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THE RENAL ELECTROGENIC Na^+:HCO_3^- COTRANSPORTER

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Summary

The electrogenic Na^+:HCO_3^- cotransporter (symporter) is the major transporter for HCO_3^- reabsorption across the basolateral membrane of the renal proximal tubule and also contributes significantly to Na^+ reabsorption. We expression-cloned the salamander renal electrogenic Na$^+$:Bicarbonate Cotransporter (NBC) in *Xenopus laevis* oocytes. After injecting poly(A)+ RNA, fractionated poly(A)+ RNA or cRNA, we used microelectrodes to monitor membrane potential (V_m) and intracellular pH (pHi). All solutions contained ouabain to block the Na^+/K^+ pump (P-ATPase). After applying 1.5 % CO_2/10 mmol l^{-1} HCO_3^- (pH 7.5) and allowing pHi to stabilize from the CO_2-induced acidification, we removed Na^+. In native oocytes or water-injected controls, removing Na^+ hyperpolarized the cell by −5 mV and had no effect on pHi. In oocytes injected with poly(A)+ RNA, removing Na^+ transiently depolarized the cell by −10 mV and caused pHi to decrease; both effects were blocked by 4,4′-diisothiocyano-2,2′-stilbenedisulfonate (DIDS) and required HCO_3^-. We enriched the signal by electrophoretic fractionation of the poly(A)+ RNA, and constructed a size-selected cDNA library in pSPORT1 using the optimal fraction. Screening the *Ambystoma* library yielded a single clone (aNBC). Expression was first obvious 3 days after injection of NBC cRNA. Adding CO_2/HCO_3^- induced a large (>50 mV) and rapid hyperpolarization, followed by a partial relaxation as pHi stabilized. Subsequent Na^+ removal depolarized the cell by more than 40 mV and decreased pHi. aNBC is a full-length clone with a start Met and a poly(A)+ tail; it encodes a protein with 1025 amino acids and several putative membrane-spanning domains. aNBC is the first member of a new family of Na^+-linked HCO_3^- transporters. We used aNBC to screen a rat kidney cDNA library, and identified a full-length cDNA clone (rNBC) that encodes a protein of 1035 amino acids. rNBC is 86 % identical to aNBC and can be functionally expressed in oocytes.

Key words: intracellular pH, acid–base balance, kidney, proximal tubule, bicarbonate transport.

Introduction

HCO_3^- reabsorption by the kidney

One of the major functions of the renal proximal tubule is to reclaim HCO_3^- that has been filtered in the glomerulus. This reabsorption of HCO_3^- from the proximal-tubule lumen to the blood helps to maintain an appropriately high [HCO_3^-] in the blood and thus helps to stabilize blood pH. Failure to reabsorb sufficient HCO_3^- would lead not only to the loss of HCO_3^- in the urine but also to the loss of Na^+ (which normally accompanies reabsorbed HCO_3^-) and osmotically obligated water. Thus, the consequences of reduced Na^+ reabsorption would be metabolic acidosis and volume depletion.

As shown in Fig. 1, HCO_3^- reabsorption by the proximal tubule is a multistep process. First, H^+ is secreted into the lumen by Na^+/H^+ exchangers (antiporters) and presumably vacuolar-type H^+ pumps (V-type ATPases). Second, this H^+ titrates luminal HCO_3^- to CO_2 and H_2O, a process accelerated by carbonic anhydrase IV (CA IV), which is tethered to the extracellular surface of the apical membrane. Third, the newly formed CO_2 and H_2O enter the proximal-tubule cell. Fourth, cytoplasmic CA II accelerates the regeneration of H^+ and HCO_3^-. Finally, this HCO_3^- exits the proximal-tubule cell across the basolateral membrane, completing the movement of HCO_3^- from lumen to blood.

