Studies on the Extracellular-signal Regulated Kinase Pathway in *Lymnaea stagnalis* Haemocytes and its Role in Molluscan Defence

Louise Danielle Plows

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February, 2005

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I have composed this thesis myself, and all results presented within this thesis are from my own investigation.

Louise Plows
February, 2005

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Abbreviations

$4,5\text{-}PIP_2$	Phosphatidylinositol-4,5-bisphosphate
ACTH	Adrenocorticotropin hormone
ADP	Adenosine diphosphate
AMP	Adenosine monophosphate
ANOVA	One-way analysis of variance
APS	Ammonium persulphate
Arg	Arginine
Asp	Aspartine
ATP	Adenosine triphosphate
BSA	Bovine serum albumin
cAMP	Cyclic adenosine monophosphate
CD	Cluster of Differentiation
DAG	Diacylglycerol
DC	Detergent compatible
DMSO	Dimethyl sulphoxide
Ds-RNA	Double stranded ribonucleic acid
ECL	Enhanced chemiluminescence
EGF	Epidermal growth factor
EGFR	Epidermal growth factor receptor
EMSA	Electrophoretic mobility shift assay
ERK	Extracellular-signal Regulated Kinase
FAK	Focal adhesion kinase
FITC	Fluoroisothiocyanate
FTase	Farnesyltransferase
GalNAc	N-acetylgalactosamine
GAP	GTPase activating protein
GDP	Guanine diphosphate
GlcNAc	N-acetylglucosamine
Gly	Glycine
GNBP	Gram negative bacteria binding protein
GNRP	Guanine nucleotide releasing factor

GPCR	G protein coupled receptor
GTP	Guanine triphosphate
GTPase	Guanine triphosphatase
HPO	Haemocyte-producing organ
HRP	Horseradish peroxidase
IFN	Interferon
IL	Interleukin
iNOS	Inducible nitric oxide synthase
JNK	c-Jun NH_2-terminal kinase
LBP	Lipopolysaccharide binding protein
LPS	Lipopolysaccharide
MAPK	Mitogen-actived Protein Kinase
MARCKS	Myristolyated alanine-rich C kinase substrate
MEK	Mitogen-activated Protein Kinase Kinase
MGD1	*Mytilus galloprovincialis* defensin 1
MKK	Mitogen-activated protein kinase kinase
MKP	MAP kinase phosphatase
NF-κB	Nuclear factor κ B
NGF	Nerve growth factor
NK Cell	Natural killer cell
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PI	Phosphatidylinositol
PI-3K	Phosphatidylinositol 3 kinase
PKA	Protein kinase A
PKC	Protein kinase C
PLC	Phospholipase C
proPO	Prophenoloxidase
proPO-AS	Prophenoloxidase-activating system
RGDS	Asganine-Glycine-Aspartine-Serine
RNA	Ribonucleic acid
RNAi	Ribonucleic acid interference
RTK	Receptor tyrosine kinase

SAPK	Stress activated protein kinase
SDS	Sodium dodecyl sulphate
SDS-PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
SEM	Standard error of the mean
Ser	Serine
SH	Src homology
SSS	Sterile snail saline
TBS	Tris buffered saline
TEMED	N,N,N',N'-tetramethylethylenediamine
TGF	Transforming growth factor
Thr	Threonine
TLR	Toll-like receptor
TNF	Tumour necrosis factor
TTBS	Tris buffered saline – Tween 20
Tyr	Tyrosine

Abstract

Haemocytes, the main type of circulating defence cell in invertebrates, functionally resemble mammalian macrophages, and are responsible for phagocytosis, endocytosis and other innate immune functions. In mammalian macrophages, the extracellular-signal regulated kinase (ERK) pathway has been shown to modulate immune responses. It was therefore hypothesised that this pathway may play a similar role in haemocytes of the gastropod mollusc, *Lymnaea stagnalis*, and intermediate host of the schistosome, *Trichobilharzia ocellata*. Two ERK-like proteins were detected by Western blotting using antibodies that recognise non-phosphorylated and phosphorylated forms of mammalian ERK 1/2; these proteins had approximate molecular weights of 44 and 43kDa respectively. In addition, the upstream regulator of ERK, MEK, was detected in haemocytes as well as the transcription factor downstream of activated ERK, Elk-1. By employing a mitogen-activated protein (MAP) kinase assay kit, the identified ERK-like proteins were found to possess kinase activity. When haemocytes were challenged with *E. coli* lipopolysaccharide (LPS), the activity of the ERK pathway was found to be significantly upregulated after 5 min challenge and perinuclear migration of phosphorylated ERK was observed by immunocytochemistry. Other compounds, including Zymosan A, adrenocorticotropin hormone, noradrenaline and challenge with heat-killed *E. coli* did not modulate haemocyte ERK pathway activity. Pharmacological inhibitors used to block signalling to the ERK pathway demonstrated that MEK, PKC, PI-3K and Ras activity are vital for phagocytosis by haemocytes. In addition, the integrin subunits $\alpha_V\beta_3$ and β_1 were found to be present on the haemocyte surface and integrin engagement was found to be necessary for phagocytosis. Monosaccharides found on the surface coat of *T. ocellata*, fucose and galactose, were applied to haemocytes in the presence and absence of haemolymph. These sugars proved to have important immunomodulatory effects, since ERK, PKC and phagocytic activity were all affected by these sugars, and differences in experiments with and without haemolymph suggest an important role for serum proteins in the molluscan immune response. In conclusion, this study provided an insight into the signalling machinery involved in the molluscan defence response, and the results should stimulate further research in snail defence responses, particularly following challenge by parasites.

CHAPTER ONE

INTRODUCTION

1.1 VERTEBRATE DEFENCE RESPONSES

The concept of cellular immunity was first reported by the Russian zoologist, Elie Metchnikoff (1845 – 1916). During his career, Metchnikoff studied mammalian leukocytes, and discovered their ability to engulf microorganisms – a process he later termed phagocytosis. Over a century later, two types of immunity are known to exist, innate and acquired, and a concerted action of both cells and humoral molecules is needed for an efficient immune response. The development of biochemical and molecular tools and their application to the study of immunity has enabled a detailed picture of the vertebrate immune system to be drawn.

