A highly polarized excitable cell separates sodium channels from sodium-activated potassium channels by more than a millimeter

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Ban Y, Smith BE, Markham MR. A highly polarized excitable cell separates sodium channels from sodium-activated potassium channels by more than a millimeter. J Neurophysiol 114: 520–530, 2015. First published April 29, 2015; doi:10.1152/jn.00475.2014.—The bioelectrical properties and resulting metabolic demands of electrogenic cells are determined by their morphology and the subcellular localization of ion channels. The electric organ cells (electrocytes) of the electric fish Eigenmannia virescens generate action potentials (APs) with Na+ currents >10 μA and repolarize the AP with Na+-activated K+ (KNa) channels. To better understand the role of morphology and ion channel localization in determining the metabolic cost of electrocyte APs, we used two-photon three-dimensional imaging to determine the fine cellular morphology and immunohistochemistry to localize the electrocytes’ ion channels, ionotropic receptors, and Na+-K+-ATPases. We found that electrocytes are highly polarized cells ~1.5 mm in anterior-posterior length and ~0.6 mm in diameter, containing ~30,000 nuclei along the cell periphery. The cell’s innervated posterior region is deeply invaginated and vascularized with complex ultrastructural features, whereas the anterior region is relatively smooth. Cholinergic receptors and Na+ channels are restricted to the innervated posterior region, whereas inward rectifier K+ channels and the KNa channels that terminate the electrocyte AP are localized to the anterior region, separated by >1 mm from the only sources of Na+ influx. In other systems, submicrometer spatial coupling of Na+ and KNa channels is necessary for KNa channel activation. However, our computational simulations showed that KNa channels at a great distance from Na+ influx can still terminate the AP, suggesting that KNa channels can be activated by distant sources of Na+ influx and overturning a long-standing assumption that AP-generating ion channels are restricted to the electrocyte’s posterior face.

three-dimensional electrolyte morphology; action potential energy efficiency; ion channel compartmentalization; sodium-activated potassium channels

ACTION POTENTIALS (APs) are transient changes in membrane voltage that are typically initiated by inward Na+ current (INa) and terminated by outward K+ current (IK). These currents are driven by ionic concentration gradients across the cell membrane (Bean 2007) and transmembrane Na+-K+-ATPases consume energy to restore the ionic gradients after each AP. Total Na+ influx during the AP determines ATPase activity and therefore the resulting metabolic cost of the AP (Attwell and Laughlin 2001; Howarth et al. 2012; Niven and Laughlin 2008). Any temporal overlap of inward INa and outward IK reduces energy efficiency, but this overlap is often necessary in fast-spiking cells, where the need to maintain brief APs requires that IK begins while INa is still active to terminate the AP quickly. This incomplete inactivation of Na+ channels during AP repolarization can result in the entry of twice as much Na+ as the theoretical minimum, significantly reducing energy efficiency at high AP frequencies (Carter and Bean 2009).

The weakly electric fish Eigenmannia virescens generates electric organ discharges (EODs) to navigate and communicate in darkness (Hopkins 1974). Because they generate APs at steady frequencies of 200–600 Hz (Scheich 1977) with underlying Na+ currents that can exceed 10 μA (Markham et al. 2013), the electric organ cells (electrocytes) create extremely high metabolic demands (Lewis et al. 2014). High rates of ATP hydrolysis by the Na+-K+-ATPases are therefore necessary to remove Na+ from the cell between APs. The simultaneous APs of >1,000 electrocytes during each EOD further magnify the metabolic cost of signal production, and as a result, EOD amplitude is highly sensitive to metabolic stress (Reardon et al. 2011).

Eigenmannia virescens electrocytes are large cells >1 mm in length, each innervated by spinal motoneurons at a cholinergic synapse on the morphologically complex posterior membrane (Fig. 1B) (Schwartz et al. 1975). Only the posterior membrane generates an AP, with the anterior membrane presumed to be electrically passive (Bennett 1961, 1971). These findings together led to a long-standing assumption that the ion channels responsible for producing the AP are restricted to the posterior region of the cell. Electrocytes express an inwardly rectifying K+ current, a voltage-gated Na+ current, and a Na+–activated K+ (KNa) current that terminates the AP. The expression of KNa channels instead of voltage-gated K+ (Kv) channels increases AP energy efficiency by reducing the overlap of INa and IK during the electrocyte AP (Markham et al. 2013). Early biochemical studies identified Na+-K+-ATPases expressed on both the anterior and posterior membranes (Denizot 1982), but the spatial distribution of the cholinergic receptors and ion channels is not yet known. A full account of the electrocyte’s fine morphology and distribution of ionotropic receptors and ion channels is a crucial first step toward understanding the interplay of the ionic currents that determine the spatiotemporal dynamics of intracellular Na+ concentrations that regulate KNa channel activation, ATPase activity, and, ultimately, the metabolic burden of EOD production.

We therefore used confocal laser-scanning fluorescence microscopy and immunohistochemistry to identify the electrocyte’s fine three-dimensional (3D) structure and the subcellular localization of their cholinergic receptors, ion channels, and...
We harvested electric organ (EO) tissue from E. virescens by cutting off a small (1–2 cm) piece of the narrow tail filament, consisting of only soft tissue and composed almost entirely of the EO (Fig. 1C). This brief procedure lasts less than 15 s and is performed without anesthesia because induction and recovery from immersion anesthesia are more harmful to the fish than the tail-clip itself. The tail and EO regenerate within 2 mo.

To obtain mouse brains for validating antibodies used in the present study, five C57BL/6J mice (stock no. 664; Jackson Laboratory) were deeply anesthetized by isoflurane inhalation [5% (vol/vol) isoflurane in oxygen] and then decapitated, and brains were quickly removed and frozen in liquid nitrogen.

All methods were approved by the Institutional Animal Care and Use Committee of The University of Oklahoma and complied with the guidelines given in the Public Health Service Guide for the Care and Use of Laboratory Animals.

Confocal Imaging and 3D Reconstructions

Microinjection. We harvested an ∼2-cm section of the tail, removed the overlying skin, and pinned the exposed EO tissue in a Sylgard (Dow Corning)-coated petri dish containing normal saline (in mM: 114 NaCl, 2 KCl, 4 CaCl2·2H2O, 2 MgCl2·6H2O, 2 HEPES, and 6 glucose; pH to 7.2 with NaOH). Temperature of the preparation was stable at room temperature (22 ± 1°C). Rhodamine B or Alexa Fluor 594 dextran (10,000 MW; Life Technologies) were prepared as a 1% (wt/vol) solution in water. Precipitate within the dextran solution was removed by centrifugation at 12,000 g for 5 min. Microinjections were performed using an automatic nanoliter injector (Nanoject II; Drummond Scientific). Micropipettes for injection were drawn from borosilicate glass capillaries (Drummond Scientific) with a laser micropipette puller (model P-2000; Sutter Instruments). We injected 13.8 nl of dextran solution into the cytoplasm of 4–5 electroctyes in each sample of EO with the constant injection speed of 23 nl/s. The EO tissue with rhodamine B or Alexa Fluor 594 dextran-injected electroctyes was then held in normal saline at room temperature (22 ± 1°C) for 15 min until the dextran fully diffused into injected electroctyes. We then proceeded immediately to image the live cells directly or to fix and section the tissue before mounting and imaging.

