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Membrane actions of male contraceptive gossypol tautomers

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The role of different gossypol tautomers in the interaction of this molecule with membranes has been investigated using the isolated hemiacetal moiety of gossypol and the pH dependency of the keto-enol tautomeric equilibrium. Our results indicate that: (a) the actions of the hemiacetal tautomer cannot explain the effects of gossypol on mitochondrial oxidative phosphorylation, lipid membrane interfacial potentials, and proton conductance of lipid bilayers; (b) the enolate forms of gossypol are the species that bind to the membrane interface and decrease the electrostatic interfacial potential; and (c) the uncharged (keto and/or enol) species in equilibrium with the enolate forms of gossypol give the molecule the ability to carry protons across biological membranes.

Introduction

The experimental male contraceptive gossypol is a binaphthalene polyalcohol aldehyde that can exist in three different tautomeric forms in aqueous solutions (Fig. 1) [1]. One of the most pronounced effects of gossypol on biological systems is its ability to uncouple mitochondrial oxidative phosphorylation (see, for example, Ref. 2). The uncoupling properties as well as other actions of gossypol on membranes can be explained by the ability of this molecule to interact with the phospholipids of biological membranes, producing changes in the electrical properties of the lipid bilayer domain [3]. However, the specific involve-

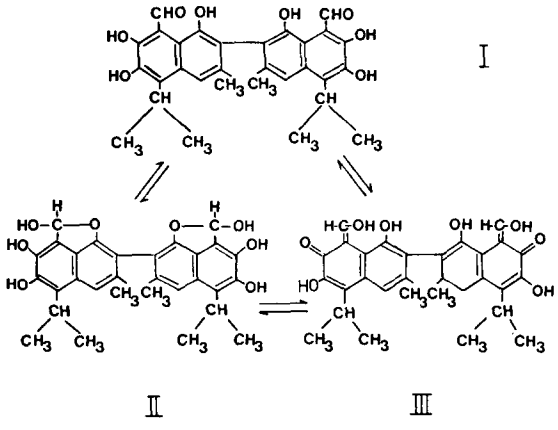

Fig. 1. Tautomeric structures of gossypol (1,1′,6,6′,7,7′-hexahydroxy-5,5′-diisopropyl-3,3′-dimethyl[2,2′-binaphthalene]-8,8′-dicarboxaldehyde) in aqueous solution. Compounds I and III are the keto and enol forms of the aldehyde and hydroxyls in positions 8,8′ and 7,7′, respectively. Compound II is the hemiacetal between the aldehyde and hydroxyl in positions 8,8′ and 1,1′, respectively.

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ment of the different tautomers of gossypol in producing these various membrane effects has not been elucidated.

In this work we report the synthesis and purification of the hemiacetal tautomer of gossypol. This tautomer is relatively stable in aqueous solutions at neutral or mildly acid pH values. Thus, the problem of the actions of the different gossypol tautomers on membranes can be approached by studying the effects on membranes of the mixed tautomers and hemiacetal gossypol. Our findings indicate that: (1) the actions of the hemiacetal tautomers of gossypol cannot explain the ability of gossypol to uncouple mitochondrial oxidative phosphorylation, to bind to phospholipids and change the interfacial potential of lipid membranes, or to induce a proton conductance in lipid bilayers; (2) the enolate anion of gossypol is the species that binds to the membrane interface and modifies the electrostatic interfacial potential; and (3) the uncharged (keto/enol) gossypol moieties also bind to lipid membranes. The uncharged species of gossypol together with the enolate forms confer upon the molecule the ability to carry protons across biological membranes.

Materials and Methods

Synthesis of hemiacetal gossypol

Gossypol acetic acid (52 mg) was dissolved in 15 ml of toluene. Glacial acetic acid (0.1 ml) was added and the solution was stirred under reflux for 24 h. The volatile substances were evaporated under vacuum and the dark residue was column chromatographed on 4 g of silica gel 60 (70–230 mesh) with CH_2Cl_2 to produce a final 25 mg of solid (m.p. 148–150°C). The isolated compound was further purified by thin-layer chromatography (TLC) at an R_f of 0.85 with CH_2Cl_2/CH_3OH (95 : 5, v/v) as solvent. ^{1}H-NMR studies revealed the absence of the reported gossypol aldehydic proton at δ 11.1 ppm and the perihydroxyl protons at 15.08 ppm. A new signal was present at 7.38 ppm which has been shown to be due to a hydrogen attached to the lactol ring [4,5]. Heating the hemiacetal gossypol at 50°C in 0.1 M HCl for 10 h quantitatively converted the hemiacetal moiety back to the aldehyde gossypol as indicated by TLC and ^{1}H-NMR studies.

