Biochimica et Biophysica Acta, 524 (1978) 231—244

BBA 68431

EFFECTS OF THIOL INHIBITORS ON HEPATIC GUANYLATE CYCLASE ACTIVITY

EVIDENCE FOR THE INVOLVEMENT OF VICINAL DITHIOLS IN THE EXPRESSION OF BASAL AND AGONIST-STIMULATED ACTIVITY

PATRICIA A. CRAVEN and FREDERICK R. DeRUBERTIS

Department of Medicine, Veterans Administration Hospital and University of Pittsburgh, Pittsburgh, Pa. 15240 (U.S.A.)

(Received September 23rd, 1977)

Summary

Several thiol blocking agents inhibit basal guanylate cyclase activity of 100 000 $\times g$ hepatic supernatant fractions and the stimulation of enzyme activity by *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (MNNG), NaN_3, $NaNO_2$ and nitroprusside. The relative potency of the thiol blockers as inhibitors was $CdCl_2$ > *p*-hydroxymercuribenzoate > *N*-ethylmaleimide > arsenite > iodoacetamide. Inhibition of basal and MNNG-responsive soluble guanylate cyclase activities by arsenite was markedly potentiated by an equimolar concentration of 2,3-dimercaprol, but not by mercaptoethanol. Inhibition of soluble guanylate cyclase by either arsenite or $CdCl_2$ was completely reversed by excess 2,3-dimercaprol. Qualitatively similar effects were observed with DE-52 cellulose purified soluble hepatic guanylate cyclase, and suggested an involvement of closely juxtaposed thiol groups in the regulation of enzyme activity. For several reasons inhibition by thiol blockers appeared to be mediated through multiple mechanisms and/or sites of interaction: (1) Concentrations of the thiol inhibitors which had no effect on basal activity strikingly inhibited the responsiveness of the enzyme to a submaximal concentration of MNNG. (2) $CdCl_2$ abolished the action of excess $MnCl_2$ to stimulate purified guanylate cyclase, but was a relatively ineffective inhibitor when $MnCl_2$ and GTP were present in equimolar concentrations. By contrast, arsenite-2,3-dimercaprol was uniformly effective in inhibiting guanylate cyclase activity in the presence or absence of excess $MnCl_2$. (3) Arsenite-2,3-dimercaprol increased the K_m for MnGTP (control, 0.13 ± 0.02 mM; 0.2 mM arsenite-2,3-dimercaprol, 0.31 ± 0.03 mM), whereas

---

Abbreviations: MNNG, *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine and cyclic GMP, guanosine 3',5'-monophosphate. 2,3-dimercaprol, 2,3-dimercaptopropanol.

$CdCl_2$ had no effect on this parameter. (4) Hepatic particulate guanylate cyclase activity was significantly inhibited by arsenite 2,3-dimercaprol but not by $CdCl_2$. Thus, the data not only indicate that vicinal dithiol groups are required for expression of basal guanylate cyclase activity and enzyme responses to agonists, but strongly suggest the involvement of more than one interacting site containing free thiol residues.

Introduction

Recent studies have shown that the thiol alkylating agents *N*-ethylmaleimide and maleimide reduce the basal activities of soluble guanylate cyclases from several tissues and inhibit enzyme activation by a number of newly identified agonists [1—3]. Moreover, dithiothreitol and other agents known to prevent thiol group oxidation suppress the O_2-dependent autoactivation of guanylate cyclase in lung [4], as well as the autoactivation process observed in platelets [5], uterus [6] and colonic mucosa [3]. Under appropriate conditions, both thiol and non-thiol antitoxidants can also block activation of soluble hepatic guanylate cyclase by several agents including *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (MNNG) [1,3,7], nitrosoureas [2], NaN_3, $NaNO_2$, NH_2OH [8] and HIO_4 [9]. Such observations have suggested that redox reactions involving free tissue thiol groups may participate both in the expression of basal guanylate cyclase activity and in the response of the enzyme to some agonists, with SH to S-S transformation as a potential mechanism of enzyme activation [1—3]. In the present study, we examined the possibility that vicinal dithiol residues were required for the expression of basal and agonist-stimulated guanylate cyclase activities of rat liver, and the suggestion of Goldberg and co-workers [6] that more than one enzyme site or tissue component containing free thiol residues is involved in this expression.

Materials and Methods

N-Ethylmaleimide, 2,3-dimercaprol, 2-mercaptoethanol, *p*-hydroxymercuribenzoate and iodoacetamide were obtained from Sigma Chemical Co., St. Louis, Mo. DE-52 cellulose was purchased from Bio-Rad, Rockville Center, N.Y. and ammonium sulfate, enzyme grade from Schwartz-Mann, Orangeburg, N.Y. The sources of all other reagents have previously been reported [1,10].

Preparation of hepatic fractions and partial purification of soluble guanylate cyclase. Male Sprague-Dawley rats (Zivic-Miller, Pittsburgh, Pa.), were anesthetized with pentobarbital (5 mg/100 g body weight intraperitoneally). Hepatic homogenates were prepared in 0.25 M sucrose/5 mM Tris, pH 7.4 (50 mg/ml) and centrifuged at 100 000 $\times g$ for 60 min. The washed particulate fraction was resuspended in the same volume as the original homogenate. For purification of soluble enzyme activity, approx. 20 g of rat liver was homogenized in 120 ml of 0.25 M sucrose/10 mM Tris/10 mM dithiothreitol/1 mM EDTA (pH 7.4) and centrifuged at 100 000 $\times g$ for 60 min. Guanylate cyclase was precipitated from the supernatant fraction with 40% $(NH_4)_2SO_4$. Following equilibration with 10 mM Tris/10 mM dithiothreitol/1 mM EDTA (pH 7.4) the resuspended ammoni-

um sulfate pellet was applied to a DE-52 cellulose column (1.5 × 25 cm) and guanylate cyclase activity eluted with a linear NaCl gradient (0—500 mM). The specific activity of the partially purified enzyme was 1850 ± 164 pmol/min per mg protein (54-fold purification) with 64% recovery of enzyme activity from the hepatic supernatant. Peak column fractions were stored at —70°C under argon. Before use, the partially purified enzyme fraction was passed through a Sephadex G-25 column (1.5 × 5 cm) equilibrated with 10 mM Tris, pH 7.4, and argon to remove residual dithiothreitol and EDTA. The enzyme was collected and stored under argon at 0—4°C until use.

