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Molecular and cellular analyses of a ryanodine receptor from hemocytes of *Pieris rapae*

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ABSTRACT

Ryanodine receptors (RyRs) are located in the sarcoplasmic/endoplasmic reticulum membrane and are a distinct class of ligand-gated calcium channels controlling the release of calcium from intracellular stores. Intracellular calcium level has a definite role in innate and adaptive immune signaling. However, very few information are accessible about calcium transients of invertebrate immunocytes, especially of insect hemocytes, the effector cells of insect immunity. In this study, we show that the RyR-stimulating agent flubendiamide inhibit hemocyte spreading and phagocytosis in the cabbage white butterfly, *Pieris rapae*. Furthermore, we cloned a cDNA encoding a ryanodine receptor (*PrRyR*) from the hemocytes of *P. rapae*. It encodes 5107 amino acids with a predicted molecular weight of 578.2 kDa. PrRyR shares a common feature with known RyRs: a well-conserved COOH-terminal domain with two consensus calcium-binding EF-hands and six transmembrane domains, and a large hydrophilic NH_2-terminal domain. In the larval stage, *PrRyR* was highly expressed in epidermis tissue and also expressed in hemocytes at a moderate level. In the adult stage, *PrRyR* was expressed at high levels in thoraces and legs, while low levels in abdomens and antennae. Quantitative real-time PCR analysis showed that its expression did not display any significant change in response to bacterial challenge. Western blot analysis and immunohistochemistry assay displayed that PrRyR was detected and presented on hemocytes. We also showed that flubendiamide, a RyR-activating insecticide, induced Ca^{2+} release and thereby confirmed functional expression of the PrRyR in the hemocytes of *P. rapae*.

1. Introduction

Calcium (Ca^{2+}) is one of the most ancient molecules and a common second messenger that regulates many cellular processes including contraction, secretion, synaptic transmission, fertilization, nuclear pore regulation and transcription of cells (Fill and Copello, 2002). Ca^{2+} is also involved in immune response (Oh-hora and Rao, 2008; Vig and Kinet, 2009). In vertebrates, Ca^{2+} signaling is essential in the activation of cellular components of innate and acquired immune responses. Ca^{2+} signals control proliferation, differentiation, apoptosis, and a variety of transcriptional programs in cells of the immune system, including T cells, B cells, mast cells, and many other cell types (Hogan et al., 2003; Hogan and Rao, 2007; Oh-hora and Rao, 2008). The importance of intracellular free Ca^{2+} levels in mammalian signal transduction pathways is well described (Case et al., 2007; Oh-hora and Rao, 2008). Invertebrate species have also evolved similar mechanisms for regulating their biochemical pathways by conserved molecular components (Crozatier and Meister, 2007). Indeed, Ca^{2+} is also involved as a second messenger in intracellular signaling of invertebrates, especially in insects and mollusk species (Whitaker, 2006).

There are two families of Ca^{2+} release channels: the ryanodine receptors (RyRs) and inositol trisphosphate receptors (IP_3Rs) (Fill and Copello, 2002). Although inositol trisphosphate (IP_3) is a key messenger regulating $[Ca^{2+}]_i$, some reports have postulated the possibility that RyRs contribute to the IP_3-insensitive component of Ca^{2+} signaling in immune cells (Bourguignon et al., 1995; Guse et al., 1999; Hakamata et al., 1994; Hosoi et al., 2001). There are three isoforms of RyR proteins (RyR1, RyR2 and RyR3) in mammals. RyR1 and RyR2 were first found in skeletal muscles and cardiac muscles, respectively. RyR3, previously referred to as the brain isoform, was expressed in many tissues including the diaphragm and brain (Hakamata et al., 1992; Lanner et al., 2010; Otsu et al., 1990; Takeshima et al., 1989). They are similar in primary structure with 66% homology at the amino acid level, except for three regions with high degrees of variability (Sorrentino and Volpe, 1993). By contrast, only one RyR encoding gene is present in *Drosophila melanogaster*, which shares only 47% homology with the mammalian isoforms of RyRs (Takeshima et al., 1994; Xu et al., 2000). Because of this, insect RyR plays an important role in developing novel and selective insecticides. Recently two classes of synthetic chemicals have emerged resulting in commercial insecticides that target

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Table 1
The primers used in this study.

Primers	Primer sequence	cDNA position in the coding area (bp)
For cDNA fragment cloning		
PrRyR-F1	5′-ATGGGGGGCTAAACATCGTTGTAT-3′	716–739
PrRyR-R1	5′-ATTTCCGCTTCACTTGTCGACCGGTT-3′	4207–4232
PrRyR-F2	5′-GATGAAGGAAGCGGTTGAAGCCCA-3′	3993–4016
PrRyR-R2	5′-TCTTTGAGAGAGAATAGTCGGGCGGA-3′	8412–8437
PrRyR-F3	5′-AAAGAACCGTATGCTGACAAAGGG-3′	8046–8069
PrRyR-R3	5′-ACGCCTCCAACTTCTCCTTGTCAC-3′	13,049–13,072
PrRyR-F4	5′-GTTTCCTGGAAACCGCCGGTTCAGT-3′	12,856–12,883
PrRyR-R4	5′-AGCTCACCGAACGCATCAATGATCAA-3′	15,010–15,035
For RACE-PCR cloning		
5′RACE outer primer	5′-CATGGCTACATGCTGACAGCCTA-3′	
5′RACE inner primer	5′-CGCGGATCCACAGCCTACTGATGATCAGTCGATG-3′	
3′RACE outer primer	5′-TACCGTCGTTCCACTAGTGATTT-3′	
3′RACE inner primer	5′-CGCGGATCCTCCACTAGTGATTTCACTATAGG-3′	
5′RACE-A	5′-GATGCTGTTGTTGCTTCCTCTCTGCT-3′	907–932
5′RACE-B	5′-ATGACAGTGGAGTCTCCATACCTGAT-3′	1018–1043
3′RACE-A	5′-GGAAGTGGAGAGGGCAGCGGTGAAA-3′	14,062–14,086
3′RACE-B	5′-CCTGATGGAGACGATTCGGAGGTGTAC-3′	14,917–14,943
For real-time PCR		
PrRyR-F	5′-AGAGGGAACTGGGATGC-3′	1680–1696
PrRyR-R	5′-ACCTGACAGCGACACCA-3′	1845–1861
18SrRNA-F	5′-ACAATTGGAGGGCAAGTCTG-3′	
18SrRNA-R	5′-CACCGCGATAGGATTTTGAT-3′	

