Hepatic Cyclic GMP Formation Is Regulated by Similar Factors That Modulate Activation of Purified Hepatic Soluble Guanylate Cyclase*

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The same factors that regulate the activation of purified hepatic soluble guanylate cyclase by diverse agents possessing distinct requirements for enzyme activation were found to modulate cyclic GMP formation in intact viable hepatic cells. A comparison was made between activation of heme-deficient or heme-reconstituted guanylate cyclase and stimulation of cyclic GMP formation in mouse hepatic slices that were 95% viable and showed no active efflux of cyclic GMP. Heme-dependent activators of guanylate cyclase elicited a greater -fold increase in hepatic cyclic GMP levels in slices from phenobarbital-pretreated than control mice. Brilliant cresyl blue and KCN inhibited both enzyme activation and hepatic cyclic GMP accumulation caused by agents that generate nitric oxide. Hepatic slices from 3,5-diethoxycarbonyl-1,4-dihy- drocolidine-treated mice, which are known to develop sharp increases in hepatic protoporphyrin IX/heme concentration ratios, showed elevated resting cyclic GMP levels whereas phenobarbital pretreatment produced decreased resting cyclic GMP levels compared to controls. Guanylate cyclase activation by azide required added catalase, and both enzyme activation and hepatic cyclic GMP formation were inhibited by amitriazole. Enzyme activation by glyceryl trinitrate and NaN02 required added thiols. Hepatic slices from acetaminophen-pretreated mice showed marked depletion of sulfhydrils and decreased cyclic GMP formation in response to these enzyme activators. Both effects were completely restored by treatment of thiol-depleted mice with N-acetylcysteine. These observations lend support to the general view that information gained from studies on the regulatory properties of purified soluble guanylate cyclase bears a close relationship to studies on regulatory mechanisms that modulate cyclic GMP formation in intact cells.

Purified soluble guanylate cyclase can be activated by several distinct chemical substances, each possessing individual requirements for the expression of maximal enzyme activation. NO1 and unstable nitrogen oxides that release NO activate soluble guanylate cyclase by heme-dependent mecha-nisms involving the formation of enzyme-bound NO-heme, which is the activating species (1-6). Stable nitrogen oxides such as organic nitrate esters and NaN02 activate guanylate cyclase also by generating NO-heme, but thiols are required to facilitate the generation of NO from the parent compounds (7, 8). Certain chemical species such as azide can also generate NO but this requires the catalytic activity of catalase, which oxidizes azide to NO with the resultant formation of catalase-bound NO-heme (2, 9). The NO-heme then exchanges with guanylate cyclase to generate the activated form of the enzyme (10). Phenylhydrazine and structurally related aromatic hydrazines cause enzyme activation by heme-dependent, but NO-independent, mechanisms involving the formation of enzyme-bound iron-heme (11). Protoporphyrin IX and structurally related porphyrins activate soluble guanylate cyclase directly by heme-independent mechanisms, where heme is a competitive inhibitor of the porphyrin (12-14).

The objective of the present study was to ascertain whether the factors that modulate the activation of purified soluble guanylate cyclase similarly influence cyclic GMP formation in intact viable cells. The purpose of this approach was to determine whether information derived from experiments on purified guanylate cyclase is truly indicative of comparable alterations in cyclic GMP formation in intact functioning cells. Hepatic slices prepared from normal and treated mice were studied, and the observations were compared with those from experiments employing purified hepatic, soluble guanylate cyclase. This kind of comparative study has not been reported previously.

The effects of guanylate cyclase inhibitors, catalase inhibitors, and cyanide on the capacity of nitroso compounds, azide, phenylhydrazine, glyceryl trinitrate, and NaN02 to stimulate hepatic cyclic GMP accumulation were evaluated. The influence of thiols on hepatic cyclic GMP accumulation elicited by guanylate cyclase activators was assessed by comparing hepatic slices from normal mice to slices from mice pretreated with high doses of acetaminophen or with both acetamizophen and N-acetylcysteine. In order to assess the influence of endogenous porphyrins and heme on hepatic cyclic GMP levels, mice were pretreated with chemical agents known to markedly alter hepatic concentrations of protoporphy- morphyrin IX and heme.

EXPERIMENTAL PROCEDURES

Materials—Sodium nitroprusside, sodium azide, sodium nitrite, phenylhydrazine-HCl, potassium cyanide, brilliant cresyl blue, amitriazole, MNNG, acetaminophen, N-acetylcysteine, sodium phenobarbital, L-cysteine, hemin, GSH, methemoglobin (2 x crystallized, bovine blood), catalase (purified, bovine liver), probersed, 5,5'-dithiobis(2-nitrobenzoic acid), and Sephadex G-25 (50-150 µm) were purchased from Sigma. Glyceryl trinitrate (10% (w/w) triturated mixture in lactose) was a gift from ICI Americas, Inc. M&B 32,946 was provided by May & Baker, Ltd., Dagenham, Essex, United Kingdom. 1-Methyl-3-isobutylxanthine and DDC were obtained from Aldrich. The DDC was recrystallized from ethanol prior to use.
Hemoglobin was prepared from methemoglobin by reduction with dithionite under N₂, followed by gel filtration as described previously (15). Hemoglobin was prepared from hemin by dissolving the hemin (1 mm final concentration) in N₂-purged 0.1 N NaOH containing 10 mm dithiothreitol and 10 mm sodium dithionite and then making a 100-fold dilution in 40 mm TEA-HCl, pH 7.4, and was used within 20 min. The synthesis and handling of S-nitroso-N-acetylpenicillamine have been described (8). Reagents for the soluble guanylate cyclase assay were described previously (5, 12).

