Slc26a6 functions as an electrogenic Cl\(^-\)/HCO\(_3\)\(^-\) exchanger in cardiac myocytes

Hyo Jeong Kim\(^1\)†, Richard Myers\(^2\)†, Choong-Ryoul Sihn\(^1\), Sassan Rafizadeh\(^2\)‡, and Xiao-Dong Zhang\(^2\)*

\(^1\)Center for Neuroscience, University of California, Davis, CA 95618, USA; and \(^2\)Division of Cardiovascular Medicine, Department of Internal Medicine, School of Medicine, University of California, One Shields Avenue, GBSF 6315, Davis, CA 95616, USA

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Aims
Alterations in cardiac acid–base balance can produce profound impact on excitation–contraction coupling and precipitate cardiac dysfunction and arrhythmias. A member of the solute carrier (SLC) family, Slc26a6, has been shown to be a chloride-hydroxyl exchanger and the predominant chloride-bicarbonate exchanger in the mouse heart. However, the exact isoforms and functional characteristics of cardiac Slc26a6 remain unknown. The objective of the present study is to determine the molecular identity of cardiac Slc26a6 isoforms, to examine their cellular expressions in the heart, and to test the function of Slc26a6 in cardiomyocytes.

Methods and results
We examined the expression and function of slc26a6 in mouse cardiomyocytes using RT–PCR, immunofluorescence confocal microscopy, and patch-clamp technique coupled with the fast solution exchange system. We identified four cardiac Slc26a6 isoforms, denoted C-a, C-b, C-c, and C-d, and detected significant expression of Slc26a6 in the plasma membrane of both atrial and ventricular myocytes. Isoforms C-a and C-b share the same sequence with the previously reported murine Slc26a6a and Slc26a6b, respectively. Isoform C-c lacks an alternate in-frame exon 12, whereas C-d is a C-terminal truncated form resulting from 102 bp exon insertion between exons 15 and 16 compared with C-b. Patch-clamp recordings demonstrated electrogenic Cl\(^-\)/oxalate and electrogenic Cl\(^-\)/HCO\(_3\)\(^-\) exchange activities in cardiomyocytes.

Conclusion
We demonstrate that cardiac myocytes express different isoforms of Slc26a6, which encode electrogenic Cl\(^-\)/HCO\(_3\)\(^-\) and Cl\(^-\)/oxalate exchangers. The electrogenic nature of the Cl\(^-\)/HCO\(_3\)\(^-\) exchange of cardiac Slc26a6 suggests important roles in regulating acid–base balance in the heart.

Keywords
Solute carrier • Slc26a6 • Cardiomyocytes • Acid–base balance • pH

1. Introduction

Acid–base balance in the heart is tightly regulated since the balance is critical to normal cardiac function. An uncompensated reduction in cytoplasmic pH results in abnormal electrical activities, acute contractile depression, disruption of intracellular Ca\(^{2+}\) signalling, and triggering of cardiac arrhythmia.\(^1,2,3\) Indeed, several H\(^+\)-equivalent transporters participate in the regulation of intracellular pH (pHi) in the heart, including Na\(^+\)–H\(^+\) exchanger (NHE), Na\(^+\)–HCO\(_3\)\(^-\) co-transporter (NBC), Cl\(^-\)–HCO\(_3\)\(^-\) exchanger (AE or CBE), Cl\(^-\)–OH\(^-\) exchanger (CHE), and monocarboxylate transporter (MCT).\(^3\) Among them, NHE and NBC mediate acid extrusion, while AE and CHE mediate acid loading.

Several solute carrier (SLC) gene families have been identified to encode for H\(^+\)-equivalent transporters in the heart, such as SLC9 for NHE, SLC4 for NBC, SLC4 and SLC26 for AE, and SLC16 for MCT.\(^4,5\) The SLC26 is a recently identified gene family of highly versatile anion exchangers encompassing 11 distinct genes. Most of the proteins in the family function as anion exchangers with diverse substrate specificity, while a few function as Cl\(^-\) channels or anion-gated molecular motors.\(^4,6\) One of the members, Slc26a6, is the most convincingly versatile anion exchanger, with reported roles in Cl\(^-\)/oxalate, Cl\(^-\)/HCO\(_3\)\(^-\),
Cl\(^{-}\)/OH\(^{-}\), Cl\(^{-}\)/SO\(_4\)\(^{2-}\), and Cl\(^{-}\)/formate exchange. It is ubiquitously and abundantly expressed in kidney, pancreas, intestines, heart, skeletal muscle, and placenta. In epithelial tissues, Slc26a6 functions as a versatile anion exchanger. However, in non-epithelial tissues, Slc26a6 functions mainly as a Cl\(^{-}\)/HCO\(_3\)\(^{-}\) (or OH\(^{-}\)) exchanger.\(^{5}\)

