Video Article

Whole-cell Patch-clamp Recordings of Isolated Primary Epithelial Cells from the Epididymis

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Abstract

The epididymis is an essential organ for sperm maturation and reproductive health. The epididymal epithelium consists of intricately connected cell types that are distinct not only in molecular and morphological features but also in physiological properties. These differences reflect their diverse functions, which together establish the necessary microenvironment for the post-testicular sperm development in the epididymal lumen. The understanding of the biophysical properties of the epididymal epithelial cells is critical for revealing their functions in sperm and reproductive health, under both physiological and pathophysiological conditions. While their functional properties have yet to be fully elucidated, the epididymal epithelial cells can be studied using the patch-clamp technique, a tool for measuring the cellular events and the membrane properties of single cells. Here, we describe the methods of cell isolation and whole-cell patch-clamp recording to measure the electrical properties of primary dissociated epithelial cells from the rat cauda epididymides.

Video Link

The video component of this article can be found at https://www.jove.com/video/55700/

Introduction

The epididymis in the male reproductive tract is an organ lined with a layer of mosaic epithelial cells. As in other epithelial tissues, the various cell types of the epididymal epithelium, including principal cells, clear cells, basal cells and cells from the immunological and lymphatic systems, work in a concerted manner to function as the barrier at the tubule frontline and as the supporting cells for sperm maturation and physiology[1,2,3]. Thus, these epithelial cells play an essential role in reproductive health.

Epithelial cells are generally regarded as non-excitable cells that are unable to generate all-or-none action potentials in response to depolarizing stimuli, due to a lack of voltage-gated Na^+ or Ca^{2+} channels[4,5]. However, epithelial cells express unique sets of ion channels and transporters that regulate their specialized physiological roles, such as secretion and nutrient transportation[6]. Different epithelial cells therefore possess characteristic electrical properties. For example, the principal cells express the CFTR for fluid and chloride transportation and express the TRPV6 for calcium reabsorption, whereas the clear cells express the proton pump V-ATPase for luminal acidification[1,7,8,9]. Some transporters and ion channels that regulate the physiological features of the epididymal epithelial cells have been reported, but the functional properties of epididymal epithelial cells are largely not yet understood[10,11,12,13].

Whole-cell patch-clamp recording is a well-established technique for examining the intrinsic properties of both excitable and non-excitable cells, and is particularly helpful for studying the functions of primarily dissociated cells in heterogeneous cell samples; the voltage-clamp is used for measuring the passive membrane properties and the ionic currents of single cells[14,15]. The passive membrane properties include input resistance and capacitance. The former parameter indicates the intrinsic membrane conductance, while the latter implies the surface area of the cell membrane (a phospholipid bilayer, where ion channels and transporters are located, that serves as a thin insulator separating extracellular and intracellular media). The membrane capacitance is directly proportional to the cell membrane's surface area. Together with the membrane resistance that is reflected by the input resistance, the membrane time constant, which indicates how fast the cell membrane potential responds to the flow of ion channel currents, can be determined. In this regard, by combining the current response characteristics from a series of voltage steps applied to the cells, the biophysical kinetics and properties of the cells are determined[15,16,17,18].

In the present paper, we describe the procedures for isolating epithelial cells from the rat cauda epididymis and the steps for measuring the membrane properties of different cell types in the dissociated cell mixture using the whole-cell patch-clamp. We show that the epididymal principal cells exhibit distinct membrane electrophysiological properties and that the conductances can be readily identified from other cell types.

Protocol

All animal experiments are carried out in accordance with the guidelines of the ShanghaiTech University Institutional Animal Care and Use Committee, which fulfill the local and international requirements.

1. Experimental Animals

1. Use adult male Sprague-Dawley rats (~300-450 g) between 8-12 weeks old. At this age in the rats, the sperm have arrived in the cauda epididymides.

2. Isolation of Epithelial Cells from Rat Cauda Epididymides

NOTE: The following steps are performed under non-aseptic conditions unless otherwise stated.

