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# β-1, 3-glucan modulates PKC signalling in *Lymnaea stagnalis* defence cells: a role for PKC in $H_2O_2$ production and downstream ERK activation

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## Summary

**Haemocytes from the gastropod snail *Lymnaea stagnalis* (Linnaeus) were used as a model to characterize protein kinase C (PKC) signalling events in molluscan defence cells. Challenge of freshly collected haemocytes with the β-1, 3-glucan laminarin resulted in a transient increase in the phosphorylation of haemocyte PKC, with maximal phosphorylation (represented by a 3.5-fold increase) occurring at 10 min; this effect was blocked by the PKC inhibitor, GF109203X. Moreover, extracellular signal-regulated kinase (ERK) was found to be a downstream target of molluscan PKC, operating *via* a MAPK/ERK kinase (MEK)-dependent mechanism. Pharmacological inhibition of PKC phosphorylation by U-73122 and ET-18-$OCH_3$ suggested that laminarin-dependent PKC signalling was modulated *via* phospholipase C (PLC); however, a role for phosphatidylinositol-3-kinase (PI-3-K) is unlikely since the PI-3-K inhibitor LY294002 was without effect. Generation of $H_2O_2$ by haemocytes in response to laminarin was also investigated. $H_2O_2$ output increased in a dose- and time-dependent manner, with 10 mg $ml^{-1}$ laminarin eliciting a 9.5-fold increase in $H_2O_2$ production after 30 min. $H_2O_2$ production was significantly attenuated by the PKC inhibitors, GF109203X and Gö 6976, and by the NADPH-oxidase inhibitor, apocynin. In conclusion, these data further our understanding of PKC signalling events in molluscan haemocytes and for the first time define a role for PKC in $H_2O_2$ production by these defence cells. Given that $H_2O_2$ is an important anti-pathogen molecule, and that haemocytes play a crucial role in the elimination of invading organisms, PKC signalling in these cells is likely to be crucial to the molluscan innate defence response.**

Key words: haemocyte, mollusc defence, PKC, ERK, hydrogen peroxide, reactive oxygen species.

## Introduction

In invertebrates, innate immunity is crucial to defence against invading organisms. Although the molecular mechanisms that regulate innate immune reactions in response to infection have been studied in detail in insects, they have received little attention in molluscs. Existing knowledge of the physiology and internal defence system of the pond snail *Lymnaea stagnalis* (Linnaeus) make it a good model organism for studies focusing on the molecular aspects of molluscan immunity (van der Knaap et al., 1993).

Innate immunity involves the cooperation of both cellular and humoral defence reactions. In molluscs, macrophage-like phagocytic cells called haemocytes are responsible for the cell-mediated response and play the predominant role in eliminating non-self *via* phagocytosis, encapsulation, and the production of reactive nitrogen intermediates (RNIs) or reactive oxygen intermediates (ROIs) (Dikkeboom et al., 1987; Adema et al., 1993). In mammalian phagocytes, ROIs are released during the respiratory burst that is often associated with phagocytosis. The mechanism of ROI production involves the participation of NADPH oxidase, which generates the superoxide anion ($O_2^-$) *via* consummation of molecular oxygen ($O_2$) (Babior et al., 2002). $O_2^-$ is then converted to hydrogen peroxide ($H_2O_2$) either spontaneously or by superoxide dismutase (SOD). *Lymnaea stagnalis* haemocytes have been shown to possess NADPH oxidase activity when challenged with zymosan, suggesting a role for ROIs in molluscan defence (Adema et al., 1993).

Triggering of cellular defence reactions relies partly on the activation of complex networks of signalling pathways. Two important signal transduction pathways involved in the innate defence response in mammalian macrophages and monocytes are the Protein Kinase C (PKC) pathway and the Mitogen-Activated Protein Kinase (MAPK) cascade. PKC and MAPK signalling pathways are activated in mammalian macrophages following challenge with the bacterial endotoxin, lipopolysaccharide (LPS) (Monick et al., 1999; Monick et al., 2000). In addition, a role for PKC and MAPK in defence reactions such as phagocytosis and the production of ROIs has been demonstrated in macrophages (Sweet and Hume, 1996;

Shapira et al., 1997). In particular, PKC has been reported to activate NADPH oxidase in human neutrophils (Curnutte et al., 1994).

PKC is expressed in all mammalian cells; 11 isoforms (67–97 kDa) have been identified and are classified into three distinct subgroups according to their structural and regulatory differences: the classical PKCs ($\alpha$, $\beta_I$, $\beta_{II}$, $\gamma$) regulated by calcium ($Ca^{2+}$), diacylglycerol (DAG) and phospholipids; the novel PKCs ($\delta$, $\epsilon$, $\eta$, $\theta$) regulated by DAG and phospholipids; and the atypical PKCs ($\xi$, $\iota/\lambda$), which do not respond to DAG or $Ca^{2+}$, but are apparently regulated by D-3 phosphoinositides (Newton, 1995; Mellor and Parker, 1998; Parker and Murray-Rust, 2004).

Characterized mammalian MAPK pathway members are divided into three main subfamilies; Extracellular signal-Regulated Kinase 1/2 (ERK 1/2), p38 MAPK, and c-Jun N-terminal Kinase (JNK); in addition other MAPK members exist including ERK 5 and ERK 3/4 (Pearson et al., 2001). Conserved through evolution, the ERK 1/2 signalling pathway is organised into a three-kinase phosphorylation cascade involving Raf (or MAPK kinase kinase), MEK (or MAPK kinase), and ERK 1/2 (or MAPK) (Kolch, 2000). Activation of ERK 1/2 in response to an array of external stimuli can promote the expression of specific genes *via* phosphorylation of many transcription factors such as Elk-1 (Janknecht et al., 1993).

Over a decade ago, studies revealed the presence of PKC-like proteins in marine molluscs; PKC Apl I, a $Ca^{2+}$-dependent PKC, and PKC Apl II, a $Ca^{2+}$-independent PKC from nerve cells of *Aplysia californica* were described (Sossin et al., 1993). More recently, a phospholipid-sensitive $Ca^{2+}$-independent protein kinase (p105) from the mantle tissue of the bivalve *Mytilus galloprovincialis* was identified (Mercado et al., 2002). However, the different processes linking signalling pathways, and more specifically those involving PKC, to the stimulation of immune processes in molluscs have only recently become a focus of investigation. In this context, the ERK 1/2 pathway has been characterized in an embryonic cell line from the gastropod snail *Biomphalaria glabrata* (Humphries et al., 2001) and in *L. stagnalis* (Plows et al., 2004). Work in our laboratory has led to the detection of PKC-like proteins in *L. stagnalis* haemocytes and has shown that PKC phosphorylation and activation are modulated following LPS challenge (Walker and Plows, 2003). Further research also showed that PKC and ERK play a role in phagocytosis (Plows et al., 2004) and the production of nitric oxide (NO) (Wright et al., 2006) by *L. stagnalis* haemocytes.

A key question that remains is whether PKC plays a broader, and perhaps more pivotal, part in *L. stagnalis* defence responses. We thus set out to characterize PKC signalling events in *L. stagnalis* haemocytes in response to the oligomeric $\beta$-1, 3-glucan, laminarin, to identify the role of PKC in ROI production, and to elucidate the mechanisms by which PKC-mediated signals are propagated to critical downstream targets in haemocytes. The activation of PKC-like proteins in response to laminarin challenge is demonstrated and ERK 1/2 is shown to be a downstream target of PKC, likely regulated through a MEK-dependent mechanism. The results also suggest that PKC activity is dependent on phospholipase C (PLC) activation. Importantly, this study also defines a role for PKC in the generation of $H_2O_2$ following laminarin challenge, thus further linking PKC signalling to functional defence responses in molluscs.

## Materials and methods

### *Reagents*

The anti-phospho PKC (pan), anti-phospho PKC$\alpha\beta_{II}$ (Thr638/641), anti-phospho ERK, anti-phospho MEK primary antibodies, and goat anti-rabbit horseradish peroxidase (HRP)-linked secondary antibody were purchased from Cell Signaling Technology (Beverly, MA, USA), whereas the anti-phospho PKC$\alpha$ (Ser 657) antibody was from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Protogel [30% (w/v) acrylamide] was from National Diagnostics (Hull, Yorks, UK), whereas Hybond nitrocellulose membrane was from Amersham Biosciences (Amersham, Bucks, UK). The Opti-4CN detection kit was purchased from Bio-Rad (Hemel Hempstead, Herts, UK). Calphostin C, Et-18-$OCH_3$ (Edelfosine), LY294002, apocynin, and Gö 6976 came from Calbiochem (Nottingham, Notts, UK) and the Qentix signal enhancer was from Perbio Sciences (Tatenhall, Cheshire, UK). Both Vectashield and the Amplex Red® hydrogen/peroxidase assay kit were purchased from Molecular Probes (AA Leiden, Netherlands). The molecular mass markers (SDS-6H), anti-actin antibody, phorbol-myristate-acetate (PMA), laminarin (from *Laminaria digitata*), zymosan, GF109203X (bisindolylmaleimide I), U-73122, FITC-conjugated goat anti-rabbit secondary antibody, rhodamine phalloidin and all other chemicals were purchased from Sigma-Aldrich (Poole, Dorset, UK).

### *Snails*

Adult *Lymnaea stagnalis* (L.) were purchased from Blades Biologicals (Edenbridge, Kent, UK). Juvenile snails were then reared from eggs laid by adults, in an aquarium at room temperature. Once they had developed into adults, they were transferred to an incubator maintained under a 12 h:12 h light:dark cycle at 20°C and housed in tanks containing continuously aerated water. Snails were regularly fed fresh round lettuce and fish flakes were given once a week. Water used in the aquarium and incubator tanks was filtered through a Brimak/carbon filtration unit (Silverline Ltd, Winkleigh, Devon, UK) and was changed weekly.

