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Synthetic, Sodium-Ion-Conducting Tris(Macrocycle) Channels that Function in a Phospholipid Bilayer Membrane: an Overview

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A family of tris(macrocyclic) compounds of the form sidearm<N18N>C_n<N18N>C_n<N18N>sidearm, has been prepared, characterized chemically, and assayed by dynamic ^{23}Na-NMR for the ability to transport sodium cations in a mixed phosphatidylglycerol/phosphatidylcholine vesicle system. A number of control experiments have been conducted that reinforce the hypothesis that cation transport occurs by a channel mechanism. The tris(macrocycle) channels proved to be functional at a level of 25–50% of that found for the natural pentadecapeptide channel gramicidin. Fluorescence data confirm the expected position of the channel within the bilayer.

Keywords: Ionophore, channel, bilayer membrane, cation transport

INTRODUCTION

Many cells and organelles are enclosed by a bilayer membrane that constitutes the boundary between the cytosol and the external aqueous environment. Because of the insulation provided by the phospholipid bilayer, the cell's interior is essentially isolated from the external medium. Cations, anions, and molecules required for the cell's survival must be transported across these barrier membranes for them to survive. This is accomplished by proteins that form transmembrane ion channels. Because of this critical cellular function, the study of ion channels is one of the most active areas in modern biological chemistry.[1,2] The fundamental importance of this area from both a chemical and biological perspective can hardly be overstated.[3]

Studies in supramolecular chemistry often involve systems that possess chemically distinct units known or thought to be present in more complex structures. By designing such "simplified" assemblies, we may gain insight into the structures and/or function of complicated, natural molecular systems.[4] Such an approach presumes that one has sufficient understanding of the natural target to prepare a compound having related function or properties. During the past ten years, we have attempted to understand the function of protein channels by the design, synthesis, and study of a completely synthetic model system.[5] Attempts have been made by other groups to mimic the transport of cations by a channel, rather than a carrier, mechanism.[6]

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These include efforts by Tabushi,[7] Menger,[8] Kobuke,[9] Nolte,[10] Fuhrhop,[11] Lehn,[12] Fyles,[13] Voyer,[14] Regen,[15] Ghadiri,[16] Reusch and Seebach,[17] Matile,[18] Mendoza,[19]and others.[6] We have developed a synthetic, tris(macrocyclic) ionophore that exhibits activity similar to that of natural channel-forming compounds.[5]

RESULTS AND DISCUSSION

Flexibility and rigidity

Synthetic organic model compounds have many advantages as mimics of natural structures. In particular, host molecules have been studied extensively as complexing agents for a variety of guest cations.[20] In general, cryptands bind appropriately sized cations much more strongly than do crown ethers designed for the same function.[21] This is because the rigid, three-dimensional placement of the donor groups in cryptands is ideal for solvating the bound cation. Cryptands do not transport cations as effectively as do crowns, however, because their inherent binding dynamics are more limited than those known for crown ethers.[22] Thus, the advantage of rigidity and organization in cation complexation is offset in this case by the disadvantage of poor dynamics. An essential feature of our channel system was to incorporate flexibility into the design.

Channel design features

Several other issues required consideration. First, the channel must function in a phospholipid bilayer membrane. The membrane possesses three distinct regimes that can be identified as (1) the insulator, (2) the mid-polar region, and (3) the headgroup. The channel must certainly span the insulator region of the membrane. This regime is sometimes referred to as the "hydrocarbon slab" and is generally considered to be 30–34Å wide.[23] This is in accord with the dimension apparent in the recently reported crystal structure of the *Streptomyces lividans* potassium-conducting channel.[24]

A second consideration was what, if any, donor groups would be required within the channel structure. Cations passing from the external to the internal leaflet of the phospholipid bilayer must traverse the bilayer's midplane. This is the least polar regime of the membrane. Intuition suggests that some "relay" group is required to be located at or near the bilayer midplane but it cannot be so strong a donor group as to fix the cation's position. Clearly, the balance between transient stabilization of the cation and strong binding of it must be carefully considered. The issues are similar to those raised above for cryptand *vs.* crown-mediated cation transport. A decision was required about the identities, strengths (donicities), and orientation of the donor groups. Moreover, since these donor groups will serve as cation relays, their position within the phospholipid bilayer must also be carefully considered. We decided to place a single donor group array at the center of the channel structure.

