Cation Conductance and Efflux Induced by Polyene Antibiotics in the Membrane of Skeletal Muscle Fiber

Nora Shvinka* and Gustav Caffier‡

*Institute of Cytology, the Russian Academy of Sciences, 194064, St. Petersburg, Russia; and ‡Federal Institute of Occupational Medicine, 1134, Berlin, Germany

ABSTRACT Cation conductance and efflux induced by polyene antibiotics amphotericin B (AMB), amphotericin B methyl ester (AME), nystatin, mycoheptin, and levorin on frog isolated skeletal muscle fibers and whole sartorius muscles were investigated. Conductance was measured under current-clamp conditions using a double sucrose-gap technique. Cation efflux was studied using flame emission photometry. Some new data were obtained concerning the effects of levorin and mycoheptin on biological membranes. The power dependence of polyene-induced cation transport on antibiotic concentration in muscle membrane was lower than that in bilayers. The decline in the equilibrium conductance caused by polyene removal (except for levorin) was very fast. There was reverse temperature dependence of AMB- and nystatin-induced conductances. Both induced conductance and efflux values demonstrated a correlation with the order of antifungal activities: levorin > AMB, mycoheptin > AME > nystatin, except for AME, which was more potent on yeastlike cells. These effects were interpreted in terms of possible differences in the kinetics of channel formation in biological and model membranes and in light of the role of nonconducting antibiotic forms in biological membranes.

INTRODUCTION

The antifungal polyene antibiotics have been shown to mediate changes in permeability by forming ion-selective channels in lipid bilayers (Marty and Finkelstein, 1975; Ermishkin et al., 1976; Kasumov et al., 1981; Borisova et al., 1988) and biological membranes (Bolard, 1986; Shvinka and Caffier, 1989, 1991). Most experiments with biological objects have been done on ergosterol-containing yeast cell membranes. Cholesterol-containing animal cell membranes, especially those of single isolated cells, are poorly investigated, and data presented on different cell types often appear to be contradictory. Thus, the activity of nystatin seems to be systematically much weaker than that of AMB on artificial membranes (Cass et al., 1970) as well as on erythrocytes (Deuticke et al., 1973), whereas the reverse efficiency is found for promoting K^+ release from both *Candida albicans* (Johnson et al., 1978) and yeast (Bolard, 1986). There is no notable difference between amphotericin B (AMB)- and amphotericin B methyl ester (AME)-induced conductance on thin lipid membranes (Cass et al., 1970), whereas potassium loss from human erythrocytes produced by AME and AME-hydrochloride is either less pronounced than that of AMB (Chen et al., 1977; Cheron et al., 1988; Gary-Bobo, 1989) or similar to that of AMB (Bolard et al., 1991). AME has been studied extensively in vivo and in vitro mainly on pathogenic microorganisms whose membranes contain ergosterol and on human red blood cells as representatives of cholesterol-containing host cells (Cheron et al., 1988; Gary-Bobo, 1989; Bolard et al., 1991). Therefore, comparison of muscle cell and tissue response to AME relative to AMB, the main drug used in human therapy against systemic mycosis, would be of interest. There has been considerable work done with aromatic polyenes such as levorin on large unilamellar lipid vesicles, yeasts and human red blood cells (Liras and Lampen, 1974; Mazerski et al., 1983; Cybulska et al., 1983), but no information is available on levorin's effect on cholesterol-containing animal cell membranes. Very little is known about mycoheptin action on biological membranes. Thus, the molecular organization of polyene antibiotic complexes responsible for the induced ion permeability in biological membranes remains obscure. The goal of this study was to investigate cation conductance and ionic flux induced by AMB, AME, nystatin, levorin, and mycoheptin in muscle cell membranes with the aim of characterizing the reversibility and dependence of steady-state conductance on concentration and temperature. Comparing these results with those reported for artificial lipid bilayer membranes and yeast cells should help in elucidating the molecular basis for the polyene action on biological membranes.

MATERIALS AND METHODS

Polyene antibiotics AMB, AME, nystatin, mycoheptin, and levorin, a kind gift from Dr. V. A. Vainsntein (Research Institute of Antibiotics and Medical Enzymes, St. Petersburg, Russia), were dissolved in dimethylsulfoxide (DMSO) to a concentration of 10^{-2} M. Antibiotics were added to experimental solution to give final concentrations (M) of 10^{-6}–10^{-5} AMB, 5×10^{-6} –4×10^{-5} AME, 2×10^{-5}–10^{-4} nystatin, 1.3×10^{-6}–10^{-5} mycoheptin, and 2.5×10^{-7}–5×10^{-5} levorin. The final concentration of DMSO did not exceed 0.8% in conductance experiments or 0.5% in efflux experiments, and had no significant effect on membrane conductance or cation effluxes. Fresh solutions were prepared daily.

To measure the temperature effect on conductance, experiments were carried out first at room temperature (22 ± 1°C) and subsequently at a temperature about 10°C higher. Temperature changes were measured with

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Address reprint requests to N. Shvinka, Institute of Cytology, Tikhoretsky Av. 4, Academy of Sciences-RAN, St. Petersburg, 194064 Russia. Tel.: 7-812-247-1829; Fax: 7-812-247-0341; E-mail: root@cell.spb.su.

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a thermistor placed in the test gap of the chamber, which recorded the difference between room temperature and the temperature in the test gap.

Conductance measurements

All conductance experiments were performed on single fibers from musculus ileofibularis and musculus semitendinosus of the *Rana esculenta* using the double sucrose-gap method (Isenberg and Küchler, 1970; Caffier et al., 1980). The membrane conductance was tested using hyperpolarizing square wave pulses (0.02–0.06 μA) of 300 ms duration applied at 10 s interval. The test compartment in our experiments was 400 μm wide. Membrane conductance was calculated by $g = If^2/V_oS$, where V_o is the voltage measured, I is the amplitude of the current pulse, S is the membrane surface area of the preparation in the test compartment and f is the short-circuiting factor. The value of f is equal to V_o/V_i, where V_o is the potential change recorded with an extracellular electrode, and V_i is the potential change recorded with an intracellular microelectrode under conditions of double sucrose gap. The short-circuiting factor in our experiments was 0.76. To calculate S both the width of the test compartment and the diameter of the preparation were measured using a microscope. The antibiotic-induced conductance g_A is equal to $g - g_0$, where g and g_0 are cation conductances in the presence of antibiotic, and before antibiotic treatment, respectively. $g_{A\infty}$ is the steady-state value of induced conductance measured at the end of every incubation period with antibiotic. The kinetics of antibiotic action were analyzed using the assumption that the conductance rises exponentially, i.e.,

$$g = g_{A\infty}(I - e^{\rho t}) + g_0 \quad (1)$$

and declines exponentially with time after removal of antibiotics from the solution, i.e.,

$$g = g_{A\infty}e^{-\rho t} + g_0 \quad (2)$$

The solution used contained (in mmol/l): 160 K^+, 8 Ca^{2+}, 88 SO_4^{2-}; and 2 Tris-maleate (pH 7.2). In this solution the resting potential was 0.1 ± 0.3 mV, and $[K]_{in}$ = 159.3 mmol/l (Leech and Stanfield, 1981). Thus, the K^+ concentration was nearly the same inside and outside the cell, and only K^+ was available for carrying a substantial current through the membrane. In some experiments K^+ was replaced by an equimolar amount of Rb^+. The time for complete solution exchange was ≤5 s, and the flow was continuous for the duration of the experiment.

