

Bicarbonate-sensing soluble adenylyl cyclase is an essential sensor for acid/base homeostasis

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pH homeostasis is essential for life, yet it remains unclear how animals sense their systemic acid/base (A/B) status. Soluble adenylyl cyclase (sAC) is an evolutionary conserved signaling enzyme that produces the second messenger cAMP in response to bicarbonate ions (HCO₃⁻). We cloned the sAC ortholog from the dogfish, a shark that regulates blood A/B by absorbing and secreting protons (H⁺) and HCO₃⁻ at its gills. Similar to mammalian sAC, dogfish soluble adenylyl cyclase (dfsAC) is activated by HCO₃⁻ and can be inhibited by two structurally and mechanistically distinct small molecule inhibitors. dfsAC is expressed in the gill epithelium, where the subset of base-secreting cells resides. Injection of inhibitors into animals under alkaline stress confirmed that dfsAC is essential for maintaining systemic pH and HCO₃⁻ levels in the whole organism. One of the downstream effects of dfsAC is to promote the insertion of vacuolar proton pumps into the basolateral membrane to absorb H⁺ into the blood. sAC orthologs are present throughout metazoans, and mammalian sAC is expressed in A/B regulatory organs, suggesting that systemic A/B sensing via sAC is widespread in the animal kingdom.

cAMP | pH | proton pump | dogfish | gill

Protons (H⁺) and bicarbonate ions (HCO₃⁻) play central roles in biology. Cellular enzymes are sensitive to pH, and CO₂/HCO₃⁻ buffer animal physiological fluids. To compensate for major acid/base (A/B) metabolic and environmental disturbances, animals have developed specialized epithelia to regulate pH and HCO₃⁻ concentrations of their extracellular fluids. The ion-transporting mechanisms in cells of A/B regulatory organs show remarkable similarities throughout animal phyla. Yet, a fundamental question has remained unsolved: How is A/B stress sensed? Like all biological sensors, an A/B sensor must be able to detect deviations from a set point and induce compensatory responses. As a signaling enzyme directly modulated by bicarbonate, soluble adenylyl cyclase (sAC) (1–3) represents a potential A/B sensor. sAC is distinct from the more widely studied, G protein-regulated sources of the ubiquitous second messenger cAMP, the transmembrane adenylyl cyclases (tmACs). sAC is not regulated by heterotrimeric G proteins and is distributed throughout the cell (1, 4). Consistent with a role as a putative A/B sensor, cAMP production by sAC (hereafter referred to as “sAC activity”) inside cells has been shown to reflect extracellular CO₂/HCO₃⁻ levels in mammalian sperm (5, 6), epididymis (7), and motile airway cilia (8).

Elasmobranchs (sharks, rays, and relatives) are cartilaginous fish that evolved ~400 million years ago (9). Their gills perform essentially all (>97%) systemic A/B relevant ion transport; neither kidney nor ventilatory adjustments play a major role (10). Elasmobranch gills (Fig. S1 A and B) have numerous acid- and base-secreting cells, which are functionally analogous to those in the mammalian kidney (11, 12). In the dogfish shark, the vacuolar proton pump (V-H⁺-ATPase; VHA) translocates from the cell cytoplasm into the basolateral membrane of gill base-secreting cells in response to blood alkalosis (12) (Fig. S1C). Basolateral VHA secretes protons into the blood and energizes apical bicarbonate secretion into seawater to counteract the alkalosis (12–14). This VHA translocation is essential for regu-

lating blood A/B during the alkaline tide (14), a natural postfeeding elevation in blood pH and bicarbonate that results from H⁺ secretion into the stomach (for food digestion) and concomitant bicarbonate absorption into the blood (15, 16). Consistent with a potential role for a sAC ortholog, the VHA translocation was shown to be dependent on generation of intracellular bicarbonate by carbonic anhydrase (CA) (14).

The aim of the current study was to identify the systemic A/B sensor in an animal. We demonstrate that an ortholog of sAC is expressed in dogfish gill and that its inhibition prevents compensatory responses to blood alkalosis both in isolated gills and in whole animals. Therefore, sAC represents a systemic A/B sensor in dogfish.

Results

Cloning Dogfish sAC. An EST from dogfish encoding dogfish soluble adenylyl cyclase (dfsAC) was identified by BLAST search using human sAC. The cDNA contained an ORF of 2,946 bp predicting a 985-aa protein of ~110 kDa (GenBank database accession no. ACA52542.1). dfsAC shares greatest sequence homology with the catalytic domains and presumptive P-loop regions of other sAC-like proteins (1, 17). Phylogenetic analysis comparing dfsAC with other nucleotidyl cyclases, including guanylyl cyclases and tmACs (Fig. 1), confirms dfsAC to be a sAC ortholog.

Recombinant dfsAC Is Bicarbonate-Responsive and Inhibited by sAC-Selective Small Molecule Inhibitors. We expressed and purified an N-terminal His-tagged recombinant protein encoding the putative two catalytic domains of dfsAC (amino acids 1–485) (Fig. 2A).

