ELSEVIER

European Journal of Pharmacology 440 (2002) 1–6

ejp

www.elsevier.com/locate/ejphar

Inhibition of T-type Ca^{2+} currents in mouse spermatogenic cells by gossypol, an antifertility compound

Junping Bai, Yuliang Shi*

Key Laboratory of Neurobiology, Institute of Physiology, Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences, 320 Yue Yang Road, Shanghai 200031, PR China

Received 17 September 2001; received in revised form 24 January 2002; accepted 29 January 2002

Abstract

Gossypol, a male antifertility compound isolated from cotton, has been proved to inhibit capacitation and the acrosome reaction in human and mammalian sperm. Here, by using whole-cell recording, we observed the effects of gossypol on Ca^{2+} and Cl^- currents in mouse spermatogenic cells obtained by mechanical dissociation. The results showed that gossypol concentration-dependently and irreversibly inhibited T-type Ca^{2+} currents in the cells. When the concentration of gossypol was ≥ 5 μM, the currents were blocked completely. The time to current block was progressively shortened as the gossypol concentration was increased from 5 to 80 μM. Moreover, the drug increased the time constant of inactivation in a concentration-dependent manner, while it did not affect the activation of the current. The inhibitory effect on the T-type Ca^{2+} current did not correlate with signaling mediated by G proteins and tyrosine phosphorylation. No obvious effect of gossypol on Cl^- currents was observed. These data suggest that the gossypol-induced inhibition of T-type Ca^{2+} currents could be responsible for the antifertility activity of the compound, indicating a possibility to use gossypol as a local contraceptive drug.

Keywords: Gossypol; Antifertility; Spermatogenic cell, mouse; Whole-cell recording; Ca^{2+} channel, T-type; Cl^- channel

1. Introduction

Gossypol, a cotton plant-derived polyphenolic compound, has attracted wide interest due to its non-steroidal antifertility action in male. Many studies have been performed to evaluate its efficacy and safety as a male contraceptive agent and to clarify its site and mechanism of antifertility action (Chang et al., 1980; Hadley et al., 1981; Stephens et al., 1983; Baccetti et al., 1986; Randell et al., 1992).

Ca^{2+} influx plays an essential role in the initiation of the acrosome reaction, and it is required for sperm capacitation and motility (Visconti et al., 1995; Darszon et al., 1999; Gonzalez-Martinez et al., 2001). It has been shown that the acrosome reaction is inhibited by Cl^- channel blockers and in Cl^- free medium, indicating an important role of the Cl^- channel in the acrosome reaction (Meizel, 1997). Previous data demonstrated that 5–100 μM gossypol inhibited sperm motility, capacitation and the acrosome reaction (Shi and Friend, 1983). The inhibition of Ca^{2+} uptake by gossypol has been observed in human spermatozoa plasma membrane vesicles (Kanwar et al., 1989). However, the effects of gossypol on Ca^{2+} channels and Cl^- channels in sperm have not been directly demonstrated by electrophysiological techniques.

Unfortunately, due to the small size, complex geometry and highly differentiated and motile nature of sperm, it is difficult to study the ion channels in mature sperm by the patch-clamp technique directly. T-type Ca^{2+} channels are expressed during the meiotic and post-meiotic stages of spermatogenesis and are retained in mature sperm. These channels play an important role in the regulation of $[Ca^{2+}]_i$ during capacitation and the acrosome reaction (Lievano et al., 1996; Arnoult et al., 1996, 1999). The existence of voltage-gated Cl^- channels in spermatogenic cells has been reported (Espinosa et al., 1998). Thus, the spermatogenic cell, a developmental precursor of mature sperm, is often used as a model system to study the effects of agents on the channel events in mature sperm (Lievano et al., 1996; Arnoult et al., 1999; Espinosa et al., 1999).

In the present study, the effects of gossypol on the Ca^{2+} and Cl^- channels in spermatogenic cells were investigated.

* Corresponding author. Tel.: +86-21-6437-0080; fax: +86-21-6433-2445.

E-mail address: ylshi@server.shcnc.ac.cn (Y. Shi).

0014-2999/02/$ - see front matter
PII: S0014-2999(02)01315-8

The results showed that gossypol concentration-dependently inhibited Ca^{2+} channel activity but did not affect Cl^- channel activity.

