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RESEARCH ARTICLE

Evidence that dopamine is involved in neuroendocrine regulation, gill intracellular signaling pathways and ion regulation in *Litopenaeus vannamei*

Lingjun Si, Luqing Pan*, Xin Zhang, Hongdan Wang and Cun Wei

ABSTRACT

The transport of ions and ammonia in gills may be regulated by neuroendocrine factors. In order to explore the mechanism of dopamine (DA) regulation, we investigated hemolymph neuroendocrine hormones, gill intracellular signaling pathways, ion and ammonia transporters, hemolymph osmolality and ammonia concentration in *Litopenaeus vannamei* after injection of 10^{-7} and 10^{-6} mol DA per shrimp. The data showed a significant increase in crustacean hyperglycemic hormone (CHH) concentration at 1–12 h and a transient significant decrease in corticotrophin-releasing hormone (CRH), adrenocorticotropic hormone (ACTH) and cortisol concentration under DA stimulation. The up-regulation of guanylyl cyclase (GC) mRNA, cyclic guanosine monophosphate (cGMP) and protein kinase G (PKG) concentration, together with the down-regulation of DA receptor D_4 mRNA and up-regulation of cyclic adenosine monophosphate (cAMP), protein kinase A (PKA), diacylglycerol (DAG) and protein kinase C (PKC) concentration suggested the activation of complicated intracellular signaling pathways. The expression of cAMP response element-binding protein (CREB), FXYD2 and 14-3-3 protein mRNA was significantly increased by PKA regulation. The increase in Na^+/K^+-ATPase (NKA) activity and the stabilization of V-type H^+-ATPase (V-rATPase) activity were accompanied by an up-regulation of K^+ channel, $Na^+/K^+/2Cl^-$ cotransporter (NKCC), Rh protein and vesicle associated membrane protein (VAMP) mRNA, resulting in an increase in hemolymph osmolality and a decrease in hemolymph ammonia concentration. These results suggest that DA stimulates the secretion of CHH and inhibits the release of cortisol, which activates intracellular signaling factors to facilitate ion and ammonia transport across the gills, and may not affect intracellular acidification.

KEY WORDS: Biogenic amine, Hormone, Second messenger, Protein kinase, Ammonia transport

INTRODUCTION

Crustacean gills primarily have two functional types of epithelia: a respiratory epithelium that is characterized by thin cells (1–2 µm thick) and an ion-transporting epithelium characterized by thick cells (10–20 µm thick) (Freire et al., 2008). The gill is a multi-functional organ, which possesses many physiological processes, such as ion transport, which is the basis for hemolymph osmoregulation and ammonia excretion (Faleiros et al., 2018; Martin et al., 2011). The primary osmolytes in the hemolymph of marine crustaceans are Na^+ and Cl^-. Euryhaline marine species are capable of inhabiting estuaries and brackish water and employ branchial pumping mechanisms to maintain ion balance (Liu et al., 2008). Active transbranchial Na^+ and Cl^- transport mainly depends on the basolateral Na^+/K^+-ATPase (NKA) and K^+ channels, as well as the apical $Na^+/K^+/2Cl^-$ cotransporter (NKCC), which play important roles in crustacean osmoregulation (Henry et al., 2012). In *Palaemonetes varians* and *Crangon crangon*, which are major ammonotelic animals, ammonia accounts for 95% of the total nitrogen, and is mainly excreted through the gills (Snow and Williams, 1971; Regnault, 1983). Because it has a similar hydrated radius, NH_4^+ can compete with K^+ for K^+-transporting proteins such as K^+ channels, NKCC and NKA (Weihrauch et al., 1998; Weihrauch and Donnell, 2015). NH_3 can potentially diffuse across any membrane down its partial pressure gradient, and also via Rh proteins that were identified as NH_3 channels in *Portunus trituberculatus* (Si et al., 2018). Furthermore, a vesicular ammonia-trapping mechanism of excretion with V-type H^+-ATPase (V-ATPase) and vesicle associated membrane protein (VAMP) were proposed in *Carcinus maenas* (Weihrauch et al., 2002) and *P. trituberculatus* (Ren et al., 2015). Importantly, biogenic amines were shown to stimulate NKA activity and NaCl uptake in *C. maenas*, *Eriochier sinesis* and *Gecarcoidea natalis* (Morris, 2001). After dopamine (DA) injection, the ion transporter activity and expression, ion concentration and osmoregulatory capacity were temporarily changed in *Litopenaeus vannamei* (Liu et al., 2008, 2009; Camacho-Jiménez et al., 2017). As outlined above, there are ion channels and specific ammonia excretion transporters in crustacean gills, but their regulation by biogenic amines is unclear.

Biogenic amines, including DA, 5-hydroxytryptamine, octopamine and noradrenaline (norepinephrine), function as neurotransmitters and neurohormones and have been identified and quantitatively measured in decapod crustaceans (Eloffson et al., 1982; Fingerman and Nagabhushnam, 1992; Kuo et al., 1995). Among the biogenic amines, DA associated with pericardial organs is widely distributed in the crustacean nervous system and has a diverse array of physiological effects, as reviewed by Tierney et al. (2003). Vertebrate corticotrophin-releasing hormone (CRH) and adrenocorticotropic hormone (ACTH) were shown to be present in *L. vannamei*, and may regulate the release of DA (Ottaviani and Franceschi, 1996; Zhao et al., 2016). Conversely, the release of ACTH is inhibited by DA in *Cyprinus carpio* (Huising et al., 2004). In addition, DA acts as a neurotransmitter, stimulating crustacean hyperglycemic hormone (CHH) secretion from the X-organ/sinus gland (XO/SG) complex in the eyestalk of *L. vannamei* (Camacho-Jiménez et al., 2017). After reaching the cells, CHH activates the

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List of abbreviations

AC	adenylyl cyclase
ACTH	adrenocorticotropic hormone
BSA	bovine serum albumin
cAMP	cyclic adenosine monophosphate
cGMP	cyclic guanosine monophosphate
CHH	crustacean hyperglycemic hormone
CREB	cAMP response element-binding protein
CRH	corticotrophin-releasing hormone
D_4	DA receptor
DA	dopamine
DAG	diacylglycerol
HPI	hypothalamic-pituitary–interrenal
GC	guanylyl cyclase
GPCRs	G protein-coupled receptors
NKA	Na^+/K^+-ATPase
NKCC	$Na^+/K^+/2Cl^-$ cotransporter
PBS	phosphate-buffered saline
PBST	phosphate-buffered saline–Tween 20
Pi	inorganic phosphate
PKA	protein kinase A
PKC	protein kinase C
PKG	protein kinase G
V-ATPase	V-type H^+-ATPase
VAMP	vesicle associated membrane protein
XO/SG	X-organ/sinus gland

cyclic guanosine monophosphate (cGMP)–protein kinase G (PKG) pathway through binding to membrane-bound guanylyl cyclase (GC). DA can itself bind to G protein-coupled receptors (GPCRs) on the membrane, and once the GPCR is activated, specific target proteins can be stimulated or inhibited, resulting in a change in the concentration of intracellular second messengers, such as cyclic adenosine monophosphate (cAMP), cGMP and diacylglycerol (DAG) (Blenau and Baumann, 2001). Finally, second messenger-dependent protein kinases are activated to regulate physiological responses (Buckley et al., 2016; Zhao et al., 2016). In particular, FXYD2 (the gamma subunit of NKA) and 14-3-3 protein phosphorylated by protein kinase A (PKA) can regulate ion transport enzyme activity in crabs (Silva et al., 2012; Jayasundara et al., 2007). As a key transcription factor, cAMP response element-binding protein (CREB) in vertebrates is mainly phosphorylated by PKA, thereby facilitating its binding to various promoters of target genes (Zhen et al., 2016). However, in crustaceans, the effects of DA on neuroendocrine hormones and the pathways of intracellular signal transduction remain elusive.

