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Bioorganic & Medicinal Chemistry
ELSEVIER
Bioorganic & Medicinal Chemistry 12 (2004) 1291–1304

Functional, synthetic organic chemical models of cellular ion channels

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Received 28 February 2003; revised 31 July 2003; accepted 1 August 2003

1. Introduction

The cell has been described as ''the basic capsule of life.''[1] It contains the nutrients required for vitality and the chemical machinery required for their use.[2] Bacteria (prokaryotes) and cells in yeast or mammals (eukaryotes) differ in many ways but both are capsules bounded by membranes. The membrane barriers of living systems are all complex but those surrounding bacteria and mammalian cells differ profoundly. Bacterial membranes may contain one or two outer barriers depending on whether they are Gram-positive or Gram-negative, respectively. The intracellular membrane system of eukaryotic cells is practically nonexistent in the bacterial cell. Bacterial cells are relatively simple; they lack defined organelles and the DNA is found in a 'nuclear area' rather than contained within a double membrane system having complex transport and regulatory roles. Eukaryotes, on the other hand, display a dramatic increase in membrane complexity that includes a surrounding plasma membrane and clearly defined subcellular components including the nucleus, mitochondria, the Golgi, lysosomes, endosomes, and the endoplasmic reticulum. Each of these membranes serves the common function of an effective barrier with remarkable selectivity.

The eukaryotic plasma membrane is a complex structure owing to the variety of individual components that comprise it. The complexity of prokaryotic membranes results from their elaborate structural features. Proteins inserted into a typical plasma membrane account for about half of its total weight. The bilayer itself may contain phospholipids having various fatty acid chains and a range of head groups. Steroids (most commonly cholesterol in mammals and ergosterol in fungi) are typically present in plasma membranes but may also be found in the bilayers surrounding organelles. Numerous other compounds such as phosphatidic acid and sphingomyelin are found in membranes as well.

The complexity of modern biological membranes has made their study a daunting task. One of the fundamental properties of membranes is the selective and regulated transport of the ions that are found in both the intra- and extracellular compartments. The study of ion transport across membranes can be simplified by using a hollow capsule formed from phospholipid monomers — usually called liposomes. These lipid bodies can be of uniform or defined composition and they may be engineered to have particular sizes and properties. The liposomes that are the most useful ones for biological studies are those that are of uniform size and that are unilamellar, that is the membrane is formed from a single phospholipid bilayer. In addition, the lipid composition of synthetic vesicles must remain in a fluid state for the transport processes to reflect the biological situation.

Synthetic membrane systems are now well enough known that phospholipid vesicles may be produced by using standard protocols. Different protocols produce vesicles of different sizes and stabilities. It is always

Keywords: Synthetic ion channel; Cation channel; Anion channel; Bilayer membrane.
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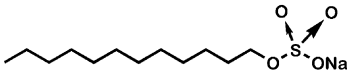
0968-0896/$ - see front matter
doi:10.1016/j.bmc.2003.08.036

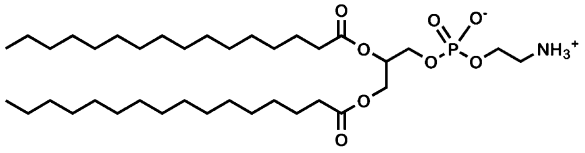
prudent, if not important, to characterize the vesicles before they are used experimentally even though the preparations are usually reproducible and the results are generally consistent.

1.1. Amphiphiles and membrane monomers

Chemists often describe membrane monomers as consisting of a polar headgroup and a nonpolar tail. This characterization is certainly true, as far as it goes. The detergent SDS (sodium dodecyl sulfate, also called sodium laurel sulfate) clearly fits this bimodal description. This amphiphile forms micelles rather than vesicles. Like other amphiphiles that form vesicles, typical membrane-forming lipids also possess polar headgroups and nonpolar tails but their overall structure is inherently complex. This is apparent in the structure of phosphatidylethanolamine (PE) shown. The two palmitic (hexadecanoic) acid chains clearly comprise the 'nonpolar tail' but the polar headgroup is really more complicated. The anionic phosphate and protonated ethanolamine groups are very polar and are expected to interact with the essentially aqueous medium that surrounds all cells.



Sodium dodecyldsulfate (1)



phosphatidylethanolamine

If the hydrocarbon tails are nonpolar and the aminoethyl phosphate is polar, where does the glyceryl unit fit? In fact, this portion of the molecule is intermediate in polarity between these two and can accurately be designated as the 'midpolar regime.' The hydrocarbon tails from monomers on either side of the bilayer form the insulator regime or 'hydrocarbon slab.' This 25–35 Å thick barrier is nonpolar. Its interior is likened to hexane, the dielectric constant (ε) of which is about 2. Water, which is in contact with the phospholipid head groups, has a dielectric constant of ~80.

The glyceryl esters are, by virtue of their chemical structure, intermediate in polarity between these two extremes. Glycerol itself has a dielectric constant of 42. Ester groups are present in this regime; methyl acetate has a dielectric constant of ~7. Of course, there are typically two ester residues in a phospholipid's midpolar regime so the polarity is expected to be higher than for methyl acetate. On the other hand, there are no free hydroxyl groups so the local dielectric should be less than glycerol's 42. Ohki and coworkers have assessed the dielectric constant of the midpolar regime by using fluorescence measurements and reported it to be about 30.[3]

1.2. Cations and anions

While the biologist may view both simply as ions, cations and anions are usually considered by chemists to be opposites. Specifically, they have exactly reversed charges and therefore opposite chemical affinities. In some ways, however, they are similar. Sodium and potassium cations are about 2.04 and 3.02 Å in diameter, respectively.[4] Chloride ion's diameter is 3.62 Å but it is negatively charged. Of course, these values are for the ions in a crystal environment and are for specific coordination numbers. In a biological milieu, the ions differ in size as well but the magnitude of this difference is unclear. Recent calculations place the sizes of hydrated Na^+, K^+, and Cl^- ions at 5.98, 5.50, and 6.48 Å, respectively.[5] Not only is the size order different from that inferred from the crystal state, but the relative sizes are more nearly equal.

An important and currently unresolved issue is the solvation state of any of these ions in their natural environment. It seems reasonable to think that a cation or anion that is passing through a channel is only partly solvated — although the meaning of 'partly' is obviously vague. Protein cation channels may pass anions if the applied potential demands it. These facts further complicate the question of selectivity.

Fortunately, the number of ions that the chemist must consider in terms of in vivo channel function is relatively limited. Although many metals are known in biological systems, relatively few are present at concentrations that demand consideration in terms of their transmembrane transport by channels. Not surprisingly, the concentrations of ions in seawater and in vivo are related. Table 1 shows the concentrations of Na^+, K^+, Mg^{2+}, Ca^{2+}, and Cl^-. Sodium is by far the most abundant cation although potassium, magnesium, and calcium are all present at significant concentrations. The cations are spherical and differ only in size and charge.

