Biochemica et Biophysica Acta, 438 (1976) 310—323

BBA67818

GUANYLATE CYCLASE

EXISTENCE OF DIFFERENT FORMS AND THEIR REGULATION BY NUCLEOTIDES IN CALF UTERUS

MARVIN I. SIEGEL * GIOVANNI A. PUCA and PEDRO CUATRECASAS *

Department of Pharmacology and Experimental Therapeutics and Department of Medicine, The Johns Hopkins University School of Medicine, Baltimore, Md. 21205 (U.S.A.)

(Received October 21st, 1975)

Summary

The activitiy of calf uterus guanylate gyclase (EC 4.6.1.2) exists in at least two and most probably three distinct forms. The cytosolic enzyme exhibits hyperbolic substrate curves with respect to GTP and Mn^{2+}, while the particulate cyclases (nuclear and microsomal) display sigmoidal (GTP) and hyperbolic (Mn^{2+}) relationships. The Hill coefficient for the GTP dependence is 0.9 for the cytosolic, 1.5 for the nuclear, and 1.4 for the microsomal enzyme. The cytosolic enzyme has a K_m for GTP of 70 μM, while half maximal velocity occurs at 90 and 100 μM GTP for the nuclear and microsomal enzymes, respectively. The K_a for Mn^{2+} is 0.57, 0.71, or 0.75 mM for the cytosolic, nuclear, or microsomal enzyme, respectively.

All three guanylate cyclase activities are stimulated by Lubrol PX, but to a varying extent. The activity of the particulate enzyme is greatly increased by detergent.

ATP is a potent inhibitor of the guanylate cyclase activity of the cytosol, while it stimulates both the nuclear and microsomal enzymes at low concentrations of substrate. Of the nucleotides tested, ATP is the best cytosolic enzyme inhibitor and the most effective stimulator of the microsomal enzyme, while UTP is the most potent nuclear enzyme activator. With the cytosolic enzyme, ATP inhibition is apparently competitive with respect to GTP and uncompetitive with respect to Mn^{2+}. In the particulate guanylate cyclases, ATP stimulates by changing the GTP substrate curves from the sigmoidal to the hyperbolic form, while also increasing the affinity of the enzymes for Mn^{2+}.

---

* Present address: Wellcome Research Laboratories, Burroughs Wellcome Co., Department of Molecular Biology, 3030 Cornwallis Road, Research Triangle Park, N.C. 27709, (U.S.A.).

Introduction

Guanylate cyclase (GTP: pyrophosphate-lyase (cyclizing), EC 4.6.1.2), the enzyme that catalyzes the conversion of GTP to guanosine 3′,5′-cyclic monophosphate (cyclic GMP), has been investigated in numerous tissues. Although several recent publications deal with particulate forms of this enzyme [1—3], most investigations have been limited to the cytosolic enzyme [4—9]. In intestinal mucosa [3], rat heart [1] and sea urchin sperm [2] the major fraction of the enzymatic activity is particulate. While the cytosolic enzyme exhibits only classical hyperbolic kinetics with respect to the substrate, GTP [4,5,10,11], particulate forms display positively cooperative kinetic patterns [1,2]. Both particulate and soluble guanylate cylcase activities exhibit manganese dependence [1,2,4,5]. Although inhibition by ATP has been described [1,3,4] for the enzyme from various tissues, stimulation by nucleotides has been described only for the enzyme from sea urchin sperm [2].

Hormones that elevate the levels of cyclic GMP in cells [12—21] might in principle regulate the activity of guanylate cyclase. While preliminary reports have described effects of various hormones on guanylate cyclase activity in vitro [11,22,23], the effects have not been consistent and the kinetic parameters affected have not been described [4,7,9]. Because of difficulties in observing hormonal modulation of guanylate-cyclase activity, the localization and the enzymatic and regulatory properties of this enzyme were examined in calf uterus. These studies suggest the presence of at least two and possibly three different forms of guanylate cyclase, and these exhibit unique regulatory phenomena with respect to nucleotides. Hitherto undescribed, distinct nuclear and microsomal enzymes whose activities are stimulated by nucleotides are reported. These observations have appeared in abstract form [24].

Materials and Methods

Materials. Creatine kinase and creatine phosphate were obtained from Boehringer Mannheim. GTP, ITP, CTP, UTP, TTP, aminophylline, 2′,3′-*O*′-isopropylidine guanosine and chromatographic alumina (neutral; Activity Grade I) were from Sigma. DEAE-cellulose was purchased from Schliecher and Schuell. Instabray counting solution was from Yorktown Research. Carrier free $H_3{}^{32}PO_4$ in 0.02 M HCl and cyclic [^{3}H]GMP were supplied by New England Nuclear. Albumin was purchased from Armour. All other reagents were of the highest grade available.

Synthesis of [α-^{32}P] GTP. [α-^{32}P]GTP is synthesized by a modification of the method of Symons [25,26]. The first step is the synthesis and purification of [^{32}P]GMP. All liquid reagents were redistilled in vacuo and stored over CaH_2. To 40 mCi of $H_3{}^{32}PO_4$ is added 3 μmol of orthophosphoric acid. The solution is evaporated to dryness at 40° C under reduced pressure. Water is added and the solution evaporated twice more to remove any residual HCl. 0.2 ml of dry, redistilled triethylamine and 1 ml of water are added and the solution is again evaporated. The residue is dried by evaporation with anhydrous, redistilled ace-

tonitrile. Approx. 100 mg (330 μmol) of 2′,3′-*O*′-isopropylidine guanosine are added and the mixture is dried twice with acetonitrile. 3 ml of redistilled anhydrous dimethyl sulfoxide are added. 0.5 ml of 5% triethylamine in dimethyl sulfoxide, followed by 0.5 ml of 5% trichloroacetonitrile in dimethyl sulfoxide, are added and allowed to react for 10 min at 40° C. The solution is then diluted with 20 ml of 4 M acetic acid and heated to 100° C in a boiling water bath for 1 h. The solution is evaporated to remove acetic acid, water is added to the residual dimethyl sulfoxide containing solution, and the solution is evaporated. The evaporation step is repeated twice with additional water. Approx. 5 ml of water are added and the solution is applied to a 1 × 15 cm DEAE-cellulose column previously washed with 4 M formic acid containing 1.0 M triethylamine formate and then with water. The column is washed with 10 ml of water and the [^{32}P]GMP is eluted with a linear gradient (200 ml) of water to 4 M formic acid containing 0.3 M triethylamine formate. 3-ml fractions are collected. The fractions containing [^{32}P]GMP are pooled and lyophilized.

