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Evaluating the role of carbonic anhydrases in the transport of HCO_3^- -related species

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Abstract

The soluble enzyme carbonic anhydrase II (CAII) plays an important role in CO_2 influx and efflux by red blood cells (RBCs), a process initiated by changes in the extracellular $[CO_2]$ (CO_2 -initiated CO_2 transport). Evidence suggests that CAII may be part of a macromolecular complex at the inner surface of the RBC membrane. Some have suggested CAII specifically binds to a motif on the cytoplasmic C terminus (Ct) of the Cl- HCO_3^- exchanger AE1 and some other members of the SLC4 family of HCO_3^- transporters, a transport metabolon. Moreover, others have suggested that this bound CAII enhances the transport of HCO_3^- -related species— HCO_3^- , CO_3^{2-} , or CO_3^- ion pairs—when the process is initiated by altering the activity of the transporter (HCO_3^- -initiated HCO_3^- transport). In this review, I assess the theoretical roles of CAs in the transport of CO_2 and HCO_3^- -related species, concluding that although the effect of bound CAII on CO_2 -initiated CO_2 transport is expected to be substantial, the effect of bound CAs on HCO_3^- -initiated HCO_3^- transport is expected to be modest at best. I also assess the experimental evidence for CAII binding to AE1 and other transporters, and the effects of this binding on HCO_3^- -initiated HCO_3^- transport. The early conclusion that CAII binds to the Ct of AE1 appears to be the result of unpredictable effects of GST in the GST fusion proteins used in the studies. The early conclusion that bound CAII speeds HCO_3^- -initiated HCO_3^- transport appears to be the result of CAII accelerating the pH changes used as a read-out of transport. Thus, it appears that CAII does not bind directly to AE1 or other SLC4 proteins, and that bound CAII does not substantially accelerate HCO_3^- -initiated HCO_3^- transport.

Keywords

Metabolon; carbon dioxide; SLC4 family; Erythrocyte; ELISA; Glutathione S-transferase

1. Introduction

The transport of HCO_3^- —and HCO_3^- -related species such as CO_3^{2-} and ion pair such as $NaCO_3^-$ —is important at the cellular level for the regulation of intracellular pH (pH_i) and at the tissue level for the role specialized cells play in the transport of acid–base equivalents across certain epithelia. At the cellular level, pH_i regulation is a critical housekeeping

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function for maintaining cytosolic pH in a range that is permissive for a wide range of processes, including biochemical reactions, channel/transporter function, and protein-protein interactions. At the epithelial level, the movement of HCO_3^- -related species plays a wide and important range of roles in such activities as regulating the pH of the blood (in the case of renal tubules) and cerebrospinal fluid (choroid plexus), promoting the digestion of foodstuffs (gastrointestinal tract), and rendering spermatozoa quiescent (male reproductive tract). In all of the above examples, the transport is highly regulated to respond to the changing demands on the cell and whole body. The erythrocyte or red blood cell (RBC) is a special case in which a cell uses a HCO_3^- transporter to enhance the carriage of CO_2 from the capillaries of the systemic circulation to those of the pulmonary circulation.

1.1. Chemical reactions

One of the factors that adds to the challenge of studying the transport of HCO_3^- -related species is that the transport triggers a series of chemical reactions. Here we arbitrarily begin with the hydration of CO_2 , leading ultimately to the formation of HCO_3^- and CO_3^{2-} :

At high pH, the reaction

becomes increasingly important for the formation of HCO_3^- from CO_2 , and is thermodynamically equivalent to the sum of the first two reactions. The α -carbonic anhydrases have the effect of speeding the first two reactions in the above sequence.

In addition to the above reactions, the presence of Na^+ leads to the formation of the $NaCO_3^-$ ion pair:

Several lines of evidence [1-4] are consistent with the hypothesis that, in the squid giant axon, the so-called Na^+ -driven Cl^- - HCO_3^- exchanger actually carries the $NaCO_3^-$ ion pair. CO_3^{2-} also forms ion pairs with Li^+ , Ca^{2+} , and Mg^{2+} [5]. Ion-pair formation is likely to be most important in non-mammalian marine species, which can have extracellular levels of

Na^+ , Ca^{2+} , and Mg^{2+} . These ion-pairs, together with CO_3^{2-} and HCO_3^- , constitute the HCO_3^- -related species¹.

Three of the above reactions involve H^+ or OH^- . Thus, any process that affects $[\text{CO}_2]$ or the concentration of a HCO_3^- -related species will ultimately impact on a multitude (m) of H^+ -buffering reactions that do not involve a HCO_3^- -related species:

Here, each weak base $\text{B}_i^{(n)}$ has its own concentration and valence n .

1.2. Modeling acid–base disturbances

Any process that results in a local change in $[\text{H}^+]$, $[\text{CO}_2]$, $[\text{H}_2\text{CO}_3]$, any HCO_3^- -related species (including any cation that can form an ion pair with CO_3^{2-}), or any member of a non- HCO_3^- buffer reaction will initiate a shift in all of the above reactions. Although each reaction is in principle quite simple, as a group they are so complex that it is impossible to obtain analytical solutions for even the simplest families of equations, even in the steady state. Various nomograms based on the $\text{CO}_2/\text{HCO}_3^-$ equilibria allow one to predict how additions (or deletions) of H^+ , HCO_3^- , or CO_2 affect steady-state pH in a system containing non- HCO_3^- buffers. Although one can computerize these approaches by replacing the nomograms with numerical solutions to the equations, these models have inherent limitations. For example, they lump all non- HCO_3^- buffers into a single equivalent buffer pair $\text{HB}^{(n+1)} \rightleftharpoons \text{B}^{(n)} + \text{H}^+$ in which $[\text{B}^{(n)}]$ rises linearly with pH (i.e., total non- HCO_3^- buffering power is invariant of pH). Although this simplifying approach yields reasonable results, it does not allow one to assess the effects of individual buffers. In the next generation of such modeling, Roos imagined what would happen—again, in the steady state, and in the absence of $\text{CO}_2/\text{HCO}_3^-$ or other buffers—if the charged species of a monoprotic buffer (e.g., lactate) could diffuse across the plasma membrane [6].

In 1976, Boron and De Weer became the first to predict the time course of pH_i , obtained by numerically integrating a simple system of two differential equations [7]. They examined two situations: (a) The influx or efflux of CO_2 in the presence of a parallel permeability to HCO_3^- , with the optional presence of a pH_i -regulating mechanism—the regulated extrusion of H^+ (the consequences would be the same for HCO_3^- uptake). And (b) the influx or efflux of NH_3 in the presence of a parallel permeability to NH_4^+ . Although this model accounted for the observed pH_i transients, and made several valuable predictions, it required several

¹In addition to the reactions listed here, several enzymatic reactions can consume or produce CO_2 or HCO_3^- .

simplifying assumptions: (a) Like the previous models, it lumps all non- HCO_3^- buffers into a single equivalent buffer pair $HB^{(n+1)}/B^{(n)}$. (b) CO_2 , HCO_3^- , and H^+ instantly equilibrate with one another (as if carbonic-anhydrase activity were infinite). (c) The extracellular fluid is an infinite and homogenous reservoir. And (4) the intracellular fluid consists of a single compartment that mixes instantly.

