SCIENTIFIC REPORTS

SUBJECT AREAS:
ANIMAL PHYSIOLOGY
CORAL REEFS
CELL SIGNALLING
ENZYMES

Received
28 December 2012

Accepted
20 February 2013

Published
5 March 2013

Correspondence and requests for materials should be addressed to M.T. (mtresguerres@ucsd.edu)

* Current address: Department of Plant Pathology and Microbiology, Hebrew University of Jerusalem, Israel.

High adenylyl cyclase activity and *in vivo* cAMP fluctuations in corals suggest central physiological role

K. L. Barott[1], Y. Helman[3]*, L. Haramaty[3], M. E. Barron[1], K. C. Hess[2], J. Buck[2], L. R. Levin[2] & M. Tresguerres[1]

[1]Scripps Institution of Oceanography, Marine Biology Research Division, La Jolla, California, United States of America, [2]Weill Cornell Medical College, Department of Pharmacology, New York City, New York, United States of America, [3]Environmental Biophysics and Molecular Ecology Program, Institute of Marine and Coastal Sciences, Rutgers, State University of New Jersey, New Brunswick, New Jersey, United States of America.

Corals are an ecologically and evolutionarily significant group, providing the framework for coral reef biodiversity while representing one of the most basal of metazoan phyla. However, little is known about fundamental signaling pathways in corals. Here we investigate the dynamics of cAMP, a conserved signaling molecule that can regulate virtually every physiological process. Bioinformatics revealed corals have both transmembrane and soluble adenylyl cyclases (AC). Endogenous cAMP levels in live corals followed a potential diel cycle, as they were higher during the day compared to the middle of the night. Coral homogenates exhibited some of the highest cAMP production rates ever to be recorded in any organism; this activity was inhibited by calcium ions and stimulated by bicarbonate. In contrast, zooxanthellae or mucus had >1000-fold lower AC activity. These results suggest that cAMP is an important regulator of coral physiology, especially in response to light, acid/base disturbances and inorganic carbon levels.

Coral reefs are among the most diverse ecosystems on the planet[1], yet have been in decline over the last several decades due in large part to human activity[2,3]. However, our ability to predict how corals will respond to pollution, eutrophication, climate change and increasingly acidified oceans is hampered by the limited information on fundamental mechanisms of coral cell biology[2]. Furthermore, as members of the basal metazoan phylum Cnidaria, corals are likely to provide important clues into the evolution of multicellular animals. Interestingly, recent sequencing efforts have revealed that cnidarians have maintained genome complexity similar to that of vertebrates, unlike the more common invertebrate model species *Drosophila melanogaster* and *Caenorhabditis elegans*[4–7]. One such conserved pathway depends on cyclic adenosine monophosphate (cAMP), a ubiquitous signal transduction molecule that is involved in the regulation of virtually every aspect of both prokaryotic and eukaryotic physiology including metabolism, cell division, ion transport, vesicle trafficking, and gene expression[8]. Adenylyl cyclases, the enzymes that catalyze the production of cAMP, are present throughout Bacteria, Archaea and Eukarya, and may therefore be conserved in corals as well. Eukaryotes have two distinct types of adenylyl cyclases: the classic G-protein regulated transmembrane adenylyl cyclases (tmACs) (reviewed in[9]) and the recently discovered bicarbonate-stimulated soluble adenylyl cyclase (sAC)[10]. Mammals have nine tmAC isoforms; though they differ in their tissue distribution, expression during development, and fine kinetic and regulatory characteristics, all are located at the cell membrane[11]. On the other hand, sAC can be found throughout the cytoplasm, in mitochondria, and in the nucleus[12]. While tmACs first appeared in metazoans[13], sAC and sAC-related enzymes have been shown to produce cAMP in response to bicarbonate in a variety of organisms ranging from cyanobacteria to chloroflexi bacteria, sea urchins, and fish[12,14–17], indicating that these enzymes are evolutionarily conserved and thus likely to be active in corals.

The cAMP pathway may regulate calcification in corals[18], and it has also been implicated as a component of coral stress responses in a few transcriptomic studies[19–21]. However, there remains a distinct lack of basic information on the presence, structure and activity of cAMP related proteins, the dynamics of cAMP levels *in vivo*, and the physiological triggers and consequences of this signal cascade in corals. A solid foundation on the basic biochemistry of cAMP production is a prerequisite for the development of mechanistic models. Additionally, characterizing the effects of mammalian-specific pharmacological drugs on coral cAMP production is necessary for designing and interpreting physiological experiments. For example, forskolin is a potent activator of tmACs in vertebrates[22,23] that has been used in a few coral studies with the aim to manipulating cAMP levels[18,24].

