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Mechanism of Bifonazole-Induced $[Ca^{2+}]_i$ Increases in MDCK Renal Tubular Cells

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Abstract

The effect of the antifungal drug bifonazole on Ca^{2+} homeostasis in Madin Darby canine kidney (MDCK) cells was investigated. Cell suspensions were loaded with the Ca^{2+}-sensitive dye fura-2, and the fluorescence changes were measured with a spectrofluorophotometer. At concentrations between 10-80 μM bifonazole increased cytosolic free Ca^{2+} levels ($[Ca^{2+}]_i$) in a concentration-dependent manner. The Ca^{2+} signals were partly inhibited by removing extracellular Ca^{2+}. Bifonazole (40 μM) released Ca^{2+} from the store sensitive to 1 μM thapsigargin, an endopolasmic reticulum Ca^{2+} pump inhibitor. Bifonazole (40 μM) per se induced capacitative Ca^{2+} entry while reduced 1 μM thapsigargin-induced capacitative Ca^{2+} entry. Inositol 1,4,5-trisphosphate may be involved in bifonazole-induced Ca^{2+} release because inhibiting phospholipase C with 2 μM U73122 partly reduced the bifonazole response. Together, bifonazole increased $[Ca^{2+}]_i$ in renal tubular cells by inducing intracellular Ca^{2+} release and extracellular Ca^{2+} influx.

Key Words: bifonazole, Ca^{2+} signalling, MDCK cells, fura-2, Ca^{2+} stores

Introduction

Bifonazole is an effective and well-tolerated imidazole antifungal drug for treating superficial fungal infections of the skin (1). *In vitro*, bifonazole was found to inhibit dermatophytes, moulds, yeast, dimorphic fungi and some Gram-positive bacteria (2). How this drug displays antifungal action is unclear. Further, bifonazole was shown to antagonize calmodulin (3,4),

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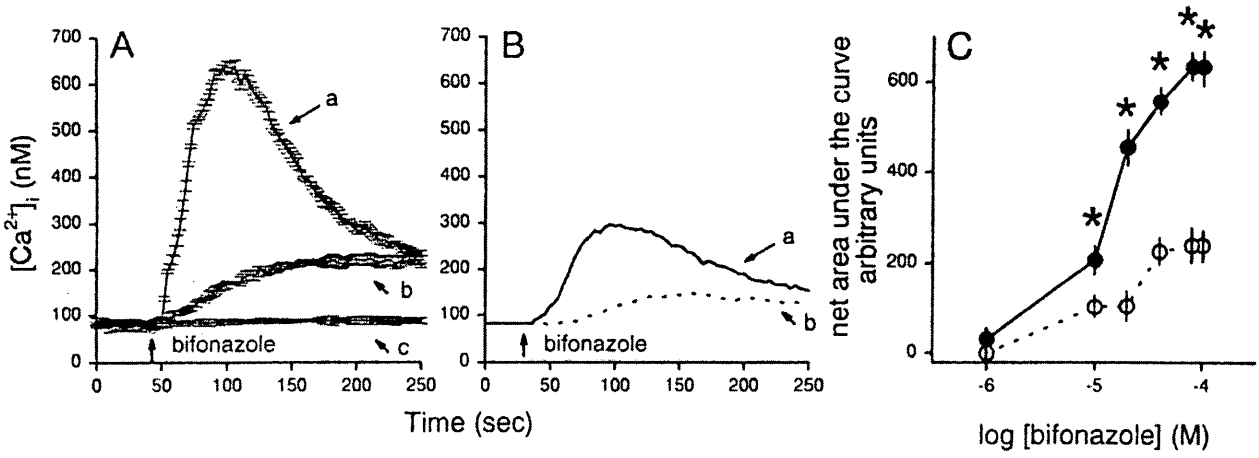

Fig. 1. A, Bifonazole-induced $[Ca^{2+}]_i$ rises. Concentration of bifonazole was 80 μM in trace a, 20 μM in trace b, and 1 mM in trace c. Experiments were performed in Ca^{2+} medium. Traces were the mean±SEM of 5-6 experiments. B, Similar to A except that the experiments were performed in Ca^{2+}-free medium. Each trace was typical of 5-6 experiments. C, Concentration-response curves of bifonazole-induced Ca^{2+} signals in the presence (filled circles) or absence (open circles) of external Ca^{2+}. They axis is the net (baseline subtracted) area under the curve of the response (50-250 sec). The data are the means±SEM of 5-6 experiments. *P<0.05.

to inhibit cytochrome P450 (5) and peroxisomal phytanic acid oxidation (6), and to induce peroxisome proliferation in rat liver (7,8).

