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Chloride Transport Across Lipid Bilayers and Transmembrane Potential Induction by an Oligophenoxyacetamide

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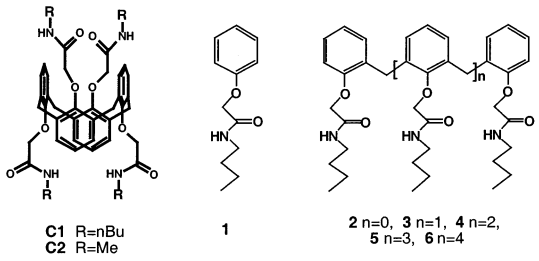
Identification of compounds that transport chloride across cell membranes has important implications in drug development. A new strategy for treating diseases caused by chloride channel malfunctions, such as cystic fibrosis, relies on synthetic Cl^- transporters.[1] Peptides based on sequences thought to line the pore of natural chloride channels can restore defective Cl^- transport.[2] Compounds that enable H^+/Cl^- cotransport also have therapeutic potential. The immunosuppressive and anticancer activities of the prodigiosin antibiotics have been attributed to their ability to co-transport H^+/Cl^-.[3] These promising therapeutic approaches are limited, however, by the relatively few compounds known to transport Cl^- across membranes.[4−6] Herein, we describe the discovery and properties of a new synthetic Cl^- transporter.

We recently showed that calix[4]arene tetrabutylamide **C1** facilitates H^+/Cl^- transport in liposomes, forms ion channels in planar lipid bilayers, and supports electric current in HEK cells.[6] A crystal structure of an HCl complex of calix[4]arene tetramethylamide **C2** provided a rationale for how Cl^- anions are moved across a membrane by **C1**. Individual calixarenes were bridged by amide NH−Cl^- and NH−H_2O hydrogen bonds to give a lattice with H_2O-filled and Cl^--filled pores. The structure revealed that the calixarene macrocycle was not directly involved in HCl complexation. We reasoned that ion transport activity might be maintained, or enhanced, if we dispensed with the calixarene and produced acyclic analogs that retained the secondary amide groups needed for Cl^- binding and self-association.



C1 R=nBu
C2 R=Me

1

2 n=0, **3** n=1, **4** n=2,
5 n=3, **6** n=4

To test our hypothesis, we synthesized the series of phenoxyacetamides **1**−**6**,[7] ranging from monomer **1** to hexamer **6** and evaluated their ability to support H^+/Cl^- transport in large unilamellar vesicles (LUVs). We report that trimer **3** is the most efficient chloride transporter among compounds **1**−**6** and calix[4]arene **C1**. Trimer **3** also has an unprecedented function for a synthetic compound. Due to its high Cl^-/SO_4^{2-} transport selectivity, **3** induces a stable potential in liposomes experiencing a transmembrane Cl^-/SO_4^{2-} gradient. These results represent encouraging developments in the search for new classes of synthetic Cl^- transporters.

The H^+/Cl^- transport activity of **1**−**6** was evaluated via the pH-stat fluorescent assay (Figure 1).[6] A significant increase in transport rates with increasing polymer chain length was observed for compounds **1**−**3**. Monomer **1** showed essentially no transport activity, even at 50 μM (10 mol %, ligand-to-lipid ratio). Dimer **2** had low transport activity at concentrations below 20 μM (4 mol %). In contrast, application of trimer **3** at concentrations as low as 5 μM (1 mol %) resulted in rapid exchange between extra- and intravesicular electrolytes (Figure 1B). Trimer **3** was an order of magnitude more active than **C1** at 5 μM (5.8×10^{-2} vs 6.8×10^{-3} s^{-1} established previously for **C1**[6]). Further elongation of the oligomer backbone resulted in activity decrease. Tetramer **4** was 6 times less active than trimer **3** at 5 μM (1 mol %), and pentamer **5** and hexamer **6** demonstrated further decreases in transport rates.

