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The electrogenic Na^+—HCO_3^- cotransporter NBCe1-B is regulated by intracellular Mg^{2+}

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ABSTRACT

NBCe1-B, a major splice variant of the electrogenic Na^+—HCO_3^- cotransporter (NBCe1) fulfills basic cellular functions including regulation of intracellular pH and epithelial HCO_3^- secretion. However, its cellular regulatory mechanism still remains elusive. Here, we provide evidence for the first time that NBCe1-B activity can be controlled by intracellular Mg^{2+} ($Mg^{2+}{}_i$), the physiologically most abundant intracellular divalent cation. Using the whole-cell patch–clamp technique, we found that recombinant NBCe1-B currents expressed in HEK293 and NIH3T3 cells were inhibited voltage-independently by $Mg^{2+}{}_i$ in a concentration-dependent manner ($K_i \approx 0.01$ mM). The $Mg^{2+}{}_i$ inhibition was partially relieved by truncation of the NBCe1-B specific N-terminal region ($K_i \approx 0.3$ mM), and was also observed for native electrogenic Na^+—HCO_3^- cotransporter current in bovine parotid acinar cells that endogenously express NBCe1-B ($K_i \approx 1$ mM). These results suggest that Mg^{2+} may be a cytosolic factor that limits intrinsic cotransport activity of NBCe1-B in mammalian cells.

The electrogenic Na^+—HCO_3^- cotransporter NBCe1, which carries one Na^+ and either two or three equivalents of HCO_3^- in one cycle across the plasma membrane not only regulates intracellular pH in various cell types, but also plays a critical role in HCO_3^- transport in epithelial cells [1,2]. Molecular cloning has so far identified three splice variants of NBCe1 (NBCe1-A, -B, and -C). NBCe1-B differs from NBCe1-A by N-terminal 85 amino acids and differs from NBCe1-C by C-terminal 46 amino acids [1]. It is believed that NBCe1-B with a 1:2 $Na^+ : HCO_3^-$ stoichiometry constitutes a major basolateral HCO_3^- influx pathway in several HCO_3^- secreting epithelia [1–3]. In support of its vital role in epithelial HCO_3^- secretion, we have previously shown that NBCe1-B mediates, at least in part, an exceptionally large electrogenic Na^+—HCO_3^- cotransporter (NBCe) current in acinar cells of bovine parotid [3], an exocrine gland secreting continuously large volumes of HCO_3^--rich fluid [4]. However, cellular regulation of NBCe-B activity in native epithelial cells remains largely unknown.

Magnesium ion, the most abundant intracellular divalent cation, is increasingly recognized as an important player modulating activities of some ion channels and transporters, directly or indirectly [5], although its cytosolic free concentration ($[Mg^{2+}]_i$) ranging between 0.5 and 1.0 mM is maintained relatively constant under normal conditions in most mammalian cells [5,6]. For example, $Mg^{2+}{}_i$ can reduce ion channel currents by direct voltage-dependent block of pores [7] or by electrostatic binding to the negative charges of membrane phosphoinositides such as PIP_2 [8–10]. Despite physiological importance of $Mg^{2+}{}_i$, it remains unknown even whether NBCe1-B can be regulated by physiological $[Mg^{2+}]_i$. To address this issue specifically, the standard (fast) whole-cell patch-clamp technique was used to monitor electrogenic Na^+—HCO_3^- cotransporter (NBCe) activity (i.e., NBCe current) heterologously and natively expressed in mammalian cells under voltage–clamp conditions at the same time as dialyzing the cells with pipette solutions containing defined concentrations of free Mg^{2+} to control their intracellular levels. A preliminary account of portions of this work has been communicated to the 85th annual meeting of the physiological society of Japan [11].