Preparation of rat liver mitochondria

Rat liver mitochondria were prepared using a homogenization and differential centrifugation technique in a medium containing 220 mM mannitol, 70 mM sucrose, 2 mM Hepes-KOH (pH 7.4), 2 mM EGTA, and 0.5 mg/ml bovine serum albumin [6]. Freshly prepared mitochondria had respiratory control ratios of approximately 4 in succinate containing medium at 25°C. Mitochondrial ADP/oxygen ratios in glutamate/malate and succinate were 2.9 and 1.9, respectively. The functional parameters of the mitochondria were tested in isolation medium supplemented with 5 mM potassium phosphate and 5 mM of each substrate. ADP was added at a final concentration of 0.4 mM.

Oxygen consumption measurements

Oxygen consumption (Q_{O_2}) measurements were performed polarographically in a sealed glass chamber (0.9 ml) surrounded by a water jacket held at 25°C. Q_{O_2} values were obtained from the slope of the O_2 tension versus time record between 15 and 60 s after each experimental addition to the Q_{O_2} chamber. Oxygen consumption of isolated mitochondria was measured by addition of an aliquot of the organelle suspension to the O_2 consumption chamber containing medium preincubated at 25°C with air. The final concentration of mitochondria was 1.0 mg protein/ml.

Lipid monolayers and lipid bilayers experiments

The interfacial potential of lipid monolayers and the conductance of lipid bilayers were measured as described by Reyes et al. [3].

Briefly, the interfacial potential of lipid monolayers and the changes induced by gossypol were measured at room temperature (20 ± 2°C) in a two-compartment chamber. An excess of lipids (10 μl, 10 mg/ml) was added in chloroform solution to the air/water interface to obtain a saturated monolayer. The experiments were performed at constant monolayer area (200 cm^2). The subphase was composed of NaCl at different concentrations and buffered at different pH values as described in the text. The concentration of gossypol was varied by addition of microliter volumes of concentrated ethanolic solution of gossypol to the subphase of the monolayer-free compartment.

The subphase was stirred with one magnetic bar positioned under a partition that separated the surface of the monolayer and monolayer-free compartments. Control experiments showed that ethanol at the concentrations used (< 200 fl 0.3%, v/v) had no effect on the surface potential of the lipid monolayers. Bilayer membranes were formed at room temperature (20 ± 2°C) according to the Montal and Mueller technique [7]. The phospholipid was spread on the surface of an electrolyte solution using 5 μl of a 10 mg/ml solution of the lipids in pentane. The approx. 0.5-mm round aperture in the Teflon partition separating the two aqueous compartments was pretreated with a 2% solution of squalene in pentane. Unless otherwise indicated, the electrolyte solutions were symmetrical and consisted of 0.1 M NaCl buffered with 50 mM phosphate. Gossypol was added from concentrated ethanolic solutions to the aqueous phases bathing the membrane. Control experiments showed that ethanol, at the concentrations used (< 0.5%, v/v), had no effect on either the bare membrane conductance or capacitance, or on the gossypol-induced conductance. We used diphytanoylphosphatidylcholine for both the monolayer and bilayer experiments.

Thin-layer chromatography

The possible formation of gossypol-phosphatidylcholine covalent complexes was investigated using silica gel G thin-layer chromatography (Analtech, Newark, DE). PC unilamellar vesicles were prepared by sonication of lipids (1 mg/ml) at 4°C in 0.1 M NaCl, 5 mM phosphate (pH 7.9) for 15 min. The vesicle suspension was incubated at 20°C for 1.5 h in the absence or presence of 2 or 100 μM gossypol. The lipids and gossypol were extracted by addition of $CHCl_3/CH_3OH$ (2:1, v/v) and 1 ml of distilled water. The mixture was vortexed vigorously followed by addition of 1 ml of $CHCl_3$. The lower phase was removed and concentrated to the desired volume by evaporation under nitrogen. Thin-layer chromatography was performed with $CHCl_3/CH_3OH/$ acetic acid (63:25:8, v/v) and $CHCl_3/CH_3OH/NH_4OH$ (59:32:4.6, v/v). The lipids and gossypol were visualized by charring with 50% H_2SO_4 (gossypol gives a scarlet red color) or by spraying with 0.1% 2′,7′-dichlorofluorescein in 95% ethanol [8,9].

