Chloride ion efflux from liposomes is controlled by sidechains in a channel-forming heptapeptide

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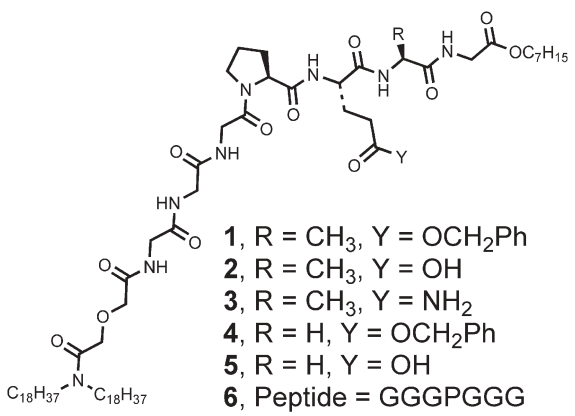
A suite of amphiphilic heptapeptides incorporating a glutamic acid derivative on the *C*-terminal side of a $(Gly)_3Pro$ sequence gives dramatically lower chloride ion release from liposomes when present in free carboxyl form rather than as an ester or amide.

Profound advances have occurred in the ion channel field during the past decade. First, solid state structures of several channels have revolutionized the study of ion transport proteins. These include structures of the mechanosensitive channel,[1] the chloride channel, the voltage gated potassium channel,[2] and the water channel.[3] The latter two of these in part resulted in the award of the Nobel Prize in Chemistry to MacKinnon[4] and Agre.[5] The second advance has been the development of several synthetic model systems that function as channels in bilayers and exhibit many, but not all, of the attributes of natural channel proteins.[6] In the anion-transporting arena, chloride has been the primary focus. It is the most common anion *in vivo* and competes for transport only with such oxygenated anions as nitrate, sulfate, and phosphate.

A solid state structure of the ClC chloride transporting protein[7] and further studies have suggested that a glutamic acid residue (E-148) may play a critical role in gating the chloride channel.[8] It should be noted that very recent work questions whether this protein is a channel or transporter.[9,10] The compound C-peptide is cleaved from proinsulin and forms ion-selective channels in bilayers.[11] C-Peptide is known in many species, including rat, human, horse, and pig. In all of these except pig, there is a conserved GxxP sequence. In rat, the most active chloride transporter, the *C*-terminal side of this sequence contains a glutamic acid, *i.e.*, the heptapeptide is GGGPEAG. The presence of glutamate in an apparently critical site of the ClC protein and its presence in remarkably active C-peptide led us to incorporate glutamate and relatives into our synthetic heptapeptide chloride transporter compounds and to assay their effects on ion flux.[12]

We prepared a suite of heptapeptides of the form $(R^1)_2NCOCH_2OCH_2CONH\text{-}(Aaa)_7\text{-}CO\text{-}R^2$, which effect the release of chloride anions from phospholipid vesicles. The *N*-terminal residue R^1 is typically octadecyl (from dioctadecylamine) but R^1 has been systematically varied from octadecyl (C_{18}) to methyl (C_1). The *C*-terminal residue, R^2, has most often been a benzyl ester but both esters and amides of varying chain lengths have been surveyed.[13] The compounds prepared for this study all had R^1 = octadecyl and $R^2 = O(CH_2)_6CH_3$. All had an *N*-terminal G-G-G-P sequence. The *N*-terminus of each was attached to $(H_{37}C_{18})_2NCOCH_2OCH_2CO\sim$ and the *C*-terminal ester was *n*-heptyl.

The *C*-peptide sequence found in rat ($\sim$GGGPEAG$\sim$), or a derivative thereof, was incorporated into compounds **1–3**. The sequence on the *C*-terminal side of proline in **1–3** was X-A-G-OC_7H_{15} in which X is a glutamic acid derivative. Glutamic acid is incorporated as its benzyl ester (**1**), the free acid (**2**), or its amide (**3**). The *C*-terminal side of proline in **4** and **5** is X-G-G-OC_7H_{15}, in which X is glutamic acid as the benzyl ester (**4**) or the free acid (**5**).



