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Effect of miconazole on the electrical conductivity of bilayer membranes

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Miconazole nitrate [1-(2,4-dichloro-8-(2,4-dichlorobenzyloxy)phenyl)imidazole nitrate] is a potent antimycotic agent, synthesized by Godefroi *et al.* [1]. The agent has a wide spectrum of antimicrobial activity. It affects, especially, the growth of dermatophytes, yeasts and Gram-positive bacteria [2–5]. Since miconazole shows no serious side-effects on human subjects, it is a valuable drug for medical use [5–7].

Evidence for the interaction of imidazole derivatives and cell membranes was provided by several workers [7–11], who showed that the ultrastructure of the cell membranes was altered by the various agents. Biochemical investigations of Swamy *et al.* [4] have indicated that miconazole affects the plasma membrane of *Candida albicans*. Furthermore, it has been shown by Yamaguchi [12] that miconazole and clotrimazole interact with specific membrane lipids.

In this publication we present data demonstrating the effects of miconazole on artificial bilayer membranes consisting of oxidized cholesterol or yeast lipids. We show that the antimycotic drug induces a significant increase of the electrical conductivity of the membrane. This effect has been shown to be reversible after removal of miconazole by washing of the membranes.

Materials and methods

Extraction of yeast lipids. Yeast lipid extracts were isolated by a modified method of Folch *et al.* [13]. Baker's yeast, 250 g, (Hamelner Hefe, Nord-West-Deutsche Hefe und Spritwerke, Hameln, F.R.G.) were lyophilized, resuspended in 700 ml of chloroform–methanol (2:1, v/v) and vortexed by means of a Tornado-emulsifier (Braun, Melsungen, Type 853512). After centrifugation for 10 min at 5000 rpm (Christ Minifuge), the supernatant was dried by rotary evaporation *in acuo* at 40°, and the residue dissolved in 40 ml of the above solution. This procedure was repeated, and the residue was resolved in 60 ml of the chloroform–methanol solution and allowed to stand for 30 min on ice. After centrifugation for 15 min at 10,000 *g* the precipitate was discarded and the supernatant mixed with 0.2 vol. of 0.73% (w/v) NaCl solution and the mixture centrifuged as before. The chloroform phase was extracted twice with chloroform–methanol–0.29% (w/v) NaCl (2:48:47), and the final chloroform extract was concentrated by rotary evaporation *in acuo* and adjusted to 50 mg/ml yeast lipids. The determination of total lipid was carried out with a Boehringer Test-Combination according to the method of Zöllner and Kirsch [14].

Preparation of bilayer membranes. For the preparation of bilayer membranes consisting of oxidized cholesterol a 'measuring double cell' was used; a device developed by Lüschow and SchulzHarder [15]. The membranes were prepared according to Mueller *et al.* [16]. To form a bilayer membrane a solution of 50 mg oxidized cholesterol/ml decane was applied on a septum with an aperture of 6 mm by means of a spatula, in a cell filled with Titrisol buffer, pH 6 (Merck, Darmstadt, F.R.G.). Membranes consisting of yeast lipids were formed in a measuring cell with two open chambers and a septum with an aperture of 1.9 mm. The cell was filled with a buffer containing 10 mM sodium citrate/100 mM NaCl, and the yeast lipid extract (50 mg/ml decane) was applied on a Teflon septum by means of a marten-hair pencil. Prior to use, the Teflon septum must be treated with yeast lipid extract and dried in a stream of nitrogen in order to ensure the formation of a stable bilayer.

The electrical conductivity of the membrane (λ) was determined by measuring the electrical membrane potential (U_m) at a constant current (I) with an amperemeter (Keithley 417) at a constant voltage (U). The results were monitored with a recorder (Metrawatt RE 520).

Chemicals. Miconazole nitrate (R 14889) was a gift of Janssen Pharmaceuticals (Beerse, Belgium). Oxidized cholesterol was kindly supplied by B. Dobias, Universität Regensburg. (The oxidation is as described in Ref. 17.) If not indicated in the text all other chemicals were purchased from Merck (Darmstadt, F.R.G.).

Results and discussion

The measurements of the conductivity of nine bilayer membranes consisting of oxidized cholesterol are summarized in Table 1. In the absence of miconazole the

Table 1. Data of the specific electrical conductivity of nine bilayer membranes consisting of oxidized cholesterol

Experiment No.	Concentration of miconazole (μM)	λ_s^0 (S × cm^{-2})	λ_s (S × cm^{-2})	λ_s/λ_s^0
1	10	2.20×10^{-8}	3.08×10^{-8}	1.4
2	10	0.99×10^{-8}	1.79×10^{-8}	1.8
3	10	0.94×10^{-8}	1.24×10^{-8}	1.3
4	10	3.75×10^{-8}	7.69×10^{-8}	2.1
5	20	1.54×10^{-8}	10.30×10^{-8}	6.7
6	20	0.94×10^{-8}	1.34×10^{-8}	1.4
7	20	11.50×10^{-8}	46.60×10^{-8}	4.1
8	20	1.04×10^{-8}	2.90×10^{-8}	2.8
9	20	0.93×10^{-8}	2.68×10^{-8}	2.9