### *Haemolymph extraction, cell stimulation and inhibition assays*

Six to eight adult *L. stagnalis* were washed with distilled water. Haemolymph was then obtained by head-foot retraction (Sminia, 1972). This natural defence reflex involves the snail withdrawing into its shell following continual prodding of its head-foot; haemolymph is then expelled through the haemal pore (Sminia, 1972). Sterile snail saline (SSS: 3 mmol $l^{-1}$ Hepes, 3.7 mmol $l^{-1}$ NaOH, 36 mmol $l^{-1}$ NaCl, 2 mmol $l^{-1}$ KCl, 2 mmol $l^{-1}$ $MgCl_2$, 4 mmol $l^{-1}$ $CaCl_2$, pH 7.8, sterilized

through a 0.22 μm disposable filter) (Adema et al., 1994) was then added to the extracted haemolymph (2 parts haemolymph: 1 part SSS) which was kept on ice to prevent haemocyte clumping.

Haemocyte monolayers were then prepared in 24-well culture plates (Nunc; 500 μl diluted haemolymph per well); cells were allowed to bind to individual wells for 30 min at room temperature, after which monolayers were washed three times for 5 min with SSS in order to remove haemolymph and non-adherent/dead haemocytes. Following equilibration in SSS (500 μl) for 1 h, cells were challenged with laminarin (10 mg $ml^{-1}$), PMA (10 μmol $l^{-1}$), or zymosan (10 μg $ml^{-1}$), or various times (0–30 min). SSS was then removed quickly and 70 μl boiling 1× SDS-PAGE sample buffer was added to the monolayers to solubilize haemocyte proteins. Samples were then briefly sonicated (40 s) and were boiled prior to electrophoresis.

Inhibition assays were performed using: GF109203X, a competitive inhibitor of the ATP binding site of PKC; calphostin C, inhibitor of the regulatory domain of PKC; U-73122 and ET-18-$OCH_3$, inhibitors of phospholipase C; and the PI-3-K inhibitor, LY294002. Monolayers were prepared as described above and haemocytes were treated for 30 min with various inhibitors at the same range of concentrations (0.001–10 μmol $l^{-1}$), or vehicle (0.1% DMSO or ethanol), prior to challenge with laminarin (10 mg $ml^{-1}$) for 10 min.

### *Electrophoresis and western blotting*

Samples (30 μl) were loaded onto discontinuous 10% SDS-PAGE gels and run at 160 V for 90 min. Separated haemocyte proteins were then transferred to nitrocellulose membranes for 90 min at 300 mA using a BioRad semi-dry electrotransfer unit and, after transfer, blots were stained with Ponceau S to confirm that homogenous transfer had taken place. In some experiments, membranes were incubated in the Qentix signal enhancer following the manufacturer's instructions before being rinsed in Tris-buffered saline containing 0.1% (v/v) Tween-20 (TTBS). Membranes were then blocked at room temperature for 1 h with 5% (w/v) non-fat dried milk in TTBS. Next, membranes were incubated with anti-phospho PKC (pan), anti-phospho PKC$\alpha\beta_{II}$, anti-phospho PKC$\alpha$, anti-phospho MEK, or anti-phospho ERK primary antibodies (1:1000 in TTBS) overnight at 4°C. Blots were then washed with TTBS and incubated for 1 h at room temperature with HRP-conjugated goat anti-rabbit secondary antibody (1:7500 in TTBS). Immunoreactive bands were visualized using colorimetric methods (Opti-4 CN detection kit). For all experiments, equal loading of proteins was checked by incubating blots with anti-actin antibodies (1:1000).

### *Immunocytochemistry*

Haemolymph was extracted and diluted in SSS as previously described and 100 μl of the diluted haemolymph was applied to individual coverslips. Haemocytes were then allowed to adhere to coverslips for 30 min and, after gently washing with SSS three times, were left to equilibrate in SSS for 1 h. Haemocytes were then challenged with laminarin (10 mg $ml^{-1}$) for 10 min; where appropriate, they were treated with the PKC inhibitor GF109203X (10 μmol $l^{-1}$) for 30 min prior to adding laminarin. Haemocytes were subsequently fixed by incubating cells in fixing/permeabilization buffer [3.7% (v/v) formaldehyde, 0.18% (v/v) Triton X-100 in phosphate-buffered saline (PBS)] for 12 min at room temperature, followed by a brief wash in PBS. Next, coverslips were incubated in blocking solution [1% (w/v) bovine serum albumin (BSA) in PBS] for a further 12 min before being incubated for 1 h in anti-phospho PKC (pan) antibody (1:200 in PBS) at room temperature. Finally, monolayers were washed with PBS and were incubated in fluorescein isothiocyanate (FITC)-conjugated goat anti-rabbit secondary antibody (1:1000 in PBS) for 30 min; after a further wash, cells were incubated in tetramethyl rhodamine isothiocyanate (TRITC)-conjugated phalloidin (0.1 μg $ml^{-1}$) for 40 min. Coverslips were mounted on microscope slides using Vectashield, and were sealed with nail varnish. Cells were visualized with a Leica TCS SP2 AOBS laser scanning confocal microscope driven by Leica software. Fluorescein was typically excited by the 488 nm line of the argon laser, and emission was collected at 543 nm; for rhodamine phalloidin, excitation and emission were 543 nm and 593 nm, respectively.

### *Production of $H_2O_2$ in haemocytes*

Haemolymph extracted from 6–10 snails was placed on ice and diluted in SSS as previously described. Haemocyte monolayers were then prepared in 96-well culture plates (Nunc; 200 μl diluted haemolymph per well) and subsequently washed three times with SSS for 5 min; cells were then left to equilibrate for 1 h in SSS. Working solutions of assay mixture (0.1 U $ml^{-1}$ HRP and 50 μmol $l^{-1}$ Amplex Red® reagent) containing different doses of laminarin (1–10 mg $ml^{-1}$) were prepared in SSS and 100 μl of this solution was added to individual wells containing haemocyte monolayers. Amplex Red® is a non-fluorescent compound that becomes fluorescent upon HRP-catalyzed oxidation by $H_2O_2$. For inhibition assays, haemocytes were incubated with GF109203X (0.01–10 μmol $l^{-1}$), Gö 6976 (0.01–10 μmol $l^{-1}$), apocynin (10–500 μmol $l^{-1}$), or vehicle (0.1% DMSO) for 30 min prior adding the working solution. The fluorescence intensity of each well was then measured using a Fluorstar Optima microplate reader (BMG Labtechnologies, Aylesbury, Bucks, UK) equipped with a 544 nm excitation filter and a 590 nm emission filter.

### *Statistical analysis*

Where appropriate, the intensities of bands on western blots were determined after scanning using Kodak 1D image analysis software; data were then analysed with SPSS software using one-way analysis of variance (ANOVA) and *post-hoc* multiple comparisons. For $H_2O_2$ assays, ANOVA and *post-hoc* multiple comparisons were also used. For all experiments, results are shown as the mean ± standard deviation (s.d.).

## Results

### *Laminarin stimulates the phosphorylation of L. stagnalis haemocyte PKC*

To evaluate the effect of the β-1, 3-glucan laminarin on the phosphorylation (activation) status of PKC-like proteins in *L. stagnalis* haemocytes, these cells were challenged with laminarin (10 mg ml$^{-1}$) over 30 min; cellular proteins were then analysed by western blotting with the anti-phospho PKC (pan) antibody. This antibody has previously been validated for use in *L. stagnalis* haemocytes (Walker and Plows, 2003) and also has been employed to study PKC phosphorylation in *M. galloprovincialis* haemocytes (Canesi et al., 2005). When *L. stagnalis* haemocytes were exposed to 10 mg ml$^{-1}$ laminarin, a rapid increase in phosphorylation of an 85 kDa PKC like-protein was observed, with maximum phosphorylation occurring at 10 min (Fig. 1A, upper panel). The increase in phosphorylation was transient and reduced to basal levels after 30 min challenge. Analysis of immunoblots revealed that after 10 min challenge, there was a 3.5-fold increase in PKC phosphorylation and this was significantly different from control values ($P \leqslant 0.001$; Fig. 1A). The use of a further two phospho-specific PKC antibodies, the anti-phospho PKCα$\beta_{II}$ antibody and the anti-phospho PKCα antibody, revealed the time course of PKC activation in haemocytes following laminarin challenge to be similar to that observed with the anti-phospho PKC (pan) antibody (Fig. 1A, middle and lower panels). Given that these antibodies all recognized a protein of similar molecular mass that had similar phosphorylation kinetics following laminarin challenge, it appears that the *L. stagnalis* haemocyte PKC-like protein might be most similar to PKCα.

Haemocytes were also challenged with the PKC activator, PMA, and the yeast cell wall glucan, zymosan. Exposure to these compounds resulted in increased phosphorylation of the haemocyte PKC-like protein after 10 min, but unlike laminarin challenge, the phosphorylation appeared more sustained (Fig. 1B,C). Throughout the experiments, the levels of phosphorylated PKC in basal (unchallenged) haemocytes were variable and in some cases appeared relatively high. This observation is likely a consequence of working with freshly collected (primary) haemocytes. Overall, the anti-phospho PKC (pan) antibody provided the clearest immunoreactive signal and was therefore used in all subsequent experiments.

