Video Article

Application of Electrophysiology Measurement to Study the Activity of Electro-Neutral Transporters

Victor Babich[1,2], Matthew K. Henry[1], Francesca Di Sole[1]

[1]Physiology and Pharmacology Department, Des Moines University

[2]School of Liberal Arts and Sciences, Mercy College of Health Sciences

Correspondence to: Francesca Di Sole at Francesca.DiSole@dmu.edu

URL: https://www.jove.com/video/56630
DOI: doi:10.3791/56630

Keywords: Biology, Issue 132, Intracellular proton, electroneutral transport, patch clamp, ion-selective electrodes phosphoinositide, Na^+/H

Date Published: 2/3/2018

Citation: Babich, V., Henry, M.K., Di Sole, F. Application of Electrophysiology Measurement to Study the Activity of Electro-Neutral Transporters. *J. Vis. Exp.* (132), e56630, doi:10.3791/56630 (2018).

Abstract

The transport of ions through cell membranes ensures the fine control of ion content within and outside the cell that is indispensable for cell survival. These transport mechanisms are mediated by the activities of specialized transporter proteins. Specifically,pH dynamics are finely controlled by plasma membrane proton (H^+) extrusion systems, such as the Na^+/H^+ exchanger (NHE) protein family. Despite extensive efforts to study the mechanisms underlying NHE regulation, our current understanding of the biophysical and molecular properties of the NHE family is inadequate because of the limited availability of methods to effectively measure NHE activity. In this manuscript, we used H^+-selective electrodes during whole-cell patch clamping recording to measure NHE-induced H^+ flux. We proposed this approach to overcome some limitations of typically used methods to measure NHE activity, such as radioactive uptake and fluorescent membrane permeants. Measurement of NHE activity using the described method enables high sensitivity and time resolution and more efficient control of intracellular H^+ concentrations. H^+-selective electrodes are based on the fact that transporter activity creates an ion gradient in close proximity to the cell membrane. An H^+-selective electrode moving up to and away from the cell membrane in a repetitive, oscillatory fashion records a voltage difference that is dependent on H^+ flux. While H^+-selective electrodes are used to detect H^+ flux moving out of the cell, the patch clamp method in the whole-cell configuration is used to control the intracellular ion composition. Moreover, application of the giant patch clamp technique allows modification of the intracellular composition of not only ions but also lipids. The transporter activity of NHE isoform 3 (NHE3) was measured using this technical approach to study the molecular basis of NHE3 regulation by phosphoinositides.

Video Link

The video component of this article can be found at https://www.jove.com/video/56630/

Introduction

Transport of ions and solutes across the plasma membrane is essential for the survival of cells and, hence, of organisms[1]. Selective transport of ions and solutes is achieved by an array of specialized channel and transporter proteins. Mutations in these proteins often result in a variety of clinical conditions, rendering channel and transporter proteins potential targets for pharmacological treatment[1]. Unfortunately, understanding the mechanisms underlying channel and transporter function and regulation is often limited by the approaches available to study their activity[2,3,4].

Specifically, transporters can be roughly sub-divided into two large groups depending on whether they alter the cell transmembrane potential during transport of solutes: the altering electrogenic ion transporters [*e.g.,* sodium-phosphate co-transporter 2a (NaPi2a), sodium-calcium exchanger (NCX), *etc.*] or the non-altering electroneutral ion transporters [*e.g.,* sodium-proton exchanger (NHE), sodium-chloride co-transporter, NaPi2c, *etc.*]. The activities of both classes of transporters have been studied extensively using uptake of radioactive isotopes and fluorescent membrane-permeant dyes[2]. Both approaches estimate the activity of transporters by measuring changes in the bulk concentration of specific cytoplasmic ions, and both methods have limitations, such as moderate sensitivity and time resolution and inadequate control of the intracellular milieu. Indeed, the activity of many transporters is dependent on the cytoplasmic concentration of the carried ions (*e.g.,* NHE3, NCX), and changes in these ion concentrations are expected to play a significant role in regulating transporter activity[2,3,5]. Precise measurement of these regulative mechanisms is limited using classical methods.

To overcome these limitations, patch clamp methods are used to study the transporter activity[2,6]. Specifically, the self-referencing ion-selective electrodes (ISEs)[7,8] combined with the patch clamping system has recently allowed the measurement of electroneutral transporter activity[3,4,5]. ISEs are based on the fact that transporter activity creates an ion gradient in close proximity to the cell membrane. An ISE moving up to and away from the cell membrane in a repetitive, oscillatory fashion records a voltage difference (μV). Voltage differences can be converted into ion flux values using a calibration method that applies Fick's first law of diffusion[2,9]. While ISEs are used to detect the flux of ions moving out of cells, the patch clamp method in both whole-cell or inside-out configurations is used to control the membrane potential and intracellular ion composition. Moreover, the application of the giant patch clamp technique allows modification of the intracellular composition of not only ions but also lipids and proteins[3,5].

