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Synthetic Ion Channel Activity Documented by Electrophysiological Methods in Living Cells

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Abstract: Hydraphiles are synthetic ion channels that use crown ethers as entry portals and that span phospholipid bilayer membranes. Proton and sodium cation transport by these compounds has been demonstrated in liposomes and planar bilayers. In the present work, whole cell patch clamp experiments show that hydraphiles integrate into the membranes of human embryonic kidney (HEK 293) cells and significantly increase membrane conductance. The altered membrane permeability is reversible, and the cells under study remain vital during the experiment. Control compounds that are too short (C_8-benzyl channel) to span the bilayer or are inactive owing to a deficiency in the central relay do not induce similar conductance increases. Control experiments confirm that the inactive channel analogues do not show nonspecific effects such as activation of native channels. These studies show that the combination of structural features that have been designed into the hydraphiles afford true, albeit simple, channel function in live cells.

Introduction

Phospholipid bilayer membranes circumscribe nearly all cells. While the membrane establishes the fundamental barrier between the interior and exterior of the cell, it must be selectively permeable to nutrients and osmolytes in order to maintain viability. Channel proteins have evolved to participate in these functions and others, including waste removal, metabolism, signal transduction, and osmolyte homeostasis. Ion channels play a critical role in the latter two processes and have, therefore, been studied extensively by both biologists and chemists. The aspiration to model ion transport behavior and to develop pharmacological agents has fueled numerous synthetic approaches to ion transport, many of which have been successful in mimicking the characteristics of native protein ion channels.

Tabushi and co-workers reported the first synthetic ion channel[1] in the early 1980s, and numerous examples have been reported since. This noteworthy list includes the peptide nanotubes of Ghadiri,[2] the peptide-linked crown ethers of Voyer,[3] our synthetic hydraphiles,[4] and others.[5,6] Each of these channel compounds has demonstrated the classic, stochastic open/closed channel behavior in planar bilayer conductance experiments[3,7,8] and has shown significant activity in synthetic liposomes.[9,10] Biological activity has also been reported for synthetic ion channels: Voyer's peptide linked crown ethers cause red blood cell haemolysis,[11] while Ghadiri's stacked cyclic peptide nanotubes and our synthetic hydraphiles are antimicrobial agents.[12,13] Notwithstanding these successes, the details of ion transport in *living* bilayers by *synthetic* channels remain elusive. We now report what is, to our knowledge, the first use of whole cell voltage clamp experiments to directly monitor the electrophysiological behavior of a completely synthetic channel in a native cell membrane.

Organic chemists wishing to model natural ion channels are faced with three significant hurdles. The synthetic channels must be designed, they must be synthesized, and finally, their activity must be characterized. There are several methods available to assay channel function, but electrophysiological methods, including whole-cell and single channel patch clamp recordings, provide the most direct way to evaluate the flow of ionic currents through membrane channels. Tabushi's early abiotic synthetic

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channels used cyclodextrins as headgroups and hydrocarbon chains terminated by amides to anchor them. They studied the transport of Cu^{2+} and Co^{2+} mediated by these channels through egg lecithin membranes. Neither of these ions is among those that occur in eukaryotes in high concentrations and that are regulated by many proteins. They were studied because the analytical methodology was available to make quantitative assessments. Transition metal ion transport was also assayed in synthetic channels reported by Fuhrhop[14] and by Nolte[15] and their co-workers.

Channel function has been assessed either by measuring proton transport directly or by competing proton transport with other ions. Menger and co-workers[16] demonstrated that a simple, long-chained ketone could transport H^+ faster than could the peptide channel-former gramicidin[17] in bilayers. Hervé, Cybulska, and Gary-Bobo[18] developed an assay system involving coupled proton transport that was used extensively by Fyles and co-workers in their synthetic ion channel studies.[19]

Various NMR methods have been used in synthetic liposome systems to evaluate ion transport. These include the use of ^{23}Na NMR,[10] ^{7}Li NMR,[20] and other approaches. In all cases, these studies have been conducted in synthetic liposomes. More recently, planar bilayer conductance methods have been used to detect characteristic channel behavior. Such studies involve synthetic bilayer-forming lipids that form so-called black lipid membranes. This technique has been used successfully to demonstrate channel activity by the groups of Kobuke,[21] Ghadiri,[8] Fyles,[22] Voyer,[23] Matile,[24] and Koert[25] and in our own laboratory.[7,26] Hall, Hall and their co-workers have used patch clamp techniques to characterize crown ether based channels that incorporate ferrocene residues.[6]

To our knowledge, no previous study has been reported in which completely synthetic channels have been studied by patch clamp methods in living cells. Such studies are of the greatest importance for two reasons. First, the patch clamp assay is the essential standard recognized in biology for channel function in a vital system. Second, it assesses whether a channel compound is compatible with a vital cellular system. The latter is critical if a drug application is contemplated for the synthetic ion channel. In addition, such studies provide a link with channel function observed in synthetic bilayers. Details of hydraphile channel function assayed by using whole-cell patch clamp recording are described below.

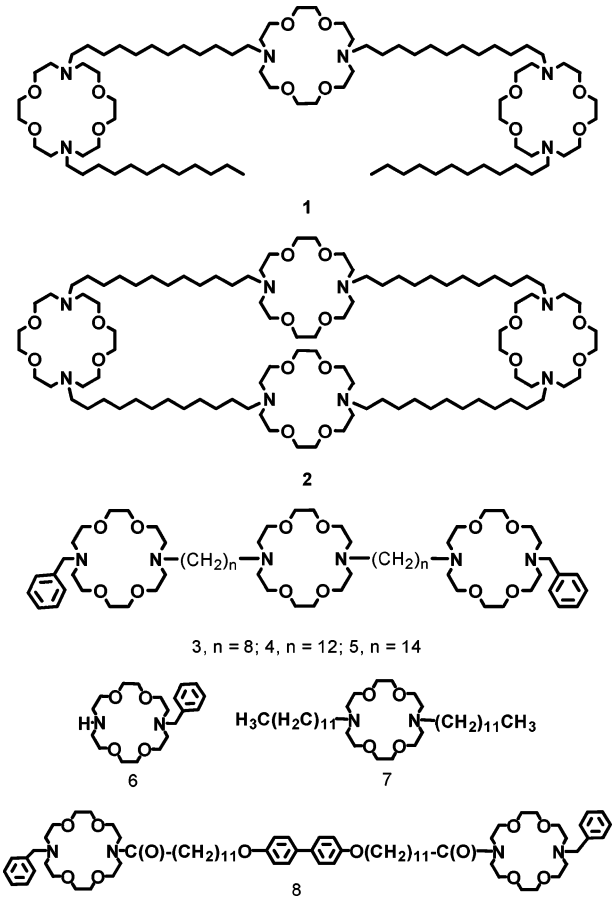

Figure 1. Structures of compounds **1**−**8**.