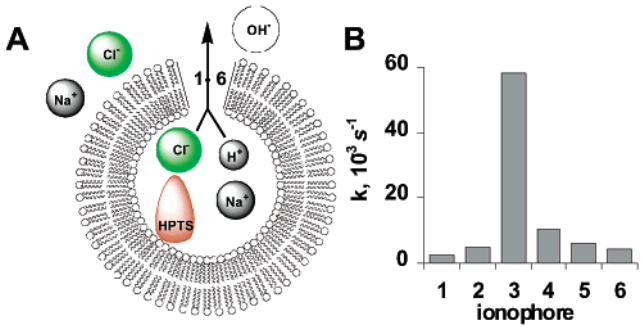


Figure 1. (A) Schematic representation of pH-stat experiments used to monitor H^+/Cl^- transport. A pH gradient results from NaOH addition to the extravesicular solution. The charge caused by H^+ efflux is compensated by Cl^- efflux, as mediated by the exogenous ligand. The increase in intravesicular pH, monitored by the entrapped pH-sensitive dye, pyranine, reflects the electrolyte exchange rate. (B) Initial pseudo-first-order rate constants in the presence of 5 μM of ionophores **1**−**6** (1 mol %), obtained from pH-stat fluorescent assays at room temperature. Suspensions of EYPC LUVs containing pyranine[9] (ex 405 and 460 nm, em 510 nm) in a phosphate buffer were used (0.5 mM of lipid, 10 mM $Na_nH_{3-n}PO_4$, $n = 1, 2$, pH 6.4, 100 mM NaCl inside and outside). Injection of 20 μL of 0.5 mM DMSO solution of ionophore **1**−**6** into 1.9 mL of vesicular suspension was followed by injection of 21 μL of 0.5 M NaOH.

In an attempt to gain insight into the transport mechanism we carried out a series of concentration-dependent studies. Whereas trimer **3** was significantly more active than the other oligomers at all concentrations, the relative transport abilities of compounds **2**−**6** varied with concentration, mostly due to the nonlinear increase of activity observed for dimer **2** (see Figure S2). This nonlinear increase in transport activity is strong evidence that H^+/Cl^- transport is mediated by a self-associated form of **2**. However, the apparent linear concentration−activity relationship observed for trimer **3** suggests that trimer **3** is either pre-assembled before membrane insertion or that it acts by a carrier mechanism.[8]

Importantly, compounds **2**−**6** mediated electrolyte exchange in the presence of Cl^- but not in the presence of SO_4^{2-} anion. In contrast to the results shown in Figure 1B, where NaCl extra- and intravesicular buffers were used, no transport activity was detected in LUVs symmetrically loaded with Na_2SO_4, even at ligand concentrations of 50 μM (10 mol %). This anion-dependent activity is strong evidence that butylamides **2**−**6** mediate Cl^- transport across the bilayer.

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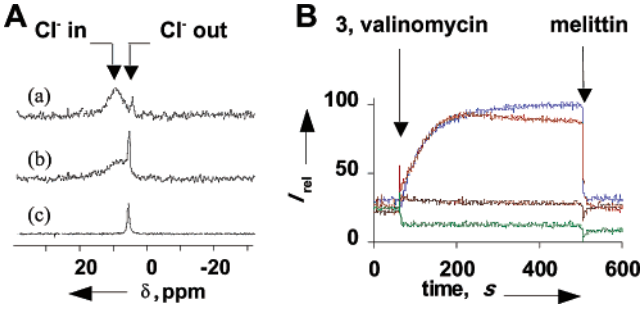

Figure 2. (A) ^{35}Cl NMR spectra of a suspension of giant vesicles (88 mM EYPC, 9:1 H_2O:D_2O, 100 mM NaCl, 10 mM $CoCl_2$,10 mM $Na_nH_{3-n}PO_4$, $n = 1, 2$, pH 5.4) suspended in 75 mM Na_2SO_4 Co^{2+}-free buffer (9:1 H_2O:D_2O, $Na_nH_{3-n}PO_4$, $n = 1, 2$, pH 6.4). Spectra correspond to (a) giant vesicles in the absence of **3**, (b) giant vesicles 1 h after application of 1 mol % **3** in DMSO, and (c) vesicles after lysis with Triton X-100. (B) Liposome potential fluorescent assays. Suspension of EYPC LUVs at room temperature was used (10 mM $Na_nH_{3-n}PO_4$, $n = 1, 2$, pH 6.4, 100 mM KCl or 75 mM Na_2SO_4 inside and 100 mM NaCl, 60 nM potential-sensitive dye safranin O, ex 480 nm, em 520 nm, outside). Color code for traces denotes the formation of potential in: (blue) KCl vesicles upon application of valinomycin, (orange) Na_2SO_4 vesicles upon application of **3**, (red) KCl vesicles upon application of **3**, (green) Na_2SO_4 vesicles upon application of valinomycin. Potentials were quenched at the end of each experiment by injecting 20 μL of a 1 mM aqueous solution of the defect-inducing peptide melittin.

