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Regulation of coral calcification by the acid-base sensing enzyme soluble adenylyl cyclase

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Abstract

Coral calcification is intricately linked to the chemical composition of the fluid in the extracellular calcifying medium (ECM), which is situated between the calcifying cells and the skeleton. Here we demonstrate that the acid-base sensing enzyme soluble adenylyl cyclase (sAC) is expressed in calcifying cells of the coral *Stylophora pistillata*. Furthermore, pharmacological inhibition of sAC in coral microcolonies resulted in acidification of the ECM as estimated by the pH-sensitive ratiometric indicator SNARF, and decreased calcification rates, as estimated by calcein labeling of crystal growth. These results indicate that sAC activity modulates some of the molecular machinery involved in producing the coral skeleton, which could include ion-transporting proteins and vesicular transport. To our knowledge this is the first study to directly demonstrate biological regulation of the alkaline pH of the coral ECM and its correlation with calcification.

Key words: cyclic AMP, biomineralization, subcalicoblastic medium, acid-base, calicodermis, coral reef

Abbreviations

cAMP: cyclic adenosine monophosphate; pHi: intracellular pH; pH_{ECM}: pH of the extracellular calcifying medium; sAC: soluble adenylyl cyclase; ECM: extracellular calcifying medium

Introduction

Precipitation of calcium carbonate ($CaCO_3$) by reef-building corals drives the formation of the largest biological structures on earth, and the physical complexity of these skeletal networks generates the habitat needed to support the large biodiversity found within coral reef ecosystems. The coral skeleton is secreted by specialized calcifying cells in the calicodermis, the tissue layer that lies directly on top of the skeleton. Underlying the calicodermis, the

extracellular calcifying medium (ECM) is at the interface between the calcifying cells and the skeleton. The fluid in the ECM has an alkaline pH and high concentrations of dissolved inorganic carbon (DIC) and calcium ions (Ca^{2+}), resulting in an hyper-saturated aragonite saturation state (Ω) that promotes skeleton biomineralization and prevents its dissolution [1–3]. The transport of DIC and Ca^{2+} from calcifying cells to the ECM is hypothesized to take place *via* recently identified SLC4 HCO_3^- transporters [4–6], CO_2 diffusion [7], plasma membrane Ca^{2+}-ATPase [4,8], and Na^+/Ca^{2+} [9]. Calcifying cells also abundantly express Na^+/K^+-ATPase [4], which presumably provides the driving force for the transport of these and other molecules to and from the ECM. In addition, calcifying cells possess abundant intracellular vesicles (reviewed in [10]), and cytoskeletal inhibitors have been shown to decrease Ca^{2+} incorporation into the skeleton suggesting a role of vesicular trafficking in coral calcification [11]. Recent evidence has suggested that intracellular vesicles within calcifying cells could deliver amorphous $CaCO_3$ to the site of calcification [4,9,12]. Finally, coral calcification requires the removal of H^+ from the ECM possibly *via* Ca^{2+}ATPases and other yet unidentified mechanisms.

While there is wide consensus that corals are able to regulate the activity of proteins involved with Ca^{2+} and DIC secretion, vesicular trafficking, and H^+ removal to promote calcification, the underlying molecular mechanisms are unknown. Thus, the goal of the current study was to establish whether the enzyme soluble adenylyl cyclase (sAC) is one of the molecular mechanisms that regulate the pH of the ECM (pH_{ECM}), and to explore its relevance for coral calcification. As an evolutionarily conserved acid-base sensor [13], sAC is stimulated by HCO_3^- to produce cyclic adenosine monophosphate (cAMP), a universal messenger molecule that can modulate a variety of downstream physiological responses (reviewed in [14]). In addition to acid-base status, sAC may act as a sensor of intracellular Ca^{2+} and ATP ([15], reviewed in [16]), two molecules critical for calcification. In the coral *Pocillopora damicornis*, sAC was recently identified to be essential for regulating intracellular pH (pHi) in response to acid-base challenges from both internal and external origin [17]. Scaling up from cells to organisms,

sAC activity has been shown essential for extracellular acid-base regulation in sharks [18], and for intestinal $CaCO_3$ precipitation in bony fishes [19,20]. The highly conserved nature of sAC makes it a likely candidate for biological regulation of the acid-base chemistry at the site of coral calcification. The current study provides experimental evidence in support of this hypothesis.