By examining the transcript levels of all known Cl\(^{-}\)/HCO\(_3\)\(^{-}\) exchangers in the mouse heart using quantitative real-time RT–PCR, a recent study reported that Slc26a6 is the predominant Cl\(^{-}\)/HCO\(_3\)\(^{-}\) exchanger and a specific Cl\(^{-}\)/OH\(^{-}\) exchanger in the mouse heart.\(^{6}\) This suggested important roles of Slc26a6 in the regulation of cardiac acid–base balance; however, the exact isoforms of Slc26a6 expressed in cardiomyocytes and the functional characteristics of cardiac Slc26a6 remain unknown. In the present study, we identified four Slc26a6 isoforms in the mouse heart and revealed that the Cl\(^{-}\)/HCO\(_3\)\(^{-}\) exchange activities of these isoforms are electrogenic. Further examination demonstrated the expression and the electrogenic Cl\(^{-}\)/HCO\(_3\)\(^{-}\) exchange activities of Slc26a6 in the plasma membrane of both ventricular and atrial myocytes.

2. Methods

An expanded Methods section is available in Supplementary material online.

2.1 Animals, protocols, and chemicals

All animal care and procedures were performed in accordance with the protocols approved by the Institutional Animal Care and Use Committee of the University of California, Davis. Animal use was in accordance with the National Institutes of Health guidelines. Mice (C57BL6) were anaesthetized by intraperitoneal injection of 80 mg/kg of ketamine and 5 mg/kg of xylazine, and the extent of anaesthesia was assessed by mouse’s reaction to the toe pinching. Mice were sacrificed by rapid heart excision. All chemicals were purchased from Sigma-Aldrich except specifically indicated.

2.2 RT–PCR and molecular cloning

Total RNA and mRNA were extracted and purified from a heart of P1 C57BL6 mouse.

2.3 Heterologous expression in Chinese hamster ovary cells

The DNA transfection followed the method we used before.\(^{9}\)

2.4 Isolation of cardiomyocytes

The isolation of mouse cardiomyocytes followed the conventional enzymatic dissociation methods.\(^{10}\)

2.5 Immunofluorescence confocal microscopy

Expressions of Slc26A6 in Chinese hamster ovary (CHO) cells and in isolated cardiomyocytes were detected by using rabbit polyclonal anti-Slc26a6 antibody (Sigma, 1:1000).

2.6 Patch-clamp recording

Whole-cell current recordings were performed using an Axopatch 200A amplifier, Digidata 1440 digitizer, and pClamp10 software (Axon Instrument/Molecular Device). The clamped and suspended whole cell was switched between two capillary tubes flowing control solution and test solution, respectively, to activate Slc26a6 as shown in Figure 3A with clamping potential of 0 mV. The fast solution exchange was controlled by SF-77 solution exchanger (Warner Instruments) and pClamp10 software. The sequential exposure to the control solution and test solution induces the exchange currents.

For the outward current recording, the pipette solution contained (in mM): 140 NaCl, 10 HEPES [4-(-2-hydroxyethyl)-1-piperazineneethanesulfonic acid], 1 EGTA [ethylene glycol-bis(2-aminoethyl)ether)-N,N,N’,N’-tetraacetic acid], pH 7.4; the bath control solution contained (in mM): 139 Na glutamate, 1 NaCl, 10 HEPES, 1 EGTA, pH 7.4; 1 mM Na oxalate was added to the bath control solution to make the Cl\(^{-}\)/oxalate test solution; the test solution for Cl\(^{-}\)/HCO\(_3\)\(^{-}\) exchange contained (in mM): 24 NaHCO\(_3\), 116 Na glutamate, 1 EGTA, 1 NaCl, pH 7.4 gassed with 5% CO2 and 95% O2 during usage. For the inward current recording, the pipette solution contained (in mM): 130 Na glutamate, 10 HEPES, 1 EGTA, 10 NaCl, 1 Na oxalate, pH 7.4; the bath control solution is the same as the pipette solution; the bath test solution contained (in mM): 130 NaGlut, 10 HEPES, 1 EGTA, 10 NaCl, and 0.1 Na oxalate (or 0.01 Na oxalate), pH 7.4. All experiments were performed at the room temperature.

