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INVOLVEMENT OF A MACROMOLECULAR ACTIVATING FACTOR IN ACTIVITY OF GUANYLATE CYCLASE PARTIALLY PURIFIED FROM SUPERNATANT OF A PIG LUNG EXTRACT

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Summary

A guanylate cyclase preparation partially purified from supernatant of a pig lung extract was subjected to affinity chromatography on an Agarose-GTP column. The major portion of the cyclase activity was adsorbed on the column and then eluted with 50 mM EDTA and 0.5 M KCl, whereas the fractions non-adsorbed on the column contained a factor which enhanced the cyclase activity. Addition of the activating factor to a cyclase reaction mixture increased the enzyme activity without a time lag, and this enhancement by the factor was dose-dependent. With concomitant presence of cyclase and the factor in the reaction mixture the apparent K_m value for GTP-Mn^{2+} of the enzyme was 56 μM, this value being the same as in absence of the factor, however, here the maximum velocity increased 4-fold. The factor was nondiffusable, heat-labile, partially sensitive to trypsin, and resistant to acid or alkali. As estimated by gel filtration, this factor had an apparent molecular weight of 85 000.

Introduction

Guanylate cyclase (GTP pyrophosphate-lyase (cyclizing), EC 4.6.1.2), an enzyme that catalyzes the formation of cyclic GMP from GTP, reportedly exists in both soluble and particulate fractions of most tissue homogenates [1—3]. Guanylate cyclase has been partially purified from sea urchin sperm [4], human platelets [5], bovine lung [6], rat lung [7] and liver [8,9]. Little is

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known, however, of the mechanism involved in regulation of guanylate cyclase activity.

The cyclases are stimulated by nonionic detergents [3], unsaturated fatty acids [10] and other agents such as azide [11]. Recently, the endogenous activators for cyclase have been found in a synaptosomal fraction from rat brain [12], and in soluble fractions from rat liver [8] and *Tetrahymena pyriformis* [13].

We describe here a macromolecular activating factor which was dissociated from the partially purified cyclase of pig lung supernatant, and the properties of the factor are briefly described.

Materials and Methods

Materials

Fresh pig lung was obtained from a slaughterhouse. [8-^{3}H]GTP was purchased from the Radiochemical Centre Amersham; GTP, cyclic GMP and creatine phosphate, were from Sigma, creatine kinase from Boehringer Mannheim, neutral Al_2O_3 from M. Woelm, 3-isobutyl-1-methylxanthine from Aldrich, DEAE-cellulose (DE 52) from Whatman, DEAE-Sepharose CL-6B and Sepharose CL-6B from Pharmacia, Agarose-Hexane-GTP from P-L Biochemicals. Other chemicals were obtained from commercial sources.

Partial purification of guanylate cyclase

Pig lungs were placed on ice immediately following excision. All subsequent manipulations were carried out at 0—4°C. After removing connective and fat tissues and resectable trachea, 200 g lung were diced and homogenized in portions with 5 vols. 0.25 M sucrose in buffer A (25 mM Tris-HCl (pH 8.0), 2 mM dithiothreitol, 1 mM EDTA and 0.1 mM EGTA) using a Polytron PT 20 for 2 min at maximum speed. The homogenate was centrifuged for 30 min at 20 000 × *g*. Solid $(NH_4)_2SO_4$ (0.14 g/ml) was added to the resultant supernatant. The precipitable materials were sedimented by centrifugation and discarded. To the resulting supernatant was added solid $(NH_4)_2SO_4$ (0.13 g/ml) and centrifugation was carried out. The collected precipitate was dissolved in 1 l buffer A. After stirring overnight, the precipitable materials were removed by centrifugation at 35 000 × *g* for 30 min, and to the resulting supernatant chilled glycerol and buffer A were added to obtain 1.5 l of the enzyme solution in buffer A with 10% glycerol. The enzyme solution (3.8 g protein) was applied to a DEAE-cellulose (DE 52) column (30 × 5.5 cm) previously equilibrated with buffer A containing 10% glycerol. Subsequently, the column was washed with 500 ml of the same buffer, and then by 1 l of the buffer containing 0.1 M KCl. Elution was carried out with a gradient of 0.1—0.5 M KCl in buffer A with 10% glycerol (total volume, 1.2 l). Guanylate cyclase activity was eluted in a broad region between 0.35 and 0.45 M KCl. The enzyme from DEAE-cellulose column was precipitated by the addition of $(NH_4)_2SO_4$ (0.28 g/ml), and redissolved in 300 ml buffer B (25 mM Tris-HCl (pH 8.2), 2 mM dithiothreitol, 1 mM EDTA, 0.1 mM EGTA and 10% glycerol). This enzyme solution was applied on a DEAE-Sepharose CL-6B column (25 × 2.7 cm) previously equilibrated with buffer B. The cyclase activity was eluted with a linear KCl