In summary, the versatility of the patch clamp method compared with that of other methods to study transporter activity has made patch clamping suitable to overcome the common limitations of these other methods. The combination of self-referencing ISEs and patch clamp techniques offers the unique possibility to measure the activity of electroneutral transporters in a tightly controlled experimental environment and to discover novel biophysical and molecular properties of cell membrane transport[3,4,5]. This approach has been successfully used to study the activity of the NHE. The mammalian NHE protein family catalyzes the electroneutral net exchange of extracellular sodium (Na^+) for intracellular proton (H^+)[10,11] utilizing an inward Na^+ gradient. In mammals, the NHE protein family includes 11 related proteins (NHE1-9 and NHA1-2) and a sperm-specific NHE[10,12,13].

NHEs (SLC9a family) are found ubiquitously in most living organisms from simple prokaryotes to higher eukaryotes and are involved in a variety of vital cell functions[10,11], including controlling the cell salinity defense in prokaryotes, maintaining acid-base homeostasis and cell volume, and regulating the absorption of salt and water in various specialized epithelia[10,12,14,15]. The key biological roles of NHEs and the significance of their functions have been determined through several studies; however, few studies have investigated the biophysical and molecular properties of mammalian NHEs because of methodological limitations[4]. Recently, the application of self-referencing ISEs during whole-cell patch clamping has revealed novel molecular mechanisms of NHE isoforms regulated by changes in the intracellular concentrations of ions, proteins and phospholipids[3,4].

Specifically, the protocol provided in this manuscript outlines the methods and approaches for studying the activity and regulation of NHE isoform 3 (NHE3), a major player in the absorption of Na^+, Cl^-, HCO_3^- and fluid in the brush border membrane of renal and intestinal epithelia[14,16]. New insight into differences in the sensitivity of NHE3 activity to intracellular phosphoinositides (phosphatidylinositide 4,5-bisphosphate [PI(4,5)P2] and phosphatidylinositide 3,4,5-triphosphate [PI(3,4,5]P3]) is reported. Cell transport proteins, such as channels and transporters, are regulated by phosphoinositides[17], and NHE3 directly binds both PI(4,5)P2 and PI(3,4,5)P3[18]. Based on the current literature, either phosphoinositide could be relevant for the physiological or pathophysiological regulation of NHE3[5,18,19]. Our findings support separate roles for PI(4,5)P2 and PI(3,4,5)P3 in the regulation of NHE3 activity. This distinction was possible because of the application of ISE techniques in combination with whole-cell patch clamp recording. This technique also allows control of the phosphoinositide cellular content via the intracellular perfusion of different phosphoinositides during the measurement of NHE3 activity.

Protocol

Note: Two amplifiers are required for recording the activity of electroneutral transporters by a self-referencing ISE in conjunction with patch clamping, a patch clamp amplifier to maintain the cell in a whole-cell configuration and a high impendence amplifier (electrometer) to record the transporter activity via the ISE (see Table of Materials). The patch clamp amplifier is directly connected to the acquisition board terminal. The electrometer is connected to the differential amplifier utilized to filter and amplify the signal. The differential amplifier is connected to the acquisition board terminal. Capmeter, patch clamp software that monitors cell capacity, has been used in previous studies[20] (see Table of Materials). The recording chamber and perfusion solutions are maintained at 37 °C.

