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IRBIT reduces the apparent affinity for intracellular Mg^{2+} in inhibition of the electrogenic Na^+-HCO_3^- cotransporter NBCe1-B

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ABSTRACT

The electrogenic Na^+-HCO_3^- cotransporter NBCe1-B can be regulated by intracellular Mg^{2+} (Mg^{2+}_i). We previously reported that under whole-cell voltage-clamp conditions, bovine NBCe1-B (bNBCe1-B) currents heterologously expressed in mammalian cells are strongly inhibited by Mg^{2+}_i, and the inhibition is likely mediated by electrostatic interaction and relieved by truncation of the cytosolic NBCe1-B specific N-terminal region. Intriguingly, NBCe1-B-like currents natively expressed in bovine parotid acinar (BPA) cells are much less sensitive to Mg^{2+}_i inhibition than bNBCe1-B currents. Here, we hypothesized that this apparent discrepancy may involve IRBIT, a previously identified NBCe1-B-interacting protein. RT-PCR, Western blot and immunofluorescence confocal microscopy revealed that IRBIT was not only expressed in the cytosol, but also colocalized with NBCe1-B in the region of plasma membranes of BPA cells. IRBIT was coimmunoprecipitated with NBCe1-B by an anti-NBCe1 antibody in bovine parotid cell lysate. Whole-cell patch-clamp experiments showed that coexpression of IRBIT lowered the Mg^{2+}_i sensitivity of bNBCe1-B currents stably expressed in HEK293 cells. Collectively, these results suggest that IRBIT may reduce the apparent affinity for Mg^{2+}_i in inhibition of NBCe1-B activity in mammalian cells.

1. Introduction

NBCe1-B, a major splice variant of the electrogenic Na^+-HCO_3^- cotransporter NBCe1 (Gene symbol; SLC4A4), with an apparent 2:1 HCO_3^--to-Na^+ coupling ratio not only regulates intracellular pH in response to an acid load in various cell types, but also plays a key role in HCO_3^- transport in epithelial cells [1,2]. However, its cellular regulatory mechanism in mammalian cells is not well understood. We previously showed that under whole-cell voltage-clamp condition, bovine NBCe1-B (bNBCe1-B) currents heterologously expressed in mammalian cells are strongly inhibited by intracellular Mg^{2+} (Mg^{2+}_i), the most abundant intracellular divalent cation [3]. The Mg^{2+}_i inhibition is likely mediated by electrostatic mechanism and is relieved by truncation of the NBCe1-B specific N-terminal region [3], which contains an auto-inhibitory domain [4,5]. Since cytosolic free Mg^{2+} concentration is kept rather constant under normal physiological conditions in most mammalian cells [6], modulation of Mg^{2+}_i sensitivity of NBCe1-B may serve as a mechanism in controlling its cotransport activity in native cellular environment.

Bovine parotid gland, which secretes continuously large volumes of HCO_3^--rich saliva [7,8], expresses NBCe1-B highly at mRNA and protein levels [3,9]. In line with the molecular evidence, acutely isolated bovine parotid acinar (BPA) cells exhibit an exceptionally large electrogenic Na^+-HCO_3^- cotransporter (NBCe) current under whole-cell voltage-clamp condition [9]. The native bovine parotid NBCe current shares common biophysical and pharmacological properties with bNBCe1-B currents [9]. Intriguingly, however, the native current has much lower sensitivity to Mg^{2+}_i inhibition compared to recombinant bNBCe1-B currents, and its Mg^{2+}_i sensitivity is rather similar to that of a mutant cotransporter lacking the NBCe1-B specific N-terminal region [3]. The reason for the apparent discrepancy remains unknown, but a potential explanation is that cytosolic regulatory protein(s) that is associated with the NBCe1-B specific N-terminal region may modulate Mg^{2+}_i sensitivity of NBCe1-B in BPA cells.

Inositol 1,4,5-triphosphate (IP_3) receptor binding protein released with IP_3 (IRBIT) was originally found to compete with IP_3 for the binding site of IP_3 receptor, a Ca^{2+}-release channel on endoplasmic reticulum [10–12]. IRBIT was identified afterward as a novel NBCe1-B-interacting protein [13] and has been also increasingly recognized to be a regulator of other ion transporting

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proteins [14]. IRBIT binds to the NBCe1-B specific N-terminal region (a.a. 1–62) and activates transport activity of NBCe1-B without changing its surface expression when they are coexpressed in *Xenopus* oocytes [13]: one determinant for IRBIT stimulation of the cotransporter activity resides in the first part of the N-terminal region (a.a. 2–16) [5]. It has been also suggested to play a role in maintaining endogenous NBCe1-B activity in mouse pancreatic ducts [15].