[α-^{32}P]GTP is synthesized enzymatically from the [^{32}P]GMP. To the purified [^{32}P]GMP are added, in a final volume of 1 ml, 100 mM Tris · HCl, pH 8, 10 mM KCl, 20 mM $MgCl_2$, 2 mM ATP, 10 mM creatine phosphate, 5 mg creatine kinase, and 2 mg of a partially purified [27] nucleotide kinase from *Escherichia coli*. The reaction is allowed to proceed at 37° C for 1.5 h. The reaction mixture is applied directly to a 1 × 15 cm DEAE-cellulose column prepared as described above, and the [α-^{32}P]GTP is eluted with a linear gradient of water to 4 M formic acid containing 0.5 M triethylamine formate. 3-ml fractions are collected and the fractions containing [α-^{32}P]GTP are pooled and lyophilized. The purified [α-^{32}P]GTP is redissolved in 1 ml of 0.1 μM EDTA, pH 7.0, and stored in small aliquots at −90° C. This preparation is stable for approx. 3 weeks and is better than 99% [α-^{32}P]GTP.

Assay of guanylate cyclase activity. Guanylate cyclase is assayed at 37° C using [α-^{32}P]GTP. The assay mixture contains, in a final volume of 0.1 ml, 25 mM Tris · HCl, pH 7.6, 5 mM aminophylline, 15 μg creatine kinase, 10 mM creatine phosphate, various concentrations of [α-^{32}P]GTP (approx. 2 μCi) and $MnCl_2$, 0.2% albumin, and the sample to be assayed. The reaction is conducted at 37° C and terminated by the addition of 0.1 ml of 0.1 M EDTA, pH 7.6. 1 ml of 25 mM Tris · HCl, pH 7.6, containing approx. 20 000 cpm of cyclic [^{3}H]GMP, is added and the solution is applied to a disposable column containing 1 g of dry alumina (activity grade I). Cyclic GMP is eluted by the addition of 2 ml of 25 mM Tris · HCl, pH 7.6. The entire eluate is collected and counted in 10 ml of Instabray solution using a Nuclear Chicago liquid scintillation counter. The amount of cyclic [^{32}P]GMP produced is corrected for the recovery of cyclic [^{3}H]GMP (approx. 70%). The product eluted eluted from the alumina has been shown to be cyclic GMP on numerous chromatographic systems (thin-layer chromatography: silica with ascending ethanol/0.5 M ammonium acetate (5 : 2, v/v); DEAE-cellulose with ascending 0.2 M ammonium bicarbonate; polyethyleneimine-impregnated cellulose with ascending 0.2 M ammonium bicarbonate; DEAE-cellulose with ascending 0.2 M ammonium formate; paper chromatography on Whatman 3 MM with descending *n*-butanol/acetic acid/water (74 : 19 : 50, v/v) or *n*-propanol/ammonia/water (6 : 3 : 1), v/v). Non-enzymatic blanks (sample boiled before additon to assay mixture) show ap-

prox. 200 cpm per 10^6 cpm of [α-^{32}P] GTP added. Assays are conducted in duplicate. Protein was determined by the method of Lowry et al. [28].

Results

Localization of guanylate cyclase. In order to determine the cellular compartmentalization of calf uterus guanylate cyclase, uteri are fractionated using a slight modification of the methods of Widnell et al. [29] and Chauveau et al. [30]. 4 g of fresh calf uterus is homogenized by three 10-s bursts of a Polytron (Brinkman; setting No. 3) using 4 ml of 0.25 M sucrose containing 25 mM Tris · HCl, pH 7.6, 3 mM $MgCl_2$, and 1 mM dithiothreitol. After filtering through three layers of cheesecloth, nuclei are prepared by centrifugation at 1000 × *g* and the pellet is resuspended in the 0.25 M sucrose buffer. This suspension is layered on 2.2 M sucrose and centrifuged at 105 000 × *g* for 1 h. Such purified nuclei are used routinely *. Mitochondria are prepared by centrifugation of the 1000 × *g* supernatant at 10 000 × *g*, and microsomes by centrifugation of the 10 000 × *g* supernatant at 105 000 × *g*. The supernatant is used as the cytosolic fraction.

Guanylate cyclase activity is found in each subcellular fraction (Table I). In the absence of detergent, nearly all of the guanylate cyclase activity is found in the cytosolic fractions. However, in the presence of 0.5% Lubrol PX, at least 8%, and occasionally as much as 17%, of the total activity is found in the purified nuclei. This is the first report of a nuclear localization of guanylate cyclase.

TABLE I

SUBCELLULAR LOCALIZATION OF GUANYLATE CYCLASE ACTIVITY IN CALF UTERUS HOMOGENATES

4 g of fresh calf uterus was homogenized and fractionated as described in Results. Guanylate cyclase activity in each subcellular fraction was determined in presence and absence of 0.5% Lubrol PX as described in Materials and Methods. The GTP concentration was 200 μM and the Mn^{2+} concentration was 1 mM. Measured in the absence (—) or presence (+) of 0.5% Lubrol PX. n.d., not detectable.

