Vol. 90, No. 1, 1979 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
September 12, 1979 Pages 261-268

ACTIVATION BY A CALCIUM-BINDING PROTEIN OF GUANYLATE CYCLASE IN TETRAHYMENA PYRIFORMIS

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Received July 16,1979

SUMMARY: A Ca^{2+}-binding protein (TCBP), which was isolated from Tetrahymena pyriformis, enhanced about 20-fold particulate-bound guanylate cyclase activity in Tetrahymena cells in the presence of a low concentration of Ca^{2+}, while the adenylate cyclase activity was not increased. The enhancement was eliminated by ethylene glycol-bis (β-aminoethyl ether)-N, N'-tetraacetic acid. The enzyme activity was not stimulated by rabbit skeletal muscle troponin-C, the Ca^{2+}-binding component of troponin, or other some proteins. In the presence of TCBP, stimulating effect of calcium ion on the enzyme activity was observed within the range of pCa 6.0 to 4.6, and was immediate and reversible.

INTRODUCTION: Guanylate cyclase (EC 4.6.1.2) has been shown to be stimulated by various agents: nonionic detergents (1, 2), azide or hydroxylamine (3, 4), prostaglandin G_2 or arachidonic acid (5), feedback regulator protein (6), nucleotides (7), free fatty acid (5, 8, 9), lysolecithin (10), hydrogen peroxide (11), or nitroso compounds (12). Furthermore, it was recently shown that rat brain synaptosomal soluble fraction contained an activating factor of guanylate cyclase (13) and that a fraction obtained during purification of guanylate cyclase from rat liver increased the activity of the enzyme (14).

In the previous paper (15), we have described that guanylate cyclase activity in a protozoan Tetrahymena pyriformis is entirely associated with

Abbreviations used were: TCBP, Tetrahymena Ca^{2+}-binding protein; cyclic GMP, guanosine 3',5'-monophosphate; cyclic AMP, adenosine 3',5'-monophosphate; EGTA, ethylene glycol-bis (β-aminoethyl ether)-N,N'-tetraacetic acid.

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0006-291X/79/170261-08$01.00/0

particulate fractions, but not with soluble fraction, and that the cytosol contains a factor which activates the enzyme in a Ca^{2+}-dependent fashion. The factor was non-dialyzable, trypsin-digestible and was excluded by Sephadex G-10. On the other hand, Suzuki et al. have recently isolated a Ca^{2+}-binding protein (TCBP) from Tetrahymena cells using preparative electrophoresis (16). This purified TCBP has prompted us to examine its effects on enzymes related with cyclic nucleotides.

The present report shows that particulate-bound guanylate cyclase of T. pyriformis is greatly stimulated by TCBP in the presence of Ca^{2+}, and that its stimulating effect is exerted in a reversible fashion.

MATERIALS AND METHODS:

Materials: [8-^{3}H] GTP and [2-^{3}H] ATP were purchased from the Radiochemical Centre. All unlabeled nucleotides used were obtained from Sigma. Creatine phosphate and creatine kinase were purchased from Boehringer Mannheim. 3-Isobutyl-1-methylxanthine was obtained from Aldrich Chemical Co. Neutral aluminum oxide was a product of M. Woelm. TCBP was prepared from Tetrahymena acetone powder as described in the preceding paper (16). Troponin-C was prepared from rabbit skeletal muscle by the method of Hirabayashi and Perry (17). Other chemicals were obtained from commercial sources.

Enzyme preparation: A thermotolerant strain of T. pyriformis (NT-I) was grown at 39.5°C in an enriched proteose-peptone medium as previously described (18). Cultures of 200 ml were harvested in the early stationary phase of growth. The cell suspension (5×10^8 cells/100 ml) in 10 mM Tris-HCl buffer, pH 7.5, containing 250 mM sucrose, 1 mM dithiothreitol and 0.5 mM EDTA (Buffer A) was sonicated at 9 kc for 2 min by a Branson Sonifier (B-12) to obtain the homogenate. The homogenate was centrifuged at 105,000 x g for 60 min and the pellet was washed by resuspension and homogenization in 200 ml of 10 mM Tris-HCl buffer, pH 7.5, containing 250 mM sucrose. 1 mM dithiothreitol and 0.5 mM EGTA (Buffer B). The homogenization and centrifugation procedure was performed a total of two times. The final pellet was resuspended and rehomogenized in the Buffer B to give appropriate protein concentrations, and then used for enzyme assays.

