0026-895X/86/030293-06$02.00/0

MOLECULAR PHARMACOLOGY, 29:293–298

Effect of the Polar Head Structure of Polyene Macrolide Antifungal Antibiotics on the Mode of Permeabilization of Ergosterol- and Cholesterol-Containing Lipidic Vesicles Studied by ^{31}P-NMR

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Received August 27, 1985; Accepted December 27, 1985

SUMMARY

Natural polyene macrolide antibiotics and their *N*-acyl and methyl ester derivatives, which differ mainly in their electric net charge, were compared for their ability to increase the ionic permeability of large unilamella vesicles, using the proton-cation exchange method and ^{31}P-NMR spectroscopy. The zwitterionic (amphotericin B, vacidin A) and negatively charged (*N-N′*-diacetyl vacidin) compounds induced permeability according to an all-or-none process on both cholesterol- and ergosterol-containing membranes. The same mechanism of permeability induction is obtained only on ergosterol-containing vesicles for positively charged antibiotics (perimycin A, vacidin A methyl ester, amphotericin B methyl ester). A different type of action is observed for the latter group of ionophores in cholesterol-containing vesicles. In this case, a progressive proton efflux occurs in which all of the vesicle population is involved. This qualitative difference in the kinetics of ionic fluxes induced by antibiotics without a free carboxyl group in cholesterol-containing as compared to ergosterol-containing membranes was ascribed to differences in polyene-sterol interactions as well as in the life time of the ionic path formed. This difference may provide a basis for the improvement of selective toxicity of this group of antifungal agents by rational modifications.

The selective toxicity of polyene macrolide antibiotics in regard to fungal and mammalian cells seems to reside in their differential affinity for the different sterols present in the two cell types. Numerous studies have shown that fungal membranes containing ergosterol are more sensitive to ionophores belonging to the group of large macrolide ring polyenes than are mammalian cell membranes containing cholesterol (1–5).

In a previous publication (6), data were obtained on both biological and model membranes with a series of aromatic heptaenes. From these data, it appears that the relative efficiency in inducing permeability in ergosterol-containing versus cholesterol-containing membrane depends upon modifications of the carboxyl and amino groups at the polar head of the antibiotic molecule, which result in the appearance of a net electric charge. On both biological and model membranes, analogs of VAC without a free carboxyl group (i.e., positively charged) exhibit the highest selectivity for ergosterol-containing membranes. More precisely, these positively charged compounds, in fact, are not more active on the ergosterol-containing membranes than the electrically neutral and negatively charged antibiotics, but are very poorly active on cholesterol-containing membranes.

Besides this quantitative difference in activity level on both membrane types, the study in LUVs by the proton-cation exchange method using the pH-stat technique shows that the neutral and negatively charged polyenes exhibit a clear difference with the positively charged polyenes in the kinetics of the induced ionic flux. The group of neutral and negatively charged polyenes was acting according to an "all-or-none" process: regardless of their activity, the ion flux induced developed very rapidly and reached a stable plateau in a few minutes. The level of this plateau corresponds to a fraction of the total amount of ions transferable (measured after vesicle destruction by Triton X-100 addition) and depends upon antibiotic concentration. It was assumed that antibiotics of this group induce a very high

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This work was supported in part by INSERM (France) Grant SAV1133 and by the Nencki Institute of Experimental Biology, Polish Academy of Science, Warsaw, Poland.

ABBREVIATIONS: VAC, vacidin A; FCCP, carbonyl cyanid -*p*-trifluoro methoxyphenyl-hydrazone; EDTA, ethylenediaminetetraacetate; P_{int}, intravesicular phosphate signal; VME, vacidin methyl ester; NAV, N,N′-diacethyl vacidin; PE, perimycin A; AMB, amphotericin B; AME, amphotericin B methyl ester; LUV, large unilamellar vesicles.

ionic permeability but (at least at low concentration) only in part of the vesicle population. The group of positively charged polyenes was designated as "slow-acting": the ion flux rates obtained were much slower, depended upon concentration, and always resulted in complete equilibration of the vesicular system. In other words, antibiotics of the "slow-acting" group induce a smaller permeability than do the antibiotics of the first group, and their effect is on the total vesicle population, whatever the concentration used.