In rat hepatocytes 500 μM bifonazole was shown to increase $[Ca^{2+}]_i$ by exclusively releasing Ca^{2+} from thapsigargin-sensitive Ca^{2+} stores (9). However, the effect of bifonazole on Ca^{2+} signaling has not been thoroughly investigated.

In the present study, the effect of bifonazole on Ca^{2+} signaling in MDCK renal tubular cells was investigated. It was shown previously that in this epithelial cell, inositol 1,4,5-trisphosphate-dependent agonists such as ATP (10) and bradykinin (11) increase $[Ca^{2+}]_i$ by depleting Ca^{2+} from the endoplasmic reticulum Ca^{2+} store followed by a Ca^{2+} refilling process termed capacitative Ca^{2+} entry (12). Also, thapsigargin (13) and 2,5-di-tert-butylhydroquinone (14) increase $[Ca^{2+}]_i$ by directly inhibiting the endoplasmic reticulum Ca^{2+} pump without elevating inositol 1,4,5-trisphosphate levels, leading to Ca^{2+} release from the endoplasmic reticulum followed by capacitative Ca^{2+} entry. In the Ca^{2+} signal induced by an agonist, the contribution of Ca^{2+} release from the endoplasmic reticulum and Ca^{2+} influx via capacitative Ca^{2+} entry appears to be equal.

Using fura-2 as a Ca^{2+} probe, this study shows that bifonazole induced a significant $[Ca^{2+}]_i$ increase in MDCK cells. The concentration-response relationships both in the presence and absence of extracellular Ca^{2+} were examined and the underlying mechanisms were explored.

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, 100U/ml penicillin and 100 μg/ml streptomycin at 37°C 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.

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

Trypsinized cells (10^6/ml) were allowed to recover in Dulbecco's modified Eagle medium for 1 hour before been loaded with 2 μM fura-2/AM for 30 min at 25°C in the same medium. The cells were washed and resuspended in Ca^{2+} medium. Fura-2 fluorecence 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 Corp., Kyoto, Japan) by continuously 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 adding 0.1% Triton X-100 (+

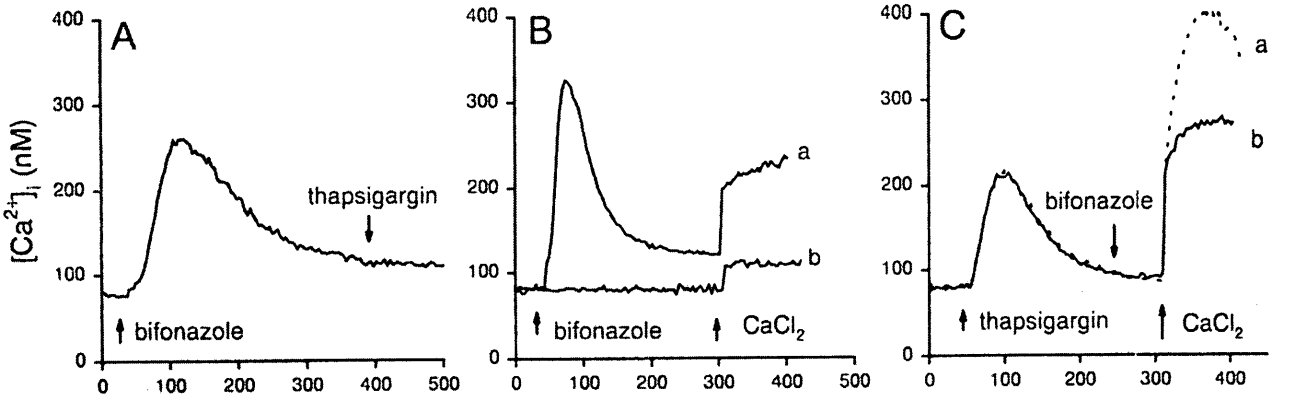

Fig. 2. A, The internal Ca^{2+} store of bifonazole-induced $[Ca^{2+}]_i$ increases. In Ca^{2+}-free medium, 40 μM bifonazole was added at 30 sec followed by 1 μM thapsigargin at 400 sec. B, Bifonazole-induced capacitative Ca^{2+} entry. Capacitative Ca^{2+} entry was induced by depleting internal Ca^{2+} stores in Ca^{2+}-free medium followed by adding 3 mM $CaCl_2$. Trace a: bifonazole (40 μM) was added at 30 sec followed by $CaCl_2$ added at 300 sec. Trace b: control $CaCl_2$ effect. C, Effect of bifonazole on thapsigargin-induced capacitative Ca^{2+} entry. Trace a: 1 mM thapsigargin was added at 30 sec followed by $CaCl_2$ added at 300 sec. Trace b: similar to trace a except that 40 μM bifonazole was added 50 sec prior to $CaCl_2$. Each trace was typical of 5-6 experiments.

