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# Novel action of lignans isolated from *Hernandia nymphaeifolia* on $Ca^{2+}$ signaling in renal tubular cells

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**Abstract**

The effect of five lignans, epi-aschantin, epi-magnolin, epi-yangambin, deoxypodophyllotoxin and yatein, isolated from *Hernandia nymphaeifolia* on $Ca^{2+}$ signaling in Madin–Darby canine kidney cells was examined using fura-2 as a $Ca^{2+}$ indicator. These lignans at concentrations between 10 and 100 μM increased $[Ca^{2+}]_i$ in a concentration-dependent manner. Removal of extracellular $Ca^{2+}$ abolished the $Ca^{2+}$ signals evoked by 50 μM of the lignans. $La^{3+}$ (50 μM) abolished the $Ca^{2+}$ signals induced by 100 μM of epi-aschantin, epi-magnolin and epi-yangambin, and 20 μM deoxypodophyllotoxin, but inhibited by 60% 50 μM yatein-induced responses. All five lignans (50–100 μM) inhibited by 42–65% thapsigargin-induced capacitative $Ca^{2+}$ entry, and inhibited by 23–61% thapsigargin-induced intracellular $Ca^{2+}$ release. Epi-yangambin (100 μM), epi-magnolin (100 μM), and epi-aschantin (100 μM) inhibited by 8–38% 10 μM ATP-induced $Ca^{2+}$ release. Trypan blue exclusion revealed that incubation with deoxypodophyllotoxin or yatein (but not the other lignans) decreased cell viability in a concentration-dependent manner. Together, the results suggest that, in renal tubular cells, these lignans exert multiple actions on $Ca^{2+}$ signaling. They caused $Ca^{2+}$ influx but reduced thapsigargin-induced capacitative $Ca^{2+}$ entry and also thapsigargin- and ATP-induced $Ca^{2+}$ release. Additionally, deoxypodophyllotoxin and yatein may be cytotoxic. 

*Keywords:* Lignan; $Ca^{2+}$; $Ca^{2+}$ store; Fura-2; MDCK cell; Thapsigargin

## 1. Introduction

Lignans are thought to be biomarkers of a 'healthy' diet (Adlercreutz, 1998). They have been shown to be toxic to cancer cells and to possess antioxidant, antiestrogenic, and antimitotic properties, making them strong candidates for natural chemo-preventive compounds (Adlercreutz et al., 1995). Lignans can be found in whole grain cereals, seeds, berries and nuts (Adlercreutz, 1998); however, they express great variability in chemical structure, which may explain the differences in their pharmacological profiles.

*Hernandia nymphaeifolia* (Presl.) Kubitzki is an evergreen tree of the Hernandiaceae family (Yang and Lu, 1996). Its seed is used as a cathartic (Kan, 1970). Plants of this genus are rich in lignans (Rahmani et al., 1995; Ito et al., 1992). Five lignans have been isolated [epi-aschantin, epi-magnolin, epi-yangambin, deoxypodophyllotoxin and yatein] from the bark of this plant (Fig. 1) and have strong anti-platelet aggregation activity in vitro. These lignans inhibited platelet aggregation factor-induced platelet aggregation at doses between 10 and 100 μg $ml^{-1}$. Furthermore, deoxypodophyllotoxin and yatein nearly abolish collagen-induced platelet aggregation at a dose of 100 μg $ml^{-1}$

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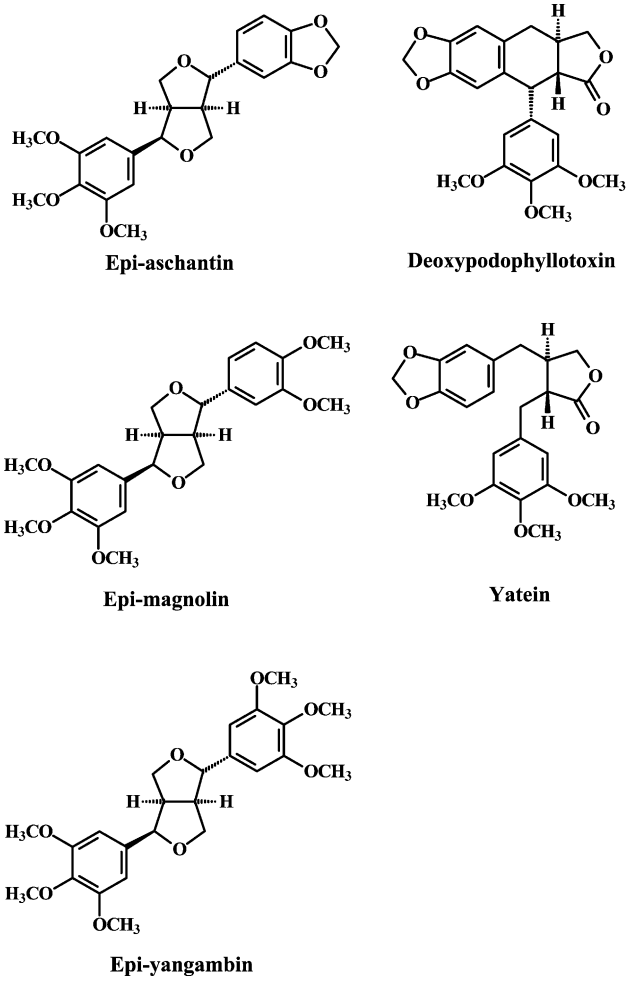


Fig. 1. Chemical structure of the five lignans.

