Gossypol Inhibition of Adenylate Cyclase

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Gossypol, a polyphenolic binaphthalene-dialdehyde reputed to exert contraceptive action in males, reversibly inhibits adenylate cyclase [ATP pyrophosphate lyase (cyclizing), EC 4.6.1.11] in a concentration-dependent manner. In membranes prepared from a variety of organs, the half-maximal inhibitory concentration (IC₅₀) ranges from 75 μM (rat Leydig tumor cells) to 250 μM (rat liver membranes). Kinetic studies using partially purified catalytic subunit isolated from bovine testis show that gossypol is competitive with ATP with an apparent Kᵢ of 110 μM. These data suggest that gossypol inhibition of adenylate cyclase is due to direct interaction at the nucleotide-binding domain of the catalytic subunit of the enzyme.

Cyclic AMP is a widely distributed second messenger involved in the modulation of multiple metabolic processes in eucaryotic cells [see (1) for a review]. Intracellular levels of cyclic AMP are regulated in large part by the activity of adenylate cyclase [ATP-pyrophosphate-lyase (cyclizing), EC 4.6.1.11]. Both the detailed understanding of the relationship of cyclic AMP levels in intact cells to physiological functions, and the purification of adenylate cyclase have proven difficult because of the lack of a suitable general, reversible inhibitor of the enzyme.

Gossypol [1,1',6,6',7,7'-hexahydroxy-5,5'-diisopropyl-3,3'-dimethyl-(2,2'-binaphthalene)-8,8'-dicarboxaldehyde] (Fig. 1), isolated from the meal and oil of cotton plants (Gossypium sp.) has been reported to be a potential nonsteroid male antifertility agent (2, 3). This compound has been shown to effectively inhibit Mg²⁺ + Ca²⁺ ATPase in sperm (4), L-lactate dehydrogenase isozyme C₄ in both testis and Trypanosoma cruzi (5, 6). Gossypol inhibition of lactate dehydrogenase is competitive with the nucleotide substrates, and it has been postulated that gossypol may function as a competitive inhibitor of a variety of nucleotide-metabolizing enzymes (6).

Because gossypol appears to be a general inhibitor of nucleotide-metabolizing enzymes, and these enzymes share common structural domains (7), we examined this compound as a potential inhibitor of adenylate cyclase in a number of systems. We also report the kinetics of inhibition at the catalytic site of adenylate cyclase using a partially purified catalytic subunit from bovine testis. This system was selected for further study because it offers the opportunity to examine effects directly at the catalytic site without interference from membranes and effector subunits.

Fig. 1. Structure of gossypol.
MATERIALS AND METHODS

Chemicals. Creatine phosphate, creatine phosphokinase, imidazole, papaverine, isopropylidine adenosine, NaCNBH3, Lubrol PX, PhMeSO4F, and gossypol acetic acid were purchased from Sigma. ATP, cyclic AMP, Tris, BSA, and Gpp(NH)p were obtained from Boehringer-Mannheim. Ion-exchange and sizing resins were from Pharmacia and Bio-Rad. H3P04 and cyclic [2,8-3H]AMP were from ICN. [32P]ATP was synthesized from [1-(a-P)]ATP by the method of Symons (10), and was purified by chromatography on DEAE-Sephadex A-25, followed by chromatography on Dowex AG-50. All other reagents were of the highest grade available commercially and were used without further purification.

Enzyme sources. Adult male rats strain CD [Charles River] of 350-550 g were sacrificed by cardiac puncture; liver and testis were removed by dissection and immediately placed in 50 mM imidazole-HCl, pH 7.5, 150 mM NaCl, 250 mM sucrose, 1 mM 2-mercaptoethanol, 0.5 mM EDTA, 1 mM PhMeSO4F (Buffer A) on ice. Epididymal sperm was obtained by cannulation of the epididymal vas deferens, immediately frozen in glycerol buffer (11), and stored in liquid nitrogen until use.

Tissues were washed in Buffer A, added to 2 vol of the same buffer, and homogenized with three strokes of a Potter-Elvehjem homogenizer. Plasma membrane fractions were obtained by centrifugation at 100,000g for 1 h at 4°C.

Rat Leydig tumor cell line LC-540 was obtained from the American Type Culture Collection [ATCC CCL 43], and grown in Eagle's minimal essential medium with nonessential amino acids and sodium pyruvate in Earle's basic salt solution supplemented with 10% fetal calf serum (GIBCO). Cultures were incubated at 37°C in a humidified atmosphere of 5% CO2. The cells were harvested at confluence and broken by sonication, and membranes were recovered by centrifugation at 100,000g for 1 h at 4°C. Membranes from bovine cerebral cortex were prepared using previously published methods (12).

