Euryhaline pufferfish NBCe1 differs from nonmarine species

NBCe1 physiology

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Marine fish drink seawater and eliminate excess salt by active salt transport across gill and gut epithelia. Euryhaline pufferfish (Takifugu obscurus, mefugu) forms a CaCO3 precipitate on the luminal gut surface after transitioning to seawater. NBCe1 (Slc4a4) at the basolateral membrane of intestinal epithelial cell plays a major role in transepithelial intestinal HCO3− secretion and is critical for mefugu acclimation to seawater. We assayed fugu-NBCe1 (fNBCe1) activity in the Xenopus oocyte expression system. Similar to NBCe1 found in other species, fNBCe1 is an electrogenic Na+/HCO3− cotransporter and sensitive to the stilbene inhibitor DIDS. However, our experiments revealed several unique and distinguishable fNBCe1 transport characteristics not found in mammalian or other teleost NBCe1-orthologs: electrogenic Li+/HCO3− cotransport; HCO3− independent, DIDS-insensitive transport; and increased basal intracellular Na+ accumulation. fNBCe1 is a voltage-dependent Na+/HCO3− cotransporter that rectifies, independently from the extracellular Na+ or HCO3− concentration, around −60 mV. Na+ removal (0Na+ pre-pulse) is necessary to produce the true HCO3−-elicited current. HCO3− addition results in huge outward currents with quick current decay. Kinetic analysis of HCO3− currents reveals that fNBCe1 has a much higher transport capacity (higher maximum current) and lower affinity (higher K0.5) than human kidney NBCe1 (hkNBCe1) does in the physiological range (membrane potential = −80 mV; [HCO3−] = 10 mM). In this state, fNBCe1 is in favor of operating as transepithelial HCO3− secretion, opposite of hkNBCe1, from blood to the luminal side. Thus, fugu-NBCe1 represents the first ortholog-based tool to study amino acid substitutions in NBCe1 and how those change ion and voltage dependence.

mefugu; euryhaline teleost; sodium-bicarbonate cotransporter; bicarbonate secretion; acid-base; Xenopus oocyte; electrophysiology; intracellular pH; membrane current

MARINE FISH LIVE IN A HYPEROSMOTIC environment, yet ionic strength of their blood plasma is similar to that of land animals. Thus these fish are in constant danger of dehydration by the inevitable osmotic loss of water through the gill. To restore body water, they drink seawater (28). However, large quantities of salts are ingested with seawater (~450 mM NaCl). This excess salt must be eliminated to maintain blood osmolarity (~350 mosM). The teleost kidney cannot produce urine more concentrated than the blood plasma (28). Therefore, the ingested seawater is partially desalted in the esophagus, which absorbs Na+ and Cl− by active and passive transport pathways (21, 24), before it enters the stomach. The desalination of the ingested seawater continues at the foregut by active transport of monovalent ions from the lumen into the blood. The desalting process reduces the osmolarity of gut fluid and allows passive absorption of water across the hindgut epithelium. The excess monovalent ions (mainly Na+ and Cl−) are eliminated by active salt transport through the salt pump located in “chloride cells” on the gills. Divalent cations (Ca2+ and Mg2+) are left behind and concentrated to extremely high levels as water absorption proceeds in the intestine.

Precipitation of a light-colored solid, i.e., “white cake,” in the marine teleost intestinal lumen, composed of Ca2+ and Mg2+ carbonates (39), was first observed decades ago (33). This phenomenon is most pronounced in highly salt-challenged fish (i.e., euryhaline species). The carbonate precipitations are a direct consequence of high intestinal lumen alkalinity (high HCO3− concentration) combined with high Ca2+ and Mg2+ concentrations (~10 mM each) from ingestion of seawater. It is recognized that intestinal HCO3− secretion via apical Cl−/HCO3− exchange is responsible for the high HCO3− concentration (6, 17). Apical anion exchangers have been considered the important components of osmoregulation in seawater-drinking marine fish, as they contribute up to 70% of net Cl− uptake and, thereby, water absorption to counteract water loss to the seawater environment (8, 17). Nevertheless, few studies have addressed the cellular mechanisms responsible for the basolateral, entrance step of intestinal HCO3− secretion.

The electrogenic Na+/HCO3− cotransporter (NBCe1, Slc4A4) at the basolateral membrane of the intestinal epithelial cell plays a major role in transepithelial intestinal HCO3− secretion in the marine teleost fish (17). We cloned, localized, and characterized NBCe1 from two freshwater fish species: Osorezan dace (Tribleodon hakonensis) (11) and zebrafish (Danio rerio) (35). NBCe1 was also cloned in the rainbow trout (Oncorhynchus mykiss), and the trout gill NBCe1 mRNA level increased during respiratory acidosis (25). Although interest in NBCe1 in freshwater fish has been growing (11, 18, 25, 35), the physiological role of NBCe1 in marine fish has only recently been studied (17, 37).

NaHCO3 absorption, a major function of the renal proximal tubule (PT), facilitates water absorption and maintains intracellular pH (pH3; renal HCO3− absorption ~350 g/day in mammalian kidney). The PT reabsorbs 80–90% of filtered HCO3− and 70% of filtered Na+. The renal NBCe1 is found at the basolateral membrane of the PT (29) and mediates almost...
all the HCO₃⁻ reabsorption and ~7% of the Na⁺ absorption in the PT. In mammals, three NBCe1 isoforms have been reported and studied functionally. Kidney NBCe1 (kNBCe1, Slc4a4-A) was reported to have an apparent 3:1 HCO₃⁻:to-Na⁺ coupling ratio, which indicates a major absorptive function (9, 34), whereas pancreatic NBCe1 (pNBCe1, Slc4a4-B), with a 2:1 HCO₃⁻:to-Na⁺ coupling ratio, is predominated by secretory function (9, 14, 34). This difference in stoichiometry is controversial (23, 30) but may involve regulation by IRBIT, an inositol 1,4,5-trisphosphate receptor-binding protein (32, 43), or phosphatidylinositol 4,5-bisphosphate (41). Curiously, even though fish must also absorb HCO₃⁻ in the kidney, kNBCe1 (NBCe1-A, Slc4a4-A) has not been positively confirmed as a transcript or protein in any teleost.

Humans with recessive NBCe1 mutations have severe kidney disease [i.e., permanent proximal renal tubular acidosis (pH <7.1, 3–12 mM HCO₃⁻)] and eye pathology [i.e., bilateral cataracts and bilateral glaucoma], but these individuals have not been reported to have intestinal or pancreatic pathology. These findings seem to indicate that the NBCe1 protein in the kidney is the major renal mechanism of HCO₃⁻ transport and systemic acid-base homeostasis. Altered NBCe1 activity caused by SLC4A4 mutations is responsible for the pathophysiology. However, the NBCe1 protein regions responsible for HCO₃⁻ and Na⁺ "binding" remain largely unknown.

