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SHORT COMMUNICATION

Mechanisms Underlying Ketoconazole-Induced Ca^{2+} Mobilization in Madin–Darby Canine Kidney Cells

Chung-Ren Jan and Ching-Jiunn Tseng*

Department of Medical Education and Research, Veterans General Hospital-Kaohsiung, Kaohsiung, Taiwan 813

ABSTRACT. The effect of ketoconazole on Ca^{2+} signaling in Madin–Darby canine kidney (MDCK) cells was investigated by using fura-2 as a Ca^{2+} probe. Ketoconazole evoked increases in cytosolic free Ca^{2+} concentration ($[Ca^{2+}]_i$) concentration dependently. The response was decreased by external Ca^{2+} removal. In Ca^{2+}-free medium, pretreatment with ketoconazole abolished the $[Ca^{2+}]_i$ rise induced by thapsigargin, an inhibitor of the endoplasmic reticulum Ca^{2+} pump. Addition of 3 mM Ca^{2+} induced a significant $[Ca^{2+}]_i$ rise after preincubation with 150 μM ketoconazole in Ca^{2+}-free medium. Pretreatment with aristolochic acid (40 μM) to inhibit phospholipase A_2 inhibited the 150-μM-ketoconazole-induced internal Ca^{2+} release by 37%, but inhibition of phospholipase C with 1-(6-((17beta-3-methoxyestra-1,3,5(10)-trien-17-yl)amino)hexyl)-1*H*-pyrrole-2,5-dione (U73122) (2 μM) had no effect. Collectively, we found that ketoconazole increases $[Ca^{2+}]_i$ in MDCK cells by releasing Ca^{2+} from thapsigargin-sensitive pools in a manner independent of the production of inositol-1,4,5-trisphosphate, followed by Ca^{2+} influx from the external space. BIOCHEM PHARMACOL **59**;8: 947–951, 2000. © 2000 Elsevier Science Inc.

KEY WORDS. ketoconazole; calcium; intracellular; fura-2; MDCK cells

Ketoconazole, the first orally active antifungal drug, and other imidazole compounds such as bifonazole, clotrimazole, econazole, isoconazole, and miconazole, were originally known as inhibitors of cytochrome P450-dependent steroidogenic enzymes [1–4]. Ketoconazole has a variety of effects both *in vivo* and *in vitro*. *In vivo*, ketoconazole was found to reduce elevated serum levels of 1,25-dihydroxyvitamin D_3 in patients suffering from hypercalcemic sarcoidosis [5] and suppress circulating calcitriol and duodenal active Ca^{2+} transport in pregnant rats [6]. *In vitro*, ketoconazole was shown to inhibit the thapsigargin-induced rise in $[Ca^{2+}]_i$† in HL-60 cells [7] and human neutrophils [8], voltage-gated Ca^{2+} entry into GH_3 and chromaffin cells [9], peroxisomal phytanic acid α-oxidation [10], and K_{ATP} currents in β cells leading to a decreased insulin secretion [11]. Ketoconazole was also shown to induce apoptosis in human colorectal and hepatocellular carcinoma cell lines [12] and increase maxi-K^+ currents in vascular smooth muscle cells [13].

The effect of ketoconazole on Ca^{2+} signaling has not been thoroughly investigated as yet. In this study, we examined the effect of ketoconazole on Ca^{2+} signaling in MDCK cells. We previously showed in this cell that IP_3-dependent agonists such as ATP [14] and bradykinin [15] increase $[Ca^{2+}]_i$ by depleting Ca^{2+} from the endoplasmic reticulum Ca^{2+} store leading to an internal Ca^{2+} refilling process termed "capacitative Ca^{2+} entry" [16]. Also, thapsigargin [17] and 2,5-di-*tert*-butylhydroquinone [18] increase $[Ca^{2+}]_i$ by inhibiting the endoplasmic reticulum Ca^{2+} pump without elevating IP_3 levels, leading to a release of Ca^{2+} from the endoplasmic reticulum followed by capacitative Ca^{2+} entry. Herein, we found that ketoconazole induced a rise in $[Ca^{2+}]_i$ in MDCK cells. We established the concentration–response relationships both in the presence and absence of external Ca^{2+} and explored the underlying mechanism.

