Planar Bilayer Conductance and Fluorescence Studies Confirm the Function and Location of a Synthetic, Sodium-Ion-Conducting Channel in a Phospholipid Bilayer Membrane

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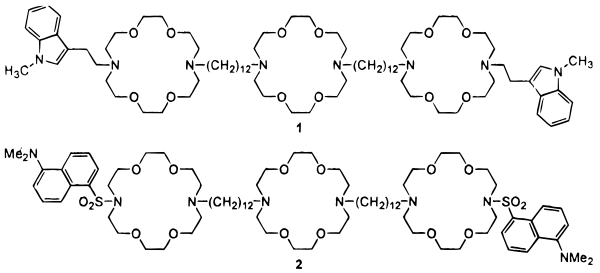
Proteins that span membranes and mediate the transport of cations, anions, and molecules across the boundaries of cells and organelles are crucial to all life. This fact has made the study of transmembrane ion channels one of the most active areas in modern biological chemistry.[1,2] The fundamental importance of this area and of the mechanistic questions concerning channel function cannot be overstated. Despite the enormous amount of information that has accumulated concerning which proteins are responsible for transport, physical chemical details of transport and selectivity remain largely obscure.[3] Supramolecular chemistry permits the design of compounds that possess properties similar to those apparent in natural systems but which can be studied in greater detail.[4] These principles have been applied in the development of synthetic channel model systems by us[5] and by others.[6] We now report two novel, synthetic, cation-conducting channel compounds that incorporate fluorescent probes.[7] This permits the first assessment of a model channel's position within a phosphilipid bilayer. We present three lines of evidence that relate to the channel compound's position within the bilayer. These are (1) solvent shift of the fluorescent headgroup, (2) attempted energy transfer, and (3) the use of doxyl-labeled lipids. Data are also included that show the membrane permeability induced by one of the channel compounds, obtained by using a patch clamp amplifier and a bilayer head stage. The latter confirms the function of the compounds under study.

Compounds **1** and **2**, prepared for the present study, were both of the form R<N18N>C_{12}<N18N>C_{12}<N18N>R in which R is 2-(3-*N*-methylindolyl)ethyl (MeInE, **1**) or 8-(dimethylamino)naphthalenesulfonyl (dansyl, Dn, **2**).[8] Synthesis of **1** was accomplished by alkylation of 3-(2-bromoethyl)indole with CH_3I (NaH, 91%, colorless oil). Reaction of the (bromoethyl)indole with 4,13-diaza-18-crown-6 gave *N*-(*N*-methyl-3-ethyl)indolyl-4,13-diaza-18-crown-6 (32% yield). Alkylation of the monosubstituted crown by using previously reported *N*,*N*′-bis(12-bromododecyl)-4,13-diaza-18-crown-6 (Na_2CO_3, KI, CH_3CN) afforded channel **1** (36% yield) as a white solid (mp 75−77 °C).[9] The dansyl residue was incorporated as follows. Previously reported H<N18N>C_{12}<N18N>C_{12}<N18N>H was treated with 3 equiv of dansyl chloride in CH_2Cl_2 (0 °C, 2 h; 25 °C, 24 h) and isolated (81%) after chromatography (Al_2O_3) as a light green oil.[10]



The activity of channel compounds **1** and **2** in phosphatidylglycerol/phosphatidylcholine bilayer vesicles was confirmed by using the dynamic ^{23}Na NMR technique devised originally by Riddell and Hayer[11] and elaborated for gramicidin by Hinton and co-workers.[12] The rate for Na^+ transport relative to gramicidin ($k_{rel} = 100$) for **1** and **2** were, respectively, 20 and 23.

