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Topical Review

Molecular Mechanisms of Electrogenic Sodium Bicarbonate Cotransport: Structural and Equilibrium Thermodynamic Considerations

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Abstract. The electrogenic Na^+-HCO_3^- cotransporters play an essential role in regulating intracellular pH and extracellular acid-base homeostasis. Of the known members of the bicarbonate transporter superfamily (BTS), NBC1 and NBC4 proteins have been shown to be electrogenic. The electrogenic nature of these transporters results from the unequal coupling of anionic and cationic fluxes during each transport cycle. This unique property distinguishes NBC1 and NBC4 proteins from other sodium bicarbonate cotransporters and members of the bicarbonate transporter superfamily that are known to be electroneutral. Structure-function studies have played an essential role in revealing the basis for the modulation of the coupling ratio of NBC1 proteins. In addition, the recent transmembrane topographic analysis of pNBC1 has shed light on the potential structural determinants that are responsible for ion permeation through the cotransporter. The experimentally difficult problem of determining the nature of anionic species being transported by these proteins (HCO_3^- versus CO_3^{2-}) is analyzed using a theoretical equilibrium thermodynamics approach. Finally, our current understanding of the molecular mechanisms responsible for the regulation of ion coupling and flux through electrogenic sodium bicarbonate cotransporters is reviewed in detail.

Key words: Sodium bicarbonate — Acid-base — Transport

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Electrogenic Na^+-HCO_3^- Cotransporters and the Bicarbonate Transporter Superfamily

NBC1 and NBC4 are the two sodium bicarbonate cotransporters among the mammalian BTS, which are known to be electrogenic (Table 1) (Burnham et al., 1997; Romero et al., 1997; Abuladze et al., 1998a; Gross et al., 2001a; Sassani et al., 2002). In addition to these two electrogenic Na^+- HCO_3^- cotransporters, other members of the BTS include the electroneutral Na^+-HCO_3^- cotransporter NBC3 (Pushkin et al., 1999a, b) [splice variant NBC2 (Ishibashi, Sasaki & Marumo, 1998); rat orthologue NBCn1 (Choi et al., 2000)], the Cl^-/HCO_3^- exchanger proteins AE1-AE3 (Alper, 1991; Casey & Reithmeier, 1998), and the Na^+-driven Cl^-/HCO_3^- exchangers NCBE and NDCBE1 (Wang et al., 2000; Grichtchenko et al., 2001). NCBE has also been reported to function as electroneutral sodium bicarbonate cotransporter (Choi, 2002). The function of AE4 is controversial since it has been reported to mediate Cl^-/HCO_3^- exchange (Tsuganezawa et al., 2001; Ko et al., 2002) and electroneutral Na^+-HCO_3^- cotransport (Parker, Boron & Tanner, 2002). BTR1 is an additional member of the BTS that has not been characterized functionally (Parker, Ourmozdi & Tanner, 2001).

The human *SLC4A4* gene encodes kNBC1 and pNBC1 (Abuladze et al., 2000). kNBC1 (116 kDa; 1035 amino acids), is transcribed from an alternative internal promoter in the *SLC4A4* gene (Abuladze et al., 2000), and is expressed in the basolateral membrane of the renal proximal tubule (Burnham et al., 1997; Romero et al., 1997; Abuladze et al., 1998b; Schmitt et al., 1999; Bok et al., 2001), and the eye (Bok et al., 2001). pNBC1 (120 kDa; 1079 amino acids), is the predominant NBC1 variant expressed in the basolateral membrane of pancreatic ducts (Abuladze et al., 1998a; Marino et al., 1999; Gross

Table 1. Classification of mammalian bicarbonate transporter superfamily genes

Human Gene	Protein	Function
SLC4A1	AE1 (band 3)	Na^+-independent Cl^--HCO_3^- exchanger
SLC4A2	AE2	Na^+-independent Cl^--HCO_3^- exchanger
SLC4A3	AE3	Na^+-independent Cl^--HCO_3^- exchanger
SLC4A4	kNBC1 (NBC1a, NBCe1a) pNBC1 (NBC1b, dNBC1, hhNBC, NBCe1b) rb2NBC[1] (NBC1c, NBCe1c)	Electrogenic Na^+-HCO_3^- cotransporter
SLC4A5	NBC4a-f[2] (NBCe2)	Electrogenic Na^+-HCO_3^- cotransporter Electroneutral Na^+-HCO_3^- cotransporter
SLC4A7	NBC3 (NBC2,NBCn1)	Electroneutral Na^+-HCO_3^- cotransporter
SLC4A8	NDCBE1	Na^+-driven Cl^--HCO_3^- exchanger
SLC4A9	AE4[3]	Na^+-independant Cl^--HCO_3^- exchanger Electroneutral Na^+-HCO_3^- cotransporter
SLC4A10	NCBE[3]	Na^+-driven Cl^--HCO_3^- exchanger Electroneutral Na^+-HCO_3^- cotransporter
SLC4A11	BTR1	Unknown

Additional names for a given protein are in parentheses.
[1]Cloned from rat only.
[2]The NBC4c splice variant functions in mammalian cells as an electrogenic sodium bicarbonate cotransporter (Sassani et al., 2002). The NBC4e splice variant has been reported to function in *Xenopus laevis* as an electroneutral sodium bicarbonate cotransporter (Xu et al., 2002).
[3]The function of these transporters is controversial (*see* text for details and references).

et al., 2001a, 2003). pNBC1 has also been identified in several other tissues such as duodenum, colon, heart, salivary gland, brain, eye, prostate, testis, and thyroid (Abuladze et al., 1998a). Although kNBC1 and pNBC1 are both electrogenic, in kidney kNBC1 normally operates with a $3HCO_3^- : 1\ Na^+$ stoichiometry (Planelles, Thomas & Anagnostopoulos, 1993; Muller-Berger, Nesteror & Fromter, 1997), whereas in pancreas pNBC1 operates with $2HCO_3^- : 1\ Na^+$ stoichiometry (Gross et al., 2001a, 2003).

Analysis of the primary structure of kNBC1 and pNBC1 reveals that the two variants are 93% identical to each other with 41 N-terminal amino acids of kNBC1 replaced by 85 distinct amino acids in pNBC1. In addition to kNBC1 and pNBC1, Bevensee et al. (2000) reported a third variant of NBC1 in rat brain, rb2NBC or NBC1c (130 kDa; 1094 amino acids), which has 61 unique C-terminal amino acids. Whether rb2NBC is expressed in other mammalian species is unknown. In addition to these NBC1 variants, the NBC4c (1121 amino acids) splice variant of NBC4 encoded by the human *SLC4A5* gene (Pushkin et al., 2001), functions as an electrogenic Na^+- HCO_3^- cotransporter in mammalian epithelial cells (Sassani et al., 2002). NBC4 transcripts are expressed in brain, heart, kidney, pancreas, testis, liver, and muscle (Pushkin et al., 2000a; Pushkin et al., 2000b; Sassani et al., 2002). The amino-acid sequence of NBC4 among all members of the BTS, has the greatest degree of similarity to NBC1 proteins.