The reason for briefly considering the history of modeling CO_2/HCO_3^- equilibria under physiological conditions is that the effects of carbonic-anhydrase (CA) activity, by their very nature, depend on time and space. For example, if two single-cell systems—one without CA activity and the other with CA at specific loci—are allowed to come into equilibrium, the two systems will be identical. The activity and the location of the CA in the second system are inconsequential. Thus, if we wish to understand the role that CAs play in physiology, we must develop mathematical models that predict—in 3-dimensional space—the time courses of concentrations of relevant solutes by considering: (a) the transport of solutes across the plasma membrane, (b) the diffusion of solutes from the bulk extra- and intracellular fluids to/from the plasma membrane, (c) the above reactions at different loci outside and inside the cell, and (d) CA activity at specific loci. To my knowledge, no such model exists. However, some of my colleagues at Case Western Reserve University are attempting to implement a first-generation model that would allow us to understand how the influx of CO_2 into a cell—with CA optionally present at the membrane surfaces or the bulk extra- or intracellular fluids—would affect pH and other parameters as a function of time in 3-dimensional space. When this model is implemented and extended, it will begin to provide a better understanding of the issues that I will address qualitatively in this review.

2. The concept of a “metabolon”

A metabolon is a complex of enzymes that catalyze a series of reactions in a metabolic pathway. Because a product of one enzyme is a substrate of the next, the “channeling” of intermediates increases their effective concentrations, permitting the overall process to proceed with a much higher efficiency than if the enzymes were spatially dispersed [8,9]. One example is a metabolon consisting of enzymes catalyzing the TCA (tricarboxylic acid) metabolon [10].

An interesting and perhaps the first-published example of a metabolon includes at least three glycolytic enzymes (GEs)—glyceraldehyde 3-phosphate dehydrogenase (GAPDH) [11,12], aldolase [13], and phosphofructokinase (PFK) [14]—that can bind to a motif on the cytosolic N terminus (Nt) of the $Cl-HCO_3^-$ exchanger AE1 (also known as SLC4A1). This transporter, with about 1 million copies per RBC [15], is by far the dominant membrane protein of erythrocytes. Two other GEs—pyruvate kinase (PK) and lactate dehydrogenase (LHD)—also cluster at the RBC plasma membrane, but apparently do not interact directly with AE1 [16]. Moreover, two physiological manipulations release the GEs from the RBC membrane: the phosphorylation of two tyrosine residues on the Nt and deoxygenation.

The physiological role of the GE-AE1 metabolon is not clear. Studies under dilute in-vitro conditions show that the enzymes bind only at nonphysiologically low values of pH and ionic strength [17]. Moreover, the interactions with AE1 inhibit—rather than stimulate—the

activity of the GEs when studied under dilute conditions [13,17]. On the other hand, it has been suggested that macromolecular crowding in the intact cell could enhance the interactions sufficiently to support binding at physiological conditions [18,19]. Moreover, binding has been observed at the membrane of freshly fixed RBCs for GAPDH [16,20,21] and the other GEs [16]. A remaining question is whether macromolecular crowding would alter the effect of an enzyme-AE1 interaction from inhibition to stimulation. An intriguing observation is that deoxygenation, which promotes assembly of the enzymes at the membrane, enhances glycolysis [22,23]. If this glycolysis indeed occurs in the AE1-glycolytic metabolon, it would be attractive to speculate that locally synthesized ATP could fuel the Na-K or other pumps [24]. If true, the glycolytic enzyme-AE1 metabolon would be an example of a transport metabolon.

3. Theoretical role of CAII in CO₂ transport by erythrocytes

3.1. Analysis

In this and the next two sections, we assume for the sake of argument that CAII is in the vicinity of the key transporters and channels at the plasma membrane. In Fig. 1A–D, we will use a semiquantitative argument to predict the effect of CAII on the rate of net CO₂ uptake by RBCs as these cells pass through systemic capillaries. The opposite reactions occur as the RBC offloads CO₂ in the pulmonary capillaries. Consistent with data from human RBCs, we will assume that the overwhelming majority of CO₂ crosses the membrane via either AQP1 or the Rh complex [25–27].

When the RBC is in a systemic artery (Fig. 1A), the system is in equilibrium. That is, (a) the CO₂ concentration is 1.2 mM (i.e., $P_{\text{CO}_2} = 40$ mm Hg) both outside (o) and inside (i) the cell, (b) the equivalent reaction $\text{CO}_2 + \text{H}_2\text{O} \rightleftharpoons \text{H}^+ + \text{HCO}_3^-$ is in equilibrium, and (c) AE1 engages in no net transport of Cl^- or HCO_3^- . For the numerical examples, we are assuming that pH_o is 7.4 and pH_i is 7.1. Because the pK of the $\text{CO}_2/\text{HCO}_3^-$ equilibrium is 6.1,

$[\text{HCO}_3^-]_i$ is 10-fold greater than $[\text{CO}_2]_i$.

When the RBC first enters a systemic capillary (Fig. 1B), the only immediate difference is that $[\text{CO}_2]_o$ abruptly rises to 1.4 mM (i.e., $P_{\text{CO}_2} = 46$ mm Hg), reflecting the metabolic production of CO₂. Thus, the gradient driving CO₂ into the RBC might be $(1.40 - 1.20) = 0.20$ mM.

Very soon, the rapid influx of CO₂ causes $[\text{CO}_2]$ to rise near the intracellular surface (is) of the gas channels (Fig. 1C). For the sake of argument, we will assume that the CAII is temporarily inactive, and that $[\text{CO}_2]_{is}$ has risen to 1.35 mM. In other words, the gradient driving CO₂ into the RBC may now be markedly reduced from its initial level of 0.20 mM to $(1.40 - 1.35) = 0.05$ mM. Thus, the net influx of CO₂ falls to 1/4 of its initial value.

Now we will activate the CAII (Fig. 1D), which consumes CO₂ and produces of H^+ and HCO_3^- . Hemoglobin buffers the vast majority of the newly produced H^+ , and AE1 exports most of the newly produced HCO_3^- in exchange for Cl^- . We will assume that $[\text{CO}_2]_{is}$ falls from 1.35 to 1.25 mM. Thus, a mere ~7% decrease in $[\text{CO}_2]_{is}$ in this example would triple

the gradient driving CO_2 into the RBC from its intermediate level of 0.05 mM to $(1.40 - 1.25) = 0.15$ mM, thereby also tripling the net influx of CO_2 .

If we imagine, for the sake of simplicity, that pH_{is} remains constant, we can see another consequence of the CAII reaction: Because $[\text{HCO}_3^-]_{\text{i}}$ is 10-fold greater than $[\text{CO}_2]_{\text{i}}$, for every 11 CO_2 molecules that enter the RBC, 1 will remain CO_2 and 10 will become HCO_3^- . Thus, the HCO_3^- concentration gradient driving the diffusion toward the center of the cell increases 10-fold more than the CO_2 concentration gradient. In other words, by converting CO_2 to HCO_3^- in the RBC during CO_2 -initiated CO_2 influx, CAII converts a small diffusion gradient for CO_2 into a large diffusion gradient for HCO_3^- , which probably has a mobility only modestly less than that of CO_2 . Thus, the carbon that does not exit the RBC as HCO_3^- (via AE1) can more readily diffuse away from the membrane as HCO_3^- than if that carbon remained CO_2 . Below, we will see that—during HCO_3^- -initiated HCO_3^- influx—this same argument, in reverse, makes the conversion of HCO_3^- to CO_2 a bad choice for the diffusion of carbon away from the membrane.

Note: Although Fig. 1 shows only the $\text{CO}_2/\text{HCO}_3^-$ buffer system, non- HCO_3^- buffers contribute to the buffering of pH in both the extra- and intracellular fluid.

