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C07219. Synthetic, Biologically Active Amphiphilic Peptides

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Abstract

Amphiphilic peptides typically consist of a peptide portion that may be 5–25 (or more) amino acids in length. The hydrophobic portion may be a single fatty acid residue, but can also be more elaborate. The main focus of this article lies on the family of synthetic anion binders (SATs) of the general structure $(R^1)_2N-COCH_2OCH_2CO-(Aaa)_n-OR^3$. The most-common R^1 groups are octadecyl ($C_{18}H_{37}$). The most studied peptide sequence in this family is $(Gly)_3-Pro-(Gly)_3$, although different sequences (and longer and shorter peptides) have been prepared as well. The C-terminal ester residue providing the most effective anion release from liposomes is heptyl (C_7H_{15}), although many others have been examined. The compound $(C_{18}H_{37})_2N-COCH_2OCH_2CO-(Gly)_3-Pro-(Gly)_3-OBn$ (Bn=benzyl) was found to mediate Cl^- transport in mouse epithelial cells.

Introduction

The protein channels that serve *inter alia* as ion, water, and pressure regulators are complex proteins that must certainly have evolved from far simpler structures. The study of channel proteins is clearly important and has been approached by scientists working in the disciplines of biochemistry, biophysics, cell biology, and neurochemistry, among others. This broad range of studies is of the greatest importance to our understanding of modern channel proteins and their function. After a half-century of intensive study, significant structural and phenomenological advances have been made in understanding channel proteins. Summary descriptions of these great advances may be found in the monographs by Hille [1], Aidley and Stanfield [2], or Stein [3], among others.

There are two issues that deserve consideration at the outset. First, modern channels certainly evolved from simpler structures that served as regulators. It seems reasonable to think that early channels were less efficient, less selective, and perhaps less durable than modern proteins. They must have functioned, however, or the remarkable proteins found in modern organisms could not have evolved from them. The second point is the other side of this coin. The design and preparation of a channel model system is actually an attempt to achieve transport function by means of a simple, low-molecular-weight structure that possesses the elements currently thought to be required. In our 'hydraphile-channel' design [4], we developed a membrane-insertable cation transporter that, as we now by know, meets the structural and functional criteria that were not recognized prior to the appearance of the KcsA channel structure [5]. The inclusion of a 'central relay' as a hydraphile-design feature was required by proper chemical understanding of distance and charge issues. It was unknown at the time the hydraphiles were designed that nature had evolved a similar module.

Amino acid sequences for channel proteins have been known for some time, but the first crystal structure of a (cation) channel protein appeared only in 1998 [5]. Additional structural information then rapidly emerged [6] [7]. Understanding the three-dimensional (3D) arrangement of a channel protein is of profound importance, but undertaking structural variations designed to probe function on natural channel proteins remains a daunting challenge.

Even today, our understanding of the chemical mechanisms that control ‘specificity’ is incomplete. The selectivity of a cation-conducting channel for Na^+ over K^+ , or *vice versa*, is of obvious importance, but the question of why a protein conducts either cations or anions remains. One hypothesis is that anion-conducting channels possess positive charges at one or more locations in their structure and that cation channels are endowed with negative charges [8]. Such speculation is certainly not unreasonable, however, to date, the experimental evidence remains limited. From a chemical perspective, it seems unreasonable that a highly charged structure could transport an oppositely charged species with reasonable throughput - *i.e.*, complexation rather than transport would be the dominant process. The cryptands, *e.g.*, are excellent alkalimetal-complexing agents that selectively encapsulate a cation of appropriate size [9]. They are, however, poor cation transporters because their cation-release rates are extremely low, even though they are neutral host molecules. The same concern presents itself with respect to channels: would a strong interaction, such as one between unlike charges, permit ion passage or would the ion be bound at the charged or strong donor site?

Natural Peptides as Models for Protein-Channel Function

Many in the biological community have accepted the value of model channels. Such channel-forming peptides as gramicidin [10], alamethicin [11], melittin [12], and others have been extensively studied in the hope that these ‘simplified proteins’ would yield functional information.

Gramicidin is an interesting example. It certainly forms robust, cation-conducting pores in bilayer membranes. It is a pentadecapeptide that dimerizes into a channel structure [13], and has been the subject of *ca.* 7,000 reports during the last 30 years. Were it not already a part of the landscape, would it be considered a good channel model? It is utterly unlike the KcsA channel. It is *not* formed from α -helices, but rather forms a π -helix. Its polar residues are not localized to a central ‘capsule’ as in the KcsA channel; rather the gramicidin pore is lined with main-chain amide donors. The negatively charged head groups of the KcsA channel are completely lacking in this accepted channel model. Gramicidin is composed of a mixture of D- and L-amino acids. Protein channels, however, comprise only L-amino acids. The KcsA potassium channel organizes a collection of α -helices that form the outer walls of the pore. Cations pass among the α -helices of the KcsA channel. Gramicidin forms a coil that probably is organized in an end-to-end arrangement. Cations pass through the inside of this coil and may interact directly with main-chain C=O groups, rather than with the amino acid side chains. Like the KcsA channel, however, gramicidin’s position in the bilayer is stabilized by a series of tryptophan residues [14]. Finally, even though gramicidin has so extensively been studied and is so well-accepted as a channel model, it has recently been the subject of a controversy concerning its functional structure [15].