Vibratome sectioning. We fixed the Alexa Fluor 594 dextran-injected EO tissue in 2% paraformaldehyde buffered with 1× phosphate-buffered saline (PBS) overnight at 4°C and washed six times for 15 min each in 1× PBS. The EO tissue was then embedded in 3% agar, trimmed, and glued to a vibratome chuck with cyanoacrylate. The chuck was mounted on a vibratome (Leica Series 1000), and 100-μm sections were cut and mounted on microscope slides using VectaShield with 4,6-diamidino-2-phenylindole (DAPI; Vector Laboratories).

Confocal imaging. Live electroctyes were imaged in situ on a Leica TCS SP8 laser scanning confocal microscope, with a Coherent Chameleon mode-locked Ti:sapphire laser and a ×25/0.95NA dipping objective. The images were acquired as serial sections through the entire electroctye using intensity compensation via increasing detector gain. The entire electroctye was imaged using a 2 × 4 tiled scan with a 30% overlap between adjacent images. The images were then rendered using a 3D shaded projection in Avizo Fire 8.0.1 (FEI Visualization Sciences Group).

Fixed electroctyes sections were imaged on a Leica TCS SP8 laser scanning confocal microscope using a ×63/1.3NA glycerol objective with a correction collar and an argon laser, a 488-nm laser line to observe tissue autofluorescence, and a DPSS 561-nm laser line to excite the Alexa 594. The images were acquired via serial sections with a voxel size of 160 × 160 × 160 nm and intensity compensation via increasing laser output. The images were then rendered using Imaris ×64 7.6.5 (Bitplane). Electroctye nuclei were counted by determining the number of DAPI-stained nuclei colocalized with Alexa 594 within an image series from the anterior end, posterior end,
or cell body and then extrapolating to the total observed volume of each electrocyte region.

**Western Blot**

EOs and mouse brains were isolated from animals and flash frozen in liquid nitrogen. Tissues were ground into fine powder using a prechilled pestle in a mortar filled with liquid nitrogen. Mouse brain (15 mg) and *E. virescens* EO tissue powder (100 mg) were dissolved in 1 ml of 1X NuPAGE lithium dodecyl sulfate (LDS) sample buffer (Life Technologies) containing 2.5% (vol/vol) 2-mercaptoethanol (Amresco) and then heated at 70°C for 10 min. After heating, protein samples were centrifuged at 17,000 g for 10 min to remove DNA. The collected supernatants were run on a NuPAGE Novex 4—12% Bis-Tris protein gradient gel (Life Technologies) and then transferred onto a polyvinylidene difluoride membrane using an iBlot dry blotting system (Life Technologies). Membranes were blocked in 1X Tris-buffered saline (TBS) containing 0.1% Tween 20 (TBST; Sigma-Aldrich) and 5% nonfat dry milk for 1 h at room temperature under agitation. After blocking, membranes were sequentially incubated with primary antibodies diluted in TBST containing 5% nonfat dry milk and 0.02% sodium azide (Sigma-Aldrich) including rabbit polyclonal anti-pan Nav (1:200; Alomone Labs) and mouse monoclonal antibody against the α-subunit of Na⁺-K⁺-ATPase [α5; 1:1,000; developed by D. M. Fambrough (Lebovitz et al. 1989) and obtained from the Developmental Studies Hybridoma Bank (DSHB) at University of Iowa] at room temperature for 2 h under agitation. After primary antibody incubation, membranes were washed three times for 5 min with TBST. Membranes were then incubated with secondary antibodies conjugated with horseradish peroxidase (HRP; Santa Cruz Biotechnology) diluted in TBST containing 5% nonfat dry milk and 1:2,000 for goat anti-rabbit IgG-HRP and goat anti-mouse IgG-HRP, respectively for 1 h at room temperature. After washing, proteins on the membrane were detected using the Amersham ECL prime Western blotting detection reagent (GE Healthcare) and imaged with a Chemi Doc XRS+ imaging system (Bio-Rad). Exposure time was selected manually depending on the observed signal intensity. The membranes stained with polyclonal anti-pan Na, and mouse monoclonal antibody against the α-subunit of Na⁺-K⁺-ATPase were cut to separate the EO and mouse brain strips and then exposed separately because of the large difference in signal intensity between *E. virescens* EO and mouse brain. Molecular weights of the detected protein were determined by loading Precision Plus Protein KaleidoScope standards (Bio-Rad) together with protein samples into the same gel. Final processing of the images was performed with ImageJ for 64-bit Windows (version 1.44B; National Institutes of Health).

**Immunohistochemistry**

Sections of EO were embedded completely in OCT compound, flash frozen in liquid nitrogen-chilled isopentane (Sigma-Aldrich), and stored at −80°C until further processing. Serial longitudinal sections (15–20 μm thick) were cut at −25°C using a cabinet cryostat (Leica CM 1900), mounted on gelatin-subbed slides, and air-dried overnight at room temperature. Tissue sections were then fixed in 4% paraformaldehyde (Electron Microscopy Sciences) buffered with 1X PBS for 20 min, subsequently washed 3 times for 5 min each in 1X PBS containing 0.05% Tween 20 (PBST; Sigma-Aldrich), blocked in PBST containing 2% bovine serum albumin and 5% goat normal serum (Jackson ImmunoResearch) for 1 h at room temperature, and then incubated overnight at 4°C with primary antibodies diluted in PBST. After primary antibody incubation, tissue sections were washed as described above and then incubated with Alexa Fluor 488- or 594-conjugated secondary antibodies (Jackson ImmunoResearch) diluted 1:200 in PBST for 1 h at room temperature in a humidified chamber. Sections were then washed and air dried. Slides were mounted using VectaShield with DAPI (Vector Laboratories) and kept in the dark at 4°C.

