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Synthetic Chloride Channel Regulates Cell Membrane Potentials and Voltage-Gated Calcium Channels

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Abstract: Synthetic ion channels are of great interest in mimicking the biological functions of natural ion channels. Although many synthetic ion channels could selectively mediate ion transport across lipid bilayer membranes, the biological roles played by these synthetic channels in living systems remain essentially obscure. In this study, we report the application of a synthetic chloride (Cl⁻) channel as a powerful tool to perturb and regulate a series of biological processes related or coupled to the changes of cell membrane potentials. This is the first example in which an artificial ion channel can regulate natural voltage-gated calcium channels, intracellular calcium concentrations, and the contraction of smooth muscle cells via modulating cell membrane potentials in living cells and tissues. The present study on the ability of the synthetic chloride channel to perturb functions of natural ion channels may open new perspectives for the applications of other synthetic ion channels in biological systems.

Introduction

Membrane potential refers to the electrical potential difference across a cell membrane, which is an intrinsic property of living cells. Changes in membrane potential are found in a wide range of important physiological processes including neuronal signaling, muscle contraction, cardiovascular function, and immune response.[1] Ion channels capable of selectively mediating ion transfer across cell membranes play a crucial role in establishing and controlling the membrane potential of a cell. Abnormal membrane potential responses arising from the malfunction of certain ion channels have been shown to be implicated in many severe human diseases such as hypertension, long-QT syndrome, epilepsy, and myotonia congenita.[2,3] At the same time, the membrane potential also directly controls and regulates a class of ion channels, i.e., voltage-dependent ion channels.[1,4]

While the majority of previous studies has focused on regulation of natural ion channels, it remains an intriguing challenge to create synthetic ion channels[5,6] that can mimic the biological functions of natural ion channels. To date, however, most reported synthetic ion channels have been characterized in artificial lipid bilayers and the roles played by these synthetic channels in setting the membrane potentials and thereby regulating natural ion channels in living systems remain

essentially obscure. In this study, we report the application of a synthetic chloride (Cl⁻) channel as a powerful tool to perturb and regulate a series of biological processes related or coupled to the changes of cell membrane potentials. This synthetic Cl⁻ channel shifts resting membrane potential toward Cl⁻ equilibrium potential in living cells by increasing the Cl⁻ permeability of cell membranes. The present study represents the first example of artificial anion channels[7-17] that can regulate natural voltage-gated ion channels, intracellular calcium (Ca²⁺) concentrations, and the contraction of smooth muscle cells via modulating cell membrane potentials in living cells and tissues.

Results and Discussion

Recently, we described a small synthetic molecule **1** (Figure 1A) that self-assembles into a new class of artificial Cl⁻ channels

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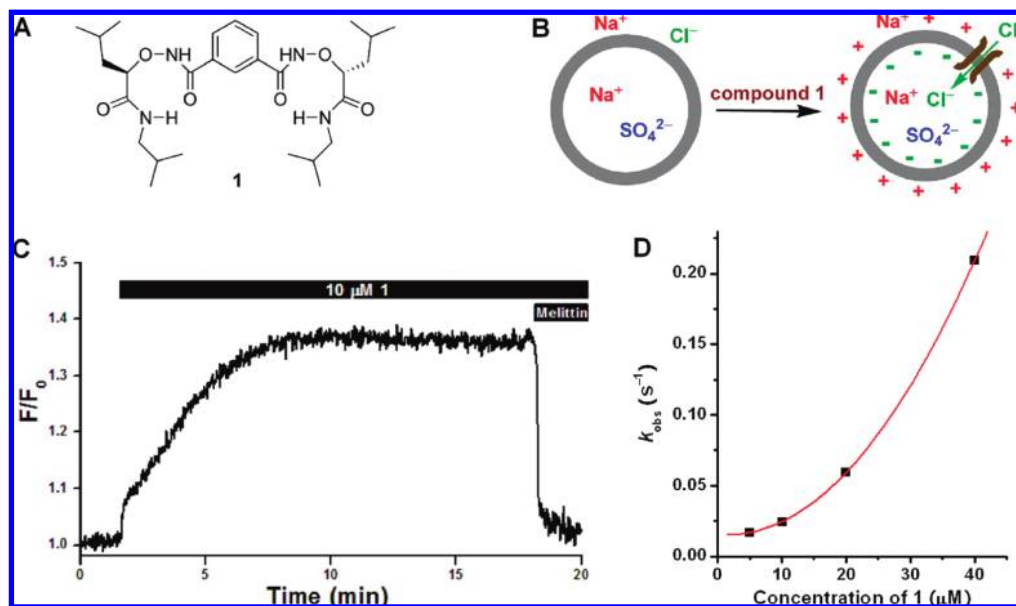
Figure 1. Synthetic Cl$^-$ channel **1** alters membrane potential of liposomes. (A) Chemical structure of compound **1**. (B) Schematic representation of the membrane potential established by compound **1** in a liposome. (C) Fluorescence assay of the membrane potential of EYPC liposomes with compound **1**. Membrane potential of liposome was monitored by the changes in emission intensity of safranin O, a potential-sensitive dye. The increase in fluorescence indicates polarization with increased negative charges inside liposomes. (D) Dependence of membrane potential polarization rate on the concentration of compound **1**.