Recombinant dfsAC activity was stimulated by HCO₃⁻ with a half-maximal effect (EC₅₀) of ~5 mM (Fig. 2B). This EC₅₀ is consistent with the physiological [HCO₃⁻] in blood plasma of normal dogfish (~4 mM; Table 1). The difference in bicarbonate EC₅₀ between dogfish and mammalian sAC (11–25 mM) (2, 3, 18) reflects the differences in plasma [HCO₃⁻] between air and water breathers. Recombinant dfsAC showed a slight stimulation at alkaline pH (Fig. 2C); however, the pH dose response was nearly insignificant compared with the stimulation that would be induced by physiologically relevant increases in HCO₃⁻.

Historically, mammalian sAC activity was thought to be dependent on the divalent cation manganese (19), and Mn²⁺ sensitivity can be used to distinguish sAC-like cyclases from

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Data deposition: The sequence reported in this paper has been deposited in the GenBank database (accession no. ACA52542.1).

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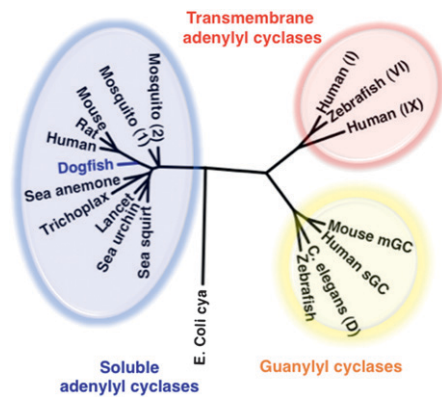


Fig. 1. Phylogenetic relation of *dfsAC* to other nucleotidyl cyclases. The GenBank accession numbers are as follows: (i) sACs: dogfish: *S. acanthias*, ACA52542.1; mouse: *Mus musculus*, NP_766617.1; rat: *Rattus norvegicus*, NP_067716.1; human: *Homo sapiens*, NP_060887.2; Trichoplax: *Trichoplax adhaerens*, XP_002117857.1 (hypothetical); Starlet sea anemone: *Nematostella vectensis*, XP_001623318.1 (predicted); mosquito (1): *Aedes aegypti*, XP_001661592.1 (hypothetical); mosquito (2): *Culex quinquefasciatus*, XP_001842661.1 (hypothetical); sea urchin: *Strongylocentrotus purpuratus*, NP_001020380.1; sea squirt: *Ciona intestinalis*, XP_002121952.1 (predicted); lancet: *Branchiostoma floridae*, XP_002214797.1 (hypothetical); (ii) guanylyl cyclases: *Caenorhabditis elegans*: guanylyl cyclase protein 28, isoform D AAC78238.2; zebrafish: *Danio rerio*, XP_001337045.2 (predicted); mouse: NP_001124165.1 guanylate cyclase 2d; human: *H. sapiens*, AAA60547.1 soluble retinal isoform; and (iii) tmACs: zebrafish (VI): XP_001922749.1 (predicted, isoform VI); human (I): *H. sapiens*, Q08828.2 (ADCY1); human (IX): *H. sapiens* O60503.4 (ADCY9); *E. coli cya*: AAA67602.1 (adenylate cyclase). Interestingly, sAC orthologs have not been identified in the genomes of some model organisms (e.g., *Drosophila melanogaster*, *D. rerio*, *C. elegans*), suggesting that they may use fundamentally distinct mechanisms for A/B sensing.

tmACs (19, 20). As expected, recombinant *dfsAC* activity is highest with millimolar Mn^{2+} as the divalent cation. Inhibitor profiles can also be used to distinguish sAC-like cyclases from tmACs; sAC-like cyclases are specifically inhibited by the small molecule KH7 (5, 21) and are more sensitive to catechol derivatives of estrogen (21, 22). Recombinant *dfsAC* activity was inhibited by KH7 and 4-catechol estrogen (4CE) with IC_{50} s of 9.6 and 46.9 μM , respectively (Fig. 2D). These values are similar to those for mammalian sAC (6, 7).

***dfsAC* Is Present and Active in Dogfish Gills.** We detected *dfsAC* mRNA in dogfish gill using RT-PCR. To confirm the presence of *dfsAC* protein in gill, we characterized the adenylyl cyclase activity in gill extracts and generated antipeptide polyclonal antisera. A subset of the total cAMP-forming activity in gill extracts qualitatively matched the biochemical characteristics of recombinant *dfsAC* (Fig. 3A and B): It is stimulated by HCO_3^- with an EC_{50} of ~ 3 mM and is inhibited by 4CE and KH7. Western blotting using antipeptide antiserum specific for *dfsAC* identified a single ~ 110 -kDa band in dogfish gills (Fig. 3C). *dfsAC* was detected in gill crude homogenates and also in gill supernatants. This anti-*dfsAC* antisera gave specific immunofluorescent staining in the cytoplasm of epithelial cells in the gill filament and lamellae (Fig. 3D–G). Some gill sections showed strong staining in pillar cells (Fig. 3G), which have been implicated with reversible hydration of CO_2 via extracellular CA and with gas exchange (23). Thus, *dfsAC* might be involved in regulating multiple physiological mechanisms in response to A/B disturbances. This widespread distribution of *dfsAC* at the gill is distinct from mammalian epididymis, where sAC is highly expressed only in the VHA-rich cells (7), but it is similar to mammalian nephron, where sAC is abundant in several segments and potentially has multiple functions (7, 24, 25).