Results and Discussion

Compounds Used in This Study. Hydraphiles are structures typically comprised of three diaza-18-crown-6 units that are separated by two hydrocarbon spacers. Structures **1**−**8** are illustrated in Figure 1. When three macrocycles are present, the two distal crowns act as headgroups for membrane stabilization, as well as portals for ions, and are positioned at opposite sides of the phospholipid bilayer. The central macrocycle is an ion relay that we believe serves the same purpose as the recently discovered "water and ion-filled capsule" identified in the solid-state structure of the KcsA channel of *Streptomyces lividans*.[27] The range of hydraphile structures used in this study is shown in Figure 1. Compounds **1**, **3**, **4**, and **5** contain three macrocycles separated by hydrocarbon spacer chains. In **1** and **4**, these spacer chains are dodecyl groups [i.e., $-(CH_2)_{12}-$]. A fourth macrocycle is present in **2**, leading to a compound that is overall symmetrical and that resembles a "tunnel." Compounds **6** and **7** are the "component parts" of hydraphile **4** but are not covalently linked. The central macrocycle of **4** has been replaced by biphenylenedioxy in **8**. In previous studies, **8** was shown to be inactive owing to the inability of this structure to coordinate water molecules at its midplane.[28]

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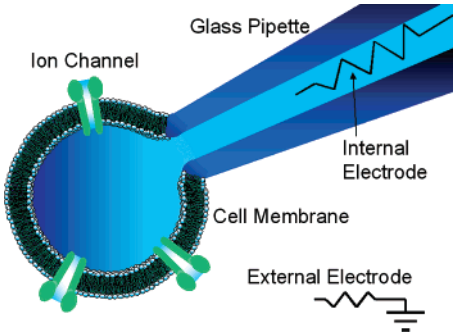

Figure 2. Schematic of the whole cell patch clamp apparatus.

Analysis of Ion Transport by Whole-Cell Patch Clamp Recording. Hydraphile-mediated transport of protons and sodium cation has been demonstrated by fluorescence,[4] NMR,[22] and planar bilayer conductance[4] measurements. As valuable and convincing as these results are, whole-cell and single channel patch clamp recording methods are currently the most stringent tests of channel activity in biological membranes. Whole-cell patch clamp recording monitors the electrophysiological behavior of all channels in the bilayer of a living mammalian cell. The name derives from the fact that a glass pipet seals onto a membrane patch, which is then ruptured to provide a low resistance connection to the cell interior. Voltage across the membrane can then be measured and held constant, or "clamped," at selected values during the experiment via passage of current from a metal electrode inserted into the glass pipet. The method provides a summation of channel activity in the entire native cell membrane. Figure 2 shows a schematic of the experimental arrangement used to undertake such studies. In practice, the glass pipet is mechanically maneuvered until it contacts the cell. Suction is applied to form a seal and to rupture the patch of membrane encircled by the pipet tip. This establishes a circuit from the internal electrode, through the membrane and its ion channels, to an external electrode in the bathing solution. At a given voltage (V), changes in current (I) correspond to alterations in membrane conductance (g), as defined by Ohm's Law: $I = g \times V$.

Control Study of Channel Function Using Kainic Acid. Whole-cell patch experiments are typically used to assess the activity of native protein channels located in the membrane of a cell. Figure 3 shows experimental data for kainate activation of endogenous glutamate receptor channels present in hippocampal nerve cells. In this experiment, the patched cell is perfused with saline solution while the voltage inside the cell is held at −20 mV with respect to the outside. This comprises the baseline for the experiment, apparent on both the left (A) and right (C) sides of the top panel graph, before (A) and after (C) exposure to the activator (B). During each phase of the experiment, voltage was briefly (10 ms) stepped in increments of 10 mV from +90 mV to −110 mV to evaluate changes in conductance. The voltage steps were repeated 6 times during each phase to produce the data points that show current amplitude recorded at each voltage and plotted in the top panel. The points merge to appear as almost continuous sweeps during phases A and C.

Channel activator (kainate) was applied directly to the cell by a local bath perfusion apparatus during phase B. When kainate binds, the endogenous channels open and the measured increase of inward current of 0.5 nA is observed (phase B). This corresponds to an increase in membrane conductance of

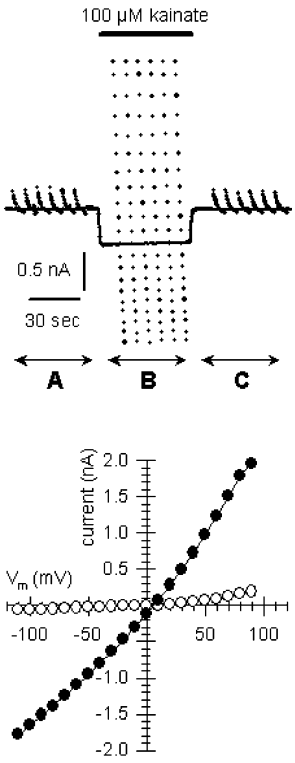

Figure 3. Whole-cell current evoked by 100 μM kainate, which activates endogenous glutamate receptor channels, in a cultured rat hippocampal nerve cell.

about 18 nanosiemens (nS) as calculated from the slope of the current voltage plot in the bottom panel of Figure 3. During phase C, the activator is removed and the cell is rinsed with the control bathing solution. As the activator unbinds, the channels close and the cell returns to its resting state. The rapid onset and recovery of current evoked by kainate illustrate the speed of experimental solution exchange, which is complete within less than 1 s. The bottom panel of Figure 3 shows the overall current−voltage ($I-V$) relationship, which plots the averages of the 6 separate current determinations at each membrane potential tested. The open circles record the membrane currents in the presence of the control saline solution, while filled circles show the whole cell currents when the channel activator, kainic acid, was present. The slope of these plots (current/voltage) is the conductance of the whole cell plasma membrane and, in this case, shows an increase from 0.2 to 20 nS or of 100-fold. The magnitude of this change indicates why channels are so important for cell signaling and ion transport processes in cellular physiology.

