Specificity of *Tetrahymena* calmodulin in activation of calmodulin-regulated enzymes

Shuzo Kudo, Yoshinori Muto, Seiji Nagao, Michiko Naka+, Hiroyoshi Hidaka+, Mamoru Sano* and Yoshinori Nozawa

Department of Biochemistry, Gifu University School of Medicine, Gifu 500, +Department of Pharmacology, School of Medicine, Mie University, Ebashi, Tsu 514 and **Section of Cytochemistry, Department of Morphology, Institute for Developmental Research, Aichi Prefectural Colony, Kasugai 713-8, Aichi, Japan*

Received 11 October 1982

Calmodulin Tetrahymena *Evolution* *Calmodulin-dependent enzyme*

1. INTRODUCTION

Calmodulin was discovered in rat brain [1,2] and subsequently identified in a variety of tissues. It now appears to play a role of multifunctional intracellular Ca^{2+} receptor [3–5]. It is thought that in general the structure and function of calmodulin is conservatively maintained throughout the animal and plant kingdoms during evolution. We have described the isolation of calmodulin from unicellular eukaryote, *Tetrahymena pyriformis* and found that it could fully activate guanylate cyclase in this organism in a Ca^{2+}-dependent fashion [6–10]. However, the activation of guanylate cyclase was specifically attributable to the calmodulins from *Tetrahymena* and *Paramecium* [9,10]. Moreover, comparison of the primary structure of calmodulin from bovine brain [11] and *Tetrahymena* [12] shows 12 amino acid differences between the 2 proteins. Therefore, calmodulin may vary in structure and function. Here, we have compared the functional properties between the two calmodulins from *Tetrahymena* and bovine brain. The data demonstrate that both *Tetrahymena* and bovine brain calmodulins are similar in their potency to activate various calmodulin-dependent enzymes such as rat brain adenylate cyclase, myosin light-chain kinase, erythrocyte (Ca^{2+} + Mg^{2+})-ATPase and plant NAD kinase.

2. MATERIALS AND METHODS

Calmodulins were prepared from *Tetrahymena* and bovine brain as in [8]. A thermotolerant strain NT-1 of *Tetrahymena pyriformis* was grown at 39.5°C in an enriched proteose peptone medium as in [13]. The plasma membrane fraction which contains guanylate cyclase was prepared as in [13]. Calmodulin-dependent phosphodiesterase was prepared from bovine brain as in [14]. Adenylate cyclase was solubilized from washed particulate preparation of rat cerebrum using 1% Lubrol PX and elute from Ultro Gel AcA 34 column with 20 mM Tris–HCl buffer (pH 7.5) containing 0.1% Lubrol PX, 0.1 mM EGTA, 0.1 M sucrose and 1 mM EDTA as in [15]. Myosin light-chain kinase was prepared from chicken gizzard as in [16]. The light-chain kinase from chicken gizzard myosin was prepared essentially as in [17]. The light chain was separated from calmodulin by DEAE-cellulose column chromatography [18]. Erythrocyte membranes for (Ca^{2+} + Mg^{2+})-ATPase assay were prepared as in [19]. Membranes were freeze–thawed 3 times just prior to incubation for ATPase assay. The preparation of NAD kinase was obtained from pea seedling (*Pisum sativum*) grown for 12 days in natural light at 20–25°C. The NAD kinase was prepared as in [20].

Guanylate cyclase activity was assayed as in [9], cyclic AMP phosphodiesterase activity as in [8]

Published by Elsevier Biomedical Press
001457593/82/0000—0000/$2.75

and adenylate cyclase activity as in [22]. The myosin light-chain kinase assay was performed at 30°C in 0.3 ml final vol. containing 50 mM Tris–HCl (pH 7.5), 10 μM [γ-^{32}P]ATP, 10 mM $MgCl_2$, 100 μM $CaCl_2$, 25 μg myosin mixed light-chain, 1 μg myosin light-chain kinase and calmodulin. Incubation was terminated at 10 min by addition of 20% trichloroacetic acid (0.5 ml). The acid-precipitable radioactivity was determined. The (Ca^{2+} + Mg^{2+})-ATPase assay used here was essentially as in [23]. P_i was determined as in [24]. The incubation medium for NAD kinase assay contained 3 mM NAD, 5 mM ATP, 5 mM $MgCl_2$, 0.5 mM $CaCl_2$, 100 mM Tricine–KOH (pH 8.0) and an appropriate amount of the enzyme in 0.5 ml final vol. The amount of NADP formed was determined spectrophotometrically (absorption 570 nm) as in [25]. The other assay procedure from [26]. Protein concentration was determined by the Lowry method [27], using bovine serum albumin as standard.

