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# TISSUE-SPECIFIC EFFECTS OF DIVALENT CATIONS AND ACTIVATORS ON SOLUBLE GUANYLATE CYCLASE

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## Summary

The interactions of divalent cations, $Mn^{2+}$, $Mg^{2+}$, and $Ca^{2+}$, with the cytosolic guanylate cyclase (GTP pyrophosphate-lyase (cyclizing, EC 4.6.1.2) from different tissues were studied. Guanylate cyclase activities of the kidney, liver, and lung were strongly dependent on $Mn^{2+}$. In contrast, the enzyme in smooth muscle of the colon, aorta, and vas diferens was active with $Mg^{2+}$ as well as with $Mn^{2+}$. $Ca^{2+}$ was ineffective in all tissues. Preincubation, at 30°C, of colon extracts, but not those of kidney and liver, increased guanylate cyclase activity. The $Mg^{2+}$-dependent activity was preferentially enhanced by this treatment. These results suggest that when the enzyme was autoactivated by endogenous factors it became more $Mg^{2+}$ dependent. Dithiothreitol strongly inhibited the $Mg^{2+}$-dependent colon enzyme, whereas activities in kidney and liver were not affected and the response of the enzyme in lung was intermediate. This suggests that autoactivation involved an oxidative-reductive alteration of the enzyme. $Ca^{2+}$ markedly inhibited the $Mg^{2+}$-dependent activity in smooth muscles but $Mg^{2+}$-dependent activities in lung, liver, and kidney were not influenced appreciably. Exogenous activators, dehydroascorbate and $NaN_3$, increased guanylate cyclase, assayed with either $Mg^{2+}$ or $Mn^{2+}$. However, the relative stimulation of the enzyme assayed with $Mg^{2+}$ was greater than with $Mn^{2+}$. When activated by these exogenous agents, guanylate cyclase in all tissues became inhibitable by $Ca^{2+}$. These findings suggest that guanylate cyclase in smooth muscle, as prepared, was in a partially activated form.

The endogenous activating factors in colon smooth muscle were heat-stable, largely extractable with chloroform/methanol, and cochromatographed with authentic fatty acids. Arachidonic acid stimulated colon guanylate cyclase and enhancement of the $Mg^{2+}$-dependent activity was blocked by $Ca^{2+}$. This

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strongly infers that a significant part of the endogenous activating factors in the colon was fatty acids or their derivatives. The colon activators had only minimal affects on the enzyme in the kidney. Similarly prepared activator extracts from the kidney increased colon guanylate cyclase but did not stimulate the renal enzyme. Thus, the ability of the enzyme to be stimulated by endogenous activators was dependent on the tissue from which the enzyme was derived.

The possibility that the interactions of $Mg^{2+}$ and $Ca^{2+}$ on guanylate cyclase in smooth muscles are of regulatory significance to the contractile response of the muscle was discussed. It was proposed as a working hypothesis that cyclic GMP and $Ca^{2+}$ might participate in reciprocal negative feedback mechanisms.

## Introduction

Guanylate cyclase (GTP pyrophosphate-lyase (cyclizing), EC 4.6.1.2), in soluble and particulate forms, was found in various animal species and tissues [1—4]. A divalent cation was required for activity. Nevertheless, the interactions of the cyclase with metals, i.e., $Mn^{2+}$, $Mg^{2+}$, and $Ca^{2+}$, either individually as the sole metal cofactor or in combination were exceedingly complex and at times seemingly inconsistant. The enzyme was usually most active with $Mn^{2+}$; however, the $K_a$ for free $Mn^{2+}$ in vitro was far in excess of the concentration normally found in tissues [5—7]. Activity with $Mg^{2+}$ as the sole divalent cation was reported to be only about 10% that with $Mn^{2+}$; $Ca^{2+}$ was even less effective [1]. When guanylate cyclase was stimulated by $NaN_3$, nitroprusside, or nitrosureas, however, $Mg^{2+}$-dependent activity, but not $Ca^{2+}$-dependent activity, was preferentially increased and approached that with $Mn^{2+}$ [8—10]. Likewise, when membranes were treated with Triton X-100, the normally low level of $Mg^{2+}$-dependent guanylate cyclase increased to a level equivalent to that with optimal concentrations of $Mn^{2+}$ [11]. In contrast, other detergents, e.g., cholate, enhanced $Mn^{2+}$-dependent activity but inhibited $Mg^{2+}$-dependent activity [11]. When the divalent cations were used in combination, the addition of $Mg^{2+}$ to a reaction mixture containing low concentrations of $Mn^{2+}$ either stimulated guanylate cyclase [12] or did not alter activity [7,13,14]. The effects of $Ca^{2+}$, in combination with $Mn^{2+}$ or $Mg^{2+}$, were similarly complicated. In several studies it was found that $Ca^{2+}$, in the presence of low concentrations of $Mn^{2+}$, increased the activity of soluble guanylate cyclase but inhibited the particulate enzyme [3,5,12,15—17]. At high concentrations of $Mn^{2+}$, there was no effect. In other reports, $Ca^{2+}$, added to a reaction containing low $Mn^{2+}$, stimulated both the soluble and particulate enzyme [18] or stimulated or inhibited the membrane enzyme, depending on conditions [19]. In the latter study, however, $Ca^{2+}$ (3 $\mu$M) significantly enhanced the $Mg^{2+}$-dependent guanylate cyclase but higher concentrations of $Ca^{2+}$ (>30 $\mu$M) were inhibitory [19]. In contrast, the activity of the soluble enzyme assayed with $Mg^{2+}$ was increased by high $Ca^{2+}$ (>0.5 mM). After $NaN_3$ activation, $Ca^{2+}$ had no effect or was somewhat inhibitory [8].