In recent decades, biogenic amines and neuroendocrine hormones have been shown to be involved in the physiological regulation of responses to environmental stressors. It has been reported that the concentration of biogenic amines in *L. vannamei*, especially DA, is significantly increased under salinity and ammonia–N stress (Zhao et al., 2016; Zhang et al., 2018). In addition, there have been many studies on the hypothalamic-pituitary–interrenal (HPI) axis of fish. Exposure to ammonia–N enhanced the levels of CRH, ACTH and cortisol in *Scophthalmus maximus* and *L. vannamei* (Jia et al., 2017; Si et al., 2019). Moreover, it has been reported that cortisol plays a major role in acclimation to hypersalinity in teleost fish (Gonzalez, 2012). Kiilerich et al. (2007) also demonstrated that cortisol was highly correlated with salinity and regulated the transcript level of ion transporters in Atlantic salmon gill. Furthermore, a notable increase of CRH and ACTH concentration was observed in *L. vannamei* under salinity stress (Zhao et al., 2016). In crustaceans, CHH acts as a stress hormone in response to changes of their environment. Chang (2005) recorded an increase in hemolymph CHH of *Homarus americanus* under conditions of acute hypoxia, elevated temperature and altered salinity. Our group also detected a significant increase in CHH concentration of hemolymph in *L. vannamei* under ammonia–N stress (Si et al., 2019). Therefore, it can be speculated that under salinity and ammonia–N stress, some hormones may participate in ion regulation together with biogenic amines and are associated with ammonia acclimation.

The white shrimp *L. vannamei* is one of the most productive aquaculture species in China, with 108,0791 tons of aquaculture production, accounting for 66.26% of marine crustaceans in 2017 (Ministry of Agriculture Fisheries and Fisheries Administration, 2018). To further understand the role of DA in the neuroendocrine regulation of ion and ammonia transport in *L. vannamei*, we determined the changes in hemolymph hormones, gill intracellular signaling pathway factors, ion and ammonia transporters, hemolymph osmolality and ammonia concentration post-DA injection. The aim of this study was to not only explore the role of DA in ion regulation but also provide a theoretical basis for understanding the neuroendocrine regulation mechanism of ion and ammonia transport in crustaceans.

MATERIALS AND METHODS

Animals

Adult shrimps of *L. vannamei* (Boone 1931) (both females and males; mean±s.e.m. length 8.5±0.5 cm, mass 10.5±1.2 g) were obtained from Shazikou farm (Qingdao, China). The shrimps were acclimated in tanks (40 cm×50 cm×60 cm) containing aerated water (salinity 31‰, pH 8.1) with an air-lift at 24±0.5°C for 7 days prior to the experiment. Only apparently healthy animals at the inter-molt stage were used for the study. The molt stage was discerned by observing partial retraction of the uropod epidermis. During the acclimation period, half of the tank water was renewed twice daily and the shrimps were fed with a commercial diet daily (Haiyue Company, Qingdao, China). The experimental protocol was approved by the Animal Research and Ethics Committees of Ocean University of China and did not involve endangered or protected species.

Experimental design

The seawater used in the experiment was consistent with that used during the acclimation period. Dopamine hydrochloride (Sigma-Aldrich) was dissolved in sterile saline (400 mmol l^{-1} NaCl, 10 mmol l^{-1} $CaCl_2{\cdot}2H_2O$, 0.4 mmol l^{-1} $Na_2HPO_4{\cdot}12H_2O$, 9 mmol l^{-1} KCl, 20 mmol l^{-1} $MgCl_2{\cdot}6H_2O$, 35 mmol l^{-1} Tris, pH 7.4) to concentrations of 2×10^{-2} and 2×10^{-3} mol l^{-1} before injection (50 µl) into the ventral sinus of the cephalothorax of individual shrimp to achieve doses of 10^{-6} and 10^{-7} mol per shrimp, respectively (Pan et al., 2008; Liu et al., 2008, 2009). Control group shrimp received a saline (50 µl) injection. There were three experimental groups (saline, 10^{-7} mol DA and 10^{-6} mol DA) and each group contained three replicates. Six shrimps were sampled randomly from each replicate group at 1, 3, 6, 12, 24 and 48 h post-injection. In addition, six shrimps that underwent no treatment were used as the initial group (0 h).

Tissue preparation

Gills were collected using RNase-treated scissors and forceps and then frozen in liquid nitrogen. Samples used for RNA extraction were lysed with 1 ml RNAiso Plus reagent (TaKaRa, Dalian, China) in 1.5 ml RNase-free tubes; after full mixing, the lysed samples were

centrifuged at 12,000 ***g*** for 15 min at 4°C and the supernatant was stored at −80°C. Samples used for enzyme activity and intracellular signal transduction factor assays were milled in liquid nitrogen, and 80–100 mg tissue powder was quickly placed into 2 ml centrifuge tubes and immediately stored at −80°C. Hemolymph samples were obtained from the first abdominal segment of each shrimp using a sterilized syringe with an equal volume of anti-coagulant (450 mmol l^{-1} NaCl, 10 mmol l^{-1} KCl, 10 mmol l^{-1} EDTA-Na_2 and 10 mmol l^{-1} Hepes, pH 7.45, osmolarity 780 mosmol kg^{-1}; modified from Söderhäll and Smith, 1983). After collection, the sample was immediately centrifuged at 700 ***g*** (4°C) for 10 min and the plasma supernatant was collected and frozen at −80°C until analysis.

Hormone and intracellular signal transduction factor assay

CHH concentration in plasma was determined by an enzyme-linked immunosorbent assay (ELISA) method. Polyclonal antibodies to purified recombinant CHH protein of *L. vannamei* were developed in two male New Zealand white rabbits (6 months old, 2–3 kg mass). The experimental protocol was approved by the respective Animal Research and Ethics Committees of Ocean University of China. Briefly, during the first week, the rabbits were injected subcutaneously at six locations with 0.5 ml of disrupted antigen (protein concentration of the antigen, 100 µg ml^{-1}) intensively mixed with complete Freund's adjuvant in a 1:1 ratio. In the third week after the initial injection, the same immunization was carried out but with incomplete Freund's adjuvant. This procedure was repeated at 2 weekly intervals. To determine anti-r*Lv*CHH antibody titer, approximately 0.1–0.5 ml serum was collected from the auricular vein of the rabbits until 1 week after the fourth injection, and assayed by ELISA. Antibody dilutions were used instead of rabbit sera as a blank control. Serum samples collected from a non-immunized rabbit were used as negative controls. The negative control revealed the non-specific, background noise of the system, which was subtracted from all values of the plate. One of the two rabbits with higher antibody titer and sensitivity was killed (anesthetized and terminally bled) and antiserum was harvested from the auricular artery with medical hemostix and stored at −20°C until use.

The ELISA was used to plot the r*Lv*CHH standard curve, and was carried out as previously described by Zou et al. (2003). The purified r*Lv*CHH suspension was serially diluted at 10^{-4}, 5×10^{-4}, 10^{-3}, 5×10^{-3}, 10^{-2}, 5×10^{-2} and 10^{-1} µg ml^{-1} in phosphate-buffered saline (PBS), and 100 µl of the solution was pipetted into the wells of an ELISA plate and incubated at 4°C overnight, then washed three times with 300 µl of PBS containing 0.05% (v/v) Tween 20 (PBST). Subsequently, the ELISA plate wells were blocked with 200 µl of PBS containing 3% bovine serum albumin (BSA, Solarbio), incubated at 37°C for 1 h, then washed with PBST as described above. The wells were then incubated at 37°C for 1 h with rabbit sera diluted 1:400 in PBST containing 0.5% (w/v) Bio-Rad Blocker (PBST-B), washed in PBST and then incubated at 37°C for 1 h with secondary antibody (HRP-labeled goat polyclonal anti-rabbit IgG, Beijing Haplen and Protein Biomedical Institute, China), diluted 1:1000 in PBST. After washing, color development was achieved by incubation with tetramethylbenzidine in the dark at 37°C for 20 min; color development was stopped by adding 50 µl of 2 mol l^{-1} H_2SO_4, and the absorbance was measured at 450 nm in a microplate reader (SpectraMax 190, Molecular Devices). The results were evaluated as an index value calculated as P/N, where $P/N=(A_{450,sample}-A_{450,blank\ control})/(A_{450,negative\ control}-A_{450,blank\ control})$. As P/N≥2.1, the sample was positive for antigen.

