The Dual Mode of Inhibition of Calmodulin-dependent Nitric-oxide Synthase by Antifungal Imidazole Agents*

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The antifungal imidazoles miconazole, ketoconazole, and clotrimazole inhibit citrulline formation by nitric-oxide synthase. These agents both increase the concentration of calmodulin required to activate the enzyme half-maximally and reduce the maximal velocity of citrulline formation. This inhibition was not reversed by increased concentrations of either the arginine substrate or (6R)-5,6,7,8-tetrahydro-L-biop terin. Miconazole, ketoconazole, and clotrimazole also inhibited the cytochrome-c reductase activity of nitric-oxide synthase competitively versus calmodulin concentration, with apparent $K_i$ values of 8, 20, and 0.8 μM, respectively. Miconazole, ketoconazole, and clotrimazole inhibited the activity of calmodulin-dependent cyclic nucleotide phosphodiesterase competitively versus calmodulin concentration, with apparent $K_i$ values of 6, 18, and 25 μM, respectively.

These observations are consistent with the proposal that the antifungal imidazoles inhibit citrulline formation by interaction with the nitric-oxide synthase at two sites. Interaction at site 1 reduces the responsiveness of the enzyme to activation by calmodulin, whereas interaction at site 2 (involving putative binding of the imidazole to the heme iron) reduces the maximal velocity of citrulline formation. The interactions of calmodulin antagonists at site 1 occur at substantially lower concentrations of drug than those at site 2 and are the principal determinant of enzyme inhibition.

Miconazole, clotrimazole, and ketoconazole are antifungal agents that inhibit ergosterol biosynthesis in yeast by inhibiting 14α-demethylase, a microsomal cytochrome P-450-dependent enzyme system. 14α-Methyl sterols that accumulate intracellularly are incorporated into the lipid bilayer, disrupting its structure and consequently cellular growth (1). Imidazole antifungal agents have been shown to inhibit a variety of cytochrome P-450-dependent enzyme activities including steroid aromatase (2) and rat liver microsomal cytochrome P-450 isoforms (3). The imidazole antifungal agents, however, do not inhibit cytochrome-P-450 reductase activity. Miconazole, ketoconazole, and itraconazole have been shown to bind to cytochrome P-450 isoforms derived from such divergent microsomal sources as human placenta (2) and Saccharomyces cerevisiae (4), producing Type II difference spectra. The formation of these spectra is consistent with the interaction of their imidazole nitrogens with the heme iron. The binding of the imidazoles to the heme iron was found to interfere with the binding of carbon monoxide to these hemoproteins and presumably similarly inhibits the ability of oxygen to bind.

Recent investigations have shown that imidazole antifungal agents including miconazole, clotrimazole, and ketoconazole inhibit the operation of receptor-operated Ca2+ channels (5), voltage-gated Ca2+ channels in GH3 and adrenal chromaffin cells (6), and Ca2+-dependent K+ channels (7). These observations have been interpreted to indicate that a hemoprotein is involved in the regulation of these ion channels.

In the accompanying paper (8), we have shown that imidazole and phenylimidazoles inhibit the activity of CaM1-dependent nitric-oxide synthase from bovine brain. This observation encouraged us to explore the effects of antifungal imidazoles on nitric-oxide synthase. We report here that miconazole, clotrimazole, and ketoconazole inhibit the CaM-dependent nitric-oxide synthase by interaction at two sites. By interaction at the first site, these drugs act as competitive CaM antagonists; by interaction at the second site, they inhibit citrulline formation in a manner not reversible by elevated concentrations of CaM, THB, or the arginine substrate.

