Supporting Information

Brams et al. 10.1073/pnas.1401917111

SI Experimental Procedures

Molecular Cloning and Protein Expression. National Center of Biotechnology Information reference numbers for protein sequences reported in this study are WP_009782512.1 (LpcK), YP_724331.1 (TerK), YP_003873862.1 (SthK), and YP_001962905.1 (LbiK). The GenBank reference number for AmaK is EDZ93391. Synthetic genes with optimized codon use for expression in *Escherichia coli* were purchased from Genscript. Each gene was subcloned into the NdeI and BamHI restriction sites of the vector pWaldo-GFPe (1, 2). Each plasmid was transformed into chemically competent OverExpress *E. coli* strains C41(DE3), C43(DE3), BL21 (DE3), and their pLysS variants (Lucigen). Bacterial suspension cultures were grown in Luria broth at a temperature of 37 °C to an optical density of 0.6–0.8 (OD_{600}). Protein expression was induced by addition of 400 μM isopropyl-thio-b-D-galactoside, and the cultures were subsequently cooled to 18 °C for overnight incubation. Cells were harvested by centrifugation at 10,000 × *g*, and the cell pellet was stored at −20 °C. Cell pellets were resuspended in lysis buffer containing 500 mM KCl, 50 mM Tris, pH 8.0, and 10% glycerol supplemented with 1 mM phenylmethanesulfonyl fluoride (PMSF), 20 mg/mL DNase, 5 mM $MgCl_2$, 1 μg/mL leupeptin, and 1 μg/mL pepstatin-A. Cells were broken by two passes at 1,000–1,500 bar through an Emulsiflex-C5 (Avestin). Membranes were isolated by ultracentrifugation in a Beckman Ti60 rotor at 125,000 × *g* at 4 °C for 1 h. Membranes were resuspended at a ratio of 1:1 (mass/vol) in buffer A, containing 500 mM KCl, 50 mM Tris, pH 8.0, and 10% glycerol supplemented with 1 mM PMSF. Membrane suspensions were snap frozen in liquid nitrogen before storage at −80 °C. Green fluorescence of GFP was monitored throughout this process using a Safe Imager blue light transilluminator (Invitrogen).

Expression and Detergent Screening. For initial expression screening, proteins were expressed in 4-mL suspension cultures under the same conditions as described above. Suspension cultures were harvested by centrifugation at 30,000 × *g* and resuspended in 400 μL of PBS. Whole-cell fluorescence was measured at room temperature in a 7500 Fast Real-Time PCR system (Applied Biosystems). For detergent screening, 270 μL of membrane suspension was mixed with 30 μL of a 20% detergent stock solution (final detergent concentration was 2% during solubilization). All detergents used in this study were obtained from Affymetrix. Next, the solubilized fraction was cleared by ultracentrifugation in a Beckman TLA 120.1 rotor at 30,000 × *g* at 4 °C for 1 h. The clear supernatant was further used for analysis by fluorescence size exclusion chromatography (FSEC) (1, 3). In brief, FSEC was carried out on a ÄKTApurifier 10 system (GE Healthcare Life Sciences) equipped with an A-900 autosampler, a Superose 6 10/300 GL column, and a RF-10AXL fluorescence detector (Shimadzu). Fluorescence of GFP was measured at an excitation wavelength of 488 nm and emission wavelength of 512 nm. Samples were run at 4 °C at a flow rate of 0.4 mL/min in running buffer containing buffer A supplemented with the corresponding detergent used during protein solubilization. Final detergent concentrations in running buffer were 0.02% Cymal-6, 0.6% lauryldimethylamine *N*-oxide (LDAO), 0.7% octyl glucoside (OG), 0.6% nonylglucoside (NG), 0.2% octylmaltoside (OM), 0.05% Fos-choline-12 (Fos-12), 0.2% decylmaltoside (DM), 0.15% undecylmaltoside (UDM), 0.05% dodecylmaltoside (DDM), 0.02% tridecylmaltoside (TDM), 0.1% lauryl maltose neopentyl glycol (LMNG), 0.05% C12E9, 0.1% CHAPS, and 0.3% HEGA-10.

