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MINIREVIEW

Interactions of intracellular calcium and immune response in earthworms

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Abstract

Intracellular calcium level has a definite role in innate and adaptive immune signaling but its evolutionary aspects are not entirely clear yet. Very few information are accessible about calcium contents of invertebrate immunocytes, especially of celomocytes, the effector cells of earthworm immunity. Different basal and induced Ca^{2+} levels characterize the various celomocyte subgroups. Intracellular calcium is mostly located in the endoplasmic reticulum and celomocytes exert intracellular Ca^{2+} ATPase activity to maintain their calcium homeostasis. Immune molecules such as phytohemagglutinin and the chemoattractant fMLP caused the elevation of intracellular Ca^{2+} level in celomocytes. All the evidence suggests that Ca^{2+} influx may play a crucial role in the signal transduction of the earthworm's innate immunity.

Key Words: annelids**;** celomocytes; intracellular signaling; lectins; chemoattractants

Introduction

Invertebrate organisms regulate their biochemical pathways by means of evolutionarily conserved molecular components (Crozatier and Meister, 2007). Calcium is one of the most ancient molecules participates in these intracellular pathways. Calcium acts as an intracellular mediator and its transient oscillations are necessary for cell activation and metabolism (Carafoli, 2002, 2005; Case *et al*., 2007). Indeed, calcium participates as a second messenger in intracellular signaling of invertebrates (Whitaker, 2006); however, the role of calcium has been investigated only in a limited number of species (Burlando *et al*., 2001; Whitaker, 2006).

Leukocyte activation in vertebrates is mediated by calcium signaling. The elevated intracellular calcium will be followed by kinase phosphorylation, activation of transcription factors and gene expression (Oh-hora and Rao, 2008). As for the role of calcium signaling in invertebrate immunity the data are scarce; an *in vitro* approach has demonstrated that elevation of cytosolic calcium induces activation of phospholipase A2 in mussel hemocytes (Marchi *et al*., 2004).

So far, there was no data available concerning the cytosolic calcium levels and its oscillations upon immune stimulus from earthworm immune competent cells (so called celomocytes). Recently, an evolutionary conserved calcium-binding protein, the calreticulin was fully cloned and characterized from *Eisenia fetida* earthworms. Calreticulin was strongly expressed in celomocytes in addition to other earthworm tissues (Kauschke *et al*., 2001; Šilerová *et al*., 2007). Moreover, another conserved calcium-binding protein, calmodulin is partially sequenced from the celomocytes of *E. fetida* (Brulle *et al*., 2006*)*. With these observations in mind we felt it is essential to clarify the possible role of calcium in earthworm immunocytes.

In our recently published report we aimed to measure calcium levels of celomocyte subpopulations and uncover the role of intracellular calcium signaling in the celomocyte's immune activation (Opper *et al*., 2010).

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Innate immunity of earthworms

Earthworms (*Oligochaeta*, *Annelida*) similarly to other invertebrates possess humoral and cellular immune responses against environmental pathogens (reviewed in Cooper *et al*., 2002). Cellular immune responses of earthworms were first evidenced through transplantation experiments by rejecting allo- and xenografts of the body wall (Cooper, 1968, 1969, 1970).

Within the body cavity (celom) of earthworm's free-floating immune cells, the celomocytes are