Effect of Dodecyl (1) and Benzyl (5) Side-Chained Compounds on Whole Cell Conductance. Hydraphiles **1** and **5** represent two highly active Na^+ transporters identified from our work in synthetic liposomes. The compounds differ in two key respects (see Figure 1 for structures). The spacer chains between the distal and medial macrocycles are 12 carbons in **1** and 14 carbons in **5**. The additional two methylene groups in each chain make **5** approximately 5 Å longer than **1**. The side chains attached to the distal macrocycles are *n*-dodecyl in **1** and benzyl in **5**. HEK 293 cells were treated with synthetic hydraphiles **1** and **5** analogous to previously described control experiments.

The electrical responses of HEK cells were recorded under whole-cell voltage clamp. Hydraphiles **1** or **5** in absolute EtOH

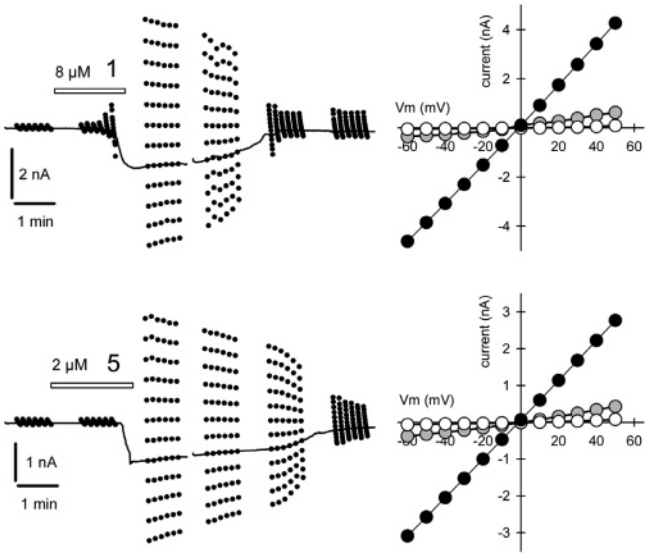

Figure 4. Response of HEK 293 cells to exposure of **1** or **5** (raw data in left panels). Concentrations shown are at 8 and 2 μM, respectively. Right panels give the current−voltage response during control (open circles), during hydraphile exposure (black circles), and after rinsing (grey circles).

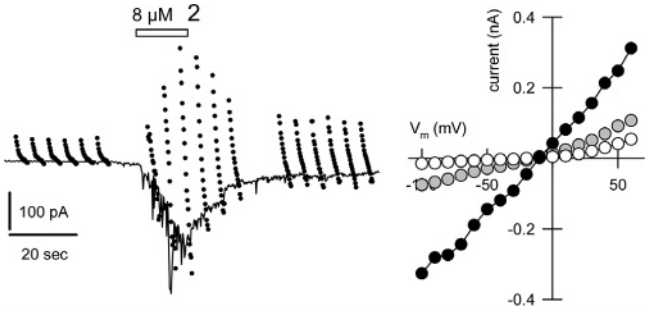

Figure 5. Current evoked by a brief application of 8 μM **2** to an HEK 293 cell.

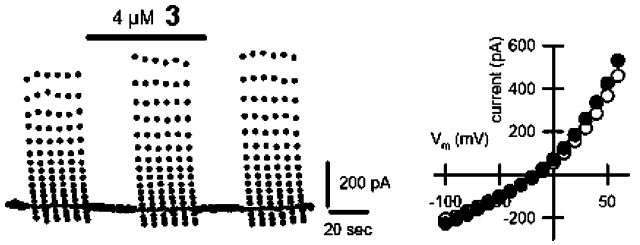

Figure 6. Prolonged exposure of an HEK 293 cell to C_8 benzyl channel, **3**, failed to evoke current.

were diluted into the extracellular solution (0.1% EtOH) and applied by local perfusion (see Experimental Section). Membrane voltage was stepped through a series of test potentials (+50 mV to −60 mV) to evaluate conductance changes caused by the hydraphile's presence. The solid horizontal traces in the left panels of Figure 4 indicate the periods of exposure to either **1** or **5**. Both the dodecyl, C_{12}-spacer (**1**, top left) and benzyl, C_{14}-spacer (**5**, bottom left) hydraphiles produced a dramatic increase in holding current associated with enhanced membrane conductance. In contrast to the rapid activation of glutamate receptor channels (Figure 3), the onset of the hydraphile-mediated conductance increase was relatively slow and followed a lag period of 60−90 s. This time was presumably required for the channel monomers to insert into the bilayer and assume their conductance states.

The behavior of hydraphiles **1** and **5** is similar in outcome but differs in magnitude. At 8 μM, **1** showed increased membrane conductance from 1 nS to ~80 nS within ~60 s after application and remained stable for several minutes. After extensive rinsing to remove **1**, some residual conductance remained (9 nS), but normal membrane behavior was restored and the cells remained vital. At [**5**] = 2 μM, C_{14}-benzyl channel showed increased membrane conductance, from 1 nS to ~52 nS, that persisted for several minutes. After washing, normal membrane function was observed and the cells were undamaged (7 nS residual conductance). On average, 2 μM **5** increased conductance by 110 ± 82 nS (n = 3) compared to 101 ± 55 nS (n = 3) for 8 μM **1**. We conclude that **5** is about twice as active as **1** in this particular context. The currents evoked by **1** and **5** are three- to 4-fold greater than those resulting from kainate activation and can, therefore, significantly modify cellular ion homeostasis (cf. Figure 3).

Similar results were obtained with other hydraphiles. Brief exposure (15 s) of HEK 293 cells to **2** resulted in a rapid increase in current (Figure 5) which involved a conductance increase from 0.4 nS to ~4 nS. This current stabilized quickly when the cells were rinsed with saline solution, and a residual baseline conductance resulted as noted for **1** and **5**. The short exposure time reduced both the magnitude and duration of the observed current. Even so, a prolonged period of stable conductance remained (1 nS residual conductance). In all cases, washing with extracellular solutions that contained 0.1% bovine serum albumin (BSA) reduced the lag period to current onset and speeded recovery during washout, suggesting that stabilization of the hydrophobic hydraphiles in aqueous solutions may facilitate their delivery to, and removal from, biological membranes.

Hydraphiles partition into the membrane, and we presume that they can also partition out of the membrane during extensive washing. Figures 4 and 5 show that washout is relatively slow and that residual conductivity is higher after exposure to the active hydraphiles. These properties suggest that some compound is retained within the membrane. The hydraphiles are hydrophobic and partition preferentially into the bilayer. There is, however, a difference in volume between the bilayer and the surrounding aqueous phase of ~10^{12} which permits the compound to be washed out.

