Biochimica et Biophysica Acta, 658 (1981) 369—376

BBA 69244

STIMULATION OF GUANYLATE CYCLASE BY EDTA AND OTHER CHELATING AGENTS

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(Received October 27th, 1980)

Key words EDTA Dopamine, Guanylate cyclase, Chelating agent, (Human caudate nucleus)

Summary

The partially purified soluble guanylate cyclase (GTP pyrophosphate-lyase(cyclizing), EC 4.6.1.2) from human caudate nucleus is stimulated from 2 to 4-fold by metal chelating agents. EDTA ($K_{1/2}$ = 4.8 μM) is more potent than CDTA ($K_{1/2}$ = 13.2 μM) or EGTA ($K_{1/2}$ = 21.8 μM) at stimulating activity Stimulation by chelating agents is apparently not due to removal of inhibitory divalent cations which contaminate the enzyme or reaction mixture. EDTA increases guanylate cyclase activity in part by increasing the affinity of the enzyme for the substrate (MgGTP) 10-fold. Dopamine inhibits partially purified guanylate cyclase in the presence or absence of EDTA Dopamine increases the K_a of guanylate cyclase for the activator, free Mn^{2+}, more than 50-fold, from 3 to 150 μM

Introduction

During an investigation of the inhibition of guanylate cyclase (GTP pyrophosphate-lyase (cyclizing), EC 4.6.1.2) from human caudate nucleus by dopamine and other catecholamines, we observed that EDTA and EGTA stimulate partially purified guanylate cyclase activity 4-fold [1]. Based on observations reported here, we suggest that the stimulation is not due to chelation of contaminating inhibitory divalent cations. Consequently, we have examined the

Abbreviations CDTA, 1,2-diaminocyclohexane-*N*,*N*'-tetraacetic acid, EGTA, ethyleneglycol bis(β-aminoethyl ether)-*N*,*N*'-tetraacetic acid

effects of EDTA on the interaction of guanylate cyclase with substrates and with the inhibitor, dopamine

Materials and Methods

Materials The following chemicals were obtained from Sigma Chemical Co. GTP, EDTA, EGTA, CDTA, diethyldithiocarbamic acid, $MgCl_2$, bovine albumin (RIA grade), γ-globulin, sucrose, dopamine and Coomassie brilliant blue G Sodium acetate, glycerol and acetic anhydride were from Mallinckrodt. Other chemicals were obtained as follows 3-isobutyl-1-methylxanthine (Aldrich Chemical Co.), Tris-HCl (Schwartz-Mann); $MnCl_2$ (Fisher Scientific Co.); DEAE-Sephacel (Pharmacia Laboratories), triethylamine (Eastman Chemical Co.), bovine serum albumin (Miles Laboratories), Chelex-100 (Bio-rad Laboratories), [8-^{3}H]GTP (Amersham Searle Co.) The anti-cyclic GMP antibody was produced in goats according to a modification of the procedure of Steiner et al. [2] Antigen for radioimmunoassays was prepared from succinyl-cyclic GMP-tyrosine methyl ester (Sigma Chemical Co.) with an ^{125}I label (New England Nuclear) according to a modification of the method of Cailla et al [3].

Enzyme preparation. Human caudate nucleus dissected at autopsy was frozen in liquid nitrogen in a solution of 25 mM Tris-HCl pH 7 4 (at 0°C)/0.32 M sucrose/1.0 mM EDTA and stored at −70°C until used. After thawing at 4°C, tissue was hand homogenized on ice in 9 vol 0.32 M sucrose using eight passes of a Teflon pestle in a glass homogenizing vessel Tissue fractionation was carried out at 0—4°C according to the procedure of Von Hungen and Roberts [4]. The 100 000 × *g* soluble fraction was purified 10-fold by chromatography on a DEAE-Sephacel column equilibrated with buffer containing 25 mM Tris-HCl, pH 7.4 at 0°C/1 mM EDTA/20% glycerol. Where specified, EDTA was omitted. After washing with column buffer, guanylate cyclase activity was eluted with a linear NaCl gradient (0—0.75 M in column buffer). Further details of this purification procedure are given elsewhere [1].

