Protein kinase C signalling in *Lymnaea stagnalis* haemocytes: a role in molluscan defence

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Declaration

I declare that the work reported in this thesis is entirely my own and has been carried out at Kingston University, UK.

The thesis has not been submitted, in whole or in part, for any other degree at this or any other university.

Audrey H. Lacchini

Abstract

The evolutionarily conserved protein kinase C (PKC) pathway plays an important role in vertebrate immunity, but its role in regulating defence processes in molluscs is poorly understood. Innate defence in the freshwater snail *Lymnaea stagnalis*, intermediate host of the avian schistosome *Trichobilharzia ocellata*, is largely assured by macrophage-like cells called haemocytes. Protein kinase C-like proteins were identified in *L. stagnalis* haemocytes using western blotting with phosphospecific anti-PKC antibodies; the proteins appeared most similar to PKCα and had an approximate molecular weight of 85 kDa. The phosphorylation (activation) of PKC increased over 5-10 minutes in response to the glucan-based compounds laminarin and zymosan, the bacterial endotoxin, lipopolysaccharide (LPS), and a PKC activator phorbol 12-myristate 13-acetate (PMA), with a 3.5-fold increase observed following challenge with laminarin. Activation of PKC was also shown by immunocytochemistry, and confocal microscopy revealed possible localisation of phosphorylated PKC to the plasma membrane following laminarin challenge. Pharmacological inhibitors of PKC reduced the phosphorylation of extra extracellular-signal regulated kinase 1/2 (ERK 1/2) and mitogen-activated protein/ERK kinase (MEK) in laminarin-challenged haemocytes demonstrating that the ERK pathway is a downstream target of PKC in these cells. Moreover, inhibitor experiments revealed phospholipase C (PLC), but not phosphoinositide-3 kinase (PI-3-K), to be upstream regulators of PKC in laminarin-challenged haemocytes. The effects of the schistosome epitopes Lewis X and Lac-di-Nac on haemocyte PKC phosphorylation were also investigated in presence of haemolymph; however, these compounds did not alter the phosphorylation status of PKC-like proteins over 30 minutes. Finally, two haemocyte defence functions were found to be partially PKC-dependent: the production of the reactive oxygen intermediate (ROI) hydrogen peroxide (H_2O_2), and cell spreading. Whereas challenge of haemocytes with laminarin resulted in a 9.5-fold increase in H_2O_2 output by haemocytes, inhibition of PKC reduced this response by up to 65 %; cell spreading was also reduced by up to 64 %. Moreover, the tyrosine kinase Src was found to play a role in haemocyte spreading; this effect could be mediated by a focal adhesion kinase (FAK)-dependent pathway. In conclusion, this study broadens our knowledge of the molecular mechanisms regulating innate defence in snails. The results should facilitate further research on molluscan immunity that could be of value in advancing our understanding of snail-schistosomes interactions.

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« A toi, petit Victor Hugo du Bachut,
Je dédie ces quelques lignes décousues,
Ces graphes et ces images saugrenus,
Le contenu te paraîtra peut-être inconnu,
Mais il représente détermination et travail assidu,
Zeste de patience et de vertue,
Pour un résultat à la valeur attendue»

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Abbreviations

ACTH	adrenocorticotropin hormone
ANOVA	analysis of variance
aPKC	atypical PKC
Bge	*Biomphalaria glabrata* embryonic
BH4	tetrahydrobiopterine
BSA	bovine serum albumin
c-AMP	cyclic- adenosine monophosphate
Ca^{2+}	calcium
cPKC	classical PKC
DAG	diacylglycerol
DMSO	dimethyl sulfoxide
DTT	dithiothreitol
ECM	extracellular matrix
EDTA	ethylenediaminetetraacetic acid
eNOS	endothelial NOS
ERK 1/2	extracellular-signal regulated kinase 1/2
ESPs	excretory/secretory products
EST	expressed sequence tags
FAD	flavine adenine dinucleotide
FAK	focal adhesion kinase
FBG	fibrinogen
FITC	fluorescein isothiocyanate
fMLP	formyl methionyl leucyl phenylalanine
FMN	flavine mononucleotide
FREP	fibrinogen-related proteins
GDP	guanosine diphosphate
GTP	guanosine triphosphate
GNBP	Gram-negative binding proteins
H_2O_2	hydrogen peroxide
$HO^{\cdot}$	hydroxyl radical
HRP	horseradish peroxidase
IFN-γ	interferon-γ
Ig	immunoglobulin
IL-1	interleukin-1
IL-1R	interleukin-1 receptor
IL-2	interleukin-2

IL-4	interleukin-4
IL-6	interleukin-6
IL-8	interleukin-8
iNOS	inducible NOS
IP_3	inositol triphosphate
JAK	janus tyrosine kinase
JNK	c-Jun N-terminal kinase
kDa	kilodalton
LDN	Lac-di-nac
LDNF	fucosylated lac-di-nac
Le^x	Lewis x
LPS	lipopolysaccharide
LSD	least significant difference
MAPK	mitogen-activated protein kinase
MARCKS	myristoylated alanine-rich C-kinase substrate
MDM	molluscan defense molecule
MEK	MAP/ERK kinase
MEKK	MEK kinase
MWM	molecular weight marker
NADPH	nicotinamide adenine dinucleotide phosphate
NF-κβ	nuclear factor-κβ
NO	nitric oxide
NOS	nitric oxide synthase
nPKC	novel PKC
O_2	molecular oxygen
O_2^-	superoxide anion
OCl^-	hypochlorite anion
PAGE	polyacrylamide gel electrophoresis
PAMP	pathogen-associated molecular pattern
PBS	phosphate-buffered saline
PDGF	platelet-derived growth factor
PDK-1	phosphoinositide-dependent kinase-1
PGRP	peptidoglycan recognition protein
PI-3-K	phosphoinositide-3 kinase
PIP_2	phosphatidylinositol-4, 5-biphosphate
PKA	protein kinase A
PKB	protein kinase B
PKC	protein kinase C

PKD	protein kinase D
PLC	phospholipase C
PMA	phorbol 12-myristate 13-acetate
PO	phenoloxidase
PPO	prophenoloxidase
PRR	pattern recognition receptor
PS	phosphatidylserine
RACK	receptor for activated protein kinase C
RAW264	murine macrophage cell line
RICK	receptors for inactived protein kinase C
RMSA	rat mesenteric small artery
RNAi	RNA interference
RNI	reactive nitrogen intermediate
ROI	reactive oxygen intermediate
RSNO	nitrosothiol
TGF-β	transforming growth factor-β
TNF	tumor necrosis factor
TRITC	tetramethyl rhodamine isothiocyanate
TTBS	Tween 20 Tris-buffered saline
SD	standard deviation
SDS	sodium dodecyl sulphate
SH1	Src-homology domain 1
SH2	Src-homology domain 2
SH3	Src-homology domain 3
SH4	Src-homology domain 4
SOD	superoxide dismutase
Sos	Son of sevenless
SSS	sterile snail saline
STAT	signal transducers and activators of transcription
STICK	substrates that interact with C-kinase
µg	microgram
µl	microlitre
nm	nanometre
M	molar
mM	millimolar
mV	millivolts
SDS-PAGE	sodium dodecyl sulphate-polyacrylamide gel electrophoresis
v/v	volume to volume

w/v	weight to volume
XO	xanthine oxidase

"Every search starts with beginner's luck. And every search ends with the victor's being severely tested."

Paulo Coelho "The Alchemist"

Chapter 1

General Introduction

1.1 The Mollusca

Molluscs are the second largest living group of invertebrates, with approximately 150,000 species described and more than 120,000 living species, ranking just below arthropods in numbers. The phylum is effectively characterised by soft bodied (Latin for soft is "mollusc"), unsegmented animals usually protected by an external shell. Seven classes of molluscs have been defined based on foot and shell differences with the most common classes being the Gastropoda, Bivalvia and Cephalopoda (Fig.1.1). The Gastropoda comprise approximately 90,000 living species that are distributed in marine, freshwater or terrestrial environments (Nicol, 1969; Myers and Burch, 2001). Sea slugs, snails (e.g. *Lymnaea stagnalis*, *Biomphalaria glabrata*), and limpets are examples of this group. The Bivalvia comprise mainly aquatic molluscs including mussels (e.g. *Mytilus edulis*, *Mytilus galloprovinciallis*), clams, and oysters (e.g. *Crassostrea gigas*); many members of this latter group are important to aquaculture and are thus of commercial importance. The Cephalopoda, likely one of the most intelligent group of invertebrates, are predominantly represented by squid and octopuses.

Fig.1.1: The freshwater snail *Lymnaea stagnalis*, the experimental model organism for this project, belongs to the order Pulmonata (The classification table was derived from http://www.ncbi.nlm.nih.gov/Taxonomy).

The extensive study of molluscs reflects their importance in ecology, their position in the food web, their use in gastronomy in countries such as in France, and their role as vectors of human and animal pathogens responsible for worldwide diseases (Mas-

Coma, 2005; Gryseels *et al.*, 2006). In addition, molluscs are crucial pests in agriculture and many species cause extensive damage to crops, causing large financial losses worldwide (Barker, 2002). Studies that further our understanding of molluscan immunology might help with efforts to develop novel control strategies for mollusc pests. Additionally, such studies may help to elucidate some of the mechanisms employed by parasites to evade the immune response of their intermediate molluscan hosts (Walker, 2006).

1.2 *Lymnaea stagnalis*, the experimental model organism for this study

Lymnaea stagnalis (Linnaeus) (the Great Pond Snail) belongs to the class Gastropoda (Fig.1.1). This snail is characterised by a yellow-brown to dark-brown/grey shell and adults can measure from 50-60 mm high and 25-30 mm across (Olsen *et al.*, 2001). Widespread in Europe but also in North America and North Africa, *L. stagnalis* are generally found in slow moving water.

Lymnaea stagnalis possesses a mantle which secretes the shell and a foot that is used for locomotion. The radula in the buccal cavity is a rough tongue that is made of chitin, a polysaccharide also found in arthropods (Merzendorfer and Zimoch, 2003). The various teeth on the radula act as scrapers to rasp away at food (Kemenes *et al.*, 1986). Pulmonate gastropods are hermaphrodite and cross-fertilization is favoured (Baily, 1931). Two weeks after mating, eggs are laid by adult snails in clusters contained in a yellowish gelatinous matrix (Dickinson and Croll, 2001). Like other gastropods, *L. stagnalis* lacks body segmentation; nevertheless it has well-developed nervous, respiratory and circulatory systems. The nervous system has been extensively studied for more than 25 years (with early research done by workers such as Roubos and Moorer-van Delft, 1979), in particular for its role in behavioural responses such as long term memory formation; *L. stagnalis* has thus become a model of choice for neurobiology research. The respiratory cavity enables the snail to come to the surface to take air, although uptake of oxygen can also occur through the skin while in water (Syed *et al.*, 1991). The circulatory system in *L. stagnalis* is open, and haemolymph (plasma) bathes the body organs. Haemocyanin, commonly found in molluscs and arthropods and widely distributed in the animal kingdom with haemoglobin (van Holde and Miller, 1995), constitutes the respiratory pigment in *L. stagnalis* (Hall *et al.*, 1975). The extrusion of haemolymph, which contains defence

cells (haemocytes), through the haemal pore (pneumostome) results from continuous stimulation of the head-foot region (Fig.1.2); the volume expelled can be quickly restored (Martin and Deyrup-olsen, 1982). The basic biology of the internal defence system of *L. stagnalis* is well-understood (Van der Knaap *et al.*, 1993), making this an appropriate species for studies into the molecular control of immune responses in gastropod molluscs.

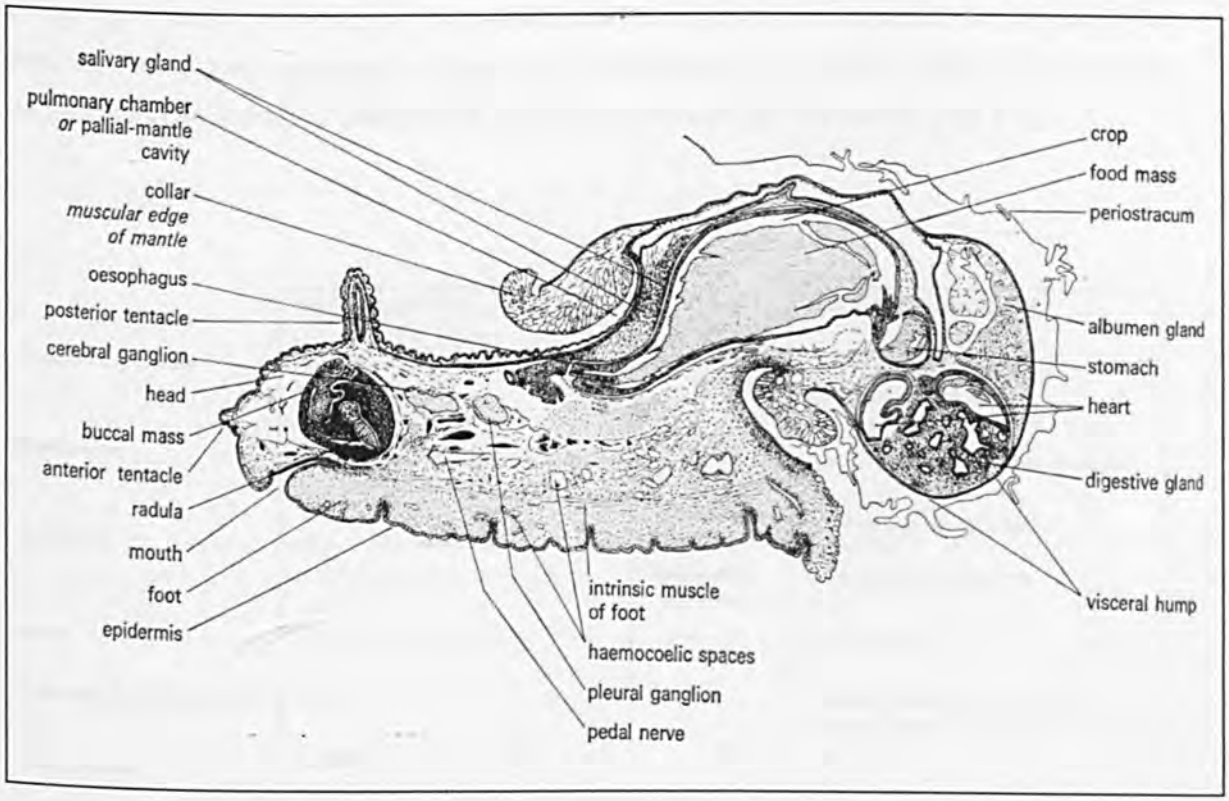

Fig.1.2: Diagram showing the generalised internal anatomy of *L. stagnalis* (reproduced from Freeman and Bracegirdle, 1988).

The defence mechanisms that *L. stagnalis* employs to kill micro-organisms or other pathogens are discussed in section 1.5.2.

1.3 Vertebrate immunity versus invertebrate immunity

Immunology is a vast field in which vertebrates have received much of the attention even though their immune systems have evolved originally from their ancestors, the invertebrates. The immune system of invertebrates is less complicated and developed than that of vertebrates, but it remains remarkably efficient. The two major animal immune systems can be described as follows: "the invertebrates possess natural, non-adaptive, innate, non-clonal, non-anticipatory responses; those of vertebrates are induced, adaptive, acquired, clonal, and anticipatory" (Cooper, 2003). Thus, unlike vertebrates, no memory component exists in invertebrate immunity (Fig.1.3).

	INNATE IMMUNE SYSTEM		ADAPTIVE IMMUNE SYSTEM
Evolutionary history	Ancient (plants, insects, mammals) Billions of years old		Modern (jawed vertebrates) 400 million years old
Recognition	PAMPS (commonly carbohydrates and lipids)		Specific detail of molecular structure
Receptors	Fixed in genome (invariant) Rearrangement not necessary Non-clonal Diverse cellular distribution	**Co-stimulation** **Education** **Cooperation** ⟷	Encoded in gene segments (variability) Rearrangement necessary Clonal Lymphocytes
Self-nonself discrimination	Perfect		Imperfect: hence, autoimmune disease, allergy and allograft rejection
Time to onset	Immediate		Delayed
Memory	No		Yes

Fig.1.3: Simplified identity card of the innate and adaptive immune systems and their respective characteristics (adapted from McGuinness *et al.*, 2003).

Their mutual co-evolution means that they are functionally dependent on each other for optimal anti-pathogen responses. Pathogen-associated molecular patterns (PAMPs) are essential compounds recognised by the pattern recognition receptors (PRRs). For further details, see section 1.5.

1.4 Overview of immunity in vertebrates

1.4.1 Adaptive immunity

The adaptive arm of immunity is thought to be found only in vertebrates with jaws and this line of defence is generally considered to have evolved from the innate system of the invertebrates (Bleyzac *et al.*, 2005). Phylogenetically younger, the lymphocytes are the main effectors of the adaptive immune response. The components of the adaptive immune system have been reviewed by many authors (see for examples Ahmed and Gray, 1996; McHeyzer-Williams *et al.*, 2006). Since the focus of the present work is the innate defence response, adaptive immunity is beyond the scope of this thesis and therefore not discussed further.

1.4.2 Innate immunity

1.4.2.1 Cellular responses

Natural killer (NK) cells and phagocytic cells mediate the second line of defence, responding immediately to invaders that breach physical barriers. Natural killer cells are lymphocytes by lineage but lack the characteristic features of lymphocytes such as immunoglobulin receptors (Rosmaraki *et al.,* 2001); they are able to kill cells infected by microbes, or tumour cells. It has been suggested that cytokines and interferons play a major role in the recognition of these abnormal cells and indirectly activate NK cells (Colucci *et al.*, 2003).

Phagocytes comprising macrophages, neutrophils, and granulocytes, fight against invading organisms by engulfing them. The phagocytic activities require chemotaxis, which enables the phagocytes to move towards pathogens through the presence of a gradient of attractant molecules; adhesion between the phagocyte and target is mediated by cell adhesion proteins, mainly transmembrane receptors (Snyderman and Pike, 1984); and destruction of pathogens trapped in phagosomes is via digestive and hydrolytic enzymes released during the fusion of lyzosomes and phagosomes (May and Machesky, 2001). Reactive oxygen intermediates (ROIs) and reactive nitrogen intermediates (RNIs) are also crucial to the destruction of the invading organism (Bogdan *et al.*, 2000). Additionally, anti-microbial peptides and the activation of the complement system help destroy pathogens. Anti-microbial peptides (of approximately 50 amino acids) such as the defensins (reviewed by Ganz, 2004) or the cathelicidins LL-37 (Zanetti *et al.*, 1995) are present in the cytoplasmic granules of neutrophils and

do not have any enzymatic activities. These peptides exert their killing action by permeabilising the membrane of the invading pathogens (Sitaram and Nagaraj, 1999). The complement system, which stimulates the immune system, is composed of approximately 30 soluble or cell-bound proteins, including the protease C3 convertase, and is mainly activated via three biochemical pathways: the classical pathway (antibody-dependent), the lectin pathway, and the alternative pathway (Song *et al.*, 2000; Mollnes *et al.*, 2002).

1.4.2.2 Reactive oxygen intermediates

1.4.2.2.1 Production of ROIs

Reactive oxygen intermediates are potent cytotoxic compounds and include molecules like hydrogen peroxide (H_2O_2), ions like the superoxide (O_2^-) or hypochlorite (ClO^-) anion and radicals such as the hydroxyl radical ($OH^\cdot$) (Babior, 2000). Three specific enzymes catalyse the generation of these oxidants: nicotinamide adenine dinucleotide phosphate (NADPH) oxidase, superoxide dismutase (SOD), and myeloperoxidase which is restricted to neutrophils only (Fig.1.4).

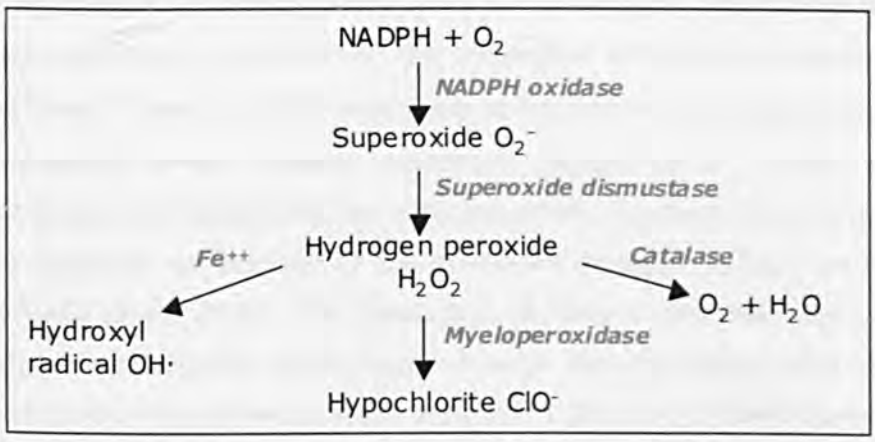

Fig.1.4: Schematic diagram showing reactions leading to the production of the four main ROIs, O_2^-, H_2O_2, OH^-, ClO^- in phagocytes.
The four enzymes involved are NADPH oxidase, SOD, catalase and myeloperoxidase. The presence of Fe^{++} is required for the formation of OH^- (Halliwell and Gutteridge, 1985).

In mammals, NADPH oxidase is composed of cytosolic and membrane-bound subunits. Upon external activation of the phagocyte, the free cytosolic subunits are targeted to

the membrane of the phagocytic vacuole and assemble with the membrane associated subunits rendering the enzyme functionally active (Morel *et al.*, 1991; Dusi and Rossi, 1993). This oxidase belongs to a transmembrane electron transporter system which enables electrons to migrate from the intracellular substrate, NADPH, to the final molecule, oxygen.

The final product of the NADPH oxidase reaction is O_2^- which subsequently dismutes to H_2O_2 via SOD (Cross *et al.*, 2004). In neutrophils, the myeloperoxidase converts H_2O_2 to the strongly antiseptic hypochlorite ion ClO^- (Harrison and Shultz, 1976). In order to prevent any damaging effects of ROIs in the cellular environment, the cell employs various enzymes such as catalase and glutathione peroxidase to detoxify them. Other anti-oxidants include vitamin E, and ascorbic or uric acid (Robinson and Babcock, 1998).

1.4.2.2.2 Regulation of ROI production

While the production of ROIs by phagocytes helps protect against infection, an uncontrolled generation may lead to classic tissue injury and damage (Henson and Johnston, 1987). Reactive oxygen intermediates propagate inflammation by helping the recruitment of other immune cells by stimulating the release of cytokines such as interleukin-1 (IL-1) (Lander, 1997). In addition, ROIs have been shown to function as intracellular messengers (Nathan, 2003); control mechanisms are therefore important to cell/tissue homeostasis. Innibition of the production of ROIs may occur via various signalling pathways[1] such as c-AMP independent activation of a serine/threonine (Thr) protein phosphatase in the plasma membrane (Revan *et al.*, 1996). Adenosine-dependent pathways in neutrophils can also impair the production of ROIs (Cronstein *et al.*, 1985) likely via an increase of the adenosine receptor A (A2), as observed in monocytes (Thiele *et al.*, 2004). The production of ROIs is also mediated via integrin-dependent signalling pathways which occur through the intracellular PML-retinoic acid receptor alpha (RARα)-regulated adaptor molecule 1 (PRAM-1) (Clemens *et al.*, 2004). The stress-activated p38 mitogen-activated protein kinase (MAPK) pathway also plays a role in integrin-dependent cell adhesion and respiratory burst in neutrophils (Detmers *et al.*, 1998; Forsberg *et al.*, 2001) whereas the role of the p42/44 MAPK pathway appears minor (Zu *et al.*, 1998).

The regulation of ROI production also depends on controlling the activity of the main enzyme, NADPH oxidase and numerous studies have reported the role of phosphorylation and cytoskeletal reorganisation in this process (Morimatsu *et al.*, 1997; Nixon & McPhail, 1999). In neutrophils, regulation is partly achieved via

[1] "For an introduction to cell signalling, refer to section 1.7"

translocation of rac GTPase along with the active components of the oxidase (Bokock and Diebold, 2002). In macrophages, arachidonic acid (AA) resulting from phospholipase A2 activity contributes to the translocation of NADPH subunits, whereas PKC is crucial in regulating NADPH oxidase by phosphorylation (Dekker *et al.*, 2000; Fontayne *et al.*, 2002; Cathcart, 2004); these events are important to the assembly or maintenance of the complex (Majumdar *et al.*, 1993). The signalling pathways that regulate ROI production are considered further in Chapter 4.

1.4.2.3 Reactive nitrogen intermediates

1.4.2.3.1 Production of RNIs

Discovered in the 1980s (Furchgott and Zawadzki, 1980), the role of nitrogen monoxide (commonly named nitric oxide (NO)), a product of nitric oxide synthase (NOS) activity, in physiological systems has been the focus of much research (Brüne and Cantoni, 2000); NO has been found to have a multifunctional role in various cellular processes. Not only does NO appear to be a critical player in host defense where NO-mediated cytotoxicity is often associated with the formation of nitrosyl-thiol complexes in enzymes within the target cell (Nathan, 1992), but it also possesses immunoregulatory properties as an intra- and inter-cellular signalling molecule (Bogdan, 2001).

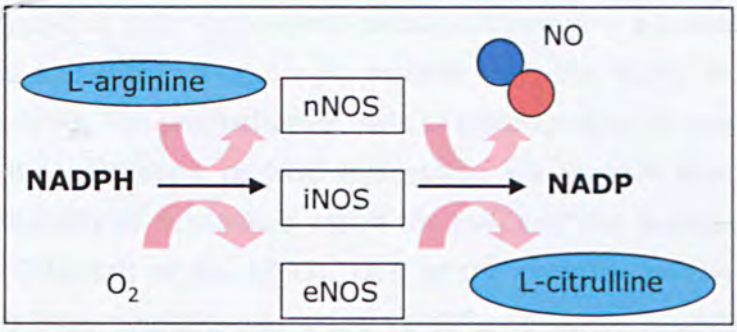

Fig.1.5: Simplified reaction scheme showing the generation of NO by NOS.
The reaction comprises a two-step conversion in which the amino acid L-arginine is first hydroxylated by O_2 and NADPH to form N-ω-hydroxy-L-arginine and then oxidised to form the final products, L-citrulline and NO. See text for definition of the different NOS isoforms.

Nitrogen monoxide exists in different redox states and the NO radical ($NO^{\cdot}$) has received much of the attention so far. Nitric oxide is produced by five successive

oxidation reactions involving L-arginine (Arg) and oxygen (O_2) (Fig.1.5). Extra cofactors/coenzymes are required for the reaction such as NADPH, flavine adenine dinucleotide (FAD), flavine mononucleotide (FMN), tetrahydrobiopterine (BH4), and protoporphyrin IX (Porasuphatana *et al.*, 2003).

Nitric oxide synthase is a membrane-anchored enzyme which must exist as homodimer to be catalytically active (Alderton *et al.*, 2001). Three distinct NOS isotypes have been identified and each possesses a tissue-specific distribution:

- type I or neuronal NOS (nNOS)/brain NOS (bNOS)
- type II or inducible NOS (iNOS)
- type III or endothelial NOS (eNOS)

Inducible NOS is produced in immune cells but also in other cells under conditions that necessitate an immunobiological response; for example, endotoxins or bacteria can induce the expression of this isoform (Kilbourn and Griffith, 1992; Wheeler *et al.*, 1997). The two other NOS isoforms are expressed constitutively by either neuronal cells or endothelial cells and the activities of these two isoforms are dependent of calcium (Ca^{2+}) influx (Bredt and Snyder, 1990). However, the presence of calmodulin as a Ca^{2+} binding protein seems to be required as a mediator for all isoforms (Cho *et al.*, 1992; Spratt *et al.*, 2006).

1.4.2.3.2 Regulation of RNI production

An effective cell-mediated defence towards pathogens requires a balance between the production of ROIs and RNIs (Fig.1.6). To achieve this, like ROIs, the production of RNIs must be regulated. The production of RNIs in macrophages is controlled by many factors including the regulation of NOS expression via various signal transduction pathways, the availability of substrates within the cell, and the feedback effects of the final product NO (Colasanti *et al.*, 1995). Due to the complex involvement of NO in immunity, it seems that regulation can occur at different levels resulting in controlled effects on both iNOS mRNA and proteins (Carpenter *et al.*, 2000). The activation of NOS and the subsequent synthesis of RNIs results from the activation of various intracellular signalling pathways such as the nuclear factor-κB (NF-κB) and janus tyrosine kinase (JAK) pathways modulated by pro-inflammatory mediators such as cytokines (Kleinert *et al.*, 1998). In this context, interferon-γ (IFN-γ), and tumor necrosis factor (TNF) act as positive regulators of iNOS expression in macrophages, whereas interleukin-4 (IL-4), interleukin-8 (IL-8), interleukin-12 (IL-12), and transforming growth factor-β (TGF-β) mediate inhibitory effects on the expression of the enzyme (Bogdan *et al.*, 2000).

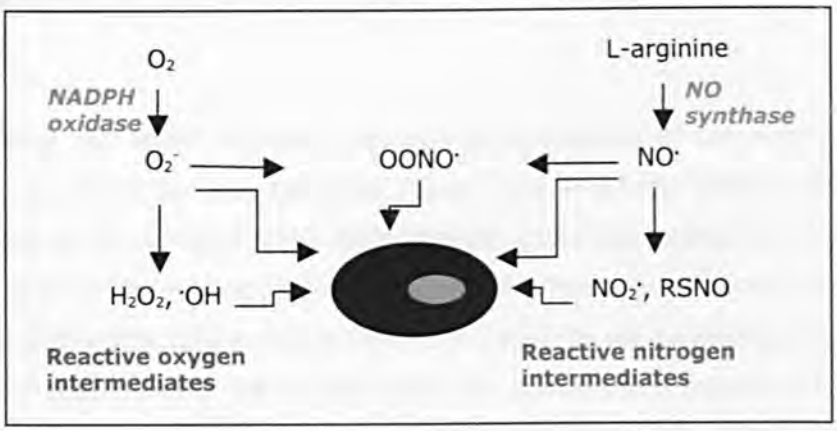

Fig.1.6: Cartoon comparing reactions leading to the production of ROIs and RNIs in response to attack from a pathogen (adapted from Chakravortty and Hensel, 2003).
On the left hand side, NADPH oxidase generates O_2^- from O_2. Superoxide either rapidly dismutates to H_2O_2 via SOD or combines with NO· to form ONOO· (Pryor and Squadrito, 2003). The hydroxyl radical OH· is thought to result from an iron-catalysed reaction from H_2O_2 and O_2^- (Kehrer, 2000). On the right hand side, NO is produced by oxidation of the terminal nitrogens on arginine via NOS. Various redox reactions convert NO into nitrosothiols (RSNOs) (Stamler *et al.*, 2001).

Various microbial compounds such as LPS also up-regulate iNOS expression in cells (Brightbill *et al.*, 1999). Protein kinase C has been reported to play a role in iNOS activation in mouse macrophage cell lines (Severn *et al.*, 1992; Diaz-Guerra *et al.*, 1996; Paul *et al.*, 1997), although MAPKs have been determined as key signal transducers: extracellular signal-regulated kinase 1/2 (ERK 1/2) up-regulates lipopolysaccharide (LPS)-induced iNOS expression in J774 mouse macrophages (Lathi *et al.*, 2000) whereas c-Jun N-terminal kinase (JNK) stimulates iNOS expression in LPS- and IFN-challenged-glial cells (Pawat and Bhat, 2006). p38 MAPK has also been shown to mediate the activation of iNOS at the transcriptional level (Bhat *et al.*, 2002).

Along with iNOS, eNOS is regulated directly at the transcriptional level (Bloch, 1999) but also via protein-protein interactions (Shah *et al.*, 1999); for example palmitoylation and myristoylation constitute translational regulatory mechanisms (Liu *et al.*, 1995) whereas phosphorylation of the enzyme of serine (Ser) residues by PKC, protein kinase A (PKA), and protein kinase B (PKB) (also termed as Akt) modulate eNOS activity (Michel *et al.*, 1993; Chen *et al.*, 1999; Michell *et al.*, 1999; Michell *et al.*, 2001). Regulation of nNOS activity can occur at the transcriptional stage (Wang *et al.*, 1999b) and various hormones, neurotransmitters, or physical stimuli can promote the expression of this isoform (Förstermann *et al.*, 1998).

1.5 Immunity in invertebrates

Although lacking the adaptive arm of immunity, analogues of the vertebrate immune system can be found in invertebrates (Beck and Habicht, 1996). Cooper (1996) considered that in a world replete with foreign material, antigens of various kinds threaten the life of invertebrates. Inherent in invertebrate immunity is the need for recognition and the assurance that antigens will bind to an appropriate receptor on an effector cell, or that there is the equivalent of an antigen processing cell that captures such antigens, enabling them to be destroyed by the effector cell; this would serve to set the immune response in motion.

In this context, invertebrate immunity is known to involve both cellular and humoral mechanisms that are synchronised to provide optimal protection from invading pathogens such as bacteria, parasites and viruses (Rinkevich and Muller, 1996; Yoshino *et al.*, 1999). The recognition of non-self, in particular via pathogen-associated molecular patterns (PAMPs), is mediated through receptors encoded in the germline and expressed in effector cells such as haemocytes (Zheng *et al.*, 2005). These pattern recognition receptors (PRRs) are able to distinguish conserved microbial features such as LPS from Gram-negative bacteria, peptidoglycan from Gram-positive bacteria, β- 1, 3 glucan from fungi, and nucleic acids (bacterial or viral DNA or RNA) (Kim *et al.*, 2000; Yu *et al.*, 2002; McGuinness *et al.*, 2003; Kim *et al.*, 2004). Much of our knowledge concerning invertebrate immunity is a consequence of work that has been done with insects.

1.5.1 Overview of immune mechanisms in insects

1.5.1.1 Cellular defence processes

As previously noted, internal defence in insects is mediated by haemocytes (Table 1.1) helped by humoral mechanisms to provide a well-coordinated and integrated response to invasion. Haemocytes are involved in phagocytosis, nodule formation, and encapsulation when foreign particles are too big to be phagocytosed (Ratner and Bradleigh Vinson, 1983). In addition, in insects, activation of the humoral response triggers a cascade of reactions, with the complex prophenoloxidase (proPO) system being a main regulator of coagulation during melanisation (Lavine and Strand, 2002). The process of phagocytosis by insect haemocytes mirrors that seen in mammalian phagocytes allowing them to engulf and digest microorganisms, cellular debris,

insoluble particles, and damaged or dead host cells (Gillepsie *et al.*, 1997). Nodule formation occurs when haemocytes aggregate to entrap invading bacterial cells or other microorganisms; during this process, recruitment of haemocytes facilitates microagreggation of these cells resulting in the generation of mature nodules attached to the body wall and various internal organs (Vilmos and Kurucz, 1998). The encapsulation response generates organised multicellular capsules comprising overlapping layers of haemocytes surrounding the foreign material (Pech and Strand, 1996). In association with melanogenetic components, killing of pathogens results from the cytotoxic activities of both ROIs and RNIs (Nappi and Vass, 1993; Kumar *et al.*, 2003) as observed in the fruit fly *Drosophila melanogaster* (Nappi *et al.*, 2000). For example, in *D. melanogaster*, both O_2^- and H_2O_2 participate in the encapsulation response after flies are infected with the parasitoid wasp *Leptopilina boulardi* (Nappi *et al.*, 1995; Nappi and Vass, 1998). Moreover, O_2^- generation constitutes an early response in the silk moth *Bombyx mori* following bacterial infection (Krishnan *et al.*, 2002).

Table 1.1: Subpopulation of immune cells involved in insect defence.
Drosophila melanogaster is a Dipteran whereas *M. sexta*, *H. cecropia*, and *B. mori* are representatives of Lepidopteran insects.

Type of insect	Subpopulation of immune cells	Functions	References
Drosophila melanogaster	• Plasmatocytes	• Resemble monocytes, role in phagocytosis	Meister and Lagueux, 2003
	• Crystal cells	• Involved in melanisation	
	• Lamellocytes	• Role in encapsulation	
Manduca sexta, Hyalophora cecropia, Bombyx mori	• Plasmatocytes	• Role in spreading, phagocytosis, nodulation	Willot *et al.*, 1994 Wang *et al.*, 1999
	• Granulocytes	• Involved in phagocytosing apoptotic cells, encapsulation, nodulation	Abu-Hakima and Faye, 1981 Lea and Gilbert, 1966
	• Oenocytoids	• Role in cuticule formation and production of melanisation enzymes	Dean *et al.*, 2004 Ling *et al.*, 2003 Ling *et al.*, 2005
	• Spherulocytes	• Role in melanisation, do not adhere to glass	
	• Hyperphagocytic cells (*M. sexta*)	• Role in phagocytosis	
	• Prohaemocytes (*B. mori*)	• Role in phagocytosis	

1.5.1.2 Humoral defence processes

In insects, humoral defence is mainly characterised by the presence of the complex prophenoloxidase (ProPO) system which is often considered as the ancient form of the complement system of vertebrates (Cerenius and Söderhäll, 1995; Zarkadis *et al.*, 2001). The ProPO system has been well characterised in *D. melanogaster* and *Manduca sexta*; it has been shown to respond to many PAMPs including LPS, β-1, 3-glucan or peptidoglycan and facilitate the production of melanin, a crucial clotting agent that traps foreign organisms (Cerenuis and Söderhäll, 2004). The transformation of the pro-form of the prophenoloxidase-activating enzyme (ProPO-A) into fully activated ProPO-A operates via serine protease cascades; although the precise mechanisms controlling these events are not fully understood (Cerenius and Söderhäll, 2004). Subsequently, phenoloxidase (PO) gets activated and catalyses the first steps which lead to the generation of quinone-dependent melanin (Ashida and Brey, 1997). Similar to melanisation in insects, the horseshoe crab *Limulus polyphemus* clotting system also involves a cascade of serine protease zymogens (Nakamura *et al.*, 1986). Interestingly, invertebrate immunoglobulin (Ig)-like proteins have been described in *D. melanogaster* (Seeger *et al.*, 1988), one of these, amalgam, contains three Ig-like domains and is a secreted protein (Frémion *et al.*, 2000). Similarly, hemolin has been identified in the insects *M. sexta* and in *Hyalophora cecropia* as a member of the Ig superfamily (Sun *et al.*, 1990). Inducible under infection, hemolin may play an active function in controlling the cellular immune response (Lanz-Mendoza *et al.,* 1996).

1.5.1.3 Overview of signalling pathways controlling immune responses in insects

Here, some of the cell signalling mechanisms that control the innate immune response of insects are described. For a general overview of the concepts of cellular signal transduction, the reader is referred to section 1.7. Our understanding of the molecular mechanisms that underpin the insect defence response is largely a consequence of excellent work that has been carried out in two insect models, *D. melanogaster* and *B. mori*.

Some of intracellular pathways regulating the *D. melanogaster* immune response (De Gregorio *et al.*, 2002) are highly conserved and are similar to the Toll-like receptor (TLR)/interleukin-1 receptor (IL-1R) and TNF-α pathways that play a role in mammalian innate immunity. During fungal or bacterial infections, two major

signalling pathways are activated in insect fat body cells: the Toll-signalling pathway and the immune deficiency (Imd) signalling pathway (De Gregorio *et al.*, 2002; Leclerc and Reichhart, 2004) (Fig.1.7). The pathways involve the participation of two types of PRRs: the peptidoglycan recognition proteins (PGRPs) and the Gram-negative binding proteins (GNBPs).

The activation of the Toll pathway results in a cascade of reactions initiating the recruitment of surface-bound proteins dMyd88/Tube/Pelle (Belvin and Anderson, 1996), and then, a cytosolic protein Cactus which ultimately induces the expression of antimicrobial peptides such as drosomycin via stimulation of transcription factors such as Dif, an NF-κB homologue (Fig.1.7) (Ip *et al.*, 1993; Rutschmann *et al.*, 2000).

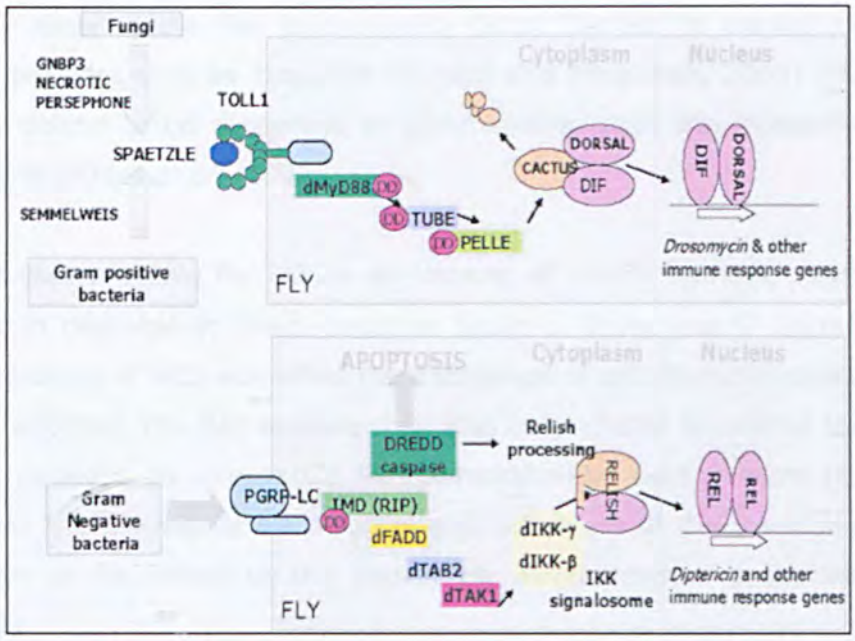

Fig.1.7: Schematic molecular representation of the Toll and Imd pathways in *D. melanogaster* induced by fungal and bacterial components (adapted from Govind and Nehm, 2004).
Drosophila host defense to fungi or Gram-positive bacteria is mediated by Spaetzle/Toll/Cactus which are the main components of the Toll pathway. Activation of the TLR receptor by the cleaved form of the cytokine Spaetzle triggers the recruitment of the adaptor complex composed of dMyd88, Tube, and the kinase Pelle. The complex induces the phosphorylation of Cactus, a protein originally bound to the nuclear factor Dif. Dif is then translocated into the nucleus which results in stimulation of the expression of various genes encoding for antimicrobial peptides such as drosomycin. Upon activation with Gram-negative bacteria, the Imd pathway is activated via Relish, a protein ultimately phosphorylated by the dTak1 cascade and the downstream IKK complex. Phosphorylated Rel becomes a substrate for the apical caspase Dredd. Removed from its inactivating domain, Rel translocates to the nucleus to trigger antimicrobial peptide expression (Wang and Ligoxigakis, 2006).

Whereas the Toll pathway is activated in response to fungi and peptidoglycans (of the Lys-type) from Gram-positive bacteria, the Imd pathway is activated by peptidoglycans (of the Meso-DAP type) from Gram-negative bacteria. Interaction of such ligands with the transmembrane receptor PGRP-LC transduces signals through the protein Imd/mammalian receptor interacting protein 1 (RIP) (Georgel *et al.*, 2001). The protein Imd subsequently associates with the adaptor *Drosophila* FADD (Naitza *et al.*, 2002) which ultimately activates the *Drosophila* MAP kinase kinase kinase dTak1 (Vidal *et al.*, 2001) via *Drosophila* dTab2 (Zhuang *et al.*, 2002). The enzyme dTak1 acts upstream of the *Drosophila* IKK complex (Vidal *et al.*, 2001) whose activation mechanism has recently been elucidated (Zhou *et al.*, 2005). In unstimulated cells, the NF-κβ homologue Relish (Rel) is held in the cytoplasm. Upon Imd-dependent stimulation, phosphorylated Rel in a *Drosophila* complex IKK-dependent fashion is then endoproteolytically cleaved by the caspase Dredd (Stöven *et al.*, 2003) allowing the Rel transcription factor further to transcribe genes for antimicrobial peptides such as diptericin (Kaneko and Silverman, 2005) (Fig. 1.7). The Imd pathway seems to be conserved in other species such the mosquito *Anopheles gambiae* (Christophides *et al.*, 2002).

Studies have shown a role for NO as an inducer of innate immune responses in *D. melanogaster* in response to Gram-negative bacteria (Foley and O' Farrel, 2003). In this study, inhibition of NOS abolished the expression of antimicrobial peptides such as diptericin. In addition, the JNK pathway has also been shown to control the release of antimicrobial peptides by *Drosophila* S2 haemocyte-like cells *in vitro* (Kallio *et al.*, 2005) whereas the JAK/signal transducers and activators of the transcription (STAT) pathway seem to be linked to the *Drosophila* developmental processes (Luo and Dearolf, 2001).

Although many studies have focused on the signalling pathways regulating the production of antimicrobial peptides in insects, other research has explored the role of signalling in functional responses, such as phagocytosis or cell adhesion in *Drosophila*. The process of *E. coli* phagocytosis by Mediterranean fruit fly *Ceratitis capitata* haemocytes involves the participation of the focal adhesion kinase (FAK)/Src complex via an integrin-dependent process, along with the ERK pathway (Foukas *et al.*, 1998; Metheniti et *al.*, 2001). In contrast, phagocytic activity in the *Aedes albopictus* mosquito cell line, C6/36, seems to involve the JNK pathway, but not ERK (Mizutani *et al.*, 2003). Furthermore, two proteins, DFak56, a homologue of vertebrate FAK, and paxillin, have been identified in *Drosophila* and are involved in promoting cell migration (Fox *et al.*, 1999; Wheleer and Hyne, 2001). Recent microarray studies

revealed that LPS treatment of *Drosophila* S2 cells stimulated the expression of a number of cytoskeletal proteins in a JNK-dependent manner (Boutros *et al.*, 2002) and analysis of the proteome of *Drosophila* upon LPS challenge identified intracellular signalling targets such as annexin IV or the Janus-like protein, cofilin (Loseva and Engström, 2004). Clearly, some of these proteins could play a crucial part in functional defence responses.

