90. Stimulation of the Activity of Cyclic 3',5'-Nucleotide Phosphodiesterase by Calcium Ion

By Shiro KAKIUCHI and Reiko YAMAZAKI

Nakamiya Mental Hospital, Hirakata, Osaka

(Comm. by Yushi UCHIMURA, M. J. A., April 13, 1970)

It has been shown recently that concentrations of 3',5'-AMP in mammalian brain tissues increased dramatically in responding to various stimuli, i.e. decapitation,¹⁾ electrical stimulation applied *in vitro*,²⁾ and certain neurohormones added *in vitro*.^{1),3)} It is generally accepted that adenylyl cyclases were responsible for regulating the level of 3',5'-AMP in the tissues.

Recently, Cheung⁴⁾ has proposed ATP and PP controls on brain cyclic 3',5'-nucleotide phosphodiesterase, the enzyme which decomposes 3',5'-AMP, suggesting a possibility of this enzyme to contribute to such regulation mechanism under certain conditions.

Results presented in this communication demonstrate the implication of the physiological concentrations of calcium ion regulating activity of cyclic 3',5'-nucleotide phosphodiesterase from rat brains.

Materials and methods. *Materials purchased are* *Crotalus atrox* from Sigma Chemical Co., glycoetherdiamine-N,N,N',N'-tetraacetic acid (GEDTA) from Dojindo, Co.

Enzyme preparation. Brains from male rats of Sprague Dowley strain were homogenized in 3 volumes of 1 mM tris-HCl (pH 7.5). The homogenate was centrifuged on Hitachi 40 A rotor for 60 min. at 40000 rpm. The precipitate was washed once by the centrifugation as above and the supernatant was combined.

A portion of the combined supernatant was dialyzed overnight against 100 volumes of 1 mM tris-HCl (pH 7.5) and referred to the "supernatant" enzyme. Another portion was subjected to GEDTA treatment and referred to the "GEDTA-washed" enzyme. The procedure was carried out by repeating the cyclic process; condensing enzyme by suction through a collodion bag and diluting it with the washing medium. 0.4 mM GEDTA in 10 mM Tris-HCl (pH 7.5) was used as such the medium, and followed by 10 mM tris-HCl alone to remove excess GEDTA.

All the procedures were done at 4°C. Enzymes were kept at 4°C for a week or more, or at -80°C for several months with no appreciable loss of the activity.

Assay for enzymatic activity. The phosphodiesterase was as-

sayed according to the method of Butcher and Sutherland⁵⁾ with a slight but important modification. The procedures consist of two separate stages of incubation at 30°C. The snake venom 5'-nucleotidase, *Crotalus atrox*, used in the second stage incubation, required for Mg^{++} or Mn^{++} and was inhibited by EDTA or GEDTA, and therefore, the concentration of free Mg ion was adjusted to more than 3 mM prior to starting the second stage incubation. It was confirmed that the excess amount of the snake venom used throughout the studies did not release any detectable amount of P from 3',5'-AMP during the incubation either in the presence or in the absence of Ca^{++} . Enzyme protein was assayed by procedures similar to those Lowry *et al.*⁶⁾

Free Ca ion concentrations in media. The concentration of free Ca ion in the absence of added $CaCl_2$, i.e. the contamination level of free Ca was roughly 10^{-6} M~ 10^{-5} M, and therefore, those in 1 mM GEDTA should be in the order of 10^{-9} M~ 10^{-8} M. Estimated concentration of free Ca ion in the mixture of GEDTA, 1 mM plus $CaCl_2$, 0.33 mM (Ca buffer) was 1×10^{-6} M. These values were calculated by adopting 5×10^5 M⁻¹ as the apparent binding constant of GEDTA according to Ogawa,⁷⁾ which is considerably lower than the formerly used.⁸⁾

Results. Since it was discovered by Sutherland and Rall⁹⁾ the requirement of the phosphodiesterase for Mg ion has been well demonstrated. It was also reported that even greater enzymatic activity was attained by Mn ion^{5),10)} and partial activity by Co ion.⁵⁾

In accordance with these reports, we observed that the "GEDTA-washed" enzyme, which retained very little activity in the absence of added cations, restored good activity by the additions of Mg, Mn or Co ions (Fig. 1). 3 mM of Mg^{++} or 0.3 mM of Mn^{++} expressed the full enzymatic activity and higher concentrations of these cations were slightly inhibitory.

The effect of these cations were stimulated when Ca ion was included in the system, as are the cases in Fig. 2, where $CaCl_2$ was added in all the reaction tubes to final 5×10^{-4} M while other experimental conditions essentially identical to those in Fig. 1. In addition to its stimulatory effect, inclusion of Ca ion changed shapes of dose response curves of the cations so that the inhibitions due to excess Mg^{++} or Mn^{++} were relived.

