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The effect of gossypol on fast axonal transport and microtubule assembly

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Gossypol at micromolar concentrations (2 μM) was found to inhibit axonal transport and a microsomal ATPase activity in the frog sciatic nerve, although axonal microtubules and the neuronal content of AMP, ADP and ATP were not affected. At slightly higher concentrations (30–40 μM), gossypol also inhibited microtubule assembly and neuronal energy metabolism. Gossypol accumulated in the nerve and the results indicate that gossypol may act as a potent neurotoxin.

Introduction

Gossypol, a polycyclic compound isolated from cotton seed oil, is currently used as a contraceptive for males [1]. The drug abolishes the motility of mammalian spermatozoa and affects spermatogenesis [2–6]. The inhibition of spermatogenesis is reversible [4] and the side-effects are reported to be infrequent. However, gossypol is not devoid of chronic toxic side-effects when taken in high doses [7]. Recently, gossypol has been shown to inhibit several nucleotide-metabolizing enzymes, e.g., dynein-ATPase [8], lactate dehydrogenase [9] and adenylate cyclase [10], although myosin ATPase, actomyosin ATPase and mitochondrial ATPase were unaffected [11]. It could thus be expected that gossypol also interferes with motility processes dependent on dynein or dynein-like ATPases other than sperm motility. Axonal transport, the translocation of substances along microtubules in neurites, may be such a process. Axonal transport requires ATP and is sensitive to at least two inhibitors of dynein ATPase: vanadate [12] and EHNA [13]. However, experimental data on particle movement in extruded axoplasm are not entirely consistent with a role for a dynein-like ATPase in axonal transport [14,15]. It was therefore of interest to investigate the effect of gossypol on axonal transport.

Material and Methods

Chemicals. Gossypol, 1,1′,6,6′,7,7′-hexahydroxy-5,5′-diisopropyl-3,3′-dimethyl-(2,2′-binaphthalene)-8,8′-dicarboxyaldehyde, was purchased from Sigma Chemical Company. The concentration of gossypol in water or methanol was estimated spectrophotometrically using $\varepsilon_{372} = 14.3\ mM^{-1} \cdot cm^{-1}$ [16]. L-[4,5-^{3}H]Leucine (130 Ci/mmol, 1 mCi/ml) was obtained from Amersham International, Amersham, U.K. All other chemicals were of reagent grade.

Protein concentration. Microtubule protein concentration was determined by Bio-Rad protein assay using Coomassie brilliant blue based on the method of Bradford [17] with tubulin as a standard. The concentration of the standard was determined from $\varepsilon_{278} = 1.2\ mg^{-1} \cdot cm^2$ and a molecular weight of 110 000 [18].

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Abbreviations: Pipes, 1,4-piperazinediethanesulfonic acid; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; EHNA, erythro-9(3-(2-hydroxynonyl))adenine.

Microtubule proteins. Microtubule proteins were prepared from bovine brain in the absence of glycerol by two or three cycles of assembly-disassembly in the presence of 0.5 mM $MgSO_4$ as previously described [19,20].

Assembly of microtubule proteins in 100 mM Pipes/0.5 mM $MgSO_4$/1 mM GTP was initiated by raising the temperature from 10 to 37°C. The increase in turbidity was monitored continuously by the change in absorbance at 475 nm, since gossypol has a strong optical absorption at 350 nm, where the increase in turbidity is usually measured. Gossypol, dissolved in dimethyl sulphoxide, or an equivalent amount of dimethylsulphoxide was added from a stock solution (2 mg/ml) to the proteins either at the start of assembly or at preincubation to the proteins at 10°C.

Electron spin resonance. In some experiments with the microtubule proteins the samples with gossypol were tested for the presence of free radicals. 1% dimethyl sulphoxide was present both in the reference sample and in the sample with the microtubule proteins. The electron spin resonance spectra were recorded with a Varian E101 at room temperature, with a microwave power of 1 mW and a modulation amplitude of 0.5 G as earlier described [21].

Axonal transport. The sciatic nerve together with the eight and ninth dorsal ganglia were dissected from frogs (*Rana temporaria*). A ligature was placed on the nerve 30 mm from the ganglia. Treated and control nerve preparations were from the same frog. The nerves were incubated in an apparatus where the ganglia could be separated from the nerve by silicon grease barriers [22]. Gossypol dissolved in dimethyl sulphoxide was added to the nerve, the ganglionic or to both compartments. The same amount of dimethyl sulphoxide was added to the control nerves. The ganglia were exposed to [^{3}H]leucine containing standard frog Ringer solution. After incubation for 17 h at 18°C, the ganglia and a 4 mm segment, cut in front of the ligature, were analysed for protein-incorporated radioactivity as previously described. The effect of gossypol on axonal transport was expressed as the ratio between the radioactivity in front of the ligature in the experimental and the control nerve.

