Calmodulin activation of rat lung adenylate cyclase is independent of the cytoplasmic factors modulating the enzyme

A. Felix OFULUE and M. S. NIJJAR

Department of Pathology, Faculty of Medicine, University of Manitoba, Winnipeg, Manitoba, Canada R3E 0W3

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Adenylate cyclase activity in the rat lung membranes washed with 150 μM -EGTA was stimulated by calmodulin in the presence of 100 μM - Ca^{2+} . The calmodulin activation of the enzyme was concentration-dependent; however, at high concentrations the activation was diminished. Activation of adenylate cyclase by calmodulin was immediate, reversible and due to an increase in the V_{max} , without apparent effect on the affinity of the enzyme for ATP. The rat lung supernatant produced additive activation of the adenylate cyclase that was already maximally stimulated by calmodulin, indicating that either calmodulin and cytoplasmic factors act at different sites on adenylate cyclase or different adenylate cyclases may be involved. The data further support our previous conclusion that calmodulin is not involved in the activation of adenylate cyclase by cytoplasmic factors in rat lungs.

A low-molecular-weight Ca^{2+} -binding protein, referred to as calmodulin or Ca^{2+} -dependent regulator, appears to influence numerous Ca^{2+} -mediated intracellular processes in a variety of tissues and is thus considered to be an important regulator of cellular metabolism (Cheung, 1980). Calmodulin binds to Ca^{2+} to form an active complex that activates cyclic AMP phosphodiesterase in most tissues (Cheung, 1971; Brostrom & Wolff, 1974; Cheung *et al.*, 1975) and adenylate cyclase in some tissues (Cheung *et al.*, 1975; Brostrom *et al.*, 1977; Valverde *et al.*, 1979; Gnegy *et al.*, 1980; Sharp *et al.*, 1980). It would thus seem that the tissue concentration of cyclic AMP can be modulated by calmodulin. However, the role of calmodulin in the regulation of lung cyclic AMP metabolism remains to be defined.

The presence of calmodulin activity and its regulation of cyclic AMP phosphodiesterase have been demonstrated in human and animal lungs (Davis & Kuo, 1977; Bergstrand *et al.*, 1978; Sharma & Wirsch, 1979). It seems important, therefore, to investigate the possible role of calmodulin in the regulation of lung adenylate cyclase. In a previous report (Nijjar, 1979), we demonstrated the presence of heat-labile cytoplasmic factor(s) in adult rat lungs that markedly enhanced basal adenylate cyclase activity in the particulate

fraction prepared from rat lungs. However, exogenous calmodulin did not alter adenylate cyclase activity in the same particulate fraction under identical conditions. It was therefore concluded that calmodulin was not involved in the activation of adenylate cyclase by the cytoplasmic factor(s) in adult rat lungs (Nijjar, 1979).

In the present paper, we report evidence to demonstrate calmodulin activation of rat lung adenylate cyclase in membrane preparations washed extensively with EGTA. Moreover, the calmodulin activation of the enzyme was independent of the activation by the lung cytoplasmic factors.

Experimental procedures

Materials

All reagents were of analytical grade and purchased from either Sigma Chemical Co. (St. Louis, MO, U.S.A.) or from Fisher Scientific locally. $[8\text{-}^{14}\text{C}]\text{ATP}$ was purchased from New England Nuclear (Boston, MA, U.S.A.). Homogeneous calmodulin, calmodulin-deficient phosphodiesterase and calmodulin-binding protein were prepared from bovine brain (Wang & Desai, 1977; Sharma & Wang, 1979) and were generous gifts from Dr. J. H. Wang and Dr. R. K. Sharma of the Department of Biochemistry.