Direct evidence for Cl^- transport by trimer **3** was obtained from ^{35}Cl NMR experiments. Giant vesicles containing NaCl and $CoCl_2$ were suspended in Co^{2+}-free Na_2SO_4 buffer. The membrane-impermeable Co^{2+} caused a downfield shift and broadening of the ^{35}Cl NMR signal for intravesicular Cl^-.[10] A separate, smaller signal was due to residual extravesicular Cl^- (Figure 2A,a). Controls showed no leakage of Cl^- from liposomes even after 3 days. Addition of trimer **3** resulted in an increased extravesicular Cl^- peak due to outwardly directed Cl^- transport (Figure 2A,b). The new intravesicular/extravesicular Cl^- equilibrium in the presence of **3** was stable for at least 3 h, or until lysis with Triton X-100 released the intravesicular Cl^- to give a single ^{35}Cl NMR resonance (Figure 2A,c).[11]

The combined pH-stat and ^{35}Cl NMR data indicate that the acyclic trimer **3**, like its predecessor calixarene **C1**, co-transports H^+/Cl^-. Addition of **2**−**6** to a suspension of NaCl-loaded liposomes in Na_2SO_4 buffer resulted in alkalinization of the vesicular aqueous compartment due to effective H^+/Cl^- co-transport down the chloride gradient. The highest activity in the series of acyclic oligomers **2**−**6**, in terms of the time required to establish equilibrium, was again demonstrated by trimer **3**. The overall activity trend was: **3** ≫ **4** ≈ **C1** > **2** > **5** ≈ **6**. As expected, reversed loading of liposomes (Na_2SO_4-loaded liposomes in NaCl buffer) resulted in acidification of the vesicular compartment upon application of trimer **3**. As mentioned above, compounds with H^+/Cl^- co-transport activity have high therapeutic potential.[3]

Although trimer **3** transports both Cl^- and H^+ into sulfate-loaded liposomes suspended in a chloride buffer, Cl^- is transported faster than H^+, and therefore the overall process is not electrically silent. Monitoring of transmembrane potential using the potential-sensitive dye safranin O[12] revealed the formation of stable negative charge inside liposomes (75 mM Na_2SO_4 inside, 100 mM NaCl outside, safranin O outside) within 2 min of applying 1 mol % of trimer **3** (Figure 2B, red trace). The magnitude of the potential induced by **3** under an inwardly directed Cl^- gradient is similar to that generated by 0.12 mol % valinomycin in liposomes with an outward K^+ gradient (Figure 2B, blue trace, 100 mM KCl inside, 100 mM NaCl outside). The lower two traces in Figure 2B verify that trimer **3** and valinomycin are functionally orthogonal. Trimer **3** creates a transmembrane potential under conditions where valinomycin cannot create a potential and vice versa.

To our knowledge, trimer **3** is the first compound to induce a stable potential in LUVs due to a transmembrane anionic gradient.[13] These data show that trimer **3** has a significant anion/cation transport selectivity. Maintenance of this potential and the stable transmembrane Cl^- equilibrium demonstrated by ^{35}Cl NMR (Figure 2) also indicate that trimer **3** does not induce membrane defects. The ability of trimer **3** to transport Cl^-, to generate and maintain a transmembrane potential, along with its high activity at low μM concentrations, its low molecular weight, and its simple preparation, make this compound a potentially valuable lead in drug development for the treatment of cystic fibrosis and cancer.[2,3,14] We do not know yet whether this chloride transporter functions as a channel or as a carrier. The mechanism by which these oligomers, particularly trimer **3**, transport Cl^- across membranes is a major focus of our ongoing research.

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Supporting Information Available: Synthetic preparations and experimental details (PDF). This material is available free of charge via the Internet at http://pubs.acs.org.

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