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MYOCARDIAL GUANYLATE CYCLASE: PROPERTIES OF THE ENZYME AND EFFECTS OF CHOLINERGIC AGONISTS IN VITRO

LEE E. LIMBIRD and ROBERT J. LEFKOWITZ

Departments of Medicine and Biochemistry, Duke University Medical Center, Durham, N.C. 27710 (U.S.A.)

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Summary

The characteristics of myocardial guanylate cyclase (GTP pyrophosphate-lyase, EC 4.6.1.2) were studied. Specific activity of the myocardial enzyme in five vertebrate species was guinea pig > man ⩾ cat > dog > rat. In the guinea pig, guanylate cyclase activity was uniformly distributed throughout the anatomical regions of the heart. The major portion of the enzyme activity was retrieved in the supernatant fraction after centrifugation at 12 000 × *g*. The K_m for GTP was similar in supernatant (0.12 mM) and particulate (0.21 mM) preparations, although the K_a for Mn^{2+} in particulate preparations (0.3—0.6 mM) was less than that observed for guanylate cyclase in the supernatant fraction (0.8—2.0 mM). ATP competitively inhibited supernatant and particulate activity. Addition of 0.005—10.0 mM Ca^{2+} to assay incubations did not enhance guanylate cyclase activity. Suspension of 105 000 × *g* supernatant guanylate cyclase preparations with membrane lipids or phosphatidylserine stimulated activity 1.4—4.3 fold, whereas similar treatment of particulate preparations caused little alteration of enzyme activity.

Addition of the cholinergic agonists acetylcholine, carbachol or methacholine (10^{-4}—10^{-8} M) to homogenate, supernatant, particulate and disrupted tissue slice preparations in the presence of 0.0012—1.2 mM GTP, 0.3—10.0 mM Mn^{2+} and 0.005—10.0 mM Ca^{2+} or 0.0012—1.2 mM ATP did not stimulate guanylate cyclase activity. Similarly, further stimulation of guanylate cyclase activity was not elicited when enzyme—lipid suspensions were assayed in the presence of cholinergic agents.

Introduction

Many hormonal effects on target tissues are mediated via stimulation of the enzyme adenylate cyclase and subsequent accumulation of cyclic adenosine 3′ : 5′-monophosphate (cyclic AMP) [1]. In particular, the positive inotropic

and chronotropic effects of beta-adrenergic agents on the myocardium may be mediated by cyclic AMP [2].

Evidence is rapidly accumulating which suggests that cholinergic effects on chronotropy and inotropy may correlate with intracellular accumulation of another cyclic nucleotide, cyclic guanosine 3′ : 5′-monophosphate (cyclic GMP), in myocardial tissue. George et al. [3] have demonstrated that infusion of acetylcholine into spontaneously beating and electrically driven isolated perfused rat hearts results in a decreased contractile force which correlates with increased intracellular cyclic GMP levels. In addition, Lee et al. [4] have reported that increased cyclic GMP concentrations in rat ventricular slices following incubation with acetylcholine are attributable to the interaction of acetylcholine with muscarinic, rather than nicotinic, receptors.

The biochemical mechanisms responsible for cyclic GMP elevations following acetylcholine administration have not been elucidated. Thus, the purpose of this investigation was twofold. First, a detailed characterization of myocardial guanylate cyclase (GTP pyrophosphate-lyase, EC 4.6.1.2) was undertaken. Studies of the enzyme to date have focused on lung preparations since the activity is reportedly higher in lung than in other tissues [5,6]. Second, an attempt was made to create in vitro conditions under which guanylate cyclase was directly stimulated by cholinergic drugs using homogenate, particulate, supernatant and disrupted tissue slice preparations from guinea pig myocardium.

Methods

Tissue preparations

Female guinea pigs weighing 450—500 grams were killed by cervical dislocation. Dogs and cats were killed by intravenous pentobarbital and rats were sacrificed by decapitation. Human atrial appendage was obtained during surgery.

