Video Article

Purification and Reconstitution of TRPV1 for Spectroscopic Analysis

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Abstract

Polymodal ion channels transduce multiple stimuli of different natures into allosteric changes; these dynamic conformations are challenging to determine and remain largely unknown. With recent advances in single-particle cryo-electron microscopy (cryo-EM) shedding light on the structural features of agonist binding sites and the activation mechanism of several ion channels, the stage is set for an in-depth dynamic analysis of their gating mechanisms using spectroscopic approaches. Spectroscopic techniques such as electron paramagnetic resonance (EPR) and double electron-electron resonance (DEER) have been mainly restricted to the study of prokaryotic ion channels that can be purified in large quantities. The requirement for large amounts of functional and stable membrane proteins has hampered the study of mammalian ion channels using these approaches. EPR and DEER offer many advantages, including determination of the structure and dynamic changes of mobile protein regions, albeit at low resolution, that might be difficult to obtain by X-ray crystallography or cryo-EM, and monitoring reversible gating transition (*i.e.*, closed, open, sensitized, and desensitized). Here, we provide protocols for obtaining milligrams of functional detergent-solubilized transient receptor potential cation channel subfamily V member 1 (TRPV1) that can be labeled for EPR and DEER spectroscopy.

Video Link

The video component of this article can be found at https://www.jove.com/video/57796/

Introduction

With recent advances in single-particle cryo-electron microscopy (cryo-EM), mammalian ion channel structures have been obtained at an extraordinary rate. Particularly, structural studies of polymodal ion channels, such as the transient receptor potential vanilloid 1 (TRPV1), have provided further understanding of its activation mechanisms[1,2,3,4,5]. However, dynamic information about ion channels embedded in a membrane environment is required to understand their polymodal gating and drug-binding mechanisms.

Electron paramagnetic resonance (EPR) and double electron-electron resonance (DEER) spectroscopies have provided some of the most definitive mechanistic models for ion channels[6,7,8,9,10,11,12,13]. These approaches have been mainly restricted to the examination of prokaryotic and archeal ion channels that yield a large amount of detergent-purified proteins when overexpressed in bacteria. With the development of eukaryotic membrane proteins production in insect and mammalian cells for functional and structural characterization[14,15,16], it is now possible to obtain biochemical amounts of detergent-purified proteins for spectroscopic studies.

The EPR and DEER signals arise from a paramagneticspin label (SL) (*i.e.*, methanethiosulfonate) attached to a single-cysteine residue in the protein. The spin-labels report three types of structural information: motion, accessibilities, and distances. This information allows determining whether residues are buried within the protein or are exposed to the membrane or aqueous environment in the apo and ligand-bound states[13,17,18,19]. In the context of a high-resolution structure (when available), the EPR and DEER data provide a collection of constraints for deriving dynamic models in their native environment while monitoring reversible gating transition (*i.e.*, closed, open, sensitized, and desensitized). Moreover, flexible regions that might be difficult to determine by X-ray crystallography or cryo-EM could be obtained by using these environmental data sets to assign secondary structures as well as location within the protein[20]. Cryo-EM structures obtained in lipid nanodiscs provided valuable information about the gating of ion channels[3,21,22,23,24,25]; however, spectroscopic approaches could provide dynamic information from conformational states (*e.g.*, thermal changes) that might be difficult to determine using cryo-EM.

Many difficulties must be overcome to implement EPR and DEER, including lack of protein function when removing all cysteine residues (especially abundant in mammalian channels), low protein yield, protein instability during purification and after spin labeling, and protein aggregation in detergent or liposomes. Here, we have designed protocols to overcome these critical barriers and have obtained DEER and EPR spectra information for a mammalian sensory receptor. The purpose here is to describe methodologies for the expression, purification, labeling, and reconstitution of a functional minimal cysteine-less rat TRPV1 (eTRPV1) construct for spectroscopic analyses. This methodology

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is appropriate for those membrane proteins that keep their function despite the removal of cysteine residues or that contain cysteine forming disulfide-bonds. This collection of protocols could be adapted for the spectroscopic analysis of other mammalian ion channels.

Protocol

1. TRPV1 Mutagenesis

Note: A minimal TRPV1 construct for spectroscopic analysis[26] was built from the full-length cysteine-less channel TRPV1[27] using the polymerase chain reaction (PCR) method (**Figure 1**). This cysteine-less minimal TRPV1 construct (referred to as eTRPV1 hereafter) consists of residues 110 - 603 and 627 - 764. eTRPV1 was cloned in pMO (a pcDNA3.1-based vector) for functional analysis and in a recombinant donor vector[28] containing 8x histidine-maltose-binding protein (MBP)-tobacco etch virus (TEV) for expression and purification (**Figure 2A**). eTRPV1 single-cysteine mutants were generated using site-directed mutagenesis. Vector and channel sequences are specified in the **Supplementary File 1: Supplementary Methods.**

1. Design mutagenesis primers with online tools[29].
2. Mix in a 0.2-mL tube: 5 µL of 10x reaction buffer, 1 µL of dNTP mix (100 mM), 1 µL each of 10 µM forward and reverse oligonucleotides, 1 µL of 100 ng/µL eTRPV1 template DNA[26], 1.5 µL of DMSO, 1 µL of mutagenesis enzyme, and 38.5 µL of deionized water. Spin the mixture before placing the tubes in the thermocycler.
3. Perform PCR mutagenesis.
    1. Perform (a) denaturation at 95 °C for 2 min, (b) denaturation at 95 °C for 20 s, (c) annealing at 60 °C for 10 s, and (d) elongation at 68 °C for 4 min (30 s per 1 kb). Repeat steps b to d for 18 cycles, then perform elongation at 68 °C for 5 min, and maintain the reaction at 4 °C until further use.
4. Add 2 µL of *Dpn*I enzyme to the reaction; mix and then incubate at 37 °C for 30 min.
5. Perform the transformation
    1. Thaw *E. coli* competent cells on ice.
    2. To a pre-cooled 14-mL sterile tube, add 75 µL of competent cells and 10 µL of *Dpn*I-treated PCR mixture. Mix gently.
    3. Incubate reaction on ice for 30 min. Maintain the tube at 42 °C for 45 s and then immediately place it on ice for 2 min. Plate the mixture on Luria Broth (LB)-agar plate containing carbenicillin (0.2 mg/mL). Incubate the plate overnight at 37 °C.
    4. Harvest at least three single colonies from the plate and inoculate each in 4 mL of LB containing carbenicillin (0.2 mg/mL) using a 14-mL sterile tube. Incubate cultures overnight at 37 °C and shake at 250 rpm.
    5. Spin down overnight cultures at 8,000 x g for 10 min and extract DNA following the instructions of commercially available mini-preparation kits.
    6. Verify the presence of single-cysteine mutants by standard automated DNA sequencing (see **Table of Materials**).

