

# V-H<sup>+</sup>-ATPase translocation during blood alkalosis in dogfish gills: interaction with carbonic anhydrase and involvement in the postfeeding alkaline tide

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**Tresguerres M, Parks SK, Wood CM, Goss GG.** V-H<sup>+</sup>-ATPase translocation during blood alkalosis in dogfish gills: interaction with carbonic anhydrase and involvement in the postfeeding alkaline tide. *Am J Physiol Regul Integr Comp Physiol* 292: R2012–R2019, 2007. First published January 4, 2007; doi:10.1152/ajpregu.00814.2006.— We investigated the involvement of carbonic anhydrase (CA) in mediating V-H<sup>+</sup>-ATPase translocation into the basolateral membrane in gills of alkalotic *Squalus acanthias*. Immunolabeling revealed that CA is localized in the same cells as V-H<sup>+</sup>-ATPase. Blood plasma from dogfish injected with acetazolamide [30 mg/kg at time (*t*) = 0 and 6 h] and infused with NaHCO<sub>3</sub> for 12 h (1,000 μeq·kg<sup>-1</sup>·h<sup>-1</sup>) had significantly higher plasma HCO<sub>3</sub><sup>-</sup> concentration than fish that were infused with NaHCO<sub>3</sub> alone (28.72 ± 0.41 vs. 6.57 ± 2.47 mmol/l, *n* = 3), whereas blood pH was similar in both treatments (8.03 ± 0.11 vs. 8.04 ± 0.11 pH units at *t* = 12 h). CA inhibition impaired V-H<sup>+</sup>-ATPase translocation into the basolateral membrane, as estimated from immunolabeled gill sections and Western blotting on gill cell membranes (0.24 ± 0.08 vs. 1.00 ± 0.28 arbitrary units, *n* = 3; *P* < 0.05). We investigated V-H<sup>+</sup>-ATPase translocation during a postfeeding alkalosis (“alkaline tide”). Gill samples were taken 24–26 h after dogfish were fed to satiety in a natural-like feeding regime. Immunolabeled gill sections revealed that V-H<sup>+</sup>-ATPase translocated to the basolateral membrane in the postfed fish. Confirming this result, V-H<sup>+</sup>-ATPase abundance was twofold higher in gill cell membranes of the postfed fish than in fasted fish (*n* = 4–5; *P* < 0.05). These results indicate that 1) intracellular H<sup>+</sup> or HCO<sub>3</sub><sup>-</sup> produced by CA (and not blood pH or HCO<sub>3</sub><sup>-</sup>) is likely the stimulus that triggers the V-H<sup>+</sup>-ATPase translocation into the basolateral membrane in alkalotic fish and 2) V-H<sup>+</sup>-ATPase translocation is important for enhanced HCO<sub>3</sub><sup>-</sup> secretion during a naturally occurring postfeeding alkalosis.

*Squalus acanthias*; bicarbonate secretion; proton reabsorption; acid-base regulation; acetazolamide; ion transport; feeding

THE GILLS OF MARINE ELASMOBRANCHS have a subpopulation of cells rich in V-H<sup>+</sup>-ATPase (29, 32). V-H<sup>+</sup>-ATPase is normally located in cytoplasmic vesicles but translocates to the basolateral membrane in a microtubule-dependent manner during blood alkalosis (33). Impairment of the translocation process by colchicine reduces the fish's ability to excrete excess HCO<sub>3</sub><sup>-</sup> loaded by intravenous infusion, indicating that V-H<sup>+</sup>-ATPase translocation is involved in enhanced HCO<sub>3</sub><sup>-</sup> secretion to the external environment (33). The proposed model for HCO<sub>3</sub><sup>-</sup> secretion included an intracellular carbonic anhydrase (CA) that serves to hydrate CO<sub>2</sub> into H<sup>+</sup> and HCO<sub>3</sub><sup>-</sup>. The basolaterally located V-H<sup>+</sup>-ATPase then reabsorbs the H<sup>+</sup> into the blood, thereby counteracting the blood alkalosis (33), whereas

the HCO<sub>3</sub><sup>-</sup> is most likely secreted to the surrounding seawater via an apical, Pendrin-like, anion exchanger (10, 30).

In the present study, we focused on identifying the nature of the stimulus that triggers the V-H<sup>+</sup>-ATPase translocation during NaHCO<sub>3</sub> infusion and on evaluating whether a similar translocation occurs during a metabolic alkalosis of natural origin associated with feeding.

A priori, two major alternatives exist for the nature of the stimulus: 1) that blood alkalosis is detected by a specific pH or HCO<sub>3</sub><sup>-</sup> sensor in a place other than the V-H<sup>+</sup>-ATPase-rich cells, resulting in a hormonal or nervous signal that ultimately results in V-H<sup>+</sup>-ATPase translocation, and 2) that intracellular H<sup>+</sup> or HCO<sub>3</sub><sup>-</sup> produced by CA is sensed inside the V-H<sup>+</sup>-ATPase-rich cells, triggering the process. Recent research demonstrating that CA inhibition affects V-H<sup>+</sup>-ATPase cellular localization (2, 6, 25) led us to investigate the potential involvement of CA in V-H<sup>+</sup>-ATPase translocation in dogfish gills. Under this second scenario, CA inhibition should prevent V-H<sup>+</sup>-ATPase translocation during blood alkalosis and also impair HCO<sub>3</sub><sup>-</sup> clearance from the blood.

Artificially induced acid-base disturbances such as intravenous acid or base infusions (1, 12, 15, 16, 32, 33, 36), exposure to extreme hypercapnia (13, 16, 27), and acidic or alkaline diet regimes (11, 28) are commonly used in fish and other animals to stimulate the acid-base secretory mechanisms. The involvement of specific ion-transporting proteins is usually inferred based on changes in protein abundance or mRNA expression. One major criticism of results obtained from these studies is that it is unclear whether they are representative of the organism's normal physiology or whether they only unveil mechanisms that are turned on after extreme stresses.

