Mechanism of acid adaptation of a fish living in a pH 3.5 lake

Taku Hirata,1 Toyoji Kaneko,2 Toshihiro Ono, Takeru Nakazato,1 Norihisa Furukawa,1 Sanae Hasegawa,2 Shigeo Wakabayashi,3 Munekazu Shigekawa,3 Min-Hwang Chang,4 Michael F. Romero,4 and Shigehisa Hirose1

1Department of Biological Sciences, Tokyo Institute of Technology, Yokohama 226-8501; 2Ocean Research Institute, University of Tokyo, Tokyo 164-8639; 3Department of Molecular Physiology, National Cardiovascular Center Research Institute, Osaka 565-8565, Japan; and 4Department of Physiology and Biophysics, Case Western Reserve University, Cleveland, Ohio 44106

Address for reprint requests and other correspondence: S. Hirose, Dept. of Biological Sciences, Tokyo Institute of Technology, 4259 Nagatsuta-cho, Midori-ku, Yokohama 226-8501, Japan (E-mail: shirose@bio.titech.ac.jp)

Submitted 13 May 2002; accepted in final form 14 January 2003

Hirata, Taku, Toyoji Kaneko, Toshihiro Ono, Takeru Nakazato, Norihisa Furukawa, Sanae Hasegawa, Shigeo Wakabayashi, Munekazu Shigekawa, Min-Hwang Chang, Michael F. Romero, and Shigehisa Hirose. Mechanism of acid adaptation of a fish living in a pH 3.5 lake. Am J Physiol Regul Integr Comp Physiol 284: R1199–R1212, 2003. First published January 16, 2003; 10.1152/ajpregu.00267.2002.—Despite unfavorable conditions, a single species of fish, Osorezan dace, lives in an extremely acidic lake (pH 3.5) in Osorezan, Aomori, Japan. Physiological studies have established that this fish is able to prevent acidification of its plasma and loss of Na⁺. Here we show that these abilities are mainly attributable to the chloride cells of the gill, which are arranged in a follicular structure and contain high concentrations of Na⁺/H⁺ exchanger (NHE3), type 1 Na⁺-HCO₃⁻ cotransporter, and aquaporin-3, all of which are upregulated on acidification. Immunohistochemistry established their chloride cell localization, with NHE3 at the apical surface and the others localized to the basolateral membrane. These results suggest a mechanism by which Osorezan dace adapts to its acidic environment. Most likely, NHE3 on the apical side excretes H⁺ in exchange for Na⁺, whereas the electrogenic type 1 Na⁺-HCO₃⁻ cotransporter in the basolateral membrane provides HCO₃⁻ for neutralization of plasma using the driving force generated by Na⁺-K⁺-ATPase, carbonic anhydrase II, and increased expression of glutamate dehydrogenase was also observed in various tissues of acid-adapted dace, suggesting a significant role of ammonia and bicarbonate transport in acid adaptation.

aquaporin; carbonic anhydrase; glutamine catabolism; sodium/bicarbonate cotransporter; sodium/proton exchanger

FISH CAN NORMALLY SURVIVE only in neutral or close-to-neutral water. A few exceptions are the Lake Magadi tilapia Oreochromis alcalicus grahami, the only fish in the alkaline lake (pH 10) (51), the Amazonian tambaqui Colossoma macropomum, which migrates between circumneutral water and dilute acidic blackwater of the Rio Negro (70, 71), and the Osorezan dace Tribolodon hakonensis, a cyprinid teleost, lives and grows in the extremely acidic (pH 3.4–3.8) Lake Osorezan and migrates to neutral streams for spawning. Lake Osorezan (formally Lake Usoriyama) is located in the Shimokita Peninsula in the northern part of Honshu, the mainland of Japan. The mechanism of adaptation of the Lake Magadi tilapia to the alkaline condition is well understood (33, 51), but the mechanism of acid adaptation of the Amazonian tambaqui and Osorezan dace has not been clarified at the molecular level.

The gill epithelium comprises five types of cells (pavement cells, mucous cells, neuroepithelial cells, undifferentiated cells, and chloride cells) and is the site of respiratory gas exchange and ion regulation (18, 26, 34, 44, 68). The gill is also believed to possess a mechanism for preventing metabolic acidosis and alkalosis (7, 8, 18). Long-term exposure of teleost fish to acidic water affects the number, distribution, and morphology of chloride cells in the gill (29, 30, 65) and can result in acute acidification of plasma and loss of NaCl, eventually leading to death. However, in Osorezan dace, these initial effects on plasma pH and Na⁺ concentration are rapidly corrected, allowing the fish to survive (25). To clarify the molecular mechanism underlying the acid tolerance, we considered it useful to identify proteins with expressions that are markedly increased in acid-adapted Osorezan dace and performed subtraction cloning. Among several clones isolated from a cDNA library enriched in acid-inducible messages, there was a clone encoding the Na⁺/H⁺ exchanger (NHE3), type 1 Na⁺-HCO₃⁻ cotransporter in the basolateral membrane provides HCO₃⁻ for neutralization of plasma using the driving force generated by Na⁺-K⁺-ATPase, carbonic anhydrase II, and increased expression of glutamate dehydrogenase was also observed in various tissues of acid-adapted dace, suggesting a significant role of ammonia and bicarbonate generation by glutamine catabolism.

The costs of publication of this article were defrayed in part by the payment of page charges. The article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.
acid-base balance in a pH 3.5 environment by excreting H⁺ and transporting HCO₃⁻ into the blood for neutralization of plasma and that these functions are supported by the driving force generated by the Na⁺-K⁺-ATPase. We further hypothesized that carbonic anhydrase (CA), which catalyzes the reversible hydration of CO₂ (CO₂ + H₂O ↔ H⁺ + HCO₃⁻) and in mammals contributes to control of pH and ion transport in the stomach, pancreas, and kidney (56), is also required for these processes in Osorezan dace. We therefore cloned the cDNAs for CA and candidate H⁺ and HCO₃⁻ transporters from this fish species and determined the localization and regulation of expression of the corresponding genes. Our results indicate that a type II CA (CA-II), a type 1 Na⁺-HCO₃⁻ cotransporter (NBC1), and a type 3 Na⁺/H⁺ exchanger (NHE3) are indeed highly expressed, with the expected polarities, in chloride cells of the acid-adapted Osorezan dace, compared with the low levels of expression apparent in fish aclimatized to neutral water.