Enzyme assays. Guanylate cyclase activity was assayed by the method of Kimura and Murad [5]. The standard guanylate cyclase reaction mixture contained 75 mM Tris-HCl, pH 7 6/4 mM 3-isobutyl-1-methylxanthine with GTP and metal as indicated in a total volume of 0.1 ml. Reactions were initiated by addition of enzyme and incubations were conducted for 10 min at 37°C unless otherwise indicated. Reactions were terminated by addition of 0 5 ml 60 mM sodium acetate, pH 4.0 and heating at 90°C for 3 min. Data presented represent the mean ± S.E. of triplicate determinations. The cyclic GMP formed was measured for each determination in duplicate by radioimmunoassay using the method of Harper and Brooker[6]. Purification of the samples prior to radioimmunoassay was unnecessary because there was no detectable cross-reactivity of the cyclic GMP antibody when GTP was used at a million times the concentration of cyclic GMP which produced 50% displacement of binding in the radioimmunoassay. Standard curves were run in reaction blanks prepared by incubating the reaction mixture without enzyme followed by addition of enzyme during boiling. The formation of cyclic GMP is a linear function of time for up to 10 min for the soluble enzyme (data not shown). Cyclic GMP formation was also a linear function of protein concentration over the range

used (0.02—0.18 mg/ml for partially purified soluble guanylate cyclase) in the presence or absence of EDTA (data not shown). [^{3}H]GTP breakdown, monitored by TLC [7], was never greater than 5% with the partially purified soluble guanylate cyclase. GTP regenerating systems were avoided since they have been reported to alter the kinetic properties of guanylate cyclase [8] and adenylate cyclase [9,10] probably by direct interaction of the regenerating system with the cyclase.

Other methods. Protein was determined as described by Bradford [11] using bovine serum albumin as a standard. Mg^{2+} concentrations were determined by atomic absorption spectroscopy. Dopamine was dissolved in water which was degassed under vacuum and extensively bubbled with N_2(g) to remove dissolved O_2. Addition of 0.01 N HCl to stabilize dopamine further did not alter results and consequently was not used routinely. Kinetic data are presented as Lineweaver-Burk plots although kinetic parameters were calculated by the Eadie-Hofstee method [12].

Results

Stimulation of partially purified guanylate cyclase by metal chelators

The guanylate cyclase activity of the 100 000 × *g* supernatant fraction of human caudate nucleus is unaffected by 1 mM EDTA or EGTA (data not shown). However, after 10-fold purification of the soluble enzyme by chromatography on DEAE-Sephacel, the guanylate cyclase activity is stimulated from 2—4-fold by metal chelators (Fig. 1) [1]. EDTA ($K_{1/2}$ = 4.8 μM) is more potent than CDTA ($K_{1/2}$ = 13.2 μM) or EGTA ($K_{1/2}$ = 21.8 μM) at stimulating guanylate cyclase activity. Furthermore, the maximum stimulated activity, (V-V_0), is greater with EDTA (21 pmol · min^{-1} · mg^{-1}) than with CDTA (16 pmol · min^{-1} · mg^{-1}) The stimulation by EGTA exhibits mild positive cooperativity (Hill coefficient = 1.7) but that by EDTA and CDTA do not (Fig. 1) The copper chelator, diethyldithiocarbamic acid, had no effect on guanylate cyclase activity (data not shown).

The observation that EDTA is a better stimulator of guanylate cyclase activity than CDTA is quite remarkable since CDTA binds all metals tested (Mg^{2+}, Ca^{2+}, Mn^{2+}, Co^{2+}, Cd^{2+}, Cu^{2+}, Pb^{2+}, Zn^{2+} and Hg^{2+}) with a higher affinity than EDTA [13]. The stability constants of metal-CDTA complexes are generally between 10 and 10^3-times greater than those of their EDTA counterparts [14]. This suggests that EDTA, EGTA, and CDTA may interact directly with guanylate cyclase, and inhibitory divalent cations may not be involved. Further evidence that EDTA stimulation is not due to removal of contaminating heavy metals from the enzyme or reaction mixture is provided by the observation that chromatography of the enzyme and reaction mixture through Chelex-100 columns did not alter the ability of EDTA to stimulate guanylate cyclase activity. After Chelex-100 chromatography, enzyme activity was stimulated from 15.6 ± 0 4 to 52.4 ± 0.5 pmol cyclic GMP · min^{-1} · mg^{-1} by 50 μM EDTA Only $MgCl_2$ (exceeds A.C.S. specifications) was added to the reaction mixture without prior purification on Chelex-100 since it binds to the resin. Preliminary results from dilution experiments indicate that the stimulation of guanylate cyclase is reversible (data not shown). We have previously reported that