Recently, Wood et al. (37) found that a pronounced blood metabolic alkalosis occurs in dogfish after force feeding via a stomach tube. This phenomenon, known as “alkaline tide” (24, 34), is the result of H<sup>+</sup> secretion into the stomach during food digestion and simultaneous HCO<sub>3</sub><sup>-</sup> reabsorption into the blood. The dogfish alkaline tide was characterized by significant rises in blood pH (~0.2 pH units) and plasma HCO<sub>3</sub><sup>-</sup> (~2.0 mmol/l) 3–6 h after feeding, which returned to control values ~17 h after feeding (37). Very recently, similar results with a more pronounced rise in plasma HCO<sub>3</sub><sup>-</sup> concentration ([HCO<sub>3</sub><sup>-</sup>]) at 6 h were obtained after dogfish were allowed to feed naturally (38). Because the gills are the main acid-base regulatory organ in marine elasmobranchs (17), it was hypothesized that the excess base load is secreted by the gills (37). Interestingly, the magnitude of the postfeeding metabolic alkaloses in these two

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feeding studies were broadly comparable to those induced by intravenous HCO<sub>3</sub><sup>-</sup> infusion in previous studies from our laboratory (32, 33). Therefore, we investigated whether the branchial V-H<sup>+</sup>-ATPase translocation to the basolateral membrane takes place during the alkaline tide in naturally fed fish.

## MATERIALS AND METHODS

**Experimental animals.** Pacific spiny dogfish (*Squalus acanthias* L.) were caught by hook and bait from the Trevor Channel (Vancouver Island, BC, Canada) and immediately transferred to the Bamfield Marine Sciences Centre, where they were held in batches of 20–60 animals in a 151,000-liter circular tank provided with flowing seawater (13°C, 32‰ salinity). Fish were fasted for at least 5 days before experimentation. All experiments were performed according to University of Alberta-approved animal care protocols.

**Surgery, NaHCO<sub>3</sub> infusion, and acetazolamide injection.** Six dogfish (mean body mass of 1.72 ± 0.11 kg) were fitted with cannulas into the caudal artery and vein following the protocol described in detail previously (32, 33). After a 24-h recovery period, a 250 mmol/l NaHCO<sub>3</sub>-250 mmol/l NaCl solution was infused via the venous cannula by a peristaltic pump. The actual infusion rate was 3.77 ± 0.17 ml·kg<sup>-1</sup>·h<sup>-1</sup>, resulting in a HCO<sub>3</sub><sup>-</sup> load of 941.90 ± 43.16 μeq·kg<sup>-1</sup>·h<sup>-1</sup>. A stock acetazolamide solution (30 mg/ml in DMSO) was prepared at the beginning of every experimentation day. Acetazolamide (30 mg/kg) was injected via the venous cannula 15 min before the start of the HCO<sub>3</sub><sup>-</sup> infusions, and an additional identical dose was injected after 6 h of infusion. Control fish were injected with an equivalent amount of DMSO alone (1 ml/kg).

**Blood sampling and analytical procedures on plasma samples.** The arterial cannula was used to withdraw blood samples (200 μl) at time (*t*) = 0, 1, 3, 6, 9, and 12 h. Blood pH was immediately measured by a thermo-jacketted Accumet microsize pH electrode model 13-620-94 (Fisher Scientific, Pittsburgh, PA). The blood samples were then centrifuged at 12,000 *g* for 3 min to obtain plasma; 40 μl of plasma were used for total CO<sub>2</sub> determination by the method of Cameron (8) in a thermostatted chamber (37°C) equipped with a CO<sub>2</sub> electrode (Radiometer, Copenhagen, Denmark). Pco<sub>2</sub> and [HCO<sub>3</sub><sup>-</sup>] were calculated by using the solubility of CO<sub>2</sub>, the apparent p*K* for dogfish at the experimental temperature, and rearrangements of the Henderson-Hasselbalch equation according to Boutilier et al. (4).

**Terminal sampling.** In the NaHCO<sub>3</sub> infusion series, cannulated dogfish were anesthetized with tricaine methanesulphonate (0.2 g/l AquaLife; Syndel Laboratories Vancouver, BC, Canada) and injected with 5 ml of a saturated KCl solution after 12 h of HCO<sub>3</sub><sup>-</sup> infusion, which killed the fish instantly. Gill samples were immediately excised and placed in fixative or were snap-frozen in liquid nitrogen for immunohistochemical and Western blot analyses, respectively. In the feeding series, noncannulated dogfish were terminally anesthetized in their isolation boxes (see below) by stopping the water flow, lowering the water level to 6 liters, and adding an overdose of MS-222 (0.2 g/l; Syndel Laboratories), a procedure that took ~3 min.

**Feeding experiments.** An entire batch of ~30 dogfish in the 151,000-liter holding tank were placed on a feeding regime in which they were fed every fifth day with freshly thawed whole hake (*Merluccius productus*), with heads removed. This is one of the most common natural preys of dogfish in British Columbia coastal waters (19). A feeding frenzy ensued, and all of the food was consumed within 30 min. The ration supplied at each feeding was ~3% of body mass, based on the estimated mass of all the dogfish in the group tank. However, not all of the dogfish ate; in a separate trial, the average mass of food consumed by those dogfish that had fed was 5–6% of body weight, based on autopsy at 24 h postfeeding. With practice, it was possible to discern which dogfish had eaten by the bulging profile of the abdomen, and, at 1 h after feeding, five of these animals were

caught by dip net and removed to isolation enclosures. The enclosures were individual 40-liter polyurethane-coated wooden boxes (seawater flow = 1 l/min) served with vigorous aeration (36, 37). Sham animals (*n* = 5) were treated identically but were removed from the holding tank immediately before feeding, i.e., after 5 days of fasting.