1.1.1 Cells involved in vertebrate innate immunity

The first line of defence in vertebrates is the innate immune system, which is capable of responding rapidly to infection, although with little specificity. A number of cell types constitute this innate system, with the majority being neutrophils (also referred to as polymorphonuclear cells). Upon infection, neutrophils leave the blood and enter tissues by following chemical gradients (chemotaxis) where they destroy invading micro-organisms. Neutrophils generally survive for only a few days since the cells are non-dividing and self-destruction often occurs with bacterial cell lysis.

Approximately, 1.5% of the population of mammalian white blood cells are eosinophils, which possess only limited phagocytic activity but contain destructive enzymes within cytoplasmic granules. The major contribution to defence by eosinophils is against larger foreign invaders such as parasites (reviewed by Dombrowicz & Capron, 2001; Klion & Nutman, 2004). Eosinophils have been linked to allergic responses, which could be due to 'cross-talk' (bi-directional regulation) with mast cells, another cell type involved in the innate immune response (reviewed by Piliponsky *et al.*, 2001). The cytokine, tumour necrosis factor α (TNFα), has recently been demonstrated to play a key role in the longevity of eosinophils (Temkin & Levi-Schaffer, 2001), which is vital to enable the cells enough time to penetrate into inflamed tissue before exocytosing destructive enzymes.

Natural killer (NK) cells, also part of the innate immune response, are capable of destroying the body's own virus infected or aberrant cells which could otherwise go on to form tumours. The primary role of NK cells is to provide an initial defence against pathological organisms during the time that the adaptive immune response is being 'formed'. NK cells are especially important in immunity to viruses (French & Yokoyama, 2003), and are capable of releasing cytokines which have a significant role in the noncytolytic control of viral infections (Guidotti & Chisari, 2001).

Platelets also play an important role in innate immunity by releasing important mediators during blood clotting, thereby activating the complement system and attracting mast cells, macrophages, and dendritic cells to the site of trauma (reviewed by Weyrich & Zimmerman, 2004). Mast cells and basophils are morphologically similar granulocytes, which release their granules as a result of binding to anaphylatoxins, allergens or lectins. Although present at very low amounts in the vertebrate circulatory system (0.2% of all granular leukocytes), mast cells play a very important role in defence against bacterial pathogens (Leal-Berumen *et al.*, 1994) and viruses (Malone *et al.*, 2001; King *et al.*, 2002). Mast cells release proteases, which are important for the recruitment of neutrophils and eosinophils (Huang *et al.*, 1998; Schmidlin *et al.*, 2002) and also express antimicrobial peptides (Di Nardo *et al.*, 2003). Complement components are especially important in regulating mast cell activity, since these cells possess multiple receptors for complement pathway molecules (Nilsson *et al.*, 1996; Prodeus *et al.*, 1997). Furthermore, complement binding gives mast cells the ability to intercept and phagocytose bacteria in a similar way to macrophages (Sher *et al.*, 1979; Malaviya *et al.*, 1994). Basophils, however, are recruited from peripheral blood during late phase responses and are responsible for the release of mediators involved in allergic inflammation (Lichtenstein & Bochner, 1991). Stimulation of basophils results in the exocytosis of inflammatory mediators which can lead to severe anaphylactic shock.

Dendritic cells are three types: Langerhans cells; interdigitating cells; and follicular dendritic cells. All three act at the interface between innate and adaptive immunity (reviewed by Castellano *et al.*, 2004a). These cells are able to interact with microbial associated molecules (e.g. lipopolysaccharide (LPS)) which results in cell activation leading to the induction of primary immune responses with the establishment of

immunological memory (Banchereau & Steinman, 1998). Dendritic cells are also important in the removal of apoptotic host cells (Nauta *et al.*, 2003), and although these cells are capable of phagocytosis, their phagocytic responses is less efficient than that of monocytes or macrophages (Netea *et al.*, 2004). An important interaction between complement and dendritic cells has recently been identified, in that immature dendritic cells are capable of producing the complement component, C1q (Castellano *et al.*, 2004b).

Constituting approximately 5% of white blood cells, monocytes provide a very effective phagocytic defence. Once mature, monocytes circulate in the blood before migrating into tissues where they subsequently enlarge and develop into macrophages. Macrophages are the largest phagocytic cells in vertebrates, they possess significant amounts of rough endoplasmic reticulum and mitochondria, and are very effective at destroying 'foreign bodies'. Microbes are destroyed by being engulfed and subsequently attacked by digestive enzymes and reactive forms of oxygen and nitrogen produced within the macrophage (Roos *et al.*, 2003). When challenged with gram-negative bacteria, it is the LPS (endotoxin) coat that stimulates and activates macrophages which then subsequently deactivate the LPS molecule Rutenburg *et al.*, 1960). Macrophages are present throughout the connective tissue and at the basement membrane of small blood vessels, particularly in the lungs (alveolar macrophages), liver (Kupffer cells), lymph node medullary sinuses (where they filter foreign material) and at various other locations such as the brain (microglia) and bone (osteoclasts).

1.1.2 Mechanisms of pathogen recognition

Macrophages express a large repertoire of cell surface receptors which recognise conserved patterns unique to microbial surfaces (reviewed by Heumann & Roger, 2002; Weber *et al.*, 2003). Activation of these pattern recognition receptors (PRR) on the macrophage causes the rearrangement of the actin cytoskeleton enabling the subsequent internalisation of the particle.

Two types of CD14, membrane bound (mCD14) and soluble (sCD14), exist and each is able to interact directly with the bacterial endotoxin, LPS. CD14 recognises various sugar or glycolipid motifs from bacteria, and therefore acts in a similar way to lectins.

mCD14 is embedded in the plasma membrane of myelomonocytic cells through a glycerophosphate inositol (GPI) anchor. Soluble CD14 results from either the shedding of mCD14 from cells, or the production of non-GPI anchored CD14 molecules. sCD14 interaction with LPS is known to be accelerated by LPS binding protein (LBP) (Hailman *et al.*, 1994), and sCD14 can also effect the transfer of LPS to high density lipoproteins (HDL) for neutralisation.

