Effect of Gossypol on Intracellular Ca^{2+} Regulation in Human Hepatoma Cells

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Abstract

Gossypol is a natural toxicant present in cottonseeds, and is hepatotoxic to animals and human. The effect of gossypol on cytosolic free Ca^{2+} levels ($[Ca^{2+}]_i$) in HA22/VGH human hepatocytes was explored using fura-2 as a fluorescent Ca^{2+} indicator. Gossypol increased $[Ca^{2+}]_i$ in a concentration-dependent manner with an EC_{50} value of 2 μM. The Ca^{2+} signal was reduced by removing extracellular Ca^{2+} or by 10 μM La^{3+}, but was not affected by nifedipine, verapamil or diltiazem. Pretreatment with 1 μM thapsigargin (an endoplasmic reticulum Ca^{2+} pump inhibitor) to deplete the endoplasmic reticulum Ca^{2+} partly reduced 10 μM gossypol-induced Ca^{2+} release; and conversely pretreatment with gossypol abolished thapsigargin-induced Ca^{2+} release. The Ca^{2+} release induced by 10 μM gossypol was not changed by inhibiting phospholipase C with 2 μM U73122 or by depleting ryanodine-sensitive Ca^{2+} stores with 50 μM ryanodine. Together, the results suggest that in human hepatocytes, gossypol induced a $[Ca^{2+}]_i$ increase by causing store Ca^{2+} release from the endoplasmic reticulum in a phospholipase C-independent manner, and by inducing Ca^{2+} influx.

Key Words: Ca^{2+} signaling, fura-2, gossypol, HA22/VGH cells, hepatocytes

Introduction

Gossypol is a polyphenol isolated from the seed, roots, and stem of the cotton plant (Gossypium sp.). It possesses diverse *in vitro* and *in vivo* effects, such as cytotoxicity against human breast, colon, and other cancer cells (22, 35), blockade of cell-to-cell communication in human and rat cells (15), induction of DNA breaks in rat lymphocytes (31), inhibition of T-type Ca^{2+} currents in mouse spermatogenic cells (1), reversible inhibition of calcineurin (2), a food toxicant for lactating dairy cows and other domestic animals (26), and a contraceptive for men (10), just to name a few. How exactly gossypol exerts so many different effects is unclear.

A rise in intracellular free Ca^{2+} concentration ($[Ca^{2+}]_i$) is a trigger for many physio-pathological events in several cell types (3, 4). However, abnormal $[Ca^{2+}]_i$ rises are cytotoxic (9). $[Ca^{2+}]_i$ is controlled by an intricate interplay of many events. In non-excitable cells, activation of receptors coupled to phospholipase C results in a $[Ca^{2+}]_i$ increase (9). The Ca^{2+} signal is caused by Ca^{2+} influx from extracellular space and/or Ca^{2+} release from intracellular stores. The Ca^{2+} stored in endoplasmic reticulum can be released by an increase in cytosolic levels of inositol 1,4,5-trisphosphate, a second messenger formed by phospholipase C (3-5). Mobilization of store Ca^{2+}

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may result in Ca^{2+} influx *via* store-operated Ca^{2+} entry (30) or other unknown pathways (21). It has been recently shown that gossypol causes significant $[Ca^{2+}]_i$ increases in renal tubular cells (18). Because in animals, gossypol can induce cytotoxicity in multiple organs, especially in liver (12), the present study was perfomed to investigate the effect of gossypol on Ca^{2+} signaling in hepatocytes.

Cultured hepatocytes have been used for evaluating the metabolism of drugs and mechanisms of drug hepatotoxicity (13). Hierarchical organization of Ca^{2+} signals plays a key role in hepatocytes (11), however, abnormal Ca^{2+} homeostasis can cause hepatotoxicity (20). In the present study, by using fura-2 as a fluorescent Ca^{2+} probe, it has been found that gossypol induced $[Ca^{2+}]_i$ increases in populations of HA22/VGH human hepatoma cells. This cell line has been successfully used as a model for evaluating the effect of drugs on Ca^{2+} signaling in human hepatocytes (7). The concentration-response relationship has been established, and the underlying mechanisms of the Ca^{2+} signal, such as the Ca^{2+} influx pathways and Ca^{2+} stores, have been evaluated.

Materials and Methods

Cell Culture

HA22/VGH cells were obtained from American Type Culture Collection and were cultured in Dulbecco's modified Eagle medium plus 10% fetal bovine serum. Cells were kept at 37°C in 5% CO_2-containing humidified air.

