**The cholinergic immune regulation mediated by a novel
muscarinic acetylcholine receptor through TNF pathway in
oyster *Crassostrea gigas***

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Abstract

Muscarinic receptors, which selectively take muscarine as their ligand, are critical for the immunological and physiological processes in animals. In the present study, the open region frame (ORF) of a homologue of muscarinic acetylcholine (ACh) receptor (mAChR) was amplified from oyster *Crassostrea gigas* (named as CgmAChR-1), whose full length was 1983 bp and the protein it encoded contained 660 amino acids with a seven transmembrane region. Phylogeny analysis suggested that CgmAChR-1 shared homology with M₅ muscarinic receptor found in invertebrates including *Habropoda laboriosa*, *Acromyrmex echinator* and *Echinococcus granulosus*. After cell transfection of CgmAChR-1 into HEK293T cells

and ACh incubation, the level of intracellular Ca^{2+} and cAMP increased significantly ($p < 0.05$). Such trend could be reverted with the addition of M_3 and M_5 muscarinic receptor antagonists DAMP and DAR. The CgmAChR-1 transcripts were ubiquitously detectable in seven different tissues with the maximal expression level in adductor muscle. When the oysters received LPS stimulation, CgmAChR-1 mRNA expression in haemocyte was increased to the highest level (6.05-fold, $p < 0.05$) at 24 h, while blocking CgmAChR-1 using receptor antagonists before LPS stimulation promoted the expression of oyster TNF, resulting in the increase of haemocyte apoptosis index. These results suggested that CgmAChR-1 was the key molecule in cholinergic neuroendocrine-immune system contributing to the regulation of TNF expression and apoptosis process.

Key words: *Crassostrea gigas*; Acetylcholine; Muscarinic acetylcholine receptor; TNF pathway; Immune regulation

1. Introduction

Acetylcholine (ACh), the neurotransmitter synthesized by choline acetyltransferase (ChAT) and hydrolyzed by acetylcholinesterase (AChE) with the products of choline and acetic acid ready for re-uptake and re-synthesis, is significant for cholinergic nervous system (Deiana et al., 2011). As the key molecule in cholinergic modulation, ACh takes part in many physiological functions in vertebrates, such as excitability, stress response and cortical modulation (Hurst et al.,

2013; Lucas-Meunier et al., 2003). It is demonstrated that ACh is critical for the immunomodulation in vertebrates (Norman et al., 2011; Rosas-Ballina and Tracey, 2009; Van der Zee et al., 2011). For example, ACh attenuates the synthesis of TNF and IL by human macrophages at the post-transcriptional stage (Borovikova et al., 2000). ACh released from the activated cholinergic nervous system of mammals can monitor the level of intracellular reactive oxygen species (ROS) by the cGMP-dependent protein kinase (PKG) pathway and the transactivation of epidermal growth factor receptors (EGFRs) (Krieg et al., 2004; Krieg et al., 2005; Wang et al., 1999). Being the first neurotransmitter ever defined (Kruse et al., 2012), ACh accomplishes its function by activating its receptors expressed on immune cells (Shi et al., 2014).

According to the relative affinities and sensitivities to different molecules, ACh receptors are classified into two major types, nicotinic ACh receptors (nAChRs) which have high affinity for nicotine, and muscarinic ACh receptors (mAChRs) which are particularly responsive to muscarine. The effects of mAChRs are mediated mainly by triggering the fluxes of intracellular second messengers (Ca^{2+} and cAMP) by activating the heterotrimeric G proteins (Dale, 1914). There are 5 subtypes (M_1 - M_5) of mAChRs in vertebrates, which could be subsequently characterized as two kinds. The M_1 , M_3 and M_5 receptors have the preference for binding $\text{G}_{q/11}$, activating phospholipase and inducing the increase of Ca^{2+} level. The M_2 and M_4 receptors inhibit the rise of adenylyl cyclase (AC) upon stimulation by binding to $\text{G}_{i/o}$, leading to down-production of cAMP (Hulme et al., 1990; Kawashima et al., 2012; Wess,

1996). Vertebrate mAChRs are related to a series of physiological processes (MacDonald et al., 2015) including the modulation of immune function. CD11a could activate intracellular signaling pathways in T cells to increase $[Ca^{2+}]_i$, and cause the increase of the expression level of ChAT genes (Fujii et al., 2003). Furthermore, in the M_1 mAChR gene-knockout mice model, $CD8^+$ T cell differentiation was severely inhibited under stimulation (Zimring et al., 2005). These findings suggested that mAChRs were indispensable during the cholinergic immunomodulation in mammals.