With the optimum concentration of Mg^{++} (3 mM) present in media, activities of the "GEDTA-washed" enzyme were tested with various additions of other components. The results presented in Table I demonstrate clearly that minute amounts of Ca ion stimulated enzymatic activity markedly. It should be noted that the stimulatory

effect of Ca ion required Mg^{++} or Mn^{++} (Co^{++}) as a basic cation in medium and Ca ion alone revealed very poor enzymatic activity as shown in Table I (Exp. 3) or in Fig. 2 (shaded bar).

Results in Fig. 3 show the stimulatory effect of Ca ion as a function of its concentration. The stimulation was observed with Ca ion below $1 \times 10^{-6}\text{M}$, within the concentrations where regulatory roles of Ca ion in physiological processes could be seen. Enzymatic activities in the presence of Ca ion lower than $1 \times 10^{-6}\text{M}$ was not analyzed directly. However, the results in Fig. 4 involve such cases of $10^{-9}\text{M} \sim 10^{-8}\text{M}$ and will be mentioned later.

Experiments using chelating agents on the "supernatant" enzyme differentiated two types of inhibitions, one caused by EDTA and the other by GEDTA (Fig. 4). Parallel relationship was found in degrees of the inhibition and concentrations of EDTA in media. The enzyme was completely inactivated as EDTA level increased over 3mM. The essential nature of this inhibition seems to be due to depriving of free Mg ion in the media. These observations coupled with those shown in Table I (Exp. 3) confirmed the absolute requirement for Mg^{++} for basic activity of the phosphodiesterase.

The inhibition caused by GEDTA was clearly seen with its concentration as low as $1 \times 10^{-4}\text{M}$ and could not be explained by possible changes in free Mg^{++} concentration in the media. GEDTA is known

Fig. 1. The effect of divalent cations on the "GEDTA-washed" enzyme. Reaction mixtures contained MgCl_2 (—●—), MnCl_2 (—○—), or CoCl_2 (---□---) as the only divalent cation. Other experimental conditions were essentially the same as those in Table I.

Fig. 2. The effect of divalent cations on the "GEDTA-washed" enzyme in the presence of $5 \times 10^{-4}\text{M}$ Ca ion. Reaction mixtures contained a fixed concentration of CaCl_2 (final $5 \times 10^{-4}\text{M}$) plus one of the following cations; MgCl_2 (—●—), MnCl_2 (—○—), or CoCl_2 (---□---). Other experimental conditions were essentially the same as those in Table I.

Table I. Activity of the "GEDTA-washed" enzyme under various conditions

Basic system	Further addition	Activity ^{†)}
Experiment 1	none ^{*)}	1.2
complete system	GEDTA, 1 μ mol ^{**)}	1.0
	GEDTA, 1 μ mol + CaCl ₂ , 0.33 μ mol ^{***)}	1.0
	CaCl ₂ , 0.03 μ mol	1.9
	CaCl ₂ , 1.0 μ mol	2.0
Experiment 2	GEDTA, 1 μ mol ^{**)}	1.0
complete system —dithiothreitol	GEDTA, 1 μ mol + CaCl ₂ , 0.33 μ mol ^{***)}	1.0
	CaCl ₂ , 0.03 μ mol	1.3
	CaCl ₂ , 1.0 μ mol	1.4
Experiment 3	GEDTA, 1.0 μ mol + CaCl ₂ , 0.33 μ mol ^{***)}	<0.1
complete system —MgCl ₂	CaCl ₂ , 0.03 μ mol	0.4
	CaCl ₂ , 1.0 μ mol	0.4
Experiment 4	GEDTA, 1.0 μ mol + CaCl ₂ , 0.33 μ mol ^{***)}	0.0
complete system —3', 5'-AMP	CaCl ₂ , 0.03 μ mol	0.0
Experiment 5	GEDTA, 1.0 μ mol + CaCl ₂ , 0.33 μ mol ^{***)}	0.0
complete system —enzyme	CaCl ₂ , 0.03 μ mol	0.0

^{†)} μ mol P in 30 min per mg protein.

As is described in the text, estimated concentrations of free Ca⁺⁺ are as follows.

^{*)}: 10^{-6} M~ 10^{-5} M, ^{**)}: 10^{-9} M~ 10^{-8} M, ^{***)}: 1×10^{-6} M.

Complete system consisted of, in one ml of the reaction mixture, 40 μ mol of Tris-maleate (pH 6.9), 3 μ mol of MgCl₂, 1 μ mol of dithiothreitol, 1.2 μ mol of 3', 5'-AMP, and the "GEDTA-washed" enzyme protein.

to have a selective affinity for Ca ion compared with Mg ion; the latter is practically not affected by GEDTA under the experimental conditions. The observations agree well with the idea that minute amounts of Ca ion are involved in the phosphodiesterase system. Comparison of Fig. 3 and Fig. 4 indicates that the degrees of the inhibition caused by GEDTA on the "supernatant" enzyme is comparable to those of the activation by Ca ion on the "GEDTA-washed" enzyme.

