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Na^+, K^+ and Cl^- selectivity of the permeability pathways induced through sterol-containing membrane vesicles by amphotericin B and other polyene antibiotics

Scott C. Hartsel [1], Sandra K. Benz [1], Woubeshet Ayenew [1], Jacques Bolard [2]

[1] The Chemistry Department, The University of Wisconsin-Eau Claire, Eau Claire, WI 54701, USA
[2] L.P.C.B. (UA CNRS 198), Université Pierre et Marie Curie, 4 Place Jussieu, F-75252 Paris Cedex, France
(Tel. 33 1 44 27 42 34, Fax. 33 1 44 27 38 82)

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Abstract. Membrane diffusion potentials induced by amphotericin B (AmB), amphotericin B methyl ester (AmE), N-fructosyl AmB (N FruAmB) and vacidin, an aromatic polyene antibiotic, in ergosterol- or cholesterol-containing egg yolk phosphatidylcholine large unilamellar vesicles (LUV), were measured in various media, in order to determine the relative selectivity of Na^+, K^+, Cl^- and other ions in these environments. Changes in the membrane potential were followed by fluorescence changes of 3,3′-dipropylthiadicarbocyanine (diS-C_3-(5)). Subtle changes in intercationic selectivity were monitored by measuring biionic potentials, using the fluorescent pH sensitive probe pyranine. In all the cases studied, *the intercationic selectivity* of the permeability pathways induced by the four antibiotics was weak compared to that of specific biological channels, though distinct differences were noted. With AmB the selectivity appeared to be concentration dependent. Above 5×10^{-7} M, the sequence determined for sterol-free small unilamellar vesicles (SUV) and cholesterol-containing SUV and LUV, $Na^+ > K^+ > Rb^+ \geq Cs^+ > Li^+$ (sulfate salts), corresponded closely to Eisenman selectivity sequence number VII. At 5×10^{-7} M and below the selectivity switched from $Na^+ > K^+$ to $K^+ > Na^+$. In contrast, Li^+ was the *most* permeant ion for AmB channels in the presence of ergosterol. *The selectivity between* Na^+ *or* K^+ vs. Cl^- varied with the antibiotic. It was very strong with vacidin at concentrations below 5×10^{-7} M, smaller with AmB, nil with AmE and N FruAmB. The selectivities observed were antibiotic, concentration and time dependent, which confirms the existence of different types of channels.

Key words: Amphotericin B – Selectivity – Vesicles – Polyene – Membranes

Introduction

The antifungal antibiotic amphotericin B (AmB) binds to membranes and may induce permeability to monovalent or divalent cations, to anions and to small molecules, the characteristics of the permeability depending on several parameters. The presence of sterols in the membrane is generally necessary for AmB to be active. However, it is not necessary with small unilamellar vesicles, which have a small radius of curvature (Hsu Chen and Feingold 1973; Archer 1976; Vertut-Croquin et al. 1983; Hartsel et al. 1988). Selective permeability to monovalent cations is observed by one-sided addition of AmB to BLM (Marty and Finkelstein 1975; Kleinberg and Finkelstein 1984) and to SUV and LUV (Hartsel et al. 1991). By two-sided addition, anion-selective permeability is observed with BLM (Andreoli 1974; Marty and Finkelstein 1975) but not with SUV or LUV (Hartsel et al. 1991). Higher sensitivity to AmB action of ergosterol- as compared to cholesterol-containing membranes is the basis of the use of the antibiotic against fungal cells, the membranes of which contain ergosterol. However, AmB-induced H^+/OH^- conductances were higher in cholesterol-containing SUV (Hartsel et al. 1988).

There is good evidence that the one-sided mechanism of action of AmB differs for ergosterol- and for cholesterol-containing membranes. Even one type of sterol may have different modes of action depending on the time of incubation, concentration (Cohen 1986, 1992) and the radius of curvature of the vesicles. AmB presumably forms transmembrane channels under all of these conditions, although their precise structure is unknown. With ergosterol, channels are thought to be formed by a sterol-

Abbreviations: AmB, amphotericin B; AmE, amphotericin B methylester; BLM, bilayer membranes; DiSC_3(5), 3,3′-dipropylthiacarbocyanine iodide; DMSO, dimethylsulfoxide; EPC, egg yolk lecithin; FCCP, carbonyl cyanide p-trifluoro methoxyphenyl-hydrazone; HEPES, N-(2-hydroxyethylpiperazine)-N′-(2-ethanesulfonic acid); LUV, large unilamellar vesicles; MOPS, 3-(N-morpholino)propanesulfonic acid; N-Fru AmB, N(1-deoxy-D-fructos-1-yl) amphotericin B; Oxonol VI, bis(3-propyl-5-oxoisoazol-4-yl)pentamethine oxonol; SUV, small unilamellar vesicles.

