

RESEARCH ARTICLE

Soluble adenylyl cyclase coordinates intracellular pH homeostasis and biomineralization in calcifying cells of a marine animal

 William Weijen Chang,¹ Angus B. Thies,²  Martin Tresguerres,² and Marian Y. Hu¹

¹Institute of Physiology, Christian-Albrechts-Universität zu Kiel, Kiel, Germany and ²Scripps Institution of Oceanography, University of California San Diego, California, United States

Abstract

Biomining cells concentrate dissolved inorganic carbon (DIC) and remove protons from the site of mineral precipitation. However, the molecular regulatory mechanisms that orchestrate pH homeostasis and biomineralization of calcifying cells are poorly understood. Here, we report that the acid-base sensing enzyme soluble adenylyl cyclase (sAC) coordinates intracellular pH (pH_i) regulation in the calcifying primary mesenchyme cells (PMCs) of sea urchin larvae. Single-cell transcriptomics, in situ hybridization, and immunocytochemistry elucidated the spatiotemporal expression of sAC during skeletogenesis. Live pH_i imaging of PMCs revealed that the downregulation of sAC activity with two structurally unrelated small molecules inhibited pH_i regulation of PMCs, an effect that was rescued by the addition of cell-permeable cAMP. Pharmacological sAC inhibition also significantly reduced normal spicule growth and spicule regeneration, establishing a link between PMC pH_i regulation and biomineralization. Finally, increased expression of sAC mRNA was detected during skeleton remineralization and exposure to CO₂-induced acidification. These findings suggest that transcriptional regulation of sAC is required to promote remineralization and to compensate for acidic stress. This work highlights the central role of sAC in coordinating acid-base regulation and biomineralization in calcifying cells of a marine animal.

calcification; cAMP; ocean acidification; primary mesenchyme cells; skeletogenesis

INTRODUCTION

The shells and skeletons of major marine calcifying animals are primarily composed of calcium carbonate (CaCO₃; 1). The required CO₃²⁻ ions are obtained from seawater in the form of HCO₃⁻ or by the hydration of metabolic CO₂ (2). Accordingly, CO₃²⁻ precipitation releases H⁺ that must be promptly removed from the site of biomineralization to protect pH homeostasis and sustain high biomineralization rates. Indeed, pH regulation and biomineralization are intrinsically linked in all calcifying invertebrates, including corals (3), mollusks (4), brachiopods (5), and echinoderms (6). Moreover, this principle also applies to the inner ear of teleost fish and their otoliths (7), to mammalian teeth (8), and even to calcifying phytoplankton (9). However, the mechanisms that coordinate acid-base-relevant ion transport and biomineralization are not completely understood, especially at the molecular and cellular levels.

The sea urchin larva develops an elaborate endoskeleton composed of magnesium-rich calcite. Radioisotopic labeling using ¹⁴C demonstrated that ~60% of the dissolved inorganic carbon (DIC) used for skeletogenesis derives from metabolic CO₂, whereas the remaining 40% is absorbed from seawater (2). Biomineralization is initiated by the formation of amorphous calcium carbonate (ACC) within the vesicles of the

calcifying primary mesenchyme cells (PMCs; 10). These cells form syncytial cables that surround the endoskeleton and are proposed to exocytose ACC to the growing spicule where the crystallization of calcite takes place (11). During this process, each mole of CO₃²⁻ that is precipitated generates 1.6 moles H⁺, which need to be removed to protect PMCs from acidification. This requires cellular proton export rates that are comparable to those of acid-secreting osteoblasts and parietal and renal tubular cells (12). Accordingly, PMCs express a suite of acid-base-relevant ion transporting proteins, including Na⁺/HCO₃⁻ cotransporter (NBC; 6, 13, 14), Na⁺/H⁺-exchangers (NHEs), V-type H⁺-ATPase (VHA; 6), and the H⁺-selective channel Otopetrin (Otop2L; 15). Gene expression analyses and live cell experiments combined with pharmacological and genetic knockdown revealed that NBC (Sp-Slc4a10) together with the extracellular carbonic anhydrase Cara7 is the main pathway for DIC uptake (16), and that Otop2L plays a central role in H⁺ removal from the cell (15). The existence of diverse acid-base transporters in PMCs implies the need to dynamically adjust DIC and H⁺ transport in PMCs to match biomineralization rates.

The soluble adenylyl cyclase (sAC; *adcy10*) has been demonstrated to coordinate cellular ion transport in acid-base regulatory epithelia and calcifying cells of marine organisms (17–19). This enzyme is stimulated by HCO₃⁻ to produce the

ubiquitous messenger molecule cyclic adenosine monophosphate (cAMP), and in association with carbonic anhydrases (CAs), sAC can also act as a CO₂ and pH sensor (20). Given its presence in diverse organisms ranging from cyanobacteria to mammals, sAC has been deemed as an evolutionarily conserved acid-base sensor (21, 22). The role of sAC as a molecular acid-base sensor seems especially prominent in aquatic organisms, perhaps due to their relatively low buffering capacity and ensuing large acid-base disturbances from metabolic and environmental origins (23, 24). The role of sAC is best characterized in fish gill ionocytes, where it contributes to blood acid-base homeostasis (19, 25–27). The sAC-dependent mechanism is independent of any autocrine or paracrine regulation; instead, sAC senses the acid-base status inside each gill ionocyte and triggers the activation of VHA to transport H⁺ from the cell into the blood (19, 25) and, potentially, the activation of anion exchangers to excrete HCO₃[−] into seawater (23, 28). Furthermore, sAC-dependent modulation of VHA has been reported in mammalian epididymis (29) and kidney (30), suggesting a universal sensor-effector-coupled mechanism in acid-base regulatory epithelial cells. In addition, sAC has been implied to regulate intestinal carbonate precipitation in marine fish (20, 31), otolith formation in the fish inner ear (17), and coral skeletal formation (18). In the latter, the functional role of sAC was evidenced by reductions in both the pH of the extracellular calcifying medium located in between the calcifying cells and the skeleton and the skeletal deposition rate upon pharmacological application of the sAC-specific small molecule inhibitor KH7 (18). After rat, mouse, and human (32), sea urchin was the first animal in which sAC was found and a physiological function was characterized (33). Similar to many other animals, sea urchin sAC (suSAC) modulates sperm motility and the acrosome reaction (33, 34), but information regarding the function of this enzyme in the calcifying PMCs remains unknown. Thus, this study investigates the potential role of suSAC in coordinating PMC intracellular pH (pH_i) regulation and skeletal formation (35). First, we examined the presence of suSAC mRNA and protein in PMCs. Next, we determined the role of suSAC in PMC pH_i regulation through live cell-imaging experiments using the pH-sensitive ratiometric dye 2',7'-bis-(2-carboxyethyl)-5-(and-6)-carboxyfluorescein acetoxymethyl ester (BCECF-AM) and two structurally unrelated small molecules that specifically inhibit sAC, KH7, and TDI-8164, respectively. Finally, we explored the functional role of suSAC in skeletal formation through biomineralization assays in control, remineralizing, and ocean acidification (OA)-exposed sea urchin larvae. Overall, the results established the role of sAC in coordinating pH_i regulation and biomineralization in sea urchin larvae.

MATERIALS AND METHODS

Experimental Animals

Adult purple sea urchins (*Strongylocentrotus purpuratus*) were collected from La Jolla (CA) and shipped to the Helmholtz Centre for Ocean Research GEOMAR (Kiel, Germany). Animals were maintained in a recirculating seawater system (10°C, pH 8.1; 12/12-h light/dark cycle); fertilization and larval cultures were performed as previously

described (13, 36). At various days post fertilization (dpf), larvae were collected for gene expression, pH_i regulation, and skeletal development analyses. The remineralization experiments involved exposing 3 dpf larvae to filtered seawater (FSW) with pH 6.0 [adjusted with 0.03 M 2-(N-morpholino) ethanesulfonic acid (MES)] for 12–15 h (37). After the dissolution of the skeleton (decalcification), the (4 dpf) larvae were transferred back to FSW (pH 8.1) to recalcify their skeletons for 3–4 days; during this period, samples were collected daily for gene expression and skeletal development analyses. cDNA samples for gene expression analyses of larvae exposed to control and CO₂-acidified seawater were obtained from a previous study (14). In brief, pH and Pco₂ of the culture seawater were maintained by a computer-controlled pH-stat system (Aqua Medic, Bissendorf, Germany), and the carbonate system was calculated based on pH (NBS scale) and total dissolved inorganic carbon measurements. The exposures were conducted in FSW bubbled with CO₂ to achieve 1) pH 8.1, 400 μatm Pco₂; 2) pH 7.7, 1,400 μatm Pco₂; and 3) pH 7.5, 2,000 μatm Pco₂.

At Scripps Institution of Oceanography, adult sea urchins were maintained in a flow-through seawater system (12°C, pH~8.0, 12:12-h light/dark cycle); fertilization was performed as previously described (35). Larvae were raised for 3.5 days in constantly stirred FSW at 12°C before Western blotting or immunocytochemistry procedures.