Structural Characteristics of Electrogenic Na^+- HCO_3^- Cotransporters

Membrane Topography

Of the known electrogenic Na^+-HCO_3^- cotransporter proteins, only the structure of pNBC1 has been characterized in detail. A recent topographical study of pNBC1, using the in vitro transcription/translation analysis and immunocytochemical studies, revealed that the cotransporter has 10 transmembrane segments with amino and carboxy-termini located intracellularly (Fig. 1; Tatishchev et al., 2003). Given that known NBC1 proteins are identical within their transmembrane regions, it is very likely that kNBC1 and rb2NBC have the same number of transmembrane segments as well as the same orientation of their N- and C-termini. The recent determination of the topographical structure of NBC1 provides further insight into the understanding of the molecular basis of Na^+-HCO_3^- cotransport. Based on the current topographical model of pNBC1 (Fig. 1), several candidate regions emerge as potential Na^+ and HCO_3^- permeation pathways.

Potential Structural Determinants of HCO_3^- Permeation

The positively charged residues pNBC1-K^{898} (TM8) and pNBC1-H^{951} (TM9) (Fig. 1) within the transmembrane segments as well as adjacent positively charged residues pNBCl-$R^{948,\ 949}$ (intracellular loop

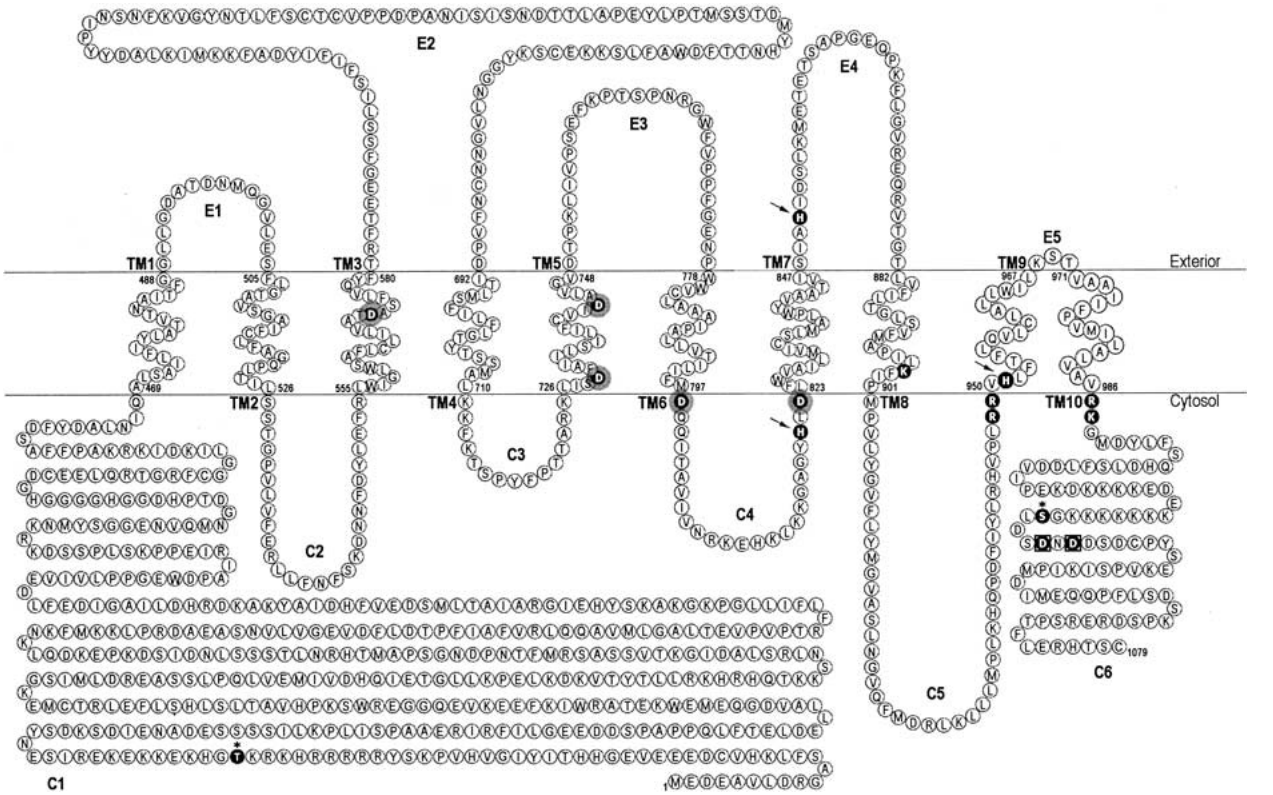

Fig. 1. Primary amino-acid sequence and predicted topographical structure of human pNBC1. Shown are the transmembrane domains in the proposed pNBC1 model, which are based on the predictions deduced from computer algorithm analyses of the pNBC1 primary structure and results obtained by the in vitro transcription/translation technique and immunocytochemical studies. The transmembrane domains of pNBC1 are numbered (TM1–10). *E* refers to extracellular loop, *C* refers to cytoplasmic loop. Residues are shaded to indicate their functional significance. *Black circles*: Amino-acid residues that are potential structural determinants for HCO_3^- permeation. *Black circles with surrounding grey area*: Aspartate residues that are potential structural determinants of Na^+ permeation pathway. *Filled circles with arrows*: Histidine residues that are potentially part of the Na^+-HCO_3^- cotransporter pH-sensor. *Black circles with asterisks*: PKA phosphorylation sites, which modulate the flux or stoichiometry of the cotransporter. *Black circles with surrounding square*: Aspartate residues that mediate the stoichiometry shift. Reproduced with changes with permission from *Biochemistry* 2003, 42(3), 755-765.

C5) and pNBC1-$K^{987,\ 988}$ (C-terminal region C6) (Fig. 1) could play an important role in regulation of binding and permeation of HCO_3^- through the cotransporter. These residues can potentially form a localized positively charged ring at the cytosolic aperture of the HCO_3^- permeation pathway or interact with adjacent negatively charged residues forming salt bridges between transmembrane segments. The crystal structure analysis of the *S. typhimurium* periplasmic sulfate-binding protein showed that salt bridges between positively and negatively charged residues stabilize the closed formation of this protein, contributing to the coordination and entrapment of the sulfate ion inside its binding cleft (Pflugrath & Quiocho, 1985, 1988). According to the model for AE-mediated anion transport proposed by Sekler and coworkers (1995), the glutamate residue E^{681} is instrumental in formation of salt bridges required for conformational changes that stabilize the carrier's transition state during anion exchange. The residue AE1-E^{681} corresponds to D^{798} in pNBC1. This aspartate residue in pNBC1 may form a salt bridge with any of the previously mentioned positively charged residues. Although it has been shown that replacement of E^{681} with an aspartate residue abolishes both monovalent and divalent anion transport in AE1 (Sekler et al., 1995), a shorter aspartate side chain in combination with specific spatial arrangements of transmembrane segments of NBCs could still be instrumental in mediating HCO_3^- transport.

