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ACTIVATION OF CORONARY ARTERIAL GUANYLATE CYCLASE BY NITRIC OXIDE, NITROPRUSSIDE, AND NITROSOGUANIDINE—INHIBITION BY CALCIUM, LANTHANUM, AND OTHER CATIONS, ENHANCEMENT BY THIOLS*

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Abstract—Although reports that certain vasodilatc s activate soluble guanylate cyclase, especially in the presence of thiols, and elevate cyclic GMP levels in smooth muscle suggest that cyclic GMP is involved in vascular smooth muscle relaxation, earlier reports that Ca^{2+} activates guanylate cyclase and that Ca^{2+}-dependent contractile agents elevate cyclic GMP levels are seemingly at odds with this hypothesis. The objective of this study was to examine the effects of Ca^{2+}, related cations, and thiols on bovine coronary arterial soluble guanylate cyclase. Guanylate cyclase activity was detected in the presence of Mg^{2+} or Mn^{2+} but not of other cations. Basal activity was greater in the presence of Mn^{2+} than of Mg^{2+}. Activity of guanylate cyclase stimulated by nitroprusside, nitric oxide, or nitrosoguanidine, however, was greater with Mg^{2+}, although the requirement of activated enzyme for Mn^{2+} was reduced about 10-fold. Ca^{2+} markedly inhibited guanylate cyclase activation in the presence of Mg^{2+} but not of Mn^{2+}. La^{3+} inhibited enzyme activation in the presence of Mg^{2+} or Mn^{2+}. Neither Ca^{2+} nor La^{3+} altered basal enzymatic activity. Results that were qualitatively similar to those indicated above were observed with partially purified, heme-free, coronary arterial soluble guanylate cyclase. Nitric oxide and nitroso compounds activated partially purified enzyme, and thiols enhanced enzyme activation by nitroprusside and nitrosoguanidine without appreciably altering basal activity. Irreversible sulfhydryl binding agents such as ethacrynic acid and gold inhibited both basal and activated guanylate cyclase. These results suggest that changes in intracellular concentrations of free Ca^{2+} and sulfhydryl groups could influence the rate of formation of cyclic GMP by vasodilators and that this, in turn, could alter smooth muscle tone.

The findings that a variety of vasodilators such as nitroglycerin, nitroprusside, and $NaNO_2$ elevate tissue levels of guanosine 3′, 5′-monophosphate (cyclic GMP)* suggested to some that smooth muscle relaxation could involve cyclic GMP [1–6]. Support for this view strengthened as reports appeared on activation of soluble guanylate cyclase from smooth muscle by vasodilators [7–9]. In addition, NO, cigarette smoke, and MNNG, which were not previously known to relax smooth muscle, were shown to activate guanylate cyclase [8–13] and relax coronary arterial smooth muscle [9]. Another apparent correlation between cyclic GMP and vascular smooth muscle relaxation stems from the observations that (1) nitroglycerin specifically requires cysteine whereas $NaNO_2$ requires one of a variety of thiols to activate coronary arterial guanylate cyclase [14], and (2) tolerance to the relaxant effect of nitroglycerin is proportional to the depletion of free sulfhydryl groups from smooth muscle [15, 16]. Finally, the reports that 8-bromo cyclic GMP markedly relaxes smooth muscle [17, 18] further strengthened the developing hypothesis that cyclic GMP is somehow involved in smooth muscle relaxation.

However, many earlier studies involving smooth muscle contractile agents and calcium are apparently inconsistent with a biological role for cyclic GMP in smooth muscle relaxation. For example, many endogenous substances which contract smooth muscle were found to elevate tissue cyclic GMP levels, an effect that was calcium-dependent [19–23]. Moreover, calcium was reported to stimulate soluble guanylate cyclase activity [24–29]. Indeed, the latter observation, together with the failure to demonstrate appreciable activation of guanylate cyclase by calcium-dependent, smooth muscle contractile agents, suggested that calcium was responsible for the tissue

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* Abbreviations used in the text are as follows: cyclic GMP, guanosine 3′ 5′-monophosphate; NO, nitric oxide; MNNG, *N*-methyl-*N*′-nitro-*N*-nitrosoguanidine; DTT, DL-dithiothreitol; GSH, reduced glutathione; GSSG, oxidized glutathione; and oxidized DTT, *trans*-4.5-dihydroxy-1.2-dithiane.

accumulation of cyclic GMP which occurred in the presence of contractile agents.

The objective of the present study was to examine the effects of calcium, related cations, and a variety of thiols on bovine coronary arterial soluble guanylate cyclase activity. The effects of these substances on both basal activity and guanylate cyclase activated by nitroprusside, NO, and MNNG were determined. In addition, preliminary data on some of the pertinent properties of partially purified coronary arterial soluble guanylate cyclase are presented.