Guanylate cyclase assay. Guanylate cyclase activity was determined as previously described [11]. Standard reaction mixtures contained 1 mM GTP and 4 mM $MnCl_2$. However, in some experiments the concentration of these latter agents were varied as described in the text. Basal soluble and particulate activities examined with or without thiol blockers were linear with respect to time for at least 15 min and with respect to added protein for crude soluble activity (50—200 μg), partially purified soluble activity (1—10 μg) and the 100 000 × *g* particulate activity (100—300 μg). Cyclic GMP formation in the presence of MNNG was also linear with time for 15 min in the presence or absence of thiol blockers. A 2 min lag phase of enzyme activation observed with nitrite and nitroprusside and a 5—8 min lag with azide was abolished by preincubating hepatic supernatant fractions with these agents for 10 min at 37°C before initiation of the enzyme assay.

Determination of GTP by thin-layer chromatography. Thin-layer chromatography of guanylate cyclase reaction mixtures was performed on polyethylenemine-impregnated cellulose sheets using a modification of the procedure described by Neuhard et al. [12] as previously described [10]. Residual GTP, calculated as percent total GTP, GDP plus GMP from chromatograms did not differ in the presence or absence of thiol blocking agents (85—89%), thus indicating that inhibition of the creatine phosphate-creatine phosphokinase GTP regenerating system did not explain effects of the thiol inhibitors on guanylate cyclase activity.

Protein content was determined by the method of Lowry et al. [13]. Statistical significance of differences between mean values was assessed using Student's *t*-test for unpaired values.

Results

Table I shows the effects of preincubating the 100 000 × *g* hepatic supernatant fraction with several thiol blocking agents on basal guanylate cyclase activity and its responsiveness to MNNG, nitroprusside, $NaNO_2$ and NaN_3. Concentrations of the agonists which gave maximal stimulation of enzyme activity in the absence of the thiol inhibitors are shown. Each of the thiol inhibitors was tested over a 100-fold concentration range. The selected concentrations given in Table I depict the relative inhibitor potency of these agents on a molar basis for basal and stimulated guanylate cyclase activities ($CdCl_2$ > *p*-hydroxymercuribenzoate > *N*-ethylmaleimide > arsenite > iodoacetamide). Higher concentrations of $CdCl_2$ (0.5 mM), *p*-hydroxymercuribenzoate (1 mM) and *N*-ethylmaleimide (5 mM) abolished basal and agonist-responsive enzyme activ-

TABLE I

THE EFFECTS OF THIOL INHIBITORS ON BASAL SOLUBLE HEPATIC GUANYLATE CYCLASE ACTIVITY AND ITS RESPONSIVENESS TO SEVERAL AGONISTS

The 100 000 X *g* soluble fraction of hepatic homogenates was initially incubated for 10 min at 0°C with the concentration of each of the thiol inhibitors indicated. A final addition of test agent was then made to the preincubation mixture where indicated and the preincubation continued for an additional 10 min at 37°C to abolish the 2—8 min lag periods of enzyme activation observed with azide, nitrite and nitroprusside [1,9,27]. Guanylate cyclase assays were initiated by adding 25 μl of the preincubation mixture to 50 μl of standard enzyme reaction mixture. Each value shown represents the mean ± S.E. of eight determinations (duplicates pooled from four separate experiments). Results are expressed in pmol/min per mg protein.

Initial additions (10 min at 0°C)	Final additions (10 min at 37°C)				
	None	0.1 mM MNNG	10 mM nitroprusside	10 mM $NaNO_2$	0.1 mM NaN_3
None	47 ± 6	2914 ± 312	1235 ± 158	1321 ± 185	924 ± 105
0.2 mM $CdCl_2$	5 ± 0.7 *	11 ± 2 *	6 ± 0.8 *	5 ± 0.7 *	5 ± 0.6 *
0.5 mM *p*-hydroxymercuribenzoate	4 ± 0.5 *	26 ± 4 *	17 ± 2 *	12 ± 2 *	9 ± 1 *
1 mM *N*-ethylmaleimide	8 ± 1 *	58 ± 7 *	28 ± 4 *	33 ± 4 *	19 ± 3 *
50 mM arsenite	11 ± 2 *	762 ± 93 *	429 ± 57 *	388 ± 48 *	286 ± 37 *
50 mM iodoacetamide	16 ± 2 *	1346 ± 161 *	781 ± 92 *	643 ± 91 *	532 ± 63 *

* $P < 0.01$ vs. corresponding value with no initial addition (degrees of freedom = 6 for statistical analysis).

TABLE II

THE EFFECTS OF THIOL INHIBITORS ON BASAL AND MNNG-RESPONSIVE SOLUBLE HEPATIC GUANYLATE CYCLASE ACTIVITY

The 100 000 X *g* soluble fraction of hepatic homogenates was initially incubated for 10 min at 0°C with the concentrations of each of the thiol inhbitors indicated. The enzyme assay was initiated by adding 25 μl of the supernatant fraction to 50 μl of standard enzyme reaction mixture which contained 1 mM GTP, 4 mM $MnCl_2$ and the final concentration of MNNG indicated. Each value shown represents the mean ± S.E. of eight determinations (duplicates pooled from four experiments). Results are expressed in pmol/min per mg protein.

Initial addition to the supernatant fraction (10 min at 0—4°C)	Addition to assay			
	None	0.01 mM MNNG	0.1 mM MNNG	3 mM MNNG
None	43 ± 5	1648 ± 252	3107 ± 312	3085 ± 511
0.025 mM $CdCl_2$	45 ± 5	142 ± 22 *	3158 ± 472	2975 ± 592
0.1 mM $CdCl_2$	18 ± 2 *	30 ± 6 *	578 ± 74 *	685 ± 84 *
0.05 mM *p*-hydroxymercuribenzoate	41 ± 6	973 ± 152 *	2863 ± 389	2881 ± 423
0.2 mM *p*-hydroxymercuribenzoate	19 ± 3 *	156 ± 19 *	351 ± 40 *	512 ± 69 *
0.5 mM *N*-ethylmaleimide	47 ± 5	82 ± 9 *	993 ± 172 *	2917 ± 604
0.75 mM *N*-ethylmaleimide	20 ± 3 *	22 ± 4 *	66 ± 9 *	363 ± 64 *
1 mM arsenite	42 ± 6	108 ± 11 *	2763 ± 391	2862 ± 523
10 mM arsenite	20 ± 3 *	21 ± 3	762 ± 83 *	1706 ± 194 *
1 mM iodoacetamide	43 ± 6	1013 ± 134 *	2932 ± 391	3115 ± 401
25 mM iodoacetamide	24 ± 3 *	325 ± 53 *	1178 ± 156 *	1934 ± 217 *

* $P < 0.01$ versus corresponding value with no initial addition (degrees of freedom = 6 for statistical analysis).

