Blockade of $K^+$ and $Ca^{2+}$ Channels by Azole Antifungal Agents in Neonatal Rat Ventricular Myocytes

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Antibiotics are widely used throughout the world. However, despite their therapeutic effectiveness, some promising antibiotics have been withdrawn from the market due to their life-threatening side effects such as ventricular arrhythmias. Among antibiotics, azole family antifungal agents have been considered relatively safe. However, recent studies suggest that azole antifungal agents such as miconazole, ketoconazole, fluconazole, and itraconazole are associated with acquired long QT (LQT) syndrome and ventricular arrhythmias. Ventricular arrhythmias caused by antifungal agents seem to be related to the human-ether-a-go-go-related gene (HERG) $K^+$ currents, is largely lacking. Using the whole cell patch-clamp technique, we investigated the effects of four azole agents (miconazole, ketoconazole, fluconazole, and itraconazole) on inward rectifying $K^+$ currents (IK$_{ir}$), voltage-gated $L$-type $Ca^{2+}$ currents (ICa$_L$), and delayed rectifier $K^+$ currents (IK$_{dr}$) in rat neonate ventricular myocytes. Strikingly, miconazole and ketoconazole strongly inhibited IK$_{ir}$, IK$_{dr}$, and ICa$_L$ at clinically relevant concentrations. The IC$_{50}$ values of miconazole for IK$_{ir}$, IK$_{dr}$, and ICa$_L$ inhibition were 2.5, 10.4, and 3.0 $\mu M$, respectively. Fluconazole and itraconazole had relatively little effect on ion currents. These findings indicate that miconazole and ketoconazole are multiple ion channel inhibitors in cardiomyocytes. We suggest that it is necessary to consider this inhibition of ion channels by azole agents when assessing cardiovascular side effects.

Key words azole antifungal agent; arrhythmia; inward rectifier $K^+$ channel; delayed rectifier $K^+$ channel; L-type $Ca^{2+}$ channel; neonate rat cardiomyocyte

The authors declare no conflict of interest. These authors contributed equally to this work.

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Some azole antifungal agents induce long QT syndrome and arrhythmias. Although composite functions of ion channels in cardiomyocytes contribute to the shaping of action potentials, information on the effects of azole antifungal agents on ion currents, except human-ether-a-go-go-related gene (HERG) $K^+$ currents, is largely lacking. Using the whole cell patch-clamp technique, we investigated the effects of four azole agents (miconazole, ketoconazole, fluconazole, and itraconazole) on inward rectifying $K^+$ currents (IK$_{ir}$), voltage-gated $L$-type $Ca^{2+}$ currents (ICa$_L$), and delayed rectifier $K^+$ currents (IK$_{dr}$) in rat neonate ventricular myocytes. Strikingly, miconazole and ketoconazole strongly inhibited IK$_{ir}$, IK$_{dr}$, and ICa$_L$ at clinically relevant concentrations. The IC$_{50}$ values of miconazole for IK$_{ir}$, IK$_{dr}$, and ICa$_L$ inhibition were 2.5, 10.4, and 3.0 $\mu M$, respectively. Fluconazole and itraconazole had relatively little effect on ion currents. These findings indicate that miconazole and ketoconazole are multiple ion channel inhibitors in cardiomyocytes. We suggest that it is necessary to consider this inhibition of ion channels by azole agents when assessing cardiovascular side effects.

