Immediately 5' to this hybrid ATG are TaqI, BamHI, and EcoRI sites which are derived from the multiple cloning site of pUC8 and could be used for optimal placement of the Tac promoter. The CaM gene from pCaM16 was positioned 3' to the Tac promoter as described in steps 3 and 4 of Fig. 2. Eight transformed bacterial colonies resulting from step 4 were selected and grown in the presence and absence of the inducer IPTG. Bacterial lysates were then prepared and assayed for the presence of immunoreactive CaM by a CaM radioimmunoassay (Chafouleas et al., 1979). The levels of CaM, produced by bacteria containing CaM expression plasmids were compared to bacteria containing either pTac2 or pCaM22, a plasmid which contains the CaM gene in an inverse orientation. All bacteria containing CaM expression plasmids produced significant quantities of CaM. One recombinant, pCaM23, yielded 2–3% of its total soluble protein as CaM (see Fig. 3). Production of CaM by this plasmid was stimulated 8–12-fold by IPTG.

The relatively high levels of CaM expression in the recombinant plasmid were somewhat unexpected. The distance between the ATG initiation codon and the endogenous Shine-Delgarno box of the Tac promoter is 14 bp in pCaM23. This is considerably greater than the 7 bp spacing present in the natural lac gene and would be expected to adversely affect the efficiency of expression even with the more efficient Tac promoter. The high levels of CaM expression may be derived from helper activity of a second Shine-Delgarno box located at the BamHI site just 7 bp 5’ from the initiation codon. To conclusively demonstrate the production of intact un-fused CaM, bacteria containing pCaM23 were metabolically labeled with [35S]Met after which CaM was immunoprecipitated with affinity-purified sheep anti-rat CaM antibody. Fig. 4 shows the results of an experiment in which extracts of bacteria containing either pTac2 or pCaM23 were compared. The bacteria containing pCaM23 produced a protein which co-migrated in an SDS gel with purified rat testis CaM and could be immunoprecipitated by the CaM antibody. This protein was not present in the control bacteria and corresponds to bacterially produced CaM which will be called CaM23. The multiple bands present in the immunoprecipitated material probably result from proteolytic digestion of CaM. Small CaM fragments can be detected if CaM is isolated from bacterial lysates by phenothiazine-Sepharose chromatography. These fragments are generally minor species; the pronouced fragment seen in Fig. 4, lane c, probably results from extended proteolysis occurring during the overnight room-temperature incubation required for immunoprecipitation.

pCaM23 was used to construct an expression plasmid for the CaM-like gene, CM1. To do this, advantage was taken of EcoRI sites which are located at homologous positions in the chicken CaM cDNA and the CM1 gene. These EcoRI sites cleave between those nucleotides which encode amino acids 11 and 12 of CaM and the putative protein product of CM1. As shown in Step 5 of Fig. 2, an EcoRI/Smal fragment was isolated from a subclone of CM1 (cCM4.3) and inserted into the EcoRI/blunt-ended PstI site of pCaM23. The resulting recombinant plasmid, pCaML1, has a CaM-like gene in which amino acids 1–11 are encoded by the chicken CaM cDNA and amino acids 12–148 are encoded by CM1. The protein product of this recombinant gene will be called CaML and has 16 of the 19 amino acid substitutions present in the predicted protein product of the CaM-like gene. Levels of expression of CaML should be equivalent to CaM23 since the transcriptional/translational determinants of pCaM23 are retained.

Fig. 5 shows SDS gels of total and heat-stable proteins from bacteria which contain either pTac2, pCaM23, or pCaML1. Both total and heat-stable extracts of bacteria containing pCaM23 (lanes B) or pCaML1 (lanes C) exhibit a protein which co-migrates with purified rat testis CaM. These major heat-stable proteins are absent in the control bacteria (lanes A) and correspond to bacterially synthesized CaM23 and CaML. There is a minor heat-stable protein present in the control bacteria which co-migrates with rat CaM. However, this protein is not immunoprecipitated with the CaM antibody and does not exhibit a Ca2+-induced electrophoretic migration shift characteristic of CaM (see Fig. 6). The concentration of CaM23 and CaML in the bacterial extracts is probably greater than would be estimated from Fig. 5 since CaM is inefficiently stained by Coomassie Blue.

