Properties of Particulate, Membrane-Associated and Soluble Guanylate Cyclase from Cardiac Muscle, Skeletal Muscle, Cerebral Cortex and Liver

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1. Guanylate cyclase of washed particles and plasma membranes showed S-shaped progress curves when titrated with either GTP or Mn^{2+} ions; similar results were obtained with Triton X-100-solubilized enzyme preparation from washed particles. Hill plots of these data revealed multiple metal–nucleotide and free-metal binding sites. 2. Guanylate cyclase of supernatant fractions displayed typical Michaelis–Menten properties when titrated with GTP. 3. Both particulate (including membrane-associated) and supernatant enzyme required excess of (free) Mn^{2+} (over GTP) for maximal activities; K_a (free Mn^{2+}) was about 0.15–0.25mM at subsaturating concentrations of GTP. 4. MnATP, MnADP, and MnGDP were found to increase the activities of both particulate and superantant enzyme, when MnGTP concentration was below saturation and free Mn^{2+} ion concentration was low ($<100\,\mu$M); MnATP ($50\,\mu$M–1mM) inhibited both these activities at high free Mn^{2+} concentration (1.5mM) and inhibition of the particulate enzyme was greater than that of supernatant enzyme. 5. Ca^{2+} ions stimulated supernatant-enzyme activity; the stimulatory concentration of Ca^{2+} ions depended on the concentration of Mn^{2+} and GTP. 6. A modest stimulation of particulate guanylate cyclase by pyrophosphate (0.02–1mM) was observed; the pyrophosphate effect appeared to be competitive with respect to GTP. At a higher concentration (2mM), pyrophosphate produced a marked inhibition of particulate enzyme; the nature of inhibitory effect appeared complex. 7. Inorganic salts (e.g. NaCl, KCl, LiBr, NaF) produced inhibition of particulate enzyme; the degree of inhibition of Triton X-100-stimulated activity was less than that of unstimulated activity. 9. Treatment of sarcolemmal or microsomal membranes with either phospholipase C or trypsin decreased, whereas phospholipase A increased, the activity of guanylate cyclase.

In the preceding paper (Sulakhe *et al.*, 1976*a*) we reported that guanylate cyclase activity (EC 4.6.1.2) was present in both particulate and soluble fractions of cardiac muscle, skeletal muscle, liver and cerebral cortex. Further, plasma-membrane fractions isolated from these tissues contained significant amounts of this enzyme activity. It was therefore of interest to know whether the properties of the particulate and soluble enzyme are similar. While this study was in progress, Kimura & Murad (1974) and Chrisman *et al.* (1975) reported that guanylate cyclase of particulate fractions of rat heart and lung showed certain differences in the kinetic properties compared with the soluble enzyme from these tissues. Garbers *et al.* (1974, 1975*a*) have made some interesting observations concerning cation and nucleotide effects on sea-urchin sperm guanylate cyclase; it is noteworthy that, in this species, guanylate cyclase is entirely particulate (Gray *et al.*, 1970) and probably associated with plasma membranes (Gray & Drummond, 1973). In the present paper we describe the kinetic properties of particulate, supernatant, membrane-associated and solubilized (Triton X-100) guanylate cyclase from

contractile tissues, liver and cerebral cortex. Effects of cations (e.g. Mn^{2+}, Ca^{2+} and Mg^{2+}), nucleotides other than GTP, and pyrophosphate, were also investigated. Since we have observed that this enzyme is associated with plasma membranes, the effects of phospholipases and trypsin were studied. Some of these findings have already appeared in abstract form (Sulakhe & St. Louis, 1975; Sulakhe *et al.*, 1975).

Experimental

Materials

$[\alpha$-^{32}P]GTP (5.34–20.4Ci/mmol), $[\alpha$-^{32}P]ATP (9.23–18.0Ci/mmol) and cyclic $[^{3}$H]GMP (2.11Ci/ mmol) were purchased from New England Nuclear Corp., Montreal, Canada. Unlabelled nucleotides, nucleosides, creatine phosphate, creatine phosphokinase, and neutral alumina were purchased from Sigma Chemical Co., St. Louis, MO, U.S.A., and P.L. Biochemicals, Milwaukee, WI, U.S.A. Disposable columns (0.7cm × 4cm) were obtained from Bio-Rad Laboratories, Richmond, CA, U.S.A., and inor-

ganic salts from Fisher Scientific Co., Montreal, Que., Canada. All reagents were prepared in, and glassware and plasticware were thoroughly rinsed with, de-ionized glass-distilled water.

