Ocean acidification stimulates alkali signal pathway: a bicarbonate sensing soluble adenylyl cyclase from oyster *Crassostrea gigas* mediates physiological changes induced by CO₂ exposure

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Abstract

Ocean acidification (OA) has been demonstrated to have severe effects on marine organisms, especially marine calcifiers. However, the impacts of OA on the physiology of marine calcifiers and the underlying mechanisms remain unclear. Soluble adenylyl cyclase (sAC) is an acid-base sensor in response to [HCO₃⁻] and an intracellular source of cyclic AMP (cAMP). In the present study, an ortholog of sAC was identified from pacific oyster *Crassostrea gigas* (designated as CgsAC) and the catalytic region of CgsAC was cloned and expressed. Similar to the native CgsAC

from gill tissues, the recombinant CgsAC protein (rCgsAC) exhibited $[\text{HCO}_3^-]$ mediated cAMP-forming activity, which could be inhibited by a small molecule KH7. After 16 days of CO_2 exposure (pH=7.50), the mRNA transcripts of CgsAC increased in muscle, mantle, hepatopancreas, gill, male gonad and haemocytes, and two truncated CgsAC forms of 45 kD and 20 kD were produced. Cytosolic CgsAC could be translocated from the cytoplasm and nuclei to the membrane in response to CO_2 exposure. Besides, CO_2 exposure could increase the production of cAMP and intracellular pH of haemocytes, which was regulated by CgsAC ($p < 0.05$), suggesting the existence of a $[\text{HCO}_3^-]$ /CgsAC/cAMP signal pathway in oyster. The elevated CO_2 could induce an increase of ROS level ($p < 0.05$) and a decrease of phagocytic rate of haemocytes ($p < 0.05$), which could be inhibited by KH7. The results collectively suggest that CgsAC is an important acid-base sensor in oyster and the $[\text{HCO}_3^-]$ /CgsAC/cAMP signal pathway might be responsible for intracellular alkalization effects on oxidative phosphorylation and innate immunity under CO_2 exposure. The changes of intracellular pH, ROS, and phagocytosis mediated by CgsAC might help us to further understand the effects of ocean acidification on marine calcifiers.

Key word: Ocean acidification; soluble adenylyl cyclase; oyster; physiological response

1. Introduction

The concentration of atmosphere CO_2 is predicted to be 550-800 ppm at the end

of this century, which would cause a 100-150% increase in hydrogen ion and a 0.3-0.4 units decrease in ocean pH value at the surface (Hönisch et al., 2012; Hoegh-Guldberg et al., 2007; Orr et al., 2005). A series of studies have demonstrated the severe consequences of OA on calcifying marine organisms, especially mollusks (Gazeau et al., 2013; Michaelidis et al., 2005; Pan et al., 2015; Parker et al., 2013; Ridgwell and Schmidt, 2010). The physiological responses of marine calcifiers such as acid-base imbalance were considered as the most direct and key effects of OA (Gutowska et al., 2010; Lannig et al., 2010; Talmage and Gobler, 2009). These physiological variations would further affect the calcification process, energy metabolism and immune response of marine calcifiers, which would lead to severe consequences such as reduction in fitness, fertilization and even genetic adaptation (Parker et al., 2013; Velez et al., 2016).

Soluble adenylyl cyclase (sAC) is an intracellular source of the ubiquitous second messenger cAMP in response to $[\text{HCO}_3^-]$ (Chen et al., 2000; Jaiswal and Conti, 2001; Zippin et al., 2013). As an evolutionally conserved signaling enzyme, sAC has been proven to reflect extracellular $\text{CO}_2/[\text{HCO}_3^-]$ levels in mammalian sperm (Jaiswal and Conti, 2001), dogfish gill (Tresguerres et al., 2010), and sea urchin sperm (Nomura et al., 2005). Its target product, cAMP, plays a central role in both vertebrate and invertebrates. For example, the cAMP-dependent signal pathways has been implied to involve in protein kinase A (PKA) activation, glycogen breakdown, gametogenesis and spawning induction, cardiac contraction, mantle and siphon movement, and adductor muscle relaxation in bivalves (Fabbri and Capuzzo, 2010).

Pacific oyster (*Crassostrea gigas*), a marine bivalve in the second largest animal phylum Mollusca (Kocot et al., 2011), is a successful colonizer in the estuaries and intertidal zones, and it must cope with many environmental fluctuations, such as those of temperature, salinity, pathogens and carbonic chemistry disturbance (Lannig et al., 2010; Zhang et al., 2012). These environmental fluctuations are always associated with increases of $[\text{HCO}_3^-]$ in the internal fluids of oyster (Tresguerres et al., 2014). In the past decades, the populations of oysters have been suffering from massive mortalities, leading to severe loss of economy and grievous destruction of ecosystem. The investigation about the CgsAC and the CgsAC-cAMP pathway would contribute to cognize the responses of marine bivalve to OA. The purposes of the present study were (1) to identify and characterize sAC from oyster, (2) to investigate the mRNA expression level, protein formats and subcellular distribution of CgsAC in response to CO_2 exposure, (3) to characterize the downstream pathway of CgsAC and its physiological function in response to CO_2 exposure, including acid-base balance, energy metabolism, and immune response, for better understanding the roles of CgsAC in systemic acid-base regulation of oyster under OA.

2. Material and methods

2.1. Animals

Oysters *C. gigas* with average shell length of 13.0 cm were collected from a local farm in Qingdao, China. They were pre-punctured by the side of the shell and then maintained in the aerated seawater at about 15 °C for one week. Female mice were purchased from the Qingdao Institute for Drug Control (Qingdao, China). Animal

experiments were approved by the Qingdao Animal Care and Use Committee with a permit number of SCXK (Shandong) 20090007, which complied with the National Institute of Health Guide for the Care and Use of Laboratory Animals.

2.2. CO₂ exposure, injection of medication and tissue collection

Thirty individuals were randomly divided into control group (pH=8.10±0.05) and CO₂ exposure group (pH=7.515 oysters each). All animal tanks were continuously bubbled with ambient air or air-CO₂ mixtures as appropriate, and the water was changed every other day to ensure adequate water quality. The pH value of the CO₂ exposure group was controlled using an acidometer (AiKB, Qingdao, China). During the whole treatment process, the temperature was controlled at 14.9±0.5 °C and 15.0±0.4 °C for the control group and CO₂ exposure group, respectively. The salinity was relative stable, and it was 32.8±0.04 and 32.6±0.05 in the control group and CO₂ exposure group, respectively. The pH value and dissolved oxygen were monitored, and they were adjusted by controlling bubbling of CO₂ and air. The dissolved oxygen in the control group and CO₂ exposure group was 7.73±0.04 mg/L and 7.83±0.02 mg/L, respectively.

