Video Article

Patch Clamp and Perfusion Techniques for Studying Ion Channels Expressed in *Xenopus* oocytes

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Abstract

The protocol presented here is designed to study the activation of the large conductance, voltage- and Ca^{2+}-activated K^+ (BK) channels. The protocol may also be used to study the structure-function relationship for other ion channels and neurotransmitter receptors[1]. BK channels are widely expressed in different tissues and have been implicated in many physiological functions, including regulation of smooth muscle contraction, frequency tuning of inner hair cells and regulation of neurotransmitter release[2-6]. BK channels are activated by membrane depolarization and by intracellular Ca^{2+} and Mg^{2+} [6-9]. Therefore, the protocol is designed to control both the membrane voltage and the intracellular solution. In this protocol, messenger RNA of BK channels is injected into *Xenopus laevis* oocytes (stage V-VI) followed by 2-5 days of incubation at 18°C[10-13]. Membrane patches that contain single or multiple BK channels are excised with the inside-out configuration using patch clamp techniques[10-13]. The intracellular side of the patch is perfused with desired solutions during recording so that the channel activation under different conditions can be examined. To summarize, the mRNA of BK channels is injected into *Xenopus laevis* oocytes to express channel proteins on the oocyte membrane; patch clamp techniques are used to record currents flowing through the channels under controlled voltage and intracellular solutions.

Video Link

The video component of this article can be found at https://www.jove.com/video/2269/

Protocol

1. Injection of mRNA into Oocytes

1. Inject 0.05 - 50 ng messenger RNA that was transcribed *in vitro* into *Xenopus laevis* oocytes (stage V-VI) using Nanoject II Auto-Nanoliter Injector (Drummond Scientific Company, model 3-000-204). Repeat the injection on a dozen oocytes for each mRNA.
2. Rinse the injected oocytes twice using ND-96 solution (96 mM sodium chloride (NaCl, Mr 58.44 g/mol), 2 mM potassium chloride (KCl, Mr 74.56 g/mol), 1.8 mM calcium chloride ($CaCl_2$•$2H_2O$, Mr 147.02), 1 mM magnesium chloride ($MgCl_2$•$6H_2O$, Mr 203.31 g/mol), 5 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES, Mr 238.31 g/mol), 2.5 mM sodium pyruvate (Mr 110.04 g/mol), and 1x Penicillin-Streptomycin. pH 7.6), then place them in a culture plate and incubate at 18°C for 2+ days.

2. Preparing the Perfusion System

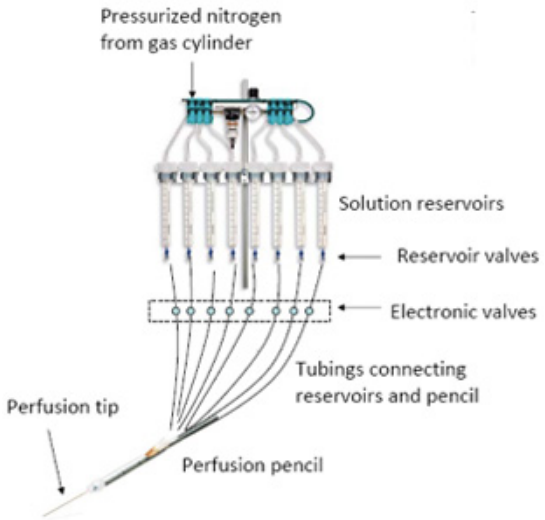


Illustration of the Automate ValveLink 16 perfusion system. The perfusion system uses pressurized nitrogen to push perfusion solutions out of the solution reservoirs, through the perfusion tubings, and to the perfusion pencil and tip. The flow of each stream of perfusion solution is controlled by one reservoir valve and one electronic valve. In this protocol, one of the reservoirs is not pressurized and functions as a waste collector as well as a pressure-release mechanism. (Picture adapted from vendor's website http://www.autom8.com)