Key words azole antifungal agent; arrhythmia; inward rectifier $K^+$ channel; delayed rectifier $K^+$ channel; L-type $Ca^{2+}$ channel; neonate rat cardiomyocyte

MATERIALS AND METHODS

Animal and Ventricular Myocyte Preparation Cardiac myocytes were isolated from neonatal Sprague-Dawley (1–2 d old) rat hearts (Nara Biotech, Seoul, Korea) using a previously reported method with some modifications. Ventricular parts of neonatal rats were excised (the approximate downstream one-third part), and ventricular tissues were minced on ice (approximately 1–2 mm). The minced tissues were treated...
with an enzyme solution containing 0.1% collagenase (Wako, Osaka, Japan), 0.1% trypsin, and 1% glucose in Ca²⁺/Mg²⁺-free phosphate buffered saline at 37°C for 10 min. After the supernatants from the first digestion were discarded, three digestions of 10 min each were performed with the same enzyme solution. The supernatants were stored in Dulbecco's modified Eagle's medium (DMEM)/F-12 culture medium containing 10% fetal bovine serum, 5% horse serum, penicillin-streptomycin (100 U/mL and 100 µg/mL, respectively) in a 4°C ice chamber and then centrifuged for 7 min at 700g. The cell pellets were incubated to attach to non-cardiac myocytes at 37°C in a 95% O₂ incubator for 1.5 h. Then, the cells were cultured on microscope cover glasses for 3 d at 37°C in a 95% O₂ incubator, and the 3–5 d cultured cells were used for ion current recordings. 

Electrophysiological Recordings Membrane currents were recorded under the conventional whole-cell patch-clamp configuration as described previously. 15) An Axopatch 200B patch-clamp amplifier and a DigiData 1200 interface (Axon Instruments, Foster City, CA, U.S.A.) were used for voltage-clamp and data acquisition, respectively. Membrane current data were digitized using pClamp 6 software (Axon Instruments) at a sampling rate of 10 kHz, low-pass filtered at 1 kHz, and stored on a computer. Voltage pulse generation was also controlled with pClamp 6 software. The patch pipettes were pulled from borosilicate capillaries (Clark Electromedical Instruments, Pangbourne, U.K.) using a puller (PP-83; Narishige, Tokyo, Japan). We used patch pipettes with a resistance of 2–4 MΩ when filled with pipette solutions. All experiments were carried out at room temperature (20–25°C).

Drugs and Solutions Normal Tyrode solution [143 mM NaCl, 5.4 mM KCl, 0.33 mM NaH₂PO₄, 1.8 mM CaCl₂, 0.5 mM MgCl₂, 5 mM 4-(2-hydroxyethyl)-1-piperazineneethanesulfonic acid (HEPES), and 11 mM glucose, adjusted to pH 7.4 with NaOH] was used as the bathing solution in the patch-clamp study. The pipette solution for recording K⁺ current contained 135 mM KCl, 5 mM NaCl, 10 mM HEPES, 5 mM ethylene glycol bis(2-aminoethyl)-ether-N,N′,N′,N′-tetraacetic acid (EGTA), 10 mM Na₂EDTA, 5 mM Mg-ATP; pH was adjusted to 7.2 with KOH. The pipette solution for recording Ca²⁺ current contained 115 mM CsCl, 10 mM HEPES, 10 mM EGTA, 5 mM Mg-ATP, 5 mM creatine phosphate disodium salt; pH was adjusted to 7.3–7.4 with CsOH.

All chemicals and drugs were purchased from Sigma-Aldrich (St. Louis, MO, U.S.A.). Miconazole, ketoconazole, fluconazole, and itraconazole were prepared as stock solutions in dimethyl sulfoxide (DMSO). The drugs were diluted in the bathing solution on the day of the experiment. The final concentration of DMSO was <1%, and DMSO at this concentration had no effect on membrane current.

Data Analysis Origin 6.0 software (Microcal Software, Northampton, MA, U.S.A.) was used for the data analysis. Half inhibition concentration (IC₅₀) and Hill coefficients (n) were obtained by fitting concentration–response data to the following Hill equation:

\[ f = \frac{100 \times (1 - 1 / (1 + (IC_{50} / [C])^n))}{1 + (IC_{50} / [C])^n} \]

where f is the % of control [100−(I_{drug} / I_{control})], and [C] represents different drug concentrations. In some cases, in which the inhibitions of membrane currents by drugs were incomplete (such as inhibitions of IK_{Ca} and IC_{aL} by ketoconazole in Fig. 4B), the logistic function of Origin 6.0, instead of the Hill equation, was used for fitting the concentration–response data.