MATERIALS AND METHODS

Materials.[^{3}H]Cyclic GMP (2–3 Ci/mmole, ammonium salt) and α-[^{32}P]GTP (10–18 Ci/mmole, triethylammonium salt) were purchased from the New England Nuclear Corp. (Boston, MA). Dowex-50(H^+) was purchased from the Sigma Chemical Co. (St. Louis, MO) (Dowex-50W, 50 × 4–400, hydrogen form), as were Dowex-1 (1 × 8–200, chloride form) and polyethyleneimine cellulose. Neutral alumina (Woelm) was obtained from ICN Nutritional Biochemicals, Cleveland, OH. Plastic straws (HP-66, 6.25 inch uncrimped), and acetate filter tips were purchased from the Maryland Paper Products Co. (Baltimore, MD.) and American Filtrona (Richmond, VA.) Corp., respectively. NO gas (99.9%) and MNNG were purchased from Matheson Gas Products (Rutherford, NJ) and the Aldrich Chemical Co. (Milwaukee, WI), respectively; all other chemical compounds used in this study were from the Sigma Chemical Co. Preparations of solutions of nitroprusside and MNNG, as well as handling and delivery of NO to reaction mixtures, were described previously [9]. The acetate salts of Mg^{2+}, Mn^{2+}, and Ca^{2+} were used, whereas the chloride salts of La^{3+}, Sr^{2+}, Co^{2+}, and Ni^{2+} were employed. Gold was in the form of either the thioglucose or sodium thiosulfate salt.

Preparation of bovine coronary arterial soluble fraction. Bovine hearts were obtained from a local slaughterhouse; segments of left descending and circumflex arteries and their branches were removed, cleaned and homogenized, and soluble fractions were prepared according to methods described previously [9]. Soluble fractions were stored at −80°, and contained 1.6–3.4 mg protein/ml, determined by the Lowry method.

Partial purification of guanylate cyclase. Guanylate cyclase was partially purified from bovine coronary arterial soluble fraction according to the procedure described previously for hepatic soluble guanylate cyclase [30]. Briefly, bovine coronary arterial soluble fraction was prepared in 10 mM Tris–HCl, pH 7.4, containing 0.25 M sucrose, 0.5 mM EDTA and 2 mM DTT (DTT-buffer). To approximately 400 ml of soluble fraction, solid ammonium sulfate was added to 40% saturation. After 3 hr at 0–4° the precipitate was collected by centrifugation, suspended in 20 ml of cold DTT-buffer, and dialyzed for 16 hr at 0–4° against 2 liters of DTT-buffer (with three changes of buffer). The dialyzed mixture was centrifuged and the clear supernatant fluid was applied to a DE52 cellulose column (1.5 × 25 cm) previously equilibrated with DTT-buffer. The column was eluted with a linear NaCl gradient (0–0.3 M) in DTT-buffer at a flow rate of 16 ml/hr (4 ml fractions). Fractions containing guanylate cyclase activity were pooled, concentrated to 2–3 ml by ultrafiltration, and applied to a Bio-Gel A-0.5 m column (1.5 × 25 cm) previously equilibrated with DTT-buffer containing 0.1 M NaCl. The column was eluted with the same buffer at a flow rate of 16 ml/hr (2 ml fractions). Fractions containing guanylate cyclase activity (partially purified enzyme) were stored at −85° for up to 10 weeks with no loss of enzymatic activity. Partially purified guanylate cyclase was assayed for heme by visible absorption spectroscopy as described by Rossi-Fanelli *et al.* [31]. The lower limit of sensitivity of this procedure is 10 nM hemoglobin. No detectable heme was found in fractions of partially purified guanylate cyclase. Since the dilution of enzyme in final reaction mixtures was 100-fold, less than 0.1 nM heme was present.

Guanylate cyclase assay. Guanylate cyclase activity was determined by measuring the formation of [^{32}P]cyclic GMP from α-[^{32}P]GTP exactly as described previously [9], including the sequential column chromatography on Dowex-50(H^+) followed by neutral alumina in order to isolate the reaction product. Dowex-50(H^+) is expensive, and since large amounts are routinely employed in this assay, a batch procedure for washing the resin completely free of traces of radioactivity was developed. A 60 cm × 5 cm column of used resin (accumulated and stored under H_2O at 4°) was eluted first with 2 liters of H_2O, followed with 2 liters of 0.1 N HCl, and followed with 4 liters of H_2O. The entire washing procedure takes 8–9 hr and no measurable ^{3}H or ^{32}P elutes (H_2O or 50 mM Tris–HCl, pH 7.6, as eluant) from the small columns of resin employed routinely in the guanylate cyclase assay. Batches of Dowex-50(H^+) have been used and washed according to the above procedure over fifty times, with no appreciable changes in the elution profiles of GTP or cyclic GMP.

Different batches of bovine coronary arterial soluble fraction were found to vary, up to 2-fold in some instances, in the sensitivity of guanylate cyclase to activation by NO, nitroprusside, and MNNG. For this reason the data that are illustrated in graphic form were obtained from a single representative experiment. In each case one or more additional experiments were conducted with a different batch of soluble fraction, and qualitatively similar data were obtained. This information is provided in the figure legends.