Adenosine nucleotide determination. Nerves were incubated for 17 h at 18°C, blotted on filter paper, weighed, dropped into liquid nitrogen and crushed. Nucleotides were extracted with 10% trichloroacetic acid. The sample was neutralised by extracting the trichloroacetic acid by trioctylamine in 1,1,2-trichlorotrifluoroethane [23] prior to injection into a Polyanion SI column (Pharmacia) attached to a Varian 5000 HPLC apparatus. Nucleotides were eluted by a linear phosphate gradient, 10–1000 mM (pH 7.0) at a flowrate of 1 ml/min for 15 min. ATP, ADP and AMP were detected by their absorbance at 254 nm and quantified using a nucleotide standard.

Electron microscopy. Negatively stained microtubule specimens for electron microscopy were prepared from 5 μl of microtubule protein samples. Fixation was performed with one drop of Karnovsky solution [24] after which the specimen was washed with distilled water and stained with 1% uranyl acetate.

Embedded specimens were prepared from the microtubule pellets after centrifugation at 35000 $\times g$ for 30 min at 35°C. The pellets were fixed with Karnovsky solution followed by 1% osmium tetroxide in 0.1 M cacodylate buffer [24]. The pellets were dehydrated in a graded series of acetone/water and embedded in Epon. Thin sections were made using an LKB Ultramicrotome and were double-stained with uranyl acetate and lead citrate. The specimens were viewed in a Zeiss 109 electron microscope.

Nerves exposed to various concentrations of gossypol were fixed in 2.5% glutaraldehyde [25] followed by 1% osmium tetroxide. Subsequently the nerves were dehydrated in a graded series of ethanol/water, blockstained in 1% phosphotungstic acid and 0.5% uranyl acetate and embedded in Epon-Araldite.

ATPase activity. The sciatic nerve was homogenized in 0.1 M KCl in 20 mM Hepes buffer at pH 7.4 and centrifuged at 15000 $\times g$ to remove mitochondria. The ATPase activity in the supernatant, which is primarily microsomal, was assayed as described by Edström et al. [26]. The final concentrations of both ATP and Mg(II) were 1.5 mM.

Sodium dodecyl sulphate gel electrophoresis. A solution (300 μl) of microtubules, assembled un-

der different conditions, was centrifuged through 5 ml 40% sucrose in assembly buffer at 37°C. The pellet of these microtubules obtained after centrifugation for 1 h at 200 000 × *g* was dissolved in the sample buffer and sodium dodecyl sulphate polyacrylamide gel electrophoresis was performed according to the method of O'Farell [27] on a linear (5–12%) gradient. Gels were stained with 0.25% Coomassie brilliant blue in methanol/acetic acid/water (5 : 1 : 5, v/v), and destained in 7% acetic acid and 5% methanol.

Results and Discussion

The male contraceptive, gossypol, was found to inhibit axonal transport markedly, measured as the accumulation of ^{3}H-labelled proteins in front of a ligature (Table I). When gossypol was present in the nerve compartment, axonal transport decreased from 52% at 2 μM to 13% at 30 μM. However, when the drug was present only in the ganglionic compartment, the inhibition was less pronounced, and at 30 μM axonal transport was still 70% of control. This is in contrast to several other axonal transport-inhibitory drugs, such as colchicine, which have better access to the cells in the ganglionic compartment due to the less developed epi- and perineurium. Gossypol is lipid-soluble [28] and its action may be related to its accumulation in myelin, which is far more abundant in the nerve compartment than in the ganglionic. To test whether gossypol accumulated in the myelin-rich nerve we incubated frog nerves in Ringer solution containing 30 μM gossypol. Following a 17 h incubation the concentration of gossypol in the nerve was at least 100-times higher than in the surrounding Ringer solution, as determined by the absorbance of the drug at 372 nm after methanol extraction.

Axonal transport is a complex process and depends on a variety of cellular activities, including energy metabolism. Since gossypol blocks the energy metabolism in spermatozoa [3,4,11], a decrease in the level of ATP could be responsible for the inhibition of axonal transport as well. Nerves incubated for 17 h with 2 μM gossypol did not show any significant changes in their content of ATP, ADP or AMP (see Table II). At higher concentrations, 30 μM, the ADP and ATP content were reduced to 25% and 60%, respectively. Protein synthesis was not affected, since gossypol at 30 μM did not block the ATP-requiring ganglionic incorporation of [^{3}H]leucine into trichloroacetic acid-insoluble material. After 17 h the ratio of [^{3}H]leucine incorporated into proteins in the control nerve and the nerve of which the ganglia was treated with 30 μM gossypol was 1.02 ± 0.57 (mean value ± S.D., $n = 8$). Thus, the inhibition of axonal transport by low concentrations of gossypol can probably not be ascribed to interference with the energy metabolism in the nerve.