EXPERIMENTAL PROCEDURES

Materials—Miconazole, clotrimazole, PIPES, affinity-purified bovine intestinal mucosal alkaline phosphatase, dimethyl sulfoxide, guanosine 3',5'-cyclic phosphate, and p-nitrophenyl phosphate were obtained from Sigma. Ketoconazole and itraconazole were obtained from Research Diagnostics (Flanders, NJ). [6,5-3H]Guanosine 3',5'-cyclic phosphate (32.8 Ci/mmol) was obtained from Du Pont-New England Nuclear. Adenosine 5'-[γ-3P]Triphosphate (10-25 Ci/mmol) was purchased from ICN Biomedicals (Irvine, CA). CaM-dependent cyclic nucleotide phosphodiesterase was prepared from bovine brain by the procedure of Brostrom and Wolff (9). CaM-dependent phosphatase (calcineurin) was prepared from bovine brain by the procedure of Wolff and Sved (10). Myelin basic protein was prepared from bovine spinal cord by the procedure of Oshiro and Eyer (11). The catalytic subunit of CaM-dependent protein kinase was prepared from bovine skeletal muscle by the procedure of Beavo et al. (12). CaM-dependent nitric-oxide synthase was prepared from bovine brain and GH3 cells as described previously (Refs. 8 and 13, respectively). All other reagents were obtained as described previously (8).

Assay of Citrulline Formation by Nitric-oxide Synthase—Standard incubations were prepared and conducted, and citrulline formation was assessed as described previously (8). Due to the limited water solubility of the antifungal imidazoles, they were added to the incubation mixtures as 5-μl portions dissolved in dimethyl sulfoxide to a 150-μl incubation. Control incubations contained dimethyl sulfoxide alone. All values are expressed relative to the dimethyl sulfoxide control.

Assay of Cytochrome-c Reductase Activity of Nitric-oxide Syst-
Citrulline formation, but did not alter the was determined at saturating concentrations of CaM (Fig. 2). Antifungal imidazoles were added to the incubation as 10-μl portions dissolved in dimethyl sulfoxide. Control incubations contained dimethyl sulfoxide alone. All values are expressed relative to the dimethyl sulfoxide control.

Assay of CaM-dependent Cyclic Nucleotide Phosphodiesterase Activity—Cyclic nucleotide phosphodiesterase activity measurements were performed by the procedure of Brostrom and Wolff (9) as modified by Brooker et al. (14) as described previously (8). Standard incubations were constructed in a reaction volume of 150 μl containing 20 mM HEPES, pH 7.5, 5 mM MgC\textsubscript{2}, 20 mM p-nitrophenyl phosphate, and 100,000 cpm [\textsuperscript{3}H]GMP. Incubations initiated by the addition of phosphodiesterase were conducted at 30 °C for 30 min, and [\textsuperscript{3}H]guanosine formation was assessed. Antifungal imidazoles were added to the incubations as 5-μl portions dissolved in dimethyl sulfoxide. Control incubations were conducted with dimethyl sulfoxide alone. Values are expressed relative to the dimethyl sulfoxide control.

Assay of CaM-dependent Phosphatase Activity—Phosphatase activity measurements were made using either p-nitrophenyl phosphate or \textsuperscript{3}P-labeled myelin basic protein as substrate. The hydrolysis of p-nitrophenol was measured in standard incubations of 1 ml in disposable plastic cuvettes containing 50 mM HEPES, pH 7.5, 100 mM KC\textsubscript{1}, 20 mM p-nitrophenol, and 1 mM MnC\textsubscript{2} with or without CaM. The formation of p-nitrophenol was assessed as the increase in absorbance at 405 nm over time. Incubations were initiated by the addition of homogenous bovine brain calcineurin. Incubations without added CaM had no detectable activity. Antifungal imidazoles were added to the incubations as 10-μl portions in methanol. Control incubations were conducted with methanol alone. Values are expressed relative to the methanol control.

The hydrolysis of \textsuperscript{3}P-labeled myelin basic protein was measured according to the procedure of Wolff et al. (15). Standard incubations of 100 μl were constructed containing 20 mM PIPES, pH 7.0, 100 mM KC\textsubscript{1}, 15 μM \textsuperscript{3}P-labeled myelin basic protein, 120 nM CaM, and either 100 μM EGTA or 200 μM Ca\textsuperscript{2+}. The presence of CaM stimulated the dephosphorylation of myelin basic protein 7-fold. Antifungal imidazoles were added as 5-μl portions dissolved in methanol. Methanol (5%) was found not to affect the myelin basic protein dephosphorylation rate.