Many organisms adapt to environmental factors affecting their physiology in ways that cannot easily be replicated by random mutagenesis. Use of divergent species adapting to environmental challenges allows one to determine protein tolerance for amino acid substitutions and how those changes affect function. We hypothesized that comparison of orthologous NBCe1 transporters from marine organisms with human NBCe1 would give us insight into how the Na⁺ or HCO₃⁻ transport processes are modified in the NBCe1 transporters involved in these different environmental challenges. Here we report functional characterization of an NBCe1 ortholog from an euryhaline teleost, mefugu. We identified NBCe1 species-specific differences and the first such different transport to aid in the identification of NBCe1 regions crucial to ion binding.

METHODS

Fugu NBCe1 Cloning

We cloned fugu NBCe1 (fNBCe1) primers based on genomic and expressed sequence tag sequences, as previously described (17). To optimize fNBCe1 functional expression in Xenopus oocytes, fNBCe1 open reading frame was subcloned into an oocyte expression vector pGEMHE (42).

Oocyte Isolation and Injection

Female Xenopus laevis were purchased from Xenopus Express (Beverly Hills, FL) or Nasco International (Fort Atkinson, WI). Oocytes were removed and dissociated with collagenase, as previously described (26). The procedure was approved by the Mayo Clinic Institutional Animal Care and Use Committee. Capped fNBCe1 cRNA was synthesized using a linearized cDNA template and the T7 mMessage mMachine kit (Ambion, Austin, TX). We routinely assessed the concentration and quality of reaction products by UV absorbance and gel electrophoresis, respectively. Oocytes were injected with 50 nl of fNBCe1 cRNA (0.5 µg/µl) or water and incubated at 16°C in OR₃ medium (26). NBCe1 from zebrafish (zNBCe1, freshwater teleost) or human kidney NBCe1 (hkNBCe1, NBCe1-A) was also injected into some oocytes for comparison. Oocytes were studied 3–10 days after injection.

Oocyte Electrophysiology

The oocyte was held on a nylon mesh in a chamber through which saline flowed continuously (5 ml/min). The standard CO₂/HCO₃⁻-free ND96 saline medium contains (in mM) 96 NaCl, 2 KCl, 1 MgCl₂, 1.8 CaCl₂, and 5 HEPES (pH 7.5, 195–200 mosM). Unless otherwise stated, 33 mM NaCl was replaced by 33 mM NaHCO₃ in 5% CO₂-equilibrated HCO₃⁻ solutions (pH 7.5). In 0 Na⁺ solutions, NaCl is isosmotic ion-substituted with choline chloride.

Ion-Selective Microelectrodes

The electrodes were pulled from borosilicate fiber capillaries, silanized with bis-(dimethylamino)-dimethyl-silane, and filled with the Fluka H⁺ ionophore I cocktail B (pH) or the Fluka Na⁺ ionophore I cocktail A. The microelectrodes were backfilled, connected, and calibrated as previously described (4, 26, 31). The finished ion-selective microelectrodes, with slopes of at least −54 mV/decade change, were used to monitor pH, and intracellular Na⁺ activity (αNa) of fNBCe1 and hkNBCe1 or zNBCe1 cRNA-injected oocytes.

Two-Electrode Voltage-Clamp Procedure

Borosilicate glass pipettes, pulled to tip diameters of 1–2 µm, were filled with 3 M KCl; their resistance was 0.2–2 MΩ. Oocytes, visualized with a dissecting microscope, were impaled with microelectrodes for measurement of membrane potential (V_m) and passing current. Oocyte membrane currents were controlled using an OC-725C voltage clamp (Warner Instruments, Hamden, CT), filtered at 2–5 kHz, digitized at 10 kHz, and recorded with Pulse software, and data were analyzed using the PulseFit program (HEKA), as previously described (4). When voltage-step protocols were not being run, oocytes were clamped at a holding potential (V_h) of −60 mV, and current was constantly monitored and recorded at 10 Hz. Current-voltage (I-V) protocols consisted of 100-ms steps from −160 to +60 mV in 20-mV steps. Voltage dependence of fNBCe1 transport was determined by plotting the mean steady-state current against voltage, as previously reported for rat kidney NBCe1 (rkNBCe1) and hkNBCe1 (2, 31).

pH Measurements During V_m Clamping

With three electrodes in the oocyte, V_m and pH were allowed to stabilize. The oocyte was then clamped to a V_h of −60 mV. At steady state, clamping current and pH were monitored, with sampling at 0.5–1 Hz. From the pH electrode voltage, we subtracted the V_m signal (from the voltage-clamp apparatus), yielding a voltage due solely to pH. The data were sampled by Pulse software (HEKA) and interfaced to a personal computer. The solution protocol was modified from previous study on hkNBC (2, 4) and zNBCe1 (35). The bath solution was changed from ND96 to 0 Na⁺ ND96 solution to access the Na⁺-dependent components of the transporter (Fig. 1). The HCO₃⁻-dependent components were studied by addition of 5% CO₂/33 mM HCO₃⁻ (pH 7.5) in the absence of Na⁺. Extracellular Na⁺ Dependence

The solution protocol was modified from a previous study on rkNBC (rkNBCe1-A) (31) for the extracellular Na⁺ dose response (see Fig. 3). Oocytes were voltage-clamped to a V_h of −60 mV. A baseline non-HCO₃⁻ I-V relation was recorded once current stabilized after initial electrode impalements. The bath solution was changed to 5% CO₂/33 mM HCO₃⁻,120 mM Na⁺ for 6 min to allow a steady-state current to be reached and the oocyte to be loaded with CO₂ (and acidify). The bath solution was changed to 0 mM Na⁺ (5% CO₂/33 mM HCO₃⁻,96 mM choline-48 mM mannitol) for an additional 6 min for a HCO₃⁻ I-V baseline. The bath solution was pulsed to a test Na⁺
solution (4.32, 12, 36, 96, and 120 mM Na\(^+\)) and then quickly back to 0 mM Na\(^+\). An I-V relation was recorded at the peak current induced by each test Na\(^+\) solution.

**Extracellular CO\(_2\)/HCO\(_3\)_ Dependence**

Oocytes expressing fNBCe1 or hkNBCe1 were voltage-clamped to a V\(_h\) of −60 mV. A baseline non-HCO\(_3\) I-V relation was recorded once current stabilized after initial electrode impalements. The extracellular perfusion solutions (pH 7.5) used in the experiment contained 5% CO\(_2\)/33 mM HCO\(_3\), 1.5% CO\(_2\)/10 mM HCO\(_3\), and 0.5% CO\(_2\)/3.5 mM HCO\(_3\). The bath solution was changed to CO\(_2\)/HCO\(_3\) solution with or without Na\(^+\) (choline replacement) to measure the HCO\(_3\)-dependent current of that specific CO\(_2\)/HCO\(_3\) concentration (see Fig. 6). Steady-state I-V relations were recorded after Na\(^+\) was added back into the bath solution as steady-state HCO\(_3\) -dependent current.