Tissue was immediately placed in cold 75 mM Tris—HCl, pH 7.6, 9 mM $MnCl_2$ (Tris—Mn^{2+} buffer), weighed and homogenized at 0—4° C, with 10 vol (V/W) of the above medium using 10 strokes of a Teflon-tipped glass Potter—Elvehjem homogenizer to prepare the homogenate. Supernatant and particulate fractions were obtained by centrifuging homogenates at 4° C in a Sorvall Model RC2-B centrifuge for 10 min at 12 000 × *g* unless otherwise indicated. Particulate fractions were washed one time with the Tris—Mn^{2+} buffer and resuspended in the same medium. Protein in these preparations was determined by the method of Lowry et al. [7].

Assay of guanylate cyclase activity

Guanylate cyclase activity in tissue preparations was assayed by a modification of the method of White et al. [8]. The final volume of the reaction mixture was 75 μl and contained: 25 mM Tris—HCl, pH 7.6, 3 mM $MnCl_2$, 5.34 mM cyclic GMP to prevent phosphodiesterase degradation of product, a GTP-regenerating system (5 mM creatine phosphate, 11.25 units creatine phosphokinase, 100 μg bovine serum albumin), 1 μCi [α-^{32}P] GTP and 0.0012—1.2 mM GTP. The enzyme preparations, containing 40—150 μg protein, and the assay

reaction mixtures were preincubated separately at 37° C for 5 min. In the usual assay system, 20 μl of hormone, drug or water (control) was added to the reaction mixture immediately prior to the addition of 25 μl of the enzyme preparations, which initiated the reaction. Reaction blanks for each set of determinations were estimated by addition of 25 μl of water or boiled tissue preparation in lieu of the fresh enzyme preparation. The blank was 0.0015% of labelled [α-^{32}P]GTP added to the incubation. 10- or 20-min incubations at 37° C were terminated by immersing the incubation tubes in ice water and adding 20 μl of 0.1 M EDTA, pH 7.6, containing approximately 30 000 cpm of cyclic [^{3}H]GMP. One ml of 0.03 M Tris—HCl, pH 7.6, was then added to each incubation tube to facilitate sample transfer.

Each reaction mixture was transferred to an 0.8 g alumina column prewashed with 6.0 ml of 0.03 M Tris—HCl, pH 7.6. The fourth, fifth and sixth ml eluted with 0.03 M Tris—HCl were collected directly into scintillation vials containing 15 ml of Triton—toluene fluor: (16.5 g PPO, 0.3 g POPOP, 1 l Triton-X 100, 2 l toluene) [9]. Samples were quantitated with double isotope counting in a Packard Tri-Carb Liquid Scintillation Counter. Recovery of product, as assessed with cyclic [^{3}H]GMP, was 45—50%. Picomoles of cyclic GMP generated were calculated from the specific activity of [α-^{32}P]GTP in the incubation mixture, cyclic [^{3}H]GMP recovery, and the amount of cyclic [^{32}P]GMP formed minus the reaction blank. Purity of cyclic GMP product was greater than 94% as assessed by thin-layer chromatography on Cellulose (PEI 300, Brinkmann) using 1 M formic acid, 1 M LiCl as solvent and on ChromAR Sheets (Mallinckrodt) developed with absolute ethanol and concentrated NH_4OH (5 : 2 V/V).

As demonstrated in Fig. 1, under the assay conditions employed, the rate of cyclic GMP production was directly proportional to time for 20 min at 37° C, (Fig. 1A) and protein concentrations from 0—150 μg per assay (Fig. 1B).

