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ACTIVATION OF RAT LUNG SOLUBLE GUANYLATE CYCLASE BY SODIUM NITROPRUSSIDE: EFFECTS OF HEMOGLOBIN AND REDUCING AGENTS

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SUMMARY: A potent vasodilator, sodium nitroprusside, activated rat lung soluble guanylate cyclase about 2.0-fold; this activation was potentiated by reducing agents such as ascorbic acid and thiols, 4.5 to 9-fold. In the presence of 2-mercaptoethanol and sodium nitroprusside maximal enzymatic activity in crude enzyme preparation was evident after a lag of several minutes, after which the activity declined. Hemoglobin blocked sodium nitroprusside activation of a partially purified enzyme by causing a lag in the activation, and this inhibition was reversed by 2-mercaptoethanol. Therefore, the extent of sodium nitroprusside activation measured is affected by the concentration of hemoglobin and reducing agent present, and the activation time.

INTRODUCTION

Soluble guanylate cyclase (EC 4.6.1.2) is activated by nitric oxide containing and generating compounds (1-8). A potent, rapid, and directly-acting vasodilator, sodium nitroprusside ($Na_2[Fe(CN_5NO]\cdot 2H_2O$; SNP) is one such compound, which is also reported to activate the soluble guanylate cyclase from most mammalian tissues (2,5). Here we report the activation of rat lung guanylate cyclase by SNP as being potentiated by thiols and ascorbic acid following a lag period of several minutes. SNP activated a partially-purified enzyme without any lag; the lag was dependent on hemoglobin concentration. A preliminary report of this work has appeared elsewhere (9).

MATERIALS AND METHODS

The commercial sources of chemicals used in the guanylate cyclase assay have been given previously (10,11). SNP and disodium EDTA were from Fisher Scientific. Sephadex gel chromatography materials were from Sigma, Chelex 100 (200-400 mesh) and Bio-Gel A-5m were from Bio-Rad.

Enzyme preparation. a) Soluble enzyme: 100,000xg supernatant fraction was prepared as described earlier (10). b) Partially purified enzyme:

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soluble enzyme prepared in buffer A (5 mM Tris-HCl, 100 mM 2-mercaptoethanol, pH 7.6) was brought to 30% of saturation with saturated ammonium sulfate solution. After 30 min, the precipitate was pelleted and supernatant was adjusted to 40% of saturation with ammonium sulfate solution and pelleted. The pellet thus obtained was suspended in buffer A (referred to as 30-40% ammonium sulfate precipitable fraction) and chromatographed on a 1x16 cm Sephadex G-100 column eluted with buffer B (5 mM Tris-HCl, 20 mM 2-mercaptoethanol, pH 7.6). The guanylate cyclase activity, eluted in the void volume, was pooled and 1 ml fractions were applied to individual 0.7x3 cm CM-23 columns; each column was eluted with 1 ml of buffer B. This enzyme was used as the essentially hemoglobin-free guanylate cyclase preparation.

Enzyme assays and other methods. Guanylate cyclase was assayed as described previously (11). Protein was determined by the method of Bradford (12), or by modified methods of Lowry et al. (13), after precipitation with silicotungstic acid; bovine serum albumin was used as the standard.

RESULTS

Potentiation of SNP stimulation by thiols and ascorbate. SNP (1 mM) activated the curde soluble guanylate cyclase less than two-fold but the activation was potentiated 4.5 to 9.3 fold in the presence of various thiols or ascorbic acid (Table 1). Since 2-mercaptoethanol resulted in the highest activation, it was used in subsequent experiments. Results obtained by varying the concentration of SNP in the presence of 5, 10 or 100 mM 2-mercaptoethanol are shown in Fig. 1. At all SNP concentrations, the activation was dependent on 2-mercaptoethanol concentration.