**Cation Dependence**

The cation selectivity of fNBCe1-mediated cotransport was tested by measurement of cation current responses in HCO\(_3\) solution. Briefly, an oocyte was perfused with 5% CO\(_2\)/33 mM HCO\(_3\) -96 mM Na\(^+\) solution for 5 min and then for 5 min with 0 mM Na\(^+\) (5% CO\(_2\)/33 mM HCO\(_3\) -96 mM choline). The same protocol was repeated again and followed by a test cation solution (5% CO\(_2\)/33 mM HCO\(_3\) -96 mM Li\(^+\)). Test solutions were isosmotic ion-stabilized with choline chloride or LiCl, as previously described (31). Similar experimental protocols were used in other experiments to monitor V\(_m\) and \(\Delta\)Na\(_i\) of oocytes in unclamped condition. Na\(^+\) microelectrodes were used as Li\(^+\) sensor in the experiment to monitor Li\(^+\) movement across the membrane during Li\(^+\) replacement.

**DIDS Inhibition**

Slc4 members are known to be sensitive to disulfonic stilbene derivatives such as DIDS (27). Oocytes were injected with fNBCe1 or hkNBCe1 and clamped at −60 mV. Inhibition of the steady-state CO\(_2\)/HCO\(_3\) -elicted currents inhibited by 200 \(\mu\)M DIDS (Sigma, St. Louis, MO) was compared between hkNBCe1 and fNBCe1.

**Kinetic Analysis**

Kinetic analyses were performed by fitting the data using GraphPad Prism (San Diego, CA) to estimate Michaelis-Menten (\(K_m\)) parameters for extracellular Na\(^+\) concentration ([Na\(^+\)]\(_o\)) of each voltage.

**RESULTS**

**HCO\(_3\) Transport by Fugu NBCe1**

To functionally characterize fugu NBCe1 (fNBCe1), we injected Xenopus oocytes with fNBCe1 cRNA and measured pH\(_i\) and membrane current with V\(_m\) clamped at −60 mV, a method previously used for other NBCe1 clones (4, 31). Our initial experiments with fNBCe1 revealed that Na\(^+\) handling was somewhat different from other NBCe1 clones. Therefore, to examine the Na\(^+\)- and HCO\(_3\)-dependent components, we modified our solution protocol to first remove Na\(^+\) (0 Na\(^+\)) then add CO\(_2\)/HCO\(_3\). Removal of Na\(^+\) from ND96 saline medium (0 Na\(^+\)-ND96) caused an acidification (−122.2 ± 22.4 \(10^{-5}\) pH units, \(n = 7\)) and 609.7 ± 82.4 nA outward current (NaHCO\(_3\) efflux) (Fig. 1A, segment ab), which were not observed in zNBCe1 (Fig. 1B) or hkNBCe1 (Fig. 1C). Addition of 5% CO\(_2\)/33 mM HCO\(_3\) (pH 7.5) in the absence of Na\(^+\) caused rapid acidification due to CO\(_2\) hydration and H\(^+\) release without obvious current changes in all three NBCe1 clones (segment bc). Readdition of Na\(^+\) in the presence of CO\(_2\)/HCO\(_3\) elicited a robust alkalinization of the cell and huge outward current (NaHCO\(_3\) influx; Fig. 1A, segment cd). The rapid rundown of the CO\(_2\)/HCO\(_3\) -elicted outward current (Table 1) gave rise to reproducible sharp peak currents (Fig. 1), which were not observed in zNBCe1 nor hkNBCe1 after the same solution maneuver. The alkalinization and acidification are also larger in response to solution change with fNBCe1 than previously observed with other NBCe1 orthologs.

**Na\(^+\) Transport by fNBCe1**

To directly test the ability to transport Na\(^+\), we used Na\(^+\) microelectrodes to measure \(\Delta\)Na\(_i\) (mM) and V\(_m\). Resting \(\Delta\)Na\(_i\) was elevated in fNBCe1 oocytes to 7 mM compared with 2 mM in hkNBCe1-injected oocytes (Fig. 2). Addition of 5% CO\(_2\)/33 mM HCO\(_3\) (pH 7.5) hyperpolarized fNBCe1 oocytes (−43.2 ± 1.1 mV) and further increased \(\Delta\)Na\(_i\) to close to 9 mM. Bath Na\(^+\) removal returned \(\Delta\)Na\(_i\) levels (Table 2). The rates of \(\Delta\)Na\(_i\) change were faster in response to solution changes in fNBCe1 oocytes than hkNBCe1-injected oocytes. However, the V\(_m\) changes in response to solution changes
were not parallel to the αNa changes. By the end of the experiments, αNa was slightly elevated in both groups.

**Kinetics of Na⁺-Dependent Currents**

The solution protocol illustrated in Fig. 3 was used to study the extracellular Na⁺ dose-response profile of NBCe1. The current (Vₘ = -60 mV) of NBCe1-expressing oocytes was recorded for the duration of the experiment at 1 Hz. The current maximized within 6 min of initial CO₂/HCO₃⁻ perfusion. The stable baseline in 0 Na⁺-CO₂/HCO₃⁻ was established after 6 min in the solution, the I-V curve was used as “0” for extracellular Na⁺, and I-V relations were recorded at the peak current in each test Na⁺ solution (4.32, 12, 36, 96, and 120 mM Na⁺). These results were used to calculate the fNBCe1 apparent affinity coefficient for extracellular Na⁺ (Kₘ) and maximum current (Iₘax) of Na⁺-HCO₃⁻ cotransport at each voltage measured. The fNBCe1-specific currents (IₓNBCe1) were obtained by subtracting the baseline non-HCO₃⁻ current at the same Vₘ from the elicited current at a given [Na⁺]ₒ and Vₘ (i.e., IₓNBCe1 = I₋ND96), as shown in Fig. 3B. This subtraction yields the full-range extracellular Na⁺-dependent fNBCe1 activity. The averaged outward currents (−60 to +60 mV in 20-mV steps) and inward currents (−160 to −80 mV in 20-mV steps) vs. [Na⁺]ₒ are plotted in Fig. 4, A and B, respectively. Kinetic analysis was performed by fitting the data to Kₘ parameters for [Na⁺]ₒ of each voltage. The averaged Kₘ for extracellular Na⁺ varies depending on the voltage (Fig. 4C), and the Kₘ values are generally lower for outward currents (24.0 ± 2.5 mM) than inward currents (43.4 ± 2.3 mM). Iₘ}_{max}