Preparation and Incubation of Hepatic Slices—Hepatic slices were prepared from male Swiss-Webster mice weighing 20-25 g. Mice were killed by cervical dislocation and their livers were rapidly perfused with ice-cold 10 mm HEPES, pH 7.4, containing 138 mm NaCl, 5 mm KCl, 1 mm CaCl₂, 1 mm MgSO₄, and 5 mm glucose, in an atmosphere of 95% O₂, 5% CO₂ at 37 °C for 20 min. Longer perfusion times were unnecessary and sometimes resulted in a loss of cell viability. Following perfusion, chemical agents were added, and reaction mixtures were further incubated at 37 °C for 2–10 min as indicated in each experiment. The pH of the incubation media remained relatively constant (7.2–7.4) throughout the perfusion and incubation periods.

Viability of Perfused and Incubated Hepatic Slices—Viability of hepatic slices incubated under the conditions described was estimated periodically by determining the extent of leakage into the incubation medium of lactate dehydrogenase activity. Incubation mixtures were rapidly cooled, clarified by filtration, and filtrates were assayed for lactate dehydrogenase activity using a diagnostic kit obtained from Sigma (No. 500). An estimate of total releasable enzymatic activity was obtained after homogenization of slices in 0.1% (w/v) Triton X-100. None of the chemical agents added to incubation mixtures interfered directly with the assay of lactate dehydrogenase activity.

Determination of Acid-soluble Thiols—Acid-soluble thiols, 95% of which is GSH in rodent liver (16), were determined in protein-free, trichloroacetic acid–extracted samples by a modification of the method reported by Beutler et al. (17) employing Ellman’s reagent (18). Briefly, 10% (w/v) homogenates of mouse hepatic slices (110-150 mg) in 6% (w/v) trichloroacetic acid were prepared at 0–4 °C and centrifuged at 5,500 × g for 15 min. Supernatants (0.8–1.0 ml) were adjusted to 5 ml with 0.3 M Na₃HPO₄, and 0.5 ml of a solution of 40 mg of 5,5'-dithiobis-(2-nitrobenzoic acid) in 100 ml of 1% (w/v) sodium citrate was added. Reaction mixtures were rapidly mixed at 25 °C and after 10-min optical densities at 412 nm were measured with a Coleman spectrophotometer. GSH made up in 5% (w/v) trichloroacetic acid was employed as the standard.

Cyclic Nucleotide and Protein Determinations—Incubations were terminated by rapidly transferring hepatic slices into small tissue grinders containing 1 ml of ice-cold 6% (w/v) trichloroacetic acid. In certain experiments 0.2 ml of 60% (w/v) trichloroacetic acid was added to incubation media after removal of hepatic slices. Tissues were homogenized and aliquots were taken for protein determinations. Homogenates and media were extracted and assayed for cyclic GMP and cyclic AMP by radioimmunoassay as described previously (8, 19). Protein concentrations of tissue samples were determined by the Bio-Rad, Coomassie Brilliant Blue G-250 method, as described by Bio-Rad Laboratories. Bovine serum albumin was used as the standard. This procedure was found to be highly reproducible and reliable because the development of color in the presence of protein is virtually unaffected by high concentrations of trichloroacetic acid, various salts, thiols, glucose, and other chemicals.

Factors Affecting Hepatic Cyclic GMP Formation

Heme Reconstitution of Guanylate Cyclase—Aliquots (0.2 ml) of purified enzyme (0.2–0.5 μg of protein) were thawed on ice and 20 μl of 50 μM heme in 25 mm TEA-HCl, pH 7.8, containing 10 mM dithiothreitol was added. The mixture was allowed to remain on ice for 20 min and was applied to a 0.7 × 15-cm column of Sephadex G-25 equilibrated with 25 mm TEA-HCl, pH 7.8. Guanylate cyclase eluted in a volume of 0.5–0.6 ml after 2.3 ml (void volume) had flowed through the column. Confirmation of heme reconstitution of enzyme was verified spectrophotometrically as described (5, 11).

Guanylate Cyclase Assay—Guanylate cyclase activity was determined as described previously (5, 12). Details of and differences in the composition of enzyme reaction mixtures are given in the appropriate table legends. All enzymatic assays were conducted under initial velocity conditions. This implies a maximal conversion of GTP to cyclic GMP of less than 10% and a linear relationship between product formation and time.