After CO₂ exposure for 16 days, six tissues, including hepatopancreas, gill, mantle, adductor muscle, male gonad and female gonad were collected to determine mRNA transcripts of CgsAC. The haemolymph of oysters was collected and centrifuged at 800 g, 4 °C for 10 min to harvest the haemocytes. The tissues and haemocytes were transferred into 1 mL of Trizol reagent and stored at -80 °C. Additional samples were snap-frozen in liquid nitrogen and kept at -80 °C until they

were used for western blotting. For RNA extraction and western blotting, the tissues from four oysters were pooled together as one sample and there were three samples for each determination.

At the same time, another thirty oysters were used for drug injection experiment, and they were pre-adapted to the CO₂-exposed environment for two days. Oysters received an injection of 100 µL of the specific inhibitor of sAC (KH7) with the concentration of 5 mM and 0.5 mM through the muscle of the oysters, to make the final content of about 50 µmol kg⁻¹ and 5 µmol kg⁻¹ in oyster tissue, respectively (10 oysters each). The oysters, which received an injection of 100 µL Dimethyl sulfoxide (DMSO), were used as negative control (10 oysters). After incubation for two weeks, gill and mantle samples of each group were collected, immersed in 0.1 M HCl and snap-frozen in liquid nitrogen for cAMP content determination. Haemocytes from different groups were collected to determine intracellular pH, rate of phagocytosis, and ROS level. For cAMP, intracellular pH, rate of phagocytosis, and ROS level determination, tissues and haemocytes from three oysters were pooled together as one sample and there were three samples for each determination.

2.3. RNA isolation and cDNA synthesis

Total RNA was extracted from tissues using Trizol reagent (Invitrogen). The first-strand synthesis was carried out with M-MLV RT using the DNase I (Promega) treated total RNA as template and oligo (dT)-adaptor as primer (Table 1, P1). The reaction mixtures were first incubated at 42 °C for 1 h, and the reaction then terminated by heating at 95 °C for 5 min. The cDNA mix was diluted to 1:50 and

stored at -80 °C for subsequent SYBR Green fluorescent quantitative real-time PCR (qRT-PCR).

2.4. cDNA cloning and sequencing of CgsAC

Sequence information of CgsAC (EKC32844.1) was retrieved from the National Center for Biotechnology Information (<http://www.ncbi.nlm.nih.gov/>). A pair of specific primers P2 and P3 (Table 1) was designed to clone the catalytic domains of CgsAC. The PCR products were cloned into pMD 19-T simple vector (TaKaRa), transformed into *Escherichia coli* trans5 α (TransGen) and confirmed by DNA sequencing with primers M13-47 and RV-M (Table 1).

2.5. Sequence, homologous and phylogenetic analysis of CgsAC

The cDNA sequence and deduced amino acid sequence of CgsAC were analyzed using the BLAST algorithm (<http://www.ncbi.nlm.nih.gov/blast>) and the Expert Protein Analysis System (<http://www.expasy.org/>). The protein domains were revealed by the simple modular architecture research tool (SMART) version 7.0 (<http://www.smart.emblheidelberg.de/>). The ClustalW Multiple Alignment program was used to create sequence alignment of CgsAC and human sAC. A phylogenetic tree was constructed based on the sequence alignment by the neighbor-joining algorithm using the Mega 5.0 program. The reliability of the branching was tested by bootstrap re-sampling 1000 times.

2.6. Recombinant expression of CgsAC

The cDNA fragments encoding the two catalytic domains of CgsAC were amplified with the primers P4 and P5 (Table 1). Nde I and Not I sites were added to

the 5' end of primers. The PCR fragment was digested with restriction enzymes Nde I and Not I (NEB), and then cloned into predigested expression vector pET-30a. The recombinant plasmid pET-30a-CgsAC was transformed into *E. coli* transetta (DE3) (TransGen). The recombinant His tagged infusion CgsAC protein (designed as His-rCgsAC) was induced and purified according to the previous description (Jia et al., 2015). The resultant protein was separated by SDS-PAGE, and visualized with coomassie brilliant blue R-250. The concentration of purified His-rCgsAC was quantified by an enhanced BCA kit (P1101, Beyotime, China).

To obtain the soluble form of recombinant CgsAC protein, the PCR fragment digested with restriction enzymes Nde I and Not I was cloned into predigested expression vector pMAL-c5X. The recombinant plasmid pMAL-c5X-CgsAC was transformed into *E. coli* transetta (DE3) (TransGen). The pMAL-c5X vector without inserting fragment was employed as a negative control, which could express a maltose binding protein (MBP) in the prokaryotic express system. The recombinant MBP tagged infusion CgsAC protein (MBP-rCgsAC) and MBP were induced and purified according to the previous description (Wang et al., 2014). The resultant protein was separated by SDS-PAGE, and visualized with coomassie brilliant blue R-250. The concentration of purified MBP-rCgsAC and MBP was quantified by BCA kit and they were stored at -80 °C.

2.7. Assays for cAMP-producing activity of MBP-rCgsAC and gill homogenates

cAMP production catalyzed by purified MBP-rCgsAC and gill homogenates was determined according to the previous description (Tresguerres et al., 2010) using the

cAMP Direct Immunoassay Kit (Abcam, Cambridge, UK). The purified MBP-rCgsAC (50 μ g) and MBP (50 μ g) were incubated with assay buffer (50 mM Tris-HCl, 2.5 mM ATP, 20 mM MgCl₂, 5 mM MnCl₂, 1 mM DTT) at 30 °C for 30 min with reaction volume of 100 μ L. To study the activation of MBP-rCgsAC, freshly prepared 1 M NaHCO₃ was added to the reaction to give a final concentration of 0 mM, 5 mM, 10 mM, 20 mM, 40 mM, 60 mM, 80 mM and 100 mM, respectively. KH7 (5 mM, dissolved in DMSO) was added to give a final concentration of 100 μ M. DMSO was used as the negative control. Tris-HCl buffer with a pH value of 7.5 was used if [HCO₃⁻] introduced a pH change. The reactions were stopped by addition of an equal volume of 0.2 M HCl.