CRH, ACTH and cortisol concentrations in plasma were measured using a shrimp CRH ELISA kit (BPE94005), a shrimp ACTH ELISA kit (BPE94055) and a shrimp cortisol ELISA kit (BPE94332), respectively. The concentration of cAMP, DAG, cGMP, PKA, protein kinase C (PKC) and PKG in the gills was measured using a shrimp cAMP ELISA kit (BPE94058), a shrimp DAG ELISA kit (BPE94095), a shrimp cGMP ELISA kit (BPE94048), a shrimp PKA ELISA kit (BPE94011), a shrimp PKC ELISA kit (BPE94012) and a shrimp PKG ELISA kit (BPE94112), respectively. All kits were obtained from Shanghai Lengton Bioscience Co., Ltd, China and steps were carried out according to the manufacturer's instructions.

Measurement of hemolymph osmolality and ammonia concentration

The hemolymph osmolality of each experimental group was measured in a Fiske Micro-Osmometer (model 210). Ammonia concentration in the hemolymph was determined according to the instructions of the hemolymph ammonia assay kit (no. A086, Nanjing Jiancheng Bioengineering Institute, Nanjing Shi, China). Hemocyanin was precipitated and the enzyme activity was disrupted with a protein precipitant to prevent the production of free ammonia, while most of the color-interfering substances were removed. Ammonia was prepared in protein-free filtrate by the Berthelot reaction, and the hemolymph ammonia concentration was measured by comparison with the standard solution.

Enzyme assay

The activities of NKA α-subunit and V-ATPase subunit B were evaluated according to the methodology of Ren et al. (2015). Gill powder samples (100 mg) were homogenized with 4 ml buffer [250 mmol l^{-1} sorbitol (Solarbio), 6 mmol l^{-1} EDTA, 25 mmol l^{-1} Tris-acetate buffer, 0.1 mmol l^{-1} DTT, 0.2 mmol l^{-1} PMSF and 100 U ml^{-1} aprotinin (Sigma), pH 7.4] on ice. The homogenate was centrifuged at 10,000 ***g*** for 30 min at 4°C. The difference in measured activity between tubes following the addition of solution A (6 mmol l^{-1} $MgCl_2$, 100 mmol l^{-1} NaCl, 10 mmol l^{-1} KCl and 25 mmol l^{-1} Tris-acetate, pH 7.4) and solution B [solution A with 5 mmol l^{-1} ouabain (Sigma) added to inhibit NKA] was taken to represent the activity of NKA. To measure the activity of V-ATPase, after adding assay buffer (34.8 mmol l^{-1} Hepes, 173.9 mmol l^{-1} KCl, 6.95 mmol l^{-1} $MgCl_2$ and 0.01 mmol l^{-1} orthovanadate, pH 7.4), the difference in measured activity between tubes following addition of solution A (assay buffer plus 1.6% DMSO) and solution B [assay buffer plus 10 µmol l^{-1} Bafilomycin A1 (Sigma) to inhibit V-ATPase] was taken to represent the activity of V-ATPase. The supernatants after reactions were assayed for inorganic phosphate (Pi) following the ammonium molybdate ascorbic acid method. The specific activity of the ATPase was calculated as µmol Pi released mg^{-1} gill protein h^{-1}. Total protein was determined using BSA as the standard, according to the method of Bradford (1976).

Reverse transcription–quantitative PCR analysis

Total RNA was extracted from 80–100 mg of gills using RNAiso Plus reagent (Takara, Kusatsu, Japan). RNA quantity, purity and integrity were examined by both native RNA electrophoresis on 1.0% agarose gel and the UV absorbance ratio at 260 and 280 nm (Multiskan Go 1510, Thermo Fisher Scientific, Vantaa, Finland). After detection, cDNA was synthesized using 1 µg of total RNA using PrimeScript RT-PCR reagent kit with gDNA Eraser (Takara). Oligonucleotide primers (Table S1) were designed by Primer Premier 5.0 and synthesized by Sangon (Shanghai, China). The

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stability of β-actin and ribosomal 18 s genes (candidate housekeeping genes) was evaluated using the BestKeeper method (Pfaffl et al., 2004). β-Actin was found to have the lower variation and was equally expressed in the DA injection groups. Therefore, it was selected as the reference gene for the rest of the analysis. PCR amplification was carried out in triplicate on a 96-well plate in a 10 µl system containing 5 µl of 2×SYBR Premix Ex Taq, 1 µl of cDNA template, 0.2 µl of 10 µmol l^{-1} forward and reverse primer and 3.6 µl of sterile water. Quantitative real-time PCR (qPCR) was carried out at 95°C for 30 s followed by 40 cycles of 95°C for 10 s, 57°C for 20 s and 72°C for 30 s. Melt curve analysis (60–95°C) was performed on the PCR products at the end of each run to ensure that only one PCR product was amplified and detected. A control lacking cDNA template was included in the qPCR analysis to determine the specificity of target cDNA amplification, and the specificity of the reverse transcription (RT)–qPCR assay was established by sequencing the reaction product. PCR efficiency (E) was determined by running standard curves with 10-fold serial dilutions of cDNA template and calculated according to $E=10^{-1/\text{slope}}$ (Rasmussen, 2001). The relative mRNA abundance ratio (R) was calculated using the equation: $R=E_{\text{target}}^{\Delta\text{CP}_{\text{target(control−sample)}}}/E_{\text{ref}}^{\Delta\text{CP}_{\text{ref(control−sample)}}}$ (Pfaffl, 2001).

Statistical analysis

The normal distribution and analysis of variance for all data were first tested by the Kolmogorov–Smirnov or Shapiro–Wilks test. A

