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Polyamine Block of Inwardly Rectifying Potassium Channels

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Abstract

Polyamine blockade of inwardly rectifying potassium (Kir) channels underlies their steep voltage-dependence observed in native cells. The structural determinants of polyamine blockade and the structure-activity profile of endogenous polyamines requires specialized methodology for characterizing polyamine interactions with Kir channels. Recent identification and growing interest in the structure and function of prokaryotic Kir channels (KirBacs) has driven the development of new techniques for measuring ion channel activity. Several methods for measuring polyamine interactions with prokaryotic and eukaryotic Kir channels are discussed.

Keywords

Inward rectifier; Potassium channel; Electrophysiology; Patch-clamp; Ion channel blockade

1. Introduction

Inwardly rectifying potassium (Kir) channels exhibit unique voltage-dependence in vivo, preferentially allowing inward conduction of potassium ions, a property commonly referred to as “inward rectification.” Kir channels influence diverse processes ranging from cardiac muscle contraction to insulin release from pancreatic β -cells (1–3), but perhaps the most obvious physiological role attributed to Kir channels is in excitable cells, where they play a fundamental role of regulating the resting membrane potential and action potential repolarization. In this milieu, the minimization of outward K^+ conductance of Kir channels is essential to permit action potentials to proceed (4, 5).

Interestingly, unlike the gating mechanisms that exemplify many other ion channel proteins, “inward rectification” is not an intrinsic property of Kir channels, but rather arises from voltage-dependent channel blockade by cytoplasmic polycations, particularly polyamines (6). The prototypical inward-rectifiers are members of the Kir2 subfamily, but importantly, different Kir channel types exhibit different sensitivities to polyamine blockade, and consequently, different degrees of inward rectification are observed in vivo, these functional differences being related to the physiological role of a specific Kir channel type. The physical principles underlying the voltage-dependence of Kir channel polyamine block have stirred interest in diverse questions such as the identification of the structural determinants of high affinity and steeply voltage-dependent polyamine binding, and the interactions between blockers and permeating ions in ion channel pores (7, 8). Methods used for characterizing polyamine interactions with Kir channels continue to be applied to illuminate the detailed features of polyamine block and the physiological effects of mutations that disrupt this critical mechanism of Kir channel regulation.

Finally, recently identified prokaryotic Kir channels (“KirBacs”) have proven very useful for crystallographic determination of Kir channel structure, but the techniques for functional

characterization of KirBac channels remain in development. Considerable effort has been invested in the development of methods for recording KirBac channel activity, which presents unique problems compared to their eukaryotic Kir channel counterparts (9, 10). In the long term, continued development of these methods will enable direct correlation of crystallographic data (11, 12) with measurable changes in ion channel function. In this chapter, we provide an overview of the electrophysiological techniques essential for characterization of polyamine interactions with eukaryotic Kir channels. In addition, methods for the purification, reconstitution, and functional characterization of prokaryotic KirBac ion channels are described.

2. Materials

2.1. KirBac Protein Purification

1. *Escherichia coli* strain BL21 Star (DE3) pLysS.
2. LB supplemented with antibiotic for selection (e.g., ampicillin or kanamycin).
3. 1 M IPTG (convenient in 1 mL aliquots).
4. Resuspension Buffer: 50 mM Tris-HCl pH 7.5, 150 mM KCl, 250 mM sucrose, 10 mM MgSO₄, one half EDTA-free mini protease inhibitor tablet per 20 mL resuspension buffer (i.e. 1/2 tablet per 1 L of bacteria) (Roche Diagnostics).
5. Wash Buffer A: 50 mM Tris-HCl pH 7.5, 150 mM KCl.
6. Wash Buffer B: 50 mM Tris-HCl pH 7.5, 150 mM KCl, 5 mM decyl maltoside, 10 mM imidazole.
7. Elution Buffer: 50 mM Tris-HCl pH 7.5, 150 mM KCl, 5 mM decyl maltoside, 500 mM imidazole.
8. Cobalt beads (Talon), and polystyrene columns (Pierce Chemical Co.).