2.7 Data analysis

Images and patch-clamp data were analysed by the Photoshop CS6 and Origin 6.0, respectively. The data points were presented as mean ± SE averaged from 4 to 8 cells. Statistical comparison was performed using the Student’s t-test with P < 0.05 considered significant.

3. Results

3.1 Identification of the different isoforms of cardiac Slc26a6

Previous studies have identified two distinct isoforms of murine intestine Slc26a6: Slc26a6a and Slc26a6b, which differ in the length of NH\(_2\) termini.\(^{11}\) The differences in the length of NH\(_2\) termini result from the inclusion of alternative splicing in exon 1. One variant containing only exon1a results in the translation of a longer 758 amino acid (a.a.) Slc26a6a, and the other variant including exon1a and exon1b leads to the translation of 735 a.a. Slc26a6b.

During the cloning of cardiac Slc26a6, several transcripts were repetitively obtained from RT–PCR amplifications, suggesting the presence of alternatively spliced variants of Slc26a6 in the heart.

We used For-long and Rev primer set (Figure 1A) for RT–PCR, and identified two bands slightly different in size on the agarose gel analysis as shown in Figure 1B. The shorter fragment was roughly three times more abundant. Fifteen clones from each product were randomly selected, compared by restriction enzyme digestion, and the full-length sequence confirmed by sequencing. Sequence analysis revealed that the shorter PCR fragment contained exon 1a encoding a longer protein containing 758 a.a., termed isoform C-a in our study, which corresponded to the previously reported Slc26a6a.\(^{11}\) No other variant was found within the randomly selected 15 clones. The longer PCR fragment of this primer set has extended exon 1 and encodes a shorter protein of 735 a.a., termed isoform C-b, corresponding to murine Slc26a6b.\(^{11}\) The sequence of the transcript with a long exon 1 (including exon 1a–intron–exon 1b) matched with the NCBI reference sequence of Slc26a6 (NM_134420.4 GI:158341685) as well as the previously cloned Slc26a6.\(^{11}\) One clone within the tested 15 clones of this longer fragment displayed a different splicing pattern, lacking the 96-bp in-frame exon 12, termed isoform C-c.

For-short primer was designed according to the sequence between exon 1 and exon 2 to verify if there were different splicing variants. We amplified the fragments without detectable multiple bands. However, there were four different patterns of splicing. The major variants (11 of 15 clones) encoded a 735 a.a. protein, the same as isoform C-b, but with a short 5′-UTR. One clone had an additional 102 bp exon inserted between exons 15 and 16, which changed the reading frame, and resulted in a 575 a.a. truncated form, termed isoform C-d.
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Figure 1 Molecular identification and sequence analysis of cardiac Slc26A6 isoforms. (A) The cloning strategy. Two different forward primers, For-long and For-short, were designed corresponding to the genomic DNA sequence in chromosome 9 (NC_000075.6 GI:372099101), one on 5′-UTR of exon 1 and the other on the intron sequence between exon 1 and exon 2 to verify isoforms having distinct 5′-UTR and/or 5′-end of coding sequence. The exon structure of the cloned isoforms was shown below. (B) RT–PCR amplification of the full-length cDNA of cardiac Slc26A6 isoforms using two sets of primers. The PCR product yield two bands using the For-long primer set and one band using the For-short primer set. Linearized pIRES-DsRed plasmid vector was also shown in the RT–PCR result. (C) Sequence analysis and alignments of the four cardiac Slc26A6 isoforms. The human SLC26A6 sequence was shown to indicate the predicted transmembrane domains labelled by the red bar under the sequence (UniProtKB/Swiss-Prot Q9BX9). The four isoforms were named as C-a, C-b, C-c, and C-d, respectively. C-a has a 23 a.a. extended NH₂-terminus. C-c lacks in-frame exon 12 and resulting protein is 32 a.a. shorter than C-b. C-d used an alternate splice site, which caused frame-shift and early termination.
Two clones had a 55-bp short exon 6, which caused early termination of translation and resulted in a 239 a.a. product. Another clone had a 239-bp long exon insertion between exons 4 and 5, also caused early termination of translation, and resulted in a 173 a.a. product. These early termination variants were not further investigated since these could not produce transmembrane proteins. The protein sequence alignment of four cardiac isoforms and the human SLC26A6 is shown in Figure 1C.

