A synthetic chloride channel regulates cell membrane potentials and voltage-gated calcium channels

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Supporting Information

Methods

Preparation of Na_2SO_4- or KCl-filled liposomes. Egg yolk L-α-phosphatidylcholine (EYPC, 91 mg, 120 μmol) was dissolved in a $CHCl_3$/MeOH mixture. The solution was evaporated under reduced pressure and the resulting thin film was dried under high vacuum for 3 h. The lipid film was then hydrated in 1.2 mL of intravesicular solution (10 mM HEPES, pH = 6.8, 75 mM Na_2SO_4 or 100 mM KCl) for 2 h. During hydration, the suspension was subjected to 5 freeze-thaw cycles (liquid nitrogen, water at r.t.). The large multilamellar liposome suspension (1 mL) was submitted to high-pressure extrusion at r.t. (25 extrusions through a 0.1 μm polycarbonate membrane afforded a suspension of LUVs with an average diameter of 100 nm). The LUV suspension was diluted with intravesicular solution to give a stock solution with a lipid concentration of 10 mM (assuming that 100% of lipid was incorporated into liposomes).

Detection of membrane potential of liposomes. Typically, 100 μL of stock solution of Na_2SO_4-filled (10 mM HEPES, pH = 6.8, 75 mM Na_2SO_4 inside and outside) or KCl-filled liposomes (10 mM HEPES, pH = 6.8, 100 mM KCl inside and outside) was suspended in 1.9 mL of NaCl-HEPES buffer (10 mM HEPES, pH = 6.8, 100 mM NaCl and 60 nM safranin O) or Na_2SO_4-HEPES buffer (10 mM HEPES, pH = 6.8, 75 mM Na_2SO_4 and 60 nM safranin O) and placed into a fluorimetric cell. The emission of safranin O at 580 nm was monitored with excitation wavelength of 520 nm. During the experiment with Na_2SO_4-filled liposomes, 20 μL of a 1 mM THF solution of compound **1** was added through an injection port. During the experiment with KCl-filled liposomes, 20 μL of a 60 μM aqueous solution of valinomycin was added through an injection port. Once stable emission was observed, 20 μL of a 1 mM THF solution of compound **1** was added through an injection port. All the experiments were completed by the injection of 50 μL of 0.1 mM aqueous solution of the defect-inducing peptide melittin. The ratio of the fluorescence intensity F/F_0 was employed to indicate the changes of membrane potential. The membrane potential depolarization rates were measured as the initial rate of fluorescence changes during the first 20s after adding compound **1**. The data were fitted according to the following equation:

$$k_{obs} = k_0 + k_{int}\,[\text{monomer}]^n/K_D$$

where K_D is the dissociation constant, k_{obs} the observed transport rate, k_0 the rate without pore, and k_{int} an intrinsic rate constant.

Calibration of fluorescence of safranin O response to membrane potential.

50 μL stock solution of KCl-filled liposomes (10 mM HEPES, pH = 6.8, 100 mM KCl inside and outside) was suspended in 1.95 mL of isotonic extravesicular solution (10 mM HEPES, pH = 6.8, 75 mM Na_2SO_4 and 60 nM safranin O) and placed into a fluorimetric cell. Safranin O emission at 580 nm was monitored with excitation wavelength of 520 nm. During the experiment, 20 μL of a 60 μM aqueous solution of valinomycin was added through an injection port. Once stable emission was observed, 50 μL of a 100 mM KCl solution was added to the extravesicular solution and the safranin O emission was recorded. Fluorescence intensities were monitored upon subsequent addition of 100, 200, 250 and 400 μL of 100 mM KCl solution. The observed fluorescence intensities were divided by the total volume (in mL) to adjust the dilution factor. Theoretical membrane potentials were calculated based on the Nernst equation: $V(\text{mV}) = 59\times\log([K^+_{out}]/ [K^+_{in}])$ at 25 °C, where $[K^+_{out}]$ and $[K^+_{in}]$ are the extravesicular and intravesicular K^+ concentrations, respectively. In the experiment, the $[K^+_{in}]$ was kept constant at 100 mM while the $[K^+_{out}]$ was varied as 2.48, 4.83, 9.22, 16.88, 24.81 and 34.77 mM, respectively. Therefore, the corresponding calculated membrane potentials were –94.8, –77.6, –61.1, –45.6, –35.7 and –27.1 mV, respectively. Linear correlation was found between adjusted fluorescence intensities and theoretical membrane potential within the tested range (Figure S1).

