Supporting Information

Acquiring Snapshots of the Orientation of Trans-membrane Protein Domains Using A Hybrid FRET Pair

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MATERIALS AND METHODS

Chemicals and Reagents

Di-8-ANEPPS ((4-{2-[6-(dibutylamino)-2-naphthalenyl]-ethenyl}-1-(3-sulfopropyl) pyridinium inner salt)), FM1-43 (N-(3-triethylammoniumpropyl)-4-(4-(dibutylamino)styryl) pyridinium dibromide), and Pluronic F-127 (nonionic polyol surfactant, MW ~ 12.5kD) were obtained from Molecular Probes (Eugene, OR, USA). Stock solutions in DMSO were kept in original vials and aliquots were diluted as needed. DPA (dipicrylamine) was obtained from City Chemicals (West Haven, CT, USA). Na-Phosphate and sucrose were obtained from Fisher (St. Louis, MO, USA). Triton X-100 was obtained from Amersham Bioscience (Uppsala, Sweden). The pulsing medium was an iso-osmotic solution consisting of 250 mM sucrose and 2 mM phosphate maintained at pH 7.2. Buffer resistivity was $\rho \cong 2.3 \cdot 10^4 \Omega \cdot cm$, measured with a conductivity meter (Amber Science, Eugene, OR).

Cell Culture, Transfection, and Labeling

PC12 cells were maintained in DMEM media (Gibco, Carlsbad, CA) supplemented with 5% horse serum, 5% fetal bovine serum, and 1% penicillin-streptomycin in a humidified 37˚C incubator. Cells were typically grown to ~ 75% confluency and lifted using Versene (Invitrogen, Carlsbad, CA) for experiments. For the mEGFP-Farnesyl (mEGFP-F) construct, the c-Ha-Ras farnesylation signal sequence (encoding SGLRSKLNPPDESGPGCMSCKCVLS) was fused to the C-terminus of the monomeric enhanced GFP gene in a CMV mammalian expression vector. Cells were transfected with the plasmid complexed with Lipofectamine 2000 (Invitrogen) diluted in Opti-MEM I (Invitrogen) media and subsequently fed with full medium 3 h after transfection. Transfected cells were lifted ~ 6-8 hrs later and typically used within ~ 1 hr.

For the pulse experiments, cells were re-suspended in growth media without serum and kept in ice for labeling procedures. Labeling in ice prevented the endocytic uptake of dyes. For Di-8-ANEPPS experiments, a 200μl aliquot of the cell suspension was incubated with 10 μM Di-8-ANEPPS and 0.05% Pluronic F-127 in ice for 10 minutes. Cells were then washed twice with cold iso-osmotic pulsing buffer and placed between parallel electrodes in a cover glass chamber for imaging and pulse experiments. The same labeling procedures were followed for FM1-43 as for Di-8-ANEPPS, except 1 μM of dye and an ~10 sec incubation was used. Optical measurements in the absence of DPA were run as control samples. Cells labeled with FM1-43 and those transfected with mEGFP-F were incubated with 10 μM DPA for ~ 5 minutes in the pulsing buffer prior to pulse applications. The incubation time was determined from separate experiments to cause ~ 20-30% quenching of the donor fluorophore due to FRET with DPA. As a positive control for field dependent acceptor mobility, we used the cells expressing mEGFP-F and labeled with DPA for further incubation with 0.05% Triton X-100 to abolish the field-induced membrane potential.

Vesicle Experiments with Membrane-Embedded Bax

To ensure site-specific labeling of Bax at Cys126, Cys62 was mutated to a Ser so that AlexaFluor546 could be labeled only at that location using maleimide reactivity. To create one reactive site at Arg145, both Cys126 and Cys62 were mutated to a Ser and Arg145 was mutated to Cys before reacting Bax with AlexaFluor 546 described previously[1]. Labeling to these specific sites was verified by analyzing pepsin fragments using LC-MS.

Giant unilamellar vesicles (GUVs) comprised of phosphatidylethanolamine (Avanti) were prepared using Pt wires described previously[2] in 20mM Tris pH 8 buffer. Before adding labeled Bax to the solution, the GUVs were incubated with 20 nM tBid prepared according to previous methods[3], then with DPA. To keep the GUV stationary during the application of the Electric Pulse, a Micropipette Cell Holder Unit (Eppendorf) held them in place during data acquisition.

Imaging And Electric Pulse Equipment

Full frame and diffraction limited spot-scanned images were acquired through a 40X, 1.3 N.A. oil immersion objective on a Zeiss LSM 5 (Carl Zeiss, Jena, Germany) confocal microscope system. The microscope is equipped with a 40 mW argon ion laser source (458, 488, 514 nm lines) and additional 1 and 5 mW, 543 and 633 nm laser sources, respectively. All excitation light sources were fiber coupled to the scope's optical path. Di-8-ANEPPS was excited with a 543 nm laser line and emission collected through a band pass 560-630 nm filter. FM1-43 and mEGFP-F were both excited with a 488 nm argon-ion laser, and emissions were collected via 560-615 nm, and 505-600 nm band pass filters, respectively. The microscope software was used to collect full frame images as well as the intensity-time data for spot scanned images.

Electric pulses were delivered across sample cells via two parallel electrodes constructed out of polished stainless steel plates and placed on a rectangular Kel-F block. The electrode separation was 600 μm as measured using a bright field image collected through a 10X objective. The holding block was designed to mount on the side walls of two-well (or one well) chambered

cover glass slides (LabTek, Winooski, VT). The electrode assembly can be used on either attached or suspended cells. Here, signals were acquired on suspended cells allowed to settle on the chambered cover glass. The electrode terminals were coupled to a square wave pulse generator, HP-8011, with variable width, and a maximum output of 16V into a $50\,\Omega$ load. Rise and fall times of output pulses were less than 50 ns.

To synchronize the acquisition of spot-scanned image intensity profiles with the external pulse application, a time delay circuit was built using a monostable 4048 IC (Integrated Circuit) timer chip (Texas Instruments, Richardson, TX) with a variable *RC* time constant of ~ 50-100 ms. The timing circuit was powered using three 1.5V AA batteries. Input to the circuit box was drawn from a 'scan' trigger event on the LSM 5 microscope supplied via a 25-pin user I/O port. The output, delayed by the *RC* time constant, was used to trigger the HP-8011 pulse generator for pulse discharge across the sample cell. The amplitude and width of the electric pulses were measured across the parallel electrodes on a 100 MHz Tektronix TDS-1012 oscilloscope (Beaverton, OR).

The intensity of the 8 bit spot-images were acquired at a rate of ~ 8 images/ms giving a time resolution of ~123 μs per image – about 5 times faster than the reported DPA translocation time of ~ 500 μs[4]. Each data trace typically contains ~ 2000 points for a total scan time of ~ 245 ms. Data shown in the figures are 60 ms subsections comprising 20 ms intervals of prior, during, and after electric pulse application. Bleaching was insignificant in the time scale of the experiments. Spot-scanned image intensity traces were analyzed and plotted using Igor Pro 5.2 (Wavemetrics, OR). The fractional fluorescence change, $\Delta F/F$, was calculated as $(F_s - F)/F$ where F_s is the mean fluorescence amplitude in the presence of the field and F is the mean signal amplitude in the 20 ms interval prior to the field onset. The signal-to-noise ratio, S/N, was calculated as $|\Delta F|$ divided by the standard deviation of F in the 20 ms interval preceding the onset of the electric pulse. Full frame images were taken with the microscope imaging software.

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