Partially purified calmodulins were gifts from: Dr M.J. Cormier (Georgia Univ., *Renilla reniformis*); Dr D.M. Watterson (The Rockefeller Univ., *Chlamydomonas* and spinach); Dr J.H. Wang (Manitoba Univ., English cucumber); Dr Bazari (Albert Einstein College of Medicine, Yeshiva Univ., *Dictyostelium discoideum*); and Dr G.B. Witman (Princeton Univ., *Chlamydomonas*).

3. RESULTS AND DISCUSSION

Calmodulins from bovine brain and *Tetrahymena* were homogenous upon electrophoresis using 15% polyacrylamide gel with addition of either Ca^{2+} or EGTA (not shown). Both proteins were effective in activating the cyclic nucleotide phosphodiesterase of bovine brain in the presence of Ca^{2+} (table 1) as in [8,9]. However, *Tetrahymena* guanylate cyclase could be activated by *Tetrahymena* calmodulin but not by brain protein. It is well documented that several enzymes from divergent tissues require calmodulin for maximal activity [3–5]. Here, the effects of *Tetrahymena* calmodulin on these enzymes were investigated. We prepared the calmodulin-regulated enzymes from mammalian tissues and cells, and from plants. They are brain adenylate cyclase, myosin light-chain kinase, erythrocyte (Ca^{2+} + Mg^{2+})-ATPase and plant NAD kinase (table 1). It was found that *Tetrahymena* calmodulin also activated all of these enzymes in the presence of Ca^{2+}. The similar activating ability indicates a close relationship between *Tetrahymena*

Table 1

Effects of calmodulins from *Tetrahymena* and bovine brain on various calmodulin-dependent enzymes

Enzymes[a]	Calmodulin minus	*Tetrahymena* calmodulin	Bovine brain calmodulin
Guanylate cyclase (*Tetrahymena*)	40	1330 (pmol.min^{-1}.mg protein^{-1})	41
Phosphodiesterase (bovine brain)	72.6	190.5 (pmol.min^{-1}.mg protein^{-1})	203.5
Adenylate cyclase (rat brain)	273.7	385.7 (pmol.min^{-1}.mg protein^{-1})	448.5
Myosin light-chain kinase (chicken gizzard)	1.0	7.3 (pmol/min)	8.1
(Ca^{2+} + Mg^{2+})-ATPase (erythrocyte)	1.78	3.40 (P_i μmol/60 min)	3.56
NAD kinase (*Pisum sativum*)	3.0	37.2 (U[b]/min)	40.9

[a] Enzyme activities were assayed in the presence or absence of calmodulin (5 μg/assay tube) with 50 μM Ca^{2+}

[b] One arbitrary unit was defined as decrease in 0.01 absorbancy at 570 nm

and brain calmodulins and suggests that the functional capabilities of calmodulin have been conserved even among evolutionary distinct species. Then the dependence of calmodulin concentration was determined for these enzymes. Representative data are presented in fig. 1–4. Increasing amounts of either brain calmodulin or *Tetrahymena* calmodulin with Ca^{2+} caused activation of these enzymes. The maximal extent of enzyme stimulation was obtained at 1–5 μg calmodulin/assay tube. However, brain calmodulin gave a slightly higher activity than *Tetrahymena* calmodulin, for which we have no explanation as yet: It could be explained in part by differences between two calmodulins in the molecular structure or the calcium-induced conformational transition, as expected from different amino acid residues. In the presence of 0.5 mM EGTA, enzyme activation by calmodulin was diminished. In addition, EGTA was found to cause 5–15% inhibition of each enzyme activity without calmodulin, indicating that the preparation of enzymes probably contained traces of calmodulin (not shown).