### *MEK and ERK phosphorylation is dependent on PKC activity in challenged haemocytes*

To explore whether PKC can modulate certain downstream signalling events in haemocytes following challenge with laminarin, inhibition assays were carried out using the PKC inhibitor, GF109203X. This inhibitor is a widely used competitive inhibitor of the PKC ATP-binding site, and is selective towards PKC α, $\beta_I$, $\beta_{II}$, γ, ϵ, δ isoforms. GF109203X significantly inhibited PKC phosphorylation following laminarin challenge in a dose-dependent manner causing a significant, 72% decrease, in PKC phosphorylation when used

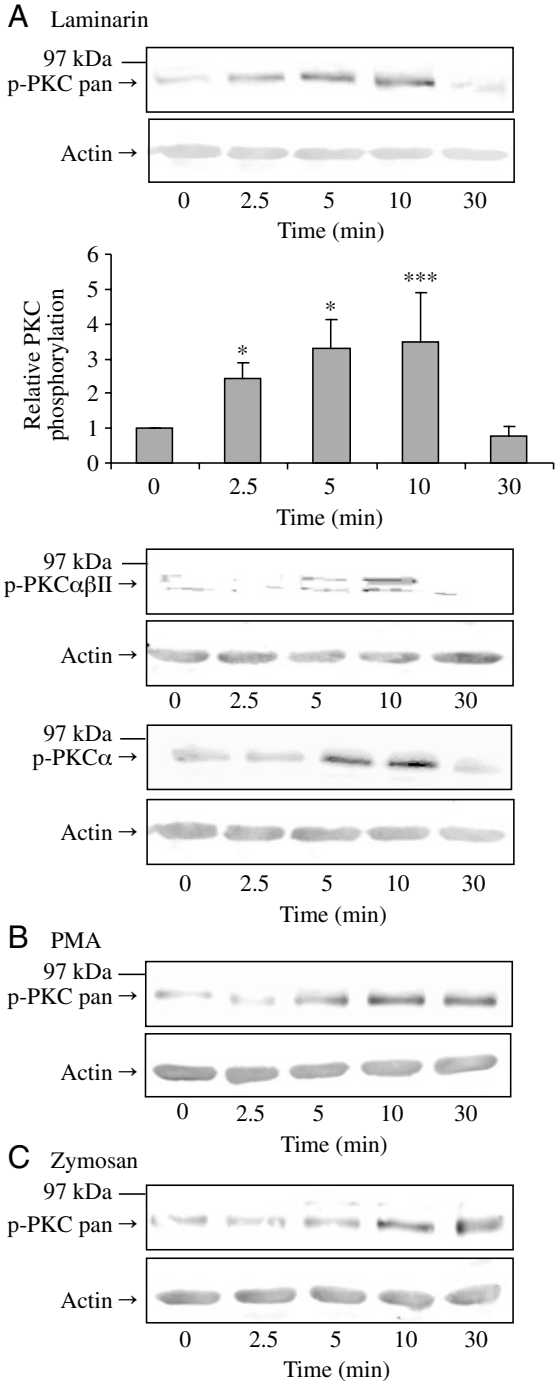


Fig. 1. The effects of laminarin, PMA and zymosan on PKC phosphorylation. *Lymnaea stagnalis* haemocytes were challenged with (A) laminarin (10 mg ml$^{-1}$), (B) PMA (10 μmol l$^{-1}$) or (C) zymosan (10 μg ml$^{-1}$) for 0–30 min and their effects on PKC phosphorylation determined by western blotting. Equal amounts of protein were loaded in each lane and membranes were probed with anti-phospho PKC (pan), anti-phospho PKCα, or anti-phospho PKCα$\beta_{II}$ antibodies as indicated in the figure. Anti-actin antibodies were used to confirm equal loading of proteins. Relative PKC phosphorylation levels (shown in the graph in A) detected with anti-phospho PKC (pan) antibodies were determined by image analysis. Values are means ± s.d. from four independent experiments. *$P \leqslant 0.05$ and ***$P \leqslant 0.001$ when compared to control values (time 0).

at a concentration of 10 μmol $l^{-1}$ ($P \leqslant 0.001$; Fig. 2A). Moreover, at this dose, PKC phosphorylation was reduced to below basal levels. Lower concentrations of GF109203X (0.01–1 μmol $l^{-1}$) also reduced laminarin-dependent PKC phosphorylation significantly ($P \leqslant 0.05$; Fig. 2A).

We then evaluated whether inhibition of PKC in laminarin-challenged haemocytes could affect MEK and ERK 1/2, since these kinases have been identified as downstream targets of PKC in mammalian macrophages (Monick et al., 2000). As shown in Fig. 2B, phosphorylation of MEK was significantly attenuated by GF109203X at all doses ($P \leqslant 0.05$), with 10 μmol $l^{-1}$ and 1 μmol $l^{-1}$ significantly reducing PKC phosphorylation levels in challenged cells by 61% and 42%, respectively ($P \leqslant 0.001$). Treatment with 10 μmol $l^{-1}$ or 1 μmol $l^{-1}$ GF109203X also significantly decreased ERK 1/2 phosphorylation (activation) by 65% ($P \leqslant 0.001$) and by 47% ($P \leqslant 0.01$), respectively (Fig. 2C). In the absence of inhibitor, laminarin produced a significant 1.9-fold increase in ERK 1/2 phosphorylation in haemocytes compared to controls ($P \leqslant 0.01$), whereas it had little effect on MEK phosphorylation (1.1-fold increase). The effects of a second PKC inhibitor, calphostin C, which targets the regulatory domain of PKC by competing for the binding site of DAG, on downstream MEK and ERK phosphorylation following challenge were also evaluated. Like GF109203X, calphostin C was able to inhibit MEK and ERK phosphorylation in a dose-dependent manner

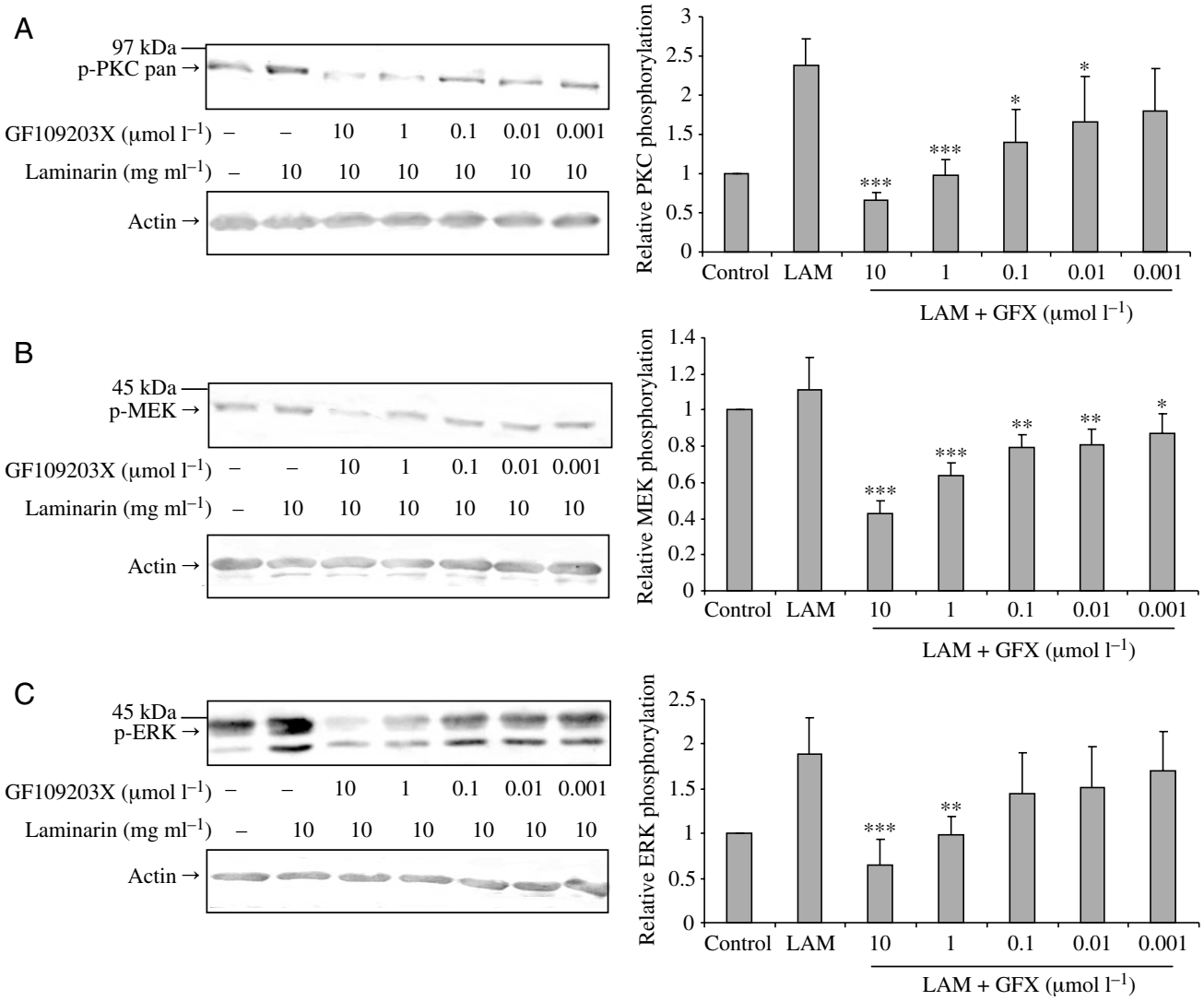


Fig. 2. GF103209X attenuates PKC, MEK and ERK 1/2 phosphorylation in laminarin-challenged haemocytes. Haemocyte monolayers were incubated with various concentrations of GF103209X (0.001–10 μmol $l^{-1}$), a competitive inhibitor of the ATP-binding site of PKC, or vehicle (0.1% DMSO), prior to challenge with laminarin (10 mg $ml^{-1}$) for 10 min. Protein extracts from haemocytes were prepared and western blotting was performed using polyclonal phospho-specific antibodies either to (A) PKC, (B) MEK or (C) ERK. Anti-actin antibodies were used to confirm equal loading of proteins. Relative PKC, MEK and ERK phosphorylation levels were determined by image analysis of blots from four independent experiments. Values are mean ± s.d. *$P \leqslant 0.05$, **$P \leqslant 0.01$, ***$P \leqslant 0.001$ when compared with phosphorylation levels observed in stimulated, uninhibited cells.