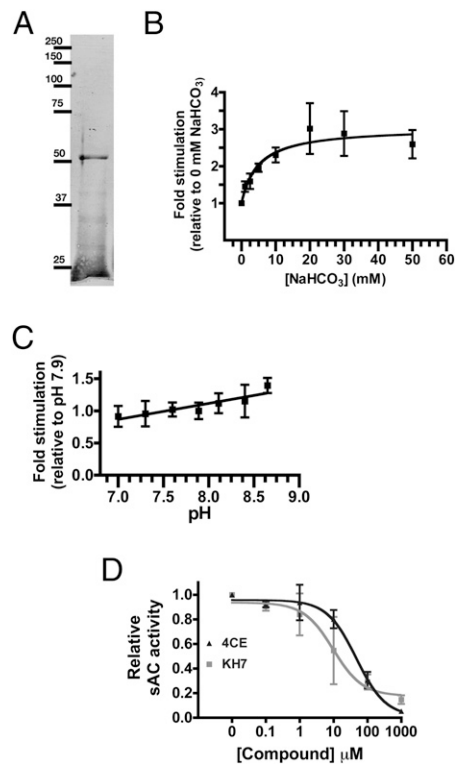


Fig. 2. Activity of recombinant *dfsAC*. (A) Coomassie blue-stained SDS/PAGE gel of His-tagged *dfsAC* (53 kDa expected) purified from *E. coli*. (B) *dfsAC* activity assayed at a constant pH of 7.75 in the presence of 2.5 mM ATP, 20 mM $MgCl_2$, 0.5 mM $MnCl_2$, and the indicated concentrations of $NaHCO_3$. $EC_{50} = 4.9$ mM $NaHCO_3$. (C) *dfsAC* activity at the indicated pH. (D) *dfsAC* activity in the presence of 2.5 mM ATP, 5 mM $MnCl_2$, and the indicated concentrations of KH7 or 4CE. IC_{50} for KH7 = 9.6 μM and IC_{50} for 4CE = 46.9 μM . Values are expressed as fold stimulation relative to the baseline in each experiment (0 mM HCO_3^- , pH 7.75, or no drug, respectively). Data points reflect the averages of four independent assays for each condition.

Pharmacological Inhibition of *dfsAC* Impairs Blood A/B Regulation In Vivo and in Isolated Gills. We used the two structurally and mechanistically unrelated inhibitors 4CE and KH7 to characterize *dfsAC* function in vivo. Basal blood pH and plasma $[HCO_3^-]$ were ~ 7.8 units and ~ 4 mM, respectively. After 12 h of i.v. $NaHCO_3$ infusion (1,000 $\mu Eq \cdot kg^{-1} \cdot h^{-1}$), control (DMSO-injected) fish reached steady-state values of pH = 7.97 and 8.3 mM $[HCO_3^-]$ (Table 1), similar to previously reported values (12–14). In fish injected with 4CE or KH7, blood pH increased to pH = 8.12 with 4CE and to pH = 8.22 with KH7 and plasma $[HCO_3^-]$ rose to 17 mM and 18 mM, respectively (Table 1). Thus, inhibition of *dfsAC* prevents dogfish from efficiently regulating their blood A/B. There was a trend for increased PCO_2 during *dfsAC* inhibition; however, it was not statistically different from that of control fish (Table 1). These experiments also demonstrate the effectiveness of sAC inhibitors in a whole-animal context.

sAC Inhibition Prevented VHA Translocation in Whole Animals and in Isolated Gills. To explore why inhibition of *dfsAC* deregulates blood A/B, we examined VHA translocation in gill cells by immunohistochemistry and Western blotting in membrane-enriched samples. In untreated fish, the predominant VHA immunostaining was cytoplasmic (Fig. 4A and B). On blood alkalosis, there was significant VHA translocation to the basolateral membrane (Fig. 4C and D) to reabsorb H^+ into the blood and compensate alkalosis (12–14). This VHA translocation was prevented by inhibition of *dfsAC* (Fig. 4E and F) by either of

Table 1. sAC inhibition prevents compensation of blood alkalosis in whole animals

	0			12 h		
	Control	KH7	4CE	Control	KH7	4CE
pH	7.77 ± 0.02 (a)	7.82 ± 0.06 (a)	7.84 ± 0.03 (a)	7.97 ± 0.03 (b)	8.22 ± 0.06 (c)	8.12 ± 0.04 (c)
[HCO ₃ ⁻]	4.1 ± 0.4 (a)	3.5 ± 1.1 (a)	3.4 ± 0.6 (a)	8.3 ± 0.8 (b)	17.9 ± 3.4 (c)	17.1 ± 4.4 (c)
PCO ₂	1.5 ± 0.2 (a)	0.9 ± 0.2 (a)	1.2 ± 0.3 (a)	2.1 ± 0.4 (b)	2.2 ± 0.4 (b)	2.8 ± 0.6 (b)