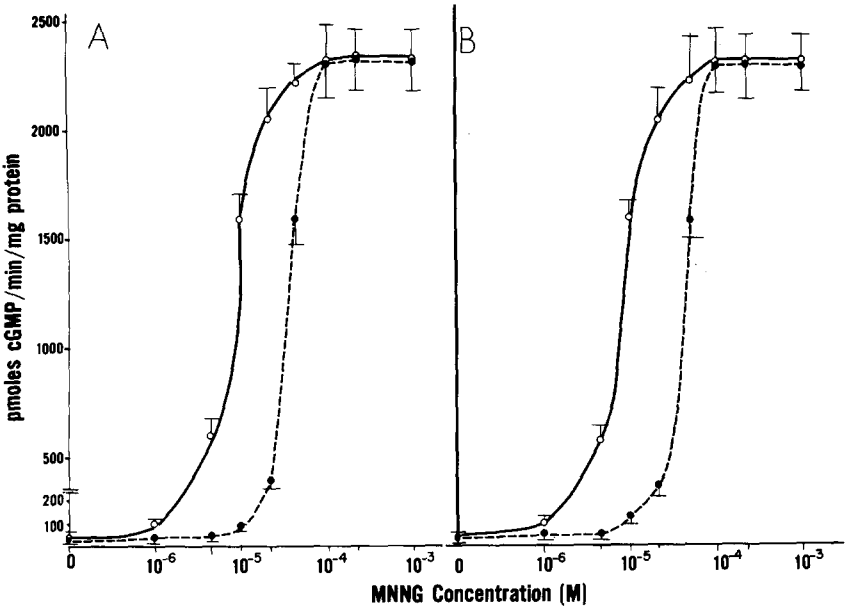

Fig. 1. The effect of preincubating the 100 000 × *g* hepatic soluble fraction for 10 min at 0—4°C with no additions (○———○), 0.025 mM $CdCl_2$ (●- - - - -●, panel A) or 1 mM arsenite (●- - - - -●, panel B) on the concentration vs. response relationship between MNNG and guanylate cyclase activity. At the end of 10 min a 25 μl aliquot of the preincubation mixture was added to a 50 μl aliquot of a standard guanylate cyclase assay mixture which contained MNNG at the final concentration indicated in the figure. The concentrations of $CdCl_2$ or arsenite employed in these studies had no effect on basal guanylate cyclase activity. Results shown are means ± S.E. of six determinations from three separate experiments.

ities, while concentrations of arsenite and iodoacetamide up to 100 mM did not abolish these activities. Two other sulfhydryl reactive agents, diamide and cystamine, also suppressed basal enzyme activity in hepatic supernatant fractions and the responses of this activity to maximally effective concentrations of MNNG, azide, nitrite and nitroprusside (not shown). As shown in Table II, concentrations of the thiol blockers that had no significant effects on basal enzyme activity, suppressed stimulation mediated by a submaximally effective concentration of MNNG (0.01 mM). Moreover, a concentration of thiol blocker which reduced basal enzyme activity by approx. 50% had proportionately much more dramatic effects on enzyme responses to submaximal or maximal (0.1 mM) MNNG. Depending upon the concentration of the thiol blocker employed, inhibition of MNNG-responsive enzyme activity was either completely or partially reversed by addition of a high concentration (3 mM) of the agonist (Table II). Thus, MNNG-responsive guanylate cyclase activity was more sensitive to inhibition by the thiol blockers than was basal activity and this inhibition appeared to be competitive relative to the concentration of MNNG. The latter possibility was further examined with arsenite and $CdCl_2$. Fig. 1 shows the concentration vs. response relationship between MNNG and guanylate cyclase activity in hepatic supernatants preincubated for 10 min at 0°C with no additions, $CdCl_2$ or arsenite. Concentrations of arsenite (1 mM) and $CdCl_2$ (0.025 mM) employed in these studies had no effect on the basal activity of soluble

guanylate cyclase (Table II). As shown in Fig. 1, MNNG stimulated soluble hepatic guanylate cyclase activity 2—60-fold when tested alone over a concentration range of 1 μM—1 mM. Prior exposure of hepatic supernatant fractions to $CdCl_2$ or arsenite inhibited guanylate cyclase responsiveness to submaximal (1 μM—0.05 mM) concentrations of MNNG, but had no effect on responsiveness to concentrations of MNNG (0.1 mM or greater) which gave maximal stimulation of enzyme activity. By contrast, a concentration of *N*-ethylmaleimide (0.5 mM) which did not alter basal enzyme activity abolished or significantly inhibited the actions of concentrations of MNNG up to 0.5 mM (not shown). Thus, when each was employed at concentrations that did not alter basal guanylate cyclase activity in hepatic supernatant fractions, $CdCl_2$, arsenite and *N*-ethylmaleimide all gave quantitatively similar inhibition of submaximal MNNG-responsive activity (Table II).

Inhibition of the activity of several enzymes by arsenite is thought to involve an interaction of this agent with closely juxtaposed thiol groups required for function of the enzyme [14—18]. Potentiation of arsenite inhibition by an

