Purification and Characterization of a Ca\textsuperscript{2+}-binding Protein in *Lumbricus terrestris*\textsuperscript{*}

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A Ca\textsuperscript{2+}-binding protein which is capable of activating mammalian Ca\textsuperscript{2+}-activatable cyclic nucleotide phosphodiesterase has been purified from *Lumbricus terrestris* and characterized. This protein and the Ca\textsuperscript{2+}-dependent protein modulator from bovine tissues have many similar properties. Both proteins have molecular weights of approximately 18,000, isoelectric points of about pH 4, similar and characteristic ultraviolet spectra, and similar amino acid compositions. Both proteins bind calcium ions with high affinity. However, the protein from *Lumbricus terrestris* binds 2 mol of calcium ions with equal affinity, \( K_{\text{diss}} = 6 \times 10^{-8} \) M, whereas the Ca\textsuperscript{2+}-dependent protein modulator from bovine tissues binds 4 mol of calcium ions with differing affinities. Although the Ca\textsuperscript{2+}-binding protein of *Lumbricus terrestris* activates the Ca\textsuperscript{2+}-activatable cyclic nucleotide phosphodiesterase from mammalian tissues, we have failed to detect the existence of a Ca\textsuperscript{2+}-activatable phosphodiesterase activity in *Lumbricus terrestris*. The activation of phosphodiesterase by the Ca\textsuperscript{2+}-binding protein from *Lumbricus terrestris* is inhibited by the recently discovered bovine brain modulator binding protein (Wang, J. H., and Desai, R. (1977) J. Biol. Chem. 252, 4175-4184). Since the modulator binding protein has been shown to associate with the mammalian protein modulator to result in phosphodiesterase inhibition, it can be concluded that the *Lumbricus terrestris* Ca\textsuperscript{2+}-binding protein also associates with the bovine brain modulator protein. Attempts to demonstrate the existence of a similar modulator binding protein in *Lumbricus terrestris* have been unsuccessful.

The protein modulator has been purified from several mammalian tissues (12-15) as well as from electroplax of the electric eel (16). Based primarily on the similarity in physical, chemical, and Ca\textsuperscript{2+}-binding properties of the protein modulator and troponin C, the Ca\textsuperscript{2+}-binding subunit of the regulatory protein of muscle, we have suggested that these two proteins are homologous proteins (17, 18). This suggestion has been confirmed by recent sequence information of the protein modulator (19). Furthermore, it has also been demonstrated that the protein modulator can substitute for troponin C in the mediation of Ca\textsuperscript{2+} activation of actomyosin ATPase (20, 21).

In a previous report (22) we investigated the phylogenetic distribution of the protein modulator in the animal kingdom. A set of criteria for the demonstration of protein modulator activity in crude animal extracts without purification was developed. Essentially, heat-treated, dialyzed, homogenate supernatants of animal species representative of major phyla were prepared and tested for Ca\textsuperscript{2+}-dependent activation of bovine heart cyclic nucleotide phosphodiesterase. Since extracts of all animal species examined activated bovine heart phosphodiesterase to a comparable maximal extent and all activations were Ca\textsuperscript{2+}-dependent and reversible, we suggested that the modulator is ubiquitous in the animal kingdom. Furthermore the activation of mammalian phosphodiesterase by extracts from lower animals was interpreted as suggesting that the structure of the protein modulator was conserved during evolution.

In the present study, the Ca\textsuperscript{2+}-dependent protein modulator has been purified from an invertebrate source, *Lumbricus terrestris*, and its physical and chemical properties have been examined. The results support previous postulates that the protein modulator is ubiquitous in the animal kingdom and that its structure is highly conserved. In addition, we have demonstrated in this study a lack of Ca\textsuperscript{2+}-activatable cyclic nucleotide phosphodiesterase in *L. terrestris*. The results suggest that the functions of the protein modulator in *L. terrestris* are not identical to those of protein modulator in mammalian tissues.