Materials and methods

Plasmid constructions and cell transfection. A cDNA of full-length bovine NBCe1-B was generated by RT-PCR from bovine parotid first-strand cDNA as described previously (Accession No. AB436382) [3]. A construct of mutant NBCe1 lacking splice variant specific N-terminal region (ΔNt-NBCe1) was generated by PCR technique using bovine NBCe1-B cDNA as a template. These constructs were subcloned into a bicistronic expression vector pIRES2-EGFP (Clontech, Mountain View, CA) and transiently transfected into HEK293 and NIH3T3 cells using Lipofectamine 2000 (Invitrogen, Carlsbad, CA). Membrane currents were recorded from EGFP positive single cells which exhibited strong fluorescence two or three days after transfection.

Cell isolation. Bovine parotid tissue was obtained from a local slaughterhouse (Hokkaido Hayakita Meat Inspection Center, Abira,

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Japan). After the tissue was removed from the slaughtered animal, it was kept at 4 °C in a NaCl-rich bath solution (145 NaCl, 5 KCl, 10 Hepes, 10 glucose, 1 $MgCl_2$, 1 $CaCl_2$, in mM, pH 7.4) until used. The animals were approved to be tested negative for bovine spongiform encephalopathy (BSE). Isolated acini and acinar cells were prepared as described previously [12,13].

Whole-cell patch–clamp experiments. The methods used for measurements and analysis of whole-cell currents were similar to those reported previously [3]. The cell potential was held at −80 mV and varied from −100 to +50 mV (with ramp pulse) over duration of 800 ms every 10 s. The pipette potential was corrected for the liquid junction potential. All experiments were performed at room temperature.

Solutions. Pipette solutions (pH 7.4 with NMDG (*N*-methyl-D-glucamine)) contained (in mM): 10 BAPTA, 100 Hepes, 4 EDTA–2Na, 2 $NaHCO_3$, 23 $cholineHCO_3$, 0–8 $MgCl_2$ (no $MgCl_2$ or appropriate amounts of $MgCl_2$ were added to yield Mg^{2+}-free or 10^{-5}–$10^{-2.5}$ M free Mg^{2+}, respectively), 0.044–0.202 $CaCl_2$ (10^{-9} M free Ca^{2+}), 15–25 NMDG-glutamate, and 0–15 NMDG-Cl. Free Mg^{2+} and Ca^{2+} concentrations were calculated using the program Maxchelator (http://www.stanford.edu/~cpatton/maxc.html). The concentrations of NMDG-glutamate and -Cl were also varied to maintain the chloride concentration (15–16 mM Cl^-). In some experiments, Mg^{2+} of pipette solutions was buffered by using 10 mM EGTA or 10 mM EGTA and 30 mM citrate. Standard bath solution contained (in mM, pH 7.4 with NMDG) 120 Na-glutamate, 25 $NaHCO_3$, 10 Hepes, 1 $MgCl_2$, and 1 $CaCl_2$. Na^+-free solution containing HCO_3^- was made by replacing Na^+ with 120 mM NMDG (or Cs^+ in a few experiments) and 25 mM choline. The solutions having HCO_3^- were bubbled with 5% CO_2/95% O_2.

Data analysis. The inhibitory constant (K_i) of Mg^{2+} was computed by fitting the dose–response curve with the following Hill equation:

$$I = I_{base} + \frac{I_{max} - I_{base}}{1 + (A/K_i)^{n_H}}$$

where A is the concentration of the inhibitor, n_H is pseudo Hill coefficient, I_{max} is the maximum current, I_{base} is the baseline of currents. When I_{base} was unable to be determined, we presumed it as 0.

All average results are presented as means ± SEM of independent experiments (n), where n refers to the number of cells tested.

Refer to Supplemental Methods for more details.

Results

Recombinant NBCe1-B expressed in mammalian cells is sensitive to intracellular Mg^{2+}

We tested whether intracellular Mg^{2+} (Mg^{2+}_i) would affect NBCe currents in HEK293 cells transfected with NBCe1-B (Fig. 1A