(Fig. 3), although in these experiments the stimulatory effects of laminarin on ERK phosphorylation were less marked.

*Visualization of phospho-PKC in L. stagnalis haemocytes*

The intracellular distribution of phosphorylated (activated) PKC in *L. stagnalis* haemocytes was studied by immunocytochemistry. Fluorescence images showed that the anti-phospho PKC (pan) antibody was able to localize activated molluscan PKC in resting and stimulated haemocytes. In unstimulated conditions, phosphorylated PKC levels were low but clustered in some areas (Fig. 4A). Challenge with laminarin (10 mg $ml^{-1}$) for 10 min triggered an increase in PKC phosphorylation in the cell body, and possibly redistribution to the plasma membrane. Exposure to laminarin also appeared to promote morphological changes in haemocytes evidenced by expansion of filopodia (Fig. 4B) and this was observed consistently in many independent experiments. GF109203X considerably reduced the phosphorylation status of PKC within haemocytes. Moreover, when treated with this inhibitor, the haemocytes appeared to possess fewer filopodia (Fig. 4C). The immunocytochemical results for stimulated and inhibited haemocytes show PKC phosphorylation levels that broadly agree with those obtained by western blotting.

*PKC phosphorylation (activation) is controlled by the upstream enzyme PLC but not by PI-3-K*

To determine whether activation of PKC by laminarin was operating *via* PLC-dependent mechanisms, the effects of the PLC inhibitors U-73122 and ET-18-$OCH_3$ on PKC phosphorylation levels were investigated in laminarin-stimulated haemocytes. Pharmacological inhibition by U-73122 significantly attenuated PKC phosphorylation following laminarin challenge, indicating that PLC acts upstream of PKC in haemocytes (Fig. 5A). Image analyses revealed that at 10 μmol $l^{-1}$, this inhibitor reduced PKC phosphorylation by approximately 85% (data not shown). A second phospholipase C inhibitor, ET-18-$OCH_3$, used at the same range of concentrations (0.001–10 μmol $l^{-1}$) also inhibited PKC phosphorylation (Fig. 5B) with 10 μmol $l^{-1}$ ET-18-$OCH_3$ reducing PKC phosphorylation by 73% (data not shown). In order to evaluate whether PI-3-K acts upstream of PKC in laminarin-challenged haemocytes, monolayers were treated with various doses of the PI-3-K inhibitor, LY294002 (0.001–10 μmol $l^{-1}$), prior to challenge; we have recently shown that this inhibitor attenuates the phagocytic activity of *L. stagnalis* haemocytes by 62% (Plows et al., 2006). Western blotting revealed that LY294002 did not reduce levels of phosphorylated PKC at any dose tested (Fig. 5C), indicating that haemocyte PKC activity is not under the control of PI-3-K in laminarin-challenged haemocytes.

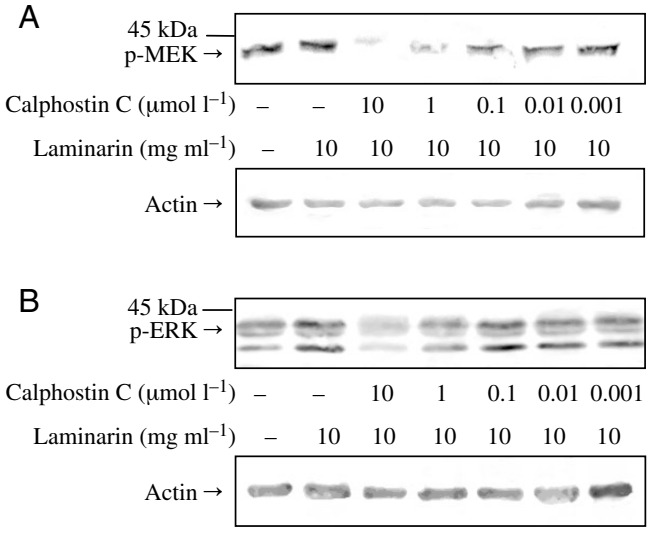


Fig. 3. The PKC inhibitor, calphostin C reduces MEK and ERK 1/2 phosphorylation in laminarin-challenged haemocytes. Haemocyte monolayers were incubated with calphostin C (0.001–10 μmol $l^{-1}$), a PKC inhibitor that interacts with the cysteine-rich zinc finger structure of the PKC regulatory domain, or vehicle (0.1% DMSO), prior challenge with laminarin (10 mg $ml^{-1}$) for 10 min. Protein extracts were subjected to SDS-PAGE followed by western blotting using polyclonal phospho-specific antibodies to (A) MEK and to (B) ERK. Anti-actin antibodies were used to confirm equal loading of proteins. Immunoblots are representative of two independent experiments.

*Production of $H_2O_2$ by laminarin-stimulated haemocytes is PKC-dependent*

To determine the effects of laminarin on $H_2O_2$ generation by *L. stagnalis* haemocytes, monolayers were treated with various doses of laminarin (1–10 mg $ml^{-1}$) for 30 min and $H_2O_2$ output determined using an Amplex Red® fluorescence-based assay. $H_2O_2$ was studied because of its relative stability compared to other by-products of cellular $O_2^-$ and its ability to permeate cell membranes. Laminarin significantly increased the production of $H_2O_2$ by haemocytes compared to control (unchallenged) cells at all of the doses tested ($P \leq 0.05$), with a 9.5-fold increase when used at 10 mg $ml^{-1}$ ($P \leq 0.001$) (Fig. 6A). Furthermore, the effects of laminarin challenge on the cellular output of $H_2O_2$ were found to be dose-dependent ($P \leq 0.001$). Haemocytes were then exposed to laminarin (10 mg $ml^{-1}$), and $H_2O_2$ production was monitored over 30 min. An increase in fluorescence signal above controls was observed at each time point (Fig. 6B), demonstrating a continuous linear increase of $H_2O_2$ production over this time period. Next, to elucidate whether $H_2O_2$ production was dependent on PKC activity, cells were treated with GF109203X or Gö 6976, a specific inhibitor of PKCα, prior to challenge and determination of $H_2O_2$ output. The PKC inhibitor GF109203X (0.01–10 μmol $l^{-1}$) significantly ($P \leq 0.001$) reduced the levels of fluorescence compared that seen in laminarin-stimulated haemocytes, with a maximum inhibition of 65% when used at a final concentration of 10 μmol $l^{-1}$ ($P \leq 0.001$) (Fig. 7A); inhibition was also significant when lower doses of inhibitor were employed ($P \leq 0.001$). In contrast to the effects of GF109203X, Gö 6976 was only effective at the highest concentration tested (10 μmol $l^{-1}$) reducing $H_2O_2$ output by 40% (Fig. 7B). Finally, the NADPH oxidase inhibitor, apocynin, dose-dependently decreased $H_2O_2$ production by haemocytes relative to

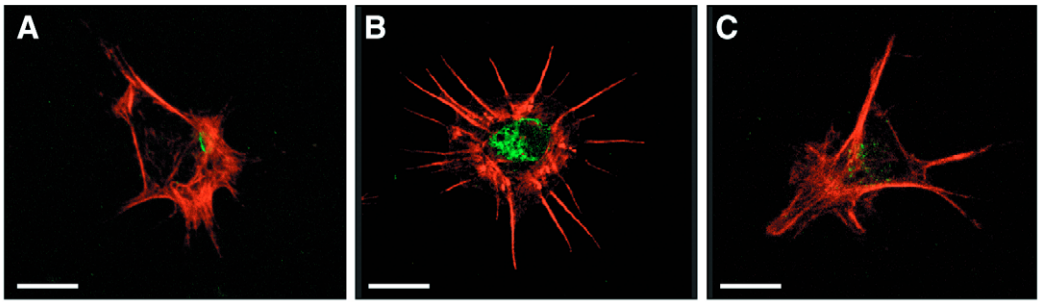


Fig. 4. Distribution and levels of phosphorylated PKC in *L. stagnalis* haemocytes investigated by immunocytochemistry. Phosphorylated PKC in (A) untreated haemocytes, (B) haemocytes challenged with laminarin (10 mg $ml^{-1}$) for 10 min, and (C) haemocytes incubated with GF109203X (10 μmol $l^{-1}$) for 30 min prior challenge with laminarin (10 mg $ml^{-1}$) for 10 min. Rhodamine phalloidin (red) stains F-actin and fluorescein (green) shows the phosphorylated PKC detected with the anti-phospho PKC (pan) antibody. Haemocytes were observed with a Leica laser scanning confocal microscope. Results are representative of three independent experiments. Bar, 20 μm.

stimulated cells not exposed to the inhibitor (Fig. 7C). When used at 500 μmol $l^{-1}$, apocynin significantly inhibited $H_2O_2$ production by laminarin-stimulated haemocytes by 57% ($P \leqslant 0.001$) whereas 43%, 36% and 13% inhibition were observed using 100 μmol $l^{-1}$, 50 μmol $l^{-1}$ and 10 μmol $l^{-1}$ apocynin, respectively.