In order to establish the chemical environment of the presumed dansyl head groups, fluorescence spectra were determined for **2** ([**2**] = 1 μM) in a range of solvents (correlated to solvent parameter E_T[13]): THF ($\lambda_{max} = 501$ nm; $E_T = 37.4$), CH_2Cl_2 (503 nm, 41.4), *n*-octanol (507 nm, 48.3), *n*-butanol (512 nm, 50.2), ethanol (517 nm, 51.9), and methanol (522 nm, 55.5). The fluorescence maximum observed in each case was plotted as a function of E_T. The fluorescence maxima vary approximately linearly (slope = 1.08, $r = 0.97$, 6 points). The dansyl channel was then incorporated into the phospholipid bilayer vesicles described above, and its fluorescence maximum was found to be 516 nm. Interpolation gave an E_T value of 52.7, a value between those of methanol and ethanol but closer to the latter. A similar result was obtained when the fluorescence data were compared to the solvent dielectric constants. Spectroscopic studies have shown that the Stern layer of a micelle has an environment similar to methanol or ethanol.[14] Studies conducted by others using vesicular membranes in which dansyl-substituted lipid monomers were incorporated showed a fluorescence shift similar to the one we observed.[15] We thus infer that the headgroups of **1** lie near the water/bilayer boundary, although they are clearly not in a water-like environment.

N-Methylindolyl channel **1** absorbs light at (λ_{max}=) 283 nm and fluoresces at (λ_{max}=) 340 nm. Dansyl channel **2** absorbs light at (λ_{max}=) 340 nm. We thus conducted the following

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(9) ^{1}H NMR (300 MHz, $CDCl_3$): 1.23 (m, 32H), 1.41 (m, 8H), 2.46 (m, 8H), 2.76 (m, 16H), 2.89 (m, 16H), 3.62 (m, 48H), 3.72 (s, 6H), 6.85 (s, indole, 2H), 7.07 (t, 2H), 7.19−7.24 (m, indole, 4H), 7.55 (d, indole, 2H). IR (KCl): 2923, 1463 1352, 1124 cm^{-1}. Anal. Calcd for $C_{82}H_{144}N_8O_{12}$: C, 68.68; H, 10.12; N, 7.81. Found: C, 68.80; H, 10.06; N, 7.84.
(10) HRMS: calcd for $C_{84}H_{148}N_8O_{16}S_2$: 1586.0219; found 1586.0242.
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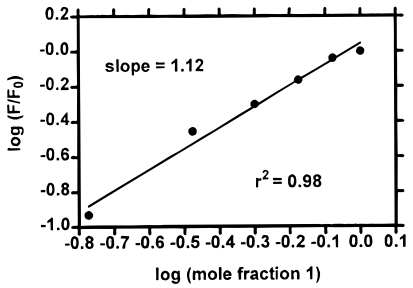
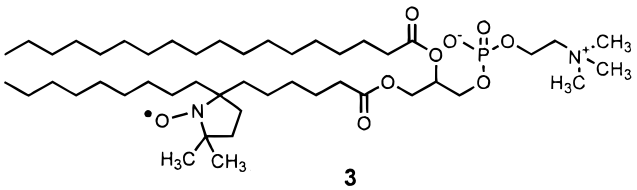


Figure 1. Plot of log F/F_0 *vs* log (mole fraction of **1**).

fluorescence energy transfer (FET) experiment. Six samples of sodium-imbued vesicles were prepared as previously reported.[5] To each was added a mixture of **1** and **2** (total in each sample $\mathbf{1} + \mathbf{2} = 10\ \mu$M). Each vesicle suspension was irradiated at 283 nm, and fluorescence emission was monitored at 340 nm. A plot of log F/F_0 *vs* log (mole fraction of **1**) is shown in Figure 1.[17] The slope of 1.12, a number that corresponds to the aggregation number, suggests that the channel molecule is essentially monomeric. This comports with the aggregation state inferred from NMR results.[5]

In order to more precisely locate the channel head groups in relation to the center of the bilayer, we used the fluorescence quenching technique developed by London *et al.*[16] that is known as the parallax method. In this experiment, a doxyl-substituted phosphatidylcholine derivative (**3**) was inserted into the bilayer along with dansyl channel **2**. The unpaired spin of the doxyl



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group quenches the channel's fluorescence in accord with the former's concentration and the equation $F/F_0 = \exp[-\pi(C/70)(R_0^2 - X^2 - Z^2)]$. In this equation,[17] F/F_0 is the fluorescence quenching, $C/70$ is the number of quencher molecules/Å^2 assuming that the lipid cross sectional area is 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. Commercially available, 7- and 12-derivatives of 1-palmitoyl-2-(*n*-doxylstearoyl)-phosphatidylcholines ($n = 7$ or 12) were used for the study.