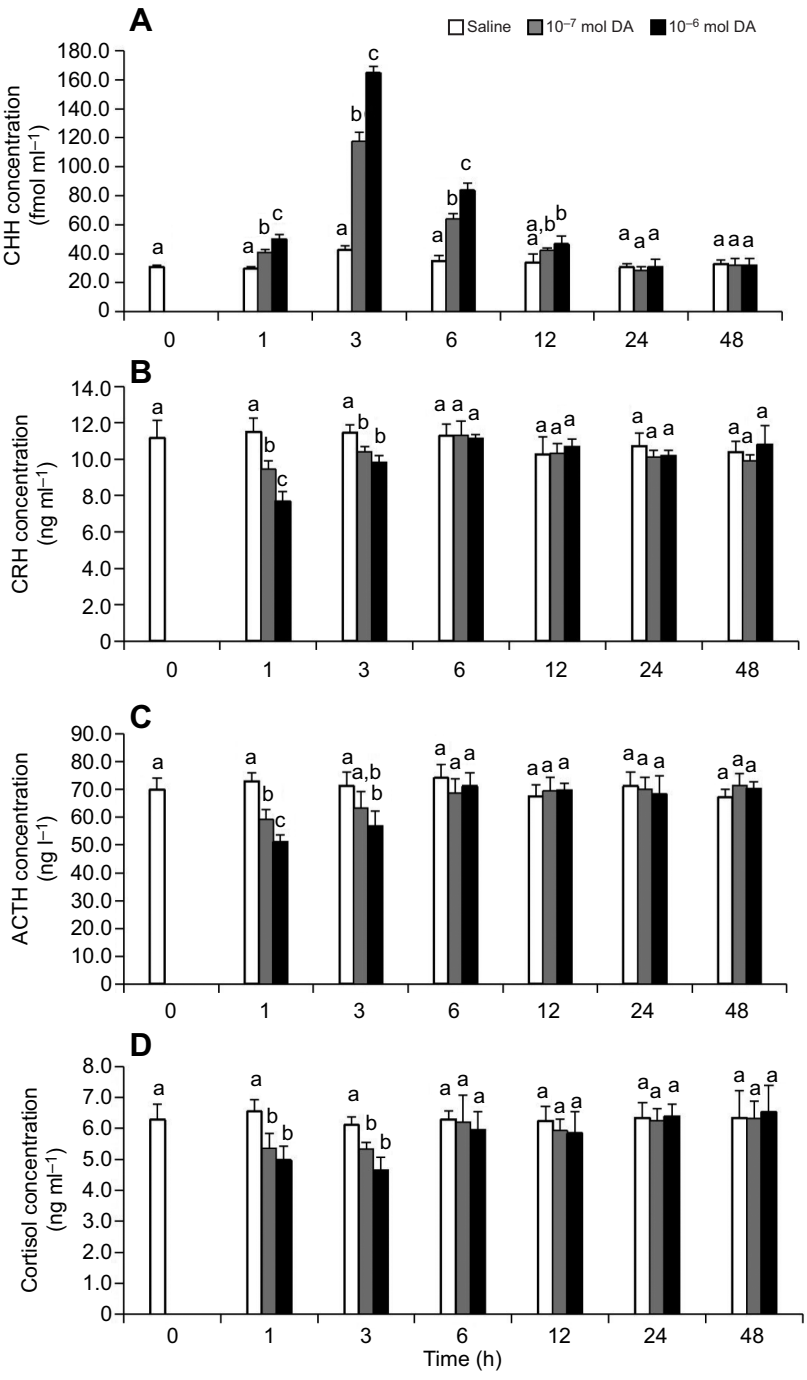

Fig. 1. Hormone concentration in hemolymph of *Litopenaeus vannamei* after dopamine injection. (A) Crustacean hyperglycemic hormone (CHH), (B) corticotrophin-releasing hormone (CRH), (C) adrenocorticotropic hormone (ACTH) and (D) cortisol concentration in the hemolymph of control (saline) and dopamine (DA)-injected (10^{-7} and 10^{-6} mol DA) groups. Each bar represents the mean (+s.e.m.) value from three independent repeats (n=6). Means not sharing the same letter are significantly different from one another (P<0.05).

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one-way analysis of variance (ANOVA) was utilized to examine the differences between control and treated groups at the same sampling time with SPSS software (version 17.0). All data are presented as means±s.e.m. Significant differences were considered at $P<0.05$. Tukey's test was used to identify the differences when significant differences were found.

RESULTS

Effect of DA on hemolymph hormone concentration

The injection of DA had remarkable effects on hormone concentration in the hemolymph (Fig. 1). The CHH concentration of 10^{-7} and 10^{-6} mol DA groups was significantly increased, and reached the highest level at 3 h. Afterwards, CHH concentration recovered to the control level gradually and tended to be stable at 24 h post-injection (Fig. 1A). Both CRH and ACTH concentration decreased significantly and reached a minimum at 1 h, then gradually returned to the control level 6 h after injection (Fig. 1B, C). The cortisol concentration in 10^{-7} and 10^{-6} mol DA groups fell dramatically within 3 h and reached the lowest level at 3 h, then recovered to the control level at 6 h (Fig. 1D).

Effect of DA on the mRNA expression of membrane-bound GC and DA receptor in the gills

Expression profiles of GC mRNA in the gills of *L. vannamei* injected with 10^{-7} and 10^{-6} mol DA are shown in Fig. 2A. Compared with the control group, GC mRNA expression in the 10^{-6} mol DA group increased significantly from 1 h to 12 h, reaching a peak (1.99-fold) at 3 h, then recovered to the control level at 24 h. However, the mRNA expression level of GC in the 10^{-7} mol DA group was significantly higher than that of the control group (1.43-fold) only at 3 h. D_4 receptor mRNA expression of DA treatment groups decreased significantly, reaching the lowest level at 3 h, then recovered to the control level at 12 h (Fig. 2B).

Effect of DA on signaling pathway factors in gills

The effects of DA injection on the concentration of cAMP, DAG and cGMP in gills of *L. vannamei* are illustrated in Fig. 3. A significant increase of cAMP (Fig. 3A) and DAG (Fig. 3C) was found in treatment groups from 1 h to 6 h, whereas the cGMP (Fig. 3E) concentration in treatment groups increased significantly at 3–6 h. All three peaked at 3 h and then returned to the control level at 12 h.

The changes of PKA and PKC concentration in DA injection groups were similar to those of cAMP and DAG, increasing significantly from 1 h to 6 h and peaking at 3 h (Fig. 3B,D). The PKG concentration in the 10^{-7} mol DA group was significantly higher than that of the control group only at 3 h, while the PKG concentration in the 10^{-6} mol DA group was significantly increased at 1–3 h, and returned to the control level at 6 h (Fig. 3F).

The mRNA expression level of nuclear transcription factor CREB in the gills of *L. vannamei* was significantly increased 1 h after injection in the 10^{-7} and 10^{-6} mol DA groups. The peak value occurred at 3 h (1.79- and 2.39-fold) and then returned to the control level at 24 h (Fig. 4A). The mRNA expression of 14-3-3 and FXYD2 was significantly up-regulated from 1 h to 6 h, with peaks occurring at 3 h and 1 h, respectively. They all recovered gradually to the control level at 12 h (Fig. 4B,C).

Effect of DA on the activity and mRNA expression of ion and ammonia transporters in gills

A significant increase in the activity of NKA was found in experimental groups post-injection (Fig. 5A). The peak of the 10^{-7} and 10^{-6} mol DA groups occurred at 3 h (1.50- and 1.87-fold of control values) and thereafter declined to the control level at 12 h. During the 48 h post-injection, the activity of V-ATPase relative to the control group did not change significantly (Fig. 5B).

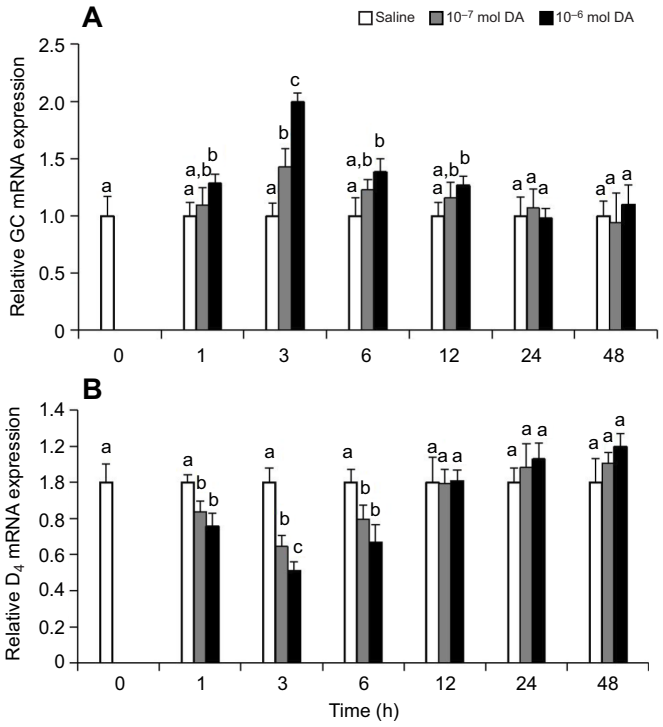

Fig. 2. Membrane-bound guanylyl cyclase and dopamine receptor D_4 mRNA expression in gills of *L. vannamei* after DA injection. (A) Guanylyl cyclase (GC) and (B) D_4 mRNA expression (using β-actin mRNA as reference) in the gills in control (saline) and 10^{-7} and 10^{-6} mol DA groups. Each bar represents the mean (+s.e.m.) value from three independent repeats (n=6). Means not sharing the same letter are significantly different from one another ($P<0.05$).

Fig. 6 shows that DA had notable effects on the mRNA expression levels of ion regulation and ammonia transport-related genes in gills of *L. vannamei*. The mRNA expression levels of the K^+ channel and NKCC increased significantly 1 h post-injection, reaching peaks at 3 and 6 h, respectively. The maximum values of the 10^{-6} mol DA treatment group were 2.06- and 2.29-fold, respectively, compared with the control group (Fig. 6A,B). A significant increase in Rh protein mRNA expression was observed with 10^{-7} and 10^{-6} mol DA treatment, reaching a peak at 3 h of 2.08- and 3.07-fold (Fig. 6C). The mRNA expression of VAMP, which shared similar trends with Rh protein, was significantly up-regulated in DA treatment groups from 1 h and reached the maximum at 3 h of 2.10-fold in the 10^{-6} mol DA group (Fig. 6D). All returned to normal status after 12 h.