(Chen et al., 2000). Evidence shows that deoxypodophyllotoxin and yatein are cytotoxic to several cancer cell lines (Chen et al., 1996; Lin et al., 1999; Ikeda et al., 1998; Wickramaratne et al., 1995; Gordaliza et al., 1994). Moreover, deoxypodophyllotoxin inhibits 12-*O*-tetradecanoylphorbol 13-acetate-induced ornithine decarboxylase in cultured mouse epidermal cells (Chang et al., 2000). However, other pharmacological characteristics of these five lignans have not been explored.

An increase in cytosolic-free $Ca^{2+}$ levels ($[Ca^{2+}]_i$) regulates numerous processes, ranging from secretion and contraction to modulation of cell growth and proliferation (Berridge, 1997; Clapham, 1995). The effect of plant lignans on $Ca^{2+}$ handling has never been investigated in any cell type. The mammalian lignan, 2,3-dibenzyl-butane-1,4-diol, has been shown to inhibit norepinephrine-induced contraction and $Ca^{2+}$ mobilization in the rat aorta (Abe et al., 1991).

In this study, using fura-2 as a $Ca^{2+}$ probe, we demonstrate for the first time that these lignans alter $Ca^{2+}$ signaling in Madin–Darby canine kidney cells in a multiple manner. Previous reports have shown that in this non-excitable renal tubular cell line, inositol 1,4,5-trisphosphate-dependent agnoists such as ATP (Jan et al., 1998a) and bradykinin (Jan et al., 1998b) increase $[Ca^{2+}]_i$ by depleting $Ca^{2+}$ from the inositol 1,4,5-trisphosphate-sensitive endoplasmic reticulum $Ca^{2+}$ store followed by capacitative $Ca^{2+}$ entry (Putney, 1986). Furthermore, thapsigargin, an endoplasmic reticulum $Ca^{2+}$ pump inhibitor (Thastrup et al., 1990), increases $[Ca^{2+}]_i$ by releasing $Ca^{2+}$ from the endoplasmic reticulum in an inositol 1,4,5-trisphosphate-independent manner (Jan et al., 1999). Thus, Madin–Darby canine kidney cells were used in the present study as a model for investigating the effect of lignans on $Ca^{2+}$ signaling in renal tubular cells.

## 2. Methods

### 2.1. Purification of lignans

Spectroscopically pure lignans (>99.9%) were extracted from *H. nymphaeifolia* (Hernandiaceae) as previously described (Chen et al., 1996). Briefly, dried trunk bark was powdered and extracted with methanol. The extract was subsequently concentrated under reduced pressure and partitioned between $H_2O$–$CHCl_3$ (1:1), leading to a $CHCl_3$-soluble fraction. This fraction was further extracted with 2% $H_2SO_4$. The acid-insoluble part was dried with $MgSO_4$ and concentrated to give a neutral $CHCl_3$-soluble fraction. A part of the neutral $CHCl_3$-soluble fraction was chromatographed on silica gel eluted with $CHCl_3$–MeOH (100:0–0:10) to produce 17 fractions. A part of fractions 5 and 7 was further purified by preparative thin layer chromatography to obtain the five lignans. The lignans were identified using spectroscopic methods: ultraviolet spectrophotometry (Shimadzu UV-160 A), infrared spectrophotometry (Hitachi 260-30), high resolution-mass spectrometry (JEOL JMX-HX 110) and $^1$H-nuclear magnetic resonance spectrometry (JEOL GSX-400). The purity of the lignans was found to be >99%.

### 2.2. Cell culture

Madin–Darby canine kidney cells obtained from American Type Culture Collection (CRL-6253) were cultured in Dulbecco's modified Eagle's medium supplemented with 10% heat-inactivated fetal bovine serum. Cells were kept at 37 °C in 5% $CO_2$-containing humidified air.

### 2.3. Solutions

$Ca^{2+}$-containing medium consisted of 140 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, 2 mM $CaCl_2$, 10 mM HEPES, and 5 mM glucose; the pH was adjusted to 7.4 with 1 N NaOH. $Ca^{2+}$-free medium had a similar composition except that $Ca^{2+}$ was replaced by 1 mM EGTA. Lignans were dissolved

in dimethyl sulfoxide and kept at −20 °C as a 0.1 M stock. The stock solutions were diluted to the final concentration before experiments. Other drugs were dissolved in water, ethanol or dimethyl sulfoxide. Organic solvents in the experimental solution were kept below 1% and were found to have no effect on $[Ca^{2+}]_i$ ($n=4$).

### 2.4. $[Ca^{2+}]_i$ measurements

Cells were detached from the flasks by trypsinization as described previously (Jan et al., 1998a,b). Trypsinized cells ($10^6$/ml) were loaded with 2 μM of the ester form of fura-2 (fura-2/acetoxy methyl) for 30 min at 25 °C in Dulbecco's modified Eagle 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 (Shimadzu, Kyoto, Japan) by recording excitation signals at 340 and 380 nm and emission signal at 510 nm at 1-s intervals. Maximum and minimum fluorescence values were obtained by addition of 10 μM digitonin (plus 10 mM $CaCl_2$) and 20 mM EGTA sequentially at the end of each experiment. $[Ca^{2+}]_i$ was calculated as previously described assuming a $K_d$ of 155 nM (Grynkiewicz et al., 1985).

### 2.5. Viability assay

Fifty microliters of cell suspension was mixed with 50 μl of trypan blue isotonic solution (0.2%; w/v) and cell viability was determined using a hemocytometer. The cell density in the assay solution was 0.5 million/ml.