## Discussion

Current knowledge of cell signalling in molluscs is poor, and much remains to be discovered concerning the specific signalling molecules that regulate defence reactions, triggered by infection, in this important group of organisms. Using the model snail *L. stagnalis*, we have recently shown that bacterial LPS modulates the phosphorylation (activation) of PKC (Walker and Plows, 2003) and ERK pathway members (Plows et al., 2004) in haemocytes, cells that play a crucial role in the molluscan defence response. The present research aimed to investigate the effects of laminarin on the activation of PKC like-proteins in *L. stagnalis* haemocytes and to identify some of the upstream regulators and downstream targets of PKC in these cells. Since PKCs help co-ordinate multiple innate immune responses in mammalian immune cells (Tan and Parker, 2003), we also wished to gain further insights into PKC-dependent defence responses in haemocytes. In this context we report here, for the first time in molluscs, that haemocyte ERK is regulated by PKC in a MEK-dependent manner in the presence of laminarin and that PLC acts upstream of PKC in these cells. Importantly, we also demonstrate a role for PKC signalling in the generation of $H_2O_2$ in haemocytes in response to challenge by laminarin.

β-1, 3-glucans are cell wall constituents of fungi and bacteria that bind to pattern-recognition receptors (PRRs) and modulate innate immune responses in invertebrate haemocytes (Vetvicka and Sima, 2004; Vetvicka and Yvin, 2004). Laminarin is an oligomeric β-1, 3-glucan that contains β-1, 6-interstrand linkages; occurring in brown algae, it is structurally analogous to an oligosaccharide involved in cell–cell recognition. Laminarin elicits various responses in a range of organisms; for example it activates cell signalling in plants (Klarzynski et al., 2000) and stimulates the production of NO and $O_2^-$ by *M. galloprovincialis* haemocytes (Arumugam et al., 2000) and NO by *L. stagnalis* haemocytes (Wright et al., 2006). It is considered that, in molluscs and insects, laminarin interacts with β-1, 3-glucan-binding proteins on haemocytes and stimulates the NADPH oxidase pathway, a component of the respiratory burst (Söderhäll and Cerenius, 1998; Arumugam et al., 2000). PMA-based studies in *Crassostrea gigas* (Toreilles et al., 1996) and *B. glabrata* (Bender et al., 2005) haemocytes suggest that this respiratory burst might be linked to PKC.

Cellular innate immune functions in vertebrates and insects are mediated by various signalling enzymes including MAPKs and PKCs (Greenberg, 1995; Soldatos et al., 2003; Nappi et al., 2004). In the present study, western blotting of *L. stagnalis* haemocyte extracts with phospho-specific anti-PKC antibodies revealed that laminarin induced a time-dependent phosphorylation (activation) of a PKC-like protein in haemocytes, with maximal phosphorylation occurring after 10 min. In contrast to the effects of PMA and zymosan, laminarin-dependent PKC phosphorylation was transient, with phosphorylation levels returning to near basal after 30 min challenge. The kinetics of PKC phosphorylation displayed following laminarin challenge were similar to those observed when haemocytes were exposed to bacterial LPS (Walker and Plows, 2003). Anti-phospho PKC (Ser 660–PKC$\beta_{II}$), anti-phospho PKC$\alpha/\beta_{II}$ (Thr 638/641) and anti-phospho PKC$\alpha$ (ser 657) antibodies all recognized the haemocyte PKC-like protein, indicating that the residues surrounding these key phosphorylation sites in the kinase domain share homology with human PKC$\alpha/\beta_{II}$. Perhaps this is not surprising since PKC$\alpha$ and $\beta_{II}$ are classical PKC isoforms, which represent the best conserved PKCs between species (Stabel and Parker, 1991; Mellor and Parker, 1998).

Experiments described here utilized freshly collected haemocytes. After preparation and washing of haemocyte monolayers, cells were left to equilibrate (rest) for 60 min in an attempt to reduce the phosphorylation of PKC prior to challenge; kinases in primary haemocytes are often found to be phosphorylated under basal (time=0) conditions (Walker and Plows, 2003; Plows et al., 2004; Canesi et al., 2002).

Nevertheless, in haemocytes unexposed to laminarin (or PMA and zymosan), PKC had variable and often high levels of phosphorylation and longer periods (up to 3 h) of equilibration did little to reduce its basal phosphorylation state (unpublished data). A remaining pool of constitutively phosphorylated PKC that is 'ready to respond to activators' often exists in mammalian cells (Newton, 2003) and our findings suggest that such a phenomenon might apply to molluscan haemocytes.

*Lynmaea stagnalis* haemocytes respond to laminarin and although a β-1, 3-glucan binding receptor has yet to be characterized in snails, the presence of carbohydrate receptors has been suggested (Horak and Deme, 1998). Identification of β-1, 3-glucan receptors such as complement receptor 3 (CR3) in a human monocyte-like cell line (Mueller et al., 2000), or Dectin-1 in bone-marrow macrophages (Brown et al., 2002), has been facilitated through binding studies. Several β-1, 3-glucan binding proteins have been purified from insects such as the tobacco hornworm *Manduca sexta* (Jiang et al., 2004) and crustaceans such as *Penaeus monodon* (Sritunyalucksana et al., 2002) or *Pacifastacus leniusculus* (Lee et al., 2000); future work will thus likely lead to the identification of a β-1, 3-glucan receptor in *L. stagnalis*.

The use of potent PKC inhibitors is crucial to help elucidate signalling events downstream of PKC and define functional roles of this enzyme. Inhibition assays using the highly selective PKC inhibitor, GF109203X, not only revealed that this inhibitor significantly attenuated PKC phosphorylation (activation) in a dose-dependent manner in laminarin-exposed cells, but also showed that phosphorylation (activation) of haemocyte MEK and ERK signalling components is at least in

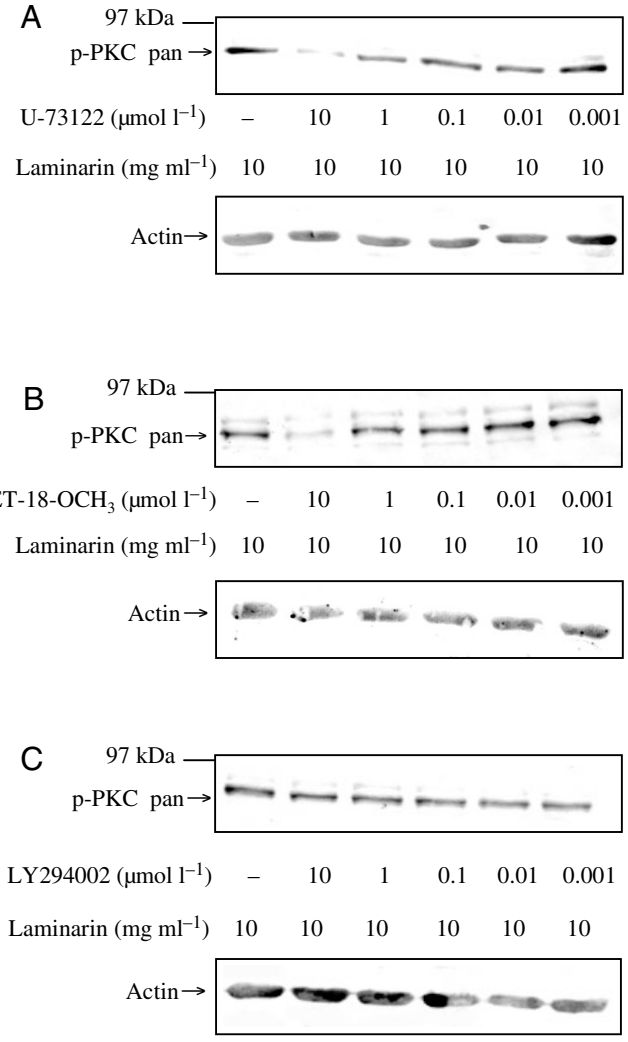


Fig. 5. PKC phosphorylation is PI-3-K-independent but phospholipase C-dependent in laminarin-stimulated haemocytes. Haemocyte monolayers were pre-treated for 30 min with the phospholipase C inhibitors U-73122 and ET-18-$OCH_3$ or the PI-3-K inhibitor LY294002 at similar range of concentrations (0.001–10 μmol $l^{-1}$), or vehicle prior to the addition of laminarin (10 mg $ml^{-1}$) for 10 min. Phosphorylated PKC was detected by immunoblotting with the anti-phospho PKC (pan) antibody. (A) For U-73122 inhibition studies, immunoblots are representative of four independent experiments. In the case of (B) ET-18-$OCH_3$ and (C) LY294002 inhibition studies, immunoblots are representative of three independent experiments.