Whole Cell Patch Clamp Control Experiments. The traces shown in Figure 4 demonstrate that hydraphiles cause persistent increases in whole cell conductance that slowly recover with time. It seemed possible that the observed activity could be caused by activation of endogenous channels or by disruptive nonspecific interactions (e.g., a detergent effect) of the organic amphiphiles with the membrane. Four compounds were thus chosen for use as controls. The first is compound **3**, C_8-benzyl, which has 8-carbon spacer chains, making it ~14 Å shorter than **5**. Otherwise, C_{14}-benzyl (**5**) and **3** are identical. When n = 8 (**3**), the octamethylene chain spans about 10 Å. We estimate the overall length of **3** to be ~30−32 Å, which is not long enough to span a phospholipid bilayer. **3** did not transport Na^+ through phospholipid liposomes, as judged by NMR analysis,[29] while **5** was successful. Conductance data for **5** are shown in the lower panel of Figure 4. Figure 6 shows that the

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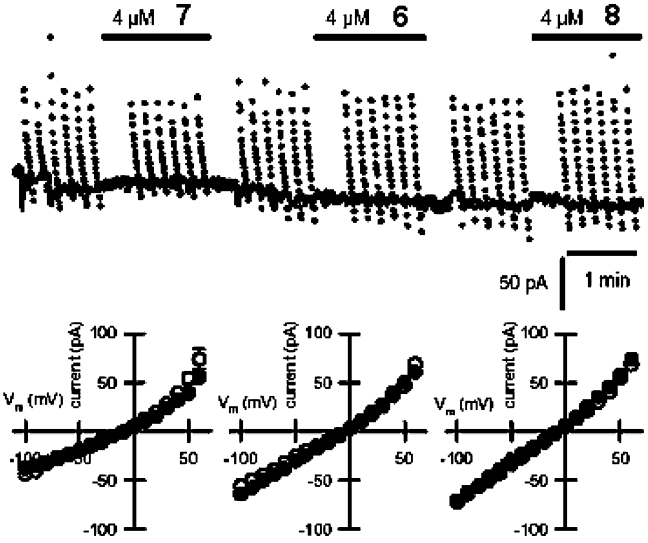

Figure 7. Control compounds **7**, **6**, and **8** tested in succession against the same cell.

"too short" C_8 benzyl channel (**3**) was inactive even during prolonged exposure of over 90 s (note *y*-axis units of pA). Compound **8** is likewise a molecule that possesses the distal macrocycles and spacer chains of successful hydraphiles such as **1** and **4**. However, the central macrocycle of **8** is replaced by a biphenyl unit that cannot effectively interact with water. Thus, the postulated "water and ion-filled capsule" that was identified in the structure of the KcsA channel[27] lacks a counterpart in this model channel. Studies on this channel showed that it failed to transport Na^+ through the bilayers of phospholipid liposomes.[30] Figure 7 shows that **8** is also inactive during prolonged exposure. Thus, **3** and **8** are structures very similar to functional hydraphiles but have failed to function as channels owing to either insufficient length (**3**) or the absence of an important structural element (**8**).

Compounds **6** and **7** are major fragments of successful hydraphiles given in Figure 1. The central crown and covalent spacers are present in **7**, but the distal macrocycles and side chains are lacking. The latter elements are present in **6**, but the central crown and spacers are missing. While concentrations as low as 2 μM C_{12}-dodecyl, **1**, and 0.5 μM C_{14}-benzyl, **5**, can elicit whole cell currents over 1 nA within about 60 s, prolonged exposure (up to 90 s) to 4 μM of **6** or **7** caused no discernible increase in membrane conductance in pA. These control compounds rule out the nonspecific activation of any ligand-gated channels within the bilayer and show that hydraphile compounds are indeed functioning as channels in the very complex landscape of the living membrane.

A discussion of membrane potentials and currents warrants defining their ionic basis. At negative membrane potentials, the inside of the cell is negatively charged with respect to the outside bathing solution. This electrical gradient favors the flow of positive ions into the cell and the movement of negative ions out of the cell, assuming a channel exists for their passage. In our experiments, 150 mM NaCl is present in the external bathing solution. Inside the cell there is less than 5 mM Cl^-; the dominant ions are K^+ and glucuronate, which is a large organic anion that is unlikely to permeate through hydraphile channels. Hence we conclude that currents observed at negative membrane potentials are primarily, if not entirely, from Na^+ influx. Meanwhile, at positive membrane voltages the scenario is reversed. The electrical gradient favors the outward flow of positively charged ions, while negatively charged ions will be drawn into the cell, again assuming a channel is present for passage of the ion. Either the outward flow of K^+ or the inward movement of Cl^- could underlie the currents observed at positive membrane potentials (Figures 4 and 5). Our future work aims to resolve these ion selectivities. However, the sizable currents observed at negative membrane potentials corroborate our previous work in lipid vesicles demonstrating efficient Na^+ flux comparable to that of gramicidin.

Conclusions

Cation transport in liposomes and planar bilayers has been demonstrated previously for hydraphile channels. We show in the present work, by whole cell patch clamp experiments, that hydraphiles integrate into the membrane of HEK 293 cells and significantly increase membrane conductance. This change in membrane permeability is reversible, and the cells under study remain vital during the experiment. Control compounds that are too short (C_8-benzyl channel) or are inactive owing to a deficiency in the central relay do not induce similar conductance increases. These inactive channel analogues do not show nonspecific effects such as activation of native channels. These studies show that the combination of structural features that have been designed into the hydraphiles afford true channel function in live cells. Of course, these compounds are purely synthetic and relatively simply structures, but their whole cell currents are comparable to protein channels that have evolved over the course of a billion or more years.

Experimental Section

General Information. Solvents were freshly distilled and reactions were conducted under N_2 unless otherwise stated. Et_3N was distilled from KOH and stored over KOH. CH_2Cl_2 was distilled from CaH_2. Column chromatography was performed on silica gel 60 (230−400 mesh). Thin-layer chromatography was performed with silica gel 60 F_{254} plates with visualization by UV light (254 nm) and/or by phosphomolybdic acid (PMA) spray. Starting materials were purchased from commercial sources and used as received unless otherwise specified. ^{1}H NMR (300 MHz) chemical shifts are reported in ppm (δ) downfield from internal Me_4Si (integrated intensity, multiplicity (br = broad; s = singlet; d = doublet; t = triplet; m = multiplet), coupling constants in Hz, assignment). ^{13}C NMR spectra (75 MHz) are referenced to $CDCl_3$ (δ 77.0). Infrared spectra were recorded in KBr unless otherwise noted and were calibrated against the 1601 cm^{-1} band of polystyrene. Melting points were determined on a Thomas−Hoover apparatus in open capillaries. Combustion analyses were performed by Atlantic Microlab, Inc., Atlanta, GA, and are reported as percents.