in lipid bilayer membranes.[18] The effect of a synthetic molecule **1**-derived Cl$^-$ channel is similar to that of natural Cl$^-$ channels as it permits the flow of Cl$^-$ ions across plasma membranes in living cells with remarkable selectivity for anions against cations.[18] This discovery inspired us to further investigate the biological behaviors and functions of the synthetic Cl$^-$ channel formed by **1** in terms of its contributions to cell membrane potentials. To avoid interferences from natural ion channels, we first used liposomes, or artificial lipid bilayer vesicles, as a model system to assess the capacity of **1** to modulate membrane potential.[19-21] Egg yolk phosphatidylcholine (EYPC) liposomes containing sodium sulfate (Na$_2$SO$_4$) were suspended in an isotonic sodium chloride (NaCl) extravesicular solution with a membrane potential-sensitive dye—safranin O.[22,23] As shown in Figure 1C, the addition of **1** to the liposome suspension induced a rapid increase in fluorescence intensity of safranin O, indicating the formation of stable negative charge inside the liposomes. This is because **1** can only mediate Cl$^-$ rather than SO$_4^{2-}$ or Na$^+$ ion transport across lipid bilayers (see the Supporting Information, Figure S4). When Cl$^-$ ions flow into the liposomes via the synthetic Cl$^-$ channels, the liposomes are polarized, that is, the interior of the liposomes becomes progressively more negative relative to the exterior (Figure 1B). Furthermore, a nonlinear dependence of polarization rate of membrane potential induced by **1** on its concentration (Figure 1D) indicated the formation of self-assembled structure responsible for polarization.[24] A Hill coefficient $n = 1.21$ can be interpreted as cooperative and/or higher-order complex formation.[25]

The finding that **1** can generate and maintain a transmembrane potential prompted us to test its capacity for modulating membrane potential in polarized liposomes.[19,20] In this assay, EYPC-liposomes encapsulating potassium chloride (KCl) were suspended in an isotonic mixture of KCl and Na$_2$SO$_4$ solution to produce a transmembrane K$^+$ concentration gradient (Figure 2A). The membrane potential of the liposomes was monitored by changes in fluorescence intensity of extravesicular safranin O. As shown in Figure 2B, exogenous addition of the selective K$^+$ carrier valinomycin resulted in K$^+$ efflux, which polarized the liposome by establishing a membrane potential with net negative charges inside the liposomes. Subsequently, the application of compound **1** rapidly depolarized the EYPC-liposomes. This depolarization process induced by **1** can be unambiguously attributed to the formation of synthetic Cl$^-$ channels by **1** which subsequently mediate the flow of Cl$^-$ ions out of the polarized liposomes to balance the electrostatic potential established by valinomycin (Figure 2A). Similar to the effect on the polarization of liposomes, compound **1** depolarized the polarized liposomes in a nonlinear concentration-dependent manner with a Hill coefficient $n = 1.22$ (Figure 2C). In addition, the rate of **1**-induced depolarization of the liposomes showed a nonlinear dependence on the transmembrane potential initialized by valinomycin (Figure 2D), i.e., the activity of **1** on modulating liposomal membrane potential is voltage-dependent.

