CYCLIC 3′,5′-NUCLEOTIDE PHOSPHODIESTERASE Ca^{++}-DEPENDENT FORMATION OF BOVINE BRAIN ENZYME–ACTIVATOR COMPLEX

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1. Introduction

A protein activator specific for bovine brain cyclic 3′,5′-nucleotide phosphodiesterase was discovered several years ago during the course of enzyme purification [1]. The existence of this activator raises the question of its physiological role in modulating phosphodiesterase activity. To answer this question, we undertook two lines of approach. One was to correlate the tissue or cellular distribution as well as tissue developmental changes of phosphodiesterase and its activator. We found that although the activities of the enzyme and the activator paralleled one another on a subcellular basis, they varied from tissue to tissue and during ontogenetic development of the tissues [2]. The variability may be a result of cellular heterogeneity or multiple forms of phosphodiesterase (for example, activator-dependent and activator-independent enzyme) [2–5]. Although parallel distribution of the two activities on a subcellular basis suggests possible physiological relevance, it does not reveal the mode of activation of phosphodiesterase by the activator.

Another approach was to characterize the activator and to elucidate its mode of action. We found that the activator increases the V_{max} of the enzyme and decreases its K_m for cyclic AMP and that stimulation of phosphodiesterase is a stoichiometric process [6,7]. We further found that the activator binds Ca^{++} and that chelation of Ca^{++} by EGTA renders the activator inactive. It was suggested that the Ca^{++}-activator complex is the active form of phosphodiesterase [8]. Recently, Teo and Wang, working with bovine heart, [9] and Kakiuchi et al., working with rat brain [10] reached a similar conclusion.

Experiments described here show that Ca^{++} causes the formation of the enzyme–activator complex, and that the rate of formation of the active complex is rapid enough to account for the fast catabolism of cyclic AMP in vivo.

2. Materials and methods

2.1. *Preparation of phosphodiesterase and its activator from bovine brain*

Phosphodiesterase was purified through DEAE-cellulose chromatography [11]; the enzyme at this stage is activator-deficient and dependent on an exogenous activator for maximum activity. The activator was purified through a DEAE-cellulose column [8,12].

2.2. *Assay of phosphodiesterase and its activator*

Phosphodiesterase was assayed with 5′-nucleotidase of snake venom as an auxillary enzyme using a two-stage procedure as described previously [7]. The activator was determined by its ability to stimulate an activator-deficient phosphodiesterase [7].

2.3. *Measurement of protein*

Protein was estimated by the spectrophotometric technique of Warburg and Christian [13].

3. Results and discussion

The protein activator of bovine brain phosphodiesterase is dissociable from the enzyme by a salt

