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The Carcinogen Safrole Increases Intracellular Free Ca^{2+} Levels and Causes Death in MDCK Cells

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Abstract

The effect of the carcinogen safrole on intracellular Ca^{2+} movement in renal tubular cells has not been explored previously. The present study examined whether safrole could alter Ca^{2+} handling in Madin-Darby canine kidney (MDCK) cells. Cytosolic free Ca^{2+} levels ($[Ca^{2+}]_i$) in populations of cells were measured using fura-2 as a fluorescent Ca^{2+} probe. Safrole at concentrations above 33 μM increased $[Ca^{2+}]_i$ in a concentration-dependent manner with an EC_{50} value of 400 μM. The Ca^{2+} signal was reduced by 90% by removing extracellular Ca^{2+}, but was not affected by nifedipine, verapamil, or diltiazem. Addition of Ca^{2+} after safrole had depleted intracellular Ca^{2+}-induced dramatic Ca^{2+} influx, suggesting that safrole caused store-operated Ca^{2+} entry. In Ca^{2+}-free medium, after pretreatment with 650 μM safrole, 1 μM thapsigargin (an endoplasmic reticulum Ca^{2+} pump inhibitor) failed to release more Ca^{2+}. Inhibition of phospholipase C with 2 μM U73122 did not affect safrole-induced Ca^{2+} release. Trypan blue exclusion assays revealed that incubation with 650 μM safrole for 30 min did not kill cells, but killed 70% of cells after incubation for 60 min. Collectively, the data suggest that in MDCK cells, safrole induced a $[Ca^{2+}]_i$ increase by causing Ca^{2+} release from the endoplasmic reticulum in a phospholipase C-independent fashion, and by inducing Ca^{2+} influx *via* store-operated Ca^{2+} entry. Furthermore, safrole can cause acute toxicity to MDCK cells.

Key Words: Ca^{2+}, Ca^{2+} stores, fura-2, MDCK, renal tubular cells, safrole

Introduction

Safrole, a rodent hepatocarcinogen, is an essential oil that has been commonly used in cosmetics and food flavoring (30). Safrole is also the main component of Piper betel inflorescence, which is frequently included in the Taiwanese betel quid (TBQ) for its aromatic flavor. More than two million people are thought to be

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in the habit of chewing TBQ (4). Fresh Piper betel inflorescence contains a high concentration of safrole (15.35 mg/g) (5). The salivary safrole concentration of TBQ chewers was as high as 420 μM (70 μg/ml) (37). The carcinogenicity of safrole is usually thought to be caused by safrole-DNA adducts formation (5, 7, 25). After safrole exposure, safrole-DNA adducts have been found in many tissues in animal models and humans (6, 8). The other important proposed mechanism of safrole in cell injury is to cause intracellular ion shifts. Trump *et al*. (35) proposed that the safrole-induced changes in intracellular free Ca^{2+} levels ($[Ca^{2+}]_i$) of mouse hepatocellular carcinoma cells are related to further ion shifts even cell death. Recently it has been shown that safrole could increase $[Ca^{2+}]_i$ and enhance proliferation in an independent manner in human oral cancer cells (13). The effect of safrole on $[Ca^{2+}]_i$ is unclear in other cell types. A more precise investigation of the effect of safrole on $[Ca^{2+}]_i$ using a Ca^{2+}-sensitive fluorescent dye is necessary in renal tubular cells.

The kidney is one of the many DNA binding targets for safrole. In pregnant mice, Lu *et al*. (26) reported high binding of safrole to kidney DNA. It is generally believed that safrole is excreted entirely *via* urine, but it is interesting to note that the metabolic disposition of safrole is dose-dependent (2). In rats and humans, it was shown that a single low dose of safrole is rapidly absorbed, metabolized and excreted in urine winthin 24 h; however, in rats, when the dose was increased to levels commonly used in chronic toxictiy or carcinogenicity studies (10), the pharmacokinetics of the compound was profoundly altered. Only 25% of the dose was excreted in the urine in 24 h and plasma/tissue concentrations of both unchanged safrole and its metabolites remained elevated for 48 h (12). This accumulation may be associated with the known toxic effects reported during chronic toxicity studies in animals and other unknown toxic effects (15).

