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Gossypol induces chloride secretion in rat proximal colon

Gisela Kuhn[a,*], Rainer Cermak[b], Kathrin Minck[a], Zoran Vujicic[a], Erwin Scharrer[a]

[a] Institute of Veterinary Physiology, University of Zurich, Winterthurerstrasse 260, CH-8057 Zurich, Switzerland
[b] Institute of Animal Nutrition, Physiology and Metabolism, Christian-Albrechts-University of Kiel, Olshausenstrasse 40, D-24118 Kiel, Germany

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Abstract

The effect of gossypol on electrolyte transport was investigated in rat colon mounted in Ussing chambers. The addition of gossypol to the mucosal or serosal side led to an increase in mucus secretion, which we did not quantify. Mucosally or serosally added gossypol also induced a rise in short circuit current (I_{sc}) and tissue conductance (G_t). Part of the mucosally added gossypol seemed to be bound to the mucus because the effects on I_{sc} and G_t were smaller when gossypol was added to the mucosal side. Serosally added gossypol had an effect on I_{sc} at a concentration of 10 μmol l^{-1}. Mucus secretion was reduced in low Ca^{2+} buffer. The increase in I_{sc} was diminished by blockers of Cl^- channels, K^+ channels, of the Na^+/K^+ ATPase and of the $Na^+/K^+/2\ Cl^-$ cotransporter. Measurements of unidirectional ion fluxes showed that gossypol added to the mucosal side had no effect on net Na^+ transport, but increased Cl^- secretion. The effect of mucosally added gossypol was significantly reduced by the use of low Cl^- buffers and abolished when the buffer was additionally depleted of HCO_3^-. Calmodulin antagonists inhibited the effect on secretion. These findings indicate that gossypol induces chloride secretion via a calmodulin-dependent mechanism. High concentrations of gossypol induced a strong increase in G_t that could be blocked by W7, a blocker of calmodulin-dependent myosin light chain kinase. This indicates that the rise in G_t is not due to an unspecific toxic effect, but instead, to specific opening of tight junctions.

Keywords: Gossypol; Colon; Cl^- secretion; Mucus; Tight junction

1. Introduction

Gossypol (2,2′ -bi-(8-formyl-1,6,7-trihydroxy-5-isopropyl-3-methylnaphthalene)) is a polyphenolic pigment produced by cotton plants (*Gossypium* sp.) to protect the plant from insect damage. It limits the use of cottonseed as cheap protein-rich animal food because it has a variety of toxic effects especially on non-ruminants. In humans, it could be used as a male antifertility agent because it reversibly damages elements of the seminiferous epithelium without having an effect on hormone production. However, this use is also limited by toxic side effects (Waites et al., 1998). Recently, gossypol was also found to be active against many cancer cell lines in vitro and in vivo. It was postulated in this context that gossypol may be the lead compound for a new class of antineoplastic drugs (Qiu et al., 2002).

The toxic effects of gossypol vary from sudden death, heart failure, cardiomyopathia, dyspnoea, oedema in lungs and many other tissues, hepatic necrosis, weakness, inappetence and diarrhoea (East et al., 1994; Haschek et al., 1989; Holmberg et al., 1988; Rogers et al., 1975; Waites et al., 1998).

Diarrhoea can be caused by an increase in chloride secretion that is mediated by the following mechanism: Chloride enters the cell through the basolaterally located $Na^+/K^+/2\ Cl^-$ cotransporter (Dharmsathaphorn et al., 1985). The driving force of this action is the sodium gradient over the membrane, which is produced by Na^+/K^+ ATPase (Heintze et al., 1983). K^+, which enters the cell via the pump and the cotransporter, leaves the cell through basolateral K^+ channels (Mandel et al., 1986). This builds up a negative membrane potential (Siemer and Gögelein, 1992; Ecke et al., 1996a,b). Cl^- leaves the cell driven by the negative membrane potential through apical Cl^- channels (Halm and Frizzell, 1991), namely cystic fibrosis transmembrane conductance regulator (CFTR) (Greger, 2000). This process can be enhanced by three different intracellular

* Corresponding author. Tel.: +41-1-6358811; fax: +41-1-6358932.
E-mail address: gkuhn@vetphys.unizh.ch (G. Kuhn).

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messengers, among them is Ca^{2+} (Böhme et al., 1991). An increase in intracellular Ca^{2+} activates basolateral K^+ channels and causes a hyperpolarisation of the cell (Strabel and Diener, 1995; Greger, 2000). Thus, the driving force for the Cl^- exit is increased.