Flux measurements

The effluxes of K^+, Li^+, Na^+ and Rb^+ were studied on whole sartorius muscles isolated from the frog *Rana temporaria*. The muscles were enriched with Li^+ or Rb^+ by leaving them overnight at 3°C in solutions containing (in mmol/l): 40 NaCl, 80 LiCl, 2.5 KCl, 1.8 $Ca(NO_3)_2F$ and Tris-HCl buffer, pH 7; or 120 NaCl, 2.5 RbCl, 1.8 $Ca(NO_3)_2$, Tris-HCl buffer, pH 7. The muscles were then immersed in sodium- and potassium-free magnesium Ringer solution (in mmol/l): 76 $MgCl_2$; 1.8 $Ca(NO_3)_2$; Tris-HCl buffer, pH 7 for 70 to 80 min at room temperature. The time required to remove all extracellular sodium in a sodium-free solution is ~1 h (Vereninov et al., 1980). The effluxes of K^+, Li^+ and Na^+ measured in sodium- and potassium-free magnesium Ringer solution. $MgCl_2$ was the best substitute for sodium and potassium, and in magnesium Ringer solution the muscles remained in a near-physiological state: the value of resting potential was normal (80–90 mV), and muscles exhibited stable ion fluxes and efflux rate constants for long periods of time (Vereninov et al., 1980; Vereninov and Marakhova, 1981). The rate constants for potassium and sodium loss in magnesium Ringer solution did not differ significantly from that in Ringer solution. The muscles were incubated individually in a series of Pyrex tubes containing 4 ml of magnesium Ringer solution and were kept for 10 min in each tube. Thus, the cation content in 4 ml of the respective solution was collected within 10 min. The cation content in the solution was determined using a Perkin-Elmer flame photometer. At the end of the experiments the muscles were dried and weighed, and the ion content in each muscle was determined. The cation content was expressed as μmol/g dry weight. Efflux μmol/g dry weight × min) was expressed as a concentration of the cation loss/min during each collection interval. Antibiotic-induced efflux E_A equaled $E - E_0$, where E_0 and E are effluxes measured at the start of the experiment without antibiotic, and after a 60-min incubation in the presence of antibiotic, respectively.

RESULTS

When polyene antibiotics were added to the external medium, they produced a concentration-dependent increase in cation conductance in muscle fiber (Figs. 1 and 2). The conductance rose exponentially to a steady state that was reached in 6 to 8 min. The time constant for the kinetics of AMB actions demonstrated in Fig. 2 was 2.70 to 4.55 min (see Fig. 3 *a*). Our data show the following order of polyene-induced steady-state conductance $g_{A\infty}$ for a given antibiotic concentration: levorin > AMB > mycoheptin > AME > nystatin (Table 1). The dose response relationship of the antibiotics used is shown in Fig. 4. On a logarithmic scale the dependence of induced cation conductance on the antibiotic concentration gives a slope of 1.3 for mycoheptin and AMB (Fig. 4, lines 2 and 3, respectively), 2 for AME (Fig. 4, line 4), and 1.7 for nystatin (Fig. 4, line 5).

The aromatic heptaene levorin, which apart from an amino sugar (mycosamine) contains a positively charged aromatic ketone *p*-aminoacetophenone, in our experiments gave a weak concentration dependence (Fig. 1 *d*; Fig. 4., line 1). In contrast, for lipid bilayers, the presence of levorin enhanced the membrane conductance with the 2nd to 5th powers of the antibiotic concentration (Kasumov and Liberman, 1973).

Our data show that polyene-induced conductance is completely reversible except for levorin, which irreversibly affects the muscle fiber membrane (Fig. 1 *d*, *arrows 2* and *7*). On the return to the antibiotic-free solution, membrane conductance falls to the pretreatment level within minutes (Fig. 1 *a*, *arrow 4*; Fig. 1 *b*, *arrow 5*; 1 *c*, *arrow 4*; Fig. 2, *arrows*). From the slopes of $\lg\{(g-g_0)/g_{A\infty}\}$ versus time plots, according to Eq 2, we calculated the values ρ (the relaxation rate constants) for the polyene removal. As shown in Fig. 5 the kinetics of the conductance decrease consists of two components, one fast and one slow. From the reciprocals of constants ρ we obtained two pairs of time constants τ: 0.74 and 1.67 min for nystatin, 2.39 and 4.64 min for mycoheptin, and 3.70 and 8.33 min for AME. The phenomenon of fast and slow components was reported earlier for the AMB removal (Shvinka and Caffier, 1991). There might be considerable variability in τ values, which increase with antibiotic concentration and application time. With AMB removal, the second slow component appeared only at the end of experiments after repeated antibiotic application (Fig. 2, *arrow 4*, which corresponds to line 4 in Fig. 3 *b*). Moreover, the τ values for the conductance rise (Fig. 3 *a*, lines 1–4) are higher than those for the fast component of conductance recovery (Fig. 3 *b*, lines 1–3).

The effect of heating on AMB- and nystatin-induced conductance is shown in Fig. 6 *a*. The AMB-induced conductance at 22°C is about 4.5 times higher than that at 29°C. Fig. 6 *b* demonstrates a decrease in nystatin-induced conductance