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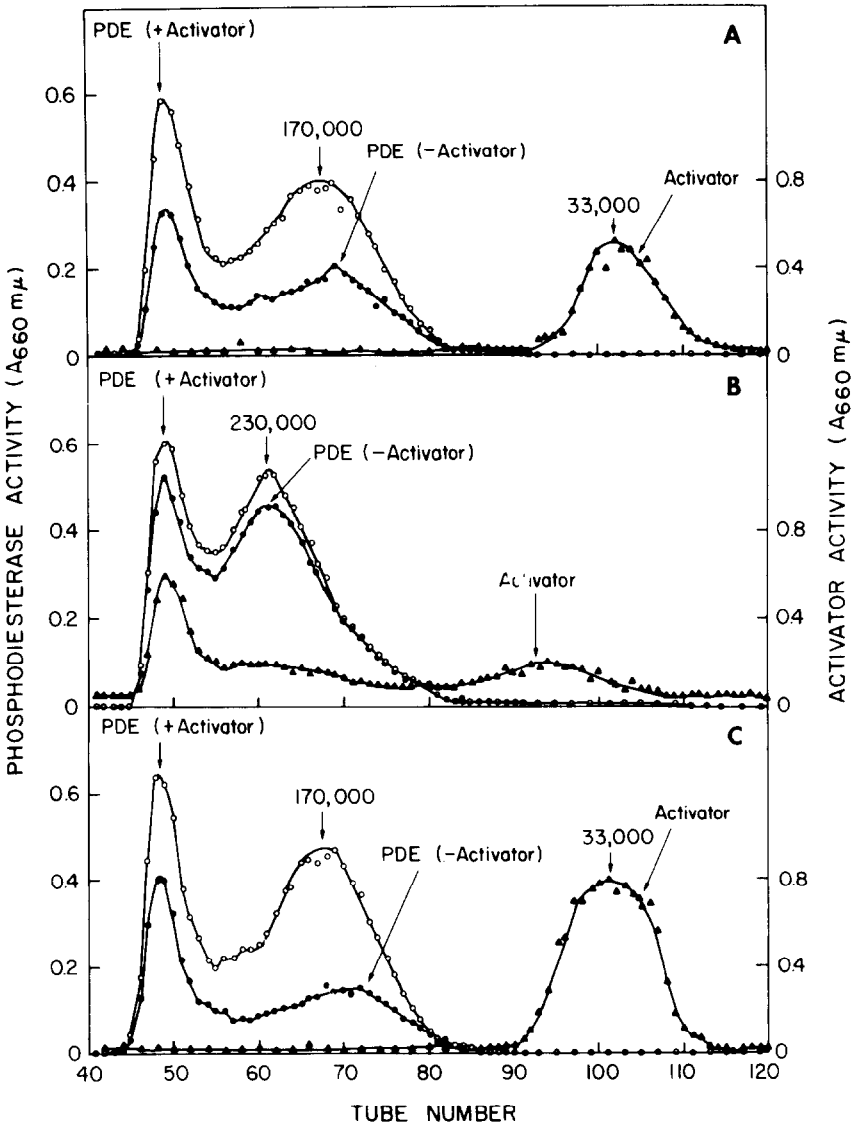

Fig. 1. Ca^{++}-dependent association of phosphodiesterase and its activator as shown by filtration in a Sephadex G-200 column (95 × 2.5 cm). Panel A.: The column was equilibrated with a buffer containing 20 mM Tris–Cl (pH 8.0), 100 mM NaCl, 0.1 mM dithiothreitol, and 0.1 mM EGTA (Buffer A) at 4°C. Thirteen milligrams of an activator-deficient phosphodiesterase and 8 μg of a highly purified activator in 2.5 ml of a buffer containing 40 mM Tris–Cl (pH 8.0), 0.1 mM dithiothreitol and 0.1 mM EGTA was incubated for 15 min at 30°C and then applied to the column. The column was eluted with Buffer A; fractions of 3.5 ml were collected at a flow rate of 24 ml/hr. Aliquots were assayed separately for the activator and for phosphodiesterase in the presence or absence of an exogenous activator by a two-stage procedure as described previously [7]. The reaction mixture contained 40 mM Tris–Cl (pH 8.0), 3 mM $MgSO_4$, 0.05 mM $CaCl_2$, 2 mM cyclic AMP and an appropriate amount of phosphodiesterase and/or activator. The trace of EGTA carried over from the elution buffer into the assay mixture did not affect either the activity of phosphodiesterase or its activator. Panel B.: The column was equilibrated with a buffer containing 20 mM Tris–Cl (pH 8.0), 100 mM NaCl, 0.1 mM dithiothreitol and 0.1 mM $CaCl_2$ (Buffer B). Thirteen milligrams of an activator-deficient phosphodiesterase and 8 μg of a highly purified activator in 2.5 ml of a buffer containing 40 mM Tris–Cl (pH 8.0), 0.1 mM dithiothreitol and 0.1 mM $CaCl_2$ was incubated for 15 min at 30°C and then loaded on the column. The column was eluted with Buffer B. Fractions were collected and assayed as in Panel A. Panel C.: Conditions were the same as those in Panel B except phosphodiesterase and the activator were applied to the column separately. The column was calibrated with apoferritin (mol. wt 460 000), pyruvate kinase (mol. wt 238 000), bovine serum albumin (mol. wt 68 000), ovalbumin (mol. wt 45 000) and soybean trypsin inhibitor (mol. wt 21 600) according to Andrews [14].