Solutions

Ca^{2+}-containing medium (pH 7.4) contained 140 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, 2 mM $CaCl_2$, 10 mM Hepes, and 5 mM glucose. In Ca^{2+}-free medium, Ca^{2+} was substituted with 1 mM EGTA.

$[Ca^{2+}]_i$ Measurements

Trypsinized cells (10^6/ml) were loaded with 2 μM fura-2/AM for 30 min at 25°C in serum-free culture medium. Fura-2 fluorescence measurements were performed in a water-jacketed cuvette (25°C) with continuous stirring; the cuvette contained 1 ml of medium and 5×10^5 cells. Fluorescence was monitored with a Shimadzu RF-5301PC spectrofluorophotometer (Shimadzu Corporation, Kyoto, Japan) by recording excitation signals at 340 nm and 380 nm and emission signal at 510 nm at 1-s intervals. Experiments were started by adding 0.1 ml cell suspension into 0.9 ml well-stirred medium in the cuvette. Reagents were applied during the experiments by pausing the fluorescence recording and adding 10 μl of drug stock solutions into the cuvette. Maximum and minimum fluorescence values were obtained by adding 0.1% Triton X-100 (+ 5 mM $CaCl_2$) and 10 mM EGTA sequentially at the end of each experiment. $[Ca^{2+}]_i$ was calculated as previously described assuming a K_d of 155 nM (6, 8, 14, 17, 19, 23-25).

Mn^{2+} quench of fura-2 fluorescence was performed in Ca^{2+}-containing medium containing 50 μM $MnCl_2$, by recording the excitation signals at 360 nm and emission signal at 510 nm at 1-s intervals.

Chemicals

The reagents for cell culture were from Gibco (Gaithersburg, MD, USA). Fura-2 was from Molecular Probes (Eugene, OR, USA). Gossypol, U73122 (1-(6-((17β-3-methoxyestra-1,3,5(10)-trien-17-yl) amino)hexyl)-1H-pyrrole-2,5-dione) and U73343 (1-(6-((17β-3-methoxyestra-1,3,5(10)-trien-17-yl) amino)hexyl)-2,5-pyrrolidine-dione) were from Biomol (Plymouth Meeting, PA, USA). The other reagents were from Sigma (St. Louis, MO, USA).

Statistics

The data are the mean ± SEM of five separate experiments. Statistical comparisons were made using Student's *t* test, and significance was accepted when $P < 0.05$.

Results

Gossypol increased $[Ca^{2+}]_i$ in a concentration-dependent manner in Ca^{2+}-containing medium. Figure 1A shows the responses induced by 1-10 μM gossypol. At a concentration of 0.1 μM, the agent had no effect (=baseline). Over a time period of 250 s, the $[Ca^{2+}]_i$ signals induced by 1-10 μM gossypol comprised an initial rise and a slow decay phase. The $[Ca^{2+}]_i$ increase induced by 10 μM gossypol expressed an initial rise that reached a net (baseline subtracted) maximum of 330±3 nM after a time lapse of 45±1 s (n=5), and a gradual decay that reached a sustained phase with a net value of 200±3 nM at the time point of 250 s. The Ca^{2+} signal saturated at 20 μM gossypol because the responses induced by 10 and 20 μM of the agent were similar in magnitude and kinetics. Figure 1C (filled circles) shows the concentration-response curve of the gossypol response. The EC_{50} value was estimated approximately at 2 μM.

Figure 1B shows that in Ca^{2+}-free medium, 10 μM gossypol induced a $[Ca^{2+}]_i$ increase by 75±1 nM above baseline (n=5). The concentration-response curve of gossypol-induced $[Ca^{2+}]_i$ increases in Ca^{2+}-free medium is shown in Figure 1C (open circles).