A channel structure requires an extended conformation and amphiphilic character. Headgroups of some sort are required to position the channel within the phospholipid bilayer. Crown ethers seemed to be good candidates for this application since they have good cation affinity. Further, Kuwamura[25] and Okahara[26] had demonstrated that several alkyl crowns could form organized assemblies (micelles). The inference was that crown ethers could serve as amphiphile headgroups. In separate studies, we have confirmed this.[27]

Our overall design concept for a cation-conducting channel was as follows. Macrocycles would serve as cation entry portals and as headgroups to stabilize the channel's termini at opposite ends of the bilayer. The overall distance spanned by the channel would be ~30Å and this would be defined by two, covalently linked

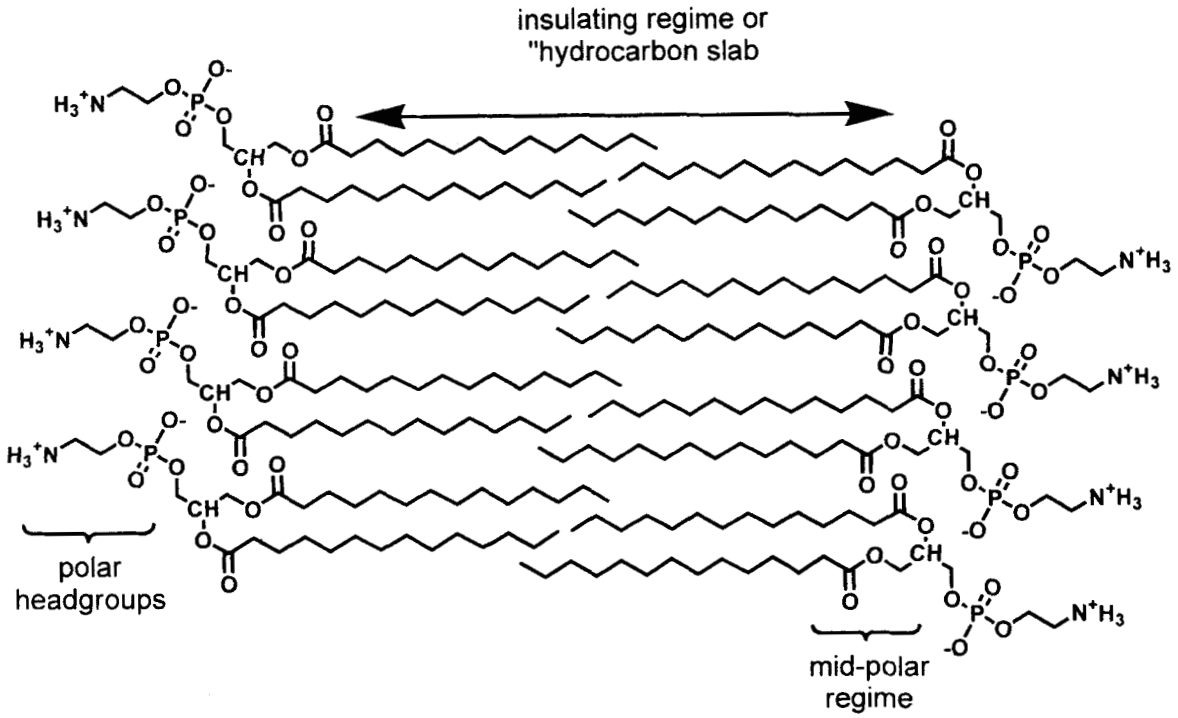


FIGURE 1 Segment of a phosphatidylethanolamine phospholipid bilayer showing the three polarity regimes

spacer chains of 12 carbons each. The linear distance spanned by a carbon-carbon bond is a little over 1Å so a dodecyl chain is about 14Å long in its extended, all *anti* conformation. A third macrocycle was incorporated to serve as a central relay unit; its thickness would make up the additional span. Flexible sidearms would be incorporated to fill in the opposite side of the "tunnel" or "groove" created within the bilayer by the channel structure. The design is illustrated schematically in figure 2.