Based on a parallel series of experiments (C. M. Wood, C. Buckingham, J. FitzPatrick, and S. Nadella, unpublished observations), we knew that postfeeding excretion of base into the external seawater started on average at 6–12 h postfeeding, reached a maximum value at 12–24 h, and continued through 48 h. Therefore, acid-base fluxes in fed and sham animals were measured overnight during the 12- to 24-h postfeeding period; animals were then killed for gill immunohistochemistry, and Western blot analysis procedures were made within a further 2 h. At the start of the 12-h flux period, the water inflow to the box was stopped, and the volume was set to a known level (~35 liters after subtraction of dogfish mass). Duplicate water samples were taken at 12 and 24 h and measured for titratable alkalinity and total ammonia. Titratable alkalinity was determined by titration of 10-ml water samples to pH 4.0, using a Radiometer-Copenhagen GK2401C combination electrode and a Gilmont microburette to dispense standardized acid (0.04 N HCl). Total ammonia concentration was measured by the indophenol blue method (18). Net acid-base fluxes were calculated as the difference between the change in ammonia concentration and the change in the concentration of titratable alkalinity in the water over the monitoring period, factored by weight, volume, and time, as outlined by McDonald et al. (22).

**Immunohistochemistry.** Gill samples were fixed in 3% paraformaldehyde in 0.1 mmol/l cacodylate buffer (pH 7.4) at 4°C for 6 h and processed as described previously (32, 33). V-H<sup>+</sup>-ATPase was immunolabeled in 4-μm paraffin sections with the use of the antibody developed by Katoh et al. (20) and the Vectastain ABC kit (Vector Laboratories, Burlingame, CA). CA immunostaining was performed in a similar manner using an anti-trout cytoplasmic CA antibody (13). To investigate whether CA is located in the same cells as V-H<sup>+</sup>-ATPase and Na<sup>+</sup>-K<sup>+</sup>-ATPase, we immunolabeled consecutive 4-μm paraffin sections as described previously (32, 33).

**Western blot analysis.** Frozen gill samples were immersed in liquid nitrogen and pulverized in a porcelain mortar. The resulting powder was combined with 1:10 wt/vol homogenization buffer (250 mmol/l sucrose, 1 mmol/l EDTA, 30 mmol/l Tris, 100 mg/ml PMSF, and 2 mg/ml pepstatin, pH 7.4) and sonicated on ice (3 times, 5 s each). Debris and nuclei were pelleted during a 10-min centrifugation (3,000 *g* at 4°C), and the supernatant was collected as “whole gill homogenate.” Part of the supernatant was further centrifuged at 20,800 *g* (4°C for 1 h), and the resulting pellet was resuspended in 200 μl of freshly made homogenization buffer. This constituted the “gill cell membrane-enriched fraction.” Aliquots of each fraction were saved separately, diluted 1:10 in homogenization buffer, and assessed for protein concentration in triplicate by the Bradford method (5).

Processed gill samples were combined with 2× Laemmli buffer (21) and heated to 70°C for 15 min, and 30 μg of total protein were separated by SDS-PAGE (45 min at 180 V) in 7.5% (V-H<sup>+</sup>-ATPase) or 12% (CA) polyacrylamide minigels. Protein was transferred to nitrocellulose membranes and incubated with the primary anti-V-H<sup>+</sup>-ATPase or CA antibody and secondary fluorescent antibodies (Li-Cor, Lincoln, NE). Bands were visualized and quantified using the Odyssey infra-red imaging system and software (Li-Cor). Values are given as arbitrary fluorometric units (afu). Differences in protein loading were corrected by quantifying the total protein concentration in each lane after Coomassie blue staining. For more details, see Refs. 32 and 33.

**Statistics.** All data are given as means ± SE. Differences between treatments were tested by Student's *t*-test. For the analyses of pH and Pco<sub>2</sub> in the 12-h NaHCO<sub>3</sub> infusion experiments, we used two-way repeated-measures ANOVA (2-way RM-ANOVA) followed by Bonferroni's posttest to compare means at each experimental times (*t* = 0,



Fig. 1. Western blot analysis of dogfish gills with the  $\alpha$ -carbonic anhydrase (CA) antibody (lane 1). The band was eliminated after preincubating the primary antibody with excess peptide antigen (lane 2).

1, 3, 6, 9, and 12 h). Statistical significance was set at  $P < 0.05$ . All statistical analyses were performed on GraphPad Prism version 3.0 (GraphPad Software, San Diego, CA). Unless otherwise mentioned, the reagents used in this study were purchased from Sigma (St. Louis, MO).

## RESULTS

**CA immunolabeling.** In accordance with a previous report that used trout (13), the anti-trout CA antibody recognized a 33-kDa band in dogfish gill samples that were subjected to SDS-PAGE Western blot analysis. The band disappeared when the anti-CA antibody was previously incubated with excess specific peptide, demonstrating specificity of the antibody (Fig. 1). The CA antibody labeled most cells in the outer layer of the gill epithelium of fasted control dogfish (Fig. 2B). CA immunolabeling was stronger in cells that labeled for Na<sup>+</sup>-K<sup>+</sup>-ATPase (Fig. 2A) and in V-H<sup>+</sup>-ATPase-rich cells (Fig. 2C) but also in a minority of cells that did not label for any of the two ATPases.

**Effect of CA inhibition on blood and plasma variables during NaHCO<sub>3</sub> infusion.** Blood pH of dogfish infused with NaHCO<sub>3</sub> ( $941.90 \pm 43.16 \mu\text{eq}\cdot\text{kg}^{-1}\cdot\text{h}^{-1}$ ) behaved as previously described (32, 33): an initial steep rise from  $7.68 \pm 0.04$  to  $7.91 \pm 0.04$  pH units followed by a plateau at  $\sim 8.04$  pH units despite the continuous infusion of base ( $n = 3$ ; Fig. 3A). Fish that were infused with base and also injected with acetazolamide (30 mg/kg) showed a similar pattern, except for at  $t = 1$  h. At this point, blood pH dropped by  $\sim 0.20$  pH units, although it was not significantly different from the control base-infused fish (Fig. 3A).

