A Synthetic Ionophore That Recognizes Negatively Charged Phospholipid Membranes

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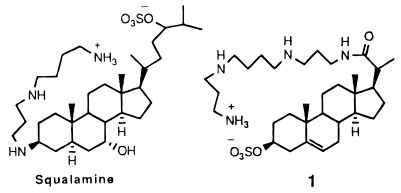
We have recently reported the synthesis of a molecule that mimics the structure and antimicrobial properties of the naturally occurring sterol, squalamine.[1−3] In this paper, we show that this mimic possesses unusual ionophoric properties. Specifically, we show that **1** favors the transport of ions across negatively charged bilayers (egg phosphatidylglycerol, egg PG) over ones that are electrically neutral (egg phosphatidylcholine, egg PC). To the best of our knowledge, this is the first example of *a synthetic ionophore that exhibits membrane as well as ion selectivity*.[4] The potential of **1** as a paradigm for the design of new classes of antibacterial agents is briefly discussed.



In order to test **1** for ionophoric activity, we first examined its ability to discharge pH gradients across phospholipid bilayers. For this purpose, a pH-sensitive dye (pyranine) was entrapped within target vesicles and its fluorescence intensity used to monitor changes in intravesicular pH; i.e., deprotonation of the dye ($pK_a = 7.2$) yields a highly fluorescent phenolate ion.[5,6] In a typical experiment, unilamellar vesicles (1000 Å diameter) were formed via extrusion using 40.0 mg of phospholipid plus 0.63 mg (2.0 mol %) **1** and 2.0 mL of an aqueous buffer that contained 50 mM NaCl, 0.1 mM pyranine, and 25 mM HEPES buffer (adjusted to pH 7.0 with NaOH).[4m,7] Non-entrapped pyranine was then removed by gel filtration (Sephadex G-25). A 200 μL aliquot of the resulting dispersion (2 mM phospholipid) was diluted with 2 mL of a similar buffer that had previously been adjusted to pH 8.0, and the fluorescence intensity monitored as a function of time at 25 °C.

In Figure 1, we show typical fluoresence versus time curves that were observed using egg PG and egg PC targets. In contrast

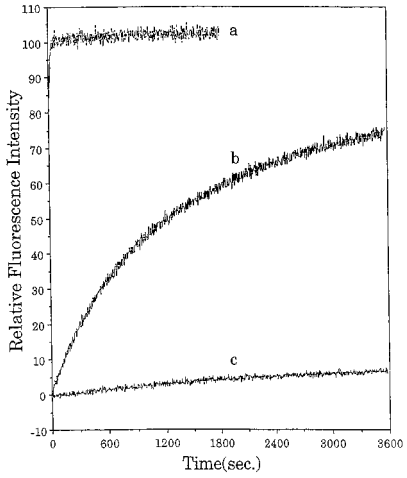


Figure 1. Changes in fluorescence intensity as a function of time for (a) pyranine released from egg PG vesicles using excess Triton X-100, (b) egg PG vesicles containing 1 mol % of **1**, and (c) egg PC vesicles containing 1 mol % of **1**.

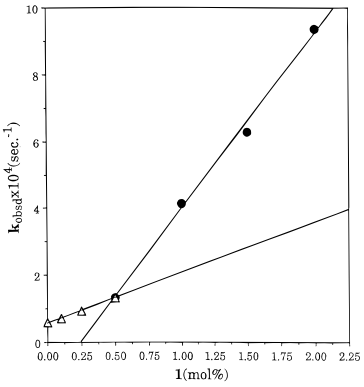


Figure 2. Plot of observed first-order rate constants for egg PG vesicles as a function of mole percent of **1**. Two different symbols have been used to highlight the two linear regions.