However, the mAChRs in invertebrate are not well investigated, and most of the researches focus on limited species, such as locust and drosophila. For instance, mAChRs have been detected in the nerves of *Drosophila melanogaster*, *Apis mellifera*, and *Manduca sexta* (Aizono et al., 1997; Blake et al., 1993; Harrison et al., 1995; Van der Zee et al., 1992). Unlike vertebrates, mAChRs in invertebrates were found to modulate various nervous and non-nervous related processes (Caulfield and Birdsall, 1998). For example, release of transmitters are often inhibited by mAChs in the presynaptic sensory terminals (Trimmer, 1995), while the neuronal responses can be enhanced when postsynaptic mAChRs are activated (Caulfield and Birdsall, 1998). Nevertheless, compared with that in vertebrates, the mechanism of mAChR in cholinergic immunomodulation in invertebrates is still far from clear.

ACh and nAChRs have been previously reported to be existed in oyster *Crassostrea gigas* (Shi et al., 2014; Sun et al., 2014a), and the investigation about mAChR will benefit for the comprehensive understanding of the cholinergic neuroendocrine-immune system as well as the immunomodulation mechanism in

marine invertebrates. The present study is conducted to (1) identify the G protein-coupled muscarinic receptor for ACh from oyster *C. gigas* (designated as CgmAChR-1), (2) investigate the fluctuation of intracellular Ca^{2+} and cAMP during the activation and inhibition of CgmAChR-1, (3) examine the phagocytosis rate and apoptosis index of oyster haemocytes, as well as the expression of CgTNFs after the activation of CgmAChR-1.

2. Materials and Methods

2.1. Oysters, antagonist incubation and LPS stimulation

Oysters *C. gigas* were collected from a breeding base in Qingdao, China. After drilling a tiny hole on the edge of the shell near the adductor muscle, oysters were put back into the tank and maintained at 18 °C for 14 days to get used to the new environment. Algae powder was added to the water every other day, and the seawater in the aquaria was replaced every day.

Five antagonists specific for M_1 to M_5 muscarinic receptors, PIR (pirenzepine, TOCRIS), AFDX (AF-DX 116, TOCRIS), DAMP (4-DAMP, TOCRIS), PD (PD102807, TOCRIS) and DAR (Darifenacin, Sigma) (Caulfield and Birdsall, 1998; Hulme et al., 1990), were employed in the antagonists incubation experiment. For example, in the CgmAChR+PIR group, one hundred microliter of M_1 muscarinic receptor antagonist PIR was added into the cultured HEK293T cells before ACh treatment. The final concentration of each antagonist was 1.0 mmol L^{-1} in both cell transfection and adult injection experiments.

For the LPS stimulation, 210 oysters were equally divided into PBS (control) and LPS groups. In the LPS group, one hundred microliter of LPS (0.5 mg mL⁻¹) from *Escherichia coli* 0111:B4 (Sigma) was injected through the tiny hole drilled on the edge of the shell, while oysters in the PBS group were treated with PBS (pH 7.4).

2.2. Sample collection, RNA isolation and cDNA synthesis

Fifteen were sampled at each time point after LPS stimulation as one replicate, and three replicates were taken for each test. The oysters in the blank group were collected at 0 h without any treatment. Hemolymph from each oyster was collected from the blood sinus and centrifuged at 800 g to gather haemocytes for RNA extraction. The serum was also harvested for the determination of the activities of immune-related enzymes. Five oysters were sampled as one parallel, and we did three parallels for each test.

Seven different kinds of tissues, such as haemocytes, gonad, mantle, gill, kidney, adductor muscle and hepatopancreas were also collected for the tissue distribution analysis. Tissues from 3 oysters were sampled as one parallel, and we did three parallels for each test. Then we extracted total RNA from haemocytes and tissues using Trizol reagent (Invitrogen, USA). The cDNA library was constructed using Promega M-MLV kit, and diluted to 1:40 for the subsequent experiments.

2.3. Gene cloning and sequence analysis

The ORF of CgmAChR-1 (CGI_10014948, EKC34110.1), which was

homologous to vertebrate mAChR, was obtained and analyzed. The Expert Protein Analysis System was applied in the amino acid analysis. The protein domain was predicted with SMART software. Multiple alignment of the CgmAChR-1 with muscarinic receptors from other species was employed by the ClustalW software. Phylogenetic tree of mAChRs was built by MEGA software using neighbor-joining (NJ) algorithm.

