Ion Conductors that Favor Passive Transport in Ergosterol-Rich over Cholesterol-Rich Phospholipid Membranes

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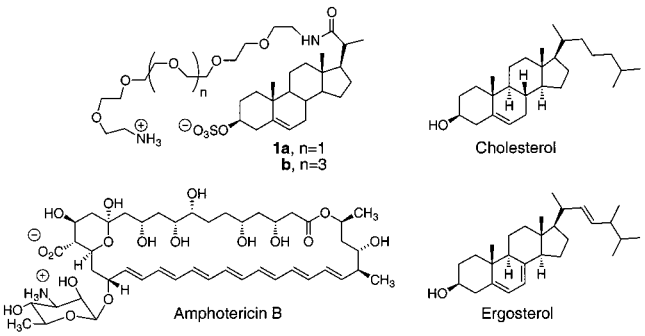
In this paper we show that sterol−polyether conjugates **1a** and **1b** exhibit greater activity in promoting passive ion transport across ergosterol-rich phospholipid bilayers as compared with cholesterol-rich analogues. The relevance of this finding to the rational design of new classes of antifungal agents is briefly discussed.



We have recently reported that a sterol−polyether conjugate (**1a**) promotes Na^+ ion transport across phospholipid bilayers.[1] This conjugate has been specifically designed, based on analogy to the antifungal agent, Amphotericin B (AmB).[2] In particular, the rigid hydrophobic unit (sterol nucleus), the flexible hydrophilic chain (polyether moiety), and the pendant polar headgroup (ammonium sulfate) mimic the heptaene, polyol, and carboxyl/mycosamine components found in Amphotericin B, respectively. On the basis of a strong activity-dependence on membrane thickness, as well as an exponential dependency of transport rates on the concentration of **1a**, a model was proposed in which membrane-spanning aggregates are the active species. Thus, if one assumes that only a small fraction of the conjugate is assembled into such aggregates, then it can be readily shown that

$$k_{obsd} = k_o + k_2[\text{monomer}]^n/K \qquad (1)$$

where k_{obsd} is the observed pseudo-first-order rate constant, K is the equilibrium constant for dissociation of the aggregate into n monomers, k_o is the rate constant for ion transport in the absence of ionophore, k_2 is an intrinsic rate constant for transport, and [monomer] is the analytical concentration of **1a** that is present in the dispersion.[1a] The fact that **1a** and AmB are capable of promoting ion transport across lipid bilayers, together with the fact that both amphiphiles appear to function in an aggregated form, lends strong support for our minimalist approach toward drug design.[1,2]

A key question that we have begun to address, and one that has important practical implications, is whether molecules such as **1a** can distinguish between fungal-like and mammalian-like membranes.[3,4] In particular, can **1a** (like AmB) exhibit significantly greater ion transport activity in phospholipid membranes that are rich in ergosterol (found in fungi) relative to ones that are rich in cholesterol (found in mammalian cells)? It should be noted, in this regard, that the therapeutic value of AmB is presumed to derive from its ability to favor pore formation, ion transport, and toxicity in ergosterol-containing cells, relative to ones that contain cholesterol.[2]

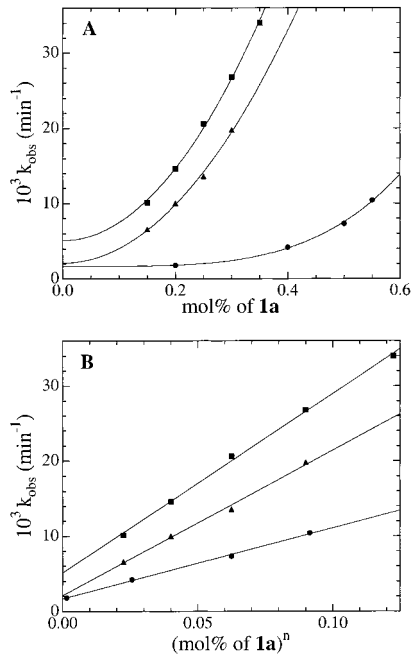


Figure 1. (A) Plot of k_{obsd} versus mol % of **1a** (50 °C) in bilayers composed of DMPC (■), DMPC + 20 mol % ergosterol (▲), and DMPC + 20 mol % cholesterol (●). (B) Plot of k_{obsd} versus (mol % of **1a**)^n in DMPC ($n = 2$) (■); DMPC + 20% ergosterol ($n = 2$) (▲); DMPC + 20% cholesterol ($n = 4$) (●).