Correspondence to: J. Bolard

AmB complex, while with cholesterol, the self-associated form of AmB is thought to be most active. In this circumstance it is assumed that cholesterol plays only a physical robe by increasing the membrane order (Kleinberg and Finkelstein 1984; Bolard et al. 1991). However, recent evidence using *ent*-cholesterol, the mirror image of cholesterol, suggests some type of specific interaction between cholesterol and AmB (Mickus et al. 1992).

In view of this complexity there is a need for a rapid test of AmB activity, in terms of intercationic selectivity and anion over cation selectivity. Measurement of the membrane diffusion potential developed in LUV under selected conditions offers such a test. We used the method proposed by Loew et al. (1985), taking advantage of the membrane potential-dependent fluorescence changes of $DiSC_3(5)$. We have extended the study to the derivative AmE, N FruAmB and the aromatic polyene vacidin. In addition, the pyranine fluorescence method was used to develop complete alkali metal selective sequences for AmB in the presence of both sterols (Whyte et al. 1989; Hartsel et al. 1991).

Material and methods

Materials

Amphotericin B and vacidin A (mepartricin B) were gifts from Squibb France (Neuilly, France) and Pr E. Borowski (Gdansk, Poland). AmE and N FruAmB were prepared as already described (Mechlinski and Schaffner 1972; Falkowski et al. 1982). The fluorescent probes $DiSC_3(5)$, oxonol VI and pyranine were purchased from Molecular Probes (Eugene, Oregon, USA), the buffers HEPES, MOPS, the phospholipid EPC, sucrose, valinomycin and gramicidin D from Sigma (Saint Louis, Missouri, USA).

Preparation and assay of lipid vesicle samples

The procedures followed for vesicle preparation and the $DiSC_3(5)$ and oxonol procedures are essentially the same as in Bolard et al. (1991). For membrane potential probe studies cholesterol-containing EPC LUV's (molecular ratio 2/8) and ergosterol-containing EPC LUV's (molar ratio 1/9) were prepared according to the method of Szoka and Papahadjopoulos (1979) at a 12 mM total lipid concentration in five different buffers: 150 mM NaCl, 10 mM HEPES; 150 mM KCl, 10 mM HEPES; 100 mM Na_2SO_4, 10 mM HEPES; 100 mM K_2SO_4, 10 mM HEPES; 300 mM sucrose, 10 mM HEPES. Their pH was adjusted to 6.8 with KOH or NaOH in order to have only one cation in the medium.

For pyranine pH-detected membrane potential fluorescence studies, the vesicles were extruded in 25 mM MOPS buffer at pH 7.20 (the pK of pyranine) and 100 mM of the alkali metal sulfate salt. pH adjustments were carried out with sulfuric acid to keep the medium clear of monovalent anions which may have an effect on channel permeation (Borisova et al. 1986). All of these vesicles contained 10 μM FCCP to ensure rapid proton equilibration. AmB was introducded to the diluting buffer from a 20 mM DMSO stock solution.

Measurement of membrane potential changes induced by the antibiotics

Membranes potential changes were determined according to the method of Loew et al. (1985), slightly modified. Briefly, to 2 mL of an aqueous solution of a membrane potential sensitive fluorescent probe was added a small aliquot (generally 10 μL) of the preparation of LUV's. After stabilization of the fluorescence signal, appropriate concentrations of antibiotic were added. If a membrane polarization resulted from the addition of this antibiotic it could be detected by the fluorescence quenching of the probe because it concentrates in the vesicles. For polarizations negative inside, the probe $DiSC_3(5)$ (stock-solution 1 mM in methanol) was used, for polarizations positive inside, oxonol VI (Apell and Bersch 1987) (stock-solution 40 μM in ethanol).

Best responses were obtained with 2.5 μM $DiSC_3(5)$ or 0.2 μM oxonol VI and 30 μM LUVs. Excitation and emission wavelengths were respectively 620 and 670 nm for $DiSC_3(5)$ and 580 and 660 nm for oxonol VI. The maximum fluorescence changes were determined by addition of gramicidin D (1 nM final) when Na^+ was the cation or valinomycin (20 nM final) when the cation was K^+. The fluorescence of the probe was not affected by the antibiotics up to a concentration of 5 μM. Experiments were done at 23 °C.

Stopped-flow techniques for measuring ion currents using the pH sensitive dye pyranine as a reporter molecule have been developed over the past few years (Whyte et al. 1989; Hartsel et al. 1991) and the concentrations, instrumentation and other experimental conditions were indentical to those in Hartsel et al. (1991) with the main difference being that 1000 Å diameter extruded LUV were used in sterol-containing vesicle studies instead of sonicated vesicles. For the current biionic potential studies, 100 mM sulfate salts of the alkali metals were exchanged for the external K_2SO_4 buffer by Sephadex G-25 column exchange. FCCP was added to all vesicle suspensions in order that the proton permeability is not rate limiting in the presence of AmB. When two identically charged species are permeant in the presence of AmB and are present on opposite sides of a membrane, as under these biionic conditions, *net* ion selecivity may be determined by the direction of the fluorescence (i.e. pH) change. In cases where biionic conditions are met, relative permeability ratios can be calculated from the equilibrium ΔpH using the following special case of the Goldman-Hodgkin-Katz equation.