Bioinformatics, RT-qPCR, and Whole Mount In Situ Hybridization

The protein sequence of sea urchin soluble adenylyl cyclase (sAC; NP_001020380) was blasted to the National Center for Biotechnology Information (NCBI) database (<https://www.ncbi.nlm.nih.gov>), and sAC homologous with 0 E-value plus four mammalian homologous (E-value e^{−114} to ^{−110}) were selected for phylogenetic analysis. Selected sequences were processed through MUSCLE alignment and trimmed using Gblocks Server. The maximum likelihood tree was generated by MegaX (38) with the best fit model LG + G and 500 bootstrap replicates.

Quantitative reverse transcriptase PCR (RT-qPCR) and cloning were performed as previously described (14). Gene expression levels were normalized to the reference gene *eflα* (LOC548620), which is stable during normal larval development and remineralization (6). Whole mount in situ hybridization was performed as previously described (14). The primers used for RT-qPCR and the probes used for in situ hybridization are listed in Table 1.

Single-Cell Transcriptomics

Single-cell transcriptomic analyses were performed as recently described (15) on larvae raised under control conditions at mesenchyme blastula [1 day post fertilization (1 dpf)], late gastrulation (2 dpf), and pluteus (3 dpf) stages. In brief, embryonic/larval cells were gently dissociated by pipetting until reaching >95% single-cell suspension (39). Dissociated single cells were washed twice in calcium-free seawater (CFSW), checked for viability (>95%) by propidium iodine staining, fixed in 90% ice-cold methanol, and stored at −20°C. Cells were rehydrated in a descending methanol series and processed according to Single Cell 3' Reagent Kits

Table 1. The primer sequences of RT-qPCR analysis and hybridization probe cloning

	Gene	Primer Sequence	Product Size, bp	Primer Efficiency Score
Hybridization probe	<i>adcy10</i> (LOC574069)	F-CGGCTTTCGACTGGAGCTTA R-CCTGCATCACTCACACCCTT	764	NA
qPCR primers	<i>adcy10</i> (LOC574069)	F-GCAGGCGATGCTTTTCTAGC R-CTATGGTCGTGACTCGGACG	184	85.55%
	<i>efla</i> (LOC548620)	F-CCGACCTTGGAAAGGGATCG R-ACAGTCGGCTGTGAGGTTT	194	71.13%

qPCR, quantitative PCR.

v. 3 User Guide (10x Genomics). Sequencing of libraries was performed on an Illumina HiSeq system resulting in an average of 23.015 raw reads per cell barcode (Illumina, San Diego, CA). Raw reads were demultiplexed, aligned to *S. purpuratus* genome 5.0 (retrieved from Echinobase; 40), and counted using the Cell Ranger pipeline from 10x Genomics (version 3.0.2). Basic uniform manifold approximation and projection (UMAP) dimensional reduction and clustering were performed using the Seurat v. 3.2 workflow (41, 42). Expression of *suSAC* mRNA levels in control and morphants for Alx1 was analyzed from data generated in Rafiq et al. (43) using the NCBI SRA BLAST service.

Western Blotting

Strongylocentrotus purpuratus larvae (~25 mg) were added to 250 μ L of 2 $\times$ Laemmli buffer (Bio-Rad, Hercules, CA) with 5% (vol/vol) β -mercaptoethanol. Larvae were homogenized via gentle pipetting and 20 s of sonication on ice before denaturing at 95°C for 5 min. Larval homogenate (32.4 μ g) was loaded into a SDS/PAGE gel. Following electrophoresis, proteins were transferred onto a PVDF membrane using a Mini Trans-Blot cell (Bio-Rad) overnight. The PVDF membrane was incubated in blocking buffer [10% powdered fat-free milk in Tris-buffered saline-Tween 20 (TBS-T; BB_{WB})] (1 h at room temperature on an orbital shaker), and then incubated with the anti-sAC antibody (0.55 μ g·mL⁻¹ in BB_{WB}) or peptide-pre-absorbed anti-sAC antibody (60 $\times$ excess peptide in BB_{WB}; 4 h at room temperature on an orbital shaker). The PVDF membrane was washed (3 $\times$ 10 min in TBS-T) before incubation with goat anti-rabbit-horseradish peroxidase (HRP) antibodies (Bio-Rad; 1:10,000 in BB_{WB}; 1 h at room temperature on an orbital shaker). The PVDF membrane was again washed (3 $\times$ 10 min in TBS-T) before development with ECL Prime Western Blot Detection Kit (GE Healthcare, Chicago, IL) and imaging in a ChemiDoc Imaging System (Bio-Rad).

Immunofluorescence

Strongylocentrotus purpuratus larvae were fixed in 4% (vol/vol) paraformaldehyde in fixation buffer [0.2 M NaCl, 0.06 M 4-(2-hydroxyethyl)-1-piperazinepropanesulfonic acid (EPPS), in FSW] on an orbital shaker (15°C, 20 min). Fixed larvae were washed in FSW (6 $\times$ 5 min), then in PBS (3 $\times$ 5 min) before incubation with blocking buffer [BB_{IHC}; 5% (wt/vol) bovine serum albumin in PBS; 1 h at room temperature]. Next, larvae were incubated with either rabbit polyclonal anti-sAC antibodies (5.5 μ g·mL⁻¹ in BB_{IHC}), peptide-preabsorbed anti-sAC antibodies (20 $\times$ excess peptide in BB_{IHC}), or BB_{IHC} alone (overnight at 4°C on a rotator). The anti-sAC antibodies recognize a 14-amino acid peptide in the second catalytic domain of sAC from the coral *Acropora digitifera*

(LPGDKHEDDPARAL; 44), which is 86% identical to the matching region in *suSAC* (LPGYKHEDDCARAL). Anti-sAC antibody and peptide were produced by Genscript Biotech (Piscataway, NJ). Larvae were washed to remove unbound primary antibody (4 $\times$ 5 min in PBS), and incubated with goat anti-rabbit Alexa Fluor 555 antibodies (4 μ g·mL⁻¹ in BB_{IHC} containing 1 μ g·mL⁻¹ DAPI to label DNA; 1 h at room temperature on an orbital shaker). After washing (4 $\times$ 5 min in PBS), larvae were mounted on a depression slide and imaged on a ZEISS Axio Observer Z1 microscope connected to a laser scanner equipped with 405- and 561-nm laser lines (ZEISS LSM 800 with Airyscan, Carl Zeiss AG). *suSAC* and DAPI signals were visualized using goat anti-rabbit Alexa Fluor 555 (Invitrogen, Waltham, MA) and DAPI stain (Invitrogen), respectively (Alexa Fluor 555—ex: 561 nm, em: 540–700 nm; DAPI—ex: 405 nm, em: 400–600 nm). Three-dimensional (3-D) reconstructions of z-stacks were generated using Imaris 9.0 (Bitplane, Zurich, Switzerland). sAC and DAPI signals are presented using the false colors orange and blue, respectively, in all figures.

pH_i Imaging

Measurements of intracellular (pH_i) were performed using the cell-permeable pH-sensitive dye 2',7'-bis-(2-carboxyethyl)-5-(and-6)-carboxyfluorescein acetoxymethyl ester (BCECF-AM) as previously described (12, 13). Larvae were mounted in a perfusion chamber with constant flow (5 mL·min⁻¹), and the temperature of the perfusion solutions was maintained at 12°C by a water jacket cooling system. The fluorescence ratio of BCECF was calibrated within PMCs using a high [K⁺] (150 mM)/10 μ M Nigericin calibration solution adjusted to different pH values. Intracellular acidosis was induced by a 20 mM NH₄Cl prepulse in FSW followed by washout in FSW during which pH_i recovery rate was determined based on the linear slope of the curve and expressed as pH units·min⁻¹. The washout solutions contained either 0.1% DMSO (carrier control) or sAC-specific specific small molecule inhibitors, KH7 (20, 45) or TDI-8164 (46). KH7 was purchased from Merck (Kenilworth, NJ), whereas TDI-8164 was a generous gift from Drs. Lonny Levin and Jochen Buck (Weill Cornell Medical College). Both sAC inhibitors were dissolved in DMSO. Experiments to rescue sAC activity used cell membrane-permeable dibutyryl cAMP (db-cAMP; Merck; 100 μ M) in conjunction with 40 μ M KH7 in the washout solution. Measurements were performed in an alternating mode between control and inhibitor-treated larvae and five to nine larvae were measured for every experimental condition.

Skeletal Development and Remineralization

To quantify skeletal development, control and remineralizing larvae were fixed in 4% PFA in FSW and imaged on an inverted microscope (ZEISS, Axiovert A1) equipped

with an Axio cam 105 camera as previously described (6, 15). Body rod length (a proxy for spicule growth) in 2–4 dpf larvae was measured and analyzed using the ZEN 3.2 blue (ZEISS) software. Growth and regeneration rates were calculated based on the average daily increase in body rod length over the 4-day period and expressed in micrometer per day. To test the role of sAC in biomineralization, control or remineralizing larvae were incubated in the presence of 0.1% DMSO or various concentrations of KH7. Three independent replicates were performed for each inhibitor concentration in six well plates. From each well, 40–166 larvae were analyzed, averaged, and treated as one replicate leading to a final $n = 3$.