Many studies indicate that nephrotoxicity is associated with Ca^{2+}-dependent processes (1, 9, 31). In this study, by using fura-2 as a Ca^{2+} probe it was demonstrated for the first time that safrole altered Ca^{2+} signaling in Madin-Darby canine kidney cells in a multiple manner. Previous reports show that in this non-excitable renal tubular cell line, inositol 1,4,5-trisphosphate-dependent agnoists such as ATP (22) and bradykinin (20) increase $[Ca^{2+}]_i$ by depleting Ca^{2+} from the inositol 1,4,5-trisphosphate-sensitive endoplasmic reticulum Ca^{2+} store followed by store-operated Ca^{2+} entry (32). Furthermore, thapsigargin, an endoplasmic reticulum Ca^{2+} pump inhibitor (33), increases $[Ca^{2+}]_i$ by releasing Ca^{2+} from the endoplasmic reticulum in an inositol 1,4,5-trisphosphate-independent manner (21). Additionally, many other reagents from different categories have been shown to increase $[Ca^{2+}]_i$ in these cells (16-19). Thus, Madin-Darby canine kidney cells were used in the present study as a model for investigating the effect of safrole on Ca^{2+} signaling and cytotoxicity in renal tubular cells.

Materials and Methods

Cell Culture

MDCK cells obtained from American Type Culture Collection (CRL-6253) were cultured in Dulbecco's modified Eagle medium supplemented with 10% heat-inactivated fetal bovine serum 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, 5 mM glucose. In Ca^{2+}-free medium, Ca^{2+} was substituted with 0.1 mM EGTA. Safrole was dissolved in ethanol as a 6.5 M stock solution which was stored at -20°C, and was diluted to the final concentrations before experiments. The other reagents were dissolved in water (ATP), ethanol (U73122, U73343, Ca^{2+} entry blockers) or dimethyl sulfoxide (thapsigargin). The concentaration of organic solvents in the solution used in experiments did not exceed 0.1%, and did not alter basal $[Ca^{2+}]_i$ (n = 3).

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

Trypsinized cells (10^6/ml) were loaded with 2 μM of the acetoxymethyl ester form of fura-2 (fura-2/AM), for 30 min at 25°C in 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 0.5 million cells. Fluorescence was monitored with a Shimadzu RF-5301PC spectrofluorophotometer by recording excitation signals at 340 nm and 380 nm and emission signal at 510 nm at 1-sec intervals. Maximum and minimum fluorescence values were obtained by adding 0.1% Triton X-100 (plus 5 mM $CaCl_2$) and 10 mM EGTA sequentially at the end of each experiment. $[Ca^{2+}]_i$ was calculated as previously described (11).

Acute Cytotoxicity Assays

Fifty μl of cell suspension in Ca^{2+}-containing medium was mixed with 50 μl of trypan blue isotonic solution (0.2%; w/v) and cell viability was determined on a hemocytometer under a microscope after cells were incubated with different concentrations of safrole for several time periods. The cell density in the assay solution was 0.5 million/ml.

Chemicals

The reagents for cell culture were from Gibco. Fura-2/AM was from Molecular Probes. 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. Safrole and other reagents were from Sigma.

Statistics

Data are reported as means ± SEM of five experiments. Data were analyzed by two-way analysis of variances (ANOVA) using the Statistical Analysis System (SAS®, SAS Institute Inc., Cary, NC, USA) on a personal computer powered by Intel Pentium IV CPU at 1.8 GHz. Multiple comparisons between group means were performed by *post-hoc* analysis using the Tukey's HSD (honestly significant difference) procedure. A *P*-value less than 0.05 was considered significant.

