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Effect of Fatty Acyl Group and Sterol Composition on Sensitivity of Lecithin Liposomes to Imidazole Antimycotics

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The specific affinity for membrane lipids and the membrane selectivity of three imidazole derivatives, clotrimazole, miconazole, and econazole, were studied using various types of liposomes with respect to the lecithin fatty acyl group composition and the liposome content and composition of sterol as membrane models. The sensitivity of liposomes to these drugs was primarily dependent upon the lecithin fatty acyl group composition. With sterol-free liposome systems, each imidazole induced maximum release of trapped glucose as a marker from the unsaturated dioleoyl lecithin liposomes, minimum release from the saturated dipalmitoyl lecithin liposomes, and intermediate release from egg lecithin liposomes. The sensitivity of the dipalmitoyl lecithin liposomes to any imidazole drug was not influenced by the incorporation of cholesterol or ergosterol. On the other hand, clotrimazole-induced permeability changes of liposomes prepared from unsaturated dioleoyl lecithin or egg lecithin were greatly enhanced by the incorporation of ergosterol, whereas they were suppressed by cholesterol incorporation. The sensitivity of liposomes prepared from these unsaturated lecithins to miconazole and econazole was also augmented by ergosterol incorporation, although it was scarcely altered by cholesterol incorporation. Negatively charged liposomes were more sensitive to the three imidazole drugs than positively charged liposomes.

Among recently developed antimycotics are three structurally related imidazole derivatives: clotrimazole, miconazole, and econazole. They are active against most species of pathogenic fungi and gram-positive bacteria and appear to share common mechanisms of action (1). Our previous studies suggest that clotrimazole interacts with certain membrane components of susceptible *Candida albicans* cells, probably phospholipids, to cause irreversible breakdown of the permeability barrier of the organism (7, 18). It has also been demonstrated that unsaturated lecithin added to the medium can protect *C. albicans* cells from clotrimazole and miconazole (16, 17). This is of special interest in view of the selective membrane toxicity of the imidazole drugs since they are well tolerated in humans and in experimental animals upon systemic administration (13, 14). However, no further information is available on the molecular basis of the imidazole-lipid interaction leading to the membrane damage.

The present studies were designed to examine: (i) whether phospholipids with different fatty acyl groups respond differently to any of the three imidazole drugs; (ii) if this is the case, what possible roles sterol existing on the membranes plays in conferring the sensitivity to such imidazoles; and (iii) whether the three drugs differ in membrane selectivity. For this purpose, experiments were conducted by using lecithin liposomes as membrane models; lecithin is found to be the major phospholipid in membranes of most species of yeasts and filamentous fungi.

The liposome preparation used in the present study was derived from three different types of lecithin: the saturated dipalmitoyl lecithin, the unsaturated dioleoyl lecithin, and the lecithin mixture isolated from egg yolk, which contains about equal amounts of saturated (namely, $C_{16:0}$, $C_{18:0}$) and unsaturated (namely, $C_{16:1}$, $C_{18:1}$, $C_{18:2}$) fatty acid residues (6). Either ergosterol or cholesterol was incorporated into these liposomes at various sterol-lecithin molar ratios. Two amphiphilic compounds, dicetyl phosphate and stearyl amine, were also incorporated to stabilize liposomes. The sensitivity of membranes of various composition to imidazole drugs was determined by the release of the trapped glucose marker.

MATERIALS AND METHODS

Lipids. A lecithin mixture of egg yolk (egg lecithin) was prepared by chromatography on alumina and silicic acid. Synthetic dipalmitoyl lecithin was obtained from Sigma Chemical Co. (St. Louis, Mo.), and dioleoyl lecithin was from P-L Biochemicals, Inc. (Mil-

waukee, Wis.). Sterols were purchased from Applied Science Laboratories Inc. (State College, Pa.), and dicetyl phosphate was from K & K Laboratories, Inc. (Plainview, N.Y.). Stearylamine was the product of Tokyo Kasei Kogyo Co. (Tokyo, Japan).

Preparation of liposomes and assay for drug sensitivity. Liposomes were prepared by dispersing 0.5 μmol of phospholipid phosphorus into 0.5 ml of aqueous glucose (0.3 M) as described by Kinsky et al. (9). Unless otherwise specified, dicetyl phosphate was used as an amphiphilic compound; the molar ratio of lecithin-dicetyl phosphate was 1:0.1. When cholesterol or ergosterol was incorporated, the molar ratio of lecithin-sterol was in the range of 1:0.25 to 1:1.