### 2.6. Chemicals reagents

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

### 2.7. Statistics

The results are given as means ± S.E.M. of 4–6 replicates. Statistical comparisons were determined by using Student's *t*-test, and significance was accepted when $P<0.05$.

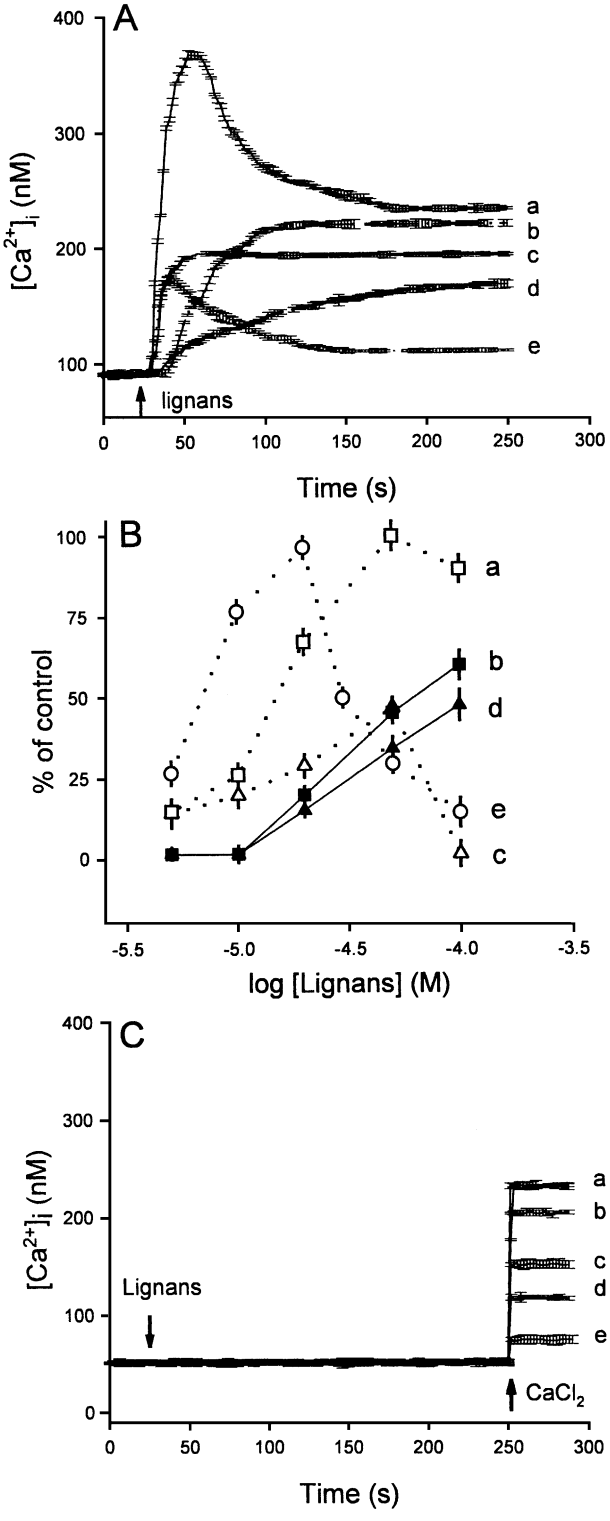


Fig. 2. (A) Lignan-induced $[Ca^{2+}]_i$ increases in $Ca^{2+}$-containing medium. Lignans were yatein (100 μM) in trace *a*, epi-yangambin (100 μM) in trace *b*, epi-aschantin (50 μM) in trace *c*, epi-magnolin (100 μM) in trace *d* and deoxypodophyllotoxin (100 μM) in trace *e*. Drugs were added at 25 s. (B) Concentration–response plots of lignan-induced $Ca^{2+}$ signals in $Ca^{2+}$-containing medium. The *y*-axis is the percentage of control. Control was the net area under the curve (25–250 s) of the $[Ca^{2+}]_i$ increase induced by 50 μM yatein. The lignan was yatein in trace *a*, epi-yangambin in trace *b*, epi-aschantin in trace *c*, epi-magnolin in trace *d*, and deoxypodophyllotoxin in trace *e*. Data are means ± S.E.M. of four to six replicates. (C) Effects of removal of extracellular $Ca^{2+}$ on lignan-induced $[Ca^{2+}]_i$ increases and the effect of readdition of $Ca^{2+}$. Lignans (50 μM) were added at 25 s followed by 3 mM $CaCl_2$ added at 250 s. Control (trace *e*): no lignan was added before addition of $Ca^{2+}$. Lignan was deoxypodophyllotoxin or epi-aschantin in trace *a*, yatein in trace *b*, epi-magnolin in trace *c*, and epi-yangambin in trace *d*.

## 3. Results

### 3.1. Effect of lignans on basal $[Ca^{2+}]_i$

Fig. 2A shows that the five lignans (trace *a* for yatein; trace *b* for epi-yangambin; trace *c* for epi-aschantin; trace *d* for epi-magnolin; trace *e* for deoxypodophyllotoxin) all increased $[Ca^{2+}]_i$ in the presence of extracellular $Ca^{2+}$. The lignans acted in a concentration-dependent manner (Fig. 2B). In the responses induced by yatein (trace *a*) and deoxypodophyllotoxin (trace *e*), the $[Ca^{2+}]_i$ signal comprised an initial rise and a gradual decay. The $[Ca^{2+}]_i$ signal induced by epi-yangambin (trace *b*), epi-aschantin (trace *c*) and epi-magnolin (trace *d*) comprised an initial increase and a sustained phase. At a concentration of 100 μM, yatein (trace *a*) caused a $[Ca^{2+}]_i$ increase which reached a maximum value after 8 ± 1 s and the $[Ca^{2+}]_i$ increase was followed by a second phase with a net value of 231 ± 5 nM at 250 s ($n = 5$). The time lapse in the $[Ca^{2+}]_i$ increases induced by other lignans was 24 ± 3 s, 28 ± 3 s, 204 ± 11 s, and 96 ± 4 s for epi-yangambin (100 μM), epi-aschantin (50 μM), epi-magnolin (100 μM) and deoxypodophyllotoxin (100 μM), respectively.