Protein Purification. Total membrane preparations of channel-GFP-Hisx8 fusion constructs from 4- to 8-L suspension cultures were solubilized by stirring for 2 h at 4 °C in buffer A supplemented with detergent and 50 mM imidazole. Insoluble material was removed by ultracentrifugation at 30,000 × *g* at 4 °C for 1 h. The supernatant was loaded onto a 1-mL HisTrap FF column (GE Healthcare) and washed with two consecutive wash steps to 50 and 70 mM imidazole in buffer A. Protein was eluted with a linear gradient from 70 to 250 mM imidazole in buffer A. The flow through, wash, and elution fractions were analyzed using FSEC to verify the amount of channel-GFP-Hisx8 in each fraction. The main elution fractions were concentrated on a Vivaspin 6 (Sartorius) concentrating column (100-kDa cutoff), and the imidazole was exchanged by concentration/dilution before protease cleavage with 150 units of thrombin (Calbiochem) at 4 °C for 36 h. For the final gel filtration, the sample was loaded onto a Superdex 200 10/300 GL gel filtration column with running buffer composed of buffer A supplemented with 0.15% UDM for AmaK and 0.05% DDM for SthK. The final elution fractions were pooled and concentrated using a Vivaspin 6 concentrating column (100-kDa cutoff) and stored at 4 °C for further analysis.

Protein Analysis. Protein fractions were analyzed on 4–15% Mini-PROTEAN TGX precast SDS/PAGE gels (Bio-Rad). A Safe Imager blue light transilluminator (Invitrogen) was used to monitor in-gel GFP fluorescence. Precision Plus All Blue and Fluorescent WesternC Protein standards were from Bio-Rad. Gels were stained using the Coomassie R-250 method. Protein concentration was estimated using the Quick Start Bradford 1× dye reagent and an Eppendorf Bio spectrophotometer.

Expression in *Xenopus* oocytes. For oocyte expression studies, SthK was cloned as a C-terminal GFP fusion into the EcoRI and XbaI restriction sites of pGEM-HE (4, 5). All constructs were verified by sequencing (LGC Genomics). The plasmids were linearized with NheI, and capped cRNA was synthesized using the T7 mMESSAGE-mMACHINE transcription kit (Ambion). Oocytes were surgically removed from adult females of *Xenopus laevis* anesthetized with 0.3% 3-aminobenzoic acid ethyl ester. After incubating the oocytes for 105 min in Ca^{2+}-free Barth's solution (in mM: 82.5 NaCl, 2 KCl, 1 $MgCl_2$, and 5 Hepes, pH 7.5) containing 3 mg/mL collagenase A (Roche), oocytes of stage IV and V were manually dissected and defolliculated. About 50 ng of cRNA encoding SthK-GFP or AmaK-GFP per oocyte was injected. Oocytes were cultured at 18 °C for 3–5 d in Barth's medium [in mM: 84 NaCl, 1 KCl, 2.4 $NaHCO_3$, 0.82 $MgSO_4$, 0.41 $CaCl_2$, 0.33 $Ca(NO_3)_2$, 7.5 Tris, cefuroxime, and penicillin/streptomycin, pH 7.4]. The vitelline membrane was manually removed from the oocyte right before imaging or electrophysiological experiments.

Confocal Laser-Scanning Microscopy. Confocal fluorescence microscopy (LSM 710; Zeiss) was used to determine protein localization in the membrane of *Xenopus* oocytes. The fluorescence signal originating from SthK-GFP was identified using excitation with the 488-nm line of an argon laser. To confirm localization to the oocyte membrane, we stained the membrane with Alexa Fluor 633-labeled wheat germ agglutinin (WGA-Alexa 633) (Invitrogen Life Technologies) from the extracellular side (6). Staining was performed by removing the vitelline membrane and

incubating the oocytes for 10 min in bath solution containing 5 μM WGA-Alexa 633. Alexa 633 was excited with the 633-nm line of helium neon laser. The laser light was projected through a main beam splitter 488/543/633 and a Plan-Neofluar 10×/0.30 or a 40×/1.2 C-Apochromat water-immersion objective onto the sample. The emitted light was collected through the objective and spatially filtered by a 37-mm pinhole resulting in a z slice of 1 μm for the 40× objective and 12.8 μm for the 10× objective. To quantify colocalization of GFP and Alexa 633, we analyzed fluorescence profiles perpendicular to the plasma membrane (10 profiles averaged per oocyte and image). Both signals were normalized with respect to the integral of their profiles. The integral of the overlap between the two profiles was used as a quantitative measure of colocalization, indicating GFP membrane localization.