MATERIALS AND METHODS

Cell Culture

MDCK cells obtained from the American Type Culture Collection (CRL-6253) were cultured in Dulbecco's modified Eagle's medium supplemented with 10% heat-inactivated fetal bovine serum, 100 U/mL penicillin, and 100 μg/mL streptomycin at 37° in 5% CO_2-containing humidified air.

Solutions

Ca^{2+} medium (pH 7.4) contained (in mM): NaCl 140; KCl 5; $MgCl_2$ 1; $CaCl_2$ 2; HEPES 10; glucose 5. Ca^{2+}-free medium contained no Ca^{2+} plus 1 mM EGTA.

* Corresponding author: Dr. Chung-Ren Jan, Dept. of Medical Education and Research, Veterans General Hospital-Kaohsiung, 386 Ta Chung 1st Rd, Kaohsiung, Taiwan 813. FAX 011-886-7-3468056; E-mail: crjan@isca.vghks.gov.tw

† *Abbreviations:* $[Ca^{2+}]_i$, cytosolic free Ca^{2+} concentration; IP_3, inositol 1,4,5-trisphosphate; MDCK cells, Madin–Darby canine kidney cells; U73122, 1-(6-((17β-3-methoxyestra-1,3,5(10)-trien-17-yl)amino)hexyl)-1*H*-pyrrole-2,5-dione); and U73343, 1-(6-((17β-3-methoxyestra-1,3,5(10)-trien-17-yl)amino)hexyl)-2,5-pyrrolidine-dione.

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

Trypsinized cells (10^6/mL) were loaded with 2 μM 1-[2-(5-carboxyoxazol-2-yl)-6-aminobenzofuran-5-oxy]-2-(2′-amino-5′-methylphenoxy)-ethane-*N*,*N*,*N*-tetraacetic acid pentaacetoxymethyl ester (fura-2/AM) for 30 min at 25° in Dulbecco's modified Eagle's medium. Cells were washed and resuspended in Ca^{2+} medium. Fura-2 fluorescence measurements were performed in a water-jacketed cuvette (25°) 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 continuously recording excitation signals at 340 and 380 nm and emission signal at 510 nm at 1-sec intervals. Maximum and minimum fluorescence values were obtained by adding Triton X-100 (0.1%) and EGTA (20 mM) at the end of an experiment. The ratio of excitation signals at 340 and 380 nm was used to calculate $[Ca^{2+}]_i$ as described previously [19].

Chemical Reagents

The reagents for cell culture were from GIBCO. Fura-2/AM was from Molecular Probes. U73122, U73343, and aristolochic acid were from Biomol. Ketoconazole was from RBI. The other reagents were from Sigma.

Statistical Analysis

All values are reported as means ± SEM of 5–6 experiments. Statistical comparisons were determined by using Student's paired *t*-test, and significance was accepted when $P < 0.05$.

RESULTS AND DISCUSSION

Sources of Ketoconazole-Induced $[Ca^{2+}]_i$ Increases

At concentrations between 50–150 μM, ketoconazole induced a rise in $[Ca^{2+}]_i$ in the presence of external Ca^{2+} (Fig. 1A). Over a time period of 5 min, the $[Ca^{2+}]_i$ rise consisted of an initial rise and an elevated phase. For example, at a concentration of 150 μM, ketoconazole induced a nearly immediate rise in $[Ca^{2+}]_i$ which peaked 100 ± 5 sec later at a net height of 185 ± 15 nM (N = 6; $P < 0.05$). This was followed by a gradual decay, with the net height remaining over 100 nM at the time point of 250 sec. The increase in the Ca^{2+} signal was slower in response to lower concentrations of ketoconazole. At a concentration of 200 μM, ketoconazole induced an immediate and persistent rise in $[Ca^{2+}]_i$; however, as this most likely reflected cell membrane damage, the result was not shown. Figure 1B shows that removal of external Ca^{2+} significantly reduced the Ca^{2+} signals induced by 100–150 μM ketoconazole while abolishing the $[Ca^{2+}]_i$ rise induced by 50 μM ketoconazole. Both the rising and sustained phases were reduced by Ca^{2+} removal, suggesting that external Ca^{2+} influx contributed to the $[Ca^{2+}]_i$ rise throughout the whole course of measurement. The concentration–response plots of the responses both in the presence and absence of external Ca^{2+} are illustrated in Fig. 1C. Ca^{2+} removal reduced the $[Ca^{2+}]_i$ rise induced by 100 and 150 μM ketoconazole in the net area under the curve by 61 ± 5% and 45 ± 9%, respectively (N = 5–6; $P < 0.05$).

