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KINETIC EFFECTS OF THE CONCENTRATION-DEPENDENT STIMULATION OF SOLUBLE GUANYLATE CYCLASE FROM RAT LUNG

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Summary

Soluble guanylate cyclase (GTP pyrophosphate-lyase (cyclizing), EC 4.6.1.2) from rat lung demonstrated concentration-dependent stimulation, that is, an increase in specific activity with increasing enzyme (protein) concentration. This phenomenon persisted through several steps of enzyme purification and was apparently due to the presence of a macromolecular activator, similar in size to the enzyme. Treatment of partially purified enzyme with *N*-ethylmaleimide destroyed catalytic activity, but did not effect the ability of the preparation to stimulate activity. Kinetic analysis demonstrated that the stimulation was due to an increased V value with no change in the apparent K_m value for MnGTP. Stimulation occurred without a time lag, the activator apparently interacting reversibly with the enzyme to increase catalytic capability. Some nonionic detergents of the Triton series inhibited enzyme activity by decreasing the V value, with no change in the K_m value, and also decreased concentration-dependent stimulation. However, the two phenomena were not directly related. While the physiological significance of the activator is unclear, its presence affects estimations of recovery during enzyme purification, V determinations, and determinations of the effect of hormone or drug treatment on the activity of tissue extracts.

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Introduction

Guanylate cyclase (GTP pyrophosphate-lyase (cyclizing), EC 4.6.1.2), the enzyme that catalyzes synthesis of guanosine 3′,5′-cyclic monophosphate (cyclic GMP) from GTP, has been found to occur in both soluble and particulate form in lung [1,2] heart [3] and liver [4]. The soluble enzyme has been found to be activated by a variety of agents including fatty acids [5—8] azide [9], nitroprusside [10,11], carcinogenic nitrosamines [10,12], nitric oxide [13] and an O_2-dependent reaction that can be duplicated by hydrogen peroxide [14]. In addition to these activators, in which the mechanisms of action are only in part understood, there is evidence for an intrinsic activating agent. In our initial attempts to purify the soluble enzyme from bovine lung, we observed that there was an increase in specific activity when the preparation was concentrated at two steps in the procedure [15,16]. Recently, two other groups have reported similar concentration-dependent activity of the soluble enzyme from rat liver [17,18]. Tsai et al. [17] were able to separate a nondialyzable, heat-labile fraction from the bulk of the guanylate cyclase activity, which stimulated the activity of the purified enzyme. In the other study Braughler et al. [18] showed that heat treatment (60°C) of their purified enzyme inactivated the catalytic activity, however, the stimulatory activity remained. The present report describes studies on the concentration-dependent stimulation of rat lung guanylate cyclase. We also obtained evidence for a macromolecular endogenous activator, and found that it increased the V of the enzyme without change in the apparent K_m value for MnGTP. The results of our effort to abolish the effects of concentration upon activity are also described.

Materials and Methods

GTP was purchased from PL Biochemicals while [α-^{32}P]GTP (20—25 Ci/mmol) and [8-^{3}H]cyclic GMP (10 Ci/mmol) were from ICN. Aluminium oxide 90, activity II-III was from EM Laboratories. Sigma supplied cyclic GMP, all detergents and other unspecified biochemicals. Sephadex G-25, G-50, and G-150 were from Pharmacia. Mallinckrodt Chemicals supplied Amberlite XAD-4.

Enzyme preparation

Male Sprague-Dawley rats (200—500 g) were decapitated and the lungs perfused in situ with cold buffer containing 50 mM Tris-HCl, pH 7.6/0.5 mM EDTA/1 mM $MgCl_2$/10 mM 2-mercaptoethanol, and removed into the same medium. The lungs were blotted dry between paper towels, weighed and homogenized for 15 s in 3 vols. cold buffer with a Willems Polytron at 10 000 rev./min. The homogenate was centrifuged at 178 000 × g for 46 min. The supernatant fraction was adjusted to 40% of saturation by adding a neutralized saturated solution of $(NH_4)_2SO_4$, and the precipitate collected by centrifugation for 20 min at 20 000 × g. The pellet was redissolved in one-half the original volume of 10 mM Tris-HCl/0.5 mM EDTA/1 mM $MgCl_2$/10 mM 2-mercaptoethanol and the resulting solution adjusted to pH 5.2 with 0.1 M acetic acid

