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Two-electrode voltage-clamp (TEVC)

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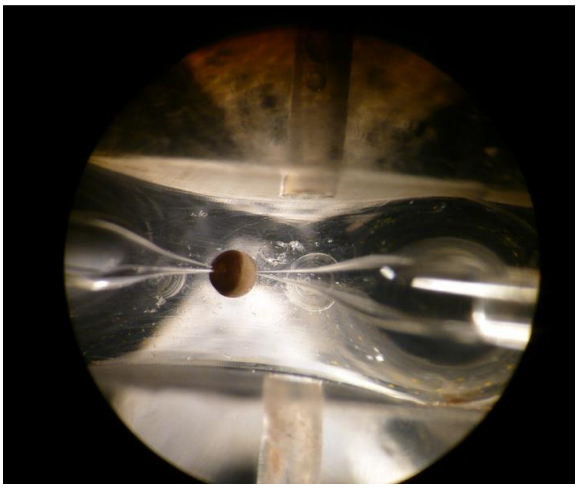
Stand: 09/01/14

1. Motivation



The function of a cell is governed to a large extent by transport across the cell membrane that is mediated by specific membrane proteins. To learn about functional characteristics of these membrane proteins, electrophysiological techniques have turned out to be a powerful method. Particularly the combination of electrophysiology with molecular biology and biochemistry allows obtaining fundamental information on structure, function and regulation of charge-translocating proteins. This can be achieved by expression of genetically modified proteins in *Xenopus* oocytes and functional characterization by electrophysiological methods. For detailed information see (Hille,2001;Schwarz and Rettinger,2003)).

This laboratory course introduces into up-to-date electrophysiological research using two-electrode voltage clamp (TEVC).

2. Background

2.1. Electrical characteristics of biological membranes

2.1.1. The membrane potential

Most electrical phenomena occurring at a cell membrane are based on asymmetrical ion distributions between cytoplasm and extracellular space and on ion-selective membrane conductances. The electrochemical gradients across the cell membrane lead to an electrical potential difference, the so-called membrane potential, that can be detected by electrodes in the intra- and extracellular space. In order to describe this potential, different approaches can be made:

(1) If we assume that a membrane is **permeable for all ions except for one species**, the membrane potential can be described by the **Donnan equation**. For instance, macromolecules like anionic proteins or nucleic acids are not able to cross the membrane. For an animal cell, the calculated Donnan potential does not match the actual membrane potential. Therefore, another approach has to be chosen.

(2) If we assume that a membrane is **impermeable for all ions except for one species**, the membrane potential can be described by the **Nernst equation:**

$$\Delta E = \frac{RT}{zF} \ln\left(\frac{a_{out}}{a_{in}}\right) \qquad \text{equ. (1)}$$

with R the universal gas constant, T absolute temperature, z valence of the ion, F the Faraday constant, and a_{in} and a_{out} the ion activities inside and outside the cell, respectively.

(3) For a real cell neither the Donnan nor the Nernst equation can describe the membrane potential. The membrane exhibits specific permeabilities for different ion species, and these permeabilities depend on the particular environmental conditions. The **Goldman-Hodgkin-Katz equation** is often used to describe the dependence of membrane potential on different ion permeabilities; if Na^+, K^+ and Cl^- were the permeable ions, the Goldman-Hodgkin-Katz potential is given by:

$$E_{GHK} = \frac{RT}{F} \ln\left(\frac{P_{Na}[Na]_{out} + P_K[K]_{out} + P_{Cl}[Cl]_{in}}{P_{Na}[Na]_{in} + P_K[K]_{in} + P_{Cl}[Cl]_{out}}\right) \qquad \text{equ. (2)}$$

However, to derive equ. 2 three assumptions have to be made:

- independent ion movement (meaning free diffusion)
- constant diffusion coefficient D_C (homogenous membrane phase)
- constant electrical field within the membrane (linearly changing potential)

2.1.2. The membrane as an electrical unit

Electrical current within a biological system is mediated by ion movements. From an electronic point of view, a biological membrane can be considered as a parallel circuit of a resistance and a capacitance (compare shaded areas in Fig. 2). Opening and closing of ion-permeable channels and activities of electrogenic carriers govern the resistance of the membrane. Therefore, current-voltage (IV) characteristics can tells us a lot about the function of the respective membrane proteins. Interestingly, the specific capacitance of a cell membrane hardly changes and is independent of the cell type. The specific capacitance of a lipid bilayer is close to 0.8 µF/cm^2, and a value of 1 µF/cm^2 is often used to calculate the surface area of a cell from electrical determinations of the cell capacitance. The capacitance can be calculated from the transient signal of charging or discharging the membrane capacitor. When a small rectangular voltage pulse is applied to the membrane (voltage clamp), the current response is composed of an exponential transient capacitive signal (I_{cap}) and a constant current (I_{res}) carried by ions moving across the membrane (see Fig. 1); I_{cap} declines exponentially with a time constant $\tau = RC$.