Hydraphile Having C_{12} Spacers and C_{12} Sidearms, 1, $CH_3(CH_2)_{10}CO\langle N18N\rangle C_{12}\langle N18N\rangle C_{12}\langle N18N\rangle CO(CH_2)_{10}CH_3$. Dodecanoic acid (206 mg, 1.03 mmol) was dissolved by slow addition of $SOCl_2$ (4 mL, 0 °C) and held at reflux for 1 h. The $SOCl_2$ was removed in vacuo and the residue was washed with toluene (2 × 5 mL) to complete azeotropic removal of the $SOCl_2$. The resulting acid chloride was dissolved in 5 mL of toluene and added dropwise to a solution of $H\langle N18N\rangle C_{12}\langle N18N\rangle C_{12}\langle N18N\rangle H$ (obtained as described in ref 11, 492 mg, 0.447 mmol), Et_3N, and catalytic (dimethylamino)pyridine (DMAP)

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at 0 °C. The reaction was stirred at ambient temperature for 2 days, filtered (Celite), and then evaporated in vacuo. The resulting residue was dissolved in $CHCl_3$ and washed with saturated $NaHCO_3$, (2 × 50 mL) followed by brine (2 × 50 mL). The product was chromatographed (SiO_2, 2% Et_3N in acetone) to afford **1** (520 mg, 78.4%) as a colorless solid, mp 49−50 °C. ^{1}H NMR: 0.88 (t, J = 6.9 Hz, *CH*$_3$, 6H), 1.24 (*pseudo*-s, *CH*$_2$, 64H), 1.43 (br quint, NCH$_2$*CH*$_2$CH$_2$, 8H), 1.61 (br quint, COCH$_2$*CH*$_2$CH$_2$, 4 H), 2.31 (t, J = 7.8, CO*CH*$_2$, 4H), 2.47 (t, J = 7.5, N*CH*$_2$CH$_2$, 8H), 2.76 (t, J = 5.4, N*CH*$_2$CH$_2$O, 16H), 3.58−3.65 (m, *CH*$_2$O, 48H). ^{13}C NMR: 14.07, 22.63, 25.33, 27.18, 27.47, 29.29, 29.46, 29.58, 31.86, 33.12, 46.89, 48.77, 53.87, 56.01, 69.62, 69.96, 70.08, 70.25, 70.32, 70.47, 70.58, 70.70, 70.96, 173.34 ppm.

CH$_3$(CH$_2$)$_{11}$⟨N18N⟩C$_{12}$⟨N18N⟩C$_{12}$⟨N18N⟩(CH$_2$)$_{11}$CH$_3$. CH$_3$(CH$_2$)$_{10}$CO⟨N18N⟩C$_{12}$⟨N18N⟩C$_{12}$⟨N18N⟩CO(CH$_2$)$_{10}$CH$_3$ (360 mg, 0.243 mmol) was added to BH_3·THF (1 M) at 0 °C under N_2 and stirred at ambient temperature (3 d). Water was added dropwise until H_2 evolution ceased. The solvent was removed in vacuo, and the residue was dissolved in HBr (24%, 10 mL) and heated to reflux for 3 h. Sodium hydroxide pellets were added (to pH ∼9) at 0 °C, and the aqueous solution was extracted with CH_2Cl_2 (3 × 50 mL), washed with brine (3 × 50 mL), dried ($MgSO_4$), and filtered through Celite. Evaporation of the solvent gave white crystals (236 mg, 67%) having mp 60−61 °C (lit. mp 61−63 °C).[11]

Compound Having Four *N,N′*-Diaza-18-crown-6 Units Connected by Four Dodecyl Spacers, "the C$_{12}$ Tunnel," 2. Br(CH$_2$)$_{11}$COOCH$_2$Ph. To a mixture of 12-bromododecanoic acid (4.0 g, 14.3 mmol), diisopropylcarbodiimide (2.3 mL, 19.0 mmol), and DMAP (0.18 g, 1.5 mmol) in CH_2Cl_2 (30 mL) at 5 °C was slowly added benzyl alcohol (1.55 g, 14.3 mmol). The reaction was stirred overnight at room temperature (rt). After evaporation, the residue was suspended in hexanes (50 mL) and filtered, and the filtrate was washed with 5% citric acid (2 × 20 mL), 5% $NaHCO_3$ (2 × 20 mL), and brine (20 mL). The hexanes layer was dried ($MgSO_4$) and concentrated to give a colorless oil (5.07 g, 96%). ^{1}H NMR: 1.20−1.35 (12H, *pseudo*-s, CO(CH$_2$)$_2$*(CH$_2$)$_6$*(CH$_2$)$_3$Br), 1.35−1.48 (2H, m, *CH*$_2$CH$_2$CH$_2$Br), 1.58−1.70 (2H, m, COCH$_2$*CH*$_2$), 1.85 (2H, quintet, J = 7.3 Hz, *CH*$_2$CH$_2$Br), 2.35 (2H, t, J = 7.5 Hz, CO*CH*$_2$), 3.40 (2H, t, J = 6.9 Hz, *CH*$_2$Br), 5.12 (2H, s,Ar*CH*$_2$O), 7.30−7.42 (5H, m, aromatics). ^{13}C NMR: 24.8, 28.0, 28.6, 29.0, 29.1, 29.2, 29.3, 32.7, 33.9, 34.2, 66.0, 128.2, 128.6, 136.2, 173.8 ppm. IR (neat film): 3066, 3034, 2927, 2854, 1737, 1498, 1456, 1383, 1353, 1256, 1214, 1164, 1120, 1003, 908, 736, 698 cm^{-1}.