Fraction	Guanylase cyclase activity						
	Total activity (pmol/min)		Percent activity		Specific activity (pmol/min per mg)		DNA * (μg)
	—	+	—	+	—	+	
Homogenate	2310	17 712	—	—	19.5	150	5136
Nuclei	177	1 422	7.7	8.0	8.2	66	2246
Mitochondria	73	2 210	3.2	12.5	10.6	321	566
Microsomes	165	4 584	7.1	25.9	13.9	385	n.d.
Cytosol	1328	4 275	57.5	24.1	15.0	48	n.d.
Total			75.5	70.5			

* DNA present in each fraction was determined by the method of Burton, K. (1968) Methods in Enzymology (Grossmann, L. and Moldave, K., eds.), Vol. XIIB, pp. 163—166, Academic Press, New York.

* The specific activity of the nuclear guanylate cyclase does not change upon further purification of calf uterus nuclei.

Guanylate cyclase activity is also found in similarly purified nuclei from Sprague-Dawley rat thymus, liver, kidney, lung, and uterus. In rat uterus, for example, the nuclei contain approx. 15% of the total guanylate cyclase activity. Based on the recovery of DNA in the nuclear fraction of calf uterus homogenates, approx. 17% of the total guanylate cyclase is nuclear. The purity of the nuclear fraction of this preparation is indicated by the following evidence: when plasma membranes of slices of fresh calf uteri are labelled with ^{125}I-labelled wheat germ agglutinin according to the method of Chang et al. [31], the nuclear pellet contains only 0.5% of the radioactivity (907 cpm) of the total bound to the slices (194 520 cpm). The fact that most of the counts are found in the cytosol (78 060 cpm) indicates that there is little or no contamination of the nuclei by cytosol. Moreover, the nuclei are not significantly (less than 4%) contaminated by microsomes which bound 21 360 cpm of the ^{125}I-labelled wheat germ agglutinin. Comparison of these results to previously reported uterine-cell fractionations [29] indicates that the contamination of nuclei by microsomes and cytosol as determined by the ^{125}I-labelled wheat germ agglutinin method [31] is an overestimation as compared to the use of marker enzymes.

The specific activity of the purified nuclei was 20% of that of the microsomal fraction and higher than the cytosol. Since contamination of the isolated nuclei by microsomes or cytosol is no more than 4 or 1%, respectively, the guanylate cyclase activity of the purified nuclei is most probably due to an enzyme originally localized in nuclei of calf uterus. Furthermore, if this nuclear cyclase is solely the result of contamination by either cytosol or microsomes, then the nuclear activity should exhibit regulatory properties characteristic of those fractions. However, as discussed below, this is not the case.

The mitochondrial pellet of calf uterine homogenates contains approx. 10% of the activity, but this fraction is highly contaminated with microsomes and has therefore not been further investigated. Microsomes and cytosol each contain about 30% of the guanylate cyclase activity (Table I).

The non-ionic detergent, Lubrol PX, stimulates the activity in nuclei approx. 10-fold, in cytosol 3-fold and in microsomes 50-fold. In detergent the specific activity of the microsomal enzyme is the highest, followed by the nuclear and cytosolic enzymes. Homogenization and fractionation of calf uterus in hypotonic medium rather than in 0.25 M sucrose, or in sucrose in the absence of Mg^{2+}, or in sucrose and 0.15 M KCl with and without Mg^{2+}, reveals essentially the same subcellular distribution of guanylate cyclase activity. Similar subcellular distribution is found using frozen rather than fresh calf uterus. Enzymatic activity is stable to freezing, and fractions can be stored at −20° C for at least 1 week with little or no loss of enzyme activity.

GTP dependence. The cytosolic and microsomal guanylate cyclase activities are linear for at least 5 min at 37° C and the nuclear enzyme activity is linear for more than 10 min (data not shown). For each enzyme the activity is linear with increasing protein concentration up to 2 mg/ml (data not shown).

The GTP concentration dependence of the nuclear, microsomal, and cytosolic guanylate cyclases is shown in Fig. 1. The cytosolic enzyme exhibits a normal hyperbolic pattern. This enzyme has an apparent K_m for GTP of 70 μM and a V of 150 pmol of cyclic GMP/min per mg of protein. The shape of the GTP concentration curve, and the K_m for GTP, are similar in the presence or

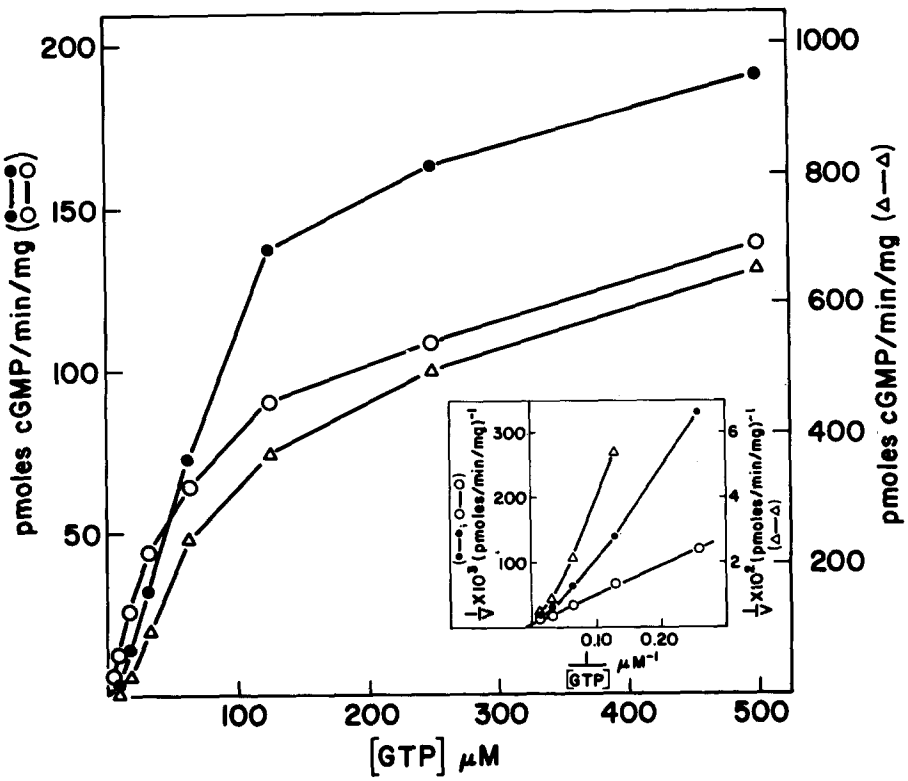

Fig. 1. Effect of GTP on the activities of calf uterus guanylate cyclases. Guanylate cyclases activities of subcellular fractions of calf uterus were assayed as described in Materials and Methods. Assays also contained 0.5% Lubrol PX. The Mn^{2+} concentration was 2 mM ○———○, cytosol; ●———●, nuclei; △———△, microsomes.