2.2. Liposome Preparation for Electrophysiology

1. Lipids (e.g., POPE, POPG, Cardiolipin, Asolectin, PIP2).
2. 8 mL polystyrene columns (Thermo Fisher Scientific).
3. MOPS buffer: 150 mM KCl and 10 mM MOPS, pH 7.5.
4. Sephadex G-50 beads (Sigma), soaked in MOPS buffer overnight.
5. Glass coverslips incubated in polylysine.

2.3. ⁸⁶Rb⁺ Liposome Uptake Assay

1. Lipids (POPE, POPG, Cardiolipin, Asolectin, PIP2).
2. 170 mM CHAPS stock.
3. Sephadex G-50 beads, soaked in the desired buffer overnight.
4. Dowex 50 X-4-100 (H⁺ form) cation exchange matrix (Sigma).
5. Polystyrene columns (Pierce Chemical Co.).
6. Buffer B: 450 mM KCl, 10 mM HEPES, 4 mM NMDG, adjust pH to 7.5 with HEPES or NMDG. All buffers are sterile-filtered and stored at 4°C.
7. Buffer C: 400 mM sorbitol, 10 mM HEPES, 4 mM NMDG, 50 μM KCl, adjust pH to 7.5 with HEPES or NMDG.

8. Sorbitol solution (400 mM).
9. BSA (5 mg/mL) in sorbitol solution.
10. Valinomycin stock: 100 μ g/mL in EtOH. Make 1 mL, and store at 4°C.

2.4. Eukaryotic Cell Culture

1. Dulbecco's modified eagle medium (DMEM) + 10% FBS, supplemented with Pen/Strep antibiotic cocktail.
2. CosM6 cells.
3. Liposome-based transfection reagent (Fugene, Lipofectamine).
4. Sterile/flamed glass coverslips.

2.5. Patch-Clamp Electrophysiology

1. Thin-walled borosilicate glass (1.5 mm OD, 1.1 mm ID).
2. Buffered KCl recording solution, typical composition: 140 mM KCl, 1 mM K-EGTA, 1 mM K-EDTA, 4 mM K_2HPO_4 , pH 7.3 (with KOH).
3. Standard patch-clamp equipment (electrode puller, patch-clamp acquisition electronics, microscope, manipulator, anti-vibration table).

3. Methods

3.1. Purification of KirBac Protein

1. Transform *E. coli* strain BL21 Star (DE3) pLysS (Invitrogen) with KirBac 1.1 DNA, and grow bacteria overnight on an LB-agar plate supplemented with an appropriate selection antibiotic. Optimal protein expression occurs only if the bacteria are newly transformed. Older bacterial stocks, even glycerol stocks, produce very low amounts of protein. Retransform every week or 2.
2. (DAY 1) Prepare a small (5 mL) seed culture of LB + selection antibiotic (e.g., ampicillin) with a single toothpick colony. Shake overnight at 37°C and 200–250 rpm.
3. (DAY 2) Inoculate 1 L of LB + selection antibiotic (e.g., ampicillin) with the 5 mL seed culture. Shake at 37°C and 250 rpm for 5 h (until $OD_{600} = \sim 1.0$).
4. Induce with 1 mM IPTG (add 1 mL of 1 M IPTG aliquot to the 1 L bacterial culture) for 3 h at 37°C and 250 rpm.
5. Pellet cells by centrifugation ($4,000 \times g$) for 15 min. Pour off supernatant. (At this stage, pellets can be frozen for up to a few days, or the experiment can immediately proceed to the protein purification steps, which typically require ~4–5 h. If proceeding immediately, lysis of the pellets is achieved by rapid freeze-thaw, 10 min at -80°C .)
6. (DAY 3) Resuspend pellets with 20 mL of resuspension buffer (per 1 L of culture) in a 50 mL Falcon tube.
7. Add 30 mM decyl maltoside (30 μ L of 1 M stock per mL of cell lysate) to the cell lysate and rock at 4°C for at least 2–3 h to solubilize the protein.
8. Centrifuge cell lysate for 30 min at 18,000 rpm ($40,000 \times g$).