Among anions, chloride and bicarbonate (HCO_3^-) are the dominant ions. These two ions have identical charges but differ substantially in shape and size. Owing in part to the difference in their charges, selectivity between K^+ and Cl^- is not nearly so hard to achieve as when the two ions are both spherical and identically charged (e.g., Na^+ and K^+).

1.3. Mechanisms of ion transport

Ions may pass through a membrane by virtue of diffusion.[6] Typically, this is a low probability process, parti-

Table 1. Ion concentration in seawater

Ion	Concentration	
	g/L	mmolar
Na^+	10.8	470
K^+	0.4	10
Mg^{2+}	1.3	54
Ca^{2+}	0.4	10
Cl^-	19.4	547

cularly for the ions that are the subject of the present discussion. Ions such as K^+ are conducted across membranes by ionophoric carriers such as valinomycin and by channel-forming proteins. Although the mechanism of channel formation and transport is not understood in chemical detail, the carrier and channel mechanisms may be contrasted by use of an analogy common in biology. The carrier mechanism of transport that is typical of an ionophore-mediated process is likened to the function of a ferryboat. The ionophore is dissolved in the membrane (a river) and shuttles between the two aqueous phases (opposite shores) that the hydrophobic portion of the membrane separates. The ionophore contacts an ion at one phase boundary and forms a complex. The complex then diffuses across the membrane at a rate determined by the ion, the ionophore, the type and strength of complex formed, and the composition of the membrane. When the complex arrives at the other membrane interface, the ion is released from the complex and the ionophore diffuses back through the membrane. Typical data obtained for a carrier-mediated cation transport experiment are shown in Figure 1.

The behavior of a channel is entirely different from that of an ionophore. A channel exhibits what is often referred to as 'open–close' behavior. When the channel is in the open state, ions flow through at a rate that may be as high as 10^8 ions per second. When closed, transport is blocked. The transition from open to closed is often remarkably fast. Further, open states are normally of the same magnitude. If two channels open, the peak will be twice as high as before, indicating that twice the number of ions are flowing through the membrane.

Figure 2 shows data that were obtained with our own chloride selective channel that we have called a 'synthetic chloride membrane transport receptor' or SCMTR ('scimitar').[7–10] The data in the figure show more than a dozen open–close transitions during the course of one second. Of course, the time course of open–close transitions for a channel depends at least on the compound that forms the channel, the type of pore, the type of ion being transported, and the membrane itself. The data observed for different channels will vary considerably but are always recognizable and the kinetic profile is significantly different from that observed when transport is mediated by a carrier (ionophore) molecule.

1.4. The phospholipid bilayer membrane

In the authors' view, there is no more remarkable or implausible structure in nature than the phospholipid bilayer. It self-assembles from thousands to millions of diverse monomers, it serves as a barrier and the shell of a capsule, yet it may accommodate an equal weight of 'impurities' such as channel proteins without collapsing or disrupting. Indeed, the plasma membranes of most cells contain about 50% by weight of proteins. Dozens of individual amphiphiles comprise membranes. Although these compositions may differ dramatically, they are characteristic for each cell type or organelle.

An obvious first question in the design of a channel compound is 'how thick is the phospholipid membrane in which it must insert?' This is a difficult question to answer with any precision even at the start of the 21st century. Initially, there were two critical questions. First, what is the thickness of the insulator regime ('hydrocarbon slab')? Second, where should the channel's headgroup be located with respect to the membrane and its aqueous boundaries?

There simply is no fixed dimension known for bilayer membranes. Small, unilamellar vesicles formed from egg phosphatidylcholine have apparent inner and outer membrane radii of 65 and 105 Å, respectively. This would place the overall membrane thickness at 40 Å. Data for some biological membranes, obtained for example by NMR measurements, suggest that the hydrocarbon barrier of the bilayer is 30–35 Å. The dynamic nature of these assemblies precludes crystal structures although data are available for some phospholipid monomers. For example, the structure of 2,3-dimyristoyl-D-glycero-1-phosphocholine dihydrate has been reported.[11]

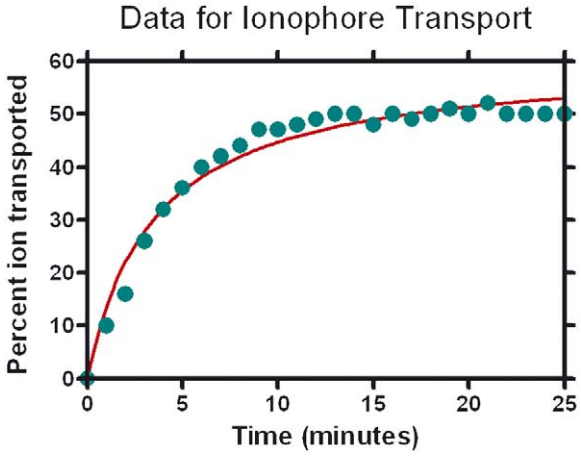


Figure 1. Typical transport data for ions transported across a bilayer by the carrier mechanism.

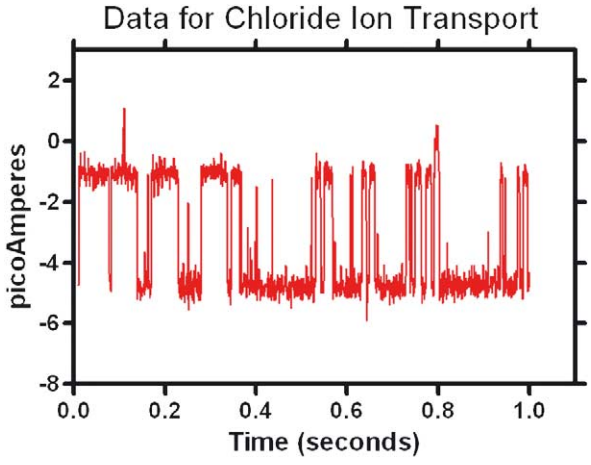


Figure 2. Data for a synthetic, chloride-transporting channel.

The overall thickness of the membrane will depend in part on how and to what extent the chains interdigitate. Other factors include the chain length, the presence of unsaturation, if any, in the acyl chains, and the orientation of the headgroups. The latter is shown in Figure 3 along with a schematic representation of headgroup size in cartoon form.

Clearly, it is the insulator regime of 30–35 Å that is critical for ion transport. Once the ion passes the insulator, it can resolvate and/or interact with a variety of polar groups. Thus, we felt that the critical distance must be a little longer than 35 Å. This would place the anchoring residues in the midpolar regime where it seems reasonable that they should be secured. We were therefore working to a distance of 35–40 Å in overall length in our initial channel designs. Separate experimental studies subsequently verified this distance requirement.[12,13]

1.5. Operational features and characteristics of channels

Lengthy monographs have been written on the subject of natural channels and their structures.[6,14] The characterization of their function has been documented in dedicated texts as well.[15] For the organic chemist wishing to design a synthetic channel, it is important to consider the questions of membrane insertion and pore formation.