10 mM $CaCl_2$), and 20 mM EGTA sequentially at the end of each experiment. $[Ca^{2+}]_i$ was calculated as described previously assuming a K_d of 155 nM (15-18).

Chemical reagents

The reagents for cell culture were from Gibco. Fura-2/AM was from Molecular Probes. U73122 and U73343 were from Biomol. Bifonazole and other reagents were from Sigma.

Statistical analysis

All values were reported as means±SEM of 5-6 experiments. Statistical comparisons were determined by using the ANOVA test and the Student's test, and significance was accepted when $P<0.05$.

Results

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

Figure 1A shows that bifonazole at concentrations between 10-80 μM increased $[Ca^{2+}]_i$ in physiological saline. The basal $[Ca^{2+}]_i$ was 85±9 nM (n=6). At a concentration of 1 mM bifonazole hardly had an effect (trace c). Over a time period of 200 s the $[Ca^{2+}]_i$ increase induced by bifonazole comprised an initial rise and a sustained phase. At a concentration of 80 mM, bifonazole induced an immediate $[Ca^{2+}]_i$ increase which reached a peak value after a latency time of 59±3 sec at a net value of 570±10 nM (baseline subtracted; n=6), followed by a decay phase which remained elevated at the time point of 250 sec (trace a). At a concentration of 20 μM, bifonazole induced a gradual $[Ca^{2+}]_i$ increase (trace b).

Effect of External Ca^{2+} Removal on the Bifonazole Response

External Ca^{2+} removal partly reduced the Ca^{2+} signals induced by 10-80 μM bifonazole (Figure 1B). The concentration-response plots of bifonazole-induced $[Ca^{2+}]_i$ increases both in the presence and absence of Ca^{2+} are shown in Figure 1C. The plots indicate that the $[Ca^{2+}]_i$ signal saturates at 80 mM bifonazole with an EC_{50} of 15 μM, calculated by fitting to the Hill equation.

Internal Ca^{2+} Sources of the Bifonazole Response

Figure 2A shows that in Ca^{2+}-free medium, after 40 mM bifonazole had induced a $[Ca^{2+}]_i$ increase, addition of 1 mM thapsigargin, an endoplasmic reticulum Ca^{2+} pump inhibitor (19) failed to increase $[Ca^{2+}]_i$ (see Figure 2C for control thapsigargin response).

Figure 2B shows that after depleting endoplasmic reticulum Ca^{2+} stores for 5 min with 40 μM bifonazole, addition of 3 mM Ca^{2+} induced a $[Ca^{2+}]_i$ increase with a net maximum of 121±5 nM (baseline subtracted; trace a, n=5) which was greater than control by 5-fold (24±5 nM, trace b, n=5, $P<0.05$).

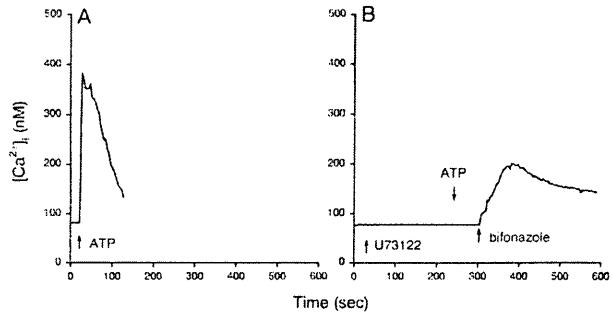

Fig.3. Effect of inhibiting phospholipase C on bifonazole-induced Ca^{2+} release. The experiments were performed in Ca^{2+}-free medium. **A**, 10 μM ATP was added at 30 sec. **B**, 2 μM U73122 was added at 30 sec followed by 10 μM ATP at 250 sec and 40 mM bifonazole at 300 sec, respectively. Each trace was typical of 5-6 experiments.

Figure 2C shows that in Ca^{2+}-free medium, 1 μM thapsigargin induced a $[Ca^{2+}]_i$ increase with a net maximum of 131±12 nM (trace a, n=5). Subsequently added 3 mM $CaCl_2$ induced a $[Ca^{2+}]_i$ increase with a net maximum of 320±9 nM (n=5). Trace b in Figure 2C shows that after 1 μM thapsigargin pretreatment, 40 μM bifonazole did not increase $[Ca^{2+}]_i$ but inhibited 38±3% of subsequently added $CaCl_2$-induced $[Ca^{2+}]_i$ increases (199±12 nM, $P<0.05$).