2. Materials and methods

2.1. Cell preparation

Individual spermatogenic cells were obtained from the testes of Kunming mice aged more than 90 days (Shanghai Experimental Animal Center, Chinese Academy of Sciences, China) by using procedures described previously (Espinosa et al., 1999). Briefly, the testes were dissected out rapidly from mice anesthetized with diethyl ether, then decapsulated in ice-cold dissociation solution (in mM: 120.1 NaCl, 4.8 KCl, 25.2 $NaHCO_3$, 1.2 KH_2PO_4, 1.2 $MgSO_4$, 1.3 $CaCl_2$, 11 glucose, 1 glutamine, pH 7.2). After removal of the *tunica albuginea*, the seminiferous tubules were isolated with forceps and then suspended in Ca^{2+}-free and DNase (100 μg/ml)-containing dissociation solution. Under a stereoscopic microscope, cells were extruded by manual trituration with forceps and pipetted repeatedly to disperse them. The dissociated cells were collected by centrifugation at 2000 rpm for 1.5 min, and then resuspended in Ca^{2+}-free dissociation solution containing 0.5% bovine serum albumin and 100 μg/ml DNase; the suspension was stored at 4–10 °C until use. The use of experimental animals was consistent with European Community guidelines.

2.2. Electrophysiological recording and data analysis

An aliquot of the cell suspension was placed in a dish and incubated at room temperature for 15 min to allow the cells to attach to the dish bottom, which was coated with 0.1% poly-L-lysine. After being washed twice with the bath solution, cells were used for whole-cell recording. Two kinds of spermatogenic cells, pachytene spermatocytes and round spermatids, were the main cell types obtained and were routinely used for ion-channel recording (Arnoult et al., 1998; Espinosa et al., 2000). Similar results were obtained with both cells, and the data were pooled for presentation.

Ca^{2+} and Cl^- currents were recorded in the whole-cell configuration of patch-clamp recording (Hamill et al., 1981). To isolate Ca^{2+} currents, cells were bathed in a solution containing (in mM): 10 $CaCl_2$, 130 NaCl, 3 KCl, 2 $MgCl_2$, 1 $NaHCO_3$, 0.5 NaH_2PO_4, 5 HEPES, 10 glucose, pH 7.3. The pipette solution was (in mM): 100 CsCl, 10 CsF, 5 EGTA, 5 HEPES, 4 ATP–Mg, 4 phosphocreatine, pH 7.3 (Espinosa et al., 1999). When Cl^- currents were recorded, the composition of the bath solution and the pipette solution was (in mM): 130 NaCl, 3 KCl, 2 $MgCl_2$, 1 $NaHCO_3$, 0.5 NaH_2PO_4, 5 HEPES, 5 glucose, 10 $CaCl_2$, 0.16 amiloride, pH 7.3, and 110 Cs–Methanesulfonate, 10 CsF, 15 CsCl, 2 EGTA, 4 ATP–Mg, 10 phosphocreatine, 5 HEPES, pH 7.3, respectively (Espinosa et al., 1998). Recording pipettes were pulled from 1.5 mm (outer diameter) capillary tubing on a two-stage vertical puller (PP-83, Narishige, Japan). The resistance of pipettes was 5–7 MΩ when filled with pipette solution. Currents were amplified with an EPC-7 amplifier (List Medical Electronics, Germany). Evocation and recording of the whole-cell Ca^{2+} currents were controlled by pCLAMP 6.02 software (Axon Instrument, USA) running on a computer through an analog-to-digital interface (Digidata 1200, Axon Instrument). A P/4 pulse protocol was used to minimize the leakage and capacitive currents. The currents were low-pass filtered at 1 kHz (eight-pole Bessel filter), digitized every 0.1 ms and analyzed off-line. All recordings were conducted at 20–25 °C room temperature.

Current density was calculated as the ratio of current to membrane capacity (pA/pF). The time constant of Ca^{2+} current inactivation was obtained by fitting the data to a single-exponential decay function of the form $f(t) = A\exp[-t/\tau] + C$, where A is an amplitude factor and τ is the time constant of current inactivation. The results are presented as means ± S.E.M. The difference between two values was determined by a paired Student's t-test and $P < 0.05$ was considered to be significant.