RESULTS

Effect of Miconazole on Formation of Citrulline by Nitric-oxide Synthase—Initial experiments exploring the effects of miconazole on citrulline formation by bovine brain nitric-oxide synthase revealed that the CaM concentration affected the apparent \textit{K}_e (IC\textsubscript{50}) value for miconazole (data not shown). A detailed examination was therefore undertaken of the effect of miconazole on the CaM concentration dependence of citrulline formation by nitric-oxide synthase (Fig. 1). Miconazole was found to antagonize the activation of nitric-oxide synthase by CaM. As measured without miconazole, the concentration of CaM required to effect half-maximal activation (\textit{K}_e) of citrulline formation was 2 nM CaM. The concentrations of CaM required to effect half-maximal activation in the presence of 10 and 33 μM miconazole (apparent \textit{K}_e values) were 7 and 15 nM CaM, respectively. Miconazole was also found to alter the maximal velocity of citrulline formation as measured at saturating concentrations (600 nM to 2 μM) of CaM. At 10 and 33 μM miconazole, the maximal rate of citrulline formation was reduced to 85 and 61%, respectively, of the value observed in the absence of miconazole. Elevated concentrations of CaM were thus able to reverse partially the inhibitory actions of miconazole on citrulline formation.

The effect of miconazole on the arginine concentration dependence of citrulline formation by nitric-oxide synthase was determined at saturating concentrations of CaM (Fig. 2). Miconazole was found to decrease the maximal velocity of citrulline formation, but did not alter the \textit{K}_a of the enzyme for the arginine substrate, a noncompetitive pattern of inhibition.

Nitric-oxide synthase derived from GH\textsubscript{3} cells has been found to exhibit much higher degrees of dependence on exogenous THB for activity than has routinely been found for the enzyme purified from bovine brain extract (13). Accordingly, we decided to examine the effect of miconazole on the apparent \textit{K}_a of bovine brain nitric-oxide synthase.
the THB concentration dependence of citrulline formation at saturating concentrations of CaM (Fig. 3) using the GH3 cell-derived enzyme. Miconazole displayed a noncompetitive pattern of inhibition, decreasing the maximal velocity of citrulline formation, but not altering the concentration of THB required for half-maximal activation (apparent $K_{m}$) of citrulline formation.

**Effect of Ketoconazole, Clotrimazole, and Itraconazole on Citrulline Formation by Nitric-oxide Synthase**—To explore whether the inhibitory properties of miconazole are unique or are shared more broadly by other antifungal agents, we examined the effects of ketoconazole, clotrimazole, and itraconazole on the CaM dependence of citrulline formation. Ketoconazole (Fig. 4A) antagonized the activation of citrulline formation by CaM. When measured without ketoconazole, a $K_{m}$ of 4 nM CaM was observed. At 150 and 300 μM ketoconazole, apparent $K_{m}$ values of 18 and 31 nM CaM were observed, respectively. Ketoconazole also reduced the maximal velocity of citrulline formation measured at saturating concentrations (600 nM to 10 μM) of CaM. At 150 and 300 μM ketoconazole, maximal velocities were 75 and 56%, respectively, of the value obtained in the absence of ketoconazole.

Clotrimazole (Fig. 4B) also antagonized the activation of citrulline formation by CaM. When measured without clotrimazole, a $K_{m}$ of 5 nM CaM was observed. In the presence of 30 and 100 μM clotrimazole, apparent $K_{m}$ values of 16 and 25 nM CaM were observed, respectively. Clotrimazole also reduced the maximal velocity of citrulline formation measured at saturating concentrations (600 nM to 10 μM) of CaM. At 30 and 100 μM clotrimazole, maximal velocities were 94 and 75%, respectively, of the value obtained in the absence of clotrimazole.

Itraconazole (data not shown) at a concentration of 100 μM did not alter the CaM concentration dependence of citrulline formation, but reduced the maximal velocity of citrulline formation to 71% of its control (without drug) value. Thus, miconazole, ketoconazole, and clotrimazole antagonized the effects of CaM on citrulline formation, whereas itraconazole did not.