1. Preparation of Pipettes for ISEs

1. Pull the pipettes from borosilicate glass capillaries to an outer diameter of 1.2 mm (see **Table of Materials**) using the pipette puller[2] .
2. Polish the pipette using the microforge. The diameter of the pipette tip should be 2-3 µm.
3. Place the pipettes in a jar holder for pipettes with the pipette tip facing up (see **Table of Materials**).
   CAUTION: Perform steps 1.4-1.6 in a well-ventilated chemical fume hood.
4. Mix the siliconization mixture that is composed of 1 drop (~150 µL) of bis(dimethylamino)dimethyl silane and 10 drops of carbon tetrachloride (see **Table of Materials**).
5. Pour the siliconization mixture into the jar and close the lid tightly.
6. Incubate the jar with the siliconization mixture for 24-48 h in a chemical fume hood at room temperature until the mixture is completely evaporated.
7. Backfill the pipette with the appropriate ion-selective resin to create a column of approximately 2-4 mm.
   NOTE: Hydrogen ionophore I cocktail B can be usedfor recording NHE activity (see **Table of Materials**).
8. Tap the tip of the pipette to distribute the ion-selective resin to the tip, and place the pipette vertically in the jar holder with the pipette tip down.
9. Checkunder the dissecting microscope that the resin cocktail has reached the end of the tip. If needed, apply positive pressure to push the resin to the end.
10. Backfill the half-pipette with the appropriate solution (~ 100 µL). Here, use 100 mM KCl and 10 mM HEPES at pH 7.0 if using ionophore I cocktail B.
11. Connect the ISE to the electrometer and place the tip of the ISE into the bath solution used for recording. Zero the electrometer.
12. Test the H^+-selective ISE response by changing the pH of the bath solution. The average H^+-selective ISE response is ~ 58 mV per unit of pH.
13. Periodically evaluate the ISE response to calibration.

2. Preparation of Patch Clamp Pipettes for Recording

NOTE: The method described by Donald Hilgemann in his article on Single-Channel Recording[21] is recommended.

1. Pull the pipettes for giant patch from borosilicate glass capillaries (2.0 mmOD / 1.5 mm ID) on the pipette puller[2,22,23].
2. Use a microforge mounted on the stage of an inverted microscope to make a wide pipette tip (10 - 22 µm)[23]. Melt a bead (~ 0.5 mL) of soft glass onto the thick platinum wire of the microforge.
   NOTE: A relatively thick platinum wire is required (~0.5 mm) to fabricate giant patch pipettes.

3. Position the patch pipette close to the soft glass bead, and apply full heat until the tip begins to recede. The platinum wire will move slightly toward the centerduring heating.
4. Turn off the heat, and push the pipette tip into the softened glass bead. The cooling wire will retract and break the pipette tip.
5. Reapply the heat to smooth the pipette tip and create a pipette tip of the desired diameter. The diameter of the tip is dependent on the size of the cell to be patched (8 - 10 µm in this study).

3. Preparation of Cells

1. Warm up phosphate buffer saline (PBS) without Ca^{2+} and Mg^{2+}, trypsin-EDTA solution and growth medium to at 37 °C.
2. Wash cells twice with PBS. Use 1 mL of the buffer for 35 mm diameter cell culture plate.
3. Add 0.5 mL of trypsin to the cells for 35 mm diameter cell culture plate.
   Note: When cells start to round-up, shake the plate to de-attach the cells.
4. Stop the trypsin action by adding 5 mL (amount for 35 mm diameter cell culture plate) of complete growth medium supplemented with 10% fetal bovine serum.
   NOTE: The reaction time in trypsin is cell-type dependent.
5. Place the cells on a shaker with rocking to prevent formation of clumps.
6. Use cells for up to 2 h after trypsinization.
   NOTE: The time might vary depending on the cell line.

4. Preparation of an Intra-Pipette Perfusion System

1. Cut an appropriate size of quartz tubing (150 µm OD / 75 µm ID) to prepare the intra-pipette perfusion line.
2. Insert one side of the quartz tubing into a 200 µL pipette tip. Glue the quartz tubing onto the tip, and cut the end of the tip with a sharp razor blade.
3. Connect the perfusion line (quartz tubing glued to the tip) to a small piece of silicon tubing that would serve as a reservoir for the intra-pipette perfusion solution.
4. Connect the silicon tubing to a syringe to create positive pressure.
5. Insert the free end of the quartz tubing through the polyethylene tube of the pipette holder of the perfusion port (see **Table of Materials**).
6. Evaluate the perfusion line with the pipette solution.

5. Preparation of Solutions

1. Prepare a heavily buffered pipette solution for recording NHE (115 mM potassium aspartate (KAsp), 1 mM EGTA, 0.5 mM $MgCl_2$, 10 mM Mg-ATP, 12.5 mM HEPES, 12.5 mM PIPES, 12.5 mM MOPS, and 12.5 mM MES, pH 6.0 adjusted with aspartic acid).
   NOTE: Prepare the stock pipette solution and filter. Add Mg-ATP before recording.
2. Prepare a fresh bath solution each time (140 mM NaCl, 2 mM $CaCl_2$, 2 mM $MgCl_2$, and 0.1 mM Tris-HCl pH 8.2).