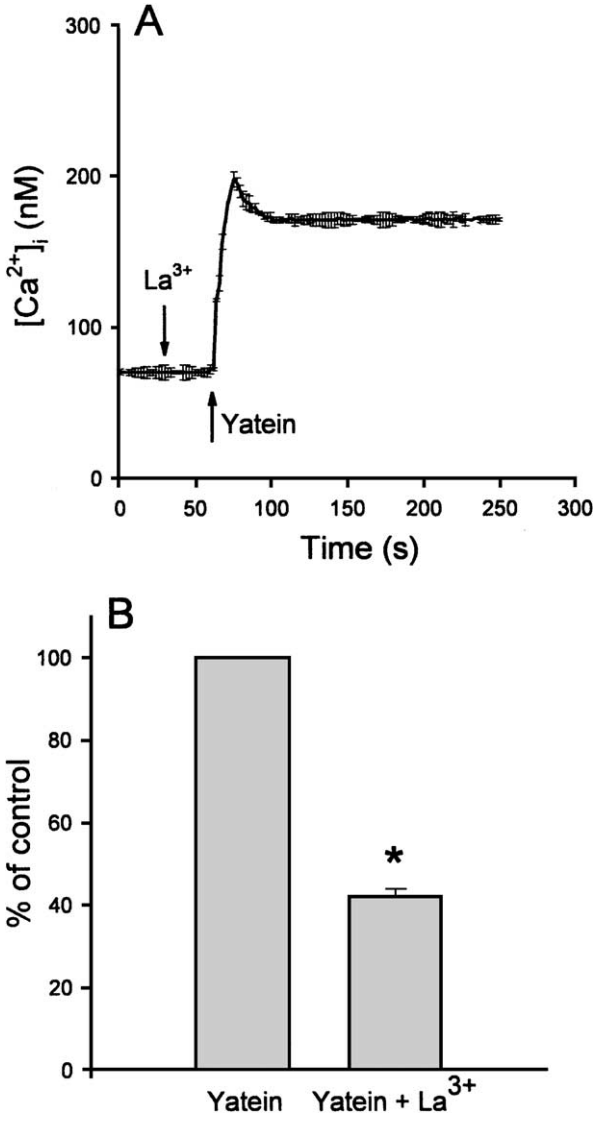


Fig. 3. Effect of $La^{3+}$ on yatein-induced $[Ca^{2+}]_i$ increases. (A) $La^{3+}$ (50 μM) was added at 30 s followed by yatein (50 μM) added at 60 s. (B) A bar graph showing the inhibitory effect of $La^{3+}$ on yatein-induced $[Ca^{2+}]_i$ increases. The experiments were performed in $Ca^{2+}$-containing medium. Data are means ± S.E.M. of four to six replicates. * $P < 0.05$.