A synthetic channel-forming compound must be sufficiently hydrophobic to insert into a bilayer. Whether the compound must penetrate deeply enough to transcend both leaflets is unclear but insertion is clearly critical. Many hydrophobic species associate with bilayers and insert into them without forming channels. It is therefore critical that the synthetic channel compound forms a pore subsequent to insertion. Such a requirement may appear obvious, but it is sadly true that putative channel-formers have been prepared and reported in the literature without data to confirm that function followed form.

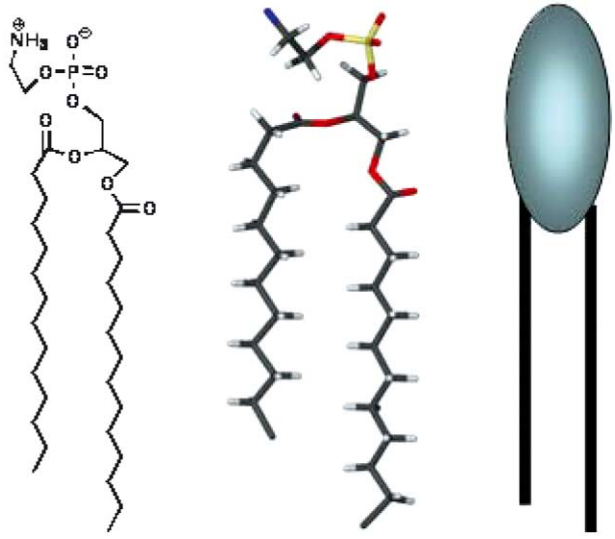


Figure 3. 2,3-Dimyristoyl-D-glycero-1-phosphocholine shown (a) in chemical structure form at left, (b) in a solid state structure (center, from the Cambridge Structural Database, LAPETM10), and (c) in a size-appropriate schematic representation.

A pore is essentially a defect in the membrane barrier that permits passage of one, some, or many species. Insertion of any channel-forming compound must occur in the bilayer without compromising it. Once the compound in question has inserted, it must be possible to assess ion transport. The techniques and expertise to prepare new compounds may be found in most chemistry laboratories but specialized analytical methods typically are not. One useful approach used to assess ion transport is the NMR technique developed by Riddell and Hayer.[16,17] This methodology gives information about transport through phospholipid bilayers but cannot distinguish between the channel and carrier (or other) mechanisms. Fluorescence studies of proton transport suffer from the same limitation. Rate comparisons with known channel-formers and carriers can help in judging whether a carrier or channel mechanism is at work but patch or bilayer clamping remains the definitive analytical method.

It is important to note that even when unequivocal evidence for open-close behavior is obtained, one still does not really know chemical details beyond the designation 'channel mechanism.' Of course, this absence of chemical detail in the mechanism by which protein channels transport ions continues to plague biological studies. Both biologists and chemists are working to understand the mechanistic details in their respective channel-forming systems. At present, clear mechanistic information is not available to either group.

Finally, once the channel inserts non-destructively in the bilayer, it must pass ions. Carriers do so as well but channels typically transport ions with rates of 10^6–10^8 ions per second. The best natural carriers can only achieve rates of 10^4 ions per second. For a synthetic compound to truly mimic the function of protein channels, it would exhibit such features as ion selectivity, voltage-dependent gating, and rectification. To our knowledge, no synthetic channel has yet demonstrated all of these properties.

2. Ion channel models

2.1. Channel model systems

What constitutes a channel mimic or a model channel? The compounds that are described in this report are completely synthetic structures that were designed de novo to exhibit channel behavior. The earliest unequivocal attempt to design, prepare, and characterize a synthetic channel was the amphiphilic cyclodextrins reported by Tabushi and coworkers two decades ago.[18] Admittedly, they studied the transport of divalent cobalt ions (rather than more bio-relevant sodium or potassium cations) through bilayers. In part, this was because they could monitor the transport process with cobalt and they did not have the techniques available to readily monitor alkali metal ion transport.

2.2. Gramicidin as a protein channel model

The peptide called gramicidin was isolated from *Bacillus brevis* four decades before Tabushi's pioneering work. As recently as the late 1990's, however, controversy remained concerning the structure adopted in the membrane that permits ion transport. Nevertheless, gramicidin has been used in literally thousands of studies since the 1940's. Is it the best model for protein channel function? It certainly has the advantage that, like proteins, it is an amino acid polymer. Unlike proteins, however, it possesses D-amino acids. The reversal of configuration allows gramicidin to form a coil (a π-helix) that is different from an α-helix. The gramicidin coil has space within that is generally thought to be the ion pathway, although recent work[19] places the exact structure in controversy.[20–23]

Another important difference between gramicidin and most protein channels is that gramicidin forms a head-to-head (also described as tail-to-tail) dimer, which spans the membrane. To the extent that it is known, it appears that protein channels form an ion pathway from multiple, parallel α-helices.[24–26] The α-helices themselves have too little space within them to accommodate alkali metal cations. Protein channels are exquisitely selective for one ion over another but gramicidin possesses a rather large pore and passes various cations. In sum, gramicidin is probably not an ideal model for the study of protein channels. It has been remarkably popular, however, because it is a peptide, it forms robust channels, and it shows open/closed gating behavior that is reminiscent of protein channels.

Amphotericin, a systemic antifungal drug, has also proved to be attractive as a model system for ion channel formation. Indeed, it has been specifically compared with the carrier valinomycin and with gramicidin in phospholipid vesicles.[27] Amphotericin is oblong and flat. It is thought to organize into a cylinder in much the same way that the staves of a barrel form a cylindrical shape.[28] These 'barrel staves' insert into a bilayer to form a pore through which ions may pass. Such channels have been much studied but bear little physical resemblance to protein channels.

2.3. Synthetic peptides

Several groups have used relatively simple peptides or peptide bundles to mimic the activity of proteins.[29–42] These compounds reveal properties similar to those of far more complex proteins and they have been used as the basis for developing functional channel models.[43,44] The peptide bundles are far closer to natural proteins than are the supramolecular models developed more recently. On the other hand, less of what might be called 'chemical design' was involved in their development. Channel function has also been achieved by cyclic peptides that appear to form nanotubes within the bilayer membrane. The amino acids in these cyclic compounds have alternating stereochemistry (i.e., D,L,D,L...) that permits the sidechains to radiate outward from the nanotube. The stack of peptides is thought to be held together by H-bond interactions along the nanotube axis.

2.4. Other synthetic channel systems

Although the first synthetic channel mimic appeared only in 1982,[18] a number of other models followed. Several attempts to prepare functioning channel mimetics appeared at the end of the 1980's and beginning of the 1990's. Several of these model systems were designed as 'half-channel elements' in the sense of the Tabushi cyclodextrin derivatives. Among them were Menger's proton transporter,[45] and those reported by Tanaka and coworkers,[46] Regen et al.,[47] and by Koert et al.[48,49] A number of structures has been reported that incorporate crown ethers. These include crown ether-containing compounds reported by Lehn,[50] Fyles,[51,52] and Voyer.[53] Cobalt-transporting oligomers formed from crown-substituted isonitriles were reported by Nolte and his coworkers.[54] Finally, Matile et al. have prepared a series of membrane-active molecules that they have designated either as 'rigid-rods'[55,56] or as 'pi-slides'[57] depending upon a combination of structure and function.

