



Title	AHCYL2 (long-IRBIT) as a potential regulator of the electrogenic Na ⁺ -HCO ₃ ⁻ cotransporter NBCe1-B
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SUPPLEMENTAL MATERIALS AND METHODS

Ethical approval

All experiments were performed in accordance with guidelines from and a protocol approved by the Committee on Animal Experimentation, Graduate School of Veterinary Medicine, Hokkaido University.

Animal Material

No animals were killed specifically for the current study. For mRNA extraction for yeast two-hybrid library production, bovine parotid was obtained from a cattle that had been humanely euthanized for educational purposes (unrelated to the present study) at Faculty of Veterinary Medicine in Hokkaido University and was stored in RNA-later (Life Technologies, Grand Island, NY, USA) at -80°C until use. For other experiments, bovine parotid tissue was obtained from a local slaughterhouse (Hokkaido Hayakita Meat Inspection Center, Hayakita, Japan). After the tissue was removed from the slaughtered animal, it was kept at 4°C in a standard NaCl-rich bath solution as described previously (Yamaguchi & Ishikawa 2005). Bovine parotid cells were dispersed by collagenase treatment as described previously (Yamaguchi & Ishikawa 2005).

Yeast two-hybrid library production

The MATCHMAKER library construction and screening kit (Takara Bio, Otsu, Japan) was used to construct a bovine parotid cDNA library. In brief, the first-strand cDNA was made using a random primer. Double-strand cDNA was amplified by

long-distance polymerase chain reaction (PCR) and cloned into pGADT7-Rec, encoding GAL4 DNA-binding domain (BD), by recombination-mediated cloning in the yeast strain AH109.

Yeast two-hybrid screening

Bovine NBCe1-B was cloned from bovine parotid as described previously (Yamaguchi & Ishikawa 2005). The bait was constructed by cloning the cytosolic N-terminal region (amino acids 1 – 444) of the bovine NBCe1-B (NBCe1-B-Nt) into the pGBKT7 vector, encoding GAL4 DNA-activation domain (AD), between EcoRI and Sall sites. The pGBK-NBCe1-B-Nt construct was transformed into the yeast strain Y187 and confirmed that it caused little autoactivation of *ADE2*, *HIS3* and *MEL1* reporters. The BD (cDNA library) and AD (NBCe1-B-Nt) constructs were introduced together through yeast mating, and positive transformants were identified by growth on synthetic dextrose (SD) quadruple essential amino acids dropout (QDO) (–Ade/–His/–Leu/–Trp) plates. The positive clones expressing *MEL1* (α -galactosidase) were further characterized by blue color in the presence of 5-bromo-4-chloro-3-indolyl- α -D-galactopyranoside (*X- α -Gal*) (Takara Bio) and grouped by restriction mapping of PCR products generated for insert DNAs. Representative clones were sequenced and blasted against GenBank sequences.

The interactions of NBCe1-B-Nt and IRBIT or AHCYL2 were re-tested using cDNA which was cloned from bovine parotid cells with high fidelity DNA polymerase as described in the next section. AH109 yeast cells were co-transfected with pGBKT7 vectors, which contained NBCe1-B-Nt, or no insert and pGADT7 vectors, which

contained IRBIT, AHCYL2, or no insert. The activation of *ADE2*, *HIS3* and *MEL1* reporters by interacting proteins were tested by growth on SD QDO and activity of α -galactosidase resulted in blue color of colony in X- α -Gal. As controls, we also tested growth on SD QDO and activity of α -galactosidase of two strains, which were produced by co-transformation with pGADT7-RecT along with pGBKT7-53 (positive control) or pGBKT7-Lam (negative control). The plate was photographed after 3 days of incubation at 30 °C.

Cloning of AHCYL2 from bovine parotid

mRNA was extracted from bovine parotid cells dispersed by collagenase treatment as described above using TRIzol reagent (Life Technologies) and 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 PCR for bovine AHCYL2 were derived from the sequences obtained from yeast two-hybrid screening. The AHCYL2 sense primer was 5'- GCG GTG ATG TCG GTG CAG GTC GTG T -3' and the antisense primer was 5'- CCA GAA AAC CCC AGA AAA CAA GGA G -3'. The size of the expected fragments of AHCYL2 was 2210 bp, which encoded their all open reading frames. The PCR reaction was performed with TaKaRa LA Taq (Takara Bio). The PCR conditions were: denaturation 94 °C for 30 sec; annealing 64 °C for 30 sec; extension 72 °C for 3 min; 35 cycles. The PCR products obtained were resolved in 1% agarose gels with ethidium bromide.

