Functional role of a putative carbonic anhydrase II-binding domain in the electrogenic Na\(^+\)-HCO\(_3^\)\(^-\) cotransporter NBCe1 expressed in Xenopus oocytes

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The electrogenic Na\(^+\)-HCO\(_3^\)\(^-\) cotransporter NBCe1 plays essential roles in the regulation of systemic and/or local pH. Homozygous inactivating mutations in NBCe1 cause proximal renal tubular acidosis associated with ocular abnormalities. We recently showed that defective membrane expression of NBCe1, caused by several mutations such as \(\Delta 65\) bp (S982NfsX4), is also associated with familial migraine. The \(\Delta 65\) bp mutant is quite unique in that it lacks a putative carbonic anhydrase (CA) II binding domain but still shows an apparently normal transport activity in Xenopus oocytes. In this addendum, we show that the co-expression of CAII together with the wild-type NBCe1 or the \(\Delta 65\) bp mutant does not enhance the NBCe1 activities in oocytes. Moreover, a carbonic anhydrase inhibitor acetazolamide fails to inhibit the wild-type or the \(\Delta 65\) bp activities co-expressed with CAII. These results indicate that a bicarbonate transport metabolon proposed for the interaction between CAII and NBCe1 does not work at least in Xenopus oocytes.

Introduction

The electrogenic Na\(^+\)-HCO\(_3^\)\(^-\) cotransporter NBCe1, encoded by \(SLC4A4\), has three spliced variants, which mediate important roles in the regulation of systemic and/or local pH. The kidney-type variant NBCe1A is expressed predominantly in kidney, where it mediates a majority of bicarbonate exit from proximal tubular cells. On the other hand, the pancreas-type transporter NBCe1B is expressed in several tissues such as pancreas, intestinal tracts, eye and brain, where it presumably mediates bicarbonate uptake into cells. The brain-type transporter NBCe1C is almost exclusively expressed in brain, but its physiological significance remains to be established.

Homozygous inactivating mutations in NBCe1 cause proximal renal tubular acidosis (pRTA) associated with ocular abnormalities such as band keratopathy, glaucoma and cataract. Functional analysis using the different expression systems suggested that at least 50% reduction of NBCe1 activities in vivo would be required to induce severe pRTA. Besides the inactivation of transport function per se, some of the NBCe1 mutants also showed aberrant membrane expression.

Recently we found that defective membrane expression of NBCe1 in C6 glioma cells is associated with familial migraine. Because NBCe1 is expressed in astrocytes and thought to play an important role in the local pH regulation, we speculated that the near total loss of NBCe1 activity in astrocytes might cause dysregulation of synaptic pH, resulting in the enhanced neuronal excitability and migraine occurrence.

Several studies have reported that carbonic anhydrase (CA) II can bind to and stimulate a variety of bicarbonate transporters including the Cl\(^-\)/HCO\(_3^\)\(^-\) exchanger AE1 and NBCe1. This phenomenon has been generally explained by the mechanism known as the bicarbonate transport metabolon, in which the bound...
CAII might enhance the bicarbonate transporters by channeling HCO₃⁻ to or away from the cytoplasmic anion translocation pathway near the CAII binding sites. Although such a model seems to be quite attractive, the results against this interpretation have been also reported.⁵,⁷,¹⁵ Consistent with the bicarbonate transport metabolon theory, the AEI mutants lacking the CAII-binding ability had significantly reduced transport activities compared to the wild-type AEI, and failed to show the transport stimulation by CAII.⁵,¹³ Unlike AEI that contains only one CAII-binding domain, NBCe1 contains two putative CAII-binding domains in its C-terminal.¹³ Pushkin et al. reported that the CAII-binding to NBCe1 required both of these domains, and that the transport stimulation by CAII was abolished when one of the CAII-binding domains was mutated.¹³ Among the NBCe1 mutations thus far identified in pRTA patients, the C-terminal flame-shift mutant Δ65 bp (S⁹⁸²N⁹⁹⁶S⁹⁸⁴X⁴⁴), associated with familial hemiplegic migraine, has quite unique clinical phenotypes caused by the Δ65 bp mutation. Despite its lack of CAII-binding ability, which was previously shown,⁸ more importantly, the co-expression of CAII failed to enhance not only the Δ65 bp mutant activity but also the wild-type activity. Furthermore, the treatment with ACZ failed to reduce the stimulatory effects of CAII on NBCe1 as shown in Figure 2B. Taken together, these results do not support the hypothesis that the lack of CAII-binding domain in Δ65 bp mutant significantly impairs the stimulation of NBCe1 by CAII.

Both positive and negative results about the stimulatory effects of CAII on NBCe1 function expressed in Xenopus oocytes have been reported. Becker and Deitmer found that CAII, either injected as the recombinant protein or expressed with NBCe1A, significantly enhanced the NBCe1A activity.¹¹ By contrast, Boron and his colleagues found that CAII, either directly injected into oocytes as the recombinant protein or expressed as the fused construct with NBCe1A, failed to enhance the NBCe1A activity.¹⁵ One of the criticisms raised by Becker and Deitmer was that Boron and his colleagues used the slope conductance for the analysis of NBCe1A activities, which might not be sensitive enough to detect a small stimulatory effect of CAII.¹¹,¹⁵ Using the voltage clamp method at the fixed membrane potentials similar to that used by Becker and Deitmer, however, we failed to detect any stimulatory effects of CAII on NBCe1A activities. Although the reasons for these conflicting results are not apparent, our co-expression strategy in Xenopus oocytes was able to detect the stimulation of NBCe1B

As shown in Figure 2A, the injection of CAII cRNA markedly enhanced the protein expression of CAII (as described in ref. 12). Figure 2B shows that the Δ65 bp mutant, despite its lack of CAII-binding ability, showed the electrogenic activity comparable to that of the wild-type NBCe1A as previously shown.⁸ More importantly, the co-expression of CAII failed to enhance not only the Δ65 bp mutant activity but also the wild-type activity as shown in Figure 2C. Taken together, these results do not support the hypothesis that the lack of CAII-binding domain in Δ65 bp mutant significantly impairs the stimulation of NBCe1 by CAII.

