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Synthetic ion transporters in bilayer membranes

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A variety of synthetic compounds mediate ion transport across bilayer membranes. The majority of reported cases involve transport across vesicle membranes, with mechanism inferred by comparison with known transporters. Single channel conductance techniques provide more direct mechanistic information. Two examples are discussed. In the first, an active unimolecular transporter $(G8TrgP)_4Tet$ is shown to induce erratic formation of transient high conductivity pores. In the second, a linear pore-former A8TrgPA8TrgA is shown produce regular multi-level channels in diphytanoylPC membranes. The pore formed shows ion selectivity in the sequence $Cs^+ > K^+ > Na^+$. A model consisting of an aggregate of a few A8TrgPA8TrgA molecules in the bilayer would be qualitatively consistent with the data.

One of the reasons that the Lock and Key concept has been such a durable and productive analogy in chemistry is due to the rich associations between "fit" and "function" which are obvious at every turn of a key. The parallels with supramolecular concepts are explicit and obvious—the supermolecule possesses functional capabilities that are absent in the separated components, in the same way that a combination of the correct key in the lock permits unlocking. Membrane transport offers the same tight relationship between recognition and function, and explorations of functional systems continue to enrich our understanding of molecular recognition phenomena.

The inspiration for artificial ion transporters has traditionally come from natural examples[1] and many systems are described as "biomimetic". The usual implication is one of *functional* similarity with a biochemical system, and this is nowhere more obvious that in transport across bilayer membranes. Natural ion transporters are massive protein aggregates containing multiple trans-membrane segments which act together to control ion transport[2]. Structural information from molecular biology is emerging apace, but the main molecular features have been inferred from low molecular weight compounds such as gramicidin, amphotericin, alamethicin, or melittin[3]. Figure 1 illustrates a range of molecular mechanisms. Discrete ion *channels*, as exemplified by gramicidin, provide a complete membrane-spanning solvation pathway for an ion in transit. *Aggregate pores* are formed by amphotericin and alamethicin, which provide a more loosely structured environment containing some water at the core of the aggregate. At the extreme are *membrane disrupting agents* such as melittin, or simple detergents. Transport in these cases would occur via deep aqueous fjords at defect structures adjacent to the agent. A *carrier* mechanism is also possible (not illustrated) in which a carrier-cation complex would cross the bilayer by diffusion.

Synthetic ion transporters active in bilayer membranes have been reported by a number of groups. Tabushi[4] was the first to report a cyclodextrin derivative which acted as a slow mediator of Co^{2+} transport across vesicle membranes. This was followed by a series of reports by Nolte[5] on crown ether isocyanide polymers. More recently Lehn[6] has reported cyclodextrin and crown ether "bouquet" molecules, and Gokel[7] has reported a simple tris-crown ether. These systems are drawn from the gramicidin paradigm of a unimolecular membrane spanning structure. Bola-amphiphillic pore-formers reported by Fuhrhop[8] are modelled on the aggregate pore. Recently, an aggregate of a cyclic peptide has been reported by Ghadiri[9]: ion transit is assumed to occur down a stack of macrocycles held face-to-face by intermolecular hydrogen bonds. A number of very simple membrane disrupting compounds have been reported by Regen[10], Kobuke[11], and by Menger[12]. This need not imply indiscriminate damage to the membrane: the Regen compounds can recognize specific membrane lipids and conditions, and the Kobuke compound forms well-behaved single channels in planar bilayers.