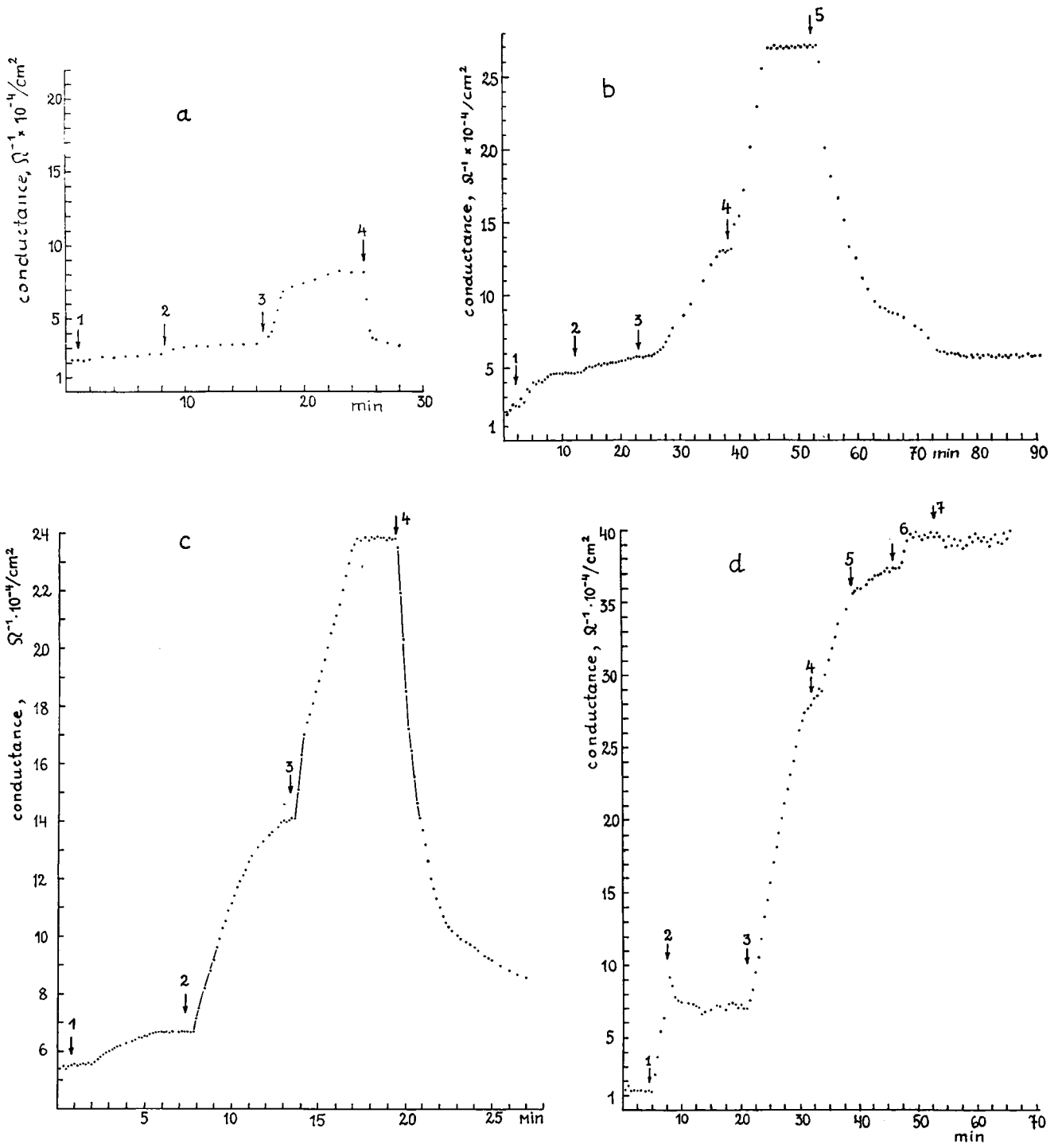

FIGURE 1 Effects of nystatin (*a*), mycoheptin (*b*), metamphocin (*c*) and levorin (*d*) on the membrane conductance of isolated frog muscle fiber. Records of conductance in 160 mmol/l K^+ isotonic solution. Points represent values obtained under hyperpolarizing constant current pulses with intensity: 0.02 μA (*a*), 0.06 μA (*b*), 0.02 μA (*c*), and 0.03 μA (*d*). (*a*) $g_0 = 2.2\,\Omega^{-1} \times 10^{-4}/cm^2$; *arrows* indicate application of nystatin to the solution in following concentrations (in mol/l): 1→ 2×10^{-5}; 2→ 4×10^{-5}; 3→ 8×10^{-5}; 4→ shows the removal of nystatin from the sodium. (*b*) $g_0 = 2.0\Omega^{-1} \times 10^{-4}/cm^2$; *arrows* indicate application of mycoheptin to the solution in following concentrations (in mol/l): 1→ 1.3×10^{-6}, 2→ 2.5×10^{-6}, 3→ 5×10^{-6}, and 4→ 10^{-5}; 5→ shows the removal of mycoheptin from the solution. (*c*): $g_0 = 5.6\ \Omega^{-1} \times 10^{-4}/cm^2$; *arrows* indicate application of metamphocin to the solution in following concentrations (in mol/l): 1–10^{-5}, 2–2×10^{-5}, and 3–4×10^{-5}. *Arrow 4* shows the removal of metamphocin from the solution. (*d*): $g_0 = 1.4\ \Omega^{-1} \times 10^{-4}/cm^2$; *arrows* indicate application of levorin to the solution in following concentrations (in mol/l): 1→ and 3→ 1.3×10^{-6}; 4→ 2.5×10^{-6}; 5→5×10^{-6}; and 6→ 10^{-5}. 2→ and 7→ indicate the removal of levorin from the solution.

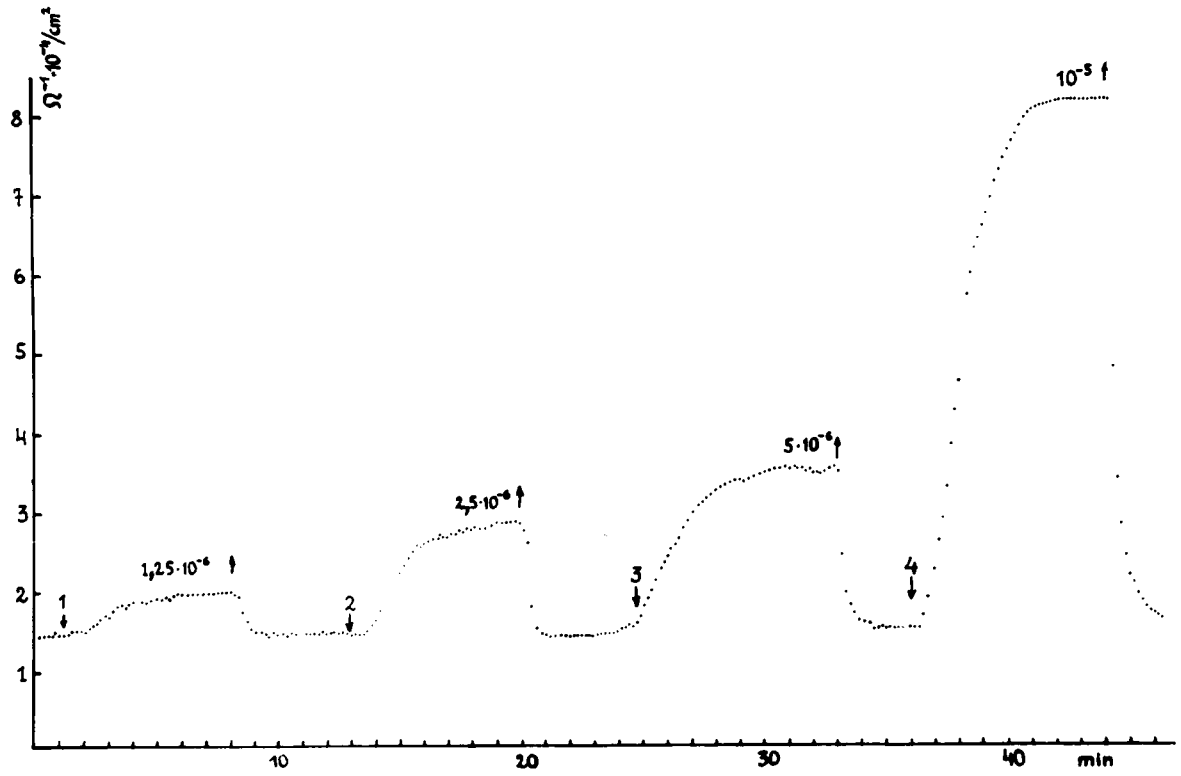

FIGURE 2 Effects of amphotericin B on the membrane conductance of isolated frog muscle fiber. Records of conductance in 160 mmol/l Rb^+ isotonic solution. Points represent values obtained under hyperpolarizing constant current pulses with intensity 0.02 $\mu A \cdot g_0 = 1.5\ \Omega^{-1} \times 10^{-4}/cm^2$; *down arrows* (↓) indicate application of amphotericin B; *up arrows* (↑) indicate removal of amphotericin B; in *intervals between arrows* the concentration of amphotericin B is shown.