absence of Lubrol PX for the cytosolic guanylate cyclase (not shown). On the other hand, the nuclear and microsomal enzymes exhibit a sigmoidal GTP concentration dependence, as has been reported for other particulate guanylate cyclases [1,2]. Lineweaver-Burke [32] plots of the GTP dependence of the three enzymatic activities (Fig. 1, insert) reveal that while the cytosolic activity is linear, the particulate enzymes exhibit apparent positive cooperativity at low GTP concentrations.

Hill plots [33] of the data for the particulate enzymes (Fig. 2) confirm cooperative behavior with respect to GTP; the Hill coefficients are 0.9, 1.4 and 1.5 for the cytosolic, microsomal and nuclear guanylate cyclases, respectively, a significant difference between the cytosolic and particulate activities. Half-maximal activity occurs at about 100 μM GTP for the microsomal enzyme and at about 90 μM GTP for the nuclear one. The apparent maximal velocities are 900 and 400 pmol of cyclic GMP/min per mg of protein for the microsomal and nuclear cyclases, respectively.

Solubilization (greater than 80% as confirmed by centrifugation at 100 000 $\times g$ for 1 h) of the particulate enzymes in 1% Lubrol PX prior to assay does not alter the sigmoidicity of the substrate curves or the concentration of GTP at which half-maximal activity is reached (data not shown).

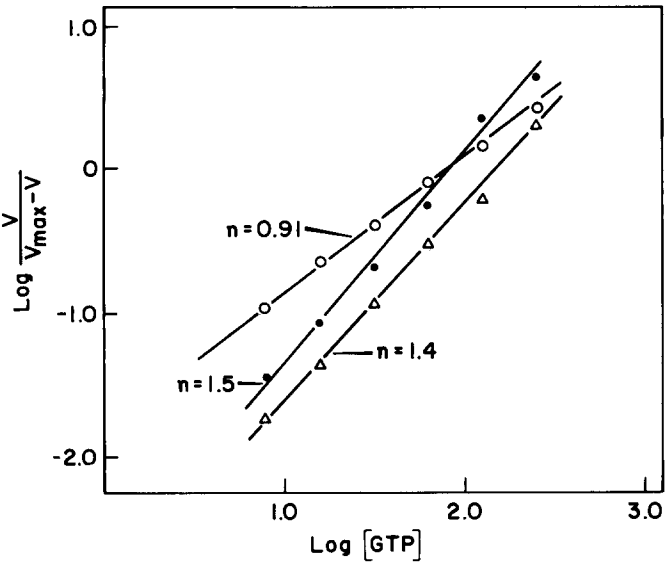

Fig. 2. Hill plots of GTP dependence of calf uterus guanylate cyclases. Replot of the guanylate cyclase activities described in Fig. 1. ○———○, cytosol; ●———●, nuclei; △———△, microsomes.

Mn^{2+} dependence. The particulate and cytosolic enzymes exhibit a hyperbolic dependence on Mn^{2+} concentration (Fig. 3). However, the nuclear and microsomal enzymes are inhibited at high concentrations of free Mn^{2+} while the cytosolic enzyme is not. The K_a for Mn^{2+} is 0.57 mM for the cytosolic, 0.71 mM for the nuclear, and 0.75 mM for the microsomal enzyme.

Detergent stimulation of activity. The non-ionic detergent, Lubrol PX, stimulates the nuclear, microsomal and cytosolic guanylate cyclases (Fig. 4). In this experiment, Lubrol PX stimulates the cytosolic enzyme about 2.5 times at a

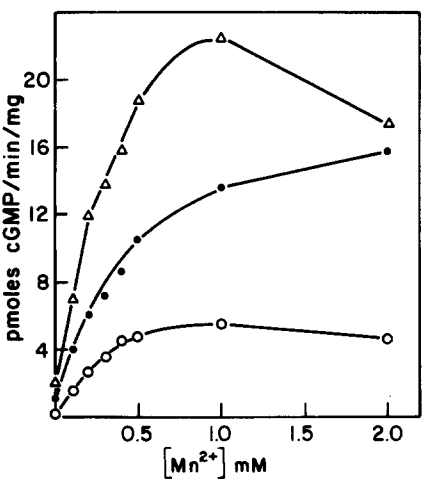

Fig. 3. Effect of Mn^{2+} on the activities of calf uterus guanylate cyclases. Assays were conducted as described in Materials and Methods. In addition to including 0.5% Lubrol PX, reaction mixtures contained 10 μM GTP, added as the GTP : Mn^{2+} (1 : 1) complex, plus increasing concentrations of free Mn^{2+}. ●———●, cytosol; ○———○, nuclei; △———△, microsomes.

