STIMULATION OF MEMBRANOUS GUANYLATE CYCLASE BY CONCENTRATIONS OF CALCIUM THAT ARE IN THE PHYSIOLOGICAL RANGE

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SUMMARY: Ca^{2+} at a concentration as low as 3 μM stimulated guanylate cyclase activity in membranes of cultured Balb 3T3 fibroblasts when Mg^{2+} was the major bivalent cation. If the enzyme was assayed with Mn^{2+} as the major cation, Ca^{2+} caused either inhibition or stimulation depending on conditions but these effects of Ca^{2+} occurred at much higher concentrations. The addition of NaCl decreased the activity with Mg^{2+} but increased the degree of stimulation by Ca^{2+}. We suggest that the activity of guanylate cyclase *in vitro* with Mg^{2+} represents its behavior *in vivo* and that Ca^{2+} regulates cyclic GMP in cells by directly stimulating guanylate cyclase activity.

INTRODUCTION

Acetyl choline, (1-7) insulin (4,6,7) and several other hormones (6,8) have bee found to raise cyclic GMP levels in animal cells, but the mechanism by which these hormones act is obscure. One factor regulating this response is the concentration of calcium. Removal of extracellular calcium prevents the rise in cyclic GMP levels produced by these agents (3,5,6-8). Not only does calcium affect the ability of hormones to control cyclic GMP levels, but calcium also controls the basal level. The basal level of cyclic GMP can be raised by increasing the extracellular calcium concentration (3) and treatment of cells with the calcium ionophore A23187 in the presence of calcium also rapidly increases cyclic GMP levels (5-7). It seems clear therefore that Ca^{2+} is a positive regulator of cellular cyclic GMP.

In cell-free extracts calcium has been reported to affect the activity of the enzymes of cyclic GMP metabolism, but the effects reported up to now are unable to account for the *in vivo* data. The activity of the cyclic GMP phosphodiesterase is increased by calcium (9); this effect should lower cyclic GMP levels. Calcium has

been reported to increase the activity of the soluble guanylate cyclase (10,11) and under the same conditions inhibit the activity of the membranous enzyme. These effects of calcium were only evident at high (millimolar) concentrations of Ca^{2+} and when Mn^{2+} was the major cation in the assay. Since the concentration of free Ca^{2+} in the cytosol is in the micromolar range (12), and the amount of Mn^{2+} in cells is two orders of magnitude less than Mg^{2+} (13), these effects of Ca^{2+} and Mn^{2+} on the activity of guanylate cyclase, are probably not relevant to how the enzyme is regulated in vivo.

We have recently reported that membranes of cultured Balb 3T3 fibroblasts con a guanylate cyclase which has high activity when assayed in the presence of Mg^{2+} and either Lubrol PX or free fatty acids (14). Here we show that when guanylate cyclase activity is assayed with Mg^{2+}, it can be stimulated by physiological concentrations of Ca^{2+}.

METHODS:

Isolation of Membranes. Balb 3T3 fibroblasts were grown in glass roller bottles, and microsomal and plasma membranes were purified as previously described (14). In preliminary experiments no difference was found between the extent of stimulation by Ca^{2+} of the guanylate cyclase in the plasma membrane fraction and in the microsomal fraction. A mixture of microsomal and plasma membranes was used, therefore, in this study. This fraction was prepared by combining the crude microsomal and plasma membrane fractions and purifying them on a Ficol cushion (14). All the guanylate cyclase activity obtained is membrane bound and could be fully recovered in a membrane pellet by diluting the sample in 50 vol of 10mM Tris-Cl pH 8.0 and resedimenting it at 100,000 g for two hr.