2.4. Cell transfection, drug treatment and second messenger determination

HEK293T cells were cultured as described by Liu et al (Liu et al., 2015a). The full length sequence of CgmAChR-1 was amplified with corresponding primers and sub-cloned into the pEASY-M₁ expression vector (TransGene), designating as pEASY-M₁-CgmAChR-1 plasmid. When the cell coverage ratio reached 60~80 % in the wells, the recombinant plasmid was transfected with Lipofectamine LTX and PlusTM Reagent (Invitrogen). After being cultured for 24 hours, the cells were digested and collected for the second messenger determination.

In the drug treatment assay, 500 μ L suspension (about 10^4 cells) of HEK293T cells transfected with pEASY-M₁-CgmAChR-1 was added into the 24-well plate with 50 μ L of ACh (final concentration of 10^{-7} mol L⁻¹) (Guo et al., 2013), designating as CgmAChR-1 group. In the CgmAChR-1+PIR group, 50 μ L of 10.0 μ mol L⁻¹ PIR was added to incubate the cells. And 3 h later, 50 μ L of ACh was then added. Similarly, specific antagonist AFDX, DAMP, PD and DAR for M₂ to M₅ muscarinic receptors were also added to the wells of transfected HEK293T cells before the addition of ACh.

The blank group referred to cells without transfection or stimulation, and the control group referred to cells with cell transfection but not stimulated by ACh. After ACh treatment for 1 h, HEK293T cells were digested and harvested. All incubation assays were carried out by 3 replicates.

Fluo-3 AM fluorescent probe (Beyotime, $1.0 \mu\text{mol L}^{-1}$) was employed in the examination of intracellular Ca^{2+} , and the cAMP Direct Immunoassay Kit (ab65355, Abcam, Cambridge, UK) was used to measure cAMP concentration as illustrated (Liu et al., 2016). Three duplicates were examined for each test.

2.5. qRT-PCR analysis of CgmAChR-1 mRNA expression

Gene relative expression of CgmAChR-1 has been analyzed using qRT-PCR. Samples from 5 different individuals were regarded as one duplicate and three technical duplicates were made for each assay. The fragment (168 bp) of oyster elongation factor (CgEF, CGI_10012474) was detected as endogenous control, and the expression level was normalized using the $2^{-\Delta\Delta\text{Ct}}$ method.

2.6. Determination of the phagocytosis rate and apoptosis index of oyster haemocytes

The apoptosis index was examined using Annexin V-FITC Apoptosis Detection Kit (KeyGEN). Before incubating with Annexin V-FITC, haemocytes were mixed with 195 μL of Binding Buffer. Then, resuspend haemocytes with 190 μL of Binding Buffer after centrifugation at 1000 g for 5 min. After incubated with Propidium Iodide (PI), the haemocytes resuspension were transferred into a polystyrene round-bottom

tube and the apoptosis index was detected by flow cytometer (BD, Aria II). The apoptosis index was calculated as Equation (1):

$$AI = \frac{\text{Number of apoptotic cells}}{\text{Number of haemocytes}} \quad (1)$$

In the phagocytosis rate assay, haemocytes were adjusted to 1.0×10^6 cell mL^{-1} with sterile seawater, and 5 μL of *Vibrio anguillarum* dyed by FITC (1.0×10^9 CFU mL^{-1}) was added to 500 μL haemocytes resuspension. After incubation at 18°C for 1 h, 10 μL of Trypan Blue (100 mg mL^{-1}) was added into the mixture, and 400 μL haemocyte resuspension was transferred to a polystyrene round-bottom tube for the determination of phagocytosis rate using BD flow cytometer. The phagocytosis rate was calculated as Equation (2):

$$PR = \frac{\text{Number of phagocytic cells with engulfed bacteria}}{\text{Number of phagocytes}} \quad (2)$$

Three parallel replicates were performed in each phagocytosis and apoptosis assay.

2.7. Statistical analysis

All values obtained were listed as means $\pm$ SD and analyzed by one-way ANOVA and a Student-Newman-Keus (S-N-K) comparison. Letters (a/b/c, etc.) were marked to illustrate the statistical significance ($p < 0.05$)

3. Results

3.1. The molecular features of CgmAChR-1 and phylogenetic relationship

The ORF of CgmAChR-1 (1983 bp, No. **EKC34110** in GenBank) was obtained

from the oyster cDNA library and the polypeptide sequence contained 660 amino acids with a predicted molecular mass of 74.08 kDa. The theoretical isoelectric point of CgmAChR-1 was 6.45, and a seven transmembrane domain (from Val51 to Ala641) was revealed using SMART program.