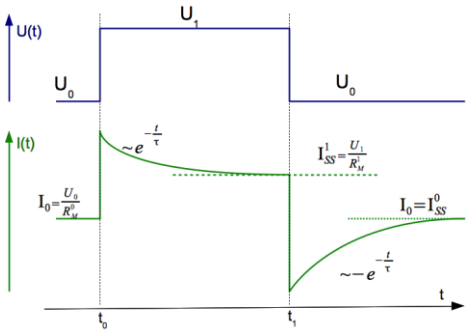


Figure 1: The time course of current (lower trace in response to a voltage-clamp pulse (upper trace). The signal is a superposition of a transient (capacitive) and a stationary (resistive) current component (I_{SS}), which is ohmic for a small voltage step:

$$I(t) = I_{cap} + I_{ss} = C\frac{dU}{dt} + I_{ss} \quad \Rightarrow \quad (I(t) - I_{SS}) \cdot dt = C \cdot dU$$

The membrane capacitance can be calculated from:

$$\int_{t_0}^{t_1}(I(t)-I_{SS})dt = C_M \int_{U_0}^{U_1} dU = C_M (U_1 - U_0) \qquad \text{equ (3)}$$

2.2. Theoretical background of voltage clamp

The most powerful electrophysiological method for basic research is the voltage-clamp technique. The method allows for a given (clamped) membrane potential the measurement and analysis of current across the cell membrane, which are mediated by specialized channels and carriers. The voltage-clamp technique was the basis for two milestones in modern electrophysiology: the Hodgkin-Huxley (1952) description of excitability and the demonstration of single-channel events by Neher and Sakmann (1976). The scientists were honoured for their work by Nobel prizes.

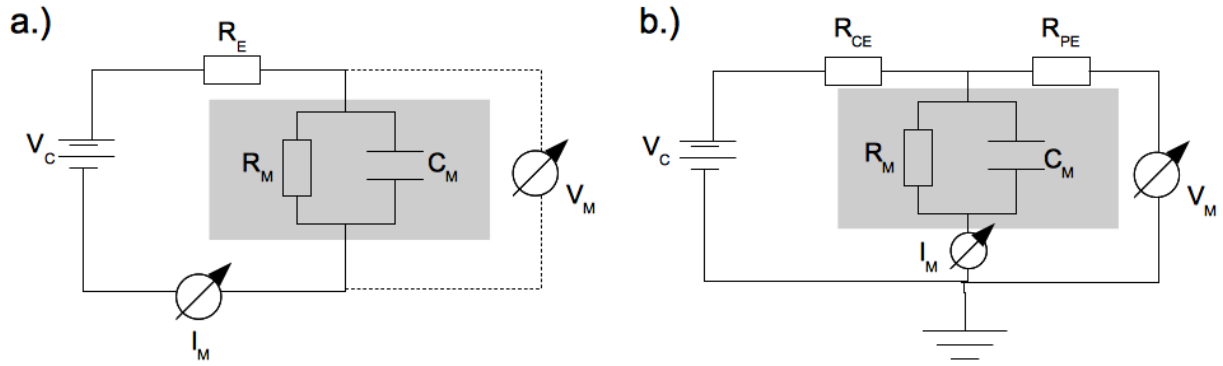


Figure 2: a.) $R_E = 0$: Ideal voltage-clamp diagram, $R_E \neq 0$: Real voltage-clamp diagram. **b.)** Voltage-clamp circuit with a current electrode (C_E) and voltage electrode (P_E). The cell membrane is shown as a parallel circuit of a capacitor C_M and a resistor R_M (grey area).