Statistical Analyses

Prism 6 (GraphPad, San Diego) was used to conduct all statistical analyses and generate all graphs. All data were normally distributed and passed equal variance tests (Levene's test). The effects of pharmacological treatments on gene expression, calcification rate, and pH_i regulation were performed using one-way ANOVA followed by Holm–Sidak post hoc test. All data are presented as mean and error bars indicate the means $\pm$ standard error (SE).

RESULTS AND DISCUSSION

Identification and Localization of s_{u} sAC Expression in the Developing Sea Urchin Larva

Phylogenetic analysis demonstrated that s_{u} sAC forms a clade with sAC orthologues from other invertebrates ranging from sponges over mollusks to basal deuterostomes. This sAC clade of invertebrates formed a separate branch from vertebrate sACs (Fig. 1A). Single-cell transcriptomics were applied to analyze spatiotemporal expression of s_{u} sAC mRNA in mesenchyme blastula (24 h post fertilization; hpf), late gastrula (40 hpf), and pluteus (72 hpf) stage larvae (Fig. 1B). Specification of the calcifying primary mesenchyme cells (PMCs) takes place during mesenchyme blastula stage (47). At this stage, s_{u} sAC mRNA expression was already evident in a few PMCs (Fig. 1B). During the late gastrulation stage, s_{u} sAC mRNA was primarily expressed in the PMC cluster that is associated with the initiation of spiculogenesis (Fig. 1B). The pluteus larval stage is characterized by spicule extension (47), and also in this stage, s_{u} sAC mRNA expression was highest in PMC clusters of the postoral tips and the middle section of the body rods (Fig. 1B). Lower expression levels of s_{u} sAC were detected in the gut and ectodermal cells. These results were supported by in situ hybridization of s_{u} sAC with an antisense probe targeting the catalytic domain

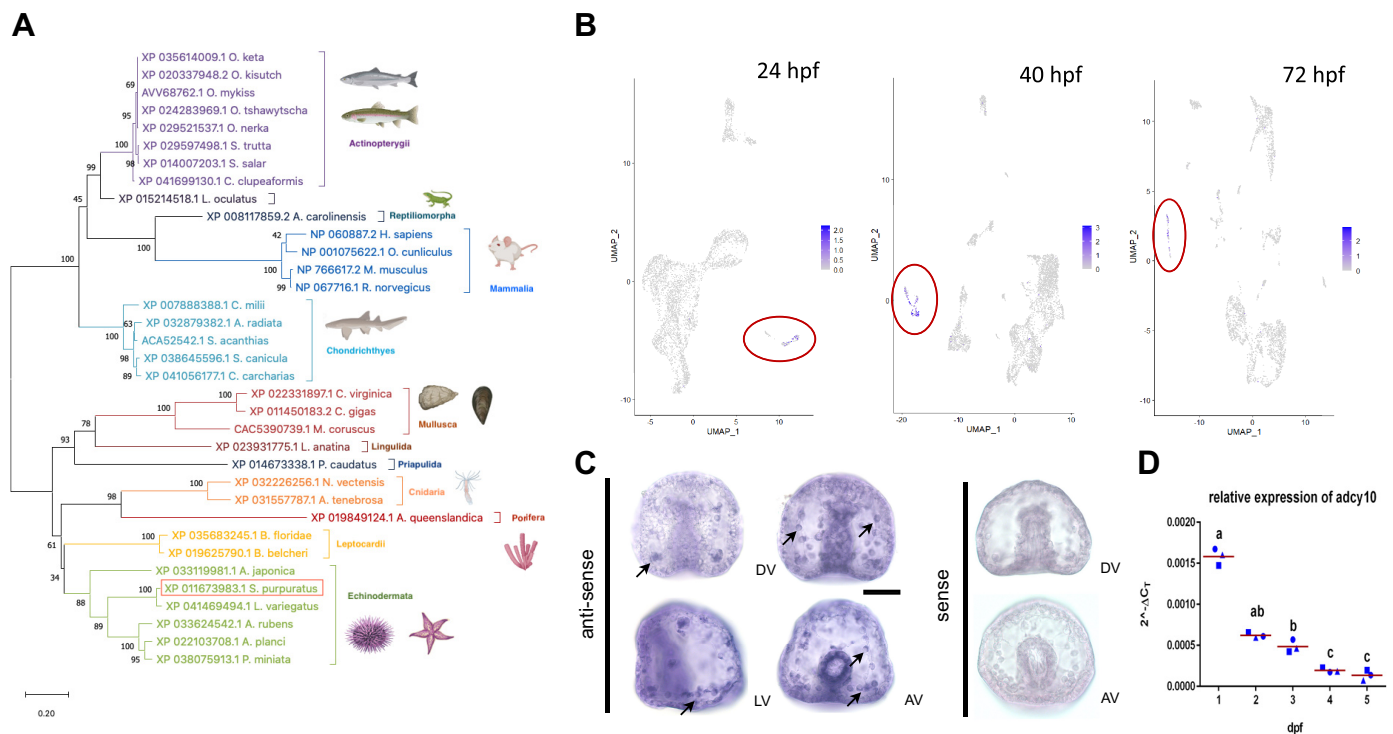


Figure 1. Phylogenetic analysis and expression of s_{u} sAC in the developing sea urchin larva. **A:** 34 homologous of s_{u} sAC, including invertebrates and vertebrates, were blasted and filtered from NCBI database to generate a maximum likelihood tree. Bootstrap values of the branches were marked on the nodes. The branch lengths are proportional to the amino acid divergence per site (see scale bar). **B:** principal component analysis of single-cell RNA-seq results from 24, 40, and 70 hpf larvae. Clusters were annotated based on overlaps between cluster-enriched peaks of s_{u} sAC (*adcy10*) and genes with known cell-specific activity. Normalized counts of *adcy10* are labeled from light blue to dark blue with increasing expression levels. PMC cluster is indicated by red oval. **C:** in situ hybridization of *adcy10* in 2 dpf larvae cultured under hypercapnic conditions of pH 7.5. An antisense probe targeting to *adcy10* catalytic domain sequence was applied to the hybridization, and a sense probe served as a negative control. Arrows indicate the PMCs with positive *adcy10* signals. Scale bar = 50 μ m. **D:** the developmental mRNA expression level of *adcy10* from 1 to 5 dpf larvae. Different letters represent significant differences. $n = 3$; one-way ANOVA; $P < 0.05$; Turkey's test. AV, anal view; dpf, day post fertilization; DV, dorsal view; hpf, hour post fertilization; LV, lateral view; NCBI, National Center for Biotechnology Information; PMC, primary mesenchyme cell; s_{u} sAC, sea urchin soluble adenylyl cyclase; UMAP, uniform manifold approximation and projection.

sequence (Fig. 1C). Along the first days of the development, gene expression analyses detected a steep decrease in $_{su}SAC$ mRNA levels between 1 and 5 dpf (Fig. 1D). This observation stands in contrast to a previous study that reported a steep increase in $_{su}SAC$ mRNA abundance in the initial hours after fertilization, peaking at the gastrula to prism stage, where development of the gut and the skeleton is initiated (35). We repeated these experiments to confirm our measurements and think that differences between these studies may be associated with the selection of different housekeeping genes or with the use of primers targeting different domains of the SAC gene. The PMC developmental gene regulatory network is well studied, and the regulatory transcription factors *Alx1* and *Ets1* are essential for the specification of calcifying primary mesenchyme cells before mesenchyme ingression and subsequent skeletogenesis (43, 48). Interestingly, $_{su}SAC$ mRNA levels are significantly decreased in *Alx1* morphants but not in *Ets1* morphants (Supplemental Fig. S1, <https://doi.org/10.6084/m9.figshare.21578724.v1>). This suggests that $_{su}SAC$ mRNA expression is under *Alx1* control and downstream $_{su}SAC$ activity is required for the proper functionality of the calcifying PMCs.

The SAC gene is characterized by extensive alternative splicing resulting in multiple protein isoforms with diverse structural domains and regulatory properties (49). In mammals,

the most active SAC isoform is truncated SAC (SAC_T), a ~48-kDa protein composed of two catalytic domains that are both essential for HCO_3^- -stimulated cAMP production (32). Here, we found that sea urchin larvae predominantly express a ~45-kDa truncated SAC protein ($_{su}SAC_T$; Fig. 2A). The anticipated size of full-length $_{su}SAC$ is ~209 kDa, and the predicted peptide size of $_{su}SAC$ catalytic domains is ~56 kDa. Although this predicted peptide is larger than what we detected by our Western blot analysis, it is very likely that alternative splicing produces $_{su}SAC_T$ of 45 kDa. Such splice variants were also frequently described in the vertebrate SAC systems (27, 50), but remain unresolved for the $_{su}SAC$. Whole mount immunostaining revealed high $_{su}SAC_T$ protein abundance in PMCs, and presence in ectodermal epithelia and cells of the ciliary bands (Fig. 2, B–E). High-resolution 3-D reconstructions from Airyscan confocal z-stacks demonstrated that $_{su}SAC_T$ is present in the cytoplasm of PMCs including the syncytial cable (Fig. 2, F and G). These molecular and immunological analyses provide evidence for the presence of $_{su}SAC$, likely as a truncated form only consisting of the catalytic C1-C2 domains in calcifying cells of the sea urchin larva.