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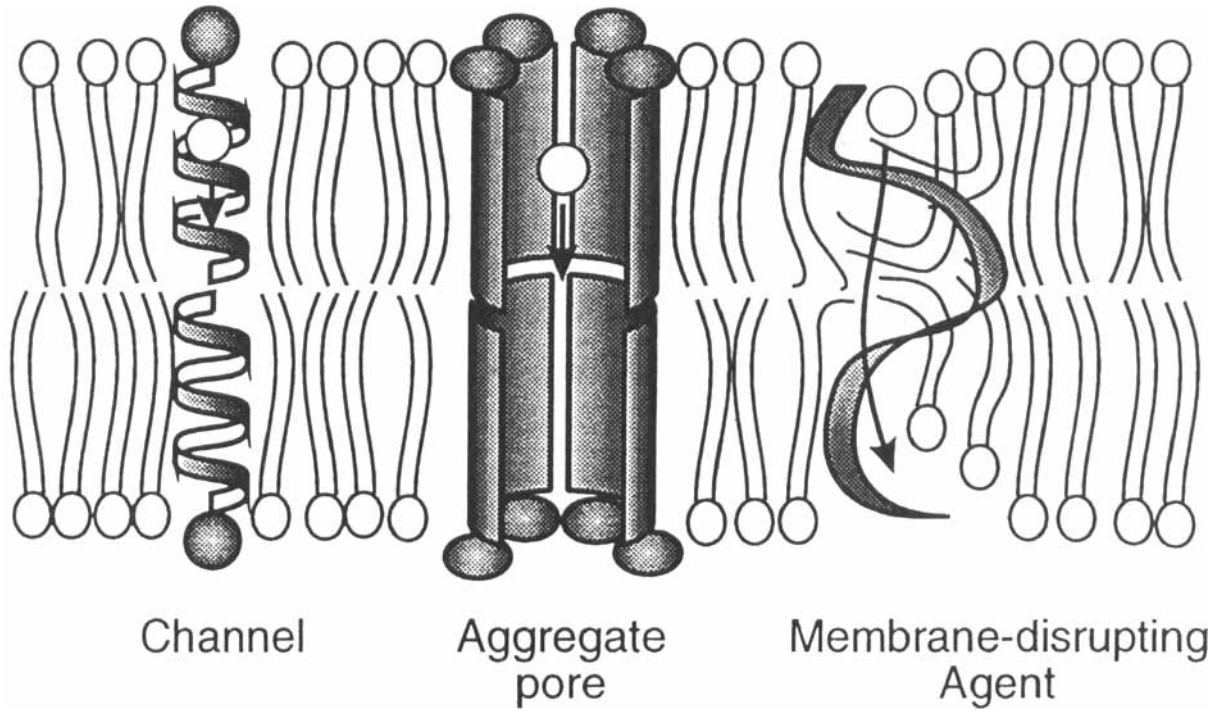


Figure 1 Schematic mechanisms for ion transport across a bilayer membrane mediated by artificial transporters.

Combining information on both natural and artificial ion transporters leads to three general criteria for membrane-active transporters. The first is a requirement for complementarity of the transporter and the lipid environment. The transporter should be amphiphillic, should have suitable columnar dimensions for accommodation in the bilayer, and might have some inherent "stiffness" to orient the transporter within the bilayer. The second requirement for a transporter is the potential to place ionophillic sites deep within the lipid core of the bilayer. The transporter must accommodate ion solvation requirements during transit, either by partial replacement of the ion solvation sphere with transporter-cation interactions, or by introduction of water within the lipid core of the bilayer. A third, practical, requirement is that the transporter have a feasible synthesis. The active structures required are large, so an efficient convergent synthesis, or a well-behaved aggregation process is a requirement.

Our own efforts in this area are summarized in Figure 2. On the left is an example of a unimolecular structure based on a crown ether carboxylic acid framework. Appended to this "core" unit are "wall" components derived from maleic acid esters. The structure is capped at either end with hydrophillic "head" groups to impart an overall amphiphillic character to the molecule. The design assumed that the crown ether would lie near the bilayer mid-plane, and would rigidify and orient the wall units towards the faces of the bilayer. A suite of compounds can be assembled from combinations of the components illustrated to explore the structural effects of the core units (2,4, or 6 carboxylic acids, R,R- or R,S-isomers), the macrocyclic tetraesters (22 to 36 membered rings, hydrocarbon, or oligoether sides), and thiol head group character[13]. A total of 21 examples of this type have been characterized.

On the right of Figure 2 is a simpler candidate structure assembled from the same suite of components. The design expectation in this series was that several molecules would aggregate in the bilayer to open an aqueous defect for ion transport. The "wall" and "head" groups are the same, but simple bifunctional "linker" units knit the structures together. A total of 14 examples of this type have been characterized[15]. Both series rely on a modular synthesis plan consisting of two optimized steps. In the first step the "core" unit carboxylates are alkylated by a "wall" unit mesylate or iodide. In the second step the "head" groups are appended by an efficient Michael addition of a thiol nucleophile to the maleic diester terminus of the wall units. This permits large structures to be assembled rapidly and with high unit efficiency. The yield for the two steps is typically 25–45% overall, which represents a single-step, single-bond coupling efficiency in excess of 95%. The larger structures are simpler to isolate and purify than the lower molecular weight counterparts, as the product mixtures are readily separated by size-exclusion chromatography[13,14].