Each compound was prepared by standard wet chemical methods as described in detail in a previous report.[14] The preparation of compound **1** is typical. The tetrapeptide Boc-Pro-Glu(OCH_2Ph)-Ala-Gly-OC_7H_{15} was constructed by (i) esterification of glycine to give glycine heptyl ester tosylate; (ii) coupling $TsOH{\cdot}H_2N\text{-}Gly\text{-}OC_7H_{15}$ with Boc-Ala-OH to afford Boc-Ala-Gly-OC_7H_{15}; (iii) Boc removal followed by coupling with Boc-Glu(OCH_2Ph)-OH to give Boc-Glu(OCH_2Ph)-Ala-Gly-OC_7H_{15}; and (iv) Boc removal and coupling with Boc-Pro-OH to afford Boc-Pro-Glu(OCH_2Ph)-Ala-Gly-OC_7H_{15}. Removal of the Boc group followed by coupling with $(C_{18}H_{37})_2NCOCH_2OCH_2CO\text{-}(Gly)_3\text{-}OH$ gave **1**. Other compounds in this series were prepared analogously. All five heptapeptides were solids that had the expected analytical properties. Their melting points are as follows: **1**, 112–114 °C; **2**, $\sim$ 126 °C (hygroscopic); **3**, 178–180 °C; **4**, 120–122 °C; and **5**, $\sim$ 103 °C (hygroscopic).

The efficacy of chloride release from liposomes was assayed by using $\sim$ 200 nm phospholipid vesicles, prepared from 1,2-dioleoyl-*sn*-glycero-3-phosphocholine (DOPC) and 1,2-dioleoyl-*sn*-glycero-3-phosphate monosodium salt (DOPA, 7 : 3, Avanti Polar Lipids)

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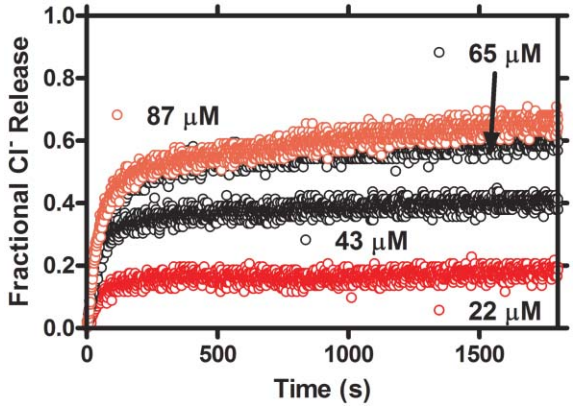

Fig. 1 Concentration dependent chloride release from liposomes mediated by compound **3** at pH = 7.0.

and an Accumet Chloride Combination Electrode (pH = 7.0) as previously described.[15] Concentration dependent chloride release for **3** (22–87 μM) is illustrated in Fig. 1.

Chloride release under these conditions follows the order **3** > **1** > **2**. If we arbitrarily select a time of 1500 seconds to compare the compounds, the percentages of total chloride released are 56% (**3**), 42% (**1**), and 11% (**2**). It is apparent that the line shapes differ to some extent as well (Fig. 2). We presume that once a pore has formed, the liposome empties rapidly and completely. Thus, the relative release reflects the dynamics of pore formation. We note that both **1** and **3** readily cause chloride release but the exponential portion of the curve shown for **3** is considerably steeper than that shown for **1**. We interpret this to mean that pore formation for **3** is more rapid than for **1**.

In compounds **1**, **2**, and **3**, the propanoyl sidechain is terminated in benzyl ester, acid, and amide residues, respectively. When glutamic acid (Glu, E) is converted into its amide, it becomes the common amino acid glutamine (Gln, Q). It is hard to compare these three sidechains in terms of polarity. Ethyl acetate and acetic acid have approximately the same dielectric constant (~ 6). Acetamide is a solid, but above its melting point of about 80 °C, it has a dielectric constant of ~ 59. Of course, glutamic acid has a pK_A of ~ 4, so it is expected to be ionized at physiologic pH. The experiments reported here were all conducted at a buffer pH value of 7.0.