In the portion of the proximal tubule furthest downstream from the glomerulus, the S3 segment, the efflux of HCO_3^- across the basolateral membrane is mediated by both a Cl^-/HCO_3^- exchanger and an electrogenic Na^+:HCO_3^- cotransporter (Nakhoul *et al.* 1990). However, in more proximal segments of the proximal tubule (S2 and S1 segments), where the bulk of HCO_3^- is in fact reabsorbed, the relative contribution of Cl^-/HCO_3^- exchange decreases and that of electrogenic Na^+:HCO_3^- cotransport increases (Kondo and Frömter, 1987). All told, the proximal tubule reabsorbs some 90 % of the filtered HCO_3^- and 70 % of the filtered Na^+. The electrogenic Na^+:HCO_3^- cotransporter carries the vast majority of this HCO_3^- and approximately 25 % of the Na^+.

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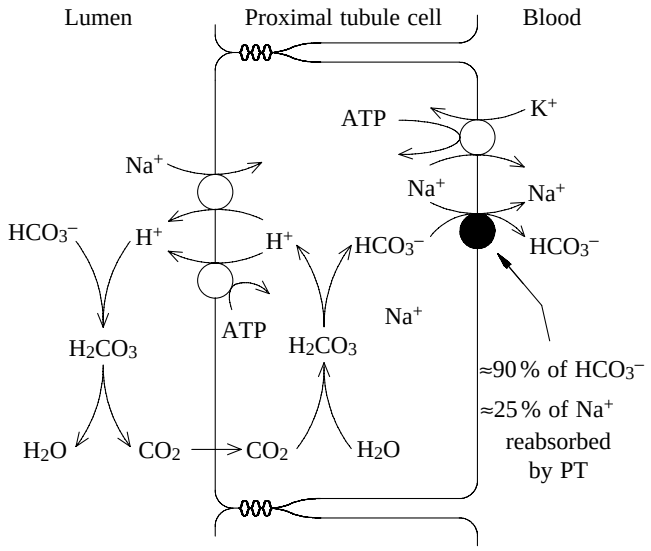


Fig. 1. Model of HCO_3^- reabsorption by the mammalian renal proximal tubule (PT). The renal electrogenic Na^+:HCO_3^- cotransporter, which has a Na^+:HCO_3^- stoichiometry of 1:3, is the major route for transferring HCO_3^- from the proximal-tubule cell to the blood and is responsible for moving as much as approximately 90 % of the HCO_3^- transported by the proximal tubule and approximately 80 % of the HCO_3^- filtered by the glomeruli. The cotransporter is also responsible for moving a substantial amount of Na^+ from cell to blood, perhaps approximately 15 %. As such, the cotransporter functions as an auxiliary Na^+ pump. The steady-state pHi of proximal-tubule cells in CO_2/HCO_3^- is approximately 7.1–7.5, being more alkaline near the glomerulus. The pH of the luminal fluid falls from 7.4 at the glomerulus to approximately 6.6 at the end of the proximal tubule. The membrane voltage across the basolateral membrane is more negative than –60 mV (Boulpaep, 1976). The lumen is a few millivolts negative with respect to the blood.

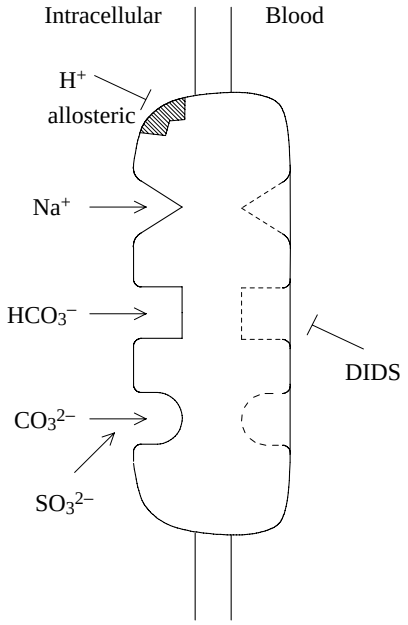


Fig. 2. Model of the renal electrogenic Na^+:HCO_3^- cotransporter, which has a Na^+:HCO_3^- stoichiometry of 1:3. Kinetic evidence (Soleimani and Aronson, 1989) suggests that there are separate binding sites for Na^+, HCO_3^- and CO_3^{2-}, with SO_3^{2-} substituting for CO_3^{2-} at the CO_3^{2-} site. H^+ is an allosteric inhibitor of Na^+ binding. DIDS inhibits the activity of the cotransporter.