[19]. After stirring for 10 min, the precipitate was collected again by centrifugation for 20 min at 20 000 × *g*. This precipitate was redissolved in one-tenth the original volume of 2.5 mM dithioerythritol/0.5 mM EDTA/1 mM $MgCl_2$/(DEM buffer) brought to pH 7.6 with Tris base. Any undissolved material was removed by centrifuging as before, and the supernatant solution was dialyzed overnight against 2 l of the same buffer. Glycerol was added to a final concentration of 10% and the partially purified enzyme quick-frozen in 1-ml portions, which were stored at −30°C. This procedure resulted in a 9—12-fold increase in guanylate cyclase specific activity, with 15% recovery. The GTPase activity of one such preparation was determined [20] in a standard reaction mixture, but without the GTP regenerating system. GTP fell only 6% after a 30 min incubation, but we found that the GTP regenerating system had to be present in order to obtain linear kinetics.

N-Ethylmaleimide-inactivated enzyme was prepared by treating 0.46 ml of partially purified enzyme with 0.04 ml 0.25 M *N*-ethylmaleimide, after which the excess *N*-ethylmaleimide was removed by chromatography on a 0.7 × 19 cm Sephadex G-25 column equilibrated in 10 mM Tris-HCl, pH 7.6.

Enzyme assay

The guanylate cyclase reaction mixture contained in a final volume of 75 µl, 1.2 mM [α-^{32}P]GTP (approx. 500 000 cpm), 5 mM cyclic GMP, 6 mM $MnCl_2$, 10 mM 2-mercaptoethanol, 50 mM Tris-HCl (pH 7.6), 3.33 units of creatine phosphokinase, 5 mM creatine phosphate and 1—50 µg enzyme protein. Kinetic assay contained the same reaction components except the noted concentrations of MnGTP with 4.8 mM excess $MnCl_2$. Incubation was at 30°C for 10 min in the standard assays, 3 min in the kinetic experiments. The cyclic [^{32}P]GMP formed was purified by sequential chromatography on Dowex 50 and aluminium oxide [21]. Protein was determined by the method of Bradford [22], using bovine plasma albumin as the standard. Double-reciprocal plots of kinetic data were made by use of the Fortran program method of Cleland [23], a weighted least-squares fit to the classical Lineweaver-Burk relationship. The program gives calculated values of V and K_m, and the standard errors of their estimates. Each determination was performed in quadruplicate, and data presented here are representative of several experiments.

Results

Rat lung guanylate cyclase exhibits concentration-dependent activity, that is, an increase in enzyme specific activity with increasing protein concentration. This is best observed in the partially purified enzyme (Fig. 1), but it is also apparent in the supernatant fraction either before or after O_2-dependent activation (data not shown). The persistence of this phenomenon after a two-step enzyme purification indicated that it was not due to a readily dissociable, low molecular weight activator. This conclusion was supported by our inability to abolish concentration-dependent stimulation in activated rat lung supernatant enzyme by treatment with cocoanut charcoal to adsorb nucleotides [24], chromatography on Amberlite XAD-4 to remove amphiphiles [25,26], or chromatography on Sephadex G-25 and G-50 columns. O_2-activated enzyme

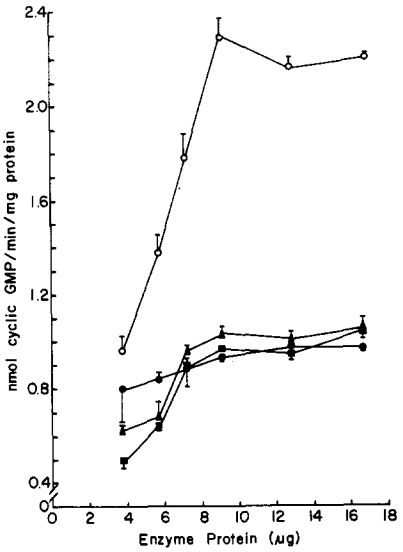

Fig. 1. Concentration-dependent activity of partially purified rat lung guanylate cyclase. Enzyme activity was determined with the indicated amount of enzyme protein with no addition (○———○), and 1% Triton X-45 (●———●), Triton X-100 (■———■) or Triton X-165 (▲———▲).

chromatographed on Sephadex G-150 in 0.8 M KCl entered the fractionation volume of the column. When the fractions containing the greatest amount of guanylate cyclase activity were pooled and desalted on Sephadex G-50, the enzyme activity was still concentration-dependent. This suggested that under