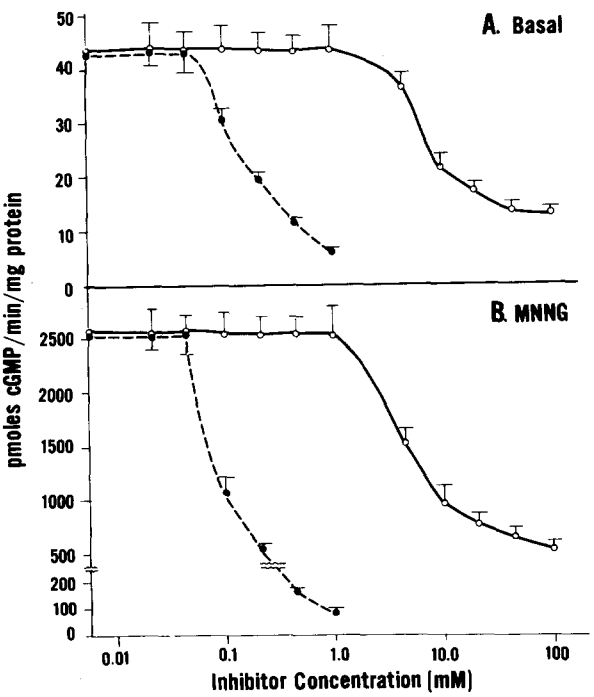

Fig. 2. The concentration vs. response relationship between arsenite and arsenite plus 2,3-dimercaprol versus basal soluble hepatic guanylate cyclase activity (panel A) or MNNG-stimulated guanylate cyclase activity (panel B). The 100 000 × *g* hepatic supernatant was preincubated for 10 min at 0—4°C with indicated concentrations of arsenite alone (o———o) or with equimolar arsenite plus 2,3-dimercaprol (•- - - - -•). At the end of the 10 min preincubation, a 25 μl aliquot of supernatant fraction was added to a 50 μl aliquot of guanylate cyclase reaction mixture with (panel B) or without (panel A) MNNG at a final concentration of 0.1 mM. Preicubation of hepatic supernatants with 0.01—1 mM 2,3-dimercaprol alone had no effect on basal or 0.1 mM MNNG-stimulated guanylate cyclase activities. Results presented are means ± S.E. of six determinations from three separate experiments.

equimolar concentration of 2,3-dimercaprol is characteristic of the vicinal dithiol mechanism of interaction [14—18]. As shown in Fig. 2, the effects of arsenite on basal and maximal MNNG-responsive soluble hepatic guanylate cyclase were strikingly potentiated by equimolar concentrations of 2,3-dimercaprol. The minimally effective concentration of arsenite which alone gave significant inhibition of basal or maximal (0.1 mM) MNNG-responsive guanylate cyclase activity was 5 mM. In the presence of an equimolar concentration of 2,3-dimercaprol the minimally effective inhibitory concentration of arsenite was reduced to 0.1 mM. At 1 mM arsenite plus 2,3-dimercaprol, loss of basal and maximal MNNG-responsive enzyme activity was pronounced and exceeded that observed with 100 mM arsenite alone (Fig. 2). Preincubation of the hepatic supernatant fraction with 2,3-dimercaprol alone at concentrations from 0.05 to 1 mM had no effect on basal or maximal MNNG-responsive guanylate cyclase activities (not shown). As with arsenite alone (Table II), arsenite-2,3-dimercaprol had proportionally greater effects on maximal MNNG-responsive enzyme activity than on basal activity (Fig. 2). Moreover a concentration of arsenite-2,3-dimercaprol (0.05 mM) which was without effect on basal activity significantly suppressed enzyme stimulation in response to submaximal MNNG (0.01 mM MNNG, 1472 ± 159; 0.01 mM MNNG plus arsenite-2,3-dimercaprol, 164 ± 19 pmol cyclic GMP/min per mg protein). Similarly, as with arsenite alone, 3 mM MNNG completely or partially reversed inhibition by arsenite-2,3-dimercaprol, depending upon the concentration of blocker employed (not shown).

Table III compares the effects of different concentrations of arsenite, 2,3-dimercaprol or mercaptoethanol on basal hepatic guanylate cyclase activity examined in crude 100 000 $\times g$ supernatant fractions and with the DE-52-purified soluble enzyme. Neither arsenite (0.5 mM) nor 2,3-dimercaprol (0.5—25 mM) alone had any effect on basal guanylate cyclase activities in these preparations. However, when added together, an equimolar concentration of 2,3-dimercaprol plus arsenite (0.5 mM) was extremely effective in inhibiting guanylate cyclase activity. The monothiol mercaptoethanol could not substitute for 2,3-dimercaprol in this regard (Table III). Moreover a 10-fold molar excess of 2,3-dimercaprol partially reversed, while a 20-fold molar excess completely reversed, the inhibitory effects of 0.5 mM arsenite in both enzyme preparations. By contrast, a 100-fold molar excess of mercaptoethanol was only partially effective in reversing the inhibition of basal soluble hepatic guanylate cyclase activity mediated by 0.5 mM arsenite-2,3-dimercaprol in both enzyme preparations.

As shown in Table IV, $CdCl_2$ (0.2 mM) inhibited basal soluble guanylate cyclase activity 89%. A 2.5-fold molar excess of 2,3-dimercaprol partially reversed the $CdCl_2$ inhibition, while a 5-fold molar excess completely reversed the effects of $CdCl_2$ on basal guanylate cyclase activity. The monothiol mercaptoethanol was much less effective than 2,3-dimercaprol in reversing $CdCl_2$ inhibition. In both crude and purified preparations of soluble guanylate cyclase, only partial restoration of enzyme activity was observed when mercaptoethanol was added at a concentration 50-fold in excess of $CdCl_2$.

Analogous to results obtained with the soluble fraction, arsenite, iodoacetamide, *N*-ethylmaleimide and *p*-hydroxymercuribenzoate inhibited basal parti-

TABLE III

EFFECTS OF 2,3-DIMERCAPROL AND MERCAPTOETHANOL ON ARSENITE-MEDIATED INHIBITION OF BASAL SOLUBLE GUANYLATE CYCLASE ACTIVITY

The 100 000 × *g* hepatic supernatant fraction or guanylate cyclase partially purified by DE-52 cellulose chromatography was initially incubated for 10 min at 0°C with or without 0.5 mM arsenite plus 0.5 mm 2,3-dimercaprol as indicated under Addition 1. Second test agents were then added where indicated and the preincubation continued for an additional 10 min at 0°C. Guanylate cyclase assays were initiated by the addition of a 25 μl aliquot of the preincubation mixture to a 50 μl aliquot of standard guanylate cyclase assay mixture which contained no further test agents. Results presented are means ± S.E. of duplicates pooled from four separate experiments (degrees of freedom = 6).