### 3.2 Expression of Slc26a6 in atrial and ventricular myocytes

The presence of the transcripts of the four cardiac Slc26a6 isoforms suggested the functional expression of these isoforms in the heart. We first tested the specificity of the anti-Slc26a6 antibodies by expressing each cloned cardiac Slc26a6 isoform in CHO cells and examined their expression by immunofluorescence confocal microscopy. As shown in Supplementary material online, Figure S1, the plasma membrane expression of the four isoforms was detected by using an anti-Slc26a6 antibody, while no immunoreactivity was detected in vector transfected control (Supplementary material online, Figure S1B). The expression of Slc26a6 in cardiomyocytes was further investigated using the same anti-Slc26a6 antibody. Both atrial and ventricular myocytes expressed Slc26a6 protein in the plasma membrane as shown in Figure 2.

### 3.3 Electrogenic Cl\(^{−}/\)oxalate and Cl\(^{−}/\)HCO\(_3\)\(^{−}\) exchange activities mediated by cardiac Slc26a6

To test the function of the cardiac Slc26a6, we first expressed the cardiac Slc26a6 isoforms in CHO cells and recorded the current generated by the anion exchanger using patch-clamp coupled with a fast solution exchange technique. The solution exchange configuration is shown in Figure 3A. Figure 3B shows the ionic concentration configuration across the cell membrane for the inward current recording when the membrane potential was clamped to 0 mV. Figure 3C shows the ionic concentration configuration for the outward current recording at 0 mV.

Slc26a6 is characterized by its electrogenic Cl\(^{−}/\)oxalate exchange,\(^{12}\) however, the electrogenicity of Cl\(^{−}/\)HCO\(_3\)\(^{−}\) exchange remains controversial. To characterize the function of cardiac Slc26a6, we designed a three-step solution exchange protocol. For the outward current recording, the first and third steps were used to activate Cl\(^{−}/\)oxalate exchange and to monitor the stability of the current, whereas the second step was applied to activate the Cl\(^{−}/\)HCO\(_3\)\(^{−}\) exchange (Figure 4A). This rapid solution exchange and current recording protocol allowed us to evaluate the Cl\(^{−}/\)HCO\(_3\)\(^{−}\) exchange in comparison with Cl\(^{−}/\)oxalate exchange in a single cell. For the inward current recording, the three-step protocol was used to test the dose-dependent activation of the current by oxalate (Figure 4B).

Figure 4A shows the outward current generated by electrogenic Cl\(^{−}/\)oxalate and Cl\(^{−}/\)HCO\(_3\)\(^{−}\) exchanges. Three isoforms (C-a, C-b, and C-d) are functional electrogenic Cl\(^{−}/\)oxalate and Cl\(^{−}/\)HCO\(_3\)\(^{−}\) exchangers, but the current generated by C-c was not substantially different from that obtained from the non-transfected CHO cells. We then reversed the current direction of the exchangers by reconfiguring the Cl\(^{−}\) and oxalate concentrations across the cell membrane. Figure 4B shows a dose-dependent inward current generated by Cl\(^{−}/\)oxalate exchange of cardiac Slc26a6 isoforms. Three isoforms (C-a, C-b, and C-d) mediated robust Cl\(^{−}/\)oxalate currents, while C-c’s current is comparable with that from the non-transfected CHO cells. Therefore, isoforms C-a, C-b, and C-d are functional electrogenic Cl\(^{−}/\)oxalate and Cl\(^{−}/\)HCO\(_3\)\(^{−}\) exchangers.

Previous studies suggested that Slc26a6 may form a structural dimer.\(^{13}\) Since different isoforms are expressed in cardiomyocytes, the four isoforms may co-assemble to form homodimers or heterodimers, and the function of the heterodimers may be altered. Since C-c is non-functional, we tested whether co-expression with the C-c may alter the function of C-a, C-b, or C-d. Figure 4C shows the outward current of Cl\(^{−}/\)oxalate exchange generated by the co-expression suggesting the functional slc26a6 isoforms in cardiomyocytes.