This regulatory system is somewhat reminiscent of that working in the mammalian renal proximal tubule cells to defend against metabolic acidosis by reabsorbing bicarbonate and returning it to the buffer pool of the body (20, 61). Other important mechanisms operating in the proximal tubules are ammoniagenesis and gluconeogenesis, which generate two ammonia and two bicarbonate from the metabolism of glutamine and facilitate the excretion of acids and partially restore normal acid-base balance (6, 10). We therefore further examined the contribution of glutamate catabolism to systemic pH homeostasis and found that expression of glutamate dehydrogenase (GDH), a mitochondrial matrix enzyme that catalyzes oxidative deamination of glutamate and enhances ammonia production, is also markedly elevated in virtually all tissues when Osorezan dace are adapted to neutral water. We therefore further hypothesized that carbonic anhydrase (CA), which catalyzes the reversible hydration of CO₂ (CO₂ + H₂O ↔ H⁺ + HCO₃⁻) and in mammals contributes to control of pH and ion transport in the stomach, pancreas, and kidney (56), is also required for these processes in Osorezan dace. We therefore cloned the cDNAs for CA and candidate H⁺ and HCO₃⁻ transporters from this fish species and determined the localization and regulation of expression of the corresponding genes. Our results indicate that a type II CA (CA-II), a type 1 Na⁺-HCO₃⁻ cotransporter (NBC1), and a type 3 Na⁺/H⁺ exchanger (NHE3) are indeed highly expressed, with the expected polarities, in chloride cells of the acid-adapted Osorezan dace, compared with the low levels of expression apparent in fish aclimatized to neutral water.

Materials and methods

Materials. The Osorezan dace were caught by netting in a neutral stream during the spawning period (June) and acclimated in a 1-ton freshwater tank for >1 mo before use. The water chemistry was as follows: pH 3.5–3.7, 0.92 mM Na⁺, 0.84 mM Cl⁻, 0.06 mM K⁺, 0.21 mM Ca²⁺, 0.07 mM Mg²⁺, and 0.49 mM SO₄²⁻ for lake water and pH 6.8–7.2, 0.40 mM Na⁺, 0.25 mM Cl⁻, 0.02 mM K⁺, 0.11 mM Ca²⁺, 0.05 mM Mg²⁺, and 0.03 mM SO₄²⁻ for neutral stream water. Control dace (25) were obtained from the Freshwater Fisheries and Environment Division, National Research Institute of Fisheries Science (Ueda, Nagano, Japan). Blood sampling and measurements of blood pH and plasma concentration of Na⁺ were performed as described previously (25).

Transfer experiment. To examine the time-course changes in the mRNA levels of vacuolar-type H⁺-translocating ATPase (V-ATPase) B subunit, Na⁺-K⁺-ATPase, CA-II, NHE3, NBC1, aquaporin-3 (AQp3), and GDH after transfer to acidic water, neutral water-adapted dace (n = 40) were transferred directly to acidic lake water, and the gills were sampled from 10 dace on days 0, 1, 2, 5, and 7 for Northern blot analysis.

Molecular cloning. Fragments of B subunit of V-ATPase, CA-II, NHE3, NBC1, and GDH cDNAs were isolated from Osorezan lake by RT-PCR with gene-specific primers as follows: 5’-ATGCGCCGACAGA-3’ (sense) and 5’-ATTCCCTGCTCTGGATG-3’ (antisense) for B subunit of V-ATPase, 5’-GTTTCTGCYATCCGATGGG-3’ (sense) and 5’-GAGTTATCAGGATGATGATG-3’ (antisense) for CA-II, 5’-GGGCTGCTGCGGCTTTGAG-3’ (sense) and 5’-GAGCGCCTAAGGCACG-3’ (antisense) for NHE3, 5’-GARAATGNTTARAGGNGG-3’ (sense) and 5’-GCTAGGACNTAYARTYGGNGT-3’ (antisense) for NBC1, and 5’-ATGACNTAYAARTYGGNGT-3’ (sense) and 5’-GCRTANGTAYATCISCATC-3’ (antisense) for GDH, where R is A or G, Y is T or C, S is C or G, N is C, G, A, or T, K is G or T, W is A or T, D is A, G, or T, H is A, C, or T, and L is inostine. Fragments of cDNAs for the α-subunit of Na⁺-K⁺-ATPase, NHE3, NBC1, aquaporin-3 (AQP3), and GDH after transfer were isolated into pGEM1Z vector (Promega). The sequence of nucleotides 767–1147 of NHE3 cDNA, nucleotides 173–480 of V-ATPase B subunit cDNA, nucleotides 315–173 of GDH cDNA, and nucleotides 1–370 of the Na⁺-K⁺-ATPase α-subunit cDNA. RNA molecular weight marker II (1.5–6.9 kb; Roche) was used as the size marker.

Cell culture and cDNA transfection. PS120 cells (47) were cultured in Dulbecco’s modified Eagle’s medium (Life Technologies) supplemented with 7.5% (vol/vol) fetal calf serum at 37°C in an atmosphere of 95% air-5% CO₂. The cloned dace NHE3 (dNHE3) and dace NBC1 (dNBC1) cDNAs were introduced into the pECE vector and transfected into PS120 cells (5 × 10⁶ cells/10-cm dish) by means of the calcium phosphate coprecipitation technique. Cell populations that stably expressed dNHE3 and dNBC1 were selected by means of the repetitive H⁺-killing selection procedure (62). In the case of dNBC1, recovery medium contained 2 mM NaHCO₃.

