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Effects of demethylzeylasteral and celastrol on spermatogenic cell Ca^{2+} channels and progesterone-induced sperm acrosome reaction

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Abstract

The male antifertility effect of a water–chloroform extract of *Tripterygium wilfordii* Hook. f. (GTW) and several monomers isolated from GTW has attracted worldwide interest. In the present study, the effects of two isolated monomers from GTW, demethylzeylasteral and celastrol, on the Ca^{2+} channels in mouse spermatogenic cells and on the sperm acrosome reaction were investigated by whole-cell patch-clamp recording and chlortetracycline staining methods, respectively. The results showed that demethylzeylasteral concentration-dependently and in a partially reversible manner inhibited the Ca^{2+} current in spermatogenic cells with an IC_{50} of 8.8 μg/ml. Celastrol decreased the Ca^{2+} current in the cells time-dependently and irreversibly. The changes in the activation and inactivation time constants of Ca^{2+} currents after application of these two compounds were also examined. Demethylzeylasteral increased both activation and inactivation time constants of Ca^{2+} currents, and celastrol had no significant effect on them. The two compounds also inhibited significantly the sperm acrosome reaction initiated by progesterone. These data suggest that inhibition of Ca^{2+} currents could be responsible for the antifertility activity of these compounds.

Keywords: Antifertility compound; (*Tripterygium wilfordii* Hook. f.); Whole-cell recording; Ca^{2+}-channel; Acrosome reaction; Spermatogenic cell; (Mouse)

1. Introduction

Tripterygium wilfordii Hook. f. (GTW) (*Celastraceae*), a perennial twining vine, has been used in Chinese traditional medicine to cure fever, edema and carbuncle for many centuries. In the last 30 years, it has been used in the treatment of rheumatoid arthritis, chronic nephritis, ankylosing spondylitis and various skin diseases (Qian, 1987). Column chromatography of a water–chloroform extract from the root xylem of the plant yields a product referred to as GTW (Qian et al., 1995). During intake of GTW tablets for treatment, a male antifertility effect was found in patients (Yu, 1983; Qian, 1987; Qian et al., 1989, 1995). It was also observed that GTW caused a pronounced decrease in sperm motility and sperm density, and significantly inhibited Ca^{2+} channels in spermatogenic cells (Qian et al., 1986; Bai and Shi, 2002a). Thus far, many monomers, including demethylzeylasteral, celastrol and L-epicatechin, etc., have been isolated from GTW, and the antifertility effects of some of them have been demonstrated (Qian, 1987; Matlin et al., 1993; Qian et al., 1995).

Ca^{2+} influx plays an essential role in the initiation of sperm–oocyte interaction, and is required for the acrosome reaction and sperm motility (Darszon et al., 1999). Previous studies showed that the acrosome reaction was inhibited by Ca^{2+} channel blockers or in Ca^{2+}-free medium (Darszon et al., 1999). However, due to the small size, complex geometry, highly differentiated and motile nature of sperm, the ion channels in mature sperm are difficult to study by the patch-clamp technique directly. T-type Ca^{2+} channels are expressed during spermatogenesis and retained in mature sperm, and play an important role in the regulation of $[Ca^{2+}]_i$ during the acrosome reaction (Lievano et al., 1996; Arnoult et al., 1996, 1999; Espinosa et al., 1999). Therefore, the spermatogenic cell, a developmental precur-

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sor of mature sperm, is usually 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., 1996, 1998, 1999; Espinosa et al., 1999, 2000).

It was reported that the intake of Ca^{2+} channel blockers could cause contraceptive effects in men (Goodwin et al., 1997). Moreover, gossypol, which was demonstrated to be an antifertility agent, inhibited the sperm acrosome reaction and T-type Ca^{2+} channels in spermatogenic cells at a similar concentration (Shi and Friend, 1983; Bai and Shi, 2002b). These results indicated that the inhibition of Ca^{2+} currents could be responsible for the antifertility activity of these drugs. In this study, the effects of two monomers purified from GTW, demethylzeylasteral and celastrol, on Ca^{2+} channels in mouse spermatogenic cells were investigated, as was the inhibitory effect of the compounds on the progesterone-evoked acrosome reaction.