Preparation of lung tissue membrane and supernatant fractions

Adult male Sprague–Dawley rats (Charles River Breeding Laboratories, Quebec, Canada) were decapitated, exsanguinated and the lungs were quickly removed and washed in 0.27M-sucrose/10mM-Tris/HCl buffer (pH 7.4) in a chilled Petri dish. The lungs were then dissected free of all bronchi and vasculature, and only the peripheral areas of lungs consisting predominantly of alveolar tissues were sliced, homogenized in 9 vol. of buffer in a Potter–Elvehjem homogenizer by 15 hand-driven strokes and centrifuged at 15000 *g* for 10 min. The supernatant was further centrifuged at 105000 *g* for 60 min. The resulting supernatant was used as the source of adenylate cyclase activator (Nijjar, 1979). The pellet was rehomogenized in a loose-fitting homogenizer (five strokes), centrifuged as before and suspended in the homogenizing buffer (10%, w/v). This fraction was designated the membrane fraction and used as a source of adenylate cyclase. A portion of this fraction was then washed in 150 μ M-EGTA by re-homogenization and recentrifugation (three times) and finally the pellet was resuspended in the previous homogenizing buffer.

Determination of calmodulin activity

The rat lung supernatant or membrane fractions were incubated in a boiling-water bath for 3 min, cooled on ice and centrifuged at 2500 rev./min for 30 min in an International refrigerated centrifuge (model B-20). The resulting supernatant was dialysed and various dilutions of the sample were analysed for calmodulin activity by measuring the activation of calmodulin-deficient cyclic AMP phosphodiesterase in the absence (0.15 mM-EGTA) and presence of 0.167 mM- Ca^{2+} (Sharma & Wang, 1979). The concentration of calmodulin present in the samples was determined by using a standard curve obtained simultaneously with homogeneous bovine brain calmodulin. Determinations were carried out at concentrations within the linear range. One unit of calmodulin activity represented the amount that produced 50% of the maximal acti-

vation of the standard phosphodiesterase (Sharma & Wang, 1979).

Adenylate cyclase assay

Adenylate cyclase activity was determined by a slight modification of the method of Drummond & Duncan (1970) as previously described (Nijjar, 1979) with $[8\text{-}^{14}\text{C}]\text{ATP}$ as substrate. The assay medium contained 25 mM-caffeine and 2 mM-cyclic AMP to prevent the degradation of radioactive cyclic AMP produced by the reaction.

Protein determination

Protein in membrane and supernatant preparations was estimated by the method of Lowry *et al.* (1951) with bovine serum albumin as standard.

Fig. 1. Effect of $[\text{Ca}^{2+}]$ on adenylate cyclase activity in rat lung membranes washed in 150 μ M-EGTA

The enzyme activity was determined in incubations containing the indicated concentrations of added CaCl_2 with 7 μ g of calmodulin (●), 14 μ g of calmodulin (□) and without calmodulin (▲). Homogeneous bovine brain calmodulin was used, where indicated, in the assay with 75 μ g of protein of the membranes. Each point is the mean of triplicate samples.

Table 1. Effect of EGTA washing of rat lung membranes on the adenylate cyclase activity and calmodulin content. Values are means $\pm$ S.D. for five experiments performed in triplicate samples. Rat lung tissues homogenate (10%, w/v) was used to prepare a 2 ml membrane fraction (3.32 mg of protein/ml). Of the fraction 1 ml was then washed in 150 μ M-EGTA and resuspended in the homogenizing buffer to 1 ml final volume (2.72 mg of protein/ml). Adenylate cyclase activity was determined with 75 μ g of protein of the membrane samples. The concentration of calmodulin in the samples was determined as described in the Experimental section.

Preparations	Total enzyme activity (pmol of cyclic AMP/min)	Calmodulin content (units)	Enzyme activity (pmol of cyclic AMP/per unit of calmodulin)
Unwashed membranes	647 $\pm$ 24	59 $\pm$ 8	11.0 $\pm$ 0.9
Washed membranes	351 $\pm$ 30	33 $\pm$ 5	10.6 $\pm$ 0.4

Results

Table 1 shows that extensive washing of rat lung membrane caused loss of the calmodulin content, with a concomitant decrease in the adenylate cyclase activity. However, the ratio of adenylate cyclase and calmodulin activity remained constant in the preparations, suggesting a relationship between calmodulin and adenylate cyclase activity in rat lung membranes.