To explore the potential applications of compound **1** in biological and pharmacological sciences, we investigated whether compound **1** can modulate cell membrane potential by increasing Cl$^-$ permeability of cell membranes. We have previously demonstrated that compound **1** can mediate chloride transport across cell membranes of the Madin–Darby canine kidney (MDCK) cells,[18] a useful model system for the study of transepithelial ion transport.[26] Thus, MDCK cells were chosen

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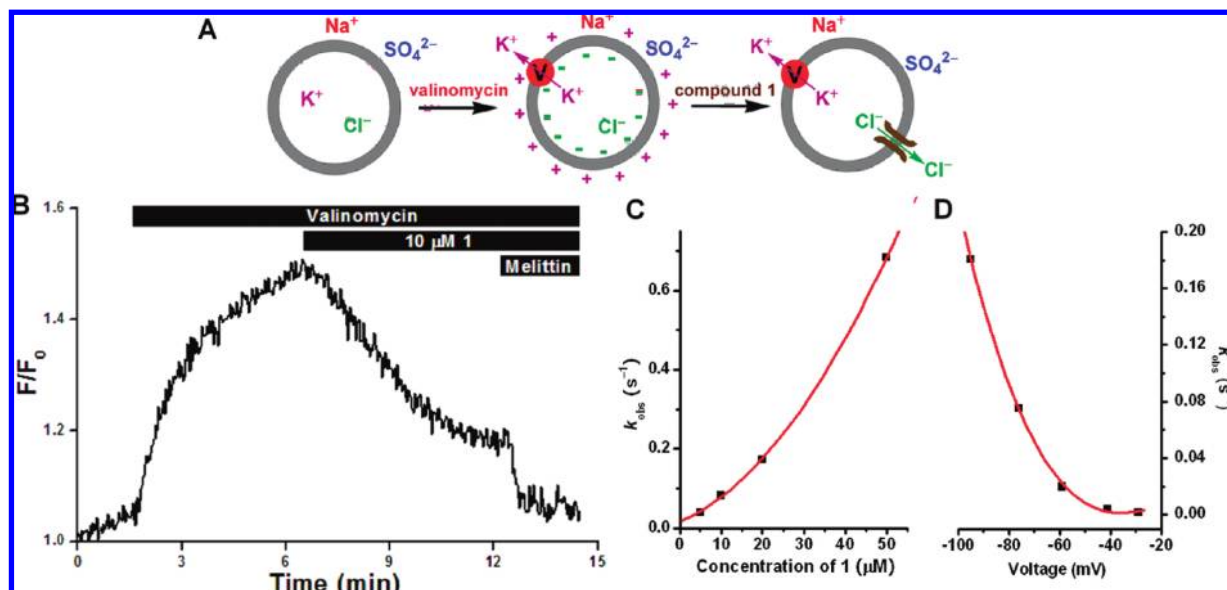(20) Sakai, N.; Matile, S. *J. Am. Chem. Soc.* **2002**, *124*, 1184–1185.
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Figure 2. Synthetic Cl$^-$ channel **1** modulates membrane potential of liposomes. (A) Schematic representation of the depolarization of a valinomycin-polarized liposome by compound **1**. (B) Fluorescence assay of the membrane potential of valinomycin polarized ($E = -76$ mV) EYPC liposomes with and compound **1**. Membrane potentials of liposomes were monitored by the changes in emission intensity of safranin O. The increase in fluorescence indicates polarization with increased negative charges inside liposomes. (C) Dependence of membrane potential depolarization rate on the concentration of compound **1** at $E = -76$ mV. (D) Dependence of membrane potential depolarization rate induced by compound **1** (10 μM) on the membrane potential.

to illustrate the effect of compound **1** in modulating cell membrane potential. We used a potential-sensitive dye *bis*-oxonol to measure the relative changes in the membrane potential of MDCK cells. An increase in fluorescence of *bis*-oxonol indicates cell depolarization while a decrease suggests hyperpolarization. The major anion conductive pathway in MDCK cells is the Cl$^-$ channels activated by cAMP-dependent phosphorylation.[27] Treatment of MDCK cells with forskolin, an agonist of intracellular cyclic AMP that can activate those Cl$^-$ channels in the cells, produced a moderate increase in fluorescence of *bis*-oxonol, indicating depolarization of the cells (Figure 3A). This depolarization effect may be accounted for by the fact that the Cl$^-$ equilibrium potential (E_{Cl}) is higher than the resting membrane potential in MDCK cells.[27−29] Thus, the opening of Cl$^-$ channels shifts the membrane potential toward the E_{Cl}. As expected, the application of **1** also showed a similar depolarization effect on the membrane potential (Figure 3B). This is consistent with the capacity of **1** to increase Cl$^-$ permeability in MDCK cells by forming synthetic Cl$^-$ channels.[18]