pH-dependence of the gossypol uncoupling

The oxygen consumption of freshly isolated liver mitochondria was determined at different pH values of a medium containing 220 mM mannitol, 70 mM sucrose, 5 mM potassium phosphate, 2 mM Hepes, 0.5 mM EGTA, 0.2 μg/ml bovine serum albumin and 5 mM succinate, or a mixture of glutamate/malate at 25°C. The functional state of the mitochondria was tested at each pH by addition of 0.4 mM ADP. Dose-response curves for gossypol stimulation of oxygen consumption at each pH were determined. A 2.2 μM gossypol concentration was chosen for repetitive experiments at different pH values. After gossypol addition and measurement of oxygen consumption, 0.4 mM ADP was added to estimate the degree of uncoupling and the extra oxidative capacity of the mitochondria. At all pH values, ADP was able to maximally stimulate oxygen consumption, indicating that the lack of maximal stimulation of Q_{O_2} by gossypol at pH values lower than 5.5 and higher than 6.5 is not due to inhibitory effect of gossypol on the mitochondria.

Mitochondrial pH gradients

The pH measurements were performed as described by Mitchell and Moyle [10]. The mitochondria (2–3 mg protein/ml) were suspended in a medium containing 250 mM sucrose, 3.3 mM glycylglycine, 10 mM choline chloride, 2.5 mM glutamate, 2.5 mM malate, 0.4 μg valinomycin/ml, 3.7 μg oligomycin/ml, catalase 33 units/ml at pH 7.1 (KOH) and 20°C. The measurements were done in a glass chamber fitted with a Radiometer pH electrode connected to a Radiometer pH meter and chart recorder.

Results

Acid-base properties of the mixed tautomers and hemiacetal gossypol

Aqueous solutions of gossypol acetic acid or free-acid gossypol presented a single inflection point in the titration curves. The inflection point occurred at an average pH of 6.6 at 20°C (see also Ref. 3). From the total amount of gossypol present in solution and the amount of acid or base needed to titrate the dissociable groups we calculated the existence of 2.1 ± 0.6 dissociable groups

per gossypol molecule. In contrast, titration with 2 mM NaOH, of hemiacetal gossypol dissolved in dilute hydrochloric acid (0.2 mM) showed no inflection point between pH 4.4 and pH 9 (Fig. 2). However, the dissolution of hemiacetal gossypol in 0.2 mM NaOH induced a time dependent drop in pH accompanied by a change in the absorbance at 400–500 nm of the solution. Subsequent titration with 2 mM HCl showed the typical inflection point of the mixed gossypol tautomers with an inflection point at pH ≈ 6.5. An example of successive acid and base titrations that illustrate this point is shown in Fig. 2.

Possible covalent complexes between gossypol and phosphatidylcholine

The interaction of gossypol with lipid monolayers to produce a change in the interfacial potential of the membranes could be the result of both non-covalent absorption and covalent reaction between PC and gossypol. The steady-state interfacial potential measurements would be a reflection of an equilibrium between aqueous gossypol and gossypol partitioned in the membrane if the formation of covalent complexes is negligible. The evidence that the interfacial potential change can be reversed more than 60% by the successive washings of the monolayer subphase does suggest that the gossypol-PC is a non-covalent interaction [3]. However, the formation of gossypol-phosphatidylcholine covalent complexes was checked by thin-layer chromatography of pure gossypol, pure PC extracted from unilamellar vesicles, and PC/gossypol extracted from incubated mixtures of gossypol and PC unilamellar vesicles.

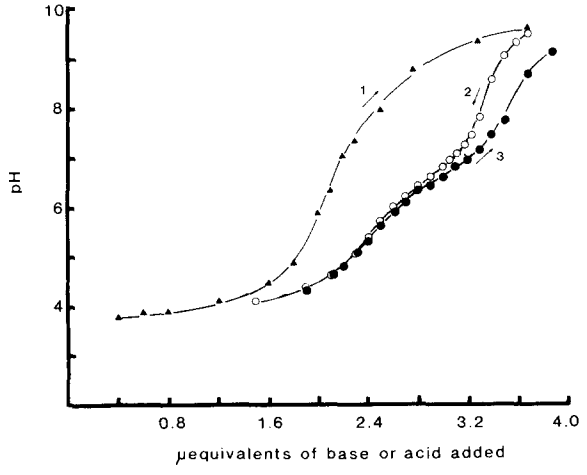

Fig. 2. Successive titrations of 0.4 μmoles of hemiacetal gossypol with NaOH or HCl in aqueous solution at 20°C.

