Protocol for recording I_h in neurons

Short title: Recording neuronal I_h

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Abstract:

The hyperpolarization-activated cyclic nucleotide-gated (HCN) channels are voltage-gated ion channels that play a crucial role in many physiological processes such as memory formation and spatial navigation. Alterations in expression and function of HCN channels have also been associated with multiple disorders including epilepsy, neuropathic pain and anxiety/depression. Interestingly, neuronal HCN currents (I_h) have diverse biophysical properties in different neurons. This is likely to be in part due to the heterogeneity of the HCN subunits expressed in neurons. This variation in biophysical characteristics is likely to influence how I_h affects neuronal activity. Thus, it is important to record I_h directly from individual neurons. Here, I describe voltage-clamp protocols that can be used to record neuronal I_h. The information obtained using this approach can be used in combination with other techniques such as computational modeling to determine the significance of I_h for neuronal function.

Materials

Institutional health and safety regulations as well as the appropriate material safety data sheets must be followed whilst carrying out this method.

I_h can be recorded under whole cell voltage-clamp conditions, in cell-attached mode or with outside-out patches.

Reagents

Biological sample. This can be a slice preparation or cultured neurons.

External recording solution (mM): 125 NaCl, 2.5 KCl, 1.25 NaH_2PO_4, 25 $NaHCO_3$, 2 $CaCl_2$, 2 $MgCl_2$, 10 glucose, 0.001 tetrodotoxin; 0.1 $CdCl_2$, 10 TEA, 1 4-AP, and 1 $BaCl_2$ bubbled with 95% O_2/5% CO_2 (pH 7.2, Osmolarity = ~320 MOsm). This solution should be ideally made-up fresh on the day of the experiment.

Internal pipette solution for whole-cell voltage-clamp mode or outside-out patch mode (mM): 120 $KMeSO_4$, 20 KCl, 10 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), 2 $MgCl_2$, 0.2 ethylene glycol-bis(2-aminoethylether)-*N*,*N*,*N*′,*N*′-tetraacetic acid (EGTA), 4 Na_2ATP, 0.3 TrisGTP, 14 Tris-phosphocreatine; pH adjusted to 7.3; Osmolarity = 300 MOsm. The solution should be aliquoted and frozen. It can be stored in this manner for 2-3 months. When using it, it should be preferably stored on ice.

Internal pipette solution for cell-attached mode (mM): 120 KCl, 20 TEA Cl, 5 4-AP, 1 $BaCl_2$, 10 HEPES, 1 $MgCl_2$, 2 $CaCl_2$, 0,001 tetradotoxin, 0.1 $NiCl_2$; pH adjusted to 7.3; Osmolarity = 300 mOsm; stored at 4^0C for not more than 1 month.

Equipment

Microscope. This should have sufficiently good optics (objectives with at least 40x magnification and a numerical aperture of 1.0) to visualize individual cultured neurons and neurons present in a slice preparation. To visualize neurons present in a slice preparation using a microscope, it is preferably to use a microscope with differential infra-red optics coupled to a good camera (e.g Rolera Bolt CMOS, QImaging) and monitor (for details of equipment specification see (Davie et al. 2006; Shah 2013).

Micromanipulator

Amplifier (e.g. Axopatch 200B or Multi-clamp 700B, Molecular Devices).

Analog-digital convertor

Computer with acquisition software (e.g. pClamp, Molecular Devices).

Pipette puller

Method

1) Place the biological sample under a microscope and perfuse the sample with the external solution. I_h is highly sensitive to temperature, with Q_{10} values ranging from 4-6 (Robinson and Siegelbaum 2003). Thus, whilst I_h can be recorded at room temperature, the biophysical characteristics are likely to be different from those recorded at near physiological temperatures. To obtain recordings at near physiological temperature, the external solution can be heated with a temperature controller such as the Model CL-100 Bipolar Temperature controller (Harvard Apparatus Ltd).

2) Identify the soma or dendrite of a healthy neuron present in culture or in a slice preparation.

3) Pull patch-pipettes. It is preferable to use thick-walled borosilicate glass for these recordings.

4) Fill a micropipette with the appropriate internal solution. The micropipette resistance should be between 3-6 MΩ. The internal solution should be filtered to remove any debris. Insert the pipette into a holder that is securely attached to the amplifier headstage and micromanipulator.

5) Add positive pressure to the pipette via tubing attached to the pipette holder. Secure the pressure within the pipette using a stopcock or a switchable valve.

6) Using the micromanipulator, lower the pipette on top of the visually identified soma or dendrite. Zero any offset recorded with the pipette before touching the cell with it. If the cell is healthy, a dimple will form on the cell membrane.

7) Release the pressure and form a giga-ohm seal. Gentle suction may need to be applied to form a good giga-ohm seal. For cell-attached recording, the voltage-clamp protocol described below can be applied to record HCN channel currents or I_h. For whole-cell recording, further gentle suction will be required to be rupture the membrane. This should be done whilst holding the cell near the normal resting membrane potential (i.e. -70 mV). Once a whole-cell recording has been established, the pipette can be gently pulled away to obtain an outside-out patch.

