Novel effects of gossypol, a chemical contraceptive in man: mobilization of internal Ca\textsuperscript{2+} and activation of external Ca\textsuperscript{2+} entry in intact cells

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Abstract

The effect of gossypol on Ca\textsuperscript{2+} signaling in Madin Darby canine kidney (MDCK) cells was investigated by using fura-2 as a Ca\textsuperscript{2+} probe. Gossypol evoked a rise in cytosolic free Ca\textsuperscript{2+} levels ([Ca\textsuperscript{2+}]i) concentration-dependently between 2 and 20 \muM. The response was decreased by external Ca\textsuperscript{2+} removal. In Ca\textsuperscript{2+}-free medium pretreatment with gossypol nearly abolished the [Ca\textsuperscript{2+}]i increase induced by carbonylcyanide m-chlorophenylhydrazone (CCCP), a mitochondrial uncoupler, and thapsigargin, an inhibitor of the endoplasmic reticulum Ca\textsuperscript{2+} pump; but pretreatment with CCCP and thapsigargin only partly inhibited gossypol-induced Ca\textsuperscript{2+} release. Addition of 3 mM Ca\textsuperscript{2+} induced a [Ca\textsuperscript{2+}]i increase after pretreatment with 5 \muM gossypol in Ca\textsuperscript{2+}-free medium. This Ca\textsuperscript{2+} entry was decreased by 25 \muM econazole, 50 \muM SKF96365 and 40 \muM aristolochic acid (a phospholipase A\textsubscript{2} inhibitor). Pretreatment with aristolochic acid inhibited 5 \muM gossypol-induced internal Ca\textsuperscript{2+} release by 55%, but suppression of phospholipase C with 2 \muM 1-(6-((17\beta-3-methoxyestra-1,3,5(10)-trien-17-yl)amino)hexyl)-1H-pyrrole-2,5-dione) had no effect. Gossypol (5 \muM) also increased [Ca\textsuperscript{2+}]i in human bladder cancer cells and neutrophils. Collectively, we have found that gossypol increased [Ca\textsuperscript{2+}]i in MDCK cells by releasing Ca\textsuperscript{2+} from multiple Ca\textsuperscript{2+} stores in a manner independent of the production of inositol-1,4,5-trisphosphate, followed by Ca\textsuperscript{2+} influx from external space. © 2000 Elsevier Science B.V. All rights reserved.

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1. Introduction

Gossypol, a polycyclic lipophilic agent naturally present in cottonseed, has been known to be a potent non-steroid antifertility agent in men and several other mammalian species. Extensive trials of gossypol as a male contraceptive has been conducted in China [1]. This compound destroys elements of the...
seminiferous epithelium but does not alter the endocrine function of the testis. Infertility develops within several months of its use and fertility is restored within several months of discontinuation of the drug. Unfortunately, gossypol causes serious side effects such as hypokalemia, weakness, diarrhea, edema, dyspnea, neuritis and paralysis. These side effects restrict the use of gossypol as a contraceptive in men [2].

In vitro, gossypol has been shown to exert different actions. These include alteration of secretory function of cultured rat sertoli cells [3], arresting of human benign prostatic hyperplastic cell growth at the G0/G1 phase of the cell cycle [4], blockade of cell-to-cell communication in human and rat cells [5] and inhibition of swelling-induced chloride channels [6]. Moreover, evidence shows that gossypol inhibits human chorionic gonadotropin-stimulated testosterone production by cultured canine testicular interstitial cells [7].

In addition, many other effects are attributed to gossypol. For example, depletion of ATP and inactivation of an ATP-sensitive taurine channel [8], anti-proliferative action on human breast cancer cells [9], in vitro and in vivo cytotoxicity against central nervous system tumor cell lines [10], induction of apoptotic DNA fragmentation and cell death in cultured canine testicular interstitial cells [7].

