

Video Article

Mutagenesis and Functional Analysis of Ion Channels Heterologously Expressed in Mammalian Cells

Bartosz Balana¹, Natalie Taylor¹, Paul A. Slesinger¹¹Clayton Foundation Laboratories for Peptide Biology, Salk Institute for Biological StudiesCorrespondence to: Bartosz Balana at balana@salk.edu, Paul A. Slesinger at Slesinger@salk.eduURL: <https://www.jove.com/video/2189>DOI: [doi:10.3791/2189](https://doi.org/10.3791/2189)

Keywords: Cellular Biology, Issue 44, Ion channels, electrophysiology, patch-clamping, site-directed mutagenesis, biophysics, neuroscience

Date Published: 10/1/2010

Citation: Balana, B., Taylor, N., Slesinger, P.A. Mutagenesis and Functional Analysis of Ion Channels Heterologously Expressed in Mammalian Cells. *J. Vis. Exp.* (44), e2189, doi:10.3791/2189 (2010).

Abstract

We will demonstrate how to study the functional effects of introducing a point mutation in an ion channel. We study G protein-gated inwardly rectifying potassium (referred to as GIRK) channels, which are important for regulating the excitability of neurons. There are four different mammalian GIRK channel subunits (GIRK1-GIRK4) - we focus on GIRK2 because it forms a homotetramer. Stimulation of different types of G protein-coupled receptors (GPCRs), such as the muscarinic receptor (M2R), leads to activation of GIRK channels. Alcohol also directly activates GIRK channels. We will show how to mutate one amino acid by specifically changing one or more nucleotides in the cDNA for the GIRK channel. This mutated cDNA sequence will be amplified in bacteria, purified, and the presence of the point mutation will be confirmed by DNA sequencing. The cDNAs for the mutated and wild-type GIRK channels will be transfected into human embryonic kidney HEK293T cells cultured *in vitro*. Lastly, whole-cell patch-clamp electrophysiology will be used to study the macroscopic potassium currents through the ectopically expressed wild-type or mutated GIRK channels. In this experiment, we will examine the effect of a L257W mutation in GIRK2 channels on M2R-dependent and alcohol-dependent activation.

Video Link

The video component of this article can be found at <https://www.jove.com/video/2189/>

Protocol

1. Site Directed Mutagenesis

We use mammalian expression plasmid pcDNA3.1 encoding a cDNA for GIRK2 and introduce a point mutation using Stratagene (Agilent Technologies) Quikchange XL II site-directed mutagenesis kit, according to the manufacturer's instructions.

Primers sequence can be easily generated using online primer design program available at:

<https://www.genomics.agilent.com/CollectionSubpage.aspx?PageType=Tool&SubPageType=ToolQCPD&PageID=15>

Note: We use standard Eppendorf 1.5 mL tubes in place of BD 14 mL tubes described in the protocol. However, the use of XL10-Gold ultracompetent *E. coli* pretreated with β -mercaptoethanol, 30s heat shock time and NZY⁺ (or NZYM⁺) media are critical for the successful mutagenesis. If possible, engineering an additional silent mutation that creates a new restriction site can be convenient for screening mutant colonies below.

2. Miniprep, DNA Sequencing and Maxiprep

2.1 Qiagen miniprep and sequencing.

Individual bacterial colonies are picked and grown in LB broth with ampicillin (AMP). We follow the manufacturer's instructions for purifying the cDNA. A simple restriction digest can be used to confirm the successful introduction of the mutation, if a restriction site is incorporated in Step 1 above. Automated DNA sequencing is done off-site, and is essential to confirm the presence of the mutation in the cDNA, as well as assess whether there are any unwanted mutations. This step is very important. We sequence the entire GIRK coding sequence using forward T7 promoter and reverse BGH sequencing primers that flank multiple cloning site of the pcDNA3.1 plasmid.

Note: It is important to carefully inspect the sequencing electropherogram to assure sufficient quality of the sequencing data. High quality electropherogram has sharp, non-overlapping peaks with little background noise.

2.2 Qiagen maxiprep.

We follow the manufacturer's instructions.