Statistical Analysis—Where appropriate, values are expressed as the mean ± S.E. and represent unpaired data. Comparisons were made using the Student’s t test for unpaired values. The level of statistically significant difference was p < 0.05.

RESULTS

Characteristics of Resting and Elevated Cyclic GMP Levels in Mouse Hepatic Slices—Initial experiments showed that incubation of hepatic slices for less than 20 min resulted in variable and declining tissue levels of cyclic GMP. The inclusion of the cyclic GMP phosphodiesterase inhibitor M&B 22,948 in incubation mixtures prevented the decline in cyclic GMP levels, but values were still variable when incubations were conducted for less than 20 min. Thus, hepatic slices were routinely preincubated in the presence of M&B 22,948 at 37 °C for 20 min prior to initiating any given protocol. Fig. 1 illustrates the effect of incubation time, following 20 min of
preincubation, on resting and elevated hepatic levels of cyclic GMP. The inclusion of M&B 22,948 in preincubation mixtures caused an increase and prevented the decline in resting cyclic GMP levels. Similarly, the stimulation of hepatic cyclic GMP accumulation by S-nitroso-N-acetylpenicillamine was enhanced, and tissue levels were maintained by M&B 22,948.

In the presence of M&B 22,948, the peak response to S-nitroso-N-acetylpenicillamine was attained in 90 s, and cyclic GMP levels remained stable at elevated values for at least 10 min.

In order to determine what fraction of intracellular cyclic GMP was released or leaked into the extracellular environment, an analysis of cyclic GMP levels in both hepatic slices and incubation media was made. The influence of probenecid on both resting and elevated cyclic GMP levels evoked by S-nitroso-N-acetylpenicillamine in normal untreated and phenobarbital-pretreated mice was studied (Fig. 2). Phenobarbital-pretreated mice were examined because of their use in another study for each experimental protocol. In no sample did the percent of total releasable enzymatic activity exceed 8%, and the range was generally 3–6% (94–97% viability). Thus, the various animal pretreatments and chemical agents included in incubation mixtures elicited no deleterious effects on incubated slices. These observations on viability of hepatic slices are in accord with the above findings that probenecid failed to alter the tissue/medium ratio of cyclic GMP. Thus, the small degree of extracellular discharge of cyclic GMP appears to be due to leakage as a result of minimal cellular disruption.

The relationship between cyclic AMP and cyclic GMP levels in hepatic slices in the absence and presence of phosphodiesterase inhibitors and a guanylate cyclase activator is illustrated in Table I. M&B 22,948 caused a 20-fold increase in resting cyclic GMP levels without appreciably affecting cyclic AMP levels. This resulted in a sharp decline in the tissue concentration ratio of cyclic AMP to cyclic GMP. The nonselective phosphodiesterase inhibitor 1-methyl-3-isobutylxanthine increased both cyclic AMP and cyclic GMP levels. S-Nitroso-N-acetylpenicillamine elicited a marked increase in hepatic cyclic GMP levels without altering cyclic AMP levels in either the absence or presence of phosphodiesterase inhibitors. Whether or not a phosphodiesterase inhibitor was employed, the potent guanylate cyclase activator caused a dramatic decline in the hepatic cyclic AMP/cyclic GMP concentration ratio to values of less than unity.

Table I

| Additions* | Cyclic AMP† | Cyclic GMP† | Cyclic AMP/cyclic GMP |
|------------|-------------|-------------|----------------------|
| Control    | 1.1 ± 0.3  | 0.03 ± 0.01 | 37                   |
| 0.5 mM M&B 22,948 (M&B) | 1.4 ± 0.2 | 0.61 ± 0.11 | 2.3                 |
| 0.5 mM 1-methyl-3-isobutylxanthine (MIX) | 5.2 ± 0.3 | 0.44 ± 0.16 | 12                  |
| 0.1 mM S-nitroso-N-acetylpenicillamine | 1.3 ± 0.4 | 6.7 ± 0.8 | 0.2                 |
| M&B + 0.1 mM S-nitroso-N-acetylpenicillamine | 1.2 ± 0.2 | 10 ± 0.7 | 0.1                 |
| MIX + 0.1 mM S-nitroso-N-acetylpenicillamine | 5.6 ± 0.5 | 8.2 ± 0.5 | 0.7                 |

* M&B 22,948 or 1-methyl-3-isobutylxanthine was added to the indicated hepatic slice reaction mixtures, which were incubated at 37°C for 22 min. Control slice mixtures were also incubated at 37°C for 22 min. Other reaction mixtures as indicated were preincubated at 37°C for 20 min in the absence or presence of phosphodiesterase inhibitors and were further incubated at 37°C for 2 min after the addition of S-nitroso-N-acetylpenicillamine. In all cases hepatic slices were rapidly removed and freeze-clamped after 22 min.

† Cyclic AMP and cyclic GMP concentrations were determined in the same hepatic slices.

Mean ± S.E. of four to six hepatic slices from each of two separate experiments.

