ARTICLE
Excitable Membranes and Action Potentials in Paramecia: An Analysis of the Electrophysiology of Ciliates

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The ciliate *Paramecium caudatum* possesses an excitable cell membrane whose action potentials (APs) modulate the trajectory of the cell swimming through its freshwater environment. While many stimuli affect the membrane potential and trajectory, students can use current injection and extracellular ionic concentration changes to explore how APs cause reversal of the cell's motion. Students examine these stimuli through intracellular recordings, also gaining insight into the practices of electrophysiology. Paramecium's large size of around 150 µm, simple care, and relative ease to penetrate make them ideal model organisms for undergraduate students' laboratory study. The direct link between behavior and excitable membranes has thought provoking evolutionary implications for the study of paramecia. Recording from the cell, students note a small resting potential around -30 mV, differing from animal resting potentials. By manipulating ion concentrations, APs of the relatively long length of 20-30 ms up to several minutes with depolarizations maxing over 0 mV are observed. Through comparative analysis of membrane potentials and the APs induced by either calcium or barium, students can deduce the causative ions for the APs as well as the mechanisms of paramecium APs. Current injection allows students to calculate quantitative electric characteristics of the membrane. Analysis will follow the literature's conclusion in a V-Gated Ca^{++} influx and depolarization resulting in feedback from intracellular Ca^{++} that inactivates V-Gated Ca^{++} channels and activates Ca-Dependent K^+ channels through a secondary messenger cascade that results in the K^+ efflux and repolarization.

Key words: paramecium; action potential; excitability; ciliates; ionic conductances; paramecium caudatum.

In 1906, Jennings set forth a comprehensive study of paramecium motion, noting the somewhat wave-like forward motion that is interrupted when the paramecium runs into an object, causing it to swim backward (Jennings, 1906). However, as early as 1934 at the birth of nascent electrophysiology, intracellular recordings were measured from paramecia; paramecium remained a popular model organism for the study of ion channels and excitable membranes into the 1960s and 1970s (Naitoh and Eckert, 1968b; Eckert and Brehm, 1979).

Living abundantly in ponds and stagnant freshwater, paramecia are ciliated protists cylindrically shaped but asymmetric between both the anterior and posterior as well as the caudal and dorsal sides (Campbell, 1987). On the caudal surface, an oral groove serves as an entry point for the bacteria it consumes. Paramecia possess two prominent contractile vacuoles that expel excess water from the cell as well as one macronucleus, which controls gene transcription despite the presence of many micronuclei. Their surface is covered in fine hair-like structures called cilia. The cilia beat because of motor proteins powering sliding filaments as well as fluid interactions between all of the cilia (Machemer, 1988b). The cilia cover around 50% of the surface area of the cell and their ion channel composition is different from the somal membrane (Dunlap, 1977; Machemer and Ogura, 1979). In addition, the membrane itself has a second layer of vesicles that provide structural support beneath the membrane, forming a larger conglomerate called the pellicle (Allen, 1988). The cilia's beat pattern causes directionality because of a "power stroke," pushing fluid opposite of the direction of motion. When a paramecium bumps into an object on its anterior side, Ca^{++} mechanoreceptors are activated and the rise in membrane potential from Ca^{++} influx causes a subsequent APs which reverses the power stroke; however, when it is touched from the posterior end, K^+ mechanoreceptors are activated and the membrane hyperpolarizes and the paramecium swims faster forward (Machemer, 1988a). This system represents one of the simplest and oldest evolutions of ionic conductances used to regulate a measurable behavior.

Throughout this series of exercises, students employ *Paramecium caudatum* to observe regenerative Ca^{++} based APs, analogous in some ways to the Na^+/K^+ based APs in human neurons. Students will measure the small resting membrane of the paramecium, and through the addition of Ca^{++} or its ionic analogue Ba^{++}, measure AP characteristics. These APs can be analyzed for amplitude and time duration as well as compared between the two divalent cations. Additionally, students can use current injection into cells paralyzed with Ni^{++} to measure the capacitance and resistance of the cell membrane (See Machemer, 1988a; Greenspan, 2007; Beale and Preer, 2008; and Martinac et al., 2008 for paramecium biology and electrophysiology). The system is simple enough for students to explore various parameters affecting AP generation and explain general models for paramecium electrophysiology but complex enough to challenge and engage students in longer advanced labs.