The activity of the suite of compounds was established using an assay in bilayer vesicles prepared from an 8:1:1

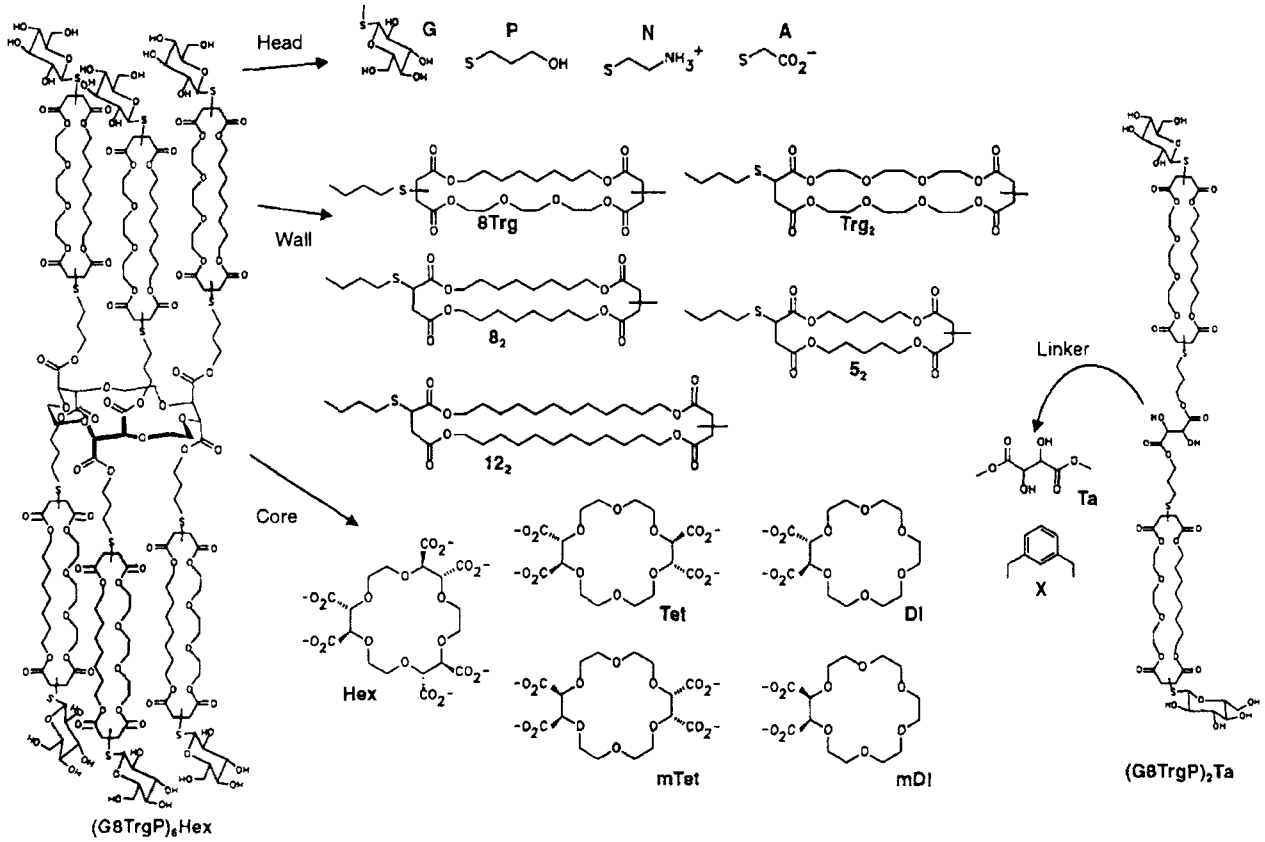


Figure 2 Components of a modular construction set for the assembly of unimolecular transporters (left) and linear pore-formers (right). Naming conventions are developed in references 13 and 14.

mixture of phosphatidyl choline (PC): phosphtidic acid (PA): cholesterol. The vesicles are prepared with an entrapped buffer, and subsequently transferred to an unbuffered medium in which the external pH is set by a pH-stat titrimeter. The transmembrane pH gradient is stable for many days due to a lack of proton and cation translocation pathways. Addition of a proton carrier and external alkali metal cation "primes" the system, but no proton efflux occurs until an active transporter is added. Proton release, via electroneutral proton-cation antiport, is quantified by the pH-stat controlled addition of standard base to maintain a set pH. Survey experiments on all 35 compounds, and extensive investigations of the more active materials established the following features[14–16]:

i) Transport activity is controlled by structural variables. The most active materials have a length complementary to the bilayer thickness, have a balance of hydrocarbon and ether groups in the walls, and neutral (unimolecular transporters) or charged (aggregate transporters) head groups.