$$\Delta\Psi = \frac{RT}{zF} \ln \frac{P_{K^+}}{P_{X^+}} \tag{1}$$

where $\Delta\Psi$ is the transmembrane potential, R is the gas constant, T is absolute temperature, F is Faraday's constant, z is the charge of the permeant ion(s) and P_{K^+} and

P_{X^+} are the permeability coefficients of potassium and the test ion, X^+. A pH gradient will build up until it is in equilibrium with $\Delta\Psi$ if protons are permeable. Since at equilibrium:

$$\Delta\Psi = \frac{RT}{zF} 2.303\ \Delta\text{pH} \tag{2}$$

then it follows that:

$$10^{\Delta\text{pH}} = \frac{P_{K^+}}{P_{X^+}}. \tag{3}$$

The conditions necessary for these approximations are met when the sulfate salts of different cations are present inside and outside of the vesicle, since sulfate is impermeant in AmB channels. The H^+ buffering capacity of the system must also be relatively low for the approximations above to hold. Since the starting pH inside is known and the experiments are within the linear range of pyranine fluorescence, calibration curves need not be constructed for each experiment. Instead, a simple data transform from fluorescence intensity to pH (internal) is carried out on the OLIS (Jefferson, GA) stopped flow data handling system.

The accuracy and sensitivity of this pyranine fluorescence method has been tested in model system in several ways. K_2SO_4 gradients versus osmolar sucrose solutions in the presence of valinomycin have generated K^+ diffusion potentials within 5% of the expected Nernst potentials. For several different gradients, dynamic K^+ ion currents with valinomycin have also been measured, giving turnover number of 10^2–10^3/s, in reasonable agreement with other experimental techniques. The sensitivity of the technique is illustrated by the fact that we can readily observe basal membrane permeability toward K^+ and other highly impermeant ions. Our typical calculated permeability coefficients (from initial currents) for K^+ and Na^+ are 10^{-15}–10^{-14} cm/s, in excellent agreement with leakage rate measurements of $^{22}Na^+$ carried out by Hauser et al. (1972).

Results

Inducement of permeability to monovalent cations

Na_2SO_4 or K_2SO_4 were entrapped in LUV's suspended in isotonic sucrose (final EPC concentration 60 μM). The fluorescence changes of $DiSC_3(5)$ observed after addition of the polyene antibiotics were indicative of the membrane potential (negative inside) developed as a consequence of the Na^+ or K^+ efflux induced by the antibiotics. In Fig. 1, given as an example, it is shown that in cholesterol-containing LUV's a fluorescence decrease was observed after addition of AmB, up to 10^{-6} M, and a plateau was reached at a rate increasing with AmB concentrations. The same plateau was reached after addition of gramicidin. For 5×10^{-6} M AmB, a minimum fluorescence was reached after 1 min, then fluorescence started to increase, which indicated that SO_4^{2-} was also permeant. It is important to notice that with the same AmB concentration

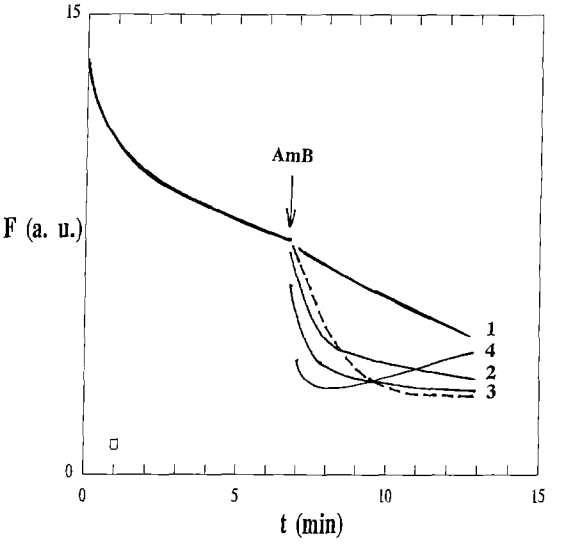

Fig. 1. Polarization resulting from AmB-induced leakage of Na^+. Time dependence of the AmB-induced fluorescence changes of $DiSC_3(5)$ in the presence of cholesterol-containing LUVs (60 μM EPC; molar ratio cholesterol/EPC: 2/8). Internal content of the LUVs: 100 mM Na_2SO_4, 10 mM HEPES adjusted to pH 6.8 with NaOH. External content of the LUVs: 300 mM sucrose, 0.05 mM Na_2SO_4, 10 mM HEPES adjusted to pH 6.8 with NaOH. *Dotted lines*: fluorescence changes after addition of 1 nM gramicidin D. Traces 1 to 4 correspond to concentrations of AmB of 10^{-7}, 5×10^{-7}, 10^{-6}, 5×10^{-6} M

but with increasing concentrations of vesicles, this second phase disappeared progressively. In the concentration range of experiments for the measurement of ΔpH with pyranine (5×10^{-6} M AmB and 1–2 mM lipid) only a plateau was observed (data not shown): SO_4^{2-} was not permeant in these conditions.