Effect of DA on osmolality and ammonia concentration in hemolymph

DA had a transient effect on hemolymph osmolality, which depended on the injection dose. Hemolymph osmolality in the 10^{-7} mol DA group increased significantly only at 3 h. The higher dose treatment group showed a more significant effect on osmoregulation, with a significant increase at 1–3 h, peaking at 3 h. After 6 h injection, DA treatment groups did not differ from saline-injected controls (Fig. 7A).

Injection of DA also had a significant effect on ammonia concentration in the hemolymph (Fig. 7B). The ammonia concentration in 10^{-7} and 10^{-6} mol DA groups was significantly decreased from 1 h to 3 h, and reached the lowest level at 3 h.

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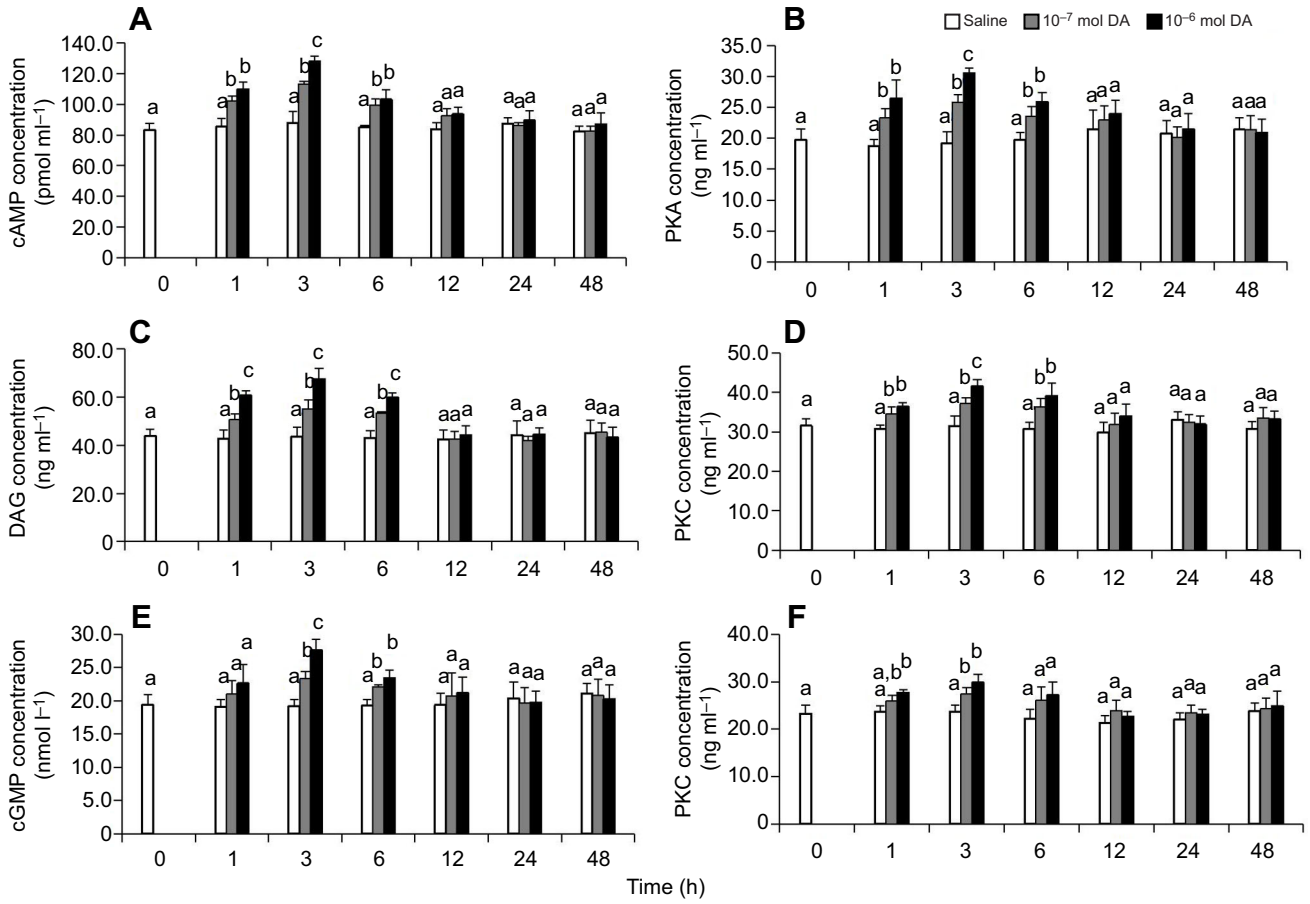

Fig. 3. Second messenger and protein kinase concentration in gills of *L. vannamei* after DA injection. (A) Cyclic adenosine monophosphate (cAMP), (B) protein kinase A (PKA), (C) diacylglycerol (DAG), (D) protein kinase C (PKC), (E) cyclic guanosine monophosphate (cGMP) and (F) protein kinase G (PKG) concentration in gills in control (saline) and 10^{-7} and 10^{-6} mol DA groups. Each bar represents the mean (+s.e.m.) value from three independent repeats (n=6). Means not sharing the same letter are significantly different from one another (P<0.05).

Afterwards, both recovered to the control level at 6 h. There was no significant difference between the two DA treatments throughout the experimental period.

DISCUSSION

Evidence that DA is involved in the regulation of neuroendocrine hormones

It is uncertain whether the HPI axis, which is the main neuroendocrine regulation axis in fish and plays a central role in acclimation to stress, is also present in crustaceans. In fish, when an environmental stress signal is perceived, it can promote the release of CRH and ACTH into the circulation and further stimulate the release of cortisol (Nardocci et al., 2014). Importantly, Zhao et al. (2016) reported that CRH-ACTH could induce the release of DA in *L. vannamei.* Our results show that DA injection had an inhibitory effect on components of the HPI axis, with a decrease in hemolymph CRH, ACTH and cortisol concentration within 3 h, possibly to maintain homeostasis. Consistent with this, investigation of the role of DA in regulating ACTH and cortisol secretion in goldfish and cows by treatment with a DA antagonist revealed that DA inhibits ACTH and cortisol secretion (Olivereau et al., 1988; Ahmadzadeh et al., 2006). In contrast, ergotamine, which has DA-like activity, increased plasma cortisol in steers and cows, suggesting that DA may stimulate cortisol release Browning et al. (1998, 2000); however, there were substantial differences between the pharmacological agents used, which may explain the difference between these results and those of the present study.

In particular, the crustacean-specific CHH functions like the HPI axis of fish. It is secreted from the neuroendocrine XO/SG complex in the eyestalk, which is dependent on the stimulation of biogenic amines (Fingerman et al., 1994). Its secretory site was confirmed by a significant increase in hemolymph glucose levels obtained in bilaterally eyestalk-ablated *L. vannamei* injected with the recombinant CHH, but not in those injected with DA (Camacho-Jiménez et al., 2017). When exposed to dilute seawater, cGMP and glucose levels in the gills and CHH levels in the hemolymph of *C. maenas* increased significantly (Chung and Webster, 2006). It was also reported that a membrane-bound GC acts as the CHH receptor, and CHH can activate cellular signaling by directly activating GC without binding to G-proteins; cGMP then acts as a second messenger and regulates the activity of PKG (Chung and Webster, 2006). Furthermore, increased intracellular cGMP leads to increased CREB phosphorylation in vascular smooth muscle cells and neuronal cells, suggesting a direct or indirect involvement of PKG in CREB phosphorylation (Johannessen and Moens, 2007). As was shown in our study, the concentration of CHH in hemolymph increased dramatically after injection of DA, indicating that DA can stimulate the secretion of CHH in *L. vannamei*. At the same time, the expression of GC mRNA and the concentration of cGMP and PKG were significantly up-regulated in a similar pattern,