Materials and methods

Coral culture: Microcolonies of *Stylophora pistillata* were grown on glass coverslips and maintained in aquaria at the Centre Scientifique de Monaco as previously described [1].

Western blotting and immunochemistry: Immunological detection of *S. pistillata* sAC (*sp*sAC) was performed using affinity-purified polyclonal anti-coral sAC antibodies described in Barott *et al.*, (2017). Specificity of the antibodies for *sp*sAC was verified by Western blotting, which was conducted following the methods described in detail in [4], with anti-coral sAC primary antibodies (0.55 µg ml^{-1}) or primary antibodies pre-incubated with 20-fold molar excess of peptide ('peptide pre-absorption control'). Localization of *sp*sAC was determined by immunohistochemistry on coral tissue sections prepared as previously described [4,21]. Briefly, coral fragments were fixed in 3% paraformaldehyde in S22 buffer overnight at 4°C, decalcified over ~7 days, and the remaining tissue was dehydrated and embedded in paraffin. Tissues were cut into 6-7 µm sections, rehydrated, and incubated for 1h at room temperature in blocking buffer (4 mL PBS-0.2% Triton-X, 80 µL normal goat serum, 0.8 µL keyhole limpet hemocyanin solution). Sections were then incubated overnight at 4°C with anti-coral sAC primary antibodies (1.8 µg ml^{-1}), antibodies preabsorbed with 20x excess peptide, pre-immune serum (1.8 µg ml^{-1}), or blocking buffer alone. After three 5-min washes in PBS-0.2% Triton-X, secondary antibodies (goat anti-rabbit-Alexa Fluor 555, 4 µg/ml; Invitrogen) were then added for 1h at room temperature, followed by 5-min room temperature incubation with DAPI DNA Stain (1 µg/ml). Sections were again washed 3 x 5 minutes PBS-0.2% Triton-X to remove unbound secondary

antibodies, and imaged using a confocal microscope with super-resolution capabilities (Zeiss LSM800 with Airyscan).

Reagents: Stock solutions Pluronic acid F-127 (Invitrogen) and KH7 were prepared in anhydrous DMSO (Invitrogen). SNARF1 (Invitrogen) was dissolved in 0.22 µm filtered seawater (FSW).

Imaging of pH_{ECM}: To avoid confounding effects of photosynthesis on calcification rate and pH_{ECM}, all experiments were conducted in the dark. pH_{ECM} was quantified as previously described [1]. Briefly, live *S. pistillata* microcolonies of ~1 cm^2 were loaded into a microscope chamber (PECON POC-R2), submerged in 2 ml FSW and held in the dark for 20 min, the final 10 min of which they were incubated with 50 µM of the cell-impermeable dye SNARF1 in FSW. After dye loading, 500 µl of FSW was removed with a pipet and mixed with 10 µl (≤0.5% v/v) of the stock solution (DMSO or KH7 in DMSO) then returned to the coral chamber and mixed by gently pipetting up and down for a final concentration of 10 µM KH7. The coral remained submerged in FSW for the duration of this procedure. Coral microcolonies were imaged using an inverted confocal laser scanning microscope (Leica SP5) with a temperature-controlled microscope stage set to 25°C. Using an excitation wavelength of 543 nm, fluorescence emission of SNARF1 was recorded at 585 ± 10 nm and 640 ± 10 nm, and chlorophyll autofluorescence was recorded at 690 ± 10 nm. A brightfield image was also captured simultaneously. Three optical sections (Z-stack) were imaged from below along the growing edge of the coral at 0, 5, 10, 15, 25, and 40 min following exposure to the treatment, and five regions of interest (ROI) were analyzed per time point across the Z-stacks (i.e. 15 measurements per time point per coral). Calibration of SNARF1 was performed in FSW containing 50 µM SNARF-1 adjusted to pH 7 – 9 as previously described [1].

