Supporting Information

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SI Materials and Methods

Materials. The lipids, 1,2-dioleoyl-*sn*-glycero-3-phosphocholine (DOPC), 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine-*N*-[4-(*p*-maleimidomethyl)cyclohexane-carboxamide] sodium salt (PE-maleimide), 1,2-distearoyl-*sn*-glycero-3-phosphoethanolamine-*N*-[carboxy(polyethylene glycol)-2000] ammonium salt (DSPE-PEG_{2000}-COOH), and 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine-*N*-[lissamine rhodamine B sulfonyl] (Rho-PE), were purchased from Avanti Polar Lipids. Fluorescent dye, Alexa Fluor 488 C_5 maleimide (Alexa 488), was from Life Technologies. Ingredients for physiologic solutions were purchased from Sigma-Aldrich.

Conjugation of pHLIP Variants with Alexa Fluor 488 and Lipids. Each pHLIP variant (WT, Var7, and kVar7) was conjugated with fluorescent dye, Alexa 488-maleimide, or lipid, PE-maleimide, at a single Cys residue in the N terminus to obtain Alexa 488-pHLIP or pHLIP-PE lipids. The conjugation of Alexa Fluor 488 with WT, Var7, and kVar7 pHLIPs was carried out in dimethylformamide with slight excess of peptide via incubation at room temperature for about 6 h and then at 4 °C until the conjugation reaction was completed. The conjugation of PE-maleimide with WT pHLIP was carried out in methanol, and the reaction mixture was left overnight while being stirred at room temperature under argon. The product was characterized by surface-enhanced laser desorption/ionization–TOF mass spectroscopy. The concentration of Alexa 488-pHLIP variants was determined by absorbance at 488 nm (ε_{488} = 71,000 $M^{-1}{\cdot}cm^{-1}$).

Liposome Preparation. Liposomes of two different compositions were prepared by extrusion: liposomes coated with pHLIP (5 mol % pHLIP-PE, 5% Rho PE, 90% DOPC) and liposomes coated with PEG (5% DSPE-PEG_{2000}-COOH, 5% Rho-PE, 90% DOPC). A chloroform solution of total 1 μmol of lipid composite of the DOPC, Rho-PE, pHLIP-PE, or DSPE-PEG_{2000}-COOH was added in above-mentioned mol % proportions into a flask and evaporated using rotary evaporator, producing an even, thin film. The film was placed under vacuum overnight to remove traces of solvent. The film was then hydrated in 1 mL of PBS, pH 7.4, buffer solution followed by 10 freeze–thaw cycles. The resulting liposome solution was extruded 31 times through a 80-nm polycarbonate filter and sterilized by filtering through a 0.2-μm filter. The concentration of lipids was quantified by diluting a small amount of the liposome solution in MeOH and measuring the OD at the rhodamine absorbance peak, 556 nm (ε_{556} = 125,000 $M^{-1}{\cdot}cm^{-1}$). Light scattering was measured at 700 nm to ensure complete dissolution of the liposomes in methanol. The hydrodynamic size of liposomes measured by dynamic light scattering (DLS) was 91.36 ± 0.56 nm.