The amino acid sequence of CgmAChR-1 shared 40.7 % similarity with that of *Drosophila melanogaster* mAChR, and 29.8 % similarity with that of *Homo sapiens* mAChR. Four conserved cysteine residues (Cys119, Cys608, Cys614 and Cys627) were identified by the alignment of CgmAChR-1 with other two mAChRs (Fig. 1).

Amino acids sequences of mAChRs from *H. sapiens*, *D. melanogaster*, *M. musculus*, *C. gigas* *et al* were employed to construct a phylogenic tree by MEGA program (Fig. 2). These mAChRs were positioned separately in five distinct groups corresponding to M₁ to M₅. The separation between different receptors was in line with their subtypes. According to the phylogenic tree, CgmAChR-1 was closely matched with M₅ muscarinic receptors found in invertebrates.

3.2. Change of Ca²⁺ and cAMP concentration after ACh incubation

Concentrations of Ca²⁺ in the CgmAChR-1 group after stimulation was increased significantly ($p < 0.05$), which was 1.38-fold of that in the control group. Dramatic decreases of intracellular Ca²⁺ concentration were observed in the CgmAChR-1+PIR, CgmAChR-1+DAMP and CgmAChR-1+DAR groups at 1 h after ACh incubation, while no significant change was observed in the CgmAChR-1+AFDX and CgmAChR-1+PD groups. On the other hand, the concentrations of intracellular

cAMP were also increased dramatically after cell transfection and ACh treatment ($p < 0.05$) (Fig. 3B and Fig. 3C)

3.3. The expression of CgmAChR-1 mRNA in different tissues and in response to LPS stimulation

The qRT-PCR was used to explore the tissue distribution of CgmAChR-1 and the change of its mRNA transcripts responsive to LPS stimulation. The mRNA transcripts of CgmAChR-1 were determined in all the tested tissues including haemocytes, mantle, kidney, gill, gonad, hepatopancreas and adductor muscle. CgmAChR-1 was highly expressed in adductor muscle, whose expression level was 7.06-fold compared with that in haemocytes ($p < 0.05$). CgmAChR-1 was also abundant in hepatopancreas and kidney, and their expression levels were 6.73 and 2.29-fold ($p < 0.05$) compared with that in haemocytes (Fig. 4).

LPS treatment could significantly induce the mRNA expression of CgmAChR-1 in haemocytes (Fig. 5). According to the qRT-PCR assay, one peak (6.05-fold) of CgmAChR-1 expression was observed ($p < 0.05$) at 24 h post stimulation.

3.4. The change of TNF mRNA expression after the treatments with CgmAChR-1 antagonists and LPS

The mRNA expression levels of three oyster TNF genes including CGI_10005109, CGI_10005110 and CGI_10006440 in haemocytes were determined by qRT-PCR (Fig. 6). After the treatments with CgmAChR-1 antagonists and LPS, the

expressions of all the three TNF genes were up-regulated significantly from 3 h to 24 h after stimulation ($p < 0.05$). Higher expression of CGI_10005109 was observed in the antagonists+LPS group at 3 h and 6 h after LPS stimulation ($p < 0.05$), while CGI_10005110 mRNA expression started to increase at 6 h and 12 h in the antagonists incubation group ($p < 0.05$). However, no obvious change of CGI_10006440 expression was detected between the LPS and antagonists+LPS groups ($p > 0.05$).

3.5. Phagocytosis rate and apoptosis index of oyster haemocyte after CgmAChR-1 antagonists incubation and LPS stimulation

In order to explore the possible immunomodulation mediated by CgmAChR-1, the phagocytosis rate and apoptosis index of oyster haemocyte were determined by flow cytometry after 12 h of CgmAChR-1 antagonist incubation and LPS stimulation (Fig. 7A and Fig. 8A). The phagocytosis rate and apoptosis index both increased significantly after LPS stimulation ($p < 0.05$). After an incubation with CgmAChR-1 specific antagonists, the apoptosis index of haemocyte increased dramatically from 13.1% (the LPS group) to 16.5% (the antagonists+LPS group) ($p < 0.05$) (Fig. 8B). However, the phagocytosis rate showed no significant change in LPS and antagonists+LPS groups (Fig. 7B).