2. Materials and methods

2.1. Spermatogenic cell preparation

Individual spermatogenic cells were obtained from the testes of ICR strain mice aged more than 90 days (Shanghai Experimental Animal Center, Chinese Academy of Sciences, China) by using procedures described previously (Espinosa et al., 1999; Bai and Shi, 2002a,b). 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 then pipetted repeatedly to disperse them. The dissociated cells were collected by centrifugation at 250 × *g* 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.

2.2. Collection and preparation of spermatozoa

Spermatozoa were obtained from the vasa deferentia and caudae epididymides of eight mice (5–6-month-old) and were released into modified Tyrode's solution (in mM: 114.54 NaCl, 2.68 KCl, 0.49 $MgCl_2$, 25 $NaHCO_3$, 0.36 NaH_2PO_4, 20 HEPES, 5.56 glucose, 1.80 $CaCl_2$, and 100 μg/ml penicillin, 4 mg/ml bovine serum albumin, pH 7.45). After incubation for 15 min at 37 °C, the motile spermatozoa were selected by centrifugation through a two-step Percoll gradient (Shi and Roldan, 1995). Briefly, aliquots of spermatozoa were layered over the upper step of a 35% and 70% Percoll gradient and centrifuged at 650 × *g* for 18 min. The sediment was diluted in 10 volumes of modified Tyrode's solution, centrifuged again at 400 × *g* for 8 min and then resuspended in the same solution. The concentration of spermatozoa was adjusted to $2-4 \times 10^7$ cells/ml. The spermatozoa were then incubated at 37 °C for 1.5 h before use. Sperm concentration and motility were determined with a hemocytometer and the sample suspensions with less than 60% motility were discarded. The use of experimental animals was consistent with European Community guidelines.

2.3. Electrophysiological recording

An aliquot of the spermatogenic 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 bath solution, cells were used for whole-cell recording. Two types 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+} currents were recorded in the whole-cell configuration of patch-clamp recording (Hamill et al., 1981). Cells were bathed in the bath 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). 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. The drugs were applied using a gravity-fed multichannel rapid solution changer (RSC-200, Bio-Logic Science Instrument, France). All recordings were made at 20–25 °C room temperature.

2.4. Evaluation of acrosomal status by chlortetracycline staining

Sperm acrosomal status was evaluated by chlortetracycline fluorescence staining as described previously (Ward and Storey, 1984; Fraser and Herod, 1990; Shi and Roldan, 1995). Briefly, the stain solution was prepared by dissolving chlortetracycline (300 mM) in TN buffer (in mM: 130 NaCl,

20 Tris, 5.0 L-cysteine, pH 7.8) and was kept on ice and in the dark until use. At the time of assay, 50 μl sperm suspension and 50 μl chlortetracycline solution were successively dropped onto a warmed (37 °C) slide and mixed. After incubation at 37 °C for 15 s, the sperm samples were fixed in 18 μl solution containing 5% formaldehyde and 16% triethanolamine. Then sperm acrosomal status was examined under a fluorescence microscope in which an Hg excitation beam passed through a 405-nm filter and fluorescence emission was monitored using a DM 455 dichroic mirror (Olympus BH-2, Japan). A total of 200 spermatozoa per experiment were counted to assess the different chlortetracycline staining patterns recognized earlier (Ward and Storey, 1984; Fraser and Herod, 1990; Shi and Roldan, 1995): "F", characteristic of uncapacitated, acrosome-intact spermatozoa; "B", representing capacitated, acrosome-intact spermatozoa; and "AR", corresponding to spermatozoa that have undergone the acrosome reaction.

2.5. Data analysis

The time constant of Ca^{2+} current activation and inactivation was obtained by fitting the data to a single-exponential decay function of the form $f(t) = A \cdot \exp[-t/\tau] + C$, where A is an amplitude factor and τ is the time constant of activation (τ_m) or inactivation (τ_h). All results obtained in the present study 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.

2.6. Drugs and chemicals

Demethylzeylasteral ($C_{29}H_{36}O_6$, FW = 480) and celastrol ($C_{29}H_{38}O_4$, FW = 450) were from Natural Chemicals Lab, Shanghai Zhong Shan Hospital (China) and their chemical structures are shown in Fig. 1. DNase, EGTA, ATP-Mg, HEPES, Tris, CsCl, CsF, phosphocreatine, poly-L-lysine, progesterone, amiloride, triethanolamine and chlortetracycline were purchased from Sigma (USA). Bovine serum albumin was from Boehringer Mannheim (Germany). Percoll was from Pharmacia (Sweden). All other chemicals were of analytical grade.