Local Myocardial Ischemia in Situ. Male C57B mice weighing 23–32 g were anesthetized with sodium pentobarbital (50–70 mg/kg, i.p.; added per need in the course of experiment) and immobilized on a heating pad. Mice were intubated for mechanical ventilation at a rate of 120 pulse/min with a tidal volume of 0.5 mL (Harvard Apparatus Respirator; model 707). Rectal temperature was maintained within 37.5–38.0 °C. Electrodes were placed to record the standard limb lead ECG. The heart was exposed through a left thoracotomy via the fourth intercostal space and 8-0 nylon thread passed around the left coronary artery using a tapered needle ∼1 mm apically from the tip of the left atrium. The ends of the nylon were threaded through a PE-10 tubing to make a snare, so that the artery could be occluded or reperfused by tightening or releasing the snare. Mice received heparin (1,000 mg/kg, i.p.). Myocardial ischemia was confirmed by regional cyanosis and ST-segment elevation; reperfusion was confirmed by a rapid color change in the surface of the myocardium. In the course of the 2 h of experiment, the artery was periodically occluded for 10 min and reperfused for 5 s to imitate an obstructed coronary flow. In sham procedure (no ischemia), the snare was left loose for 2 h. Tested pHLIP variants (80 μM in 100 μL of PBS) were injected 3 min before the first occlusion into the femoral vein. With this protocol, we did not observe ventricular arrhythmia in the absence or presence of pHLIP. At the end of the protocol, the heart was excised with the left coronary artery still occluded, the aorta was cannulated, and 1% Evans Blue was injected into the aorta to delineate the area at risk. When the heart was intended for fluorescence microscopy, atria were trimmed and ventricles were frozen in dry ice powder and stored at −80 °C. Later, the frozen ventricles were sectioned perpendicular to the apico-basal axis (six to seven slices, 1 mm thick) and placed in PBS on slides under coverslips for obtaining fluorescent and white-light images with a Nikon SMZ1000 UV dissection microscope. The hearts intended for confocal microscopy were frozen in OCT compound and 20-mm sections were prepared for inspection with a NikonA1RMP microscope. Image J (National Institutes of Health) software was used to measure the area at risk and mean intensity of fluorescence. The contrast of fluorescence was measured as the relationship of mean intensity of fluorescence in the area at risk and in the intact myocardium. Measurements from individual slices were pooled to yield the mean value for each heart that went into final statistics.

Low-Flow Myocardial Ischemia in Isolated Hearts. Mice were anesthetized and received heparin as described above. When the appropriate depth of anesthesia had been established, the heart was excised and placed into a cold (4 °C) physiologic solution of the following composition (in mmol/L): 150 NaCl, 4 KCl, 1.8 $CaCl_2$, 1.0 $MgCl_2$, 5.5 dextrose, and 10 Hepes equilibrated with 100% oxygen (pH 7.4). The aorta was cannulated and perfused in the Langendorff mode at a constant flow rate of 4 mL/min at 37 °C resulting in a typical pressure of ∼80 mmHg. Perfusion solutions were passed through a 5-μm filter and a bubble trap. The heart was immersed in a 3-mL chamber and superfused at 10 mL/min through a separate line; the temperature of superfusate was also 37 °C. The heart was paced through a pair of platinum electrodes placed at the base of right ventricle with 2-ms square current pulses of 3× threshold amplitude at a cycle length of 200 ms. A pair of 5-mm Ag-AgCl tablet electrodes placed near the right and left ventricles ∼2 mm away from the heart provided a quasi-ECG signal used to monitor the electrical activity of the heart. To induce low-flow ischemia, the flow rate was reduced to 0.2 mL/min for 15 min. In sham experiments, the rate of perfusion remained normal for 15 min. During ischemia, the heart was perfused either with physiologic solution containing 1 μM pHLIP variants or liposomes containing fluorescent lipids (Rho-PE) coated with pHLIP or PEG. At the end of the experiment, hearts were frozen for microscopy and processed as described above.

Isolated Preparations of Left Atrial Appendages. Hearts were excised from anesthetized mice; left atrial appendages were dissected and mounted with the epicardial surface up in a 3-mL chamber perfused with physiologic solution described in the