Guanylate and adenylate cyclase assay: Unless otherwise indicated, the standard assay mixture for guanylate cyclase contained 1 mM [^{3}H] GTP (5 Ci/mol), 1 mM cyclic GMP, 15 mM creatine phosphate, 20 µg of creatine kinase, 1 mM dithiothreitol, 1.5 mM isobutylmethylxanthine, 3 mM $MgCl_2$, 0.1 mM EGTA, 25 mM Tris/maleate (pH 6.8) and 100 µg of enzyme protein in a total volume of 0.20 ml. For adenylate cyclase assay the same assay conditions as for guanylate cyclase were employed except that 1 mM [^{3}H] ATP (4 Ci/mol), 0.8 mM cyclic AMP and 10 mM $MgCl_2$ were substituted for GTP, cyclic GMP and 3 mM $MgCl_2$, respectively. After the assay mixture was incubated at 37°C for 15 min, the reaction was terminated by heating for 2 min in a boiling bath, following the addition of 1 N HCl (40 µl). The radioactive cyclic GMP or cyclic AMP was isolated by the serial use of neutral aluminum oxide-Dowex 1-X2 column and the radioactivity was determined, as described elsewhere (19).

TABLE 1

Effects of TCBP and Ca^{2+} on guanylate and adenylate cyclase activities in Tetrahymena pyriformis

Guanylate and adenylate cyclases were assayed under standard conditions. EGTA (0.1 mM) was present in all incubations. Five µg of TCBP was added to the indicated incubations.

Additions	Cyclase activity	
	Guanylate	Adenylate
	(pmol/min/mg protein)	
EGTA (0.1 mM)	2.3	62.5
Ca^{2+} (0.2 mM)	31.0	23.4
EGTA (0.1 mM) + TCBP	2.7	53.2
Ca^{2+} (0.2 mM) + TCBP	551.6	20.6

Ca^{2+}-EGTA buffer was prepared essentially as described by Ogawa (20) and to calculate the concentration of free Ca^{2+}, the apparent binding constant of Ca^{2+}-EGTA of 5×10^5 M^{-1} was used.

Protein was determined by the method of Lowry et al. (21) with bovine serum albumin as a standard.

RESULTS AND DISCUSSION: Particulate fractions prepared as described in MATERIALS AND METHODS were used for all enzyme assays. Effects of Ca^{2+} and TCBP on Tetrahymena guanylate cyclase activity were examined (TABLE 1). The enzyme activity was higher in the presence of 0.2 mM $CaCl_2$ and 0.1 mM EGTA than that in the presence of 0.2 mM EGTA.

TCBP did not influence the guanylate cyclase activity when $Ca^{2+}$was not added, and also TCBP plus GTP did not form any measurable amounts of cyclic GMP. However, when Ca^{2+} was added, the activity was markedly increased by TCBP. This cyclase activity enhanced by Ca^{2+} in the absence of TCBP might be due to incomplete removal of endogenous TCBP even by thorough washing of the particulate fractions with EGTA-containing buffer. However, any TCBP was not detected in the particulate fractions by alkaline polyacrylamide gel electrophoresis which was used for isolation of TCBP. Similar Ca^{2+}-dependencies have been reported for guanylate cyclases from rat adipocytes plasma membranes (22) and fibroblast membranes (23).

TABLE 2

Specificity of TCBP activation on Tetrahymena guanylate cyclase

The reaction mixture contained the standard components, guanylate cyclase, 0.1 mM $CaCl_2$ added in excess of EGTA, and the test protein as indicated in the Table.