Results

Effect of Safrole on $[Ca^{2+}]_i$ in MDCK Cells

Safrole at concentrations above 33 μM increased $[Ca^{2+}]_i$ in a concentration-dependent manner in Ca^{2+}-containing medium. Figure 1A shows the responses induced by 33-650 μM safrole. At a concentration of 10 μM, the agent had no effect (= baseline; not shown). The $[Ca^{2+}]_i$ increase induced by 650 μM safrole expressed a rapid initial rise that reached a net (baseline subtracted) maximum of 351 ± 3 nM after a time lapse of 30 ± 1 sec (n = 5), and a gradual decay that reached a sustained phase of 201 ± 2 nM at the time point of 250 sec. The Ca^{2+} signal saturated at 650 μM safrole because 1000 μM safrole induced a similar response. The $[Ca^{2+}]_i$ rise induced by 33-325 μM safrole was composed of a slow initial increase and a sustained phase lacking a decay phase. Figure 1C (filled circles) shows the concentration-response curve of the safrole response. The curve suggests an EC_{50} value of 400 μM.

Effect of Removing Extracellular Ca^{2+} on the Safrole

Response Further experiments were performed to determine the relative contribution of extracellular Ca^{2+} influx and intracellular Ca^{2+} release in safrole-induced $[Ca^{2+}]_i$ increases. The $[Ca^{2+}]_i$ increases induced by 325 and 650 μM safrole in Ca^{2+}-free medium are shown in Figure 1B (time points between 0-250 sec). Removal of extracellular Ca^{2+} did not alter baseline, suggesting that extracellular fura-2 was negligible in the experiments performed in Ca^{2+}-containing medium. Safrole increased $[Ca^{2+}]_i$ by 22 ± 1 nM and 49 ± 2 nM above baseline at the concentrations of 325 μM and 650 μM, respectively (n = 5). The concentration-response curve of safrole-induced $[Ca^{2+}]_i$ increases in Ca^{2+}-free medium is shown in Figure 1C (open circles). The two curves in Figure 1C suggest that Ca^{2+} influx contributed to 33-650 μM safrole-induced $[Ca^{2+}]_i$ increases by more than 50%. Figure 1B also shows that addition of 3 mM Ca^{2+} after 325 μM and 650 μM safrole treatment induced an immediate $[Ca^{2+}]_i$ increase in a manner dependent on the concentration of safrole. The Ca^{2+}-induced $[Ca^{2+}]_i$ increase was 102 ± 2 nM and 55 ± 3 nM (n = 5) after 325 μM and 650 μM safrole treatment, respectively. Addition of Ca^{2+} without safrole treatment induced a $[Ca^{2+}]_i$ increase of 25 ± 1 nM (n = 5). This suggests that safrole caused the opening of cell surface Ca^{2+} channels.

Lack of Effect of Ca^{2+} Entry Blockers on Safrole-Induced $[Ca^{2+}]_i$ Increase

In Ca^{2+}-containing medium, 650 μM safrole-induced $[Ca^{2+}]_i$ increase was not affected by pretreatment with 1 μM of nifedipine, verapamil, or diltiazem (n = 5; $P > 0.05$; not shown).

Intracellular Sources of Safrole-Induced Ca^{2+} Release

Efforts were made to explore whether safrole released Ca^{2+} from the endoplasmic reticulum, the major Ca^{2+} stores in MDCK cells. Figure 2A shows that in Ca^{2+}-free medium, after addition of 650 μM safrole for 370 sec, 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 (33), failed to increase $[Ca^{2+}]_i$ (n = 5). In contrast, Figure 2B shows that thapsigargin induced a $[Ca^{2+}]_i$ increase by 45 ± 3 nM (n = 5) followed by a gradual decay that returned to baseline. Addition of 650 μM safrole at 400 sec did not release more stored Ca^{2+} (n = 5).

