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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

synthetic dually phosphorylated peptides corresponding to amino acid residues surrounding TXY regulatory motif of human MAPKs. The anti-phospho-p38, anti-phospho-ERK1/2 and anti-phospho-JNK antibodies were purchased from Cell Signalling Technology (USA) and used according to the manufacturer's instructions. Immunoreactive proteins were visualised by the enhanced chemiluminescence method using Western lightning reagents from Perkin Elmer.

After probing with anti-phospho-MAPK antibody each membrane was stripped for 30 min at 50 °C with stripping buffer (50 mM Tris–HCl, pH 6.8, 2% SDS, 10 mM β-mercaptoethanol) and then re-probed with anti-actin antibodies (1:1000 v:v) to confirm equivalent protein loading. After exposure to horseradish peroxidase-conjugated protein A (0.1 mg ml^{-1}; Institute of Epidemiology and Microbiology, St-Petersburg, Russia) the complexes were visualised as described above.

The intensity of phospho-MAPK immunoreactive signal on individual blots was analysed using Gel-Pro 3.1 (Media Cybernetics) software. Values presented on the graphs were calculated as a relative amount of phosphorylated enzyme at every time point in control and challenged cells. To avoid an influence of loading errors on the results of experiments the corresponding actin band densities were measured and assigned a standardised value of 1, and all changes in phospho-MAPK band density were normalised for that of the corresponding actin band. At least three replicate experiments were performed for the analysis of each MAPK pathway (p38, ERK and JNK), each replicate was carried out on a different day with a different batch of snails.

The values of phospho-MAPK band optical density obtained for each time point for different treatments and corresponding control values were compared using the two-tailed *t*-test for paired measurements. Differences were considered to be significant at $P < 0.05$. Mean values and 95% confidence intervals are shown in figures.

3. Results

Effects of various compounds on MAPK-mediated signalling pathways were studied by evaluating the activation of MAPKs belonging to three different subfamilies. The activation state of these enzymes was assessed by detection of dually phosphorylated (hence activated) p38, ERK1/2 and JNK enzymes in haemocyte extracts. Equal loading of proteins was confirmed in each case by use of anti-actin antibodies applied to the membranes after MAPK antibodies had been stripped. At least three independent experiments were carried out to assess the phosphorylation status of each MAPK after the challenge of *L. littorea* haemocytes with different stimuli. In all cases the general trends (activation or inhibition of the particular MAPK) were reproducible in experiments dealing with the same inductor substance and the most representative Western blot picture was selected for presentation. In case there were statistically significant differences in phospho-MAPK band densities between control and challenged haemocytes at any time point the graphs with results of densitometric analysis are shown in figures besides Western blot panels.

As a first step in this study the effect of PMA treatment on MAPK phosphorylation level was examined to test the presence of active MAPK cascades triggered by this compound in periwinkle cells. The results showed an activation of p38 and one of the ERK isoforms (ERK2) in PMA exposed cells as compared to control preparations (Fig. 1A,B). The increase in p38 phosphorylation was clearly observed within 10 min from the addition of the inductor and remained significantly higher ($P < 0.05$) than in control cells incubated with plasma alone until the end of the 45 min incubation time. In control samples, detectable amounts of phosphorylated p38 were found only at the two first time points – after 5 and 10 min of incubation. The PMA treated haemocytes demonstrated a significant increase in level of phosphorylated ERK2 as compared to control ($P < 0.05$); the dynamics of ERK2 activation was similar to that for p38. No differences were detected in the phosphorylation level of ERK1 in PMA-treated and control cells at any time. It is worth noting that the activated ERK1 kinase could be found in control cells during the whole course of experiment. PMA treatment only slightly modulated activation of JNK kinases. In both experimental and control samples a slow decline in the amount of phosphorylated forms of JNK was observed over time. However, at the first time point (5 min) PMA-treated cells demonstrated significantly higher level of JNK54 phosphorylation than control haemocytes ($P < 0.05$).