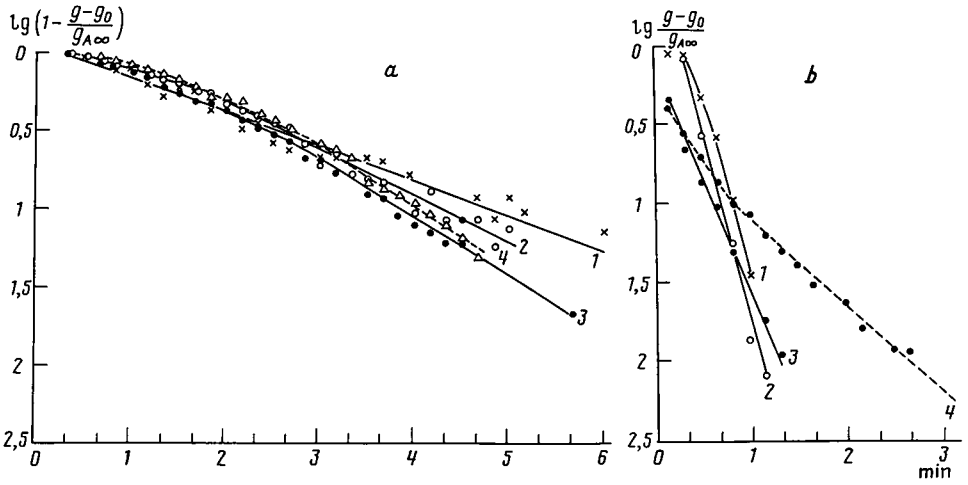

FIGURE 3 Time dependence of $\lg\{I(g - g_0/g_{A\infty})\}$ (*a*) and $\lg\{(g - g_0)/g_{A\infty}\}$ (*b*). The analysis of the conductance records shown in Fig. 2. g_0, g, and $g_{A\infty}$ are the conductances before the antibiotic treatment, during the treatment, and at the steady-state, respectively. The numbers at curves correspond to those over the arrows in Fig. 2. The values of $\lg\{(I(g - g_0(/g_{A\infty})\}$ are calculated from formula 1 for the conductance rise in the presence of amphotericin B; the values of $\lg\{(g - g_0(/g_{A\infty})\}$ are calculated from formula 2 for the conductance decrease after the removal of amphotericin B. The slope of the linear sections of the curves is equal to ρ.

by a factor of 1.2 resulting from heating (Fig. 6 *b*, interval between arrows 5 and 6). Thus, our results indicate that Q_{10} for nystatin-induced conductance is negative in contrast to the situation reported for the *Aplysia* neurons (Russel et al., 1977). The rate of the conductance decrease after AMB removal increases with heating ($Q = 2.8$). It appears, therefore, that the decrease in the steady-state conductance with temperature is caused both by an increase of the destruction rate constant and a decrease in the formation rate constant, the latter being the predominant effect.

Our data obtained by measuring the potassium, rubidium, and sodium effluxes and cation content in sartorius muscle support the results of the conductance experiments reported above. Fig. 7 *a* shows an increase in the potassium efflux and Fig. 7 *c* an increase in sodium efflux upon the addition of 10^{-5} M polyenes. The induced efflux values for K^+ and Rb^+ fol-

TABLE I Ionoforic activities of polyene antibiotics on muscle membrane

Polyene Antibiotic	Concentration (mol/l)	$^{*}g_{A\infty}$ ($\Omega^{-1} \times 10^{-4}/cm^2$)	$^{\ddagger}E_A$ ($\mu mol \times 10^{-3}/g$ dry weight $\times$ min)
	10^{-5}	§29.0 ± 8.3	232.2 ± 40.0
AMB		(n = 6)	(n = 8)
AME	10^{-5}	1.6 ± 0.2	66.6 ± 7.6
		(n = 8)	(n = 9)
Nystatin	2.0×10^{-5}	0.8 ± 0.1	
		(n = 6)	
	2.5×10^{-5}		54.9 ± 5.5
			(n = 7)
Mycoheptin	10^{-5}	25.3 ± 1.1	177.3 ± 13.1
		(n = 6)	(n = 6)
Levorin	10^{-5}	42.3 ± 2.1	356.6 ± 28.4
		(n = 6)	(n = 6)

* $g_{A\infty}$ is the steady-state value of induced K^+ conductance.
‡ E_A is antibiotic-induced potassium efflux.
§ Data appear as means ± SEM.

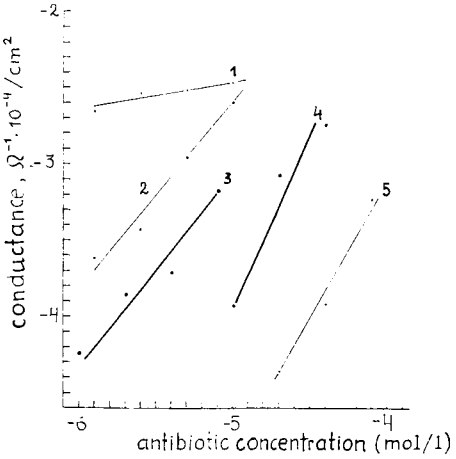

FIGURE 4 lg conductance versus lg antibiotic concentration plot. The data from Figs. 1 and 2 are used. (*1*) Levorin; (*2*) Mycoheptin; (*3*) Amphotericin B; (*4*) Metamphocin; (*5*) Nystatin.

low this order (see Table 1): levorin > AMB > mycoheptin > AME > nystatin, and for Na^+: levorin ≫ AMB > mycoheptin > nystatin > AME. Thus, the orders are close to those for induced conductance. The lithium efflux is less affected by polyene-treatment in this situation (Fig. 7 *e*); only levorin/AMB/mycoheptin shows remarkable effect. Figs. 7 *b*, *d*, and *f* illustrate the corresponding changes of cation content.

DISCUSSION

Our results, demonstrating almost complete reversibility of AMB-, AME-, mycoheptin- and nystatin-induced conductance after an approximately 1-min wash in antibiotic-free solution, show that these polyene antibiotics can readily exchange between the aqueous phase and the biological membrane. Our data support those obtained with nystatin on *Aplysia* neurons (Russel et al., 1977), rat lacrimal gland cells (Horn and Marty, 1988) and red cells (Cass and Dalmark, 1973). With one-sided application of AMB to lipid bilayers Brutyan (1982) also reported very fast conductance decrease after reincubation in AMB-free medium. On the other hand, the results obtained on lipid bilayers, when polyenes are applied from two sides, show much slower relaxation kinetics. Thus, on thin lipid membranes, conductance decreases in antibiotic-free medium with a half-time of about 20 min for nystatin, 120 min for AMB, and 1 min for AME (Cass et al., 1970). It may be that the permeability pathways induced by one- and two-sided addition of polyenes are different. It seems quite probable that the polyene channels in biological membranes are mobile "half-pores" spanning the entire membrane, which has been postulated earlier for lipid bilayers with one-sided nystatin (Marty and Finkelstein, 1975; Kleinberg and Finkelstein, 1984) and AMB (Van Hoogevest and De Kruijff, 1978; Brutyan, 1982) effects. The "half-pore" must be a highly dynamic structure with a short lifetime.