Time course of SNP activation. Based on the results shown in Fig. 1, final concentrations of 1 mM SNP and 10 mM 2-mercaptoethanol were used to determine the time course of activation; the enzyme was incubated with SNP for increasing periods of time and then enzymatic activity was assayed for 2 min (Fig. 2). In the absence of 2-mercaptoethanol, maximal activation (2.5-fold) was immediate and the activity decreased slowly thereafter. In the presence of 10 mM 2-mercaptoethanol, however, maximal (19.5-fold) activation was evident only after about 10 min preincubation and the activity declined rapidly thereafter. The reason for this decay of the activated state is not known. In mammals, SNP is known to be toxic *in situ*. It releases cyanide through a postulated nonenzymatic reaction of the iron atom of nitroprusside

TABLE I. Effects of various thiols and ascorbic acid on SNP activation of guanylate cyclase.

Additions	pmoles cGMP formed/min/mg protein		
	No SNP	0.1 mM SNP	1.0 mM SNP
None	416.1 ± 12.9	604.4 ± 20.2	727.9 ± 49.8
2-mercaptoethanol	235.9 ± 3.5	1293.2 ± 18.4	6901.4 ± 344.4
Monothioglycerol	257.5 ± 5.9	1361.1 ± 36.0	6717.2 ± 26.1
Dithioerythritol	225.8 ± 6.2	3706.6 ± 70.6	6074.9 ± 60.0
Cysteine	217.9 ± 0.7	2498.1 ± 9.1	5761.7 ± 189.0
Dimercaptopropanol	205.7 ± 16.2	4522.7 ± 38.3	5180.8 ± 190.8
Mercaptoethylamine	201.7 ± 6.7	2373.7 ± 150.9	4066.1 ± 166.4
Glutathione (reduced)	263.1 ± 8.6	2038.5 ± 139.3	3278.9 ± 108.0
Ascorbic Acid	521.4 ± 27.0	1838.2 ± 26.0	5600.6 ± 39.7

Twenty-five µl aliquots of rat lung supernatant containing 30 mM thiol or ascorbate were added to tubes containing 25 µl of reaction mixture and 25 µl sodium nitroprusside at the indicated final concentration and assayed for 10 min. Values for guanylate cyclase activity are the mean ± S.E.M.

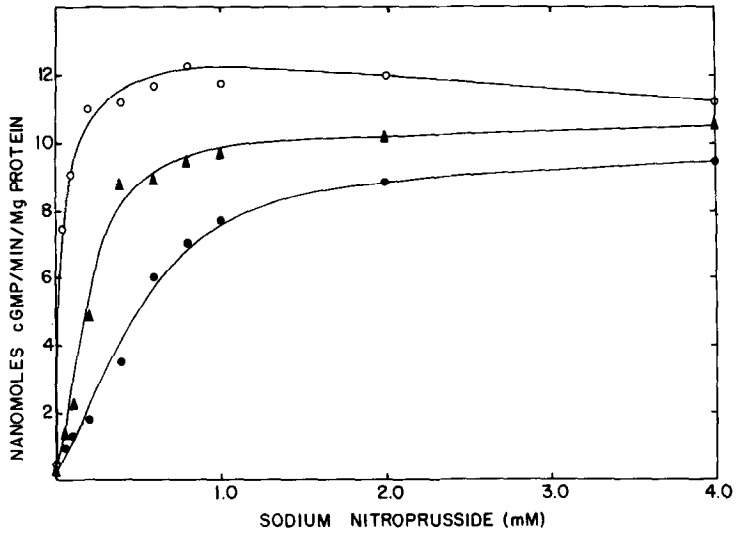

Fig. 1. Effect of SNP concentration on the activation of guanylate cyclase. Twenty-five µl aliquots of rat lung supernatant (78 µg) containing 2-mercaptoethanol were added to reaction mixtures containing SNP. Final concentratons of 2-mercaptoethanol were 5 mM (●), 10 mM (▲), or 100 mM (○).