The data were calculated from experiments as shown in Fig. 1, in which the membrane potential (U_m) was measured at a constant current (I). Electrical conductivity: $\lambda(U_m) = I/U_m$, specific electrical conductivity: $\lambda_s = \lambda$/surface of the bilayer, (λ_s^0, before treatment with miconazole, λ_s after treatment with miconazole).

average conductivity was 2.65×10^{-8} S/cm^2. After addition of 10 μM and 20 μM miconazole the conductivity increased by a factor of 1.65 ± 0.35 and 3.57 ± 1.98, respectively. Higher concentrations of the drug led to a destruction of the membrane. However, the effect of miconazole at low concentrations (less than 20 μM) has been found to be reversible (Fig. 1). After changing the buffer in the 'measuring double cell' the conductivity of the membrane returned to the original value. The same effect has been demonstrated with yeast cells, when ^{3}H-labelled miconazole was removed by several washings with citrate buffer [18].

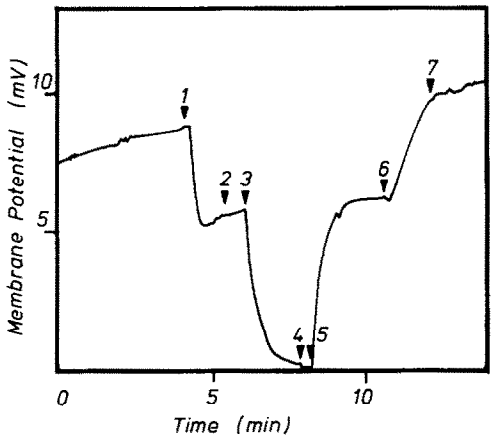

Fig. 1. Effect of miconazole on the electrical conductivity of a bilayer membrane consisting of oxidized cholesterol. The membrane potential (U_m) was measured at constant current (I) of 10^{-8} mA during addition and removal of miconazole. The experiment was carried out in a 'measuring double cell', which permits changing of buffer during the measurement. After an equilibration time of several minutes the Titrisol buffer, pH 6, was substituted by a buffer containing 10 μM miconazole (1). The following steps were: (2) end of buffer exchange, (3) disconnection of the current, (4) short-circuit of the 'measuring double cell', (5) reconnection of the current, (6) removal of miconazole and (7) end of buffer exchange.

In order to prepare bilayer membranes which resemble the yeast plasma membrane, we have isolated a yeast lipid extract. However, bilayer membranes consisting of the yeast lipid extract proved to be unstable in the 'measuring double cell' because the diameter of the septum aperture is too wide (minimum diameter 3 mm). Therefore, we carried out our experiments in an open measuring cell with a septum aperture of 1.9 mm.

The results in Fig. 2 show the effect of miconazole on artificial yeast lipid membranes. The conductivity of the membranes increased during successive addition of miconazole. In contrast to cholesterol membranes (Table 1) yeast lipid bilayers remained intact up to a concentration of 50 μM miconazole (results not shown). The different resistance of the two membrane types against the effect of the antimycotic agent is probably due to the different lipid compositions of the membranes. In this context, we cannot rule out a stabilizing effect of the smaller aperture in the measuring cell system which was used in the yeast lipid bilayer membrane experiments.

Miconazole has been found to interact with both kinds of bilayer membranes, consisting of oxidized cholesterol or yeast lipid extracts, causing an increase of its electrical conductivity. This result is consistent with investigations in which a low miconazole concentration (2 μM, 2×10^7 cells/ml) diminished the H^+-gradient in the plasma membrane of *Saccharomyces cereisiae* [18]. Similar effects were observed by Foury *et al.* [19], indicating that miconazole has an almost immediate effect on the exchange of K^+ for extracellular H^+ across the cellular membrane of *Schizo-*

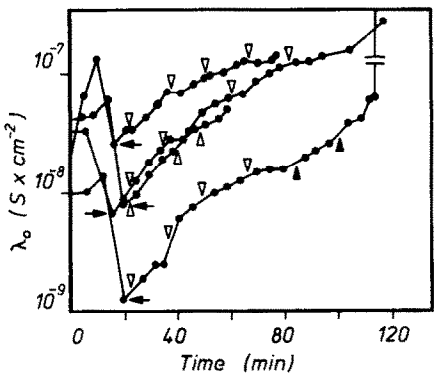

Fig. 2. Effect of miconazole on the electrical conductivity of four bilayer membranes consisting of yeast lipids. Miconazole (5 mM, dissolved in ethanol) was directly pipetted in the measuring cell, and the increase of the current (I) was measured at a constant voltage (U) (−40 mV) in a measuring cell with an aperture diameter of 1.9 mm and specific conductivity was calculated from: $\lambda_s = I\ (U \times \text{surface of the bilayer})^{-1}$. (→), start of the experiment; (△), increase of the miconazole concentration by 6.25 μM; (▲), increase of the miconazole concentration by 12.5 μM.

saccharomyces pombe. However, a vitiation of the proton-gradient of the plasma membrane impairs the uptake of pyrimidine and purine bases [20, 21], amino acids [22, 23] and carbohydrates [23]. Also, miconazole has been found to inhibit the uptake of purine bases [18, 24]. Thus, the fungicidal effect of miconazole may be partially due to a change of the ion-permeability of the plasma membrane. On the other hand, a change of ion-permeability may be explained by a direct binding of miconazole with free and esterified fatty acids (van den Bossche and Ruysschaert, manuscript in preparation). Yamaguchi and Iwata [25] reported on a destabilization of unsaturated dioleoyl lecithin liposomes induced by miconazole, which is enhanced by the incorporation of ergosterol.