Primarily a lipid transfer molecule, the LPS binding protein (LBP), is present in plasma and transforms LPS to monomers for binding to CD14 receptors (Thomas *et al.*, 2002). LBP mediates the movement of phospholipids, in particular LPS monomers to membrane bound CD14 (mCD14) and soluble CD14 (sCD14) and phospholipids such as HDL. The transfer of LPS to CD14 results in cell activation (through the CD14 receptor) or through the neutralisation of LPS by HDL. Kinetic studies have demonstrated that LPS/LBP complex binding to CD14 occurs before the neutralisation of LPS via HDL, which suggests that LPS activates immune cells before it is neutralised (Yu & Wright, 1996).

Toll-like Receptors (TLRs) have an extracellular leucine-rich repeat domain, a cysteine rich cytoplasmic domain that mediates intracellular signal transduction, and the Toll/interleukin-1 receptor (IL-1R) homology domain. Toll-like receptors were first identified in *Drosophila melanogaster* (Hashimoto *et al.*, 1988) and the first human homologue was cloned in 1997 (Medzhitov, 1997). One TLR that is important in the induction of cytokines in vertebrates is TLR4, which also binds LPS and affects intracellular signalling pathways. Different toll proteins are able to recognise molecular patterns of pathogens and distinguish between different pathogen groups (Underhill & Ozinsky, 2002). It is now thought that different TLRs discriminate between the major molecular signatures of pathogens, including peptidoglycan, teichoic acids (Gram-positive bacteria), LPS (Gram-negative bacteria), arabinomannans and glucans. Although TLRs are considered to be primarily involved in macrophage intercellular signalling in response to gram-negative LPS (reviewed by Dobrovolskaia & Vogel, 2002), TLR2 has become increasingly linked with the recognition of Gram-positive bacteria (reviewed by Weber *et al.*, 2003b). TLRs not only signal the presence of a pathogen, but also trigger expression of co-stimulatory molecules and effector

cytokines and thus prepare the cell for its involvement in the development of the adaptive immune response.

A group of transmembrane cell-surface molecules known as scavenger receptors mediate binding and internalisation of microbes, along with certain modified, damaged or apoptotic self-cells (reviewed by Mukhopadhyay & Gordon, 2004). Indeed, scavenger receptors have an important role in the recognition of teichoic acid on Gram-positive bacteria. Scavenger receptors are expressed on macrophages and dendritic cells, and have the ability to recognise multiple ligands and regulate a large range of cellular functions (reviewed by Gough & Gordon, 2000). They are capable of binding bacterial, viral, fungal or parasitic coats as well as soluble or particulate ligands. Many scavenger receptors exist, with one such receptor (SR-A) having specificity for the lipid A component of lipopolysaccharide and of lipoteichoic acid associated with bacteria. SR-A has been proven to be involved in the recognition of parasitic infections when SR-A negative mice were infected with the murine form of malaria (Nogami *et al.*, 1998).

The mannose receptor, another transmembrane receptor expressed on macrophages, dendritic cells and subsets of endothelial cells, has eight carbohydrate recognition domains (CRDs) that allow it to bind to a broad range of ligands (reviewed by Stahl & Ezekowitz, 1998; Allavena *et al.*, 2004). The Ca^{2+}-dependent, mannosyl/fucosyl recognition pattern permits it to interact with a variety of pathogens that enter through mucosal surfaces. Because the mannose receptor is expressed on macrophages throughout the body, it is likely to be one of the first innate receptors to interact with microbes, which, once bound, are internalised and degraded by endosomes. This receptor is extremely important as it mediates phagocytosis (demonstrated by Koziel *et al.*, 1998), by binding to opsonins, and the destruction of microbes even before the adaptive immune response is triggered.

1.1.3 Molecules of innate immunity

Clearly, the innate immune system comprises many cellular components that give rise to the characteristic rapid immune response following infection by bacteria or larger

organisms, such as parasites. However, not only cellular responses are involved. Many molecules interact with immune cells to facilitate an efficient immune response.

1.1.3.1 Complement

Innate immunity also involves the complement system, which consists of over 20 inter-dependent proteins that undergo sequential activation to mediate protection against infection. These proteins are synthesised by hepatocytes and monocytes and can be activated through either 'classical', 'alternative' or the more recently discovered 'lectin' pathways (Fig 1.1.1; Matsushita & Fujita, 1996). Molecules associated with microorganisms activate the alternative pathway; while antibodies bound to a microbe or antigen activate the classical pathway. The lectin pathway is activated upon carbohydrate recognition by mannose-binding proteins and ficolins (pattern recognition molecules). C3 is the central component of the complement system and is equipped with a unique thioester bond that is exposed to the molecular surface upon activation; it then forms a covalent bond with hydroxyl or amine groups in cell surface molecules present on the invading microorganism (Law *et al.*, 1980).

The complement system facilitates phagocytosis and mediates an acute inflammatory reaction in vertebrates. Mast cells play a central role in the complement pathway, releasing mediators that, together with complement components, also recruit polymorphonuclear phagocytes and further plasma complement components to sites of microbial infection.

The major functions of the complement system are to initiate inflammation by direct activation of mast cells (components C3a and C5a), to attract neutrophils (by chemotaxis) to the site of microbial attack (C5a), to enhance attachment of the microbe to the phagocyte (opsonisation; C3b) and to kill the microbe (lysis; C9; MAC). The ultimate aim of complement is, however, the formation of a membrane attack complex (MAC or C5b-9 complex) which disrupts and forms a pore in the phospholipid bilayer in order to lyse cells (Morgan, 1989). Some viruses and bacterial pathogens, however, take advantage of the complement system by binding to complement receptors to facilitate their entry into cells and gain shelter from innate and adaptive immunity (Speth *et al.*, 2002).