2.3. Drugs and chemicals

Gossypol, DNase, CsF, CsCl, EGTA, ATP–Mg, HEPES, phosphocreatine, poly-L-lysine, amiloride, Cs–methanesulfonate, tryphostin 47, GTP-γs and GDP-βs were purchased from Sigma (USA), and bovine serum albumin from Boehringer Mannheim (Germany). All other chemicals were of analytical grade.

Stock solutions of gossypol and tyrphostin 47 were prepared in dimethylsulfoxide (DMSO), and those of NiCl, GTP-γs and GDP-βs were prepared in deionized water. They were stored at −20 °C, and freshly diluted with bath or pipette solution to the desired concentration before experiments. The drugs were applied using a gravity-fed multichannel rapid solution changer (RSC-200, Bio-Logic Science Instrument, France). The final concentration of DMSO in the bath solution did not exceed 0.1% (v/v). Control experiments showed that 0.2% DMSO did not affect Ca^{2+} and Cl^- currents in spermatogenic cells, $n = 3$.

3. Results

When a spermatogenic cell was given designed depolarizing test pulses, a series of evoked current traces was recorded, and then the I–V curve, i.e., the amplitude of the peak current-holding potential relationship, was obtained. As shown in Fig. 1A and B, the inward Ca^{2+} current started to be activated at −60 mV; it reached a maximum near −20 mV and with a mean current density of 11.41 ± 0.81 pA/pF ($n = 30$). There were few steady state components in

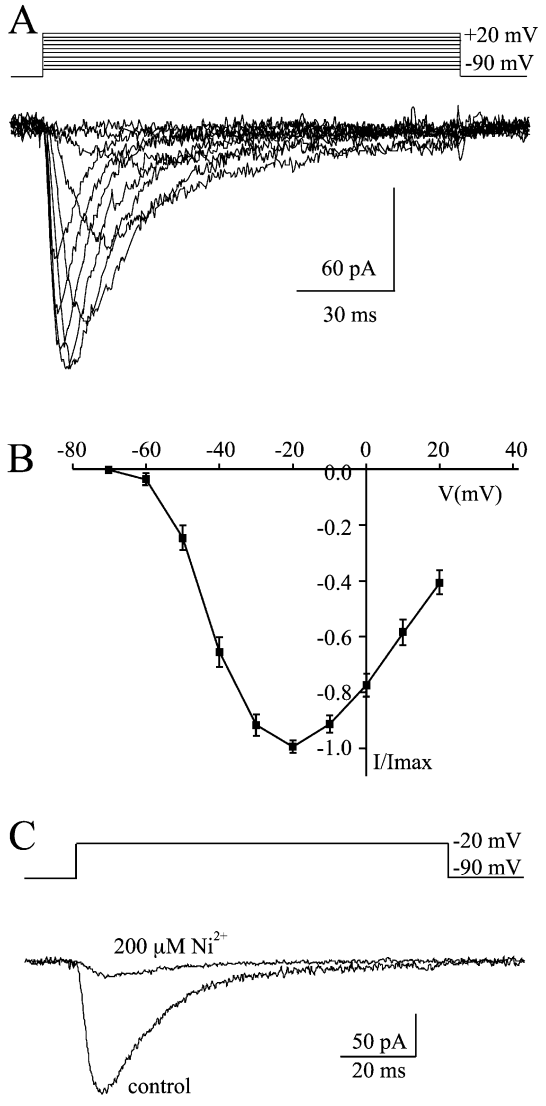

Fig. 1. Whole-cell T-type Ca^{2+} currents in mouse spermatogenic cells. (A) Original records of the currents evoked by a series of depolarizing pulses from a holding potential of -90 mV to test potentials between -70 and $+20$ mV, in 10-mV increments. (B) $I-V$ curve for the currents ($n=10$). (C) Inhibitory effect of 200 μM Ni^{2+} on the current.

the current. After perfusion with 200 μM Ni^{2+}-containing solution, the current was significantly inhibited ($n=3$, Fig. 1C). The low voltage-activated, fast inactivating, steady state component-free and Ni^{2+}-sensitive current showed properties typical of the T-type current. This is in accordance with previous reports that there are only T-type Ca^{2+} channels in spermatogenic cells (Arnoult et al., 1996, 1998; Lievano et al., 1996; Santi et al., 1996; Espinosa et al., 2000). To isolate the Cl^- current, the cell was given a prepulse of -40 mV to inactivate inward currents before a series of depolarizing test pulses was given. The evoked Cl^- currents with outward rectification and a reversal potential of near -10 mV were similar to those previously reported (Espinosa et al., 1998).