The electrogenic Na^+:HCO_3^- cotransporter

First described by Boron and Boulpaep (1983) in the proximal tubule of the salamander *Ambystoma tigrinum*, electrogenic Na^+:HCO_3^- cotransporters have subsequently been studied in several epithelial cell lines (Jentsch *et al.* 1984, 1985), intact rat and rabbit proximal tubules (Alpern, 1985; Sasaki *et al.* 1987), as well as in a number of other cell types (Deitmer and Schlue, 1989; Gleeson *et al.* 1989; Fitz *et al.* 1989; Weintraub and Machen, 1989; Rajendran *et al.* 1991; M. O. Bevensee and W. F. Boron, in preparation; M. O. Bevensee, M. Apkon and W. F. Boron, in preparation). The four hallmarks of the renal Na^+:HCO_3^- cotransporter are its electrogenicity, Na^+-dependence, HCO_3^--dependence and blockade by stilbene derivatives such as DIDS. Probably the most reliable assay for detecting the electrogenic Na^+:HCO_3^- cotransporter in intact cells is to remove extracellular Na^+. This maneuver causes an abrupt positive shift (i.e. depolarization) in the cell membrane voltage (V_m). In the absence of HCO_3^-, in the presence of DIDS or in cells lacking the cotransporter, removing Na^+ generally causes a small negative shift in V_m, reflecting the normal Na^+ conductance of the cell. In the presence of a functional electrogenic Na^+:HCO_3^- cotransporter, Na^+ removal also causes a slower fall in intracellular pH (pHi) as well as a DIDS-sensitive fall in $[Na^+]_i$.

The electrogenicity of the cotransporter implies that each Na^+ moves with two or more HCO_3^- or HCO_3^- equivalents (e.g. CO_3^{2-}). The Na^+:HCO_3^- stoichiometry has not been directly measured. However, it has been deduced by Soleimani *et al.* (1987) on the basis of thermodynamic data obtained from membrane vesicles prepared from the basolateral membranes of rabbit proximal tubules. The principle underlying these experiments is that the net direction of cotransport depends on the stoichiometry as well as on the Na^+ and HCO_3^- electrochemical gradients. Varying these gradients while measuring the net direction of transport, the authors concluded that the renal electrogenic Na^+:HCO_3^- cotransporter has a Na^+:HCO_3^- stoichiometry of 1:3. Further work (Soleimani and Aronson, 1989) was consistent with the hypothesis that the renal electrogenic Na^+:HCO_3^- cotransporter has binding sites for 1 Na^+, 1 HCO_3^- and 1 CO_3^{2-}, and that sulphite (SO_3^{2-}) can replace CO_3^{2-} at the CO_3^{2-} site (Fig. 2).

The physiological family of HCO_3^- transporters

The HCO_3^- transporters shown in Fig. 3 might be thought of as a functional family of transporters that may or may not be related molecular genetically. The renal electrogenic Na^+:HCO_3^- cotransporter, with a Na^+:HCO_3^- stoichiometry of 1:3, is one of as many as four Na^+-coupled HCO_3^- transporters, as well as a Cl^-/HCO_3^- exchanger and a K^+:HCO_3^-

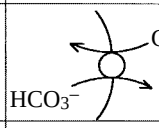
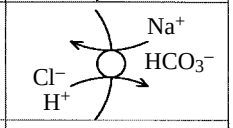
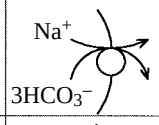
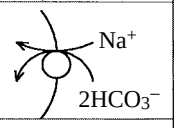
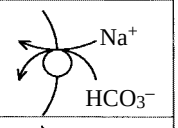
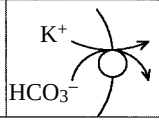
Name	ΔpHi	Process
Cl^-/HCO_3^- exchanger	↓	
Na^+-driven Cl^-/HCO_3^- exchanger	↑	
1:3 Na^+:HCO_3^- cotransporter	↓	
1:2 Na^+:HCO_3^- cotransporter	↑	
1:1 Na^+:HCO_3^- cotransporter	↑	
K^+:HCO_3^- cotransporter	↓	