Methods

Isolation of various fractions. Washed particulate and supernatant fractions from rabbit heart ventricles were isolated as described in the preceding paper (Sulakhe *et al.*, 1976*a*). Sarcoplasmic reticulum from rabbit heart was isolated by the method of Sulakhe & Dhalla (1970*a,b*); sarcolemma and sarcoplasmic reticulum from rabbit leg muscle were isolated as described by Sulakhe *et al.* (1973). Plasma membranes from rat liver were isolated by a modification of the method of Smigel & Fleischer (1974) as described in the preceding paper (Sulakhe *et al.*, 1976*a*). Plasma membrane-enriched fractions from guinea-pig heart ventricles were isolated by the procedure of Sulakhe *et al.* (1976*b*). A synaptic-plasma-membrane-enriched fraction from rat cerebral cortex was isolated by the method of Jones & Matus (1974). In most studies, fractions isolated from these tissues were used within 1 h of their isolation. Marker-enzyme activities of these membrane fractions were determined periodically as described in the preceding paper (Sulakhe *et al.*, 1976*a*).

Guanylate cyclase assay. Details of the guanylate cyclase assay are given in the preceding paper (Sulakhe

et al., 1976*a*). Variations in the assay protocol are specified with the appropriate Figure.

Protein determination. Protein was determined by the method of Lowry *et al.* (1951), bovine serum albumin being used as a standard.

Results

Titration with GTP

Guanylate cyclase activity increased in rectangular hyperbolic fashion for the supernatant fraction, whereas that of the particulate fraction showed S-shaped kinetic profiles, when the total GTP concentrations (0.05–0.4mM) in the assay mixture were increased; these patterns were observed at various fixed total Mn^{2+} concentrations (0.25, 0.50, 1.0 and 2.0mM) in the assay. These findings were obtained with fractions isolated from rabbit heart ventricles and skeletal muscle, rat liver and rat cerebral cortex (results not shown). The Triton X-100-solubilized enzyme from the particulate fraction displayed S-shaped progress curves; the plots of reciprocal of velocity against the squared reciprocal of the substrate were linear, whereas the Hill plots revealed multiple GTP-binding sites. Mn^{2+} ions increased the velocity of the reaction for both the particulate (including solubilized) and the soluble enzyme, although Mn^{2+} ions decreased the second-order dissociation constant for GTP of the particulate enzyme (results not shown).
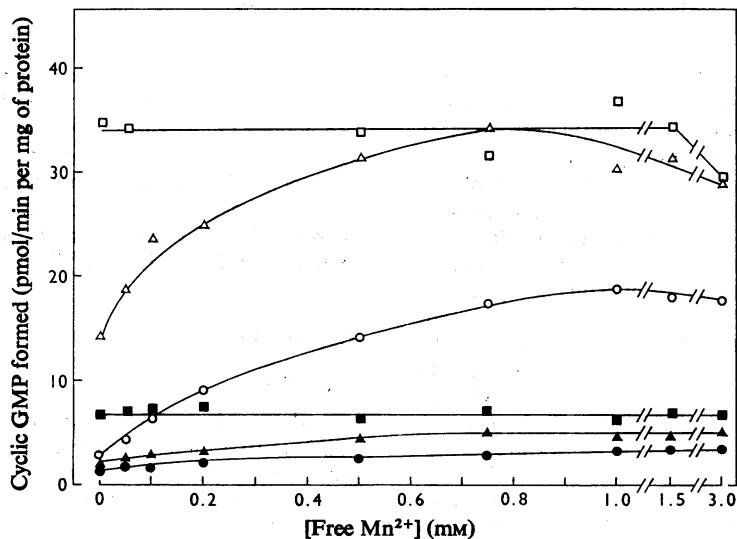

Fig. 1. *Titration with free Mn^{2+} ions*

Washed particles (□, △, ○) and supernatant fluid (■, ▲, ●) obtained from heart ventricles (both containing 127μg of protein) were incubated under standard assay conditions except that the MnGTP (initial) concentrations were 0.174 (○, ●), 0.38 (△, ▲) and 1.0mM (□, ■) and free Mn^{2+} concentrations were varied as shown.