These findings led us to hypothesize that IRBIT may reduce the apparent affinity for $Mg^{2+}{}_i$ in inhibition of bNBCe1-B. As a first step toward addressing this hypothesis, we used RT-PCR, Western blot, immunofluorescence confocal microscopy and coimmunoprecipitation analyses to examine expression of IRBIT and its colocalization and interaction with NBCe1-B in BPA cells. We also used the standard (i.e. fast) whole-cell patch-clamp technique to investigate whether IRBIT would reduce $Mg^{2+}{}_i$ inhibition of NBCe1-B heterologously expressed in HEK293 cells.

2. Materials and methods

See Supplemental materials and methods for more details.

2.1. Animal material

Bovine parotid tissue was obtained from a local slaughterhouse (Hokkaido Hayakita Meat Inspection Center, Abira, Japan) and kept until used in a NaCl-rich solution as described elsewhere [3].

2.2. Cloning of IRBIT from bovine parotid

Using TRIzol reagent (Life Technologies, Grand Island, NY, USA), total RNA was extracted from bovine parotid cells dispersed by collagenase treatment as described previously [9,16,17]. mRNA was purified by BioMag mRNA purification kit (Polysciences, Warrington, PA, USA) following the producer's instructions. First-strand cDNA was generated from mRNA using SuperScript II RT (Life Technologies). The specific oligonucleotide primers for polymerase chain reaction (PCR) for bovine IRBIT were derived from the preliminary sequences obtained from a yeast two-hybrid screening of a bovine parotid cDNA library using the N-terminus of NBCe1-B as a bait (data not shown). IRBIT sense primer was 5′-CGG GGG ATG TCG ATG CCT GAC GCG ATG C-3′ and the antisense primer was 5′-GTG GTT CAT GTG TTC TGG TCC TTG G-3′. The size of the expected fragments of IRBIT was 1636 bp and they encode all of their open reading frames. The PCR reaction was performed with TaKaRa LA Taq (Takara Bio, Otsu, Japan). The PCR conditions were: denaturation 94 °C for 30 s; annealing 72 °C for 30 s; extension 72 °C for 1 min; 35 cycles. The PCR products obtained were resolved in 1% agarose gels with ethidium bromide.

PCR products of IRBIT amplified using a high fidelity enzyme, Pfu-Turbo (Stratagene, LaJolla, CA, USA) were cloned into the pGEM-T Easy vector. Four clones of IRBIT were sequenced, and one of the clones verified to be with no error was subcloned into pIRES2-EGFP vector (bicistronic EGFP (enhanced green fluorescence protein) expression vector, clontech, Mountain View, CA, USA) or pEGFPC1 vector (EGFP is tagged at N-terminal, clontech) and used for following experiments.

2.3. Cell transfection

HEK293 cells stably expressing NBCe1-B (pCIneo mammalian expression vector, Promega, Madison, WI, USA) were generated and maintained as described previously [9]. Using Lipofectamine 2000 (Life Technologies), the plasmid encoding IRBIT or empty vector (pIRES2-EGFP) was transiently transfected into HEK293 cells stably expressing NBCe1-B. After 4–6 h, the cells were seeded on glass coverslips. Patch-clamp recordings were made 2 days after transfection from EGFP positive single cells, which exhibited strong fluorescence.

2.4. Western blot

Isolated bovine parotid cells and HEK293 cells stably expressing NBCe1-B were transfected with each construct (i.e. bovine IRBIT subcloned into pIRES2-EGFP vector or vector alone) were lysed in a buffer (50 mM HEPES, 150 mM NaCl, 1.5 mM $MgCl_2$, 1 mM EGTA (ethylene glycol-bis(2-aminoethylether)-*N*,*N*,*N*′,*N*′-tetraacetic acid), 10% glycerol, 1.0% Triton X-100, pH 7.4) containing a protease inhibitor cocktail (1/100 dilution, Sigma–Aldrich, St. Louis, MO, USA, P8340). Bovine parotid protein (5 μg) and transfected HEK293 cell protein (20 μg) were separated by SDS–PAGE with a 3.4% stacking and 7.7% separating gel and transferred to nitrocellulose paper. After incubation in blocking buffer, the blots were treated with mouse anti-IRBIT antibody (1:5000, epitope: a.a. 1–102 of human IRBIT, which are completely conserved in bovine IRBIT, Abnova, H00010768-A01) and then with horseradish peroxidase-conjugated anti-mouse IgG antibody (1:10,000, GE Healthcare, Buckinghamshire, UK) as the secondary antibody. The signal was detected by an ECL Plus system and ECL mini-camera (GE Healthcare).