The effect of imidazole drugs and amphotericin B on liposomes was determined by comparing the amount of glucose released from liposomes in the presence and absence of the drug tested. The assay system was essentially the same as that developed by Kinsky et al. (8) and modified by HsuChen and Feingold (2).

Clotrimazole was generously supplied by Bayer Yakuhin, Ltd. (Osaka, Japan), miconazole was supplied by Mochida Pharmaceutical Co. (Tokyo, Japan), and econazole was supplied by Otsuka Pharmaceutical Factory, Inc. (Tokyo). Stock solutions of these antimycotics were made in dimethyl sulfoxide and stored at −20°C. Dimethyl sulfoxide was present to a final concentration of 1% (vol/vol) in all the experiments with liposomes.

RESULTS

Figures 1, 2, and 3 illustrate the effects of lecithin fatty acyl group composition and the incorporation of sterol on the permeability change of the liposome systems induced by clotrimazole, miconazole, and econazole, respectively. The liposomes used in these experiments were prepared from the three different types of lecithin, in the presence of dicetyl phosphate as an amphiphile, with or without sterol (molar ratio of lecithin-dicetyl phosphate-sterol, 1:0.1:1). Clotrimazole significantly affected liposomes prepared from the unsaturated dioleoyl lecithin without sterol, leading to an appreciable release of trapped glucose (Fig. 1). This clotrimazole sensitivity of the dioleoyl lecithin liposomes was slightly suppressed by the incorporation of cholesterol, whereas it was pronouncedly augmented by ergosterol incorporation. On the contrary, the sterol-free saturated dipalmitoyl lecithin liposomes were almost insensitive to clotrimazole, and this property of the liposomes was not altered by the incorporation of cholesterol or ergosterol. The reactivity of the sterol-free egg lecithin liposomes was intermediate; regardless of the existence and the nature of sterol, they were less sensitive to clotrimazole than the liposomes derived from dioleoyl lecithin but far more sensitive than those derived from dipalmitoyl lecithin.

The sensitivity of liposomes to miconazole and econazole was influenced by lecithin fatty acyl group composition and the incorporation of sterol in a similar fashion to that observed for clotrimazole. In sterol-free systems, both miconazole and econazole maximally induced release of trapped glucose from the dioleoyl lecithin liposomes, to a lesser extent from the egg lecithin liposomes, and minimally from the dipalmitoyl lecithin liposomes. Although liposomes of the former two types of lecithin prepared with ergosterol were more sensitive to these imidazoles than the comparable sterol-free liposomes, the incorporation of cholesterol resulted in almost unchanged sensitivity (Fig. 2 and 3). These results suggest that there is no significant difference among the three imidazole drugs under study in the action on lecithin liposomes.

To confirm the effects of lecithin fatty acyl substituents on the imidazole-induced permeability alteration, experiments were performed using liposomes prepared from various proportions of saturated dipalmitoyl lecithin and unsaturated dioleoyl lecithin in the presence and

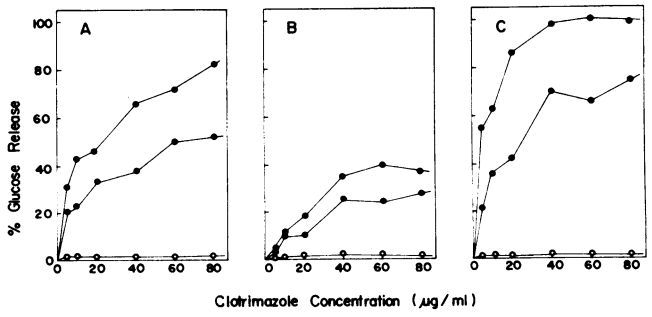

FIG. 1. *Clotrimazole action on liposomes prepared from dioleoyl lecithin (●), dipalmitoyl lecithin (○), and egg lecithin (◑) in the presence of dicetyl phosphate at 10 mol%, with or without sterol at 100 mol% (relative to lecithin). The results depicted in (A), (B), and (C) were obtained with sterol-free liposomes, cholesterol-containing liposomes, and ergosterol-containing liposomes, respectively. Liposomes were incubated with varying concentrations of the drug at 25°C for 2 h, and glucose marker release was enzymatically determined.*

absence of sterol (cholesterol or ergosterol); the final composition of total phospholipid-dicetyl phosphate-sterol was 1:0.1:1 in a molar ratio. It is clear from Fig. 4 that regardless of the existence and the nature of sterol, the liposomes increased their sensitivity to the imidazole drugs with increasing proportions of the unsaturated lecithin.