In part because of the extensive studies undertaken on gramicidin, peptide channels have gained a greater credibility during the past decade and, accordingly, have been more extensively studied [16] than any synthetic system. Gramicidin has the great advantage that it is a naturally occurring material and, therefore, possesses an inherent legitimacy. It has the further advantage of robust function, but this function does not really correspond to what is known about protein channels. Notwithstanding these issues, the thousands of studies on gramicidin have certainly advanced our understanding of channel phenomena.

Ions that Occur *in vivo* and Their Transport

Both the cytosol and periplasm are rich in ions of various types. These include metallic or inorganic cations and anions, organic cations and anions, and ionized cellular building blocks such as amino acids, ATP, and proteins. The most-common metallic cations are sodium (Na^+) and potassium (K^+). Both calcium (Ca^{2+}) and magnesium (Mg^{2+}) cations are also present, but they are found at much lower concentrations. The concentrations of Na^+ and K^+ are regulated such that $[\text{K}^+]$ is typically 150 mM within the cell, and *ca.* 10 mM outside. The reverse is true for Na^+ , which is present within the cell at concentrations near 10 mM, and outside at *ca.* 150 mM.

The predominant anions *in vivo* are chloride (Cl^-), bicarbonate (HCO_3^-), inorganic phosphate ($\text{H}_2\text{PO}_4^- - / \text{HPO}_4^{2-}$), ionized amino acids, proteins with negative side chains, and ATP. The extracellular concentration of Cl^- is often *ca.* 110 mM. Unlike Na^+ and K^+ , for which there is a large electrochemical gradient across the bilayer, Cl^- distribution is more subtly related to cellular function. The activation of Cl^- channels at the plasma membrane normally does not significantly change either the membrane potential or the intracellular concentrations of Cl^- . Consequently, the many known chloride transporters [17] are not associated with signal transduction, but rather involve the regulation of cellular processes.

Biomedical Importance of Chloride Channels

The biomedical importance of chloride channels became apparent when studies on the pathogenesis of cystic fibrosis demonstrated that CFTR, the cystic fibrosis transport regulator, is a Cl^- channel as well as a regulator of other transport systems. Mutations in this protein that give rise to cystic fibrosis, the most common fatal genetic disease of Caucasian humans. More recently, *myotonia congenita*, *Dents'* disease, and *Bartters'* syndrome have been shown to be serious disorders involving chloride-channel dysfunction [18]. In addition, the potential for disrupted intracellular vesicular function, as indicated above, is an area that is just beginning to be understood. There is nothing to be gained from contrasting the importance of cation vs. anion channels. In the end, charge balance must be maintained and both must be important. Our goal must be to understand each particular instance to answer biomedical questions.

The Cl^- distribution across membranes is presently an area of intense interest, and of some controversy. In many cells, E_{Cl} approximates E_{M} , so that Cl^- distribution appears predominantly passive. Thus, the opening of Cl^- channels generally stabilizes the membrane potential [19] [20]. One example is the importance of excessive epithelial salt secretion in cystic fibrosis. It has long been known that chloride permeability and the cystic fibrosis transport receptor are related [21]. The transport of Cl^- is prominent in a variety of processes that are fundamental to all cellular activity, including pH and volume regulation [22][23]. The potential importance of these channels in cellular biology are already broad, and are just beginning to be appreciated in detail.

Synthetic-Channel Models

A number of channel model systems have been developed over the past two decades. These can generally, but not exclusively, be divided into peptide and nonpeptide model systems. Examples of synthetic peptides include the so-called Asynporins', developed by *Montal* and co-workers [24]. *Mutter* and co-workers [25] [26] used so-called 'template-assembled synthetic proteins' (TASPs), which are expected to associate to a four-helix bundle in a globular conformation that forms ion channels in lipid bilayers. *DeGrado* and co-workers [27] developed 'Ser-Leu' peptides that were shown to exhibit cation-transport properties remarkably similar to those found in proteins. In particular, they prepared model peptides

containing only Leu and Ser residues. A 21-residue peptide, $\text{H}_2\text{N}-(\text{Leu-Ser-Ser-Leu-Leu-Ser-Leu})_3-\text{CONH}_2$ was reported to form ion channels, with ion-permeability and lifetime (opening and closing) characteristics resembling the acetylcholine receptor. This work has been reviewed by Åkerfeldt *et al.* [28].