6. Recording of Transporter Activity

1. Maintain the recording chamber and all solutions at 37 °C, because transporter activity is temperature-dependent.
2. Place the cells into the recording chamber. Wait until they drop to the bottom of the chamber but do not let them attach to the chamber.
3. Choose an appropriate cell. Measure the diameter of the cell with the ocular micrometer. Choose cells with similar diameters.
   NOTE: Generally, a large cell will have more cellular surface and possibly more transporters expressed on its plasma membrane, thus also having more transporter activity. However, this is not necessarily correct, as transporter activity depends on the specific transporter and the cell type under study.
4. Mount the ion-selective microelectrode pipette to the micromanipulator, backfill the patch-clamping pipette with the intracellular solution, make sure that the solution reaches the tip of the pipette[2].
   NOTE: It might require several gentle taps to eliminate bubbles in the tip of the pipette.
5. Mount the patch-clamping pipette to the head stage of the electrophysiology set up[2,22,23].
   NOTE: It is important to keep the head stage at about a 45-degree angle to the horizontal axis, as this ensures an entry angle for the pipette that is suitable for the formation of a highly resistant seal.
6. Apply a small positive pressure (~ 5 cm H_2O) to the patch pipette to reduce the chance of blocking the tip, and place it into the bath solution.
7. Stop moving the patch clamp pipette once the tip is close to the cell.
8. Relieve the positive pressure while pipette touches the cell and apply slight negative pressure (~ 5 cm H_2O) to obtain a >1000 MΩ (a Giga-ohm) seal on the cell membrane with the patch pipette[23].
9. Place the patch pipette with the cell in the same focal plane as the ISE, and move the patch pipette with the cell in proximity to the ISE (~ 5 - 10 µm)[2,5] but avoid allowing the cell to contact the ISE (**Figure 1A**).
10. Assure that the holding potential is 0 mV.
11. Rupture the seal with series of short suctions to obtain the whole-cell configuration.
12. Move the ISE (or patch clamp pipette) slowly left-right or up-down to record the activity of the transporter (**Figure 1B**).
13. Record at least 5-8 peaks (**Figure 1C, from A to B**).
14. Apply square wave perturbations during the recording to monitor the quality of the seal and the cell membrane capacitance (**Figure 1**).
15. Estimate the H^+ flux (J_{H^+}) from the difference in free H^+ concentration between the cell surface and the bulk solution (ΔH^+)

$$J_{H^+}\left(\frac{mols}{s}\right) = \Delta H^+ \cdot 4 \cdot \pi \cdot r \cdot \left(D_{H^+} + D_B \cdot \frac{\Delta B_H}{\Delta H^+}\right)$$

$$\frac{\Delta B_H}{\Delta H} = \frac{B_T \cdot K_D}{(K_D + H^+)^2}$$

NOTE: D_{H^+} and D_B are H^+ and buffer diffusion coefficients respectively, B_T is the total buffer concentration, K_D is the dissociation constant of the buffer, r is the cell radius, and $\frac{\Delta B_H}{\Delta H}$ determines the steady-state change in the concentration of bound H^+ for a small change in free H^+. H^+ indicates a free proton in solution. To be consistent with previous studies, H^+ fluxes are converted to electrophysiological flux units by calculating the current equivalents of the fluxes (J_{H^+}/faraday) in pA[3,5].

16. Normalize the proton flux to the cell surface that can be calculated from the cell size or to the membrane capacitance.

Representative Results

A self-referencing ISE during whole-cell patch clamp recording was applied to study the regulation of NHE3 activity by phosphoinositides. PS120 fibroblast-like cells[24], which lack the expression of endogenous plasma membrane NHEs, were used. NHE3 wild-type (NHE3-wt) or NHE3 mutants that do not bind phosphoinositides [tyrosine 501, arginine 503 and lysine 505 were substituted with alanine, Y501A/R503A/K505A (NHE3-YRK)] were stably expressed in PS120 fibroblast-like cells[18].