AE1 studies using Woodward's reagent K/$NaBH_4$ modification, which mimics permanent protonation of carboxyl groups, showed inhibition of Cl^-/Cl^- exchange while stimulating SO_4^{2-}/SO_4^{2-} exchange (Jennings & Al-Rhaiyel, 1988). Based on these findings Jennings and Smith (1992) proposed that the protonation of E^{681} appears to switch AE1 between monovalent and divalent anion selectivity, suggesting that E^{681} is a titratable site. In sodium bicarbonate cotransporters, however, the aspartate residue corresponding to AE1-E^{681} may accommodate Na^+ ions instead of H^+. The fact that the side chain of the aspartate residue is shorter could be crucial in allow-

ing a larger Na^+ ion to fit into a putative allosteric binding site located in this region of Na^+-HCO_3^- cotransporters and possibly cause conformational changes that affect HCO_3^- transport. The accessibility of AE1-E^{681} to membrane-impermeant Woodward's reagent K from either side of the membrane suggests that this residue is localized at the transmembrane permeability barrier (Jennings & Anderson, 1987; Jennings & Al-Rhaiyel, 1988; Jennings & Smith, 1992; Tang et al., 1999). Given that AE1-E^{681} and pNBC1-D^{798} reside in similar positions relative to the membrane bilayer (Fig. 1 & Tang et al., 1999), it is likely that pNBC1-D^{798} is also found at the transmembrane permeability barrier. In addition, Tang et al. (1999) proposed that the anion-permeation pathway of AE1 forms an outward-facing funnel that narrows to a permeability barrier at E^{681}. The same could be true for NBC proteins, where the HCO_3^- permeation pathway would form a funnel narrowing at a corresponding aspartate residue. A negative charge on the aspartate's side chain would seem to impede HCO_3^- transport. However, an aspartate residue at this particular location could bind H^+ or Na^+ ions, changing the net charge of the permeability barrier, which in turn may trigger conformational changes necessary for HCO_3^- translocation. The aspartate residue may also interact with a histidine residue on the adjacent transmembrane segments, as has been proposed in the case of mouse AE1-H^{752} and AE1-E^{699} (Muller-Berger et al., 1995), which could be crucial for the pH dependence of HCO_3^- transport.

Using site-directed mutagenesis Muller-Berger et al. (1995) showed that mouse AE1 histidine residues $H^{721, 752, 837, 852}$ (human AE1-$H^{703, 734, 819, 834}$) are required for anion translocation. Substitution of any of these amino acids by glutamine or serine residues inhibited Cl^- flux through the exchanger. Under physiological conditions a complex of allosteric interactions between the four histidine residues, rather than each residue on its own, is required to maintain the exchanger in a functional state. Sequence alignments of well characterized BTS proteins show that the histidine residues AE1-$H^{703, 734, 834}$ are highly conserved throughout all the bicarbonate transporters among various species and correspond to pNBCl-$H^{820, 851, 951}$ (Fig. 2*A*), which makes these residues putative participants in HCO_3^- permeation by NBC members. In addition, it should be noted that these three histidines of AE1 ($H^{703, 734, 834}$) and pNBC1 ($H^{820, 851, 951}$) reside in approximately the same position in relation to the membrane topography of these transporters (Fig. 1; Taylor, Zhu & Casey, 2001; Zhu, Lee & Casey, 2003).

Potential Structural Determinants of Na^+ Permeation

Wiebe, Dibattist and Fliegel (2001) proposed that conservation of polar residues along with their specific spatial orientation represents a general mechanism for binding, coordination and subsequent translocation of cations by various transport proteins. Based on the eukaryotic and prokaryotic alignments of selected regions of Na^+/H^+ exchangers, a relatively small number of conserved polar amino acids that may be sufficient to carry out and regulate Na^+/H^+ exchange was identified. Polar side chains of the identified conserved amino acids from different transmembrane segments form a crown-ether like arrangement at the cytoplasmic surface of the exchanger that may be instrumental in coordination and permeation of Na^+ ions (Wiebe et al., 2001). Based on the topographical structure of pNBC1 (Fig. 1), the negatively charged residues pNBC1-$D^{729, 798, 822}$ could form a crown-ether like structure similar to the one found in Na^+/H^+ exchangers. A sequence alignment of different well characterized members of the BTS shows that the residue corresponding to pNBCl-D^{798} is found only in Na^+-transporting proteins (Fig. 2*B*), whereas residues corresponding to pNBCl-$D^{729, 822}$ are highly conserved. This finding might explain the differences between Na^+-transporting and non-Na^+-transporting members of the BTS.

In addition to the crown-ether-like structure, Na^+-transport may depend on specific 3D-arrangements of transmembrane segments that form the Na^+-conducting pathway. Recent determination of the crystal structure of K^+ channels and molecular bases of K^+ conduction revealed a relatively large water-filled cavity near the center of the membrane (Doyle et al., 1998). Surrounding water dipoles inside this cavity are necessary to overcome the electrostatic destabilization that the cation experiences within the low dielectric lipid bilayer (Parsegian, 1975). Similar electrostatic considerations hint to a possible spatial arrangement of transmembrane segments that form a Na^+-conducting pathway in members of the BTS that transport Na^+. It is less likely that AE1-AE3 proteins possess a water-filled cavity to stabilize Na^+ ions.

Alternatively, the destabilizing effect of the hydrophobic milieu surrounding an ion permeation pathway may be overcome by ion binding sites along the entire permeation pathway forming a polarizable surface through which ions move in a single file (McCleskey, 1997, Doyle et al., 1998). Negatively charged residues pNBC1-D^{572} (TM3) and pNBC1-$D^{729, 743}$ (TM5) (Fig. 1) have a potential to bind and electrostatically stabilize a Na^+ ion inside its permeation pore. These three aspartate residues are conserved amongst all well characterized BTS proteins that are known to transport Na^+ (Fig. 2*C*). In the AE proteins, however, the pNBC1-D^{572} residue corresponds to a negatively charged glutamate residue (Fig. 2*C*). Glutamate's longer side chain protruding inside the putative permeation pore may hinder free movement of Na^+ ions in the anion exchangers. In fact, it has previously been demonstrated that mutagenesis of