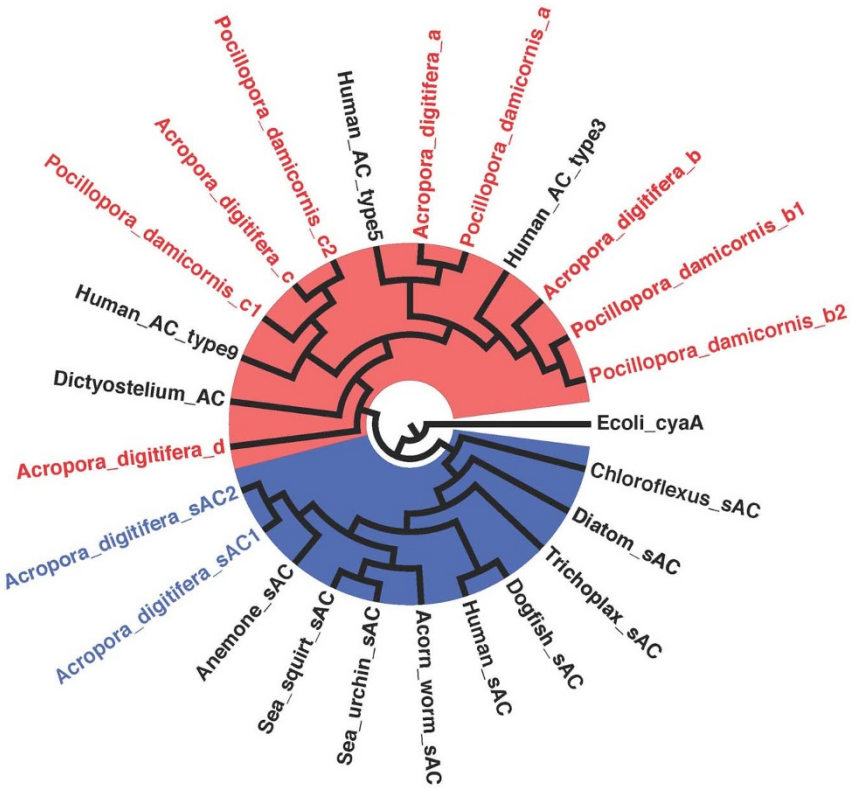

Figure 1 | Phylogenetic tree of putative adenylyl cyclases identified in *A. digitifera* and *P. damicornis*. Coral tmAC isoforms are colored in red and coral sAC isoforms are colored in blue.

However, while forskolin stimulates tmACs in some invertebrates such as mollusks[25], it does not have any effect on some cnidarians[26,27], and its effect on corals has not been characterized.

The aim of this work was to perform an initial characterization of cAMP production in the coral holobiont. Our results demonstrate that coral tissues, but not zooxanthellae or bacteria-harboring mucus, have an unprecedentedly high capacity for producing cAMP that is consistent with the presence of both tmACs and sAC. Furthermore, cAMP levels in live corals fluctuate in a diel manner, suggesting that the cAMP pathway may play a role in light-enhanced calcification, circadian rhythms, and metabolic communication between corals and symbiotic zooxanthellae.

Results

Coral adenylyl cyclase homologs. Several putative tmAC and sAC genes were identified through BLAST searches of the available coral genomic and transcriptomic databases (*Acropora digitifera* and *Pocillopora damicornis*, respectively; Figure 1). These searches revealed the presence of several tmAC isoforms (four in *A. digitifera* and five in *P. damicornis*) and at least two sAC genes (both from *A. digitifera*; Figure 1). Coral tmAC genes clustered into four different groups, which were most similar to human tmACs 3 (coral type b), 5 (coral type a), or 9 (coral type c), with the fourth not grouping as closely with other tmACs (coral group d; Figure 1). The two coral sAC genes were most similar to anemone sAC, followed by sAC from other invertebrates, including the sea urchin, sea squirt, and acorn worm (Figure 1).

Diurnal fluctuations of endogenous cAMP in live corals. Endogenous cAMP levels in coral tissue were highest during the day (Figure 2). Within one hour after dark (7:45 pm), endogenous cAMP decreased from the previous time point (6:40 pm), although this difference was not statistically significant (Figure 2). A minimum of cAMP was reached after 10 hr of darkness (5:00 am, $p = 0.01$; Figure 2). No cAMP was detected in coral-free populations of *Symbiodinium* (either freshly isolated from corals or cultured) when prepared in the same manner as the coral tissue homogenates (resuspended and vortexed in 0.1 N HCl), while cAMP in coral mucus was ~25-fold lower than in tissue. Therefore, the majority of cAMP measured from coral fragments is likely derived from cells of the cnidarian host and not the associated microorganisms.

Characterization of cAMP production by corals. Basal production of cAMP from coral tissue homogenates ranged from 17,000 pmol

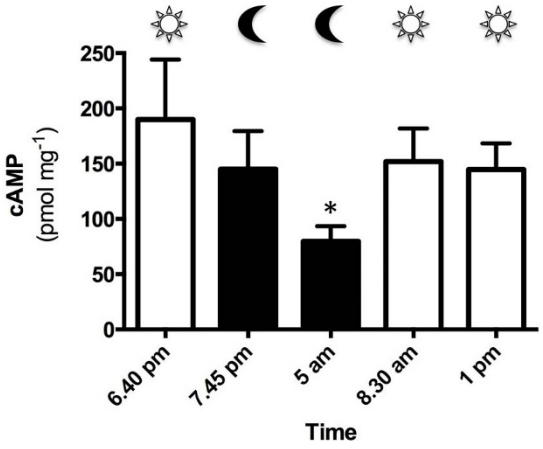

Figure 2 | Daily fluctuations of endogenous cAMP levels in the coral *P. damicornis* normalized to total protein. N = 9 (3 fragments per time point per day on 3 different days spanning 2 months). Error bars indicate SEM; asterisk indicates statistical significance ($p < 0.01$).

Table 1 | Production of cAMP from coral tissue (*Pocillopora damicornis* and *Acropora youngei*) or zooxanthellae (mean ± SEM) determined by ELISA. Letters indicate statistically significant groups as determined by ANOVA and Tukey post-hoc test (*P. damicornis*) or *t*-test (*A. youngei*)

Treatment	cAMP (pmol mg_{prot}^{-1} min^{-1})	Fold stimulation	*N*
Pocillopora damicornis			
40 mM NaCl	28,690 ± 2,212	1.00[a]	6
40 mM $NaHCO_3$	46,700 ± 9,053	1.63[b]	6
2.5 mM $CaCl_2$	7,177 ± 982	0.25[c]	6
2.5 mM $CaCl_2$ + 40 mM $NaHCO_3$	19,189 ± 2,602	0.67[a]	6
1% DMSO	41,956 ± 6,237	1.00	4
50 μM Forskolin (in DMSO)	46,923 ± 9,211	1.12	4
40 mM NaCl	19,409 ± 3,018	1.00[a]	5
40 mM NaCl + 50 μM KH7	15,801 ± 2,639	0.81[a]	5
40 mM $NaHCO_3$	27,830 ± 4,152	1.43[b]	5
40 mM $NaHCO_3$ + 50 μM KH7	16,776 ± 2,931	0.86[a]	5
Acropora youngei			
40 mM NaCl	17,333 ± 437	1.00[a]	3
40 mM $NaHCO_3$	23,578 ± 1,135	1.36[b]	3
Zooxanthellae			
40 mM NaCl	28.74 ± 0.55	1.00	5
40 mM NaCl + 50 μM KH7	27.96 ± 0.75	0.97	5
40 mM $NaHCO_3$	30.25 ± 0.80	1.05	5
40 mM $NaHCO_3$ + 50 μM KH7	29.02 ± 1.00	1.01	5