Preparation of guanylate cyclase—lipid suspensions

Aqueous suspensions of 105 000 × *g* supernatant or 12 000 × *g* particu-

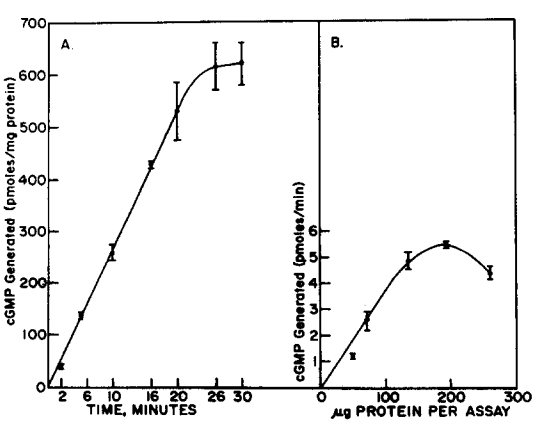

Fig. 1. Time dependence of guanylate cyclase activity (A) and relation of guanylate cyclase activity to protein concentrations (B). Guanylate cyclase activity in supernatant preparations from guinea pig myocardium was determined in the presence of 0.3 mM GTP and 3.0 mM $MnCl_2$ at 37° C. Values are the mean of triplicate determinations ±S.D.

late guanylate cyclase preparations with commercial phospholipids or membrane lipids isolated from guinea pig myocardium [10] were prepared as follows. (1) Chloroform: methanol (2 : 1) solutions of lipid were dried as a thin coat on the bottom of screw-capped polyethylene tubes (2 cm circumference) by rotating the tubes in ice under a stream of nitrogen. (2) One ml of supernatant or particulate guanylate cyclase in the Tris—Mn^{2+} buffer was added to the lipid-coated vessel. (3) Suspension of the lipid into the aqueous medium containing guanylate cyclase was accomplished by swirling the capped tubes in a New Brunswick Rotary Shaker water bath at 25° C for 45 min at 300 rev./min [11].

Materials

GTP, ATP, ITP, cyclic GMP, creatine phosphate, creatine phosphokinase, acetylcholine, carbamylcholine chloride (carbachol) and acetyl-β-methylcholine chloride (methacholine) were obtained from Sigma. [α-^{32}P]GTP (1—10 Ci/mmole) and cyclic [^{3}H]GMP, ammonium salt, (1—5 Ci/mmole) were obtained from New England Nuclear. Phospholipids were from various commercial sources: Phosphatidyl-L-serine (Schwarz Mann), bovine phosphatidylethanolamine (Supelco), bovine brain phosphatidylinositol (General Biochemicals), β-γ-dipalmitoyl-DL-α-lecithin (Sigma). Alumina, neutral grade, was obtained from Nutritional Biochemicals.

Results

Myocardial guanylate cyclase in different vertebrate species

Guanylate cyclase activity in 12 000 × *g* supernatant and particulate preparations was determined in guinea pig, cat, rat, dog and human myocardium. Fig. 2 is a summary of the specific activity and apparent K_m for GTP observed in these preparations. On the basis of the greater guanylate cyclase activity observed in the guinea pig myocardium, particularly in the supernatant fraction, further characterization of the enzyme was pursued in this species.

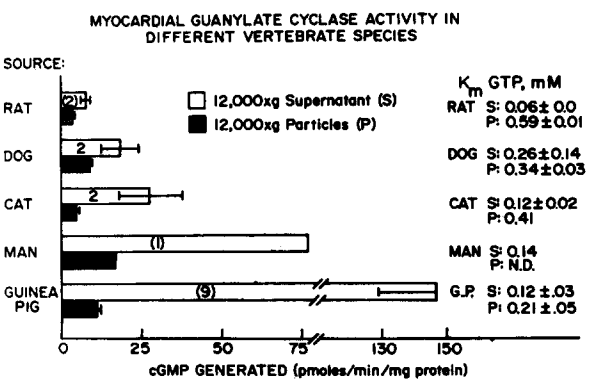

Fig. 2. Myocardial guanylate cyclase activity in different vertebrate species. Specific activity of guanylate cyclase in 12 000 × *g* supernatant and particulate preparations from the five species shown was determined in the presence of 0.6 mM GTP and 3 mM $MnCl_2$. Incubations were for 10 min at 37° C. (N) refers to the number of experiments represented ±S.E. Calculation of apparent K_m for GTP was carried out in experiments where GTP was varied from 0.0012—1.2 mM. N.D. means "not determined".

