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# SLC4A Transporters

**Inyeong Choi***

Department of Physiology, Emory University, Atlanta, Georgia, USA

## Abstract

SLC4A gene family proteins include bicarbonate transporters that move $HCO_3^-$ across the plasma membrane and regulate intracellular pH and transepithelial movement of acid–base equivalents. These transporters are Cl/$HCO_3$ exchangers, electrogenic Na/$HCO_3$ cotransporters, electroneutral Na/$HCO_3$ cotransporters, and $Na^+$-driven Cl/$HCO_3$ exchanger. Studies of the bicarbonate transporters in vitro and in vivo have demonstrated their physiological importance for acid–base homeostasis at the cellular and systemic levels. Recent advances in structure/function analysis have also provided valuable information on domains or motifs critical for regulation, ion translocation, and protein topology. This chapter focuses on the molecular mechanisms of ion transport along with associated structural aspects from mutagenesis of particular residues and from chimeric constructs. Structure/function studies have helped to understand the mechanism by which ion substrates are moved via the transporters. This chapter also describes some insights into the structure of SLC4A1 (AE1) and SLC4A4 (NBCe1) transporters. Finally, as some SLC4A transporters exist in concert with other proteins in the cells, the structural features associated with protein–protein interactions are briefly discussed.

## 1. INTRODUCTION

In most cells, the internal ionic environment is regulated by membrane proteins that move ions across the cell membranes. The SLC4A bicarbonate transporters are a subset of these proteins that are specialized to move $HCO_3^-$. $HCO_3^-$ buffers $H^+$, and therefore, $HCO_3^-$ transport is equivalent to $H^+$ transport in the opposite direction from a pH standpoint. The primary function of the bicarbonate transporters is to regulate intracellular $H^+$. Some bicarbonate transporters move $HCO_3^-$ into the cytosol and raise intracellular pH (acid extruders), whereas others remove $HCO_3^-$ from the cytosol and lower intracellular pH (acid loaders). The activity of acid extruders and loaders affects steady-state pH in the cells. Cells also utilize these pH regulators to transfer large amounts of $Na^+$ and $HCO_3^-$ from one body compartment to another to maintain extracellular and total body acid–base balance. The bicarbonate transporters are also widely distributed in many epithelial tissues where they play a role in transepithelial absorption and secretion of $HCO_3^-$. This transepithelial $HCO_3^-$ movement contributes to tissue-specific physiological processes such as reabsorbing $HCO_3^-$ or excreting acid into urine (kidney), neutralizing intestinal gastric acid (pancreatic ducts), or maintaining the cerebrospinal fluid pH (choroid plexus).

*Corresponding author: ichoi@emory.edu.

Many studies have been done to determine the functional and physiological significance of the SLC4A bicarbonate transporters in the body and to understand their pathological involvement in acid–base disorders. Cellular signals responsible for transport regulation have also been examined. In addition, the members of SLC4A transporters exhibit high homology in amino acid sequence, and this homology has enabled researchers to investigate domains or residues important for distinct molecular and functional properties of ion movement. Such efforts have produced valuable results to advance our knowledge of the underlying molecular mechanisms of ion transport. Furthermore, similar structural features among bicarbonate transporters have facilitated our understanding of transporter structure and the macromolecular complexes with other proteins. This chapter provides some insight into ion transport properties, structure, and protein–protein interactions of SLC4A bicarbonate transporters. This chapter focuses on anion exchangers AE1–3 and $Na/HCO_3$ cotransporters NBCe1 and NBCn1 because less has been done with other $Na^+$-coupled transporters.

## 2. SLC4A TRANSPORTERS

The SLC4A transporters (Table 1) are $Cl/HCO_3$ exchangers, $Na/HCO_3$ cotransporters, $Na^+$-driven $Cl/HCO_3$ exchanger, and Na/borate transporter. These are distinct from the SLC26A anion exchangers that transport a variety of monovalent and divalent anions (Sindic, Chang, Mount, & Romero, 2007). Some SLC26A proteins transport $HCO_3^-$ in exchange for $Cl^-$ or other anions.

### 2.1. $Cl/HCO_3$ Exchangers (AEs)

**2.1.1. AE1 (SLC4A1), AE2 (SLC4A2), AE3 (SLC4A3)—**The three $Cl/HCO_3$ exchangers (AE1–3) primarily move external $HCO_3^-$ in exchange for internal $Cl^-$ (Alper, 2009; Bonar & Casey, 2008). AE1 is the first cloned $HCO_3^-$ transporter (Kopito & Lodish, 1985). The human AE1 contains 911 amino acids with the predicted structure of 13 transmembrane (TM) (The TM topology of AE1 is defined according to the 13-TM model (Bonar & Casey, 2008), in which 13 TMs and one unusual membrane-spanning segment (called extended structure) between TM11 and TM12 are present. The topology of NBCe1 is defined according to the 14-TM model (Zhu et al., 2010), in which 14 TMs are present.) segments and cytoplasmic N- and C-terminal domains (Zhu, Lee, & Casey, 2003). The large N-terminal domain (~400 amino acids) plays a regulatory role, while the transmembrane domain is responsible for anion exchange activity. The N-terminal domain interacts with other proteins such as ankyrin, glycolytic enzymes, and hemoglobin, whereas the C-terminal domain interacts with carbonic anhydrase II (CA II) (Vince & Reithmeier, 1998). AE1 exists as homodimers (Wang, Sarabia, Reithmeier, & Kuhlbrandt, 1994). AE1 is the most abundant membrane protein in red blood cells and plays a key role in $CO_2$ delivery from tissues to the lungs. AE1 is also localized to the basolateral membrane of type-A intercalated cells in the kidney collecting ducts (van Adelsberg, Edwards, & Al Awqati, 1993). The renal kAE1 lacks the first 65 amino acids because of an alternate promoter site. AE1 also transports other anions such as $SO_4^{2-}$ at a very low rate (Milanick & Gunn, 1982).

AE2 was originally isolated from the kidney (Alper, Kopito, Libresco, & Lodish, 1988). AE2 is ~300 amino acids larger than AE1 and exists as multiple variants because of

alternative promoter sites. Similar to AE1, the N- and C-terminal domains of AE2 play regulatory roles in its pH dependence, as well as response to $NH_4^+$ and hypertonicity (Chernova et al., 2003; Stewart, Chernova, Shmukler, Wilhelm, & Alper, 2002), while the transmembrane domains are responsible for exchanger function (Stewart, Kurschat, Vaughan-Jones, Shmukler, & Alper, 2007). AE2 is widely distributed in the body, particularly in epithelial cells including the thick ascending limb and cortical collecting duct intercalated cells in the kidney (Brosius et al., 1995), and contributes to transepithelial movement of $HCO_3^-$ and salt.