Statistical Analysis Results are presented as mean± standard error of the mean. Paired t-tests were used to test for significance, as appropriate. Statistical significance was set at \(a=0.05\).

RESULTS

Ion Currents in Neonatal Rat Ventricular Myocytes We elicited membrane currents using brief voltage ramps from −120 mV to +50 mV at a holding potential of −80 mV to examine whether the ion channels of neonatal rat ventricular myocytes have normal function (Fig. 1, inset). Representative current tracings in the absence and presence of various ion channel inhibitors are shown in Fig. 1A. Control current tracings in Figs. 1A and B are very similar to those of ventricular myocytes that were elicited by similar voltage ramps in previous reports. BaCl₂ (100 µM), a relatively selective cardiac K⁺ channel inhibitor, markedly suppressed the inward current during hyperpolarization without effects on other currents. Nifedipine, a selective Ca_{aL} inhibitor, also selectively suppressed the bell-shaped inward current during depolarization. The voltage-gated K⁺ channel inhibitors 4-aminopyridine (5 mM) and tetraethylammonium (1 mM) mainly suppressed the outward current during depolarization. The sharp and large inward currents, which appeared before the bell-shaped inward currents, were completely abolished by N-methyl-d-glutamine substitution of Na⁺ in the bathing solution (Fig. 1B). These results suggest that neonatal rat ventricular myocytes functionally expressed cardiac specific ion channels such as K_{m}, K_{dL}, and Ca_{aL}, as well as voltage-gated Na⁺ channels.

Effects of the Azole Antifungal Agents on Ion Currents in Neonatal Rat Ventricular Myocytes Typical cardiac ion currents were also recorded with voltage steps (Fig. 2, inset). Representative control current traces are presented in the left panel of Fig. 2. To examine the acute effects of the four azole antifungal agents, we applied each drug at a single concentration of 10 µM for 10 min and compared the currents traces in the absence and presence of each azole agent.
Miconazole and ketoconazole markedly inhibited the ion currents elicited by the voltage steps (Figs. 2A, B). In contrast to miconazole and ketoconazole, fluconazole and itraconazole had little effect on ion currents (Figs. 2C, D). The average current–voltage ($I$–$V$) relationships in the absence and presence of the azole agents are displayed in the right panel of each Fig. The current amplitudes were measured from $-130$ mV to $+70$ mV at the end of the voltage steps. The raw current traces and $I$–$V$ relationships in Figs. 2A and B suggest that miconazole and ketoconazole inhibited IK$_{ir}$ and ICa$_L$ as well as IK$_{dr}$.

**Concentration-Dependent Effects of the Azole Antifungal Agents on the IK$_{ir}$, IK$_{dr}$, and ICa$_L$ in Neonatal Rat Ventricular Myocytes**

We examined the effects of cumulatively applying the azole agents on ion currents to analyze the concentration–response relationship of the azole agent-induced inhibition of each ion current type. The shape of the voltage steps for eliciting the IK$_{ir}$ and IK$_{dr}$ is shown as an inset in Fig. 3 (top of left panel). The IK$_{ir}$ was assessed at $-120$ mV, and the IK$_{dr}$ was assessed at $+50$ mV. Miconazole and ketoconazole concentration-dependently inhibited the IK$_{ir}$ and

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**Fig. 2.** Effects of Azole Family Antifungal Agents on Whole Cell Currents in Neonatal Rat Ventricular Myocytes