Significantly different (p < 0.01) from corresponding control values.
hepatic cyclic GMP accumulation (Table II). In addition, the
effects of the guanylate cyclase inhibitor brilliant cresyl blue,
the catalase inhibitor aminotriazole, and KCN were evaluated
in the absence and presence of guanylate cyclase activators.
Brilliant cresyl blue caused a slight decrease in resting cyclic
GMP levels whereas KCN and aminotriazole were without
effect. All of the guanylate cyclase activators tested markedly
stimulated cyclic GMP accumulation, and these responses
were inhibited by both brilliant cresyl blue and KCN. Amin-
otriazole inhibited only the stimulatory response to NaN3.

Influence of Hepatic Thiol Depletion and Repletion on Cyclic
GMP Accumulation in Hepatic Slices—Pretreatment of mice
with acetaminophen resulted in a marked depletion of hepatic
thiol content. Acid-soluble thiols in hepatic slices were deter-
mined as outlined under “Experimental Procedures.” Hepatic
slices from control mice gave values of 197 ± 11 mg of GSH/100
g of liver. Acetaminophen pretreatment resulted in values of
45 ± 8 mg of GSH/100 g of liver whereas pretreatment of
mice with both acetaminophen and N-acetylcyesteine did not
significantly alter control values (189 ± 14 mg of GSH/100 g
of liver). The above values are expressed as the mean ± S.E.
of five separate determinations from three mice and are
comparable to those reported by others (20).

Hepatic thiol depletion did not appreciably alter resting
hepatic levels of cyclic GMP (Table III). The stimulation of
hepatic cyclic GMP accumulation elicited by glyceryl trinit-
rate and NaNO3, however, was inhibited in thiol-depleted
hepatic slices. Complete restoration of responses to both
agents was afforded by treatment of acetaminophen-pre-
treated mice with N-acetylcyesteine. Hepatic thiol depletion
produced a small inhibitory effect on hepatic cyclic GMP
accumulation elicited by sodium nitroprusside (Table III) and
MNNG (not shown) but not by NaN3 (Table III) or by S-
nitroso-N-acetylpenicillamine (not shown).

A time-course analysis of hepatic cyclic GMP accumulation
stimulated by various agents in the absence and presence of
cysteine was made. Only the data for NaN3 and sodium
nitroprusside are illustrated in Fig. 3. In the absence of added
cysteine, the response to sodium nitroprusside was sluggish
during the first 2 min of incubation. Cysteine accelerated the
response to sodium nitroprusside to approximate that for
NaN3 conducted in the absence of added cysteine (Fig. 3).
The rate of response to NaN3 was not altered by cysteine (not
shown). Cysteine did not alter the rates of hepatic cyclic GMP
accumulation in response to S-nitroso-N-acetylpenicillamine
or glyceryl trinitrate but the responses to NaN3 and MNNG
were accelerated in a manner that was similar to that for
sodium nitroprusside (not shown). The slower responses to
MNNG, NaNO3, and sodium nitroprusside in the absence of
added cysteine were evident only at incubation times of less
than 2 min.

Influence of Phenobarbital and DDC Pretreatment on Cyclic
GMP Accumulation in Hepatic Slices—The responses to var-
ious guanylate cyclase activators were evaluated in hepatic
slices prepared from mice that were untreated or pretreated
with phenobarbital alone or with phenobarbital and DDC.
Pretreatment with phenobarbital causes elevated hepatic
levels of free heme as well as hemoproteins. The administration
of DDC to phenobarbital-pretreated animals is well known to
cause a marked increase in the hepatic concentration ratio
of protoporphyrin IX to heme by virtue of its capacity to inhibit
ferrochelatase (21-23). Phenobarbital pretreatment caused a
significant decrease in hepatic resting levels of cyclic GMP
whereas treatment of phenobarbital-pretreated mice with
DDC caused a pronounced increase in cyclic GMP levels when
compared with untreated controls (Table IV). When the -fold
increase in hepatic cyclic GMP accumulation over corre-
sponding control is considered, each activator produced a
significantly greater response (p < 0.01) in slices from phen-
obarbital-pretreated mice but produced a significantly
smaller response (p < 0.01) in slices from DDC-treated,
phenobarbital-pretreated mice. With the exception of phen-
ylhydrazine, the absolute values for hepatic cyclic GMP levels,
however, were similar among slices from untreated and
treated mice. The reason for this difference is that the basal
or resting levels of hepatic cyclic GMP were significantly
altered (p < 0.01) by pretreatment of mice with phenobarbital
or DDC (Table IV). The most dramatic difference was the
response to phenylhydrazine, which was virtually unmasked
in hepatic slices from phenobarbital-pretreated mice. DDC
pretreatment appeared to abolish the capacity of phenylhy-
drazine to stimulate hepatic cyclic GMP accumulation.