**Effect of Antifungal Imidazoles on Cytochrome-c Reductase Activity of Nitric-oxide Synthase**—Since miconazole, ketoconazole, and clotrimazole were found to antagonize the activation of nitric-oxide synthase by CaM, we examined whether these drugs would also affect the activation by CaM of the cytochrome-c reductase activity of this enzyme. Miconazole (Fig. 5A), ketoconazole (Fig. 5B), and clotrimazole (Fig. 5B) were each found to inhibit the CaM-dependent stimulation of cytochrome c reduction. Since the effects of these drugs on citrulline formation had been found to be largely reversible by saturating concentrations of CaM, these measurements were conducted at subsaturating concentrations of CaM. Apparent $K_{i}$ (IC$_{50}$) values of 8, 0.8, and 20 μM were observed for miconazole, clotrimazole, and ketoconazole, respectively.

To explore further the mechanism of this inhibition, we examined the CaM concentration dependence of cytochrome c reduction in the absence and presence of either 10 or 33 μM miconazole (Fig. 6). Miconazole was found to be a competitive inhibitor of cytochrome c reduction, that is, the inhibition could be completely reversed by elevated concentrations of CaM. This contrasts with its behavior in inhibiting citrulline formation, where the effect of miconazole on activity could be overcome only partially by elevated concentrations of CaM.

**Reversibility of Inhibitory Properties of Antifungal Imidazoles**—Since the inhibition of cytochrome-c reductase activity by the antifungal imidazoles was eliminated in incubations containing elevated concentrations of CaM, we decided to examine whether CaM was able to reverse inhibition or whether its concurrent presence prevented these drugs from exerting their actions. Standard incubations containing 60 nM CaM were initiated with enzyme, and reduction was allowed to proceed for 2 min. At this point, miconazole, ketoconazole, or clotrimazole was added to the incubations at inhibitory concentrations near their observed IC$_{50}$ values. Activity was found to be immediately inhibited, and the degree of inhibition did not progress with time. After an additional 2 min, incubations were adjusted to contain 6 μM CaM, and cytochrome c reduction was allowed to continue. Cytochrome c reduction rates were routinely found to be restored to values 90–100% of their initial rate prior to drug addition (data not shown).
Fig. 5. Effect of miconazole (A) and ketoconazole and clotrimazole (B) on CaM-dependent cytochrome-c reductase activity of bovine brain nitric-oxide synthase. Standard incubations were constructed as described under "Experimental Procedures" with a sub saturating concentration of CaM (20 nM) and the indicated concentrations of miconazole (A, ●), clotrimazole (B, ○), or ketoconazole (B, △). Incubations were initiated with 9 μg of bovine brain nitric-oxide synthase, and the reduction of cytochrome c was measured. Values are expressed as the percentage of that observed in the control incubation without drug (555 nmol of cytochrome c reduced per minute/milligram).

Fig. 6. Effect of miconazole on CaM concentration dependence of cytochrome-c reductase activity of bovine brain nitric-oxide synthase. Standard incubations were constructed as described under "Experimental Procedures" without (●) or with either 10 (△) or 33 (□) μM miconazole and the indicated concentrations of CaM. Incubations were initiated with 9 μg of bovine brain nitric-oxide synthase, and reduced cytochrome c formation was measured as the change in absorbance at 550 nm. Values are expressed as the percentage of control values (988 nmol of cytochrome c reduced per minute/milligram) observed in incubations containing a saturating concentration of CaM (6 μM).

 shown). Similar experiments employing the citrulline formation assay revealed that CaM could partially restore activity. In experiments in which nitric-oxide synthase was preincubated in the absence or presence of CaM with the antifungal agents present at concentrations >5-fold in excess of their IC₅₀ values and diluted up to 300-fold in the subsequent assay, complete reversal of their inhibition of citrulline formation could be achieved (data not shown). These observations are consistent with the interpretation that the antifungal imidazoles interact with the enzyme system in a manner fully reversible by their dilution. In the presence of drug at inhibitory concentrations, their inhibitory actions versus cytochrome c reduction are fully reversible by CaM, whereas their inhibitory actions versus citrulline formation are only partially reversible by CaM.