1. Connect the tubings to the perfusion pencil. Turn on the electronic valve controller and open the electronic valves.
2. To assure that the tubings and the perfusion pencil are free of clog and air bubbles, push deionized (DI) water through each tubing using a syringe filled with deionized water.
3. Connect the tubings to the solution reservoirs and open the valve of gas cylinder to apply pressurized nitrogen.
4. Open the reservoir valve to fill the tubing with reservoir solution. Flick the reservoir valve to remove bubbles in the solution if needed. Close the valve after the tubing is filled with solution.
5. Fill the perfusion tip with water and screw it onto the perfusion pencil. Be careful not to trap any bubbles when connecting the tip.
6. Fix the perfusion pencil to the bath stage using modeling clay. Make sure the perfusion tip is in the bath space. The perfusion system is now set up.
7. Add DI water in the bath. Make sure the perfusion tip is submerged in the water.
8. Test that each perfusion solution comes out of the perfusion tip correctly. Close the electronic valve connected to the tubing of waste collector and open the valve for one perfusion solution at a time (140 mM potassium hydroxide (KOH, M_r 56.1 g/mol), 20 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES, M_r 238.31 g/mol), 2 mM potassium chloride (KCl, M_r 74.56 g/mol), 1 mM Ethylene glycol-bis(2-aminoethylether)-N,N,N′,N′-tetraacetic acid (EGTA, M_r 380.35 g/mol), pH is adjusted to 7.1-7.2 by methanesulfonic acid($MeSO_3$, M_r 96.1 g/mol), Ca^{2+} or Mg2+ is added to desired concentration when needed). Under the microscope, observe the jet of perfusion fluid coming out of the perfusion tip. Close the valve for perfusion solution and open the valve for the waste collector. The perfusion jet should almost disappear. Repeat the same test for all the perfusion solutions.
9. When you have verified that all the perfusion solutions flow correctly, replace DI water in the bath with bath solution (140 mM potassium hydroxide (KOH, M_r 56.1 g/mol), 20 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES, M_r 238.31 g/mol), 2 mM potassium chloride (KCl, M_r 74.56 g/mol), pH is adjusted to 7.1-7.2 by methanesulfonic acid($MeSO_3$, M_r 96.1 g/mol)).

3. Patch Clamping

1. Recoat the Ag recording electrode with AgCl before each patch clamping session. First, remove the electrode wire from the pipette holder and submerge the tip half of the electrode wire in a vial containing fresh bleach for at least 15 minutes. This deposits a layer of AgCl on the wire.
2. Rinse the electrode wire with deionized water and blot dry. Then install the Ag/AgCl electrode back in the pipette holder.
3. Connect the bath (reference) electrode to the headstage and place it in the bath. This prepares the electrodes for recording.
4. Now prepare for data acquisition. Turn on your computer and plug the hardware key into the printer port of your computer. The hardware key must be plugged for you to use the data acquisition software, which is HEKA pulse.
5. Switch on the amplifier (Axon Instruments, AXOPATCH 200B) and start HEKA Pulse. Load the protocol file and adjust the configuration settings, such as the Stimulus Scale. The goal for adjusting the configuration settings is to assure that the test potential is exactly equal to the command potential. This prepares the data acquisition equipment.
6. Prepare oocytes. Submerge the oocyte in stripping solution (200 mM N-Methyl-D-glucamine (NMG, M_r 195.22 g/mol), 200 mM Aspartate (no K^+) (M_r 133.1 g/mol), 2 mM potassium chloride (KCl, M_r 74.56 g/mol), 10 mM Ethylene glycol-bis(2-aminoethylether)-N,N,N′,N′-tetraacetic acid (EGTA, M_r 380.35 g/mol), 1 mM magnesium chloride ($MgCl_2$•$6H_2O$, M_r 203.31 g/mol), 10 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES, M_r 238.31 g/mol), pH is adjusted to 7.4 by sodium hydroxide (NaOH, M_r 40.0 g/mol) for 5 - 10 min. The stripping solution detaches the vitelline membrane from the plasma membrane which makes it possible to strip the vitelline membrane.
7. Gently strip the vitelline membrane from the oocyte using two pairs of forceps. A devitellinized oocyte is extremely fragile and therefore should be moved as little as necessary.