9. While the cell lysate is centrifuging, prepare cobalt beads by washing a batch of beads with 5 bed volumes of Wash Buffer A. Use 0.2 mL (bed volume) of beads for each 1 L of culture.
10. Taking the centrifuged cell lysate, discard the pellet and put the washed beads into the supernatant. Rock at 4°C for 1 h to bind the protein to the beads.
11. Centrifuge the extract + beads at very low speed (100–200 rpm) for 5 min to collect the beads at the bottom. Pour off most of the supernatant and pipette the beads and remaining cell extract into the columns. Allow the extract to flow through.
12. Wash the beads with 40 bed volumes of Wash Buffer B.
13. Elute the protein with 4 bed volumes of Elution Buffer.
14. Store eluted protein at 4°C. WT KirBac1.1 is highly stable and remains active beyond 1 year of storage. Protein can be concentrated for future applications using Amicon columns, or other comparable products.

3.2. Storage of Lipids

1. Solubilize lipids in chloroform and aliquot the desired amount (2–4 mg of lipid) into glass culture tubes. Use a stream of nitrogen gas to dry the lipids.
2. Fill glass tubes containing dried lipids with nitrogen, and seal with parafilm. Store tubes in a container filled with nitrogen at –20°C.

3.3. Functional Assay for KirBac, ⁸⁶Rb⁺ Liposome Uptake Assay

1. Swell sephadex beads by incubating in buffer B or buffer C overnight at 4°C. One batch of beads should be swelled in each buffer.
2. Rinse polystyrene column bodies with double distilled water (ddH₂O), and push one polyethylene disk all the way to the end (with a Pasteur pipette). Rinse again with ddH₂O.
3. Fill each column about half-way with swollen Sephadex beads, and wash several times with the appropriate buffer (B or C). If storage is desired, apply 1–2 mL of buffer solution over beads to prevent drying, and cap the top and bottom of the column. (Capped columns can be stored at 4°C for weeks.)
4. Prepare lipids for the assay by resuspending stored lipids (in glass tubes, from Subheading 3.2) in buffer B and 35 mM CHAPS (for 4 mg of lipid, add 80 µL of 170 mM CHAPS stock and 320 µL of buffer B) to a concentration of 10 mg/mL. Use brief sonication of ~5–10 s and vortex to help with resuspension. Inspect the solution and re-sonicate if any dried lipids are visible in the bottom of the tube. Mix lipids to the desired composition (i.e., 3:1 POPE:POPG), and incubate for 2 h at room temperature. Allow 100 µL of lipid (10 mg/mL) per sample in the ⁸⁶Rb⁺ flux assay.
5. Dowex columns are prepared by placing ~0.5–1 mL of Dowex beads in serum separator columns. Wash the Dowex column with 1 mL of BSA–Sorbitol solution, and rinse with sorbitol solution (fill column). Next, wash the Dowex column with ~100 µg of lipid solution (resuspend 2 mg of POPG in 1 mL of sorbitol solution and add 50 µL) and rinse again with sorbitol solution.
6. Mix purified KirBac protein with each 100 µL sample of lipid solution, and incubate for 20 min. Protein:lipid ratios in the range of 6 µg KirBac per mg lipid

are typical and may be adjusted depending on the activity of a particular protein prep.