To elucidate the signalling pathway triggered by LPS, detection of phospho-MAPKs was carried out using LPS-stimulated *L. littorea* haemocytes. Incubation of the cells with LPS induced an activation of p38 and ERK2 (Fig. 2A,B). Maximum differences in intensities of the band corresponding to phospho-p38 in experimental and control haemocytes were detected at both 5 and 10 min of incubation. Over the following 35 min a slow decrease in p38 activation occurred but the amount of the activated enzyme remained still significantly higher in LPS-treated cells at

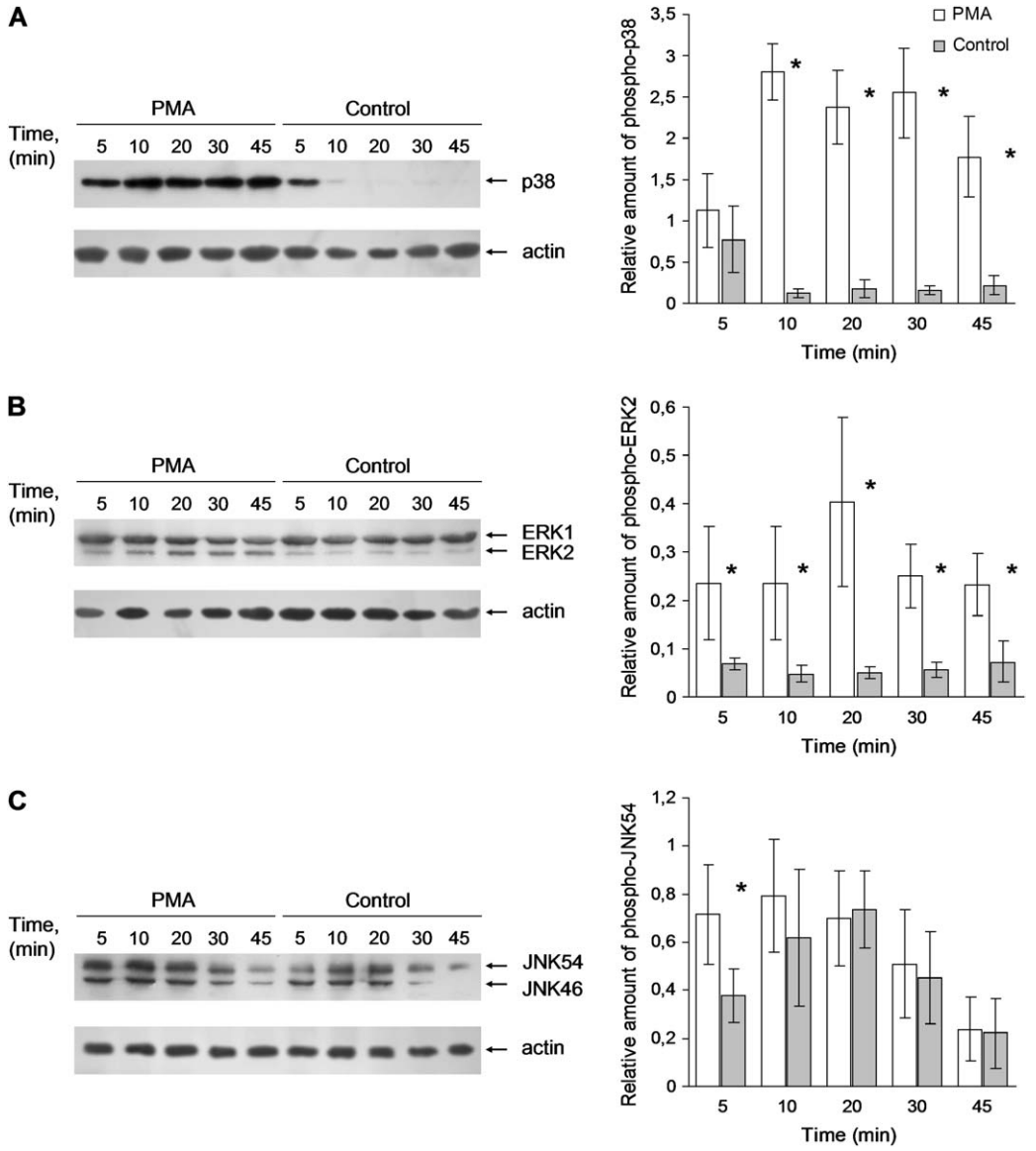

Fig. 1. Modulation of MAPK phosphorylation in *L. littorea* haemocytes by PMA treatment (A – p38, B – ERK1/2, C – JNK). Cells in suspension were incubated with PMA for different periods of time in the presence of plasma (PMA) or with plasma alone (Control). Actin was probed as a protein loading control. Western blots shown are representative of at least three independent experiments. On the graphs showing results of densitometric analysis means ±95% confidence intervals of phospho-MAPK band densities with reference to those for actin are presented. * — $P < 0.05$ when compared to control values.