The inhibitory effect of acetazolamide on CA was apparent in plasma [HCO<sub>3</sub><sup>-</sup>]. From  $t = 3$  h, [HCO<sub>3</sub><sup>-</sup>] was significantly higher than in base-infused control fish, showing a continual increase to  $t = 12$  h ( $28.72 \pm 0.41$  vs.  $6.57 \pm 2.47$  mmol/l; Fig. 3B) with no plateau noticed. Pco<sub>2</sub> was also significantly higher in acetazolamide-treated, base-infused fish, reaching a maximum value by 9 h ( $6.36 \pm 0.14$  vs.  $1.51 \pm 0.29$  Torr; Fig. 3C). By  $t = 12$  h, Pco<sub>2</sub> reached a plateau in acetazolamide-treated fish, but it was still elevated compared with control base-infused fish ( $5.79 \pm 1.00$  vs.  $1.21 \pm 0.38$  Torr).

**Effect of CA inhibition on V-H<sup>+</sup>-ATPase translocation and abundance.** Immunolabeling of gill sections from fish infused with NaHCO<sub>3</sub> revealed that V-H<sup>+</sup>-ATPase had translocated into the basolateral membrane (Fig. 4A), similar to our previous reports (32, 33). Acetazolamide clearly prevented V-H<sup>+</sup>-ATPase translocation (Fig. 4B), indicating that functional CA is required for the process to occur. The effect of acetazolamide on V-H<sup>+</sup>-ATPase translocation was also assessed from Western blot analysis (Fig. 5). There were no statistical differences in V-H<sup>+</sup>-ATPase abundance in whole gill homogenates

from acetazolamide-treated fish compared with controls ( $1.00 \pm 0.13$  vs.  $1.43 \pm 0.34$  afu,  $n = 3$ ;  $P > 0.05$ ). However, V-H<sup>+</sup>-ATPase in cell membrane-enriched samples from fish injected with acetazolamide was significantly lower than that

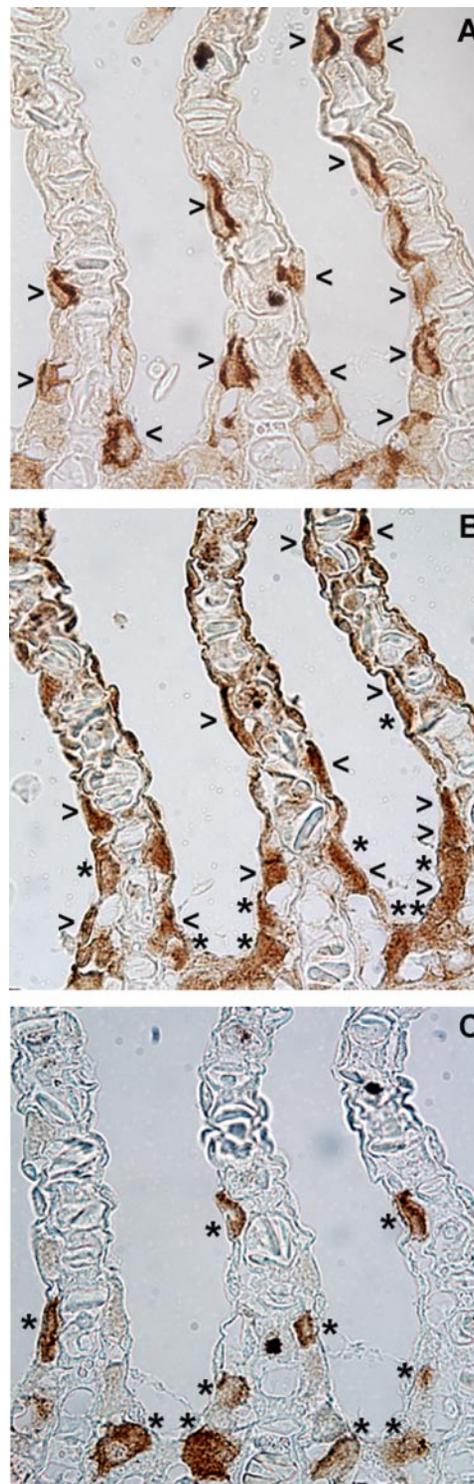


Fig. 2. Representative micrographs of 4- $\mu\text{m}$  consecutive sections of dogfish gills immunolabeled for Na<sup>+</sup>-K<sup>+</sup>-ATPase (A), CA (B), and V-H<sup>+</sup>-ATPase (C). Arrowheads indicate Na<sup>+</sup>-K<sup>+</sup>-ATPase labeling; asterisks indicate V-H<sup>+</sup>-ATPase labeling. Notice how CA immunoreactivity takes place in cells that are positive for one or the other ATPase. Magnification =  $\times 400$ .