<table>
<thead>
<tr>
<th>Condition</th>
<th>Units</th>
<th>fNBCe1 (n = 7)</th>
<th>hNBCe1 (n = 5)</th>
<th>hNBCe1 (n = 4)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Initial pH</td>
<td></td>
<td>7.48 ± 0.06</td>
<td>7.66 ± 0.07</td>
<td>7.43 ± 0.01</td>
</tr>
<tr>
<td>Final pH</td>
<td></td>
<td>7.18 ± 0.06</td>
<td>7.69 ± 0.04</td>
<td>7.52 ± 0.09</td>
</tr>
<tr>
<td>ΔΗᵢ (0 Na⁺-ND96⁻)</td>
<td>mM</td>
<td>-0.117 ± 0.026</td>
<td>-0.582 ± 0.027</td>
<td>-0.517 ± 0.023</td>
</tr>
<tr>
<td>ΔΗᵢ (0 Na⁺-CO₂/HCO₃⁻)</td>
<td>mM</td>
<td>0.459 ± 0.055</td>
<td>0.135 ± 0.022</td>
<td>0.415 ± 0.048</td>
</tr>
<tr>
<td>ΔΗᵢ (0 Na⁺-CO₂/HCO₃⁻)</td>
<td>mM</td>
<td>-0.359 ± 0.07</td>
<td>-0.093 ± 0.012</td>
<td>-0.241 ± 0.041</td>
</tr>
<tr>
<td>[HCO₃⁻]</td>
<td>mM</td>
<td>13.17 ± 0.85</td>
<td>17.26 ± 2.39</td>
<td>18.41 ± 2.32</td>
</tr>
</tbody>
</table>

ΔdpHi/dt (×10⁻⁵ pH units · s⁻¹)

0 Na⁺-ND96⁻ | ΔdpHi/dt | -122.2 ± 22.4 |
CO₂/HCO₃⁻ | ΔdpHi/dt | 764.57 ± 238.61 |
0 Na⁺-CO₂/HCO₃⁻ | ΔdpHi/dt | -400.98 ± 142.88 |
ND96 wash | ΔdpHi/dt | -547.71 ± 340.21 |

Values are means ± SE; n, number of experiments. Voltage-clamped data were collected using 3 electrodes to allow clamping at -60 mV with simultaneous intracellular pH (pHi) measurement. For pHi, and ΔpHi, although there are actually 4 significant values, 3 are shown for readability. Intracellular HCO₃⁻ concentration ([HCO₃⁻]ₙ) was calculated from the Henderson-Hasselbalch equation. fNBCe1 and hNBCe1, fugu and human kidney Na⁺ - HCO₃⁻ cotransporter. Im membrane current.
is also voltage-dependent (Fig. 4D) and decreased as the voltage approached the reversal potential ($E_{rev}$).

$E_{rev}$, Nernst Equilibrium Potential for Na$^+$, and Stoichiometry

$E_{rev}$ is the voltage where the inward movement of Na$^+$-nHCO$_3^-$ equals the outward movement of Na$^+$-nHCO$_3^-$. $E_{rev}$ values for NBCe1-expressing oocytes were determined by calculating the $x$-coordinate where the $I$-$V$ curve (Fig. 3, B and D) crosses the voltage axis (Table 3). The fNBCe1 $E_{rev}$ does not change much ($-64.5 \pm 2.9$ mV) as Na$^+$ increases. In contrast, $E_{rev}$ (hkNBCe1) shows a negative shift (from $-30$ to $-120$ mV) with the increase of bath Na$^+$ concentration. The Nernst equilibrium potential for Na$^+$ ($\Delta \Psi_{Na}$) was calculated using the Nernst equation

$$\Delta \Psi_{Na} = -\frac{RT}{Z_{Na}F} \ln\left(\frac{[Na^+]_i}{[Na^+]_o}\right)$$

where $Z_{Na}$ is the Na$^+$ valence, $T$ is temperature (K), $R$ is the gas constant, and $F$ is Faraday’s constant. The $aNa_i$ values used in the calculation are listed in Table 2. The estimated $E_{rev}$ is not in agreement with the calculated $\Delta \Psi_{Na}$ (Table 3), meaning that

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Fig. 3. A and C: experimental protocol used to study Na$^+$-dependent currents of fNBCe1 ($I_{fNBCe1}$) and hkNBCe1 ($I_{hkBCE1}$). Preparation was perfused with 5% CO$_2$/33 mM HCO$_3^-$ (pH 7.5, red, 96 mM Na$^+$). After 5–8 min for the oocyte to load with CO$_2$ (and acidify), extracellular Na$^+$ concentration ([Na$^+$]$_o$) was changed as indicated. Current-voltage ($I$-$V$) relations were measured at peak current. fNBCe1 current was $\sim 10$ times greater than hkNBCe1 current. For these experiments, Na$^+$ was replaced with choline. Current traces [holding potential ($V_h$) = $-60$ mV] were recorded for the duration of the experiment at 1 Hz. B and D: $I$-$V$ relations from 1 representative oocyte expressing fNBCe1 and hkNBCe1. Current and $I$-$V$ relations of fNBCe1 were different from those of hkNBCe1 in response to Na$^+$ manipulation. hkNBCe1 shows a negative shift of reversal potential ($E_{rev}$, from $-30$ to $-120$ mV) with readdition of Na$^+$ to the bath. In contrast, fNBCe1 $E_{rev}$ does not change as much ($-64.5 \pm 2.9$ mV) as Na$^+$ increases.

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Fig. 4. Kinetics for Na$^+$-dependent currents of fNBCe1-expressing oocytes. A and B: averaged inward ($A$, $V_h = -160$ to $-80$ mV) and outward ($B$, $V_h = -60$ to $+60$ mV) currents plotted against [Na$^+$]$_o$. Data were subjected to kinetic analysis to calculate Michaelis-Menten ($K_m$) parameters for [Na$^+$]$_o$ at each voltage. C and D: averaged $K_m$ and maximum current ($I_{max}$) at $V_h = -160$ to $+60$ mV. Values are means $\pm$ SE.
the Na+ gradient across the membrane alone does not dictate the transport direction. The apparent HCO3\textsuperscript{−}-to-Na\textsuperscript{+} coupling ratio through the NBCe1 cotransporter is known to range from 3:1 in the kidney to 2:1 in the pancreas and brain to 1:1 in the heart. The transport ratio \(n\), i.e., stoichiometry, can be calculated from the substrate concentration gradients across the membrane and the \(E_{\text{rev}}\) when the inward-to-outward (\(i-o\)) and the outward-to-inward electrochemical potentials are equal, i.e., when

\[
\Delta \mu_{\text{Na}}^{i-o} = -n\Delta \mu_{\text{HCO3}}
\]