8) For whole-cell voltage-clamp recordings, appropriate series resistance compensation (at least 60 % -70 %) should be carried out before commencing the recordings. For whole-cell voltage-clamp and outside-out patch recordings, the protocol shown in Fig 1A, should be applied. Briefly, the voltage-potential should be stepped up to -40 mV and 2 s hyperpolarizing square steps applied in 10 mV increments to -120 mV or beyond to fully activate the current. The voltage is stepped between successive increments to a potential such as -60 mV to record the tail I_h current (Fig 1A). Once the recorded current is stable, the pharmacological inhibitor ZD7288 (10 μM) or Cs^+ (1-2 mM) should be applied for 15 min and the protocol repeated in the presence of this compound. The currents in the presence of the inhibitor should be subtracted from those under control conditions to obtain I_h.

9) For cell-attached recordings, it is important to ensure that the background noise levels are between 1-5 pA. The protocol described above is also used to elicit I_h in cell-attached conditions too (Fig 1B). However, since the internal side of the cell membrane is not accessible to the pipette, the internal resting membrane potential (RMP) must be initially estimated. This then should be subtracted from the required potentials. For example, if the RMP is known to be on average -70 mV, the cell-attached potential will be +25 mV to achieve a membrane potential of -45 mV. In addition, since under cell-attached conditions, it is difficult to inhibit the recorded current with externally applied pharmacological inhibitors, it is advisable to measure the leak current by applying a +10 mV step from a holding potential of -45 mV. This leak current can then be subtracted from the current acquired using the protocol in Fig 1 to record I_h. At the end of the cell-attached recording, the patch can be ruptured to obtain the RMP. This can then be used to determine the membrane potential values at which I_h was recorded. For example if upon rupturing the patch, the RMP was -68 mV, then a potential of +25 mV would be equivalent to holding the cell at -43 mV.

10) The currents obtained should be filtered at 1-2 kHz and acquired using appropriate software.

11) Liquid junction potentials are a potential source of error for whole-cell voltage-clamp or outside-out patch recordings and can be measured by determining the difference in offset of an intact micropipette with that of a micropipette with a broken tip. Other methods for determining the liquid junction potential are described in (Neher 1992).

12) From the acquired records, the tail current amplitudes elicited at the various voltages can be expressed as a fraction of the maximal tail current amplitude (Magee 1998; Poolos et al. 2002; Shah et al. 2004). The fractional current amplitudes can then be plotted against the voltage potential from which they were evoked and fitted using a Boltzmann function.

13) To estimate the reversal potential, the current amplitudes at a given voltage are plotted against the voltage (Magee 1998). The best line fit can be extrapolated to the x-axis to obtain an approximate value for the reversal potential.

14) The activation and de-activation kinetics are obtained by fitting the steady-state currents and tail currents respectively with exponential functions as described by Magee (1998).

Discussion.

The voltage-clamp protocols described are useful for obtaining the biophysical characteristics of I_h. Whilst the presence of a 'sag' obtained under current-clamp conditions (see Introduction Fig 1) is indicative that the neuron expresses HCN channels, this will not provide an insight into the biophysical characteristics of I_h. This information can then be used in computational models to determine the effect of I_h on neuronal and network activity.

Whole-cell voltage-clamp conditions have been used by many laboratories to record I_h from neuronal somata. There are, though, several limitations of the method including run-down of the current, space-clamp and series resistance issues. A solution to reducing series resistance errors is to measure the applied voltage using a second patch-pipette from the cell. This information can then be used to estimate membrane potential errors and to construct a more accurate activation curve.

The voltage errors associated with the whole-cell voltage-clamp method can be avoided using the cell-attached protocol and outside-out patch method. With the outside-out patch method, the intracellular mileu is disturbed and so the biophysical properties may not be representative of the native current. This is avoided using the cell-attached method (Williams and Wozny 2011). There are, though, several drawbacks of cell-attached method too. Errors associated with the transmembrane voltage changes caused by activation of voltage-gated ion channels in the patch in contact with the tip of the micropipette may still occur (Williams and Wozny 2011). An additional limitation is that the HCN channel density may not be uniform in all parts of the cell membrane. Thus, the current recorded from a single patch may not fully represent the density of the current in the particular subcellular compartment of the neuron. Therefore, a large number of recordings from a given cell might be necessary to accurately determine the amplitude and kinetics of the current. Despite the shortcomings, the methods described can be useful for estimating I_h densities and characteristics within a given neuron.

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Figure Legends

Fig 1 **A**, Example showing a whole-cell voltage-clamp recording of current from a hippocampal pyramidal neuron somata present in a rat brain slice under control conditions and in the presence of ZD7288 (15 μM) when a series of 2 s hyperpolarizing steps between -40 mV and -120 mV were applied as shown. The ZD7288-subtracted current, I_h, is shown on the left. **B**, Example of a cell-attached recording of I_h recorded from an entorhinal cortical layer III pyramidal neuron present in a rat brain slice when a step from -40 mV to -150 mV was applied (adapted from Shah et al. 2004). The trace was leak subtracted. The leak current was obtained by applying a step from -40 mV to -20 mV.

Fig 1, Protocol