As shown in Figure 3C, reducing extracellular Cl$^-$ concentration from 140 mM to 100 mM elicited intracellular Cl$^-$ efflux through a positive shift in the E_{Cl} and, therefore, depolarized the membrane potential. Interestingly, subsequent addition of compound **1** clearly facilitated this depolarization process as compound **1**-derived synthetic Cl$^-$ channels increased cell Cl$^-$ permeability. In addition, despite of the presence of a natural Cl$^-$ channel blocker, diphenylamine-2-carboxylate (DPC), which entirely inhibited the depolarization induced by low-Cl$^-$ extracellular solution, compound **1** still depolarized the cells by restoring chloride permeability of the cell membranes (Figure

3, parts D and E). These results confirmed the ability of **1** to modulate membrane potentials of living cells through forming artificial Cl$^-$ channels independent of the natural ones.

In biological systems, changes in membrane potential induced by opening a certain ion channel invariably affects the behavior of other ion channels.[1,30] The contraction of muscle cells is a classic example of this. It is well-known that a rise in intracellular Ca^{2+} concentration ([Ca^{2+}]$_i$) triggers a variety of cellular responses in biological processes such as the release of neurotransmitters from nerve terminals and muscle contraction.[4] The voltage-gated Ca^{2+} channels that mediate Ca^{2+} influx across the plasma membrane serve as one of the important pathways for regulating the [Ca^{2+}]$_i$ in vascular smooth muscle cells.[3,31] It is therefore interesting to investigate whether the synthetic Cl$^-$ channel formed by **1** can regulate natural voltage-gated Ca^{2+} channels by modulating membrane potential in vascular smooth muscle cells.

To this end, the rat thoracic aortic smooth muscle cell line A7r5 was used to elucidate the effect of **1** on the membrane potential and [Ca^{2+}]$_i$ of vascular smooth muscle cells. As shown in Figure 4, parts A and B, treatment of smooth muscle cells with 60 mM K$^+$ (high-K$^+$) extracellular solution strongly depolarized the membrane potential and stimulated a dramatic increase in [Ca^{2+}]$_i$. This observation is predictable as it is known that high-K$^+$ extracellular solution, through a positive shift in the K$^+$ equilibrium potential (E_K), can depolarize cell membrane potential to activate voltage-gated L-type Ca^{2+} channels. This then raised the [Ca^{2+}]$_i$. However, the subsequent addition of compound **1** to this high-K$^+$ extracellular solution resulted in a slow decrease in [Ca^{2+}]$_i$ to almost the resting level (Figure 4B), suggesting that **1** may repolarize the cell membrane to deactivate the voltage-gated calcium channels. This assumed repolarization process was further confirmed by measuring the cell membrane potential of A7r5 cells under the same experimental conditions (Figure 4A). The results suggest that the high-K$^+$ extracellular