4. Discussion

The molecular features and biological functions of the important components

cholinergic nervous system, such as ChAT, AChRs, ChT, ACh, AChE and VAcHT, have been well investigated in vertebrates (Shi et al., 2015), while some of them (such as ACh, AChE and ChAT) have also been identified in nematodes (Sattelle, 2009), molluscs (Burbring et al., 1953; Shi et al., 2012) and arthropods (Sattelle, 2009). For example, the activation of nAChRs could inhibit the innate immune regulation in insects (Czura et al., 2003; Razani-Boroujerdi et al., 2004; Sopori, 2002). So far, the information about the immune regulation of cholinergic nervous system, especially the function that mAChRs mediates in immunomodulation is still very limited in invertebrates (Razani-Boroujerdi et al., 2008).

Here in this research, we amplified the ORF of a mAChR (CgmAChR-1) from *C. gigas*. The full length of CgmAChR-1 was 1983 bp, and it encoded a protein of 660 amino acids. CgmAChR-1 displayed high similarity of 40.7% with that of *Drosophila melanogaster* mAChR. Multiple alignment results suggested that there were four conserved cysteine residues (Cys119, Cys608, Cys614 and Cys627) in CgmAChR-1 with other two mAChRs (Fig. 1). Moreover, according to the phylogenetic tree, CgmAChR-1 was closely matched with M₅ muscarinic receptors found in invertebrates. The molecular features of CgmAChR-1 suggested that it might belong to the GPCRs family with a similar structure as M₅ muscarinic receptor, and it might be critical for calcium signaling, c-fos gene expression, and the immune function of immune cells.

Binding of neurotransmitters to their receptors will trigger the concentration change of various second messengers, relaying signals into downstream pathways

(Fabbri and Capuzzo, 2010). The intracellular fluxes of Ca^{2+} and cAMP were determined in this research to explore the binding activity of CgmAChR-1. Besides, antagonists of M_1 to M_5 muscarinic receptors, including PIR, AFDX, DAMP, PD and DAR, were used in the blocking experiment to ascertain the subtype of CgmAChR-1. The concentration of intracellular Ca^{2+} increased dramatically ($p < 0.05$) after CgmAChR-1 transfection and ACh treatment. Similarly, cAMP level was also significantly up-regulated. The significant increase of intracellular Ca^{2+} and cAMP concentration suggested that CgmAChR-1 should belong to the mAChRs. Moreover, incubation of DAMP and DAR caused a dramatic decrease of intracellular Ca^{2+} concentration, while the concentrations of Ca^{2+} in the CgmAChR-1+PIR, CgmAChR-1+AFDX and CgmAChR-1+PD groups did not change significantly ($p > 0.05$). Such result further indicated that CgmAChR-1 might be M_3 or M_5 subtype of mAChRs. It has been announced that the intracellular Ca^{2+} fluxes would enhance once M_1 , M_3 and M_5 muscarinic receptors were activated (Berridge, 1993; Elaev et al., 1984; Felder, 1995; Hulme et al., 1990). cAMP accumulation also occurred in neuroblastoma cells when the M_3 mAChR pathway was triggered (Baumgold and Fishman, 1988). The phylogeny analysis indicated that CgmAChR-1 might be one of the M_5 muscarinic receptors, and the present biological validation using antagonists further implied that CgmAChR-1 might be M_3 or M_5 subtypes. Collectively, CgmAChR-1 was regarded as a mAChR in oyster, whose function was mediated by intracellular Ca^{2+} and cAMP.

qRT-PCR was employed in the tissue distribution assay of CgmAChR-1 for a

308 better exploration of its physiological role in oyster. In the present study, the mRNA
309 transcripts of CgmAChR-1 were expressed in every detected oyster tissue, suggesting
310 that CgmAChR-1 was the key hub-gene in marine molluscs. Besides, since
311 hepatopancreas is thought to be an important immune tissue in molluscs (Sun et al.,
312 2014b; Wootton et al., 2003), the high CgAChR-1 expression in hepatopancreas
313 indicated that CgmAChR-1 should be critical for the immune regulation in oyster.