1. **Preparation of dissection instruments and reagents**
    1. Disinfect the dissection tools by immersion in 70% ethanol and let them air dry.
    2. Turn on the warming bath (32 °C); Prepare and pre-warm 500 mL of 1x Roswell Park Memorial Institute 1640 Medium (RPMI) supplemented with 1% (v/v) antibiotics (100 U/mL penicillin and 100 µg/mL streptomycin final), and labeled as "RPMI (+P/S 1:100)". Perform this step in a clean air-flow controlled working station.
    3. Prepare and pre-warm 1x 500 mL Iscove's Modified Dulbecco's Medium (IMDM) containing non-essential amino acids (0.1 mM) and sodium pyruvate (1 mM), and supplemented with 5-α-dihydrotestosterone (1 nM), 10% fetal bovine serum, 1% (v/v) antibiotics (100 U/mL penicillin and 100 µg/mL streptomycin final), and labeled as "Full-IMDM". Create 50 mL aliquots and seal with parafilm; store at 4 °C. Use aseptic conditions.
    4. Prepare a collagenase enzyme digestion solution by dissolving collagenase Type I and collagenase Type II in RPMI (+P/S 1:100) resulting in 1 mg/mL of each collagenase in the solution. Filter through a 0.22-µm membrane and mark as "Collagenase Solution". Keep at room temperature (RT) until use. Adjust the volume of the enzyme solution based on the weight of the enzyme; the minimal volume required for both cauda epididymides from a single rat is 2 mL.
    5. Fill a 35 mm dish with RPMI (+P/S 1:100).

2. **Dissection of rat cauda epididymides**
    1. Sacrifice the animal by either using sodium pentobarbital 85 mg/kg i.p. or using an isoflurane chamber until the animal does not respond to tail-pinching stimulation; follow by cervical dislocation.
    2. Disinfect the lower abdomen by wiping with 70% ethanol, gently push the two testes down to the lower abdomen, and then open the lower abdomen near the scrotum.
    3. Pick up the epididymal fat, dissect out the whole reproductive organs (testes, epididymides, and vas deferens) and immerse in the dish with RPMI (+P/S 1:100).
    4. Transfer the reproductive organs in the dish with RPMI (+P/S 1:100) to an aseptic working station.
    5. Dissect out the cauda epididymides from the connective and fatty tissues and the epididymal capsule. Place one epididymis with ~0.2 mL of Collagenase Solution in a 1.5 mL tube.Perform this step in a clean air-flow controlled working station.

3. **Dissociation of single cells from rat cauda epididymides**
    1. Cut the epididymides in the Collagenase Solution using fine scissors until the tissue becomes a paste-like fluid. Rinse the scissors gently with the rest of (~0.8 mL) enzyme solution in the 1.5 mL tube.
    2. Place the tube on a metal thermomixer for 30 min at 37 °C with a shaking speed of 1,000 rpm.
    3. Centrifuge the enzyme-tissue mixture at 30 x g at RT for 3 min and decant the sticky supernatant that contains mostly the sperm.
    4. Resuspend the pellet in 1 mL Full-IMDM to quench all the enzymatic activity. Transfer the cell suspension to a 50 mL tube containing 49 mL RPMI (+P/S 1:100).
       NOTE: Optionally, filter the cell suspension through a 100 µm mesh membrane with constant trituration to avoid large, cell aggregates. However, do not use a mesh filter if cell suspension is needed for growing cell monolayers.
    5. Centrifuge the cellular mixture at 30 x g at RT for 10 min; decant the supernatant.
    6. Resuspend the pellet in 1 mL Full-IMDM with gentle trituration for at least 5 min to dissociate single cells from the enzymatic treated epididymal tissue mixtures.

4. **Separation of epithelial cells from other cells under aseptic conditions**
    1. Culture the cell suspension on a 10 cm Petri dish containing Full-IMDM for at least 8 h or overnight, in an incubator at 32 °C in 5% CO_2.
    2. Prepare sterile coverslips in advance by immersion in 100% alcohol. Air dry and dip in a small volume of the culture medium. Place the coverslips in 6 cm culture dishes or in single wells of a 24-well plate.
    3. Next morning, harvest the dissociated epithelial cells by gently collecting the cell suspension from the Petri dish, which consists mostly of epithelial cells. Centrifuge the cell suspension at 30 x g at RT for 5 min, and then decant the supernatant.
    4. Resuspend the cell pellet in ~2 mL Full-IMDM.
    5. Seed 0.2 mL of the harvested cell suspension onto the center of each sterile coverslip.
    6. Let the cell suspension settle in the liquid droplet for at least 10 min to allow cells to loosely adhere to the glass coverslips. Carefully add 1 mL Full-IMDM at the edge of 10 cm dish, or 0.3 mL Full-IMDM to each well of a 24-well plate; do not disturb cells.
    7. Keep the isolated single cells on coverslips in the incubator at 32 °C in 5% CO_2 until the patch-clamp experiments.

3. Recording Solutions and Micropipettes

NOTE: For the patch-clamp experiments, use the best quality chemicals and solutions.

1. **Preparation of stock solutions**
    1. Autoclave all the bottles for stock storage and filter all the stock solutions (except the corrosive solutions) and filter through 0.22-µm membranes before use.
    2. Prepare all stock solutions in advance at RT, and store at 4 ^{o}C: 5 M NaCl; 1 M KCl; 100 mM $MgCl_2$; 100 mM $CaCl_2$; 200 mM NaH_2PO_4; 100 mM EGTA (pH 7.0 with KOH). Handle 5 M NaOH, 1 M HCl and 1 M KOH stocks as corrosive solutions.