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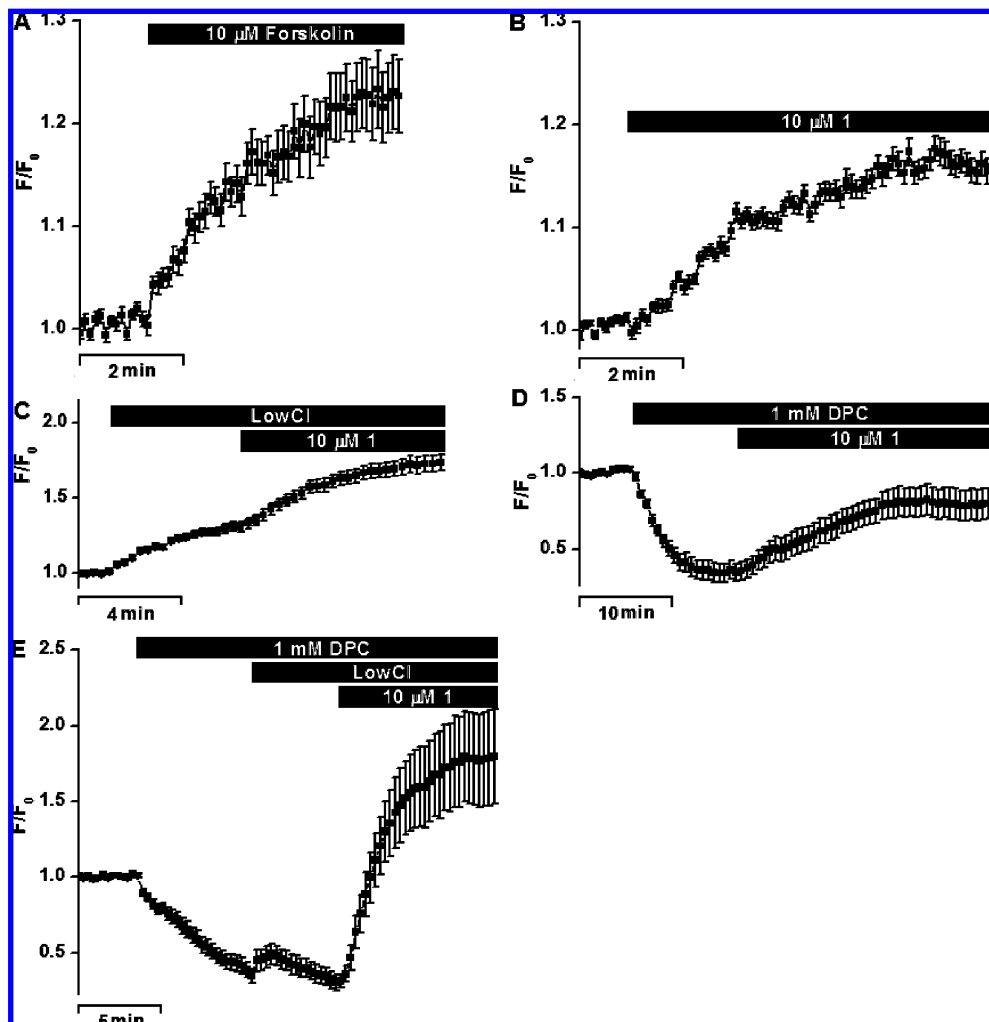(27) Breuer, W. V.; Mack, E.; Rothstein, A. *Pflügers Arch.* **1988**, *411*, 450–455.
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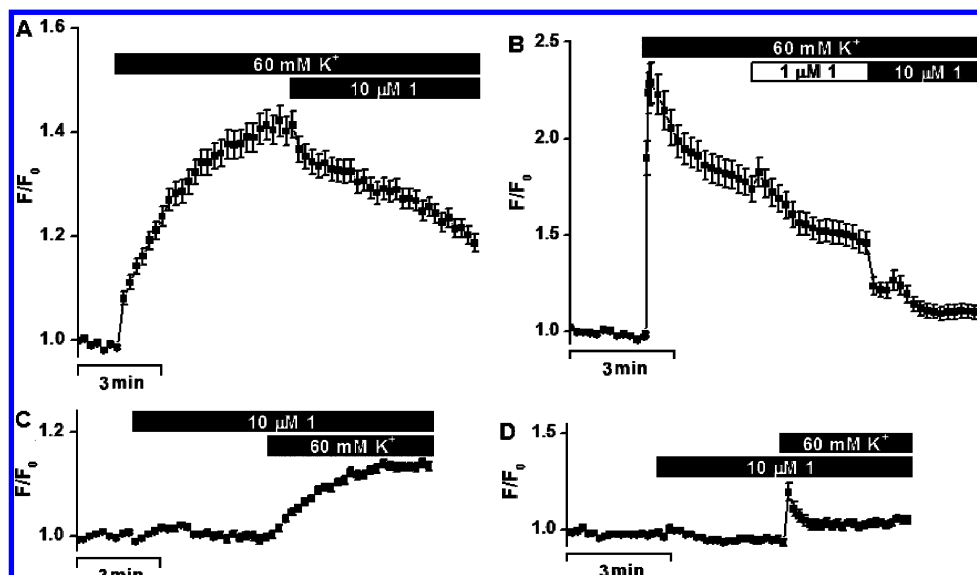(31) Morgan, K. G. *Am. J. Med.* **1987**, *30*, 9–15.

