Memory in the Hirudo Medicinalis retzius cell: spiking rate as function of past membrane potential

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Abstract

Dramatic advances in electrophysiological technologies combined with the resilience of leech preparation have made experimental neuroscience accessible to the classroom. The Retzius cell in Hirudo Medicinalis is a prime target for current-dependent frequency studies. Fundamental spiking dynamics were explored in Retzius by examining short term memory effects of voltage clamping. Two successive voltages were clamped for half second or full second intervals. Activity during the second phase was monitored for dependence on the previous phase. Experimental results of this assay revealed noticeable memory effects in cellular activity. In addition, memory was found to be influenced by the substitution of isoelectric Ba^{2+} for Ca^{2+} in extracellular solution. This study is an investigation of action potential dynamics unaccounted for by the Hodgkin-Huxley model and is potentially illuminating on the cellular dynamics of Retzius in Hirudo Medicinalis.

Introduction

The theory of action potentials rests on the classical Hodgkin-Huxley model. This model remains one of the most significant breakthroughs in neuroscience and is quite apt in describing the basic spiking dynamics in the leech Retzius cell. The model accounts for spiking of a neuron by a triggered inward current followed by an outward current. This current flow can be separated into two distinct components: a rapid influx of Na^+ ions and a slower outflux of K^+ ions. Conductances of Na^+ and K^+ are independent mechanisms as functions of time and membrane potential.



Voltage gated ion channels and leak currents are physiologically responsible for the spiking in nerve cells. The Hodgkin-Huxley model accounts for this biological process by statistically determining when the channels are open or closed. The net formula for membrane current and potential is given by:



The *m*, *h*, and *n* variables are functions of cell voltage and time. These variables govern the activation or inactivation of Na⁺ and K⁺ conductances, respectively g_{Na} and g_{K} . Conversely, these conductances describe the kinetics of an action potential. The individual ionic potential gradients, E_k and E_{Na} , are obtained from the Nernst equation and are based on the ionic concentrations inside and outside of the cell.

A wide range of electrical cell responses can be described by minor parameter adjustment in the Hodgkin-Huxley model; these include threshold spiking, refractory periods, and subthreshold oscillations. However, the model is imperfect and does not provide an explanation for phenomenological learning in nerve cells. Memory mechanisms that extend well beyond the refractory period of an action potential govern the future response of cells such as the Reztius in Hirudo Medicinalis. It is the exploration of these mechanisms that is the primary objective of this project.

Memory in neural systems remains a largely unknown phenomenon. Many factors may be involved in eliciting a memory response in single cells; some influencing factors include: Ca^{2+} ion channels, K^+/Ca^{2+} codependent ion channels, neuromodulators, calcium/calmodulin dependent protein kinase, and a number of biochemical properties involved in synapse plasticity². Calcium dynamics in leech Retzius is of particular interest because the cell membrane contains slowly inactivating Ca^{2+} channels that depend on extracellular pH, extracellular surface potential, and the K⁺ gradient across the membrane³. The effect of these channels on action potentials and hysteresis is not well understood; the issue is probed by replacing extracellular Ca^{2+} with isoelectric Ba^{2+} and monitoring changes in cell activity and memory.

The Retzius cell in Hirudo Medicinalis is resilient and accessible for use with intracellular recording. There are 29 ganglia along the leech central nervous bundle, each containing two Retzius cells in the anteromedial packet. One leach can provide numerous studies and remain viable for many hours after initial surgery. The Retzius cells are the largest neuronal bodies in the ganglion and reside centered and just anterior to the lateral roots; all bodies in the ganglion are resolvable using dark field microscopy. Additionally, the Retzius is a stable cell that has a firing rate highly correlated with injected current. This combination of physical and electrical properties makes the Retzius a prime target for electrophysiological study.



Experimental

Surgery

Leeches used for these experiments were obtained from the Kristan lab. Leeches were transported in small jars containing diluted Instant Ocean and then placed in a refrigerator where they could be stored in excess of a week. To extract a ganglion, the leech was first stretched and pinned out in a custom-made dish using two 21G injection needles. The leech was pinned dorsal-side up, distinguished by its pair of longitudinal orange bands. The dish consisted of a hollow base containing ice and a recessed dissection area lined with bees wax and filled with leech saline. After pinning, the leech was placed under a dissection microscope. An initial incision of approximately 4 centimeters (~20 ridges) was made along the midline. The skin was peeled back and pinned with 25G injection needles. Blood was sucked out of the body cavity using a vacuum, exposing the ganglion. A stocking of vasculature encases the ganglion and nerves. This was cut open and removed except along the lateral nerves. The ganglion and short segments of attached nerve were freed and placed in a small Petri dish containing Silguard. The nerves were pinned out using short segments of bare .002 tungsten wire. The stocking on the lateral nerves was used as a net for pinning. The nerves should be stretched so that the ganglion is held taught with the ventral (concave) side up.

Instant Ocean in the Petri dish should be replaced every half hour. The solution is made of the following ingredients and set to a pH of 7.4.