2. **Preparation of standard external recording physiological salt solution (PSS)**
    1. Warm the stock solutions to RT on the morning of the patch-clamp recording.
    2. Pipette the ingredients from each stock according to the desired final volume, except $CaCl_2$, *e.g.* for preparing 500 mL of PSS: 140 mM NaCl = 14 mL of 5M; 5 mM KCl = 2.5 mL of 1M; 1.2 mM $MgCl_2$ = 6 mL of 100 mM; 1.2 mM NaH_2PO_4 = 3 mL of 200 mM.
    3. Add double-distilled water (ddH_2O) to the final volume of 400 mL and equilibrate.
    4. Weigh 0.9 g glucose and 1.19 g HEPES and dissolve completely in the solution mixture.
    5. Add the $CaCl_2$ stock (2.5 mM = 12.5 mL of 100 mM) with stirring.
    6. Add up to 99% of final volume.
    7. Adjust the pH to 7.4 using NaOH or HCl.
    8. Check the osmolarity and adjust using 5 M NaCl or glucose, if necessary.
    9. Add ddH_2O to the final volume of 500 mL in a cylinder.

3. **Preparation of micropipette internal solutions (low EGTA K^+ -based solutions)**
    1. Weigh or pipette the correct volume of the reagents from each stock according to the desired final volume and concentration, *e.g.* for preparing 50 mL low EGTA K^+-based intracellular solution to a volume of ~ 30 mL ddH_2O: 100 mM K-gluconate = 1.17 g; 35 mM KCl = 1.75 mL of 1 M; 2 mM $MgCl_2$ = 1 mL of 100 mM; 0.1 mM EGTA = 0.05 mL of 100 mM; 10 mM HEPES = 0.072 g.
    2. Add enough water for ~ 95% of final volume and allow the solution to equilibrate at RT. Make sure that the solution is clear.
    3. While constantly stirring the solution, adjust the pH to 7.2 using KOH.
    4. Weigh and add 0.078 g Mg-ATP to the solution until it is dissolved completely.
    5. Place the solution on ice and use a small aliquot for the measurement of osmolarity; typically, the solutions measures ~290 mOsmol and does not need adjustment. If the osmolarity differs significantly from 280-295 mOsmol, prepare a new solution.
    6. Add ddH_2O to final volume.
    7. Divide the solution into 500 µL aliquots, filter with a 0.2 µm syringe filter, tightly seal and immediately store at ≤ -20 °C.
    8. On the date of the patch-clamp experiment, thaw one aliquot of intracellular solution on ice and keep chilled during the patch-clamp experiment to prevent degradation.

4. Pull the patch pipettes from glass capillaries (following pipette puller user's manual) to obtain micropipette sizes with resistance of 5-10 M when filled with intracellular solution.

4. Setting up the Patch-Clamp Experiment and Establishing Whole-Cell Configuration with Cells

1. **Setting up the patch-clamp experiment**
    1. Turn on the patch-clamp set up (computer, computer-controlled amplifier, digitizer, *etc.*)
    2. Open the patch-clamp software (*e.g.* AXON pCLAMP10 or HEKA PatchMaster) and set up the protocols for electrophysiological recordings. Set the filter for the signal to low pass at 1-3 kHz and the digitizer at 10-20 kHz.
    3. Turn on the camera, micromanipulator and light source.
    4. When the computer-controlled amplifier is on, ground the body of experimenter by touching with hands the patch-clamp rig that has been grounded, before touching the headstage, in order to protect it from electrical shocks.
    5. Transfer the culturing epithelial cells on the glass coverslip to the recording chamber filled with ~1 mL of standard PSS at RT. Carefully change the bathing PSS at least two times using a pipette before any patch-clamp experiments.
    NOTE: Optionally, fill the perfusion system with standard PSS or another external solution according to the planned experiment. Perfuse the microscope-mounted recording chamber (RC-26G or RC-26GLP) with PSS a few times at a speed of ~2 mL/min before the start off the patch-clamp experiments. Make sure that there are no air bubbles trapped along the perfusion system.
    6. View and select the cells under an inverted microscope using 10X and 40X objectives equipped with a differential interference contrast optical system. Look for large single isolated cells for recording. Identify the isolated epididymal epithelial cells by their spherical shape with rough microvilli on one end of the membranes and polarized distribution of intracellular contents (**Figure 1**).
    7. Using a 1 mL syringe (a home-made nonmetallic microsyringe needle), fill a micropipette with the internal solution (low EGTA K^+-based solution, see step 3.3). Make sure that there are no air bubbles in the micropipette, which can increase the resistance of the micropipette. Use enough solution so that the internal solution submerges the chloride-coated silver wire electrode within the micropipette holder.
    8. Mount the micropipette in the electrode holder, and apply a low positive pressure (~0.2 mL syringe volume). Keep the low positive pressure continuous until touching the cell membrane in the later steps.

