Transmembrane Channels

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Voltage-Driven Reversible Insertion into and Leaving from a Lipid Bilayer: Tuning Transmembrane Transport of Artificial Channels**

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Abstract: *Three new artificial transmembrane channel molecules have been designed and synthesized by attaching positively charged Arg-incorporated tripeptide chains to pillar[5]arene. Fluorescent and patch-clamp experiments revealed that voltage can drive the molecules to insert into and leave from a lipid bilayer and thus switch on and off the transport of K^+ ions. One of the molecules was found to display antimicrobial activity toward Bacillus subtilis with half maximal inhibitory concentration (IC_{50}) of 10 μM which is comparable to that of natural channel-forming peptide alamethicin.*

The transition of the voltage-gated ion channel between the close and open states can be regulated by changing the transmembrane potential, which is responsible for a variety of crucial metabolic and signaling functions in biological systems.[1] The development of artificial systems displaying similar voltage-gated capability is not only fundamentally important but also may generate signal processing and transduction devices.[2] Currently, a large number of non-regulable artificial transmembrane transport systems, including gramicidin-like channels[3] and shuttle-like transporters,[4] have been developed. Examples of controllable artificial channels are also available.[5,6] In this context, voltage-gated systems are useful models for mimicking the transport processes in life,[6,3e] which had been realized by constructing unsymmetric scaffolds for invoking dipole reorientation[6a,b,d] or dipole-potential interaction[6c,e] in response to an applied potential. We herein describe a new strategy of controlling transmembrane transport by using voltage to drive positively charged channel molecules to insert into and leave from a lipid bilayer.

Natural voltage-gated K^+ channels possess a unique transmembrane segment, the S4 domain, which contains four to eight positively charged Arg units.[7] Under an electric field, this domain can move across the cell membranes to cause the channel to open and close.[8] Pillar[5]arene represents a new family of paracyclophanes that exhibit an unique column-like structure,[9] which could be used to build artificial transmembrane channels.[10] We envisioned that, by introducing positively charged Arg units into the peptide chains of our previously reported pillar[5]arene-based transmembrane channel **1a** (Figure 1a),[10c] they might undergo similar voltage-driven movement. If the interaction between the channel molecules and the lipid is strong enough, they could stay within the lipid bilayer to enable ion transport. Imposing a higher reversed potential might drag the molecules to move out and thus stop the transport (Figure 1b). We therefore prepared compounds **1b–d** by attaching ten Arg-contained tripeptide chains to a pillar[5]arene backbone. The peptide chains are composed of alternative L- and D-amino acids to ensure the assembly of tubular structures.[10c]

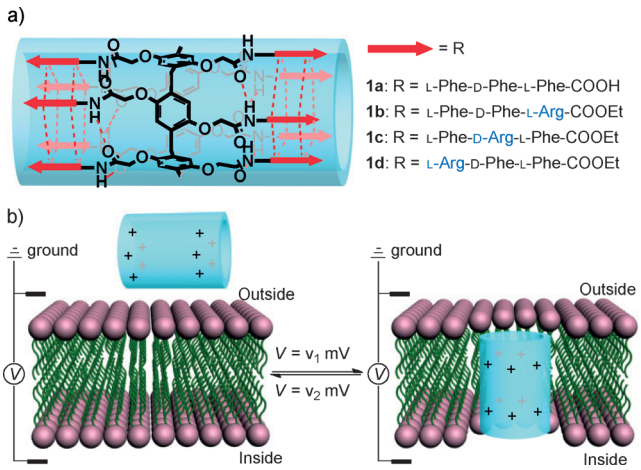

Figure 1. a) Structure of channels **1a–d**. b) Schematic presentation for the voltage-driven channel inserting into and leaving of lipid bilayer.

It had been demonstrated that **1a** had a high membrane-insertion capability and could transport cations and anions in a symport manner.[10c] Because **1b–d** have the same backbone, they were expected to display a similar transport activity if they can be inserted into the bilayer. Thus, their KCl transport experiments were performed with large unilamellar vesicles (LUVs) made from egg yolk L-α-phosphatidylcholine (EYPC; see Figure S19 in the Supporting Information),[11] which showed that, different from **1a**, these molecules only possessed a very weak activity toward Cl^-. This is not unexpected because the introduction of the ten hydrophilic Arg units should remarkably weaken their ability to insert into a hydrophobic lipid bilayer. We thus exploit the possibility of inserting the molecules into a lipid bilayer by imposing a potential across the bilayer.