ii) Transporter mechanism can be inferred from a combination of kinetic measurements including apparent kinetic order in transporter, dependence on cation type and concentration, apparent activation energy, and inhibition by competing ions[14–16]. Channel-like behaviours were suggested by significant inhibitions and unusual selectivity patterns among the alkali metal cations[16]. The formation of aggregate pores was suggested by high apparent kinetic orders in transporter, and by apparent selectivity between hydrated protons and hydrated alkali metal cations[14].

iii) Transport mechanism is also under structural control. Channel-like activity is associated with unimolecular structures bearing four or six wall units on cores which support a columnar orientation.

The vesicle assay technique is well suited to this type of structure-activity survey. However, the inference of mechanism is inherently indirect, relying on an comparison with known transporter activity and behaviour in the same vesicle system. More direct mechanistic information can be obtained from the bilayer conductance techniques known collectively as single-channel recording techniques[17]. In our hands this involves the formation of a lipid bilayer across a 0.25 mm hole in a polystyrene barrier by direct application of the lipid in decane with a fine paint brush. The lipid thins to a bilayer as the excess decane is removed by brushing above the hole. The bilayer electrically isolates the two sides of the cell, and electrical contact is made via Ag/AgCl electrodes inserted into the electrolyte on either side of the bilayer. A bilayer clamp amplifier is used to apply a potential and to measure the current across the bilayer as

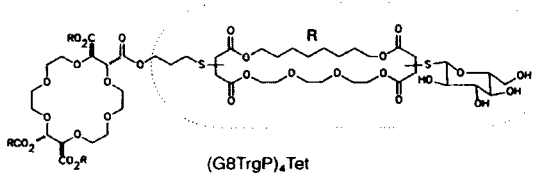
function of time. Channel activity is unambiguously detected by the presence of step changes in the current as a channel switches from a "closed" to an "open" state. Subsequent analysis of the current-time record can establish the mean amplitude of the channel currents, the current-voltage response function of the transporter, ion selectivity of the ion transit event, and the lifetime of channel openings as a function of the potential and chemical compositions.

Single channel techniques are a powerful mechanistic toolbox[18]. They also offer chemists a unique opportunity—to directly observe the behaviour of a single molecule. In the case of an aggregate-pore the behaviour of a small group of molecules can be observed, including the effect of single molecule addition to the properties of the aggregate[3]. Two examples will illustrate the potential of the technique for the evaluation of synthetic ion transporters.

Example 1: Activity of the unimolecular transporter $(G8TrgP)_4Tet$

One of the most active transporters uncovered in the vesicle survey was the four-armed transporter $(G8TrgP)_4Tet$ (structure below). In addition to very high specific activity it showed a marked Na^+ selectivity that fell outside any of the Eisenman selectivity sequences and the transport of Na^+ was strongly inhibited by K^+. The inference from the vesicle survey was that this compound is capable of forming ion channels[16].

Figure 3 shows a portion of a single-channel record spanning about 2.5 seconds for $(G8TrgP)_4Tet$ in an 8:1:1 PC:PA:cholesterol bilayer separating 0.5 M CsCl solutions, at an applied potential of −160 mV. In comparison to well behaved systems such as gramicidin this record is awful. The record was chosen to illustrate the general behaviour of this compound; this is neither a best nor a worst case, but a randomly selected example of fundamentally irregular behaviour. After addition of the transporter in methanol to the electrolyte a variable lag-time eventually leads to sporadic openings such as those in the initial trace at the top of Figure 3. These openings become more pronounced, and of longer duration, eventually leading to major conductivity events such as those indicated with A or B in the Figure. The current at these points exceeds 100 pA. As rapidly and as randomly as the openings build up, they collapse to a predominantly closed state (near the middle of the Figure). Subsequent bursts of openings follow.