Additions	Activity
	(pmol/min/mg protein)
None	30.4
Cytochrome (mol. wt. 12,500; 5 μg)	29.6
Chymotrypsinogen (mol. wt. 25,000; 5 μg)	31.0
Ovalbumin (mol. wt. 45,000; 5 μg)	33.3
Bovine serum albumin (mol. wt. 67,000; 5 μg)	29.1
Troponin-C (mol. wt. 18,000; 5 μg)	38.8
TCBP (mol. wt. 14,000; 5 μg)	547.7

In contrast, Ca^{2+}inhibited the adenylate cyclase activity which is also particulate-bound in this cell, and TCBP did not increase the activity as measured with or without addition of Ca^{2+} (TABLE 1). Moreover, phosphodiesterase activities hydrolyzing cyclic AMP and cyclic GMP were detected in both soluble and particulate fractions from Tetrahymena cells, when assayed as described elsewhere (24). A significant change of cyclic nucleotide phosphodiesterase activities in each fraction was not observed by addition of TCBP at various concentrations of Ca^{2+} (data not shown).

TABLE 2 showed the effects of some proteins, including rabbit skeletal muscle troponin-C, the Ca^{2+}-binding component of troponin, on Tetrahymena guanylate cyclase activity. None of these proteins mimicked TCBP stimulation of the enzyme. The proteins of other concentrations (2.5-20 μg) were also tested but found ineffective.

Degrees of activation of the guanylate cyclase were examined as a function of TCBP (Fig. 1). Half-maximal activation of the enzyme occurred about 0.5-0.6 μg of TCBP per 0.2 ml of incubation contents. TCBP did not alter the apparent Km for GTP, which was 8×10^{-5} M regardless of the presence of TCBP (data not shown). The dependency of guanylate cyclase activity

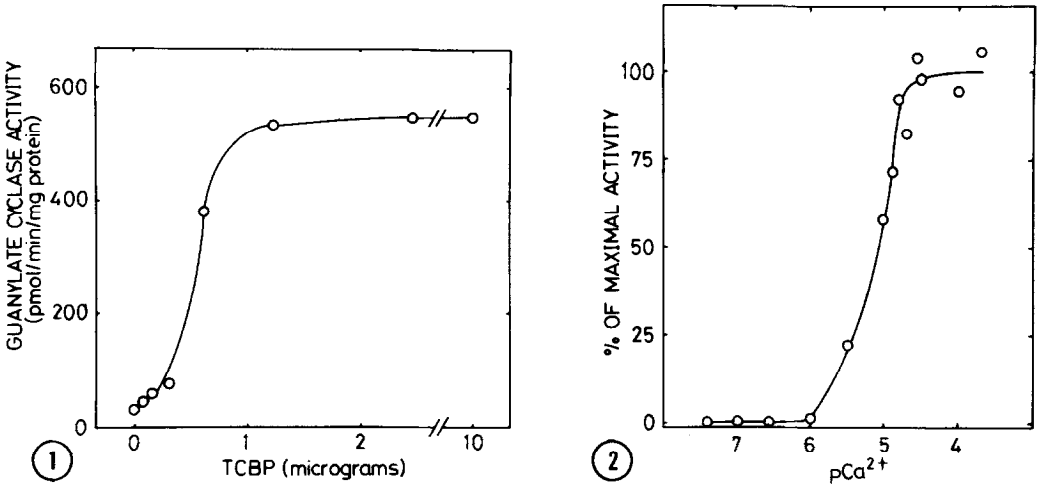

Fig. 1. Variation of Tetrahymena guanylate cyclase activity with concentration of TCBP. Guanylate cyclase was assayed under standard conditions except that 0.2 mM Ca^{2+} and various amounts of TCBP were added.

Fig. 2. Effect of addition of TCBP on Tetrahymena guanylate cyclase at various concentrations of Ca^{2+}. Guanylate cyclase was assayed under standard conditions except that 3 µg of TCBP and different concentrations of Ca^{2+} were added. Free Ca^{2+} concentration was varied by use of a Ca^{2+}/EGTA buffer containing 1 mM EGTA, assuming that contaminating Ca^{2+} (20×10^{-6} M) was contained in a reaction mixture (20). Values for the ordinate were expressed as percentages of the guanylate cyclase activity observed at pCa 4.6 and were corrected for the enzyme activity without addition of TCBP.

on $MgCl_2$ was not affected by TCBP, the maximal activity being obtained at 3 mM $MgCl_2$.