Additions to enzyme preparation		Guanylate cyclase activity (pmol/min per mg protein)	
Addition 1 (0.5 mM arsenite plus 0.5 mM 2,3-dimercaprol)	Addition 2	100 000 × *g* supernatant fraction	DE-52-purified soluble enzyme
—	None	42 ± 5	1873 ± 212
—	0.5 mM arsenite	43 ± 6	1956 ± 268
—	0.5 mM 2,3-dimercaprol	41 ± 5	1909 ± 224
—	25 mM 2,3-dimercaprol	36 ± 4	1894 ± 205
—	0.5 mM arsenite plus 0.5 mM 2,3-dimercaprol	9.1 ± 2 *	135 ± 22 *
—	0.5 mM mercaptoethanol	38 ± 5	1981 ± 258
—	0.5 mM arsenite plus 0.5 mM mercaptoethanol	43 ± 4	1943 ± 220
—	50 mM mercaptoethanol	39 ± 5	2005 ± 295
+	None	8.7 ± 1	124 ± 21 *
+	5 mM 2,3-dimercaprol	18 ± 2 *,**	688 ± 74 *,**
+	10 mM 2,3-dimercaprol	45 ± 6	1851 ± 241 **
+	25 mM 2,3-dimercaprol	40 ± 6 **	1917 ± 248 **
+	25 mM mercaptoethanol	11 ± 2 *	511 ± 66 *,**
+	50 mM mercaptoethanol	23 ± 3 *,**	1152 ± 139 *,**

* $P < 0.01$ compared to value in the absence of any additions to the 100 000 × *g* soluble fraction or partially purified enzyme preparation.

** $P < 0.01$ compared to value obtained with equimolar arsenite plus 2,3-dimercaprol (0.5 mM) as the first or second addition.

culate guanylate cyclase activity 48—100% (not shown). Moreover, 2,3-dimercaprol when added at a concentration equimolar to that of arsenite, strikingly potentiated the inhibitory effects of arsenite on particulate guanylate cyclase activity (no additions, 8.9 ± 1; 50 mM arsenite, 4.6 ± 0.6; 0.5 mM arsenite, 9.2 ± 1; 0.5 mM 2,3-dimercaprol, 8.7 ± 1.5; 0.5 mM arsenite plus 0.5 mM 2,3-dimercaprol, 1.1 ± 0.2 pmols/min per protein ± S.E.). The monothiol mercaptoethanol did not substitute for 2,3-dimercaprol in this regard. Qualitatively similar inhibitory effects of the thiol agents were observed with a preparation of particulate guanylate cyclase which had been treated with 1% Lubrol PX for 1 h at 0°C before addition of the thiol inhibitors. These results suggested that inhibition of particulate guanylate cyclase by thiol blockers did not require an intact cell membrane. However, in striking contrast to the effects of $CdCl_2$ on soluble guanylate cyclase activity, $CdCl_2$ (0.05—5 mM) had no effect on activity in the particulate fraction (not shown).

Since $CdCl_2$ failed to inhibit particulate guanylate cyclase, the effects of this agent and arsenite-2,3-dimercaprol on expression of soluble guanylate cyclase

TABLE IV

EFFECTS OF 2,3-DIMERCAPROL AND MERCAPTOETHANOL ON $CdCl_2$-MEDIATED INHIBITION OF BASAL SOLUBLE HEPATIC GUANYLATE CYCLASE ACTIVITY

The 100 000 × *g* hepatic soluble fraction or guanylate cyclase partially purified by DE-52 cellulose chromatography was initially incubated for 10 min at 0°C with or without 0.2 mM $CdCl_2$ as indicated under Addition 1. A second test agent was then added where indicated and the incubation continued for an additional 10 min at 0°C. Guanylate cyclase assays were initiated by the addition of a 25 μl aliquot of the preincubation mixture to a 50 μl aliquot of standard guanylate cyclase assay mixture which contained no other test agents. Results presented are means ± S.E. of duplicates pooled from four separate experiments (degrees of freedom = 6 for statistical analysis).

Additions to enzyme preparation		pmol/min per mg protein	
Addition 1 (0.2 mM $CdCl_2$)	Addition 2	100 000 × *g* supernatant fraction	DE-52-purified soluble enzyme
—	None	41 ± 5	2021 ± 304
—	0.2 mM $CdCl_2$	4.3 ± 0.5 *	305 ± 42 *
—	5 mM 2,3-dimercaprol	36 ± 5	1953 ± 212
+	None	3.8 ± 0.5 *	281 ± 39 *
+	0.5 mM 2,3-dimercaprol	19 ± 2 *,**	1062 ± 152 *,**
+	1 mM 2,3-dimercaprol	37 ± 5 **	1989 ± 231 **
+	5 mM 2,3-dimercaprol	45 ± 5 **	2006 ± 312 **
+	5 mM mercaptoethanol	5.5 ± 1 *	483 ± 52 *,**
+	10 mM mercaptoethanol	21 ± 3 *,**	958 ± 164 *,**

* $P < 0.01$ compared to value in the absence of any additions to the enzyme preparation.
** $P < 0.01$ compared to value with $CdCl_2$ alone.

were further studied to assess the possibility that important differences existed in the means by which these thiol blockers suppressed soluble activity. Fig. 3 (upper panel) shows the effects of arsenite plus 2,3-dimercaprol on guanylate cyclase activity over a wide range of MnGTP concentrations (0.015—1 mM) and at a constant excess $MnCl_2$ concentration of 3 mM. Results from a representative experiment, repeated twice are presented in Fig. 3, while the apparent K_m values for MnGTP represent the mean ± S.E. of values pooled from three separate experiments. Incubation of the enzyme preparation with arsenite plus 2,3-dimercaprol resulted in inhibition of enzyme activity at all concentrations of MnGTP tested. The V was decreased (control, 2018; 0.1 mM arsenite plus 2,3-dimercaprol, 909; 0.2 mM arsenite plus 2,3-dimercaprol, 588 pmol/min per protein). The apparent K_m for MnGTP was modestly increased (control, 0.13 ± 0.02; 0.1 mM arsenite plus 2,3-dimercaprol, 0.20 ± 0.02; 0.2 mM arsenite plus 2,3-dimercaprol, 0.31 ± 0.03 mM) after incubation of the enzyme preparation with arsenite plus 2,3-dimercaprol. Thus, the inhibition could not be overcome by an excess of substrate and appeared to be non-competitive with respect to MnGTP. As shown in the lower panel of Fig. 3, $CdCl_2$ also non-competitively inhibited guanylate cyclase activity over the wide range of MnGTP concentrations tested (0.015—1 mM) and decreased the V compared to that of a control enzyme preparation with no prior exposure to $CdCl_2$ (control, 1989; 0.1 mM $CdCl_2$, 833; 0.2 mM $CdCl_2$, 294 pmol/min per mg protein). By contrast, $CdCl_2$ had no effect on the K_m of guanylate cyclase for MnGTP (0.12 ± 0.02 mM). Fig. 4 (panel A) shows the effects of arsenite plus 2,3-dimercaprol on partially purified guanylate cyclase activity at various concentrations