Finally, the role of a class of prostaglandins, the eicosanoids, in mediating insect defense responses (Stanley-Samuelson *et al.*, 1991) has also been demonstrated in many insect species such as *M. sexta* (Miller *et al.*, 1994) and *B. mori* (Stanley-Samuelson *et al.*, 1997). In *B. mori*, the production of other antibacterial peptides, such as attacin and cercropin, are induced following bacterial injection (reviewed by Ponnuvel and Yamakawa, 2002) with cercropin possibly being regulated by PKC signalling (Shimabukuro *et al.*, 1996). In addition, protease cascades that are initiated upon recognition of β-1, 3-glucan or bacterial compounds constitute an intracellular signalling pathway, likely triggering the activity of immune effectors in addition to the production of melanin around pathogens (Ochiai and Ashida, 2000).

1.5.2 Overview of defence mechanisms in molluscs

The internal defence mechanisms employed by molluscs against non-self share similarities with those used by insects. Apart from the existence of PO activity in gastropods (Bai *et al.*, 1996; Siddiqui *et al.*, 2006) and bivalves (Cong *et al.*, 2005), the proPO-activating system and melanisation have not been identified. The most prominent cellular defence reactions used to eliminate pathogens are phagocytosis, encapsulation and cell-mediated cytotoxicity. Humoral responses also play a fundamental role in the innate immunity of molluscs. An overview of the different types of haemocytes present in molluscs is outlined in Table 1.2.

1.5.2.1 Cellular defense processes

Haemocytes are the principal effectors of the cell-mediated defence response in molluscs (Van der Knaap *et al.*, 1993). The connective tissue, present throughout the snail and the circulating haemolymph (plasma), contains an interchangeable pool of haemocytes. These mobile macrophage-like cells participate in a variety of defence reactions including stimuli-dependent migration towards sites of infection, phagocytosis of foreign material, encapsulation of larger invading organisms, and the

generation of cytotoxic compounds, including ROIs and RNIs, and lysosomal enzymes (Sminia, 1972; Van der Knaap *et al.*, 1983; Van der Knaap *et al.*, 1993; Yoshino and Vasta, 1996; Bayne *et al.*, 2001). Haemocytes also play a role in wound healing (Yamaguchi *et al.*, 1989). In *L. stagnalis*, three other types of defence cell have been identified in the haemolymph: the pore cells, which produce the blue respiratory pigment haemocyanin, the phagocytic reticulum cell, and lectin-bound cells (Van der Knaap *et al.*, 1993). In the context of parasitic infections, infection of *L. stagnalis* with the avian schistosome *Trichobilharzia ocellata* induces a general activation of the internal defence system resulting in enhanced phagocytic and peroxidase activity (Amen *et al.*, 1991). Moreover, haemocytes from a strain of *B. glabrata* that are resistant to *Schistosoma mansoni* are capable of killing *S. mansoni* larvae via encapsulation-dependent responses (Loker *et al.*, 1982).

Table 1.2: Subpopulation of immune cells involved in molluscan defence; *L. stagnalis* and *B. glabrata* are gastropod molluscs, whereas *M. edulis* and *M. galloprovinciallis* are representatives of the Bivalvia.

Type of mollusc	Subpopulation of immune cells	Functions	References
Lymnaea stagnalis	• Pore cells • Phagocytic reticulum cells • Endothelial cells • Haemocytes	• Produce haemocyanin • Undetermined function • Trap micro-organisms • Mobile phagocytes	Van der Knaap *et al.*, 1993 Sminia *et al.*, 1979
Biomphalaria glabrata	Three types of haemocyte based on size, description: • Large haemocytes with glycogen aggregates • Medium-size haemocytes poor in glycogen • Small haemocytes	• Phagocytosis of foreign material	Matricon-Gondran and Letocart, 1999a Matricon-Gondran and Letocart, 1999b
Mytilus edulis	• Granular eosinophils • Agranular haemocytes • Basophilic granular haemocytes	• Role in phagocytosis and RO release • Unclear function • Unclear function	Pipe *et al.*, 1997 Friebel *et al.*, 1995
Mytilus galloprovinciallis	• Haemocytes comparable to hyalinocytes and granulocytes	• Role in cell spreading and phagocytosis	Carballal *et al.*, 1997 Ottaviani *et al.*, 1998

1.5.2.2 Humoral defence processes

In bivalve molluscs, the humoral response initially involves the release of various antimicrobial, bacteriostatic, and antiviral substances into the plasma (Mitta *et al.*, 2000b). *Mytilus galloprovinciallis* possesses cysteine-rich proteins, myticins (Mitta *et al.*, 1999), a defensin-like peptide, *M. galloprovinciallis* defensin-1 (Hubert *et al.*, 1996), and various types of mytilins (Mitta *et al.*, 2000a). In addition, two types of defensin-like molecules and mytilins (A and B isoforms) have been identified in *M. edulis* (Charlet *et al.*, 1996).

In gastropods, sequencing work has identified a putative defensin in *B. glabrata* haemocytes (Schneider and Zelck, 2001). Agglutinins and/or lectins also act as potent defense mediators in molluscs (Horák and Van der Knaap, 1997). Lectins are carbohydrate-recognition proteins with specific binding properties for polysaccharides and lipopolysaccharides; they do not posses enzymatic- or immunoglobulin-like activities (Ni and Tizard, 1996). As lectins are soluble in the plasma or bound to the haemocyte surface, where they act as cytophilic receptors, they can serve as bridging molecules between the saccharides present on haemocytes and those present on pathogens. They also participate in opsonisation, enabling the recognition of non-self particles (Horák and Deme, 1998); this coating process renders microorganisms more susceptible to phagocytosis. Among the lectins, fibrinogen-related proteins (FREPs) have been identified in *B. glabrata* following infection with the trematode *Echinostoma paraensei* (Adema *et al.*, 1997). Such proteins are produced by haemocytes and released in the haemolymph following trematode infection (Couch *et al.*, 1990). They are mainly characterised by one or two Ig superfamily (IgSF) domains at the N-terminal extremity and a fibrinogen (FBG) domain at their C-terminus (Léonard *et al.*, 2001; Zhang *et al.*, 2001) although structural characteristics of FBG-related proteins are better known from vertebrate systems (Doolittle, 1992).

In *L. stagnalis*, FREP-like proteins may be present, although no direct evidence exists; only one type of lectin (60 kDa) has been isolated from the haemolymph of this snail species (Van der Knaap *et al.*, 1982). Additionally, a molecule termed the "molluscan defense molecule", which contains five IgSF domains, was identified and its role seems to be similar to that of hemolin in insects (Hoek *et al.*, 1996). Recent work in *L. stagnalis* has also led to the identification of a novel defense peptide, granularin, a new opsonin that is induced by parasitation (Smit *et al.*, 2004). Interestingly, an Ig-like protein has also been described in squid (Williams *et al.*, 1988), so perhaps such molecules are conserved across the Mollusca.

1.5.2.3. Overview of signalling pathways controlling immune responses in molluscs

As observed in vertebrates (Blalock, 1989), a pool of common and highly conserved molecules are thought to operate between the immune and neuroendocrine systems, and regulate molluscan immune responses (Ottaviani and Franceschi, 1997). These molecules include cytokine-like molecules such as IL-1, hormones such as adrenocorticotropin hormone (ACTH) and estrogens, and growth factors such as TGF-β and platelet-derived growth factor (PDGF) (Ottaviani and Franceschi, 1998).

Cytokine-like molecules have been detected in various molluscs including the freshwater snails *Planorbarius corneus* and *Viviparus ater*, in which the presence of IL-1, IL-2, IL-6, and TNF-α have been shown (Ottaviani *et al.*, 1993). Moreover, IL-1 and TNF-α like molecules have been detected in the bivalve *M. edulis* (Hughes *et al.*, 1990). Cytokines have been shown to affect the immune responses in a range of mollusc species; for example, IL-1 and TNF-α seemed to have significant effects in *P. corneus* haemocyte migration experiments (Ottaviani *et al.*, 1995), whereas phagocytic activity and NO induction were increased by IL-1, IL-2, and TNF-α (Ottaviani *et al.*, 1995). Similarly, IL-1 has been shown to increase O_2^- production and phagocytic activity in *B. glabrata* haemocytes (Connors *et al.*, 1995). Recent research has also shown that cytokines can influence the activities of haemocyte signal transduction pathways. For example, stimulation of *M. galloprovinciallis* haemocytes with TNF-α activates the p38 MAPK pathway and the STAT1 transcription factor (Betti *et al.*, 2006); STAT1 was also activated in these cells by human recombinant IFN-γ and subsequent bacterial challenge (Canesi *et al.*, 2003). Given that TNF-α and IFN-γ are both potent activators of mammalian macrophages and innate immune defences (Schreiber *et al.*, 1986; Kato and Kitagawa, 2006), the importance of these molecules to molluscan defence reactions requires further study.

In mammals, chemokines are a family of cytokines which enhance cell motility and activate leukocytes via specific membrane receptors (Rollins, 1997). Their release is usually IL-1 and TNF-α mediated (Matsukawa *et al.*, 2000). In *M. galloprovinciallis*, IL-8-like molecules have been detected (Franchini *et al.*, 1996) and cell shape, chemotaxis and phagocytosis are influenced by this chemokine via PKC and PKA signalling pathways (Ottaviani *et al.*, 2000). Additionally, the presence of PDGF and/or TGF-β have been reported in *P. corneus* (Ottaviani *et al.*, 1993), *V. ater* (Franchini *et al.*, 1996), *M. galloprovinciallis* (Ottaviani *et al.*, 2000), *C. gigas* (Lelong *et al.*, 2000)

and *L. stagnalis* (Fainzilber *et al.*, 1996). These cytokines are able to induce cell shape changes in *M. galloprovinciallis* haemocytes via various signalling pathways that include the cyclic-adenosine monophosphate (c-AMP) pathway (Kletsas *et al.*, 1998). Phagocytosis by molluscan haemocytes is also affected by PDGF and TGF-β (Ottaviani *et al.*, 1997). In vertebrates, the pathway induced by TGF-β involves intracellular signalling molecules called Smads (Attisano and Wrana, 2000). Interestingly, the translocation of a Smad4-like molecule to the nucleus upon TGF-β challenge was demonstrated in *M. galloprovinciallis* haemocytes (Ottaviani *et al.*, 1999). Finally, the reproductive hormone, estrogen E2, was recently found able to modulate cell shape changes, activate the lysosomal machinery, and stimulate bactericidal activity of *M. galloprovinciallis* haemocytes through stimulation of the ERK and p38 MAPK pathways and the phosphatidylinositol-3 kinase (PI-3-K) pathway (Canesi *et al.*, 2004).

A further discussion of kinase-mediated signalling in molluscs appears in Chapter 2 and subsequent chapters.

1.6 Molluscs, vectors of parasites

Molluscs are important intermediate hosts for many trematode parasites including *Schistosoma* spp. and *Fasciola* spp., causative agents of the human and veterinary diseases schistosomiasis and fascioliasis, respectively (Sturrock, 1993). Amazingly, molluscs can act as hosts for over 4000 species of digenean trematodes (Adema and Locker, 1997).

1.6.1 Schistosomiasis and fasciolasis

Schistosomiasis is estimated to affect approximately two hundred million people worldwide (see http://www.who.int/tdr/dw/schisto2004.htm) and recent studies have indicated that, in terms of morbidity rates, it is the second most socio-economically devastating parasitic disease after malaria (Chitsulo *et al.*, 2000). Three main species of human schistosome have been defined, *S. haematobium, S. japonicum,* and *S. mansoni;* and two other species, more localized geographically, are also important in human diseases: *S. mekongi* and *S. intercalatum*. Schistosomiasis infections are mainly found in developing and tropical countries including Africa, South America, the Caribbean, the Middle East (such as Iran, Iraq), Southern China and Southeast Asia

(including Laos, Indonesia) (Corachan, 2002). Human schistosomiasis results in two different types of clinical lesions in the human body, depending on the infecting parasite: the urinary system (*S. haematobium*) and the intestinal area (*S. mansoni* and other types of schistosomes). The eggs generated by the worm are largely responsible for the host immune response. In *S. mansoni*, the release of organic substances by the deposition of eggs in the rectal mucosa or colon induces granuloma formation and macrophage accumulation in the tissue which can lead to fibrosis (Wynn and Cheevers, 1995; Wilson *et al.*, 2007). With *S. haematobium*, eggs also promote granulomatous inflammation and ulceration (Gryseels *et al.*, 2006). Katayama fever is often one of the clinical features of acute schistosomiasis and travellers often seem to be the most affected (Jensen *et al.*, 1995). The pathology appears to be associated with severe and chronic effects for approximately 5% of the infected individuals. For the remaining population, the parasite survives in the body without triggering a heavy manifestation of symptoms (Pearce and Mac Donald, 2002). The presence of a suitable snail host, often a species of *Biomphalaria*, *Bulinus*, *or Oncomelania*, facilitates the maintenance and transmission of these schistosomes, with *B. glabrata* being the major host of *S. mansoni*, the most economically important schistosome species infecting humans.

Fasciolasis is caused by the parasitic digenean *Fasciola hepatica*. The adult stage usually resides in the bile ducts of ruminants although humans also act as definitive hosts. Infection in humans is age-independent and two million people worldwide are infected, although this number increases daily (Mas-Comas, 2005). The intermediate hosts for this parasite include many species of the genus *Lymnaea* (Saba *et al.*, 2004; Mas-Coma *et al.*, 2005).

Many schistosome species that have animals as definitive hosts can infect non-natural hosts such as humans. These include *Schistosoma mattheei* and *Schistosoma bovis* which normally infect cattle, and *Schistosoma rhodhaini*, which infects rodents. In addition, different species of schistosomes that are parasites of birds or mammals can cause cercarial dermatitis (swimmers itch) by accidentally penetrating human skin causing itching (Horák and Kolářová, 2001). The release of excretory/secretory products (ESPs) by the penetration glands of the cercaria trigger an immune response from the host which induces the immediate allergic reaction (hypersensitivity) and late phase inflammation (Kouřilová *et al.*, 2004; Jenkins *et al.*, 2005).

1.6.2 *Trichobilharzia ocellata*, a schistosome that infects *Lymnaea stagnalis*

Lymnaea stagnalis is intermediate host to the avian schistosome *Trichobilharzia ocellata/regenti*. The *Trichobilharzia* genus is the largest of the Schistosomatidae and includes approximately 40 species, although the precise taxonomy remains unclear due to scarcity of molecular information and the lack of detailed morphological descriptions (Kock, 2001; Rudolfová *et al.*, 2005). *Trichobilharzia ocellata* has a typical trematode vertebrate-invertebrate life cycle, with birds (commonly a duck) being the definitive hosts and snails the vectors. Close similarities with mammalian schistosomes have been observed in the type of larvae produced and the successive steps of the life cycle (Horák *et al.*, 2002) (Fig.1.8).

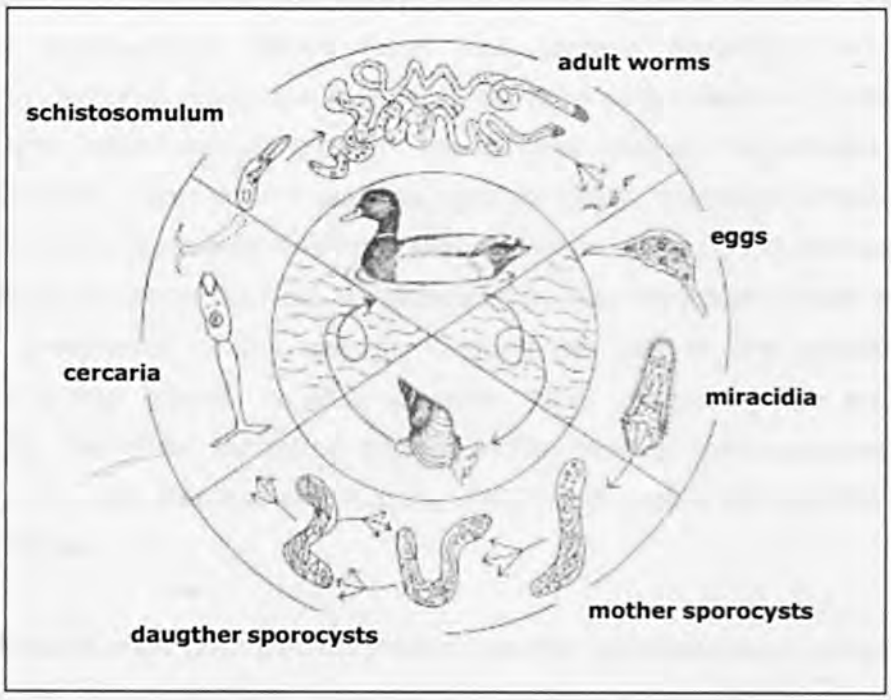

Fig.1.8: Life cycle of *T. ocellata* in a duck and *L. stagnalis* (adapted from http://www.biologie.uni-erlangen.de/parasit/contents/research/tricho.html).

The adult worms, residing in the duck, release eggs that pass into the water via the faeces. Eggs contain fully developed miracidia that are free-swimming larvae measuring approximately 70 µm in length (Horák *et al.*, 1998); the miracidia are released when the eggs contact the water. Miracidia possess ciliated plates which allow movement and facilitate the search of a suitable snail. Host finding is guided by the environment (light and temperature) and by various secreted/excreted products

(SEPs) released by the snails. Among SEPs, miracidia-attracting glycoproteins seem to enable the miracidia to locate their specific intermediate host (Kalbe *et al.*, 2000). The behaviour of miracidia seems to be positively influenced by the chemical-rich microenvironment of the snail (Hertel *et al.*, 2006) enabling miracidia to increase the rate of change of direction and accumulate near the snails (Kalbe *et al.*, 1997). The penetration of miracidia into to the snail is probably due to proteolytic enzymes synthesised by the apical penetration glands, as seen in *S. mansoni* (Yoshino *et al.*, 1993).

After penetration, miracidia develop into mother sporocysts, which then develop into daughter sporocysts via asexual reproduction in the head-foot region of the snail (Amen and Meuleman, 1992). Daughter sporocysts produce cercaria, which represent the second free-living infective larval stage, are then released from the snail into the water and search for a suitable vertebrate (duck) host. *In vitro* studies have shown that parasite development (which takes into account production of daughter sporocysts and cercaria) could continue up to 83 days post-infection (Sluiters *et al.*, 1981). The large forked swimming tail of the cercaria enables the parasite to locate the vertebrate host; other stimuli such as light intensity, chemical attractants, and skin surface lipids facilitate host-finding (Feiler and Haas, 1988a). The contact and the attachment of the larvae to the host are enhanced by free fatty acids (Feiler and Haas, 1988b). The penetration of the cercaria through the skin of the vertebrate host operates in a similar manner to that observed for *S. mansoni* (Haas and van de Roemer, 1998). The gland secretions present in the head of the larvae secrete lytic enzymes onto the skin (Bahgat and Ruppel, 2002) and enable the cercaria to move into the host tissue.

Then, each cercaria undergoes transformation into the schistosomulum stage which is able to leave the skin three days post-infection (Bourns *et al.*, 1973) and migrate to localised regions of the host, such as the lungs (Haas and Pietsch, 1991). The navigation of the parasite through deeper host tissues appears to be dependent on chemical gradients (Grabe and Haas, 2004b) and photo-orientation (Grabe and Haas, 2004a).

Maturation into filiform adult (female and male) worms occurs when the schistosomula reach the blood vessels and veins (Horák *et al.*, 2002) and the cycle starts again. Although *L. stagnalis* represents the most favorable intermediate host for *Trichobilharzia*, the parasite is able to infect other snails belonging to the Physidae family (Horák *et al.*, 2002).

1.6.3 Schistosome glycans and their functions

The importance of schistosomiasis to human health has led researchers to search for anti-parasite drugs or potential targets that could eradicate the diseases associated with them. Many studies have focused on the glycoconjugates expressed on the surface of schistosomes and/or released by them and the role of these compounds in the modulation of the host immune response (Cumming and Nyame, 1996). Since glycoconjugates represent the primary target of the humoral immune response in the definitive host (Hokke and Deelder, 2001; Hokke and Yazdanbakhsh, 2005), researchers have suggested that they could become key factors in the development of potential vaccine candidates for treating human infections (Nyame *et al.*, 2004).

1.6.3.1 Structure of glycans

Helminth parasites express a broad spectrum of glycoproteins bearing O-glycans, N-glycans and glycosphingolipids. The presence of recurrent carbohydrate based-antigens on the tegument of the parasite, or secreted as glycoconjugates, have been demonstrated for some important helminth species, including *S. mansoni, S. haematobium, S. japonicum* or *F. hepatica* (Nyame *et al.*, 2004). Characterisation of these molecules has revealed diverse mannose-rich glycoproteins or complex-type biantennary N-glycans mainly bearing the terminal structures Lac-di-Nac (LDN) and fucosylated LDN (LDNF) (Nyame *et al.*, 1989), whereas the antigen Lewis X (Le^X) is present in both O- and N-glycans (Srivatsan *et al.*, 1992) (Fig.1.9).

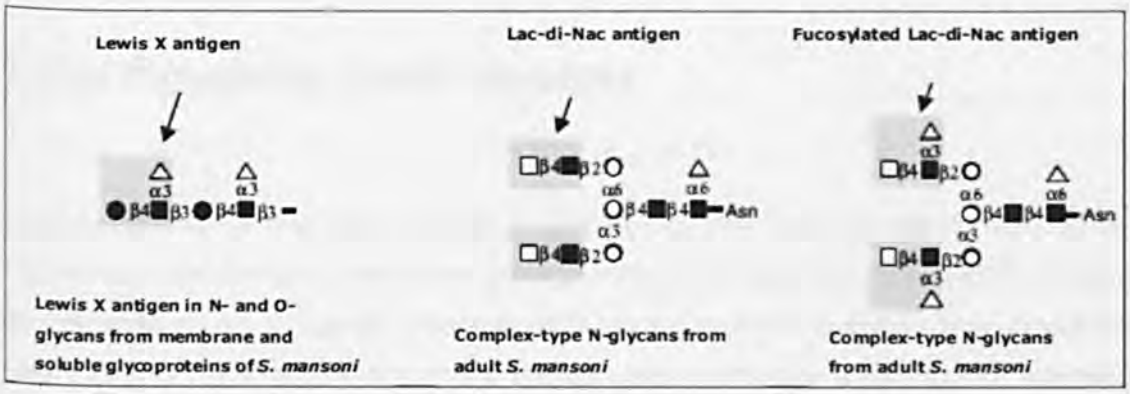

Fig.1.9: Representation of schistosome glycans including Le^x, LDN and fucosylated LDN (adapted from Varki *et al.*, 1999).

Glucose is represented by □, galactose by ●, and fucose by ■.

1.6.3.2 Expression of Lac-di-Nac, fucosylated Lac-di-Nac, and Lewis X during the developmental stages of the parasite

The presentation of LDN, LDNF, and Le^X epitopes at the surface of the parasite varies according to the life cycle stage of the parasite (Cummings and Nyame, 1999). For instance, LDN, a motif that occurs in both vertebrate and invertebrate complex-type *N*-glycans (Van den Eijnden *et al.*, 1998) is present on eggs, mother sporocysts, and daughter sporocysts (Nyame *et al.*, 2002); also it occurs on adult worms (Van Remoortere *et al.*, 2000). Lewis X, another terminal structure commonly found in galactose-rich *N*-glycans, is highly restricted to schistosomes and has not been found among other helminths tested (Nyame *et al.*, 1998). In *S. mansoni*, Le^X is not expressed on the mother or daughter sporocysts whereas it is widely present on the vertebrate stages of the parasite (Koster and Strand, 1994) and on the cercaria and eggs (Srivatsan *et al.*, 1992; Nyame *et al.*, 2002).

Work concerning the potential of schistosome glycoconjugates to modulate immunity in the host has focused solely on the definitive human host. The effects of such glycoconjugates on snail defence cells have never been explored. In mammals, recent research has shown the production of antibodies, specifically towards LDN or LDNF by patients infected with *S. mansoni*, demonstrating the immunoregulatory properties of these compounds (Nyame *et al.*, 2003). Lewis X is found in mammalian cells but is highly restricted. This antigen, identified as the trisaccharide a-fucosyl-N-acetyllactosamine present on both glycoproteins and glycolipids, is present on monocytes but is masked by sialation.

1.7 Cell Signalling- Basic concepts

Signal transduction is the basic process in cell biology that involves the conversion of a signal outside the cell to a functional change within the cell (Hancock, 1997). A signal (often referred to as a ligand) interacts with a cell surface receptor; this interaction causes a change in intracellular second messengers eventually triggering an alteration in cell function, activity, and/or gene expression by sequential activation of protein kinase cascades (Kholodenko, 2003).

1.7.1 Signals, receptors, and transducers

Biological signals can be local; these include neurotransmitters and hormones secreted by other cell types, but they can also include chemical molecules such as carbon dioxide (Lake *et al.*, 2001), NO, or H_2O_2 resulting from the metabolic activities of other cells (Schindler and Bogdan, 2001; Nathan, 2003; Rhee; 2006). Most signalling molecules do not penetrate the plasma membrane of the cell because of their charge and structure, although steroid hormones can do so and bind cytosolic and nuclear receptors (Mangelsdorf *et al.*, 1995). Cell surface receptors specifically recognize extracellular ligands and the interaction between them allows the transfer of information across the plasma membrane. These receptors usually have an extracellular domain which acts as a signal binding site and an intracellular domain which becomes activated due to conformational changes in the receptor following ligand binding (Ullrich and Schlessinger, 2004; Abramow-Newerly *et al.*, 2006). The main role of signal transducers (kinases) is then to initiate and amplify the information brought by ligands through a series of protein reactions. Their action is facilitated by the presence of second messengers which include various molecules such as cyclic AMP, diacylglycerol (DAG) (Licosvitch and Cantley, 1994) or Ca^{2+} (Bootman *et al.*, 2001).

1.7.2 Protein kinase cascades

Signal transduction largely involves phosphorylation events in which upstream kinases phosphorylate downstream targets which in turn activate transcription factors that control gene expression (Giovane *et al.*, 1994; Strahl *et al.*, 1996; Janknecht and Hunter, 1997); cytoplasmic targets also exist. Post-translational protein modifications represent a common control mechanism of controlling signals, and phosphorylation controls a multitude of cellular processes including development, differentiation multiplication, apoptosis, migration, inflammation, survival, and gene expression (Thomas *et al.*, 2000). Phosphorylation generally changes protein activity which is linked to its specific conformation; the addition of a phosphate group to one or more amino acid side chains of a protein modulates its structure and then activity in a few seconds (Cohen, 1992). Protein phosphatases are able to dephosphorylate the substrate protein largely resulting in protein deactivation (Ingebritsen and Cohen, 1983) (Fig.1.10). Protein kinases are classified into many families, many of which have been conserved through evolution (Kruse *et al.*, 1996; Hanks *et al.*, 1988; Manning *et al.*, 2002a).

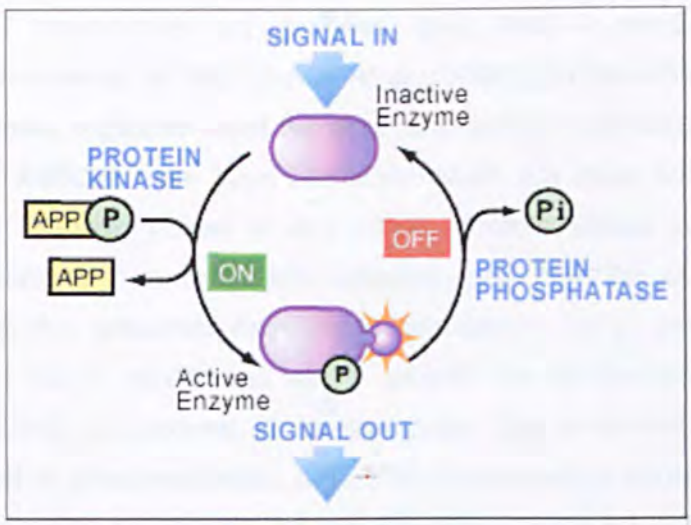

Fig.1.10: A diagramatic representation of the transmission of signal in a cell by activation of proteins through phosphorylation/dephosphorylation mechanisms (adapted from www.biotech.ucb.ca/CellBiology).

1.8 Protein kinase C

Protein kinase C is a Ser/Thr kinase which is recognized as a key regulatory element in transducing signals resulting from the receptor-mediated hydrolysis of plasma membrane phospholipids (Wakelam, 1998). This enzyme was discovered more than thirty years ago by Nishizuka and co-workers (Takai *et al.*, 1977; Inoue *et al.*, 1977) and has been widely studied since. Molecular cloning and biochemical analyses indicate that this ubiquitous enzyme comprises multiple subspecies that are structurally related (Knopf *et al.*, 1986; Coussens *et al.*, 1986; Ono *et al.*, 1988). Involved in many physiological functions in a number of different systems, PKC seems to play an important role in mitogenesis and cell proliferation, cell differentiation, apoptosis, activation of ion fluxes, secretion, and smooth muscle contraction (Mellor and Parker, 1998) but also in immune responses (Greenberg, 1995; Monick *et al.*, 2000; Spitaler and Cantrell, 2004).

1.8.1 An archetypal PKC

In addition to being present in higher organisms, PKC has been isolated from various lower eukaryotes. In the yeast *Saccharomyces cerevisiae*, there is a sole PKC, PKC 1;

at 132 kDa, it is much larger than mammalian PKCs because all the different modules in mammalian PKC isoenzymes are present, thus PKC 1 could constitute the archetypal, common ancestor of PKC (Levin *et al.*, 1990). In the African clawed frog *Xenopus laevis*, a model organism used for gene and protein expression and knockout studies, X-PKCI and X-PKCII have been identified which are close homologues of the mammalian PKCα/β isoforms (Chen *et al.*, 1989). Protein kinase C has also been cloned in the nematode *Caenorhabditis elegans* and the fly *D. melanogaster*, organisms for which the genomes have been sequenced. In *C. elegans*, four PKC genes named tpa-1, pkc-1, pkc-2 and pkc-3 control the synthesis of multiple PKC isoforms (Tabuse, 2002). In contrast, *D. melanogaster* has three PKC enzymes: eye-specific PKC, involved in photoreception, dPKC53F (expressed in adults) and dPKC98F (expressed through development) both mainly expressed in the brain (Schaeffer *et al.*, 1989). Protein kinase C has also been found in plants. For example, a Ca^{2+}-dependent homologue of mammalian PKC has been isolated from potato plants (Subramaniam *et al.*, 1997) and a PKC-like kinase present in tomato plants has been shown to be involved in cellular immune defence reactions in response to fungal invasion (Xing *et al.*, 1996).

1.8.2 Classification of PKC isotypes

Protein kinase C is a member of the broad AGC (cAMP-dependent PKA, PKB, PKC) family (Manning *et al.*, 2002b). Eleven isoforms (78-95 kDa) have been identified in mammals and classified into three distinct subgroups according to their structural and regulatory differences: the classical PKCs (cPKCs) comprise PKCα, β_I, β_{II}, and γ and are regulated by Ca^{2+}, DAG and phospholipids; the novel PKCs (nPKCs) PKCδ, ε, η, and θ which are regulated by DAG and phospholipids; and the atypical PKCs (aPKCs) PKCξ , and ι/λ 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). Each mammalian PKC isoenzyme is a unique polypeptide chain, resulting from the transcription of a single gene. Isoforms PKCβI and PKCβII are alternative spliced variants of the same genes (Ono *et al.*, 1986).

Closer examination of protein sequence alignments of PKCs reveals the presence of regions of homology between the family members with specific motifs distributed within the regulatory N-terminal and catalytic C-terminal domains (Mochly-Rosen and Gordon, 1998) (Fig.1.11). Four highly conserved regions (C1-C4) alternate with five variable regions (V1-V5) (Liu, 1996). A hinge region (also called V3) serves as a

connector between the regulatory domain and the catalytic domain (Liu, 1996) and is sensitive to proteolytic cleavage by cellular proteases such as trypsin and calpain, which results in the liberation of the protein kinase M (free kinase domain) (Parker *et al.*, 1986).

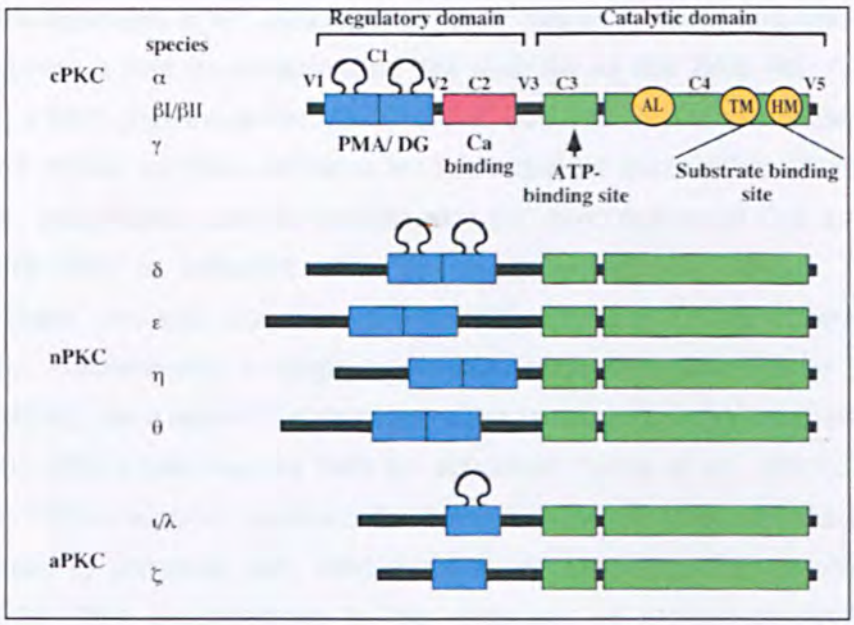

Fig.1.11: Schematic representation of the primary structure of the PKC subfamilies, the conventional or calcium-dependent PKCs, the novel or calcium-independent PKCs and the atypical PKCs. The categorization of the PKC isotypes is based on their regulatory modules and their cofactor dependence (adapted from Mochly-Rosen and Gordon, 1998).
Conventional PKCs, aPKCs, and nPKCs possess an auto-inhibitory pseudosubstrate sequence at the extreme N-terminus of the regulatory domain. The kinase core is common to all classes. The activation loop (AL), the turn motif (TM) and the hydrophobic motif (HM) include the phosphorylation sites within the enzyme.

1.8.3 The different regions of PKC

1.8.3.1 The pseudosubstrate region

The pseudosubstrate region is present in each subclass of PKC, at the N-terminal side of the C1 domain. This region interacts with the substrate binding site in the catalytic domain, maintaining PKC in an inactive state (House and Kemp, 1987). Studies have shown that in the pseudosubstrate region, Arg22 has a strong inhibitory role (House and Kemp, 1990) and that alanine has replaced the amino acid that is normally phosphorylated (Thr or Ser) in the substrate (House and Kemp, 1987).

1.8.3.2 The C1 domain

The C1 domain (approximately 50 amino acid residues) is present in all PKC isotypes (Fig.1.11). In cPKCs and in nPKCs, this DAG/phorbol ester binding structure is localised at the C-terminal end of the protein. Commonly named C1a and C1b, each motif is mainly composed of two successive zinc-finger-like motifs which fold into a globular form (Canagarajah *et al.*, 2005). Each motif has a cysteine- and histidine-rich composition and has a role in translocating the enzyme to the DAG-rich membrane (Burns and Bell, 1991). However, the exact role of C1a and C1b may not be identical within cPKCs and nPKCs as their affinities for DAG/phorbol ester differ (Slater *et al.*, 1996). Moreover, mutational analysis reveals that the contribution of C1a and C1b to the activation of PKC is different (Ananthanarayanan *et al.*, 2003). The non-equivalence of these two sub-domains was recently discussed (Colon-Gonzales and Kazanietz, 2006). Furthermore, a single tryptophan residue appears to be crucial in enhancing the affinity that novel C1b domains have for the plasma membrane, which could explain why nPKCs only require DAG for activation (Dries *et al.*, 2007). It is well established that PKCs respond to phorbol esters such as phorbol-myristate-acetate (PMA) which seem to compete with DAG for the same binding site (Hurley *et al.*, 1997); affinity for PMA is enhanced in the presence of phosphatidylserine (PS) (Johnson *et al.*, 2000). In contrast to cPKCs and nPKCs, aPKCs contain only a single cysteine-rich conserved pattern, and surprisingly, do not respond to phorbol esters (Hurley *et al.*, 1997). Structural comparison between the different zinc-finger-like motifs confirms that this unpaired motif is more analogous to the C1a motif than the C1b motif (Mellor and Parker, 1998). Finally, PS has an important role in PKC activation and its specificity is conferred by the C1 domain in all subclasses (Orr and Newton, 1992).

Various studies have demonstrated that other kinases or non-kinase mammalian phorbol ester receptors contain C1 domains, single, or in tandem, and consequently are stimulated by DAG and PMA. These proteins include protein kinase D (PKD), chimaerin, and Munc-13 (Ron and Kazanietz, 1999; Hall *et al.*, 2005).

1.8.3.3 The C2 domain

In cPKCs, the C2 domain (approximately 130 amino acid residues) which binds Ca^{2+} is within the regulatory part of the enzyme, on the carboxy side of the C1 domain (Fig.1.11). The interaction with Ca^{2+} is caused by the presence of five conserved residues of aspartic acid situated in two loops (Corbalán-García *et al.*, 1999). The C2

domain also has a role in membrane targeting (Stahelin and Cho, 2001). The nPKCs possess a C2-like domain but it is unable to bind Ca^{2+} due to the absence of acidic amino acids (Ono *et al.*, 1987). Moreover, aPKCs lack the C2 domain and are consequently Ca^{2+} insensitive. The C2 domain is not unique to PKC since it has been found in other proteins including synaptotgamins, Munc-13, and phospholipase C (PLC) (Ron and Kazanietz, 1999). The receptor for activated PKC (RACK) proteins, interacts with the C2 domain, targeting the activated kinase to specific cellular compartments; RACKs therefore coordinate the localisation of PKCs (Mochly-Rosen and Gordon, 1998).

1.8.3.4 The C3 domain

The C3 domain contains the ATP-binding consensus sequence, which allows PKC to become phosphorylated; it is therefore responsible for PKC activity. This site is highly conserved and resembles that found in other protein kinases such as cAMP-dependent protein kinases (Hanks *et al.*, 1988; Taylor *et al.*, 1992). The activity of PKC isotypes is under the control of three distinct and conserved phosphorylation sites on specific Ser or Thr residues within the activation loop (AL), turn motif (TM), and hydrophobic motif (HM) (Newton, 1997) (Fig.1.11). The aPKCs have a glutamic acid residue in the hydrophobic region, and thus unlike other PKCs are not subject to phosphorylation in this region.

1.8.3.5 The C4 domain

The C4 domain is located at the extreme C-terminal part of the protein, just prior the V5 region, and it contains the substrate binding site (Fig.1.11). It is assumed that the structure of PKC is folded such that the pseudosubstrate and the substrate binding cavity are proximate to one another (House and Kemp, 1987).

1.8.3.6 The V5 region

Present in all PKC isoforms, the C-terminal V5 region (approximately 50 amino acid residues) seems to regulate the kinase domain of the protein, as its modification alters kinase activity of some isoforms (Coussens *et al.*, 1986) (Fig.1.11). It plays a critical role in PKC isoform-specific localisation (Stebbins and Mochly-Rosen, 2001; Wang *et al.*, 2004); this finding is based on the fact that $PKC\beta_I$ and $PKC\beta_{II}$ isoforms, for which the sequence differs only in the V5 region (Blobe *et al.*, 1996), translocate differently

in human HL60 and U937 leukemia cells (Hocevar and Fields, 1991; Kiley and Parker, 1995). The region also contains at least two phosphorylation sites, mostly identified in cPKCs (Bonancin and Parker, 1997).

1.8.4 The mechanisms of PKC activation

The function of PKC relies on three important events; these are all essential to cPKC function, the class which has been the most studied (Newton, 2003). First, the protein undergoes a series of phosphorylations which, in addition to PKC activation or inactivation contribute to the maturation of the enzyme (Parekh *et al.*, 2000). Second, PKC activity depends on the presence of cofactors such as DAG, Ca^{2+}, or PS which facilitate the translocation of phosphorylated, mature, PKC to the plasma membrane and pseudosubstrate release (Newton, 1997). Association of activated PKC with scaffolding proteins further dictates its subcellular distribution (Jaken and Parker, 2000). These events are further described below.

1.8.4.1 PKC maturation: the role of successive phosphorylations

Phosphorylation constitutes the first and initial step in the maturation of PKC. Crucially, only phosphorylated PKCs play a role in the transduction of signals, within the cell. Newly synthesised species of PKC bind to the cytoplasmic face of the plasma membrane through weak energy interactions between the phospholipid bilayer and the C1/C2 domain (Chow, 2001). The first processing phosphorylation involves the participation of a universal PKC kinase, the phosphoinositide-dependent protein kinase-1 (PDK-1) with intrinsic activity that is constitutive and dependent on specific characteristics (location, conformation) of the substrates (Dutil *et al.*, 1998). The hydrophobic motif seems to act as a docking module for PDK1 (Gao *et al.*, 2001). The pseudosubstrate region of PKC does not bind the substrate-binding cavity allowing PDK-1 to phosphorylate Thr497 or Thr500 in PKCα and PKCβ_{II} respectively (Dutil and Newton, 2000). Transphosphorylation of the activation loop by PDK-1 is then followed by the intramolecular phosphorylation of the turn motif and the hydrophobic site within the carboxyl domain, which allow PKC to become catalytically competent (Ben-Kappra and Newton, 1999) (Fig.1.12). Interestingly, the suppression of the phosphorylation at THr500 of the activation loop of PKCβII does not affect the maximal catalytic activity of mature PKCs when they are autophosphorylated on the turn motif (Keranen *et al.*, 1995). PKC is catalytically competent but not active yet, as

the pseudosubstrate re-engages the substrate binding cavity. Phosphorylated PKC is then released into the cytosol (Newton, 2003).

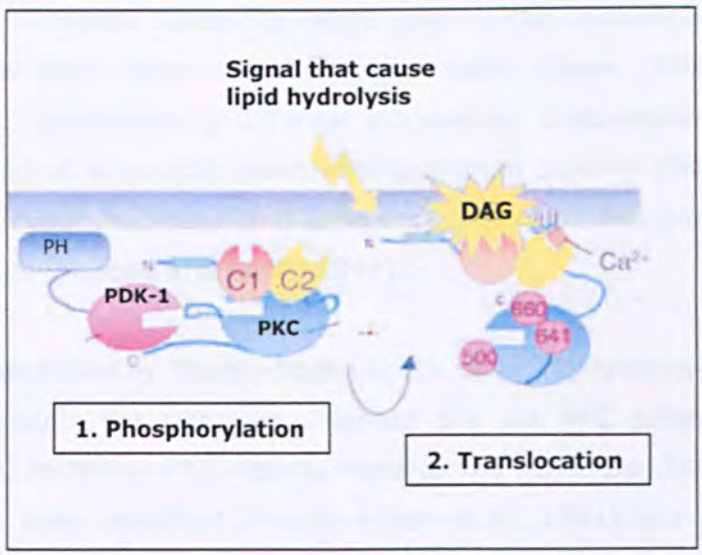

Fig.1.12: Cartoon representing the different steps of cPKC activation involving first the phosphorylation of PKC activation loop by pleckstrin homology (PH) linked-PDK-1, and then the translocation of PKC at the membrane upon the interaction of the C2/C1 domain with diacylglycerol (adapted from Newton, 2003).

1.8.4.2 Catalytic activation of PKC by cofactors

Under resting conditions, cells seem to have a "pool" of mature phosphorylated PKC dispersed in the cytoplasm "ready to be used" (Keranen *et al.*, 1995). Inactive PKCs may bind to A-kinase anchoring proteins (AKAPs) (Klauck *et al.*, 1996; Colledge and Scott, 1999), in addition to PKA, and target PKC isoforms at specific intracellular locations such as postsynaptic sites in neurons (Faux *et al.*, 1999). Upon external stimulus, different cofactors such as Ca^{2+} and DAG make PKC catalytically active by targeting the enzyme at the phospholipid bilayer (Newton and Johnson, 1998). The autoinhibitory sequence is then released from the substrate-binding site allowing PKC to phosphorylate downstream targets (Keranen and Newton, 1997; Newton, 2003).

1.8.4.3 Distribution of PKC: role of binding proteins

The regulation of PKC activity is also determined by binding proteins which direct PKC to intracellular compartments such as the plasma membrane or perinuclear region (Kiley *et al.*, 1995). Substrate specificity might result in the colocalisation of activated PKC isoenzymes and their respective shuttling proteins (Jaken, 1996). Indeed, the translocation of PKC isoenzymes to different intracellular compartments is facilitated by receptors which serve as specific isoenzyme-anchoring proteins (Mochly-Rosen and Gordon, 1998); moreover PKC may bind different types of protein partners according to its activation status (Pawson and Scott, 1997).

The first receptors identified by Mochly-Rosen in the early 1990s were RACKs (Mochly-Rosen, 1995). Receptors for activated C-kinase are not PKC substrates but their association with PKC increases PKC activity towards the substrate. Two RACKS, RACK 1 and RACK 2, have been described (Mochly-Rosen *et al.*, 1991) and they interact via direct protein-protein interaction (involving the WD40 repeats of RACK and the C2 domain (or C2-like domain) of PKC) with the PKCβ_{II} and PKCε respectively (Ron *et al.*, 1995). Receptors for activated C-kinase belong to the large family of PKC-interacting proteins (C-KIPs) which include other classes of PKC binding partners (Fig.1.13). The model proposed by Mochly-Rosen (Mochly-Rosen and Gordon, 1998) suggests that the differences observed in the localization of inactive PKCs within a resting cell could be due to another type of receptor, receptors for inactived protein kinase C (RICKs) which anchor individual PKCs in an inactive state. In this model, the mechanism by which PKC shuttles from RICKs to RACKs has still not been elucidated but the translocation is probably facilitated by activators of PKC such as PMA (Mochly-Rosen and Gordon, 1998).