Verification of reaction product. Cyclic GMP was verified as the product of the enzymatic reaction by comparison with [^{3}H]cyclic GMP by column chromatography and by rapid hydrolysis to GMP by beef heart phosphodiesterase. Dowex-1, chloride form was converted to the formate form by suspending the resin in 4 N formic acid and agitating gently for 1 hr. The fines and supernatant fractions were decanted, and the resin was stored in 0.5 N formic acid at 4°. Columns (4 cm × 0.7 cm) of Dowex-1 formate were prepared and immediately washed with sufficient H_2O (15–20 ml) to raise the pH of the eluate to 4. Two milliliters of neutral alumina eluate (from the guanylate cyclase assay) was applied to a Dowex-1 formate column, and the eluate was dis-

carded. Ten milliliters of H_2O was added and the eluate was discarded. The column was then eluted first with five individual 2-ml aliquots of 2 N formic acid followed with seven individual 2-ml aliquots of 4 N formic acid. Column eluates were added to 10 ml of Multisol (Isolab), and radioactivity was measured by liquid scintillation spectrometry. In certain experiments, beef heart phosphodiesterase and Mg^{2+} [0.1 ml of 90 mM $Mg(C_2H_3O_2)_2$ containing 300 μg of phosphodiesterase] were added to 3 ml of neutral alumina eluate and the samples were incubated for 10 min at 37°. Reaction mixtures were cooled rapidly to 0–4°, and 2 ml was immediatly chromatographed on columns of Dowex-1 formate as described above.

RESULTS

Activation of guanylate cyclase in the presence of Mg^{2+} *or* Mn^{2+}. NO (0.1 μl), nitroprusside (0.1 mM), and MNNG (0.1 mM) activated bovine coronary arterial soluble guanylate cyclase (0.1 mM GTP substrate) in the presence of 3 mM Mg^{2+} in a time-dependent (linear for 10 min) and tissue protein concentration-dependent (linear up to 0.3 mg) manner. Cyclic GMP was verified as the only ^{32}P-product of the reaction by comparison with [^{3}H]cyclic GMP. Figure 1 illustrates that the ^{32}P-product displayed elution profiles identical to those of [^{3}H]cyclic GMP on columns of Dowex-1 formate, both before and after hydrolysis of cyclic GMP with added phosphodiesterase. Phosphodiesterase-treated samples displayed a shift in elution profiles from 4 to 2 N formic acid, where GMP is eluted from the column (data for GMP are not shown). The reaction product was identified as cyclic GMP also by chromatography on columns of polyethyleneimine cellulose (data not shown). Optimal concentrations of Mg^{2+} and Mn^{2+} for activation of guanylate cyclase by NO (Fig. 2) or by 0.1 mM nitroprusside or 0.1 mM MNNG (not illustrated) were approximately 3 mM and 0.3 mM, respectively, when the GTP concentration was 0.1 mM. At optimal concentrations of divalent cation, enzymatic activity in the presence of Mg^{2+} was twice that with Mn^{2+}. In the absence of activators (basal activity), optimal concentrations of both Mn^{2+} and Mg^{2+} were 3–4 mM, and guanylate cyclase activity in the presence of Mn^{2+} [12 pmoles cyclic GMP·min^{-1}·mg protein^{-1}] exceeded that with Mg^{2+} [4 pmoles cyclic GMP·min^{-1}·mg protein^{-1}] by 3-fold. Soluble guanylate cyclase from some, but not from all, tissues undergoes activation upon preincubation at 37° for several minutes to 1 hr [26, 32]. Preincubation of bovine coronary arterial soluble guanylate cyclase for up to 1 hr with Mg^{2+} or Mn^{2+}, in the

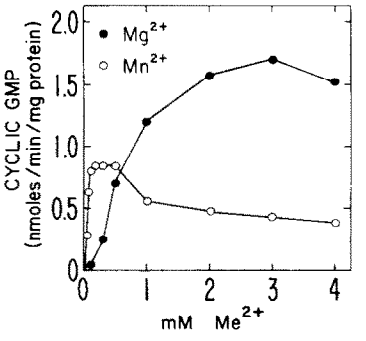

Fig. 2. Activation of guanylate cyclase by NO in the presence of various concentrations of Mg^{2+} or Mn^{2+}. Reaction mixtures, containing various concentrations of either Mg^{2+} or Mn^{2+}, were incubated for 10 min at 37° as described in Materials and Methods. Reactions were started by addition of soluble fraction followed 1 min later by 0.1 μl NO (contained in 50 μl N_2). Me^{2+} signifies either Mg^{2+} or Mn^{2+}. Data are the averages of triplicate determinations from a single experiment. Two to three additional experiments using two different batches of soluble fraction yielded qualitatively similar data.