Axonal transport also depends on an intact microtubular system. If the main part of the microtubules are destroyed by a cold-block or by colchicine treatment, axonal transport is inhibited, [29]. However, the axonal morphology and the density of the microtubules appeared to be normal

TABLE I

EFFECTS OF GOSSYPOL ON RAPID AXONAL TRANSPORT

[Gossypol] (μM)	Exposed area	Axonal transport ratio[a] (exp/control (%))	*P*	*n*
2	ganglia + nerve	52 ± 17	0.05	8
10	nerve	24 ± 8	0.001	6
30	nerve	13 ± 4	0.001	8
30	ganglia	70 ± 9	0.05	8
100	nerve	5 ± 3	0.001	6

[a] Mean value ± S.E. Student's *t*-test was used for statistical evaluation. The ratio expresses the accumulation of radioactivity in a 4 mm segment proximal of the ligature in the gossypol-exposed nerves to that of the accumulation in the control nerve. The incorporation of [^{3}H]leucine in the ganglia was unaffected.

TABLE II

EFFECTS OF GOSSYPOL ON ADENINE NUCLEOTIDE LEVELS IN FROG SCIATIC NERVE

Values, mean ± S.D. ($n = 4$) are the ratio of the concentrations in the exposed vs. control nerve. * $P < 0.05$.

[Gossypol] (μM)	AMP	ADP	ATP
2	0.98 ± 0.17	1.01 ± 0.06	1.09 ± 0.17
20	1.05 ± 0.21	0.97 ± 0.07	1.03 ± 0.18
30	1.57 ± 0.29 *	0.60 ± 0.04 *	0.26 ± 0.08 *

after treatment of the nerve with gossypol for 17 h at concentrations at or below 10 μM (not shown). Therefore we examined the effect of gossypol on isolated brain microtubule proteins. We found that gossypol had no effect at concentrations below 10 μM on microtubule assembly in vitro, see Fig. 1. This is consistent with the finding that cultured cells exposed to low concentrations of gossypol (10 μM) were blocked in the S-phase [30,31] rather than in mitosis as found with potent microtubule inhibitors like colchicine. Microtubules assembled in the presence of 10 μM gossypol had normal morphology and no difference in protein composition could be detected, as determined by SDS-polyacrylamide gel electrophoresis (not shown). However, 17% inhibition of microtubule assembly did occur at higher gossypol concentrations (40 μM) (see Fig. 1) (similar inhibition has been observed by Medrano, F.J. and Andreu, J.M., personal communication). We found that preincubation of the microtubule proteins with the drug for either 1 min or 1 h, enhanced the inhibitory effect of 40 μM gossypol to 33%. If 40 μM gossypol was added to preformed microtubules at 37°C, the decrease in assembly level was also 33%.

Attempts to isolate the complex between gossypol and the microtubule proteins by gel filtration were not successful because of the very strong interaction of gossypol with the gel-filtration media used, Sephadex G-25 Medium or Bio-Gel®P-6DG. We also tried to use this strong interaction of gossypol for affinity chromatography. However, microtubule proteins were not retarded on a Sephadex G-25 column preloaded with gossypol.

It has been proposed that gossypol may exert its effect via the production of free radicals [32]. This was tested with electron spin resonance measurements. We could not detect any free radical formation from gossypol in the presence of the microtubule proteins.

The lack of effects of gossypol on energy metabolism and microtubule assembly at concentrations which inhibited axonal transport suggested that gossypol had another site of action. Drugs which block dynein-ATPase, including vanadate and the nucleotide derivative EHNA are effective inhibitors of axonal transport [13,33,34]. Furthermore, high concentrations of gossypol have been reported to affect dynein-ATPase activity,