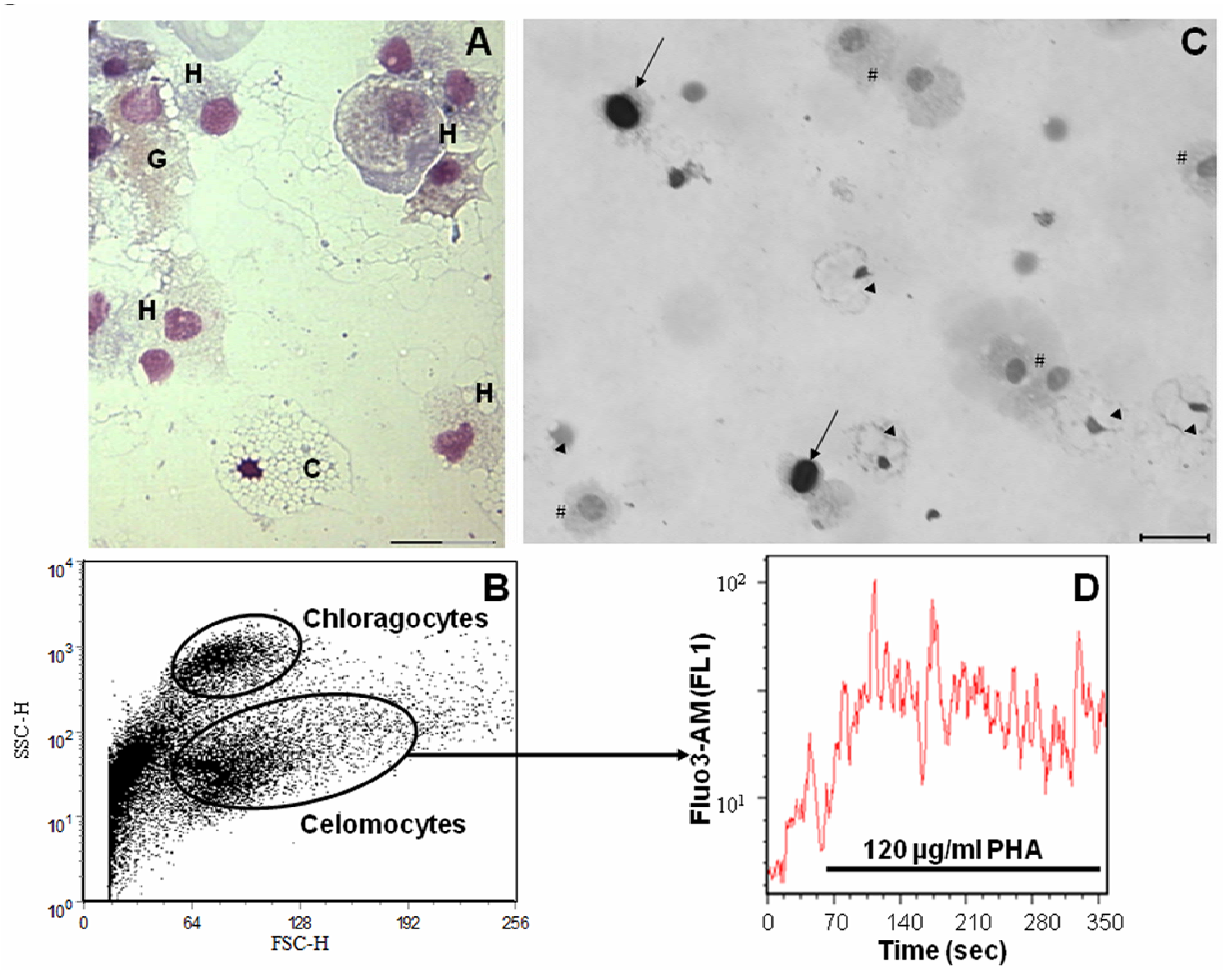

Fig. 1 Intracellular calcium harbored by earthworm celomocytes is responsible for mitogen stimuli. (A) Various subgroups of celomocytes are identified by May-Grünwald/Giemsa staining (H, hyaline amebocytes; G, granular amebocytes; C, chloragocytes/eleocytes). (B) Physical distribution of earthworm immunocytes (celomocytes/chloragocytes) analyzed by means of flow cytometry. Only effector celomocytes were responsive in terms of Ca^{2+} influx. (C) Ca^{2+} ATPase enzyme activity was detected by cytochemistry in different celomocytes of *E. fetida* (arrows). Note the Ca^{2+} ATPase negative celomocytes (amebocytes, number sign) and chloragocytes (arrowheads). Bars: 20 µm (A, C). (D) Addition of 120 µg/ml phytohemagglutinin caused Ca^{2+} influx in celomocytes demonstrated by using Fluo-3AM Ca^{2+} sensitive fluorescent dye.

circulated. Earthworm celomocytes are responsible for self/non-self recognition, phagocytosis, encapsulation (called brown body formation in earthworms), and production of antimicrobial/cytotoxic molecules.

