Electronic Supplementary Material (ESI) for ChemComm.

K^+ preference at the NaK channel entrance revealed by fluorescence lifetime and anisotropy analysis of site-specifically incorporated (7-hydroxycoumarin-4-yl)ethylglycine

Sanling Liu,‡ Pei Lv,‡ Dong Li, Xiaoqi Guo, Bo Zhang, Mu Yu, Dandan Li, Ying Xiong, Longhua Zhang* and Changlin Tian*

High Magnetic Field Laboratory, Chinese Academy of Sciences, and School of Life Sciences, University of Science and Technology of China, Hefei 230026, Anhui, P.R.China

*E-mail: zlhustc@ustc.edu.cn or cltian@ustc.edu.cn

Plasmid construction

The open-state NaK channel from *B. cereus* (residues 20–114) was cloned into a modified pET28a vector (Novagen) between *Nde*I and *Xho*I restriction endonuclease sites, resulting in an N-terminal MGHHHHHHMA tag. Site-directed mutagenesis was used to introduce the F69TAG mutation into the NaK gene. The pEVOL plasmid for incorporating (7-hydroxycoumarin-4-yl)ethylglycine (HC) into proteins (pEVOL-HC), which included a mutant *Methanococcus jannaschii* tyrosyl amber suppressor tRNA (*Mj*tRNATyr CUA) /tyrosyl-tRNA synthetase (*Mj*TyrRS) pair that responses to the TAG codon, was constructed as previously described.[1] All constructs were confirmed by DNA sequencing.

Protein expression and purification

Plasmids pET28a-NaKF69TAG and pEVOL-HC were cotransformed into *Escherichia coli* BL21 (DE3)-gold cells for further protein expression. Cells were grown at 37°C in Luria-Bertani medium containing 30 μg/mL kanamycin and 34 μg/mL chlormycetin to an optical density at 600 nm (OD_{600}) of 0.8, then 1 mM HC (synthesized as previously described)[2] and 0.02% arabinose (dissolved in deionized H_2O) were added to the culture. After incubation at 37°C until the OD_{600} reached 1.0, the cell culture was induced by the addition of 0.02% arabinose and 0.2 mM isopropyl-β-Dthiogalactopyranoside at 25°C for an additional 20 h. The cells were harvested by centrifugation at 4000 rpm for 20 min at 4°C (Beckman Coulter, Allegra X-15R). The cells were resuspended in 20 mM Tris–HCl, pH 8.0, 200 mM chloride salts at different K^+/Na^+ molar ratios (0/100; 20/80; 40/60; 60/40; 80/20; 100/0) and lysed by sonication. Cell debris was removed by centrifugation at 14000 rpm for 20 min at 4°C (Hitachi, CR21GII). The supernatant was collected and incubated with 1% (w/v) n-decyl-β-D-maltoside (DM) for 2 h at 4°C. Expressed proteins were purified on a Ni^{2+}-NTA affinity column (Qiagen). After elution, the proteins were concentrated and further purified on a Superdex 200 10/300 GL column (GE Healthcare) in a buffer of 20 mM Tris–HCl, pH 8.0, 200 mM chloride salts at different K^+/Na^+ molar ratios detailed above, and 0.2% (w/v) DM. Purified target proteins were subjected to further experimental analysis. The results of each purification step were monitored by SDS–PAGE.

Protein reconstitution into liposomes

A mixture of 1.5 mg 1-palmitoyl-2-oleoyl-phosphatidylcholine (POPC) and 0.5 mg 1-palmitoyl-2-oleoyl-phosphatidylglycerol (POPG) (Avanti Polar Lipids) in chloroform was dried under a nitrogen stream and residual chloroform removed by vacuum desiccation overnight.

Lipids were well dispersed in 1 mL reconstitution buffer (20 mM Tris-HCl, pH 8.0, 200 mM NaCl, 0.2% DM) through five freeze-thaw cycles by alternately placing the sample in liquid nitrogen and a bath sonicator. Purified protein and liposomes were mixed at a molar ratio of 1:600 and incubated at 4°C for 1 h with gentle rotation. The detergent/lipid/protein mixture was dialyzed against buffer (20 mM Tris, pH 8.0, 200 mM NaCl) for 3 d at 4°C to slowly remove the detergent. To obtain unilamellar liposome vesicles, samples were extruded with a 0.4 μm polycarbonate filter using an Avanti Mini-Extruder.

Single-channel electrophysiology

For electrophysiology measurements, the Ionovation Compact V02 system (Ionovation GmbH, Osnabrück, Germany) was used. Two compartments of the chamber (cis and trans) were separated by a Teflon septum with a 120 μm microhole. The cis chamber contained 150 mM KCl, the trans chamber contained 150 mM NaCl, both of which were buffered to pH 7.0 with 5 mM Tris-MOPS (Morpholinopropanesulfonic acid). A 3:1 mixture of POPC and POPG dissolved in *n*-decane was painted over the hole in the trans chamber. Lipid bilayers were formed upon repetitive lowering and raising of the buffer level. The planar lipid bilayer formation was monitored optically and by measuring the capacitance (a stable bilayer with a capacitance of about 70 pF). After a stable bilayer had formed, proteoliposomes were added into the cis chamber next to the bilayer with a micropipette. During the experiments an EPC10 patch-clamp amplifier (HEKA Elektronik) was used to detect the channel conductance. All measurements were performed at room temperature. Currents were filtered at 2 kHz and sampled at 10 kHz. Data were analyzed using the pCLAMP 10.0 software (Axon Instruments). Traces were low-pass filtered at 1 kHz.