zinsect RyRs: the phthalic acid diamides flubendiamide, and the anthranilic diamides chlorantraniliprole (Sattelle et al., 2008). The action of flubendiamide is highly specific with selective toxicity to lepidopterous insect pests (Tohnishi et al., 2005). By contrast, chlorantraniliprole induces Ca^{2+} response not only in lepidopterous insects but also in mammals at concentrations exceeding 10 μM (Lahm et al., 2007). Hence, flubendiamide is regarded as a better experimental tool for exploring the physiological effects of insect RyR activity, even though the exact binding sites for flubendiamide have not yet been identified (Kato et al., 2009). The sequence and distribution of several insect RyRs have been documented (Guo et al., 2012; Wang et al., 2012a,c), however, little is known about the immunological role of insect RyRs in insect hemocytes.

In the present study, we report cloning and characterization of a gene encoding ryanodine receptor (*PrRyR*) in hemocytes of the cabbage white butterfly, *Pieris rapae*. Considering various roles of RyRs in physiological processes, PrRyR expressed in the hemocytes might play an important role in insect immune response. *PrRyR* mRNA was constitutively expressed in several tissues, including hemocytes. Real-time PCR analysis showed that PrRyR mRNA in hemocytes was not significantly up-regulated by the challenge of the bacterium, *Escherichia coli*. Western blot analysis and immunohistochemistry assay confirmed that the protein of PrRyR existed in *P. rapae* hemocytes. Finally, to verify the functional expression of the PrRyR, Ca^{2+} release by RyR-stimulating agent was assessed using *P. rapae* hemocytes *in vitro*. A global view of RyR expression in insect hemocytes was addressed.

2. Materials and methods

2.1. Insects and antibodies reagents

The larvae of *P. rapae* were collected primarily from cabbage fields in the experimental farmland of Zhejiang University, Hangzhou, China. The laboratory colony of *P. rapae* was maintained as described previously (Zhang et al., 2005). Briefly, *P. rapae* larvae were fed on fresh cabbage leaves in a stainless steel-mesh cage (55 cm × 55 cm × 55 cm, 1.0 mm × 1.0 mm mesh) at 25 ± 1 °C with a photoperiod of 10:14 h (light:darkness).

Bioinformatics show that the insect RyR gene is highly conserved, such as *P. rapae* and *Plutella xylostella*. Comparison analysis of the predicted amino acid sequences between PrRyR (NCBI sequence viewer, accession No.: JX481776) and *P. xylostella* RyR (PxRyR) (NCBI sequence viewer, accession No.: AER25354) showed 90% identity and 94% positivity in amino acid sequences. Polyclonal antibodies against PxRyR, which was produced by a 19 amino acid peptide (LRARAILRSLVPLEDLQGV) (Guo et al., 2012), which are also well conserved in PrRyR, were kindly offered by Prof. Pei Liang (Department of Entomology, China Agricultural University, Beijing, China). Flubendiamide (Dr. Ehrenstorfer, Augsburg, Germany) dissolved in dimethyl sulfoxide (DMSO) was diluted to its tested concentration in Ringer's buffer.

2.2. In vitro hemocyte-spreading assay

The bioassay *in vitro* was modified from an established method (Huang et al., 2012). Briefly, the fourth instar larvae of *P. rapae* were surface-sterilized using 70% ethanol. Total hemolymph was collected in Grace's medium (1:10, v/v; Invitrogen, Carlsbad, CA) by cutting its proleg. Hemocyte monolayer was prepared with 50 μl of hemocyte suspension (≈5×10^3 cells) containing 5 μl of test chemical (flubendiamide) or solvent (control) and this mixture was incubated in a 96 well tissue culture plate (Nunc, Roskilde, Denmark) for 30 min at 26 °C. Spreading granular cells and plasmatocytes were determined by counting the number of cells that displayed cytoplasmic expansion under the phase contrast microscope at 400× magnification. The proportion of hemocytes that spread was scored by counting 200 cells from 10 randomly chosen fields.

2.3. In vitro hemocyte-phagocytosis assay

The assay for phagocytosis was performed according to the method described by (Huang et al., 2012). The monolayers of hemocytes were prepared as described above for the spreading assays. After washing each well three times with Grace's medium, 45 μl (≈5×10^5 bacterial cells) FITC-labeled *E. coli* suspension (Invitrogen) was added to 5 μl of the test chemical. After incubation in darkness for 30 min in a moist chamber, the monolayers