Initial target

The initial target of our synthetic effort was the tris(macrocycle) having three 4,13-diaza-18-crown-6 ether residues. The plan was to connect the macrocycles with dodecyl chains. The latter would also serve as the flexible sidearms. The structure may be conveniently illustrated using the following shorthand:
C_{12}<N18N>C_{12} <N18N>C_{12}<N18N>C_{12}, **1**.

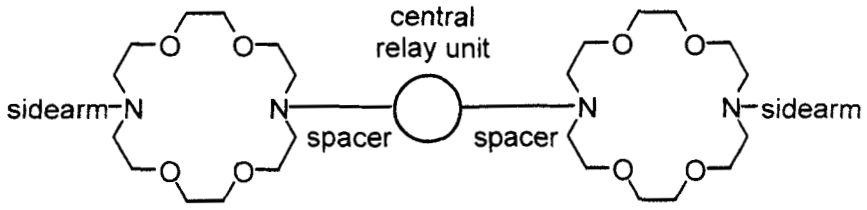


FIGURE 2 Schematic of the synthetic channel design

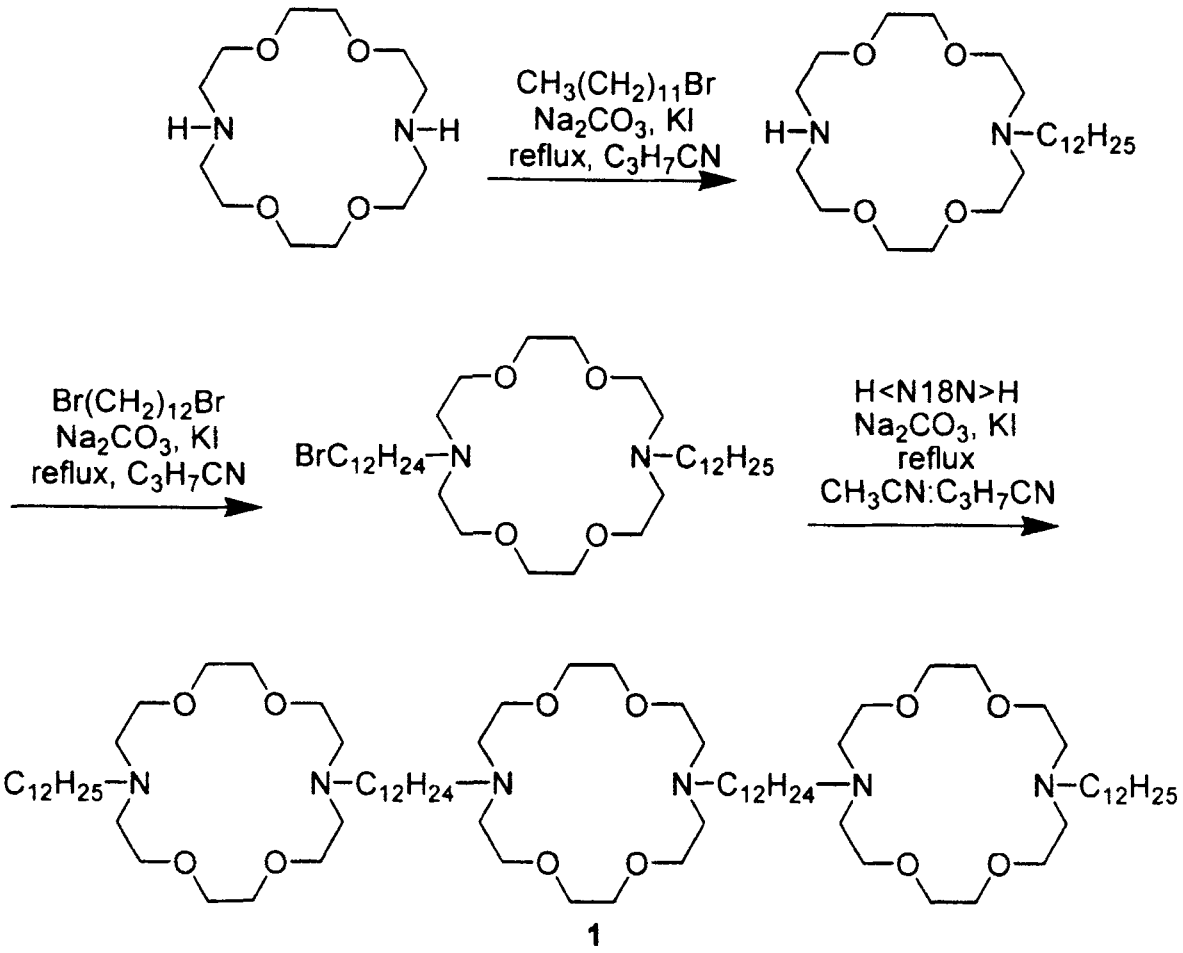


FIGURE 3 Synthetic sequence for the preparation of **1**

Channel syntheses

The synthesis of tris(macrocycle) **1** presents certain challenges. The molecule is two-fold symmetric about the central macroring but the distal macrocycles are asymmetrically (though similarly) substituted. Our strategy for synthesis was executed as follows. 4,13-Diaza-18-crown-6 (H<N18N>H) was monoalkylated using 1-bromododecane. The monoalkylated derivative, C_{12}<N18N>H, was then further alkylated using 1,12-dibromododecane to give C_{12}<N18N>C_{12}Br. Reaction between two equivalents of this bromide and H<N18N>H afforded **1**.

The preparations of other molecules in this family were conducted in a conceptually similar fashion.

Assessment of transport efficacy