Figure 2 demonstrates the antibiotic concentration dependence of the $DiSC_3(5)$ fluorescence changes 3 min after addition of antibiotic corresponding to polarization resulting from Na^+ leakage. It can be seen that in cholesterol-containing LUVs, vacidin is more active than AmB, by a factor of 2 or 3. In contrast, AmE is much less active. Ergosterol-containing LUV's are more sensitive to antibiotic action than cholesterol-containing LUV's. In this case vacidin is less active than AmB. AmE is only slightly less active than AmB.

Intercationic selectivity

For this determination it was necessary to develop experiments which could be done on the same batch of LUV's. Indeed the results depend on the concentration of vesicles and their nature, parameters which vary from one sample to another whatever the care used in the preparation. Comparison could be done on the selectivity of the permeability pathways to K^+ and Na^+ with LUV's containing sucrose and dispersed in K_2SO_4 or Na_2SO_4. Using the fluorescent potential probes $DiSC_3(5)$ or oxonol VI, no clear selectivity could be observed. This determination was also carried out with LUV's containing Na_2SO_4 and suspended in isotonic K_2SO_4, or the reverse. In this case

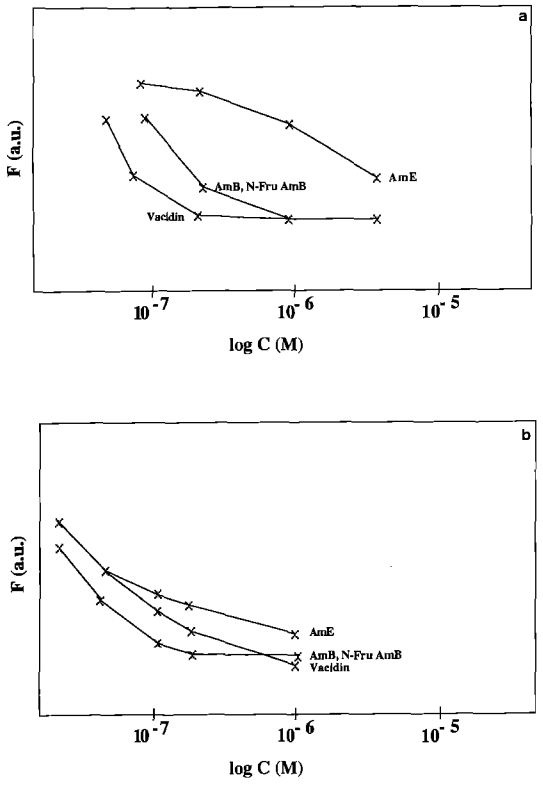

Fig. 2a, b. AmB, AmE, N FruAmB and vacidin concentration dependence of inducement of leakage of Na^+. Antibiotic-induced fluorescence changes of $DiSC_3(5)$ in the presence of sterol-containing EPC LUVs, 3 min after addition of antibiotic. **a** Cholesterol-containing LUVs (60 μM EPC; molecular ratio cholesterol/EPC: 2/8). **b** Ergosterol-containing LUVs (60 μM EPC; molar ratio ergosterol/EPC: 1/9)

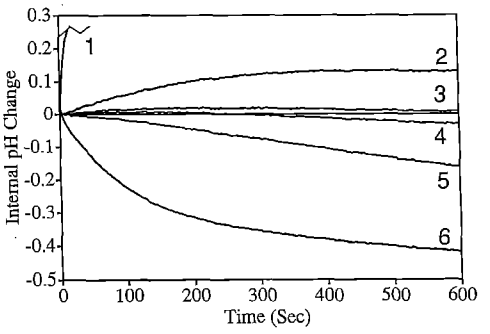

Fig. 3. AmB concentration dependence of the Na^+ over K^+ selectivity. Experimental fluorescence traces (baseline subtracted) were converted to pH changes for the AmB induced ion selectivity profile of cholesterol-containing LUV (3–4 mM EPC; molar ratio cholesterol/EPC: 2/8) with 100 mM K_2SO_4 outside and 100 mM Na_2SO_4 inside, both with 25 mM MOPS, initial pH 7.20. Traces 1 to 6 correspond to AmB concentrations of respectively: control 0 M AmB with 1×10^{-7} M valinomycin; 5×10^{-7} M; 1×10^{-7} M; 1×10^{-6} M; 5×10^{-6} M. Notice that between 5×10^{-7} and 10^{-6} M there is a rather abrupt transition from $K^+ > Na^+$ to $Na^+ > K^+$. This concentration transition corresponds to the CMC of aqueous AmB