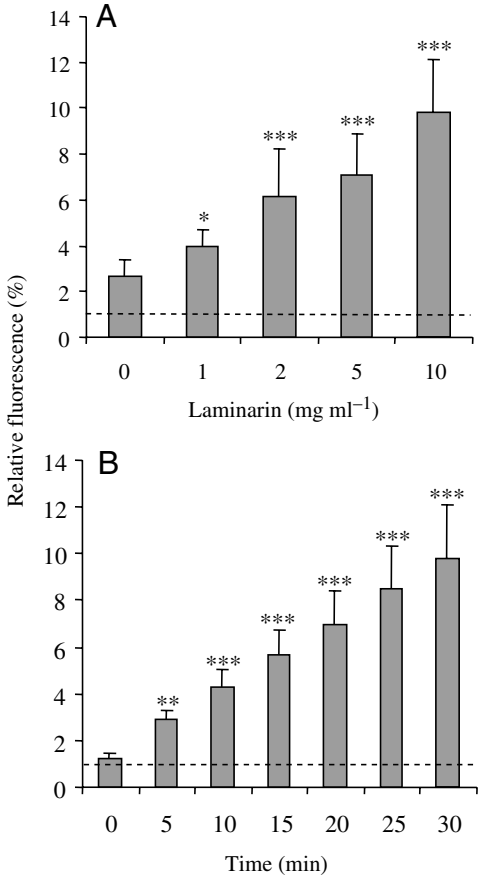


Fig. 6. Stimulation of $H_2O_2$ production in *L. stagnalis* haemocytes by laminarin. The generation of $H_2O_2$ was investigated in (A) haemocytes stimulated with different doses of laminarin (1–10 mg $ml^{-1}$) for 30 min, and (B) in haemocytes stimulated with laminarin (10 mg $ml^{-1}$) over 30 min. Values are relative fluorescence (mean ± s.d.) of two independent experiments, each done in triplicate. *$P \leqslant 0.05$, **$P \leqslant 0.01$ and ***$P \leqslant 0.001$ when compared to control (unstimulated) values.

part PKC-dependent. Experiments with calphostin C also revealed that MEK and ERK lie downstream of PKC. Results from a study employing a haemocyte-like embryonic cell line (Bge) derived from the gastropod snail *B. glabrata*, suggest that ERK activity might also be under the control of PKC in *B. glabrata* defence cells (Humphries et al., 2001). Although the study by Humphries and co-workers employed PMA as a stimulant, the present work opens the possibility that PKC-dependent modulation of haemocyte ERK activity following immune challenge might be a feature conserved between mollusc species. PKC-dependent activation of MEK and ERK also occurs in a range of mammalian cell types (Schönwasser et al., 1998; Weinstein-Oppenheimer et al., 2000), although different cells and tissues display differences in PKC specificity and targeting.

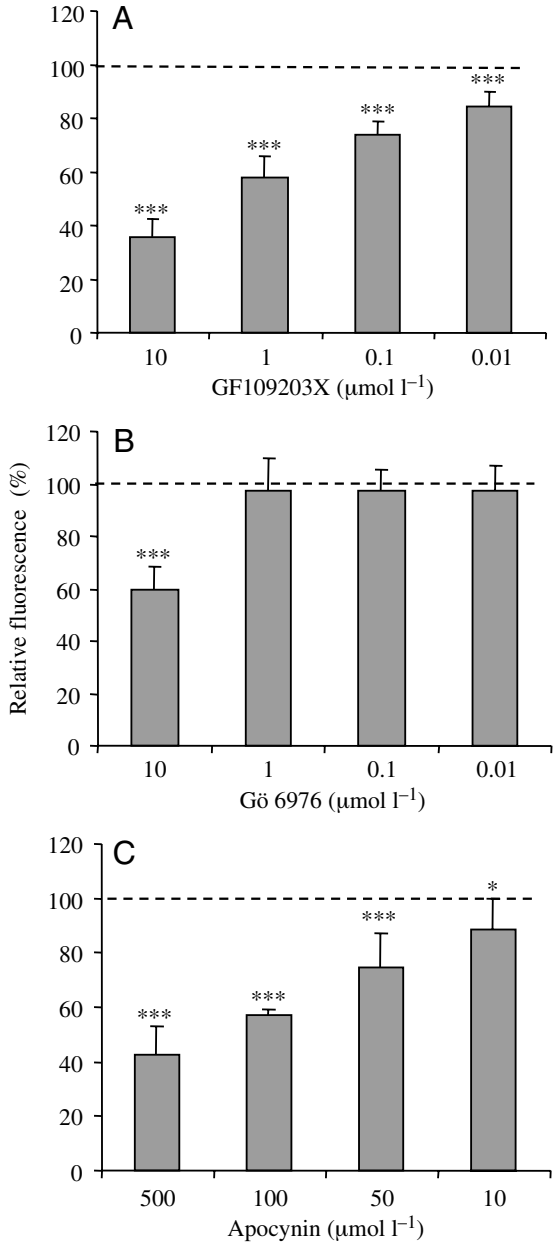


Fig. 7. Inhibition of laminarin-mediated $H_2O_2$ generation in haemocytes. The production of $H_2O_2$ was investigated in haemocytes pre-incubated with the PKC inhibitors (A) GF109203X (0.01–10 μmol $l^{-1}$) and (B) Gö 6976 (0.01–10 μmol $l^{-1}$), or (C) the NADPH oxidase inhibitor apocynin (10–500 μmol $l^{-1}$), for 30 min prior to stimulation with laminarin (10 mg $ml^{-1}$). Values are means ± s.d. of two independent experiments each performed in triplicate. $*P \leqslant 0.05$, $**P \leqslant 0.01$ and $***P \leqslant 0.001$ when compared to $H_2O_2$ production in stimulated haemocytes not exposed to either inhibitor (shown as 100%, broken line).

In order to elucidate possible upstream regulators of PKC, inhibition assays were carried out using the PLC inhibitors, U-73122 and ET-18-$OCH_3$, and the PI-3-K inhibitor, LY294002, in laminarin-challenged haemocytes. In macrophage-like U937 cells, U-73122 effectively blocks PLC activity (Matsui et al., 2001). Although previously used in *Drosophila* (Estacion et al., 2001) and in the fleshfly *Boettcherisca peregrina* (Koganezawa and Shimada, 2002), U-73122 was used for the first time in molluscan haemocytes in the present study. Pre-treatment of *L. stagnalis* haemocytes with either U-73122 or ET-18-$OCH_3$ resulted in a significant reduction in PKC phosphorylation, identifying PLC as an upstream regulator of PKC activity. In LPS-treated macrophages, 3-phosphoinositide-dependent kinase 1 (PDK1) is a downstream target of phosphorylated inositides produced in response to PI-3-K activation (Monick et al., 2000). PDK1 can activate classical PKCs since it is responsible for the phosphorylation of the activation loop, a key site belonging to the catalytic domain of this group of PKCs (Dutil et al., 1998); such phosphorylation initiates the complete process of PKC activation (Balendran et al., 2000). In laminarin-challenged *L. stagnalis* haemocytes, LY294002 did not affect PKC phosphorylation (activation) at any dose studied, implying that PI-3-K is not an upstream regulator of the haemocyte PKC. This agrees with the general mechanism of PKC phosphorylation by PDK1, described elsewhere (Sonnenburg et al., 2001), in which PI-3-K does not play a role. Our present findings also corroborate previous work in which we showed that ERK activation in *L. stagnalis* haemocytes was unaffected by LY294002 (Plows et al., 2004), implying that the regulation of PKC/MEK/ERK phosphorylation in these cells is PI-3-K independent. It can therefore also be concluded that the recently reported inhibitory effect of LY294002 on phagocytosis by *L. stagnalis* haemocytes (Plows et al., 2006), is likely mediated by PKC- and ERK-independent mechanisms.

Immunocytochemistry enabled visualization of the intracellular distribution of phosphorylated (activated) PKC in haemocytes. In unchallenged cells, the fluorescence of phosphorylated PKC was low and appeared dispersed in the centre of the cell. Challenge with laminarin resulted in a large increase in the phosphorylation of PKC within the cytoplasm where it appeared to cluster. These clusters might result from the association of phosphorylated PKC with cytoskeletal components or receptors for activated C kinase (RACK). Mammalian PKCs can associate with various cytoskeletal

proteins such as actin, vinculin and talin, which anchor contractile filaments to integrins within the plasma membrane (Liu, 1996). In the marine mollusc *Aplysia*, binding of PKC isoform Apl II to actin is favoured when PKC is dephosphorylated, whereas for Apl I binding is enhanced when phosphorylated (Nakhost et al., 1998). That *L. stagnalis* haemocyte PKC might interact with RACK remains a possibility since RACK has been identified in the snail *B. glabrata* (Lardans et al., 1998). Stimulation of cells often leads to phosphorylation of classical PKCs, accompanied by their translocation to cellular membranes, a sequence triggered by the presence of cofactors such calcium ($Ca^{2+}$) and diacylglycerol (DAG). Further experimental investigation is needed to help define whether stimulation of haemocytes with laminarin leads to the physical translocation of PKC to the plasma membrane.