Human AE3 comprised the cardiac variant (cAE3) and the brain variant (bAE3) with different N-terminal domains (Kopito et al., 1989). Both variants are found in many different tissues in addition to the heart and brain. AE3 has a lower exchange activity than AE1 and AE2 because its membrane expression is weak (Fujinaga, Loiselle, & Casey, 2003).

**2.1.2. AE4 (SLC4A9)—**Human AE4 contains 983 amino acids and has three alternative splice variants according to the NCBI database. AE4 is more closely related to Na/ $HCO_3$ transporters than AEs in amino acid sequence. AE4 is functionally characterized as a $Cl/HCO_3$ exchanger (Tsuganezawa et al., 2001; Ko, Luo et al., 2002), but it is also suggested to be an electroneutral NBC without $Cl/HCO_3$ exchange activity (Parker et al., 2002). AE4 is localized to renal collecting duct type-A intercalated cells, but its membrane polarity appears to be species-specific. It is localized to the basolateral membrane of the intercalated cells in rats and mice, but to the apical and lateral membranes in rabbits (Ko, Luo et al., 2002).

## 2.2. $Na^+$-Coupled Bicarbonate Transporters (NCBTs)

**2.2.1. NBCe1 (SLC4A4), NBCe2 (SLC4A5)—**Romero and Boron cloned the electrogenic NBCe1 from the salamander kidney (Romero, Hediger, Boulpaep, & Boron, 1997). On the basis of predicted topology and similarity to AE1, NBCe1 (1035 amino acids) was proposed to have a cytoplasmic N-terminal domain, 12–14 TM segments, and a cytoplasmic C-terminal domain. The most widely distributed variant is NBCe1-B containing the unique N-terminal 85 amino acids, which replace the first 41 amino acids of the renal variant NBCe1-A (Abuladze et al., 1998; Choi, Romero, Khandoudi, Bril, & Boron, 1999). The brain-specific NBCe1-C is identical to NBCe1-B except for the C-terminal 61 amino acids (Bevensee, Schmitt, Choi, Romero, & Boron, 2000). NBCe1-A heterologously expressed in *Xenopus* oocytes produces an electrogenic $HCO_3^-$ current with a $1Na^+$ and $2HCO_3^-$ stoichiometry (Ducoudret, Diakov, Muller-Berger, Romero, & Frömter, 2001; Grichtchenko et al., 2001; Sciortino & Romero, 1999), mediating net Ducoudret, Diakov, Muller-Berger, Romero, & Frömter, 2001 influx. In contrast, the transporter in the proximal tubules moves $1Na^+$ and $2HCO_3^-$ across the basolateral membrane, mediating net $HCO_3^-$ efflux. Studies show that the same transport molecule presumably changes its stoichiometry depending on the cellular environment and parameters such as intracellular $Ca^{2+}$ levels (Muller-Berger, Ducoudret, Diakov, & Frömter, 2001) or phosphorylation activated by cAMP (Gross et al., 2003). Similar to AEs, NBCe1 forms a homodimer composed of two individually functional subunits (Gill & Boron, 2006a; Kao et al., 2008).

NBCe2 is the second type of the electrogenic $Na/HCO_3$ cotransporter (Pushkin et al., 2000; Virkki, Wilson, Vaughan-Jones, & Boron, 2002). NBCe2 is strongly expressed in hepatocytes and bile duct cholangiocytes in the liver and renal pelvis uroepithelial cells in the kidney (Abuladze et al., 2004). It is also found in the renal collecting ducts, choroid plexus epithelia (Bouzinova et al., 2005; Damkier, Nielsen, & Praetorius, 2007), and sarcolemmal membranes in skeletal muscles (Kristensen, Kristensen, & Juel, 2004). NBCe2 produces a $HCO_3^-$-induced current with the $1Na^+:2HCO_3^-$ stoichiometry when expressed in *Xenopus* oocytes (Virkki et al., 2002). However, it operates with the $1Na^+:3HCO_3^-$ stoichiometry when expressed in the renal proximal tubule cell line (Sassani et al., 2002) and in intact cells prepared from choroid plexus epithelia (Millar & Brown, 2008).

**2.2.2. NBCn1 (SLC4A7), NBCn2 (SLC4A10)—**The electroneutral NBCn1 was isolated from rat vascular smooth muscle (Choi, Aalkjaer, Boulpaep, & Boron, 2000) and human skeletal muscle (Pushkin et al., 1999). Human NBCn1 contains 1214 amino acids with a predicted structure similar to that of NBCe1. NBCn1 exists as multiple variants because of N- and C-terminal splice events. The transporter is widely distributed in the body (Boedtkjer, Praetorius, Fuchtbauer, & Aalkjaer, 2008). NBCn1 is expressed in vascular smooth muscle cells and endothelial cells and modulates artery tone and blood pressure control (Boedtkjer et al., 2011). The variant containing the N-terminal cassette of 123 amino acids (cassette II) is predominantly found in the cardiovascular system (Cooper et al., 2006). Among SLC4A bicarbonate transporters, NBCn1 is least sensitive to the anion channel/transporter blocker 4,4′-diisothiocyanatostilbene disulfonate (DIDS) (Choi et al., 2000). In addition, NBCn1 has $HCO_3^-$-independent, $Na^+$ channel-like activity (Cooper et al., 2005).

NBCn2 is the second cloned electroneutral $Na/HCO_3$ cotransporter (Wang, Yano, Nagashima, & Seino, 2000). This transporter is predominantly expressed in neurons and choroid plexus epithelia (Damkier et al., 2007). NBCn2 operates with the $1Na^+:1HCO_3^-$ stoichiometry with $Cl^-$ self-exchange activity (Parker, Musa-Aziz et al., 2008). Other studies show that NBCn2 directly transports $Cl^-$ in exchange for $Na/HCO_3$ cotransport and functions as a $Na^+$-driven $Cl/HCO_3$ exchanger NCBE (Damkier, Aalkjaer, & Praetorius, 2010; Giffard, Lee, Ouyang, Murphy, & Monyer, 2003).

**2.2.3. NDCBE1 (SLC4A8)—**NDCBE1 is the $Na^+$-driven $Cl/HCO_3$ exchanger (Grichtchenko et al., 2001; Parker, Bouyer, Daly, & Boron, 2008). This transporter is predominantly expressed in neurons (Chen, Haddad, & Boron, 2008; Sinning et al., 2011), where $Na^+$-driven $Cl/HCO_3$ exchange has been known to primarily govern $HCO_3^-$-dependent acid extrusion (Schwiening & Boron, 1994). NDCBE moves external $1Na^+$ and $2HCO_3^-$ in exchange for internal $Cl^-$.

### 2.3. Sodium Borate (NaBC) Transporter

NaBC1 (SLC4A11) transports $Na^+$ and borate (Park, Li, Shcheynikov, Zeng, & Muallem, 2004). This transporter has substantial sequence homology to SLC4A bicarbonate transporters and was assigned as SLC4A11 before its function was characterized. NaBC1 operates with the stoichiometry of $1Na^+$ and at least $2B(OH)_4^-$ (Park et al., 2004) The transporter moves $Na^+$ and $OH^-$ ($H^+$) in the absence of borate.