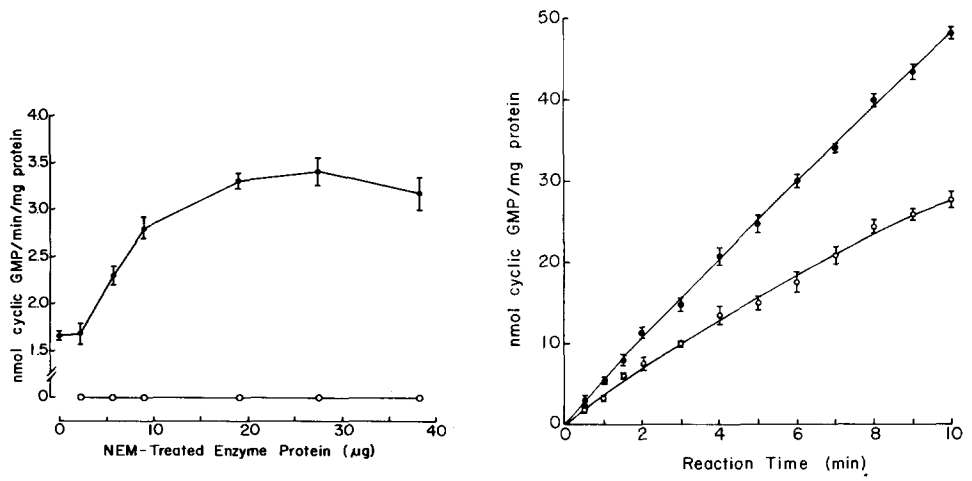

Fig. 2. Stimulation of guanylate cyclase activity by *N*-ethylmaleimide-treated enzyme. Activity of 0.98 μg of partially purified enzyme was determined in the presence of the indicated amount (●———●) of an identical enzyme preparation after *N*-ethylmaleimide (NEM) treatment. The lack of activity of the *N*-ethylmaleimide-treated enzyme is also shown (○———○).

Fig. 3. Time course of guanylate cyclase activation by *N*-ethylmaleimide-treated enzyme. Reactions were initiated by the addition of 0.88 μg of partially purified enzyme to reaction mixtures either containing (●———●) or not containing (○———○), 15.89 μg *N*-ethylmaleimide-treated enzyme protein.

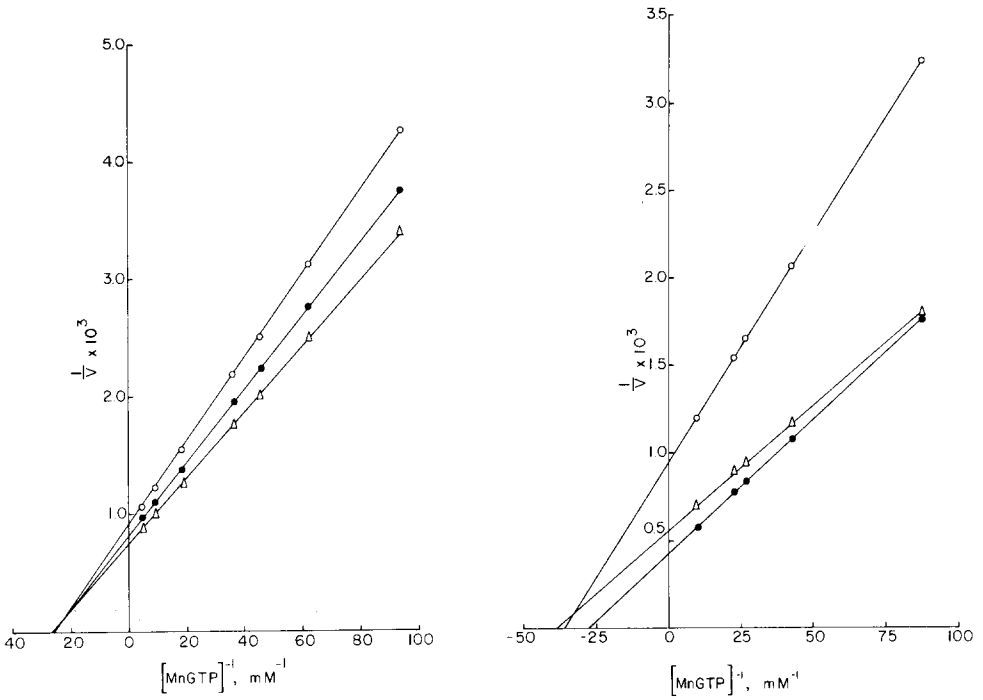

Fig. 4. Double-reciprocal plots of substrate kinetics at three protein concentrations. Reactions contained 4.00 (○———○), 8.20 (●———●) or 12.50 μg (△———△) of partially purified enzyme protein. Activity (v) is expressed as pmol of cyclic GMP formed/min per mg protein. Double-reciprocal plots were determined as described in Materials and Methods.