The time constant of the fast component of conductance recovery by antibiotic removal probably reflects the kinetics of the channel disassembly reactions. The appearance of the slow component seems to result from nonconductive forms of antibiotics such as nonconducting "half-pores" (Marty and Finkelstein, 1975) and/or micelles within the membrane (O'Neill et al., 1986), which would slow down the relaxation kinetics. The increased values of τ reported in this study are consistent with the situation in which the concentration of nonconducting forms in the membrane may be enhanced, namely, at the end of long experiments and after prolonged incubation with a large concentration of antibiotic. This sug-

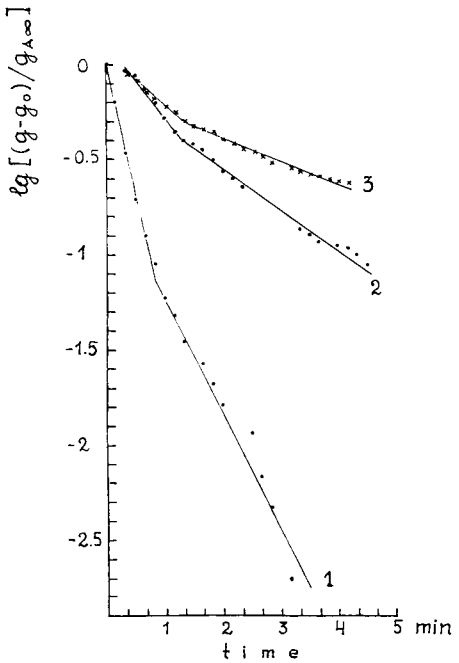

FIGURE 5 Plot of lg $\{(g - g_0)/g_{A\infty}\}$ versus time for the conductance decrease after the removal of antibiotics. (*1*) Nystatin; (*2*) Mycoheptin; (*3*) Metamphocin. Records of conductance in 160 mmol/l Rb^+ (1 and 160 mmol/l K^+ (2, 3) isotonic solutions. Note that the decline of conductance is a double-exponential process.

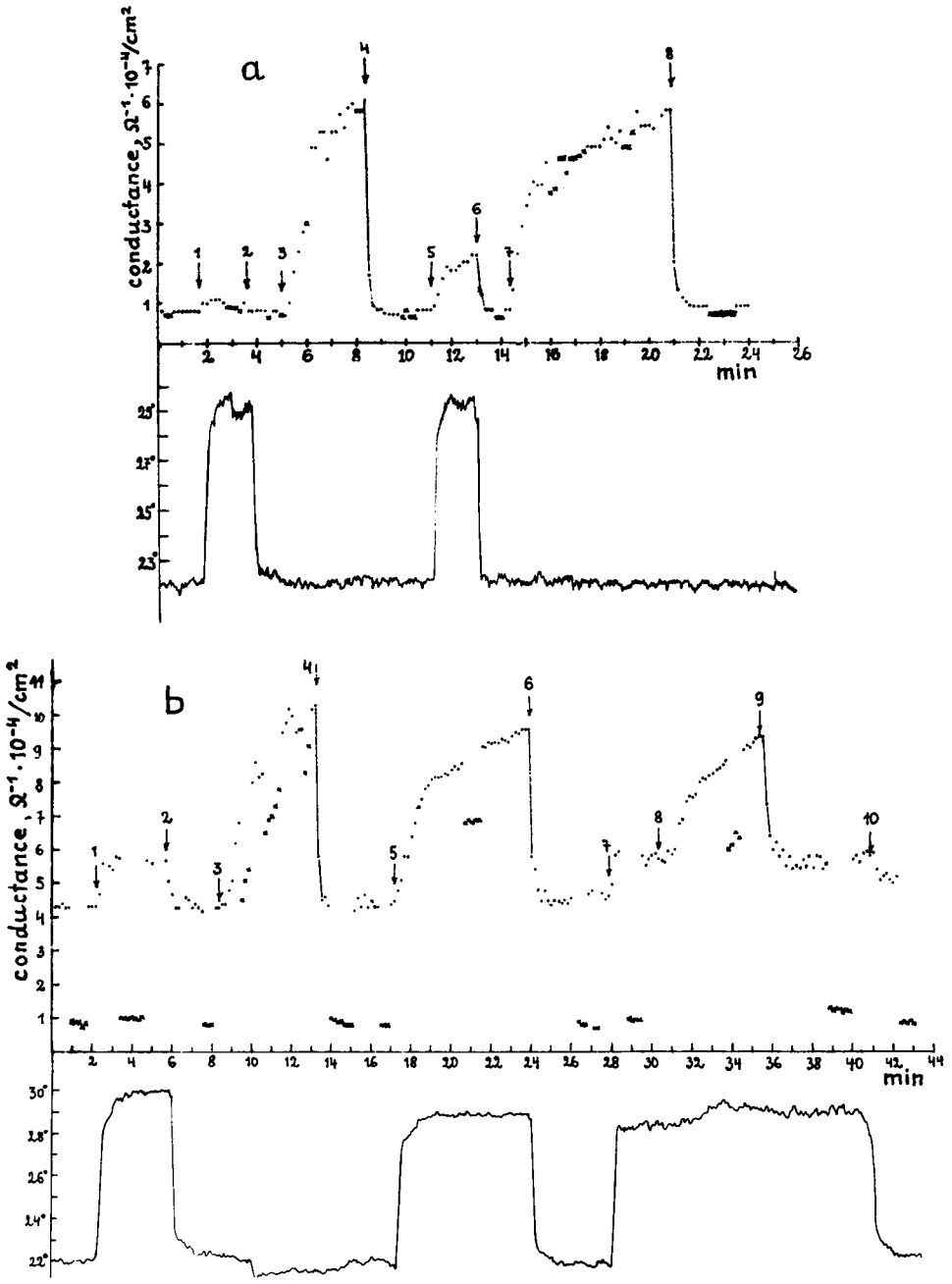

FIGURE 6 Effect of temperature on polyene-induced conductance. *Top graphs* in (*a*) and (*b*) show the conductance under hyperpolarizing (•) and depolarizing (×) stimulus. *Bottom graphs* show the temperature records. (*a*) The muscle fiber in 160 mmol/l Rb^+ isotonic solution; the intensity of constant current pulses 0.02 μA; g_0 for hyperpolarizing pulses is 0.8 $\Omega^{-1} \times 10^{-4}/cm^2$. The *intervals between arrows* indicate the following: (between 1 and 2) slight effect of heating on the untreated membrane conductance; (between 3 and 4, 7 and 8) the conductance increase induced by 10^{-6} mol/l amphotericin B at 22°C; (between 5 and 6) the conductance increase induced by 10^{-6} mol/l amphotericin at temperature rise by about 10°C. (*b*) The muscle fiber in 160 mmol/l K^+ isotonic solution; the intensity of constant current pulses 0.015 μA. Note the difference between the conductance values at hyperpolarizing and depolarizing stimulus (anomalous rectification). g_0 for hyperpolarizing pulses is 4.3 $\Omega^{-1} \times 10^{-4}/cm^2$. The *intervals between arrows* indicate the following: (between 1 and 2, 7 and 8, 9 and 10) effect of heating on the membrane conductance in 160 mmol/l K^+ isotonic solution without antibiotic; (between 3 and 4) the conductance increase induced by 5×10^{-5} mol/l nystatin at 22°C; (between 5 and 6, 8 and 9) the conductance increase induced by 5×10^{-5} mol/l nystatin at increased temperature.

gests that the channel assembly-disassembly reactions are not always rate-limiting in biological membranes.

The dose-response curves of lg conductance against lg antibiotic concentration in our experiments have slopes which are similar to the slope range (1.5–2.5) obtained for the amphotericin-induced enhancement of erythrocyte permeability (Deuticke et al., 1973). The nystatin-induced conductance in *Aplysia* neurons (Russel et al., 1977) and exchange of K^+ for Li^+ in nystatin-treated red cells (Cass and Dalmark, 1973) are proportional to the 3rd power of the nystatin concentration, whereas in rabbit bladder the conductance of nystatin varies as the 4.6th power of its concentration (Lewis et al., 1977). On the other hand, the power dependence of induced conductance on antibiotic concentration in our experiments is lower than that in artificial membranes. In thin lipid membranes, the steady-state conductance is proportional to the 6th through 12th (Cass et al., 1970), 10th (Kasumov and Liberman, 1972), and 4th through 5th (Kleinberg and Finkelstein, 1984) powers of the nystatin concentration and 5th through 10th powers of amphotericin concentration (De Kruijff and Demel, 1974).