**EXPERIMENTAL PROCEDURES**

Materials and Protein Preparations—Chemicals and protein samples used in this work are described in the miniprint supplement.\textsuperscript{1}

\textsuperscript{1} Portions of this paper (including Figs. 1s, 2s, 3s, 4s, Table 1s, and additional Refs. 1-3) are presented in miniprint following the
**Phosphodiesterase Assay**—When millimolar concentrations of cyclic nucleotides were used, the enzyme activity was normally measured by the colorimetric method described previously (12) unless indicated otherwise. At low concentrations of the nucleotides, the assay was carried out according to the procedure of Wicken et al. (23). Essentially, each incubation tube contained 44 mM Tris-HCl, 3 mM magnesium acetate, and 1 mM or 10 mM cyclic nucleotide in a final volume of 0.2 ml. The reaction was started by the addition of substrate. After incubation at 30° for 30 min, the reaction was stopped by addition of 0.01 ml of 55% trichloroacetic acid; an aliquot of the supernatant was spotted along with appropriate carriers (0.1 ml of cyclic nucleotide, nucleotide, and nucleoside) on Whatman No. 3MM paper and chromatographed (descending) for 19 h using 1 M ammonium acetate, 0.01 ml of 55% trichloroacetic acid; an aliquot the method of Lowry et al. (24) using bovine serum albumin as the standard. The reaction velocity was determined by scintillation spectrometry. Reaction velocity was determined from the amino acid composition of the standard.

**Amino Acid Analysis**—Samples (0.3 to 0.6 mg) of reduced and alkylated protein were hydrolyzed with 6 N HCl containing 2 μl of thioglycolic acid and 50 μl of 5% phenol at 110° in sealed evacuated tubes for 21, 48, and 72 h. Analyses were performed on a Spinco 120/139 amino acid analyzer as outlined in the Spinco manual.

**Proteins and Alkaloids**—Protein samples were dissolved in 0.1 M Tris-HCl pH 8.5, containing 6 mM guanidine-HCl, 1 mg of EDTA/ml, and 0.1 M diethanol. After 2 h at room temperature an equal volume of 0.2 M Tris-HCl, pH 8.5, containing 0.05 M iodoacetic acid was added to the reaction mixture. After 2 h at room temperature the reaction mixture was extensively dialyzed versus distilled water and freeze-dried.

**Digestion with Trypsin and Peptide Mapping**—The protein sample, about 0.5 mg, was dissolved in 100 μl of 0.1 M ammonium bicarbonate, 0.1 mM EGTA. The solution was saturated with nitrogen and 5 μl of N-tosyl-l-phenylalanine chloromethyl ketone (1 mg/ml in 0.1 M ammonium bicarbonate) were added: the tube was covered, mixed and incubated at 37° for 2 h. A sample (50 μl) of the digest was applied to Whatman No. 3MM paper and subjected to peptide mapping. High voltage electrophoresis was performed in a Savant electrophoresis tank at pH 4.7 according to Tan and Stevens (31) using methyl green (1%) as marker. The digest was then run on a 25-cm column of Sephadex G-25 (4.5 x 15 cm) to remove the chloramine-T. The column used was a plastic Pharmacia K9 column (30). The protein was eluted and analyzed with a Biochrom 19 absorbance spectrophotometer model 960 with the aid of a spinco manual.

**Removal of Ca++ from Reagents**—Chelex 100, a resin specific for chelating divalent cations, was used for removing contaminating Ca++. The resin was washed once with 1 N HCl and then with 1 N NaOH prior to the packing of the column. The packed column was then washed with double-distilled water. Double-distilled water, Tris/HCl (0.5 M), and imidazole (10 mM) were separately passed through Chelex 100 column (6 x 1.5 cm) to remove Ca++. Plastic columns and connections were used in the chromatography. Purified reagents were always stored in plastic containers and all reactions were carried out in plastic vessels. A Perkin-Elmer atomic absorption spectrophotometer model 303 was used to monitor the calcium concentration. After Chelex 100 treatment, the calcium content of stock reagents was below the limit of detection (4 ppm). Calcium was removed from protein modulator and phosphodiesterase by treatment with 1.0 mM EGTA for 30 min at 4° followed by gel filtration on Sephadex G-25 (4.5 x 1.5 cm) to remove the chelating agent. Chelex 100-treated water and buffer were used in all steps.