Titration with Mn²⁺ ions

Guanylate cyclase of various particulate fractions from these tissues displayed S-shaped progress curves when the total Mn^{2+} concentrations in the assay were varied (total GTP held constant); Hill plots of these data revealed h values ranging from 1.35 to 1.75 (results not shown). Guanylate cyclase of plasma membranes from these tissues also showed similar progress curves (results not shown).

Titration with free Mn²⁺ ions

The experiments described above were carried out with various amounts of GTP and Mn^{2+} ions in the assay mixture. An $MnGTP^{2-}$ species would predominate at the pH of these assays. However, free Mn^{2+} and GTP^{4-} concentrations were variable in these assays, and this complicates interpretation of these data. To gain further insight into the kinetic behaviour of guanylate cyclase, experiments were carried out in which $[MnGTP^{2-}]$ was held constant and the free $[Mn^{2+}]$ was varied. The results are shown in Fig. 1. Interestingly, when MnGTP was kept at 0.174 and 0.38 mM, the particulate-enzyme activity increased in a rectangular hyperbolic fashion with increasing free
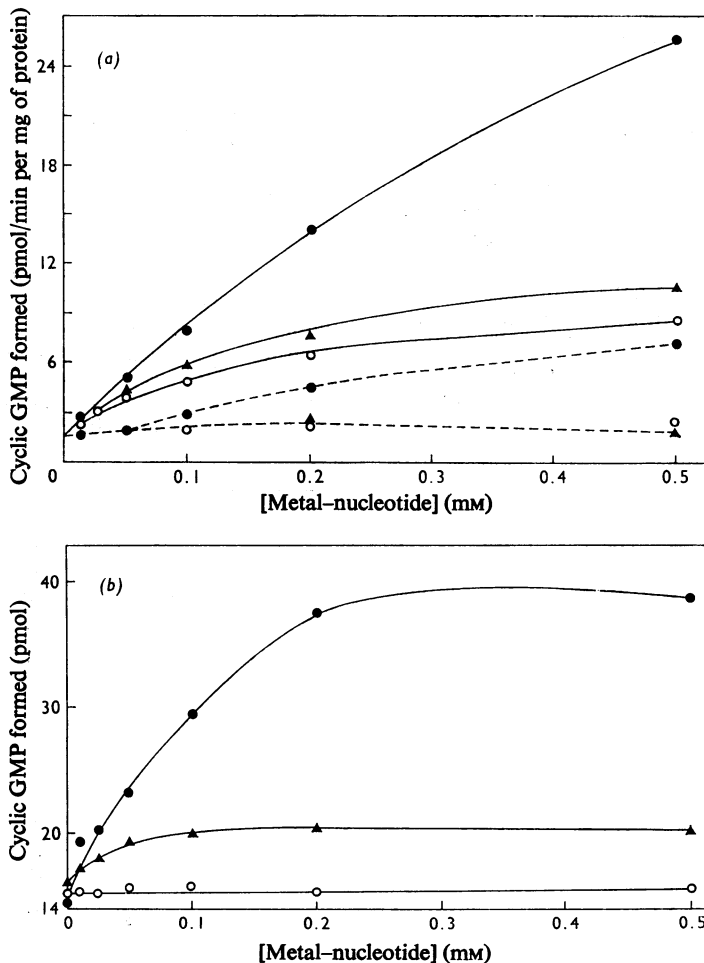

Fig. 2. *Effect of nucleotides*

(a) Washed particles (169 μg, ———) and supernatant fluid (99 μg, - - - -) obtained from heart ventricles were incubated under standard assay conditions except that initial MnGTP concentration was 257 μM and the free Mn^{2+} concentration was 75 μM. (b) Washed particles (122 μg) were incubated for 10 min under standard assay conditions except that initial MnGTP concentration was 262 μM and Mn^{2+} (free) was 20 μM. In both (a) and (b) Mn–nucleotides were added as shown: ●, MnGTP; ○, MnADP; ▲, MnATP. Triton X-100 (1%) was also present.