3.1. Concentration-dependent and irreversible inhibition of the T-type Ca^{2+} current

Based on the previous data that 5–100 μM gossypol significantly inhibited the acrosome reaction, capacitation and motility of human sperm (Shi and Friend, 1983), in this study we chose a similar concentration to observe the effect of the drug on T-type Ca^{2+} currents in mouse spermatogenic cells. It was found that when cells were perfused with a gossypol (≥ 5 μM)-containing bath solution, the currents evoked by depolarization to -20 from -90 mV were progressively reduced with prolongation of the drug application time, until they disappeared completely. Taking the effect of 40 μM gossypol shown in Fig. 2A as an example, the current decreased to 55% of control 1 min after drug application and was completely blocked within 3 min. Even

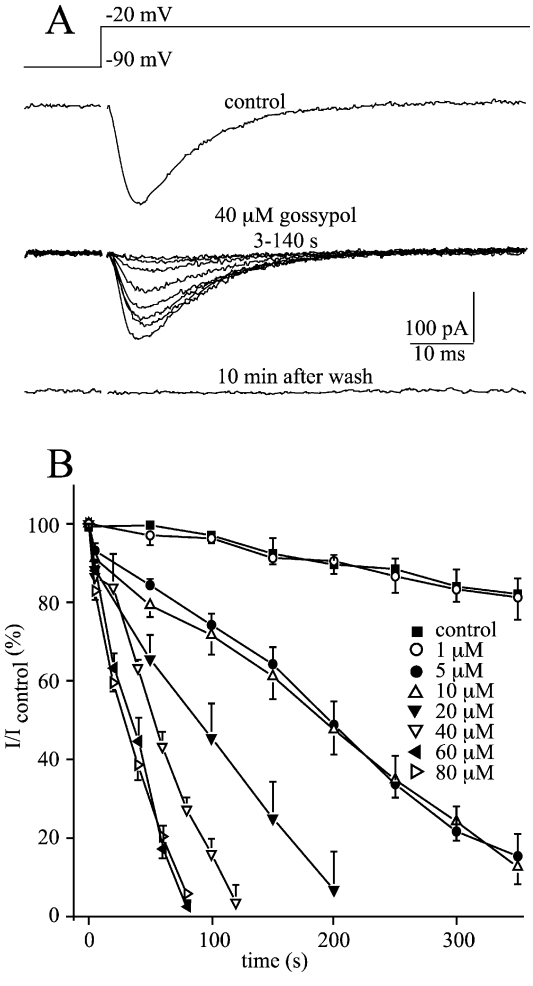

Fig. 2. Gossypol-induced inhibition of T-type Ca^{2+} currents. (A) An example showing the original records (every 20 s) in a spermatogenic cell before and after gossypol application and after washing. (B) Time course of the current decrease after the application of different concentrations of gossypol. Each point represents the mean ± S.E.M. of 3–5 experiments similar to that shown in panel (A).