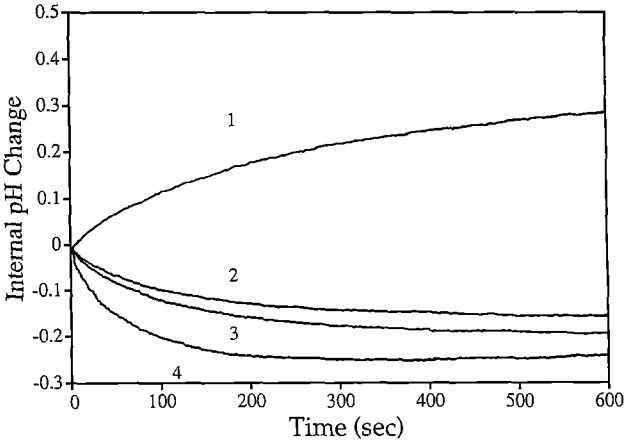

Fig. 4. Typical ion selectivity data of K^+ vs. X^+ using the pyranine fluorescence technique. Experimental fluorescence traces were converted to pH changes for the AmB induced ion selectivity profile of 10% cholesterol-containing sterol LUV with 100 mM K_2SO_4 inside and 100 mM X_2SO_4 outside, both with 25 mM MOPS, initial pH = 7.20. AmB was added to 5×10^{-6} M. External cations are Na^+ (trace 1), Cs^+ (trace 2), Rb^+ (trace 3) and Li^+ (trace 4). Hence the selectivity sequence for this set of experiments is $Na^+ > K^+ > Rb^+ > Cs^+ > Li^+$

no significant and reproducible changes were observed in the fluorescence changes of the potential probes after addition of the antibiotics, in the range of concentrations compatible with the rapidity of the method. For instance with AmB and (10 or 20%) cholesterol-containing LUV this determination was performed in a concentration range of 5×10^{-8} to 10^{-6} M. There was an exception: with AmE and cholesterol-containing LUV's suspended in K_2SO_4 a polarization (positive inside) was observed.

Measurement of potential driven pH changes with pyranine, however, did yield some interesting results with AmB. Indeed this method, in comparison with the preceeding one, enabled measurements in a larger concentration of antibiotic owing to the possibility of recording data at shorter reaction times, that is at high concentrations. Figure 3 shows a set of typical data traces for biionic potential measurements made using entrapped pyranine as a probe, for AmB concentrations between 1×10^{-7} and 5×10^{-6} M (EPC concentration 3–4 mM, cholesterol/EPC = 2/8). The Na^+ versus K^+ selectivity was determined in Fig. 3 relative to internally trapped Na_2SO_4 where a downward fluorescence deflection indicates Na^+ is the more permeable ion and an upward deflection indicates Na^+ is the less permeant ion. It is apparent that at a concentration significantly below the CMC of AmB (Fig. 3, traces 3 and 4) there is little preference, probably because there is little overall activity against cholesterol-containing LUV at these concentrations (Bolard et al. 1991). However, at 5×10^{-7} M (trace 2) there is a distinct $K^+ > Na^+$ selectivity which then shifts to $Na^+ > K^+$ at AmB concentrations $> 10^{-6}$. This fascinating shift in selectivity also occurs with EPC/cholesterol (ratio 9/1) LUV (data not shown) but the selectivities are smaller. These data clearly indicate that different channel structures may predominate depending on the solution concentrations, and therefore structure of AmB (Cohen 1986, 1992; Bolard et al. 1991).

A similar approach was taken for the determination of the intercationic selectivities of K^+ versus Na^+, Li^+, Rb^+ and Cs^+ where this time K_2SO_4 was the entrapped salt. Typical traces are shown in Fig. 4 for cholesterol-contain-