The comparison of the power dependence of polyene conductance effects between muscle and planar bilayers has some serious problems. Most power-dependence studies on planar bilayers have been done at or below the polyene concentration of about 10^{-6} mol/l. It has been shown that as long as the total concentration of AMB in aqueous media is less than 10^{-6} mol/l, AMB exists in a monomeric form (Mazerski

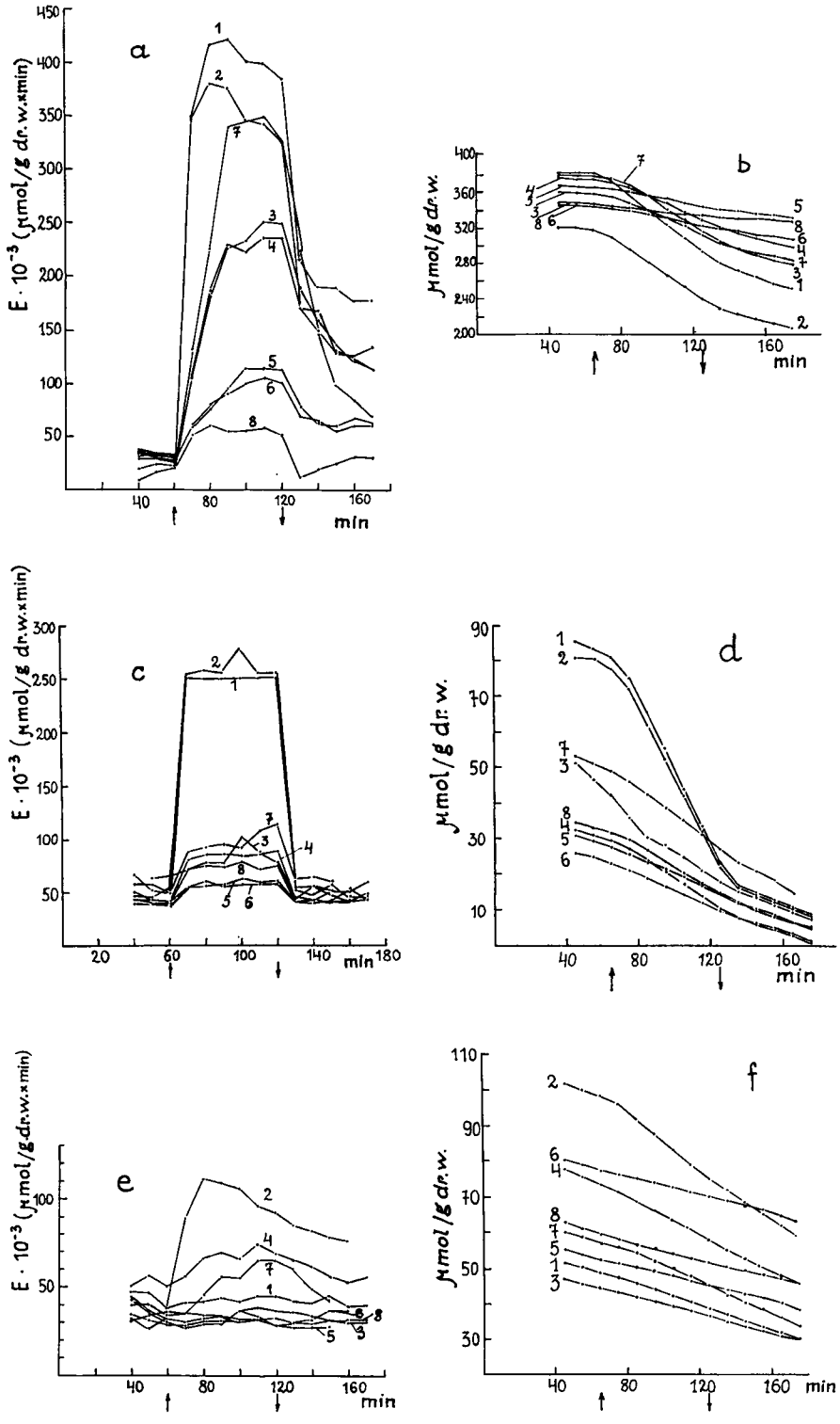

FIGURE 7 Effects of polyene antibiotics on the cation efflux and concentration in the frog sartorius muscle. The muscles were initially incubated for 75 min in sodium- and potassium-free magnesium Ringer solution. *Abscissa*: time (min). *Ordinate*: (*a*), (*c*) and (*e*) effluxes (μmol/g dry weight $\times$ min) $\times 10^{-3}$ for potassium, sodium and lithium, respectively; (*b*), (*d*) and (*f*) potassium, sodium and lithium concentration (μmol/g dry weight) in the same experiment. The values of E_0 are measured in sodium- and potassium-free magnesium Ringer solution at 60 min. Up arrows indicate application, and down arrows indicate removal of polyene antibiotics (10^{-5} mol/l). (*1, 2*) Levorin; (*3, 4*) Mycoheptin; (*5, 6*) Metamphocin; (*7*) amphotericin B; (*8*) nystatin. The antibiotic-induced effluxes $E-E_0$ are higher for K^+ than those for Na^+ and Li^+ in the case of all polyenes used.

et al., 1990). At higher concentrations AMB, AME, nystatin, and other polyenes become aggregated (Mazerski et al., 1982, 1990 Cheron et al., 1988; Bolard et al., 1991). Our studies focus almost exclusively on concentrations within the range of 10^{-6} to 10^{-4} mol/l except levorin, which is applied at a lower concentration. This means that the effects of both monomeric and aggregated forms have been investigated. The effect of aggregation on nystatin and AMB activity has been clarified considerably by Bolard and coworkers (1991). It is proposed that polyenes, which respond by inducing permeability in cholesterol-containing membranes, are incorporated into the membranes mainly in self-associated form of small oligomers (Bolard et al., 1991), most probably dimers (Mazerski et al., 1990). However, it cannot be ex-

cluded that monomers, which have a strong tendency to undergo lipophilic interaction, also bind to cholesterol-containing membranes and subsequently aggregate within the membranes forming permeability pathways. If both forms of antibiotics are active in channel formation, the power dependence may be in the range obtained in our experiments, depending on the mole fractions of monomeric and dimeric forms responsible for the induced cation conductance. Therefore, in our studies, no information about the "molecularity" of the polyene channels can be deduced from the slope values, in contrast to studies of planar bilayer systems.

An observed decreased in AMB and nystatin effect with temperature rise has been reported in thin lipid membranes (Cass et al., 1970), liposomes and *Acholeplasma laidlavii* cells (De Kruijff et al., 1974), *C. albicans* (Johnson et al., 1978), and nystatin-treated (Cass and Dalmark, 1975) and AMB-treated (Bolard et al., 1991) red cells. An increase in polyene effectivity with temperature rise was observed in *Aplysia* neurons (Russel et al., 1977), sterol-containing vesicles and *Mycoplasma* PG3 (Archer, 1976) and *Saccharomyces cerevisiae* cells (Bolard et al., 1991). In liposomes, the temperature dependence of polyene-induced cation transport is related to membrane sterol content (Singer, 1975). The effect of temperature in model membranes is much more pronounced compared with that in our experiments. Thus, the rate of conductance decrease after polyene removal increases in bilayers with a Q_{10} of 10 for AMB and 5 for nystatin (Cass et al., 1970). It may be assumed that the temperature dependence of the destruction kinetics reflects the breakup of the conductance site, whereas the decrease in the formation rate with temperature depends on the melting of the aggregates from which channels are formed. It has been shown that heating results in a shift toward higher concentration of both induced K^+ leakage and aggregation of AMB in the solution (Bolard et al., 1991).