Measurement of calcification rates: Microcolony calcification rates were measured by incorporation of fluorescent calcein dye into $CaCO_3$ crystals at the growing edge of colonies on glass coverslips as described in [22]. Colonies were fitted into a perfusion chamber and placed on the confocal microscope in darkness. Temperature was maintained at 25°C. Each colony was incubated in 20 µM calcein dye in 3 ml FSW for 30 minutes in dark, during which time images of crystals at the growing edge were captured every 3 min. Following this first period each colony was then incubated for a second 30 min period in FSW containing 20 µM calcein, with the addition of either 10 µM KH7 (from a DMSO stock) or equivalent DMSO volume. Images were captured every three minutes. Acquisition parameters were as follows: magnification 40X, excitation 488 nm, emission 510-530 nm, resolution 512 x 512, scan speed 500 Htz. 12 images were captured for each time point in a Z-stack of about 12 µm height. These images were combined to provide a total fluorescence value for each time point. The time series of Z-stacks were analysed using the object analyser in Huygens Essential (Scientific Volume Imaging, Netherlands). The rate of increase in total calcein fluorescence of all the crystals combined in the image was used as a measurement of calcification rate. The DMSO treatment induced a slight reduction in calcification rate compared to FSW; however, the difference was not significant (one sample t-test, p=0.2). The data are presented relative to the increase in calcein fluorescence in the DMSO treatment.

Statistical analyses: pH_{ECM} time series data were analyzed using two-way repeated measures ANOVAs with a Boferroni multiple comparisons test. Calcein incorporation rates were analyzed using a two-tailed *t*-test.

Results and Discussion

Soluble adenylyl cyclase (sAC) is expressed throughout coral tissues.

Anti-coral sAC antibodies specifically recognized *sp*sAC for both WB and IF (Fig. 1). Western blot showed that *S. pistillata* expressed a ~90 kD isoform of sAC (Fig. 1A), which was similar in size to the dominant isoform expressed in the closely related coral species *P. damicornis* [17]. Immunofluorescence staining on histological sections showed that *sp*sAC protein was expressed throughout *S. pistillata* tissues (Fig. 1B). Higher magnification images revealed that *sp*sAC was present in all cell types and tissues, and more abundant in the gastrodermis compared to the epidermis for both the oral and aboral tissues (Fig. 2A). Within the gastrodermis, *sp*sAC was abundant in all cell types, including those with and without intracellular symbionts (Fig. 2B). This expression pattern is similar to what has been observed in *P. damicornis,* where sAC was shown necessary for regulating pHi in response to both internal and external acid-base perturbations [17]. The widespread localization of sAC described in the current study indicates similar roles in *S. pistillata*, and possibly in corals in general. Furthermore, *sp*sAC expression throughout the calicodermis (Fig. 2C) suggests a role in regulating skeletal formation. Thus, we next investigated a potential role of sAC in regulating the pH_{ECM} and calcification.

sAC activity promotes ECM alkalinization

To test the hypothesis that sAC is important for maintaining an elevated pH_{ECM}, we monitored pH_{ECM} through time in microcolonies loaded with the dye SNARF1 (Fig. 3A) following application of 10 μM KH7, a small molecule that specifically inhibits coral sAC activity [17,23]. Compared to controls, KH7-treated corals experienced a drop in pH_{ECM} of ~0.45 pH units (equivalent to an increase in $[H^+]$ from 5 to 13 nM; Fig. 3B). These data confirm that sAC in the calcifying cells indeed plays a regulatory role on the cellular mechanisms that alkalinize the ECM. Previous studies have reported that similar decreases in coral pH_{ECM} occur in response to abiotic factors, and these declines were also associated with depressed calcification rates [24,25]. Thus, we next tested the effect of sAC inhibition on coral calcification.

sAC activity promotes calcification rate

To test whether the observed sAC-dependent pH_{ECM} regulation had an effect on coral calcification, we performed additional experiments on *S. pistillata* microcolonies and quantified calcification *via* the incorporation of calcein into growing $CaCO_3$ skeletal crystals (Fig. 4A). Indeed, inhibition of sAC with 10 μM KH7 resulted in a significant decrease in calcification rate of ~40% ($p<0.05$, two-tailed *t*-test; Fig. 4B). Put together, the SNARF1 and calcein results indicate that sAC activity plays an important regulatory role in maintaining the alkaline pH_{ECM}, which in turn promotes coral calcification.