Figure 5 and 6 depict the results of experiments using liposomes of the three different types of lecithin prepared with cholesterol or ergosterol at various lecithin-sterol ratios. They show that regardless of the amount of sterol incorporated, the dipalmitoyl lecithin liposomes were minimally affected by the three imidazoles, although there was a slight increase in the sensitivity to each drug when 20 to 40 mol% (relative to lecithin) of cholesterol or ergosterol was incorporated. On the other hand, the response of liposomes prepared from dioleoyl lecithin or egg lecithin to clotrimazole decreased with increasing amounts of cholesterol incorporated, whereas their response to miconazole and econazole was influenced to a lesser extent (Fig. 5). The results of experiments with the dioleoyl lecithin liposomes also demonstrate that the liposome systems prepared with ergosterol were more sensitive to the three imidazoles than the cholesterol-containing liposome systems over a wide range of liposome sterol content and that the sensitivity of the former type of liposomes increased with the amount of sterol incorporated.

Figure 7 illustrates the results of experiments performed to determine how the amount of negative or positive charge influences the imidazole sensitivity of the liposomes. It indicates that the incorporation of increasing amounts of dicetyl phosphate as a molecule with negative charge significantly enhanced the imidazole sensitivity of cholesterol-containing egg lecithin liposomes, whereas the incorporation of positively charged amphiphilic stearylamine suppressed the sensitivity of the liposomes.

DISCUSSION

In previous studies of the clotrimazole action on *C. albicans* cells, we reported that this im-

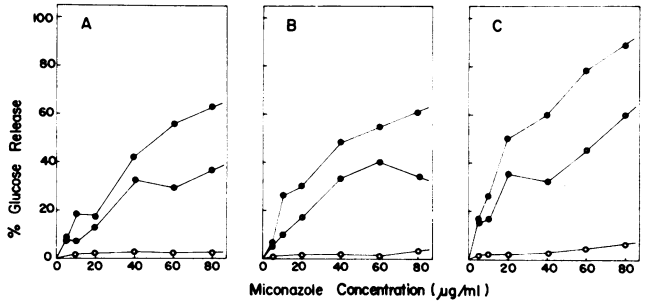

FIG. 2. *Miconazole action on liposomes prepared from dioleoyl lecithin (●), dipalmitoyl lecithin (○), and egg lecithin (◑) in the presence of dicetyl phosphate at 10 mol%, with or without sterol at 100 mol% (relative to lecithin). The results depicted in (A), (B), and (C) were obtained with sterol-free liposomes, cholesterol-containing liposomes, and ergosterol-containing liposomes, respectively. Experimental conditions were identical to those described for Fig. 1.*

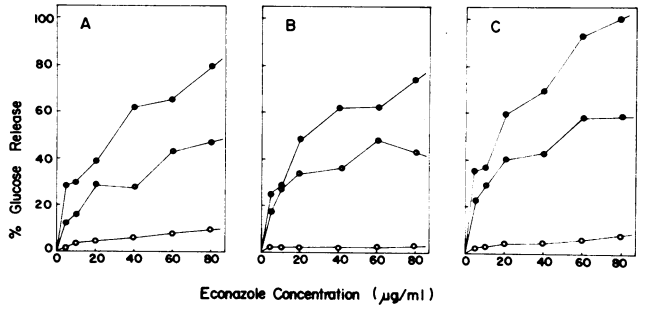

FIG. 3. *Econazole action on liposomes prepared from dioleoyl lecithin (●), dipalmitoyl lecithin (○), and egg lecithin (◑) in the presence of dicetyl phosphate at 10 mol%, with or without sterol at 100 mol% (relative to lecithin). The results depicted in (A), (B), and (C) were obtained with sterol-free liposomes, cholesterol-containing liposomes, and ergosterol-containing liposomes, respectively. Experimental conditions were identical to those described for Fig. 1.*