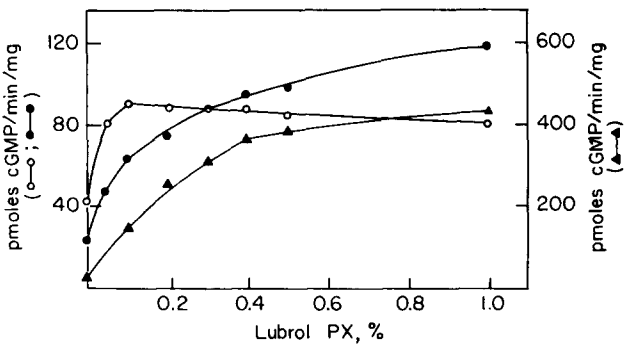

Fig. 4. Stimulation of calf uterus guanylate cyclases by Lubrol PX. Guanylate cyclase activities were measured as described in Materials and Methods. The GTP concentration was 100 μM, and Mn^{2+} was 2 mM. ○———○, cytosol; ●———●, nuclei; ▲———▲, microsomes.

concentration of 0.1%, while 1% Lubrol PX activates the nuclear cyclase approx. 6-fold and the microsomal enzyme about 50-fold. Half-maximal stimulation of the particulate enzymes occurs at a detergent concentration of 0.3%.

Effect of nucleotides. It has been reported that sea urchin sperm [2] guanylate cyclase is inhibited by ATP. In order to determine whether the regulation of guanylate cyclase by nucleoside triphosphates is a common feature of all these enzymes, the effect of various nucleotides on the three guanylate cyclase activities of calf uterus was investigated.

The cytosolic enzyme is inhibited by most of the nucleotides tested, although ATP is the most potent inhibitor (Table II). On the other hand, the effect of nucleotides on the particulate enzymes differs markedly from that seen with the cytosolic cyclase. At low concentrations of GTP, where the particulate enzymes exhibit positive cooperativity with respect to substrate (Figs. 1 and 2), nucleotides stimulate the microsomal and to a lesser extent the nuclear enzymes. UTP and TTP appear to be the most potent activators of the nuclear enzyme, while ATP is the best stimulator of the microsomal activity. However,

TABLE II

EFFECT OF NUCLEOSIDE TRIPHOSPHATES ON CALF UTERUS GUANYLATE CYCLASES

The guanylate cyclase activity of subcellular fractions of calf uterus was determined in the presence of 0.5% Lubrol PX using 10 μM GTP and 0.4 mM Mn^{2+} as described in Materials and Methods. The effect of various nucleotides was determined by assaying each fraction in the presence of 50 μM of the nucleoside triphosphate indicated. The relative effectiveness of these nucleotides was reproducible in subsequent experiments. Duplicates were within 2%. Values are expressed as specific activity ± S.D.

Addition	Fraction (pmol/min per mg of protein)		
	Cytosol	Nuclei	Microsomes
None	7.0 ± 0.1	12.2 ± 0.2	7.8 ± 0.1
ATP	5.2 ± 0.1	12.1 ± 0.2	51.5 ± 0.7
UTP	7.0 ± 0.1	14.6 ± 0.2	31.0 ± 0.4
ITP	5.1 ± 0.1	13.1 ± 0.2	46.5 ± 0.7
CTP	5.7 ± 0.1	12.3 ± 0.3	33.6 ± 0.5
TTP	6.1 ± 0.1	14.0 ± 0.2	36.1 ± 0.5

concentrations of ATP greater than 300 μM inhibit the particulate cyclases (data not shown).

Since ATP is both an inhibitor of the cytosolic guanylate cyclase and an activator of the particulate cyclases, the effect of this nucleotide on various kinetic parameters was investigated.

Effect of ATP on GTP concentration dependence. The cytosolic enzyme is inhibited by ATP at each concentration of GTP tested (Fig. 5a). The extent of inhibition by 50 μM ATP decreases as the concentration of GTP is increased. As expected from this observation, double-reciprocal plots (Fig. 5b) reveal that, for the cytosolic enzyme, ATP behaves approximately as a competitive inhibitor with respect to the substrate, GTP. 50 μM ATP nearly doubles the apparent K_m for GTP.

The slight activation of the nuclear guanylate cyclase by ATP occurs at low and high GTP concentrations (Fig. 6a). This activation is very pronounced at high GTP concentrations because the control activity is strongly inhibited by substrate while that of the stimulated enzyme is not. At low concentrations of GTP, where the nuclear enzyme exhibits positive cooperativity with respect to substrate (Figs. 1 and 2), ATP stimulation is also observed (Fig. 6b). In fact, in the presence of ATP the apparent affinity for substrate is increased. If ATP were not inhibitory at higher concentration, the GTP substrate curve would probably be hyperbolic in the presence of the activator, ATP, while it is sigmoidal (Fig. 1) in its absence.

The microsomal guanylate cyclase is stimulated by 50 μM ATP at all concentrations of GTP tested (Fig. 7a). This activation is more pronounced at low concentrations of GTP (Fig. 7b), where the microsomal enzyme exhibits positive cooperativity with respect to GTP (Figs. 1 and 2). As with the nuclear enzyme, ATP reduces the apparent cooperativity towards GTP. The sigmoidal substrate curve approaches a hyperbolic plot with increasing concentrations of

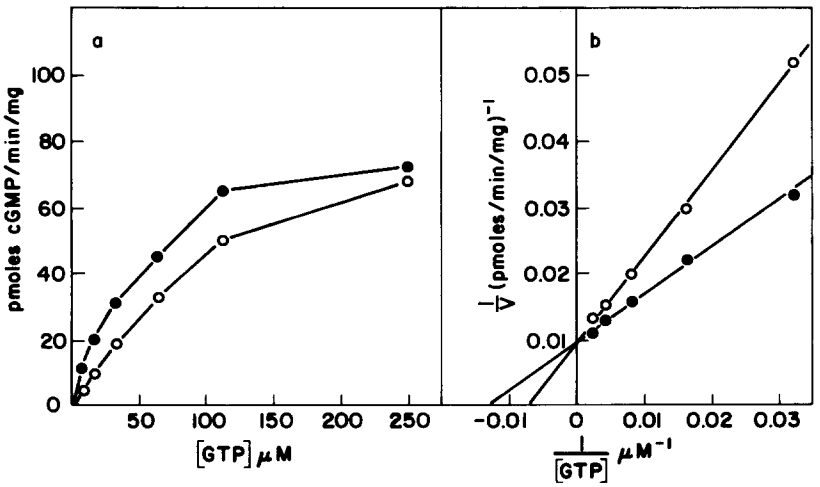

Fig. 5. Effect of ATP on the GTP dependence of the cytosolic guanylate cyclase. The cytosolic enzyme was assayed in 0.5% Lubrol PX as described in Materials and Methods in the absence (●———●) and presence (○———○) of 50 μM ATP, added as the ATP : Mn^{2+} (1 : 1) complex.