We observed significant inactivation of the outward and inward current of Cl\(^{−}/\)oxalate exchange in cardiac Slc26a6. The left panel of Figure 5A shows the inactivation of the outward current of cardiac C-a, C-b, and C-d, whereas the right panel shows the inactivation of the inward current of the three cardiac Slc26a6 isoforms. The inactivation time course was fitted with a single exponential function to obtain the

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**Figure 2** Expression of Slc26a6 in atrial and ventricular myocytes. Single atrial and ventricular cardiomyocytes from the heart of an adult mouse were isolated and fixed. The cells were treated by the anti-Slc26a6 antibody and subjected to immunofluorescence confocal microscopy. The nucleus of the cell was labelled by 4′,6-diamidino-2-phenylindole. The significant expression of slc26a6 in both atrial and ventricular myocytes was indicated by the strong fluorescence signal on the plasma membrane of the cells.
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4. Discussion

4.1 Potential roles of Slc26a6 in the regulation of cardiac acid–base balance

The most well-accepted functional importance of Slc26a6 is in mediating the diverse anion transport in proximal tubular cells and pancreatic ductal cells. In particular, Slc26a6 is the dominant Cl⁻/oxalate exchanger of the proximal brush border. Regulated secretion of pancreatic HCO₃⁻ requires the expression of Slc26a6 in the early portion of the pancreatic duct. However, Slc26a6 is also expressed in abundance in other tissues such as heart, intestine, muscle, and placenta. Here, we directly document four different cardiac Slc26a6 isoforms and characterize their function in both heterologous expression system and cardiomyocytes. Specifically, using the patch-clamp technique coupled with rapid solution exchange, we demonstrate that the Cl⁻/HCO₃⁻ exchange activity via cardiac Slc26a6 is electrogenic. Moreover, we directly demonstrate similar electrogenic Cl⁻/HCO₃⁻ and Cl⁻/oxalate exchange activities in mouse cardiomyocytes, and the Cl⁻/HCO₃⁻ exchange activity is inhibited by the activation of α₁-adrenergic receptors consistent with the previously documented regulation of α₁-adrenergic receptors on Slc26a6. Taken together, our results suggest important roles of cardiac Slc26a6 in Cl⁻/HCO₃⁻ exchange activities and pH regulation in the heart.

In physiological conditions, the extracellular Cl⁻ concentration is high (~110 mM), whereas the intracellular Cl⁻ concentration is low (~20 mM). The concentrations of extracellular and intracellular HCO₃⁻ are ~24 and ~20 mM, respectively. The reversal potential ($E_{rev}$) for the Cl⁻/HCO₃⁻ exchange can be estimated as follows:

$$E_{rev} = \left(\frac{r}{r-1}\right)×E_{HCO_3^-} + \left[\frac{1}{(1-r)}\right]×E_{Cl^-}$$

(1)

3.4 Endogenous Cl⁻/oxalate and Cl⁻/HCO₃⁻ exchange currents in cardiomyocytes

Heterologous expression of three of the four isoforms clearly showed the electrogenic Cl⁻/oxalate and Cl⁻/HCO₃⁻ exchange activities of cardiac Slc26a6. We also confirmed the expression of Slc26a6 in the plasma membrane of both atrial and ventricular myocytes. We therefore predicted the electrogenic activities of Slc26a6 in cardiomyocytes. As shown in Figure 6A and B, we recorded the outward currents generated by the Cl⁻/oxalate and Cl⁻/HCO₃⁻ exchanges as well as the inward currents generated by the Cl⁻/oxalate exchange. Compared with the currents from the heterologously expressed Slc26a6 isoforms in CHO cells, the current in cardiomyocytes is smaller, but the electrogenicity of both the Cl⁻/oxalate and Cl⁻/HCO₃⁻ exchanges is similar to that of the heterologously expressed cardiac Slc26a6. Moreover, the dose-dependent inward current generated by Cl⁻/oxalate exchange further supported the function of Slc26a6 in cardiomyocytes.

Previous studies have shown that the activation of α₁-adrenergic receptors inhibited Slc26a6 Cl⁻/HCO₃⁻ exchange activity. We therefore examined the effect of the activation of α-adrenergic receptors on the cardiac Slc26a6 Cl⁻/HCO₃⁻ exchange. Consistent with previously documented effects of α₁-adrenergic receptors on Slc26a6, 10 μM phenylephrine inhibited the Cl⁻/HCO₃⁻ exchange current by 44 ± 3% (n = 5) in ventricular myocytes, further supporting the roles of Slc26a6 in cardiac myocytes (Figure 6C).