Fig. 1 shows the dependence of the adenylate cyclase activity on $[Ca^{2+}]$. The enzyme activity in the EGTA-treated membranes was slightly stimulated by Ca^{2+} , followed by inhibition at higher concentrations. The addition of homogeneous bovine brain calmodulin further augmented the adenylate cyclase response to Ca^{2+} . In the presence of $100\mu M$ - Ca^{2+} , calmodulin stimulated the enzyme in a concentration-dependent manner, though at high concentrations of calmodulin the enzyme activation was diminished (Fig. 2).

In the EGTA-treated membranes that had been pre-incubated with $100\mu M$ - Ca^{2+} , addition of the homogenous bovine brain calmodulin enhanced adenylate cyclase activity readily and linearly until the addition of $100\mu M$ -EGTA when the enzyme activation was decreased (Fig. 3). The kinetics of adenylate cyclase activation (Fig. 4) revealed that calmodulin did not alter the affinity of the enzyme for ATP but increased the maximum velocity of the reaction.

As shown in Fig. 5, boiled rat lung supernatant, containing calmodulin activity, did not stimulate adenylate cyclase activity in unwashed (non-EGTA-treated) rat lung membranes and thus confirms our previous observations with a crude preparation of

rat lung membranes (Nijjar, 1979). In contrast, adenylate cyclase activity in the EGTA-treated membranes was stimulated by the same boiled lung supernatant (Fig. 5).

Fig. 6 shows that addition of fresh lung supernatant to adenylate cyclase that was maximally stimulated by calmodulin produced further activation of the enzyme in a concentration-dependent manner. In contrast, addition of the boiled lung supernatant diminished the activation of adenylate cyclase by the homogeneous bovine brain calmodulin.

Table 2 shows that fresh lung supernatant similarly enhanced the adenylate cyclase activities in both the EGTA-treated and -untreated membranes, whereas calmodulin in the presence of Ca^{2+} only affected the EGTA-treated membrane enzyme. The data further reveal that in the presence of calmodulin and Ca^{2+} , the rat lung supernatant produced additive activation of the enzyme in EGTA-treated membranes. Although calmodulin and Ca^{2+} did not stimulate adenylate cyclase in the untreated lung membranes, they did not alter activation of the

Fig. 2. Calmodulin activation of adenylate cyclase in EGTA-washed rat lung membrane in the presence of $100\mu M$ - Ca^{2+}

A membrane preparation equivalent to $75\mu g$ of protein was used in the assay. Each point represents the mean of triplicate determinations.

Fig. 3. Time course of the calmodulin stimulation of the EGTA-washed lung membrane adenylate cyclase

In a total volume of 5 ml, the assay medium contained 40 mM-Tris/HCl buffer (pH 7.4), 25 mM-caffeine, 5.5 mM-KCl, 10 mM-MgCl₂, 20 mM-phosphoenolpyruvate, pyruvate kinase (130 μg /ml), 2 mM-cyclic AMP and 0.56 mM-[8-¹⁴C]ATP (sp. radioactivity 10 μCi /mol). The reaction was initiated by addition of about 100 μg of protein of the membrane preparation. Incubation was maintained at 37°C. At indicated times, 100 μl portions (in duplicate) were withdrawn from the reaction medium into test tubes and boiled for 3 min to stop the reaction. At 10 min, 1 ml of the reaction was removed and transferred to another assay tube, serving as control, and then CaCl₂ was added. At 20 min, homogeneous bovine brain calmodulin (CM) was added. Finally at 30 min, EGTA was included and the reaction was allowed to continue for another 5 min. The concentrations of Ca^{2+} and EGTA shown represent the final concentrations in the reaction medium.