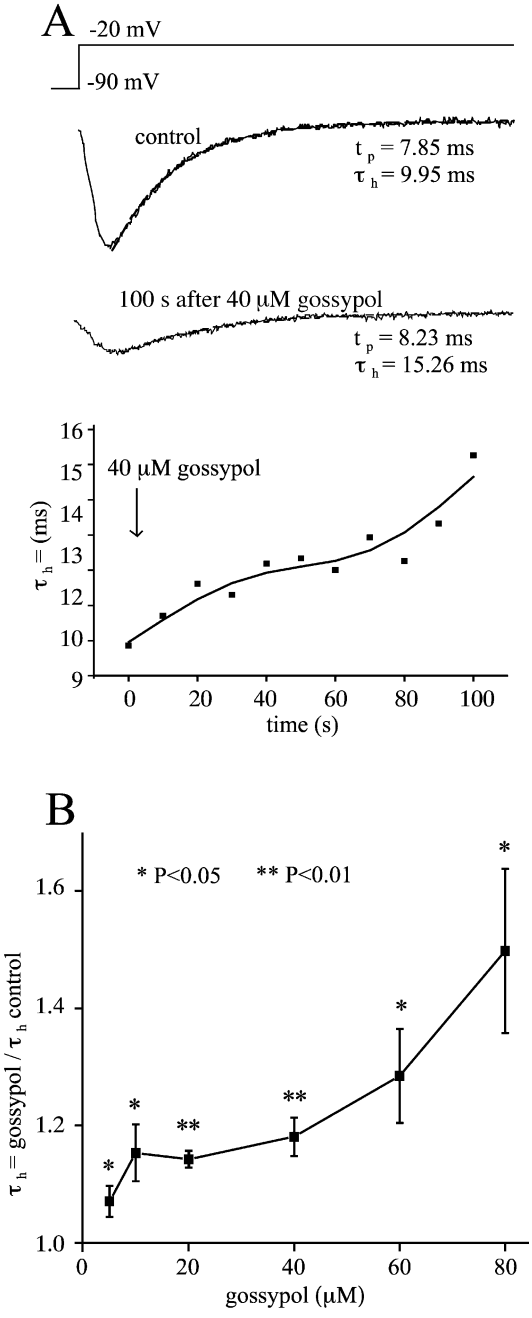

Fig. 3. Concentration-dependent prolongation of inactivation of Ca^{2+} currents induced by gossypol. (A) An example shows progressive enhancement of the inactivation time constant (τ_h) and no change of the time to peak (t_p) 100 s after 40 μM gossypol application. The dashed lines are the fitted results with single exponential equation. (B) Change of τ_h vs. concentration of gossypol. Each point is the mean ± S.E.M. of five experiments 30 s after application of gossypol as shown in panel (A). * $P<0.05$, ** $P<0.01$ vs. control.

a 10-min washing period with drug-free solution did not reverse the blockade, demonstrating the irreversibility of the effect (Fig. 2A).

The effect was concentration-dependent. At a concentration of 1–2 μM, the current did not change, but it almost disappeared within 7 min of 5 μM gossypol application. This indicates that the threshold concentration of the drug to inhibit T-type Ca^{2+} current was very near its concentration to completely block the current. When the concentration of gossypol was higher than 5 μM, the time to block the current sharply shortened, and it was difficult to reach a stable inhibition level, as shown in Fig. 2B.

3.2. Delaying inactivation of the current

In spermatogenic cells, the time constant of inactivation (τ_h), measured by fitting a single exponential to the decay phase of the current evoked by a depolarizing pulse from − 90 to − 20 mV, was 10.84 ± 0.42 ms ($n=30$). Accompanying the decrease of the current amplitude, a gossypol-induced prolongation of inactivation of the current was observed (Fig. 3). For example, 100 s after addition of 40 μM gossypol, τ_h increased from 9.95 to 15.26 ms (Fig. 3A). The effect was concentration-dependent. As shown in Fig. 3B, 80 μM gossypol caused a 1.5-fold increase within 30 s. However, the activation of the current was not affected significantly. Before and 100 s after gossypol (5–80 μM) application, the time to peak (t_p) of the current was 7.42 ± 0.36 and 7.67 ± 0.51 ms ($P>0.05$, $n=30$), respectively.

3.3. Drug-induced effects are not related to G proteins or tyrosine kinase

Previous data showed that Ca^{2+} channels were modulated by G protein-mediated pathways of signal transduction (Hille, 1994), and that T-type Ca^{2+} channels in mouse

