Developmental and Comparative Immunology 65 (2016) 139–148

Contents lists available at ScienceDirect

Developmental and Comparative Immunology

journal homepage: www.elsevier.com/locate/dci

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

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ARTICLE INFO

Article history:
Received 21 April 2016
Received in revised form
3 July 2016
Accepted 4 July 2016
Available online 6 July 2016

Keywords:
Crassostrea gigas
Acetylcholine
Muscarinic acetylcholine receptor
TNF pathway
Immune regulation

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 M_5 muscarinic receptor found in invertebrates including *Habropoda laboriosa*, *Acromyrmex echinatior* 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.

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, 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

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http://dx.doi.org/10.1016/j.dci.2016.07.003
0145-305X/

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 $G_{q/11}$, 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 $G_{i/o}$, 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 (See Table 1).

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-M_1 expression vector (TransGene), designating as pEASY-M_1-CgmAChR-1 plasmid. When the cell coverage ratio reached 60–80% in the wells, the recombinant plasmid was transfected with Lipofectamine LTX and Plus™ Reagent (Invitrogen). After being cultured for 24 h, the cells were digested and collected for the second messenger determination.

In the drug treatment assay, 500 μL suspension (about 10^4 cells) of HEK293T cells transfected with pEASY-M_1-CgmAChR-1 was added into the 24-well plate with 50 μL 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 μL of 10.0 μmol L^{-1} PIR was added to incubate the cells. And 3 h later, 50 μL of ACh was then added. Similarly, specific antagonist AFDX, DAMP, PD and DAR for M_2 to M_5 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 μmol L^{-1}) was employed in the examination of intracellular Ca^{2+}, and the cAMP Direct Immunoassay Kit (ab65355, Abcam, Cambridge, UK) was