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# PARTICULATE GUANYLATE CYCLASE OF SKELETAL MUSCLE

## EFFECTS OF $Ca^{2+}$ AND OTHER DIVALENT CATIONS ON ENZYME ACTIVITY

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## Summary

The properties of particulate guanylate cyclase (GTP pyrophosphate-lyase (cyclizing), EC 4.6.1.2) from purified rabbit skeletal muscle membrane fragments were studied. Four membrane fractions were prepared by sucrose gradient centrifugation and the fractions characterized by analysis of marker enzymes. Guanylate cyclase activity was highest in the fraction possessing enzymatic properties typical of sarcolemma, while fractions enriched with sarcoplasmic reticulum had lower activities. In the presence of suboptimal $Mn^{2+}$ concentrations, $Mg^{2+}$ stimulated particulate guanylate cyclase activity both before and after solubilization in 1% Triton X-100. Guanylate cyclase activity was biphasic in the presence of $Ca^{2+}$. Increasing the $Ca^{2+}$ concentration from $10^{-8}$ to $10^{-5}$ M decreased the specific activity. As the $Ca^{2+}$ concentration was further increased to $5 \cdot 10^{-4}$ M enzyme activitiy again increased. After solubilization of the membranes in 1% Triton X-100, $Ca^{2+}$ suppressed enzyme activity. Studies utilizing ionophore X537A indicated that the altered effect of $Ca^{2+}$ upon the solubilized membranes was independent of asymmetric distribution of $Ca^{2+}$ and $Mg^{2+}$.

## Introduction

Contractile processes of muscular tissues are regulated by the intracellular distribution of $Ca^{2+}$. The myofibrils of muscle cells contract when the free $Ca^{2+}$ concentrations of the sarcoplasm reaches $10^{-6}$—$10^{-5}$ M [1]. Recently,

Abbreviations: Hepes, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N,N'*-tetraacetic acid.

evidence has accumulated that cyclic nucleotides, cyclic AMP and cyclic GMP, are involved in modifying the response of myofibrils to $Ca^{2+}$ as well as regulating the availability of $Ca^{2+}$ [2—5]. While a specific role for cyclic AMP has been postulated, a direct relationship between cyclic GMP levels and changes in muscle tension has not been shown. In fact, with appropriate stimuli, increases in cyclic GMP concentrations have been associated with both contraction and relaxation [6—10]. Tissue levels of cyclic GMP are determined by the relative activities of guanylate cyclase (GTP pyrophosphate-lyase (cyclizing), EC 4.6.1.2), phosphodiesterase (3′:5′-cyclic-AMP 5′-nucleotido-hydrolase, EC 3.1.4.17), and secretion into the extracellular space [11]. There appears to be both a soluble and a particulate form of guanylate cyclase with distinct biochemical characteristics [12,13]. Effects of divalent cations on guanylate cylase activity have been studied in some detail and the activities of both the soluble and particulate forms of the enzyme have been shown to be optimal in the presence of supraphysiological concentrations of $Mn^{2+}$. $Ca^{2+}$ has generally been found to stimulate soluble guanylate cyclase and has, in most systems, an inhibitory effect upon the particulate enzyme [12—14]. However, occasional reports have demonstrated a stimulation of particulate guanylate cyclase by $Ca^{2+}$ [15,16].

In the present report we show that guanylate cyclase of a sarcolemma-enriched fraction derived from rabbit skeletal muscle is affected by $Ca^{2+}$ in a complex manner dependent upon the specific concentrations of $Ca^{2+}$, $Mg^{2+}$, and $Mn^{2+}$. In addition, solubilization of the particulate enzyme dramatically alters the response to $Ca^{2+}$. This may help to explain some of the previous inconsistencies in the literature concerning the effect of $Ca^{2+}$ upon particulate guanylate cyclase.

## Methods

*Preparation of membranous fractions.* Membranous fractions were prepared by zonal centrifugation of rabbit skeletal muscle homogenates as previously described [17] with the following modifications: 50 g ground muscle, were homogenized in 250 ml 0.3 M sucrose containing 5 mM maleate/10 mM Tris (pH 7.1) at 0°C for 45 s in a Waring Blendor. The homogenate was centrifuged at 6000 rev./min for 10 min in a HS-4 rotor (Sorvall Model RC 2-B centrifuge). Membranes present in the supernatant fraction were then enriched, purified and resolved into fractions of differing buoyant density by three subsequent zonal centrifugations. Fractions with buoyant densities corresponding to 23—28% (Fraction I), 28—31% (Fraction II), 31—38% (Fraction III) and 38—43% (Fraction IV) were recovered from the final gradient. The four fractions were diluted with an equal volume of 1.2 M KCl in 5 mM Hepes (pH 7.4) to remove small amounts of remaining extraneous muscle proteins. After standing for 1 h on ice, membranes were pelleted by centrifugation for 90 min at 130 000 $\times g$, resuspended in a small volume of 0.3 M sucrose, 1 mM Hepes (pH 7.4) quick-frozen, and stored at −70°C.

