Acid-Base Transport by the Renal Proximal Tubule

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One of the major tasks of the renal proximal tubule is to secrete acid into the tubule lumen, thereby reabsorbing approximately 80% of the filtered HCO$_3^-$ as well as generating new HCO$_3^-$ for regulating blood pH. This review summarizes the cellular and molecular events that underlie four major processes in HCO$_3^-$ reabsorption. The first is CO$_2$ entry across the apical membrane, which in large part occurs via a gas channel (aquaporin 1) and acidifies the cell. The second process is apical H$^+$ secretion via Na-H exchange and H$^+$ pumping, processes that can be studied using the NH$_4^+$ prepulse technique. The third process is the basolateral exit of HCO$_3^-$ via the electrogenic Na/HCO$_3^-$ co-transporter, which is the subject of at least 10 mutations that cause severe proximal renal tubule acidosis in humans. The final process is the regulation of overall HCO$_3^-$ reabsorption by CO$_2$ and HCO$_3^-$ sensors at the basolateral membrane. Together, these processes ensure that the proximal tubule responds appropriately to acute acid-base disturbances and thereby contributes to the regulation of blood pH.

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the formation of titratable acid (14). The tiny amount of HCO₃⁻ that, in parallel, moves into the blood is the “new HCO₃⁻.”

In this review, for the sake of simplicity, I refer to PT trans-epithelial acid-base transport as “HCO₃⁻/H₂CO₃ reabsorption” (J HCO₃). In fact, I mean the sum of J HCO₃ and the creation of new HCO₃⁻. We will see that these processes are under the powerful control of a system that we are only beginning to understand, one that acutely monitors plasma [CO₂] and [HCO₃⁻/H₂CO₃] but not plasma pH.

Apical CO₂ Entry
Effect of CO₂ on pHᵢ

Since the work of Jacobs on Symphytum flower petals (the pigment of which is a pH indicator) in 1920 (15), it has been appreciated that CO₂ can rapidly cross cell membranes and acidify the cytoplasm (reviewed in reference [16]). Thomas (17) and Boron and De Weer (18), both working with pH-sensitive microelectrodes, did the first work with physiologic levels of CO₂ on animal cells. Both studies confirmed that CO₂ does indeed cause a rapid fall in pHᵢ (Figure 2A), as shown for the squid axon by the initial part of the record in Figure 2B. If CO₂ simply equilibrated across the cell membrane, as shown in Figure 2A, then pHᵢ would have fallen—by an amount determined by the initial pHᵢ, [CO₂], and intracellular buffering power (16,19,20)—and stabilized. However, pHᵢ recovers from this acute acid load via a mechanism that requires the input of energy. The experiment in Figure 2B was the first to demonstrate such a pHᵢ recovery, that is, dynamic pHᵢ regulation. We now know that a Na⁺/H⁺ exchanger (discussed below) is responsible for this pHᵢ recovery—a metabolic compensation (i.e., HCO₃⁻ uptake) to a respiratory acidosis (i.e., CO₂ influx). The withdrawal of CO₂ causes pHᵢ to rise substantially above its initial level, this overshoot being a direct reflection of the preceding metabolic compensation. Subsequent work by scores of authors on countless cell types, including the PT and other renal cells, shows that the response of virtually all cells to CO₂/HCO₃⁻ is some variation on the theme first demonstrated with the squid giant axon (for review, see reference [21]).

Membranes with Negligible Gas Permeability

One of the core dogmas of physiology has been that all gases rapidly cross all membranes by dissolving in the membrane lipid. In the case of CO₂, the gas produces the effects that are shown in Figure 2B. For example, perfusing the lumen of a PT with CO₂/HCO₃⁻ causes pHᵢ to fall rapidly. Thus, when perfusing single gastric glands as in Figure 3A (22), using the...
technique that was introduced originally by Burg et al. (23), we were surprised by the parietal cell data in Figure 3B (and chief cell data; data not shown), which demonstrate that adding CO₂/HCO₃⁻ to the luminal perfusate causes no detectable pH change, even though far lower [CO₂] levels—when presented to the basolateral surface—evoke easily detectable pH decreases (24). The perfusate with 100% CO₂ maintained its acidity along the entire length of the lumen, ruling out the possibility that the CO₂ had totally escaped in the first few milliseconds of its journey. Furthermore, luminal CO₂/HCO₃⁻ failed to alter pH₁ over a wide range of [CO₂]/[HCO₃⁻] ratios, ruling out the possibility that the separate influxes of CO₂ and HCO₃⁻ might have precisely compensated for one another and yielded a null pH₁ shift. These were the first membranes shown to have negligible gas permeability. We also found that lowering luminal pH to 1 failed to alter pH₂ (22).

We similarly observed null pH₁ shifts, rather than the expected pH increase, when perfusing lumens of gastric gland (24) or colonic crypts (25) with NH₃/NH₄⁺. Kikeri et al. (26), when perfusing the lumen of a mouse myocardial thick ascending limb with NH₃/NH₄⁺, previously observed a substantial and paradoxic pH₁ decline, which proves that the pH₁ effects of the NH₄⁺ influx overwhelmed those (if any) of an NH₃ influx. In fact, it would be interesting to see whether the apical membranes of the medullary thick ascending limb, like those of the gastric gland, indeed are impermeable to CO₂ and NH₃.

