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A hydrocarbon anchored peptide that forms a chloride-selective channel in liposomes†

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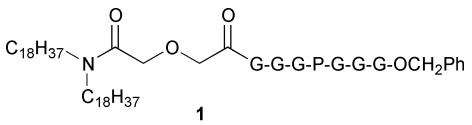
The heptapeptide sequence Gly-Gly-Gly-Pro-Gly-Gly-Gly, when anchored to diglycolic acid derived $(C_{18}H_{37})_2NCOCH_2OCH_2COOH$, forms chloride-selective ion channels in phospholipid liposomes but the related heptapeptide Gly-Gly-Gly-Leu-Gly-Gly-Gly, and tripeptide Gly-Gly-Gly do not.

The transport of ions through phospholipid bilayers is mediated by a variety of channels. Recent solid state studies of potassium,[1,2] sodium,[3] mechano-sensitive,[4] and water channels[5] have greatly advanced our understanding.[6] Until this year, no structural evidence had appeared that would correspondingly aid our comprehension of transmembrane chloride channels (ClC).[7] Even the remarkable solid state structure of the ClC channel raises nearly as many questions as it resolves because it is so inherently complex. Naturally occurring peptides such as alamethicin,[8] melittin,[9] gramicidin[10] and a number of synthetic organic models have been developed to mimic cation channel function.[11] No organic chemical model of chloride channel function has yet appeared although an anion-selective analogue of the channel-forming peptide alamethicin[12] has been reported. Tomich and coworkers have developed a chloride-selective peptide by modifying a known glycine-gated Cl-channel peptide.[13]

The challenge to develop a functional, synthetic chloride channel is great, especially considering the dearth of structural information available on which to base a model. One possibility was to modify our hydraphile cation channel model[14] compounds in accord with the electrostatic analyses of Dawson and coworkers.[15] Instead, we chose to devise a novel model system consisting of three parts. A twin-tailed amine would serve as the equivalent of the phospholipid's fatty acyl chains. Diglycolic acid, $HOCOCH_2OCH_2COOH$ would connect the hydrophobic residues to the headgroup and approximate the phospholipid's midpolar (acyl glycerol) regime.[16] The overall length of the 'anchor' or phospholipid mimic would be determined by the alkyl chains attached to the acid, *i.e.*, R in R_2NCOCH_2O-CH_2COOH.

We considered using such previously incorporated 'portal elements' as crown ethers and cyclodextrins but ultimately chose a different approach. It is known that proline plays a critical role in the chloride selectivity of naturally occurring chloride transporters.[17] We further noted that all members of the ClC family of chloride protein channels contain the conserved motif GKxGPxxH in the putative anion pathway.[18] It is known that substitution of a proline into the intrinsic channel selectivity filter of nicotinic acetylcholine receptors reverses the ion selectivity.[19] Proline may form a 'hinge-bend' regime (GxxP)[20] or it may induce a surface 'kink' in membrane transport proteins.[21] Finally, proline is at the apex of the helix–loop–helix motif in C-peptide and this arrangement is required for ion channel activity.[22]

We therefore set as our target $(C_{18}H_{37})_2NCOCH_2OCH_2CO$-G-G-G-P-G-G-G-$OCH_2Ph$, **1**. Diglycolic anhydride was heated at reflux with dioctadecylamine in toluene for 48 h. The monoamide $(C_{18}H_{37})_2NCOCH_2OCH_2COOH$ ([18]$_2$DGA-OH) was obtained in 87% yield after crystallization from $CHCl_3$ (mp 81–82 °C). The acid, [18]$_2$DGA-OH, was coupled to $TsOH{\cdot}H_2N$-Gly-Gly-Gly-OCH_2Ph ($Me_2N(CH_2)_3N{=}C{=}NEt$ (EDCI), Et_3N, CH_2Cl_2, 0–25 °C, 30 h) to afford **3**. Hydrogenolysis of **3** (H_2, Pd/C, 95% EtOH) afforded [18]$_2$DGA-G-G-G-OH (96%, mp 163–164 °C). Coupling (EDCI, Et_3N, CH_2Cl_2, 0–25 °C, 30 h) of [18]$_2$DGA-G-G-G-OH with either H_2N-L-G-G-G-OCH_2Ph or H_2N-P-G-G-G-OCH_2Ph gave **2** (83%, mp 164–165 °C) or **1** (82%, mp 116–118 °C) respectively.[23]



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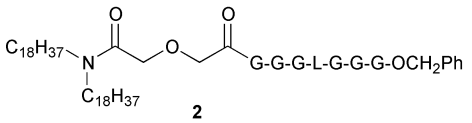

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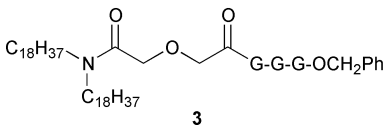