At equilibrium, the Nernst equation expression becomes

\[
RT\ln([\text{Na}^+]_i/[\text{Na}^+]_o) + FV_m = -nRT\ln([\text{HCO3}\textsuperscript{−}]_i/[\text{HCO3}\textsuperscript{−}]_o - FV_m)
\]

where \(V_m\) is the reversal potential, or \(E_{\text{rev}}\). Intracellular Na\textsuperscript{+} and HCO3\textsuperscript{−} concentrations ([Na\textsuperscript{+}]\textsubscript{i} and [HCO3\textsuperscript{−}]\textsubscript{i}) were measured or determined from the previous experiments (Tables 1 and 2). The transport stoichiometry \(n\) for NBCe1 for each experimental condition can be calculated by substitution of these values into Eq. 3. The calculated \(n\) for NBCe1 at 33 mM extracellular HCO3\textsuperscript{−} increases from 1:1 to 4:1 HCO3\textsuperscript{−}-Na\textsuperscript{+} with the increase of Na\textsuperscript{+} gradient (Table 3). On the contrary, at 33 mM extracellular HCO3\textsuperscript{−}, \(n\) for NBCe1 remains fixed at 2:1 HCO3\textsuperscript{−}-Na\textsuperscript{+} and is insensitive to transmembrane Na\textsuperscript{+} gradient.

**Kinetics of HCO3\textsuperscript{−}-Dependent Currents**

NBCe1-expressing oocytes were voltage-clamped (−60 mV) in the non-HCO3\textsuperscript{−} solution. After stabilization, HCO3\textsuperscript{−} solutions were introduced to study the extracellular CO2/HCO3\textsuperscript{−} dose-response profile of the NBCe1s. Current traces (\(V_h = -60 mV\)) recorded at 1 Hz in response to the solution protocol are shown in Fig. 5. The steady-state HCO3\textsuperscript{−}-dependent currents were determined by subtraction of the baseline non-HCO3\textsuperscript{−} current from the HCO3\textsuperscript{−}-elicited current. Two I-V relations of HCO3\textsuperscript{−}-dependent current at the specific CO2/HCO3\textsuperscript{−} concentration after Na\textsuperscript{+} readdition are shown in Fig. 5, B and D. The first peak is the outward current. The second I point was recorded when the outward current stabilized (after current decay) immediately before exposure to the next extracellular CO2/HCO3\textsuperscript{−} concentration.

As mentioned above, kinetic analyses were performed by fitting the HCO3\textsuperscript{−}-dependent currents to \(k_m\) parameters for extracellular HCO3\textsuperscript{−} concentration (\([\text{HCO3}\textsuperscript{−}]_o\)) at −160, −60, and 60 mV \(V_h\) (Fig. 6). At +60 mV \(V_h\), \(I_{\text{max}}\) values for both NBCe1 transporters were very similar. Nevertheless, \(I_{\text{rev}}\) saturated at ~10 mM HCO3\textsuperscript{−}, and its \(k_m\) was much lower than that of hkBNCe1. NBCe1 transports oppositely from hkBNCe1 at −60 mV, yet the current magnitudes are equal. The HCO3\textsuperscript{−} affinities are also similar for both NBCe1 transporters. At negative \(V_h\), only inward currents are detected. The estimated \(I_{\text{max}}\) of HCO3\textsuperscript{−}-dependent NBCe1 at −160 mV was much greater than the \(I_{\text{max}}\) of hkBNCe1. Interestingly, NBCe1 had a higher \(k_m\) at more negative \(V_h\) (below −60 mV) than at positive \(V_h\), indicating that the transport affinity and capacity of fNBCe1 are voltage-dependent. It appears that the CO2/HCO3\textsuperscript{−} concentration affects fNBCe1 and hkBNCe1 differently, depending on voltage range.

**Li\textsuperscript{+} Replacement of Na\textsuperscript{+}**

Two-electrode voltage clamp was used to determine whether Li\textsuperscript{+} could replace Na\textsuperscript{+} as the alternative cation transported by fNBCe1. A brief 0 Na\textsuperscript{+} pulse (choline replacement) in 5% CO2/33 mM HCO3\textsuperscript{−} at the beginning of the protocol was used to confirm Na\textsuperscript{+} dependence of fNBCe1 activity (Fig. 7A).

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**Table 3. \(E_{\text{rev}}, \Delta \Psi, \text{and stoichiometry}\)**

<table>
<thead>
<tr>
<th>[Na\textsuperscript{+}]\textsubscript{o, mM}</th>
<th>[HCO3\textsuperscript{−}]\textsubscript{o, mM}</th>
<th>NBCe1 (n = 4)</th>
<th>hkBNCe1 (n = 6)</th>
</tr>
</thead>
<tbody>
<tr>
<td>4</td>
<td>33</td>
<td>Δ(\Psi_{\text{Na}}), mV</td>
<td>(E_{\text{rev}}, mV)</td>
</tr>
<tr>
<td>12</td>
<td>33</td>
<td>−14.6336379</td>
<td>−63.33 ± 9.58</td>
</tr>
<tr>
<td>36</td>
<td>33</td>
<td>13.59257482</td>
<td>−71.08 ± 6.74</td>
</tr>
<tr>
<td>96</td>
<td>33</td>
<td>41.81878755</td>
<td>−67.35 ± 4.67</td>
</tr>
<tr>
<td>120</td>
<td>33</td>
<td>67.01884714</td>
<td>−61.59 ± 5.80</td>
</tr>
<tr>
<td>290</td>
<td>33</td>
<td>72.75198649</td>
<td>−59.45 ± 6.98</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>[Na\textsuperscript{+}]\textsubscript{o, mM}</th>
<th>[HCO3\textsuperscript{−}]\textsubscript{o, mM}</th>
<th>NBCe1 (n = 6)</th>
<th>hkBNCe1 (n = 6)</th>
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</thead>
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<tr>
<td>96</td>
<td>33</td>
<td>23.60035554</td>
<td>−50.12 ± 3.89</td>
</tr>
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<td>96</td>
<td>10</td>
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<td>96</td>
<td>3.3</td>
<td>−35.5905614</td>
<td>−66.17 ± 6.5</td>
</tr>
</tbody>
</table>

Values are means ± SE; \(n\), number of experiments. HCO3\textsuperscript{−} and Na\textsuperscript{+} activities are shown in tables 1 and 2. [Na\textsuperscript{+}]\textsubscript{o} and [HCO3\textsuperscript{−}]\textsubscript{o}, extracellular Na\textsuperscript{+} and HCO3\textsuperscript{−} concentration, respectively; \(E_{\text{rev}}\), reversal potential; \(\Psi\), Nernst equilibrium potential.
During the second 0 Na+ pulse, choline replacement was transiently substituted by Li+ replacement. A spike and very fast-decaying outward current were associated with the Li+ replacement. The I-V relation at the peak current and steady state in various perfusion solutions is presented in Fig. 7B. Transiently, Li+ was able to replace Na+ as transported substrate via fNBCe1 at all tested voltages; however, the outward current decayed quickly and reversed direction (Fig. 7B). In the steady state, Li+ was able to replace Na+ only at more negative voltages (below −100 mV). Similar results were observed in a non-voltage-clamp configuration, where voltage and dNa of oocytes were measured simultaneously (Fig. 7C). The elevated dNa and hyperpolarized V_m during Li+ replacement further demonstrate that fNBCe1 can indeed transport Li+ as Na+.