The possibility was then tested by assessing the ability of **1a–d** to depolarize polarized LUVs.[6c] EYPC-LUVs with

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internal KCl (100 mM) and external NaCl (100 mM) and Safranin O (180 nM), a fluorescent probe for membrane potential,[12] were first prepared. External addition of valinomycin (20 nM), a selective K^+ carrier,[13] led to significant increase in the emission of Safranin O after six minutes, indicating an internal negative potential from inside to outside was established (Figure 2a). Then, aliquots of

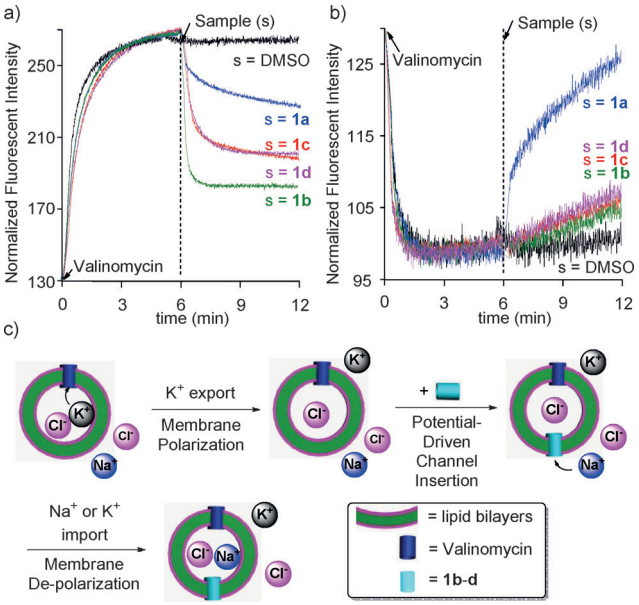

Figure 2. a,b) Changes in fluorescence intensity of Safranin O (180 nM; $\lambda_{ex}=522$ nm; $\lambda_{em}=581$ nm) loaded outside of the vesicles (Mes buffered at pH 6.4. a: $[KCl]_{inside}=100$ mM, $[NaCl]_{outside}=100$ mM; b: $[NaCl]_{inside}=100$ mM, $[KCl]_{outside}=100$ mM) with time after addition of valinomycin (20 nM) and **1a–d** ($x=0.1\%$). c) Schematic presentation of the membrane polarization induced by the valinomycin mediated K^+ exportation and subsequent depolarization induced by the potential-driven **1a–d** insertion into the bilayer.

DMSO solutions of **1a–d** [molar ratio relative to lipid (x) = 0.1%] were added and the fluorescence of Safranin O was recorded accordingly. It was found that, with the addition of **1a–d**, the fluorescent intensity of Safranin O decreased significantly, by 28%, 63%, 51%, and 50%, respectively, after 6 minutes, indicating that the polarized bilayer was depolarized because of the incorporation of the molecules, which allowed for the fluxing of Na^+ or K^+ ions from outside into vesicles (Figure 2c). It is reasonable to propose that **1a** depolarized the polarized bilayer through spontaneous insertion into the bilayer to form channels. Because without a membrane potential, **1b–d** did not exhibit any transport ability, the above efficient de-polarization of the bilayer by these positively charged molecules supported that they were forced by the internal negative potential to insert into the bilayer to form channels.[14] The effective concentration needed to reach 50% depolarization (EC_{50}) for **1b–d** were calculated to be 0.1%, 0.2%, and 0.2%, respectively, by using Hill analysis (see Figure S20), demonstrating that the channels became active after the bilayer was polarized. When vesicles with internal NaCl (100 mM) and external KCl (100 mM) were employed, the addition of valinomycin caused important quenching of the emission of Safranin O, indicating a positive potential from inside to outside was produced (Figure 2b). Further addition of **1a** ($x=0.1\%$) led to 91% recovery of the fluorescence, reflecting the depolarization of the vesicles. In contrast, adding the same amount of **1b**, **1c** or **1d** caused only slight increase of the fluorescence (Figure 2b) and thus a weak depolarization of the vesicles, indicating that the ability of the molecules to spontaneously insert into the bilayer, if any, was quite low.

The voltage-responding insertion behaviors of compounds **1b–d** were then investigated by performing patch-clamp experiments on planar lipid bilayer.[15] For all these experiments, two compartments containing KCl solution (1.0 M, pH 6.4, Mes buffered) were separated by a planar lipid bilayer composed of diphytanoylphosphatidylcholine (diPhyPC). Compound **1b** was firstly investigated. In a typical experiment, **1b** (3 μM) was added to the *cis* compartment which was grounded. To determine the minimum potential (V_{min}(in)) which could drive **1b** to insert into the bilayer, alternative negative–positive potential pulsing was applied (Figure 3a).

