COMMUNICATION

ChemComm
www.rsc.org/chemcomm

Anchor chain length alters the apparent mechanism of chloride channel function in SCMTR derivatives

Paul H. Schlesinger,*[a] Natasha K. Djedovič,[b] Riccardo Ferdani,[b] Jolanta Pajewska,[b] Robert Pajewski[b] and George W. Gokel*[b]

[a] *Department of Cell Biology and Physiology, Washington University School of Medicine, 660 S. Euclid Ave., St. Louis, MO 63110, USA*

[b] *Program in Bioorganic Chemistry, Division of Bioorganic Chemistry, and Department of Molecular Biology & Pharmacology, Washington University School of Medicine, 660 S. Euclid Ave., St. Louis, MO 63110, USA*

Received (in Columbia, MO, USA) 2nd November 2002, Accepted 21st November 2002
First published as an Advance Article on the web 7th January 2003

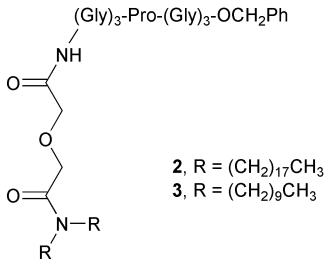
Two membrane-anchored heptapeptides have been prepared and their pore-formation behavior in phospholipid bilayer membranes has been found to differ profoundly as a result only of alkyl chain length.

Chloride ion is critical for cellular function but the mechanism by which it is transported remains obscure.[1] The recent solid state structures of prokaryotic ClC Cl^- channels from *Salmonella typhimurium* and *Escherichia coli*[2] have dramatically enhanced our understanding of structural relationships in these elaborate proteins. The channels are dimeric; each nearly autonomous monomer unit possesses 18 helical segments. The inherent complexity of such a molecule is obvious. Surely, compounds having far simpler structures must have functioned as chloride channels, albeit less effectively, at an earlier stage of evolution.

We recently reported the preparation of a synthetic membrane-insertable, chloride transporter ('SCMTR').[3,4] The compound was designed to possess (1) a membrane anchoring unit, (2) a heptapeptide that serves as headgroup, entry portal, and selectivity filter, and (3) a connector for (1) and (2) that mimics a phospholipid's midpolar regime.[5] We noted that among modern chloride transporters, all ClC chloride protein channels have, in their anion pathway, the conserved sequence GKxGPxxH.[6,7] Further, the presence of proline is known to affect channel selectivity,[8,9] and to induce a 'kink' or 'bend' (hinge-bend regime, GxxP) in a protein chain.[10] Proline's critical presence at the apex of C-peptide's helix-loop-helix motif[11] additionally inspired its use in our anion channel design.

We chose a symmetrical dialkylamine ($R_2N\sim$) to serve as the membrane anchor in this family of compounds. For the preparation of **2**, dioctadecylamine and diglycolic anhydride were heated ($C_6H_5CH_3$, reflux, 48 h) to give $(C_{18}H_{37})_2NCOCH_2OCH_2COOH$, **1**. When coupled to GGGPGGG-OCH_2Ph (in a sequence of steps previously reported), $(C_{18}H_{37})_2NCOCH_2OCH_2CO$-GGGPGGG-$OCH_2Ph$, **2**, was obtained.[3] When inserted into phospholipid liposomes, chloride was released in a concentration dependent fashion. The analog of **2** in which Leu replaced Pro was much less active. Remarkably, **2** showed voltage dependent gating when assessed in bilayer clamp experiments.[3] We have now prepared an exact analog of **2** in which the octadecyl chains are replaced by decyl groups to give $(C_{10}H_{21})_2NCOCH_2OCH_2CO$-GGGPGGG-$OCH_2Ph$, **3**. This novel compound transports Cl^- but exhibits a range of properties quite different from those observed with **2**. Specifically, the pore sizes of channels formed by **2** and **3** are the same but the apparent ionophoretic mechanism differs.

The synthesis of **3** was undertaken as follows. Diglycolic anhydride was heated with didecylamine in THF (reflux, 48 h). After removal of the solvent and crystallization of the crude product from Et_2O, $(C_{10}H_{21})_2NCOCH_2OCH_2COOH$ was obtained (88%) as a white solid, mp 51–52 °C. This was coupled to $TsOH\cdot H_2N$-GGG-OCH_2Ph to give $(C_{10}H_{21})_2NCOCH_2OCH_2CO$-GGG-$OCH_2Ph$ (76%, light yellow oil). As previously reported,[3] the PGGG fragment was added according to the following sequence: (a) coupling $TsOH\cdot H_2N$-GGG-OCH_2Ph with Boc-proline to give Boc-PGGG-OCH_2Ph, and (b) Boc group removal to give ClH_3N-PGGG-OCH_2Ph. Deprotection of $(C_{10}H_{21})_2NCOCH_2OCH_2CO$-GGG-$OCH_2Ph$ gave the acid, which was coupled (EDCI, HOBt, Et_3N, CH_2Cl_2, 0 °C→rt, 30 h) with $ClNH_3$-PGGG-OCH_2Ph to give **3** (60%, white solid, mp 127–128 °C). Proton and ^{13}C-NMR spectra corresponded to the assigned structures.