We have examined calmodulins isolated from bovine brain, scallop and sea anemone, and observed that they did not activate *Tetrahymena* guanylate cyclase [8], but the calmodulin purified from *Paramecium* could stimulate the cyclase activity [9]. Calmodulin isolated from *Renilla reniformis, Chlamydomonas, Dictyostelium*, spinach and English cucumber was also tested here. However, under identical assay conditions where *Tetrahymena* calmodulin activates guanylate cyclase in this organism, none of the calmodulins could stimulate the cyclase activity (fig. 5). Therefore calmodulins from *Tetrahymena* and *Paramecium* are the only proteins known so far which implicated in guanylate cyclase activation.

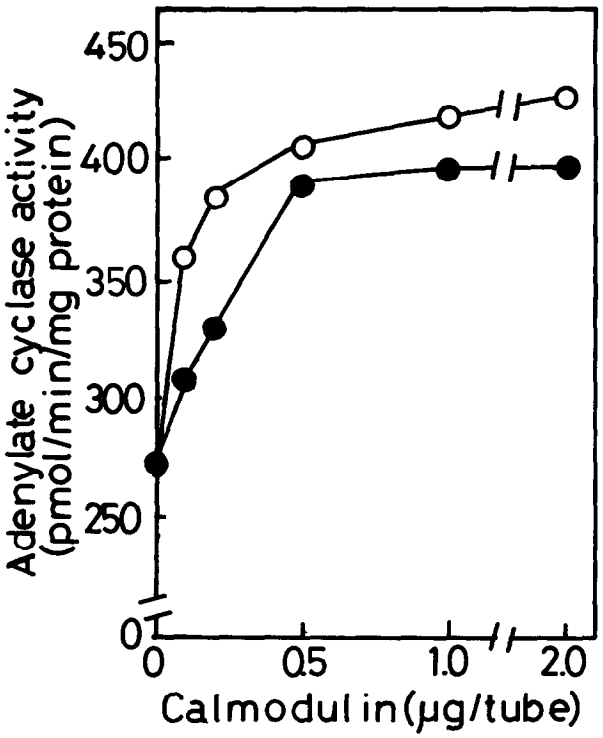

Fig. 1. Activation of brain adenylate cyclase by calmodulin. The incubation was done as in [22] in 0.15 ml final vol. The assay tube contained increasing concentration of either *Tetrahymena* (●) or bovine brain (○) calmodulin and 50 μg enzyme protein.

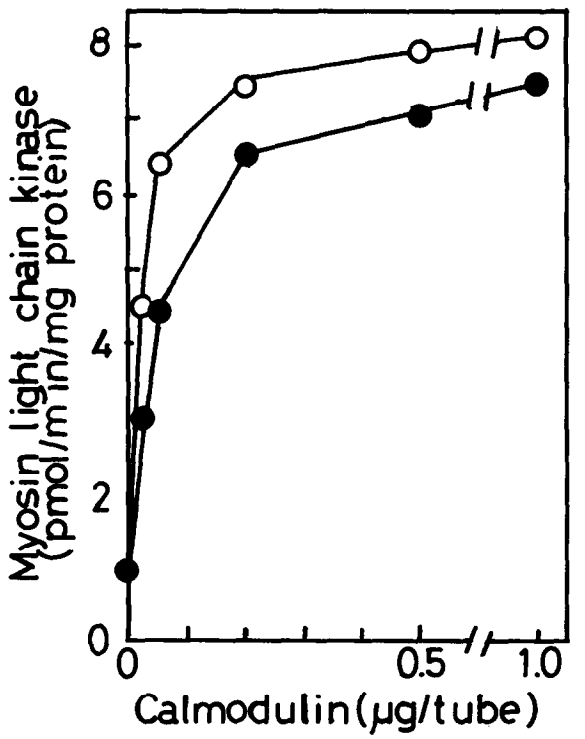

Fig. 2. Activation of myosin light-chain kinase by calmodulin. The enzyme assay was performed as described in the text. Reaction mixture in 0.3 ml final vol. contained increasing concentrations of either *Tetrahymena* (●) or bovine brain (○) calmodulin.