314 The circling haemocytes mediates a complex process in which immune and
315 neuroendocrine responses are intermixed to help the lower forms of life successfully
316 live and survive from environments full of potentially agents and substances (Costa et
317 al., 2009; Travers et al., 2008). Haemocytes mediate the immune regulation by
318 neurotransmitters mainly with the involvement of receptors expressed on the cell
319 surface. The mRNA expression level of CgmAChR-1 significantly increased ($p < 0.05$)
320 at 24 h post immune stimulation. Our previous study proved that the release of ACh
321 could be positively induced under LPS stimulation in scallop (Shi et al., 2014). Thus,
322 the increase of both ACh and its receptor might indicate that during the immune
323 regulation, CgmAChR-1 play important role in the cholinergic neuroendocrine system.
324 Besides, when the pathogens are recognized by haemocytes, cytokines are produced
325 to enhance the immunomodulation (Guo et al., 2013). In this research, the recognition
326 of LPS by pathogen recognition receptors was found to enhance the synthesis of
327 cytokines (e.g., TNF- α) and transcription factors (e.g., NF- κ B), and then induce a
328 higher expression of CgmAChR-1, which might up-regulate the immune regulation to
329 accelerate the elimination of pathogens. However, the sustained high-level immune

response would in turn cause immune injury. In order to avoid killing normal cells and maintain homeostasis, more receptors with inhibitory effect such as α -adrenergic receptor (Zhou et al., 2013) will be expressed on the haemocyte surface to make the immunomodulation come down when the pathogens are totally eliminated (Sternberg, 2006). The fall of CgmAChR-1 mRNA expression level at 48 h should be the indication of immune response switching from higher to the normal level. The above results implied that CgmAChR-1 was critical for the immune regulation in oyster and its expression could be regulated to balance the cholinergic modulation during the immune activities.

Appropriate innate immune response and inflammation control depend on the interaction between nervous and immune systems (Pavlov and Tracey, 2005; Tracey, 2002). As the key molecule in cholinergic nervous system, ACh inhibited the production of TNF, (IL)-1 β , IL-6 and IL-18 in invertebrates [7]. Previous experimental evidences have indicated that α 7 nicotinic acetylcholine receptors are required for cholinergic anti-inflammatory signaling (Gallowitsch-Puerta and Pavlov, 2007), but the involvement of mAChRs in such process remains unclear in marine invertebrates. In this paper, the expression of three oyster TNFs (CGI_10005109, CGI_10005110 and CGI_10006440) as well as haemocyte phagocytosis rate and apoptosis index were determined to further understand the immunomodulation mediated by CgmAChR-1. The expression of CGI_10005109 and CGI_10005110 was up-regulated after LPS treatment, and the up-regulation was further enhanced by mAChR antagonist. LPS stimulation could activate the

neuroendocrine system to release neurotransmitters including enkephalin (ENK) (Liu et al., 2015a; Liu et al., 2015b), norepinephrine (NE) (Zhou et al., 2011) and ACh (Shi et al., 2014) to regulate the immune responses through cytokines, such as IL-17 (Xin et al., 2015), IFN- γ (Zhang et al., 2015) and TNF- α (Sun et al., 2014a) in mollusc. The change of TNF expression might due to the effects of neurotransmitter regulation. The up-regulation of TNF after the treatment with antagonist suggested that ACh could inhibit the expression of TNF by binding with CgmAChR-1, which was in coherent with that in mammals. Moreover, phagocytosis rate and apoptosis index both increased significantly after LPS stimulation ($p < 0.05$), and CgmAChR-1 specific antagonists could induce significant increase of apoptosis index, indicating that the blockage of mAChR in oyster could promote TNF expression, and then improve haemocyte apoptosis to regulate cellular response against infection.

In conclusion, an ACh specific receptor CgmAChR-1 was identified from oyster *C. gigas*, which was distributed mainly in gill, adductor muscle and hepatopancreas. LPS could enhance the expression of CgmAChR-1 in haemocytes, while the blockage of CgmAChR-1 using receptor antagonists before LPS stimulation promoted the expression of oyster TNF, resulting in the increase of haemocyte apoptosis index. These results suggested that mAChRs in oyster also played important roles as nAChRs in cholinergic neuroendocrine-immune regulation to protect the host from pathogen infection and maintain homeostasis.

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Figure legend

Fig.1. Multiple alignment of CgmAChR-1 compared with other mAChRs from model species based on their deduced amino acid sequences.

Fig.2. Neighbor-joining (NJ) phylogeny tree of mAChRs from various creatures, such as *H. sapiens*, *D. melanogaster*, and *C. gigas*.

Fig.3. Change of Ca^{2+} and cAMP after muscarinic receptor antagonist incubation and ACh treatment. (A) Ca^{2+} concentration determined by flow cytometry. (B) Concentration change of Ca^{2+} after cell transfection, M_1 to M_5 muscarinic receptor antagonist incubation and acetylcholine stimulation. (C) Concentration change of cAMP after cell transfection, M_1 to M_5 muscarinic receptor antagonist incubation and acetylcholine stimulation.