The traces are presented in **Figure 2**. The cell was heldin a whole-cell configuration by a pipette for patch clamping, and the ISE was placed in front of the cell. In this position, the ISE recorded the concentration of free H^+ close to the cell membrane (**Figure 1A**); then, the ISE (or patch clamp pipette) was manually moved laterally 50 µm and recorded the concentration of free H^+ in the solution (**Figure 1B**). The recorded voltage differences, corresponding to the two positions of the patch pipette with the cell in front of or far from the ISE, are representative of NHE3 activity (**Figure 1C**). The intra-pipette perfusion of apyrase (ATP-diphosphatase) decreases the intracellular concentration of ATP, subsequently decreasing the phosphoinositide content[5]. In agreement with previously reported studies[5,18,19], the decreased phosphoinositide content mediated by apyrase treatment reduced NHE3 activity (~50%), which was recorded as a decrease in the voltage oscillation amplitude (**Figure 2**). The effect of apyrase (given at 5 units/ml) on NHE3 activity was completely reversed by the intra-pipette perfusion of apyrase in combination with 2 µM PI(3,4,5)P3 (**Figure 2** and 3A). These findings were supported by the NHE3 activity recorded in PS120 cells stably expressing NHE3 mutants (NHE3-YRK) that were unable to bind phosphatidylinositides[18]. The perfusion of apyrase alone or in combination with PIP3 did not affect the activity of NHE3 in NHE3-YRK mutants (**Figure 3B**). The activity of endogenous NHE3 was also inhibited by apyrase treatment in opossum kidney (OK) cells[3]. The intra-pipette perfusion of PI(3,4,5)P3 reversed the inhibition of NHE3 activity induced by apyrase in OK cells (**Figure 3C**). These findings suggest that the effect of PI(3,4,5)P3 on NHE3 activity is not cell type-specific.

Next, we aimed to test the specificity of PIP3 on regulating apyrase-mediated changes in NHE3 activity. We did this by assaying the effect on apyrase-dependent reduction on NHE3 activity after the perfusion of other phosphoinositides, such as PI(4,5)P2. PI(4,5)P2 (at 10 µM) did not affect the decreased NHE3 activity mediated by apyrase (**Figure 4A**). Finally, the perfusion of AMP-PNP, a non-hydrolysable ATP analog, did not block theapyrase-dependent inhibition of NHE3 activity (**Figure 4B**). These findings suggest a role of PI(3,4,5)P3, but not PI(4,5)P2, in the regulation of NHE3 after ATP depletion. The identification of separate roles for PI(4,5)P2 and PI(3,4,5)P3 in NHE3 regulation was possible only because of the intercellular perfusion of phosphoinositides during ISE measurements combined with whole-cell patch clamp recording[5]. A specific action of PI(3,4,5)P3 rather than PI(4,5)P2 in the regulation of NHE3 activity is valuable to determine whether different intracellular phosphoinositides regulate the function of NHE3 differently.

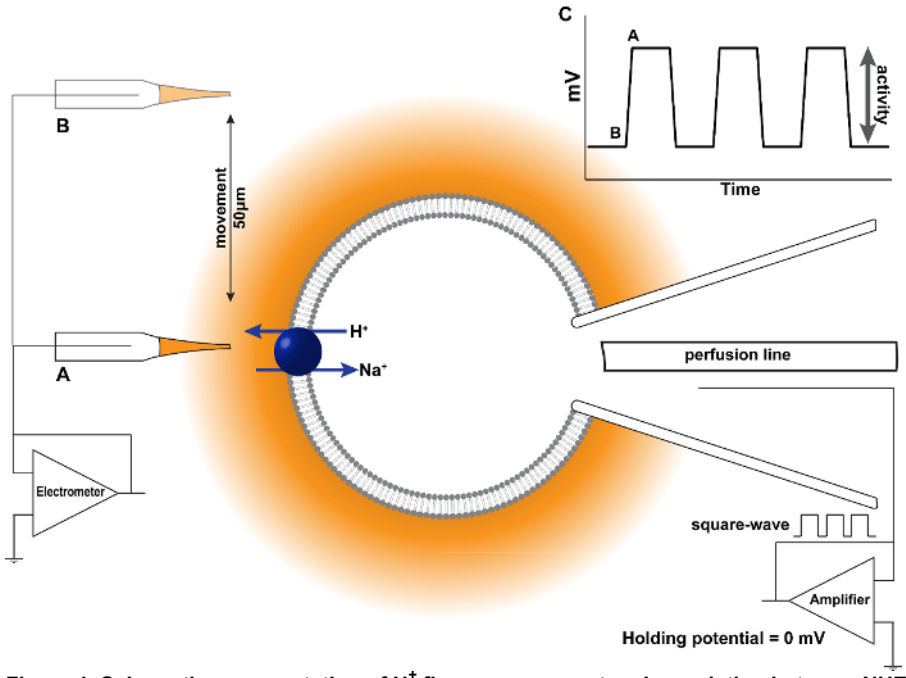

Figure 1. Schematic representation of H^+ flux measurement and correlation between NHE activity and potential differences recorded by the pH microelectrode.
Schematic representation of the recording setting. **A.** Diagram of the initial experimental setting. The cell is held in the whole-cell configuration by the patch clamp pipette and placed in front of the ISE. In this position, the ISE records the concentration of free H^+ close to the cell membrane. **B.** The ISE (or patch clamp pipette) is manually moved laterally ~50 µm and records the concentration of free H^+ in the solution in this position. **C.** Graphic representation of voltage differences recorded using the ISE. The oscillations from position A (cell in front of the ISE) to B (cell far from the ISE) are representative of NHE activity. The software used registered the voltage differences recorded by the ISE and generated square wave perturbations (20 mV, 0.2 kHz) to monitor the cell membrane capacitance and quality of the patch clamp seal. Please click here to view a larger version of this figure.