The range of compounds that has appeared during the past two decades is remarkable indeed. We have reviewed the models and approaches in two articles that go far beyond the breadth of the present discussion.[58,59]

3. Tris(macrocycle)s as cation channel models

Our efforts to develop a cation-conducting channel were inspired in part by the fact that biological transport occurs almost exclusively by a channel mechanism. Extensive studies involving crown ethers as carriers have been reported and much has been learned from these efforts. Still, the development of completely synthetic cation channels that insert directly into bilayer membranes presented an exciting challenge.

Our first question concerned the state of knowledge of transport proteins. We began to consider channel design strategies in the late 1980's and reported our first success early in 1990. In that same year, a book on natural channel compounds written by Stein contained the following statement:[60]

> We know a good deal about transport kinetics and about what regulates transport through the channels, carriers, and pumps. But we do not have, even in one instance, a clear understanding of how these molecules function. We do not know how they distinguish so effectively between their substrates and other similar ions or molecules. We do not know how the transported substrates move through the transporting proteins, nor how these transporting proteins catalyze those movements and, in some cases, link transport to the consumption of metabolic energy.

The inevitable question that arises concerning channel mimetics is 'how do they function at the chemical level?'

It is important to bear in mind that after a century of study, natural channels are only now beginning to give up details about their action. On balance, however, far less is known than remains unknown about protein channel function, rectification, gating, and selectivity. What is clear to the modern chemist and what we believed at the outset of this project is well stated in a recent biochemistry text by Berg, Tymoczko, and Stryer.[61]

> Before the appearance of life, simple molecular systems must have existed that subsequently evolved into the complex chemical systems that are characteristic of organisms.

Our goal, then, was to develop a simple molecular system that could insert in a bilayer and permit the transport of a cation through it. We took into consideration the issue of cation selectivity but our thinking was not sophisticated enough to contemplate anions. In retrospect, it seems obvious that when a 'hole' forms in a bilayer, it may be able to pass anions as well as cations. Had we been wise enough to consider this problem, we might well have dismissed it because sensing of anions was then and remains problematic. Despite the difficulty, great progress has been made in anion complexation and recognition during the past decade.[62]

What is certainly known about transport proteins is that they insert into phospholipid bilayers, they permit the selective transport of cations, anions, or molecules, and transport is typically unidirectional (the channels exhibit rectification). At a minimum, we needed to develop a synthetic molecule that could insert in the bilayer without completely disrupting it, form a stable pore, and transport a biologically relevant ion that we could detect quantitatively.

3.1. Flexibility as a design philosophy

The complexation and transport of cations by macrocyclic polyethers were well known by the time we began our efforts in the synthetic channel area.[63] It was apparent that one could enhance complexation strength by 10-fold by increasing the free energy of binding by less than 1.5 kcal/mol. The problem with channel design is that although strong binding may afford selectivity (as in cryptands), this may be counterproductive in a system that requires ion flow of 10^7 or 10^8 ions per second. Indeed, biological activity is a dynamic phenomenon in which great stability is, in some senses, a dead end. If the hydrogen bond network of DNA was infinitely stable, how could reproduction take place?

There was another, somewhat simpler consideration with respect to a flexible design. Obviously, we did not know how a channel functioned. We would make the most informed guesses possible but these might be wrong. If the latter case, a rigid system was likely to compound the error. A structurally flexible system, on the other hand, might be able to adjust enough to function. As soon as a functional model system is in hand, structure activity studies can be undertaken. If no activity can be coaxed from the model system, it will probably be unclear where to go next.

A peripheral observation is that the risk of failure must play a role in synthetic design. An elaborate and elegant synthesis of a potential model system that has no activity is a costly failure. If a model with at least some function can quickly be brought to hand, the synthesis can be optimized as the activity evolves. An active model system is clearly worth the investment of additional synthetic effort whereas a non-functional model is not.

3.2. The essential elements for a synthetic channel compound

Although the absolute prerequisites for channel function were obscure at the outset of our effort, several requirements were obvious.[64] First, the channel would have to be amphiphilic, like the membrane monomers in which it would reside. Second, some sort of headgroup and/or entry portal was necessary. If the same chemical element could serve both requisites, economy would clearly be enhanced. Third, we felt that some kind of polar central unit would be required at or near the midplane of the bilayer. The membrane is least polar at its midplane and the energetic cost of traversing a 30–35 Å, nonpolar barrier must surely be significant. The latter structural feature was based upon chemical intuition because no such structure was known at that time to exist in protein channels. Eventually, of course, the exact analogue of this design feature was revealed in the structure of the KcsA channel of *Streptomyces lividans*.[24]

We decided that diaza-18-crown-6 would serve as our channel's headgroup. The ability of crown ethers to serve as amphiphile headgroups was established from the work of Kuwamura,[65–68] Okahara,[69] and from our own studies.[70,71] The ability of these compounds to complex cations was established as was their dynamic behavior. We anticipated that if a cation passed through a macrocycle headgroup, some selectivity for one cation over another would be observed.

The basic design for our cation channel is shown in Figure 4. The top panel shows how the structure was originally envisioned. The two distal macrocycles were expected to function both as headgroups in the amphiphilic sense and as 'selectivity filters.' It was unclear to us exactly how the known complexation constants for diaza-18-crown-6 would relate to selectivity in transport. Channel function is a process that must be dynamic to be successful. Such strong cation binding compounds as cryptands are poor prospects for channel elements and, indeed, have proved to be pH dependent and only modestly successful carriers.[72,73]

The central macrocycle was envisioned to align parallel to the bilayer leaflet (as would the distal macrocyclic headgroups). It was thought that cations would pass through the former, providing a polar interaction at the midplane of the bilayer. We anticipated that this would reduce the energy, and therefore enhance the efficacy, of

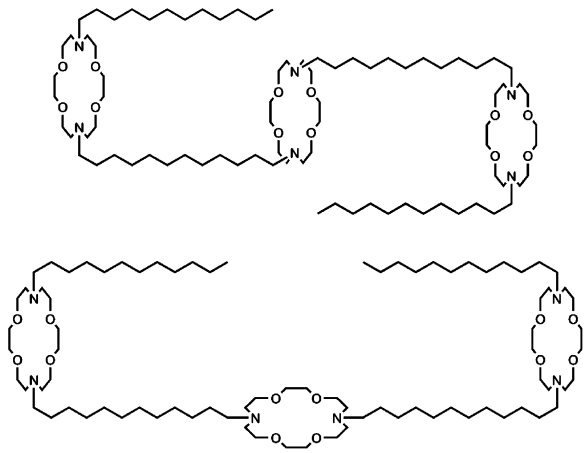


Figure. 4. The first tris(macrocyclic) cation channel shown as originally conceived (top) and in the experimentally confirmed operating conformation.

transport. This has recently been demonstrated to be the case for the KcsA potassium selective protein channel. In a sense, this design notion was foolish within the overall context of a flexible channel.[74] Transport of a cation that involves its passage through a third macrocycle must surely be slower than if the cation passed by it. The stabilization might be lower in the latter case but the dynamics should be enhanced.