CHEMICAL	mM	g/6L	g/L
NaCl	115	40.26	6.71
KCl	4	1.8	.3
CaCl ₂ 2H ₂ 0	1.8	1.62	.27
MgCl ₂ 6H ₂ 0	1.5	1.8	.3
Glucose	10	10.8	1.8
Trismalente	4.6	6.6	1.1
TrisBase	5.4	3.9	.65

- pH to 7.4 usually takes 4-6 drops of saturated NaOH

- BaCl .42 g/L

Setup

The basic optical setup consists of a microscope with dark field illuminating beardath the sample field condenser consisting of a ring of LED's focuses light into a hollow cone, avoiding direct transmission into the microscope. Only scattered light from illuminated tissue is used to resolve the ganglion. The sample sits on a floating breadboard table that eliminates vibrations. An electrode clamp, positioned next to the microscope on the air table, can be controlled with micro-calibrators with four degrees of freedom. Adjustments can be made in the x-y-z planes and rotationally about the base of the clamp. A fifth degree of freedom is provided by diagonal extension of the electrode mount. This motion can be fine controlled with hydraulic-manipulators. The electrode is wired to an amplifier. Saline solution in the sample Petri dish is grounded with a ground wire placed directly in the solution. The wire is twisted in a helix and placed in solution to produce more effecting grounding.

Glass pipette microelectrodes were used for intracellular recording of action potentials. The electrodes were made from 1.5mm diameter borosilicate glass capillaries in a laser electrode puller. In order to pierce the cell membrane the electrode must have a fine tip. The electrode should taper gradually so as not to be too short or too long. Error in either direction can lead to excessive resistance or fragility. A good taper length is between 5mm and 10mm. The resistance of the electrode should ideally fall between 20 and 100 M Ω (error on the lower range is preferable). The electrodes are filled with 3mM KCl and a bleach treated platinum wire is inserted through the end. The wire is pre-galvanized with bleach to coat it with chloride anions. This allows for a transmission medium for current between the platinum wire and electrode solution.

The signal from the electrode is passed through a 10X amplifier in order to resolve the signal. An oscilloscope provides real-time observation of the signal. The electrode tip is placed in solution and causes the oscilloscope to display a finite voltage reading. This voltage is grounded so the potential should be corrected to zero. The resistance of the electrode is then tested by passing a 1nA current and noting the resulting potential. The current is switched off and the electrode is positioned close to the ganglion. At this point, the potential is reset to zero because of its proximity to the ganglion. Next, the resistance of the electrode is compensated. This is done by passing a current of 1nA and using the resistance compensation on the amplifier to set the potential to zero. By doing this, offsets due to the electrode's intrinsic properties are compensated for.

At this point the Retzius cell is impaled. The best way to do this is by positioning the tip of the electrode on top of the cell in the center so that it causes the cell to pucker. This requires good visibility and a strong electrode. Piercing the cell can then be done through a few methods. The most effective method seems to be slightly tapping the diagonal adjustment knob. This pushes the electrode through the glial sheathing and plasma membrane. Once the tip is inside the cell a resting potential and cell activity can be read on the oscilloscope. If this technique doesn't work, the micromanipulator can be used to further along the electrode tip and tapping can be tried again. Care must be taken not to advance the tip too far or it may come into contact with the bottom of the dish and break. It is also possible to break the tip on the ganglion sheath. If the tip is dragged carelessly over the sheath it will most likely break. The sheath should be pierced just like the cell. It is a rare to pierce a cell on the first or second try. This process become easier with experience.

After entering the cell it is important to verify that the cell is viable and healthy. There are several parameters from which to judge this. First, the membrane potential should be about -20mV or lower. If the cell's resting potential is higher than this, it could indicate that the cell is unhealthy. It could also indicate that the tip is not in fact inside of a cell. It may simply have pierced the sheath without entering a cell. Second, there may be tonic activity. If the cell is firing action potentials, it is a sign of health. Ideally, the action potentials should have a peak-to-peak voltage of about 15mV and they should have a nice textbook shape to them. At times, the action potentials appear small or smoothed out. This may indicate a deteriorating cell. A third parameter from which to gauge cell vitality is the membrane resistance. The most important thing about the membrane resistance is its stability over time. If the resistance fluctuates then the cell may be undergoing some drastic changes. The membrane resistance is checked by injecting a current of 1nA and noting the change in membrane potential. Typical values for the membrane resistance vary between 10M Ω and 75M Ω . One final, and perhaps the most relevant, characteristic to note is the firing rate of action potentials. If a positive current is injected into the cell the spike rate should increase. If a negative current is injected the spike rate should be suppressed. If no action potentials fire when as much as +2nA is injected, the cell is either unusable or it is an inadequate piercing.

Sometimes a cell may be pierced but it may not be immediately apparent if it is a Retzius cell. Because the Retzius cells are electrically coupled by a gap junction the action potential train may exhibit doublets. This occurs when the firing of one cell sets of the firing of the other cell. It will display on the oscilloscope as two action potentials which occur over a time scale (<3ms) that is too small for both spikes to be from the same cell. One of the spikes will probably have a smaller amplitude than the other because of the physical distance of the electrode from the far cell. If doublets are observed the cell is a Retzius cell.