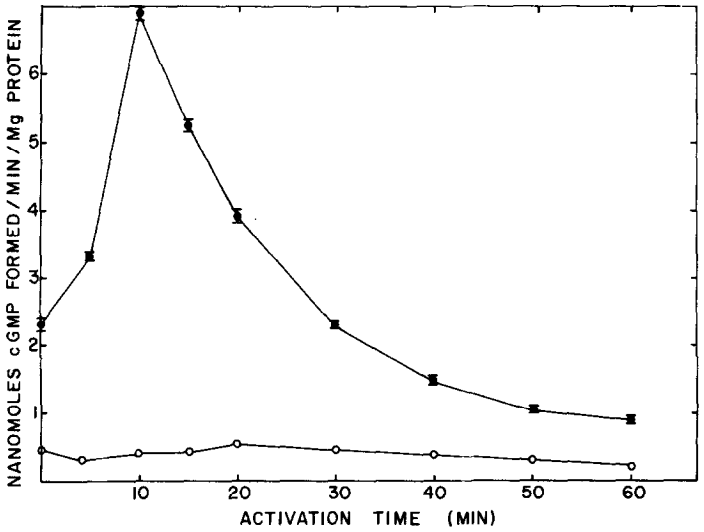

Fig. 2. Time course of SNP activation. Twenty-five µl aliquots of rat lung supernatant (120 µg) without (○) or with (●) 30 mM 2-mercaptoethanol were incubated with 25 µl of 3 mM SNP for the indicated times, after which 25 µl of reaction mix was added and the enzyme activity assayed for 2 min.

complex with free sulfhydryl groups (presumably of hemoglobin) (14). However, 1 mM KCN did not alter the pattern evident in Fig. 2. Another possibility is that the continued presence of a short-lived intermediate is necessary for sustained activation. The occurrence of such a molecule due to the reaction of SNP with reducing agents has been suggested (15). However, once maximally activated enzyme had begun to decay, it was not reactivated by further addition of SNP with or without 2-mercaptoethanol.

Gel chromatography studies. When 100,000xg supernatant was chromatographed on a Bio-Gel A-5m column, guanylate cyclase activity (SNP sensitive) was eluted in the fractions separated from the total volume and the void volume. Pretreatment of enzyme with SNP did not alter the elution profile, and SNP reactivated the eluted enzyme. Almost no lag was evident when enzyme partially purified by Bio-Gel A-5m chromatography or by ammonium sulfate precipitation was activated by SNP; as seen in Fig. 3, the activity declined after a 1 min preincubation.

Effect of hemoglobin on SNP activation. Hemoglobin has been reported to inhibit activation of cerebral and liver guanylate cyclase by various agents

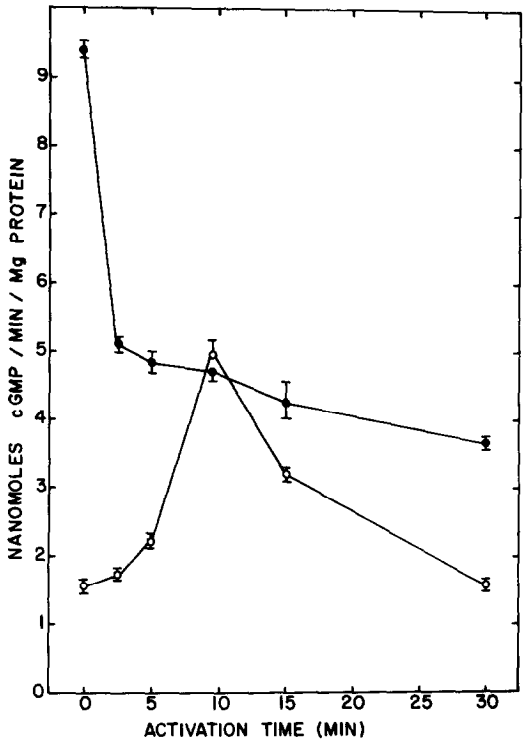

Fig. 3. Effect of Bio-Gel A-5m chromatography on the time course of SNP activation. Twenty-five µl aliquots of rat lung supernatant before (○, 60 µg) and after (●, 7.5 µg) gel filtration on a Bio-Gel A-5m column were incubated with SNP as in Fig. 2. Enzyme activity was assayed for 2 min.

