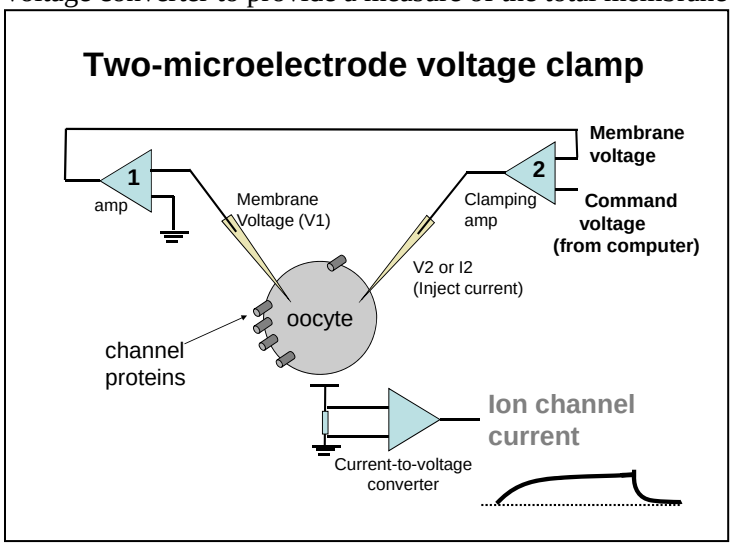
Two microelectrode voltage clamp (TEVC) of *Xenopus* oocytes

Introduction

The voltage clamp technique is a method that allows ion flow across the cell membrane to be measured as an electric current as the transmembrane potential is held under constant experimental control with a feedback amplifier. Ion channels expressed in *Xenopus* oocytes can be studied using the two-microelectrode voltage clamp (see figure below). The membrane of the oocyte is penetrated by two microelectrodes, one for voltage sensing and one for current injection. The membrane potential as measured by the voltage-sensing electrode and a high input impedance amplifier (amp1) is compared with a command voltage, and the difference is brought to zero by a high gain feedback amplifier (amp 2). The injected current is monitored via a current-to-voltage converter to provide a measure of the total membrane current.



In this lab exercise you will learn how to inject oocytes with nL quantities of cRNA, impale oocytes with glass microelectrodes and record whole cell ionic currents of heterologously expressed channels using the two-microelectrode voltage clamp technique. A Geneclamp 500 amplifier will be used to record K^+ channel currents in oocytes 3 days after injection with cRNA encoding Kv11.1 (hERG) channels. Data acquisition is performed using a personal computer and an A/D interface. Pulse protocols are preset and are executed using CLAMPEX module of pCLAMP software.

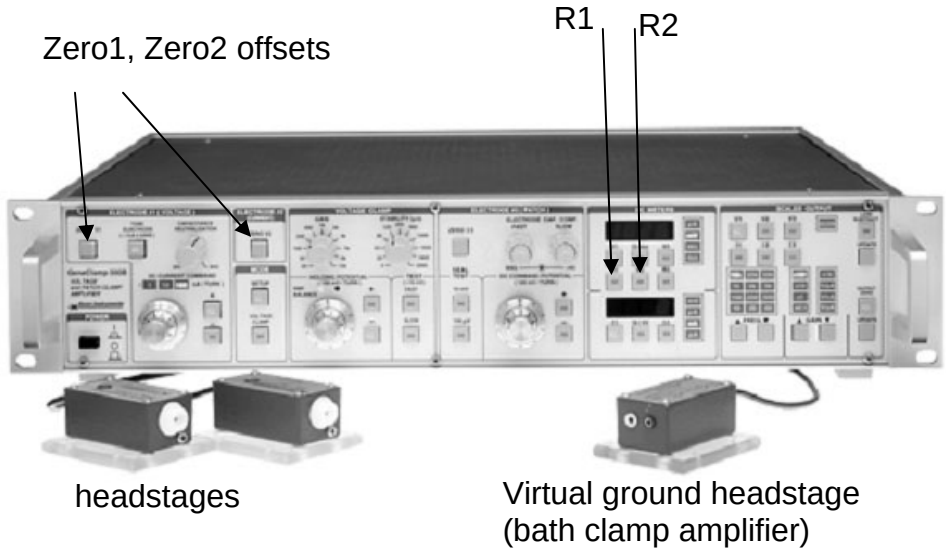
Electrophysiological measurements

Fabrication and testing resistance of glass micropipettes

Microelectrodes are pulled from 1.0 mm o.d. borosilicate glass tubing using a Flaming/Brown micropipette puller. Backfill pipettes with 3M KCl solution using the special syringe needle and place pipettes in storage jar until ready for use.