AQP1: A Bifunctional Water/Gas Channel

In a seminar at the University of Pennsylvania, in which I presented the data in Figure 3B, I suggested that the unknown specialization of gastric-gland apical membranes that allows them to resist luminal pH values below 1 also renders these membranes impermeable to CO₂ and NH₃. Paul De Weer asked me whether I had considered the possibility that the difference between apical and basolateral gastric-gland membranes might be that the basolateral membranes possess “gas channels” that the apical membranes lack. At first blush, I thought that the gas-channel suggestion was outlandish. De Weer soon forgot his comment, but I did not. After returning home, I began to imagine where gas channels might exist, if they existed at all. As luck would have it, Peter Agre, after a seminar at Yale, had generously sent us the cDNA encoding AQP1—discovered in red blood cells (RBC)—so that we might confirm the observation (27) that the water channel AQP1 is not permeable to H⁺ (see also reference [28]). The combination of De Weer’s comment and Agre’s seminar provoked us to think about why RBCs—which transport gas, not water, for a living—should be a rich reservoir of AQP1. Although there is no doubt that AQP1 is a water channel, might its physiologic role in RBCs be as a gas channel?

To test this hypothesis, Nakhoul injected cRNA encoding human AQP1 (or water as a control) into Xenopus oocytes, which he later injected with CA II protein. The purpose of the CA II was to catalyze the intracellular reaction CO₂ + H₂O → HCO₃⁻ + H⁺ and thereby keep [CO₂] low near the inner surface of the cell membrane, maximizing the CO₂ influx. Nakhoul found that when he exposed oocytes to CO₂/HCO₃⁻, those that expressed AQP1 exhibited a CO₂-induced fall in pH₁ that was 40% faster than the control cells (29). This was the first evidence that the presence of a protein could enhance the movement of a dissolved gas across a cell membrane.

Gordon Cooper extended Nakhoul’s observations in experiments such as those shown in Figure 4. An important difference in Cooper’s experiments is that, rather than inject CA II, he enhanced CO₂ influx by removing the oocyte’s vitelline membrane (30). Focusing on the purple record in Figure 4, we see that switching the extracellular solution from one buffered with HEPES to one buffered with 1.5% CO₂/10 mM HCO₃⁻ caused pH₁ to fall slowly, at a rate of -9.6 × 10⁻⁴ pH units/s. When Cooper subsequently transferred the oocyte to deionized water, osmosis caused it to lyse in 180 s. This oocyte had a relatively low level of AQP1 expression. The oocyte that is represented by the orange record had a much higher rate of acidification and a more rapid lysis. Finally, the oocyte that is represented by the green record had an even higher acidification rate and an even quicker lysis. These results showed that CO₂ entry paralleled AQP1 expression. Cooper additionally demonstrated that p-chloromercuribenzene sulfonate (pCMBS), an organic mercury that blocks AQP1’s water permeability, also eliminates the statistical difference between AQP1 and control oocytes. Finally, Cooper found that the C189S mutant, which Preston et al. (31) showed to be mercury insensitive in terms of water permeability, also is pCMBS insensitive in terms of CO₂ permeability.

Prasad et al. (32) confirmed that AQP1 enhances CO₂ permeability, studying purified AQP1 protein that was reconstituted into vesicle that was made from Escherichia coli membranes. Yang et al. (33) published experiments that seem to disprove the hypothesis that AQP1 acts as a conduit for CO₂. However, as discussed by Cooper et al. (34), these experiments were designed in a way that precluded detection of any enhancement of CO₂ permeability by AQP1.

Using a mass-spectroscopy technique to measure the permeability of RBC to ¹²C¹⁸O¹⁶O, Forster et al. (35) in 1998 made the...
intriguing observation that 4,4′-diisothiocyanostilbene-2,2′-disulfonate (DIDS)—which, among other things, blocks the Cl-HCO₃ exchanger AE1—greatly reduces CO₂ permeability. In 2003, Blank and Ehmke (36), using the fluorescent dye BCECF to monitor pH in RBC ghosts, showed that HgCl₂ (presumably by blocking AQP1) and DIDS (presumably by blocking AE1) greatly reduced CO₂ permeability. Also in 2003, Uehlein et al. (37) demonstrated that a homologue of AQP1 enhances CO₂ permeability in plants, enhancing photosynthesis (the rate-limiting step for which is the availability of CO₂) and the growth of leaves. More recently, Gros et al. (38), using the mass-spectroscopy approach with normal and AQP1-null human RBCs, found that AQP1 is responsible for approximately 60% of the cells’ CO₂ permeability. An unidentified RBC protein, possibly the Rh complex or AE1, is responsible for an additional approximately 30%. It is interesting that parallel experiments on oocytes showed that DIDS blocks approximately 50% of the CO₂ flux through AQP1.

A final example of a physiologic role for AQP1 as a gas channel is provided by Zhou et al. (39), who worked with PT from wt versus AQP1-null mice (generously provided by Alan Verkman). Zhou et al. found that lack of AQP1 leads to a substantial deficit in HCO₃⁻ reabsorption, as would be predicted by the model in Figure 1. He also performed another set of experiments that rely on a novel rapid-mixing technique that was developed by Zhao et al. (40) for creating out-of-equilibrium (OOE) CO₂/HCO₃⁻ solutions. We describe this approach in more detail below. When Zhou perfused the PT lumen with a CO₂/HCO₃⁻-free solution and exposed the basolateral surface to a “pure” HCO₃⁻ solution (i.e., one with a physiologic [HCO₃⁻] and pH but virtually no CO₂), it made no difference whether the PT was from a wt or AQP1-null mouse: The back-flux of “carbon” was the same. However, when he exposed the basolateral surface to a “pure” CO₂ solution (i.e., one with a physiologic [CO₂] and pH but virtually no HCO₃⁻), the back-flux, compared with wt tubules, was 60% lower in AQP1-null than in wild-type tubules. These data are consistent with the hypothesis that the CO₂ permeability of AQP1 plays a major role in the reabsorption of HCO₃⁻.