The knowledge about the origin of earthworm celomocytes is rather limited, because there is no evidence of any active hematopoietic organs/glands in oligochaeta annelids with some exceptions (Vetvicka and Sima, 2009). Possible maturation sites for celomocytes could be the mesoderm-related celomic epithelium and the metanephridial tissue (Engelmann *et al*., 2005a, Vetvicka and Sima, 2009).

Earthworm celomocytes can be distributed into several subpopulations. Earlier studies characterized celomocyte subgroups based on histochemical and microscopical properties (Adamowicz, 2005). Nowadays, physico-chemical characteristics of these cells are taken more into account. Two or three main populations of earthworm leukocytes are distinguished using a flow cytometry-based approach (Quagliano *et al*., 1996; Engelmann *et al*., 2005a). According to the morphological terminology, those subpopulations are the granular-, hyaline amebocytes and chloragocytes/eleocytes distinguished previously by microscopy (Cooper *et al*., 2002 and Fig. 1A). Our group has been characterized three antigenically different populations of celomocytes (R1, R2 and R3) using specific monoclonal antibodies, which could correspond to these previously identified celomocyte populations (Engelmann *et al*., 2005a). These data were confirmed independently by other researchers (Fuller-Espie *et al*., 2010, Vernile *et al*., 2007).

Initiation of invertebrate innate immune response is based on germ-line pattern recognition molecules. Recently, a pattern recognition receptor (PRR) molecule, called celomic cytolytic factor (CCF) binding to lipopolysaccharide (LPS)-, β-1,3-glucan-, muramic acid-, and *N,N'*-diacetylchitobiose, was identified from *E. fetida*. This protein is expressed in celomocytes and presents in the celomic fluid (Beschin *et al*., 1998, 1999).

On the other hand Toll proteins have not been found yet in earthworms (Engelmann *et al*., 2005b), however just recently an *in silico* approach proved that Toll genes are present in annelid phyla (Davidson *et al*., 2008). Moreover, a transcriptomic analysis provided evidence about the expression of MyD88 homologue in *E. fetida* (Gong *et al.,* 2008). So far, it is known that CCF has unique antigen recognition characteristics; although might other receptors also contribute in non-self recognition accelerating various signal transduction pathways still unknown in annelids.

Calcium contents of celomocytes

Concerning about the calcium content and signaling involved in invertebrate immunocyte activation only limited information is available since studies have been carried out mainly on the classical model organism *Drosophila melanogaster* (Yagodin *et al*., 1999).

Similarly to those experiments performed on hemocytes from the *Crassotrea gigas* oyster (Aton *et al*., 2006), we assessed the intracellular calcium contents of earthworm celomocytes. Earlier, we characterized three subpopulations of earthworm celomocytes based on physical parameters and immunological properties using monoclonal antibodies (Engelmann *et al*., 2005a). During Ca^{2+} measurements we included only two populations for celomocytes. The first gate represents the effector celomocytes corresponds to hyaline and granular amebocytes, the second gate was applied for chloragocytes (Fig. 1B). Comparing the intracellular calcium levels of celomocyte subpopulations from *E. fetida* we observed characteristic differences. Effector celomocytes such as granular and hyaline amebocytes had lower intracellular calcium contents, while chloragocyte/eleocyte subpopulation harbors elevated calcium levels (Opper *et al*., 2010).

In oysters only a low percentage (20-25 %) of hemocytes proved to be fluorescent after preincubation with the Fluo-3 AM Ca^{2+} sensitive dye (Aton *et al*., 2006). In contrast, among earthworm celomocytes we observed 70-90 % Fluo-3 AM positive cells. This discrepancy could be due to species-specific differences and the different experimental conditions. *C. gigas* hemocytes were stained in hemolymph or artificial sea water while celomocytes were labeled in cell culture media or in PBS similarly to mammalian cells.