Using experimental protocols similar to those previously described, **1a** was incorporated into both leaflets (double-sided addition) of 200 nm unilamellar 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine (DMPC) vesicles that were prepared in LiCl solution.[1] After dilution with an aqueous NaCl solution containing a shift reagent ($DyCl_3$), the rate of entry of Na^+ into the vesicles was monitored using $^{23}Na^+$−NMR spectroscopy at 50 °C. Gel filtration and analysis of the vesicle fractions established that more than 90% of the ionophore was bound to these membranes. Values of k_{obsd} that were calculated from these data showed a second-order dependency on the mole percent of **1a** (Figure 1). Similar experiments that were carried out in the presence of 20 mol % ergosterol also showed a second-order dependency on the mole percent of **1a**. When 20 mol % cholesterol was present in the

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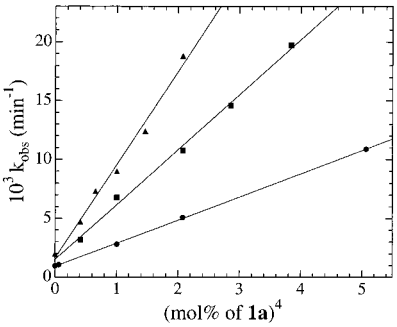


Figure 2. Plot of k_{obsd} versus (mol % of **1a**)^4 in DPPC (■), DPPC + 20% ergosterol (▲), and DPPC + 20% cholesterol (●).

Table 1. Kinetic Parameters for Passive Ion Transport Mediated by **1a**[a]

phospholipid	sterol	$10^3 k_o$ (min^{-1})	$10^3 k_2/K$ (min^{-1} (mol %)$^{1-n}$)	n
DMPC		6 ± 1	240 ± 40	2
	Ergo	2.1 ± 0.3	190 ± 30	2
	Cho	1.6 ± 0.2	90 ± 10	4
DPPC		1.5 ± 0.4	4.7 ± 0.7	4
	Ergo	1.6 ± 0.5	8 ± 1	4
	Cho	0.9 ± 0.1	2.0 ± 0.3	4

[a] Ergo = ergosterol; Cho = cholesterol. All measurements were made at 50 °C. The error in k_{obsd} is estimated to be ±15%.

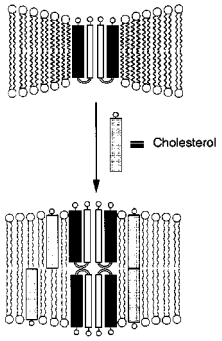
membrane, however, a *fourth*-order dependency of k_{obsd} on the mole percent of **1a** was found. Within the concentration range examined, the presence of cholesterol resulted in a substantial reduction in k_{obsd} relative to pure DMPC bilayers; ergosterol also appeared to lower k_{obsd}, but the effects were only modest.

Related experiments that were carried out using 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine (DPPC) in place of DMPC gave results that are summarized in Figure 2. In all cases, the observed Na^+ transport activity showed a fourth-order dependency on the mole percent of **1a**. In sharp contrast to DMPC-based bilayers, the presence of 20 mol % of ergosterol resulted in an *increase* in transport activity relative to pure DPPC. Similar to DMPC-based membranes, cholesterol-rich bilayers of DPPC showed substantially diminished activity on the basis of the mol % of **1a** that was present.

Because our kinetic analysis does not permit us to separate k_2 from K, our discussion is limited to an operational comparison of the activity of **1a**, and to the apparent size of the aggregates that are responsible for Na^+ transport. What is readily apparent from Figures 1 and 2 is that **1a** exhibits significant selectivity such that ion transport is favored in the ergosterol-containing membranes relative to cholesterol analogues. Specific k_o, k_2/K, and n values that have been obtained for each system are summarized in Table 1. The second- and fourth-order dependencies of k_{obsd} on the mole percent of **1a** that is present implies the existence of transport-active dimers and tetramers, respectively.