We evaluated Cl^- release from unilamellar liposomes of defined size and composition (150 ± 16 nm, 30% 1,2-dioleoyl-*sn*-glycero-3-phosphate, 70% 1,2-dioleoyl-*sn*-glycero-3-phosphocholine). Concentration dependent chloride release was observed with both **2**[3] and **3** (data not shown). The apparently greater Cl^- release activity of $(C_{10}H_{21})_2NCOCH_2OCH_2CO$-GGGPGGG-$OCH_2Ph$, **3**, encouraged us to characterize the pores further. This was done by using the 'dextran block' method, involving carboxyfluorescein (CF) release from liposomes. In this experiment, transport of CF is attempted in the presence of oligomerized sugars of various, known effective diameters. Carboxyfluorescein self-quenches when trapped within a vesicle but is readily detected by fluorescence when it emerges from the liposome.[12,13] When CF transport is blocked, reduced fluorescence (corresponding to dye release) is observed. Note that the diameter of the pore must be slightly larger than the dextran used to block it.

Either **2** or **3** (100 μM) was inserted into vesicles, prepared as above except that carboxyfluorescein (20 mM, 10 mM HEPES, pH = 7.0) was trapped within the liposomes. The extravesicular medium contained 100 mM KNO_3 (10 mM HEPES, pH = 7.0). The concentration dependent CF release data are shown in Fig. 1(a) and (b) for **2** and **3**, respectively. Similar CF release rates are observed for **2** at 25.3 μM and for **3** at 1.67 μM. This implies that **3**, which has shorter anchor chains, is ~15-fold more active than is **2** under the conditions of this experiment.

The concentration dependent activities for **2** and **3** are summarized in the log–log Hill[14] plots (Fig. 1(c)). In these plots, the slopes represent the molecularity of the limiting step. Both plots have a slope of 2.0 ± 0.3 at 50% of the maximum rate of pore activation. At lower concentration, the slope of **3** (curved line) decreases to 0.89 ± 0.3 suggesting that dimer formation no longer limits the rate of pore activation. The apparent pore sizes

DOI: 10.1039/b211629b

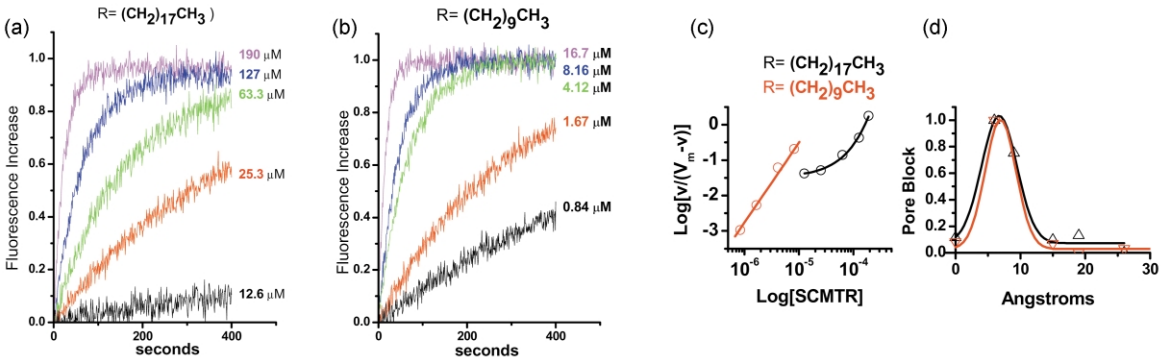


Fig. 1 (a) Carboxyfluorescein release for SCMTR (**2**). (b) Carboxyfluorescein release for **3**. (c) Hill plots for **2** and **3**. (d) Apparent pore diameters for **2** and **3**.

for **2** and **3** were obtained using size-specific block by dextran polymers (Fig. 1(d)). The apparent pore activation is diminished by dextrans of a size appropriate to block the pore.[15] When the blocker becomes too large, the pore activity is no longer affected.[16] Fig. 1(d) shows the near identity in sizes of the pores formed by **2** and **3**: 8.2 ± 0.44 Å. The formation of dimer pores having nearly identical diameters supports a model in which the dimerization step does not limit pore activation at low concentrations of **2**.

It occurred to us that these anchored heptapeptides might form solution aggregates that fuse into liposomes affording preformed pore structures. The aggregation behavior of both **2** and **3** were studied from ~2–60 μM by dynamic light scattering. Compound **3** consistently formed 350 ± 70 nm liposomes throughout this concentration range. Compound **2** formed 500 nm aggregates at < 30 μM but larger and more complex aggregates were noted as concentration increased. In the liposome assay, fluorescence dequenching is dominated by the initial pore activation[15] and is therefore sensitive to a shift from a bi- to monomolecular mechanism.

