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A Comparison of Phenyl- and Naphthyl-substituted tris(Macrocycle), Cation-conducting Channels to Assess the Effect of Extended Aromaticity

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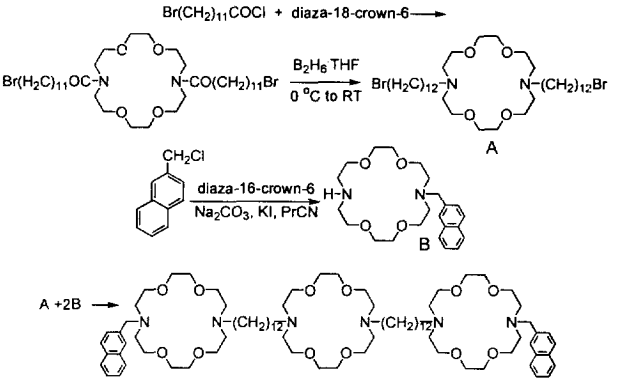
Abstract Two sodium-cation-conducting, tris(macrocycle) channels, differing only in the size of the terminal aryl groups, have been prepared, characterized, and shown by the planar bilayer conductance method to exhibit qualitative differences in channel activity attributable to the π-electron surface area difference. © 1997 Elsevier Science Ltd.

The possibility that the aromatic sidechains of amino acids play a special role in the function of naturally-occurring proteins that conduct cations across phospholipid bilayers[1] has been much discussed in recent years. It was postulated, for example, that the potassium cation selectivity exhibited by the *Drosophila melanogaster* "shaker" channel might be due to direct interactions between K^+ and the aromatic sidechains of phenylalanine or tyrosine.[2] This intriguing postulate was based in part on calculated interaction energies for sandwich complexation of Li^+, Na^+, or K^+ by benzene. MacKinnon and coworkers[3] showed by site-directed mutagenesis, specifically a Y7→F mutation in the signature sequence, that selectivity was lost by virtue of removing the tyrosine hydroxyl group even though an aromatic residue was retained in that position. Our own interest in the possible effect of aromatic residues in channel function[4] led us to prepare tris(macrocycle) channels[5] having either phenyl[5b] or naphthyl terminal groups. The goal was to see what, if any, effect would be observed on Na^+ transport rate if the aromatic surface area of the pliant sidechain was approximately doubled. The transport rate would be evaluated by the planar bilayer conductance (pbc) method.

The two tris(macrocycle)s required for the present study were constructed in an essentially similar fashion. Diaza-18-crown-6 was condensed with a substoichiometric amount of either benzyl bromide or β-chloromethylnaphthalene. This afforded the monosubstituted macrocycle represented in our standard shorthand as H<N18N>CH_2Ph[5b] or H<N18N>CH_2Np (24%). The central macroring unit was assembled by treating diaza-18-crown-6 with the acid chloride of 12-bromododecanoic acid to give $Br(CH_2)_{11}$CO<N18N>$CO(CH_2)_{11}Br$ (61%). The bis(amide) was then reduced (BH_3•THF) to the symmetrical dibromide, $Br(CH_2)_{12}$<N18N>$(CH_2)_{12}Br$ (43%). Alkylation of the two terminal units (H<N18N>CH_2Ar) by the dibromide afforded the tris(macrocycle). The final alkylation was accomplished for naphthyl channel **2** in 11% yield. Both compounds were fully characterized by IR, NMR, and high resolution mass spectrometry.[6]



The efficacy of these compounds as cation transporters was assessed by using the planar bilayer conductance (pbc) technique. This method is similar to the whole cell patch clamp method used in electrophysiology. Rather than using a patch of cellular membrane, a planar phospholipid bilayer is "painted" over a miniscule orifice drilled in a Teflon disk. When the bilayer forms in the hole, it turns opaque giving rise to the cognomen "black lipid membrane." A voltage differential may be applied across the phospholipid bilayer and changes in current can be detected using a standard patch clamp amplifier equipped with a bilayer headstage. Channel activity is apparent as regular opening and closing of the transporter. These openings are observed as square waves of integral and multiple integral height although they may be of different duration (dwell time). Multiple channel openings are apparent as superimposed square waves. An important characteristic of channel behavior is apparent in the so-called I-V (or "i-E") curve. A plot such as that shown in figure 1, of amperage determined as a function of applied (holding) voltage, is typical of many protein channels.[7] A comparable I-V curve has previously been reported for benzyl channel, **1**.[5f]

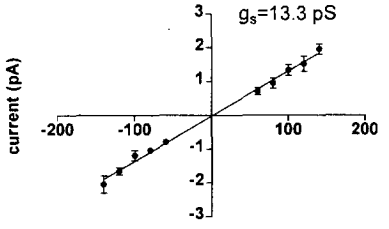


Figure 1. I-V curve for **2** (500 mM NaCl)

The current-time traces for **1** and **2** are shown in figure 2.[7] The data in the figure show only about 2 min each of recording but are representative of a minimum of 1 h of transport activity through a phospholipid bilayer membrane in each case, over which time the membranes were stable and the data proved to be consistent and reproducible.