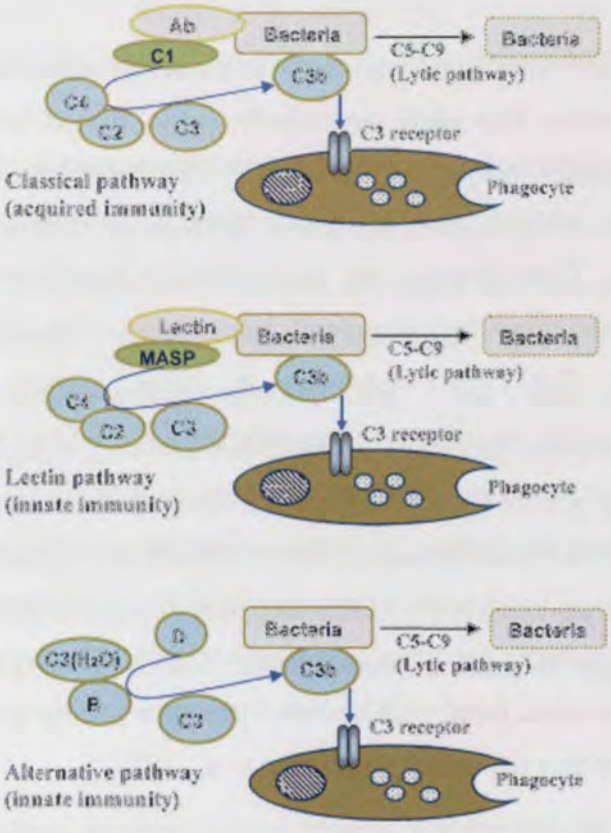

Figure 1.1.1. The complement pathway. The 20 proteins comprising the complement pathway opsonise pathogens to aid phagocytosis/endocytosis by cells of the innate immune response. The alternative, classical (acquired immunity triggered by antibody (Ab) binding to the complement component C1) and lectin pathways are shown. All pathways involve the complement components C3, C3b and C5 – C9, whilst the lectin pathway also involves the MBL-associated serine protease (MASP). Diagram is taken from Fujita *et al.* (2004).

1.1.3.2 Acute phase proteins

Acute phase proteins are also important in the innate defence of vertebrates, mainly acting against bacteria and protozoa. These proteins also act to limit tissue damage and trauma. Acute phase proteins include C-reactive proteins (CRP), complement components, opsonic proteins (such as mannose-binding protein (MBP)), metal-binding proteins and protease inhibitors (reviewed by Murata *et al.*, 2004). The proteins are mainly produced by the liver and are either produced *de novo,* or are present at low levels which rapidly increase following infection. The proteins are produced by

hepatocytes in response to the cytokines IL-1, IL-6, TNFα and IFNγ, which are released by activated macrophages and NK cells. The acute phase proteins have many functions, the most predominant of which is to maximise activation of the complement system and opsonisation of microbes. C-reactive proteins are known to specifically bind to the phosphatidylcholine (PC) moiety of pneumococcal C-polysaccharide in a calcium-dependent manner (Volanakis, 2001), although recently, protein A specific binding has also been reported (Das *et al.*, 2004).

1.1.3.3 Cytokines

Another group of molecules involved in innate immunity are the cytokines, which can induce growth, differentiation, chemotaxis, and activation/enhanced cytotoxicity. Cytokines are classified according to the cell that they are released from. Interleukins (ILs) are predominantly produced by leukocytes, monokines by myeloid cells, lymphokines by lymphocytes, with interferons (IFNs) being produced by a wide variety of cells in response to viral infection.

Divided into two sub-groups, type I IFN (α and β) and type II IFN (γ; also referred to as immune IFN) interferons can mediate protection against viral infection. IFN α and β are produced by many cell types in response to viral or bacterial infection, especially when cells are invaded by intracellular bacteria (Poston & Kurlander, 1992). At least 12 different homologous species of IFNα are produced, primarily by infected leukocytes, as well as by epithelial cells and fibroblasts (Zhao *et al.*, 1998). In contrast, a single species of IFN β exists and is normally secreted by fibroblasts and epithelial cells. The proinflammatory cytokines, IL-1 and TNF α, are potent inducers of IFN α and β secretion, as are endotoxins derived from the cell wall of gram-negative bacteria (Poston & Kurlander, 1992; Hunter *et al.*, 1995).

Monokines have many different local and systemic activities that are critical to immune defence. As a result of a particular stimulus, including ingestion of Gram-negative bacteria and subsequent activation by LPS, macrophages secrete many interleukins and TNFα. In particular, TNFα, IL-1 and IL-6 increase body temperature, lymphocyte activity, mobilisation of neutrophils for phagocytosis, and induce the release of acute phase proteins that are involved in complement activation and opsonisation (Simms *et*

al., 1991). IL-8 (produced by leukocytes upon LPS stimulation) is stimulated by integrin binding, which aids the binding of neutrophils to epithelial cells and migration into tissues (Garcia-Velasco & Arici, 1999). In addition, TNFα, IFNγ and LPS all stimulate macrophages to produce a key immunomodulatory molecule, nitric oxide, *via* activation of inducible nitric oxide synthase (iNOS), (Fecker *et al.*, 2002). Mast cells have been shown to produce IL-6 when induced by bacterial LPS (Leal-Berumen *et al.*, 1994), and monokine production is elevated in patients with infection and cancer (Shin *et al.*, 2003).

Chemokines, of which there are more than 50, are small closely-related cytokines involved in chemoattraction of lymphocytes, monocytes and neutrophils. They are primarily produced by monocytes/macrophages, but also by platelets and some endothelial cells in response to infection or physical damage. Chemokines direct cells to the source of infection/damage by promoting chemotaxis. The receptors for chemokines are G-protein coupled receptors. Most of these receptors bind more than one chemokine and are distributed in particular cell populations which gives rise to the selective activity of these molecules (Chen *et al.*, 2004).

Other cytokines important in innate immune defences are granulocyte monocyte colony stimulating factor (GM-CSF), granulocyte CSF (G-CSF) and monocyte CSF (M-CSF), which drive the development, differentiation and expansion of cells of the myeloid series. Transforming growth factor (TGF) is produced by a variety of cells including monocytes, macrophages, T cells and chondrocytes, and plays an important role in suppressing immune responses, as it can inhibit activation of macrophages and the growth of B and T cells. The role of TGF in the down-regulation of the immune response aids in the survival of the intracellular parasite, *Leishmania chagasi* (Gantt *et al.*, 2003).

1.1.3.4 Collectins

Collectins are a group of carbohydrate-binding proteins structurally related to the complement component C1q. These molecules act as opsonins and are important in the innate immune response to infections (reviewed by Hoppe & Reid, 1994). Receptors for collectins are present on macrophages, facilitating the removal and destruction of

microorganisms. The mannose-binding protein, an example of a collectin which binds to the mannose receptor, is able to activate complement via the classical pathway and to engage host inflammatory, lytic and phagocytic responses (Hoppe & Reid, 1994).