It is here that our 'flexible framework' approach paid off. Nature and the cations took the path of least resistance to transport across the bilayer. The actual conformation adopted by the channel is shown in the lower panel of Figure 4. The central macrocycle lay perpendicular to the bilayer plane and parallel to the lipid chains. This had two consequences. First, it presumably made the transport faster because the cation did not pass through the central macrocycle. Second, this conformation extended the channel's overall length. The latter was important because we had apparently underestimated the membrane's distance requirements.

3.3. Cation channel activity

A molecule is not a channel just because it was designed to be one or because it may look to a chemist as if it should be. The verification of channel function requires that two criteria be met. First, transport of the species of interest must be demonstrated. Second, there should be some confirmation that the transport actually occurs as a result of the channel mechanism rather than that the molecule functions as a carrier or in some other fashion.

3.4. Construction of the tris(macrocycle) channels: 'Channel 1'

The first target in the cation channel family was the tris(macrocycle) pictured in Figure 4. For convenience in writing these relatively complex structures, we have adopted a shorthand notation in which we represent macrocycles as units framed by angle brackets.[75] Thus, 15-crown-5 is represented by ⟨15⟩ and 4,13-diaza-18-crown-6 by ⟨N18N⟩. Using this shorthand, channel **1** becomes $C_{12}\langle N18N\rangle C_{12}\langle N18N\rangle C_{12}\langle N18N\rangle C_{12}$.

An important challenge faced in the preparation of this structure concerns symmetry. The molecule was designed to be symmetrical so that insertion of either end of the molecule into the bilayer would be effective. Channel 1 is symmetrical about the central macrocycle and nearly symmetrical about the two distal macrocycles. It was therefore necessary to design the synthesis so that the same macrocycle could be substituted the same way (central crown) or differently (distal crowns).[76] The successful synthetic sequence is shown in Scheme 1. It is interesting to note that rather vigorous alkylation conditions were required. Potassium iodide is used to exchange the starting bromide to increase reactivity and the first reaction is conducted at the reflux temperature of butyronitrile (~115 °C). In the first step, some dialkylated macrocycle is obtained as a by-product.

3.5. Measuring ion transport activity

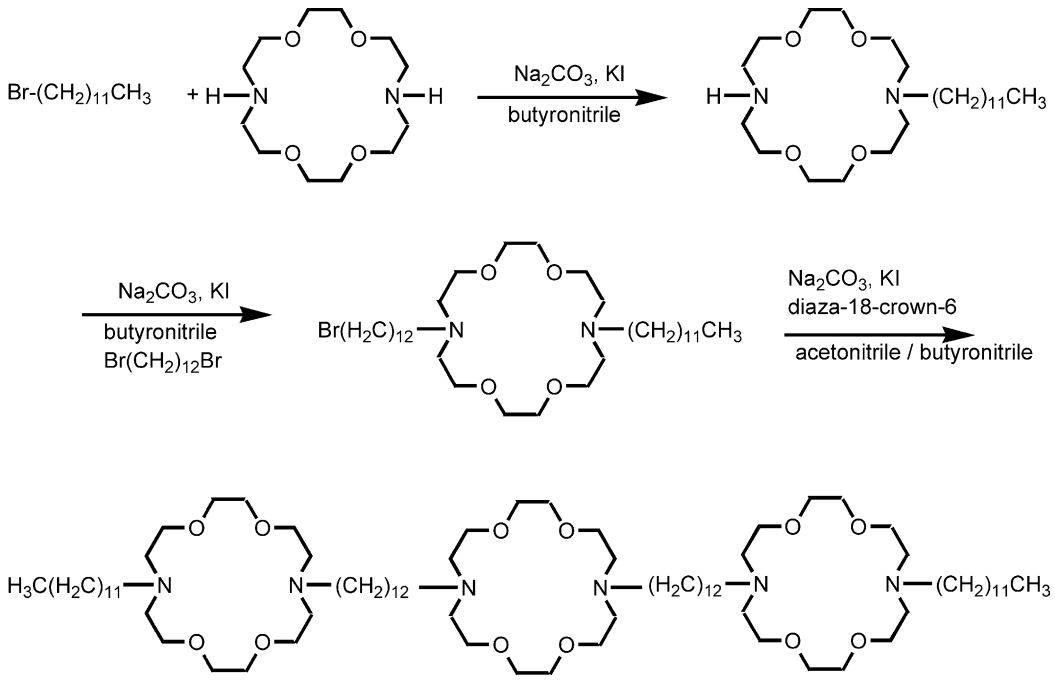
We have assayed the activity of the tris(macrocyclic) channel formers by various methods. Our earliest studies measured proton transport by following changes in a fluorescent dye.[77] Other studies used the electrophysiologists' planar bilayer conductance technique.[78,79] Most of our studies, however, used a ^{23}Na-NMR method mentioned above.[80] In this method, phospholipid liposomes (vesicles) are created in the presence of 200–400 mM NaCl. As expected, NaCl is trapped within the vesicles and is also present in the same concentration in the bulk aqueous phase surrounding them. NMR detects one ^{23}Na signal. Addition of Dy^{3+} causes the signal of the bulk Na^+ to shift. Addition of a channel-former allows exchange of the sodium cation. From the observed linewidths, an exchange constant can be calculated.

Working in the range 1–20 μM of pore-former added, we determined the exchange constant for channel **1** to be about 30% of that determined for the channel-forming peptide gramicidin (parallel experiments). A number of other compounds in this family have been studied and their transport rates generally correlate with expectations based upon their structures.

3.6. Control experiments

The gratifying observation that ion transport occurred in the presence of tris(macrocyclic) channels was necessary but not sufficient evidence of channel formation. Several important control experiments were undertaken to rule out other possible mechanisms.[77]

One such experiment was to replace the crown-ether-based ionophores with the detergents sodium dodecyl sulfate or Triton X-100. Both of these molecules are capable of inserting in the bilayer and could, in principle, foster defect formation and ion transport. Sodium-23 NMR experiments similar to those described above were conducted with these two detergents. An important difference was that concentrations of the detergents were as high as 200 μM compared to a maximum of



Scheme 1.

only 20 μM of the tris(macrocycle)s. In no case did membrane disruption occur. Moreover, no ion transport was observed.

Calculation of octanol–water partition coefficients for the family of tris(macrocycles) showed that they preferred the membrane over water by at least a billionfold.[81] An experiment that assayed for carrier-mediated ion transport was done by using a conventional concentric tube apparatus in which the two membrane layers were water and chloroform. Sodium transport by ten different compounds was studied by using the bulk membrane system. Transport rates measured in liposomes were determined for the same group of structures. A comparison of the transport rates showed no correlation between the two groups. This rules out the carrier mechanism for the channel-forming compounds although it does not unequivocally confirm the channel mechanism.

