Supporting Information

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SI Materials and Methods

Molecular Biology. Ci-VSP in the pSD64TF vector was kindly provided by Y. Okamura (Osaka University, Osaka, Japan). rIRK1 in pGEM-HE was kindly provided by E. Reuveny (Weizmann Institute of Science, Rehovot, Israel). The EGFP-PH_{PLC} construct was kindly provided by T. Meyer (Stanford University, Palo Alto, CA) and subcloned into pGEM-HE. The EGFP-PH_{OSH1} construct was a kind gift from T. Balla (National Institutes of Health, Bethesda, MD) and subcloned into pGEM-HE. The ptagRFP vector was a kind gift from T. Hughes (Montana State University, Bozemann, MT). The coding sequence for tagRFP was subcloned into the NheI and XhoI sites of the pGEM-HE-EGFP-PH_{PLC} vector to create the tagRFP-PH_{PLC} construct. The S158T mutation (for increase photostability) was added to tagRFP in the pGEM-HE-tagRFP-PH_{PLC} construct using Quikchange (Stratagene). The EGFP-Ct_{TREK1} was constructed by subcloning the C terminus of mTREK1 (residues 293–411) into ApaI and XbaI sites of the pGEM-HE-EGFP-PH_{PLC}. The EGFP-Ct_{TREK1} mutants were introduced into pGEM-HE-EGFP-Ct_{TREK1} using Quikchange (Stratagene). The EGFP-Ct_{IRK1} construct was constructed by subcloning the C terminus of rIRK1Q (residues 177–428) into the ApaI and XbaI sites of the pGEM-HE-EGFP-PH_{PLC}.

Electrophysiology and Fluorometry. For experiments with Ci-VSP, cells were voltage clamped at −80 mV in the dark for at least 2 min before recording to obtain baseline fluorescence protein (FP) fluorescence. The PMT gain was adjusted so that the resting fluorescence signal was ~3 V. Cells were voltage clamped at −80 mV for 5–30 s with the shutter open to record baseline fluorescence. The shutter remained open for the remainder of the experiment and data were recorded every 500 μs. For experiments with EGFP-Ct constructs and GPCRs, cells were voltage clamped at −80 mV with the shutter open during the entire duration of the experiment. GPCR ligand was applied using a Valve Bank 8 II perfusion system (Automate Scientific). EGFP fluorescence was monitored during the entire experiment.

Total Internal Reflection Microscopy on *Xenopus laevis* Oocytes. Defolliculated *Xenopus* oocytes were injected with 50 nL of cRNA at 0.001 μg/μL. Oocytes were used 24 h following injection. Total internal reflection microscopy was done as described earlier (1). Devitellinized *Xenopus* oocytes were attached to a coverslip, and movies of 500 frames (10–30 fps) were acquired with 488-nm illumination for GFP through an Olympus 100×/NA1.65 objective.

Experiments on HEK293 Cells. The EGFP-Ct_{TREK1} and mutants were transiently expressed in HEK293 cells. After 48 h of expression, cells were fixed for 20 min with paraformaldehyde (4% at room temperature) or with ethanol (70% on ice for 3–12 h). After mounting, cells were observed using an epifluorescence microscope (IX71, Olympus) equipped with 20× objective and appropriate combinations of filters. Images were recorded with a cooled CCD camera (Luca; Andor Technology).

1. Ulbrich MH, Isacoff EY (2007) Subunit counting in membrane-bound proteins. *Nat Methods* 4:319–321.

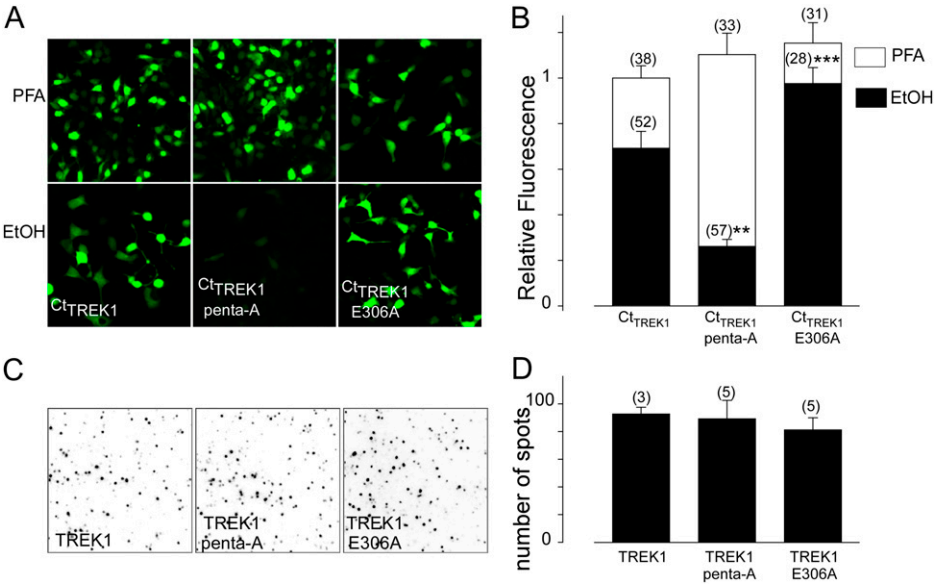

Fig. S1. The differences in fluorescence and current amplitude are not due to differences in proteins expression levels. (*A*, *B*) Quantification of fluorescence in EGFP- Ct_{TREK1} construct-transfected HEK cells fixed with ethanol or without permeabilization (PFA). (*C*) Expression of TREK1-EGFP constructs in Xenopus oocytes in TIR. (*D*) Number of TREK1, TREK1-penta-A, and TREK1-E306 channels observed as green spots in TIR in 13 × 13 μm^2 field of view. Student's t test (**$P < 0.01$, ***$P < 0.001$) show the difference between TREK1 and TREK1 mutants (or EGFP-Ct_{TREK1} and EGFP-Ct_{TREK1} mutants). The numbers of cells tested are indicated in parentheses.