Cermak et al. (2001) showed in Ussing chamber studies that gossypol increases the short circuit current (I_{sc}) as well as tissue conductance (G_t) in rat colon. An increase in I_{sc} in Ussing chambers can be caused by an increase in chloride secretion. Interestingly, Jan et al. (2000) showed that gossypol increases the intracellular Ca^{2+} concentration by releasing Ca^{2+} from internal stores followed by Ca^{2+} influx from the extracellular space in different cell types, among them are epithelial cells (Madin Darby canine kidney cells, T24 bladder carcinoma cells and human neutrophils). These observations led us to the hypothesis that gossypol could induce chloride secretion by an increase in intracellular Ca^{2+}.

The aim of this study was to investigate whether the increase in I_{sc} induced by gossypol in rat colon is indeed caused by activation of chloride secretion. Furthermore, we wanted to look at whether Ca^{2+} is involved in this effect and how gossypol influences the tissue conductance. The experiments were performed with proximal colon because this part of the intestine showed the largest reaction in preliminary experiments.

2. Materials and methods

2.1. Tissue preparation

Male Wistar rats (RCC, Füllinsdorf, Switzerland), weighing 190–220 g, were used for the experiments. The animals were kept and treated according to institutional guidelines approved by the Kantonales Veterinäramt Zürich and had free access to water and a standard diet (Eberle Nafag, Gossau, Switzerland) until approximately 30 min before the experiment. They were stunned by a blow on the head and killed by exsanguination. The proximal colon was taken out immediately and the serosa and muscularis were stripped away by hand.

2.2. Determination of electrophysiological parameters

Sheets of tissue were mounted in a modified Ussing chamber bathed with 4 ml of a buffer solution on both sides. The exposed surface of the tissue was 1 cm^2. The epithelium was continuously short circuited by an automatic voltage clamp device (Aachen Microclamp, AC Copy Datentechnik, Aachen, Germany) with correction for solution resistance. The short circuit current (I_{sc}) was continuously reported. Every 5, a current pulse of $I_{sc} \pm 100$ μA was applied for 1 s. From the resulting change in the transepithelial potential, the tissue conductance (G_t) was calculated according to Ohm's law ($G_t = I/U$). The values of I_{sc} and G_t were printed out every minute as mean of this minute.

The equilibration time before the addition of drugs was at least 50 min. Colon sheets with a basal G_t higher than 30 mS cm^{-2} were excluded from the experiments. When the effects of blockers were tested, control experiments were performed with tissue of the same animal. The baseline of the electrical parameters was determined as the mean over a 5-min period immediately before administration of a drug. The maximal change in I_{sc} induced by a drug was expressed as the difference from baseline (ΔI_{sc}). Changes in G_t were calculated as difference (ΔG_t) between basal G_t (mean over 5 min) and G_t 30 min (mean of min 30–35) after drug application.

2.3. Measurement of unidirectional ion fluxes

Ten to fifteen minutes after the tissue was mounted in the chambers, $^{22}Na^+$(59 kBq) and $^{36}Cl^-$ (29 kBq) were added to one side of the epithelium (labelled side). After an additional 60 min to allow isotope fluxes to reach a steady state and I_{sc} to stabilize, unidirectional ion fluxes were determined over seven sequential 10-min periods. After the first three periods, gossypol was added. The mean of these three periods was taken as basal value without the drug. It was compared with the mean of period five to seven (10–40 min after gossypol administration). The net ion flux (J_{net}) was calculated as the difference between the mucosal to serosal flux (J_{ms}) and the serosal to mucosal flux (J_{sm}). Control experiments were conducted with the same time schedule with application of the vehicle dimethyl sulfoxide (DMSO) only.

2.4. Solutions

The standard buffer contained (in mmol l^{-1}) 107 NaCl, 4.5 KCl, 25 $NaHCO_3$, 1.8 Na_2HPO_4, 0.2 NaH_2PO_4, 1 $MgCl_2$ and 12 glucose. $CaCl_2$ was either 0.25 (low Ca^{2+} buffer) or 1.25 mmol l^{-1}. The solution was gassed with 5% CO_2 in 95% O_2 and kept at 37 °C; pH was adjusted to 7.4. In the low Cl^- solution, NaCl was replaced by sodium gluconate and the Ca^{2+} concentration was 1.25 mmol l^{-1}. In the low Cl^-/HCO_3^-, free solution HCO_3^- was replaced by 10 mmol l^{-1} HEPES and the sodium gluconate increased to 132 mmol l^{-1}. This solution was gassed with O_2. Experiments with barium were performed in a HCO_3^--free solution containing 132 mmol l^{-1} NaCl and 10 mmol l^{-1} HEPES, gassed with O_2.