Blood pH, plasma [HCO₃⁻] (mM), and PCO₂ (torr) of dogfish infused i.v. with NaHCO₃ (1,000 μEq·kg⁻¹·h⁻¹) and injected with DMSO (control, *n* = 11), KH7 (*n* = 9), or 4CE (*n* = 7). KH7 and 4CE were injected as a 5-μmol·kg⁻¹ bolus at *t* = -0.5 and 6 h. Data were analyzed using repeated-measures two-way ANOVA. Different letters indicate significant differences (*P* < 0.05) within each variable using Bonferroni's posttest (if the interaction between treatment and time was significant) and the Tukey–Kramer planned least square means test (if the interaction was not significant). A summary of the statistics is shown in Table S1.

two structurally unrelated inhibitors, KH7 or 4CE. Inhibition of sAC during alkalosis resulted in fewer cells with VHA translocation (Fig. 4G) and less abundant VHA in cell membranes (Fig. 4H) compared with fish injected with DMSO alone.

To test whether VHA translocation in gills from alkalotic animals is the result of autonomous sensing at the gill or a response to signals originating at another organ, we selectively perfused and isolated gill fragments. As in the whole-animal experiments, VHA translocated to the basolateral membrane in response to perfusion of alkaline solutions (50 mM HCO₃⁻, pH 8.50) on the blood side but not on the seawater side (Fig. 5A and B). Also similar to the whole-animal experiments, VHA translocation was significantly reduced by sAC inhibition (Fig. 5A and C). Therefore, sAC-dependent A/B sensing appears to be autonomously controlled within the gill.

Discussion

In this paper, we have identified sAC to be a sensor for systemic A/B status in the dogfish shark by means of the following criteria:

- (i) HCO₃⁻-responsive dfsAC is abundantly expressed in the gill, the organ almost exclusively responsible for systemic A/B regulation in dogfish. In particular, it is expressed in the external epithelial layer, where the base-secreting and H⁺-reabsorbing VHA-rich cells are located.
- (ii) dfsAC in vitro affinity for HCO₃⁻ predicts that its activity will reflect physiologically relevant changes in plasma [HCO₃⁻].
- (iii) Two structurally and mechanistically unrelated pharmacological inhibitors prevented recovery from alkalosis in whole animals. This effect can be attributed at least partially to diminished translocation of VHA into the basolateral membrane of gill cells, as observed in whole animals and isolated gills.

In the absence of genetic manipulation, which is not yet possible in the dogfish shark, these data represent the strongest possible evidence pointing to sAC as an intrinsic gill sensor essential for systemic A/B homeostasis.

The His-tagged recombinant dfsAC that we generated consists almost exclusively of the two catalytic domains, which have been shown in all class III adenylyl cyclases to be important for conversion of ATP into cAMP. Because it lacks the C-terminus region present in full-length native dfsAC, it is possible that native dfsAC has additional regulatory properties compared with recombinant dfsAC. However, as seen with mammalian and bacterial sAC-like enzymes (2, 3, 17, 18, 22), the catalytic domains of dfsAC dictate its affinity for HCO₃⁻ and its sensitivity to the inhibitors used in this study.

Inhibition of dfsAC mimicked the effects on systemic A/B regulation and VHA translocation previously observed when CA was

inhibited (14) or when microtubule assembly and vesicle translocation were interrupted (13). Both CA (14) and sAC are widespread throughout the gill epithelium and likely present in the cytoplasm of the same cells. Therefore, we propose a combined role for CA, sAC, and VHA movement in A/B regulation. In our model (Fig. S1C), dfsAC is an intrinsic A/B sensor that detects elevations in intracellular [HCO₃⁻] generated from CO₂ by CA and dfsAC-generated cAMP triggers the microtubule-dependent translocation of VHA to the basolateral membrane. Other as yet unidentified mechanisms that are constitutively active and/or up-regulated during dfsAC inhibition (or incomplete dfsAC inhibition during our study) may also be present and could have moderated the A/B disturbances observed.

The cellular mechanism for A/B regulation in VHA cells of dogfish gills (Fig. S1C) is the mirror image (i.e., similar mechanism with opposite cellular polarity) to the one described in clear cells of isolated rat epididymis. Mammalian sAC inside clear cells senses elevations in luminal pH/HCO₃⁻ and, similar to dfsAC, triggers membrane accumulation of VHA (7). However, in the clear cells, sAC-generated cAMP induces VHA translocation to the apical membrane, where it secretes H⁺ to restore an acidic pH in the fluid of the lumen necessary to keep stored sperm quiescent (26). As in dogfish gill, inhibiting CA (7) or interrupting microtubule assembly (27) also blocked the A/B response in epididymis.

The kidney is the organ primarily responsible for metabolic A/B regulation in mammals, and it possesses both acid- and base-secreting cells (28). These cells are functionally analogous to epididymal clear cells and dogfish VHA-rich cells, respectively. Although there are no in vivo or in vitro functional studies reported for sAC in kidney, mammalian sAC has been shown to colocalize with VHA in both renal cell types (24). This association supports the model that CA, sAC, and VHA form a regulatory complex in diverse A/B sensing organs.