These findings indicate the complexity of metal ion interactions with guanylate cyclase but also suggest that $Mg^{2+}$ and/or $Ca^{2+}$ could have important roles in the regulation of the enzyme. Some of this apparent controversy and con-

fusion in the various studies may be due to the use of different tissues. Accordingly, we have examined the interactions of divalent cations with the enzyme in several tissues and report here that the effects of the metals are tissue specific and dependent, in part, on the state of activation of the guanylate cyclase in that tissue.

## Materials and Methods

GTP from P-L Biochemicals was used in the guanylate cyclase assay medium and GTP from Sigma Chemical Co. was used in the quench medium to stop the incubation. Cyclic GMP, creatine phosphate, creatine kinase, theophylline, and indomethacin were obtained from Sigma Chemical Co. Dehydroascorbic acid was from K and K Labs. and arachidonic acid was from Supelco, Inc. [$\alpha$-$^{32}$P]-GTP (2—20 Ci/mmol) was from New England Nuclear and [8-$^{3}$H]cyclic GMP (10—30 Ci/mmol) was from Amersham.

The kidneys, lungs and portions of the colon and liver were excised from New Zealand white male rabbits. The colon smooth muscle was obtained by scraping away the mucosa and the renal medulla was dissected from the cortex. The tissues were homogenized in 10 ml of 0.25 M sucrose, 10 mM Tris-HCl, pH 7.5, and 1 mM EDTA with a Potter-type glass vessel and Teflon pestle. The homogenates were centrifuged at 4000 $\times g$ for 10 min. The supernatants were then centrifuged at 105 000 $\times g$ for 1 h. The resultant supernatants were diluted with three vol. of 10 mM Tris-HCl, pH 7.5, and used to measure guanylate cyclase activity.

Guanylate cyclase activity was assayed as described previously [20]. The reaction mixture contained 50 mM Tris-HCl buffer, pH 7.5, various concentrations of $MgCl_2$ or $MnCl_2$, 0.4 mM [$\alpha$-$^{32}$P]GTP, 5 mM theophylline, 1 mM cyclic GMP containing 10 nCi of [8-$^{3}$H]cyclic GMP, 10 mM creatine phosphate, 12.5 $\mu$g creatine kinase, 10 $\mu$l of enzyme preparation (20—30 $\mu$g of protein) in total vol. of 50 $\mu$l. The mixture was incubated for 20 min at 30°C. The reaction was stopped by the addition of 0.15 ml of ice-cold quench solution consisting of 50 mM Tris-HCl buffer, pH 7.5, 0.93 mM GTP and 0.5 mM cyclic GMP, followed immediately by heating in boiling water for 2 min. After heating, the mixture was adjusted to 1 ml with 10 mM Tris-HCl buffer. The [$^{32}$P]cyclic GMP formed was separated from [$^{32}$P]GTP with a two column chromatography method modified [21] from that described by Nesbitt et al. [22]. Radioactivity was measured in ACS (Amersham) with a Packard TriCarb liquid scintillation counter. Under the conditions of the assay, activities were proportional to time and amount of enzyme. Experiments were replicated with at least two tissue preparations, each carried out in triplicate.

## Results

*Divalent cation dependence, autoactivation and dithiothreitol sensitivity*

Fig. 1 compares the effects of different concentrations of $Mn^{2+}$, $Mg^{2+}$, and $Ca^{2+}$ on the cytosolic guanylate cyclase activities from colon smooth muscle, lung, liver, and renal cortex after the enzyme preparations were: (A) preincubated at 0°C for 30 min, control; (B) preincubated at 30° for 30 min, autoactivated; and (C) pretreated at 0°C for 30 min in the presence of 5 mM dithio-

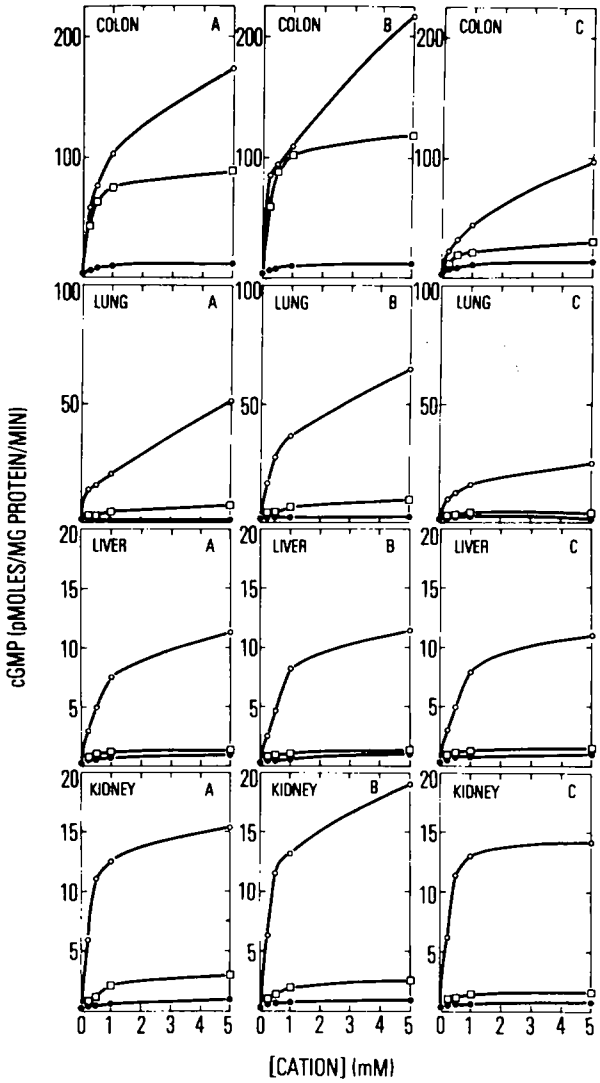


Fig. 1. The effects of $Mn^{2+}$ (○), $Mg^{2+}$ ([]), and $Ca^{2+}$ (●) on the cytosolic guanylate cyclase activities from colon smooth muscle, lung, liver, and renal cortex after the enzyme preparations were (A) preincubated at 0°C for 30 min, control; (B) preincubated at 30°C for 30 min, autoactivated; and (C) pretreated at 0°C for 30 min in the presence of 5 mM dithiothreitol, reduced. After the preincubation treatments, incubations were initiated with the addition of the reaction mixture. The final concentration of dithiothreitol in (C) was 1 mM. The data represent the average of two experiments, each replicated in triplicate.