1.8.5 PKC substrates

Each PKC isoform displays a distinctive tissue distribution and substrate specificity, suggesting that individual isoforms have specific functions *in vivo* (Nishikawa *et al.*, 1997, Teruel and Meyer, 2000). The primary function of PKC is to catalyse the transfer of phosphate from ATP to the free hydroxyl group of Ser or Thr residues of substrate proteins. In mammals, many substrates of PKC have been identified belonging to different classes of proteins; these include myristoylated alanine-rich C-kinase substrate (MARCKS), neuromodulin, G-proteins, proto-oncogene products, nuclear proteins and cytoskeletal proteins (Jaken and Parker, 2000). Many C-KIPs include substrates that interact with C-kinase (STICKs) (Liu, 1996). The list of PKC substrates is broad and

only a few of them have been extensively studied and are considered primary targets of PKC.

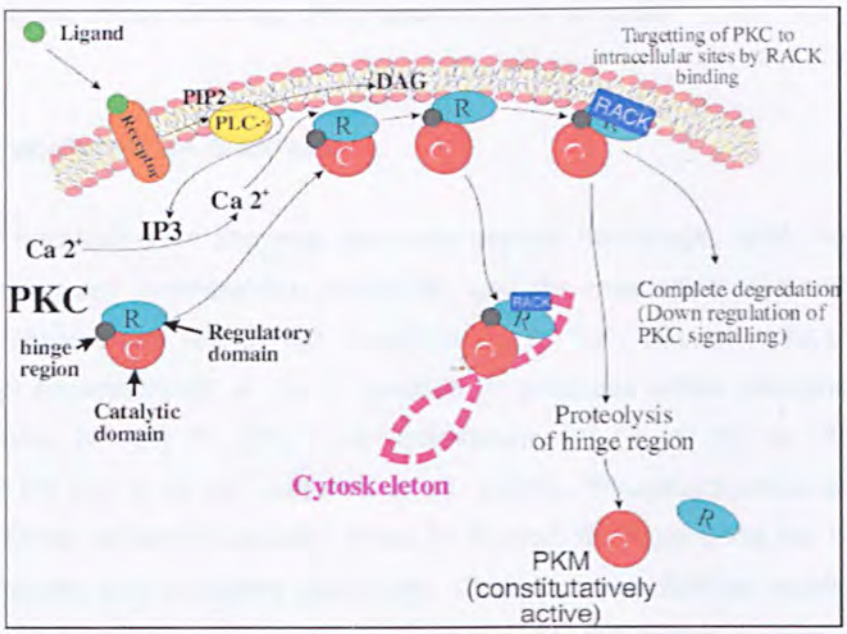

Fig.1.13: Role of RACK in PKC activation.
Upon ligand binding and receptor activation, PKC is targeted to the membrane via the C1 module which binds DAG. The docking of PKC to the membrane is also facilitated by the interaction between RACKs and the C2 domain which localise PKC at the appropriate intracellular site according to the availability of substrates and cellular requirements.

1.8.6 Other key proteins involved in PKC signalling

The integration of various signals within the whole cell occurs via a web of pathways cross-reacting with each other in which each individual pathway has a specific input. Such "cross-talk" occurs in PKC signalling. Phospholipase C, PI-3-K, PDK1, the ERK 1/2 pathway and PKD are some important intracellular signalling components which directly or indirectly interact with PKC to coordinate biological responses.

1.8.6.1 Phospholipase C

Members of the PLC family are key enzymes in phosphatidylinositol 4, 5-biphosphate (PIP_2) metabolism and are divided in to six groups according to their structure: PLCβ, PLCγ, PLCδ, PLCζ, PLCε and PLCη (Rebecchi and Pentyala, 2000; Cockcroft, 2005). These membrane-associated enzymes are activated following receptor binding and

convert PIP_2 to the second messengers inositol 1, 4, 5-triphosphate (IP_3) and DAG (Rhee, 2001). Given that Ca^{2+} is mobilised from intracellular stores by IP_3 (Berridge, 1993) and that Ca^{2+} and DAG are key modulators of PKC activity (Newton, 1997), PLC plays a crucial role in coordinating cPKC responses in the cell.

1.8.6.2 Phosphoinositide-3 kinase

This group of heterodimeric enzymes generate second messenger lipids and transduce signals mediating cell proliferation, apoptosis, and the regulation of membrane traffic in mammals (Fruman *et al.*, 1998). Specifically, PI-3-Ks phosphorylate the inositol ring in inositol phospholipids at the 3′ position to generate either phosphatidylinositol (PI) 3-phosphate (PI (3) P), (PI) 3, 4-biphosphate (PI (3, 4) P_2) or (PI) 3, 4, 5 - trisphosphate (PI (3, 4, 5) P_3) (Leevers *et al.*, 1999). Phosphoinositide-3 kinases are divided into three different classes: class I, II and III depending on their domain structure, function, and substrate specificity. Class I can be further subdivided in two groups: type IA enzymes that have a p85 or p50-55 regulatory subunit and a p110 catalytic subunit (Hawkins *et al.*, 2006) which associates with an adaptator molecule possessing a Src-homology-2 (SH2) domain (Vanhaeseboeck *et al.*, 1997); and type IB enzymes that have a p101 or p84 regulatory subunit and a p110 catalytic subunit (Hawkins *et al.*, 2006) but do not bind to adaptator molecule (Vanhaeseboeck *et al.*, 1997). Class I PI-3-Ks are activated by various extracellular stimuli (Wyman and Pirola, 1998) and influence the activity of p70 S6 kinase (Cheatham *et al.*, 1994), PKB (Datta *et al.*, 1996), and PKC isoforms via the regulation of the PDK-1 activity (Filippa *et al.*, 2000) (Fig.1.14). Class II PI-3-Ks are larger in size (200 kDa) and possess a C2-like domain at the C-terminus similar to the one found in cPKCs (Domin *et al.*, 1997) whereas class III PI-3-Ks are considered to be the mammalian homologues of the yeast VPS34 PI-3-K (Schu *et al.*, 1993). The lipid products of PI-3-Ks have been shown by various workers to act as second messengers for PKC isoforms (Toker *et al.*, 1994; Derman *et al.*, 1997; Chou *et al.*, 1998).

1.8.6.3 Phosphoinositide-dependent protein kinase-1

Phosphoinositide-dependent protein kinase-1, encoded by only one gene in mammals, is a Ser/Thr kinase comprising of 556 amino acids (63 kDa), and also belongs the AGC family. It possesses a pleckstrin homology (PH) domain in the C-terminal region whereas the catalytic domain is located on the N-terminus (Belham *et al.*, 1999). Its activity is closely related to activation of an upstream kinase, PI-3-K and the products

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of its substrates, but also requires phosphorylation at its activation T-loop (Casamayor *et al.*, 1999). Studies on bacteria suggest that PDK-1 may be autophosphorylated (Vanhaesebroeck and Alessi, 2000).

The importance of PDK-1 has increased in the past decade because of its role in the phosphorylation of the activation loop (on Ser or Thr residue) of many proteins belonging to the AGC kinase family (Parker and Parkinson, 2001) such as cAMP-regulated PKA, PKB and PKC (Gold *et al.*, 2006). In the context of PKC signalling, PDK1 catalyses the phosphorylation of the activation loop of mature cPKC and aPKC isoforms (Dutil *et al.*, 1998; Le Good *et al.*, 1998) and plays a role in the translocation of PKC to the plasma membrane (Chou *et al.*, 1998; Seki *et al.*, 2005). Phosphoinositide-dependent protein kinase-1 also has other targets such as p70 S6 kinase, p21-activated kinase, and ribosomal S6 kinase (Biondi, 2004) (Fig.1.14).

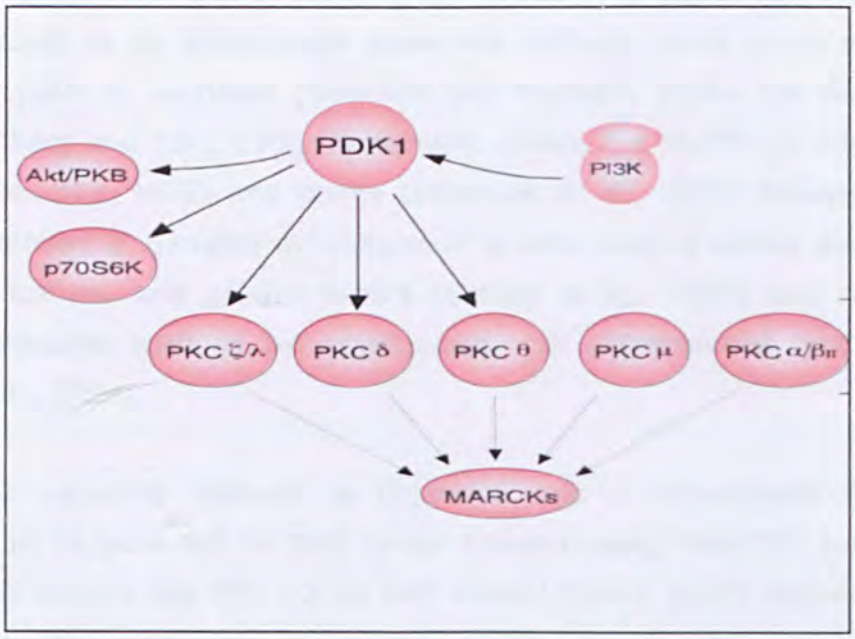


Fig.1.14: Simplified overview of PDK1 in signalling in mammals (adapted from www.cellsignal.com). Most of the PKC isoforms are phosphorylated by PDK-1.

1.8.6.4 Ras

Ras is a monomeric G protein with a molecular weight of 21 kDa, anchored on cytoplasmic face of the cell membrane via its C-terminal moiety. Encoded by three different genes, it seems that the four known mammalian Ras proteins, H-Ras, N-Ras,

K-Ras4A and K-Ras4B (the last two result from alternative splicing) have similar functions (Reuther *et al.*, 2000). Originally discovered as an oncogene in human tumors (reviewed by Bos, 1989), Ras acts as a key switch (off/on) protein in the MAPK pathway (Marshall, 1996) and many other pathways including the PI-3-K pathway (Rodriguez-Viciana *et al.*, 1996; Kolch, 2000). Ras is activated by different extracellular stimuli; it is a guanine nucleotide binding protein and the exchange of guanine diphosphate (GDP) and guanine triphosphate (GTP) modifies Ras to an activated conformation (Dumaz *et al.*, 2005). Such activation is facilitated by guanine nucleotide exchange factors (GEF) such as Son of sevenless (Sos) (Downward, 1994; Innocenti *et al.*, 2002).

1.8.6.5 The extracellular signal-regulated kinase 1/2 pathway

Extracellular signal-regulated kinase 1/2 is a member of the MAPK family which also includes p38 MAPK, JNK, ERK 5, and ERK 3/4 (Pearson *et al.*, 2001). The ERK 1/2 signalling cascade is an evolutionary conserved pathway found in all eukaryotes, including the yeast *S. cerevisae* (Schwartz and Madhani, 2004), the nematode *C. elegans* (Stenberg and Han, 1998; Sundaram, 2006), the fruitfly *D. melanogaster* (Biggs and Zipursky, 1992) and plants (Cardinale *et al.*, 2000; Nakagami *et al.*, 2005). The pathway is activated in response to diverse array of stimuli which include hormones, cytokines, and growth factors (Caffrey *et al.*, 1999) and regulates a number of processes such as cell proliferation, cell differentiation, and apoptosis (Widmann *et al.*, 1999).

The ERK 1/2 signalling pathway is organised into a three-kinase hierarchical architecture that includes Raf (or MAP kinase kinase kinase), MAP/ERK kinase (MEK) (or MAP kinase kinase), and ERK 1/2 (or MAP kinase) (Kolch, 2000). Activation of ERK 1/2 promotes the expression of specific genes via phosphorylation of many transcription factors such as Elk-1, c-jun and c-Myc (Janknecht *et al.*, 1993; Hill and Treisman, 1995) (Fig.1.15). Transmission of the signal through the cascade is optimised by the presence of additional factors such as adapters and scaffolding proteins. Protein kinase C has been linked to the ERK pathway following stimulation of cells with phorbol esters (Marquardt *et al.*, 1994); it has been shown to act upstream of ERK signalling in a Ras-independent manner (Ueda *et al.*, 1996) or Raf-dependent manner (Schönwasser *et al.*, 1998) in fibroblasts. In growth factor-stimulated neuronal cells, PKCζ and PKCδ activate the ERK pathway at the level of MEK and lead either to mitogenesis or differentiation (Corbit *et al.*, 2000).

The individual ERK pathway components are discussed further below.

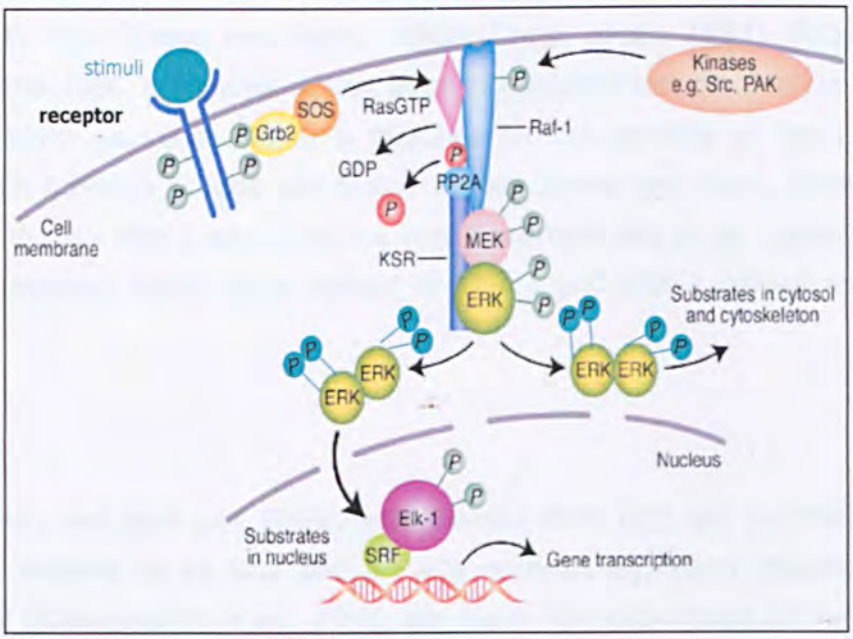


Fig.1.15: Organisation of the Ras/Raf/MEK/ERK pathway (adapted from Expert Reviews in Molecular Medicine http://www.expertreviews.org/02004404a.pdf). See text for a description of the components.

• Raf

First identified as an oncogene transduced by transforming retroviruses (Rapp *et al.*, 1983), the role of Raf in activating the ERK 1/2 pathway has become reasonably well established. This Ser/Thr protein kinase comprises a family of three different isoforms, cRaf-1, B-Raf, and A-Raf. Although the activation process of Raf is not completely understood, Raf proteins undergo activation through recruitment by Ras from the cytoplam to the membrane (Dickson *et al.*, 1992). After phosphorylation of Raf's activation segment, activated B-Raf is probably responsible, *in vivo*, for the phosphorylation of MEK (Baccarini, 2005); activation of MEK by Raf-1 has, so far, been mainly studied *in vitro*. Specific localisation of Raf-1 results in its phosphorylation, via the intervention of p21-activated kinases (King *et al.*, 2001) and PKC isoforms such as PKCα (Kolch *et al.*, 1993) and PKCε (Hamilton *et al.*, 2001). Recent studies revealed that Raf might regulate other signalling pathways such as NF-κB (Mayo *et al.*, 2000) and those involving the cell cycle protein p53 and the pro-apoptotic kinase RIP2 (Navas *et al.*, 1999). Besides functioning as a kinase, Raf may also act as a scaffold protein (Hindley *et al.*, 2002).

• MEK

Two isoforms of MEK exist, MEK 1 and MEK 2 with a molecular weight of approximately 45 kDa (Zheng and Guan, 1993a; Crews *et al.*; 1992). Substrates of Raf-1, MEK 1 and MEK 2 become active once phosphorylated on Ser/Thr residues within a proline-rich sequence which is required for the binding of Raf-1 and the regulation of MEK function (Zheng and Guan, 1993b; Zheng and Guan, 1994; Catling *et al.*, 1995). Although MEK 1 and 2 have a very important role in cell signalling, their physiological substrates seem to be limited to ERK 1 and ERK 2 (Shaul and Seger, 2007).

• ERK 1/2

The two isoforms, p42 and p44 MAPK (also named ERK 1/2) are Ser/Thr kinases. With molecular weights of 42 kDa and 44 kDa respectively, ERK1 (Boulton *et al.*, 1990) and ERK2 (Canagarajah *et al.*, 1997) are dually phosphorylated on Tyr 185 and Thr 183 residues within the TEY motif (Payne *et al.*, 1990) by the upstream kinase, MEK (Robinson *et al.*, 1996). The subcellular localisation of ERK 1/2 is regulated by various scaffolding proteins such as kinase suppressor of Ras (Yu *et al.*, 1998) and MEK partner 1 (MP1) (Sharma *et al.*, 2005). In response to pathway activation, phosphorylated ERK 1/2 migrates rapidly to the nucleus of the cell (Khokhlatchev *et al.*, 1998) before being translocated back to the cytoplasm (Adachi *et al.*, 2000). The regulation of the nuclear traffic of ERK 1/2 is a critical step for the generation of adaptive biological responses in different cell types (Pouyssegur *et al.*, 2002). The biological outcomes of ERK 1/2 activation are therefore cell-type dependent and vary with the duration of pathway stimulation. Downstream targets of ERK 1/2 also include the cytoskeletal networks (Klemke *et al.*, 1997). For example, adhesion-mediated activation of ERK 1/2 is dependent on integrin engagement (Miyamoto *et al.*, 1996).

1.8.6.6 Protein kinase D

More than a decade ago, researchers identified a novel class of DAG/phorbol ester-dependent protein kinases, namely PKD (also known as PKCμ) (Valverde *et al.*, 1994). For a long time considered part of the PKC family, studies have revealed that PKD possesses structural and enzymological properties that are divergent from PKCs (Rozengurt *et al.*, 2005); PKD therefore constitutes a new sub-family of Ser/Thr protein kinases within the calcium/ calmodulin-dependent kinases (CAMK) family (Hanks, 2003). Three isoforms of PKD have been characterised, PKD1 (Valverde *et al.*,

1994; Johannes *et al.*, 1994), PKD2 (Sturany *et al.*, 2001), and PKD3 (Hayashi *et al.*, 1999), although to date PKD1 (115 kDa) remains the most studied (Rykx *et al.*, 2003). The three isoforms are characterised by an N-terminal regulatory domain with two cysteine-rich zinc fingers, a PH domain, and a kinase catalytic domain (Rykx *et al.*, 2003). Research has revealed that PKD is present in various cell types such as fibroblasts (Yuan et *al.*, 2002) and lymphocytes (Matthews *et al.*, 2000) and is activated in an nPKC-dependent manner via successive phosphorylations at specific residues (Ser744 and Ser748) present within the catalytic domain (Iglesias *et al.*, 1998; Waldron and Rozengurt, 2003).

1.8.6.7 Integrins

Integrins are cell surface heterodimeric glycoproteins that act as cell adhesion receptors. At least 16 distinct α subunits and 8 β subunits have been isolated and identified in vertebrates and they can associate in various combinations to form more than 20 different types of integrin (Tamkun *et al.*, 1986; Hynes 1992). Two functions for integrins have been determined. First, where protein complexes form at focal adhesion sites, integrins link extracellular matrix (ECM) components such as fribronectin and collagen to intracellular actin filaments within the cytoskeleton. Second, in response to ligands which contain the minimal integrin binding sequence, Arg-Gly-Asp (RGD), integrins are able to transduce signals inside the cell and regulate various cellular processes such as cell motility, cell adhesion and cell spreading, or survival (Pierschbacher and Ruoslahti, 1984; Aplin *et al.*, 1998). In carcinoma cells, the β1 integrin subunit and PKCα have been shown to colocalise. Furthermore, the cellular distribution of β1 integrin is regulated in a PKCα-dependent fashion (Ng *et al.*, 1999). Finally, studies have shown that integration of cytokine and integrin signalling operates via PKCε (Ivaska *et al.*, 2003). Thus integrin function seems to be intimately linked to PKC dependent processes.

1.8.6.8 Focal adhesion kinase

Focal adhesion kinase is a 125 kDa cytoplasmic non-receptor protein tyrosine kinase that is conserved through species (Cary and Guan, 1999). It is often colocalized at focal adhesion sites, via a focal adhesion targeting-domain, with other proteins such as Src, paxillin (Pax) and p130Cas (Schaller *et al.*, 1999; Panetti, 2002). Integrin-mediated signalling events enhance tyrosine phosphorylation of FAK in many cell types (Burridge *et al.*, 1992) (Fig.1.16). Although the exact mechanisms of FAK activation

remain unclear, autophosphorylation on Tyr397 is required (Calalb *et al.*, 1995), enabling the binding of the SH2 domain of Src which ultimately triggers phosphorylation of other FAK tyrosine residues such as 407, 576, 577, 861 and 925 (Cary and Guan, 2000). Tyr925 has been identified as the binding site of the adaptor protein Grb2 (Schlaepfer and Hunter, 1995). A direct link between FAK phosphorylation and PKC activity has been reported in platelets (Haimovich *et al.*, 1996), in murine mast cells (Bhattacharyya *et al.*, 1999), and in Swiss 3T3 (mouse) fibroblasts (Hunger-Glaser *et al.*, 2003). Additionally, PKC is involved in focal adhesion formation (Woods and Couchman, 1992), cell spreading, and cell migration (Haller *et al.*, 1998; Rigot *et al.*, 1998; Vuori and Ruoslahti; 1993), all of which require modification of integrin-cytoskeleton-ECM interactions.

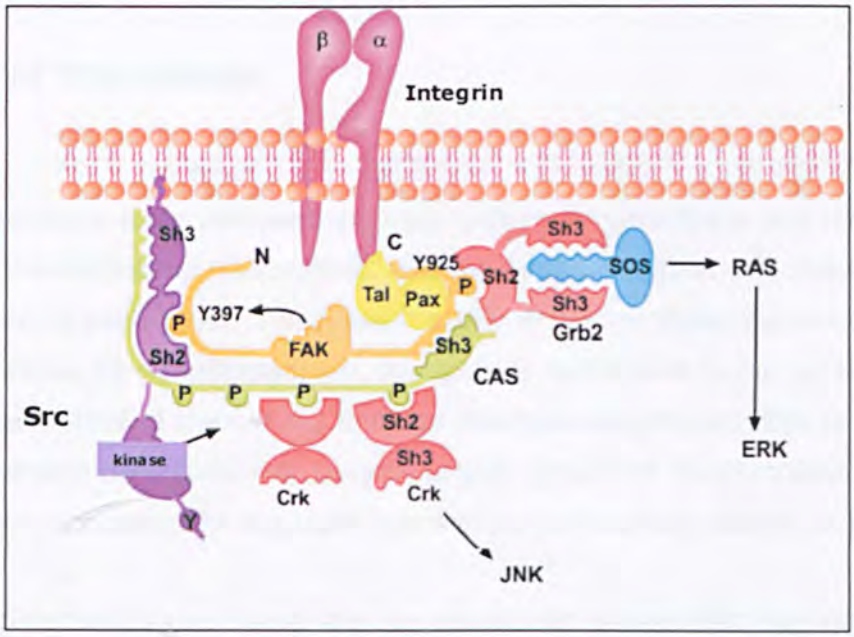


Fig.1.16: Integrin-mediated phosphorylation of FAK ultimately leading to the activation of MAPK pathways such as those involving ERK and JNK, and modification of the cytoskeleton.
This involves the docking protein Crk-associated substate, p130^{Cas} (Cas) (Polte and Hank, 1995), Src kinase (Schaller *et al.*, 1999), Crk (Vuori *et al.*, 1996) and the adaptor proteins tallin (Tal), and Pax (adapted from Giancotti and Ruoslahti, 1999).

1.8.6.9 Src protein kinase

Src family kinases are non-receptor protein tyrosine kinases of approximately 60 kDa. Nine members have been identified in mammals and appear to be proto-oncogenic proteins (Brown and Cooper, 1996; Martin, 2001). Src is composed of seven different domains including SH4, the unique domain, SH3, SH2, the SH2-Kinase linker, and the

kinase domain (comprising SH1) (Williams *et al.*, 1998). Src is maintained in a repressed state when Tyr527 is phosphorylated (MacAuley and Cooper, 1989) by C-terminal Src kinase (Okada and Nakagawa, 1989) whereas its activation is due to autophosphorylation on Tyr416 in the catalytic domain (Stover *et al.*, 1994). The activity of Src is also regulated by its interaction with FAK (Tatosyan and Mizenina, 2000) (Fig.1.16). Src has been found to be involved in gene transcription, cell adhesion and migration, apoptosis and differentiation (Tatosyan and Mizenina, 2000; Courtneidge, 2002). The substrates of Src include the protein 1, p36 (Johnsson *et al.*, 1988), the p85 subunit of PI-3-K (Haefner *et al.*, 1995), p130Cas (Pellicena and Miller, 2001), and PKCs (Gschwendt *et al.*, 1994). It has also been reported that PKC can phosphorylate Src (Gould *et al.*, 1985; Moyers *et al.*, 1993).

1.9 Scope of this thesis

The previous sections have reviewed a global picture of vertebrate and invertebrate immunity, snail-schistosome interactions, and signal transduction and the way these disciplines relate to each other. The present study draws on these subject areas and can thus be considered multidisciplinary. Particularly incomplete is our understanding of the molecular control of the various defense reactions of molluscs; this is surprising given that molluscs constitute the second largest group of invertebrates and that many species are economically important pests of agriculture and vectors of diseases.

Aim: The principal aim of this thesis was to explore and unravel PKC signalling events in *L. stagnalis* defence cells to further our knowledge of the regulatory mechanisms controlling defence responses in molluscan intermediate hosts. Since the physiology and general characteristics of the internal defence system of *L. stagnalis* are well understood (Van der Knaap *et al.*, 1993), this snail constituted an excellent model of study for this research.

Structure: The layout of this thesis is in the form of a series of interconnecting chapters. The General introduction (**Chapter 1**) describes the background. Then, each subsequent chapter contains its own independent sections (Introduction, Experimental procedures, Results, and Discussion). Findings, conclusions, and future areas of study are then summarised in a final chapter entitled General Discussion (**Chapter 6**). Literature cited is listed in the final part of the thesis (References).

Published work resulting from this thesis, primarily derived from Chapter 2 and 4, appears in Appendices (Appendix II):

Lacchini, AH; Davies, AJ; Mackintosh, D; Walker, AJ (2006) 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. *The Journal of Experimental Biology*, 209: 4829-4840.

Wright, B; Lacchini, AH, Davies, AJ; Walker, AJ (2006) Regulation of nitric oxide production in (*Lymnaea stagnalis*) defence cells: a role for PKC and ERK signalling pathways. *Biology of the Cell*, 98: 265-278.

Chapter 2 provides evidence for the presence of specific PKC isotypes in *L. stagnalis* haemocytes and their response to challenge with the β-1, 3-glucan, laminarin. The use of pharmacological inhibitors enabled dissection of the underlying molecular mechanism(s) of PKC activation by laminarin, and the characterisation of upsteam- and downstream PKC-dependent signalling events in these cells.

Chapter 3 examines the signal-based haemocyte responses to schistosome antigenic components that have been shown to be present on the parasite surface (Nyame et *al.*, 2002). *Lymnaea stagnalis* served a good model for these studies since it is an intermediate host for avian schistosomes of the *Trichobilharzia* genus.

Knowledge gained in Chapter 2 provided a framework for further studies that focused on the role of PKC signalling in functional defence responses:

Chapter 4 investigates the role of PKC in the cytotoxic activities of *L. stagnalis* haemocytes by describing the generation of H_2O_2 following challenge with laminarin and blockade of signalling.

Chapter 5 explores how PKC might be involved in *L. stagnalis* haemocyte migration and spreading, essential processes during the innate defence response. Work in this chapter also aims to define how PKC might play a role in haemocyte adhesion, mediated by signalling interactions between PKC and integrins.

Chapter 2

The PKC pathway in *L. stagnalis* haemocytes

2.1 Introduction

Activation of a network of signalling pathways involving protein kinase- and lipid kinase-dependent reactions is crucial in mammalian innate immunity. Upon different external stimuli, a plethora of signalling molecules such as the highly conserved PLC, PKC, and MAPKs become activated and take part in complex cascades which transduce information into functional defence responses (Paul *et al.*, 1997; Downey *et al.*, 1998; Perskvist *et al.*, 2000). For example, the bacterial endotoxin LPS is known to activate several signal transduction pathways in macrophages; these often involve PKC and ERK and ultimately lead to the modulation of defence reactions such as phagocytosis and cytotoxic activities (Larsen *et al.*, 2000, Monick *et al.*, 2000; Castrillo *et al.*, 2001).

While much of the research concerning the molecular control of innate immunity has focused on mammalian and insect systems, knowledge of the molecular events that regulate molluscan immune responses remains limited. In this context, it is not known which receptors mediate defence reactions in molluscs and also which signal transduction pathways play a major role in the elimination of pathogens. Given that molluscan haemocytes functionally resemble mammalian macrophages, and that PKC is crucial to macrophage defence responses (Majumdar *et al.*, 1993; Greenberg, 1995; Tan and Parker; 2003) an investigation into PKC signalling events in molluscan haemocytes following challenge might offer novel insights into the regulation of molluscan defence strategies.

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 the sea slug *Aplysia californica* were described (Sossin *et al.*, 1993). The mechanisms of activation and translocation of PKC Apl I and II within the cell have been studied (Sossin *et al.*, 1994; Nakhost *et al.*, 1999). Protein kinase C Apl I and II appear to have a role in synaptic plasticity (Yanow *et al.*, 1998) although, to date, these proteins have not been reported in the *Aplysia* immune system. Recently, a phospholipid-sensitive Ca^{2+}-independent protein kinase (p105) from the mantle tissue of the bivalve *Mytilus galloprovincialis* was identified (Mercado *et al.*, 2002a). This protein was characterised as a mussel nPKC like-protein (Mercado *et al.*, 2002b) and its PMA-dependent translocation to haemocyte membranes was demonstrated (Mercado *et al.*, 2003). The first report of a PKC-like protein being present in molluscan defence cells was recently published (Walker and Plows, 2003).

Many signalling components are known to be linked either upstream or downstream of PKC signalling (see Chapter 1, section 1.8.4.5), these include PLC, PI-3-K, MEK, and ERK. In snails, the ERK 1/2 pathway has been characterised in an embryonic cell line from *B. glabrata* (see Humphries *et al.*, 2001) and in *L. stagnalis* haemocytes (Plows *et al.*, 2004). Recent work has also 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) identifying a role for these pathways in functional defence responses. The work described here characterises the PKC pathway in *L. stagnalis* haemocytes in response to various external compounds and elucidates the mechanisms by which PKC-mediated signals are propagated to critical downstream targets in haemocytes. Moreover, the work identifies upstream regulators of PKC activation in these cells.

2.2 Experimental procedures

2.2.1 Reagents

Protogel (30% (w/v) acrylamide) was from National Diagnostics (Hulls, Yorks, UK), whereas Hybond nitrocellulose membrane and enhanced chemiluminescence (ECL) Hyperfilm were from Amersham Biosciences (Little Chalfont, Bucks, UK). The Qentix signal enhancer and the Super Signal West Pico Chemiluminescent substrate were from Perbio Sciences (Cramlington, Northumberland, UK), and the Opti-4CN detection kit and detergent compatible (DC) protein assay kit were from Bio-Rad (Hemel Hempsted, Herts, UK). The anti-phospho PKC (pan), anti-phospho PKCαβII (Thr 638/641), anti-phospho PKCθ (Thr 538), anti-phospho PKCδ (Ser 643), anti-phospho PKCδ (Thr 505), anti-phospho PKCζ/λ (Thr 410/403), anti-phospho PKD/PKCμ (Ser 744/748), anti-phospho PKD/PKCμ (Ser 916), anti-phospho ERK 1/2, anti-phospho MEK1/2, anti-phospho Elk-1 (Ser 383), anti-PKCα and goat anti-rabbit horseradish peroxidase (HRP)-linked, antibodies were all purchased from Cell Signaling Technology (Beverly, MA, USA). The anti-phospho PKCα (Ser 657) antibody was from Santa Cruz Biotechnology (Santa Cruz, CA, USA) whereas the anti-phospho PKCε (Ser 729) antibody was purchased from Upstate Biotechnology (Temecula, CA, USA). The PLC inhibitor, ET-18-OCH_3 or edelfosine (1-O-Octadecyl-2-O-methyl-*rac*-glycero-3-phosphorylcholine); the PI-3-K inhibitor, LY294002 (2-(4-morpholinyl)-8-phenyl-4H-1-benzopyran-4-one hydrochloride); and the PKC inhibitor, calphostin C (from *Cladosporium cladosporioides*) came from Calbiochem (Nottingham, Notts, UK). Vectashield was purchased from Vector Laboratories (Burlingame, CA, USA). Molecular weight markers (SDS-6H), protease inhibitor cocktail (AEBSF, 100 mM; aprotinin, 0.08 mM; leupeptin, 2.2 mM; bestatin, 4 mM pepstatin A, 1.5 mM; E-64, 1.4 mM), phosphatase inhibitor cocktail (sodium orthovanadate, sodium molybdate, sodium tartrate, imidazole), anti-actin antibody, *E. coli* LPS (serotype 0111: B4), PMA, laminarin (from *Laminaria digitata*), zymosan, GF109203X (GFX) or bisindolylmaleimide 1, U-73122, fluorescein isothiocyanate (FITC)-conjugated goat anti-rabbit secondary antibody, tetramethyl rhodamine isothiocyanate (TRITC)-conjugated phalloidin, 4'-6-diamidino-2-phenylindole (DAPI), and all other chemicals were purchased from Sigma-Aldrich (Poole, Dorset, UK). Rat brain was also obtained from Sigma-Aldrich (Poole, Dorset, UK) and rat mesenteric artery (RMSA) extracts were kindly provided by Dr Jaqueline Ohanian (University of Manchester). All antibodies and reagents were stored as per the manufacturer's instructions.

2.2.2 Maintenance of snails in the laboratory

Adult snails were purchased from Blades Biologicals (Edenbridge, Kent, UK). Juvenile snails were then obtained by allowing eggs laid by adults to hatch in aquaria at room temperature. When these juvenile snails developed into adults, they were transferred to an incubator, maintained under a 12 h light-12 h dark cycle at 20°C, and kept in separated tanks containing water. Snails were fed with lettuce, *ad libitum*, and some fish food flakes once a week. A daily check was undertaken to remove any dead snails, since these might infect water and lead to the death of other snails sharing the same tank. Water used in aquaria and tanks within the incubator was filtered through a Brimak/carbon filtration unit (Silverline Ltd., Winkleigh, Devon, UK), was continuously aerated, and was changed every week to remove faeces and decaying lettuce.

2.2.3 Haemolymph extraction

Adult *L. stagnalis* were washed with distilled water and any mucus was removed with paper tissue. Haemolymph was obtained from six to eight snails by head-foot retraction (Sminia, 1972) using a 200 µl pipette tip as a tool. 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 (Lever and Bekius, 1965). Sterile snail saline (SSS) (SSS: 3 mM (4-(2-hydroxymethyl piperazine) (HEPES), 3.7 mM NaOH, 36 mM NaCl, 2 mM KCl, 2 mM $MgCl_2$, 4 mM $CaCl_2$, pH 7.8, sterilised through a 0.22 µm disposable filter) (Sminia, 1972) was then added to the extracted haemolymph (2 parts haemolymph: 1 part SSS) which was kept on ice to prevent haemocytes from clumping.

2.2.4 Haemocyte signalling assays

2.2.4.1 Haemocyte monolayers

Haemocyte monolayers were prepared by adding 500 µl of diluted haemolymph to individual wells of a sterile 24-well culture plate (Nunc). Cells were then allowed to bind to the wells for 30 min at room temperature. Afterwards, monolayers were washed three times with 500 µl SSS to remove haemolymph and non-adherent/dead haemocytes; cells were then left to equilibrate for 1 h (Walker *et al.*, 2003) in 500 µl SSS.

2.2.4.2 Challenge of haemocytes

After equilibrating in SSS, haemocytes were challenged with bacterial LPS (1 μg/ml), laminarin (10 mg/ml), PMA (10 μM), or zymosan (10 μg/ml) in SSS for various durations (0-30 min). The SSS containing the stimulants was then immediately removed and 70 μl of boiling 1X sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS–PAGE) sample buffer (62.4 mM Tris-HCl (hydroxymethyl) methylamine–hydrochloride) pH 6.8, 2 % (w/v) SDS, 2 % (v/v) 2-mercaptoethanol, 5% (v/v) glycerol, 0.003 % (w/v) bromophenol blue) was added to the monolayers to solubilise the haemocyte proteins. Samples were then briefly sonicated (40s) and were boiled for 5 min prior to electrophoresis or storage at -20°C for subsequent analysis.

2.2.4.3 Inhibition assays

The effect of various inhibitors on PKC phosphorylation was determined in laminarin-challenged haemocytes. Inhibition assays were performed using: the PKC inhibitors, GF109203X and calphostin C; PLC inhibitors, U-73122 and ET-18-OCH_3; and the PI-3-K inhibitor, LY294002. Haemocytes were treated for 30 min with GF109203X (0.001 μM-10 μM), calphostin C (0.001 μM-10 μM), U-73122 (0.001 μM-10 μM), ET-18-OCH_3 (0.001 μM-10 μM), or LY294002 (0.001 μM-10 μM) prior challenge. Where inhibitors were solubilised in DMSO or ethanol, the final DMSO/ethanol concentration in the assay was below 0.1% (v/v) and control samples were exposed to DMSO/ethanol alone. GF109203X is a widely used competitive inhibitor of PKC at the ATP binding site in the catalytic domain (Toullec *et al.*, 1991) preventing PKC phosphorylation (i.e. activation), and is selective towards PKCα, βI, βII, γ, ε, and δ isoforms. Calphostin C has the ability to bind the zinc-finger-like motifs of the regulatory domain of PKC (Tamaoki *et al.*, 1990). U-73122 is an inhibitor of PLC but its exact mechanism of action remains unclear (Bleasdale *et al.*, 1990). LY294002 is a cell-permeable synthetic inhibitor that selectivity blocks the ATP binding site in the p85 subunit of the different classes of PI-3-K, preventing the activation of the enzyme (Walker *et al.*, 2000). Finally, the ether lipid ET-18-OCH_3 is a cell-permeable cytotoxic molecule that selectively inhibits the phosphatidylinositol-specific PLC (Powis *et al.*, 1992).

2.2.5 Haemocyte lysis and protein determination

In some experiments, haemocyte monolayers were lysed and protein concentrations of the extracts were determined. Rat brain extracts were used as an control. Monolayers were either prepared in 24-well culture plates (as described in section

2.2.4.1) or Petri dishes to which 3 ml of diluted haemolymph was added and 3 ml SSS used to wash the cells. Cell lysis was achieved by adding either 70 µl (in 24-wells culture plates) or 150 µl (in Petri dish) of extraction buffer (50 mM Tris-HCl, pH 7.4, 250 mM sucrose, 150 mM NaCl, 250 µM EDTA, 1% (v/v) Triton X-100, 1% (w/v) SDS, 0.25% (w/v) deoxycholic acid) containing protease and phosphatase inhibitor cocktails at the manufacturer's recommended concentration to the monolayer. Protein extraction was performed on ice and protein concentrations subsequently determined using the Bio-Rad DC protein assay kit following the manufacturer's instructions with bovine serum albumin (BSA) as the standard. After protein determination, an appropriate volume of 5X SDS–PAGE sample buffer was added to samples; they were then briefly sonicated (40 s) and boiled prior to electrophoresis or storage at -20°C for subsequent analysis.

2.2.6 Polyacrylamide gel electrophoresis

Sodium dodecyl sulphate-polyacrylamide gel electrophoresis was carried out in discontinuous vertical slab mini gels. The resolving gel was composed of 100 mM Tris (pH 8.8), 10% (w/v) acrylamide (from a 30% stock solution of Protogel), 0.1% (w/v) SDS, 0.3 % (v/v) N, N, N′, N′ –tetramethylethylenediamine (TEMED) and 0.3% (w/v) ammonium persulphate (APS). The stacking gel comprised 100 mM Tris (pH 6.8), 6.6% (w/v) acrylamide (from a 30% stock solution of Protogel), 0.1% (w/v) SDS, 0.25% TEMED and 0.3% (w/v) APS. Samples were loaded onto the stacking gel in addition to the molecular weight markers (MWMs) (SDS-6H: 10 µl per lane). The MWMs comprised the following proteins: myosin (from rabbit muscle; 205 kDa), β-galactosidase (from *E. coli*; 116 kDa), phosphorylase b (from rabbit muscle; 97.4 kDa), bovine albumin (66 kDa), egg albumin (45 kDa), and carbonic anhydrase (from bovine erythrocytes; 29 kDa). Bromophenol blue, present in the SDS-PAGE sample buffer, served as an electrophoresis tracking dye. The gel was run at constant 160 V and 35 mA (per gel) for approximately 90 min in running buffer (24 mM Tris, 193 mM glycine, 0.1% (w/v) SDS) until the tracking dye had migrated through the whole gel.

2.2.7 Western blotting

Separated haemocyte proteins were transferred to nitrocellulose membranes (0.45 µm pore size) using a Bio-Rad semi-dry electrotransfer unit for 90 min at 300 mA and 15 V; the blotting buffer comprised 24 mM Tris, 200 mM glycine, and 20% (v/v) methanol. Membranes were then washed briefly in distilled water. In some

experiments, the Qentix signal enhancer kit was used following the manufacturer's instructions to enhance the immunoreactive signal on blots: briefly, membranes were immersed for 2 min in enhancer reagent 1, then following three rinses with distilled water, were incubated for 10 min in enhancer reagent 2. Membranes were then rinsed five times with distilled water. Blots were stained with (10%) (w/v) Ponceau S working solution (30% (w/v) trichloroacetic acid, 30% (w/v) sulfosalicylic acid, 2% (w/v) Ponceau S in distilled water) for 5 min to confirm that homogenous transfer of proteins had taken place. After staining, membranes were briefly rinsed in distilled water to reveal the protein bands and the location of each MWM was marked with a biro. Ponceau S is a reversible stain and was removed by rinsing the membrane in Tween 20 Tris-buffered saline (TTBS) (15 mM Tris-HCl (pH 8.0), 150 mM NaCl containing (0.1%) (v/v) Tween 20). The membranes were then blocked at room temperature for 1 h with 5% (w/v) non-fat dried milk in TTBS to prevent non-specific binding of antibodies to the membranes. Membranes were subsequently incubated with appropriate polyclonal antibodies (see below) overnight at 4°C. In early experiments, different dilutions of the primary antibody were used to find the optimum concentration of antibodies required to provide a good immunoreactive signal.

The commercially obtained polyclonal primary anti-phospho antibodies were raised in rabbits against a short sequence (of approximately 10-12 amino acids) containing the targeted phosphorylated specific amino acid residue(s) as indicated below:

- Anti-phospho PKC (pan) antibody (1:1000 in TTBS) which detects PKCα, β_I, β_{II}, ε, η, and δ only when phosphorylated on a carboxy-terminal residue homologous to the Ser 660 within the human PKCβII hydrophobic motif.

- Anti-phospho PKC$\alpha\beta$II (Thr 638/641) antibody (1:1000 in TTBS) which recognises PKCα when phosphorylated on Thr 638 and PKCβII when phosphorylated on Thr 641.

- Anti-phospho PKCα (Ser 657) antibody (1:1000 in TTBS) which detects PKCα when phosphorylated on Ser 657.

- Anti-phospho PKCδ (Ser 643) antibody (1:1000 in TTBS) which detects PKCδ when phosphorylated on Ser 643.

- Anti-phospho PKCδ (Thr 505) antibody (1:1000 in TTBS) which recognises PKCδ when phosphorylated on Thr 505.

- Anti-phospho PKCε (Ser 729) antibody (1:1000 in TTBS) which detects PKCε when phosphorylated on Ser 729.

- Anti-phospho PKCθ (Thr 538) antibody (1:1000 in TTBS) which detects PKCθ when phosphorylated on Thr 538.

- Anti-phospho PKCζ/λ (Thr 410/403) antibody (1:1000 in TTBS) which recognises PKCζ isoforms when phosphorylated on Thr 410 and PKCλ isoforms when phosphorylated on Thr 403.

- Anti-phospho ERK 1/2 (or p42/44 MAPK) (Thr 202/Tyr 204) antibody (1:1000 in TTBS) which detects p42 (ERK 2) and p44 (ERK 1) MAP kinase only when phosphorylated on Thr 202 and Tyr 204 of human ERK, or on Tyr183 and Tyr 185 of rat ERK2.

- Anti-phospho MEK1/2 (Ser 221) antibody (1:1000 in TTBS) which recognises MEK1/2 only when phosphorylated on Ser 221.

- Anti-phospho Elk-1 (Ser 383) antibody (1:1000 in TTBS) which detects Elk-1 only when phosphorylated on Ser 383.

- Anti-phospho PKD/PKCμ (Ser 916) antibody (1:1000 in TTBS) which detects PKD only when phosphorylated on Ser 916.

- Anti-phospho PKD/PKCμ (Ser744/748) antibody (1:1000 in TTBS) which detects PKD only when dually phosphorylated at Ser 744 and Ser 748.

For the above anti-phosphospecific antibodies, phosphorylation at the respective sites (numbering for the mammalian isoforms) results in enzyme activation; therefore the antibodies are designed to detect their respective, activated kinases in cell extracts.

The anti-PKCα (total) antibody (1:1000 in TTBS) was also used to detect PKCα irrespective of their phosphorylation state.

In all experiments, equal loading of proteins between lanes was checked by incubating blots with an anti-actin antibody (1:1000 in TTBS) which shows a broad reactivity towards actin isoforms by recognising the N-terminal region of the protein.

The next day, immunoblots were washed four times for 5 min each with TTBS and incubated for 1 h at room temperature with secondary HRP-conjugated goat anti-rabbit antibody (1:7500 in TTBS). Following four washes with TTBS for a further 5 min each, immunoreactive bands were visualised using either enhanced chemiluminescence (ECL) detection with ECL Hyperfilm, or colorometric (Opti-4CN, Bio-Rad) methods following the manufacturer's instructions.