The carbonyl groups in the glutamic sidechains of **1**, **2**, and **3** are expected to serve as Lewis base donors. The amide hydrogens of **2** can directly hydrogen bond to a chloride anion, as could the OH of **2**, depending on the pH. The data show that chloride ion transport is greater for **1** and **3** than for **2**. The carboxyl group, which is presumably ionized, will repel a proximate chloride ion rather than interact favorably with it. Thus, incorporation of a carboxyl residue in or near the "ion path" of this synthetic channel has the expected, deleterious effect.

Compounds **4** and **5** are similar to **1** and **2** except that the sixth amino acid is glycine in **4** and **5**, rather than alanine. The availability of **1**, **2**, **4**, and **5** permits a comparison of the amino acid sidechains in slightly different heptapeptide sequences. Fractional chloride release data from phospholipid vesicles are shown in Fig. 3. For all four compounds, chloride release from vesicles is greater for the GGGPEGG sequence compared to the GGGPEAG peptide. This difference is small, but reproducible. It is also clear that when glutamate's sidechain is benzylated, activity is considerably higher than when the free acid is present. These trends are apparent in the graph of Fig. 3, in which the ordinate shows fractional chloride release only to the extent of 50% to emphasize the differences.

The release profile of **6**, which has the sequence ~$(Gly)_3Pro(Gly)_3$~, is included on the graph of Fig. 3 for comparison. From the exponential portion of the curve, it appears that insertion of **6** occurs more rapidly than either **1** or **4**, but chloride release by all three compounds is identical within experimental error after 1500 s.

In all of the data accumulated for the compounds disclosed here, the most significant finding is clearly the effect of the ionizable glutamic acid. We interpret this to mean that the presence of a negative charge (ionized Glu) has a repulsive effect on chloride proximate to the entry portal of the channel or pore. If the pores formed by these heptapeptides are dimeric,[12a] two negative charges are likely to be present at the pore's entry portal when Glu is ionized. This will present chloride ion with a significant electrostatic barrier. When neutralized, the residue alters insertion dynamics but not ultimate ion release. The presence of glutamate in the ion pathway of the ClC chloride transporter proteins will exhibit similar electrostatics if deprotonated. In

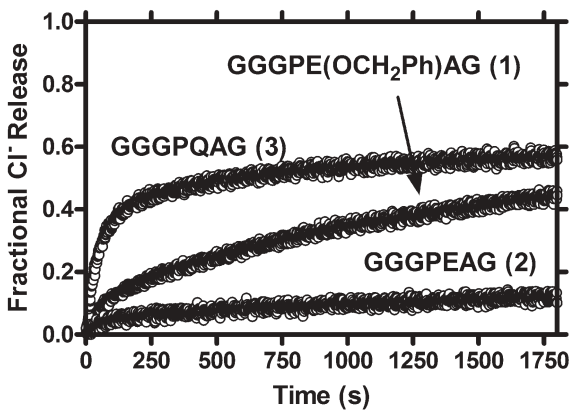

Fig. 2 Chloride release from phospholipid liposomes ([lipid] = 0.31 mM) mediated by **1**, **2** and **3** at a peptide concentration for each of 65 μM at pH = 7.0.

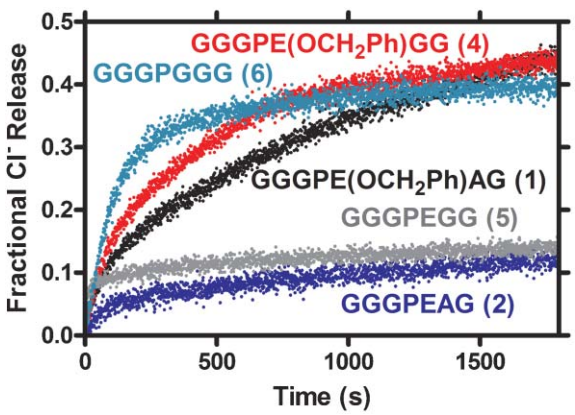

Fig. 3 Comparison of fractional chloride ion release from phospholipid vesicles, mediated by **1**, **2**, **4**, **5**, and **6**.

principle, at least, protonation and deprotonation of the carboxyl group could comprise a gating mechanism for chloride channel proteins as currently speculated.

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