The data presented here are unequivocal. Their interpretation, however, is complicated. To our knowledge, no other family of "point mutated" synthetic channels has been studied by the planar bilayer clamp technique. The first important result, shown in figure 1, is that the current depends linearly upon the holding voltage. The excellent linear fit shows that the channel is behaving as an ohmic resistor.[8] Second, the open-close behavior is characteristic of cation-conducting protein channels. The conductances calculated (0.5 M NaCl, pH=7) for these two compounds are: **1**, 13.4 pS and **2**, 13.3 pS. Indeed, all but one of the half-dozen or so tris(macrocycle) channels we have studied so far have exhibited very similar conductances.[9] This is not surprising in the sense that they presumably form similarly sized, water-filled pores within the bilayer. It should be noted that the data for **2** were obtained on 2 pmoles of compound compared to 8 pmoles for **1**. Thus, although the single-channel conductances of the two compounds are the same, the amount of compound required to give comparable signal levels differs. This clearly suggests that naphthyl channel **2** is more "effective" than **1**. We choose the term effective deliberately because the source of this efficacy is unclear and certainly undefined. The possibility that the more hydrophobic naphthyl channel partitions better into the

phospholipid bilayer does not account for functional differences because previous studies[5f] have shown that log P values for tris(macrocycle)s are at least 10.

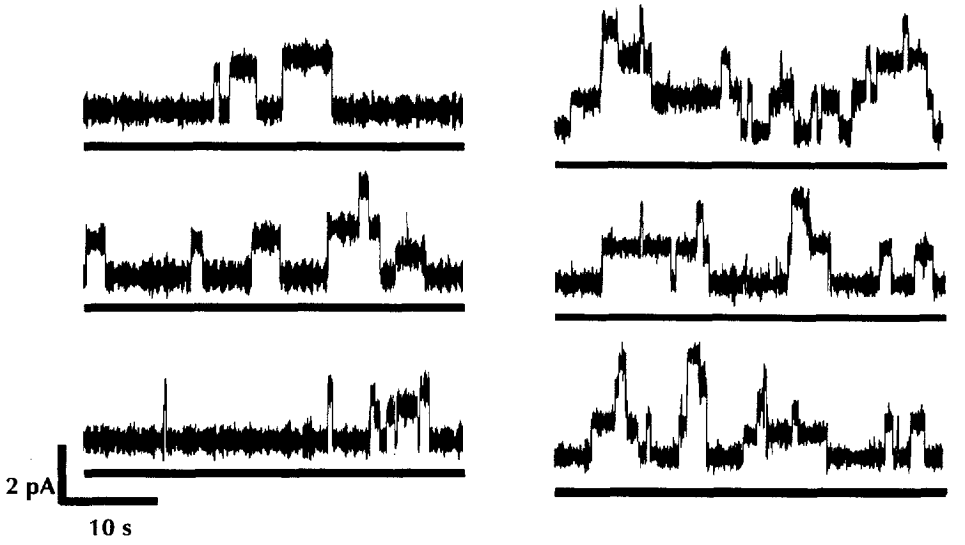


Figure 2. Conductivity traces for (left) benzyl (**1**) and naphthylmethyl (**2**) channels

A significant difference in the pbc results is apparent from inspection of the traces shown in figure 2. Although the channel open peak heights are similar, qualitatively, the naphthyl channels are open more often and undergo a greater number and/or multiple transitions than do the benzyl channels. As noted above, these traces are representative of much longer data acquisitions. While these observations are admittedly not quantitative, they are both extensive and internally consistent with the other experimental data.

As the breadth of our model systems expands, our ability to quantitate these results will no doubt follow. At present, we can say (1) that these model channel systems behave in many respects like natural protein channels, and (2) that the increase in π-surface area within the channel model has an observable effect on Na^+ cation flux although this effect is difficult to quantitate. Thus, the naphthyl channel is as "effective" as the benzyl channel at about a fourth of the concentration. This is anticipated from the open-close behavior which, for **2**, appears qualitatively to occur with a higher frequency and a higher probability of multiple open transitions. Future studies will address whether the extended π-system affects cation selectivity.

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6 **Synthesis of naphthalene channel 2.** A solution of the monosubstituted crown **(8)** (0.58 g, 1.4 mmol), *N,N'*-bis(12-bromododecyl)-diaza-18-crown-6 (BrC_{12}<N18N>$C_{12}Br$) (0.549 g, 0.7 mmol), Na_2CO_3 (0.296 g, 2.8 mmol), KI (0.02g), and butyronitrile (75 mL) was set to reflux in a round-bottomed flask for 48h. The reaction mixture was filtered and the solvent removed under vacuum. The oil obtained was dissolved in $CHCl_3$ (100 mL). The organic layer was washed with water (3×25 mL), dried ($MgSO_4$), filtered, and the solvent removed using rotary evaporation. On addition of acetone the product precipitated as a white solid, in 11% yield, (0.11 g), mp 52-54 °C. ^{1}H-NMR (300 MHz, $CDCl_3$): 1.25 (m, -*CH*$_2$-, 24H), 1.44 (m, NCH_2CH_2, 8H), 1.76 (m, $NCH_2CH_2CH_2$, 8H), 2.49 (m, NCH_2CH_2, 8H), 2.89 (m, N-CH_2CH_2O, 24H), 3.61 (m, N-CH_2CH_2O, 48H), 3.83 (s, N-CH_2-naphthalene, 4H), 7.44 (dd, 4H, naphthalene), 7.52 (d, 2H, naphthalene), 7.77 (m, 8H, naphthalene). High resolution mass spectrometry: calculated for $C_{82}H_{138}N_6O_{12}$: 1400.0224. Found 1400.0278.

7 The planar bilayer chamber was prepared with 0.5 M NaCl, 0.001 M sodium phosphate (pH=7.0) solution on each side of the membrane. The membrane was formed by the painting method using a solution (30 mg/mL) of L-α-lecithin in decane. The channels were added as 1.0 μM solutions in trifluoroethanol and stirred for 10 minutes. After a 5 minute 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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9 These channels function within the range 13±1 pS. The outlier has a Na^+ conductance of ~9 pS.

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