threitol, reduced. Preincubation at 0°C for 30 min (control) did not affect the specific activities nor their responses to metals when compared to extracts not preincubated. As shown in Fig. 1A guanylate cyclase activities of the lung, liver, and kidney were strongly dependent on $Mn^{2+}$, as the sole metal cation cofactor. $Mg^{2+}$ and, particularly, $Ca^{2+}$ were poor substitutes. In contrast, the enzyme from colon was active with $Mg^{2+}$ as well as with $Mn^{2+}$; $Ca^{2+}$ was ineffective, however. Indeed, at concentrations up to 1 mM the activities with $Mg^{2+}$ approached those with $Mn^{2+}$. Activity in the presence of 1 mM $Mg^{2+}$ reached a

plateau, whereas with $Mn^{2+}$ it continued to increase with increased $Mn^{2+}$ to 5 mM, and then activity decreased at higher concentrations (not shown). Since the GTP concentration in the reaction mixture was 0.4 mM, the greater cyclase activity found with the higher $Mn^{2+}$ concentrations was probably due to stimulation by excess free $Mn^{2+}$.

When the extracts were preincubated at 30°C (Fig. 1B), guanylate cyclase activities in lung and colon were significantly enhanced. This was especially marked when assayed with $Mg^{2+}$ or low concentrations of $Mn^{2+}$. On the other hand, guanylate cyclase activities in kidney and liver were not affected by the preincubation. Dithiothreitol strongly inhibited the activities in lung and colon (Fig. 1C). The activities in kidney and liver were not influenced. These findings indicate that the tissues whose activities were readily increased by endogenous factors during preincubation were also those whose activities were most susceptible to inhibition by the reducing agent. Further, when the ratios of the $Mg^{2+}$- to $Mn^{2+}$-dependent activities in colon, at 1 mM cation, were calculated, values of 0.52 for the dithiothreitol treated, 0.75 for the control, and 0.02 for the autoactivated enzyme were found. Similar correlations were found when the $Mg^{2+}$- to $Mn^{2+}$-dependent activity ratios were calculated from values using other concentrations of cation. These results suggest that $Mg^{2+}$-dependent activity was stimulated to a greater extent than was $Mn^{2+}$-dependent activity, and that the enzyme when activated by endogenous factors was able to utilize $Mg^{2+}$ as well as $Mn^{2+}$. On the other hand, $Mg^{2+}$-dependent activity was more sensitive to inhibition by dithiothreitol.

*Effect of $Ca^{2+}$ on $Mn^{2+}$- and $Mg^{2+}$-dependent guanylate cyclase*

Fig. 2 illustrates the effects of $Ca^{2+}$ on guanylate cyclase activities in different tissues, assayed in the presence of $Mn^{2+}$ or $Mg^{2+}$. With $Mn^{2+}$, $Ca^{2+}$ markedly increased the activity in the colon, maximum enhancement being obtained with about 1 mM $Ca^{2+}$. This stimulation was found when the concentrations of $Mn^{2+}$ was either relatively low (0.5 mM) or in great excess (5.0 mM). The $Mn^{2+}$-dependent activity in lung was moderately increased by $Ca^{2+}$, at both concentrations of $Mn^{2+}$. $Ca^{2+}$ had little affect on the $Mn^{2+}$-dependent activities in liver and kidney. The $Mg^{2+}$-dependent activities in lung, liver, and kidney were not influenced appreciably by the presence of $Ca^{2+}$. In contrast, $Ca^{2+}$ strongly inhibited the $Mg^{2+}$-dependent activity in the colon. These findings provided additional evidence that colon guanylate cyclase responded to divalent cations differently from the enzyme in other tissues.

The question arose as to whether the singular effects of divalent cations on the colon enzyme were unique to this tissue or representative of smooth muscles in general. That the latter is probably correct is seen from the data in Table I. Similar to the guanylate cyclase in the colon, the enzyme in the aorta and vas deferens was almost fully active when $Mg^{2+}$ replaced $Mn^{2+}$ in the reaction mixture. Furthermore, $Ca^{2+}$ inhibited the $Mg^{2+}$-dependent activity in all three smooth muscles. The $Mn^{2+}$-dependent cyclase in colon and aorta was stimulated by $Ca^{2+}$, but the activity in the vas deferens was not significantly changed, under these conditions.

The possibility was considered that the inhibition of the $Mg^{2+}$-dependent or stimulation of the $Mn^{2+}$-dependent activity by $Ca^{2+}$ in the colon guanylate