A rabbit polyclonal antibody (1:100) against an intracellular epitope of Na₉.1.x channels (anti-pan Na; Alomone Labs) was used to label voltage-gated Na⁺ channels. A mouse monoclonal antibody (1:100) against α5 [developed by D. M. Fambrough (Lebovitz et al. 1989), and obtained from the DSHB at University of Iowa] was used to label Na⁺-K⁺-ATPase. A rat antibody (1:10) against the muscle-type acetylcholine nicotinic receptor [mAb 35; developed by J. Lindstrom (Tzartos et al. 1981) and obtained from DSHB at University of Iowa] was used to label acetylcholine receptors. A mouse monoclonal antibody (1:100) against neurofilament-associated antigen (3A10; developed by T. Jessel and J. Dodd, and obtained from DSHB at University of Iowa) was used to label axon terminals. A rabbit polyclonal antibody (1:200) against Kan (channel 6.2 (B-9; Santa Cruz Biotechnology) was used to label ATP-sensitive inward rectifier K⁺ channels.

Immunohistochemistry slides were imaged on a Zeiss AxioTome.2 microscope with ×50.16NA and ×100.45NA dry objectives. Images were acquired with a Zeiss AxioCam MRm camera and then processed by Zeiss AxioVision Rel. 4.8. We used structured illumination to create optical sections of our fluorescent samples. Final processing of the images was performed with ImageJ for 64-bit Windows (version 1.44B; National Institutes of Health).

**Computational Simulations**

We modeled the *E. virescens* electrocyte as a three-compartment cell consisting of an active posterior compartment, a passive central compartment, and an active anterior compartment. We used the Hodgkin-Huxley formalism to simulate ionic currents and changes in membrane voltage. We also applied a simplified model of Na⁺ entry, diffusion, and pumping to simulate changes in Na⁺ concentrations in the three compartments. Simulated cholinergic synaptic current was applied only to the posterior compartment. The capacitance was 48.0 nF for the posterior compartment and 18.0 nF for the anterior compartment. We based these values on the surface areas of the posterior 0.35 mm and anterior 0.2 mm of the electrocyte, respectively, with surface areas determined from confocal 3D reconstructions of electrocytes. The central compartment capacitance was 18 nF, estimated as the surface area of a cylinder 0.95 mm long and 0.6 mm in diameter, dimensions determined by our imaging data. Differential equations were coded in Matlab (The MathWorks) and integrated using Euler’s method with integration time steps of 5 × 10⁻⁶ s. All model parameters are shown in Table 1.

The passive central compartment’s current balance equation included only passive leak (**I**ₗ), fixed at 5 μS, and coupling to the two active compartments:

\[
C_m \frac{dV_C}{dt} = -I_l + g_w (V_A - V_C) + g_w (V_p - V_C).
\]

where \(C_m\) is membrane capacitance, \(V_A\), \(V_C\), and \(V_p\) are the anterior, central, and posterior compartment membrane voltage, and \(g_w\) is the coupling conductance, fixed at 322 μS.

The current balance equations for the posterior and anterior active compartments were, respectively,

\[
C_{np} \frac{dV_p}{dt} = I_{syn}(t) - I_{Na} - I_l + g_w (V_C - V_p)
\]

\[
C_{na} \frac{dV_A}{dt} = -I_{KNa} - I_R - I_l + g_w (V_C - V_A),
\]

where \(I_{syn}\) represents synaptic current, \(I_{Na}\) is Na⁺ current, \(I_{KNa}\) is the Na⁺-activated K⁺ current, and \(I_R\) is the inward-rectifier K⁺ current.

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Table 1. Parameter values for the electrolyte model

<table>
<thead>
<tr>
<th>Parameter Value</th>
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<tr>
<td>$E_L$</td>
<td>20 $\mu$S</td>
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<tr>
<td>$\tilde{g}_{\text{syn}}$</td>
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<tr>
<td>$\tau$</td>
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<td>$g_{\text{f}}$</td>
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<tr>
<td>$\mu$</td>
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<td>$k_{\text{off}}$</td>
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<tr>
<td>$\lambda_{\text{on}}$</td>
<td>50 $\text{mM}^{-1} \text{ms}^{-1}$</td>
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<tr>
<td>$\eta_{\text{off}}$</td>
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</tr>
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<td>$\tilde{g}_{\text{f}}$</td>
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<tr>
<td>$\eta_{\text{f}}$</td>
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<td>$k_{\text{f}}$</td>
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<td>$\lambda_{\text{f}}$</td>
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<tr>
<td>$h_{\text{f}}$</td>
<td>0.0019 $\text{mm}^2/\text{s}$</td>
</tr>
<tr>
<td>$b_{\text{f}}$</td>
<td>0.7 $\text{mm} \cdot \text{ms}^{-1}$</td>
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<tr>
<td>$p$</td>
<td>5 $\text{mM} \cdot \text{ms}^{-1}$</td>
</tr>
<tr>
<td>$\delta$</td>
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</tr>
<tr>
<td>$b_{\text{p}}$</td>
<td>0.3 $\text{mM} \cdot \text{ms}^{-1}$</td>
</tr>
</tbody>
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See text for definitions.

For all three compartments, $I_L$ is the leak current, which is given by Eq. 4:

$$I_L = \bar{g}_L(V + 95). \quad (4)$$

where $\bar{g}_L$ is the leak conductance. The posterior compartment synaptic current, $I_{\text{Syn}}$, is given by Eq. 5:

$$I_{\text{Syn}} = \bar{g}_{\text{Syn}} \tilde{g}_{\text{Syn}(n)}(V_p - 15), \quad (5)$$

where $\bar{g}_{\text{Syn}}$ is the synaptic conductance and the time series $\tilde{g}_{\text{Syn}(n)}$ is a series of 10 alpha waveforms generated using the discrete time equation (Graham and Redman 1993):

$$\tilde{g}_{\text{Syn}(n+2)} = 2 \left( 1 - \frac{T}{\tau} \right) \tilde{g}_{\text{Syn}(n+1)} - \left( 1 - \frac{T}{\tau} \right)^2 \tilde{g}_{\text{Syn}(n)} + \left( \frac{T}{\tau} \right)^2 x(n), \quad (6)$$

where $T$ is the integration time step and $\tau$ is the time constant. The binary series $x(n)$ specifies the onset times of the synaptic inputs, and the resulting time series $\tilde{g}_{\text{Syn}(n)}$ was normalized such that $0 \leq \tilde{g}_{\text{Syn}(n)} \leq 1$.