2.5. Coimmunoprecipitation

Isolated bovine parotid cells were suspended and homogenized in lysis buffer for coimmunoprecipitation (150 mM NaCl, 2 mM EDTA-2Na, 10 mM HEPES, 1.0% Triton X-100, 1% protease inhibitor cocktail (Sigma–Aldrich), pH 7.4, cooled to 4 °C). The homogenized suspension was centrifuged and the supernatant was mixed with equivalent amount of Laemmli sample buffer (Bio-Rad) with 5% β-mercaptoethanol (Sigma–Aldrich), and used as an input. The samples (1 ml) were incubated with mouse anti-IRBIT antibody (5 μl of serum, Abnova), rabbit anti-NBCe1 antibody (5 or 1 μl of serum, Chemicon, International, Temecula, CA, USA, AB3204, [3]), or control normal rabbit IgG (5 μg, Sigma–Aldrich). The mixtures were then added to the homogeneous protein A-suspension (50 μl) and incubated for overnight at 4 °C. Immunoprecipitated proteins were analyzed by SDS–polyacrylamide gel electrophoresis and Western blot.

2.6. Immunofluorescence confocal microscopy

Isolated bovine parotid cells plated on coverslips coated with poly-L-lysine were fixed in PLP fixation (2% paraformaldehyde, 75 mM lysine, 37 mM Na phosphate, 10 mM Na periodate, pH 7.4) overnight at 4 °C, and permeabilized in 1% SDS in phosphate-buffered saline (PBS) for 5 min. After blocked in image-iT signal enhancer (Life Technologies) and in PBS containing 5% normal goat serum and 0.2% BSA, the cells were then stained with mouse anti-IRBIT antibody (1:500, Abnova) and rabbit anti-NBCe1 antibody (1:1000, Chemicon) overnight at 4 °C, and Alexa 488-conjugated goat anti-mouse IgG and/or Alexa 594-conjugated goat anti-rabbit IgG (1:500, Life Technologies) were applied. As negative controls, antibodies incubated with immunizing antigen peptides were used instead of the primary antibodies. Nuclei of bovine parotid cells were additionally stained with Hoechst 33342 (2 μg/ml, Life Technologies). The coverslips were mounted on glass slides with ProLong Gold Antifade Reagent (Life Technologies) and observed using an IX-70 confocal fluorescence microscope (Olympus, Tokyo, Japan) with a 60× oil-immersion objective.

2.7. Patch-clamp experiments

The methods used for measurements of whole-cell currents were similar to those described elsewhere [3,9]. Pipette solutions (pH 7.4 with NMDG (*N*-methyl-D-glucamine)) contained (in mM): 10 BAPTA (1,2-Bis (2-aminophenoxy) ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid), 100 HEPES, 4 EDTA-2Na (ethylenediaminetetraacetic acid disodium salt), 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^{-7} to $10^{-2.5}$ M free Mg^{2+}, respectively), 0.044–0.202 $CaCl_2$ (10^{-9} M free Ca^{2+}), 13–25 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 NMDG-Cl were also varied to maintain the chloride concentration (14–16 mM Cl^-). 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 containing HCO_3^- were bubbled with 5% CO_2/95% O_2. NBCe1-B currents were evaluated as the extracellular Na^+-dependent currents at 0 mV, because under the present experimental conditions the current amplitudes were found to be almost the same as those elicited by addition of HCO_3^- to the nominally HCO_3^--free bath solutions containing Na^+ as described previously [3].