Effect of Antifungal Imidazoles on CaM-dependent Cyclic Nucleotide Phosphodiesterase and Phosphatase Activities—Since the antifungal imidazoles had been found to act as antagonists of the effects of CaM on nitric-oxide synthase, we decided to examine whether this property was restricted to nitric-oxide synthase or was shared with other CaM-regulated enzyme activities. Miconazole, ketoconazole, clotrimazole, and itraconazole were examined for effects on CaM-dependent cyclic nucleotide phosphodiesterase activity at subsaturating concentrations of CaM (Fig. 7A). Itraconazole had no effect on activity at concentrations as high as 300 μM. Miconazole, ketoconazole, and clotrimazole were found to inhibit activity profoundly, with apparent IC₅₀ values of 6, 18, and 25 μM, respectively. To characterize further the mechanism by which these antifungal agents inhibited activity, we examined the CaM concentration dependence of cyclic nucleotide hydrolysis in incubations with or without either 10 or 33 μM miconazole (Fig. 7B). Miconazole was found to inhibit CaM-dependent cyclic nucleotide hydrolysis in a competitive manner, with complete reversal of inhibition by elevated concentrations of CaM.

Miconazole, ketoconazole, clotrimazole, and itraconazole were also examined for effects on CaM-dependent hydrolysis of both p-nitrophenyl phosphate and ³²P-labeled myelin basic protein by bovine brain calcineurin. No effects on either activity by any of the drugs were observed.

Comparison of Inhibition of Diverse CaM-dependent Activities by Antifungal Imidazoles—The ability of miconazole, ketoconazole, and clotrimazole to inhibit citrulline formation by nitric-oxide synthase appears to be exerted by interactions at two sites. These agents appear to act at one site to diminish the sensitivity of the enzyme to activation by CaM and at a second site to reduce the maximal velocity of citrulline formation. Miconazole also acts both to inhibit the CaM-dependent activation of the cytochrome-c reductase activity of nitric-oxide synthase (Figs. 5 and 6) and to inhibit the CaM-dependent activation of cyclic nucleotide phosphodiesterase (Fig. 7) in a competitive manner. Ketoconazole and clotrimazole presumably behave similarly. The apparent Kᵢ (IC₅₀) values for inhibition of these activities measured at subsaturating CaM concentrations are provided in Table I (first and second columns). Miconazole, ketoconazole, and clotrimazole also alter the apparent Kᵢ values for activation of citrulline formation by CaM (Fig. 1 and 4, A and B). This effect is presumably produced by interaction at the same site responsible for competitive inhibition of cytochrome c reduction by
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Fig. 7. A, effect of antifungal imidazoles on activity of CaM-dependent cyclic nucleotide phosphodiesterase. Standard incubations were constructed as described under "Experimental Procedures" with 60 nM CaM (subsatrating) and the indicated concentrations of miconazole (●), ketoconazole (△), clotrimazole (□), and itraconazole (○). B, effect of miconazole on CaM concentration dependence of bovine brain cyclic nucleotide phosphodiesterase activity. Standard incubations were constructed without (●) or with either 10 (△) or 33 (□) μM miconazole and the indicated concentrations of CaM. Incubations were initiated with 14 μg of bovine brain cyclic nucleotide phosphodiesterase. Values are expressed as the percentage of control activity in incubations without added drug. For the experiment depicted in A, which was conducted at 60 nM CaM, 22.4 nmol of 5′-GMP were produced per min/ml; for the experiment depicted in B measured at a saturating CaM concentration (4 μM), control activity was 54 nmol of 5′-GMP produced per min/ml.