The equation for $I_{\text{Na}}$ was divided into a transient component ($I_{\text{NaT}}$) and a persistent component ($I_{\text{NaP}}$) as in Eqs. 7 and 8:

$$I_{\text{NaT}} = \tilde{g}_{\text{NaT}} n^3 m^4 (V_p - E_{\text{Na}}), \quad (7)$$

$$I_{\text{NaP}} = \tilde{g}_{\text{NaP}} h^3 m^3 (V_p - E_{\text{Na}}), \quad (8)$$

where $\tilde{g}_{\text{NaT}}$ is the sodium conductance and $0 < \gamma < 1. E_{\text{Na}}$, the sodium equilibrium potential, was allowed to vary with changing Na$^+$ concentrations in the posterior compartment ($[\text{Na}]_p$) according to the equation $E_{\text{Na}} = 25.7 \ln (114/[\text{Na}]_p)$, assuming 114 mM extracellular Na$^+$ and a temperature of 25°C.

The anterior compartment K$^+$ currents are given by Eqs. 9 and 10:

$$I_{\text{KNa}} = \tilde{g}_{\text{KNa}} n^4 s^4 (V_A + 95), \quad (9)$$

$$I_K = \tilde{g}_K \left( \frac{1}{1 + \exp \left[ \eta_K (V_m + 110) \right]} \right) (V_A + 95). \quad (10)$$

The gating variables $m$, $n$, and $h$ in Eqs. 7–9 are given by Eqs. 11–13, where $j = m$, $n$, or $h$ and $\alpha$, $\beta$, and $k$ are rate constants:

$$\frac{dj}{dt} = \alpha_j (1 - j) - \beta_j (j) \quad (11)$$

$$\alpha_j = k_{\alpha_j} \exp(\eta_{\alpha_j} V) \quad (12)$$

$$\beta_j = k_{\beta_j} \exp(\eta_{\beta_j} V). \quad (13)$$

We modeled the Na$^+$ dependence of $I_{\text{KNa}}$, with the gating variable, $s$, which is determined by the Na$^+$ concentration in the bulk cytoplasm in the anterior compartment ($[\text{Na}]_A$), according to Eq. 14:

$$\frac{ds}{dt} = k_{\text{[Na]}} (1 - s) - k_{\beta s}. \quad (14)$$

We modeled Na$^+$ concentrations in each compartment on the basis of the compartmental volumes, which were $4.2 \times 10^7$, $2.7 \times 10^8$, and $1.7 \times 10^7 \mu\text{m}^3$ for the posterior, central, and anterior compartments, respectively. The posterior and anterior compartment volumes were measured from our 3D reconstructions of electrocytes, whereas the central compartment volume was estimated as the volume of a cylinder 0.95 mm long and 0.6 mm in diameter. The initial Na$^+$ concentration in all three compartments was 15 mM. The equation for Na$^+$ concentration in the posterior compartment is

$$\frac{d[\text{Na}]_p}{dt} = p + \frac{q}{V_p} - \delta([\text{Na}]_p - [\text{Na}]_T), \quad (15)$$

where $V_p$ is the posterior compartment volume, $p$ represents sodium leak, and $q$ is moles of Na$^+$ ions entering through the cholinergic receptors and Na$^+$ channels, given by Eq. 16:

$$q = \frac{\text{d} (2 I_{\text{Syn}} + I_{\text{Na}})}{e L}. \quad (16)$$

wherein the integrated Na$^+$ current, $\text{d}(2I_{\text{Syn}} + I_{\text{Na}})$ in nanoamperes-milliseconds (nA·ms), is multiplied by $10^{-12}$ to yield coulombs, divided by the elementary charge on a monovalent cation, $e$, to yield the number of Na$^+$ ions, and divided by Avogadro’s constant, $L$, to yield moles of Na$^+$. $I_{\text{Syn}}$ was multiplied by 2 in Eq. 16 to account for Na$^+$ entry associated with $I_{\text{Syn}}$ where $\tilde{g}_{\text{Syn}}$ arises from cholinergic receptors, assuming the Na$^+$ permeability is twice that of the K$^+$ permeability. Diffusion of Na$^+$ between compartments is governed by $\delta$ (the diffusion rate constant), the difference in Na$^+$ concentration between the compartments, and the ratio of their volumes ($\lambda$). Na$^+$ removal is modeled by the fractional pumping rate $b_p$, representing the rate at which Na$^+$ is pumped out of the posterior compartment to the extracellular space.

The central compartment’s Na$^+$ concentration was affected only by diffusion to and from the posterior and anterior compartments as in Eq. 17:

$$\frac{d[\text{Na}]_T}{dt} = \delta([\text{Na}]_p - [\text{Na}]_T) \frac{\lambda_p}{\lambda_C} - \delta([\text{Na}]_C - [\text{Na}]_A) \frac{\lambda_C}{\lambda_A}. \quad (17)$$

The posterior compartment Na$^+$ concentration is given by Eq. 18:

$$\frac{d[\text{Na}]_A}{dt} = \delta([\text{Na}]_A) \frac{\lambda_C}{\lambda_A} - b_p [\text{Na}]_A, \quad (18)$$

which includes diffusion to and from the central compartment, as well as $b_a$, which gives the fractional rate at which Na$^+$ is pumped from the anterior compartment to the extracellular space.

RESULTS

Gross Electrocyte Morphology

In *E. virescens*, the EO runs longitudinally along the body and extends into the caudal tail filament (Fig. 1A). Several rows of electrocytes are densely packed in the EO (Fig. 1C). We injected rhodamine B dextran (10,000 MW) into single electrocytes within an isolated section of EO, allowed 15 min for the dextran to fully diffuse, and then imaged the cell on a Leica ECS7 SP8 laser scanning confocal microscope. Three-dimen-
sional reconstructions of these cells show that electrocytes are large cylinder-like cells ~1.5 mm in length and 600 µm in diameter (Fig. 2A). The electrocyte’s surface structure is not uniform, and based on differences in surface structure, we divided the cell into three regions: the posterior face, the main body, and the anterior face (Fig. 2A; Supplementary Movie 1). (Supplementary data for this article is available online at the Journal of Neurophysiology website.) The space between adjacent electrocytes along the rostral-caudal axis is ~30 µm, and the posterior face of each electrocyte is surrounded by the anterior face of the adjacent cell (Fig. 2B).

The surface of the posterior face was densely occupied by narrow invaginations that extended longitudinally into the cell ~300 µm, resulting in a pronounced increase in membrane surface area (Fig. 2C; Supplementary Movie 2). The anterior face usually contained three large lobes with smaller papillary extensions. In addition, a network of capillaries was observed embedded immediately underneath the ruffled anterior membrane (Figs. 2D and 3B2; Supplementary Movie 3). In contrast to the posterior and anterior faces, the surface of the main body is relatively smooth, with an array of spherical structures just beneath the membrane (Fig. 2E).