2. Functional Analysis of eTRPV1 and Single-cysteine Mutants

1. **HEK293 cell line transfection[30]**
    1. Culture HEK293 cells in 6 -well plates in 2 mL of high glucose medium (DMEM) per well (37 °C, 5% CO_2, 95% Humidity). Prepare HEK293 cells that are 70 – 80% confluent.
    2. Prepare the transfection mixture in two separate 1.5-mL micro-centrifuge tubes.
        1. For reaction A, add 0.5 µg eTRPV1 or cysteine mutant-containing pMO to 250 µL of minimal essential medium (*e.g.* Opti-MEM). For reaction B, add 3 µL of transfection reagent to 250 µL of minimal essential medium. Incubate each reaction for 5 min at room temperature (RT).
        2. Mix reactions A and B with a 1-mL pipette. Incubate the mixture for 45 min at RT.
    3. Remove 500 µL of culture media from the 70 - 80% confluent well and add 500 µL of the reaction mixture. Store plates overnight for Ca^{2+} imaging (37 °C, 5 % CO_2, and 95% humidity).
2. **Ca^{2+} imaging in HEK293 cells[30]**
    1. Clean 12-mm diameter glass coverslips with ethanol and water and place them into a 24-well plate.
    2. Add 75 µL of poly-l-lysine in the center of each coverslip and store the plate for 45 min (37 °C, 5% CO_2, 95% humidity).
    3. Remove the excess poly-l-lysine and wash coverslips three times with phosphate-buffered saline (PBS) to remove any leftover.
    4. Once the coverslips are ready, proceed to detach cells from the 6-well plate.
    5. Remove the media and add 500 µL of warm PBS (37 °C) for washing.
    6. Remove PBS, add 500 µL of trypsin, and incubate for 1 min.
    7. Add 500 µL of warm culture media (37 °C) to stop the digestion reaction and mix with the pipette; avoid forming bubbles. If needed, adjust the cell concentration diluting this resuspension with more culture media.
    8. Seed 250 µL of transfected HEK293 cells (70% confluent) plus 250 µL of culture media (500 µL final volume per well) on the pre-treated coverslips and let them settle down (for attachment) for 1 - 2 h in the incubator (37 °C, 5 % CO2, 95% humidity).
    9. In the meantime, prepare a loading solution by adding 0.02% pluronic acid and 1 µM Fluo-4-AM to a Ringer's buffer. Mix the solution well.
    10. Remove the HEK293 culture media from the well and add 500 µL of the loading solution. Incubate cells for 1 h (37 °C, 5% CO_2, 95% humidity). Wash the Fluo-4-loaded cells twice with warm Ringer's buffer.
    11. Place a coverslip with loaded cells into a 10-mm Petri dish in a microscope stage (using a 10X objective; 494 nm excitation and 506 nm emission) to perform intracellular Ca^{2+} measurements.

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12. Measure changes in fluorescence intensity after perfusing respective ligands (*e.g.*, 10 µM capsaicin) using commercially available software (see **Table of Materials**).

3. **mRNA and *Xenopus laevis* oocytes preparation**[30]
    1. Linearize eTRPV1 and single-cysteine mutants-containing pMO with *Pme*I for 1 h at 37 °C. Clean DNA following the instructions of commercially available purification kits.
    2. Use linearized and cleaned DNAs to synthesize capped RNAs with a commercially available kit compatible with T7 RNA polymerase.
    3. Clean capped RNAs according to commercially available purification kits. Quantify the RNA amount by measuring the absorbance at 260 nm wavelength.
    4. Transfer 5 – 10 mL of *X. laevis* oocytes (commercially available) into a 50-mL conical tube containing 20 mL of ND96 solution (96 mM NaCl, 2 mM KCl, 5 mM HEPES, 1 mM $MgCl_2$; pH 7.4) with 1 mg/mL collagenase and nutate for 50 min at RT.
    5. Rinse the oocytes in ND96 until the solution is clear; add fresh collagenase and shake for 30 min at RT. Examine the oocytes under a stereo microscope and stop the digestion when the outer follicular layer (glossy layer with red blood vessels) is absent in most oocytes (90%). Rinse oocytes in ND96 until the solution is clear.
    6. Transfer oocytes to a 50-mL polystyrene tube with ND96 plus 1 mM $CaCl_2$ and shake for 1 h. Under-digested oocytes will stick to the polystyrene tube.
    7. Wash oocytes with ND96 plus 1 mM $CaCl_2$ and supplement solution with 50 µg/mL of gentamicin and 50 µg/mL of tetracycline.
    Note: Protect tetracycline-containing solutions from light exposure.
    8. Select large oocytes without damaged membranes and displaying a clear separation between the animal and vegetable poles. Let oocytes recover for 4 h before microinjection at 16 °C.
    9. Use glass pipettes (O.D.: 1.11 mm, I.D.: 0.5, and length 8.8 cm) to inject 1-5 ng of mRNA from eTRPV1 and single-cysteine mutants into oocytes using a nanoliter-injector system (46 nL/s) and a stereo microscope (2X magnification). Store oocytes at 16 °C in ND96 supplemented with $CaCl_2$ and antibiotics (gentamycin/tetracycline 1x).
    10. After 72 h, measure macroscopic current using a two-electrode voltage-clamp (TEVC) acquisition system[31].
    11. Pull borosilicate glass pipettes (0.3 MΩ) and fill them with 3 M KCl.
    12. Place the oocyte in the recording chamber using a transfer pipette and perfuse the bath solution (120 mM NaCl, 2 mM KCl, 2 mM $MgCl_2$, 1 mM EGTA, and 10 mM HEPES solution; pH 7.4) at low speed.
    13. Immerse both electrodes in the bath solution to adjust their pipette offsets. Gently push the pipette electrodes (borosilicate glass pipettes; O.D.: 1.5 mm, I.D.: 1.10, and length 10 cm) into the oocyte until a tiny indentation is observed under a stereo microscope (2X magnification), as well as a change in the membrane potential.
    14. Change the amplifier settings to recording mode and measure macroscopic currents while applying a voltage-clamp ramp from -80 to +80 mV for 1 s. Load the perfusion system with the solution used in step 2.3.12 at pH 7.4 (as a control) and at pH 5 to check eTRPV1 activity.