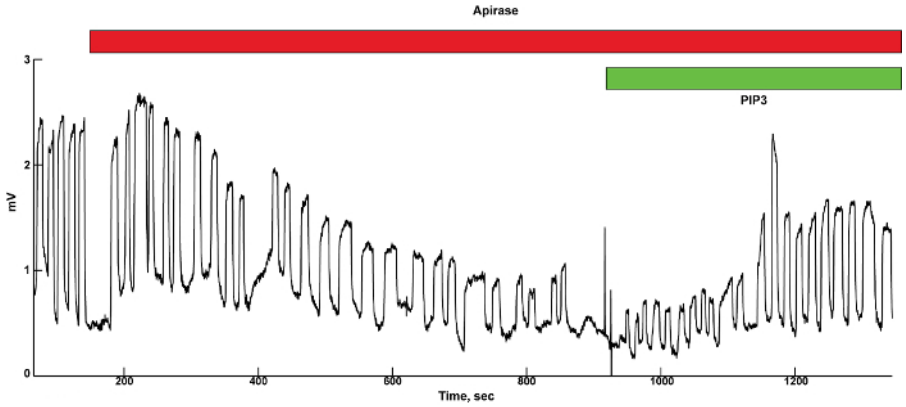

Figure 2. Example of a recording set for NHE3 transporter activity in a single PS120 cell stably expressing NHE3-wt before and after perfusion with apyrase and PI(3,4,5)P3 (PIP3).
After the initial recording, the cell was depleted of ATP by the intra-pipette perfusion of apyrase. NHE3 activity gradually decreased and was restored by PIP3 intra-pipette perfusion. Apyrase addition is indicated by the red bar, and PIP3 addition is indicated by the green bar. Please click here to view a larger version of this figure.

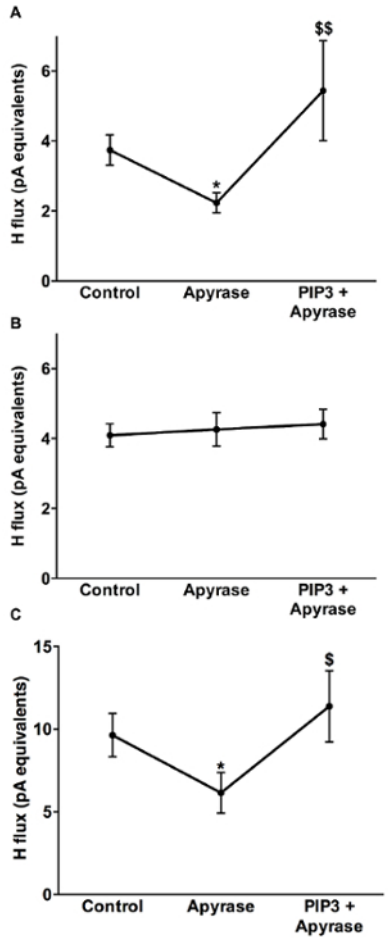

Figure 3. Effect of ATP depletion and PIP3 perfusion on NHE3 transporter activity in PS120 and OK cells.
Effect of apyrase-mediated ATP depletion on NHE3 activity and reserving the effect of apyrase on NHE3 activity induced by PIP3 intra-pipette perfusion: **A.** PS120 cells expressing NHE3-wt or **B.** PS120 cells expressing NHE3-YRK, NHE3 mutants unable to bind phosphoinositides. **C.** OK cells expressing endogenous NHE3. Solid circles show average data from individual experiments (four experiments performed in identical conditions). Error bars represent the means ± standard errors (SE). *P < 0.05 vs. control, ANOVA. $^{\$}$P< 0.5, $^{\$\$}$P < 0.01 apyrase vs. apyrase + PIP3, ANOVA. Please click here to view a larger version of this figure.