# 3. ION TRANSPORT PROPERTIES

## 3.1. Ion Dependence and Selectivity

**3.1.1. AEs—**AEs are permissive to many anions in addition to $HCO_3^-$ and $Cl^-$. For example, AE1 has the capacity to move several monovalent and divalent anions in either direction or via anion self exchange (Knauf & Pal, 2003). AE2 also mediates the exchange of anions including $NO_3^-$, $Br^-$, and $I^-$ with low affinity when expressed in *Xenopus* oocytes (Humphreys, Jiang, Chernova, & Alper, 1994). The amino acid residue best characterized for anion selectivity is $Glu^{681}$ (TM8) in erythrocyte AE1 and the corresponding Glu site in AE2. Chemical modification of $Glu^{681}$ to an alcohol leads to electrogenic $SO_4^{2-}/Cl^-$ exchange and electroneutral $SO_4^{2-}/SO_4^{2-}$ self exchange (Jennings, 1995; Jennings & Al Rhaiyel, 1988). Similar changes are also observed from the site-directed mutagenesis of the corresponding Glu in cloned AE1 and AE2 (Chernova et al., 1997; Sekler, Lo, & Kopito, 1995), indicating that the role of $Glu^{681}$ is conserved among AEs. The underlying mechanism involves an interaction of sulfate with $H^+$ that is donated by the Glu residue lining the pore. Therefore, $Glu^{681}$ contributes to anion translocation, as discussed below. Other residues in AE are identified to serve as the anion selectivity sites in addition to $Glu^{681}$ of TM8. By scanning cysteine accessibility mutagenesis (SCAM) of AE1, Casey and his colleagues (Zhu & Casey, 2004) found that the region of $Ser^{852}$–$Leu^{857}$ in the extracellular loop between the last two TMs serves as an anion selectivity filter. Whether the residues $Ser^{852}$–$Leu^{857}$ are mechanistically associated with $Glu^{681}$ for function is unclear.

While it is generally accepted that the transmembrane domain plays an essential role in anion selectivity, the cytoplasmic domains may also contribute to maintaining $HCO_3^-$ selectivity. Deletion and missense mutants without the C-terminal putative CAII-binding site lose $Cl^-/HCO_3^-$ exchange while retaining $Cl^-/Cl^-$ self-exchange (Dahl et al., 2003).

**3.1.2. NCBTs—**All NCBTs are dependent upon $Na^+$ (Amlal, Wang, Burnham, & Soleimani, 1998; Sciortino & Romero, 1999). The current–voltage relationship for electrogenic $HCO_3^-$ transport shows a positive and linear slope with outward currents at positive voltages. The current is highly selective to $HCO_3^-$ and is not elicited by other anions (Fig. 1). The charge-conserved substitution of $Glu^{555}$ with an Asp (i.e. D555E) produces dose-dependent $Cl^-$ currents in the absence of $HCO_3^-$ (Yang, Kim et al., 2009). Nonetheless, D555E produces a $HCO_3^-$ current when both $Cl^-$ and $HCO_3^-$ are present. Anion selectivity experiments reveal that D555E is broadly permissive to other anions including $NO_3^-$, $SCN^-$, $I^-$, and $Br^-$, whereas wild-type NBCe1 shows a high selectivity to $HCO_3^-$. D555 may act to distinguish $HCO_3^-$ from other trigonal planar polyanions such as $NO_3^-$, as well as provide steric hindrance for small anions and $SCN^-$.The mechanism appears to involve a salt bridge between $Asp^{555}$ and adjacent charge residues in TM5 (unpublished observation by Soojung Lee). NBCe1 may also transport $OH^-$ at high extracellular pH (Amlal et al., 1998).

The apparent affinity of NBCe1-A for $Na^+$ is about 30 mM, and independent of different voltages and external [$HCO_3^-$] (Sciortino & Romero, 1999). Similar affinity values were also observed for all three NBCe1 variants, -A, -B, and -C (apparent $K_m$ of 21–36 mM)

(McAlear & Bevensee, 2006). The apparent $K_m$ of NBCe1 for $HCO_3^-$ is 4–6.5 mM (Grichtchenko, Romero, & Boron, 2000; McAlear & Bevensee, 2006).

### 3.2. Ion Translocation

**3.2.1. AEs—**In addition to anion dependence and selectivity, $Glu^{681}$ plays a role in ion translocation. Chemical modification or site-directed mutagenesis of this residue alters anion exchange kinetics (Chernova et al., 1997; Sekler et al., 1995), indicating that it is located in the anion translocation pathway. An SCAM study on TM8 (Tang, Kovacs, Sterling, & Casey, 1999) also identified residues that are predicted to line the translocation pore adjacent to $Glu^{681}$. These are $Ala^{666}$, $Ser^{667}$, $Leu^{669}$, $Leu^{673}$, $Leu^{677}$, and $Leu^{680}$ located in one face of α-helical TM8. Furthermore, cysteine mutants at $Ile^{684}$ and $Ile^{688}$ in the intracellular loop are also inhibited by sulfhydryl reagents and thus may be parts of the pore. Other TMs have been implicated as part of anion translocation. $Lys^{539}$ in TM5 is the site where the anion channel/transporter blocker 4,4′-diisothiocyanostilbene-2,2′-disulfonate (DIDS) binds (Bartel, Hans, & Passow, 1989). Mutations of $His^{703}$, $Arg^{731}$, $His^{735}$, $His^{816}$, and $His^{834}$ located in the region of TM10–13 result in inhibition of $Cl^-$ flux (Muller-Berger, Karbach, Konig et al., 1995). The His residues, particularly $His^{735}$, are proposed to interact with $Glu^{681}$ and form an access channel (Muller-Berger, Karbach, Kang et al., 1995). Several other TMs from 9 to 13 are required for transport function or contribute to ion translocation. Cysteine substitutions G714C, S725C, S731C, S762C, G790C, and F806C all reduced transporter activity to <40% of wild-type AE1 (Fujinaga, Tang, & Casey, 1999). The authors also found that the sulfhydryl reagent decreases the activity of AE constructs S852C (TM12) and A858C (TM13).