mg^{-1} min^{-1} in *A. youngei* to ~30,000 pmol mg^{-1} min^{-1} in *P. damicornis* (Table 1). This production was stimulated by bicarbonate, with a half maximal effect concentration (EC_{50}) of ~10 mM bicarbonate for both coral species (Figure 3). To achieve maximal stimulation, 40 mM bicarbonate was used for subsequent experiments. Treatment with 40 mM bicarbonate significantly increased cAMP production in both *A. youngei* and *P. damicornis* by ~1.4 and ~1.6-fold, respectively (paired *t*-test: $p = 0.024$ and repeated measures [RM] one-way ANOVA [$p < 0.0001$] with Tukey post-hoc: $\alpha = 0.05$, $p < 0.001$, respectively; Table 1). To validate cAMP measurements, we performed a subset of experiments and measured cAMP production using both ELISA and a radioactivity-based two-column assay[28]. Both methods used for measuring cAMP production by coral homogenates yielded similar values (Figure 4A,B). Bicarbonate-stimulated production of cAMP was abolished in the presence of 50 μM KH7 (Figure 4A,B), a specific inhibitor of sAC-like adenylyl cyclases that is inert towards mammalian tmACs at this concentration[12,29]. KH7 reduced basal cAMP production by ~20% as measured both by ELISA and the 2-column assay; however, this result was statistically significant only in the latter case (RM one-way ANOVA: $p = 0.0006$, Tukey post-hoc: $\alpha = 0.05$, $p < 0.01$; Figure 3A,B). Production of cAMP in all subsequent experiments was measured using ELISA.

Production of cAMP from freshly isolated or cultured zooxanthellae could not be detected after cells were homogenized by vortexing or sonication. Zooxanthellae cAMP production was detected when algal cells were homogenized by glass bead beating, and was ~1000-fold lower than cAMP production by coral tissues (30 vs. 30,000 pmol cAMP mg^{-1} min^{-1}; Table 1, Figure 4C). Production of cAMP by zooxanthellae was not responsive to bicarbonate or KH7 (Figure 4C). The total amount of cAMP produced by the mucus of one coral fragment was ~10^6-fold lower than coral tissue from similarly sized fragments (~10 vs. 10^7 pmol). Production of cAMP in mucus was not stimulated by 40 mM $NaHCO_3$, neither was it inhibited by 50 μM KH7 ($N = 3$).

Treatment of coral homogenates with 2.5 mM $CaCl_2$ significantly decreased cAMP production relative to controls (RM one-way ANOVA, $p = 0.0087$; Tukey post-hoc: $\alpha = 0.05$, $p < 0.01$). However, 40 mM $NaHCO_3$ still stimulated cAMP production by ~2.7 fold in the presence of 2.5 mM $CaCl_2$ (relative to 40 mM NaCl + 2.5 $CaCl_2$) (Table 1, Figure 5A). Treatment with 50 μM forsoklin, a potent activator of vertebrate tmACs, had no effect on basal cAMP production relative to the DMSO control (Table 1, Figure 5B).

Discussion

This is the first time, to our knowledge, that endogenous cAMP or adenylyl cyclase activity has been measured in any coral. We found that endogenous cAMP levels were significantly lower at night than during the day, though this decrease appears to take some time, as levels were lower but not significantly so after 45 minutes of darkness. It is possible that more time is required for cAMP levels to reach a minimum, particularly given that it takes isolated symbiotic coral cells at least 35 minutes for pH to reach a stable minimum after dark is initiated[30]. Coral endogenous cAMP levels were several fold higher than cAMP levels in any other cnidarian examined to date, including anemones (7–20 fold[31]), hydra (2–15 fold[32,33]), and the sea pansy (5–13 fold[34]). In addition, corals had higher basal adenylyl cyclase activity than other cnidarians, ranging from ~200 to over 10^5-fold

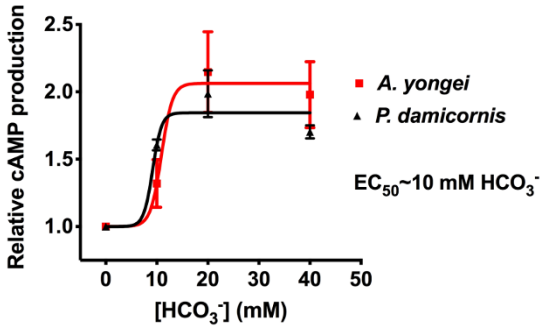

Figure 3 | Dose response curve of the effect of bicarbonate on cAMP production in coral tissue homogenates (*P. damicornis*, N = 5; *A. youngei*, N = 3). Lines were drawn from a non-linear curve fit model. Error bars indicate SEM.