Ca^{2+} ATPase activity in celomocytes

Intracellular calcium content harbored in celomocytes must be controlled by several mechanisms. One major component is the Ca^{2+} ATPase protein family balancing the Ca^{2+} content among the intracellular cell organelles and the cytoplasm. Ca^{2+} ATPases are membrane located transport proteins of invertebrate and vertebrate cells (Ballarin *et al*., 1997; Yagodin *et al*., 1998; Yagodin *et al*., 1999; Granfeldt *et al*., 2002; Baron *et al*., 2009). Separate Ca^{2+} ATPase molecules can be distinguished in the plasma membrane (PMCA) and in the endoplasmic reticulum (SERCA). Functionally active SERCA pumps can be demonstrated by thapsigargin (TG) treatments. TG promptly inhibits this ATP dependent ion pumps, moreover later TG induces unfolded protein response and autophagy (Sakaki *et al*., 2008). Celomocytes were responsive upon this treatment, because we observed a delayed rise of intracellular calcium level after 2 μM TG addition. Besides, we localized the Ca^{2+} ATPases in the celomocytes. The cells were stained for Ca^{2+} ATPase activity using enzyme cytochemical approach (Opper *et al*., 2010 and Fig. 1C). Previously, annelid mesenchymal tissues proved to be positive for ATPase cytochemistry (De Eguileor *et al*., 1999; Gastaldi *et al*., 2007).

Inhibition of Ca^{2+} ATPase activity can diminish sufficient phagocyte response as it was demonstrated in the immunocytes of *Botryllus schlosseri* (Ballarin *et al*., 1997), which further strengthen the immunological relevance of calcium homeostasis in invertebrates.

Celomocytes exert Ca^{2+}-independent PKC activation

Invertebrate immunocyte activation involves signal transduction events similarly to vertebrates. Emerging data shows that cytosolic enzymes, which are important in kinase mediated signal transductions such as mitogen activated protein kinases (MAPK), protein kinase C (PKC), phospatidyl-inosytol 3 kinase (PI-3K), are well conserved molecules from invertebrates to vertebrates (Canesi *et al*., 2006; Engelmann *et al*., 2011).

Earthworm PKC1, PKC2 and mitogen-activated protein kinase kinase kinase 1 (MAP3K1 or MEKK1) were partially cloned and characterized from *E. fetida* (Brulle *et al*., 2006; Gong *et al*., 2008). According to the report of Brulle *et al.* (2006) worms challenged with cadmium-spiked soil have biased gene expression involved in oxidative stress response and metal detoxification while there was no alteration in kinase pathway genes.

We tested that phorbol 12-myristate 13-acetate (PMA) as an agonist of PKC proteins is able to evoke Ca^{2+} response in earthworm celomocytes. PMA did not induce Ca^{2+} mobilization of celomocytes; however addition of various concentrations of PMA, just right after ionomycin challenge, caused a concentration-dependent decrease of Ca^{2+} signal (Opper *et al*., 2010). Similar results were obtained from vertebrate leukocytes, where PMA treatment abolished the observed Ca^{2+} influx (McCarthy *et al*., 1989; Mahomed and Anderson, 2000). Moreover, experiments on mussel hemocytes proved the existence of dual, PMA resistant and sensitive isoforms of PKCs in invertebrates (Gonzales-Riopedre *et al*., 2009).