Testes from freshly slaughtered bulls were obtained from Arena & Sons, Hopkinton, Massachusetts, and immediately placed on ice. The epididymis and testicular capsule were removed by dissection and the seminiferous tubular mass was isolated. The tissue was homogenized in 2 vol of Buffer A in a Waring blender. The mixture was further homogenized with three strokes of a Potter-Elvehjem homogenizer. The resulting homogenate was subjected to centrifugation at 7700g for 20 min. The pellet was discarded and the supernatant containing soluble adenylate cyclase was further purified by the procedure of Kornblüth et al. (13) through the sizing step.

Assay procedures. Adenylate cyclase activity was measured in a final volume of 50 μl using purified ATP as the substrate and cyclic [3H]AMP to monitor recovery. The assay mixture contained 1 mM [α-32P]ATP (120-200 cpm/pmol), 19 mM MgCl2 or 5 mM MgCl2, cyclic [2,8-3H]AMP (200,000 cpm/ml), 1 mM EDTA, 1 mM 2-mercaptoethanol, 100 μM papaverine, and an ATP-regenerating system consisting of 20 mM creatine phosphate and 120 units of creatine phosphokinase/ml in a 20 mM imidazole-HCl buffer, pH 7.5. Assays were carried out in triplicate for 10 min at 37°C with a standard error of less than 5%. The reaction was stopped and [32P]cyclic AMP formed was recovered by the method of Salomon et al. (14).

Gossypol solutions were freshly prepared by dissolving gossypol acetic acid in 10 mM sodium bicarbonate and adjusting the pH to 8.3 with 0.5 N HCl. Gossypol concentrations were estimated spectrophotometrically at 372 nm in a Perkin-Elmer Lambda 3 spectrophotometer using an absorbivity of 14.3 mM (15). Addition of gossypol solutions did not alter the pH of the adenylate cyclase assay.

Protein concentration was estimated by a modification of the Lowry method described by Peterson (16) using BSA as a standard.

All calculations and linear regression analyses were performed using a Tandy TRS-80 Model 4 computer (17).

RESULTS

Adenylate cyclase activity in both membranous and solubilized preparations was inhibited by gossypol in a concentration-dependent manner (Fig. 2). The concentration dependence of inhibition of enzymatic activity was examined in various tissues. As shown in Table I, gossypol was a general inhibitor of adenylate cyclase activity, with apparent IC50 values in the range of 75 to 500 μM. The higher IC50 value observed in the presence of Lubrol PX may reflect nonspecific interactions of gossypol with the detergent.

Adenylate cyclase is a complex enzyme system comprised of at least three non-identical subunits (1). In order to test whether gossypol inactivates the enzyme by interacting directly with the catalytic unit of adenylate cyclase, we isolated soluble adenylate cyclase from bovine testis. This form of the enzyme appeared to lack guanine nucleotide effector subunits based

\[ \text{Abbreviations used: BSA, bovine serum albumin; CaM, calmodulin; Gpp(NH)p, guanosine-5'[(beta,gamma-imido)triphosphate; PhMeSO4F, phenylmethysulfonfyl fluoride.} \]
Fig. 2. Inhibition of adenylate cyclase as a function of gossypol concentration. Adenylate cyclase activity was measured as described under Materials and Methods. The data shown represent the mean of triplicate measurements; (●) bovine cerebral cortex membranes; (△) partially purified adenylate cyclase from bovine testis; (□) adenylate cyclase in Lubrol PX-solubilized extracts from bovine cerebral cortex.

on the data in Table II, and the absence of ADP ribosylation in the presence of cholera toxin (Mary Jo Young, personal communication), and allowed us to examine direct effects on the catalytic site of the enzyme. Soluble adenylate cyclase from bovine testis showed a strict requirement for Mn$^{2+}$ similar to that observed in other soluble mammalian systems (8, 9). Kinetic analysis of the substrate requirements showed an apparent $K_m$ for Mn$^{2+}$ of

6 mM, and for ATP of 580 μM (Fig. 3). Kinetics of gossypol inhibition of adenylate cyclase activity, shown in the Dixon plot of Fig. 4, demonstrated that gossypol is a competitive inhibitor with ATP, with an apparent $K_i$ value of 110 μM.