3.7. The channel's conformation

We sought to determine whether or not the central macrocycle was parallel to the distal macrocycles. When we reduced the size of the middle macrocycle to 15 or 12 members, the transport rate was diminished but efficacy was not lost. Likewise, replacement of diaza-18-crown-6 by an $O(CH_2CH_2O)_3$ chain did not prevent sodium transport. Based on these data, we concluded that the central macrocycle was parallel to the lipid axis rather than to the two distal macrocyclic headgroups. This conformation is illustrated in the lower panel of Figure 4.

3.8. The position of the channels within the bilayer

We found that dansyl residues could replace the dodecyl side chains of Channel 1 without significant loss of transport activity. The fluorescent dansyl group could serve as a probe of position and environment by the application of several different types of experiments.[82] The dansyl channel may be abbreviated as $Dn\langle N18N\rangle C_{12}\langle N18N\rangle C_{12}\langle N18N\rangle Dn$.

The first step was to determine the intensity and wavelength (λ_{max}) maxima for the dansyl channel in a variety of solvents as well as in phospholipid vesicles. A comparison of fluorescence maxima and dielectric constants gave a straight-line plot. According to these experimental data, the dansyl group in a bilayer experienced an environment intermediate in polarity between ethanol and methanol. This is a range of dielectric constants (ε) of about 20–40. The dielectric constant of a hydrocarbon is typically below 10 so the dansyl group is clearly not in the bilayer's insulator regime. The dielectric constant of water is about 80 so the dansyl group does not protrude into the bulk aqueous phase. We conclude that the dansyl 'headgroups' are in the midpolar regime rather than in the hydrocarbon slab or penetrating the aqueous phase.

Doxyl groups that contain unpaired electrons can be attached at specific positions on a phospholipid's fatty acyl chain. The radical quenches fluorescence. By using lipids that have doxyl groups in different positions, the distance between them and the fluorescent dansyl group can be triangulated. This fluorescence depth quenching experiment reported that the dansyl groups were 14 Å from the midplane of the bilayer. If the conformation is an extended one, the headgroups will be as shown in Figure 3 and about 30 Å apart. Again, this does not prove the channel mechanism but it is consistent with Figure 4.

We prepared an additional channel that had a fluorescent headgroup. The compound represented in short-

hand in which MeIn represents an *N*-methylindolyl group attached at its 3-position to the distal macrocycle: $MeInCH_2CH_2<N18N>C_{12}<N18N>C_{12}<N18N>C_{12}<N18N>CH_2CH_2InMe$. The indolyl channel may be excited at about 280 nm and emits near 340 nm. This is approximately the wavelength at which the dansyl channel absorbs. By using mixtures of the dansyl and indolyl channels, fluorescence resonance energy transfer (FRET) permits a determination of the aggregation state. For these two channels, the aggregation state was determined to be 1.1.

3.9. Other experimental confirmation

A close relative of the methylindolyl channel described above was also prepared and studied. It has the structure $InCH_2CH_2<N18N>C_{12}<N18N>C_{12}<N18N>CH_2CH_2In$. In this compound, indole's NH is not methylated. Infrared studies confirmed that an intramolecular hydrogen bond had formed in this compound. Monte Carlo computational studies and an analysis of molecular models suggested that indole could form a hydrogen bond across the face of the distal macrocycle. In contrast to the methylindolyl channel, the indole channel was inactive as a transporter.[83] We inferred that the distal macrocycles were blocked by the H-bonded indole residue.

Channel activity was high when the headgroups attached to the distal macrocycles were benzyl: $PhCH_2<N18N>C_{12}<N18N>C_{12}>N18N>CH_2Ph$. If cations passed through the distal macrocycles, we anticipated that the transport process should be affected by substituent electronic effects. Thus, the Na^+ transport rates for three benzyl channels were compared. These were *p*-nitrobenzyl, *p*-methoxybenzyl, and benzyl. The nitro group is electron withdrawing and the methoxy group is electron donating. Although the Hammett plot obtained by comparing the transport rates contained only three compounds, it showed a negative slope and a good correlation coefficient. The negative slope was expected for an interaction with a cation.[80]

Shrinking the ring size of the distal macrocycles was also expected to reduce the ion transport rates. Sodium cation is about 2 Å in diameter and can pass through the hole in the 18-membered ring macrocycle. It can also pass through the hole of a 15-membered ring crown but the fit is much closer. Two channels having fluorobenzyl headgroups were prepared. In one case, the distal macrocycles were 15-membered and in the other, 18-membered. When the ^{23}Na NMR method was used to assay cation transport, the 15-membered ring channel was found to be about 60% as active as the compound that had 18-membered distal macrocycles.[84] These data suggest that the cation passes through the distal macrocycles.

3.10. Planar bilayer conductance

A planar bilayer may be formed in a (typically $\sim$100 μm) hole in a Teflon plate that separates two chambers. A solution of the phospholipid membrane monomers is painted over the hole. The phospholipids organize into a membrane in this hole. The membrane is called a black lipid membrane or simply a bilayer lipid membrane. We have previously reported data for a variety of cation channels using such a system.[79] The amplitudes and time courses vary, of course, but they are generally similar to that shown in Figure 2 above.

3.11. Conclusions concerning cation channels

We have outlined here the studies undertaken in our laboratory to design, prepare, and characterize artificial channels. Our family of working cation channels, which we call 'hydraphiles,' has also demonstrated significant biological activity. They are active against *Escherichia coli*[85] and are under study in other organisms. We continue to explore new methods to assess transport efficacy that may permit us to directly compare cation and anion transport. Our development of synthetic anion-conducting channels is discussed below.

4. Anchored peptides as anion channels

If alkali metal cations are featureless, positively charged spheres, then halide anions are the negatively charged equivalents. Anions and their complexation behavior have been studied intensively in recent years[62] but much remains elusive about them. One simple question that persists is the hydrated diameter of chloride. The size is obviously critical if transport through any pore or chloride channel is to be achieved.[86] Chloride hydration has been studied in water, methanol, and DMF.[87–89] A recent computational study has placed the hydrated diameter of Cl^- at 6.48 Å.[5] The latter compares with chloride's crystallographic diameter of 3.62 Å.

An important modern challenge for the chemist is to understand and mimic anion complexation. A further venture is to achieve and understand anion transport.[90] In a sense, the most daunting task is to develop a synthetic anion channel when the structure of the ClC family of proteins is barely outlined.[85] We undertook the summons to develop de novo an anion channel.

C-Peptide,[91] a peptide having about 30 residues depending on the species from which it is isolated, transports chloride anion through bilayer membranes.[92] A model of the structure suggests that the central proline residue imparts a bend in the overall chain. Indeed, the proline is present in all of the species in which the C-peptides are active chloride transporters but absent in the pig sequence. A homology study shows that leucine replaces proline at this central position and that chloride transport by the porcine peptide is low. This information, coupled with the general observation that the motif GKxGPxxH is conserved in the anion pathway of all members of the ClC family of chloride protein channels,[93,94] suggested a potentially active headgroup for an anchored peptide channel model.

Our general strategy was to use a heptapeptide possessing a membrane anchor. The proline noted above

would occupy the fourth (middle) position. Molecular models of a xxxPxxx chain suggested that the heptapeptide would be bent. Two such L-shaped peptides could form a parallelogram 6–8 Å across. If the heptapeptide served as the channel headgroup, the opening formed by a dimer would be similar in size to the hydrated diameter of a chloride ion.