Electrocytes are multinucleated cells formed by the fusion of skeletal myocytes during development (Bennett 1970; Unguez and Zakon 1998a, 2002; Zakon and Unguez 1999), resulting in a syncytium with nuclei localized to the surface of the electrocyte (Machado et al. 1976). Our images of DAPI-stained nuclei colocalized with Alexa Fluor 594 within our 3D reconstructions confirm that the nuclei of *E. virescens* electrocytes are densely distributed in a thin layer near the membrane (Fig. 2F; see Fig. 3A for colocalization). The combination of whole cell 3D imaging and high-resolution imaging of smaller electrocyte sections in the present study allowed us to estimate the number of nuclei within a single electrocyte by two distinct methods, both of which indicated that each electrocyte has ~30,000 nuclei.

The first method we used was to create an isosurface around the whole cell image of the electrocyte (Fig. 2A). This allowed us to measure the surface area of the entire electrocyte, which was $4.5 \times 10^3 \, \mu m^2$. We then counted the number of nuclei per unit surface area in a single high-resolution image of the cell body and found that there were 220 nuclei per $2.8 \times 10^4 \, \mu m^2$ of cell surface area. This method thereby yielded $(4.5 \times 10^3 \, \mu m^2)(220 \, \text{nuclei}/2.8 \times 10^4 \, \mu m^2) = 35,000 \, \text{nuclei}$ in the electrocyte.

To validate the above estimation, we used an alternate approach to estimate the number of nuclei within an electrocyte by modeling the electrocyte with simple geometries, with the anterior end and cell body approximated as a hollow cylinder with a paraboloid scoped out at the anterior end, and the posterior end as a solid paraboloid. The anterior end had a cupped shape, with nuclei along the surface, so the surface area was approximated as a paraboloid with a measured radius of 365 µm and a depth of 144 µm, giving a surface area of $4.8 \times 10^3 \, \mu m^2$. We then counted the number of nuclei in a single high-resolution image of the anterior region with $1.5 \times 10^5 \, \mu m^3$ of surface area and found 170 nuclei, giving a total of $(4.8 \times 10^3 \, \mu m^2)(170 \, \text{nuclei}/1.5 \times 10^5 \, \mu m^3) = 5.4 \times 10^4 \, \text{nuclei}$ in the anterior end. The central compartment end was approximated as a hollow cylinder with a measured length of $1.0 \times 10^3 \, \mu m$ and a measured diameter of 600 µm. Therefore, the total number of nuclei on the cell body would be $(600 \, \mu m)(\pi(1,000 \, \mu m))(220 \, \text{nuclei}/2.8 \times 10^4 \, \mu m^2) = 1.5 \times 10^5 \, \text{nuclei}$ in the central compartment. For the posterior end, since the invaginations penetrate back to the cell body, resulting in nuclei being located throughout the posterior end, we modeled the posterior end as a solid paraboloid. The paraboloid had a measured length of 590 µm and a radius of 215 µm, giving a total volume of $8.6 \times 10^7 \, \mu m^3$. We then measured the number

*Fig. 2. Gross morphology of *E. virescens* electrocytes. A: 3-dimensional (3D) reconstruction from serial confocal scanning through a live electrocyte injected with rhodamine B dextran (10,000 MW). Arrow indicates site of dextran injection. Darkened horizontal and vertical lines (arrowheads) are artifacts caused by the image tile overlap. Full 3D image is shown in Supplemental Movie 1. B: a segmented autofluorescence image of the junction between two adjoining electrocytes with the posterior face of one electrocyte at left (cyan) and the anterior face of the adjacent electrocyte (pink) at right. C: a single optical section of the posterior face from a rhodamine B dextran-injected electrocyte. The posterior face contains deep invaginations, dramatically increasing the surface area of the cell. Additional detail is shown in Supplemental Movie 2. D: A single optical section of the anterior face from a rhodamine B dextran-injected electrocyte. Arrows indicate penetrating capillary structures within the anterior face. See also Supplemental Movie 3. E: orthogonal sectional views of the spherical structures beneath the membrane of the cell body. E1 shows view looking down at the cell surface. E2 shows a single Y-Z image orthogonal to view in E1, and E3 is a single X-Z image orthogonal to views in E1 and E2. F: a single 20 µm-thick electrocyte section stained with 4,6-diamidino-2-phenylindole (DAPI) to label nuclei (posterior is at right).*
of nuclei in a 7.0 \times 10^6 \mu m^3 image. To ensure that we only measured electrocyte nuclei, we only counted nuclei that were colocalized with Alexa Fluor 594. In the sample volume, we counted 1,000 nuclei. Extrapolated over the whole posterior end, there would therefore be \((8.6 \times 10^7 \mu m^3)(1,000 \text{nuclei/7.0} \times 10^6 \mu m^3) = 1.2 \times 10^8 \text{nuclei in the posterior region.}

Taken together, these quantities sum to 32,000 nuclei per electrocyte, which is in close concordance with our estimate based on the first method.

**Fine Structure of the Electrocyte**

We investigated the fine structure of the posterior and anterior faces by imaging 100-\mu m-thick serial sections of a paraformaldehyde-fixed EO sample that contained a single target electrocyte filled with Alexa Fluor 594 dextran. Electrocytes, vasculature, and pigment cells within the EO also emit a broad autofluorescence spectrum when excited by a 488-nm laser line. We took advantage of this autofluorescence to image tissue adjoining the Alexa Fluor 594 dextran-injected cell.

Our images of the posterior face showed that the surface of each invagination contained many small spine-like structures \(\sim 50 \mu m\) in length. The spines terminate in an enlarged sphere \(\sim 20 \mu m\) in diameter (Fig. 3A). Blood vessels occupy the space between electrocytes, with the majority penetrating into the invaginations of the posterior face, although a smaller number contact the anterior face (Fig. 3, A2 and B2, respectively). Capillaries occupy much of the space within each posterior-face invagination, and the spines within the invaginations are largely enveloped by these capillaries. The fine structure of the anterior face (Fig. 3B) is generally less complex than that of the posterior face, with the unique feature that capillaries appear to reside within enclosed, tubelike structures proximal to the anterior face membrane (Fig. 3B2). An additional and striking difference between the anterior and posterior faces is that the posterior-face membrane is densely occupied by vesicles, which are exceedingly less abundant on the anterior face (Fig. 3, C and D).

**Subcellular Localization of Cholinergic Receptors, Ion Channels, and Ion Transporters**

In gymnotiforms, electrocyte APs are controlled by a medullary pacemaker nucleus via spinal electromotor neurons that innervate each electrocyte (Fig. 1A). Labeling of spinal nerves with the antibody 3A10 against neurofilament-associated antigen (Unguez and Zakon 1998b) showed that only the posterior face is innervated and that the innervation occurs throughout the posterior face (Fig. 4A). We also found that acetylcholine receptors were clustered only on the posterior face (Fig. 4B). Given that the cholinergic synapses are restricted to the posterior membrane, we hypothesized that the other ion channels also would be localized on the posterior membrane.

