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Ca^{2+}-DEPENDENT CYCLIC NUCLEOTIDE PHOSPHODIESTERASE FROM RABBIT LUNG

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SUMMARY

Studies are presented which demonstrate that rabbit lung contains both Ca^{2+}-activated cyclic nucleotide phosphodiesterase and calmodulin activity. The Ca^{2+}-activatable cyclic nucleotide phosphodiesterase is different from the common type in that it contains tightly bound calmodulin. The bound calmodulin is not dissociated from the enzyme even in very low concentrations of Ca^{2+} after DEAE-cellulose and Sephadex G-200 column chromatography.

INTRODUCTION

A Ca^{2+}-dependent cyclic nucleotide phosphodiesterase was discovered in rat brain extracts by Kakiuchi and Yamazaki [1], and later shown to be present also in other mammalian tissues [2]. The activation of the enzyme depends on the presence of a Ca^{2+}-binding protein [3,4] which was originally discovered by Cheung [5] as an activator protein of phosphodiesterase and recently this protein was renamed calmodulin by Cheung. The mechanism of the enzyme activation has been proposed as according to Scheme I:

$$CM + Ca^{2+} \rightleftharpoons CM - Ca^{2+} \rightleftharpoons CM^* - Ca^{2+} \qquad (I)$$

$$CM^* - Ca^{2+} + E \rightleftharpoons \begin{matrix} E \\ | \\ CM^* \end{matrix} - Ca^{2+} \rightleftharpoons \begin{matrix} E^* \\ | \\ CM^* \end{matrix} - Ca^{2+} \qquad (II)$$

Scheme I

where E and CM stand for the enzyme and the calmodulin, respectively, and E^* and CM^* denote the active forms of the respective proteins. The main feature of the scheme is that the calmodulin exists as an independent protein molecule which associates with the enzyme only upon binding of Ca^{2+}. Recently, Hidaka et al. [6] have shown the separation of two Ca^{2+}-dependent phosphodiesterases

Abbreviations used are EGTA, ethylene glycol bis (β-amino-ethyl ether) N,N'-tetraacetic acid; cAMP, cyclic adenosine 3':5'-monophosphate; cGMP, cyclic guanosine 3'5'-monophosphate; CM, calmodulin.

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from human aorta, and both forms are activated by the calmodulin. Also Dedman et al. [7] have shown the presence of a calmodulin independent, but Ca^{2+}-activatable cyclic nucleotide phosphodiesterase among testis sertolis proteins. These observations indicate that not all Ca^{2+}-dependent cyclic nucleotide phosphodiesterases are regulated by calmodulin.

In the present study, we have found the existence of a Ca^{2+}-dependent phosphodiesterase in rabbit lung which is not activated by the addition of calmodulin. The enzyme preparations, however, contain the calmodulin activity even after several column chromatographies. The result suggests that the enzyme contains tightly bound calmodulin which mediates the Ca^{2+}-activation of the enzyme. Thus, scheme I should not be considered as a general mechanism for the calmodulin mediated enzyme activation.

MATERIALS AND METHODS

Bovine heart calmodulin deficient phosphodiesterase and bovine brain calmodulin were prepared according to the previously described methods [8-10]. The assays of phosphodiesterase and calmodulin were also as previously described [10]. To prepare the $(NH_4)_2SO_4$ fraction of rabbit lung extracts, frozen rabbit lung (Pel Freeze) was thawed and homogenized in 2.5 volumes of a buffer containing 20 mM Tris-HCl and 1 mM EDTA, pH 7.5. The homogenate was centrifuged at 3,500 rpm for 30 min, the supernatant was collected and brought to 55% saturation of $(NH_4)_2SO_4$. The solution was then centrifuged at 8,000 rpm for 20 min and the pellet was suspended in minimal volumes of 20 mM Tris, 1 mM imidazole buffer, pH 7.5, containing 1 mM Mg^{2+} 0.1 mM EGTA and 10 mM 2-mercaptoethanol (buffer A). The solution was dialyzed against the same buffer overnight. The dialyzed sample was centrifuged at 40,000 rpm for 30 min and the clear supernatant was collected.

RESULTS AND DISCUSSION

Table I shows that rabbit lung contains both Ca^{2+}-activated cyclic nucleotide phosphodiesterase and calmodulin activities. The presence of these activities in human and guinea pig lungs [11-13] has been shown before. However the mechanism of Ca^{2+} activation of the phosphodiesterase and the relationship between the enzyme and the calmodulin have not been investigated.