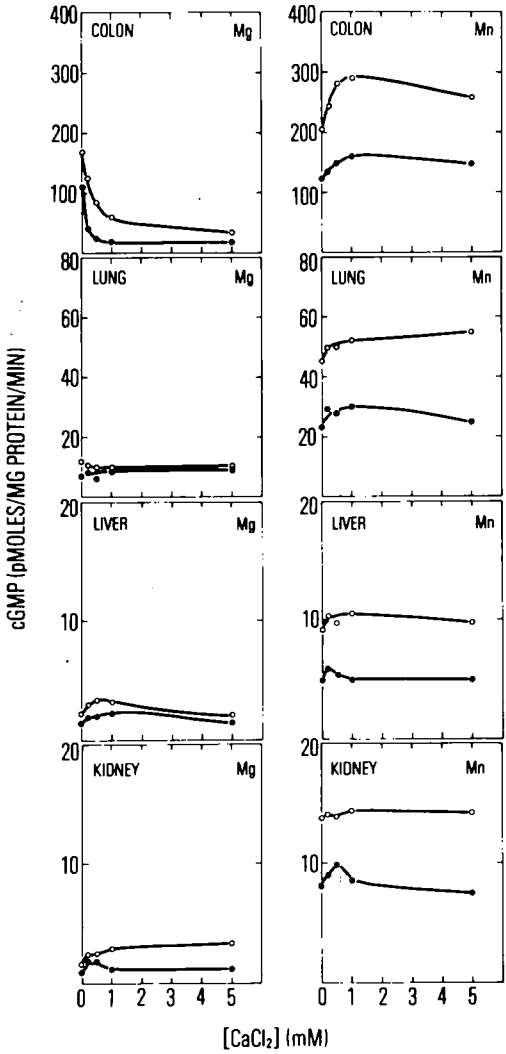


Fig. 2. The effects of $Ca^{2+}$ on guanylate cyclase activities in different tissues, assayed in the presence of $Mg^{2+}$ and $Mn^{2+}$ at concentrations of 5 mM (○) and 0.5 mM (●).

cyclase preparation was due to the presence in the crude extract of a $Ca^{2+}$-dependent enzyme, e.g., phospholipase, unrelated to guanylate cyclase, which was stimulated by the added $Ca^{2+}$ and that the product of this hypothetical reaction affected guanylate cyclase. If this were the case, then responses initiated by the addition of $Ca^{2+}$ at zero time might have a lag period. However, time course of the guanylate cyclase reactions in the presence and absence of $Ca^{2+}$ were linear (data not shown), thus arguing counter to this alternative. Also supporting a direct effect of $Ca^{2+}$ were experiments in which the crude colon guanylate cyclase preparation was preincubated at 30°C for 20 min with or without 2.5 mM $CaCl_2$, and at zero time the incubation started by the addition of reaction mixtures, comprised so that the final concentration of $CaCl_2$ in all

TABLE I

EFFECT OF DIVALENT CATIONS ON THE GUANYLATE CYCLASE ACTIVITIES IN VARIOUS SMOOTH MUSCLES

Soluble fractions of the colon, aorta, and vas deferens were assayed for guanylate cyclase, in the absence of divalent cation or in the presence of 0.5 mM $MnCl_2$ or $MgCl_2$ (experiment I). In determining the effect of $Ca^{2+}$ on the $Mn^{2+}$- and $Mg^{2+}$-dependent activities (experiment II), the $CaCl_2$ concentration was 0.5 mM and the $MnCl_2$ or $MgCl_2$ concentration was 2.0 mM. Each datum represents the mean ±S.E. of three determinations.

| | Cation | Guanylate cyclase (pmol/min per mg protein) | | |
|---|---|---|---|---|
| | | Colon | Aorta | Vas deferens |
| I. | None | 7.4 ± 0.2 | 18.2 ± 1.3 | 16.4 ± 2.1 |
| | $Mn^{2+}$ | 74.1 ± 2.1 | 123.8 ± 7.2 | 65.2 ± 3.2 |
| | $Mg^{2+}$ | 65.0 ± 2.3 | 99.7 ± 2.6 | 50.3 ± 1.5 |
| II. | $Mn^{2+}$ | 108.8 ± 4.5 | 100.2 ± 3.2 | 47.2 ± 2.3 |
| | $Mn^{2+}$ + $Ca^{2+}$ | 154.0 ± 4.6 | 123.2 ± 10.5 | 45.4 ± 4.5 |
| | $Mg^{2+}$ | 79.0 ± 2.3 | 85.3 ± 7.8 | 70.2 ± 2.5 |
| | $Mg^{2+}$ + $Ca^{2+}$ | 21.8 ± 1.0 | 35.3 ± 1.7 | 52.4 ± 3.2 |

reactions was 2.5 mM. In the $Mg^{2+}$-dependent system, the inhibition by $Ca^{2+}$ was 50%, whether $Ca^{2+}$ was added to the preincubation or incubation medium. In the $Mn^{2+}$-dependent system, the magnitude of activation by $Ca^{2+}$ was also approximately the same in the two experiments. These findings suggest that the actions of $Ca^{2+}$ in inhibiting the $Mg^{2+}$-dependent or stimulating the $Mn^{2+}$-dependent guanylate cyclase were effective immediately and probably not mediated by an intermediate reaction.

*Effect of exogenous activators on the $Mn^{2+}$- and $Mg^{2+}$-dependent guanylate cyclase*

Dehydroascorbate and $NaN_3$ were known to enhance guanylate cyclase activity [23,24]. The effects of these activators on the enzyme from the different tissues, in the presence of several concentrations of either $Mn^{2+}$ or $Mg^{2+}$, as sole cation cofactor, are illustrated in Fig. 3. In general: (a) guanylate cyclase activity in all tissues examined was stimulated by dehydroascorbate and $NaN_3$, although the various tissues responded differently. For example, the magnitude of the increase effected by dehydroascorbate with the kidney enzyme was twice that with the lung preparation. The enhancement by $NaN_3$ was notably small especially when activity was measured in the presence of $Mn^{2+}$. The difference between the minimal $NaN_3$ stimulations found in this study using rabbit tissues and the large increases reported previously using tissues from the rat [24], appears to be due to a species distinction, as noted earlier [20]. (b) The exogenous activators increased guanylate cyclase, assayed with either $Mg^{2+}$ or $Mn^{2+}$. However, the relative stimulation of the enzyme assayed with $Mg^{2+}$ was greater than that assayed with $Mn^{2+}$. This was more evident when the concentrations of metals were 1 and 5 mM. (c) With $Mg^{2+}$, stimulation by the two exogenous activators increased as the concentration of divalent cation was raised from 0.5 to 5.0 mM. In contrast, with $Mn^{2+}$ the opposite was found; the higher the $Mn^{2+}$ concentration the smaller was the relative enhancement.