HCO3^- Independence of fNBCe1

A HCO3^- independent component of I_fNBCe1 seems to be associated with Na+ removal, which was studied using the solution protocol illustrated in Fig. 5. Under voltage-clamped condition (V_h = −60 mV), outward currents elicited by 0 Na+ or CO2/HCO3^- were recorded and compared between fNBCe1- and hkNBCe1-expressing oocytes. The recorded raw (unsubtracted) currents are shown in Fig. 9, A and C. The adjusted current was obtained by subtraction of the baseline ND96 I-V relation from the 0 Na+ and CO2/HCO3^- I-V relation (see Fig. 9, B and D). The HCO3^- independent outward current elicited by 0 Na+ was only observed in fNBCe1 at more negative testing voltages (below −20 mV) with no E_rev. This outward current elicited by 0 Na+ was even more distinct at the second solution maneuver (Fig. 9).

Inhibition of fNBCe1

The current elicited by CO2/HCO3^- addition of fNBCe1- and hkNBCe1-expressing oocytes was immediately blocked (~95%) by 200 μM DIDS (Fig. 8). The inhibition effects of DIDS on the HCO3^- elicited currents were very similar for fNBCe1 and hkNBCe1 (Fig. 8). The pure I_DIDS was calculated as the difference between I_co2/HCO3^- and I_co2/HCO3^- + DIDS. I_DIDS of fNBCe1 and hkNBCe1 were voltage-dependent. The HCO3^- elicited current of fNBCe1 did not recover after DIDS was removed from the HCO3^- solution (Fig. 8A), whereas the HCO3^- elicited current of hkNBCe1 slowly, but incompletely, recovered after DIDS was removed (Fig. 8B). To determine if the HCO3^- independent currents elicited by 0 Na+ could be inhibited by DIDS, 200 μM DIDS was added to 0 Na^-ND96 using the solution protocol illustrated in Fig. 9A. Addition of 200 μM DIDS did not inhibit the 0 Na^- elicited current of fNBCe1 (Fig. 9B).

DISCUSSION

Taking advantage of niche adaptation of animals that cope with different environmental challenges, we hypothesized that transport characteristics of NBCe1 from a marine species, in constant danger of dehydration, would be different from those of human/freshwater NBCe1 orthologs. We cloned and characterized NBCe1 from a euryhaline pufferfish (T. obscurus, mefugu) by overexpression of fNBCe1 in Xenopus oocytes (17). Our data demonstrate that the molecular transport properties and inhibitor sensitivity of fNBCe1 are similar to those of other NBCe1 orthologs. However, there are unique and distinguishable fNBCe1 characteristics that are not found in mammalian or other teleost NBCe1 orthologs.

Shared Characteristics

The fNBCe1 protein has high identity to NBCe1 orthologs: 84% to dace, 89% to zebrafish, and 76% to hkNBCe1. There are transport properties shared with human and NBCe1 orthologs from freshwater fish. Electrophysiology experiments...
Fig. 7: Na⁺ and HCO₃⁻ dependence of fNBCe1. A: experimental protocol used to study Na⁺ dependence (Li⁺ replacement) of CO₂/HCO₃⁻-elicited currents in fNBCe1. Current trace was recorded at Vh = −60 mV (n = 4). Inset: inward current (off the chart) trace when CO₂/HCO₃⁻ was removed from the solution at the end of the recording. Symbols on trace in A represent I-V curves (B) collected from Li⁺-replacement experiment. CO₂/HCO₃⁻-elicited current (red) after readdition of Na⁺ into the HCO₃⁻ solution is higher than the first CO₂/HCO₃⁻-elicited current (black). transiently, Li⁺ was able to replace Na⁺ as transported substrate by fNBCe1 (light blue). C and D: experimental protocol similar to that used in A and B was used in non-voltage-clamp configuration (n = 4) to monitor membrane voltage and intracellular Na⁺ activity of fNBCe1-expressing oocyte (C) and water-injected oocyte (D).
observed in a seawater European flounder (Platichthys flesus), which does not require serosal Na\(^+\) for luminal HCO\(_3\) secretion and is not sensitive to serosal DIDS (6, 7).

In the extracellular Na\(^+\) dose-response study, we examined the Na\(^+\) dependence of the fNBCe1 cotransporter and revealed properties quite different from hkNBCe1 (Fig. 3). The fNBCe1 I-V relation rectifies at the \(E_{rev}\) (−64.5 mV) independent of [Na\(^+\)]\(_o\). \(E_{rev}\) of fNBCe1 does not change, yet \(E_{rev}\) of hkNBCe1 shows a negative shift (from −30 to −120 mV) with the increase of bath [Na\(^+\)] (Table 3). The \(K_m\) values for [Na\(^+\)]\(_o\) from fNBCe1 can be divided into outward and inward transport groups, depending on the testing voltage (\(V_0; \text{Fig. 4, A and B}\)), and the estimated \(K_m\) is 24 and 43 mM, respectively. The reported \(K_m\) for [Na\(^+\)]\(_o\), from the rkNBCe1 was voltage- and HCO\(_3\)−-independent at −30 mM (31). Similar \(K_m\) values for Na\(^+\) (21–36 mM) were reported for all three NBCe1 isoforms (NBCe1-A, -B, and -C) (22). \(I_{max}\) of fNBCe1 is also voltage-dependent; it is lowest at the \(E_{rev}\), and its voltage dependence is different from that of other NBCe1 orthologs (\(I_{max}\) typically increased with testing voltage) (22, 31).

**HCO\(_3\) transport**. Using pH microelectrodes, we monitored HCO\(_3\) transport in response to solution changes. The rate of pH change was faster in fNBCe1 than in hkNBCe1 and much faster than in zNBCe1 upon solution changes (Fig. 1), indicating that HCO\(_3\) handling is fundamentally different. Further evidence was shown in the kinetic study of CO\(_2\)/HCO\(_3\)−-dependent currents (Fig. 5). fNBCe1 is a much higher-capacity (\(I_{max}\) at −100 mV = 3.62 \(\mu\)A) but lower-affinity (\(K_m\) for HCO\(_3\)−~20 mM) HCO\(_3\)− transporter than hkNBCe1, especially around the \(V_m\) of teelost fish intestinal epithelial cell, which ranges from −80 to −100 mV (19). This result, however, is different from the HCO\(_3\) affinity of the NBCe1 isoform from seawater gulf toadfish (Opsanus beta), which is reported as 8.5–10.2 mM (37). The discrepancy may be due to differences in experimental conditions (e.g., [Na\(^+\)]\(_o\), and CO\(_2\) concentration). Alternatively, kinetic discrepancies could indicate additional interesting species differences.