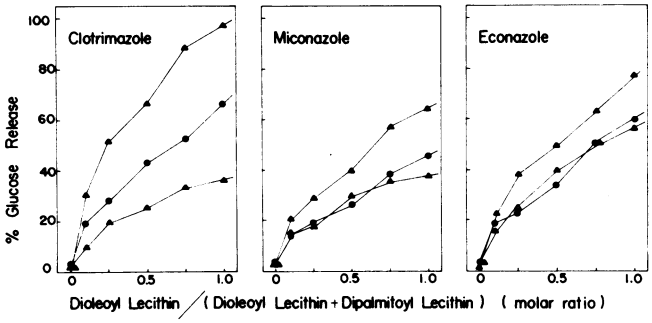

FIG. 4. *Action of imidazole derivatives on liposomes prepared from various combinations of the saturated dipalmitoyl lecithin and the unsaturated dioleoyl lecithin in the presence of dicetyl phosphate at 10 mol%, with or without sterol at 100 mol% (relative to lecithin). The results were obtained with sterol-free liposomes (●), cholesterol-containing liposomes (△), and ergosterol-containing liposomes (▲). Experimental conditions were identical to those described for Fig. 1.*

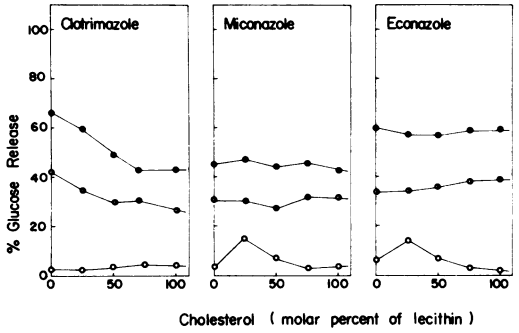

FIG. 5. *Effect of cholesterol incorporation into liposomes prepared from dioleoyl lecithin (●), dipalmitoyl lecithin (○), and egg lecithin (◑), in the presence of dicetyl phosphate at 10 mol% (relative to lecithin), on the sensitivity to imidazole derivatives. Experimental conditions were identical to those described for Fig. 1.*

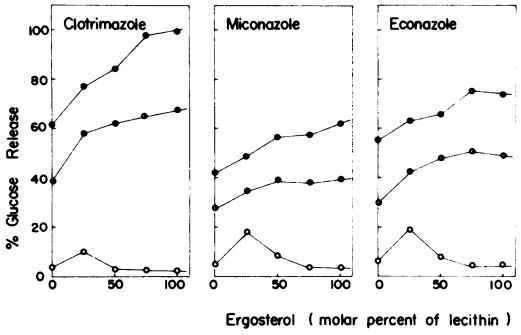

FIG. 6. *Effect of ergosterol incorporation into liposomes prepared from dioleoyl lecithin (●), dipalmitoyl lecithin (○), and egg lecithin (◑), in the presence of dicetyl phosphate 10 mol% (relative to lecithin), on the sensitivity to imidazole derivatives. Experimental conditions were identical to those described for Fig. 1.*

idazole causes a permeability alteration through a binding of the drugs to certain lipid components, probably phospholipids, of the membranes (7, 18). Regarding a relationship between phospholipids and the action of imidazoles, our further studies have demonstrated that an anti-*Candida* activity of clotrimazole and miconazole is significantly antagonized by naturally occurring phospholipids (e.g., egg lecithin) and synthetic unsaturated phospholipids (e.g., dioleoyl lecithin) added to the medium, although none of the saturated phospholipids (e.g., reduced egg lecithin and dipalmitoyl lecithin) or sterols (e.g., cholesterol and ergosterol) was effective as an antagonist (16). These studies imply that this reversal of the imidazole effect resulted from a physicochemical interaction between the imidazole derivatives and the added unsaturated phospholipids, which prevented the drugs from interacting with the unsaturated phospholipids of the organisms. More direct evidence for the interaction between unsaturated phospholipids and imidazole derivatives has been provided by spectrophotometric analyses (17). These analyses showed that when egg lecithin was added to aqueous solutions of clotrimazole or miconazole the ultraviolet absorption profile of the drug was changed. All these results strongly suggest that the effects of imidazole drugs may be dependent upon the presence of unsaturated phospholipids in the membranes. This postulation is favorably supported by data obtained in the present study with a lecithin liposome membrane model system, which showed that all of the three imidazoles (clotrimazole, miconazole, and econazole) can preferentially interact with liposomes of unsaturated lecithin, regardless of the presence and the nature of the sterol.

