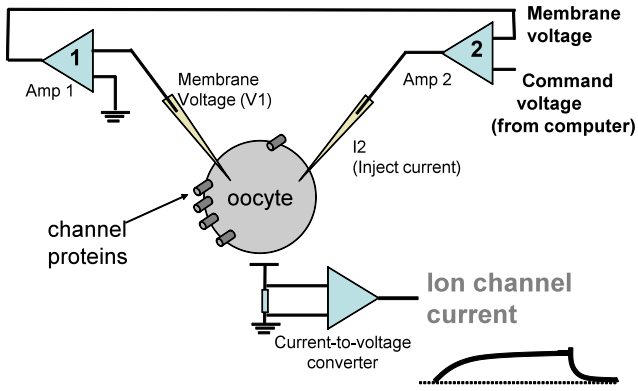
Two microelectrode voltage clamp (TEVC) of *Xenopus* oocytes

Introduction

The voltage clamp technique is used to measure ionic currents in response to precisely controlled changes in the transmembrane potential of an isolated cell. Large cells (e.g., frog oocytes) can be studied using two-microelectrode voltage clamp (Fig. 1). The oocyte is impaled by two glass micropipettes, one for voltage sensing and one for current injection. The transmembrane potential is measured by the voltage-sensing electrode (V1) connected to a high input impedance amplifier (amp1). This signal is compared to a command voltage generated by a computer at the input of amp 2. The output of the high gain feedback amp 2 is a current delivered to the cell interior by the second micropipette. This current is sufficient to force the transmembrane potential to equal the command voltage. The current delivered by micropipette 2 is monitored as "I2" via a current-to-voltage converter.

Fig. 1: Two-microelectrode voltage clamp



In this lab exercise you will record whole cell ionic currents conducted by K^+ channels heterologously expressed in *Xenopus* oocytes (Fig. 3) using the two-microelectrode voltage clamp technique. A Geneclamp 500 amplifier will be used to record currents in oocytes that were injected 3 days ago with cRNA encoding Kv11.1 (hERG) channels. Data acquisition is performed using a personal computer and an analog-to-digital (A/D) interface.

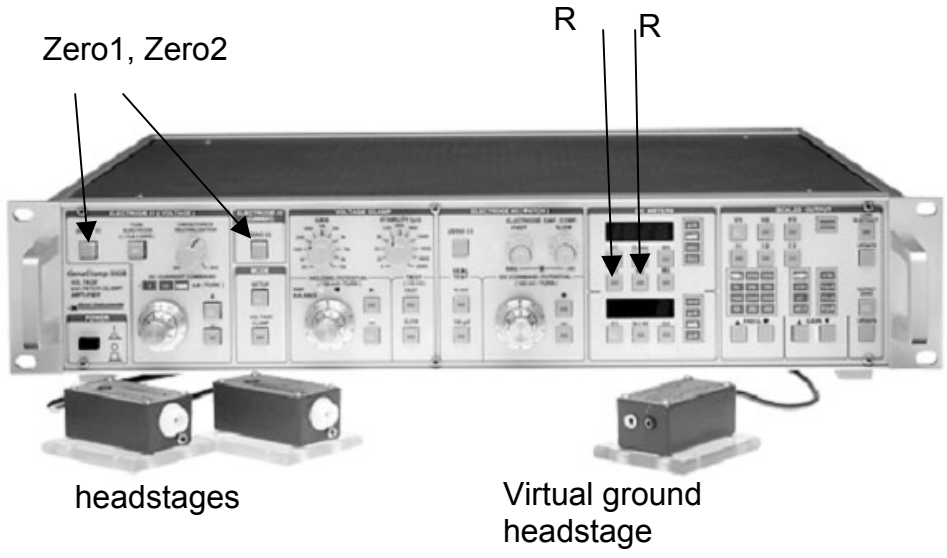
Electrophysiological measurements

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 a 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 (Fig. 2).