*Assay of guanylate cyclase activity.* Guanylate cyclase activity was determined by measuring the conversion of GTP to cyclic GMP by the method of Kimura and Murad [18] with minor modifications. The reaction mixture had a

total volume of 0.150 ml and contained 50 mM Tris-HCl (pH 7.8), 0.1 mM isobutylmethylxanthine, 20 μg creatine phosphokinase (140 units/mg), 15 mM phosphocreatine, 1 mM GTP, 2—8 μg protein, and $MnCl_2$, $MgCl_2$ and $CaCl_2$ in varying concentrations, as specified in the individual experiments. The constitutents were preincubated at 37°C for 10 min in the absence of GTP. The reaction was initiated by the addition of 1 mM GTP, incubated at 37°C for 5, 10, or 15 min and terminated by the addition of 0.9 ml 0.05 M sodium acetate (pH 4.0) followed by heating at 90°C for 2 min. All samples were run in triplicate. The cyclic GMP generated in the reaction was measured by the radioimmunoassay as described by Steiner et al. [19]. In experiments where $Mg^{2+}$ or $Ca^{2+}$ were present, samples were acetylated by the procedure of Harper and Brooker [20]. While these divalent cations can alter the affinity of the antibody for cyclic GMP in the assay as originally described, no such effect can be demonstrated if the samples are acetylated prior to addition to the radioimmunoassay (Levine, S.N. and Steiner, A.L., unpublished data). When samples were solubilized, rabbit skeletal muscle membranous fractions were incubated for 1 h at 4°C in 1% Triton X-100 prior to addition to the guanylate cyclase assay. Cyclic GMP production was linear with respect to time and protein concentrations. The standard errors were less than 10% for a given preparation.

Proteins were measured by the method of Lowry et al. [21]. Adenylate cyclase was determined by the method of Salomon et al. [22], leucine aminopeptidase by that of Goldberg and Rutenberg [23] and [$^{32}$P]phosphoenzyme as described by Meissner [17].

## Results

*Localization of guanlyate cyclase*

When particulate guanylate cyclase activity was measured under optimal conditions (3.0 mM $Mn^{2+}$ and 1.0 mM GTP) the specific activity was highest in the membranous fraction with the lowest buoyant density, Fraction I (Table I). Fractions II—IV had lower activities. Adenylate cyclase activity and leucine aminopeptidase activity, two enzymes generally accepted to be associated with surface membranes [24,25] were also highest in Fraction I, suggesting that particulate guanylate cyclase was mainly localized to the sarcolemma. All fractions were capable of forming a $^{32}$P-labeled phosphoenzyme intermediate (Table I), had a $Ca^{2+}$-stimulated ATPase activities and could accumulate $Ca^{2+}$. These three activities are typically associated with sarcoplasmic reticulum [17]. Fraction I had a lower content of [$^{32}$P]phosphoenzyme than Fractions II—IV and since [$^{32}$P]phosphoenzyme content correlates with $Ca^{2+}$-ATPase protein concentration of the preparations [17], it appears that Fraction I contained other membranous fractions such as sarcolemma. As previously reported, contamination of the four fractions with inner and outer mitochondrial membrnase was low [17].

Solubilization of the membranes with 1% Triton X-100 resulted in an 8—15 fold stimulation of guanylate cyclase activity (Table II). After solubilization, activity remained highest in Fraction I compared to Fractions II—IV. All subsequent experiments, unless otherwise stated, were therefore performed with Fraction I.

TABLE I

ENZYMATIC PROPERTIES OF MEMBRANOUS FRACTIONS ISOLATED FROM RABBIT SKELETAL MUSCLE

Membranous fractions were isolated from rabbit skeletal muscle and assayed for enzymatic activity. Guanylate cyclase activity was assayed in the presence of 3.0 mM $MnCl_2$ and 1.0 mM GTP. Adenylate cyclase activity was measured in the presence of 10 mM NaF. The data are the average of at least three determinations ± S.E.