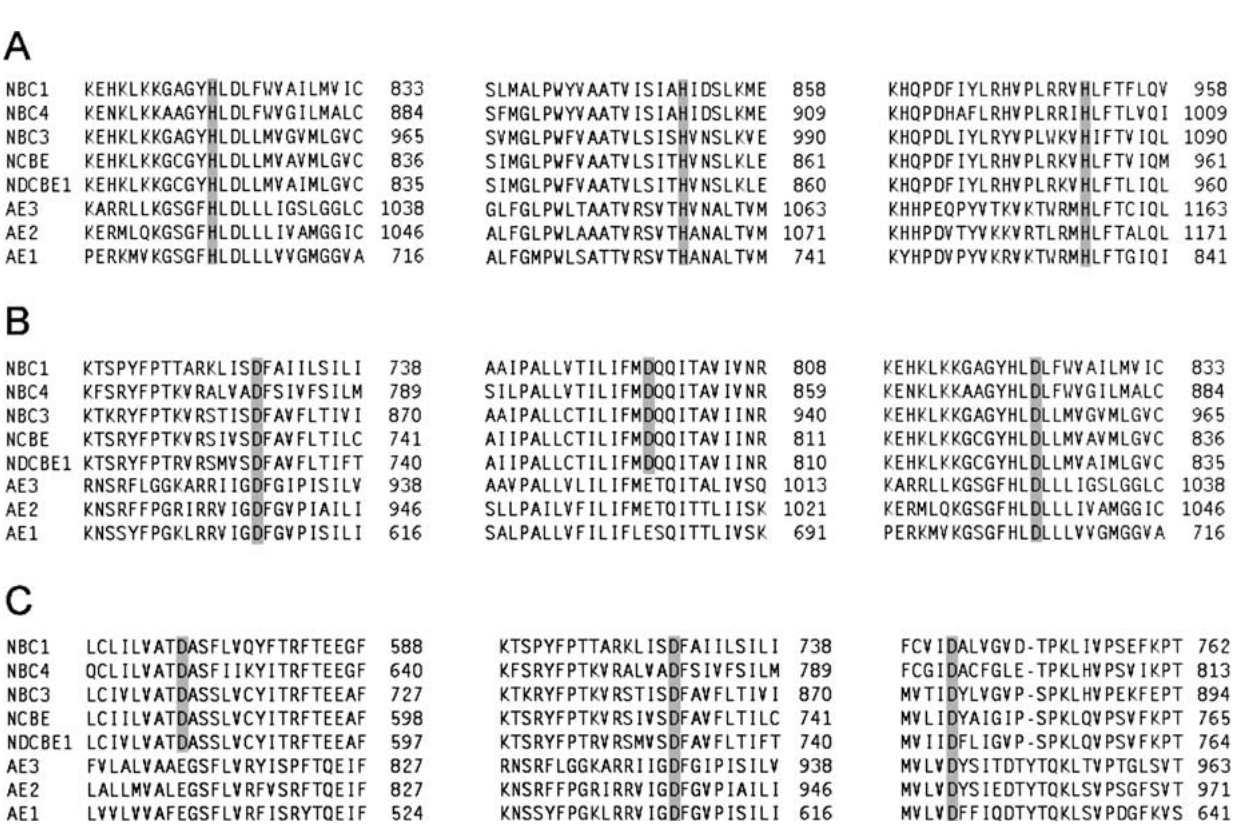

Fig. 2. Multiple protein-sequence alignment of human members of BTS in which the Na^+ dependence has been well characterized. Shaded regions designate conserved amino-acid residues that can potentially be a part of (*A*) the HCO_3^- permeation pathway, (*B*) form a crown-ether like arrangement instrumental in co-ordination and permeation of Na^+ ions, and (*C*) bind and electrostatically stabilize Na^+ ions inside the putative permeation pore. GenBank accession numbers for the aligned sequences are: NBC1 (pNBC1, AF011390); NBC4 (NP_597812); NBC3 (AAD38322); NCBE (BAB18301); NDCBE1 (AF069512); AE3 (NP_005061); AE2 (AAC50964); AE1 (AAA35514).

the aspartate residues to glutamate in prokaryotic melibiose permease reduces its efficiency and binding affinity for Na^+ (Poolman et al., 1996).

It should also be noted that Na^+ selectivity and coordination could be mediated by carbonyl oxygens of the backbone rather than by amino-acid side chains, as has also been proposed for K^+ channels (Doyle et al., 1998). If this were the case, the charged residues within and on the periphery of transmembrane segments of the BTS transporters may only function in stabilizing and maintaining the tertiary structure (Sahin-Toth & Kaback, 1993; Lee, Hwang & Wilson, 1993; Dunten, Sahin-Toth & Kaback, 1993).

Potential Structural Determinants of pH-Sensing

Previously, it has been suggested that the activity of the Na^+-HCO_3^- cotransporters is regulated by an intracellular pH-sensitive site (Soleimani et al., 1991). In addition, Gross and Hopfer (1999) proposed that configuration of the channel-like structure of the Na^+-HCO_3^- cotransporter is modified by intracellular pH, affecting the net flux of Na^+ and HCO_3^- ions. Several studies have shown the importance of histidine residues in the pH-induced modulation of membrane transporter function (Todt & McGroarty, 1992; Coulter et al., 1995; Rimon et al., 1995; Sekler et al., 1996; Fei et al., 1997; Dibrov et al., 1998; Echtay et al., 1998; Chanchevalap et al., 2000; Cha et al., 2003). Inter-molecular hydrogen bonds formed by histidine residues can induce changes in protein conformation, pore size, and rate of an ion transport (Wiebe et al., 2001). Rimon et al. (1995) demonstrated that NhaA-H^{225} is a part of a pH-sensitive site that regulates the activity of the Na^+/H^+ exchanger in *E. coli*, while Dibrov et al. (1998) indicated that sod2-H^{367} plays a similar role in the *S. pombe* Na^+/H^+ exchanger. Based on a recent analysis of the role of histidine residues in NHE3, Cha et al. (2003) suggested that NHE3-H^{479} and H^{499} located in the juxtamembrane cytoplasmic region are part of the NHE3 pH-sensor. Histidine residues pNBC1-$H^{820,\ 851,\ 951}$, conserved among well-defined members of the BTS (Fig. 1; Fig. 2*A*), are potential candidates for pH-sensing region(s) in the vicinity of the plasma membrane/aqueous interface. As has been demonstrated in various classes of transporters, amino-acid residues other than histidine (Choe et al., 1997; Jordt & Jentsch, 1997; Stewart et al., 2002), and

in particular glutamate residues (Stroffekova et al., 1998; Xu et al., 2000; Jordt, Tominaga & Julius, 2000; Sekler et al., 1995; Stewart et al., 2002), may also contribute to pH sensitivity.

Equilibrium Thermodynamics and Stoichiometric Considerations

In the basolateral membrane of the renal proximal tubule, kNBC1 mediates the cellular efflux of Na^+ and HCO_3^- against their respective concentration gradients, by exploiting the negative membrane potential. In contrast, pNBC1 mediates the cellular influx of Na^+ and HCO_3^- across the basolateral membrane of the pancreatic duct. The net charge carried by each cotransporter varies with its transport stoichiometry and is an important determinant of the direction of Na^+ and HCO_3^- flux. In the proximal tubule of the kidney, kNBC1 is thought to normally function in the 3:1 mode, whereas pNBC1 in the pancreatic duct functions in the 2:1 mode.