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

3. Results

3.1. IRBIT colocalizes and closely associates with NBCe1-B in bovine parotid acinar (BPA) cells

RT-PCR analysis showed that bovine parotid cells expressed transcripts of IRBIT (Fig. 1A). Subsequent analysis revealed that the nucleotide sequence of open reading frame of the IRBIT cDNA was identical to the reported sequence of bovine IRBIT (GenBank Accession No.: NM_001101052.2), and the translated amino acid sequence was also exactly the same as those reported for human (NP_006612.2) and mouse (NP_663517.2) orthologues. Protein expression of IRBIT in bovine parotid extract was confirmed by Western blotting (Fig. 1B). The protein extracts of bovine NBCe1-B-expressing HEK293 cells transiently transfected with bovine IRBIT or mock-transfected cells as a positive or negative control, respectively, were also loaded to show that the band detected in bovine parotid lane was derived from IRBIT (Fig. 1B).

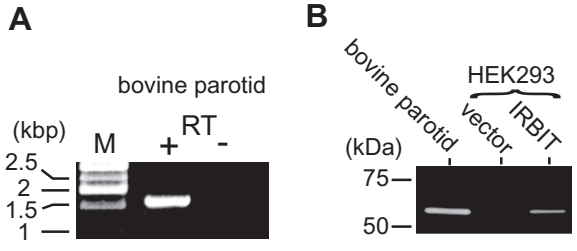

Fig. 1. Bovine parotid gland expresses IRBIT. (A) RT-PCR analysis of IRBIT expression in bovine parotid cells. RT, reverse transcription. (B) Protein expression of IRBIT was detected in bovine parotid cells or bovine NBCe1-B-expressing HEK293 cells which were transiently transfected with IRBIT. As a negative control, protein extract of bovine NBCe1-B-expressing HEK293 cells that were transfected with empty vector was loaded.

Using confocal microscopy and double immunolabeling for IRBIT and NBCe1-B, we found that isolated BPA cells expressed both IRBIT and NBCe1-B (Fig. 2). The IRBIT immunoreactivity was detected mainly in the region of plasma membranes of BPA cells and to a lesser extent in cytoplasm. IRBIT expression also overlapped with NBCe1-B, which appeared to be expressed in the region of plasma membranes, presumably in the basolateral domain assuming that the polarity of these isolated cells was kept intact (Fig. 2A). The specificity of the staining was confirmed by control antigen-preabsorption experiments (Fig. 2B). In heterologous expression experiments in HEK293 cells, IRBIT also colocalized with NBCe1-B in the region of plasma membranes of the cells, but it retained in intracellular compartments when expressed alone (Supplemental Fig. S1). Therefore, the plasma membrane expression of IRBIT may depend upon NBCe1-B expression.

Having suggested the colocalization of IRBIT and NBCe1-B in isolated BPA cells, we next examined possible interaction between IRBIT and NBCe1-B endogenously expressed in BPA cells by coimmunoprecipitation analysis. As shown in Fig. 3, IRBIT was coimmunoprecipitated with NBCe1-B by an anti-NBCe1-B antibody from bovine parotid cell lysates. Similar results were obtained in three other experiments. The precipitation of IRBIT protein was not due to non-specific binding to beads, tubes, or IgG, because IRBIT was not precipitated with control IgG (Fig. 3 IP: control rabbit IgG) and a lower concentration of anti-NBCe1 antibody, which failed to precipitate enough amount of NBCe1-B protein to follow IRBIT (Fig. 3 IP: anti NBCe1 (1 μl)). These results together with immunocytochemical findings suggest that endogenous IRBIT and NBCe1-B closely associates in isolated BPA cells.

We were unable to demonstrate that NBCe1-B was coimmunoprecipitated with IRBIT using an anti-IRBIT antibody (n = 4, data not shown). Given only a small amount of IRBIT being coimmunoprecipitated with NBCe1-B (Fig. 3), however, these results may suggest that a large fraction of IRBIT might be free form or bind to IP_3 receptor under normal conditions as described in other cell types [11]. Alternatively, this antibody might interrupt binding of NBCe1-B to IRBIT.