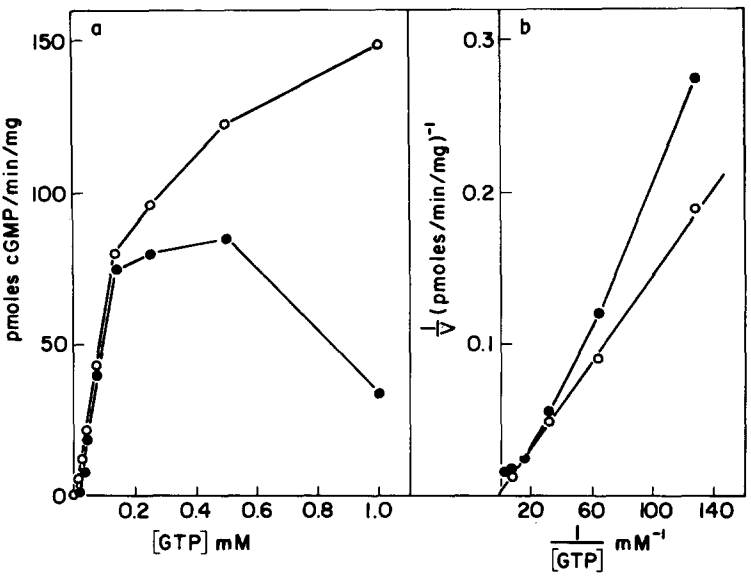

Fig. 6. Effect of ATP on the GTP dependence of the nuclear guanylate cyclase. The nuclear enzyme was assayed in the absence (●——●) and presence (○——○) of 50 μM ATP as described in Fig. 5.

ATP. However, as in the nuclear enzyme, ATP at high concentrations inhibits the microsomal cyclase. In contrast to the nuclear enzyme, the microsomal enzyme is not inhibited by high concentrations of GTP.

Effect of ATP on Mn^{2+} concentration dependence. The cytosolic nuclear, and microsomal guanylate cyclases exhibit hyperbolic kinetics with respect to Mn^{2+} (Fig. 3). ATP affects the Mn^{2+} dependence of each of these enzymes. In the presence of 50 μM ATP, the cytosolic enzyme is inhibited at all Mn^{2+} concentrations tested (Fig. 8a). Since ATP is added as ATP : Mn^{2+} (1 : 1), the activity observed in the absence of excess Mn^{2+} appears elevated due to an in-

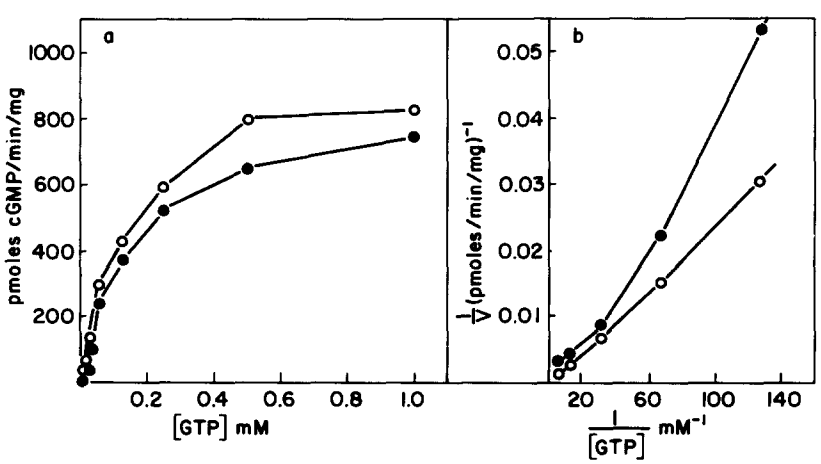

Fig. 7. Effect of ATP on the GTP dependence of the microsomal guanylate cyclase. The microsomal enzyme was assayed in the absence (●——●) and presence (○——○) of 50 μM ATP as described in Fig. 5.

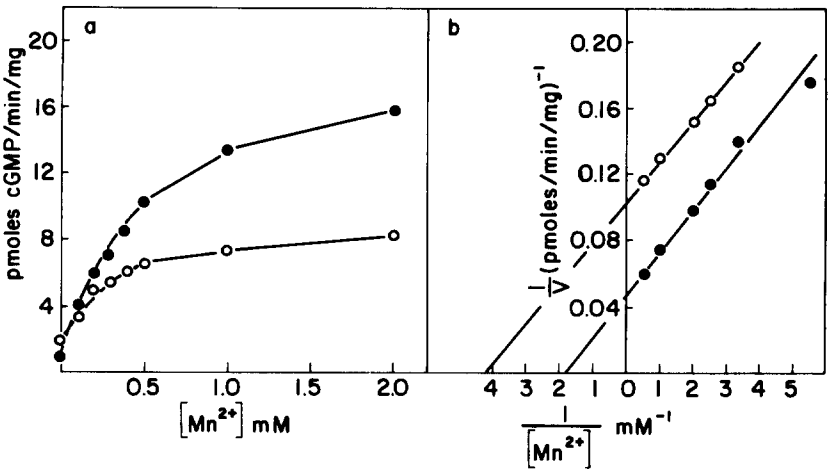

Fig. 8. Effect of ATP on the Mn^{2+} dependence of the cytosolic guanylate cyclase. The cytosolic enzyme was assayed as described in Materials and Methods. GTP, which was added as the GTP : Mn^{2+} (1 : 1) complex, was maintained at 10 μM while free Mn^{2+} concentrations were varied. Activity was determined in 0.5% Lubrol PX in the absence (●——●) and presence (○——○) of 50 μM ATP, added as the ATP : Mn^{2+} (1 : 1) complex.

crease in total Mn^{2+} present relative to the control enzyme. Double-reciprocal plots (Fig. 8b) indicate that ATP increases the apparent affinity of the cytosolic cyclase for Mn^{2+} by about 2.3-fold while decreasing its V. This is, ATP is an uncompetitive inhibitor of the cytosolic enzyme with respect to Mn^{2+}.