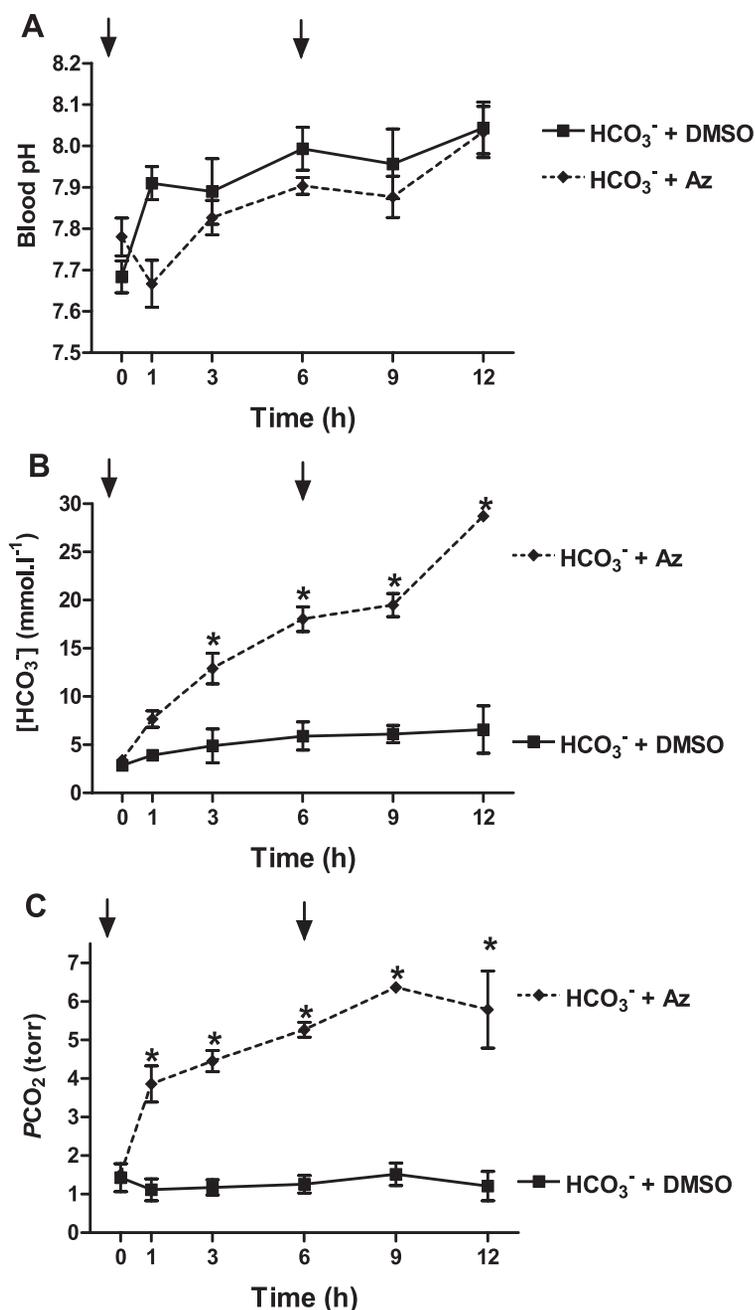


Fig. 3. Blood parameters of fish infused intravenously with NaHCO<sub>3</sub> (941.90 ± 43.16 μeq·kg<sup>-1</sup>·h<sup>-1</sup>) and injected with either DMSO (control; solid line) or acetazolamide (Az; 30 mg/kg, dash line); *n* = 3. Arrows indicate bolus injection of DMSO or acetazolamide. *A*: arterial blood pH. *B*: arterial plasma HCO<sub>3</sub><sup>-</sup> concentration ([HCO<sub>3</sub><sup>-</sup>]). *C*: arterial plasma PCO<sub>2</sub>. \**P* < 0.05 compared with the control value of the respective time (2-way repeated-measures ANOVA, Bonferroni's posttest).

shown in controls (0.24 ± 0.08 vs. 1.00 ± 0.28 afu, *n* = 3; *P* < 0.05), providing further evidence for the CA involvement in V-H<sup>+</sup>-ATPase translocation.

**Base efflux and V-H<sup>+</sup>-ATPase translocation and abundance during postfeeding alkalosis.** Dogfish (*n* = 5) that had fed ad libitum in the housing tank and were transferred to individual boxes displayed a net efflux of metabolic base to the water at a rate of 294.9 ± 63.7 μeq·l<sup>-1</sup>·kg<sup>-1</sup>·h<sup>-1</sup> between 12 and 24 h after feeding. In comparison, sham fish (*n* = 5) that had not fed exhibited no net acid-base fluxes at all during this time (-14.7 ± 25.9 μeq·l<sup>-1</sup>·kg<sup>-1</sup>·h<sup>-1</sup>). The contribution of ammonia excretion, which is negative in the net base flux calculation, was identical in the two groups (-70.2 ± 34.7 μeq·l<sup>-1</sup>·kg<sup>-1</sup>·h<sup>-1</sup> in fed animals vs. -70.8 ± 30.6 μeq·l<sup>-1</sup>·kg<sup>-1</sup>·h<sup>-1</sup> in sham animals). These

data are very similar to a more extensive examination of postfeeding acid-base flux, which demonstrated that this elevation of net base efflux continues for at least 48 h after a meal (C. M. Wood, C. Bucking, J. Fitzpatrick, and S. Nadella, unpublished observations).

V-H<sup>+</sup>-ATPase immunolabeling in gills of fasted fish occurred in the cytoplasm (Fig. 6A) (also see Refs. 32 and 33). However, V-H<sup>+</sup>-ATPase in gills of fed fish was distinctly located in the cell basolateral membrane of gill (Fig. 6B). To substantiate these findings, we quantified V-H<sup>+</sup>-ATPase in gill samples of both fed and fasted fish (Fig. 7). V-H<sup>+</sup>-ATPase abundance in whole gill homogenates was similar in samples from fasted (1.00 ± 0.29 afu) and fed fish (1.18 ± 0.16 afu) (*n* = 4–5; *P* > 0.05). However, the cell membrane-enriched fraction from fed fish demonstrated increased V-H<sup>+</sup>-ATPase

