MECHANISM OF STIMULATION OF Ca^{2+} PLUS Mg^{2+}-DEPENDENT PHOSPHODIESTERASE FROM RAT CEREBRAL CORTEX BY THE MODULATOR PROTEIN AND Ca^{2+}.

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Summary : The activity of phosphodiesterase ("Ca^{2+} plus Mg^{2+}-dependent" phosphodiesterase) of a preparation from brain was found to depend on the presence of both Ca^{2+} and a protein factor called modulator. It was shown by gel filtration that the active enzyme-modulator complex (MW, about 200,000) was formed from the modulator (MW, 28,000) and an inactive enzyme (MW, about 150,000) in the presence of Ca^{2+}. When EGTA was added, this active enzyme-modulator complex dissociated into inactive enzyme and modulator. These results, together with the finding of Teo and Wang that Ca^{2+} binds to the modulator, could explain the stimulatory effect of Ca^{2+} on this enzyme as follows: The "Ca^{2+} plus Mg^{2+}-dependent" phosphodiesterase may exist as the inactive free form in equilibrium with the active enzyme-modulator (Ca^{2+}) complex, and Ca^{2+}, through binding to the modulator, may shift the equilibrium towards formation of the active enzyme-modulator (Ca^{2+}) complex, thereby increasing the activity of the mixture. On decreasing the concentration of Ca^{2+}, the process is reversible.

Hydrolysis of cAMP[1] and cGMP to their corresponding 5'-nucleotides catalyzed by phosphodiesterase is the only well established mechanism through which the biological actions of these nucleotides are terminated. Work from this laboratory has demonstrated that phosphodiesterase activity in the soluble fraction of the brain homogenate was stimulated by micromolar concentrations of Ca^{2+} in the presence of Mg^{2+} (1-3), and that the stimulation of this enzyme activity by Ca^{2+} required the presence of a protein factor called modulator (4,5). Recent work by Teo and Wang (6) has identified this protein factor with the protein activator first described by Cheung (7). Although the physiological significance of the stimulation of the activity of this enzyme by Ca^{2+} and the modulator is still unclear, the findings that the concentration of Ca^{2+} that regulates the activity of

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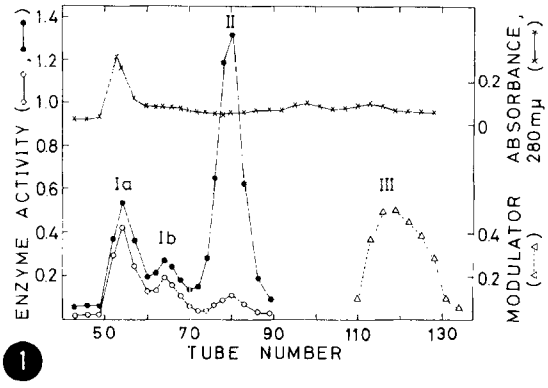
1 Abbreviations used are: cAMP, adenosine cyclic 3',5'-monophosphate; cGMP, guanosine cyclic 3',5'-monophosphate; EGTA, ethyleneglycol bis (β-aminoethylether)-N,N'-tetraacetic acid.

phosphodiesterase seems to be within the physiological range (2,8), and that these effects of Ca^{2+} and the modulator are greatest at low concentrations (1 μM or less) of cyclic nucleotides (5), which are close to the concentrations of these nucleotides found in tissues, strongly suggest that this mechanism actually functions *in vivo*. We proposed calling the enzyme responsible for this activity "Ca^{2+} plus Mg^{2+}-dependent" phosphodiesterase. Teo and Wang (6) have also shown that the activation of a preparation of cAMP phosphodiesterase from bovine heart was completely dependent on the presence of a protein activator and Ca^{2+} in the reaction system.

Stimulation of the enzyme activity by the modulator was stoichiometric and was not due to a catalytic process requiring Ca^{2+} (5,9). The modulator seemed to have a specific effect and could not be replaced by other proteins (5,9). A hypothesis has been advanced that the modulator may reversibly bind to the inactive form of enzyme to form an active enzyme-modulator complex and that Ca^{2+} shifts the equilibrium of this reaction towards formation of the active enzyme-modulator complex (5). This paper provides substantial evidence supporting this hypothesis.

