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The C-terminal ester of membrane anchored peptide ion channels affects anion transport

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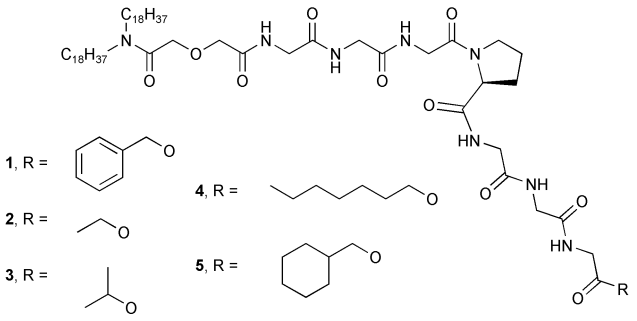
Five heptapeptide derivatives, $[CH_3(CH_2)_{17}]_2NCO$-CH_2OCH_2CO-Gly-Gly-Gly-Pro-Gly-Gly-Gly-OR, in which R = ethyl, 2-propyl, heptyl, benzyl, and cyclohexylmethyl, were found to transport chloride anion through a phospholipid bilayer to varying extents dependent on the identity of R. It was concluded that the R group is a membrane anchor for the synthetic chloride channels.

Modern ion-selective protein channels are remarkable for both their structural complexity and for the subtle regulatory control that they achieve.[1] Despite more than a century of study, mechanistic details in most cases are lacking. This is especially true of chloride-conducting channels, which have been studied much less intensively than have cation channels.[2,3] Using the known selectivity filter sequences of the ClC family channels, we designed an artificial, chloride-selective channel that we have called SCMTR for "synthetic chloride membrane transport receptor."[4] This membrane-anchored heptapeptide showed substantial Cl^-/K^+ selectivity. The first X-ray structure of a chloride channel appeared recently.[5] Notwithstanding, the chemical details of how this protein transports ions remain obscure.[6]

As the number of solid state structures increases, the complexity of channel proteins becomes more obvious, as does the need for simple, functional models.[7,8] This is especially important for chloride channels, which are critical for cellular pH control and osmotic regulation. To our knowledge, only two functional synthetic chloride channels have been reported. Tomich and coworkers reported a modification of a known peptide sequence and engendered chloride secretion from cells.[9–13] Our membrane anchored, synthetic heptapeptide (**1**)[4,14] shows remarkable,[15,16] but anchor-chain-length dependent, ion selectivity.[16] Our design of the latter molecule assumed that a pair of adjacent hydrocarbon chains at the peptide's N-terminus would comprise a single membrane-anchoring element. We show here that the C-terminal ester of the heptapeptide is also critical to ion transport and apparently serves as a secondary membrane anchor.



Compound **1** (SCMTR, "scimitar") was designed to incorporate four critical elements: an anchor, a connector chain, a heptapeptide, and a proline residue within the heptapeptide chain. In the examples illustrated here (**1–5**), dioctadecylamine comprises the membrane-anchoring hydrocarbon chains. These are expected to insert in the bilayer and align with the phospholipid's fatty acyl chains. The connector unit derives from diglycolic acid, which was chosen because it is synthetically manageable and because it approximates the shape and polarity of the acyl glycerol midpolar regime. The heptapeptide sequence shown, Gly-Gly-Gly-Pro-Gly-Gly-Gly, was the simplest peptide that symmetrically incorporated the GxxP motif known in various ClC channel proteins.[17,18] The proline-enforced bend in the peptide chain is also known in ClC channel proteins. What we did not recognize as part of our design was the importance of the C-terminal ester. Our first successful Cl^--transporter had a C-terminal benzyl ester (**1**).[4] We now report that a study of **1–5** reveals that the identity of the C-terminal ester group significantly affects Cl^- transport.

The family of structures studied was prepared from $(C_{18}H_{37})_2NCOCH_2OCH_2CONH$-Gly-Gly-Gly-Pro-OH by coupling with the appropriate tripeptide of the form H_2N-Gly-Gly-Gly-O-R. Compounds **1–5** were all white solids that have the following melting points: **1**, 164–165 °C;[4] **2**, 127–129 °C; **3**, 129–131 °C; **4**, 99–101 °C; and **5**, 100–102 °C. These compounds had proton and carbon NMR spectra and combustion analyses consistent with the assigned structures.