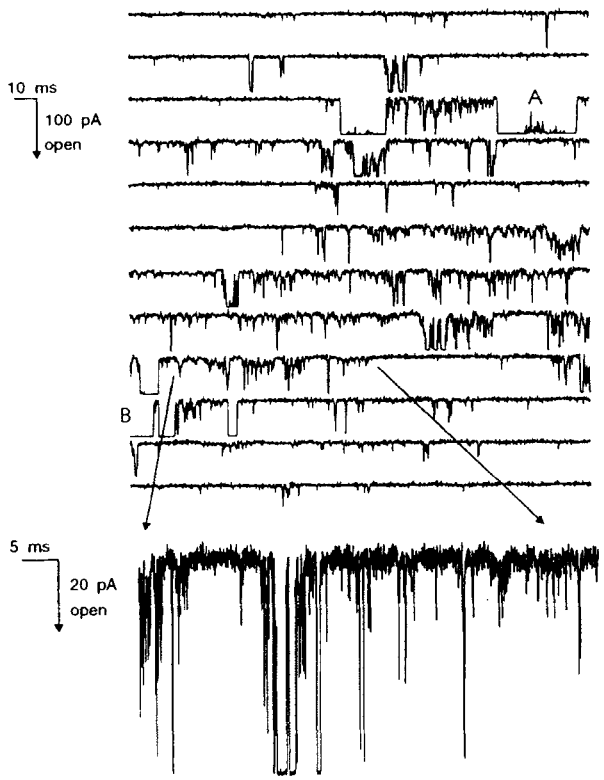
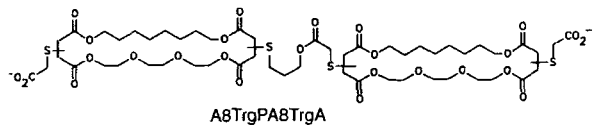


Figure 3 Single channel recording of $(G8TrgP)_4Tet$ in a 8:1:1 PC:PA:cholesterol membrane, 0.5 M CsCl electrolyte, applied potential -160 mV. This 2.5 second section is taken from 28–30 seconds into a record spanning 2 minutes approximately 5 minutes after introduction of the transporter to the cell. The lower trace is an expansion of the section indicated.

On an expanded scale, illustrated at the bottom of Figure 3, some of the openings appear, to the eye-of-faith, to have some regularities. Any objective analysis of this section fails to find a regular behaviour and the record is apparently erratic at all resolution scales. The same is true for NaCl and KCl as electrolytes, and for diphytanoylPC as lipid.

What can be made of the mechanism of action of $(G8TrgP)_4Tet$ based on a set of results akin to Figure 3? There is no doubt that "channel" openings and closures occur, some of which move very significant amounts of ionic charge across the membrane. Thus the high activity in vesicles is not surprising. We were unable to detect any pattern in the behaviours, so the ion selectivity question is unanswered for these planar bilayers. The unusual selectivity, and the inhibition effect of K^+ might be a result of ion-selective initiation of the erratic transport evident in the single-channel record. It is very likely that this early part of the record represents only one or two molecules, behaving randomly from the outset. At longer times the current rises, and the quiescent states become less frequent. This would be consistent with a slow buildup of molecules in the bilayer. One mechanistic possibility is that a molecule is acting on its



A8TrgPA8TrgA

own, but is so poorly organized that it cannot establish a channel of defined properties. Another possibility is that a molecule seeds a transient defect in the membrane, but cannot stabilize the required aqueous fjord effectively. Whatever the mechanisms it is clear that active, reproducible macroscopic behaviour is based on molecular chaos.

Example 2: Activity of the linear pore-former A8TrgPA8TrgA

The linear pore-former A8TrgPA8TrgA is a derivative in a series which includes zwitterionic examples of potential interest as voltage-gated transporters[19]. The synthesis follows directly from the methods used previously[13,14].

The single channel records for this compound under a variety of conditions are regular, and are particularly well-behaved in the presence of CsCl electrolyte. Figure 4 shows a two minute section from a single-channel record of A8TrgPA8TrgA in a diphytanolyPC bilayer at an applied potential of +50 mV. Several conductance levels are obvious, and appear to be separated by a relatively constant step height. The openings vary in duration between 0.5 and 15 seconds, but these appear to be independent of which conductance state is considered.