Note: This step is included in our experiments to improve the purity of the cDNA, which we find is important for efficient transfection.

3. Culture and Transfection of HEK293T Cells

All of these steps should be done in a sterile tissue culture hood.

3.1 Cell culture

HEK293T cells are convenient to use because they are easy to culture (37°C., 5%CO₂ humidified incubator), have fast replication time, high transfection efficiency and are amenable to whole-cell patch-clamp electrophysiology. HEK293T cells express SV40 large T antigen that ensures episomal replication of pcDNA3.1 plasmid.

HEK293T cells are cultured in low-glucose DMEM media supplemented with L-glutamine and 10 % Fetal Bovine Serum (FBS). For passaging cells, add 0.25% trypsin/EDTA to confluent (90-95%) HEK293T cells, wait until cells detach from the tissue dish, then stop the enzyme activity by addition of fresh media containing FBS/. Plate $\sim 1 \times 10^5$ cells/well into a 12 well cell culture dish together with 1 mL of antibiotic-free culture media per well. (1×10^5 cells is $\sim 350 \mu\text{L}$ of cell suspension obtained from confluent T25 flask resuspended with 10 mL media).

3.2 Transfection

After 8-24 hrs, HEK cells will adhere to the plastic and are ready for transfection. Transfect cells with 0.2 μg channel cDNA, 0.4 μg M2R receptor cDNA and 0.04 μg YFP cDNA using Invitrogen Lipofectamine 2000. Note: we use a small amount of YFP cDNA to determine which cells are successfully transfected (green cells will likely contain both GIRK channels and M2 receptors). The concentration of cDNA to use will need to be adjusted for each clone.

3.3 Fluorescence visualization of transfected cells.

Place 12-well plate on stage of an inverted fluorescence microscope, and examine cells for YFP expression. Compare to DIC image and make note of approximate number of green cells. This provides an estimate of the transfection efficiency.

3.4 Preparation of 24-well dishes and Poly-D-lysine coated cover slips

Clean 12 mm glass cover slips (Warner, CS-12R) by shaking them overnight with Radiacwash, followed by washing with deionized water and overnight shaking in 95% ethanol. Store the clean cover slips in 70% ethanol until use.

Place cover slips in a Petri dish with ethanol, flame off ethanol using forceps and burner, and place in one well of a sterile 24 well plate.

Cover each well with 250 μL of poly-D-lysine (PDL) solution [0.2 mg/mL] and let the cover slips sit overnight immersed in PDL solution under sterile hood. Wrap the 24 well plate in Parafilm to avoid evaporation.

Next morning, aspirate PDL and wash 2x with sterile water. Leave plates open to dry under the hood, then cover. The 24 well dishes with coated cover slips can be wrapped in with sterile aluminum foil and stored at room temperature for weeks.

3.5 Plating transfected HEK293T cells on the cover slips

24h after transfection, split cells into 24-well dish ($\sim 4 \times 10^3$ cells/well) containing glass cover slips coated with poly-D-lysine. Note: transfected cells can be used up to 24-72 hrs post transfection. The cells are split to provide individual cover slips for patch clamp electrophysiology experiment.

4. Electrophysiology

4.1 Preparing Solutions

Extracellular (bath) solution: 20 mM KCl, 140 mM NaCl, 0.5 mM CaCl₂, 2 mM MgCl₂ and 10 mM HEPES (pH 7.4 set with NaOH).

Aliquot 20K bath solution and add appropriate modulators

- 20K + Ba²⁺: solution containing 1 mM BaCl₂: a specific inhibitor of inward rectifying currents
- 20K + Carbachol: solution with 5 μM Carbachol: a specific M2R agonist; M2R couples to G-proteins that open GIRK channels
- 20K + Ethanol: bath solution containing 100 mM EtOH to activate GIRK channels directly

Place bath solutions into syringes (solution containers) of the rapid exchange perfusion system. Note: We use a Warner pinch valve system to control the bath solutions. PE tubing is recommended.

Internal/electrode solution: 140 mM KCl, 20 mM NaCl, 5 mM EGTA, 5.4 mM MgCl₂, 10 mM HEPES (pH 7.4) with 2.5 mM K₂ATP and 0.3 μM Li₂GTP.