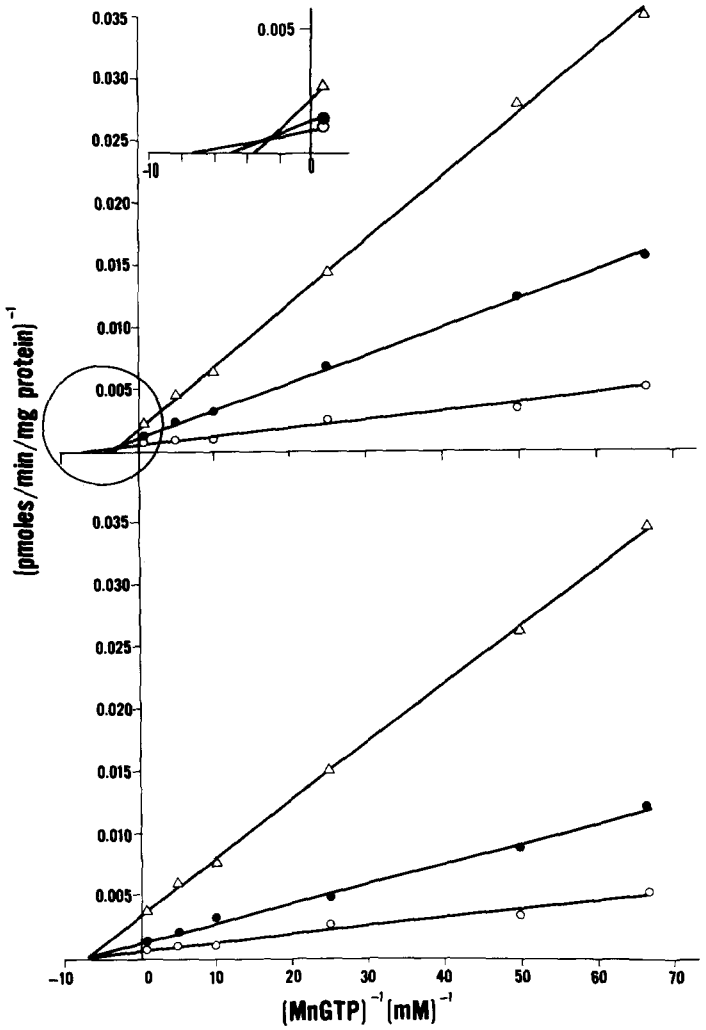

Fig. 3. Effects of arsenite plus 2,3-dimercaprol (upper panel) and $CdCl_2$ (lowerpanel) on the double reciprocal plot of MnGTP vs. guanylate cyclase activity. DE-52-purified hepatic soluble guanylate cyclase was preincubated for 10 min at 0°C with no additions (○———○), 0.1 mM arsenite plus 0.1 mM 2,3-dimercaprol (●———●) or 0.2 mM arsenite plus 0.2 mM 2,3-dimercaprol (△———△) as shown in the upper panel or with 0.1 mM $CdCl_2$ (●———●) or 0.2 mM $CdCl_2$ (△———△) as shown in the lower panel. Guanylate cyclase assays were begun by the addition of 25 μl of the preincubation media to 50 μl of a guanylate cyclase assay mixture which contained MnGTP (0.015—2 mM) at the final concentration indicated in the assay mixture. The concentration of $MnCl_2$ present in excess of substrate (MnGTP) was held constant at 3 mM. Results presented are averages of duplicate determinations from a representative experiment, repeated twice.

of excess $MnCl_2$ and a fixed concentration of substrate (1 mM MnGTP) in the enzyme assay mixture. As shown, increasing the concentration of excess $MnCl_2$ from 0.5 to 8 mM resulted in a linear increase in guanylate cyclase activity in control preparations as well as in enzyme preparations which had been preincubated with arsenite plus 2,3-dimercaprol for 10 min at 0°C before assay. The curves were not linear when the excess $MnCl_2$ concentration was varied below

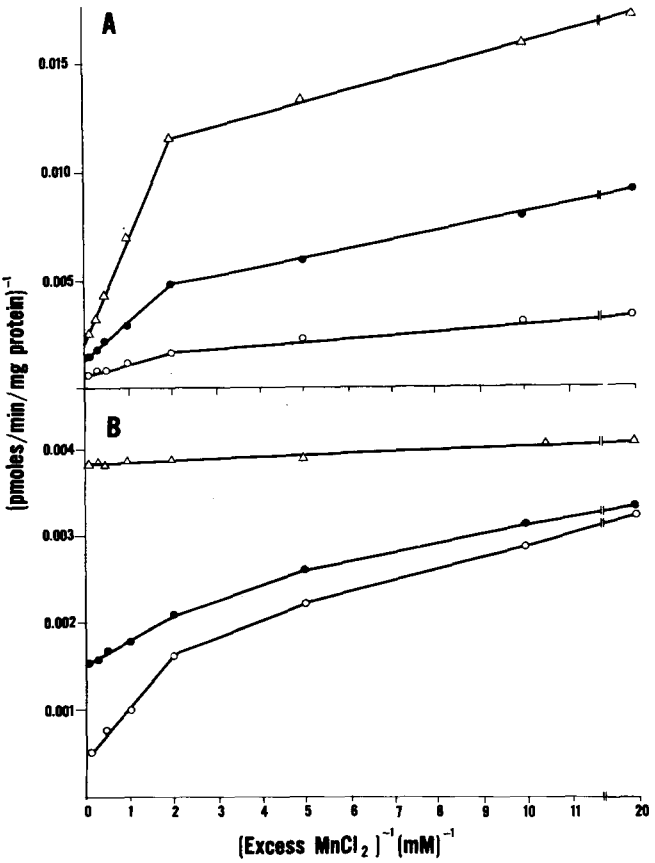

Fig. 4. Effects of arsenite plus 2,3-dimercaprol (panel A) and $CdCl_2$ (panel B) on the double reciprocal plot of excess $MnCl_2$ concentration vs. guanylate cyclase activity. DE-52-purified guanylate cyclase was preincubated for 10 min at 0°C with no additions (○———○), 0.1 mM arsenite plus 0.1 mM, 2,3-dimercaprol (●———●) or 0.2 mM arsenite plus 2,3-dimercaprol (△———△) as shown in panel A. Effects of 0.1 mM $CdCl_2$ (●———●) or 0.2 mM $CdCl_2$ (△———△) are shown in panel B. Guanylate cyclase assays were then begun by the addition of 25 μl of the preincubation media to 50 μl of a guanylate cyclase assay mixture which contained various concentrations of excess $MnCl_2$ (0—8 mM) and a fixed and saturating concentration (1 mM) of MnGTP as substrate. Results presented are averages of duplicate determinations from a representative experiment, repeated twice.