Although the precise structure of these transport-active aggregates and the conformation of **1a** in its active form remain to be established, one plausible model is illustrated in Scheme 1; here, a hairpin conformation of **1a** is assumed. Thus, face-to-face dimers that span thinned regions of DMPC bilayers provide a contiguous passage way for Na^+ transport. In the presence of cholesterol, a *thickened* membrane requires back-to-back dimers (i.e., membrane-spanning tetramers) for transport activity.[5,6] Such a model bears a resemblance to the hypothesis advanced by Finkelstein and co-workers in describing the single- and double-sided action of AmB.[7] The finding that the active species is a dimer in ergosterol-rich membranes would then imply that ergosterol does not cause membrane thickening to the same extent as cholestserol.[6] Results that have been obtained with the longer

Scheme 1



DPPC molecule are consistent with this model. Thus, even in the absence of sterol, membrane-spanning tetramers are required for Na^+ transport across this thicker membrane. The relative differences in the rate of ion transport between ergosterol- and cholesterol-containing DPPC bilayers are a likely consequence of differences in (i) membrane thickness, (ii) the position of the monomer−aggregate equilibrium, and/or (iii) the microenvironment.

Finally, in an effort to demonstrate generality, we have measured the rate of Na^+ transport across *unsaturated* bilayers made from 1,2-dimyristoleoyl-*sn*-glycero-3-phosphocholine [PC (14:1)], using a homologue of **1a** (i.e., **1b**); here, both the phospholipid matrix and the structure of the ion conductor were varied, as well as the temperature used (i.e., 35 °C). Thus, using 0.08 mol % of **1b**, values of k_{obsd} across pure PC (14:1) bilayers, PC (14:1) bilayers containing 20% ergosterol, and PC (14:1) bilayers containing 20% cholesterol were found to be 14.8×10^{-3}, 4.39×10^{-3}, and 0.54×10^{-3}, respectively, at 35 °C. These results clearly show that the ergosterol/cholesterol-based selectivity reported herein is not an artifact of the DMPC− and DPPC− sterol systems that have been investigated.[8,9]

To the best of our knowledge, the findings reported herein represent the first example of significant ergosterol/cholesterol selectivity by a synthetic ionophore. Detailed structure−activity studies, which are currently in progress, are aimed at understanding and maximizing ion transport activity as well as membrane selectivity. The results of these efforts will be reported in due course.

Acknowledgment. We are grateful to the National Science Foundation (Grant CHE-9612702) for support of this research.

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(6) Although, to the best of our knowledge, no data exist for the influence of ergosterol on membrane thickness, it is not unreasonable to assume that it acts analogously to cholesterol, but is less effective. For example, ergosterol has been found to be less effective than cholesterol in reducing the permeability of egg PC membranes toward glucose: Demel, R. A.; DeKruijff, B. *Biochim. Biophys. Acta* **1976**, *457*, 109.

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(8) While DMPC bilayers that contain 20 mol % cholesterol are maintained in the fluid state at 50 °C, the broadening effect that 20 mol % cholesterol has on the melting DPPC bilayers is expected to leave a portion of the membrane in the gel phase: Mabrey, S.; Mateo, P. L.; Sturtevant, J. M. *Biochemistry* **1978**, *17*, 2464. The melting temperature of PC (14:1) is estimated to be ca. −40 °C: Lewis, B. A.; Engelman, D. M. *J. Mol. Biol.* **1983**, *166*, 211.

(9) Values of k_{obsd} for Na^+ transport across egg PC bilayers containing 20 mol % ergosterol and 20 mol % cholesterol in the presence of 2 mol % **1b** were 11.7×10^{-3} and 6.8×10^{-3}, respectively, at 35 °C; with 0.005 mol % of AmB, the values were 5.3×10^{-3} and $<1 \times 10^{-4}$, respectively. Using 0.001 mol % of gramicidin A, rate constants in ergosterol- and cholesterol-rich membranes were 2.8×10^{-2} and 2.4×10^{-2}, respectively.