A more rigour analysis is presented in Figure 5. For the file of Figure 4, a list of each opening/closing event was prepared and a histogram of the amplitude of the events was constructed. The result for Figure 4 is given in Figure 5, showing a regular spacing of the observations. Fitting a series of Gaussians to the histogram gives values of the mean amplitudes (± standard deviation) for the levels as: 2.3 ± 0.3 pA; 4.0 ± 0.9 pA; 6.1 ± 1.7 pA;

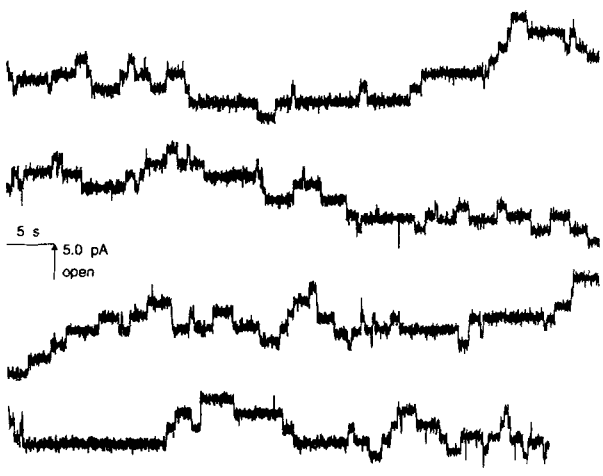


Figure 4 Single channel recording of A8TrgPA8TrgA in a diphytanoylPC membrane, 0.5 M CsCl electrolyte, applied potential +50 mV.

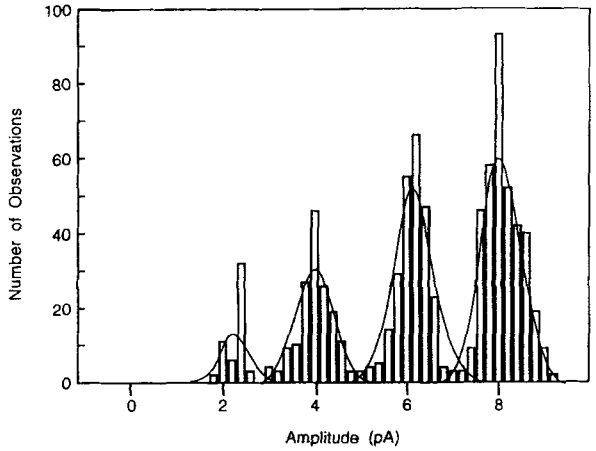


Figure 5 Amplitude histogram for the record of Figure 4.

8.0 ± 2.6 pA. This short series suggests that the mean channel opening is just a multiple of 2 pA, and the levels correspond to 1, 2, 3, and 4 occurrences of the same type of opening. Alamethicin also shows multiple openings[3] which can be modelled as a series of pores formed by addition and deletion of individual molecules from a core of 3 or 4 molecules. The individual conductance increments due to addition increase with the number of molecules in the pore bundle[3]. The noise in Figure 4, and the relatively low number of levels observed masks this effect in our system, if it is indeed present.

Histograms similar to Figure 5 can be constructed for a range of values of applied potential. The mean conductance at each potential can then be plotted as illustrated in Figure 6 for 3 different electrolytes: NaCl, KCl, CsCl (all 1M). Two features are evident. The less interesting one is that the channels are ohmic, and symmetrically disposed about the origin, indicating a complete lack of voltage dependence. More striking is the result that this pore is ion selective in the order $Cs^+ > K^+ > Na^+$. The

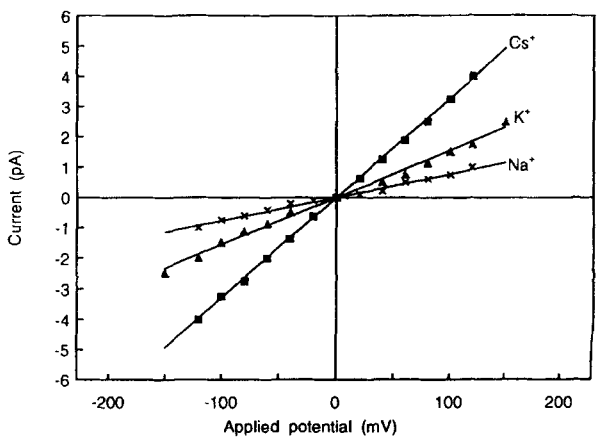


Figure 6 Current-voltage diagram for mean amplitude of pores formed by A8TrgPA8TrgA in diphytanoylPC bilayers for three electrolytes.

specific conductances of the pore with the different electrolytes are: Na^+, 9.9 pS; K^+ 14.3 pS; Cs^+ 33 pS.