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397 Fig.4. Tissue distribution of CgmAChR-1 determined by qRT-PCR.

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399 Fig.5. Expression of CgmAChR-1 in oyster haemocytes under LPS stimulation.

400

401 Fig.6. Relative expression of oyster TNF genes (CGI_10005109 (A); CGI_10005110
402 (B) and CGI_10006440(C)) in haemocytes after mAChRs antagonists incubation and
403 LPS stimulation.

404

405 Fig.7. Effects of *in vivo* incubation with CgmAChR-1 antagonist and stimulation of
406 LPS on haemocyte phagocytosis rate. (A) Determination of haemocyte phagocytosis
407 rate by flow cytometry. (B) The overall change of haemocyte phagocytosis rate after
408 the incubation of CgmAChR-1 antagonist and LPS stimulation.

409

410 Fig.8. Effects of *in vivo* incubation with CgmAChR-1 antagonist and stimulation of
411 LPS on haemocyte apoptosis index. (A) Determination of haemocyte apoptosis index
412 by flow cytometry. (B) The overall change of haemocyte apoptosis index after the
413 incubation of CgmAChR-1 antagonist and LPS stimulation.

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Reference

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Crassostrea gigas -----MAGLLTTAPVSDI 13
Homo sapiens ----- 0
Drosophila melanogaster MRSLNKSAGEEELTGLNGNSSDSIYTIKNKWITDWSLRERERDKLCSFIFKRVLTHTECKRLLEAAKIYGESSDFNSWFPVNEQLQWYRNFVNNINLLAV 100

Crassostrea gigas STSVVDMNSTENCTSSSGVTVAADIPFP-----HELWLTII LGVLVAFIVVTVAGNVLVLLSFALERTIRQPTNYFIA 86
Homo sapiens -----MNTSAPPAVSPNITVLAPGKG-----PWQVAFIGITTGLLSLATVTGNLLVLISFKVNTLKTVMNYFLL 65
Drosophila melanogaster RNQSSDGGSTMSGSSDSEILGPVLFP-----FALWQTIILIAICLAICILTVGGNILVLLAFIVDRNIRQPSNYFIA 173

Crassostrea gigas SLAVS^{*}DL^{*}LIGSFSMP^{*}CF^{*}TL^{*}YLL^{*}LG-YWFLGELVCDLWLALDWTVCLASQYTVFFITVDREFSVKIPAKYRHWRT^{*}EKKVVTMVSLTWIFPTLIFFTSIVGW 185
Homo sapiens SLACADLIIGTFSMNLYTT^{*}YLLMG-HWALGTLACDLWLALDYVASNASVMNLLISFDRYFSVTRPLSYRAKRTFERRAALMICLAWLVSVFLWAPAILFW 164
Drosophila melanogaster SLAATDMLIGTVSM^{*}PFY^{*}TIYVLKG-YWDLGPMLCDLWLSVDYTVCLVSQYTVLLITIDRYCSVKIAAKYRSWRT^{*}TRVIYMV^{*}TI^{*}WI^{*}I^{*}PALLFFISIFGW 272

Crassostrea gigas QYFVGK-RTVKPE^{*}QCEVQFM^{*}SDPLE^{*}FT^{*}LLTIGYYWIEPCVMIGLYGGIYYVALT^{*}LQWKAEAKHKMTTAMELSKNKQSSKS-NRNASGADNNTLGTENN 283
Homo sapiens QYLVGE-RTVLAGQCYIQFLSQPIITFGTAMAAFYLPVTVMCTLYWRIYRETENRARELAALQGSSETPGKG-----GG 236
Drosophila melanogaster EHFTGK-RDLLPGQCAVQFLKDP^{*}IENTALIIGYYWTT^{*}LI^{*}VL^{*}FVLYAGIYK^{*}TAYDMQKRSEAKQ^{*}RKM^{*}QSMVALSAGAMSGMAGHAAGIGVIEEKILKTKVE 371

Crassostrea gigas GR^{*}MRPDEST^{*}SFTKAP-----EEERS^{*}SSSPVFESDEENNSSQGACGSSKTHFRHADVDDDEQTCFVNSAVQTDTTYRPSLK 358
Homo sapiens SSSS-----SERSQP-----GAEGSPETPPGRCCRCRAPRLQAYSWKEEEED----EGSMESLTS-----SE 292
Drosophila melanogaster LAGDQTDLDSACTTVI-----KRLSGSGQANPLAVATEEVEKMTPEQRRASAAKIQEEAKKREAAVDAEKSSSSPAFD 446