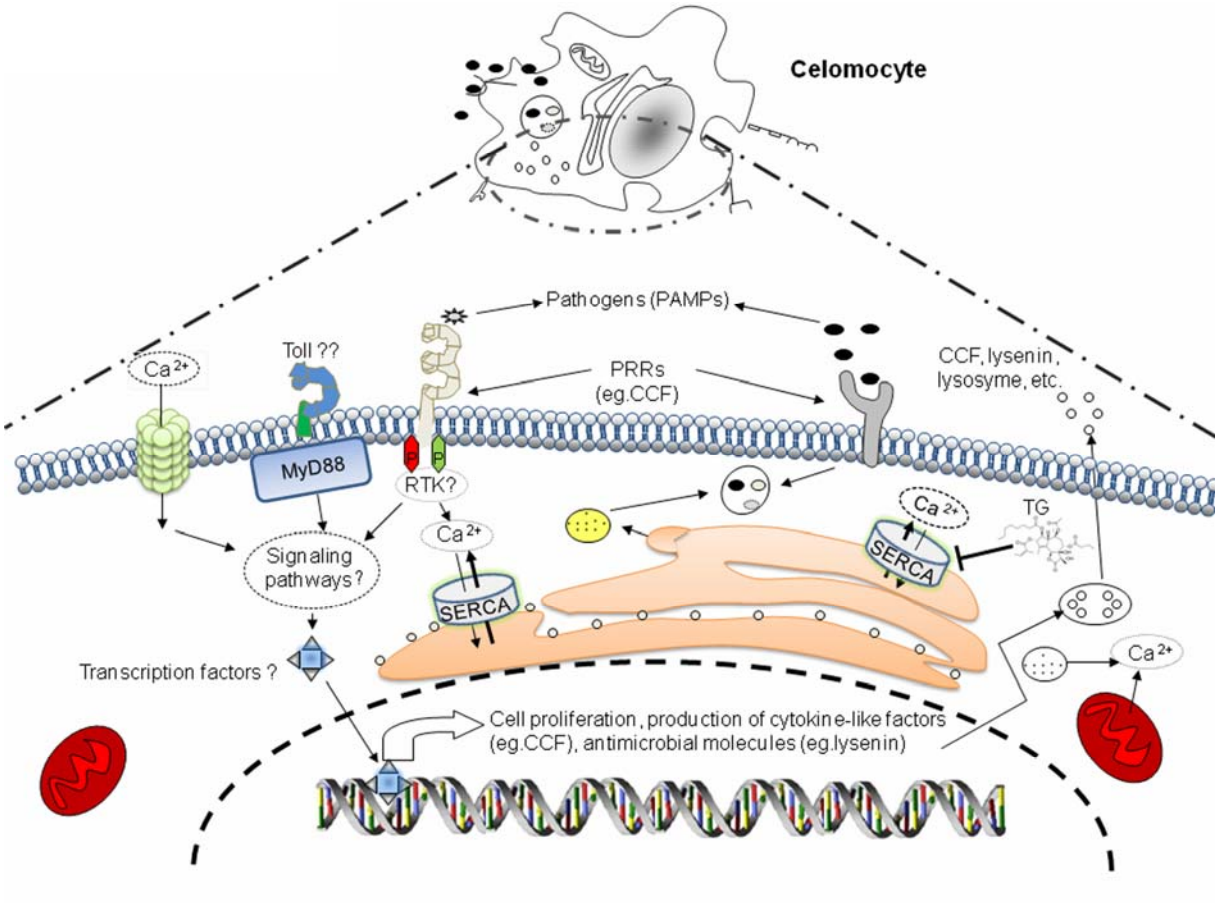

Fig. 2 The hypothetical model shows intracellular signaling events after activations of earthworm celomocytes. It proposes possible, yet uncovered signal transduction events that follow the recognition of pathogens or pathogen associated molecules (PAMPs). These activate several effector mechanisms such as phagocytosis and production of bioactive molecules (CCF, lysenin, lyzosyme, etc.). This modified figure is reproduced with kind permission of Springer Science + Business Media from Engelmann *et al.* (2011).

Ca^{2+} influx induced by a plant lectin

Microbial cell wall components and plant lectins are widely used mitogenic reagents in immuno-biological research. These mitogens such as phytohemagglutinin (PHA), concanavalin A (ConA), pokeweed mitogens (PWM), wheat germ agglutinin (WGA) lectins and lipopolysaccharide (LPS) endotoxin cause signal transduction events and proliferation of vertebrate leukocytes (Lichtman *et al*., 1983; Sei and Arora, 1991; Siegl *et al*., 1998). It is known that some of these mitogens evoke activation and cell proliferation of invertebrate immunocytes as well (Holm *et al*., 2008).

Earthworm celomocytes were increased in numbers upon treatment with LPS, ConA and PHA mitogens, however highest proliferation rate was observed upon ConA stimulation (Roch *et al*., 1975; Roch, 1977). Flow cytometry-based cell cycle analysis (BrdU uptake and propidium iodide staining) of mitogen-treated celomocytes complemented these findings (Quaglino *et al*., 1996, Engelmann *et al*., 2011).

We were interested in whether mitogen stimulus of celomocytes involves calcium influx or not. Different concentrations of PHA caused an increase of intracellular calcium levels in celomocytes, but we were not able to detect Ca^{2+} influx using other mitogens (Opper *et al*., 2010 and Fig. 1D). Based on this data we suggest that PHA may lead to proliferation based on a Ca^{2+}-dependent signaling, while other mitogens such as ConA and LPS may cause celomocyte proliferation through other, non Ca^{2+}-related mechanisms in earthworms.