Spectral Imaging. To confirm that the emission spectrum obtained form SthK-GFP–expressing oocytes is GFP-related and not affected by autofluorescence, we recorded the autofluorescence spectrum of control oocytes and a GFP emission spectrum from HEK293 cells expressing cytosolic GFP. Control oocytes for autofluorescence imaging were harvested from *Xenopus laevis* as described in *Experimental Procedures*. HEK cells were transfected using the calcium phosphate transfection method. Excitation in oocytes and HEK cells was realized using the 488-nm line of an argon laser of the confocal laser-scanning microscope LSM710 (Zeiss). For SthK-GFP imaging, the applied laser power was 2.5 times lower than for autofluorescence imaging; thus, autofluorescence was not detectable in SthK experiments. Spectral detection was performed in the range of 472–686 nm for oocytes and in the range of 501–686 nm for HEK cells. The step width was 9.7 nm. In all cases, a Zeiss 40×/1.2-W Korr M27 objective was used.

Spectral imaging of AmaK-GFP–injected oocytes was performed in the same way as of SthK-GFP–injected oocytes, but with a 2.5 times higher laser power of the 488-nm line of an argon laser. For experiments performed to test the expression in *Xenopus* oocytes, the membrane was additionally counterstained with 5 μM WGA-Alexa 633.

Electrophysiology. Patch-clamp recordings were carried out in the inside-out configuration using macropatches excised from *Xenopus laevis* oocytes expressing SthK-GFP. Patch pipettes were pulled from borosilicate glass tubing (Hilgenberg) with outer and inner diameters of 2.0 and 1.0 mm, respectively. The resistance of the solution-filled pipettes was between 0.7 and 1.1 MΩ. The bath and the pipette solutions contained the following (in mM): 150 KCl, 5 EGTA, 5 Hepes (pH 7.4). In experiments upon the selectivity of SthK channels, KCl was substituted by 150 mM NaCl in the bath and/or pipette solution. All recordings were obtained at room temperature. Concentration–activation relationships for cAMP were obtained by applying different cAMP concentrations to the bath solution. Currents were recorded with an Axopatch 200B amplifier (Axon Instruments) controlled by the patch-clamp software ISO2 (MFK). Because of a run-up of the current amplitude in the first tens of seconds after ligand application, recordings were started 1 min after patch excision. For the experiments upon voltage dependence and ion selectivity of SthK, voltage steps of 2-s duration from −100 to +100 mV with 20-mV increments were applied. For cAMP concentration–activation relationships, the voltage was first stepped from a holding potential of 0 to −100 mV, then to +100 mV and back to the holding potential. The voltage steps lasted 2.1 s. Single-channel recordings were obtained under the same experimental conditions. All macroscopic currents were corrected for the capacitive and the leak components by subtracting respective currents in the absence of ligands in the bath solution. Concentration–activation relationships were determined from the steady-state current at +100 mV by normalizing the current I at the actual [cAMP] with respect to the current I_{max} at saturating [cAMP] and fitting the data points with a Hill equation of the following form:

$$\frac{I}{I_{max}} = \frac{1}{1 + \left(\frac{EC_{50}}{[cAMP]}\right)^{H}}, \quad \textbf{[S1]}$$

yielding values for the concentration of half-maximum activation, EC_{50}, and the Hill coefficient, H. Curves were fitted to the data with appropriate nonlinear approximation algorithms using Origin9.0G (OriginLab Corporation) software. Single-channel analysis was performed using the ISO2 software (MFK). Amplitude histograms were fitted with the sum of Gaussian functions using Origin9.0G software (OriginLab Corporation). Statistical data are given as mean ± SEM.