2.2.1. The ideal voltage clamp

The ideal voltage clamp (Fig. 2a with $R_E = 0$) consists of a voltage source providing the clamp potential V_C, the model membrane and an ampere meter for measuring membrane current I_M. This circuit is "ideal" if the intrinsic resistances of wires, ampere meter and battery are neglected. Therefore, the potential drop across the model membrane V_M equals the potential of the battery ($V_M = V_C$).

2.2.2. The real voltage clamp

In real voltage clamp the connections within the electronic circuit cannot be treated with negligible resistances. In particular, the electrode resistance R_{CE} (Fig. 2b) cannot be neglected, and the two resistors, R_{CE} and R_M, in series act as voltage divider. The potential drop across the membrane is only:

$$V_M = \frac{R_M}{R_M + R_{CE}} V_C \qquad \text{equ (4)}$$

If R_{CE} cannot be neglected compared to R_M, a second electrode is needed to determine the actual membrane potential (see Fig. 2b). In the laboratory course glass microelectrodes are used to penetrate the cell membrane to access the cell interior. These electrodes have tip resistances in the range of MΩ similar to the input resistance of large cells such as *Xenopus* oocytes.

2.2.3. Two-electrode voltage-clamp

For large cells with low input resistances ($R_M \approx R_E$) we use the so-called Two-Electrode Voltage Clamp (TEVC). Since the membrane resistance R_M changes in response to various stimuli during an experiment, the membrane potential V_M is continuously monitored via the potential electrode PE, and then can be readjusted by changing the command potential V_C (see Fig. 2B). Instead of doing this manually, electronic devices are used that allow exact and rapid communication between command and measured membrane potential. The basic devices of such an electronic set-up are operational amplifiers (*op-amp*).

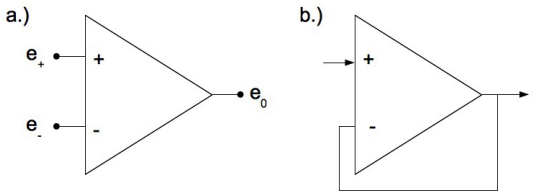


Figure 3: Schematic drawing of **a.)** an op-amp and **b.)** a voltage follower (op-amp with unity gain)

The main characteristic of an op-amp (Fig. 3a) is its ability to amplify a difference ($e_+ - e_-$) between its two inputs by a factor A (gain).

$$e_0 = A\,(e_+ - e_-) \qquad \text{equ (5)}$$

As a negative feedback amplifier this kind of op-amp forms the central part of the voltage-clamp system (see Fig. 4). The positive input is connected to the potential V_{VC} that is to be clamped to the memberane, the negative input to the signal provided by the potential measuring electrode. These two input signals define the potential at the output, and hence allow the cell to be clamped fast and accurately to V_{VC}. The current flow from the feedback amplifier is identical to the membrane current and can be measured either at the output of the op-amp (see Fig. 4) or at the grounded bath electrode.

Another essential op-amp of the voltage-clamp system is the voltage follower (Fig. 3b) where the negative input is directly connected to the output (i.e. $e_0 = e_-$). At the typically high gain factor ($A \approx 10^4 - 10^6$), according to equ (5), the output signal will follow the signal at the positive input ($e_0 \approx e_+$). The voltage follower is used to uncouple the sensitive signal of the potential electrode from the following recording devices, and to serve as a high resistance input in order to minimize current flow through the voltage electrode.

Very often two bath electrodes are used, one current-passing grounded electrode and one bath electrode serving as a reference electrode for the intracellular potential electrode (virtual ground). The use of the virtual-ground electrode has the advantage that this bath electrode will not polarize due to current flow.

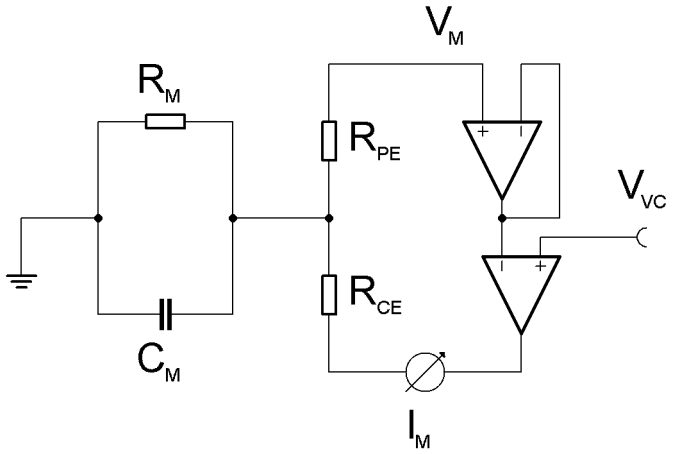


Figure 4: Two-electrode voltage-clamp circuit with op-amps for measuring the membrane potential via the voltage follower and performing voltage clamp by the negative-feedback amplifier. R_{PE} and R_{CE} represent the resistances of the potential measuring and the current supplying electrode, respectively.