MATERIALS AND METHODS

Preparation

P. caudatum can be easily purchased from suppliers such as Carolina Biological Supply. They can be kept in a jar or culture dish for several months provided that the temperature is around room temperature (22 ˚C), and they are kept in dim lighting. Adding wheat seeds to the jar feeds the bacteria the paramecia consume, maintaining their food supply. Removing some of the dirtier water with a pipette and replacing it with spring water maintains ion concentrations and keeps the water oxygenated. Proliferating the bacteria and refreshing the water with spring water about weekly maintains conditions that are suitable for the paramecia. For recording, dilute a small volume of the paramecium filled pond water with 0.1 mM $CaCl_2$, 0.2 mM KCl saline solution to form adequate paramecia filled recording solution with the necessary ions and known concentrations (Naitoh and Eckert, 1968b). We recommend placing approximately 0.5 mL of this paramecia filled recording solution onto a petri dish lid. They can be visually confirmed to be alive and moving if viewed under a bright light by eye or by dissecting microscope. Students should observe and record the standard behavior of the cell swimming in the solution before proceeding. We paralyzed the paramecia using 0.6 mL of 1 mM $NiCl_2$ per 1 mL of paramecia filled recording solution, generally 5-8 drops of $NiCl_2$ from a transfer pipette per 0.5 mL of paramecium water (Eckert and Naitoh, 1970). The paramecium should be slow or still enough to impale and record after 20-30 minutes and students should observe and record their behavior.

In the meantime, students should prepare two microelectrodes of 15-80 MΩ of resistance using a glass puller and 3 M KCl. Electrodes with a short, sharp tip and large funnel are easiest to fill and penetrate. Each electrode is connected to an intracellular amplifier, at least one of which must be capable of current injection, as seen in *Figure 1 Left*. Although recording can take place with one electrode fitted with a bridge balance, two electrodes are needed to stably penetrate the cell. The angle of the recording electrodes must be fairly low (≤30˚) in order to more easily penetrate the cell. A chloridated silver wire in the solution is used as a reference ground.

Standard intracellular recording techniques are used while observing the paramecium through an inverted microscope (Nikon Eclipse TS100 Inverted Microscope) or good-quality dissecting microscope with the capability of at least a 40x long working distance objective. (Inverted microscopes work well because of their larger working distances, high optical resolution, and lack of directional inversion.) Electrodes are advanced (Narashige manipulators) until each forms a fairly significant dimple on the paramecium as shown in *Figure 1*. Because there is both a membrane and alveoli to penetrate, mechanical pressure as well as tapping to cause vibrations and oscillating electrical stimulation (our amplifiers are equipped with a tickler or buzzer that provided high amplitude feedback) help to ease the electrodes inside the paramecium. The electrodes do not need to go very far into the paramecium, and the membrane resting voltage may vary slightly but should remain close to -30 mV. A healthy cell will still wiggle, evacuate its vacuoles, maintain an intact cell membrane, and have a resting potential around -30 mV.

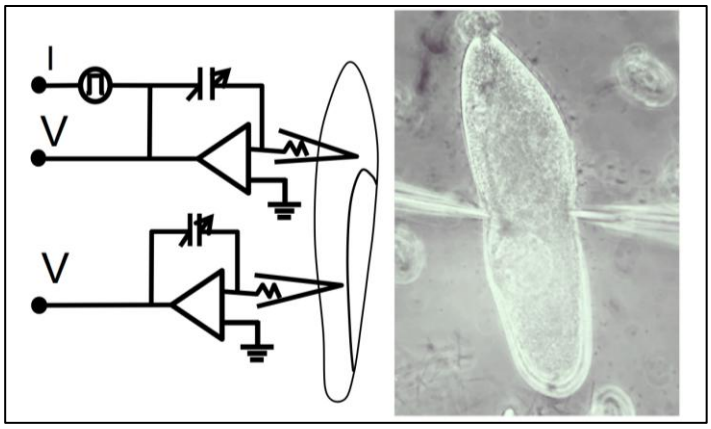

Figure 1. Left. The two electrodes both use measured voltage to confirm the electrode is in the cell, but one retains the ability to inject current. Note that while they are shown on the same side, in reality they must be opposite to anchor the entry of the opposite electrode. *Right*. The electrodes are slightly out of focus at the tips because they are slightly above the paramecium's midsection. The electrodes are not approaching either the vacuoles or the oral groove because these are more challenging and harder three-dimensional shapes.