3.2. Effects on pH

It is worth noting that a side effect of CO_2 influx is a fall in pH_{i} . The activity of CAII should speed the rate at which bulk pH_{i} falls, but should have no effect on the magnitude of bulk pH_{i} decrease. Near the inner surface of the membrane, CAII activity might exaggerate a transient fall in pH_{is} . On the other hand, the RBC is ideally suited to resist such pH_{is} transients because the non- HCO_3^- intracellular buffering power of RBCs—due mainly to hemoglobin—is ~50 mM, perhaps the highest of any cell in the body at resting pH_{i} . Moreover, the Nt of AE1 binds deoxygenated hemoglobin (which is a better H^+ buffer than oxygenated hemoglobin), thereby increasing the local buffering power and tending to muffle pH_{i} transients.

3.3. Conclusions

In the absence of a quantitative model, we can only guess at values for $[\text{CO}_2]_{\text{is}}$. Thus, the above $[\text{CO}_2]_{\text{is}}$ values of 1.35 and 1.25 mM, though reasonable, are for illustration only. The important message is that even by producing a seemingly minor fall in $[\text{CO}_2]_{\text{is}}$ from 1.35 to 1.25 mM (i.e., a decrease of slightly more than 7%), CAII could produce an immense increase in net CO_2 influx (i.e., a tripling in this example), and this stimulus comes without an untoward effect on pH_{i} .

4. Theoretical role of carbonic anhydrases in HCO_3^- transport

4.1. Analysis

In Fig. 1, the prime mover was a sudden increase in $[\text{CO}_2]_{\text{o}}$, which drove CO_2 into the cell.

The action of CAII, in turn, produced an increase in $[\text{HCO}_3^-]_{\text{i}}$ that, finally, provoked AE1 to move HCO_3^- out of the cell in exchange for Cl^- . Here in Fig. 2, the players will introduce

themselves in the opposite order. The prime mover will be a sudden decrease in $[Cl^-]_o$ from 102 to 0 mM to mimic the experiment that provided the impetus for concluding that the catalytic activity of CAII enhances the activity of AE1 [28]. Although we assume that the cell is an RBC, in fact, this could be any cell.

Under the initial, control conditions (Fig. 2A), an RBC in arterial blood is in equilibrium, as in the previous example.

When we remove extracellular Cl^- (Fig. 2B), AE1 begins to move Cl^- out of the cell in exchange for extracellular HCO_3^- . Note that AE1 is not driven by a Cl^- or HCO_3^- gradient, but by a complex set of kinetic parameters. It is worth noting that—because it is challenging to study $Cl^-HCO_3^-$ exchange—practitioners of the art have generally chosen to probe AE1 in RBCs by studying how it mediates Cl^-Cl^- exchange or other more tractable processes. Much less data is available on the details of how AE1 mediates $Cl^-HCO_3^-$ exchange. Thus, to my knowledge, it is not possible to predict precisely how AE1 in RBCs will respond to the challenge outlined in Fig. 2B (e.g., how the net efflux of Cl^- depends on $[Cl^-]_i$, $[HCO_3^-]_i$, and on $[HCO_3^-]_o$). Nevertheless, we can be sure that AE1—one of the fastest known transporters—is moving Cl^- out of the cell with great speed.

Very soon, the rapid action of AE1 causes $[Cl^-]$ near the inner surface of the membrane to fall, and the local $[HCO_3^-]$ to rise (Fig. 2C). For the sake of argument, we will assume that the CAII is temporarily inactive, and that $[Cl^-]_{is}$ has fallen from 51 to 50 mM, and that $[HCO_3^-]_{is}$ has risen from 12 to 13 mM. As noted in the preceding paragraph, we do not know precisely how these concentration changes affect the rate of $Cl^-HCO_3^-$ exchange. It is possible that, if the concentrations were near their saturating effects, the net effect would be negligible. Even if $[Cl^-]_{is}$ were at its effective K_m , we would expect only a ~2% decrease in rate. And even if $[HCO_3^-]_{is}$ were at its K_i , we would expect an inhibition of only ~4%. One could imagine more complicated kinetic calculations, but these are not unreasonable. Thus, an 8% increase $[HCO_3^-]_{is}$ would inhibit AE1 by only 4%.

Finally, we will activate the CAII (Fig. 2D), which consumes HCO_3^- and H^+ and produces CO_2 and H_2O . Buffers would replenish most of the consumed H^+ , and gas channels would dispose of most of the newly formed CO_2 . We will assume that CAII causes $[HCO_3^-]_{is}$ to fall from 13 to 12.3 mM. However, following the logic of the preceding paragraph, we might expect this fall in $[HCO_3^-]_{is}$ to increase the rate of $Cl^-HCO_3^-$ exchange by perhaps 3%.

If we imagine, for the sake of simplicity, that pH_{is} remains constant, we can see another consequence of the CAII reaction: Because $[HCO_3^-]_i$ is 10-fold greater than $[CO_2]_i$ in this example ($pH_{is}=7.1$, $pK=6.1$), for every 11 HCO_3^- molecules that enter the RBC, 10 will remain HCO_3^- and 1 will become CO_2 . Thus, the CO_2 concentration gradient driving

diffusion toward the center of the cell increases only 1/10 as much as does the HCO_3^- gradient. In other words, by converting HCO_3^- to CO_2 during HCO_3^- -initiated HCO_3^- influx, CAII converts a large diffusion gradient for HCO_3^- into a small gradient for CO_2 , which probably has a mobility only modestly higher than that of HCO_3^- . The conversion from HCO_3^- to CO_2 is expected to markedly slow the diffusion of carbon from the transport machinery. According to this argument, the hypothesized advantage of converting HCO_3^- to CO_2 during HCO_3^- -initiated HCO_3^- influx—or converting CO_2 to HCO_3^- during HCO_3^- -initiated HCO_3^- efflux—is illusory. The interconversion that is so advantageous when a change in $[CO_2]$ is the prime mover is a liability when a change in HCO_3^- transport rate is the prime mover.

Note: Although Fig. 2 shows only the CO_2/HCO_3^- buffer system, non- HCO_3^- buffers contribute to the buffering of pH in both the extra- and intracellular fluid. Before concluding, I would like to make two additional points.

4.2. Possible allosteric effects

All arguments made thus far have been based on the assumption that any effects of CAII on AE1 would be due only to the catalytic activity of the CAII. In fact, one could imagine that CAII or other CAs—whether extra- or intracellular, whether binding directly or indirectly to a transporter—could have allosteric effects that modulate transport.

4.3. Effects of CAII on local pH

The activity of CAII would lead to an exaggerated, transient pH increase near the intracellular surface of the cell membrane during HCO_3^- -initiated HCO_3^- influx and, conversely, an exaggerated, transient pH_{is} decrease during HCO_3^- efflux.

4.4. Conclusions

Again, the concentration values in this example are not meant to be accurate, but to illustrate a principle that is quite clear: Whereas CAII near the membrane can have an immense effect on CO_2 transport when the prime mover is a change in $[CO_2]$, the enzymatic activity of this CAII is likely to have only minor—and perhaps unmeasurable—effects on HCO_3^- transport when a change in HCO_3^- transport is the prime mover. In addition, CAII near the membrane would produce exaggerated pH_{is} transients that could have a deleterious effect.

5. Theoretical role of carbonic anhydrases in $CO_3^{=}$ or H^+ transport

As noted above, data on the squid giant axon are consistent with the idea that the substrate for transport is actually the $NaCO_3^-$ ion pair [1-4]. The electrogenic Na/HCO_3 cotransporter NBCe1—when operating in the renal proximal tubule—appears to mediate the efflux of 1 Na^+ and the equivalent of 3 HCO_3^- , that is, a $Na^+:HCO_3^-$ stoichiometry of 1:3. However, in several other natural settings as well as when expressed heterologously in the *Xenopus* oocyte—human NBCe1 has a $Na^+:HCO_3^-$ stoichiometry of 1:2. Preliminary data from our laboratory [29,30] suggest that, as expressed in *Xenopus* oocytes, NBCe1—as well as the Na^+ -driven $Cl-HCO_3$ exchanger NDCBE—transports $CO_3^{=}$ or $NaCO_3^-$ rather than HCO_3^- .