Surprisingly, the calculated HCO\(_3\)−-to-Na\(^+\) transport ratio of fNBCe1 increases with Na\(^+\) gradient and is as high as 4:1 at [Na\(^+\)]\(_o\) = 120 mM, 5% CO\(_2\)/33 mM HCO\(_3\)− (Table 3). The same experimental approach showed 2:1 HCO\(_3\)−-Na\(^+\) stoichiometry of hkNBCe1, which is independent of the Na\(^+\) gradient. Similar results were found in rkNBCe1 (10, 31). Nevertheless, the HCO\(_3\)−-to-Na\(^+\) transport ratio of fNBCe1 also depended on the extracellular CO\(_2\)/HCO\(_3\)− concentrations when we kept [Na\(^+\)]\(_o\) constant (Table 3). The high HCO\(_3\)−-to-Na\(^+\) transport ratio and variable stoichiometry may be responsible for the quick rundown, spike-like CO\(_2\)/HCO\(_3\)−-elicited current only found in fNBCe1 (Fig. 1A).

fNBCe1 can also function as an electrogenic Li\(^+\)/HCO\(_3\)− cotransporter, at least transiently (Fig. 7). While small pH changes were reported with hkNBCe1 (1), voltage-clamp experiments with hkNBCe1 and other NBCe1 orthologs did not show current with cation (K\(^+\), Li\(^+\), and choline) replacements (31). However, the question remains whether Li\(^+\) can be cotransported with carbonate as an ion pair or Li\(^+\) merely substitutes for the Na\(^+\) binding site. Na\(^+\) microelectrodes can also sense Li\(^+\). Because there is almost no Li\(^+\) inside the oocyte, the Na\(^+\) electrodes are exquisitely sensitive to Li\(^+\) movement across the cell membrane. (Fig. 7, C and D). The elevated Na\(^+\) electrode signal, i.e., increased cellular Li\(^+\), and hyperpolarized membrane during Li\(^+\) replacement further demonstrate that Li\(^+\) can indeed be cotransported with carbonate as an ion pair by fNBCe1.

Our experiments revealed another unique fNBCe1 characteristic, a fast-decay Na\(^+\)-dependent, outward current elicited by HCO\(_3\)− (Figs. 1A, 5A, and 7A). The current peaked quickly and transiently, resulting in a sharp peak in the fNBCe1 current. This fNBCe1 also displayed a huge inward current (off-scale in Fig. 1A) and acidification after CO\(_2\)/HCO\(_3\)− is removed from the bath solution (Figs. 1A, 3A, and 7A). How-
ever, the huge outward current (Na+/HCO₃⁻ cotransport into the cell) occurred only after readdition of Na⁺ to the HCO₃⁻ solution. That is, the first HCO₃⁻ addition did not cause the huge outward current. Na⁺ removal (0 Na⁺ prepulse) seems necessary to produce the second HCO₃⁻ peak current (Fig. 7A). These data suggest that Na⁺ binding to fNBCe1 is much tighter than to other NBCe1 orthologs, requiring virtually complete emptying of this site. These fast-decay data may also suggest that total removal of Na⁺ from its binding site controls the turnover number of the fNBCe1 cotransporter, such that the transport rate is maximal when Na⁺ is completely removed. Alternatively, in the voltage-clamped state, intracellular Na⁺ was depleted quickly during the 0 Na⁺-HCO₃⁻ period to create greater chemical gradient across the cell membrane. Readdition of Na⁺ to the HCO₃⁻ solution reversed the transport direction and created large outward currents.

The stilbene inhibitor DIDS is known as an anion transport blocker (for channels and transporters). Slc4 members are at least partly inhibited by DIDS. This DIDS inhibition of fNBCe1 was not different from that observed with other NBCe1 orthologs (Figs. 8 and 10). However, the HCO₃⁻-elicited currents of hkNBCe1 partially recovered after DIDS was washed away, while there was no obvious current recovery for fNBCe1. In AE1 and NBCe1, DIDS has been shown to interact with lysine residues near the extracellular end of transmembrane (TM) 5 (20, 27). The putative DIDS binding motifs KKMIK (609–613) and KLKK (808–811) are also conserved in fNBCe1 amino acid sequence (20, 25). The fNBCe1 HCO₃⁻-independent, outward current elicited by Na⁺ removal was not sensitive to DIDS (Fig. 11), suggesting that a structural-molecular difference is less likely in the TM5 region of fNBCe1 than other NBCe1. These data and our previous human S427L-hkNBCe1 mutation data (4) indicate that HCO₃⁻ affinity and Na⁺ handling are associated with a region other than TM5 (likely TM1).

Reported affinity constants of NBCe1 for Na⁺ (~30 mM) (31) are generally much lower than physiological Na⁺ concentrations (145 mM); hence, NBCe1 activity is more likely dictated by the CO₂/HCO₃⁻ and Vₘ. Given a typical intestine serosal HCO₃⁻ concentration of 4–10 mM in teleost fish (21, 40) and cytosolic Vₘ difference of nearly ~80 mV (19), one could predict from the results in Fig. 5 that fNBCe1 and hkNBCe1 will favorably be transporting Na⁺ and HCO₃⁻ in opposite directions under these same experimental conditions.

**Fig. 10.** DIDS effects on CO₂/HCO₃⁻-elicited currents of NBCe1 proteins. A and B: DIDS inhibition of CO₂/HCO₃⁻-elicited currents in oocytes expressing fNBCe1 and hkNBCe1. Experimental protocols are described in Fig. 6 legend. I-V relationships show CO₂/HCO₃⁻-elicited currents, DIDS inhibition, and recovery after DIDS removal. Pure DIDS inhibition = kCO₂/HCO₃⁻ – kCO₂/HCO₃⁻ + DIDS. HCO₃⁻-elicited current slowly and incompletely recovered after DIDS removal only from hkNBCe1.

