J|A|C|S
COMMUNICATIONS

Published on Web 08/27/2002

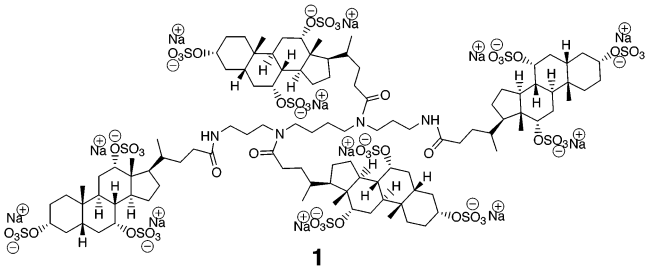
An Ion Conductor That Recognizes Osmotically-Stressed Phospholipid Bilayers

Prasun Bandyopadhyay, Punam Bandyopadhyay, and Steven L. Regen*

Department of Chemistry, Lehigh University, Bethlehem, Pennsylvania 18015

Received May 14, 2002

In this work, we show how a synthetic ion conductor is capable of recognizing a phospholipid bilayer that has been placed under osmotic stress. Specifically, we show that the Na^+/Li^+ transport activity of a spermine/sulfated-cholic acid conjugate (**1**), in liposomal membranes derived from 1,2-dipalmitoleoyl-*sn*-glycero-3-phosphocholine [(C16:1)PC], increases as the liposomes are converted from an isotonic to a hypotonic state. The potential for using osmotic stress in bacterial membranes as a "therapeutic target" is noted.



1

Previous studies have shown that certain conjugates derived from cholic acid and spermine promote Na^+/Li^+ transport across phospholipid bilayers and that the transport activity can be strongly dependent upon membrane thickness. For example, the activity of **1** in bilayers made from (C16:1)PC is ca. 700 times greater than in bilayers derived from 1,2-dioleoyl-*sn*-glycero-3-phosphocholine [(C18:1)PC].[1] On the basis of its ability to recognize small changes in membrane thickness, it occurred to us that such a compound might also be able to recognize osmotically stressed bilayers. In particular, when liposomes are exposed to hypotonic solutions, osmotic swelling leads to an increase in surface area and to a higher occupied area per molecule.[2] For neighboring phospholipids to maintain hydrophobic contact, the number of gauche conformers in the acyl chains must increase, and the bilayer must undergo thinning. Here, we provide the first evidence for *osmotic stress recognition of a phospholipid bilayer by a synthetic ion conductor*.[3,4]

In this study, we examined the activity of **1** for Na^+/Li^+ transport in liposomal membranes made from (C16:1)PC under varying osmotic conditions. Thus, large unilamellar vesicles (200-nm average diameter) were prepared using standard extrusion methods, a 450 mM aqueous (10% D_2O) NaCl solution and varying mole percentages of **1**. Gel filtration, via minicolumn centrifugation (prepacked Sephadex G-25, PD-10, Pharmacia Biotech), removed the NaCl that was present in the external aqueous phase. Subsequent mixing with appropriate salt solutions afforded liposomal dispersions that were osmotically balanced, having an external aqueous phase that was 440 mM in LiCl, 2.4 mM in $DyCl_3$, and 7.3 mM in $Na_5P_3O_{10}$; the latter two salts function as a shift reagent. Exact osmolarities were determined experimentally for each salt solution used. These dispersions were then analyzed for Na^+ efflux by use of ^{23}Na NMR spectroscopy at 35 °C.[5] Similar dispersions that were analyzed for Li^+ influx by ^7Li NMR gave pseudo-first-order rate constants, k_{obsd}, that were identical to those observed for Na^+ efflux. A plot of k_{obsd} versus (mol % of **1**)^2 was found to be linear, indicating that transport-active dimers are involved (Figure 1). Here, it is assumed that only a small fraction of the conjugate is aggregated, where it can be shown that:

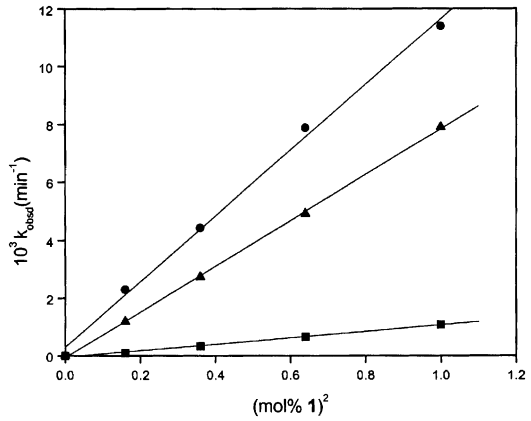


Figure 1. Plot of k_{obsd} versus (mol % **1**)^2 for 200-nm vesicles made from (C16:1)PC at 35 °C under (■) isotonic conditions, and hypotonic conditions having initial osmolarity gradients of (▲) 417 and (●) 517 mosM, respectively.