**Equilibrium Ca++ Binding**—The gel filtration method of Hummel and Dreyer (33) as modified by Fairleigh and Fruton (34) was used. A column (45 x 0.9 cm) of Sephadex G-25 was equilibrated at 22° with buffer containing 25 mM Tris/HCl, 25 mM imidazole, and 0.1 M magnesium acetate with a known concentration of Ca++. A sample of desalted modulator protein (48 μg in 0.6 ml) was then applied to the column and the column was eluted with the equilibrating buffer. Gel filtration was carried out at 22° at a flow rate of 5 ml/h, and 0.6-ml fractions were collected. Aliquots (100 μl) of each fraction were analyzed for radioactivity in a Beckman LS-55 liquid scintillation spectrometer. The column used was a plastic Pharmacia F40 column. Chelex 100-treated reagents were used throughout. The scintillator mixture was composed of 125 g of naphthalene, 7.5 g of 2,5-diphenyloxazole, and 0.375 g of 1,4-bis[2-(5-phenyloxazolyl)]benzene filter of dioxane.

**RESULTS**

**Purity of Earthworm Protein Modulator**—The protein modulator was purified 650-fold from the earthworm homogenate. The purified protein has a specific activity of 76,000 units/mg and appears essentially homogeneous on the analytical urea-sodium dodecyl sulfate-polyacrylamide disc gel electrophoresis (Fig. 1). The presence of faint bands of impurity were observed.
when 75 µg of sample were electrophoresed in 15% polyacrylamide (Fig. 1C). Denaturation of this gel suggests the impurity to be less than 5%. Polyacrylamide gel electrophoresis in sodium dodecyl sulfate of the purified earthworm sample (Fig. 1D) revealed the presence of two major bands of equal intensity. Since electrophoresis of earthworm modulator on sodium dodecyl sulfate gels in the presence of urea (Fig. 1, F and G) showed only one band, anomalous behavior for the earthworm modulator on sodium dodecyl sulfate-polyacrylamide gels is suggested. Further evidence of homogeneity was suggested by the presence of a single protein band on gel isoelectric focusing (results not shown); the isoelectric point determined for the protein modulator by this method is pH 4.0.

Physical Parameters—In sedimentation velocity experiments, the purified protein modulator exhibited a single symmetrical peak in its schlieren pattern with an extrapolated sedimentation constant of 1.95 S. The diffusion constant determined using the ultracentrifuge has a value of $9.25 \times 10^{-7}$ cm$^2$/s. Using these values and a partial specific volume determined for the protein modulator by this method is pH 4.0.

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A comparison of the physical parameters of bovine heart and earthworm protein modulators is presented in Fig. 2. From the amino acid analysis (Table II) a total of 12 residues of lysine and arginine were found/mol of protein. If the preparation is homogeneous and consists of a single polypeptide chain the trypic digest should contain a maximum of 13 peptides. The number of clearly visible peptides observed was 20. It therefore appears that some nonspecific peptide bond hydrolysis has taken place during digestion.

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Tryptic Peptide Mapping—A comparison of the tryptic maps of the earthworm and bovine heart modulators is presented in Fig. 2. From the amino acid analysis (Table II) a total of 12 residues of lysine and arginine were found/mol of protein. If the preparation is homogeneous and consists of a single polypeptide chain the trypic digest should contain a maximum of 13 peptides. The number of clearly visible peptides observed was 20. It therefore appears that some nonspecific peptide bond hydrolysis has taken place during digestion.

| Parameter | Earthworm modulator | Bovine heart modulator$^a$ |
|-----------|---------------------|-----------------------------|
| $S_{0.2}$ | 1.95                | 2.0                         |
| $U_{0.2}$ ($\times 10^7$ cm$^2$/s) | 9.25 | 9.0 |
| Molecular weight | 15,700-18,200 | 18,500 |
| Sodium dodecyl sulfate-gel electrophoresis | | |
| $E_{275-280}$ (1% protein, 1 cm) | 3.2 | 1.9 |
| $\psi$ | 0.73 | 0.72 |

$^a$ From Wang et al. (17) and Stevens et al. (18)

A comparison of the amino acid composition of earthworm modulator is shown in Table II. The notable features of the amino acid composition include the low content of histidine and tyrosine, the high content of acidic residues, and the high phenylalanine to tyrosine ratio.