The transport of sodium cation through a phospholipid bilayer membrane was assessed by using the ^{23}Na-NMR-based method of Riddell.[28] This method has the advantage of permitting ready comparison of rates among the ionophores studied. In this method, vesicles were prepared from phosphatidylcholine and phosphatidylglycerol

(4:1 w/w) in the presence of 100 mM NaCl (phosphate buffer, pH = 7.3).[29] Vesicle size (typically 1750–2000Å) was determined by laser light scattering. The ^{23}Na-NMR spectrum showed a singlet. When dysprosium tris(polyphosphate) shift reagent was added to the vesicle suspension,[30] two ^{23}Na signals were observed. The signal for $^{23}Na^{+}_{outside}$ was larger than for $^{23}Na^{+}_{inside}$ because of its greater concentration.

Incorporation of an active ionophore into the vesicles, induced line-broadening of both signals in the ^{23}Na-NMR spectrum. In the slow exchange region, the rate constant, $k = 1/\tau$, is directly proportional to the line broadening observed, $\pi[(\Delta\nu - \Delta\nu_o)]$, where $\Delta\nu$ is the linewidth at half-height of the observed resonance line in the presence of the ionophore and $\Delta\nu_o$ is the corresponding value in its absence. The exchange rate constant in each case was determined from the slope of a plot of $1/\tau$ *vs.* channel concentration.[31] A simultaneous assay of gramicidin was done as a control. By setting the rate observed for gramicidin[32] to an arbitrary value of 100, we found that **1** transports Na^+ with a relative rate of 28.