Crassostrea gigas GMLGMSFNHVS^{*}KIALTITGTSGSETETDGNEDTNATKALIKEPPPPYNHVVPNTYKASKQTDKLLENDVLEGCKYIEEDSLKTLTSNDNLKIYPDGSIS 458
Homo sapiens GEE-PG---SEVVI-KMPMVDPEAQA----- 313
Drosophila melanogaster SDEESSVNQAQQ^{*}LITQQKLNNMRKRSSIGLVFGAQAALLATRGKIEDFAKVRRES^{*}CIEAICLLDVPGKLAADCPHRRASSPMETSSKDAILYSEMPMP 546

Crassostrea gigas FDATIDESCSFVWKRREGRYLPIPIAED-----SSNTISTTLEGDSSIRHASNDDEMSKRI^{*}PSNGTS^{*}AVHSRDRNEDEDVDS^{*}DANREKPSIMAPLQNLV 552
Homo sapiens -----PTKQPP--RSSPNTVKRP^{*}TKK-----G 333
Drosophila melanogaster LSPKVK^{*}TSTPQITGTPQSAAGVTPPVPLERIEERETS^{*}SDSNTNKMPSTSGTSS^{*}TGGVGGGGEQDGVGATKKNAGDD^{*}DANREEKSLAASRNSSKRAFIHSIG 646

Crassostrea gigas QNMR^{*}AKRKK--SKTRQ-----KVNSKTENRARKA^{*}RTIT^{*}IFILGAYILCWTPYHIMVLIIGVCDG-GWNCVNMKLYQFTYWL^{*}CYLNSPI 633
Homo sapiens RDRAGKGQKPRGKEQLA-----KRKTFSLVKEKKAARTLSAILLAFILTWTPYNIMVLVSTFCK---DCVPETLWELGYWLCYVNSTI 413
Drosophila melanogaster KHFKSKKALPLILGVGG-----RQKSKSEN^{*}RARKA^{*}FR^{*}ILSFILGCEVACWTPYHVLALVEGFCR--HPFCINEHLYMFSYELCYANSPM 728

Crassostrea gigas NPFCYALANQQFKETFLRILRLDCHKT----- 660
Homo sapiens NPMCYALCNKAFRDTERLLLLCRWDKRAWRKIPKRPGS---VHRTPSRQC 460
Drosophila melanogaster NPFCYALANQQFKETFLRILKGD^{*}LHMT----- 755

Figure 1

Figure 2

A

B

C

Figure 3

Figure 4

Figure 5

Figure 6

Figure 7

Figure 8

Table 1

Sequences of the primers used in the experiment.

Primer	Sequence (5'-3')	Sequence information
P1 (forward)	ATGGCTGGACTATTGACCAC	CgmAChR-1 specific primer
P2 (reverse)	TTATGTCTTATGACAGTCCAATCTC	CgmAChR-1 specific primer
P3 (forward)	TTGAGAACGATGTATTAGAAGG	Real-time CgmAChR-1 primer
P4 (reverse)	GTAGATACCTTCCCTCTCGCCG	Real-time CgmAChR-1 primer
P5 (EF-RTF)	AGTCACCAAGGCTGCACAGAAAG	Real-time CgEF primer
P6 (EF-RTR)	TCCGACGTATTTCTTTGCGATGT	Real-time CgEF primers
P7 (forward)	CGCAATGGTCGCTTGGTGGTC	Real-time TNF (CGI_10005109) primer
P8 (reverse)	CGTAGGGGCGGAAGGTCTCG	Real-time TNF (CGI_10005109) primer
P9 (forward)	CAACGGTCTAACTTACCATCCAAAC	Real-time TNF (CGI_10005110) primer
P10 (reverse)	TGGTGGTAGATAAAATGGGACAGTG	Real-time TNF (CGI_10005110) primer
P11 (forward)	ATTGGAGCACCTGGAGGATAAG	Real-time TNF (CGI_10006440) primer
P12 (reverse)	CAGTCTTCCGTGCTGGTATTTTC	Real-time TNF (CGI_10006440) primer
M13-47	CGCCAGGGTTTTCCCAGTCACGAC	pMD18-T simple vector primer
RV-M	GAGCGGATAACAATTTACACAGG	pMD18-T simple vector primer
P13 (forward)	GGCCACGCGTCGACTAGTACT ₁₇	Oligo(dT)-adaptor