Oyster gill tissues were homogenized in 100 mM Tris buffer (pH=7.5) for 30 s, and the supernatants with 100 μ g total protein were incubated in cyclase assay buffer containing 100 mM Tris-HCl (pH 7.5), 0.5 mM isobutylmethylxanthine (IBMX, Sigma), 1 mM dithiothreitol (DTT, Solarbio, Beijing, China), 20 mM creatine phosphate (CP, Sigma), 100 U mL⁻¹ creatine phosphokinase (CPK, Sigma), 5 mM MgCl₂, and 5 mM ATP at 30 °C for 30 min with a total volume of 100 μ L. The appropriate amounts of [HCO₃⁻], DMSO, and KH7 (5 mM, dissolved in DMSO) were added as described above. The reactions were stopped by addition of an equal volume of 0.2 M HCl (giving a final concentration of 0.1 M). The experiments were performed in triplicate and the initial concentration of cAMP in protein homogenates was determined.

2.8. The determination of cAMP in oyster tissues

Oyster tissues were immersed in 0.1 M HCl and homogenized using an IKA homogenizer at 10,000 rpm (IKA Works Asia, GuangZhou, China) for 30 s. The supernatants were collected and the protein concentrations of each sample were determined by bicinchoninic acid (BCA) method. The cAMP level of each sample was determined using the cAMP Direct Immunoassay Kit after adjusting the protein concentration by diluting the samples with 0.1 M HCl.

2.9. Quantitative real-time PCR analysis of CgsAC mRNA expression

The mRNA expression level of CgsAC was measured by SYBR Green fluorescent RT-PCR on an ABI 7500 Real time Thermal Cycler. One pair of specific primers P6 and P7 (Table 1) for CgsAC were used to amplify a fragment of 108 bp. The *C. gigas* elongation factor 1 alpha (CgEF) fragment amplified with primers P8 and P9 (Table 1) was used as the internal reference. The relative expression level of CgsAC was determined by $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001) with female gonad of the control group as the reference tissue in the assay.

2.10. Preparation of a polyclonal antibody and western blotting

His-rCgsAC was used for the generation of a polyclonal antibody as described previously (Cheng et al., 2006). Briefly, 100 μ L mixture of His-rCgsAC and Freund's adjuvant was injected to immunize each female mouse four times and the interval between two injections was 10 days. The serum was harvested and stored at -80 °C until use.

Oyster tissue samples were homogenized for 30 seconds in lysis buffer (Beyotime biotechnology, Shanghai, China). The supernatants were collected, and the protein

concentrations were quantified by BCA method. About 40 µg protein was loaded for SDS-PAGE according to previous report (Jia et al., 2015). After SDS-PAGE, the protein was electrophoretically transferred onto a nitrocellulose membrane. The membrane was blocked with 5% milk (dissolved in TBST) at room temperature for 1 h. After washing with TBST for three times, the membrane was incubated with polyclonal antibody (diluted at 1:500) at 4 °C overnight, and then washed three times with PBST and incubated with goat-anti-mouse IgG (AS003, Abclonal, 1:2000) for 1 h at room temperature. Finally the membrane was washed three times and incubated in Western lighting-ECL substrate system (Perkin Elmer) before exposure to X-OMAT AR X-ray film (Eastman Kodak, Rochester, NY).

2.11. Immunohistochemistry assay

Immunohistochemistry assay was performed according to previous description with some modification (Jia et al., 2015). The haemolymph samples collected from 6 oysters were immediately centrifuged at 800 g, 4 °C for 10 min. The haemocytes were re-suspended in modified Leibovitz's L-15 mediums (M-L15) (Cao et al., 2003) and the suspension was added onto positively charged slides. After incubation at room temperature for 3 h, the supernatant was discarded and 4% PFA (Paraformaldehyde diluted in PBS) was added to fix the haemocytes for 15 min. After three times of washing in PBST (0.1% Tween 20 in PBS, pH 7.5), the slides were blocked with 3% BSA in PBS at room temperature for 30 min. Then the supernatant was removed and the slides were incubated with polyclonal antibody of CgsAC (diluted 1:500 in blocking buffer) at 37 °C for 1 h. After three times of washing in PBST, the slides

were incubated with Alexa Fluor 488-labeled goat-anti-mouse antibody (diluted 1:1000 in locking buffer) at 37 °C for 1 h. Finally, 4',6-Diamidine-2'-phenylindole dihydrochloride (DAPI, Roche, 10 mg mL⁻¹, diluting in PBS by 1:2500) was added onto the dishes to stain the nucleus. After the last three times of washing, 50% glycerol was added to seal the slides before observation under Laser Scan Confocal Microscope (ZEISS).

Mantle and gill tissues were immersed in Bouin solution (saturated picric acid solution: formaldehyde: acetic acid = 15:5:1) for 24 h and 70% (vol/vol) ethanol was used for decolorization. The samples were then dehydrated in 80%, 95% and 100% successive ethanol baths. After dehydration with Xylene/ethanol (1:1), and Xylene, respectively, all the tissue samples were embedded in paraffin and cut at 6 µm thickness. Paraffin was eliminated in Xylene bath and the sections were rehydrated in successive 95% to 30% ethanol baths and finally in distilled water. For antigen retrieval, the slides were immersed in 0.01 M sodium citrate buffer (pH=6) and sterilized at 121 °C for 6 min. After cooling down at room temperature, the slides were first blocked in BSA and then incubated with the polyclonal antibody of rCgsAC and Alexa Fluor 488 labeled goat-anti-mouse antibody. The other procedures were the same as described above.

2.12. Intracellular pH, rate of phagocytosis and ROS level assay by FACS Arial II flow cytometer

The haemolymphs were collected and mixed immediately with equal volume of pre-chilled anticoagulant (8 g L⁻¹ citric acid, 22 g L⁻¹ sodium citrate, 24.5 g L⁻¹

glucose), and then centrifuged at 800 g, 4 °C for 10 min. The haemocytes were re-suspended in M-L-15 medium and the cell concentration of each sample was adjusted to 10^6 mL^{-1} .