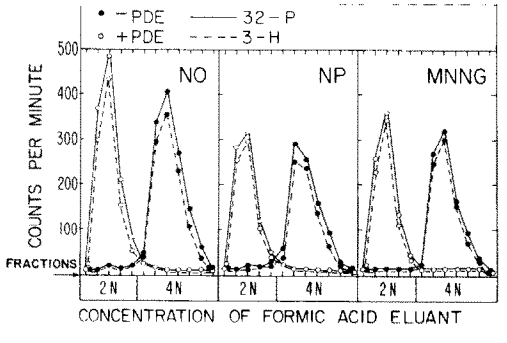

Fig. 1. Identification of cyclic GMP as product of the guanylate cyclase reaction by Dowex-1 formate column chromatography and hydrolysis of product to GMP by phosphodiesterase. Reactions, using 3 mM Mg^{2+} as the required divalent cation, and chromatography were conducted as described in Materials and Methods. The concentrations of activators tested were 0.1 μl NO, 0.1 mM nitroprusside (NP), and 0.1 mM MNNG. PDE signifies phosphodiesterase. Data are the averages of duplicate determinations (two separate columns) from a single experiment. One additional experiment yielded qualitatively similar data.

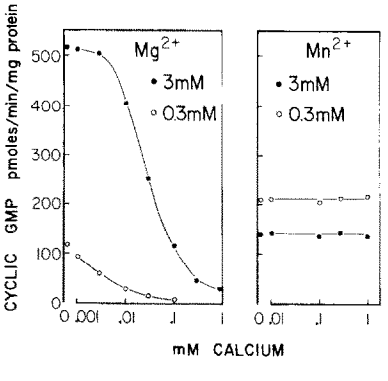

Fig. 3. Effects of Ca^{2+} on activation of guanylate cyclase by NO in the presence of Mg^{2+} or Mn^{2+}. Reaction mixtures were incubated for 10 min at 37° as described in Materials and Methods. The divalent cation requirement was satisfied by either Mg^{2+} or Mn^{2+} at the concentrations indicated. NO (0.1 μl contained in 50 μl N_2) was injected into reaction mixtures exactly 1 min after starting reactions with soluble fraction. Note that the abscissa represents a log scale. Data are the averages of duplicate determinations from a single experiment. Two additional experiments, each with a different batch of soluble fraction, yielded qualitatively similar data.

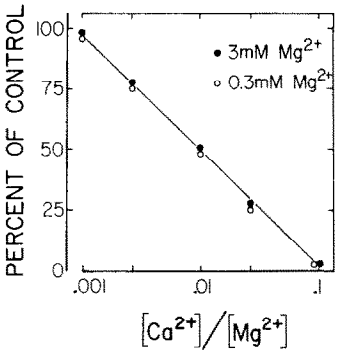

Fig. 4. Relationship between the concentration ratio of Ca^{2+} to Mg^{2+} and the inhibition of guanylate cyclase activation by NO. Reaction mixtures were incubated for 10 min at 37° as described in Materials and Methods. The divalent cation requirement was satisfied by 3 or 0.3 mM Mg^{2+}. The Ca^{2+} concentration was adjusted according to the desired ratio of Ca^{2+} to Mg^{2+}, as indicated on the abscissa (log scale). NO (0.1 μl contained in 50 μl N_2) was injected into reaction mixtures exactly 1 min after starting reactions with soluble fraction. Basal guanylate cyclase activity was 5.0 ± 0.4 (for 3 mM Mg^{2+}) and less than 1 (for 0.3 mM Mg^{2+}) pmole cyclic GMP·min^{-1}·mg protein)$^{-1}$. Data are the averages of six determinations from three separate experiments. Standard errors (not shown) were less than 10 per cent of the corresponding mean, and the correlation coefficient was 0.98.

absence or presence of thiols (2 mM DTT or cysteine), failed to activate the enzyme.

Effects of Ca^{2+}, La^{3+}, *and other cations on guanylate cyclase activation.* Mg^{2+} or Mn^{2+} could not be substituted with 0.1–3 mM concentrations of Ca^{2+}, La^{3+}, Sr^{2+}, Co^{2+}, or Ni^{2+} for the expression of either basal or activated (0.1 mM nitroprusside) guanylate cyclase [enzymatic activity never exceeded 1 pmole cyclic GMP·min^{-1}·mg protein)$^{-1}$. Also, it is important to emphasize that neither Ca^{2+} nor La^{3+} at concentrations of 0.001–3 mM significantly altered basal guanylate cyclase activity in the presence of either 0.1–3 mM Mg^{2+} or 0.1–3 mM Mn^{2+} when the GTP concentration was 0.1 mM. Concentration ratios of GTP to either Mg^{2+} or Mn^{2+} in excess of unity were not tested. In the presence of Mg^{2+}, Ca^{2+} markedly inhibited guanylate cyclase activation by NO (Fig. 3), nitroprusside (Table 1), and MNNG (not shown). Inhibition occurred even at Ca^{2+} concentrations that were over 100-fold smaller than that of Mg^{2+}. When Mn^{2+} was used, however, Ca^{2+} failed to inhibit enzyme activation even at concentrations that were more than 3-fold greater than those of Mn^{2+}. Inhibition of cyclic GMP formation by Ca^{2+}, in the presence of excess Mg^{2+}, varied directly with the concentration ratio of Ca^{2+} to Mg^{2+} (Fig. 4) and was independent of the concentrations of GTP or enzyme (not shown). The identical relationship between percent inhibition and concentration ratio of Ca^{2+} to Mg^{2+} resulted when 0.1 mM nitroprusside or 0.1 mM MNNG was used as activator (data not shown).