2.5. Chemicals

Barium chloride, diphenylamin-2-carboxylate (DPC), gossypol, ophiobolin A, quinine, and W7 (*N*-(6-aminohexyl)-5-chloro-1-naphthalenesulfonamide hydrochloride) were obtained from Sigma, Buchs, Switzerland. Bumetanide and ouabain were obtained from Fluka, Buchs, Switzerland. Quinine was dissolved in ethanol. All other drugs were dissolved in dimethyl sulfoxide (DMSO).

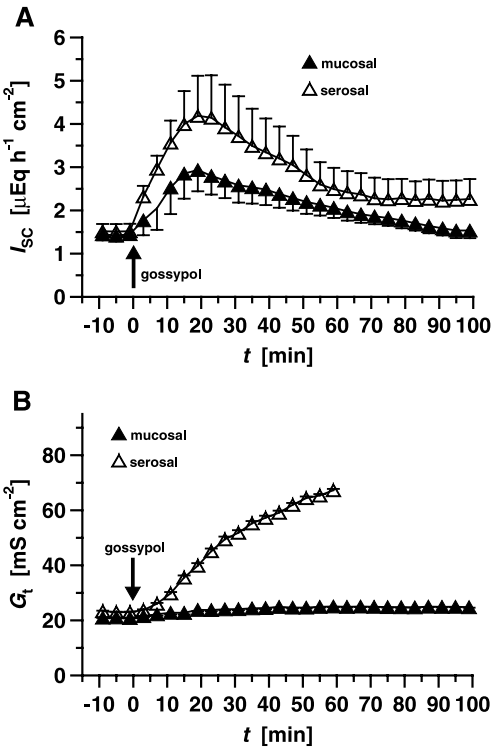

Fig. 1. Effect of gossypol on basal I_{sc} (A) and G_t (B) after mucosal and serosal application in rat proximal colon. Gossypol (200 µmol l^{-1}) was added at time 0 to the mucosal ($n=20$) or serosal ($n=12$) compartment of the Ussing chamber. Since G_t left the measurable range when gossypol was added serosally, data beyond 60 min is not indicated after serosal application in B.

2.6. Statistics

Data are presented as mean ± S.E.M. Statistical significance of the effects was determined with Student's *t*-test pairing the drug effect against baseline. Comparisons between two experimental groups under various conditions were made with the unpaired *t*-test. Comparisons between more than two experimental groups were carried out using ANOVA with subsequent pair comparison by the method of Student, Newman and Keuls.

3. Results

3.1. Effects of gossypol on short circuit current and tissue conductance

The first experiments were performed to test the effect of gossypol in proximal colon of rats in Ussing chambers. For these experiments, a standard buffer containing 1.25 mmol l^{-1} Ca^{2+} was used. Gossypol was added at a concentration of 200 µmol l^{-1} on respectively mucosal or serosal side of the tissue.

The first effect to be seen after mucosal application of gossypol was an increased secretion of mucus, which induced foaming of the mucosal buffer solution. Gossypol seemed to be bound to the mucus because the mucus became yellow during the experiment. Additionally, gossypol increased the basal I_{sc} from 1.7 ± 0.1 to 3.7 ± 0.3 µEq h^{-1} cm^{-2} ($P<0.001$, $n=20$). The maximum of I_{sc} was reached after 22.8 ± 1.8 min. Thereafter, the I_{sc} decreased again and after 100 min, it reached basal values (1.8 ± 0.1 µEq h^{-1} cm^{-2}) (Fig. 1A).

Gossypol also induced an increase in G_t. Thirty minutes after gossypol administration to the mucosal medium G_t changed from 19.5 ± 1.6 to 22.9 ± 1.2 mS cm^{-2} ($P<0.001$, $n=20$) (Fig. 1B).

When gossypol (200 µmol l^{-1}) was added to the serosal side, there was also an increase in mucus secretion visible. However, gossypol was not bound to the mucus as it was added to the other side of the tissue. The increase in I_{sc} was stronger compared to mucosal addition, since it increased from 1.5 ± 0.5 to 5.2 ± 0.6 µEq h^{-1} cm^{-2} ($P<0.001$, $n=12$), with the maximum having been reached 17.4 ± 3.6 min after addition of gossypol (Fig. 1A). G_t started to rise constantly when gossypol was added. Thirty minutes after addition of gossypol, it reached values of 31.4 ± 5.1 mS cm^{-2} (Fig. 1B). The increase in G_t further continued until it exceeded the voltage clamp's maximum range of 99.9 mS cm^{-2}.

3.2. Concentration-response curve

Because undefined amounts of mucosally added gossypol appeared to be bound to the mucus, the concentration–response curve was determined solely following serosal application of gossypol.