In $CHCl_3/CH_3OH$/acetic acid, phosphatidylcholine appeared at an R_f of 0.19 ± 0.03 ($N = 10$). Gossypol runs with the solvent front ($R_f = 1$). The incubation of gossypol with unilamellar PC vesicles did not produce a change in the R_f for PC or the appearance of new spots in the TLC plates. Using $CHCl_3/CH_3OH/NH_4OH$, as the solvent system, PC ran with an R_f value of 0.32 ± 0.01. Gossypol presented a definite spot that migrated at an R_f of 0.50 ± 0.02. Other more diffuse gossypol spots were also observed at higher R values in this solvent system. As in the previous solvent system, incubation of gossypol and PC unilamellar vesicles did not produce any change in the gossypol or PC R_f values or the appearance of new spots. Dichlorofluorescein labelling of PC did not show any significant presence of lipids at any position other than that position expected for phosphatidylcholine.

Mitochondrial oxidative phosphorylation

Addition of micromolar concentrations of the mixed gossypol tautomers to state 4 liver mitochondria produced a stimulation of the mitochondrial oxygen consumption and a concomitant release of the mitochondrial Q_{O_2} stimulation by 0.6 mM ADP (Fig. 3A and B). As described for other uncouplers of oxidative phosphorylation (see, for example, Ref. 11), at concentrations above 2 μM, gossypol inhibited the oxygen consumption of liver mitochondria. In contrast, the hemiacetal gossypol moiety only increased the mitochondrial oxygen consumption at concentrations higher than 10 μM (Fig. 3A). At concentrations lower than 10 μM, hemiacetal gossypol did not affect significantly the ability of 0.6 mM ADP to stimulate mitochondrial Q_{O_2}. This result indicates that the approximately 15-fold shift in the hemiacetal gossypol Q_{O_2} vs. concentration curve as compared to the mixed gossypol tautomers is not the result of an inhibition of reducing equivalent delivery into the mitochondria or inhibition of the mitochondrial electron transport chain.

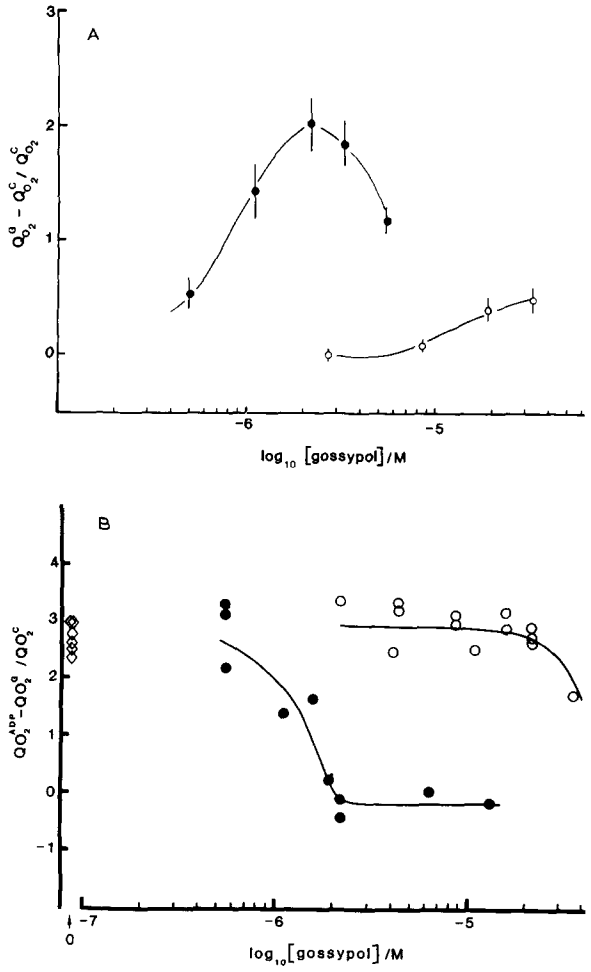

Fig. 3. (A) Fractional increase in the oxygen consumption (Q_{O_2}) of rat liver mitochondria (1 mg protein/ml) at different gossypol concentrations. The mitochondria were suspended in mannitol-sucrose medium with succinate as substrate at 25°C. $Q_{O_2}{}^{G}$ and $Q_{O_2}{}^{C}$ represent the mitochondrial oxygen consumption in the presence and absence of gossypol, respectively. ●, mixed tautomers; ○, hemiacetal. (B) Stimulation of mitochondrial O_2 consumption by 0.6 mM ADP ($Q_{O_2}{}^{ADP}$) after addition of different gossypol concentrations. The ratio $(Q_{O_2}{}^{ADP} - Q_{O_2}{}^{G})/Q_{O_2}{}^{C}$ is a measurement of mitochondrial coupling. ●, mixed tautomers; ○, hemiacetal.