Fig. 3. Major HCO_3^- transporters in animal cells. Three of these transporters normally cause pHi to decrease: the Cl^-/HCO_3^- exchanger, the 1:3 electrogenic Na^+:HCO_3^- cotransporter and the K^+:HCO_3^- cotransporter. Three others normally cause pHi to increase: the Na^+-driven Cl^-/HCO_3^- exchanger, the 1:2 electrogenic Na^+:HCO_3^- cotransporter and the 1:1 electroneutral Na^+:HCO_3^- cotransporter.

cotransporter. There is at least one, and perhaps two, other Na^+:HCO_3^- cotransporter that normally mediates a net influx, rather than a net efflux, of HCO_3^- equivalents (see Boron and Boulpaep, 1989). That is, they are acid extruders rather than acid loaders. In invertebrate glial cells (Deitmer and Schlue, 1989), mammalian astrocytes (M. O. Bevensee and W. F. Boron, in preparation; M. O. Bevensee, M. Apkon and W. F. Boron, in preparation) and other cells, an electrogenic Na^+:HCO_3^- cotransporter mediates a net influx of Na^+ and HCO_3^-. On the basis of analyses of electrochemical gradients of Na^+ and HCO_3^-, it is believed that such acid-extruding electrogenic Na^+:HCO_3^- cotransporters have a 1:2 stoichiometry. Thus, it is the stoichiometry of the cotransporter, as well as the electrochemical gradients of the transported ions, that determines the net direction of transport and whether the cotransporter behaves as an acid loader (as is generally the case with a 1:3 stoichiometry) or as an acid extruder (as is generally the case with a 1:2 stoichiometry). In addition to cotransporters with stoichiometries of 1:3 and 1:2, work by Vaughan-Jones and colleagues (Dart and Vaughan-Jones, 1992) on heart cells suggests that these cells have an electroneutral Na^+:HCO_3^- cotransporter with a Na^+:HCO_3^- stoichiometry of 1:1. Camilion de Hurtado *et al.* (1995), however, concluded that this cotransporter is an electrogenic 1:2 Na^+:HCO_3^- cotransporter.

The Na^+-driven Cl^-/HCO_3^- exchanger is the major acid extruder in a wide variety of animal cells. In contrast to the two electrogenic Na^+:HCO_3^- cotransporters, the Na^+-driven Cl^-/HCO_3^- exchanger is electroneutral and has an absolute requirement for Cl^-. Like the 1:2 and 1:1 Na^+:HCO_3^- cotransporters, the Na^+-driven Cl^-/HCO_3^- exchanger normally moves HCO_3^- into cells. Aside from the work described here on the cloning of the 1:3 electrogenic Na^+:HCO_3^- cotransporter, there is no published molecular information on any of the Na^+-coupled HCO_3^- transporters.

The Cl^-/HCO_3^- exchanger, sometimes referred to as the Na^+-independent Cl^-/HCO_3^- exchanger to distinguish it from the Na^+-driven Cl^-/HCO_3^- exchanger, was the first HCO_3^- transporter to be described. There is now considerable information on the molecular biology and biochemistry of Cl^-/HCO_3^- exchangers. The first of these to be cloned was the band 3 protein of red blood cells, also known as AE-1 (for Anion Exchanger) (Kopito and Lodish, 1985). AE-1 plays a major role in CO_2 carriage by erythrocytes. Two related Cl^-/HCO_3^- exchangers, AE-2 and AE-3, are present in a wide variety of non-erythroid cells. Because they normally mediate the exchange of extracellular Cl^- for intracellular HCO_3^-, AE-2 and AE-3 function as intracellular acid loaders.