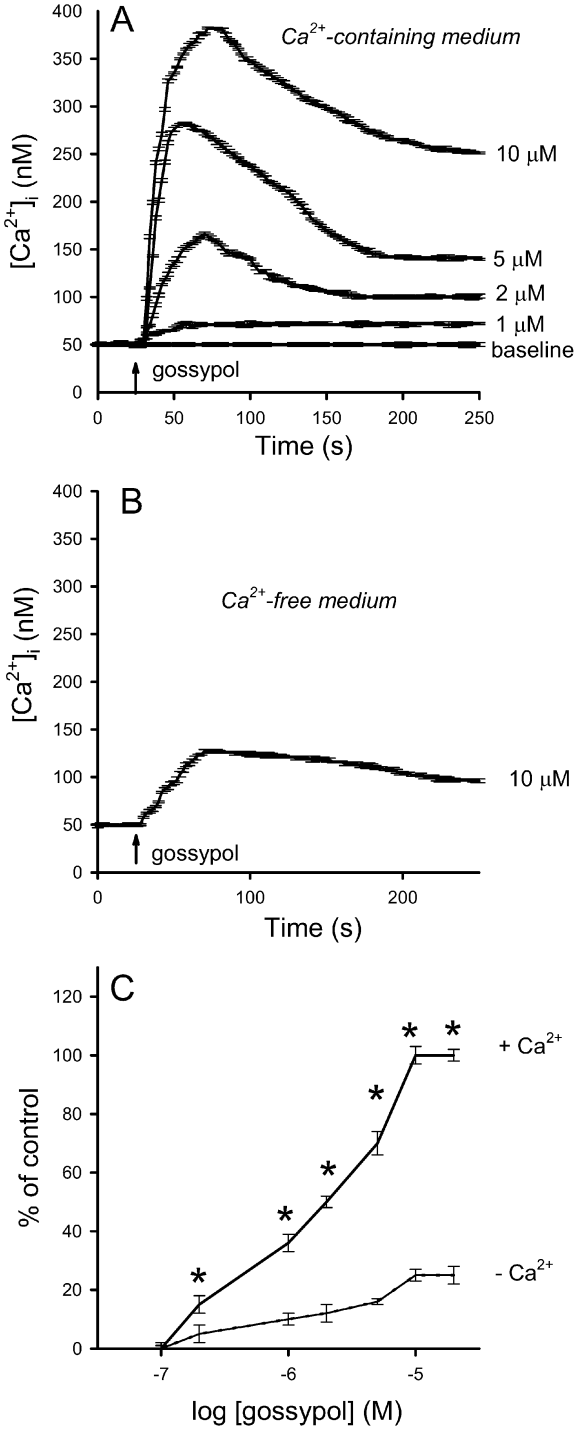

Fig. 1. Effect of gossypol on $[Ca^{2+}]_i$ in HA22/VGH cells. (A) $[Ca^{2+}]_i$ increase induced by gossypol in Ca^{2+}-containing medium. The concentration of gossypol was 1-10 μM. Gossypol was added at 25 s. (B) Effect of removing extracellular Ca^{2+} on gossypol-induced $[Ca^{2+}]_i$ increase. Gossypol (10 μM) was added at 25 s in Ca^{2+}-free medium. (C) Concentration-response curves of gossypol-induced Ca^{2+} signals in the presence and absence of extracellular Ca^{2+}. Y axis is the percentage of control which is the net maximum value (baseline subtracted) of the $[Ca^{2+}]_i$ increase induced by 10 μM gossypol in Ca^{2+}-containing medium. Data are mean ± SEM of five experiments. *$P < 0.05$ compared with open circles.

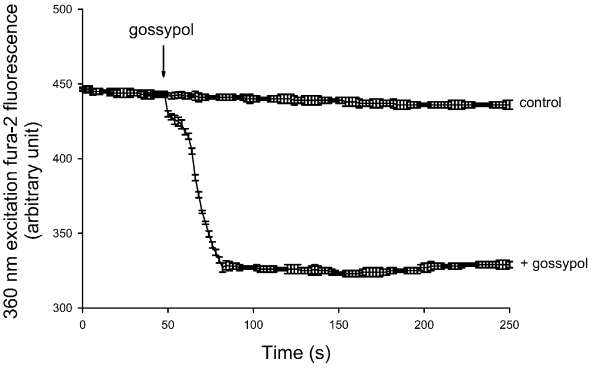

Fig. 2. Effect of gossypol on Ca^{2+} influx by measuring Mn^{2+} quench of fura-2 fluorescence. Experiments were performed in Ca^{2+}-containing medium. $MnCl_2$ (50 μM) was added to cells before fluorescence measurements. Control: in the absence of gossypol. Gossypol (10 μM) was added at 50 s. Data are mean ± SEM of five experiments.