5.1. Model of HCO_3^- influx

Fig. 3A shows how NBCe1, if it actually moved 1 Na^+ plus 2 HCO_3^- into the cell, would work in the context of the extracellular carbonic anhydrase CAIV and the cytosolic carbonic anhydrase CAII if the transporter actually moved 1 Na^+ plus 2 HCO_3^- . Note that the function of CAIV under these conditions is to help—along with the diffusion of HCO_3^- from the bulk extracellular fluid—in the replenishment of HCO_3^- near the outer surface (os) of the cell membrane. In the process, the CAIV would cogenerate H^+ . Even if CAIV were not present, the uncatalyzed cogeneration of H^+ would tend to push pH_{os} below the bulk extracellular pH ($pH_{o/Bulk}$). However, CAIV would accentuate this surface acidosis. Thus, blocking CAIV would be expected to allow $pH_{o/Bulk}$ to drift up, closer to (but still below) $pH_{o/Bulk}$.

On the inside of the cell, CAII would tend to consume incoming HCO_3^- and, in the process, consume H^+ . Even in the absence of CAII, the uncatalyzed reaction would tend to raise pH_{is} above that of the bulk intracellular fluid ($pH_{i/Bulk}$). However, CAII would accentuate this surface alkalosis—as we outlined for AE1 in Fig. 2D. Note: in all models of Fig. 3, non- HCO_3^- buffers contribute to the buffering of pH in both the extra- and intracellular fluid.

5.2. Model of $CO_3^{=}$ influx

Fig. 3B shows how NBCe1, if it actually moved 1 Na^+ plus 1 $CO_3^{=}$ into the cell, would work in the context of CAIV and CAII. (Note that, in terms of acid–base equivalents, the influx of 1 $CO_3^{=}$ is equivalent to that of 2 HCO_3^- .) As for our hypothetical example of the uptake of 2 HCO_3^- in Fig. 3A, what approaches the membrane from the bulk extracellular fluid is 1 Na^+ plus 2 HCO_3^- . However, here in Fig. 3B, the 2 HCO_3^- ions undergo a disproportionation reaction near the transporter, ultimately yielding 1 $CO_3^{=}$ (to replenish that lost to transport), 1 CO_2 and 1 H_2O . The role of the CAIV is to catalyze the formation of the CO_2 plus H_2O , thereby consuming 1 H^+ . Even without the CAIV, the regeneration of $CO_3^{=}$ would result in the formation of H^+ , and thus tend to push pH_{os} below $pH_{o/Bulk}$. However, as recognized by Grichtchenko and Chesler [31], the extracellular CA reduces the buildup of H^+ and thus tends to minimize the fall in pH_{is} . They also recognized that blocking the extracellular CA would remove this tempering, and exaggerate the fall in pH_{os} . To complete the extracellular side of the transport process, the newly formed CO_2 and H_2O must enter the cell.

On the inside of the cell, incoming $CO_3^{=}$ would react with 1 local H^+ to form 1 HCO_3^- . The result would be an immediate rise in pH_{is} that would eventually spread to the rest of the cell. The depleted H^+ would be replenished by local non- HCO_3^- buffers and, in the absence of an intracellular CA, by the slow reaction $CO_2 + H_2O \rightarrow H^+ + HCO_3^-$. However, CAII would greatly speed this reaction, and thereby minimize the immediate rise in pH_{is} . The starting materials for CAII would be the CO_2 and H_2O that entered from the outside. These reactions near the inner surface of the membrane would yield 2 HCO_3^- , which—along with the Na^+ —would diffuse into bulk intracellular fluid.

5.3. Model of H⁺ efflux

Fig. 3C shows how an H⁺ extruder such as the Na-H exchanger NHE1 (SLC9A1)—the same would be true for the vacuolar H⁺ pump—would work in the context of CAIV and CAII. The role of the CAIV is to catalyze the consumption of the newly exported H⁺, along with existing HCO_3^- , thereby forming CO_2 plus H_2O .

On the inside of the cell, the job of the CAII is to help replenish the depleted H⁺. The reactions catalyzed by CAIV would establish a countercurrent of CO_2 and HCO_3^- in the extracellular unstirred layer, with some CO_2 entering the cell across the plasma membrane.

5.4. Analysis of three flux models

Examining the three cases in Fig. 3, we see that the CAs play fundamentally different roles—mediating opposite reactions—for HCO_3^- uptake on the one hand vs. $\text{CO}_3^{=}$ uptake or H⁺ extrusion on the other. In the case of HCO_3^- uptake, CAIV accentuates the transient rise in pH_{os} ; in the case of $\text{CO}_3^{=}$ uptake or H⁺ extrusion, CAIV minimizes the rise in pH_{os} . On the inside of the cell, CAII accentuates the transient rise in pH_{is} produced by HCO_3^- influx, but minimizes the rise in pH_{is} produced by $\text{CO}_3^{=}$ uptake or H⁺ extrusion. If the direction of transport were reversed, we would reach similar conclusions for HCO_3^- efflux on the one hand vs. $\text{CO}_3^{=}$ efflux or H⁺ influx on the other.

Preliminary data from our Lab shows that CAIV minimizes changes in pH_{os} produced by NBCe1 as well as NDCBE—evidence in favor of $\text{CO}_3^{=}$ rather than HCO_3^- transport [29,30]. Moreover, these same preliminary experiments show that CAIV has no substantial effect on transport rate, as assessed by voltage clamp.

In summary, we would predict that the catalytic activity of a CA located near the transport machinery—whether the CA be extra- or intracellular—would have only modest effects on transport rate. In the case of transport where HCO_3^- movement is the prime mover, CA activity (per se) near the transport machinery would actually be detrimental for the stabilization of local pH. However, in the case of transport where HCO_3^- or H⁺ movement is the prime mover, CA activity (per se) near the transport machinery would play a potentially beneficial role by stabilizing local pH.

Two notes of caution. First, the argument in the previous paragraph pertains to CA activity per se, and does not speak to the issue of allosteric effects that the CA protein may have on other components of the transport machinery. Second, the arguments presented thus far in this review pertain to CA activity that is restricted to the vicinity of the transport machinery (i.e., part of a proposed transport metabolon). We will see later that CA activity distributed throughout the bulk intracellular fluid could enhance transport by promoting the diffusion of substrates from this bulk fluid to the unstirred layer near the transport machinery.

6. Is CAII part of a membrane-associated metabolon?

6.1. Supportive evidence from experiments on RBCs

Four lines of evidence are consistent with the hypothesis that the soluble, cytosolic enzyme carbonic anhydrase II (CAII) can interact with the plasma membrane of the RBC. First, mixing human RBC fragments with bovine CAII leads to increased enzymatic activity [32]. Of course, these data do not address the issue of which components of the RBC fragments were responsible for the observed enhancement.

Second, the binding of DIDS—regarded as a specific inhibitor of AE1—to human RBC pink ghosts causes a ~20% increase in the rate constant and a ~50% increase in the apparent K_d for the binding of dansylsulfonamide (DNSA) to CAII [33]. As a note of caution, we now appreciate that DIDS also interacts with the water channel aquaporin 1 (AQP1) and RhAG [25,26,34-37], both of which are expressed at very high levels in the RBC membrane.