$$k_{obsd} = \frac{k_2[\text{monomer}]^2}{K}$$

where K is the equilibrium constant for dissociation of the dimer, k_2 is the rate constant for ion transport, and [monomer] is the analytical concentration of the ion conductor that is present in the dispersion.[6]

Analogous dispersions that were prepared under two different hypotonic conditions were also examined for Na^+/Li^+ transport. In one preparation, the initial osmolarity gradient and osmotic pressure were 417 mosM and 10 atm, respectively. A second preparation had an initial gradient and pressure of 517 mosM and 13 atm, respectively. An analysis of the 10-atm dispersion showed ion-transport characteristics that were very similar to those found under nonstressed conditions, except that the activity was significantly greater; that is k_2/K increased from 0.0011 min^{-1} mol $\%^{-2}$ (isotonic) to 0.0079 min^{-1} mol $\%^{-2}$ (hypotonic) (Figure 1).[7] With the 13-atm dispersion, the value of k_2/K increased to 0.011 min^{-1} mol $\%^{-2}$.

Related experiments that were carried out, in which only the shift reagent was present in the external aqueous phase (i.e., LiCl

* To whom correspondence should be addressed. E-mail: SLR0@Lehigh.edu.

10.1021/ja026895s CCC: $22.00

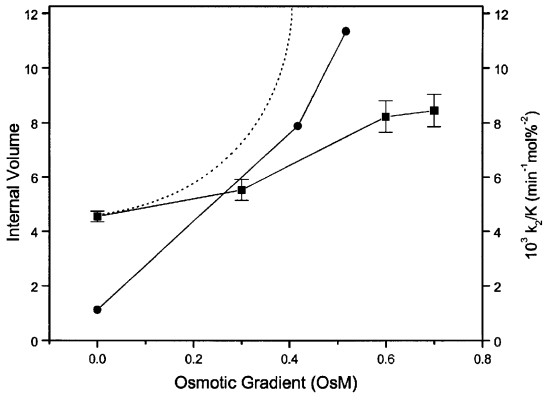


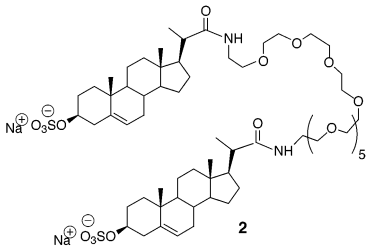
Figure 2. Plot of specific internal volume (■, L/mol of phospholipid) and $10^3k_2/K$ (●, min^{-1} mol %$^{-2}$) as a function of applied osmotic gradient for 200-nm vesicles made from (C16:1)PC; the dotted line represents a hypothetical curve for vesicles exhibiting ideal osmotic behavior.

was absent) and 1.5 mol % of **1** was present in the liposomes, showed *negligible Na*$^+$ *efflux*; addition of LiCl to the dispersion resulted in rapid Na^+/Li^+ transport. Such a finding is significant for two reasons. First, it establishes that the presence of **1** does not destroy the integrity of the bilayer; that is, the movement of Na^+/Li^+ across the membrane takes place by a transport process and not via leakage through defects.[3] Second, it lends strong support for an antiport mechanism in which Na^+ efflux is compensated by Li^+ influx.

To confirm the osmotic responsiveness of liposomes made from (C16:1 PC), we measured their changes in internal volume when exposed to varying hypotonic solutions. Thus, using procedures similar to those previously described, 5(6)-carboxyfluorescein (CF) was encapsulated within large unilamellar vesicles (200 nm), and its self-quenching efficiency (Q) plotted as a function of the internal CF concentration under isotonic conditions.[3,4] Here, Q is defined as a percentage such that $Q(\%) = 100[1 - (I/I_o)]$, where I_o is the total fluorescence intensity of the dispersion after complete release by Triton X-100, and I is the fluorescence intensity of the entrapped CF. Using this plot as a calibration curve, the changes in internal volume (as indicated by changes in internal CF concentrations) could then be determined under hypotonic conditions. A plot of internal volume as a function of the initial osmotic gradient is shown in Figure 2. Also shown in this figure is a hypothetical curve for liposomes exhibiting ideal osmotic behavior. Similar to what has been observed previously, the osmotic properties of such liposomes were found to be nonideal.[2] These results clearly show that a considerable amount of osmotic pressure has been converted into osmotic stress.