The Internal Ca^{2+} Store of the Ketoconazole Response

We examined the internal Ca^{2+} source of the ketoconazole-induced Ca^{2+} response. Figure 1D shows that in the absence of external Ca^{2+} and after the 150-μM-ketoconazole-induced rise in $[Ca^{2+}]_i$ had decayed to the baseline, addition of 1 μM thapsigargin, an endoplasmic reticulum Ca^{2+} pump inhibitor [20], did not release additional Ca^{2+}. In contrast, Fig. 2B shows that pretreatment with 1 μM thapsigargin in Ca^{2+}-free medium abolished the 150-μM-ketoconazole-induced $[Ca^{2+}]_i$ rise. These results suggest that the thapsigargin-sensitive Ca^{2+} store is the source of ketoconazole-induced internal Ca^{2+} release.

Effects of Ketoconazole on Capacitative Ca^{2+} Entry

In MDCK cells, mobilization of internal Ca^{2+} often results in capacitative Ca^{2+} entry [14, 15, 17, 18]. Thus, we tested whether ketoconazole-induced Ca^{2+} influx occurs via capacitative Ca^{2+} entry. Capacitative Ca^{2+} entry was measured by addition of 3 mM Ca^{2+} to cells pretreated with ketoconazole in Ca^{2+}-free medium. Figure 2A shows that after depletion of the internal Ca^{2+} store for 7–8 min with 150 μM ketoconazole, addition of Ca^{2+} induced a $[Ca^{2+}]_i$ rise with a net maximum height of 65 ± 4 nM (*trace a*; N = 6), which was 3.6-fold higher than control (18 ± 3 nM; *trace b*; N = 6; $P < 0.05$). We next examined whether ketoconazole could alter the capacitative Ca^{2+} entry induced by thapsigargin. Figure 2B shows that 1 μM thapsigargin induced capacitative Ca^{2+} entry with a net peak height of 395 ± 12 nM (*trace a*; N = 5; $P < 0.05$), which was inhibited by 35 ± 4% in the area under the curve (400–500 sec) by adding 150 μM ketoconazole 80 sec prior to Ca^{2+} (*trace b*; N = 6; $P < 0.05$). This is consistent with the result found in HL-60 cells [7] and human neutrophils [8].

Effects of Inhibition of Phospholipase C or A_2 on Ketoconazole-Induced Internal Ca^{2+} Release

The question arose as to how ketoconazole releases Ca^{2+} from the thapsigargin-sensitive store. We investigated whether the internal Ca^{2+} release induced by ketoconazole was mediated by a rise in IP_3 levels. We used U73122, a phospholipase C inhibitor, to suppress IP_3 formation. We have previously shown that ATP induces significant internal Ca^{2+} release in an IP_3-dependent manner [21]. *Trace a* in Fig. 2C shows a typical $[Ca^{2+}]_i$ rise induced by 10 μM ATP. Incubation with U73122 (2 μM), a phospholipase C inhibitor [22], for 210 sec induced a slight $[Ca^{2+}]_i$ rise,