In the presence of Ca2+, CaM will bind to both phenyl-Sepharose (Gopalakrishna and Anderson, 1982) and phenothiazine-Sepharose (Charbonneau and Cormier, 1979). The bacterially synthesized CaM23 and CaML also bound to both resins in a Ca2+-dependent manner. This indicates that the 16 amino acid substitutions present in CaML do not prevent the molecule from binding the Ca2+ necessary to induce a conformational change and they do not destroy the hydrophobic drug binding site(s).

The affinity of bacterially produced CaM23 and CaML for phenyl-Sepharose permits a very simple procedure for the purification of these proteins from crude bacterial lysates. The bacterial lysates were first passed over a phenyl-Sepharose column in the presence of EDTA. This separates the CaML or CaM23 from those bacterial proteins which bind to the column in a calcium-independent manner and could contaminate the proteins if a 1-column purification procedure were utilized. The nonabsorbed fraction from this first column was then made 5 mM in CaCl2 and reapplied to a second phenyl-Sepharose column. The column was washed with 0.5 M NaCl, and the bound CaM23 or CaML was then eluted with 1 mM EDTA. By this procedure, 3–4 mg of CaM23 or CaML can be isolated per liter of bacterial culture in 1 day with greater than 85% recovery. The purity of the isolated proteins can be judged by the gel shown in Fig. 6. Phenyl-Sepharose has the advantage over phenothiazine-Sepharose in that it is more stable and under the conditions employed here, it will not bind the small M, CaM fragments that are present in lysates of bacteria containing pCaM23. Phenothiazine-Sepharose will bind CaM fragments present in the bacterial extracts.

The availability of the purified proteins allowed a more detailed comparison of CaM23 and CaML and a demonstration that the proteins were faithfully synthesized. A preliminary amino acid analysis confirmed the predicted amino acid differences between CaM23 and CaML and the identity between CaM23 and rat CaM. Amino acid analysis also indicated that neither CaM23 nor CaML have a trimethyllysine. Two-dimensional gel electrophoresis showed CaM23 to be less acidic than CaM23 which is consistent with the predicted amino acid differences. Finally, when aliquots of both CaM23
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**Fig. 2. Construction of bacterial expression plasmids for CaM and CaML.** Maps of the three primary constructs are shown with the order of construction indicated by the large arrows. The open regions are derived from the plasmid pUC8, the black regions are derived from the chicken CaM cDNA, the striped areas indicate DNA from the CM1 gene. As indicated, these plasmids were constructed in five cloning steps. Step 1: the chicken CaM cDNA clone pCB12 was digested with BglII and treated with T₄ DNA polymerase in the presence of all four deoxyribonucleotides to generate a blunt end. The plasmid was then digested with PstI, and a 300-bp fragment containing the proximal two-thirds of the CaM gene was isolated from a polyacrylamide gel. The cloning vector pUC8 was digested with Sall, blunt ended with the Klenow fragment, and then digested with PstI. The large pUC8 fragment was separated from the small 30-nucleotide fragment by chromatography on Sepharose 4B. The 300-bp fragment from pCB12 was ligated into the pUC8 vector to yield the plasmid pCaM4.