**3.2.2. NCBTs—**Regarding ion translocation of NBCe1, site-directed mutagenesis of amino acid residues in NBCe1 has been performed to identify the structural requirements for cotransport function (Abuladze et al., 2005). Many charged and polar residues, such as Glu, Asp and Arg, scattered throughout all the TMs were found critical for transport function. Nonetheless, while these residues contribute to charge interaction with ion substrates, it is unclear whether they are responsible for ion translocation or structural components of the protein. Furthermore, noncharged or nonpolar residues may play a more important role in ion translocation. An SCAM analysis of residues in TM8 identified $Leu^{750}$ among others involved in ion translocation (McAlear & Bevensee, 2006). The sulfhydryl reagent *p*CMBS accessibility of L750C is strongly influenced by $Na^+$ and $HCO_3^-$, DIDS binding, and membrane potential. Kurtz and his colleagues used a similar approach to evaluate amino acid residues in TM1 and found that T442C, A435C, and A428C have substantial sensitivity to the methylsulfonate reagents (Zhu et al., 2009). The α-helical structure of TM1 suggests that these residues would be located on the same face of the TM and serve as binding sites for ion substrates or lining the pore. The functional significance of TM1 is also evident for S427L, which has a defect in either the voltage sensor or $Na^+$-binding site and fails to mediate $Na/HCO_3$ efflux (Dinour et al., 2004). An SCAM analysis of residues in TM10–14 reveals that the C-terminal TMs are tightly folded with limited access to the extracellular aqueous environment (Zhu et al., 2010). D894C (in a loop between TM12 and TM13) and L937C (in TM14) appear to display a severe decrease in NBC function. Figure 2

summarizes amino acid residues that have been identified as residues critical for ion translocation via NBCe1. These residues are located in TM1, TM5, TM7, TM8, and TM14.

While structure/function studies continue to investigate the transmembrane domain for ion translocation, the cytoplasmic domain may also play a role. The naturally occurring mutant R298S is located in the cytoplasmic N-terminal domain of NBCe1 and has reduced transport activity. By modeling NBCe1 onto the crystal structure of the AE1 N-terminal domain, Romero and his colleagues hypothesized that $Arg^{298}$ interacts with $Glu^{91}$ or $Glu^{295}$ via hydrogen bonds or charge–charge interactions (Chang, Dipiero, Sonnichsen, & Romero, 2008). The double mutation E91R/R298E rescues transport activity, indicating that the N-terminal domain contributes to $HCO_3^-$ permeation.

### 3.3. Mechanisms of Ion Transport

The following two different mechanistic models are considered for SLC4A transporters: a sequential (or ping-pong) mechanism and a simultaneous mechanism (Fig. 3). In the sequential mechanism, one ion substrate binds to the transporter, causes a conformational change, and is released at the *trans* side of the membrane. Then, a second ion substrate binds to the transporter, regenerates the original conformation, and is released on the original *cis* side of the membrane. Therefore, the sequential mechanism involves a series of association–dissociation steps and conformational changes. However, the simultaneous mechanism proposes that the transporter forms a ternary complex with substrates before translocation occurs. The first and second ion substrates bind to the transporter and form a tertiary complex. This causes a conformational transition that makes the bound substrates face the *trans* side of the membrane. The two substrates are subsequently released.

The sequential mechanism has been proposed to account for the exchange kinetics of $Cl^-$ and $HCO_3^-$ by AEs (Frohlich & Gunn, 1986). $H_2$-DIDS can bind preferentially to the outward-facing state (Jennings, Whitlock, & Shinde, 1998). The exchange rates for $HCO_3^-$ or $Cl^-$ are different depending upon the inward facing or outward facing state (Knauf, Law, Leung, Gehret, & Perez, 2002). In addition, for the E699Q mutation of mouse AE1 (corresponding to E681Q in human AE1), elevating intracellular $[SO_4^{2-}]$ increases the apparent $K_m$ for extracellular $Cl^-$ and $SO_4^{2-}$ (Chernova, Stewart, Barry, Jennings, & Alper, 2008). All these data are consistent with the sequential kinetics.

It is unclear if the mechanism used by NCBTs is simultaneous or sequential. The simultaneous mechanism can be distinguished from the sequential mechanism experimentally by studying the relationship of $K_m$ and $V_{max}$ as shown for the Na/alanine transporter (Jauch & Lauger, 1986). The simultaneous mechanism exhibits a variable $K_m/V_{max}$ ratio for the variable substrate at different concentrations of the fixed substrate, whereas the sequential mechanism shows a constant $K_m/V_{max}$ ratio. The apparent $K_m$ and $V_{max}$ of NBCe1 for $Na^+$ are unaffected at 10 and 33 mM $[HCO_3^-]$ (Sciortino & Romero, 1999), implying that NBCe1-mediated transport may occur via a sequential mechanism although additional experiments are needed to confirm this. The ability of D555E to produce a $Cl^-$ current (Yang, Kim et al., 2009) provides some insights into the binding order. The mutant transporter favorably selects $HCO_3^-$ when $HCO_3^-$ and $Cl^-$ are present, but this $HCO_3^-$ selection requires $Na^+$. In the absence of $Na^+$, D555E fails to select $HCO_3^-$ and

instead selects $Cl^-$. This indicates that $HCO_3^-$ binding requires the precondition of $Na^+$ binding from the perspective of the ion binding order.

### 3.4. Electrogenicity and Stoichiometry

The electrogenicity of a given ion transporter determines whether it is affected by the membrane voltage in addition to the ion gradient across the cell membrane. Among SLC4A transporters, NBCe1 and NBCe2 are electrogenic despite their sequence similarity with other transporters. The molecular basis of electrogenicity is poorly understood. Studies on chimeric transporters constructed from NBCe1 and NBCn1 reveal that the NBCe1 electrogenicity is conferred by the conserved transmembrane domain, while the cytoplasmic N and C termini and the large third extracellular loop (EL3) between TM5 and TM6 are not critical (Choi, Yang, & Boron, 2007). Furthermore, electrogenicity is mediated via interactions between TM1–5 and TM6–14 because swapping these two regions with NBCe1 and NBCn1 results in electroneutral $Na/HCO_3$ transporters. The EL4 (32 amino acids) between TM7 and TM8 has been found to be critical for electrogenicity (Chen, Liu, & Boron, 2011). Chimeric transporters with NBCe1–EL4 exhibit electrogenic properties, while those with NBCn1– EL4 exhibit electroneutral properties. EL4 may directly constitute a part of the ion-binding vestibule or coordinate with TMs to modulate ion binding.