To our knowledge, this is the first functional characterization of a molecular mechanism that regulates both coral pH_{ECM} alkalinization and calcification. These results confirm the chemistry of the ECM is under coral biological control and is intrinsically linked to calcification. Future research should address two immediate questions. The first question is about the acid-base parameters that modulate sAC activity and pH_{ECM} *in vivo*, including during the steep daily fluctuations in CO_2, pH and HCO_3^- [24,26] and in response to the more gradual changes predicted to take place as a result of ongoing anthropogenic ocean acidification [2,27–29]. A second question concerns the downstream targets of sAC activity. Since sAC produces the messenger molecule cAMP, sAC activity could regulate virtually every cellular process through protein kinase A-dependent phosphorylation, channel gating, or exchange protein activated by cAMP signaling (reviewed in [30,31]). Based on research from vertebrate animals, potential downstream targets of sAC activity in coral calcifying cells include HCO_3^- transporters like in the eye ciliary body [32] and airway epithelial cells [33], Na^+/K^+-ATPase as in kidney collecting duct cells [34], and vesicular trafficking, HCO_3^- secretion and H^+ absorption as shown in shark gill epithelial cells [35]. However, identifying the targets of sAC activity in coral requires a deeper understanding of the molecular and cellular mechanisms responsible for coral calcification, as

well as further developing essential experimental tools such as specific pharmacological drugs, cell cultures and genetic manipulation, to name a few.

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Competing interests: The authors declare no competing interests.

Author contributions

Designed the study: KB, MT, AV, ST

Performed experiments: KB, AV, MT, AT

Analyzed data: KB, MT, AV, ST

Wrote the paper: KB, MT

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Figure/Table legends

Table 1. Effect of sAC inhibition on coral pH_{ECM}. Summary values. See Figure 3 and text for details. Data are shown as mean ± SEM, n=3, *p<0.05.

Figure 1. Presence of sAC in the coral *S. pistillata*. (A) Western blot in coral tissue homogenates showing a specific band of ~90 kDa. *Left:* anti-*spsAC* antibodies; *Right*: peptide pre-absorption control. The total amount of protein loaded in the gel is shown above each lane. (B-D) Immunohistochemistry in coral tissue sections. sw: seawater; sk: skeleton. The green signal is from coral endogenous green fluorescence protein (GFP) and chlorophyll from symbiotic algae.

Figure 2. Cellular localization of sAC in *S. pistillata*. (A) Representative coenosarc section demonstrating widespread *sps*AC localization throughout the coral tissues. (B) Detail of oral tissues showing abundant *sps*AC expression in cells of the oral gastroderm (OG) and limited expression throughout the oral ectoderm (OE). (C) Detail of aboral tissues showing abundant *sps*AC expression in cells of the aboral gastrodermal (AG) and calicodermis (CD). co, coelenteron; sk, skeleton; sw, seawater.

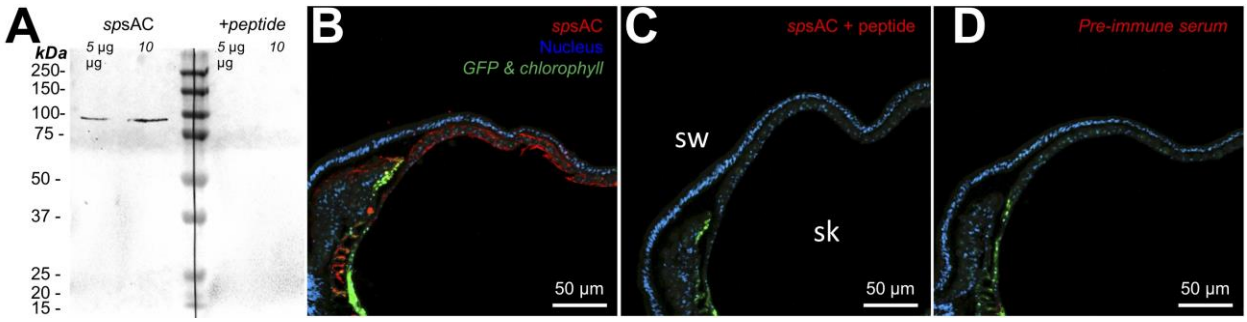
Figure 3. Effect of sAC inhibition on coral pH_{ECM}. (A) Overlay of brightfield and fluorescence images of a *S. pistillata* microcolony loaded with SNARF1 (orange). pH_{ECM} was quantified within 5 distinct extracellular calcifying medium (ECM) pockets (arrowhead) within each microcolony from an average of three z-planes within each ECM pocket. Asterisks indicate newly formed $CaCO_3$ crystals (gray with defined edges); calicodermis appears gray; sw, seawater. (B) Inhibition of sAC by KH7 (10 μM) caused acidification of the ECM under dark conditions. Data are shown as mean ± SEM. (n=3, *p<0.05 compared to the corresponding DMSO time point).