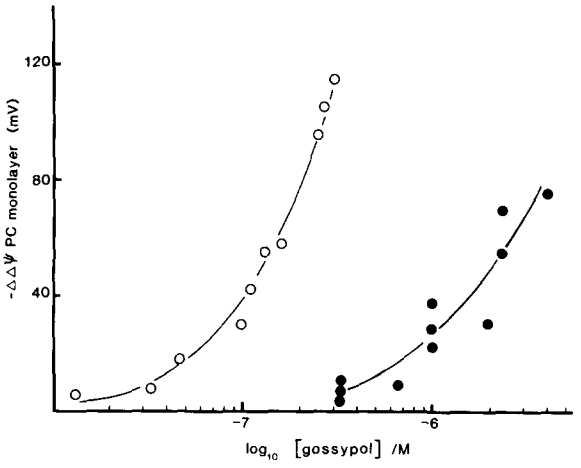

Fig. 4. Changes in phosphatidylcholine monolayer interfacial potentials versus gossypol concentration in the subphase. The experiments were performed at 20°C, 0.1 M NaCl, 5 mM phosphate (pH 7.2). ○, mixed tautomers; ●, hemiacetal.

Interaction of the gossypol tautomers with phosphatidylcholine monolayers

The mixed gossypol tautomers interacted with phospholipid monolayers and induced changes in the electrostatic interfacial potentials of the lipids (Fig. 4; see also Ref. 3). Fig. 4 also shows that hemiacetal gossypol did not produce changes in the interfacial potential of phosphatidylcholine monolayers when present in the aqueous solution at concentrations below 0.3 μM. At this aqueous concentration the mixed gossypol tautomers induced a 120 mV decrease in the PC monolayer interfacial potential. Thus, the dose-response curve for interfacial potential changes vs. hemiacetal gossypol concentration is shifted toward about 10–15-fold higher concentrations as compared to the mixed gossypol tautomers dose-response curve.

The pH dependence of the interfacial potential change induced by the mixed gossypol tautomers is illustrated in Fig. 5. A characteristic saturation of the effect of high pH values was observed. The mid-titration point for this effect of gossypol occurred at a pH of ≈ 6.3. A substantial change in interfacial potential remained after the pH of the aqueous subphase had been lowered to pH 4.0. The magnitude of this interfacial potential change at pH 4.0 was relatively independent of the initial pH of the monolayer subphase. For discussion purposes, the calculated aqueous concentrations of the uncharged, univalent and divalent forms of gossypol as a function of pH are shown in Fig. 6.

Effect of gossypol on phosphatidylcholine bilayer conductance

As shown by Reyes et al. [3], the mixed tautomers of gossypol can increase by about two

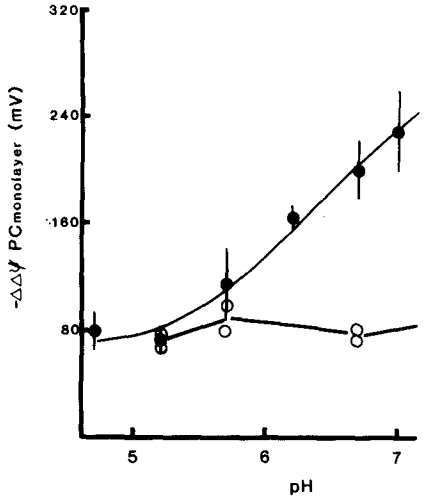

Fig. 5. Changes in phosphatidylcholine monolayer interfacial potential induced by 1.3 μM gossypol at different pH values and 20°C (closed circles). The bars represent the standard deviation of at least four determinations. The residual interfacial potential changes after the subphase pH has been lowered to pH 4 are shown as open circles. 0.1 M NaCl, 5 mM phosphate.

orders of magnitude planar phosphatidylethanolamine bilayer conductance when added in micromolar concentrations to the aqueous phases (pH 7.0). A similar effect was observed in diphytanoylphosphatidylcholine bilayers (Fig. 7). In