Related to the issue of electrogenicity is the ion transport stoichiometry. The $Na^+$:$HCO_3^-$ stoichiometry of NBCe1 is particularly essential for $HCO_3$ reabsorption in the proximal tubules of the kidney, where the basolateral NBCe1-A moves $1Na^+$ and $3HCO_3^-$ into the interstitium (Soleimani, Grassl, & Aronson, 1987). If the stoichiometry were $1Na^+$ and $2HCO_3^-$, then the transporter would instead move ions in the opposite direction (i.e. into the cell) because the equilibrium potential for $1Na^+/2HCO_3^-$ transport is estimated to be more negative than the resting potential, typically −70 mV (Boron & Boulpaep, 1983). Indeed, NBCe1 in astrocytes has a $1Na^+$:$2HCO_3^-$ stoichiometry and transports ions into the cells (Bevensee, Apkon, & Boron, 1997; Bevensee, Weed, & Boron, 1997). Similarly, NBCe1 in pancreatic ducts has a $1Na^+$:$2HCO_3^-$ stoichiometry and transports ions into the cells (Ishiguro, Steward, Lindsay, & Case, 1996; Zhao, Star, & Muallem, 1994). Given the different N-terminal amino acids between NBCe1-A and NBCe1-B/C, it was initially expected that the stoichiometry would be determined by the N-terminal domain of the transporter. However, NBCe1-A expressed in *Xenopus* oocytes mediates $Na/HCO_3$ influx with a $1Na^+$:$2HCO_3^-$ stoichiometry (Ducoudret et al., 2001; Grichtchenko et al., 2001; Sciortino & Romero, 1999). The stoichiometry changes from $1Na^+$:$2HCO_3^-$ to $1Na^+$:$3HCO_3^-$ when the cytosolic $Ca^{2+}$ concentration increases (Muller-Berger et al., 2001). Both NBCe1-A and -B show a $1Na^+$:$3HCO_3^-$ stoichiometry when expressed in a proximal tubule cell line and a $1Na^+$:$2HCO_3^-$ stoichiometry when expressed in a mouse collecting tubule cell line (Gross et al., 2001). These findings provide a strong argument that the same transport molecule can express different stoichiometries, depending on the cellular environment. Gross and his colleagues demonstrate that the stoichiometry can be changed by a cAMP-dependent protein kinase A phosphorylation site ($Ser^{982}$ in NBCe1-A and $Ser^{1026}$ in NBCe1-B) in the C-terminal domain (Gross et al., 2003).

### 3.5. Pharmacological Inhibition of $HCO_3^-$ Transport

All SLC4A $HCO_3^-$ transporters except NBCn1 are inhibited by stilbene derivatives such as DIDS, 4-acetamide-4′-isothiocyanatostilbene-2,2′-disulfonic acid (SITS) and 4,4′-dinitrostilbene-2,2′ disulfonate (DNDS). These derivatives are broad inhibitors of anion transporters/channels (Cabantchik, Knauf, & Rothstein, 1978). DIDS binds to the pore entry of anion channels/transporters and occludes the pore vestibule (Tombola, Del Giudice, Papini, & Zoratti, 2000). The inhibition constant $K_i$ of AE1 in erythrocytes for DIDS is as low as 0.04 μM (Knauf, Law, & Hahn, 1995), while the values of cloned AEs are much larger. For NBCe1, the inhibition constant is 36 μM (Liu, Williams, Sumpter, & Bevensee, 2007). DIDS reversibly and covalently binds to the first lysine (K) in the SKLIK motif in TM 5 of AE1 (Bartel et al., 1989). NBCe1 has the homologous KKMIK motif, and mutation analysis of this motif shows the second K (i.e. $K^{559}$) with the most profound effect on the reversible DIDS binding (Lu & Boron, 2007). However, in the absence of DIDS, the mutation at $K^{559}$ produces a $HCO_3^-$ conductance similar to those of other mutations in the KKMIK motif. Thus, $K^{559}$ does not appear to serve directly as a $HCO_3^-$ translocation site. Other NCBTs contain similar DIDS motifs in the corresponding region of TM5, but their role in DIDS sensitivity has not been tested.

In addition to DIDS, fluorescent oxonol dyes such as WW-781, diBA(3) C4 and diBA(5)C4 are potent inhibitors for AEs (Alper et al., 1988). The inhibition potency of these oxonol dyes is higher than that of DIDS and varies among AE isoforms. The oxonol dyes diBA(3)C4 and diBA(5)C4 also inhibit NBCe1 (Liu et al., 2007). The oxonol dyes and DIDS compete with each other, and therefore appear to share at least part of the same external binding site. It is suggested that SLC4A transporters may contain a relatively large inner vestibule, in which a number of large anions bind and block $Cl^-$ permeation. Other chemicals that inhibit AEs and/or NBCs include niflumic acid (Cousin & Motais, 1976; Liu et al., 2007), benzamil and tenidap (Ducoudret et al., 2001; Lu & Boron, 2007), and an N-cyanosulphonamide inhibitor S8059 (Ch'en, Villafuerte, Swietach, Cobden, & Vaughan-Jones, 2008).

### 3.6. $HCO_3^-$ vs $CO_3^-$ Transport

The possibility that $HCO_3$ transporters would carry $CO_3^{2-}$ is based on an unexpected observation by Grichtchenko and Chesler (1994). They monitored extracellular pH in the brain slices and found a pH decrease that was caused by electrogenic $Na/HCO_3$ influx via NBCe1 into the astrocytes. They anticipated that inhibiting extracellular carbonic anhydrase (CA) would reduce the supply of extracellular $HCO_3^-$ and thus blunt the pH decrease. However, CA inhibition had the opposite effect and stimulated the pH decrease. They hypothesized that NBCe1 transports $CO_3^{2-}$ instead of $HCO_3^-$. At physiological pH (i.e. pH 7.4 in the extracellular space), $HCO_3^-$ is in the millimolar range, while $CO_3^{2-}$ is in the micromolar range and $H^+$ is in the nanomolar range. If $CO_3^{2-}$ is consumed via NBCe1, the reaction $HCO_3^- \rightarrow CO_3^{2-} + H^+$ would produce a large increase in $[H^+]$. However, $[HCO_3^-]$ would remain relatively unchanged as it is in the millimolar range. From the reaction $CO_2 + H_2O \leftrightarrow HCO_3^- + H^+$, one can expect that an increase in $[H^+]$ makes the reaction run leftward. Therefore, inhibiting extracellular carbonic anhydrase would blunt this leftward reaction and consequently enhance a decrease in extracellular pH. Boron and his colleagues

(Grichtchenko & Boron, 2002; Lee, Grichtchenko, & Boron, 2011) tested this model by coexpressing NBCe1 and GPI-linked CAIV in *Xenopus* oocytes and measuring surface pH ($pH_s$). They found that stimulating NBCe1 caused pHs to change as predicted from $CO_3^{2-}$ transport and inhibiting CAIV augmented all $pH_S$ changes. These results are consistent with $CO_3^{2-}$ transport rather than $HCO_3^-$ by NBCe1.