Table I

Apparent \( K_i \) values for antifungal imidazoles versus diverse CaM-dependent activities

| Drug     | Cytochrome-reductase\( ^a \) | Phosphodiesterase\( ^a \) | Calculated citrulline formation\( ^a \) | GH4 extract citrulline formation\( ^a \) | Citrulline formation at saturating [CaM]\( ^a \) | Calculated value at saturating [CaM]\( ^a \) |
|----------|-------------------------------|-----------------------------|----------------------------------------|----------------------------------------|----------------------------------------|----------------------------------------|
| Miconazole | 6                              | 6                           | 44                                     | 38                                     | 350                                    | 410                                    |
| Ketoconazole | 20                             | 18                          | 19                                     | 20                                     | 240                                    | 390                                    |
| Clotrimazole | 0.8                            | 25                          | 19                                     | 20                                     | 240                                    | 390                                    |

\( ^a \) Apparent \( K_i \) values for the inhibition of CaM-dependent cytochrome c reduction (Fig. 5).

\( ^b \) Apparent \( K_i \) values for the inhibition of CaM-dependent phosphodiesterase (Fig. 6A).

\( ^c \) \( K_i \) values calculated for CaM competition using the expression \( K_{app} = K_{cat}(1 + [I]/K_i) \) using data from Figs. 1 and 4 (A and B).

\( ^d \) Apparent \( K_i \) values measured for the inhibition of citrulline formation in GH4 crude extract.

\( ^e \) Apparent \( K_i \) values measured for citrulline formation by bovine brain nitric-oxide synthase measured at 2 μM CaM.

\( ^f \) \( K_i \) values calculated for noncompetitive inhibition of citrulline formation at saturating [CaM] using the expression \( v_{(max)} = V_{max}/1 + [I]/K_i \) using data from Figs. 1 and 4 (A and B).

the enzyme. For a competitive inhibition (16) of CaM-dependent activation by drug, \( K_{act(app)} = K_{cat}(1 + [I]/K_i) \), where \( K_{act(app)} \) is the concentration of CaM required to activate the enzyme half-maximally in the presence of inhibitor, \( K_{cat} \) is the concentration of CaM required to activate the enzyme half-maximally in the absence of inhibitor, \( I \) is the concentration of inhibitor, and \( K_i \) is the dissociation constant of inhibitor from its site of inhibition. Using this relationship and employing the data from Figs. 1 and 4 (A and B) depicting the CaM dependence of citrulline formation in the presence of miconazole, ketoconazole, and clotrimazole, it is possible to calculate the \( K_i \) values for each drug to a site responsible for the shift of CaM sensitivity. Values of 7, 44, and 19 μM for miconazole, ketoconazole, and clotrimazole were calculated, respectively (Table I, third column). For miconazole and ketoconazole, the calculated \( K_i \) values are in reasonably close agreement with the measured apparent \( K_i \) values for the inhibition of the activation by CaM of both the cyclic nucleotide phosphodiesterase and cytochrome-c reductase activities of bovine brain nitric-oxide synthase. These comparisons suggest that these inhibitions are mediated by binding to a similar site. The calculated \( K_i \) value for clotrimazole agrees well with the \( K_i \) value measured for inhibition of cyclic nucleotide phosphodiesterase activity. The inhibition of CaM-dependent cytochrome c reduction by clotrimazole is, however, exerted at much lower concentrations (IC\( _{50} = 0.8 \) μM). This potent inhibition has been confirmed repeatedly. The basis for this high potency is not known, but may reflect an ability of clotrimazole to interact with the cytochrome c substrate.