| Fraction | Buoyant density (% sucrose) | Guanylate cyclase (pmol/mg protein per min) | Adenylate cyclase (pmol cyclic AMP/mg protein per min) | Leucine amino-peptidase (nmol/ mg protein per min) | $^{32}$P-labelled phos-phoenzyme (nmol $^{32}$P/mg protein) | Yield (mg protein per 1000 g muscle) |
|---|---|---|---|---|---|---|
| I | 23—28 | 31.01 ± 2.64 | 210 ± 50 | 37 ± 10 | 2.5 ± 0.6 | 20 ± 10 |
| II | 28—31 | 7.42 ± 0.20 | 60 ± 20 | 7 ± 4 | 4.9 ± 0.7 | 100 ± 50 |
| III | 31—38 | 4.53 ± 0.27 | 12 ± 8 | 2.4 ± 1 | 4.5 ± 0.7 | 500 ± 150 |
| IV | 38—43 | 5.62 ± 0.58 | 8 ± 5 | 3.0 ± 2 | 4.1 ± 0.5 | 75 ± 25 |

TABLE II

EFFECTS OF $MnCl_2$ AND $MgCl_2$ ON FRACTION I GUANYLATE CYCLASE ACTIVITY

Guanylate cyclase activity of Fraction I (0.134 mg protein/ml) was determined in the presence of 1.0 mM GTP, and the indicated concentrations of $MnCl_2$ and $MgCl_2$. Samples assayed after solubilization were preincubated in 1% Triton X-100 for 60 min at 4°C.

| Additions | pmol cyclic GMP/mg protein per min | |
|---|---|---|
| | Fraction I | Fraction I after 1% Triton X-100 |
| 0.1 mM $MnCl_2$ | 0.30 ± 0.10 | 25.91 ± 3.46 |
| 3.0 mM $MgCl_2$ | 0.50 ± 0.06 | 105.04 ± 5.47 |
| 0.1 mM $MnCl_2$ plus 3.0 mM $MgCl_2$ | 3.39 ± 0.29 | 244.50 ± 19.72 |
| 3.0 mM $MnCl_2$ | 27.89 ± 3.31 | 229.46 ± 36.45 |

*$Mn^{2+}$ and $Mg^{2+}$ requirements*

When $Mn^{2+}$ (0.1—6.0 mM) was provided as the sole divalent cation, the optimal concentration for Fraction I was 3.0 mM. A similar pattern of $Mn^{2+}$-dependence was evident when Fractions II—IV were tested. Table II compares the $Mn^{2+}$ and $Mg^{2+}$ requirement of guanylate cyclase activity in both particulate and solubilized samples. Incubation with 3.0 mM $Mg^{2+}$ alone or 0.1 mM $Mn^{2+}$ alone resulted in minimal activity of the particulate fraction. However, when both cations were added together marked synergism was observed with the resulting activity exceeding, by more than 4-fold, the activities seen in the presence of either cation alone. Solubilization of the membrane in 1% Triton X-100 markedly stimulated the activity. Again, the effect of 0.1 mM $Mn^{2+}$ and 3.0 mM $Mg^{2+}$ was more than additive, in this case yielding a specific activity equal to that seen under optimal conditions (3.0 mM $Mn^{2+}$).

Effects of $Mn^{2+}$ and $Mg^{2+}$ on guanylate cyclase were further investigated by maintaining the concentration of one of the cations at a constant level while the concentration of the other was varied. Fig. 1 shows the results of an experiment in which the $Mn^{2+}$ concentration was held constant at 0.1 mM and the $Mg^{2+}$ concentration was varied. There was a progressive increase in activity of guanylate cyclase as the $Mg^{2+}$ concentration was increased, reaching a maximum activity at 3.0 mM $Mg^{2+}$. Higher $Mg^{2+}$ concentrations were inhibitory. Fig. 2 demonstrates the effects of varying $Mn^{2+}$ concentrations at a fixed $Mg^{2+}$ concentration of 3.0 mM. The specific activity increased as the $Mn^{2+}$ was increased from $10^{-6}$ to $5 \cdot 10^{-4}$ M and then declined as the $Mn^{2+}$ concentration was raised to higher levels. These results would suggest a potential mechanism for the regulation of in vivo enzyme activity by variations in the intracellular $Mg^{2+}$ concentrations in the presence of small, but perhaps physiologic, $Mn^{2+}$ concentrations.