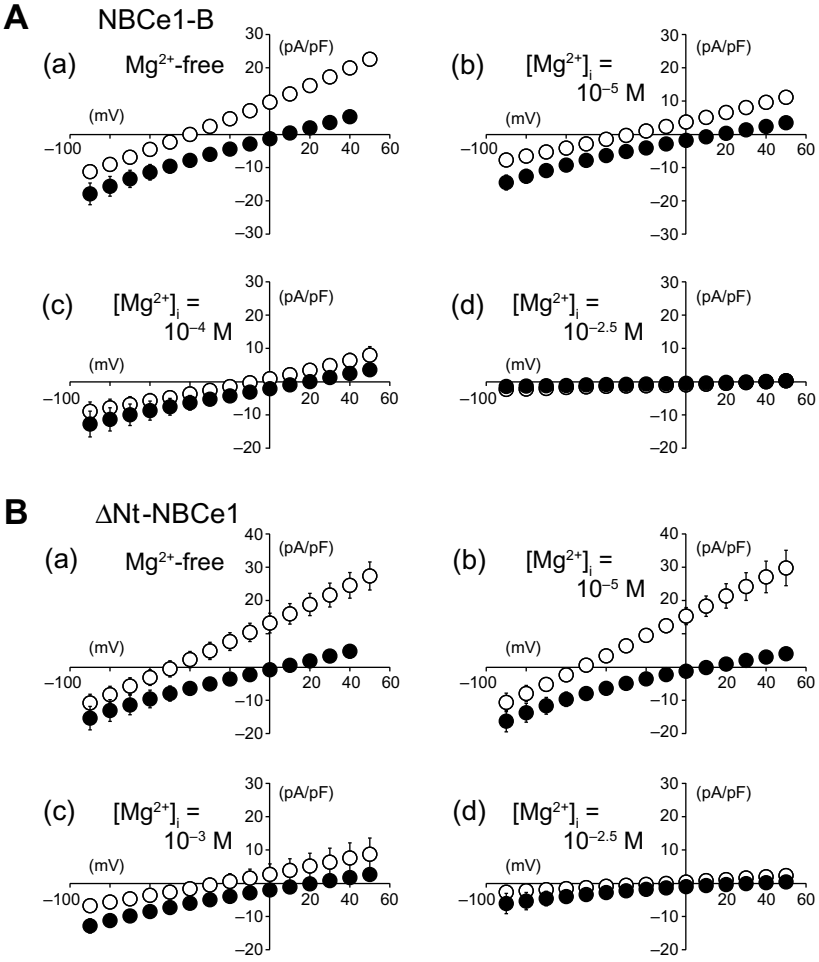

Fig. 1. Modulation by intracellular free Mg^{2+} concentrations ($[Mg^{2+}]_i$) of wild-type NBCe1-B or splice variant specific N-terminal truncated NBCe1 (ΔNt-NBCe1). Summary of currents recorded from HEK293 cells transiently transfected with NBCe1-B (A) or ΔNt-NBCe1 (B) using pipette solutions containing various concentrations of free Mg^{2+} (Mg^{2+}-free or 10^{-5}–$10^{-2.5}$ M) and bath solutions containing HCO_3^- in the presence (open circles) or absence (filled circles) of Na^+. Each data point represents the average amplitude of currents measured at voltages ranging from −90 to +50 (or 40) mV with 10 mV intervals. Data shown were obtained after currents were stabilized. Shown are means ± SEM. Error bars smaller than symbols are not shown. (A), n = 5–10; (B), n = 5–8.

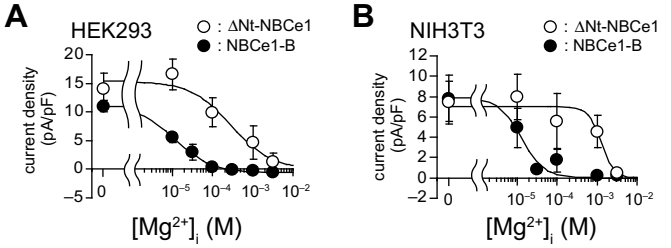

Fig. 2. Dose-dependent inhibition of NBCe1-B and ΔNt-NBCe1 currents by intracellular Mg^{2+}. Extracellular Na^+-dependent current densities at 0 mV in the presence of HCO_3^- were measured in HEK293 (A) or NIH3T3 (B) cells transfected with NBCe1-B (filled circles) or ΔNt-NBCe1 (open circles). Pipette solutions containing various concentrations of free Mg^{2+} (Mg^{2+}-free or 10^{-5}–$10^{-2.5}$ M) were used. Shown are mean of current densities (pA/pF) ± SEM. Error bars smaller than symbols are not shown. The lines are fits to the Hill equation. (A), NBCe1-B, n = 5–10; ΔNt-NBCe1, n = 5–8; (B), NBCe1-B, n = 7–10; ΔNt-NBCe1, n = 10–14.