2.3. Hypothesis testing - the paired-sample t-test [1]

For evaluation of data we want to test the hypothesis whether the measured K^+-sensitive ($I_{K\text{-}sens}$) current is mainly composed of the electrogenic current mediated by the Na^+/K^+-ATPase (I_{pump}) and the current through K^+-selective channels (I_K) i.e. whether:

$$I_{K-sens} \cong I_K + I_{pump}$$

The t-test is to be applied for testing the above hypothesis. Basis for hypothesis testing is that the data form a sup-population of an unknown source population can be supposed to have normal distribution. The sup-population of the source must have the same mean value as the source; however, the standard deviation of the source is unknown.

When taking random samples of size N from the source, the mean variance of the samples is given by the variance of the source population multiplied by 1/N.

The samples do not follow normal distribution, but rather t-distribution. This distribution is also bell-shaped, but depending on its degrees of freedom (df = N-1), it is flatter than the normal distribution, hence more prone to producing values far away from the mean (see Fig. 5, left). For $N \rightarrow \infty$ it becomes the normal distribution. A t-value can be calculated, indicating in terms of standard deviation σ_S, how significantly the sample mean M_S differs from the test mean μ, which is assumed to be the mean value of the source (**one sample t-test**). For N samples m_i :

$$t = \frac{M_S - \mu}{\sigma_S}\sqrt{N} \quad \text{with} \quad M_S = \frac{1}{N}\sum_{i=1}^{N} m_i \ , \ \sigma_S = \sqrt{\frac{1}{N-1}\sum_{i=1}^{N}(m_i - \mu)^2}$$

Usually M_S is considered significantly different at 0.05 level if M_S is located in the 2.5% area of the t-distribution on either tail (two-tailed t-test) or significantly greater/less than the test mean if it is in the 5% area in the right/left tail (one-tailed t-test) (see Fig. 5, right). The **paired-sample t-test** is used for comparing two sets of sample populations in which the individual subjects are related in pairs (here the sum $I_{K\text{-}sens}$ and the two current components $I_K + I_{pump}$). Hence, the pair-wise difference can be calculated, and then a one sample t-test can be performed on the difference values with the test mean being zero ($\mu = 0$).

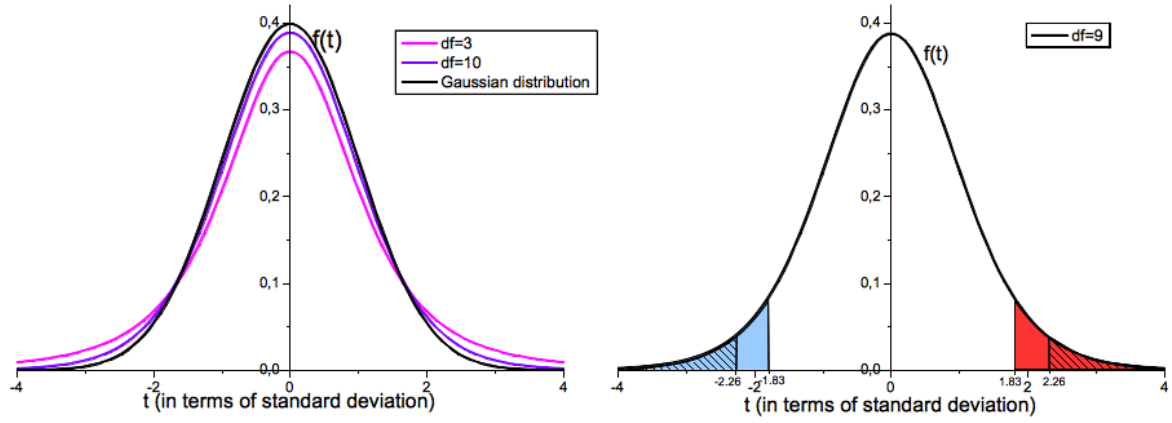


Figure 5: Probability density function f(t, df) for df = 3, df = 9, df = 10 and Gaussian normal distribution. The blue / red areas mark 5% areas for the one-tailed t-test with |t| > 1.83 denoting that the sample mean is significantly less / greater than the test mean.. The striped areas mark the 2.5% areas for the two-tailed t-test with |t| > 2.26 denoting that the sample mean is significantly different from the test mean.