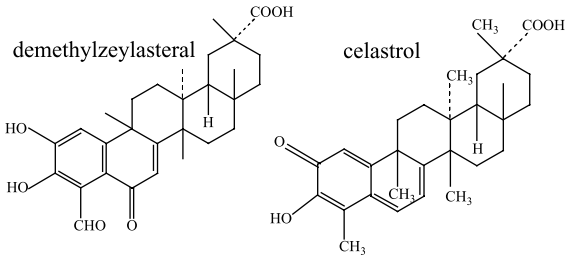

Fig. 1. Chemical structures of demethylzeylasteral and celastrol.

Stock solutions of demethylzeylasteral, celastrol and progesterone were prepared in dimethylsulfoxide (DMSO); the stock of amiloride was prepared in deionized water. All stock solutions were stored at −20 °C and freshly diluted to the desired concentration before use. The maximal final concentration of DMSO in the recording bath solution was lower than 0.2%. Control experiments showed that 0.2% DMSO did not affect Ca^{2+} currents in spermatogenic cells ($n = 3$).

3. Results

3.1. Effects of demethylzeylasteral and celastrol on Ca^{2+} currents

When a spermatogenic cell held at −90 mV was given designed depolarizing test pulses, a series of evoked inward currents was recorded, and then the I–V curve, i.e., the amplitude of the peak current-holding potential relationship, was obtained. As determined in a previous study (Bai and Shi, 2002a,b), the low voltage-activated, fast-inactivating, steady-state component-free and Ni^{2+}-sensitive current showed properties of the T-type Ca^{2+} current. This is in accordance with the previous reports that only T-type Ca^{2+} channels exist in spermatogenic cells (Arnoult et al., 1996, 1998; Lievano et al., 1996; Espinosa et al., 2000). Inhibition of the currents after application of demethylzeylasteral was observed in our recent study (Bai and Shi, 2002a).

3.1.1. Demethylzeylasteral inhibited Ca^{2+} currents concentration-dependently and partially reversibly

After control Ca^{2+} currents were recorded, the cell was perfused with demethylzeylasteral-containing bath solution (10 μg/ml, 20.8 μM). Demethylzeylasteral significantly decreased the currents (Fig. 2A). The amplitude of the currents evoked by −20 mV depolarizing pulses from −90 mV was decreased to 48.7 ± 1.8% of the control 30 s after drug application ($n = 5$). The currents were restored to 72.3 ± 7.4% ($n = 5$) 1 min after withdrawing the drug (Fig. 2A), indicating that the inhibition was partially reversible. Demethylzeylasteral caused a concentration-dependent increase in the inhibitory effect and an IC_{50} of 8.8 μg/ml was obtained. The inhibitory effects of demethylzeylasteral on the currents showed no obvious voltage dependence (Fig. 2B).

Both the time constants of activation (τ_m) and inactivation (τ_h) of the channels were measured by single-exponential expression. The results showed that both τ_m and τ_h significantly increased after administration of demethylzeylasteral (Fig. 2C).

3.1.2. Celastrol inhibited Ca^{2+} currents time-dependently and irreversibly

Similar experiments were performed to observe the effect of celastrol on the Ca^{2+} currents in spermatogenic cells.