For intracellular pH (pH_i) determination, haemocytes were incubated with 2',7'-bis-(2-carboxyethyl)-5-(and-6)-carboxyfluorescein, acetoxymethyl ester (BCECF AM) at a final concentration of 10 μM at 37 °C for 20 min. Median fluorescence intensity of 10,000 cells was recorded as relative pH_i value. The intracellular ROS content was determined by incubating the haemocytes with 2',7'-dichloro-fluorescein diacetate (DCFH-DA) at a final concentration of 10 μM at 37 °C for 20 min, and ROS level was quantified by the median fluorescence intensity of 10,000 cells by FACS Arial II flow cytometer. Rate of phagocytosis was determined according to previous description with some modification (Jia et al., 2015). Haemocytes were incubated with 20 μL fluorescent yellow-green latex beads (L4530, Sigma) at room temperature for 1 h with rotation. The rate of phagocytosis by haemocytes was evaluated by FACS Arial II flow cytometer (Becton-Dickinson Company).

2.13. Statistical analysis

All the data were expressed as mean $\pm$ standard deviation ($N = 3-5$), and analyzed by Statistical Package for Social Sciences (SPSS) 16.0. Significant differences among groups were evaluated by one-way analysis of variance (ANOVA) and multiple comparisons. Differences were considered significant at $p < 0.05$ and extremely significant at $p < 0.01$.

3. Results

3.1. Sequence features and phylogeny of CgsAC

The coding sequence of CgsAC was identified in the *C. gigas* genome (<http://www.oysterdb.cn/>) with GenBank accession EKC32844.1. There was an open reading frame (ORF) of 3396 bp encoding a protein of 1131 amino acids with a calculated molecular mass of about 126 kD (Fig. 1.). The two catalytic domains in CgsAC, residues 55-212 and residues 215-549, were predicted by SMART (residue marked with thin rectangular in Fig. 1). Blast analysis revealed that the whole sequence of CgsAC shared 28% identity with that of human sAC, while the two catalytic domains in CgsAC (residues 55-549) shared 40% identity with that of human sAC (residues 30-444). The presumptive P-loop regions of human sAC (residues 622-629) were also identified in CgsAC (residues 619-626). Moreover, the whole sequence of CgsAC was 479 residues, which was shorter than that of human sAC.

Phylogenetic relationship of CgsAC with other sACs and tmACs was analyzed by the neighbor joining method (Fig. 2). The first and second catalytic domains of sAC (sAC-C1, sAC-C2), and the catalytic domains of tmAC were separated into three distinct groups. The two catalytic domains of oyster (sAC-C1 and sAC-C2) were clustered into sAC-C1 and sAC-C2, respectively.

3.2. The adenylyl cyclase activity of CgsAC

A recombinant MBP labeled protein (MBP-rCgsAC) and a His-tagged protein (His-rCgsAC) containing two catalytic domains were expressed and purified. The whole cell lysate and the purified protein were analyzed by SDS-PAGE, and two

protein bands were revealed with molecular mass of approximately 100 kD and 60 kD respectively, which were in accordance with predicted molecular weight of MBP-rCgsAC and His-rCgsAC, respectively (Fig. 3A).

The adenylyl cyclase activity was determined by detecting the increase of cAMP product using the cAMP Direct Immunoassay Kit. The activities of both MBP-rCgsAC and gill homogenates could be stimulated by $[\text{HCO}_3^-]$ (Fig. 3B, D). The cAMP production catalyzed by MBP-rCgsAC increased by 2.5 fold with 40-100 mM $[\text{HCO}_3^-]$ (Fig. 3B). The half maximal effective concentration (EC50) was defined as the concentration of $[\text{HCO}_3^-]$ which stimulated the cAMP production halfway between the baseline (1.0) and maximum (2.5) after 30 min reaction. The EC50 of MBP-rCgsAC was about 12.5 mM (Fig. 3B). The purified MBP was used as a negative control and showed no ability to produce cAMP. The ability of MBP-rCgsAC to produce cAMP could be inhibited by the specific inhibitor KH7, and its activity decreased 62.1% after inhibition with 50 μM KH7 (Fig. 3C). Bicarbonate-stimulated cAMP production activity of MBP-rCgsAC decreased by 18.8% after inhibition with 50 μM KH7. The adenylyl cyclase activity of gill extracts was examined to confirm the presence of CgsAC protein. The cAMP production catalyzed by gill extract increased to 1.25-fold maximum with 40-100 mM $[\text{HCO}_3^-]$ (Fig. 3D), and its EC50 was about 5 mM (Fig. 3D). The cAMP-forming activity of gill extract decreased by 48.6% after inhibition with 50 μM KH7 (Fig. 3E). Bicarbonate-stimulated cAMP production activity of gill extracts decreased by 44.3% after inhibition with 50 μM KH7 (Fig. 3E).

3.3. The mRNA expression of CgsAC after CO₂ exposure

The mRNA expression levels of CgsAC in seven tissues of oysters in both control and CO₂ exposure groups were examined using RT-PCR (Fig. 4 A). The CgsAC mRNA transcripts could be detected in all tissues including muscle, gill, hepatopancreas, mantle, female gonad, male gonad and haemocytes. The highest expression level was found in male gonad, which was 96.43-fold of that in female gonad. After CO₂ exposure for 16 days, the mRNA expression levels of CgsAC in muscle, gill, hepatopancreas, mantle, haemocytes and female gonad were all up-regulated, and were 8.23-fold ($p < 0.01$), 5.54-fold ($p < 0.01$), 4.06-fold ($p < 0.01$), 2.14-fold ($p < 0.05$), 3.74-fold ($p < 0.01$) and 1.42-fold higher than those of the control group, respectively. In male gonad, however, the mRNA expression level decreased by 50% ($p < 0.01$).

3.4 The response of CgsAC protein against CO₂ exposure

Polyclonal antibody to CgsAC was prepared by immunizing mice with purified His-rCgsAC and the specificity of the polyclonal antibody was examined by western blotting. Two distinct bands of about 100 kD and 60 kD were revealed (Fig. 3A, lane 7 and lane 8), indicating the high specificity of the antibody. The tissue samples of the control group and CO₂ exposure group were collected for western blotting. For the control group, a 126-kD band was identified in all the seven tissues, while a 45 kD band was only observed in muscle and mantle tissues. After CO₂ exposure, multiple bands were identified in the tissue homogenates (Fig. 4B). The 126 kD band was only identified in muscle, mantle and male gonad, while the 45 kD band was identified in

almost all the tissues in the CO₂ exposure group. Besides, a 20 kD band was identified in all the tissues except the haemocytes in the CO₂ exposure group.