Lack of Effect of Inhibiting Phospholipase C on Safrole Response

Because safrole was able to deplete Ca^{2+} in thapsigargin-sensitive endoplasmic reticulum stores, the role of inositol 1,4,5-trisphosphate in this release was examined. U73122, a phospholipase C inhibitor (34), was applied to see whether inositol 1,4,5-trisphosphate was required for safrole-induced Ca^{2+} release. Figure 3B shows that, in Ca^{2+}-free medium, incubation with 2 μM U73122 did not alter basal $[Ca^{2+}]_i$ but abolished ATP (10 μM; an inositol-1,4,5-trisphosphate-dependent purinergic receptor agonist)-induced $[Ca^{2+}]_i$ increase. Conversely, Figure 3A shows

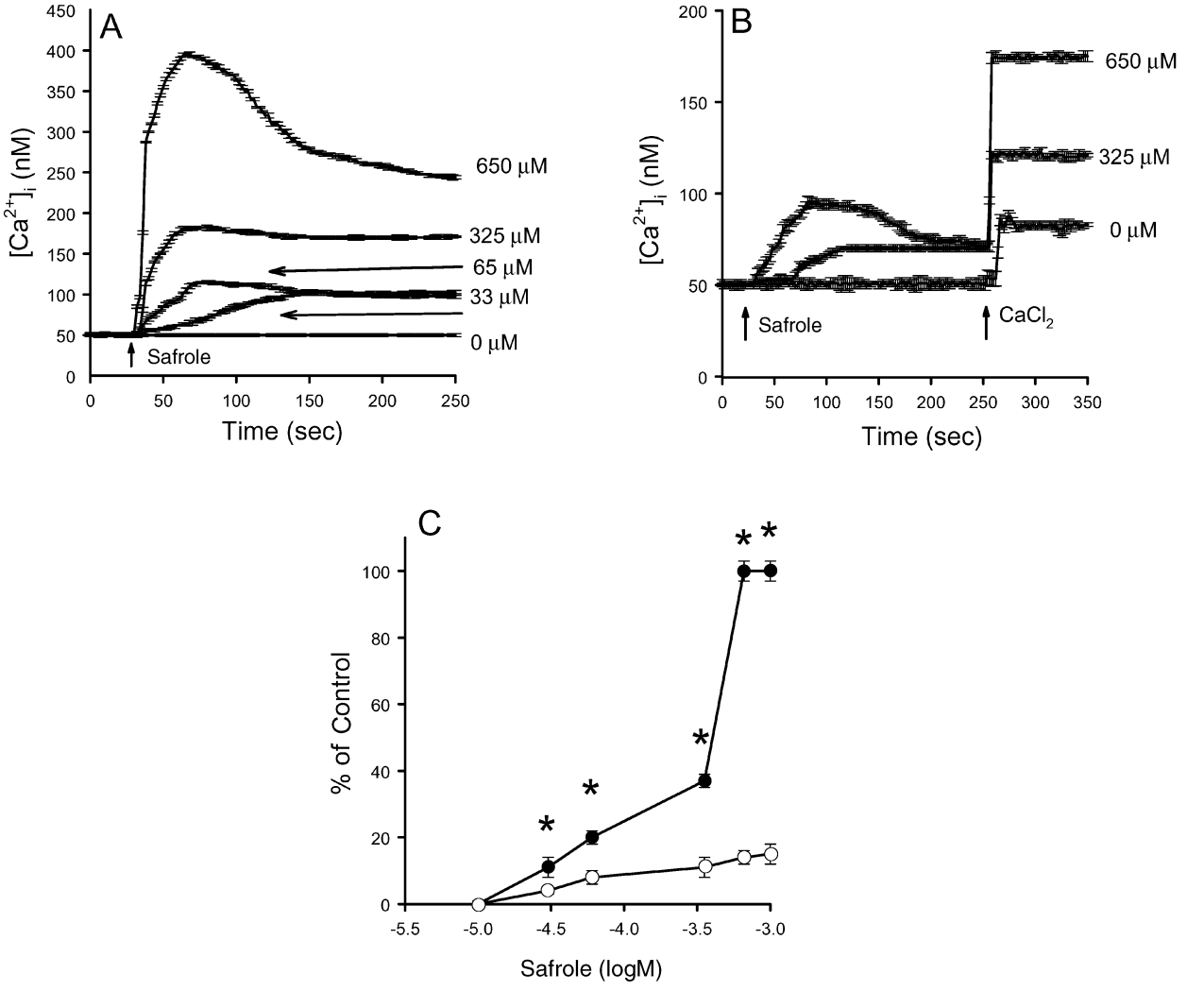

Fig. 1. (A) Effect of safrole on $[Ca^{2+}]_i$ in fura-2-loaded MDCK cells. Safrole was added at 30 sec. The concentration of safrole was indicated. (B) Effect of extracellular Ca^{2+} removal on safrole-induced $[Ca^{2+}]_i$ increases and effect of readdition of Ca^{2+}. The experiments were performed in Ca^{2+}-free medium. Safrole was added at 25 sec. The concentrations of safrole were 0, 325 and 650 μM, respectively. $CaCl_2$ (3 mM) was added at 250 sec. (C) Concentration-response plots of safrole-induced Ca^{2+} signals in the presence (solid circles) and absence (open circles) of extracellular Ca^{2+}. Y axis is the percentage of control which is the net (baseline subtracted) maximum $[Ca^{2+}]_i$ response induced by 650 μM safrole in Ca^{2+}-containing medium. Data are mean ± SEM of five experiments. *$P < 0.05$ by two-way ANOVA .