An analogy may be drawn between this difference in ion flux kinetics between the two groups of polyene antibiotics and the comparable difference observed between mobile carrier and channel-forming ionophores acting on vesicular systems. Such a difference has been shown in small unilamellar vesicles by Clement and Gould (7) and, more recently, on LUVs by Hervé *et al.* (8). In this latter study, a systematic comparison of ion flux kinetics induced on LUVs by the mobile carrier ionophore, valinomycin, and by the channel-forming antibiotics, gramicidin D and AMB, has been carried out by the proton-cation exchange method using, concurrently the pH-stat technique and ^{31}P-NMR spectroscopic pH measurements. The ^{31}P-NMR method, by monitoring intravesicular pH, allows the direct observation of coexisting subpopulations in vesicular suspensions. This comparison has demonstrated that, in the presence of valinomycin, the vesicle population as a whole shifts toward equilibrium at a relatively slow rate which depends upon antibiotic concentration. In contrast, the channel-forming ionophores, gramicidin D and AMB, behave according to the "all-or-none" process. The NMR measurements indicate that, after antibiotic addition, the intravesicular phosphate signal rapidly splits in two parts corresponding to two subpopulations of vesicles: one population reaches equilibrium at a fast rate, and the other remains in the initial condition, apparently unaffected by the antibiotic, at least in the short time range.

In view of these results, ^{31}P-NMR spectroscopy was applied to the study of the mode of permeabilization of cholesterol- and ergosterol-containing vesicles using representatives of positively charged, zwitterionic, and negatively charged polyene macrolides. As will be shown, the large difference in the action of positively charged compounds on two types of membranes is related to differences in the properties of the ionic pathways they form in the presence of ergosterol and cholesterol. Therefore, these positively charged polyenes seem to be particularly interesting from the point of view of selective toxicity.

Materials and Methods

Antibiotics and derivatives. Six polyene macrolide antibiotics were used in this study: VAC, VME, NAV, PE (for the structural formulae see Ref. 6), AMB, and AME. AMB was a Squibb product. Sources of other antibiotics and methods of preparation of their derivatives were reported previously (6). All compounds were used as freshly made solutions in dimethyl formamide. Stock solution contained 1–5 mg of antibiotic/ml of solvent.

FCCP was from Boehringer Mannheim (Indianapolis, IN) and was used as an ethanolic solution.

Lipids. L-α-Phosphatidylcholine was prepared from egg yolk according to the method of Patel and Sparrow (9). Phosphatidic acid was enzymatically prepared from it (10). Cholesterol was from Fluka and ergosterol was from Sigma Chemical Co. (St. Louis, MO). Both sterols were twice recrystallized in ethanol before use.

Preparation of vesicles. The preparation of LUVs by reverse phase evaporation according to the method of Szoka and Papahadjopoulos (11) has been described previously (6, 8). The lipid composition of LUVs was L-α-phosphatidylcholine, phosphatic acid, sterol, in a molar ratio of 80:10:10. The composition of aqueous medium was: 200 mM sodium phosphate, 200 mM sodium sulfate, and 1 mM EDTA, dissolved in 40% D_2O, pH 5.50. Seventy μmol of lipid/ml of aqueous medium were used for the preparation.

After vesicle formation, suspensions diluted four times in 400 mM sodium sulfate were sequentially filtered through polycarbonate porous membranes (Nuclepore Corp., Pleasanton, CA) of 1-μm and 0.4-μm pore size.

^{31}P-NMR spectroscopy. Filtered vesicle suspension (2.5 ml), pH 5.5 (about 45 μmol of lipids) was brought to pH 7.5 with sodium hydroxide. Ten μl of FCCP solution were added to a final concentration of 4 μM; this was followed by the addition of the desired amount of antibiotic solution in dimethyl formamide. The protonophore (FCCP) was added to prevent any restriction in proton movement, as protonophoretic properties of polyene macrolides differ from one antibiotic to the other. After various times of incubation at room temperature, 20 μl of 100 mM $MnCl_2$ solution were added to eliminate the external medium phosphate signal. The suspension was then transferred into a 10-mm bore NMR tube and the spectrum was recorded. The accumulation time was 9 min.