The sensitivity of **1** toward Na^+/Li^+ transport in osmotically stressed bilayers is signficant. How much of this recognition is due to a proper matching of the *length* of the transport-active species with the thickness of the bilayer or to a decrease in the packing density of the membrane is not apparent from these results. It was of interest, therefore, to compare the stress-recognition behavior of an ion conductor that shows only a modest dependency on bilayer thickness. One such compound is the sterol−polyether conjugate, **2**. Similar to that for **1**, k_{obsd} exhibits a second-order dependency on the mol % of **2** that is present (see Supporting Information). The ion-transport activity of **2**, however, shows a relatively modest dependence on bilayer thickness; its activity in (C16:1)PC membranes is ca. 20 times greater than in (C18:1)PC liposomes.

When (C16:1)PC liposomes containing **2** were placed under isotonic and hypotonic (517 mosM, 13 atm) conditions, the value of k_2/K for Na^+/Li^+ transport was the same within experimental error (i.e., k_2/K was 0.66 ± 0.06 min^{-1} mol %$^{-2}$). These results imply that the ability of **1** to recognize osmotically stressed phospholipid bilayers has more to do with the length of the transport-active species than changes in the packing density of the membrane. At the same time, they indicate that the ability of an ion conductor to recognize osmotically stressed phospholipid bilayers depends, significantly, upon its structure.



2

In preliminary studies, we have examined the activity of **1** for Na^+/Li^+ transport across liposomes of (C16:1)PC under isotonic and hypotonic conditions when **1** is added, externally, to preformed liposomes; that is, the ion conductor is introduced under "single-sided" conditions. Qualitatively, the results that were obtained were similar to those found under "double-sided" conditions, where the ion conductor was present during liposome formation. Thus, the values of k_2/K under isotonic and hypotonic (517 mosM, 13 atm) conditions were 0.0044 and 0.041 min^{-1} mol %$^{-2}$, respectively.

Efforts currently in progress are aimed at examining, in greater detail, the relationships that exist between the structure of an ion conductor and its osmotic stress-recognition behavior with a view toward drug design.[8] The results of these studies will be reported in due course.

Acknowledgment. We are grateful to the National Science Foundation (Grant CHE-9986704) for support of this research.

Supporting Information Available: Procedures for the synthesis of **2** and ion transport measurements; plots of k_{obsd} versus (mol % **2**)^2 (PDF). This material is available free of charge via the Internet at http://pubs.acs.org.

References

(1) Bandyopadhyay, P.; Janout, V.; Zhang, L.; Regen, S. L. *J. Am. Chem. Soc.* **2001**, 123, 7691.
(2) (a) Bummer, P. M.; Zografi, G. *J. Pharm. Sci*. **1988**, *77*, 1021. (b) Naka, K.; Sadownik, A.; Regen, S. L. *J. Am. Chem. Soc*. **1993**, *115*, 2278.
(3) Melittin and certain wedge-shaped surfactants disrupt the integrity of osmotically stressed liposomal membranes, releasing calcein and 5(6)-carboxyfluorescein, more efficiently than similar membranes held under isotonic conditions.[2b,4] In contrast, liposomes that contain **1** do not release Na^+ until Li^+ is added, externally; i.e., **1** is not membrane-disrupting.
(4) Benachir, T.; Lafleur, M. *Biophys. J*. **1996**, *70*, 831.
(5) Pregel, M. J.; Jullien, L.; Lehn, J. M. *Angew. Chem., Int. Ed. Engl*. **1992**, *31*, 1637.
(6) Otto, S.; Osifchin, M.; Regen, S. L. *J. Am. Chem. Soc*. **1999**, *121*, 7276.
(7) A similar experiment that was carried out using 1 mol % of **1** and a 10-atm osmotic gradient, with *lower salt concentrations* (the internal NaCl and external LiCl concentrations were reduced from 450 and 208 mM to 250 and 49 mM, respectively) gave the same rate constant, within experimental error (±10%). Thus, ionic strength is not a significant factor in these experiments.
(8) Hypotonicity produces osmotic pressures of ca. 15−20 and 0.9−5 atm in Gram positive and Gram negative bacteria, respectively. In contrast, mammalian membranes are relatively stress-free: Csonka, L. N. *Microbiol. Rev*. **1989**, *53*, 121.

JA026895S