Place one electrode into each of the two holders, making sure that Ag/AgCl wire makes contact with KCl solution in the pipette. Insert holders into the two headstages of the Geneclamp 500 amplifier (photo below).

GENECLAMP 500 amplifier:



Using the micropositioners, immerse tips of both electrodes into the KCM411 solution within the oocyte chamber. Look through the compound microscope to visualize the tips of the electrodes. Break the tip off the pipettes with fine forceps until a tip resistance of ~1 MΩ is achieved. To determine resistance of pipette #1, depress "R1" on DC METER; for pipette #2, depress "R2" on DC METER.

Press "Zero 1" and "Zero 2" buttons on amplifier; this will remove voltage offset; panel meter should now read "0 mV" for each electrode.

Measurement of resting membrane potential

Initial settings of amplifier:
MODE: "SETUP"
DC METER: "V1" and "I2/V2"
SCALED OUTPUT: "I2"
FREQ (low-pass filter): "500"; GAIN: "x1"

Impale oocyte with both microelectrodes. DC meter will read resting membrane potential (in mV) for electrode #1 (V1) and #2 (V2). The numbers should be about the same and will vary from -40 to -70 mV for healthy oocytes.

Voltage clamp (whole cell currents)

Now switch MODE to "VOLTAGE CLAMP"
VOLTAGE CLAMP controls should be as follows:
GAIN (controls loop gain of voltage clamp): "9k"
STABILITY (introduces phase lag into feedback loop): "200 μs"
HOLDING POTENTIAL (dial): off – full counter clockwise

The DC meter will now display the holding potential in mV (V1) and the holding current in μA (I2). The reading for I2 is the current required to "clamp" the membrane potential to V1. A virtual ground headstage amplifier is used to actively clamp the bath potential to zero.

Data acquisition and analysis software

PCLAMP software will be used; CLAMPEX for data acquisition and CLAMPFIT for analysis. In addition, you will use ORIGIN software to plot and analyze current-voltage relationships. Lab Instructors will walk you through the basics of using these programs.

Lab Exercise: biophysical properties of wild-type and mutant hERG K^+ channels

You will be supplied with oocytes injected with cRNA encoding either wild-type or a mutant form of hERG. The mutant form contains a single point mutation that will alter one or more biophysical properties of the channel.

1. Under current clamp (SETUP MODE), record resting membrane potential of the oocyte._____ mV

2. Under VOLTAGE CLAMP MODE, record currents elicited in response to test voltages that range from -60 mV to +40 mV, applied in 10-mV increments from a holding potential of -80 mV. First use a pulse duration of 0.3 sec; next use a pulse duration of 5 sec. Measure currents at the end of each pulse and plot the "NORMAL" current-voltage (I-V) relationships for both sets of data.

3. Measure peak "tail" currents and plot as a function of test voltage for both sets of data. These plots define the voltage dependence of channel activation. Fit the relationship with a Boltzmann function (using ORIGIN software) to determine the half-point for activation ($V_{0.5}$) and the slope factor (k, a measure of steepness of the relationship).

   For 0.3 s pulses $V_{0.5}$: _____mV; k: _____mV
   For 5.0 s pulses $V_{0.5}$: _____mV; k: _____mV

4. Under VOLTAGE CLAMP MODE, record currents using a "FULLY-ACTIVATED I-V" protocol. From a holding potential of -80 mV, apply a prepulse to +40 mV for 1 sec, followed by test pulses that range in voltage from -130 mV to -10 mV, applied in 10-mV increments. Measure peak tail currents and plot as a function of test potential. Measure the reversal potential (Erev) of the I-V relationship.

5. Repeat steps 1-4 using oocytes injected with cRNA encoding "mutant hERG" channels.

***Questions:*

a) At what potential does hERG current first appear to activate, and how does this relate to the resting membrane potential of the oocyte when first impaled by an electrode? How does it relate to Erev of the fully-activated I-V relationship?

b) How does the shape of the normal I-V relationship differ from the fully-activated I-V relationship?

c) What causes the I-V relationships to deviate from simple linearity? Explain the basis for this difference between the Normal and Fully-Activated I-V relationships.

d) How do the biophysical properties of the mutant hERG channels differ from the wild-type channels?

e) Based on the altered biophysical properties, what part of the channel protein do you suppose harbors the mutation?

f) How would the altered properties of the mutation affect the shape of a cardiac action potential?