Of even greater interest is the observations that the enzyme still retains about a half the original activity even in the presence of excess amount of GEDTA (1~4 mM), where the estimated concentrations of free Ca ion should be in the order of 10^{-9} M~ 10^{-8} M. The results would suggest a possible occurrence of two separate enzymatic activities in the preparation, the calcium dependent and the calcium independent.

The effect of dithiothreitol seems to enhance the calcium dependent activity (Exp. 2 in Table I). The effect of Ca ion could be replaced with strontium or barium ions in some extent.

Comments. It is now widely accepted and well documented¹¹⁾

that the calcium ion is the final activator of the contractile system under physiological conditions. Ebashi and Endo¹¹⁾ reported that the maximum tension was attained at pCa less than 4.9 and threshold pCa was around 5.9 by using skinned fibres. These values are close to those in Fig. 3, where the maximum stimulation of the phosphodiesterase was observed at pCa near 4.5 and threshold pCa was observed around 5.5 (with 3mM Mg present). It was observed both in the contractile system¹¹⁾ and on the phosphodiesterase (Fig. 3) that the increase in free Mg ion concentration up to 8~10 mM decreased the Ca sensitivities.

Taking the above comparisons into consideration, it would be highly probable that the calcium ion is controlling the phosphodiesterase activity under physiological status and thus affecting 3',5'-AMP level in the tissues *in vivo*. It is of interest that Namm *et al.*,¹²⁾ by using isolated and perfused rat hearts, have shown the changes in the tissue level of 3',5'-AMP as the concentration of Ca in the medium changed. They have proposed a hypothesis that Ca ion either inhibits adenyl cyclase activity or stimulates the phosphodiesterase activity.

This communication provides the first direct evidence of the calcium ion acting upon the phosphodiesterase, and supplies another

Fig. 3. Activity of the "GEDTA-washed" enzyme as a function of concentrations of Ca ion.

CaCl_2 were added into reaction mixtures to produce the indicated concentrations, except $1 \times 10^{-6}\text{M}$ which was achieved by the addition of GEDTA, 1.0 μmol plus CaCl_2 , 0.33 μmol . Other experimental conditions were essentially the same as those in Table I.

Fig. 4. The effect of chelating agents on the "supernatant" enzyme.

One ml of reaction mixtures contained 40 μmol s of Tris-maleate (pH 6.9), 5 μmol s of dithiothreitol, 3 μmol s of MgSO_4 , 2 μmol s of 3',5'-AMP, the "supernatant" enzyme, and indicated amounts of EDTA (---●---) or GEDTA (—○—).

clue to the probable interrelationship between the 3',5'-AMP system and the calcium ion.¹³⁾⁻¹⁶⁾

We would like to acknowledge the continuing encouragement of Dr. H. Nakajima, Nakamiya Mental Hospital. Our thanks are also due to Drs. H. Wada and I. Sano, Osaka University, Medical School and Dr. M. Kurokawa, Institute of Brain Research, University of Tokyo for many helpful suggestions. The work was supported in part by Research Grant from Pharmacopsychiatry Research Foundation.

References

- 1) Kakiuchi, S., and Rall, T. W. (1968): *Mol. Pharmacol.*, **4**, 379.
- 2) Kakiuchi, S., Rall, T. W., and McIlwain, H. (1969): *J. Neurochem.*, **16**, 485.
- 3) Kakiuchi, S., and Rall, T. W. (1968): *Mol. Pharmacol.*, **4**, 367.
- 4) Cheung, W. Y. (1966): *Biochem. Biophys. Res. Commun.*, **23**, 214.
- 5) Butcher, R. W., and Sutherland, E. W. (1962): *J. Biol. Chem.*, **237**, 1244.
- 6) Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J. (1951): *J. Biol. Chem.*, **193**, 265.
- 7) Ogawa, Y. (1968): *J. Biochem.*, **64**, 255.
- 8) Schwarzenbach, G., Senn, H., und Anderegg, G. (1957): *Helv. Chim. Acta.*, **40**, 1886.
- 9) Sutherland, E. W., and Rall, T. W. (1958): *J. Biol. Chem.*, **232**, 1077.
- 10) Cheung, W. Y., and Jenkins, A. (1969): *Fed. Proc.*, **28**, 473.
- 11) Ebashi, S., and Endo, M. (1968): "Progress in Biophysics and Molecular Biology" (Butler, J. A. V., and Noble, D. eds.) p.123, Pergamon Press, Oxford and New York.
- 12) Namm, D. H., Mayer, S. E., Maltbie, M. (1968): *Mol. Pharmacol.*, **4**, 522.
- 13) Ozawa, E., and Ebashi, S. (1967): *J. Biochem.*, **62**, 285.
- 14) Rasmussen, H., and Tenenhouse, A. (1968): *Proc. N. Y. Acad. Sci.*, **59**, 1364.
- 15) Bär, H. P., and Hechter, O. (1969): *Biochem. Biophys. Res. Commun.*, **35**, 681.
- 16) Vigdahl, R. L., Marquis, N. R., and Tavormina, P. A. (1969): *Biochem. Biophys. Res. Commun.*, **37**, 409.