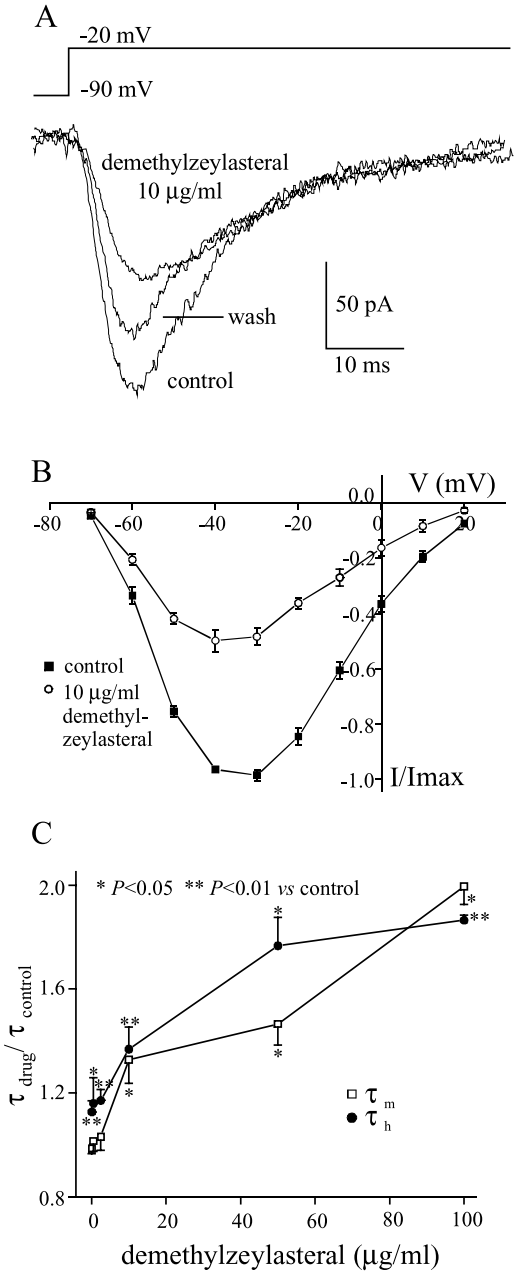

Fig. 2. Demethylzeylasteral-induced inhibition of T-type Ca^{2+} currents in mouse spermatogenic cells. (A) Original traces of the Ca^{2+} current before and after 10 μg/ml demethylzeylasteral application and washing. (B) *I–V* curves before and after exposure to demethylzeylasteral. Each point represents mean ± S.E.M. of five experiments. (C) Effects of demethylzeylasteral on inactivation and activation of the currents, showing concentration-dependent increase in activation and inactivation time constants (τ_m and τ_h) after drug application. Each point is mean ± S.E.M. of five experiments.

Based on the previous data showing that celastrol (~ 3 μg/ml) significantly inhibited the acrosome reaction of guinea pig sperm (Yuan et al., 1995), in this study, a similar concentration of the drug was used to observe its effect on the Ca^{2+} current. It was observed that when the cells were perfused with celastrol (≥ 5 μg/ml)-containing solution, the currents evoked by depolarization from − 90 to − 20 mV were progressively reduced along with prolongation of the drug application time, until they completely disappeared (Fig. 3). Taking the effect of 100 μg/ml (222 μM) celastrol as an example, the currents decreased to 47.4 ± 3.5% of the control in 60 s after drug application and were completely blocked within 150 s. Washing for 3 min with drug-free solution did not reverse the blockade, demonstrating the irreversibility of the effect.

The inhibitory effect was time- and concentration-dependent. The time to 80% inhibition of the currents was 255.7 ± 10.7 s for 5 μg/ml celastrol and 83.3 ± 7.5 s for 100 μg/ml. Fig. 3B shows the time course of the Ca^{2+} current decrease after application of celastrol. As mentioned above, τ_m and τ_h were measured by fitting the