Fig. 5. Effect of *N*-ethylmaleimide-treated enzyme on substrate kinetics. Activity was determined with 1.44 (○———○) or 16.96 (△———△) μg partially purified enzyme protein, or 1.44 μg enzyme protein plus 15.36 μg *N*-ethylmaleimide-treated enzyme protein (●———●). Activity (v) calculations are as in Fig. 3.

these conditions any activator had a molecular weight not much different from that of the enzyme.

The aforementioned results led us to the hypothesis that concentration-dependent stimulation was due to a macromolecular activator, which interacted reversibly with the enzyme. The presence of an activator could be demonstrated in rat lung enzyme, which had no remaining guanylate cyclase activity, after storage for 10 days at 5°C. Addition of this preparation to fresh enzyme stimulated activity. Thiol reagents such as *N*-ethylmaleimide or the mercurial mersalyl provided a more rapid and reproducible method for destroying catalytic activity, without loss of concentration-dependent stimulation. Fig. 2 shows an experiment performed with *N*-ethylmaleimide-treated, partially purified enzyme. Increasing amounts of this preparation were added to a fixed amount of the untreated enzyme, which resulted in stimulation of the activity of the enzyme, although the *N*-ethylmaleimide-treated preparation had no activity when assayed by itself. Other experiments have supported the protein-like nature of the activator, including heat lability, observed during subsequent attempts at purification. Fig. 3 shows the time course of the activation. Enzyme was added to reaction mixtures either containing or not containing

additional *N*-ethylmaleimide-treated enzyme, and activation was detectable at the shortest reaction time used (30 s). The absence of a time lag suggests that activation did not involve an enzyme catalyzed event.

Fig. 4 depicts the effects of enzyme concentration upon MnGTP kinetics. As shown, an increase in the amount of partially purified enzyme in the reaction mixture from 4.00 to 8.20 to 12.50 μg protein produced a concomitant increase in the apparent V (from 1126 ± 72 at 4.00 μg to 1368 ± 34 at 12.50 μg), while there was no significant change in apparent K_m (about 0.039 mM). Fig. 5 shows the same effect induced by the addition of 15.36 μg *N*-ethylmaleimide-treated enzyme to 1.44 μg active enzyme, the V increasing from 1059 ± 77 to 2351 ± 129.

Among compounds tested for the ability to abolish concentration-dependent stimulation, certain nonionic detergents were found to be partially effective. Fig. 1 shows results obtained with Triton X-45, Triton X-100 and Triton X-165, which have hydrophile-lipophile balance values of 10.4, 13.5 and 15.8, respectively [27]. These values represent the relative ratio of hydrophilic to hydrophobic regions within a surfactant, and in the Triton series vary with the length of the ethylene oxide chain. All three detergents decreased, but did not abolish, concentration-dependent stimulation to virtually the same extent. Triton X-45 had the same effect on the stimulation of activity by *N*-ethylmaleimide-treated enzyme (data not shown).

The effect of Triton X-45, the most hydrophobic of the three detergents, on

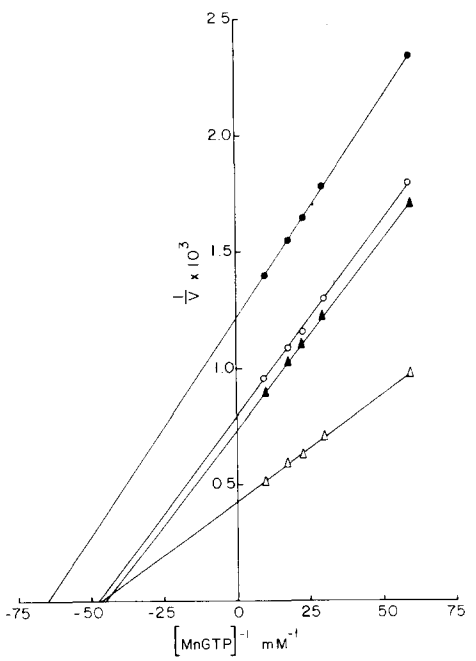

Fig. 6. Effect of Triton X-45 on substrate kinetics. Activity was determined with 2.06 (○, ●) or 15.40 (△, ▲) μg partially purified enzyme protein, both in the absence (○, △) or presence (●, ▲) of 1% Triton X-45. Activity (v) calculations are as in Fig. 3.