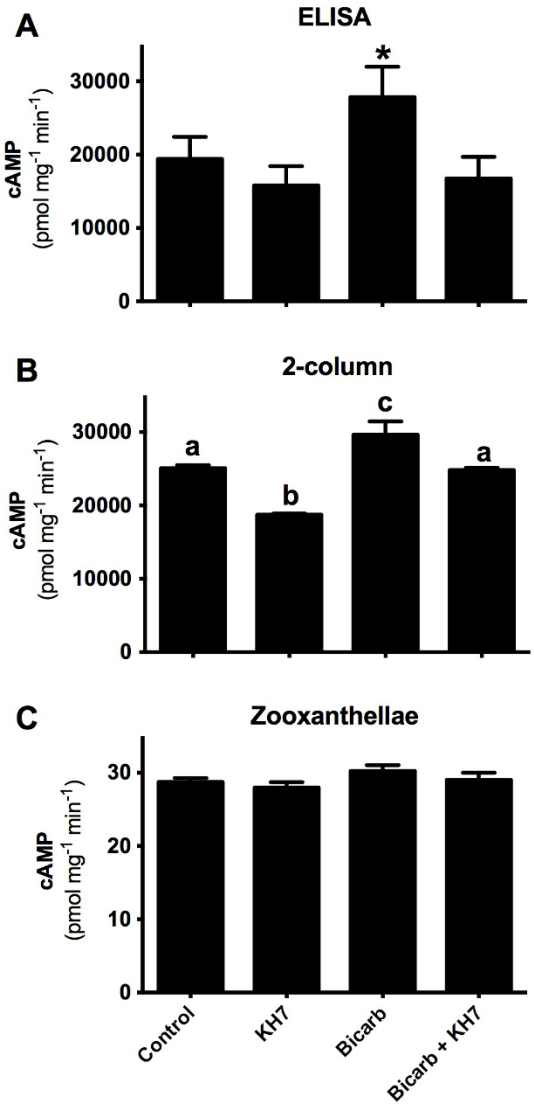

Figure 4 | Production of cAMP in *P. damicornis* tissue homogenates as measured by A) ELISA (N = 5) or B) 2-column assay (N = 3) or C) freshly isolated zooxanthellae (N = 5) as measured by ELISA. Note different scale for panel C. Treatments: Control = 40 mM NaCl; KH7 = 40 mM NaCl + 50 μM KH7; Bicarb = 40 mM $NaHCO_3$; Bicarb + KH7 = 40 mM $NaHCO_3$ + 50 μM KH7. Symbols indicate statistically significant differences. Error bars indicate SEM.

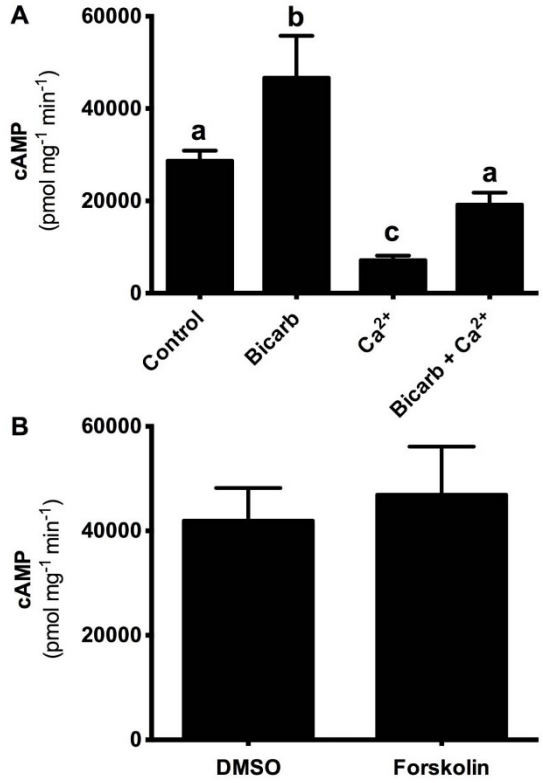

Figure 5 | Cyclic AMP production in *P. damicornis* tissue homogenates in response to A) bicarbonate and/or calcium (N = 6) or B) forskolin (N = 4). Treatments: Control=40 mM NaCl; Bicarb=40 mM $NaHCO_3$; Ca^{2+} = 2.5 mM $CaCl_2$; Bicarb + Ca^{2+} = 40 mM $NaHCO_3$ with 2.5 mM $CaCl_2$; 50 μM forskolin. Letters indicate statistically significant differences. Error bars indicate SEM.

higher[26,35,36]. To the best of our knowledge, coral cAMP production rates measured here are higher than any other animal observed to date. It is important to note that corals are host to a variety of microorganisms that could potentially contribute to cAMP production, including endosymbtiotic zooxanthellae, prokaryotes, fungi and viruses (i.e. the coral holobiont[37]). However, given that cAMP production from isolated zooxanthellae and coral mucus was 10^3-fold and 10^5-fold lower, respectively, than whole tissue homogenates, we can rule out any significant contribution from these microorganisms.

Our bioinformatic analysis revealed that *A. digitifera* has at least four tmAC and two sAC isoforms, while *P. damicornis* has at least five tmACs but no sAC mRNA transcripts. However, further characterization of coral adenylyl cyclase activity was consistent with the presence of active forms of both tmACs and sAC in both species. Adenylyl cyclase activity in *A. youngei* and *P. damicornis* was stimulated by bicarbonate, and the sAC inhibitor KH7 abolished this stimulation. These responses indicate that both coral species have one or more active forms of sAC, and that the absence of a sAC ortholog in the *P. damicornis* transcriptome database is likely due to incomplete coverage.

The physiological role of sAC in corals remains an outstanding question, but it likely acts as a $pH/CO_2/HCO_3$ sensor as it does in other animals. For example, mammalian sAC is stimulated by bicarbonate to trigger sperm flagellar movement[29]; it also regulates embryo development, epithelial salt and fluid transport, gene transcription, and acid-base homeostasis in epididymis and kidney, among other processes (reviewed in[16]). Knowledge on the roles of sAC in aquatic organisms is much more limited; however, they seem to be similar to those in mammals: sAC regulates sperm function in sea urchin[15,38], epithelial salt transport across the intestine of bony fish[39,40], and modulates acid-base homeostasis in shark gills[41]. There are many potential roles for sAC as a $pH/CO_2/HCO_3$ sensor in corals. For example, photosynthetic activity by symbiotic zooxanthellae increases pH in zooxanthellae-hosting gastrodermal cells[42], in the sub-calicoblastic medium[43], and in the coelenteron[42]; photosynthesis also significantly increases bicarbonate concentration in tissues[44]. Calcification in zooxanthellate corals is stimulated by light, a phenomenon referred to as 'light-enhanced calcification'[45]. The mechanism is unknown, but it is thought to be related to availability of energy from photosynthesis, production of photosynthates that serve as substrates for the organic matrix of the skeleton[45], increased oxygen to support aerobic metabolism, titration of protons produced by calcification[46] and/or by associated changes in pH and $[HCO_3^-]$[42–44]. The presence of bicarbonate-sensitive adenylyl cyclase activity in corals demonstrated here, as well as the potentially light-driven changes in endogenous cAMP, point to sAC as a promising possibility for sensing acid-base disturbances in corals and triggering light

enhanced calcification and other homeostatic physiological responses.