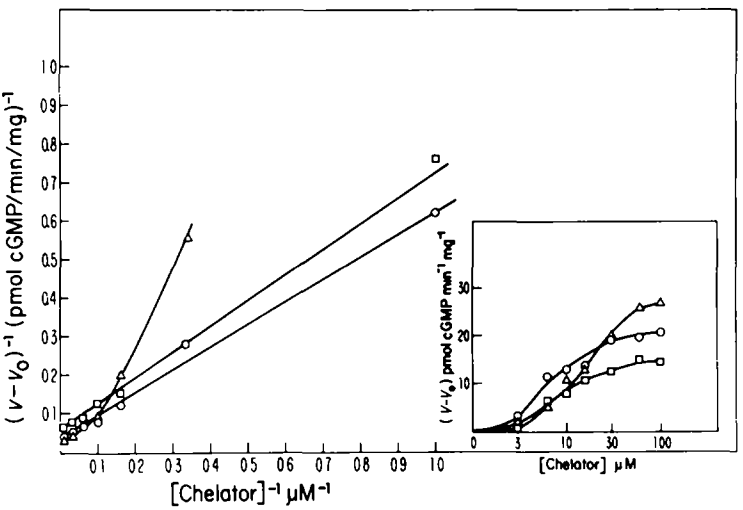

Fig 1 Double-reciprocal plot of the stimulation of guanylate cyclase by chelating agents The reaction mixture contained 75 mM Tris-HCl, pH 7.6/4 mM 3-isobutyl-1-methylxanthine/0 5 mM MgGTP/1 mM excess $MgCl_2$/EDTA (○), CDTA (□) and EGTA (△) as indicated in a total volume of 100 μl Reactions were initiated with the addition of 4 μg partially purified guanylate cyclase with a specific activity, in the absence of chelator (V_0) of 12 0 pmol/min per mg Insert Increase in guanylate cyclase activity as a function of chelator concentration

dopamine and other catecholamines inhibit the partially purified guanylate cyclase of human caudate nucleus [1]. Using enzyme and reaction mixtures previously chromatographed on Chelex-100, inhibition of guanylate cyclase by dopamine is observed in the presence or absence of 50 μM EDTA (data not shown).

The stimulation of guanylate cyclase by chelating agents differs from stimulation by sodium azide since enzyme purification reduces azide stimulation [15] but is required for stimulation by chelators such as EDTA. Furthermore, we have observed that addition of catalase is required to restore azide stimulation of partially purified guanylate cyclase from human caudate (data not shown) but catalase is not required for stimulation by chelating agents (Fig. 1)

EDTA decreases the K_m for MgGTP

The unpurified soluble guanylate cyclase exhibits a K_m for MgGTP of 330 μM [1]. There is no significant change in the affinity of guanylate cyclase for MgGTP (K_m = 409 μM) following partial purification of the soluble enzyme on DEAE-Sephacel, when the chromatography is conducted in the presence of 1 mM EDTA (Fig. 2A). However, when the enzyme is partially purified and assayed in the absence of EDTA, the affinity for MgGTP is decreased more than 21-fold (K_m = 8.7 mM) (Fig. 2B). The addition of 50 μM EDTA to the assay alone increases the affinity for substrate about 10-fold (K_m = 0 82 mM) (Fig. 2C) Therefore, EDTA increases guanylate cyclase activity in part by increasing the affinity of the enzyme for substrate Frey et al. [16] have reported a similar high K_m value for MgGTP for the particulate guanylate cyclase from sheep kidney outer medulla Consequently, cellular concentrations