[1] A very detailed introduction into statistical methods in general can be found on the internet webpage "*Concepts & Applications of Inferential Statistics*" (Lowry, 1999).

3. Questions

In preparing for the lab course you should be familiar with the following questions:

(a) What are the approximate activities of dominating intra- and extracellular ions of a vertebrate cell?

(b) What is the specific membrane capacitance (capacitance per unit area), and why is it an important biophysical parameter? What is the expected total capacitance of a *Xenopus* oocyte with a diameter of 1 mm (if the oocyte were assumed to have the shape of a perfect sphere)?

(c) Calculate the Nernst potential for a typical K^+ distribution at a biological membrane. How can the Nernst equation be used to determine the intracellular K^+ activity?

(d) Write down the Goldman-Hodgkin-Katz equation. What are the assumptions, the GHK equation is based on?

(e) Describe the mechanism of ion translocation of the Na^+/K^+-ATPase (sometimes also referred to as "sodium pump"), and explain why the transport mediated by this ion pump is electrogenic.

(f) Describe the main features of the voltage-clamp technique. How does it work (circuit diagram)? What is the function of the operational amplifiers needed to perform voltage clamp?

(g) During the lab course you have to prepare microelectrodes. How is the electrical connection between solution and the electronic achieved? Give the reaction formula.

Since you are also asked to hand in a "pre-protocol" - consisting of an introduction and a description of method and procedure - it may be helpful for your preparation to include answers to the above questions into the pre-protocol.

4. Set-up and basic instructions

4.1. Experimental set-up (see Fig. 6)

Defolliculated oocytes of the clawed toad *Xenopus laevis* are positioned in the cell chamber **(1)** that is mounted under a microscope and connected to a stopcock for changing the solutions perfusing the chamber. The cell is impaled by two microelectrodes. A clamp potential V_C is applied to the membrane by the voltage-clamp system Turbo TEC (NPI, Germany). **(2)** The Turbo TEC is under control of a personal computer **(3)** on which the program CellWorks is running. For control of the quality of the voltage clamp, the time courses of membrane current I_M and membrane potential V_M are monitored on an oscilloscope **(4)**. In addition, holding potential and holding current are continuously recorded by a pen recorder **(5).**

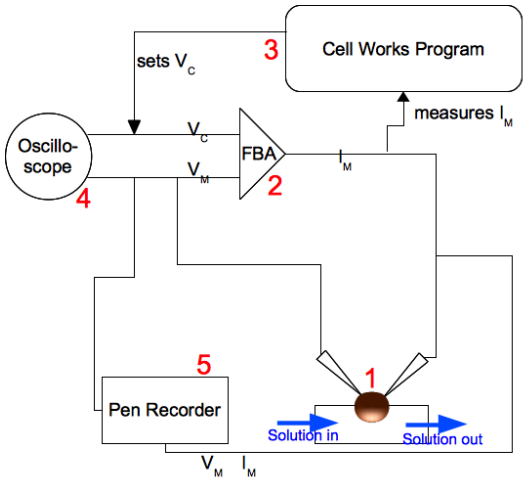


Figure 6: Schematic diagram of experimental set-up (see text)

4.2. Preparation of microelectrodes

First, glass micropipettes are pulled from filament-containing capillaries of borosilicate glass using a vertical puller. The pipettes are filled with 3-M KCl using a syringe with a fine needle. It is crucial to eliminate all air bubbles from the tip of the electrode. The capillary is inserted into an electrode holder mounted to a micromanipulator. A silver wire covered with AgCl serves as connection to the electronics. A resistance in the range of 1 MΩ can be check by an ohmmeter built-in to the Turbo TEC system.

4.3. Instructions for the use of CellWorks program for Turbo TEC

(1) After starting CellWorks choose the appropriate user (usually DEF).