and Supplemental Fig. S1). Protein expression of NBCe1-B was confirmed by Western blotting (Supplemental Fig. S2). HEK293 cells were dialyzed with pipette solutions containing different concentrations of free Mg^{2+} (buffered by EDTA) in bath solutions containing HCO_3^-. Under these conditions, as also described elsewhere [3], NBCe currents were defined as the extracellular Na^+-dependent currents (Fig. 1A), whose amplitudes were almost the same as those elicited by addition of HCO_3^- to the nominally HCO_3^--free bath solutions containing Na^+ (data not shown). The NBCe currents were decreased by increasing intra-pipette (i.e., intracellular) free Mg^{2+} concentrations ($[Mg^{2+}]_i$) from 0 to $10^{-2.5}$ M (Figs. 1A and 2). The apparent K_i value and pseudo Hill coefficient of $Mg^{2+}{}_i$ at 0 mV were 11.8 μM and 0.96, respectively. There was little, if any, voltage-dependence of the $Mg^{2+}{}_i$ inhibition, so that K_i values were estimated to be 14.6 μM at −20 mV, 11.1 μM at +20 mV, or 10.9 μM at +40 mV, respectively. Similar results were also obtained from NIH3T3 cells expressing NBCe1-B (Fig. 2). No NBCe currents were detected in mock-transfected HEK293 (−0.4 ± 0.3 pA/pF at 0 mV, n = 8) and NIH3T3 cells (−0.3 ± 0.2 pA/pF at 0 mV, n = 10) even with the Mg^{2+}-free pipette solution.

$Mg^{2+}{}_i$ inhibition of NBCe1-B is partially relieved by truncation of the variant specific N-terminal region

A previous study in *Xenopus* oocyte expression system has shown that the cytosolic NBCe1-B (and -C) specific N-terminal region may lower their activities [14]. The evidence supporting this mechanism is based on the observations that NBCe1-B and -C are less active than NBCe1-A and that the truncation of NBCe1-C (and thus -B) specific N-terminal region activates NBCe1-C activity [14]. Intriguingly, the observations were made in two-electrode voltage–clamp experiments using intact oocytes, where resting $[Mg^{2+}]_i$ has been reported to be 0.3 mM [15] and in inside-out macropatch experiments, where the cytosolic solution contained 1 mM $MgCl_2$ with no added chelators for divalent cations [14]. We thus hypothesized that regardless of whether it is direct or indirect, $Mg^{2+}{}_i$ inhibition of NBCe1-B may at least in part involve the variant specific N-terminal region and evaluated the effect of $Mg^{2+}{}_i$ on currents generated by the mutant NBCe1 lacking the N-terminal region (ΔNt-NBCe1) in HEK293 cells. Western blot analysis confirmed the protein expression of ΔNt-NBCe1 (Supplemental Fig. S2). ΔNt-NBCe1 currents ($K_i \approx 0.3$ mM) appeared to be much less sensitive to $Mg^{2+}{}_i$ than the wild-type NBCe1-B currents (Figs. 1B and 2). Comparable observations were made in NIH3T3 cells (Fig. 2).