1. Kawate T, Gouaux E (2006) Fluorescence-detection size-exclusion chromatography for precrystallization screening of integral membrane proteins. *Structure* 14(4):673–681.
2. Drew D, Lerch M, Kunji E, Slotboom D-J, de Gier J-W (2006) Optimization of membrane protein overexpression and purification using GFP fusions. *Nat Methods* 3(4):303–313.
3. Cortes DM, Perozo E (1997) Structural dynamics of the *Streptomyces lividans* K^+ channel (SKC1): Oligomeric stoichiometry and stability. *Biochemistry* 36(33): 10343–10352.
4. Rath A, Glibowicka M, Nadeau VG, Chen G, Deber CM (2009) Detergent binding explains anomalous SDS-PAGE migration of membrane proteins. *Proc Natl Acad Sci USA* 106(6):1760–1765.
5. Liman ER, Tytgat J, Hess P (1992) Subunit stoichiometry of a mammalian K^+ channel determined by construction of multimeric cDNAs. *Neuron* 9(5):861–871.
6. Nache V, et al. (2012) Differential regulation by cyclic nucleotides of the CNGA4 and CNGB1b subunits in olfactory cyclic nucleotide-gated channels. *Sci Signal* 5(232):ra48.

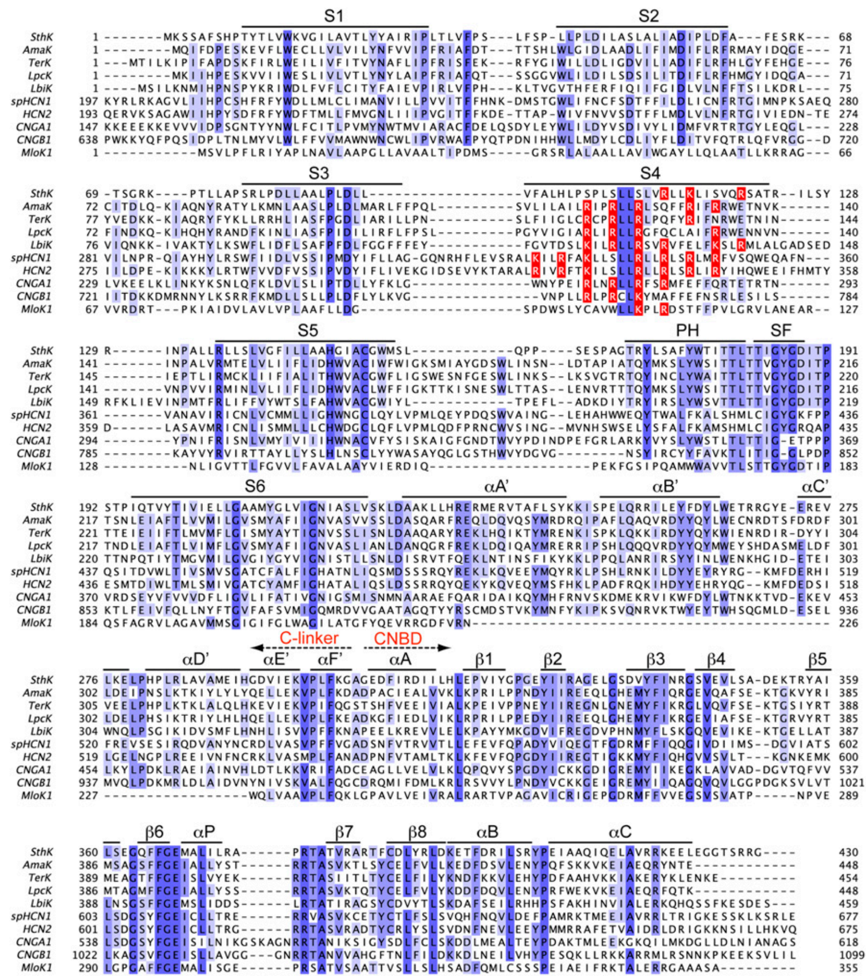

Fig. S1. Full-sequence alignment of prokaryote cyclic nucleotide-modulated ion channels with different eukaryote HCN and CNG channels.