4.1. Synthetic access to membrane-anchored peptides

It was decided that a hydrophobic anchor, compatible with a phospholipid bilayer, would be required. Twin hydrophobic chains were desired and the connector between them and the heptapeptide was required to fulfill two criteria. First, the connector should have polar elements similar to those in the glyceryl ester midpolar regime of a typical phospholipid bilayer. Second, we knew from previous experience that synthetic subunits and preparative approaches must be both modular and economical.[95]

It was decided that the twin anchor chains of a typical phospholipid bilayer would be mimicked by and constructed from dialkylamines, R_2NH. The amine would be treated with diglycolic anhydride to form $R_2NCOCH_2OCH_2COOH$, abbreviated R_2[DGA]-OH. The latter could be coupled with the heptapeptide to give the membrane-insertable monomer unit. The sequence is shown in Scheme 2. The twin-tailed acid is obtained by heating the amine and anhydride in the absence of solvent above their melting points. A convenient procedure for the preparation of $(C_{18}H_{37})_2NCOCH_2OCH_2COOH$ (or 18_2[DGA]-OH) involves heating the dialkylamine and anhydride in toluene for 48 h. The crude product is obtained by evaporation of the solvent; the product is purified by a single crystallization.[7] Owing to practical considerations, the heptapeptide was completed after addition of a first fragment to the anchor. Notwithstanding, the compound was assembled with reasonable speed and economy.

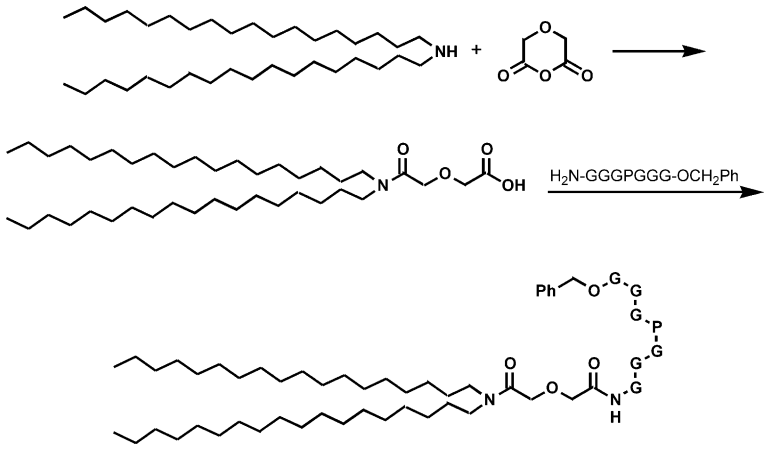
4.2. Confirmation of ion transport

Our goal at the outset of this project was to synthesize molecules that had anion selective, channel-like activity similar to that identified in eukaryotic cells. It seemed reasonable that, at a minimum, a successful compound would need to transport ions across the membrane by an electromotive diffusion mechanism. Such a mechanism must be rapid and would presumably be based upon a water pathway traversing the membrane. Our view excluded mobile carriers and assumed that a successful compound would remain in the membrane and enable the transmembrane diffusion of ions across this barrier.

Although small molecules and gasses do diffuse through bilayers, ions and water (and perhaps even gasses) are also transported by pore-forming proteins.[61] After more than 100 years of study, we are only beginning to understand three-dimensional structures and the chemical mechanisms of pore formation and transport. For channels that form dynamically from specific monomers, diffusion may play a greater role in the overall transport phenomenon. Dynamic membrane partitioning by, and oligomerization of, channel-forming monomers may occur simultaneously with pore-mediated transport. This physical complexity will require more complex mechanistic paradigms to be understood completely.[96–99]

Several methods were used to assay ion release. One involves direct measurement of chloride in the external solution surrounding phospholipid vesicles. This is done with a chloride selective electrode. The second method uses the fluorescent dye carboxyfluorescein (CF), which is encapsulated in the vesicles during formation. When CF is concentrated within a vesicle, it self-quenches and its fluorescence is weak. Addition of a pore-forming agent permits release of CF into the bulk phase. At the lower concentrations present in the aqueous phase, fluorescence can readily be detected. Experiments have also been done using the planar bilayer conductance method but we use this less extensively as it is both complex and time-consuming.

4.2.1. Ion specific electrode studies to establish anion selectivity. Our first evidence for chloride ion transport was obtained in phospholipid bilayer vesicles using 18_2[DGA]-GGGPGGG-OCH_2Ph, a SCMTR. We fol-



Scheme 2.

lowed the pore-forming activity of SCMTR using unilamellar liposomes (200 nm diameter). The vesicles contained 200 mM KCl and the surrounding medium was 200 mM KNO_3 buffer. If the SCMTR pore is selective for anions over cations, the electrogenic release of chloride should result in a positive membrane potential inside the vesicle, which would slow the anion's release.

Direct observation of open-close behavior obtained by planar bilayer conductance studies would give a plot such as shown in Figure 2. Planar bilayer clamp measurements are complicated and tedious to perform. We thus resorted to the use of an ion selective electrode method to obtain concentration dependent ion release data. Because this method is different from a patch clamp or planar bilayer conductance experiment, we anticipated that a plot similar in shape to that shown in Figure 1 would be obtained. The kinetics would reflect the rate of channel formation in the membrane rather than actual ion release. The release of Cl^- from phospholipid vesicles is shown in Figure 5. The most important observation is that chloride release is detected at sub-millimolar concentrations. Additionally, the chloride release rate is clearly dependent on the concentration of pore-former.

In the presence of nitrate as the external ion, chloride rapidly exited the liposomes.[7] The kinetics of chloride release reflect the rate of pore activation rather than the kinetics of chloride efflux per se,[97] which are very fast. When permeant nitrate was replaced by impermeant sulfate, the SCMTR-mediated chloride release was slowed 10-fold. The release of chloride in the absence of SCMTR but the presence of nitrate occurs with a half-time $\leq$100 ms.[100] We infer that the SCMTR-induced permeability is selective for chloride over both potassium and sulfate.[8] The strong SCMTR concentration dependence apparent in Figure 5 suggests that the activated pore involves more than one SCMTR monomer.

4.2.2. Confirmation of pore formation by carboxyfluorescein release. The ion specific electrode determination of pore activation is an important direct measure of macroscopic ion release but it has a limited sensitivity and a slow response time. Pore formation was thus confirmed by a study using the fluorescent dye carboxyfluorescein (CF, see Fig. 6).[97,101,102] A model, produced by molecular mechanics calculations, of the dye is shown in Figure 7. Liposomes were prepared in the presence of CF. The dye was removed from the external medium by dialysis. Addition of the anchored heptapeptide leads to immediate CF release as assayed by the appearance of fluorescence. Concentration dependent release of CF is shown in Figure 6. The results from these studies support those obtained by using the ion specific electrodes, shown above in Figure 5.