A lingering issue has been whether it is reasonable to expect a dissolved gas such as CO₂ to pass through AQP1. Recent molecular dynamics simulations by Wang et al. (41) suggested that CO₂ could pass (1) through each of the four aquaporines of an AQP1 tetramer, single file with water and (2) through the central pore formed by the four monomers. This central pore seems to be a vacuum through which CO₂ and O₂ can move with great speed.

**Apical H⁺ Secretion**

**The NH₄⁺ Prepulse**

From his original work on the membrane-permeability properties of *Spirogyra* in the late 19th century, Overton understood that NH₃ in a solution that contains NH₄⁺ (NH₃ + H⁺ ⇌ NH₄⁺)—as well as various neutral amines (R-NH₂ + H⁺ ⇌ R-NH₃⁺) in solutions that contain their charged ammonium ions—crosses cell membranes predominantly in their uncharged form. Working with *Rhododendron* flower petals (the pigment of which shifts from red to blue in response to alkalinity) and starfish eggs that contain neutral red, Jacobs (42) in 1922 recognized that the influx of NH₃—even in acidic solutions of NH₄Cl—causes a rise in pH (Figure 5A). Many decades later, Thomas (17) used a pH-sensitive microelectrode to monitor the rise in pHi that was caused by exposing a snail neuron to a solution that contained NH₄Cl. The left side of Figure 5B shows an experiment from 1976 in which Boron and De Weer (18) exposed a squid giant axon for a relatively brief period to a solution that contained 10 mM NH₄Cl. Although the NH₃ in the solution was present at very low levels compared with the NH₄⁺, the influx of this NH₃ nevertheless led to a consumption of cytoplasmic H⁺ and thus an increase in pHi. Removing the NH₄Cl caused the pHi changes to reverse. However, pHi always fell to a value slightly below the initial one.

The explanation for the pHi undershoot in the first part of the experiment becomes clear with a more protracted exposure to NH₄Cl. The rapid initial NH₃-induced pH increase was followed by a much slower but sustained pHi decrease, which reflects the influx of the weak acid NH₄⁺. Subsequently removing the NH₄Cl, after this long exposure, caused pHi to undershoot the pre-NH₄Cl value by an exaggerated amount. The magnitude of this undershoot reflects the degree of NH₄⁺ influx during the previous NH₄Cl exposure. This was the first example of what has come to be known as the “ammonium prepulse” technique for acid loading cells. In fact, the axon will use its pHi-regulatory machinery to recover from the intracellular acid load in Figure 5B but only in the presence of CO₂/HCO₃⁻, as Boron and De Weer (43) subsequently demonstrated. This later work was the first practical use of an NH₄⁺ prepulse as a tool to acid load a cell and the first demonstration...
that cells can use HCO₃⁻ uptake to neutralize an acid load and thereby regulate pHᵢ.

**Apical Na-H Exchange**

One of the first vertebrate cells to be the focus of a study that exploited the NH₄⁺ prepulse technique was the PT of the salamander. Figure 6 shows the results of an experiment by Boron and Boulpaep (44), performed on an isolated, perfused tubule, cells of which were impaled with microelectrodes for monitoring pHᵢ and basolateral membrane potential (basolateral Vₘ). At first, both the lumen and the “bath” (i.e., the basolateral solution) contained a Na⁺-free solution to block Na⁺-dependent processes for regulating pHᵢ. In addition, the bath contained 4-acetamido, 4'-isothiocyanato-2,2'-stilbene disulfonate (SITS; an analog of DIDS) to block the electrogenic Na/HCO₃ co-transporter on the basolateral membrane. Applying NH₄⁺ caused a rapid rise in pHᵢ followed by a slower fall. Washing away the NH₄⁺ caused pHᵢ to fall substantially and then recover only very slightly. Subsequently returning 100 mM Na⁺ to the lumen caused pHᵢ to return to its initial value. Other experiments showed that this pHᵢ recovery is inhibited by amiloride, demonstrating that it was an apical Na-H exchanger that was responsible for the pHᵢ recovery. Murer et al. (45), in their studies of brush border membrane vesicles, had been the first to observe Na-H exchange. The groups of Aronson (46) and Saktor (47) also made seminal contributions to our understanding of Na-H exchange by working with brush border membrane vesicles. The experiment shown Figure 6 was the first demonstration of apical Na-H exchange by a living epithelial cell.