Using nitroprusside to activate guanylate cyclase, La^{3+} markedly inhibited enzyme activation in the presence of either Mg^{2+} or Mn^{2+}, although inhibition was greater in the presence of Mg^{2+} (Table 1). Concentrations of La^{3+} that were 30,000-fold lower than that of Mg^{2+} inhibited enzyme activation by almost 50 per cent. Inhibition by Sr^{2+} resembled qualitatively that by Ca^{2+}, whereas inhibition by Co^{2+} and Ni^{2+} resembled qualitatively that by La^{3+}. Mn^{2+} also inhibited guanylate cyclase activation in the presence of much larger concentrations of Mg^{2+}. Au^{2+}, which readily forms covalent bonds with free sulfhydryl groups, inhibited guanlyate cyclase activation (Table 1) and basal enzymatic activity (not shown) equally well in the presence of either Mg^{2+} or Mn^{2+}. Qualitatively similar inhibitory effects of the various cations were obtained when 0.1 μl NO or 0.1 mM MNNG was used as activator. Barium and various monovalent cations (Na^+, K^+, Li^+) and monovalent anions (Cl^-, F^-, CH_3COO^-) elicited no effects on guanylate cyclase activation by nitroprusside or NO (data not shown).

Effects of thiols, other reductants, disulfides and ethacrynic acid on guanylate cyclase activation. DTT enhanced activation of guanylate cyclase by nitroprusside and MNNG (Fig. 5). DTT decreased guan-

Table 1. Effects of various cations on activation of guanylate cyclase by nitroprusside in the presence of Mg^{2+} or Mn^{2+}*

Cation	Conc. (mM)	Percent inhibition of activation: 3 mM Mg^{2+}	0.3 mM Mn^{2+}
Ca^{2+}	0.1	74 ± 5	0
	0.03	48 ± 3	0
	0.01	25 ± 1	0
La^{3+}	0.003	98 ± 4	66 ± 5
	0.001	93 ± 3	47 ± 3
	0.0003	79 ± 6	10 ± 1
	0.0001	43 ± 4	0
Sr^{2+}	0.1	35 ± 2	0
	0.03	18 ± 1	0
	0.01	9 ± 1	0
Co^{2+}	0.1	53 ± 6	23 ± 3
	0.03	19 ± 2	9 ± 1
	0.01	8 ± 1	0
Ni^{2+}	0.1	34 ± 3	16 ± 1
	0.03	20 ± 2	9 ± 1
	0.01	7 ± 1	0
Mn^{2+}	0.1	47 ± 4	
	0.03	32 ± 2	
	0.01	16 ± 1	
Au^{2+}	0.01	99 + 2	93 + 4
	0.003	74 ± 6	68 ± 5
	0.001	31 ± 2	33 ± 2

* Reaction mixtures were incubated for 10 min at 37° as described in Materials and Methods. Reactions were started by addition of soluble fraction followed immediately by 0.1 mM nitroprusside. Basal guanylate cyclase activity was 4.8 ± 0.4 (for 3 mM Mg^{2+}) and 8.4 ± 0.8 (for 0.3 mM Mn^{2+}) pmoles cyclic GMP·min^{-1}·mg protein)$^{-1}$. Guanylate cyclase activity in the presence of nitroprusside was 686 ± 23 (for 3 mM Mg^{2+}) and 403 ± 13 (for 0.3 mM Mn^{2+}) pmoles cyclic GMP·min^{-1}·mg protein)$^{-1}$.

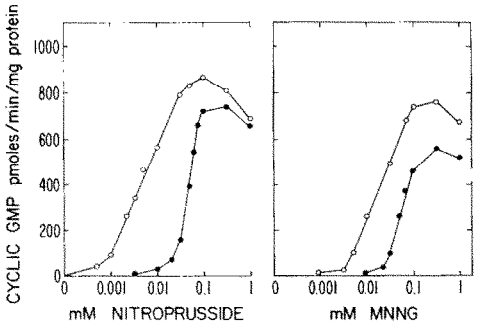

Fig. 5. Effect of DTT on activation of guanylate cyclase by nitroprusside and MNNG. Reaction mixtures, containing 3 mM Mg^{2+} as the required divalent cation, were incubated for 10 min at 37° as described in Materials and Methods. Reactions were started by addition of soluble fraction followed immediately by either nitroprusside or MNNG at the concentrations indicated on the abscissa (log scale). Open and closed circles signify the presence and absence, respectively, of 2 mM DTT. Basal guanylate cyclase activity was 5.7 ± 0.5 pmoles cyclic GMP·min^{-1}·mg protein)$^{-1}$. Data are the averages of duplicate determinations from a single experiment. Two additional experiments using a different batch of soluble fraction yielded qualitatively similar data.