The effect of gossypol on Ca\(^{2+}\) signaling has not been investigated previously. In this study we examined the effect of gossypol on Ca\(^{2+}\) signaling in Madin Darby canine kidney cells (MDCK) cells. Previous results showed that in this renal tubular cell, inositol 1,4,5-trisphosphate (IP\(_3\))-dependent agonists such as ATP [13] and bradykinin [14] increase \([\text{Ca}^{2+}]\), by depleting \(\text{Ca}^{2+}\) from the endoplasmic reticulum \(\text{Ca}^{2+}\) store followed by an internal \(\text{Ca}^{2+}\) refilling process termed ‘capacitative \(\text{Ca}^{2+}\) entry’ [15]. Also, thapsigargin [16] and 2,5-di-tert-butylhydroquinone [17] increase \([\text{Ca}^{2+}]\), by inhibiting the endoplasmic reticulum \(\text{Ca}^{2+}\) pump without elevating IP\(_3\) levels, leading to a release of \(\text{Ca}^{2+}\) from the endoplasmic reticulum followed by capacitative \(\text{Ca}^{2+}\) entry.

Here we show that gossypol induced an increase in \([\text{Ca}^{2+}]\), in MDCK cells and two other cells: T24 human bladder cancer cells and human neutrophils.

2. Materials and methods

2.1. Cell culture

MDCK and T24 bladder carcinoma cells obtained from the American Type Culture Collection (CRL-6253) were cultured in Dulbecco’s modified Eagle medium supplemented with 10% heat-inactivated fetal bovine serum, 100 U/ml penicillin and 100 μg/ml streptomycin at 37°C in 5% CO\(_2\)-containing humidified air.

2.2. Isolation of human neutrophils

After obtaining informed consent, whole blood was taken by venous puncture from a healthy human volunteer with no history of infections 2 weeks before the experiments. Blood was mixed with heparin (20 units/ml) and the erythrocytes were allowed to sediment for 50 min at room temperature following a 1:6 (v/v) Hespan:blood blend. The leukocyte-rich plasma was harvested and centrifuged at 300×g for 20 min. The supernatant was aspirated and centrifuged at 2170×g for 15 min to produce platelet-poor plasma. The pellet from centrifugation of leukocyte-rich plasma was resuspended in 2.5 ml platelet-poor plasma and was transferred to a 15 ml tube, where it was underlayered with 2 ml freshly prepared 42% Percoll in platelet-poor plasma. This mixture was in turn underlayered with 2 ml of freshly prepared 52% Percoll in platelet-poor plasma. The gradients were centrifuged for 10 min at 280×g. The neutrophils were collected at the 42-51% Percoll interface. The final cell population was determined to contain >95% neutrophils by Wright’s staining. The neutrophils were 98% viable assayed by trypan blue exclusion.

2.3. Solutions

\(\text{Ca}^{2+}\) medium (pH 7.4) contained (in mM): 140 NaCl; 5 KCl; 1 MgCl\(_2\); 2 CaCl\(_2\); 10 HEPES and 5 glucose. \(\text{Ca}^{2+}\)-free medium contained no Ca\(^{2+}\) plus 1 mM EGTA.

2.4. Optical measurements of \([\text{Ca}^{2+}]\),

Trypsinized MDCK (or T24) cells and freshly iso-
lated neutrophils (10^6/ml) were allowed to recover in Dulbecco’s modified Eagle medium for 1 h before being loaded with 2 µM 1-[2-(5-carboxyoxazol-2-yl)-6-aminobenzofuran-5-oxyl]-2-(2’-amino-5’-methylphenoxy)-ethane-N,N,N,N-tetraacetic acid pentaece-
toxyethyl ester (fura-2/AM) for 30 min at 25°C in the same medium. The cells were washed and resus-
cended in Ca^{2+} medium. Fura-2 fluorescence mea-
surements were performed in a water-jacketed cu-
vette (25°C) with continuous stirring; the cuvette contained 1 ml medium and 0.5 million cells. Fluor-
escence was monitored with a Shimadzu RF-
5301PC spectrofluorophotometer by continuously re-
cording excitation signals at 340 and 380 nm and 
emission signals at 510 nm at 1-s intervals. Maxi-
mum and minimum fluorescence values were ob-
tained by adding Triton X-100 (0.1%) and EGTA
(20 mM) sequentially at the end of an experiment. 