A number of Ca^{2+}-dependent cellular events is known to be controlled by a transient increase in the cytoplasmic Ca^{2+} concentration from an unstimulated level of approximately 10^{-7} M to a stimulated value of approximately 10^{-5} M (25). The effect of various concentrations of Ca^{2+} on TCBP activation of the guanylate cyclase was examined (Fig. 2). Increasing degrees of stimulation of the enzyme were observed over a range of pCa from 6.0 to 4.6 in the presence of saturating TCBP. Moreover, the Ca^{2+} requirement of TCBP activation of the enzyme was examined to determine whether the enzyme was activated in a reversible or irreversible fashion (Fig. 3). A sample of Tetrahymena guanylate cyclase incubated with 0.2 mM EGTA and 6.25 µg/ml TCBP exhibited only traces of activity. As soon as Ca^{2+} was added to the reaction mixture, the rate of cyclic GMP formation was increased immediately.

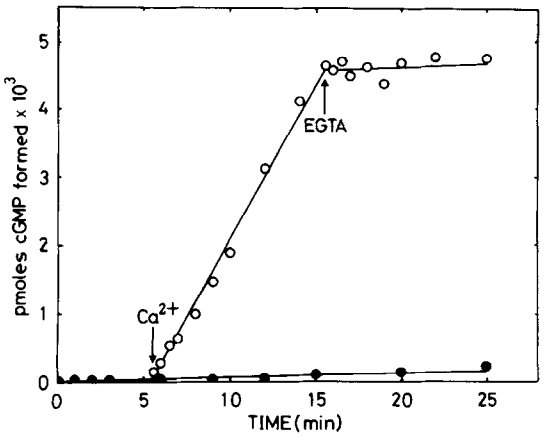

Fig. 3. Effect of Ca^{2+} on Tetrahymena guanylate cyclase. A reaction mixture of 8 ml contained the components and concentrations of the standard reaction except for the addition of TCBP (6.25 µg/ml) and EGTA (0.1 mM). The reaction was initiated by addition of the enzyme (4 mg of protein). At appropriate times, 0.2 ml aliquots were withdrawn from the reaction system and were transferred to tubes containing 40 µl of 1 N HCl. At 5.5 min of incubation the reaction mixture was divided into two portions, one of which was adjusted to 0.3 mM Ca^{2+} with 150 mM $CaCl_2$. Further additives to the Ca^{2+}-treated tubes were 3 mM EGTA (final concentration) at 15.5 min. Open circles (O-O) represent aliquots from the sample containing the additives; closed circles (●-●) represent aliquots from the untreated samples.

The enhanced rate was maintained until excess EGTA was added, thereby reducing the rate of formation to its prestimulated level. These data combined with the observations of Figs. 1 and 2, indicate that TCBP activation of the enzyme responds directly to changes in free Ca^{2+} in an immediate and reversible fashion, although the precise mechanism by which the enzyme is activated in the presence of TCBP and Ca^{2+} remains to be elucidated.

A protein modulator (CDR), which was originally discovered as protein activator of cyclic nucleotide phosphodiesterase (26, 27), has been shown to exhibit multiple, Ca^{2+}-dependent, regulatory functions, i.e. activations of Ca^{2+}-activatable adenylate cyclase (28), $(Ca^{2+} + Mg^{2+})$-ATPase (29, 30) and myosin light chain kinase (31), and relief of troponin-I inhibition of actomyosin ATPase (32). TCBP shows a striking resemblance to CDR in some properties such as molecular weight, acidic nature, heat stability, conformational change caused by Ca^{2+}, and binding to troponin-I in the presence of Ca^{2+} (16). However, in order to conclude whether or not TCBP is identical

to CDR, it should be necessary to disclose more detailed physico-chemical properties of TCBP, together with the cross reactivities of TCBP and CDR in the activations of mammalian CDR-dependent cyclic nucleotide phosphodiesterase and Tetrahymena guanylate cyclase. Further extensive experiments are in progress to obtain information regarding TCBP's functions in Tetrahymena cell.