Guanylate cyclase activity in different regions of the myocardium

The activity of guanylate cyclase was measured in several anatomical regions of the guinea pig heart: atrial appendage, right ventricle, intraventricular septum, left ventricular free wall and apex. Since guanylate cyclase was observed to be uniformly distributed throughout the myocardium, all subsequent studies were carried out on ventricular preparations.

Activity of guanylate cyclase in different subcellular fractions

To assess the apparent subcellular distribution of guanylate cyclase activity in the guinea pig myocardium, the specific activity of the enzyme was determined in different centrifuged fractions. The specific activity of guanylate cyclase was greatest in the 105 000 × *g* supernatant fraction, approximately 150 pmoles/min/mg protein. However, measurable guanylate cyclase activity was always detected in the 600 × *g*, 12 000 × *g* and 105 000 × *g* particulate fractions, varying from 5—25 pmoles/min/mg protein in each of the fractions. Since particulate preparations were washed with homogenizing buffer, it is unlikely that this pelleted activity was due to contamination with supernatant activity.

Effects of GTP and Mn^{2+}

(A) Supernatant guanylate cyclase preparation. It has been previously established that guanylate cyclase catalyzes the formation of cyclic GMP utilizing GTP as a substrate and that this activity is dependent on the presence of Mn^{2+} [5]. The effects of varying GTP concentration on the 12 000 × *g* supernatant guanylate cyclase preparation at fixed Mn^{2+} concentrations are shown in Fig. 3A. The apparent K_m calculated for GTP in the presence of 1—10 mM Mn^{2+} in a series of four experiments was 0.08—0.20 mM GTP.

Fig. 3B demonstrates the effects of varying Mn^{2+} concentrations at fixed GTP levels on supernatant activity. Calculation of the apparent K_a for Mn^{2+} gave values ranging from 0.8—2.0 mM Mn^{2+}.

(B) Particulate guanylate cyclase preparation. Figs 4A and 4B demonstrate the effects of GTP and Mn^{2+} on the 12 000 × *g* particulate guanylate cyclase

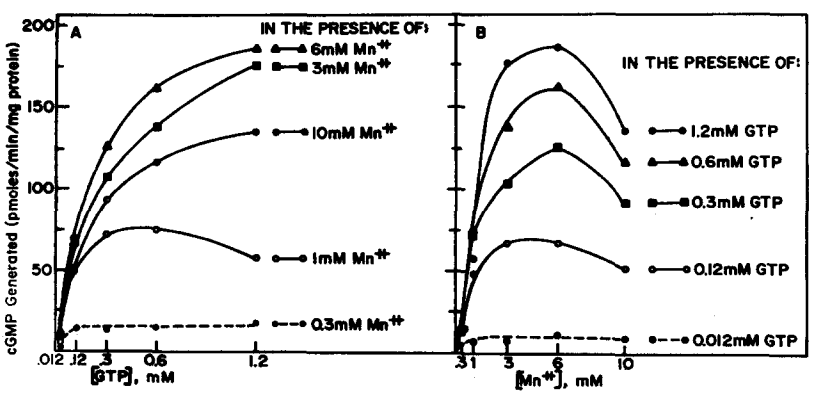

Fig. 3. Kinetic properties of 12 000 × *g* supernatant guanylate cyclase activity. (A) Effect of varying the concentration of GTP at fixed Mn^{2+} concentrations, (B) Effect of varying the total Mn^{2+} concentration at fixed GTP concentrations. All incubations were for 10 min at 37°C.