*Effects of $Ca^{2+}$ on the particulate enzyme*

When $Ca^{2+}$ (1.0—3.0 mM) was provided as the sole cation no activity was detected. When enzyme activity was measured in the presence of 0.1 mM $Mn^{2+}$ and 3.0 mM $Mg^{2+}$, increases in $Ca^{2+}$ concentration in the assay medium from 10 to 500 $\mu$M resulted in a progressive increase in specific activity (Fig. 3). At $Ca^{2+}$ concentrations greater than 0.5 mM a decline in activity was noted. The lower

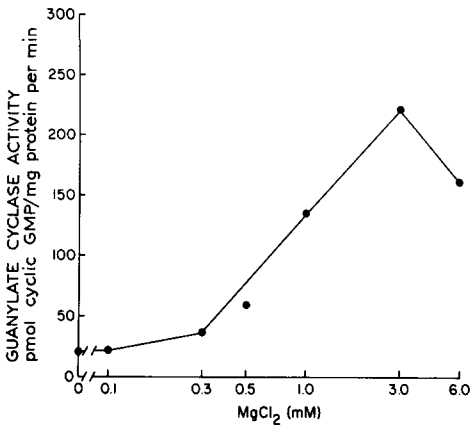


Fig. 1. Effect of varying $MgCl_2$ concentrations on guanylate cyclase activity in the presence of 0.1 mM $MgCl_2$. Fraction I membranes (0.067 mg protein/ml), solubilized in 1% Triton X-100 for 1 h at 4°C, were assayed for guanylate cyclase activity in the presence of 0.1 mM $MnCl_2$ and varying concentrations of $MgCl_2$. GTP was present at a final concentration of 1.0 mM.

limit of $Ca^{2+}$ (10 $\mu$M) was determined by endogeneous $Ca^{2+}$ concentration of the sample as measured by atomic absorption spectroscopy. In the presence of 3.0 mM $Mn^{2+}$, the activity (25 pmol/mg per min) was independent of the $Ca^{2+}$ concentration (10 $\mu$M to 3.0 mM).

The concentration of free $Ca^{2+}$ in sarcoplasm is thought to fluctuate between $10^{-7}$ and $5 \cdot 10^{-5}$ M, thereby controlling muscle relaxation and contraction. $Ca^{2+}$ concentrations in the assay medium were decreased to $10^{-7}$ M and less by the addition of EGTA. Since EGTA also complexes $Mn^{2+}$ with an affinity comparable to $Ca^{2+}$ these experiments were carried out in the presence of 3.0 mM $Mg^{2+}$ without added $Mn^{2+}$. Under these conditions, we found that guanylate

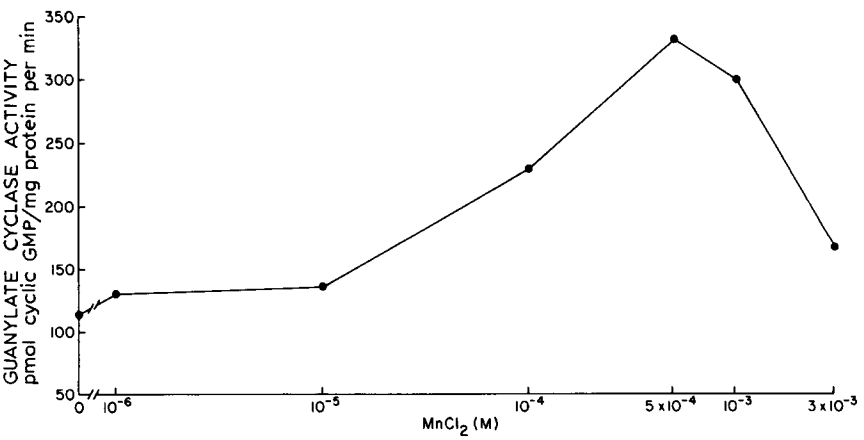


Fig. 2. Effect of varying $MnCl_2$ concentrations on guanylate cyclase activity in the presence of 3.0 mM $MgCl_2$. Fraction I membranes (0.067 mg protein/ml), solubilized in 1% Triton X-100 for 1 h at 4°C, were assayed for guanylate cyclase activity in the presence of 3.0 mM $MgCl_2$ and varying concentrations of $MnCl_2$. GTP was present at a final concentration of 1.0 mM.