Mn^{2+} ions in the assay; on the other hand, when MnGTP was held at 1 mM, no change in activity was demonstrable with increasing free Mn^{2+} ions (up to 1.5 mM); higher concentrations of Mn^{2+} ions were, as expected, inhibitory. The soluble enzyme revealed similar properties, except that the high Mn^{2+} concentration (3 mM, free) was not inhibitory, as shown previously (Sulakhe *et al.*, 1976*b*). It is evident that free Mn^{2+} ions are necessary for maximal activity as long as the enzyme is not saturated with MnGTP. From these studies and those described above, it can be concluded that a free-metal site as well as nucleotide-binding sites exist on the enzyme (both particulate and soluble); further, both of these sites must be occupied for the maximal activity. In the case of the particulate (or membrane-associated) enzyme, multiple nucleotide-binding sites are apparently present.

Effect of other nucleotides

Under defined assay conditions (Fig. 2*a*), guanylate cyclase activity of particles was increased by MnATP, MnADP and MnGDP; all were tested between 10 and 500 μM. In this experiment, soluble enzyme was stimulated by MnGDP, although to a smaller extent than the particulate enzyme; both MnATP and MnADP showed only minimal stimulatory effects. When the free-Mn^{2+} concentration was decreased to 20 μM (Fig. 2*b*), MnADP was ineffective in stimulating the particulate enzyme, although stimulation by MnATP and MnGDP were observed. By contrast, when free-Mn^{2+} ion concentration was 1.5 mM (Fig.

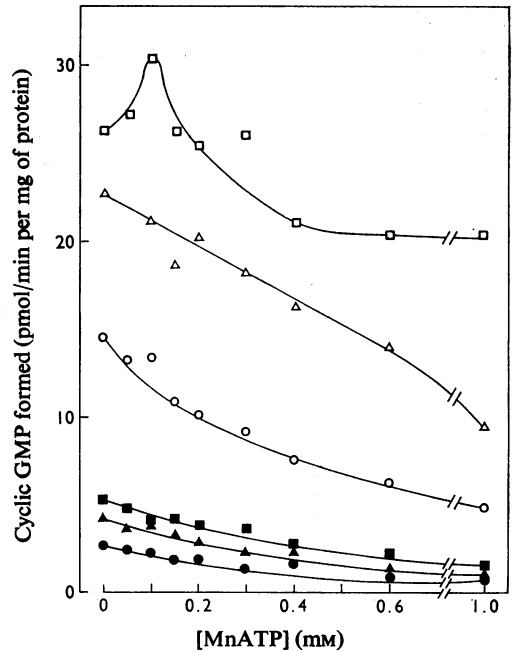Fig. 3. *Titration with MnATP*

Washed particles (112 μg; □, △, ○) and supernatant fluid (130 μg; ■, ▲, ●) obtained from heart ventricles were incubated under standard assay conditions except that initial MnGTP concentrations were either 0.17 mM (○, ●), 0.40 mM (△, ▲) or 0.98 mM (□, ■); the free Mn^{2+} concentration was 1.5 mM and MnATP was varied as shown.
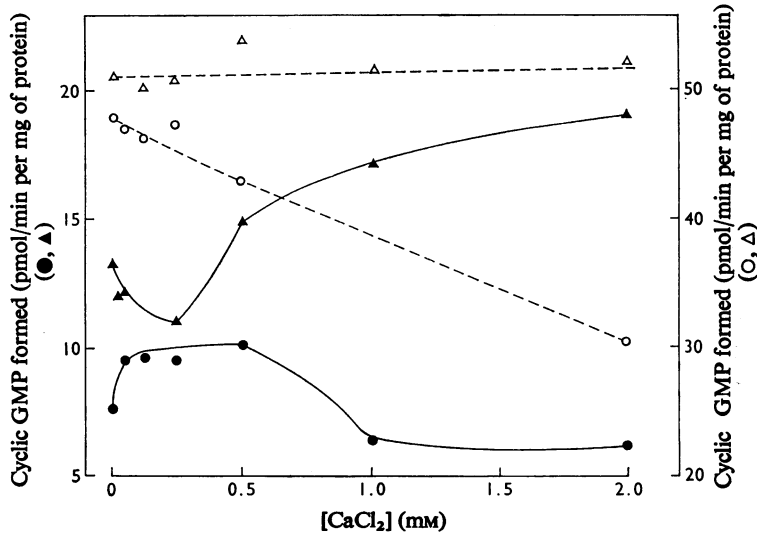

Fig. 4. *Effect of Ca^{2+} ions*

Washed particles (118 μg, - - - -) and supernatant fluid (124 μg, ——) obtained from heart ventricles were incubated under standard assay conditions, except that 0.4 mM-GTP (total) was present and the total Mn^{2+} concentrations were either 0.5 (●, ○) or 3.0 mM (▲, △).