D,L -Cyclic-Peptide Nanotubes

Ghadiri and co-workers [29] reported channel formation from self-assembling peptide ‘nanotubes’. Cyclic peptides incorporating alternating D - and L -amino acids such as *cyclo*[(Trp- D -Leu) $_3$ Gln- D -Leu] were prepared. When planar, the diameter of the internal orifice is 7.5 Å. The stacking of these cyclopeptides is envisioned as involving H-bond formation similar to those found in a β pleated sheet. The electric conductances of Na^+ and K^+ chlorides were found to be 60 ± 5 pS. These nanotube channels have now been shown to function as antibiotics [30]. The latter observation clearly enhances the value of model systems.

Non-Peptide Channel Models

We have recently reviewed the development of channel models [31] [32], so only a brief recounting of the history will be presented here. The earliest non-peptidic, synthetic organic channel model was reported by Tabushi, Kuroda, and Yokota [33]. In this effort, β -cyclodextrin was used as a ‘head group’ in concert with hydrocarbon tails that comprised the channel’s ‘tunnel’ or pore element. Menger and co-workers [34] prepared $\text{Me}(\text{CH}_2)_{10}\text{COO}(\text{CH}_2\text{CH}_2\text{O})_5\text{CH}_2\text{Ph}$ and demonstrated that this compound gives rise to a more effective proton (H^+) transport than was observed for gramicidin under the same conditions, when using the method of Kano and Fendler [35]. Kobuke and co-workers [36], using a mixture of glycolate ethers of monoalkyl-oligo(1,4-butylene glycol) complexed with the dioctadecyl(dimethyl) ammonium cation, reported Na^+ and K^+ transport in a phospholipid bilayer. Regen and co-workers [37] esterified a hydroxylated androst-5-ene derivative at positions 3β and 17β with $\text{HOOC}(\text{CH}_2\text{CH}_2\text{O})_n\text{H}$, and demonstrated that the resulting oligomeric ethyleneglycol-modified steroid acts as a modest Na^+ transporter. Kobuke and Nagatani [38] recently reported a channel based on cholic acid that transports cations, as judged by electrophysiological methods.

An interesting family of molecular structures that conduct cations has been reported by Matile and co-workers [39]. Molecules in this family have been referred to variously as ‘rigid rods’ and ‘p-slides’. Ionophoretic activity has been demonstrated in phospholipid membranes by means of a valinomycin-coupled transport system.

Crown Ethers as Structural Elements in Synthetic Channels

Crown ethers have played a prominent role in the development of channel models. Nolte and co-workers [40] obtained an oligomer of a crown-isonitrile that appeared to be tunnel-like and exhibited Co^{2+} transport. 21-Crown-7 ethers comprised the ‘tunnel’ through which alkali-metal cations pass in membrane-active compounds, as reported by Voyer and co-workers [41]. The macrocycles were attached to a peptide inspired by DeGrado, *i.e.*, $\text{Leu}-(\text{CrF-Leu-Leu-Leu-CrF-Leu})_3$, which is known to adopt an α -helical conformation¹⁾. Crowns derived from tartaric acid residues were the basis of two systems developed almost simultaneously in the groups of Lehn and Fyles. Jullien and Lehn and co-workers [42] used a central, tartaric acid-derived crown to anchor radiating dendrimer-like chains. Fyles and co-workers [43] built an amphotericin-inspired tunnel using a similar crown central unit with bolaamphiphilic walls.

¹⁾CrF represents a crown-ether-substituted phenylalanine (F, Phe).

The same group ultimately produced a variety of ion transporters, and assessed their efficacy by means of an H⁺-transport system [44].

The efforts made in our laboratory [4] have led to a family of compounds that insert in bilayer membranes and transport cations at a rate competitive with some protein channels. The significance of these crown-ether-containing cation channels is that the functional, synthetic channel is modular and simple. Because of this, we may be able to isolate functions and to probe chemical issues directly within the bilayer. The synthetic-channel structures developed thus far have molecular weights up to 2,000 Da, but accomplish some of the functions of natural protein channels having much higher molecular weights and structural complexity.