gradient (0.1—0.5 M) in a broad region between 0.31 and 0.40 M. The enzyme was precipitated by the addition of $(NH_4)_2SO_4$ and redissolved in 100 ml buffer B, followed by the re-application to a DEAE-Sepharose CL-6B column (25 × 1.7 cm). The enzyme activity was again eluted with a linear KCl gradient (0.1—0.5 M) in a peak at 0.27 M. The peak tubes were combined, $(NH_4)_2SO_4$ (0.28 g/ml) was added and centrifugation was carried out. The resultant precipitate (0.19 g protein) was dissolved in 100 ml buffer A containing 0.25 M sucrose and denoted as crude cyclase. This enzyme contained 60% of the original activity of 20 000 × *g* supernatant from pig lung with a 60-fold increase in the specific activity, which in the 20 000 × *g* supernatant was 14 pmol of cyclic GMP formed/min per mg protein. Alternatively, the precipitate was dissolved in 100 ml buffer C (25 mM glycylglycine (pH 7.5), 2 mM dithiothreitol and 10 mM NaN_3) with 2 mM $MnCl_2$ for the following chromatography on an agarose-GTP column.

Separation of cyclase and activating factor on an agarose-GTP column

Crude cyclase (100 ml) was applied on an agarose-GTP column (10 ml) previously equilibrated with buffer C containing 2 mM $MnCl_2$. The fractions that were not retained on the column were pooled and $(NH_4)_2SO_4$ (0.28 g/ml) was added, followed by centrifugation. The resulting precipitate was dissolved in 100 ml buffer A containing 0.25 M sucrose and designated as the activating factor fraction. After washing the column by buffer C with 2 mM $MnCl_2$, the cyclase activity was eluted with buffer C containing 50 mM EDTA and 0.5 M KCl. The enzyme was precipitated by the addition of $(NH_4)_2SO_4$ (0.28 g/ml) and redissolved in 100 ml buffer A with 0.25 M sucrose. This fraction was denoted as purified cyclase.

For further purifications of activating factor and purified cyclase, each fraction from the agarose-GTP column was subjected to gel filtration on Sepharose CL-6B column (140 × 1.5 cm) with buffer A containing 0.25 M sucrose. The purified activating factor contained a small amount of guanylate cyclase activity (approx. 50 pmol cyclic GMP/min per mg protein). All values of guanylate cyclase activity assayed in the presence of the factor were corrected for the cyclase activity of the factor. When the purified cyclase was frozen at —20°C for 3 months, no loss of the activity was observed, although the enzyme activity was markedly decreased by repeated freezing and thawing.

Guanylate cyclase assay

The standard assay mixture contained 0.2 mM GTP (12.5 Ci/mol), 1 mM cyclic GMP, 15 mM creatine phosphate, 40 μg creatine kinase, 1 mM dithiothreitol, 1.5 mM isobutylmethylxanthine, 3 mM $MnCl_2$, 25 mM Tris-HCl (pH 7.7) and an enzyme preparation in a total volume of 0.20 ml. After the reaction mixture was incubated at 37°C for 8 min, the reaction was terminated by heating for 2 min in a boiling water bath, following the addition of 1 N HCl (40 μl). The radioactive cyclic GMP was isolated by the serial use of neutral Al_2O_3-Dowex 1-X2 column, and the radioactivity was determined as described elsewhere [3].

Protein was determined by the method of Lowry et al. [14] with bovine serum albumin as standard.

Results and Discussion

Crude cyclase partially purified from pig lung supernatant with a 60-fold increase in the specific activity as described in Methods was applied to an agarose-GTP column. The major portion of the cyclase activity was eluted by buffer C containing 50 mM EDTA and 0.5 M KCl, and this fraction (purified cyclase) contained 24% of the original activity of crude cyclase with a 3-fold increase in the specific activity, whereas the fractions nonadsorbed on the agarose-GTP (activating factor fraction) showed 4% of the original activity with much lower specific activity as presented in Table I. The addition of activating factor fraction to the reaction mixture containing purified cyclase was observed to increase the cyclase activity to 50% of the original activity in crude cyclase (Table I, upper panel). To eliminate the possibility that the contaminated cyclase activity in the factor fraction may be stimulated by the addition of purified cyclase fraction, each fraction was exposed to 50 mM HCl at room temperature for 30 min, followed by neutralization with NaOH. Each cyclase activity of purified cyclase and activating factor fractions was lost after exposure to acid, but the activating factor exposed to acid still enhanced the activity of the purified cyclase as shown in Table I (lower panel). These observations suggested that the activating factor fraction contained a factor which stimulated guanylate cyclase, and that the factor may play a role in the activity of crude cyclase.