Measurement of ²²Na⁺ uptake for dNHE3 activity. The rates of ethylisopropylamiloride (EIPA)-sensitive ²²Na⁺ uptake by PS120 cells expressing dNHE3 were measured using cells clamped at intracellular pH (pHi) 5.6 by the standard
K\(^+\)/nigericin method. For measurement of \(^{22}\)Na\(^+\) uptake, cells were preincubated for 1 h in NH\(_4\)Cl medium (50 mM NH\(_4\)Cl, 1.8 mM CaCl\(_2\), 1 mM MgCl\(_2\), 5 mM KCl, 70 mM choline chloride, 5 mM glucose, and 15 mM HEPES-Tris, pH 7.4), washed twice with choline chloride medium (1.8 mM CaCl\(_2\), 1 mM MgCl\(_2\), 5 mM KCl, 120 mM choline chloride, 5 mM glucose, and 15 mM HEPES-Tris, pH 7.4), and then incubated for 15 min in the same medium additionally containing 1 mM \(^{22}\)NaCl (370 kBq/ml), 1 mM ouabain, and various concentrations of EIPA. Cells were washed four times with ice-cold, nonradioactive choline chloride medium, and then \(^{22}\)Na\(^+\) radioactivity was counted.

**pH measurement for dNBC1 activity.** Changes in pH\(_i\) were monitored using the acetoxymethyl ester of the pH-sensitive fluorochrome \(2',7'\)-bis(2-carboxyethyl)-5(6)-carboxyfluorescein (BCECF-AM), as described previously (2, 58, 59). Transfected cells were grown to 70–80% confluent density on coverslips and serum starved for 10 h to arrest growth. At the start of the experiment, the culture medium was removed, incubated in the presence of 2 \(\mu\)M BCECF in a choline chloride medium for 5 min, and washed to remove extracellularly localized BCECF. Coverslips were mounted in a cuvette containing a mixture of pH electrodes were pulsed in NH\(_4\)Cl medium in the presence of 30 mM Na\(^+\) or 30 mM HCO\(_3\)\(^-\) for 8 min. Removal of NH\(_4\)Cl and perfusion with choline chloride medium resulted in stable acidification of the cells. The cuvette was then perfused with 30 mM Na\(^+\) and 30 mM HCO\(_3\)\(^-\)-containing medium, and pH\(_i\) recovery was measured. pH\(_i\) was measured in a thermostatically controlled holding chamber (37°C) in a Deltameter dual-excitation spectrofluorometer (Photon Technology International, South Brunswick, NJ). The fluorescence ratio at excitation wavelengths of 500 and 450 nm was utilized to determine pH\(_i\); values in the experimental groups were compared with the calibration curve that was generated by the standard KCl/HEPES \(\pm\)AcCl method.

**Immunohistochemistry and immunofluorescence.** Ten dace were acclimated in neutral water for 2 wk and then transferred to acidic water. On day 5 after transfer, gills were removed and fixed for 2 h in PBS containing 4% (wt/vol) paraformaldehyde at 4°C. After fixation, they were separated into two groups and embedded in Tissue Tek OCT compound or in paraaffin. Frozen sections (6 \(\mu\)m) were separated and embedded in Tissue Tek OCT compound or in paraaffin. Frozen sections (6 \(\mu\)m) and paraaffin-embedded sections (4 \(\mu\)m) were prepared, immersed in PBS containing 1% H\(_2\)O\(_2\), exposed to 2% normal goat serum, and incubated with the dace CA-II antibodies. Primary antibodies were generated in rabbits in response to injection with B subunit of dace V-ATPase (a full-length 260-residue recombinant protein fused to a histidine tag), dace CA-II (a full-length 260-residue recombinant protein fused to a histidine tag), dace CA-II (a full-length 260-residue recombinant protein fused to a histidine tag), dace CA-II (a full-length 260-residue recombinant protein fused to a histidine tag), dace CA-II (a full-length 260-residue recombinant protein fused to a histidine tag), dace CA-II (a full-length 260-residue recombinant protein fused to a histidine tag), dace CA-II (a full-length 260-residue recombinant protein fused to a histidine tag), dace CA-II (a full-length 260-residue recombinant protein fused to a histidine tag), dace CA-II (a full-length 260-residue recombinant protein fused to a histidine tag), dace CA-II (a full-length 260-residue recombinant protein fused to a histidine tag), dace CA-II (a full-length 260-residue recombinant protein fused to a histidine tag), and dace CA-II (a full-length 260-residue recombinant protein fused to a histidine tag). Cells were incubated with peroxidase-conjugated secondary antibodies (Dako), and the peroxidase was detected with diaminobenzidine.

Western blot analysis. Neutral water and acidic water dace gills were homogenized in 100 mM Tris buffer containing 0.9% NaCl, 0.2 mM phenylmethylsulfonyl fluoride, 10 mg/ml pepstatin, and 10 mg/ml leupeptin. The homogenates were centrifuged at 5,000 g for 20 min, and the pellets were resuspended in the same buffer. To prepare cell homogenates, gills were homogenized in 100 mM Tris buffer containing 0.9% NaCl, 0.2 mM phenylmethylsulfonyl fluoride, 10 mg/ml pepstatin, and 10 mg/ml leupeptin. The homogenates were centrifuged at 5,000 g for 20 min, and the pellets were resuspended in the same buffer. The membrane proteins were separated by SDS-PAGE and transferred to Immobilon polyvinylidene difluoride membrane (Millipore). After the membranes were blocked in Tris-buffered saline with Tween 20 [150 mM NaCl, 0.05% (vol/vol) Tween 20, and 10 mM Tris-HCl, pH 8.0] containing 5% (vol/vol) nonfat milk for 1 h at room temperature, it was incubated with anti-B subunit of V-ATPase antibody at 1:3,000 dilution for 10 h at 4°C. After it was washed, the membrane was incubated for 1 h at room temperature with an alkaline phosphatase-conjugated antibody (Sigma) diluted 1:5,000 in Tris-buffered saline-Tween 20. The membrane was then developed with 0.4 mM 5-bromo-4-chloro-3-indolyl phosphate and 0.4 mM nitro blue tetrazolium chloride (Wako Pure Chemicals) in 0.1 mM Tris-HCl, pH 9.5, containing 50 mM MgCl\(_2\) and 150 mM NaCl.