The subcellular distribution of CgsAC in haemocytes of both the control and CO₂ exposure groups was further explored by immunohistochemistry (Fig. 4C). In the control group, positive immunoreactivity of CgsAC (green signal) was observed in both nuclei and cytoplasm. However, for the CO₂ exposure group, although the immunoreactivity of CgsAC could also be found in nuclei and cytoplasm, most of it was localized on the cell membrane and aggregated at some particular part of the cytoplasm, which was different from that of the control group.

3.5. The localization of CgsAC in mantle and gill

The localization of CgsAC in mantle and gill was detected by immunohistochemistry. The positive signal (green) occurred mainly as an aggregate inside the fold structure (Fig. 5 A, B) and in the epithelial areas of the mantle edge (Fig. 5 B, C). In the gill, positive signal was dominantly located in the outer epithelium cell of the gill filament while the signal inside the filament was quite weak (Fig. 5 D, E).

3.6. The changes of cAMP, intracellular pH and ROS levels after CO₂ exposure and CgsAC inhibition

The cAMP levels in gill and mantle of oyster were examined using the cAMP Direct Immunoassay Kit (Fig. 6, A). After CO₂ exposure, the cAMP level increased by 10.7 % in gill (from 9.33 to 10.32 pmol/mg protein) and 6.2 % in mantle (from 10.81 to 11.47 pmol/mg protein). The injection of 5 mM KH7 reduced the cAMP

levels in both gill and mantle tissues significantly ($p < 0.05$). The cAMP levels in mantle and gill tissue tended to be reduced also after treatment with 0.5 mM KH7, but the changes were not significant. Injection of DMSO (as a control) had no effect on the cAMP level relative to that in the CO₂ exposure group.

BCECF AM was used to determine the intracellular pH changes by detecting the relative fluorescence intensity of haemocytes with a flow cytometer. After CO₂ exposure, the fluorescence intensity of oyster haemocytes increased 7.66 fold ($p < 0.05$) relative to the control group (Fig. 6 B), indicating an increase of pHi. In oysters injected with 100 μ L 0.5 mM and 5 mM KH7, the relative fluorescence intensity of haemocytes decreased by 14.3% and 44.4% compared to that of the CO₂ exposure group, respectively. DMSO had no effect on pHi of haemocytes.

The ROS level in haemocytes was determined with flow cytometer using DCFH-DA probe. The relative fluorescence intensity of the haemocytes of the CO₂ exposure group increased by 25% relative to the control group ($p < 0.05$), indicating an increase of intracellular ROS level (Fig. 6 C). Such an ROS increase was inhibited by a treatment with KH7, and the fluorescence intensity decreased by 28.5% ($p < 0.05$) relative to the CO₂ exposure group after the injection of 0.5 mM KH7. Injection of 5 mM KH7 resulted in a further decrease in the level of ROS: the fluorescence intensity was reduced by 38.9% ($p < 0.05$). No significant change of ROS was observed after the treatment with DMSO.

3.7. The rate of phagocytosis in haemocytes after CO₂ exposure and CgsAC inhibition

Fluorescent yellow-green latex beads were used to detect the ability of haemocytes to phagocytose after CO₂ exposure and CgsAC inhibition. The rate of phagocytosis of haemocytes was determined by flow cytometry (%/h, Fig. 7 A, B), and it decreased from 20.2% in the control group to 13.1% in the CO₂ exposure group ($p < 0.05$) (Fig. 7 C). Such a decrease was inhibited by KH7 injection, and the rates of phagocytosis of oysters treated with 0.5 mM KH7 (24.3%) and 5 mM KH7 (26.9%) were both higher than that in the CO₂ exposure group (13.1%). DMSO had no significant effect on the rate of phagocytosis of haemocytes.

4. Discussion

Compared with vertebrates, most invertebrates exhibit lower capacity for acid-base regulation and therefore they could be easily affected by environmental changes (Parker et al., 2013). Facing decreasing ocean pH, increasing [HCO₃⁻] concentration and exacerbated environmental stress, marine invertebrates must have developed strategies to adapt to the global climate changes if they are to survive the changes. sAC is a signal enzyme directly stimulated by [HCO₃⁻], and its product is the ubiquitous second messenger cAMP. Although [HCO₃⁻] accumulation in response to ocean acidification has been typically reported in marine invertebrates such as oyster (Lannig et al., 2010), the knowledge on the physiological roles of sAC in such environmental stress is still limited.

In the present study, a sAC gene was identified and characterized from oyster *C. gigas*. Its ORF was of 3396 bp encoding a polypeptide of 1131 amino acids. The

amino acid sequence of CgsAC shared 40% identity with human sAC. It contained two catalytic domains, a conserved P-loop structure and residues for ATP and bicarbonate binding (Fig. 1), which were confirmed by the recent released high-resolution crystal structure of human sAC (Saalau-Bethell et al., 2014). The residues for ATP binding in human sAC are Asp 47, Ile 48, Ala 97, Asp 99, Ala 100, Arg 176, Phe 296, Phe 336, Phe 338, Leu 345, Val 406, Val 411, Asn 412 and Arg 416, which are the same and in identical positions in CgsAC except for Arg 176 (Fig. 1). For bicarbonate binding sites, Lys 95, Ala 97 and Asp 99 were also conserved in identical positions (Fig. 1). These similarities exemplified the evolutionary conservation of the predicted sAC protein in mollusk. However, three other residues for bicarbonate binding, Met 337, Arg 176 and Val 167, were replaced by Phe, Asn, and Glu, respectively, in CgsAC (Fig. 1). Thr 405, the potential bicarbonate recognition site in human sAC, was replaced by Ser in CgsAC (marked with red ‘▼’ in Fig. 1). This serine was different from the threonine in all the known sACs from other animals, but matched the motifs of $[\text{HCO}_3^-]$ sensing bacterial sAC-like enzymes (Tresguerres et al., 2014). These differences might be the key to explain the variances in activity and function between CgsAC and human sAC. Moreover, phylogenetic analysis of sAC and tmAC demonstrated that the two catalytic domains of oyster CgsAC were more closely matched to sACs in other species rather than to tmACs. According to the results of a previous report, the two catalytic domains of human sAC were clustered with cyanobacterial ACs rather than with mammalian tmACs (Chen et al., 2000). Collectively, these results confirmed that CgsAC is a sAC ortholog.