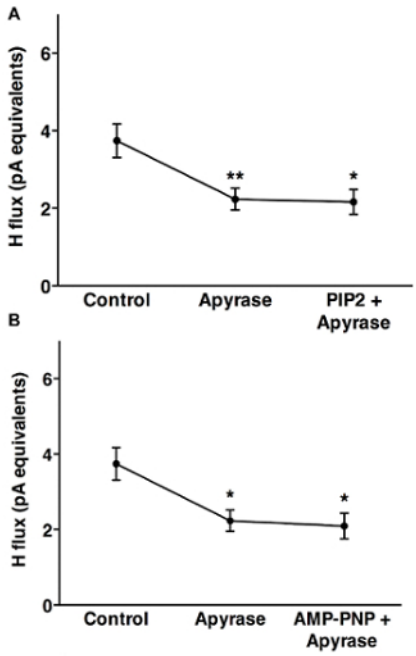

Figure 4. Effect of PI(4,5)P2 (PIP2) and ANP-PNP perfusion on NHE3 activity after ATP depletion.
Effect of **A.** PIP2 or **B.** ANP-PNP intra-pipette perfusion on the apyrase-dependent reduction of NHE3 activity in PS120 cells expressing NHE3-wt. Solid circles show average data from individual experiments (four experiments performed in identical conditions). Error bars represent the means ± SE. *P < 0.05, **P < 0.01 vs. control, ANOVA. Please click here to view a larger version of this figure.

Discussion

Despite the crucial functions of transporters, the methods available to study their activity are ineffective and inadequate. One limitation is that the available methods measure ion movement mediated by transporter activity without considering fluctuations in intracellular ion composition during the experiment[4]. The presented method ensures precise control of extracellular and intracellular ion compositions and offers a powerful tool for modifying intracellular ion, protein and lipid compositions[3,4,5].

The most important step in this protocol is the preparation of an ISE that is stable for a long period[25]. In our experience, an unstable ISE is not well-siliconized (*e.g.,* the ion-selective resin is redistributed on the ISE pipette sides), which can be detected by a constant and uncontrollable drift in the ISE signal. Additionally, the resin distribution in the ISE should be closely monitored during the experiment. The bath solution may push the ion-selective resin inside the ISE pipette, creating a column of bath solution before the resin[25]. In this case, the ISE will record the ion concentration from this column and become nonresponsive to changes in the ion gradients during the experiment. Adjusting the geometry of the ISE is key to obtain an ISE response in the range of 100 ms[9] and overcome possible delays in ISE time-responses compared with changes in ion gradients.

The major limitation of the presented method is inherited from the patch clamping measurement of transporter activity in epithelial cells. When detached from the support and maintained in suspension, polarized epithelial cells might lose the polarized localization of transporters on the apical or basolateral membrane.

A modified patch clamping measurement should be used when studying channels and transporters in polarized epithelia. Considering that cells might approximate into a cylinder rather than a sphere is important when transforming the H^+ gradient into flux via the formula provided. In this case, the formula should be changed to $J_{H^+}\left(\frac{mols}{s}\right) = \Delta H^+ \cdot 2 \cdot \pi \cdot \lambda \cdot \ln\left(\frac{r}{x}\right) \cdot \left(D_{H^+} + D_B \cdot \frac{\Delta B_H}{\Delta H^+}\right)$, where λ is the length of the cylinder, r is the cylinder radius, ad x is the radial distance from the cylinder midpoint over which the gradient is measured[5]. Future development of this powerful technique for studying electroneutral transporters should focus on modifying the method to optimize recording cells grown in a monolayer.

Disclosures

The authors have no conflicts of interest to disclose.

Acknowledgements

The authors would like to thank Eric Fishback (Des Moines University, Des Moines, Iowa, USA) for his assistance with shooting and editing the video. The PS120 fibroblast-like cells stably expressing NHE3-wt or NHE3-YRK were kindly provided by Dr. Mark Donowitz (Johns Hopkins University School of Medicine, Baltimore, MD, USA).