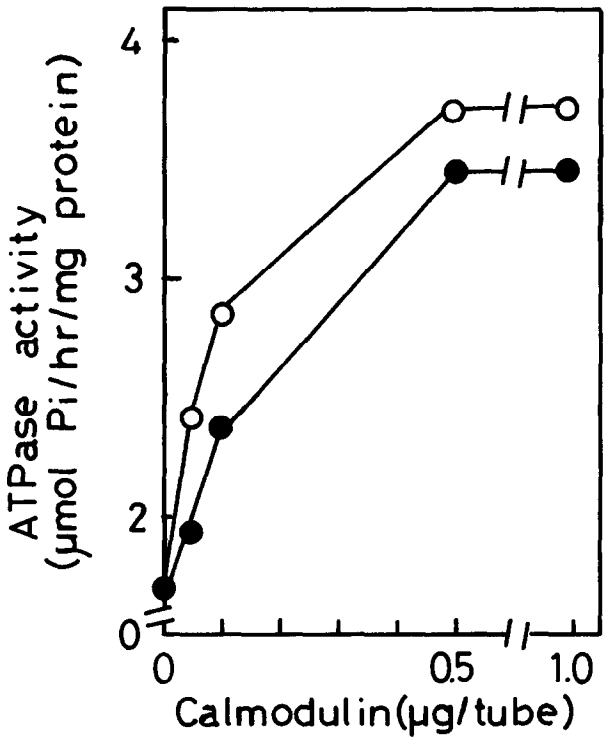

Fig. 3. Activation of erythrocyte (Ca^{2+} + Mg^{2+})-ATPase by calmodulin. Reaction mixture in 1.0 ml final vol. contained 110 μg erythrocyte ghost proteins and increasing concentrations of either *Tetrahymena* (●) or bovine brain (○) calmodulin. The assay procedure used here was essentially as in [33].

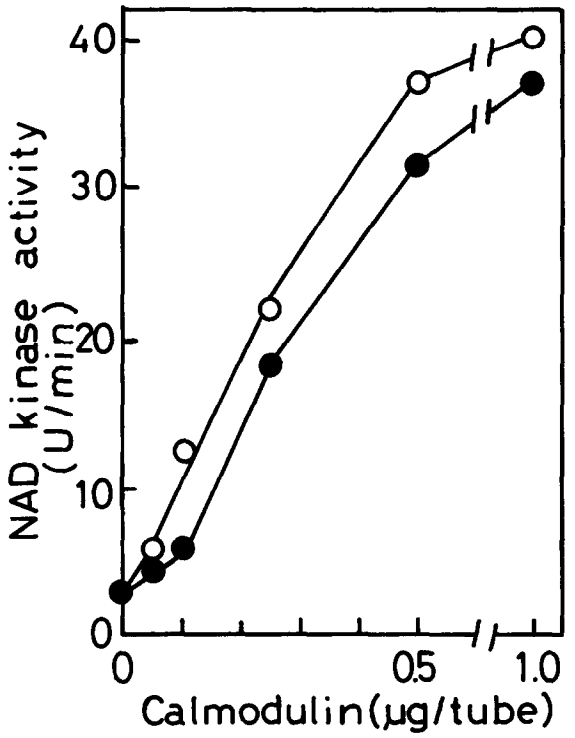

Fig. 4. Activation of plant NAD kinase by calmodulin. The enzyme activity was assayed as described in the text. Reaction mixture in 0.5 ml final vol. contained 28 μg enzyme preparation and increasing concentrations of either *Tetrahymena* (●) or bovine brain (○) calmodulin.

The activation of calmodulin-dependent enzyme such as cyclic nucleotide phosphodiesterase, myosin light-chain kinase, (Ca^{2+} + Mg^{2+})-ATPase and NAD kinase by *Tetrahymena* calmodulin may support the ubiquitous and highly conservative nature of calmodulin.