PCR products of AHCYL2 amplified using a high fidelity enzyme, PrimeSTAR HS

DNA Polymerase (Takara Bio) or Pfu-Turbo (Stratagene, LaJolla, CA, USA), were cloned into the pGEM-T Easy vector. Seven clones of AHCYL2 were sequenced, respectively, and the clones without error were subcloned into pGADT7, pIRES2-EGFP (bicistronic enhanced green fluorescence protein (EGFP) expression vector), or pEGFP-C1 (expression vector encoding fusion protein tagged with EGFP at its N-terminus) vector and used for following experiments.

Western Blot

Bovine parotid cells isolated as described above were incubated in lysis buffer (50 mM HEPES, 150 mM NaCl, 1.5 mM MgCl₂, 1 mM EGTA (ethylene glycol - bis (2 - aminoethylether) - N,N,N',N' - tetraacetic acid), 10% glycerol, 1.0% Triton X-100) containing a protease inhibitor cocktail (1/100 dilution, Sigma P8340) for 30 min. The HEK293 cells stably expressing NBCe1-B were transfected with each construct (i.e. bovine AHCYL2 or IRBIT subcloned into pIRES2-EGFP vector or vector alone) as described before, and the cells were scraped in lysis buffer containing the protease inhibitor cocktail. These cell lysates were centrifuged at 13,000 × g for 5 min at 4 °C. The supernatants were diluted with equal amount of Laemmli sample buffer (Bio-Rad, Hercules, CA, USA) containing 5% β-mercaptoethanol and incubated for 15 min at 50 °C. Their protein concentrations were determined colorimetrically using a RC DC protein Assay (Bio-Rad) with bovine serum albumin as a standard. 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 the diluted rabbit anti-NBCe1 antibody (1:10,000, epitope: a.a. 990-1035 of rat NBCe1-A

(kNBC), most of which are also conserved in bovine NBCe1-B, Chemicon International, Temecula, CA, USA), rabbit anti-AHCYL2 antibody (1:10,000, epitope: a.a. 1-50 of human AHCYL2, most of which are also conserved in bovine AHCYL2, Bethyl laboratories, Inc., Montgomery, TX, USA), or mouse anti-IRBIT antibody (1:5,000, epitope: a.a. 1-102 of human IRBIT, which are completely conserved in bovine IRBIT, Abnova, Taipei, Taiwan) and then with horseradish peroxidase-conjugated anti-rabbit or 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).

Co-immunoprecipitation

Isolated bovine parotid cells were suspended in lysis buffer for co-immunoprecipitation (150 mM NaCl, 2 mM EDTA-2Na, 10 mM HEPES, 1.0% Triton X-100, 1% protease inhibitor cocktail (Sigma), pH 7.4, cooled to 4 °C) to achieve a concentration of 20 mg/ml and homogenized by vortex for 10 sec and incubation for 30 min at 4 °C on a rocking platform. The homogenized suspension was centrifuged at $12,000 \times g$ for 10 min at 4 °C to remove debris and the supernatant was transferred to fresh microfuge tubes. The resultant supernatant was mixed with equivalent amount of Laemmli sample buffer (Bio-Rad) with 5% β -mercaptoethanol (Sigma), heated at 50 °C for 15 min, and used as an input. To reduce background due to non-specific adsorption of irrelevant cellular proteins to protein A-agarose, 50 μ l of the homogeneous protein A-agarose suspension (25 μ l bed volume) was mixed with the sample (1 ml) and incubate for 3 h at 4 °C on a rocking platform. The beads were removed by centrifugation and supernatants were transferred to fresh tubes. By gentle rocking for 1

h at 4 °C on a rocking platform, the samples (1 ml) were incubated with the following antibodies; rabbit anti-AHCYL2 antibody (5 µg, Bethyl), mouse anti-IRBIT antibody (5 µl of serum, Abnova), rabbit anti-NBCe1 antibody (5 µl of serum, Chemicon), control normal mouse IgG (5 µg, Sigma), or control normal rabbit IgG (5 µg, sigma). The mixtures were added to the homogeneous protein A-suspension (50 µl) and incubated for overnight at 4 °C on a rocking platform. The complexes were collected by centrifugation at 12,000 × g for 20 s in a microfuge tube at 4 °C. After three washing steps with lysis buffer, immunoprecipitated proteins were eluted with Laemmli sample buffer with 5% β-mercaptoethanol by heating to 100 °C for 3 min, analyzed by SDS-polyacrylamide gel electrophoresis and western blot.