2.2.8 Immunocytochemistry

Haemolymph was extracted from four to six adult snails and was diluted in SSS as previously described in section 2.2.3, 100 µl of the diluted haemolymph was then applied to individual glass coverslips which were placed in 6-well culture plates to facilitate subsequent washing. Haemocytes were allowed to adhere to coverslips for 30 min and, after gently washing with 1ml SSS three times, were left to equilibrate in 1 ml SSS for 1 h. Haemocytes were then either left in SSS or were challenged with laminarin (10 mg/ml in SSS) for 10 min; where appropriate, cells were treated with the PKC inhibitor GF109203X (10 µM) for 30 min prior to adding laminarin. Cells were then fixed by adding 1 ml of fixing 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. Triton X-100 removes cell membrane lipids allowing the antibodies into the cells whereas formaldehyde fixes the cells on the support and the proteins within the cell. Fixed haemocytes were then incubated in 1 ml blocking solution (1% BSA in PBS) for a further 12 min before being incubated in anti-phospho PKC (pan) antibody (1:200 in PBS), anti-phospho PKCαβII (1:200 in PBS), or anti-phospho PKCα (1:200 in PBS) for 1 h at room temperature. Finally, after three washes in PBS, cells were incubated with FITC-conjugated goat anti-rabbit secondary antibody (1:1000 in PBS) for 30 min at room temperature. The cells were then washed in PBS a further three times and incubated in TRITC-conjugated phalloidin (0.1 µg/ml in PBS) for 40 min at room temperature. This compound stains actin filaments. If appropriate, the nucleus was stained using DAPI (1 µg/ml in PBS) for 5 min. DAPI is known to form fluorescent complexes with natural double-stranded DNA, showing fluorescence specificity for AT, AU and IC clusters. After washing a final three times with PBS, the coverslips were mounted with one drop of Vectashield (to prevent fluorescence fading during microscope examination) onto a microscope slide and were sealed with clear nail polish. Cells were then visualised with a Leica laser scanning confocal microscope SP2 AOBS running Leica software and optical sections and total images were captured on the computer. The FITC-conjugated antibody has excitation peak at 495 nm (blue)

and emission peak at 525 nm (yellow/green) whereas TRITC is excited at 550 nm (green) and emits at 580 nm (red).

2.2.9 Data analysis

Where appropriate, the intensity of individual bands on scanned immunoblots were determined using Kodak 1D image analysis software; data were then analysed with SPSS software using one way analysis of variance (ANOVA) and *post-hoc* multiple comparison (LSD) tests. For all experiments, results are shown as the mean standard deviation (± SD).

2.3 Results

2.3.1 Initial identification of PKC and PKD-like proteins in *L. stagnalis* haemocytes by western blotting

The identification of a PKC-like protein in *L. stagnalis* haemocytes has been established (Walker and Plows, 2003). In order to identify other PKC-like proteins, protein extracts from LPS-challenged haemocytes were first screened against a panel of commercially available anti-PKC/PKD and anti-phospho PKC/PKD antibodies to ascertain which other PKC/PKD isoforms might be present in the cells. These anti-phospho antibodies recognise their respective, activated kinases in cell extracts and were raised against epitopes surrounding the following activation sites: PKCα, Ser 638; PKCβII, Ser 641; PKCε, Ser 729; PKCδ, Ser 643 and Thr 505; PKCθ, Thr 538; PKCλ, Ser 410/403; PKD, Ser 744/748 and Ser 916.

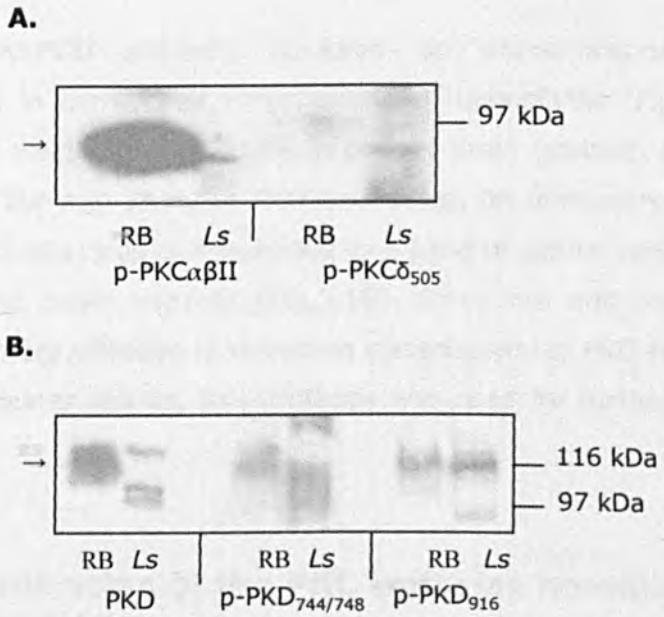


Fig.2.1: Initial identification of different PKC/PKD-like proteins in *L. stagnalis* haemocytes after challenge with LPS (1 µg/ml) for 5 min using western blotting.
(A) Anti-phospho PKCαβII, anti-phospho $PKC\delta_{505}$, (B) anti-PKD, anti-phospho $PKD_{744/748}$, and anti-phospho PKD_{916} antibodies were used to detect the respective phosphorylated PKC-like and PKD-like proteins present in *L. stagnalis* haemocyte protein extracts (*Ls*) (20 µg). Rat brain extract (RB) (6 µg) was used as a positive control.

Western blotting with anti-phospho $PKC\delta_{643}$, anti-phospho $PKC\theta_{538}$ and anti-phospho $PKC\lambda_{410/403}$ antibodies did not give any convincing immunoreactive signals in *L.*

stagnalis haemocyte extracts; this suggests that the enzymes are not present, that there are differences in the residues surrounding the phosphorylation sites between molluscan and mammalian isoforms, or that they were not activated by LPS in haemocytes. Although these isoforms were expected to be found in the control rat brain, no significant immunoreactive bands were revealed. Other anti-PKC antibodies were used but they did not appear to be reliable as fluctuation of results between experiments often occurred: these include the anti-phospho PKCε antibody (from Upstate), and the anti-PKCα antibody (from Cell Signaling Technology).

One immunoreactive band was, however, detected in *L. stagnalis* haemocyte extracts with the anti-phospho PKCαβII antibody, corresponding to a protein with approximate molecular weight of 80 kDa (Fig.2.1A). A single large band corresponding to PKCαβII was also detected in the control rat brain extract at the expected molecular weight for this isoform (80 kDa) (Fig.2.1A). The anti-phospho $PKC\delta_{505}$ detected a relatively diffuse band in both RB and *Ls* (Fig.2.1A).

Blotting with the anti-PKD antibody revealed an immunoreactive band of approximately 115 kDa in extracts of LPS-challenged haemocytes (Fig.2.1B) which was a similar molecular weight to that found in the rat brain (control) extract. When blots were probed with the anti-phospho PKD antibodies, an immunoreactive protein was also detected at 115 kDa; this corresponded to a band of similar molecular weight that was detected in rat brain extracts (Fig.2.1B). Since the anti-phospho PKD_{916} antibody seemed to be more effective at detecting phosphorylated PKD-like proteins in haemocytes and gave clearer results, this antibody was used for further experiments (see section 2.3.5).

2.3.2 Further identification of the PKC isoforms homologues in *L. stagnalis* haemocytes

In order to ascertain whether the 80 kDa immunoreactive band detected in *L. stagnalis* haemocytes with the anti-phospho PKCαβII antibody (Fig.2.1A) corresponded to a PKCαβII-like protein, other western blotting analyses were done using a further antibody and rat mesenteric small artery (RMSA) extracts as a PKC control (Fig.2.2). Blots with RMSA and *L. stagnalis* haemocyte proteins were probed with the anti-phospho PKC (pan) antibody, and the anti-phospho PKCαβII antibody. The advantage of using RMSA extracts over rat brain extracts was that they had been stimulated with noradrenaline to activate PKC signalling pathways. Many PKC isoforms

are known to be present in RMSA's (Ohanian *et al.*, 1996) including PKCα, PKCγ, PKCδ, PKCε and PKCζ.

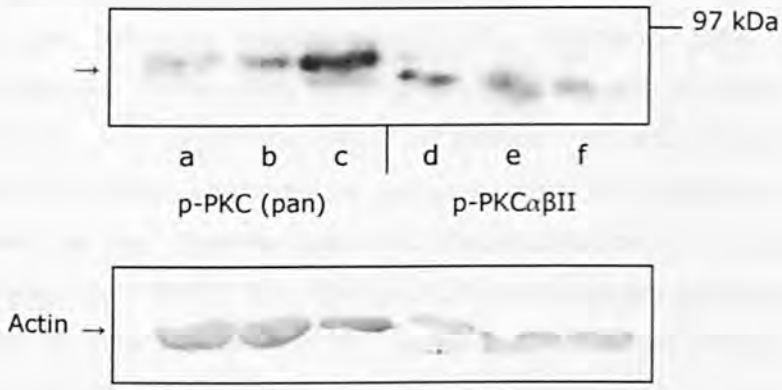


Fig.2.2: Detection of PKC-like proteins in *L. stagnalis* haemocytes.
The upper immunoblot shows the presence of a PKCαβ-like protein in *L. stagnalis* haemocytes. Lanes a and e (each with 8 μg protein) contain unstimulated RMSA extracts whereas lanes b and f (8 μg) contain extracts of RMSAs stimulated with noradrenaline. Lanes c and d contain 30 μg protein obtained from haemocytes that had been stimulated with LPS (1 μg/ml) for 5 min. Blots were then probed with anti-phospho PKC (pan) and anti-phospho PKCαβII antibodies. The immunoblot was also probed with anti-actin antibodies. Immunoblots are representative of two independent experiments.

The anti-phospho PKC (pan) antibody detected two phosphorylated proteins on western blots, with approximate molecular weights of 80 and 85 kDa in haemocytes (Fig.2.2); proteins of similar molecular weight were also detected in the RMSA extracts. Three bands were sometimes detected in haemocyte samples with both anti-phosphospecific antibodies although often, only two bands appeared. In *L. stagnalis* haemocytes, the anti-phospho PKC (pan) antibody seems to recognise the middle band more effectively (corresponding to the 85 kDa PKC) whereas the anti-phospho PKCαβII antibody better detects the lower band (corresponding to an 80 kDa isoform) (Fig.2.3).

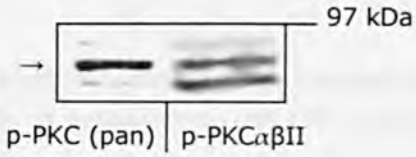


Fig.2.3: Respective immunoreactive bands detected by the anti-phospho PKC (pan) and anti-phospho PKCαβII antibodies in *L. stagnalis* haemocytes. Each well was loaded with 30 μg protein obtained from haemocyte extracts that had been stimulated with LPS (1 μg/ml) for 5 min. The immunoblot is representative of two independent experiments.

2.3.3 Effects of LPS, laminarin, PMA and zymosan on the phosphorylation status of haemocyte PKC

The kinetics of PKC phosphorylation (activation) over 30 min were evaluated in *L. stagnalis* haemocytes following challenge with LPS, laminarin, PMA, or zymosan. Samples were processed for western blotting using either anti-phospho PKC (pan), anti-phospho PKCαβII, or anti-phospho PKCα antibodies. The anti-phospho PKC (pan) antibody has previously been validated for use in *L. stagnalis* haemocytes (Walker *et al.*, 2003) and also has been used to study PKC phosphorylation in *M. galloprovinciallis* haemocytes (Canesi *et al.*, 2005). The effects of LPS on signalling pathways have been briefly investigated in insect (Foukas *et al.*, 1998) and molluscan (Walker and Plows, 2003; Plows et *al.*, 2004) haemocytes.

2.3.3.1 Challenge with LPS

The bacterial endotoxin LPS is a well-known stimulant of mammalian macrophages (Ulevitch and Tobias, 1999) and it has been shown to activate a number of intracellular signalling pathways including those involving ERK 1/2 and PKC (Liu *et al.*, 1994; Sweet and Hume, 1996).

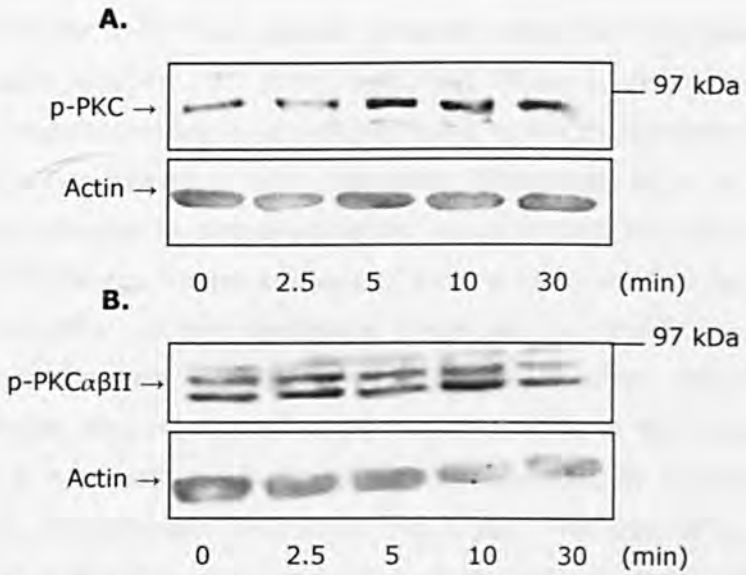


Fig.2.4: The effects of LPS on PKC phosphorylation levels: immunodetection of phosphorylated PKC-like proteins following challenge of haemocytes with LPS (1 μg/ml) for various durations (0-30 min).

Samples were obtained as described in "Experimental procedures". Equal amounts of protein were loaded in each lane and membranes were probed with phosphospecific antibodies (A) to PKC (pan) and (B) to PKCαβII. Anti-actin antibodies were used to confirm equal loading of proteins. Immunoblots are representative of two experiments.

Lymnaea stagnalis haemocytes were challenged with LPS (1 μg/ml) for 0-30 min and PKC phosphorylation was assessed with the anti-phospho PKC (pan) antibody. Exposure to LPS resulted in increased phosphorylation of the 85 kDa haemocyte PKC-like protein after 5-10 min; after 30 min challenge, phosphorylation levels remained higher than in unchallenged haemocytes (Fig.2.4A, 0 min). However, the use of a second phosphospecific anti-phospho PKCαβII antibody did not reveal any obvious changes of PKC phosphorylation over 30 min (Fig.2.4B).

2.3.3.2 Challenge with laminarin

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 superoxide by *M. galloprovincialis* haemocytes (Arumugam *et al.*, 2000), and NO by *L. stagnalis* haemocytes (Wright *et al.*, 2006).

To evaluate the effect of laminarin on the phosphorylation (activation) status of PKC-like proteins in *L. stagnalis* haemocytes, haemocyte monolayers were challenged with laminarin (10 mg/ml) for 0–30 min; cellular proteins were then analysed by western blotting with the anti-phospho PKC (pan) antibody. When *L. stagnalis* haemocytes were exposed to 10 mg/ml laminarin, a rapid increase in the phosphorylation of the 85 kDa PKC-like protein was observed with maximum phosphorylation occurring at 10 min (Fig.2.5A). The increase in phosphorylation was transient and reduced to basal levels after 30 min challenge. Image analysis of several immunoblots and subsequent ANOVA revealed that after 10 min challenge, there was a 3.5-fold increase in PKC phosphorylation and this was significantly different to control values (Fig.2.5A). Shorter exposure times also resulted in a significant change in PKC phosphorylation levels with 2.5 and 5 min challenge increasing phosphorylation by 2.5 and by 3 times that of the control, respectively ($P \leq 0.05$; Fig.2.5A). The use of a further two phosphospecific PKC antibodies, the anti-phospho PKC$\alpha\beta_{II}$ 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.2.5B and C).

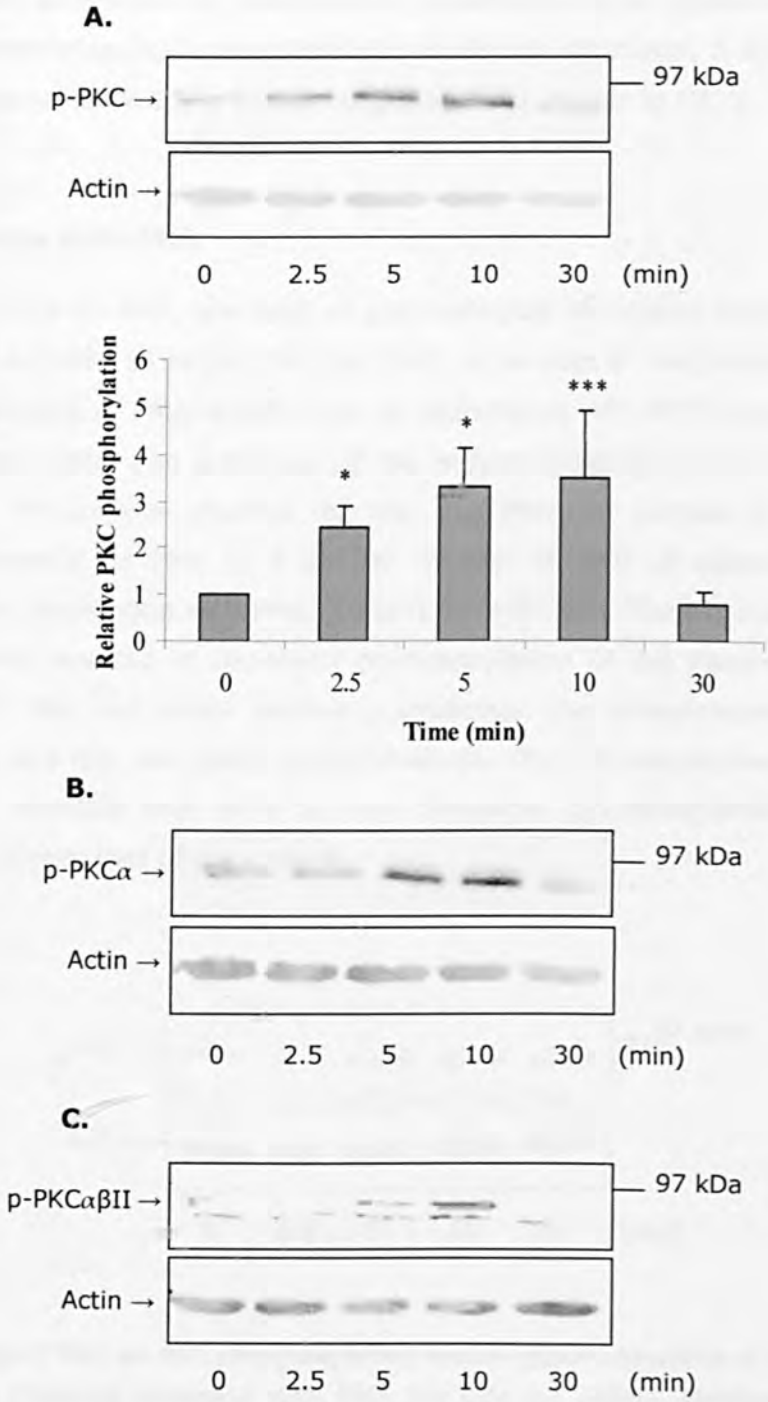


Fig.2.5: The effects of laminarin on PKC phosphorylation levels: immunodetection of phosphorylated PKC-like proteins following challenge with laminarin (10 mg/ml) for various durations (0-30 min).

Samples were obtained as described in "Experimental procedures". Equal amounts of protein were loaded in each lane and membranes were probed with phosphospecific antibodies (A) to PKC (pan), (B) to PKCα and (C) to PKCαβII. Anti-actin antibodies were used to confirm equal loading of proteins. Immunoblots are representative of four independent experiments. Relative PKC phosphorylation levels (shown in the graph) detected with anti-phospho PKC (pan) antibody (A) were determined by image analysis. Values are shown as mean (± SD) from n=4 experiments. *$p \leq 0.05$ and ***$p \leq 0.001$ when compared with control values (time 0).

Given that these antibodies all recognized a protein of similar molecular weight that had similar phosphorylation kinetics following laminarin challenge, it appears that the *L. stagnalis* haemocyte PKC-like protein might be most similar to PKCα.

2.3.3.3 Challenge with PMA

Phorbol esters, such as PMA, are used as pharmalogical stimulants since they are cell permeable and are able to mimic the lipid DAG, a product of PIP_2 hydrolysis by PLC. The biological activity of PMA results from its association with PKC which leads to the membrane translocation and activation of the enzyme (Castagna *et al.*, 1982; Niedel *et al.*, 1983). To confirm whether or not the PKC-like protein in *L. stagnalis* haemocytes responds to PMA in a similar manner to that of mammalian cPKCs, haemocytes were challenged with PMA (10 μM) for 0-30 min. The exposure of the cells to this compound resulted in increased phosphorylation of the haemocyte PKC-like protein after 10 min, but unlike laminarin challenge, the phosphorylation appeared more sustained and did not return to basal levels after 30 min challenge (Fig.2.6). Image analysis revealed that after 10 min challenge, phosphorylation levels were approximately 5 times that of the control.

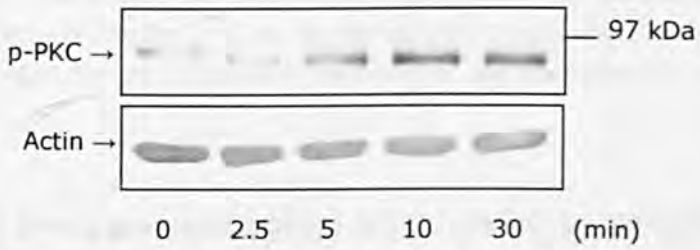


Fig.2.6: The effects of PMA on PKC phosphorylation levels: immunodetection of phosphorylated PKC-like proteins following challenge with PMA (10 μM) for various durations (0-30 min). Samples were obtained as described in "Experimental procedures". Equal amounts of protein were loaded in each lane and membranes were probed with the anti-phospho PKC (pan) antibody. Anti-actin antibodies were used to confirm equal loading of proteins. Immunoblots are representative of two independent experiments.

2.3.3.4 Challenge with zymosan

Zymosan is a yeast (*Saccharomyces cerevisiae*) cell wall glycan, containing mannan and approximately 55% β-1, 3 glucan (Di Carlo and Fiore, 1958). Studies have shown that zymosan activates immune functions in macrophages (Tapper and Sundler,

1995). Furthermore, this compound has been shown to stimulate the production of ROIs by molluscan haemocytes (Toreilles and Guérin, 1999; Zelck *et al.*, 2004). *Lymnaea stagnalis* haemocytes were therefore challenged with zymosan (10 µg/ml) for 0-30 min to determine the effects of exposure to this compound on haemocyte PKC phosphorylation levels. Challenge with zymosan resulted in an increase of PKC phosphorylation after 10 min, similar to that observed with PMA. Moreover, PKC phosphorylation appeared more sustained than that observed with laminarin challenge and no transient effects were observed over 30 min (Fig.2.7). After 10 min challenge, phosphorylation levels were approximately 2 times that of the control.

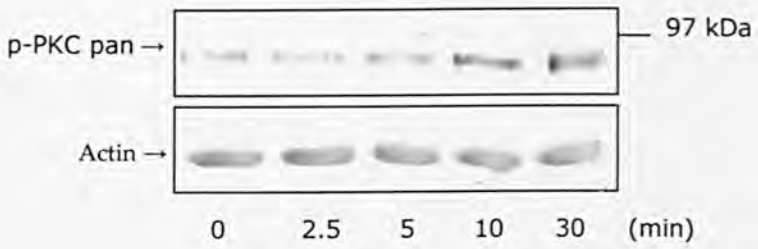


Fig.2.7: The effects of zymosan on PKC phosphorylation levels: immunodetection of phosphorylated PKC-like proteins following challenge with zymosan (10 µg/ml) for various durations (0-30 min).

Samples were obtained as described in "Experimental procedures". Equal amounts of protein were loaded in each lane and membranes were probed with the anti-phospho PKC (pan) antibody. Anti-actin antibodies were used to confirm equal loading of proteins. Immunoblots are representative of two independent experiments.

2.3.4 Effects of the pharmalogical PKC inhibitor GF109203X on PKC phosphorylation

GF109203X (GFX) is known to inhibit different isoforms of PKC (see section 2.2.4.3) by acting as a competitive inhibitor of the ATP binding site of PKC. This inhibitor was therefore used in an attempt to block PKC phosphorylation in laminarin (10 mg/ml)-stimulated haemocytes. GF109203X significantly inhibited PKC phosphorylation following laminarin challenge in a dose-dependent manner (Fig.2.8A) causing a significant 72% decrease in PKC phosphorylation when used at a concentration of 10 µM ($P \leq 0.001$; Fig.2.8B). Moreover, at this dose, PKC phosphorylation was reduced to approximately 33% below basal levels. Lower concentrations of GF109203X (0.01 µM–1 µM) also reduced laminarin-dependent PKC phosphorylation significantly ($P \leq 0.05$; Fig.2.8B) with 1 µM reducing phosphorylation levels to those seen in unchallenged haemocytes.

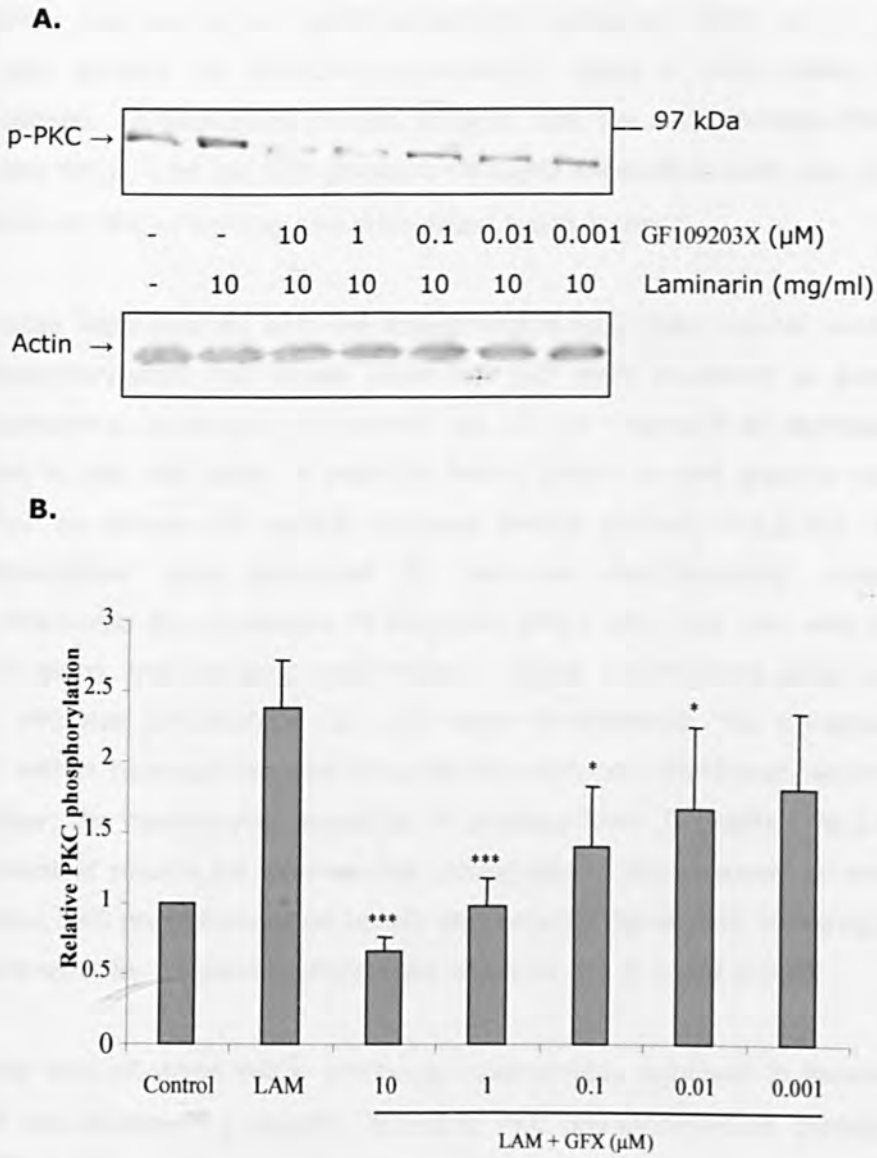


Fig.2.8: GF103209X reduces PKC phosphorylation in laminarin-stimulated haemocytes.
(A) Haemocyte monolayers were incubated with various concentrations of GF103209X (GFX; 0.001 μM-10 μM), or vehicle (0.1% DMSO), prior challenge with laminarin (10 mg/ml) for 10 min. Protein extracts from haemocytes were prepared, subjected to SDS-PAGE, and western blotting which was performed using the anti-phospho PKC (pan) antibody. Similar amounts of protein were loaded onto each lane and anti-actin antibodies were used to confirm equal loading of proteins. (B) Relative PKC phosphorylation levels following treatment were determined by image analysis of blots from n=4 independent experiments. Values shown are mean values (± SD). $*P \leq 0.05$ and $***P \leq 0.001$ when compared with phosphorylation levels observed in stimulated, uninhibited, cells.

2.3.5 Distribution of phosphorylated PKC in *L. stagnalis* haemocytes

The intracellular distribution of phosphorylated (activated) PKC in *L. stagnalis* haemocytes was studied by immunocytochemistry using a Leica laser scanning confocal microscope. Fluorescence images showed that the anti-phospho PKC (pan), the anti-phospho PKCα, and the anti-phospho PKCαβII antibodies were able to localize activated molluscan PKC in resting and stimulated haemocytes.

When haemocytes were stained with the anti-phospho PKC (pan), under unstimulated conditions, phosphorylated PKC levels were low but were clustered in some areas (Fig.2.9A). Exposure to laminarin (10 mg/ml) for 10 min triggered an increase in PKC phosphorylation in the cell body. A possible redistribution to the plasma membrane seems to occur as shown on optical sections (white arrows; Fig.2.10). In some instances, stimulation also appeared to promote morphological changes in haemocytes, evidenced by expansion of filopodia (Fig.2.9B), and this was observed consistently in many independent experiments. When haemocytes were incubated with the PKC inhibitor GF109203X (10 μM) prior to challenge, the phosphorylation status of PKC within haemocytes was considerably reduced. Moreover, when treated with this inhibitor, the haemocytes appeared to possess fewer filopodia (Fig.2.9C). The immunocytochemical results for haemocytes challenged in the presence or absence of GF109203X show PKC phosphorylation levels that broadly agree with those obtained in previous experiments by western blotting (see sections 2.3.3.2 and 2.3.4).

When using the anti-phospho PKCα antibody, haemocytes exposed to laminarin (10 mg/ml) for 10 min displayed a slightly increased PKC phosphorylation compared with the control (Fig.2.11A). However, the level of PKC phosphorylation in control cells often appeared relatively high; this could reflect the reasonably high basal level of haemocyte PKC sometimes observed in western blotting analyses (Fig.2.5B). Upon laminarin challenge, immunoreactivity appeared to be slightly stronger in the vicinity of the plasma membrane, but phosphorylated PKC often appeared to be predominantly associated with the nucleus (Fig.2.11B). The PKC phosphorylation levels were reduced in presence of GF109203X (10 μM) (Fig.2.11C).

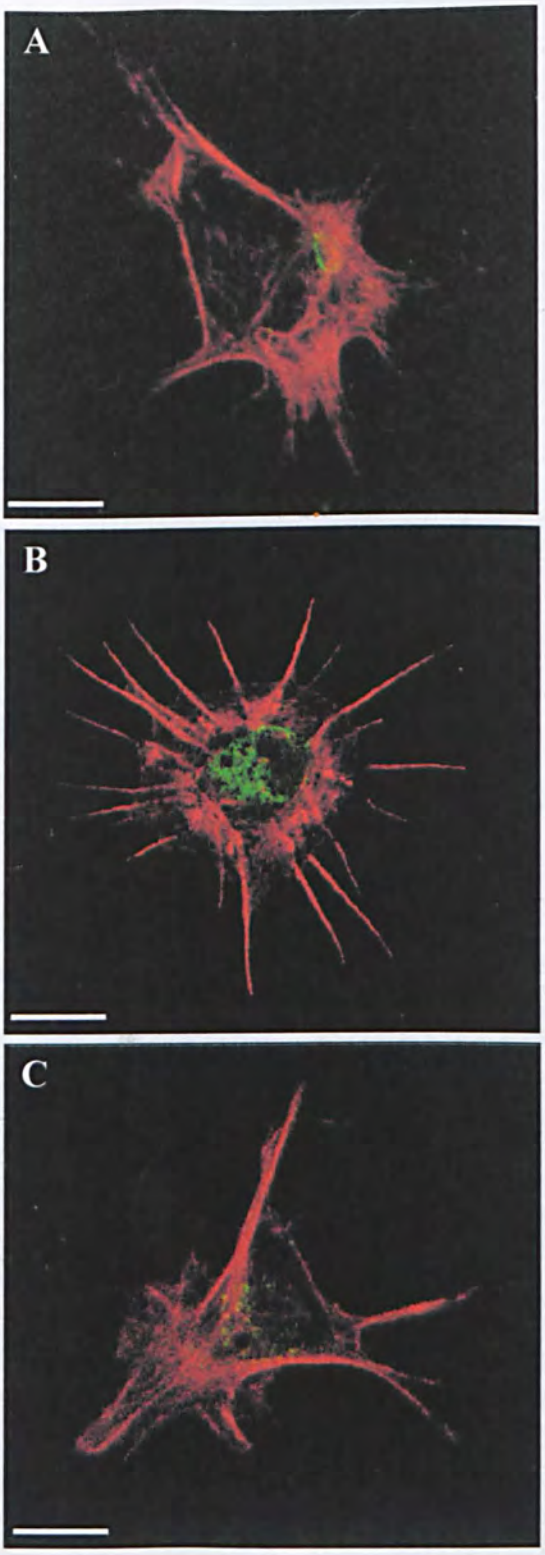


Fig.2.9: Distribution and levels of phosphorylated PKC in *L. stagnalis* haemocytes investigated by immunocytochemistry using the anti-phospho PKC (pan) antibody.

Phosphorylated PKC in (A) untreated haemocytes, (B) haemocytes challenged with laminarin (10 mg/ml) for 10 min, and (C) haemocytes incubated with GF109203X (10 μM) for 30 min prior to challenge with laminarin (10 mg/ml) 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. Images are representative of three independent experiments. Bar = 20 μm.

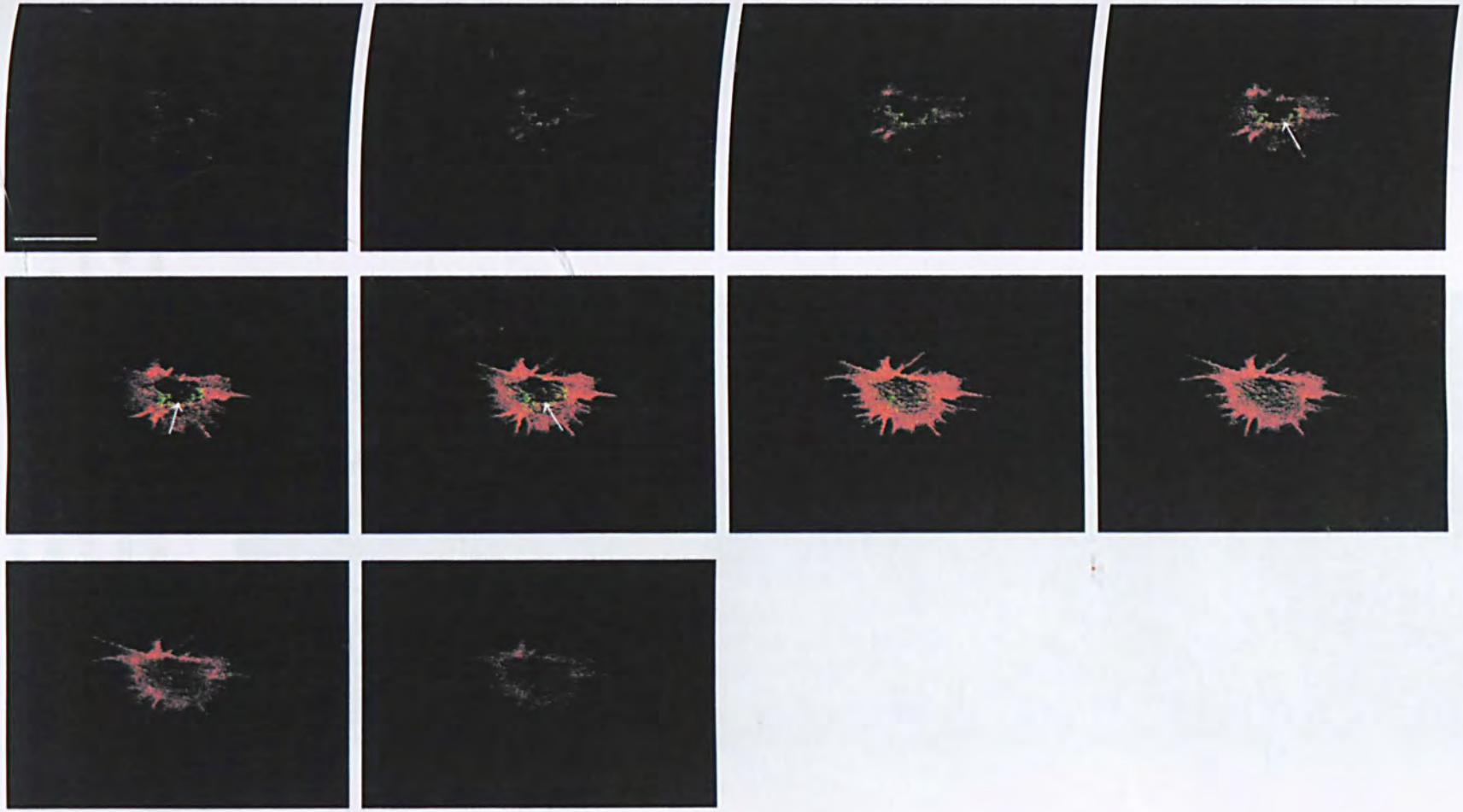


Fig.2.10: Optical sections through a single *L. stagnalis* haemocyte challenged with laminarin (10 mg/ml) for 10 min using the Leica laser scanning confocal microscope. Haemocytes have been labelled with the anti-phospho PKC (pan) antibody (green). Rhodamine phalloidin (red) stains F-actin. White arrows show clusters of phosphorylated PKC possibly located at plasma membrane. Images are representative of three independent experiments. Bar = 60 µm.

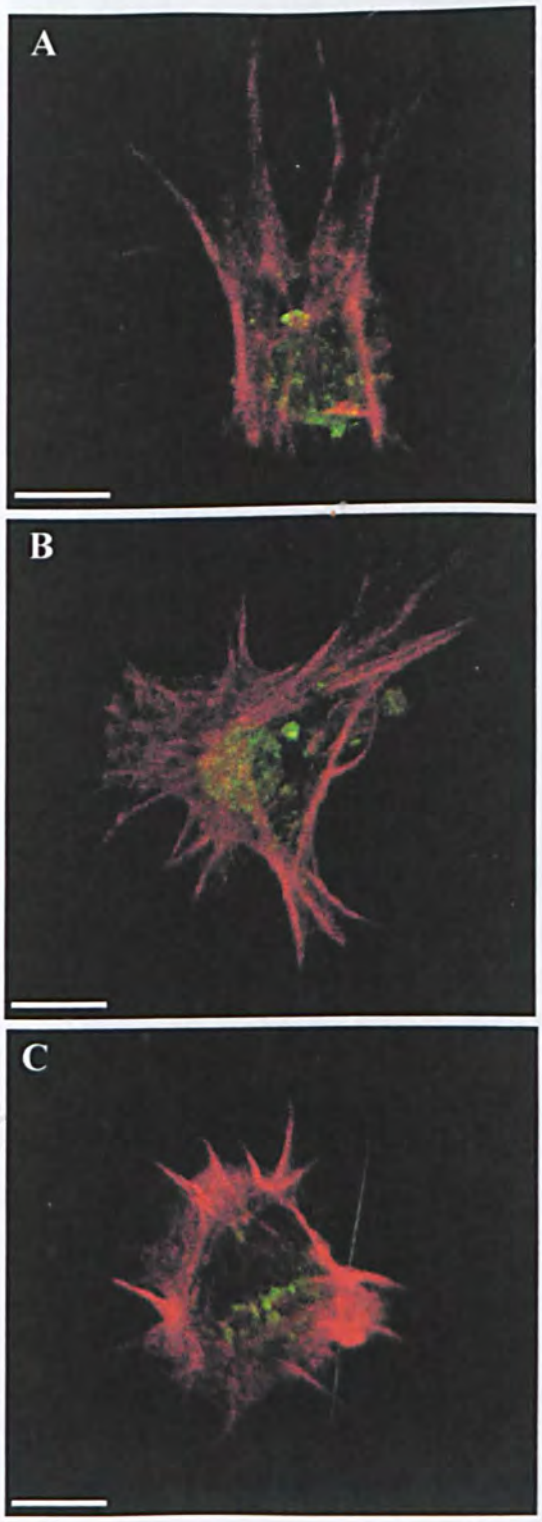


Fig.2.11: Distribution and levels of phosphorylated PKC in *L. stagnalis* haemocytes investigated by immunocytochemistry using the anti-phospho PKCα antibody.

Phosphorylated PKC in (A) untreated haemocytes, (B) haemocytes challenged with laminarin (10 mg/ml) for 10 min and (C) haemocytes incubated with GF109203X (10 μM) for 30 min prior to challenge with laminarin (10 mg/ml) for 10 min. Rhodamine phalloidin (red) stains F-actin and fluorescein (green) shows the phosphorylated PKC detected with the anti-phospho PKCα antibody. Haemocytes were observed with a Leica laser scanning confocal microscope. Images are representative of three independent experiments. Bar = 20 μm.

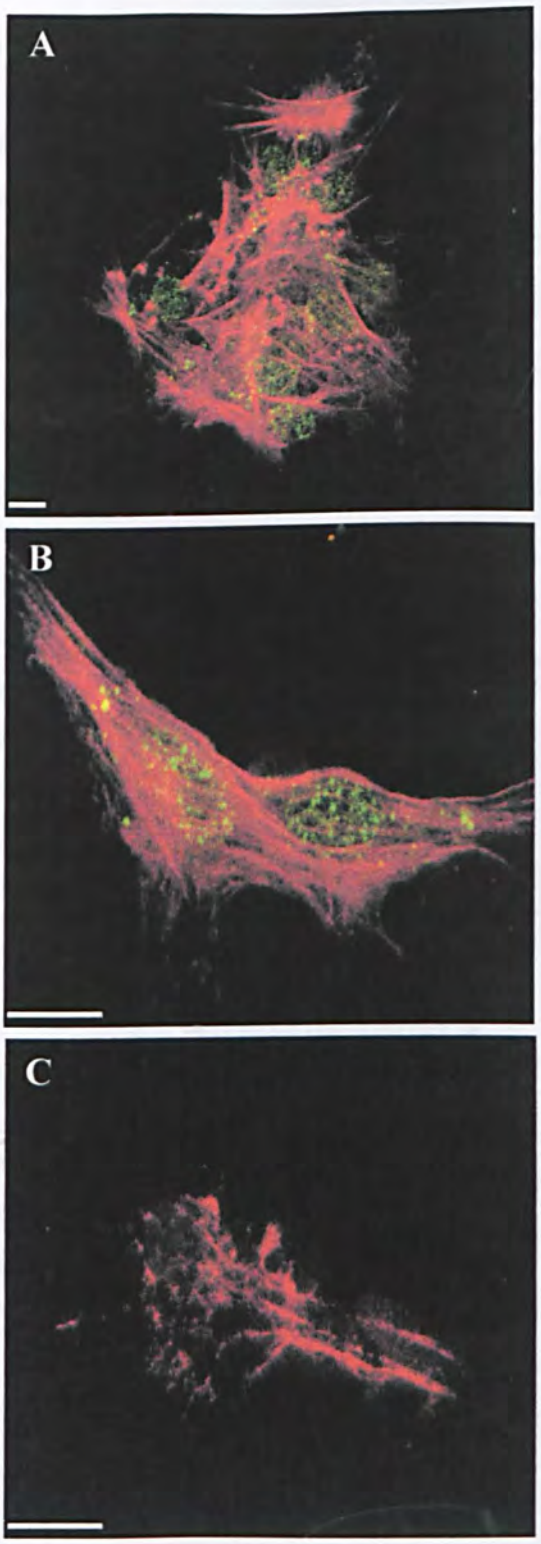


Fig.2.12: Distribution and levels of phosphorylated PKC in *L. stagnalis* haemocytes investigated by immunocytochemistry using the anti-phospho PKCαβII antibody.

Phosphorylated PKC in a group of (A) and two (B) untreated haemocytes, and in (C) haemocytes incubated with GF109203X (10 μM) for 30 min prior to challenge with laminarin (10 mg/ml) for 10 min. Rhodamine phalloidin (red) stains F-actin and fluorescein (green) shows the phosphorylated PKC detected with the anti-phospho PKCαβII antibody. Haemocytes were observed with a Leica laser scanning confocal microscope. Images are representative of two independent experiments. Bar = 20 μm.

Immunocytochemistry was also performed using the anti-phospho PKCαβII antibody. When stained with this antibody, control (unstimulated) cells displayed a relatively high level of phosphorylated PKC (Fig.2.12A, B) which could be explained by the fact that the antibody recognises more than one protein as seen by western blot analyses (see section 2.3.2). GF109203X (10 μM) was able to attenuate the phosphorylation of PKC-like proteins in laminarin-stimulated haemocytes suggesting that the antibody is detecting phosphorylated PKCs in these cells (Fig.2.12C).

2.3.6 Effects of LPS and PMA on the phosphorylation status of PKD

Protein kinase D phosphorylation (activation) has been shown to be PKC-dependent in fibroblasts (Zugaza *et al.*, 1996) and in lymphocytes (Matthews *et al.*, 2000); such activation (Waldron *et al.*, 2003) has been shown to occur following PMA stimulation (Valverde *et al.*, 1994; Van Lint *et al.*, 1995). As PKC-like proteins were phosphorylated (activated) after 5-10 min in *L. stagn*alis haemocytes following challenge with LPS or PMA (see section 2.3.3.3), the effects of these compounds on the phosphorylation of the PKD-like protein were studied over 20 min.