It is generally accepted that the effect of polyene antibiotics on the membranes of susceptible organisms depends upon the interaction between the polyene and sterols in the mem-

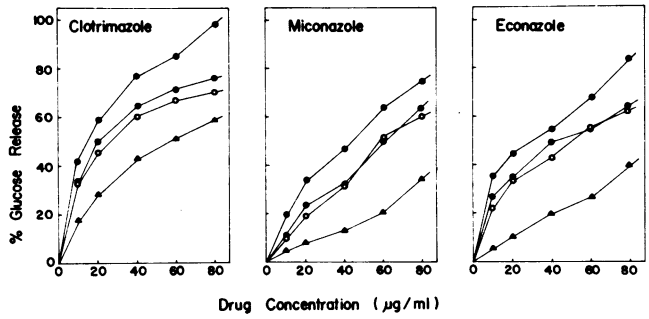

FIG. 7. *Effect of incorporation of amphiphilic compounds into liposomes prepared from egg lecithin, in the presence of ergosterol at 100 mol% (relative to lecithin), on the sensitivity to imidazole derivatives. Amphiphilic compounds incorporated were: dicetyl phosphate at 5 mol%* (○), *10 mol%* (●), *and 20 mol%* (◑); *and stearylamine at 10 mol%* (▲) *(relative to lecithin). Experimental conditions were identical to those described for Fig. 1.*

branes. HsuChen and Feingold (2, 3) have studied the action of amphotericin B and nystatin on various types of lecithin liposomes and demonstrated that the response of liposome systems to these polyenes is influenced not only by the nature of the sterol incorporated but also by the lecithin fatty acyl group composition. Comparing their data with ours, there is a qualitative difference between polyene antibiotics and imidazole derivatives in the membrane selectivity.

It is apparent that sterol may not be essential for the membrane to interact with imidazoles, since the sterol-free unsaturated lecithin liposomes showed a substantial reactivity with the three imidazole drugs. However, results were also obtained in the present study indicating that the sensitivity of unsaturated lecithin liposomes to these drugs was influenced by the liposome content and the nature of the sterol; the incorporation of ergosterol enhanced the imidazole sensitivity of the liposomes, whereas the incorporation of cholesterol was either without effect or suppressive. Such a difference between ergosterol and cholesterol in conferring imidazole sensitivity is considered to be due to the distribution of a double bond in the sterol nucleus and/or the presence of a double bond in the C17 side chain. It is possible that the effect of sterol on the imidazole sensitivity of the membranes may be exerted through sterol-lecithin interaction that controls the overall state of the membrane, in particular, the conformation of phospholipid molecules. Although the response of cholesterol-containing liposomes to clotrimazole seems to be somewhat different from their response to miconazole and econazole, it is our contention that these three imidazoles share a common molecular basis for an interaction between the drug and the membrane in which unsaturated lecithin provides an avidity for the drug. Preferentially high sensitivity of unsaturated lecithin liposomes, especially those prepared with ergosterol, to imidazole derivatives might be compatible with their selective toxicity toward fungal cells, since the membranes of fungi contain lecithins with unsaturated fatty acyl groups at a considerable density and ergosterol as the major sterol (11, 15).

As expected from the cationic nature of imidazole drugs, the results of experiments using negatively charged liposomes prepared with dicetyl phosphate and positively charged liposomes with stearylamine suggest that the imidazoles more strongly interact with the former type of lecithin liposomes than with the latter. In this respect, the effect of imidazole derivatives is similar to that of the membrane-active peptide antibiotic polymyxin B. Working with various liposomes derived from naturally occurring and synthetic phospholipids, HsuChen and Feingold (4) and Imai et al. (5) have demonstrated that a negative charge is required for the reactivity of liposomes with polymyxin B and that the sensitivity of lecithin liposomes to polymyxin B is much lower than that of liposomes prepared from phosphatidylethanolamine. Pache et al. (12) reported that the amino group of this antibiotic selectively interacts with the phosphate group of certain phospholipids. A study is in progress to examine whether the action of imidazole derivatives is also influenced by the nature of the polar head of the phospholipid molecule.

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