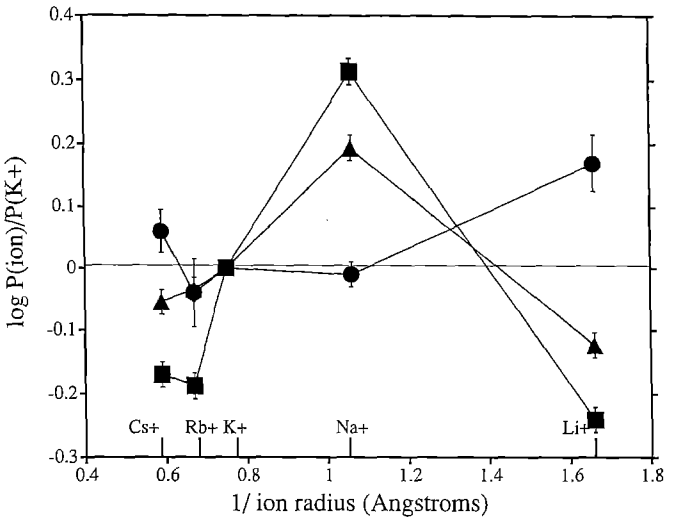

Fig. 5. Topology of ion selectivity of AmB in sterol free egg PC SUV (▲) and 10% cholesterol LUV (■) or ergosterol LUV (●), where AmB was added to 5×10^{-6} M via the stopped-flow syringes. Cations were present as sulfate salts and gradients were created by column dialysis. Permeability ratios were determined from the equilibrium ΔpH values obtained from experimental data typified by Fig. 4. Where equilibrium was not reached after 10 min, final pH values were calculated from curve fitting of the data with the OLIS software. Note that of Li^+ is the most permeant ion with ergosterol-containing vesicles and least permeant in sterol-free or cholesterol-containing vesicles. Data points represent duplicate runs of at least three different vesicle preparations. The total lipid concentrations ranged from about 3 to 4 mM. Temperature was 22 °C ± 1°

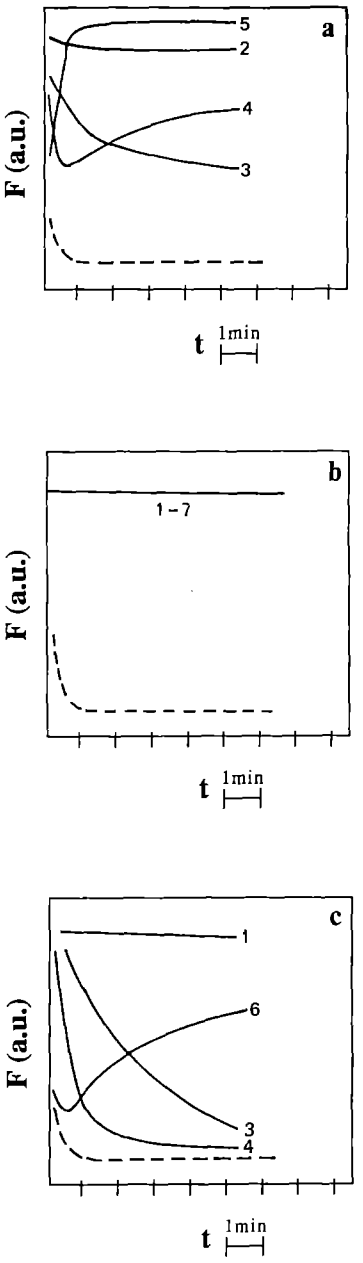

Fig. 6a–c. K^+ over Cl^- selectivity of the permeability pathways induced by AmB, AmE, NFruAmB and vacidin. Time dependence of the antibiotic-induced fluorescence changes of $DiSC_3(5)$ in the presence of sterol-containing EPC LUVs (60 μM EPC; molar ratio cholesterol/EPC: 2/8 and 60 mM EPC. Internal content of the LUVs: 150 mM KCl, 10 mM HEPES adjusted to pH 6.8 with KOH. External content of the LUVs: 300 mM sucrose, 0.05 mM K_2SO_4, 10 mM HEPES adjusted to pH 6.8 with KOH. *Dotted line*: fluorescence changes after addition of 20 nM valinomycin. **a** AmB; **b** AmE and NFruAmB; **c** vacidin. Traces 1 to 7 correspond to antibiotic concentrations of respectively: 2×10^{-8}, 5×10^{-8}, 10^{-7}, 2×10^{-7}, 5×10^{-7}, 10^{-6}, 2×10^{-6} M

ing LUV and 5×10^{-6} M AmB. The equilibrium pH changes can be converted to permeability ratios as seen in Fig. 5. Two noteworthy findings emerge from these data. First, the major difference in selectivity profiles between 10 mol percent cholesterol and ergosterol-containing LUV confirms the existence of different AmB channel structures at these concentrations of AmB and lipid. Secondly, the close correspondence between the selectivity profiles of sterol-free sonicated vesicles (SUV) and cholesterol-containing vesicles suggests that the same structure may be responsible for membrane activity in both systems, as proposed by Bolard et al. (1991). Considering that the actual selectivity ratios are small (see Discussion), it is likely that the pyranine fluorescence method is more sensitive than the methods with membrane potential sensitive dyes, which depend upon redistribution and aggregation (unpublished results).

Monovalent cation over chloride selectivity

Figure 6 shows the membrane potential, as measured by the fluorescence of DiSC(3)5, developed by the polyene antibiotics in KCl containing LUVs suspended in isotonic sucrose. Strong differences appeared between the three antibiotics considered.

AmE and N FruAmB induced no change in fluorescence whatever the type of LUVs, the cation or the antibiotic concentration, even in the concentration ranges in which the antibiotics induced cation leakage, as deduced from experiments performed with Na_2SO_4 or K_2SO_4.

With vacidin, a strong polarization was observed up to 2×10^{-7} M in ergosterol-containing LUVs and 10^{-6} M in cholesterol-containing LUVs. For the highest concentrations studied, after having reached a maximum the polarization decreased.