The HCO_3^- to Na^+ transport stoichiometry can be determined by solving the Nernst equation for z (Eq. 1).[1]

$$z = \frac{RT}{E_{rev}F} \ln \frac{c_i}{c_o} \quad (1)$$

A z of 2 is compatible with the transport of one Na^+ and three HCO_3^- ions, as in the case of kNBC1 in the proximal tubule; while a z of 1 implies that two HCO_3^- ions are being transported with one Na^+ ion. There is, however, an important limitation to this kind of analysis. Equation 1 dictates the total valence being transported but not the ionic species. Therefore, when using Eq. 1, one cannot distinguish, for example, between the transport of $2HCO_3^-$ and $1CO_3^{2-}$ since in both cases the total transported negative charge is 2.

Use of Equilibrium Thermodynamics to Determine the Transported Anionic Species

A 3:1 cotransporter stoichiometry predicted by Eq. 1 could result from the transport of either $3HCO_3^- + 1Na^+$ or $1CO_3^{2-} + 1HCO_3^- + 1Na^+$. Similarly, a 2:1 stoichiometry could result from the transport of $2HCO_3^- + 1Na^+$ or $1CO_3^{2-} + 1Na^+$ (Planelles et al., 1993; Muller-Berger et al., 1997 & 2001).[2] Thus far, equilibrium thermodynamics has not been used directly to determine the anionic species transported by electrogenic Na^+-HCO_3^- cotransporters. Intuitively, one would expect that the substitution of $1CO_3^{2-}$ in place of $2HCO_3^-$ ions in Eq. 1 would result in a different E_{rev}, enabling a distinction between the transported species. When there is no PCO_2 gradient across the cell membrane, Eq. 1 gives the same E_{rev} independently of whether $3HCO_3^- + 1Na^+$ or $1CO_3^{2-} + 1HCO_3^- + 1Na^+$ are transported. (*See* Fig. 3 for a detailed mathematical analysis).

Theoretically, equilibrium thermodynamics could be used to distinguish between CO_3^{2-} *vs.* HCO_3^- flux by establishing a stable PCO_2 difference across a plasma membrane. Under these conditions, the logarithm of the ratio between $[HCO_3]_o$ and $[HCO_3]_i$ is no longer equal to the difference between pH_o and pH_i (*see* Fig. 3). This is exemplified in Fig. 4*A*, which demonstrates graphically the theoretical difference between the respective E_{rev} values for a $3HCO_3^- + 1Na^+$ transport mode versus a $1CO_3^{2-} + 1HCO_3^- + 1Na^+$ transport mode at various PCO_2 gradients. Another approach, whereby equilibrium thermodynamics could be used for determining the transported anionic species, is by establishing a stable pK difference in the solutions on each side of the membrane. This is theoretically possible to accomplish because the pK of the $HCO_3^- \rightarrow CO_3^{2-} + H^+$ reaction varies with ionic strength in a predictable manner[3], as shown graphically in Fig. 4*B*. There are, however, experimental limitations to these approaches. First, establishing and maintaining a known PCO_2 difference is difficult given the high permeability of biological membranes to CO_2. Second, formulas characterizing the dependence of pK on ionic strength are empirical and must be evaluated separately in each case. Whether these technical and theoretical difficulties can be resolved in the near future remains to be determined.

Modulation of HCO_3^- : Na^+ Stoichiometry

Role of NBC1 Carboxy-Terminus

It has been shown that electrogenic Na^+-HCO_3^- cotransporters operate in either a 3:1 mode or a 2:1 mode (Gross et al., 2001b). Expression of two N-terminal variants of NBC1, kNBC1 and pNBC1, in different mammalian cell lines, demonstrated that either of the NBC1 variants can function with both a 3:1 and 2:1 stoichiometry, depending on the cell type

[1] R, T and F have their usual meaning; c_i and c_o represent intra- and extracellular concentrations of a given ion, respectively. E_{rev} is the reversal membrane potential and z is the charge number of a transported species.

[2] It should also be indicated that based on the concentration of CO_3^{2-}, which is 500 and 1000 times lower relative to that of HCO_3^-, at an extracellular pH of 7.4 and intracellular pH of 7.1, respectively; the flux of CO_3^{2-} would be negligible compared with that of HCO_3^-. This assumption, however, doesn't hold true if the binding constant of the cotransporter for CO_3^{2-} is 500- to 1000-fold higher than that for HCO_3^-.

[3] $pK = -\log K_o - 0.52\sqrt{I}$; where K_o is the original value of the dissociation constant and I is the ionic strength defined as $I = \frac{1}{2}\sum c_i z_i^2$ where c_i is molar concentration and z_i is a charge number of an ion.

E_{rev} for Transport of $3HCO_3^- + 1Na^+$ versus $1CO_3^{2-} + 1HCO_3^- + 1Na^+$

Using the Nernst Equation (Eq. 1) one can determine whether or not E_{rev} values for two alternative modes of 3:1 electrogenic sodium bicarbonate cotransport ($3HCO_3^- + 1Na^+$ or $1CO_3^{2-} + 1HCO_3^- + 1Na^+$) are equal. We begin by equating these two alternatives:

$$E_{rev} = 2.303\frac{RT}{zF}\log\frac{[Na^+]_o}{[Na^+]_i}\frac{[HCO_3^-]_o^3}{[HCO_3^-]_i^3} = 2.303\frac{RT}{zF}\log\frac{[Na^+]_o}{[Na^+]_i}\frac{[HCO_3^-]_o}{[HCO_3^-]_i}\frac{[CO_3^{2-}]_o}{[CO_3^{2-}]_i} \quad \text{(A1-1)}$$

Canceling like terms:

$$\log\frac{[HCO_3^-]_o^2}{[HCO_3^-]_i^2} = \log\frac{[CO_3^{2-}]_o}{[CO_3^{2-}]_i} \quad \text{(A1-2)}$$

Expressing the Henderson-Hasselbach equation in terms of $[CO_3^{2-}]$:

$$[CO_3^{2-}] = [HCO_3^-]10^{pH-pK_a} \quad \text{(A1-3)}$$

Substitution of Eq. A1-3 into Eq. A1-2 gives the following expression:

$$\log\frac{[HCO_3^-]_o^2}{[HCO_3^-]_i^2} = \log\frac{[HCO_3^-]_o}{[HCO_3^-]_i}\frac{10^{pH_o-pK_{ao}}}{10^{pH_i-pK_{ai}}} \quad \text{(A1-4)}$$

Canceling like terms and rearranging:

$$\log\frac{[HCO_3^-]_o}{[HCO_3^-]_i} = pH_o - pH_i \quad \text{(A1-5)}$$

Given that:

$$pH = -\log\frac{24PCO_2}{[HCO_3^-]} \quad \text{(A1-6)}$$

The equality A1-5 can be expressed as follows:

$$\log\frac{[HCO_3^-]_o}{[HCO_3^-]_i} = \log\frac{[HCO_3^-]_o}{24PCO_{2o}}\frac{24PCO_{2i}}{[HCO_3^-]_i} \quad \text{(A1-7)}$$

If $PCO_{2o} = PCO_{2i}$, then the equality A1-5 is true and consequently the two alternative modes of cotransport ($3HCO_3^-$ vs. $1CO_3^{2-} + 1HCO_3^-$) are indistinguishable. Conversely, if there is a PCO_2 gradient across the plasma membrane (see text and Fig. 4 for details), then the equality A1-5 is not valid, and the two alternative modes of cotransport are thermodynamically distinguishable.