^{31}P-NMR spectra of phosphate ions, with proton noise decoupling, were recorded at 36.4 MHz on a Bruker WH 90 spectrometer. The chemical shifts of the phosphate signals were measured from 85% H_3PO_4 as an external reference. For more detailed information, see Ref. 8.

Results

Proton-cation exchange measured by ^{31}P-NMR spectroscopy. Under the conditions of obligatory proton-cation exchange, the cation permeability induced by ionophores in lipidic vesicles can be measured by monitoring pH changes in the intravesicular or external medium. Whereas pH changes of the external medium can be easily followed by the pH-stat method (6, 12), ^{31}P-NMR spectroscopy has proved to be especially convenient for monitoring intravesicular pH using orthophosphate ions as a probe (8, 13).

In the ^{31}P-NMR spectra of lipidic vesicles prepared and suspended in the medium buffered with phosphate and submitted to a pH gradient, three signals of phosphate ions arising from phospholipids, intravesicular pH and external pH should be detected. The phosphate signal of membrane phospholipids, which is centered at $\delta = -0.9$ ppm in the case of small unilamellar vesicles (13), cannot be seen in LUVs due to their slower mobility. Also, due to the addition of manganese ions to the external medium, the linewidth of the external medium phosphate signal is broadened to such an extent that it becomes undetectable. Therefore, in the conditions used, only signals arising from P_{int} were detected. The position and intensity of these signals show simultaneously both the total phosphate concentration and the pH distribution in the whole population of vesicles. The ionophore-induced permeability can be easily followed by monitoring in time the position and intensity of P_{int} signals.

Large unilamellar lipidic vesicles prepared by reverse phase evaporation and composed of egg yolk phosphatidylcholine are characterized by their relatively high basic permeability to protons (8). However, proton movement is observed only in the case when electroneutrality is ensured. In the case of vesicles prepared and suspended in a medium containing only Na, phosphate, and sulfate, since none of these two anions can cross the membrane, any proton movement can occur only by electroneutral proton/sodium exchange (12). Without ionophore, vesicles could maintain a pH difference up to 2 pH units across their membranes for many hours. Only one signal of phosphate ion, indicating intravesicular pH, is detected during that time. Addition of a proton carrier (FCCP) alone did not induce any significant proton flux. Under these conditions there was no significant NMR chemical shift of the internal medium phosphate signal even after 24 hr. Only a slight decrease in the intensity of the signal was observed, indicating the destruction of a small fraction of the vesicles. Shifts of the P_{int} were initiated by the addition of any ionophore at a sufficient concentration. Under the experimental conditions used, the initial position of the intravesicular phosphate signal was $\delta = 0.25$ ppm, corresponding to pH 5.5. After complete pH equilibration due to H^+/Na^+ exchange mediated by the antibiotic and the protonophore, the P_{int} signal had the same intensity and was detected at $\delta = 2.2$ ppm corresponding to pH 7.5.

Effect of positively charged compounds. The results presented in Fig. 1 indicate that the mode of action of polyene macrolide antibiotics that lack a free carboxyl group is different in vesicles that contain cholesterol compared to those that contain ergosterol. Treatment of vesicles with PE, VME, or AME causes completely different changes in the shape and position of the P_{int} signal, depending on which sterol is incorporated into the membrane. The process of permeabilization of ergosterol-containing vesicles by these three compounds is typical for channel-forming ionophores (8).

In ergosterol-containing LUVs shortly after addition of PE, VME or AME, the initial P_{int} signal splits into two peaks, one ($\delta = 0.25$ ppm) corresponding to the initial pH equal to 5.5 and the second one ($\delta = 2.20$ ppm) corresponding to the pH of equilibrium equal to 7.5. With time, the peak at $\delta = 2.20$ ppm increases in intensity at the expense of the peak at $\delta = 0.25$ ppm. The area covered by these two peaks is always equal either to the initial peak or the peak at equilibrium, showing no destruction of vesicles.