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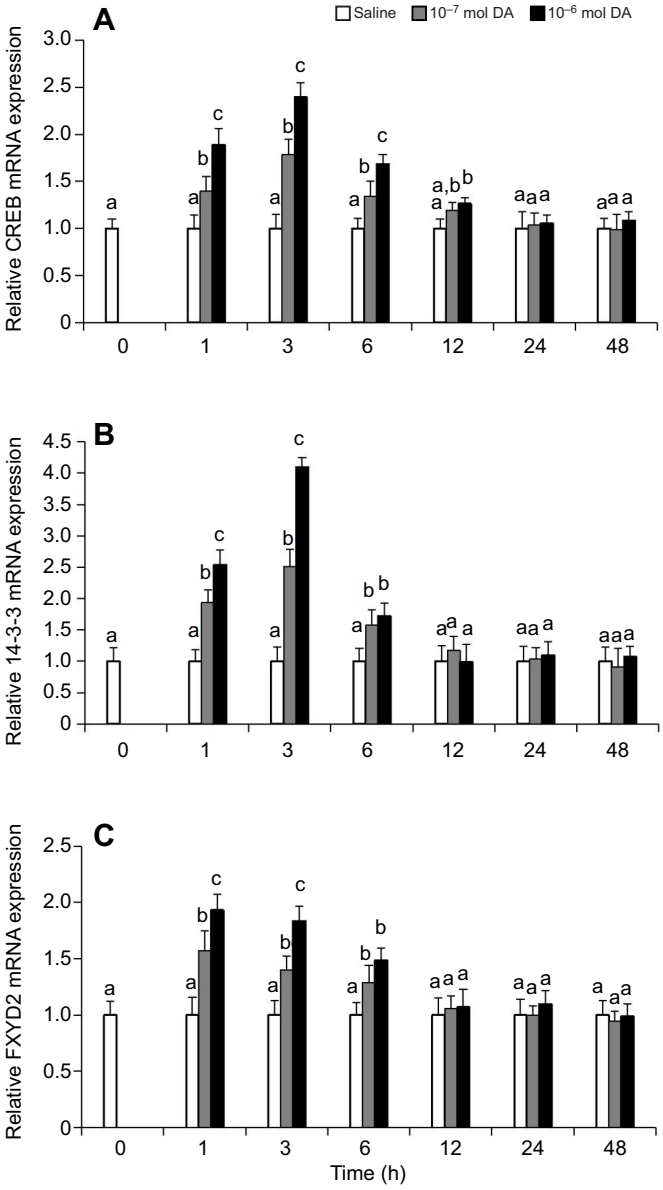

Fig. 4. Nuclear transcription factor CREB and downstream effector protein mRNA expression in gills after DA injection. (A) cAMP response element-binding protein (CREB), (B) 14-3-3 and (C) FXYD2 mRNA expression (using β-actin mRNA as reference) in gills in control (saline) and 10^{-7} and 10^{-6} mol DA groups. Each bar represents the mean (+s.e.m.) value from three independent repeats (n=6). Means not sharing the same letter are significantly different from one another (P<0.05).

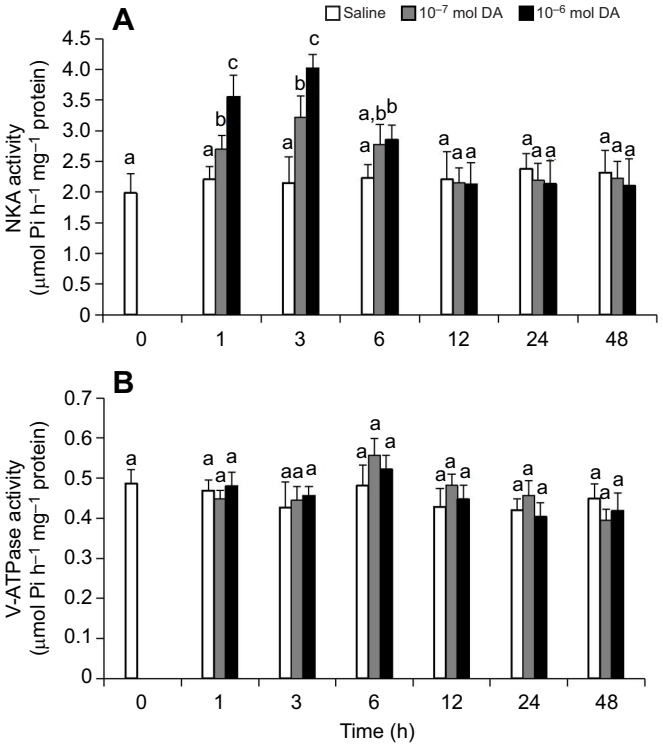

Fig. 5. Na^+/K^+-ATPase and V-ATPase activity in gills of *L. vannamei* after DA injection. (A) Na^+/K^+-ATPase (NKA) and (B) V-type H^+-ATPase (V-ATPase) activity in gills in control (saline) and 10^{-7} and 10^{-6} mol DA groups. Pi, inorganic phosphate. Each bar represents the mean (+s.e.m.) value from three independent repeats (n=6). Means not sharing the same letter are significantly different from one another (P<0.05).

which is consistent with the changes in CHH. We suggested that the up-regulation of CHH can activate GC on the membrane and leads to an increase in PKG by activating cGMP activity and binding to the transcription factor CREB to ultimately regulate transcription of the target genes.

DA-mediated intracellular signal transduction in gills

There is evidence that the concentration of hemolymph DA in crustaceans was significantly increased under salinity or ammonia–N stress (Zhao et al., 2016; Zhang et al., 2018; Pan et al., 2014; Yue et al., 2010). The release of DA may be a response to environmental stress, indicating that DA plays a regulatory role in the physiological adaptation. In addition to the regulation of the above-mentioned neuroendocrine hormones, DA can modulate physiological effects through specific GPCRs. In the present study, the mRNA expression level of the D_4 receptor in DA injection groups decreased significantly, while the concentration of cAMP and DAG increased significantly. Simultaneously, the concentration of PKA and PKC in the gills significantly increased to the highest levels at 3 h, and the changes in the different treatment groups depended on the dose of DA injected. The DA receptors in mammals are broadly classified into two subfamilies: D_1-like receptors (D_1/D_{1A}, D_{1B}/D_5, D_{1C}, D_{1D}) and D_2-like receptors (D_2, D_3, D_4). Only a partial sequence of the D_4-type receptor was obtained from our transcriptome library. It belongs to D_2-like family, which is associated with the G_i subtype of GPCR. The activated G_i protein inhibits the activity of adenylyl cyclase (AC), thereby reducing the intracellular concentration of cAMP (Neve et al., 2004). In addition, activation of D_2 receptors causes a decrease in the level of inositol phosphates, and D_2 receptors are also known to act through DAG to activate PKC, which also functions in calcium-dependent pathways (Rodrigues and Dowling, 1990; Titlow et al., 2013). Combining these studies with our results, we speculate that the down-regulated D_4 receptor that binds to G_i proteins may have depressed the inhibition of AC. As a consequence of the joint effect, the cAMP and PKA concentrations were successively increased. Furthermore, the results suggest that DA can modulate physiological responses by the activation of PKC via DAG.