Approach for cloning the renal electrogenic Na^+:HCO_3^- cotransporter

There are no molecular tools available for uniquely identifying any of the cation-coupled HCO_3^- transporters. By forming covalent derivatives with its targets, the stilbene derivative DIDS usually blocks all of the cation-coupled HCO_3^- transporters other than the K^+:HCO_3^- cotransporter. DIDS also inhibits a host of other transporters, channels and proteins. It is clear that DIDS, as well as the related compound 4-acetamido-4′-isothiocyano-2,2′-stilbenedisulfonate (SITS), is hardly specific. Therefore, in embarking on our attempt to clone the renal electrogenic Na^+:HCO_3^- cotransporter, we decided on an expression-cloning approach. Others have used *Xenopus laevis* oocytes to expression-clone cDNAs as widely ranging as those that encode the Na^+:glucose cotransporter (Hediger *et al.* 1987), the H^+:oligopeptide cotransporter (Fei *et al.* 1994), the epithelial amiloride-sensitive Na^+ channel (Canessa *et al.* 1994) and the Ca^{2+} receptor from the parathyroid gland (Brown *et al.* 1993).

We isolated poly(A)+ RNA from either the cortex of rabbit kidneys or whole kidneys of tiger salamanders, and microinjected this RNA into stage 5/6 oocytes from the frog *Xenopus laevis.* After waiting several days, we assayed the oocytes for expression of an electrogenic Na^+:HCO_3^- cotransporter, using the approach schematized in Fig. 4. An oocyte, which is approximately 1.2 mm in diameter, is impaled with both a conventional voltage electrode filled with 3 mol l^{-1}

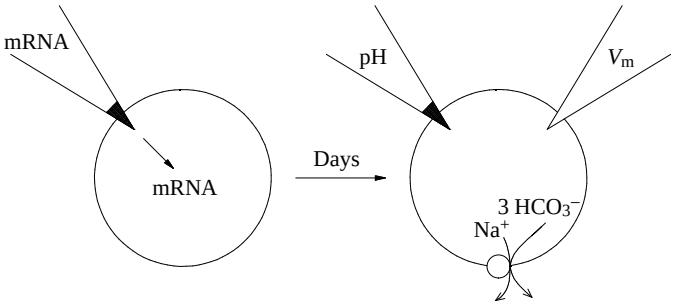


Fig. 4. Injecting RNA and assaying it for activity of an electrogenic Na^+:HCO_3^- cotransporter. First, poly(A)+ RNA (or cRNA synthesized from NBC cDNA) is microinjected into a *Xenopus laevis* oocyte. After several days, the oocyte is impaled with pH and voltage electrodes in order to monitor the pHi and V_m changes characteristic of the electrogenic Na^+:HCO_3^- cotransporter.

KCl and a pH-sensitive microelectrode filled with a liquid pH sensor.

The assay

Perhaps the two most important aspects of an expression-cloning project are to identify a good source of the mRNA that encodes the protein of interest (see below) and to develop a good assay. Our assay was to monitor the V_m and pHi changes caused by removing extracellular Na^+ in the presence of CO_2/HCO_3^-. In the presence of an active electrogenic Na^+:HCO_3^- cotransporter, this maneuver is expected to cause Na^+, HCO_3^- and net negative charge to leave the cell *via* the electrogenic Na^+:HCO_3^- cotransporter and thus to cause a rapid depolarization (i.e. positive shift in V_m) and a slower pHi decrease. To make the assay more specific, we required that these V_m and pHi changes be blocked by DIDS and be dependent upon the presence of CO_2/HCO_3^-. By itself, the depolarization induced by Na^+ removal is nearly diagnostic of electrogenic Na^+:HCO_3^- cotransport. Bear in mind that a conventional Na^+ conductance would produce a hyperpolarization, not the depolarization characteristic of the electrogenic Na^+:HCO_3^- cotransporter. A decrease in electrogenic Na^+ pumping will also produce a depolarization, albeit a slow one that should be prevented by pretreating the cell with ouabain (Boron and Boulpaep, 1983). Indeed, we conducted all of our screening experiments in the presence of ouabain to eliminate the potential complication coming from the Na^+/K^+ pump. A Na^+-dependent K^+ conductance (Kameyama *et al.* 1984) would lead to a depolarization in response to Na^+ removal, and this effect may have complicated the identification of a 1:2 electrogenic Na^+:HCO_3^- cotransporter in mammalian astrocytes (M. O. Bevensee, M. Apkon and W. F. Boron, unpublished results). However, by requiring that the depolarization induced by Na^+ removal be blocked both by DIDS and by removing CO_2/HCO_3^-, we should have eliminated potential confusion from Na^+-dependent K^+ conductances or any other known mechanisms. By further requiring that the V_m changes be accompanied by HCO_3^--dependent and DIDS-sensitive pHi changes, we should have made the assay virtually specific for either a 1:3 or 1:2 electrogenic Na^+:HCO_3^- cotransporter.