2.3.6.1 Challenge with LPS

Lymnaea stagnalis haemocytes that were challenged with LPS (1 μg/ml) for 0-20 min were analysed for phosphorylated PKD-like proteins by western blotting. The anti-phospho PKD_{916} sometimes recognised three bands, but all three were not always visible on the immunoblots.

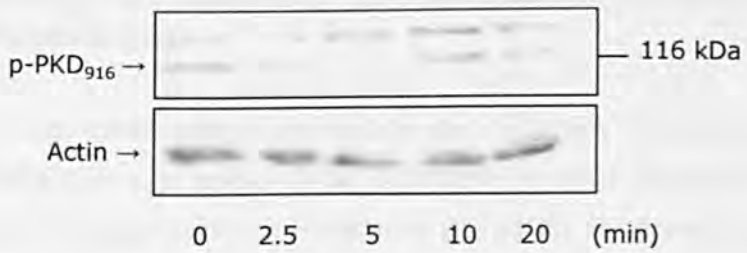


Fig.2.13: The effects of LPS on PKD phosphorylation levels: immunodetection of phosphorylated PKD-like proteins following challenge with LPS (1 μg/ml) for various durations (0-20 min). Samples were obtained as described in "Experimental procedures". Equal amounts of protein were loaded in each lane and membranes were probed with the anti-phospho PKD_{916} antibody. Anti-actin antibodies were used to confirm equal loading of proteins. Immunoblots are representative of two independent experiments.

In unchallenged haemocytes (time 0), the PKD phosphorylation levels were low (Fig.2.13). Analyses of the western blots revealed that the phosphorylation status of PKD did not change substantially over 20 minutes of LPS challenge; however an apparent slight increase in phosphorylation occurred at 10 min as seen in duplicate experiments (Fig.2.13) but no image analysis has been done on these blots.

2.3.6.2 Challenge with PMA

Similar to the results obtained following LPS challenge, exposure of *L. stagnalis* haemocytes to PMA (1 μM) for 0-20 min may have induced a slight increase in PKD phosphorylation at 10 min (Fig.2.14) although no substantial differences were revealed.

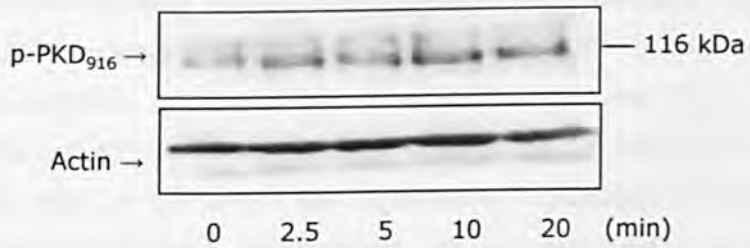


Fig.2.14: The effect of PMA on PKD phosphorylation levels: immunodetection of phosphorylated PKD-like proteins following challenge with PMA (1 μM) for various durations (0-20 min). Samples were obtained as described in "Experimental procedures". Equal amounts of protein were loaded in each lane and membranes were probed with were probed with the anti-phospho PKD_{916} antibody. Anti-actin antibodies were used to confirm equal loading of proteins. Immunoblots are representative of two independent experiments.

2.3.7 Delineating PKC pathway components in laminarin-challenged haemocytes

Protein kinase C has been shown to act as an upstream component of the ERK pathway, along with PLC, and seems to be important to many mammalian, insect and possibly molluscan immune functions (Foukas *et al.*, 1998; Monick *et al.*, 2000, Canesi *et al.*, 2005). In an attempt to establish whether communication between such signalling modules has been conserved in *L. stagnalis* haemocytes, pharmacological inhibitors of PKC were used to dissect downstream signalling events in haemocytes in response to laminarin challenge; laminarin was chosen because it was able to modulate PKC activity in *L. stagnalis* haemocytes (see section 2.3.2.2). The possible

activation of PKC by PLC and PI-3-K was also investigated using inhibitors of these enzymes in an attempt to identify upstream regulators of molluscan PKC.

2.3.7.1 MEK

The activation of the ERK pathway has already been demonstrated in LPS-challenged haemocytes (Plows *et al.*, 2004) although a role for PKC as an upstream kinase following challenge was not investigated. To explore whether PKC can modulate the activity of the ERK pathway in haemocytes following challenge with laminarin, inhibition assays were carried out using the PKC inhibitor, GF109203X. Western blotting was used to determine the levels of phosphorylated MEK-like proteins in laminarin-treated haemocytes that had either been pre-incubated with GF109203X (0.001 μM–10 μM) for 30 min or not.

Results from four independent experiments revealed that laminarin (10 mg/ml) had little effect on MEK phosphorylation in haemocytes with phosphorylation being increased to approximately 1.1 times that of control haemocytes (Fig.2.15). However, the phosphorylation of MEK was significantly attenuated by GF109203X at all doses tested ($P \leq 0.05$) with 10 μM and 1 μM significantly reducing MEK phosphorylation levels in laminarin-challenged haemocytes by approximately 61% and 42% respectively ($P \leq 0.001$; Fig.2.15B). Even at the lowest dose tested (0.001 μM), GF109203X significantly reduced MEK phosphorylation to 13% below basal levels ($P \leq 0.05$; Fig.2.15B).

The effects of PKC inhibition on MEK phosphorylation in laminarin-challenged haemocytes were then tested with a second PKC inhibitor, calphostin C, which targets the regulatory domain of PKC by competing at the binding site of DAG. Although the mechanisms by which this inhibitor blocks PKC activation are different to GF109203X, calphostin C was able to inhibit MEK phosphorylation in a dose-dependent manner in a similar way to that observed for GF109203X (Fig.2.16). Image analysis of duplicate blots revealed that when used at 1 μM and 10 μM, calphostin C was able to inhibit laminarin-dependent MEK phosphorylation by approximately 52% and 73%, respectively.

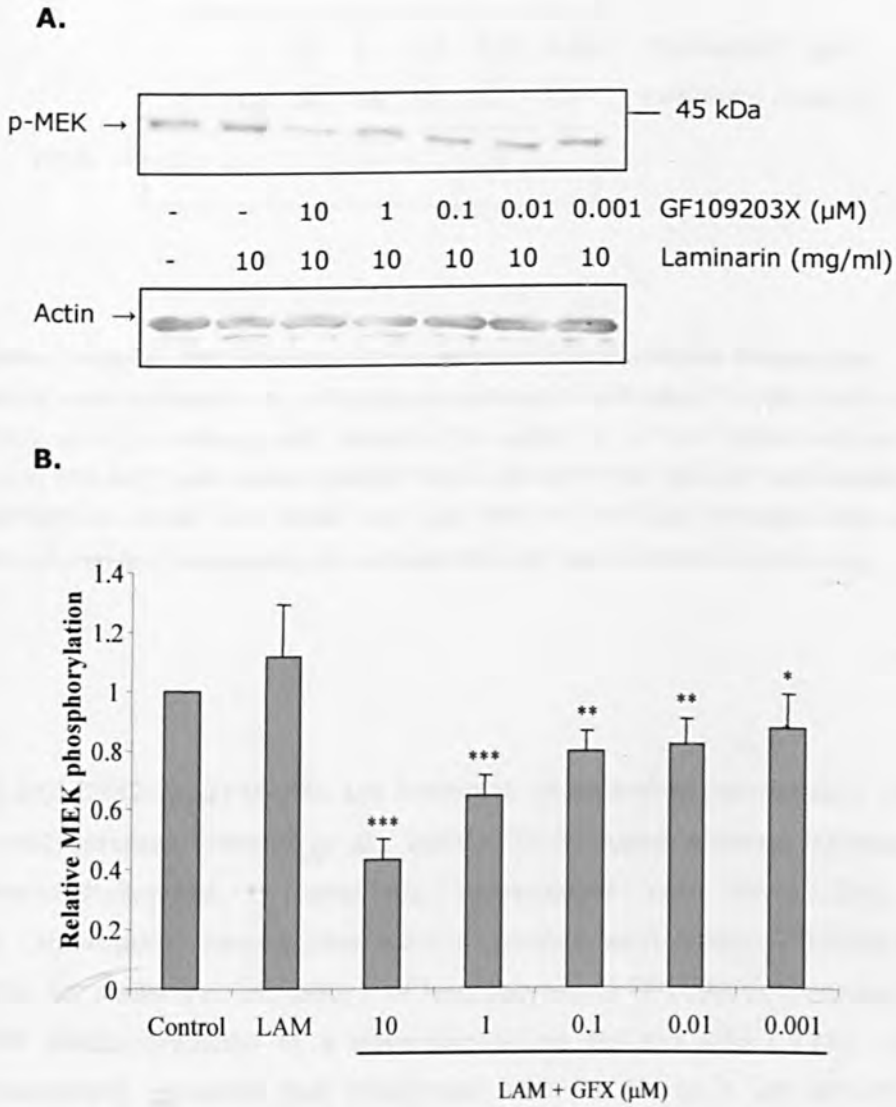


Fig.2.15: GF103209X reduces MEK phosphorylation in laminarin-stimulated haemocytes
(A) Haemocyte monolayers were incubated with various concentrations of GF103209X (0.001 μM-10 μM), or vehicle (0.1% DMSO), prior to challenge with laminarin (10 mg/ml) for 10 min. Protein extracts from haemocytes were prepared, subjected to SDS-PAGE and western blotting which was performed using the anti-phospho MEK antibody. Similar amounts of protein were loaded onto each lane and anti-actin antibodies were used to confirm equal loading of proteins. (B) Relative MEK phosphorylation levels following treatment were determined by image analysis of blots from n=4 independent experiments. Values shown are mean values (± SD). $*P \leq 0.05$, $**P \leq 0.01$, and $***P \leq 0.001$ when compared with phosphorylation levels observed in stimulated, uninhibited, cells.

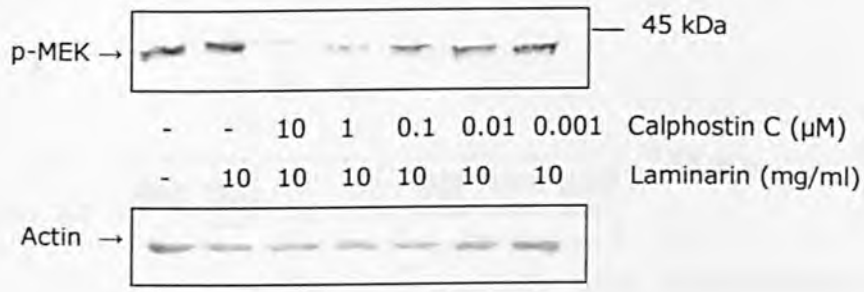


Fig.2.16: Calphostin C reduces MEK phosphorylation in laminarin-stimulated haemocytes
Haemocyte monolayers were incubated with various concentrations of calphostin C (0.001 μM-10 μM), or vehicle (0.1% DMSO), prior to challenge with laminarin (10 mg/ml) for 10 min. Protein extracts were prepared, subjected to SDS-PAGE and western blotting which was performed using the anti-phospho MEK antibody. Similar amounts of protein were loaded onto each lane and anti-actin antibodies were used to confirm equal loading of proteins. Immunoblots are representative of two independent experiments.

2.3.7.2 ERK

The ERK1 (p44) and ERK2 (p42) MAPKs are known to be activated downstream of PKC in mammalian macrophages (Monick *et al.*, 2000). To evaluate whether inhibition of PKC in laminarin-challenged *L. stagnalis* haemocytes can affect ERK 1/2 phosphorylation (activation), haemocytes were incubated with either GF109203X or calphostin C, prior to challenge. Incubation of haemocytes in GF109203X resulted in a reduction of ERK phosphorylation in a dose-dependent fashion (Fig.2.17A). Image analysis of immunoblots revealed that treatment with 10 μM or 1 μM GF109203X significantly attenuated ERK 1/2 phosphorylation (activation) by 65% ($P \leq 0.001$) and by 47% ($P \leq 0.01$), respectively (Fig.2.17B); moreover, 10 μM GF109203X reduced ERK phosphorylation to 36% below basal levels (Fig.2.17B). In the absence of inhibitor, laminarin produced a significant 1.9-fold increase in ERK 1/2 phosphorylation in haemocytes when compared with controls ($P \leq 0.01$; Fig.2.17).

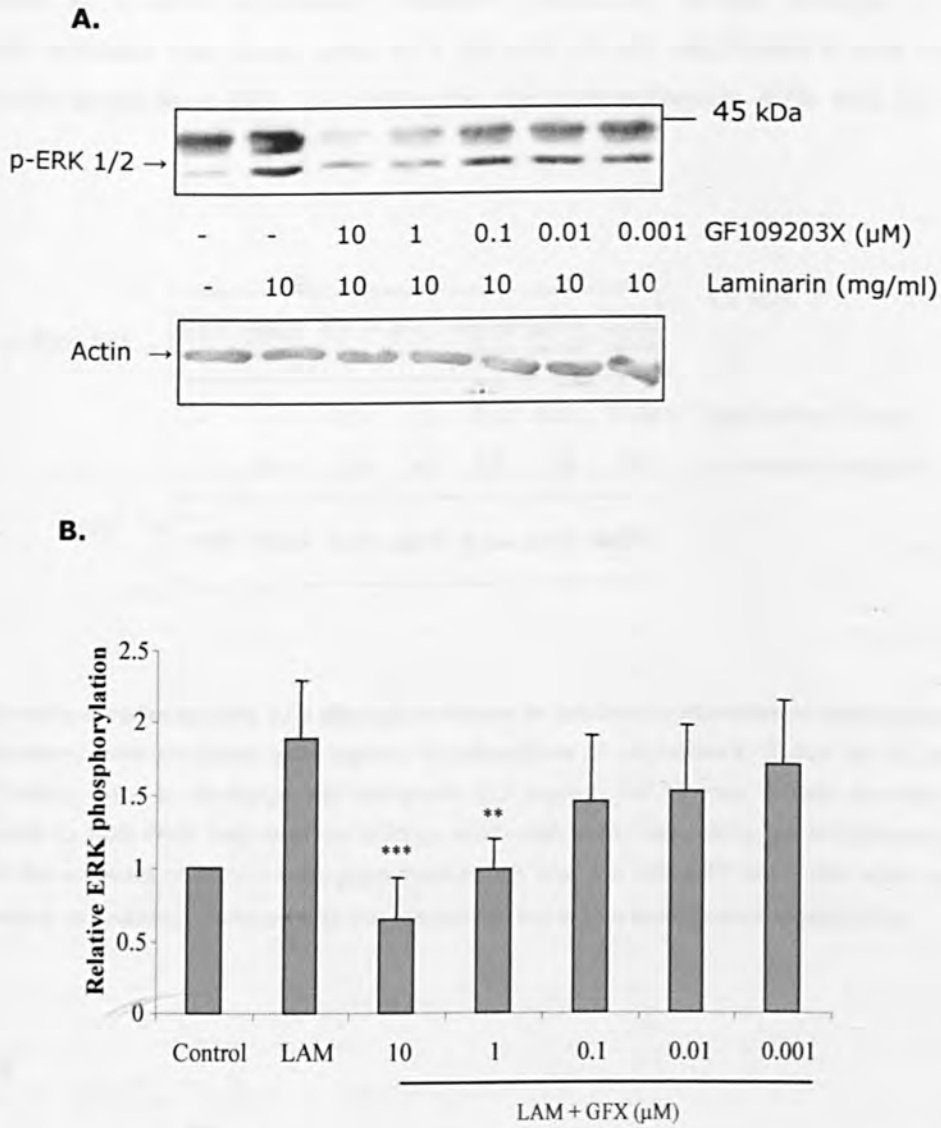


Fig.2.17: GF103209X reduces ERK 1/2 phosphorylation in laminarin-stimulated haemocytes
(A) Haemocyte monolayers were incubated with various concentrations of GF109203X (0.001 μM-10 μM), or vehicle (0.1% DMSO), prior to challenge with laminarin (10 mg/ml) for 10 min. Protein extracts from haemocytes were prepared, subjected to SDS-PAGE and western blotting which was performed using the anti-phospho ERK1/2 antibody. Similar amounts of protein were loaded onto each lane and anti-actin antibodies were used to confirm equal loading of proteins. (B) Relative ERK phosphorylation levels following treatment were determined by image analysis of blots from n=4 independent experiments. Values shown are mean values (± SD). $*P \leq 0.05$, $**P \leq 0.01$, and $***P \leq 0.001$ when compared with phosphorylation levels observed in stimulated, uninhibited, cells.

The effects of calphostin C on downstream ERK phosphorylation following challenge were also evaluated. Similar to GF109203X, calphostin C was able to inhibit ERK phosphorylation in a dose-dependent manner (Fig.2.18). Image analysis of the duplicate blots revealed that when used at 1 μM and 10 μM, calphostin C was able to inhibit laminarin-dependent ERK 1/2 activation by approximately 40% and by 49% respectively.

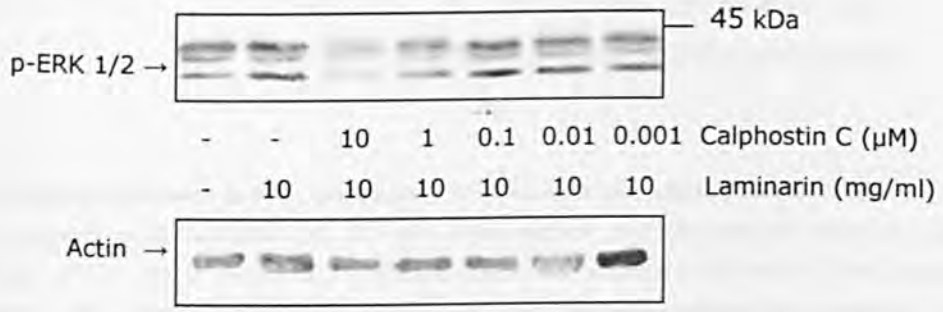


Fig.2.18: Calphostin C reduces ERK 1/2 phosphorylation in laminarin-stimulated haemocytes
Haemocyte monolayers were incubated with various concentrations of calphostin C (0.001 μM-10 μM), or vehicle (0.1% DMSO), prior to challenge with laminarin (10 mg/ml) for 10 min. Protein extracts were prepared, subjected to SDS-PAGE and western blotting which was performed using the anti-phospho ERK 1/2 antibody. Similar amounts of protein were loaded onto each lane and anti-actin antibodies were used to confirm equal loading of proteins. Immunoblots are representative of two independent experiments.

2.3.7.3 Elk-1

In mammals, the transcription factor Elk-1 is one of the nuclear targets of ERK 1/2 (Yang *et al.*, 1998). The Ser 383 residue constitutes the major phosphorylation site of Elk-1 by ERK 1/2 (Marais *et al.*, 1993). In *L. stagnalis* haemocytes, the kinase activity of ERK 1/2 against an Elk-1 fusion protein has been demonstrated using a non radioactive MAP-kinase activity assay (Plows *et al.*, 2004). Moreover, using the anti-phospho Elk-1 (Ser 383) antibody, the phosphorylation of Elk-1 increased in *L. stagnalis* haemocytes when challenged by LPS (Plows, PhD thesis, 2005). Since ERK 1/2 phosphorylation (activation) was reduced by GF109203X (0.001 μM–10 μM) in a dose-dependent manner in laminarin-stimulated haemocytes (Fig.2.19), the phosphorylation of Elk-1 was investigated in haemocytes incubated with GF109203X prior to laminarin challenge. Interestingly, the higher doses of GF109203X (10 μM) did not seem to substantially reduce the level of phosphorylated Elk-1 whereas lower doses (0.001 μM-0.1 μM) seemed to have an inhibitory effect (Fig.2.19). Image

analysis of the duplicate blots revealed that when used at 0.001 μM and 0.01 μM, GF109203X was able to inhibit laminarin-dependent Elk-1 activation by approximately 72% and 65%, respectively.

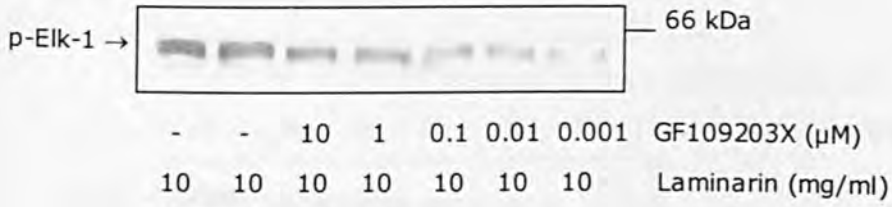


Fig.2.19: Elk-1 phosphorylation is PKC-dependent in laminarin-stimulated haemocytes
Haemocyte monolayers were incubated for 30 min with various concentrations of the PKC inhibitor GF109203X (0.001 μM-10 μM), or vehicle (0.1% DMSO) prior to the addition of laminarin (10 mg/ml) for 10 min. Phosphorylated Elk-1 was detected by immunoblotting with the anti-phospho Elk-1 antibody. Similar amounts of protein were loaded onto each lane and anti-actin antibodies were used to confirm equal loading of proteins. Immunoblots are representative of two independent experiments.

2.3.7.4 PI-3-K

Activation of PKC in mammalian cells is dependent on PI-3-K products (Moryia *et al.*, 1996; Kolanus and Seed, 1997; Chou *et al.*, 1998). In order to evaluate whether PI-3-K acts upstream of PKC in laminarin-challenged haemocytes, monolayers were treated with various doses of an inhibitor of PI-3-K, LY294002 (0.001 μM–10 μM) for 30 min, prior to challenge with laminarin (10 mg/ml) for 10 min.

Western blotting revealed that this inhibitor did not reduce levels of phosphorylated PKC at any of the doses tested (Fig.2.20) indicating that haemocyte PKC activation is not under the control of PI-3-K in laminarin-challenged haemocytes.

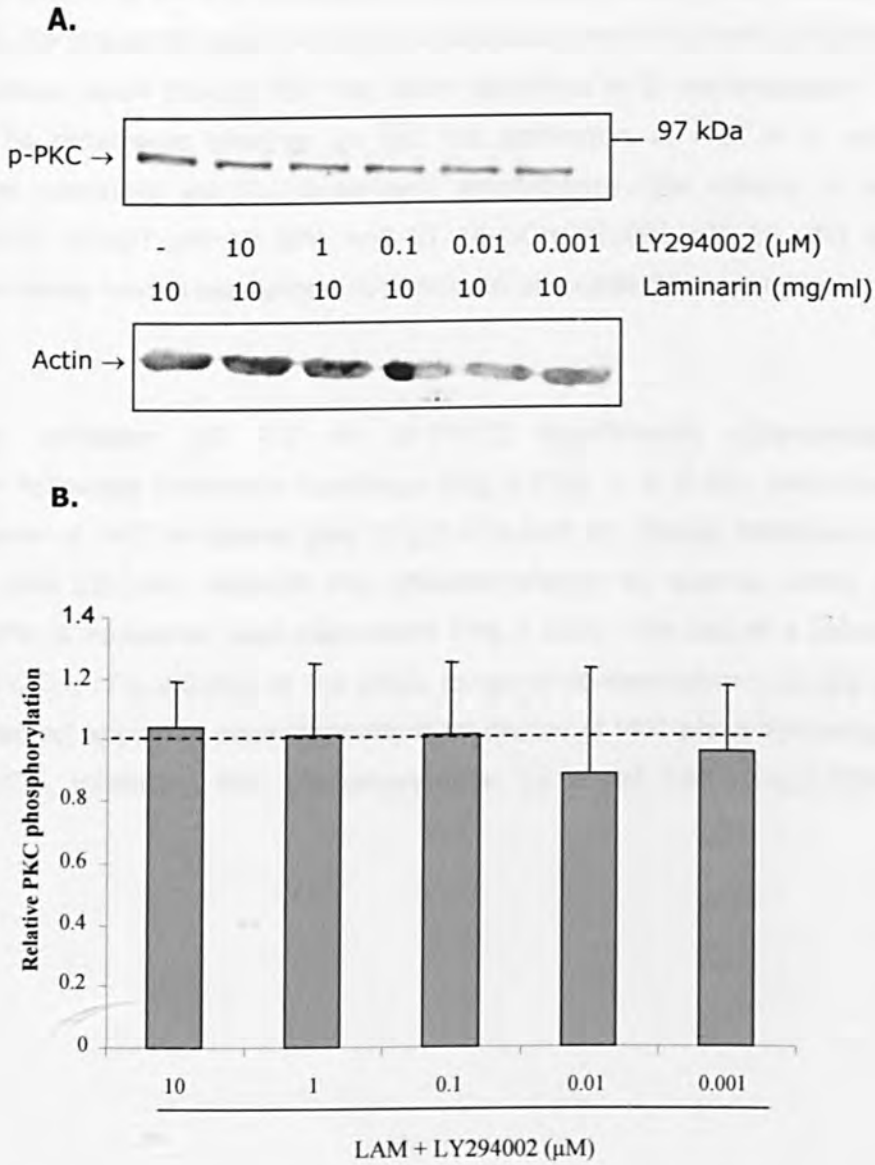


Fig.2.20: PKC phosphorylation is PI-3-K-independent in laminarin-stimulated haemocytes
(A) Haemocyte monolayers were pre-treated for 30 min with various concentrations of the PI-3-K inhibitor LY294002 (0.001 μM-10 μM), or vehicle (0.1% DMSO) prior to the addition of laminarin (10 mg/ml) for 10 min. Protein extracts were prepared, subjected to SDS-PAGE and western blotting which was performed using the anti-phospho PKC (pan) antibody. Similar amounts of protein were loaded onto each lane and anti-actin antibodies were used to confirm equal loading of proteins. (B) Relative PKC phosphorylation levels following treatment were determined by image analysis of blots from n=3 independent experiments. Values shown are mean values (± SD). The broken line shows the PKC phosphorylation levels in haemocytes stimulated with laminarin (10 mg/ml) for 10 min.

2.3.7.5 PLC

Phospholipase C is critical for the activation of PKC in mammalian cells (Rebecchi and Pentyala, 2000). Its characterisation and role in signalling remain poorly understood in insects and molluscs even though PLC has been identified in *D. melanogaster* (Emori *et al.*, 1994). To determine whether or not the activation of PKC in *L. stagnalis* haemocytes was operating via PLC-dependent mechanisms, the effects of the PLC inhibitors U-73122 (0.001 μM–10 μM) and ET-18-OCH_3 (0.001 μM–10 μM) on PKC phosphorylation levels were investigated in laminarin-stimulated haemocytes.

Pharmacological inhibition of PLC by U-73122 significantly attenuated PKC phosphorylation following laminarin challenge (Fig.2.21B; $P \leq 0.01$) indicating that PLC acts upstream of PKC in haemocytes (Fig.2.21A and B). Image analyses showed that at 10 μM, this inhibitor reduced PKC phosphorylation by approximately 76 %; moreover the effects appeared dose dependent (Fig.2.21B). The use of a second PLC inhibitor, ET-18-OCH_3 (Fig.2.22A), at the same range of concentrations (0.001 μM–10 μM) also revealed an apparent dose-dependent inhibition of PKC phosphorylation with 10 μM ET-18-OCH_3 inhibiting PKC phosphorylation by about 73% (Fig.2.22B; $P \leq 0.01$).

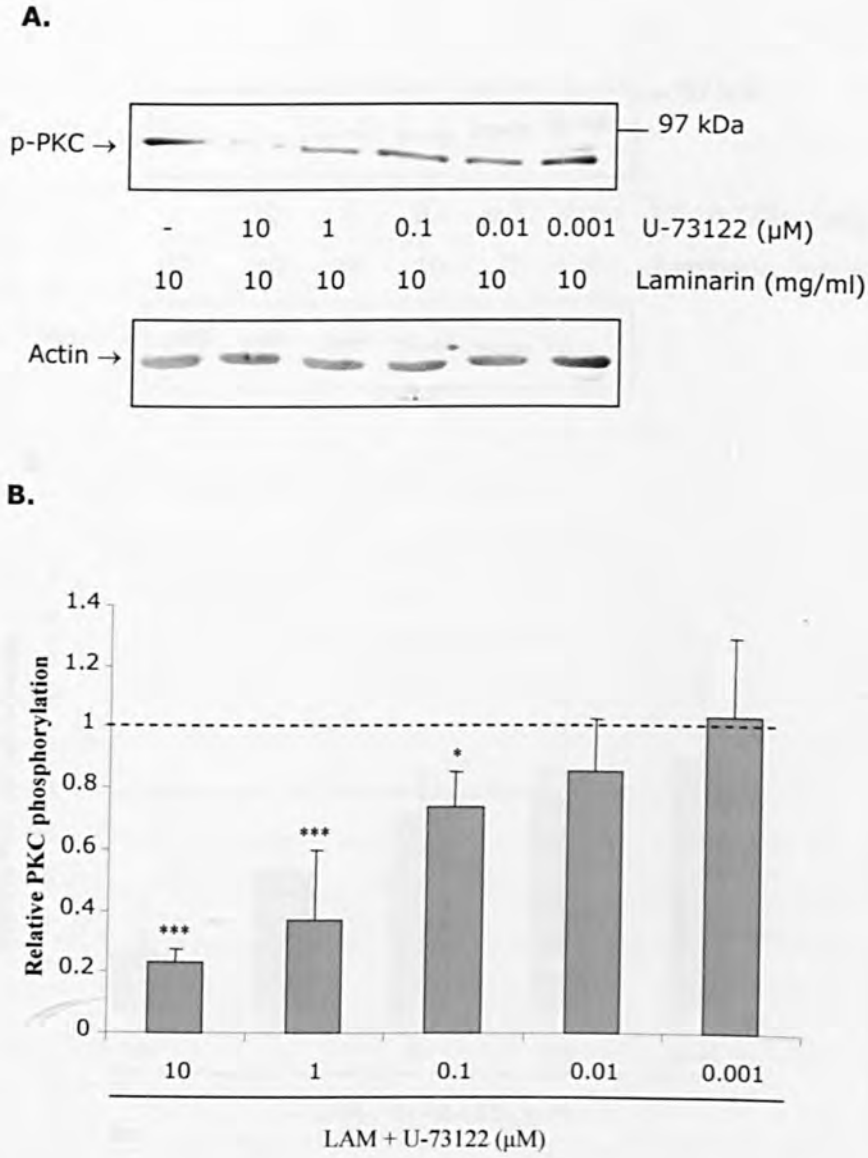


Fig.2.21: U-73122 reduces PKC phosphorylation in laminarin-stimulated haemocytes. (A) Haemocyte monolayers were treated for 30 min with the PLC inhibitor U-73122 at various concentrations (0.001 μM-10 μM), or vehicle (0.1% ethanol) prior to the addition of laminarin (10 mg/ml) for 10 min. Protein extracts from haemocytes were prepared, subjected to SDS-PAGE and western blotting which was performed using the anti-phospho PKC (pan) antibody. Similar amounts of protein were loaded onto each lane and anti-actin antibodies were used to confirm equal loading of proteins. (B) Relative PKC phosphorylation levels following treatment were determined by image analysis of blots from n=4 independent experiments. Values shown are mean values (± SD). $*P \leq 0.05$, $**P \leq 0.01$, and $***P \leq 0.001$ when compared with phosphorylation levels observed in stimulated, uninhibited, cells (broken line).

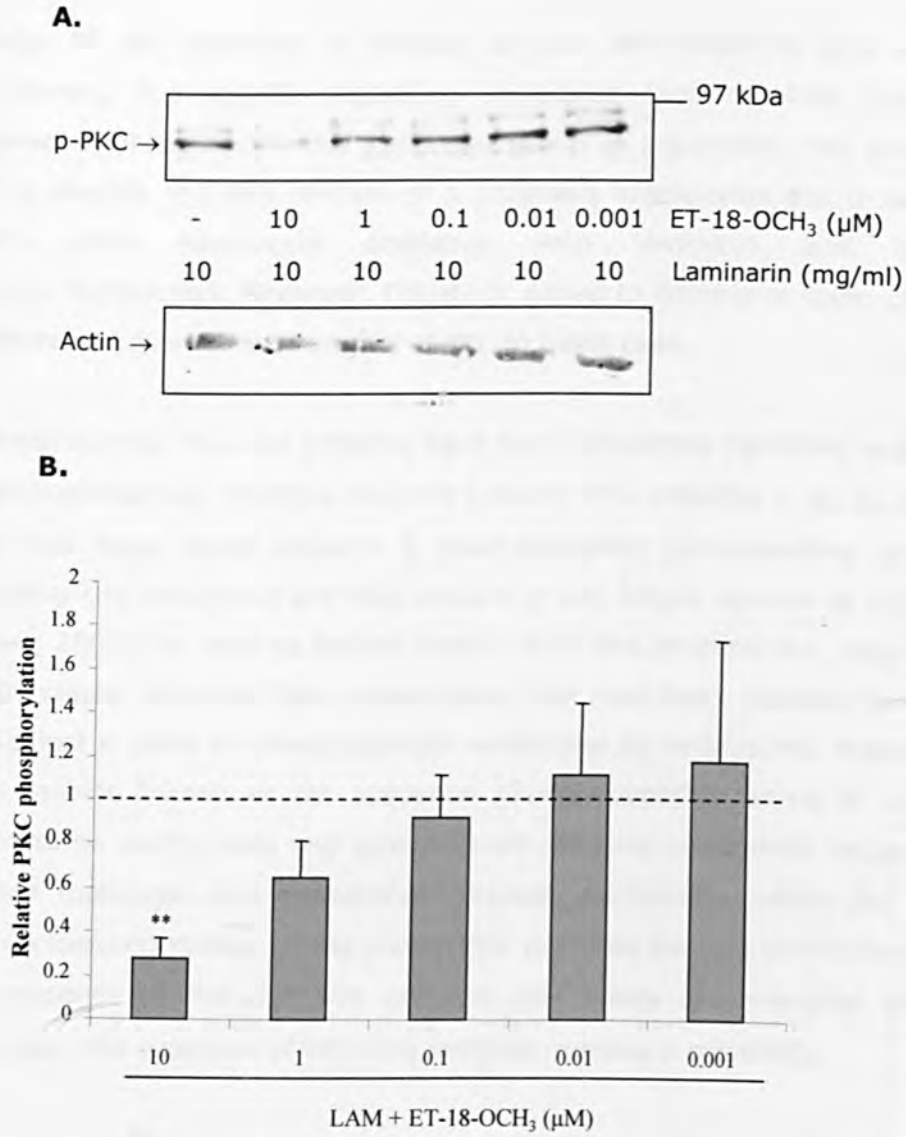


Fig.2.22: ET-18-OCH$_3$ reduces PKC phosphorylation in laminarin-stimulated haemocytes. (A) Haemocyte monolayers were treated for 30 min with the PLC inhibitor ET-18-OCH$_3$ at various concentrations (0.001 μM-10 μM), or vehicle (0.1% DMSO) prior to the addition of laminarin (10 mg/ml) for 10 min. Protein extracts from haemocytes were prepared, subjected to SDS-PAGE and western blotting which was performed using the anti-phospho PKC (pan) antibody. Similar amounts of protein were loaded onto each lane and anti-actin antibodies were used to confirm equal loading of proteins. (B) Relative PKC phosphorylation levels following treatment were determined by image analysis of blots from n=3 independent experiments. Values shown are mean values (± SD). $^{**}P \leq 0.01$ when compared with phosphorylation levels observed in stimulated, uninhibited, cells (broken line).

2.4 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. The present research aimed to identify PKC-like proteins in *L. stagnalis* haemocytes and to define their activation upon haemocyte challenge with laminarin and other immunomodulatory compounds. Moreover, the study aimed to determine some of the upstream regulators and downstream targets of PKC in these cells.

In *L. stagnalis* haemocytes, PKC-like proteins have been previously identified using a polyclonal anti-phosphospecific antibody reactive towards PKC isoforms α, β_I, β_{II}, η, ε, δ; the antibody has been raised towards a phosphopeptide corresponding to the residues surrounding the conserved Ser 660 present in the kinase domain of PKCβII (Walker and Plows, 2003). In order to further identify PKC-like proteins in *L. stagnalis* haemocytes, cell lysates obtained from haemocytes that had been exposed to LPS were screened against a panel of phosphospecific antibodies to various PKC isoforms. Although raised against human or rat sequence phosphopeptides, some of these antibodies proved to be useful tools and strengthened the idea of common sequence homology between molluscan and mammalian kinases, particularly within the key phosphorylation (activation) motifs. Of the eleven PKC isoforms existing in mammalian cells, only the presence of PKCα/βII-like proteins was firmly demonstrated in *L. stagnalis* haemocytes; the presence of PKD-like proteins remains a possibility.

Knowledge of signalling pathways that are triggered by a variety of immunomodulatory agents is essential to understand the biological events that underlie certain cellular defence responses. In this regard, the kinetics of PKC phosphorylation in *L. stagnalis* haemocytes were studied using: LPS, laminarin, and zymosan; PMA was also used since it is known to mimic DAG and thus act as a PKC activator. Laminarin belongs to the β-1, 3-glucans, polymers of D-glucose which are cell wall constituents of fungi and bacteria (Manners *et al.*, 1973). β-1, 3-glucans bind to PRRs, and are known to modulate innate immune responses in macrophages (Kataoka *et al.*, 2002) and invertebrate haemocytes (Vetvicka and Sima, 2004; Vetvicka and Yvin, 2004). Western blotting of *L. stagnalis* haemocyte extracts with anti-phosphospecific 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 (see Walker and Plows, 2003 and section 2.3.1.1). In LPS-stimulated mammalian macrophages, PKC reaches its activation peak 15-30 min post-challenge and activity returns to basal levels after 30 min (Shapira *et al.*, 1994; Shapira *et al.*, 1997). The slight differences in PKC kinetics observed between mammalian immune cells and *L. stagnalis* haemocytes following LPS challenge might be PKC isotype-related and also suggest evolutionary variation in the efficiency of signalling. Anti-phospho PKC (pan), anti-phospho PKCα/βII (Thr 638/641) and anti-phospho PKCα (Ser 657) antibodies all recognised the haemocyte PKC-like protein, indicating that the residues surrounding these key phosphorylation sites in the kinase domain share homology with human PKCα/βII. Perhaps this is not surprising since PKCα and βII 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, since 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 LPS, PMA and zymosan), PKC had variable and often high levels of phosphorylation, and in preliminary experiments, longer periods (up to 3 h) of equilibration did little to reduce its basal phosphorylation state (personal communication Dr A.J. Walker). A remaining pool of constitutively phosphorylated PKC that is "ready to respond to activators" often exists in mammalian cells (Newton, 2003) and the present findings suggest that such a phenomenon might also apply to molluscan haemocytes.

Clearly, *L. stagnalis* haemocytes respond to laminarin and although a β-1, 3-glucan binding receptor has yet to be characterised in snails, the presence of carbohydrate recognition receptors has been hypothesised (Fryer *et al.*, 1989; 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. In invertebrates, several β-1, 3-glucan binding proteins have been purified including these 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*. In terms of other receptors, immulectin-2 has been identified as a PPR in *M. sexta* (Yu and Kanost, 2004), and in *Drosophila melanogaster*, peptidoglycan recognition protein-LE recognizes peptidoglycan and activates cell signalling (Takehana *et al.*, 2002). Given the conservation of innate defence, similar receptors are also likely to be present on snail haemocytes.

The use of potent PKC inhibitors is crucial to help elucidate signalling events downstream of PKC and ultimately to enable functional roles of this enzyme to be defined (see later Chapters). Experiments were thus designed to define the early signalling events associated with laminarin activation of PKC in haemocytes. 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 part PKC-dependent. The transcription factor Elk-1 which is a downstream target of ERK belongs to the Ets family and is well conserved among the metazoan species (Degnan *et al.*, 1993). Although GF109203X was able to partially block ERK phosphorylation when used at 1 μM or 10 μM in haemocytes, the phosphorylation of Elk-1 was not impaired by the use of GF109203X at these doses; lower doses of GF109203X did, however, appear to have an effect. This suggests that the activation of the Elk-1 transcription factor might be PKC-independent following laminarin challenge and is probably the result of the integration of multiple upstream signalling pathways. Studies in mammalian fibroblasts have also shown that Elk-1 phosphorylation can be PKC-independent (Duan *et al.*, 2002), or PKCα- and PKCε-dependent (Soh *et al.*, 1999).

Experiments with calphostin C also revealed that MEK and ERK lie downstream of PKC in *L. stagnalis* haemocytes. Results from a study employing a haemocyte-like embryonic cell line (Bge) derived from the gastropod snail *Biomphalaria glabrata* and calphostin C, suggest that ERK activation 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 activation following immune challenge might be a feature conserved between mollusc species. Protein kinase C-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.

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 *D. melanogaster* (Estacion *et al.*, 2001) and in the fleshfly, *Boettcherisca peregrina* (Koganezawa and Shimada, 2002), U-73122 was employed 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 activation. This is in line with the well understood general mechanisms of activation for cPKCs (see Chapter 1, Section 1.8.4.5). In LPS-treated macrophages, PDK-1 is a downstream target of phosphorylated inositides produced in response to PI-3-K activation (Monick *et al.*, 2000). PDK-1 can activate cPKCs since it is responsible of 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 by Sonnenburg *et al.* (2001), in which PI-3-K does not play a role. The present findings also corroborate previous work in which ERK activation in *L. stagnalis* haemocytes was shown to be unaffected by LY294002 (Plows *et al.*, 2004), implying that the regulation of PKC/MEK/ERK phosphorylation in these cells is PI-3-K independent. Another explanation could also be that PKC downregulates the kinase activity of PI-3-K; such a mechanism has recently been shown for PKCα in Sf21 cell lines (Sipeki *et al.*, 2006). In a recent report, Plows *et al.* (2006) demonstrated an inhibitory effect of LY294002 on phagocytosis by *L. stagnalis* haemocytes. Results of the present work suggest that such inhibition is likely mediated by PKC- and ERK-independent mechanisms in these cells.

Immunocytochemistry with the anti-phospho PKC (pan) antibody 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 around the nucleus. Challenge with laminarin resulted in a large increase in the phosphorylation of PKC within the cytoplasm where it appeared in clusters. These

clusters might result from the association of phosphorylated PKC with cytoskeletal components or 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 Ca^{2+} and 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. However, in experiments using the anti-phosphospecific PKCα antibodies, PKC-like proteins in *L. stagnalis* haemocytes appear to be compartmentalised slightly in the nucleus. Although surprising, this localization has been observed in mammalian cells such as Swiss 3T3 fibroblasts or brain cells, in which translocation of PKCα to the nucleus operated in response to various stimuli (Leach *et al.*, 1989; Buchner, 1995; Rosenberger *et al.*, 1995).

Chapter 3

Exploring the effect of carbohydrate epitopes on PKC signalling in *Lymnaea stagnalis* haemocytes

3.1 Introduction

Glycan moieties expressed at the surface of schistosome parasites constitute a predominant target for the defence system of the mammalian host (Omer Ali *et al.*, 1988; Cumming and Nyame, 1999). Such glycan moieties may also serve as a way for the parasite to modulate the cellular and humoral defence mechanisms of the intermediate and definitive hosts in order for the parasite to survive and perpetuate in their suitable environment (Nyame *et al.*, 1999). The quest to find new drugs to control and eradicate blood fluke diseases has led researchers to focus on identifying parasite-derived glycan structures and analyse the immune host defense to schistosomes (Khoo, 2001; van Die and Cummings, 2006).

A considerable amount of research on schistosome glycobiology has therefore helped to further our knowledge of the potential of schistosome glycans to act as immunogens (van den Berg *et al.*, 2004). The surface carbohydrate distribution among the invertebrate stages of *S. mansoni* is known to differ (Hokke and Deelder, 2001) although detailed information regarding expression on the vertebrate stages of the parasite still remains to be obtained. Interestingly, some of the crucial glycan determinants on *S. mansoni* appear to be identical to those found in mammalian glycoproteins (Kawar *et al.*, 2005); these include Lewis X (Le^X), LDN, and fucosylated LDN (LDNF) antigens, whose structure and role first started to be explored in helminth parasites (van Remoortere *et al.*, 2000). Additionally, two major antigens, namely the circulating cathodic and anodic antigens (CCA and CAA respectively), which are released from the gut of *S. mansoni* worms, have been shown to contain repeating Le^X units (Van Dam *et al.*, 1994). In the avian parasite, *Trichobilharzia ocellata* which uses *L. stagnalis* as an intermediate host, the presence of LDN structures has been determined in oligosaccharides using western blotting (Nyame *et al.*, 2000). Similar LDN structures have also been found in the haemocyanin of *L. stagnalis* (Van Kuik *et al.*, 1987). Furthermore, the key enzyme involved in the biosynthesis of oligosaccharides present in schistosome glycoconjugates, the *N*-acetyl galactosaminyltransferase, has been shown to be present in *T. ocellata* miracidia, mother sporocysts and cercariae (Neelman *et al.*, 1994).

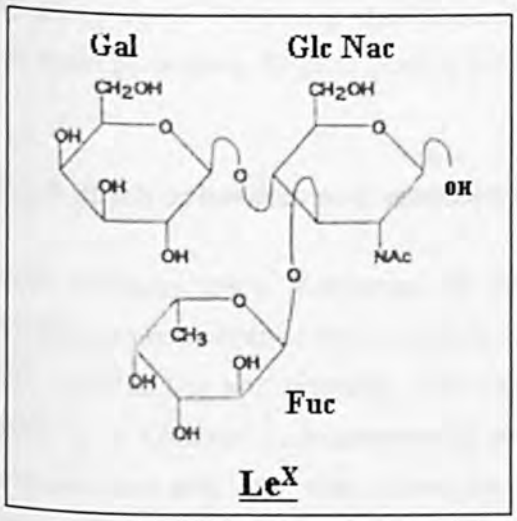
Molluscan innate defence responses depend on the ability to recognise infectious non-self via interactions between PRRs present on the haemocyte surface and PAMPs located on the surface of pathogenic agents (see Chapter 1, section 1.5). Recognition of specific carbohydrate structures known to be present on the surface of invading schistosomes have been shown to regulate internal defence mechanisms of *B. glabrata*

(Hahn *et al.*, 2000). In this study, three types of carbohydrate conjugates comprising either galactose, mannose, or fucose were able to stimulate the production of ROIs by *B. glabrata* haemocytes (Hahn *et al.*, 2000). Surprisingly however, whether schistosome glycans modulate signalling pathways that control innate immunity in molluscs remains unclear. Recently, the effects of albumin-linked fucose and albumin-linked galactose (that mimic schistosome surface coat components) on *L. stagnalis* haemocyte signalling were investigated; challenge of haemocytes with these neoglyconjugates was found to affect ERK and PKC pathways (Plows *et al.*, 2005). Armed with further knowledge of the PKC pathway in *L. stagnalis* haemocytes (see Chapter 2), and based on the findings of Plows and co-workers, the present study was undertaken to determine whether Le^X and LDN structures modulate PKC signalling in *L. stagnalis* haemocytes. The work also aimed to provide knowledge of the initial signalling events that could occur at the host/parasite interface when *T. ocellata* infects *L. stagnalis*.