Fig. 3. Derivation illustrating that the E_{rev} calculated for transport of three HCO_3^- ions and one Na^+ ion is precisely the same as for transport of one CO_3^{2-} ion, one HCO_3^- ion, and one Na^+ ion. Similarly, the E_{rev} for transport of two HCO_3^- ions and one Na^+ ion, is the same as for transport of one CO_3^{2-} ion and one Na^+ ion (*not shown*).

in which they were expressed. These findings indicated that the difference in $Na^+ : HCO_3^-$ stoichiometry previously measured in various epithelia and expression systems was not an inherent property of NBC1 proteins and was unrelated to structural differences between cotransporters. Rather, these results suggested that stoichiometric differences were potentially regulated by cell-specific factors. In conjunction with these findings, a study of potential second messengers that might mediate the shift in stoichiometry indicated that the cAMP agonist, 8-Br-cAMP, caused the stoichiometry to shift from 3:1 to 2:1 in a mouse proximal tubule mPCT cell line transfected with either kNBC1 or pNBC1. The switch between these two alternative stoichiometries was found to be mediated by PKA phosphorylation of kNBC1-Ser^{982} (Gross et al., 2001c) or pNBC1-Ser^{1026} (Gross et al., 2003). In a separate study, Muller-Berger et al. (2001) have recently shown that kNBC1 expressed in *Xenopus laevis* oocytes undergoes a shift in stoichiometry from

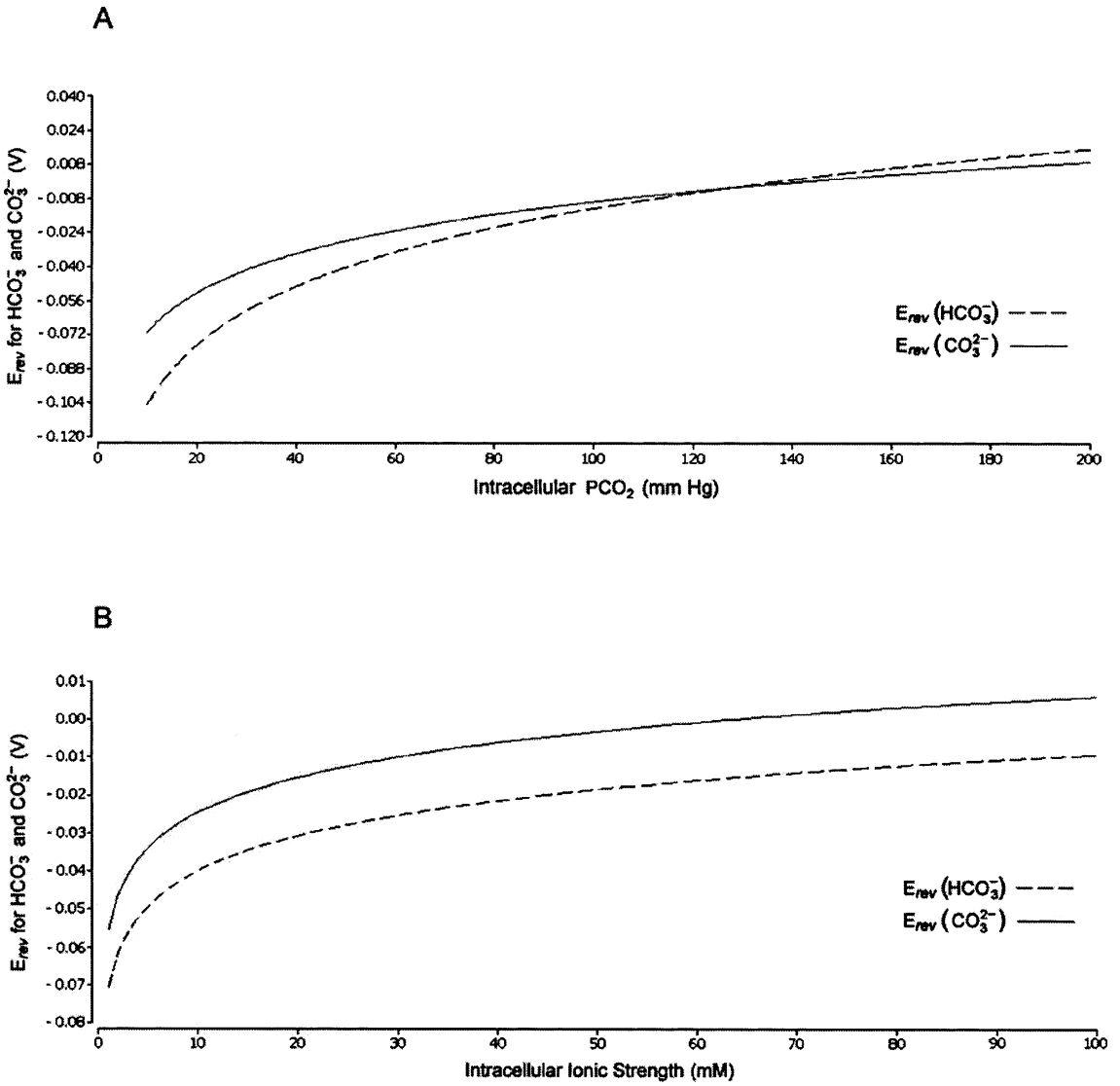

Fig. 4. (*A*) Theoretical dependence of the E_{rev} of the cotransporter under conditions of a PCO_2 gradient across the cell membrane ($PCO_{2(out)}$ = 125 mm Hg and varying the $PCO_{2(in)}$). The dependence of E_{rev} on the a PCO_2 gradient differs when the cotransporter functions with a 3:1 stoichiometry in the $3HCO_3^- + 1Na^+$ mode versus the $1CO_3^{2-} + 1HCO_3^- + 1Na^+$ mode. (*B*) Theoretical dependence of the E_{rev} of the cotransporter under conditions of an ionic strength gradient that was utilized to generate a pK gradient for the $HCO_3^- \rightarrow CO_3^{2-} + H^+$ reaction across the cell membrane. The dependence of E_{rev} on the ionic strength differs when the cotransporter functions with a 3:1 stoichiometry in the $3HCO_3^- + 1Na^+$ mode versus the $1CO_3^{2-} + 1HCO_3^- + 1Na^+$ mode.

