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Modulation of mitogen-activated protein kinases (MAPK) activity in response to different immune stimuli in haemocytes of the common periwinkle *Littorina littorea*

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Abstract

The modulation of mitogen-activated protein kinase (MAPK) activity in haemocytes of the common periwinkle (*Littorina littorea*) in response to immune challenges by lipopolysaccharide from *Echerichia coli* (LPS), mannan from baker's yeast *Saccharomyces cerevisiae* and secretory-excretory products (SEP) of trematodes *Himasthla elongata* (Echinostomatidae) or after the treatment with phorbol ester (PMA) has been studied by Western blotting using affinity purified rabbit polyclonal antibodies. Exposure of the cells in suspension to PMA, LPS and mannan triggered an activation of p38 and ERK2. The JNK-mediated cascade was modulated differently by the elicitors examined. PMA treatment caused a transient activation of the JNK54 isoform, LPS exposure resulted in a decrease in activity of JNK46, and mannan had no effect on JNK phosphorylation status. Incubation of periwinkle haemocytes in culture medium containing trematode SEP did not affect the activity of any MAPK.

Keywords: *Littorina littorea*; Mollusca; Haemocyte; Invertebrate; Innate immunity; Mitogen-activated protein kinase; Signal transduction

1. Introduction

In molluscs the main line of defence against invading pathogens consists of the mobile haemolymph cells. Haemocytes are capable of the phagocytosis of microorganisms and encapsulation of metazoan parasites accompanied by production of various bioactive and toxic compounds resulting in the destruction and elimination of non-self materials [1–4]. Despite the vast amount of data on morphology and functional characteristics of molluscan haemocytes, the molecular mechanisms underlying the processes of non-self recognition and destruction by these cells are still poorly understood.

The intracellular signal transduction pathways involved in the response of molluscan haemocytes to different immune challenges are still largely uncharacterised. In most eukaryotic cells, from yeasts to mammals, various forms of cellular stress lead to the activation of conserved intracellular enzymes – the mitogen activated protein kinases

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(MAPKs) [5]. In mammalian cells there are three subfamilies of MAPKs: the extracellular signal-regulated kinases ERK1 and ERK2 (with molecular weights of 44 and 42 kDa respectively); the c-Jun NH_2-terminal kinases (JNK) including up to ten JNK isoforms, usually migrating on electrophoresis as two bands corresponding to proteins with molecular weights of 54 and 46 kDa; and p38 enzymes. MAPKs phosphorylate target protein substrates to turn on or off their activities and in such a way regulate many intracellular processes of various kind – gene expression, cell division, movement, metabolic reactions, programmed cell death [5].

In molluscan haemocytes only a few MAPK homologs have been identified to date solely by immunoblotting using cross-reactive antisera raised against their mammalian counterparts [6,7]. Recently we have partially characterised three MAPK homologs belonging to different subfamilies (ERK, p38 and JNK) from haemocytes of the common periwinkle *Littorina littorea* at both nucleotide and protein levels by the methods of RT-PCR and Western blotting [8]. The present study focuses on the role of MAPK-mediated signalling pathways in the response of *L. littorea* haemocytes to different immune-related stimuli. This study aimed to clarify the modulation of phosphorylation and activation of three MAPKs – p38, ERK, JNK – after the challenge of periwinkle haemocytes by cell free lipopolysacharide (LPS), mannan, and secretory-excretory products (SEP) released in vitro by rediae of trematode *Himasthla elongata* (Echinostomatidae) naturally infecting periwinkles or after the treatment of the cells with phorbol ester (PMA). To our knowledge this is the first work assessing the contribution of different MAPK into the responses triggered in molluscan cells by different foreign substances.

2. Materials and methods

Common periwinkles, *Littorina littorea*, 6–13 years old, were collected during low tide in Kruglaya bay of the Chupa inlet (Biological station ''Kartesh'', Kandalaksha Bay of the White Sea). The animals were maintained in cages until use and given adequate food supplies (fucoid seagrass). Before the experiments were started, all the snails were examined for trematode infections – only individuals that did not shed cercaria were chosen for the experiments. In addition, after haemolymph sampling each snail was dissected and its soft tissues carefully examined under a dissection microscope for the presence of trematode parthenitae. Any haemolymph samples derived from infected individuals were excluded from the experiments. Prior to haemolymph collection the animals were placed individually in small closed jars filled with sea water and maintained under such conditions for several hours in order to evoke analgesia. Haemolymph was withdrawn from the buccal sinus of the relaxed snails using a 25-gauge needle and the sample was held on ice until use. For each experiment pooled haemolymph collected from 10–15 uninfected individuals was used.

To collect trematode secretory-excretory products (SEP) rediae derived from hepatopancreas of naturally infected snails were cultivated under in vitro conditions as described elsewhere [9]. The protein concentration in the parasites-conditioned medium was measured according to Bradford [10]. All the chemicals used in the experiments were purchased from Sigma (St-Louis, USA) unless indicated otherwise.

Each sample of pooled haemolymph (about 10 ml) was divided in two parts one of which was supplied with inductor solution up to the following final concentrations: 0.5 μg/ml for phorbol myristate acetate (PMA), 2 mg ml^{-1} for mannan from cell walls of yeasts *Saccharomyces cerevisiae*, 0.2 mg ml^{-1} for lipopolysaccharide from *Escherichia coli* strain B:055, and 5 μg ml^{-1} for SEP released by rediae of *H. elongata* during in vitro cultivation for 48 h. The other part of the haemolymph sample served as a control and received an equal volume of filtered sea water or in the case of SEP pure medium L-15.

At different time points (5, 10, 20, 30 and 45 min) after the addition of the inductor 1 ml aliquots (that corresponds to approximately 3 million cells) were taken from experimental and respective control samples. Cells were settled by centrifugation for 7 min at 360 g, the supernatant was discarded and the haemocytes were lysed for 15 min at 4 °C in the lysis buffer containing 20 mM Tris–HCl (pH 6,8), 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, 1 mM PMSF and 100-fold diluted phosphatase inhibitor cocktails 1 and 2 (Sigma cat numbers P2850 and P5726). The resulting samples were then mixed 3:1 v:v with the sample buffer (100 mM Tris–HCl, pH 6,8, 5% SDS, 40% glycerol, 3% β-mercaptoethanol and 0.05% bromphenol blue), boiled for 5 min, centrifuged for 5 min at 10 000 g to remove insoluble debris and then resolved by 12.5% SDS-PAGE according to Laemmli [11]. Prestained molecular mass markers (Fermentas, Vilnius, Lithuania) were run in adjacent lanes.

The gels were electro-blotted onto PVDF membrane (Millipore) in a wet protein transfer unit for 2 h at 250 mA in Towbin's buffer. Membranes were then probed with affinity purified rabbit polyclonal antibodies raised against