Recording Membrane Voltage and Action Potentials

Students should record the membrane voltage in order to discuss the possible ion conductances and measure passive electrical properties. The Ni^{++} treatment blocks V-Gated Ca^{++} channels, so current injection will not initiate action potentials. However, students should try current injections of 3-15 nA to find the passive electrical properties of each paramecium tested.

Without current injection to cause action potentials, students can raise the extracellular Ca^{++} concentration to compete with the Ni^{++}, which is reversibly bound to V-Gated Ca^{++} channels, as well as to slowly raise the membrane voltage. In order to keep track of the number and timing of drops, we recommend inserting a chloridated silver wire into the dropper used for the 10 mM $CaCl_2$. Attaching the wire to a 330 Ω resistor, ground in the solution, and AA battery, a complete circuit should form when a drop of Ca^{++} solution is lowered onto the recording solution surface. This produces a noticeable electrical artifact to determine the number and timing of Ca^{++} drops. Students can determine at what membrane voltage and Ca^{++} concentration action potentials begin to be induced. Action potentials will spontaneously occur after raising Ca^{++} concentration. Students should record for long periods of time, 2-5 minutes, to record action potentials (Naitoh and Eckert, 1968a). We had a Labview program developed for recording.

In another paramecium bath, students can use the same procedure as above with the calcium analogue barium to cause action potentials. After recording passive

properties of the cell with current injection, 1 mM $BaCl_2$ solution is used to increase the Ba^{++} concentration in the solution and cause spontaneous action potentials (Naitoh and Eckert, 1968b).

RESULTS

Current Injection

Using a Ni^{++} block, students will observe that they cannot initiate action potentials by simply depolarizing the membrane voltage. A typical current injection is shown in *Figure 2*. Using 3 nA of current injection, students will find depolarizations of ≥40 mV. From the height of the steady state plateau, students can calculate the resistance of the cell membrane. Then using Excel, students fit the repolarization curve and find the time constant τ in an exponential decay. This allows students to come up with a data set to find the resistance and capacitance of the cell membrane. Our results agree with the literature of resistances on the scale of >10 MΩ and capacitances of the scale of <1 nF (Machemer and Ogura, 1979).

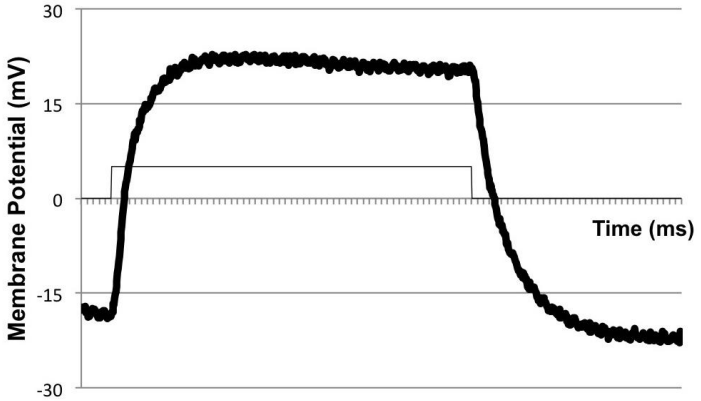

Figure 2. 3 nA of current was injected for 600 ms, represented by the grey line forming the square pulse. The membrane voltage rose and fell as expected for voltage gated channels to be blocked. For this trial, we calculated the steady state ΔV=43 mV and therefore the resistance of the membrane would be 14 MΩ. Fitting the exponential curve resulted in a capacitance calculated at 970 pF.

Students should be able to deduce the Ohmic relationship for the charging lipid membrane acting as a capacitor while ion channels act as non-zero resisting conductors at steady state. Using Ohms law as an approximation in the steady state because of the constant voltage difference and constant current input, $\Delta V_m = I*R_{input}$ describes the plateau, where R_{input} is the total input resistance dominated by $R_{membrane}$. This allows students to easily calculate the resistance simplified over "long time" at the plateau of the injection curve.