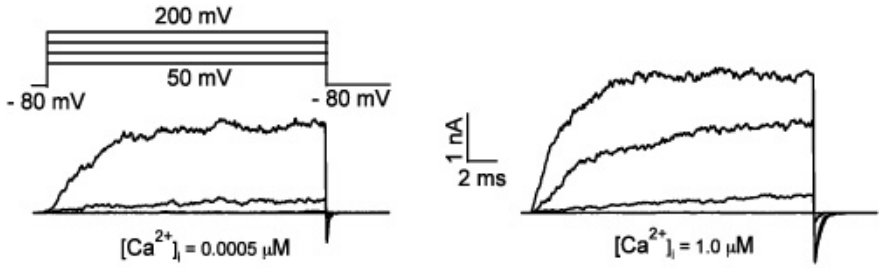
8. Taking care to prevent exposing the devitellinized oocyte to air or bubbles, use a glass pipette filled with enough solution to transfer the oocyte to the bath. This prepares the oocyte for recording.
9. Prepare patch pipettes. Pull the glass pipettes after inserting a glass capillary tube (VWR International, Cat # 53432-921) into a Sutter P-97 Flaming/Brown Micropipette Puller. Observe the pipettes under microscope to determine the tip shape and opening diameter. The opening diameter should be 2-4 micrometers.
10. To reduce capacitive current during recording and to ensure a smooth tip, coat the tip with wax and then fire-polish it.
11. Fill the pipette tip by placing the tip of the pipette inside the pipette solution (140 mM potassium hydroxide (KOH, M_r 56.1 g/mol), 20 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES, M_r 238.31 g/mol), 2 mM potassium chloride (KCl, M_r 74.56 g/mol), 2 mM magnesium chloride ($MgCl_2 \cdot 6H_2O$, M_r 203.31 g/mol), pH is adjusted to 7.1-7.2 by methanesulfonic acid($MeSO_3$, M_r 96.1 g/mol)) and drawing the plunger. This wets the tip.
12. Fill pipette 1/3 full with pipette solution using a syringe and place the pipette in the pipette holder with the Ag/AgCl electrode inside and contacting with the pipette solution. This prepares the patch pipette.
13. To excise an inside-out patch from the prepared oocyte, find the clear edge of oocyte under microscope. Move the patch pipette close to the oocyte using manipulator. Record the serial resistance of pipette. The ideal series resistance of the patch pipette is between 1-1.5 MΩ when filled with pipette solution and submerged in bath solution.
14. Slowly push the pipette against the oocyte until the resistance approximately doubles.
15. Apply gentle suction to the membrane by mouth through a suction tube which is connected to the pipette holder until a giga-ohm seal is obtained. Stabilize the patch by holding it at voltage -30 mV for a couple of minutes.
16. To obtain an inside-out patch, excise the patch by quickly pushing the pipette further into the oocyte and then gently pulling it out.
17. The inside-out patch is now ready for clamping. Move the pipette holding the inside-out patch to about 100 microns away from the opening of the perfusion tip.
18. Close the electronic valve for waste collecting and open the reservoir valve for the desired perfusion solution
19. Begin recording currents across the patch. Change the voltage and perfusion solutions as desired.
20. When you are finished recording, clean the perfusion tubings, pencil and tip by pushing through deionized water to avoid clogs.

4. Representative Results

During the preparation of oocytes for patch clamp, the 5-10 min treatment of stripping solution would detach the vitelline membrane from the plasma membrane, which makes possible stripping the vitelline membrane.

The ideal series resistance of the patch pipette is between 1-1.5 MΩ when filled with pipette solution and submerged in bath solution.

Following is a representative recording of wild-type BK channels on an oocyte membrane patch. On the left top, the square waves indicate the voltage applied to the patch - the second step of voltage increases from 50 mV to 200 mV with increment of 50 mV. Below is the corresponding current traces when the perfusion solution has nominal 0 Ca^{2+} (free $[Ca^{2+}]$ is about 0.5 nM). The increase of current amplitude indicates that the open probability of BK channels increases with voltage. On the right side, the same patch is perfused with 1.0 μM Ca^{2+} when applied with the same voltage protocol. The current amplitude increases more under the same voltage, indicating that the open probability increases with $[Ca^{2+}]$.



Discussion

The oocyte expression system is ideal for electrophysiological characterization of voltage-dependent ion channels due to relatively low background of endogenous channels. Furthermore, since it is a transient expression system, it provides an efficient method of performing mutagenesis study of these channels. However, it should be noted that the oocyte expression system is different from that in mammalian cells, thus there may be differences in post-translational modification and association with different subunits. Furthermore, the lipid concentration may differ between the two cells which may affect the channel's functional properties.

The perfusion tubings, pencil and tip should be cleaned with DI water every time after experiment to avoid clog. Always use fresh bleach to treat the Ag electrode wire to make sure it is coated with AgCl, otherwise recording will be inaccurate. Adjust the configuration settings for data acquisition so that the test potential is exactly equal to the command potential. It would be helpful to keep the patch pipettes clean so use a lid to protect them from dirt and fire-polish them only before use.

Disclosures

No conflicts of interest declared.

Acknowledgements

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