gradient on a DEAE-cellulose column [11]. Dissociation of the two proteins reduces the activity of phosphodiesterase to its basal level. Chelation of Ca^{++} in the activator by EGTA also reduces phosphodiesterase to its basal activity [8]. The possibility that chelation of Ca^{++} by EGTA may cause dissociation of the two proteins and hence the reduction of enzymic activity was examined. A mixture of phosphodiesterase and its activator was passed through a Sephadex G-200 column that had been equilibrated with EGTA. Fig. 1A shows that phosphodiesterase was resolved into two active fractions: the first peak coincided with the exclusion volume whereas the second peak corresponded to a mol. wt of 170 000. Phosphodiesterase activity was considerably higher when assayed in the presence of an exogenous activator than in its absence. This was due to the fact that the activator, originally mixed with the enzyme, was eluted after both peaks of enzyme activity and none was detected with the enzyme. This experiment shows that chelation of Ca^{++} by EGTA dissociates the activator from the enzyme, making it less active. In this experiment, the activator was eluted at a position corresponding to a mol. wt of 33 000. It has been shown elsewhere that the molecular weight of the activator obtained by gel filtration under this condition is approximately twice that obtained with other methods [8].

In a separate experiment (fig.1B), the enzyme-activator mixture was passed through the same column that had been equilibrated with Ca^{++} instead of EGTA. In contrast to the result shown in fig. 1A, the activator was eluted with the enzyme and phosphodiesterase activity was virtually the same whether assayed in the presence or absence of an exogenous activator. Further, the second activity peak was shifted to a position corresponding to a higher mol. wt of 230 000, suggesting that this form of the enzyme retained approximately two moles of the activator [15]. In addition, the activator which was not associated with the enzyme was eluted ahead of its usual position as a broad peak. This presumably resulted from association-dissociation of the enzyme and the activator during the course of filtration through the column.

Fig. 1C illustrates a control experiment in which the enzyme and the activator were passed separately through the column previously equilibrated with Ca^{++}. Both the enzyme and the activator were eluted at positions identical to those in Fig. 1A, demonstrating that Ca^{++} did not affect the behavior of these proteins on gel filtration.

It may be concluded from these experiments that in the presence of Ca^{++} the enzyme and the activator form a complex, whereas in its absence the two proteins remain dissociated. Since the activator binds Ca^{++} [8,16], the cation may function as follows:

$$Ca^{++} + \text{Activator} \rightleftharpoons Ca^{++}\text{-Activator}$$

$$\underset{\text{inactive}}{[\text{Enzyme}]} + Ca^{++}\text{-Activator} \rightleftharpoons \underset{\text{active}}{[\text{Enzyme-}Ca^{++}\text{–Activator}]}$$

Although the experiments depicted in fig. 1 clearly show the Ca^{++}-dependent formation of the enzyme–activator complex, they do not indicate the time for this process to take place. The rate of formation or dissociation of the enzyme–activator complex was next examined. Since the enzyme–activator complex is the active species, the rate of increase of phosphodiesterase activity by Ca^{++} would be a measure of the rate of complex formation. Similarly, the rate of decrease of enzymic activity by EGTA would be a measure of the rate of complex dissociation. Fig. 2 shows the change of phosphodiesterase activity upon the addition or the chelation of Ca^{++}. The initial portion of the curve depicts the basal rate of cyclic AMP hydrolysis in the absence of Ca^{++}. As soon as $Ca^{++}$was added to the reaction mixture, the rate of hydrolysis was increased immediately. The enhanced rate was maintained until excess EGTA was added, thereby reducing the rate of hydrolysis to its prestimulated level. This experiment demonstrates that both the formation and the dissociation of the enzyme–activator complex are rapid.

Kakiuchi and his coworkers described a Ca^{++}- and Mg^{++}-dependent phosphodiesterase from rat brain and found that a micromolar Ca^{++} enhanced the stimulation of phosphodiesterase by the activator [17,18]. Recently, they reported that the association of the enzyme with the activator was dependent on Ca^{++} [15].