PhCH$_2$OOC(CH$_2$)$_{11}$⟨N18N⟩(CH$_2$)$_{11}$COOCH$_2$Ph. A mixture of 4,13-diaza-18-crown-6 (0.79 g, 3 mmol), Br(CH$_2$)$_{11}$COOCH$_2$Ph (2.22 g, 6 mmol), Na_2CO_3 (6.36 g, 60 mmol), and KI (30 mg, 0.18 mmol) in *n*-PrCN (40 mL) was heated at reflux for 24 h. After the mixture was cooled and filtered, the resulting solution was concentrated to an oil. Toluene (30 mL) was added and evaporated to ensure complete removal of *n*-PrCN. The crude, oily product was purified by chromatography (SiO_2, 100% acetone) and afforded PhCH$_2$OOC(CH$_2$)$_{11}$⟨N18N⟩(CH$_2$)$_{11}$COOCH$_2$Ph (2.14 g, 85%) as a colorless oil. ^{1}H NMR: 1.20−1.35 (28H, *pseudo*-s, N(CH$_2$)$_2$*(CH$_2$)$_7$*(CH$_2$)$_2$CO), 1.36−1.48 (4H, br, NCH$_2$*CH$_2$*CH$_2$), 1.63 (4H, quintet, J = 7.4 Hz, COCH$_2$*CH$_2$*CH$_2$), 2.34 (4H, t, J = 7.6 Hz, CO*CH$_2$*CH$_2$), 2.47 (4H, t, J = 7.8 Hz, N*CH$_2$*CH$_2$CH$_2$), 2.76 (8H, t, J =) 6.0 Hz, N*CH$_2$*CH$_2$O), 3.56−3.64 (16H, m, *CH$_2$OCH$_2$*), 5.11 (4H, s, Ar*CH$_2$*O), 7.30−7.42 (10H, m, aromatics). ^{13}C NMR: 24.8, 27.1, 27.4, 29.0, 29.1, 29.3, 29.4, 29.5, 34.2, 53.8, 56.0, 66.0, 70.0, 70.7, 128.2, 128.6, 136.2, 173.9 ppm. IR (neat film): 3065, 3034, 2927, 2854, 1737, 1456, 1352, 1255, 1163, 1127, 994, 751, 698 cm^{-1}.

***N,N′*-bis(11-Carboxyundecyl)-4,13-diaza-18-crown-6, HOOC(CH$_2$)$_{11}$⟨N18N⟩(CH$_2$)$_{11}$COOH.** PhCH$_2$OOC(CH$_2$)$_{11}$⟨N18N⟩(CH$_2$)$_{11}$COOCH$_2$Ph (0.95 g, 1.13 mmol) was dissolved in EtOH (40 mL) and added to 10% Pd on carbon (0.10 g) and hydrogenated in a Parr bottle (H_2, 60 psi) at rt overnight. After filtration through Celite, the mixture was evaporated to afford HOOC(CH$_2$)$_{11}$⟨N18N⟩(CH$_2$)$_{11}$COOH (0.73 g, 98%) as a colorless solid, mp 77−79 °C. ^{1}H NMR 5% CD_3OD in $CDCl_3$: 1.22 (28H, *pseudo*-s, N(CH$_2$)$_2$*(CH$_2$)$_7$*(CH$_2$)$_2$CO), 1.48−1.52 (8H, br, NCH$_2$*CH$_2$*CH$_2$, COCH$_2$*CH$_2$*CH$_2$), 2.17 (4H, t, J = 7.3 Hz, CO*CH$_2$*CH$_2$), 2.69 (4H, t, J = 7.8 Hz, N*CH$_2$*CH$_2$CH$_2$), 2.99 (8H, t, J = 5.4 Hz, N*CH$_2$*CH$_2$O), 3.53 (8H, s, NCH$_2$*CH$_2$*O), 3.65 (8H, t, J = 5.4 Hz, O*CH$_2$CH*O). ^{13}C NMR: 25.1, 25.4, 28.4, 28.6, 28.7, 28.8, 35.6, 52.4, 54.6, 67.5, 70.3, 178.8 ppm. IR ($CHCl_3$): 3435, 2924, 2853, 1717, 1567, 1466, 1354, 1254, 1115 cm^{-1}.

***N,N′*-bis(11-Chlorocarbonylundecyl)-4,13-diaza-18-crown-6, ClOC(CH$_2$)$_{11}$⟨N18N⟩(CH$_2$)$_{11}$COCl.** Oxalyl chloride (0.07 mL, 0.80 mmol) was added dropwise to a suspension of HOOC(CH$_2$)$_{11}$⟨N18N⟩(CH$_2$)$_{11}$COOH (0.10 g, 0.14 mmol) in freshly distilled CH_2Cl_2 (20 mL) cooled to 5 °C. The ice bath was removed, the reaction stirred for 2 h, the solvent was removed in vacuo, and the residue dried on a vacuum pump. The oily product was dissolved in CH_2Cl_2 (10 mL) and used without purification.

Compound 2, "Tunnel," ((CH$_2$)$_{12}$⟨N18N⟩)$_4$. A mixture of H⟨N18N⟩(CH$_2$)$_{12}$⟨N18N⟩(CH$_2$)$_{12}$⟨N18N⟩H (0.15 g, 0.14 mmol) and Et_3N (0.10 mL, 0.72 mmol) in CH_2Cl_2 (20 mL) was cooled to 5 °C. The CH_2Cl_2 solution of ClOC(CH$_2$)$_{11}$⟨N18N⟩(CH$_2$)$_{11}$COCl (see above) was added dropwise. After stirring for 48 h at room temperature, the mixture was washed with $NaHCO_3$ (5%, 2 × 20 mL) and brine (20 mL). The organic layer was dried ($MgSO_4$) and concentrated to give *cyclo*[C$_{12}$⟨N18N⟩C$_{12}$⟨N18N⟩COC$_{11}$⟨N18N⟩C$_{11}$CO⟨N18N⟩] as a yellow oil (0.20 g, 86%), which was used in the subsequent step without further purification.