3), MnATP (50 μM–1 mM) inhibited both particulate- and supernatant-enzyme activities. MnATP (1 mM) inhibited the soluble enzyme by about 70–80 %, when assayed at three fixed concentrations of MnGTP (0.17, 0.40 and 0.98 mM); inhibition by MnATP of the particulate enzyme appeared to depend on the
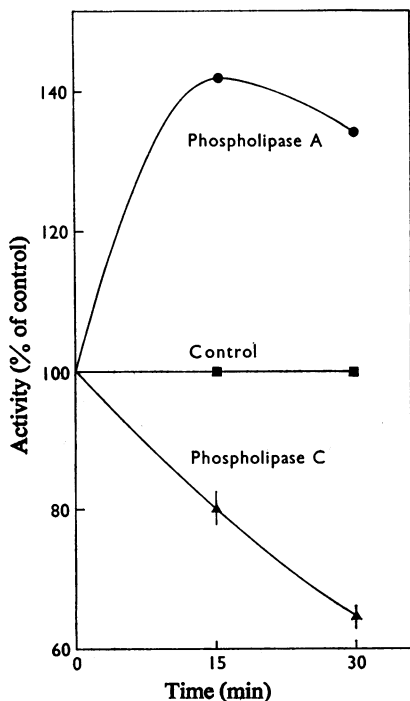

Fig. 5. *Effect of phospholipase A (●) and phospholipase C (▲) treatments*

(a) Phospholipase A treatment: muscle sarcolemmae (2.41 mg) were incubated at 30°C in total volume of 1.3 ml containing 0.25 M-sucrose, 0.2 mM-dithiothreitol, 10 mM-Tris/HCl, pH 7.0, and 200 μg of snake (*Naja naja*) venom (treated at 70°C for 15 min to destroy proteinase activity) in the presence of 1.0 mM-CaCl$_2$. Digestion was terminated by the addition of EGTA [ethanedioxybis(ethylamine)tetra-acetic acid] (3.3 mM, final concn.) and placing the tubes on ice. Control tubes were incubated simultaneously without snake venom. Guanylate cyclase was determined under standard assay conditions except that CaCl$_2$ (0.27 mM), EGTA (0.89 mM), Mn^{2+} (3.0 mM) and GTP (0.8 mM) were present. (b) Phospholipase C treatment: muscle sarcol-emmae (2.41 mg) were incubated as described above in the presence of 200 μg of lecithinase C (Sigma) and 0.4 mM-CaCl$_2$. Reaction was terminated by the addition of EGTA (1.6 mM, final concn.) and placing the tubes on ice. Control tubes without lecithinase C addition were incubated simultaneously. The guanylate cyclase assay was carried out under standard assay conditions, except that CaCl$_2$ (0.08 mM), EGTA (0.32 mM), Mn^{2+} (3.0 mM) and GTP (0.8 mM) were present.

initial MnGTP concentration. For example, 1 mM-MnATP inhibited particulate activity by 70, 60 and 25 % at 0.17, 0.40 and 0.98 mM-MnGTP respectively. These findings suggest that other nucleotides can activate or inhibit enzyme activity, depending on the assay conditions.

Effect of calcium ions

By using rabbit cardiac-muscle supernatant frac-tion, we observed that the enzyme activity was stimu-lated by Ca^{2+} ions, although very modestly (Fig. 4); the stimulating action of Ca^{2+} ions depended on the Mn^{2+} concentration, i.e. 0.05 mM-Ca^{2+} ions stimu-lated, whereas at 1–2 mM they inhibited the enzyme, when Mn^{2+} was 0.5 mM. In the presence of 3 mM-Mn^{2+}, stimulation of the supernatant enzyme was apparent between 0.5 and 2 mM-Ca^{2+}. With the particulate fraction, this enzyme activity was inhibited steadily with increasing calcium concentrations, when 0.5 mM-Mn^{2+} was present; however, when the Mn^{2+} concen-tration was 3 mM, no significant effect (inhibitory or stimulatory) was noted with Ca^{2+} (0.05–2 mM). Stimu-lation of the supernatant enzyme activity by 3 mM-Ca^{2+} in the presence of various Mn^{2+} ion concentra-tions (0.2–2.0 mM) was also observed at several GTP concentrations (0.012–0.3 mM) (results not shown).