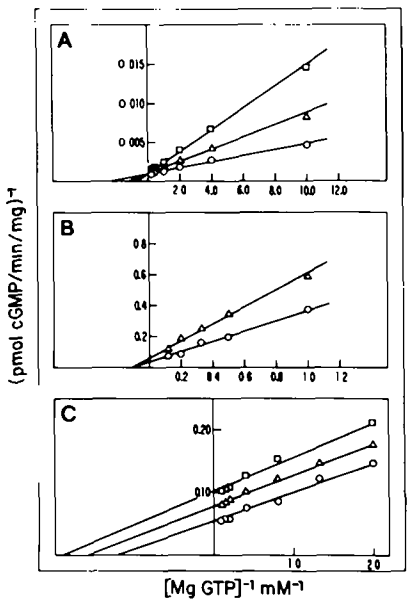

Fig 2 Double-reciprocal plots of the effect of chelating agents on the kinetics of dopamine inhibition of guanylate cyclase The reaction mixture is as described in Fig 1, except that the concentration of MgGTP is varied as indicated A Guanylate cyclase was partially purified in the presence of 1 mM EDTA and 4 μg were assayed in the presence of 50 μM EDTA with no addition (○), 1 μM dopamine (△) or 5 μM dopamine (□) B Guanylate cyclase was partially purified in the absence of EDTA and 18 μg were assayed in the absence of EDTA with no addition (○) and in the presence of 50 μM dopamine (△) C Guanylate cyclase was partially purified in the absence of EDTA and 10 μg were assayed in the presence of 50 μM EDTA with no additions (○), 10 μM dopamine (△) and 50 μM dopamine (□).

of MgGTP, approx 0 4 mM [17], may represent an important determinant of both soluble and particulate guanylate cyclase activity.

Effect of EDTA on the kinetics of dopamine inhibition

As reported previously [1] when guanylate cyclase is partially purified and assayed in the presence of EDTA, dopamine inhibition is competitive with respect to MgGTP (Fig. 2A). However, when enzyme is partially purified and assayed in the absence of EDTA, dopamine inhibition is noncompetitive (Fig. 2B). Addition of EDTA to the reaction mixture alone does not restore competitive inhibition but rather yields a kinetic pattern more consistent with uncompetitive inhibition (Fig. 2C). These results indicate that, whereas dopamine inhibition is observed in the presence or absence of EDTA, the interaction of guanylate cyclase with EDTA during purification and assay markedly affects the ability of increasing concentrations of MgGTP to compete with dopamine.

Interaction of guanylate cyclase with Mg^{2+} and Mn^{2+}

The dependence of partially purified guanylate cyclase on free Mg^{2+} in the presence of EDTA is biphasic (Fig. 3). Free Mg^{2+} is a required activator as indicated by the linear double-reciprocal plot (slope > 0) obtained with increasing concentrations of free Mg^{2+} up to 1 mM (Fig. 3, insert). Increasing free Mg^{2+}

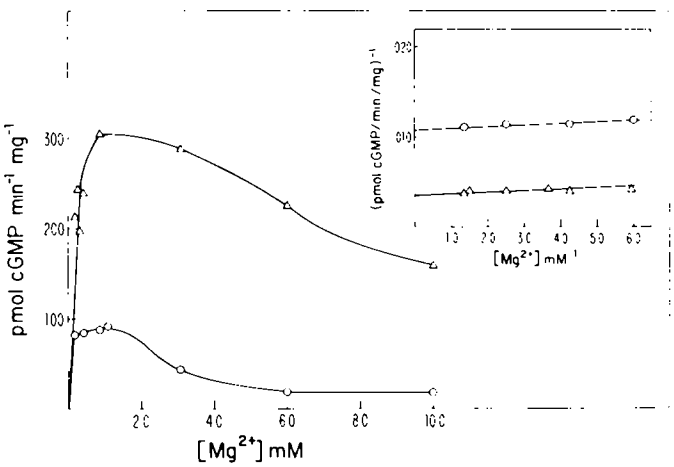

Fig 3 Dependence of guanylate cyclase activity on the concentration of free Mg^{2+} The reaction mixture is as described in Fig 1 except that free Mg^{2+} is varied as indicated The reaction was initiated with 4 μg partially purified guanylate cyclase in the presence (○) or absence (△) of 50 μM dopamine Lower concentrations of free Mg^{2+} could not be tested without increasing the concentration of free GTP, a possible effector, in the reaction mixture Insert Double-reciprocal plot

above 2 mM decreases guanylate cyclase activity. The addition of 50 μM dopamine inhibits guanylate cyclase activity 73% with 1 mM free Mg^{2+} while decreasing the apparent $K_{1/2}$ for free Mg^{2+} from 60 to 21 μM. Similar results were obtained in the absence of EDTA (data not shown). The concentration of free Mg^{2+} which results in optimal guanylate cyclase activity (1 mM) is very similar to the concentration of free Mg^{2+} reported for brain and other tissues, 0 6—1.3 μmol/g wet weight [18]. Since the cellular concentration of free Mg^{2+} is well above the $K_{1/2}$ values, the effect of dopamine on the affinity for free Mg^{2+} may have no physiological importance.