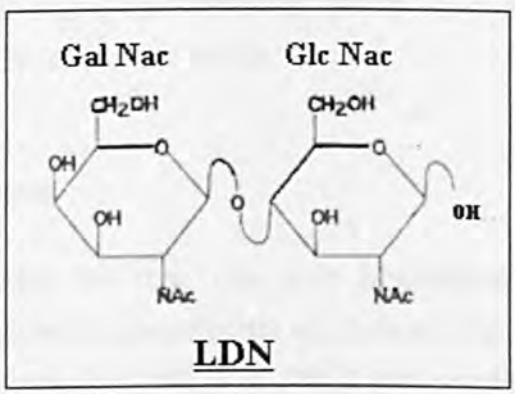
3.2 Experimental procedures

3.2.1 Reagents

Lac-di-Nac (LDN: 4-O-(2-acetamido-2-deoxy-β-D-galactopyranosyl)-β-D-2-acetamido-2-deoxy-D-glucose) and Le^X linked to PA (polyacrylamide conjugate) were obtained from Lectinity Holdings (Moscow, Russia). PA is a poly N-(2-hydroxyethyl) acrylamide, a flexible polymer chain which serves as spacer molecule. All others chemicals were purchased from Sigma-Aldrich (Poole, Dorset, UK).



Galβ1-4[Fucα1-3]Glc NAc
Trisaccharide **Le^X**



Gal Nacβ1-4Glc Nac
Disaccharide **LDN**

3.2.2 Effects of Lewis X and LDN on PKC signalling in *L. stagnalis* haemocytes

3.2.2.1 Haemocyte monolayers

Haemolymph was extracted from six to ten adult snails, then placed on ice and diluted with SSS as described in Chapter 2, Experimental procedures (section 2.2.3). Haemocyte monolayers were then prepared in 24-well culture plates (200 µl diluted haemolymph per well). Cells were not washed following binding and were then left to equilibrate for 1 hour as described in Chapter 2, Experimental procedures (section

2.2.3). Due to the cost of the carbohydrate-derived products, the volume of diluted haemolymph/per well was reduced to 200 µl (instead of 500 µl).

3.2.2.2 Challenge of haemocytes

After equilibrating, haemocytes were challenged with 200 µl LDN-PA (400 nM–1600 nM), Le^x-PA (400 nM-1600 nM), or PA (control, 200 nM-1600 nM) in diluted haemolymph for various durations (0–3 h). The MW of each carbohydrate is approximately 30 kDa and the carbohydrate content is 20% mol of the MW of each PA-conjugate. The concentrations chosen for the present experiments were based on those used in previous work employing BSA-conjugated saccharides (Plows, PhD thesis, 2005; Plows *et al.*, 2004) and represent the final molarities of the PA-conjugates used. Following challenge, haemolymph was removed quickly and cells were lysed by adding 70 µl of boiling 1X SDS-PAGE sample buffer.

3.2.2.3 Electrophoresis and western blotting

Protein extracts were subjected to SDS-PAGE on 10% gels and transferred to nitrocellulose membranes as described in Chapter 2, Experimental procedures (section 2.2.6; 2.2.7). The anti-phospho PKC (pan) antibody (1:1000 in TTBS) (see antibody specificity in Chapter 2, Experimental procedures (section 2.2.7)) was used to probe the membrane and actin was employed as a loading control. Immunoreactive proteins were then visualised using Opti-4CN or ECL detection.

3.2.3 Data analysis

No statistical analyses were done on these data.

3.3 Results

3.3.1 Effects of LDN-PA on the phosphorylation status of PKC

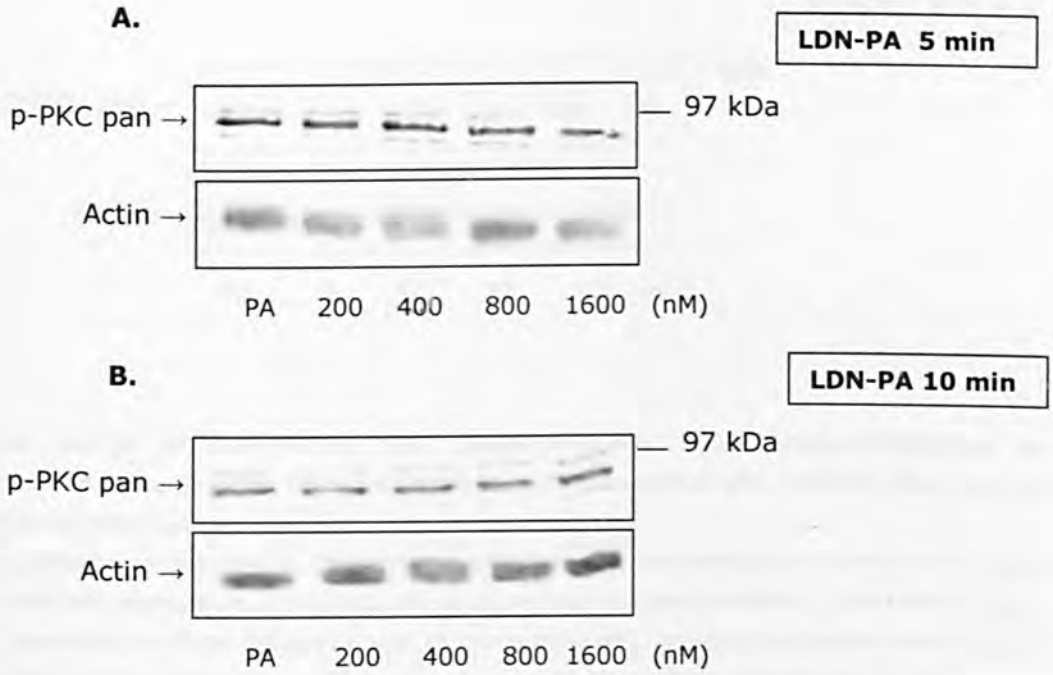


Fig.3.1: The effects of LDN-PA on PKC phosphorylation levels: immunodetection of phosphorylated PKC-like proteins following challenge of haemocytes with LDN for (A) 5 min and (B) 10 min at various concentrations (200 nM-1600 nM).

Samples were obtained as described in "Experimental procedures ". Equal amounts of protein were loaded in each lane and membranes were probed with the anti-phospho PKC (pan) antibody. The control sample consisted of haemocyte extracts challenged with PA alone (1600 nM). Anti-actin antibodies were used to confirm equal loading of proteins. Immunoblots are representative of two independent experiments.

In an attempt to determine a dose of conjugated LDN which modulates PKC phosphorylation (activation) in *L. stagnalis* haemocytes, a dose-response experiment was carried out. Lysates from *L. stagnalis* haemocytes not exposed to glycans and from haemocytes that had been exposed to LDN-PA for various times (5 min, 10 min), were analysed by western blotting using the anti-phospho PKC (pan) antibody (Fig.3.1). These time periods were chosen because previous results demonstrated that PKC phosphorylation in laminarin-stimulated haemocytes peaked after 10 min of challenge (see Chapter 2, section 2.3.3.2). Also, when LPS was used as a stimulant, PKC phosphorylation levels were also highest after 5-10 min exposure (see Chapter 2,

section 2.3.3.1). Treatment of haemocytes with different doses (200 nM-1600 nM) of conjugated LDN had little effect on PKC phosphorylation; no increases or decreases were observed compared to control levels (PA) after either 5 min (Fig. 3.1A) or 10 min (Fig.3.1B) of exposure.

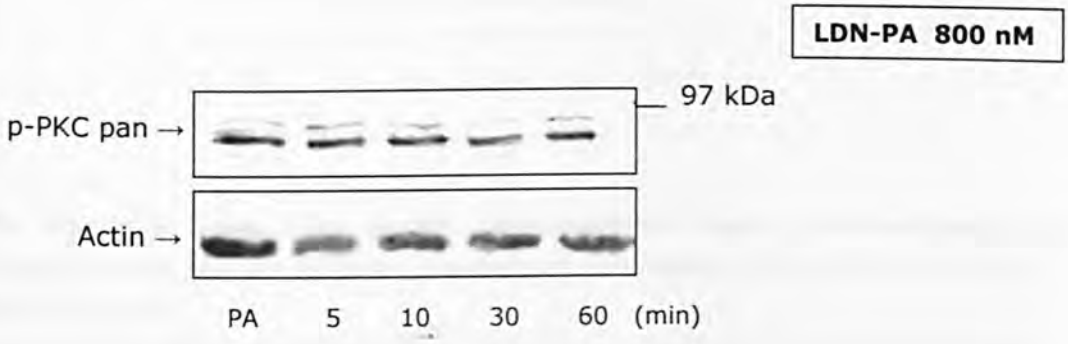


Fig.3.2: The effects of LDN-PA on PKC phosphorylation levels: immunodetection of phosphorylated PKC-like proteins following challenge of haemocytes with LDN-PA (800 nM) for various durations (0-60 min).

Samples were obtained as described in "Experimental procedures ". Equal amounts of protein were loaded in each lane and membranes were probed with the anti-phospho PKC (pan) antibody. The control sample consisted of haemocyte extracts challenged with PA alone (800 nM). Anti-actin antibodies were used to confirm equal loading of proteins. Immunoblots are representative of two independent experiments.

In the study by Plows *et al.*, 2005, PKC phosphorylation was found to be inhibited when *L. stagnalis* haemocytes were challenged with 800 nM BSA-galactose, BSA-fucose or a combination of both for 60 min. Based on this finding, similar concentrations of LDN-PA was used to challenge *L. stagnalis* haemocytes over 60 min. The levels of PKC phosphorylation did not appear to be affected by LDN-PA at any of the time points used and phosphorylation remained sustained after 60 min of challenge (Fig.3.2).

3.3.2 Effects of Lewis X-PA on the phosphorylation status of PKC

To confirm whether or not PKC signalling in *L. stagnalis* haemocytes is affected following exposure of these cells to conjugated Le^X, haemocytes were challenged with Le^X-PA (800 nM) for 0-60 min. Exposure of the cells to this compound did not alter the phosphorylation status of the haemocyte PKC-like proteins compared to the PA control, and like LDN-PA challenge, the phosphorylation appeared sustained even after 60 min challenge (Fig.3.3).

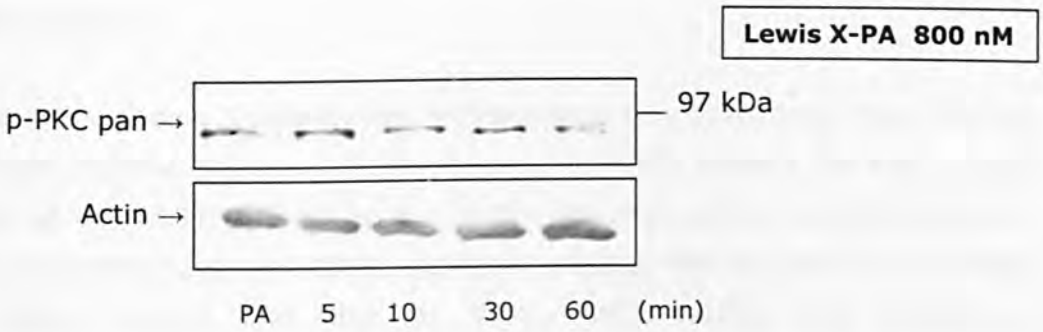


Fig.3.3: The effects of Lewis X-PA on PKC phosphorylation levels: immunodetection of phosphorylated PKC-like proteins following challenge of haemocytes with Le^X-PA (800 nM) for various times (0-60 min).

Samples were obtained as described in "Experimental procedures ". Equal amounts of protein were loaded in each lane and membranes were probed with the anti-phospho PKC (pan) antibody. The control consisted of haemocyte extracts challenged with PA (800 nM). Anti-actin antibodies were used to confirm equal loading of proteins. Immunoblots are representative of two independent experiments.

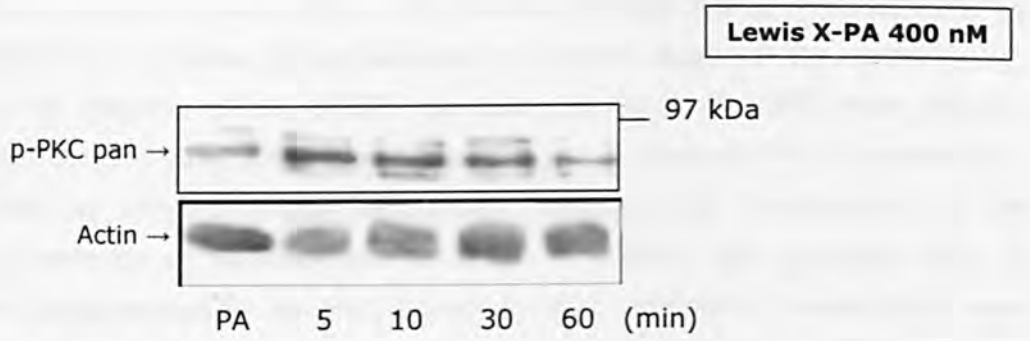


Fig.3.4: The effects of Lewis X-PA on PKC phosphorylation levels: immunodetection of phosphorylated PKC-like proteins following challenge of haemocytes with Le^X-PA (400 nM) for various durations (0-60 min).

Samples were obtained as described in "Experimental procedures ". Equal amounts of protein were loaded in each lane and membranes were probed with the anti-phospho PKC (pan) antibody. Control consisted of haemocyte extracts challenged with PA (400 nM). Anti-actin antibodies were used to confirm equal loading of proteins. Immunoblots are representative of two independent experiments.

Exposure of *L. stagnalis* haemocytes to a lower dose of Le^X-PA (400 nM) appeared to result in a slight increase of PKC phosphorylation after 5 min challenge compared to the control (Fig.3.4). While this indicated that Le^X might stimulate the PKC pathway in haemocytes, the effect was not entirely reproducible in subsequent experiments (n=2). Moreover, challenge of haemocytes with Le^X (200 nM or 1600 nM) did not reveal any PKC phosphorylation changes over 60 min.

3.4 Discussion

Many parasites including *Trypanosoma*, *Schistosoma*, and *Leishmania* may interfere with the host internal defence system via specific PAMPs present on their surface (Appelmelk *et al.*, 2003; Giorgio *et al.*, 2003). As such, glycan and carbohydrate molecules represent important PAMPs, and their antigenic role has been demonstrated (Nyame, 2000; Thomas and Harn Jr, 2004). Many studies have focused on investigating the immunomodulatory effects of carbohydrate epitopes present on *S. mansoni* egg glycoproteins on the innate immune response of the mouse model (Okano *et al.*, 1999; Van der Kleij *et al.*, 2002, Faweeuw *et al.*, 2003), but little is currently known about the regulation of defence mechanisms by parasite carbohydrates within the molluscan intermediate host. The alteration of the defence system of *L. stagnalis* by *T. ocellata* has been relatively well studied *in vitro* (Amen *et al.*, 1992), but the parasite carbohydrate recognition by haemocytes and the possible modulation of intracellular signalling pathways, which regulate functional defence responses in the snail by glycans, still remain poorly explored. Investigations of the carbohydrates present at the surface of the human schistosome *S. mansoni* have led to the identification of three glycan moieties on various stages of the parasite, Le^X, LDN, and LDNF (Nyame *et al.*, 2002). Moreover, Plows *et al.*, 2005 have recently demonstrated that the phosphorylation (activation) of *L. stagnalis* PKC-like proteins is downregulated by albumin-bound saccharides (L-fucose and D-galactose), in the presence or absence of haemolymph. In order to address the possible effect of schistosome glycoconjugates on PKC signalling in *L. stagnalis*, haemocytes were exposed to chemically-synthesised compounds, Le^X–PA and LDN-PA. These glycan structures could have some antigenic properties as they have been shown to be present on the schistosome surface; however, their effect on cells has not been extensively tested *in vitro* in mammalian or invertebrate systems and there are no reports of the effects of these glycans on invertebrate defence cells.

All experiments were conducted in the presence of haemolymph as it was more representative of the *in vivo* environment. The two glycan compounds, Le^X and LDN, were chosen for this study because these determinants have both been identified on the surface of *S. mansoni* (Nyame *et al.*, 2002). Contrary to the LDN epitope which is present on *S. mansoni* eggs, mother sporocysts, and daughter sporocysts, Le^X is predominant on eggs and cercariae but not detectable in mother and daughter sporocysts (Nyame *et al.*, 2002). LDN is an epitope mainly composed of galactose and glucose whereas Le^X possesses an additional fucose residue. The glycans bearing the LDN or LDNF motifs that have been identified in *S. mansoni*, are either membrane-

bound or eventually form part of the parasite ESPs (Srivatsan *et al.*, 1992; Nyame *et al.*, 2002). Given that Le^X is not present on the sporocysts stages whereas LDN is, the hypothesis that LDN, but not Le^X, could modulate PKC signalling in haemocytes was tested. Challenge of *L. stagnalis* haemocytes with LDN-PA at various concentrations (200 nM-1600 nM) did not seem to modulate PKC phosphorylation at any of the time points studied and similar results were obtained with Le^X. In both cases, the level of PKC phosphorylation was sustained and did not appear to differ significantly from basal levels even after 60 min of challenge. The likely presence of carbohydrate bearing L-fucose and D-galactose has been reported on *T. ocellata* surfaces (Gerhardus *et al.*, 1991), and phosphorylation of *L. stagnalis* PKC-like proteins appeared to be reduced in the presence of albumin–linked sugars, such as BSA-fucose (Plows *et al.* 2005). Given that LDN is part of the *S. mansoni* sporocyst surface structure, it was disappointing that no changes in PKC phosphorylation were observed when *L. stagnalis* haemocytes were challenged with LDN-PA. We might hypothesise that the carbohydrate surface composition of *T. ocellata* sporocysts differs from that of *S. mansoni* sporocysts. The specificity of the repertoire of *L. stagnalis* haemocyte membrane receptors might also explain the difference in PKC responses observed when haemocytes were challenged with either conjugated saccharides (Plows *et al.*, 2005) or with LDN-PA and Le^X-PA as the carbohydrate structure of the glycans used varied; Le^X and LDN are composed of various glycans whereas fucose and galactose are only monomers. Perhaps, conjugated LDN and Le^X carbohydrates might activate other signalling pathways in a PKC-independent fashion in *L. stagnalis* haemocytes. Interestingly, recent studies have shown that common carbohydrate epitopes containing LDN and fucosylated LDN are shared by *S. mansoni* and its intermediate host *B. glabrata* (Lehr *et al.*, 2006). Further investigations are crucial to see whether or not similar glycan sharing occurs in *L. stagnalis* and *T. ocellata*. Immune evasion represents a possible mechanism for the parasite to survive within its host; such evasion could occur by the parasite interfering with the host cell signalling pathways via carbohydrate-receptor interaction (Walker *et al.*, 2006). Clearly, any resemblance of molecules between the parasite and its host (molecular mimicry) could be used as a parasite's strategy to not be recognised by the host immune system.

Although present on the parasite and on human cells, studies have shown that Le^X containing glycans and more particularly the α, 1-3 linked fucose group have immunomodulatory properties in schistosomiasis patients and downregulate their immune defences (Velupillai *et al.*, 2000; Van der Kleij *et al.*, 2002). In mammalian systems, Le^X is also known as CD15. CD15 is found on numerous proteins involved in cell adhesion and has a broad cellular distribution, occurring mainly on blood cells (as

monocytes or neutrophils), but also on leukemic cells. A role for CD15 in initiating intercellular adhesion molecule-1 (ICAM)/leukocyte function-associated antigen-1 (LFA-1) adhesion signalling pathways has been found in neutrophils (Forsyth *et al.*, 1989). In monocytes, CD15 cross-linking has been associated with nuclear signalling events involving the activator protein-1 (AP-1) (Lo *et al.*, 1997). The investigations of the presence of glycoproteins and more specifically of the Le^X epitope have been carried out in different species of parasite including *S. mansoni* (Nyame *et al.*, 2002; Faveeuw *et al.*, 2003), *S. bovis* (Ramajo-Hernández *et al.*, 2007), *Haemonchus contortus* (Debose-Boyd *et al.*, 1998) and the nematode *Dictyocaulus viviparous* (Haslem *et al.*, 2000). The expression of various fucosylated epitopes (including Le^X and LDNF) in glycoconjugates on different stages of the parasite *S. mansoni* have also been screened using anti-carbohydrate monoclonal antibodies (Robijn *et al.*, 2005).

Interestingly, binding assays done *in vitro* between molluscan Bge cells and *S. mansoni* sporocysts were not inhibited in the presence of L-fucose or N-acetyl galactosamine, residues found to be present on the larval stage of the schistosome parasite (Castillo and Yoshino, 2002). Functional defence responses in *L. stagnalis* haemocytes such as phagocytosis of "bioparticles" are thought to be PKC-dependent (Plows *et al.*, 2004). Recent studies done in *B. glabrata* have shown that BSA-fucose, -mannose and -galactose stimulated the generation of H_2O_2 by haemocytes (Hahn *et al.*, 2001). In the presence of haemolymph, *L. stagnalis* haemocyte phagocytic activities were reduced in presence of BSA-galactose and combined sugars, BSA-galactose and BSA-fucose (Plows *et al.*, 2005). However, BSA-fucose alone enhanced phagocytosis (Plows *et al.*, 2005) in the presence of haemolymph, therefore, this carbohydrate appeared to be crucial in this process.

Fucosylated LDN-PA was not tested in the present study since it was not commercially available. Clearly, the effects of LDNF-PA on PKC signalling could differ from the results obtained using conjugated Le^X and conjugated LDN. Fucosylated LDN has been identified on glycoproteins from *T. ocellata* cercariae and adult *S. mansoni* using the monoclonal antibody SMLDNF1 (Nyame *et al.*, 2000). In addition, *S. mansoni* eggs, mother sporocysts and daughter sporocysts also express the LDNF antigen (Nyame *et al.*, 2002), whereas the *S. mansoni* cercarial glycocalix has been shown to express an unusual glycan complex bearing fucosylated terminal motifs (Khoo *et al.*, 1997). Further investigation into the effects of LDNF on signalling would be crucial to our understanding of host-schistosome interactions.

Glycobiology is a vast and complex discipline and the field of research concerning the effects of glycans on signalling pathways remains rudimentary. The lack of knowledge concerning the surface components of *L. stagnalis* haemocytes and the structural differences that probably occur between snail species means that there is likely to be much to discover concerning parasite-host-ligand interactions at the snail-schistosome interface. Further work is thus warranted on the *L. stagnalis* model.

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Chapter 4

Hydrogen peroxide generation by *Lymnaea stagnalis* haemocytes

4.1 Introduction

Cytotoxic activities represent one of the major components of the innate immune defence response in vertebrate immunity. Phagocytes undergo 'the respiratory burst' in which large amounts of oxygen are transiently consumed (El-Benna *et al.*, 2005) leading to the production of RNIs and ROIs (Bogdan *et al.*, 2000). These highly reactive intermediates that include NO, O_2^-, and H_2O_2, play an essential role in helping to eliminate invading pathogens by damaging DNA, and interacting with various macromolecules including lipids or proteins (Nathan and Shiloh, 2000). Interestingly, cytotoxic activities are also conserved among invertebrates. Observed in insects (Nappi and Vass, 1993), in annelids (Geracitano *et al.*, 2004), in nematodes (Kaiser *et al.*, 1998), and even in flagellates (Oda *et al.*, 1997), the production of ROIs and RNIs is also considered important to molluscs. The oxidative burst has been reported in several molluscan species including the hard clam *Mercenaria mercenaria* (Buggé *et al.*, 2006), *Mytilus edulis* (Pipe, 1992), *Mytilus galloprovinciallis* (Ordas *et al.*, 2000) and *Biomphalaria glabrata* (Hahn *et al.*, 2000).

The outcome of pathogen invasion, that is the destruction of the parasite or establishment of a successful infection, relies on the ability of the host to trigger defence reactions; this partly relies on activation of complex networks of signalling pathways. Hence, understanding the mechanisms controlling molluscan immunity is essential to gaining a comprehensive knowledge of how schistosome parasites might survive in, or be destroyed by, the snail intermediate host. In this context, ROI generation by *B. glabrata* haemocytes was recently shown to be important in the killing of *Schistosoma mansoni* (Hahn *et al.*, 2000). In earlier studies, the production of O_2^- by *B. glabrata* haemocytes was found to be stimulated by *S. mansoni* miracidia (Shozawa *et al.*, 1989) and inhibited by *S. mansoni* ESPs (Connors and Yoshino, 1990). Moreover, encapsulation and elimination of *S. mansoni* in the incompatible host, *L. stagnalis*, is facilitated by ROIs, whereas *T. ocellata* is able to evade the defence response of this compatible snail and survive (Dikkeboom *et al.*, 1988; Adema *et al.*, 1994). Clearly, therefore, interactions between schistosomes and reactive intermediates produced by host haemocytes appear crucial to the outcome of infection. In *L. stagnalis*, free radicals and oxidizing agents have been shown to be produced by haemocytes during phagocytosis (of zymosan and bacteria) using chemiluminescence studies (Dikkeboom *et al.*, 1987). It has been also suggested that an NADPH oxidase activity, similar to that found in mammals, is likely involved in the generation of ROIs by *L. stagnalis* haemocytes (Adema *et al.*, 1993).

The different processes linking early signalling events to defence reactions has only started to be recently investigated in snails. Work in our laboratory has led to the detection of PKC-like proteins in *L. stagnalis* haemocytes and has shown that phosphorylation (activation) of these proteins can be modulated following LPS (Walker and Plows, 2003 and Chapter 2) and laminarin challenge (Chapter 2). Other research also showed that PKC and ERK play a role in phagocytosis (Plows *et al.*, 2004) and in the production of nitric oxide (Wright, 2005) by *L. stagnalis* haemocytes. Given that ROIs appear to play a vital role in the elimination of pathogens such as schistosome parasites, this study was undertaken to examine the importance of PKC in the generation of H_2O_2 in *L. stagnalis* haemocytes. The work was also undertaken to establish whether PKC plays a broad, and perhaps more pivotal, part in *L. stagnalis* defence responses.

4.2 Experimental procedures

4.2.1 Reagents

The Amplex® Red hydrogen peroxide/peroxidase assay kit was purchased from Molecular Probes (AA, Leiden, Netherlands) and comprises the HRP enzyme, the Amplex® Red reagent (10-acetyl-3,7-dihydroxyphenoxazine), reaction buffer (0.25 M sodium phosphate, pH 7.4), DMSO and H_2O_2. The NADPH oxidase inhibitor, apocynin and the PKCα/β inhibitor, Gö 6976, came from Merck Biosciences Ltd (Nottingham, Notts, UK). Laminarin, PMA, and GF109203X were purchased from Sigma-Aldrich (Poole, Dorset, UK).

4.2.2 Production of H_2O_2 by haemocytes

4.2.2.1 Haemocyte monolayers

Haemolymph was extracted from six to ten adult snails, then placed on ice and diluted in SSS as described in Chapter 2, Experimental procedures (section 2.3). Haemocyte monolayers were then prepared in 96-well culture plates (Nunc; 200 µl diluted haemolymph per well) and, after the 30 min binding period, were washed three times with SSS for 5 min each wash; haemocytes were then left to equilibrate for 1 h in 200 µl SSS.

4.2.2.2 Stimulation of H_2O_2

Amplex® Red is a non-fluorescent compound which becomes a fluorescent product (named resorufin) upon HRP-catalyzed oxidation by H_2O_2. The reaction is stoichiometric: one molecule of resorufin is produced for each molecule of H_2O_2 used (Zhou *et al.*, 1997). Stock solutions of Amplex® Red and HRP were initially made up and stored following the manufacturer's instructions. Working solutions of assay mixture (0.1 U/ml (HRP) and 50 µM Amplex® Red reagent) containing different doses of laminarin (1-10 mg/ml) or PMA (0.01 µM-10 µM) were prepared in SSS (instead of the 1X reaction buffer). One unit (U) is defined as the amount of HRP that will form 1.0 mg purpurogallin from pyrogallol in 20 s at pH 6.0 and 20°C. Snail sterile saline was removed from the individual wells of the 96-well culture plate and 100 µl of this working solution was added to wells containing haemocyte monolayers. Immediately after the addition of the stimulants (laminarin or PMA), the fluorescence intensities of each well were measured over 1 h at 5 min intervals using a Fluorstar Optima microplate reader with 544 nm excitation filter and 590 nm emission filter. Fluorescence tests were also performed with laminarin, PMA and cells alone for 1 h to ensure that the observed changes in fluorescence were solely due to resorufin

produced. The control fluorescence values derived from unchallenged haemocytes at time 0 were normalised to 1 and all other averaged fluorescence values at 30 min related to that in each experimental condition.

4.2.2.3 Inhibition of H_2O_2

The effects of inhibitors on the generation of H_2O_2 by laminarin-stimulated *L. stagnalis* haemocytes were determined. Haemocyte monolayers were incubated with the PKC inhibitor, GF109203X (0.01 μM–10 μM), the PKCα/β specific inhibitor, Gö 6976 (0.01 μM–10 μM), the NADPH oxidase inhibitor, apocynin (10 μM–500 μM), or vehicle (0.1 % DMSO) for 30 min. GF109203X blocks PKC signalling in haemocytes following laminarin challenge (see Chapter 2) and potentially affects multiple PKC isoforms, whereas Gö 6976 inhibits PKCα/β isoenzymes by binding competitively to the ATP binding site of the catalytic domain of the enzyme (Martiny-Baron *et al.*, 1993). Apocynin, for which the mechanisms of action are not well established, is a non-toxic compound derived from the roots of the herb *Picrorhiza kurroa* (Vejražka *et al.*, 2004). After the 30 min incubation, the assay mixture working solution (containing 10 mg/ml laminarin) was added and the fluorescence intensities of each well were then determined as described above (see section 4.2.2.2). The solvents used for the inhibitors did not influence the fluorescence intensities derived from the experiments. The control fluorescence values derived from 10 mg/ml laminarin-challenged haemocytes at 30 min were normalised to 100% and all other averaged fluorescence values at 30 min related to that in each experimental condition.

4.2.3 Data analysis

Multiple comparisons of data were subjected to one-way ANOVA and LSD *post-hoc* using the statistical software package SPSS to determine the effects of laminarin and time on the generation of H_2O_2 and the effects on inhibitors on H_2O_2 output. Results are shown as the mean ± SD.

4.3 Results

4.3.1 Production of H_2O_2 by laminarin-stimulated haemocytes

To determine the effects of laminarin on H_2O_2 generation by *L. stagnalis* haemocytes, monolayers were treated with various doses of laminarin (1 mg/ml–10 mg/ml) for 30 min and H_2O_2 output was determined using the Amplex Red® fluorescence-based assay. H_2O_2 was studied because of its relative stability compared to other by-products of cellular $O_2^{\cdot-}$.

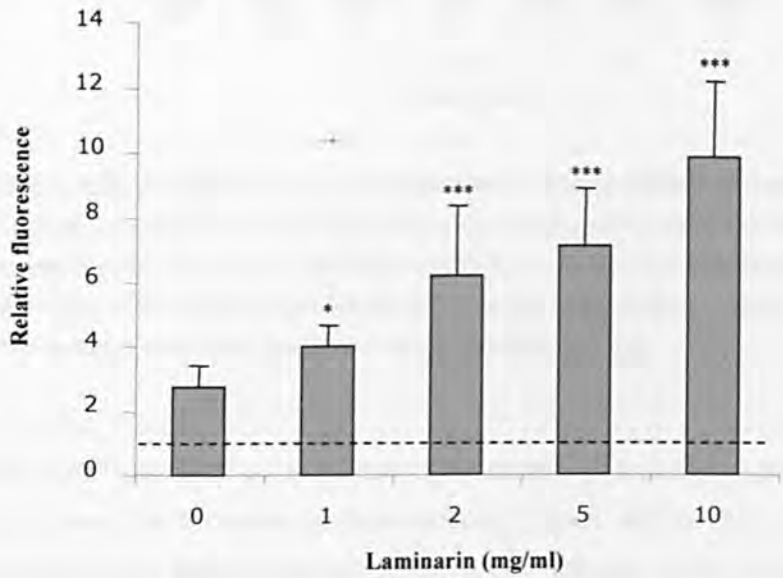


Fig.4.1: Stimulation of H_2O_2 production in *L. stagnalis* haemocytes by various doses of laminarin. The generation of H_2O_2 was investigated in haemocytes stimulated with different doses of laminarin (1 mg/ml–10 mg/ml) for 30 min. Bars represent mean relative fluorescence (± SD) of two independent experiments, each done in triplicate (n=6). $*P \leq 0.05$ and $***P \leq 0.001$ when compared to control (unstimulated) values. The dotted line represents relative fluorescence value at 0 min derived from unchallenged cells.

Laminarin significantly increased the production of H_2O_2 by haemocytes compared to control (unchallenged) cells at all of the doses tested ($P \leq 0.05$). There was a 9.5-fold increase in fluorescence over 30 min when used at 10 mg/ml, which was significantly different from the increase observed in the absence of laminarin (2.5-fold) over this period ($P \leq 0.001$; Fig.4.1). The effects of laminarin challenge on the cellular output of H_2O_2 were also found to be dose-dependent ($P \leq 0.001$; Fig.4.1); 2 mg/ml and 5 mg/ml laminarin significantly increased H_2O_2 generation by 6-fold ($P \leq 0.001$) and 7-fold ($P \leq 0.001$) respectively, over 30 min. The lowest dose of laminarin (1 mg/ml) also significantly increased H_2O_2 output by 4-fold compared to that of the control (2.5–fold) ($P \leq 0.05$).

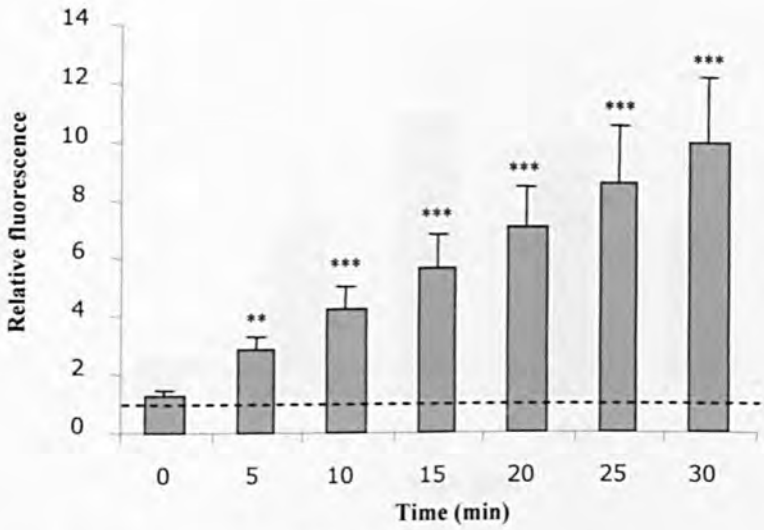


Fig.4.2: Stimulation of H_2O_2 production in *L. stagnalis* haemocytes by laminarin over time. The generation of H_2O_2 was investigated in haemocytes stimulated with laminarin (10 mg/ml) over a 30 min time period. Bars represent mean relative fluorescence (± SD) of two independent experiments, each done in triplicate. $^{**}P \leq 0.01$ and $^{***}P \leq 0.001$ when compared to control (unstimulated) values. The dotted line represents relative fluorescence value at 0 min derived from unchallenged cells.

Haemocytes were then exposed to laminarin (10 mg/ml), and H_2O_2 production was monitored over 30 min. An increase in fluorescence signal above that of the control was observed at each time point demonstrating a continuous linear increase of H_2O_2 production over this time period (Fig.4.2). The relative fluorescence was significantly greater than the control level as early as 5 min after challenge ($P \leq 0.01$). The generation of H_2O_2 was significantly amplified by approximately 4-, 5.5-, 7-, and 8-fold when haemocytes were challenged with laminarin for 10, 15, 20, and 25 min, respectively ($P \leq 0.001$; Fig.4.2).

4.3.2 Production of H_2O_2 by PMA-stimulated haemocytes

The effects of the PKC activator, PMA, on H_2O_2 generation by *L. stagnalis* haemocyte monolayers were investigated. Haemocytes were treated with various doses of PMA (0.01 μM-10 μM) for 30 min.

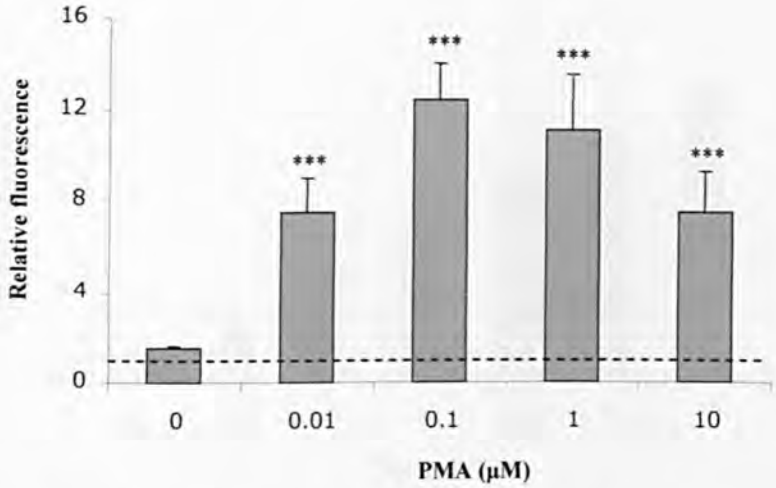


Fig.4.3: Stimulation of H_2O_2 production in *L. stagnalis* haemocytes by various doses of PMA
The generation of H_2O_2 was investigated in haemocytes stimulated with different doses of PMA (0.01 μM–10 μM) for 30 min. Bars represent mean relative fluorescence (± SD) of two independent experiments, each done in triplicate. ***$P \leq 0.001$ when compared to control (unstimulated) values. The dotted line represents relative fluorescence value at 0 min derived from unchallenged cells.

Stimulation of haemocytes with PMA (0.01 μM-1 μM) resulted in a significant increase in H_2O_2 generation compared to unchallenged controls ($P \leq 0.001$; Fig.4.3). The highest level of fluorescence was observed when haemocytes were challenged with 0.1 μM PMA; the change in relative fluorescence was almost 8.5 times greater than that observed for the control ($P \leq 0.001$). However, when PMA was used at 1 μM and 10 μM, the level of change in fluorescence started to decrease but remained 7.4 and 4.7 times higher than control (unchallenged) values respectively ($P \leq 0.001$; Fig.4.3).

Haemocytes were then exposed to 0.1 μM PMA, and H_2O_2 production was monitored over 30 min (Fig.4.4). Similarly to laminarin challenge, an increase in fluorescence signal above controls was observed at each time point demonstrating a continuous linear increase of H_2O_2 production over 30 min (Fig.4.4). After 5 min challenge, the relative fluorescence was already significantly greater than that of unchallenged controls; an increase of almost 2.5 times was observed ($P \leq 0.05$). The generation of H_2O_2 was significantly amplified by approximately 4.5-, 7-, 9- and 10-fold when haemocytes were challenged with PMA for 10, 15, 20, and 25 min respectively ($P \leq 0.001$; Fig.4.4). After 30 min, the increased H_2O_2 generation in 0.1 μM PMA-stimulated haemocytes was greater than that observed when haemocytes were treated with 10mg/ml laminarin (Fig.4.2 and Fig.4.4).

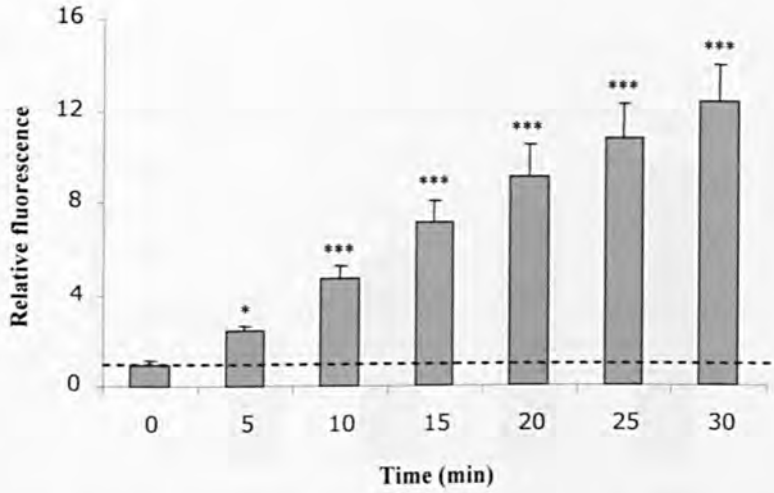


Fig.4.4: Stimulation of H_2O_2 production in *L. stagnalis* haemocytes by PMA over time
The generation of H_2O_2 was investigated in haemocytes stimulated with PMA (0.1 μM) over a 30 min time period. Bars represent mean relative fluorescence (± SD) of two independent experiments, each done in triplicate. $*P \leq 0.05$ and $***P \leq 0.001$ when compared to control (unstimulated) values. The dotted line represents relative fluorescence value at 0 min derived from unchallenged cells.

4.3.3 Inhibition of laminarin-induced H_2O_2 generation by haemocytes using PKC inhibitors

The finding that PMA stimulated H_2O_2 output suggested a role for PKC in the production of the ROIs. To further elucidate whether H_2O_2 production is critically dependent on PKC activity, cells were treated with GF109203X or a specific inhibitor of PKCα, Gö 6976, prior to challenge with laminarin and determination of H_2O_2 output. The results presented below are those obtained when haemocytes were challenged with 10mg/ml laminarin for 30 min; however similar degrees of inhibition of H_2O_2 output also occurred after 20 min, 40 min, and 60 min.

4.3.3.1 Effect of GF109203X on H_2O_2 generation in laminarin-stimulated haemocytes

The PKC inhibitor GF109203X (0.01 μM–10 μM) significantly ($P \leq 0.001$) reduced the levels of fluorescence compared to that seen in laminarin-stimulated haemocytes with a maximum inhibition of 65% when used at a final concentration of 10 μM ($P \leq 0.001$) (Fig.4.5). The inhibition of H_2O_2 by GF109203X was also dose-dependent; 42%, 26% and 16% inhibition was observed when this inhibitor was used at 1 μM, 0.1 μM, and 0.01 μM respectively (Fig.4.5).

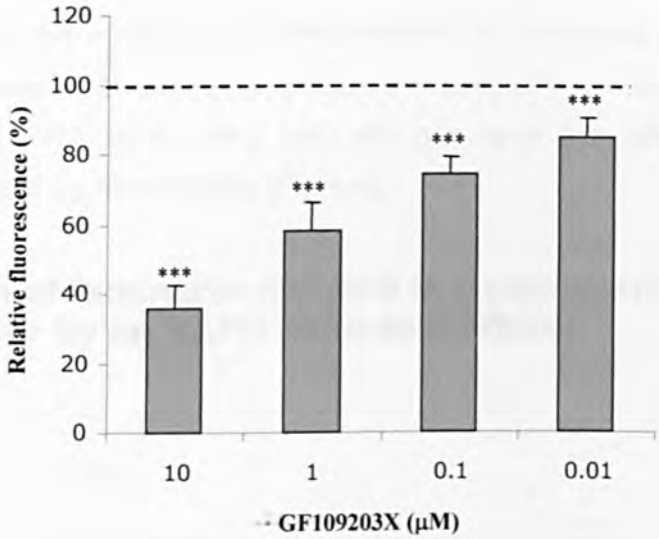


Fig4.5: Inhibition of laminarin-mediated H_2O_2 generation in haemocytes by GF109203X

The production of H_2O_2 was investigated in haemocytes incubated with the PKC inhibitor, GF109203X (0.01 μM-10 μM) or vehicle (DMSO) for 30 min prior to stimulation with laminarin (10 mg/ml) for 30 min. Bars show as means (± SD) of two independent experiments each performed in triplicate. ***$P \leq 0.001$ when compared to H_2O_2 production in stimulated haemocytes not exposed to inhibitor (shown as 100%, ---).

4.3.3.2 Effect of Gö 6976 on H_2O_2 generation in laminarin-stimulated haemocytes

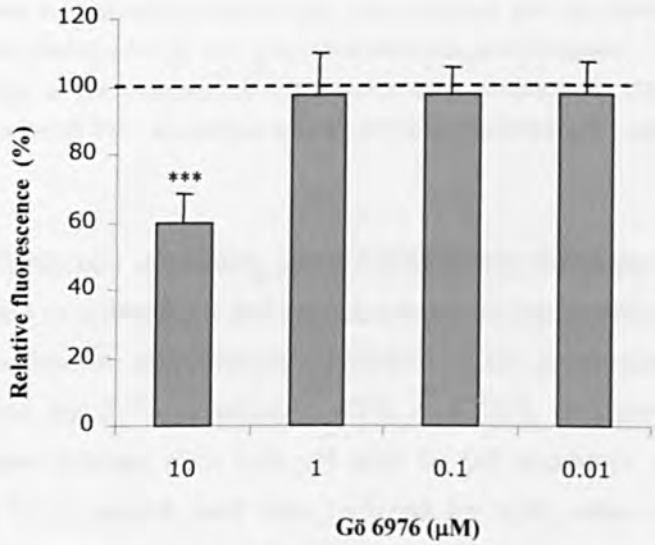


Fig4.6: Inhibition of laminarin-mediated H_2O_2 generation in haemocytes by Gö 6976.

The production of H_2O_2 was investigated in haemocytes incubated with the PKCα inhibitor, Gö 6976 (0.01 μM-10 μM) or vehicle (DMSO) for 30 min prior to stimulation with laminarin (10 mg/ml) for 30 min. Bars show as means (± SD) of two independent experiments each performed in triplicate. ***$P \leq 0.001$ when compared to H_2O_2 production in stimulated haemocytes not exposed to inhibitor (shown as 100%,---).