Moreover, studies have shown that some downstream effector proteins are involved in the regulation of ion transporter activity, including 14-3-3 protein and FXYD2. It has been reported that the 14-3-3 family of proteins are highly conserved and associate with

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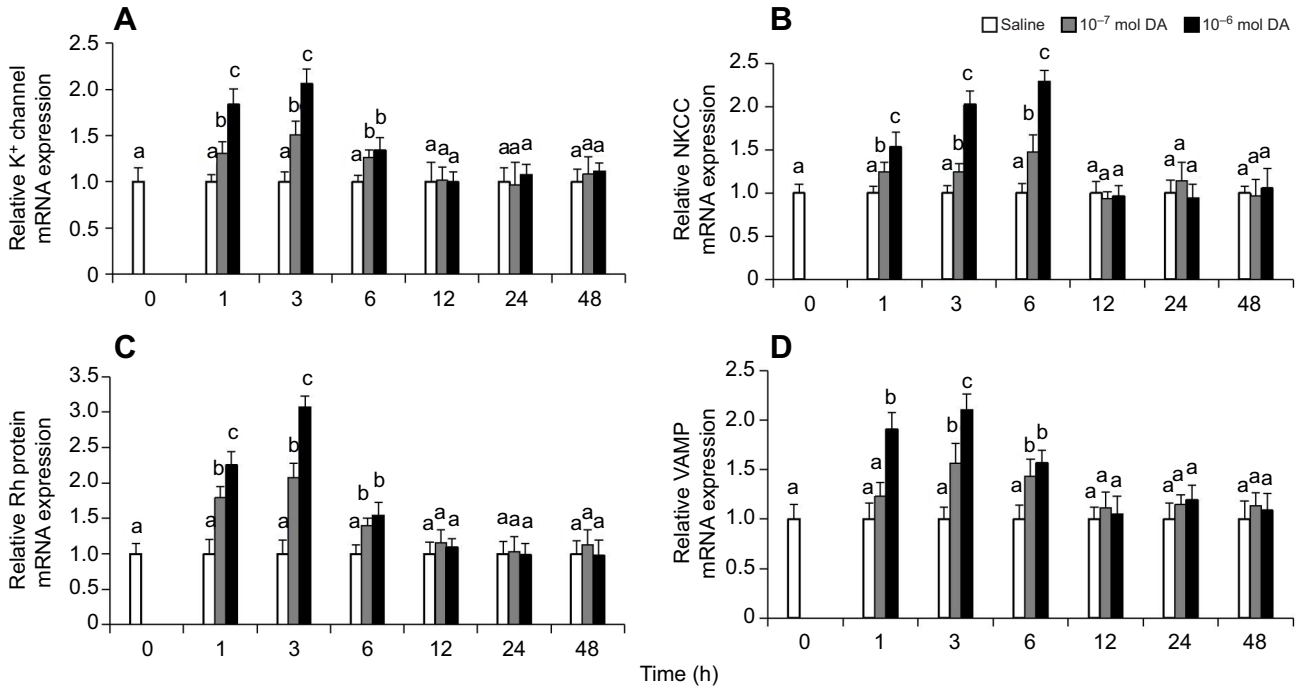

Fig. 6. Ion and ammonia transporter mRNA expression in gills of *L. vannamei* after DA injection. (A) K^+ channel, (B) $Na^+/K^+/2Cl^-$ cotransporter (NKCC), (C) Rh protein and (D) vesicle associated membrane protein (VAMP) mRNA expression (using β-actin mRNA as reference) in gills in control (saline) and 10^{-7} and 10^{-6} mol DA groups. Each bar represents the mean (+s.e.m.) value from three independent repeats (n=6). Means not sharing the same letter are significantly different from one another (P<0.05).

ion channels in a PKA-dependent phosphorylation manner, which has been detected in some crustaceans (Kagan et al., 2002; Wanna et al., 2012; Zhu et al., 2014; Shu et al., 2015). In addition, the FXYD2 protein has been identified in the posterior gills of *Callinectes danae*, and was shown to be phosphorylated by PKA but not PKC (Silva et al., 2012). At the transcription level, CREB can be considered as a nuclear transcription factor that regulates the expression of ion transporters. CREB is directly mediated by PKA, while PKC does not appear to directly phosphorylate it (Johannessen and Moens, 2007). Also, it has been reported previously that PKG phosphorylates CREB at a slower rate than does PKA (Colbran et al., 1992). In this study, DA injection was shown to have a dose-dependent effect on the expression of 14-3-3, FXYD2 and CREB. However, the expression of FXYD2 peaked at 1 h, which may be related to the rapid enhancement of NKA activity under DA stimulation. Therefore, we assume that the exogenous DA influences the PKA pathway in the gills, and then activates the effector proteins 14-3-3 and FXYD2, as well as the transcription factor CREB to regulate the activity and expression of the target genes.

Regulation by DA of ion and ammonia transporters in gills

Recently, the neuroendocrine regulation mechanism of osmoregulation has received increasing attention. Chiu et al. (2006) found that *L. vannamei* receiving 10^{-6} mol DA per shrimp showed transient elevation of osmolality, Na^+ and Cl^- levels, while the K^+ level remained unchanged. Similar results were obtained in *Penaeus monodon*, but the K^+ level was reduced (Chang et al., 2007). Our study was consistent with the work of Chiu et al. (2006) and Liu et al. (2008): DA had a transient effect on hemolymph osmolality, which was significantly higher than the control level by 3 h post-injection. In addition, the effects on the 10^{-6} mol DA group were marked compared with those on the 10^{-7} mol DA group, indicating an obvious dose-dependent effect of neuroendocrine control. It was observed that 80% of the hemolymph osmotic pressure of crustaceans was accounted for by the Na^+ and Cl^- concentration (Lima et al., 1997). Na^+ absorption and K^+ discharge via the basolateral membrane of epithelial cells usually occur through transporters: NKA and K^+ channels. Our study showed a similar trend, with a peak in NKA activity and K^+ channel expression 3 h after DA injection. This is consistent with previous findings for *Leptograpsus variegatus* and *L. vannamei* (Morris and Edwards, 1995; Liu et al., 2009), indicating that DA can enhance NKA activity. Little is known about the direct control of K^+ channels by DA. In combination with the above studies, we hypothesize that DA can up-regulate the basolateral K^+ channel to transport K^+ into the epithelial cells, but this requires further investigation. On the apical membrane, NKCC acts as a key channel for NaCl absorption in gill epithelia (Henry et al., 2012). Previous reports have suggested that the activation of NKCC is modulated by DA (Aoki et al., 1996; Hattori and Wang, 2006). In this study, the expression of NKCC was significantly up-regulated under DA stimulation, indicating that active transbranchial Na^+ and Cl^- uptake dependent on NKCC was enhanced by DA. In particular, hydrated NH_4^+ and K^+ ions have the same ionic radius: 1.45 Å. Theoretical considerations argue that basolateral K^+ channels, NKA and NKCC can transport NH_4^+ into the epithelial cells, because the hemolymph NH_4^+ may substitute for K^+ (Choe et al., 2000; Wood et al., 2013; Wilkie, 1997), which could play an important role in ammonium transport.

In the present study, we provided the first evidence on the ammonia excretion mechanism under DA stimulation in *L. vannamei*. The hemolymph ammonia concentration was significantly reduced at the beginning of the experiment, which may be related to the effects of protein metabolism, but it also suggested that DA may enhance ammonia excretion. It was directly related to the uptake of Na^+ and the discharge of NH_4^+ (K^+) by ion

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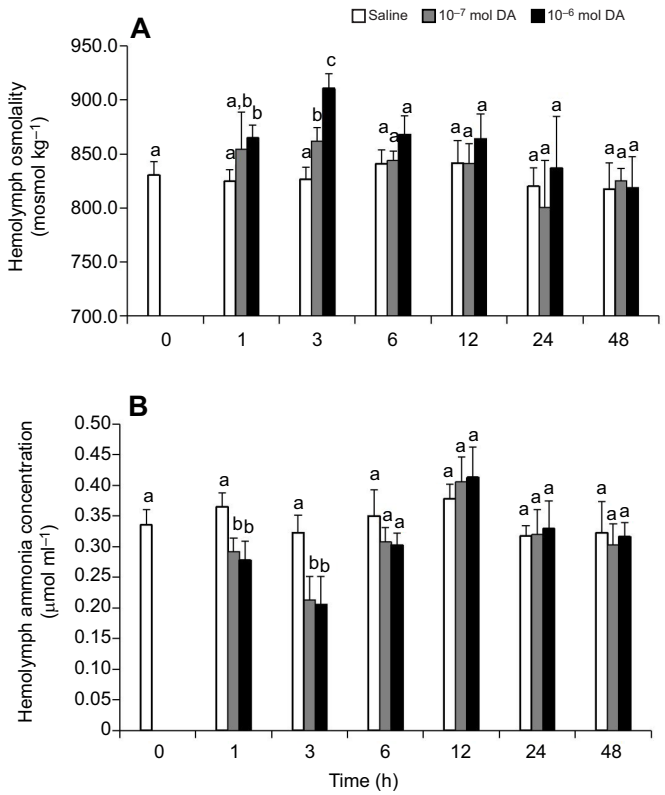