In situ hybridization histochemistry was performed with paraffin-embedded sections (4 \(\mu\)m) according to the protocol recommended by the manufacturer of a digoxigenin RNA labeling kit (http://biochem.roche.com/biochemistry/nol_98/p10.pdf). Sections were subjected to hybridization with a digoxigenin-labeled cRNA probe of 315 bp that is complementary to the mRNA sequence encoding a central portion of CA-II that includes the signature sequence; the corresponding 315-bp sense probe was used as a control.
Fig. 1. Changes in blood pH (A) and plasma Na\(^+\) concentration ([Na\(^+\)], B) during adaptation to an acidic environment. Changes in blood pH and plasma [Na\(^+\)] in Osorezan dace (●) and control Ueda dace (○) were measured after their transfer from neutral (pH 6.8–7.2) to acidic (pH 3.5) water. Survival rate of Osorezan dace was 100\% when they were transferred from neutral water to water at pH 3.5, whereas only 66\% of control dace survived for >24 h, and most of them died within 36 h under such acidic conditions. Values are means ± SE from 7–11 fish. *Significant differences between 2 groups of dace, \(P < 0.05\) (Student’s t-test). #Significantly different from initial values within the same species, \(P < 0.05\).

RESULTS AND DISCUSSION

Extraordinary abilities of Osorezan dace to prevent acidification and loss of Na\(^+\). Previous physiological studies have demonstrated remarkable acid tolerance of the Osorezan dace (25, 35). We confirmed this by measuring changes of blood pH and plasma Na\(^+\) after transfer of the Osorezan dace from neutral to acidic (pH 3.5) water and by comparing them with those of control dace (Fig. 1). The blood pH and plasma Na\(^+\) concentration of the control dace declined continuously in the acidic environment, and most of them died within 36 h. In contrast, in the case of the Osorezan dace, the blood pH and Na\(^+\) concentration, which were once lowered, were restored to close-to-normal levels in 24 h, and they all survived (Fig. 1).

Relatively small changes in V-ATPase levels. The gills of the fish are the primary site of ionic and acid-base regulation. The favored model proposes that an apically oriented V-ATPase plays a major role in acid-base regulation and uptake of Na\(^+\) from the environment by excreting acid (\(H^+\)) and providing a driving force to apical membrane Na\(^+\) channels (32, 45, 46). To begin to understand the molecular mechanisms of the acid tolerance of the Osorezan dace, the expression levels of V-ATPase were examined using a specific cDNA probe and antiserum with the expectation that if V-ATPase is the key player in the acid adaptation, its activity is regulated by, for example, phosphorylation in a pH-dependent manner or, alternatively, it plays a significant but not a central role in the adaptation of the dace to a low-pH environment.

Poly(A)\(^+\) RNA was isolated from the dace gill, reverse transcribed, and used as a template for PCR. A 480-bp cDNA fragment coding for the V-ATPase B subunit was obtained by PCR (DDBJ/EMBL/GenBank accession no. AB094793) and used for Northern blot analysis as a probe. On Northern analysis using mRNA preparations from the gills of dace in neutral and acidic water, a single mRNA species of 2.6 kb was detected, but its band intensities were relatively weak and exhibited only a slight increase in the samples from acidified dace gills (Fig. 2A). Also at the protein level, we were unable to detect a large difference between gill extracts of dace acclimated to neutral and those of dace acclimated to acidic water (Fig. 2B); the amount of the B subunit increased only slightly (<50\%) during acid adaptation of the Osorezan dace.

Contrary to our expectation, neither at the message level nor at the protein level was a dramatic alteration observed in the V-ATPase levels when compared before and after acidification of the dace. This fact may indicate that if V-ATPase is the key player in the acid adaptation, its activity is regulated by, for example, phosphorylation in a pH-dependent manner or, alternatively, it plays a significant but not a central role in the adaptation of the dace to a low-pH environment.

![Image](http://ajpregu.physiology.org/doi/abs/10.1152/ajpregu.00007.2003)

Fig. 2. Significant but relatively low contribution of vacuolar-type H\(^+\)-translocating ATPase (V-ATPase) to acid adaptation of Osorezan dace. A: time course of mRNA expression of B subunit of V-ATPase in the gill after transfer of Osorezan dace to pH 3.5 water. Northern blot analysis was performed with 20 \(\mu\)g of total RNA, which revealed a single band at 2.6 kb. Exposure time (4 days) was much longer than for Na\(^+\)-K\(^+\)-ATPase, type II carbonic anhydrase (CA-II), type 3 Na\(^+\)/H\(^+\) exchanger (NHE3), type 1 Na\(^+\)-HCO\(_3\) cotransporter (NBC1), aquaporin-3 (AQP3), and glutamate dehydrogenase (GDH), i.e., 6–12 h (see Figs. 4 and 10). B: Western blot analysis of B subunit of V-ATPase in gills of dace adapted to neutral and acidic water. A specific antiserum was raised against recombinant B subunit of dace V-ATPase. Antiserum recognized a band at 55 kDa; size of this band is consistent with that reported for B subunit of other species (14, 15).
Marked elevation of Na⁺-K⁺-ATPase mRNA in the gill but not in the kidney. In a parallel attempt, we tried to identify genes that are highly expressed in acid-adapted Osorezan dace by constructing a subtraction cDNA library from mRNA preparations of the gills of the Osorezan dace acclimated to neutral and pH 3.5 water. Sequencing of cDNA clones of the subtraction library indicated the presence of Na⁺-K⁺-ATPase (DDBJ/EMBL/GenBank accession no. AB055615) in a relatively high proportion, suggesting that the Na⁺-K⁺-ATPase message is highly elevated in the gill of acid-adapted Osorezan dace. Indeed, Northern blot analysis indicated a marked induction of Na⁺-K⁺-ATPase mRNA in the gill on acidification (Fig. 3). However, its levels in the kidney, one of the richest sources of Na⁺-K⁺-ATPase, were not significantly affected by acidification (Fig. 3B). This marked induction of branchial Na⁺-K⁺-ATPase mRNA, together with the accumulating evidence for the role of the fish gill in ion transport beginning with Smith’s study in 1930 (57), provided the initial clue that the ion transport systems in the gill might be responsible for the acid tolerance of the Osorezan dace, because Na⁺-K⁺-ATPase has been demonstrated to have an important role in driving a variety of ion-transporting processes. We believed that CA and the downstream ion transporters that have long been recognized to participate in the control of pH in a variety of tissues including the branchial epithelium (21, 69) may play major roles and initiated the studies described below.