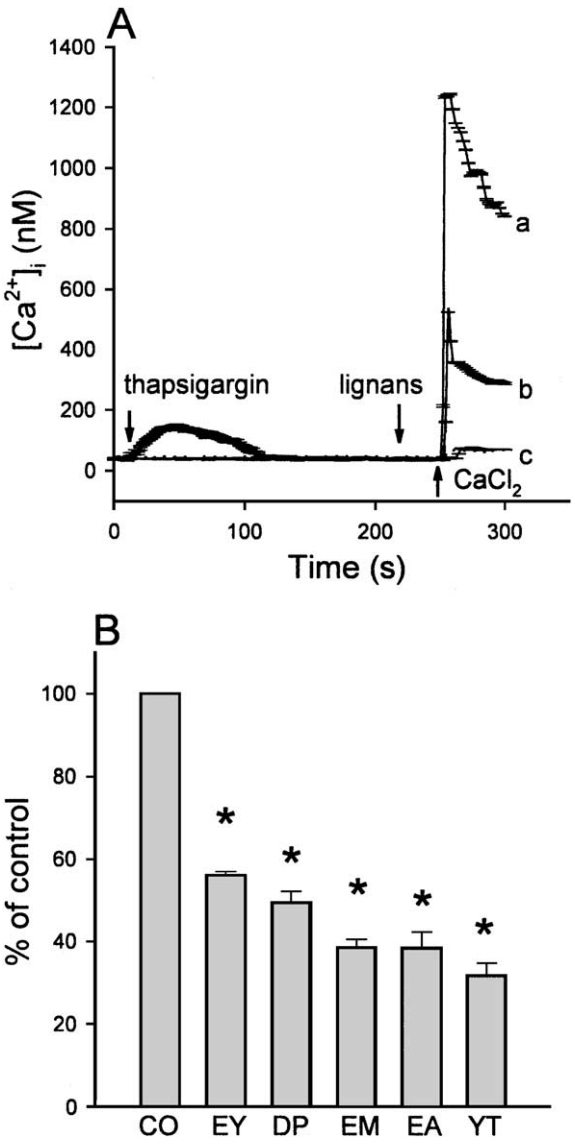


Fig. 4. Effect of lignans on thapsigargin-induced capacitive $Ca^{2+}$ entry. The final concentration was 100 μM for epi-yangambin (EY), epi-magnolin (EM), and epi-aschantin (EA); it was 50 μM for deoxypodophyllotoxin (DP) and yatein (YA). (A) In $Ca^{2+}$-free medium, 1 μM thapsigargin was added at 10 s followed by a lignan and 3 mM $Ca^{2+}$ added at 230 and 250 s, respectively. (B) A bar graph showing the inhibitory effect of lignans on thapsigargin-induced capacitive $Ca^{2+}$ entry. Data are expressed as percentages of control. Control (CO) was the net (baseline subtracted) maximum of 3 mM $Ca^{2+}$-induced $[Ca^{2+}]_i$ increase. Data are means ± S.E.M. of four to six replicates. * $P < 0.05$ compared to control.

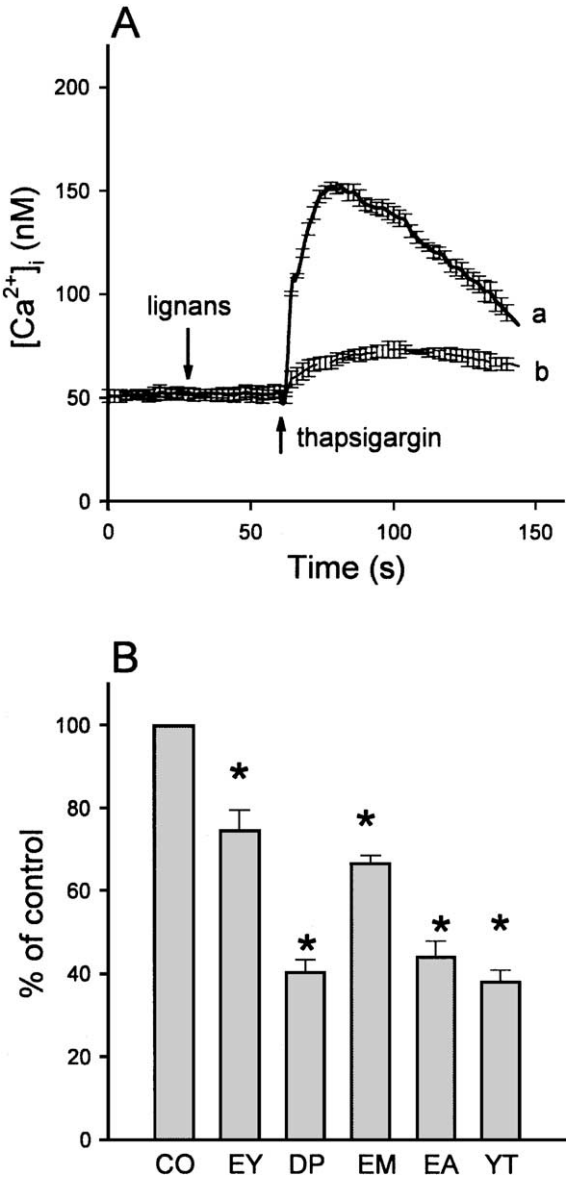


Fig. 5. Effect of lignans on thapsigargin-induced $[Ca^{2+}]_i$ increases. The final concentration was 100 μM for epi-yangambin (EY), epi-magnolin (EM), and epi-aschantin (EA); it was 50 μM for deoxypodophyllotoxin (DP) and yatein (YA). (A) In $Ca^{2+}$-free medium, a lignan was added at 30 s followed by 1 μM thapsigargin added at 60 s. Trace *a*: control thapsigargin response with no lignan pretreatment. Trace *b*: the lignan was 50 μM yatein. (B) A bar graph showing the inhibitory effect of lignans on thapsigargin-induced $[Ca^{2+}]_i$ increases. Data are expressed as percentages of control. Control was the net (baseline subtracted) maximum of 1 μM thapsigargin-induced $[Ca^{2+}]_i$ increase. Data are means ± S.E.M. of four to six replicates. * $P<0.05$ compared to control.

Fig. 2B shows the concentration-dependent plots for the $[Ca^{2+}]_i$ increases induced by the lignans. Epi-aschantin, deoxypodophyllotoxin and yatein induced maximum $[Ca^{2+}]_i$ increases at a concentration of 50, 20 and 50 μM, respectively. Higher levels of these three lignans induced smaller responses. Epi-yangambin and epi-magnolin-induced $[Ca^{2+}]_i$ increases did not saturate at 100 μM.

### *3.2. Effect of removal of extracellular $Ca^{2+}$ on lignan-induced $[Ca^{2+}]_i$ increases*

Fig. 2C shows that removal of extracellular $Ca^{2+}$ abolished 50 μM lignan-induced $[Ca^{2+}]_i$ increases ($n=4$; $P<0.05$ compared with the responses obtained in $Ca^{2+}$-containing medium). When 3 mM $Ca^{2+}$ was added afterwards, $[Ca^{2+}]_i$ increased to a magnitude comparable to the lignan-induced $[Ca^{2+}]_i$ increase in $Ca^{2+}$-containing medium.