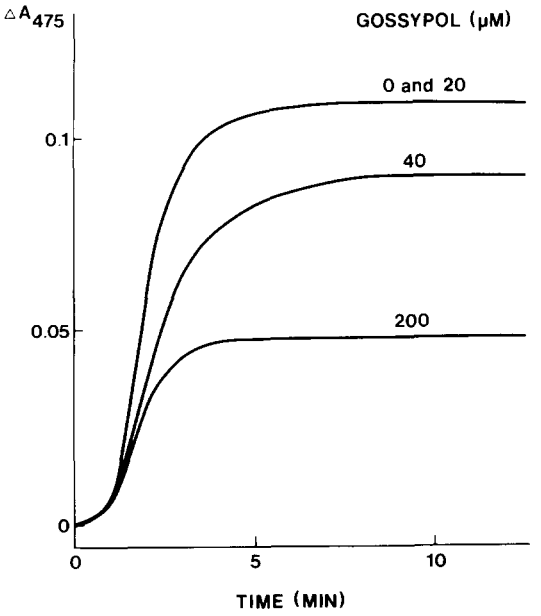

Fig. 1. Effect of gossypol on microtubule assembly. Microtubule proteins were assembled in 0.1 M Pipes/1 mM GTP/0.5 mM $MgSO_4$ (pH 6.8) by raising the temperature from 10 to 37°C and monitored by the absorbance at 475 nm (A_{475}), versus time. The reference cell contained the same additions as the experimental cell but was kept at 10°C. Gossypol was added to the samples at initiation of assembly at 37°C at the concentrations indicated on the curves.

although no effect was found on mitochondrial ATPase [11]. We therefore examined the effect of gossypol on two ATPase activities in a homogenate of the frog sciatic nerve. The ATPase recovery in the microsomal fraction, which is not a dynein-ATPase [26], was effectively inhibited by gossypol at the same concentrations that blocked axonal transport (Fig. 2). At 2 μM gossypol the ATPase activity decreased to 68% of the control, and at 10 μM to 32%. In contrast, the soluble ATPase activity in a 100000 $\times g$ supernatant of the nerve was not blocked by 10 μM gossypol. The finding that the ATP levels decreased at gossypol concentrations which also suppressed the microsomal ATPase activity may seem contradictory. However, it shows that the two separate events, production of ATP and the consumption of ATP, are both inhibited by gossypol, as also has been found in spermatozoa [3].

It is tempting to relate the effect of gossypol on axonal transport to the effect on the microsomal

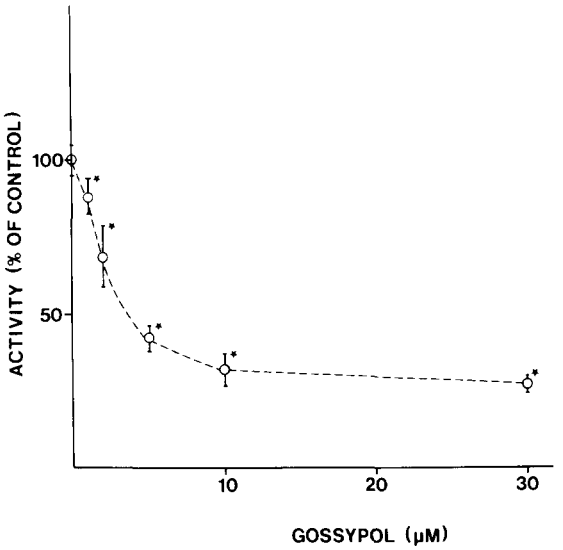

Fig. 2. Effect of gossypol on frog nerve ATPase. The ATPase activity in a homogenate of the frog sciatic nerve was measured in the presence of 1.5 mM ATP and 1.5 mM $MgSO_4$. The samples were incubated at 25°C for 20 min. Released phosphate was estimate spectrophotometrically as the blue ammonium molybdate complex. ATPase activity was expressed in percent of released phosphate in the absence of gossypol. Each point represents the mean ± S.D. of four experiments. * $P < 0.05$.

ATPase activity. There is some evidence that this specific ATPase is of importance for axonal transport. The enzyme is recovered in the microsomal fraction together with the rapidly transported material. Furthermore, the enzyme is stimulated by the same low concentrations of Zn^{2+} which are also known to stimulate rapid axonal transport [35]. However, the nucleotide specificity of this ATPase is distinct from that of axonal transport. Translocation of microscopic particles in permeabilized axons requires ATP, but other nucleotides are poor substrates, whereas the microsomal ATPase degrades other nucleotides as effectively as ATP [26,36].

The present study showed that gossypol inhibits rapid axonal transport at micromolar concentrations. Only batrachotoxin [37] or ionophores [38] inhibit at comparable concentrations. The finding that gossypol accumulates in the nerve therefore merits attention to the possible neurotoxicity of the drug. Inhibition of axonal transport could not be linked directly to an inhibition of a dynein-ATPase by gossypol. Gossypol was less specific than anticipated, since it was found to block a microsomal non-dynein ATPase at concentrations which inhibited axonal transport (Table II).

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