3. Generating the Recombinant Bacmid and Baculovirus for Protein Expression

1. **Bacmid**
    1. Transform 100 µL of DH10Bac *E. coli* competent cells with 7.5 ng of the recombinant donor vector containing eTRPV1 and/or single-cysteine mutants.
    2. Add 900 µL of SOC media (20 mg/mL Tryptone, 5 mg/mL yeast extract, 2.5 mM KCl, 10 mM $MgCl_2$, 10 mM $MgSO_4$, and 20 mM glucose) and incubate for 6 - 7 h at 37 °C and 225 rpm.
    3. Seed 100 µL directly from the mixture, and from serial dilutions (1/10 and 1/100), in LB-agar plates containing 100 µg/mL X-gal, 40 µg/mL IPTG, 7 µg/mL gentamicin, 10 µg/mL tetracycline and 50 µg/mL kanamycin. Incubate the plates for at least 48 h at 37 °C.
    4. Select white colonies that are 2 mm in diameter, since blue colonies lack the insert of interest. Use a stereo microscope to verify that white colonies do not contain any blue spots.
    5. Harvest at least three single colonies and transfer them into a 14-mL sterile tube containing 4 mL of LB media supplemented with 7 µg/mL gentamicin, 10 µg/mL tetracycline, and 50 µg/mL kanamycin. Incubate cells overnight (~16 h) at 37 °C and 250 rpm.
    6. Take 1.5 mL of the overnight cultures and isolate recombinant bacmid DNA (refer to the Supplementary File 1 for detailed protocol). Store the bacmid at 4 °C for up to two months.
    Note: Prepare the glycerol stocks of DH10Bac *E. coli* containing the bacmid DNA. Take 300 µL of culture and add 200 µL of glycerol at 50% (final glycerol concentration of 20%). Store the aliquot at -80 °C until further use.
    7. Verify the presence of eTRPV1 in the recombinant bacmid using PCR (refer to **Supplementary File 1** for detailed protocol).

2. **Baculovirus**
Note: For this procedure, transfect Sf9 insect cells in a 6-well format. All amounts and volumes are given on a per-well basis. Avoid the use of antibiotics.
    1. Plate 1 x 10^6 Sf9 cells in 2 mL of insect cell media. Allow cells to attach for at least 45 min.
    2. Dilute 1 µg of recombinant bacmid DNA in 100 µL of insect cell media. Mix gently.
    3. Dilute 6 µL of transfection reagent (TR) in 100 µL of insect cell media. Mix gently.
    4. Combine the DNA and TR solutions (from steps 3.2.2 and 3.2.3, respectively). Mix gently and incubate for 30 min at RT.
    5. Add 0.8 mL of insect cell media to the DNA/TR mixture; mix gently.
    6. Remove the insect cell media from the Sf9 cells and wash once with 1 mL of fresh medium.
    7. Remove the wash medium and add DNA/TR mixture (3.2.2 – 3.2.5) to the cells. Incubate cells at 27 °C for 5 h (without agitation).
    8. Remove the transfection mixture and replace with 2 mL of insect cell media containing 0.5% fetal bovine serum (FBS). Incubate cells at 27 °C for five days.
    CAUTION: Prevent evaporation of the cell media by filling the empty wells with media and covering the 6-well plate with two layers of paraffin film.

9. Transfer the cell culture media into a 15-mL centrifuge tube. Isolate the first passage (P1) of viral stock by spinning the tube at 8,000 x g for 15 min at 4 °C. Transfer the supernatant to a clean 15-mL centrifuge tube and immediately store it at 4 °C.
   Note: Protect the virus from light exposure by covering the tube with aluminum foil.
10. For the second generation (P2) of viral stock, put a 25-mL suspension culture of insect cell media at 1 x 10^6 Sf9 cells/mL containing 2% FBS into a 125-mL flask (plain bottom and vented). Add 25 µL of the P1 virus to the 25 mL culture.
11. Incubate the culture at 27 °C for 72 h.
12. To harvest the P2 viral stock, transfer the culture to a new 50-mL tube and centrifuge it at 8,000 x g for 15 min at 4 °C.
13. Transfer the supernatant to a new 50-mL tube and immediately store it at 4 °C.
    Note: Protect the virus from light exposure by covering the tube with aluminum foil. Perform a small-scale experiment to titrate the P2 virus/Sf9 ratio for optimal TRPV1 expression (refer to **Supplementary File 1** for a detailed protocol, Section 4 titled “Small-scale experiment for P2 virus.”)
14. For large-scale protein expression, set a 1-L Sf9 cell suspension culture at 2 x 10^6 cells/mL in insect cell media supplemented with 0.5% FBS into a 2.8-L borosilicate glass flask.
15. Add 1 mL of P2 virus stock to the 1-L culture and incubate it at 27 °C for 72 h.
    Note: On the third day, cell density should be at approximately 1.5 to 2.5 x 10^6 cells/mL.

4. eTRPV1 and Single-cysteine Mutant Purification

1. Membranes isolation[28]
   1. Harvest the 1-L insect cell suspension culture by spinning at 4,500 x g, 4 °C, for 20 min. Weigh the cell pellet (it is expected to be around 6 - 9 g).
   2. Resuspend the cell pellet in 25-mL of ice-cold Buffer A (36.5 mM sucrose, 2 mM tris(2-carboxyethyl)phosphine (TCEP), and 50 mM Tris; pH 7.4) in the presence of protease inhibitors (1 mM phenylmethyl sulfonyl fluoride (PMSF), 3 µg/mL leupeptin, 3 µg/mL aprotinin, and 1 µg/mL pepstatin). Disaggregate cell clumps to obtain a homogeneous suspension and rotate at 4 °C for 20 min.
   3. Break the cells using a manual (dounce tissue grinder) or a high-pressure homogenizer. Remove the cell debris by centrifugation at 8,000 x g for 20 min at 4 °C.
      Note: Keep the lysed cells and tubes on ice during the whole process.
   4. Collect supernatant and centrifuge at 100,000 x g for 30 min at 4 °C. Discard supernatant and resuspend the membrane pellet in a total volume of 20 mL of ice-cold Buffer B (150 mM NaCl, 10% glycerol, 2 mM TCEP, 50 mM HEPES; pH 7.4), supplemented with protease inhibitors (as in step 4.1.2).
   5. Aliquot 20 mL of membrane suspension in 50-mL tubes and flash-freeze in liquid nitrogen. Store samples at -80 °C until further use.
2. Protein purification[26,28]
   1. Thaw the membrane aliquots on ice and add 3 mL of n-Docecyl-β-D-Maltopyranoside (DDM) per tube (200 mM stock). Rotate the tube for 2 h at 4 °C.
   2. Centrifuge the sample at 100,000 x g for 30 min at 4 °C. Collect the supernatant and add 1 mL of clean wet amylose resin. Rotate the mixture for 2 h at 4 °C. Load the protein/amylose mixture in gravity flow chromatography columns.
      Caution: Do not allow the resin to dry during washes.
   3. Wash the protein-bound amylose resin with 10 times the bed volumes of ice-cold Buffer C (150 mM NaCl, 10% glycerol, 50 mM HEPES, 0.5 mM DDM, 0.1 µg/mL asolectin, 0.5 mM TCEP; pH 7.4). Wash with 10 bed volumes of ice-cold Buffer D (150 mM NaCl, 10% glycerol, 50 mM HEPES, 0.5 mM DDM, 0.1 µg/mL asolectin, pH 7.4).
      Note: Prior to washing, degas Buffer D without detergent or lipids, purging with nitrogen. After degasification, add DDM and asolectin.
   4. Elute eTRPV1 protein with 0.5 mL fractions, up to 5 mL, of ice-cold degassed Buffer D, supplemented with 20 mM maltose. If possible, perform the washes and elution at 4 °C.
   5. Quantify the protein amount by measuring the absorbance at 280 nm wavelength.
      Note: This protocol yields 0.5 to 1.0 mg of protein per liter of culture. Protein yield could vary for each eTRPV1 single-cysteine mutant. For EPR and DEER experiments, grow 4 – 6 L of culture.