Results and Discussion

Dahl et al. reported that the endogenous CAII in Xenopus oocytes was able to enhance the activity of AEI.¹² However, conflicting data had been reported regarding the effects of CAII on the NBCe1 activity expressed in Xenopus oocytes.¹¹,¹⁵ We therefore decided to co-express CAII together with the NBCe1A constructs. Oocytes were removed from Xenopus laevis, dissociated with collagenase, and were injected with cRNA solution containing cRNAs expressing the wild-type human NBCe1A or the Δ65 bp mutant with or without human CAII. The total injection volume was adjusted to 50 nl (5 ng of each cRNA). Electrophysiological analysis using the two-electrode voltage-clamp method was performed three to five days after cRNA injection (as described in refs. 4, 5, 8 and 18). HCO₃⁻-free ND96 solution contained 5 mM Hepes, pH 7.4/96 mM NaCl/2 mM KCl/1 mM MgCl₂/1.8 mM CaCl₂, HCO₃⁻-containing solution contained 5 mM Hepes, pH 7.4/66 mM NaCl/30 mM NaHCO₃/2 mM KCl/1 mM MgCl₂/1.8 mM CaCl₂, which was equilibrated with 5% CO₂ in oxygen (pH 7.4). Oocytes were initially perfused with ND96 solution, and the NBCe1 currents induced by solution changes from ND96 to HCO₃⁻-containing solution were measured by the two-electrode voltage-clamp method at a holding potential of -25 mV (as described in refs. 4 and 5). To avoid a large shift in intracellular ion concentrations, which might have been caused by the prolonged voltage clamping in the presence of large NBCe1-mediated ion fluxes, voltage was clamped only for a brief period (less than 1 min) during the current measurements. Voltage clamp was immediately released after the first current measurements, and oocytes were perfused with ND96 solution containing a carbonic anhydrase inhibitor acetazolamide (ACZ, 0.3 mM). After the perfusion with ACZ for 30 min, voltage was clamped again and the second current measurements were performed in the continued presence of ACZ.
activity by an inositol 1,4,5-trisphosphate receptor-binding protein IRBIT, which was subsequently confirmed in mammalian cells. Theoretically, the CAII binding, irrespective of its enzymatic activity, could induce the conformational changes of NBCe1A and thereby increase its transport, though Boron recently pointed out the methodological problems in the previous studies which had demonstrated the physical interaction between CAII and bicarbonate transporters.

Alternatively, the enzymatic activity of CAII could be essential in the transport stimulation, even without the physical binding to NBCe1A. However, our negative data for both the wild-type NBCe1A and the Δ65 bp mutant rather support the conclusion by Boron and his colleagues that the transport metabolon based on such mechanisms do not work at least for NBCe1A and CAII co-expressed in Xenopus oocytes.

One possible explanation for the lack of CAII-mediated NBCe1A stimulation in Xenopus oocytes would be the variable functional modes of NBCe1A depending on the environmental factors. For example, NBCe1A is known to function with 1Na+ to 2HCO3− stoichiometry in Xenopus oocytes. On the other hand, the electrogenic NBCe1A activity is markedly inhibited by carbonic anhydrase inhibitors in rat renal proximal tubules in vivo, where NBCe1A activities with 1Na+ to 3HCO3− stoichiometry. Interestingly, ACZ failed to inhibit the electrogenic NBCe1A activity in isolated rabbit renal proximal tubules incubated in the conventional medium, where NBCe1A functions with 1Na+ to 2HCO3− stoichiometry. This is not due to species differences, because the improved incubation conditions in isolated rabbit renal proximal tubules converted the NBCe1A transport stoichiometry to 1Na+ to 3HCO3− as in rat renal proximal tubules in vivo, and rendered the NBCe1A activity quite sensitive to ACZ.

Another possible explanation for the negative effects of CAII in Xenopus oocytes would be the different rates of changes in intracellular HCO3− concentrations. For example, rat renal proximal tubular cells in vivo can change cell HCO3− concentrations by several mmol/liter per second in response to sudden changes in peritubular HCO3− concentrations. On the other hand, Xenopus oocytes expressing NBCe1A can change cell Na+ concentrations only by several mmol/liter per minute in response to bath CO2/HCO3− application.

As recently discussed by Boron, CAII may potentially have a stimulatory effect on NBCe1A activity only if the conversion between CO2− and HCO3− is involved in the transport process. NBCe1A may transport 1Na+ and 2HCO3− in renal proximal tubules incubated in the conventional conditions, but may actually transport 1Na+, 1CO3−, and 1HCO3− in renal proximal tubules in vivo and in isolated renal proximal tubules incubated in the improved conditions. Furthermore, the effect of CAII should depend on the conversion rates between CO2− and HCO3− required to match the NBCe1A-mediated transport rates, though no mathematical models exit that can precisely predict the real effects of CAII on the NBCe1A-mediated transport. From these considerations, we cannot exclude the possibility that the bicarbonate transport metabolon affects the NBCe1A activity in vivo. Nevertheless, the present study
indicates such a mechanism is unlikely to work in Xenopus oocytes.

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