Currents were elicited by voltage steps from a holding potential of $-80$ mV. The shape of the voltage steps is shown in the inset at the top of the figure. (A) Representative recording of whole cell currents in the absence and presence of miconazole (10 µM) and their average steady state current–voltage ($I$–$V$) relationship. (B) Representative recording of whole cell currents in the absence and presence of ketoconazole (10 µM) and their average steady state $I$–$V$ relationship. (C) Representative recording of whole cell currents in the absence and presence of fluconazole (10 µM) and their average steady state $I$–$V$ relationship. (D) Representative recording of whole cell currents in the absence and presence of itraconazole (10 µM) and their average steady state $I$–$V$ relationship. *$p<0.05$, **$p<0.01$ vs. control. Numbers in parentheses indicate numbers of cells examined.
Inhibition of IK_dr was also evident in the tail currents, which reflect mainly HERG type K\textsuperscript{+} currents, during repolarizations to \(-40\) mV following step depolarizations to \(+50\) mV. Although fluconazole significantly inhibited both the IK_dr and tail currents (Fig. 3C), the degree of inhibition was small (Fig. 4).

Effects on IC\textsubscript{aL} were also analyzed. A CsCl-rich pipette solution was used to elicit the IC\textsubscript{aL}. The voltage-step protocol for eliciting IC\textsubscript{aL} is shown as an inset in Fig. 3 (top of right panel). The voltage step to 0 mV followed the prepulse to \(-40\) mV from the holding potential of \(-80\) mV to inactivate voltage-gated Na\textsuperscript{+} currents. The peak IC\textsubscript{aL} was measured at a voltage of 0 mV. Representative current tracings in the absence and presence of eachazole agent are shown in the right panel of Figs. 3A–C. Both miconazole and ketoconazole concentration-dependently inhibited IC\textsubscript{aL}, whereas fluconazole had little
Because of a solubility limitation, itraconazole was examined up to a concentration of 10 \( \mu M \), and the effects on the IK_{ir}, IK_{dr}, and ICa_{L} are summarized in Fig. 4.

The concentration-dependent effects of eachazole agent are summarized as concentration–response curves in Fig. 4. The IC_{50} of miconazole was 2.5 \( \mu M \) for IK_{dr}, 10.4 \( \mu M \) for IK_{ir}, and 3.0 \( \mu M \) for ICa_{L} (Fig. 4A). The IC_{50} of ketoconazole was 3.2 \( \mu M \) for IK_{dr}, 20.8 \( \mu M \) for IK_{ir}, and 3.5 \( \mu M \) for ICa_{L} (Fig. 4B). Because the inhibition by fluconazole and itraconazole was minimal (Figs. 4C,D), we did not obtain the correct IC_{50} values for the ion currents at the drug concentrations examined.
DISCUSSION

The major finding of this study was that miconazole and ketoconazole strongly inhibited the IK_{dr}, IK_{ir}, and IK_{m} at clinically relevant concentrations in rat neonatal ventricular myocytes. This is the first report of inhibition of IK_{dr} and IK_{m} by these two azole antifungal agents and should be considered when assessing their cardiovascular side effects. In contrast to these two azole agents, fluconazole and itraconazole had little effect on the ion currents in rat neonate myocytes.

The inhibition of the IK_{dr} by miconazole (IC_{50}=2.5µM) in the present study was similar to that of HERG K^{+} currents previously reported by Kikuchi et al. in which the IC_{50} of miconazole for HERG K^{+} current inhibition was 2.2µM. The clinical range of miconazole is 2.3–8.4µM.

In addition to IK_{dr}, IK_{ir} activity is also closely related to the QT interval; inhibition of IK_{dr} prolongs the action potential whereas activation of IK_{dr} shortens it. Short QT syndrome is a recently accepted disorder associated with atrial fibrillation. Recently, K_{ir} (KCNJ2) gain-of-function mutation has been reported to cause short QT syndrome by decreasing inward rectification of IK_{ir}.

Therefore, IK_{ir} inhibition (IC_{50}=10.4µM in this study) by miconazole may potentiate the inhibition of repolarization and prolong the QT interval. Although miconazole has been regarded as relatively safe among the azole antifungal agents, the results of this study suggest that miconazole is associated with prolongation of the QT interval and/or arrhythmias by inhibiting both the IK_{dr} and IK_{ir} in cardiomyocytes.