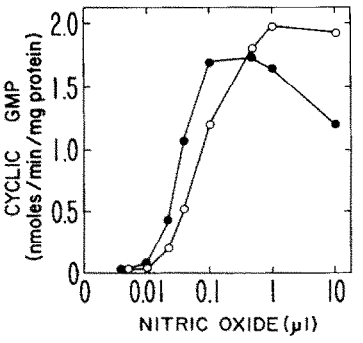

Fig. 6. Effect of DTT on activation of guanylate cyclase by NO. Reaction mixtures, containing 3 mM Mg^{2+} as the required divalent cation, were incubated for 10 min at 37° as described in Materials and Methods. Reactions were started by addition of soluble fraction, followed 1 min later by the amounts of NO (contained in 50 μl N_2) indicated on the abscissa (log scale). Open and closed circles signify the presence and absence, respectively, of 2 mM DTT. Basal guanylate cyclase activity was 5.2 ± 0.4 pmoles cyclic GMP·min^{-1}·mg protein)$^{-1}$. Data are the averages of triplicate determinations from a single experiment. Two additional experiments using a different batch of soluble fraction yielded qualitatively similar data.

ylate cyclase activation by small amounts of NO (up to 0.1 μl) and enhanced activation by larger amounts (Fig. 6). Experiments with 0.3 mM Mn^{2+} yielded qualitatively similar data with activities that were 50–60 per cent of those observed with 3 mM Mg^{2+}. Like DTT, other thiols, such as cysteine, GSH, penicillamine, 2-mercaptoethanol, 2-mercaptoethylamine and 3-mercaptopropionic acid, enhanced guanylate cyclase activation by nitroprusside and MNNG but not by NO (Table 2). The oxidized forms of cysteine, GSH, and DTT failed to enhance enzyme activation by nitroprusside or MNNG. In fact, cystine and GSSG were inhibitory. Ethacrynic acid, a sulfhydryl alkylating agent, practically abolished guanylate cyclase activation. The nonthiol reductants, ascorbate and dithionite, did not enhance

Table 2. Effects of thiols, other reductants, disulfides, and ethacrynic acid on guanylate cyclase activation by NO, nitroprusside, and MNNG*

	Guanylate cyclase activity [pmoles cyclic GMP·min^{-1}·(mg protein)$^{-1}$]			
Additions	Basal activity	0.1 μl NO	0.1 mM Nitroprusside	0.1 mM MNNG
None	5.1 ± 0.4	912 ± 43	571 ± 23	606 ± 21
5 mM Cysteine	4.5 ± 0.3	903 ± 37	984 ± 25†	1042 ± 39†
5 mM GSH	5.0 ± 0.4	936 ± 46	908 ± 32†	906 ± 37†
5 mM Penicillamine	4.2 ± 0.5	921 ± 49	812 ± 28†	971 ± 32†
20 mM 2-Mercaptoethanol	5.3 ± 0.4	898 ± 35	839 ± 21†	884 ± 23†
5 mM 2-Mercaptoethylamine	4.8 ± 0.2	902 ± 44	855 ± 30†	923 ± 41†
5 mM 3-Mercaptopropionic acid	5.0 ± 0.5	885 ± 26	893 ± 26†	827 ± 25†
2 mM Ascorbate		762 ± 37†	583 ± 21	895 ± 30†
5 mM Ascorbate	5.4 ± 0.4	451 ± 21†	594 ± 28	972 ± 27†
0.1 mM Dithionite		626 ± 19†	405 ± 17†	209 ± 9†
0.5 mM Dithionite	4.1 ± 0.3	177 ± 12†	266 ± 11†	31 ± 2†
2 mM Cystine	4.8 ± 0.2	220 ± 9†	267 ± 14†	208 ± 11†
5 mM GSSG	4.3 ± 0.3	593 ± 28†	390 ± 16†	347 ± 10†
5 mM Oxidized DTT	5.6 ± 0.4	869 ± 41	537 ± 20	551 ± 22
2 mM Ethacrynic acid	<1†	13 ± 2†	32 ± 1†	29 ± 2†

* Reaction mixtures, containing 3 mM Mg^{2+} as the required divalent cation, were incubated for 10 min at 37° as described in Materials and Methods. Reactions were started by addition of soluble fraction followed immediately by either nitroprusside or MNNG. NO (contained in 50 μl N_2) was added at exactly 1 min of incubation. Data are means ± S.E.M. for four to eight determinations from two to four separate experiments.

† Significantly different ($P < 0.01$) from activation in absence of additions (Student's t-test for unpaired values).

enzyme activation by NO or nitroprusside (Table 2). Dithionite inhibited activation by NO, nitroprusside or MNNG, whereas ascorbate inhibited activation by NO and enhanced that by MNNG. Ascorbate enhancement of MNNG was attributed to the direct release of NO from MNNG in the presence of ascorbate [33]. Basal guanylate cyclase activity was unaffected by the agents tested except ethacrynic acid, which abolished activity.