** Answers due one week after lab.

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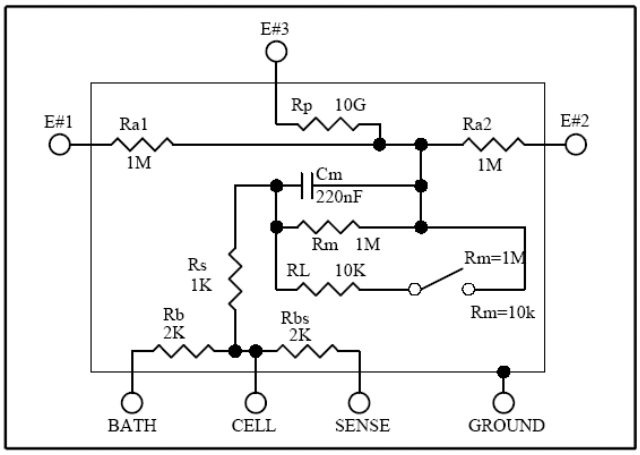
Basic electronics for TEVC:

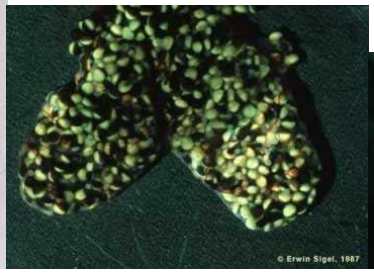
MCO-2U Model Cell



Isolation of oocytes *(for your information only; oocytes will already be isolated)*

1) Anesthetize frog ~10 min in ice-cold 0.2% tricaine (3-aminobenzoic acid ethyl ester, Sigma) solution.
2) Make small incision in the abdominal wall; remove several ovarian lobes through abdominal wall. Suture incision closed (approximately 3-4 sutures, size 6-0).
3) Place oocytes in ND-96 Ca^{2+} Free solution (in petri dish); cut apart ovarian lobes into clumps containing 5-6 oocytes each using fine forceps.
4) Wash oocytes with ND-96 Ca^{2+} free 3-5x.
5) Remove follicle cell layer: pour the oocytes and 2 mg/ml Type 1A collagenase solution into the 50ml plastic tube. Gently shake for ~ 1 hour @ room temperature.
6) Rinse the oocytes 5 times with Barth's solution.
7) Sort oocytes (usually keeping only stage IV and V oocytes) into Barth's solution. Incubate in petri dishes (30-60 oocytes/dish) at 16-19 °C overnight before injecting RNA.

Xenopus laevis frog



ovarian lobes

single oocyte (~1 mm diameter)

Injection of cRNA *(for your information only; oocytes have already been injected for you)*

1) Use large (approx. 1 mm diameter; stage V, VI) oocytes that have had follicle cell layer removed.
2) Pull injection needles on Flaming/Brown micropipette puller at settings indicated on puller. Only use WPI 1B100-4 glass pipettes.
3) After pulling needles, break tip so that outer diameter is approx. 20 μm. Use microforge scope with high power objective to check tip size.
4) Back-fill pipette tip with mineral oil (using 30 ga needle and 5 ml syringe) before attaching to the injector head. Mount pipette to the injector as instructed on page 3 of WPI Nanoliter Injector instruction manual.
5) Purge a small amount of oil from tip of pipette using EMPTY button.
6) Stretch parafilm over small petri dish. Deposit RNA sample onto parafilm.

7) Fill the pipette with RNA solution by maneuvering (with micromanipulator) tip into the solution. Depress the FILL button until all RNA solution is aspirated into pipette.
8) Select desired injection volume. The injection volume depends on the concentration of RNA you wish to inject. The volume of RNA injected should not exceed 60 nl to avoid oocyte rupture.
If necessary, dilute RNA and inject a smaller volume.
9) Impale oocyte, depress INJECT button, release button; retract pipette; do again for each oocyte.

Solutions

ND96-Ca^{2+}-free (in mM): 96 NaCl, 2 KCl, 1 $MgCl_2$, 5 HEPES (pH 7.6).
Barth´s oocyte storage solution (in mM): 88 NaCl, 1 KCl, 0.4 $CaCl_2$, 1 $MgCl_2$, 10 HEPES (pH 7.4) + gentamycin (50 mg/L) + 1 mM pyruvate.
KCM 411 (extracellular bath solution): 94 NaCl, 4 KCl, 1 $CaCl_2$, 1 $MgCl_2$, 5 HEPES (pH 7.6).
Micropipette: 3 M KCl

REFERENCE:
Methods in Enzymology. Volume 207 ION CHANNELS. Bernardo Rudy and Linda Iverson, eds. Academic Press, 1992. (Section IIA. Expression of ion channels in *Xenopus* oocytes). pp 225-390.