to the former, which showed a rapid pH discharge that obeyed pseudo first-order kinetics, relatively little activity was observed with the latter; i.e., the rate was very similar to that observed in the absence of **1** (not shown).[8] A plot of the observed pseudo first-order rate constant (k_{obsd}) as a function of ionophore concentration generated *two discrete linear regions* with a discontinuity occurring at ca. 0.5 mol % of **1** (Figure 2). Gel filtration of a fully-discharged dispersion, and analysis of the void volume fraction, confirmed that >95% of the dye remained entrapped within the vesicles. Thus, the observed increase in fluorescence intensity clearly reflects ionophoric activity and not the release of entrapped dye to the bulk aqueous phase. In striking contrast, **1** *showed negligible activity in egg PC vesicles over this same concentration range*. Estimates of binding of **1** to egg PG and egg PC membranes were made using multilamellar vesicles that were prepared with 2.0 mol % of the ionophore. Thin layer chromatographic analysis (ninhydrin, densitometry) before and after pelleting indicated that less than 5% of the sterol was partitioned into the aqueous phase in both systems; i.e., analysis of the supernatant showed a negligible concentration of phospholipid and **1**.

In order to test for Na^+ transport activity, $^{23}Na^+$ NMR measurements were carried out using procedures similar to those previously described.[4m] In brief, *no activity* was observed for either egg PG or egg PC vesicles over a 48 h period (3 and

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(7) Phospholipid and **1** were codesposited onto a glass surface using $CHCl_3/CH_3OH$ (80/20, v/v).
(8) Qualitatively, very similar selectivities were observed when **1** was added, externally, to preformed vesicles. In this case, however, the kinetics of the pH discharge for egg PG vesicles was complex.

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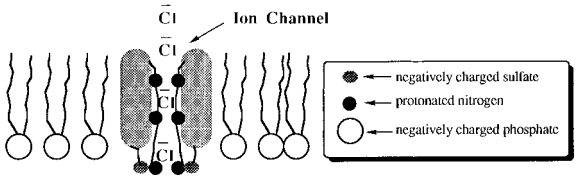

Figure 3. Hypothetical scheme depicting an aggregated form of **1** bearing zwitterionic head groups and a channel, which allows the passage of anions such as chloride ion; in this model, a second channel in the adjoining monolayer leaflet (not shown) would be required in order to produce a contiguous channel across the bilayer.

also 5 mol % **1**); i.e., dilution of dispersions that were initially prepared in LiCl with aqueous NaCl containing a paramagnetic shift reagent, and subsequent NMR analysis, showed that all of the Na^+ remained in the bulk aqueous phase. Analysis of these same dispersions for $^7Li^+$ further showed that the percentage of entrapped Li^+ was unchanged after dilution. This finding not only confirms that the vesicles have remained intact, but also establishes that **1** is inactive for Li^+ transport as well.

The inability of **1** to promote Na^+ transport across egg PG bilayers indicates that either proton and/or anion transport controls the rate of pH discharge. In order to distinguish between these two possibilities, a pH discharge experiment was carried out in the presence of 5 mol % of a protonophore; i.e., carbonyl cyanide *p*-trifluoromethoxy phenylhydrazone (FCCP).[9,10] This weak organic acid has been shown to effectively increase the permeability of lipid membranes toward protons.[5,9] The fact that the rate of discharge was unaffected by the presence of FCCP implies that anion transport is rate-limiting. Since it is not possible to experimentally distinguish between proton permeability and hydroxide permeability, both H^+/Cl^- symport and Cl^-/OH^- antiport pathways must be considered as being equally likely.[9]

The two linear regions that are apparent in Figure 3 provide compelling evidence for the existence of of *two discrete forms of active ionophore*.[11] The simplest interpretation of this result, we believe, is that a "cross-over" occurs from a monomer-active to a monomer- plus aggregate-active form of the ionophore. Specifically, we propose that monomers of **1** (favoring the inner and outer surface of the bilayer) are solely responsible for promoting the pH discharge in the low concentration regime. At 0.5 mol % of **1**, a critical micelle concentration is reached on the membrane surface, which leads to a cooperative insertion of an *aggregate-active* form.[12] In order to test our hypothesis that an aggregate-based ion channel is involved at ionophore concentrations that exceed 0.5 mol %, we have compared the activity of **1** in fluid egg PG membranes relative to analogous gel-phase membrane made from 1,2-dipalmitoyl-*sn*-glycero-3-phosphatidylglycerol (DPPG). The basis for such an experiment may be briefly summarized as follows: An ion carrier is expected to show greatly diminished activity in gel-phase membranes since the rate of diffusion across the bilayer is significantly reduced.[13,14] In sharp contrast, the activity of a membrane-spanning ion channel is expected to be almost independent of membrane viscosity since transbilayer movement of the ionophore is not required. A recent study lends strong support for this hypothesis, where a comparision of the rates of Na^+ transport across egg PC (fluid phase, 25 °C) and 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine (DPPC) membranes (gel phase, 25 °C) has confirmed the carrier mechanism for the natural ionophore, monensin, and a channel mechanism for gramicidin.[4a] Thus, the fact that the activity associated with 1.5 mol % of **1** (6.3×10^{-4} s^{-1}) in egg PG is very similar to that which is found in gel phase vesicles made from DPPG (3.1×10^{-4} s^{-1}), using the same ionophore concentration, strongly suggests that ion channels are involved.[4a,13]