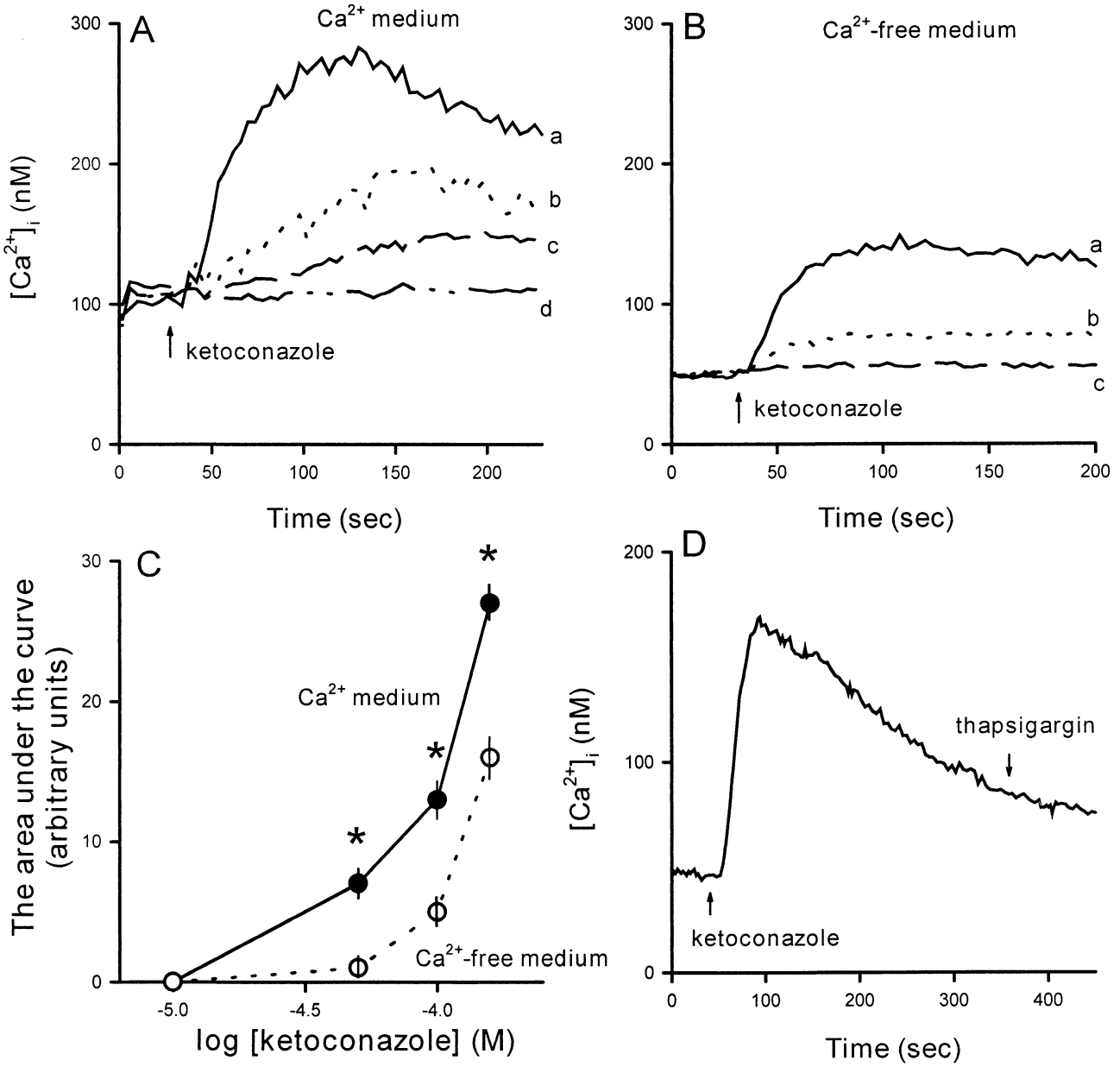

FIG. 1. (A) Concentration-dependent effects of ketoconazole on $[Ca^{2+}]_i$. [Ketoconazole] = 150 μM in *trace a*, 100 μM in *trace b*, 50 μM in *trace c*, and 10 μM in *trace d*. The experiments were performed in Ca^{2+} medium. (B) Similar to A except that the cells were bathed in Ca^{2+}-free medium. (C) Concentration–response plots of ketoconazole-induced Ca^{2+} signals in the presence (filled circles) or absence (open circles) of external Ca^{2+}. The y axis is the net area under the curve of the $[Ca^{2+}]_i$ rise. The data are means ± SEM of 5–6 experiments. *P < 0.05. (D) In Ca^{2+}-free medium, 150 μM ketoconazole and 1 μM thapsigargin were added as indicated. Traces are typical of 5–6 experiments.