A comparison of the amino acid composition of the earthworm and bovine heart modulator indicates that the two proteins are remarkably similar. They have identical numbers of residues of threonine, methionine, isoleucine, leucine, and tyrosine and both lack tryptophan. Furthermore, both proteins contain approximately 35% acidic residues. The major differences between the earthworm and bovine heart modulator appear to be the presence of 1 residue of cysteine and a significantly higher proline content in the earthworm modulator.

| Amino acid composition of earthworm and bovine heart protein modulators |
|-----------------------------|-----------------------------|----------------------------- |
| Amino acid | 21-h hydrolysate | 48-h hydrolysate | 72-h hydrolysate | Average or extrapolated value | Nearest integer | Bovine heart |
| Lysine | 7.12 | 7.03 | 7.28 | 7.14 | 7 | 9$^a$ |
| Histidine | 1.48 | 1.41 | 1.48 | 1.46 | 1-2 | 1 |
| Arginine | 5.28 | 5.26 | 5.39 | 5.31 | 5 | 6 |
| Aspartic acid | 23.98 | 23.79 | 23.86 | 23.88 | 24 | 25 |
| Threonine | 11.54 | 10.84 | 10.75 | 11.7 | 12 | 12 |
| Serine | 6.00 | 5.71 | 5.24 | 6.3 | 6 | 3 |
| Glutamic acid | 99.06 | 98.28 | 98.86 | 98.95 | 29 | 30 |
| Proline | 5.39 | 6.01 | 6.18 | 5.86 | 6 | 2 |
| Glycine | 13.20 | 12.92 | 13.05 | 13.02 | 13 | 12 |
| Alanine | 10.76 | 10.03 | 10.91 | 10.87 | 11 | 12 |
| Cysteine$^b$ | 0.73 | 0.71 | 0.65 | 0.70 | 1 | 0 |
| Valine | 7.13 | 7.51 | 7.44 | 7.36 | 7 | 9 |
| Methionine $^c$ | 8.63 | 9.21 | 8.96 | 8.93 | 9 | 9 |
| Isoleucine | 8.23 | 8.47 | 8.33 | 8.34 | 8 | 8 |
| Leucine | 9.70 | 9.97 | 9.84 | 9.84 | 10 | 10 |
| Tyrosine | 1.89 | 1.78 | 1.84 | 1.84 | 2 | 2 |
| Phenylalanine | 7.92 | 7.70 | 8.08 | 7.9 | 8 | 9 |
| Tryptophan$^c$ | 0 | 0 | | 0 | 0 | 0 |

$^a$ This value for lysine includes 1 residue of 3-methyl-lysine which does not separate from lysine in the acid hydrolysate method. From Stevens et al. (18) and Watterson et al. (19).

$^b$ Determined as carboxymethylcysteine in the hydrolysate of the reduced carboxymethylated protein.

$^c$ By the spectrophotometric method of Goodwin and Morton (35).
with trypsin. This has been shown to be the case for the bovine brain modulator (18).

Electrophoretic Analysis of Protein Modulator in Animal Extracts—It has been suggested that invertebrates are a rich source of the protein modulator since heated and dialyzed extracts of many representative species are highly potent in the Ca\(^{2+}\)-dependent activation of mammalian phosphodiesterase (22). As is shown in Fig. 3, when several of the heated and dialyzed animal extracts were subjected to electrophoresis on 15% polyacrylamide gels according to the procedure of Davis (25), the phosphodiesterase activating activity could be located as a single band on the gel. The mobilities of these activity bands are all similar (R\(_{v}\) value ranging from 0.59 to 0.62). The protein band of the purified earthworm protein modulator has a R\(_{v}\) value 0.60. In addition to those presented in Fig. 3, extracts of sponge, blue crab, and mystery snail were analyzed on polyacrylamide gel electrophoresis and similar results were obtained. The results strongly suggest that the phosphodiesterase activating activity of these invertebrate extracts are attributable specifically to the protein modulator. Furthermore, modulators from all these animals appear to have similar physical and chemical properties since the mobility of proteins on analytical disc gel electrophoresis is dictated by both the size and charge properties of the proteins.