**Basolateral Na/HCO₃ Co-Transport**

**Initial Description**

In the late 1970s and early 1980s, the mechanism of HCO₃⁻ exit across the PT basolateral membrane was a subject of considerable interest (reviewed in references [2,3]). One suggestion was that HCO₃⁻ exited via Cl-HCO₃ exchange, although the dominant view seems to have been that HCO₃⁻ exited via a conductive pathway, the simplest explanation for which would be a HCO₃⁻ channel. While exploring these options in an isolated, perfused salamander PT, Boulpaep and Boron (48) found that lowering basolateral [HCO₃⁻] ([HCO₃⁻]ᵢ) at a fixed [CO₂]ᵢ causes pHᵢ to fall rapidly—an observation that was consistent with both models. In addition, they found that this maneuver causes basolateral Vₘ to shift rapidly in the positive direction, consistent with the HCO₃⁻ conductance model. However, when they removed basolateral Na⁺, they made a startling observation (Figure 7): Although pHᵢ fell as expected (e.g., reversing the basolateral Na-H exchanger would have caused an internal acidification), the basolateral Vₘ shifted rapidly in the positive direction. Lowering the concentration of an extracellular cation should have caused basolateral Vₘ to shift in the negative direction. The observation that lowering [HCO₃⁻]ᵢ and removing basolateral Na⁺ each caused basolateral Vₘ to shift in the positive direction—together with other data—pointed to a new kind of transporter, an electrogenic Na/HCO₃ co-transporter that moves more negative charge as HCO₃⁻ (or CO₃²⁻) ions than positive charge as Na⁺. Like the previously discovered Cl-HCO₃ exchanger of RBCs and the Na⁺-driven Cl-HCO₃ exchanger of invertebrates, the Na+/HCO₃ co-transporter is blocked by the disulfonic stilbenes SITS and DIDS. However, in contrast to the other two transporters, the electrogenic Na/HCO₃ co-transporter is independent of Cl⁻. Later work by Soleimani and Aronson on basolateral membrane vesicles from rabbit renal cortex pointed to a Na⁺: HCO₃⁻ stoichiometry of 1:3 (49), which ensures that the transporter normally mediates the exit of Na⁺ and HCO₃⁻ across the basolateral membrane—the basolateral step of HCO₃⁻ reabsorption. Later work by Frömter and his colleagues (50–52) showed that the electrogenic Na/HCO₃ co-transporter is the dominant basolateral pathway for HCO₃⁻ in the S₁ and S₂ segments of the PT, where the vast majority of the HCO₃⁻ reabsorption occurs, but that a basolateral Cl-HCO₃ exchanger also is important in the S₃ segment.

**Figure 6.** Use of an NH₄⁺ prepulse to acid-load an isolated, perfused salamander PT cell. pHᵢ was measured with a recessed-tip glass pH microelectrode (17), in conjunction with a KCl-filled microelectrode. The tubule was exposed throughout the experiment, at both the luminal and the basolateral sides, to HEPES-buffered solution that lacked CO₂/HCO₃⁻. During the indicated time, the tubule was exposed from both the luminal and the basolateral (i.e., “bath”) surfaces to a solution in which 20 mM NH₄⁺ replaced 20 mM Na⁺, an NH₄⁺ prepulse designed to acid-load the cell. Extracellular [Na⁺]ᵢ was either 0 or 100 mM, as indicated. Vₘᵢ, basolateral membrane potential; SITS, 4-acetamido, 4'-isothiocyanato-2,2'-stilbene disulfonate. Data from reference (44).

**Figure 7.** The effect of basolateral Na⁺ removal in an isolated, perfused salamander PT, the electrogenic Na/HCO₃ co-transporter. pHᵢ was measured with a recessed-tip glass pH microelectrode (17), in conjunction with a KCl-filled microelectrode. The tubule was exposed throughout the experiment, at both the luminal and the basolateral sides, to a solution buffered with 1.5% CO₂/10 mM HCO₃⁻. During the indicated times, basolateral Na⁺ was replaced with an organic cation. Dotted red lines indicate expected Vₘᵢ response in the absence of an electrogenic Na/HCO₃ co-transporter. Data from reference (48).
Cloning

The absence of a naturally abundant and highly enriched source of the electrogenic Na/HCO₃⁻ co-transporter—unlike the Cl/HCO₃⁻ exchanger AE1, which is highly expressed in RBCs—frustrated early attempts to obtain the cDNA that encodes the electrogenic Na/HCO₃⁻ co-transporter. Michael Romero joined our laboratory in 1992 with the goal of expression-cloning the transporter in Xenopus oocytes. Matthias Hediger in the laboratory of Ernest Wright had perfected this approach in cloning the Na/glucose transporter (53–55). Later, as an independent investigator, Hediger had already expression-cloned a wide range of membrane proteins, including a glutamate transporter (56), a subunit of heteromeric amino-acid transporters (57), a urea transporter (58), and an H-driven peptide transporter (59). We were most fortunate that Hediger was interested in collaborating with Romero.

The first step in expression cloning is to inject size-selected mRNA from a tissue source (rabbit kidneys in our case) into Xenopus oocytes and then assay for expression of the desired function. Unfortunately, repeated attempts with rabbit mRNA were fruitless. Before giving up, we decided to inject mRNA from salamander kidneys, reasoning that amphibian oocytes might better express an amphibian mRNA. To our delight, this approach was successful. Another key element in this study was our assay. We chose to remove extracellular Na⁺ in the presence of CO₂/HCO₃⁻ and then look for a small, positive shift in $V_m$ that is characteristic of the co-transporter (Figure 7), rather than the usual negative shift. With Hediger, Romero generated a cDNA library from the salamander mRNA and methodically searched through ever-smaller groups of clones to arrive at a single clone that can serve as the template for cRNA that encodes the protein that we named NBC, for Na⁺ bicarbonate co-transporter (60).