Figure 4. Effect of sAC inhibition on coral calcification rate. (A) Image of calcein incorporation (green) into calcium carbonate crystals of the coral skeleton. Dashed line indicates the outer edge of live tissue of the colony; sw, seawater (B) Inhibition of sAC by KH7 (10 μM) reduced calcification rate by ~60% relative to DMSO carrier controls (N=4). Data are shown as mean ± SEM (*p<0.05; two-tailed *t*-test).

Figures/Tables

Table 1.

Time (min)	**DMSO**	**KH7**
0	8.11 ± 0.06	8.30 ± 0.09
5	8.05 ± 0.07	8.10 ± 0.07
10	8.10 ± 0.12	7.93 ± 0.07
15	8.12 ± 0.10	7.93 ± 0.03
25	8.33 ± 0.16	7.87 ± 0.08*
40	8.34 ± 0.18	7.89 ± 0.07*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20 -
15 -
B
spsAC
Nucleus
GFP & chlorophyll
50 µm
C
spsAC + peptide
sw
sk
50 µm
D
Pre-immune serum
50 µm

Figure 1.

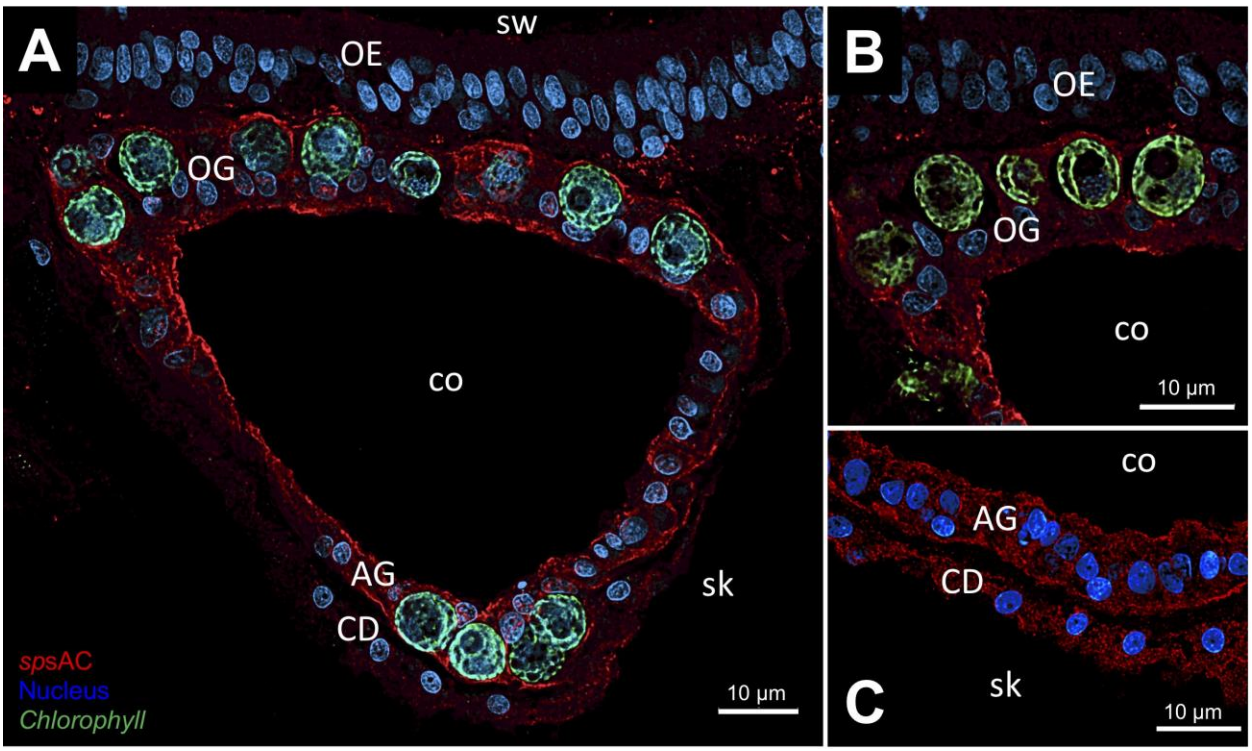

A
SW
OE
OG
CO
AG
CD
sk
spsAC
Nucleus
Chlorophyll
10 µm
B
OE
OG
CO
10 µm
CO
AG
CD
sk
C
10 µm