consistent with our previous report [21], but prevented subsequently applied ATP (10 μM) from increasing $[Ca^{2+}]_i$ (*trace b*; N = 6; $P < 0.05$). This most likely suggests that U73122 effectively inhibited phospholipase C-dependent IP_3 formation. After U73122 pretreatment for 270 sec, application of 150 μM ketoconazole induced a $[Ca^{2+}]_i$ rise with a net peak height indistinguishable from control (*trace c*, ketoconazole effect without U73122 pretreatment; N = 6; $P > 0.05$). U73343, an inactive U73122 analogue, neither altered the resting $[Ca^{2+}]_i$ nor inhibited the $[Ca^{2+}]_i$ rises induced by ATP and ketoconazole. We examined the effect of inhibition of phospholipase A_2 on ketoconazole-induced Ca^{2+} release. Figure 2D shows that pretreatment with aristolochic acid (40 μM), a phospholipase A_2 inhibitor [23], for 250 sec inhibited the 150-μM-ketoconazole-induced $[Ca^{2+}]_i$ rise by 37 ± 5% in net peak height (compared to *trace c* in Fig. 2C). Aristolochic acid did not alter the resting $[Ca^{2+}]_i$. Thus, our data suggest that ketoconazole-induced internal Ca^{2+} release most likely does not require a rise in IP_3 levels. However, ketoconazole-