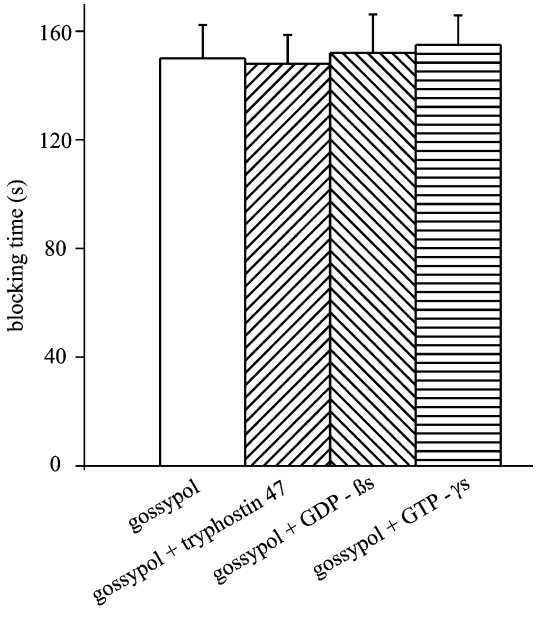

Fig. 4. Lack of correlation of the inhibitory effect of gossypol with G proteins and tyrosine kinase. The time to block Ca^{2+} currents by gossypol (40 μM) was not affected by GTP-γs and GDP-βs (500 μM in pipette) or by tryphostin 47 (100 μM in bath), $n=3$, $P>0.05$.

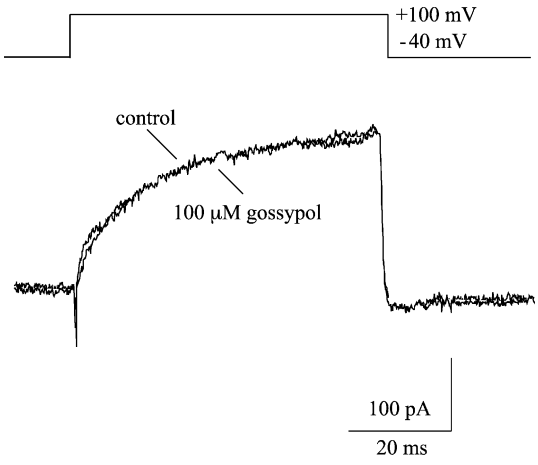

Fig. 5. No change in Cl^- current in spermatogenic cells before and 2 min after gossypol application.

spermatogenic cells were down-regulated by activation of tyrosine kinase (Arnoult et al., 1997). In this study, we investigated the effects of GTP-γs and GDP-βs, an activator and an inhibitor of G proteins, respectively, on the gossypol-induced inhibition of T-type Ca^{2+} currents. The results showed that intracellular addition of 500 μM GTP-γs or GDP-βs did not change the time to current block by gossypol ($P>0.05$). Another group of experiments was performed to observe the effect of tyrphostin 47, an inhibitor of tyrosine kinase, on the gossypol-induced inhibition. Bath application of 10 μM tyrphostin 47 caused a slight increase of the Ca^{2+} currents (data not shown), as reported in a previous study (Arnoult et al., 1997). However, the time to current block by gossypol alone or by gossypol and tyrphostin 47 was not significantly different, indicating that tyrphostin 47 did not affect the gossypol-induced inhibition of Ca^{2+} channels ($P>0.05$). These results are summed up in Fig. 4.

3.4. No effect on Cl^- currents

When a cell held at -40 mV was given a series of designed test pulses ranging from -60 to $+100$ mV, Cl^- currents were recorded and then the $I–V$ curve was plotted. The effect of gossypol on Cl^- channels was tested by observing the amplitude change of the current after perfusion of the drug. As shown in Fig. 5, the amplitude of the currents before and 2 min after 100 μM gossypol application was 25.85 ± 6.74 and 27.05 ± 7.97 pA/pF, respectively ($P>0.05$, $n=3$). The results showed that gossypol did not affect Cl^- channel activity.

4. Discussion

Previous data showed that gossypol inhibited the motility, capacitation and acrosome reaction of sperm, and affected spermatogenesis in the testes and hormone levels in males (Shi and Friend, 1983; Baccetti et al., 1986; Vranova et al., 1999). It was found that as a key step in sperm–oocyte membrane fusion, the acrosome reaction was inhibited by Ca^{2+} channel blockers and in Ca^{2+}-free solution (Visconti et al., 1995; Darszon et al., 1999; Gonzalez-Martinez et al., 2001). In the present study, a concentration-dependent blockade of the T-type Ca^{2+} current by gossypol was demonstrated. The results suggested that the gossypol-induced blockade of Ca^{2+} channels could be responsible for its inhibition of the acrosome reaction and the capacitation of sperm and hence for the antifertility capacity of the drug.