Figure 2.

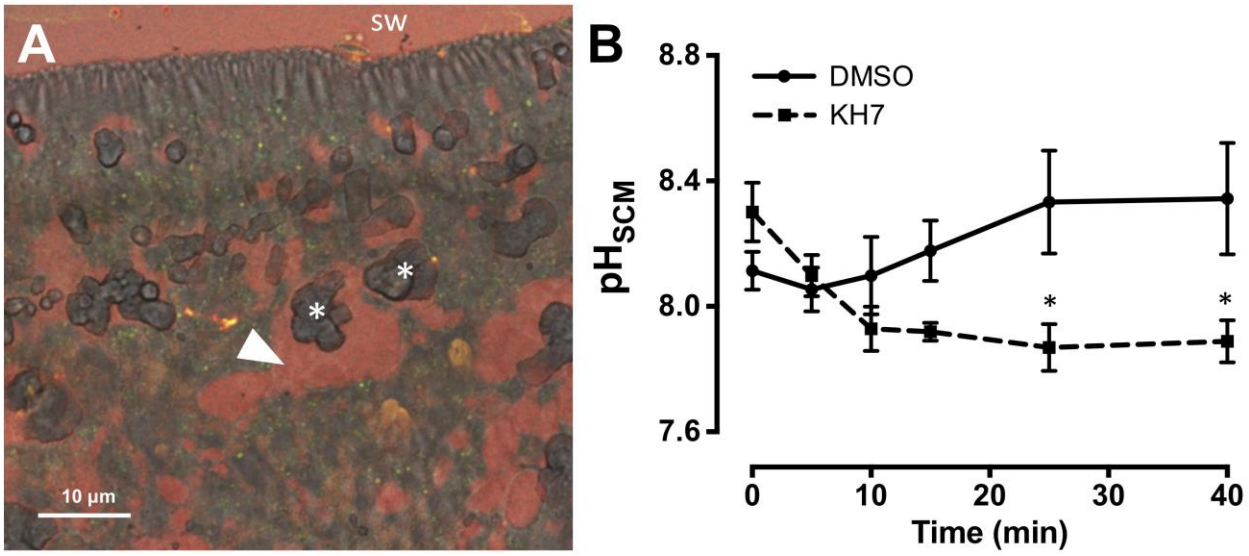


Figure 3.