To a solution of BH_3·THF (1.0 M, 10 mL) was added *cyclo*[C$_{12}$⟨N18N⟩C$_{12}$⟨N18N⟩COC$_{11}$⟨N18N⟩C$_{11}$CO⟨N18N⟩] (0.19 g, 0.11 mmol) dissolved in THF (5 mL) at 5 °C. The mixture was stirred at room temperature for 72 h. Water was added dropwise until the liberation of hydrogen ceased. The mixture was concentrated in vacuo, and 2 N HCl (40 mL) was added. The solution was heated at reflux for 4 h, cooled, and adjusted to pH = 9 with 2 M NaOH. The aqueous phase was diluted with water (50 mL) and then extracted with $CHCl_3$ (3 × 40 mL). The combined organic layer was washed with $NaHCO_3$ (5%, 2 × 20 mL) and brine (20 mL), dried ($MgSO_4$), and concentrated. Crystallization of crude product from acetone gave **2** as a slightly yellow solid (0.15 g, 79%). ^{1}H NMR: 1.15−1.35 (64H, s, NCH$_2$CH$_2$(C*H$_2$)$_8$CH$_2$CH$_2$N), 1.35−1.5 (16H, s, NCH$_2$C*H$_2$*(CH$_2$)$_8$C*H$_2$*CH$_2$N), 2.4−2.55 (16H, t, J = 7.5 Hz, NC*H$_2$*(CH$_2$)$_{10}$C*H$_2$*N), 2.7−2.9 (32H, t, J = 6.0, OCH$_2$C*H$_2$*N), 3.5−3.7 (64H, overlapping signals due to C*H$_2$CH$_2$*OC*H$_2$*CH$_2$N). ^{13}C NMR: 27.41, 27.67, 27.73, 29.76, 29.84, 54.11, 54.17, 56.21, 56.27, 70.22, 70.95. IR ($CHCl_3$): 2925, 2853, 1635, 1465, 1352, 1298, 1124 cm^{-1}. Calculated for $C_{96}H_{192}N_8O_{16}$·3H$_2$O (see IR and NMR for evidence of water): C, 65.19; H, 11.28; N, 6.34%. Found: C, 65.31; H, 11.56; N, 6.63%.

Channel with C$_8$ Spacers and Benzyl Sidearms, 3, PhCH$_2$⟨N18N⟩(CH$_2$)$_8$⟨N18N⟩(CH$_2$)$_8$⟨N18N⟩CH$_2$Ph. This compound was prepared as described previously.[2] The crude material (51%) was crystallized from acetone to give **3** as a slightly tan/white solid, mp 39−41 °C and spectral properties as reported previously.[2] We note that **3** was previously obtained as a thick oil rather than a low melting solid.

Channel with C$_{12}$ Spacers and Benzyl Sidearms, 4, PhCH$_2$⟨N18N⟩(CH$_2$)$_{12}$⟨N18N⟩(CH$_2$)$_{12}$⟨N18N⟩CH$_2$Ph. This compound was prepared as described previously.[2]

Channel with C$_{14}$ Spacers and Benzyl Sidearms, 5, PhCH$_2$⟨N18N⟩(CH$_2$)$_{14}$⟨N18N⟩(CH$_2$)$_{14}$⟨N18N⟩CH$_2$Ph. This compound was prepared as described previously.[29b] The oily product (49%) was crystallized from acetone to give a slightly yellow solid, mp 46−48 °C (lit.[32] oil) and spectral properties as reported previously.

Preparation of *N*-Benzyl-4,13-diaza-18-crown-6, 6, H⟨N18N⟩CH$_2$Ph. A solution of PhCH$_2$⟨N18N⟩CH$_2$Ph (10.0 g, 22.6 mmol) and Pd/C (10%, 0.35 g) in EtOH (100 mL) was shaken in a Parr series 3900 hydrogenation apparatus at 60 psi of H_2 pressure and 25 °C for 2.5 h. The mixture was filtered through Celite and concentrated in vacuo. Crystallization from hexanes gave diaza-18-crown-6 (1.92 g, 32%), as a colorless solid, mp 114−115 °C, having spectral properties as reported previously.[31]

The mother liquor was concentrated in vacuo. Column chromatography (SiO_2, 0−2% Et_3N/acetone) gave two products. The first fraction contained $PhCH_2\langle N18N\rangle CH_2Ph$ (1.90 g, 19%), which was isolated after evaporation of the solvent as a colorless solid. The second fraction contained 3.24 g (41%) of $H\langle N18N\rangle CH_2Ph$, as a pale yellow oil, the spectral properties of which were the same as previously reported.[2]

Central Relay Analogue *N,N′*-Didodecyl-4,13-diaza-18-crown-6, 7, $CH_3(CH_2)_{10}CO\langle N18N\rangle CO(CH_2)_{10}CH_3$. Dodecanoic acid (2 g, 0.01 mol) was heated at reflux with $SOCl_2$ (15 mL, 0.2 mol) for 1 h and then evaporated. Residual $SOCl_2$ was removed by azeotropic distillation with toluene (2 × 15 mL). The residue, in 10 mL of toluene, was added dropwise to a toluene solution (10 mL) containing 4,13-diaza-18-crown-6 (1.0 g, 3.8 mmol), Et_3N (1 mL), and pyridine (10 mg) at 0 °C. The solution was stirred at room temperature for 48 h, filtered through Celite, and evaporated in vacuo. The residue was dissolved in $CHCl_3$ (100 mL), washed with saturated aq $NaHCO_3$ (2 × 75 mL), and brine (2 × 100 mL). The organic layer was dried over $MgSO_4$, concentrated in vacuo, and chromatographed over alumina (25% acetone in hexanes) to give **7** (860 mg, 36%) as a white solid, mp 74−75 °C. ^{1}H NMR: 0.72−0.76 (6H, t, J = 6.6 Hz, CH_2CH_3), 1.05−1.25 (32H, s, CH_2CH_2), 1.4−1.5 (4H, t, J = 6.9 Hz, $COCH_2CH_2$), 2.05−2.15 (4H, t, J = 7.2 Hz, $COCH_2$), 3.25−3.6 (24H, overlapping signals due to CH_2CH_2O CH_2CH_2N). ^{13}C NMR: 14.21, 22.77, 25.43, 25.47, 29.42, 29.59, 29.72, 32.00, 33.19, 33.26, 46.95, 47.10, 48.83, 48.96, 173.39, 173.45.

$CH_3(CH_2)_{11}\langle N18N\rangle(CH_2)_{11}CH_3$. $CH_3(CH_2)_{10}CO\langle N18N\rangle CO(CH_2)_{10}CH_3$ (0.5 g, 0.8 mmol) was added to BH_3·THF (1 M, 15 mL) at 0 °C. The reaction was brought to room temperature and stirred for 96 h. The reaction was again cooled to 0 °C, and water was added slowly until the liberation of H_2 had ceased. The mixture was concentrated in vacuo, and HCl (2 M, 15 mL) was added. The aqueous mixture was refluxed for 4 h and then cooled and brought to pH = 9 using NaOH pellets, followed by dropwise addition of 1 M NaOH. Distilled water was added to dissolve any remaining salt, and the aqueous phase was extracted with $CHCl_3$ (3 × 75 mL), which was then washed with brine (2 × 50 mL). After drying ($MgSO_4$), filtration (Celite), and evaporation, didodecyldiaza-18-crown-6 (370 mg, 77%) was obtained as a white solid, mp 46−48 °C (lit.[34] 46−48 °C), the spectral properties of which agreed with those reported previously.[32]