Role of $_{su}SAC$ in PMC pH_i Regulation

To specifically look at the role of $_{su}SAC$ in PMC pH_i regulation, we performed live cell imaging experiments in combination

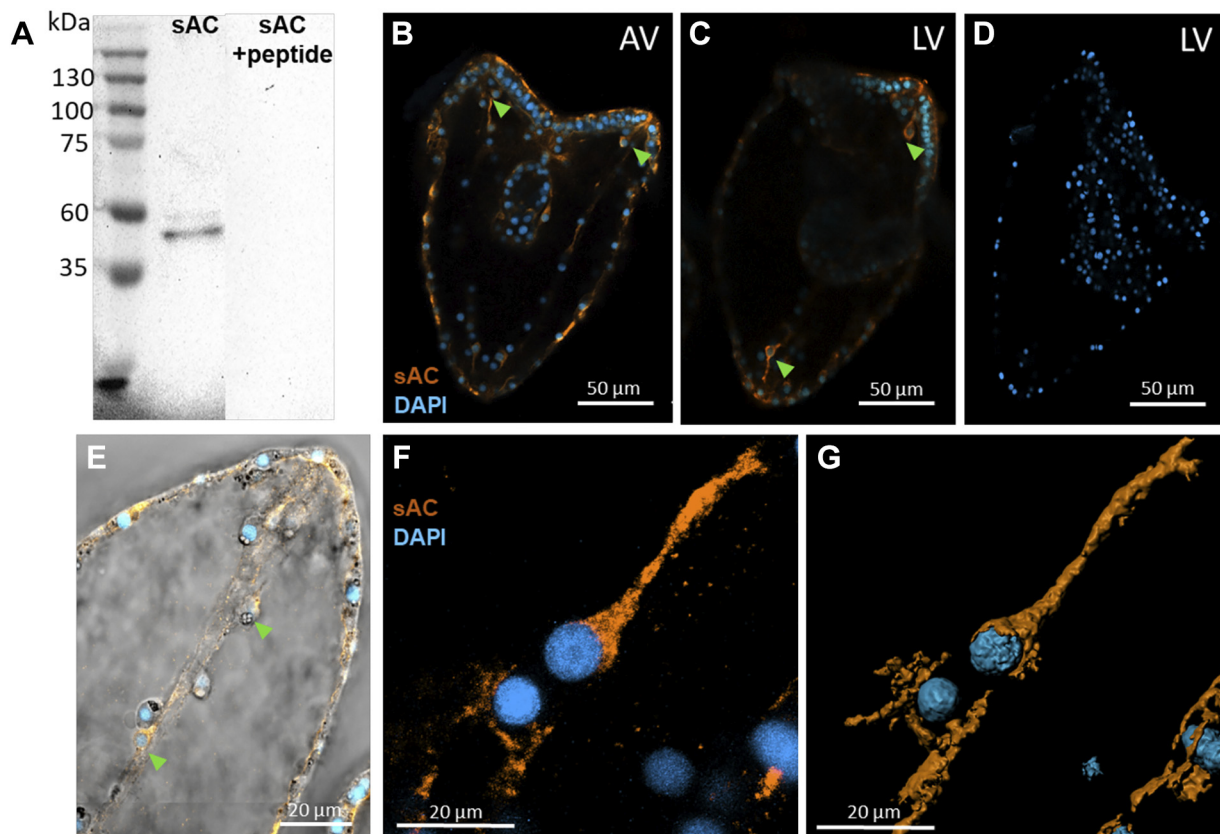


Figure 2. Immunocytochemical analyses of SAC in the sea urchin larva. **A:** Western blot analysis using the anti-coral SAC antibody against crude protein extracts of sea urchin larvae. The right lane shows the blotting result using the primary antibody preabsorbed with the immunization peptide. **B** and **C:** immunolocalization of $_{su}SAC$ in 3 dpf sea urchin larva. Pseudocolors were applied on signals of SAC protein (orange) and nuclei (blue). $_{su}SAC$ -positive PMCs were indicated by arrowhead. **D:** negative control without addition of the primary antibody. **E:** merged image of SAC immunostaining and bright field. $_{su}SAC$ (orange); nuclei (blue). $_{su}SAC$ -positive PMCs attaching to spicule were indicated by arrowhead. **F:** reconstituted 3-D image of $_{su}SAC$ staining. $_{su}SAC$ (orange); nuclei (blue). The $_{su}SAC$ signal followed the shape of PMC syncytial cables and is highlighted in **G**. AV, anal view; dpf, day post fertilization; LV, lateral view; SAC, soluble adenylyl cyclase; $_{su}SAC$, sea urchin soluble adenylyl cyclase; PMC, primary mesenchyme cell; 3-D, three-dimensional.

with the NH_4Cl prepulse technique to measure the ability of PMCs to recover from an induced acidosis (Fig. 3). We have extensively used this technique to assess acid-base regulatory mechanisms in PMCs based on their pH_i recovery rate (6, 15), and here we performed similar experiments in the presence of two sAC-specific pharmacological inhibitors.

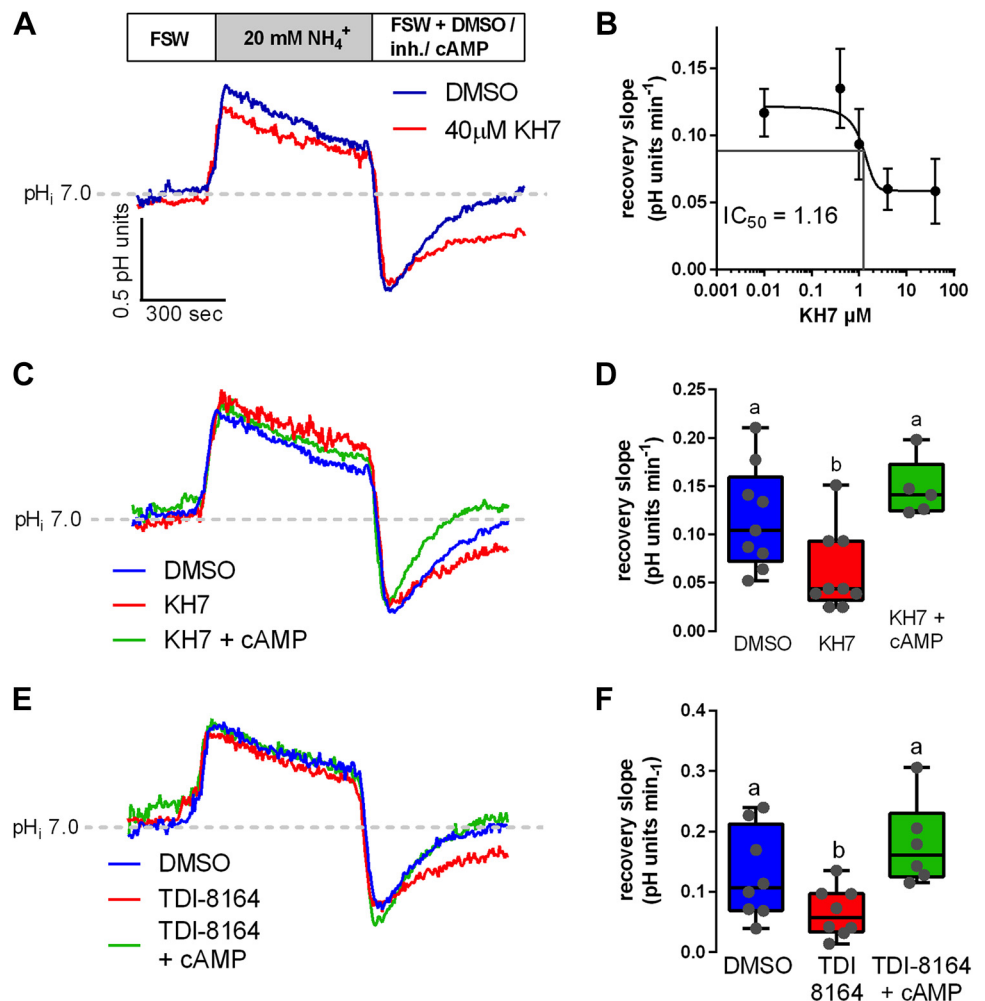
The first inhibitor, KH7, decreased PMC pH_i recovery in a dose-response manner ($\text{IC}_{50} \sim 2 \mu\text{M}$; Fig. 3, A and B). At the highest KH7 concentration (40 μM), PMC pH_i recovery was significantly decreased to half of that seen in DMSO-treated controls (0.06 ± 0.02 vs. 0.12 ± 0.02 pH units $\cdot \text{min}^{-1}$, respectively). Rescue of KH7-induced inhibition of pH_i recovery by db-cAMP (0.15 ± 0.01 pH units $\cdot \text{min}^{-1}$) confirmed the involvement of s_{su} sAC (Fig. 3, C and D). The second sAC inhibitor, TDI-8164, had identical effects on PMC pH_i recovery, namely, significant inhibition compared with controls and full rescue by db-cAMP (0.09 ± 0.03 , 0.13 ± 0.03 , and 0.15 ± 0.04 pH units $\cdot \text{min}^{-1}$, respectively; Fig. 3, E and F). Other than genetic knockdown, which is not possible in this case due to the role of sAC in gastrulation (35), comparable inhibition by two structurally unrelated pharmacological inhibitors and rescue of the resulting phenotype by cAMP are the strongest possible evidences for the involvement of s_{su} sAC in sea urchin larva PMC pH_i regulation.