1.1.4 The Acquired Immune Response

The innate immune response provides the non-specific element of immunity. However, the acquired immune response is specific, with memory cells such as lymphocytes providing specificity.

1.1.4.1 Cells involved in Acquired Immunity

Two types of lymphocytes, T and B cells, that mature in the thymus and bone marrow, respectively, are involved in the acquired response and each possess different antigen receptors on their cell surface. These include molecules required for lymphocyte activation and for movement in and out of body tissues. NK cells are also considered a cell of the adaptive immune system, but they also participate in innate protection against viruses and some tumours.

T cells are of two classes, T helper (Th) and T cytotoxic (Tc), and they both possess antigen receptors that determine their specificity and CD3 which is crucial for cellular activation (de Saint Basile *et al.*, 2004). In contrast, B cells produce and present antibodies as their specific antigen receptor, however, they do have molecules similar to CD3 that are important in cell activation. When activated by antigen, and often T cells, B cells proliferate and mature into memory cells or plasma cells. Whilst memory cells produce antibodies for expression on their cell surface and remain able to respond to an antigen if it is reintroduced, plasma cells do not express cell surface antibody receptors (although they do have receptors for other immune-related molecules such as chemokines; Natayama *et al.*, 2003). Instead, the plasma cells produce and secrete large amounts of antibody of the same specificity as the antigen receptor on the stimulated parent B cell, and these antibodies are always of the same specificity.

1.1.4.2 Antibodies

Antibodies, or immunoglobulins, are glycoproteins that bind antigens with high specificity and affinity (antibody structure is shown in Fig 1.1.2). In humans there are five chemically and physically distinct classes of antibodies (IgG, IgA, IgM, IgD and IgE). The affinity of the antibody to the antigen is important in the immune response, since the antibody will be less likely to dissociate from the antigen as affinity gets greater. Interestingly, antibodies produced by a memory response have higher affinity than those produced from a primary response (Neuberger *et al.*, 2000). The valency of the antibody is also important for the immune response, since this is the number of antigenic determinants with which the antibody can bind. Having multiple binding sites for an antigen therefore dramatically increases the efficiency of recognition.

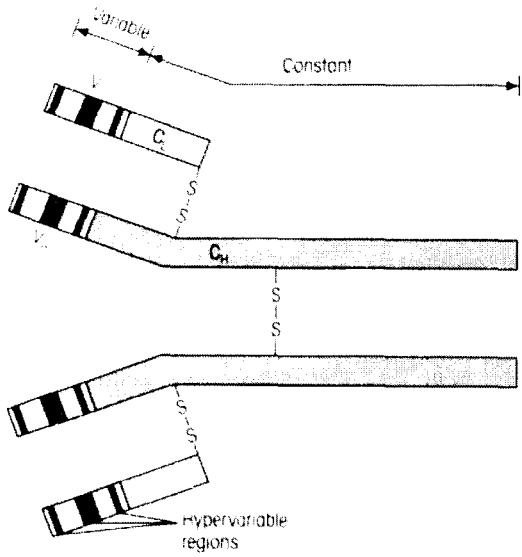

Figure 1.1.2. Antibody structure. The terms 'V region' and 'C region' are used to designate the variable and constant regions, respectively; 'V_L' and 'C_L' are generic terms for these regions on the light chain and 'V_H' and 'C_H' specify the variable and constant regions on the heavy chain. One L chain is bound to a H chain by a disulphide bridge and noncovalent interactions. Two H chains are also bound by covalent and non-covalent bonds. The antibody class is determined by the type of H chain the antibody possesses, of which there are five (μ, δ, γ, ε and α). Additionally, there are two types of L chain (κ and λ), of which an antibody can only possess one. Diagram taken from Roitt (2001).

Antibody diversity is generated by the random selection of genes by B cells during their development into mature cells (Collins *et al.*, 2003; Li *et al.*, 2004a). During

development, the B cell randomly selects from its H-chain gene group and then from the κ or λ gene groups. After successful rearrangement, the cell is then committed to the expression and production of antibodies that will have the same specificity.

1.2 INVERTEBRATE DEFENCE MECHANISMS

An interest in comparative immunology appears to have been triggered by the comparative immunologist, William Hildemann (1927 – 1983), who published his work on immune-phylogeny over two decades ago (Hildemann *et al.*, 1979; Hildemann *et al.*, 1981). Since these initial experiments, much is now known about the immune responses in invertebrate species, especially insects due to the use of *Drosophila melanogaster* as a model organism (reviewed by Leclerc & Reichhart, 2004). This increase in knowledge is reflected in the number of review articles recently published (Vilmos & Kurucz, 1998; Mushegian & Medzhitov, 2001; Salzet, 2001; Bayne, 2003; Humphries & Yoshino, 2003; Schulenburg *et al.*, 2004).

Immunity in invertebrates can be divided into humoral and cellular aspects of the immune system. Humoral immunity in insects involves melanisation, and haemolymph (serum) clotting and immune proteins such as antimicrobial peptides; whereas cellular immune responses include phagocytosis and encapsulation by haemocytes (the invertebrate equivalent of the mammalian macrophage), and nodule formation.

1.2.1 Cellular aspects of invertebrate immunity

The success of invertebrate species belonging to the phyla Arthropoda and Mollusca is thought to be partially due to them possessing efficient defence systems against foreign invaders such as bacteria. Haemocytes, the main phagocytic defence cells of invertebrate species, greatly resemble mammalian macrophages both functionally and morphologically. These cells are responsible for the phagocytosis of microbes, or encapsulation of larger invading organisms such as parasites. Haemocytes are either found attached to endothelial cells lining the haemocoel or are mobile in the haemolymph. The mobile haemocytes are also capable of moving into tissues by chemotaxis, similarly to cells involved in the vertebrate innate immune response. In fact, cell-free *E. coli* LPS has been demonstrated to stimulate chemotaxis of *Mytilus edulis* haemocytes using a Boyden chamber (Schneeweiss & Renwrantz, 1993).