The initial piercing of a cell can sometimes be detrimental to its health. For this reason the cell may not appear viable at first. It may have a high resting potential and it may not fire any action potentials. However, if given time to rest it may renormalize. The mechanism by which it does this may be a simple resealing of its membrane and resetting of the ion concentrations. Often times a small (> -.5nA) hyperpolarizing current was injected for a few minutes to help the cell reset its ion concentrations. Afterwards, the cell may be perfectly viable. If after a few minutes the cell does not demonstrate the characteristics of a healthy cell it is probably unusable and the other Retzius cell should be attempted.



Difficulties

The dark field condenser is not ideal. Imperfect illumination causes lower contrast between cell bodies and difficulty in visualizing the specimen. Some leeches have more opaque ganglia than others; targeting Retzius cells in opaque ganglia can be difficult. Healthy leeches seem to provide better optical contrast and good spiking. Additionally, the electrode puller did provide precise reproducibility when identical parameters were used. This inconsistency is largely responsible for lost experimental time.

Noise is an ever important issue in data acquisition. Shielding is imperative and can be provided by wrapping grounded foil around the electrode clamps and stands. Grounding the air table also reduces noise. A properly grounded and shielded experimental setup can provide a noise of less than ~2mV. This may be improved with better shielding or a faraday cage.

Results

Frequency response to injected current was observed in voltage clamped Retzius cells. Two consecutive potentials were held for either half or full second intervals and cellular activity was recorded during the second phase. The potentials were established by initial current injection A followed immediately by current injection B. A neuron's "memory" can be observed by running multiple A currents for each B current. Variance in frequency response for the same B current is indicative of cell memory of variable A currents. This memory is not accounted for by the Hodgkin-Huxley model and may have partial dependence on calcium dynamics in the cell. A rest period of five seconds was allowed between each trial to discourage accustoming the cell to injected currents. Additionally, voltage clamping was randomized within a specified range to discourage any memory of previous trials. A single test trial is shown below and clearly indicates frequency response to different injected currents. Each test run contained a randomized sequence of 100 to 150 trials. Data acquisition was implemented through Labview and analyzed with Matlab. Programming was completed by teaching assistants Jeff Gauthier and Dan Hill.



Each sequence of trials is analyzed and plotted in a set of three graphs. The first graph gives frequency as a function of B current (fig. 6). Each line corresponds to a particular baseline A current. Note that there should be no variance between these lines if frequency during B phase was independent of A current. The second graph establishes particular trends in the memory of the first graph (fig. 7). Each point on this graph is an average spike count of the lines in figure 6 (average of frequency across the entire B spectrum). Particular up and down trends in frequency can be monitored through the profile of the second graph. No memory in the cell would result in points with equal value and the joining line would be horizontal. The third graph is a measure of membrane resistance throughout a test sequence (fig. 8). This graph is a good indicator of the cells health and hence the viability of acquired data. An increase in membrane resistance can indicate resealing of the plasma membrane around the puncture wound of the electrode. Conversely, increasing resistance can indicate ion channel deterioration resulting in decreased membrane conductance.



Recollection of past spiking activity in neurons remains a largely unknown process. Likely factors involved in cell memory are Ca^{2+} ion channels and K^+/Ca^{2+} codependent ion channels. In order to test this hypothesis, Ca^{2+} in the extracellular medium is replaced by Ba^{2+} . These ions share electrical properties and exchanging the cell medium minimally changes the cell's electric gradient. However, Ba^{2+} does not share the same chemical properties as Ca^{2+} and elicits a different memory response from the Retzius cell. The relevant graphs for a Retzius cell in barium solution are shown below. A distinctive high frequency spreading is observed in the first graph. Note the decreasing frequency trend for high B current values. This is a recursive phenomenon that has occurred in many of the barium trials. The original Ca^{2+} trend can be recovered by replacing the Ba^{2+} solution with new Ca^{2+} cell medium.



Conclusion

Study of spiking rate as a function of prior potential in the Retzius cell of leech has proven to be a resilient and reproducible assay. Action potential frequency shows definite dependence on previous membrane potential and extracellular Ca^{2+} concentration. This study indicates cellular dynamics involved in memory not accounted for by the Hodgkin-Huxley model. Specific cellular function is beyond the scope of this project; however, repetitive trends in experimental results suggest that further study may illuminate memory dependent processes. Additionally, well established experimental and analysis techniques provide a good structure for future classroom investigation.

References

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Notes for Future Students

All of our analyzed data is shown below subsection per ganglia and by study (e.g. calcium or barium). The images are high resolution and can be enlarged for better viewing Microsoft word.



Cell 1 (6/3/04) Calcium Solution

Cell 1 (6/3/04) Barium Solution







Cell 2 Barium Solution (6/3/04)





















Cell 5 Replaced Calcium Solution (6/8/04)



Cell 5 Replaced Calcium Solution Measured Again (6/8/04)