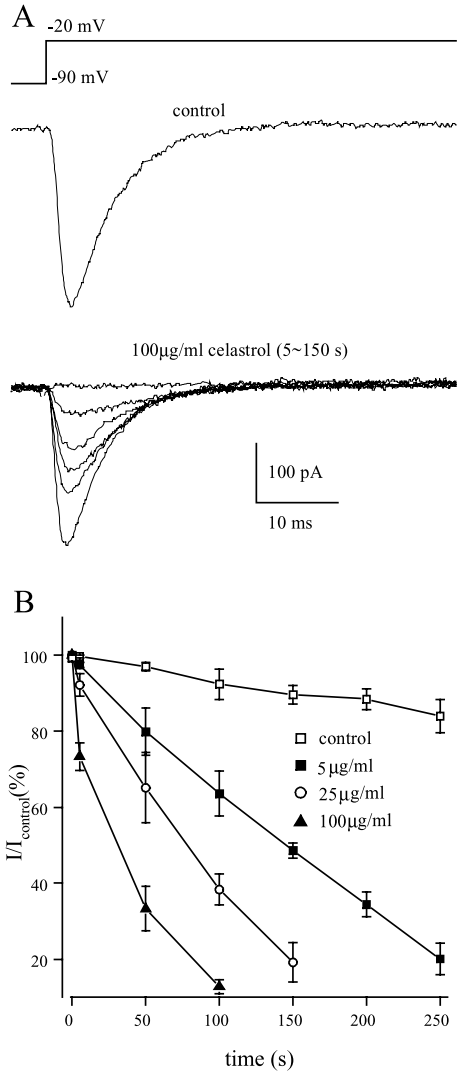

Fig. 3. Celastrol-induced inhibition of Ca^{2+} currents. (A) An example showing the original records (every 30 s) in a spermatogenic cell before and after application of 100 μg/ml celastrol. (B) Time course of the current decrease after celastrol application. Each point represents mean ± S.E.M. of three experiments.

activation or inactivation phase to a single-exponential function. Before and after celastrol (5–100 μg/ml) application, τ_m and τ_h were 3.6 ± 0.2, 15.0 ± 0.5 ms and 3.8 ± 0.4, 15.0 ± 0.5 ms ($P>0.05$, $n=9$), respectively, showing no significant change in activation and inactivation of the currents.

3.2. Inhibition of sperm acrosome reaction by demethylzeylasteral and celastrol

Aliquots of sperm suspension were added to an equal volume of modified Tyrode's solution without or with different concentrations of demethylzeylasteral or celastrol and then incubated at 37 °C for 5 min. Progesterone, a physiological agonist of the acrosome reaction, was used to evoke the acrosome reaction. Progesterone (15 μM) was added and after an additional 15-min incubation, the acrosome reaction was assessed.

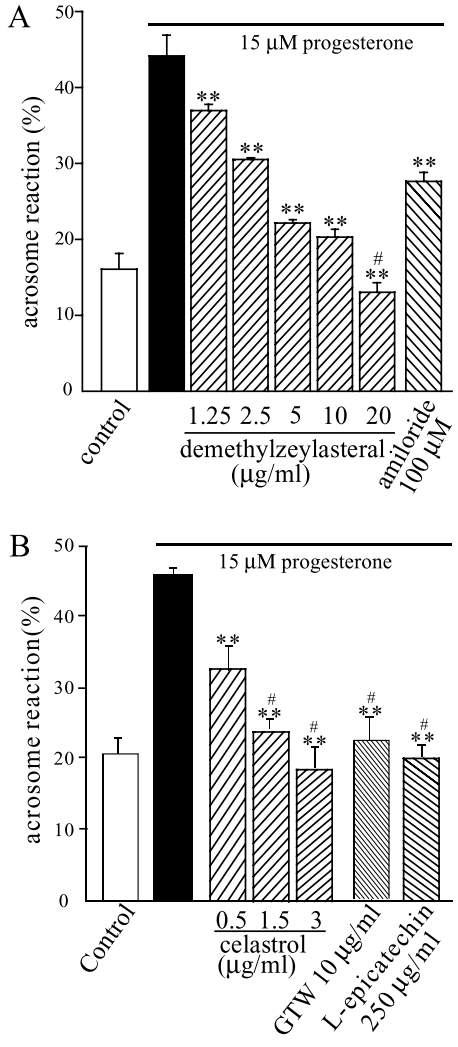

Fig. 4. Inhibition of progesterone-evoked acrosome reaction by demethylzeylasteral and amiloride (A) and celsatrol, GTW and L-epicatechin (B). Mice spermatozoa were preincubated in modified Tyrode's solution (mT s) at 37 °C for 1.5 h. Samples (0.2 ml each) of capacitated sperm suspension in mTs were treated without or with the compounds for 5 min (or their solvent as control), respectively, and then challenged with 15 μM progesterone for another 15 min under similar conditions before assessment of the acrosome reaction. Data in each column were obtained from three experiments with total 600 spermatozoa and represent means ± S.E.M. $^{**}P<0.01$ vs. acrosome reaction evoked by progesterone, #$P>0.05$ vs. control.