2. **Establishing whole-cell configuration with cells for recordings**
    1. Immerse the pipette into the bath solution at the highest speed of the micromanipulator. Find the pipette tip in the screen connected to the digital camera; slow down the micromanipulator speed to the medium-high mode.

2. Quickly check the micropipette resistance (5-10 MΩ) using the data acquisition interface command (*e.g.* "Membrane Test" in the AXON system) by applying a voltage step (*e.g.* 5 mV for 100 ms) generated from the computer-controlled amplifier. Change to a new micropipette if the resistance is significantly out of this range.
3. Start to move down the objective mounted on the microscope; gradually guide the micropipette toward the selected cell. Always lower the objective first, and then lower the micropipette to the plane of focus, untilthe micropipette is above the center surface of the selected cell.
4. Cancel the liquid junction potential between the pipette and bath solutions to zero using the "pipette offset" command in the commander interface of software.
5. Set the computer-controlled amplifier commander to the voltage-clamp and the membrane test to the "Bath" mode.
6. Fine focus for a clearer view of the cell, then gradually lower the micropipette using the micromanipulator at the low-medium speed.
7. When the micropipette is close to the cell (demonstrated by a decreased current when triggered by the membrane test command), remove the low positive pressure immediately and apply a weak negative pressure (0.1 mL syringe volume) to form the gigaseal (>1 GΩ).
8. Monitor the resistance with the membrane test. If the resistance is >500 MΩ but <1 GΩ, apply a negative potential (usually as the holding potential which is set to -60 mV), which can help form the gigaseal. Compensate the transient capacitive current of the micropipette.
9. If the seal is >1 GΩ and stable (as shown in the software interface), apply a brief and strong suction in order to break the cell membrane. Do not apply compensation for the series resistance and the cell capacitance.
10. Immediately after achieving a successful whole-cell configuration, apply a 10 mV hyperpolarizing step (5-traces with minimal time intervals, 20 ms duration, signal sample at 20 kHz) from a holding potential of -60 mV.
11. Switch the voltage-mode to the zero-current mode and mark down the readings from the software interface or perform a gap-free recording (10-60 s) for the membrane potential readings of the cell.
12. Quickly switch back to and remain in the voltage-mode and apply the voltage protocols according to the planned experiments and measure the current responses. Subtraction is not applied to the intrinsic leak current during the recordings.
13. Monitor the stability of the responses during the recordings, or the different parameters of the cell. For example, use the "Membrane Test" command interface to check the input resistance (R_i), the series resistance (R_s) and the cell membrane capacitance (C_m) in the membrane test at the "Cell" mode during the switch of protocols.
    NOTE: Sudden drops in input resistance is usually indicative of a loose patch, and a dramatic increase in the series resistance may be indicative of micropipette tip clogging by the intracellular organelles or membrane fragments. During recording, regularly monitor the micropipette location to check if drift occurs, which may lead to the loss of the patch. If drift is a problem, carefully lift together the micropipette and the patching cell away from the bottom of recording chamber. This step may sometimes lead to the loss of the patch, in which case, it is necessary to repeat the whole-cell patch-clamp procedure.

5. Analysis of Passive Electrophysiological Properties of Cells

1. After obtaining the data from the patch-clamp experiments, open the gap-free data in the Clampfit software and measure the mean value for the resting membrane potential for each cell. Alternatively, use the value as marked down from the zero-current voltage at the current mode. Correct the values with the liquid junction potential (12.4 mV in this study).
2. Open the data from the 10-mV hyperpolarizing step (ΔV) obtained from a cell, measure the current difference before and during the step (ΔI_{step}), and calculate the input resistance (R_i) using the equation as:

$$R_i = \frac{\Delta V}{\Delta I_{step}}$$

3. Calculate the cell capacitance (C_m, in unit of pF) (use the same current data from the 10-mV hyperpolarizing step by integrating the total area under the membrane capacitor current during the initial transient decay current raised upon the voltage step trigger), to obtain the value of the total accumulated charge (Q, in unit of pA•ms) of the cell. Use the following equation:

$$C_m = \frac{Q}{\Delta V}$$

4. Calculate the value of the series resistance (R_s) for each cell by fitting the initial transient current from the 10-mV negative step with a standard exponential algorithm to obtain the time constant decay current (*T*, in unit of ms) and use the following equation:

$$R_s = \frac{\tau}{C_m}$$

Representative Results

The described enzymatic digestion procedure for the isolation of epithelial cells from the rat cauda epididymides is a modified protocol from our previous studies[9,12]. This method produces a mixture of single cells with over 90% viability and without surface blisters or swollen cell volume. The heterogeneous cell mixture consists mainly of principal cells, clear cells and basal cells, as we have described previously[1]. In this protocol, relatively pure samples of epididymal epithelial cells can be obtained by culturing the primary dissociated cells overnight on a Petri dish to allow for the adhesion of the non-epithelial cells (such as, fibroblasts and smooth cells) on the dish, as demonstrated in **Figure 1A** (before harvest). The cell types can then be preliminarily distinguished by their phenotypes under the microscope and categorized according to their specific passive membrane properties. In the dissociated cell mixture, there is a group of cells that have microvilli or a rough membrane on one end of their surface membrane, and polarized cellular contents; these are termed the principal cells or the clear-like cells (**Figure 1B**, arrows). These polarized epithelial cells are indistinguishable by their morphological features but can be identified according to their passive membrane properties and conductance responses obtained from the whole-cell patch-clamp recordings. The other group of cells is smaller in size with no stereocilia on one end of the membrane and no obvious polarized cellular contents; these are termed the no-microvilli cells, and consist of mostly basal cells (**Figure 1B**, asterisks).

The primary epithelial principal cells can be distinguished from other cells by their distinct passive membrane properties (**Figure 2**) and current patterns (**Figure 3**) in response to the applied voltage protocol using the whole-cell patch-clamp technique. The passive membrane electrophysiological properties of cells can help to assess the initial health status of individual cells and of cell type groups. A summary of the individual dot plots of the membrane capacitance (C_m), the input resistance (R_m) and the membrane potential (V_m) of each cell group is given in **Figure 2A**. There is no difference in the time constant for the membrane capacitance amongst the different cell types (~0.4 ms) (data are not demonstrated in this manuscript). Using the low EGTA K^+-based solution, the average measured membrane capacitance for the principal cells is 9.4 0.5 pF (n = 32) and for the clear-like cells is 9.7 1.9 pF (n = 12). The membrane capacitance of the no-microvilli cells is 5.2 0.8 pF (n = 17), which is statistically smaller than the principal and the clear-like cells membrane capacitances.

The input resistance of principal cells in **Figure 2A** (middle panel) is 1.1 ± 0.2 GΩ (n = 32) and that of the clear cells is 2.2 ± 0.8 GΩ (n = 12), which is significantly lower (P <0.001) than that of the no-microvilli cells (8.9 ± 2.1 GΩ; n = 17). As depicted in **Figure 2A** (right panel), the zero-current membrane potential (*i.e.* the resting membrane potential V_m) was measured shortly after establishing the whole-cell configuration and used for comparing the groups after the correction with liquid junction potential (12.4 mV). Cells were routinely bathed in standard PSS and dialyzed with 0.1 mM EGTA K^+-based pipette solution containing ATP. The mean membrane potential of the principal cells is -26 ± 2 (n = 28), between -51 mV and +1 mV, and the mean membrane potential of the clear-like cells is -30 ± 3 mV (n = 10), between-47 mV and -17 mV. The no-microvilli cells possess the highest mean membrane potential with a value of -33 ± 5 mV (n = 17), between -63 mV and -13 mV.

The low input resistance of the principal cells suggests that there are intrinsic conductances in these cells, but it could indicate leaking of the seal between the pipette and the patch cell membranes. To address this concern, we first excluded the data from those cells with sudden changes (indicative of a seal leak) of the electrical properties during recordings. Then we analyzed the correlation between the sealing resistance at a threshold of 1 giga-ohm with the input resistance, as demonstrated in **Figure 2B**. The result was a very low correlation value ($R^2 \approx 0.02$). This suggests that the sealing resistance has negligible influence on the input resistance, and that the reported passive electrical properties of the various cells (such as the low input resistance of the principal cells) are the intrinsic properties of these cells.

Figure 3A is the typical current response recorded from the principal cells under quasi-physiological conditions with the cells bathing in normal physiological salt solution (PSS), dialyzed with a low EGTA K-based pipette solution and stimulated from a holding potential of -60 mV to a series of 500 ms testing voltages (ranging from -120 mV to +60 mV as indicated in the insert of **Figure 3B**). The two tracings in **Figure 3C** show the typical resting membrane responses of an identified principal cell under the zero-current clamp in the presence of, or absence of ATP in the pipette solution. In the presence of ATP, the resting potential is relatively stable. Whereas a progressive hyperpolarization was observed when cells were dialyzed with a pipette solution without ATP. The initial resting membrane potential measured at the beginning of the cell dialysis with pipette solutions shows no significant difference in the cells with or without ATP (**Figure 3D**), suggesting that the measured values were still intact and minimally influenced by the pipette solutions.