Fig. 7. Hemolymph osmolality and ammonia concentration of *L. vannamei* after DA injection. (A) Osmolality and (B) ammonia concentration in hemolymph in control (saline) and 10^{-7} and 10^{-6} mol DA groups. Each bar represents the mean (+s.e.m.) value from three independent repeats (n=6). Means not sharing the same letter are significantly different from one another (P<0.05).

transporters. It is not only the above-mentioned ion channels that may be involved in ammonium transport: crustaceans also have a specific ammonia transporter-Rh protein (Si et al., 2018; Martin et al., 2011; Wright and Wood, 2009). A significant increase was observed in the mRNA expression level of Rh protein post-DA injection, suggesting that DA may activate Rh protein to promote NH_3 excretion. In addition, the existence of a vesicular ammonia-trapping strategy cannot be disregarded. The cytoplasmic V-ATPase was thought to acidify cytosolic NH_3 as NH_4^+ and trap it in vesicles for exocytotic release from the gill (Weihrauch et al., 2002). Here, DA injection had no significant effect on the activity of V-ATPase, which was consistent with the findings of Liu et al. (2009), who speculated that V-ATPase is regulated by other endocrine factors and DA may not affect intracellular acidification. However, a significant up-regulation of VAMP was noted in our treatment groups. This suggests that DA may cause an increase in intracellular vesicles and vesicle transport. These studies highlight the multiple pathways for osmoregulation and ammonia transport in *L. vannamei.*

It is unclear how DA acts on ion channels and ammonia transporters of gills directly or through the neuroendocrine hormones. There is evidence that some ion channels, including NKA, K^+ channels and NKCC, are regulated by PKA and PKC pathways. Moreover, FXYD2 and 14-3-3 protein that were phosphorylated by PKA can activate NKA (Zhang et al., 2008; Yuan et al., 2002; Yang et al., 2001; Silva et al., 2012; Jayasundara et al., 2007). CHH has also been demonstrated to exert ion regulation on the gill by regulating hemolymph Na^+ and Cl^- and the expression of NKA in *L. vannamei* (Camacho-Jiménez et al., 2018). Kiilerich et al. (2007) demonstrated the important role of cortisol in upregulating the transcript levels of NKA and NKCC in Atlantic salmon gill. Cortisol also stimulated mRNA levels of Rh protein and V-ATPase, as well as ammonia permeability in gill epithelium of cultured trout (Tsui et al., 2009). In addition, exocytosis can be stimulated by direct activation of PKA and PKC as well as 14-3-3 protein (Jung et al., 2004; Burgoyne et al., 1993). These findings together with those of the present study suggest that reduced cortisol probably helps to maintain a balance of the neuroendocrine system. The activity of NKA and the expression of K^+ channels and NKCC may be regulated by DA-activated PKA and PKC. NKA is also mediated by PKG, while FXYD2 and 14-3-3 protein play multiple regulatory roles. The mRNA expression of K^+ channels is also probably regulated by 14-3-3 protein (Kagan et al., 2002). Rh protein may be regulated by DA or CHH, and a study has shown

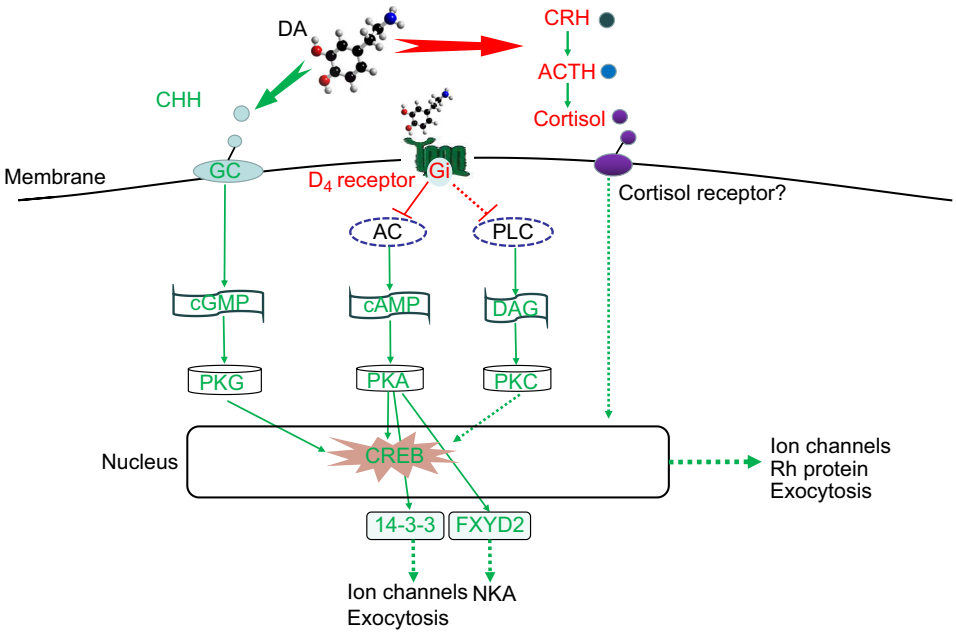

Fig. 8. Proposed model of neuroendocrine regulation of signaling pathways in gills under DA stimulation. Signaling pathway factors that are highlighted in green indicate up-regulation, while those highlighted in red indicate down-regulation. As only the D_4-type DA receptor was obtained from our transcriptome library, only this receptor was detected in the present study. Cortisol receptors have not been found in our transcriptome library, and the downstream region of the cortisol-regulated signaling pathway is not yet clear. Ion channels include NKA, K^+ channels and NKCC. V-ATPase and VAMP are involved in the process of exocytosis, whereas the activity of V-ATPase did not change significantly after DA injection.

that RHBG acts as a direct target of Wnt/β-catenin signaling in mammals (Merhi et al., 2015), which requires further study. Stimulation of V-ATPase may be inhibited by reduced cortisol or by other pathways, while VAMP is up-regulated by multiple effects of PKA, PKC and 14-3-3 protein. This is a preliminary study on the signal transduction mechanism of neuroendocrine regulation of ion and ammonia transport in *L. vannamei*, and we propose a hypothetical model of signal transduction pathways in gills under DA stimulation (Fig. 8). This may turn out to be a fertile ground for further research.

In summary, the present study demonstrated the important role of DA in regulating ion and ammonia transport in the gills of *L. vannamei*. After injection of DA, *L. vannamei* secreted CHH into the hemolymph and reduced the release of CRH, ACTH and cortisol. DA receptor and GC transduced the signals from DA and CHH into gill cells and increased the concentration of intracellular factors such as cAMP-PKA, DAG-PKC and cGMP-PKG. 14-3-3 protein, FXYD2 and CREB controlled the activity and transcription of target genes mainly through PKA. Under the regulation of these signaling molecules, ion transporters (NKA, K^+-channel and NKCC) were activated, but the activity of V-ATPase did not change significantly. Both the ammonia transporter Rh protein and the exocytosis-associated VAMP expression were up-regulated under DA stimulation. Eventually, as a result of the activation of ions and NH_3 transport, an increase in hemolymph osmolality and a decrease in hemolymph ammonia concentration occurred.

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Competing interests
The authors declare no competing or financial interests.

Author contributions
Conceptualization: L.S., L.P.; Methodology: L.S., L.P., X.Z.; Software: L.S., X.Z.; Validation: L.S., X.Z., H.W., C.W.; Formal analysis: L.S., X.Z., H.W., C.W.; Investigation: L.S., X.Z., H.W., C.W.; Resources: L.S., X.Z., H.W., C.W.; Data curation: L.S., H.W., C.W.; Writing - original draft: L.S.; Writing - review & editing: L.S., L.P.; Visualization: L.P.; Supervision: L.P.; Project administration: L.P.; Funding acquisition: L.P.

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Supplementary information
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