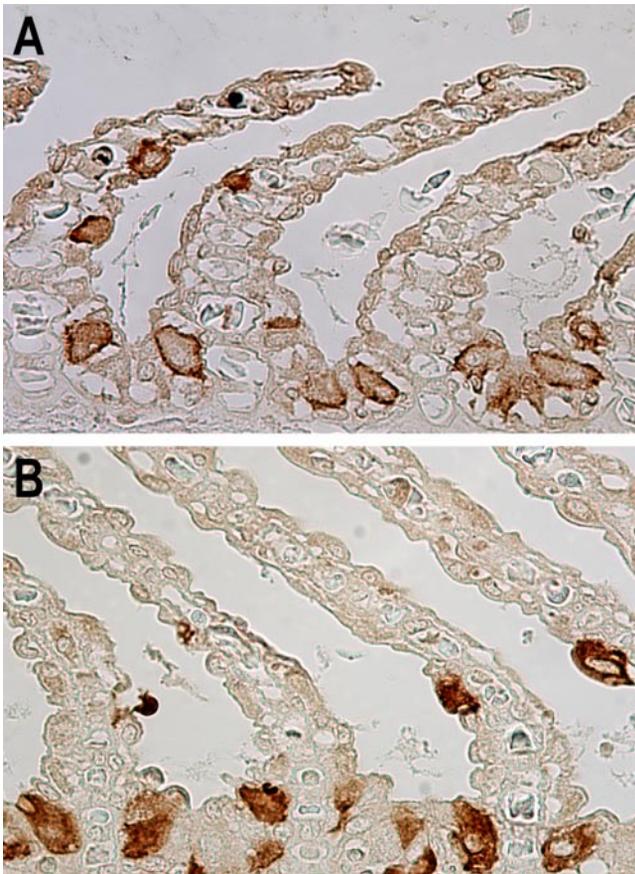


Fig. 4. Representative micrographs of V-H<sup>+</sup>-ATPase immunolabeling in gills from fish infused with NaHCO<sub>3</sub> for 12 h. A: control fish (injected with DMSO). B: fish injected with acetazolamide (30 mg/kg) at 0 and 6 h. Magnification = ×640.

abundance compared with fasted fish ( $3.10 \pm 0.54$  vs.  $1.00 \pm 0.16$  afu,  $n = 4$  or  $5$ ;  $P < 0.05$ ).

## DISCUSSION

We have previously shown that, during blood alkalosis, V-H<sup>+</sup>-ATPase translocates to the basolateral membrane of specific gill cells of the Pacific dogfish (32, 33). This process is dependent on functional cytoskeleton microtubules and correlates with the ability to recover from blood alkalosis (33). Therefore, we proposed that branchial cellular remodeling involving V-H<sup>+</sup>-ATPase translocation into the basolateral membrane was necessary for H<sup>+</sup> reabsorption and HCO<sub>3</sub><sup>-</sup> secretion. Here, we present novel findings about the cellular mechanism responsible for V-H<sup>+</sup>-ATPase translocation, its involvement in HCO<sub>3</sub><sup>-</sup> secretion and H<sup>+</sup> reabsorption, and the role of this mechanism under a normal physiological situation, the postfeeding alkaline tide.

**Role of CA.** On the basis of the estimated molecular size of the band from PAGE-Western blot analysis and the peptide preabsorption experiment, the  $\alpha$ -CA antibody demonstrated good specificity in dogfish gills. CA seems to be widely distributed throughout the gill epithelium, but it is found in higher abundance in gill cells that are also rich in Na<sup>+</sup>-K<sup>+</sup>-ATPase and V-H<sup>+</sup>-ATPase. This localization is consistent with the previously proposed role of CA in branchial acid-base

regulation (26, 32, 33, 35). This model predicted that CA hydrates CO<sub>2</sub> into H<sup>+</sup> and HCO<sub>3</sub><sup>-</sup> inside the Na<sup>+</sup>-K<sup>+</sup>-ATPase- and V-H<sup>+</sup>-ATPase-rich cells. The Na<sup>+</sup>-K<sup>+</sup>-ATPase-rich cells are responsible for secreting H<sup>+</sup> into the water in exchange for Na<sup>+</sup>, probably via an Na<sup>+</sup>/H<sup>+</sup> exchanger (32), whereas the V-H<sup>+</sup>-ATPase-rich cells reabsorb H<sup>+</sup> into the blood through basolateral V-H<sup>+</sup>-ATPases (32, 33) and apically located Cl<sup>-</sup>/HCO<sub>3</sub><sup>-</sup>, pendrin-like anion exchangers (10, 30).

The effect of CA inhibition by acetazolamide during blood alkalosis on blood pH, plasma [HCO<sub>3</sub><sup>-</sup>], and plasma Pco<sub>2</sub> mimicked previously reported results in dogfish that used a similar CA inhibitor, methazolamide (31). The lack of significant change in blood pH compared with fish infused with HCO<sub>3</sub><sup>-</sup> alone is due to the simultaneous increase of plasma [HCO<sub>3</sub><sup>-</sup>] and Pco<sub>2</sub>, which counteract each other in acid-base terms. Here, we must mention that the acetazolamide treatment inhibits not only gill intracellular CA but also other CAs in the body, including red blood cell (RBC) CA and the extracellular CA IV recently reported to be present at the basolateral membrane of gill pillar cells (14). Inhibition of RBC and type IV CA would slow down gaseous CO<sub>2</sub> diffusion into the water, which is evident from the plasma Pco<sub>2</sub> readings in our study. Thus we cannot rule out that the elevated Pco<sub>2</sub> in the acetazolamide-treated, HCO<sub>3</sub><sup>-</sup>-infused fish inhibits V-H<sup>+</sup>-ATPase