Early biochemical studies with haemocytes suggested that they lack oxygen generating systems (Chain & Anderson, 1983), which are important in the mammalian immune

response (reviewed by MacMicking *et al.*, 1997). However, recent studies have confirmed that molluscan haemocytes are capable of generating reactive oxygen species (Conte & Ottaviani, 1995; Adema *et al.*, 1991; Hahn *et al.*, 2000). Other similarities between mammalian macrophages and haemocytes lie in the mechanisms of phagocytosis. Although haemocyte phagocytosis is a fairly recent area of research, evidence is accumulating which suggests that the mechanisms phagocytosis employed by haemocytes are similar to those of macrophages. It is known that the haemocyte cell surface receptors for recognition of microbes are homologous to those found on the macrophage cell surface as discussed in section 1.1.

Internalisation of bacteria or particles by molluscan haemocytes has largely been studied using *E. coli*, latex beads or cell-free LPS, and similar approaches to those used in studies with mammalian macrophages. Lacoste *et al.* (2002a), Lane & Birkbeck (1999) and Canesi *et al.* (2002a; 2002b; 2002c) used live bacteria to challenge haemocytes from *Crassostrea gigas*, *M. edulis* and *Mytilus galloprovincialis* respectively, and these workers found that haemocytes from these species appeared to phagocytose bacteria in similar way to mammalian macrophages. Cell-free LPS has been used to challenge haemocytes from a wide range of invertebrates (see for example, Charalambidis *et al.*, 1996b; Foukas *et al.*, 1998; Metheniti *et al.*, 2003; Cao *et al.*, 2004), to determine the specific effect of this endotoxin on the function of invertebrate defence cells.

1.2.1.1 Invertebrate Pattern Recognition Receptors

Toll-like receptor proteins were first identified in *D. melanogaster* (Hashimoto *et al.*, 1988), and those found in invertebrate species, and indeed plant species, are highly homologous to those find in mammalian cells (Jones *et al.*, 2004). However, toll-like receptors in invertebrates appear to also have roles in development as well as immunity (Kambris *et al.*, 2002; Ligoxygakis *et al.*, 2002). Although the *Drosophila* toll like receptor is a major player in the immune response, it does not act as a pattern recognition receptor, instead binding cytokines. Signalling of toll receptors in invertebrate cells appears to parallel that of mammalian IL-1 receptor signalling which leads to the activation of the necrosis factor kappa B (NF-κB) pathway, therefore suggesting a possible role of TLRs in the immune response. Homologies to the

Drosophila and the mammalian toll pathway have also recently been found in *Caenorhabditis elegans* (reviewed by Schulenburg *et al.*, 2004).

Apart from the Toll-like proteins, other haemocyte cell-surface molecules, including those involved in the phagocytic response, have a striking resemblance to mammalian macrophage receptors. For example, in the mid-1990s insect homologues to the class-A macrophage specific scavenger receptor were identified (Pearson *et al.*, 1995). Similarly, invertebrate homologues to CD36 have also been found on the insect haemocyte surface (Franc *et al.*, 1996).

Recent studies have utilised LPS to stimulate invertebrate haemocytes, in an attempt to decipher mechanisms of haemocyte action and function, and to compare findings with those derived from studies on mammalian macrophages (Conte & Ottaviani, 1995; Stefano *et al.*, 1999; Cao *et al.*, 2004). LPS binding protein (LBP) is an important protein in the immune system of invertebrates and vertebrates alike. While mammalian LBP has been extensively characterised, investigations into invertebrate LBP are relatively new. It has been found, however, that LPS-binding proteins differ greatly in their chemical and structural properties. A Gram-negative bacteria binding protein (GNBP) has been identified in the silkworm *Bombyx mori* (Lee *et al.*, 1996) and an LPS-specific binding protein has also been reported from the freshwater crayfish *Pacifastacus leniusculus* (Lee *et al.*, 2000). In addition, hemolin (a protein that stimulates phagocytosis) has been identified in the haemolymph of *Hyalophora cecropia* (Faye *et al.*, 1975) and has more recently been found to specifically bind to the lipid A part of LPS (Daffre *et al.*, 1997), acting as an opsonin to enhance phagocytosis (Lanz-Mendoza *et al.*, 1996). An LPS-binding protein found on the surface of medfly, *Ceratitis capitata*, haemocytes has been implicated in LPS internalisation by these cells (Metheniti *et al.*, 2003), and plays a similar role to the haemocyte surface protein, calreticulin, in the insect *Pieris rapae*, although this is not an LPS-binding protein (Asgari *et al.*, 2003). Proteins that bind *E. coli* LPS have also been characterised in the horseshoe crab, *Tachypleus tridentatus,* and well as proteins that bind to protein A (the Gram-positive equivalent to LPS) from *Staphylococcus aureus* (Chiou *et al.*, 2000). Furthermore, a major non-self-recognition protein, which may be related to the LPS-binding protein, is released from haemocytes of *C. capitata* during degranulation following stimulation with LPS (Charalambidis *et al.*, 1995).

Lectins could also be described as pattern recognition molecules since they bind to carbohydrates at specific sugar motifs, similarly to many LPS binding proteins. Lectins have been characterised in many invertebrate species, and have been found to be one of the main groups of molecules involved in the humoral immune response. Galactose specific lectins have been isolated from a wide range of invertebrate species including the ascidian *Halocynthia roretzi* (Azumi *et al.*, 2000), the horseshoe crab *Tachypleus tridentatus* (Chiou *et al.*, 2000), the abalone *Haliotis laevigata* (Mann *et al.*, 2000), *Drosophila* (Pace *et al.*, 2002), and the worm *Lumbricus terrestris* (Hirabayashi *et al.*, 1998). Binding of lectins can be calcium dependent or –independent, with both types of lectin known to exist in invertebrate species (Richards & Renwrantz, 1991). Recently, it has been speculated that a mannan-binding protein may exist in the ascidian, *H. roretzi* (Ji *et al.*, 1997), and serum lectins have recently been found in the cockroach, *B. discoidalis* (Wilson *et al.*, 1999). The existence of lectins in molluscs (reviewed by Horak & Van der Knaap, 1997) is particularly important due to lectins being involved in the recognition of parasites (discussed in section 1.3).