Activation of Mammalian Phosphodiesterase—The activation of phosphodiesterase by the purified earthworm protein modulator was characterized using a modulator-deficient phosphodiesterase from bovine heart. Fig. 4A shows that this enzyme activation, like that by the bovine protein modulator, can be completely abolished by EGTA. The extent of the enzyme activation by the earthworm modulator is identical to that achieved by the bovine modulator. In addition, the similarity in specific activities of the earthworm and bovine modulator (5) suggests that the two proteins have similar affinity toward the enzyme. Similar results were obtained when a preparation of the bovine brain phosphodiesterase was used to characterize the activation properties of the earthworm protein modulator (results not shown).

Fig. 4B depicts the activation of the modulator-deficient bovine heart phosphodiesterase by Ca\(^{2+}\) both in the presence and absence of added earthworm protein modulator. The enzyme activation can be observed only in the presence of added protein modulator. The amount of Ca\(^{2+}\) required for 50% activation of the enzyme is determined to be 2.0 \(\mu\)M, this value may be compared with that obtained for the enzyme activation by bovine heart protein modulator under similar conditions, 2.3 \(\mu\)M (5).

Equilibrium Ca\(^{2+}\) Binding—The equilibrium interaction between the earthworm protein modulator and Ca\(^{2+}\) has been
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FIG. 4. A, dose-response curve for the activation of bovine heart phosphodiesterase by purified earthworm protein modulator in the presence (△) or absence (○) of 800 μM EGTA. EGTA was used to chelate Ca²⁺ which normally included in the enzyme assay at a concentration of 100 μM. B, activation of bovine heart modulator-deficient phosphodiesterase by varying concentrations of Ca²⁺ indicated. The activation was assayed either in the presence of 25 units of earthworm modulator (△) or in the absence of added modulator (○).

studied by the gel filtration method (33). The stoichiometry of the Ca²⁺ binding and the dissociation constant of the Ca²⁺-protein complex were calculated from a Scatchard plot (Fig. 5). The plot consists of a single straight line and from the slope of this line the dissociation constant is calculated to be 6 μM. The intersection of the line at 1.7 mol/mol of modulator indicates two Ca²⁺ binding sites per molecule of the modulator. Furthermore, the linearity of the line suggests that the binding sites behave as independent, noncooperative sites.

Interaction with Modulator Binding Protein—A protein which forms a Ca²⁺-dependent complex with the bovine modulator protein has been recently reported in bovine brain (36, 37). This protein, called the modulator binding protein, has no known function and is therefore assayed on the basis of its ability to counteract the activation of phosphodiesterase by the protein modulator. Fig. 6 shows that the activation of bovine brain phosphodiesterase by earthworm protein modulator can be completely overcome by bovine brain modulator binding protein. The results suggest that the earthworm modulator, like the bovine modulator, is capable of specific interactions with the modulator binding protein.

Search for Ca²⁺-activatable Phosphodiesterase and Phosphodiesterase Inhibitory Protein—In order to establish the function of the Ca²⁺-dependent protein modulator in the earthworm, the possible existence of a Ca²⁺-activatable cyclic nucleotide phosphodiesterase was investigated. As is shown in Table III, both cAMP and cGMP phosphodiesterase activities can be demonstrated in the crude earthworm extract under a variety of conditions but they do not appear to be inhibited by EGTA. In a separate experiment 1 μM cGMP was used as the substrate and similar results were observed. Furthermore, EGTA appears to stimulate the enzyme at millimolar concentrations of cAMP.

To further test the possible existence of a Ca²⁺-activatable cyclic nucleotide phosphodiesterase, an earthworm extract was chromatographed on a DEAE-cellulose column (2.6 × 30 cm). The column was pre-equilibrated with a pH 7.5 buffer containing 40 mM Tris(HCl), 1 mM MgAc₂, 0.050 mM CaCl₂, and 10 mM β-mercaptoethanol and eluted with 750 ml of a NaCl gradient of 0.1 to 0.8 M in the same buffer. Two peaks
containing cyclic nucleotide phosphodiesterase activity were eluted at salt concentrations of about 0.25 and 0.6 M. Both peaks were assayed at 10 μM cAMP as well as 10 μM cGMP at pH 8.5 and found to be insensitive to EGTA. These results suggest that the earthworm does not contain a Ca"+-activatable cyclic nucleotide phosphodiesterase.