We anticipated that NBC could be genetically related to any of several known transporters, including the Na/glutamate transporters (subsequently grouped as part of the SLC1 family of solute-linked carriers [61]), the Cl/HCO₃⁻ exchangers AE1 through 3 (now part of the SLC4 family [11]), the Na/mono-carboxylate co-transporters (which turned out to be part of the SLC5 family that includes the Na/glucose co-transporters [62]), the Na/bile-salt transporters (SLC10 [63]), the cation-coupled Cl⁻ co-transporters such as the Na/Cl co-transporter (SLC12 family [64]), the Na/carboxylate co-transporters (SLC13 [65]), and the Na/phosphate co-transporters (SLC17 and SLC34 [66,67]). Upon sequencing the cDNA clone that encodes NBC, we were surprised to learn that the deduced amino-acid sequence of salamander NBC is approximately 30% identical to that for the three anion exchangers AE1 though AE3, which group closely in the dendrogram in Figure 8 (blue region) and also are known as SLC4A1 through SLC4A3. The cloning of the original NBC (also now known as SLC4A4) led to the discovery of several other Na⁺-coupled members of the SLC4 family, which group together in the gray region of Figure 8.

Burnham et al. (68) subsequently cloned human ortholog of the salamander NBC, and Romero et al. (69) obtained the rat clone. It is interesting to note that even the pure rat clone failed to express robustly in Xenopus oocytes until subcloned into a Xenopus expression vector that flanks both the 5’ and 3’ ends of the rat NBC with the corresponding untranslated regions of the Xenopus β-globin mRNA. This result probably explains why we and others failed in our attempts to expression clone NBCe1 using mammalian mRNA. Bernhard Schmitt raised the first antibodies to the electrogenic NBC, and he and his colleagues demonstrated that the protein is expressed heavily at the basolateral membranes of the S1 and likely the early S2 segments of the renal PT (70).

Other Members of the SLC4 Family

With the discovery of at least one electroneutral NBC—the clone was identified by Pushkin et al. (71), and the encoded protein was characterized as an electroneutral NBC by Choi et al. (72)—we appended the suffix “e” to designate electronegative and “n” to designate electroneutral. Moreover, Virkki et al. (73) and Sassani et al. (74) demonstrated that one of four cDNA clones that previously were isolated by Pushkin et al. (75,76) actually encodes a second electronegative NBC. Thus, the original, renal electronegative NBC is known as NBCe1-A. A second splice variant of the same gene—identified by Abuladze et al. (77) in pancreas and by Choi et al. (78) in heart— is known as NBCe1-B. This splice variant seems to be the most widely expressed NBCe1 variant. A brain-specific splice variant that was identified by Bevensee et al. (79) is known as NBCe1-C. The second electronegative NBC gene is NBCe2, and the electroneutral NBC is NBCn1.

In addition to NBCn1, at least two other electroneutral Na⁺-coupled HCO₃⁻ transporters exist. NDCBE, cloned and characterized by Grichchenko et al. (80), is a Na⁺-driven Cl-HCO₃⁻ exchanger that is expressed heavily in brain but also in other tissues, including kidney. NCBE, cloned by Wang et al. (81), is electroneutral, but whether it transports Cl⁻ still is controversial. Form follows function among mammalian transporters.
Na\(^{+}\)-coupled HCO\(_3\)^\(-\) transporters, with the electrogenic NBC family members grouping together in the peach-colored subregion in the dendrogram in Figure 8 and the electroneutral Na\(^{+}\)-coupled HCO\(_3\)^\(-\) transporters grouping together in the green subregion.

Two members of the SLC4 family have deduced amino acid sequences that lie apart from the rest. AE4 originally was named as a Cl-HCO\(_3\) exchanger (82). However, its function is unsettled, and key areas of its amino acid sequence are more reminiscent of a Na\(^{+}\)-coupled HCO\(_3\)^\(-\) transporter. BTR1, originally cloned by Parker et al. (83), seems to be a Na\(^{+}\)-coupled borate (i.e., boron!) transporter (84), renamed NaBC1. Human mutations in NaBC1 cause a corneal condition that is known as congenital hereditary endothelial dystrophy (85).

Structure

Figure 9 is a model of the topology of NBCe1-A, based on studies of the Cl-HCO\(_3\) exchanger AE1 (86). All members of the family have a large cytoplasmic N terminus (Nt) and a much smaller cytoplasmic C terminus (Ct). The crystal structure of most of the Nt of AE1 has been solved (87) and shows that the Nt is a dimer. We now have crystallized the Nt of NBCe1-A, and a preliminary x-ray diffraction analysis indicates that this Nt, too, is a dimer (88).

Various approaches (e.g., see references [89,90]) generally have yielded similar conclusions about the amino acid residues that contribute to transmembrane segments 1 through 5 (TM1 through TM5). Therefore, investigators feel reasonably confident about these assignments. However, investigators are far less comfortable about the topology of the remainder of the molecule because the data sometimes are conflicting, presumably because the later TMs are more flexible.

One characteristic of the Na\(^{+}\)-coupled HCO\(_3\)^\(-\) transporters is that they all have a long extracellular loop between TM5 and TM6 (11). Moreover, this loop is the site of consensus glycosylation sites—proven in the case of NBCe1-A (91). Another curiosity is the four heavily conserved cysteine residues in these loops but not those of other SLC4 family members. (It is interesting that AE4 has these for Cys residues.) Work on AE1 shows that lysine residues near the end of TM5 and TM12 can bind covalently the inhibitor DIDS. Preliminary work from our laboratory (92) indicates that these same regions are important for the reversible, noncovalent binding of DIDS.