In addition to the membrane, miconazole has another target in the cell, as reported by van den Bossche *et al.* [26]. Their findings indicate that miconazole affects the ability of the cell to form the plasma membrane by an accumulation of ^{14}C-methyl sterols and an inhibition of the ergosterol synthesis.

Miconazole induced a significant increase of the electrical conductivity of bilayer membranes consisting of oxidized cholesterol or yeast lipids. Membranes consisting of oxidized cholesterol burst at miconazole concentrations higher than 20 μM, while membranes consisting of yeast lipids resisted concentrations up to 50 μM. The effect of miconazole on the electrical conductivity of the membranes proved to be reversible when the agent was washed out.

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The effect of beta-adrenoceptor blocking drugs on 'ecto-ATPase' activity of rat blood platelets

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The mechanism of action of beta-adrenoceptor blocking drugs in affecting blood platelet aggregation is still controversial. It seems that this effect is not related to the beta-blocking activity but rather to the nonspecific alteration of platelet membrane [1, 2]. Since platelet 'ecto-ATPase' or the ability of whole washed blood platelet suspensions to dephosphorylate ATP is considered to be involved in the mechanisms of platelet aggregation [3–6], this study examined the effect of ten beta-adrenoceptor blocking drugs on the 'ecto-ATPase' activity of rat blood platelets.

Blood was collected from male Wistar albino rats (350–400 g) in ether anesthesia via polyethylene cannula from common carotid artery into plastic tubes containing citrate anticoagulant (9 ml blood + 1 ml 129 mM trisodium citrate). Platelet rich plasma (PRP) was obtained by centrifugation at 200 *g* for 20 min at room temperature. The platelets were separated by centrifugation at 900 *g* for 20 min at room temperature and washed two times with citrated saline solution (12.9 mM trisodium citrate, 137 mM sodium chloride, pH adjusted to 6.5 with HCL). The washed packed platelets were then resuspended in a Tris-buffered saline (50 mM Tris–HCl in 137 mM NaCl, pH 7.4) and platelet count adjusted to 5×10^8/ml (counted manually). Aliquots from platelet suspension were incubated with beta-adrenoceptor blocking drugs at 37° for 5 min and then mixed with an equal volume of medium containing ATP (50 mM Tris–HCl, 137 mM NaCl, 2 mM ATP, 4 mM $MgCl_2$, pH 7.4). Mixing of these solutions represented the beginning of the enzyme assay. The latter was terminated at 15 min by addition of the reaction solution (ammonium molybdate 8.09 mM, ammonium metavanadate 2.01 mM, sodium dodecylsulphate 69.3 mM, ammonia 13.2 mM, nitric acid 650 mM) according to Lin and Morales [7]. The extinction of colour complex formed by released inorganic phosphorus (P_i) and molybdovanadate was measured at 350 nm using a cell with a 1 cm path. Ecto-ATPase activity was defined as nmole P_i cleaved from added ATP per 10^9 platelets per hr.

The average ATPase activity (± S.E.M.) of a suspension of intact washed rat blood platelets was 365.1 ± 7.5 nmole P_i per 10^9 platelets per hour. The activity was linearly proportional to time and platelet concentration (Fig. 1). It was not inhibited by oubain (0.5 mM) and was about twice as great at 37° (365.1 ± 7.7) as at 22° (188.4 ± 4.6). The enzyme showed divalent cation dependence with magnesium and calcium (Fig. 2). Stimulation by magnesium was greatest at 2 mM and stimulation by calcium increased with increasing concentration up to 4 mM. At optimal magnesium concentration addition of calcium diminished the ecto-ATPase activity.

Figure 3 demonstrates the effect of alprenolol (AB Hässle, Sweden), atenolol (ICI, Macclesfield, U.K.), doberol (Boehringer, Ingelheim, F.R.G.), Ko 1124 (Boehringer), metipranol (SPOFA, Praha, Czechoslovakia), oxprenolol (Ciba-Geigy, Basel, Switzerland), pricoron (VULM, Modra, Czechoslovakia), pronethalol (ICI) and propranolol (ICI) on the ecto-ATPase activity of rat blood platelets. No appreciable changes in the enzyme activity were observed after 5 min incubation with beta-adrenoceptor blocking drugs at a concentration of 0.1 mM. Increasing the concentration of tested drugs to 1 mM led