Fig. 2: GENECLAMP 500 amplifier:



Using the micropositioners, immerse tips of both electrodes into the KCM211 extracellular 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.

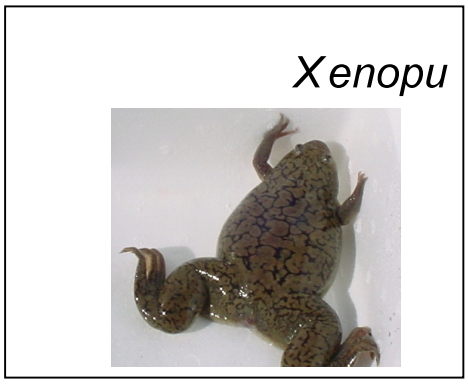


Fig. 3: *Xenopus* frog and oocytes

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

You will be supplied with oocytes injected with cRNA encoding wild-type and two mutant forms of hERG. The mutant channels contain a single point mutation (introduced by site-directed mutagenesis) that alters one or more biophysical property of the channel.

Include the following data in the RESULTS of your LAB REPORT:

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 -130 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.
   Pulse protocols: "hERG 300ms IV" and "hERG 5s IV"

3. Measure peak "tail" currents (I_{tail}) and plot as a function of test voltage (V_t) for both sets of data. These plots define the voltage dependence of channel activation. Fit the relationship with a Boltzmann function to determine the half-point for activation ($V_{1/2}$) and the slope factor (k, a measure of steepness of the relationship; k = RT/zF):

   $$I_{tail} / I_{tail\text{-}max} = 1/\{1 + \exp[(V_{1/2} - V_t)/k]\}$$

   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 (E_{rev}) of the I-V relationship.
   Pulse protocol: "hERG fully activated IV"

5. Estimate the time- and voltage-dependent contribution of hERG current to a cardiac action potential.
   Pulse protocol: hERG_AP"

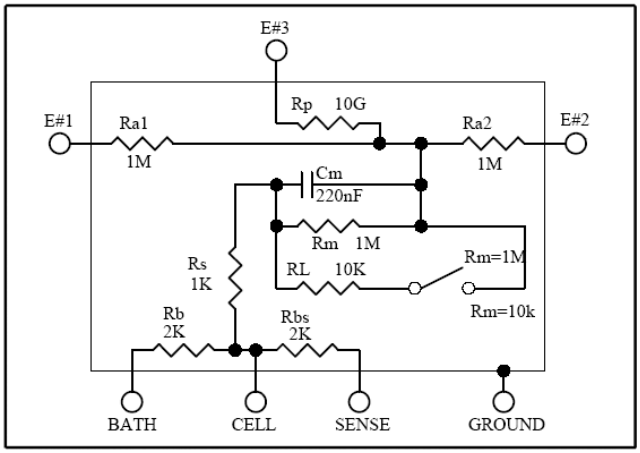
6. Repeat steps 1-4 using oocytes injected with cRNA encoding the two different mutant hERG channels.

Issues to consider when writing Discussion section of LAB REPORT:

a) At what potential does hERG current first appear to activate, and how does it compare to the resting membrane potential of the oocyte when first impaled by an electrode?
b) How and why does the shape of the "normal" I-V relationship differ from the "fully-activated" I-V relationship?
c) How do the biophysical properties of the mutant hERG channels differ from the wild-type channels?
d) Based on the altered biophysical properties, what part of the channel protein do you suppose harbors each of the two mutations?
e) How would the altered properties of the mutation affect the shape and duration of a cardiac action potential?
f) IN ADDITION, answer the following two essay questions. Please insert in lab Report after your Discussion section (these two essays will account for ½ your grade for this lab exercise):
   1) Based on information revealed by the x-ray crystal structure of the KcsA bacterial K^+ channel, what is the molecular basis of K^+ selectivity over Na^+ in this channel?
   2) Describe the origin of gating current and the structural basis of electromechanical coupling.

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.

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.