After incubation of the sperm suspension in modified Tyrode's solution, $16.0 \pm 2.1\%$ of cells exhibited spontaneous acrosome reaction pattern, while $44.2 \pm 2.7\%$ of cells exhibited the acrosome reaction after exposure to 15 μM progesterone in three experiments with a total of 600 spermatozoa. The progesterone-evoked acrosome reaction was inhibited by demethylzeylasteral at concentration of ≥2.5 μg/ml. At a concentration of 20 μg/ml, demethylzeylasteral completely blocked the progesterone-evoked sperm acrosome reaction. Fig. 4A shows the concentration dependence of inhibition of the acrosome reaction by demethylzeylasteral.

Amiloride, an antagonist of T-type Ca^{2+} channels, inhibits T-type Ca^{2+} channels in spermatogenic cells with an IC_{50} of 245 μM (Arnoult et al., 1996). In this studies, the effect of amiloride on the acrosome reaction was also examined. Amiloride significantly inhibited the progesterone-evoked acrosome reaction, to $26.7 \pm 1.2\%$ in 100 μM amiloride-containing solution ($P<0.01$, see Fig. 4A also).

Another group of experiments with similar procedures was performed to observe the effects of celastrol, GTW and L-epicatechin on the progesterone-induced sperm acrosome reaction. The results showed that celastrol (≥0.5 μg/ml) caused a significant decrease in the acrosome reaction, which was related to the concentration of the compound used in the medium. Similarly, the progesterone-induced acrosome reaction was also inhibited markedly by 10 μg/ml GTW or 250 μg/ml L-epicatechin (see Fig. 4B).

4. Discussion

After a male antifertility effect was found, interest in GTW as a potential contraceptive for men increased (Qian, 1987; Qian et al., 1995). To date, several monomers have been isolated from GTW and their antifertility effects demonstrated in mice, rats and men (Qian et al., 1986, 1989; Yuan et al., 1995). Given the pivotal role of Ca^{2+} and Ca^{2+} channels in the sperm acrosome reaction and the finding that spermatogenic cells are a target of GTW (Zheng et al., 1985), it is worthwhile to investigate the action of GTW and these monomers on Ca^{2+} channels in mouse spermatogenic cells and the potential clinic use of these compounds.

The acrosome reaction is an essential event in sperm–oocyte fusion (Yanagimachi, 1994). In the present study, amiloride significantly inhibited the progesterone-evoked acrosome reaction. This result is consistent with the established importance of Ca^{2+} channels and Ca^{2+} influx in the sperm acrosome reaction.

Table 1
A comparison of effective dosages of some male antifertility compounds to inhibit both sperm acrosome reaction and spermatogenic cell Ca^{2+} current

	Demethylzeylasteral (μg/ml)	Celastrol (μg/ml)	L-Epicatechin (μg/ml)	GTW (μg/ml)	Gossypol (μM)	Amiloride (μM)
Ca^{2+} current	≥ 1, IC_{50} = 8.8[a]	≥ 5[a]	≥ 1000[b]	≥ 1, IC_{50} = 6.4[b]	≥ 5[c]	IC_{50} = 245[d]
AR	≥ 2.5[a]	≥ 0.5[a]	250[a]	10[a]	≥ 5[e]	≥ 100[a]
Oral contraception	–	–	2000 mg/kg day[f]	10 mg/kg day[g]	12–40 mg/kg day[h]	–

[a] Shows the data from the present study.
[b] From Bai and Shi, 2002a.
[c] From Bai and Shi, 2002b.
[d] From Arnoult et al., 1996.
[e] From Shi and Friend, 1983.
[f] From Qian et al., 1995.
[g] From Zheng et al., 1985.
[h] From Shi et al., 1987.

Comparison of effective concentrations of these male antifertility compounds showed that L-epicatechin, which has a lower antifertility effect, inhibited Ca^{2+} currents at higher concentrations than the other compounds. Similarly, it inhibited the acrosome reaction only at a relatively high concentration (250 μg/ml). However, other compounds, such as GTW and gossypol, which had an oral contraceptive effect at lower concentrations, inhibited Ca^{2+} currents at lower concentrations. Their inhibitory effects on the sperm acrosome reaction and Ca^{2+} channels in mouse spermatogenic cells are consistent with their antifertility effects (see Table 1). These data show that inhibition of Ca^{2+} channels is closely involved in and could be responsible for the male antifertility effects of these compounds.