s_{su} sAC Is Required for Biomineralization under Control and Acidified Conditions

In the next step, we looked at the effect of pharmacological downregulation of sAC activity on sea urchin larvae biomineralization. To prevent an off-target effect on gastrulation (35), KH7 was applied at the late gastrula stage (1.5 dpf), where skeletal development begins. Tracking spicule growth in 2–4 dpf larvae demonstrated a dose-dependent decrease in the biomineralization rate of the growing spicules from $80.46 \pm 13.94 \mu\text{m} \cdot \text{day}^{-1}$ in control larvae down to 36.99 ± 4.37 , 25.14 ± 5.89 , and $20 \pm 3.81 \mu\text{m} \cdot \text{day}^{-1}$ in larvae treated with 1, 2, and 4 μM KH7, respectively (Fig. 4, A and B).

To further disentangle potential collateral effects of KH7 on other developmental processes that could indirectly impair skeletogenesis, we performed a remineralization assay with 3 dpf larvae. This experiment demonstrated that pharmacological s_{su} sAC inhibition induced a dose-dependent decrease in spicule remineralization from $14.95 \pm 3.61 \mu\text{m} \cdot \text{day}^{-1}$ down to 5.14 ± 1.23 , 1.07 ± 1.26 , and $0.5 \pm 0.81 \mu\text{m} \cdot \text{day}^{-1}$ in larvae treated with 0, 1, 2, and 4 μM KH7, respectively (Fig. 4C). Recalcifying larvae subsequently demonstrated roughly double the s_{su} sAC mRNA levels compared with controls (Fig. 4D). These results indicate that s_{su} sAC activity is essential, for both normal biomineralization

Figure 3. Effects of sAC activity on pH_i regulatory capacities of PMCs. **A:** average traces from real-time intracellular pH recordings in PMCs under control conditions (blue line) or in the presence 40 μM KH7 (red line). **B:** dose dependency of recovery slopes under different doses of KH7 treatments (0.04, 0.4, 1, 4, and 40 μM). IC_{50} of KH7 on PMC pH_i regulation is 1.16 μM . **C:** PMCs' pH_i regulatory capacities in the presence of DMSO (blue line), 40 μM KH7 (red line), or 40 μM KH7 + 100 μM dibutyl cAMP (green line). **D:** average slope of PMC pH_i capacities at the given pharmacological treatment, including control (blue box), sAC inhibition (red box), and cAMP rescue (green box). **E:** real-time trace of pH_i regulatory capacities of PMCs that were treated with DMSO (blue line), 40 μM TDI-8164 (red line), or 40 μM TDI-8164 + 100 μM dibutyl cAMP (green line). **F:** average slopes of pH_i recovery in PMCs treated with DMSO (blue box), TDI-8164 (red box), and TDI-8164 + dibutyl cAMP (green box). Different letters represent significant differences. $n = 5$ –9; one-way ANOVA: $P < 0.05$; Turkey's test. FSW, filtered seawater; PMC, primary mesenchyme cell; sAC, soluble adenylyl cyclase.



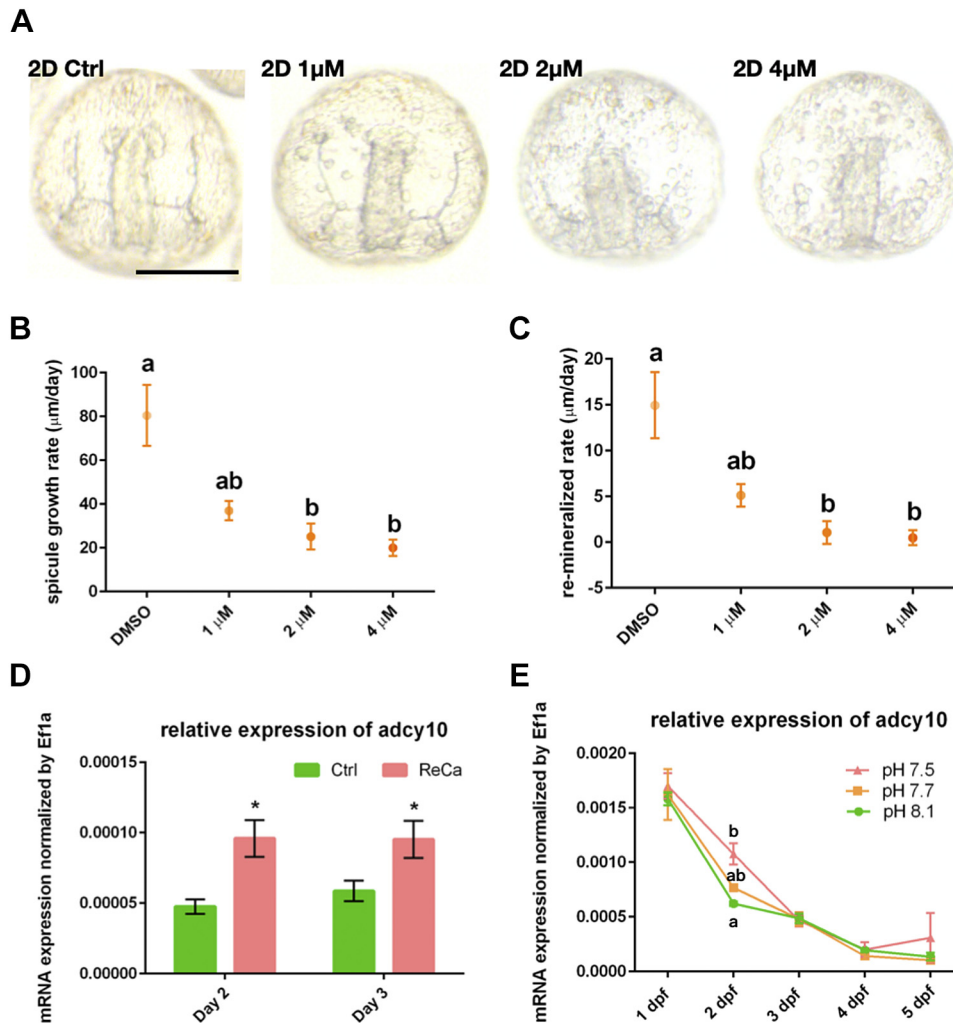


Figure 4. Role of sAC activity for the calcification process in the sea urchin larva. **A:** representative images of 2 dpf larvae demonstrating the effects of the sAC inhibitor, KH7, on the spicule development. Scale bar = 100 μm. **B:** growth rate of spicules (μm/day) in normally developing 2 to 4 dpf sea urchin larvae treated with 0, 1, 2, or 4 μM KH7. Different letters represent significant differences. $n = 3$; one-way ANOVA: $P < 0.05$; Turkey's test was performed. **C:** effect of different concentrations of KH7 on the skeleton regeneration rate after the decalcification treatment. Spicule length of 2–5 days remineralized larvae (6–9 dpf larvae) was measured and applied to calculate for the regeneration rate. $n = 3$; one-way ANOVA: $P < 0.05$; Turkey's test was performed. **D:** transcription levels of *adcy10* in control (Ctrl) and remineralizing (ReCa) larvae. Sampling from 2 to 3 days of recover from the decalcification treatment (6/7 dpf larvae). *Significant differences. $n = 5–6$, Student's *t* test: $P < 0.05$. **E:** the mRNA level of *su*sAC in 1–5 dpf larvae treated with pH 8.1 seawater (SW) (400 μatm P_{CO_2} , green), pH 7.7 SW (1,400 μatm P_{CO_2} , orange), or pH 7.5 SW (2,000 μatm P_{CO_2} , pink). $n = 3$; values are presented as means $\pm$ SE. Statistical differences were analyzed by one-way ANOVA: $P < 0.05$; Turkey's test. Different letters denote significant differences. dpf, day post fertilization; sAC, soluble adenylyl cyclase.

and remineralization following skeleton dissolution by an extreme acidic challenge.