CA-II levels and locations. A CA cDNA probe was obtained by RT-PCR performed with Osorezan dace gill mRNA and a set of primers based on the highly conserved regions of CAs cloned previously. A full-length cDNA was then isolated from a dace gill cDNA library. Sequence analysis indicated that the cDNA encodes CA-II, which is composed of 260 amino acid residues (DDBJ/EMBL/GenBank accession no. AB055617; Fig. 4A); such isoforms are cytosolic and possess the highest catalytic activity of the 11 different active isoforms of CA identified to date, which include cytoplasmic, mitochondrial, and membrane-bound forms (38, 56). CA-II is expressed in a wide variety of mammalian tissues. However, Northern blot analysis revealed that CA-II mRNA was present in substantial amounts only in the gill and anterior intestine of acid-exposed Osorezan dace; RNA preparations from these organs of control fish maintained in neutral water yielded only a faint hybridizing band under the same conditions (Fig. 4C). Transfer of fish to acidic water resulted in a marked induction of CA-II mRNA in the gill within 1 day (Fig. 4B). Immunohistochemistry with gill sections revealed that CA-II is localized to the chloride cells (see Fig. 8, A and B). In situ hybridization demonstrated that CA-II mRNA is highly expressed in the chloride cells of the gill (data not shown).

Cloning and Northern analysis of NHE3 and NBC1. Similar approaches were adopted to identify candidates for the proteins that mediate the transport of H⁺ and HCO₃⁻, yielding cDNAs that encode the A and B subunits of V-ATPase, three isoforms of NHE (NHE1–NHE3), and an NBC. Among the transcripts encoding these candidate proteins, NHE3 and NBC1 mRNAs were readily detected in the gill of acid-adapted Osorezan dace by Northern blot analysis of total RNA (Fig. 4, F and I). The amounts of NHE3 and NBC1 mRNAs in the gill were markedly increased within 1 day of transfer of fish from neutral to acidic water (Fig. 4, E and H). Whereas NHE3 mRNA was also relatively abundant in the kidney of acid-adapted fish (Fig. 4F), NBC1 mRNA was present at a high concentration only in the gill (Fig. 4I). In situ hybridization demonstrated that NHE3 and NBC1 mRNAs are localized to chloride cells in the gill (data not shown).

Structural and functional properties of dNHE3 and dNBC1. The nucleotide sequence of the full-length open reading frame of dNHE3 cDNA indicated that it encodes an integral membrane protein of 827 amino acid residues with 12 transmembrane spans (DDBJ/EMBL/GenBank accession no. AB055466; Fig. 4D). The dNHE3 shares a relatively high similarity with the mammalian counterparts in its membrane-spanning domains, but it is quite divergent in the intracellular COOH-terminal region (Fig. 4D). For determining its functional property, a cell line stably expressing dNHE3 was established by transfecting PS120 cells, a cell line lacking NHEs (47), with the expression vector pECE carrying the coding region of dNHE3, and its Na⁺/H⁺ exchange activity was measured using ²²Na⁺ (Fig. 5). The activity was seen only in the transfected cells and was sensitive to EIPA (Fig. 5A), a derivative of amiloride. Compared with mammalian NHE1 and NHE3 (63), the dace exchanger was closer to the NHE3 isoform in its amiloride sensitivity (Fig. 5B), consistent with the classification based on the sequence similarity.

NBC1 cDNA clones were also isolated from the dace gill cDNA library. Their sequence analysis revealed an open reading frame coding for a protein of 1,077 amino acid residues (DDBJ/EMBL/GenBank accession no. AB055467) with 12 predicted transmembrane spans and hydrophilic intracellular NH₂- and COOH-termi-
nal regions (Fig. 4G). Dace NBC1 is ~80% similar to mammalian NBC1 and is most closely related to pNBC1/NBCe1-B, which has an extended NH₂ terminus (1, 52). The NBC activity was first assayed in PS120 cells transfected with a dNBC1 expression construct by monitoring changes in pH with the pH-sensitive fluorescent dye BCECF-AM (Fig. 6). Cells were prepulsed with NH₄⁺ for 7 min to lower, on removal of NH₄⁺, pH to 6.2–6.4 to maximally stimulate transporters activated by intracellular acidosis after the 7-min NH₄⁺ pulse. In the first series of experiments (Fig. 6A), cells were incubated with their NH₄⁺-containing solution, loaded with the fluorescent dye BCECF in an Na⁺-free solution, exposed to NH₄⁺ for 7 min, and acid loaded by switching to an NH₄⁺-free solution. By this treatment, pH became 6.2, and when the cells were perfused with an Na⁺- and HCO₃⁻-containing medium there was rapid recovery from acidosis in transfected cells, which was completely inhibited with 0.3 mM DIDS. Nontransfected cells showed very slow recovery. This result demonstrates the Na⁺ dependency of the dNBC1 activity. In the second series of experiments (Fig. 6B), similar measurements were done using HCO₃⁻ in place of Na⁺, and rapid recovery of pH was also observed. Taken together, these results clearly indicate the Na⁺ and HCO₃⁻ dependency of the cloned dNBC1.

We next expressed dNBC1 in Xenopus oocytes to determine whether this clone was electrogenic while measuring pH with microelectrodes. Figure 7A shows the response of a water-control oocyte. As previously reported, addition of CO₂/HCO₃⁻ acidifies the oocyte (CO₂ hydration, then formation of H⁺ and HCO₃⁻) with an obvious change in membrane potential (Vₘ). Removal of Na⁺ in CO₂/HCO₃⁻ caused no change in pH and a slight hyperpolarization (normal Na⁺ conductance). Subsequent removal of CO₂/HCO₃⁻ caused pH to return to the pre-CO₂ value. Figure 7B illustrates...
that dNBC1 mediates electrogenic Na\(^{+}\)-HCO\(_3\)\(^{-}\) cotransport activity observed with other NBC1 clones (52, 53). Specifically, Fig. 7B shows that the addition of 10 mM HCO\(_3\)\(^{-}\) elicits a rapid and sustained hyperpolarization (ΔV\(_m\) 40 mV; i.e., negative charge entering the cell) while the oocyte acidifies. Unlike the control (Fig. 7A), removal of Na\(^{+}\) in CO\(_2\)/HCO\(_3\)\(^{-}\) immediately depolarizes the oocyte (ΔV\(_m\) 40 mV; i.e., negative charge leaving the cell) and causes an additional acidification (loss of HCO\(_3\)\(^{-}\); Fig. 7B). Readdition of Na\(^{+}\) hyperpolarizes and alkalinizes the oocyte, both indicating HCO\(_3\)\(^{-}\) entry into the cell. Removal of CO\(_2\)/HCO\(_3\)\(^{-}\} depolarizes and alkalinizes the oocyte. The final pHi for this dNBC1 oocyte is 0.2 pH unit above the initial pHi, indicating that the oocyte has been loaded with HCO\(_3\)\(^{-}\). Finally, even the initial pHi of the dNBC1 oocyte is elevated (7.4) compared with the control (7.2), implying that trace air CO\(_2\) has entered the dNBC1 oocyte. These results were typical of eight oocytes from three different Xenorhynchus tested at 1.5% CO\(_2\)/10 mM HCO\(_3\)\(^{-}\) or 5% CO\(_2\)/33 mM HCO\(_3\)\(^{-}\).