Figure 3 Fast solution exchange and ion configuration to activate Slc26a6 exchange currents. (A) Fast solution exchange schematic diagram of the suspended whole-cell recording. The clamped whole cell was switched between two capillary tubes flowing control solution and test solution, respectively, to activate Slc26a6. Diameters of the solution exchange pipe and the cell are not proportionally drawn. (B) Ion configurations across the cell membrane to activate inward Cl⁻/oxalate currents. (C) Ion configurations across the cell membrane to activate outward Cl⁻/oxalate and Cl⁻/HCO₃⁻ exchange currents.
where \( r \) represents the stoichiometry of the exchanger \((\text{HCO}_3^- : \text{Cl}^-)\). \( E_{\text{HCO}_3^-} \) and \( E_{\text{Cl}^-} \) are the reversal potentials of \( \text{HCO}_3^- \) and \( \text{Cl}^- \), respectively. In physiological condition, \( E_{\text{Cl}^-} = -46 \text{ mV} \) and \( E_{\text{HCO}_3^-} = -5 \text{ mV} \) at 37°C. If \( r = 2 \), \( E_{\text{Rev}} = 2E_{\text{HCO}_3^-} - E_{\text{Cl}^-} = 36 \text{ mV} \). The driving force is \( E_m - E_{\text{Rev}} = E_m - 36 \text{ mV} \), and hence, the \( \text{Cl}^-/\text{HCO}_3^- \) exchange will generate either outward or inward currents depending on the membrane potential \( E_m \). As a result, the pH will be increased by membrane depolarization (when \( E_m > 36 \text{ mV} \)) or decreased by membrane hyperpolarization (when \( E_m < 36 \text{ mV} \)). Therefore, the cardiac slc26a6 contributes to the pH regulation, and the \( \text{Cl}^-/\text{HCO}_3^- \) exchange activity of cardiac slc26a6 may be regulated by the membrane potential.

Similarly, the reversal potential for the \( \text{Cl}^-/\text{oxalate} \) exchange can be estimated by using the following equation:

\[
E_{\text{Rev}} = \left[2r/(2r - 1)\right] \times E_{\text{oxalate}^-} + \left[1/(1 - 2r)\right] \times E_{\text{Cl}^-},
\]

where \( r \) represents the stoichiometry of the exchanger \((\text{oxalate}^- : \text{Cl}^-)\), and \( E_{\text{oxalate}^-} \) and \( E_{\text{Cl}^-} \) are the reversal potentials of oxalate ions and \( \text{Cl}^- \), respectively. The physiological extracellular oxalate concentration is \(~2 \mu\text{M}\), whereas the intracellular concentration is \(~1 \mu\text{M}\).\(^{22}\) \( E_{\text{Cl}^-} = -46 \text{ mV} \) and \( E_{\text{oxalate}^-} = -9 \text{ mV} \) at 37°C. If \( r = 1 \), \( E_{\text{Rev}} = 2E_{\text{oxalate}^-} - E_{\text{Cl}^-} = 28 \text{ mV} \). The driving force is \( E_m - E_{\text{Rev}} = E_m - 28 \text{ mV} \), and hence, the \( \text{Cl}^-/\text{oxalate} \) exchange will generate either outward or inward currents depending on the membrane potential, \( E_m \). On the other hand, the physiological role of the \( \text{Cl}^-/\text{oxalate} \) exchange by cardiac slc26a6 remains unclear and needs to be further studied.