We evaluated the slope conductances of **2** and **3** in planar lipid bilayers under steady-state conditions of pore activity in order to determine ion conduction characteristics (see Fig. 2). In this experiment, 5.2 μM of **3** was added to the preformed bilayer until the current stabilized; current was then determined as a function of voltage. The same protocol was followed for **2**, except the compound was studied at both 23.6 and 89.7 μM. The conductance of bis(C_{18}) peptide **2** was 349 ± 69 pS under steady state conditions (compared to 1.3 nS under ion gradient conditions[3]). Shorter-chained **3** showed a conductance of 573 ± 13 pS (steady state). Both compounds displayed rectification at voltages below E_{rev}. This is in accord with the voltage

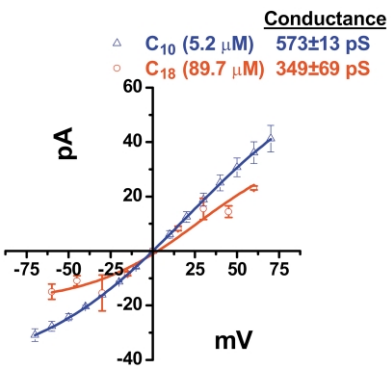


Fig. 2 Comparison of conductance in bilayers from insertion of **3** or **2**. For **3** (C_{10}, blue), the bilayer was exposed to 5.2 μM until the current stabilized; current was determined as a function of voltage. The same protocol was followed for **2**, except the compound was at 23.6 or 89.7 μM. Each experiment was repeated 3 times and error bars are shown.

dependent gating behavior previously reported for **2**.[3] Less curvature was observed in the data plot for **3** than for **2**. Both compounds produced stable pores of constant steady-state conductance. The per-mole size of this conductance was consistent with the concentration dependence of pore activation in liposomes: **3** was 17-fold greater than **2**. We conclude that at least two mechanisms of pore activation occur. One involves dimerization of monomeric, membrane-inserted, pore-forming molecules. A second reflects the membrane insertion of a preorganized pore unit. The latter occurs at a very low rate and is observed only when the former pathway becomes slow.

The complex properties of these two simple molecules are remarkable. Even more unexpected are the striking differences in their insertion, transport, and gating properties. These chemically simple channel-formers exhibit an astonishing complexity in their membrane functions. They demonstrate properties previously associated only with proteins and peptides of substantial size. Their value as prototypes for channel evolution is apparent from this astounding complexity.

We gratefully acknowledge support of this work by the NIH (GM-36262) and by the Kilo Foundation.

Notes and references

1 C. Miller, *Curr. Opin. Chem. Biol.*, 2000, **4**, 148.
2 R. Dutzler, E. B. Campbell, M. Cadene, B. T. Chait and R. MacKinnon, *Nature*, 2002, **415**, 287.
3 P. H. Schlesinger, R. Ferdani, J. Liu, J. Pajewska, R. Pajewski, M. Saito, H. Shabany and G. W. Gokel, *J. Am. Chem. Soc.*, 2002, **124**, 1848.
4 P. H. Schlesinger, R. Ferdani, R. Pajewski, J. Pajewska and G. W. Gokel, *Chem. Commun.*, 2002, 840.
5 G. W. Gokel, *Chem. Comm.*, 2000, 1.
6 C. Fahlke, H. T. Yu, C. L. Beck, T. H. Rhodes and A. L. George Jr., *Nature (London)*, 1997, **390**, 529.
7 C. Fahlke, R. R. Desai, N. Gillani and A. L. George, *J. Biol. Chem.*, 2001, **276**, 1759.
8 P. J. Corringer, S. Bertrand, J. Galzi, A. Devillers-Thiery, J.-P. Changeux and D. Bertrandt, *Neuron*, 1999, **22**, 831.
9 J. L. Galzi, A. Devillers-Thièry, N. Hussy, S. Bertrand, J. P. Changeux and D. Bertrand, *Nature*, 1992, **359**, 500.
10 C. J. Brandl and C. M. Deber, *Proc. Natl. Acad. Sci. USA*, 1986, **83**, 917.
11 Y. Ido, A. Vindigni, K. Chang, L. Stramm, R. Chance, W. F. Heath, R. D. DiMarchi, E. DiCera and J. R. Williamson, *Science*, 1997, **277**, 563.
12 B. Hille, *J. Gen. Physiol.*, 1968, **51**, 199.
13 D. Levitt, *Curr. Top. Membranes Transport*, 1984, **21**, 181.
14 I. Segel, *Enzyme Kinetics. Behavior and Analysis of Rapid Equilibrium and Steady-State Enzyme Systems*, John Wiley & Sons, New York, 1975.
15 M. Saito, S. J. Korsmeyer and P. H. Schlesinger, *Nat. Cell Biol.*, 2000, 553.
16 N. Sakai, C. Ni, S. M. Bezrukov and S. Matile, *Bioorg. Med. Chem. Lett.*, 1998, **8**, 2743.