Abuladze et al. (93) have mutated a large number of charged residues that are conserved among SLC4 members and have identified several mutations that interfere with function. Choi et al. (unpublished observations) have constructed a series of chimeras between NBCe1-A and one splice variant of NBCn1 (NBCn1-B) in which they exchanged the cytoplasmic Nt domain, third extracellular loop, and/or the cytoplasmic Ct domain. They found that none of these three domains is important for determining the electrogenic of the transporter but that both TM1 through 5 and TM6 through 13 of NBCe1 must be present for the chimera to function as an electrogenic co-transporter.

Naturally Human Mutations of NBCe1 (SLC4A4)

Investigators thus far have identified 10 mutations (94–100) in the human SLC4A4 gene that are associated with a variety of defects that may include severe autosomal recessive, pRTA, ocular abnormalities, growth and mental retardation, and abnormal dentition. The sites of these mutations are indicated by green circles in Figure 9. Three of these are nonsense mutations that lead to premature truncation of the protein, and seven are missense mutations.

Of the three missense mutations, the first is Q29X (95), numbered for the renal splice variant NBCe1-A, which results in a truncation very early in the cytoplasmic Nt. The second is the deletion of nucleotide 2311 (97), which presumably causes a frameshift in codon 721 and, after 27 anomalous amino acids, a premature stop in the extracellular loop between TM7 and TM8. The third missense mutation is a 67-nucleotide deletion at the boundary of exon 23 and the following intron (100); it would lead to a truncated cytoplasmic Ct.

Of the seven missense mutations, R298S in the cytoplasmic Nt, S427L in TM1, and R510H near the extracellular end of TM4 all seem to reduce targeting of the co-transporter to the basolateral membrane in polarized renal epithelial cells in culture (101). All three also exhibit decreased functional expression in Xenopus oocytes, although it is not clear to what extent this effect reflects decreased protein delivery to the plasma membrane (94,96,98,101,102).

Work from our laboratory suggests that R881C most likely causes a trafficking defect with normal intrinsic function (103), and A799V seems to cause defects in both trafficking and intrinsic function (104). The most recently discovered NBCe1 mutation is L522P in TM4, which does not traffic to the plasma membrane in oocytes (99).

Basolateral CO\(_2\) and HCO\(_3\)^\(-\) Sensors

Initial Hints

More than a half century ago, Brazeau and Gilman (105) and Pitts and his colleagues (106) published classical experiments demonstrating that respiratory acidosis elicits a rapid, compen-
satory increase in renal H\(^+\) secretion. However, the problem of determining how the kidney senses acid-base disturbances had been refractory to further dissection, mainly because the equilibrium CO\(_2\) + H\(_2\)O \(\rightleftharpoons\) HCO\(_3^-\) + H\(^+\) makes it difficult to attribute effects to changes in [CO\(_2\)] or to [HCO\(_3^-\)] or to [H\(^+\)]. We became interested in this problem in a very indirect way.

Figure 10 shows the arrangement of an isolated, perfused PT. Figure 11 shows two experiments in which Nakhoul et al. (107) used the absorbance spectrum of a fluorescein dye to monitor pH\(_i\) in the cells of an isolated, perfused S3 segment of a rabbit PT. In Figure 11A, switching only the luminal buffer from HEPES to CO\(_2\)/HCO\(_3^-\) caused the expected decrease in pH\(_i\); the influx of CO\(_2\) produced the initial pH\(_i\) decline, and the efflux of HCO\(_3^-\) across the basolateral membrane sustained the intracellular acidification. In Figure 11B, we see that making the same solution change on the basolateral side has a very different effect. For a few seconds, pH\(_i\) declined rapidly, as it did when we added CO\(_2\)/HCO\(_3^-\) to the lumen. However, within 5 s, pH\(_i\) began a rapid increase to a value that is far higher than the initial pH\(_i\). One might argue that the sustained pH\(_i\) increase could have been due to HCO\(_3^-\) uptake. However, adding basolateral CO\(_2\)/HCO\(_3^-\) triggers a similar pH\(_i\) increase even when CO\(_2\)/HCO\(_3^-\) is already present in the lumen, and under these conditions, the tubule actively reabsorbs HCO\(_3^-\). That is, the net traffic of HCO\(_3^-\) across the basolateral membrane is outward. Therefore, as unlikely as it seems, CO\(_2\) and/or HCO\(_3^-\) on the basolateral side of the cell somehow must trigger the secretion of H\(^+\) across the membrane on the apical side.

Further experiments by Chen and Boron (108, 109) showed that adding CO\(_2\)/HCO\(_3^-\) to the bath or to the bath and the lumen markedly stimulated both apical Na-H exchangers and proton pumps. However, adding CO\(_2\)/HCO\(_3^-\) to the only lumen was without effect. These experiments directly demonstrated that a sensor for CO\(_2\) and/or HCO\(_3^-\)—located at or near the basolateral membrane—can stimulate H\(^+\) extrusion at the opposite side of the cell. These experiments provided another surprise: Decreases in pH\(_i\) produce only very small increases in the rates of apical Na-H exchange and H\(^+\) pumping in a living PT cell. The real regulation of H\(^+\) transport rates comes not from intracellular protons but from basolateral CO\(_2\) and/or HCO\(_3^-\). But which?