previous section (37 °C). Preparations were paced with 2-ms square current pulses of 2× threshold amplitude at a cycle length of 250 ms through a pair of platinum wires placed close to the preparation. One end of the preparation was pinned to the bottom and another connected to an isometric force transducer with a fine stainless-steel hook. Preparations were stretched to the maximum of the force–length relationship and allowed to equilibrate for 1 h. Conventional microelectrode techniques were used to register transmembrane potential from the epicardial side of left atrial appendages. In the first type of experiments, to study the effects of pHLIP at normal pH, registrations of developed force and transmembrane potential were taken before and 30 min after administration of 10 μM pHLIP variants. In another type of experiment, intended to study the effects of pHLIP in a low-pH environment, a low-pH physiologic solution (in mmol/L: 150 NaCl, 4 KCl, 1.8 $CaCl_2$, 1.0 $MgCl_2$, 5.5 dextrose, and 10 Mes equilibrated with 100% oxygen; pH 6.5) was introduced first, and 20 min later pHLIP variants were added and remained for 30-min exposure time. At the end of the experiments, preparations were frozen in dry ice powder for microscopy.

1. Reshetnyak YK, Andreev OA, Lehnert U, Engelman DM (2006) Translocation of molecules into cells by pH-dependent insertion of a transmembrane helix. *Proc Natl Acad Sci USA* 103(17):6460–6465.

Table S1. Baseline parameters of lead II ECG in predrug control and 3 min after injection of saline or pHLIP variants kVar7, WT, and Var7

	N	RR, ms	P-wave, ms	PR, ms	QRS, ms	QT, ms
Control	17	125.2 ± 2.6	13.2 ± 0.4	38.2 ± 0.6	12.2 ± 0.3	70.0 ± 1.5
Saline	4	123.9 ± 4.7	13.0 ± 0.7	37.9 ± 0.9	12.1 ± 0.4	70.3 ± 2.4
kVar7	5	127.5 ± 3.9	13.5 ± 0.5	38.0 ± 1.1	12.0 ± 0.5	69.2 ± 2.9
WT	4	126.0 ± 4.6	13.1 ± 0.6	38.2 ± 1.2	12.1 ± 0.5	70.1 ± 3.0
Var7	4	124.1 ± 4.2	13.2 ± 0.5	38.3 ± 1.2	12.0 ± 0.4	70.1 ± 2.8

P-wave, P-wave duration; PR, PR interval duration; QRS, QRS interval duration; QT, QT interval duration; RR, RR interval duration. In each mouse, 10 consecutive PQRST complexes were measured and averaged.

Table S2. Effects of pHLIP variants at 10 μM on the parameters of transmembrane potential and the developed force in preparations of murine left atrial appendage paced at a cycle length of 250 ms at the normal and low pH

	MDP, mV	V_{max}, V/s	APA, mV	APD30, ms	APD50, ms	APD90, ms	*F*, mN
Normal pH = 7.4							
Control	−81 ± 2	202 ± 18	100 ± 2	6 ± 1	14 ± 2	46 ± 5	1.9 ± 0.2
kVar7	−82 ± 2	203 ± 18	101 ± 3	6 ± 1	14 ± 2	45 ± 6	2.0 ± 0.3
WT	−83 ± 1	190 ± 23	102 ± 3	7 ± 1	14 ± 2	46 ± 7	2.0 ± 0.3
Var7	−81 ± 2	201 ± 14	102 ± 2	6 ± 2	15 ± 3	48 ± 6	2.0 ± 0.2
Low pH = 6.5							
Control	−82 ± 1	204 ± 22	104 ± 3	8 ± 2	15 ± 2	49 ± 4	0.5 ± 0.1*
kVar7	−83 ± 1	206 ± 24	105 ± 3	7 ± 1	13 ± 1	46 ± 3	0.6 ± 0.1*
WT	−81 ± 1	222 ± 29	104 ± 4	6 ± 1	14 ± 2	45 ± 4	0.5 ± 0.2*
Var7	−81 ± 2	212 ± 19	106 ± 3	7 ± 2	14 ± 3	48 ± 7	0.6 ± 0.1*

APA, action potential amplitude; APD30, 50, 90, action potential duration to 30, 50, and 90%, respectively; *F*, developed force of contraction; MDP, maximum diastolic potential; V_{max}, maximum upstroke velocity. Mean ± SEM values are shown; $n = 5$ per group.
*$P < 0.05$ vs. respective normal pH = 7.4.