3.2. IRBIT may regulate intracellular Mg^{2+} sensitivity of NBCe1-B

Because the results described so far suggest that IRBIT closely associates with NBCe1-B in BPA cells, we next specifically examined whether IRBIT would reduce the sensitivity of NBCe1-B to $Mg^{2+}{}_i$ inhibition. To this end, IRBIT was transiently transfected into HEK293 cells stably expressing bovine NBCe1-B, which displayed Na^+ and HCO_3^--dependent currents (i.e. NBCe1-B currents) [9]. We took this approach, because we thought it was difficult to control and ensure the expression levels of both IRBIT and NBCe1-B relatively constant by their transient coexpression at the single-cell level. When the cells were dialyzed with a nominally Mg^{2+}-free pipette solution, NBCe1-B current densities at 0 mV of the cells transfected with vector alone (control) and IRBIT (+IRBIT) were 5.5 ± 1.3 pA/pF (n = 8) and 7.6 ± 2.7 pA/pF (n = 9), respectively (Fig. 4A and B). When free Mg^{2+} concentration of the intracellular solution ($[Mg^{2+}]_i$) was 10^{-6} M, NBCe1-B current densities of the control cells were decreased to 2.3 ± 1.0 pA/pF at 0 mV (n = 8), whereas the values of the IRBIT transfected cells remained almost unchanged (8.1 ± 1.5 pA/pF (n = 9) at 0 mV, Fig. 4A and B). As shown in Fig. 4C, coexpression of IRBIT caused a rightward shift of the dose-inhibition curve for $Mg^{2+}{}_i$, but under the present experimental conditions NBCe1-B current densities of control and IRBIT-transfected cells were comparable at $Mg^{2+}{}_i$ concentration higher than 1 mM or lower than 0.1 μM, respectively. The apparent K_i value and pseudo Hill coefficient of $Mg^{2+}{}_i$ at 0 mV were estimated to be 5.6×10^{-7} M and 0.71 (control) or 1.1×10^{-5} M and 1.24 (+IRBIT), respectively.

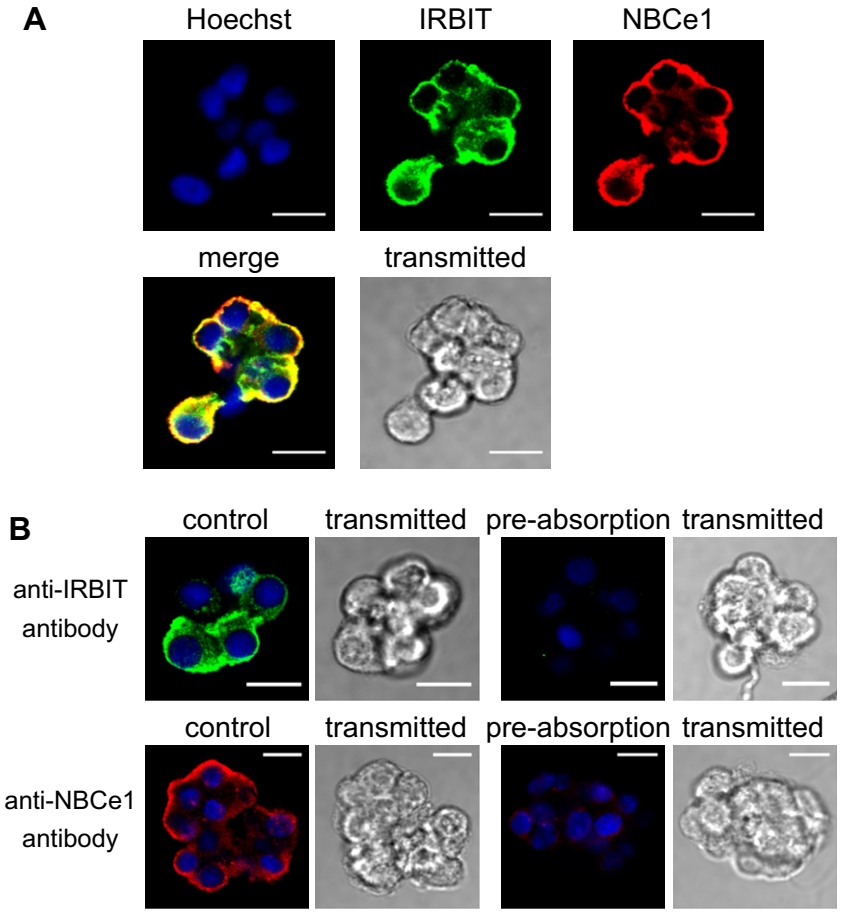

Fig. 2. IRBIT and NBCe1-B are coexpressed in bovine parotid acinar cells. (A) The double staining images of IRBIT (Green) and NBCe1-B (Red) in bovine parotid acinar cells were obtained by confocal microscopy. Nuclei (Blue) were stained with Hoechst 33342. (B) Specificities of the antibodies were confirmed by pre-absorbing the antibodies with corresponding antigen peptides. Both images of bovine parotid acinar cells stained with the antibodies (control) and absorbed antibodies (pre-absorption) were taken under the same conditions of confocal microscopy. Scale bars: 10 μm (A and B).