The \(V_m\) waveforms of dNBC1 appear different from those observed with amphibian and mammalian NBC1. Specifically, \(V_m\) changes are sustained, rather than peaking and rapidly decaying. This difference could indicate a leftward shift of the Na\(^{+}\) and/or HCO\(_3\)\(^{-}\) apparent affinity of dNBC1 vs. rKNBC1/NBCe1-A (19, 54). Such a shift (increased ion affinity) would ensure that dNBC1 could operate in a low-tonicity (fresh water) or acidic environment (pH 3.5 lake water).

**Immunohistochemical localization of NHE3 and NBC1.** To clarify the physiological roles of NHE3 and NBC1, we investigated whether these proteins are located in the apical or basolateral membrane of chloride cells. We therefore first prepared specific antisera to the most COOH-terminal fragments (20–42-residue long) of these proteins. Immunohistochemical analysis with the resulting antibodies revealed the presence of marked NHE3 immunoreactivity on the apical side of chloride cells (Fig. 8, C and D), whereas NBC1 immunoreactivity was localized to the basolateral membrane that forms the tubular network (and thereby increases the cell surface area) by extensive infolding into the cytoplasmic space (Fig. 8E). This latter structural feature renders the staining patterns of proteins in the

---

**Fig. 5. Functional characterization of dace NHE3.** A: initial rates of H\(^{+}\)-activated \(^{22}\)Na\(^{+}\) influx measured in untransfected PS120 cells and in a clonal isolate (PS120\(^{\text{NHE3}}\)) expressing dace NHE3 in the absence or presence of 1 mM ethylisopropylamiloride (EIPA, \(n = 4\)). B: concentration-response curves for inhibition of \(^{22}\)Na\(^{+}\) uptake by EIPA in human NHE1 (hNHE1), human NHE3 (hNHE3), and dace NBC1 stably expressed in PS120 cells (\(n = 4\)). hNHE1- and hNHE3-expressing PS120 cell lines have previously been established by Wakabayashi et al. (63).

**Fig. 6. Functional characterization of dace NBC1.** PS120 cells stably transfected with dace NBC1 were acid loaded by NH\(_4\)\(^{+}\) withdrawal and monitored for intracellular pH (pHi) recovery. Presence or addition of key ion species, for determining Na\(^{+}\) and HCO\(_3\)\(^{-}\) dependency of dace NBC1 activity, is indicated above A and B (open rectangles). After acidification, cells were exposed to an Na\(^{+}\)-containing solution (A, \(n = 4\)) or an HCO\(_3\)\(^{-}\}-containing solution (B, \(n = 4\)), in the presence or absence of 300 \(\mu\)M DIDS.
basolateral membrane indistinguishable from those of cytoplasmic proteins. The Na\(^{+}\)-K\(^{+}\)-ATPase, a marker protein of the basolateral membrane of the chloride cell (55), yielded a staining pattern similar to that of NBC1 (Fig. 8F). Their colocalization was, however, not complete; NBC1-positive cells appear to represent a subpopulation of the Na\(^{+}\)-K\(^{+}\)-ATPase-positive chloride cells. We therefore performed double-immunofluorescence staining for NBC1 and Na\(^{+}\)-K\(^{+}\)-ATPase. Indeed, a subpopulation of Na\(^{+}\)-K\(^{+}\)-ATPase-positive cells were stained with anti-NBC1 (Fig. 9). Also noteworthy is a follicular arrangement of chloride cells in the acid-adapted dace gill (Fig. 8D), which was not seen in the neutral water-acclimated Osorezan dace (data not shown) (25). This follicular arrangement may be one of the strategies to exclude H\(^{+}\) efficiently.