Control experiments

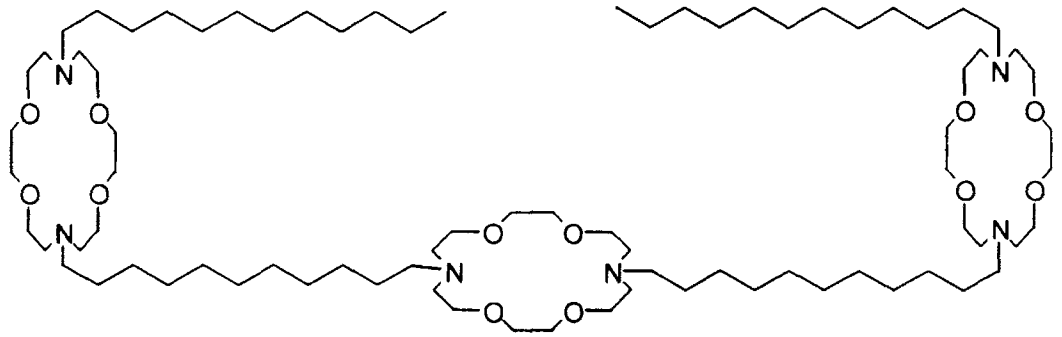
We have undertaken certain controls to eliminate other transport mechanisms and other phenomena that might be interpreted as channel activity. First, it was possible that structural fragments of the channel could transport Na^+ as effectively as does **1**. Thus, neither C_{12}<N18N>C_{12}<N18N>C_{12} nor <18N>C_{12}<N18N>C_{12}<N18> showed Na^+ cation transport at a rate detectable by the ^{23}Na-NMR method. The former is a major fragment of **1** and the latter is identical to **1** except that the sidearms and attached distal nitrogen atom have been replaced by oxygen. Two known carrier molecules, $PhCH_2$<N18N>CH_2Ph and C_{12}<N18N>C_{12} both failed to transport sufficient Na^+ to be detected under these conditions. Carrier molecules, even if relatively complex, do not seem to function in the present case.[5]

A channel is effectively a membrane defect. It was possible that the molecules prepared as ionophores were creating a defect in the phospholipid bilayer much as a detergent might. This seemed unlikely since the transport rates were reproducible and varied with changes in structure. As a control, we examined the efficacy of sodium dodecyl sulfate and Triton X-100 in place of **1**. The former is an ionic detergent and the latter is nonionic. The concentration of ionophore added in the case of **1** and its relatives is typically 0–15 μM. In the control experiments, the concentration range was increased to 0–200 μM but no cation transport could be detected using either detergent.[5]

It seemed to us that the tris(macrocycle)s might be an unusually active group of carrier molecules because they are flexible and have multiple binding sites. Sodium transport rates were measured using a bulk $CHCl_3$ membrane. Transport observed in a concentric tube apparatus of this sort occurs by a carrier mechanism. When the sodium transport rates observed for a family of tris(macrocycles) of the form R<N18N>C_{12}<N18N>C_{12}<N18N>R obtained in bulk membrane and phospholipid bilayer were compared, no correlation was discernable. This does not confirm the channel mechanism but it certainly discounts the obvious carrier mechanism.

Function of the central macroring

An important question was whether the central macroring was parallel to the membrane surfaces as the distal macrocycles are thought to be. The sodium transport rates of three macrocycles were compared. They were C_{12}<N18N>C_{12}<N18N>C_{12}<N18N>C_{12} (**1**), C_{12}<N18N>C_{12}<N15N>C_{12}<N18N>C_{12} (**2**), and C_{12}<N18N>$C_{12}(OCH_2CH_2O)_3C_{12}$<N18N>$C_{12}$ (**3**). If Na^+ passes through the central ring, reducing its size from 18 to 15 members is expected to



1

FIGURE 4 Presumed active conformation of **1**

reduce the transport rate. In fact, Na^+ transport by **1** and **2** occurred at the same rate within experimental error. When the ring was cleaved (**3**), the relative rate dropped from 28 to 14. This is a significant change but no activity would be expected if the macrocycle were absolutely required. We infer that a polar residue is required at the bilayer midplane to serve as a cation relay but that it need not be a macrocycle. Further, the identity of transport rates observed for **1** and **2** suggests that the central macroring is aligned with the long axis of the lipid monomers as shown in figure 4.