Figure 3. Effect of synthetic Cl^- channel **1** on the membrane potential of MDCK cells. Membrane potentials of MDCK cells were monitored by the changes of emission intensity of the potential-sensitive dye *bis*-oxonol (DiBAC$_4$(3)). The increase in fluorescence indicates depolarization. Applications of (A) 10 μM foskolin and (B) 10 μM compound **1** slightly depolarized the membrane potential of MDCK cells. (C) Perfusion with relatively low Cl^- (100 mM Cl^-) solution resulted in depolarization, which was facilitated in the presence of 10 μM compound **1**. (D) Application of 1 mM DPC hyperpolarized the membrane potential and the subsequent addition of 10 μM compound **1** shifted the hyperpolarized membrane potential toward the original resting potential. (E) Perfusion with low Cl^- (60 mM Cl^-) Ringer's solution failed to depolarize the membrane potential in the presence of 1 mM DPC, whereas the final application of 10 μM compound **1** resulted in the depolarization. Each point represents the mean $\pm$ s.e. ($n = 20-80$ cells in 4-6 experiments).

solution depolarized the membrane potential to a level that was not only sufficient to activate the L-type Ca^{2+} channels but also greater than the E_{Cl} of these cells. Consequently, compound **1**, which forms artificial Cl^- channels in the cell membranes, could increase the Cl^- permeability and thus shift the membrane potential back (i.e., repolarize the membrane potential) toward E_{Cl} that is likely to be lower than the activation potential of L-type Ca^{2+} channels in these cells.

Furthermore, the prior application of **1** showed no obvious effect on both the resting membrane potential and the $[Ca^{2+}]_i$ of A7r5 cells (Figure 4C and 4D). This result ruled out the possibility that **1** blocks the native potassium channels in these cells because the inhibition of native potassium channels can always cause depolarization of the cells. It also implies that the E_{Cl} of A7r5 cells is close to the resting cell membrane potential. In addition, the high-K^+ induced depolarization effect on the membrane potential and the changes in $[Ca^{2+}]_i$ was remarkably inhibited by the pretreatment of the cells with **1** (Figure 4, parts C and D). This result revealed that by increasing Cl^- permeability, **1** could balance the depolarization effect elicited by the positively shifted E_K. Taken together, compound **1** is shown to

be the first synthetic ion channel that can regulate $[Ca^{2+}]_i$ by modulating membrane potential in living cells. Compared with using other agents that activate or inhibit certain natural ion channels through direct binding to them, the application of synthetic small molecule **1** (which self-assembles to form artificial Cl^- channels) clearly stands out as a novel strategy for regulating the physiological functions of natural ion channels.

We expected that the artificial Cl^- channels formed by **1** can promote vasorelaxation during vasoconstriction induced by depolarization because the contraction of vascular smooth muscle cells depends on an increase in $[Ca^{2+}]_i$.[31] Thus, we performed experiments on mouse thoracic aortic rings mounted in an organ bath apparatus and measured their muscular activity. Figure 5A shows a typical experiment in which increasing the concentrations of compound **1** from 1 to 30 μM induced the complete relaxation of endothelium-denuded mouse aortic rings preconstricted with high-K^+ solution. Compound **1** produced a concentration-dependent relaxation of preconstricted aortic rings with half-maximal relaxation value IC$_{50}$ of 8.42 $\pm$ 0.18 μM ($n = 5$).