Following the calculation of the resistance, fitting of the discharge curve $V_d(t) = V_0 \cdot e^{-t/\tau}$ will produce an experimental value for τ. Since $\tau = R_{input}*C_{input}$, students can collect data for resistances and capacitances of many cells and form an average data set.

Students use this data from paramecia to compare their results to known data of the membranes of paramecia. Additionally, students can compare their results to known data from other animals, plants, and neuronal membrane characteristics as well as considering the implications of cilia on the resistance and capacitance.

Extracellular Ionic Changes

Changing the concentrations of the extracellular solution will allow students to explore paramecia's action potential mechanism, different from the neuronal model but still similar to SA Node and smooth muscle APs. Slowly raising the calcium concentration in the solution will cause Ca^{++} to leak into the cell. This allows it to both compete with Ni^{++} for binding sites in the V-Gated Ca^{++} channel but also to raise the resting membrane voltage. Together this will allow induced but spontaneous action potentials (Naitoh and Eckert, 1968a). As shown in *Figure 3*, the increased Ca^{++} concentration, even with a Ni^{++} block present, will cause spontaneous action potentials with depolarizations to around +5 mV, or ΔV of about 35 mV. They are approximately 25 ms in duration for the spike followed by a much longer repolarization of around 300 ms. These results match the data from the literature (Machemer, 1988a).

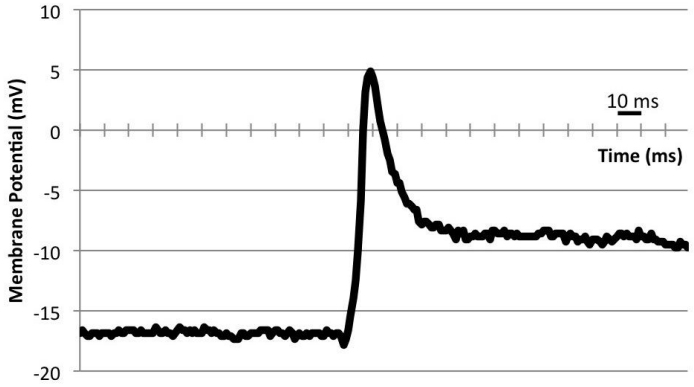

Figure 3. Ca^{++} induced action potential. Since the action potential was spontaneous, the elevated resting membrane potential is shown as the flat signal at -17 mV prior to the spike. Then, action potentials will have a steep depolarization of 20-25 mV followed by a wide spike, in this case 25 ms. There is a lengthy repolarization period of >100 ms.

Adding barium to the extracellular solution will however cause a much different action potential. Increasing the barium concentration of the solution causes a depolarization and repolarization, but over the course of seconds and minutes. As shown in *Figure 4*, depolarizations will peak at approximately the same height as Ca^{++} depolarizations but the width of the spike is hard to differentiate from the repolarization. Instead the overall repolarization will take between 10s and several minutes depending on the concentration of barium. Also similar to the Ca^{++} AP, the membrane potential at which both divalent cations cause Ni^{++} blocked paramecium to have spontaneous action potentials occurs at values between -15 and -20 mV, higher than expected for current induced APs (Naitoh and Eckert, 1968b; Machemer, 1988a).

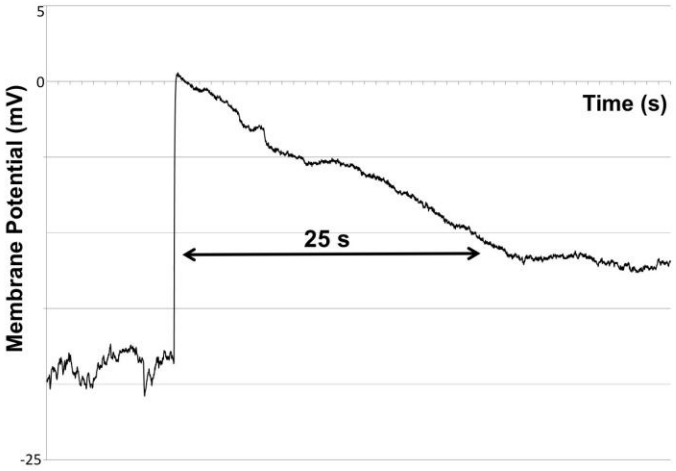

Figure 4. Ba^{++} induced action potential. These action potentials are also spontaneous and fluctuations in the membrane voltage can be seen prior to the spike. The sharp increase is about 20 mV, similar to the Ca^{++} AP. Barium action potentials can last for several minutes but still retain similar changes in voltage from similar resting potentials as the calcium induced action potentials.