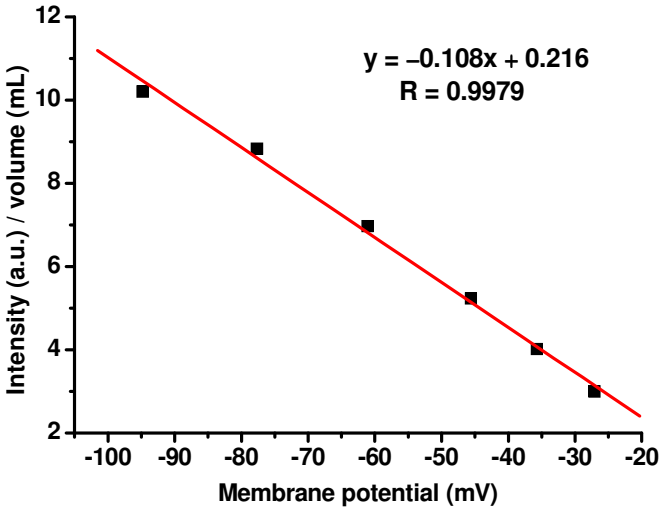

Figure S1. Plot of emission intensity of safranin O as a function of applied membrane potential.

Mammalian cell culture. The type I MDCK cells were a generous gift of Dr. Leo LM Poon (Department of Microbiology, The University of Hong Kong, Hong Kong). The cells were cultured in DMEM supplemented with 10% fetal bovine serum (FBS) and 100 U/mL penicillin and 0.1 mg/mL streptomycin. Cells were grown in plastic culture flasks at 37℃ in a 5% CO_2 humidified incubator and passaged every 3–5 days.

Rat aorta smooth muscle (A7r5) cells were from American Type Culture Collection. The cells were cultured in DMEM supplemented with 10% fetal bovine serum (FBS) and 100 U/ml penicillin and 0.1 mg/ml streptomycin. Cells were grown in plastic culture flasks at 37℃ in a 5% CO_2 humidified incubator and passaged every 3–5 days.

Measurement of Membrane Potential of MDCK and A7r5 cells. Membrane

potential of MDCK and A7r5 cells were monitored by the changes in emission intensity of the potential-sensitive dye *bis*-oxonol ($DiBAC_4(3)$). The cells cultured on coverslips were placed into Ringer's solution containing 140 mM NaCl, 5 mM KCl, 2 mM $CaCl_2$, 1 mM $MgCl_2$, 10 mM glucose, 5 mM HEPES (pH 7.4 with NaOH) and 100 nM *bis*-oxonol and incubated in 37℃ for 10 min to equilibrate the dye between intracellular cytosol and extracellular solution. Subsequently, the effects of the different reagents on membrane potential were determined. The stock solution of compound **1** in DMSO (0.1% at final) was used in all the experiments. The fluorescence signals were measured by FluoView 1000 (Olympus America Inc.) laser scanning confocal imaging system whose excitation and emission wavelengths were set at 488/520 nm, and the data were analyzed by FV10-ASW 1.5 software. The readings were collected at 8-second intervals. The ratio of the fluorescence intensity F/F_0 was employed to indicate the changes of membrane potential. Each experiment involved 10–20 cells and was performed at room temperature (22–25℃).

Calibration of fluorescence of *bis*-oxonol response to cell membrane potential.

The procedure for calibration of *bis*-oxonol ($DiBAC_4(3)$) was previously described in Breuer W. V.; Mack E.; Rothstein A. *Pflugers Arch*. **1988**, *411*, 450. and Dall'Asta V.; Gatti R.; Orlandini G.; Rossi P. A.; Rotoli B.M.; Sala R.; Bussolati O.; Gazzola G. C. *Exp Cell Res*. **1997**, *231*, 260. In presence of the Na^+/K^+ ionophore gramicidin (2 μg/ml) in Na^+-free Ringer's solution (140 mM NMDG-Cl, 5 mM KCl, 2 mM $CaCl_2$, 1 mM $MgCl_2$, 10 mM glucose, and 5 HEPES, pH 7.4), the membrane potential under these conditions is determined by the Nernst equation at 25□:

$E_m = -59 \log ([K^+ + Na^+]_e/[K^+ + Na^+]_i)$

where E_m is the membrane potential, and $[K^+ + Na^+]_i$ and $[K^+ + Na^+]_e$ are the intracellular and extracellular concentrations, respectively. In the equation, $[K^+ + Na^+]_i$ was assumed to be 140 mM. In the presence gramicidin in the Na^+-free solution, the fluorescence intensity of the cell equilibrated with *bis*-oxonal named F_0. When stepwise elevations in $[K^+]_e$ induced depolarization, the fluorescence intensity (F) increased gradually. The osmolality was maintained constant by reduction of NMDG-Cl. Thus, membrane potential were calculated from the Nernst equation with the different $[K^+ + Na^+]_e$. The calibration curves of the relationship between the fluorescence intensity ratio of F/F_0 and the membrane potential was constructed and used to estimate the changes in the membrane potential of MDCK cells (Figure S2) and A7r5 cells (Figure S3). An increase in fluorescence by 1% corresponds to a depolarization of 0.24 mV for MDCK cells and 0.19 mV for A7r5 cells, respectively, as calculated from the mean calibration curves.

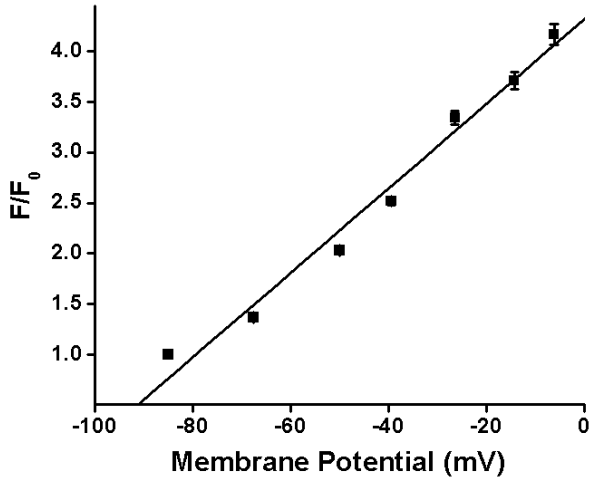

Figure S2. The calibration curve of the relationship between the ratio of the fluorescence intensity F/F_0 and the membrane potential of MDCK cells (Each point represents the

mean ± s.e. (n = 40 cells in 3 experiments).

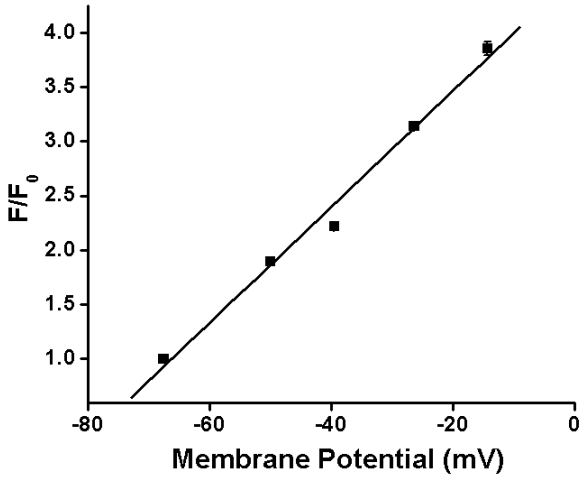

Figure S3. The calibration curve of the relationship between the ratio of the fluorescence intensity F/F_0 and the membrane potential of A7r5 cells (Each point represents the mean ± s.e. (n = 60 cells in 3 experiments).

Measurement of intracellular Ca^{2+} concentration of A7r5 cells.

Intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) of A7r5 cells was measured by using a calcium-sensitive fluorescent dye Fluo-4. Briefly, cells grown on coverslips were loaded with 2 μM Fluo-4/AM in a Ringer's solution containing 140 mM NaCl, 5 mM KCl, 2 mM $CaCl_2$, 1 mM $MgCl_2$, 10 mM glucose, and 5 HEPES (pH 7.4 with NaOH) at 37□ for 20 minutes, and then rinsed with the Ringer's solution. $[Ca^{2+}]_i$ was recorded by a FluoView 1000 laser scanning confocal imaging system with excitation/emission at 488/520 nM, and the data were analyzed by FV1000 Viewer software. The ratio of the fluorescence intensity F/F_0 was calculated. Each experiment had 10–20 cells and was done at room temperature (22℃–25□).

Isolation of Thoracic Aortic Strip Segment and Tension Measurement.

All animal experiments were conducted in accordance with the regulation of the U.S.