We make the electrode solution without ATP and GTP. We separately prepare aliquots of ATP and GTP, which are stored at -80°C, and add to the electrode solution on the day of the experiment. We keep syringe with final electrode solution on ice to preserve the ATP/GTP. We sterile filter (0.2 μm) the internal/external solutions to inhibit microbial growth and reduce possibility of clogging the tubes and the electrode.

4.2 Pulling electrodes

We use Narishige two-step vertical puller and borosilicate glass electrode from Warner Instrument (1.5 mm outer diameter, 0.86 mm inner diameter and 7.5 cm length) to obtain electrodes with 3-7 M Ω of resistance when filled with intracellular solution.

Note: It is necessary to experimentally determine parameters for the electrode puller and type of electrode glass in your lab. For our vertical puller, we use a heat setting of 60.2 for the first pull and ~43 for the second pull.

4.3 Whole-cell patch clamping

1. Mount cover slip in a 35 mm dish using small amount of vaseline, cover with external solution and place dish on stage of an inverted fluorescence microscope. Use DIC to focus and find cells, switch to YFP fluorescence to locate YFP positive cells.
2. Position tip of perfusion manifold near a green cell (20-30 μ m from the cell of interest=2-3 cell length apart).
3. Fill electrodes with solution, attach it to the headstage of Axopatch 200B amplifier (Axon Instruments) with the following initial settings: Gain 1, Configuration: whole-cell β =1, mode V-clamp.
4. Apply positive pressure on electrode to prevent clogging of the pipette, and use the manipulator to place electrode tip just above the cell.
5. To Zero the pipette potential, select the Seal Test button to deliver a test voltage step. The 5 mV rectangular voltage step will appear in the Seal Test window of Clampex. Adjust the Pipette Offset potentiometer on the amplifier to bring the base-line current to 0 nA. Alternatively, zero the pipette potential by switching the amplifier mode selector to V-track mode and the meter knob to Vtrack and use the Pipette Offset potentiometer on the amplifier to bring the voltage on the amplifier meter display to "0.00".
6. Check resistance of electrode using seal test command of the Clampex 8.2 software (should be 3-7 M Ω).
7. Open a saved protocol file or create a new protocol. To open protocol file select from the main menu Acquire then Open Protocol and choose a saved voltage protocol file. For this experiment, the protocol has been created to hold membrane potential at -40mV, deliver a single 50 ms voltage step to -100 mV and ramp to +40 mV over 200 ms. This protocol is executed every 2 seconds. The ramp protocol allows for observation of the reversal potential and inward rectification of GIRK channels. For voltage-gated ion channels, a rectangular voltage step may be more appropriate.
8. Approach cell using fine adjustment of the manipulator, release positive pressure, after touching the cell surface apply negative pressure, and monitor increase in resistance in the Seal Test oscilloscope window. Once resistance is in the G Ω range, the electrode has sealed onto membrane (Gigaseal has formed).
9. Apply a pulse of negative pressure to break the cell membrane and get access into the cell. Once in the whole-cell configuration, you will register increased capacitive currents.
10. Switch to Membrane Test in Clampex and write down the access resistance R_a , membrane resistance R_m , membrane capacitance C_m and verify by eye the exponential fit of the theoretical curve to the real capacitive current. Membrane capacitance is proportional to cell size and will be used later to calculate current density.
11. Switch back to Seal Test, set V-Out (membrane potential) to -40 mV and filter at 10 kHz
12. Compensate for membrane capacitance and series resistance on the amplifier with the following steps:
 - a. Switch the WHOLE CELL CAP switch on. Adjust WHOLE CELL CAP and SERIES RESISTANCE dials back and forth, until you get a flat test pulse. You may also need to adjust pipette capacitance FAST CAP knob.
 - b. Turn on % PREDICTION knob to about 60-80%, again re-adjusting WHOLE CELL CAP and the SERIES RESISTANCE dial (you may have to adjust pipette capacitance compensation FAST CAP knob as well).
 - c. Turn on % COMP- to 100% without oscillating the cell, while trying to keep the pulse flat by re-adjusting WHOLE CELL CAP and SERIES RESISTANCE (you may have to adjust pipette capacitance compensation FAST CAP knob as well). Adjust the lag by reducing the time constant.
 - d. Write down values for C_m (membrane capacitance), R_s (series resistance), %COMP, %PRED and Lag time set on the amplifier in the notebook. The C_m and R_s should be approximately the same as the C_m and R_m measured in the Membrane Test above.
13. Turn on external solution valve to control the bath solution. Set the amplifier 8-pole Bessel filter to 2kHz, open a voltage clamp protocol and begin recording currents.
14. We record GIRK currents using a ramp protocol that steps to -100 mV for 50 ms then ramps from 100 mV to +40 mV over 200 ms and is delivered every 2 seconds. The ramp protocol allows for observation of the reversal potential and for inward rectification property of GIRK channels. At room temperature (22-25 $^{\circ}$ C), the GIRK current should reverse near -50 mV (with 20 mM KCl in the bath), which is near the calculated equilibrium potential for K ($E_K = -50$ mV), then remain fairly flat at potentials positive to E_K .
15. Experimental Protocol: After recording a stable baseline, switch to solution 20K + Carbachol and wait for peak of response, switch back to 20K bath solution, then apply 20K + Ethanol and wait for peak response, then switch to 20K bath solution, and finally to 20K + Ba $^{2+}$ solution. During application of these drugs you will see changes in the amplitude of GIRK currents (most pronounced at -100 mV). Note the trace number corresponding to the application of different extracellular solutions in the notebook. A single Clampfit file will contain all of the sweeps for the entire experiment.