Several concentrations of gossypol between 5 and 200 µmol l^{-1} were added on the serosal side of the tissue. Gossypol had a significant effect on the I_{sc} at a concentration of 10 µmol l^{-1}. The maximum response was reached at about 150 µmol l^{-1} (Fig. 2). The G_t started to increase at a

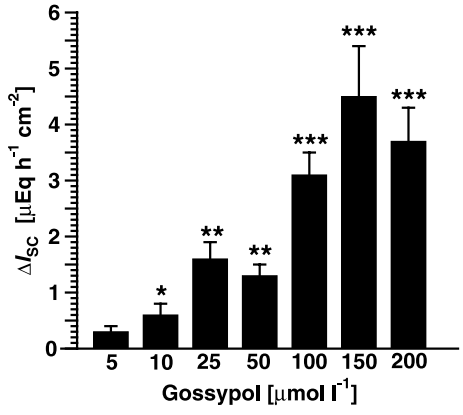

Fig. 2. Concentration–response effects of serosally applied gossypol on I_{sc} across rat proximal colon. Gossypol was added at concentrations between 5 and 200 µmol l^{-1} to the serosal side of the tissue ($n=6$ for gossypol concentrations of 5–100 µmol l^{-1}, $n=5$ for 150 µmol l^{-1} and $n=12$ for 200 µmol l^{-1}). *$P<0.05$, **$P<0.01$, ***$P<0.001$ vs. basal values.

concentration of 100 μmol l^{-1}. It was not possible to estimate a maximal effect for this parameter because G_t values at the highest gossypol concentrations used were beyond the measurable range of the voltage clamp device.

3.3. Involvement of Ca^{2+}

In the following experiments, we investigated the involvement of Ca^{2+} in gossypol reaction. Ca^{2+} was reduced in the buffer from 1.25 to 0.25 mmol l^{-1}. We avoided a complete depletion of Ca^{2+} from the buffer because this causes an increase in G_t due to an increase of tight junction permeability (Cermak et al., 2000). Gossypol was added to the mucosal or serosal side at a concentration of 200 μmol l^{-1}. The mucus secretion was less striking than in standard buffer. With mucosal gossypol application, the increase in I_{sc} was 3.4 ± 0.6 μEq h^{-1} cm^{-2} (n = 8), which was significantly (P < 0.05) higher than in standard buffer (ΔI_{sc} = 1.4 ± 0.4 μEq h^{-1} cm^{-2}, n = 7). When gossypol was added serosally, I_{sc} increased by 4.2 ± 0.6 μEq h^{-1} cm^{-2} (n = 7), which was not different from the control group (ΔI_{sc} = 3.3 ± 0.8 μEq h^{-1} cm^{-2}, n = 6). The course of G_t was not influenced by the Ca^{2+} content of the buffer.

All following experiments with mucosally added gossypol were performed in low Ca^{2+} buffer in an effort to reduce the mucus secretion and binding of gossypol to mucus.

One possibility to inhibit signal transduction via Ca^{2+} is to block the calcium binding protein calmodulin, which mediates the further signal transduction. To do this, we used the calmodulin antagonists ophiobolin A (Leung et al., 1984) and W7 (Kanamori et al., 1981). Ophiobolin A (100 μmol l^{-1} serosal) increased the basal I_{sc} temporarily by 2.3 ± 0.2 μEq h^{-1} cm^{-2}, n = 9. Forty-five minutes after ophiobolin A application, the I_{sc} stabilised 1.0 ± 0.4 μEq h^{-1} cm^{-2} above basal values. At the same time, ophiobolin A increased the basal G_t by 18.0 ± 2.8 mS cm^{-2}. Gossypol, added 45 min after ophiobolin A, had no significant effect on the I_{sc} (ΔI_{sc} = − 0.1 ± 0.1 μEq h^{-1} cm^{-2}, n = 9, control: ΔI_{sc} = 3.4 ± 0.6 μEq h^{-1} cm^{-2}, n = 7) (Fig. 3). G_t increased further beyond measurable values.

W7 (100 μmol l^{-1} mucosal and serosal) increased basal I_{sc} by 0.9 ± 0.1 μEq h^{-1} cm^{-2} and blocked the gossypol-induced rise in I_{sc} completely (ΔI_{sc} = 0.2 ± 0.1 μEq h^{-1} cm^{-2}, n = 6, control: ΔI_{sc} = 3.0 ± 0.5 μEq h^{-1} cm^{-2}, n = 6, P < 0.001). G_t started to rise slowly when W7 was added. After 60 min, it was increased by 3.7 ± 0.8 mS cm^{-2} (control: ΔG_t = − 1.2 ± 0.2 mS cm^{-2}, P < 0.001). Thirty minutes after gossypol was added, it rose further by 4.4 ± 1.2 mS cm^{-2}. This increase was not significantly different from the gossypol-induced increase in control group (ΔG_t = 6.0 ± 1.3 mS cm^{-2}).