**Role of NHE3.** Members of the NHE family of proteins are present in the plasma membranes of virtually all animal cells, where they mediate the electroneutral exchange of intracellular H\(^{+}\) for external Na\(^{+}\) with a 1:1 stoichiometry (9, 43, 64). Seven NHE isoforms (NHE1–NHE7) have been identified in mammalian cells and share a membrane topology characterized by 12 transmembrane domains in the NH2-terminal region and a large COOH-terminal tail located in the cytosol. The isoforms NHE1–NHE4 are expressed in epithelial cells; NHE5 is abundant in the brain; NHE6 (42) is an endoplasmic reticulum/recycling endosomal isoform (5, 37); and NHE7 is in the trans-Golgi network (41). NHE1, NHE6, and NHE7 are widely expressed and are believed to perform housekeeping functions such as the control of pH and maintenance of ionic homeostasis in subcellular organelles. Among the epithelial isoforms, NHE2 is preferentially expressed in the gastrointestinal tract. NHE3 (60) is confined to the apical membrane of polarized epithelial cells of the kidney (3), gastrointestinal tract (22), and fish gill (13), and it is therefore believed to perform specialized functions. The structure (Fig. 4D) and predicted function of dNHE3 are consistent with this notion. The relatively divergent COOH-terminal tail of dNHE3 suggests that a specific mechanism may be responsible for regulation of its function. Chloride cells are rich in mitochondria, are metabolically active, and contain large amounts of Na\(^{+}\)-K\(^{+}\)-ATPase, a membrane-bound enzyme that couples ATP hydrolysis to the exchange of Na\(^{+}\) and K\(^{+}\) with a 3:2 stoichiometry to maintain a low intracellular Na\(^{+}\) concentration (7, 26, 34, 68). This Na\(^{+}\) pump activity and the protons generated by CA-II may provide the driving force for NHE3 (see Fig. 11A). Recently, Edwards et al. (12) isolated a partial cDNA clone of NHE3 from the gills of the Atlantic hagfish and showed by RT-PCR that its message levels in the gill were elevated more than four times after an induced metabolic acidosis. In this marine fish, downhill Na\(^{+}\) flux can be used for H\(^{+}\) flux; but in the case of Osorezan dace, such a large concentration gradient of Na\(^{+}\) is not available; furthermore, a steep (>1,000-fold) uphill H\(^{+}\) transport is necessary. Whether this problem can be overcome by strong induction alone of the driving-force providing CA-II and Na\(^{+}\)-K\(^{+}\)-ATPase remains to be established. The follicular arrangement of the chloride cells in acid-acclimated Osorezan dace may make some contribution by partially sealing the lumen of the follicle from low-pH lake water.
Role of NBC1. NBC mediates the coupled transport of Na⁺ and HCO₃⁻ across the plasma membrane and, thereby, contributes to the secretion and absorption of HCO₃⁻ and the regulation of pHₐ (52). NBC was first identified in the salamander kidney (4) and was subsequently cloned from the same source (53). Four types of NBCs (NBC1–NBC4) have been identified in mammals (24, 49, 50). The NBC now cloned from the acid-adapted dace gill (Fig. 4G) is similar to NBC1, which transports Na⁺ and HCO₃⁻ with a stoichiometry of ≈1:2 (an electrogenic process). The electrogenic nature of NBC1-mediated ion transport (Fig. 7B) suggests that the efflux of HCO₃⁻ from chloride cells is stimulated not only by the supply of HCO₃⁻ provided by CA-II but also by the hyperpolarization of these cells caused by the sustained activity of Na⁺-K⁺-ATPase. These
observations are consistent with the model proposed in Fig. 11. dNBC1 is most similar to pNBC1/NBCe1-B, which has an extended NH2 terminus (52). Our model of dace gill transport is similar to the cellular model of mammalian proximal tubule HCO3\(^{-}\)/H\(^{+}\) absorption. Figure 4I indicates that there is no detectable dNBC1 mRNA in the kidney, even though a probe common to NBC1 isoforms was used. Thus, at least for acidosis, the gill, rather than the kidney, is crucial for systemic acid-base homeostasis. At the molecular level, pNBC1/NBCe1-B, rather than kNBC1/NBCe1-A, is the major homeostatic HCO3\(^{-}\)/H\(^{+}\) transporter in the Osorezan dace.

Potential role of AQP3. An attempt to identify genes that are specifically expressed in dace gill under acidic conditions by screening a subtracted cDNA library suggested that the gene for the water channel AQP3 (DDBJ/EMBL/GenBank accession no. AB055465) might be one such candidate (for the initial cloning and review of aquaporins, see Refs. 16, 23, 48). Northern blot analysis revealed that AQP3 mRNA is enriched in the gill compared with other tissues of acid-adapted dace (Fig. 4L) and that its abundance in this tissue is markedly increased within 1 day of the transfer of fish to acidic water (Fig. 4K). We therefore determined the predicted amino acid sequence of dace AQP3 (Fig. 4J), prepared antibodies to a synthetic peptide based on this sequence, and examined the expression of this protein in the gill by immunohistochemistry. Marked AQP3 staining was observed in the chloride cells (Fig. 8G) in a pattern that resembled that of Na\(^+/\)H\(^{-}\)-K\(^{+}\)/H\(^{+}\)-ATPase immunoreactivity (Fig. 8H). This observation suggests that acid-induced expression of AQP3 results in stimulation of water transport across the basolateral membrane and, thereby, provides a substrate for CA-II (see Fig. 11A). Although not illustrated in Fig. 11, the possibility also exists that AQP3 is somehow involved in osmoregulation (e.g., indirect involvement in Na\(^+/\)H\(^{-}\) uptake), because Cutler and Cramb (11) and Lignot and coworkers (31) recently demonstrated a marked downregulation of AQP3 in eel gill chloride cells after seawater acclimation. Another potential role of AQP3 may be acceleration of ammonia secretion, because its relative, AQP1, has been demonstrated to transport ammonia (40), and it is known that freshwater fish excrete a higher percentage of total body ammonia across the gills than marine teleosts (66, 67) and 2)
plasma ammonia levels increase in the Amazonian tambaqui on transfer from neutral to acidic water (71).

Role of glutamine catabolism. In mammals, it has been established that increased renal ammoniagenesis and gluconeogenesis (see Fig. 11B) constitute an adaptive response for restoring acid-base balance during metabolic acidosis (10). To determine whether the system plays a role in acid adaptation of the Osorezan dace, we measured induction of its component GDH (Fig. 10) by Northern blot analysis. A partial 173-bp cDNA for dace GDH exhibiting 97% sequence similarity to human GDH was obtained by RT-PCR (DDBJ/EMBL/GenBank accession no. AB094342) and used as a hybridization probe. Large inductions of GDH mRNA were observed in all tissues examined when dace were exposed to acid (Fig. 10B). This increase in ubiquitous tissues is a marked contrast to the tissue- and cell-specific increases of CA-II, NHE3, NBC1, Na⁺-K⁺-ATPase, and AQP3 described above (Figs. 3 and 4).

The result suggests that glutamine metabolism is activated in virtually all tissues of the Osorezan dace in response to acidification, and the increased generations of ammonia and bicarbonate are sustained by increased expression of the enzymes involved in this adaptive catabolic response. It is noteworthy that, in mammals, only the renal catabolism of glutamine is acutely activated in response to the onset of metabolic acidosis, in contrast to other tissues where glutamine metabolism is largely constitutive. In the Osorezan dace, however, glutamine catabolism appears to be regulatory in a variety of tissues and contribute to a greater extent to maintaining acid-base homeostasis. Although this ubiquitous response can alternatively be considered a side effect of acidosis, we prefer the interpretation of the results as reflecting the regulatory response by analogy to the Lake Magadi tilapia that synthesizes, as a strategy for living in an alkaline lake, large amounts of urea in whole body muscles in addition to the liver, which is normally the sole organ of urea synthesis (33). The question as to the regulatory mechanism vs. simple side effects would be addressed by, for example, analyzing the promoter regions of the genes involved in the ammoniagenesis and identifying acid responsive elements.