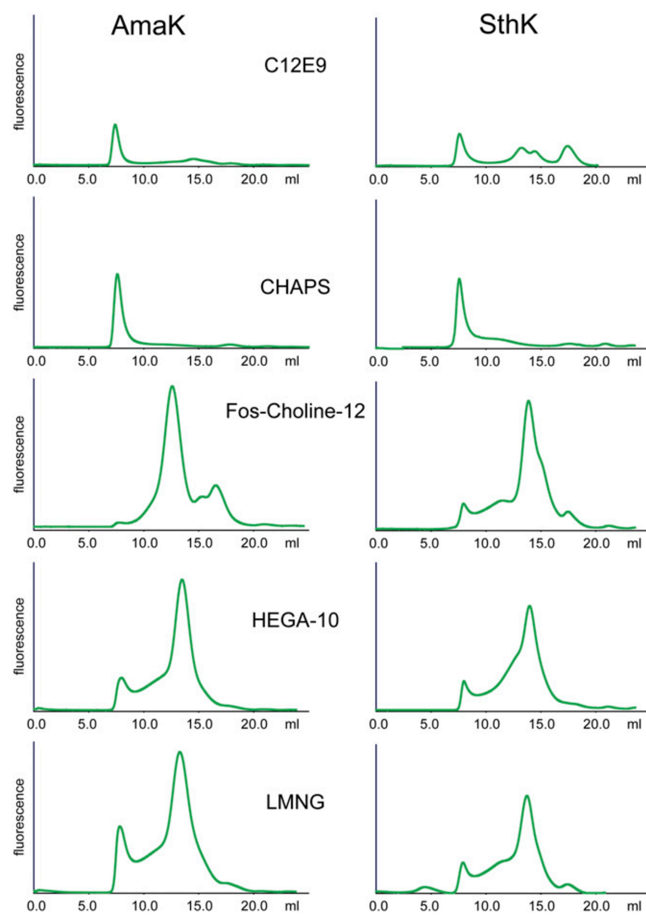

Fig. S2. Detergent screen for AmaK and SthK using FSEC. FSEC profiles show that C12E9 and CHAPS are unable to stably extract AmaK or SthK from membranes. For Fos-12, HEGA-10, and LMNG, relatively symmetric fluorescent peaks could be observed, indicating that AmaK and SthK are extracted from membranes in a monodisperse state.