Assessing the channel's position within the bilayer

The experiments described above imply that channel **1** exists in the conformation illustrated. A number of additional experiments were undertaken to further define the conformation and the channel's position within the phospholipid bilayer.

Compound **4** is an analog of **1**. In **4**, the dodecyl sidearms of **1** are replaced by the fluorescent dimethylaminonaphthylsulfonyl or dansyl group.[33] Changing the sidearm groups did not preclude channel activity: the rate for Na^+-transport relative to gramicidin ($k_{rel} = 100$) for **4** is 23. The fluorescence spectrum of dansyl, and therefore of **4**, is sensitive to environment.[34] In general, λ_{max} will be observed at longer wavelength in more polar solvents. If previous designs and inferences were correct, the approximate conformation of **4** and its position in the bilayer are as shown schematically in figure 5.

The fluorescence spectrum of **4** was determined in methanol, ethanol, *n*-butanol, *n*-octanol, dichloromethane, and tetrahydrofuran (THF). The correlation of λ_{max} with Reichardt's solvent polarity parameter[35] is good ($R^2 = 0.93$). The fluorescence spectrum for **4**, determined in a mixed phospholipid bilayer (see above), gave a value for λ_{max} of 516 nm. This value suggests that the dansyl groups reside in an environment slightly more polar than ethanol. Precise values for the polarity of a bilayer are not, to our knowledge, available. One may reasonably estimate that the phosphoethanolamine headgroup will be in an environment nearly as polar as water. The hydrocarbon chains must be a nonpolar regime. The span of the channel compared to the span of the membrane suggests that the dansyl headgroups will be near the "mid-polar" regime comprised of glycerol esters. We estimate that the polarity of this region should be between that of ethanol and methanol: certainly more than a hydrocarbon but less than water.[36] Stud-

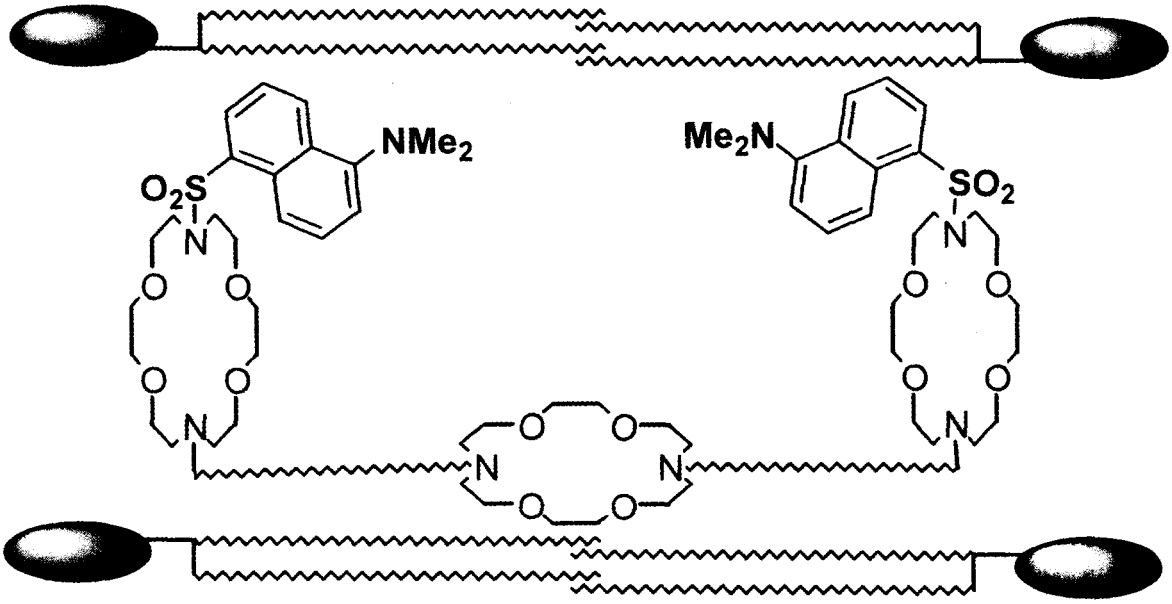


FIGURE 5 Schematic of dansyl channel **4** as it is thought to be positioned in the phospholipid bilayer

ies conducted by others using vesicular membranes in which dansyl-substituted lipid monomers were incorporated showed a fluorescence shift similar to the one we observed.[37] We infer that the headgroups of **4** are near the water/bilayer boundary, but not in a water-like environment.