Voltage-gated Ca^{2+} channels can be regulated through G protein-mediated signaling (Hille, 1994), and a down-modulation of T-type Ca^{2+} channel activity by activation of tyrosine kinase was reported in spermatogenic cells (Arnoult et al., 1997). However, in this study, neither GTP-γs and GDP-βs, nor an inhibitor of tyrosine kinase was found to affect the blocking effect of the drug on T-type Ca^{2+} channels in spermatogenic cells. Our findings indicate that the gossypol-induced blockade of channels is not mediated by these signal transduction pathways. Considering that gossypol is liposoluble and its blocking effect is time-dependent, we believe its mechanism of action could involve drug-induced microenvironment and conformational changes that inhibit the channels. Consistent with this possibility, a previous study, which reported an inhibition of Ca^{2+} uptake in human spermatozoa plasma membrane vesicles by gossypol, suggested that the inhibition was similar to that of phosphatidyl choline and could result from a change of the lipid microenvironment of the membrane (Kanwar et al., 1989).

The idea of using gossypol as a clinical long-term contraceptive drug administrated orally has been given up due to its side effects, such as hypokalemia, weakness and irreversible azoospermia (Shi et al., 1987). This is in spite of a few studies that suggested the possibility that gossypol causes infertility without side effects at lower dosages (Gu et al., 2000). However, since gossypol blocks T-type Ca^{2+} channels at lower concentrations, similar to those effective against the acrosome reaction and capacitation of a sperm, it would be possible to use the drug locally as an emergency vaginal contraceptive drug. Furthermore, the method to measure the effect of a drug on Ca^{2+} currents in spermatogenic cells could be used for preliminary screening of a male contraceptive compounds.

In summary, gossypol blocked T-type Ca^{2+} channels in spermatogenic cells at concentrations similar to those that inhibit the sperm acrosome reaction and capacitation. These results suggest the possibility that gossypol could be administrated locally as an emergency contraceptive drug.

Acknowledgements

This work was supported by the National Natural Science Foundation of China (39870197).

References

Arnoult, C., Cardulio, R.A., Lemos, J.R., Florman, H.M., 1996. Activation of mouse sperm T-type Ca^{2+} channels by adhesion to the egg zona pellucida. Proc. Natl. Acad. Sci. U. S. A. 93, 13004–13009.

Arnoult, C., Lemos, J.R., Florman, H.M., 1997. Voltage-dependent modulation of T-type calcium channels by protein tyrosine phosphorylation. EMBO J. 16, 1593–1599.

Arnoult, C., Villaz, M., Florman, H.M., 1998. Pharmacological properties of the T-type Ca^{2+} current of mouse spermatogenic cells. Mol. Pharmacol. 53, 1104–1111.

Arnoult, C., Kazam, I.G., Visconti, P.E., Kopf, G.S., Villaz, M., Florman, H.M., 1999. Control of the low voltage-activated calcium channel of mouse sperm by egg ZP3 and by membrane hyperpolarization during capacitation. Proc. Natl. Acad. Sci. U. S. A. 96, 6757–6762.

Baccetti, B., Bigliardi, E., Burrini, A.G., Renieri, T., Selmi, G., 1986. The action of gossypol on rat germinal cells. Gamete Res. 13, 1–17.

Chang, M.C., Gu, Z.P., Saksena, S.K., 1980. Effects of gossypol on the fertility of male rats, hamsters and rabbits. Contraception 21, 461–469.

Darszon, A., Labarca, P., Nishigaki, T., Espinosa, F., 1999. Ion channels in sperm physiology. Physiol. Rev. 79, 481–510.

Espinosa, F., De La Vega-Beltran, J.L., Lopez-Gonzalez, I., Delgado, R., Labarca, P., Darszon, A., 1998. Mouse sperm patch-clamp recordings reveal single Cl^- channels sensitive to niflumic acid, a blocker of the sperm acrosome reaction. FEBS Lett. 426, 47–51.

Espinosa, F., Lopez-Gonzalez, I., Serrano, C.J., Gasque, G., De La Vega-Beltran, J.L., Trevino, C.L., Darszon, A., 1999. Anion channel blockers differentially affect T-type Ca^{2+} currents of mouse spermatogenic cells, α1E currents expressed in *Xenopus oocytes* and the sperm acrosome reaction. Dev. Genet. 25, 103–114.