Chloride release from liposomes was assayed directly on ~200 nm phospholipid vesicles, prepared from 1,2-dioleyl-*sn*-glycero-3-phosphocholine (DOPC) and 1,2-dioleyl-*sn*-glycero-3-phosphate monosodium salt (DOPA, 7:3, Avanti Polar Lipids) by using an Accumet Chloride Combination Electrode. Vesicles were prepared in the presence of 600 mM KCl, 10 mM HEPES (pH = 7). The liposomes were extruded and the external solution was exchanged for a chloride-free buffer (400 mM K_2SO_4, 10 mM HEPES, pH = 7). The vesicles were then suspended in the same external buffer (final phospholipid concentration about 0.7 mM), the electrode was introduced, and the system was allowed to equilibrate. The voltage output was recorded, and after 5 min, aliquots of **1–5** (5–9 mM in 2-propanol) were added. A maximum of 20 μL of 2-propanol was added in any experiment in order to avoid damage to the liposomes. At the conclusion of each experiment, 2% aq. Triton X100 (100 μL) was added and the data were normalized to this maximum release value. Data were collected by Axoscope 9.0 using a DigiData 1322A interface. The data obtained by this method for **1–5** are summarized in Fig. 1.

The five anchored heptapeptide derivatives show different chloride release rates. It should be noted that this analytical method reflects the kinetics of insertion and pore formation rather than the open-close dynamics of the resultant channels.[19] The short chain esters **2** (ethyl) and **3** (isopropyl) are measurably less active than is benzyl ester **1**. In planar bilayer conductance analyses, however (data not shown), **2** and **3** fail to transport Cl^- while **1** is effective.[4] The cyclohexylmethyl (**5**) and *n*-heptyl (**4**) esters are far more active as Cl^- transporters. It is interesting to note that the molecular weights of ethyl ester **2**

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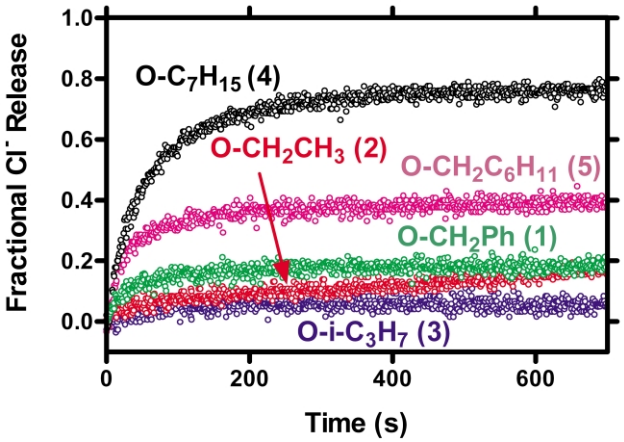

Fig. 1 Chloride release from phospholipid liposomes mediated by **1–5**.

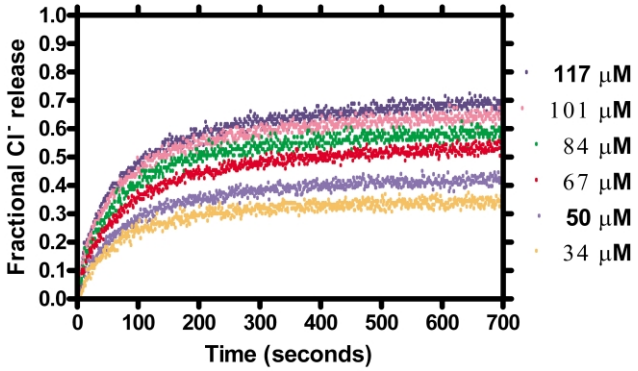

Fig. 2 Concentration dependent chloride release from liposomes mediated by compound **4**.

(1105 Da) and heptyl ester **4** (1176 Da) differ by only 6% and, with the exception of the ester terminus, are identical. The efficacy of **4** suggests that the C-terminus serves as a secondary anchor for the transport receptor. The trends shown in Fig. 1 were confirmed by a similar study of carboxyfluorescein release (data not shown). At the present time, it is not known if this increased activity of longer chain esters is accompanied by any change in transport selectivity. It does seem clear from a comparison of benzyl (**1**) and cyclohexylmethyl (**5**) esters that the benzyl group's aromaticity plays no significant role in the transport.

In order to test whether the benzyl group's aromaticity played any role, compound **5** was synthesized. The essentially isostructural but non-aromatic ester proved to be about twice as active as **1**. It had seemed possible that a cation–pi interaction[20] between the benzyl group's pi-system and a cationic choline headgroup in the phospholipid headgroup might influence the system's organization and efficacy. The experimental evidence fails to support this notion.

In all systems of this type, it is important to confirm that the transporter acts in the expected way. We thus undertook the concentration dependence study of our most active ester, heptyl ester **4**, which is shown in Fig. 2. It was assayed over a nearly 4-fold concentration range from 34 μM to 117 μM. The release curves show well-behaved chloride transport and the fractional ion release increases regularly with increasing concentration.

In previously reported studies, we found that both anchor chain length[16] and a slight change from proline to pipecolic acid[15] significantly altered the behavior of this simple but remarkable channel compound. The present study reveals that the C-terminal ester group is critical as well. We speculate that the ester residue functions as a "secondary" anchor chain and assists either in insertion of the channel or in stabilizing the heptapeptide's active conformation. Studies to determine if the previously established dimer stoichiometry and Cl^-/K^+ selectivity are retained or altered are currently underway and will be reported in due course.

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