A model which is qualitatively consistent with the data is an aggregate of few molecules which can increase in size by accretion of individual molecules. The mean properties of the aggregate cannot vary greatly with aggregation number. Ion selectivity is consistent with a head group effect where the ion with the most diffuse surface charge is least retarded by the anionic head groups of the aggregate. Considering the simple structure of A8TrgPA8TrgA, the remarkable regularity of this pore is strong incentive to continue to explore the very simplest of pore-former candidates.

EXPERIMENTAL

The synthesis of $(G8TrgP)_4Tet$ was described previously[13]. The synthesis of A8TrgPA8TrgA follows from the obvious route reported previously[19] by the alkylation of $A_2$8Trg (as the bis tetramethylammonium carboxylate) with 8TrgPOMs. The resultant ester-alkene was isolated by gel permeation chromatography, and the final head group was added using conditions as previously described[13]. Purification by gel permeation chromatography (Sephadex LH-20, 4:3 $CHCl_3$:$CH_3CH(OH)CH_3$) gave A8TrgPA8TrgA as an oil in 42% yield: ^1H NMR 360 MHz (δ, $CDCl_3$): 5.12 (br. s, 2H), 4.28-4.02 (m, 18H), 3.92-3.26 (m, 26H), 3.00-2.65 (m, 10H), 1.94 (m, 2H), 1.61 (m, 8H), 1.30 (br. s.., 16H); ^{13}C NMR 90.57 MHz (δ, $CDCl_3$): 172.1, 171.8, 171.5, 71.2, 171.1, 170.9, 170.5, 170.3, 170.0, 169.9, 169.6 (C=O); 77.2, 70.5, 70.3, 70.1, 69.3, 69.1, 68.9, 65.6, 65.4, 65.0, 64.7, 64.2, 64.1, 63.9, 63.7, 42.4, 41.7, 41.6, 41.5, 36.4, 35.9, 33.9, 33.6, 33.5, 33.2, 32.9, 29.6, 29.3, 28.6, 28.3, 28.1, 27.9, 27.9, 25.5, 25.3; MS (-FAB, mNBA): 1261.4 $(M-H)^+$ 100%. The sample was contaminated with the corresponding isopropyl ester as seen in the ^1H NMR (1.26-1.22 ppm), the ^{13}C NMR (21.7 and 14.1 ppm) and the MS (1303.4 (M+isopropyl-H)$^-$).

All lipids were obtained from Avanti Polar Lipids and were used without further purification. Water was deionized and doubly distilled in quartz. Chloroform was washed three times with water, dried over $MgSO_4$, stabilized with 1% absolute ethanol, and stored over molecular sieves. Other salts and solvents were analytical grade.

Instrumentation

Single channel currents were measured with a bilayer clamp (Warner Instruments BC-525A) using Ag/AgCl electrodes inserted into electrolyte held in a bilayer chamber (Warner Instruments, BCH-22; polystyrene, 0.25 mm hole size). Bilayer formation was monitored with an oscilloscope using the capacitance test function of the clamp. Single channels were directly recorded on an X-Y recorder. The signal was also filtered and amplified using a Model 113 pre-amp/RC filter (PAR), and digitized using a Digidata 1200 ATD board and the pClamp6 suite of programs (Axon Instruments). Early experiments, including the data of Figure 6, were analysed manually from the X-Y recordings.

Bilayer Formation

Stock solution of lipid in $CHCl_3$ (15 mg/mL, 1mL) was evaporated and the lipid was redissolved in decane (1 mL) using sonication if required. The hole area of the dry bilayer cell was pre-painted with the decane solution of the lipid, and dried under a nitrogen stream. Electrolyte was added to the cell and the electrical circuit was completed. The bias of the bilayer clamp was adjusted to compensate for junction potentials, and a drop of lipid solution was painted across the orifice using a fine brush. If necessary the lipid layer was brushed with a dry brush until any excess was removed. The capacitance of the membrane was monitored until stable (0.3-0.45 $\mu F/cm^2$), followed by a period of baseline monitoring. Methanolic solutions of transporters ($1\text{-}5 \times 10^{-4}$ M) were added by microliter syringe. Channels of $(G8TrgP)_4Tet$ and A8TrgPA8Trg formed spontaneously in virtually every attempt.

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