Native NBCe1-B-like current in bovine parotid acinar cells also displays sensitivity to intracellular Mg^{2+}

We have previously identified and characterized an extremely large NBCe current in bovine parotid acinar (BPA) cells that express transcripts of NBCe1-B but not NBCe1-A [3], NBCe1-C (Supplemental Fig. S3), and NBCe2 (Supplemental Fig. S3). Thus, we examined whether the novel $Mg^{2+}{}_i$ inhibition would be also observed for native NBCe1-B like current in BPA cells. Protein expression of NBCe1-B was confirmed by Western blotting (Supplemental Fig. S2). Native NBCe1-B like currents were inhibited by increasing $[Mg^{2+}]_i$ from 10^{-5} to $10^{-2.5}$ M in a concentration-dependent manner (Fig. 3 and Supplemental Fig. S1). The apparent K_i value and pseudo Hill coefficient of $Mg^{2+}{}_i$ at 0 mV were 817 μM and 1.64,

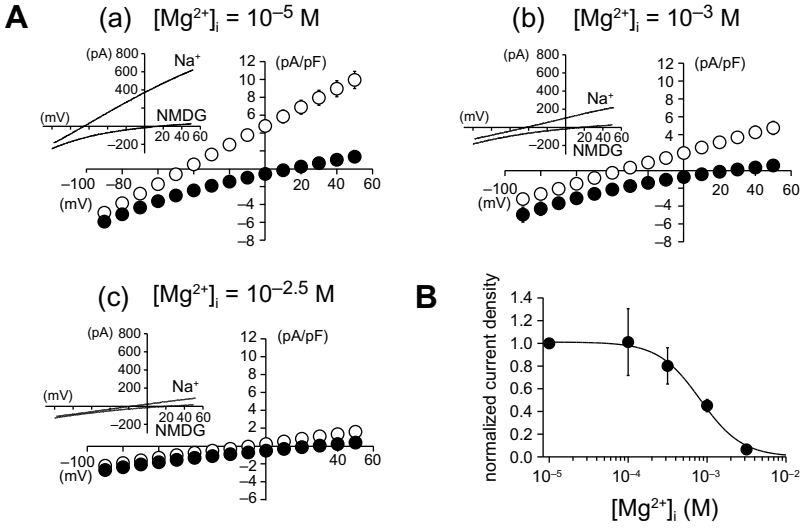

Fig. 3. Modulation by $[Mg^{2+}]_i$ of NBCe currents natively expressed in bovine parotid acinar (BPA) cells. (A) summary of currents recorded using pipette solutions containing various concentrations of free Mg^{2+} (10^{-5}–$10^{-2.5}$ M) and bath solutions containing HCO_3^- in the presence (open circles) or absence (filled circles) of Na^+. Other experimental conditions are same as described in Fig. 1. Shown are means ± SEM. n = 12–23. Error bars smaller than symbols are not shown. (Insets) typical traces of currents recorded from acinar cells derived from a bovine parotid gland. (B) Dose–response relations between free Mg^{2+} concentrations (10^{-5}–$10^{-2.5}$ M) and normalized NBCe current densities. NBCe current densities at 10^{-4}–$10^{-2.5}$ M free Mg^{2+} were normalized to that at 10^{-5} M free Mg^{2+} obtained from a BPA cell derived from the same bovine parotid gland. Other experimental conditions are same as described in Fig. 2. Shown are means ± SEM. The line is a fit to the Hill equation. n = 12 each.

respectively. Interestingly, the native currents were less sensitive to $Mg^{2+}{}_i$ than recombinant NBCe1-B currents (Figs. 2 and 3). There was no indication of voltage-dependent block, so that K_i values were 895 μM at −20 mV, 818 μM at +20 mV, or 847 μM at +40 mV, respectively. Increasing $[Mg^{2+}]_i$ buffered by either EGTA alone or EGTA and citrate also inhibited the native currents in a similar manner (data not shown).

Discussion

In the present study, we have provided several lines of evidence that NBCe1-B can be regulated by intracellular Mg^{2+} in mammalian cells. First, recombinant NBCe1-B currents expressed in mammalian HEK293 and NIH3T3 cells were similarly inhibited by increasing $[Mg^{2+}]_i$, suggesting that the inhibition is not restricted to NBCe1-B expressed in a particular cell type. Second, the mutant NBCe1 (ΔNt-NBCe1) was less sensitive to $Mg^{2+}{}_i$, compared to the wild-type NBCe1-B, finding being consistent with the view that the $Mg^{2+}{}_i$ inhibition may be associated, directly or indirectly, with the N-terminal region. Finally, NBCe1-B-like currents expressed in BPA cells were also inhibited by $Mg^{2+}{}_i$ in a concentration-dependent manner, indicating that the novel $Mg^{2+}{}_i$ inhibition may be valid for NBCe1-B expressed in a native cellular environment.