The two catalytic domains of sAC are essential for the conversion of ATP into cAMP (Chen et al., 2000; Tresguerres et al., 2010). In the present study, the activities of two catalytic domains in CgsAC (residues 1-549) were examined after treatment with $[\text{HCO}_3^-]$ and KH7. The MBP-rCgsAC could be activated by $[\text{HCO}_3^-]$ with EC50 of 12.5 mM (Fig. 3B, D). The value was similar with sACs from mammals (10-25 mM) but higher than that of dogfish sAC (5 mM) (Tresguerres et al., 2010). The oyster gill homogenates could be stimulated by $[\text{HCO}_3^-]$ with EC50 of 5 mM, which was quite consistent with the physicochemical $[\text{HCO}_3^-]$ concentration in oyster serum ($2.09 \pm 0.09 \text{ mmol kg}^{-1}$) (Lannig et al., 2010). This value was also similar to the EC50 (about 3 mM) in dogfish (Tresguerres et al., 2010). However, the EC50s of the gill homogenates and MBP-rCgsAC were different from each other. The MBP label and the lack of C-terminus region in the recombinant protein were suspected to affect the affinity of MBP-rCgsAC to $[\text{HCO}_3^-]$. On the other hand, the full-length and the truncated protein might have different regulatory properties, which could also influence their affinity to $[\text{HCO}_3^-]$ and sensitivity to KH7. These results illustrate the evolutionary conservation of CgsAC, and the sAC activity in gill tissue of oyster suggests that CgsAC might be involved in regulating multiple physiological activities.

The changes of CgsAC at both mRNA and protein level were determined in different tissues of oysters after CO_2 exposure (Fig. 4A). The up-regulation of CgsAC in muscle, mantle, hepatopancreas, gill, female gonad, haemocytes suggest that it might participate in acid-base regulation. One interesting phenomenon was that the mRNA level of CgsAC in the male gonad decreased significantly ($p < 0.01$) but

increased in female gonad after CO₂ exposure. Such changes of CgsAC might greatly affect the fertilization of oyster, because sAC was usually highly expressed in male gonad and the sAC in the sperm was closely related to the maturation, motility regulation and the acrosome reaction (Beltran et al., 2007; Schmid et al., 2014; Vacquier et al., 2014). In the control group, a 126 kD band protein was identified in all the seven tissues, while a 45 kD band was observed only in muscle and mantle tissues (Fig. 5). Multiple bands were identified in the tissue homogenates of the CO₂ exposure group including a 126 kD, a 45 kD, and a 20 kD band (Fig. 4B), and the 45 kD and 20 kD bands were identified in almost all the tissues (Fig. 4B). Previously, a 187 kD and a 48 kD band were observed in rat testis by western blotting (Chen et al., 2000), while three bands of 180 kD, 48 kD and 35 kD were detected in the liver homogenate (Acin-Perez et al., 2009). Our present results also indicated that CgsAC could be proteolytically processed into different forms. In mammalian sperm, the truncated form of 48 kD sAC exhibited 10-20 times higher activity than that of the full-length sAC (Chen et al., 2000). The truncated 48 kD sAC was reported to prevail in mitochondria, and the smaller one (35 kD) to exist mainly in ER-rich fractions of rat liver (Acin-Perez et al., 2009). It was suggested that the elevated CO₂ altered the internal concentration of [HCO₃⁻] which might trigger the activation and truncation of CgsAC, as the 45 kD and 20 kD bands were strongly expressed in all the tissues after CO₂ exposure. The immunohistochemical assay revealed that the immunoreactivity of CgsAC was located along the cell membrane of the haemocytes after CO₂ exposure (Fig. 4C), which demonstrated that CgsAC might interact directly with some

membrane proteins like vacuolar proton pump (VHA) or Na^+/H^+ exchanger (NHE) and participate in acid-base regulation under CO_2 exposure (Espejo et al., 2015; Pastor-Soler et al., 2003; Wang et al., 2007). The distribution of CgsAC in the haemocytes also indicated its translocation inside the cell after CO_2 exposure. The truncated CgsAC (45 kD) might enter the mitochondria and trigger the cAMP pathway which subsequently regulated the downstream pathways such as mitochondrial apoptotic pathway and the oxidative phosphorylation pathway (Acin-Perez et al., 2009; Appukuttan et al., 2012; Kumar et al., 2009). The still smaller fragments (20 kD) might enter into endoplasmic reticulum (ER) fraction (Acin-Perez et al., 2009). These results demonstrate that the mRNA expression of CgsAC could be induced by elevated CO_2 and CgsAC protein could be truncated and translocated in response to CO_2 exposure, but the mechanism related to the truncation to 45 kD and 20 kD CgsACs should be further investigated.

Mantle and gill are the main tissues of oyster for substance exchange with the environment. In the mantle, CgsAC was mainly observed in the outer epithelium cells and inside the fold structure (Fig. 4 A, B, C. Red arrow). The mantle and the outer epithelium cells were reported to be related with calcification (Marie et al., 2012). The present results suggest a potential role for CgsAC in the regulation of acid-base status, as the calcification process is always associated with changes of pH and $[\text{HCO}_3^-]$ (Parker et al., 2013). Because oyster lives in intertidal zones, it must cope with many environmental fluctuations, such as temperature, salinity and dry exposure which might bring about carbonic chemistry disturbance (Lannig et al., 2010; Zhang et al.,

2012). The distribution of CgsAC in such structure might be related to mantle movement in response to environmental stresses (Fabbri and Capuzzo, 2010). It has been reported that cAMP production enzyme from the mussel mantle is insensitive to forskolin (specific activator of tmAC) (Fabbri and Capuzzo, 2010). Thus, the main cAMP production enzyme is likely to be sAC, because tmACs are sensitive to forskolin (Tresguerres et al., 2014). In the present study, CgsAC was also found to be located in the gill epithelium, which agreed with the previous report about dogfish sAC (Tresguerres et al., 2010). In dogfish, dfsAC could promote the insertion of vacuolar proton pumps into the basolateral membrane to absorb $[H^+]$ into the blood and thereby function as an acid-base sensor (Tresguerres et al., 2010). Thus, the distribution of CgsAC in mantle and gill epithelium implies its vital role in acid-base regulation and stress response.