all time points, as compared with control cells ($P < 0.05$). In a similar way, a higher content of active ERK2 isoform was detected in LPS-stimulated cells at all time points examined as compared with controls ($P < 0.05$), the maximum being reached in the first 10 min. With longer incubation times the level of phosphorylated ERK2 decreased in LPS-stimulated cells. In control cells the phosphorylation level of ERK2 was significantly reduced and detectable amount of phospho-ERK2 was observed only at the 5 min time point. LPS-treated haemocytes also showed a significant decrease in the phosphorylation level of JNK46 as compared to the control ($P < 0.05$). No time-dependent changes in band intensities corresponding to activated JNK54 were revealed in either experimental and control samples.

In order to clarify the possible intracellular pathways involved in signal transduction from surface receptors binding components of fungi cell walls the effect of incubation of *L. littorea* haemocytes with mannan on MAPK

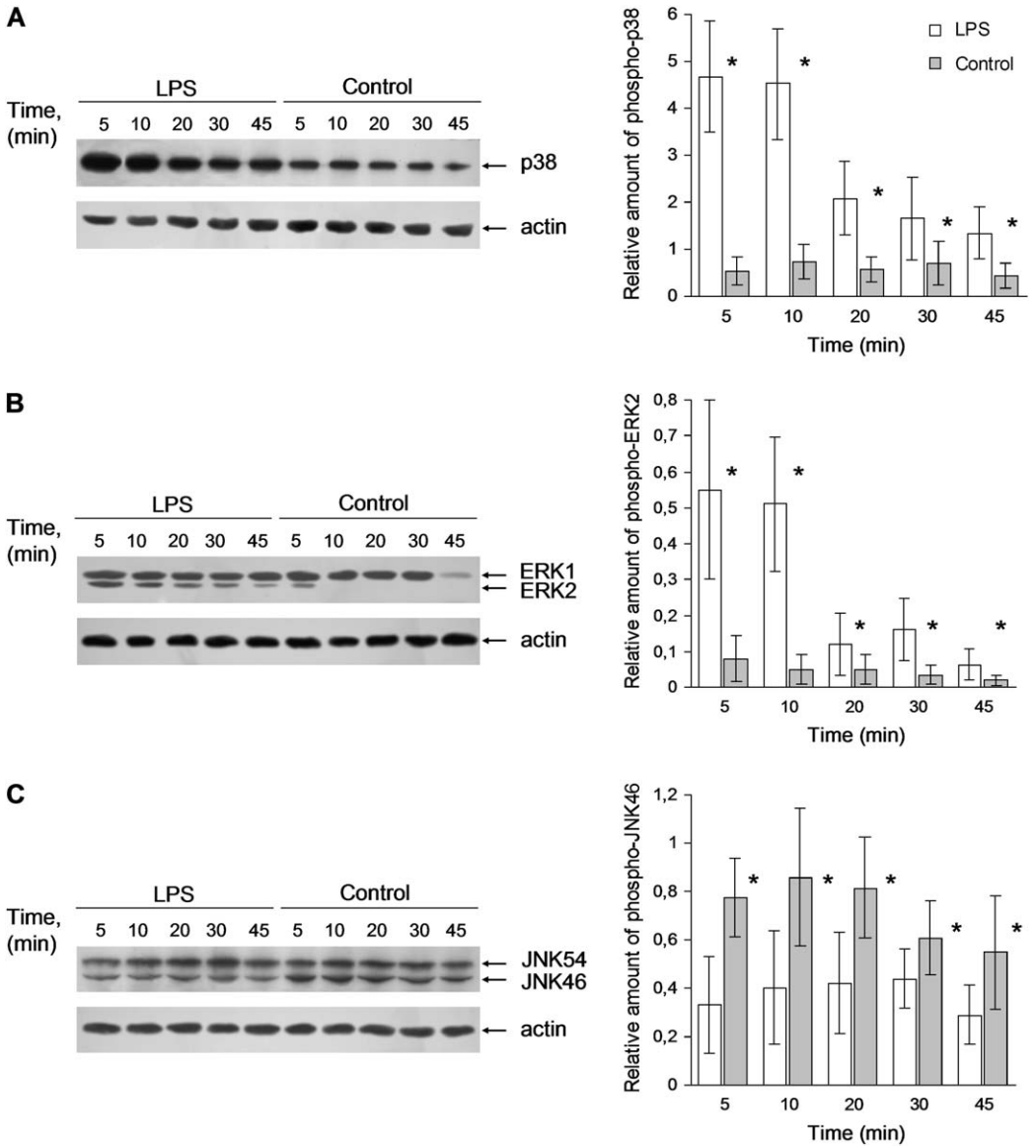

Fig. 2. Modulation of MAPK phosphorylation in *L. littorea* haemocytes by LPS treatment (A – p38, B – ERK1/2, C – JNK). Cells in suspension were incubated with LPS for different periods of time in the presence of plasma (LPS) or with plasma alone (Control). Actin was probed as a protein loading control. Western blots shown are representative of at least three independent experiments. On the graphs showing results of densitometric analysis means ±95% confidence intervals of phospho-MAPK band densities with reference to those for actin are presented. * — $P < 0.05$ when compared to control values.

phosphorylation was studied (Fig. 3). In mannan-stimulated cells an increase in the phosphorylation of p38 was seen (Fig. 3A). The amount of active enzyme in mannan-treated cells remained significantly higher than in controls at all time points ($P < 0.05$). In control haemocyte samples the visible bands corresponding to phospho-p38 were present only for the first 20 min of incubation. Mannan treatment also resulted in prolonged activation of ERK2 as suggested by a significant increase in optical density of immunoreactive bands in experimental versus control samples observed until the end of the experiment ($P < 0.05$) (Fig. 3B). No changes in the levels of phosphorylation of the ERK1 form or of JNK were seen after the challenge of periwinkle haemocytes with mannan.

An assessment of the effect of compounds produced by metazoan parasites on MAPK activation in *L. littorea* haemocytes was carried out utilising SEP released by trematode rediae during in vitro cultivation. In contrast to