5. eTRPV1 Single-cysteine Mutant Site-Directed Spin Labeling

1. Concentrate the eTRPV1-containing fractions using a centrifugal filter unit (cutoff = 100 kDa) up to 2 – 2.5 mg/mL for labeling (cycles of 7,000 x g for 2 min at 4 °C).
2. Add a 10-fold molar excess (3 times, every 30 min) of (1-oxyl-2,2,5,5-tetra-methylpyrrolidin-3-yl) methyl methanethiosulfonate (MTSSL) spin label from a 100-mM stock solution in DMSO to the concentrated protein (eTRPV1 monomer: MTSSL in 1:10 molar ratio)[17]. Keep the reaction in the dark at RT for 1 h 30 min, followed by overnight incubation at 4 °C.
   Note: In this step, MBP could be removed by adding TEV protease during the overnight spin-labeling incubation.
3. Load spin-labeled eTRPV1 on a size exclusion chromatography column equilibrated in Buffer E (150 mM NaCl, 20 mM HEPES, 0.5 mM DDM; pH 7.4), controlled by a fast protein liquid chromatography (FPLC) system. Collect fractions containing eTRPV1 tetramer.
   Note: Do not include 10% glycerol in this step, as it reduces the reconstitution efficiency.
4. Quantify the protein amount by measuring the absorbance at 280 nm wavelength.
5. Evaluate the purity of the sample by running a SDS-PAGE gel (stain-free gel, 4 – 20%). The sample is now ready for spectroscopic measurements in solution and/or proteoliposomes.

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6. eTRPV1 Spin-labeled Single Cysteine Mutant Reconstitution

1. Dry 10 mg of asolectin using a rotary evaporator under vacuum (≤100 mbar) for 1 h at 40 °C. To make asolectin liposomes, add 1 mL of Buffer F (200 mM NaCl, 5 mM MOPS; pH 7.4) and sonicate the mixture for 15 min or until the sample is homogeneous.
2. Destabilize the liposomes by adding DDM to a final concentration of 2 mM and incubate for 30 min at RT.
3. Concentrate the labeled protein at 2 mg/mL and add it to the liposomes using a 1:5 protein/Lipid ratio (mass:mass).
   CAUTION: It is important to keep the aforementioned protein concentrations, since spin-labeling efficiency decreases at low concentrations and at high the protein might precipitate.
4. Add Buffer F to adjust the protein/Liposome mixture to the DDM critical micelle concentration (CMC) and incubate overnight at 4 °C with gentle agitation.
5. Double the volume of the protein/Liposome mixture with Buffer F and remove detergent by sequentially adding three aliquots of nonpolar polystyrene adsorbent beads (30 mg, 50 mg, and 80 mg) at 1-h intervals with gentle agitation at RT.
6. Load the mixture onto a column filter (gravity flow chromatography column) to remove the beads and transfer the proteoliposomes mixture to an ultracentrifuge tube. Centrifuge the samples at 100,000 x g for 1 h at 4 °C. Discard supernatant and resuspend the pellet in 30 µL of Buffer F.
   Note: Samples are ready for spectroscopic analysis and injection into *Xenopus* oocytes. If the samples cannot be used the same day, flash-freeze them in liquid nitrogen and store at -80 °C.
7. Proteoliposomes-*Xenopus* oocyte electrophysiology[26,32]
   1. Inject 50 nL of different dilutions of spin-labeled proteoliposomes into *Xenopus* oocytes as described in section 2.2.
   2. Perform TEVC measurements after 12 h of injection as described in section 2.3.10.
8. Proceed to perform spectroscopic measurements and analysis (section 7).

7. DEER and EPR Spectroscopies

1. **Double Electron-Electron Resonance (DEER) Spectroscopy.**
   1. Perform DEER measurements in a pulsed EPR spectrometer operating at Q-band frequency (34 GHz) and equipped with a 10-W amplifier with the dead-time free four-pulse sequence at 83° using manufacturer-supplied software[33].
   2. Supplement 50 µM of detergent-purified eTRPV1 spin-labeled cysteine mutants in Buffer E (step 5.5) with 30% (v/v) glycerol for cryo-protection.
   3. Load the sample into a sealed quartz capillary tube and spin to the bottom of the tube by brief low-speed centrifugation (100 x g).
   4. Place the capillary tube into liquid nitrogen to freeze the sample. The samples can be stored at this point at -80 °C for later measurement.
   5. Place the sample directly into the microwave resonator and let it re-equilibrate at -83° for 10 – 20 min.
   6. Measure the sample using a standard four-pulse DEER protocol, (π/2)mw1–τ1–(π)mw1–τ1–(π)mw2–τ2–(π)mw1–τ2–echo[34]. The pulse lengths for (π/2)mw1 and (π)mw1 are 10 and 20 ns, respectively, and 40 ns for (π)mw2. Set the frequency separation at 63 MHz.
      Note: Primary DEER decay data can be analyzed with a home-built software (*e.g.* in Matlab) that assumes a sum of Gaussian distributions to describe the distances between spin labels[19,35].