We labeled voltage-gated Na\(^+\) channels (Na\(_a\)) with a pan-Na\(_a\) antibody raised against an epitope identical in all isoforms of Na\(_a\), and found that Na\(_a\) channels were expressed only on the electrocyte’s posterior face (Fig. 4C). To ensure these signals are not from the innervating axons, we co-stained Na\(_a\) channels with 3A10 and found no colocalization between them (Fig. 4D). The expression pattern of acetylcholine receptors and Na\(_a\) channels indicates that the posterior face is the only entrance site for Na\(^+\) influx and the site of AP initiation.

Based on electrophysiological and molecular evidence that electrocytes express inward-rectifier and K\(_Na\) channels (Markham et al. 2013), we immunolabeled both channels and, to our surprise, found that both are localized only to the anterior face (Fig. 4, E and F). Immunolabeling of Na\(^+\)-K\(^+\)-ATPase \(\alpha\)-subunits showed that these are found on both the anterior and posterior faces, another unusual arrangement given that the sources of Na\(^+\) influx are restricted to the posterior membrane (Fig. 4G).
Given the counterintuitive spatial separation of the Na\(^+\) channels, K\(_{\text{Na}}\) channels, and Na\(^+\)-K\(^+\)-ATPases, we performed Western blot analyses to ensure that these antibodies indeed specifically labeled the proteins of interest. Western blot of \(E.\) \(virescens\) EO and mouse brain whole cell lysate labeled with polyclonal anti-Na, detected specific bands of \(\sim 250\) kDa (the predicted molecular mass of Na\(_{\text{a}}\) channels) in both tissues (Fig. 4H). Similarly, polyclonal anti-\(k\text{cnt1}\) (K\(_{\text{Na}}\)) and monoclonal anti-Na\(^+\)-K\(^+\)-ATPase \(\alpha\)-subunit labeled bands of \(\sim 130\) and \(100\) kDa, respectively, in both EO and mouse brain (Fig. 4H). These molecular masses correspond with the predicted molecular masses of K\(_{\text{Na}}\) channels and Na\(^+\)-K\(^+\)-ATPase \(\alpha\)-subunits.

### Numerical Simulations of Electrocyte Function

Given the unusually large separation of K\(_{\text{Na}}\) channels from potential Na\(^+\) sources revealed by our imaging data, we used computational simulations of electrocyte action potentials and Na\(^+\) dynamics to test whether the high-frequency firing typical of \(E.\) \(virescens\) electrocytes could be reproduced in a model cell where the spatial arrangement of ion channels matched our imaging data. Our model cell had three compartments: an active posterior compartment, a passive central compartment, and an active anterior compartment. The passive properties of these compartments were guided by morphological measurements of their volumes and membrane areas. We estimated the coupling conductances between compartments on the basis of our measurements of the electrocyte’s length and diameter.

The posterior compartment had linear leak current, synaptic current via cholinergic receptors, and voltage-gated Na\(^+\) current (\(I_L\), \(I_{\text{Syn}}\), and \(I_{\text{Na}}\), respectively). The central compartment had only \(I_L\), and the anterior compartment had \(I_L\) inward-rectifier K\(^+\) current (\(I_R\)), and K\(_{\text{Na}}\) current (\(I_{K_{\text{Na}}}\)). Unlike previous simulations of \(I_{K_{\text{Na}}}\) where the Na\(^+\) sensitivity of the current arises from direct access to a localized persistent Na\(^+\) current (Brown et al. 2008; Markham et al. 2013), we modeled \(I_{K_{\text{Na}}}\) in this study such that its Na\(^+\) sensitivity was determined by the Na\(^+\) concentration in the bulk cytoplasm of the anterior compartment. Our model also simulated changes in intracellular Na\(^+\) in each compartment on the basis of Na\(^+\) influx resulting from a static Na\(^+\) leak, \(I_{\text{Syn}}\) and \(I_{\text{Na}}\) in the posterior compartment, a fractional Na\(^+\) pumping rate in both the...
posterior and anterior compartments, and Na\(^+\) diffusion between all three compartments.

In the full model with all currents present according to the parameters in Table 1, the model cell maintained repetitive firing in response to 500-Hz synaptic stimulation (Fig. 5A). During these APs, \(I_{Na}\) reached peak currents of \(\sim 15\) µA, consistent with earlier experimental results (Lewis et al. 2014), and \(I_{KNa}\) exhibited current magnitudes exceeding 5 µA (Fig. 5B), suggesting an important role in shaping the cell’s firing pattern. Na\(^+\) concentrations in the three compartments did vary in response to Na\(^+\) influx, pumping, and diffusion, but only over a range of 1–2 mM (Fig. 5C). As a result, the fractional activation of \(I_{KNa}\) due to Na\(^+\) concentrations in the anterior compartment remained fairly steady around 0.7 throughout the action potential train.

To evaluate whether \(I_{KNa}\) is a necessary component of AP repolarization in the model cell, we made a model cell with all of the same parameters as in Fig. 5, but set the conductance for \(I_{KNa}\) to zero. This model cell could not maintain repetitive firing at 500 Hz (Fig. 5, D and E) and instead remained in a state of depolarized oscillation. These results provide evidence that \(I_{KNa}\) in our model cell is necessary for AP repolarization and plays a role in AP termination even when the \(K_{Na}\) channels are at a great distance from transient Na\(^+\) sources.

**DISCUSSION**

The most striking finding from this study is the extreme compartmentalization of ion channels and ion transporters across vast distances in \(E.\) \(virescens\) electrocytes. The \(K_{Na}\) channels that repolarize the electrocyte AP and a substantial portion of the Na\(^+-K^+\)-ATPases responsible for removing Na\(^+\) after each AP are separated by more than a millimeter from the only identified sources of Na\(^+\) influx.

The presence of \(K_{Na}\) channels at such a great distance from Na\(^+\) sources raises the important question of how these channels are activated during the AP. In previous work with \(K_{Na}\) channels from other taxa, channel activation required intracellular Na\(^+\) concentrations ([Na\(^+\)]\(_i\)) that far exceed those normally found in bulk cytoplasm (Dryer et al. 1989; Kameyama et al. 1984; Yuan et al. 2003). In mammalian neurons, \(K_{Na}\) channels are clustered within just a few micrometers of Na\(^+\) channels in microdomains that allow localized elevation of Na\(^+\) concentrations sufficient to activate \(K_{Na}\) channels without changing [Na\(^+\)]\(_i\) in the bulk cytoplasm (Budelli et al. 2009; Hage and Salkoff 2012). However, we show in the present study that Na\(^+\) and \(K_{Na}\) channels are separated by great distances in \(E.\) \(virescens\) electrocytes, suggesting that \(K_{Na}\) channels in \(E.\) \(virescens\) electrocytes do not require proximal sources of Na\(^+\) influx.