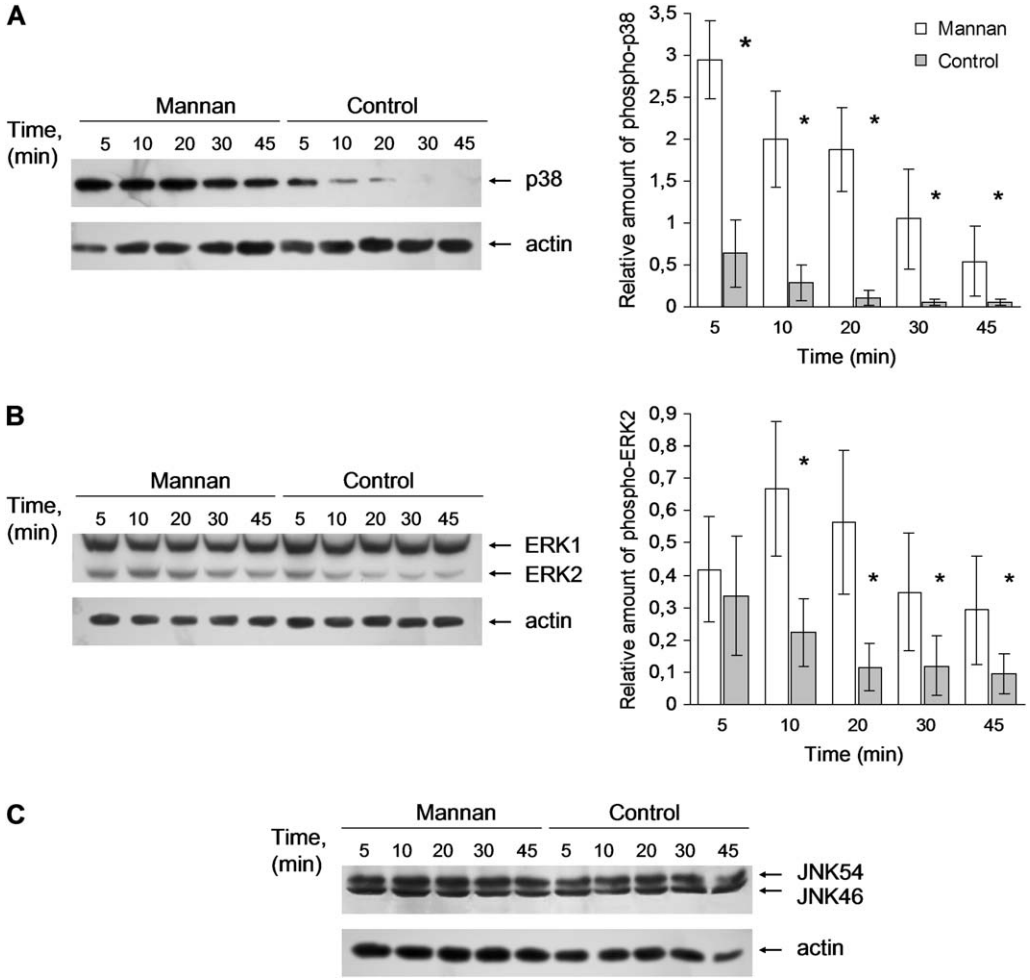

Fig. 3. Modulation of MAPK phosphorylation in *L. littorea* haemocytes by mannan treatment (A – p38, B – ERK1/2, C – JNK). Cells in suspension were incubated with mannan for different periods of time in the presence of plasma (Mannan) or with plasma alone (Control). Actin was probed as a protein loading control. Western blots shown are representative of at least three independent experiments. On the graphs showing results of densitometric analysis means ±95% confidence intervals of phospho-MAPK band densities with reference to those for actin are presented. * — $P < 0.05$ when compared to control values.

the other substances examined, the presence of trematode SEP did not cause any alterations in MAPK activity. All three enzymes showed similar levels of phosphorylation under control and experimental conditions (Fig. 4).

4. Discussion

We have previously reported that haemocytes of *L. littorea* possess MAPKs being representatives of the three different subfamilies described so far for all multicellular organisms. Three cDNAs coding periwinkle MAPKs were partially sequenced and submitted to the GenBank (accession numbers DQ078309, DQ078310, and DQ078311). The deduced amino acid sequences of *L. littorea* MAPKs demonstrate high degree of similarity with their counterparts from mammals and other invertebrates for which these data are available [8]. The region surrounding enzyme regulatory TXY motif seems to be one of the most conservative parts of the molecules showing 85–95% identity among several species analysed and therefore using the antibodies that bind specifically peptides corresponding to that region is of great advantage when studying *L. littorea* MAPK. A comparison of amino acid sequences of