Synthesis of $PhCH_2\langle N18N\rangle Co(CH_2)_{11}<$biphenyl$>(CH_2)_{11}CO\langle N18N\rangle CH_2Ph$, 8. $PhCH_2\langle N18N\rangle CO(CH_2)_{11}Br$. 12-Bromododecanoic acid (0.60 g, 2.15 mmol) and $SOCl_2$ (4 mL, 200 mmol) were heated at reflux for 2 h and then evaporated. Residual $SOCl_2$ was removed by azeotropic distillation (toluene, 2 × 5 mL). The residue was dried at high vacuum for 2 h. *N*-Benzyl-4,13-diaza-18-crown-6 (0.75 g, 2.13 mmol), *N*,*N*-(dimethylamino)pyridine (DMAP, 0.045 g, 0.37 mmol), and Et_3N (0.5 mL) were dissolved in anhydrous toluene (15 mL), and the resulting solution was cooled to 0 °C. The 12-bromododecanoyl chloride obtained in the preceding step was dissolved in anhydrous toluene and added dropwise to the reaction over 30 min. After the solution was stirred at rt for 48 h, the solvent was evaporated, and the residue was dissolved in CH_2Cl_2, washed with 5% $NaHCO_3$, evaporated, and then chromatographed over silica gel (eluant: hexanes/acetone 9:1 → 1:1) to give $PhCH_2\langle N18N\rangle CO(CH_2)_{11}Br$ (1.12 g, 86%) of the desired product as a pale yellow oil. ^{1}H NMR: δ 1.24 (14H, m, $COCH_2CH_2(CH_2)_7CH_2CH_2Br$), 1.58 (2H, m, $COCH_2CH_2(CH_2)_7CH_2CH_2Br$), 1.80 (2H, m, $COCH_2CH_2(CH_2)_7CH_2CH_2Br$), 2.28 (2H, t, J = 7.8 Hz, $COCH_2CH_2(CH_2)_7CH_2CH_2Br$), 2.75 (4H, m, $PhCH_2NCH_2$), 3.36 (2H, t, J = 6.9 Hz, $COCH_2CH_2(CH_2)_7CH_2CH_2Br$), 3.56 (22H, m, $PhCH_2NCH_2CH_2OCH_2CH_2OCH_2CH_2N-CO$), 7.25 (5H, m, ArH). ^{13}C NMR: δ 25.4, 28.2, 28.8, 29.5, 32.9, 33.2, 34.1, 47.0, 48.9, 53.8, 53.9, 60.0, 69.7, 70.1, 70.3, 70.4, 70.5, 70.7, 70.8, 71.0, 127.0, 128.2, 128.9, 139.6, 173.4.

$PhCH_2\langle N18N\rangle CO(CH_2)_{11}<C_6H_4\text{-}C_6H_4>(CH_2)_{11}CO\langle N18N\rangle CH_2Ph$, 8. A mixture of $PhCH_2\langle N18N\rangle CO(CH_2)_{11}Br$ (0.40 g, 0.65 mmol), 4,4′-dihydroxybiphenyl (0.06 g, 0.32 mmol), and Na_2CO_3 (0.30 g, 2.83 mmol) in CH_2Cl_2 (30 mL) was stirred at reflux for 48 h. The mixture was filtered and evaporated, and the residue was chromatographed over silica gel (eluant: acetone/hexanes 1:1 → 8:2 → 8:2 + 2% NEt_3) to give **8** (0.25 g, 62%) as a pale yellow wax. ^{1}H NMR: δ 1.29 (24H, m, $C(O)CH_2CH_2CH_2(CH_2)_6CH_2CH_2O$), 1.42, (4H, m, $C(O)CH_2CH_2CH_2(CH_2)_6CH_2CH_2O$), 1.63 (4H, m, $C(O)CH_2CH_2CH_2(CH_2)_6CH_2CH_2O$), 1.79 (4H, m, $C(O)CH_2CH_2CH_2(CH_2)_6CH_2CH_2O$), 2.32 (4H, t, J = 7.2 Hz, $C(O)CH_2CH_2CH_2(CH_2)_6CH_2CH_2O$), 2.80 (8H, m, $PhCH_2NCH_2$), 3.65 (44H, m, $PhCH_2NCH_2CH_2OCH_2CH_2OCH_2CH_2N-C(O)$), 3.96 (4H, t, J = 6.3 Hz, $C(O)CH_2CH_2CH_2(CH_2)_6CH_2CH_2O$), 6.93 (4H, d, J = 9 Hz, ArH), 7.28 (8H, m, Ph), 7.45 (4H, d, J = 8.7 Hz, ArH). ^{13}C NMR ($CDCl_3$, 75 MHz): δ 25.5, 26.2, 29.4, 29.5, 29.6, 29.7, 33.3, 47.1, 48.9, 53.9, 54.0, 60.1, 68.2, 69.8, 70.2, 70.4, 70.5, 70.6, 70.7, 70.8, 71.0, 114.8, 127.0, 127.7, 128.3, 128.9, 133.4, 139.7, 158.3, 173.5. IR ($CHCl_3$): 3468, 2922, 2853, 1644, 1607, 1499, 1468, 1454, 1352, 1289, 1271, 1243, 1174, 1122, 1041, 921, 823, 734, 699 cm^{-1}. Elem. Anal. Calcd for $C_{74}H_{114}N_4O_{12}$·H_2O: C, 70.00; H, 9.21; N, 4.41%. Found: C, 70.36; H, 9.25; N, 4.50%.

Electrophysiology. HEK 293 cell cultures were bath-perfused with Tyrode's solution, containing (in mM) the following: NaCl (150), KCl (4), $MgCl_2$ (2), $CaCl_2$ (2), D-glucose (10), and HEPES (10), pH 7.4, with NaOH. Whole-cell recordings were established using borosilicate pipets with a tip resistance of 3−8 MΩ when filled with a solution containing (in mM): Cs-glucuronate (140), CsCl (5), $MgCl_2$ (5), EGTA (10), HEPES (10), pH 7.4, with CsOH. Hydraphiles were prepared as 16 mM stock solutions in absolute ethanol, diluted into Tyrode's solution (final EtOH concentrated = 0.1%), and applied by local perfusion from a multibarreled pipet. For some experiments, hydraphiles were diluted into Tyrode's solution containing 0.5% BSA. Cells were typically held at −20 mV and stepped to different test potentials to monitor whole cell conductance. Currents were recorded with an Axopatch 200A amplifier, filtered at 2 kHz, and digitized at 10 kHz for off-line analysis.

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