3.4. Blockers of chloride secretion

To investigate whether the increase of I_{sc} induced by gossypol was due to an enhanced secretion of chloride, we used specific blockers of the transporters involved in Cl^- secretion.

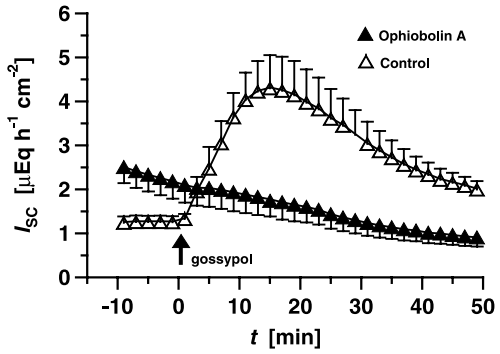

Fig. 3. Effect of ophiobolin A (100 μmol l^{-1} serosal) on gossypol-induced increase of I_{sc} across rat proximal colon. Gossypol (200 μmol l^{-1} mucosal) was added at time 0 either without any pre-treatment (n = 7) or in the presence of ophiobolin A (n = 9).

Ouabain (1 mmol l^{-1} serosal), a blocker of the Na^+/K^+ ATPase (Heintze et al., 1983), caused a transient peak in I_{sc} after addition (ΔI_{sc} = 1.1 ± 0.2 μEq h^{-1} cm^{-2}, n = 5). After 30 min, the current reached values not significantly different from control values (Fig. 4A). DPC (1 mmol l^{-1} mucosal), a blocker of CFTR chloride channels (Di Stefano et al., 1985; Schultz et al., 1999), bumetanide (100 μmol l^{-1} serosal), a blocker of the $Na^+/K^+/2$ Cl^- cotransporter (Dharmsathaphorn et al., 1985) and quinine (1 mmol l^{-1} serosal), a blocker of basolateral K^+ channels (Heinke et al., 1998) decreased basal I_{sc} significantly (Fig. 4A). Barium (10 mmol l^{-1} serosal), another blocker of K^+ channels (Böhme et al., 1991), led to a significant transient increase in basal I_{sc} by 0.7 ± 0.2 μEq h^{-1} cm^{-2}, n = 6. After 30 min, I_{sc} reached a plateau significantly higher than basal values (Fig. 4A).

The effect of gossypol was blocked completely by DPC and barium. Ouabain and quinine nearly abolished it. Bumetanide reduced the effect tendentially, but not significantly (Fig. 4B).

The fact that bumetanide could not block the gossypol reaction shows that there must still be either a possibility for chloride to enter the cell independently of $Na^+/K^+/2$ Cl^- cotransport or secretion of another anion. To exclude the first possibility, experiments in low Cl^- buffer were performed. Under these conditions, gossypol still increased basal I_{sc} by 0.7 ± 0.1 μEq h^{-1} cm^{-2} (n = 9), but this increase was significantly smaller than in control experiments (ΔI_{sc} = 2.0 ± 0.3 μEq h^{-1} cm^{-2}, n = 23, P < 0.01) (Fig. 5). It is known that the chloride channel responsible for Cl^- secretion (CFTR) also regulates the secretion of bicarbonate (Illek et al., 1998). To test whether the remaining secretion is caused by bicarbonate, we used a low chloride and bicarbonate free buffer. Here, gossypol no longer caused an increase in I_{sc} (ΔI_{sc} = 0.1 ± 0.1 μEq h^{-1} cm^{-2}, n = 8) (Fig. 5).

To determine which ions contribute to the change in I_{sc}, unidirectional fluxes of $^{22}Na^+$ and $^{36}Cl^-$ were measured. In