0.5 mM. This could be due to a negative cooperative effect at low excess $MnCl_2$ availability or to residual enzyme heterogeneity. The degree of inhibition of guanylate cyclase activity observed with arsenite plus 2,3-dimercaprol was identical over the wide range of excess $MnCl_2$ concentrations tested (0.050—8 mM). In contrast to the effects of arsenite plus 2,3-dimercaprol on guanylate cyclase activity at various excess $MnCl_2$ concentrations, $CdCl_2$(Fig. 4, panel B) was a much more potent inhibitor of enzyme activity when examined at high concentrations of excess $MnCl_2$ than at low or zero excess concentrations of this cation. Thus, 0.1 mM $CdCl_2$ had no significant effect on enzyme activity assayed with zero excess $MnCl_2$, but inhibited guanylate cyclase activity by 61% when the latter was assayed at 3 mM excess $MnCl_2$. Moreover, as shown in Fig. 4, preincubation of the partially purified enzyme with 0.1 mM $CdCl_2$ for

10 min at 0°C largely suppressed, while 0.2 mM completely abolished the stimulation of enzyme activity by excess $MnCl_2$ (control response to excess Mn^{2+}, 6.4-fold increase; 0.1 mM $CdCl_2$, 2.2-fold increase; 0.2 mM $CdCl_2$, no increase). However, concentrations of $CdCl_2$ higher than 0.2 mM significantly inhibited basal soluble guanylate cyclase activity in the absence of excess $MnCl_2$. Complete inhibition of soluble guanylate cyclase activity was observed with 1 mM $CdCl_2$ in the presence or absence of excess $MnCl_2$ (not shown). These results imply that the suppressive effects of $CdCl_2$ on guanylate cyclase, unlike those of arsenite-2,3-dimercaprol, are at least in part due to inhibition of the effects of excess metal ion to activate the enzyme. However, the action of $CdCl_2$ was complex, since high concentrations of this agent abolished soluble enzyme activity in the presence or absence of excess $MnCl_2$.

Discussion

The present study demonstrates that free thiol groups are required for the expression of basal and agonist-responsive soluble hepatic guanylate cyclase activity. Several thiol inhibitors of differing size, charge and mode of interaction with SH groups inhibited basal soluble guanylate cyclase activity and its responsiveness to MNNG, $NaNO_2$, NaN_3 and nitroprusside in a concentration-dependent fashion. Further, a requirement for vicinal dithiol groups in the expression of basal and MNNG-responsive soluble enzyme activity was strongly supported by studies of the inhibitory actions of arsenite. There is considerable evidence that arsenite inhibition of enzyme activity requires the presence of two closely juxtaposed thiol groups [15—19]. Moreover, several studies have demonstrated potentiation of arsenite inhibition by equimolar concentrations of 2,3-dimercaprol [14—18]. This is thought to be due to the formation of a cyclic dithioarsenite complex, which has enhanced accessibility to hydrophobic regions of proteins [18]. High concentrations of 2,3-dimercaprol have been shown to reverse the inhibitory effects of arsenite on the activity of several enzymes by competing for free arsenite [14,15]. Monothiols do not substitute or substitute poorly for 2,3-dimercaprol in either potentiating the effects of arsenite at equimolar concentrations or in reversing arsenite inhibition at high concentrations [14—17]. In the present study, the inhibition of basal and MNNG-responsive soluble hepatic guanylate cyclase activity by arsenite was markedly potentiated by equimolar 2,3-dimercaprol but not by 2-mercaptoethanol. Moreover, arsenite inhibition of basal soluble hepatic guanylate cyclase activity was completely reversed by a 20-fold molar excess of 2,3-dimercaprol, while a 100-fold molar excess of mercaptoethanol only partially reversed this effect. These results provide support for the hypothesis that the actions of arsenite to inhibit basal and MNNG-responsive soluble guanylate cyclase activity involve an interaction of this agent with vicinal dithiol groups. $CdCl_2$ is also thought to react with vicinal dithiols [14—16], although the specificity for such an interaction of $CdCl_2$ is less well established than that for arsenite. In the present study, $CdCl_2$ was a potent inhibitor of basal and agonist-responsive soluble guanylate cyclase activity. Similar to arsenite effects, the action of $CdCl_2$ on basal soluble guanylate cyclase activity was completely reversed by a 5-fold molar excess of 2,3-dimercaprol but only partially reversed by a 50-fold molar excess of 2-mer-

captoethanol. Arsenite-2,3-dimercaprol and $CdCl_2$ also inhibited DE-52 cellulose-purified soluble guanylate cyclase activity. The latter observations suggest direct interactions of the blockers with closely juxtaposed thiol groups on the enzyme. However, the possibility of effects of these agents on other tissue components involved in the expression of guanylate cyclase activity cannot be definitively excluded until the enzyme is purified to homogeneity.