Third, immunoprecipitation of solubilized AE1—with an anti-AE1-Nt but not an anti-AE1-Ct antibody—from human RBC ghosts resulted in the co-precipitation of CAII [38]. The authors interpreted a negative result with anti-AE1-Ct as reflecting the displacement of the CAII from the Ct by the antibody. As a note of caution, the reverse co-immunoprecipitation is not presented.

Fourth, immunofluorescence studies of RBC ghosts shows the enhanced presence of CAII near the plasma membrane [16,38]. Additional immunofluorescence data shows that tomato lectin causes clustering of both AE1 and CAII. As a note of caution, the AE1 and CAII were not doubly labeled, so that no “merge” image was possible. Although none of the above data unambiguously points to a specific interaction of CAII with AE1, the results strongly support the hypothesis that CAII binds to a site in the neighborhood of the inner surface of the plasma membrane.

One would like to be more specific. For example, does CAII binds indirectly or indirectly to an integral membrane protein? I believe that the answer is likely to be “yes,” though the definitive answer must await the clear identification of that protein.

More specifically, does CAII bind to a complex of proteins that includes AE1? Again, I believe that the answer is likely to be “yes” because of the decided advantage (discussed above) that such a metabolon would offer for CO₂ transport—when CO₂ is the prime mover—across the RBC membrane. The DNSA and AE-1 immunoprecipitation data cited above are also supportive. Again, the definitive answer awaits the clear identification of the CA-II target protein.

Still more specifically, does CAII bind directly to AE1? This extremely attractive hypothesis is the subject of the next sub-section.

6.2. Supportive evidence from ELISA assays

The fundamental tool used to study the interaction of CAII with AE1 [38-40] has been a solid-phase binding assay in which immobilized CAII interacts with a liquid-phase construct

consisting of glutathione S-transferase (GST) from a trematode fused to the N-terminal end of the Ct of AE1 (GST-AE1-Ct). The initial ELISA assays demonstrated that GST-AE1-Ct binds with high affinity to CAII [38]. A second study then narrowed the binding site on AE1 to a reasonably well conserved LDADD motif on the proximal portion of the AE1-Ct [38]. Additional evidence indicated that the requirements are a leucine followed by at least two negatively charged residues in the next four. Finally, a third study concluded that the negatively charged residues on AE1-Ct bind to a positively charged area near the Nt of CAII [40]. An interesting aspect of the system is that, although GST-AE1-Ct binds to solid-state CAII with $K_{1/2}$ of 20 nM, an AE1-Ct peptide (without the GST) prevents binding of the GST-AE1-Ct with a $K_{1/2}$ that is 5000-fold higher, 100 μ M. The authors offered two explanations for this difference: (a) The human AE1-Ct is not, by itself, sufficiently structured to bind to human CAII. According to this explanation, it would be the trematode GST that provides this structure for one human protein to bind to another. (b) Because GST is a dimer, a two-point attachment mechanism (whereby either of two coupled AE-Cts could bind to a lawn of CAII molecules) could increase the avidity of the interaction.

6.3. Negative evidence from ELISA assays ... absence of GST or reversed orientation

We found the metabolon hypothesis so attractive that Peter Piermarini in my laboratory decided to cocrystallize CAII with the AE1-Ct. Although he was able to obtain nicely diffracting CAII crystals—with the advice of Rob McKenna (University of Florida) and Elias Lolis (Yale University)—we were never able to detect differences when we cocrystallized with the AE1-Ct at molar ratios over 10:1. After several attempts, an advisor asked if we were certain that CAII binds to the AE1-Ct.

Piermarini, together with Eugene Kim, began a series of experiments [41] in which they attempted to replicate the earlier ELISA, not only with GST-AE1-Ct, but also with the comparable constructs for two other human members of the SLC4 family, NBCe1 (GST-NBCe1-Ct) and the human Na^+ -driven $\text{Cl}^-/\text{HCO}_3^-$ exchanger (GST-NDCBE-Ct). Piermarini et al. verified the earlier conclusion that GST-AE1-Ct (detected with anti-GST) binds with high affinity to immobilized CAII², and extended the observation to the other two members of the SLC4 family (Fig. 4). However, when Piermarini et al. repeated the study with pure Ct peptides (i.e., without the GST), they could detect no binding to immobilized CAII for any of the three SLC4-Ct peptides. The white squares along x -axis in Fig. 5 show these data for AE1-Ct (detected with anti-AE1-Ct). The red squares in Fig. 5 show the paired “control” data for GST-AE1-Ct (detected with anti-AE1-Ct). Thus, the binding of the C termini to immobilized CAII requires GST.

The next step was to invert the orientation of the components in the ELISA—making it more physiological—by putting the CAII in the liquid phase and immobilizing the Ct of the transporter. The upper curve (white diamonds) in Fig. 6 shows that CAII binds with high affinity to immobilized GST. The middle curve (yellow squares) shows that the addition of the AE1-Ct actually reduces binding by about half. Finally, the lower curve along the x -axis

²Some experimental details were omitted in the original papers.

(red squares) shows that liquid-phase CAII is unable to bind to the pure AE1-Ct peptide. Piermarini et al. obtained similar results (not shown) for NBCe1 and NDCBE.

From Figs. 4–6, we can conclude that SLC4-Ct fusion proteins can bind to CAII only when fused to GST, and even then only when the GST-SLC4-Ct is in the liquid phase. These results suggest that the earlier conclusion that CAII binds specifically to a motif on the Ct of AE1 was the result of the presence of GST and the orientation of the components in the ELISA. However, the data of Piermarini et al. presented thus far leave open the possibility—not addressed in the original work—that CAII may indeed bind to SLC4-Cts, but that the dissociation between the two may be too rapid to detect by ELISA. For this reason, as discussed in the next subsection, Piermarini et al. examined the potential interactions by an assay with a more rapid time resolution.

6.4. Negative evidence from SPR assays

In Fig. 7, Piermarini et al. immobilized CAII to a surface plasmon resonance (SPR) chip. Fig. 7A shows the effect of introducing in the liquid phase various levels of acetazolamide (ACZ), a reversible CAII inhibitor, and then washing out the drug. These data yield an equilibrium constant for the ACZ-CAII interaction of 8.7 nM, which is virtually identical to that originally reported by Maren [42]. Fig. 7B–D are the results with pure SLC4-Ct peptides in the liquid phase. Given the response to the small ACZ molecule (222.2 Da), the SPR signals for the much larger peptides should have been massive. However, they were indistinguishable from background, indicating that none of the pure peptides—AE1-Ct, NBCe1-Ct, or NDCBE-Ct—bind rapidly to immobilized CAII.

Not shown are SPR data obtained with the orientation of the components inverted. In each case, Piermarini et al. were able to detect the immobilized pure SLC-Ct peptide using the appropriate antibodies in the liquid phase. However, CAII in the liquid phase did not produce a signal distinguishable from background. Thus, CAII cannot bind rapidly to any of the three immobilized C termini.

6.5. Conclusions

In summary, Piermarini et al. were able to reproduce the original binding of liquid-phase GST-AE1-Ct to immobilized CAII—and extend these positive results to GST-NBCe1-Ct and GST-NDCBE-Ct in an ELISA. However, we were unable to detect binding of any of the three GST fusion proteins by ELISA using a reversed orientation of the components. Similarly, we were unable to detect binding of CAII to any of the pure SLC4-Ct peptides by ELISA or SPR, regardless of the orientation of the components. Thus, we are left to conclude that CAII does not bind directly to any of the three C termini that we tested. I hasten to point out that our data do not rule out the direct binding of CAII to another portion of the molecules in question (e.g., to the Nt of AE1), nor do our data rule out the indirect binding of CAII to these proteins. As noted above, because CAII would markedly enhance CO₂-initiated CO₂ transport, it is attractive to hypothesize that CAII is part of a macromolecular complex that puts the enzyme in sufficiently close proximity and with the proper orientation to AE1 and/or the gas channels AQP1 and the Rh complex.