(2) Open the following modules

(2.1) "*Execution*" (executing valve settings and the pulse protocol for determination of IV curves)

(2.2) "*Online A*" (displaying time course of the VC pulses and current responses, steady-state potential and current, and IV relationship)

(3) Only if a valve system is installed (at present only available in Shanghai laboratory): Run from *Execution* the *Manual* option. Start the desired solution. The respective valve will open and the pump will start running.

If the valve system has not been installed to your set-up, you have to chance the solutions and to turn on the pump manually.

(4) Run from *Execution* the *Pulse* option and start *IV curve*, but abort immediately. This will set the holding potential of -60 mV and default parameters in the *Online* monitor. **Check, whether the *Export on* option in the *Online* monitor is activated.** Now an IV curve can be recorded.

4.4. Solutions

During the experiment, the chamber is perfused with four different solutions (see Tab. 1).

Solution #	NaCl	$CaCl_2$	MOPS	TMA-Cl	$BaCl_2$ / TEA-Cl	KCl
1	100	1	5	25	0	10
2	100	1	5	25	0	0
3	100	1	5	0	5 / 20	10
4	100	1	5	0	5 / 20	0

Table 1: Ionic composition of external solutions (in mM). MOPS (pH buffer, pH=7.2), TMA: tertramethyammonium, TEA: tetraethylammonium.

Solution 1 is a solution to mimic roughly physiological extracellular ion composition.

In **Solution 2** the KCl is missing, and hence, current mediated by the Na/K pump, I_P, and inward current through K^+ channels, I_K, should be blocked.

In **Solution 3**, the TMA-Cl is replaced by $BaCl_2$ and TEA-Cl. Ba^{2+} and TEA^+ are specific inhibitors of currents through K^+ channels.

In **Solution 4** the KCl of solution 3 is missing, and hence, current mediated by the Na/K pump should again be blocked.

5. Experiments and data analysis

5.1. IV characteristics

5.1.1. Procedure

Since we are interested in detecting current mediated by the Na/K pump, we choose conditions that stimulate pump activity. That is why we have extracellularly 10 mM K^+; the intracellular Na^+ activity is increased by incubating the cells in Na^+ -loading solution for about 30 min. Thereafter, the oocytes are placed into post-loading solution for at least 15 min before the experiments is started.

After the two microelectrodes have been prepared and mounted to the manipulators, the perfusion for each solution should be adjusted to about 1 drop/s. In the VC –off mode of the Turbo TEC the offsets of potential and current electrode should be adjusted to zero. It is crucial to do so right before each single experiment.

Now the oocyte can be impaled with the two microelectrodes. It is helpful to turn on the built-in audio monitor of the Turbo TEC which converts the value of the potential at the electrode tip into an acoustic signal. Once the tip penetrates the cell membrane, the resting potential of the cell is registered indicated by a chance in audio frequency.

After both microelectrodes have been inserted properly, the amplifier can be set to *VC* mode. Wait until the holding current has stabilized before starting measurements of IV dependencies (by starting *IV curve* in the CellWorks *Execution* window). Superfuse the cell with the different solutions in the following sequence:

1 → 2 → 1 → 3 → 1 → 3 → 4 → 3

After having switched to another solution, wait for complete exchange (about 2 min) before recording the next IV dependency.

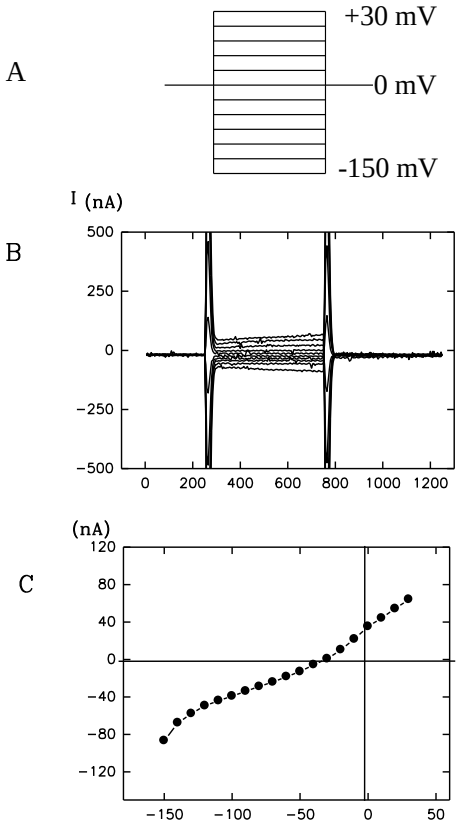


Figure 7: Currents (**B**) in response to a series of rectangular voltage steps (**A**). Steady-state current-voltage dependence (**C**). Steady-state values are obtained from data averaged close to the end of the pulse during a period of 20 ms (in order to correct for 50-Hz noise).