**OOE CO\(_2\)/HCO\(_3^-\) Solutions**

In the 1993 paper by Nakhoul et al. (107) is an appendix that analyzes the CO\(_2\)/HCO\(_3^-\)-induced alkalinization of Figure 11B in the context of the reactions that are responsible for the interconversion of CO\(_2\) and HCO\(_3^-\). The first of these reactions is very slow, and the second is extremely fast:

\[
\begin{align*}
\text{CO}_2 + \text{H}_2\text{O} & \rightleftharpoons \text{H}_2\text{CO}_3 \rightleftharpoons \text{HCO}_3^- + \text{H}^+ \\
k_1 & \quad k_{-1} \\
& \quad k_2 \\
& \quad k_{-2}
\end{align*}
\]

Before submitting the paper, I wanted to review my calculations with Robert Berliner, who at the time was Professor Emeritus in our department. After considering the above reaction rates for a couple of hours—and thinking about Figure 11B—Bob wistfully looked up at the ceiling and said that it was too bad that we had to add CO\(_2\) and HCO\(_3^-\) together, rather than one at a time. In an instant—primed by our discussion of the slow reaction governed by \(k_1\) and \(k_{-1}\)—the thought came to me, “Of course we can!” All we need to do is to take advantage of that slow reaction… and thus were born out-of-equilibrium (OOE) solutions.

Figure 12A shows the approach for generating a solution with physiologic levels of HCO\(_3^-\) and pH but virtually no CO\(_2\) (“pure” HCO\(_3^-\)), and Figure 12B shows the comparable approach for generating a solution with physiologic levels of CO\(_2\) and pH but virtually no HCO\(_3^-\) (“pure” CO\(_2\)). However, implementing OOE solutions proved to be more challenging than conceiving of them. Shortly after my conversation with Bob, Jinhua Zhao arrived as a new postdoctoral fellow. For 2 yr, she: (1) labored with ever more powerful syringe pumps to deliver evenly two streams of liquid, (2) employed various tricks for adequately mixing these two streams, (3) dealt with issues of temperature regulation, and (4) accommodated the chemistry of CO\(_2\) and HCO\(_3^-\). We tested the system on squid giant axons (40), in which we were able to create “pure” CO\(_2\) solutions that could acidify the cell and increase intracellular buffering power as predicted as well as “pure” HCO\(_3^-\) solutions that could supply the substrate for a HCO\(_3^-\) transporter but not increase intracellular buffering power.
Zhao also applied her new technology to the isolated perfused rabbit PT (110) and then introduced Yuehan Zhou to the project. He monitored \( J_{\text{HCO}_3} \) as well as the rate of volume absorption \( (J_V) \) as he systematically used OOE solutions to vary basolateral composition one parameter at a time (Figure 13A). The results show that raising \([\text{HCO}_3/\text{H}_2\text{CO}_3]_B\) (at a fixed \([\text{CO}_2]_B\) of 5% and a fixed \(p\text{H}_B\) of 7.40) causes \( J_{\text{HCO}_3} \) to fall (Figure 13B, left)—an appropriate response for the “metabolic” part of metabolic acid-base disturbances. That is, the greater the plasma \([\text{HCO}_3/\text{H}_2\text{CO}_3]_B\), the less \( \text{HCO}_3/\text{H}_2\text{CO}_3 \) the kidney ought to reabsorb. Moreover, raising \([\text{CO}_2]_B\) (at a fixed \([\text{HCO}_3/\text{H}_2\text{CO}_3]_B\) of 22 mM and a fixed \(p\text{H}_B\) of 7.40) causes \( J_{\text{HCO}_3} \) to rise (Figure 13B, middle)—an appropriate compensation for the “respiratory” part of respiratory acid-base disturbances. That is, the greater the plasma \([\text{CO}_2]_B\), the more \( \text{HCO}_3^- \) the kidney ought to reabsorb. However, we were surprised to find that raising \( p\text{H}_B \) (at a fixed \([\text{HCO}_3^-]_B\) of 22 mM and a fixed \([\text{CO}_2]_B\) of 5%) elicits no change in \( J_{\text{HCO}_3} \) (Figure 13B, right).

In parallel experiments, we measured \( p\text{H}_L \) under the conditions of Figure 13B and found that, indeed, \( p\text{H}_L \) changes substantially as one increases \( p\text{H}_B \) from 6.8 to 8.0. However, these changes in \( p\text{H}_B \) and \( p\text{H}_L \) do not evoke changes in \( J_{\text{HCO}_3} \). Instead, at least in terms of its acute response to acid-base disturbances, the PT regulates plasma \( p\text{H} \) not by monitoring \( p\text{H} \) but by monitoring two surrogates: the main buffer components of the body, \( \text{HCO}_3^- \) and \( \text{CO}_2 \) (Figure 14). This tactic of regulating a parameter that the body does not measure directly is not unique to acid-base parameters and the PT. For example, the body uses stretch receptors in vessels to gauge the adequacy of the effective circulating blood volume.

An extremely interesting observation in the above study is that the changes in \( J_{\text{HCO}_3} \) occurred without the expected parallel changes in \( J_V \). Because the fluid that the PT reabsorbs is approximately isosmotic, an increase in \( J_{\text{HCO}_3} \), for example, should have been accompanied by a sizable increase in \( J_V \). It seems that the PT cell compensates for changes in NaHCO3 reabsorption by making reciprocal changes in the reabsorption of other solutes, thereby keeping \( J_V \) relatively constant (Figure 14). This response would help to maintain a stable BP as the tubule responds to acid-base disturbances.

It is worth noting that in the above experiments, we applied the challenges only to the basolateral surface of the tubule. Therefore, it is possible that PT have a \( p\text{H} \) sensor at the apical
Figure 14. Model of the regulation of JHCO₃ and Jₙa by basolateral CO₂ and HCO₃⁻ in a renal PT. The green arrows represent stimulation, and the red arrows represent inhibition. We propose that basolateral CO₂ stimulates the acid-base transporters but reciprocally inhibits the transporters that are responsible for the reabsorption of other solutes. HCO₃⁻ would have the opposite effect. Glc, glucose; Lac, lactate; Gln, glutamine; /JOther rate of reabsorption of solutes other than NaHCO₃.