METHODS

Adult male rats of the Sprague Dawley strain were sacrificed by decapitation under ether anesthesia. Then their cerebral cortices were rapidly excised and homogenized in 3 volumes of cold medium consisting of 20 mM Tris-HCl (pH 7.5), 1 mM $MgCl_2$, 2 mM 2-mercaptoethanol, and 0.1 mM EGTA. The homogenate was then contrifuged at 100,000 × g for 60 min. The supernatant fluid was applied to a Sephadex G-200 column and fractionated as illustrated in Fig. 1. Fr. II containing the "Ca^{2+} plus Mg^{2+}-dependent" phosphodiesterase was concentrated using a collodion filter under reduced pressure. The modulator was prepared from the hog brain extract after heat treatment (95°, 5 min). The modulator protein was purified as described by Teo *et al.* (10) with a slight modification. The media used for DEAE-



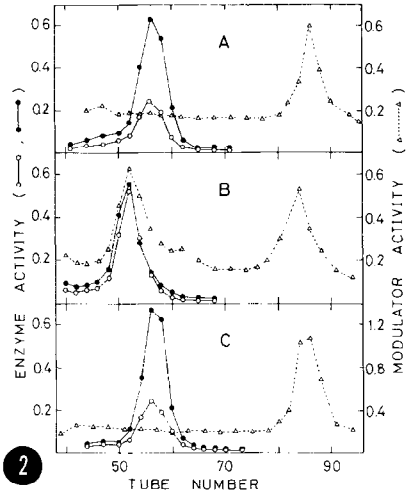


Fig. 1. Fractionation of brain supernatant on a Sephadex G-200 column. Fifteen ml of the supernatant fluid, containing approximately 100 mg of protein, was applied on a Sephadex G-200 column (5 x 85 cm). The column was then eluted with 20 mM Tris-HCl (pH 7.5), 1 mM $MgCl_2$, 100 mM NaCl, 0.1 mM dithiothreitol, and 0.1 mM EGTA, at a flow rate of 18.3 ml per hour, and fractions of 10.4 ml were collected. Phosphodiesterase activity was assayed in the presence of the modulator and either 0.1 mM $CaCl_2$ (●—●), or 1 mM EGTA (○—○). Modulator activity (△--△) was assayed after heating aliquots of the fractions for 3 min in boiling water. Enzyme activity and modulator activity are expressed as absorbance at 790 mμ of Pi colour.

Fig. 2. Ca^{2+}-dependent association of the modulator and enzyme. For panel (A) (top), 80 μl of modulator (114 units) was mixed with 2 ml of Fr. II (2 units of enzyme activity) containing EGTA at a final concentration of 0.1 mM. The mixture was incubated for 30 min at 30°, and then applied on a Sephadex G-200 column (2.5 x 90 cm) which had been equilibrated with medium consisting of 20 mM Tris-HCl (pH 7.5), 1 mM $MgCl_2$, 100 mM NaCl, 0.1 mM DTT, and 0.1 mM EGTA. The column was eluted with the same medium at a flow rate of 7 ml/hour, and fractions of 4.1 ml were collected. Aliquots from fractions were assayed for phosphodiesterase activity with (●—●) or without (○—○) added modulator protein. $CaCl_2$, at a final concentration of 0.05 mM was added to all assay tubes. Aliquots of 0.05 ml were assayed for modulator activity (△---△). Results are expressed as absorbance at 790 mμ of Pi colour. For panel (B) (middle), EGTA in the elution medium was replaced by 0.2 mM $CaCl_2$. The other components of the medium were as for (A). A mixture of 80 μl of the modulator, 2 ml of Fr. II, and $CaCl_2$ at a final concentration of 0.025 mM was incubated as for (A) and then applied to the column. The column was eluted with the above medium containing $CaCl_2$. Other conditions were as for (A), except that 0.15 ml aliquots were used for assay of modulator. For panel (C) (bottom), the modulator and Fr. II were not mixed but applied on the column successively. The medium was the same as for (B). The results obtained with modulator and Fr. II are shown on the same panel.

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cellulose and Sephadex G-100 columns contained 0.1 mM EGTA. Phosphodiesterase activity was determined by a modification (5) of the method of Butcher and Sutherland (11) using incubation medium consisting of 80 mM

imidazole-HCl (pH 6.9). 3 mM $MgSO_4$, 0.3 mM dithiothreitol, and 0.9 mM cAMP as substrate. The modulator was determined from its ability to enhance the activity of diluted 40,000 rpm supernatant fluid of brain homogenate in the presence of 2.5 μM Ca^{2+}. The amount of the modulator which doubled the enzyme activity in the standard system was defined as 10 units. Units of phosphodiesterase activity are in terms of μmoles of cAMP hydrolyzed per min at 30° in the presence of both the modulator and Ca^{2+}.

RESULTS AND DISCUSSION

The "Ca^{2+} plus Mg^{2+}-dependent" phosphodiesterase free from the endogenous modulator was obtained from the supernatant fluid by gel filtration. This enzyme was eluted in Fr. II, while the modulator was eluted in Fr. III

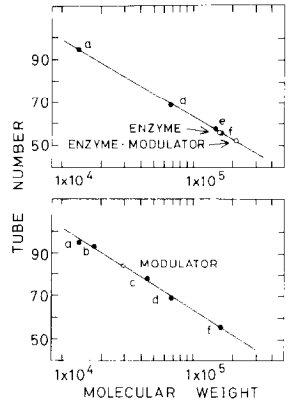

Fig. 3. Determination of the molecular weights of the modulator, enzyme, and the enzyme-modulator complex using a Sephadex G-200 column. Gel filtration was performed as in Fig. 2. The molecular weight markers used were cytochrome c, (a); myoglobin, (b); ovalbumin, (c); bovine plasma albumin, (d); aldolase, (e); and γ-globulin, (f).