Fig. 4. Effect of calmodulin stimulation of adenylate cyclase in washed lung membranes at different substrate concentrations

Enzyme activities were performed without calmodulin (▲), with 7 μg of calmodulin (●) and with 14 μg of calmodulin (■). The insert is a Lineweaver-Burk plot of the data with (■) and without (▲) calmodulin (14 μg). Estimated K_m of the enzyme was 0.31 mM without calmodulin and 0.33 mM with calmodulin; V_{max} (expressed as pmol of cyclic AMP/min per mg of protein) was 278 without and 455 with calmodulin. Values are averages of triplicate determinations.

Fig. 5. Effect of dialysed, boiled lung supernatant (containing 0.2 μg of calmodulin/ μg of protein) on adenylate cyclase activities in EGTA-washed (●) and unwashed (Δ) membranes

Enzyme activities were determined in the presence of 100 μM - Ca^{2+} . Each point represents the mean of triplicate samples.

enzyme by the supernatant in these membrane preparations (Table 2).

Since calmodulin-binding protein abolishes calmodulin-stimulated enzyme reaction (Sharma *et al.*, 1978), the effect of this protein on calmodulin-

Fig. 6. Effect of fresh lung supernatant (▲) and dialysed, boiled lung supernatant, containing 0.5 μg of calmodulin/ μg of protein (□) on maximal Ca^{2+} -calmodulin stimulated lung membrane adenylate cyclase activity (●)

Each point is the average of triplicate determinations.

activated adenylate cyclase was examined. As shown in Fig. 7, calmodulin-binding protein diminished the calmodulin stimulation of the rat lung

Table 2. Effects of calmodulin and lung supernatant on adenylate cyclase activity in rat lung membranes

Values are means ± s.d. for five experiments performed in triplicate samples. Rat lung supernatant was prepared from the tissue homogenate as described in the Experimental section.

Additions	Adenylate cyclase activity (pmol of cyclic AMP/min per mg of protein)	
	Washed membranes	Unwashed membranes
None	135 ± 17	192 ± 20
Ca ²⁺ (100 μM) + calmodulin (21 μg)	322 ± 25	184 ± 24
Supernatant (23 μg)	389 ± 15	455 ± 19
Ca ²⁺ (100 μM) + calmodulin (21 μg) + supernatant (23 μg)	574 ± 22	449 ± 23

Fig. 7. Effect of calmodulin-binding protein on maximal Ca²⁺-calmodulin stimulated adenylate cyclase activity in EGTA-washed lung membranes

Each value represents the average of triplicate determinations.

membrane adenylate cyclase in a concentration-dependent manner.

Discussion

It is now well established that calmodulin plays a pivotal role in cellular regulation (Cheung, 1980). The regulation of adenylate cyclase by calmodulin has only been described in mammalian brain (Cheung *et al.*, 1975; Brostrom *et al.*, 1977; Gnegy *et al.*, 1980) and in the pancreatic islets of Langerhans (Valverde *et al.*, 1979; Sharp *et al.*, 1980). Although other tissues contain calmodulin activity, they do not appear to possess calmodulin-stimulated adenylate cyclase activity (Cheung *et al.*, 1978). The results reported in the present paper demonstrate for the first time that calmodulin in the presence of Ca²⁺ stimulates adenylate cyclase activity in rat lung membranes only after the

membranes had been washed with EGTA. Calmodulin alone was without effect. The concentration of Ca²⁺ was critical for the calmodulin stimulation of the enzyme.