Figure 15. Effect of tyrosine-kinase inhibitors on the CO₂-induced increase in JHCO₃. Data from reference (121).

Role of Tyrosine Kinases
A critical question is how the tubule is able to sense changes in [CO₂]b and transduce them into altered cell function. The bacterium Rhizobium meliloti senses O₂ (113) and the plant Arabidopsis thaliana senses ethylene (114–119) using membrane proteins that signal through a histidine kinase. Because animal cells lack histidine kinases, we hypothesized that PT cells might use a tyrosine kinase to signal an increase in CO₂. Indeed, as shown in Figure 15, 35 nM PD168393, which alkylates a Cys residue in the ATP binding pocket of tyrosine kinases, blocks the ability of the tubule to respond to increased [CO₂]b. Similarly, 10 nM BPIQ-I, which also targets members of the erbB family (122), blocks the ability of the tubule to respond to increased [CO₂]b. Indeed, preliminary work suggests that exposing tubule suspensions to CO₂/HCO₃⁻ leads to the phosphorylation of erbB1 (i.e., EGF receptor) at Tyr residues (123). Although we do not yet know the molecule that senses CO₂, it seems that this dissolved gas signals through a receptor tyrosine kinase, perhaps in part through erbB1.

Role of Angiotensin II
The hormone that has the greatest stimulatory effect on JHCO₃ is angiotensin II (AngII), applied at a “low” dosage to either the luminal or basolateral surface (124–127). When applied at a “high” dosage to either surface, however, AngII actually reduces JHCO₃ (126,128).

We examined the effect low-dosage (10⁻¹¹ M) and of high-dosage (10⁻⁹ M) AngII on the response of JHCO₃ to alterations in [CO₂]b (129). We found that low-dosage AngII, added to either the lumen or the bath, tends to shift the JHCO₃-versus-[CO₂]b relationship to the left, so that lower levels of [CO₂]b tend to stimulate HCO₃⁻ reabsorption. Conversely, high-dosage AngII, added to either the lumen or the bath, tends to blunt the JHCO₃-versus-[CO₂]b relationship.

The PT has all of the molecular machinery to generate its own AngII (130–136), which actually appears in the tubule lumen. It is not yet established whether the tubule secretes angiotensinogen, AngI, or AngII. However, preliminary data from our group on Prinivil, an inhibitor of the angiotensin-converting enzyme, suggest that the tubule actually secretes preformed AngII (137). We decided to examine the effect of AngII receptor blockers on the JHCO₃-versus-[CO₂]b relationship. We were surprised to find that in the absence of added AngII, 10⁻⁸ saralasin, a peptide that blocks both AT₁ and AT₂ receptors, reduces JHCO₃ to levels that normally are observed at 0% CO₂ (see Figure 13B) and renders the tubule insensitive to changes in [CO₂]b between 0 and 20%. The AT₁-specific blocker candesartan has a similar effect. Finally, in tubules from AT₁a-null mice, we find a moderate depression of JHCO₃ at 5% CO₂ and a total insensitivity to changes in [CO₂]b.

Endogenous luminal AngII plays at least a critical permissive role in the tubule’s response to alterations in [CO₂]b. It would be interesting to know whether basolateral CO₂ somehow accentuates the endogenous AngII system, perhaps by increasing the secretion of AngII, increasing the density or the sensitivity of apical AT₁ receptors, or enhancing downstream signaling from the AT₁ receptor to the acid-base transporters.

Conclusions and Outlook
Looking back over the past 60 yr of progress in understanding acid secretion by the PT, it is sobering to realize that the pioneers in the field, working with technologies that are far more primitive than those that are at our disposal today, were able to assemble a good picture of the fundamental processes (138–141): The tubule exchanges luminal Na⁺ for cytosolic H⁺, carbonic anhydrase catalyzes the secretion of H⁺ to titrate filtered HCO₃⁻ to CO₂ and H₂O, and these substances enter the cell...
and regenerate $\text{HCO}_3^-$ that then moves into the blood. Today, we understand many more of the details, including the dynamics of electrical and chemical gradients and the molecular identities of the transporters. We know their amino acid sequences, and we even know many examples in which specific mutations of these proteins lead to human disease.

These advances notwithstanding, much remains to be learned. We still do not understand how the transporters function at an atomic level or the mechanisms of disease-causing mutations. Such understanding will come eventually through advances in structural biology. However, reading between the lines of this review, one can sense that many mysteries remain even at the cellular and molecular levels. Just how do $\text{CO}_2$ and other gases move through aquaporins and other gas channels? Which molecules does the PT cell use to sniff basolateral $\text{CO}_2$ and $\text{HCO}_3^-$? How does the cell transduce these signals to the acid-base transporters? How does the cell reciprocally regulate the reabsorption of $\text{NaHCO}_3$ and of other solutes so as to stabilize $j_{\text{V}}$? What role does locally generated AngII play in these processes? How does the PT cell integrate information from $\text{CO}_2$ levels, locally versus systemically generated AngII, and other hormones such as parathyroid hormone and endothelin? With continued support from the National Institutes of Health and other agencies such as the American Heart Association and the National Kidney Foundation, the renal community can look forward to the day when other winners of the Homer W. Smith Award will answer these questions.

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