### 3.7. Channel-Like Activity

Historically, ion transporters have been considered mechanistically and structurally different from ion channels (Hille, 2001). Ion transporters move ions slowly and exhibit distinct substrate coupling (i.e. stoichiometry) and kinetics, whereas ion channels move ions rapidly (>$10^6$ ions per second) and exhibit gating and permeation. A typical view is that ion transporters structurally undergo rather marked conformational changes between inward-facing and outward-facing states, whereas ion channels undergo more subtle gating changes that open and close the pore. However, this distinction between transporters and channels became increasingly unclear as some membrane proteins share functional characteristics of both groups. Neurotransmitter transporters, including serotonin transporters, norepinephrine transporters, glutamate transporters and dopamine transporters, have imbedded channel properties (DeFelice & Blakely, 1996; Torres & Amara, 2007). Similarly, NBCn1 has robust $Na^+$ channel-like activity ($Na^+ \gg K^+ = Cs^+ = NMDG^+$) (Choi et al., 2000; Cooper et al., 2005). This activity can cause a positive shift in the membrane potential, and raise intracellular $Na^+$ when NBCn1 is expressed in HEK 293 cells or *Xenopus* oocytes. The channel-like activity is not coupled to $Na/HCO_3$ cotransport. The domain responsible for the channel-like activity of NBCn1 is the region between TM6 and TM14 (Choi et al., 2007). NBCn1 is not the only $HCO_3^-$ transporter exhibiting this activity, other SLC4A proteins also have similar activities. Erythrocyte AE1 mediates an anion flux that is stimulated by cell hyperpolarization, implicating a conductive pathway (Freedman & Novak, 1997). Trout AE1 expressed in *Xenopus* oocytes induces $Cl^-$ currents (Fievet, Gabillat, Borgese, & Motais, 1995). The electroneutral NDAE1 in *Drosophila* has a small inward current (Romero et al., 2000). NBCe1 variants display a $Na^+$-independent $HCO_3^-$ current (McAlear, Liu, Williams, McNicholas-Bevensee, & Bevensee, 2006). Furthermore, the $Cl/HCO_3$ exchangers Slc26a3 and Slc26a6 produce large $NO_3^-$ and $SCN^-$ currents (Ko, Shcheynikov et al., 2002; Shcheynikov et al., 2008).

The channel-like activity is more than just an oddity of the transporter because it affects cellular membrane potential and controls entry of ions at least in heterologous expression systems. For example, the $Na^+$ channel activity of NBCn1 lowers an electrochemical $Na^+$ gradient across the cell membrane. The lowered $Na^+$ gradient may alter activities of other $Na^+$-conducting ion channels if such channels and NBCn1 are present together. The significance of this effect has yet to be explored.

## 4. STRUCTURE OF SLC4A TRANSPORTERS

AE1 is predominantly composed of dimers although both monomers and tetramers are known to exist (Reithmeier, Chan, & Popov, 1996). A structural analysis by electron microscopy and three-dimensional image reconstruction at 20 Å resolution reveal the dimeric membrane domain in the U-shaped structure (Dolder, Walz, Hefti, & Engel, 1993;

Wang et al., 1994). The two monomers are in contact at the lower part (called the basal domain) embedded in the membrane, while forming a pair of protrusions at the upper part that faces the cytosol. The protrusions form the sides of a canyon, which surrounds a wide space and then converges into a depression in the basal domain. This depression may represent the pore entry or the opening to a transporter vestibule located at the dimer interface.

In support of the above model, the three-dimensional structure of the AE1 membrane domain shows V-shaped densities near the center of the dimer at 7.5 Å resolution (Hirai, Hamasaki, Yamaguchi, & Ikeda, 2011; Yamaguchi et al., 2010). Interestingly, similar V-shaped densities exist in the previously reported structure of a prokaryotic chloride channel (ClC) protein. The ClC protein contains 14 TMs with two internal repeats of 7 TMs inserted into the membrane in the transverse orientation (Dutzler, Campbell, Cadene, Chait, & MacKinnon, 2002). A V-shaped pair of helical repeats (TM1 +2 and TM8 +9) in AE1 are similar to corresponding repeats in the ClC. This similarity in the density and projection maps coincides with a sequence identity of 20%. Nonetheless, there are difficulties with this model. All the TMs cannot be clearly accounted for at 7.5 Å. The beginning of TM1 predicted from NMR studies (Chambers, Bloomberg, Ring, & Tanner, 1999) does not align with the beginning of that for ClC.

The cytoplasmic N-terminal domain of NBCe1-A has been crystallized at 3 Å (Gill & Boron, 2006b). The domain forms two monomers related by a 2-fold axis. The dimer architecture is similar to that observed for the N-terminal crystals of AE1 (Zhang, Kiyatkin, Bolin, & Low, 2000).

## 5. INTERACTION WITH OTHER PROTEINS

Similar to many other membrane proteins, some SLC4A transporters exist in concert with other proteins and form macromolecular complexes. It would be worth discussing the structural features associated with protein– protein interactions, which mainly occur in the cytoplasmic N- and C-terminal domains of the transporters.

### 5.1. AEs

The N-terminal domain in AE1 has an ankyrin-binding motif in residues 175–185 (Chang & Low, 2003). The AE1/ankyrin interaction tethers the cytoskeletal protein spectrin to the microdomains near the plasma membrane. Tyrosine phosphorylation promotes dissociation of AE1 from the spectrin-actin skeleton and may enable erythrocytes to undergo adaptive changes in response to stimuli such as malaria parasite invasion, cell shrinkage, and aging (Ferru et al., 2011). AE1 also binds to glycolytic enzymes, hemoglobin and protein 4.1 and 4.2.

The cytoplasmic C-terminal domain of AE1 interacts with CAII (McMurtrie et al., 2004; Vince & Reithmeier, 2000). The consensus sequence for the CAII binding site is DADD, where the first Asp makes an electrostatic interaction with His or Lys residues in the N-terminal region of CAII. Other AEs and different CA isoforms also interact with each other (Casey, Sly, Shah, & Alvarez, 2009). The AE1/CAII interaction significantly increases the

rate of $HCO_3^-$ transport (Sterling, Reithmeier, & Casey, 2001). $Cl^-/HCO_3^-$ exchange via AE1 in erythrocytes is a rate-limiting step for $CO_2$ delivery from tissues to the lungs (Wieth, Andersen, Brahm, Bjerrum, & Borders, 1982). Maximizing the $HCO_3^-$ transport rate helps fully utilize the $CO_2$-transport capacity of the blood.

### 5.2. NCBTs

$IP_3$ receptor binding protein released with $IP_3$ (IRBIT) is a signaling protein that is released from $IP_3$ receptors and regulates downstream target molecules. IRBIT binds to the N terminus of NBCe1-B, but not NBCe1-A, and increases $HCO_3^-$ transport activity (Shirakabe et al., 2006). IRBIT-mediated NBCe1-B stimulation has been demonstrated in pancreatic ducts (Yang, Shcheynikov et al., 2009a). The N-terminal 87 amino acids of NBCe1-B is an autoinhibitory domain that, when deleted, stimulates transport activity (McAlear et al., 2006). Deleting the N-terminal residues 2–16 of NBCe1-B abolishes the IRBIT-stimulated effect while maintaining autoinhibition (Lee, Boron, & Parker, 2012). Thus, autoinhibitory and IRBIT-binding determinants are not identical.