Immunofluorescence confocal microscopy

Isolated bovine parotid cells plated on glass coverslips coated with poly-L-lysine were washed once in phosphate-buffered saline (PBS), fixed in Paraformaldehyde/Lysine/Periodate (PLP) fixative (2% paraformaldehyde, 75 mM lysine, 37 mM Na phosphate, 10 mM Na periodate, pH 7.4) overnight at 4 °C, permeabilized with 1% SDS in PBS for 5 min, and washed again. After blocked in image-iT signal enhancer (Life Technologies) for 30 min and in PBS containing 5% normal goat serum and 0.2% BSA for 60 min, the cells were then stained with rabbit anti-AHCYL2 antibody (1 µg/ml, Bethyl), mouse anti-IRBIT antibody (1:500, Abnova), and/or mouse anti-NBCe1 antibody (1:1,000, Abnova, H00008671-A01). As negative controls, antibodies incubated with immunizing antigen peptides for two hours at room temperature were used instead of the primary antibodies. Following four times 5-min PBS washes, Alexa 488-conjugated goat anti-mouse IgG and/or Alexa 594-conjugated

goat anti-rabbit IgG (1:500, Life Technologies) were applied for 1 hr at room temperature. Following four times 5-min PBS washes, nuclei of the cells were additionally stained with Hoechst 33342 (2 $\mu\text{g/ml}$, Life Technologies) for 15 min and washed. The coverslips were mounted with ProLong Gold Antifade Reagent (Life Technologies) and observed under IX-70 confocal fluorescence microscopy (Olympus, Tokyo, Japan) with a 60 \times oil-immersion objective.

Electrophysiology

Whole-cell patch-clamp experiments were performed as described elsewhere (Yamaguchi & Ishikawa 2008; Yamaguchi & Ishikawa 2012). In brief, an Axopatch-1D patch-clamp amplifier and the pCLAMP6 software (Axon Instruments, Union City, CA, USA) were used to perform voltage clamp, data storage, and analysis. A reference Ag-AgCl electrode was connected to the bath solution via an agar bridge filled with a NaCl-rich solution. The whole-cell currents were filtered at 500 Hz with an internal four-pole Bessel filter, and sampled at 2 kHz. The cell potential was held at -80mV and varied from -100 to $+50$ mV (with ramp pulse) over duration of 800 ms every 10 sec. The pipette potential was corrected for the liquid junction potential between the pipette solution and the external solution, and between the external solution and the agar bridge as described elsewhere (Barry & Lynch 1991; Neher 1992). The average series resistance of HEK293 cells, which was not electrically compensated, was 25.1 ± 0.5 M Ω ($n = 56$). The cell capacitance was 14.5 ± 0.7 pF ($n = 56$). All experiments were performed at room temperature.

Pipette solutions (pH = 7.4 adjusted 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 Na-glutamate, 0-16.3 MgCl₂ (no MgCl₂ or appropriate amounts of MgCl₂ were added to yield Mg²⁺-free or 10⁻⁶ to 10⁻² M free Mg²⁺, respectively), 0.037 - 0.202 CaCl₂ (10⁻⁹ M free Ca²⁺), 32-43 NMDG-glutamate, and 0-15 NMDG-Cl. Free Mg²⁺ and Ca²⁺ 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 within a range from 15 mM to 33 mM. Standard bath solution contained (in mM, pH = 7.4 adjusted with NMDG) 120 Na-glutamate, 25 NaHCO₃, 10 HEPES, 1 MgCl₂, and 1 CaCl₂. Na⁺-free solution containing HCO₃⁻ was made by replacing Na⁺ with 120 mM NMDG and 25 mM choline. The solutions having HCO₃⁻ were bubbled with 5% CO₂/95% O₂. Under the present standard “fast” whole-cell conditions, where the cells were dialyzed with the pipette solutions (pH 7.4) containing 100 mM Hepes, cytosolic pH change due to the cotransporter-mediated HCO₃⁻ fluxes should have been minimized. NBCe1-B currents were defined as the extracellular Na⁺-dependent currents at 0 mV as described previously (Yamaguchi & Ishikawa 2008; Yamaguchi & Ishikawa 2012).

Curve fitting

The inhibitory constant (K_i) of Mg²⁺ 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, and I_{base} is the baseline of currents. The fits were done using Igor Pro (Wave Metrics, Portland, OR, USA).

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

SUPPLEMENTAL REFERENCES

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