One hypothesis as to how \(K_{Na}\) channels are activated in electrocytes is that electrocytes experience a significant in-

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**Fig. 5.** Computational simulations of electrocyte APs in a model electrocyte with (A–D) and without \(K_{Na}\) channels (E–G). A: membrane potential (\(V_m\)) of the posterior (red), central (black), and anterior (blue) compartments of a model cell with \(K_{Na}\) during a train of 10 APs elicited at 500 Hz by simulated synaptic conductances. B: time course of the Na\(^+\) current (\(I_{Na}\)), \(K_{Na}\) current (\(I_{KNa}\)), and synaptic current (\(I_{Syn}\)) during the AP train shown in A, C: time course of internal Na\(^+\) concentrations ([Na\(^+\)]\(_i\)) in the posterior (red), central (black), and anterior (blue) compartments during the AP train shown in A. The different initial Na\(^+\) concentrations reflect the equilibrium of resting Na\(^+\) leak, pumping, and diffusion rates as shown in D, D: posterior \(V_m\) and time course of [Na\(^+\)]\(_i\) in the posterior (red), central (black), and anterior (blue) compartments during the same simulation shown in A, but on an expanded timescale that shows the initial changes in Na\(^+\) concentrations as the Na\(^+\) leak, pumping, and diffusion processes reach equilibrium. E: \(V_m\) of the posterior (red), central (black), and anterior (blue) compartments of a model cell without \(K_{Na}\) during a train of 10 APs elicited at 500 Hz by simulated synaptic conductances. F: time course of \(I_{Na}, I_{KNa}\), and \(I_{Syn}\) during the AP train shown in E. G: time course of [Na\(^+\)]\(_i\) in the posterior (red), central (black), and anterior (blue) compartments during the AP train shown in E. The different initial Na\(^+\) concentrations reflect the equilibrium of resting Na\(^+\) leak, pumping, and diffusion rates as shown in D.
crease in $[\text{Na}^+]_i$ during each AP or during sustained high-frequency firing, a distinct possibility given the magnitude of $I_{\text{Na}}$ during the AP. We found a high density of Na$^-$/K$^-$/ATPases on the anterior face, consistent with earlier biochemical data (Denizot 1982), suggesting that Na$^+$ influx from the posterior face ultimately increases $[\text{Na}^+]_i$ in the anterior region. Our computational simulations suggest that only small changes in $[\text{Na}^+]_i$ occur during high-frequency firing in *E. virescens* electrocytes. This suggests instead a second hypothesis, that the electrocyte’s $K_{\text{Na}}$ channels are more sensitive to $[\text{Na}^+]_i$ than most other $K_{\text{Na}}$ isoforms identified to date. Indeed, our simulated $I_{K_{\text{Na}}}$ was based on Na$^+$-dependent rate constants that created partial activation with Na$^+$ concentrations of $\sim 15$ mM. Multiple factors determine the Na$^+$ sensitivity of $K_{\text{Na}}$ channels. Single amino-acid substitutions can shift the Na$^+$ sensitivity of $K_{\text{Na}}$ channels over a range of 200 mM (Zhang et al. 2010). Given the elevated rates of evolution for other ion channels in gymnotiform electrocytes (Zakon et al 2006, 2008), it seems possible that $K_{\text{Na}}$ channels also could have undergone evolutionary changes that would enhance Na$^+$ sensitivity. Additionally, intracellular factors such as NAD$^+$ can modulate the Na$^+$ sensitivity of $K_{\text{Na}}$ channels such that the EC$_{50}$ is $\sim 17$ mM (Tamsett et al. 2009). It is possible that such molecular evolution or functional modulation in *E. virescens* $K_{\text{Na}}$ channels could allow their activation at Na$^+$ concentrations in the 15 mM range. These possibilities can only be directly addressed through cloning and heterologous expression of *E. virescens* electrocyte $K_{\text{Na}}$ channels.

Our numerical simulations in the present study suggest that the electrocyte $K_{\text{Na}}$ channels remain in a state of partial activation during repetitive firing, rather than responding to transient increases in $[\text{Na}^+]_i$ as concluded in earlier work (Markham et al. 2013). If electrocyte $K_{\text{Na}}$ channels indeed are maintaining a steady level of activation during normal repetitive firing, this raises the question of what function the channels’ Na$^+$ sensitivity serves in this system. Further experimental investigation and computational simulations of Na$^+$ dynamics in the electrocyte are needed to clarify the functional significance of $K_{\text{Na}}$ channels’ Na$^+$ sensitivity in *E. virescens* electrocytes.

Additionally, the finding that ion channels are expressed on both the anterior and posterior electrocyte faces contradicts a long-standing assumption, originating with some of the earliest electrophysiological studies of *E. virescens* electrocytes, that only the innervated face is active (Bennett 1961, 1971) and that all ion channels which produce the AP should therefore be localized to the posterior face (e.g., Assad et al. 1998). The presence of $K_{\text{Na}}$ and inward-rectifier K$^+$ channels on the electrocyte anterior membrane indicates that both the posterior and anterior faces of the electrocyte are involved in AP production.

A second important finding here is the shear magnitude and complexity of the electrocyte’s morphology. Our two-photon live-cell and fixed-tissue imaging of *E. virescens* electrocytes extends earlier electron microscopy studies (Schwartz et al. 1975) that first reported the 2D morphological polarization of these cells. Our 3D reconstructions of *E. virescens* electrocytes also show that they are extremely large multinucleated cells with striking differences in the ultrastructural features of the posterior and anterior faces. Because of the electrocytes’ large diameter, the laser scanning confocal microscope could not resolve the submicrometer detail of the medial region of the cells, preventing us from obtaining a detailed 3D reconstruction of this region of the electrocyte. However, we observed a discrete lower boundary of the fluorescence on the medial side of the electrocyte at a depth of 600 μm, suggesting that the cell is 600 μm across on both the lateral-medial axis and the ventral-dorsal axis.