2:1 to 3:1 in response to elevation of cytosolic Ca^{2+}. The change in stoichiometry was hypothesized to be due to a shift from a $1Na^+ + 2HCO_3^-$ mode to a $1Na^+ + 1CO_3^{2-} + 1HCO_3^-$ mode of transport, wherein a conformational change in the protein alters the relative affinity for CO_3^{2-} and HCO_3^-.

Electrostatic Interactions with NBC1

A better understanding of the interaction between HCO_3^- and the cotransporter is required to further elucidate how phosphorylation of kNBC1-Ser^{982} and pNBC1-Ser^{1026} at the carboxy termini shifts the stoichiometry from 3:1 to 2:1. A mathematical model has previously been formulated, which describes the interaction of HCO_3^- and Na^+ with the cotransporter (Gross & Hopfer, 1998). This model assumes that HCO_3^- and Na^+ ions bind to the cotransporter on one side of a membrane and are released sequentially on the other side. The finding that the current through the cotransporter as a function of either HCO_3^- or Na^+ concentration can be described by the Michaelis-Menten kinetics further supported this simplification (Gross & Hopfer, 1998). In addition, to make the mathematical derivations of the model simpler, the binding and release of all three negative

Free Energy Change Following Phosphorylation of the Cotansporter

The electrochemical potential (μ) of a species i is given by the following formula:

$$\mu_i = \mu_i^* + RT \ln a_i + z_i FE \qquad \text{(A2-1)}$$

where μ_i^* is an unknown additive constant or reference level; R, T, and F have their usual meaning; E is the electrical potential; z is the charge number of the species i and a_i is the activity. Since the electrochemical potential of a species i is the partial molal Gibbs free energy (G) of a system with respect to that species (Nobel, 1999):

$$\mu_i = \left(\frac{\partial G}{\partial n_i} \right)_{\mu_i, n_i}, \qquad \text{(A2-2)}$$

the electrochemical potential can be expressed in terms of G , so that

$$G = G^o + RT \ln a_i + z_i FE \qquad \text{(A2-3)}$$

Considering that the movement across the membrane depends on ΔG ($G_{out} - G_{in}$) and $a_i = \gamma_i c_i$ (γ_i is an activity coefficient and for an ideal solute is equal to 1) we obtain the following expression:

$$\Delta G = RT \ln \frac{c_o}{c_i} + zF\Delta E \,^{1} \qquad \text{(A2-4)}$$

which gives the Nernst equation when $\Delta G = 0$.

If Eq. A2-3 is modified to account for free energy lost or gained by phosphorylation[2] (see text), then:

$$G = G^o + RT \ln a_i + z_i FE + G_{phosphorylation} \qquad \text{(A2-5)}$$

and

$$\Delta G = RT \ln \frac{c_o}{c_i} + zF\Delta E + \Delta G_{phosphorylation} \qquad \text{(A2-6)}$$

[1] $\Delta E = E_o - E_i$

[2] $\Delta G_{phosphorylation}$ is the energy resulting from a cascade of processes (cAMP $\rightarrow$ protein kinase and phosphorylation of serine by ATP $\rightarrow$ ADP). This total process is estimated to be 5kJ/mole.

Fig. 5. Calculation of the free energy change following phosphorylation of NBC1 ($\Delta G_{phosphorylation}$).

charge equivalents were grouped into one step. The binding of $3HCO_3^-$ ions (or $1CO_3^{2-}$ and $1HCO_3^-$) to the cotransporter was voltage dependent, with an electrical coefficient of 0.2 at pH = 7.5, which was indicated by fitting the model to a series of *I-V* relationships obtained at different concentrations of HCO_3^- and Na^+. Furthermore, this data suggested that HCO_3^- "senses" ~ one-fifth of the membrane's electric field on binding to the cotransporter, or that the binding site for HCO_3^- is located ~ one-fifth of the electrical distance into the membrane. This result implied that modifying the electric field around its binding site might regulate the binding of HCO_3^- to the cotransporter.

Recently it was proposed that phosphorylation of kNBC1-Ser^{982} and pNBC1-Ser^{1026} causes a 3:1 to 2:1 stoichiometric shift by modifying the local electric field around the HCO_3^- binding site, thereby disrupting the binding of a HCO_3^- ion (Gross et al., 2001c, 2002). In keeping with this hypothesis, the stoichiometry of a kNBC1-S982D mutant was shown to be 2:1 in the presence or absence of 8-Br-cAMP (Gross et al., 2002). This finding is compatible with the idea that the charge on kNBC1-Ser^{982} plays an important role in shifting the transport stoichiometry. Furthermore, mutagenesis studies of a nearby negatively charged kNBC1 carboxy-terminal D986NDD motif indicated an important role for additional specific residues in mediating the stoichiometry shift. Specifically, kNBC1-Asp^{986} and kNBC1-Asp^{988} were found to be essential for the shift in stoichiometry to occur following kNBC1-Ser^{982} phosphorylation (Gross et al., 2002). The finding that these negatively charged aspartate residues near

kNBC1-Ser^{982} are essential for the phosphorylation-induced shift in stoichiometry further strengthens the argument in favor of an electrostatic effect, whereby one HCO_3^- binding site is blocked. The data are compatible with a model wherein Asp^{986} along with Asp^{988} is able to electrostatically block a HCO_3^- binding site when kNBC1-Ser^{982} is phosphorylated. Whether phosphorylation of Ser^{982} favors the electrostatic interaction of Asp^{986} and Asp^{988} with a HCO_3^- binding site by inducing a local conformational change in the kNBC1 carboxy-terminus remains to be determined.