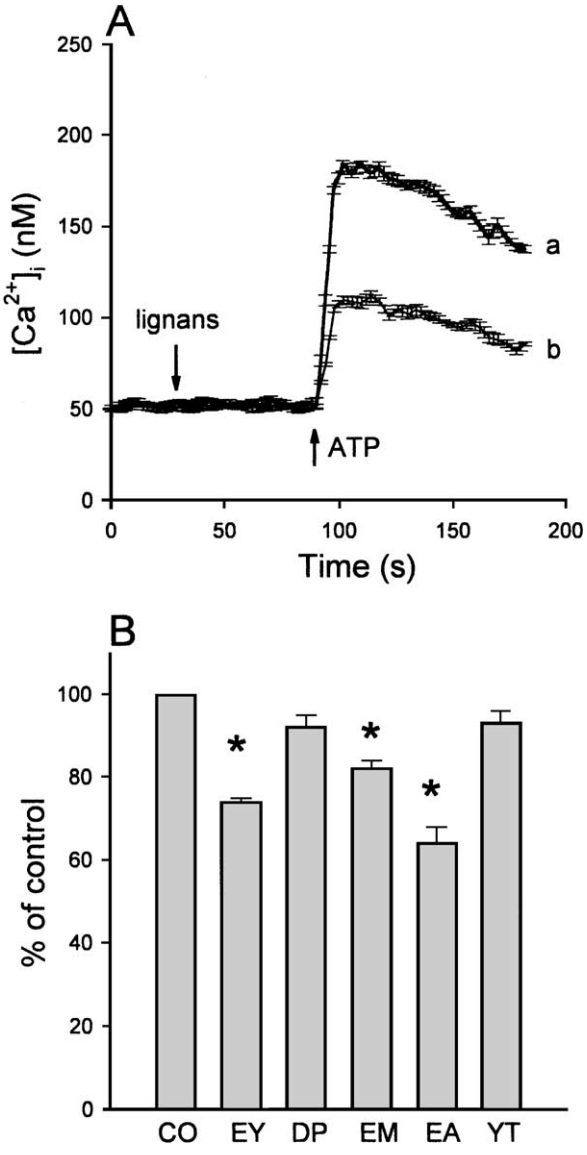


Fig. 6. Effect of lignans on ATP-induced $[Ca^{2+}]_i$ increases. The concentration was 100 μM for epi-yangambin (EY), epi-magnolin (EM), and epi-aschantin (EA); it was 50 μM for deoxypodophyllotoxin (DP) and yatein (YA). (A) In $Ca^{2+}$-free medium, the lignan was added at 50 s followed by 10 μM ATP added at 80 s. Trace *a*: control ATP response without lignan pretreatment. Trace *b*: the lignan was 100 μM epi-aschantin. (B) A bar graph showing the inhibitory effect of lignans on ATP-induced $[Ca^{2+}]_i$ increases. Data are expressed as percentages of control. Control was the net (baseline subtracted) maximum of 10 μM ATP-induced $[Ca^{2+}]_i$ increase. Data are means ± S.E.M. of four to six replicates. * $P<0.05$ compared to control.

### 3.3. Effect of $La^{3+}$ on lignan-induced $[Ca^{2+}]_i$ increases

In Madin–Darby canine kidney cells, it has been shown that $Ca^{2+}$ influx is composed of $La^{3+}$-sensitive and $La^{3+}$-insensitive components (Jan et al., 1998a,b, 1999). Experiments were performed to examine the pathway of lignan-induced $Ca^{2+}$ influx by testing the effect of $La^{3+}$. In $Ca^{2+}$-containing medium, 50 μM $La^{3+}$ abolished the $[Ca^{2+}]_i$ increases induced by epi-yangambin (100 μM), epi-aschantin (50 μM), epi-magnolin (100 μM), or deoxypodophyllotoxin (20 μM) ($n=5$; not shown). Fig. 3A shows the $[Ca^{2+}]_i$ increase induced by 50 μM yatein in the presence of 50 μM $La^{3+}$. Fig. 3B shows that the maximum value of the yatein-induced $Ca^{2+}$ signal was reduced by 60 ± 3% by $La^{3+}$ as compared to control (Fig. 2A, trace *a*) ($n=5$; $P<0.05$).

### 3.4. Effect of lignans on thapsigargin-induced capacitative $Ca^{2+}$ entry

Thapsigargin, an endoplasmic reticulum $Ca^{2+}$ pump inhibitor (Thastrup et al., 1990), has been shown to cause marked $Ca^{2+}$ influx via capacitative $Ca^{2+}$ entry (Jan et al., 1999). Thus, the effects of lignans on thapsigargin-induced capacitative $Ca^{2+}$ entry were investigated. Fig. 4A shows that in $Ca^{2+}$-free medium, 1 μM thapsigargin induced a $[Ca^{2+}]_i$ transient with a net maximum value of 151 ± 5 nM ($n=5$). Subsequently added 3 mM $CaCl_2$ at 250 s induced a $[Ca^{2+}]_i$ increase of 1202 ± 12 nM (trace *a*; $n=5$). Conversely, addition of $Ca^{2+}$ without thapsigargin pretreatment only induced a $[Ca^{2+}]_i$ increase of 25 ± 2 nM ($n=5$; Fig. 4A, trace *c*). This confirms that thapsigargin induced significant capacitative $Ca^{2+}$ entry. When lignans were added 20 s prior to $Ca^{2+}$, the $Ca^{2+}$-induced $Ca^{2+}$ influx was inhibited. Trace *b* in Fig. 4A shows the effect of 50 μM yatein on thapsigargin-induced capacitative $Ca^{2+}$ entry. Fig. 4B summarizes the inhibitory effects of these lignans. Epi-yangambin (100 μM), deoxypodophyllotoxin (50 μM), epi-magnolin (100 μM), epi-aschantin (100 μM), and yatein (50 μM) inhibited thapsigargin-induced capacitative $Ca^{2+}$ entry by 42 ± 3%, 51 ± 3%, 61 ± 4%, 62 ± 3%, and 65 ± 5%, respectively ($n=6$; $P<0.05$).