In the quest to understand further the molecular control of haemocyte defence, the role of PKC-like proteins in the generation of extracellular $H_2O_2$ by haemocytes was explored. This work was prompted because $H_2O_2$ is a highly cytotoxic molecule that participates in the elimination of pathogens. *Lymnaea stagnalis* is intermediate host to schistosomes of the genus *Trichobillharzia* and $H_2O_2$ might possess schistosomicidal activity (Dikkeboom et al., 1987; Adema et al., 1994); thus knowledge of the molecular control of $H_2O_2$ production by molluscan haemocytes is crucial to our understanding of snail–schistosome host–parasite interactions. Laminarin stimulated $H_2O_2$ production by *L. stagnalis* haemocytes, effects were dose-dependent and, when used at 10 mg $ml^{-1}$, a tenfold increase in $H_2O_2$ output occurred after 30 min challenge. In *M. galloprovinciallis*, a similar concentration of laminarin triggered maximal $O_2^-$ generation (Arumugam et al., 2000). Additionally, phagocytosis of zymosan by *L. stagnalis* haemocytes was associated with an increase of $H_2O_2$ produced over 45 min (Zelck et al., 2005). Importantly, PKC appears to regulate laminarin-induced $H_2O_2$ generation by *L. stagnalis* haemocytes because the PKC inhibitor, GF109203X, significantly attenuated $H_2O_2$ generation in a dose-responsive manner. Whereas GF109203X (10 μmol $l^{-1}$) reduced $H_2O_2$ generation by 65%, the PKCα inhibitor Gö 6976 was less effective, reducing $H_2O_2$ production by only 40% at the highest dose tested (10 μmol $l^{-1}$). This differential effect is likely a consequence of the sensitivity of haemocyte PKC to the different inhibitors and/or the existence of multiple PKC-like proteins in haemocytes that are sensitive to GF109203X, an inhibitor that targets more PKC isoforms. In haemocytes, increased extracellular $H_2O_2$ production was evident before PKC was maximally phosphorylated (activated) (10 min). This could be explained in two ways: $H_2O_2$ generation might be supplemented *via* xanthine oxidase (XO) as in mammalian phagocytes (Segal et al., 1999); alternatively, early production of $H_2O_2$ could be a consequence of basal cellular activity due to other unidentified signalling events. In human neutrophils, activation of PKCα and PKCβ correlates with the assembly of the NADPH oxidase complex (Sergeant and McPhail, 1997). Moreover, in mammalian leucocytes, PKC can phosphorylate p47-*phox*, a cytosolic component of NADPH oxidase, and promote its translocation, enabling it to assemble with other membrane-associated subunits, ultimately making p47-*phox* functionally active (El-Benna et al., 2005). Interestingly, in RAW 264.7 mouse macrophages, part of the respiratory burst has been shown to be mediated by classical PKCs, since Gö 6976 reduced the production of $H_2O_2$ by a similar amount (50%) (Larsen et al., 2000) to that observed in *L. stagnalis* haemocytes.

Taken together, the results of this study show for the first time that laminarin is able to modulate PKC phosphorylation (activation) in *L. stagnalis* haemocytes and that signalling in response to this β-1, 3-glucan can occur *via* the PLC-PKC-MEK-ERK 1/2 pathway. In addition, the increased production of $H_2O_2$ observed following laminarin challenge seems to be, at least in part, intimately linked to PKC signalling. PKC also regulates NO output in *L. stagnalis* haemocytes (Wright et al., 2006). Given that $H_2O_2$ and NO are key anti-pathogen defence molecules, and that haemocytes are considered the sentinels of molluscan defence, haemocyte PKC is likely to play a critical role in limiting infection in *L. stagnalis in vivo*. Although we have characterized distinct elements of PKC signalling in haemocytes following challenge, it must be emphasised that cells respond to immunomodulatory compounds *via* a network of signalling proteins, integrated in a cooperative manner to confer an adapted response. Therefore, responses of haemocytes to laminarin are likely to be complex and pathways other than PKC might be crucial to $H_2O_2$ production. Nevertheless, by taking an integrative approach to the study of molluscan defence (Walker, 2006), this research has furthered our understanding of cell signalling in molluscs and has helped elucidate the functional relevance of PKC to innate defence reactions in this important group of organisms.

**List of abbreviations**

| | |
|---|---|
| DAG | diacyl glycerol |
| ERK | extracellular signal-regulated kinase |
| $H_2O_2$ | hydrogen peroxide |
| LPS | lipopolysaccharide |
| MAPK | mitogen-activated protein kinase |
| MEK | MAPK/ERK kinase |
| NO | nitric oxide |
| PKC | protein kinase C |
| PLC | phospholipase C |
| PMA | phorbol-myristate-acetate |
| PRR | pattern-recognition receptor |
| RACK | receptor for activated C kinase |
| RNI | reactive nitrogen intermediate |
| ROI | reactive oxygen intermediate |

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## References


**Adema, C. M., van Deutekom-Meulder, E. C., van der Knaap, W. P. W. and Sminia, T.** (1993). NADPH-oxidase activity: the probable source of reactive oxygen intermediate generation in haemocytes of the gastropod *Lymnaea stagnalis*. *J. Leukoc. Biol.* **54**, 379-383.

**Adema, C. M., van Deutekom-Meulder, E. C., van der Knaap, W. P. W. and Sminia, T.** (1994). Schistosomicidal activities of *Lymnaea stagnalis* haemocytes: the role of oxygen radicals. *Parasitology* **109**, 479-485.

**Arumugam, M., Romestand, B., Torreilles, J. and Roch, P.** (2000). In vitro production of superoxide and nitric oxide (as nitrite and nitrate) by *Mytilus galloprovincialis* haemocytes upon incubation with PMA or laminarin or during yeast phagocytosis. *Eur. J. Cell Biol.* **79**, 513-519.

**Babior, B. M., Lambeth, J. D. and Nauseef, W.** (2002). The neutrophil NADPH oxidase. *Arch. Biochem. Biophys.* **397**, 342-344.

**Balendran, A., Hare, G. R., Kieloch, A., Williams, M. R. and Alessi, D. R.** (2000). Further evidence that 3-phosphoinositide-dependent protein kinase-1 (PDK1) is required for the stability and phosphorylation of protein kinase C isoforms. *FEBS Lett.* **484**, 217-223.

**Bender, R. C., Broderick, E. J., Goodall, C. P. and Bayne, C.** (2005). Respiratory burst of *Biomphalaria glabrata* haemocytes: *Schistosoma mansoni*-resistant snails produce more extracellular $H_2O_2$ than susceptible snails. *J. Parasitol.* **91**, 275-279.

**Brown, G. D., Taylor, P. R., Reid, D. M., Willment, J. A., Williams, D. L., Martinez-Pomares, L., Wong, S. Y. C. and Gordon, S.** (2002). Dectin-1 is a major β-glucan receptor on macrophages. *J. Exp. Med.* **196**, 407-412.

**Canesi, L., Betti, M., Ciacci, C., Scarpato, A., Citterio, B., Pruzzo, C. and Gallo, G.** (2002). Signaling pathways involved in the physiological response of mussel haemocytes to bacterial challenge: the role of stress-activated p38 MAP kinases. *Dev. Comp. Immunol.* **26**, 325-334.

**Canesi, L., Betti, M., Ciacci, C., Lorusso, L. C., Gallo, G. and Pruzzo, C.** (2005). Interactions between *Mytilus* haemocytes and different strains of *Escherichia coli* and *Vibrio cholerae O1 El Tor*: role of kinase-mediated signalling. *Cell. Microbiol.* **7**, 667-674.

**Curnutte, J. T., Erickson, R. W., Ding, J. and Badwey, J. A.** (1994). Reciprocal interactions between protein kinase C and components of the NADPH oxidase complex may regulate superoxide production by neutrophils stimulated with a phorbol ester. *J. Biol. Chem.* **14**, 10813-10919.

**Dikkeboom, R., Tijnagel, J. M., Mulder, E. C. and van der Knaap, W. P. W.** (1987). Hemocytes of the pond snail *Lymnaea stagnalis* generate reactive forms of oxygen. *J. Invert. Path.* **49**, 321-331.

**Dutil, E. M., Toker, A. and Newton, A. C.** (1998). Regulation of conventional protein kinase C isozymes by phosphoinositide-dependent kinase 1 (PDK-1). *Curr. Biol.* **8**, 1366-1375.

**El-Benna, J., My-Chang Dang, P., Gougerot-Pocidalo, M. A. and Elbim, C.** (2005). Phagocyte NADPH oxidase: a mulicomponent enzyme essential for host defenses. *Arch. Immunol. Ther. Exp.* **53**, 199-206.

**Estacion, M., Sinkins, W. G. and Schilling, W. P.** (2001). Regulation of *Drosophila* transient receptor potential-like (TrpL) channels by phospholipase C-dependent mechanisms. *J. Physiol.* **530**, 1-19.

**Greenberg, S.** (1995). Signal transduction of phagocytosis. *Trends Cell Biol.* **5**, 93-99.

**Horak, P. and Deme, R.** (1998). Lectins and saccharides in *Lymnaea stagnalis* haemocyte recognition. *Comp. Haematol. Int.* **8**, 210-218.

**Humphries, J. E., Elizondo, L. and Yoshino, T. P.** (2001). Protein kinase C regulation of cell spreading in the molluscan *Biomphalaria glabrata* embryonic (Bge) cell line. *Biochim. Biophys. Acta* **1540**, 243-252.

**Janknecht, R., Ernst, W. H., Pingoud, V. and Nordheim, A.** (1993). Activation of ternary complex factor Elk-1 by MAP kinases. *EMBO J.* **12**, 5097-5104.

**Jiang, H., Ma, C., Lu, Z. Q. and Kanost, M. R.** (2004). β-1, 3-Glucan recognition protein-2 (βGRP-2) from *Manduca sexta*: an acute-phase protein that binds β-1,3-glucan and lipoteichoic acid to aggregate fungi and bacteria and stimulate prophenoloxidase activation. *Insect Biochem. Mol. Biol.* **34**, 89-100.

**Klarzynski, O., Plesse, B., Joubert, J. M., Yvin, J. C., Kopp, M., Kloareg, B. and Fritig, B.** (2000). Linear β-1, 3 glucans are elicitors of defense responses in tobacco. *Plant Physiol.* **124**, 1027-1037.

**Koganezawa, M. and Shimada, I.** (2002). Inositol 1,4,5-trisphosphate transduction cascade in taste reception of the fleshfly, *Boettcherisca peregrina*. *J. Neurobiol.* **51**, 66-83.

**Kolch, W.** (2000). Meaningful relationships: the regulation of the Ras/Raf/MEK/ERK pathway by protein interactions. *J. Biochem.* **351**, 289-305.

**Lardans, V., Serra, E., Capron, A. and Dissous, C.** (1998). Characterization of an intracellular receptor for activated protein kinase C (RACK) from the mollusc *Biomphalaria glabrata*, the intermediate host for *Schistosoma mansoni*. *Exp. Parasitol.* **88**, 194-199.