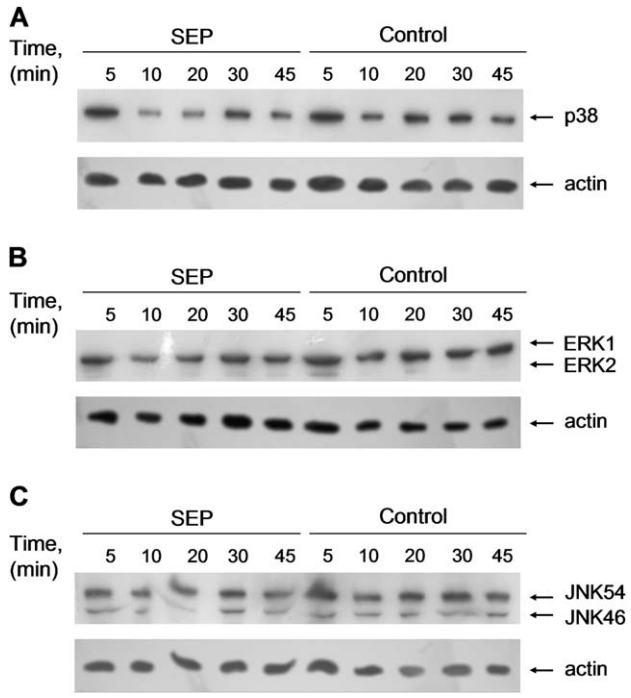

Fig. 4. Modulation of MAPK phosphorylation in *L. littorea* haemocytes by trematode SEP treatment (A – p38, B –ERK1/2, C – JNK). Cells in suspension were exposed to trematode-conditioned medium for different periods of time in the presence of plasma (SEP) or incubated in pure culture medium with plasma (Control). Actin was probed as a protein loading control. Western blots shown are representative of at least three independent experiments.

phosphorylated peptides from human MAPK used by manufacturer to obtain antiserum with the corresponding regions of periwinkle MAPK molecules revealed almost 100% identity among them (Fig. 5). Thus, the method of Western blotting for periwinkle MAPK detection with polyclonal antibodies from Cell Signalling Technology is absolutely valid and suitable for the estimation of activation status of the snail enzymes. Moreover, to our knowledge this study is

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ERK

L.littorea (DQ078309)       HTGFLTEYVATRW
H.sapiens  (M84490)         HTGFLTEYVATRW
                            *************

p38

L.littorea (DQ078310)       AEDEMTGYVATRW
H.sapiens  (L35253)         TDDEMTGYVATRW
                            ::***********

JNK

L.littorea (DQ078311)       TSFMMT-------
H.sapiens  (L26318)         TGFMMTPYVVTRY
                            *.****
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Fig. 5. Comparison of *L. littorea* MAPK predicted amino acid sequences with their human counterparts in the regions surrounding the conserved dual phosphorylation motif TXY (underlined) with CLUSTAL W alignment (http://www.ebi.ac.uk/clustalw). "*" – indicates identical residues, ":" – conserved substitutions, "." – semi-conserved substitutions. GenBank accession numbers are given in brackets.

the first one in which the Western blotting detection is underlain by strict evidences on structural similarity between snail MAPKs and determinants recognised by antibodies applied.

Previous immunoblotting experiments demonstrated that the molecular weights of periwinkle MAPK were similar to those reported for their mammalian counterparts [8]. In contrast to our previous results we have found in this study the ERK isoform of molecular weight 42 kDa corresponding to mammalian ERK2 in periwinkle haemocytes. This finding can be attributed to better protein resolution on SDS-PAGE achieved in the later experiments or to the fact that this ERK form seems to be more labile than p44 and can be detected only after the cells have been subjected to the certain treatments.

Three (PMA, LPS, mannan) out of four compounds tested caused alterations in the phosphorylation status of different MAPK-family members. Two kinases (p42 ERK and p38) were activated after the exposure of *L. littorea* haemocytes to PMA, LPS and mannan. A similar increase in p38 phosphorylation has also been described in mussel haemocytes after bacterial challenge [6], osmotic or oxidative stress [12], or exposure to environmental pollutants [13]. The fact that p38 activation may be triggered by many different stimuli suggests the convergence of various intracellular signalling pathways on this enzyme. On the other hand, the broad reactivity of this MAPK implies that the p38-mediated pathway targets proteins whose induction is rather non-specific in the cell stress response.

Together with the activation of p38 kinase in response to LPS, PMA or mannan treatment an augmentation in phosphorylation of ERK2 was observed. Challenge of haemocytes from the pond snail *Lymnaea stagnalis* with LPS resulted in a transient activation of ERK, but because both ERK isoforms (p44 and p43) identified in *L. stagnalis* often migrate in SDS-PAGE as one band it was impossible to determine whether the increase in band intensity observed was due to activation of p44 or p43 [7]. An activation of ERK has previously been reported in haemocytes from *Ceratitis capitata* when treated with *E. coli* LPS, but in this case the MAPK activation was solely due to its p44 isoform [14]. In *L. littorea* haemocytes the amount of phospho-ERK1 was stable in both control and experimental preparations. The strong activation of p44 observed in control haemocytes might be attributed to an activation of ERK stimulated by the contact of cells with foreign surfaces followed by adhesion during the haemolymph collection and in vitro incubation. An increase in tyrosine-phosphorylated proteins has been revealed during attachment of isolated insect haemocytes to cell culture wells [14].