In mammalian systems, the LPS-binding protein transforms LPS to monomers for binding to CD14 receptors on the leukocyte surface (Yu & Wright, 1996). CD14 also recognises various sugar or glycolipid motifs from bacteria, thus acting similarly to lectins. Cluster of differentiation (CD) receptors have been found on the surface of haemocytes, for example, the insect cell surface protein, croquemort, is part of the CD36 superfamily and mediates recognition of apoptotic cells in a similar fashion to mammalian CD36 (Franc *et al.*, 1996).

1.2.2 Humoral aspects of invertebrate immunity

Three immediate defence mechanisms contribute to invertebrate humoral immunity. These mechanisms involve the production of antimicrobial peptides/proteins, haemolymph clotting, and a process that solely belongs to insects, melanisation.

Melanisation is the result of phenoloxidase activity, which catalyses the formation of melanin. As a result of phenoloxidase activity, an invading organism is 'blackened' within the host haemolymph by melanin deposition. Before phenoloxidase is activated,

recognition of 'foreign' material occurs via recognition molecules in the haemolymph, which induce activation of the prophenoloxidase (proPO)-activating system (proPO-AS) causing haemocytes to release the inactive enzyme (Jiang *et al.*, 2003). This proPO-A system is extremely efficient, responding to concentrations of LPS, peptidoglycans or β-1,3-glucans from yeast as low as picograms per litre (Söderhäll, 1982). The proPO activating system has been found to exist in many insects including the silk moth *Bombyx mori* (Ashida, 1971), the tobacco moth *Manduca sexta* (Aso *et al.*, 1985), the cockroach *Blaberus discoidalis* (Durrant *et al.*, 1993), and the wax moth *Galleria mellonella* (Kopacek *et al.*, 1995). It has been suggested that phenoloxidase is also involved in crosslinking LPS to a yet uncharacterised haemocyte surface receptor in *C. capitata* (Charalambidis *et al.*, 1996b).

Prophenoloxidase is activated in a stepwise manner, which involves serine proteinases; proteolytic cleavage by these proteinases converts the inactive prophenoloxidase to the active enzyme. Prophenoloxidase-activating serine proteases have been isolated from the crayfish *Pacifastacus leniusculus* (Aspán *et al.*, 1990), *D. melanogaster* (Chosa *et al.*, 1997) and *B. mori* (Ashida *et al.*, 1980). Low molecular weight proteinase inhibitors, with two such inhibitors being characterised in the insect *Locusta migratoria* (Kromer *et al.*, 1994), provide inhibition and thus physiological regulation of the proPO-A system.

Antimicrobial peptides/proteins are principally synthesised in the fat body of invertebrates upon injury, but also in haemocytes, epithelial cells, the gut and the reproductive tract (Boulanger *et al.*, 2001). These proteins act on the cell membrane of microorganisms causing cell lysis. Although antimicrobial peptides have some activity against Gram-negative bacteria and viruses, they are mainly active towards Gram-positive bacteria (Nakajima *et al.*, 2003). The fat body of invertebrates is analogous to the mammalian liver and prostaglandins, known mediators of vertebrate inflammatory responses, are synthesised by the fat body of the armyworm, *Pseudaletia unipuncta* (Tunaz *et al.*, 2001). Moreover, perforins are mammalian immune-regulatory proteins of lymphocytes, and a perforin-like protein has recently been isolated from the marine mollusc, *Haliotis rufescens* (Mah *et al.*, 2004). One of the proteins that perforin is homologous to is released from mature macrophages, and the molluscan perforin-like protein is probably secreted from the haemocytes (Mah *et al.*, 2004). A striking

resemblance in structure and function has been found between penaeidins, antimicrobial peptides of the shrimp, *Penaeus vannamei*, and components of innate immunity found in vertebrates and plants (reviewed by Bachère *et al.*, 2000). Penaeidins are predominantly active towards Gram-positive bacteria. This demonstrates the high degree of conservation of antimicrobial molecules. Dunphy & Halwani (1997) carried out an extensive study of two haemolymph proteins in larvae of the wax moth *Galleria mellonella*, which were shown to bind to insect pathogenic bacteria and reduce the endotoxic effect on the haemocytes. The proteins studied by Dunphy & Halwani (1997) also increased prophenoloxidase activation by LPS, therefore aiding the immune response towards the pathogen. The antibacterial peptide *Mytilus galloprovincialis* defensin 1 (MGD1) was first isolated and partially characterised from haemolymph of the mollusc, *M. galloprovincialis* (Hubert *et al.*, 1996). However, MGD1 has also recently been isolated in the plasma of arthropod spp., being produced in granulocytes and released into the plasma following challenge by Gram-positive bacteria (Mitta *et al.*, 1999).

In vertebrates, the complement system facilitates phagocytosis and mediates an acute inflammatory reaction (see section 1.1.3.1). Although it was initially thought that the complement system was exclusive to vertebrates, complement components have recently been found in invertebrate species. Haemocytes of the tunicate, *Styela plicata*, have been shown to express a homologue of the main complement component, C3 (Raftos *et al.*, 2004). The C3 homologue is stored within sub-cellular vesicles and is exocytosed after bacterial challenge. This is of interest since it is widely hypothesised that the complement system was established at the point of emergence of jawed fish, evidence for which lies in the fact that complement components of bony and cartilaginous fish are nearly identical to the mammalian complement system (reviewed by Nonaka & Yoshizaki, 2004). A C3-like lectin has also been shown to exist in *H. roretzi*, with its function being linked to opsonisation and enhanced phagocytosis (Azumi *et al.*, 2000).

Haemolymph clotting plays a key role in the immune response of invertebrates. Two types of clotting have been demonstrated, the first being where a gel is formed through the polymerisation of clottable proteins, a process catalysed by a calcium-dependent transglutaminase released from haemocytes (Wang *et al.*, 2001). A three-step serine

protease cascade activates the second type of coagulation (Iwanaga *et al.*, 1992). Components of the reaction comprise, amongst others, proteins with complement-related and C-type lectin domains. Li *et al.* (2002) have provided evidence that the prophenoloxidase activating cascade is involved in the coagulation of insect haemolymph, which involves recognition of the microbial organisms by pattern recognition proteins. Lipophorin has been identified as the major humoral procoagulant in many insect species (Brehélin, 1979). Cloning and characterisation of a haemolymph clottable protein from the haemolymph of the shrimp, *Penaeus monodon* has interestingly revealed this protein to be present in most shrimp tissue except for mature haemocytes (Yeh *et al.*, 1999). Such molecular studies will ultimately help identify the molecular basis of haemolymph clotting.