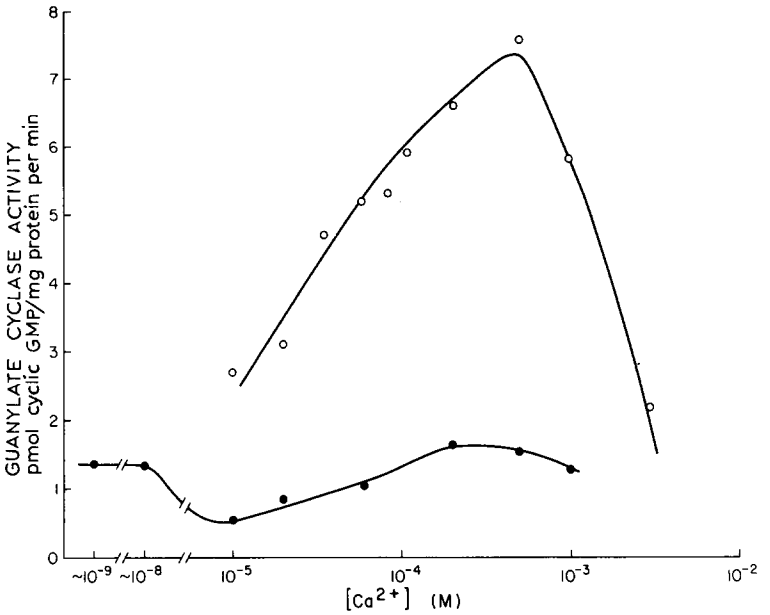


Fig. 3. Effect of $Ca^{2+}$ on Fraction I particulate guanylate cyclase. Guanylate cyclase activity was determined in the presence of 0.1 mM $MnCl_2$ and 2.0 mM $MgCl_2$ (o———o), or 3.0 mM $MgCl_2$ alone (•———•) and varying concentrations of $Ca^{2+}$. Fraction I membranes were present at a final concentration of 0.144 mg protein/ml (o———o) or 0.580 mg protein/ml (•———•) and 1.0 mM GTP. The free $Ca^{2+}$ concentrations of approx. $10^{-8}$ and $10^{-9}$ M were obtained by carrying out the assay in the presence of 0.1 mM EDTA plus 3.1 mM $MgCl_2$ or 1.0 mM EGTA plus 4.0 mM $MgCl_2$ respectively. The $K_D$ of the Ca-EGTA complex was assumed to be $5 \cdot 10^6$ $M^{-1}$ [36].

cyclase activity was affected by $Ca^{2+}$ in a rather complex manner (Fig. 3). Of possible physiologic importance was the observation that lowering of $Ca^{2+}$ concentration from $10^{-5}$ to approx. $10^{-8}$ M resulted in a more than 2-fold increase in guanylate cyclase activity, from 0.40 to 1.05 pmol/mg protein per min. As the $Ca^{2+}$ concentration was raised from 10 to 500 $\mu$M enzyme activity was stimulated, from 0.40 to 1.03 pmol/mg protein per min. This response to concentrations of $Ca^{2+}$ greater than $10^{-5}$ M was similar to that seen in the presence of 3.0 mM $Mg^{2+}$ and 0.1 mM $Mn^{2+}$ except that the activities were lower in the absence of $Mn^{2+}$.

*Effect of $Ca^{2+}$ on the solubilized particulate enzyme*

Membranous fragments of Fraction I were solubilized in 1% Triton X-100 and the activity determined under indentical conditions as used for the unsolubilized particulate enzyme. In the presence of optimal $Mn^{2+}$, activity was unaffected by concentrations of $Ca^{2+}$ between 10 and 60 $\mu$M, however, at $Ca^{2+}$ levels greater than 100 $\mu$M a profound inhibition was evident. A similar $Ca^{2+}$-dependent inhibition of the solubilized particulate enzyme was observed when the assay was performed in the presence of 0.1 mM $Mn^{2+}$ and 3.0 mM $Mg^{2+}$ (Fig. 4). In order to determine the effect of $Ca^{2+}$ concentrations less than 10 $\mu$M on the solubilized guanylate cyclase activity it was necessary again to utilize EGTA buffers and 3.0 mM $Mg^{2+}$ as the sole cation. The omission of

