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A Small Synthetic Molecule Forms Chloride Channels to Mediate Chloride Transport across Cell Membranes

Xiang Li,[†] Bing Shen,[§] Xiao-Qiang Yao,[§] and Dan Yang*,[†]

Department of Chemistry, The University of Hong Kong, Pokfulam Road, Hong Kong, China, and Department of Physiology, The Chinese University of Hong Kong, Shatin, N.T., Hong Kong, China

Received April 3, 2007; E-mail: yangdan@hku.hk

Chloride (Cl^-) ions are the most abundant anions found in organisms. The Cl^- channels, unique anion channels that mediate the transfer of Cl^- ions across cell membranes, play crucial roles in controlling membrane excitability, transepithelial transport, cell volume, and intracellular pH.[1,2] In fact, dysfunctional Cl^- channels are responsible for many severe human diseases, including cystic fibrosis, inherited kidney stone diseases, myotonia, and epilepsy.[2,3] As such, studies into Cl^- channels have attracted the attention of scientists from a diverse range of disciplines. While most studies have focused on natural Cl^- channels,[4,5] it remains an intriguing challenge to create synthetic systems that mimic biological functions of natural Cl^- channels.[6−15] To date, however, most of those synthetic Cl^- channels have relatively complicated structures and high molecular weights, which restrict their further applications in drug discovery.[16,17] Here we report our discovery of a small organic molecule that can form synthetic Cl^- channels in lipid bilayer membranes.

In Nature, the high specificity of Cl^- channels originates from recognition sites in which the anion is completely desolvated and bound exclusively through hydrogen bonds.[2,3] The simulation of such a chloride recognition process was, therefore, our first consideration when designing our synthetic Cl^- channels. α-Aminoxy acids[18] are a class of unnatural analogues of α-amino acids (Figure 1). Because the amide NH units of α-aminoxy acids are more acidic than are regular amide NH groups, they are better hydrogen bond donors when interacting with anions. Previously, we described the incorporation of α-aminoxy acids as building blocks for the synthesis of macrocyclic peptides that bind to Cl^- ions selectively.[19,20] Considering these systems' preference for Cl^- ions, we also employed α-aminoxy acid units for the construction of synthetic Cl^- channels.

The present design is based on a C_2-symmetric isophthalamide scaffold, featuring two α-aminoxy acid units (Figure 1). Starting from L-leucine, a natural amino acid, we prepared compound **1** in five steps in an overall yield of 68% (Supporting Information). The analyses of solution-phase anion binding properties of **1** were carried out using the standard ^1H NMR spectroscopic titration technique in $CDCl_3$. Quantitative assessments of the anion binding affinities of **1** toward different anions reveal that **1** is not only an effective 1:1 anion binding agent in solution but also a selective one, which shows a remarkable preference for Cl^- relative to other anions[21] (Br^-, I^-, NO_3^-, and $H_2PO_4^-$) (Table 1).

To assess the ability of compound **1** to facilitate Cl^- transport across lipid bilayer membranes, we prepared liposomes encapsulating sodium nitrate ($NaNO_3$) and suspended them in a sodium chloride (NaCl) bulk solution. We studied the Cl^- influx into liposomes by monitoring the fluorescence intensity of an entrapped Cl^--sensitive indicator, 6-methoxy-*N*-(3-sulfopropyl)quinolinium

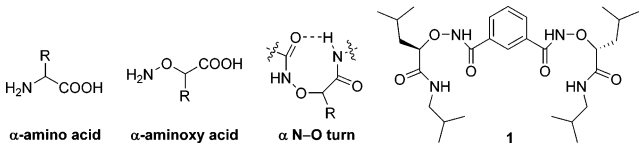

Figure 1. Representative chemical structures of **1** and relevant compounds.

Table 1. Association Constants for the Binding of **1** with Anions[a] in $CDCl_3$ at 25 °C

anions	K (M^{-1})[b]	$\Delta\delta_{max}$ (O-NH)[c]	$\Delta\delta_{max}$ (NH)[c]
Cl^-	>100000	2.17	0.37
Br^-	18000	1.73	0.31
I^-	1500	1.44	0.28
NO_3^-	1100	1.28	0.32
$H_2PO_4^-$	1400[d]	*e*	0.81

[a] Anions were added as concentrated $CDCl_3$ solutions of Ph_4PCl, Ph_4PBr, Bu_4NI, Bu_4NNO_3, and $Bu_4NH_2PO_4$, respectively. To account for the dilution effect, these anion solutions also contained receptor **1** at its initial concentration (1−5 mM). [b] Determined by following the changes that occurred to the aminoxy amide NH protons' resonances. [c] Estimated maximum change in chemical shift (ppm). [d] Determined by following regular amide NH protons' resonances. [e] The signal became too broadened to be followed.

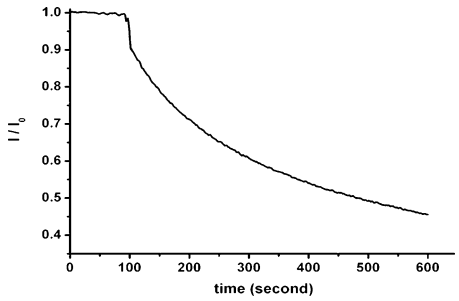

Figure 2. Fluorescence assay of chloride transport into liposomes. Inside vesicles: 200 mM $NaNO_3$, 0.5 mM SPQ. Outside vesicles: 200 mM NaCl. At 100 s, add compound **1** at 5 μM (final concentration).