Unlike tmACs from vertebrate animals[22], adenylyl cyclase activity in coral homogenates was insensitive to forskolin. This lack of response has been observed in other invertebrates, including the sea pansy[26], hydra[27], and sea urchins[47], suggesting that the structure of invertebrate cyclases is different from those in vertebrates. However, forskolin does have biological effects on corals and other cnidarians, including inhibition of coral metamorphosis[24] and sensitization of mechanoreceptors for nematocyst discharge in anemones[48], a process that is stimulated by cAMP[31,35,48,49]. It is important to note, however, that neither study measured the effect of forskolin on cAMP levels, so whether the responses observed are the result of forskolin stimulation of tmACs is not known. In any case, it is clear that future research cannot rely on inhibitors and activators of adenylyl cyclases developed for mammals without first verifying their effects on corals.

The inhibitory effect of calcium on cAMP production in corals was consistent with tmAC activity. Moreover, bicarbonate stimulation was retained in the presence of calcium, indicating that calcium inhibition was specific to tmACs and not sAC. In mammals, calcium ions significantly affect the activity of tmACs, but the effect differs depending on the concentration and the isoform involved. For example, micromolar concentrations of calcium activate some tmACs[50–52] but inhibit others[50,52], while millimolar concentrations inhibit all mammalian tmAC isoforms[50–53]. Here we found that coral adenylyl cyclase activity was inhibited by millimolar concentrations of calcium. It remains to be determined how the four individual isoforms of coral tmACs identified here respond to different levels of calcium, and it is possible that each may respond differently, particularly to lower (micromolar) concentrations of calcium. The interaction between adenylyl cyclases and calcium may be particularly important for coral calcification, which requires calcium uptake from the surrounding seawater and transport across the epithelia[18,54]. This process involves both calcium ion channels[55] and Ca-ATPases[56,57], and leads to an increasing concentration gradient of calcium in coral tissue going from the outer oral ectoderm, through the oral and aboral endoderm, to the skeleton-facing calicoblastic ectoderm[58]. Interestingly, the activity of these calcium transport enzymes is not regulated at the level of transcription[59], suggesting that they are post-translationally modulated.

The cAMP pathway has been somewhat characterized in other cnidarians: it is involved in the regulation of mechanoreceptors and nematocyst discharge in anemones[31,35,48]; promotion of regeneration in anemones[60] and hydra[27,61]; promotion of cilia beating and feeding in anemones[35] but inhibition of feeding in hydra[33,62]; progression of metamorphosis in jellyfish[63]; and modulation of muscle contractions[34] and bioluminescence[64] in sea pansies. Many of these processes may also be regulated through the cAMP pathway in corals, and there are several additional and possibly coral-specific roles open to discovery. For example, a variety of coral stressors (e.g. temperature, allelochemicals) have been shown to cause upregulation of cAMP-dependent transcription factors such as CREB[19,20], ATF-2[20], ATF-4[19,20], and CRE modulators[19], as well as PKAs[21], the upstream mechanisms and downstream effects of which are unknown. The diurnal fluctuations of cAMP in corals observed here suggest that coral sAC may be involved in regulating acid-base homeostasis during photosynthesis and light-enhanced calcification. Coral gametogenesis and gamete release, which are coordinated to daily and annual light cycles[65], are other physiological processes potentially modulated by adenylyl cyclase activity (for example, *via* light-sensitive GPCRs or cryptochromes and downstream effects linked to circadian rhythms).

In summary, our study demonstrates that corals have a uniquely high capacity for cAMP production that appears to involve both tmACs and sAC, and that cAMP levels in host coral tissues fluctuate on a diel basis. These results make the cAMP pathway in corals a promising new avenue of research. If we are to predict how corals will respond to environmental perturbations, including but not limited to eutrophication, rising sea surface temperatures, and ocean acidification[2,66], it is essential to understand the cellular mechanisms regulating coral physiology.

Methods

Bioinformatics. Homologs for adenylyl cyclases in corals were identified by querying mammalian tmAC and sAC nucleotide sequences via BLASTn versus the *Acropora digitifera* genome[7] and a *Pocillopora damicornis* transcriptome database (http://cnidarians.bu.edu/PocilloporaBase/cgi-bin/index.cgi). Putative coral adenylyl cylases were aligned with representatives from various phyla using Clustal W2 and a phylogenetic tree of the alignment was generated using the neighbor joining method[67].

Specimens. *Pocillopora damicornis* fragments were obtained from coral colonies growing at aquaria at Rutgers University or at the Birch Aquarium at Scripps. *Acropora youngei* fragments were obtained from Dimitri Deheyn at the Scripps Institution of Oceanography. All fragments were from different colonies. Coral tissue was removed from the skeleton and homogenized using an airbrush (80 psi) or by scraping with a toothbrush into 100 mM Tris (pH 7.5) supplemented with a protease inhibitor cocktail (Sigma-Aldrich). Protein concentration in each homogenate was measured by the method of Bradford in triplicate. Zooxanthellae were removed via differential centrifugation (1000 × *g* for 2 min) and the supernatant was divided into aliquots and frozen at −20°C. All cAMP assays were performed with first thaw homogenates, since samples subjected to more than one freeze-thaw lost HCO_3^--stimulation of cAMP production. Mucus release from *A. youngei* fragments was stimulated by brief exposure to air and was collected in a sterile syringe. Mucus was concentrated by transferring strands and clumps of mucus to a new tube with 500 µL of 0.2 µm filtered seawater. Mucus was then broken up by passing it through a small pipet tip multiple times, causing sheering, and was assayed for cAMP production and protein content immediately. Because protein content within the mucus was undetectable, specific activity could not be calculated.

cAMP levels in live corals. At least one week before sampling, *P. damicornis* fragments of ~5 mm in length were severed from coral colonies and placed in individual stands. Corals were maintained under a 12:12 hr light:dark cycle. At different times throughout the day, coral fragments were removed from the tank by hand, immediately immersed in 200 µL 0.1 N HCl, vortexed for 15 sec, and stored at −20°C. This treatment bursts the cells and causes the release of cytoplasmic molecules (including cAMP) into the surrounding 0.1 N HCl, which was then used in the ELISA (acetylated protocol) for cAMP quantification. Total protein concentration in each sample was measured by the method of Bradford and used to standardize cAMP concentration. Values of cAMP are an average from nine coral fragments at each time point, which were sampled in groups of three per sampling time on three different days over a two-month period. The effect of time of day was analyzed as a fixed effect in a linear mixed-effects model fit by maximum likelihood test with "sampling day" treated as a random effect using R 2.11.1[68].