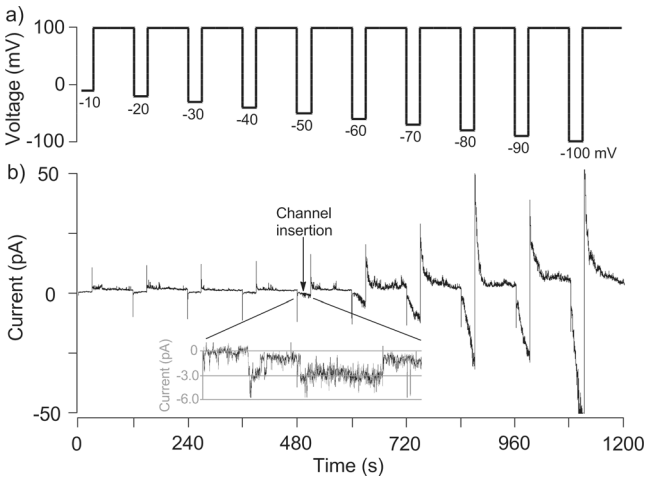

Figure 3. a) The alternative negative potential (30 s) and +100 mV (90 s) potential pulse protocol. The negative potential (30 s) increased from −10 to −100 mV in ten steps, while the positive potential was kept constantly at +100 mV. b) The macroscopic current trace of **1b** by applying the pulse in (a). Inset: The expanded current trace (30 s) at −50 mV showing the single-channel current.

The negative potential was set to increase from −10–100 mV in ten steps, with every pulse lasting 30 s, while the positive potential was kept constantly at +100 mV, with the pulsing time being 90 s,[16] which was found to be able to drive the inserted channels away from the bilayer (see below). The macroscopic ion current was recorded with time and the results are shown in Figure 3b. When the potential was increased to −50 mV, a single-channel current was observed, which vanished after 90 s of +100 mV pulsing. With further increase of the negative potential, the current increased gradually with time, suggesting that more and more molecules of **1b** were driven to insert into the bilayer to form channels. The tendency that the mean current remarkably increases

with increasing negative potential indicates that a higher negative potential could more efficiently drive the molecules to insert into the bilayer. For all the intervals of the +100 mV potential pulsing, the current decreased quickly with time, indicating a quick dragging-out of the molecules from the bilayer.

The minimum potential (V_{min}(out)) for driving the inserted channel **1b** to leave from the bilayer was also determined. We conjectured that, if two or more channel molecules were inserted into the bilayer, it would be difficult to accurately detect the leaving of one channel molecule. Thus, the best way of evaluating the V_{min}(out) would be to keep one channel molecule in the bilayer and then to measure the potential of forcing it to leave out of the bilayer. For this, one channel molecule was first forced to insert into the bilayer from the *cis* compartment by applying −50 mV, as established in Figure 3, and then the current trace was recorded. The result showed typical single-channel currents, as evidenced by the generation of a relatively stable channel current around 2 pA (Figure 4a). The erratic channel currents may be

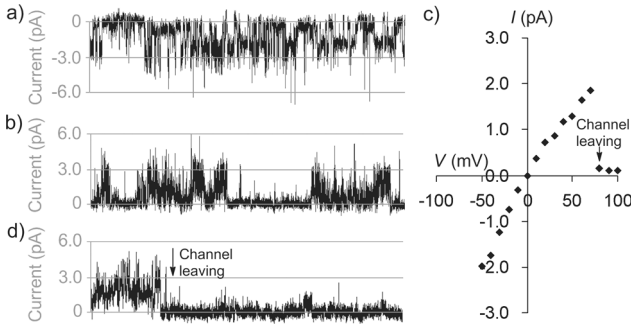

Figure 4. V_{min}(out) determination by recording the single-channel conductance. The typical single-channel current traces (30 s) at a) −50 mV and b) +70 mV. c) The *I-V* curve indicating the linear voltage-current relationship in the range of −50-+70 mV and channel leaving with voltage being +80 mV and higher. The current value represents the mean value generated by the program Fitmaster. d) The current trace (30 s) at +80 mV indicating the presence of single-channel current signals during 0–7 s and the absence of them after that.