(SPQ).[22] Addition of compound **1** to such liposome suspensions induced a rapid decrease in the fluorescence of SPQ (Figure 2), indicating that compound **1** did indeed transport Cl^- into the liposomes.

Single-channel recording, using patch clamp techniques, is the most critical test for distinguishing ion channels from other ion transport mechanisms such as ion carriers and quantifying the ion transport efficiency of a membrane channel.[23] The characteristic single-channel currents that we observed for compound **1** in giant liposomes[24] indicated that it formed functional ion channels. We detected two primary conductances (54 and 108 pS) in a bath solution of symmetric 0.2 M *N*-methylglucamine hydrochloride (NMDG-Cl) incorporating 10 nM of compound **1** (Figure 3a and b). These data suggest that compound **1**, intrinsically different from

[†] The University of Hong Kong.
[§] The Chinese University of Hong Kong.

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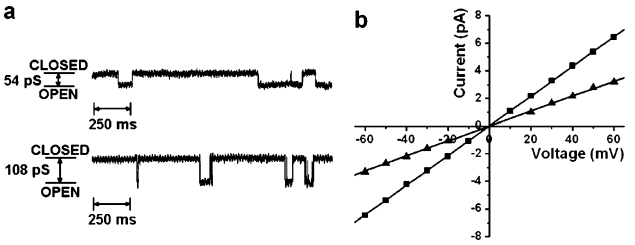

Figure 3. (a) Single-channel traces of the self-assembled channels with two primary conductances recorded in lipid bilayers of liposomes composed of EYPC and cholesterol with a 10:1 molar ratio at −60 mV. The currents were recorded in symmetric 0.2 M NMDG-Cl in the presence of 10 nM of **1**. (b) Current−voltage relationships for the channels. The conductances of the channels were 54 (▲) and 108 pS (■), respectively. Currents are means ± SEM; $n \geq 10$.

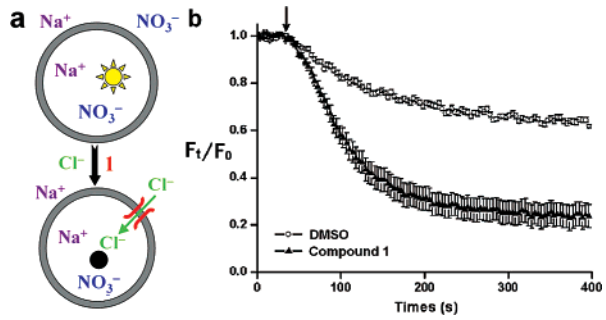

Figure 4. (a) Graphical representations of the SPQ assay. (b) SPQ assay of Cl^- transport. MDCK cells loaded with SPQ were preincubated with NO_3^- buffer for 30 min to allow the depletion of intracellular chloride. At the beginning of the experiment, the cells were first perfused with NO_3^- buffer until the fluorescence was stable. Perfusion solution was then switched to Cl^- buffer (shown by the arrow) containing 1 μM of compound **1** (dissolved in DMSO) or equal volume of DMSO. Data obtained from 55 cells ($n = 4$) are means ± SEM of fluorescence at time t (F_t) relative to baseline fluorescence (F_0).

previously reported chloride transporters with isophthalamide scaffold,[25] automatically assembles into efficient ion channels within the lipid bilayers of liposomes. Various conductance substates are anticipated for molecules that self-assemble into ion channels of various sizes.[6,7] We investigated the ion selectivity of our designed channels by incorporating various ions into the bath solutions. There was no measurable change in either the conductance or the reversal potential when NMDG-Cl was replaced in the bath solution by KCl or NaCl, suggesting that these channels are not permeable to K^+ or Na^+ ions.[23] Taking the SPQ fluorescence and patch clamp studies together, we believe that the observed single-channel currents are indeed Cl^- currents.

To explore the potential of compound **1** for applications in biomedical science, we investigated the ability of the compound to facilitate Cl^- transport in plasma membranes of living cells. The Madin−Darby canine kidney (MDCK) cells were treated with the compound, and Cl^- transport was then monitored by a Cl^--sensitive fluorescent indicator SPQ (see Supporting Information).[11,26] In this assay, MDCK cells loaded with SPQ were preincubated with NO_3^- extracellular buffer for at least 30 min to replace most of intracellular Cl^- ions with NO_3^- ions. The NO_3^- extracellular buffer was then replaced with Cl^- buffer to establish an inwardly directed Cl^- gradient. The decrease in SPQ fluorescence is attributed to a dynamic quenching as an influx of extracellular Cl^- ions in exchange for NO_3^- efflux from the cells. As shown in Figure 4, addition of 1 μM compound **1** induced a significant decrease in SPQ fluorescence, indicating increased anion permeability. To our knowledge, compound **1** represents the smallest synthetic molecule that can mediate Cl^- ion transport efficiently in plasma membranes of living cells.

In summary, small organic molecule **1** forms ion channels in the lipid bilayers to mediate the passage of Cl^- ions. It has the potential to become a novel lead compound for the treatment of human diseases associated with Cl^- channel dysfunctions. We believe that such systems will help design other transmembrane channels and have a broad range of applications in chemistry, biochemistry, biology, and materials science.

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Supporting Information Available: Procedures for the preparation of **1** and its characterization data; a list of the observed and calculated binding profiles for NMR titrations; Job plots; SPQ assay in liposomes; single-channel recording; and SPQ assay in MDCK cells. This material is available free of charge via the Internet at http://pubs.acs.org.

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