(16-18). SNP activated an essentially hemoglobin-free enzyme from rat lung about 2.6-fold; the activation was increased to 3-fold when 0.1 and 1 µg of crystalline hemoglobin was present (Fig. 4). Greater than 1 µg hemoglobin caused a decrease in the activation, but did not affect the basal activity. The presence of 2-mercaptoethanol decreased the inhibition and potentiated SNP activation. Thus, 10 µg hemoglobin strongly reduced the SNP activation in the presence of less than 0.1 mM 2-mercaptoethanol but had no effect when greater than 1 mM 2-mercaptoethanol was present (Fig. 4). Inhibition produced by 500 µg hemoglobin was only partially reversed by 10 mM 2-mercaptoethanol.

Partially purified enzyme which was essentially free of hemoglobin had linear reaction rates (Fig. 5). Addition of 1.0 mM SNP caused a 4.5-fold increase in cGMP formation rate but in presence of hemoglobin the reaction rate was non-linear; initially the reaction progressed at the rate of un-

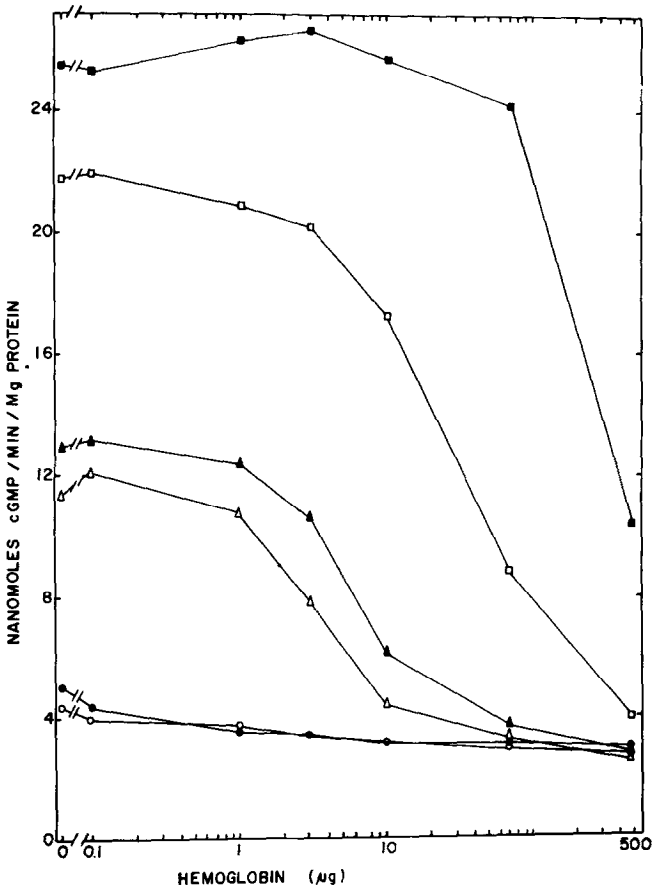

Fig. 4. Hemoglobin inhibition of guanylate cyclase activation by SNP. Essentially hemoglobin-free enzyme aliquots (20 µg) containing various 2-mercaptoethanol concentrations [0 mM (0,Δ) 0.1 mM (▲), 1 mM (□), and 10 µM (■,●)] were added to the tubes containing indicated amount of hemoglobin. The reactions were initiated by adding reaction mixture with (Δ,▲,□,■) or without (0,0) SNP at a final concentration of 1 mM. Reaction time was 6 min.

treated enzyme and later at the rate of SNP-treated enzyme. The period for which the reaction progressed at the rate of untreated enzyme lengthened with increasing concentration of hemoglobin (Fig. 5).