Some SLC4A transporters contain a motif in their C terminus to which PDZ proteins bind (PDZ stands for Post synaptic density protein PSD95, Drosophila disc large tumor suppressor Dlg1, and Zonula occludens-1 protein zo-1). PDZ proteins are proteins that interact with a variety of membrane and cytosolic proteins, modulate activities of their binding partners, associate the partners with cytoskeletal protein complexes near the membrane, and recruit signaling proteins (Kim & Sheng, 2004). NBCn1 contains the PDZ-binding motif (amino acid sequence ETSL) and interacts with the 56 kDa subunit of H-ATPase (Pushkin et al., 2003). The two proteins colocalize to the collecting duct intercalated cells of the kidney. NBCn1 also interacts with ezrin-binding protein 50 (EBP50; *alias* Na/H exchanger regulatory factor 1 NHERF-1) in the apical membrane of the pancreatic ducts and salivary glands (Park et al., 2002). The NBCn1/EBP50 interaction helps scavenge luminal $HCO_3^-$ at rest but facilitates $Cl^-$-mediated $HCO_3^-$ release during stimulation. NBCn1 also interacts with the postsynaptic density protein PSD-95 at synapses in the hippocampus (Park et al., 2010). The NBCn1/PSD-95 interaction appears to increase channel-like activity of the transporter. NBCn1 also interacts with harmonin to form a protein complex in the ear (Reiners et al., 2005). Other SLC4A bicarbonate transporters capable of interacting with PDZ proteins include NBCn2-C, which contains ETCL at its C terminus and interacts with the cytoskeleton and EBP5 (Lee, Ouyang, & Giffard, 2006).

Similar to AEs, NCBTs are also reported to interact with CAII isoforms. For example, the cytoplasmic C-terminal domain of NBCe1 contains $D^{986}$NDD for the CA II binding and the interaction enhances transport function (Becker & Deitmer, 2007; Pushkin et al., 2004). In contrast, other studies reveal no interaction between NBCe1 and CA II (Lu et al., 2006) or normal transport activity of a mutant lacking the CAII-binding domain (Yamada, Horita, Suzuki, Fujita, & Seki, 2011).

## 6. CONCLUSION

SLC4A transporters regulate intracellular pH in a variety of cells. Almost all cells in the body possess at least one of these transporters, and changes in pH influence activities of

other physiologically important proteins. For example, pH alters ion permeation, sensitivity, and response to agonists of ion channel receptors in the nervous system. Slc4a-knockout mice exhibit aberrant neuronal activities that are caused by acid–base disturbance. In addition, the bicarbonate transporters are essential for transepithelial movement of acid–base equivalents in epithelial tissues such as the kidney where the transporters contribute to regulating plasma pH. Studying the molecular mechanism of how ions are selected and carried via the transporters helps better understand acid–base homeostasis at the cellular and whole-body levels. Studying the transporter structure is valuable for drug designs for transporter-related pathophysiology. Future molecular studies of SLC4A bicarbonate transporters will certainly advance our understanding of transporter physiology.

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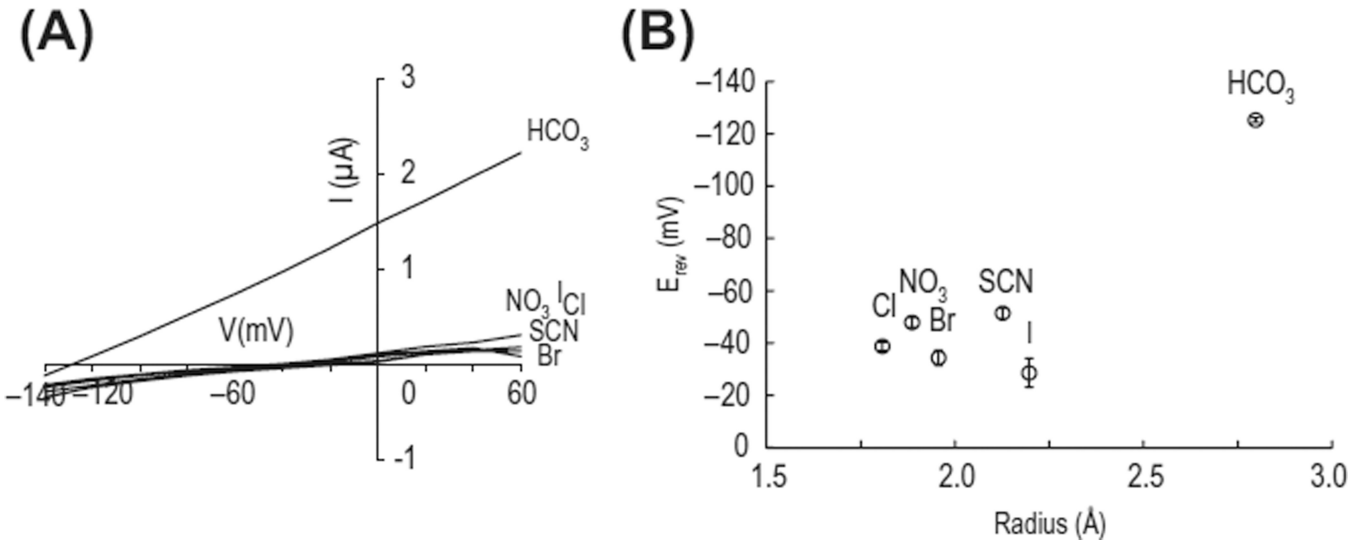


**Figure 1.**

Anion selectivity of NBCe1. A) NBCe1 expressed in *Xenopus* oocytes produces electrogenic outward currents in response to $HCO_3^-$, but not other anions. Recording was done while oocytes were exposed to different anions (25 mM for each). B) Zero current voltage ($E_{rev}$) versus anion radius. Note that $HCO_3^-$ has a large effective radius, but produces a current. The molecular or effective radius (in Å) is 1.81 $Cl^-$, 1.89 $NO_3^-$, 1.96 $Br^-$, 2.13 $SCN^-$, 2.20 $I^-$ and 2.8 $HCO_3^-$. $n$ = 6 for each.