The calmodulin activation of the rat lung adenylate cyclase was immediate, reversible and due to an increase in the maximum velocity of the reaction with no apparent alteration in the affinity of the enzyme for ATP. The Ca²⁺-calmodulin complex, rather than free calmodulin or Ca²⁺, was apparently the adenylate cyclase activator. It would thus seem that the molecular mechanism of rat lung adenylate cyclase regulation by calmodulin resembles that proposed for the Ca²⁺-dependent phosphodiesterase and adenylate cyclase of brain (Brostrom & Wolff, 1974; Brostrom *et al.*, 1977). Free calmodulin is inactive and thus unable to stimulate adenylate cyclase, but on binding to Ca²⁺ it forms an active Ca²⁺-calmodulin complex that associates reversibly with the enzyme to enhance the enzyme activity (Cheung *et al.*, 1975; Brostrom & Wolff, 1974; Brostrom *et al.*, 1977). The results of the present study are consistent with this model and also indicate that calmodulin bound to lung membranes probably saturated the adenylate cyclase; hence the addition of exogenous Ca²⁺ plus calmodulin did not enhance the enzyme activity (Nijjar, 1979; Table 2). The observation that EGTA treatment of the membrane caused removal of some of the bound calmodulin with a concomitant decrease in adenylate cyclase activity (Table 1), which was then stimulated by exogenous Ca²⁺ plus calmodulin (Table 2), clearly demonstrates the need for depletion of membrane calmodulin before the stimulatory effect on adenylate cyclase can be evident. Similar observations have been made in the brain tissue (Gnegy *et al.*, 1980).

Since the adult rat lung cytosol contains calmodulin activity (Fig. 5) as well as some other factors that markedly enhanced adenylate cyclase activity (Nijjar, 1979; Nijjar & Ho, 1980), we found it necessary to differentiate the effects of the two activators on adenylate cyclase. In the present study, we demonstrated that adenylate cyclase in EGTA-washed membranes was activated by the boiled rat

lung supernatant, whereas the enzyme in the unwashed membranes was not responsive to the boiled supernatant. This finding was consistent with that observed with homogeneous bovine brain calmodulin and therefore demonstrates a role of lung calmodulin in regulation of the adenylate cyclase activity. However, fresh lung supernatant markedly enhanced the enzyme activity in both the washed and unwashed membranes and produced further activation of the enzyme, which was already maximally stimulated by calmodulin. These findings confirm our previous conclusion (Nijjar, 1979) that calmodulin was not involved in the activation of rat lung adenylate cyclase by the cytoplasmic factors, and further demonstrate that the cytoplasmic factors activate adenylate cyclase independent of calmodulin. Other investigators have also reported the presence of cytoplasmic factors that can activate adenylate cyclase in different tissues and these are clearly different from calmodulin (Doberska & Martin, 1977; Sanders *et al.*, 1977; Beaumont *et al.*, 1979). The partially purified lung cytoplasmic factors activating adenylate cyclase are composed of two distinct proteins of mol.wts. 14 500 and 65 000, and their relative mobilities on sodium dodecyl sulphate/polyacrylamide-gel electrophoresis was quite different from that of calmodulin (Nijjar *et al.*, 1981).

One of us (Nijjar, 1979, 1980) has previously described some differences in the properties of adenylate cyclase from rat lungs and brain in terms of their development and sensitivity to various agonists including GTP, NaF, calmodulin and cytoplasmic factors. Thus it would appear that the present finding of calmodulin activation independent of the cytoplasmic factors in the EGTA-treated rat lung membranes may be specific for rat lungs and not applicable to the cyclases in other tissues.

There are several possibilities for the independent activations of rat lung adenylate cyclase by calmodulin and the cytoplasmic factors observed in the present study. The findings here suggest that the activators may stimulate the same enzyme through different sites. However, it is also possible that there are two separate enzymes that respond differentially to their specific stimulants. Calmodulin-dependent and -independent forms of adenylate cyclase have been demonstrated in rat brain (Brostrom *et al.*, 1978). Moreover, the enzyme responding to calmodulin may be present in one cell type of the lung tissue, whereas the same enzyme in another cell type responds to the cytoplasmic factors. The data do not allow differentiation of these possibilities. Further experiments where different isolated cell types are studied individually or where different forms of adenylate cyclase are resolved by affinity or Sephadex gel chromatography would provide a clarification of these possibilities.

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