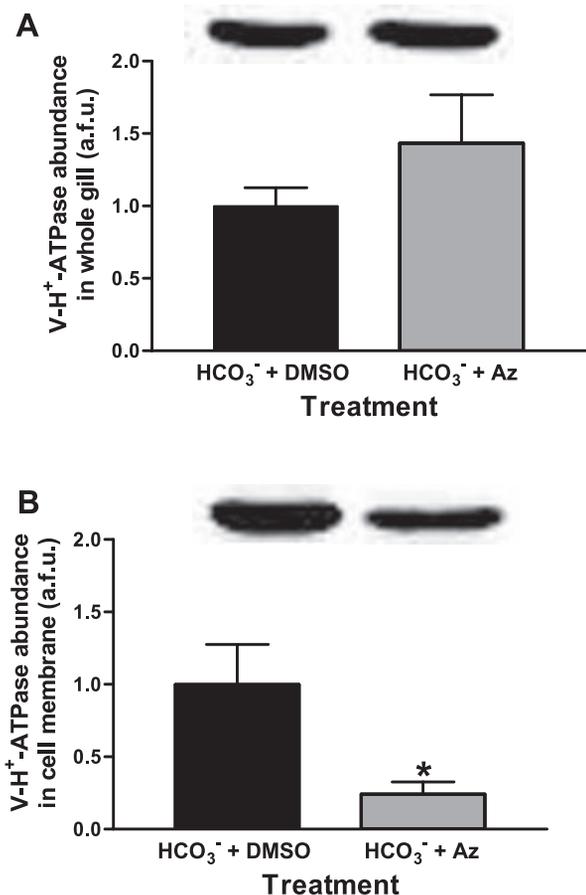


Fig. 5. Fluorometric analysis of V-H<sup>+</sup>-ATPase abundance in gills from fish infused with NaHCO<sub>3</sub> for 12 h;  $n = 3$ . A: whole gill homogenates. B: membrane fraction. Top: representative bands. HCO<sub>3</sub><sup>-</sup> + DMSO = control fish. HCO<sub>3</sub><sup>-</sup> + Az = fish injected with acetazolamide (30 mg/kg). afu, Arbitrary fluorometric units. \* $P < 0.05$  (Student's *t*-test).

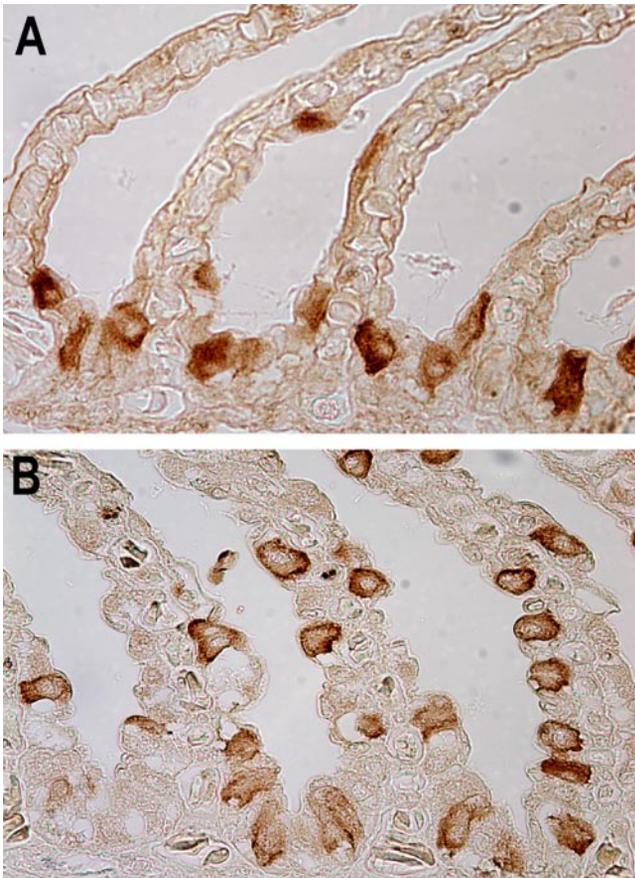


Fig. 6. Representative micrographs of V-H<sup>+</sup>-ATPase immunolabeling in gills from fasted and fed fish. A: fasted fish. B: fish 24–26 h after feeding. Magnification =  $\times 640$ .

translocation. However, elevated Pco<sub>2</sub> in the acetazolamide-treated HCO<sub>3</sub><sup>-</sup>-infused fish also indicates that delivery of CO<sub>2</sub> to the cytoplasm of the V-H<sup>+</sup>-ATPase-rich cells is not a problem during RBC CA inhibition. It would be extremely difficult to inhibit intracellular CA alone while also controlling for all the blood parameters during these prolonged whole animal HCO<sub>3</sub><sup>-</sup> infusion experiments.

A priori, one of the alternatives was that V-H<sup>+</sup>-ATPase translocation was triggered by increased blood pH and/or [HCO<sub>3</sub><sup>-</sup>]. However, when the gill samples were taken after 12 h of HCO<sub>3</sub><sup>-</sup> infusion, acetazolamide-treated, base-infused fish had the same blood pH as and higher plasma [HCO<sub>3</sub><sup>-</sup>] than fish that were infused with HCO<sub>3</sub><sup>-</sup> alone. At this point, branchial V-H<sup>+</sup>-ATPase translocation was negligible in the acetazolamide-treated, HCO<sub>3</sub><sup>-</sup>-infused fish, suggesting that intracellular H<sup>+</sup> or HCO<sub>3</sub><sup>-</sup> generated by CA is the stimulus that triggers V-H<sup>+</sup>-ATPase translocation. Alternatively, it is also possible that CA is involved in the sensing mechanism somewhere else in the body.

Assuming that intracellular pH or [HCO<sub>3</sub><sup>-</sup>] is the stimulus that triggers V-H<sup>+</sup>-ATPase translocation, the nature of the intracellular sensor remains to be determined. A distinct possibility is that the sensor is a soluble adenylyl cyclase (sAC). sAC is known to be activated by [HCO<sub>3</sub><sup>-</sup>], resulting in increased cAMP production (7, 9, 23). sAC has also been shown to mediate the translocation of V-H<sup>+</sup>-ATPase into the apical

membrane of clear cells at the rat epididymis in response to increased luminal pH (25). This process is mediated by modulation of the actin cytoskeleton and appears to result in H<sup>+</sup> secretion into the lumen (3). Moreover, CA inhibition by acetazolamide prevented the V-H<sup>+</sup>-ATPase translocation into the apical membrane (25). The results of these two studies on epididymis strongly suggest that intracellular HCO<sub>3</sub><sup>-</sup> generated by CA activates sAC, resulting in increased cAMP production. This in turn modulates the actin cytoskeleton polymerization, which ultimately results in V-H<sup>+</sup>-ATPase accumulation in the apical region of clear cells and subsequent H<sup>+</sup> secretion into the lumen of the epididymis.