MnGTP kinetics was assessed at two protein concentrations (Fig. 6). As in Fig. 3, increasing the enzyme protein from 2.06 to 15.40 μg increased the V from 1269 ± 77 to 2370 ± 112 pmol/min per mg protein, and had no effect on the apparent K_m (about 0.022 mM). The addition of 1% Triton X-45 decreased the V value at both protein concentrations, (to 823 ± 46 at 2.06 μg protein and to 1360 ± 43 at 15.40 μg protein), with again, no significant change in K_m. The ratio of maximum velocities at the two protein concentrations was 1.87 without Triton X-45, and 1.65 in its presence.

Discussion

We have presented evidence here that there is a macromolecular factor present in the supernatant fraction, prepared from rat lung homogenates, that is responsible for the increase in specific activity of guanylate cyclase as the supernatant protein concentration is increased. The factor remained present after catalytic activity disappeared as a result of aging or treatment with *N*-ethylmaleimide. It copurified with the enzyme through $(NH_4)_2SO_4$ fractionation and isoelectric precipitation and was not separated from the enzyme by gel filtration on Sephadex G-150. It is, therefore, apparently of similar molecular dimensions as the enzyme. Similarly, Braughler et al. [18] were able to demonstrate concentration-dependent activity through several purification steps using soluble rat liver enzyme. The kinetic experiments (Figs. 4—6) showed that the V value increased when either the enzyme concentration was increased or when *N*-ethylmaleimide-treated enzyme was added to active enzyme, without change in the apparent K_m. Tsai et al. [17] recently reported the purification of liver guanylate cyclase, and showed that an activator fraction was separated from cyclase activity at an early step in the procedure. Their activator, like the one discussed here, was nondialysable and heat labile. The purified enzyme gave a nonlinear Lineweaver-Burk plot, but when activator was added, the plots became linear. The diagram of these results in their article also shows that the activator increased the V; however, no conclusions can be drawn about the apparent K_m. In contrast with their results, the plots we obtained with the semi-purified lung enzyme were invariably linear, which suggests that activator was always present in this preparation.

Because early experiments indicated that nonionic detergents decreased the effects of enzyme concentration on activity, we hypothesized that the activator interacted hydrophobically with the enzyme, and could be displaced by detergent at the hydrophobic binding site. However, the effects upon activity of the three nonionic detergents used in Fig. 1 were all similar, although they differed in hydrophobicity [27]. Each inhibited enzyme activity, and decreased but did not abolish concentration-dependent stimulation. Furthermore, the experiment depicted in Fig. 6 demonstrated clearly that the inhibition of activity by Triton X-45 did not parallel its inhibition of concentration-dependent stimulation. Triton X-45 decreased the V value about 60%, while the stimulation of activity induced by increasing enzyme protein from 2.06 to 15.40 μg protein was only inhibited 21%. We concluded that activator and detergent bound to the enzyme at different sites, and exerted separate although interacting effects on activity.

The physiological significance of the activator is unknown. It is obvious that

the existence of such a regulatory device could be important, if physiologically relevant agents could be shown to affect the enzyme-activator association. Calcium is an attractive candidate because of its ability to regulate adenylate cyclase and cyclic nucleotide phosphodiesterase [28,29]. However concentration-dependent stimulation was unaffected by 0.1 mM calcium, EDTA or EGTA in both semipurified enzyme preparations and crude supernatant fractions. Nucleotides, likewise, do not appear to be possible dissociating agents since charcoal treatment of a supernatant fraction, which would adsorb nucleotides [24] did not decrease concentration-dependent stimulation. Also ATP, a known inhibitor of the enzyme [30], did not decrease stimulation of activity by *N*-ethylmaleimide-treated enzyme when present at 0.1 or 0.5 mM.

Apart from its possible, although unsupported role in regulation, the principal immediate importance of the activator lies in its' effects upon guanylate cyclase determinations. As we have previously pointed out [16], estimations of enzyme recovery during purification will be distorted because of concentration-dependent stimulation. Braughler et al. [18] attempted to compensate for this by calculating recoveries on the basis of maximally observed activities. Tsai et al. [17] showed that activity was proportional to enzyme concentration when activator fraction was present in excess. The same direct proportionality should also result if no activator was present. In addition, kinetic determinations of V will vary as a function of protein concentration, making comparisons among different preparations difficult. Perhaps a more important problem arises out of attempts to determine the guanylate cyclase activity of tissue extracts as a consequence of drug or hormone treatment. Until a method is developed for controlling the effect of concentration upon activity, activity comparisons must be made at equivalent protein concentrations.

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