Crude and purified cyclase fractions as well as activating factor fraction were each subjected to gel filtration on Sepharose CL-6B (Fig. 1). As estimated by

TABLE I

DISSOCIATION OF GUANYLATE CYCLASE AND ITS ACTIVATING FACTOR IN CRUDE CYCLASE PARTIALLY PURIFIED FROM PIG LUNG SUPERNATANT

To 100 ml of crude cyclase in buffer A containing 0.25 M sucrose, 28 g $(NH_4)_2SO_4$ were added and centrifugation was carried out. The resultant precipitate was redissolved in buffer C with 2 mM $MnCl_2$, and was then chromatographed on an agarose-GTP column as described in Methods. The activating factor fraction (Factor) which was not adsorbed on the column and the purified cyclase fraction (Cyclase) which was eluted by EDTA-KCl in buffer from the column, were precipitated by addition of $(NH_4)_2SO_4$ and sedimented. Each fraction was dissolved in 100 ml buffer A containing 0.25 M sucrose. Exposure to HCl was carried out for 30 min at room temperature after addition of 10 µl 1 N HCl to 200 µl of each fraction; 10 µl 1 N NaOH were then added to effect neutralization before assay (acid-Fraction). 30 µl of each fraction, exposed or not to acid, were used for each assay. One unit is defined as the amount of enzyme which produces 1 pmol cyclic GMP per min. Values in parentheses are per cent total activity

Fractions	Protein (mg/ml)	Cyclic GMP formed	
		Spec. act. (units/mg protein)	Total activity (units)
Crude Cyclase	1.85	859	158 000 (100)
Factor	1.32	49	6 300 (4)
Cyclase	0.16	2410	38 600 (24)
Cyclase + Factor	—	—	80 200 (51)
Crude Cyclase + Factor	—	—	221 000 (140)
Acid-Cyclase + Factor	—	—	6 000 (4)
Cyclase + acid-Factor	—	—	62 600 (40)

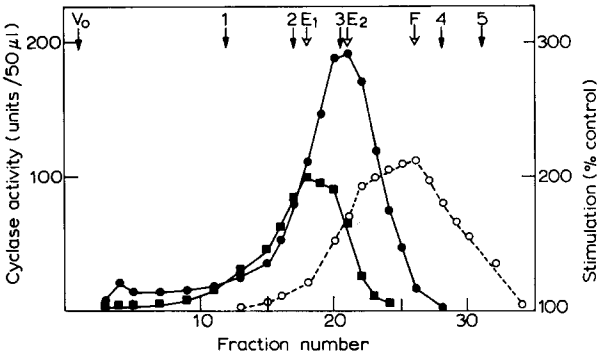

Fig. 1. Gel filtrations of guanylate cyclase preparations and activating factor on Sepharose CL-6B column. 500 µl each of crude cyclase, purified cyclase and activating factor fractions in buffer A containing 0.25 M sucrose were applied to the column (140 × 1.5 cm). Elutions were carried out with the same buffer and 2-ml fractions were collected. The enzyme activities of crude cyclase (■———■) and purified cyclase (●———●) were assayed. To measure the stimulating activity (○- - - - -○) of activating factor, the cyclase purified by the present gel filtration was used. The stimulating activity of the factor is presented as per cent stimulation, where 100% means the basal activity of the purified gel filtered cyclase (15 pmol cyclic GMP formed/min per 15 µg protein) itself. The marker proteins (Boehringer Mannheim) were: 1, ferritin; 2, bovine liver catalase; 3, rabbit muscle aldolase; 4, bovine serum albumin; 5, ovalbumin. The arrows at E_1, E_2 and F indicate the elution volumes of crude cyclase, purified cyclase and activating factor, respectively. The definition of unit is described in Table I.

gel filtration, guanylate cyclase in crude and purified cyclase fractions had apparent molecular weights of approx. 230 000 and 140 000, respectively, while the molecular weight of the activating factor was assessed to be about 85 000. These observations suggest that a macromolecular activating factor for cyclase was dissociated by affinity chromatography from crude cyclase to form the purified cyclase, and that this enzyme has a smaller molecular weight. Whether the combination of the purified cyclase and activating factor, under certain conditions, may reproduce a cyclase with the same molecular weight as crude cyclase is now under investigation.