7. Prespin a Sephadex column containing Buffer B by placing the column in a 15 mL conical tube and centrifuging in a Beckman TJ6 Centrifuge at maximum speed and stopping when 3,000 rpm ($\sim 1,000 \times g$) is reached.
8. Place the prespun column B in a glass collection tube (glass culture tubes work well for this purpose) and add lipid/protein mixture to the top of the column. One column is needed for each sample.
9. Recover liposomes by spinning the Sephadex column (with a collection tube) until the centrifuge reaches 2,500 rpm ($\sim 700 \times g$). Liposomes are contained in the flow-through, and a normal yield is $\sim 50 \mu\text{L}$ for each $100 \mu\text{L}$ loaded. Store the recovered liposomes at room temperature and use within 3 h (see Note 1).
10. Prespin a Sephadex column containing Buffer C as done previously with the Buffer B column.
11. Place the prespun column in a collection tube and add liposomes to the column.
12. Recover liposomes by spinning into the collection tube at 2,500 rpm ($\sim 700 \times g$) for 60 s. Use the recovered liposomes as soon as possible (at this point, liposomes are stable for hours, but dissipation of the gradient is noticeable within 20–30 min).
13. Mix recovered liposomes with 4–5 aliquot volumes ($50 \mu\text{L}$ aliquots will be taken in subsequent steps) of Buffer C containing $^{86}\text{Rb}^+$ at a final specific activity of $\sim 1\text{--}1.5 \mu\text{Ci/mL}$, and start a timer (this is time = 0).
14. At each desired time point, remove a $50 \mu\text{L}$ aliquot from the mixture. Pipette the aliquot onto a Dowex column to remove extraliposomal $^{86}\text{Rb}^+$, and allow aliquot to enter beads. For KirBac1.1, useful time points are within the range of 15 s to 1 min, with uptake saturating in 2–4 min.
15. Elute liposomes with 1 mL of sorbitol solution. Allow the flow-through to drain into a scintillation vial. Elute a second time with another 1 mL of sorbitol solution.
16. Add scintillation fluid and count.
17. After all desired time points have been collected, add $1 \mu\text{L}$ of valinomycin stock, wait ~ 10 min, and process a final aliquot as above. Valinomycin is an ionophore that will allow $^{86}\text{Rb}^+$ to equilibrate inside and outside the liposomes. This provides an assessment of maximum possible uptake in the assay.
18. For characterization of polyamine interactions with KirBac channels (or mutant KirBac channels) using the ^{86}Rb uptake assay, several options are possible. The most straightforward is to include polyamines in $^{86}\text{Rb}^+$ -containing Buffer C during the $^{86}\text{Rb}^+$ uptake step of the assay (step 4). If the reconstituted channel is polyamine-sensitive, uptake of $^{86}\text{Rb}^+$ into liposomes will be hindered (see Note 1). When interpreting this type of data (for polyamines or other blocker types), it is important to remember that reconstituted ion channels likely insert randomly into

¹Liposomes are formed in the presence of Buffer B, leading to liposomes with high internal KCl. For certain experimental questions, it may be desirable to switch the identity of the cation used during the formation of liposomes, to use a cation mixture, or to supplement the solution with a channel blocker/modifier such as a polyamine.

After the liposomes are exposed to Buffer C (400 mM Sorbitol, 0 KCl), permeant ions exit the liposomes via available conduction pathways (i.e., ion channels), generating a strong electrical gradient across the liposome membrane. In subsequent steps, when the liposomes are added to solutions that contain permeant ions, liposomes immediately begin to take up permeant ions.

the liposome bilayer, so the “sidedness” of liposomes will not be uniform (see Note 2).

3.4. Reconstitution of KirBac Channels in Giant Liposomes for Patch-Clamp Electrophysiology

1. As in the preparation of liposomes for uptake assays, polystyrene columns (Thermo Fisher Scientific, 8 mL columns) are packed with Sephadex G-50 beads, soaked in the desired buffer overnight, and spun until reaching $1,500 \times g$ on a Beckman TJ6 Centrifuge (3,000 rpm). For KirBac electrophysiology, a MOPS buffer (150 mM KCl and 10 mM MOPS, pH 7.5) is typically used.
2. While the column is packing, mix KirBac protein with an appropriate lipid mixture (resuspend lipids as described above). A typical lipid mixture is 3:1 POPE:POPG (200–400 μL , 10 mg/mL in MOPS buffer with 35 mM CHAPS) with a small volume of the desired protein (depending on concentration). For KirBac1.1, 30–50 μg of protein per 1 mg of lipid can yield macroscopic currents, whereas 1–5 μg can be sufficient to observe single channel openings. The composition of lipids may change depending on the experimental question being addressed, but 3:1 POPE:POPG has proven generally reliable for the formation of giant liposomes suitable for patch-clamping. We have found no significant difference in recordings generated after reconstitution using Bio-Beads or Sephadex – both methods seem to effectively remove CHAPS detergent.
3. Spin the entire (~200–300 μL) protein–lipid solubilized mixture through the prespun sephadex column (step 1) until reaching $1,000 \times g$ (~2,500 rpm).
4. Collect the flow-through, and spin the liposome solution at $100,000 \times g$ for 1 h.
5. The key step for the formation of giant liposomes is to dry a high concentration of resuspended liposomes onto a glass slide. Thus, the pelleted liposomes should be resuspended in as small a volume as possible (usually ~3–8 μL of buffer depending on how much lipid was used). The quality of giant liposomes also depends on how much the liposome spots are spread out or smeared on the glass slide. By trial and error, one has to find the best balance here. Typically, 3–5 μL of liposome solution is applied onto the slide and smeared out slightly with the pipette tip. The spot of solution should be cloudy white and homogeneous.
6. Dry the liposomes in a petri dish containing a moderate amount of calcium sulfate dessicant for ~1–2 h at 4°C. Liposome spots have to be completely dried before moving on to the next rehydration step, but it is best to rehydrate as soon as drying is complete.
7. Rehydrate each liposome spot with the same buffer used for liposome formation (e.g., 150 mM KCl, 10 mM MOPS, pH 7.5). A buffer with lower osmolarity may