The inhibition of the maximal velocity of citrulline formation appears to be mediated by interaction of drug at a second site. This inhibition could not be overcome by saturating concentrations of CaM, arginine, or THB and appeared noncompetitive in nature (Figs. 1–3 and 4, A and B). For a noncompetitive inhibitor (16), \( v_{(max)} = V_{max}/1 + [I]/K_i \), where \( v_{(max)} \) is the maximal velocity expressed in the presence of inhibitor, \( V_{max} \) is the maximal velocity expressed in the ab-
sence of inhibitor, $[I]$ is the concentration of inhibitor, and $K_i$ is the dissociation constant of inhibitor from its site of inhibition. Using this expression and the data for the inhibition of citrulline formation measured at saturating concentrations of CaM provided in Figs. 1 and 4 (A and B), we calculated the $K_i$ value for noncompetitive inhibition of citrulline formation by miconazole, ketoconazole, and clotrimazole. Values of 55, 410, and 300 μM for miconazole, ketoconazole, and clotrimazole were calculated, respectively (Table I, sixth column). To confirm these values calculated from measurements conducted at only two inhibitor concentrations, we measured experimentally the concentration dependence of inhibition by miconazole, ketoconazole, and clotrimazole of citrulline formation by nitric-oxide synthase in incubations containing a saturating concentration of CaM (2 μM). Half-maximal inhibition of citrulline formation was observed at 50, 350, and 240 μM miconazole, ketoconazole, and clotrimazole, respectively (fifth column). These values are in reasonable agreement with the calculated values. It is especially difficult to measure the effects of these drugs at concentrations in excess of 100 μM due to the limited water solubility of the agents and their tendency at high concentrations to form metastable supersaturated aqueous solutions from which they precipitate.

It is clear from the data presented previously that the concentrations of antifungal imidazoles necessary to inhibit half-maximally citrulline formation by nitric-oxide synthase depend critically on the CaM concentrations at which the measurements are conducted. It is known that the concentration of trifluoperazine necessary to exert a 50% inhibition of diverse CaM-regulated enzymes is governed by the molar ratio of excess CaM to CaM-dependent enzyme (17). Our measurements were made using purified nitric-oxide synthase and purified CaM reconstituted at ratios controlled by the experimenter. We were interested in examining the behavior of nitric-oxide synthase in a crude extract of GH3 cells, in which CaM and CaM-dependent nitric-oxide synthase are found at their native ratios. Measurements of the miconazole, ketoconazole, and clotrimazole concentration dependence of inhibition of Ca$^{2+}$-dependent citrulline formation by an unfraccionated GH3 crude extract (10,000 x g x 20 min supernatant) provided apparent $K_i$ values of 6, 38, and 20 μM, respectively (Table I, fourth column). These values very closely resemble those determined for the competitive inhibition of the CaM stimulation of activity. These measurements support the proposal that these antifungal agents would exert their effects in intact cells predominantly, if not exclusively, by interfering with the actions of CaM. Presumably, these effects would not be restricted exclusively to formation of nitric oxide by nitric-oxide synthase, but would be exerted on other CaM-regulated processes as well.

**DISCUSSION**

Miconazole, clotrimazole, and ketoconazole antagonize the activation of nitric-oxide synthase by CaM, exerting effects on cytochrome c reduction that are completely reversible by CaM and on citrulline formation that are partially reversible by CaM. All inhibitory effects of the agents are reversible by dilution. Cytochrome c is reduced by rat liver cytochrome-P-450 reductase using electrons derived from FMN (18). Given the extensive structural homology between nitric-oxide synthase and cytochrome-P-450 reductase (19), it is reasonable to assume that FMN is also the electron donor for nitric-oxide synthase that reduces cytochrome c. The failure of CaM to reverse completely the effects of the antifungal imidazoles on citrulline formation by nitric-oxide synthase while reversing completely those on cytochrome c reduction indicates that these agents possess a site of action distal to FMN in the electron transfer sequence necessary to generate citrulline. This distal site of action is neither the arginine- nor the THB-binding site since inhibition versus these substances was noncompetitive. The combined observations that the imidazole nitrogens of miconazole and ketoconazole bind to the heme iron of cytochrome P-450 isomers (2, 4) to form a Type II difference spectrum and that imidazole and phenylimidazoles prevent the reduction of oxygen by CaM-dependent nitric-oxide synthase (8) support the contention that the imidazole portion of these antifungal agents similarly binds to the heme iron of nitric-oxide synthase (site 2) to reduce the maximal velocity of citrulline formation. These interactions, however, occur at relatively high concentrations (Table I, fifth and sixth columns).