sAC enzyme can catalyze the conversion of ATP to cAMP. In the present study, cAMP content in gill and mantle was determined to understand the downstream signaling pathway of CgsAC in response to CO₂ exposure. The cAMP level increased by 10.7 % in gill and 6.2 % in mantle after CO₂ exposure (Fig. 6), and the basal endogenous cAMP production was significantly reduced ($p < 0.05$) after a 14-day treatment with 5 mM KH7. Although the changes of cAMP seemed to be minor after CO₂ exposure, a previous study demonstrated that minor increases of cAMP concentration were sufficient to cause maximal phosphorylation due to the high efficiency of downstream enzyme PKA and Epac (exchange factor directly activated by cAMP) (Dao et al., 2006; Wojcikiewicz and Luo, 1998). The AC/cAMP system

was also reported to respond to environmental stress factors, such as heavy metals or polycyclic aromatic hydrocarbons (Fabbri and Capuzzo, 2010). For mussel, the cAMP content in digestive gland cells increased by 158.5% after cadmium (50 μ M) exposure for 60 min (Dailianis et al., 2005), increased by ~20% in mantle and gland tissue complex after cadmium (10 μ g/L) treatment for 9 days and increased by ~80% after cadmium (100 μ g/L) treatment for 9 days (Dailianis and Kaloyianni, 2007). A Cr^{6+} exposure for 7 days also increased cAMP content by 28% in the gills of mussels (Fabbri and Capuzzo, 2006). After incubation with polycyclic aromatic hydrocarbons for 18 h in vitro, the cAMP level in goldfish testis pieces increased by about 11% ($p < 0.05$) (Evanson and Van Der Kraak, 2001). The difference in effects of heavy metals, organic pollutants and CO_2 exposure on cAMP level could be related to differences in the physicochemical properties of the contaminant, treatment time, concentration and signal transduction pathway. Cadmium could interact with β -adrenoreceptor, increase the activity of tmAC to induce a significant and instant increase of cAMP (Dailianis and Kaloyianni, 2004, 2007; Koutsogiannaki et al., 2014). Some mollusks such as oysters and mussels could accumulate heavy metals in their tissues, and the higher concentration of heavy metals with longer residence time in vivo might cause higher cAMP level in the tissues (Dailianis and Kaloyianni, 2007; Fabbri and Capuzzo, 2006; Gret et al., 2002). In the present study, the increase of cAMP in gill and mantle of oyster after CO_2 exposure of 16 days was mainly induced by CgsAC and not by tmAC. Although the increase of cAMP induced by some environmental factors was not as significant as that induced by a neurotransmitter, such an increase could

stimulate downstream pathway and then facilitate the adaptation of the organism to the changing environment. The present results indicate that the elevations of intracellular $[\text{HCO}_3^-]$ could activate CgsAC to generate more cAMP which would further trigger the downstream pathway, and a $[\text{HCO}_3^-]$ /CgsAC/cAMP regulation model could be proposed in oyster.

The intracellular pH (pH_i) was further determined to understand the regulation of physiological activities mediated by CgsAC in oyster. After CO_2 exposure, the pH_i value increased significantly, and the increase could be inhibited by KH7 (Fig. 7 A), indicating that the increase of pH_i was facilitated by CgsAC. In a previous report, the absorption of $[\text{H}^+]$ into the blood by vacuolar proton pump was promoted by dfsAC in dogfish (Tresguerres et al., 2010). Some environmental factors such as cadmium could cause the elevation of pH_i and rise of Na^+ influx by the activation of NHE, which would then regulate intracellular signal transduction (Dailianis and Kaloyianni, 2004). In oyster, elevated CO_2 in the environment could diffuse across the epithelial surfaces to be hydrolyzed into $[\text{H}^+]$ and $[\text{HCO}_3^-]$ (Lannig et al., 2010), and such increases of $[\text{H}^+]$ and $[\text{HCO}_3^-]$ would cause the activation of CgsAC or NHE. The proton secretion facilitated by CgsAC or NHE would be accelerated and more $[\text{HCO}_3^-]$ be left inside the haemocytes, leading to the increase of pH_i . Thus, CgsAC was supposed to participate in intracellular pH regulation by interaction with membrane proteins such as NHE or proton pumps a suggestion consistent with our immunohistochemistry results.

The increase of pH_i and $[\text{HCO}_3^-]$ caused intracellular alkalization. The acid-base

status of marine organisms is often related to energy metabolism, because ion transport is energy-consuming. In many bivalves, elevated CO₂ increases standard metabolic rate, a change associated with an increased ROS level (Parker et al., 2013). In the present study, the ROS level in haemocytes increased significantly after CO₂ exposure compared to that in the control group ($p < 0.05$). However, it decreased after treatment with KH7, a change corresponding to the change of intracellular pH value. sAC has been reported to be a metabolic sensor in both cytoplasm and mitochondria monitoring glycolysis, Krebs cycle and oxidative phosphorylation (Chang and Oude-Elferink, 2014). A mitochondrial sAC pathway, sAC-cAMP-PKA signaling cascade, was reported to serve as a metabolic sensor to modulate ATP generation and ROS production in mouse liver (Acin-Perez et al., 2009). Previously in mussel, elevated ROS production after cadmium and zinc exposure was shown to be regulated by NHE; an inhibition of NHE could prevent an increase of ROS level (Dailianis et al., 2005; Koutsogiannaki et al., 2006). The key role of NHE in the signal transduction was probably via NO synthesis and PKC (protein kinase C)-mediated NADPH oxidase activation (Dailianis, 2009; Dailianis et al., 2009). Thus, the increase of ROS caused by CO₂ exposure might be mediated by the sAC-cAMP-PKA signaling cascade or other signal pathways involving NHE. The exact mechanism needs to be further explored and the present results could still indicate that the excessive ROS production was induced by CgsAC-dependent pathway, where cAMP could be involved.

In addition to energy metabolism, the cAMP-dependent signaling also accounted

for the control of cellular cascades involved in immune responses. After CO₂ exposure, the rate of phagocytosis by oyster haemocytes decreased significantly compared to that in the control group ($p < 0.05$). This result agreed with previous reports about acid-base disturbances caused by CO₂ exposure reducing immune responses (Dupont and Thorndyke, 2012; Hernroth et al., 2012). Inhibition of phagocytic rate by KH7 demonstrated that CgsAC could regulate that immune response probably by its product cAMP. In mollusks, the modulation of the haemocyte phagocytosis and antibacterial function by the second messenger cAMP has been reported for neuroendocrine and immune system. For example, noradrenaline (NA) could inhibit haemocyte phagocytosis by a β -adrenoceptor/cAMP/PKA signaling pathway in oyster (Serezani et al., 2008), and an α -adrenergic receptor in scallop *Chlamys farreri* could modulate phagocytic and antibacterial activity of haemocytes through cAMP (Zhou et al., 2013). It can thus be suspected that the cAMP/PKA signaling pathway may be the underlying mechanism behind the reduced rate of phagocytosis induced by CO₂ exposure.