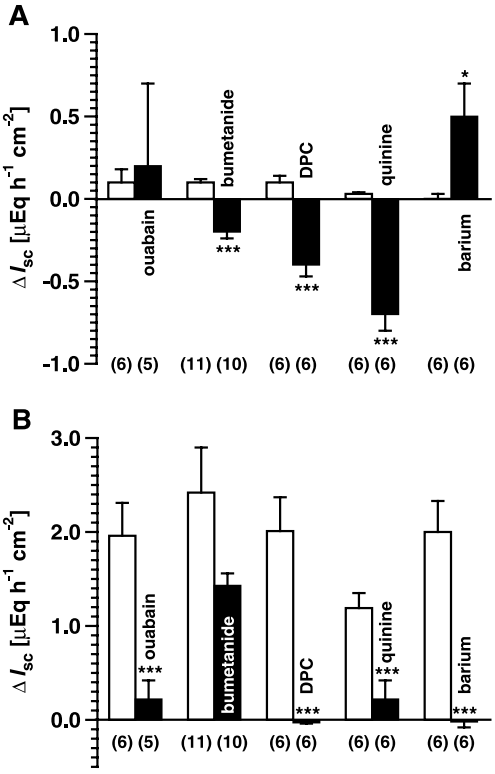

Fig. 4. Effects of various blockers on basal I_{sc} and on gossypol-induced increase of I_{sc} across rat proximal colon. Part A shows effects of the indicated blockers (black bars) or their vehicle (white bars) on basal I_{sc}. Part B shows the maximum effect of gossypol on I_{sc} in presence (black bars) or absence (white bars) of the respective blocker. Ouabain (1 mmol l^{-1} serosal), bumetanide (100 μmol l^{-1} serosal), DPC (1 mmol l^{-1} mucosal) and quinine (1 mmol l^{-1} serosal) were added 20 min, barium (10 mmol l^{-1} serosal) 30 min before gossypol. Numbers in parentheses indicate number of experiments. *$P<0.05$, ***$P<0.001$ vs. respective controls.

these experiments, gossypol was used at a concentration of 100 μmol l^{-1} mucosally to prevent large effects on tissue conductance. Gossypol increased the I_{sc} by 1.3 ± 0.2 μEq h^{-1} cm^{-2} ($P<0.001$, $n=16$) and G_t by 3.9 ± 0.5 mS cm^{-2} ($P<0.001$). J^{Na}_{ms} was not influenced by gossypol. J^{Na}_{sm} increased from 9.4 ± 1.3 to 11.3 ± 1.0 μmol h^{-1} cm^{-2} ($P<0.01$, $n=8$) but J^{Na}_{net} did not change significantly

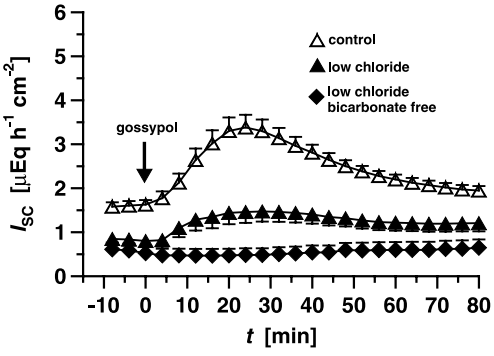

Fig. 5. Effect of gossypol (200 μmol l^{-1} mucosal) on I_{sc} across rat proximal colon in standard buffer ($n=23$), low Cl^- buffer ($n=9$) and low Cl^-/HCO_3^- free buffer ($n=8$). Gossypol was added at time 0.

($\Delta J^{Na}_{net} = -1.6 \pm 1.4$ μmol h^{-1} cm^{-2}, $P>0.05$, $n=8$). J^{Cl}_{ms} slightly decreased after gossypol administration from 14.8 ± 1.9 to 12.0 ± 0.8 μmol h^{-1} cm^{-2} ($P>0.05$, $n=8$). J^{Cl}_{sm} increased from 13.7 ± 1.1 to 15.8 ± 1.1 μmol h^{-1} cm^{-2} ($P<0.05$, $n=8$). J^{Cl}_{net} changed significantly from basal absorption (1.1 ± 1.5 μmol h^{-1} cm^{-2}) to secretion (-3.8 ± 1.4 μmol h^{-1} cm^{-2}) (Fig. 6). In control experiments performed with addition of DMSO only, the vehicle had no effect on I_{sc}, G_t or any of the fluxes (data not shown).

These data indicate that gossypol activates a net Cl^- secretion. The increase of J^{Na}_{sm} might be caused by an increase in paracellular conductance as indicated by the higher G_t.

3.5. Effect of gossypol on G_t

Serosal application of gossypol caused a strong increase in tissue conductance. This could be due to an increase in transcellular or paracellular conductance, which are regulated actively by the epithelial cells. But tissue conductance is also increased when the tissue is damaged by mechanical or cytotoxical influences. It has been shown that gossypol has cytotoxic properties (Ye et al., 1983). Therefore, we wanted to check whether the strong increase in G_t caused by gossypol is due to cytotoxicity or whether it is a specific effect on paracellular conductance. Increase of paracellular conductance can be blocked by the calmodulin antagonist W7 (Kishi et al., 1996).