5. Data Analysis

We use Clampfit 9.2 software to analyze data GIRK currents at -100 mV.

1. Open the file with the recording, set cursor at -100 mV. By default you will see all the traces overlaid on the screen. By pressing "Write cursors" icon you will create Excel-like spreadsheet with numerical data.
2. By assigning X to the trace number column and Y to the cursor column and pressing "Create Graph" icon you can quickly draw a time-course plot of the experiment.
3. Calculate basal current by subtracting current in presence of Ba $^{2+}$ from current recorded in external perfusion solution alone ("20K current" minus "20K + Ba $^{2+}$ current"). Calculate induced currents by subtracting basal current in 20K from the activated currents ("20K + Carbachol current" minus "20K current"; "20K + Ethanol current" minus "20K current"). Calculate current density (pA/pF) by dividing the current by

membrane capacitance (read directly from the amplifier). Calculate percent activation by dividing induced currents by basal currents and multiplying by 100.

6. Representative Results

You should be able to record current from cells transfected with wild-type channels. For GIRK channels measured in the extracellular solution containing 20 mM K^+ (and 145 mM K^+ intracellular) they should exhibit strong inward rectification and a reversal potential of ~ -50 mV, which is an essential property of potassium channels. The wild-type current will increase strongly upon carbachol application (3-5 fold increase over basal current) and respond similarly to the 100 mM ethanol. Note: the sign convention for electrophysiology is that inward current is negative and outward current is positive. For the L257W mutation in the alcohol binding pocket of GIRK2, you will see attenuated responses to both carbachol and ethanol. This suggests that L257 is involved in alcohol and G protein activation of GIRK2 channels. For further examples of the results of GIRK2 mutagenesis, see recent publication by Aryal *et al.*¹