### 3.5. Effect of lignans on thapsigargin-induced $Ca^{2+}$ release

Efforts were made to evaluate the effect of lignans on thapsigargin-induced $Ca^{2+}$ release in order to understand how lignans interact with $Ca^{2+}$ release induced by inositol 1,4,5-trisphosphate-insensitive inhibition of the endoplasmic reticulum $Ca^{2+}$ pump. Fig. 5A shows that in $Ca^{2+}$-free medium, addition of lignans 30 s prior to thapsigargin (1 μM) significantly reduced thapsigargin-induced $Ca^{2+}$ release (trace *b* vs. control = trace *a*; $n=5$; $P<0.05$). The effects of the lignans are shown in Fig. 5B. Epi-yangambin (100 μM), deoxypodophyllotoxin (50 μM), epi-magnolin (100 μM), epi-aschantin (100 μM), and yatein (50 μM) inhibited the thapsigargin response by 23 ± 3%, 61 ± 4%, 33 ± 4%, 58 ± 3%, and 62 ± 4%, respectively ($n=6$; $P<0.05$).

### 3.6. Effect of lignans on ATP-induced $Ca^{2+}$ release

Experiments were performed to investigate the effect of lignans on inositol 1,4,5-trisphosphate-sensitive $Ca^{2+}$ release. ATP was used to release $Ca^{2+}$ from inositol 1,4,5-trisphosphate-sensitive stores in the endoplasmic reticulum

Table 1
Effects of lignans on cell viability

| | Incubation time (s) | [Lignan] 100 μM | [Lignan] 50 μM | [Lignan] 20 μM | [Lignan] 10 μM |
|---|---|---|---|---|---|
| Control | 10 | 97.7 ± 0.4 | 98.3 ± 0.3 | 98.9 ± 0.3 | 98.7 ± 0.3 |
| | 250 | 97.3 ± 0.2 | 98.1 ± 0.1 | 98.0 ± 0.2 | 97.9 ± 0.2 |
| | 500 | 96.4 ± 0.4 | 97.5 ± 0.4 | 98.0 ± 0.3 | 96.4 ± 0.3 |
| Epi-aschantin | 10 | 98.3 ± 0.2 | 98.5 ± 0.5 | 98.3 ± 0.1 | 98.3 ± 0.1 |
| | 250 | 97.5 ± 0.4 | 93.9 ± 0.4[a] | 98.2 ± 0.1 | 97.5 ± 0.4 |
| | 500 | 79.5 ± 0.4 | 90.1 ± 0.4 | 91.8 ± 0.5 | 92.8 ± 0.4 |
| Epi-magnolin | 10 | 96.3 ± 0.1 | 98.3 ± 0.1 | 98.3 ± 0.1 | 98.3 ± 0.1 |
| | 250 | 90.2 ± 0.1 | 98.2 ± 0.1 | 98.2 ± 0.1 | 98.2 ± 0.1 |
| | 500 | 74.8 ± 0.5 | 81.8 ± 0.5 | 91.8 ± 0.5 | 91.8 ± 0.5 |
| Epi-yangambin | 10 | 96.7 ± 0.4 | 97.2 ± 0.3 | 95.9 ± 0.4[a] | 96.7 ± 0.6 |
| | 250 | 92.9 ± 0.7 | 95.4 ± 0.5[a] | 96.1 ± 0.6[a] | 96.9 ± 0.7 |
| | 500 | 78.8 ± 0.5 | 86.8 ± 0.5 | 95.9 ± 0.4[a] | 92.8 ± 0.5 |
| Deoxypodophyllotoxin | 10 | 70.3 ± 4.8[a] | 65.9 ± 3.8[a] | 96.7 ± 0.4 | 96.7 ± 0.2[a] |
| | 250 | 59.0 ± 3.6[a] | 59.8 ± 3.9[a] | 77.2 ± 2.7[a] | 95.5 ± 0.2[a] |
| | 500 | 49.8 ± 3.8[a] | 53.8 ± 3.4[a] | 73.2 ± 2.9[a] | 90.5 ± 1.2[a] |
| Yatein | 10 | 89.4 ± 0.7[a] | 91.4 ± 0.9[a] | 98.1 ± 0.2 | 97.2 ± 0.4 |
| | 250 | 73.6 ± 3.5[a] | 73.0 ± 3.4[a] | 96.9 ± 0.2[a] | 95.9 ± 0.6[a] |
| | 500 | 63.0 ± 2.4[a] | 70.0 ± 3.6[a] | 87.0 ± 3.1[a] | 83.0 ± 2.4[a] |

Data are means ± S.E.M. of 4–6 replicates. Trypan blue exclusion assay was described in Methods. Control cell viability is defined as 100%. The values in the Table are the percentage of cells alive after the Trypan blue exclusion assay was performed.