After application of demethylzeylasteral, a stable inhibition of the Ca^{2+} current was obtained in several seconds. However, the celastrol-induced inhibition of the current developed progressively. It was difficult to reach a stable inhibition, similar to that in the case in gossypol (Bai and Shi, 2002b). This difference implies that demethylzeylasteral and celastrol inhibit Ca^{2+} channels by different mechanisms.

In conclusion, demethylzeylasteral and celastrol significantly inhibit T-type Ca^{2+} channels in mouse spermatogenic cells and the progesterone-evoked sperm acrosome reaction, indicating that inhibition of Ca^{2+} channels may be a mechanism for the antifertility effects of the compounds and that investigation of the effect of agents on Ca^{2+} channels in mouse spermatogenic cells is a useful method to select male contraceptives.

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References

Arnoult, C., Cadullo, 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., 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.

Bai, J.P., Shi, Y.L., 2002a. Inhibition of Ca^{2+} channels in mouse spermatogenic cells by male antifertility compounds from *Tripterygium wilfordii* Hook. f.. Contraception 65, 441–445.

Bai, J.P., Shi, Y.L., 2002b. Inhibition of T-type Ca^{2+} currents in mouse spermatogenic cells by gossypol, an antifertility compound. Eur. J. Pharmacol. 440, 1–6.

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

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.

Fraser, L.R., Herod, J.E., 1990. Expression of capacitation-dependent changes in chlortetracycline fluorescence patterns in mouse spermatozoa requires a suitable glycolysable substrate. J. Reprod. Fertil. 88, 611–621.

Goodwin, L.O., Leeds, N.B., Hurley, I., Mandel, F.S., Pergolizzi, R.G., Benoff, S., 1997. Isolation and characterization of the primary structure of testis-specific L-type calcium channel: implications for contraception. Mol. Hum. Reprod. 3, 255–268.

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. Pflügers Arch. 391, 85–100.

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.

Matlin, S.A., Belenguer, A., Stacey, V.E., Qian, S.Z., Xu, Y., Zhang, J.W., Sanders, J.K.M., Amor, S.R., Pearce, C.M., 1993. Male antifertility compounds from *Tripterygium wilfordii* Hook f.. Contraception 47, 387–400.

Qian, S.Z., 1987. *Tripterygium wilfordii*, a Chinese herb effective in male fertility regulation. Contraception 36, 335–345.

Qian, S.Z., Zhong, C.Q., Xu, Y., 1986. Effect of *Tripterygium wilfordii* Hook. f. on the fertility of rats. Contraception 33, 105–110.

Qian, S.Z., Hu, Y.Z., Tong, J.S., Ding, J.H., Wang, S.Y., Tao, J.Z., Qi, A.P., Guan, F., 1989. Studies on the effect of *T. wilfordii* on the reproduction of men. Chin. J. Androl. 3, 129–132.

Qian, S.Z., Xu, Y., Zhang, J.W., 1995. Recent progress in research on *Tripterygium*: a male antifertility plant. Contraception 51, 121–129.

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., Roldan, E.R.S., 1995. Evidence that a $GABA_A$-like receptor is involved in progesterone-induced acrosomal exocytosis in mouse spermatozoa. Biol. Reprod. 52, 373–381.

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 and Breach Sci. Publ., New York, pp. 389–408.

Ward, C.R., Storey, B.T., 1984. Determination of the time course of capacitation in mouse spermatazoa using a chlortetracycline fluorescence assay. Dev. Biol. 104, 287–296.

Yanagimachi, R., 1994. Mammalian fertilization. In: Knobil, E., Neill, J.D. (Eds.), The Physiology of Reproduction, 2nd ed. Raven Press, New York, pp. 189–317.

Yu, D.Y., 1983. One hundred and forty-four cases of rheumatoid arthritis treated with *Tripterygium wilfordii*. J. Trad. Chin. Med. 3, 125–129.

Yuan, Y.Y., Gu, Z.P., Shi, Q.X., Qin, G.W., Xu, R.S., Cao, L., 1995. In vitro inhibition of celastrol on spermatozoa fertilization ability of guinea pig. Acta Pharm. Sin. 30, 331–335.

Zheng, J.R., Fang, J.L., Xu, L.F., Gao, J.W., Guo, H.Z., Li, Z.R., Sun, H.Z., 1985. Effect of total glycoside of *T. wilfordii* on reproductive organs of male rats. Acta Acad. Med. Sin. 7, 256–259.