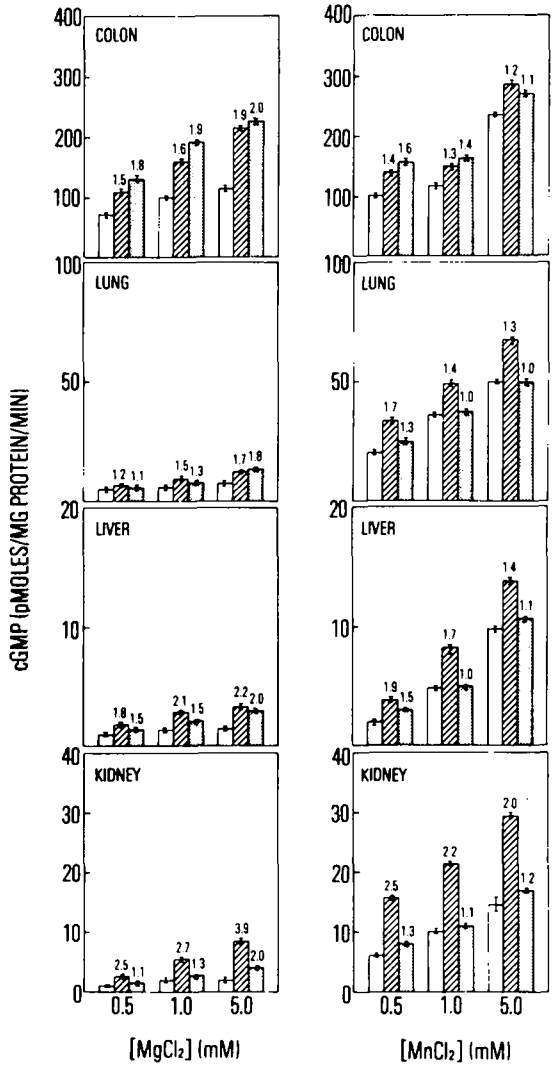


Fig. 3. The effects of exogenous activators, dehydroascorbate and $NaN_3$, on guanylate cyclase activities in different tissues assayed in the presence of several concentrations of $Mg^{2+}$ and $Mn^{2+}$. The open columns represent basal values. The bars with diagonal lines represent values obtained with 1 mM dehydroascorbate and the stippled bars represent values with 1 mM $NaN_3$. The number over the column indicates the ratio of the activated to basal activities.

The effect of $Ca^{2+}$ on guanylate cyclase activity stimulated by exogenous activators depended on whether $Mn^{2+}$ or $Mg^{2+}$ was the metal cofactor and on the tissue (Fig. 4). With $Mn^{2+}$ as the sole cation, the presence of dehydroascorbate, $NaN_3$, or $Ca^{2+}$ increased colon guanylate cyclase activity. When $Ca^{2+}$ was combined with either dehydroascorbate or $NaN_3$ the enhancements were additive. In the lung and kidney, the stimulations by the exogenous activators were not appreciably affected by the further addition of $Ca^{2+}$. In the liver, the relatively small increases provoked by dehydroascorbate and $NaN_3$ were decreased when the activators were combined with $Ca^{2+}$. In contrast, with the $Mn^{2+}$-

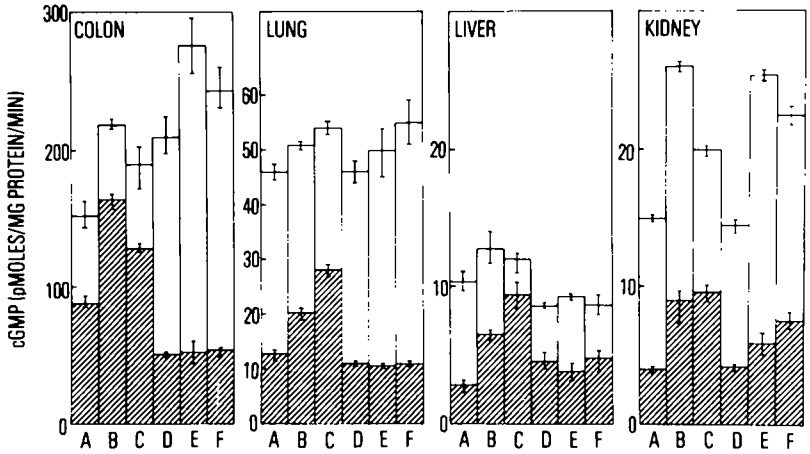


Fig. 4. The effects of $Ca^{2+}$ on the exogenous activator-stimulated guanylate cyclase activities in different tissues assayed in the presence of 5 mM $Mn^{2+}$ (open columns) or 5 mM $Mg^{2+}$ (columns with diagonal lines). The various experimental conditions are represented by: (A) basal activities; (B) activities in the presence of 1 mM dehydroascorbate; (C) activities in the presence of 1 mM $NaN_3$; (D) activities in the presence of 1 mM $Ca^{2+}$; (E) activities in the presence of 1 mM dehydroascorbate and 1 mM $Ca^{2+}$; and (F) activities in the presence of 1 mM $NaN_3$ and 1 mM $Ca^{2+}$. The mean and the S.E. are indicated for duplicate experiments, each replicated in triplicate.

dependent guanylate cyclase from colon, the presence of $Ca^{2+}$ completely inhibited the dehydroascorbate and $NaN_3$ stimulated activities. The same response to $Ca^{2+}$ was found for the $Mg^{2+}$-dependent, exogenously activated enzyme from lung and liver, but the response was less marked with the kidney enzyme.