[a] $P<0.05$ compared to control.

as described previously (Jan et al., 1998a). Fig. 6A shows that in $Ca^{2+}$-free medium, 10 μM ATP induced a $[Ca^{2+}]_i$ increase with of 158 ± 10 nM ($n = 6$). This ATP response was significantly inhibited by pretreatment for 30 s with epi-yangambin (100 μM), epi-magnolin (100 μM) and epi-aschantin (100 μM) by 23 ± 4%, 20 ± 4%, and 39 ± 3% ($n = 6$; $P < 0.05$), respectively. Trace *b* in Fig. 6A shows the effect of 100 μM epi-aschantin on the ATP-induced $[Ca^{2+}]_i$ increase. Fig. 6B shows a bar graph summarizing the effect of the lignans. Deoxypodophyllotoxin (50 μM) and yatein (50 μM) had no effect ($n = 6$; $P > 0.05$).

### 3.7. Effect of lignans on cell viability

To test whether lignans were cytotoxic, the effect of lignans on cytotoxicity was examined by Trypan blue exclusion. The data are summarized in Table 1.

## 4. Discussion

Lignans can be characterized as oxidatively coupled products of two or more phenyl propanoids, and can be distinguished by their structural variation and complexity. The lignans tested in this study included the tetrahydrofurofuran type (epi-aschantin, epi-magnolin, epi-yangambin), the aryltetralin lactone type (deoxypodophyllotoxin) and the dibenzylbutyrolactone type (yatein). The present study is the first to demonstrate that tetrahydrofurofuran lignans (epi-aschantin, epi-magnolin, epi-yangambin), an aryltetralin lactone lignan (deoxypodophyllotoxin) and a dibenzylbutyrolactone lignan (yatein) increase $[Ca^{2+}]_i$ in renal tubular cells at concentrations used in previous studies to inhibit platelet aggregation. It is rather unlikely that the increase in $[Ca^{2+}]_i$ induced by the five lignans resulted from alterations of platelet aggregation factor receptors because antagonists of platelet aggregation factor receptors, such as 1,4-dihydro-2,4,6-trimethyl-3,5-pyridinedicarboxylic acid methyl 2-(phenylthio) ethyl ester (PCA-4248; 1–20 μM) and 4-[3-[4-(*o*-chlorophenyl)-9-methyl-6*H*-thieno[3,2-*f*]-*s*-triazolo[4,3-*a*][1,4] diazepin-2-yl]proprionyl]-morpholine (WEB2086; 5–50 μM), did not affect basal $[Ca^{2+}]_i$ ($n = 5$; not shown).

The data suggest that the five lignans increased $[Ca^{2+}]_i$ solely by triggering $Ca^{2+}$ influx because the $Ca^{2+}$ signal disappeared after removal of extracellular $Ca^{2+}$ and was restored after addition of $Ca^{2+}$. Epi-aschantin, epi-magnolin, epi-yangambin, and deoxypodophyllotoxin appeared to cause $Ca^{2+}$ influx primarily via $La^{3+}$-sensitive pathways, whereas yatein was able to cause $Ca^{2+}$ influx through $La^{3+}$-sensitive (60%) and $La^{3+}$-insensitive pathways (40%). This is consistent with previous evidence that Madin–Darby canine kidney cells possess three major types of $Ca^{2+}$ entry mechanisms: $La^{3+}$-sensitve capacitative $Ca^{2+}$ entry; $La^{3+}$-sensitive, non-capacitative $Ca^{2+}$ entry; and $La^{3+}$-insensitive $Ca^{2+}$ entry (Jan et al., 1998a,b, 1999). The lignan-induced $Ca^{2+}$ entry was unlikely to be via capacitative $Ca^{2+}$ entry because the lignans did not cause the release of $Ca^{2+}$ from intracellular stores.

Additional to the stimulating effect of lignans on basal $[Ca^{2+}]_i$, the data show that the lignans significantly reduced thapsigargin-induced capacitative $Ca^{2+}$ entry. The exact mechanism underlying the dual action of the lignans is unclear. These five lignans were found to inhibit $Ca^{2+}$ release from both inositol 1,4,5-trisphosphate-dependent stores and inositol 1,4,5-trisphosphate-independent (thapsigargin-sensitive) stores.

It seems that deoxypodophyllotoxin and yatein may be cytotoxic to renal tubular cells, because 37–50% of cells were killed by acute incubation with the two drugs (100 μM) for 500 s. This is consistent with previous findings that these two lignans are toxic to several other cell types (Chen et al., 1996; Lin et al., 1999; Ikeda et al., 1998; Wickramaratne et al., 1995; Gordaliza et al., 1994). There appears to be a structure–cytotoxicity relationship for these lignans because epi-aschantin, epi-magnolin, and epi-yangambin (tetrahydrofurofuran type) were not cytotoxic, whereas deoxypodophyllotoxin (aryltetralin lactone type) and yatein (dibenzylbutyrolactone type) were. The cytotoxic effect may not be directly linked to the $Ca^{2+}$-elevating effect since the other three $Ca^{2+}$-elevating lignans only caused mild cytotoxicity.

Previous studies focused on the anti-platelet aggregation effect of the five lignans, and the present study is the first to show that these natural products can cause abnormal $Ca^{2+}$ signaling and cytotoxicity in renal tubular cells. The lignans were found to possess the following multiple actions: (1) elevation of $[Ca^{2+}]_i$ by causing $Ca^{2+}$ influx; (2) inhibition of thapsigargin-induced capacitative $Ca^{2+}$ entry; (3) inhibition of thapsigargin- and ATP-induced $Ca^{2+}$ release; and (4) possible cytotoxicity. Because abnormal $Ca^{2+}$ signaling may affect numerous cell events leading to cell death (Berridge, 1997; Clapham, 1995), the effect of these lignans on $Ca^{2+}$ signaling should be considered when investigating the in vivo and other in vitro effects of these drugs.

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