A Semi-Synthetic Peptide that Functions as a Chloride-Conducting Channel

Tomich and co-workers [26] [45] have modified the 23-residue sequence of the M2 segment of the glycine-gated Cl⁻ channel. The sequence is H₂N-PARVG-LGITT-VLTMT-TQSSG-SRA-COOH. They have extended this sequence by the addition of four Lys (K) residues at the C-terminus, the resulting sequence being H₂N-PARVG-LGITT-VLTMT-TQSSG-SRAKK-KK-COOH. They have given this peptide the abbreviated name C-K₄-M2GlyR for the C-terminally modified tetralysyl M2 glycine receptor. It is important to note that *Tomich* and co-workers have demonstrated *i*) chloride-channel activity, *ii*) enhanced solubility by dint of the tetralysyl terminus, and *iii*) increased capacity for epithelial anion secretion when studied in *Madin-Darby* canine-kidney and T84 cell monolayers. These are important findings, demonstrating the potential of chloride-channel models in therapeutic applications.

Biological Activity of Synthetic Cation Channels

Several families of compounds, originally designed as cation-conducting ion channels, were also explored as antibiotics. The first such example was reported by *Fernandez-Lopez* and *Ghadiri* and their co-workers [30]. *Voyer* and co-workers [46], as noted above, developed a family of compounds in which crown ethers were appended to α -helical peptide chains. These 'crown peptides' did not exhibit activity against *Gram*-positive bacteria, but proved to be cytotoxic to breast cancer (MDA) cells and to mouse leukemia (P388) cells.

The hydrophile channels developed in our laboratory were extensively characterized by their transport efficacy in liposomes [4]. In previous work with the synthetic cation-conducting channels called 'hydraphiles', we explored their antibiotic activity in *Escherichia coli*, *Bacillus subtilis*, *Saccharomyces cerevisiae*, *Cryptococcus neoformans*, *Pseudomonas aeruginosa*, and human embryonic kidney (HEK) cells, among others [47]. We have also noted antibacterial effects in lipophilic lariat ethers and used membrane fluorescence depolarization (with the DiSC₃(5) dye) to explore the underlying mechanism [48].

The great interest in amphiphilic peptides and in antibiotic compounds shows that this is a significant area for exploration. The development of novel antibiotics is unquestionably an important current goal, but developing simple compounds that can exploit certain biological functions is as great a challenge.

Biological Activity of Amphiphilic Peptides

Numerous peptides isolated from a variety of sources have proved to exhibit antibiotic activity. This field has been described generally in recent review articles [49]. In addition, *Tossi* has created a website (<http://www.bbcm.univ.trieste.it/~tossi/>) listing antibiotic peptides, which can be accessed *via* the Antimicrobial Sequences Database (termed 'AMSDb' on the website). There is also a nascent message board available called Synthetic Antimicrobial Peptides Database (SAPD; see <http://oma.terkko.helsinki.fi:8080/~SAPD/>). The existence of these

resources serves to emphasize the growing importance of this area of research. The need for novel antibiotic compounds can hardly be overstated [50].

Several approaches have been taken in this area. One is to conjugate a peptide of known biological activity with a hydrophobic residue such as a fatty acid [51]. Alternately, an antibacterial peptide may be fused to a peptide sequence having known membrane affinity. Several examples of amphiphilic peptides have been prepared and studied by the groups of *Tirrell* and *Fields* [52]-[54].

There has been considerable recent interest in developing novel antibiotics by these 'hybrid' approaches, and there has also been invested much effort in understanding how the combinations of segments cooperate to achieve efficacy [55]. Indeed, it has recently been shown that certain peptides exhibit new antibacterial activity upon lipidation [51c, d].

Synthetic Anion Transporters (SATs)

The amphiphilic peptides developed in our laboratory have the general structure $(R^1)_2N-COCH_2OCH_2CO-(Xxx)_7-OR^3$ (**A**), as shown in Fig. 1. We may consider the basic molecular structure of **A** as consisting of four subunits: *i*) the N-terminal anchor, *ii*) the connector chain, *iii*) the actual peptide, and *iv*) the C-terminal ('secondary') anchor. A typical representative is peptide **1**, which is shown together with two other amphiphilic peptides, **2** and **3**. The connector unit in **1** was designed to mimic the midpolar regime of phospholipids (*i.e.*, the glyceryl ester fragment). Although we have undertaken some variations in the 'connector chain' subunit, the studies are currently incomplete and unpublished.

The peptide chain in **1** and most of the studied analogues have seven amino acid residues ($-NH-CHR^2-CO-$). Further, the peptide chain was generally $(Gly)_3-Pro(Gly)_3$. Shorter and longer chains have also been studied (see below) [56], as have sequences in which Gly and/or Pro were replaced with other residues. The C-terminal residue, originally and frequently a benzyl (Bn) ester, was also varied extensively. The effect of structural changes was assayed by recording the release of Cl^- or the carboxyfluorescein anion from phospholipid vesicles. To a more limited extent, planar-bilayer voltage-clamping experiments were used to characterize ion transport.