The order of affinity of polyenes for muscle cell membrane (levorin > AMB, mycoheptin > AME > nystatin) is the same as that for bilayers (levorin > AMB > nystatin) (Kasumov and Liberman, 1975). Minimal fungistatic concentrations (μg/ml), determined on *C. albicans*, *Candida tropicalis* and *Cryptococcus neoformans* for the polyenes used in our experiments, follow the sequence levorin > AME > AMB > mycoheptin > nystatin. The order differs from that of potassium conductance and efflux in place of AME, which exerts higher activity in ergosterol-containing fungal cells than in cholesterol-containing muscle membranes, AME, a positively charged alkyl derivative of AMB, has a $—CH_3$ group that replaces the hydrogen in the carboxyl group.

Higher sensitivity of yeast cells to AMB has been clearly related to the presence of ergosterol in this membrane (Gary-Bobo, 1989). The lack of a free carboxyl group in the molecule favors the differentiation between cholesterol- and ergosterol-containing cells. Thus, ergosterol/cholesterol selectivity, expressed as antifungal versus haemolytic activity as well as relation of concentration resulting in 50% potassium release from yeasts versus erythrocytes, is better exhibited for AME than for AMB (Cheron et al., 1988). Furthermore, on cholesterol- and ergosterol-containing lipid vesicles the qualitative difference in the kinetics of ionic fluxes induced by antibiotics without a free carboxyl group is ascribed not only to differences in polyene-sterol interactions but also to the lifetime of the ionic path (Cybulska et al., 1986). Single channels formed in phospholipid-cholesterol bilayers by AME, as opposed to those formed by AMB itself, become nonconducting for some time and the mean lifetime of the active state is much shorter than that of the AMB channels (Kasumov et al., 1979). In order to understand the difference between AME and AMB action by one-sided application, the hypothesis about the different nature of "half-pores" formed in ergosterol- and cholesterol-containing membranes might be useful (Bolard et al., 1991). AME has been claimed to act as a transporter through cholesterol-containing membranes and as a channel former through ergosterol-containing membranes (Gary-Bobo, 1989). At least it must be kept in mind that in the case when the main conducting species are dimers, the difference in the aggregated state of AMB and AME in the solution might play a role (Mazerski et al., 1990). It should be mentioned that aromatic heptaene macrolide antibiotics such as levorin are more potent than nonaromatic polyenes not only in inducing permeability of muscle membrane but also in inducing yeast growth inhibition, red blood cell lysis, and increase in the ionic permeability of large unilamellar lipid vesicles (Cybulska et al., 1983; Mazerski et al., 1983). It appears that the activity of aromatic heptaenes mainly depends upon the structure of their polar head groups (carboxyl in position C_{18} and amino sugar). However, the influence of the hydrophilic part of the ring cannot be disregarded. The ideal selectivity for K^+ of levorin in lipid membranes seems to be associated with the absence of two hydroxyl groups in the hydrophilic chain of the molecule (Kasumov et al., 1981). The irreversible nature of levorin-induced conductance in our experiments is to be expected due to the extremely low solubility of levorin in water. Unlike AMB and nystatin, the aromatic antibiotics are thought to be adsorbed on the surface of the lipid bilayer in monomeric form (Mazerski et al., 1983). It may be that the greater length of levorin, caused by the presence of the aromatic group enables the antibiotic to form a transmembrane channel even in monomeric form. The low sensitivity of muscle membrane to nystatin might be attributed to the flexibility of the molecule at points where the double bonds break, which makes it more difficult for a complex with a cholesterol molecule to form.

In our experiments with the measurement of direct cation efflux, cation leakage is accompanied by Cl^- leakage through the polyene-induced channels. It has been reported that the nystatin-induced pathway is essentially impermeable for divalent ions, and the same is likely to be true for AMB (Cass and Dalmark, 1973; Russel et al., 1977). In the case of magnesium Ringer solution when Mg^{2+} is the only external cation, passive loss of K^+, Rb^+, Na^+, and Li^+ could occur only by simultaneous loss of Cl^-. Although polyene-induced K^+ and Rb^+ effluxes seem to be of a passive nature, the exact

mechanism of Na^+ and Li^+ effluxes in the presence of antibiotics may be more complicated. It has been shown that active Na^+ efflux via the Na^+/K^+ pump in magnesium Ringer solution is to be taken into consideration (Vereninov et al., 1980). There is evidence that AMB (5×10^{-6} mol/l) causes complete inhibition of the Na^+/K^+ pump of human erythrocytes (Vertut-Doi et al., 1988). Furthermore, the role of Na^+/Ca^{2+} exchanger for Na^+ efflux cannot be ruled out. The possible interactions of cation efflux in the pores formed by polyene antibiotics are to be examined in our subsequent studies.

In summary, this study demonstrates the following: a), the kinetics of conductance decrease after removal of polyenes in muscle membrane is much faster than that with two-sided polyene-treatment in bilayers and similar to that obtained with one-sided application; b), the power dependence of polyene-induced conductance in muscle membrane is lower than that in artificial membranes, which shows that processes limiting the rate of channel formation are different in biological membranes and BLM; c), there is a reverse temperature dependence of polyene-induced conductance in muscle membranes, which is less pronounced compared with model membranes; d), levorin exerts high and irreversible effect on muscle cell membranes, indicating a probable toxic influence in antifungal therapy; e) higher activity of amphotericin B compared with metamphocin in muscle membrane and a reverse relation between them in yeastlike microorganisms confirm the data obtained earlier (Cheron et al., 1988; Gary-Bobo, 1989) by comparison of polyene effects on erythrocytes and fungi cells.

In general, our results seem to suggest that the steady-state characteristics of polyene complexes responsible for the permeability induction in biological membranes, and especially the kinetics of channel formation, might differ from those in model membranes.

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REFERENCES

Archer, D. B. 1976. Effect of the lipid composition of *Mycoplasma mycoides* subspecies Capri and phosphatidylcholine vesicles upon the action of polyene antibiotics. *Biochim. Biophys. Acta.* 436:68–76.

Bolard, J. 1986. How do the polyene macrolide antibiotics affect the cellular membrane properties? *Biochim. Biophys. Acta.* 864:257–304.

Bolard, J., P. Legrand, F. Heitz, and B. Cybulska. 1991. One-sided action of amphotericin B on cholesterol-containing membranes is determined by its self-association in the medium. *Biochemistry.* 30:5707–5715.

Borisova, M. P., L. N. Ermishkin, and R. Shlegel. 1988. Role of ketogroups in block of mycoheptin channels by tetramethylammonium ions. *Stud. Biophys.* 123:63–71.

Brutyan, R. A. 1982. One-sided amphotericin B-induced channels in a lipid bilayer. *Biofizika.* 27:646–649. In Russian

Caffier, G., F. Kössler, and G. Küchler. 1980. The influence of free fatty acids on functional properties of isolated skeletal muscles. Octanoate action on membrane resistance. *Pflüegers Arch. Eur. J. Physiol.* 383: 87–89.

Cass, A., and K. Dalmark. 1973. Equilibrium dialysis of ions in nystatin-treated red cells. *Nature: New Biology.* 244:47–49.

Cass, A. A., A. Finkelstein, and V. Krespi. 1970. The ion permeability induced in thin lipid membranes by the polyene antibiotics nystatin and amphotericin. *Br. J. Gen. Physiol.* 56:100–124.