In contrast to the effects of GF109203X, Gö 6976 was only effective at the highest concentration tested (10 μM) ($P \leq 0.001$; Fig.4.6) reducing H_2O_2 output by 40%. This PKC inhibitor targets the α isoforms of the classical PKCs and was less effective than GF109203X when used at 10 μM (65% inhibition was observed with GF109203X). The lower doses of Gö 6976 (0.01 μM-1 μM) did not have any effect on laminarin-stimulated H_2O_2 output by haemocytes (Fig.4.6).

4.3.4 Inhibition of laminarin-induced H_2O_2 generation in haemocytes by an NAPH oxidase inhibitor

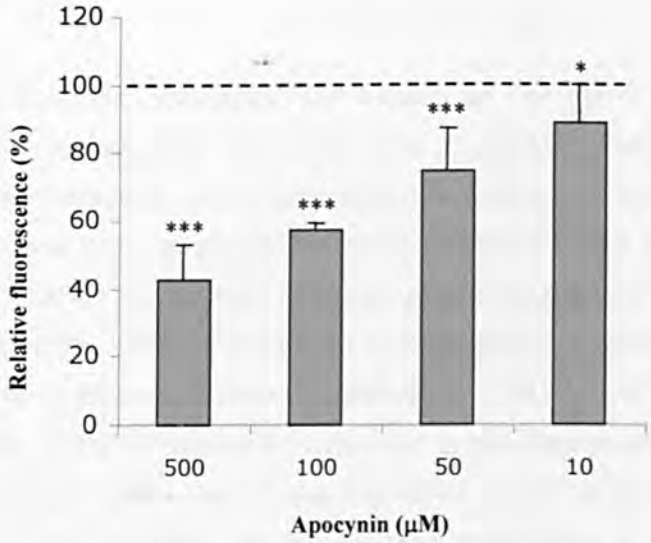


Fig4.7: Inhibition of laminarin-mediated H_2O_2 generation in haemocytes by apocynin
The production of H_2O_2 was investigated in haemocytes pre-incubated with the NADPH inhibitor, apocynin (10 μM–500 μM) or vehicle (DMSO) for 30 min prior to stimulation with laminarin (10 mg/ml) for 30 min. Bars show as means (± SD) of two independent experiments each performed in triplicate. *$P \leq 0.05$ and ***$P \leq 0.001$ when compared to H_2O_2 production in stimulated haemocytes not exposed to either inhibitor (shown as 100%,---).

The NADPH oxidase inhibitor, apocynin, dose-dependently decreased H_2O_2 production by haemocytes relative to stimulated cells not exposed to the inhibitor (Fig.4.7). When used at 500 μM, apocynin significantly inhibited H_2O_2 production by laminarin-stimulated haemocytes by 57% whereas a 43% and 36% reduction were observed when haemocytes were treated with 100 μM and 50 μM apocynin, respectively ($P \leq 0.001$; Fig.4.7). The H_2O_2 output was only reduced by 13% when haemocytes were pre-treated with 10 μM apocynin ($P \leq 0.05$; Fig.4.7).

4.4 Discussion

In the quest to further understand the molecular control of haemocyte defence responses, the role of PKC signalling in the generation of H_2O_2 by haemocytes was explored. This work was prompted because H_2O_2 permeates cell membranes and acts as a highly cytotoxic molecule participating in the elimination of pathogens. Given that *L. stagnalis* is an intermediate host to the avian schistosome *T. ocellata* and H_2O_2 is thought to possess schistosomicidal activity (Dikkeboom *et al.*, 1988; Adema *et al.*, 1994), knowledge of the molecular control of H_2O_2 production by molluscan haemocytes is likely to be crucial to our understanding of snail-schistosome host-parasite relationships.

In mammals, basic immune processes are known to be partly governed by cell signalling pathways involving ERK and PKC (see Chapter 1, section 1.8). NADPH oxidase is an enzyme composed of cytosolic subunits and membrane-bound subunits; activation of this enzyme through phosphorylation of specific sites has been shown to involve PKC. In mammalian leucocytes, PKC can phosphorylate p47-*phox*, a cytosolic NADPH oxidase component, and promote its translocation, enabling it to assemble with other membrane-associated subunits, ultimately making p47-*phox* functionally active (El-Benna *et al.*, 2005). Proteins homologous to the mammalian NADPH oxidase subunits p47-*phox* and p67-*phox* have been identified in the moth *Galleria mellonella* (Bergin *et al.*, 2005) but, to-date, no information concerning a molluscan NADPH complex has been published. Laminarin (10 mg/ml) was found to increase the phosphorylation (activation) status of *L. stagnalis* PKC-like proteins with maximum phosphorylation occurring at 10 min (see Chapter 2). In the present study, laminarin was a potent stimulator of H_2O_2 production by *L. stagnalis* haemocytes; the effect was dose-dependent and, when used at 10 mg/ml, an almost ten-fold increase in H_2O_2 levels was observed 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 found to be associated with increased H_2O_2 production over 45 min (Zelck *et al.*, 2004). Although laminarin elicits H_2O_2 production in defence cells of molluscs, it fails to elicit the oxidative burst by phagocytic hyaline cells in the shore crab *Carcinus maenas* (Bell and Smith, 1993). The different results observed in studies on invertebrates regarding the respiratory burst might result from the different methods of identification used. Although flow cytometry has been employed to investigate the respiratory burst in *C. gigas* (Lambert *et al.*, 2003), this method remains poorly exploited in molluscan systems. The methods of investigation for ROIs are often based on the use of

compounds which become fluorescent upon cell activation; the localisation of the reactive species studied (extracellular or intracellular) also influences the approach chosen (Myrhe *et al.*, 2003). As such, luminol whose oxidation is triggered by various ROIs (Rathakrishnan *et al.*, 1992), lucigenin (Maskiewicz *et al.*, 1979), 2′, 7′-dicholorodihydrofluorescein diacetate (DCFH-DA) (Bland *et al.* 2001), or dihydrorhodamine 123 (DHR) (Henderson and Chappell, 1993) are commonly used in invertebrate systems, but the Amplex® Red, employed in the present study, is known to have had the greater stability. This fluorescent probe cannot penetrate haemocyte membranes and thus only allows only the detection of extracellular H_2O_2 (Zhou *et al.*, 1997).

Lymnaea stagnalis haemocytes were also challenged with the PKC activator PMA. Although all of the PMA concentrations (0.01 μM-10 μM) used induced H_2O_2 generation after 30 min stimulation, the magnitude of the response seemed to decline when the concentration of PMA was above 1 μM. Nevertheless, over 30 min, the H_2O_2 output of haemocytes after exposure to PMA (0.1 μM) was greater than that when haemocytes were challenged with laminarin (10 mg/ml). Other work, investigating the production of H_2O_2 in resistant and susceptible strains of *B. glabrata* involved challenging haemocytes with PMA; increased H_2O_2 generation by haemocytes was observed after 20 min of exposure to 500 nM PMA (Bender *et al.*, 2005). In early studies, exposure of *L. stagnalis* haemocytes to this compound (0.4 μM) resulted in an increase of luminol-enhanced chemiluminescence, with a peak occurring between 30 and 60 min (Dikkeboom *et al.*, 1987). The differences observed in the kinetics of the respiratory burst in laminarin-activated or PMA-activated *L. stagnalis* haemocytes could be explained by the nature of the stimuli used and their mechanisms of action: laminarin probably interacts with a membrane receptor which transduces signals via intracellular pathways, whereas PMA likely bypasses the haemocyte membrane and activates PKC directly.

Although the effects of PMA suggested a role for PKC in H_2O_2 production, inhibitors were employed to more clearly define this phenomenon. Experiments with GF109203X showed that this PKC inhibitor significantly attenuated H_2O_2 generation in a dose-responsive manner. Whereas GF109203X (10 μM) reduced H_2O_2 generation by 65%, the PKCα inhibitor Gö 6976 was less effective, significantly reducing H_2O_2 production by 40% only at the highest dose tested (10 μM). Although these results clearly define a role for PKC in H_2O_2 production, the differential effects of the inhibitors are likely a consequence of the sensitivity of haemocyte PKC to the different inhibitors and/or the presence of multiple PKC-like proteins existing in haemocytes that are sensitive to GF109203X (an inhibitor that targets more PKC isoforms). In laminarin-stimulated

haemocytes, increased extracellular H_2O_2 generation appeared 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 in a similar manner to that which occurs in mammalian phagocytes (Segal *et al.*, 1999), or early production of H_2O_2 could be a consequence of basal cellular activity coordinated by unidentified signalling events. In human neutrophils, activation of PKCα and PKCβ has been shown to correlate with the assembly of the NADPH oxidase complex (Sergeant and McPhail, 1997). Moreover, 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.

We cannot exclude the possibility that the release of H_2O_2 by *L. stagnalis* haemocytes might have been diminished by the actions of detoxifying mechanisms like via scavenging enzymes. As such, catalase has been identified in the snails *Biomphalaria alexandrina* (Mahmoud and Rizk, 2004) and *Bulinus truncatus* (Nabih and el Ansary, 1993), and in the shore crab *C. maenas* (Bell and Smith, 1993). Interestingly, similar antioxidant enzymes appear to be produced by parasites such as *S. mansoni* and their role is likely to be to subvert ROI-mediated cytotoxicity in the snail vector (Hong *et al.*, 1993; Mei and Lo Verde, 1997; Zelck *et al.*, 2004).

Chapter 5

Molecular control of *Lymnaea stagnalis* haemocyte spreading and migration

5.1 Introduction

Motility is a critical and universal phenomenon shared by various cell types from multi-cellular organisms such as mammals to single-celled organisms such as amoebae (Lauffenburger and Horwizt, 2000). Human macrophages, dendritic cells, but also malignant cells, undergo this multistep process, occurring in a variety of normal and pathological conditions including embryonic development, wound healing, inflammation, and metastasis (Ridley *et al.*, 2003). Cell motility involves the reorganisation of various proteins, including filamentous actin (Yamada and Geiger, 1997), which ultimately leads to cytoskeletal remodelling and an increase in the number of protrusions at the leading edge of the cell, with retraction at the rear. Cell adhesion and spreading are associated with migration and are important to various immune functions including encapsulation. Adhesion and spreading are mediated by cell adhesion receptors such as integrins (Kumar, 1998), which are localised at focal adhesion sites; these are critical since they anchor extracellular matrix (ECM) proteins to actin filaments (Horwitz and Parson, 1999). Integrin binding enhances the phosphorylation of focal adhesion kinase (FAK) (Kornberg *et al.*, 1992) in the focal adhesion site (Burridge *et al.*, 1992) via a Src-dependent mechanism (Cary *et al.*, 2002). Protein kinases such as PKC, PKA, and PI-3-K have been shown to play a role in controlling RAW264 macrophage spreading (Petty, 1989; Munugalavadla *et al.*, 2005).

The directional movement of cells along a gradient of chemoattractant is an early event in cell migration and is regulated by the presence of specific receptors on the cell membrane (Katanaev, 2001). Common chemoattractants which elicit migration patterns in mammalian immune cells comprise the peptide N-formylmethionine-leucine-phenylalanine (fMLP), the monocyte chemoattractant protein 1 (MCP-1), and LPS. The fMLP peptide is derived from *E. coli* (Schiffmann *et al.*, 1975; Marasco *et al.*, 1984) and, since its discovery, many formyl peptides have been synthesised based on known bacterial peptides. The MCP-1 is a 76 kDa polypeptide, which possesses an N-glycosylation linked site and belongs to the family of C-C chemokines (Carr *et al.*, 1994). Lipopolysaccharide has been shown to induce chemotaxis in leukocytes (Creamer *et al.*, 1991). Cell motility studies have been conducted with insect haemocytes, and results have revealed similarities between invertebrates and vertebrate cells in their responses to chemoattractants. The Boyden chamber, commonly used to quantify cell migration comprises two chambers that are separated by a filter through which cells migrate (Boyden, 1962). Multi-well chambers and use of fluorochromes have recently been commercially developed to increase sensitivity of

migration experiments. Such chambers and fluorochromes were used in the present work.

Much research aimed at understanding cell migration has focused on the amoebae *Dictyostelium discoideum* (Bonner, 1977; Manahan *et al.*, 2004), mammalian cells such as fibroblasts (Owen *et al.*, 1999; Munevar *et al.*, 2001), cancer cells (Jones *et al.*, 2006), and immune cells (Vanhaesebroeck *et al.*, 1999; Filippi *et al.*, 2007). In insects and molluscs, studies on cell spreading and migration have been carried out on organisms such as the fruit fly *Drosophila melanogaster* (Lehmann, 2001), the mussel *Mytilus edulis* (Schneeweiss and Renwrantz, 1993), the hard clam *Mercenaria mercenaria* (Fawcett and Tripp, 1994), and the oyster *Crassostrea madrasensi* (Ittoop *et al.*, 2006). Migratory responses of *M. edulis* haemocytes have been shown to be enhanced by endogenous stimulatory factors such as neuropeptides (Stefano *et al.*, 1989), or nitric oxide (Magazine *et al.*, 1996). Cell spreading has also been investigated in haemocytes from *Biomphalaria glabrata* (Fryer and Adema, 1993; Humphries *et al.*, 2001), the freshwater snail *Viviparus ater* (Ottaviani, 1989) and the European limpet *Patella vulgata* (Jones and Partridge, 1974); these studies have largely determined the ability of the cells to spread on glass.

Generally, our knowledge of the molecular mechanisms controlling cell migration and cell spreading in molluscan haemocytes is limited. In a recent report, *L. stagnalis* haemocyte phagocytosis and spreading have been shown to be integrin-dependent (Plows *et al.*, 2006) and in *B. glabrata* embryonic (Bge) cells, spreading might rely on PKC and ERK1/2 activities (Humphries *et al.*, 2001). Results obtained in Chapter 4 show that PKC like-proteins play an essential role in the generation of ROIs by *L. stagnalis* haemocytes. The effects of various inhibitors on migration were also determined. Inhibition assays were performed using GF109203X, U0126 and cytochalasin D. GF109203X blocks PKC signalling in haemocytes (Chapter 3), reduces H_2O_2 generation by laminarin-stimulated haemocytes (Chapter 4) and potentially affects multiple PKC isoforms. U0126 targets both MEK isoforms, MEK 1 and MEK 2, and prevents ERK 1/2 phosphorylation (Favata *et al.*, 1998). Numerous reports have shown that PKC acts upstream of Src (Brandt *et al.*, 2003) whereas PKCδ has been shown to be tyrosine phosphorylated by Src in fibroblasts (Gschwendt *et al.*, 1994; Zang *et al.*, 1997). Other studies have revealed that FAK is phosphorylated on various Ser in addition to Tyr residues (Ma *et al.*, 2001; Hunger-Glaser *et al.*, 2003). The work described here investigates whether PKC like-proteins are key to *L. stagnalis* haemocyte spreading and migration; it also explores the role of other key signalling molecules, such as FAK and Src, in these cellular functions.

5.2 Experimental procedures

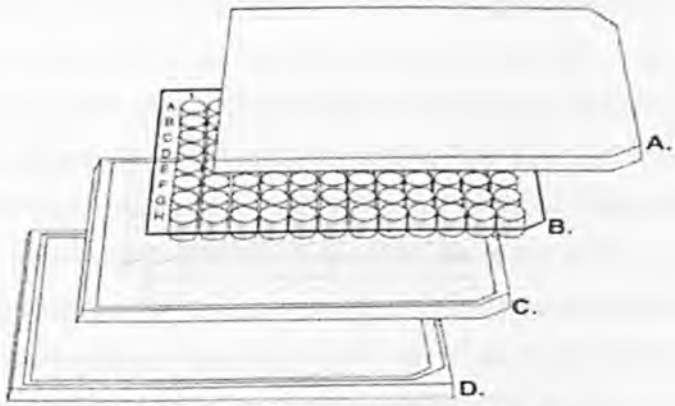
5.2.1 Reagents

The QCM™ chemotaxis 96-well cell migration assay kit was ordered from Chemicon Europe Ltd (Chandlers Ford, Hampshire, UK). It comprises a sterile 96-well (5 μm) cell migration plate assembly, a 96-well cell culture tray, cell detachment solution, 4X cell lysis buffer and CyQuant GR Dye®. The peptide, N-formyl-methionyl-leucyl-phenylalanine (fMLP), the Src kinase inhibitor 1 (SrcI) (4-(4'-Phenoxyanilino)-6, 7-dimethoxyquinazoline), herbimycin A (Herb) (from *Streptomyces sp.*), and Gö 6976 were purchased from Calbiochem (Nottingham, Notts, UK) together with the inhibitors U0126 (1, 4-diamino-2, 3-dicyano-1, 4-bis [2-aminophenylthio] butadiene) and cytochalasin D. The anti-phospho PKC (pan) and the anti-phospho FAK^{925} antibodies were from Cell Signaling Technology (Beverly, MA, USA). Vectashield came from Molecular Probes (AA Leiden, Netherlands). Tetramethyl rhodamine isothiocyanate (TRITC)-conjugated phalloidin, 4'-6-diamidino-2-phenylindole (DAPI), GF109203X (GFX) and all other reagents were from Sigma-Aldrich (Poole, Dorset, UK).

5.2.2 Haemocyte migration assay

5.2.2.1 Basic principle of the assay

The QCM™ cell migration assay kit has been developed to provide more rapid and accurate alternative to the more traditional Boyden chamber for determining rates of cell migration. The principle of this assay is straightforward: cells are added to the wells of the chamber plate (Fig.5.1) and those that migrate through the insert membrane towards the feeder tray are detached from the underside of the membrane using a cell detachment buffer. These cells are subsequently lysed with lysis buffer and are detected by the CyQuant GR Dye®. The size of the membrane (5 μm) chosen for haemocyte assays was the same as that recommended for experiments on mammalian macrophages and monocytes.



A. Lid B. Cell Migration Chamber Plate
C. 96-well Feeder Tray D. Base

Fig.5.1: Components of the QCM™ cell migration assay kit (from Chemicon Ltd)

5.2.2.2 Haemocyte migration assays

The different plates and reagents of the migration assay kit were brought to room temperature prior to starting the assay. Haemolymph was extracted from six to ten adult snails, pooled and diluted in SSS as described in Chapter 2 (section 2.3). Diluted haemolymph (100 µl) was then placed in individual wells of the migration chamber plate (B in Fig.5.1).

Cell-free haemolymph for the feeder tray (C in Fig.5.1) was prepared by centrifuging diluted haemolymph from the same batch of snails for 5 min at 800 *g* to pellet the haemocytes. Possible chemoattractants including LPS (final concentrations: 1 µg/ml; 10 µg/ml; 100 µg/ml), fMLP (final concentrations: 1 µM; 10 µM), were then added to the supernatant (cell-free haemolymph) and 150 µl of this solution was placed in individual wells of the feeder tray (C). Control wells were also set up to establish any effects of the SSS and haemolymph alone on the final fluorescence readings. The kit was then assembled and incubated for 1-3 h at 26°C to allow the haemocytes to migrate. After this period, the migration chamber plate was removed and non-adherent cells/media were discarded by flipping out the remaining cell suspension; this plate was then placed in a new 96-well feeder tray containing 150 µl of pre-warmed cell detachment buffer in each well. The assembly was then incubated for 30 min at 26°C. Haemocytes could be dislodged completely from the underside by gently tilting the migration chamber plate back and forth. From the original feeder tray (C), 75 µl of the chemoattractant solution (or control) was transferred to a well of a new 96-well plate containing 75 µl cell detachment buffer with haemocytes (from above). This served to combine cells that migrated through the membrane into the medium in the lower chamber with those adherent haemocytes detached from the underside of

the membrane by the detachment buffer. The lysis buffer containing the dye was then prepared (CyQuant GR Dye® 1:75 in 4X lysis buffer) and 50 µl of this mixture added to each well containing the 150 µl suspension of migratory haemocytes, and incubated for 15 min. The CyQuant GR Dye® exhibits strong fluorescence when bound to nucleic acids; fluorescence was thus measured using a Fluorstar Optima microplate reader with an excitation wavelength of 480 nm and an emission wavelength of 520 nm. The control fluorescence values derived from wells containing only cell-free haemolymph in the feeder plate were normalised to 1 and all other averaged fluorescence values derived from migrated cells related to that in each experimental condition.

5.2.2.3 Inhibition of haemocyte migration

Haemocytes (in the migration plate chamber B) were treated for 30 min with GF109203X (0.1 µM; 10 µM), U0126 (10 µM) or cytochalasin D (1 µM) prior adding them to the migration chamber plate. Cytochalasin D is a cell permeable fungal toxin that blocks the association and dissociation of actin units (Goddette and Frieden, 1986). Where inhibitors were solubilised in DMSO/methanol, the final DMSO/methanol concentration in the assay was kept below 0.1% (v/v) and control samples were exposed to DMSO/methanol alone. The control fluorescence values derived migrated haemocytes in control wells (containing 10 µg/ml LPS in haemolymph) were normalised to 1 and all other averaged fluorescence values derived from migrated cells related to that in each experimental condition.

5.2.3 Haemocyte spreading assays

Haemolymph was extracted from four to six adult snails and was diluted with SSS as described in Chapter 2 (section 2.3); 100 µl was then added to individual coverslips and cells were allowed to adhere for 40 min at room temperature. In some experiments, the diluted haemolymph was incubated on ice for 30 min with the PKC inhibitors, GF109203X (10 µM) and Gö 6976 (10 µM), and the Src kinase inhibitors, herbimycin A (5 µM) and Src kinase inhibitor I (5 µM) before it was applied to coverslips; control cells were treated with the vehicle (0.1% DMSO) alone. Herbimycin A is a cell permeable, potent, inhibitor of protein tyrosine kinases including some protein kinases belonging to the Src family (Fukazawa *et al.*, 1991). Src kinase inhibitor 1 is a selective, dual-site, competitive inhibitor of Src tyrosine kinase and simultaneously interacts with both the ATP- and peptide-binding sites (Tian *et al.*, 2001). Following cell attachment, coverslips were rinsed in 6-well culture plates three times with SSS to remove dead or non-adherent haemocytes, large haemocyte

clumps, and any remaining haemolymph. Coverslips were then immersed in fixing buffer (3.7% (v/v) formaldehyde, 0.18% (v/v) Triton X-100 in PBS) for 12 min to fix and permeabilise the cells. After a further wash in PBS, haemocytes were incubated in TRITC-conjugated phalloidin (1 µg/ml) and DAPI (1 µg/ml) for 40 min. Cells were then washed with several volumes of PBS to remove unbound dye before coverslips were mounted onto slides with Vectashield and sealed with nail varnish. Visualisation of the cellular fluorescence was performed using a Zeiss Axiophot 20 photomicroscope equipped with a triple filter; excitation wavelengths were 410, 505 and 585 nm (with beamsplitters: 395, 485 and 560 nm; and barriers: 460, 530 and 610 nm, respectively). Fields of view were randomly chosen and 40 images (at x 40 or x 100 objective magnification) of individual cells were captured from three independent experiments (15 images per experiment) using a Nikon DN100 camera linked to Nikon Eclipse Net image analysis software. The area analysis function of this software was then used to determine the extent of cell spreading following the different treatments.

5.2.4 Haemocyte cell signalling studies

5.2.4.1 Effects of fMLP on activation of PKC- and ERK-like proteins

Haemolymph was extracted from six to ten adult snails, placed on ice and diluted with SSS; haemocyte monolayers were then prepared in 24-well culture plates (500 µl diluted haemolymph per well) as described in Chapter 2, Experimental procedures (section 2.2.3). After equilibrating in SSS, haemocytes were challenged with fMLP (10 µM). Following challenge, haemolymph was quickly removed and cells were lysed by adding 70 µl of boiling 1X SDS-PAGE sample buffer to the wells. Protein extracts were then subjected to SDS-PAGE on 10% gels and transferred to nitrocellulose membrane as described in Chapter 2, Experimental procedures (sections 2.2.6 and 2.2.7). The anti-phospho PKC (pan) antibody (1:1000 in TTBS) was used to probe the membrane and actin was used as a loading control. Immunoreactive proteins were then visualised using ECL detection.

5.2.4.2 Effects of Src kinase inhibitors on phosphorylation of PKC- and FAK-like proteins during cell attachment

Haemolymph was extracted and haemocyte monolayers were prepared in 6-well culture plates (1000 µl diluted haemolymph per well) according to Chapter 2, Experimental procedures (section 2.2.3). Haemocytes were, however, exposed to GF109203X (10 µM), SrcI (5 µM) or vehicle (DMSO) during the 30 min cell attachment period. Haemolymph was subsequently removed and cells were lysed by adding 40 µl

of boiling 1X SDS-PAGE sample buffer. Cells were scraped off the wells and placed on ice. Protein extracts were subjected to SDS-PAGE on 10% gels and transferred to nitrocellulose membranes as described in Chapter 2, Experimental procedures (sections 2.2.6 and 2.2.7). Anti-phospho PKC (pan) (1:1000 in TTBS) and anti-phospho FAK^{925} (1:800 in TTBS) antibodies were used to probe membrane and actin was probed as a loading control. The anti-phospho FAK^{925} antibody detects FAK when phosphorylated on Tyr 925. Immunoreactive proteins were then visualised using ECL detection.

5.2.5 Data analysis

Multiple comparisons of raw data were subjected to one-way ANOVA and LSD *post-hoc* using the statistical software package SPSS to determine the effects of treatment on cell spreading. Results are shown as the mean ± SD. The student t test was used to compare the tested data with the control data in both migration and spreading assays.

5.3 Results

5.3.1 Cell migration

Exogenous factors such as LPS and fMLP have been shown to elicit chemotactic reactions in human neutrophils (Creamer *et al.*, 1991; Marasco *et al.*, 1984) and in molluscan haemocytes (Schneeweiss and Renwrantz, 1993; Fawcett and Tripp, 1994). To assess the role of PKC-like proteins and other signalling molecules in cell migration by *L. stagnalis* haemocytes, migration assays were performed using various pharmological inhibitors. In some experiments, the rate of cell migration was low; therefore, when determining the effects of different inhibitors, only experiments where migration of control haemocytes was clearly observed were analysed and shown. The fluorescence signal detected by the microplate reader was normalised to the signal detected in control well for each experiment.

5.3.1.1 Haemocyte migration to LPS and fMLP

5.3.1.1.1 Haemocyte migration to LPS

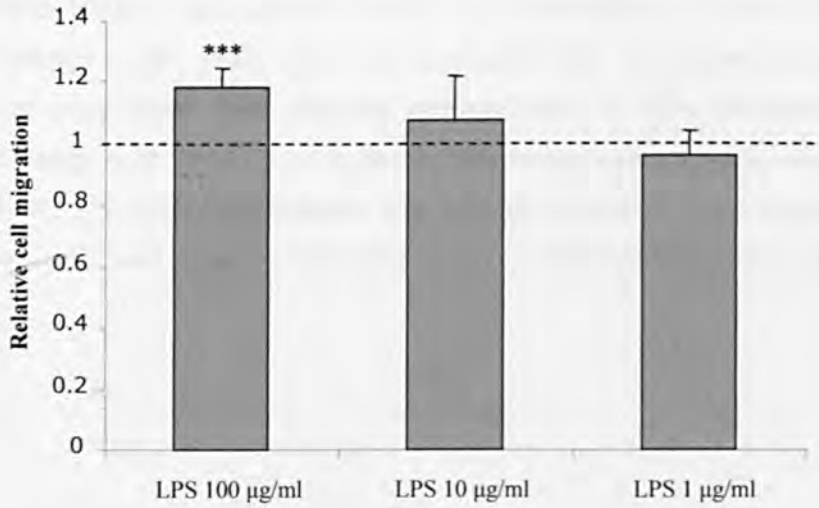


Fig.5.2: Migration of haemocytes to various concentrations of LPS diluted in cell-free haemolymph.

Haemolymph was placed in wells of the migration chamber plate whereas the feeder plate contained various concentrations of LPS (0 µg/ml-100 µg/ml) in cell-free haemolymph. Migration was allowed to take place for 1h at 26°C. Bars represent the relative fluorescence derived from cells that had migrated through the pores of the migration chamber plate in nine independent experiments. The dotted line represents the mean haemocyte migration observed in control wells (containing only cell-free haemolymph in the feeder plate). ***$P \leq 0.001$, when compared to control (---).

At high doses, LPS appeared to slightly increase the rate of migration of *L. stagnalis* haemocytes. When LPS was used at 100 μg/ml, the haemocyte migration rate was significantly increased to 1.2 times that of the control (with cell-free haemolymph in feeder tray) ($P \leq 0.001$; Fig. 5.2). No significant effects were observed when LPS was used at 10 μg/ml and 1 μg/ml suggesting that these concentrations were to low to induce further migration (Fig.5.2). Haemocyte migration was also found to occur in the absence of LPS. In *L. stagnalis* haemocytes, PKC- (Chapter 2) and ERK 1/2- (Plows *et al.*, 2004) like proteins were shown to be activated by LPS after 5 min of challenge suggesting a possible link between LPS-dependent signalling events and haemocyte migration.

5.3.1.1.2 Haemocyte migration to fMLP

Interestingly, the relative migration rates of *L. stagnalis* haemocytes were unaffected by fMLP when it was applied to the feeder plate. Neither 10 μM nor 1 μM fMLP acted as a chemoattractant because both relative migration values were similar to that of the control (Fig.5.3A). In parallel experiments, *L. stagnalis* haemocytes were challenged with fMLP (10 μM) for 0-30 min to determine the effects of exposure to this compound on the phosphorylation (activation) status of haemocyte PKC and ERK 1/2–like proteins. Challenge with fMLP did not increase PKC phosphorylation; however, phosphorylation may have been slightly reduced after 5 min exposure (top panel, Fig.5.3B). Challenge with fMLP at a similar concentration appeared to result in a slight increase in ERK 1/2 phosphorylation; the immunoreactive band corresponding to phosphorylated p42 was slightly stronger after 10 min of exposure (bottom panel, Fig.5.3B).

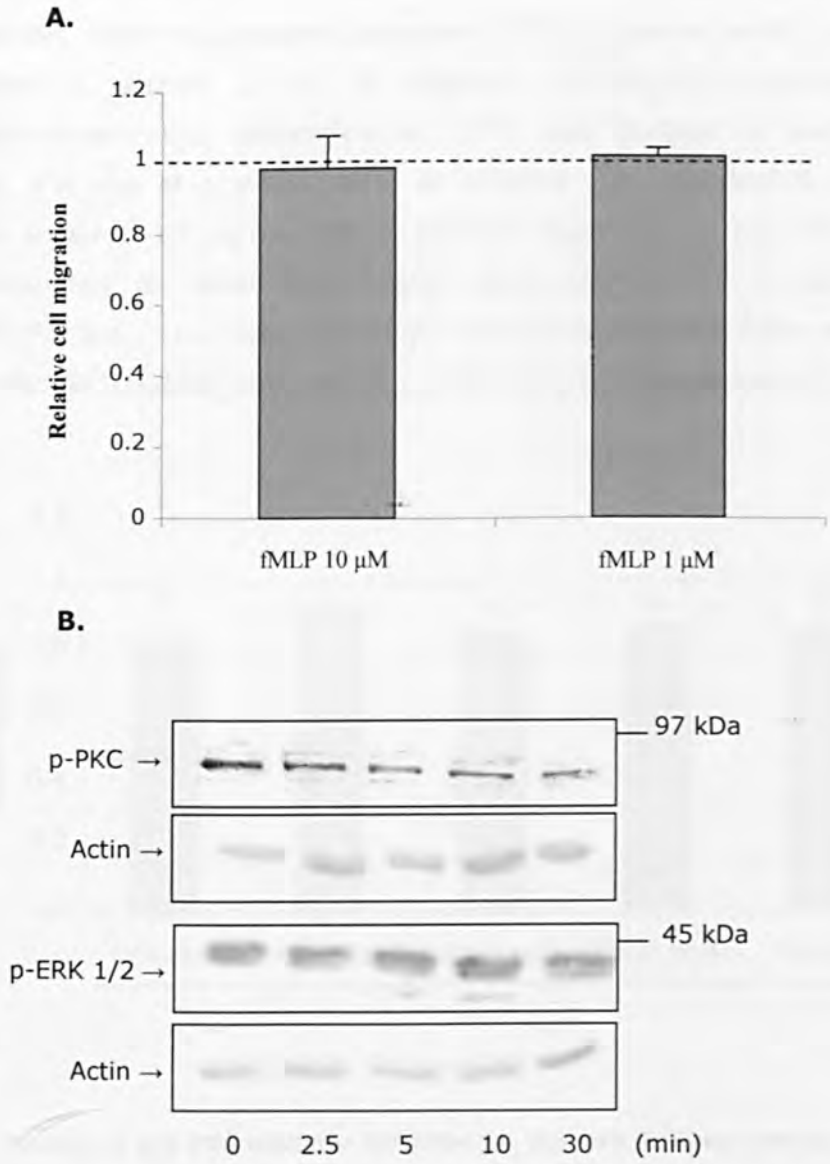


Fig.5.3: Migration of haemocytes to various concentrations of fMLP diluted in cell-free haemolymph and the effects of fMLP on the phosphorylations of PKC and ERK-like proteins.
(A) Haemolymph was placed in wells of the migration chamber plate whereas the feeder plate contained two concentrations of fMLP (1 μM; 10 μM) in cell-free haemolymph. Migration was allowed to take place for 1h at 26°C. Bars represent the relative fluorescence derived from cells that had migrated through the pores of the migration chamber plate in n=6 and n=3 independent experiments when fMLP was used at 10 μM and 1 μM, respectively. The dotted line represents the mean haemocyte migration observed in control wells (containing only cell-free haemolymph in the feeder plate). (B) Phosphorylated PKC-like and ERK 1/2 -like proteins following challenge with fMLP (10 μM) for various durations (0-30 min) were detected by western blotting. Samples were obtained as described in "Experimental procedures". Equal amounts of protein were loaded in each lane and membranes were probed with the anti-phospho PKC (pan) and the anti-phospho ERK 1/2 antibodies. Anti-actin antibodies were used to confirm equal loading of proteins. Immunoblots are representative of two independent experiments.

5.3.1.2 Effects of pharmological inhibitors on haemocyte migration

In previous experiments, GF109203X was able to significantly attenuate PKC phosphorylation, with the greatest reduction (72%) observed when used at 10 μM (see Chapter 2; section 2.3.4). In addition, GF109203X significantly reduced laminarin-stimulated H_2O_2 generation by 65% (see Chapter 4; section 4.3.2.1). Surprisingly, the use of a similar dose of inhibitor only impaired the migration of haemocytes towards 10 μg/ml LPS in cell-free haemolymph by 17% ($P \leq 0.01$; Fig.5.4) compared to when experiments were carried out in the absence of GF109203X (Fig.5.4). The mean migration rates of haemocytes were reduced by 7% and 16% when GF109203X was used at 1 μM and 0.1 μM, respectively (Fig.5.4).

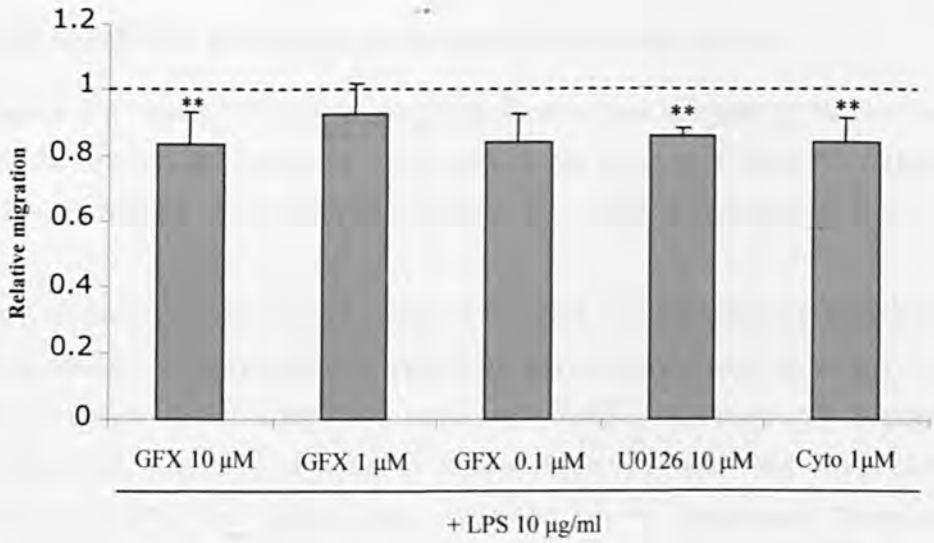


Fig.5.4: The effects of the PKC inhibitor GF109203X, the MEK inhibitor U0126, and the actin disruptor cytochalasin D, on LPS-induced migration of haemocytes.
Haemocytes were pre-incubated for 30 min in GF109203X (GFX; 0.1, 1, and 10 μM), U0126 (10 μM), cytochalasin D (Cyto; 1 μM) or vehicle (DMSO or methanol) before placing in the migration plate. The feeder tray contained 10 μg/ml LPS in cell-free haemolymph. Migration was allowed to take place for 1h at 26°C. Bars represent the relative fluorescence derived from cells that had migrated through the pores of the migration chamber in n independent experiments (GFX: 10 μM, n=8; 1 μM, n=4; 0.1 μM, n=4; U0126, n=5; Cyto, n=5). The dotted line represents the mean of migrated haemocytes observed in control wells (containing 10 μg/ml LPS in haemolymph). $^{**}P \leq 0.01$, when compared to control (---).

The MEK inhibitor U0126 (10 μM) significantly reduced haemocyte migration towards LPS (10 μg/ml) by 14% ($P \leq 0.01$; Fig.5.4) when compared to controls. Given that PKC-like proteins lay upstream MEK and ERK in laminarin-challenged *L. stagnalis*

haemocytes (see Chapter 2), the effects seen here could be mediated via PKC (Fig.5.4).

Cytochalasin D only significantly reduced haemocyte migration to LPS-diluted haemolymph by 16% ($P \leq 0.01$; Fig.5.4). This is surprising given the role of actin in cell migration in mammalian and invertebrate defence cells (Cheng and Howland, 1982; Stossel, 1993; Panara *et al.*, 1996). Although the concentration of inhibitor chosen was based on that used in other studies (Matheny *et al.*, 2000; Matsubayashi *et al.*, 2004), it may be possible that a higher dose of cytochalasin D would inhibit *L. stagnalis* haemocyte migration to a greater extent.

5.3.2 Cell spreading

5.3.2.1 Effects of PKC inhibitors on haemocyte cell spreading

To investigate the role of PKC in *L. stagnalis* haemocyte spreading, haemolymph was treated for 30 min on ice (prior to being placed on coverslip) with GF109203X which targets a broad range of PKC isoforms, or Gö 6976, which is specific for PKCα.

Observation of haemocytes revealed that treatment of haemolymph with GF109203X reduced the ability of haemocytes to spread on glass coverslips (Fig.5.5A-C). Although haemocytes treated with this inhibitor appeared more ovoid, they still displayed some filopodia (Fig.5.5B, C). Area analysis of haemocytes revealed that when GF109203X was used at 10 µM, the mean area occupied by an individual haemocyte was significantly less than that occupied by a control haemocyte; a 64% reduction in spread was seen ($P \leq 0.001$; Fig.5.5). A lower dose of GF109203X (1 µM) also significantly reduced the spread of individual haemocytes by 38% ($P \leq 0.001$; Fig.5.5) compared to control haemocytes.

The incubation of haemolymph with the PKCα inhibitor Gö 6976 (10 µM) also impaired the spreading of haemocytes on glass coverslips (Fig.5.6A, B). Area analysis of individual cells revealed that the mean area occupied by an individual cell treated with 10 µM Gö 6976 was 46% less than that observed for control cells ($P \leq 0.001$; Fig.6.3). Thus, the effects of Gö 6976 on haemocyte spreading are less marked than with GF109203X (Fig.5.5).

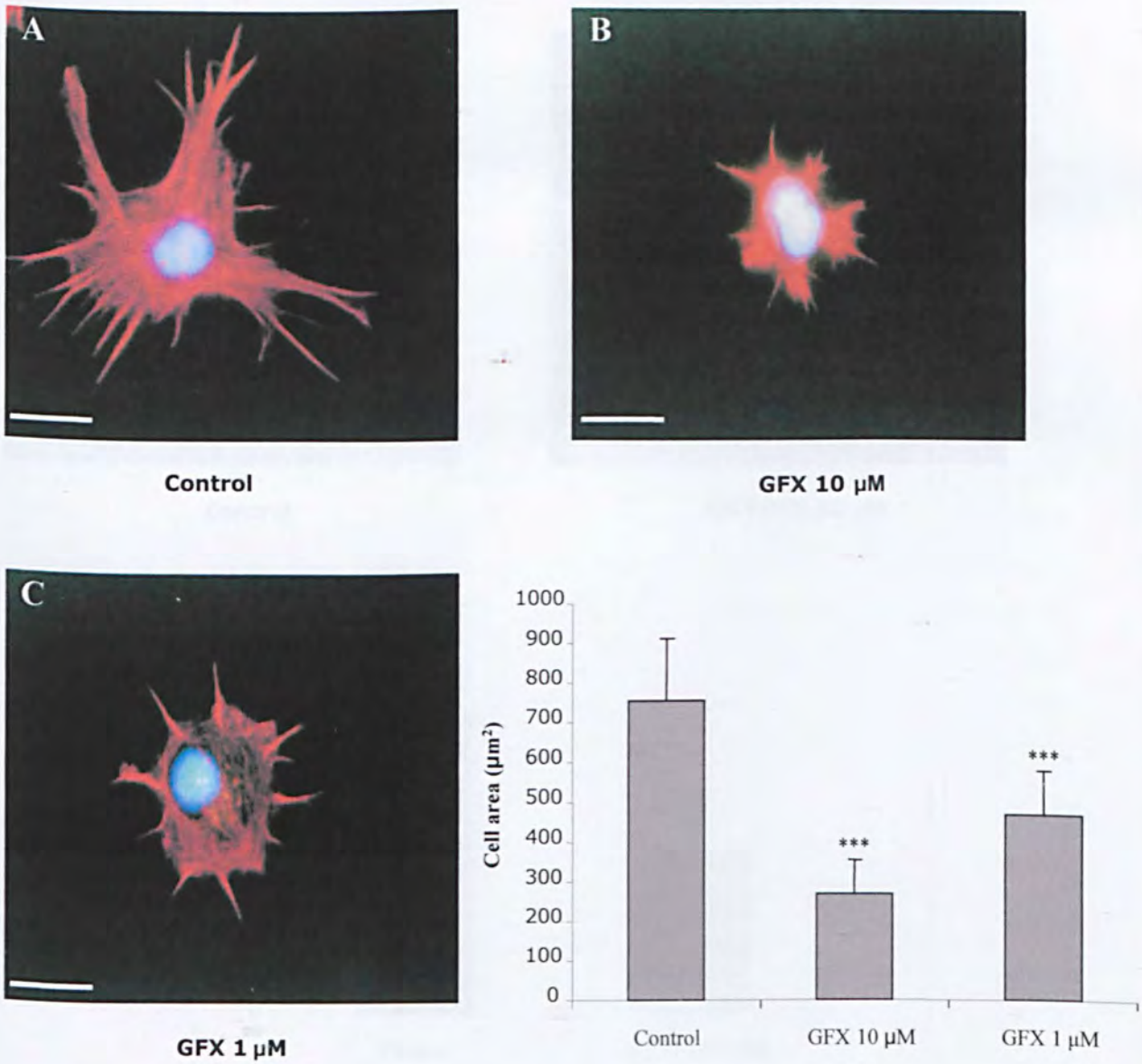


Fig.5.5: Reduction of haemocyte cell spreading by the PKC inhibitor GF109203X.

To assess the effect of GF109203X, haemocytes were incubated with this inhibitor (GFX; 10 μM; 1 μM) or vehicle (DMSO) for 30 min. Cells were then applied to glass coverslips, fixed and stained with rhodamine phalloidin (for F-actin) and DAPI (for nuclei). Images of individual cells were captured (A-C) and the area occupied by each cell determined using Eclipse Net cell area analysis software. Values shown in the graph are mean cell area (± SD) from n= 40 haemocytes. A-C are representative images of 40 haemocytes. ***$P \leq 0.001$, when compared to the cell area of control samples. Bar = 20 μm.

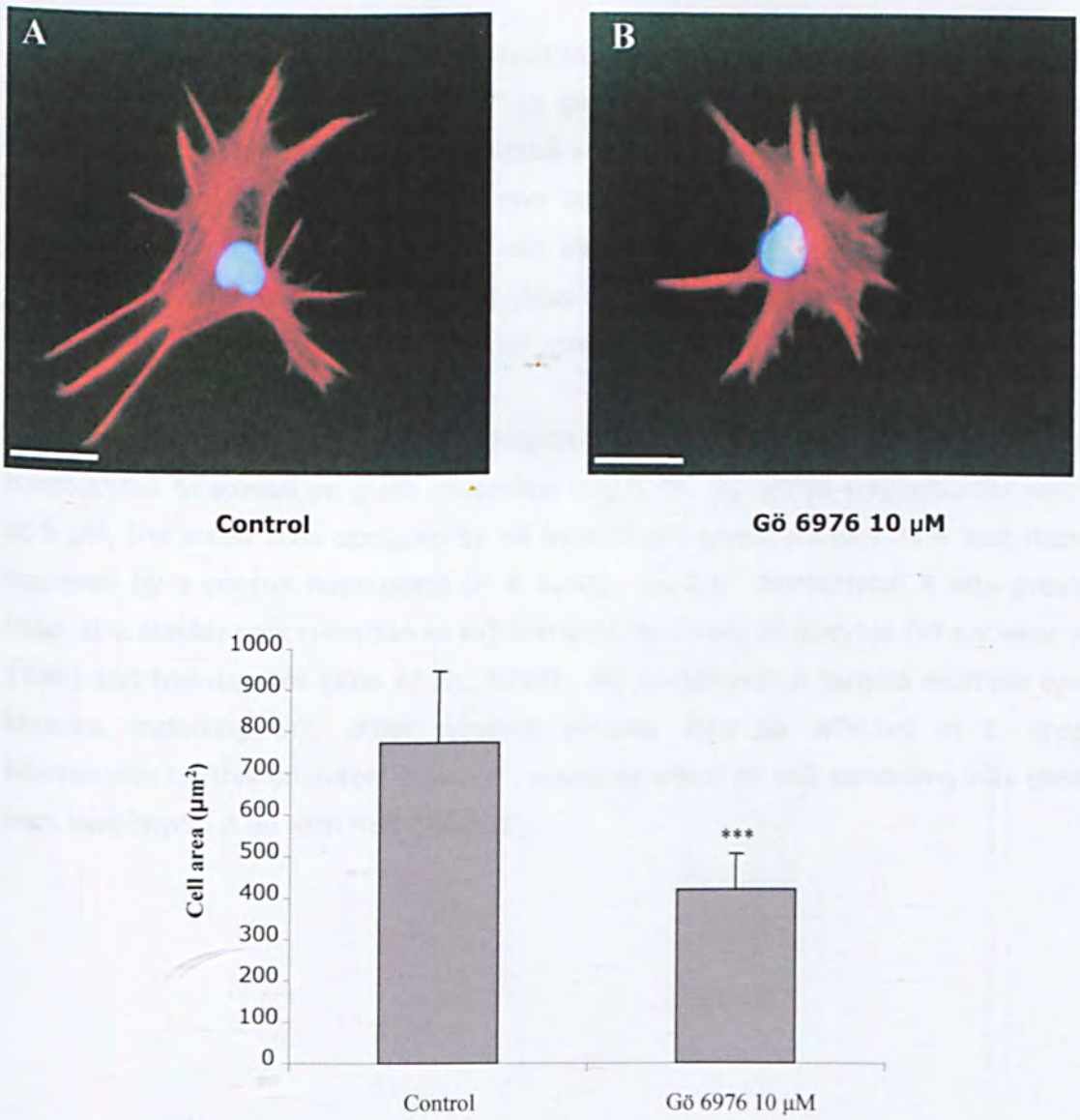


Fig.5.6: Reduction of haemocyte cell spreading by the PKC inhibitor Gö 6976.