Carboxyfluores

Initial, Successful Model System

It is possible to design and synthesize many potentially mimetic systems, but the real challenge is to achieve at least some level of the desired function. Our initial success with anion transporters [57] was realized with the molecule $(C_{18}H_{37})_2NCOCH_2OCH_2CO-(Gly)_3-Pro-(Gly)_3-OBn$. The twin C_{18} chains were intended to mimic the hydrocarbon tails of fatty acids in phospholipids. As noted above, the $COCH_2OCH_2CO$ connector chain was designed to mimic the portion of the phospholipid glyceryl chain ('midpolar regime'). A survey of sequences known for the CIC family of chloride-transporting channels [58] and for the C-peptide [59] suggested that a G-X-X-P sequence might be successful for achieving anion transport. The simplest, symmetrical system of this type is G_3PG_3 . Finally, the Bn group was used at the C-terminus because a free COOH group was expected to confer too much polarity upon the system.

Characterization of Anion Release from Liposomes

We have used planar-bilayer voltage-clamp [58], ion-selective-electrode (ISE) [60], and fluorescence methods [61] to characterize the ion transport by synthetic anion transporters (SATs). Release from liposomes mediated by the ionophore may be monitored in a variety of ways. When the ISE method is used with Cl^- , vesicles are prepared so that Cl^- is present in the liposomes, but not in the surrounding buffer. When the ionophore is added, an increase in Cl^- concentration is detected as an electrical response. Ideally, this response is concentration-dependent, as shown in Fig. 2, a for $(C_{18}H_{37})_2NCOCH_2OCH_2CO-(Gly)_3-Pro-(Gly)_3-OBn$.

In the planar-bilayer experiment, the ionophore is introduced to a membrane separating two aqueous ion reservoirs. A voltage is applied, and the response of the membrane, normally an insulator, is recorded. Channel activity is apparent in an open/closed response. The channel (or pore) is either open or closed and the openings are uniform, reflecting a uniform open size. When the channel is closed, there is no ion flux. This response is shown in Fig. 2, b for $(C_{18}H_{37})_2NCOCH_2OCH_2CO-(Gly)_3-Pro-(Gly)_3-OBn$.

Ion-transport efficacy was originally assayed by measuring Cl^- release from phospholipid vesicles. In the presence of $(C_{18}H_{37})_2NCOCH_2OCH_2CO-(Gly)_3-X-(Gly)_3-OBn$ ($X = Pro$) at concentrations of 24-154 μM , the anion was released in a concentration-dependent fashion; as a control, we also tested the analogue with $X = Leu$. The central Pro residue appears to be important in the ion-conducting peptide sequence. Thus, transport of Cl^- by $(C_{18}H_{37})_2NCOCH_2OCH_2CO-(Gly)_3-Pro-(Gly)_3-OBn$ (at 154 μM) was almost identical to that observed for peptide **2** (at 24 μM). Leu and Pro have a similar number of C-atoms, but Pro is a cyclic and Leu is an open-chain amino acid. It is important to note that $(C_{18}H_{37})_2NCOCH_2OCH_2CO-(Gly)_3-OBn$ failed to transport anions [63].

Planar-bilayer voltage-clamp experiments confirmed the Cl^- transport by $(C_{18}H_{37})_2NCOCH_2OCH_2CO-(Gly)_3-Pro-(Gly)_3-OBn$ [62], the open/closed behavior of this transporter being shown in Fig. 2, b. A remarkable and unexpected observation was that the open/closed frequency changed with the applied voltage. This voltage-dependent gating is a property not anticipated for such simple amphiphilic peptides. Analysis of the planar-bilayer clamp data (current (I) vs. voltage (V) curve) led to the conclusion that the pore has a more than tenfold selectivity for Cl^- over K^+ .

Variations in the N-terminal anchor chains

The dual hydrocarbon chains R^1 in peptides of type **A** (Fig. 1), comprising the N-terminus of $(R^1)_2N-COCH_2OCH_2CO-(Gly)_3-Pro-(Gly)_3-OBn$, were identical in all cases, but were varied in length from methyl (CH_3) to octadecyl ($C_{18}H_{37}$) [62]. Anion release was monitored either

by means of ISE (Cl^-) or by fluorescence methods (carboxyfluorescein). Release of an anionic dye such as carboxyfluorescein may be monitored by fluorescence methods. When liposomes are loaded with the dye, the high concentration causes self-quenching. When the ionophore mediates the passage of the dye through the bilayer, the dye concentration in the external medium will be much lower than in the interior of the liposome. Quenching will no longer occur, and the fluorescence will be proportional to the release of the dye ('fluorescence quenching').