The similarities of this model with our results are evident. We hereby propose a role of sAC in the V-H<sup>+</sup>-ATPase translocation mechanism that takes place in gills of dogfish experiencing blood alkalosis. To further support this hypothesis, identification of dogfish sAC homologue using tools of molecular biology, biochemistry, or both is absolutely required.

*Physiological role of the V-H<sup>+</sup>-ATPase translocation.* Although the alkalosis-induced V-H<sup>+</sup>-ATPase translocation is readily evident, it correlates well with recovery from alkalosis (33) and it is in accordance with base secretory mechanisms from other physiological systems (see Ref. 6); it was unclear whether this mechanism is of physiological relevance for normal (noninfused) dogfish. Recently, it was demonstrated that dogfish show a characteristic alkaline tide whereby blood

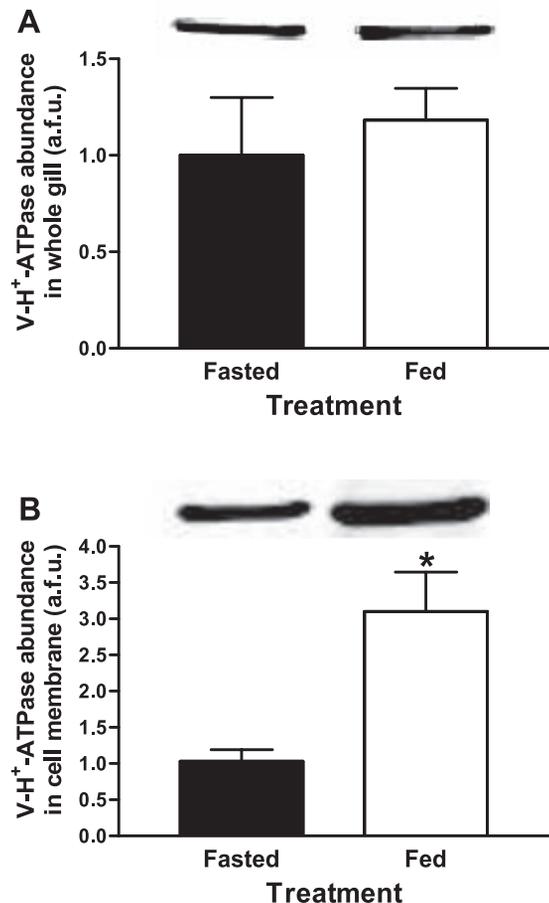


Fig. 7. Fluorometric analysis of V-H<sup>+</sup>-ATPase abundance in gills from fasted and fed fish (24–26 h after feeding);  $n = 4$  or  $5$ . A: whole gill homogenates. B: membrane fraction. Top: representative bands. \* $P < 0.05$  (Student's  $t$ -test).

pH and [HCO<sub>3</sub><sup>-</sup>] are significantly elevated a few hours after feeding (37, 38). We took advantage of this situation to address whether this naturally induced blood alkalosis results in branchial V-H<sup>+</sup>-ATPase translocation.

Dogfish in the present study were sampled 24–26 h after feeding. Between 12 and 24 h after feeding, these fish were excreting net base (i.e., HCO<sub>3</sub><sup>-</sup> equivalents) into the water. The measured rate was ~30% of the net NaHCO<sub>3</sub> infusion rate used in the first set of experiments. We show using immunohistochemistry that V-H<sup>+</sup>-ATPase has translocated to the basolateral membrane at *t* = 24–26 h. V-H<sup>+</sup>-ATPase abundance in gill cell membranes was also significantly higher than that shown in fasted fish, supporting the histological results. These results strongly suggest that branchial V-H<sup>+</sup>-ATPase insertion into the basolateral membrane is responsible for enhanced HCO<sub>3</sub><sup>-</sup> secretion during the natural postfeeding period.

It is possible that the same factor that activates HCl secretion into the stomach lumen during digestion is also responsible for triggering the branchial V-H<sup>+</sup>-ATPase translocation or for enhancing the triggering signaling mechanism. On the basis of the mammalian system, some candidates would be histamine, acetylcholine, and gastrin (24). However, our NaHCO<sub>3</sub> infusion experiments (32, 33; this paper) clearly demonstrate that V-H<sup>+</sup>-ATPase translocation can take place without the participation of any feeding-related hormone. Although we cannot discount the possibility that base infusion results in release of an unknown hormone or factor, we propose that the elevated blood [HCO<sub>3</sub><sup>-</sup>] during the postfeeding blood alkalosis results in an intracellular increase in [HCO<sub>3</sub><sup>-</sup>] inside the V-H<sup>+</sup>-ATPase-rich cells, which triggers branchial V-H<sup>+</sup>-ATPase translocation.

We conclude that a physicochemical variable (probably [HCO<sub>3</sub><sup>-</sup>]) inside the V-H<sup>+</sup>-ATPase-rich cells triggers the translocation of V-H<sup>+</sup>-ATPase into the basolateral membrane to reabsorb the CA-generated H<sup>+</sup> into the blood. This mechanism is important for maintaining blood acid-base balance during the naturally occurring postfeeding alkaline tide. If our model is confirmed, it would be a clear example of two physiological functions (digestion and acid-base regulation) interacting with each other based on simple positive-negative feedback loops mediated by CO<sub>2</sub>/HCO<sub>3</sub><sup>-</sup>.

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