Assays for cAMP-producing activity. Coral tissue homogenates were incubated in "cyclase assay buffer" consisting of 100 mM Tris (pH 7.5), 0.5 mM isobutylmethylxanthine (IBMX), 1 mM dithiothreitol (DTT), 20 mM creatine phosphate (CP), 100 U mL^{-1} creatine phosphokinase (CPK), 5 mM $MgCl_2$, and 5 mM ATP for 30 min at room temperature on an orbital shaker (300 rpm). Treatments were performed in triplicate for each homogenate along with one reaction to determine the initial concentration of cAMP (t = 0). Treatments included a control (40 mM NaCl), increasing concentrations of $NaHCO_3$, 2.5 mM $CaCl_2$, DMSO, 50 µM forskolin (in DMSO), or 50 µM KH7 (in DMSO). $NaHCO_3$ solutions were prepared fresh before addition to the assay. Reactions were stopped with the addition of one volume of 0.2 N HCl. Production of cAMP was measured by ELISA using the acetylated protocol of the Direct cAMP ELISA kit (Enzo Life Sciences).

Freshly isolated zooxanthellae pellets were resuspended in 100 mM Tris (pH 7.5), sonicated on ice, and assayed for cAMP production as described above. However, these samples did not produce any detectable cAMP. Additional zooxanthellae pellets were combined with glass beads in Tris buffer and homogenized using a bead beater. These homogenates were then assayed for cAMP production as described above, and had detectable cAMP production (data shown in 'Results'). Since zooxanthellae cAMP production could only be detected when the cells were prepared with bead beating, this method was used to determine if zooxanthellae cAMP production was sensitive to bicarbonate or KH7. Protein concentration in each homogenate was determined by the Bradford method. All cAMP production rates are reported in pmol cAMP produced per mg total protein per minute.

Selected treatments with coral tissue homogenates were repeated using a radioactivity-based two-column adenylyl cyclase assay according to the method of Salomon[28]. Homogenates were incubated in cyclase assay buffer supplemented with ~1,000,000 cpm [α-^{32}P]ATP (Perkin Elmer) and ~5,000 cpm [^{3}H]cAMP (Perkin Elmer). Reactions were performed in 100 µL for 30 min at room temperature and stopped with 150 µL 1.5% SDS. Product [^{32}P]cAMP was separated from substrate [α-^{32}P]ATP by sequential column chromatography over dowex 50WX4-400 resin

(Fluka) followed by aluminium oxide resin (Sigma). Product [^{32}P]cAMP was eluted from dowex directly onto the alumina by water, and cAMP was eluted from alumina by 0.1 M imidazole (pH = 7.3).