Finally, we analyzed *su*sAC mRNA expression in sea urchin larvae that had been exposed to control (pH 8.1 and 400 μatm P_{CO_2}), mild (pH 7.7 and 1,400 μatm P_{CO_2}), and extreme (pH 7.5 and 2,000 μatm P_{CO_2}) CO_2 -induced seawater acidification between 0 and 5 dpf (14). These OA-exposed larvae were reported to experience reduced body length growth; however, they were able to biomineralize their skeletons at a rate that was comparable to controls (based on body size-specific Ca^{2+} accumulation; 14). Analysis of *su*sAC mRNA levels by RT-qPCR in the same samples revealed significant approximately twofold upregulation in 2 dpf larvae exposed to the lowest pH treatment (Fig. 4E), and the whole mount in situ hybridization confirmed the presence of *su*sAC mRNA in PMCs and in ectodermal and developing gut cells in larvae raised under acidified conditions (Fig. 1B). These results provide a link between PMC *su*sAC and compensatory responses to OA that maintain biomineralization during an environmentally relevant acidic challenge.

Conclusions

The various lines of evidence presented in this study indicate that *su*sAC is a molecular acid-base sensor that coordinates

pH_i regulation and biomineralization in sea urchin larvae PMCs during both normal conditions and acidic challenges. It has been demonstrated that during active mineralization, pH_i of PMCs is increased due to an enhanced uptake of bicarbonate that is needed for the calcification process (6). In fact, this carbon concentration mechanism has been recently resolved, demonstrating an interaction between the extracellular carbonic anhydrase, Cara7, with the Na^+ -dependent HCO_3^- cotransporter, Sp_Slc4a10 (16). It has been demonstrated that HCO_3^- stimulates *su*sAC and initiates cAMP production (34). Accordingly, we propose that this elevation of the cytosolic carbonate pool stimulates *su*sAC to activate cellular proton export pathways (Fig. 5). This is essential to maintain intracellular pH homeostasis during precipitation of $CaCO_3$, where large amounts of protons are liberated inside the cell.

However, the exact mechanisms that stimulate *su*sAC activity in PMCs need to be confirmed experimentally and the subsequent downstream signaling pathways and effectors are an exciting topic of future investigation. Based on previous experiments (6, 14, 15), the most promising candidates include the Na^+/HCO_3^- cotransporter (Sp_Slc4a10), Na^+/H^+ -exchanger (Sp_Slc9a2), and the proton channel (Otop21); in addition, regulation of Na^+/K^+ -ATPase cannot be ruled out (Fig. 5). For example, sAC in rat cardiomyocytes is

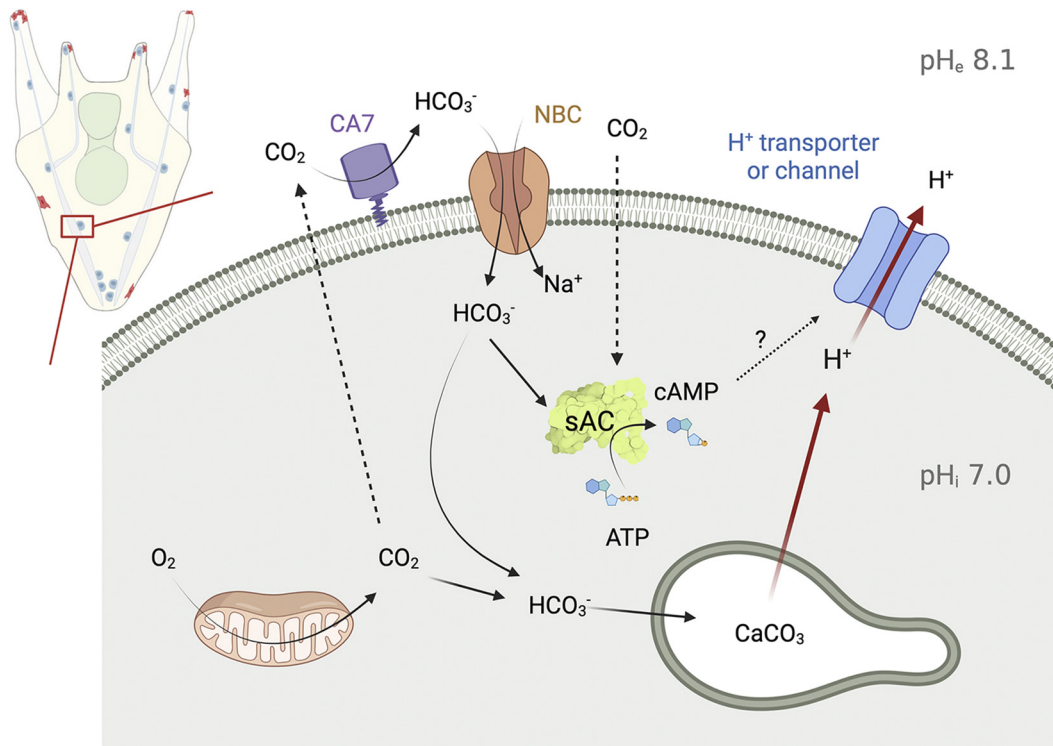


Figure 5. Proposed model for $_{su}SAC$ -dependent coordination of sea urchin PMC pH_i regulation and biomineralization. During active PMC biomineralization, $_{su}SAC$ is stimulated by an increase in cytosolic HCO_3^- levels resulting from NBC-mediated HCO_3^- import, which was supported by Cara7 (CA7) converting metabolic CO_2 to HCO_3^- . The subsequent $_{su}SAC$ -dependent increase in cAMP regulates the activity of downstream effector H^+ -transporting proteins; the most likely candidates include Otop2L, VHA, and NHE. The effectors were triggered by cAMP through an uncertain stimulation pathway. NBC, Na^+/HCO_3^- cotransporter; NHE, Na^+/H^+ -exchanger; $_{su}SAC$, sea urchin soluble adenylyl cyclase; VHA, V-type H^+ -ATPase. Figure was created with BioRender.com.

stimulated by HCO_3^- that enters the cell via Slc4 (51), a mechanism that may also take place in hagfish and lamprey (52). Interestingly, Slc4-stimulated sAC activity promotes, via protein kinase A (PKA), the transport of Ca^{2+} into the rat cardiomyocyte cytosol and sarcoplasmic reticulum (53). Thus, it is possible that $_{su}SAC$ in PMCs simultaneously regulates the activity of multiple acid-base and Ca^{2+} transporters to ultimately coordinate pH_i and biomineralization.

A comparative analysis reveals striking similarities in the molecular machinery of diverse biomineralizing cells. In addition to sea urchin larva PMCs, Slc4 family Na^+/HCO_3^- cotransporters are also present in the calicoblastic cells that make the coral skeleton (54, 55), the ameloblasts that produce dental enamel (56), and matrix vesicles of human osteoblast-like cells (57). Analogous to NBC, sAC activity promotes biomineralization in sea urchin PMCs (this study), coral calcifying cells (44), and fish inner ear epithelial cells that make the otoliths (17), as well as mammalian osteoblasts (58, 59) and ameloblasts (56). The strong association of sAC with biomineralizing systems points toward a conserved physiological role of this enzyme in marine calcifying organisms. Here, the sAC-mediated activity between pH_i regulation and biomineralization should be more prominently considered when assessing the potential effects of environmental disturbances on the vulnerability or resilience of marine organisms and the assumptions used to reconstruct Earth's climates based on skeleton-bound proxies.

DATA AVAILABILITY

Data will be made available upon reasonable request.

SUPPLEMENTAL DATA

Supplemental Fig. S1: <https://doi.org/10.6084/m9.figshare.21578724.v1>.

ACKNOWLEDGMENTS

We thank Drs. Amro Hamdoun and Kate Nesbit (Scripps Institution of Oceanography at University of California, San Diego) for providing sea urchin specimens and Drs. Lonny Levin and Jochen Buck (Weill Cornell Medical College) for providing TDI-8164. The authors further thank J. Brandenburg and D. Garfield for providing access to single-cell transcriptomic database and F. Thoben and R. Lingg for the maintenance of the sea urchin culture systems.

GRANTS

M.Y.H. and W.W.C. are funded by the Emmy Noether Program (403529967) of the German Research Foundation (DFG). This work was partially supported by National Science Foundation (NSF) Graduate Research (GRFP 2019271478), SIO Doctoral Scholar, and Achievement Rewards for College Students Fellowships (to A.B.T.) and NSF Grant no. 1754994 (to M.T.).

DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS

W.W.C. and M.Y.-A.H. conceived and designed research; W.W.C., A.B.T., and M.Y.-A.H. performed experiments; W.W.C., A.B.T., and M.Y.-A.H. analyzed data; W.W.C., M.T., and M.Y.-A.H. interpreted results of experiments; W.W.C., A.B.T., and M.Y.-A.H. prepared figures; W.W.C. and A.B.T. drafted manuscript; W.W.C., A.B.T., M.T., and M.Y.-A.H. edited and revised manuscript; W.W.C., M.T., and M.Y.-A.H. approved final version of manuscript.