Similar to miconazole, ketoconazole also inhibited ion currents. The IC_{50} of ketoconazole was 3.2µM for IK_{dr} inhibition and 20.8µM for IK_{ir} inhibition (Fig. 4). The IC_{50} value (3.2µM) of ketoconazole for IK_{dr} inhibition was lower than values from a previous report. Dumaïne et al. reported IC_{50} values of 49µM and 107µM for the HERG and Kv1.5 currents. This difference probably results from differences between the expression system (to Xenopus oocytes) and native cells. Similar to our results, Chen and Woosley reported that ketoconazole blocks the IK tail current with an IC_{50} of 2.5µM in cat ventricular myocytes. They also reported that the transient outward K^{+} current is inhibited by ketoconazole and that the action potential plateau is extended by around 15% in cat ventricular myocytes. Interestingly, the IC_{50} (20.8µM) of ketoconazole for IK_{ir} inhibition in this study was similar to the reported value for HERG or Kv1.5 current inhibition.

We report the potent blocking effects of miconazole (IC_{50}=3.0µM) and ketoconazole (IC_{50}=3.5µM) on IK_{ir} for the first time. Blocking IK_{ir} decreases cardiac contractility by decreasing both the Ca^{2+} influx across plasmalemmal membranes and the subsequent Ca^{2+}-induced Ca^{2+} release from sarcoplasmic reticulum in cardiomyocytes. Accordingly, an IC_{ir} blocker verapamil was reported to produces negative inotropic effects in hearts, indicating that miconazole and ketoconazole may also cause negative inotropic effects in a similar way. Although the chronotropic effect of miconazole and ketoconazole is not evident, IK_{dr} blockers such as nifedipine and diltiazem are recognized to have negative chronotropic effect on the isolated sinoatrial node cells, suggesting that miconazole and ketoconazole could cause similar effects.

In clinical cases, however, the IK_{ir} blockers such as nifedipine and diltiazem cause the sympathetic reflex tachycardia because of their hypotensive effects.

Because IK_{ir} contribute to maintaining the plateau phase of ventricular action potential, inhibition of IK_{ir} results in shortening of the action potential. We speculate that this effect of IK_{ir} inhibition may compensate for the blocking effects of miconazole and ketoconazole on the IK_{dr}, of which their effect is prolongation of action potential. In this regard, Xu et al. reported that drugs with a dual blocking action against HERG K^{+} and Ca^{2+} channels have relatively lower risks for arrhythmias than drugs with selective blocking action against HERG K^{+} current. This may explain the relative safety of miconazole and ketoconazole despite their blocking effects on multiple K^{+} channels such as HERG/K_{ir} and K_{dr}.

In contrast to miconazole and ketoconazole, fluconazole and itraconazole had little effect on the ion channels of rat neonate ventricular myocytes. Although fluconazole inhibited the IK_{dr} significantly, the inhibition was small (Fig. 4). However, Han et al. reported that fluconazole inhibits HERG K^{+} channels expressed in HEK293 cells both by directly blocking them and by disrupting protein trafficking. The reason for the discrepancy is not clear but it does not seem to be due to differences between the expression system and native cells, because fluconazole did not inhibit currents through HERG K^{+} channels that were expressed in HEK293 cells (unpublished observation).

Azole family antifungal agents are antibiotics prescribed widely for subcutaneous and superficial mycosis. Although they are relatively safe compared to amphotericin, a growing body of evidence suggests that they are associated with cardiac arrhythmias including long QT syndrome. Accordingly, the results of the present study indicate that miconazole and ketoconazole are potent inhibitors of ion channels such as K_{ir}, Ca_{dr}, and K_{dr}. In particular, because such an inhibition was observed in their clinical prescription concentration range, careful and extensive research is required for safe drug prescriptions in the future.

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