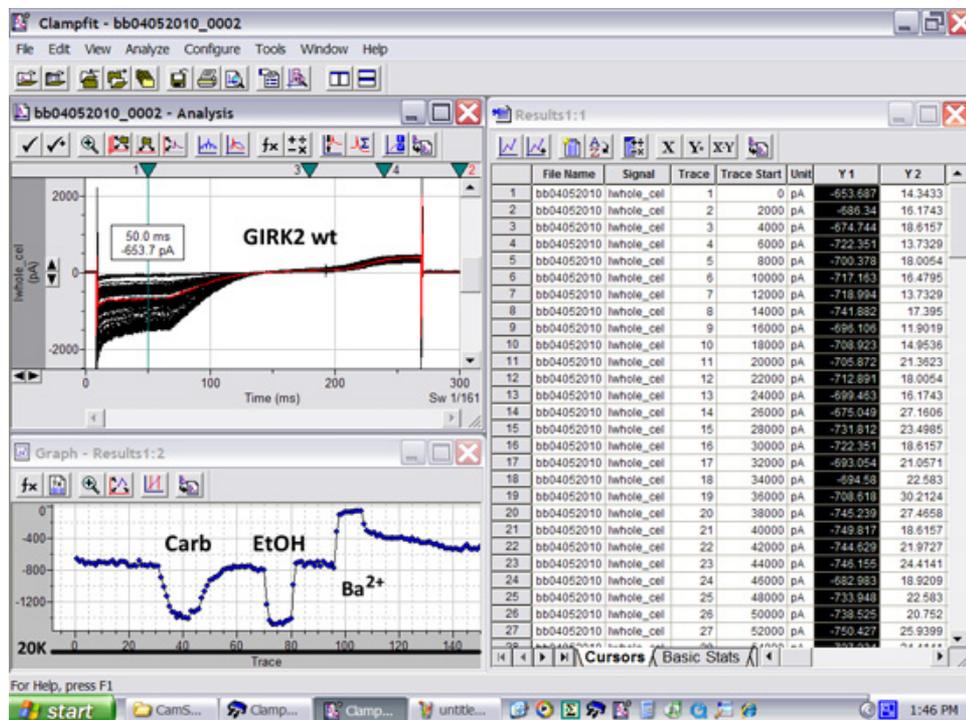


Figure 1. Window of Clampfit 8.0 shows data file for wild-type GIRK2 recording. Upper left panel shows superimposed sweeps recorded in response to a ramp voltage protocol in the presence of different external bath solutions. Cursor 1 is positioned at -100 mV. The cursor values are written to the spreadsheet (Right panel). Lower left panel shows the plot of sweep number vs., current. Note the increase in current (downward deflection) with ethanol (EtOH) and carbachol (Carb) and current inhibition with Ba^{2+} .

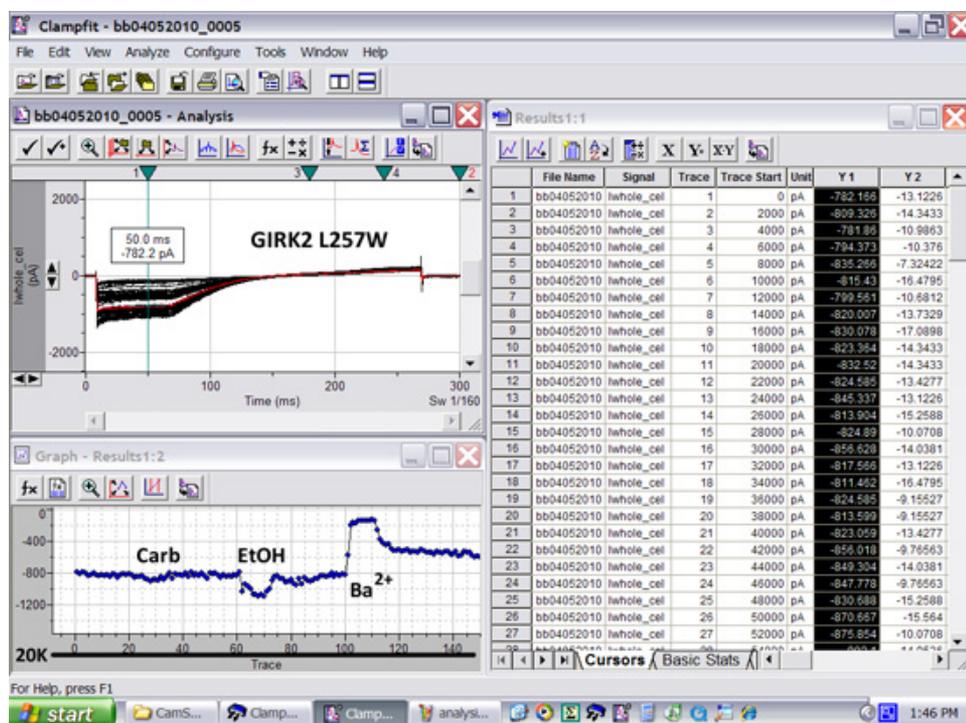


Figure 2. Window of Clampfit 8.0 shows data file for a mutant GIRK2-L257W recording. Descriptions are same as in Figure 1. Note the loss of carbachol (Carb) activation and reduction of EtOH-induced currents in the mutant channels.