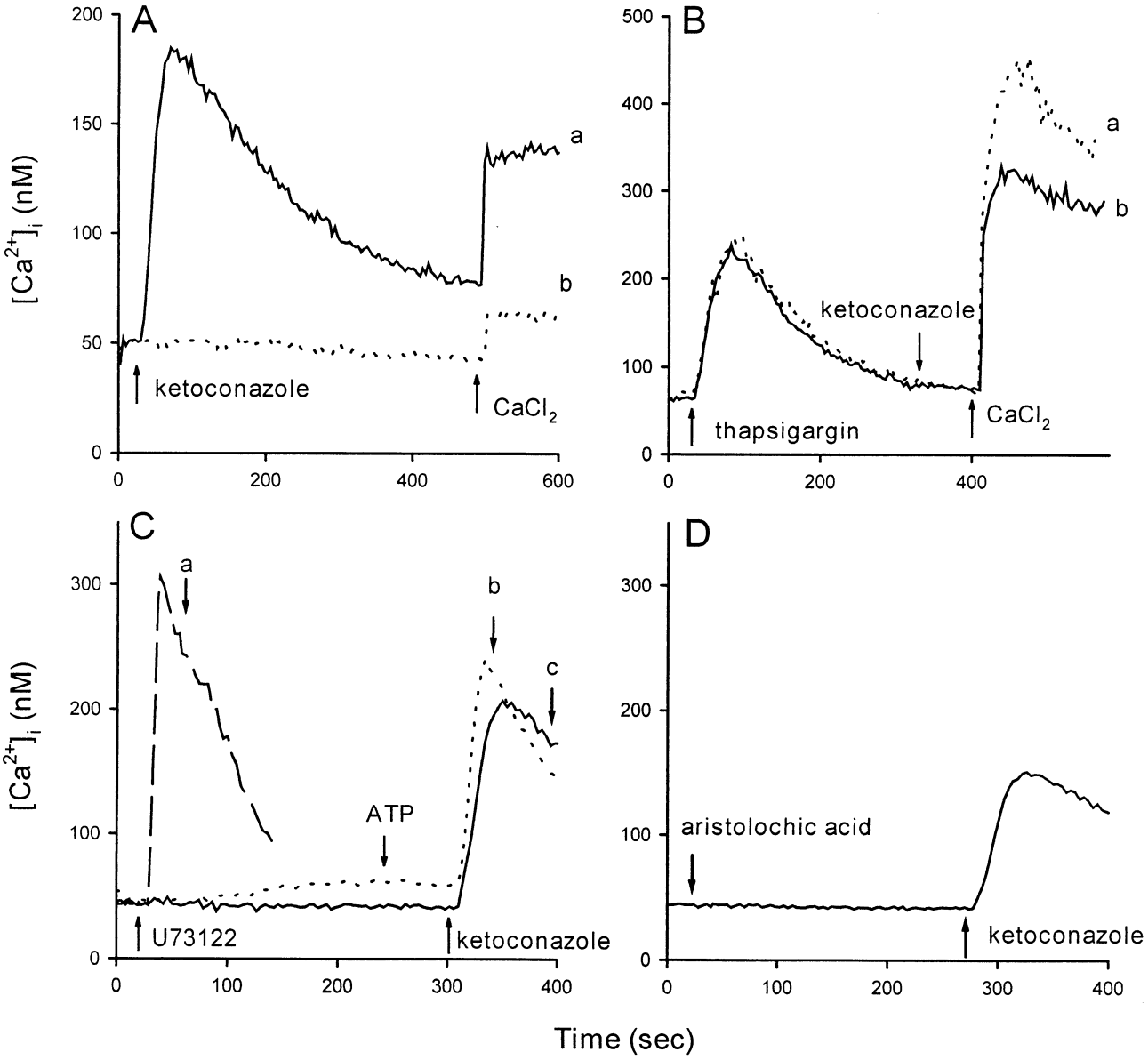

FIG. 2. Effects of ketoconazole on capacitative Ca^{2+} entry. (A) Capacitative Ca^{2+} entry induced by ketoconazole. *Trace a*: 150 μM ketoconazole was added at 30 sec followed by 3 mM $CaCl_2$ at 500 sec. *Trace b*: control $CaCl_2$ effect. (B) *Trace a*: 1 μM thapsigargin was added at 30 sec followed by 3 mM $CaCl_2$ at 400 sec. *Trace b*: similar to *trace a* except that 150 μM ketoconazole was added 80 sec prior to $CaCl_2$. (C) *Trace a*: 10 μM ATP was added at 30 sec in Ca^{2+}-free medium. *Trace b*: 2 μM U73122, 10 μM ATP, and 150 μM ketoconazole were added as indicated. *Trace c*: control effect of ketoconazole. (D) Aristolochic acid (40 μM) was added at 30 sec followed by 150 μM ketoconazole at 280 sec. Traces are typical of 5–6 experiments.

induced internal Ca^{2+} release might be regulated by phospholipase A_2-coupled events.

Ketoconazole's action is not necessarily coupled to inhibition of a cytochrome P450, because we found that several other cytochrome P450 inhibitors with imidazole structures such as lansoprazole, metronidazole, antazoline, benzylimidazole, and 1-aminobenzotriazole had no effect on the resting $[Ca^{2+}]_i$ (N = 6; not shown). Compared with econazole, ketoconazole is at least 10 times less potent in inducing a rise in $[Ca^{2+}]_i$ in MDCK cells. As we reported in a previous study, econazole induced a significant rise in $[Ca^{2+}]_i$ at a concentration range between 5–50 μM [24]. At a concentration of 10 μM, econazole induced a rise in $[Ca^{2+}]_i$ with a peak height of nearly 400 nM, whereas 10 μM ketoconazole did not increase $[Ca^{2+}]_i$ and 150 μM ketoconazole induced a $[Ca^{2+}]_i$ rise with a peak height of less than 300 nM.

In summary, we have characterized the $[Ca^{2+}]_i$ rise induced by ketoconazole in MDCK cells and have investigated the underlying mechanism. Our data suggest that ketoconazole increased $[Ca^{2+}]_i$ by releasing Ca^{2+} from thapsigargin-sensitive pools in a phospholipase A_2-regu-

lated, IP_3-independent manner, followed by capacitative Ca^{2+} entry. Because ketoconazole is clinically used as an oral medicine, the effects of this drug at higher concentrations should not be ignored, especially during acute or chronic intoxication. The new findings in this study are: 1) the concentration-dependent effects of ketoconazole in the presence and absence of external Ca^{2+}; 2) ketoconazole released internal Ca^{2+} solely from thapsigargin-sensitive pools, leading to capacitative Ca^{2+} entry. A previous study [7] demonstrated that ketoconazole released internal Ca^{2+}, but did not show that this Ca^{2+} release was followed by capacitative Ca^{2+} entry and; 3) ketoconazole-induced internal Ca^{2+} release was not via IP_3, but could be modulated by aristolochic acid, a phospholipase A_2 inhibitor. Thus, our results may contribute to the current pharmacology and toxicology of ketoconazole. Because prolonged $[Ca^{2+}]_i$ rises or altered Ca^{2+} handling lead to cytoxicity, it would be of interest to determine whether the effect of ketoconazole on Ca^{2+} signaling plays a significant role in its antifungal action and side effects.

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