Espinosa, F., Lopez-Gonzalez, I., Munoz-Garay, C., Felix, R., De La Vega-Beltran, J.L., Kopf, G.S., Visconti, P.E., Darszon, A., 2000. Dual regulation of the T-type Ca^{2+} current by serum albumin and β-estradiol in mammalian spermatogenic cells. FEBS Lett. 475, 251–256.

Gonzalez-Martinez, M.T., Galindo, B.E., de De La Torre, L., Zapata, O., Rodriguez, E., Florman, H.M., Darszon, A., 2001. A sustained increase in intracellular Ca^{2+} is required for the acrosome reaction in sea urchin sperm. Dev. Biol. 236, 220–229.

Gu, Z.P., Mao, B.Y., Wang, Y.X., Zhang, R.A., Tan, Y.Z., Chen, Z.X., Cao, L., You, G.D., Segal, S.J., 2000. Low dose gossypol for male contraception. Asian J. Androl. 2, 283–287.

Hadley, M.A., Lin, Y.C., Dym, M., 1981. Effects of gossypol on the reproductive system of male rats. J. Androl. 2, 190–199.

Hamill, O.P., Marty, A., Neher, E., Sakmann, B., Sigworth, F.J., 1981. Improved patch-clamp techniques for high-resolution current recordings from cell and cell-free membrane patches. Pflüegers Arch. 391, 85–100.

Hille, B., 1994. Modulation of ion-channel function by G-protein-coupled receptors. Trends Pharmacol. Sci. 17, 531–536.

Kanwar, U., Batla, A., Sanyal, S., Minocha, R., Majumdar, S., Ranga, A., 1989. Gossypol inhibition of Ca^{2+} uptake and Ca^{2+}-ATPase in human ejaculated spermatozoal plasma membrane vesicles. Contraception 39, 431–445.

Lievano, A., Santi, C.M., Serrano, C.J., Trevino, C.L., Bellve, A.R., Hernandez-Cruz, A., Darszon, A., 1996. T-type Ca^{2+} channels and α_{1E} expression in spermatogenic cells, and their possible relevance to the sperm acrosome reaction. FEBS Lett. 388, 150–154.

Meizel, S., 1997. Minireview: amino acid neurotransmitter receptor/chloride channels of mammalian sperm and the acrosome reaction. Biol. Reprod. 56, 569–574.

Randell, R.D., Chase Jr., C.C., Wyse, S.J., 1992. Effects of gossypol and cottonseed products on reproduction of mammals. J. Anim. Sci. 70, 1628–1638.

Santi, C.M., Darszon, A., Arturo, H., 1996. A dihydropyridine-sensitive T-type Ca^{2+} current is the main Ca^{2+} current in mouse primary spermatocytes. Am. J. Physiol. 271, C1583–C1593.

Shi, Q.X., Friend, D.S., 1983. Gossypol-induced inhibition of guinea pig sperm capacitation in vitro. Biol. Reprod. 29, 1027–1032.

Shi, Q.X., Tso, W.W., Friend, D.S., 1987. Gossypol inhibition of spermatogenesis, sperm motility and metabolism. In: Mohri, H. (Ed.), New Horizons in Sperm Cell Research. Japan Sci. Soc. Press, Tokyo/Gordon & Breach, New York, pp. 389–408.

Stephens, D.T., Critchlow, L.M., Hoskins, D.D., 1983. Mechanism of inhibition by gossypol of glycolysis and motility of monkey spermatozoa in vitro. J. Reprod. Fertil. 69, 447–452.

Visconti, P.E., Bailey, J.L., Moore, G.D., Pan, D., Olds-Clarke, P., Kopf, G.S., 1995. Capacitation of mouse spermatozoa: I. Correlation between the capacitation state and protein tyrosine phosphorylation. Development 121, 1129–1137.

Vranova, J., Jezova, M., Scsukova, S., Kolena, J., 1999. Inhibitory effect of gossypol on basal and luteinization factor-stimulated progesterone synthesis in porcine granulosa cells. Physiol. Res. 48, 119–128.