Calmodulin is the most widely distributed protein among a class of evolutionarily related Ca^{2+}-binding proteins that include troponin C and parvalbumin. It is believed that these Ca^{2+}-binding proteins are derived from a common ancestor [5]. However, the cyclic nucleotide phosphodiesterase was reported to be stimulated by 600-fold excess of troponin C over calmodulin [28]. In addition, parvalbumin which is present in all vertebrates, lacks the ability to activate any calmodulin-dependent enzymes [29]. This may suggest that troponin C and parvalbumin are highly specialized derivatives of the more generalized calcium receptor, calmodulin. We have shown that *Tetrahymena* calmodulin is very similar to bovine brain calmodulin in many respects such as heat stability, isoelectric point and Ca^{2+}-dependent electrophoretic mobility [7,8,10]. As shown here, *Tetrahymena* calmodulin is almost as effective as brain calmodulin in activating many calmodulin-dependent enzymes (table 1). A particularly specific feature of *Tetrahymena* calmodulin is to be able to activate guanylate cyclase. Postulating that the ability of mammalian calmodulins to activate guanylate cyclase would be lost during the course of evolution, it can be considered that *Tetrahymena* calmodulin could be an ancestral form of mammalian calmodulins.

However, in general, calmodulin-dependent enzymes such as phosphodiesterase and myosin light-

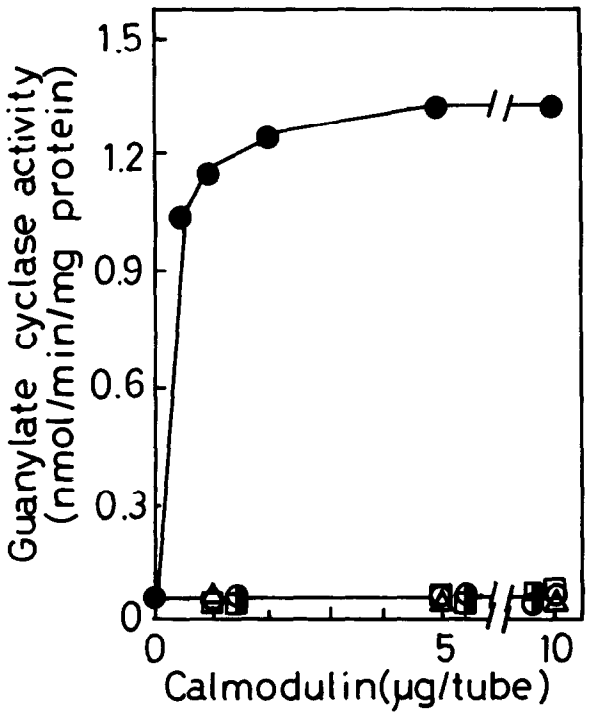

Fig. 5. Activation of *Tetrahymena* guanylate cyclase activity by calmodulin. The assay was performed as in [9]. Each assay tube in 0.2 ml final vol. contained 100 μg enzyme protein, 100 μM $CaCl_2$ and increasing concentrations of *Tetrahymena* (●), *Renilla reniformis* (△), *Chlamydomonas* (○), *Dictyostelium* (□), spinach (◑) and English cucumber (◨).

chain kinase can be activated by calmodulin from any source, but the activation of guanylate cyclase by calmodulin is limited to the protozoan calmodulins. Guanylate cyclases from other sources, including rat tissue, human platelet [8] and *Paramecium* [9], could be activated by neither *Tetrahymena* calmodulin nor brain calmodulin. Accordingly, guanylate cyclase in *Tetrahymena* per se seemed to be specific.

In *Tetrahymena* cells, there are at least two types of endogenous calmodulin-sensitive enzymes. One is guanylate cyclase and the other is dynein ATPase in ciliary fraction of the cell. Surprisingly, while the former enzyme is specific for protozoan calmodulins in its activation, the latter was activated by both calmodulin from *Tetrahymena* and rat testis [30]. The protozoan, *T. pyriformis*, may be one of the most primitive forms among eukaryotic animal cells which have calmodulin, and therefore extensive investigation, including protein chemical analysis, would offer further useful information regarding structure–function relationship of this ancestral calmodulin.

ACKNOWLEDGEMENTS

We express our appreciation to Drs Cormier, Watterson, Wang, Bazari and Witman for supplying us with calmodulin. This study was supported in part by the grant from the Ministry of Education, Culture and Science, Japan.