The two curves in Figure 1C suggest that Ca^{2+} influx contributs to 1-20 μM gossypol-induced $[Ca^{2+}]_i$ increases by 80%. In order to confirm that the smaller gossypol-induced Ca^{2+} signal in Ca^{2+}-free medium was not caused by store Ca^{2+} depletion by substitution of Ca^{2+} with 1 mM EGTA, further experiments were performed. Mn^{2+} enters cells through similar pathways as Ca^{2+} but quenches fura-2 fluorescence at all excitation wavelengths (27). Thus, quench of fura-2 fluorescence excited at the Ca^{2+}-insensitive excitation wavelength of 360 nm by Mn^{2+} indicates Ca^{2+} influx. Figure 2 shows that 10 μM gossypol induced an immediate decrease in the 360 nm excitation signal by 119±3 (n = 5) arbitrary units within 30 s, which was significantly lower than control ($P < 0.05$). The gossypol-induced Mn^{2+} influx sustained for at least 200 s.

Figure 3 shows that in Ca^{2+}-containing medium, the $[Ca^{2+}]_i$ increase induced by 10 μM gossypol was inhibited by 53±3% (n = 5; $P < 0.05$) by pretreatment with 10 μM La^{3+}, but was not affected by 10 μM of nifedipine, verapamil, or diltiazem (n = 5; $P > 0.05$).

Experiments were performed to explore whether gossypol releases Ca^{2+} from the endoplasmic reticulum, the major Ca^{2+} stores in non-excitable cells. Figure 4A shows that in Ca^{2+}-free medium, addition of thapsigargin (1 μM), a selective endoplasmic reticulum Ca^{2+} pump inhibitor that increases $[Ca^{2+}]_i$ by passively depleting the endoplasmic reticulum Ca^{2+} store (36), increased $[Ca^{2+}]_i$ by 41±2 nM (n = 5). The Ca^{2+} peak signal was followed by a gradual decay. Subsequently added (at 500 s) gossypol (10 μM) increased $[Ca^{2+}]_i$ by 30±2 nM (n = 5) which was 60% smaller than control (Fig. 4B; 75±1 nM; $P < 0.05$). Conversely, Figure 4B shows that, in Ca^{2+}-free medium, depletion of store

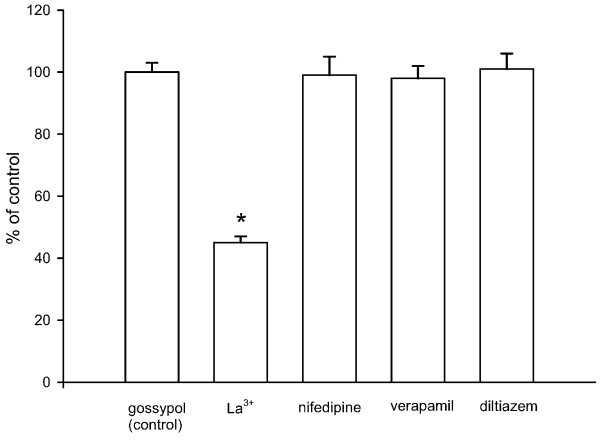

Fig. 3. Effect of Ca^{2+} entry blockers on gossypol-induced $[Ca^{2+}]_i$ increases. La^{3+}, nifedipine, verapamil or diltiazem (all at 10 μM) was added 30 s prior to 10 μM gossypol in Ca^{2+}-containing medium. Data are presented as percentage of control which was the net maximum value of 10 μM gossypol-induced $[Ca^{2+}]_i$ increases in the absence of blockers, and are the mean ± SEM of five experiments. *$P < 0.05$ compared with control.

Ca^{2+} with 10 μM gossypol for 8 min prevented thapsigargin (1 μM) from releasing more Ca^{2+}. The role of another Ca^{2+} source, the ryanodine-sensitive store, in gossypol-induced Ca^{2+} release was explored. Figure 4C shows that addition of 50 μM ryanodine increased $[Ca^{2+}]_i$ by 24±2 nM (n = 5) above baseline. After pretreatment with ryanodine for 8 min, addition of gossypol (10 μM) increased $[Ca^{2+}]_i$ by 73±1 nM (n = 5), which was comparable to the control gossypol response (Fig. 4B).