Effect of phospholipases and trypsin

Fig. 5 shows that treatment of isolated sarcolem-mal membranes (rabbit leg muscle) with phospho-lipases C and A decreased (35 %) and increased (40 %) respectively the guanylate cylase activity of this fraction. Triton X-100 was present in these assays, hence a detergent-like effect of free fatty acids (liberated during phospholipase A digestion) cannot account for these observations; further, excess of de-fatted albumin was also present during phospho-lipase A treatment. Trypsin treatment (Fig. 6) inac-tivated guanylate cyclase of cardiac microsomal fractions and skeletal-muscle sarcolemmal mem-branes; the trypsin inhibitor had no effect.

Effect of pyrophosphate

A moderate stimulation of particulate guanylate cyclase was observed in the presence of pyrophos-phate (0.02–1.0 mM). The action of pyrophosphate (1 mM) was found to be competitive with respect to the substrate (GTP). Guanylate cyclase of skeletal-muscle sarcolemma was inhibited significantly by 2 mM-pyrophosphate. However, this inhibitory effect was not competitive in nature and appeared to be more complex (result not shown).

Effect of NaCl, KCl, NaF and LiBr

Cardiac particulate guanylate cyclase activity (minus Triton X-100 in the assay) was inhibited (30 to 80 %) by high concentrations (50–500 mM) of salts such as NaCl, KCl, NaF and LiBr. The Triton X-100-
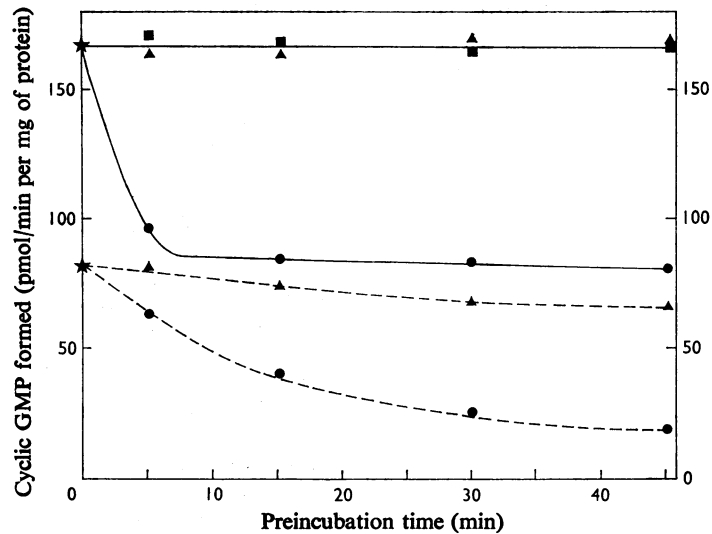
Fig. 6. *Effect of trypsin treatment*

Cardiac microsomal fractions (1.12 mg; ———) or skeletal-muscle sarcolemmae (0.70 mg; –––––) were incubated at 30°C in total volume of 0.4 ml containing 0.25 M-sucrose, 0.2 mM-dithiothreitol, 10 mM-Tris/HCl, pH 7.0, in the presence of either trypsin (60 μg of protein; ●) or trypsin inhibitor (300 μg; ▲). At the end of the desired incubation time, trypsin inhibitor (300 μg) was added to the experimental tubes (trypsin-treated), and the tubes were chilled. Control tubes (■), in which water was added instead of trypsin or trypsin inhibitor, were incubated simultaneously. Guanylate cyclase assays were carried under standard assay conditions except that Mn^{2+} was 2 mM and GTP was 0.6 mM.

stimulated activity was inhibited to a smaller extent. Stimulation of guanylate cyclase (with or without Triton X-100 in the assay) by NaF (5–100 mM) was not observed. In these particles, adenylate cyclase was present (80 pmol/min per mg at 37°C) and was stimulated threefold with 8 mM-NaF. This finding suggests that adenylate cyclase activity was not contributing to the observed cyclic GMP synthesis when GTP was used as a substrate.