*Stimulation of guanylate cyclase by endogenous activating factors in colon and the effects of $Ca^{2+}$*

The findings that (a) guanylate cyclase in the different tissues, when activated by exogenous activators, i.e., dehydroascorbate and $NaN_3$, became relatively more $Mg^{2+}$ dependent (Fig. 3); (b) $Ca^{2+}$ inhibited the activated enzyme assayed in the presence of $Mg^{2+}$ but enhanced or had little effect on the activated enzyme assayed in the presence of $Mn^{2+}$ (Fig. 4); (c) $Mg^{2+}$ effectively replaced $Mn^{2+}$ as the metal cofactor in colon, but not in other tissues (Fig. 1); and (d) $Ca^{2+}$ inhibited $Mg^{2+}$-dependent activity only in colon while activating or having little effect on $Mn^{2+}$-dependent activities (Fig. 2), suggested the hypothesis that the colon enzyme, as well as the enzyme from different smooth muscles (Table I), was already activated, perhaps by an endogenous activator. To test this possibility, the colon extract was placed in a boiling water bath for 1 min, centrifuged, the precipitated protein discarded, and the supernatant fraction, devoid of guanylate cyclase activity, added to preparations of the enzyme from colon, lung, liver, and kidney, and activities determined with $Mg^{2+}$ or $Mn^{2+}$, in the presence and absence of $Ca^{2+}$. As shown in Fig. 5, the boiled extract from colon had the same effect on $Mg^{2+}$-dependent guanylate cyclase as did the exogenous activators; namely, it stimulated in the order colon > lung > liver > kidney. Moreover, these activations were strongly

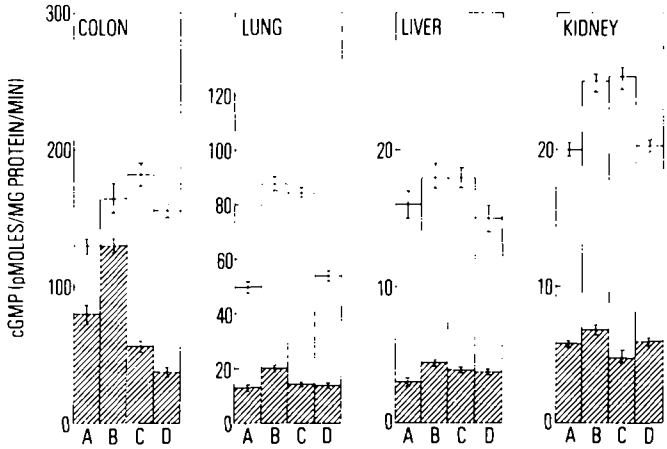


Fig. 5. The effects of $Ca^{2+}$ on the endogenous activator-stimulated guanylate cyclase activities in different tissues assayed in the presence of 5 mM $Mn^{2+}$ (open columns) or 5 mM $Mg^{2+}$ (columns with diagonal lines). The endogenous activator was from colon smooth muscle and was obtained by boiling the 105 000 × *g* supernatant fraction (30 μg of protein/10 μl) for 1 min. The preparation was devoid of guanylate cyclase activity. When indicated 10 μl of activator preparation and 1 mM $Ca^{2+}$ was added to the incubations. The various experimental conditions are represented by; (A) basal activities; (B) activities in the presence of endogenous activator; (C) activities in the presence of $Ca^{2+}$; and (D) activities in the presence of endogenous activator and $Ca^{2+}$. The mean and S.E. are indicated for duplicate experiments, each replicated in triplicate.

inhibited by $Ca^{2+}$, especially in colon and lung. The boiled extract only moderately stimulated $Mn^{2+}$-dependent enzymic activity. However, $Ca^{2+}$ blocked these stimulations, in contrast to the lack of an effect of $Ca^{2+}$ on the activation by dehydroascorbate and $NaN_3$, in the $Mn^{2+}$ system (Fig. 4). Thus, these results generally support the view that the guanylate cyclase in the colon extract, as prepared, was in an activated form and that the smooth muscle contained endogenously activating factors capable of stimulating the enzyme in the different tissues.

In an attempt to identify the activating factors in the boiled colon supernatant, the preparation was extracted with chloroform/methanol (2 : 1), the lower phase removed, solvents evaporated, and the residue resuspended in buffer. The material which was extracted with organic solvents was then compared with the substances in the upper aqueous phase for stimulatory activity. An aliquot of the chloroform/methanol extract activated $Mg^{2+}$-dependent colon guanylate cyclase 84%. An equivalent aliquot of the aqueous fraction activated the enzyme only 10%. The response was tissue specific with respect to the source of the enzyme, however, for the chloroform/methanol extract from colon had no effect on kidney guanylate cyclase. On the other hand, a chloroform/methanol extract of a boiled renal cortex supernatant fraction increased the activity of the colon enzyme, assayed with $Mg^{2+}$, 78%, whereas it did not stimulate the activity of the renal enzyme.

Fig. 6 describes the effects of the chloroform/methanol extracted activator from colon on colon and kidney guanylate cyclase activities, determined with $Mg^{2+}$ or $Mn^{2+}$ as sole metal cofactor. The extract increased activity of the colon enzyme, measured with both $Mg^{2+}$ and $Mn^{2+}$. Activation of the $Mg^{2+}$-dependent enzyme was blocked by the addition of $Ca^{2+}$, but $Ca^{2+}$ did not affect the