One final point in this section concerns the proposed CAII-binding motif: LXXXX, where at least two of the X residues are negatively charged (e.g., LDADD in AE1). It is interesting to note that although NBCe1 (LDDVI) and NDCBE (LDDL M) have at least one such motif in their Ct, the related SLC4 member NBCe2 (IDN IL) lacks the motif. Even more curious, the SLC4 member BTR1/BOR1 (LDVMD)—reported to transport borate, not a HCO_3^- -related species [43,44]—has the hypothesized CAII-binding motif. Similarly, the sponge SLC4 homolog (LDSEE), which apparently transports silicate, has the proposed motif [45]. In fact, even the most primitive known SLC4 homolog—“Nitro” [46] from the bacterium *Nitrococcus mobilis*—has the proposed CAII-binding motif, even though to our knowledge this species has no α -type carbonic anhydrases. It will be interesting to see if this LXXXX motif plays some other important role in the structure, trafficking, or regulation of the SLC4 family.

7. Does CAII enhance HCO_3^- or CO_3^{2-} transport?

As discussed from a theoretical perspective in an earlier section, when an increase in $[\text{CO}_2]_o$ leads to the influx of CO_2 into an RBC, we expect that the presence of CAII in the vicinity of AE1 and/or the RBC gas channels (i.e., AQP1 and the Rh complex) would markedly enhance this CO_2 initiated CO_2 influx, and secondarily promote HCO_3^- efflux via AE1 (i.e., CO_2 -initiated HCO_3^- efflux). In the wake of the original studies on the binding of GST-AE1-Ct to immobilized CAII [38-40], others hypothesized that the enzymatic activity of CAII would similarly enhance AE1 activity when the prime mover is the removal of extracellular Cl^- (i.e., HCO_3^- -initiated HCO_3^- efflux). As discussed above from a theoretical perspective, we should not expect the enzymatic activity of CAII to make a substantial contribution in this situation. Nevertheless, several studies have concluded that the enzymatic activity of CAII or other CAs attached to the outer surface of the membrane (CAIV and CAXIV) do indeed stimulate acid-base transporters under conditions in which changes in $[\text{CO}_2]$ are not the prime mover [28,47-56].

7.1. Supportive evidence

In the first of these studies [28], the authors co-expressed CAII together with AE1, AE2, or AE3 in HEK293 cells. The fundamental observation was that the removal of extracellular Cl^- (see Fig. 2) causes a rapid rise in pH_i (monitored with the fluorescent dye BCECF), and that this alkalization is markedly slowed by using ACZ to inhibit CAII, by mutating the LDADD motif of AE1 to preclude CAII binding, or by co-expressing a catalytically inactive (i.e., dominant-negative, DN) CA. Ideally, one would prefer to assay AE1 by a direct method, such as one based on Cl^- . Nonetheless, the Cl^- -removal assay is adequate when the variable is AE1. However, the Cl^- -removal assay provides no insight whatsoever when the variable is CAII or anything else that can alter the linkage between Cl^- - HCO_3^- exchange activity and the pH_i change reported by an intracellular dye. For example, even if ACZ would have doubled the activity of AE1 per se, the massive inhibition of CAII per se would slow the consumption of H^+ to such an extent that the rate of the pH_i increase would plummet. The expression of DN-CAII would also have decreased the effective CA activity in the vicinity of the membrane. It was proposed that the mutations to the LDADD motif—which did not affect the expression of the AE1 mutant at the cell surface—slowed transport

by reducing the binding of endogenous CAII to AE1. However, because we now appreciate that CAII does not bind to the Ct of AE1 (see previous section), we must entertain the alternate hypothesis that the LDADD mutations disrupted a portion of the AE1 molecule critical for baseline function.

The same Cl^- -removal assay led to the conclusion that CAII enhances the HCO_3^- transport mediated by the DRA protein (SLC26A3) [53].

A third study addressed the possibility that the extracellular enzyme CAIV binds to AE1 [54]. In HEK293 cells expressing CAIV, the authors were able to pull down CAIV from cell lysates, using as bait the immobilized fourth extracellular loop (EL4) of AE1—fused to GST. In spite of aforementioned negative results from the data by Piermarini et al. with CAII and GST-AE1-Ct [41], it is possible that the pull-down of CAIV with GST-AE1-EL4 yields valid data. In any case, it is too early to conclude that the proposed interaction is direct. The authors also found that, intact HEK293 cells, overexpression of DN-CAII greatly slows the pH_i increase elicited by removal of extracellular Cl^- , and the co-expression of CAIV restores the rate of pH_i increase to control levels. This result is not easily dismissed because the experimental maneuver (i.e., expression of CAIV) produces a change on the opposite side of the membrane from the pH_i measurement (i.e., we have no trivial reason to expect CAIV to accelerate a pH_i change). On the other hand, the physiological role of the proposed CAIV-AE1 association is not immediately clear. RBCs do express CAIV [57]. However, the CAIV in RBCs is only about 1/600 as abundant as CAII, which is present at about a 1:1 ratio with AE1 monomers [38]. Thus, at most, only a tiny fraction of the AE1 could bind to a CAIV molecule.

A study much like that in the previous paragraph concludes that CAXIV, the catalytic domain of which is also extracellular, interacts with AE3 in the brain [47]. Indeed, antibodies to CAXIV and AE3 co-immunoprecipitate the opposite protein. In intact HEK293 cells, those transfected with AE3 exhibited a markedly augmented rate of pH_i increase in the Cl^- -removal assay, indicating the presence of endogenous CA, presumably CAII. The co-expression of CAXIV produced a further augmentation of the rate of pH_i increase, and 300 μM ACZ greatly reduced this rate. The authors interpreted this last result as if ACZ produced its effect by blocking CAXIV, when in fact ACZ is equally effecting in blocking CAII.

We are still left to explain how CAIV apparently stimulates AE1 [54] or how CAXIV stimulates AE3 [47] in the Cl^- -removal assay. Either the theoretical analysis presented

above (i.e., a CA cannot change $[\text{HCO}_3^-]$ by an amount that is substantial in a paradigm of HCO_3^- -initiated HCO_3^- transport) is incorrect, or the interpretation of the data is incorrect. Perhaps more detailed mathematical modeling will disprove the theoretical argument. On the other hand, it is worth noting that, even when the CA domain is on the outside of the cell, the monitoring of pH_i remains an indirect measure of Cl-HCO_3 exchange activity. Moreover, as noted above, it is possible that CAIV or CAXIV could enhance transport via an allosteric effect unrelated to catalytic activity per se. Finally, I would suggest that even though the CAIV or CAXIV is targeted, via the secretory pathway, to the exterior surface of

the plasma membrane, it is possible that—with substantial over-expression of the protein—the lysis of vesicles could result in the release of small amounts of the enzymes into the cytosol. Because the enzymes such a high activity, only a small amount would be needed to accelerate pH_i changes.

The data discussed above in this section all deal with Cl-HCO_3 exchange. One study focused on the electroneutral Na/HCO_3 cotransporter NBCn1 [52]. The authors used a GST-NBCn1-Ct to assess the binding to immobilized CAII. Given the negative results of Piermarini et al. [41] with both NBCe1 and NDCBE (very closely related to NBCn1), we must view these data with caution. The key physiological assay, performed on HEK-293 cells transfected with NBCn1, was to examine the rate of pH_i recovery from an acute acid load imposed by the NH_4^+ -prepulse technique [7]. The authors found that the co-expression of dominant-negative CAII greatly reduced the rate of pH_i recovery, as did the mutation of the LXXXX motif on Ct (LDDLM→LNNLM). As noted above in the context of AE1, DN-CAII likely reduces CA activity and thus by itself would slow pH_i changes, whereas it remains to be proven that the mutation of the LXXXX motif does not reduce baseline activity independent of CA activity.