Guanylate Cyclase assay. The reaction mixture contained, in a final volume of 0.05 ml 50mM Tris-HCl pH 7.8, [α-^{32}P] GTP (2 - 5 x 10^6 cpm), 0.05% defatted albumin, 2mM dithiothreitol, 15 µg of creatine kinase, 5mM creatine phosphate, 2.4mM cyclic GMP, membranes (1-2 µg protein), 0.2mM GTP and Lubrol PX 5mM (1.15 mg/ml). In addition the reaction mixture contained various amounts of Mg^{2+} as indicated. The reaction was carried out at 37°, for 10 minutes and stopped with trichloroacetic acid, excess of GTP and cyclic GMP and cyclic [^{3}H] GMP (15). The cyclic [^{32}P] GMP produced was determined as previously described (14,15). In addition the product cochromatographed with authentic cyclic GMP in the following chromatographic systems: 1) 0.75M KH_2PO_4 on PEI cellulose sheets (Brinkmann) 2) 0.2M LiCl on PEI cellulose sheet. 3) Ethanol/0.5M ammonium acetate (5:2) on cellulose sheets (Eastman).

RESULTS AND DISCUSSION

Guanylate cyclase activity with Mn^{2+}, Mg^{2+}, or Ca^{2+}. The guanylate cyclase activity, in membranes of cultured Balb 3T3 fibroblasts is shown as a function of Mn^{2+}, or Mg^{2+}, or Ca^{2+} concentration in Fig. 1. In this experiment and all others in this paper the GTP concentration was 0.2mM, and Lubrol PX at 5mM was included in the assay. The

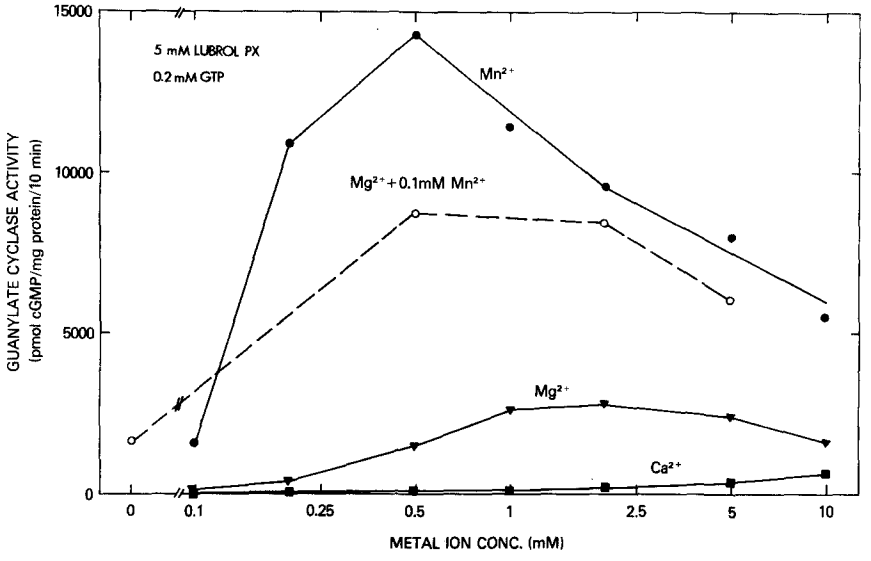

Fig. 1. Guanylate cyclase activity with various metals.

guanylate cyclase, like the enzyme from the membranes of other cells, shows highest activity with Mn^{2+} and very little activity with Ca^{2+}. The activity with Mg^{2+} is intermediate. With 0.2mM GTP the activity at the optimal Mg^{2+} concentration is about 20% of the maximal activity with Mn^{2+} (Fig. 1). We previously showed that at higher GTP concentrations the activity with Mg^{2+} approaches the activity with Mn^{2+} (14)

Mg^{2+} has three different effects on guanylate cyclase activity: 1) Up to a concentration of about 3mM,Mg^{2+} increases enzyme activity. 2) Above 3mM, Mg^{2+} inhibits. Since the dissociation constant of MgGTP is about 15 x 10^{-6}M, at the optimal Mg^{2+} concentration most of the Mg^{2+} is free. Therefore, both the stimulation and the inhibition probably occur by interaction of free Mg^{2+} ions with the enzyme. 3) Mg^{2+} strongly stimulates the activity of the enzyme when added in the presence of low and suboptimal concentrations of Mn^{2+} (Fig. 1). This stimulation may occur by the formation of MgGTP and release of free Mn^{2+}.