During the experiment the membrane holding potential is clamped to -60 mV. For each IV measurement the program applies rectangular voltage pulses (of 200 ms duration) from -150 mV to +30 mV in 10 mV increments and records the steady-state values of membrane potential and current close to the end of the test pulse (see Fig. 7).

After having recorded all 8 IV dependencies (IV1 to IV8) in the respective solutions, repeat the experiment with another oocyte. Eventually, you should have at least 5 successfully performed experiments.

5.1.2. Tasks

For data analysis, a software is recommended that provides features such as data manipulation, calculation of mean values and errors, plotting, fitting and hypothesis testing. In the lab course, the application of ORIGIN will be demonstrated.

The files of the recorded data can be found in the *Export* folder in the CellWorks directory. Every time you took an IV dependency, a new ASCII file was produced by CellWorks (so, there should be 8 files per experiment). Each file consists of six columns of which you need the first (potential in mV) and the second (current in nA) column.

The following current components should be determined from the measured steady-state data for each cell: $I_{K\text{-}sens}$, I_K and I_{Pump} (see below). Calculate the mean values and SEMs (standard error of the mean). Plot the IV characteristics of the three current components of each experiment into respective graphs, and the calculated mean currents with SEMs as error bars into a separate graph.

(a) Determine the total K^+-sensitive current as the difference between currents measured in solution 1 and 2. Sometimes the current during the experiment tends to drift linearly in time. Therefore, a drift correction is performed by taking the arithmetic mean of I_1 and I_3 (recall that both have been measured in solution 1).

$$I_{K-sens} = \frac{I_1 + I_3}{2} - I_2$$

(b) Determine the K^+ current through the K^+ channels as the current difference in solution 1 and 3.

$$I_K = \frac{I_3 + I_5}{2} - I_4$$

(c) Determine the current that was mediated by the Na^+/K^+-ATPase as the difference between the currents measured in solution 3 and 4.

$$I_{Pump} = \frac{I_6 + I_8}{2} - I_7$$

(d) Demonstrate that the K^+ channels and the sodium pump are the major sources for K^+-activated currents. In order to do so, compare the sum $I_K + I_{Pump}$ with $I_{K\text{-}sens}$. Select at least three potentials (for instance -120 mV, -60 mV and 0 mV) and perform a paired-sample t-test at these potentials (present in your protocol the t values calculated by the data analysis program). Determine whether $I_K + I_{Pump}$ is significantly different from $I_{K\text{-}sens}$, discuss the result.

(e) Calculate from the reversal potential of the current mediated by K^+ channels (I_K) the intracellular K^+ activity using Nernst equation (equ. 1).

5.2. Determination of the membrane capacitance

5.2.1. Procedure

Open the window *Export settings* in the *Online* monitor. Tick the "Export raw data" option. This will result in an exported file that does not only contain the IV dependencies, but also the time courses of current and voltage of the single pulses.

Take an unloaded oocyte, place it into the cell chamber and impale the two microelectrodes as in the previous experiments. Since the membrane capacity is independent of the external solution used, perfuse the chamber with the solution of your choice.

Run from *Execution* the *IV-curve_cap* option. Now the program applies just a single voltage pulse to the membrane from the holding potential of -60 mV to -70 mV (duration 200 ms).

5.2.2. Tasks

(a) Measure the diameter of the oocyte used for this experiment under the microscope, and calculate the expected total membrane capacitance assuming a ball-shaped cell.

(b) Determine the capacitance of the oocyte by analysing the transient current. The file of recorded data of the single pulse consists of three columns: time (ms), current (nA), and potential (mV). At the bottom of the file, you find a differing data structure containing the steady-state potential in the first and the steady-state current in the second column. Plot the time course of the current. Use equ. 3 to calculate the membrane capacitance for the potential step from -60 mV to -70 mV and back to -60 mV (the integral may be calculated by the data analysis software you are using). Determine the mean value of the "on" and "off" response. What is the corresponding surface area of the oocyte?

(c) Compare results (a) and (b). Discuss the result.

6. References and further reading

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