REFERENCES

- Knoll AH. Biomineralization and evolutionary history. *Rev Mineral Geochemistry* 54: 329–356, 2003. doi:10.2113/0540329.
- Sikes CS, Okazaki K, Fink RD. Respiratory CO₂ and the supply of inorganic carbon for calcification of sea urchin embryos. *Comp Biochem Physiol A Physiol* 70: 285–291, 1981. doi:10.1016/0300-9629(81)90181-X.
- Sevilgen DS, Venn AA, Hu MY, Tambutté E, De Beer D, Planas-Bielsa V, Tambutté S. Full in vivo characterization of carbonate chemistry at the site of calcification in corals. *Sci Adv* 5: 1–10, 2019. doi:10.1126/sciadv.aau7447.
- Ramesh K, Hu MY, Thomsen J, Bleich M, Melzner F. Mussel larvae modify calcifying fluid carbonate chemistry to promote calcification. *Nat Commun* 8: 1–8, 2017. doi:10.1038/s41467-017-01806-8.
- Jurikova H, Liebetrau V, Gutjahr M, Rollion-Bard C, Hu MY, Krause S, Henkel D, Hiebenthal C, Schmidt M, Laudien J, Eisenhauer A. Boron isotope systematics of cultured brachiopods: response to acidification, vital effects and implications for palaeo-pH reconstruction. *Geochim Cosmochim Acta* 248: 370–386, 2019. doi:10.1016/j.gca.2019.01.015.
- Hu MY, Petersen I, Chang WW, Blurton C, Stumpp M. Cellular bicarbonate accumulation and vesicular proton transport promote calcification in the sea urchin larva. *Proc Biol Sci* 287: 20201506, 2020. doi:10.1098/rspb.2020.1506.
- Kwan GT, Tresguerres M. Elucidating the acid-base mechanisms underlying otolith overgrowth in fish exposed to ocean acidification. *Sci Total Environ* 823: 153690, 2022. doi:10.1016/j.scitotenv.2022.153690.
- Lacruz RS, Nanci A, Kurtz I, Wright JT, Paine ML. Regulation of pH during amelogenesis. *Calcif Tissue Int* 86: 91–103, 2010. doi:10.1007/s00223-009-9326-7.
- Taylor AR, Chrachri A, Wheeler G, Goddard H, Brownlee C. A voltage-gated H⁺ channel underlying pH homeostasis in calcifying coccolithophores. *PLoS Biol* 9: e1001085, 2011. doi:10.1371/journal.pbio.1001085.
- Beniash E, Aizenberg J, Addadi L, Weiner S. Amorphous calcium carbonate transforms into calcite during sea urchin larval spicule growth. *Proc Biol Sci* 264: 461–465, 1997. doi:10.1098/rspb.1997.0066.
- Vidavsky N, Addadi L, Mahamid J, Shimoni E, Ben-Ezra D, Shpigiel M, Weiner S, Addadi L. Initial stages of calcium uptake and mineral deposition in sea urchin embryos. *Proc Natl Acad Sci USA* 111: 39–44, 2014. doi:10.1073/pnas.1312833110.
- Liu L, Schlesinger PH, Slack NM, Friedman PA, Blair HC. High capacity Na⁺/H⁺ exchange activity in mineralizing osteoblasts. *J Cell Physiol* 226: 1702–1712, 2011. doi:10.1002/jcp.22501.
- Stumpp M, Hu MY, Melzner F, Gutowska MA, Dorey N, Himmerkus N, Holtmann WC, Dupont ST, Thorndyke MC, Bleich M. Acidified seawater impacts sea urchin larvae pH regulatory systems relevant for calcification. *Proc Natl Acad Sci USA* 109: 18192–18197, 2012. doi:10.1073/pnas.1209174109.
- Hu MY, Yan JJ, Petersen I, Himmerkus N, Bleich M, Stumpp M. A SLC4 family bicarbonate transporter is critical for intracellular pH regulation and biomineralization in sea urchin embryos. *Elife* 7: e36600, 2018. doi:10.7554/eLife.36600.
- Chang WW, Matt AS, Schwede M, Musinszki M, Grüssel S, Brandenburg J, Garfield D, Bleich M, Baukowitz T, Hu MY. An otopetrin family proton channel promotes cellular acid efflux critical for biomineralization in a marine calcifier. *Proc Natl Acad Sci USA* 118: e2101378118, 2021. doi:10.1073/pnas.2101378118.
- Matt AS, Chang WW, Hu MY. Extracellular carbonic anhydrase activity promotes a carbon concentration mechanism in metazoan calcifying cells. *Proc Natl Acad Sci USA* 119: e2203904119, 2022. doi:10.1073/pnas.2203904119.
- Kwan GT, Smith TR, Tresguerres M. Immunological characterization of two types of ionocytes in the inner ear epithelium of Pacific Chub Mackerel (*Scomber japonicus*). *J Comp Physiol B Biochem* 190: 419–431, 2020. doi:10.1007/s00360-020-01276-3.
- Barott KL, Venn AA, Thies AB, Tambutté S, Tresguerres M. Regulation of coral calcification by the acid-base sensing enzyme soluble adenylyl cyclase. *Biochem Biophys Res Commun* 525: 576–580, 2020. doi:10.1016/j.bbrc.2020.02.115.
- Tresguerres M, Parks SK, Salazar E, Levin LR, Goss GG, Buck J. Bicarbonate-sensing soluble adenylyl cyclase is an essential sensor for acid/base homeostasis. *Proc Natl Acad Sci USA* 107: 442–447, 2010. doi:10.1073/pnas.0911790107.
- Tresguerres M, Levin LR, Buck J. Intracellular cAMP signaling by soluble adenylyl cyclase. *Kidney Int* 79: 1277–1288, 2011. doi:10.1038/ki.2011.95.
- Kobayashi M, Buck J, Levin LR. Conservation of functional domain structure in bicarbonate-regulated “soluble” adenylyl cyclases in bacteria and eukaryotes. *Dev Genes Evol* 214: 503–509, 2004. doi:10.1007/s00427-004-0432-2.
- Chen Y, Cann MJ, Litvin TN, Iourgenko V, Sinclair ML, Levin LR, Buck J. Soluble adenylyl cyclase as an evolutionarily conserved bicarbonate sensor. *Science* 289: 625–628, 2000. doi:10.1126/science.289.5479.625.
- Tresguerres M. sAC from aquatic organisms as a model to study the evolution of acid/base sensing. *Biochim Biophys Acta* 1842: 2629–2635, 2014. doi:10.1016/j.bbdis.2014.06.021.
- Tresguerres M, Clifford AM, Harter TS, Roa JN, Thies AB, Yee DP, Brauner CJ. Evolutionary links between intra- and extracellular acid-base regulation in fish and other aquatic animals. *J Exp Zool Part A Ecol Integr Physiol* 333: 449–465, 2020. [Erratum in *J Exp Zool A Ecol Integr Physiol* 333: 607, 2020]. doi:10.1002/jez.2367.
- Roa JN, Tresguerres M. Soluble adenylyl cyclase is an acid-base sensor in epithelial base-secreting cells. *Am J Physiol Cell Physiol* 311: C340–C349, 2016. doi:10.1152/ajpcell.00089.2016.
- Roa JN, Tresguerres M. Bicarbonate-sensing soluble adenylyl cyclase is present in the cell cytoplasm and nucleus of multiple shark tissues. *Physiol Rep* 5: e13090, 2017. doi:10.14814/phy2.13090.
- Salmerón C, Harter TS, Kwan GT, Roa JN, Blair SD, Rummer JL, Shiels HA, Goss GG, Wilson RW, Tresguerres M. Molecular and biochemical characterization of the bicarbonate-sensing soluble adenylyl cyclase from a bony fish, the rainbow trout *Oncorhynchus mykiss*. *Interface Focus* 11: 20200026, 2021. doi:10.1098/rsfs.2020.0026.
- Roa JN, Munévar CL, Tresguerres M. Feeding induces translocation of vacuolar proton ATPase and pendrin to the membrane of leopard shark (*Triakis semifasciata*) mitochondrion-rich gill cells. *Comp Biochem Physiol A Mol Integr Physiol* 174: 29–37, 2014. doi:10.1016/j.cbpa.2014.04.003.
- Pastor-Soler NM, Hallows KR, Smolak C, Gong F, Brown D, Breton S. Alkaline pH- And cAMP-induced V-ATPase membrane accumulation is mediated by protein kinase A in epididymal clear cells. *Am J Physiol Cell Physiol* 294: C488–C494, 2008. doi:10.1152/ajpcell.00537.2007.
- Gong F, Alzamora R, Smolak C, Li H, Naveed S, Neumann D, Hallows KR, Pastor-Soler NM. Vacuolar H⁺-ATPase apical accumulation in kidney intercalated cells is regulated by PKA and AMP-activated protein kinase. *Am J Physiol Renal Physiol* 298: F1162–F1169, 2010. doi:10.1152/ajprenal.00645.2009.
- Carvalho ES, Gregório SF, Power DM, Canário AV, Fuentes J. Water absorption and bicarbonate secretion in the intestine of the sea bream are regulated by transmembrane and soluble adenylyl cyclase stimulation. *J Comp Physiol B* 182: 1069–1080, 2012. doi:10.1007/s00360-012-0685-4.
- Buck J, Sinclair ML, Schapal L, Cann MJ, Levin LR. Cytosolic adenylyl cyclase defines a unique signaling molecule in mammals. *Proc Natl Acad Sci USA* 96: 79–84, 1999. doi:10.1073/pnas.96.1.79.
- Nomura M, Beltrán C, Darszon A, Vacquier VD. A soluble adenylyl cyclase from sea urchin spermatozoa. *Gene* 353: 231–238, 2005. doi:10.1016/j.gene.2005.04.034.
- Beltrán C, Vacquier VD, Moy G, Chen Y, Buck J, Levin LR, Darszon A. Particulate and soluble adenylyl cyclases participate in the sperm acrosome reaction. *Biochem Biophys Res Commun* 358: 1128–1135, 2007. doi:10.1016/j.bbrc.2007.05.061.
- Shipp LE, Hill RZ, Moy GW, Gökirmak T, Hamdoun A. Abcc5 is required for camp-mediated hindgut invagination in sea urchin

