Molecular Immunology 47 (2010) 2047–2056

Contents lists available at ScienceDirect

Molecular Immunology

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

Calcium is required for coelomocyte activation in earthworms

Balázs Opper[a,b], Péter Németh[a], Péter Engelmann[a,*]

[a] Department of Immunology and Biotechnology, Clinical Center, University of Pécs, Szigeti u. 12, H-7643 Pécs, Hungary
[b] Department of Anatomy, Faculty of Medicine, University of Pécs, Szigeti u. 12, H-7643 Pécs, Hungary

ARTICLE INFO

Article history:
Received 10 February 2010
Received in revised form 12 April 2010
Accepted 13 April 2010
Available online 1 May 2010

Keywords:
Innate immunity
Coelomocytes
Ca^{2+} influx
Flow cytometry
Ca^{2+} ATPase
Lectins

ABSTRACT

The role of calcium signaling in activation of both innate and adaptive immunity is basically important, however, the evolutionary aspects are not clarified yet. Currently limited data are available about calcium levels of coelomocytes, cellular mediators of earthworm immunity. We aimed to observe basal and induced Ca^{2+} levels of coelomocyte subgroups after various stimulations in *Eisenia fetida* and *Allolobophora caliginosa* using a Ca^{2+}-sensitive dye. *E. fetida* chloragocytes had the highest basal Ca^{2+} levels among subpopulations; however there was no detectable Ca^{2+} influx after any stimuli, while coelomocytes showed strong Ca^{2+} increase after ionomycin treatment, which could be attenuated using phorbol ester. *A. caliginosa* coelomocytes showed a weak response to ionophore, while chloragocytes, similar to those in *E. fetida*, exhibited no changes after this stimulation. Intracellular calcium is mainly stored in the endoplasmic reticulum of coelomocytes as proved by thapsigargin treatments. Among several mitogens only phytohemagglutinin caused increased Ca^{2+} level in *E. fetida* coelomocytes, but not in *A. caliginosa* coelomocytes. Moreover, the chemoattractant fMLP revealed calcium influx of *Eisenia* coelomocytes. For the first time we observed various basal Ca^{2+} levels and sensibility to Ca^{2+} influx inducers (including mitogens and chemoattractant) of coelomocyte subgroups using flow cytometry. These observations suggest that Ca^{2+} influx and signal transduction may play crucial roles in the innate immunity of the earthworm.

1. Introduction

Calcium acts as a second messenger in a variety of cellular processes (Carafoli, 2002, 2005). Transient variations of cytoplasmic Ca^{2+} are necessary for cell activation and to ensure homeostasis (Case et al., 2007). Ca^{2+} is also involved in various processes including muscle contraction (Panfoli et al., 1999), reproduction (Whitaker, 2008), and immune response (Oh-hora and Rao, 2008; Vig and Kinet, 2009). The importance of intracellular free calcium levels in mammalian signal transduction pathways is well described (Case et al., 2007; Langenbacher and Chen, 2008; Oh-hora and Rao, 2008).

Calcium signaling is essential in the activation of cellular components of innate and adaptive immune response in vertebrates. The increase of intracellular calcium level will be followed by the activation of many signaling pathways resulting chemotaxis, degranulation, phagocytosis, production of cytokines and proliferation.

Invertebrate species have evolved similar mechanisms for regulating their biochemical pathways by means of evolutionarily conserved molecular components (Crozatier and Meister, 2007). Indeed, calcium is involved as a second messenger in intracellular signaling of invertebrates (Whitaker, 2006); however, the role of calcium has been investigated mainly in insect and mollusk species (Burlando et al., 2001; Whitaker, 2006). An *in vitro* approach has demonstrated that cytosolic calcium induces activation of phospholipase A2 in mussel hemocytes (Marchi et al., 2004). Heavy metals induce alteration of calcium homeostasis in mussel hemocytes and earthworm coelomocytes (Homa et al., 2007; Marchi et al., 2004; Viarengo et al., 1993).