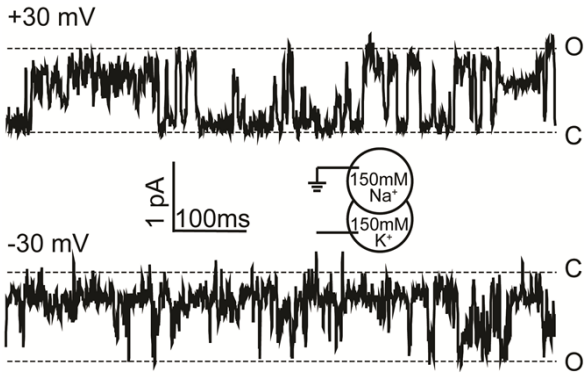

Figure S1. Single-channel traces of wild-type NaK reconstituted into lipid bilayers (POPC:POPG = 3:1) at ±30 mV.

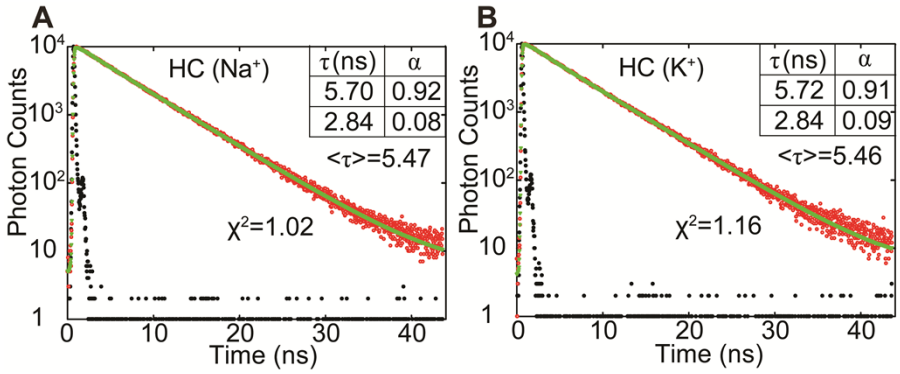

Figure S2. Discrete analysis of fluorescence intensity decays of fluorescent compound, HC, in buffer containing (A) 0% K^+/100% Na^+, (B) 0% Na^+ /100% K^+. Decays were fitted using Eq. 1.

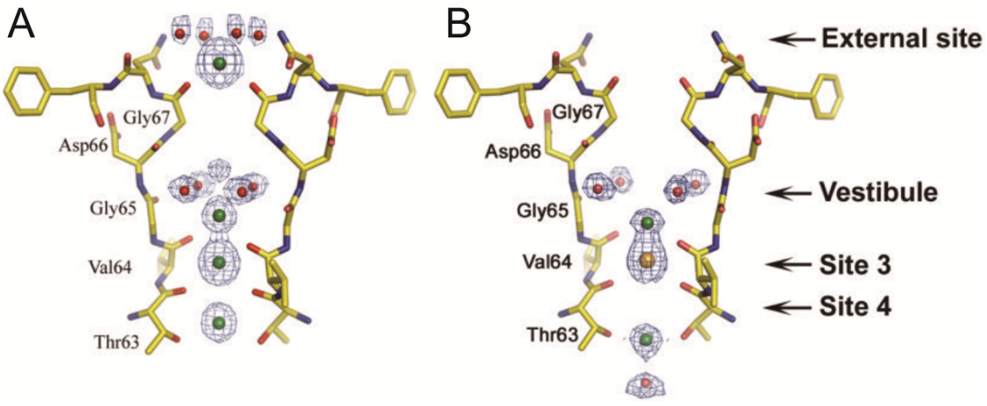

Figure S3. Binding sites of K^+ or Na^+ at different sites of the NaK channel revealed by the crystallographic studies.[3] (A) The electron density of K^+ was stabilized by the carbonyl oxygen of Gly67 and H_2O. (B) No electron density of Na^+ observed in the Gly67 site.

Table S1 Multi-exponential analysis of NaK-F69HC in buffers at different K^+/Na^+ molar ratios[a]

K^+/Na^+ molar ratio	τ_1[b]	τ_2	τ_3	α_1[c]	α_2	α_3	$\langle\tau\rangle$[d]	χ^2
0% / 100%	5.64	1.72	0.11	0.43	0.22	0.35	2.83	1.16
20% / 80%	5.66	1.75	0.17	0.42	0.23	0.35	2.86	1.19
40% / 60%	5.83	1.81	0.22	0.42	0.22	0.36	2.93	1.10
60% / 40%	5.75	1.62	0.14	0.43	0.21	0.36	2.88	1.17
80% / 20%	5.67	1.59	0.12	0.35	0.21	0.44	2.39	1.12
100% / 0%	5.61	1.50	0.15	0.28	0.27	0.45	2.01	1.19

[a] Lifetimes were obtained at the steady-state peak emission wavelength (450 nm) for all samples. All the decays were fitted to a three exponential function using Eq. 1. [b] Lifetimes are presented in ns; errors were ±3% for τ_1 and τ_3, and ±10% for τ_2. [c] The weighting factors errors were ±3%. [d] Average lifetimes are presented in ns, and calculated using Eq. 2.

References

1 T. S. Young, I. Ahmad, J. A. Yin and P. G. Schultz, *J. Mol. Biol.*, 2010, **395**, 361-374.

2 T. Koopmans, M. van Haren, L. Q. van Ufford, J. M. Beekman and N. I. Martin, *Bioorg. Med. Chem.*, 2013, **21**, 553-559.

3 A. Alam and Y. Jiang, *Nat. Struct. Mol. Biol.*, 2009, **16**, 35-41.