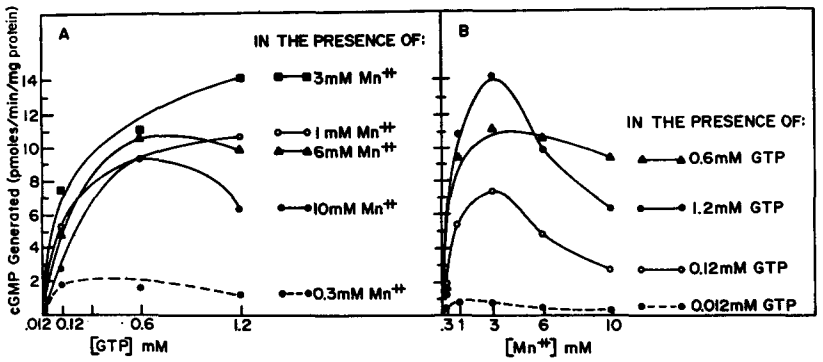

Fig. 4. Kinetic properties of 12 000 × *g* particulate guanylate cyclase activity. (A) Effect of varying GTP concentrations at fixed Mn^{2+} concentrations, (B) Effect of varying the total Mn^{2+} concentration at fixed GTP concentrations. All incubations were for 10 min at 37°C.

preparation. In Fig. 4A, the effects of varying the GTP concentration at fixed Mn^{2+} concentrations are shown. The calculated K_m for GTP in particulate preparations in four experiments varied from 0.12—0.3 mM GTP, comparable to calculated substrate K_m values for supernatant activity.

The effects of varying the Mn^{2+} concentration in the presence of fixed GTP concentrations are shown in Fig. 4B for one particulate preparation. The apparent K_a in four experiments varied from 0.3—0.6 mM Mn^{2+} which is less than the mean K_a (1.1 mM) observed for Mn^{2+} in supernatant preparations.

Effects of ATP

ATP competitively inhibited both 12 000 × *g* supernatant and particulate guanylate cyclase enzyme preparations. Fig. 5 is a Lineweaver—Burk double reciprocal plot [12] of supernatant guanylate cyclase activity in the absence and presence of ATP. Calculation of K_i for the inhibitor—supernatant enzyme complex from Fig. 5 and similar plots from other experiments yielded a value for $K_i = 2.2 \pm 0.4 \cdot 10^{-4}$ M ($n = 12$). The constancy of K_i at different concen-

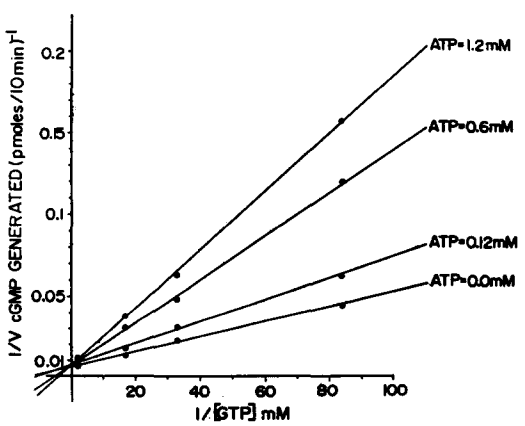

Fig. 5. Lineweaver—Burk double reciprocal plot showing the competitive inhibition of 12 000 × *g* supernatant guanylate cyclase activity by ATP. Incubations were in the presence of 0.012—0.6 mM GTP and 3.0 mM Mn^{2+} for 10 min at 37°C. Values are the mean of triplicate determinations.

trations of GTP indicates that ATP interacts with only one site on the enzyme, presumably the GTP substrate site. Although inhibition of particulate guanylate cyclase was also competitive in nature (Lineweaver—Burk plots intersected on the $1/V$ axis), calculation of K_i yielded different values at different ATP concentrations suggesting a more complex ATP—enzyme interaction in the particulate preparations. Since total Mn^{2+} cation concentration was held constant at 3.0 mM for these experiments, it is possible some of the inhibitory effects of ATP may be due to a lowering of free Mn^{2+} concentrations due to ATP—Mn^{2+} complexation.