In a manner similar to that of gramicidin D or AMB (8), we also observed for these antibiotics a rapid phase followed by a much slower phase, during which vesicles unaffected at the beginning of drug action were permeabilized after a longer time of incubation. No vesicles of intermediate pH were detected, indicating that ion fluxes occur according to the all-or-none mechanism even during the second slow phase. From the NMR results, a correction has to be introduced to the interpretation of the process of the permeability induction based on pH-stat measurements (6). By the pH-stat method, only the first step of rapid permeabilization was detected. Now it is clear that the stability of the plateau observed in the pH-stat measurements was only apparent. NMR shows that permeabilization is continued, although at a rate too slow to be detected using the pH-stat method.

The rate at which the relative proportion of the two signals varies with time depends upon the compound and its concentration. For PE at a concentration corresponding to 20 molecules/vesicle, the pH equilibration was completed in 2 hr. For the two esters, AME and VME, used at the same concentration,a longer time period was required. For the classical channel-forming agent, gramicidin D, the biphasic process of permeabilization, according to an "all-or-none" mechanism, was observed by another NMR procedure (14).

In cholesterol-containing vesicles, immediately after the addition of the antibiotic, the sharp P_{int} signal centered at $\delta = 0.25$ ppm (corresponding to pH = 5.5) broadens and progressively shifts toward $\delta = 2.2$ ppm, which corresponds to the equilibrium, pH = 7.5. This broadening of the initial peak appears to be due to a heterogeneity of vesicle size and hence to a distribution of ion flux rates (8). After a longer time of incubation the signal is narrowed to the initial linewidth, and only one peak corresponding to pH = 7.5 is detected. The area of the P_{int} signal remains unchanged, indicating that the intravesicular phosphate does not leak out during the whole process.

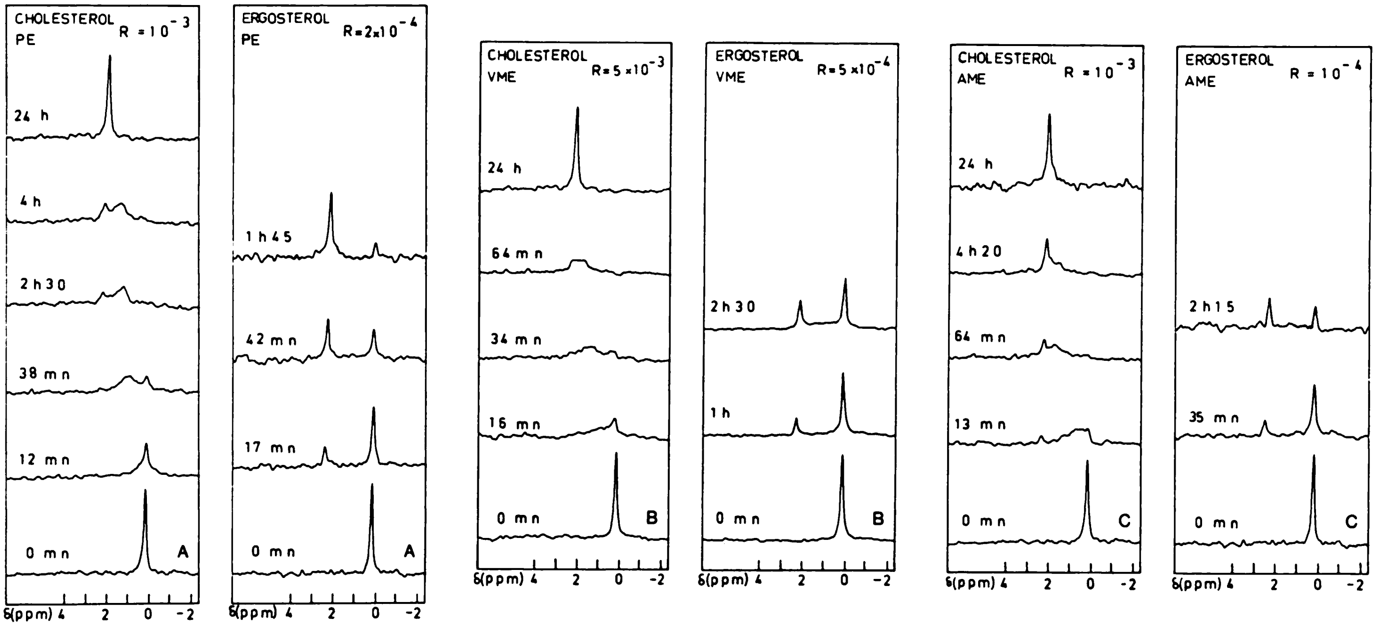

Fig. 1. Ionic permeability induced by positively charged polyene antibiotics studied by ^{31}P-NMR. The evolution with time of the chemical shifts of the intravesicular phosphate is given comparatively in cholesterol- and ergosterol-containing vesicles, for three polyenes: A, PE; B, VME; and C, AME. *R* is the antibiotic/lipid molar ratio. Indicated times are the times of incubation with antibiotic at the midpoints of the accumulation period. The accumulation time was 9 min. The temperature was 295° K.