**Fig. 11.** Effect of DIDS on Na⁺-dependent currents of NBCe1 proteins. A: traces of HCO₃⁻-independent outward current elicited by 0 Na⁺ in fNBCe1 (Vₑ = −60 mV, n = 3). Experimental protocol is indicated by horizontal bars above traces. Symbols in A correspond to symbols in B. DIDS at 200 μM does not have an inhibitory effect on outward current elicited by 0 Na⁺.
In a comparison of fNBCe1 and hkNBCe1, it is not at all surprising to see their difference in transport properties, if we consider their distribution and physiological role in osmoregulation and acid-base regulation. hkNBCe1 at the basolateral membrane of the kidney PT is responsible for HCO$_3^-$/H$^+$/OH$^-$ reabsorption from the lumen to the blood side. On the other hand, fNBCe1 at the basolateral membrane of the fish intestinal epithelial cell (17) is responsible for transepithelial HCO$_3^-$/$\text{H}^+$/OH$^-$ secretion from the blood to the luminal side in concert with apical HCO$_3^-$/$\text{H}^+$/OH$^-$ secretion (Slc26a6A and Slc26a6B). However, fNBCe1 and hkNBCe1 can move Na$^+$ and HCO$_3^-$ in both directions (into to or out of the cell). The direction of the transport depends on the electrochemical gradient at its site of operation. In other words, fNBCe1 and hkNBCe1 will operate (transport) in the same direction if the circumstances are right. For example, in the non-voltage-clamp experiment (Fig. 2), electrical gradients are not manipulated (artificially controlled), and fNBCe1 and hkNBCe1 can move Na$^+$ and HCO$_3^-$ in a similar fashion in response to solution maneuvers.

Northern blot analysis also indicated high fNBCe1 transcript levels in several absorptive/secretory epithelia, including kidney and gill, and significant levels in liver, spleen, and skin (17). Similar findings were reported for other fish species, such as dace (11), rainbow trout (25), and zebrafish (18, 35). Little is known about the molecular physiology of renal HCO$_3^-$ reabsorption in fish species, but we speculate that, as in mammals, basolateral NBCe1 at the renal PT is responsible for systemic acid-base regulation and Na$^+$ reabsorption. NBCe1 expression was elevated in the teleost kidney during respiratory acidosis (11, 25), as well as in seawater-acclimated euryhaline mefugu (17). These findings are consistent with a role of NBCe1 in systemic acid-base regulation.

The fish gill is important in maintaining acid-base and osmotic homeostasis (12). It is manifested by elevated NBCe1 expression level in the gill when fishes are challenged by a pH change (11, 25) or salinity (17). Long-term exposure of teleosts to acidic water can result in acute plasma acidification and loss of NaCl, eventually leading to death. Physiological studies
have established that Osorezan dace is able to prevent plasma acidification and Na\(^+\) loss (16). These characteristics are mainly attributable to the gill chloride cells, where Na\(^-\)K\(^+\) ATPase, carbonic anhydrase II, Na\(^+/H^+\) exchanger-3, NBCe1, and aquaporin-3 increase mRNA expression after environmental acidification (11). This regulatory system resembles the function of the mammalian renal PT, yet the gill is more analogous to the lungs, as it is the organ of gas exchange (4). That is, these cells defend against metabolic acidosis by reabsorbing HCO\(_3\)\(^-\) and returning it to the systemic circulation (11). However, fish, like other teleosts, has only one NBCe1 isoform (NBCe1-B), which has been verified with functional data (35). Yet this NBCe1-B isoform in teleosts is used for systemic ion and acid-base homeostasis (11). Transport mediated by dace and zebrafish NBCe1 and human NBCe1-B (pNBCe1) is not as robust as that mediated by hNBCe1 (NBCe1-A), while protein abundance is similar. By contrast, this study illustrates that the fugu NBCe1-B transporter has higher transport capacity/turnover and different apparent affinities for Na\(^+\) than human or freshwater NBCe1 orthologs. Analogously, two forms of Na-K-Cl cotransporter (NKCC1 and NKCC2) have different Na\(^+\) affinities (13).

Nevertheless, recently, Lee et al. (18) demonstrated that zebrafish has two genes for NBCe1, zebrafish NBCe1a (zslc4a4a) and NBCe1b (zslc4a4b). In light of these new data, we used the information from previous studies and genome databases to perform phylogenetic analysis of teleost NBCe1s (Fig. 12). We found that teleost NBCe1s consist of two paralogs probably produced during a teleost-specific genome duplication (15). For clarity, we propose to name these paralogs NBCe1.1 (Slc4a4a.1; previously NBCe1a) and NBCe1.2 (Slc4a4a.2; previously NBCe1b), respectively, so that splicing isoforms of each gene may be unambiguously indicated. All the teleost-genome databases searched (fugu, Tetraodon, medaka, 3-spined stickleback, zebrafish, Nile tilapia, and cod) contain each NBCe1 paralog. The fNBCe1 analyzed in this study belongs to the NBCe1.2 subfamily, which contains dace NBCe1 (11), rainbow trout NBCe1 (25), Z. wergandicus NBCe1 (3), Mozambique tilapia NBCe1 (5), and zebrafish NBCe1b (18), and was studied in the gill, skin ionocyte, and intestine. In contrast, only NBCe1.1 (35) was studied in the kidney PT of zebrafish. Further studies are required to establish differential function of NBCe1 paralogs in freshwater and seawater fishes. Interestingly, all the genome sequences for fish NBCe1.1 encode a putative translational initiation site of the "A" isoform, yet its authenticity remains to be elucidated.

The unique transport characteristics of fNBCe1 provide a versatile tool to study amino acid substitutions in orthologs of NBCe1 and how those changes affect function. We hypothesize that NBCe1 chimeras composed of NBCe1 and NBCe1-A orthologs will allow us to pinpoint structure and membrane domains for binding, transporting, and/or "gate-keeping" of Na\(^+\) and HCO\(_3\)\(^-\). This strategy will also allow us to easily make chimeric protein cDNA constructions, which should be 100% functional, since we are merely "splicing" natural variations of the same protein. Such chimeric proteins will allow us to determine NBCe1 TM regions important for Na\(^+\) and HCO\(_3\)\(^-\) transport. Understanding the Na\(^+\) and HCO\(_3\)\(^-\) binding/transport capacity of NBCe1 will provide crucial new insights for acid-base, as well as Na\(^+\), homeostasis mediated by the proximal nephron.

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DISCLOSURES

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

AUTHOR CONTRIBUTIONS

M.-H.C. and M.F.R. are responsible for conception and design of the research; M.-H.C., C.P., and A.K. performed the experiments; M.-H.C. analyzed the data; M.-H.C. and S.H. interpreted the results of the experiments; M.-H.C., C.P., and A.K. prepared the figures; M.-H.C. drafted the manuscript; M.-H.C., Y.K., A.K., S.H., and M.F.R. edited and revised the manuscript; M.-H.C. and M.F.R. approved the final version of the manuscript.

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but not sodium-bicarbonate cotransporter 1b, plays a role in transport

of rat Na


\( / HCO_3 \) cotransporter (NBCe1) variants expressed in Xenopus oocytes: functional comparison and roles of the amino


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cotransporter (rKNBC) overexpressed in