7.2. Negative evidence from expression of NBCe1 in oocytes

In order to perform a direct assessment of the effect of CAII on the activity of an SLC4-family member, Lu et al. [58] used the two-electrode voltage-clamp technique to measure the electrical current carried by NBCe1 (SLC4A4) expressed in *Xenopus* oocytes. Fig. 8A illustrates the steps. With the oocyte exposed to ND96 (a HEPES-buffered solution that is nominally free of $\text{CO}_2/\text{HCO}_3^-$), Lu et al. obtained a the current–voltage (I–V) relationship of the oocyte by stepping for 60 ms each from -160 mV to $+20$ mV in steps of 20 mV. This steady-state I–V relationship is virtually a straight line (black), the slope of which—the slope conductance—represents the ease with which current moves through the membrane. Even though this oocyte was expressing human NBCe1-A (the renal variant), the slope conductance in ND96 was only slightly higher than that of control oocytes injected with H_2O rather than the cRNA encoding NBCe1-A. However, after the switch to a $\text{CO}_2/\text{HCO}_3^-$ solution, the slope conductance increases markedly (red), reflecting the HCO_3^- -dependent activity of this electrogenic transporter. Finally, adding the blocker tenidap (green) in the continued presence of $\text{CO}_2/\text{HCO}_3^-$ reduces the slope conductance to a value that is nearly the same as in the absence of $\text{CO}_2/\text{HCO}_3^-$. The difference between the I–V plots, $\pm$ tenidap, is a reasonable estimate of the NBCe1-dependent current (Fig. 8B).

The gray bars in Fig. 9A represent the slope conductances of oocytes, 3 days after they were injected with cRNA encoding NBCe1-A, but before any other maneuvers. Immediately after these assays, Lu et al. injected one group of oocytes with recombinant human CAII dissolved in Tris, and the other group with just the Tris. The blue hatched bars represent the slope conductances—on the same oocytes—1 day after injecting CAII+Tris (left side) or simply Tris (right side). Compared to the “Pre-injection” data, the NBCe1 activity 1 day after injection was 12% lower, both for CAII+Tris and Tris oocytes. However, CAII+Tris oocytes were not significantly different from the Tris oocytes 1 day after injection—indicating that the injected CAII had no effect on NBCe1 activity.

Lu et al. then examined the effect of blocking the CAII with ethoxzolamide (EZA) by treating the oocytes for 3 h and obtaining a third set of I–V relationships on the same cells. The two solid blue bars in Fig. 9B represent the same oocytes as the two blue hatched bars—except that the numbers are smaller because not all oocytes survived the third I–V determination. In both the CAII+Tris oocytes and the Tris oocytes, the EZA treatment caused a tiny but significant increase in NBCe1 activity (pink bars). However, the important point is that the increase was the same for both populations, and the means represented by the pink bars are not significantly different. Although not illustrated here, Lu et al. have shown that the CAII+Tris oocytes had a much faster CO₂-induced fall in pH_i than the Tris oocytes—a demonstration that CAII enhances CO₂ influx (see Fig. 1). Moreover, Lu et al. showed that this stimulatory effect of CAII on the rate of pH_i decline was blocked by EZA, which had no effect in the Tris oocytes.

Thus, we can conclude that the injected CAII enzyme was active, but had no effect on NBCe1 activity. Because one might object that the CAII might not have bound to the NBCe1—although this was the whole point of the metabolon hypothesis—Lu et al. [58] commenced another series of experiments in which they fused CAII to the Ct of NBCe1-A (i.e., near the LXXXX motif). To verify that the transporters trafficked to the plasma membrane, Lu et al. fused GFP, via a 20aa linker, to the Nt side of all constructs. Using a CO₂-influx/pH_i assay, they confirmed that the fused CAII was catalytically active, and blocked by EZA (not shown). The two solid blue bars in Fig. 10 represent the mean slope conductance of GFP-NBCe1-CAII (left side) and GFP-NBCe1 without the CAII (right side); these values were indistinguishable. EZA treatment had no effect on either construct (pink bars).

Thus, even when fused to the Ct of NBCe1, CAII has no effect on slope conductance, as assessed in a traditional voltage-clamp experiment.

7.3. Afly in the ointment

After the paper by Lu et al. [58], another group performed experiments with a protocol similar to that of the first part of the Lu study [56]. However, they came to entirely different conclusions, namely, that CAII does indeed increase the slope conductance of NBCe1-A.

How does one account for such different data? Because the data are likely to be reliable, one must examine the methodology, which was substantially different in one regard.³ Whereas Lu et al. performed a standard I–V analysis, using a computer to control the length (60 ms) and size (20 mV) of the voltage steps, the other group changed the holding voltage by hand, sometimes maintaining membrane potential (V_m) many tens of mV away from the reversal potential (E_{rev}) of the transporter for many tens of seconds. A consequence of this protocol, which engenders very large currents, is that the ionic composition of the cell changes markedly, as revealed in Fig. 1 of ref. [56], where the NBC current decays markedly and $[Na^+]_i$ rises markedly due to the NBCe1-mediated influx of Na⁺. We must assume that pH_i also increased markedly. In the standard protocol employed by Lu et al., the voltage steps

³Ref. [56] did not provide the critical experimental details, although these were revealed at a lecture at a CA meeting.

away from the reversal potential are brief (the whole I–V relationship being obtained in far less than 1 sec) so that cell composition is minimally disturbed.

I hypothesize that, when V_m is held far from E_{rev} for long periods, large intracellular spatial gradients develop for Na^+ , HCO_3^- -related species, and pH_i . Under these conditions, CAII—not the CAII near the transporter but the CAII distributed throughout the cytoplasm—helps to dissipate long-distant gradients for HCO_3^- -related species and pH_i , and thereby enhance transport. According to this hypothesis, CAII would indeed promote large and sustained—and probably not-so-physiological—fluxes of HCO_3^- -related species. However, mine is not the HCO_3^- -metabolon model, but the opposite ... proposing that CAII confined to the vicinity of the transporter has only minor effects. Advances in mathematical modeling would provide a theoretical framework for this hypothesis. Moreover, the hypothesis could easily be tested using the GFP-NBCe1-CAII fusion proteins developed by Lu et al. I predict that if the only CAII is that fused to NBCe1, the stimulatory effect on transport would be nil for standard I–V protocols, and only modest even with large and sustained NBCe1 currents. On the other hand, if soluble CAII were added to the cytosol in the continued presence of the fused CAII, I predict that the effect would be nil for standard I–V protocols, but increasingly substantial as the size and duration of the NBCe1 currents increase.

8. Future directions

CAII clearly plays an important role in CO_2 transport by RBCs. Further elucidating this role will require progress in modeling the system at the macroscopic level and, ultimately, at the level of macromolecular complexes. However, the latter will not only require the structures of each of the relevant proteins, for example, at the RBC membrane—AE1, AQP1, the Rh complex, and the proteins that contact them—but also will require detailed information on how these proteins assemble to form a transport metabolon.