**Larsen, E. C., DiGennaro, J. A., Saito, N., Mehta, S., Loegering, D. J., Mazurkiewicz, J. E. and Lennartz, M. R.** (2000). Differential requirement for classic and novel PKC isoforms in respiratory burst and phagocytosis in RAW 264.7 cells. *J. Immunol.* **165**, 2809-2817.

**Lee, S. Y., Wang, R. and Söderhäll, K.** (2000). A lipopolysaccharide- and beta-1, 3-glucan-binding protein from hemocytes of the freshwater crayfish *Pacifastacus leniusculus*. Purification, characterization, and cDNA cloning. *J. Biol. Chem.* **275**, 1337-1343.

**Liu, J. P.** (1996). Protein kinase C and its substrates. *Mol. Cell. Endocrinol.* **116**, 1-29.

**Matsui, S., Adachi, R., Kusui, K., Yamaguchi, T., Kasahara, T., Hayakawa, T. and Suzuki, K.** (2001). U-73122 inhibits the dephosphorylation and translocation of cofilin in activated macrophage-like U937 cells. *Cell. Signal.* **13**, 17-22.

**Mellor, H. and Parker, P. J.** (1998). The extended protein kinase C superfamily. *Biochem. J.* **332**, 281-292.

**Mercado, L., Cao, A., Barcia, R. and Ramos-Martinez, J. I.** (2002). Regulatory properties of p105: a novel PKC isoenzyme in mantle tissue from marine mussels. *Biochem. Cell Biol.* **80**, 771-775.

**Monick, M. M., Carter, A. B., Gudmundsson, G., Mallampalli, R., Powers, L. S. and Hunninghake, G. W.** (1999). A phosphatidylcholine-specific phospholipase C regulates activation of p42/44 Mitogen-Activated Protein Kinases in lipopolysaccharide-stimulated human alveolar macrophages. *J. Immunol.* **162**, 3005-3012.

**Monick, M. M., Carter, A. B., Flaherty, D. M., Peterson, M. W. and Hunninghake, G. W.** (2000). Protein kinase C ζ plays a central role in activation of the p42/44 Mitogen-Activated Protein Kinase by endotoxin in alveolar macrophages. *J. Immunol.* **165**, 4632-4639.

**Mueller, A., Raptis, J., Rice, P. J., Kalbfleisch, H., Stout, R. S., Ensley, H. E., Browder, W. and Williams, D. L.** (2000). The influence of glucan polymer structure and solution conformation on binding to (1→3)-β-D-glucan receptors in a human monocyte-like cell line. *Glycobiology* **10**, 339-346.

**Nakhost, A., Forcher, P. and Sossin, W. S.** (1998). Binding of protein kinase C isoforms to actin in *Aplysia*. *J. Neurochem.* **71**, 1221-1231.

**Nappi, A. J., Kohler, L. and Mastore, M.** (2004). Signalling pathways implicated in the cellular innate immune responses of *Drosophila*. *Invert. Survival J.* **1**, 5-33.

**Newton, A. C.** (1995). Protein kinase C: structure, function and regulation. *J. Biol. Chem.* **270**, 28495-28498.

**Newton, A. C.** (2003). Regulation of the ABC kinases by phosphorylation: protein kinase C as a paradigm. *J. Biochem.* **370**, 361-371.

**Parker, P. J. and Murray-Rust, J.** (2004). PKC at a glance. *J. Cell Sci.* **117**, 131-132.

**Pearson, G., Robinson, F., Gibson, T. B., Xu, B. E., Karandikar, M., Berman, K. and Cobb, M. H.** (2001). Mitogen-Activated Protein (MAP) Kinase pathways: regulation and physiological functions. *Endocr. Rev.* **22**, 153-183.

**Plows, L. D., Cook, R. T., Davies, A. J. and Walker, A. J.** (2004). Activation of etracellular-signal regulated kinase is required for phagocytosis by *Lymnaea stagnalis* haemocytes. *Biochim. Biophys. Acta* **1692**, 25-33.

**Plows, L. D., Cook, R. T., Davies, A. J. and Walker, A. J.** (2006). Phagocytosis by *Lymnaea stagnalis* haemocytes: a potential role for phosphatidylinositol 3-kinase but not protein kinase A. *J. Invert. Path.* **91**, 74-77.

**Schönwasser, D. C., Marais, R. M., Marshall, C. J. and Parker, P. J.** (1998). Activation of the Mitogen-Activated Protein Kinase/Extracellular signal-regulated kinase pathway by conventional, novel, and atypical Protein Kinase C isotypes. *Mol. Cell. Biol.* **18**, 790-798.

**Segal, B. H., Sakamato, N., Patel, M., Maemura, K., Klein, A. S., Holland, S. M. and Bulkley, G. B.** (1999). Xanthine oxidase contributes to host defense against *Burkholderia cepacia* in the $p47^{phox-/-}$ mouse model of chronic granulomatous disease. *Infect. Immun.* **68**, 2374-2378.

**Sergeant, S. and McPhail, L. C.** (1997). Opsonized zymosan stimulates the redistribution of protein kinase C isoforms in human neutrophils. *J. Immunol.* **159**, 2877-2885.

**Shapira, L., Sylvia, V. L., Halabi, A., Soskolne, W. A., van Dyke, T. E., Dean, D. D., Boyan, B. D. and Schwartz, Z.** (1997). Bacterial lipopolysaccharide induces early and late activation of protein kinase C in inflammatory macrophages by selective activation of PKCϵ. *Biochem. Biophys. Res. Commun.* **240**, 629-634.

**Sminia, T.** (1972). Structure and function of blood and connective tissue of the freshwater pulmonate *Lymnaea stagnalis* studied by electron microscopy and enzyme histochemistry. *Z. Zellforsch. Microsk. Anat.* **130**, 497-526.

**Söderhäll, K. and Cerenius, L.** (1998). Role of the prophenoloxidase-activating system in invertebrate immunity. *Curr. Opin. Immunol.* **10**, 23-28.

**Soldatos, A., Metheni, A., Mamali, I., Lambropoulou, M. and Marmaras, V.** (2003). Distinct LPS-induced signals regulate LPS uptake and morphological changes in medfly hemocytes. *Insect Biochem. Mol. Biol.* **33**, 1075-1084.

**Sonnenburg, E. D., Gao, T. and Newton, A. C.** (2001). The Phosphoinositide-dependent Kinase, PDK-1, phosphorylates conventional protein kinase C isoenzymes by a mechanism that is independent of phosphoinositide 3-Kinase. *J. Biol. Chem.* **276**, 45289-45297.

**Sossin, W. S., Diaz-Arrastia, R. and Schwartz, J. H.** (1993). Characterization of two isoforms of protein kinase C in the nervous system of *Aplysia californica*. *J. Biol. Chem.* **268**, 5763-5768.

**Sritunyalucksana, K., Lee, S. Y. and Söderhäll, K.** (2002). A β-1, 3-glucan binding protein from the black tiger shrimp, *Penaeus monodon*. *Dev. Comp. Immunol.* **26**, 237-245.

**Stabel, S. and Parker, P. J.** (1991). Protein kinase C. *Pharmacol. Ther.* **51**, 71-95.

**Sweet, M. J. and Hume, D. A.** (1996). Endotoxin signal transduction in macrophages. *J. Leukoc. Biol.* **60**, 8-26.

**Tan, S. L. and Parker, P. J.** (2003). Emerging and diverse roles of protein kinase C in immune cell signalling. *J. Biochem.* **376**, 545-552.

**Toreilles, J., Guerin, M. C. and Roch, P.** (1996). Reactive oxygen species and defense mechanisms in marine bivalves. *C. R. Acad. Sci. III Sci. Vie* **319**, 209-218.

**van der Knaap, W. P. W., Adema, C. M. and Sminia, T.** (1993). Invertebrate blood cells – morphological and functional aspects of the haemocytes in the pond snail *Lymnaea stagnalis*. *Comp. Haematol. Int.* **3**, 20-26.

**Vetvicka, V. and Sima, P.** (2004). β-Glucan in invertebrates. *Invert. Survival J.* **1**, 60-65.

**Vetvicka, V. and Yvin, J. C.** (2004). Effects of marine β-1,3 glucan on immune reactions. *Int. Immunopharmacol.* **4**, 721-730.

**Walker, A. J.** (2006). Do trematode parasites disrupt defence-cell signalling in their snail hosts? *Trends Parasitol.* **22**, 154-159.

**Walker, A. J. and Plows, L. D.** (2003). Bacterial lipopolysaccharide modulates protein kinase C signalling in *Lymnaea stagnalis* haemocytes. *Biol. Cell* **95**, 527-533.

**Weinstein-Oppenheimer, C. R., Blalock, W. L., Steelman, L. S., Chang, F. and McCubrey, J. A.** (2000). The Raf signal transduction cascade as a target for chemotherapeutic intervention in growth factor-responsive tumors. *Pharmacol. Ther.* **88**, 229-279.

**Wright, B., Lacchini, A. H., Davies, A. J. and Walker, A. J.** (2006). Regulation of nitric oxide production in *Lymnaea stagnalis* defence cells: a role for protein kinase C and extracellular signal-regulated kinase signalling pathways. *Biol. Cell* **98**, 265-278.

**Zelck, U. E., Janje, B. and Schneider, O.** (2005). Superoxide dismutase expression and $H_2O_2$ production by hemocytes of the trematode intermediate host *Lymnaea stagnalis* (Gastropoda). *Dev. Comp. Immunol.* **29**, 305-314.