All modulator-regulated proteins exhibit specific association with the protein modulator (8, 9, 11, 17). The possible existence in earthworm of proteins capable of binding to the protein modulator was therefore investigated. Such proteins are then expected to counteract the activation of phosphodiesterase by the protein modulator, and therefore, may be detected as inhibitory proteins of the Ca"+-activatable phosphodiesterase. To analyze its inhibitory activity, the sample has to be freed of its endogenous protein modulator first. To this end, a batch DEAE-cellulose fractionation method has been previously developed to remove protein modulator from modulator binding protein in crude bovine brain extract (36). This method was used in this study; in one experiment, crude earthworm was treated with DEAE-cellulose according to the batch DEAE-cellulose fractionation procedure, and the treated sample was then assayed for phosphodiesterase inhibitory activity; no inhibitory activity was detected. In another experiment, an extract of 50 g of earthworm was chromatographed on a DEAE-cellulose column (2.1 × 19 cm) under conditions identical to those employed for the search of Ca"+-activatable phosphodiesterase (see above). The modulator activity was found to be eluted as a single peak with maximum activity occurring at 0.3 M NaCl. Thus, the crude and the highly purified protein modulator appear to have identical behavior on DEAE-cellulose columns. After the modulator protein activity had been located, fractions both preceding and following the modulator activity peak were assayed for phosphodiesterase inhibitory activity; again no inhibitory activity was detected.

### DISCUSSION

In a previous study (22), crude extracts of more than 10 representative invertebrate species were examined and all were found to activate bovine heart cyclic nucleotide phosphodiesterase in a Ca"+-dependent and reversible manner. Based mostly on this observation, we have suggested that the Ca"+-dependent protein modulator is ubiquitous in the animal kingdom (22). The present study describes the purification of the protein modulator from one of those invertebrates: Lumbricus terrestris. The purified earthworm protein modulator is shown to be very similar to bovine protein modulator in many physico-chemical properties such as molecular weight, amino acid composition, disc-gel electrophoretic mobility, etc. In addition, when extracts of several other invertebrates are subjected to disc gel electrophoretic analysis, the modulator activities are all located in a single band with mobility essentially the same as that of the purified protein modulator. Thus, present results substantiate the previous suggestion of a ubiquitous distribution of Ca"+-dependent protein modulator in the animal kingdom.

The purified earthworm protein modulator has essentially the same potencies as bovine modulator in the activation of mammalian cyclic nucleotide phosphodiesterase. In addition, earthworm modulator appears capable of specific interaction with bovine brain modulator binding protein. These results, along with the general similarity in physico-chemical properties of earthworm and bovine modulators, suggest that protein modulator is highly conserved. In this respect, it is noted that rabbit skeletal muscle troponin C, which has a high degree of homology with bovine protein modulator, has been shown by some investigators to be inactive as a phosphodiesterase activator (17, 18, 37) and by others as a very poor substitute for modulator in phosphodiesterase activation (21). The apparent lack of similarity in tryptic maps of earthworm and bovine protein modulators does not necessarily argue against the notion that the Ca"+-dependent protein modulator structure is conserved to a high degree during evolution since highly homologous proteins giving rise to dissimilar peptide maps have been previously observed (15).

The Ca"+-dependent protein modulator belongs to a family of homologous proteins with diverse functions. The family of proteins includes troponin C, myosin light chains, and the parvalbumins. Evolutionary relationships among these proteins are being actively studied in several laboratories (38, 42). Among these proteins, protein modulator appears to be the most widely distributed in nature; in addition to being present in all animal species examined, modulator activity has been detected in several species of higher plants which have been examined. Since modulator is widely distributed and reasonable quantities of pure samples of the protein can be readily obtained, it is suggested that this protein is especially suitable for phylogenetic developmental studies.

Two groups of investigators (20, 21) have recently found that protein modulator in mammalian tissues can substitute for troponin C in the mediation of rabbit skeletal muscle actomyosin ATPase. This observation has raised the possibility that the Ca"+-dependent protein modulator found in these lower forms of animal is in fact troponin C. Several observations, however, appear to argue against such a possibility: (a) troponin C from skeletal muscle of rabbit is at best a poor substitute for protein modulator in phosphodiesterase activation; (b) modulator activity is mostly found in the supernatant of the low ionic strength extract of the animal; (c) the modulator in crude extracts is eluted from DEAE-cellulose column at the same position as the purified modulator, suggesting that the protein exists as a single protein in the extract whereas troponin C is a subunit of a protein complex, troponin; (d) animal species whose muscle is controlled by a myosin-linked rather than a troponin-linked Ca"+-regulation also have high amounts of the modulator activity in their muscle extracts. However, none of the above observations argue against a possibility that the protein modulator plays the role of troponin C as one of its functions in these animals. Such a possibility should be further studied.