As to the role of CAs in the transport of HCO_3^- -related species, we will again require progress in modeling at the macroscopic level. In addition, investigators need to address several specific questions. For example, can CAs produce allosteric effects on transporters, independent of enzymatic activity? What is the real role of the LXXXX motif in the cytoplasmic Ct of many of the SLC4 proteins? Why do mutations of this motif decrease activity? Does CA activity in the bulk intracellular fluid enhance transporter activity independent of binding to the transport machinery? How many of the Na^+ -coupled HCO_3^- transporters in the SLC4 family actually transport $CO_3^{=}$? And do CAs at the extra- and intracellular surfaces of the membrane play a physiological role in minimizing pH changes in these local regions during large changes in transport activity?

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Fig. 1.

Predicted effect of intracellular CAII on CO₂ influx into red blood cells. (A) Equilibrium before a change in [CO₂]. (B) Increase in extracellular [CO₂] from 1.20 to 1.40 mM. (C) CO₂ influx leads to an increase in intracellular [CO₂]. The specific value of 1.35 mM is for illustration only. Up until this point, we assume that CAII is inactive. (D) Hypothetical sudden activation of CAII causes the conversion of CO₂ to HCO₃⁻. Note that, although the fractional rise in $[HCO_3^-]_i$ is the same as the fractional rise in $[CO_2]_i$, the absolute rise in $[HCO_3^-]_i$ is an order of magnitude larger. Thus, we would expect HCO₃⁻ to contribute far more than CO₂ in the diffusion of “carbon” from the membrane into the center of the cell. Although not shown, non-HCO₃⁻ buffers would contribute to the buffering of H⁺ in both the extra- and intracellular fluid.

Fig. 2.

Predicted effect of intracellular CAII on HCO_3^- -initiated HCO_3^- influx into red blood cells.

(A) Equilibrium before a change in $[\text{CO}_2]$. (B) Removal of extracellular Cl^- promotes

sudden activation of transport by the Cl-HCO_3 exchanger AE1. (C) HCO_3^- influx leads to

an increase in intracellular $[\text{HCO}_3^-]$. The specific value of 13 mM is for illustration only.

Up until this point, we assume that CAII is inactive. (D) Hypothetical sudden activation of

CAII causes the conversion of HCO_3^- to CO_2 . Note that, although the fractional rise in

$[\text{HCO}_3^-]_i$ is the same as the fractional rise in $[\text{CO}_2]_i$, the absolute rise in $[\text{HCO}_3^-]_i$ is an

order of magnitude larger. Although not shown, non- HCO_3^- buffers would contribute to the buffering of H^+ in both the extra- and intracellular fluid.

Fig. 3. Predicted effect of extra- and intracellular CAs during the transport of HCO_3^- vs. $\text{CO}_3^{=}$ vs. H^+ . (A) Hypothetical model of NBCe1 mediating the uptake of 1 Na^+ and 2 HCO_3^- . (B) Hypothetical model of NBCe1 mediating the uptake of 1 Na^+ and 1 $\text{CO}_3^{=}$. (C) Model of a Na-H exchanger mediating the uptake of 1 Na^+ and the efflux of 1 H^+ . Although not shown, non- HCO_3^- buffers would contribute to the buffering of H^+ in both the extra- and intracellular fluid.

Fig. 4.

Binding of liquid-phase GST-SLC4-Ct fusion proteins to immobilized CAII. (A) GST-AE1-Ct (red). (B) GST-NBCe1-Ct (green). (C) GST-NDCBE-Ct (blue). The inset schematizes the binding of GST-AE1-Ct to immobilized CAII. Each curve represents SLC4-specific binding, that is, the difference between binding of the GST-SLC4-Ct construct (probed with anti-GST and a secondary antibody) and the binding of GST to solid-phase CAII. These difference data were then fitted by a non-linear least-squares method to the Michaelis Menten equation. For GST-AE1-Ct, the apparent affinity (K_d) was 597 ± 186 nM and the maximal binding (B_{max}) was 0.88 ± 0.14 relative GST immunoreactivity units. For GST-NBCe1-Ct, $K_d = 322 \pm 76$ nM and $B_{max} = 2.06 \pm 0.20$. For GST-NDCBE-Ct, $K_d = 330 \pm 165$ nM and $B_{max} = 0.60 \pm 0.13$. Data from ref [41].

Fig. 5.

Binding of liquid-phase “pure” SLC4-Ct peptides (i.e., not fused to GST) to immobilized CAII. The insets schematize the binding of GST-AE1-Ct vs. AE1-Ct to immobilized CAII. In both cases, the bound material was detected with an antibody to the AE1-Ct (rather than with anti-GST, as in Fig. 4). Similar results were obtained with NBCe1-Ct (detected with anti-NBCe1-Ct) and His-tagged NDCBE-Ct (detected with anti-His). All values are relative to 1000 nM GST-AE1-Ct. Data from ref [41].

Fig. 6. Binding of liquid-phase CAII to immobilized GST, GST-AE1-Ct fusion protein, or “pure” AE1-Ct peptide (i.e., not fused to GST). The insets schematize the binding of CAII to immobilized GST, GST-AE1-Ct, or AE1-Ct. The bound CAII was detected with anti-CAII. Anti-AE1-Ct confirmed equal amounts of immobilized GST-AE1-Ct fusion protein and “pure” AE1-Ct peptides. Similar results were obtained with NBCe1 and NDCBE. All values are relative to 1000 nM GST-AE1-Ct. Data from ref. [41].

Fig. 7.

Surface plasmon resonance (SPR) with immobilized CAII. Each panel shows an initial baseline, the wash-in of the liquid-phase “analyte,” and the washout of the analyte (A) Acetazolamide (ACZ) as the analyte. Increasing concentrations of ACZ (15, 62.5, and 250 nM) produced faster and increased levels of binding, fitted by a 1:1 Langmuir model. (B) Pure AE1-Ct peptide as the analyte. (C) Pure NBCe1-Ct peptide as the analyte. (D) Pure NDCBE-Ct peptide as the analyte. Responses in the last three panels are consistent with shifts in bulk refractive index (i.e., nonspecific interactions) caused by high peptide concentrations. The insets schematize the binding of ACZ, AE1-Ct, NBCe1-Ct, or NDCBE-Ct to immobilized CAII. Data from ref [41].

Fig. 8.

Determination of the slope conductance of NBCe1-A. (A) Data are from a two-electrode voltage-clamp of a *Xenopus* oocyte expressing human NBCe1-A. The sequence of measurements was ND96 (HEPES-buffered, $\text{CO}_2/\text{HCO}_3^-$ -free), $\text{CO}_2/\text{HCO}_3^-$ without inhibitor, $\text{CO}_2/\text{HCO}_3^-$ with tenidap to block the transporter. (B) NBCe1 current. The symbols are the result of subtracting the green symbols in panel A from the red symbols. The dashed line is the result of a linear fit, which yielded a slope conductance of 14 μS . Data from ref [58].

Fig. 9.

Effect of injected CAII and ethoxzolamide (EZA) on the slope conductance of NBCe1-A. (A) Comparison of the same oocytes, before and after injection of CAII+Tris (or just Tris). (B) Comparison of the same oocytes from panel A, before and after exposure to EZA. Measurements of pH_i (not shown) indicated that the injected CAII was catalytically active, and blocked by EZA. Data from ref [58].

Fig. 10.

Effect of CAII—fused to the C terminus of the cotransporter—on the slope conductance of NBCe1-A. For the two bars at the left, the oocytes were expressing a construct consisting of GFP fused (via a 20aa linker) to human NBCe1-A, which was fused at its C terminus to human CAII. For the two bars at the right, the oocytes were expressing a construct consisting of GFP fused (via a 20aa linker) to human NBCe1-A, but without the CAII. Measurements of pH_i (not shown) indicated that the fused CAII was catalytically active. Data from ref [58].