We emphasize the specificity of the assay because others have attempted to identify electrogenic Na^+:HCO_3^- cotransporters using much less specific physiological criteria. For example, applying DIDS would be expected to cause a depolarization if an electrogenic Na^+:HCO_3^- cotransporter were operating in the inward direction and a hyperpolarization if the cotransporter were operating in the outward direction. However, DIDS is notoriously non-specific; it can inhibit a wide variety of membrane proteins, including certain Cl^- channels, that could influence V_m. Thus, a DIDS-induced V_m change can hardly be regarded as specific for an electrogenic Na^+:HCO_3^- cotransporter. Another approach that has been used to identify electrogenic Na^+:HCO_3^- cotransporters is to monitor the V_m changes that occur when a CO_2/HCO_3^- solution is applied. In principle, applying CO_2/HCO_3^- should cause an initial hyperpolarization (due to electrogenic Na^+/HCO_3^- influx) that wanes as $[HCO_3^-]_i$ slowly increases, secondary to the influx of CO_2. In the new steady state, the predicted V_m would be either more negative or more positive than the initial V_m, depending on whether the net direction of transport was inward or outward, respectively. However, the application of CO_2/HCO_3^- can cause non-specific V_m changes. In the first place, CO_2 diffuses rapidly across most cell membranes and could cause pHi decreases in the bulk cytoplasm, preceded by perhaps even larger pH changes in the unstirred layer on the inner surface of the cell membrane. Because virtually every ion channel and transporter is at least somewhat pH-sensitive, and some exquisitely so, these pH changes are expected to cause V_m changes. A second mechanism for CO_2-induced V_m changes is the presence of conductance pathways for HCO_3^-. A third potential mechanism for V_m effects is carbamino formation, analogous to the rapid and reversible formation of carbamino hemoglobin, which leads to major conformational changes. Any or all of these CO_2/HCO_3^--induced effects could lead to V_m changes, some of which would probably be blocked or masked by the effects of DIDS. For these reasons, we believe that, when working with an undefined system, it is extremely dangerous to employ non-specific assays for electrogenic Na^+:HCO_3^- cotransport, such as simply the application of DIDS and/or CO_2/HCO_3^-.

Source of mRNA

Our initial approach was to isolate poly(A)+ RNA from the renal cortex of rabbits. Unfortunately, the V_m record in our Na^+-removal assay showed no sign of a transient depolarization. Instead, removing extracellular Na^+ simply caused V_m to become more negative, rapidly and reversibly. These V_m changes are characteristic of a classical Na^+ conductance.

Reasoning that mRNA isolated from another amphibian might express better in *Xenopus* oocytes, our next approach was to isolated poly(A)+ RNA from the kidneys of the tiger

salamander *Ambystoma tigrinum*. This was the species in which the electrogenic Na^+:HCO_3^- cotransporter was first identified (Boron and Boulpaep, 1983). Indeed, we found that Na^+ removal led to a rapid, but transient, depolarization and a slow pHi decrease. These effects were blocked by DIDS and did not occur in the nominal absence of CO_2/HCO_3^-. Nor did these effects occur until several days after the injection of the RNA.