CA and VHA are implicated in A/B regulation throughout metazoans (28–30), and the presence of sAC in metazoans ranging from placozoans to mammals (Fig. 1) suggests that systemic A/B homeostasis via CA-sAC-VHA is conserved throughout animal physiology.

Materials and Methods

Animals. Pacific spiny dogfish *Squalus acanthias* L. were caught from the Trevor channel (Vancouver Island, BC, Canada) and immediately transferred to a 151,000-L circular tank provided with flowing seawater at the Bamfield Marine Sciences Centre (9–13°C, 30–32‰ salinity, normal photoperiod). Fish were offered dead hake, herring, and flatfish once a week until 3 days before the experiment, at which point fish were fasted. Forty-two dogfish were used for this study (mean ± SEM: 1.93 ± 0.12 kg). All experiments were performed following animal care protocol 531702 approved by the Biological Sciences Animal Care Committee at the University of Alberta.

Infusions. Dogfish were catheterized in the caudal vein and artery under anesthesia (12–14). To induce blood alkalosis without producing major osmotic or ionic disturbances, a 250-mM NaHCO₃ solution supplemented with

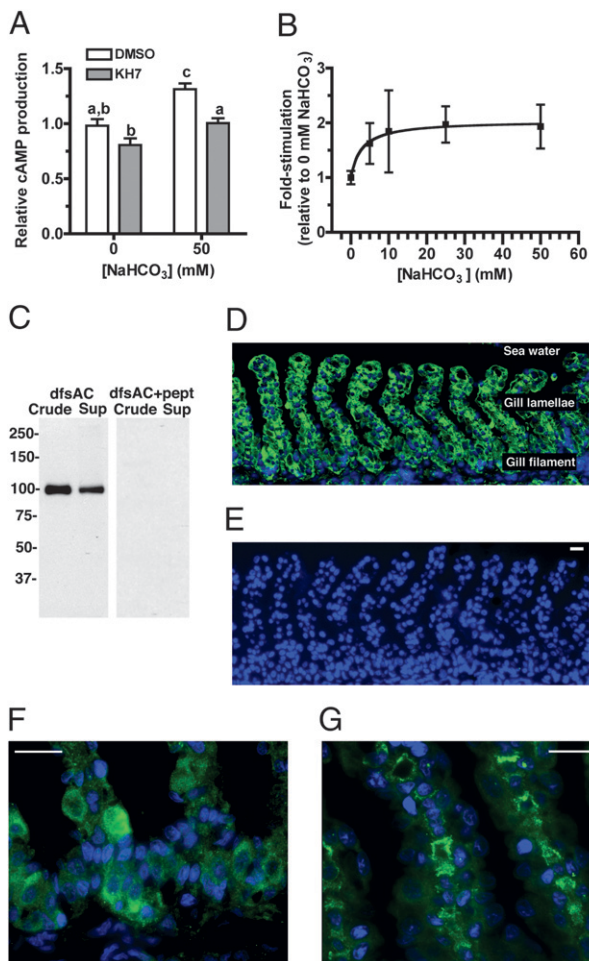


Fig. 3. dfsAC is present in the gill, and cAMP-producing activity in the gill matches recombinant dfsAC. (A) Adenylyl cyclase activity in gill homogenates measured in the presence 50 mM NaCl or NaHCO₃ in the presence or absence of 50 μM KH7. Shown are the averages of quadruple determinations with SEM. Equivalent results were obtained with 4CE. Lowercase letters denote levels of activity that are not significantly different from each other by repeated-measures two-way ANOVA ($P < 0.05$ for treatment, time, and interaction), Bonferroni's posttest. (B) KH7-sensitive cAMP activity in gill homogenates measured as indicated in A. $EC_{50} = 2.9$ mM NaHCO₃. (C) Western blot of dogfish shark gill homogenate (crude) and high-speed supernatant (sup) using anti-dfsAC in the absence or presence of immunizing peptide. (D) Immunofluorescence using anti-dfsAC (green) in dogfish gill. Nuclei were labeled with DAPI (blue). (E) Staining using anti-dfsAC antisera preabsorbed with excess immunizing peptide; similar results were obtained with preimmune serum. (F and G) Higher magnification pictures of dfsAC immunolabeling. (G) Immunolabeling in pillar cells. (Scale bars = 10 μm.)

250 mM NaCl was continuously infused into the vein using a peristaltic pump at a rate of 1,000 μEq·kg⁻¹·h⁻¹ (12). 4CE and the small molecule inhibitor KH7 (5 μmol·kg⁻¹) were also injected through the caudal vein catheter from a concentrated 5-mM stock in DMSO as a 1-mL·kg⁻¹ bolus. The targeted final concentration in the blood was 100 μM [based on ~5% blood volume per mass in marine elasmobranchs (31)]. A first dose was injected 30 min before the infusions, and a second dose was injected at $t = 6$ h to compensate for drug penetration into tissues, breakdown, and/or excretion. Control fish were injected with the same volume of DMSO alone (2% of blood volume, 0.1% of body mass).