The JNK cascade appeared to react differently on cell exposure to PMA, LPS and mannan. Only a slight transient augmentation in phosphorylation of p54 was observed when the cells were treated with PMA. Activation of JNK triggered by phorbol esters has been previously described for certain mammalian cancer cells [15]. The co-incubation of periwinkle haemocytes with LPS caused a decrease in p46 phosphorylation suggesting inhibition of the JNK signalling pathway. These data disagree with those obtained for mammalian macrophages and neutrophils where c-jun kinase is often activated after challenge of the cells with LPS [16,17]. However, the inhibition of p46 in LPS-treated periwinkle haemocytes may be a consequence of the activation of other signal cascades aimed at the prevention of apoptosis as is the case in some mammalian cell types in which the proapoptotic effect of JNK activation has been clearly demonstrated [18,19]. This explanation for the observed phenomenon seems to be very likely because LPS treatment causes the production of reactive oxygen intermediates in *L. littorea* haemocytes as it was measured by NBT-test after 1 h of exposure (unpublished observation) and overproduction of these compounds may trigger cell death cascades, as is well known for some human cell lines [20,21].

Co-incubation of *L. littorea* haemocytes with SEP produced by rediae of *H. elongata* had no effect on the phosphorylation status of MAPKs. Similarly neoglycoproteins resembling the surface molecules of schistosome trematodes did not influence the activity of ERK in *L. stagnalis* haemocytes in presence of the snail plasma [22]. In case of the *L. littorea-H. elongata* combination, parasite SEPs are apparently capable of interacting with cell surface molecules because the presence of these compounds decreased the phagocytic activity of host haemocytes for zymosan particles in a dose-dependent manner [23] suggesting the direct competition for cell surface receptors between target particles and trematode SEP. Although *H. elongata* SEPs demonstrate an ability to inhibit phagocytosis of periwinkle haemocytes, this effect seems to be attributed merely to blocking of cell surface receptors responsible for the recognition of target cells and is not accompanied by any dramatic changes in haemocyte physiology under the experimental conditions used. The binding of *Schistosoma mansoni* SEP to the surface of hemocytes of its specific host snail *Biomphalaria glabrata* has been previously reported [24]. A similar mechanism of surface receptor blocking appears to underlie the phenomenon of inhibition of the adhesion of *B. glabrata* embryonic cells (Bge) to the *S. mansoni* mother sporocysts by carbohydrates [25]. On the other hand in another study concerning molecular interactions in trematode-mollusc systems changes in gene expression in host haemocytes caused by parasite have been reported

[26] implying the ability of trematode SEP to directly induce cell response in host cells. Taking all these data together and keeping in mind the real complexity of interactions between host and parasite, one must not exclude the possibility of involvement of other signalling cascades and other mechanisms of modulation of host defence reaction by parasites but direct action of SEP on snail defence cells. One such mechanism involved may be the modulation of activity of host steering systems [27,28] resulting in systemic effects on snail defence system.

The different pattern of MAPK activation observed in response to various immune stimuli suggests the special type of cell reaction elicited in periwinkle haemocytes by each of them. Although p38 and ERK2 activation was rather unspecific and occurred after the cell treatments with PMA, LPS and mannan, JNK reacted differently to these substances. Since other intracellular signalling cascades like Toll/NF-kappaB, Janus/STAT are presumably involved in the response of haemocytes to immune challenges it is quite possible that invertebrate haemocytes can discriminate between different stimuli and generate rather specific responses. However, the precise mechanisms of inter-stimuli discrimination by these cells remain to be elucidated. The absence of any effect of parasite SEP on *L. littorea* MAPK signalling could represent the result of parasite adaptation for survival in the periwinkle organism without eliciting any stress response in host cells. However, to check this hypothesis the influence of SEP on other signalling cascades in snail haemocytes needs to be studied.

Acknowledgements

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