Earthworms became a model for comparative immunologists by demonstrating graft rejections through transplantation experiments (Cooper, 1968, 1969, 1970). These pioneer experimental results showed the existence of self/non-self recognition and immune mechanisms, before more extensive analyses of immunity were even initiated. Numerous studies on humoral and cellular properties of earthworm immune systems have been published (reviewed in Bilej et al. (2000) and Cooper et al. (2002)). Based on their ultrastructural and cytochemical properties, three main populations of earthworm leukocytes, so called coelomocytes (immune cells of body cavity), can be defined and confirmed: granular, hyaline coelomocytes and chloragocytes (Cooper et al., 2002). Our own flow cytometric measurements have likewise identified three different populations of coelomocytes (R1, R2, R3), which

Abbreviations: CCF, coelomic cytolytic factor; EFCC, *Eisenia fetida* coelomocyte clusters; LBSS, *Lumbricus* balanced salt solution; TG, thapsigargin.

* Corresponding author at: Department of Immunology and Biotechnology, Clinical Center, University of Pécs, Pécs, H-7643, Szigeti u. 12, Hungary. Tel.: +36 72 536 288; fax: +36 72 536 289.
E-mail address: peter.engelmann@aok.pte.hu (P. Engelmann).

0161-5890/$ – see front matter © 2010 Elsevier Ltd. All rights reserved.
doi:10.1016/j.molimm.2010.04.008

correspond to these previously identified coelomocyte populations (Engelmann et al., 2002). Recently, a lipopolysaccharide (LPS)-, β-1,3-glucans-, muramic acid-, and *N*,*N*'-diacetylchitobiose-binding protein named coelomic cytolytic factor (CCF) has been identified and characterized. This molecule is a pattern recognition receptor (PRR) present in earthworm's coelomic fluid and coelomocytes. Following binding to pathogen associated molecular patterns (PAMPs), CCF triggers the prophenoloxidase cascade, an important invertebrate immune mechanism (Beschin et al., 1998, 1999; Bilej et al., 1998, 2001, 2006; Bloc et al., 2002) and also exerts opsonising properties (Bilej et al., 1995) that aid in phagocytosis. The prophenoloxidase (proPO) system is well known from analyses of other invertebrates, mainly arthropods (insects, crustaceans) (Cerenius et al., 2008). These multiple biological activities point to a crucial role of CCF in innate immune reactions in earthworms and evidence accumulates that lectin-like interactions serve as initial recognition events. So far, it is known that CCF has unique antigen recognition characteristics; although non-self recognition by CCF may accelerate various signal transduction pathways still unknown in annelids. There are few data available concerning cytosolic calcium levels of earthworm coelomocytes and the effect of ionophores or mitogens. Recently, an evolutionary conserved calcium-binding protein the calreticulin was cloned and characterized from *Eisenia fetida* earthworms. Calreticulin was highly expressed in various earthworm tissues including coelomocytes (Kauschke et al., 2001; Šilerová et al., 2007). With these observations in mind it is essential to clarify the role of calcium in earthworm leukocytes. This is fundamental in order to augment understanding of earthworm immune responses.

Our aim in this present study was to measure intracellular calcium levels of coelomocyte subpopulations and to reveal the role of calcium in coelomocyte activation. Recently, we have characterized three subpopulations of peripheral coelomocytes using specific monoclonal antibodies. Two of these subpopulations are the immune effector coelomocytes (Engelmann et al., 2005). However during measurements of intracellular calcium, we observed no significant differences between these two subgroups and thus included them as one population for calcium measurements. In the following experiments we tested how Ca^{2+} ionophores like ionomycin effect the intracellular calcium levels. Furthermore, we applied various mitogens (plant lectins and lipopolysaccharide) used for leukocyte stimulations and also the bacterial formyl peptide to test their calcium mobilization in earthworm immune-competent cells.

2. Materials and methods

2.1. Coelomocyte extrusion

Prior to experimental procedures earthworms were placed on moist paper towels for at least two days until there was no more visible gut content. This was done to minimize large scale contamination. Then whole earthworms were placed into cold coelomocyte extrusion buffer in Petri dishes (71.2 mM NaCl; 5 mM EGTA; 50.4 mM guiacol–glycerol–ether; 5% (v/v) ethanol; pH 7.3). After a brief period, coelomocytes were rapidly shed, harvested and washed twice in *Lumbricus* balanced salt solution (LBSS; 71.5 mM NaCl; 4.8 mM KCl; 4.2 mM $NaHCO_3$; 1.1 mM $MgSO_4{\cdot}7H_2O$; 0.4 mM KH_2PO_4; 0.3 mM NaH_2PO_4; pH 7.3).