Effects of Ca^{2+}

Since Schultz et al. have emphasized the importance of calcium in the regulation of cyclic GMP in vivo [13] and Hardman et al. have described an apparent synergism between Ca^{2+} and Mn^{2+} on rat lung guanylate cyclase in vitro [14], we tested the effects of Ca^{2+} on guinea pig myocardial guanylate cyclase. No enzyme activity could be detected in the absence of Mn^{2+} even in the presence of 10 mM Ca^{2+}, thus Ca^{2+} did not appear to be able to substitute for Mn^{2+}. When Ca^{2+} concentrations were varied from 0.005—10 mM in the presence of 0.3—3.0 mM Mn^{2+} and 0.006—0.6 mM GTP, no increase in guanylate cyclase activity was observed above that in the presence of Mn^{2+} alone in either homogenate, 12 000 × *g* supernatant or particulate preparations. The only consistent effect of Ca^{2+} noted was inhibition of guanylate cyclase activity at concentrations of 10 mM.

Effects of lipids

The effects of membrane lipid preparations and commercial phospholipids on 105 000 × *g* supernatant and 12 000 × *g* particulate guanylate cyclase were evaluated. By swirling the enzyme preparations in a lipid-coated vessel, enzyme—lipid interaction could occur in an aqueous medium without prior sonication of the lipids. It was found, using this technique, the lipids markedly stimulated 105 000 × *g* supernatant guanylate cyclase activity. As seen in Fig. 6A, supernatant enzyme activity was stimulated 1.8—3.8 fold when enzyme preparations were suspended with 0.05—5.0 mg/ml of membrane lipid. When 12 000 × *g* particulate preparations were suspended with similar lipid concentrations, negligible stimulation of enzyme activity was observed. In similar experiments phosphatidylserine stimulated 105 000 × *g* supernatant activity 1.8—4.3 fold, as demonstrated in Fig. 6B. The effects of other commercial phospholipids on supernatant and particulate guanylate cyclase activity were compared. Phosphatidylcholine, phosphatidylethanolamine and phosphatidylinositol were much less effective in stimulating supernatant guanylate cyclase than phosphatidylserine or membrane lipid preparations and had negligible effect on enzyme activity in particulate preparations.

Attempts to directly stimulate myocardial guanylate cyclase with cholinergic agents

Since levels of myocardial cyclic GMP have been reported to rise after cholinergic stimulation of isolated rat hearts [3] and rat ventricular slices [4], it seemed appropriate to determine if guanylate cyclase in broken cell preparations could be directly stimulated by cholinergic agents.