Several investigators have shown the great abundance of the protein modulator activity over that of Ca"+-activatable

### TABLE III

| pH  | Substrate | Rate of Hydrolysis* |
|-----|-----------|---------------------|
|     |           | +Ca""               | +EGTA                |
|     |           | pmol/min/mg         |                      |
| 5.5 | 10 μM cAMP  | 0.2                 | 0.2                  |
| 7.6 | 1 mM cAMP  | 120.0               | 160.0                |
| 8.5 | 10 μM cAMP  | 1.8                 | 1.9                  |
| 8.5 | 1 mM cAMP  | 260.0               | 330.0                |
| 8.5 | 10 μM cGMP  | 2.0                 | 2.0                  |
| 7.5 | 1 mM cGMP  | 10.0                | 10.0                 |

* EGTA, 500 μM, or 50 μM CaCl₂, was added.

D. M. Waisman, unpublished observation.
phosphodiesterase in various mammalian tissues, animal species, and cultured cells (15, 22, 43–45). This observation has led to the suggestion that the protein modulator has function(s) in addition to the regulation of cyclic nucleotide metabolism. The failure to detect Ca²⁺-activatable phosphodiesterase in earthworm extract under a variety of assay conditions further supports this suggestion. Although the function or functions of the protein modulator in the lower animals is not yet known, it appears to require the conservation of both the Ca²⁺-dependent modulator structure since this more primitive modulator has retained the structural feature for both the Ca²⁺-dependent activation of bovine phosphodiesterase and specific interaction with bovine brain modulator binding protein. It seems plausible to suggest from the present results that the protein modulator has a more fundamental function which is expected to be operative in all animal and plant species and that the regulation of cyclic nucleotide metabolism by the protein modulator is a more recent development in the animal kingdom.

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Ca\textsuperscript{2+}-binding Protein in Lumbricus terrestris

Experimental details on the purification and characterization of the Ca\textsuperscript{2+}-binding protein from Lumbricus terrestris are provided. The protein was isolated using ion exchange chromatography and characterized using SDS-PAGE and Western blotting.

Table 1: Purification of Ca\textsuperscript{2+}-binding protein

| Fraction | Total Activity (units) | Specific Activity (units/mg protein) | Recovery |
|----------|-----------------------|-------------------------------------|----------|
| Elution  | 4.2                   | 277                                 | 100      |
| Maximal  | 3.4                   | 73                                  | 54       |
| Immunoprecipitation | 3.05  | 13.4                               | 47       |
| Immunoprecipitation | 3.10  | 18.1                               | 50       |
| Immunoprecipitation | 3.13  | 18.9                               | 6         |

Figure 1: Chromatography of Ca\textsuperscript{2+}-binding protein on a calcium column. The protein was applied to the column in 0.5 M NaCl buffer and eluted with a gradient of 0-1 M NaCl. Fractions were analyzed for Ca\textsuperscript{2+}-binding activity.

Figure 2: SDS-PAGE analysis of purified Ca\textsuperscript{2+}-binding protein. The protein was run on an 8% SDS-PAGE gel and stained with Coomassie Blue. The molecular weight of the protein was estimated to be 30 kDa.

Figure 3: Western blot analysis of Ca\textsuperscript{2+}-binding protein with anti-Ca\textsuperscript{2+}-binding protein antibody. The protein was transferred to a nitrocellulose membrane and probed with the antibody. The band was detected with horseradish peroxidase-conjugated secondary antibody.

Table 2: Summary of experimental conditions for Ca\textsuperscript{2+}-binding protein purification

1. The Ca\textsuperscript{2+}-binding protein was purified from the extract by size exclusion chromatography.
2. The protein was dialyzed against 0.1 M Tris-HCl buffer, pH 7.4, and concentrated using a Centricon 100 membrane.
3. The protein was analyzed by SDS-PAGE and Western blotting.

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