When W7 (100 μmol l^{-1} mucosal and serosal) was added to our tissue preparation, basal I_{sc} increased continuously during 60 min by 1.2 ± 0.2 μEq h^{-1} cm^{-2} ($P<0.01$, $n=6$). The basal G_t was increased by 3.1 ± 0.4 mS cm^{-2} ($P<0.001$). Gossypol (200 μmol l^{-1} serosal) added 60 min after W7 increased the I_{sc} by 2.3 ± 0.3 μEq h^{-1} cm^{-2} ($n=6$), which was significantly ($P<0.05$) less than gossypol effect in the control group ($\Delta I_{sc} = 4.1 \pm 0.7$ μEq h^{-1} cm^{-2}, $n=6$). G_t increased 30 min after gossypol addition by 9.5 ± 1.2 mS cm^{-2} ($n=6$), which was signifi-

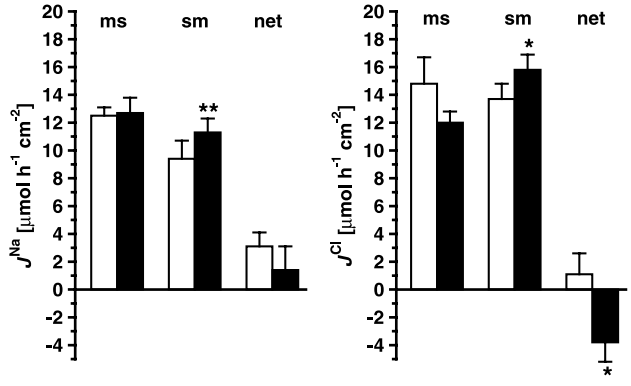

Fig. 6. Effect of gossypol on unidirectional fluxes of Na^+ (left part) and Cl^- (right part of picture) across proximal colon of the rat. White bars indicate basal fluxes, black bars indicate fluxes in presence of gossypol. $n=8$ for all experiments, *$P<0.05$, **$P<0.01$ vs. basal values.

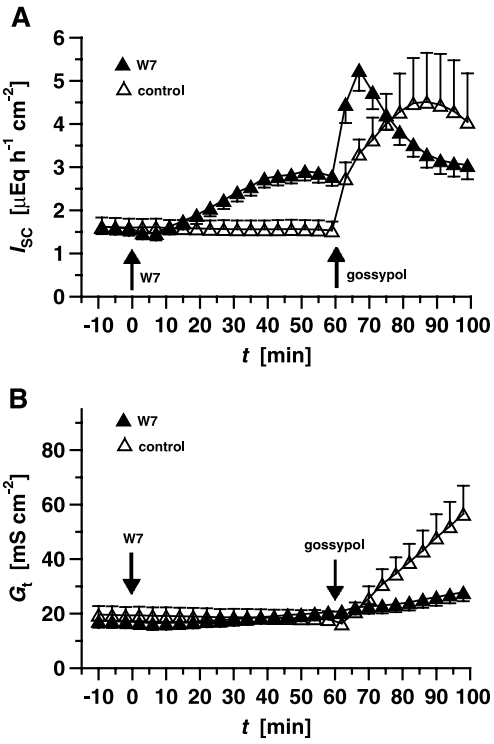

Fig. 7. Effect of W7 (100 µmol l^{-1} serosal and mucosal) on gossypol-induced increase of I_{sc} (A) and G_t (B) across rat proximal colon. W7 was added on time 0, gossypol (200 µmol l^{-1} serosal) 60 min later ($n=6$ for both groups).

cantly ($P<0.01$) less than the increase in G_t in the control group ($\Delta G_t = 31.2 \pm 6.2$ mS cm^{-2}, $n=6$) (Fig. 7). These data may lead to the assumption that gossypol increases the paracellular conductance by a specific calmodulin-dependent mechanism.

4. Discussion

The polyphenolic compound gossypol, which is produced by cotton plants, has a variety of effects on humans and animals. Cermak et al. (2000) showed in Ussing chamber studies that gossypol increases the short circuit current and the tissue conductance in the colon of rats. An increase in I_{sc} indicates increased active electrolyte transport. A rise in G_t can be caused by specific upregulation of paracellular or transcellular conductance as well as by cytotoxic damage to the tissue. In this study, the underlying mechanisms of the gossypol effects were investigated.

In our experiments, gossypol showed three effects. It increased I_{sc}, G_t and mucus secretion. Mucus secretion was not quantified in our experiments, but the effect was clearly visible. The rise in I_{sc} and G_t was stronger when gossypol was added to the serosal side. This can be explained by the binding of gossypol to the mucus when it was added mucosally, thereby reducing its effective concentration.