A common type of Ca^{2+}-activatable phosphodiesterase that is found in many mammalian tissues has been shown to depend on the presence of the calmodulin for its activation. The enzyme and calmodulin exist as separate pro-

TABLE 1

CYCLIC NUCLEOTIDE PHOSPHODIESTERASE AND CALMODULIN IN CRUDE EXTRACT OF RABBIT LUNG

	Activity	
	Units/mg protein	Units/KG (Wet tissue weight)
Calmodulin activity	45.0[a]	2,750,000
Phosphodiesterase activity		
$+Ca^{2+}$	0.011[b]	221.0
$+Ca^{2+}$ + CM	0.011[b]	221.0
$-Ca^{2+}$ (EGTA)	0.004[b]	78.0

20 gms rabbit lung was homogenized in 20 mM Tris-HCl containing 1 mM EDTA, pH 7.5. The homogenate was centrifuged as described in "Materials and Methods." The supernatant was analyzed for phosphodiesterase activity in the presence 0.1 mM Ca^{2+} or 0.1 mM Ca^{2+} with 60 units of exogenous bovine brain calmodulin, when in the absence of Ca^{2+} 0.1 mM EGTA was used. For the assay of calmodulin, an aliquot from the supernatant was boiled for two minutes, centrifuged to remove the precipitate and then dialyzed prior to use.

[a]One unit of the calmodulin is defined as the amount which give rise to 50% of the maximal enzyme activation of the standard phosphodiesterase from bovine brain.

[b]One unit of phosphodiesterase is defined as the amount of enzyme which, when fully activated, hydrolyzes 1 μ mole cAMP per minute at 30°.

teins in buffers containing low concentrations of Ca^{2+}. Upon increase in Ca^{2+} concentration in the medium, Ca^{2+} binds to the calmodulin and the protein-metal complex thus associates with the enzyme resulting in enzyme activation (see scheme I in Introduction). When a crude preparation of this enzyme is chromatographed on a DEAE-cellulose column or by gel filtration in buffers containing EGTA, the enzyme and the calmodulin are separated from each other. Consequently, the fractionated enzyme is no longer activatable by Ca^{2+} unless calmodulin is added in the assay.

The Ca^{2+}-activatable cAMP phosphodiesterase from rabbit lung appears different from the common type of Ca^{2+}-activatable phosphodiesterase. When an $(NH_4)_2SO_4$ pellet fraction of lung extract was chromatographed on DEAE-cellulose column in a buffer containing 0.1 mM EGTA, three peaks of cAMP phosphodiesterase activity could be resolved (Fig. 1A). All three peaks of activity could be activated by Ca^{2+} without the addition of calmodulin. When the calmodulin

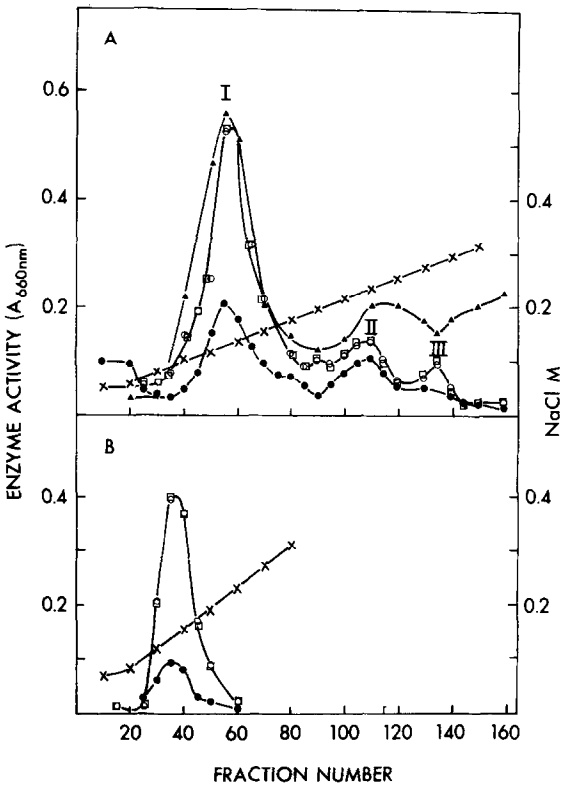

Figure 1. Elution profile of phosphodiesterase from DEAE-cellulose column. A) Supernatant was applied on a DEAE-cellulose column (2.8 x 40 cm) previously equilibrated with buffer A. The column was washed with two column volumes of buffer A containing 0.05 M NaCl followed by a linear salt gradient (x—x) from 0.05 to 0.35 M in the same buffer. Fractions were analyzed for phosphodiesterase activity in the presence of 0.1 mM Ca^{2+} with 60 units of bovine brain calmodulin (o—o) or without calmodulin (□—□) and with 0.1 mM EGTA (●—●). For the assay of the calmodulin, an aliquot of the fraction was diluted 100 fold and incubated in a boiling water bath for 2 min, then 30 µl of the boiled supernatant was used to activate the calmodulin-deficient phosphodiesterase (0.015 units) from bovine brain (▲—▲). B) Peak I, from the first DEAE-cellulose, was dialyzed against buffer A overnight and rechromatographed on a DEAE-cellulose column under the same conditions.

activity was monitored in the eluents, it was found to be distributed in all the fractions containing phosphodiesterase activity.