Step 2: a 600-bp PstI fragment from pCB15 which contained the distal one-third of the amino acid coding region of chicken CaM was isolated and inserted into the PstI site of pCaM4 to generate pCaM16 which contains the complete amino acid coding region for chicken CaM. The nucleotide sequence bordering the ATG initiation codon in pCaM16 is shown. Those residues derived from pCB12 are underlined. The residues which contain the BamHI and TaqI sites are derived from pUC8. Step 3: the Tac promoter was isolated as an EcoRI/BamHI fragment from the plasmid pDR540 (Russell and Bennett, 1982) and inserted into the EcoRI/BamHI site of pUC8 to form pTac2. Step 4, the CaM gene was isolated from pCaM16 by a partial digestion with PstI followed by digestion with BamHI and inserted into the BamHI/PstI site of pTac2 to yield pCaM23 which has two Shine-Delgarno boxes located 7 and 14 bp 5' from the initiation codon. Step 5: pCaM23 was digested with PstI and treated with the Klenow fragment. The plasmid was then partially digested with EcoRI, and the second largest fragment was isolated. A subclone of CM1, cCM4.3, was used as a source of the CaM-like gene. pCM4.3 contains a 4.3-kilobase pair BamHI fragment from CL1 and has all the putative amino acid coding region of CL1 together with 2.0 kilobase pairs each of 3' and 5' flanking region. The CaM-like gene was isolated from pCM4.3 as a 471-bp EcoRI/SalI fragment which encodes all but the first 10 amino acids of the putative protein product of CM1. This fragment was then inserted into pCaM23 to yield pCaML1.
and CaML were reduced with dithiothreitol and passed over thiol-Sepharose columns, only the CaML with its two Cys residues was retained and could be eluted with Cys (data not shown).

The electrophoretic migration rate of CaM and other calcium-binding proteins such as troponin C is markedly affected by the presence of calcium. In an SDS-gel system, calcium-binding proteins such as troponin C is markedly affected by the presence of calcium. In an SDS-gel system, calcium is commonly used as one indicator of functional similarity between CaMs from a variety of species using a similarly prepared antibody (Chafouleas et al., 1979) and suggests that a common antigenic determinant(s) found in a variety of CaMs is retained in CaML.

The relative ability to activate brain cAMP phosphodiesterase in vitro is commonly used as one indicator of functional similarity between CaMs from a variety of species (Ruben et al., 1983; Schlicher et al., 1984; Head et al., 1983; Jamison and Frazier, 1983). This assay was, therefore, employed to assess the relative functional properties of rat CaM, CaM23, and CaML. The kinetics of activation of rat brain phosphodiesterase by these proteins was determined under conditions of CaM dependence (Fig. 8) and Ca2+ dependence (Fig. 9). The kinetic parameters derived from Figs. 8 and 9 are shown in Table I. Under all conditions tested the kinetics of activation of phosphodiesterase by rat CaM and CaM23 are identical.
with a \( K_{\text{CaM}} \) of 3.1 nM which agrees well with the value of 2.9 obtained for bovine testis CaM under similar conditions (Newton et al., 1984). The kinetics of CaM-dependent activation of phosphodiesterase by CaML are different than that observed for rat CaM or CaM23. While the Hill coefficients for rat CaM, CaM23, and CaML are not significantly different, the \( K_{\text{CaM}} \) for CaML is almost 3-fold greater than the \( K_{\text{CaM}} \) for rat CaM and CaM23. This suggests that the nature of the interaction between phosphodiesterase and CaM or CaML is similar but that CaML has less affinity for phosphodiesterase than does CaM. Alternatively CaM and CaML may have similar affinities for phosphodiesterase but, once bound, CaML may have a decreased ability to activate the enzyme.

The Hill coefficient for Ca\(^{2+}\)-dependent activation of phosphodiesterase by rat CaM or CaM23 is 4–5 and suggests the presence of multiple positively cooperative Ca\(^{2+}\)-binding sites required for activation. This is consistent with other studies indicating that 3 or 4 Ca\(^{2+}\) must be bound to fully activate phosphodiesterase (Crouch and Klee, 1980). The Hill coefficient for Ca\(^{2+}\)-dependent activation of phosphodiesterase by CaML is less than half that observed for activation by rat CaM or CaM23. This suggests that either Ca\(^{2+}\) binding differs
protein activator at varying CaCl₂ concentrations as described under phodiesterase activation may be found in Table 8. The data for rat testis CaM and CaM23 could be fitted between the two proteins or that the amino acid substitutions in CaML impose structural constraints on the molecule that evolved from a CaM processed gene (Stein et al., 1983). The second expression plasmid contains a CaM-like gene prepared to CaM. If the subsequent activation process was altered, then maximal activation of phosphodiesterase by both CaML and CaM would not be equivalent.