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For the reasons noted above, we hypothesize that proline is critical to the channel forming activity of **1**. Assessing the release of chloride from liposomes mediated by **1**, **2**, and **3** tested this hypothesis. Phospholipid liposomes were prepared in 200 mM KCl. A chloride selective resin electrode[24] was used to measure Cl^- concentration after extravesicular chloride had been chromatographically exchanged for non-interfering nitrate.[25] The data are shown in the two graphs of Fig. 1. The top panel presents data for [18]$_2$DGA-GGGPGGG-OCH_2Ph (**1**, 147 μM) and [18]$_2$DGA-GGGLGGG-OCH_2Ph (**2**, 154 μM). The slight concentration difference results from experimental conditions and is not significant. The bottom panel presents the same data for [18]$_2$DGA-GGGPGGG-OCH_2Ph (**1**, 147 μM) and compares it with [18]$_2$DGA-GGG-OCH_2Ph (**3**, 154 μM). Compound **3**, in which the peptide chain is truncated compared to **1**, showed substantially reduced chloride release. When proline in **1** was replaced by leucine (**2**), chloride release was again greatly reduced (Fig. 1B). We infer from these results that

† Electronic supplementary information (ESI) available: analytical data for **1**, **2** and **3**. See http://www.rsc.org/suppdata/cc/b2/b200126h/

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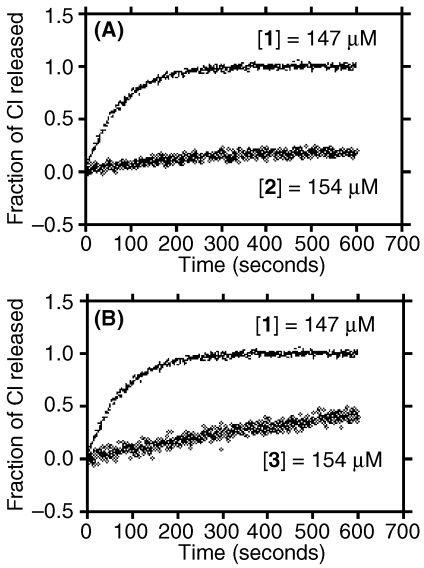

Fig. 1 (A) Chloride release by 147 μM **1** (upper trace) and 154 μM **2**. (B) Chloride release by 147 μM **1** (upper trace) and 154 μM **3**.

the twin hydrophobic tails, in the absence of the peptide, are not sufficient to form a channel. Further, the difference in activity between **1** and **2** suggests a critical function for the kink or 'hinge-bend' provided by proline.[8,26]

We have previously shown that the pore of **1** is at least 10-fold selective for Cl^- over K^+:[27] KCl transport is therefore not possible. For rapid, complete chloride release to occur, the system must remain electroneutral. The external anion must enter the vesicle as chloride exits. Non-interfering NO_3^- or SO_4^{2-} was employed in the extravesicular medium to determine the anion selectivity of **1**. In Fig. 2 and in previous work,[27] we have shown that NO_3^- effectively permeates the pore of **1**, permitting rapid chloride release.

Fig. 2 shows that SO_4^{2-} does not support Cl^- release as well as does NO_3^-. Chloride release must be compensated by another anion and the vesicles are less permeable to SO_4^{2-} than to NO_3^-. Addition of valinomycin increased the release of chloride (see Fig. 2) by allowing K^+ to exit the liposome in concert with Cl^- release mediated by **1**. Taken together, these studies indicate a relative ion permeability order of $Cl^- \sim NO_3^- > SO_4^{2-} \gg K^+$ for **1**. This sequence of relative anion permeabilities indicates that extravesicular monovalent anions are more effective than divalent SO_4^{2-} in supporting Cl^- release. We draw the hopeful inference from this that when **1** is applied to living cells, it will increase permeability to Cl^-, the major physiologic anion, more effectively than it will affect phosphate permeability. This selectivity is critical for use of **1** *in vivo*, which is a long-term goal of this effort.

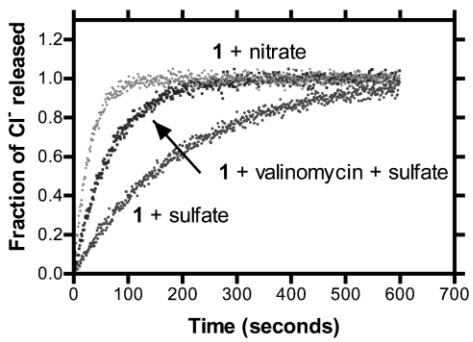

Fig. 2 Fraction of Cl^- released with respect to time by **1** in the presence of NO_3^-, valinomycin, and SO_4^{2-}.

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