DISCUSSION

Paramecium demonstrates ionic conductances that directly control movement. The ability to both see live cells perform the actions as well as be able to induce that electrical activity makes paramecia an effective teaching tool. Paramecium signaling encompasses only the one cell and is large enough for inexperienced electrophysiologists to develop the techniques to penetrate and record from cells. It possesses a balance of both the practical challenges of electrophysiological experimentation as well as being easy enough for entry level experimentation that demonstrate a number of factors of evolutionarily derived excitable membrane characteristics common to ciliates, plants, and animals: ion gradients, passive ion channels, and a variety of active or facilitated ion channels. Considering the common evolution of these membrane characteristics, paramecium electrophysiology provides an example from an organism that is neither a plant or animal but with them shares vital electrical properties. The experiment supplements generally studied animal models so that students can understand and form conclusions about the process of their action potentials through their own observations with the greater context of the commonality and age of membrane excitability. This balance allows paramecium experimentation to provide experimentation for a wide range of student abilities and scale of experiments. Additionally, the low cost, little maintenance, and ease of recording make paramecia ideal for a teaching laboratory.

In many ways, recording membrane voltages and calculating membrane characteristics following current injection provide the basic skills and techniques for intracellular recording to undergraduates. Although difficult for beginners, it is well within the capacity of undergraduates to learn the process within a few lab sessions and come out of the experiment understanding more about microscopy, electronic recording equipment, and the process of penetrating cells. Alongside these valuable skills, measuring total capacitances and input resistances allows students to directly compare neurons and paramecia, noting that differences in shape, length, and size all affect the input resistance and total capacitance as well as the increase in surface area and segregation of ion channels due to cilia.

Indeed the cell is reliant on the spatial segregation of ion channels to function as an excitable membrane, as with neurons. The ability of the cell to produce action potentials leads students to conclude there must be some form of voltage-gated ion channel. The failure of current injection to induce action potentials but the success of divalent cations demonstrates to students that their immobilization of the paramecia inhibits the mechanism for direct action potential formation when only the membrane potential rises. Students will see that the action of Ca^{++} directly results in the formation of action potentials. When comparing to Ba^{++} action potential, students will see, while the depolarization is similar, the repolarization differs drastically (Naitoh et al., 1972). This exhibits evidence for two known mechanisms for calcium involvement: the blockage of Ca^{++} ion channels because of the failure of a spike followed by a fast repolarization and the activation of a secondary ion via Ca^{++} because of the slow long repolarization (Machemer, 1988a).

Whereas the Ca^{++} action potential has a swift repolarization as Ca^{++} deactivates V-Gated Ca^{++} channels and activates Ca^{++}–Gated K^{+} channels, the Ba^{++} action potential remains elevated. Furthermore, the Ca^{++} action potential returns to resting membrane potential after a few hundred milliseconds whereas the Ba^{++} takes up to several minutes. This suggests that Ca^{++} controls a mechanism to activate the polarizing ion, in this case K^{+}, because Ba^{++} fails to induce a swift repolarization. The long time scale for the repolarization also suggests that the repolarization mechanism is not voltage gated, or is at least very slow acting. This fits with the known model of secondary messenger activation of K^{+} efflux (Machemer, 1988a). This analysis allows students to explore and possibly use their data to make conclusions about the nature of paramecium action potentials with minimal literature aid.

Additionally, paramecium's similar ionic channels and mechanisms to neurons make them an excellent example of the evolution of excitable membranes. Inherently, as a divergence prior to plants and animals, paramecium's excitable membrane demonstrates that ionic gradients and channels are common to all plants and animals. In this experimental preparation, paramecium are "swimming neurons" with their own spatial distribution of multiple ion channels and controlled gradient changes to modulate behavior. Paramecia demonstrate some of the oldest evolutionarily derived common membrane and ion channel features of excitable cell membranes (See Greenspan, 2007 Chapter 1; Hille, 2001 pages 12-13, 664-665, and Chapter 22 for information on paramecia electrical evolution).

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