Because gossypol mainly releases Ca^{2+} from thapsigargin-sensitive stores, the role of inositol 1,4,5-trisphosphate in this release was examined. U73122, a phospholipase C inhibitor (37), was applied to investigate whether inositol 1,4,5-trisphosphate was responsible for gossypol-induced Ca^{2+} release. Histamine has been shown to release Ca^{2+} in HA22/VGH cells via inositol 1,4,5-trisphosphate (7). Figure 5A shows that, in Ca^{2+}-free medium, histamine (10 μM) rapidly increased $[Ca^{2+}]_i$ by 150±2 nM (n = 5) above baseline, and the Ca^{2+} signal returned to a level close to baseline within 3 min. Figure 5B shows that histamine failed to increase $[Ca^{2+}]_i$ after cells were incubated with 2 μM U73122 for 3 min (n = 5). U73122 did not alter basal $[Ca^{2+}]_i$. In contrast, U73343, a biologically inactive analogue of U73122, did not affect histamine-induced $[Ca^{2+}]_i$ increases (n = 5; not shown). This suggests that U73122 effectively suppressed phospholipase C activity. Figure 5B further shows that addition of gossypol (10 μM) afterwards caused a $[Ca^{2+}]_i$ increase indistinguishable from the control gossypol response shown in Figure 4B (n = 5).

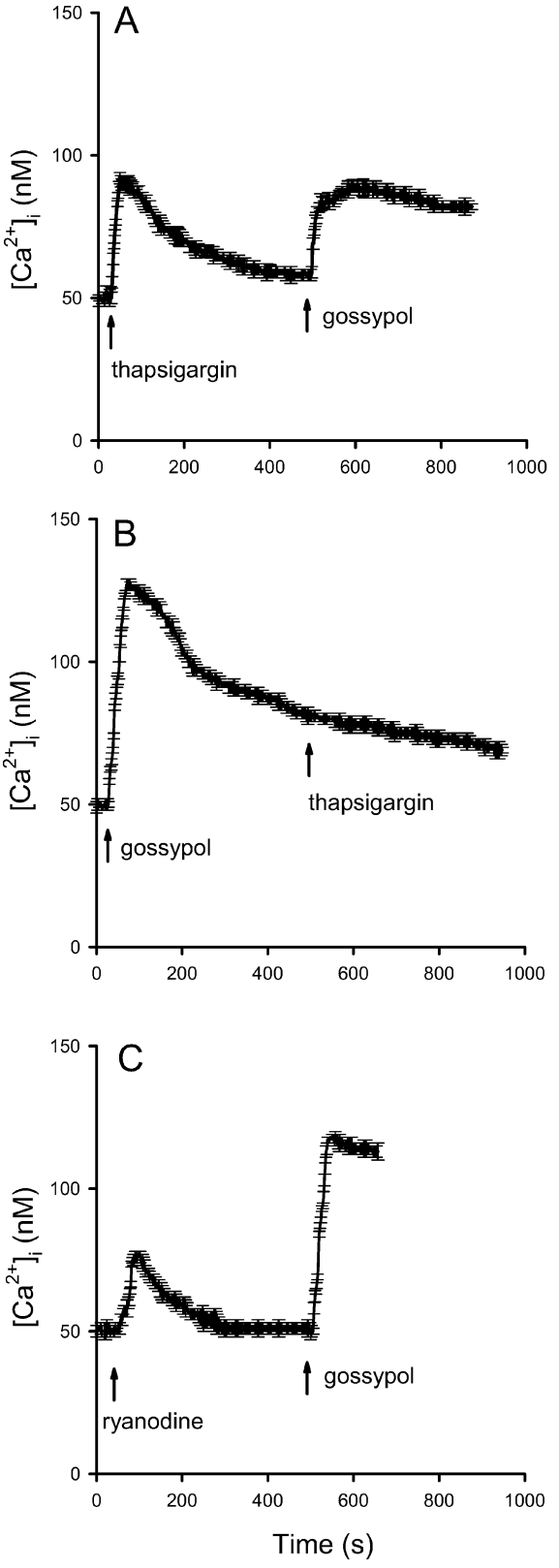

Fig. 4. Intracellular Ca^{2+} stores of gossypol-induced Ca^{2+} release. (A)-(C) In Ca^{2+}-free medium, thapsigargin (1 μM), gossypol (10 μM) or ryanodine (50 μM) was added at the time indicated by arrows. Data are mean ± SEM of five experiments.