²It is important to recognize that many blockers interact with ion channels with specific “sidedness.” Polyamine blockade is far more potent from the intracellular side of the channel, and this is a complication that must be taken into account when analyzing $^{86}\text{Rb}^+$ uptake into liposomes and potentially patch-clamp recordings from giant liposomes. In the case of polyamine exposure during the $^{86}\text{Rb}^+$ uptake assay, we have observed that polyamine-sensitive mutants of KirBac1.1 exhibit a biphasic dose–response to spermine (Fig. 2). This likely corresponds to a 50:50 mix of ion channels oriented with their cytoplasmic side on either the outside or inside of the liposome (channels oriented outwards will exhibit a strong sensitivity to spermine, while channels with the cytoplasmic entrance inside the liposome will only be weakly sensitive to spermine). In the case of patch-clamp electrophysiology, we have used a variety of approaches to determine sidedness of channels in the liposome bilayer. One approach is to introduce a reactive cysteine (KirBac1.1 has no native cysteine) at a pore-lining position. This results in current reduction upon exposure to cysteine reactive compounds (e.g., MTSET), so long as the channel is oriented appropriately. A second approach (somewhat elegant in principle, but difficult in practice) is to use cysteine mutant channels in the KirBac “slide helix” region which are inactive when unmodified, but become active after MTSET modification (9, 13). In this scenario, application of MTSET to the cytoplasmic face ensures that all recorded activity arises from channels with the same orientation.

also work, but using a buffer with a higher osmolarity will lead to poorly formed liposomes. The volume used should simply be enough to cover the dehydrated spots.

8. Keep these rehydrated liposome spots at 4°C overnight – this stalls giant liposome formation.
9. The next day, put the liposomes in room temperature, after which there is a time window between ~1 and 3 h where they are best used for patch-clamp. After at least 1 h at room temperature, add an additional ~40–80 µL of buffer (150 mM KCl, 10 mM MOPS, pH 7.5) to one of the spots, depending on the size of the spot. Pipette up and down to dislodge the liposomes from the glass, making a homogeneous-looking solution.
10. Place a shard of glass coverslip incubated in polylysine overnight at the bottom of the patch-clamp recording chamber. Pipette ~15 µL of giant liposome solution onto the coated coverslip with no solution flow in the chamber, allow liposomes to settle for ~2–5 min (depending on the density of liposomes), and then start solution exchange to wash off any debris that is not adhering to the coverslip.
11. We have not identified any consistent rules for obtaining giga-ohm seals with giant liposomes. Generally, best success seems to be achieved by sealing onto liposomes that are smaller and have a relatively thin membrane border (closer to being unilamellar). To obtain seals more easily, pipettes with a resistance of ~1–3 MΩ are typically used. Avoid adding EGTA/EDTA to solutions unless absolutely necessary because trace contaminating ions such as Ca²⁺ may help seals. Patch-clamp recording from liposomes is difficult, but with persistence and care, these recordings can be obtained routinely (see Subheading 3.6 for details on techniques for inside-out patch-clamp recordings).