Two series of experiments were conducted with dansyl channel **2** and either of the two doxyl lipids. In each case, the concentration of fluorophore was held constant at 10 μM but the amount (mole fraction) of each doxyl lipid was varied. A plot of ln F/F_0 as a function of (mole fraction of doxyl lipid quencher) (Figure 2) gave two straight lines having r^2 values of 0.92 and 0.95 (7 points each). From these, a distance from the bilayer midplane to the fluorescent head group of 14 Å was calculated. Assuming that the bilayer is 30 Å overall, this places the dansyl headgroup near the surface of the bilayer.

In Figure 3 is shown a conductance trace[18] for **1**, obtained in a phospholipid bilayer at an applied voltage of −100 mV (500 mM NaCl). An essentially similar trace was obtained at +100 mV. The characteristic behavior of a channel compound is apparent in these traces and comports with results obtained by the ^{23}Na NMR method. Similar, but not identical, results (not shown) were obtained for dansyl channel **2**, also confirming its channel activity. The conductance (g_S) for dansyl channel **2** was calculated to be 14 pS. Gramicidin, under these conditions, is reported[19] to have a conductance of 17 pS.

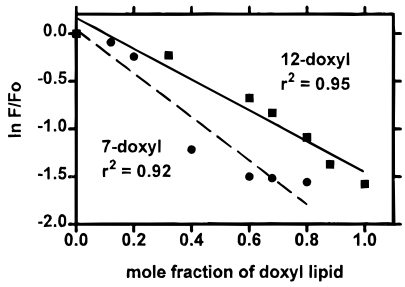

Figure 2. Fluorescence depth quenching for **2** with 7- and 12-doxyl lipids.

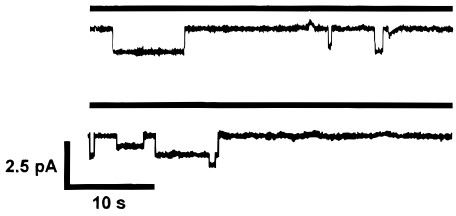

Figure 3. Conductance of Na^+ *vs* time for **1**. Data acquired at −100 mV, 500 mM NaCl, pH = 7.0, 5 pmol of channel added.

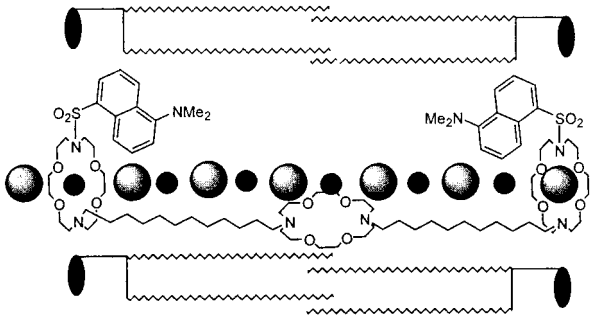

Figure 4. Postulated structure of **1** in a phospholipid bilayer. Sodium cations and water are represented by the shaded and solid spheres, respectively.

The results of the present study indicate the following: (1) The channel headgroup is in an environment intermediate in polarity between methanol and ethanol as expected for the headgroup region of a phosphatidylcholine bilayer. (2) Fluorescence energy transfer studies between **1** and **2** show that the channel tris(macrocycle) is monomeric. (3) The fluorescent headgroup is located about 14 Å from the midplane of the bilayer. If the membrane is 30 Å, this places the headgroup essentially at the bilayer surface. Finally, bilayer clamp data show conductance traces that are unequivocal evidence for channel function by these molecules. Our postulate of the structure assumed by compound **2** in a phospholipid bilayer is shown in Figure 4.

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(18) The planar bilayer chamber was prepared with 0.5 M NaCl, 0.001 M sodium phosphate (pH = 6.8) solution on each side of the membrane. The membrane was formed by the painted method using a solution (30 mg/mL) of L-α-lecithin in decane. The channels were added (2−40 μL) as 1.0 μM solutions in trifluoroethanol and stirred for 5−10 min. After a 5 min equilibration period, a holding voltage was applied and the channel responses were recorded using a Warner PC-505 patch clamp amplifier, a DigiData A/D converter, and the aquisition software Axoscope.

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