This means that none of these three compounds at any of the concentrations used exerts any detergent-like effect, even when applied at a relatively high concentration.

Monitored in time, changes of the P_{int} signal reflect the progressive alkalinization of the intravesicular medium, due to the antibiotic-induced Na^+/H^+ exchange. The whole population is permeabilized simultaneously, but complete equilibration of the internal medium pH is reached slowly. The results presented in Fig. 1 are for concentrations of around 100 antibiotic molecules/vesicle for PE and AME and around 500 for VME. This lower efficiency of positively charged compounds in cholesterol-containing membranes is in agreement with pH-stat data (6). It is striking that these compounds in cholesterol-containing membranes exhibit a behavior identical to that observed with the classical mobile carrier, valinomycin (8).

Effect of zwitterionic and negatively charged compounds. The permeability induction by antibiotics having a free carboxyl group, zwitterionic (VAC and AMB) and negatively charged (NAV), is not dependent upon sterol structure. It is the same in both kinds of vesicles and similar to the effect described for positively charged antibiotics in ergosterol-containing membranes. Typically, for channel-forming ionophores (8), the permeabilization is an "all-or-none" and biphasic process. The results obtained for VAC, NAV, and AMB are shown in Fig. 2. In both ergosterol- and cholesterol-containing vesicles, two signals of P_{int} were detected, one corresponding to the initial pH and another corresponding to the equilibrium pH.

The progress of the permeabilization reflected by the changes of the proportions of the two signals of P_{int} in time is dependent upon antibiotic concentration and the kind of sterol. VAC was more effective on ergosterol-containing vesicles but the difference was small in comparison with that observed for VME or PE. For NAV, a slightly higher rate of pH equilibration was observed in cholesterol-containing vesicles. The pattern of permeabilization does not depend upon the antibiotic effectiveness. It was the same for VAC and for its *N*-acetyl derivative which was 10 times less active.

Discussion

Results obtained by ^{31}P-NMR spectroscopy provide information essential for understanding the ionic permeability induced by polyene macrolide antibiotics. This information derives mainly from the ability to distinguish subpopulations of vesicles on the basis of intravesicular pH.

Zwitterionic and negatively charged polyenes (AMB, VAC, NAV) induce permeability in sterol-containing LUVs according to the pattern characteristic of channel-forming ionophores. The process of ionic gradient equilibration occurs according to the "all-or-none" mechanism and is biphasic. The first phase is very fast and, for low and moderate antibiotic concentrations, results in the appearance of two subpopulations of vesicles, one permeabilized and characterized by the pH of equilibrium, and the second unaffected and characterized by the initial pH. This phase was the only one observed for polyene macrolides (6, 8) as well as for gramicidin (7, 8) in the short-term experiments.

In the second phase, permeabilization of the unaffected part of the vesicle population at a very slow rate is observed. In both phases no phosphate signals other than those indicating the initial pH or pH of the equilibrium were detected. This means that there were no vesicles in an intermediate situation, i.e., with a partially discharged pH gradient.

This means that the ionic flux through the channel proceeds at a rate too fast to be observed in the time scale of the NMR experiment and even too fast to be detected by fluorescence spectroscopy, which can measure the kinetics in a fraction of second (7). In other words, in the case of channels characterized by a very high ionic permeability, the measured rate of proton-cation exchange reflects the rate of pore formation and not the ionic permeability of the channel once formed. The coexistence of two subpopulations of vesicles and the very slow rate of disappearance of the "unaffected" subpopulation reflects the statistical distribution of ionophores among the vesicles and the slow exchange of ionophore molecules between vesicles resulting in formation of new pores (15, 16).