Effects of Various Metabolites on Hepatic Cyclic
GMP Formation stimulated by nitrogen oxides and NaN3

| Additions* | Cyclic GMP |
|------------|-----------|
|            | None | Brilliant cresyl blue (0.5 mM) | KCN (10 mM) | Aminotriazole (10 mM) |
| Control    | 0.78 ± 0.15 | 0.46 ± 0.075 | 0.72 ± 0.1 | 0.87 ± 0.2 |
| 0.1 mM S-nitroso-N-acetylpenicillamine | 12 ± 0.9 | 2.1 ± 0.25 | 2.5 ± 0.3 | 13 ± 1.2 |
| 1 mM MNNG | 10 ± 0.7 | 1.9 ± 0.3 | 2.1 ± 0.4 | 10 ± 0.8 |
| 1 mM NaN3 | 11 ± 0.9 | 2.4 ± 0.4 | 3.0 ± 0.4 | 2.2 ± 0.8 |
| 0.1 mM glyceryl trinitrate | 10 ± 0.6 | 2.0 ± 0.3 | 2.5 ± 0.5 | 11 ± 0.8 |

* Agents were added to hepatic slice reaction mixtures 20 min after preincubation at 37 °C in the presence of 0.5
mM M&B 22,948. Some preincubates also contained brilliant cresyl blue, KCN, or aminotriazole as indicated.
Slices were rapidly removed and freeze-clamped after 2 min of incubation at 37 °C. Control slices contained M&B
22,948 and were incubated at 37 °C for 22 min before freeze-clamping.

| Mean ± S.E. of four to six hepatic slices from each of four separate experiments. |
| Significantly different (p < 0.05) from corresponding values listed under “None.” |
N-acetylpenicillamine or NaN, (not shown). KCN inhibited viable cells. Cell viability was consistently about absence of additions to incubation mixtures.

That accurate and consistent determinations of cyclic GMP values.

Enzyme activation produced by all activators tested (Table VI). Aminotriazole selectively inhibited enzyme activation addition, the acetaminophen-pretreated mice were administered 500 min after each injection of N-acetylcysteine.

Influence of acetaminophen- and pretreatment on cyclic GMP formation in hepatic slices

| Additions | Control | Acetaminophen-pretreated | Acetaminophen + N-acetylcysteine-pretreated |
|-----------|---------|--------------------------|--------------------------------------------|
|           | pmol GMP/mg protein |                           |                                            |
| None      | 0.74 ± 0.09f | 0.68 ± 0.07 | 0.67 ± 0.06 |
| Glyceryl trinitrate | 0.5 mM | 6.7 ± 0.5 | 1.0 ± 0.3 | 5.4 ± 0.8 |
|           | (9) | (1.5) | (8) |
|          | 0.1 mM | 9.6 ± 1.0 | 1.1 ± 0.2 | 10 ± 1.1 |
|          | (15) | (1.6) | (15) |
|          | 1.0 mM | 11 ± 0.8 | 2.2 ± 0.7 | 11 ± 0.9 |
|          | (15) | (3.2) | (16) |
|          | 1 mM NaN3 | 8.7 ± 0.9 | 3.4 ± 0.5 | 8.1 ± 0.6 |
|          | (12) | (5) | (12) |
|          | 0.1 mM sodium nitroprusside | 10 ± 0.7 | 7.1 ± 0.8 | 9.4 ± 0.7 |
|          | (13) | (11) | (14) |
|          | 0.1 mM NaN3 | 8.2 ± 0.7 | 9.8 ± 0.8 | 10 ± 1.0 |
|          | (11) | (14) | (15) |

* Agents were added to hepatic slice reaction mixtures 20 min after preincubation at 37 °C in the presence of 0.5 mM M&B 22,948. Slices were rapidly removed and freeze-clamped after 2 min of incubation at 37 °C. Control slices contained M&B 22,948 and were incubated at 37 °C for 22 min before freeze-clamping.

* Mice were pretreated with 250 mg/kg of acetaminophen in saline (10 ml/kg) by intraperitoneal injection at 24, 48, and 2 h prior to death.

* Mice were pretreated with acetaminophen as described above. In addition, the acetaminophen-pretreated mice were administered 500 mg/kg of N-acetylcysteine (10 ml/kg) by intraperitoneal injection 30 min after each of the three injections of acetaminophen. Mice were sacrificed 90 min after the third injection of N-acetylcysteine.

* Mean ± S.E. of four to six hepatic slices from each of three separate experiments. Numbers in parentheses signify -fold increase in cyclic GMP levels when compared with values obtained in the absence of additions to incubation mixtures.