With AmB, for low concentrations, polarization weaker than with vacidin, was observed. For concentrations

higher than 10^{-6} M in cholesterol-containing LUVs and 10^{-7} M in ergosterol-containing LUVs, after having reached a maximum the polarization decreased. The exact value of the transient polarization in cholesterol-containing LUVs depended on the LUV preparation.

The pyranine fluorescence method was also used to determine relative K^+ vs Cl^- permeability by converting the pH change to the corresponding driving voltage. Cholesterol containing vesicles loaded with 100 mM KCl were exposed to 5.0×10^{-6} M AmB via stopped-flow dilution (with osmolar sucrose, dilution: 5.7:1). A voltage of 30 mV $\pm$ 2 was estimated from the maximum ΔpH as compared to a theoretical voltage of 45 mV expected for ideal K^+ over Cl^- permeability (data not shown). This corresponds to a P_{K^+}/P_{Cl^-} of about 7:1 in general agreement with the results above.

Discussion

The K^+ leakage from LUVs, induced by the polyene antibiotics, has been studied several times. The corresponding appearance of a membrane potential, observed in the present study, occurred in a concentration range expected from the former studies. With AmB it is between 10^{-7} and 10^{-6} M for cholesterol-containing LUVs and between 10^{-8} and 10^{-7} M for ergosterol-containing LUVs (Bolard et al. 1991). The activity of vacidin against cholesterol-containing LUVs was higher than that of AmB in a ratio corresponding to what could be expected from previous results (Gary-Bobo and Cybulska 1982; Cybulska et al. 1983). The increase in activity of vacidin when going from cholesterol-containing LUV's to ergosterol-containing LUVs was weaker than the increase for AmB, a result also expected from former studies. As a consequence, the activity of vacidin against ergosterol-containing LUVs is weaker than that of AmB. For AmE or N FruAmB, there is no prior data on LUVs for comparison.

The intercationic selectivity of the permeability pathways induced by the polyene antibiotics was weak and could not be observed with confidence by the membrane potential fluorescence measurements for concentrations below 10^{-6} M, except for AmE which showed a slight $K^+ > Na^+$ preference. The pyranine fluorescence method was more sensitive to subtle changes in intercationic selectivity and we observed major differences between AmB alkali metal selectivity sequences in ergosterol and cholesterol containing LUV. It should be noted that experiments with pyranine were performed at much higher lipid concentration than experiments with $DiS(C_3)5$ (50 fold). We have seen that in the former case no SO_4^{2-} leakage was observed. As a matter of fact for comparison of the effects of AmB on vesicles at different concentrations, one could consider the data presented on the lipid concentration dependance of the AmB-induced leakage of K^+ in Bolard et al. (1991). For instance, an AmB concentration of 5×10^{-6} M with 75 µM lipids corresponds approximately to a concentration of 3×10^{-7} M with 2 mM lipids.

There have been several other studies on AmB channel cation selectivity which have yielded different results, although none of them were carried out under comparable conditions. Differences in the alkali metal selectivity sequences between Fig. 5 and the partial sequences ($K^+ > Na^+ > Li^+$) derived from the BLM studies (with cholesterol) of Kleinberg and Finkelstein (1984) could be due to a number of factors, including use of nystatin rather than AmB, membrane lipid composition, intrinsic differences between BLM and vesicle systems, different antibiotic concentrations, temperature, or the fact that Cl^- salts were used in that study. AmB concentration seems to be a likely explanation for this discrepancy since at or below 5×10^{-7} M AmB (Fig. 3) the selectivity does indeed switch from $Na^+ > K^+$ to $K^+ > Na^+$. The BLM studies were generally carried out at sub-micromolar concentrations of AmB. To our knowledge, our results show the first known case in which a channel forming compound has demonstrated a concentration dependent switch in ion selectivity. The more recent study of Schell et al. (1989) employed renal brush border membrane vesicles as a model system for nephrotoxicity by AmB. These authors observed a very different cation selectivity of $K^+ > Rb^+ > Cs^+ > Li^+ > Na^+$ at an AmB concentration of ca. 2×10^{-6} M, also with Cl^- salts. However, anions were far more permeant in AmB channels in this vesicle system, a result never previously observed for single sided antibiotic addition (Hartsel et al. 1991). Because of the unique nature of this system and the presence of many intrinsic transporters in these brush border membranes, it is difficult to make a comparison with our more controlled studies. Similar studies were carried out on cholesterol-containing *Acholeplasma* cells in an early study by de Kruijff et al. (1974). In osmotic swelling experiments using a concentration of AmB of about 20 µM they observed alkali metal permeability corresponding to the lyotropic sequence with Li^+ the least and Rb^+ the most permeant ion. With AmB, however, the differences in swelling rates were very minor among these ions. Also, their experimental conditions were very different from ours (e.g. whole *Acholeplasma* cells versus LUVs of known composition, low cell concentrations versus high lipid concentrations, Cl^- salts versus sulfate salts, unknown internal ion concentrations and osmolality versus true biionic conditions and 0 °C versus 20 °C). Considering the influence that temperature, concentration and aggregation state have on AmB activity (Bolard et al. 1991; Lambing et al. 1993), these differences alone could account for these variations. The uncertainty of the concentrations of various other ionic species within the cells (Ca^{2+}, Na^+, K^+, Cl^- etc...) complicates the interpretation of these cellular osmotic swelling rates even further. The BLM studies of Borisova et al. (1986) on the two sided action of AmB did not directly compare different cation permeabilities but did seem to demonstrate that almost no cation permeation could occur in the absence of a permeant anion (e.g. Cl^-, NO_3^-). This is clearly not the case for single sided addition protocols such as ours where we see very significant permeation of cations as their sulfate salts.