- embryos. *Development* 142: 3537–3548, 2015. doi:10.1242/dev.126144.
36. Stumpp M, Hu M, Casties I, Saborowski R, Bleich M, Melzner F, Dupont S. Digestion in sea urchin larvae impaired under ocean acidification. *Nat Clim Change* 3: 1044–1049, 2013. doi:10.1038/nclimate2028.
37. Pennington JT, Strathmann RR. Consequences of the calcite skeletons of planktonic echinoderm larvae for orientation, swimming, and shape. *Biol Bull* 179: 121–133, 1990. doi:10.2307/1541746.
38. Kumar S, Stecher G, Li M, Knyaz C, Tamura K. MEGA X: molecular evolutionary genetics analysis across computing platforms. *Mol Biol Evol* 35: 1547–1549, 2018. doi:10.1093/molbev/msy096.
39. McClay DR. Methods for embryo dissociation and analysis of cell adhesion. *Methods Cell Biol* 74: 311–329, 2004. doi:10.1016/s0091-679x(04)74014-5.
40. Arshinoff BI, Cary GA, Karimi K, Foley S, Agalakov S, Delgado F, Lotay VS, Ku CJ, Pells TJ, Beatman TR, Kim E, Cameron RA, Vize PD, Telmer CA, Croce JC, Ettensohn CA, Hinman VF. Echinobase: leveraging an extant model organism database to build a knowledgebase supporting research on the genomics and biology of echinoderms. *Nucleic Acids Res* 50: D970–D979, 2022. doi:10.1093/nar/gkab1005.
41. Butler A, Hoffman P, Smibert P, Papalexi E, Satija R. Integrating single-cell transcriptomic data across different conditions, technologies, and species. *Nat Biotechnol* 36: 411–420, 2018. doi:10.1038/nbt.4096.
42. Stuart T, Butler A, Hoffman P, Hafemeister C, Papalexi E, Mauck WM 3rd, Hao Y, Stoeckius M, Smibert P, Satija R. Comprehensive integration of single-cell data. *Cell* 177: 1888–1902.e21, 2019. doi:10.1016/j.cell.2019.05.031.
43. Rafiq K, Shashikant T, McManus CJ, Ettensohn CA. Genome-wide analysis of the skeletogenic gene regulatory network of sea urchins. *Development* 141: 950–961, 2014 [Erratum in *Development* 141: 2542, 2014]. doi:10.1242/dev.105585.
44. Barott KL, Barron ME, Tresguerres M. Identification of a molecular pH sensor in coral. *Proc Biol Sci* 284: 20171769, 2017. doi:10.1098/rspb.2017.1769.
45. Hess KC, Jones BH, Marquez B, Chen Y, Ord TS, Kamenetsky M, Miyamoto C, Zippin JH, Kopf GS, Suarez SS, Levin LR, Williams CJ, Buck J, Moss SB. The “soluble” adenylyl cyclase in sperm mediates multiple signaling events required for fertilization. *Dev Cell* 9: 249–259, 2005. doi:10.1016/j.devcel.2005.06.007.
46. Körschen HG, Hamzeh H, Pascal R, Alvarez L, Bönigk W, Kaur N, Levin LR, Buck J, Kambach C, Michino M, Jennings A, Sato A, Seifert R, Strünker T, Steegborn C, Kaupp UB. External fertilization is orchestrated by a pH-regulated soluble adenylyl cyclase controlling sperm motility and chemotaxis (Preprint). bioRxiv doi:10.1101/2021.06.18.448929.
47. Moreno B, DiCorato A, Park A, Mobilia K, Knapp R, Bleher R, Wilke C, Alvares K, Joester D. Culture of and experiments with sea urchin embryo primary mesenchyme cells. *Methods Cell Biol* 150: 293–330, 2019. doi:10.1016/bs.mcb.2019.01.002.
48. Smith J, Davidson EH. A new method, using cis-regulatory control, for blocking embryonic gene expression. *Dev Biol* 318: 360–365, 2008. doi:10.1016/j.ydbio.2008.02.056.
49. Tresguerres M, Salmerón C. Molecular, enzymatic, and cellular characterization of soluble adenylyl cyclase from aquatic animals. *Methods Enzymol* 605: 525–549, 2018. doi:10.1016/bs.mie.2018.02.022.
50. Chen X, Baumlin N, Buck J, Levin LR, Fregien N, Salathe M. A soluble adenylyl cyclase form targets to axonemes and rescues beat regulation in soluble adenylyl cyclase knockout mice. *Am J Respir Cell Mol Biol* 51: 750–760, 2014. doi:10.1165/rcmb.2013-0542OC.
51. Espejo MS, Orłowski A, Ibañez AM, Di Mattia RA, Velásquez FC, Rossetti NS, Ciancio MC, De Giusti VC, Aiello EA. The functional association between the sodium/bicarbonate cotransporter (NBC) and the soluble adenylyl cyclase (sAC) modulates cardiac contractility. *Pflügers Arch* 472: 103–115, 2020. doi:10.1007/s00424-019-02331-x.
52. Wilson CM, Roa JN, Cox GK, Tresguerres M, Farrell AP. Introducing a novel mechanism to control heart rate in the ancestral Pacific hagfish. *J Exp Biol* 219: 3227–3236, 2016. doi:10.1242/jeb.138198.
53. Lo M, Shahriari A, Roa JN, Tresguerres M, Farrell AP. Differential effects of bicarbonate on severe hypoxia- and hypercapnia-induced cardiac malfunctions in diverse fish species. *J Comp Physiol B* 191: 113–125, 2021. doi:10.1007/s00360-020-01324-y.
54. Barott KL, Perez SO, Linsmayer LB, Tresguerres M. Differential localization of ion transporters suggests distinct cellular mechanisms for calcification and photosynthesis between two coral species. *Am J Physiol Regul Integr Comp Physiol* 309: R235–R246, 2015. doi:10.1152/ajpregu.00052.2015.
55. Tresguerres M, Barott KL, Barron ME, Deheyn DD, Kline DI, Linsmayer LB. Cell biology of reef-building corals: ion transport, acid/base regulation, and energy metabolism BT. In: *Acid-Base Balance and Nitrogen Excretion in Invertebrates: Mechanisms and Strategies in Various Invertebrate Groups with Considerations of Challenges Caused by Ocean Acidification*, edited by Weihrauch D, O'Donnell M. Cham, Switzerland: Springer International Publishing, 2017, p. 193–218.
56. Yin K, Paine ML. Bicarbonate transport during enamel maturation. *Calcif Tissue Int* 101: 457–464, 2017. doi:10.1007/s00223-017-0311-2.
57. Thouveney C, Malinowska A, Balcerzak M, Strzelecka-Kiliszek A, Buchet R, Dadlez M, Pikula S. Proteomic characterization of biogenesis and functions of matrix vesicles released from mineralizing human osteoblast-like cells. *J Proteomics* 74: 1123–1134, 2011. doi:10.1016/j.jprot.2011.04.005.
58. Ichikawa S, Koller DL, Curry LR, Lai D, Xuei X, Edenberg HJ, Hui SL, Peacock M, Foroud T, Econs MJ. Association of adenylyl cyclase 10 (ADCY10) polymorphisms and bone mineral density in healthy adults. *Calcif Tissue Int* 84: 97–102, 2009. doi:10.1007/s00223-008-9200-z.
59. Shi W, Gao Y, Wang Y, Zhou J, Wei Z, Ma X, Ma H, Xian CJ, Wang J, Chen K. The flavonol glycoside icariin promotes bone formation in growing rats by activating the cAMP signaling pathway in primary cilia of osteoblasts. *J Biol Chem* 292: 20883–20896, 2017. doi:10.1074/jbc.M117.809517.

Supplementary materials

Figure S1 The graphic summary of SRA database BLAST results. Full-length *adcy10* mRNA sequence (NM_001025209.1) was blast to transcriptome of control (SRX366892) and Alx1 morphants (SRX385939). RNA sequencing reads matched with input sequences were labeled by color blocks.