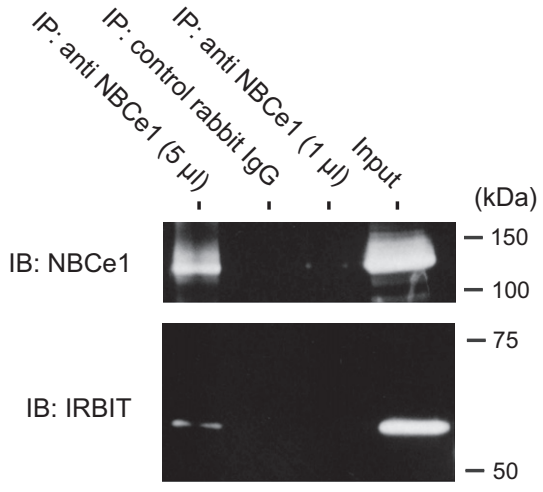

Fig. 3. IRBIT is coimmunoprecipitated with NBCe1-B from bovine parotid lysate. Bovine parotid cell lysates were immunoprecipitated with anti-NBCe1 or control antibody and were probed with anti-NBCe1 (upper) or anti-IRBIT (lower) antibody to detect NBCe1-B or IRBIT. Total lysate was also loaded (input). IP, immunoprecipitation; IB, immunoblot.

4. Discussion

Here, we show that IRBIT colocalizes and closely associates with NBCe1-B in isolated BPA cells, where endogenous NBCe1-B-like currents are much less sensitive to $Mg^{2+}{}_i$ inhibition compared to recombinant bNBCe1-B currents [3], and that coexpression of IRBIT relieves $Mg^{2+}{}_i$ inhibition of bNBCe1-B currents in HEK293 cells. These results suggest that IRBIT may be a cytosolic factor that modulates the apparent affinity for $Mg^{2+}{}_i$ in inhibition of bNBCe1-B in mammalian cells.

In the present study, we found that bNBCe1-B currents stably expressed in HEK293 cells are inhibited by increasing $Mg^{2+}{}_i$, and the $Mg^{2+}{}_i$ sensitivity is evidently reduced (about a 20-fold decrease) by coexpression of IRBIT. Although cell surface localization of NBCe1-B has been reported to be unaffected by IRBIT coexpression in HEK293 cells (see Discussion in Ref. [13]), a recent study has shown that IRBIT can increase NBCe1-B activity in part by stabilizing expression of NBCe1-B at the plasma membrane of these cells under some experimental conditions [18]. Thus, we cannot completely rule out the possibility that a small increase (by 38%) of NBCe1-B current density upon IRBIT coexpression under the virtually Mg^{2+}-free condition might reflect changes in surface expression level of NBCe1-B.

In the course of these experiments, we noticed that the sensitivity to $Mg^{2+}{}_i$ inhibition ($K_i = 5.6 \times 10^{-7}$ M) of NBCe1-B stably expressed in HEK293 cells was higher than that ($K_i = 1.2 \times 10^{-5}$ M)