National Institute of Health (NIH publication No.8523). The isolated thoracic aortic strip segment and tension measurement was the same as that described previously. Briefly, after the mice were killed by cervical dislocation, at room temperature (22℃ –25□) thoracic aorta of mouse was quickly dissected free and placed in Krebs Henseleit solution containing 118 mM NaCl, 4.7 mM KCl, 2.5 mM $CaCl_2$, 1.2 mM KH_2PO_4, 1.2 mM $MgSO_4$, 25.2 mM $NaHCO_3$, and 11.1 mM glucose (pH 7.4 with NaOH). Under a dissecting microscope, adhering perivascular tissue was carefully removed, and the descending thoracic aorta was cut into 2-mm long rings. The endothelium was mechanically rubbed off with a small piece of plastic tubing. The vessels were mounted onto two thin stainless steel holders in 5 mL organ baths containing Krebs Henseleit solution at 37℃, and continuously bubbled with a gas mixture of 95 % O_2 and 5 % CO_2 to maintain a pH of 7.4. A movable device allowed the application of a passive tension of 500–550 mg, which were determined to be the optimal resting tension for obtaining the maximal active tension induced by high-K^+ solution containing 42.7 mM NaCl, 80 mM KCl, 2.5 mM $CaCl_2$, 1.2 mM KH_2PO_4, 1.2 mM $MgSO_4$, 25.2 mM $NaHCO_3$, and 11.1 mM glucose (pH 7.4 with NaOH). The isometric tension was recorded on a Multi Myograph System (Danish Myo Technology A/S, Denmark). After an equilibration period of 1 h, the contractile function of vessel was tested twice by replacing the Krebs Henseleit solution with the high K^+ solution. After washout, the vessel was contracted once with phenylephrine (PE) 10 μmol/L for 10 min and then relaxed with acetylcholine (ACh) 10 μmol/L for 4 min. The integrity or functional removal of endothelium was verified by the relaxant

response to 1 μmol/L ACh at the start of each experiment. After another washout period, the cumulative dose-response of compound **1** was tested after preconstrictions evoked by the high-K^+ solution or 10 μmol/L PE.

Ion selectivity studied by pH-stat fluorometric assay

We used the liposome-based pH-stat fluorometric assay (Matile, S. & Sakai, N. The characterization of synthetic ion channels and pores. In *Analytical Methods in Supramolecular Chemistry*, ed Schalley C (Wiley, Weinheim, 2007) pp 391–418.) to study the ion transport activities of compound **1**. In this assay, a controlled amount of NaOH was added to a suspension of liposomes containing a pH-sensitive dye, 8-hydroxypyrene-1,3,6- trisulfonate (HPTS). The resulting pH gradient across the bilayer membrane causes H^+ efflux or OH^- influx and a built-up an electrostatic potential, which may be balanced by the efflux of anions or the influx of cations mediated by the potential synthetic channels. We monitored the increase in the intravesicular pH, which reflected the degree of cation or anion transport across the membrane, through the increase in the relative fluorescence (I_{460}/I_{403}). For NaCl-filled liposomes suspended in an isotonic NaCl solution, addition of **1** resulted in a rapid pH-induced ion flux across the lipid bilayers of the liposomes (Fig. S4). In contrast, addition of **1** in the symmetrically loaded Na_2SO_4 liposomes did not induce intravasicular pH changes, indicating **1** is only able to mediate Cl^- rather than Na^+ or SO_4^{2-} transport across lipid bilayer.

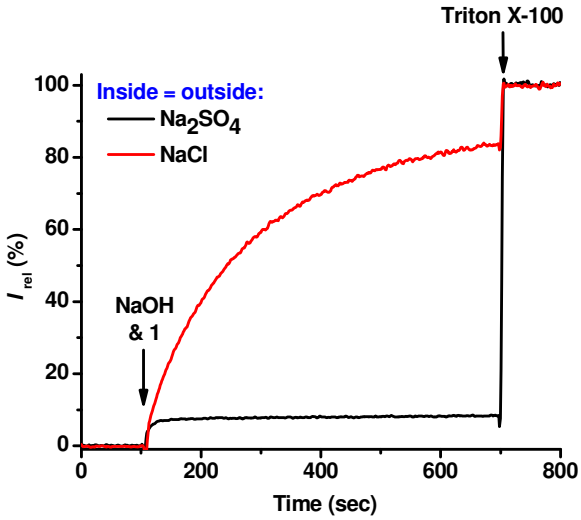

Figure S4. pH-stat ion transport assays illustrating the ion transport activities of compound **1**. All experiments employed suspensions of EYPC liposomes containing the pH-sensitive dye HPTS in a HEPES buffer. Both the intra- and extravesicular solutions contained 10 mM HEPES (pH 6.8) and 100 or 75 mM Na_nX (X = Cl^-, or SO_4^{2-}). At t = 100 s, a THF solution (20 μL) of the tested compound at a final concentration of 10 μM was added to the extravesicular solution; 0.5 M NaOH solution (20 μL) was then added. At t = 700 s, 5% Triton X-100 (40 μL) was added to lyse the liposomes.