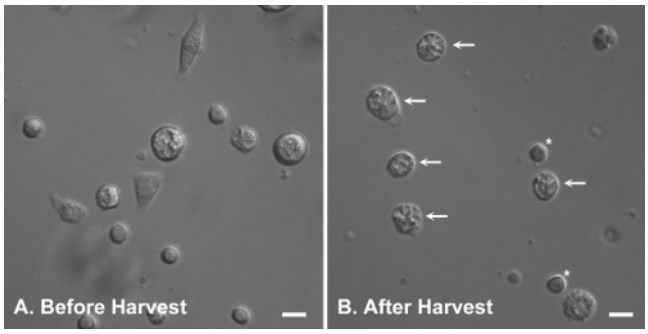

Figure 1: Morphological features of isolated rat cauda epididymal epithelial cells. (**A, B**) Examples of the epithelial cells isolated from the rat cauda epididymides before (**A**) and after (**B**) re-harvest of overnight culture on the dish prior to the patch-clamp experiments. Arrows indicate the microvilli-cells, which mainly consist of the principal cells and the clear-like cells. Asterisks indicate the no-microvilli cells. Only single cells were selected for patch-clamp recording. Scale bar = 10 µm. Please click here to view a larger version of this figure.

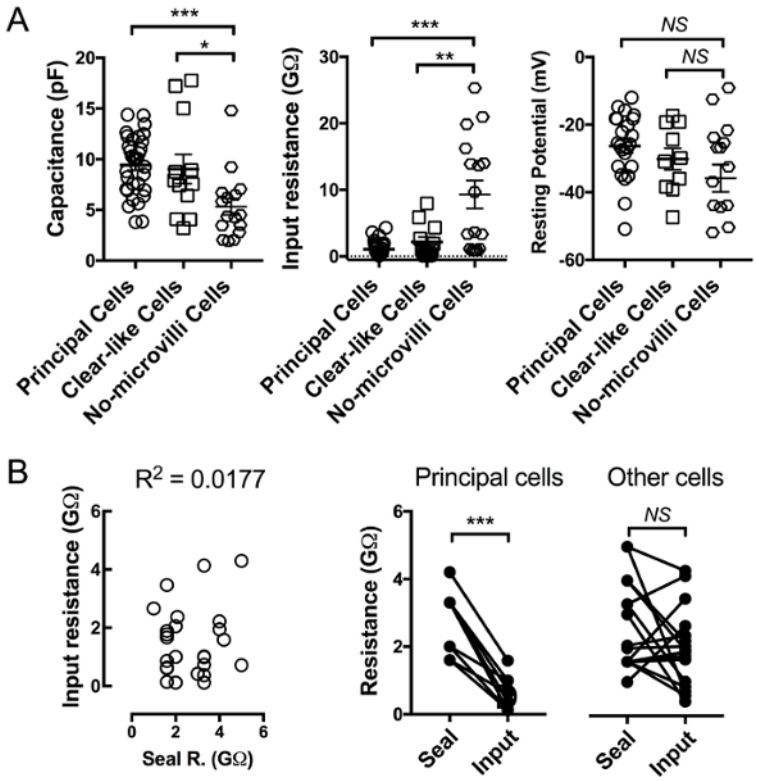

Figure 2: Passive membrane properties of single rat cauda epididymal epithelial cells. (**A**) Dot plots of the passive membrane properties of single rat cauda epididymal epithelial cells. Pipette solution is low EGTA K^+-based and bathing solution is standard PSS. (**B**) Correlation analysis of the input resistance with the seal resistance of the cells. *NS*: no significant difference, * P <0.05, ** P <0.01 *** P <0.001 significant difference vs. appropriate controls using one-way ANOVA with Bonferroni post-hoc test. Please click here to view a larger version of this figure.