The data obtained with the above two methods differed, but so did the anions under study and the analytical methods. Still, similar trends were observed for both anions. The data for the release of Cl^- were reported previously [60]. Fig. 3, thus, shows the release of carboxyfluorescein for the different SAT compounds, R^1 ranging from C_8H_{17} to $\text{C}_{18}\text{H}_{37}$. The most-active compounds in the series are those having medium chain lengths. Subsequent studies revealed that the greater activity of $(\text{C}_{10}\text{H}_{21})_2\text{N-CO-CH}_2\text{OCH}_2\text{CO-(Gly)}_3\text{-Pro-(Gly)}_3\text{-OBn}$ compared to $(\text{C}_{18}\text{H}_{37})_2\text{N-COCH}_2\text{OCH}_2\text{CO-(Gly)}_3\text{-Pro-(Gly)}_3\text{-OBn}$ resulted, at least in part, from a loss of selectivity [61].

Anchoring Effect of the C-Terminal Residue

In the initial design, the C-terminal ester was intended to avoid deprotonation of the COOH group and to reduce polarity. As above, a family of SAT molecules was prepared having the general structure $(\text{C}_{18}\text{H}_{37})_2\text{NCOCH}_2\text{OCH}_2\text{CO-(Gly)}_3\text{-Pro-(Gly)}_3\text{-OR}^3$, the alkyl group R^3 of the ester moiety including ethyl (Et), isopropyl (i-Pr), heptyl (C_7H_{15}), cyclohexylmethyl ($\text{CH}_2\text{C}_6\text{H}_{11}$), decyl ($\text{C}_{10}\text{H}_{21}$), and octadecyl ($\text{C}_{18}\text{H}_{37}$). The ester residue was replaced in six cases, R^1 being octadecyl, and YR^3 being NR^4 (see Fig. 1); the C-terminal amides (NR^4) were $\text{N}[(\text{CH}_2)_6\text{Me}]_2$, $\text{NH}(\text{CH}_2)_9\text{Me}$, $\text{N}[(\text{CH}_2)_9\text{Me}]_2$, $\text{NH}(\text{CH}_2)_{17}\text{Me}$, and $\text{N}[(\text{CH}_2)_{17}\text{Me}]_2$. The corresponding fractional-chloride-release data are shown in Fig. 4 for 200-nm phospholipid vesicles at lipid and ionophore concentrations of 0.20 mM and 65 mM, respectively.

The curves in Fig. 4 may be placed in three groups. The top trace refers to the heptyl ester, which gave the highest rate of Cl^- release. The next two lines (from top to bottom) are nearly superimposed, resulting from Cl^- release by $(\text{C}_{18}\text{H}_{37})_2\text{N-COCH}_2\text{OCH}_2\text{CO-(Gly)}_3\text{-Pro-(Gly)}_3\text{-OR}^3$ compounds in which the esters are cyclohexylmethyl and decyl. The three esters that exhibit the poorest ion-release rates (three bottom curves) were the corresponding Et, i-Pr, and octadecyl esters, respectively [62]. We surmise that a long C-terminal chain has an affinity for the *N*-alkyl chains and constricts the pore. Smaller esters play little anchoring role because they do not penetrate deeply enough into the membrane. A heptyl (C_7H_{15}) moiety seems to strike a useful balance. In any event, it is clear that the C-terminal residue plays an anchoring function.

Studies of Peptide-Sequence Variation

We had found in our initial studies [62] that Cl^- release from liposomes diminished sixfold when the 4th amino acid (Aaa) in $(\text{C}_{10}\text{H}_{21})_2\text{NCOCH}_2\text{OCH}_2\text{CO-(Gly)}_3\text{-Aaa-(Gly)}_3\text{-OBn}$ was Leu rather than Pro (Fig. 5). A dramatic loss of activity was also observed when pipecolic acid replaced Pro, even though the two compounds are nearly identical in size, shape, and polarity [62]. We surmise that the differences in activity relate to the different conformations adopted by the two triglycine chains when the central amino acid differs. We further presume that Leu provides a less-organized framework for the triglycine chains, owing to its more flexible, open-chain structure.

Covalent Linkage of Synthetic Anion Transporters

Several lines of evidence suggested that the peptides of type **A** (Fig. 1) function at least as dimers to transport anions through a phospholipid bilayer. *Hill* plots showed a slope of *ca.* 2, suggesting a minimum of a dimeric aggregation state. Dextran-block experiments [63] suggested that the pore is *ca.* 7-8 Å in diameter, consistent with a dimeric structure, as determined by molecular modeling. If the pore is dimeric indeed, then covalent linkage of two molecules of **A** should produce a compound having the activity similar to twice the concentration of the peptide. To test this hypothesis, we, thus, prepared the covalently linked pseudo-dimeric compounds **4** and **5** [64].