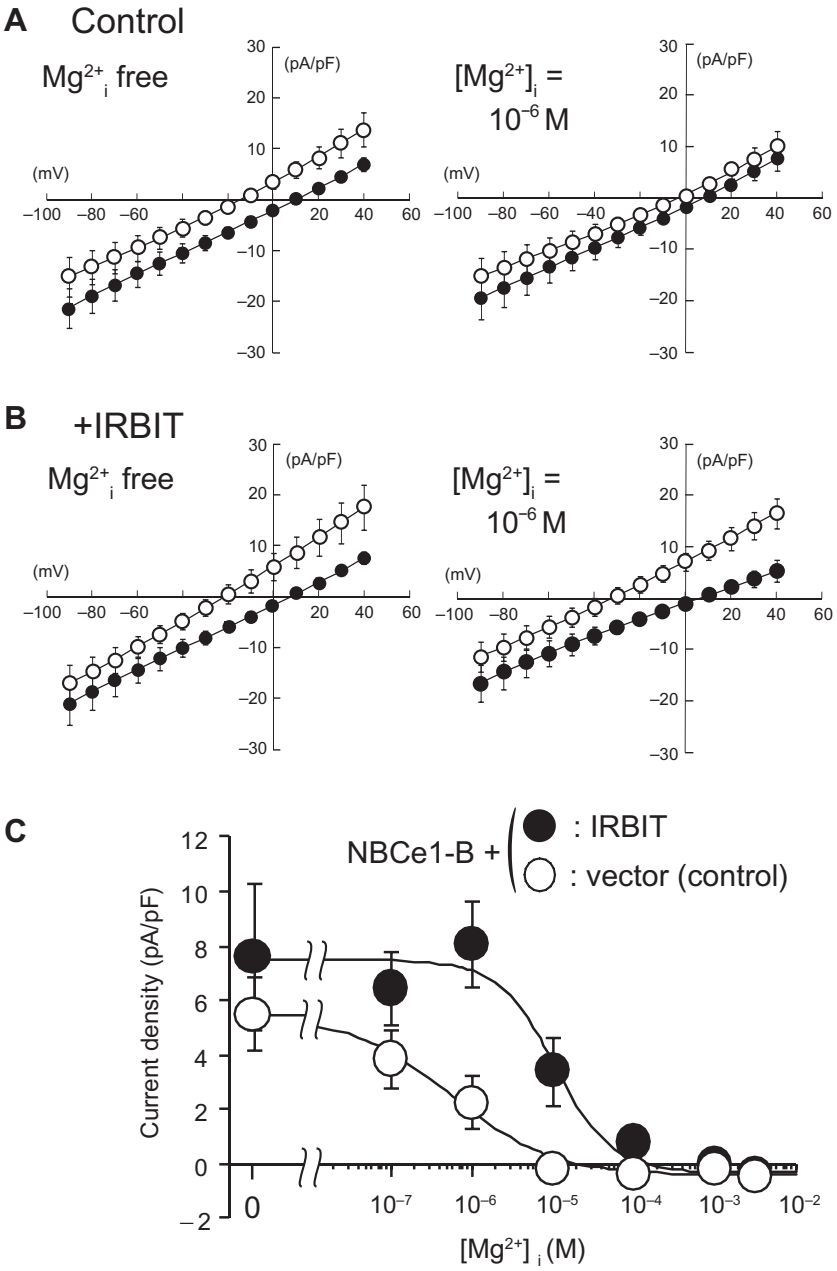

Fig. 4. Coexpression of IRBIT relieves inhibition of NBCe1-B by intracellular Mg^{2+}. (A and B) Whole-cell current density evoked by a ramp pulse protocol was recorded in bath solutions containing HCO_3^- in the presence (open circles) or absence (solid circles) of Na^+. Data are shown at 10 mV intervals from −90 to 40 mV. An empty vector (A, pIRES2-EGFP) or a vector encoding IRBIT (B) was transiently transfected into HEK293 cells stably expressing NBCe1-B (pCIneo vector). Pipette solutions contained no Mg^{2+} (left) or 10^{-6} M Mg^{2+} (right). Shown are mean ± SE (n = 8 or 9). (C) Comparison of dose–response curves for the inhibition of NBCe1-B current by increasing free $Mg^{2+}{}_i$ concentrations in the cells transiently transfected with vector alone (open circle) and with IRBIT (solid circle). Shown are mean ± SE (n = 8–10). The lines were fits to the Hill equation.

reported for NBCe1-B transiently expressed in HEK293 cells [3]. The reason for the discrepancy remains unclear. When heterologously over-expressed, NBCe1-B would overload both Na^+ and HCO_3^- into the cells and thus toxic to the cells. The stably transfected cells might have adapted themselves to these conditions through making NBCe1-B highly sensitive to $Mg^{2+}{}_i$ (e.g. via modification of NBCe1-B itself or induction of regulatory factor(s)). Such a higher $Mg^{2+}{}_i$ sensitivity would also provide an explanation for both low incidence and variable stability (i.e. rapid rundown of the current) of the cotransporter currents recorded from these cells with pipette solutions containing approximately 0.5 mM free Mg^{2+} as we reported previously [9]. Besides, we found that even when IRBIT was coexpressed, the NBCe1-B current still had a higher sensitivity to $Mg^{2+}{}_i$ (Fig. 4C: $K_i = 1.1 \times 10^{-5}$ M) than native NBCe1-B-like current in BPA cells ($K_i = 8.2 \times 10^{-4}$ M, [3]). The findings may suggest that other factor(s), which modulates $Mg^{2+}{}_i$ sensitivity of NBCe1-B might exist in BPA cells. Further studies will be indeed required to establish primary cultures of BPA cells and to investigate how knock-down of endogenous IRBIT affects the properties of NBCe1-B in these cells and whether other interacting protein(s) that modulates $Mg^{2+}{}_i$ sensitivity of the cotransporter would exist in BPA cells.