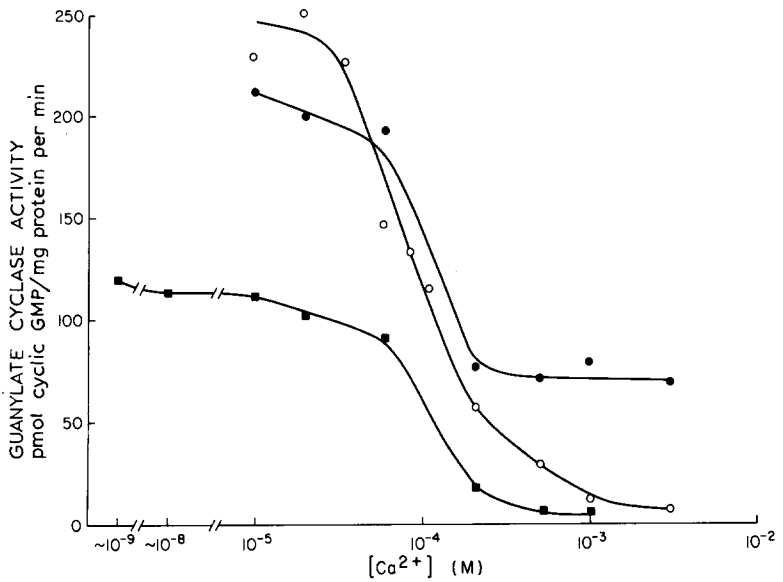


Fig. 4. Effect of $Ca^{2+}$ on Fraction I solubilized particulate guanylate cyclase activity. Fraction I membranes (final concentrations 0.012—0.023 mg protein/ml) were solubilized in 1% Triton X-100 for 1 h at 4°C and then assayed for guanylate cyclase activity in the presence of 3.0 mM $MnCl_2$ (●———●), 0.1 mM $MnCl_2$ and 3.0 mM $MgCl_2$ (○———○), or 3.0 mM $MgCl_2$ (■———■). GTP was present at a final concentration of 1.0 mM. The free $Ca^{2+}$ concentrations of approx. $10^{-8}$ and $10^{-9}$ M were obtained by carrying out the assay in the presence of 0.1 mM EGTA and 3.1 mM $MgCl_2$ or 1.0 mM EGTA and 4.0 mM $MgCl_2$, respectively. The $K_D$ of the Ca-EGTA complex was assumed to be $5 \cdot 10^6$ $M^{-1}$ [36].

$Mn^{2+}$ resulted in a lower basal activity but in contrast to the unsolubilized membranes, reducing the $Ca^{2+}$ concentrations from $10^{-5}$ to $10^{-9}$ M, did not appreciably change the activity. Under these conditions, (3.0 mM $Mg^{2+}$) the solubilized preparation was inhibited by concentrations of $Ca^{2+}$ between 0.1 and 1.0 mM, whereas the unsolubilized enzyme was stimulated by $Ca^{2+}$ in identical concentrations (Fig. 3 and 4). Thus, solubilization of the membranes dramatically altered the response to $Ca^{2+}$.

Electron microscopic examination of the native particulate fractions showed them to be partially present in the form of closed vesicles (Malouf, N. and Meissner, G., unpublished data). Solubilization will disrupt these structures. A possible explanation for the anomalous behavior in the presence of $Ca^{2+}$ could be that part of the $Ca^{2+}$ was taken up by the unsolubilized (but not the solubilized) membranes. To test this hypothesis, $Ca^{2+}$ uptake capacity of Fraction I membranes was measured at $Ca^{2+}$ concentrations of 0.05—3.0 mM under conditions of the guanylate cyclase assay as described above, with the exception that trace amounts of $^{45}Ca^{2+}$ were added to the incubation media. $^{45}Ca^{2+}$ bound to the membranes was separated from free $Ca^{2+}$ by centrifugation for 15 min at 45 000 rev./min. Less than 10% of total $^{45}Ca^{2+}$ was bound to the membranes indicating that most of the added $Ca^{2+}$ was present in free form during the guanylate cyclase assay.

An alternative explanation for the differential effect of $Ca^{2+}$ might be that the enzyme activity depended on whether the cation was made available to the

inner or outer surface of the vesicles. To examine this possibility, and also to further test the $Ca^{2+}$ uptake hypothesis, experiments were repeated with and without the addition of ionophore X537A. The ionophore was dissolved in acetone at an initial concentration of 10 mg/ml and was present in the guanylate cyclase assay at a final concentration of 25 $\mu$g/ml. Control experiments could demonstrate no effects of the solvent alone. The addition of ionophore X537A did not effect the stimulation of the unsolubilized particulate guanylate cyclase by $Ca^{2+}$. Thus, the effect of $Ca^{2+}$ on increasing enzyme activity does not appear to be dependent upon $Ca^{2+}$ uptake or its exclusion from the inner surface of the vesicle.

*GTP kinetics*

Studies of the sarcolemma enriched membranous fragments from rabbit skeletal muscle did not demonstrate simple Michaelis-Menton kinetics (Fig. 5 and 6). This was true at 10 and 200 $\mu$M $Ca^{2+}$ for both the particulate enzyme and its solubilized counterpart. Aas shown with the particulate guanylate cyclase isolated from other tissues [14,16,18] the enzyme isolated from rabbit skeletal muscle displayed a concave upward configuration when plotted on