attributed to the conformational flexibility of the channel molecule in the lipid bilayer.[17] By stepwise increasing the clamped voltage from −50 to +70 mV (10 mV per step), single-channel conductance state was always observed for all of the pulsing voltages, indicating that the channel molecule stayed in the bilayer throughout the experiment. The conductance traces at +70 mV is provided in Figure 4b as an example. The plot of the applied voltage versus the mean channel current shows a linear relationship in the range of −50–+70 mV (Figure 4c), which corresponded to a channel conductance (γ) of 38 ± 5 pS. When applying a pulse of +80 mV, the conductance trace still showed single-channel currents during the first 7 s (Figure 4d). After that, no current signals were observed, suggesting that the channel molecule was driven out of the bilayer from the *cis* side. No current was observed when the potential was increased to +100 mV, further confirming the absence of the channel molecule in the bilayer. The fact that V_{min}(out) was higher than V_{min}(in) implied that, once inserted, the channel molecule and the surrounding lipid molecules might adapt their conformation to maximize their interaction. Theoretically, applying a −80 mV potential might also drive the channel to leave from the *tans* side of the bilayer. However, it was difficult to differentiate this process from its insertion from the *cis* side by evaluating the changing of the current.

To exploit if the above insertion/leaving of the channel molecules was recyclable, we also performed the patch-clamp experiments at different alternate pulses. The results obtained using −100 (30 s) and +100 mV (60 s) pulses are provided in Figure 5. Under the negative pulse, a negative current was

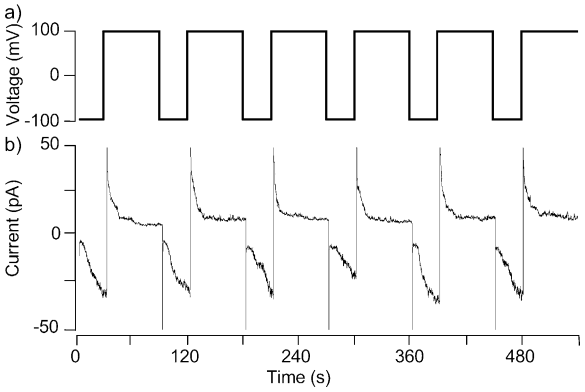

Figure 5. a) The alternate −100 mV (30 s) and +100 mV (60 s) potential pulse protocol. b) The macroscopic current trace of **1b** by applying the pulse in (a).

always observed, which increased significantly with time, whereas under the positive pulse, a positive current was observed, which quickly decreased with time (Figure 5b). These observations clearly supported that alternate channel insertion and leaving took place. The cycle could be run for up to six times. When **1b** was added to the *trans* compartment, reverse voltage-responding was observed under the same conditions.

The voltage-responding behaviors of **1c** and **1d** were then investigated by increasing the voltage from 0 to −140 mV with a step of 10 mV. Both compounds could also be driven by the applied voltage to insert into the lipid bilayer and V_{min}(in) was determined to be −90 and −50 mV, respectively (see Figure S21). However, different from **1b**, once being inserted into the bilayer, the two molecules could not be driven out from the bilayer even under +140 mV which was the highest potential tolerated by the bilayer. One possible reason is that their positively charged Arg units aggregate in the hydrophobic inner of the bilayer, which made them more difficult to move out. As expected, neutral **1a** did not show any voltage-driven insertion in the range of −100–+100 mV. By fitting the *I–V* curves with the exponential method,[18] the gating charges (z_g) for **1b**, **1c**, and **1d** were calculated to be 1.82 ± 0.09, 2.7 ± 0.06, and 1.64 ± 0.08, which were even higher than that of the natural voltage-gated channel melittin (z_g = 1.50)[18b] and represented significant voltage dependences. The z_g value of **1c** is clearly higher than **1b** and **1d**, indicating that **1c** is more voltage sensitive.

It has been established that metabolically active bacteria maintain negative potential in their cell interior and their cell membranes consist of a large fraction of negatively charged lipids which display a great affinity for cationic compounds.[19,20] We envisioned that our new positively charged channels might be able to inhibit the growth of the bacteria by inserting into their membranes.[21] Thus, the antimicrobial activities of **1b–d** were evaluated on three typical bacterial strains, Gram-negative *Salmonella typhimurium*, Gram-positive *Bacillus subtilis*, and *Staphylococcus epidermidis*, using the standard microdilution procedure.[22] It was found that **1c** displayed antimicrobial activity toward *Bacillus subtilis* (see Figure S22). The half maximal inhibitory concentration (IC_{50}) was achieved at 10 μM, which is close to that (2.0 μM) of alamethicin, a natural voltage-gated channel-forming peptide.[23] Under the same conditions, **1b** and **1d** did not show any antimicrobial activity toward the three bacteria.

In conclusion, we have developed a new strategy for controlling the transmembrane transport of artificial channels using a voltage to drive a positively charged channel compound to insert into and leave from a lipid bilayer. One of the channel compounds exhibited antimicrobial activity, which is the important function of many natural channel-forming peptides. This finding holds great promises for the design of more efficient voltage-responding artificial channel inhibitors for bacteria.

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