Phosphodiesterase is only one of many CaM-dependent enzymes. It is probable that CaML will be incapable of substituting for CaM in one or more of these activation processes. We are currently evaluating the effectiveness of CaML in stimulation of several other CaM-dependent enzymes. Also, the CaM protein described here has only 16 of the 19 amino acid substitutions that are encoded by the CM1 gene. The three amino acid substitutions which CaML does not contain are clustered in the amino-terminal portion of the molecule at positions 2, 3, and 5. The inclusion of these amino acid substitutions could adversely affect the ability of CaML to

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**TABLE 1**

| CaM dependence | Ca²⁺ dependence |
|----------------|-----------------|
| K<sub>CaM</sub>, n<sub><sub>CaM</sub></sub>, r<sup>2</sup> | K<sub>CaM23</sub>, n<sub><sub>CaM23</sub></sub>, r<sup>2</sup> |
| Rat testis CaM | 3.1, 1.2, 0.99  | 4.6, 0.99 |
| CaM23         | 3.1, 1.3, 0.99  | 4.8, 5.3, 0.97 |
| CaML          | 8.8, 1.2, 0.99  | 0.58, 2.1, 0.97 |

between the two proteins or that the amino acid substitutions in CaML impose structural constraints on the molecule which prevent it from assuming a fully active configuration similar to CaM.

**DISCUSSION**

In this paper we describe the construction of expression plasmids for two chicken CaM genes. One plasmid contains a normal gene derived from the cDNA for chicken CaM (Putkey et al., 1983). This plasmid directs the synthesis of chicken CaM which is not N-acetylated and lacks a trimethyllysine at position 115 but is otherwise physically and functionally indistinguishable from purified rat CaM by all criteria tested. The second expression plasmid contains a CaM-like gene obtained from a chicken genomic clone which appears to have evolved from a CaM processed gene (Stein et al., 1983). The bacterially synthesized protein product of the CaM-like gene has been called CaML and contains 16 amino acid substitutions as compared to normal CaM. The purpose for constructing these expression plasmids was to compare and contrast the physical and biological properties of the respective protein products with each other and with purified rat CaM.

Several lines of evidence reveal that CaML will bind Ca²⁺ and undergo a conformational change. First, CaML undergoes a Ca²⁺-dependent electrophoretic migration shift, yet the magnitude of this shift was only half that seen for CaM. This indicates that either the nature or the degree of Ca²⁺ binding is different or that structural constraints in CaML prevent a larger shift even with equivalent numbers of bound Ca²⁺.

A second demonstration of the calcium-binding properties of CaML was the fact that the protein bound to both phenothiazine-Sepharose and phenyl-Sepharose in a Ca²⁺-dependent manner. Studies with proteolytic fragments have demonstrated the presence of at least two hydrophobic drug-binding sites in CaM and that there is at least one site in both the carboxyl- and amino-terminal halves of the molecule (Brzeska et al., 1983; Vogel et al., 1983; Newton et al., 1984). This indicates that either the nature or the degree of Ca²⁺ binding is different or that structural constraints in CaML prevent a larger shift even with equivalent numbers of bound Ca²⁺.