Blood and Terminal Sampling. Blood sampling was performed as described previously (12–14). Briefly, 200-μL blood samples were withdrawn via the caudal vein catheter; blood pH was measured immediately using a thermostated Accumet microsize pH electrode model 13-620-94 (Fischer Scientific), plasma

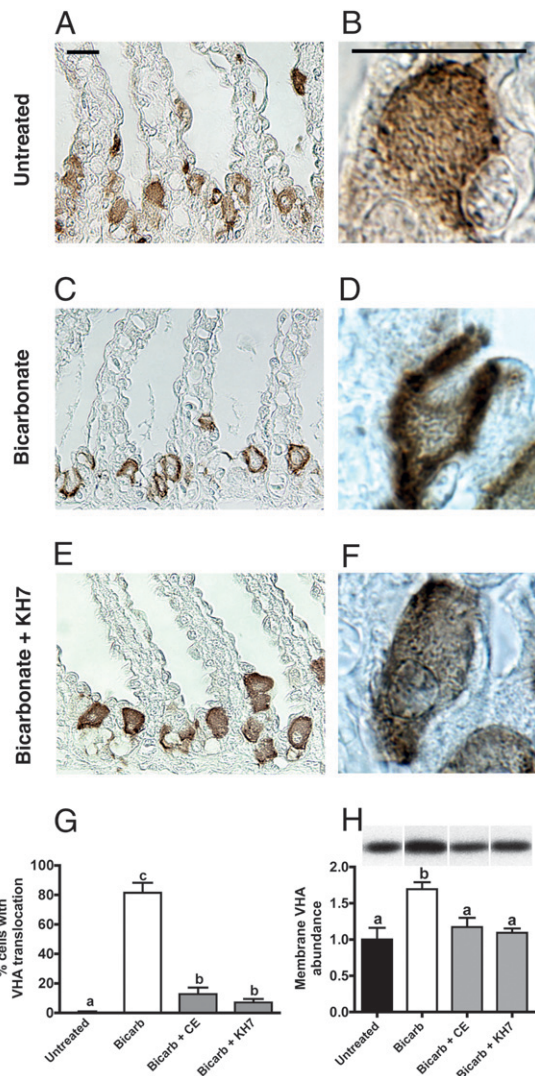


Fig. 4. sAC-dependent VHA translocation during systemic alkalosis in whole fish. Anti-VHA immunolabeling in dogfish gills. Low-power (A) and high-power (B) magnification of gills from untreated dogfish sharks. Low-power (C) and high-power (D) magnification of gills taken from dogfish shark after 12 h of continuous NaHCO₃ infusion. Low-power (E) and high-power (F) magnification of gills taken from dogfish shark after 12 h of continuous NaHCO₃ infusion and injected with KH7 (5 μmol·kg⁻¹ bolus at $t = -0.5$ and 6 h). (Scale bars = 10 μm.) (G) Percentage of cells showing VHA translocation in gill fragments ($n = 4$). (H) VHA abundance in membrane-enriched gill samples ($n = 5-8$). (Top) Bands are representative of each treatment. In G and H, letters indicate different levels of statistical significance by one-way ANOVA, Bonferroni's multiple comparison test.

was obtained after a 2-min spin, and total CO₂ was determined in a chamber (37°C) equipped with a CO₂ electrode (Radiometer) by the method of Cameron (32). [HCO₃⁻] was calculated using the solubility of CO₂, the apparent pK for dogfish at the experimental temperature, and rearrangements of the Henderson–Hasselbalch equation (33). Dogfish were killed by an overdose of tricaine methanesulphonate (TMS, AquaLife; Syndel Laboratories). Gill samples were snap-frozen in liquid N₂ and kept at -80°C until they were used in Western blotting experiments. Additional gill samples were immersed in ice-cold 3% (weight/vol) paraformaldehyde in 0.1 M Na⁺-cacodylate (pH 7.4) overnight at 4°C and stored in 70% (vol/vol) ethanol for immunohistochemistry.

Isolated Gill Experiments. Dogfish were anesthetized with TMS (1 g·L⁻¹), and the heart was exposed via pectoral/pericardial incision. Gills were perfused for at least 10 min via the bulbus arteriosus with ~1.5 L of dogfish saline (280 mM NaCl, 6 mM KCl, 5 mM NaHCO₃, 5 mM CaCl₂, 3 mM MgCl₂, 0.5 mM NaSO₄, 1 mM

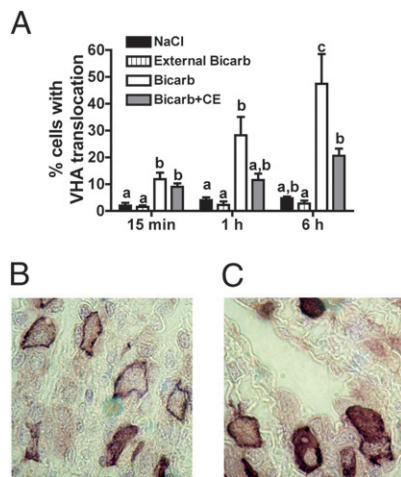


Fig. 5. sAC-dependent VHA translocation during localized alkalosis in isolated gill. (A) Percentage of cells showing VHA translocation in gill fragments internally perfused and incubated in alkaline saline with or without 100 μM 4CE ($n = 5$). A repeated-measures two-way ANOVA ($P < 0.05$ for treatment, time, and interaction) or the Tukey–Kramer planned least square means test was used. Letters indicate different levels of statistical significance. Bicarb, bicarbonate. Representative VHA immunostaining of gills internally perfused and incubated in alkaline saline and DMSO (B) or 4CE (C).