ATP activates the nuclear (not shown) and microsomal (Fig. 9a) guanylate cyclases in a similar fashion at all Mn^{2+} concentrations. The stimulation occurs by an increased apparent affinity for Mn^{2+} (Fig. 9b) without any apparent change in V. In contrast to the cytosolic enzyme, both particulate enzymes exhibit inhibition by high concentrations of divalent cation. ATP stimulation does not reverse this inhibition.

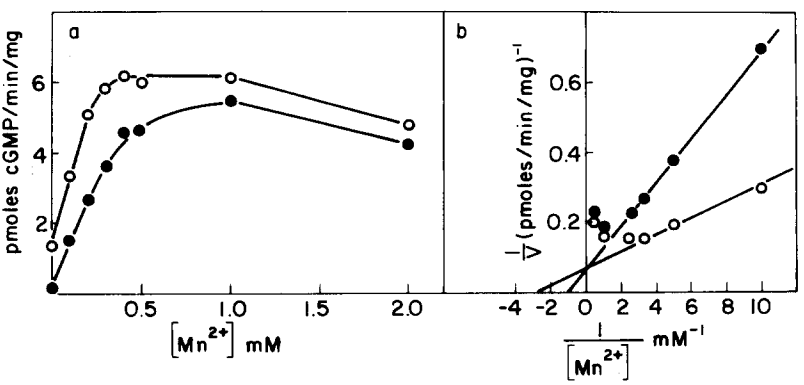

Fig. 9. Effect of ATP on the Mn^{2+} dependence of the microsomal guanylate cyclase. The microsomal enzyme was assayed in the absence (●——●) and presence (○——○) of 50 μM ATP as described in Fig. 8.

Discussion

Guanylate cyclase activity of calf uterus is localized in cytosolic, nuclear, and microsomal fractions of homogenates (Table I). While the presence of guanylate cyclase in cytosolic and microsomal fractions of various tissues has been reported [1—3], no evidence of a nuclear enzyme has appeared previously. In the present studies the occurrence of nuclear guanylate cyclase was also confirmed in various other tissues (thymus, liver, lung, kidney) in rats. However, nuclear adenylate cyclase activity has been previously reported [34,35]. Although some recent publications [1—3] have studied the particulate enzyme, most studies of guanylate cyclase have been confined to the cytosolic fraction [4—9]. It is clear that when assayed in the presence of detergent, approximately half of the enzymatic activity is found in particulate fractions of calf uterus (Table I). The non-ionic detergent, Lubrol PX, stimulates the microsomal and nuclear enzymes more than 50- and 8-fold, respectively, thereby unmasking the high activity in these fractions (Table I and Fig. 4). This stimulation may be due to solubilization of the enzyme as well as increased substrate accessibility. The cytosolic enzyme is also stimulated by Lubrol PX, but only 3-fold.

Evidence for the different nature of the three guanylate cyclases of calf uterus described here can be derived from their GTP concentrations dependence and from their regulation by nucleotides. The fact that the cytosolic enzyme exhibits a hyperbolic GTP curve while the particulate enzymes show positive cooperativity with respect to this substrate (Fig. 1) is a distinguishing characteristic. Hill coefficients (Fig. 2) for the nuclear, microsomal, and cytosolic guanylate cyclases are 1.5, 1.4, and 0.91, respectively. The effects of nucleotides on these enzymes further supports the hypothesis that the cytosolic and particulate cyclases are not identical. Nucleotides inhibit the cytosolic and activate the particulate enzymes (Table II). The stimulation of the particulate calf uterus guanylate cyclases by nucleotides is consistent with the ATP activation reported for the sea-urchin sperm guanylate cyclase [2], but contrast with the inhibition observed with the rat-heart particulate enzyme [1]. Inhibition of the cytosolic enzyme by ATP is apparently competitive with respect to GTP (Fig. 5) and uncompetitive with respect to Mn^{2+} (Fig. 8). Stimulation of the particulate guanylate cyclase by nucleotides is due to an increased affinity for Mn^{2+} (Fig. 9) and to a reduction in the apparent positive cooperativity observed with GTP (Figs. 6 and 7). That is, as the ATP concentration is raised, the Hill coefficients for the particulate enzymes approach 1. The particulate guanylate cyclases, therefore, appear to have an allosteric activator site for nucleotides in addition to the GTP substrate site. Finally, the particulate enzymes are inhibited by high concentrations of Mn^{2+} (Fig. 9) while the cytosolic enzyme is not (Fig. 8).

While the properties of the cytosolic and particulate guanylate cyclases contrast markedly, the difference between the nuclear and microsomal enzymes are more subtle. The following evidence indicates that the guanylate cyclase activity found in purified nuclei is not a result of contamination by microsomal enzyme: (1) the specific activity of the nuclear fraction cannot be explained in the light of the low contamination by microsomes indicated by the ^{125}I-labelled

wheat germ agglutinin marker or by previous reports using this method of fractionation [29,30]; (2) the non-ionic detergent Lubrol PX activates these enzymes to different extents; (3) the nuclear enzyme is inhibited by high concentrations of GTP (Fig. 6) while the microsomal enzyme is not (Fig. 7); (4) there is a much higher nucleotide stimulation of the microsomal guanylate cyclase than that observed with nuclear activity; (5) the apparent nucleotide specificity for activation of these enzymes (Table II) is intriguing, since UTP and TTP, the nucleotides uniquely involved in the pathways of RNA and DNA biosyntheses, appear to be the most potent stimulators of the nuclear guanylate cyclase, while ATP exhibits the most pronounced activation of the microsomal enzyme. While this evidence supports the hypothesis that the nuclear and microsomal guanylate cyclases of calf uterus are different, definite conclusions cannot be drawn without further physical-chemical studies on the individually purified enzymes. This work is now in progress.