2.2. Chemicals

Stock solutions of phytohemagglutinin (PHA-P, Sigma L1668, St. Louis, MO, USA), lipopolysaccharide (LPS, Sigma L6529), concanavalin A (ConA, Sigma C0412), and pokeweed mitogen (lectin from *Phytolacca americana*, PWM, Sigma L8777) were prepared in LBSS. 1 mM stock solution of fMLP was provided by Orpegen Pharma (Heidelberg, Germany). Ionomycin (Sigma I0634) was dissolved in absolute alcohol and phorbol 12-myristate 13-acetate (Sigma P8319) was prepared in DMSO (Sigma D8418). 1 mg/ml Fluo-3AM (Invitrogen Molecular Probes, F-1242, Eugene, OR, USA) stock solution was dissolved in DMSO containing 15% Pluronic-F-127 (Sigma P2443). Thapsigargin was used in 1 mM stock concentration dissolved in DMSO. All reagents were kept in −20 °C until used in experiments.

2.3. Loading of coelomocytes with Fluo-3 AM

After harvesting, coelomocytes were resuspended in Dulbecco's Modified Eagle Medium (DMEM, Sigma) or Ca^{2+}-free PBS at 10^7/ml concentration and loaded with 1% Fluo-3 AM for 30 min at room temperature with gentle shaking (Boldizsar et al., 2002; Minta et al., 1989). Afterwards tubes were filled with 10 ml DMEM or Ca^{2+}-free PBS and incubated additional 30 min. After this 1 h incubation period coelomocytes were centrifuged for 5 min at 1000 rpm and resuspended at 10^6/ml concentration in DMEM or Ca^{2+}-free PBS. Fluo-3 AM fluorescent signal was measured in FL1 channel with flow cytometry.

2.4. Detection of intracellular calcium levels at basal state and upon treatments with ionophore, thapsigargin, mitogens and chemoattractant

Aliquots of 700 μl cell suspension were measured with Becton-Dickinson FACSCalibur flow cytometer. After 50 s measurement of the basal Ca^{2+} level, ionomycin, thapsigargin, phorbol 12-myristate 13-acetate (PMA), different concentrations of mitogens (LPS, PHA ConA, PWM) and the bacterial peptide *N*-formyl-L-methionyl-L-leucyl-L-phenylalanine (fMLP) were added to the tubes. To verify that the signal in FL1 channel was observed from living coelomocytes, 1 μg/ml propidium iodide (PI) was added to the samples before measurements and stimulations. Data was collected from PI negative, viable coelomocytes. Absolute ethanol and DMSO (used for preparing ionomycin, thapsigargin and PMA solutions) were tested as controls in Ca^{2+} influx measurements. None of these tested reagents caused elevation of the intracellular Ca^{2+}.

2.5. Ca^{2+} ATPase enzyme cytochemistry

Coelomocytes were spread onto glass slides using Cytospin 3 (SHANDON, Thermo Labsystems, Waltham, MA, USA) and dried overnight. Then, coelomocytes were incubated for 20 min at 37 °C in Na-barbital buffer, pH 9.4, containing 3 mM ATP and 0.18% anhydrous $CaCl_2$. Slides were rinsed in 0.1% $CaCl_2$ and 2% $CoCl_2$ and then immersed in 0.5% ammonium sulfide for reaction development. After incubation coelomocytes were counterstained with Mayer's haematoxylin (Reanal, Budapest, Hungary). Slides were observed with Nikon Eclipse 80i microscope (Auroscience Kft., Budapest, Hungary) equipped with a cooled CCD camera. Images were taken with SPOT imaging software package (Diagnostic Instruments Inc., Sterling Heights, MI, USA).

2.6. Data analysis and statistics

The collected flow cytometry data were analyzed with CellQuest and FCS Express softwares (De Novo Software, Los Angeles CA, USA). Kinetic plots were constructed with FlowJo 7.5 software (Tree Star, Inc., Ashland, OR, USA). Statistical analysis was performed with Microcal Origin (Microcal Software Inc., Northhampton, MA, USA). Results are presented as mean and all error bars represent the standard error of the mean. The effect of treatments was analyzed statistically by Student's *t*-test (two