(Fig. 1.)[2]. In earlier work (3,8), the column was eluted with medium without EGTA (10 mM Tris-HCl, pH 7.5, and 1 mM $MgCl_2$). Fr. II obtained with this medium contained a minute amount of the modulator and therefore, its activity increased when the concentration of Ca^{2+} in the assay mixture was

2 Two other phosphodiesterase activities, Frs. Ia and Ib, were also separated, but these were not stimulated by Ca^{2+}.

raised (3,8). Later, it was found that inclusion of EGTA in the elution medium completely eliminates such contaminating modulator from Fr. II. The activity of Fr. II obtained with medium containing EGTA, and used throughout the present study, was found to be completely dependent on the addition of modulator. These observations suggested the possibility of formation of an enzyme-modulator complex in the presence of Ca^{2+} and its dissociation into free enzyme and modulator on addition of EGTA. This possibility was examined in the experiments presented below.

When a mixture of Fr. II and excess modulator was applied to a gel column and the column was eluted with medium containing EGTA, the enzyme and the modulator were eluted separately (Fig. 2A). The positions of their peaks corresponded to those obtained on gel filtration of the enzyme and the modulator separately (Fig. 2C). No modulator (Δ---Δ) was found in the enzyme peak and activation of this peak by Ca^{2+} was dependent on the addition of the modulator to the assay tubes (cf. (●——●) and (○——○)). On the other hand, as shown in Fig. 2B, when the column, which was charged with a mixture of Fr. II and the modulator, was eluted with medium containing Ca^{2+}, the enzyme was eluted with the modulator and did not require addition of modulator for activity (cf. (●——●) and (○——○) of Fig. 2B). Two peaks of modulator are shown in Fig. 2B. This is because the amount of modulator used was in excess of enzyme and unbound free modulator was also eluted[3]. With medium containing Ca^{2+} (Fig. 2B), the enzyme peak was eluted before that shown in Fig. 2A, indicating that a higher molecular weight species had been formed. These results are consistent with the idea that the enzyme-modulator complex is formed in the presence of Ca^{2+} and that, when the concentration of Ca^{2+} is reduced by the addition of EGTA, the complex dissociates into the free enzyme and the modulator. Identical results were obtained with cGMP as substrate. Gel filtration showed that the molecular weight

3 It looks that the total amount of modulator shown in Fig. 2B is greater than in Fig. 2A. This apparent difference is due to the difference in the amounts of aliquots used for modulator assay in both experiments.

of the modulator was 28,000 (Fig. 3)[4], which agrees well with the value reported by Teo et al. (10). The molecular weights of the free enzyme and the enzyme-modulator complex were estimated to be about 150,000 and 200,000, respectively (Fig. 3). Therefore, it is likely that two modulator molecules may bind to the enzyme, though not conclusive at moment.

The present results, combined with the findings of Teo and Wang (6) that Ca^{2+} binds to the modulator protein with dissociation constants of 3 and 12 μM, strongly suggest that the activity of the "Ca^{2+} plus Mg^{2+}-dependent" phosphodiesterase is regulated as follows:

$$\left[\text{Enzyme}\right]_{\text{inactive}} + \text{Modulator} + Ca^{2+} \rightleftharpoons \left[\text{Enzyme} \bullet \text{Modulator} \bullet Ca^{2+}\right]_{\text{active}}$$

The modulator was shown to be present in the brain in excess of enzyme activity (9). The above equilibrium may be influenced by a variety of factors in the cell, such as pH and salts, but the intracellular concentration of Ca^{2+} is probably the main factor determining this equilibrium and thereby regulating enzyme activity. By analogy with the muscle system (12, 13), the intracellular concentration of Ca^{2+} in brain tissue may be controlled by Ca^{2+} flux through the cell membrane and also by uptake and release of Ca^{2+} by cellular components. Recently, a microsomal component was obtained from brain, which, like the sarcoplasmic reticulum, accumulates Ca^{2+} (14, 15).

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4 Previously, when gel filtration was performed by using Sepharose 6 B with low ionic strength medium (10 mM Tris-HCl, pH 7.5, and 1 mM $MgCl_2$, either in the presence or absence of dithiothreitol), the molecular weight of the modulator was estimated to be about 68,000 by using bovine serum albumin as a marker (4, 8). Later study revealed, however, that the calibration of molecular weight with such low ionic strength medium gave incorrect values.

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