2. **Continuous-wave (CW) EPR spectroscopy**
   1. Perform CW EPR experiments at RT on an X-band (9.6 GHz) spectrometer using the manufacturer software.
   2. Start the instrument by turning on the water chiller, the console, and the magnet power supply. Connect the software to the instrument, place the instrument in tune and wait at least 30 min for the instrument to warm up.
   3. Load 20 µL of the sample from step 5.5 into a 25-µL glass capillary tube using capillary action and seal the end of the tube with sealant.
   4. Load the capillary tube into the microwave cavity and critically couple the resonator either manually or automatically using the auto-tune function of the software.
   5. Collect the first derivative spectra under standard instrument conditions: 100 kHz microwave modulation, 1.6 G magnetic field modulation, and 10 mW microwave power.
   6. For data analysis and presentation, correct the spectra against the background and normalize them by dividing the spectra by the peak-to-peak value of the double integral.
   7. Determine the spin-label mobility by measuring the inverse of the central line width of the first derivative absorption spectra (ΔH_o^{-1})[36].

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Representative Results

Functional Characterization of the Minimal Cysteine-less TRPV1 Construct (eTRPV1) and Single-cysteine Mutants

The first step toward spectroscopic studies is to engineer and characterize cysteine-less protein constructs (**Figure 2A**) that are functional and yield biochemical amounts of proteins. eTRPV1 is functional as determined by Ca^{2+} imaging and TEVC (**Figure 2B-C**). Moreover, eTRPV1 provides sufficient amounts of detergent-purified protein for EPR and DEER experiments (0.5 - 1 mg per liter of Sf9 cells)[26]. Introducing single cysteines in particular protein regions might affect their function. **Figure 2B** shows some examples of single-cysteine mutants (E651C and A702C) on eTRPV1 that behave like wild type (WT), since their fluorescence intensity increases when TRPV1 agonist (*e.g.*, capsaicin) is added to eTRPV1-containing HEK293 cells, as shown by Ca^{2+} imaging[26]. On the other hand, A680C is the typical example of a non-functional cysteine mutant, as the fluorescence is indistinguishable from the background. The A680C mutant was excluded for further analysis. After Ca^{2+} imaging experiments, single-cysteine mutants' function is tested using TEVC or patch clamp to evaluate their biophysical properties. **Figure 2C** shows that mutants recapitulate the outward rectification characteristic of TRPV1 when challenged with pH 5, as determined by TEVC[26]. Functional analysis of single-cysteine mutants is the first checkpoint before undertaking expression and purification protocols.

Biochemical Characterization of the eTRPV1 and Single-cysteine Mutants

The purification protocol described above yields detergent-solubilized protein that can be labeled via cysteine covalent modification (*e.g.*, fluorophores, spin-labeled [SL] methyl-methanethiolsulfonate) along the TRPV1 sequence for spectroscopic analysis. Minimal TRPV1 channel-containing cysteine residues migrate as stable and monodisperse species (~ 13.6 mL), as determined by size-exclusion chromatography (**Figure 3**). eTRPV1 and single-cysteine spin-labeled mutants (E651C-SL and A702C-SL) recapitulate the elution profile and stability featured by the minimal TRPV1 construct (**Figure 3**)[26]. The elution profile could vary among different mutants, as single cysteines might affect protein stability. In some instances, mutants form aggregates; and a fraction of the sample could elute at the void volume of the column (8 - 9.5 mL), reducing the amount of protein that migrates as a tetramer (**Figure 3**, bottom red arrow). In this case, cell culture volumes can be scaled up to compensate for the protein lost in the aggregated fraction, or one can exclude this mutant from further analysis and proceed to test the neighboring residue. Other examples include a broadening of the peak that corresponds to the tetramer. Main peaks wider than 3 mL are not recommended for spectroscopic analysis, as they contain multi-disperse species. Biochemical characterization of spin-labeled single-cysteine mutants is the second checkpoint before undertaking reconstitution and spectroscopic analysis.

Functional Characterization of Reconstituted Spin-labeled eTRPV1 Single-cysteine Mutants

Because the EPR and DEER signals rely on the attachment of a paramagnetic spin-label to a cysteine residue, it is important to verify whether the location of the spin-label alters protein function. There are several ways to test for function after reconstituting the spin-labeled protein, including planar lipid bilayer experiments, patch clamp, and TEVC. TEVC allows the evaluation of a large number of spin-labeled channels while recording macroscopic currents. To assess the functionality of the spin-labeled single-cysteine mutants, channels are reconstituted in pre-formed asolectin liposomes. During this step, it is important to exclude the 10% glycerol that is present throughout purification, since glycerol decreases the efficiency of protein reconstitution into liposomes. **Figure 4** shows a representative result of A702C-SL TRPV1 reconstituted in asolectin liposomes and microinjected into *Xenopus* oocytes. As expected, pH 5 elicits robust outwardly rectifying currents[37] that are blocked by co-application of the TRPV1 antagonist capsazepine (CPZ, blue trace)[26]. Mutants that do not retain functionality after spin labeling and reconstitution should be excluded from further analysis. Functional characterization of reconstituted spin-labeled single-cysteine mutants is the third checkpoint before undertaking spectroscopic analysis.

Structural Dynamics of Spin-labeled eTRPV1 Mutants Monitored by CW-EPR and DEER

Nitroxide spin labels are sensitive to the surrounding environment and the flexibility of the protein backbone to which the label is attached[17]. Hence, CW-EPR allows determination of the dynamic regimen of a given spin-labeled cysteine; namely, positions exposed to the aqueous media or the membrane are more dynamic than those restricted to protein-protein interactions[17]. **Figure 5A** shows positions Glu651, Ile679, and Ala702 in TRPV1 chosen to monitor mobility parameters. To calculate the mobility parameter of the spin label probe, one calculates the inverse of the central line width of the first derivative absorption spectra (**Figure 5B**, black line ΔH_o^{-1}). For instance, E651C-SL (**Figure 5B**) displays a spectral line shape and mobility value (0.24) commonly found on dynamic positions at the membrane interface[26]. On the other hand, I679C-SL and A702C-SL exhibit broadening of the spectra (see dotted lines) and a decrease in the mobility values (0.16 and 0.18, respectively; **Figure 5B**)[26] that are consistent with their constrained surrounding environment (protein-protein), according to the cryo-EM structure[1].

Figure 5C shows the spectra of spin-labeled TRPV1 mutants after reconstitution in asolectin liposomes. As expected, the dynamic features of the spectra for E651C-SL and A702C-SL did not change after reconstitution, as their shape and mobility values are the same in solution as in a membrane environment[26]. On the other hand, I679C-SL illustrates a noisy spectrum; consequently, the lower (blue arrow) and higher (red arrow) field components become less obvious. Noisy spectra are not usually desired, as they tend to underestimate the mobility of the spin-labeled position. This spectrum type could be the product of inefficient protein reconstitution rather than under-labeling, since the I679C-SL signal in solution is robust.