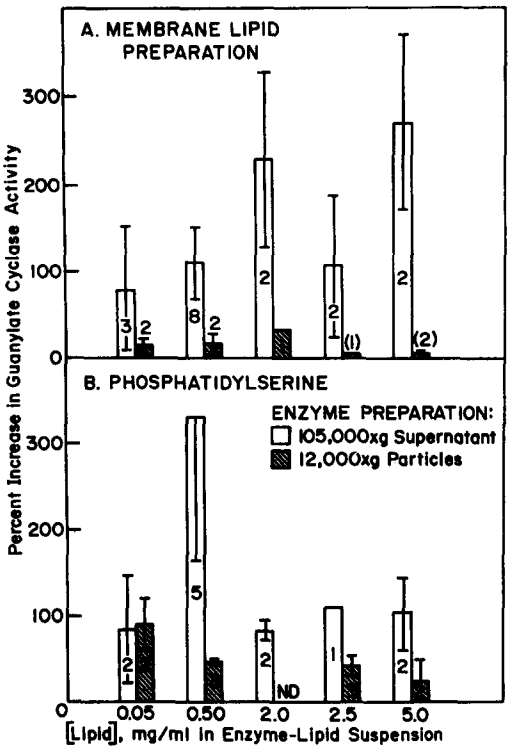

Fig. 6. Effects of lipids on myocardial guanylate cyclase activity. The stimulation of 105 000 X *g* supernatant and 12 000 X *g* particulate guanylate cyclase preparations suspended with guinea pig membrane lipids (A) and commercial phosphatidylserine (B) is shown. The data are represented as percent increase in guanylate cyclase activity, where 0% represents control activity of guanylate cyclase in the absence of added lipid. Enzyme activity in the suspensions was assayed in the presence of 0.3 mM GTP, 3.0 mM Mn^{2+} for 10 min at 37°C. (N) refers to the number of experiments ±S.E.

(A) Addition of cholinergic agonists to enzyme incubation media. In a large series of experiments, the effects of a variety of incubation conditions on the hormone sensitivity of guanylate cyclase were evaluated. The addition of 10^{-8}—10^{-4} M acetylcholine, carbachol or methacholine to homogenate, supernatant or particulate preparations of guanylate cyclase did not enhance enzyme activity in the presence of 0.0012—1.2 mM GTP and 0.3—10.0 mM $MnCl_2$. Addition of 0.005—10.0 mM Ca^{2+} to the assay incubations was also without effect in eliciting cholinergic stimulation of the guanylate cyclase enzyme in homogenate or subcellular preparations. The activity of guanylate cyclase was also not altered by the addition of cholinergic agonists in the presence of the competitive inhibitors ATP (0.0012—1.2 mM) or ITP (0.012—1.2 mM). Similarly, sensitivity to cholinergic drugs was not observed when supernatant and particulate guanylate cyclase preparations were suspended with membrane lipids or any of the commercial phospholipids studied.

(B) Incubation of guinea pig ventricular slices with carbachol. In an attempt to examine guanylate cyclase activity under conditions where exposure to cholinergic drugs occurred prior to cell disruption, Stadie—Riggs ventricular slices (100 mg aliquots) were bathed in Tyrode's solution in the presence of 10^{-4} M and 10^{-6} M carbachol or acetylcholine, conditions previously reported

to cause measurable cyclic GMP elavations in similarly prepared slices [4].

Following 15-s—3-min incubation at 37°C in the presence or absence of cholinergic agonists, the slices were rapidly homogenized in the usual Tris—Mn^{2+} buffer and assayed for guanylate cyclase activity. Preincubation of intact cell preparations with cholinergic agents prior to tissue disruption did not enhance guanylate cyclase activity assayed in the presence of 0.12 and 0.012 mM GTP.

Discussion

Characteristics of guanylate cyclase from guinea pig myocardium are similar to those previously reported for the lung enzyme in terms of GTP substrate and Mn^{2+} cation requirements and the largely soluble subcellular distribution [5,6]. These characteristics differ markedly, however, from the exclusively particulate mammalian adenylate cyclase enzyme which utilizes ATP—Mg^{2+} as its physiologic substrate.

The observation that a major portion of guanylate cyclase is consistently retrieved in the supernatant fraction following homogenization is puzzling. Comparison with the adenylate cyclase—cyclic AMP model for mediation of hormonal effects suggests that if cholinergic actions are mediated via guanylate cyclase and cyclic GMP, the enzyme might be more likely to be located in particulate fractions where preliminary data indicates the muscarinic cholinergic receptors are likely to exist [15]. Although guanylate cyclase may have been dissociated from in vivo membrane sites with homogenization, it is also possible that flux of the enzyme between cytosol and membrane compartments may be of potential regulatory significance in vivo [16]. Our observation that suspension of supernatant guanylate cyclase preparations with membrane lipids markedly enhanced enzyme activity may suggest that soluble enzyme is capable of in vivo association with lipid-containing structures such as plasma or intracellular membranes.