REFERENCES:

1. Kimura, H., and Murad, F. (1974) J. Biol. Chem. 249, 6910-6916.
2. Chrisman, T.D., Gabers, D.L., Parks, M.A., and Hardman, J.G. (1975) J. Biol. Chem. 250, 374-381.
3. Deguchi, T. (1977) J. Biol. Chem. 252, 596-601.
4. Kimura, H., Mittal C.K., and Murad, F. (1975) J. Biol. Chem. 250, 8016-8022.
5. Glass, D.B., Frey, W., Carr, D.W., and Goldberg, N.D. (1977) J. Biol. Chem. 252, 1279-1285.
6. Asakawa, T., Johnson, C., Ruiz, J., Scheinbaum, I., Russell, T.R., and Ho, R-j. (1976) Biochem. Biophys. Res. Commun. 72, 1335-1342.
7. Siegel, M.I., Puca, G.A., and Cuatrecases, P. (1976) Biochim. Biophys. Acta 438, 310-323.
8. Asakawa, T., Scheinbaum, I., and Ho, R-j. (1976) Biochem. Biophys. Res. Commun. 73, 141-148.
9. Wallach, D., and Pastan, I. (1976) J. Biol. Chem. 251, 5802-5809.
10. Shier, W.T., Baldwin, J.H., Nilsen-Hamilton, M., Hamilton, R.T., and Thanassi, N.H. (1976) Proc. Natl. Acad. Sci. U.S.A. 73, 1586-1590.
11. White, A.A., Crawford, K.M., Patt, C.S., and Lad, P.J. (1976) J. Biol. Chem. 251, 7304-7312.
12. DeRubertis, F.R., and Craven, P.A. (1976) Science 193, 897-899.
13. Deguchi, T. (1977) J. Biol. Chem. 252, 7617-7619.
14. Tsai, S-C., Manganiello, V.C., and Baughan, M. (1978) J. Biol. Chem. 253, 8452-8457.
15. Nakazawa, K., Shimonaka, H., Nagao, S., Kudo, S., and Nozawa, Y. (1979) J. Biochem. in press.
16. Suzuki, Y., Hirabayashi, T., and Watanabe, Y. (1979) Biochem. Biophys. Res. Commun. preceding paper.
17. Hirabayashi, T., and Perry, S.V. (1974) Biochim. Biophys. Acta 351, 273-289.
18. Nozawa, Y., and Thompson, G.A. (1971) J. Cell Biol. 49, 712-721.
19. Nakazawa, K., Sano, M., and Saito, T. (1976) Biochim. Biophys. Acta 444, 563-570.
20. Ogawa, Y. (1968) J. Biochem. 64, 255-257.
21. Lowry, O.H., Resebrough, N.J., Farr, A.L., and Randall, R.J. (1951) J. Biol. Chem. 193, 265-275.
22. Levilliers, J., Lecot, F., and Pairault, J. (1978) Biochem. Biophys. Res. Commun. 84, 727-735.
23. Wallach, D., and Pastan, I. (1976) Biochem. Biophys. Res. Commun. 72, 859-865.
24. Nakazawa, K., and Sano, M. (1974) J. Biol. Chem. 249, 4207-4211.
25. Kretsinger, R.H. (1977) in "Calcium Binding Proteins and Calcium Function", pp. 63-73 Elsevier North-Holland, Inc., New York.
26. Cheung, W.Y. (1970) Biochem. Biophys. Res. Commun. 33, 533-538.
27. Kakiuchi, S., Yamazaki, R., and Nakajima, H. (1970) Proc. Jap. Acad. 46, 587-592.

28. Brostrom, C.O., Huang, Y-C., Breckenridge, B.M., and Wolff, D.J. (1975) Proc. Natl. Acad. Sci. U.S.A. 72, 64-68.
29. Gopinath, R.M., and Vincenzi, F.F. (1977) Biochem. Biophys. Res. Commun. 77, 1203-1209.
30. Jarrett, H.W., and Penniston, J.T. (1977) Biochem. Biophys. Res. Commun. 77, 1210-1216.
31. Yagi, K., Yazawa, M., Kakiuchi, S., Ohshima, M., and Uenishi, K. (1978) J. Biol. Chem. 253, 1338-1340.
32. Amphlett, G.W., Vanaman, T.C., and Perry, S.V. (1976) FEBS Lett. 72, 163-168.