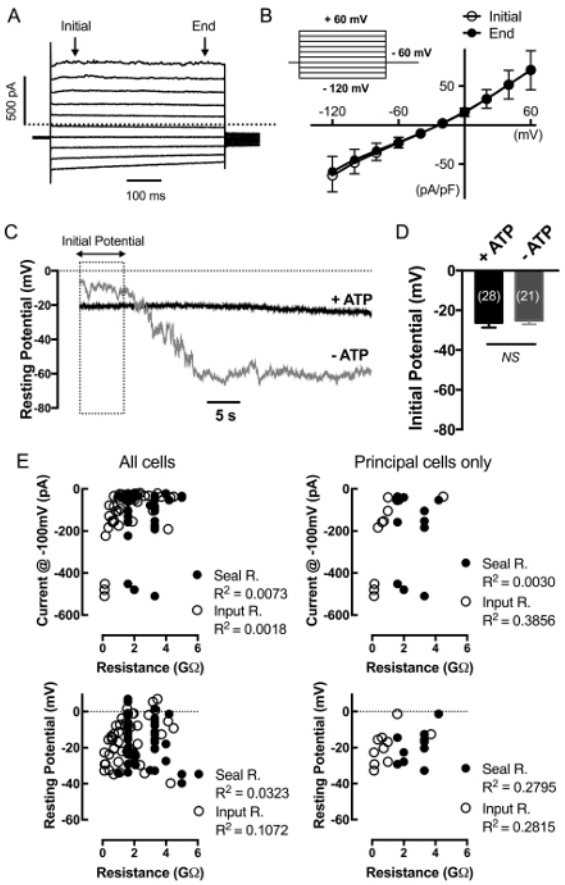

Figure 3: Typical whole-cell currents in single epididymal principal cells. (**A**) Typical whole-cell currents recorded from single epididymal epithelial cells recorded under quasi-physiological conditions using a pulse-eliciting protocol as shown in the inset of panal B. The dotted line indicated the zero current levels. (**B**) The current-voltage relationship of the current responses measured at the indicated time points as in A. Data are the means ± SEM of eight principal cells from at least three animals. (**C**) Representative tracings of the resting membrane potential of the principal cells dialyzed with a pipette solution, either with ATP (+ATP) or without ATP (-ATP). (**D**) A bar graph showing no significant difference between the initial resting membrane potential of +ATP and -ATP. Values are means ± SEM measured at the time that is indicated in panel C. Numbers in bracket within the bars indicate the number of tested cells from at least three animals. *NS*: no significant difference. (**E**) Correlation analyses of the seal resistance and the input resistance of all the cells, or the principal cells only, versus the resting membranes measured at the zero-current clamp shortly after the whole-cell establishment and the current magnitudes measured at the hyperpolarizing step to -100 mV from holding potential. Please click here to view a larger version of this figure.

Discussion

In this protocol, the enzymatic dispersion of the rat cauda epididymides consistently yielded healthy epithelial cells. The quality of the epididymal epithelial cells for the patch-clamp experiments is dependent on a few critical steps in the protocol. For instance, the centrifugation of the cell mixture at a low centrifugal force (30 x g) is important for removing the spermatozoa and the epididymal luminal content; the epididymal epithelial cells become unhealthy in the presence of the spermatozoa in the cell culture. In addition, culturing the dissociated cell mixture on a Petri dish for several hours is an important step in order to remove the fibroblasts and the smooth muscle cells; the non-epithelial cells adhere faster on the culture dish and thus, leaving the epithelial cells in the suspension, which can be re-harvested by gentle aspiration. Furthermore, collagenase digestion and trituration with a fine glass pipette are the two important steps to release single cells during the cell dissociation procedure. Finally, the enzymatic digestion of the epididymal cells is critical for the patch-clamp experiment because under-digestion by inefficient enzymatic activity or over-digestion by prolonged incubation can both disrupt the giga-ohm seal formation between the pipette and cell membranes.

In the cell mixtures, the cells in a larger, columnar shape with a rough membrane bearing stereocilia on one end and polarized distribution of the intracellular contents are presumably the principal cells or the clear cells. Other cells that do not possess prominent microvilli and polarized cell contents may include the basal cells and some cells from the immune system. In addition to describing basic morphological features of epididymal epithelial cells, this protocol employs the whole-cell patch-clamp method to measure the passive membrane property of each cell. These membrane properties allow for preliminary distinction of the cell types. This method has some limitations such as washout of the intracellular contents with the pipette solution and electronic variations in relation to the cellular architecture modifications during continuous recordings[19,20]. We provide only the parameters that we measured immediately following the establishment of the whole-cell configuration, which reflect the initial, relatively intact conditions of the cells. The passive membrane properties of each cell include the membrane capacitance, the input resistance and the resting membrane potential.