DEER data are a sum of oscillating, sinusoidal signal decays containing information about the distance distribution of interacting spin-labels[38,39]. The period and complexity of the decay directly reflects the underlying distance distribution. The distance distribution is comprised of the number of structural components present in the sample and their disorder. Hence, the time-domain signal derives from the dipolar coupling between non-fixed, distant spin labels in solution which usually cause an exponential background decay, andrigidly coupled spins within the same protein[19,40]. Specifically, DEER allows the determination of intra-protein long-range distances (20 - 70 Å) between spin-labeled residues[19]. **Figure 6** shows the signal decay and the corresponding distance distribution of E651C-SL. The distribution of Glu651 shows three peaks corresponding to 24, 36, and 58 Å[26]. The two shorter distances are consistent with the closed TRPV1 structure (23 and 32 Å for the Cβ-Cβ distances, respectively)[1], whereas the third 58 Å peak might correspond to protein aggregation.

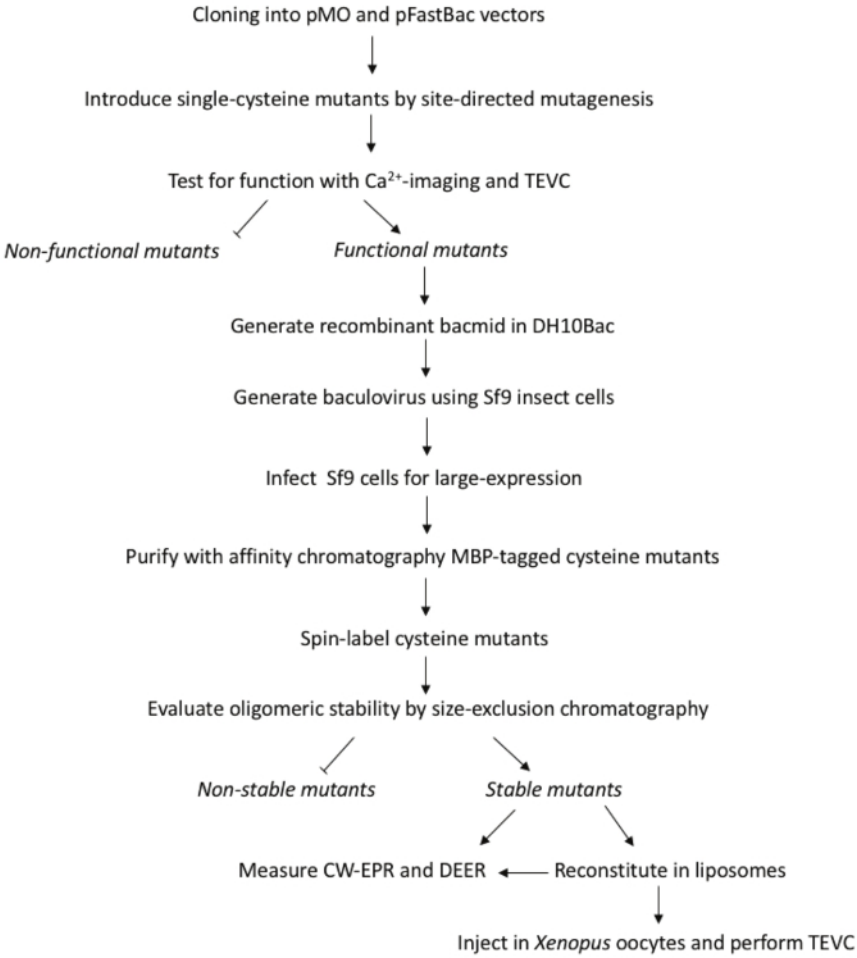

Figure 1. Experimental outline. Diagram of the experimental outline required to express, purify, and reconstitute eTRPV1 for EPR and DEER. Please click here to view a larger version of this figure.

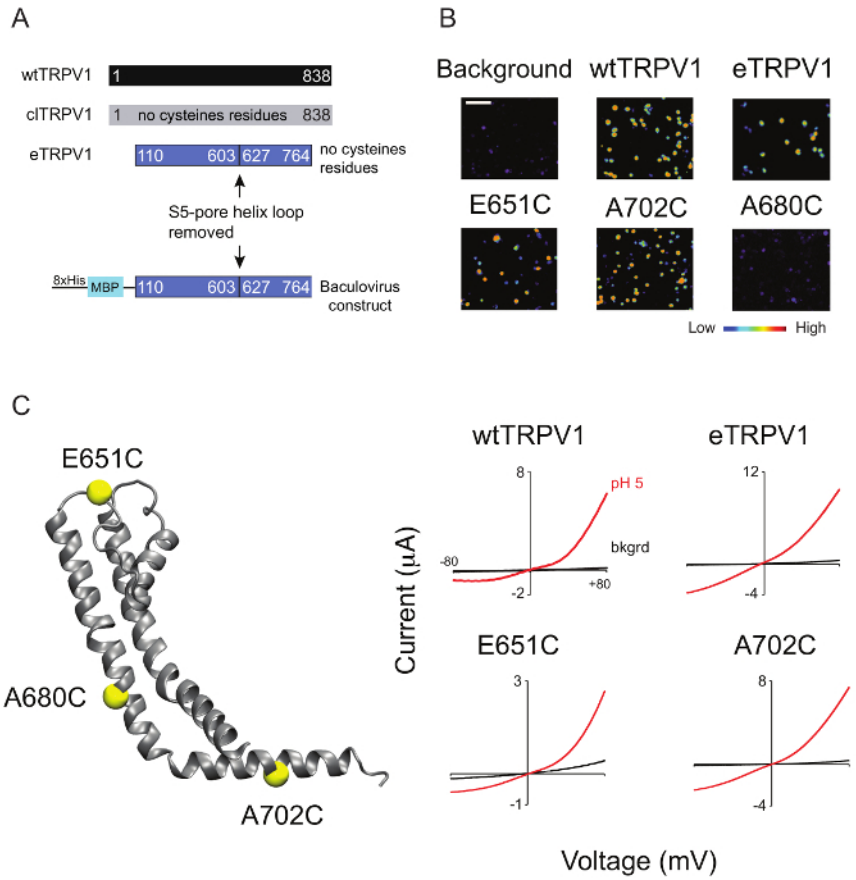

Figure 2. Functional characterization of eTRPV1 and single-cysteine mutants. (**A**) Schematic representation of the TRPV1 constructs used for spectroscopic analysis (eTRPV1: cysteine-less TRPV1 110-603/627-764). (**B**) HEK293 cells expressing WT TRPV1, eTRPV1, and mutants (loaded with Ca^{2+}-sensitive Fluo-4-AM) were analyzed for capsaicin (10 μM)-evoked responses using fluorescence Ca^{2+} imaging. Color bar indicates relative changes in fluorescence intensity, with blue and red denoting the lowest and highest cytoplasmic Ca^{2+}, respectively. White bar represents 100 μm. (**C**) Left, one subunit (S5, pore helix, S6 and TRP domain) of TRPV1 tetramer structure highlighting single-cysteine residues (yellow spheres) introduced along the channel sequence. Right, current-voltage relationships determined by TEVC recordings from *Xenopus* oocytes expressing WT TRPV1, eTRPV1, and single-cysteine mutants challenged with pH 5. Background currents (bkgrd). Modified from the original figure[26]. Please click here to view a larger version of this figure.