The Effect of Ca^{2+} on the activity in the presence of Mg^{2+}. Guanylate cyclase activity in the presence of Mg^{2+} is stimulated by low concentrations of Ca^{2+} (Fig. 2). The stimulation occurs at all Mg^{2+} concentrations tested (Fig. 2). With 0.5 mM Mg^{2+}

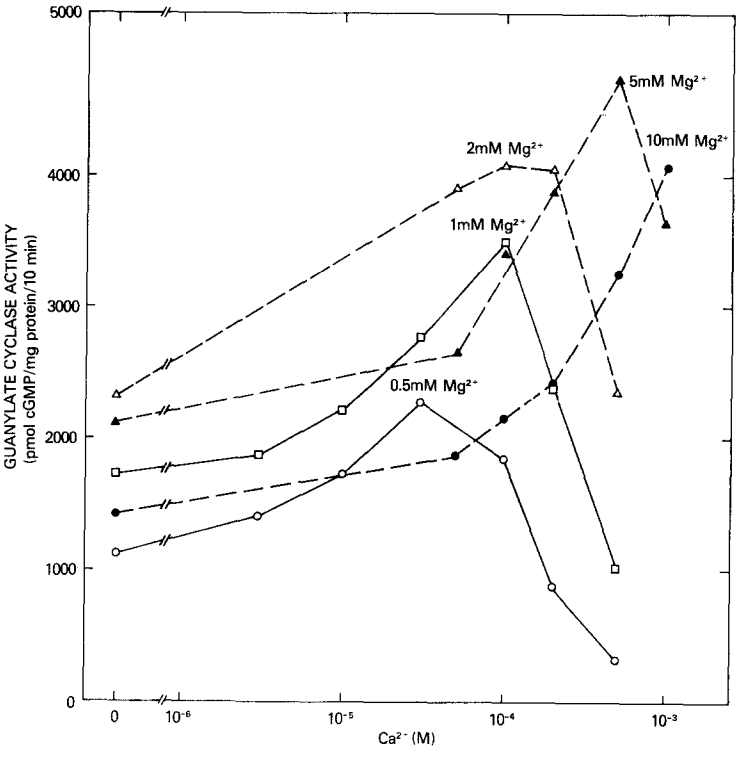

Fig. 2. Effect of varying the concentration of calcium in the presence of five different concentrations of magnesium.

there is a 25% stimulation by 3μM Ca^{2+} and about a 100% stimulation by 30 μM Ca^{2+}. Higher Ca^{2+} is inhibitory. Similar stimulation of guanylate cyclase activity in the presence of Mg^{2+} occurs with lanthanum (data not shown). As the concentration of Mg^{2+} in the assay is increased, higher concentrations of Ca^{2+} are necessary to stimulate but greater degrees of stimulation are achieved. This shift in the optimal calcium concentration might result from competition between Mg^{2+} and Ca^{2+} for common binding sites.

The effects of Ca^{2+} on the activity in the presence of Mn^{2+}. Ca^{2+} has previously been reported to affect the activity of guanylate cyclase when the enzyme was assayed in the presence of Mn^{2+} (10,11,16). The effect of increasing Ca^{2+} concentrations on guanylate cyclase activity in fibroblast membranes with