The monomers had to be linked in these pseudo-dimers such that compounds that were otherwise identical to two monomers were simply not possible. Thus, there are minor structural differences between **4** and **5** beyond the linkage position. For example, the COCH₂OCH₂CO linkage was converted to COCH₂NHCH₂CO in **4** so that an ethylene chain would connect the two monomers. Likewise, construction of the C-linked dimer **5** required replacement of the C-terminal ester groups by an ethylenediamine linkage.

A comparison was made of the ability of **4** and **5** to release Cl⁻, the concentrations of the dimers being adjusted to compensate for the 'covalent pre-organization'. The fractional Cl⁻ release for either the dimer **6** or **7** was greater than that observed for twice the concentration of (C₁₈H₃₇)₂NCOCH₂OCH₂CO-(Gly)₃-Pro-(Gly)₃-OBn under identical conditions.

Variations in Peptide Chain Length

Nearly all of the studies conducted thus far in this system involved heptapeptides. A limited study was conducted to determine the effect of incorporating shorter or longer sequences. In addition, the peptide sequence G₃PG₃ has been rearranged to see if the placement of Pro within the chain alters the efficacy of Cl⁻ transport [56]. In short, the five internal sequences explored were G₂PG₂, G₃PG₃, G₂PG₄, G₄PG₂, and G₄PG₄. The fractional Cl⁻ release from liposomes mediated by the first three compounds was within experimental error of each other. When the heptapeptide sequence was rearranged from G₃PG₃ to G₂PG₄, a somewhat higher release rate was observed. The largest difference, however, was observed when the G₃PG₃ chain was extended to the nonapeptide sequence G₄PG₄. As noted, these results were obtained in a limited study, and further exploration is currently in progress.

Charged Amino Acids in the Peptide Sequence

It has been proposed that a glutamic acid residue (Glu¹⁴⁸), present in the putative ion conduction pathway of the CIC protein, may play a role in mediating the passage of Cl⁻ [8]. The amphiphilic heptapeptides also form a Cl⁻ conducting pathway. Replacement of a Gly residue with a Glu would place a negative charge within the conducting pore. Based simply on electrostatic considerations, the transport of Cl⁻ should be diminished by the presence of another negative charge.

Several amphiphilic peptides derived from $(C_{18}H_{37})_2N-COCH_2OCH_2CO-(Gly)_3-Pro-(Gly)_3-O(CH_2)_6Me$ were prepared. The peptide sequence GGGPGGG was altered to GGGPEGG, GGGPGE, or GGGPGGE to test whether there would be an effect on Cl^- transport from vesicles [65]. In short, Cl^- release from liposomes was impeded when a Glu residue was present at any of the positions on the C-terminal side of proline; but the effect was minimal when the Glu side chain was esterified to remove the negative charge. At physiological pH, the Glu $COOH$ side-chain moiety is ionized, its negative charge being within the peptides formed by aggregation of amphiphilic monomers. An interesting observation is that the greatest transport retardation was found for GGGPGGE. The reason for this positional difference remains unclear.

Chloride Binding by Amphiphilic Heptapeptides

To establish that the synthetic anion transporters directly interact with Cl^- , we conducted detailed NMR studies in CDCl_3 [66]. The first step in this study required the preparation of six amphiphilic, labeled peptides of the form $(\text{C}_3\text{H}_7)_2\text{N-COCH}_2\text{OCH}_2\text{CO-(Gly)}_3\text{-Pro-(Gly)}_3\text{-OBn}$, each differing in the replacement of two H-atoms of a Gly residue with deuterium (D) atoms. The NMR spectrum of each deuterated compound was compared with that of the parent compound, and individual NMR shifts were unequivocally assigned. Finally, association constants (K_a) were determined by titrating $(\text{C}_3\text{H}_7)_2\text{N-COCH}_2\text{OCH}_2\text{CO-(Gly)}_3\text{-Pro-(Gly)}_3\text{-OBn}$ (host) with Bu_4NCl (guest).

Three key findings emerged from this work. First, peptides of the form $\text{R}_2\text{N-COCH}_2\text{OCH}_2\text{CO-(Gly)}_3\text{-Pro-(Gly)}_3\text{-OR'}$ bound Bu_4NCl in CDCl_3 with K_a $1,800 \text{ M}^{-1}$. This value is nearly the same as that reported for the much-studied *Crabtree* triarene [67]. Second, the magnitude of Cl^- complexation was found to depend significantly upon the counter cation. Thus, the binding constant for Cl^- was $1,800 \text{ M}^{-1}$ when the counter ion was Bu_4N^+ , but ca. $16,000 \text{ M}^{-1}$ when the cation was Ph_3P^+ (substrate: $\text{Ph}_3\text{P}^+ \text{Cl}^-$). This difference of nearly an order of magnitude suggests significant interactions between the host and the guest.