[Ca^{2+}] was calculated as described previously [18].

2.5. Chemical reagents

The reagents for cell culture were from Gibco. 
Fura-2/AM was from Molecular Probes. Gossy-
pol, 1-6-((17β-3-methoxyestra-1,3,5(10)-trien-17-yl)- 
amino)hexyl)-1H-pyrrole-2,5-dione) (U73122), 1-(6-
((17β-3-methoxyestra-1,3,5(10)-trien-17-yl)amino)hex-
yl)-2,5-pyrrolidine-dione (U73343) and aristolochic
acid were from Biomol. The other reagents were from Sigma.

2.6. Statistical analysis

The traces were typical of 4–6 experiments. All 
values were reported as means±S.E.M. of 4–6 ex-
periments. Statistical comparisons were determined 
by using Student’s paired t-test and significance was accepted when P<0.05.

3. Results

3.1. Effects of gossypol on [Ca^{2+}]

At concentrations between 2–20 µM, gossypol in-
creased [Ca^{2+}], in the presence of Ca^{2+} (Fig. 1A).
Over a time period of 5 min the responses were monophasic but exhibited different velocities. For example, at a concentration of 5 µM, gossypol in-
duced a nearly immediate increase in [Ca^{2+}], fol-
lowed by a gradual rising phase which had a net 
[Ca^{2+}] of 320±14 nM (trace b; n=6; P<0.05) at the time point of 250 s. The increase of the Ca^{2+}
signal was slower in response to lower concentrations 
of gossypol. The [Ca^{2+}], response saturated at a con-
centration of 10 µM gossypol because 20 µM gossy-
pol did not induce a greater response (trace b versus 
trace a; n=5; P>0.05).

Fig. 1B shows that removal of external Ca^{2+} re-
duced the Ca^{2+} signals induced by 3–20 µM gossypol 
and abolished the [Ca^{2+}], increases induced by 2 µM 
gossypol. The concentration–response plots of the 
responses both in the presence and absence of exter-
nal Ca^{2+} are illustrated in Fig. 1C. Ca^{2+} removal 
reduced the [Ca^{2+}], increase induced by 3, 5, 10 
and 20 µM gossypol by 87±6, 85±4, 82±6 and 
81±3%, respectively, at the maximum value (n=5– 
6; P<0.05).

3.2. The internal Ca^{2+} stores of gossypol-induced 
[Ca^{2+}], increase

Fig. 2A shows that after pretreatment with 2 µM 
CCCP, a mitochondrial uncoupler, and 1 µM thap-
sigargin, an endoplasmic reticulum Ca^{2+} pump in-
hibitor [19], in Ca^{2+}-free medium to deplete the 
Ca^{2+} stores in mitochondria and the endoplasmic 
reticulum, respectively, 5 µM gossypol still induced a [Ca^{2+}], increase with a net maximum value of 
61±5 nM (n=5; P<0.05). In contrast, Fig. 2B 
shows that 5 µM gossypol induced a [Ca^{2+}], increase with a net maximum value of 120±10 nM (n=5; 
P<0.05). Subsequently added CCCP only induced a tiny increase in [Ca^{2+}], and thapsigargin failed to 
increase [Ca^{2+}].

3.3. Effect of gossypol on capacitative Ca^{2+} entry

In MDCK cells mobilization of internal Ca^{2+} 
often results in capacitative Ca^{2+} entry [13,14, 
16,17]. Thus, the possibility that 5 µM gossypol in-
duced Ca^{2+} influx via capacitative Ca^{2+} entry was tested. Capacitative Ca^{2+} entry was measured by 
adding 3 mM Ca^{2+} to cells pretreated with gossypol 
in Ca^{2+}-free medium. Fig. 2C shows that after deple-
tion of the internal Ca^{2+} stores for 15 min with 5 µM
gossypol, addition of Ca\(^{2+}\) induced a \([\text{Ca}^{2+}]_i\) increase with a net maximum value of 789 ± 21 nM (trace a; \(n = 6\)) which was 39-fold higher than the control (22 ± 3 nM; trace e; \(n = 6\); \(P < 0.05\)).