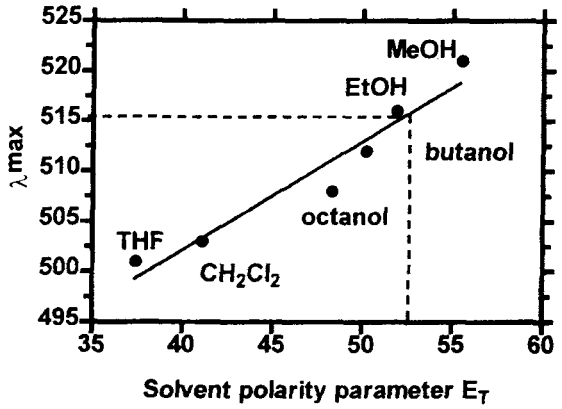


FIGURE 6 Plot of λ_{max} for **4** as a function of medium polarity

Fluorescence quenching

In order to further clarify the position of the putative channel in relation to the lipid bilayer, we used the fluorescence quenching technique developed by Chattopadhyay and London.[38] This approach is known as the parallax method. In this experiment, dansyl channel **4** was incorporated into the bilayer along with doxyl-substituted phosphatidylcholine derivatives. The doxyl group's unpaired electron spin quenches the dansyl group's fluorescence according to the equation $F/F_0 = \exp[-\pi(C/70)(R_0^2-X^2-Z^2)]$. In this expression[39] F/F_0 is fluorescence quenching, $C/70$ is the number of quencher molecules/Å^2 (lipid cross – sectional area is assumed to be 70 Å^2), R_0 is the "hard sphere" critical radius for quenching, X is the closest possible lateral distance between quencher and fluorophore, and Z is the corresponding vertical distance.

We conducted these experiments[40] using commercially available, 7- and 12-derivatives of

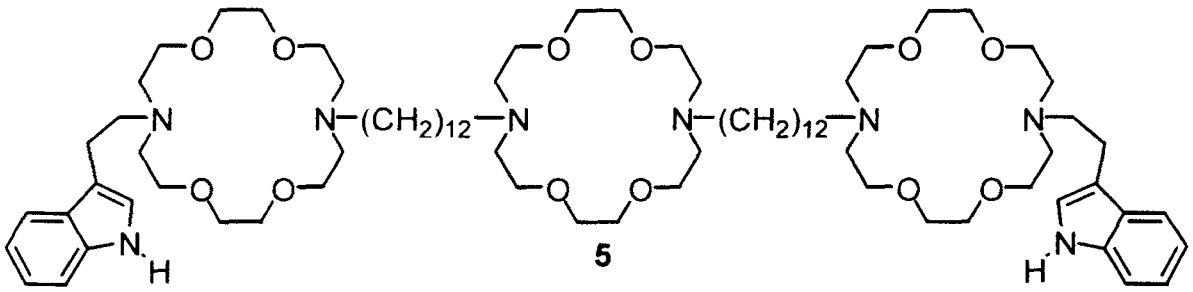


FIGURE 7 Structure of **5**

1-palmitoyl-2-(*n*-doxylstearoyl)phosphatidylcholines (n= 7 or 12). The experiment was conducted by holding constant the amount of channel **4** and varying the concentration of doxyl lipid quencher in the phospholipid bilayer. The data obtained were then plotted as in F/F_0 vs. mole fraction of doxyl lipid quencher. In both cases, the 7 data points plotted as straight lines ($R^2 = 0.92, 0.95$). From these, a distance from the bilayer midplane to the fluorescent headgroup of 14 Å was calculated. Assuming that the bilayer's insulator ("hydrocarbon slab") is 30 Å overall, this places the dansyl headgroup near the mid-polar region of the bilayer. This is the situation shown in figure 5.