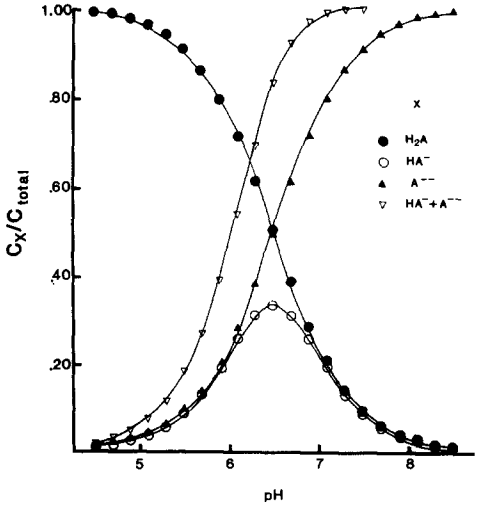

Fig. 6. Calculated concentrations of uncharged, univalent and divalent gossypol in aqueous solution at 20°C and different pH values (pK_a 6.6).

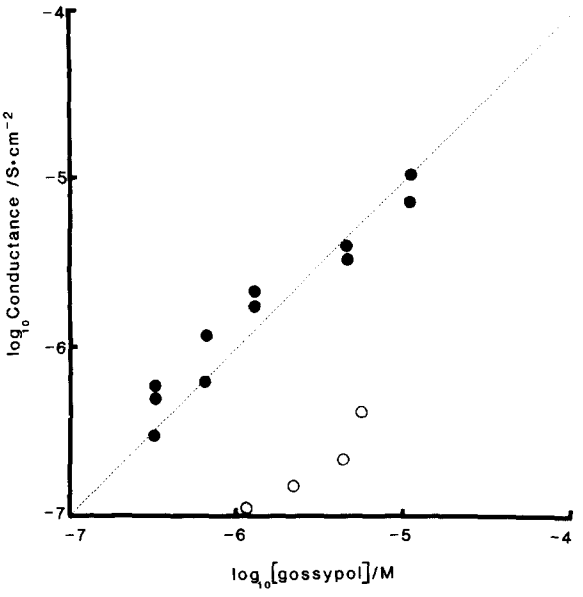

Fig. 7. The effect of gossypol on the conductance of solvent-free PC bilayer membranes. Log-log of the zero-voltage conductance of diphytanoyl-PC membranes vs. gossypol concentrations at 20°C. The broken line is for reference purposes and has a slope equal to 1. The zero-voltage bilayer conductance in the absence of gossypol was $(1.5 \pm 1.0) \cdot 10^{-8}$ S/cm^2. 0.1 M NaCl, 50 mM phosphate (pH 7.0).

contrast to the effect of the mixed tautomers of gossypol, the hemiacetal gossypol moiety only began to modify the bilayer conductance when present at concentrations about 10-fold higher.

pH-dependence on gossypol uncoupling

The pH-dependence of the mitochondrial uncoupling of 2.2 μM gossypol at 25°C is shown in Fig. 8. Gossypol was tested a concentration of 2.2 μM after characterization of the uncoupling versus concentration curves at different pH values (not shown). A maximum uncoupling effect of gossypol at about pH 6.0 was observed.

The pH dependence of the gossypol uncoupling compares well with the pH dependence of the conductance changes induced by gossypol in lipid bilayers [3].

Effects of gossypol on mitochondrial pH gradients

Addition of oxygen to an anoxic mitochondrial suspension in the presence of electron chain substrates and valinomycin induces a pH gradient

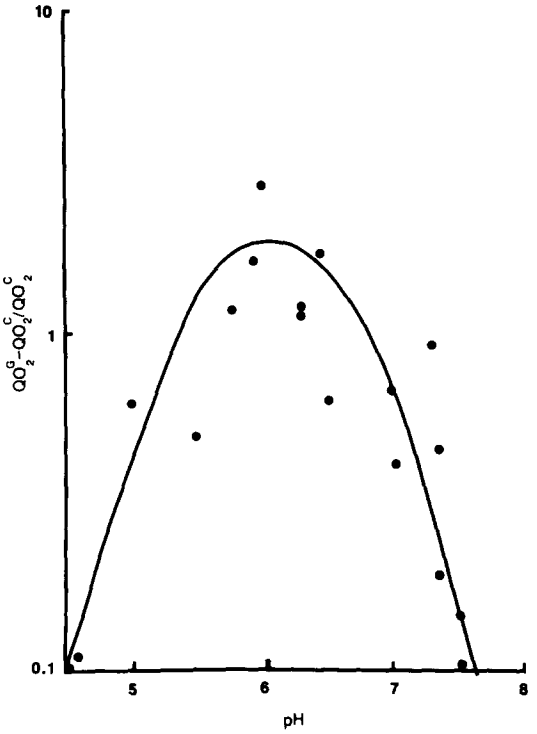

Fig. 8. pH-dependence of the gossypol uncoupling of isolated liver mitochondria (0.5 mg protein/ml) at 25°C. The mitochondria were suspended in a mannitol-sucrose medium with 5 mM glutamate/malate or succinate as metabolic substrate. The gossypol concentration at each pH was 2.2 μM.