References

1. Rives, M. L., Javitch, J. A., & Wickenden, A. D. Potentiating SLC transporter activity: Emerging drug discovery opportunities. *Biochem Pharmacol.* (2017).
2. Kang, T. M., Markin, V. S., & Hilgemann, D. W. Ion fluxes in giant excised cardiac membrane patches detected and quantified with ion-selective microelectrodes. *J Gen Physiol.* **121** (4), 325-347 (2003).
3. Babich, V., Vadnagara, K., & Di Sole, F. The biophysical and molecular basis of intracellular pH sensing by Na+/H+ exchanger-3. *FASEB J.* **27** (11), 4646-4658 (2013).
4. Fuster, D., Moe, O. W., & Hilgemann, D. W. Steady-state function of the ubiquitous mammalian Na/H exchanger (NHE1) in relation to dimer coupling models with 2Na/2H stoichiometry. *J Gen Physiol.* **132** (4), 465-480 (2008).
5. Fuster, D., Moe, O. W., & Hilgemann, D. W. Lipid- and mechanosensitivities of sodium/hydrogen exchangers analyzed by electrical methods. *Proc Natl Acad Sci U S A.* **101** (28), 10482-10487 (2004).
6. Forster, I. C., Virkki, L., Bossi, E., Murer, H., & Biber, J. Electrogenic kinetics of a mammalian intestinal type IIb Na(+)/P(i) cotransporter. *J Membr Biol.* **212** (3), 177-190 (2006).
7. Smith, P. J., & Trimarchi, J. Noninvasive measurement of hydrogen and potassium ion flux from single cells and epithelial structures. *Am J Physiol Cell Physiol.* **280** (1), C1-11 (2001).
8. Parker, M. D., Musa-Aziz, R., & Boron, W. F. The use of extracellular, ion-selective microelectrodes to study the function of heterologously expressed transporters in Xenopus oocytes. *Am J Physiol Cell Physiol.* **296** (5), C1243; author reply C1244 (2009).
9. Smith, P. J. S., Sanger, R. H., & Messerli, M. A. in *Electrochemical Methods for Neuroscience.* eds A.C. Michael & L.M. Borland) 373-406 CRC Press/Taylor & Francis, (2007).
10. Orlowski, J., & Grinstein, S. Diversity of the mammalian sodium/proton exchanger SLC9 gene family. *Pflugers Arch.* **447** (5), 549-565 (2004).
11. Brett, C. L., Donowitz, M., & Rao, R. Evolutionary origins of eukaryotic sodium/proton exchangers. *Am J Physiol Cell Physiol.* **288** (2), C223-239 (2005).
12. Bobulescu, I. A., Di Sole, F., & Moe, O. W. Na+/H+ exchangers: physiology and link to hypertension and organ ischemia. *Curr Opin Nephrol Hypertens.* **14** (5), 485-494 (2005).
13. Donowitz, M., Ming Tse, C., & Fuster, D. SLC9/NHE gene family, a plasma membrane and organellar family of Na(+)/H(+) exchangers. *Mol Aspects Med.* **34** (2-3), 236-251 (2013).
14. Zachos, N. C., Tse, M., & Donowitz, M. Molecular physiology of intestinal Na+/H+ exchange. *Annu Rev Physiol.* **67** 411-443 (2005).
15. Girardi, A. C., & Di Sole, F. Deciphering the mechanisms of the Na+/H+ exchanger-3 regulation in organ dysfunction. *Am J Physiol Cell Physiol.* **302** (11), C1569-1587 (2012).
16. Bobulescu, I. A., & Moe, O. W. Na+/H+ exchangers in renal regulation of acid-base balance. *Semin Nephrol.* **26** (5), 334-344 (2006).
17. Hilgemann, D. W., Feng, S., & Nasuhoglu, C. The complex and intriguing lives of PIP2 with ion channels and transporters. *Sci STKE.* **2001** (111), re19 (2001).
18. Mohan, S. *et al.* NHE3 activity is dependent on direct phosphoinositide binding at the N terminus of its intracellular cytosolic region. *J Biol Chem.* **285** (45), 34566-34578 (2010).
19. Alexander, R. T. *et al.* Membrane surface charge dictates the structure and function of the epithelial Na+/H+ exchanger. *EMBO J.* **30** (4), 679-691 (2011).
20. Wang, T. M., & Hilgemann, D. W. Ca-dependent nonsecretory vesicle fusion in a secretory cell. *J Gen Physiol.* **132** (1), 51-65 (2008).
21. Hilgemann, D. W. in *Single-Channel Recording.* eds B. Sakmann & E. Neher) 307-328 Plenum Press, (1995).
22. Hilgemann, D. W., & Lu, C. C. Giant membrane patches: improvements and applications. *Methods Enzymol.* **293** 267-280 (1998).
23. Matsuoka, S., & Takeuchi, A. in *Patch Clamp Techniques.* (ed Y. Okada) 207-218 Springer, (2012).
24. Pouyssegur, J., Sardet, C., Franchi, A., L'Allemain, G., & Paris, S. A specific mutation abolishing Na+/H+ antiport activity in hamster fibroblasts precludes growth at neutral and acidic pH. *Proc Natl Acad Sci U S A.* **81** (15), 4833-4837 (1984).
25. Voipio, J., Pasternack, M., & MacLeod, K. in *Microelectrode Techniques - The Plymouth Workshop Handbook* .(ed D. Ogden) 275-316 The Company of Biologists Ltd., (1994).

