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The immunomodulation of nicotinic acetylcholine receptor subunits in Zhikong scallop *Chlamys farreri*

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ABSTRACT

Nicotinic acetylcholine receptor (nAChR), the best-studied ionotropic neuron receptor protein, is a key player in neuronal communication, and it has been reported to play an important role in immunomodulation of vertebrates. Although nAChRs have also been identified in most invertebrates, the knowledge about their immunomodulation is still limited. In the present study, two scallop nAChR genes were identified from *Chlamys farreri* (designed as *Cf*nAChR1 and *Cf*nAChR2), which encoded 384 and 443 amino acids, respectively. The conserved disulfide-linked cystines, ion selectivity residues and the hydrophobic gating residues (L251, V255 and V259) were identified in *Cf*nAChR1 and *Cf*nAChR2. The immunoreactivities of *Cf*nAChR1 and *Cf*nAChR2 were observed in all the tested scallop tissues, including adductor muscle, mantle, gill, hepatopancreas, kidney and gonad. After LPS (0.5 mg mL^{-1}) stimulation, the expression of *Cf*nAChR1 mRNA in haemocytes increased significantly by 9.83-fold ($P < 0.05$) and 12.93-fold ($P < 0.05$) at 3 h and 24 h, respectively. While the expression level of *Cf*nAChR2 mRNA increased 43.94% at 12 h after LPS stimulation ($P < 0.05$). After TNF-α (50 ng mL^{-1}) stimulation, the expression levels of *Cf*nAChR1 and *Cf*nAChR2 both increased significantly at 1 h, which were 21.33-fold ($P < 0.05$) and 2.44-fold ($P < 0.05$) of that in the PBS group, respectively. The results collectively indicated that the cholinergic nervous system in scallops could be activated by immune stimulations through *Cf*nAChR1 and *Cf*nAChR2, which function as the links between the cholinergic nervous system and immune system.

1. Introduction

Nicotinic acetylcholine receptors (nAChRs) are prototypical members of the Cys-loop ligand-gated ion channel (cysLGIC) superfamily which also includes ionotropic receptors for GABA, glycine and serotonin (5-hydoxytryptamine) [1,2]. nAChRs mediate the fast actions of acetylcholine (ACh) in the nervous system, and act as molecular switches which change conformation upon binding to an agonist such as ACh to allow a net influx of ions into the cell [3,4].

Since the nAChR subunit was reported firstly from the electric rays *Torpedo californica* and *Torpedo marmorata* in the early 1980s [5], nAChRs subunit have been identified in most vertebrates and invertebrates, such as mammals, arthropod, mollusk and nematode. In mammals, there are eight α (α2-7 and α9-10) and three β (β2-4) nAChR subunits, which are selectively assembled into distinct type of nAChRs with different pharmacology, cation conductance and cellular localization [6,7]. In arthropods, 10 nAChR subunits have been identified in insects, and the nAChR subunits in *Drosophila melanogaster* are commonly referred to as Dα1-Dα7 and Dβ1-Dβ3 [8–10]. In mollusk, majority of nAChRs have been identified in gastropods, and 12 subunits have been observed in the central nervous system of *Lymnaea stagnalis*, of which 10 are α-type, one is β-type, and one has not been classified because of insufficient sequence information [11]. In nematodes, 27 nAChR genes have been identified by genomic sequence analysis, which are divided into five classes: UNC-29, UNC-38, ACR-8, ACR-16 and DEG3 [12,13]. All the nAChRs share the basic structural features in

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vertebrates and invertebrates. They were composed of five homologous subunits which arranged around a central ion channel, and each subunit has four transmembrane domains (TM1-4) and possesses an N-terminal extracellular domain, and the extracellular domain contains the characteristic Cys-loop motif consisting of two disulfide bond-forming cysteines separated by 13 amino acid residues [3,14]. The Cys-loop plays crucial role in nAChR assembly as well as the kinetics of ion channel gating [15,16].

nAChRs in vertebrates involved in a series of physiological functions through binding ACh, such as development, synaptic plasticity, learning, memory, immunity, and attention [17–22]. Decline, disruption, or alterations of nicotinic cholinergic regulation network would contribute to dysfunctions such as epilepsy, schizophrenia, Parkinson's disease, autism, dementia with Lewy bodies, Alzheimer's disease, inflammation and addiction [21–25]. In invertebrates, nAChRs play crucial roles in escape behaviours, learning, memory and olfactory in insects [26–28]. The nAChRs might be involved in ciliary movement [29,30], heart beat [31], rectum twitch [31] and immunomodulation in mollusks [32]. And the nAChRs are reported to involve in many behaviours directly or indirectly, including locomotion, egg laying, feeding and male mating in nematode [33]. Furthermore, experimental studies have established a strong interconnection between nACRs and inflammation in vertebrates. In mammals, α7 nicotinic acetylcholine receptor (nAChRα7) has been confirmed to be the key component in cholinergic anti-inflammatory pathway, which can control the inflammatory reflex through modulating the production of proinflammatory cytokines from immune cells [34]. The immunomodulation of nAChRs has been reported in invertebrates. For example, the nicotine responses were found to be dependent on nAChRs, and nicotine could affect the immunosuppressive of in nematodes [35–37]. In mollusk, although nAChRs have also been identified in *Aplysia californica* [38] and *Lymnaea stagnails* [11,39,40], the knowledge about their immunomodulation is still limited in mollusk.