NaHPO_4 , 4 mM NaHCO_3 , 350 mM urea, 70 mM Trimethylamine N-oxide, 5 mM glucose, pH 7.80), with either 50 mM NaCl (control) or 50 mM NaHCO_3 (which raised pH to 8.43 ± 0.04) added. The final 50 mL contained 4CE (100 μM in DMSO) or carrier [DMSO alone, 2% (vol/vol)]. To test the effect of external $[\text{HCO}_3^-]$ on VHA translocation, gills were perfused with normal dogfish saline (5 mM NaHCO_3 , pH 7.80) and incubated in dogfish saline with 50 mM NaHCO_3 (pH: 8.42 ± 0.05). For cloning and activity experiments, gill samples were snap-frozen in liquid nitrogen. For quantification of VHA translocation in isolated gills, samples were dissected into three to four fragments of isolated filaments, which were placed in a small plastic basket and suspended inside an Erlenmeyer beaker almost completely filled with the corresponding saline solution (i.e., containing 4CE or DMSO). Beakers were sealed and maintained at 12°C , and the solutions were stirred using a magnetic agitator. At the end of the incubations (6 h), pH and TCO_2 in the saline solutions did not vary significantly between treatments and incubation times (repeated-measures two-way ANOVA). Gill samples were fixed for immunohistochemistry at the indicated times.

Cloning of dfsAC. A putative sAC homolog was identified via BLAST search from the *S. acanthias* multiple tissues EST library maintained by the Mount Desert Island Biological Laboratory (MDIBL). The putative dfsAC EST clone was sequenced by primer walking from a pcMV SPORT6.1 vector (Invitrogen). Sequences were aligned using Clustal W2 (34). Phylogenetic analysis on the alignment was performed using the maximum likelihood method from the PHYLIP software package (1,000 bootstrap replicates) (35). Treillustrator (Department of Molecular Biotechnology, Ghent University, Belgium) was used to draw the phylogenetic tree in a radial logarithmic format.

To demonstrate dfsAC expression in gill, total RNA was extracted from frozen gill samples using TRIzol (Invitrogen). cDNA was prepared by standard methods using gene-specific as well as oligo-dT primers. An initial round of PCR (35 cycles) was performed using 5'-AGTAAGATGAAGTTCGGAAAGCT-3' (base pairs 577–599) and 5'-ACAGAATAGCGGACCACTTGAACG-3' (base pairs 1219–1243) as primers. This PCR yielded a band of the expected size (666 bp) whose identity was confirmed to be dfsAC by nested PCR using 5'-CAACTGAATCTGTCTTCG-3' (base pairs 840–859) and 5'-AGATGCTCACTGACATATC-3' (base pairs 1173–1193) as primers (expected size of 353 bp) and direct nucleotide sequencing.

Production of Polyclonal Antibodies Against dfsAC. Antiserum against the peptide INNEFRNYQGRINKC (amino acids 342–355, located in putative catalytic domain 2) was generated in rabbits and affinity-purified (GeneScript). Specificity of the antiserum was confirmed by immunoblots of *Escherichia coli* BL21-AI transfected with the His-dfsAC plasmid before (control) and after induction with arabinose. Further specificity controls were performed on gill samples using peptide preabsorption and preimmune serum in immunoblots and immunohistochemistry.

Heterologous Expression and Purification of Recombinant dfsAC. All vectors used were from Invitrogen. The region corresponding to the putative catalytic domains of dfsAC (amino acids 1–485) was amplified from the pcMV SPORT6.1 vector containing full-length dfsAC by PCR, recombined into pENTR/TEV/D-TOPO vector, and shuttled into the pDEST17 vector, thus adding a 6 \times His tag to the N-terminus of the protein. Recombinant protein was produced in *E. coli* BL21-AI cells (pRARE) by induction with 0.1% arabinose for 3–4 h at 25°C . Cells were harvested by centrifugation, washed in 50 mM Tris and 1 mM EDTA (pH 8.0), and stored at -80°C . Pelleted cells were suspended in lysis buffer [50 mM Tris, 2 mM 3-thioglycerol, 300 mM NaCl, 2 mM imidazole, 1 mM PMSF, 20% (vol/vol) glycerol, pH 8.0] and lysed using an air-pressure cell disruptor (Avestin). The whole-cell extract was centrifuged at $31,000 \times g$ for 40 min, and the supernatant was passed through agarose nickel-resin (Qiagen). Recombinant dfsAC was eluted by increasing concentrations of imidazole. The eluates were subjected to SDS/PAGE, and recombinant dfsAC was identified by Coomassie blue staining, immunoblotting using the anti-dfsAC antisera, and detection with Ni^{2+} -HRP (HisProbe; ThermoScientific).

cAMP Assays Using Recombinant dfsAC and Gill Homogenates. cAMP production by purified recombinant protein and in gill homogenates was determined using the Correlate-EIA Direct Assay (Assay Designs, Inc.).