that 10 μM ATP induced a $[Ca^{2+}]_i$ increase of 67 ± 2 nM (n = 5). U73343, a biologically inactive analogue of U73122, did not affect basal or ATP-induced $[Ca^{2+}]_i$ increase (n = 5; not shown). This suggests that U73122 effectively suppressed phospholipase C activity. Figure 3B shows that addition of safrole (650 μM) after U73122 and ATP treatment caused a $[Ca^{2+}]_i$ increase indistinguishable from the control safrole response shown in Figure 2A (n = 5; $P > 0.05$).

Effect of Safrole on the Viability of MDCK Cells

Given that acute incubation with safrole induced substantial $[Ca^{2+}]_i$ increases, and that unregulated $[Ca^{2+}]_i$ increases often lead to cytotoxicity (16), trypan blue exclusion assays were performed to examine the effect of safrole on cell viability. Figure 4 shows that control cell viability was approximately 88 ± 2% (n = 5). Incubation with 325 μM or 650 μM safrole for 30 min did not decrease the viability (n = 5; $P > 0.05$). However, incubation with 650 μM safrole for 60 min decreased cell viability by 63 ± 3% of control (n = 5; $P < 0.05$). In contrast, incubation with 325 μM safrole for 60 min did not affect cell viability (n = 5; $P > 0.05$).