FIG. 9. The Ca²⁺ dependence of calmodulin activation of phosphodiesterase. Phosphodiesterase activity was assayed at a final concentration of 10⁻¹⁰ M, in the presence of 100 nM of each protein activator at varying CaCl₂ concentrations as described under "Experimental Procedures." The data were calculated and graphed as noted in Fig. 8. Each data point represents the average of three experiments. The data for rat testis CaM and CaM23 could be fitted to a single line with a K<sub>CaM</sub> of 0.47 μM, n<sub><sub>CaM</sub></sub> = 4.3, and r<sup>2</sup> = 0.97. A summary of the kinetic parameters for the Ca²⁺ dependence of phosphodiesterase activation may be found in Table 1.
activate phosphodiesterase. An expression plasmid which encodes a CaM molecule with all 19 amino acid substitutions has now been constructed. Initially it was felt that the amino acid substitutions in CaML would interfere considerably with the CaM-like functions of the protein. This was based on an inspection of the amino acid sequences of Dictyostelium CaM (Marshak et al., 1984), Tetrahymena CaM (Yazawa et al., 1981), and spinach CaM (Burgess et al., 1983). A comparison of the sequences of these proteins to the 148 amino acids of chicken CaM reveal direct confirmed amino acid substitutions of 13, 11, and 12, respectively, while CaML has 16 substitutions. The position of these substitutions is also a distinguishing feature of CaML. Of the 36 combined substitutions in the 3 naturally occurring CaMs 80% occur in domains III and IV, and only 10 are at unique sites. In CaML, domain III is devoid of substitutions, 8 of 16 substitutions are in domains I and II, and 9 substitutions are at unique sites. By this comparison the 16 substitutions in CaML appear more random than those present in naturally occurring CaMs and might be expected to dramatically alter the properties of this highly conserved protein. Yet, the properties of CaML described here are very similar to CaM. This forces us to conclude that either the functional domains of CaM are more tolerable to mutation than previously thought or that the mutations in the CM1 gene are not random and that it serves some useful purpose to the cell to retain CaM-like properties in the putative protein product of CM1.

An inspection of the nucleic acid and predicted amino acid sequence of CM1 implicates a nonrandom mechanism of mutation. Of the 117 point mutations in CM1, 88 occur in the third codon position and all but five of these are silent. This is similar to the human metallothionine II processed gene in which 7 of 8 point mutations are silent and in the third codon position (Karim and Richards, 1982). Fig. 1 reveals that the spacial distribution of the amino acid substitutions in CM1 is not random. Domain III of CM1 is devoid of substitutions, and most are clustered in groups of 2 or 3. An indication of the potential functional conservation of the amino acid substitutions encoded by CM1 can be appreciated by comparing these substitutions to amino acids in homologous positions of other Ca"+-binding proteins. There are 4 amino acid substitutions in CaML which are identical to those found in Tetrahymena CaM or spinach CaM. In addition, 9 substituted positions in CM1 encode amino acids which are found in homologous positions in rabbit skeletal muscle or bovine cardiac muscle troponin C (Collins et al., 1977; Van Eerd and Takahashi, 1976). In total, 13 of the 19 amino acid substitutions encoded by CM1 are identical to amino acids found at homologous positions in 4 other functional Ca"+-binding proteins. Thus, the nucleic acid mutations seen in the CM1 gene seem to result in nonrandom amino acid substitutions as judged by both spacial distribution and functional criteria.

The question that naturally arises is what evolutionary pressure exists that would induce selective mutations in a gene which is apparently a CaM processed gene. Stein et al. (1983) showed that a hybridization probe derived from CM1 recognized a mRNA species present in chicken muscle. Therefore, the CM1 gene could have undergone selective mutations and evolved to encode a protein that is expressed and performs a subset of CaM activities or has acquired new activities which are vital to the survival of the organism. The presence of the mRNA species in muscle is intriguing since 9 of 19 amino acid substitutions encoded by CM1 are identical to those found in homologous positions in troponin C. This could impart troponin C-like functions to the protein which could be utilized in muscle. If the protein exists in tissues as a minor CaM species it would have properties similar to bacterially synthesized CaML and be virtually indistinguishable from the bulk of the CaM by techniques which are usually employed to assess CaM. The aberrant electrophoretic migration of CaML as compared to CaM could be interpreted to be a small fraction of CaM molecules which migrate abnormally in the presence and absence of Ca"+ due to an artifact of the isolation procedure. Burgess et al. (1980) have demonstrated that such artifacts occur when CaM is heated. We are now exploiting the properties of the bacterially synthesized CaML in an effort to detect a similar protein in chicken tissue.

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