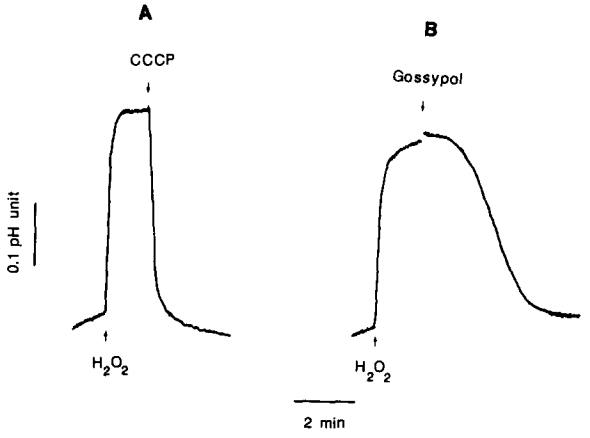

Fig. 9. pH changes induced by oxygen pulses in mitochondrial suspensions (3 mg protein/ml) in a medium containing 0.25 M sucrose, 3.3 mM glycylglycine, 10 mM choline chloride, 2.5 mM glutamate, 2.5 mM malate, 7 mM potassium, 0.4 μg/ml valinomycin, 3.7 μg/ml oligomycin, 33 units/ml catalase at pH 7.1 and 20°C. CCCP (A) and gossypol (B) were added from concentrated ethanolic solutions to a final concentration of 0.4 and 16 μM, respectively. Mitochondria were preequilibrated without oxygen for 10 min at pH 6.9–7.0. Upward traces represent external acidification.

across the mitochondria membrane [10]. Fig. 9 depicts this kind of phenomenon with rat liver mitochondria suspended in sucrose medium supplemented with valinomycin, oligomycin and catalase. The mitochondrial suspension was preequilibrated without oxygen for 10 min at 20°C. Addition of H_2O_2 produces an external acidification that is reversed by addition of the uncoupler carbonyl cyanide *m*-chlorophenylhydrazone (CCCP, 0.4 μM, Fig. 9A). Addition of gossypol (16 μM, Fig. 9B) mimics the effect of CCCP in collapsing the pH gradient induced by the O_2 pulse. Gossypol dose-response uncoupling effects in rat liver mitochondria are dependent on the mitochondrial concentration. Thus, maximal uncoupling effects at 3–4 mg mitochondrial protein/ml can be observed at 15–30 μM gossypol (Reyes, Allen and Benos, unpublished results). From the right shift in the dose-response curve of gossypol uncoupling with increasing liver mitochondria concentrations, we can estimate that approx. 1.8–2.0 nmoles of gossypol bind per mg of mitochondrial protein (see, for example, Ref. 11).

Discussion

Reyes et al. [3] suggested that the tautomers involved in the interaction of gossypol with lipid membranes were the keto and enol forms of the compound. However, the participation of the hemiacetal tautomer of gossypol on the interactions of gossypol with membranes was not completely excluded. In this work we have used the stable hemiacetal tautomer of gossypol to address this issue.

Thin-layer chromatography and ^{1}H-NMR studies of the hemiacetal moiety of gossypol have shown that its properties are in agreement with the structure presented in Fig. 1(II). The aqueous acid-base behavior of the hemiacetal tautomer also demonstrates that its aqueous properties are clearly different from the mixed tautomers (Fig. 2). Our results also point to the importance of the al-

dehyde group in gossypol in conferring to the molecule the properties of being a weak acid with two dissociable groups and a pK_a of ≈ 6.5. The weak acid properties of gossypol arise from the keto-enol tautomerism of the aldehydes and hydroxyls in positions 8,8′- and 7,7′-, respectively. The enol tautomer would therefore contain weak acid protons in positions 7 and 7′ (see, for example, Ref. 5). Similar acid-base properties to those presented by gossypol have been shown for other molecules that also display a similar keto-enol tautomerism [12,13].