It may be instructive to speculate regarding the possible involvement of the different guanylate cyclases in the response of tissues to different hormonal stimuli which may give rise to increased levels of cyclic GMP. Possibly the plasma membrane guanylate cyclase might be anticipated to respond to peptide hormones, acetylcholine, and plant lectins [12], while either the cytosolic or nuclear enzyme might be regulated by steroid hormones, which raise the level of cyclic GMP in the uterus [13]. Further investigations into the possible regulation of calf uterus guanylate cyclases by various hormones as well as nucleotides are in progress.

Acknowledgements

This research was supported by grants from the National Institutes of Health (AM-18035), (AM-14956), (AM-07422) and the Kroc Foundation. M.I.S. is a recipient of an N.I.H. postdoctoral fellowship (AM-53928). G.A.P. is a recipient of a W.H.O. Travel Fellowship and a fellowhip from the Lalor Foundation; on leave from the Institute of General Pathology, I. Medical School, University of Naples. P.C. is a recipient of U.S.P.H.S. Research Career Development Award AM-31464.

References

1 Kimura, H. and Murad, F. (1974) J. Biol. Chem. 249, 6910—6916
2 Garbers, D.L., Hardman, J.G. and Rudolph, F.B. (1974) Biochemistry 13, 4166—4171
3 Ishikawa, E., Ishikawa, S., Davis, J.W. and Sutherland, E.W. (1969) J. Biol. Chem. 244, 6371—6376
4 Hardman, J.G. and Sutherland, E.W. (1969) J. Biol. Chem. 244, 6363—6370
5 White, A.A. and Aurbach, G.D. (1969) Biochim. Biophys. Acta 191, 686—687
6 Hardman, J.G., Beavo, J.A., Gray, J.P., Chrisman, T.D. Patterson, W.D. and Sutherland, E.W. (1971) Ann. N.Y. Acad. Sci. 185, 27—35
7 McMillian, B.H., Ney, R.L. and Schorr, I. (1971) Endocrinology 89, 281—283
8 Schultz, G., Jakobs, K.H., Böhme, E. and Schultz, K. (1972) Eur. J. Biochem. 24, 520—529
9 Marks, F. (1973) Biochim. Biophys. Acta 390, 349—356
10 Böhme, E. (1970) Eur. J. Biochem. 14, 422—429
11 Thompson, W.J., Williams, R.H. and Little, S.A. (1973) Biochim. Biophys. Acta 302, 329—337
12 Illiano, G., Tell, G.P.E., Siegel, M.I. and Cuatrecasas, P. (1973) Proc. Natl. Acad. Sci. U.S. 70, 2443—2447
13 George, W.J., Polson, J.B., O'Toole, A.G. and Goldberg, N.D. (1970) Proc. Natl. Acad. Sci. U.S. 66, 398—403

14 Ferrendelli, J.A., Steiner, A.L., McDougal, D.B. and Kipnis, D.M. (1970) Biochem. Biophys. Res. Commun. 41, 1061—1067
15 Kuo, J.F., Lee, T.P., Reyes, P.L., Walton, K.G., Donnelley, Jr., T.E. and Greengard, P. (1972) J. Chem. 247, 16—22
16 Yamashita, K. and Field, J.B. (1972) J. Biol. Chem. 247, 7062—7066
17 Schultz, G., Hardman, J.G., Schultz, K., Davis, J.W. and Sutherland, E.W. (1973) Proc. Natl. Acad. Sci. U.S. 70, 1721—1725
18 Murael, F. and Kimura, H. (1974) Biochim. Biophys. Acta 343, 275—286
19 Kimura, H., Thomas, E. and Murael, F. (1974) Biochim. Biophys. Acta 343, 519—528
20 Stoner, J., Manganiello, V.C. and Vaughan, M. (1974) Mol. Pharmacol. 10, 155—161
21 Kuehl, Jr., F.A., Ham, E.A., Zanetti, M.E., Sanford, C.H., Nicol, S.E. and Goldberg, N.D. (1974) Proc. Natl. Acad. Sci. U.S. 71, 1866—1870
22 White, L.E., Ignarro, L.H. and George, W.J. (1972) The Pharmacologist 15, 157
23 Amer, M.S. and McKinney, G. (1973) The Pharmacologist 15, 157
24 Siegel, M.I., Puca, G.A. and Cuatrecasas, P. (1975) Fed. Proc. 34, 232
25 Symons, R.H. (1968) Biochim. Biophys. Acta 155, 609—610
26 Symons, R.H. (1973) Biochim. Biophys. Acta 320, 535—539
27 Hubert, R.B. and Furlong, N.B. (1967) Methods in Enzymology (Grossman, L. and Moldave, K., eds.), Vol. XIIA, 193—202, Academic Press, New York
28 Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1961) J. Biol. Chem. 193, 265—275
29 Widnell, C.C., Hamilton, T.H. and Tata, J.R. (1967) J. Cell Biol. 32, 766—770
30 Chauveau, J., Moule, Y. and Roviller, C. (1956) Exp. Cell Res. 11, 317
31 Chang, K.-J., Bennett, V. and Cuatrecasas, P. (1975) J. Biol. Chem. 250, 488—500
32 Lineweaver, H. and Burke, D. (1934) J. Am. Chem. Soc. 56, 658—666
33 Hill, A.V. (1910) J. Physiol. Lond. 40, IV—VIII
34 Sofer, D. and Hechtor, O. (1971) Biochim. Biophys. Acta 230, 530—542
35 Liao, S., Lin, A.H. and Tymuczko, J.L. (1971) Biochim. Biophys. Acta 230, 535—538