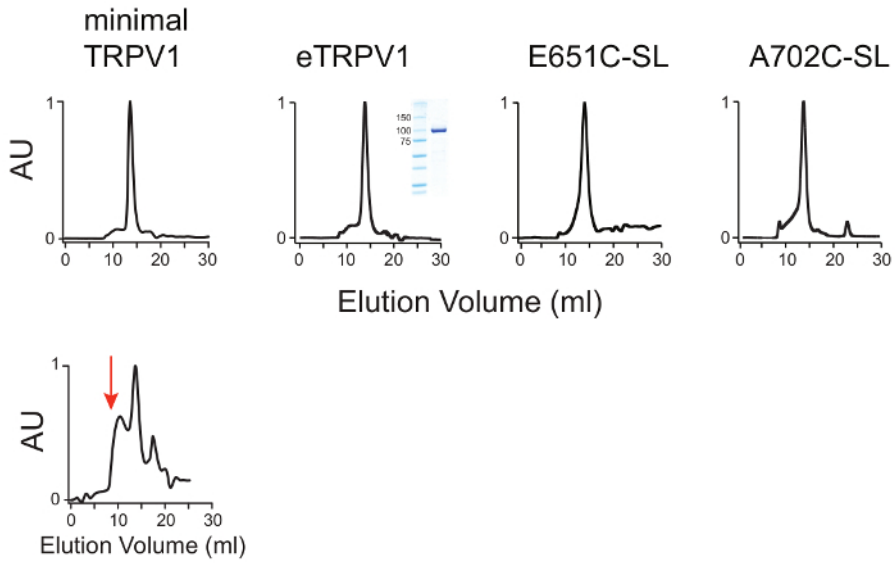

Figure 3. Biochemical characterization of eTRPV1 and single-cysteine mutants.
Size-exclusion chromatography profile of DDM-solubilized eTRPV1 and single-cysteine spin-labeled mutants after expression and purification from Sf9 cells. Inset: SDS-PAGE gel showing the monomeric eTRPV1-MBP fusion protein (modified from the original figure[26]). Please click here to view a larger version of this figure.

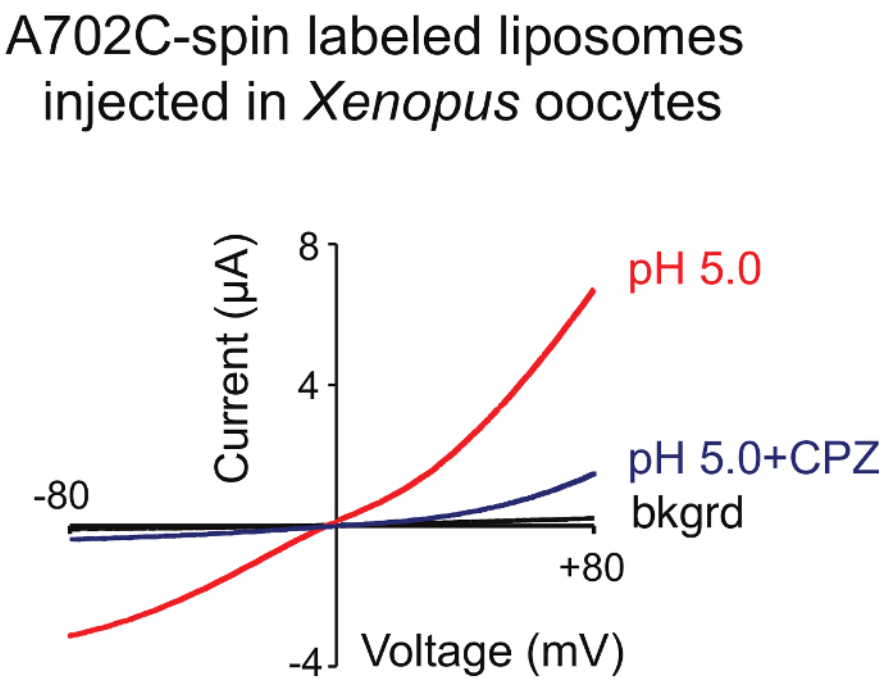

Figure 4. Functional characterization of a spin-labeled eTRPV1 mutant.
Current-voltage relationships determined by TEVC from *Xenopus* oocytes microinjected with proteoliposomes containing spin-labeled A702C challenged with pH 5 (red) and blocked by capsazepine (CPZ, blue: 40 μM). Background currents (bkgrd). Modified from the original figure[26]. Please click here to view a larger version of this figure.