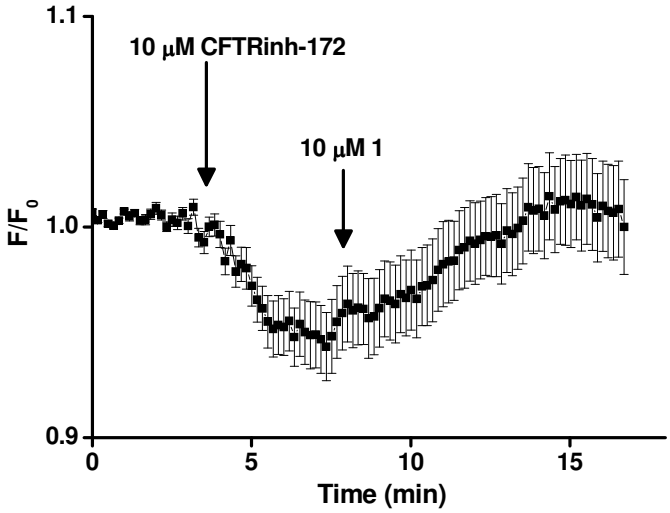

Figure S5. Effect of compound **1** on the membrane potential of MDCK cells. Membrane potentials of MDCK cells were monitored by the changes of emission intensity of the potential-sensitive dye *bis*-oxonol ($DiBAC_4(3)$). The increase in fluorescence indicates

depolarization. Application of 10 μM CFTRinh-172 hyperpolarized the membrane potential and the subsequent addition of 10 μM compound **1** shifted the hyperpolarized membrane potential toward the original resting potential. Each point represents the mean ± s.e. (n = 60 cells in 4 experiments).

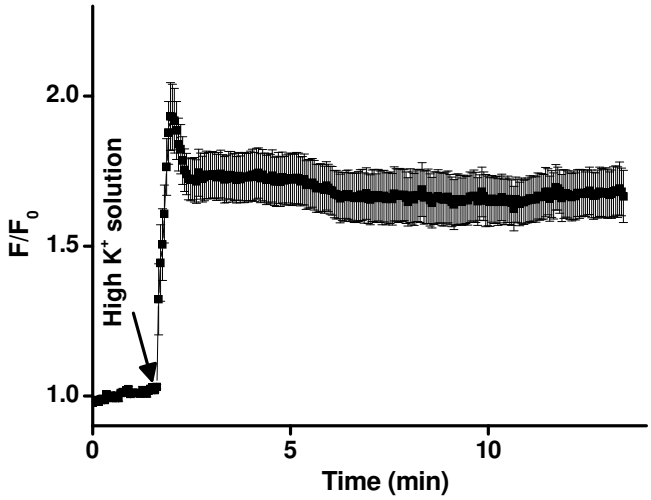

Figure S6. Effect of high K^+ solution on the $[Ca^{2+}]_i$ of A7r5 cells. Intracellular Ca^{2+} concentration of A7r5 cells was monitored by the changes of emission intensity of calcium-sensitive fluorescent dye Fluo-4. The increase in fluorescence indicates the rise in the intracellular Ca^{2+} concentration. Perfusion with high-K^+ solution Each point represents the mean ± s.e. (n = 50 cells in 4 experiments).

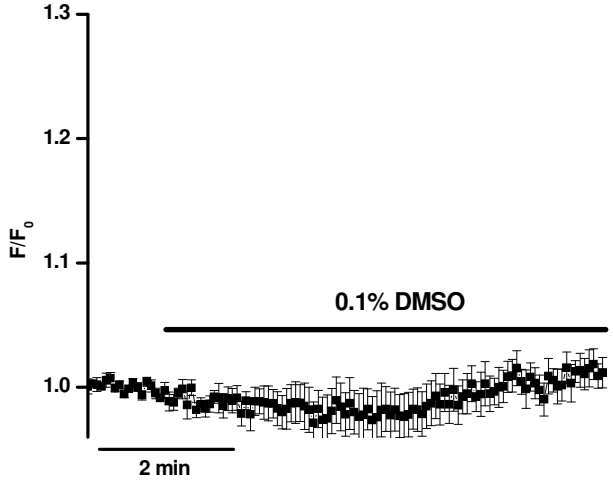

Figure S7. Effect of the vehicle of compound **1** (DMSO) on the $[Ca^{2+}]_i$ of A7r5 cells. Each point represents the mean ± s.e. (n = 40 cells in 3 experiments).