Experiments with partially purified bovine coronary arterial soluble guanylate cyclase. Partial purification of soluble guanylate cyclase, resulting in a stable preparation that was devoid of detectable heme, had 85–100 times the basal specific activity (0.3 mM GTP, 3 mM Mg^{2+}) of the unpurified enzyme. Enzymatic reactions containing 5–10 μg protein were linear for 10 min of incubation. Product formation, however, showed a sigmoid relationship with respect to protein concentration (2–20 μg, 10-min incubation), although no preincubation activation was apparent. NO, nitroprusside, and MNNG activated guanylate cyclase in the presence of excess Mg^{2+} but not excess Mn^{2+} (Table 3). However, enzyme activation was evident when the Mn^{2+} concentration was reduced to that of GTP (0.3 mM). At a concentration that was 300-fold smaller than that of Mg^{2+}, Ca^{2+} markedly inhibited guanylate cyclase activation without affecting basal activity (Table 3). Inhibition by Ca^{2+} varied directly with the concentration ratio of Ca^{2+} to Mg^{2+}. On the other hand, enzyme activation in the presence of 0.3 mM Mn^{2+} was not appreciably altered by Ca^{2+}. La^{3+} markedly inhibited guanylate cyclase activation, but not basal activity, in the presence of Mg^{2+}. Although neither cysteine nor DTT affected basal activity, both of these thiols significantly enhanced enzyme activation by NO, nitroprusside, and MNNG ($P < 0.01$, compared with activation in the absence of added thiol). Ethacrynic acid markedly inhibited both basal and activated guanylate cyclase (Table 3).

DISCUSSION

The seemingly discordant observations that elevated intracellular concentrations of free Ca^{2+} and cyclic GMP [19–23] are associated with smooth muscle contraction and that decreased free Ca^{2+} but elevated cyclic GMP levels [1–6] are associated with drug-elicited relaxation prompted an analysis of the interactions between Ca^{2+} and vasodilators on vascular smooth muscle guanylate cyclase. Nitroprusside was used to activate unpurified and partially purified soluble guanylate cyclase from bovine coronary artery because this potent vasodilator elevates vascular smooth levels of cyclic GMP [6]. In addition, NO and MNNG were employed because these potent activators of guanylate cyclase [8, 10] also relax coronary arterial smooth muscle [9]. Interactions between thiols and nitroso compounds were examined in view of recent reports from this laboratory suggesting that many different vasodilators react with thiols to form *S*-nitrosothiols, which are potent guanylate cyclase activators [14, 33] and vascular smooth muscle relaxants [34]. The results of this study indicate clearly that Ca^{2+}, La^{3+}, and related cations markedly inhibit, whereas thiols enhance, activation of unpurified and partially purified soluble guanylate cyclase without altering basal activity.

Similar to soluble guanylate cyclase from many other tissues [8, 26, 35–38], that from bovine coronary artery displayed basal activity that was greater in the presence of Mn^{2+} than of Mg^{2+}, whereas the activated enzyme was more effective in the presence of Mg^{2+}, and other divalent cations could not substitute. Since mammalian tissue concentrations of Mg^{2+} exceed those of Mn^{2+} by two to three orders of magnitude [39], basal guanylate cyclase activity may be very low, dependent on Mg^{2+}, and highly sensitive to activation. This view is supported by the observations that coronary arterial guanylate cyclase was very sensitive to activation by nitroprusside,

Table 3. Effects of cations, thiols, and ethacrynic acid on activation of partially purified guanylate cyclase by NO, nitroprusside, and MNNG*

Additions	Divalent cation requirement	Guanylate cyclase activity [pmoles cyclic GMP·min^{-1}·mg protein^{-1}] Basal activity	0.1 μl NO	0.1 mM Nitroprusside	0.1 mM MNNG
None	3 mM Mg^{2+}	0.34 ± 0.04	2.8 ± 0.2	1.3 ± 0.1	0.82 ± 0.07
	3 mM Mn^{2+}	0.62 ± 0.05	0.66 ± 0.07	0.60 ± 0.04	0.65 ± 0.04
	0.3 mM Mn^{2+}	0.15 ± 0.01	0.87 ± 0.1	0.45 ± 0.02	0.55 ± 0.08
0.01 mM Ca^{2+}	3 mM Mg^{2+}	0.31 ± 0.03	1.4 ± 0.1	0.54 ± 0.04	0.39 ± 0.02
0.1 mM Ca^{2+}	3 mM Mg^{2+}	0.32 ± 0.04	0.66 ± 0.04		
	0.3 mM Mn^{2+}	0.15 ± 0.02	0.83 ± 0.1	0.44 ± 0.02	0.59 ± 0.06
0.001 mM La^{3+}	3 mM Mg^{2+}	0.36 ± 0.02	0.63 ± 0.05	0.33 ± 0.02	0.30 ± 0.01
5 mM Cysteine	3 mM Mg^{2+}	0.37 ± 0.04	3.9 ± 0.5	2.4 ± 0.1	2.7 ± 0.2
5 mM DTT	3 mM Mg^{2+}	0.28 ± 0.03	4.3 ± 0.4	2.3 ± 0.1	4.1 ± 0.2
2 mM Ethacrynic acid	3 mM Mg^{2+}	< 0.01	0.04 ± 0.01	< 0.01	< 0.01

* Reaction mixtures, containing 6–12 μg protein, 0.3 mM GTP and either Mg^{2+} or Mn^{2+} as indicated, were incubated for 10 min at 37° as described for unpurified soluble fraction. Reactions were started by addition of enzyme followed immediately by either nitroprusside or MNNG. NO (contained in 50 μl N_2) was added at exactly 1 min of incubation. Data are means ± S.E.M. for four to eight determinations from two to four separate experiments using three different fractions of partially purified enzyme.