Bacterial chemoattractant peptides evoked Ca^{2+} oscillations in celomocytes

Microbial products such as LPS, zymosan and N-formylmethionine-leucine-phenylalanine (fMLP) can recruit immunocytes into the site of infection (Heit *et al*., 2002). This migration requires the events of intracellular activation and cytoskeletal reorganization in both invertebrate and vertebrate immunocytes (Mahomed and Anderson, 2000).

Recent evidence show that fMLP receptors are involved in the migration and engulfment of foreign particles mediated by the hemocytes of mollusks and arthropods (Yip *et al*., 2001; Malagoli and Ottaviani, 2004; Garcia-Garcia *et al*., 2009).

Until now there was no experimental data about fMLP receptor expression or fMLP mediated activation of earthworm celomocytes. However, celomocytes from the sipunculan *Themiste petricola* worms were reactive for fMLP stimulus (Cabrera *et al*., 2002). In earthworm celomocytes, we were able to detect a transient intracellular calcium rise after fMLP treatment. This effect was evoked by similar concentration (5 μM) of fMLP (Opper *et al*., 2010) which was effective for the migration of sipunculan celomocytes and for the activation of vertebrate leukocytes (Tintinger *et al*., 2005). Our data claim for a functional evidence of fMLP receptor-like molecule in celomocytes, however we have not got direct nucleic acid information yet. The mammalian chemoattractant receptor is a G-protein coupled receptor (GPCR) sharing functional and sequence similarities with other GPCRs such as pituitary adenylate cyclase-activating polypeptide type I receptor (PAC1) and vasoactive intestinal polypeptide receptor 1 (VPAC1) proved by molecular communication (El Zein *et al*., 2008). More interestingly, earthworm celomocytes express an immune-reactive PAC1 receptor-like structure (Somogyi *et al*., 2009). This data may suggest further evidence for the immune-neuroendocrine cross-talk in invertebrate immune systems discussed by Ottaviani *et al*. (2007).

Conclusions and future prospects

Our results emphasize the variation of calcium levels in the celomocyte subpopulations and the activation induced calcium influx mediated by different immunogen molecules such as lectins and bacterial chemoattractants. These data lead us to propose a model where stress events cause activation/Ca^{2+} mobilization along with other possible signaling events in earthworm's immune cell subpopulations (Fig. 2) proving that intracellular calcium release is the most ancient signaling mechanism which is required for sufficient immune response in all organisms (Opper et al., 2010; Engelmann *et al*., 2011).

There are further applications which could have benefit from our observations and flow cytometry is an easily adaptable tool that can be used for many cellular-based functional assays. It is widely accepted that cellular homeostasis and intracellular signaling are strongly influenced by environmental pollutions (Worth *et al*., 2001; Kim *et al*., 2002; Marchi *et al*., 2004; Franco *et al*., 2009; Vergani *et al*., 2009). For instance earthworms are a well established model organism for ecotoxicology. Celomocytes can be isolated from toxicants exposed worms and the functional status of celomocytes (cell survival, ROS production, Ca^{2+} levels) could be quantitatively assessed using assay-specific fluorescent probes for flow cytometry. However, we have very limited information how environmental toxicants such as pesticides and heavy metals affect the calcium homeostasis. Moreover we have no information so far how these toxicants influence the dynamic changes of calcium levels in celomocytes and other invertebrate immunocytes. Heavy metals alter the intracellular calcium homeostasis in earthworm celomocytes demonstrated by X-ray microanalysis (Homa *et al.*, 2007). Just recently it has been showed that chronic Cu^{2+} exposure of *Penaeus monodon* shrimp caused impaired hemocyte counts, biased the immune functions and elevated the intracellular calcium contents (Xian *et al*., 2010). These data further supports that our proposed method could be applied by toxicologists to study the toxic effects of heavy metal-, chemical pollutions or the possible harmful effects of emerging nanomaterials using functional assays such as intracellular calcium level measurements by fluorescence dyes.

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