The effect of econazole and SKF96365, two inhibitors of capacitative Ca\(^{2+}\) entry in MDCK cells [20,21] and aristolochic acid, a phospholipase A\(_2\) inhibitor [22] on 5 µM gossypol-induced capacitative Ca\(^{2+}\) entry was explored. Fig. 2C shows that addition of econazole (25 µM; trace b), SKF96365 (50 µM; trace c) and aristolochic acid (40 µM; trace d) 300 s prior to CaCl\(_2\) inhibited 5 µM gossypol-induced capacitative Ca\(^{2+}\) entry by 28 ± 4, 73 ± 8 and 82 ± 5%, respectively, in the net \([\text{Ca}^{2+}]_i\) value at the time-point of 1100 s.

3.4. Effect of inhibiting phospholipase C or A\(_2\) on gossypol-induced Ca\(^{2+}\) release

The question arose as to how gossypol releases Ca\(^{2+}\) from internal pools. The role of IP\(_3\) in the response was examined. U73122, a phospholipase C inhibitor [23], was used to suppress IP\(_3\) formation. Previous studies have shown that ATP releases internal Ca\(^{2+}\) in MDCK cells in an IP\(_3\)-dependent manner [24]. Fig. 3A, trace a, shows a typical \([\text{Ca}^{2+}]_i\) increase induced by 10 µM ATP. Incubation with U73122 (2 µM) for 210 s induced a small \([\text{Ca}^{2+}]_i\) increase consistent with previous reports [24], but prevented subsequently applied ATP (10 µM) from increasing \([\text{Ca}^{2+}]_i\) (trace b; \(n = 6\); \(P < 0.05\)). This most likely suggested that U73122 had effectively inhibited phospholipase C-dependent IP\(_3\) formation. After U73122 pretreatment for 270 s, application of 5 µM gossypol induced a \([\text{Ca}^{2+}]_i\) increase with an area under the curve (300–450 s) indistinguishable from control (trace b versus trace c, 200 ± 12 versus 195 ± 5 arbitrary units, \(n = 6\); \(P > 0.05\)). U73343, an inactive U73122 analogue, neither altered basal \([\text{Ca}^{2+}]_i\) nor inhibited the \([\text{Ca}^{2+}]_i\) increases induced by ATP and gossypol. Because aristolochic acid was found to alter Ca\(^{2+}\) fluxes in MDCK cells [25],
Fig. 2. (A and B) Internal Ca\(^{2+}\) sources of the gossypol-induced \([\text{Ca}^{2+}]_{i}\) rise. (A) In Ca\(^{2+}\)-free medium, 2 \(\mu\)M CCCP, 1 \(\mu\)M thapsigargin and 5 \(\mu\)M gossypol were added as indicated. (B) In Ca\(^{2+}\)-free medium, 5 \(\mu\)M gossypol, 2 \(\mu\)M CCCP and 1 \(\mu\)M thapsigargin were added as indicated. (C) Gossypol-induced capacitative Ca\(^{2+}\) entry. Trace a: 5 \(\mu\)M gossypol was added at 30 s followed by 3 mM CaCl\(_2\) at 950 s. Traces b-d: similar to trace a except that 25 \(\mu\)M econazole, 50 \(\mu\)M SKF96365 or 40 \(\mu\)M aristolochic acid were added 300 s prior to CaCl\(_2\), respectively. Trace e: control CaCl\(_2\) effect. CaCl\(_2\) was added as shown without pretreatment with other drugs.