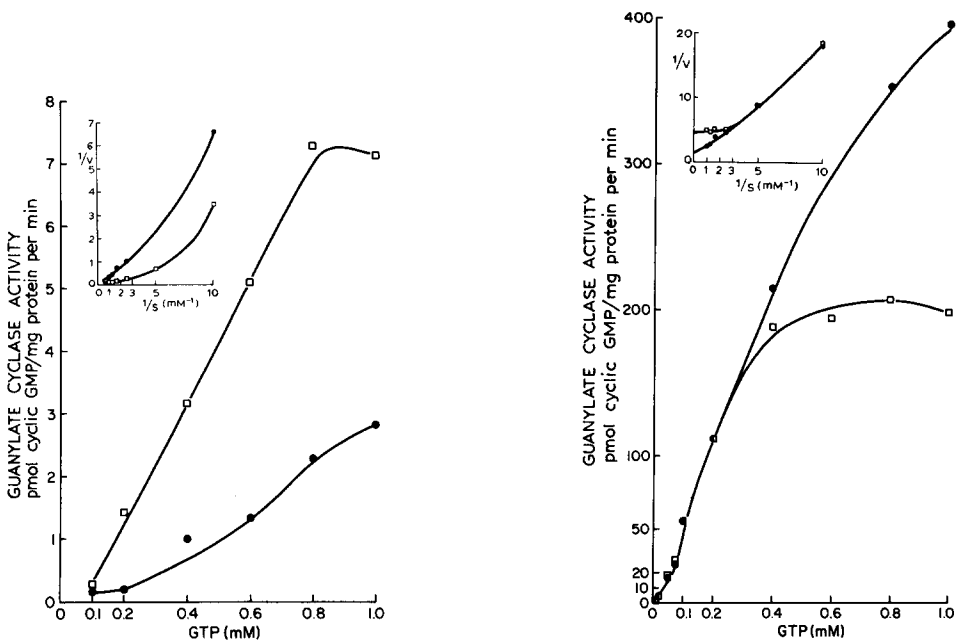


Fig. 5. Dependence of particulate Fraction I guanylate cyclase activity on GTP concentration. Samples of Fraction I membranes (0.216 mg protein/ml) were assayed in the presence of 0.1 mM $MnCl_2$, 3.0 mM $MgCl_2$, and either 10 $\mu$M (●——●) or 200 $\mu$M (□——□) $Ca^{2+}$. Inset shows the data in the form of a Lineweaver-Burk plot.

Fig. 6. Dependence of solubilized particulate Fraction I guanylate cyclase activity on GTP concentration. Samples of Fraction I membranes were solubilized in 1% Triton X-100 for 1 h at 4°C and assayed at a final concentration of 0.013 mg protein/ml in the presence of 0.1 mM $MnCl_2$, 3.0 mM $MgCl_2$, and either 10 $\mu$M (●——●) or 200 $\mu$M (□——□) $Ca^{2+}$. Inset shows the data in the form of a Lineweaver-Burk plot.

double reciprocal coordinates. It may be noted, however, that the influence of GTP on guanylate cyclase activity was tested in the presence of three metal ions ($Mn^{2+}$, $Mg^{2+}$, and $Ca^{2+}$). The total concentration of each ion remained the same while that of GTP was variable. It is thus possible that various metal-GTP complexes were formed and free metal ion concentrations were variable. This may have contributed to the complexity of the data.

## Discussion

It has generally been accepted that two separate forms of guanylate cyclase with distinct biochemical properties exist in most tissues: one soluble and the other particulate [12—14]. We have limited our present investigation to characterizing the effects of divalent cations on the particulate enzyme which has been prepared by sucrose density centrifugation from rabbit skeletal muscle. In these preparations, guanylate cyclase activity was predominantly localized in a membranous fraction which also had appreciable adenylate cyclase and leucine aminopeptidase activities, suggesting that these membranes are enriched with sarcolemma. It appears that because of the low enzyme activity of Fractions II—IV the higher specific activity seen in the membranous Fraction I is not due to contamination with the soluble enzyme.

As shown in studies with other tissues, the optimal activity obtained with a single metal occurs with $Mn^{2+}$ as the cofactor [12], but a supraphysiologic concentration of 3.0 mM is required. Also in agreement with other workers, we found that the particulate guanylate cyclase is markedly stimulated when solubilized with non-ionic detergents. In solubilized samples, activity with 0.1 mM $Mn^{2+}$ and 3.0 mM $Mg^{2+}$ was equal to that seen with optimal $Mn^{2+}$ alone. During the preparation of this manuscript a publication appeared confirming this observation in solubilized cardiac particles [26]. Of possibly greater physiologic significance is the observation that in the absence of detergents, the particulate enzyme was also stimulated by $Mg^{2+}$ in the presence of suboptimal (0.1 mM) $Mn^{2+}$. This stimulation was 4-fold greater than expected if the activities were simply additive. It is, therefore, likely that in the presence of low $Mn^{2+}$ concentrations, availability of $Mg^{2+}$ may be a rate determining factor.