In the following experiments the cyclase (Fractions 18—22, in Fig. 1) and activating factor (Fractions 23—26, in Fig. 1) obtained by the above described gel filtration, were employed. The stimulatory effect of the factor on cyclase activity was dependent on quantity of the factor, and the maximum effect was obtained with the addition of 30—40 µg protein of the factor per assay (Fig. 2). When the factor was added to the reaction mixture together with enzyme, stimulation occurred with no time lag and the concentration of Mn^{2+} required for maximal cyclase activity was the same as in its absence. As illustrated in Fig. 3, the apparent K_m value for GTP-Mn^{2+} in the cyclase reaction, as determined at various concentrations (10—100 µM), using a Lineweaver-Burk plot, was approx. 56 µM under the standard assay conditions in the presence or absence of the factor, whereas the maximal reaction velocity increased 4-fold with addition of the factor. Addition of EGTA or Ca^{2+} to the reaction mixture had no effect on the stimulation of cyclase by the activating factor. The factor was nondialyzable, heat-labile, partially sensitive to trypsin and resistant to acid or alkali. These properties are different from those described for activator in a synaptosomal fraction from rat brain [12] and in soluble fractions from *Tetra-*

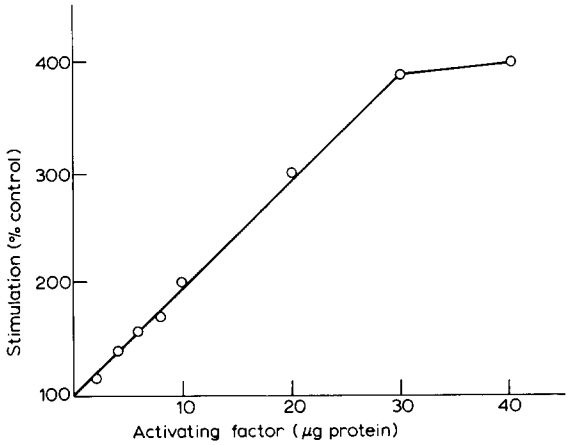

Fig. 2. Effect of activating factor on the stimulation of guanylate cyclase activity. Both activating factor and cyclase (15 μg protein per assay), which were purified by gel filtration as shown in Fig. 1, were used. The stimulating activity of the factor is defined as described in the legend to Fig. 1. The basal activity was 50 pmol cyclic GMP formed/min per 15 μg of protein.

hymena pyriformis [13]. Recently an activator for guanylate cyclase has been reported to be present in rat liver supernatant and to be similar to the present lung activator, except that the liver activator is dissociable from the cyclase by chromatography on DEAE-Bio-Gel [8]. In the present studies, the activity of

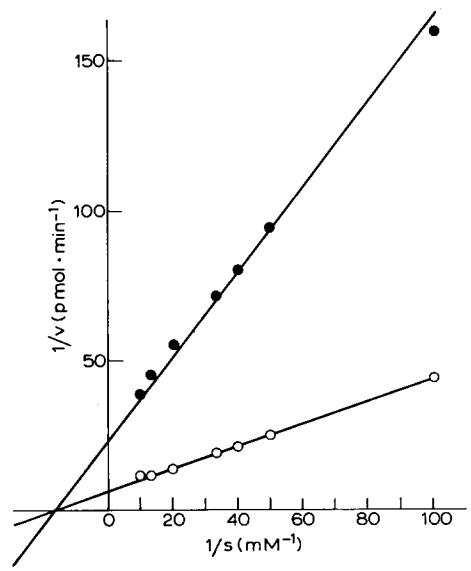

Fig. 3. Lineweaver-Burk plots of the reciprocals of reaction rate and substrate concentration in the presence (○———○) and absence (●———●) of activating factor (30 μg protein per assay). Both activating factor and cyclase (15 μg protein per assay), which were purified by gel filtration as described in Fig. 1, were employed. The standard assay conditions were used except that various concentrations (10—100 μM) of GTP-Mn^{2+} were added.

crude cyclase was also stimulated by the factor (Table I), but less so when the more purified enzyme was used, suggesting that the crude cyclase preparation contains a relatively limited amount of the factor. Therefore, the activating factor in lung supernatant may be partially dissociated from the cyclase by chromatographies on DEAE-cellulose and DEAE-Sepharose CL-6B. To determine the chemical properties of this activating factor and to elucidate the mechanism by which the factor may activate the cyclase, further purification of the activating factor and the cyclase is now under way.

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