However, the seemingly radical shifts in ion selectivity seen in Fig. 4 should be interpreted with some caution. The *absolute* X^+/K^+ selectivity ratios were generally

small, indicating a rather unselective (i.e. large diameter) channel. However, the fact that the observed selectivity sequences are significantly different from the lyotropic sequence we expect in large water-filled channels *does* indicate that channel fine structure is influencing ion permeation. Specifically, the major selectivity shift between ergosterol (Li^+ is most permeant among the alkali metals) and cholesterol (Li^+ is least permeant) containing vesicles seems surprising in light of the prevailing view that AmB channels are similar or identical in these environments. However, a large shift of this sort could be caused by a relatively small structural change in the channel ligand rigidity, as predicted by recent molecular dynamics simulation of simplified gramidicin channels (Polymeropoulos and Brickman 1985). Since it is likely that hydroxyl groups determine the selectivity of channels in both cholesterol and ergosterol containing membranes, differences in the supramolecular structure of these channel types (such as direct incorporation of sterols) are likely to be the determining factors in selectivity. This fits with the recent models for the different possible modes of sterol dependent and independent AmB aggregation (Bolard et al. 1991; Legrand et al. 1992) and with studies demonstrating major sterol dependent differences in H^+/OH^- permeation (Hartsel et al. 1988).

The experiments with NaCl or KCl entrapped in LUVs suspended in sucrose show a monovalent cation over chloride selectivity which varies with the antibiotic considered. With AmE and N FruAmB, no selectivity could be observed by the fluorescence measurements. In contrast, with vacidin, an almost ideal Na^+ or K^+ over Cl^- selectivity was observed. AmB represented an intermediate situation. For this compound the value of 7 found for the ratio P_{K^+}/P_{Cl^-} fits well with that determined with human erythrocytes (Deuticke et al. 1984). In our experiments, the polarization observed generally decreased after having reached a maximum, at least for the highest concentrations studied. This is in agreement with Cohen's results (1986, 1992) in which species different from the first ones were formed with increasing antibiotic concentrations or longer incubation times.

Our results raise questions about the interpretation of polyene antibiotic activity on cells. The polyene antibiotic-induced membrane polarizations observed in mammalian cells have generally been attributed to the polyene antibiotic-induced fluxes of monovalent cations (K^+ or Na^+). In the light of our results that appears to be true for vacidin but it is less clear for AmB and still less so for AmE. AmB-induced Cl^- leakage cannot be neglected for the interpretation of toxicity in mammalian cells, especially in the case of AmE. In particular, with human erythrocytes suspended in sucrose, it was shown (Glaser 1982) that AmB induces a hyperpolarization (negative inside). The origin of the polarization changes cannot simply be assigned to K^+ leakage, particularly in view of the fact that cells suspended in sucrose instead of PBS already have strongly increased membrane permeability to K^+ (Sambavisaro et al. 1986). The similar activities of AmB and N FruAmB in inducing movements of K^+ or Na^+ are particularly striking, in view of the fact that N FruAmB and not AmB, has strong immunomodulating properties (Henry-Toulmé et al. 1989a). The different cation over Cl^- selective could then be the basis of this difference, although internalization of the drugs appeared not to be the same (Henry-Toulmé et al. 1989b).

As far as fungal cells are concerned vacidin is much more toxic than AmB (Schaffner 1984). This is in contrast with data obtained in ergosterol-containing LUVs. This would indicate that either the strong selectivity for monovalent cations or the high protonophoric activity of vacidin (Cybulska et al. 1992) is important in the mechanism of toxicity.

In conclusion, we have obtained information on some important characteristics of the permeability pathways induced by polyene antibiotics across membranes, in conditions relevant to biological studies, that is one-sided addition of the antibiotic and lipid model membranes with a large radius of curvature. These results allow correct interpretations of the first step of polyene antibiotic action on cells, that is passive membrane permeability modulation. It gives a firm basis for the interpretation of other events related to the activity of membrane components such as Na^+/K^+ ATPase (Vertut-Doï et al. 1988; Capuozzo et al. 1990), Na^+/H^+ exchanger (Cybulska et al. 1992) or H^+ ATPase (Surarit and Shepherd 1987).

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