REFERENCES

[1] Kakiuchi, S., Yamazaki, R. and Nakajima, H. (1970) Proc. Japan Acad. 46, 587–592.
[2] Cheung, W.Y. (1970) Biochem. Biophys. Res. Commun. 38, 533–538.
[3] Cheung, W.Y. (1980) Science 207, 19–27.
[4] Means, A.R. and Dedman, J.R. (1980) Nature 285, 73–77.
[5] Klee, C.B., Crouch, T.H. and Richman, P.G. (1980) Annu. Rev. Biochem. 49, 489–515.
[6] Nagao, S., Suzuki, Y., Watanabe, Y. and Nozawa, Y. (1979) Biochem. Biophys. Res. Commun. 90, 261–268.
[7] Suzuki, Y., Hirabayashi, T. and Watanabe, Y. (1979) Biochem. Biophys. Res. Commun. 90, 253–260.
[8] Kakiuchi, S., Sobue, K., Yamazaki, R., Nagao, S., Umeki, S., Nozawa, Y., Yazawa, Y. and Yagi, K. (1981) J. Biol. Chem. 256, 19–22.
[9] Kudo, S., Ohnishi, K., Muto, Y., Watanabe, Y. and Nozawa, Y. (1981) Biochem. Internat. 3, 255–263.
[10] Watanabe, Y. and Nozawa, Y. (1982) in: Calmodulin and Cell Function (Cheung, W.Y. ed) vol. II, pp. 296–323, Academic Press, New York.
[11] Watterson, D.M., Sharief, F. and Vanaman, T.C. (1980) J. Biol. Chem. 255, 962–975.
[12] Yazawa, M., Yagi, K., Toda, H., Kondo, K., Narita, K., Yamazaki, R., Sobue, K., Kakiuchi, S., Nagao, S. and Nozawa, Y. (1981) Biochem. Biophys. Res. Commun. 99, 1051–1057.
[13] Nozawa, Y. and Thompson, G.A. (1971) J. Cell Biol. 49, 712–721.
[14] Teo, T.S. and Wang, J.H. (1973) J. Biol. Chem. 248, 588–595.
[15] Sano, M. and Drummond, G.I. (1981) J. Neurochem. 37, 558–566.

[16] Adelstein, R.S. and Klee, C.B. (1981) J. Biol. Chem. 256, 7501–7509.
[17] Perrie, W.T. and Perry, S.V. (1970) Biochem. J. 119, 31–38.
[18] Klee, C.B. (1977) Biochemistry 16, 1017–1024.
[19] Luthra, M.G., Hildenbrandt, G.R. and Hanahan, D.L. (1976) Biochim. Biophys. Acta 419, 164–179.
[20] Anderson, J.M., Charbonneau, H., Jones, H.P., McCann, R.O. and Cormier, M.J. (1980) Biochemistry 19, 3113–3120.
[21] Nakazawa, K., Sano, M. and Saito, T. (1976) Biochim. Biophys. Acta 444, 563–570.
[22] Salomon, Y., Londas, C. and Rodbell, M. (1974) Anal. Biochem. 58, 541–548.
[23] Gopinath, R.M. and Vincenzi, F.F. (1977) Biochem. Biophys. Res. Commun. 77, 1203–1209.
[24] Fiske, C.H. and Subbarow, Y. (1925) J. Biol. Chem. 66, 375–400.
[25] Apps, D.K. (1970) Eur. J. Biochem. 13, 223–230.
[26] Muto, S. and Miyachi, S. (1977) Plant Physiol. 59, 55–60.
[27] Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951) J. Biol. Chem. 193, 265–275.
[28] Dedman, J.R., Jackson, R.L., Schreiber, W.E. and Means, A.R. (1977) J. Biol. Chem. 252, 2437–2440.
[29] LeDonne jr, N.C. and Coffee, C.J. (1979) J. Biol. Chem. 254, 4317–4320.
[30] Blum, J.J., Hayes, A., Jamieson, G.A. jr and Vanaman, T.C. (1980) J. Cell Biol. 87, 386–397.