Because of the observed competitive inhibition of adenylate cyclase activity and the dialdehyde nature of gossypol, we examined whether inhibition of enzymatic activity was reversible, or the result of a covalent interaction with an ε-amino group in the catalytic site. This possibility has been put forth to explain the competitive inhibition of adenylate cyclase activity by 2,3-dialdehyde-ATP, which could be rendered irreversible on reduction with Na-CNBH$_3$ (18). Partially purified catalytic subunit was incubated with 400 μM gossypol for 20 min at 4°C. Under these conditions, enzyme activity was reduced to 3 to 5% of the control (8.2 ± 0.01 vs 279 ± 4 pmol cAMP formed mg$^{-1}$ min$^{-1}$). After incubation, the adenylate cyclase-gossypol complex was passed over a 1 X 5-cm Bio-Gel P-4 column and eluted into 20 mM imidazole-HCl, pH 7.5, 250 mM sucrose, 0.5 mM EDTA, 1 mM 2-mercaptoethanol, and assayed for activity. This treatment restored activity of the enzyme to 96% of control samples treated in a similar manner (269 ± 2.0 vs 279 ± 4.0 pmol cAMP formed mg$^{-1}$ min$^{-1}$). Unlike inhibition with 2,3-dialdehyde-ATP, gossypol inhibition of

<table>
<thead>
<tr>
<th>Enzyme source</th>
<th>IC$_{50}$ (μM)</th>
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<tbody>
<tr>
<td>Rat liver</td>
<td>250$^b$</td>
</tr>
<tr>
<td>Rat sperm</td>
<td>200</td>
</tr>
<tr>
<td>Rat Leydig tumor cells</td>
<td>75</td>
</tr>
<tr>
<td>Bovine testis</td>
<td>160</td>
</tr>
<tr>
<td>Bovine cerebral cortex</td>
<td>100</td>
</tr>
<tr>
<td>Solubilized bovine cerebral cortex</td>
<td>600</td>
</tr>
</tbody>
</table>

$^a$ Membranes were prepared from several tissues, and the concentration dependence of gossypol inhibition of adenylate cyclase activity was measured as described under Materials and Methods.

$^b$ IC$_{50}$ values were obtained from concentration-dependent inhibition curves and calculated using the computer program described by Tallarida and Murray (17).

**TABLE I**

**GOSSYPOL INHIBITION OF ADENYLATE CYCLASE**

<table>
<thead>
<tr>
<th>Addition</th>
<th>Cyclic AMP formed (pmol mg$^{-1}$ min$^{-1}$)</th>
<th>Effect (-fold)</th>
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<tbody>
<tr>
<td>None</td>
<td>196 ± 3</td>
<td>1.0</td>
</tr>
<tr>
<td>CaM, 2 μg</td>
<td>198 ± 2</td>
<td>1.0</td>
</tr>
<tr>
<td>NaF, 15 mM</td>
<td>200 ± 2</td>
<td>1.1</td>
</tr>
<tr>
<td>Gpp(NH)p, 10 μM</td>
<td>198 ± 1</td>
<td>1.0</td>
</tr>
<tr>
<td>Gpp(NH)p, 100 μM</td>
<td>204 ± 3</td>
<td>1.1</td>
</tr>
<tr>
<td>Mg-ATP$^b$</td>
<td>0</td>
<td>—</td>
</tr>
</tbody>
</table>

$^a$ Effector molecules were added to the assay at the final concentrations shown.

$^b$ 5 mM MgCl$_2$ was used in place of MnCl$_2$.  

**TABLE II**

**EFFECTS OF MODULATORS OF ADENYLATE CYCLASE ACTIVITY ON PARTIALLY PURIFIED BOVINE TESTIS ADENYLATE CYCLASE**
FIG. 3. Initial velocities of adenylate cyclase activity with variations in Mn$^{2+}$ and ATP. (A) Adenylate cyclase activity was measured in the presence of varying concentrations of Mn$^{2+}$ with ATP held constant at 1 mM. (B) Enzyme activity was measured as described previously except Mn$^{2+}$ was constant at 19 mM and ATP concentrations were varied as shown in the plot. Data for the double-reciprocal plots (inset) were analyzed by linear regression analysis ($r = 0.99$).

The results of this study show that gossypol is an effective inhibitor of both membrane-bound and soluble adenylate cyclases. This inhibition appears to result from direct interaction with the catalytic subunit, as demonstrated with partially purified enzyme from the testis. Inhibition is competitive with ATP (Fig. 4), indicating that gossypol binds at the nucleotide-binding domain common to adenylate cyclase and other nucleotide-metabolizing enzymes (7, 19). Several other inhibitors have been reported for adenylate cyclase (18, 20).
ADENYLATE CYCLASE INHIBITION BY GOSSYPOL

The competitive, reversible nature of gossypol inhibition of adenylate cyclase, this compound may prove to be a useful tool to achieve this goal.

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REFERENCES