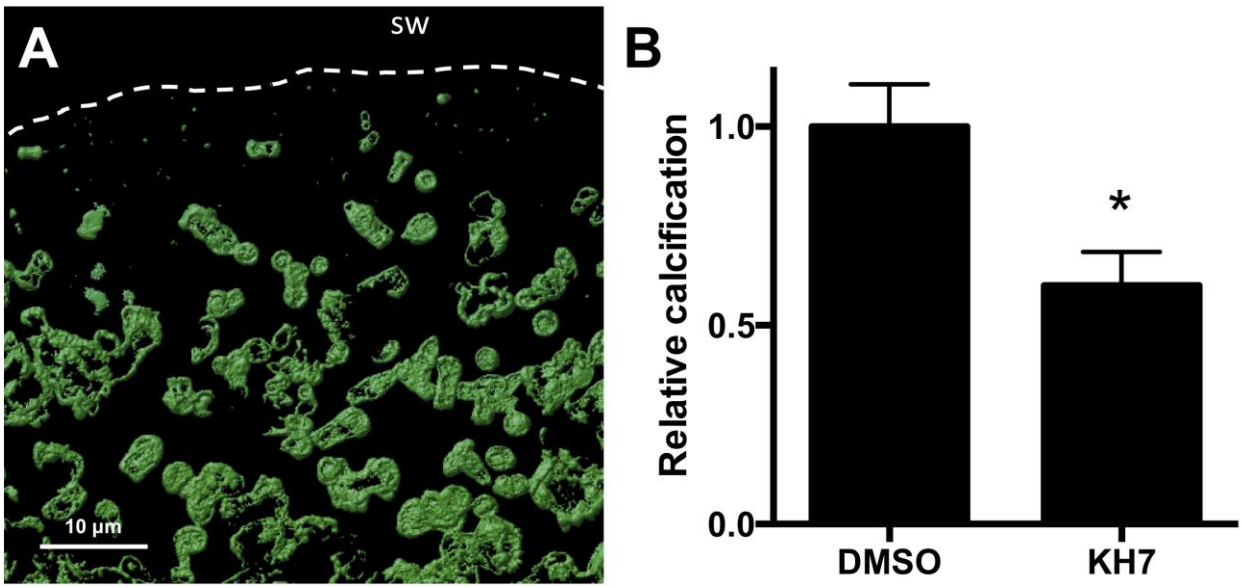
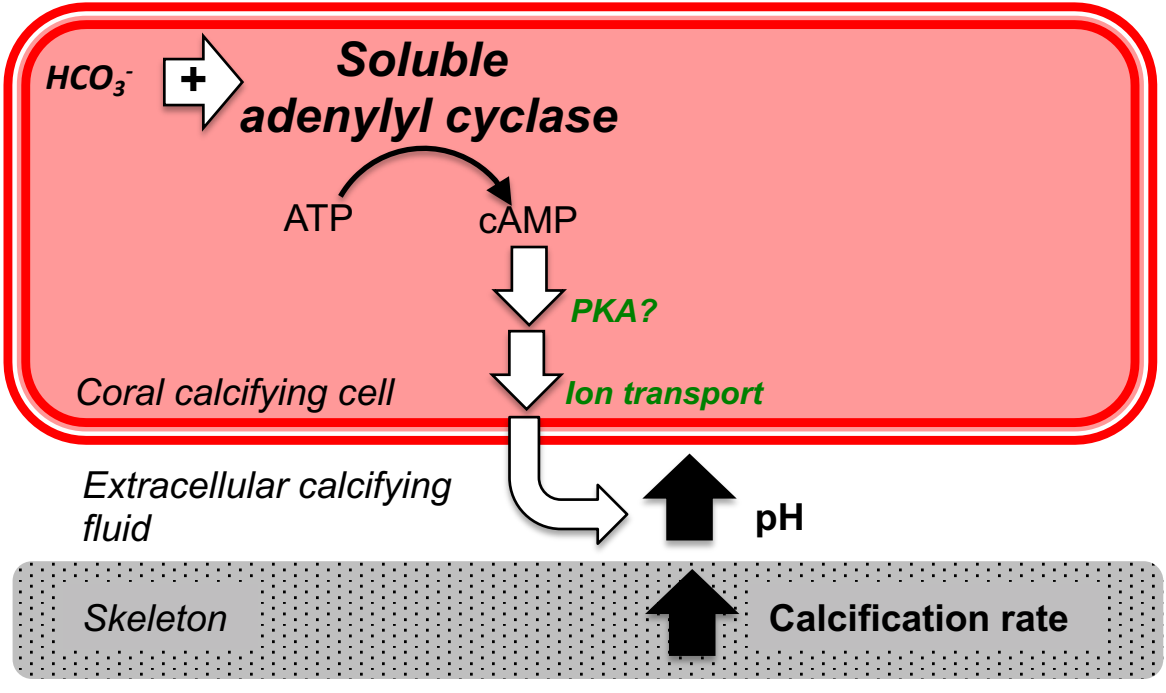


Figure 4.

Highlights

- Soluble adenylyl cyclase (sAC) is an acid-base sensing enzyme
- sAC is present in coral calcifying cells
- sAC activity helps maintain an elevated pH at the site of coral calcification
- sAC activity also influences calcification rate
- Thus, sAC is a molecular mechanism linking coral acid-base sensing to calcification


HCO_3^-
+
Soluble adenylyl cyclase
ATP
cAMP
PKA?
Ion transport
Coral calcifying cell
Extracellular calcifying fluid
pH
Skeleton
Calcification rate