At this stage, we do not know how IRBIT relieves $Mg^{2+}{}_i$ inhibition of NBCe1-B. The $Mg^{2+}{}_i$ inhibition has been shown to be

mimicked by polyvalent cationic compounds such as neomycin [3] and spermine (Yamaguchi and Ishikawa, unpublished data), implying possible involvement of electrostatic mechanism in the inhibition [3]. Thus, most simply, a Mg^{2+}-binding site might exist in the NBCe1-B specific N-terminal region that contains acidic amino acids, and IRBIT might mask the site. However, it does not need to be the case that such a putative Mg^{2+}-binding site in NBCe1-B is necessary for binding of IRBIT. In fact, phosphorylation of serines in the N-terminal PEST motif of IRBIT is shown to be required for its binding to NBCe1-B, suggesting that the negatively charged and phosphorylated N-terminal region of IRBIT may be important in the interaction with the NBCe1-B-specific N-terminal region, which contains basic amino acids [13,15].

We have previously reported that compared to wild type NBCe1-B, a mutant cotransporter lacking the NBCe1-B-specific N-terminal region was less sensitive to $Mg^{2+}{}_i$ ($K_i = 2.9 \times 10^{-4}$ M), a K_i value also falling within a range to that inhibits native NBCe1-B in BPA cells [3]. Thus, $Mg^{2+}{}_i$ inhibition with higher K_i of the mutant was probably mediated by mechanism(s) distinct from that can be affected by IRBIT. It has been reported that phosphatidylinositol 4,5-bisphosphate (PIP_2) stimulates NBCe1-A activity expressed in *Xenopus* oocytes [19]. In that report, NBCe1-A currents were shown to be run-down by addition of 2 mM Mg^{2+} to the bath solution exposing cytosolic surface of the membrane excised from *Xenopus* oocytes expressing NBCe1-A [19]. The authors argued that the Mg^{2+}-induced run-down may be mediated by Mg^{2+}-dependent PIP_2 phosphatase, because application of PIP_2 recovered the NBCe1-A currents. They also noticed that Mg^{2+} inhibits NBCe1-A directly, although the dose-inhibition relationship for $Mg^{2+}{}_i$ was not shown (See Discussion of Ref. [19]). Therefore, the inhibition by high $Mg^{2+}{}_i$ concentrations observed for the N-terminally truncated NBCe1-B and for the native bovine parotid NBCe1-B could be due to PIP_2 depletion by dephosphorylation or due to direct competition with Mg^{2+}, because PIP_2 binding to some membrane ionic channels is suggested to be mediated by electrostatic mechanism through its negative charge of acidic head [20].

From a physiological view point, IRBIT interaction with NBCe1-B in unstimulated BPA cells would be advantageous to maintain continuous basolateral influx of HCO_3^-, which might support resting secretion that is observed especially in ruminant parotid [7,21,22]. In fact, endogenous NBCe current can contribute significantly to resting membrane potential in BPA cells [9]. Given that physiological concentrations of IP_3 release IRBIT from IP_3 receptor [11], muscarinic receptor activation by acetylcholine, a major secretagogue of parotid salivary secretion [23] might make more IRBIT available for binding to NBCe1-B and thus enhance its cotransport activity during stimulated fluid secretion in BPA cells, although such a situation depends upon how fast IRBIT diffuses in the cytosol.

In conclusion, the present work suggests a potential role of IRBIT in releasing $Mg^{2+}{}_i$ inhibition of NBCe1-B and thus may provide an insight into the mechanism(s) in controlling and diversifying NBCe1-B activity in mammalian cells.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2012.06.127.

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