A significant portion of guinea pig myocardial guanylate cyclase activity was invariably detected in the 12 000 × *g* particulate preparations, even after repeated buffer washings. Although the K_a for Mn^{2+} of the particulate enzyme (0.3—0.6) was less than that observed for the supernatant enzyme, (0.8—2.0), it is not clear whether this indicates two separate enzyme species or whether in vivo Mn^{2+}, still strongly bound to membranes after homogenization, is contributing some of the necessary cation. The apparent substrate K_m calculated for particulate (0.10—0.30 mM GTP) and supernatant (0.08—0.18 mM GTP) preparations were comparable for guinea pig myocardial guanylate cyclase, although other investigators have reported that the supernatant enzyme requires fourfold less GTP for half-maximal activity in rat heart [17]. More detailed characterization will be required to determine if one or two species of enzyme exist in supernatant and particulate myocardial guanylate cyclase preparations.

A number of laboratories have now reported that exposure of isolated organs or tissue slices to acetylcholine results in the rapid accumulation of intracellular cyclic GMP. Goldberg refers to the "Yin-Yang" philosophy to describe the emerging evidence that cyclic GMP mediates physiological phe-

nomena antagonistic to effects mediated by cyclic AMP [1]. Accordingly, a major interest in this investigation was to determine if myocardial guanylate cyclase could be directly stimulated in broken cell preparations by cholinergic agonists. Such a demonstration would be analogous to one of the criteria established by Sutherland [18] for confirming that a hormone action on a target cell is mediated by adenyl cyclase and cyclic AMP. A number of in vitro conditions were evaluated to test the sensitivity of guanylate cyclase to cholinergic drugs. The cholinomimetic agonists carbachol and methacholine were tested in addition to acetylcholine because of their reported resistance to acetylcholinesterase. The study of homogenate preparations permitted the presence of all intracellular components, should a number of factors be necessary for the expression of hormone-sensitive guanylate cyclase activity. Although guanylate cyclase activity was greatest in the supernatant fraction, particulate guanylate cyclase seemed a likely candidate for hormonal stimulation and thus particulate preparations were similarly studied. A wide range of GTP substrate and Mn^{2+} concentrations was added to incubation media and the inclusion of sub-optimal levels was intended to create conditions under which hormonal stimulation might be more likely, or at least more easily detectable. Direct stimulation of guanylate cyclase was attempted in the presence of the inhibitory effectors ATP and ITP to determine if cholinergic agonists could alter this nucleotide effect. ATP was specifically evaluated in this regard since GTP is known to enhance the sensitivity of myocardial adenylate cyclase to hormonal stimuli [19]. The effects of cholinergic agonists on supernatant and particulate enzyme preparations suspended with lipids were studied since successful reconstitution of hormone-sensitive adenylate cyclase following phospholipid treatment has been reported [20,21]. Finally, cholinergic drugs were preincubated with ventricular slices prior to enzyme assay. Although this approach permitted hormone—tissue interaction prior to cell disruption, the method employed for the assessment of guanylate cyclase activity ultimately required homogenization prior to assay and thus evaluation of enzyme activity in the absence of cellular integrity. The uniformly unsuccessful attempts to directly stimulate guanylate cyclase under in vitro conditions in broken cell preparations suggest three possible explanations: (1) the unique in vitro conditions required for guanylate cyclase stimulation by acetylcholine were not found during the course of this investigation, (2) cell viability and intact architecture are required to elicit stimulation of guanylate cyclase by cholinergic drugs, or (3) elevations of cyclic GMP levels observed following administration of cholinergic agents are not mediated by direct stimulation of guanylate cyclase and enhanced cyclic GMP synthesis, but rather are mediated by one or more other intracellular processes, such as phosphodiesterase inhibition and reduced cyclic GMP degradation or activation of guanylate cyclase by another intracellular effector.

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