Figure 4. Effect of synthetic Cl^- channel **1** on regulating the membrane potential and the $[Ca^{2+}]_i$ of A7r5 cells. Membrane potentials of A7r5 cells were monitored by the changes of emission intensity of the potential-sensitive dye *bis*-oxonol (DiBAC$_4$(3)). The increase in fluorescence indicates depolarization. Intracellular Ca^{2+} concentration of A7r5 cells was monitored by the changes of emission intensity of calcium-sensitive fluorescent dye Fluo-4. The increase in fluorescence indicates the rise in the intracellular Ca^{2+} concentration. Perfusion with high-K^+ solution (A) depolarized the membrane potential and (B) raise the $[Ca^{2+}]_i$, and the subsequent application of 10 μM compound **1** resulted in (A) the repolarization and (B) the concentration-dependent decrease in the $[Ca^{2+}]_i$. In the presence 10 μM compound **1**, perfusion with high-K^+ solution resulted in (C) much weaker depolarization of the membrane potential and (D) much lower increase in the $[Ca^{2+}]_i$ than those in the absence of **1**. Each point represents the mean $\pm$ s.e. (n = 20–80 cells in 4–6 experiments).
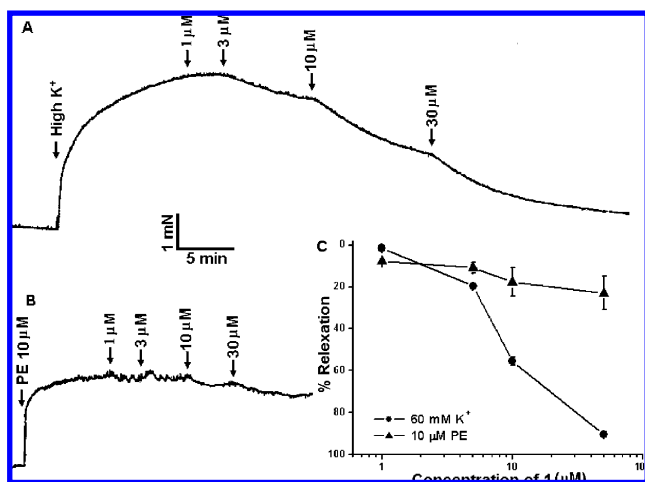
Figure 5. Vasorelaxant effect of synthetic Cl^- channel **1** on mouse aorta. The original traces of vasorelaxation induced by **1** on the aortic rings preconstricted by (A) 60 mM K^+ and (B) the α-adrenergic agonist phenylephrine (PE, 10 μM). (C) Concentration-dependent curves for the vasorelaxant effect of **1** on the mouse aortic rings preconstricted by 60 mM K^+ ($\bullet$) and 10 μM PE ($\blacktriangle$). Each point represents the mean $\pm$ s.e. (n = 4–5).

Besides depolarizing membrane potential, another approach commonly used to induce muscle contraction is to activate the α-adrenergic receptor. To verify whether the vasorelaxant action of **1** is solely due to the regulation of membrane potential of smooth muscle cells, we also examined the effect of **1** on the relaxation of mouse aortic rings constricted by the α-adrenergic receptor agonist phenylephrine (PE). Figure 5B presents a typical experiment wherein **1** failed to relax those aortic rings (n = 4). Therefore, we propose that compound **1** relaxes the preconstricted mouse aortic rings by repolarizing membrane potential to deactivate voltage-gated Ca^{2+} channels in smooth muscle cells, which is similar to the proposed functional role played

by cystic fibrosis transmembrane conductance regulator (CFTR) Cl^- channels in smooth muscle cells in controlling the vascular tone.[32] This effect may also underly the possibility of using synthetic Cl^- channels to treat hypertension.

In summary, the Cl^- channels formed by small synthetic molecule **1** represents the first example of artificial ion channels that can modulate cell membrane potential by shifting the membrane potential toward the Cl^- equilibrium potential. This synthetic Cl^- channel also regulates the voltage-gated Ca^{2+} channels and the $[Ca^{2+}]_i$ of smooth muscle cells and promotes vasorelaxation of preconstricted mouse aorta through modulating the cell membrane potentials. Compared with the core of the field of chemical biology, in which small molecules as utilized as ligands to activate or inhibit the functions of natural proteins, the present study represents a novel strategy to perturb biological processes. Instead of direct interaction with natural ion channel proteins, the synthetic small molecule self-assembles into a functional ion channel that not only mediates ions flow across cell membrane, but also regulates the physiological functions of relevant natural ion channel proteins in a biological system. Therefore, we believe that the present study on the synthetic Cl^- channel will help to explore the novel functional roles played by natural Cl^- channels in physiological processes and open new perspectives for the applications of other artificial ion channel mimics in regulating natural ion channels in biological systems.

Supporting Information Available: Detailed experimental methods. This information is available free of charge via the Internet at http://pubs.acs.org.

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