Even if the residues involved are located on the enzyme, it is clear from the present study that the mode of interaction of blocking agents with thiol groups of the guanylate cyclase system is quite complex. A single mechanism or site of action is unlikely. These observations both support the concept originally advanced by Goldberg and Haddox [6], that more than one oxidizable site is involved in the expression of guanylate cyclase activity and provide further evidence that these critical sites are free thiol residues. Thus, the actions of the thiol inhibitors on soluble enzyme activity were not uniform under all assay conditions. Concentrations of the thiol inhibitors that did not alter basal soluble guanylate cyclase activity markedly inhibited the responsiveness of the enzyme to a submaximal concentration of MNNG. Moreover, striking differences were observed in the effects of $CdCl_2$ versus equimolar arsenite plus 2,3-dimercaprol when the actions of these inhibitors were examined using a partially purified preparation of the soluble enzyme. The inhibitory effects of equimolar arsenite plus 2,3-dimercaprol on basal soluble guanylate cyclase activity were uniformly expressed over a wide range of excess $MnCl_2$ concentrations (0—8 mM); arsenite plus 2,3-dimercaprol modestly increased the apparent K_m of the partially purified enzyme for substrate (MnGTP). By contrast, $CdCl_2$ was much more effective as an inhibitor of basal enzyme activity at zero or low concentrations of excess $MnCl_2$. Under appropriate conditions, $CdCl_2$ abolished the stimulatory effect of excess $MnCl_2$ on the soluble enzyme, but had no effect on the K_m of the soluble enzyme for MnGTP. A further indication of differences between the actions of $CdCl_2$ and the other thiol blockers came from studies of their effects on 100 000 $\times g$ particulate guanylate cyclase activity. Unlike arsenite alone, arsenite plus 2,3-dimercaprol, iodoacetamide, *N*-ethylmaleimide or *p*-hydroxymercuribenzoate, $CdCl_2$ had no inhibitory effect on basal particulate enzyme activity when tested over a wide range of concentrations (0.05—5 mM). The lack of inhibitory effect of $CdCl_2$ on the particulate enzyme could be related to the lower excess $MnCl_2$ requirement of the latter compared to that of the soluble enzyme [20—26]. However, the differences found between the effects of $CdCl_2$ on soluble versus particulate guanylate cyclase, or $CdCl_2$ versus arsenite inhibition of soluble activity, cannot be completely explained by an action of $CdCl_2$ to modify enzyme stimulation by excess $MnCl_2$. High concentrations of $CdCl_2$ abolished basal and stimulated soluble enzyme activity with or without excess $MnCl_2$. A concentration-dependent interaction of $CdCl_2$ with multiple thiol groups of differing affinities could be involved.

Recently, Spiegel et al. [14] reported that vicinal dithiol groups are required for the expression of fluoride- and isoproterenol-stimulated adenylate cyclase activity in turkey erythrocyte membranes. Arsenite and $CdCl_2$ inhibited both membrane-bound and detergent-solubilized adenylate cyclase activities. Although $CdCl_2$ inhibition of adenylate cyclase activity may involve more than one mechanism and/or site of interaction [14], $CdCl_2$ shared with arsenite the

ability to inhibit both the holocatalytic and the non-holocatalytic forms of adenylate cyclase [14]. Comparison of the present results with those of Spiegel and co-workers [14] suggests that similarities exist in the functional groups required for expression of both adenylate and guanylate cyclase activities. In this regard, it is of considerable interest that Mittal and Murad [27] have recently shown that under some conditions of assay soluble hepatic guanylate cyclase can generate cyclic AMP from ATP. Structural similarities between guanylate and adenylate cyclases may ultimately explain these findings.

Acknowledgements

We are indebted to Ms. Candice Mauney and Mr. Ronald St. Clair for expert technical assistance and to Mrs. Delauriese Bridgett for secretarial support. This work was supported by funds from the Medical Research Services of the Veterans Administration.

References

1 DeRubertis, F.R. and Craven, P.A. (1977) J. Biol. Chem. 252, 5804—5814
2 DeRubertis, F.R. and Craven, P.A. (1977) Biochim. Biophys. Acta 499, 337—351
3 DeRubertis, F.R. and Craven, P.A. (1978) Cancer 40, 2600—2608
4 White, A.A., Crawford, K.M., Patt, C.S. and Lad, P.J. (1976) J. Biol. Chem. 251, 7304—7312
5 Glass, D.B., Frey, W., Carr, D.W. and Goldberg, N.D. (1976) J. Biol. Chem. 252, 1279—1285
6 Goldberg, N.D. and Haddox, M.K. (1977) Annu. Rev. Biochem. 46, 823—896
7 Craven, P.A. and DeRubertis, F.R. (1978) Cancer Res. 37, 4088—4097
8 Craven, P.A. and DeRubertis, F.R. (1977) Fed. Proc. 36, Abstract 2182
9 Haddox, M.K., Furcht, L.T., Gentry, S.R., Moser, M.F., Stephenson, J.H. and Goldberg, N.D. (1976) Nature 262, 146—148
10 DeRubertis, F.R. and Craven, P.A. (1976) J. Biol. Chem. 251, 4651—4658
11 Craven, P.A. and DeRubertis, F.R. (1976) Anal. Biochem. 72, 455—459
12 Neuhard, J., Randerath, F. and Randerath, K. (1965) Anal. Biochem. 13, 211—222
13 Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951) J. Biol. Chem. 193, 265—275
14 Spiegel, A.M., Brown, E.M. and Aurbach, G.D. (1976) J. Cyclic Nucl. Res. 2, 393—404
15 Gaber, B.P. and Fluharty, A.L. (1968) Q. Rep. Sulfur Chem. 3, 318—337
16 DeLuca, M., Wirtz, G.W. and McElroy, W.D. (1964) Biochemistry 3, 935—939
17 Jakoby, W.B. (1958) J. Biol. Chem. 232, 89—97
18 Fluharty, A.L. and Sanadi, D.R. (1961) J. Biol. Chem. 236, 2772—2778
19 Stocken, L.A. and Thompson, R.H.S. (1946) Biochem. J. 40, 535—548
20 Craven, P.A. and DeRubertis, F.R. (1976) Biochemistry 15, 5131—5137
21 Kimura, H. and Murad, F. (1974) J. Biol. Chem. 249, 6910—6916
22 Kimura, H. and Murad, F. (1975) Metabolism 24, 439—445
23 Kimura, H. and Murad, F. (1975) J. Biol. Chem. 250, 4810—4817
24 Garbers, D.L., Dyer, E.L. and Hardman, J.G. (1975) J. Biol. Chem. 250, 382—387
25 Chrisman, T.D., Garbers, D.L., Parks, M.A. and Hardman, J.G. (1975) J. Biol. Chem. 250, 374—381
26 Gray, J.P. and Drummond, G.I. (1976) Arch. Biochem. Biophys. 172, 31—38
27 Mittal, C.K. and Murad, F. (1977) J. Biol. Chem. 252, 3136—3140