Both the membrane selectivity and ion selectivity of **1** can be accounted for by use of an ion channel model. If one assumes that **1** is drawn into the membrane as a "macrocyclic salt", having its sterol moiety and polyamine chain aligned perpendicular to the membrane's surface, then its head group would be zwitterionic. Since this zwitterionic head group may then favor other zwitterionic head groups (e.g. those of egg PC) as nearest neighbors, internalized aggregates (channels) of **1** might be expected to be more stable in a "sea" of a negatively charged phospholipids than ones that are zwitterionic (Figure 3).[15] Thus, we hypothesize that micelles that form on the surface of negatively charged phospholipid membranes are partitioned into their hydrocarbon interior to a greater extent than those that are formed at electrically neutral phospholipid surfaces, which results in a greater percentage of internalized ionophore. Results that have been obtained from high-sensitivity differential scanning calorimetry analysis of multilamellar vesicles of DPPG and 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine (DPPC) are consistent with this hypothesis. Thus, whereas inclusion of 10 mol % of **1** in DPPG significantly lowers the gel to liquid-crystalline phase transition temperature (T_m) of the main transition (40.7 to 40.4 °C) and eliminates the pre-transition peak, similar inclusion of **1** had no effect on the thermotropic properties of DPPC. Finally, the inability of **1** to promote Na^+ and Li^+ transport can be readily accounted for by charge repulsion by proton-ionized channels that permit the passage of only protons and anions.

The ability of **1** to recognize negatively charged bilayers should make it attractive as a paradigm for the design of new classes of antibacterial agents. In particular, the fact that the outer monolayer leaflet of the plasma membrane of bacterial cells is negatively charged, while those of mammalian cells are electrically neutral suggests that sterol conjugates that are modeled after **1** may be able to selectively destroy the integrity of bacterial membranes.[16] Efforts aimed at controlling the aggregation behavior of related sterol-based ionophores within negatively charged and electrically neutral bilayers, with a view towards drug design, are now under intensive investigation in our laboratories.

Acknowledgment. We are grateful the National Institutes of Health (PHS Grant AI28220) for support of this research.

Supporting Information Available: Schematic representations of the selectivity of **1** when added, externally, to preformed egg PC and egg PG vesicles and the influence of **1** on the endotherms (DSC) of DPPG and DPPC (3 pages). See any current masthead page for ordering and Internet access information.

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(12) A monomer-active species would be expected to have a first-order dependency on the concentration of ionophore that is present. An aggregate-active form would be expected to show an exponential dependency of the concentration of **1** that is present, *only if* the monomer/aggregate equilibrium strongly favored the monomeric form.[11] If the equilibrium were to strongly favor the aggregate form, however, then a first-order dependency on the "analytical" concentration (i.e., the total concentration of **1** that is present in the dispersion) would be expected, which is what we observe.

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(14) Although conductance experiments can clearly distinquish between carriers and channels, such measurements do not have sufficient sensitivity for determining the ion flux associated with **1**. For this reason, we have chosen what is considered by most researchers to be "the next best experiment" (i.e., to measure ionophoric activity in fluid-phase versus gel-phase vesicles).[13]

(15) In preliminary studies, we have found that removal of the sulfate group of **1** (leaving a hydroxyl moiety at the C-3 position) essentially eliminates its ionophoric activity in egg PG membranes at 1 mol %.

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