* Significantly different (p < 0.01) from corresponding control values.

discussion

The mouse hepatic slice system described appears to be a valid model for assessing the effects of various activators of soluble guanylate cyclase on cyclic GMP formation in intact viable cells. Cell viability was consistently about 95%. Cyclic GMP hydrolysis in the hepatic cells was extensive enough so that accurate and consistent determinations of cyclic GMP levels could not be made. This problem was resolved by inclusion of the selective cyclic GMP phosphodiesterase inhibitor, M&B 22,948 in incubation mixtures. Resting levels of cyclic AMP were unaffected by M&B 22,948 whereas the levels of both cyclic nucleotides were elevated by the nonselective phosphodiesterase inhibitor, 1-methyl-3-isobutyryl xanthine. Therefore, in order to avoid any possible confounding influence of elevated cyclic AMP levels on hepatic

**TABLE IV**

| Additions | Cyclic GMP | Phenobarbital-pretreated | Phenobarbital-and-DDC-pretreated |
|-----------|------------|-------------------------|---------------------------------|
|           | pmol GMP/mg protein |                          |                                 |
| None      | 0.74 ± 0.09f | 0.38 ± 0.07 | 2.9 ± 0.4 |
| 0.1 mM S-nitroso-N-acetylpenicillamine | 11 ± 1.4 | 8.1 ± 0.9 | 16 ± 1.3 |
|           | (14) | (21) | (5.7) |
| 0.1 mM sodium nitroprusside | 9.6 ± 1.1 | 9.2 ± 0.9 | 14 ± 1.5 |
|           | (13) | (24) | (4.8) |
| 0.1 mM NaN3 | 10 ± 0.8 | 9.1 ± 0.6 | 11 ± 1.2 |
|           | (14) | (24) | (4) |
| 0.1 mM glyceryl trinitrate | 2.2 ± 1.0 | 7.5 ± 0.8 | 10 ± 0.8 |
|           | (12) | (20) | (3.5) |
| 1 mM NaN3 | 8.9 ± 0.7 | 8.1 ± 0.9 | 5.2 ± 0.7 |
|           | (12) | (21) | (1.8) |
| 1 mM phenylhydrazine | 0.77 ± 0.05 | 3.8 ± 0.4 | 2.7 ± 0.2 |
|           | (0) | (10) | (0) |

* Agents were added to hepatic slice reaction mixtures 20 min after preincubation at 37 °C in the presence of 0.5 mM M&B 22,948. Slices were rapidly removed and freeze-clamped after 2 min of incubation at 37 °C. Control slices contained M&B 22,948 and were incubated at 37 °C for 22 min before freeze-clamping.

* Mice were administered 80 mg/kg of phenobarbital sodium in saline (10 ml/kg) by intraperitoneal injection once daily for four days. Mice were sacrificed 24 h after the last injection.

* Mice were pretreated with phenobarbital sodium as described above. In addition, mice were administered 400 mg/kg of DDC in corn oil (10 mg/kg) by intraperitoneal injection 2 h prior to sacrifice.

* Mean ± S.E. of four to six hepatic slices from each of three separate experiments. Numbers in parentheses signify -fold increase in cyclic GMP levels when compared with values obtained in the absence of additions to incubation mixtures.
TABLE V

Effects of activators and inhibitors on heme-reconstituted soluble guanylate cyclase purified from rat liver

| Additions* | Guanylate cyclase activity |
|------------|---------------------------|
|            | None | 0.1 mM brilliant creasel blue | 5 mM KCN | 1 mM amino-triazole |
|            | μmol cGMP/min/mg |

|            | 0.12* |
|------------|-------|
| None       | 0.08 |
| 0.1 mM S-nitroso-N-acetylpenicillamine | 0.12 |
| 0.1 mM MNNG | 0.12 |
| 1 mM NaN₃  | 0.12 |
| + 1 mM catalase | 0.12 |
| 1 mM NaN₃  | 0.12 |
| + 2 mM cysteine | 0.12 |
| 1 mM nitroprusside | 0.12 |
| + 2 mM cysteine | 0.12 |

* Activators were added to reaction mixtures at incubation immediately after enzyme. Inhibitors and other agents were added just prior to incubation. Reaction mixtures (0.2 ml) contained 40 mM TEA-HCl, pH 7.4, 1 mM GTP, 3 mM MgCl₂, 10 ng of heme-reconstituted enzyme, and the agents indicated. Incubations were at 37°C for 5 min.

**Average of triplicate determinations from a single experiment.

**Two additional experiments yielded very similar values to those illustrated.

TABLE VI

Properties of the activation of heme-deficient and heme-reconstituted soluble guanylate cyclase by phenylhydrazine and glyceryl trinitrate

| Additions* | Guanylate cyclase activity |
|------------|---------------------------|
|            | Heme-deficient enzyme | Heme-reconstituted enzyme |
|            | μmol cGMP/min/mg |

|            | 0.22* |
|------------|-------|
| None       | 0.11 |
| 0.1 mM phenylhydrazine | 0.36 |
| + 1 mM heme | 0.12 |
| + 1 μM hemoglobin | 0.12 |
| 0.3 mM glycerol trinitrate | 0.12 |
| + 2 mM cysteine | 0.12 |
| + 2 mM glutathione | 0.12 |

* Phenylhydrazine and glycerol trinitrate were added to reaction mixtures at incubation immediately after enzyme. The other agents were added just prior to incubation. Reaction mixtures (0.2 ml) contained 40 mM TEA-HCl, pH 7.4, 1 mM GTP, 3 mM MgCl₂, 28 ng (heme-deficient) or 10 ng (heme-reconstituted) of enzyme, and the agents indicated. Incubations were at 37°C for 5 min.