3.5. Eukaryotic Cell Culture

1. CosM6 cells (see Note 3) are grown in standard T-25 (25 cm²) square tissue culture flasks. These cells are very adherent, and no measures are taken to maintain adherence. Flasks are passaged every 4–5 days, with standard tissue culture protocols, using trypsin/EDTA to dislodge cells from the bottom of the flask. We have not encountered problems with expression or reproducibility of ion channel function even at relatively high passage number (~50), but make it practice to discard cells after ~50 passages.
2. To prepare for experiments (DAY 1), cells are first split into 35 mm petri dishes or 6-well plates. A fairly high level of confluence is acceptable at this stage, to optimize transfection efficiency with liposomal transfection reagents (see manufacturer's instructions regarding the optimal confluence of cells for transfection with a particular reagent).
3. (DAY 2) Plated cells are transfected with plasmids containing the cDNA for the desired ion channel and any necessary auxiliary subunits. Standard liposome-based

³Detailed characterization of polyamine interactions with eukaryotic Kir channels is most conveniently done in a heterologous expression system. The two basic options in this regard are immortalized mammalian cell lines or *Xenopus* oocytes, and the selection of an expression system will depend primarily on the relative expression of the ion channel of interest. In our own experiments, we have found that CosM6 cells (a mammalian cell line) work especially well for expression of KATP (Kir6.2 + SUR1), Kir2.1, and Kir4.1 channels – the model ion channels most frequently used in our experiments. We consistently achieve extremely high expression of all of these channels in CosM6 cells. In addition, this cell line has proven especially amenable to “inside-out” patch-clamp experiments, as CosM6 cells adhere very well to standard plastic tissue culture dishes. Other groups have successfully employed *Xenopus* oocytes for studies of similar channels.

transfection reagents generally work well for this purpose. Some optimization may be necessary to maximize expression and efficiency. In addition, a plasmid encoding GFP or some other useful marker is also included in the transfection, to help assess efficiency and identify transfected cells while recording.

4. (DAY 3) Transfected cells are split and plated onto sterile glass coverslips, in 35 mm petri dishes (or a 6-well plate). Soaking coverslips in ethanol and flaming them is sufficient for sterilization. Trypsin/EDTA is used to dislodge cells from the bottom of tissue culture plates, and cells are gently triturated to break up clumps of cells. The density of plating may be varied according to the planned experiment. For characterizing the effects of polyamines, inside-out patch-clamp recordings are the typical method of choice. This recording configuration is sometimes easier at somewhat high levels of confluence (~50% on the day of recording), because cells tend to adhere better to the dish, making it easier to excise a membrane patch (other recording configurations, such as whole cell mode, require isolated individual cells). Lower and higher degrees of confluence are also tolerable, but not ideal. Typically, cells are useful for recording up to 3 days after plating onto cover slips.
5. (DAY 4–6) Electrophysiological recordings (see Subheading 3.6).

3.6. Functional Assay: Patch-Clamp Electrophysiology

High resolution characterization of polyamine effects on Kir channels necessitates the “inside-out” patch-clamp technique. In this technique, a giga-ohm seal is formed between a patch pipette and a cell or liposome, and a membrane patch is excised, thereby exposing the cytoplasmic membrane face to the bathing solution. This configuration allows the application of specific polyamines, at specific concentrations. The generation of high-quality inside-out patch-clamp recordings presents many challenges, especially with regard to polyamine interactions, and we will share some of the detailed methods we and others have used to overcome these experimental hurdles.

1. Patch-clamp pipettes are manufactured from thin-walled borosilicate glass, using a micropipette puller (e.g., Sutter P-97). Using symmetrical 150 mM KCl solutions, the resistance of pipettes used is typically in the range of 0.5–1.5 M Ω . For measurement of kinetics of blockade, it may also be helpful to coat pipettes with Sylgard.
2. The recording chamber we employ is depicted in Fig. 1, although many possible configurations of recording chambers are used. This recording chamber is designed to accommodate up to five different solutions and enables relatively easy solution exchange, by moving the patch pipette between the lanes of the chamber (by moving the translatable microscope stage).
3. With solutions flowing in the recording chamber, glass cover-slips plated with cells are broken into small shards, and a shard is transferred to the recording chamber using delicate forceps.
4. A transfected cell is selected for recording by green GFP fluorescence or some other marker of transfection.
5. Using a micromanipulator, a patch pipette is moved close to the vicinity of the cell, and fine adjustments are used to bring the pipette into contact with the cell. As the pipette contacts the cell, there is an increase in resistance across the tip of the pipette (patch-clamp acquisition programs have routines such as the “Seal Test” that monitor tip resistance to alert the operator that the pipette has contacted the cell). Application of a small amount of suction (using a syringe, or mouth) through a tube connected to the patch-clamp pipette holder will ideally result in a dramatic

increase in resistance, and formation of a giga-ohm seal. Seal formation is not an easy task for a beginner, and requires practice.