Phosphodiesterase in the brain is localized in the nerve endings [19,20]. Increasing evidence indicates that cyclic AMP is closely involved with nervous activity [21]. Addition of a depolarizing agent such as K^+ to brain slices resulted in a marked elevation of tissue level of cyclic AMP [22]. Depolarizing squid axons by increasing the external K^+ concentration caused a pronounced increase of Ca^{++} influx [23]. The increase of cyclic AMP may be temporally related to

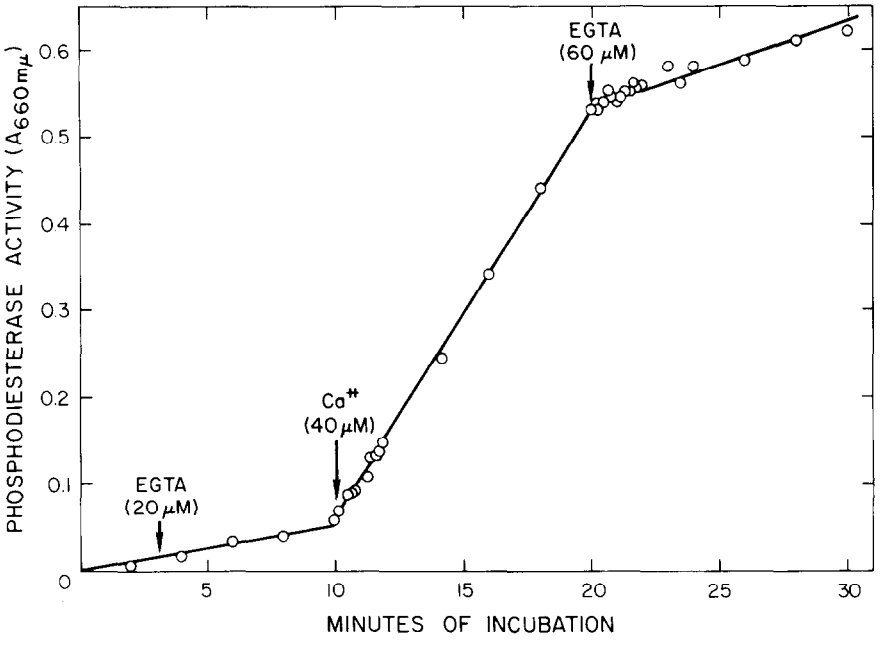

Fig. 2. Effect of Ca^{++} or EGTA on the rate of cyclic AMP hydrolysis catalyzed by bovine brain phosphodiesterase. A reaction mixture of 25 ml contained 40 mM Tris–Cl (pH 8.0), 3 mM $MgSO_4$, 0.02 mM EGTA, 2.6 mg of an activator-deficient phosphodiesterase and 0.05 mg of a highly purified activator. The reaction at 30°C was initiated by 2 mM cyclic AMP; at 10 and 20 min, Ca^{++} (40 μM) and EGTA (60 μM) were added to the reaction mixture, respectively. At times indicated, 0.5 ml of the reaction mixture was transferred to a tube kept at 100°C to terminate the enzyme activity. Phosphodiesterase was assayed by a two-stage procedure [7].

the enhanced Ca^{++} influx when the nervous tissue is stimulated. The increase of intracellular Ca^{++} may activate phosphodiesterase, which then restores the concentration of cyclic AMP to its prestimulated level.

A protein activator specific for phosphodiesterase has now been described by several other groups of workers [9,17,24]. The work presented here as well as that from other laboratories suggests that the in vivo regulation of phosphodiesterase by the activator may be related to the Ca^{++} flux, as first suggested by Kakiuchi et al. [18]. This notion appears credible from the following considerations: first, stimulation of phosphodiesterase requires micromolar Ca^{++} which is within the physiological range of this cation [23]; second, the activator is generally in excess of phosphodiesterase [7] so that the Ca^{++} flux could be rate-determining; and third, the response of the enzyme–activator system to Ca^{++} is rapid (fig. 2) and therefore is compatible with the fast catabolism of cyclic AMP [25,26]. Although direct in vivo experimental data in support of this notion is still lacking, the observation of Namm et al. [27] that cyclic AMP level in rat heart was increased or decreased by perfusion of the heart with a Ca^{++}-free or a Ca^{++}-rich medium, respectively, is in accord with this view.