The scallop *Chlamys farreri* is one of important mollusk species cultured widely in the northern coastal provinces of China. The investigation of nAChRs in *C. farreri* will help further cognizing the cholinergic nervous system in invertebrate, as well as its modulation in the innate immune system. The main objective of this study were (1) to characterize the structural feature of nAChRs from *C. farreri* (designated as *Cf*nAChR1 and *Cf*nAChR2), (2) to examine the expression of *Cf*nAChR1 and *Cf*nAChR2 in the major tissues, and (3) to investigate the responses of *Cf*nAChR1 and *Cf*nAChR2 in haemocytes against immune stimulation (LPS and TNF-α) to better understand their roles of immunomodulation.

2. Materials and methods

2.1. Animals, stimulations and sample collection

Zhikong scallops *C. farreri* (averaging 55 mm in shell length) were collected from a commercial farm in Qingdao, China, and acclimatized in filtered seawater at 15 °C for 10 days before proceeding.

One hundred and sixty scallops were employed and randomly divided into four groups to receive the injections of 50 μL phosphate buffered saline (PBS, 377 mmol L^{-1} NaCl, 2.7 mmol L^{-1} KCl, 8.09 mmol L^{-1} Na_2HPO_4, 1.47 mmol L^{-1} KH_2PO_4, pH 7.4, osmolarity 780 mOsm L^{-1}), 50 μL LPS from *Escherichia coli* 0111:B4 (Sigma Aldrich, 0.5 mg mL^{-1} in PBS), and 50 μL recombinant human TNF-α (Sigma Aldrich, 50 ng mL^{-1} in PBS), respectively. Eighty scallops received PBS injection were chosen as control group, while the other two groups were employed as LPS stimulation group and TNF-α stimulation group, respectively. These scallops were return to water tanks after treatment, and 6 individuals were randomly sampled at 3, 6, 12, 24, 48 and 96 h post-injection from the control and LPS stimulation groups. For the TNF treatment experiment, 6 individuals were randomly sampled at 1, 3, 6, 9 and 12 h post-injection from the PBS control and TNF-α stimulation groups. The haemolymph was collected, and centrifuged at 800 × g, 4 °C for 10 min to harvest the haemocytes. All these samples were stored at −80 °C after addition of 1.0 mL TRIzol reagent (Invitrogen) for subsequent RNA extraction.

Six scallops without any treatment were sampled at 0 h along with the LPS and TNF-α experiments, and they were employed as the blank group. The haemocytes were harvested for subsequent RNA extraction, while the adductor muscle, mantle, gill, hepatopancreas, kidney and gonad of scallops were collected and fixed in Bouin's fluid for tissue slide.

2.2. Cloning the full-length cDNA of CfnAChR1 and CfnAChR2

BLAST analysis of scallop EST sequences revealed that two contigs (GI: 115429102 and 31903080) were homology to the other identified nAChRs. Sense primers P1, P2, P3 (P7, P8, P9) and reverse primers P4, P5, P6 (P10, P11, P12) were designed based on the EST sequences to clone the full-length cDNA of *Cf*nAChR1 (*Cf*nAChR2) by rapid amplification of cDNA ends (RACE) approach (Table 1). PCR amplifications to clone the 3′ ends of *Cf*nAChR1 and *Cf*nAChR2 were carried out using sense primer P1, P2, P3, P7, P8 or P9 and antisense primer Oligo(dT)-adaptor P13, while sense primer Oligo(dG)-adaptor P14 and antisense primer P4, P5, P6, P10, P11, P12 were used to get the 5′ end according to the Usage Information of 5′ RACE system (Invitrogen) (Table 1). All PCR amplifications were performed in a PCR Thermal Cycle (TAKARA, GRADIENT PCR).

The PCR products were gel-purified and cloned into pMD18-T simple vector (Takara, Japan). After being transformed into the competent cells of *E. coli* Top10F, the positive recombinants were identified through anti-Ampicillin selection and PCR screening with sense vector primer RV-M and antisense vector primer M13-47 (Table 1). The positive clones were sequenced on an ABI 3730 XL Automated Sequencer (Applied Biosystems). The sequencing results were verified and subjected to cluster analysis. PCR amplification with sense primer P15 and P17, and antisense primer P16 and P18 (Table 1, designed according to *Cf*nAChR1 and *Cf*nAChR2 ORF sequence) were performed to verify the LBD domain of *Cf*nAChR1 and *Cf*nAChR2, respectively.

2.3. Sequence analysis

The similarity searches were performed with the BLAST program at the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/blast). The deduced amino acid sequence was analyzed with the Expert Protein Analysis System (http://www.expasy.org). SignalP 3.0 program was utilized to predict the presence and location of signal peptide, and the cleavage sites in amino acid sequences (http://www.cbs.dtu.dk/services/SignalP). The protein domain, transmembrane domain (TM), phosphorylation sites and possible intramolecular disulfide were predicted by Simple Modular Architecture Research Tool (SMART) version 5.1 (http://smart.Embl-heidelberg.de/), TMHMM Server 2.0, NetPhos, and DiANNA 1.1 web server (http://clavius.bc.edu/~clotelab/DiANNA/), respectively. Multiple sequence alignment of *Cf*nAChR1 and *Cf*nAChR2 with other nAChRs were performed with the ClustalW multiple alignment program (http://www.ebi.ac.uk/clustalw/) and multiple alignment show program (http://www.biosoft.net/sms/index.html). An unrooted phylogenic tree was constructed based on the deduced amino acid sequences of *Cf*nAChR1, *Cf*nAChR2 and other known nAChRs by the Neighbor-Joining (NJ)