Properties of the cDNA clone, NBC

After screening a cDNA library by expression in *Xenopus* oocytes, we identified a single clone, which we have named NBC (for Na$^+$:Bicarbonate Cotransporter). It is a full-length clone with a start Met and a poly(A)+ tail. The deduced amino-acid sequence of the encoded protein is 1025 amino acids long. Hydropathy analysis predicts multiple membrane-spanning domains. Comparing the DNA sequence of NBC with the GenBank data base reveals that NBC has no substantial homology to known proteins and is thus the first in a new family of Na^+-coupled HCO_3^- transporters.

When cRNA made from the pure NBC clone is injected into *Xenopus* oocytes, the V_m and pHi changes are those predicted above. However, the magnitudes of the changes are substantially larger than when we injected oocytes with poly(A)+ RNA.

Using the NBC clone from *Ambystoma*, which we refer to as aNBC, we cloned its homologue from a rat kidney library. The rat clone, which we refer to as rNBC, is 86 % identical to aNBC. It encodes a protein with a predicted length of 1035 amino acids.

Future trends

Cloning of cDNAs encoding other HCO_3^- transporters

We predict that the 1:3 and 1:2 electrogenic Na^+:HCO_3^- cotransporters will be sufficiently homologous at the base-pair level for it to be possible to use aNBC or rNBC (which presumably represent 1:3 electrogenic Na^+:HCO_3^- cotransporters) to identify various 1:2 electrogenic Na^+:HCO_3^- cotransporters. If there is a discrete 1:1 Na^+:HCO_3^- cotransporter, then aNBC and rNBC may aid in the identification of that cDNA as well. We would expect that each of the major classes of Na^+:HCO_3^- cotransporter (i.e. 1:3, 1:2 and 1:1) would have multiple isoforms, just as the Na^+/H^+ exchangers (NHEs) and AEs have multiple isoforms. The other two uncloned HCO_3^- transporters, the Na^+-driven Cl^-/HCO_3^- exchanger and the K^+:HCO_3^- cotransporter, are more different, physiologically, from the various Na^+:HCO_3^- transporters. In particular, the Na^+-driven Cl^-/HCO_3^- exchanger differs from the Na^+:HCO_3^- cotransporter in that it has an absolute requirement for Cl^-. The K^+:HCO_3^- cotransporter differs from the Na^+:HCO_3^- cotransporters in that it transports K^+ rather than Na^+ and that it is inhibited by quaternary alkyl ammonium ions, such as tetraethylammonium (B. A. Davis, E. M. Hogan, J. Zhao and W. F. Boron, unpublished observations), but is insensitive to DIDS. Thus, it will be interesting to see whether the NBCs can be used to identify the cDNAs encoding either of these other two HCO_3^- transporters.

Structure–function analyses

As the cDNAs encoding the various HCO_3^- transporters become identified, comparing the structures and functions of the wild-type transporters may yield important clues as to which parts of the respective molecules might be important for determining such parameters as Na^+:HCO_3^- stoichiometry and Cl^- binding. Truncations, deletions and point mutations to the cDNAs encoding these transporters, as well as chimeras made among their cDNAs, could yield additional insights into structure–function relationships of the HCO_3^- transporters. It is intriguing to speculate that, if the 1:3 electrogenic Na^+:HCO_3^- cotransporter has separate binding sites for Na^+, CO_3^{2-} and HCO_3^-, the 1:2 cotransporter might lack the HCO_3^- binding site and the 1:1 cotransporter might lack the CO_3^{2-} binding site. Chimera experiments may make it possible to test this hypothesis.

Distribution and localization of the transporters

As the cDNAs encoding the HCO_3^- transporters become available, it will be possible to establish the tissue distribution of their mRNAs. The development of antibodies specific for various HCO_3^- transporters and their isoforms may make it possible to determine the tissue distribution of the transporter proteins and to determine their topology. Antibodies suitable for immunocytochemistry could establish the cellular distribution (e.g. apical *versus* basolateral membrane of epithelial cells) of the transporter proteins. Finally, antibodies suitable for immunoprecipitation could permit biochemical analyses of transporter proteins.

In short, the next few years could be an exciting time for those interested in HCO_3^- transport, with the promised development of a host of molecular tools making it possible to probe the molecular physiology of HCO_3^- transporters to an unprecedented degree.

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