The imidazole antifungal agents miconazole, ketoconazole, and clotrimazole, but not the triazole itraconazole, act as competitive antagonists of the ability of CaM to activate nitric-oxide synthase. This competency was not restricted to nitric-oxide synthase since these agents also inhibit the stimulation of cyclic nucleotide phosphodiesterase activity by CaM. However, the drugs do not affect all CaM-dependent enzymes since no effects of these agents on the phosphatase activity of calcineurin were observed.

The CaM antagonistic actions of the antifungal imidazoles are presumably exerted by binding either to CaM or to the CaM-binding domains of nitric-oxide synthase and phosphodiesterase. In contrast to CaM, whose sequence is well conserved from *Paramecium* to humans (17), the CaM-binding domains of its targets display marked variability in sequence (20–22). CaM is capable of recognizing positively charged amphiphilic α-helical peptides independent of their precise amino acid sequence. The CaM-binding domains of many CaM-regulated enzymes have been narrowed down to ~20 amino acid residues. These assignments have been confirmed by solid-state peptide synthesis, deletion mutagenesis, and chemical modification procedures (23–25). Thus, the amino acid sequences of CaM-binding domains display broad structural homology, but sufficient variability that ligands such as the antifungal imidazoles might bind to some, but not all, such domains. The ability of the antifungal imidazoles to inhibit both CaM-dependent nitric-oxide synthase and phosphodiesterase activities, but not the phosphatase activity, is compatible with the differences in the sequence of their CaM-binding domains.

Not all CaM-regulated enzymes recognize a single common structural feature of calmodulin (26–28). Individual tryptically generated CaM fragments (26) and genetically engineered calmodulins (27) have each been found to activate different CaM-regulated enzymes with dramatically different potencies and efficacies. The covalent adduct of the drug norchlorpromazine and CaM is a competitive antagonist of CaM in stimulating phosphodiesterase and myosin light chain kinase activities, but activates the CaM-dependent phosphatase (28). Thus, different structural features of CaM are involved in the activation of different CaM-dependent activities. Given these considerations, it is not possible from the kinetic data presented in this report to decide unequivocally whether the CaM antagonistic actions of the antifungal imidazoles are exerted by binding to the CaM-binding domains of phosphodiesterase and nitric-oxide synthase or by binding to CaM itself. Additional experiments will be required to resolve this interesting issue.

Miconazole and ketoconazole are used systemically in the treatment of diverse fungal infections, whereas clotrimazole
is used topically (29). Miconazole (30) and ketoconazole (31) inhibit the cytochrome P-450 drug metabolizing system in vivo, whereas itraconazole does not (32). Ketoconazole inhibits steroid hydroxylation reactions involved in the biosynthesis of testosterone, estrogen, glucocorticoids, and 1,25-dihydroxycholecalciferol (33) and accordingly has been used to treat prostate and breast carcinomas, hyperadrenalinism, and hypercalcemia. Miconazole conventionally achieves a maximal therapeutic plasma concentration of 10 µg/ml, of which 92% is bound to albumin, while 8% is found free in the water phase of plasma (34). Ketoconazole conventionally achieves a maximal therapeutic plasma concentration of 7 µg/ml, of which 95% is bound to plasma albumin (35). Despite this extensive albumin binding, both drugs achieve high volumes of distribution, exceeding 150 liters/70 kg, indicating that most of the drug in the body is associated either with lipids or macromolecular cellular constituents. The maximal free concentration of ketoconazole distributed throughout the water phase of body tissues is 0.7 µM, whereas that for miconazole is 2 µM. Given the apparent Ki (IC50) values determined for ketoconazole inhibition of CaM-dependent activities (18-44 µM), ketoconazole would not be expected to affect CaM-dependent processes throughout the range of dosages used clinically. Miconazole exhibited apparent Ki values ranging from 6 to 8 µM for diverse CaM-regulated processes. At the normal maximal therapeutic concentration of miconazole (2 µM), only minimal effects on CaM-regulated processes would be expected. However, effects of miconazole on CaM-regulated processes might contribute to the severe cardiovascular, respiratory, and central nervous toxicities that accompany drug overdosage.

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