Although the molecular mechanism of the inhibition of NBCe1-B by $Mg^{2+}{}_i$ is beyond the scope of this report, our data would also give some clue to it. A lack of clear voltage-dependence of the current inhibition suggests that $Mg^{2+}{}_i$ binding site, if exists, may be outside the transmembrane electric field. Given the lower Mg^{2+} sensitivity of ΔNt-NBCe1 than NBCe1-B, a binding site might be within NBCe1-B specific N-terminal region that contains acidic amino acids. However, the residual sensitivity of ΔNt-NBCe1 to high concentrations of $Mg^{2+}{}_i$ might also imply the existence of other binding sites and/or different mechanisms. Furthermore, our findings that a polyvalent cationic compound neomycin inhibited both recombinant and native currents (Supplemental Fig. S4) as Mg^{2+} did may suggest that the positive charge of Mg^{2+} and thus electrostatic mechanism might be involved in its inhibitory action. Finally, we cannot completely rule out the possibility that the $Mg^{2+}{}_i$ inhibition could involve Mg^{2+} sensitive enzymes [5]. However, we can perhaps exclude enzymes requiring ATP-Mg such as many kinases, because we minimized the influence of ATP-Mg by using pipette solutions not added ATP. Further experiments are necessary to specifically address these possibilities.

We did not investigate the relationship between $Mg^{2+}{}_i$ and the cotransport stoichiometry in the present study. To address specifically this issue requires more detailed experiments as described in our previous report [3], but would be interesting since cytosolic Ca^{2+} has been shown to change the stoichiometry of NBCe1-A when expressed in *Xenopus* oocytes [16].

At least under the whole-cell voltage–clamp conditions, NBCe1-B currents were almost negligible under the physiological $[Mg^{2+}]_i$ reported for mammalian cells [5,6]. However, native NBCe1-B-like currents in BPA cells, like ΔNt-NBCe1, were much less sensitive to $Mg^{2+}{}_i$ than the wild-type NBCe1-B, so that K_i values of $Mg^{2+}{}_i$ (approximately 1 mM, irrespective of three different buffers used) fell within the physiological range of $[Mg^{2+}]_i$. The reason for these phenomena remains unclear at present, but one potential explanation is that regulatory factors (could be related to the NBCe1-B specific N-terminal region) might reduce $Mg^{2+}{}_i$ sensitivity of NBCe1-B in BPA cells. The reduction might enable NBCe1-B to be active enough to significantly contribute to the electrical properties and thus continuous HCO_3^- transport of unstimulated BPA cells [3]. Furthermore, such a modulation of $Mg^{2+}{}_i$ sensitivity might determine NBCe1-B activity in other cell types under physiological conditions, because cytoplasmic free Mg^{2+} is largely buffered and thus unlikely to act as an intracellular second messenger as Ca^{2+} [5,6].

It should be stressed, however, that the regulation of NBCe1-B activity by the change of $[Mg^{2+}]_i$ could be also relevant particularly under pathophysiological conditions. In ventricular myocytes, for example, NBCe including NBCe1-B [17] plays a role in modulating intracellular pH and Na^+ concentration [18]. Thus, a significant increase in $[Mg^{2+}]_i$ during ischemia [19,20] might inhibit Na^+ influx via NBCe1-B. This inhibition may in turn reduce post-ischemic dysfunction in heart, possibly by keeping intracellular Na^+ concentration lower and thereby preventing Ca^{2+} overload, as suggested in a previous study [21].

In conclusion, the results of this study indicate for the first time that $Mg^{2+}{}_i$ can be an important regulator of NBCe1-B activity. Further understanding of this $Mg^{2+}{}_i$ inhibition will provide important insights into diverse functions and activities of NBCe1-B in various native cell types.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2008.08.104.

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