Effect of Inhibiting Inositol 1,4,5-Trisphosphate Formation on the Bifonazole Response

The possibility that inositol 1,4,5-trisphosphate was involved in bifonazole-induced Ca^{2+} release was tested. It was shown that ATP (10 μM) released Ca^{2+} via inositol 1,4,5-trisphosphate (10). Figure 3A shows a 10 μM ATP-induced $[Ca^{2+}]_i$ increase in Ca^{2+}-free medium. Figure 3B shows that addition of 2 μM U73122, a phospholipase C inhibitor (20), for 220 sec did not alter baseline while prevented subsequently added 10 μM ATP from increasing $[Ca^{2+}]_i$ (n=6, $P<0.05$). U73343 (10 μM), an inactive U73122 analogue, did not alter basal $[Ca^{2+}]_i$ or the ATP response (n=5, data not shown). This suggests that U73122 effectively suppressed the activity of phospholipase C. Subsequently added 40 μM bifonazole induced a $[Ca^{2+}]_i$ increase with a net peak value of 100±3 nM (n=6) which is 45±2% smaller than control (Fig. 2A, 181±9 nM).

Discussion

In this study it was found that the antifungal drug bifonazole induced a significant $[Ca^{2+}]_i$ increase in renal tubular cells. Our data suggest that bifonazole induced both external Ca^{2+} influx and internal Ca^{2+} release because the Ca^{2+} signals induced by 1-10 μM bifonazole was abolished by external Ca^{2+} removal while that induced by 20-100 μM bifonazole was decreased by 63-78% by external Ca^{2+} removal. Note that both the rising and decay phases were reduced by Ca^{2+} removal, suggesting that external Ca^{2+} influx contributes to the $[Ca^{2+}]_i$ signal throughout the whole course of measurement.

The internal Ca^{2+} source for bifonazole-induced $[Ca^{2+}]_i$ increases is the thapsigargin-sensitive endoplasmic reticulum store because in Ca^{2+}-free medium, pretreatment with 40 μM bifonazole completely depleted the Ca^{2+} store sensitive to 1 μM thapsigargin. The question arose as to how bifonazole releases Ca^{2+} from the thapsigargin-sensitive store. The two primary mechanisms by which Ca^{2+} could be released from the endoplasmic reticulum in MDCK cells are mediated by inositol 1,4,5-trisphosphate formation, such as bradykinin- or ATP-induced $[Ca^{2+}]_i$ increases (10,11) and by directly inhibiting the endoplasmic reticulum Ca^{2+} pump, such as thapsigargin- or 2,5-di-tert-butylhydroquinone-induced $[Ca^{2+}]_i$ increases (13,14). The data suggest that inositol 1,4,5-trisphosphate may be partly involved in bifonazole-induced Ca^{2+} release because the bifonazole response was partly reduced by inhibition of phospholipase C activity.

Bifonazole-induced Ca^{2+} influx may occur via capacitative Ca^{2+} entry because readmission of Ca^{2+} to cells pretreated with 40 μM bifonazole induced a $[Ca^{2+}]_i$ increase with a maximum value 5-fold of control. Additionally, when added after thapsigargin, bifonazole inhibited 38% of thapsigargin-induced capacitative Ca^{2+} entry. This suggests that bifonazole had dual effect on capacitative Ca^{2+} entry: inducing capacitative Ca^{2+} entry *per se* but inhibiting the capacitative Ca^{2+} entry induced by another substance.

A study conducted in rat hepatocytes showed that bifonazole at a single concentration of 0.5 mM induced a $[Ca^{2+}]_i$ rise (9) by releasing Ca^{2+} from thapsigargin-sensitive Ca^{2+} store without causing Ca^{2+} influx. In our study it was found that in MDCK cells bifonazole at a 10-fold lower concentration range (20-80 μM) was enough to trigger a great $[Ca^{2+}]_i$ increase by releasing internal Ca^{2+} followed by capacitative Ca^{2+} entry. This suggests that bifonazole induced $[Ca^{2+}]_i$ increases via different mechanisms in different cell types.

Collectively, this study is the first to show that bifonazole induced significant increases in renal tubular cells, and to explore the underlying mechanisms. How

imidazoles kill fungi and how these drugs cause side effects in human are unclear. However, one hypothesis is that these drugs act by increasing membrane permeability. This is consistent with our finding that bifonazole can increase $[Ca^{2+}]_i$ because prolonged $[Ca^{2+}]_i$ increases or altered Ca^{2+} handling lead to cytotoxicity in all kinds of cell types (22-24). Also the data may provide a clue for elucidating the mechanism underlying bifonazole's antifungal action which still remains elusive. Although the data about the plasma level that bifonazole can achieve after oral taking is lacking, it was shown that ketoconazole, an antifungal imidazole very similar to bifonazole, can reach a plasma level of about 20 μM 2 hours after an oral dose of 400 mg (25). Because the data in this study show that 10 μM bifonazole induced significant $[Ca^{2+}]_i$ increases, the *in vivo* effect of bifonazole on $[Ca^{2+}]_i$ may contribute to its antifungal action and side effects.

Acknowledgments

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