Discussion

Site-directed mutagenesis is a classical approach to study structure-function relationships of ion channels. It is based on the premise that by changing a critical residue of the ion channel one should be able to observe pronounced changes in the channel function. In contrast, many mutations in non-critical position can be introduced without major perturbations to the channel function. Typically, the residue in question is first mutated to alanine, a small non-polar amino acid, followed by mutation to tryptophan, the largest of amino acids². If the residue is important to the channel function, both mutations will change channel activity. If a less essential residue is mutated, the effects of mutation, especially to alanine are often unremarkable. Tryptophan mutations are more likely to cause changes due to steric hindrances associated with the tryptophan size. Often, when the structural information is lacking, systematic mutation to alanine or tryptophan within a fragment of channel (called alanine or tryptophan scan) can be used to locate critical residues. It is important to note that multiple clones with different point mutations can be prepared in parallel allowing for testing multiple residues within relatively short time. Recently, multiple crystal structures of the channels have become available^{3,4,5}. They can be used to propose potential key residues to be tested using the approach described in this protocol.

Measurements on wild-type channel should always accompany the measurement of a mutant channel to serve as a positive control to assure that transfection and electrophysiological experiments were done correctly. Point mutations may lead to lack of a functional current, a current similar to the wild-type or altered regulation of the channel activity. Lack of measurable current may be due to improper folding or altered trafficking. In such case it may be useful to find out whether the channel traffics correctly to the surface using a biotinylation assay or immunofluorescence staining against extracellular tag (without cell permeabilization) to distinguish between non-functional channels on the plasma membrane and channels retained inside the cell. Finally, it is advisable to investigate not only basal activity of the channel but also consequences of mutation on the channel regulation. Different mechanisms of modulation of the channel function could be studied depending of the ion channel of interest including regulation by G proteins, PIP2, ions, ATP concentration, phosphorylation (using tyrosine, serine or threonine mutation), ubiquitination or sumoylation (using lysine mutation) and direct channel openers or blockers.

Disclosures

No conflicts of interest declared.

Acknowledgements

Our work was made possible by financial support from McKnight Endowment Fund for Neuroscience (PAS), the National Institute on Drug Abuse (R01 DA019022; PAS) and a pre-doctoral NIH NRSA fellowship to P.A. (F31AA017042) from the National Institute on Alcohol Abuse and Alcoholism. The GIRK2- L257W mutant was provided by Dr. Prafulla Aryal. Fluorescence microscope and sponsorship kindly provided by Leica Microsystems.

References

1. Aryal, P., Dvir, H., Choe, S. & Slesinger, P.A. A discrete alcohol pocket involved in GIRK channel activation. *Nature Neurosci* 12, 988-995 (2009).
2. Panchenko, V.A., Glasser, C.R. & Mayer, M.L. Structural similarities between glutamate receptor channels and K⁺ channels examined by scanning mutagenesis. *J Gen Phys* 117, 345-360 (2001).
3. Doyle, D.A., Morais Cabral, J., Pfuetzner, R.A., Kuo, A., Gulbis, J.M., Cohen, S.L., Chait, B.T. & MacKinnon, R. The structure of the potassium channel: molecular basis of K⁺ conduction and selectivity. *Science* 280, 69-77 (1998).
4. Nishida, M., Cadene, M., Chait, B.T. & MacKinnon, R. Crystal structure of a Kir3.1-prokaryotic Kir channel chimera. *EMBO J* 26, 4005-4015 (2007).
5. Tao, X., Avalos, J.L., Chen, J. & MacKinnon, R. Crystal structure of the eukaryotic strong inward-rectifier K⁺ channel Kir2.2 at 3.1 Å resolution. *Science* 326, 1668-1674 (2009).