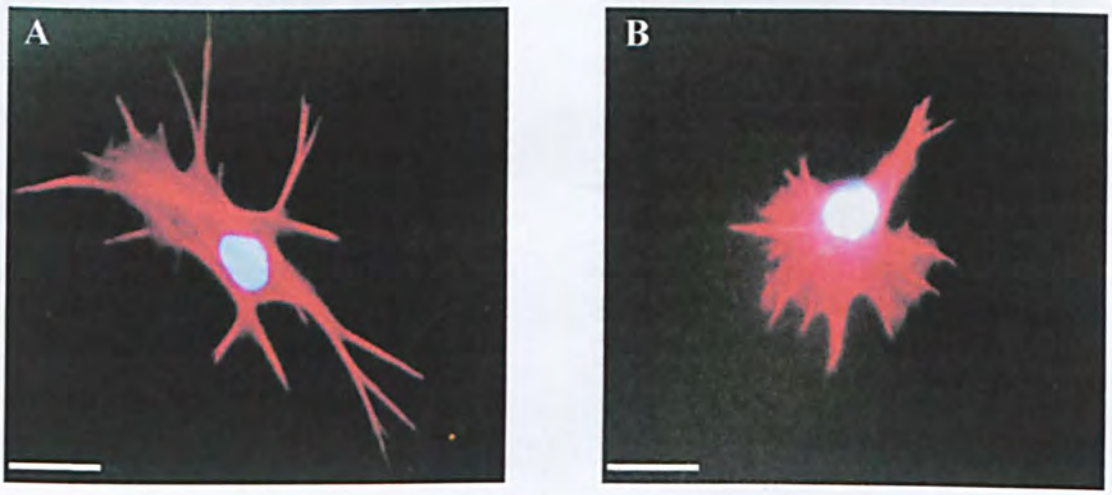
To assess the effect of Gö 6976, haemocytes were incubated with Gö 6976 (10 μM) or vehicle (DMSO) for 30 min. Cells were then applied to glass coverslips, fixed and stained with rhodamine phalloidin (for F-actin) and DAPI (for nuclei). Images of individual cells (A, B) were captured and the area occupied by each cell determined using Eclipse Net cell area analysis software. Values shown in the graph are mean cell area (± SD) from n= 40 haemocytes. A, B are representative images of 40 haemocytes. ***$P \leq 0.001$, when compared to the cell area of control samples. Bar = 20 μm.

5.3.2.2 Effects of Src inhibitors on haemocyte cell spreading

To investigate if the tyrosine kinase Src is required for *L. stagnalis* haemocyte spreading, haemolymph was treated for 30 min on ice with the Src inhibitor I (SrcI) and the tyrosine kinase inhibitor, herbimycin A, prior to being applied to coverslips.

Observation of haemocytes revealed that treatment of haemolymph with SrcI reduced the ability of haemocytes to spread on glass coverslips (Fig.5.7A, B). Whereas the control haemocytes appeared well spread with extensive filopodia, those treated with SrcI were less spread; they did however still display some filopodia. Area analysis of haemocytes revealed that when SrcI was used at 5 μM, the mean area occupied by an individual haemocyte was significantly less than that occupied by a control haemocyte ($P \leq 0.001$) and a 31% reduction in cell spread was observed (Fig.5.7).

Treatment of haemocytes with herbimycin A also significantly reduced the ability of haemocytes to spread on glass coverslips (Fig.5.8A, B). When this inhibitor was used at 5 μM, the mean area occupied by an individual haemocyte was 31% less than that occupied by a control haemocyte ($P \leq 0.001$; Fig.5.8). Herbimycin A was previously used at a similar concentration in experiments involving leukocytes (Khandaker *et al.*, 1998) and hepatocytes (Kuo *et al.*, 1997). As herbimycin A targets multiple tyrosine kinases, including Src, other tyrosine kinases may be affected in *L. stagnalis* haemocytes by this inhibitor; however, a similar effect on cell spreading was observed with herbimycin A as with SrcI (Fig.5.7).



Control **SrcI 5 μM**

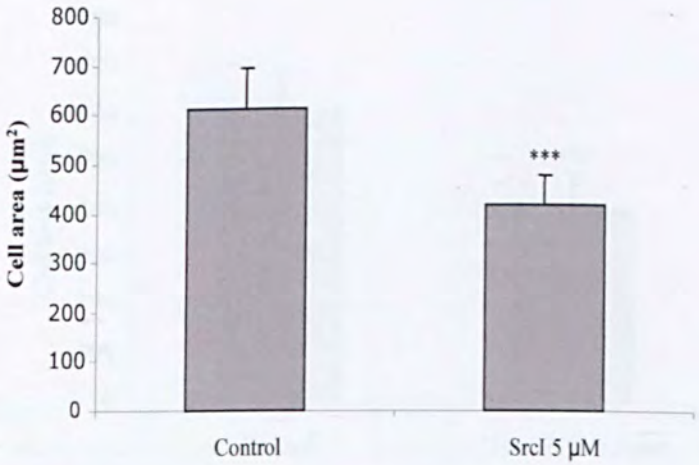
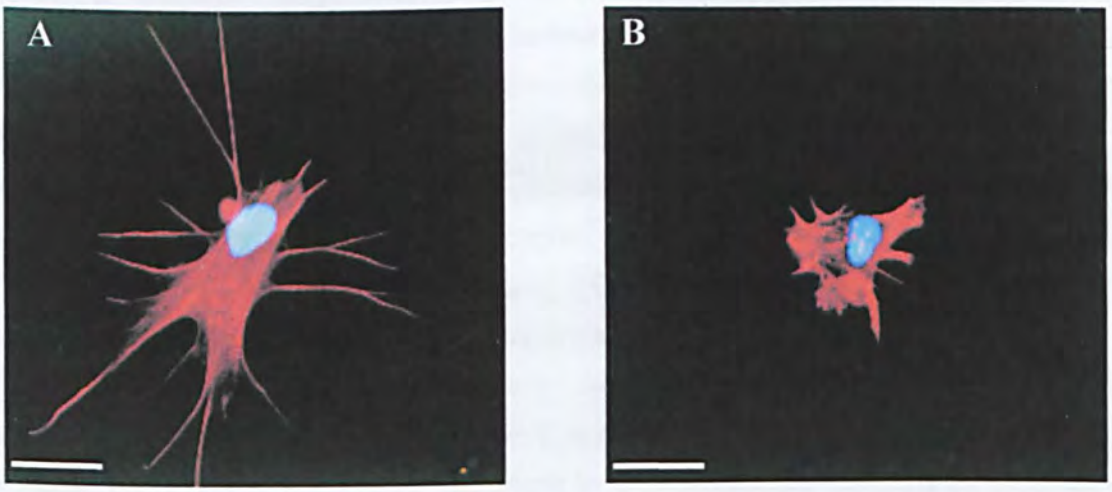


Fig.5.7: Reduction of haemocyte cell spreading by the Src inhibitor Src inhibitor I.
To assess the effect of SrcI, haemocytes were incubated with SrcI (5 μM) or vehicle (DMSO) for 30 min. Cells were then applied to glass coverslips, fixed and stained with rhodamine phalloidin (for F-actin) and DAPI (for nuclei). Images of individual cells were captured (A-B) and the area occupied by each cell determined using Eclipse Net cell area analysis software. Values shown in the graph are mean cell area (± SD) from n= 40 haemocytes. A, B are representative images of 40 haemocytes. ***$P \leq 0.001$, when compared to cell area of control samples. Bar = 20 μm.



Control **Herbimycin 5 μM**

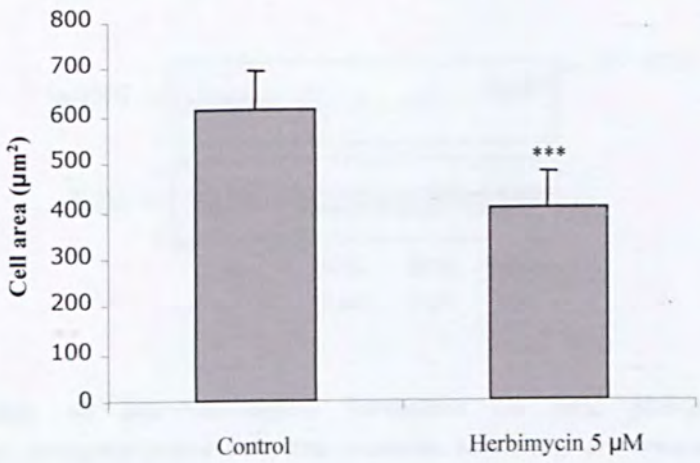


Fig.5.8: Reduction of haemocyte cell spreading by the Src inhibitor herbimycin A.
To assess the effect of herbimycin A, haemocytes were incubated with Herbimycin (5 μM) or vehicle (DMSO) for 30 min. Cells were then applied to glass coverslips, fixed and stained with rhodamine phalloidin (for F-actin) and DAPI (for nuclei). Images of individual cells were captured (A-B) and the area occupied by each cell determined using Eclipse Net cell area analysis software. Values shown in the graph are mean cell area (± SD) from n= 40 haemocytes. ***$P \leq 0.001$ when compared to cell area of control samples. Bar = 20 μm.

5.3.2.3. Effects of inhibitors of haemocyte spreading on the phosphorylation of FAK- and PKC-like proteins

To assess the possible link between the activation of PKC and Src/FAK following haemocyte adhesion, western blotting was performed on haemocytes that had been pre-treated with GF109203X, SrcI, and herbimycin A; blots were then probed with the anti-phospho PKC (pan) and anti-phospho FAK^{925} antibodies. The anti-phospho FAK^{925} antibodies have been previously used to detect FAK phosphorylation in *L. stagnalis* haemocytes (Plows *et al.*, 2006). In duplicate experiments, PKC phosphorylation was reduced below basal levels when haemocytes were treated with GFX (10 μM) and SrcI (5 μM) for 30 min. Image analysis revealed the reduction in PKC phosphorylation to be approximately 55 %. However, when haemocytes were incubated with herbimycin A (5 μM) for 30 min, the phosphorylation status of PKC was unaffected (Fig.5.9). Herbimycin A has been used at a similar concentration to that chosen here to prevent the downregulation of chemokine receptors in LPS-stimulated neutrophils (Khandaker *et al.*, 1998). Herbimycin A has also been previously used in *L. stagnalis* neurons to assess the effects of tyrosine kinases on calcium channels (Pafford *et al.*, 1995).

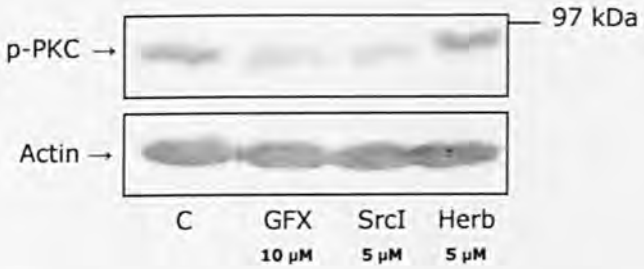


Fig.5.9: The effects of pharmacological inhibitors on PKC phosphorylation levels: immunodetection of phosphorylated PKC-like proteins following pre-treatment with the PKC inhibitor GF109203X (GFX; 10 μM), the Src inhibitor I (SrcI; 5 μM), or the tyrosine kinase inhibitor herbimycin A (Herb; 5 μM) for 30 min.

Samples were obtained as described in "Experimental procedures". Equal amounts of protein were loaded in each lane and membranes were probed with the anti-phospho PKC (pan) antibody. Anti-actin antibodies were used to confirm equal loading of proteins. Immunoblots are representative of two independent experiments.

In duplicate experiments, the PKC activator PMA appeared to slightly increase FAK tyrosine phosphorylation levels in *L. stagnalis* haemocytes when compared to haemocytes not exposed to PMA (Fig.5.10). Treatment of haemocytes with GF109203X (10 μM) appeared to have a little effect on FAK phosphorylation whereas treatment with SrcI (5 μM) appeared to have a greater effect; although difficult to

analyse given the diffuse p-FAK signal, image analysis of duplicate blots suggested that SrcI reduced FAK phosphorylation by approximately 54 % (Fig.5.10).

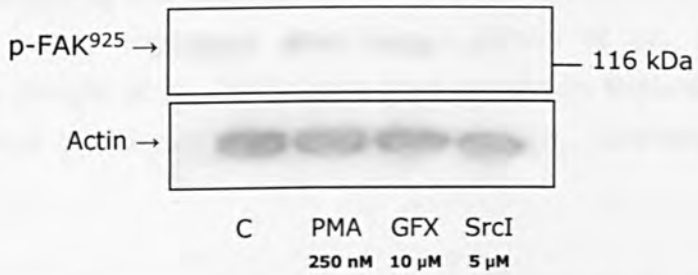


Fig.5.10: The effects of PMA and pharmacological inhibitors on FAK phosphorylation levels: immunodetection of phosphorylated FAK-like proteins following pre-treatment with PMA (250 nM) for 10 min, the PKC inhibitor GF109203X (GFX; 10 µM) or Src inhibitor I (SrcI; 5 µM) for 30 min.

Samples were obtained as described in "Experimental procedures". Equal amounts of protein were loaded in each lane and membranes were probed with the anti-phospho FAK^{925} antibody. Anti-actin antibodies were used to confirm equal loading of proteins. Immunoblots are representative of two independent experiments.

5.4 Discussion

The present study was carried out to investigate the role of selected signalling molecules in cell spreading and cell migration of *L. stagnalis* haemocytes. While some of the molecular events regulating phagocytosis (Plows *et al.*, 2004), and the production of RNIs (Wright *et al.*, 2006) have been previously explored, knowledge of the molecular control of cell migration and spreading by *L. stagnalis* haemocytes is poor.

Migration by *L. stagnalis* haemocytes was slightly enhanced in the presence of high concentrations of LPS; 100 µg/ml LPS attracted more haemocytes than lower concentrations of this compound. Variability of chemotactic responses towards chemoattractants has been observed in molluscan species. For instance, haemocyte migration in the hard clam *Mercenaria mercenaria* was not induced by LPS or by 50 mM acetyl-D-glucosamine, a major component of bacterial cell walls, whereas 10 µM fMLP resulted in haemocyte migration (Fawcett and Tripp, 1994). Contrasting results were observed in the mussel *Mytilus edulis* where haemocytes were attracted to LPS and a lower concentration (0.1 µM) of *N*-fMLP (Schneeweiss and Renwrantz, 1993). The fMLP synthetic chemotactic peptide mimics the activity of bacterially-derived peptides with formylated N-terminal methionine groups. Its effect on cell signalling in immune cells such as neutrophils has recently been reviewed (Selvatici *et al.*, 2006) and the receptor for fMLP (N-formyl peptide receptor) has been shown to be linked to a G-protein (Prossnitz *et al.*, 1997). Furthermore, this compound has been shown to stimulate the production of ROIs by neutrophils via the activation of ERK 1/2 and PKC (Dewas *et al.*, 2000; Dang *et al.*, 2001). It was therefore important to establish any effect of the peptide on cell migration and PKC/ERK signalling in haemocytes. The different concentrations of fMLP used in the present study failed to induce *L. stagnalis* haemocyte migration. Also, this peptide did not enhance the phosphorylation status of PKC. The lack of migration towards fMLP could be due to a lack of fMLP receptors on the haemocyte surface. The PKC signalling pathway has been delineated in *L. stagnalis* haemocytes (See Chapter 2) and MEK has been found to lie downstream of PKC in laminarin-challenged haemocytes. Cell migration of *L. stagnalis* haemocytes appeared to be partly PKC-dependent as the PKC inhibitor GF109203X was able to particularly reduce haemocyte migratory activity with the greatest effect observed at 10 µM. The use of the MEK inhibitor U0126 (10 µM) also revealed that haemocyte migration is partially dependent on MEK-like proteins. Further studies would be crucial to establish whether ERK-like proteins in *L. stagnalis* haemocytes are involved in cell migration. The signalling pathways controlling *Drosophila* haemocyte migration include PI-3-K

(Wood *et al.*, 2006), but no evidence has yet been shown for the involvement of PKC and MAPK molecules. Partial inhibition of *L. stagnalis* haemocyte chemotaxis by cytochalasin (1 μM) confirmed the importance of the cytoskeleton in the migratory processes, similar to what has been observed in other molluscan species (Cheng and Howland, 1982); however, inhibition of haemocyte migration by this compound was expected to be more marked.

Integrins are cell adhesion molecules and adhesion receptors that are critical in many cellular immune processes. For example, cell spreading (Taddei *et al.*, 2007), cell migration (Ridley *et al.*, 2003) and the production of ROIs are mediated by integrins in macrophages (Berton and Lowell, 1999). The cytoplasmic signalling molecules focal adhesion kinase and Src colocalize at focal adhesion sites in mammalian systems (Schaller *et al.*, 1998). The FAK-Src complex is activated via integrin-binding and transduces signals through multiple routes (Mitra and Schlaepfer, 2006). In vertebrates, other signalling molecules such as PKC are known to be crucial to macrophage (Petty, 1989; Phaire-Washington *et al.*, 1980) and integrin-mediated muscle cell (Disatnik and Rando, 1999; Disatnik *et al.*, 2002) spreading. Specifically, studies have shown that translocation of PKCα and PKCε to focal adhesions occurred through integrin-mediated signalling (Haller *et al.*, 1998). Moreover, several reports have shown that FAK phosphorylation is mediated by PKC in platelets, ovary cells (Vuori and Ruoslathi, 1993; Haimovitch *et al.*, 1996), and in fibroblasts (Hunger-Glaser *et al.*, 2003). The presence of integrin-like $\alpha_v\beta_3$ and β_1 subunits has been reported in *L. stagnalis* haemocytes and their role in cell spreading on glass has been demonstrated (Plows *et al.*, 2006). The RGD-like binding receptors have also been identified in *B. glabrata* haemocytes and are involved in cell motility (Davids and Yoshino, 1998). In the study by Plows *et al.* (2006), integrin engagement also led to the phosphorylation of a FAK-like protein in haemocytes whereas ERK activity was not up-regulated following integrin binding. In the present study, spreading of *L. stagnalis* haemocytes was partly mediated by PKC and, more specifically PKCα as 10 μM Gö 6976 reduced spreading haemocytes by 41%. A slightly greater inhibition (64%) was observed with GF109203X implying that additional homologues of PKC isoform might be required for cell spreading by *L. stagnalis* haemocytes. Similarly, *Biomphalaria glabrata* haemocyte spreading might be PKC-dependent, because in the presence of 10 μM calphostin C, spreading of Bge cells on glass slides was reduced by 75% compared to the controls (Humphries *et al.*, 2001). In the current work, incubation of *L. stagnalis* haemocytes with pharmological inhibitors (ScrI and herbimycin A) of the tyrosine kinase Src resulted in a significant 30% reduction in the mean cell area, although some haemocytes appeared less affected than others. Haemocytes from the

insect *Malacosoma disstria* were shown to adhere to glass slides in a PKC- and PKA-dependent fashion (Giannoulis *et al.*, 2005) whereas similar kinases seemed to impair the adhesion of *Galleria mellonella* haemocytes (Zakarian *et al.*, 2003). The spreading of haemocytes from *Manduca sexta* is under influence of Ca^{2+} (Willot *et al.*, 2002). In mammals, the use of pharmological PKC inhibitors such as sphingosine or H-7 resulted in a decrease of the mean macrophage perimeter (Petty, 1989). The regulation of mammalian cell spreading by Src following integrin binding is currently controversial. Some *in vitro* studies suggest that fibroblast spreading may not require the scaffolding functions of Src (Cary *et al.*, 2002): also, in recent studies, Src and FAK were shown to be not required for integrin-mediated cell spreading and downstream activation of MAPK (Meng and Lowell, 1998). Interestingly, the role of ERK 1/2 in integrin-mediated signalling events is quite controversial in mammalian cells because some reports have demonstrated that integrin-mediated MAPK activation can occur in a FAK-independent manner (Lin *et al.*, 1997). In cytotoxic T lymphocytes, the binding of β1 or β3 integrins by antibodies did not lead to activation of ERK1 and ERK2 (Puente and Ostergaard, 2003). In eosinophils, fMLP triggered an increase of ERK 1/2 between 1-5 min compared to control, before declining over 15 min (Zhu *et al.*, 2001).

In the experiments described here, the Src family kinases, and additional signalling molecules such as PKC, were found to be involved in *L. stagnalis* haemocyte spreading; this suggests that these cells employ similar mechanisms to those of mammals to regulate spreading. The phosphorylation (activation) of *L. stagnalis* FAK-like and PKC-like proteins may also be dependent on Src, as revealed through the use of Src inhibitor SrcI and western blotting. Although herbimycin did not appear to affect PKC phosphorylation, it was also ineffective at altering PKC activity in mammalian fibroblasts (Fukazawa *et al.*, 1991).

In mammals, activation of PKC via tyrosine kinases does not appear to be a mechanism of activation universally observed, although some cPKC and nPKC have been shown to be catalytically activated by tyrosine phosphorylation (Konishi *et al.*, 1997). Clearly, in *L. stagnalis*, the observed effects of Src inhibition on PKC phosphorylation would be indirect and only further elucidation of *L. stagnalis* haemocyte signalling pathways will reveal the extent of the molecular cross-talk between enzymes. Interestingly, a Src homologue and FAK are present in *D. melanogaster* (Kussick and Cooper, 1992; Palmer *et al.*, 1999) and play a role in cell migration during embryogenesis (Fox *et al.*, 1999). The role of the FAK/Src complex has also been demonstrated in *E. coli* phagocytosis by *Ceratitis capitata* haemocytes (Metheniti *et al.*, 2001), however, studies are yet to investigate integrin-mediated

events. It would be particularly interesting to verify whether Src and FAK play a similar role in *L. stagnalis* haemocyte migration, and whether integrins are crucial to this process. A recent study has demonstrated that, in mammalian epithelial cells, PKC acts upstream FAK and Src and facilitates the formation of adherence junctions (Ozaki *et al.*, 2007). These results highlight the involvement of PKC in the integrin mediated-event previously described (Lewis *et al.*, 2003).

Chapter 6

Final Discussion

The aim of the research described in this thesis was to delineate the PKC pathway in molluscan haemocytes and to characterise some of the principal defence responses regulated by this pathway in these cells. *Lymnaea stagnalis* proved to be a suitable model in which to study PKC signalling in molluscan innate immunity. *Lymnaea stagnalis* was originally established as an animal model for the study of learning and memory (Lukioviak *et al.*, 2003), but it has become an excellent model to investigate snail-schistosome host-parasite interactions (Horák *et al.*, 2002) and, more recently, cell signalling in molluscan defence (Walker, 2006). The ease of snail maintenance and the ability to extract reasonably large quantities of primary haemocytes without killing the snail also make *L. stagnalis* ideal for studies on the cell biology of molluscan immunity. Many years ago, the *Biomphalaria glabrata* embryonic (Bge) cell line was established (Hansen, 1976) and the cells appear to possess similar defence functions to *B. glabrata* haemocytes (Yoshino *et al.*, 1999). However, the Bge cell line has been continuously maintained in the laboratory and the cells are probably physiologically less relevant. Another great advantage in working with *L. stagnalis* is that the amount of haemolymph that can be extracted from each snail is considerably greater than from *B. glabrata*.

Different questions have been addressed in the chapters of this thesis: is PKC present in *L. stagnalis* haemocytes? How is PKC signalling regulated in these cells? Which defence processes does PKC control and, how might these processes be important in a parasitological context? Following this study, other questions remain: how does PKC integrate with other uncharacterised signalling pathways in molluscan haemocytes? How conserved are PKC signalling mechanisms across the mollusca, and how can the results of this study be used in the future?

6.1 The PKC pathway in *L. stagnalis* haemocytes

Numerous studies on mammalian PKC have helped us to gain a comprehensive understanding of PKC's structure, enzymology and regulation (Newton, 1995; Parker and Murray-Rust, 2004). In this thesis, similarities between PKC activation and regulation were observed between mammalian immune cells and *L. stagnalis* haemocytes. The schematic diagram in Fig.6.1 summarises the research in this thesis, and shows the connections between signalling molecules in *L. stagnalis*

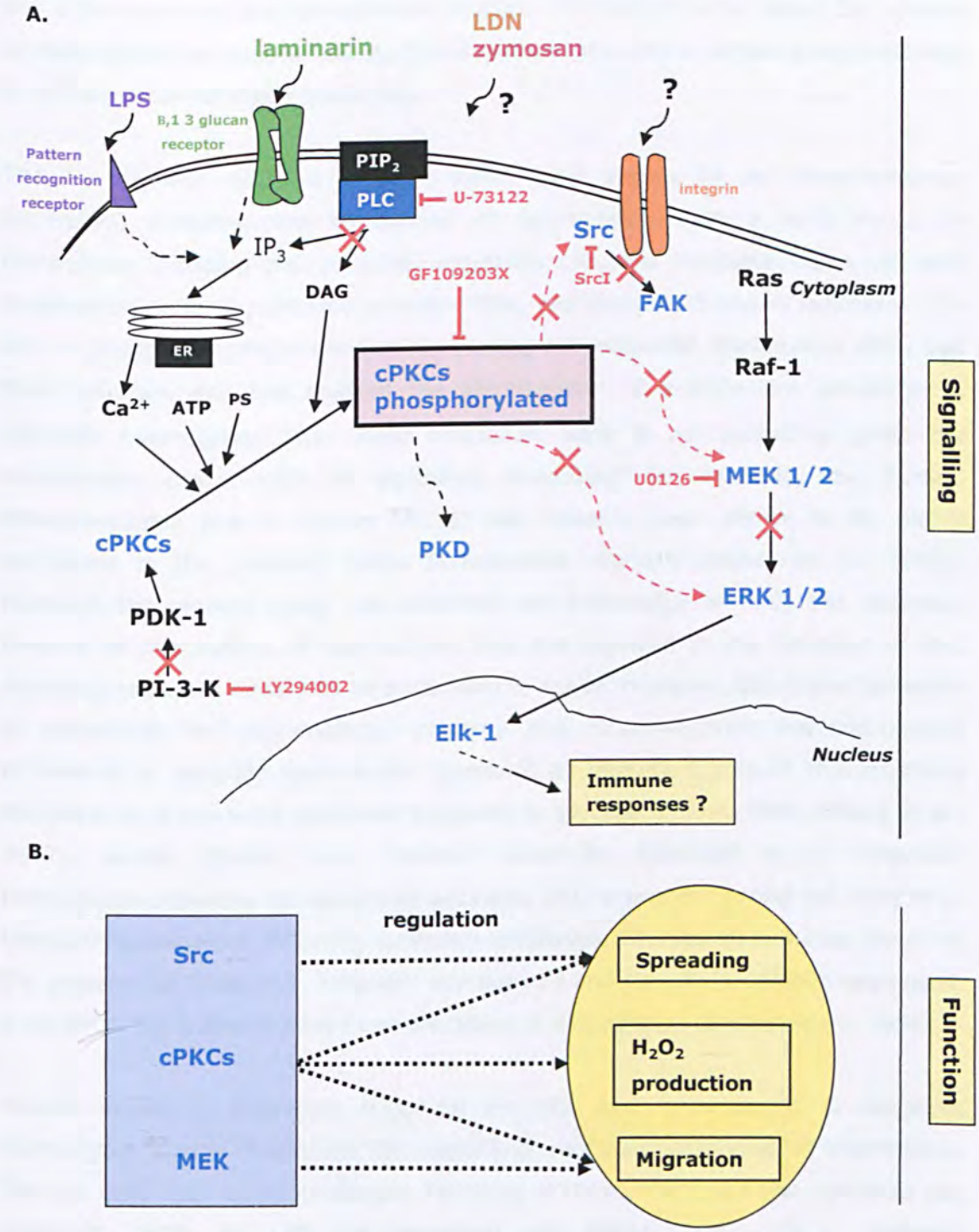


Fig.6.1: Summary of the studies on *L. stagnalis* haemocytes in this thesis

(A) Schematic diagram displaying the PKC-dependent signalling events in *L. stagnalis* haemocytes following laminarin, LPS and zymosan challenge. Pharmacological inhibitors (in red) prevent the activation of protein kinases and block further downstream signalling pathways (red crosses and dotted lines). Molecules in black are present in mammalian immune cells whereas molecules in blue have also been identified in *L. stagnalis* haemocytes; black arrows identifying downstream activation. (B) Defence responses in *L. stagnalis* haemocytes are regulated by Src, cPKCs and MEK according to the arrows.

haemocytes, determined through the use of pharmological inhibitors targeting PKC and other upstream and downstream kinases. The diagram also shows the various immune functions explored during this study, and the role of certain protein kinases in the regulation of these processes.

The *L. stagnalis* PKC-like proteins have been shown to be phosphorylated (activated) following the stimulation of haemocytes with a wide range of compounds including the bacterial endotoxin LPS, the insoluble yeast cell wall preparation zymosan, the PKC activator PMA, and the β-1, 3-glucan laminarin. The use of phosphospecific antibodies that recognise activated mammalian PKCs has been valuable, and has enabled the identification of a PKCα-like protein in *L. stagnalis* haemocytes. That these antibodies work is not surprising given the evolutionary conservation of signalling molecules; for example, the Ser660 phosphorylation site in human PKCβII has recently been shown to be highly conserved in the parasitic worm *Schistosoma mansoni* (Bahia *et al.*, 2006). Although the present study has extended our knowledge of PKC, the receptors present on the surface of haemocytes that are involved in the initiation of PKC signalling cascades remain to be elucidated in snails. However, the characterisation of transcripts for peptidoglycan proteins and Gram-negative bacteria-binding proteins in *B. glabrata* haemocytes represent an important step in characterising components of signalling pathways triggered by glycans in these cells (Zhang *et al.*, 2007); similar studies may hopefully soon be extended to *L. stagnalis*. Immunocytochemistry revealed that activated PKC is present in the cell body of *L. stagnalis* haemocytes following laminarin challenge. Studies should now focus on the presence of RACK in *L. stagnalis* haemocytes and its role in defence responses; such RACK-like proteins have been identified in *B. glabrata* (Lardans *et al.*, 1998).

Protein kinase C activation triggered by LPS and zymosan in *L. stagnalis* haemocytes partly resembles the signalling reactions occurring in mammalian defence cells such as macrophages following immune challenge (for zymosan see Underhill, 2003; for LPS see Heumann and Roger, 2002). In *L. stagnalis* haemocytes, the activation of PKC-like proteins in response to laminarin challenge was clearly demonstrated and ERK 1/2 was shown to be a downstream target of PKC, likely regulated through a MEK-dependent mechanism. Results also suggest that PKC activity is dependent on PLC activation, whereas PI-3-K does not seem be involved. In another mollusc, *Aplysia californica*, ERK activation in neurons was not linked to PKC (Nakhost *et al.*, 2002). We can then speculate that differences might exist among molluscan species, and/or tissue-specific effects occur. Since LDN and

fMLP failed to activate PKC, other pathways might be involved in response to these compounds or receptors for such compounds are absent on the surface of *L. stagnalis* haemocytes. Other research in molluscs has focused on two other MAPK pathways, the p38 MAPK and the SAPK/JNK pathways, since these pathways are also involved in the immune response of mammals (Yamamori *et al.*, 2000) and invertebrates such as *C. elegans* (Troemel *et al.*, 2006). In this context, Canesi and co-workers have demonstrated a role for the stress-activated p38 MAPK in the physiological response of mussel haemocytes to bacterial challenge (Canesi *et al.*, 2002). Activation of p38 MAPK in Bge cells has also been observed in response to ESPs from *S. mansoni* (Humphries and Yoshino, 2005). Interestingly, the SAPK/JNK pathway has been shown to be activated in *Mytilus galloprovinciallis* haemocytes in response to bacteria (Canesi *et al.*, 2005) and TNF-α (Betti *et al.*, 2006). It is conceivable that similar stress signalling pathways exist in *L. stagnalis* haemocytes and that they may be modulated by compounds such as laminarin. Comparative studies using *L. stagnalis* haemocytes would be valuable, allowing us to establish the degree of conservation of signalling mechanisms between molluscs.

Activation of cell signalling pathways by various molecules also occurs in schistosome parasites. As such, a few receptors have been identified in *S. mansoni*; these include the TGF-β receptor II (Osman *et al.*, 2006) and nuclear receptors (de Mendonca *et al.*, 2000). Protein kinase C has also been identified in the tegument and the acetabular gland of *S. mansoni* (Wiest *et al.*, 1992; Bahia *et al.*, 2006) and new challenges for parasitology would be to determine the extent to which signalling pathways are modulated between the snail host and the parasite during host-parasite interplay.

6.2 PKC signalling and functional defence responses of *L. stagnalis* haemocytes

Dissection of the PKC pathway in *L. stagnalis* haemocytes facilitated investigations into the role of this pathway in various immune functions including cell spreading, migration and the production of ROIs. These studies were carried out using pharmological inhibitors that were shown to block PKC activation. It can be concluded that both *L. stagnalis* haemocyte spreading and the production of ROIs appear to be PKC-dependent. The generation of H_2O_2 following laminarin challenge and the spreading behaviour of haemocytes on glass coverslips are likely to be

mediated by the PKCα-like protein. Additionally, the spreading activities are dependent on Src-like activation, although Src has not yet been characterised in snail haemocytes. The results obtained from the haemocyte migration assays were surprising as the reduction of haemocyte migration by PKC and MEK inhibitors was minimal. Laminarin was not used as a chemoattractant in migratory experiments, maybe this would warrant further investigation. Although similarities in migration mechanisms between vertebrate and mollusc immune cells have been observed, we cannot exclude potential evolutionary discrepancies between species.

Nitric oxide and H_2O_2 have been defined as signalling molecules and have been shown to initiate signalling cascades in mammalian cells (Suzuki *et al.*, 1997). In molluscs, similar phenomena have been observed (Nappi and Ottaviani, 2000). Hydrogen peroxide is known to modulate PKC activity in mammalian endothelial cells (Taher *et al.*, 1993); it is therefore possible that H_2O_2 physiologically contributes to the activation of the PKC pathway in *L. stagnalis* haemocytes.

The use of the glycan epitopes, LDN and Le^X as potential elicitors of PKC signalling in *L. stagnalis* haemocytes was explored; however, these compounds did not affect PKC phosphorylation. As components of the schistosome surface coat, it would be particularly interesting to investigate the effect of LDN and Le^X on the generation of ROIs and RNIs in *L. stagnalis* and to ascertain whether or not such molecules are critical in helping schistosomes subvert the defence mechanisms of their snail intermediate hosts. Apparently, *S. mansoni* may escape the immune defence of its vertebrate host by diminishing the levels of hydrogen peroxide produced by the infected mice (Smith *et al.*, 1989). Whether a similar phenomenon exists in snails should certainly be explored. Further research is also required to establish the effects of other schistosome components on the molluscan defence response: compounds secreted or excreted by the parasite are likely to be important in modulating the activities of the snail defence system (Walker, 2006).

Spreading of *L. stagnalis* haemocytes relies on both PKC and Src-like proteins. Haemocyte spreading may also be dependent on FAK as Src inhibitors slightly impaired the activation of FAK; this is supported by the recent work done by Plows *et al.* (2006) linking integrins and FAK activation. Other work done by Plows *et al.* (2006) has also explored the mechanisms underlying phagocytosis following integrin engagement in *L. stagnalis* haemocytes. In endothelial cells, FAK phosphorylation is dependent on PKC and F-actin (Zhang *et al.*, 1996) and relies on Ca^{2+} when cells are stimulated with collagen (Achison *et al.*, 2001). The work in this

thesis is the first to show a possible link between FAK phosphorylation (activation) and Src in snail haemocytes. However, further work is crucial to picture the global connections between PKC, FAK and Src and their role in defence responses such as phagocytosis of bacteria and encapsulation of parasites.

6.3 Concluding remarks

Overall, the findings of this thesis have demonstrated an important role for PKC in the mediation of key innate immune responses in *L. stagnalis*. Belonging to the same molluscan class (gastropoda) as *B. glabrata*, *L. stagnalis* is considered as a model organism and further molecular investigation on this snail would be worthwhile. This is particularly important since our knowledge of the biology of the *Lymnaea-Trichobilharzia* snail-schistosome model is considerable (Horák *et al.*, 2002). Numerous studies have investigated the molecular biology of *B. glabrata* as it is the major intermediate host of *S. mansoni*. Various gene libraries have already been established and collaboration between different scientific laboratories aims to determine the whole genome sequence for *B. glabrata* (Knight *et al.*, 2000; Raghavan *et al.*, 2003; Mitta *et al.*, 2005 and http://biology.unm.edu/biomphalaria-genome/index.html). A first microarray manufactured commercially has been developed for schistosomes, based on expressed sequence tags (EST) known for *S. mansoni* and *S. japonicum* (Gobert *at al.*, 2005). Although less research effort has been directed towards the molecular biology of *L. stagnalis*, recent EST projects in this snail have revealed genes that might be of interest to our study of snail-schistosome in host-parasite relationships (Davidson and Baxter, 2005).

Proteomics with the help of the protein microarray techniques represents a valuable tool to analyse direct products of genes and changes in protein levels, therefore bringing new insights in the expression of proteins in a particular organism under normal and pathological conditions (Templin *et al.*, 2002). As the full genome sequence of *B. glabrata* and other snails is still incomplete, the investigation of expression levels following host-parasite interplay must rely on other techniques. Such a technique could comprise two-dimensional gel electrophoresis followed by mass spectrometry. A model study has been performed with *B. glabrata* to identify some of the proteins differentially expressed in *Echinostoma caproni*-susceptible and -resistant snails (Bouchut *et al.*, 2006). A similar approach could be used in *L.*

stagnalis; for example, it would be interesting to determine the effects of ESPs from *T. ocellata* on the protein expression profile of *L. stagnalis* haemocytes.

The RNA interference (RNAi) technique has been used to down-regulate the generation of NO by disrupting the synthesis of the NO synthase in *L. stagnalis* which correlates with the reduction of the feeding behaviour of adult snails (Korneev *et al.*, 2002). This technique could be a valuable to research on molluscan immunity and knock-down of PKC in *L. stagnalis* by RNAi could enable the importance of this enzyme to defence responses to be firmly established *in vivo*.

Investigation of the effects of *T. ocellata* ESPs on PKC signalling in *L. stagnalis* haemocytes would be useful since such molecules could block PKC activation and this blunt certain defence response. In addition, it would be interesting to look at the biochemical molecules and signalling proteins in *L. stagnalis* which may control other defence processes such as encapsulation. Another study investigated the encapsulation of *S. mansoni* sporocysts by *L. stagnalis* haemocytes and found that this was mediated by MAPK and PI-3-K signalling (Zelck *et al.*, 2006). Exploring further the encapsulation response would determine additional signalling molecules that contribute to important anti-parasite defence reactions.

To conclude, improving our knowledge of haemocyte signalling pathways and particularly the involvement of PKC as a signal transducer is crucial for a better comprehension of the molecular mechanisms that regulate molluscan defence. The understanding of PKC signalling in *L. stagnalis* gained from the present study will facilitate further research in this and related organisms and will help researchers to determine evolutionary conservation of cell signalling pathways between phyla. Knowledge of signalling in *C. elegans* (Stern and DeVore, 1994; Kim *et al.*, 2004; Kondo *et al.*, 2005) and *D. melanogaster* (Tanji and Ip; 2005) is extensive compared to molluscs. However, by taking a comparative approach, it is possible to identify potential signalling pathways for further study in molluscs, some of these might be crucial to snail defence against trematode parasites such as *S. mansoni*.

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Appendices

Appendix I

Conference abstracts

Lacchini, AH; Davies, AJ; Mackintosh, D; Walker, AJ (2007) Release of reactive oxygen intermediates by *Lymnaea stagnalis* haemocytes: a pivotal role for protein kinase C. *The Malacologist*, 48, 10. Molluscan Forum, London, November 2006.

Lacchini, AH; Davies, AJ; Mackintosh, D; Walker, AJ (2006) PKC signalling in molluscan immune response. *The Malacologist*, 46, 14. Molluscan Forum, London, November 2005.

Lacchini, AH; Davies, AJ; Mackintosh, D; Walker, AJ. Release of reactive oxygen intermediates by *Lymnaea stagnalis* haemocytes: a pivotal role for protein kinase C. Poster presented at the International Congress of Parasitology (ICOPA XI), Glasgow, August 2006.

Lacchini, AH; Davies, AJ; Mackintosh, D; Walker, AJ. Protein kinase C (C) signalling in molluscan defence cells. Poster presented at the British Society for Parasitology, Nottingham, April 2005.

Release of reactive oxygen intermediates by *Lymnaea stagnalis* haemocytes: a pivotal role for protein kinase C

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In molluscs, reactive oxygen intermediates (ROI), produced by macrophage-like cells called haemocytes, play a major role in innate immune defence and participate in the elimination of pathogens such as parasites. The NADPH oxidase enzyme, responsible for the successive formation of cytotoxic oxidative molecules including hydrogen peroxide (H_2O_2), is in part activated by protein kinase C (PKC) in mammalian phagocytes. We have reported the existence of PKC-like protein(s) in haemocytes from the freshwater snail *L. stagnalis*, host for the avian schistosome *Trichobilharzia ocellata*, and have found that the activity of this signalling enzyme is modulated following challenge with lipopolysaccharide (LPS) and laminarin, a β-1, 3- glucan that occurs in fungal cell walls. The purpose of the present study was to investigate the generation of H_2O_2 by *L. stagnalis* haemocytes, and to determine whether or not the molecular mechanisms underlying "the respiratory burst" involve the PKC pathway. Laminarin promoted extracellular H_2O_2 output by *L. stagnalis* haemocytes in a dose- and time-dependent manner, with 10 mg/ml laminarin stimulating H_2O_2 production - approximately 5-fold. The PKC inhibitor, GF109203X (10 μM), significantly attenuated laminarin-dependent H_2O_2 production by 65 % ($P \leq 0.001$); moreover, the NADPH oxidase inhibitor, apocynin (500 μM), reduced stimulated H_2O_2 levels by 57 % ($P \leq 0.001$). These results demonstrate that PKC is at least in part responsible for the synthesis of ROI by molluscan haemocytes following immunological challenge and is therefore likely to be important in snail anti-parasite responses.

PKC signalling in the molluscan immune response

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Although molluscan biology has been studied for many years, little is currently known about the molecular mechanisms that regulate the immune system in molluscs thus preventing infection. This is particularly surprising given that molluscs serve as intermediate hosts to a range of trematode parasites including species of *Schistosoma* and *Fasciola* that cause the diseases schistosomiasis and fascioliasis.

In mammals, the protein kinase C (PKC) family consists of 11 isoforms. Some of them play a key role in a range of biological innate immune responses including phagocytosis, the production of reactive oxygen intermediates, and the release of nitric oxide.

The overall goal of my study is to elucidate the role of the PKC signalling pathway in haemocytes, macrophage-like defence cell of the freshwater snail *Lymnaea stagnalis*, which is host to the avian schistosome *Trichobilharzia ocellata*. The study will provide a better understanding of PKC modulation following haemocyte challenge and will also bring new insights into PKC isoform-dependent immune responses in *L. stagnalis*.

Protein kinase C (PKC) signalling in molluscan defence cells

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Lymnaea stagnalis is the intermediate host for the avian schistosome *Trichobilharzia ocella*. We have recently shown that carbohydrate moieties known to be present on the schistosome surface affect protein kinase (PKC) signalling and downstream functional responses in *L. stagnalis* defence (haemocytes) (Plows et al, 2005). Given the role of the PKC pathway in mammalian innate immunity and the conservation of cell signalling through evolution, it is now important to further characterise PKC signalling in molluscan haemocytes and define its role in the molluscan innate defence response. When haemocytes are challenged with laminarin, a β-1,3-glycan present in fungal cell walls, a PKC-α like protein is activated transiently over 30 minutes. Immunocytochemistry has revealed the localisation of active PKCα within haemocytes and specific PKC inhibitors (e.g.GF109203X) are able to block PKC activation. Studies now aim to define upstream regulators and downstream targets of PKC in haemocytes. Future work will also focus on the effects of schistosome coat components on the regulators of the PKC pathway.

Appendix II

Peer-reviewed publications arising from PhD

Lacchini, AH; Davies, AJ; Mackintosh, D; Walker, AJ (2006) 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. *The Journal of Experimental Biology*, **209**: 4829-4840.

Wright, B; **Lacchini, AH**, Davies, AJ; Walker, AJ (2006) Regulation of nitric oxide production in (*Lymnaea stagnalis*) defence cells: a role for PKC and ERK signalling pathways. *Biology of the Cell*, **98**: 265-278.