Fig. 3. (A) Effect of U73122 on gossypol-induced internal Ca\(^{2+}\) release. In Ca\(^{2+}\)-free medium, trace a: 10 \(\mu\)M ATP was added at 30 s. Trace b: 2 \(\mu\)M U73122, 10 \(\mu\)M ATP and 5 \(\mu\)M gossypol were added as indicated. Trace c: control effect of gossypol. (B) Effect of aristolochic acid on gossypol-induced internal Ca\(^{2+}\) release. Trace a: control effect of gossypol, added at 250 s. Trace b: aristolochic acid (40 \(\mu\)M) was added at 30 s followed by 5 \(\mu\)M gossypol at 250 s. (C) Gossypol-induced \([\text{Ca}^{2+}]_{i}\) rises in human T24 bladder cancer cells and neutrophils.
its effect on gossypol-induced \( \text{Ca}^{2+} \) release was explored. Fig. 3B shows that pretreatment with aristolochic acid (40 \( \mu \text{M} \)) for 250 s inhibited a 5 \( \mu \text{M} \) gossypol-induced \([\text{Ca}^{2+}]\) increase by 55 ± 5% \((n = 5; P < 0.05)\) at the maximum value.

The effect of gossypol on \([\text{Ca}^{2+}]\) in other cells was investigated. Fig. 3C shows that 5 \( \mu \text{M} \) gossypol induced a significant increase in \([\text{Ca}^{2+}]\) in T24 human bladder cancer cells and human neutrophils with a net maximum value of 581 ± 12 and 89 ± 5 nM \((n = 6; P < 0.05)\), respectively.

4. Discussion

Gossypol has been used as a non-steroid antifertility agent in vivo, however the mechanism of its action is unclear. The present study is the first to show that gossypol increased \([\text{Ca}^{2+}]\) in intact cells. Based on the data from \( \text{Ca}^{2+} \)-removal experiments, gossypol appeared to increase \([\text{Ca}^{2+}]\) by both causing \( \text{Ca}^{2+} \) influx and releasing internal \( \text{Ca}^{2+} \), with the former playing a major role.

Gossypol induced internal \( \text{Ca}^{2+} \) release from \( \text{Ca}^{2+} \) stores located in mitochondria, the endoplasmic reticulum and other unidentified pools because this drug still released some internal \( \text{Ca}^{2+} \) after the \( \text{Ca}^{2+} \) stores in the endoplasmic reticulum and mitochondria had been discharged by thapsigargin and CCCP, respectively. Consistently, gossypol was shown to release \( \text{Ca}^{2+} \) from rat liver mitochondria [26]. MDCK cells do not appear to possess ryanodine-sensitive internal \( \text{Ca}^{2+} \) stores because previous reports demonstrated that neither ryanodine nor caffeine increased basal \([\text{Ca}^{2+}]\) [14].

Our findings suggest that gossypol induced external \( \text{Ca}^{2+} \) influx most likely via capacitative \( \text{Ca}^{2+} \) entry, based on the \( \text{Ca}^{2+} \) readmission experiment. This hypothesis was supported by the data that econazole and SKF96365 (two capacitative \( \text{Ca}^{2+} \) entry inhibitors) partly inhibited the gossypol-induced \( \text{Ca}^{2+} \) influx.

It seems that IP\(_3\) did not play a dominant role in gossypol-induced internal \( \text{Ca}^{2+} \) release because the \( \text{Ca}^{2+} \) release was not altered when IP\(_3\) formation was suppressed by U73122. Interestingly, aristolochic acid, a phospholipase A\(_2\) inhibitor, was effective in inhibiting both gossypol-induced \( \text{Ca}^{2+} \) release and capacitative \( \text{Ca}^{2+} \) entry, thus the possibility that phospholipase A\(_2\) might have a regulatory role in gossypol-induced \([\text{Ca}^{2+}]\) increase cannot be excluded.

Gossypol did not increase \([\text{Ca}^{2+}]\) in renal tubular cells alone as it also did so in human bladder cancer cells and neutrophils. Collectively, we have characterized the \([\text{Ca}^{2+}]\) increase induced by gossypol in MDCK cells and have investigated the underlying mechanisms. Because altered \( \text{Ca}^{2+} \) homeostasis changes many cellular functions and prolonged \([\text{Ca}^{2+}]\) increases might cause cytotoxicity, this study addresses an important issue that gossypol may exert its diverse in vivo and in vitro effects through induction of marked \([\text{Ca}^{2+}]\), increases independent of other mechanisms and these effects on \([\text{Ca}^{2+}]\) are often overlooked in the process of data interpretation. Researchers are cautioned to be aware of the existence of such effects.

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