The ammonia produced in extrarenal tissues may be secreted through the gill, as demonstrated in many fish species (66, 67). The acidic condition of the lake is favorable for this process, known as a diffusion-trapping mechanism (39), because the lower the pH of the surrounding medium, the faster the diffusion of ammonia across the membrane of the gill epithelial cells. The protonated form of ammonia, ammonium ion (NH₄⁺ ↔ NH₃ + H⁺), cannot directly cross the cell membrane, but it can be transported across the basolateral membrane by substitution for K⁺ on the Na⁺-K⁺-ATPase, because the hydrated radius of NH₃⁺ is similar to that of K⁺ (17, 28). NH₄⁺ secretion could occur downhill on the apical NHE3 (27) in exchange of Na⁺, the concentration of which is kept low inside the cell by the Na⁺ pump or Na⁺-K⁺-ATPase. It is therefore likely that NH₃ and NH₄⁺ transport contribute to ammonia secretion in Osorezan dace.

Proposed mechanism. In survival of the Osorezan dace, the combined roles of 1) CA and its upstream

---

Fig. 11. Two mechanisms essential for overcoming acid challenges. A: model for concerted actions of CA-II, NHE3, NBC1, AQP3, and Na⁺-K⁺-ATPase in chloride cell of Osorezan dace. Expressions of these molecules are strongly induced on acidification and reach levels that can be readily detected by Northern blot analysis using total RNA preparations. Electrogenic natures of NBC1 and Na⁺-K⁺-ATPase are represented by unequal arrow sizes. Bay-like structures of basolateral membrane represent numerous infoldings of membrane forming tubular system. For explanation of the mechanism and functional role of each component, see RESULTS AND DISCUSSION. B: generation of ammonia and bicarbonate through metabolism of glutamine, which is activated on acidification in various tissues of Osorezan dace and is expected to play a significant role in survival in acidic water. In mammals, similar induction of components of glutamine metabolism has also been well established and shown to be sensitive to small pH changes but can be seen only in renal proximal tubule cells during metabolic acidosis. Mito, mitochondria; Q, glutamine; Glnase, glutaminase; E, glutamic acid; Mal, maleic acid; OAA, oxaloacetic acid; PEPCK, phosphoenolpyruvate carboxykinase; Pep, phosphoenolpyruvate.
(AQP3) and downstream molecules (NHE3 and NBC1) in chloride cells of the gill and 2) ammoniagenesis and gluconeogenesis in various tissues are schematically shown in Fig. 11. As a coarse adjustment taking place in the chloride cell, CA-II, a Zn2+-containing enzyme, provides HCO3− and H+ for the regulation of pH homeostasis. Protons are excreted across the apical membrane by NHE3 in exchange of Na+, whereas HCO3− is cotransported with Na+ across the basolateral membrane into the blood by NBC1 (Fig. 11A). Na+-K+-ATPase, an electrogenic Na+ pump, provides the driving force for NHE3 and NBC1 by reducing the intracellular concentration of Na+ and by hyperpolarizing the cell, respectively. AQP3 may also contribute to this system by supplying H2O to CA-II.

Fine adjustment is achieved by activation of glutamine catabolism, leading to ammoniagenesis and gluconeogenesis (Fig. 11B). For the continued generation of ammonia and bicarbonate, glutamine undergoes sequential deamination reactions catalyzed by glutaminase and GDH to form α-ketoglutarate, which enters the tricarboxylic acid cycle and is sequentially converted to malate, which leaves the mitochondria. Malate is then oxidized in the cytosol to oxaloacetate, which is subsequently converted to phosphoenolpyruvate and then to glucose. Conversion of glutamine to α-ketoglutarate generates two ammonium ions, and further catabolism of α-ketoglutarate yields two bicarbonate ions, which help compensate for reduced systemic pH, which is not completely accomplished by the chloride cells. Ammonia may be directly excreted from the gill without being metabolized by the liver to urea in a process that consumes bicarbonate and ATP. Additionally, part of the resultant HCO3− could provide more HCO3− for NBC1 absorption, thereby increasing systemic buffering.

Perspectives

We have shown, at the molecular level, that the chloride cells of the gill play an important role in maintaining acid-base balance and that this ability is greatly exaggerated in the Osorezan dace. The expression of the molecules, identified here and expected to be involved in such pH and ion regulation, appears to be finely tuned. Analysis of their gene structures, especially of the promoter regions, and identification of corresponding trans-acting regulators of transcription will help us understand the mechanism of the orchestration and the enhanced expression in the chloride cells of the Osorezan dace. For the analysis of the coordinated expression of multiple genes that encode the regulatory enzymes in the glutamine catabolic pathway, the information obtained from studies on an analogous system in the mammalian renal proximal tubules (10) will be helpful. Another interesting feature of the Osorezan dace is the acid-induced formation of the follicular arrangement of chloride cells that is not seen in neutral water. The Osorezan dace will therefore also be useful for studying the mechanism of follicle formation; among the clones identified by subtraction cloning were cDNAs encoding the ezrin-radixin-moesin family of proteins, key regulatory molecules in linking F-actin to specific membrane proteins, especially in cell surface structures.

We thank Dr. Tetsuya Hirano for discussions and encouragement and Akira Kato, Fumi Kato, Ako Takamori, Yoko Saruta, Susanti Hidayat, Azzania Fibriani, Setsuko Sato, Shojiro Kuroda, Sato Kuroda, Akira Oyagi, and Nathan Angel for technical and secretarial assistance.

This work was supported by Ministry of Education, Culture, Sport, Science, and Technology of Japan Grants-in-Aid for Scientific Research 09102000 and 140400, Ministry of Health, Labour, and Welfare of Japan Research Grant for Cardiovascular Diseases 11C-1, and National Institute of Diabetes and Digestive and Kidney Diseases Grants DK-56218 and DK-60845 (to M. F. Romero).

REFERENCES