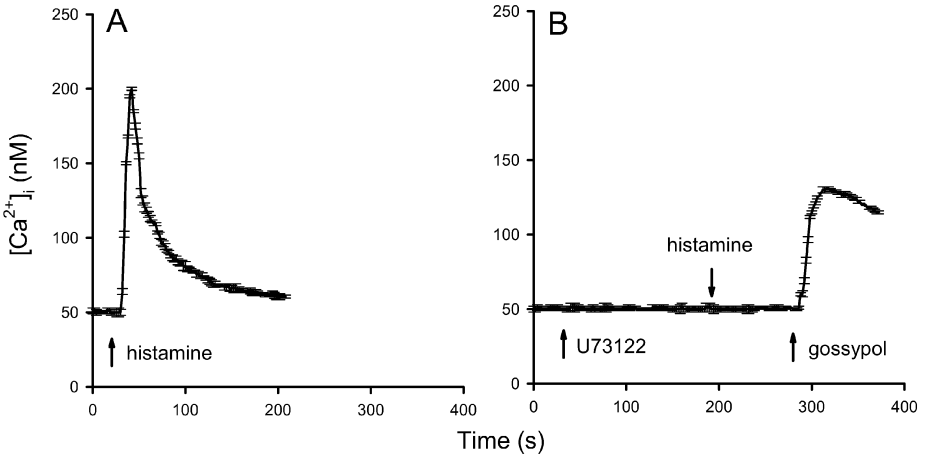

Fig. 5. Effect of inhibiting phospholipase C activity on gossypol-induced $[Ca^{2+}]_i$ increase. In Ca^{2+}-free medium, (A) 10 μM histamine was added at 15 s; (B) 2 μM U73122, 10 μM histamine and 10 μM gossypol were added at the time indicated by arrows. Data are mean ± SEM of five experiments.

Discussion

In hepatocytes, a $[Ca^{2+}]_i$ increase can regulate many cell functions such as activatiton of volume-sensitive SK(Ca^{2+}) channels (34), glucose release, bile flow (39), ATP secretion (29), and recovery from swelling (33). In this study it is shown that in human hepatoma cells, the natural toxicant gossypol induces significant $[Ca^{2+}]_i$ increases in a concentration-dependent manner at concentrations above 1 μM. Similar effective concentration ranges of gossypol were found in a canine renal tubular cell line (18). In human receiving chronic administatrtion of gossypol as a contraceptive, the plasma concentration of gossypol may attain to 3 μM (10). The actual level of gossypol in liver may be much higher because evidence shows that in broilers fed with cottonseed meals, the concentration of gossypol in plasma and liver may reach 26 μM and 600 μM, respectively (12). Thus the possible effect of gossypol on Ca^{2+} signaling in different organs *in vivo* should be considered in the evaluation of the pharmacology of gossypol. The gossypol-induced $[Ca^{2+}]_i$ increase sustained for several min. Because prolonged elevations in $[Ca^{2+}]_i$ is cytotoxic (3-5), gossypol may have a regulatory or cytotoxic effect *in vivo*, depending on its concentrations and the cell types.

The Ca^{2+} signal induced by gossypol results from store Ca^{2+} release and extracellular Ca^{2+} influx because: 1] removing extracellular Ca^{2+} inhibited 80% of the signal; and 2] gossypol induced Mn^{2+} quench of cytosolic fura-2 fluorescence. The exact mechanism underlying gossypol-induced Ca^{2+} influx is unclear, but the influx is sensitive to the non-selective Ca^{2+} entry blocker La^{3+}, and is insensitive to L-type Ca^{2+} entry blockers, although the effectiveness of dihydropyrines in blocking Ca^{2+} influx has been reported previously in rat hepatocytes (28, 32).

In hepatocytes, several Ca^{2+} stores have been thought to contribute to a Ca^{2+} releasing event including inositol 1,4,5-trisphosphate-sensitive stores, ryanodine-sensitive stores, nucli, etc. (16). A Ca^{2+}-induced Ca^{2+} release mechanism mediated by neither inositol 1,4,5-trisphosphate nor ryanodine receptors has also been proposed (38). Gossypol appears to release Ca^{2+} mainly from the thapsigargin-sensitive endoplasmic reticulum store, because pretreatment with gossypol abolished thapsigargin-induced Ca^{2+} release. This argument is supported by the finding that depletion of ryanodine-sensitive stores did not effectively reduce gossypol-induced Ca^{2+} release. It has been shown that inositol 1,4,5-trisphosphate receptors can trigger Ca^{2+} waves in hepatocytes (38). The mechanism underlying gossypol-induced Ca^{2+} release from the endoplasmic reticulum is unknown, but it appears that a preceding increase in the cytosolic inositol 1,4,5-trisphosphate level is unnecessary because gossypol induced normal Ca^{2+} release when phospholipase C was inhibited. Collectively, this study has demonstrated that gosssypol causes significant $[Ca^{2+}]_i$ increases in human hepatocytes. These results may contribute to the understanding of the *in vivo* action of this natural compound.

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