Blocking the channel

In other work, we have attempted to define the ability of tryptophan's indole residue to function as a "membrane anchor."[41] This work involved the formation of vesicular assemblies from alkylated indoles. The success of such channel headgroups as benzyl and dansyl (see **4**, above) encouraged us to prepare **5**, the analog of **1**, in which the distal dodecyl groups were replaced by 3-(2-ethylindolyl) groups.

An examination of CPK molecular models suggested that the indolyl NH was capable of forming a hydrogen bond to the distal macrocycle to which it was attached. Such hydrogen bond formation would block entry of a cation into the macrocycle rendering the channel inactive. Monte Carlo simulations conducted for *N*-[3-(2-indolylethyl)]-*N'*-*n*-hexyl-4,13-diaza-18-crown-6 showed that all ten of the lowest energy conformations were blocked by a hydrogen-bonded indole. In contrast, similar calculations conducted for $PhCH_2$<N18N>C_6 showed that the ten lowest energy conformations were all "open."[42]

Channel function was assayed using the dynamic NMR method. No ionophoretic activity could be detected by this technique. When the indolyl NH was replaced by NCH_3, however, hydrogen bond formation was precluded and the resulting tris(macrocycle) showed full sodium transport activity.

CONCLUSIONS

Based upon the data presented here and other information accumulated in this project, we believe that the tris(macrocycle) channels form a cation and water filled pore through the phospholipid bilayer. The structures studied here are not proteins but they transport cations at rates that are similar to those reported for some proteins. The tris(macrocycle)s appear to adopt a conformation in which the distal macrorings serve as headgroups and the central macroring serves as a relay. The channel headgroups are at a distance from the midplane of the bilayer and

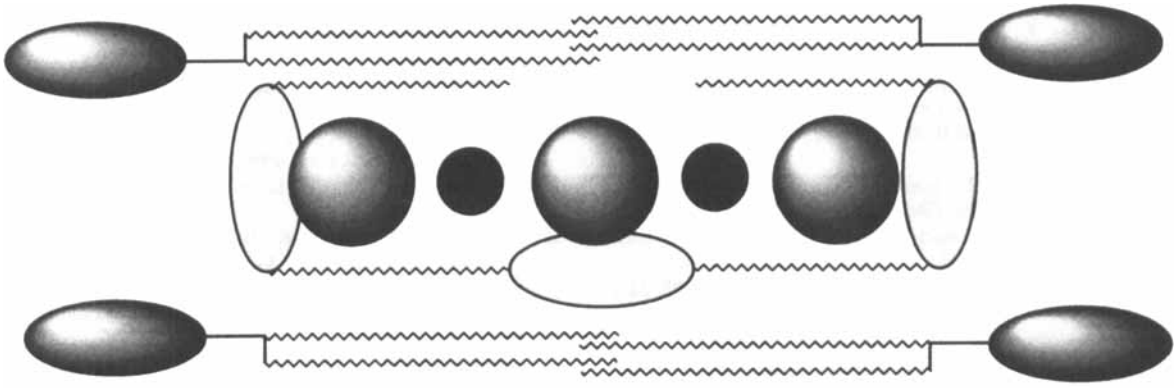


FIGURE 8 Schematic of the active channel conformation supported by current evidence

in an environment of appropriate polarity to have the conformation shown in figure 8.

The tris(macrocycle) is a flexible and dynamic structure. It inserts spontaneously into a phospholipid bilayer and adopts a conformation appropriate for transport. It is symmetrical, however, and therefore not rectifying. Assaying, understanding, and improving selectivity as well as incorporating rectification remain as great challenges in this area.

Acknowledgements

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