Discussion

Safrole is commonly used as a tool to induce

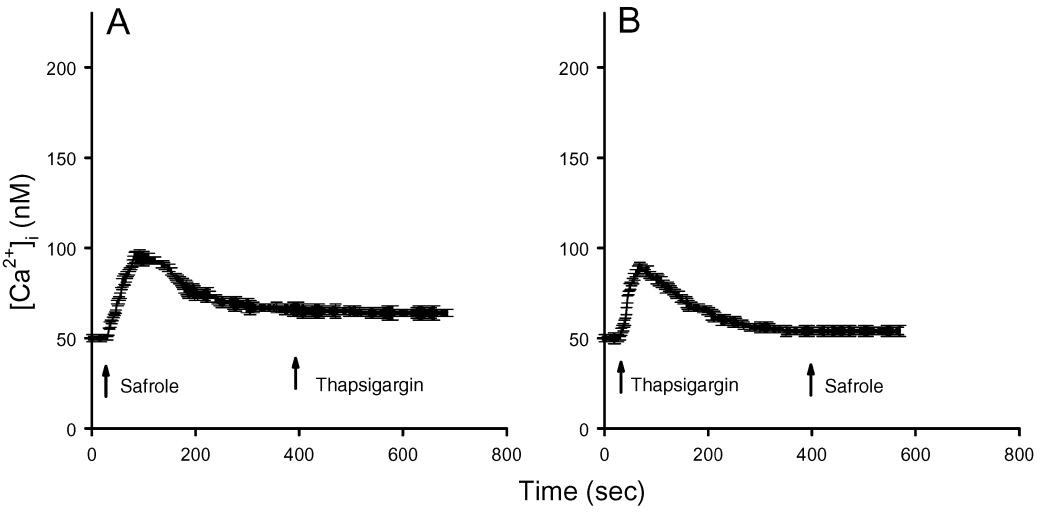

Fig. 2. Intracellular Ca^{2+} stores of safrole-induced Ca^{2+} release. Experiments were performed in Ca^{2+}-free medium. (A) Safrole (650 μM) was added at 30 sec followed by thapsigargin (1 μM) added at 400 sec. (B) Thapsigargin (1 μM) was added at 30 sec followed by safrole (650 μM) added at 400 sec. Data are mean ±SEM of five experiments.

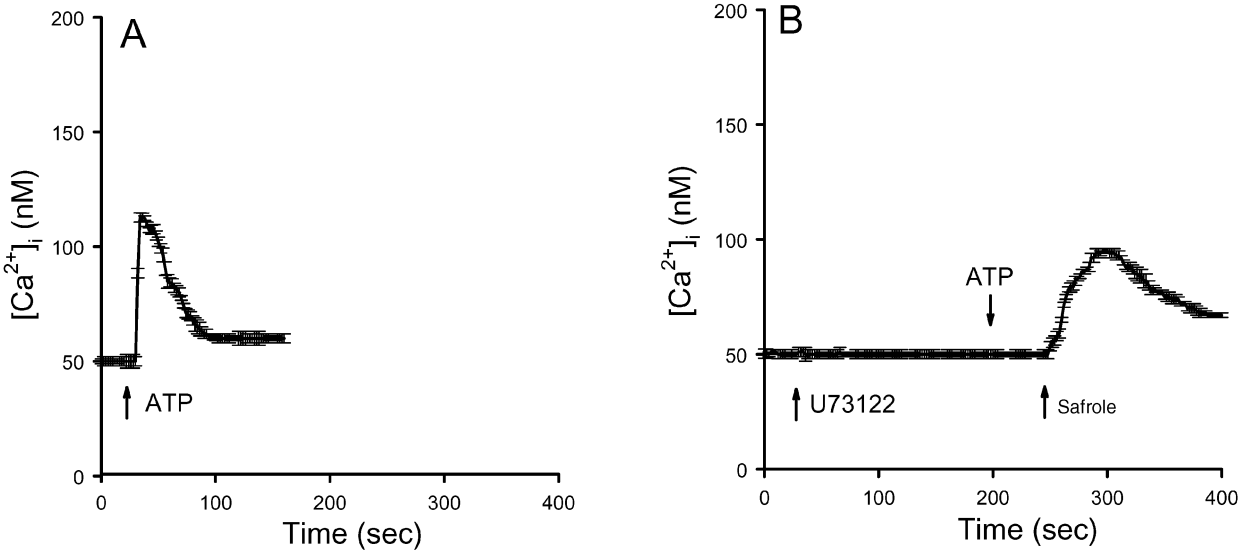

Fig. 3. Lack of effect of inhibition of phospholipase C on safrole-induced Ca^{2+} release. Experiments were performed in Ca^{2+}-free medium. (A) ATP (10 μM) was added at 25 sec. (B) U73122 (2 μM), ATP (10 μM), and safrole (650 μM) were added at time points indicated by arrows. Data are mean ± SEM of five experiments.

tumors in various experimental settings (27). Studies on safrole are of special geological interest in Taiwan. Many people die of oral cancer (23, 24) because of chewing betel quid in which safrole is a major carcinogen. Although the molecular mechanisms that underlie the safrole's carcinogenic action has been widely investigated (3, 28), the cytotoxicity and malignant impact of this compound on signal transduction are largely unclear. Although safrole is known to be entirely excreted via urine (26), its carcinogernic and cytotoxic effect on renal tubular cells is unknown.