### 4.2 Cardiac Slc26a6 mediated electrogenic \( \text{Cl}^-/\text{HCO}_3^- \) exchange activities

The electrogenicity of \( \text{Cl}^-/\text{HCO}_3^- \) exchange by Slc26a6 remains controversial.\(^{11,14,23,24}\) By using Xenopus oocyte expression and isotopic flux studies, Chernova et al. examined the \( \text{Cl}^-/\text{HCO}_3^- \) exchange using human and mouse Slc26a6. They found that the reversal potential of the induced current by Slc26a6 was not shifted by sequential bath solution changes from \( \text{Cl}^- \) to other monovalent anions including gluconate, gluconate/\( \text{HCO}_3^- \), and \( \text{Cl}^-/\text{HCO}_3^- \). They concluded that both human and mouse Slc26a6 mediated electroneutral \( \text{Cl}^-/\text{HCO}_3^- \) exchanges.\(^{24}\) However, Ko et al. and Xie et al. also used the oocyte expression system and measured the membrane potential upon the removal of
extracellular Cl\(^{-}\). They found that oocytes expressing mouse Slc26a6 were hyperpolarized in response to bath Cl\(^{-}\) removal, an indicator of the electrogenic Cl\(^{-}\)/HCO\(_3\)\(^{-}\) exchanges.\(^{11,14}\) The study by Shcheynikov et al.\(^{23}\) further investigated the stoichiometry of Cl\(^{-}\)/HCO\(_3\)\(^{-}\) exchanges by mouse Slc26a6, and found that mouse Slc26a6 functions as an electrogenic coupled 1Cl\(^{-}\)/2HCO\(_3\)\(^{-}\) exchanger.

Our study used CHO cells to express the three different cardiac Slc26a6 isoforms and applied suspended whole-cell patch-clamp and the fast solution exchange techniques to activate and record the exchanger currents. There are several advantages of our techniques. First, the ion concentrations in the intracellular and extracellular solutions can be precisely controlled; secondly, the fast solution exchange can be accomplished in tens of milliseconds to record the transient exchange currents; thirdly, the multiple voltage-clamp protocols can be designed and performed to document stable current recordings. By using the electrogenic Cl\(^{-}\)/oxalate exchange of Slc26a6 as control, we applied three-step solution exchange protocols to activate Cl\(^{-}\)/oxalate exchange and Cl\(^{-}\)/HCO\(_3\)\(^{-}\) exchange sequentially in a single cell. We observed that three cardiac Slc26a6 isoforms, C-a, C-b and C-d, generated the Cl\(^{-}\)/HCO\(_3\)\(^{-}\) exchange current similar to Cl\(^{-}\)/oxalate exchange, demonstrating the electrogenic Cl\(^{-}\)/HCO\(_3\)\(^{-}\) exchange activities. Even though our experiment did not provide the information of the precise stoichiometry of this electrogenic Cl\(^{-}\)/HCO\(_3\)\(^{-}\) exchange, our data support a 1Cl\(^{-}\)/nHCO\(_3\)\(^{-}\) exchange (n ≥ 2) mediated by mouse cardiac Slc26a6 isoforms.

In addition, our data support the hypothesis that electrogenic Cl\(^{-}\)/HCO\(_3\)\(^{-}\) exchange currents from the cardiomyocytes are mediated by Slc26a6. AE1, AE2, AE3, and slc26a3 are also expressed in cardiomyocytes and function as Cl\(^{-}\)/HCO\(_3\)\(^{-}\) exchangers.\(^{8}\) However, the Cl\(^{-}\)/HCO\(_3\)\(^{-}\) exchange by AE is electroneutral and will not contribute to the Cl\(^{-}\)/HCO\(_3\)\(^{-}\) exchange current.\(^{25}\) Slc26a3 mediates electrogenic Cl\(^{-}\)/HCO\(_3\)\(^{-}\) exchanges, but the stoichiometry of Cl\(^{-}\)/HCO\(_3\)\(^{-}\) is opposite to that of Slc26a6, i.e. 2Cl\(^{-}\)/1HCO\(_3\)\(^{-}\),\(^{23}\) and the expression of slc26a3 is much lower than that of slc26a6 in the adult mouse heart.\(^{8}\) Therefore, Slc26a3 will not significantly contribute to the outward currents generated by the Cl\(^{-}\)/HCO\(_3\)\(^{-}\) exchange. Additionally, the Cl\(^{-}\)/oxalate exchange current recorded from cardiomyocytes further supports the functional expression of Slc26a6 in the heart.

The electrogenic Cl\(^{-}\)/HCO\(_3\)\(^{-}\) exchanges of cardiac Slc26a6 suggest a membrane potential-dependent cardiac pH regulation mechanism by Slc26a6. The depolarization of the cell membrane may reduce the acid-loading process mediated by Cl\(^{-}\)/HCO\(_3\)\(^{-}\) exchanges. Therefore, acid
extrusion may be enhanced by changes in membrane potentials, not only through acceleration of NBC, but also through reducing acid loading via Slc26a6.