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References

[1] Cheung, W. Y. (1967) Biochem. Biophys. Res. Commun., 29, 478–482.
[2] Smoake, J. A., Song, S. Y. and Cheung, W. Y. (1974) Biochim. Biophys. Acta, 341, 402–411.
[3] Uzunov, P. and Weiss, B. (1972) Biochim. Biophys. Acta, 284, 220–226.
[4] Thompson, W. J. and Appleman, M. M. (1971) Biochemistry, 10, 311–316.
[5] Appleman, M. M., Thompson, W. J. and Russel, T. R. (1973) in: Advances in Cyclic Nucleotide Research (Greengard, P. and Robison, G. A., eds.), Vol. 3, pp. 65–98, Raven Press, New York.
[6] Cheung, W. Y. (1970) in: Advances in Biochemical Psychopharmacology Vol. 3, pp. 51–66, Raven Press, New York.
[7] Cheung, W. Y. (1971) J. Biol. Chem., 243, 2859–2869;
[8] Lin, Y. M., Liu, Y. P. and Cheung, W. Y. (1974) J. Biol. Chem. (in press).
[9] Teo, T. S. and Wang, J. H. (1973) J. Biol. Chem., 248, 5950–5955.
[10] Kakiuchi, S., Yamazaki, R., Teshima, Y. and Uenishi, K. (1973) Proc. Natl. Acad. Sci. (USA) 70, 3526–3530.
[11] Cheung, W. Y. (1969) Biochim. Biophys. Acta, 191, 303–315.
[12] Lin, Y. M., Liu, Y. P. and Cheung, W. Y. in: Methods in Enzymology (O'Molley, B. W. and Hardman, J. G., eds.), Academic Press, New York (in press).
[13] Warburg, O. and Christian, W. (1941) Biochemistry, 121, 428–436.
[14] Andrews, P. (1964) Biochem. J., 91, 222–233.
[15] Teshima, Y. and Kakiuchi, S. (1974) Biochem. Biophys. Res. Commun., 56, 489–495.
[16] Teo, T. S., Wang, T. H. and Wang, J. H. (1973) J. Biol. Chem. 248: 588–595.
[17] Kakiuchi, S. and Yamazaki, R. (1970) Biochem. Biophys. Res. Commun., 41, 1104–1110.
[18] Kakiuchi, S., Yamazaki, R. and Teshima, Y. (1972) in: Advances in Cyclic Nucleotide Research (Greengard, P. and Robison, G. A., eds.), Vol. 1, pp. 455–477, Raven Press, New York.
[19] Cheung, W. Y. and Salganicoff, L. (1967) Nature, 214, 90–91.
[20] DeRobertis, E., Arnaiz, G. R. D. L., Alberici, A., Butcher, R. A. and Sutherland, E. W. (1967) J. Biol. Chem., 242, 3487–3493.
[21] McAfee, D. A., Schorderet, M. and Greengard, P. (1971) Science, 171, 1156–1158.
[22] Schimizu, H., Creveling, C. R. and Daly, J. W. (1970) Mol. Pharmacol., 6, 184–188.
[23] Hodgkin, A. L. and Keynes, R. D. (1957) J. Physiol., 138, 253–281.
[24] Goren, E. N. and Rosen, O. M. (1971) Arch. Biochim. Biophys., 142, 720–723.
[25] Kakiuchi, S. and Rall, T. W. (1968) Mol. Pharmacol., 4, 367–378.
[26] Kakiuchi, S. and Rall, T. W. (1968) Mol. Pharmacol., 4, 379–388.
[27] Nam, D. H., Mayer, S. E. and Maltbie, M. (1968) Mol. Pharmacol., 4, 522–530.