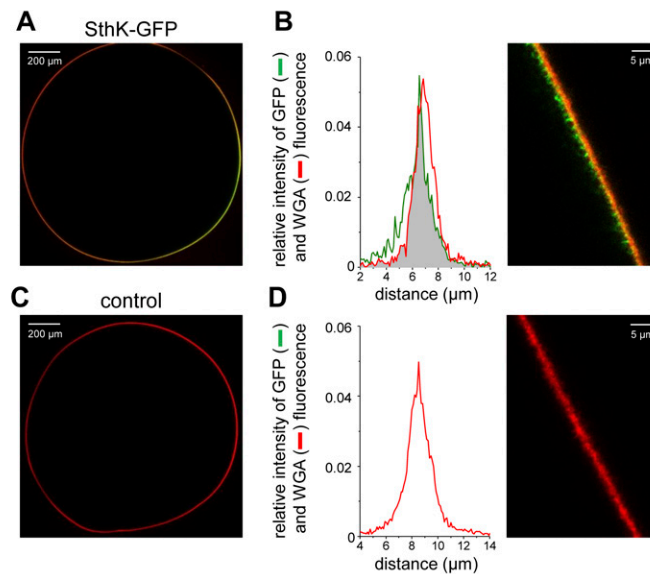

Fig. S3. Membrane localization of SthK-GFP in *Xenopus* oocytes. (*A*) Confocal image of an intact oocyte expressing SthK-GFP. The outer side of the membrane was counterstained with WGA-Alexa 633 to define the position of the membrane (red signal). GFP emission signal is shown in green. Shades of yellow are caused by superimposed red and green signals. (*B*) Confocal image of an oocyte expressing SthK-GFP at higher magnification. The fluorescence profiles are averages of 10 profiles perpendicular to the membrane surface for GFP (green) and Alexa 633 (red). Normalization of the profiles was performed by setting the area under the curves to unity. The gray area marks the portion of colocalization. (*C*) Confocal image of an intact noninjected control oocyte. Counterstaining the outer side of the membrane caused a red signal similar to *A*. A green signal is missing due to the absence of SthK-GFP. There was no autofluorescence signal under these experimental conditions. (*D*) Confocal image of a control oocyte at higher magnification. The fluorescence of the Alexa 633 profile is similar to SthK-GFP–expressing oocytes, shown in *B*.

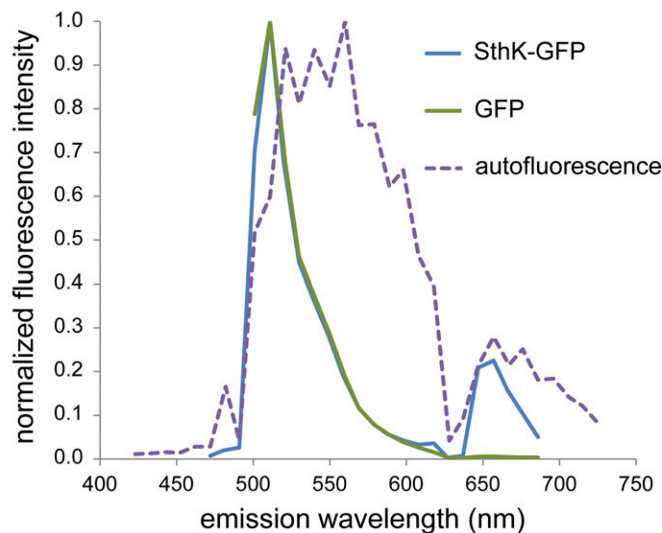

Fig. S4. Comparison of emission spectra for SthK-GFP, cytosolic GFP, and autofluorescence. Shown are the emission spectra of SthK-GFP expressed in *Xenopus laevis* oocytes, cytosolic GFP expressed in HEK293 cells, and the spectrum of the autofluorescence in control oocytes. All three emission spectra were normalized with respect to the maximum values for comparison. The superimposition of the SthK-GFP and GFP spectra indicates that the spectrum recorded in SthK-GFP–expressing oocytes is not affected by autofluorescence.

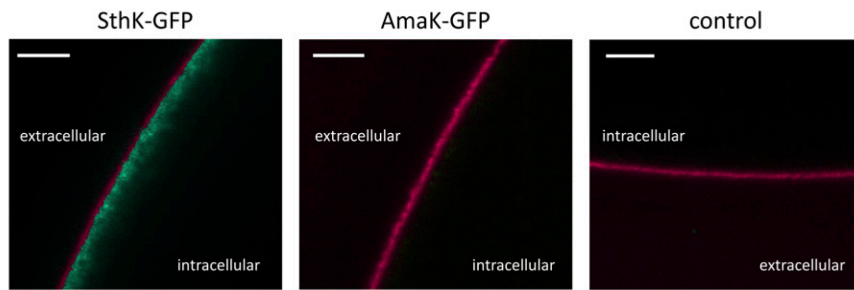

Fig. S5. Spectral imaging of *Xenopus laevis* oocytes. Shown are spectral images (*SI Experimental Procedures*) of oocytes 3 d after injection of SthK-GFP and AmaK-GFP cRNA, respectively, in comparison with a noninjected control oocyte. Whereas in SthK-GFP oocytes a strong GFP fluorescence could be detected, there was no GFP-related signal in AmaK-GFP or control oocytes. The red fluorescence arose from WGA-Alexa 633 counterstaining to confirm the position of the plasma membrane (*SI Experimental Procedures*). (Scale bar: 20 μM in all three images.)