Energetic Considerations

In the proximal tubule, the direction of NBC1-mediated Na^+ and HCO_3^- flux is normally in the absorptive direction (basolateral membrane cellular efflux). Following cAMP/PTH treatment, the magnitude of HCO_3^- absorption is reduced, however, the direction remains in the absorptive mode (McKinney & Myers 1980a, 1980b). Assuming that following cAMP/PTH treatment basolateral HCO_3^- transport is mediated by kNBC1, and that the HCO_3^- : Na^+ stoichiometry is 2:1, cellular efflux of HCO_3^- and Na^+ is achievable as long as the E_{rev} value is more positive than the membrane potential. It has been hypothesized that in rabbit proximal tubules this can occur because the intracellular sodium concentration is sufficiently increased (Seki et al., 1993). It has not been previously considered, however, that as a result of PKA-dependent phosphorylation, the system could gain sufficient free energy independent of ion gradients to make E_{rev} more positive than the membrane potential. This would require that the expression $\Delta G = RT\ln\frac{c_o}{c_i} + zF\Delta E$ (Fig. 5, Eq. A2-4) be modified. The new formula (Eq. A2-6) includes an additional term, which represents the phosphorylation of kNBC1-Ser^{982} or pNBC1-Ser^{1026} and the consequent free energy change: $\Delta G = RT\ln\frac{c_o}{c_i} + zF\Delta E + \Delta G_{phosphorylation}$ (Fig. 5, Eq. A2-6). This modification of Eq. A2-4 can account for the free energy lost or gained by phosphorylation of the cotransporter (Fig. 5). Consistent with this hypothesis is the data from Hurley et al. (1990) where phosphorylation of Ser^{113} isocitrate dehydrogenase was estimated to add 4.5 kcal/mole of free energy to the system.

In addition, previous studies have shown that a S982D substitution shifts the stoichiometry from 3:1 to 2:1 (Gross et al., 2001c). It would be of interest to determine whether or not the free energy change is comparable to the change induced by serine phosphorylation. Molecular dynamics experiments would allow a theoretical calculation of the phosphorylation and its energetic consequences. This, however, requires knowledge of the crystal structure of the cotransporter, which is currently not available.

Modulation of the HCO_3^- : Na^+ Flux

Interaction of NBC1 and Carbonic Anhydrase II: A Transport Metabolon

It has previously been demonstrated that carboxy-terminal aspartate residues of AE1 interact electrostatically with amino-terminal basic residues of carbonic anhydrase II (CAII) (Vince & Reithmeier 1998, 2000; Vince, Carlsson & Reithmeier, 2000). CAII stimulates the transport function of AE1, AE2, and AE3 and inhibition of CAII by acetazolamide (ACZ) in HEK-293 cells transfected with AE1 decreases the function of the exchanger by 50–60% (Sterling, Reithmeier & Casey, 2001). It is thought that the electrostatic interactions between CAII and AEs minimize the distance for HCO_3^- diffusion between the two proteins and form a transport metabolon (Kifor et al., 1993; Sterling et al., 2001). Furthermore, it should be noted that the absence of functional CAII, which is normally expressed in the cytoplasm of proximal tubule cells and intercalated cells, leads to proximal and distal renal tubular acidosis and depletion of collecting duct intercalated cells (Breton et al., 1995; Nagai et al., 1997; Lai et al., 1998). NBC1 proteins and AE1 share a homologous region of negative aspartate residues at the carboxy-terminus. Recently, it has been demonstrated that the kNBC1 carboxy-terminus can interact with CAII (Gross et al., 2002). Interestingly, the same study showed that, unlike the essential role for the carboxy-terminal aspartate residues in shifting the stoichiometry, inhibition of CAII with ACZ neither altered the stoichiometry nor prevented the shift in stoichiometry induced by 8-Br-cAMP. However, when the stoichiometry was 3:1, inhibition of carbonic anhydrase activity decreased the current through the transporter by ~60%. In the 2:1 mode, inhibition of carbonic anhydrase by ACZ did not affect the flux through the transporter significantly. As a possible explanation for this finding, it was proposed that the interaction of CAII with the cotransporter may be decreased following phosphorylation of cotransporter (Gross et al., 2002). This model is based on the concept of a dynamic phosphorylation-dependent interaction of CAII with the C-terminus of kNBC1. Specifically, when the transporter is functioning in the 3:1 mode (kNBC1-Ser^{982} or pNBC1-Ser^{1026} unphosphorylated), CAII interacts with the carboxy-terminus of kNBC1. Following the PKA-dependent phosphorylation of kNBC1-Ser^{982} or pNBC1-Ser^{1026}, CAII dissociates from the transporter, which accounts for the decreased effect of CAII inhibition.

Muller-Berger et al. (1997) have suggested an alternative explanation for the finding that carbonic anhydrase inhibition alters flux through the cotransporter only in the 3:1 mode, but not in the 2:1 mode. Furthermore, in the same study, the finding that ACZ alters the flux through the cotransporter in the 3:1 and not in the 2:1 mode was utilized as a means to distinguish whether HCO_3^- or CO_3^{2-} was the transported species. The authors suggested that the ratio of $[CO_3^{2-}]_o$ to $[CO_3^{2-}]_i$ would be altered significantly following ACZ treatment, whereas the ratio of $[HCO_3^-]_o$ to $[HCO_3^-]_i$ would not. As such, the inhibition of flux through the transporter in the 3:1 mode argues in favor of the stoichiometry being $1Na^+ + 1CO_3^{2-} + 1HCO_3^-$, in contrast to the 2:1 mode, where $1Na^+ + 2HCO_3^-$ are being transported.

Role of NBC1 Amino-Terminus

Gross et al. (2003) have recently shown that an additional consensus PKA phosphorylation site (Thr^{49}) found in the pNBC1 ammo-terminus plays an essential role in the increase of electrogenic flux through the cotransporter in response to 8-Br-cAMP. 8-Br-cAMP, in addition to shifting the stoichiometry, caused a significant increase in G_{DS} of cells expressing pNBC1. The findings differed from the data obtained in cells expressing the kNBC1 variant, where only a stoichiometry shift was detected. Although the effect did not involve phosphorylation of Thr^{49}, which was endogenously phosphorylated, replacing this residue with Asp or Ala abolished the 8-Br-cAMP-induced increase in G_{DS}. These data provide the first evidence of the distinct roles of the amino and carboxy termini in regulating the function the NBC1 cotransporters.

Conclusion

Members of the BTS have the unique property of mediating bicarbonate transport across biological membranes. The transport of HCO_3^- is coupled to the flux of Na^+ and/or Cl^- in an electroneutral or electrogenic fashion. Until recently the structure of electrogenic Na^+-HCO_3^- co-transporters remained elusive. Significant progress has been made in determining the structural properties of the electrogenic Na^+-HCO_3^- cotransporter NBC1. Motifs in the carboxy terminus have been shown to play an important role in modulating the $Na^+ : HCO_3^-$ transport stoichiometry and in mediating the interactions of the co-transporter with CAII. Moreover, the role of the amino terminus in modulating the magnitude of the flux through the cotransporter has been characterized. Furthermore, the change in free energy associated with the PKA-dependent phosphorylation of a specific carboxy-terminal serine residue (kNBC1-Ser^{982} or pNBC1-Ser^{1026}) provides an additional mechanism for continuous cellular bicarbonate efflux when the cotransporter operates in a 2:1 transport mode. The recent topographic characterization of NBC1 permits putative residues that determine the ion specificity, coordination, and permeation through the cotransporter to be identified. In addition, an equilibrium thermodynamic approach can provide further insight into the specific anionic species (HCO_3^- or CO_3^{2-}) that is cotransported with Na^+.

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