While $Ca^{2+}$ has generally been demonstrated to stimulate soluble guanylate cyclase, the effect of this cation on the particulate form of the enzyme has usually been shown to be inhibitory [12—14,18]. However, exceptions to this generalization have been documented in sea urchin sperm [16], rat cerebellum [15], and Balb 3T3 fibroblasts [27]. We found that the effect of $Ca^{2+}$ upon the particulate enzyme present in a membranous fraction enriched with sarcolemma is complex, and in addition, dependent upon whether membranes are used in particulate form or are solubilized with a non-ionic detergent. The unsolubilized particulate guanylate cyclase is inhibited as the $Ca^{2+}$ concentrations is increased from $10^{-9}$ to $10^{-5}$ M, while further increases of $Ca^{2+}$ to $5 \cdot 10^{-4}$ M are again stimulatory. These effects are evident at suboptimal $Mn^{2+}$ concentrations. When optimal (and supraphysiological) concentrations of $Mn^{2+}$ (3.0 mM) are utilized higher specific activities are observed but the effect of $Ca^{2+}$ is no longer evident. When membranous fractions were solubilized in 1% Triton X-100, $Ca^{2+}$ did not significantly effect guanylate cyclase activity up to a concentration of about $5 \cdot 10^{-5}$ M. At higher $Ca^{2+}$ concentrations an inhibi-

tory effect was evident. It is clear that alterations in the guanylate cyclase activity of the particulate and solubilized particulate preparations are related to changes in $Ca^{2+}$ concentration at levels greater than $10^{-5}$ M. The data at concentrations of $Ca^{2+}$ less than $10^{-5}$ M must be interpreted with some caution as the EGTA employed to achieve such concentrations could have also complexed and altered the concentration of other trace metal ions.

Two previous reports have also investigated the effects of $Ca^{2+}$ on both a particulate and a solubilized particulate guanylate cyclase isolated from the same tissue. In rat cerebellum $Ca^{2+}$ stimulates the native particulate preparation and inhibits its solubilized counterpart. It should be noted that the levels of $Ca^{2+}$ used in this study far exceeded those attainable within the intracellular milieu [15]. Garbers et al. [16] have similarly shown that, under the appropriate Mn : GTP ratios, $Ca^{2+}$ would stimulate the guanylate cyclase from sea urchin sperm and that with the Triton dispersed enzyme there was no such stimulation. However, a report demonstrating stimulation of a solubilized particulate guanylate cyclase by $Ca^{2+}$ was published by Wallach and Pastan [27]. It seems evident that the conditions for assaying guanylate cyclase are critical in determining how the activity is altered in the presence of $Ca^{2+}$. In fact, the original reports of the inhibition of particulate guanylate cyclase from rat heart and liver were done after solubilization with Triton X-100 [18,28].

At the present time it remains unclear why solubilization of rabbit skeletal muscle membranes results in an altered response of guanylate cyclase to $Ca^{2+}$. $^{45}Ca^{2+}$ uptake studies and use of the ionophore X537A suggest that differences seen between particulate and solubilized samples are not likely due to an asymmetric distribution of $Ca^{2+}$ across the unsolubilized membranes. It is then more likely that membrane solubilization results in a structural alteration of the guanylate cyclase or possibly a change in its interaction with a $Ca^{2+}$ regulatory protein. Although a $Ca^{2+}$ regulatory protein has not yet been demonstrated to effect the activity of guanylate cyclase, several such proteins have been now isolated. These have been found to have similarities to troponin C and to effect the activities of a number of enzymes including adenylate cyclase, phosphodiesterase, and $Ca^{2+}$ ATPase [29—32].

As in the case of cyclic AMP, there appears to exist a complex interrelationship between intracellular $Ca^{2+}$ and cyclic GMP levels [32]. Data presented in this communication suggest that the particulate guanylate cyclase may be controlled by $Ca^{2+}$ in more than one way. For example, changes in the intracellular $Ca^{2+}$ concentration between $10^{-8}$ and $10^{-5}$ M brought about during the contraction-relaxation cycle of muscle would be sufficient to alter particulate cyclase activity several fold. Activation of guanylate cyclase by higher concentrations of $Ca^{2+}$ raises the possibility that $Ca^{2+}$ fluxes across the sarcolemma may result in an increase in cyclic GMP levels in certain localized areas of the cell. In this regard it is of interest that a number of hormones including histamine [34] and acetylcholine [35] effect cyclic GMP levels only in the presence of extracellular $Ca^{2+}$ but not in its absence. Unfortunately, the mechanisms by which these hormones exert their influence is not known. A better knowledge of the molecular properties and of the physiological role of guanylate cyclases in muscle function will be necessary before we can hope to clarify the precise relationship between $Ca^{2+}$ and these enzymes.

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