As shown in Figs. 3A and B, 4 and 7, the hemiacetal moiety of gossypol interacts differently with biological and artificial membranes than do the mixed tautomers. Thus, the existence of hemiacetal gossypol cannot explain the capacity of the mixed gossypol tautomers to uncouple mitochondrial oxidative phosphorylation, to induce changes in the interfacial potential of lipid bilayers and monolayers, or to increase the conductance of lipid bilayers. Combining the mixed tautomers of gossypol with the hemiacetal tautomer does not modify the magnitude of the effects that the mixed tautomers themselves exert on the membranes. Furthermore, conversion of high pH of the hemiacetal tautomer to the keto-enol tautomers shifts the dose response curve in mitochondria and lipid monolayers back toward the mixed tautomer dose response relation. The 10–15-fold higher concentration of hemiacetal gossypol required to produce changes in the above mentioned characteristics of the membranes could be explained either by a slow conversion (5–10%) of hemiacetal to the keto-enol tautomers of gossypol which could occur either with storage or slowly at neutral pH in aqueous solution; or by a 10–15-fold difference in affinity of the two tautomers for the phospholipids (see Results). Hence, the keto-enol moieties of gossypol are the main tautomers responsible for the membrane actions of this compound.

The role of the uncharged keto-enol or the enolate anions can also be elucidated utilizing the acid-base properties of gossypol. Thus, it is the mono and/or divalent enolate species of gossypol that interact with the lipid solution interface and modify the electrostatic interfacial potential of the membranes. This conclusion is supported by the following experimental evidence. First, gossypol does not form appreciable amounts of covalent complexes with PC and hence, the steady-state measurements of electrical properties of lipid bilayers and monolayers reflect an equilibrium between aqueous gossypol and gossypol in the membrane phase. Second, from the pH dependence of the gossypol-induced monolayer interfacial potential (compare Figs. 6 and 7), it cannot be the uncharged keto and/or enol gossypol species that is (are) producing the changes in monolayer interfacial potentials. On the other hand, if only the monovalent gossypol species were involved in the monolayer interfacial potential change, a maximum in the interfacial potential change vs. pH curve would be expected at a pH of ≈ 6.5. A similar prediction could be made for the induction of zeta potentials by gossypol in PC vesicles [3].

Thus, our results strongly support the idea that either the divalent or the mono and divalent gossypol moieties are producing the change in interfacial potential of the PC monolayers. This conclusion does not rule out the existence of the uncharged gossypol species in the membrane. It only implies that the changes induced by the binding of the mono/divalent charged species of gossypol at the lipid/solution interfacial region are the main cause of the interfacial potential changes. In fact, the uncharged gossypol moieties (keto and/or enol) do appear to bind to membranes. An indication of this binding of the uncharged gossypol is the 60–80 mV change in the interfacial potential shown at pH values less than 5.2 (Fig. 5) where less than 0.5% of the gossypol is in the charged form. As discussed by Reyes et al. [3], the ability of gossypol to transport protons across lipid bilayers can only be explained by a proton transport mechanism where both the uncharged and the mono- and divalently charged species can partition into the membranes. The plausibility of this mechanism of proton transport and its applicability to the uncoupling mechanism of gossypol on mitochondria is also supported by the results of the pH-dependence of the gossypol uncoupling in liver mitochondria (Fig. 8), and by our direct measurements of the ability of gossypol to collapse the mitochondrial membrane pH gradient (Fig. 9). The slower drop in mitochondrial membrane pH gradient induced by gossypol as

compared to CCCP could, in principle, be attributed to both the comparatively low proton conductance induced by gossypol because of the low permeability of the uncharged gossypol moiety in lipid membranes (see also Refs. 14–17), as well as to the marked dependency on pH (and pH gradients) of the gossypol-induced proton conductance due to the existence of the relatively impermeable A^{2-} gossypol moiety [17]. Gossypol also appears to be able to collapse the mitochondrial membrane potential of Sertoli cells in culture as evidenced by the distribution of the cationic fluorescent dye, Rhodamine 123 [18].

In conclusion, we can state that: (a) hemiacetal gossypol either does not participate or participates only marginally in the effects that gossypol produces on biological and lipid membranes; (b) the effects of gossypol on membranes that are the result of changes in polar/apolar interfacial properties will be mediated primarily by the binding and action of the enolate moieties of gossypol; and (c) it is the keto-enol tautomerism together with the partition and permeabilities of the uncharged and charged gossypol species which enable the gossypol molecule to transfer protons across the lipid domain of biological membranes.

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