1. Knowlton, N. *et al.* in *Life in the World's Oceans: Diversity, Distribution, and Abundance 65–77* (John Wiley and Sons, 2010).
2. Hoegh-Guldberg, O. *et al.* Coral reefs under rapid climate change and ocean acidification. *Science* **318**, 1737–1742 (2007).
3. Knowlton, N. & Jackson, J. B. C. Shifting baselines, local impacts, and global change on coral reefs. *PLoS Biol* **6**, e54 (2008).
4. Miller, D. J. *et al.* The innate immune repertoire in Cnidaria-ancestral complexity and stochastic gene loss. *Genome Biol* **8**, R59 (2007).
5. Technau, U. *et al.* Maintenance of ancestral complexity and non-metazoan genes in two basal cnidarians. *Trends Genet* **21**, 633–639 (2005).
6. Putnam, N. H. *et al.* Sea anemone genome reveals ancestral eumetazoan gene repertoire and genomic organization. *Science* **317**, 86–94 (2007).
7. Shinzato, C. *et al.* Using the *Acropora digitifera* genome to understand coral responses to environmental change. *Nature* **476**, 320–323 (2011).
8. Sossone-Corsi. The cyclic AMP pathway. *Cold Spring Harbor Perspectives Biol* **4**(12), 1–3 (2012).
9. Cooper, D. M. F. Regulation and organization of adenylyl cyclases and cAMP. *Biochem J* **375**, 517 (2003).
10. Buck, J., Sinclair, M., Schapal, L., Cann, M. & Levin, L. Cytosolic adenylyl cyclase defines a unique signaling molecule in mammals. *Proc Natl Acad Sci* **96**, 79–84 (1999).
11. Hanoune, J. & Defer, N. Regulation and role of adenylyl cyclase isoforms. *Annu Rev Pharmacol* **41**, 145–174 (2001).
12. Tresguerres, M., Levin, L. R. & Buck, J. Intracellular cAMP signaling by soluble adenylyl cyclase. *Kidney Int* **79**, 1277–1288 (2011).
13. Kamenetsky, M., Middelhaufe, S., Bank, E. M., Levin, L. R., Buck, J. & Steegborn, C. Molecular details of cAMP generation in mammalian cells: a tale of two systems. *J Molec Biol* **362**, 623–639 (2006).
14. Chen, Y. *et al.* Soluble adenylyl cyclase as an evolutionarily conserved bicarbonate sensor. *Science* **289**, 625–628 (2000).
15. Nomura, M., Beltrán, C., Darszon, A. & Vacquier, V. D. A soluble adenylyl cyclase from sea urchin spermatozoa. *Gene* **353**, 231–238 (2005).
16. Tresguerres, M., Buck, J. & Levin, L. R. Physiological carbon dioxide, bicarbonate, and pH sensing. *Pflü Arch Eur J Phy* **460**, 953–964 (2010).
17. Kobayashi, M., Buck, J. & Levin, L. R. Conservation of functional domain structure in bicarbonate-regulated 'soluble' adenylyl cyclases in bacteria and eukaryotes. *Dev Genes Evol* **214**, 503–509 (2004).
18. Marshall, A. T. Calcification in hermatypic and ahermatypic corals. *Science, New Ser* **271**, 637–639 (1996).
19. DeSalvo, M., Estrada, A., Sunagawa, S. & Medina, M. Transcriptomic responses to darkness stress point to common coral bleaching mechanisms. *Coral Reefs* **31**, 215–228 (2012).
20. DeSalvo, M. K., Sunagawa, S., Voolstra, C. R. & Medina, M. Transcriptomic responses to heat stress and bleaching in the elkhorn coral *Acropora palmata. Mar Ecol Prog Ser* **402**, 97–113 (2010).
21. Shearer, T., Rasher, D., Snell, T. & Hay, M. Gene expression patterns of the coral *Acropora millepora* in response to contact with macroalgae. *Coral Reefs* **31**, 1177–1192 (2012).
22. Seamon, K. B., Padgett, W. & Daly, J. W. Forskolin: unique diterpene activator of adenylate cyclase in membranes and in intact cells. *Proc Natl Acad Sci* **78**, 3363–3367 (1981).
23. Schorderet-Slatkine, S. & Baulieu, E.-E. Forskolin increases cAMP and inhibits progesterone induced meiosis reinitiation in *Xenopus laevis* oocytes. *Endocrinology* **111**, 1385–1387 (1982).
24. Tran, C. & Hadfield, M. G. Are G-protein-coupled receptors involved in mediating larval settlement and metamorphosis of coral planulae? *Biol Bull* **222**, 128–136 (2012).
25. Tensen, C. P. *et al.* Molecular cloning and characterization of an invertebrate homologue of a neuropeptide Y receptor. *Eur J Neurosci* **10**, 3409–3416 (1998).
26. Awad, E. W. & Anctil, M. Positive coupling of beta-like adrenergic receptors with adenylate cyclase in the cnidarian *Renilla koellikeri*. *J Exp Biol* **182**, 131–146 (1993).
27. Galliot, B., Welschof, M., Schuckert, O., Hoffmeister, S. & Schaller, H. C. The cAMP response element binding protein is involved in hydra regeneration. *Development* **121**, 1205–1216 (1995).
28. Salomon, Y. Adenylate cyclase assay. *Adv Cyclic Nucleotide Res* **10**, 35–55 (1979).
29. Hess, K. C. *et al.* The 'soluble' adenylyl cyclase in sperm mediates multiple signaling events required for fertilization. *Dev Cell* **9**, 249–259 (2005).
30. Laurent, J., Tambutté, S., Tambutté, É., Allemand, D. & Venn, A. The influence of photosynthesis on host intracellular pH in scleractinian corals. *J Exp Biol* (2012). doi:10.1242/jeb.082081.
31. Ozacmak, V. H., Thorington, G. U., Fletcher, W. H. & Hessinger, D. A. N-acetylneuraminic acid (NANA) stimulates in situ cyclic AMP production in tentacles of sea anemone (*Aiptasia pallida*): possible role in chemosensitization of nematocyst discharge. *J Exp Biol* **204**, 2011–2020 (2001).
32. Cobb, M. H., Heagy, W., Danner, J., Lenhoff, H. M. & Marshall, G. R. Effect of glutathione on cyclic nucleotide levels in *Hydra attenuata*. *Comp Biochem Phys C* **65**, 111–115 (1980).
33. Venturini, G. & Carolei, A. Dopaminergic receptors in Hydra. Pharmacological and biochemical observations. *Comp Biochem Phys C* **102**, 39–43 (1992).
34. Anctil, M. Modulation of a rhythmic activity by serotonin via cyclic AMP in the coelenterate *Renilla köllikeri*. *J Comp Physiol B* **159**, 491–500 (1989).
35. Gentleman, S. & Mansour, T. E. Adenylate cyclase in a sea anemone: Implication for chemoreception. *Biochim Biophys Acta* **343**, 469–479 (1974).
36. Venturini, G., Silei, O., Palladini, G., Caroieli, A. & Margotta, V. Aminergic neurotransmitters and adenylate cyclase in Hydra. *Comp Biochem Phys C* **78**, 345–348 (1984).
37. Knowlton, N. & Rohwer, F. Multispecies microbial mutualisms on coral reefs: the host as a habitat. *Am Nat* **162**, S51–62 (2003).
38. Beltrán, C. *et al.* Particulate and soluble adenylyl cyclases participate in the sperm acrosome reaction. *Biochem Biophys Res Commun* **358**, 1128–1135 (2007).
39. Carvalho, E. S. M., Gregório, S. F., Power, D. M., Canário, A. V. M. & 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).
40. Tresguerres, M., Levin, L. R., Buck, J. & Grosell, M. Modulation of NaCl absorption by [HCO_3^-] in the marine teleost intestine is mediated by soluble adenylyl cyclase. *Am J Physiol Regul Integr Comp Physiol* **299**, R62–71 (2010).
41. Tresguerres, M. *et al.* Bicarbonate-sensing soluble adenylyl cyclase is an essential sensor for acid/base homeostasis. *Proc Natl Acad Sci* **107**, 442 (2010).
42. Venn, A. A. *et al.* Imaging intracellular pH in a reef coral and symbiotic anemone. *Proc Natl Acad Sci* **106**, 16574–16579 (2009).
43. Venn, A., Tambutté, E., Holcomb, M., Allemand, D. & Tambutté, S. Live tissue imaging shows reef corals elevate pH under their calcifying tissue relative to seawater. *PLoS One* **6**, e20013 (2011).
44. Furla, P., Galgani, I., Durand, I. & Allemand, D. Sources and mechanisms of inorganic carbon transport for coral calcification and photosynthesis. *J Exp Biol* **203**, 3445–3457 (2000).
45. Moya, A. *et al.* Study of calcification during a daily cycle of the coral *Stylophora pistillata*: implications for 'light-enhanced calcification'. *J Exp Biol* **209**, 3413–3419 (2006).
46. Furla, P., Bénazet-Tambutté, S., Jaubert, J. & Allemand, D. Functional polarity of the tentacle of the sea anemone *Anemonia viridis*: role in inorganic carbon acquisition. *Am J Physiol Regul Integr Comp Physiol* **274**, R303–R310 (1998).
47. Garbers, D. L. & Kopf, G. S. The regulation of spermatozoa by calcium cyclic nucleotides. *Adv Cyclic Nucleotide Res* **13**, 251–306 (1980).
48. Watson, G. M. & Hessinger, D. A. Receptors for N-acetylated sugars may stimulate adenylate cyclase to sensitize and tune mechanoreceptors involved in triggering nematocyst discharge. *Exp Cell Res* **198**, 8–16 (1992).
49. Mire-Thibodeaux, P. & Watson, G. M. Cyclical morphodynamics of hair bundles in sea anemones: second messenger pathways. *J Exp Zool* **270**, 517–526 (1994).
50. Guillou, J. L., Nakata, H. & Cooper, D. M. Inhibition by calcium of mammalian adenylyl cyclases. *J Biol Chem* **274**, 35539–35545 (1999).
51. Wang, H. & Storm, D. R. Calmodulin-regulated adenylyl cyclases: cross-talk and plasticity in the central nervous system. *Molec Pharmacol* **63**, 463–468 (2003).
52. Willoughby, D. & Cooper, D. M. F. Organization and Ca2+ regulation of adenylyl cyclases in cAMP microdomains. *Physiol Rev* **87**, 965–1010 (2007).
53. Paterson, J. M. *et al.* Characterisation of human adenylyl cyclase IX reveals inhibition by Ca2+/calcineurin and differential mRNA polyadenylation. *J Neurochem* **75**, 1358–1367 (2000).
54. Tambutté, E., Allemand, D., Mueller, E. & Jaubert, J. A compartmental approach to the mechanism of calcification in hermatypic corals. *J Exp Biol***199**, 1029–1041 (1996).
55. Zoccola, D. *et al.* Cloning of a calcium channel α1 subunit from the reef-building coral, *Stylophora pistillata. Gene* **227**, 157–167 (1999).
56. Ip, Y. K., Lim, A. L. L. & Lim, R. W. L. Some properties of calcium-activated adenosine triphosphatase from the hermatypic coral *Galaxea fascicularis. Mar Biol* **111**, 191–197 (1991).
57. Zoccola, D. *et al.* Molecular cloning and localization of a PMCA P-type calcium ATPase from the coral *Stylophora pistillata. BBA-Biomembranes* **1663**, 117–126 (2004).
58. Marshall, A. T., Clode, P. L., Russell, R., Prince, K. & Stern, R. Electron and ion microprobe analysis of calcium distribution and transport in coral tissues. *J Exp Biol* **210**, 2453–2463 (2007).
59. Moya, A. *et al.* Cloning and use of a coral 36B4 gene to study the differential expression of coral genes between light and dark conditions. *Mar Biotechnol* **10**, 653–663 (2008).
60. Lenicque, P. M., Féral, J. P. & Doumenc, D. Effects of some cyclic nucleotides on the wound healing and the regeneration of the sea-anemones, *Cereus pedduncula*tus *penn.* and *Metridium senile*. *Comp Biochem Phys C* **61**, 341–346 (1978).
61. Hill, S. K. & Lesh-Laurie, G. E. Cyclic AMP concentrations during distal regeneration in *Hydra oligactis*. *J Exp Zool* **218**, 233–238 (1981).
62. Venturini, G. The hydra GSH receptor. Pharmacological and radioligand binding studies. *Comp Biochem Phys C* **87**, 321–324 (1987).