In summary, a bicarbonate-stimulated enzyme CgsAC was characterized in oyster. It appears to be an important acid-base sensor in regulation of pHi value, and the [HCO₃⁻]/CgsAC/cAMP signaling pathway could be stimulated by CO₂ exposure. This signal pathway might be responsible for effects of intracellular alkalization on oxidative phosphorylation and innate immune functions in response to CO₂ exposure. CgsAC-mediated changes of physiological status such as intracellular pH, ROS level and changes in phagocytosis might help us to further understand the effects of ocean

acidification on marine calcifiers.

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Author contributions: Xiudan Wang designed the study, conducted most of the experiments, analyzed the results, and wrote the manuscript. Mengqiang Wang and Lingling Wang participated in the design of the study and revised the manuscript. Zhihao Jia participated in the design of the study and preparation of the antibody. Hao Wang and Shuai Jiang participated in the design of the study. Chen Hao participated in the sample preparation and RNA extraction. Linsheng Song was responsible for overall design of the study and revised the manuscript. All authors reviewed the results and approved the final version of the manuscript.

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Figure captions

Fig. 1. The amino acid sequence of CgsAC aligned with human sAC. Identical amino acid residues are shaded in red, and similar amino acids are labeled with red characters. The two catalytic domains are marked with thin rectangle. The 8 residues of P-loop structure are emphasized with a black bracket. Functional residues related to ATP binding are marked with “*” (Residues that are the same in human and oyster are

marked with black ‘*’, others with red ‘*’). Residues for bicarbonate activation were marked with “▼” (Residues that are the same in human and oyster are marked with black ‘▼’, others with red ‘▼’).

Fig. 2. Phylogenetic tree comparing the protein sequence of catalytic domains (C1 and C2) in CgsAC and other adenylyl cyclases. The abbreviations: AC1-C1: the first catalytic domain of adenylyl cyclase type 1; sAC-C1: the first catalytic domain of soluble adenylyl cyclase. The gene bank accession numbers were as follows: human AC1 (NP_066939.1); human AC2 (NP_065433.2); human AC5 (NP_899200.1); cattle AC2 (XP_587884.4); zebrafish AC5 (NP_001165056.1); zebrafish AC9 (XP_003201145.2); oyster AC1 (EKC21679.1); oyster AC2 (EKC42885.1); human sAC (NP_060887.2); mouse sAC (NP_766617.2); dogfish sAC (ACA52542.1); sea urchin sAC (NP_001020380.1); oyster sAC (EKC32844.1); fowl sAC (XP_004949700.1); lancelet sAC (XP_002214797.1); trichoplax sAC (XP_002117857.1); mosquito sAC (EFN85820.1).

Fig. 3. Recombinant expression and adenylyl cyclase activity of CgsAC. A: SDS-PAGE analysis of MBP-rCgsAC and His-rCgsAC. B: cAMP production by MBP-rCgsAC assayed in the presence of indicated concentrations of NaHCO₃. Data are expressed as relative stimulation compared to that without NaHCO₃. C: cAMP production of MBP-rCgsAC in the presence or absence of 50 μM KH7. D: cAMP production of gill homogenates assayed in the presence of indicated concentrations of

NaHCO₃. E: cAMP production of gill homogenates in the presence or absence of 50 μ M KH7. The results are shown as mean $\pm$ SD (n=3). Statistically significant differences are indicated by asterisks (**: $p < 0.01$; *: $p < 0.05$).

Fig. 4. The responses of CgsAC to CO₂ exposure. A: CgsAC mRNA expression level in different tissues of oyster *C. gigas* in the control group and CO₂ exposure group (N=3). B: Western blotting of CgsAC and β -tubulin in oyster tissue homogenate of the control group and CO₂ exposure group. C. Immunohistochemistry of CgsAC (green) in oyster haemocytes of the control group and CO₂ exposure group. Statistically significant differences are indicated by asterisks (**: $p < 0.01$; *: $p < 0.05$).

Fig. 5. Immunohistochemistry of CgsAC in oyster mantle and gill of control group. A: Positive signal (green) in fold structure of mantle; B: Magnification of the red square in A; C: Positive signal (green) in epithelial areas of the mantle edge; D: Positive signal (green) in the gill filament; E: Magnification of the red square in D.

Fig. 6. The cAMP level, intracellular pH and ROS level changes in the control group, CO₂ exposure group, DMSO injection group, 0.5 mM KH7 injection group and 5 mM KH7 injection group. A: Endogenous cAMP levels in the mantle and gill; B: Intracellular pH of haemocytes; C: ROS levels of haemocytes. The cAMP levels were normalized to the protein concentration. The intracellular pH and ROS level were

recorded as relative fluorescence intensity with that of the control group as the reference value. Each value is given as mean $\pm$ SD (N=3). Statistically significant differences are indicated by asterisks and letters (a-d) ($p < 0.05$); the bars not sharing the same letter differ significantly from each other.

Fig. 7. Rate of phagocytosis of haemocytes of oysters in the control group, CO₂ exposure group, DMSO injection group, 0.5 mM KH7 injection group and 5 mM KH7 injection group. A: Flow cytometry result of phagocytotic ability of haemocytes towards latex beads in the control group; B: Flow cytometry result of phagocytotic ability of haemocytes towards latex beads in the CO₂ exposure group; C: Rates of phagocytosis (%/h) of different groups (axis label: phagocytic rate of haemocytes (%/h)). The values are shown as mean $\pm$ SD (N=3), and statistical significances ($p < 0.05$) are indicated by different letters; the bars not sharing the same letter differ significantly from each other.

[illegible]

A **M:** Protein molecular standard**1:** Negative control for MBP-rCgsAC**2:** Induced MBP-rCgsAC**3:** Purified MBP-rCgsAC**4:** Negative control for rCgsAC**5:** Induced rCgsAC**6:** Purified rCgsAC**7:** Western blot of lane 3**8:** Western blot of lane 6**B** **C** **D** **E**

DAPI

CgsAC

Merge

A

B

C

DAPI

CgsAC

Merge

D

E

A **B** **C**

Seawater
acidification

extracellular

Intracellular