### 4.3 Functional features of the cardiac Slc26a6 isoforms

We observed a significant current inactivation mediated by Cl⁻/oxalate exchange of cardiac Slc26a6 isoforms in our study. The inactivation of other exchangers has been reported before.\(^{26–29}\) The Na\(^+\)-dependent inactivation of Na\(^+\)/Ca\(^{2+}\) exchanger was attributed to the competitive binding of Na\(^+\) and Ca\(^{2+}\) to a translocation site on the exchanger.\(^{27}\) A similar mechanism was also suggested for the inactivation of Na\(^+\)/Ca\(^{2+}\)/K\(^+\) exchanger current.\(^{29}\) The inactivation of the outward current of slc26a6 may utilize a similar mechanism. However, the working machinery of Slc26a6 is far less explored and understood. We cannot rule out the possibility that the direction of the exchange may be altered by the local accumulation of intracellular oxalate on the intracellular side, which may reduce the exchanger activity and the early termination.

4.4 Splice variants of cardiac Slc26a6

Slc26a6 was the first member of the slc26 family identified exclusively through database mining.\(^{5}\) Full-length human SLC26A6 was first identified by Lohi et al.\(^{18}\) It encodes a predicted 738 a.a transmembrane protein. Murine Slc26a6 was then cloned as a primary candidate for the apical Cl⁻/formate exchanger on the brush border membrane of the renal proximal tubule cells.\(^{30}\) Several alternative spliced variants of both human SLC26A6 and murine Slc26a6 were identified and characterized suggesting the significant heterogeneity of this gene.\(^{11,18,19,31,32}\)

Two isoforms of murine Slc26a6, Slc26a6a and Slc26a6b, were cloned from mouse intestine total RNA.\(^{31}\) Slc26a6a is the longer isoform with 758 a.a., and Slc26a6b is a shorter isoform with 735 a.a. corresponding to human SLC26A6 and murine Slc26a6 were identified and characterized suggesting the significant heterogeneity of this gene.\(^{11,18,19,31,32}\)

Another two human isoforms were reported by Lohi et al.\(^{32}\) that Slc26a6c lacks 38 a.a. by missing exon 6 and lacks 1 a.a. by using an alternative splice donor and acceptor site at the beginning of exon 17; Slc26a6d had spliced intron after exon 16 resulting in frame-shift and early termination.

Even though the previous study found that Slc26a6 transcripts were abundant in the heart,\(^{11,30}\) few studies have examined these transcripts and their functions. Our studies identified four isoforms of Slc26a6 in the mouse heart. Isoforms C-a and C-b correspond to the previously reported murine Slc26a6a and Slc26a6b, respectively. Isoform C-c lacks 32 a.a. by missing exon 12, whereas C-d is a C-terminal truncated form of C-b. Functional studies found negligible activities of isoform C-c.
suggested the key roles of the 32 a.a. in the function of Slc26a6. Isoforms C-a, C-b, and C-d are all functional Cl⁻/HCO₃⁻ exchangers in the heart, suggesting their important roles in the cardiac pH regulation.

4.5 Future directions

We identified Slc26a6 isoforms encoding the functional Cl⁻/HCO₃⁻ exchanger in the heart. The electrogenic Cl⁻/HCO₃⁻ exchange of cardiac Slc26a6 isoforms suggests the potential roles of these molecules in the regulation of acid–base balance in the heart. Further studies are required to examine the functional contribution of these isoforms to the regulation of cardiac pH and cardiac excitability under physiological and pathological conditions. Additionally, the functional significance of the alternative splicing of slc26a6 gene and the physiological role of Cl⁻/oxalate exchange of Slc26a6 in the heart remain to be determined.

Supplementary material

Supplementary material is available at Cardiovascular Research online.

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References

10. C-a, C-b, and C-d are all functional Cl⁻/HCO₃⁻ exchangers in the heart. The electrogenic Cl⁻/HCO₃⁻ exchange of cardiac Slc26a6 isoforms suggests the potential roles of these molecules in the regulation of acid–base balance in the heart. Further studies are required to examine the functional contribution of these isoforms to the regulation of cardiac pH and cardiac excitability under physiological and pathological conditions. Additionally, the functional significance of the alternative splicing of slc26a6 gene and the physiological role of Cl⁻/oxalate exchange of Slc26a6 in the heart remain to be determined.