Purified recombinant dfsAC protein was incubated in assay buffer (50 mM Tris, 2.5 mM ATP, 20 mM MgCl_2 , 0.5 mM MnCl_2 or 5 mM MnCl_2 , 1 mM DTT) at 30°C for 30 min. Changes in pH produced by increasing $[\text{HCO}_3^-]$ were compensated by altering the pH of the Tris buffer to ensure that added bicarbonate resulted in a final pH of 7.75 in all cases. Reactions were stopped by addition of an equal volume of 0.2 N of HCl. Recombinant dfsAC from two different protein purifications were each assayed twice at the indicated pH or in the presence or absence of the indicated concentrations of HCO_3^- and DMSO, KH7, or 4CE (both of the latter dissolved in DMSO).

Aliquots of gill “crude homogenates” were incubated for 30 min at room temperature in an orbital shaker (500 rpm) in 150 mM Tris (pH 7.5), 2.5 mM ATP, 20 mM MgCl_2 , 0.25 mM isobutylmethylxanthine (IBMX), 3 mM DTT, 20 mM creatine phosphate, and 100 $\text{U}\cdot\text{mL}^{-1}$ creatine phosphokinase. NaHCO_3 was added from freshly prepared concentrated stocks. The incubations were stopped by addition of HCl to 0.1 N, and cAMP produced was quantified as described above.

Immunohistochemistry. Fixed gill samples were dehydrated in an increasing ethanol series, embedded in paraffin, and sectioned at 6 μm (12–14). Deparaffinized sections were treated with 1% SDS in PBS, followed by 2×5 -min washes in PBS. The anti-VHA antibodies (mouse A-subunit) were a kind gift from Dennis Brown (Massachusetts General Hospital and Harvard Medical School, Harvard University, Boston, MA). Incubation with the anti-VHA antibodies (1:1,000 dilution in 2% (vol/vol) normal goat serum in PBS) was performed overnight at 4°C ; detection was performed using the diaminobenzidine-based kit ImmPress (Vector Labs). Qualitatively similar results were obtained with antibodies against killifish VHA A and B subunits (gifts from Fumi Katoh, University of Alberta, Edmonton, AB, Canada, and J.B. Claiborne, Georgia Southern University, Statesboro, GA, respectively), but no quantification was performed. For dfsAC detection by immunofluorescence, samples were incubated in 50 mM NH_4Cl in PBS for 5 min (after 1% SDS treatment and wash in PBS) to quench autofluorescence. Sections were incubated with the anti-dfsAC antibodies (1:200) overnight at 4°C , detection was performed using highly cross-absorbed Alexa-fluor 488-conjugated goat anti-rabbit antibodies (1:500; Invitrogen), and sections were mounted using DAPI-containing medium (Vector Labs).

Western Blotting of dfsAC and Quantification of VHA Translocation. Frozen gill samples were homogenized in 10 volumes of lysis buffer (250 mM sucrose, 1 mM EDTA, 30 mM Tris, 100 $\mu\text{g}\cdot\text{mL}^{-1}$ PMSF, 10 $\mu\text{g}\cdot\text{mL}^{-1}$ leupeptin, 10 $\mu\text{g}\cdot\text{mL}^{-1}$ aprotinin, pH 7.75). Tissue was ground in liquid N_2 , sonicated 2×15 s, and centrifuged ($3,000 \times g$, 10 min, 4°C) to remove debris. Supernatant aliquots (“crude homogenate”) were saved for cAMP assays (see above). The remaining supernatant was centrifuged ($20,000 \times g$, 30 min, 4°C), and pellets were resuspended in lysis buffer and used for Western blotting. Samples were mixed with an equal volume of $2 \times$ Laemmli buffer (with 2 mM fresh DTT) and heated (15 min, 70°C). Twenty μg of total protein from each sample (estimated by Coomassie blue stain of SDS/PAGE) was separated by SDS/PAGE; transferred to PVDF membranes, which were blocked in 5% (weight/vol) milk-TBST for 30 min at room temperature; and incubated with anti-VHA antibodies (1:5,000 in 5% milk in TBST) or anti-dfsAC (1:5,000 in 5% milk in TBST) overnight (4°C), followed by 1 h of incubation with HRP-conjugated goat anti-rabbit antibodies. Membranes were washed (3×20 min) between steps. Bands were detected by chemiluminescence, and VHA was quantified by densitometry.

Quantification of Cells with VHA Translocation. Dogfish gill sections from the different experiments (whole animal and isolated gills) were immunostained for VHA, coded, and examined by an experimenter in a “blind” fashion. The total number of VHA-rich cells and those displaying distinct VHA translocation (defined as cells with strong basolateral VHA staining and a clear cytoplasm; see Fig. 4C for examples) were recorded. At least 500 cells from several gill filaments from five different fish from each treatment were analyzed. Percentages were transformed to the arcsine of the squared root of the value before statistical analysis.

Statistics. All data are given as means \pm SEM. Statistical significance was set at $P < 0.05$. Statistical analyses were performed using GraphPad Prism v.4.0a for Macintosh (Graphpad Software) and Statistica 7 (Statsoft Pacific).

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