The above host-guest interactions were studied by additional NMR experiments [68]. The results of these 2D-NMR and computational studies showed that the amide H-atoms of the 5th and 7th amino acid residues (GGGPGGG) are the key donors for Cl^- complexation. A computational model placed the Bu_4N^+ ion adjacent to the peptide- Cl^- complex. At present, however, it remains unknown what (if any) role such cations as Na^+ and K^+ play in the transport of Cl^- through the self-assembled pores formed by amphiphilic peptides.

Biological Studies

As noted above, extensive studies were undertaken, showing that the hydrophile cation channels are cytotoxic. Encouraged by these results, similar studies were undertaken with $(\text{C}_{18}\text{H}_{37})_2\text{N-COCH}_2\text{OCH}_2\text{CO-(Gly)}_3\text{-Pro-(Gly)}_3\text{-OBn}$. No toxicity to *E. coli* was observed, and the results of these studies are unreported. Other possible biological applications for these compounds have been successfully explored, however.

An obvious potential application for synthetic anion transporters is to produce a pharmaceutical drug against cystic fibrosis [69]. This hereditary disease affects one in 2,500 humans of Northern-European descent. The maintenance of ion homeostasis in multicellular organisms requires vectorial ion transport across the cell layers in lung epithelia. The regulation of anion concentrations and transport is critical in the function of epithelial layers. We, thus, explored the effect of $(\text{C}_{18}\text{H}_{37})_2\text{N-COCH}_2\text{OCH}_2\text{CO-(Gly)}_3\text{-Pro-(Gly)}_3\text{-OBn}$ on epithelial cell layers [70] by use of an *Ussing* chamber [71]. This study resulted in the first example in which Cl^- transport was stimulated by a synthetic, amphiphilic peptide in mouse trachea epithelial cells. Similar results were not observed when identical experiments were undertaken with $(\text{C}_{18}\text{H}_{37})_2\text{N-COCH}_2\text{OCH}_2\text{CO-(Gly)}_3\text{-Leu-(Gly)}_3\text{-OBn}$. It appears that a new pathway for Cl^- transport has been established by the above Pro-containing amphiphilic heptapeptide. The fact that this peptidic compound has a molecular weight of ca. 1,200 D might militate against its utility *in vivo*. Still, introduction into lungs could be effected by a simple inhaler such as used to administer steroids. In any event, the critical finding is that these compounds alter Cl^- transport in mammalian cells.

Conclusions

Amphiphilic peptides comprise a potentially vast family of compounds that could exhibit a broad range of biological activities. The synthetic anion transporters that are the focus of this

article form pores that conduct ions. The individual peptides recognize and complex Cl^- ions. Indeed, they mediate Cl^- transport in vital epithelial cells. Although the study of amphiphilic peptides is still in its infancy, these compounds offer a vast potential as host molecules, complexing agents, transporters, and drugs.

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Fig. 1. Structures of selected amphiphilic peptides

A shows the general design principle of an amphiphilic peptide that functions as a synthetic anion transporter. Compound **1** is an RDG-based cell-adhesion peptide. Compound **2** is a self-assembling amphiphilic peptide. Compound **3** is known as the C18-SC4 peptide.

Fig. 2. Experimental data for Cl^- release mediated by the synthetic peptide $(\text{C}_{18}\text{H}_{37})_2\text{N}-\text{COCH}_2\text{OCH}_2\text{CO}-(\text{Gly})_3-\text{Pro}-(\text{Gly})_3-\text{OCH}_2\text{Ph}$
a) Fractional Cl^- release measured with the aid of ion-selective electrodes; b) planar-bilayer-clamp recording.

Fig. 3. Carboxyfluorescein release from liposomes mediated by different synthetic peptides of the type $(R^1)_2N-COCH_2OCH_2CO-(Gly)_3-Pro-(Gly)_3-OBn$ as a function of time. The corresponding R^1 substituents are indicated beneath each curve.

Fig. 4. Fractional Cl^- release for compounds of the type $(\text{C}_{18}\text{H}_{37})_2\text{N-COCH}_2\text{OCH}_2\text{CO-(Gly)}_3\text{-Pro-(Gly)}_3\text{-OR}^3$

For R^3 , see text. Conditions: phospholipid vesicles: 200 nm; [lipid]=0.20 mM; [ionophore]=65 μM .

Fig. 5. Amino acid (Aaa) residues incorporated in the synthetic peptide $(C_{10}H_{21})_2NCOCH_2OCH_2CO-(Gly)_3-Aaa-(Gly)_3-OBn$