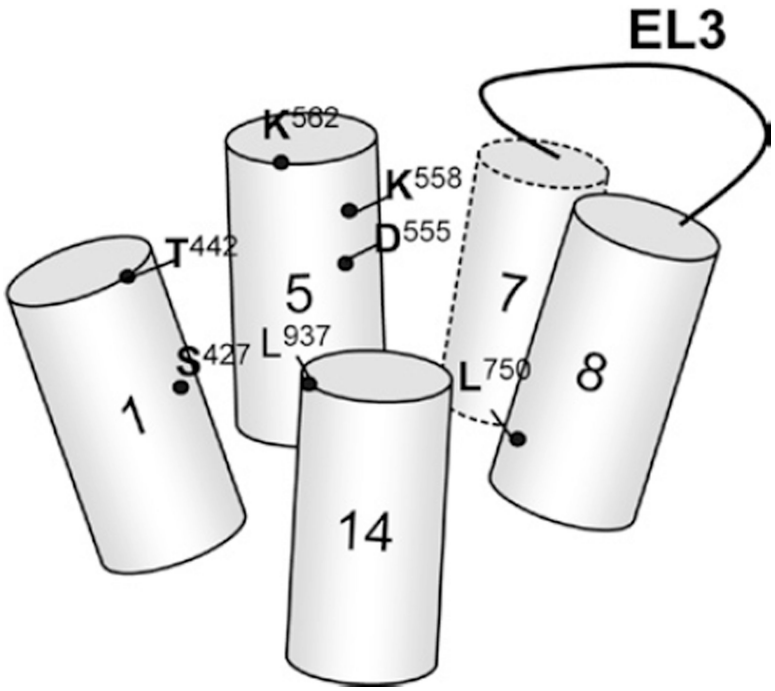


**Figure 2.**

Amino acid residues critical for ion translocation through NBCe1. The residues are located in TM1, TM5, TM7, TM8, and TM14. Identification and characterization of these residues are described in the text.

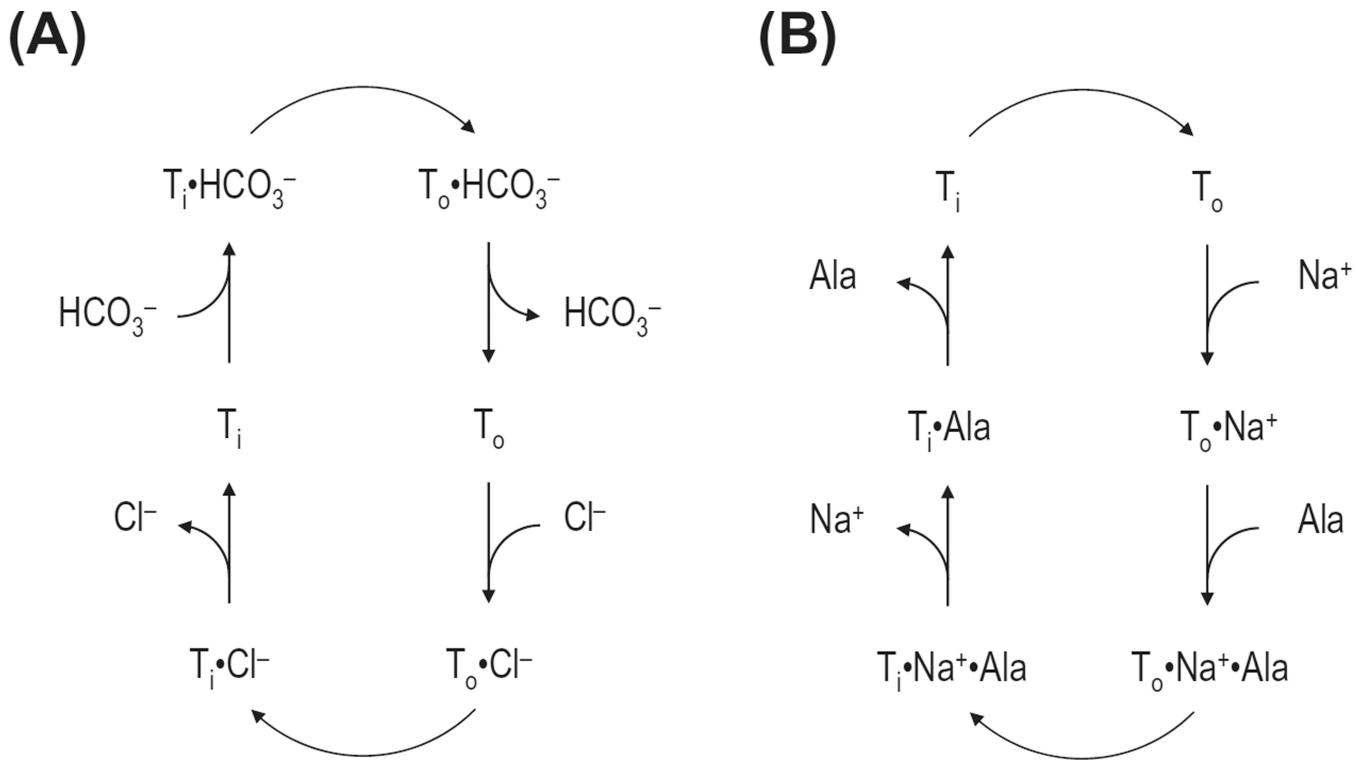


**Figure 3.**

Sequential (ping pong) vs simultaneous transport mechanisms. A) $Cl^-/HCO_3$ exchange via a sequential mechanism. In this mechanism, the transporter (T) accepts only one ion substrate at a time at each membrane surface. $T_i$ and $T_o$ represent the transporter in the inward- and outward-facing states, respectively. B) $Na^+$/alanine cotransport via a simultaneous mechanism. Both $Na^+$ and alanine (Ala) bind to the transporter before translocation occurs. The tertiary complex $T \cdot Na^+ \cdot Ala$ undergoes a conformational change. Releasing the two substrates on the opposite side of the membrane causes the transporter to be returned to the original state.

## Table 1

Human SLC4A transporters

| Human gene | Protein | Function | $pH_i$ regulation | Human chromosome |
|---|---|---|---|---|
| SLC4A1 | AE1 Band 3 | $Cl^-/HCO_3^-$ exchange | Acid loader | 17q21–q22 |
| SLC4A2 | AE2 | $Cl^-/HCO_3^-$ exchange | Acid loader | 7q35–36 |
| SLC4A3 | AE3 | $Cl^-/HCO_3^-$ exchange | Acid loader | 2q36 |
| SLC4A4 | NBCe1 NBC1 | $Na^+/HCO_3^-$ cotransport (electrogenic) | Acid extruder (except NBCe1-A) | 4q21 |
| SLC4A5 | NBCe2 NBC4 | $Na^+/HCO_3^-$ cotransport (electrogenic) | Acid extruder | 2p13 |
| SLC4A6 | – | | | – |
| SLC4A7 | NBCn1 NBC3 | $Na^+/HCO_3^-$ cotransport | Acid extruder | 3p22 |
| SLC4A8 | NDCBE | $Na^+/Cl^-/2HCO_3^-$ cotransport/exchange* | Acid extruder | 12q13 |
| SLC4A9 | AE4 | $Cl^-/HCO_3^-$ exchange | Acid loader | 5q31 |
| SLC4A10 | NBCn2 NBCE | $Na^+/HCO_3^-$ cotransport | Acid extruder | 2q23–q24 |
| SLC4A11 | NaBC BTR1 | $Na^+/B(OH)_4^-$, $OH^-$ cotransport | Acid extruder for $OH^-$ transport | 20p12 |

*NDCBE cotransports external $1Na^+$ and $2HCO3^-$ in exchange for internal $Cl^-$.