Discussion

Guanylate cyclase from several tissues is present in both particulate and supernatant fractions and, although both require Mn^{2+} for activity, they exhibit several differences, e.g. number of nucleotide-binding sites, effects of Ca^{2+}, temperature and nucleotides, and activation by non-ionic detergents. Kimura & Murad (1974) and Chrisman et al. (1975) have suggested that two different forms of guanylate cyclase are present in rat heart and lung. Our results suggest that this enzyme is mainly present in the plasma membrane and may be partly released on homogenization.

Guanylate cyclase activity of the original washed particles is likely to be due to the presence of plasma membranes in these fractions. For example, multiple

nucleotide-binding sites and a free-metal site were present on both membrane-associated and particulate enzymes. Kinetic data obtained with such fractions, however, must be interpreted with caution. Partially purified guanylate cyclase from rat lung soluble fraction (Chrisman et al., 1975) and the supernatant enzyme of the present study showed similar properties.

Stimulation of the particulate enzyme activity with MnATP is noteworthy. It has been shown in a number of laboratories that the particulate adenylate cyclase activity, especially its responsiveness to hormones, can be enhanced by GTP and other guanine nucleotides (Rodbell et al., 1975). Whether ATP and other nucleotides function to 'couple' stimulation of the plasma-membrane receptor (cholinergic) to the catalytic site of guanylate cyclase remains to be answered. Limbird & Lefkowitz (1975) have failed to observe any effect of cholinergic agents on cardiac particulate enzyme in the absence and presence of other nucleotides; however, these investigators could not observe a stimulatory effect of ATP on guanylate cyclase. The latter finding is not surprising, since stimulation of this enzyme by ATP can be shown only under certain assay conditions (such as very low [free Mn^{2+}] and low [MnGTP]) (Fig. 2). Garbers et al. (1974) have reported con-

ditions for ATP (and other nucleotides) stimulation of sea-urchin sperm enzyme activity.

Guanylate cyclase catalyses the reaction, GTP → cyclic GMP + PP_i, as shown by Garbers *et al.* (1975*a*) for the soluble lung enzyme. Pyrophosphate ions caused a modest stimulation of sarcolemmal guanylate cyclase in contrast with its reported inhibitory action on adenylate cyclase (Severson *et al.*, 1972).

Many membrane-bound enzymes exhibit dependency on membrane components for full activities: adenylate cyclase and ouabain-sensitive $Na^+ + K^+$-dependent adenosine triphosphatase fall into this category. Numerous reports have shown that both of these enzymes can be inactivated by phospholipase C, lipid removal or trypsin treatment (Skou, 1965, Rodbell *et al.*, 1975). Guanylate cyclase also appeared to be inactivated by such treatments. On the other hand, phospholipase A treatment activates NaF-stimulated adenylate cyclase of rat liver plasma membranes (Pohl *et al.*, 1971) and of cardiac sarcotubular membranes (Sulakhe & Dhalla, 1973); this was also observed for sarcolemmal guanylate cyclase (Fig. 5).

Reports show that many inorganic salts are capable of activating adenylate cyclase (Dousa & Hechter, 1970; Dousa, 1972; Tada *et al.*, 1975). We have previously observed the stimulation of cardiac sarcotubular adenylate cyclase by Na^+ or K^+ ions (P. V. Sulakhe & N. S. Dhalla, unpublished work). Guanylate cyclase of cardiac washed particles, or particulate fractions of other tissues, was inhibited by high salt concentrations. The significance of this observation is not clear at present.

From the present findings it is difficult to discern how guanylate cyclase activity might be regulated within the cell. Although Mn^{2+} ions appeared to catalyse the reaction most effectively, the content of these ions within cells is very much lower than that required for the effect *in vitro*. It is possible that Mn^{2+} ions are an integral part of this enzyme, and that Ca^{2+} or Mg^{2+} ions modulate its activity in the cell. At the same time other nucleotides such as ATP, ADP and GDP could influence the catalytic reactivity and hence their regulatory role cannot be ignored. Obviously, further work is required to clarify the physiological properties and regulation of guanylate cyclase.

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