The specific capacitance of cell membranes is generally agreed to be 1 μF/cm^2 and this holds true for cells (such as, immune cells and neurons) with limited folding on their cellular membranes[16,18]. However, the specific capacitance constant tends to be greater for cells with more complex, cellular architectures, like the epithelial cells, which have many cellular membrane folds in distinct compartments. Reports indicate that the MDCK epithelial cell line values are 4 μF/cm^2 and 3 μF/cm^2 for the apical and basal membrane specific capacitances, respectively [17]. In the present study, the measured average membrane capacitances are ~8 pF and ~12 pF for the no-microvilli cells group and the principal cells and clear-like cells group (*i.e.* microvilli-cells), respectively. Using the specific capacitance of 1 μF/cm^2 for our calculations, the estimated cell surface areas are 500 μm^2 and 1,000 μm^2 for the no-microvilli cells and the microvilli cells, respectively, which corresponds to the diameters of 13 μm and 18 μm. However, these estimations are greater than what was expected based on the cells sizes under the microscope, which are ~8 μm and ~12 μm for no-microvilli cells and microvilli-cells, respectively, and these cells are about 2.4 μF/cm^2 and 1.9 μF/cm^2, respectively. Our results indicate that the membrane landscape of no-microvilli cells is more complex than that of the micro-villi cells, which is consistent with their secretion and transportation functions.

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In the whole-cell recordings, the leak conductance across the membrane and at the seal between the membrane and pipette both contributed to the measured membrane properties. The seal has a significantly higher resistance than the membrane, and so it has minimal influence on the measurement of the passive membrane properties, such as the input resistance and the zero-current membrane potential. Consistent with this notion, the correlation analysis of the sealing resistance at a threshold of 1 giga-ohm against the input resistance of each cell resulted in a value of ~0.02, suggesting a very weak influence. Further correlation analyses of either the seal resistance or the input resistance versus the resting membrane potentials or the current magnitude measured at -100 mV also gave low values for all tested cells or for the principal cells only. Our results demonstrate that the input resistance of the epididymal principal cells and the clear-like cells is significantly lower than the no-microvilli cells, thus providing a preliminary parameter for the judgement for differentiating the cell types. These results suggest that the low input resistance in the principal cells is an intrinsic physiological property of the cells. The single epithelial cells also possess distinct current patterns in response to the applied protocols and the experimental conditions. We observed that each defined cell type exhibited unique current patterns under the same applied voltage protocol. An example shown in **Figure 3** demonstrates the typical current responses recorded from the principal cells under quasi-physiological conditions, as we have reported previously[9]. The electrophysiological characteristics of each specialized cell type are readily differentiated (data are not shown in this paper). Based on these parameters, the different cell types can be roughly categorized into the principal cells, the clear-like cells and the no-microvilli cells, as described in the results.

The input resistance is the membrane resistance in response to the applied potential step (10 mV hyperpolarizing step in this study) elicited from a holding potential of -60 mV. This reflects the extent of open channel activity in response to the applied potential. In this regard, a low resistance implies a high intrinsic "leak" conductance of the cells, while a high resistance implies closed channels. The low input resistance value in principal cells corresponds to the prominent membrane conductance, whereas the clear-like cells that possess a small conductance at the testing membrane potential implies their moderate conductance under the recording conditions. The significantly high input resistance of the no-microvilli cells implies that they have no open channels in this status. In the zero-current clamp, the average resting membrane potentials of the measured, isolated epithelial cells are in the range of 26-33 mV for the three cell groups. These values are comparable with the reported membrane potential (-30 mV) using the microelectrode method[21]. However, the measured resting potentials vary widely in individual cells (from +3 mV to -63 mV) despite the absence of spontaneous spike potentials. This variation may reflect the interaction of different ion channel activities in various cell types, such as the coupled interplay of TRPV6 and TMEM16A channels in the principal cells, as we have reported recently[9]. It is worthwhile to note that when cells were dialyzed with the ATP pipette, we observed no spontaneous electrical potentials in the principal cells; this agrees with the classification of the epithelial cells as non-excitable[4]. A progressive hyperpolarization when the cells were dialyzed with the pipette solution without ATP may have reflected the gradual appearance of an ATP-sensitive potassium current. It has been reported that the ATP-sensitive potassium channels are expressed in the Golgi apparatus of the principal cells of the rat epididymis and that the depletion of the intracellular ATP leads to the activation of K_{ATP} channels[14,22]. This observation suggests that the inclusion of ATP in the pipette solution favors the stable physiological conditions of the epididymal principal cells.

The whole-cell patch-clamp technique is a well-established method that is commonly used to study the electrophysiology of excitable cells, such as neurons. In this paper, we showed that it also a useful tool for the characterization of bioelectrical properties of non-excitable cells, such as epithelial cells. In addition, this protocol enables functional investigations of primary isolated epididymal epithelial cells to further elucidate their physiological role in the epididymis. This study provides some preliminary electrical properties of the epithelial cells isolated from rat cauda epididymis for aiding the future investigations of the physiological behavior of these cells and their underlying biological relevance in the epididymis.

Disclosures

The authors have nothing to disclose.

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