Mucus secretion is regulated via the second messengers cAMP as well as Ca^{2+} (Marcon et al., 1990; McCool et al., 1990; Bradbury, 2000). A reduction of the Ca^{2+} concentration of the buffer appeared to decrease gossypol-induced mucus secretion. This effect is obviously dependent on the extracellular Ca^{2+} level. Reducing the Ca^{2+} content of the buffer had no effect on G_t. ΔI_{sc} of serosally added gossypol was also not influenced, but ΔI_{sc} of mucosally added gossypol was enhanced. This can be explained by less binding of gossypol to mucus.

As gossypol reaches the colonic epithelium with the digesta at the mucosal side, we decided to perform further experiments to investigate the effect of the polyphenol, with the exception of the concentration–response curve, with mucosal application of gossypol. These experiments were performed in low Ca^{2+} buffer to reduce the binding of gossypol to the mucus. The chosen Ca^{2+} concentration of 0.25 mmol l^{-1} was obviously sufficient for the rise in I_{sc}.

A rise in I_{sc} can be caused by different effects. For gossypol, it seemed to be most likely caused by an increased Cl^- secretion that is mediated by an increase in the intracellular Ca^{2+} level. In rat colon, an increase of intracellular Ca^{2+} activates basolateral rSK4 K^+ cannels. This leads to a hyperpolarisation of the cell and an enhanced Cl^- exit on the apical side (Warth et al., 1999). These Ca^{2+} regulated K^+ channels have no binding site for calcium. Instead, the signal is transmitted via calmodulin, which forms a complex with a subunit of the channel (Fanger et al., 1999; von Hahn et al., 2001). Ophiobolin A and W7, two antagonists of calmodulin, abolished the effect of mucosally applied gossypol on I_{sc}. This demonstrates that gossypol activates a calcium-calmodulin-mediated mechanism. The effect of serosally added gossypol on I_{sc} was only partly inhibited by W7. This was maybe due to the higher efficiency of serosally added gossypol.

Experiments with blockers of chloride transport as well as measurements of unidirectional ion fluxes demonstrated that the increase in I_{sc} induced by gossypol is indeed caused by Cl^- secretion. Besides Cl^-, bicarbonate is also secreted, as was shown by ion substitution experiments. It is known that CFTR, the channel responsible for intestinal Cl^- secretion, is also permeable for bicarbonate (Illek et al., 1998).

Collectively, all these findings show that gossypol stimulates Cl^- secretion via a calmodulin-dependent mechanism. Possibly, gossypol acts through hyperpolarisation of the cell membrane produced by a calmodulin-dependent activation of basolateral K^+ channels.

When gossypol was added to the serosal side in concentrations of 100 µmol l^{-1} or higher, it caused a strong increase in G_t. In intact tissue, the paracellular transport is limited by the tight junctions. Their permeability is controlled by the cells by modulation of the contraction of the perijunctional actomyosin ring (Nusrat et al., 2000). A critical step in regulation of tight junction permeability is the phosphorylation of the myosin light chain by myosin light chain kinase. The activation of this enzyme by Ca^{2+}

calmodulin can be blocked by the calmodulin antagonist W7 (Kishi et al., 1996; Kanamori et al., 1981). W7 indeed blocked the gossypol-induced increase in G_t. This observation suggests that the increase in G_t is due to an active upregulation of paracellular transport and not caused by cytotoxicity of gossypol.

Serosally added gossypol showed a significant effect at a concentration of 10 μmol l^{-1}. Noftsger et al. (2000) showed that plasma gossypol in cows can reach levels of about 4 μg ml^{-1}, equivalent to 7.7 μmol l^{-1}, or more when feeding diets containing 20% processed cotton seed. In ruminants, the level of free gossypol and the toxic form, are reduced by bacterial processing in the rumen (Reiser and Fu, 1962). It can be assumed that higher gossypol blood levels can be reached with lower gossypol intake in non-ruminants. In the colon, gossypol is additionally present on the mucosal side. Therefore, it is presumable that gossypol in vivo reaches levels that can affect electrolyte and fluid transport in the colon.

In lower concentrations, gossypol increases chloride, bicarbonate and fluid secretion. This may increase digestibility of plant foods by bringing more liquid and buffer into the colon, thus providing a better environment for bacteria.

At higher concentrations, gossypol also increases paracellular permeability. The tight junctions are the barriers for uncontrolled liquid and salt exchange. When this barrier leaks, diarrhoea may occur. Thus, the diarrhoea observed after gossypol intoxication may result from this mechanism.

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