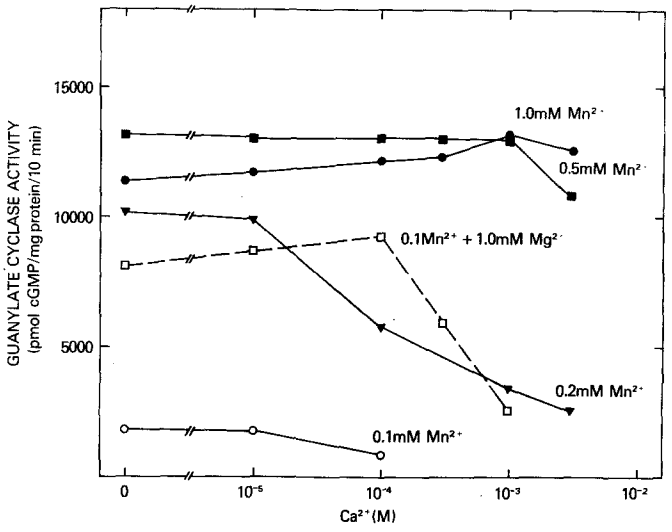

Fig. 3. Effect of varying the concentration of calcium in the presence of maganese or manganese and magnesium.

various Mn^{2+} concentrations is shown in Fig. 3. The extent and the nature of the effect of Ca^{2+} varies with the concentration of Mn^{2+}. With 0.5mM Mn^{2+} or less, Ca^{2+} causes inhibition. At higher Mn^{2+} concentrations or with low Mn^{2+} + high Mg^{2+} there is also some stimulation by Ca^{2+}, (Fig. 3). The concentrations at which Ca^{2+} stimulates or inhibits in the presence of Mn^{2+} are much higher than the concentrations at which Ca^{2+} stimulates guanylate cyclase when Mg^{2+} is present.

Effects of ionic strength on the stimulation by Ca^{2+}. The ionic composition of the guanylate cyclase assay mixture is very different from that *in vivo*. Therefore we examined whether increasing the ionic strength affected guanylate cyclase activity and the stimulatory effect of Ca^{2+}. NaCl (Table 1) as well as KCl (not shown) inhibited guanylate cyclase activity. Na_2SO_4 was more inhibitory than NaCl (Table 1). None-the-less in the presence of 100mM NaCl, Ca^{2+} was still stimulatory and on a percentage basis was even more effective. Without NaCl 0.2mM Ca^{2+} stimulated 2.6 fold; with 100mM NaCl, 0.2mM Ca^{2+} stimulated 4.2 fold. When Mn^{2+} was the sole divalent cation, NaCl and Na_2SO_4 increased enzyme activity.

The ability of Mg^{2+} to serve as the divalent cation for guanylate cyclase is not unique to the enzyme from cultured fibroblasts. The particulate guanylate cyclase

Table I: The effect of NaCl and Na_2SO_4 on guanylate cyclase activity

Addition to assay	Activity with bivalent cations in the assay		
	3mM Mg^{2+}	3mM Mg^{2+} + 0.2mM Ca^{2+}	3mM Mn^{2+}
	pmol cGMP/mg protein/10 min		
---	1700	4400	8700
NaCl 20mM	1300		10000
NaCl 50mM	700		11200
NaCl 100mM	400	1700	11500
Na_2SO_4 5mM	1100		---
Na_2SO_4 15mM	600		10600
Na_2SO_4 30mM	300		10000

of kidney medulla, when treated with Triton-X-100, shows high activity with Mg^{2+} (17). Free fatty acids (14) and azide (18) increase the activity of the enzyme with Mg^{2+}. Since both fatty acids and Mn^{2+} can increase guanylate cyclase activity, we considered the possibility that the effect of Ca^{2+} was secondary to release of these substances from the membranes used in the assay. Since only small amounts of membrane were employed and these were extensively washed with EDTA during their preparation, ver little Mn^{2+} should be present. It is also unlikely that the activation by Ca^{2+} is due to release of fatty acids; this activation would have been prevented by the defatted albumin included in the assay.

CONCLUSION

Since the activation of guanylate cyclase by calcium we observe occurs at physiological concentrations of calcium and magnesium, changes in intracellular calcium brought about by hormones or other substances may be one way cellular cyclic GMP levels are controlled.

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