**Average of triplicate determinations from a single experiment.

**Two additional experiments yielded very similar values to those illustrated.

Cyclic GMP formation or on the radioimmunoassay for cyclic GMP, M&B 22,948 was employed throughout the study. Mouse hepatic slices released only small amounts of cyclic GMP into the extracellular medium, and this release was unaffected by probenecid. The percent of total cyclic GMP discharged from the cells was the same as or slightly greater than the percent of total lactate dehydrogenase activity released. Thus, the small degree of cyclic GMP extrusion is probably the result of a loss in cell viability. A small component may be due to passive diffusion but is not attributed to any active carrier system. These observations are unlike those reported for rat hepatic slices, where greater than 50% of the total cell-derived cyclic GMP was discharged from the cells, largely by a probenecid-sensitive active carrier system (27).

An important objective of this study was to ascertain whether the individual requirements for or properties of hepatic guanylate cyclase activation by select substances applied also to the stimulation of cyclic GMP accumulation in intact hepatic cells. S-Nitroso-N-acetylpenicillamine and MNNG activated guanylate cyclase in a heme-dependent manner and elevated hepatic cyclic GMP levels, both of which were inhibited by brilliant creasel blue and cyanide but not aminotriazole. Brilliant creasel blue is a lipophilic inhibitor of soluble guanylate cyclase (28) whereas cyanide binds to the heme of guanylate cyclase, thereby rendering heme inaccessible to NO (13, 29). In either case, guanylate cyclase activation and hepatic cyclic GMP accumulation elicited by NO or chemicals that release NO were inhibited. In addition, brilliant creasel blue produced a small inhibitory effect on both basal guanylate cyclase activity and resting hepatic levels of cyclic GMP. Azide had an absolute requirement for native catalase in order to activate guanylate cyclase, a finding that is in accord with previous reports (2, 11, 30). Catalase catalyzes the oxidation of azide to NO with the resultant formation of the nitrosyl-heme adduct of catalase (2, 9). The NO-heme moiety then exchanges with guanylate cyclase to yield the nitrosyl-heme derivative (10), which represents the activated form of the enzyme (1, 6, 10). Therefore, the activation of guanylate cyclase and the stimulation of hepatic cyclic GMP accumulation caused by azide were inhibited not only by brilliant creasel blue and cyanide but also by the catalase inhibitor aminotriazole.

Free sulfhydryls were required to observe the heme-dependent activation of guanylate cyclase by NaN₃. A variety of thiols satisfied this requirement and the mechanism involved is the rapid reaction of thiol and nitrite to yield an S-nitrosotiol (or thionitrite), which is a potent guanylate cyclase activator because of the extreme lability of the sulfurbound NO group (7, 8). Hepatic cells have a plentiful supply of thiols, primarily GSH, and NaN₃ caused marked cyclic GMP accumulation in hepatic slices. Hepatic slices prepared from acetaminophen-pretreated mice had markedly reduced free thiol levels and were less responsive to NaN₃. Full responsiveness to NaN₃ was restored in acetaminophen-pretreated mice that had been given N-acetylcysteine to regenerate hepatic thios. High doses of acetaminophen cause hepatic free sulfhydryl depletion by stimulating their oxidation (20). N-Acetylcysteine acts as a thiol-reducing agent by itself and after hepatic conversion to cysteine (31–33). Sodium nitroprusside and MNNG activate guanylate cyclase through the release of NO and their reactivity is increased by thiols, which accelerate the availability of NO by generating highly unstable S-nitrosothiols (94, 35). Hepatic slices from thiol-depleted mice were less responsive than slices from controls or N-acetylcysteine-treated mice to the cyclic GMP accumulating action of sodium nitroprusside or MNNG. Both enzyme activity and tissue cyclic GMP accumulation were inhibited by brilliant creasel blue and cyanide. These observations again highlight the striking similarity of the factors regulating both activation of purified guanylate cyclase and stimulation of hepatic cyclic GMP accumulation.

Organic nitrate esters such as glyceryl trinitrate require the presence of cysteine in order to activate crude and partially purified guanylate cyclase (7, 8), and this is attributed to the formation of S-nitrosocysteine which is a potent enzyme activator (34, 35). In the present study glyceryl trinitrate activated purified hepatic guanylate cyclase in both a cysteine- and heme-dependent manner. The capacity of glyceryl trinitrate to stimulate cyclic GMP accumulation was greatly impaired when livers from sulfhydryl-depleted but not sulfhydryl-repleted mice were employed. Although GSH is the principal thiol present in liver, small amounts of cysteine are present, and this is apparently sufficient to enable glyceryl trinitrate to stimulate hepatic cyclic GMP formation. Hepatic
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Free thiol depletion did not influence either resting hepatic levels of cyclic GMP or cyclic GMP accumulation stimulated by agents not requiring the presence of sulfhydryls, such as azide and the preformed S-nitrosothiol, S-nitroso-N-acetylpenicillamine. These observations support the view (8) that certain activators of guanylate cyclase, particularly nitrates and organic nitrate esters, utilize free sulfhydrils in cells to generate potent enzyme activators that stimulate cellular cyclic GMP accumulation. Large doses of organic nitrate esters cause hepatic thiol depletion (36) and the development of tolerance to both the vasodilator and cyclic GMP accumulating responses to the nitrate esters (37–40). Complete restoration of responsiveness to glyceryl trinitrate was produced in nitro by administration of sulfhydryl compounds (37–40) and in humans by administration of N-acetylcysteine (41). Thus, the present observations with hepatic slices mimicked those described above.