The deep invaginations of the posterior region create a significant expansion of membrane surface area in a comparatively small volume. This membrane area is almost entirely occupied by vascularization on the extracellular surface and by vesicles on the intracellular surface. It is perhaps possible that some of the fibrillary structures we identified as capillaries were instead portions of the innervating spinal nerves, but this is unlikely because blood vessel walls, unlike nerve, contain autofluorescent elastin and collagen (Deyl et al. 1980), and the narrowest vessels we observed were $\sim 3$ μm in diameter with a hollow center. Additionally, the vessels branched from a large central hollow tube that was almost 20 μm in diameter (Fig. 3B2), making it extremely unlikely that these are axons. The dense vascularization of the posterior face is likely necessary to provide efficient nutrient supply and waste removal, consistent with reports that high concentrations of mitochondria are present in the posterior region (Schwartz et al. 1975). We hypothesize that the densely packed vesicles on the posterior membrane are associated with constitutive trafficking of Na$^+$ channels and Na$^+$-$K^+$-ATPases. In a related electric fish, Na$^+$ channels are constitutively cycled into and out of the electrocyte membrane, and upregulation of channel exocytosis by hormonal factors can increase $I_{\text{Na}}$ magnitude by more than 50% within minutes (Markham et al. 2009), as has been reported for *E. virescens* (Markham et al. 2013).

The proliferation of membrane surface area on the posterior face also provides a substrate for high numbers of Na$^+$-$K^+$-ATPases, which are expressed throughout the posterior membrane. Active transport of Na$^+$ by the Na$^+$-$K^+$-ATPase occurs at the rate of $\sim 10^3$ ions per second (Holmgren et al. 2000), whereas selective ion channels pass $10^5$ to $10^6$ ions per second (Morais-Cabral et al. 2001). In *E. virescens* electrocytes, $-6 \times 10^6$ Na$^+$ ions enter the electrocyte with each AP (Lewis et al. 2014) with only 1 ms between APs at 500 Hz. Accordingly, efficient removal of Na$^+$ during the brief interspike interval would depend on extremely high densities of Na$^+$-$K^+$-ATPases. The extensive posterior-face membrane area also would increase membrane capacitance and decrease resistance (assuming a constant membrane resistivity). This combination of high capacitance and low resistance would increase current flow during the AP (Schwartz et al. 1975), and the tuning of resistance relative to capacitance would determine the membrane time constant potentially influencing AP duration (Mills et al. 1992).

The complex organization of the posterior face in *E. virescens* electrocytes contrasts sharply with the morphology of the anterior face, which is relatively featureless with sparse vascularization and few detectable vesicles. Within the relatively simple organization of the anterior face, $K_{\text{Na}}$ channels, inward-rectifier K$^+$ channels, and Na$^+$-$K^+$-ATPases were densely and apparently evenly distributed across the membrane surface. The paucity of anterior-face exocytotic vesicles suggests that $K_{\text{Na}}$ channels are perhaps not cycled or trafficked in the same manner as Na$^+$ channels on the posterior face. These results
are consistent with our earlier studies of the hormonal regulation of ionic currents in *E. virescens* electrocytes. Application of adrenocorticotropic hormone (ACTH) increased the magnitudes of *I*_{Na} and *I*_{KNa}. The increase in *I*_{Na} was a direct effect of ACTH application regulating vesicular trafficking of Na\(^{+}\) channels, but the increased conductance of K\(Na\) channels was found to be a secondary effect of the hormone-induced increase in *I*_{Na} magnitude (Markham et al. 2013).

The bioelectrical properties of all excitable cells are determined by their morphology and the subcellular localization of ion channels. Electrocyte morphology is an important determinant of species- and individual-specific EOD waveforms (Bass 1986; Gallant et al. 2011; Hopkins et al. 1990; Mills et al. 1992), and the subcellular distributions and densities of ionic currents also help determine EOD waveform (Ferrari and Zakon 1993; Markham and Zakon 2014; Shenkel and Sigworth 1991). Some of the ion channels, ionotropic receptors, and ion transporters responsible for electrocytes’ biophysical properties have been localized in other gymnotiform and mormyridiform electric fish (Cuellar et al. 2006; Gallant et al. 2011; Liu et al. 2007). The present work is, to our knowledge, the first comprehensive presentation of detailed electrocyte morphology together with subcellular localization of all ionic mechanisms responsible for an electrocyte’s electrical excitability. It is of course possible that additional key membrane proteins are present but not yet detected. Of particular concern is the possibility that a second undetected isoform of K\(Na\) channel is expressed in proximity to the voltage-gated Na\(^{+}\) channels. We believe this is unlikely, because an earlier study detected only a single isoform of K\(Na\) channel in *E. virescens* electrocytes by RT-PCR, the KCNT1/Slack isoform (Markham et al. 2013), supporting the conclusion that our immunolabeling in the present study detected all K\(Na\) channels expressed in electrocytes. Moreover, the present immunolabeling study detected ion channels corresponding to all known ionic currents in *E. virescens* electrocytes, and these ionic currents are sufficient to reproduce completely the electrical behavior of these cells as shown by our computational simulations in this study and in earlier work (Markham et al. 2013).

Ultimately, the energetic demands of electrocyte APs and the ability to maintain firing rates exceeding 500 Hz throughout the animal’s life span stem from the spatiotemporal dynamics of Na\(^{+}\) entry during the AP and the subsequent Na\(^{+}\) removal within a millisecond by the Na\(^{+}\)-K\(^{+}\)-ATPases. For these peripheral excitable cells, the metabolic cost of AP generation is likely a major force governing their biophysical properties, as is the case for central neurons (Hasenstaub et al. 2010). Future studies on the temporal and spatial dynamics of Na\(^{+}\) entry and removal in electrocytes will be necessary for understanding how the ion channels and Na\(^{+}\)-K\(^{+}\)-ATPases coordinate to maintain high firing rates while managing the extremely large inward Na\(^{+}\) currents. Furthermore, additional exploration of the interaction between electrocyte Na\(^{+}\) channels and K\(Na\) channels will likely lead to new insights on the many important roles that K\(Na\) channels play in excitable cell physiology (Bhattacharjee and Kaczmarek 2005).

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS

Y.B. and M.R.M. conception and design of research; Y.B., B.E.S., and M.R.M. performed experiments; Y.B., B.E.S., and M.R.M. analyzed data; Y.B. and M.R.M. interpreted results of experiments; Y.B., B.E.S., and M.R.M. prepared figures; Y.B., B.E.S., and M.R.M. drafted manuscript; Y.B., B.E.S., and M.R.M. edited and revised manuscript; Y.B., B.E.S., and M.R.M. approved final version of manuscript.

REFERENCES


Bhattacharjee A, Kaczmarek LK. For K\(^{+}\) channels, Na\(^{+}\) is the new Ca\(^{2+}\). Trends Neurosci 28: 422–428, 2005.