DISCUSSION

Rat lung soluble guanylate cyclase is activated by SNP after a lag period, which seems to be due to hemoglobin present in the preparation; there was no lag during the activation of partially-purified enzyme. Also, as

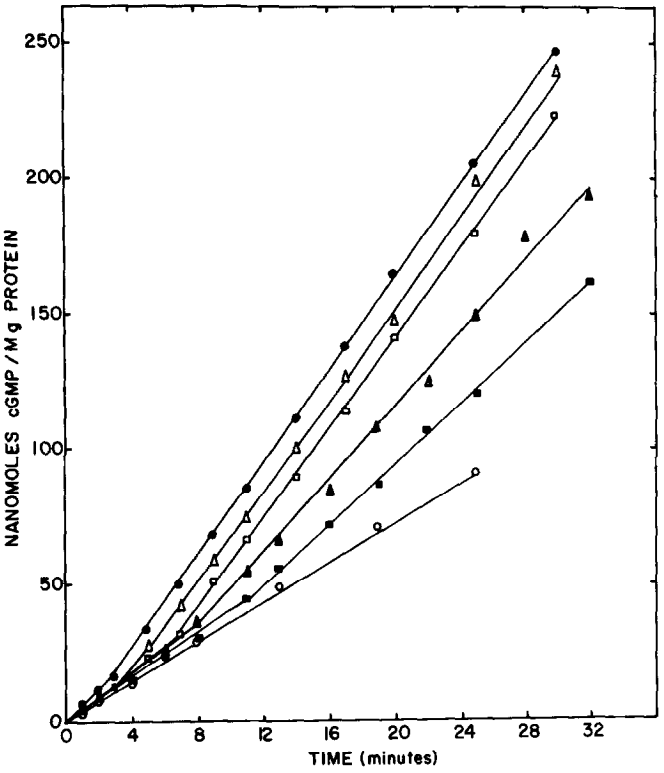

Fig. 5. Effect of hemoglobin on the progress curve of nitropruside activation. Aliquots of essentially hemoglobin-free enzyme (18 µg protein) were incubated with (O,●), 20 (Δ), 40 (□), 80 (Δ) or 200 µg (■) hemoglobin. The tubes contained 1 mM 2-mercaptoethanol and either no SNP (O) or 1 mM SNP (●,Δ,□, Δ,■) in addition to the components of the guanylate cyclase reaction mixture. Reactions were terminated at the indicated times.

reported earlier, no lag was observed for SNP activation of rat lung particulate enzyme treated with filipin (19).

Reducing agents, such as 2-mercaptoethanol and possibly ascorbate affect SNP activation in two different ways; first, they potentiate SNP activation in a concentration-dependent manner and second, 2-mercaptoethanol reverses hemoglobin inhibition as greater amounts of hemoglobin were required to cause inhibition in the presence of 2-mercaptoethanol. Results in Fig. 5 demonstrate that hemoglobin causes inhibition by delaying the activation, while 2-mercaptoethanol shortened such a delay and in turn caused reversal of inhibition. These observations and the absence of activation after prolonged incubation

(Fig. 2) may explain conflicting observations reported with respect to hemoglobin and reducing agents (7,16-18). The inhibitory effects of reducing agents reported by DeRubertis and Craven (7) are seen only when the enzyme is preincubated with the activating agents for 30 min at 4°. In such a system, hemoglobin is implicated in activation via a NO-hemoglobin complex. However, later data of Craven and DeRubertis obtained by using shorter incubation periods (20) and Ignarro, et al. (17,18), show that reducing agents potentiate SNP activation.

A postulate which includes SNP activation via an active intermediate, whose formation is enhanced by reducing agents, but is toxic to the enzyme upon prolonged exposure, may explain the conflicting observations. Such an intermediate may be scavanged by hemoglobin and thus hemoglobin would have the two-fold effect of delaying and also supporting activation. The exact mechanism of SNP activation is not known, but free radicals (7) or reactive radicals (nitroxyl or hydroxyl) have been implicated (21). Ignarro et al. (17,18) have suggested that S-nitrosothiols formed by the reaction of nitroprusside and thiols are the proximate activators of guanylate cyclase. In addition, SNP is known to cause auto-oxidation of free thiols in alkaline solution, presumably through the formation of thiol-free radicals, $RS^{\bullet}$ (15). However, whether the formation of an analogous moiety in the reaction of SNP and ascorbic acid takes place is unknown. A common product formed in both the ascorbate and thiol reactions with SNP is the anion $(Fe(CN)_5N{=}O)^{3-}$ (15), but a correlation between the activation curve and presence of any of these species is not known.

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