Since peak I phosphodiesterase contained most of the enzyme activity, it was pooled and rechromatographed on a DEAE-cellulose column in a buffer containing EGTA. Fig. 1B shows that the enzyme was eluted from the column at the same salt concentrations, about 0.15 M NaCl. As the chromatographed enzyme was still activatable by Ca^{2+}, addition of the calmodulin to the assay mixture did

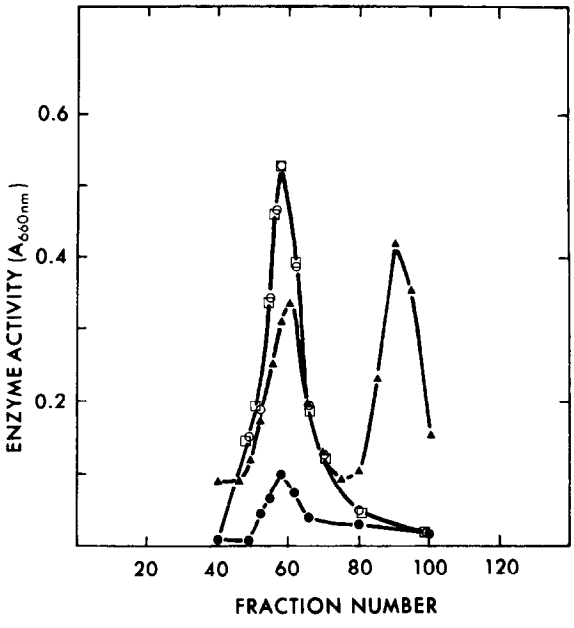

Figure 2. Gel filtration of phosphodiesterase on Sephadex G-200. Fractions from the second DEAE-cellulose column containing the enzyme and calmodulin were collected (75 ml pooled fraction was concentrated to 4.0 ml by diaflo ultrafiltration using an Amicon PM 10 membrane). The concentrated sample was applied to a Sephadex G-200 column (2.5 x 80 cm) which had been equilibrated and eluted with buffer A containing 0.1 M NaCl and 10% sucrose. Phosphodiesterase activity in the presence of 0.1 mM Ca^{2+} with 60 units of calmodulin from bovine brain (o—o) or without calmodulin (□—□), phosphodiesterase activity in the presence of 0.1 mM EGTA (●—●) and calmodulin activity (▲—▲), other conditions were the same as in Fig. 1.

not result in changes in the Ca^{2+}-activation. When the fractions containing the enzyme activity were pooled and assayed, they were found to contain the calmodulin activity. These results show that rabbit lung Ca^{2+}-activatable cyclic nucleotide phosphodiesterase differs from the common type of the enzyme in that it does not depend on the added calmodulin for activity. Since the calmodulin activity was found in the enzyme preparation even in the rechromatographed sample, it is possible that the enzyme contains tightly bound calmodulin which mediates the Ca^{2+} effect.

To further test this suggestion, the pooled fraction from the second DEAE-cellulose column was concentrated and applied to a Sephadex G-200 column. Figure 2 shows that, like the previous column chromatographies, gel filtration also failed to render the cyclic nucleotide phosphodiesterase Ca^{2+} insensitive.

The enzyme was eluted in a single peak and the enzyme activity was enhanced by Ca^{2+} to the same extent either with or without the additions of the purified bovine brain calmodulin. The calmodulin activity was found to elute in two peaks, one of them correlated closely with the enzyme activity whereas the other was eluted at a position identical to that of the free bovine brain calmodulin. The result further supports the notion that the Ca^{2+}-activatable phosphodiesterase from rabbit lung binds the calmodulin tightly irrespective of the presence of Ca^{2+}. The reason for the presence of free calmodulin in the enzyme preparation (Fig. 2) is not known. However, since only about 45% of the enzyme activity of the second DEAE-cellulose column pooled fraction was recovered from the gel filtration column, it suggests that about 55% of the enzyme was inactivated. Consequently, the free calmodulin might have been dissociated from the inactivated enzyme.

In conclusion, the present study suggests that the Ca^{2+}-activatable cyclic nucleotide phosphodiesterase in rabbit lung is different from the common type in that it contains tightly bound calmodulin. This bound calmodulin is not dissociated from the enzyme even in very low concentrations of Ca^{2+}. Thus, it appears that the mechanism of action of the calmodulin described in scheme I should not be considered as generally applicable to all the enzyme systems. A second mechanism is proposed as follows (scheme II):

$$Ca^{2+} + E - CM \rightleftharpoons E - CM - Ca^{2+} \rightleftharpoons E^* - {}^*CM - Ca^{2+}$$

Scheme II

where E and CM stand for the enzyme and the calmodulin respectively and the asterisk indicates the activated state of the protein species.

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