It is very important in the present study to note that the

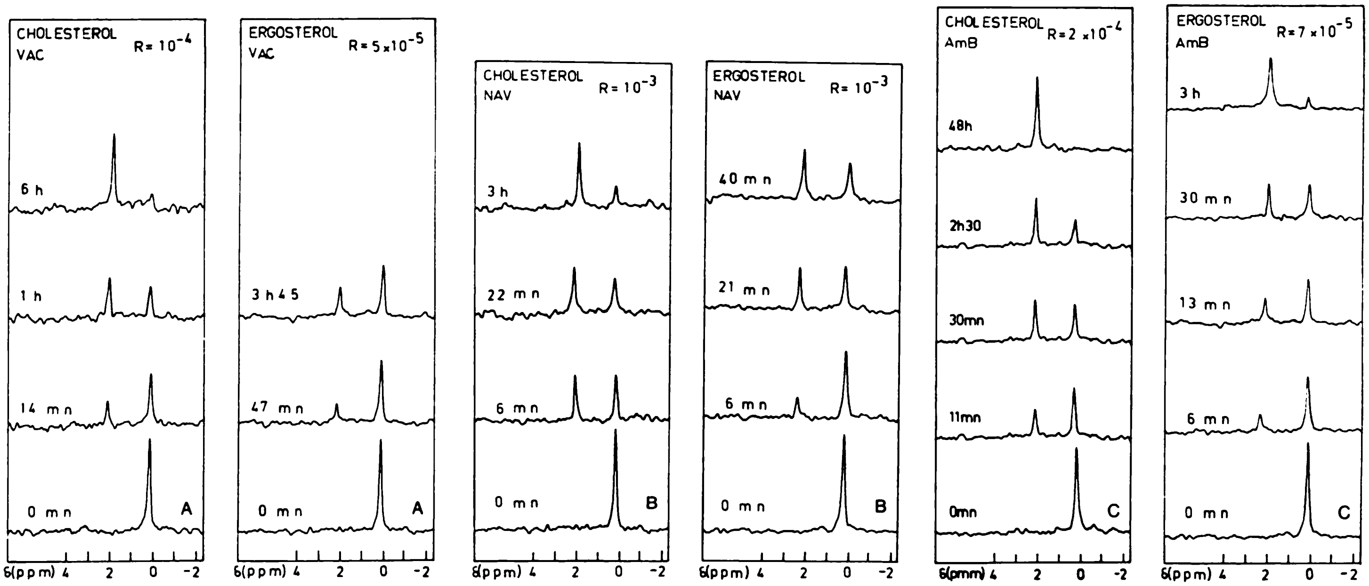

Fig. 2. Ionic permeability induced by zwitterionic and negatively charged antibiotics studied by ^{31}P-NMR. The evolution with time of the chemical shifts of the intravesicular phosphate is given comparatively in cholesterol- and ergosterol-containing vesicles for three polyenes: A, VAC; B, NAV; and C AMB. Specifications are the same as in Fig. 1.

behavior of this group of electrically neutral or negatively charged polyenes is alike whether the vesicles contain cholesterol or ergosterol. Therefore, the higher efficiency of these polyenes in ergosterol-containing membranes (6) appears to be due only to a better interaction with these membranes, but the mode of permeabilization is the same in both types of membrane. This is not the case for the positively charged polyenes. A qualitative difference in the mode of permeability induction of cholesterol and ergosterol-containing membranes is observed in the case of VME, PE, and AME. This confirms the importance of the lack of a free carboxyl group in the C_{18} or C_{16} position in both aromatic and non-aromatic polyenes, respectively.

In ergosterol-containing vesicles, these positively charged compounds were acting according to the all-or-none mechanism described above. In cholesterol-containing ones, the same polyenes induce permeability according to a totally different pattern. Upon addition of these polyenes to a vesicle suspension, the P_{int} signal does not split but slowly shifts toward the equilibrium value. Therefore, the whole population is immediately permeabilized, but the ion flux rate is slow enough to be observed by NMR spectroscopy. This situation is like that observed in typical mobile carriers such as valinomycin (8) and may be summerized as follows.