Pretreatment of mammals with phenobarbital is well known to stimulate hepatic synthesis of heme and hemoproteins such as cytochrome P-450. The administration of DDC and related chemical agents causes a marked decrease in hepatic levels of heme which is accompanied by the accumulation of hepatic protoporphyrin IX (21–23). DDC is metabolized by cytochrome P-450 to active intermediates that form adducts of protoporphyrin IX derived from the heme of cytochrome P-450 and perhaps other hepatic hemoproteins (22, 23). One such active intermediate is N-methylprotoporphyrin IX, which is a potent inhibitor of ferrochelatase (22, 23), resulting in inhibition of heme synthesis from protoporphyrin IX. These effects are greatly exaggerated in animals pretreated with phenobarbital. Hepatic slices from phenobarbital-pretreated mice showed significantly lower resting cyclic GMP levels than slices from untreated controls, and this may have been due to elevated heme concentrations as heme is an inhibitor of soluble guanylate cyclase (13). Similarly, heme-reconstituted guanylate cyclase showed a lower basal activity than did heme-deficient enzyme. In contrast, hepatic slices from DDC-treated mice showed a 4-fold increase in resting cyclic GMP levels, which may have been attributed to elevated protoporphyrin IX and decreased heme levels in liver. These findings are consistent with the knowledge that protoporphyrin IX is a potent activator of guanylate cyclase whereas heme is a competitive inhibitor (13). The apparent increase in the cyclic GMP accumulation in hepatic slices from phenobarbital-pretreated mice caused by nitroso compounds and other heme-dependent activators of guanylate cyclase (Table IV) may have been due to the presence of elevated heme levels.

The most noticeable effect of phenobarbital pretreatment was on the influence of phenylhydrazine, which produced a 10-fold increase in hepatic cyclic GMP accumulation using pretreated mice and was without effect on slices from untreated mice. This is consistent with the findings that the further addition of heme or hemoglobin to heme-reconstituted hepatic guanylate cyclase enhanced enzyme activation by phenylhydrazine. Guanylate cyclase activation by phenylhydrazine is markedly enhanced in the presence of excess hemoproteins, which react with the former to generate the iron-phenyl-heme species that is responsible for the enzyme activation (11). Consistent with this interpretation is the observation that decreasing the heme/protoporphyrin IX concentration ratio by treatment of mice with both phenobarbital and DDC resulted in the apparent abolition of hepatic cyclic GMP accumulation in response to phenylhydrazine. The 10-fold increase in hepatic cyclic GMP accumulation in response also to nitroso compounds and other guanylate cyclase activators was depressed in slices obtained from DDC-treated mice. This may be attributed to deficient hepatic levels of heme and/or to increased levels of guanylate cyclase-bound protoporphyrin IX. Although protoporphyrin IX is an enzyme activator, it also acts as a competitive inhibitor of NO since both species bind at a common site on guanylate cyclase (10–14).

In summary, the present observations reveal that the information gained from numerous studies by several groups of investigators on the regulatory properties of purified soluble guanylate cyclase bears a direct and close relationship to biochemical mechanisms that regulate cyclic GMP formation in intact functioning cells. The requirement of additional components in reaction mixtures for the full expression of guanylate cyclase activation by select agents was substantiated to a large extent in intact hepatic cells by manipulating hepatic levels of such components. Thus, free sulfhydrils permitted or enhanced enzyme activation by glyceryl trinitrate, NaNO_2_ and certain other agents, and hepatic slices from thiol-depleted but not thiol-repleted mice were also less responsive to the same agents. The requirement of catalase activity for enzyme activation by azide was substantiated in hepatic slices by the observation that slices pretreated with aminotriazole were much less responsive than control slices to the cyclic GMP accumulating effect of azide. Further, the marked enhancement of phenylhydrazine-elicited guanylate cyclase activation by excess heme or hemoproteins was consistent with the finding that hepatic slices only from phenobarbital-pretreated but not control or DDC-treated mice responded to phenylhydrazine with increases in cyclic GMP levels. Moreover, all heme-dependent enzyme activators produced greater 10-fold increases in hepatic cyclic GMP levels in slices obtained from phenobarbital-pretreated than from control or DDC-treated mice.

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