6. Formation of an inside-out patch requires excision of a small patch of membrane from a cell. This is achieved by quickly moving the patch pipette away from the cell after formation of a giga-ohm seal. The best possible outcome is formation of a useful excised patch; however, this is not always the case. On occasion, the entire cell may lift from the bottom of the recording chamber, or alternatively, a vesicle may form in the tip of the patch pipette (by excess pieces of membrane sealing together). In these situations, a common approach is to briefly (<1 s) remove and return the patch pipette to the recording solution using the micromanipulator. This brief exposure to air sometimes ruptures the cell/vesicle and exposes the cytoplasmic face of the membrane patch to bathing solution. Another approach is to use a small bead of mineral oil in the recording chamber (as in Fig. 1). Brief exposure of a cell/vesicle to oil rapidly disintegrates cellular materials outside the patch, but typically leaves the excised patch (protected by the patch pipette) intact.
7. Excised patches are perfused with control solution. During this time, polyamines diffuse away from the patch, until little rectification is apparent at positive voltages. It is often difficult to fully wash polyamines away from patches, and some residual polyamine block may be tolerable, depending on the goal of the experiment (see Note 4). Application of desired voltage-step or voltage-ramp protocols in the presence or absence of polyamines can be used to determine the voltage and dose-dependence of blockade for particular pairs of ion channels and polyamines.

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⁴A quantitative description of polyamine blockade requires reliable measurement of channel currents in the absence and presence of polyamines. In the inside-out patch configuration, it is certainly possible to wash away polyamines from patches, so that virtually no blockade/inward rectification is detectable in polyamine-free solutions. However, a hurdle that may be encountered is extremely slow washout of polyamines from patches. We envision that this issue arises due to formation of recessed patches in the tip of the patch pipette. To minimize this problem, we strive for “flat patches,” by using very little negative pressure during seal formation. When using cultured mammalian cell lines, it may help to experiment with different plating protocols (trypsinization time, cell density, etc.) to develop one that results in good seal formation with minimal negative pressure, but ultimately, patience, practice, and persistence are the prime requirements.

While much of this is viewed as simply good practice, other details are worth mentioning. Notably, spermine has proven to be especially “sticky” in our recording chambers and perfusion systems. Frequent replacement of plastic tubing and fastidious cleaning of the recording chamber will assist in the generation of cleanly washed patches. In addition, various impurities in commercially available HEPES buffer have proven to cause modest inward rectification and stand in the way of generating cleanly perfused patches. We and others have had greater success using phosphate-buffered solutions, together with EDTA (~1 mM), to ensure that no contaminating divalent cations contribute to rectification.

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Fig. 1. Schematic diagram of patch-clamp recording chamber for studying polyamine interactions with inwardly rectifying potassium (Kir) channels. Control (polyamine-free) and polyamine solutions flow into distinct lanes separated by physical barriers. The barriers are manufactured to contain small reservoirs that are filled with mineral oil. Small slots on either side of the reservoir are used to allow patch pipettes to be moved briefly into contact with oil. Solutions can be changed with relative ease by moving the chamber (on a translatable stage) relative to the patch pipette.

Fig. 2. Biphaseic spermine dose-response relationship of KirBac1.1 channels in $^{86}\text{Rb}^+$ uptake assay. Plot of relative $^{86}\text{Rb}^+$ uptake of WT KirBac1.1 and KirBac1.1 I131C/I138D (a mutant that confers strong polyamine sensitivity) in liposomes at different concentrations of externally applied spermine, normalized to uptake without spermine ($n = 9 \pm \text{SEM}$). The I131C/I138D data are fit with the sum of two Hill functions, and the WT data with a single Hill function. Most importantly, only 50% of $^{86}\text{Rb}^+$ uptake is inhibited with high affinity, because the I131C/I138D mutation generation of high affinity block only to internal/cytoplasmic spermine and channels are bidirectionally oriented.