In the absence of EDTA, free Mn^{2+} can replace free Mg^{2+} as the required activator of guanylate cyclase when MnGTP is used as substrate (Fig. 4) While linear double-reciprocal plots are obtained below 1 mM free Mn^{2+}, increasing free Mn^{+} above 1 mM decreases guanylate cyclase activity. The addition of 50 μM dopamine dramatically increases the $K_{1/2}$ for free Mn^{2+} from 3 to 159 μM while inhibiting guanylate cyclase activity (78% at 0.6 mM Mn^{2+}). Thus, dopamine inhibits guanylate cyclase in part by reducing its affinity for the activator, free Mn^{2+}, more than 50-fold This effect of dopamine may be of physiological importance since the cytosolic concentration of Mn^{2+} has been reported to be 1.8 μM [19].

The effects of EDTA on the kinetic properties of guanylate cyclase are noteworthy since a number of authors prepare [15,20,21], purify [20—22] and assay [15,20,21] guanylate cyclase in the presence of EDTA while others do not use chelating agents during preparation [8,16,23—25], purification [25] and assay [8,16,23—25]. Our observation that the unpurified guanylate cyclase activity of the 100 000 × g supernatant fraction is not stimulated by EDTA is in agreement with two earlier reports [26,27]. The reason purification is required to observe the effects of chelators or catecholamines on guanylate

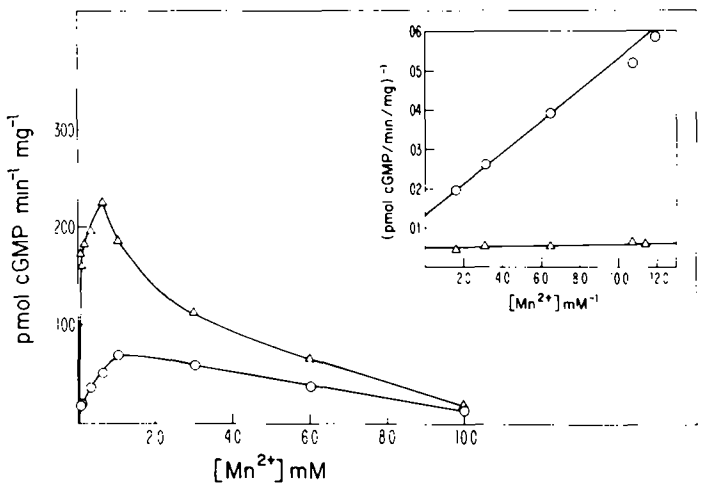

Fig 4 Dependence of guanylate cyclase activity on the concentration of free Mn^{2+} The reaction mixture contained 75 mM Tris-HCl, pH 7 6/4 mM 3-isobutyl-1-methylxanthine/0.5 mM MnGTP/free Mn^{2+} was varied as indicated The reaction was initiated with 4 μg partially purified guanylate cyclase in the presence (○) or absence (△) of 50 μM dopamine Insert Double-reciprocal plot

cyclase remains unknown. Olson et al. [28] have reported a 2-fold stimulation of partially purified rat brain guanylate cyclase by 0.2 mM EGTA, in agreement with the effect of EGTA and other chelating agents on partially purified human brain guanylate cyclase reported here. They suggested that the activation by EGTA may possibly be due to chelation and removal of endogenous Ca^{2+} [28]. The stimulation of human brain guanylate cyclase by EDTA following purification of the enzyme and reaction mixture on Chelex-100 and the greater stimulation achieved with EDTA than CDTA (Fig. 1) make it highly unlikely that stimulation results from removal of free inhibitory divalent cations A direct interaction of the chelating agents with the enzyme or with a tightly bound metal on the enzyme (which is not accessible to Chelex-100 and has only limited accessibility to CDTA, perhaps because of steric hinderance) remains a viable alternative

Acknowledgements

This work was supported by Grants NIH NS-14583, NIMH MH-30324 from the U.S A. Public Health Service and by grants from the Medical Education and Research Foundation of St. Paul-Ramsey Medical Center No. 8250 and No. 8208

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