Taking into account that the polyene concentrations involved are higher in cholesterol than in ergosterol systems, the fact that the whole vesicle population is slowly permeabilized means that the exchange rate of polyene molecules between cholesterol-containing vesicles is, first, much faster than that between ergosterol-containing ones and, second, is fast enough to average the individual ionic flux rates. It is worth noting that the broadening of the P_{int} signal during its shift toward the equilibrium value indicates a rather large dispersion of these individual flux rates. Unlike the "all-or-none" channel-like pattern, ion flux rates measured in the mobile carrier-like pattern are true reflections of the permeability induced by polyenes, since the interaction parameters seem not to be the limiting factor.

In spite of the similarities in the actions of valinomycin and positively charged polyene antibiotics, it is difficult to imagine that the same antibiotic forms channels in ergosterol-containing membranes, whereas in cholesterol-containing ones it acts as a mobile carrier. In fact, classification of ionophores as carriers or channel-forming is based mainly on differences in the kinetics of ionic conductance in black lipid membranes (17). Polyene antibiotics without free carboxyl group probably form channels in both kinds of vesicles. However, the differences detected by NMR spectroscopy demonstrate that ion-transporting properties of the channels formed are strongly influenced by sterol structure.

The rate of ion flux induced by any ionophore depends on two factors: the number of functioning permeability units per vesicle and their ion transporting efficiency. The first factor depends upon the affinity of the polyene antibiotic for a given sterol, and the second factor depends upon the intrinsic permeability properties of the channel and its stability. Both factors play a role, since the concentration of positively charged polyenes required in ergosterol-containing membranes is 10 times lower than that in membranes containing chlolesterol and since only two subpopulations in the initial and equilibrium state are obtained without any intermediate.

The channels formed in ergosterol-containing membranes must have a lifetime long enough for complete equilibration at once. The progressive, slow process of equilibration in the whole population of cholesterol-containing vesicles indicates a lower permeability of the channel, probably due to a much shorter lifetime. This interpretation is supported by the studies of ionic conductance in black lipid membranes performed using many polyene antibiotics (18–21). It has been shown that the channels formed by these antibiotics fluctuate between conducting and nonconducting states. Their lifetime is mainly dependent on the ionic character of the antibiotic molecule (20) but is also influenced by sterol structure and concentration (21). In the studies on AMB modified with respect to the carboxyl and amino groups, it has been found that neutralization of one or both charges in the molecule decreases the probability of the channel to be in a conducting state. In particular, a drastic decrease of 2 orders of magnitude has been found for AME: the mean lifetime of the open channel was 260 sec and 2.5 sec for AMB and AME, respectively (20). Channels formed by AMB in ergosterol-containing membranes were characterized by longer mean lifetimes of both the open and closed states (21). There is evidence in vesicular membranes that complexes formed by polyene macrolide antibiotics with membrane components have to be dynamic (15, 16, 22), and the mobility of the antibiotic is affected by sterol structure (22).

Although pore theory does not account for all of the biological effects of polyene macrolides, it explains well the permeabilizing effects of these antibiotics on model and cell membranes, at least in experiments *in vitro*. Pore properties are dependent on the structure of antibiotic molecules and on the structure of the membrane components with which complexes are formed. The NMR results suggest that quantitative differences in channel properties, such as channel lifetime, may result in qualitative differences in the distribution of permeabilized vesicles in the population. Whether a comparable difference observed in LUVs can be observed in ergosterol- or cholesterol-containing cells is under examination.

It should be stressed that the improvement of selective toxicity of polyene macrolides is always associated with a decrease of their effectiveness toward cholesterol-containing membranes. In addition to their diminished affinity for cholesterol, polyene macrolides that lack a free carboxyl group also exhibit a different mode of permeabilization which is observed in cholesterol-containing LUVs. This suggests that both features, affinity for cholesterol and the properties of the channel determining the ionic conductance, could be essential for the improvement of selective toxicity of this group of compounds. It may be that the absence of the ionizable carboxyl group rather than the net charge of the antibiotic molecule is important for the differentiation between cholesterol- and ergosterol-containing membranes. Studies on other polyene macrolides modified in their ionizable polar groups are under way.

Acknowledgments

We are indebted to Dr. A. W. Rutherford for revising the manuscript.

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