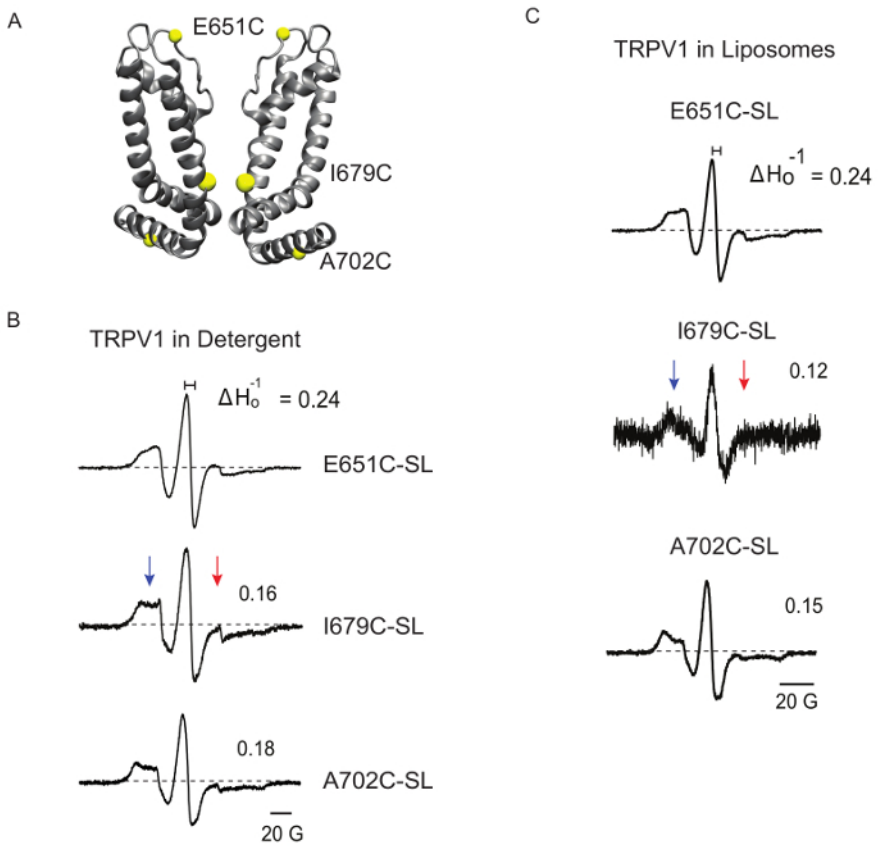

Figure 5. Mobilities of spin-labeled eTRPV1 mutants determined by CW-EPR.
(**A**) Two subunits (S5, pore helix, S6 and TRP domain) of TRPV1 tetramer structure highlighting the amino acid residues (yellow spheres) probed through site-directed spin-labeling spectroscopies. (**B**) First derivative of CW EPR spectra of spin-labeled cysteine mutants in DDM-solution. (**C**) First derivative of CW EPR spectra of spin-labeled cysteine mutants reconstituted in asolectin liposomes. Spectra were obtained at pH 7.4 (closed state). ΔHo^{-1} denotes the magnitude of the mobility parameter. The black dotted line highlights the broadening of the spectra. Blue and red arrows denote the low and high field components of the spectra, respectively. EPR spectra were normalized to the total number of spin labels. Modified from the original figure[26]. Please click here to view a larger version of this figure.

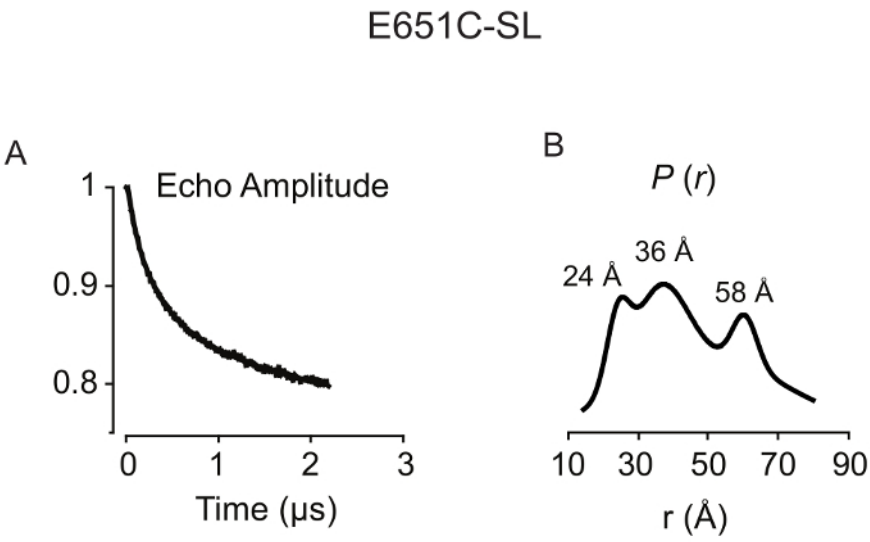

Figure 6. Distance distribution of an eTRPV1 mutant in detergent.
DEER echo (**A**) and distance distribution (**B**) of spin-labeled mutant E651C; a sum of Gaussians was fitted to the DEER data. *P(r)* denotes distance distribution of the spin pairs[41]. Spectra were obtained at pH 7.4 (closed state). Modified from the original figure[26]. Please click here to view a larger version of this figure.

Discussion

Current technologies for expression and purification of mammalian membrane proteins have made it possible to obtain sufficient amounts of protein for spectroscopic studies[14,15,16,42]. Here, we have adapted these technologies to express, purify, reconstitute, and perform spectroscopic analyses in TRPV1.

Among the critical steps in the protocol, below are the ones that we have troubleshot for TRPV1 and that might be adjusted for other proteins. Modify the DNA sequence to generate a template that yields 0.5 - 1 mg/mL of detergent-purified protein; templates that yield less than 0.5 mg/mL of protein are challenging, since growing more than 6 liters of insect cells cultures would be required per mutant. Protein must be concentrated, no less than 2 mg/mL, without TCEP to increase spin-labeling efficiency. Labeling at low protein concentrations and/or in the presence of TCEP traces will generate noisy EPR spectra. Although the proteins are kept at 4 °C during purification, it is essential to perform spin labeling at RT to improve efficiency. During purification and labeling, glycerol improves protein stability; however, it must be completely removed before reconstitution, as it decreases the amount of protein in liposomes and generates noisy EPR spectra. Decreasing the detergent concentration below the CMC is a common practice during reconstitution; however, TRPV1 tends to precipitate before incorporating into the liposomes. To solve this problem, TRPV1 was incubated overnight with pre-formed liposomes exactly at the CMC to avoid protein precipitation and increase the quantity of channels in the proteoliposome preparation. TRPV1 is fully functional in asolectin liposomes[28]; hence, it was the preferred lipid mixture used in this protocol. However, it is essential to determine the lipid composition in which a particular protein is functional (*e.g.*, cholesterol) before proceeding with spectroscopic analyses.

Spectroscopic approaches such as EPR and DEER present several limitations, including: changes in protein template sequence that improve biochemical stability might have an impact in function[26]; introduction of non-native single cysteines, as well as the spin label, might not be well tolerated in certain regions of the protein; obtaining large amounts of labeled proteins; and limited conformational changes when determining distances with DEER in detergent micelles. However, the latter limitation could be overcome by collecting the spectra, at Q-band frequency, of TRPV1 mutants reconstituted in nanodiscs[19,43]. Nonetheless, the advantage of these approaches mainly comes from the pattern of global dynamics, accessibilities, and distances more than from the absolute value of a given position.

Temperature sensitivity is one of the most fascinating and less understood gating mechanisms; hence, using spectroscopic approaches, it would be possible to determine how membrane proteins translate thermal energy into protein motion in a membrane environment. Importantly, it would be challenging to determine structural changes during thermal gating using X-ray crystallography or cryo-EM, as these techniques are performed at freezing temperatures. Future experiments will be directed towards determining TRPV1 conformational changes compatible with thermal-dependent gating using EPR, DEER, or fluorescence. EPR spectroscopy has provided detailed mechanistic models for prokaryotic ion channels, so we expect that by using the protocols described above, we will gain insight into the mechanisms of activation and drug-binding of mammalian ion channels using spectroscopic approaches.

Disclosures

The authors have nothing to disclose.

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