63. Fitt, W. K., Hofmann, D. K., Wolk, M. & Rahat, M. Requirement of exogenous inducers for metamorphosis of axenic larvae and buds *of Cassiopea andromeda* (Cnidaria: Scyphozoa). *Mar Biol* **94**, 415–422 (1987).
64. New, D. C. & Wong, J. T. Y. The evidence for G-protein-coupled receptors and heterotrimeric G proteins in protozoa and ancestral metazoa. *Neurosignals* **7**, 98–108 (1998).
65. Levy, O. *et al*. Light-responsive cryptochromes from a simple multicellular animal, the coral *Acropora millepora. Science* **318**, 467–470 (2007).
66. Orr, J. C. *et al*. Anthropogenic ocean acidification over the twenty-first century and its impact on calcifying organisms. *Nature* **437**, 681–686 (2005).
67. Larkin, M. A. *et al*. Clustal W and Clustal × version 2.0. *Bioinformatics* **23**, 2947–2948 (2007).
68. R Development Core Team. R: a language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria (2010).

Acknowledgements

Many thanks to Fernando Nosratpour (Birch Aquarium at Scripps) and Dimitri Deheyn (Scripps Institution of Oceanography) for generously providing coral fragments, and Frank Natale (Rutgers) for help maintaining corals. We also thank Dr. Paul Falkowski (Rutgers) for useful comments and for logistical support, and Dr. Jonathan Shurin (UCSD) for advice with statistical analyses. This work was supported by the National Science Foundation grants EF-1220641 to MT and OCE-1226396 to KB.

Author contributions

Conceived the study: M.T., Y.H., L.L., J.B. Performed experiments: M.T., K.B., Y.H., L.H,. M.B., K.H. Analyzed data: M.T., K.B., Y.H., K.H., L.L., J.B. Wrote paper: K.B., M.T.

Additional information

Competing financial interests: The authors declare no competing financial interests.

How to cite this article: Barott, K.L. *et al*. High adenylyl cyclase activity and *in vivo* cAMP fluctuations in corals suggest central physiological role. *Sci. Rep.* **3**, 1379; DOI:10.1038/srep01379 (2013).