Chen, W. C., J. J. Sud, D. L. Chou, and D. S. Feingold. 1977. Selective toxicity of the polyene antibiotics and their methyl ester derivatives. *Biochem. Biophys. Res. Commun.* 74:480–487.

Cheron, M., B. Cybulska, J. Mazerski, J. Grzybowska, A. Czerwinski, and E. Borowski. 1988. Quantitative structure-activity relationships in amphotericin B derivatives. *Biochem. Pharmacol.* 37:827–836.

Cybulska, B. 1986. Effect of the polar head structure of polyene macrolide antifungal antibiotics on the mode of permeabilization of ergosterol- and cholesterol-containing lipid vesicles studied by ^{31}P-NMR. *Mol. Pharmacol.* 29:293–298.

Cybulska, B., T. Ziminski, E. Borowski, and C. M. Gary-Bobo. 1983. The influence of electric charge of aromatic heptaene macrolide antibiotics on their activity on biological and lipid model membranes. *Mol. Pharmacol.* 24:270–276.

De Kruijff, B., and R. A. Demel. 1974. Polyene antibiotic-sterol interaction in membranes of *Acholeplasma laidlawii* cells and lecithin liposomes. *Biochim. Biophys. Acta.* 339:57–70.

De Kruijff, B., W. J. Gerritsen, A. Oerlemans, P. W. M. van Dijck, R. A. Demel, and L. L. M. van Deenen. 1974. Polyene antibiotic-sterol interaction in membranes of *Acholeplasma laidlawii* cells and lecithin liposomes. *Biochim. Biophys. Acta.* 339:44–56.

Deuticke, B., M. Kim, and Chr. Zöllner. 1973. The influence of amphotericin B on the permeability of mammalian erythrocytes to nonelectrolytes, anions and cations. *Biochim. Biophys. Acta.* 318:345–359.

Ermishkin, L. N., K. M. Kasumov, and V. M. Potseluyev. 1976. Single channels induced in lipid bilayers by polyene antibiotics amphotericin B and nystatin. *Nature.* 262:698–699.

Gary-Bobo, C. M. 1989. Polyene-sterol interaction and selective toxicity. *Biochimie.* 71:37–47.

Horn, R., and A. Marty. 1988. Muscarinic activation of ionic current measured by a new whole-cell recording method. *J. Gen. Physiol.* 92: 145–159.

Isenberg, G., and G. Küchler. 1970. Zur Ableitung bioelectrischer Potentiale von einzelnen Skelettmuskelfasern mit der Sacharosetrennwandmethode. III. Membran-Widerstandsmessungen. *Acta Biol. Med. Ger.* 24: 625–638.

Johnson, B., R. I. White, and G. M. Williamson. 1978. Factors influencing the susceptibility of *Candida albicans* to the polyenoic antibiotics nystatin and amphotericin. *Br. J. Gen. Microbiol.* 104:325–333.

Kasumov, K. M., and E. A. Liberman. 1972. Ionic permeability of bimolecular membranes in the presence of polyenic antibiotics. I. Nystatin and amphotericin B. *Biofizika.* 17:1024–1031. (In Russian)

Kasumov, K. M., and E. A. Liberman. 1973. Ionic permeability of bimolecular membranes in the presence of polyenic antibiotics. II. Lavorine, trychomycine, candycydine. *Biofizika.* 18:264–271. (in Russian).

Kasumov, K. M., M. P. Borisova, L. N. Ermishkin, V. M. Potseluyev, A. Y. Silberstein, and V. A. Vainshtein. 1979. How do ionic channel properties depend on the structure of polyene antibiotic molecules? *Biochim. Biophys. Acta.* 551:229–237.

Kasumov, K. M., N. K. Mekhtiev, and S. D. Karakozov. 1981. Potential-dependent formation of single conducting ion channels in lipid bilayers induced by the polyene antibiotic levorin A_2. *Biochim. Biophys. Acta.* 644:369–372.

Kleinberg, M. E., and A. Finkelstein. 1984. Single-length and double-length channels formed by nystatin in lipid bilayer membranes. *J. Membr. Biol.* 80:257–269.

Leech, C. A., and P. R. Stanfield. 1981. Inward rectification in frog skeletal muscle fibres and its dependence on membrane potential and external potassium. *J. Physiol. Lond.* 319:295–309.

Lewis, S. A., D. C. Eaton, O. Clausen, and J. M. Diamond. 1977. Nystatin as a probe for investigating the electrical properties of a tight epithelium. *J. Gen. Physiol.* 70:427–440.

Liras, P., and J. O. Lampen. 1974. Sequence of candicidin action on yeast cells. *Biochim. Biophys. Acta.* 372:141–153.

Marty, A., and A. Finkelstein. 1975. Pores formed in lipid bilayer membranes by nystatin. *J. Gen. Physiol.* 65:515–526.

Mazerski, J., J. Bolard, and E. Borowski. 1982. Self-association of some polyene macrolide antibiotics in aqueous media. *Biochim. Biophys. Acta.* 719:11–17.

Mazerski, J., J. Bolard, and E. Borowski. 1983. Circular dichroism study of the interaction between aromatic heptaene antibiotics and small unilamellar vesicles. *Biochem. Biophys. Res. Comm.* 116:520–526.

Mazerski, J., J. Grzybowska, and E. Borowski. 1990. Influence of net charge on the aggregation and solubility behaviour of amphotericin B and its derivatives in aqueous media. *Eur. Biophys. J.* 18:159–164.

O'Neill, L. J., J. G. Miller, and N. O. Petersen. 1986. Evidence for nystatin micelles in L-cell membranes from fluorescence photo-bleaching measurements of diffusion. *Biochemistry.* 25:177–181.

Russel, J. M., D. C. Eaton, and M. S. Brodwick. 1977. Effects of nystatin on membrane conductance and internal ion activities in Aplysis neurons. *J. Membr. Biol.* 37–137–156.

Shvinka, N. E., and G. Caffier. 1989. Effect of amphotericin B and nystatin on cation transport in muscle fibre membrane. *Biol. Membr.* 6:1216–1221. (In Russian)

Shvinka, N. E., and G. Caffier. 1991. The kinetics of amphotericin B interaction with the muscle fibre membrane. *Biol. Membr.* 8:1292–1303. (In Russian)

Singer, M. A. 1975. Interaction of amphotericin B and nystatin with phospholipid bilayer membranes: effect of cholesterol. *Can. J. Physiol. Pharmacol.* 53:1072–1079.

Van Hoogevest, P., and B. De Kruijff. 1978. Effect of amphotericin B on cholesterol-containing liposomes of egg phosphatidylcholine and didocosenoyl phosphatidylcholine. A refinement of the model for the formation of pores by amphotericin B in membranes. *Biochim. Biophys. Acta.* 511:397–407.

Vereninov, A. A., and I. I. Marakhova. 1981. The comparative study of sodium and potassium fluxes across muscle membrane in sodium- and potassium-free media. *Tsitologiia.* 23:312–322. (In Russian)

Vereninov, A. A., T. A. Vinogradova, I. I. Marakhova, and F. V. Toropova. 1980. Sodium fluxes through muscle membrane in sodium and potassium deficient media. *Tsitologiia.* 22:781–791. (In Russian)

Vertut-Doi, A., P. Hannaert, and J. Bolard. 1988. The polyene antibiotic amphotericin B inhibits the Na^+/K^+ pump of human erythrocytes. *Biochem. Biophys. Res. Comm.* 157:692–697.