Our study is the first to demonstrate that safrole increases $[Ca^{2+}]_i$ in renal tubular cells. The data suggest that safrole increased $[Ca^{2+}]_i$ by releasing stored Ca^{2+} and causing Ca^{2+} influx because removing extracellular Ca^{2+} partly reduced safrole-induced $[Ca^{2+}]_i$ increase. Furthermore, adding back Ca^{2+} after safrole treatment in Ca^{2+}-free medium evoked immediate Ca^{2+} influx, suggesting that safrole could open plasma membrane Ca^{2+} channels. Our findings suggest that safrole mainly released Ca^{2+} from thapsigargin-sensitive endoplasmic reticulum store, a dominant Ca^{2+} store in MDCK cells as demonstrated

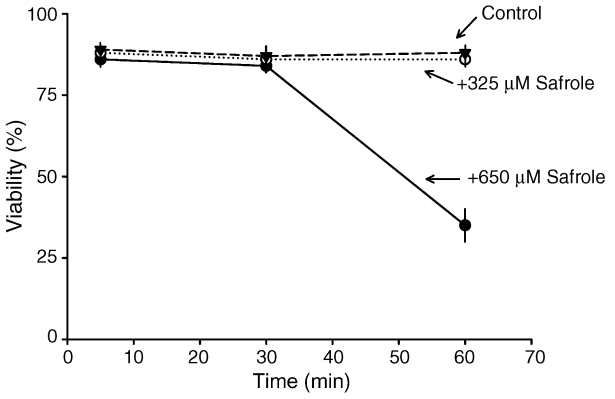

Fig. 4. Trypan blue exclusion assays. The concentration of safrole was 0 (control), 325 μM and 650 μM in the three groups. Data are mean ± SEM of five experiments. *P < 0.05 by two-way ANOVA. The effect of the vehicle ethanol was examined and was found not to alter control values (not shown).

previously (23).

It seems that phospholipase C did not participate in safrole-induced Ca^{2+} release, since safrole-induced Ca^{2+} release remained the same when phospholipase C was suppressed. Because ryanodine-sensitive Ca^{2+} stores are absent in MDCK cells (20), how exactly safrole releases Ca^{2+} remains unknown. Safrole may release Ca^{2+} by inhibiting the endoplasmic reticulum Ca^{2+} pump like thapsigargin does. The pathway underlying safrole-induced Ca^{2+} influx is unclear except that it was insensitive to voltage-gated Ca^{2+} entry blockers. It has previously been shown that L-type Ca^{2+} channel blockers could block Ca^{2+} entry induced by some agents such as PAF (38) in MDCK cells. The data that addition of Ca^{2+} after safrole's depletion of Ca^{2+} stores in Ca^{2+}-free medium induced an immediate Ca^{2+} influx implicate that safrole may cause Ca^{2+} influx *via* the pathway of store-operated Ca^{2+} entry, a Ca^{2+} refilling mechanism that is turned on by depletion of stored Ca^{2+} (21), or it may just directly open some Ca^{2+} channels on plasma membranes.

Our data suggest that safrole becomes cytotoxic at rather low concentrations (650 μM). Safrole decreased antimicrobial activity in human neutrophils at high concentrations (5-10 mM) but didn't show cytotoxicity (14). Uhl *et al*. (36) demonstrate that safrole causes a significant increase in DNA migration in human hepatoblastoma cells after 24 h treatment at a concentration of 4 mM. Liu *et al*. (25) show that safrole (500-1000 mg/kg body weight) induced a dose-dependent increase in hepatic lipid hydroperoxides and 8-hydroxy-2'-deoxyguanosine levels in rat. These results indicate that safrole exerts its toxic effects on different tissues at a wide concentration range. Interestingly, in human oral cancer cells, safrole has been shown to induce $[Ca^{2+}]_i$ rises and 50% increase in cell proliferation in an independent manner. However, because of habitual, long-term exposure to safrole, betal quid chewers could be more susceptible to renal damage. Collectively, safrole's *in vitro* $[Ca^{2+}]_i$ elevating and cytotoxic effect may be physiologically significant in people who comsume large amounts of betal quids daily. In rats, it has been shown that betal quids contain components that can induce tumors of the lung, liver, nasal cavity, forestomach and kidneys (29).

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