4.2.3. SCMTR pores in planar lipid bilayers. Our most detailed analysis of the SCMTR channels has been obtained in planar phospholipid bilayers. These studies are sensitive at the level of single active pores and the time resolution is at the sub-millisecond level. We have employed planar lipid bilayers to study ion transport by SCMTR channels.[7,9] Only permeant ions can be studied; an impermeant ion will affect neither membrane currents nor membrane potential.

When the concentrations of ions are identical on both sides of a bilayer, there is no ion gradient. When there is an ion gradient, the observed current–voltage (I–V) relationship is not linear and crosses the abscissa at a point offset from 0 by an amount that reflects the difference in ion permeabilities. In voltage clamp studies, this offset can be used to compare ion selectivities. The data we obtained show that SCMTR is at least 10-fold selective for Cl^- over K^+. The SCMTR-dependent current is stable and reflects the transport of 1,000,000 Cl^- per second per SCMTR molecule. This level of transport is consistent with a pore 6 Å in diameter.[6] Further studies showed that SCMTR exhibited voltage dependent gating (not shown).[7] This is a phenomenon

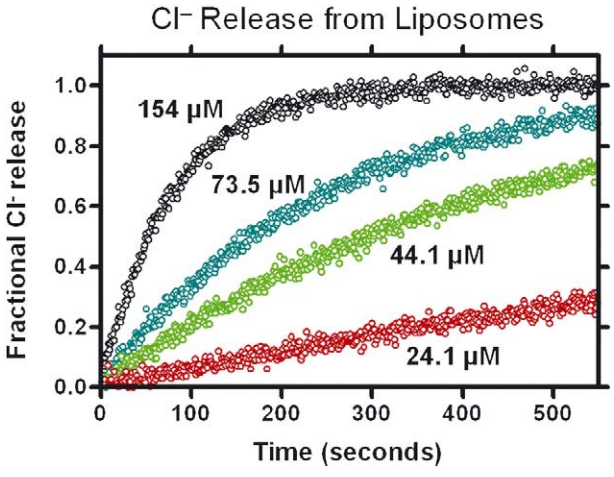


Figure 5. Chloride release from liposomes mediated by 18_2[DGA]-GGGPGGG-OCH_2Ph, 'SCMTR.'.

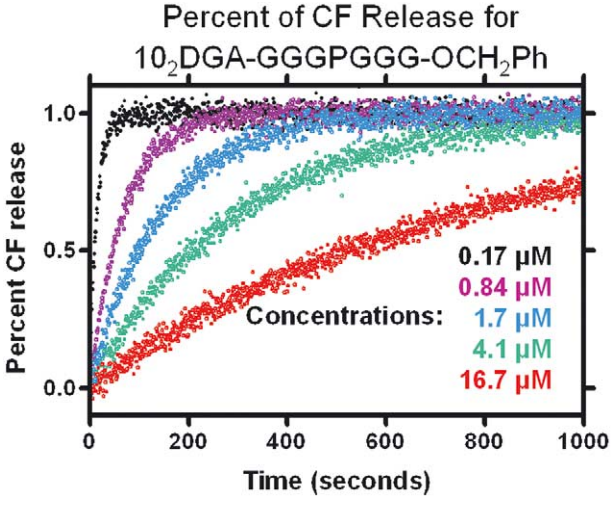


Figure 6. Release of entrapped carboxyfluorescein (CF) dye from liposomes, mediated by 10_2[DGA]-GGGPGGG-OCH_2Ph.

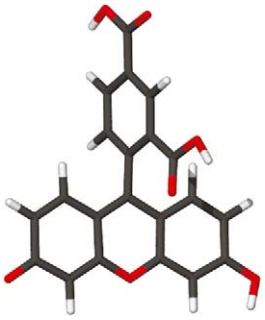
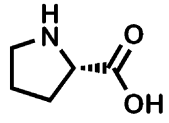


Figure 7. Molecular model of carboxyfluorescein in the tube representation.

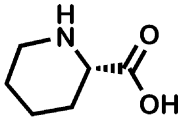
that we did not expect to observe in such a simple molecular structure.

4.3. Variations in the proline residue

Simple, modular synthetic channel models permit us to ask pointed questions about how changes in specific elements affect overall channel function. A striking example of this was found in the seemingly minor change from proline to pipecolic acid. Proline is a 5-membered ring amino acid. Pipecolic acid differs only in the ring size: six-members rather than five.[9]



Pyrrolidine-2-carboxylic acid
Proline



Piperidine-2-carboxylic acid
Pipecolic acid

Admittedly, proline and pipecolic acid are not identical; they differ in several respects. The presence of an additional carbon in pipecolic acid compared to proline means that the former is more hydrophobic and the N–C–CO angles change slightly in proline from ~112.5 to ~110.5° in pipecolic acid. The two compounds, 18_2[DGA]-GGG-Pro-GGG-OCH_2Ph and 18_2[DGA]-GGG-Pip-GGG-OCH_2Ph have molecular weights of 1168 and 1182 Da, respectively. This difference is only about 1% and is not significant in terms of hydrophobicity.[9]

The carboxyfluorescein (CF) dequenching method has been used to assay the difference between 18_2[DGA]-GGG-Pro-GGG-OCH_2Ph and 18_2[DGA]-GGG-Pip-GGG-OCH_2Ph. We surveyed the activity of these two compounds over a range of concentrations and found that 18_2[DGA]-GGG-Pro-GGG-OCH_2Ph was 10–20-fold more active than 18_2[DGA]-GGG-Pip-GGG-OCH_2Ph at similar concentrations.[9]

4.4. Differences in activity when the twin anchor chains are 10 or 18 carbon atoms

Our original model for the anion channel monomer involved a heptapeptide anchored in the bilayer by two 18-carbon chains. We thus varied the anchor chains from 18 to 10 carbons to assess the effect of this alteration on channel efficacy. Again we assayed channel function by carboxyfluorescein release from liposomes. We compared 18_2[DGA]-GGGPGGG-OCH_2Ph with 10_2[DGA]-GGGPGGG-OCH_2Ph. This is a significant alteration to the anchor but the presumed channel headgroup is unaltered. The rates of CF release were assessed at several concentrations. When the CF release rate was the same, the difference in activity was equal to the difference in concentration. Identical rates were found when the concentration of 10_2[DGA]-GGGPGGG-OCH_2Ph was 0.167 mM compared to 63.3 mM for 18_2[DGA]-GGGPGGG-OCH_2Ph. This converts to a transport rate difference of ~400–fold.[9]

5. Conclusions

Although the SCMTR family of compounds was designed to exhibit channel-like activity, we have been surprised by the efficacy of these compounds. In addition, the subtlety and sophisticated properties of these apparently simple compounds has proved to be remarkable. The formation of an anion selective channel with properties of voltage gating is more than we predicted for the first synthetic example. Notwithstanding the properties already observed, we still have significant work before us to understand the chemical mechanisms that account for these properties.

Acknowledgements

The cation channel work described here was supported by the NIH under GM-36262 (to G.W.G.). This grant supported the early phases of the chloride channel studies, now supported separately by GM 63190 (to G.W.G. and P.H.S.). We are grateful to the NIH for support of these projects.

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