NO, and MNNG in the presence of Mg^{2+} and that these activators markedly elevated tissue cyclic GMP levels [1–6, 10, 11]. Like guanylate cyclase from other tissues [40], that from coronary artery appears to possess free sulfhydryl groups required for catalytic activity, because ethacrynic acid and gold abolished both basal and stimulated guanylate cyclase activity. Partially purified, heme-free, soluble guanylate cyclase was activated by NO, nitroprusside, and MNNG in the absence of added heme, and this activation was enhanced by thiols. These data are more in line with those reported by Braughler *et al.* [41] than by Craven and DeRubertis [12] for hepatic guanylate cyclase.

Ca^{2+} markedly inhibited activation of unpurified and partially purified guanylate cyclase by nitroprusside, NO, and MNNG in the presence of Mg^{2+} but not Mn^{2+}. Inhibition by Ca^{2+} was directly dependent on the Ca^{2+} to Mg^{2+} concentration ratio and independent of either GTP or enzyme concentrations when Mg^{2+} was in excess of GTP. Thus, Ca^{2+} may have competed with Mg^{2+}, but not with Mn^{2+}, for common binding sites on GTP, perhaps rendering GTP less suitable as substrate for activated guanylate cyclase. Alternative explanations however, are possible. As in our observations, Ca^{2+} was reported recently to inhibit activation of unpurified guanylate cyclase from rabbit tissues by azide in the presence of Mg^{2+}, but not of Mn^{2+} [42]. Ca^{2+} also has been shown to inhibit azide activation of hepatic soluble guanylate cyclase in the presence of either Mg^{2+} or Mn^{2+} [38]. The present report illustrates the remarkable potency of La^{3+} as an inhibitor of guanylate cyclase activation. At concentrations that were four orders of magnitude lower than, that of Mg^{2+}, La^{3+} inhibited enzyme activation by nitroprusside by over 50 per cent. In contrast to Ca^{2+}, larger concentrations of La^{3+} inhibited enzyme activation in the presence of Mn^{2+} as well as of Mg^{2+}.

The biological significance of the inhibition by low Ca^{2+} concentrations of coronary arterial guanylate cyclase activated by vasodilators is not clear. This lack of clarity derives from previous observations that Ca^{2+} stimulated guanylate cyclase activity [24–29] and that smooth muscle contractile agents were dependent on Ca^{2+} to elevate tissue levels of cyclic GMP [19–23]. Guanylate cyclase stimulation however, occurred only with large Ca^{2+} concentrations in the presence of a small concentration ratio of Mn^{2+} to GTP [24–27, 38], and no enzyme stimulation occurred when Mg^{2+} was substituted for Mn^{2+} [43]. Moreover, elevations of cyclic GMP levels in smooth muscle that was challenged with contractile agents were suggested to be the result of indirect rather than direct effects of Ca^{2+}, such as increased formation of arachidonic acid metabolites capable of activating guanylate cyclase [44]. Nevertheless, the proposed ultimate effect of Ca^{2+} was elevation of cyclic GMP levels, a view that appears to be inconsistent with our findings that low concentrations of Ca^{2+} inhibit activation of guanylate cyclase by vasodilators. Resolution of this apparent discrepancy may result from additional experimentation, which is in progress, with several sources of vascular smooth muscle that vary in their dependency for contraction on extracellular and/or intracellular pools of Ca^{2+}.

Enhancement by many thiols of coronary arterial guanylate cyclase activation by nitroprusside, together with previous findings that nitroglycerin and $NaNO_2$ required thiols to activate coronary arterial guanylate cyclase [14], suggest that thiols may be involved in smooth muscle relaxation by vasodilators. Indeed, free sulfhydryl groups in vascular smooth muscle were required for drug-elicited relaxation [15], and their depletion resulted in tolerance to relaxation by certain vasodilators [15, 16]. Cysteine and DTT have been reported to enhance activation of guanylate cyclase from liver and other tissues by nitroso compounds [7, 9, 11, 45, 46]. Thiols may enhance activation by nitroso compounds by reacting with the latter to liberate NO [33] and/or to form *S*-nitrosothiols, which are potent activators of guanylate cyclase [14]. The possibility that thiols served merely as reductants to facilitate formation of nitrosyl-ferroheme, which itself activates guanylate cyclase, as suggested by Craven and DeRubertis [12], is unlikely in the present study because other reductants such as ascorbate and dithionite failed to enhance enzyme activation by NO or nitroprusside, and thiols enhanced activation of heme-free, partially purified guanylate cyclase.

These observations suggest that stimulation of cyclic GMP formation in coronary artery by nitroprusside and other vasodilators could be attenuated or enhanced, respectively, by elevated intracellular concentrations of free Ca^{2+} or sulfhydryl groups. The precise relationship, however, among Ca^{2+}, sulfhydryl groups, guanylate cyclase and cyclic GMP in vascular smooth muscle, as well as the biologic role of cyclic GMP in relaxation, remain to be determined.

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