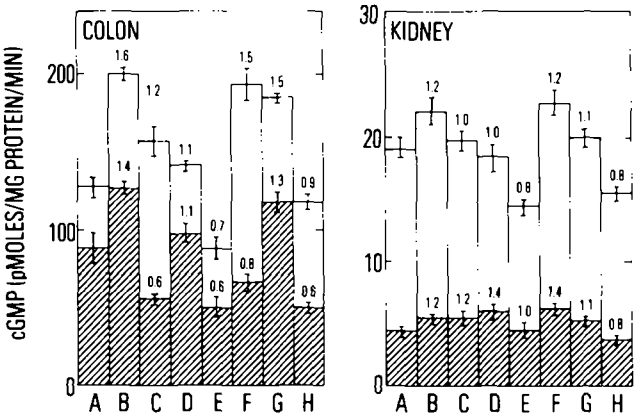


Fig. 6. The effects of the chloroform/methanol extracted endogenous activator from colon smooth muscle on the colon and renal cortex guanylate cyclase, determined in the presence of 5 mM $Mn^{2+}$ (open columns) or 5 mM $Mg^{2+}$ (columns with diagonal lines). The activator, prepared as described in Fig. 5, was extracted with chloroform/methanol (2 : 1) after adjusting the pH of the supernatant to 5.0 with 1 M acetate buffer. The organic lower layer was removed and solvents were evaporated. The residue was suspended by sonication in 10 mM Tris buffer, pH 7.4, using the same volume as in the original extract. 20 $\mu$l of the suspension was added to the reaction mixture, when indicated. The various experimental conditions are represented by: (A) basal activities; (B) activities in the presence of extracted activator; (C) activities in the presence of 1 mM $Ca^{2+}$; (D) activities in the presence of 50 $\mu$M indomethacin; (E) activities in the presence of 1 mM dithiothreitol; (F) activities in the presence of extracted activator and $Ca^{2+}$; (G) activities in the presence of extracted activator and indomethacin; and (H) activities in the presence of extracted activator and dithiothreitol. The mean and S.E. are indicated for duplicate experiments, each replicated in triplicate. The number over the column indicates the ratio of the determined activity relative to the basal value.

activation of the $Mn^{2+}$-dependent cyclase. Dithiothreitol inhibited the activation of the enzyme assayed with $Mg^{2+}$ or $Mn^{2+}$, but the inhibition was more significant in the presence of $Mg^{2+}$ than $Mn^{2+}$. The chloroform/methanol extract had relatively small effects on the renal enzyme, determined with either $Mg^{2+}$ or $Mn^{2+}$, and these were not altered appreciably by the presence of $Ca^{2+}$ of dithiothreitol.

*Stimulation of guanylate cyclase by fatty acids and the effects of $Ca^{2+}$*

Fatty acids were known to stimulate guanylate cyclase in fibroblast membranes [25] and platelets [26,27]. Since the activating factors in colon were heat stable and extractable with chloroform/methanol, suggesting that the factors may, in part, be lipids, the effect of arachidonic acid on the colon enzyme was examined directly (Fig. 7). When assayed in the presence of $Mn^{2+}$, activity increased with increasing concentrations of arachidonic acid, reaching a plateau at 100 $\mu$M fatty acid. In the presence of $Mg^{2+}$, however, the activity vs. concentration curve had a fairly sharp peak at 50 $\mu$M arachidonic acid. These findings would be consistent with the tentative view that the heat stable, chloroform/methanol extracted activators from the colon were at least in part, fatty acids.

Additional support for this hypothesis came from a study of the effect of $Ca^{2+}$ on the activation by arachidonic acid (Fig. 8). The fatty acid stimulation of the colon $Mg^{2+}$-dependent cyclase was blocked by $Ca^{2+}$. In contrast, the $Mn^{2+}$-dependent enzyme activity was augmented. These responses to $Ca^{2+}$ were

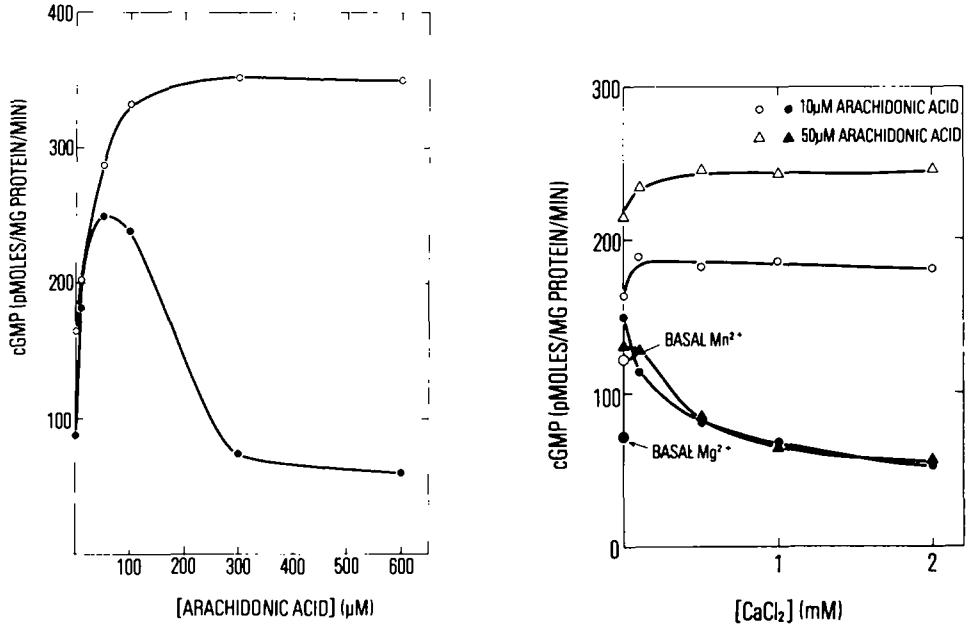


Fig. 7. The effects of arachidonic acid on guanylate cyclase from colon muscle, assayed in the presence of 5 mM $Mn^{2+}$ (○) or 5 mM $Mg^{2+}$ (●). The fatty acid was suspended by sonication in 10 mM Tris buffer, pH 7.4, and added to the reaction mixture.

Fig. 8. The effects of $Ca^{2+}$ on the arachidonic acid-stimulated guanylate cyclase from colon smooth muscle determined in the presence of 5 mM $Mn^{2+}$ (○,△) and 5 mM $Mg^{2+}$ (●,▲). Basal activities for the $Mn^{2+}$- and $Mg^{2+}$-dependent activities in the absence of arachidonic acid and $Ca^{2+}$ are indicated by the arrows.

similar to those seen with the endogenous colon activator (Figs. 2 and 5).

Arachidonic acid was reported to be a precursor of prostaglandins and prostaglandins were shown previously to increase guanylate cyclase activity [26]. Therefore, to ascertain whether arachidonic acid activated the colon enzyme directly or had to be converted to prostaglandins, the effect of indomethacin on the fatty acid induced activation was determined. In this experiment basal colon guanylate cyclase activities were 83 and 149 pmol/mg protein per min, measured with $Mg^{2+}$ and $Mn^{2+}$, respectively. The arachidonic acid (50 μM) stimulated activities were 220 and 224, respectively. The presence of indomethacin (50 μM) had little effect, activities being decreased slightly to 184 and 216, in the $Mg^{2+}$- and $Mn^{2+}$-dependent systems, respectively. This small inhibition by indomethacin was also seen for basal guanylate cyclase, in the absence of arachidonic acid. Thus, we conclude that the fatty acid, itself, stimulated colon guanylate cyclase and that its conversion to prostaglandins was not a prerequisite. A similar conclusion was reached with the platelet guanylate cyclase system [26].

Direct evidence that the heat stable, chloroform/methanol extractable activator from colon contained fatty acids came from chromatography of the extract on silica thin layers, using a solvent system of petroleum ether/ethyl ether/acetic acid, 80 : 30 : 1. The fatty acid region was scraped and then extracted

with chloroform/methanol. The colon material that cochromatographed with authentic fatty acids stimulated the colon guanylate cyclase (data not shown). This strongly suggests that at least part of the endogenous activating factors in the colon was fatty acids or their derivatives.

## Discussion

The findings described in this paper show that the effects of divalent cations in regulating cytosolic guanylate cyclase were tissue specific and dependent, in part, on the state of activation of the enzyme in that tissue. Thus, in smooth muscles, i.e., colon, aorta, and vas deferens, $Mg^{2+}$ effectively replaced $Mn^{2+}$ as the sole metal cofactor. The activities in other tissues, e.g., lung liver, and kidney, were strongly dependent on $Mn^{2+}$. Preincubation of smooth muscle extracts increased guanylate cyclase activity and when autoactivated in this fashion by endogenous factors or when stimulated by exogenous agents, as found here and as reported previously [8—10], the activated enzyme became relatively more $Mg^{2+}$ dependent. These findings suggest that guanylate cyclase in smooth muscle extracts, as prepared, was in a partially activated form. That this autoactivation may involve oxidative-reductive alterations [4,20,27,28] is indicated by the increased sensitivity of the $Mg^{2+}$-dependent activity to inhibition by dithiothreitol.

The hypothesis that the enzyme in smooth muscle was in a partially activated state is also supported by the actions of $Ca^{2+}$ on the $Mg^{2+}$- and $Mn^{2+}$-dependent activities. $Ca^{2+}$ strongly inhibited the $Mg^{2+}$-dependent activity of smooth muscles but had little affect on this activity in other tissues. However, when guanylate cyclase in colon as well as in lung, liver, and kidney were activated by exogenous agents, such as dehydroascorbate and $NaN_3$, then $Ca^{2+}$ inhibited the $Mg^{2+}$-dependent activities in all tissues. Additionally, $Ca^{2+}$ enhanced the activity of the $Mn^{2+}$-dependent enzyme in colon, even at high concentrations of $Mn^{2+}$. $Ca^{2+}$ had the same effect on the $Mn^{2+}$-dependent guanylate cyclase after activation by exogenous activators.

The endogenous activating factors in the colon smooth muscle were heat stable and largely extractable with chloroform/methanol. The extracted material cochromatographed with authentic fatty acids. Also, arachidonic acid stimulated colon guanylate cyclase and this enhancement of the $Mg^{2+}$-dependent enzyme was blocked by $Ca^{2+}$. Since fatty acids were found previously to activate fibroblast membrane and platelet guanylate cyclase [25—27], the present findings strongly suggest that a significant fraction of the activating factors in the colon was fatty acids or their derivatives. It is important to note, however, that the colon activating factors, although they markedly increased guanylate cyclase activity in the colon, had only minimal effects on the enzyme in the kidney. Thus, the ability of the enzyme to be stimulated by endogenous activators was dependent on the tissue from which the enzyme was derived. But, other studies (Takenawa, T., Liang, C.T. and Sacktor, B., unpublished observations) indicate that the kidney contained a potent inhibitor of guanylate cyclase; thus, the possibility that a potential stimulation of the renal enzyme by the endogenous colon activator was precluded by this inhibitor was not ruled out.

It is intriguing to consider whether the special interactions of $Mg^{2+}$ and $Ca^{2+}$ on guanylate cyclase in smooth muscle have a regulatory role on the contractile response of the muscle. It was found that agents known to be capable of contracting and relaxing smooth muscle produced marked increases in cyclic GMP levels [29,30] and it was proposed that these increases in cyclic GMP might be part of a negative feedback mechanism tending to reduce $Ca^{2+}$ influx or to increase $Ca^{2+}$ efflux. The results of the present study indicate that $Mg^{2+}$, at in vivo concentrations, could serve as the sole metal cofactor for guanylate cyclase in various smooth muscles and that the addition of $Ca^{2+}$ effectively inhibited the synthesis of cyclic GMP by the $Mg^{2+}$-dependent enzyme. Accordingly, an influx of $Ca^{2+}$ or a mobilization of the divalent cation which leads to an increase in its concentration in the cytosol would tend to decrease the level of cyclic GMP. Thus, cyclic GMP and $Ca^{2+}$ might participate in reciprocal negative feedback mechanisms. Further investigations are needed to test this working hypothesis.

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