1.2.3 Immunity in Gastropod Molluscs

Sminia and co-workers carried out extensive research into gastropod immunity in the early 1970s, with studies focused on the morphology and function of *Lymnaea stagnalis* haemocytes (Sminia, 1972; Sminia *et al.*, 1973; Sminia *et al.*, 1974). Owing to recent advances, knowledge of gastropod antimicrobial proteins and immune responses has dramatically improved, although when compared with insects, research into gastropod defence mechanisms is still in its infancy.

The defence responses of gastropod molluscs have been investigated largely as a consequence of these snails being intermediate hosts for helminth parasites, with *L. stagnalis* being the intermediate host for the schistosomes, *Trichobilharzia ocellata, Trichobilharzia szidati* and *Echinostoma* spp.. *Lymnaea stagnalis* is a pond-dweller but also inhabits slow moving streams. Owing to the relative ease of keeping gastropod mollusc cultures in the laboratory, and the easy access to the blood cells and serum of these animals, the number of studies emerging from the use of these snails are increasing. Studies on immunity in marine bivalve molluscs, such as *Mytilus* spp., are mainly related to toxicological effects of pollution, as they are a major food source and therefore economically important in many countries.

The humoral arm of the molluscan immune system is very similar to that of insects, in that they both have anti-microbial proteins, except that melanisation does not occur in

molluscs, although components of the prophenoloxidase cascade have been identified in *Biomphalaria glabrata* eggs (Bai *et al.*, 1997). The role of lectins has been studied in gastropods due to their function as pattern recognition molecules to intramolluscan stages of parasite larvae (discussed in section 1.3).

Horák and co-workers led these lectin-based studies in the 1990s (Horák & van der Knaap, 1997; Horák *et al.*, 1998; Horák & Deme, 1998), and found that the introduction of sugars to bind lectins altered the immune responses of *L. stagnalis* considerably. As in mammalian and insect systems, calcium-dependent binding mechanisms for lectins have been shown in gastropod molluscs such as *B. glabrata* (Fryer *et al.*, 1989) and *L. stagnalis* (Horák & Deme, 1998).

Glycosidases, enzymes which modify and degrade glycoproteins, have been detected in the haemolymph of *B. glabrata*, and have been suggested to play a role in the humoral immune defence system (Zelck *et al.*, 1996). These enzymes which are probably synthesised by the digestive gland or head/foot tissues, may modify molecular patterns of the surface of invading pathogens, thus facilitating subsequent phagocytosis or encapsulation by haemocytes. Hydrolytic enzymes such as alkaline and acid phosphatases, peroxidase, lysozyme, lipase and amylase have also been found in the haemolymph of molluscan species (see for example Luna-Gonzalez *et al.*, 2004).

Studies on the cellular aspects of gastropod defence mechanisms have revealed that the main phagocytes, haemocytes, are functionally and morphologically very similar to mammalian macrophages (Van der Knaap *et al.*, 1993). Gastropod haemocytes are thought to derive from the haemocyte-producing organ (HPO), situated between the mantle cavity and the pericardium. The HPO has been studied in many gastropod species, including *Lymnaea palustris* (Rachford, 1976). Four types of defence cell have been found in *L. stagnalis*: antigen-trapping endothelial cells; foreign-engulfing pore cells; phagocytic reticulum cells and mobile haemocytes. Haemocytes contribute most to the internal defence system (reviewed by Van der Knaap *et al.*, 1993). Two types of circulating haemocytes, referred to as spreading and rounded cells, have been classified in the freshwater gastropod, *Planorbarius corneus* (Ottaviani, 1992). However, in *L. stagnalis*, only a single circulating haemocyte has been identified, which has been reported to be round when 'immature', and spreading when 'mature'.

Many CD antigens, markers that are used to differentiate between lymphocyte subsets in mammals, have been found on the surface of gastropod haemocytes (Francheschi *et al.*, 1991). However, since these molecules were detected with mouse 'anti-human' monoclonal antibodies, positive staining with the antibody could have been due to the epitope recognised by the antibody being present, and not the whole molecule. CD antigens are also referred to as integrin subunits or chains, for example, CD29 is also referred to as the integrin β_1 chain. The β_1 integrin has been cloned from the *B. glabrata* embryonic (Bge) cell line (Davids *et al.*, 1999).

1.3 MOLLUSC-TREMATODE INTERACTIONS

Gastropod molluscs are intermediate hosts for a range of helminth parasites, which are known to be the cause of many diseases of medical and veterinary importance. One such mollusc of the Lymnaeid family, *Lymnaea truncatula*, is host to the liver fluke, *Fasciola hepatica*, a food-borne trematode which is an important medical and veterinary parasite as it is capable of infecting both humans and other mammals such as sheep and cattle (Hurtrez-Boussès *et al.*, 2001). A particularly important family of helminths are the Schistosomatidae, which are digenean trematodes. *Lymnaea stagnalis* is the intermediate host to *Trichobilharzia ocellata*, an avian schistosome that uses ducks as its definitive hosts (van der Knaap *et al.*, 1985; Joosse *et al.*, 1986; Horak *et al.*, 2002). *Trichobilharzia ocellata* is known to cause swimmers itch in humans, resulting from larvae burrowing into the upper layers of the skin (Horak *et al.*, 2001). The most extensively studied schistosome, however, is probably *Schistosoma mansoni* (which has *B. glabrata* as its intermediate host) (Miller *et al.*, 2001; Stirewalt *et al.*, 1983). The importance of this parasite has recently been highlighted since it has been estimated that over 83 million people are infected with *S. mansoni* in 54 countries (Chitsulo *et al.*, 2000).

1.3.1 Schistosomiasis

1.3.1.1 The schistosome life cycle

Unlike most other helminth parasites, schistosomes are not hermaphroditic. The adult male worm possesses a gynaecophoric canal which is a deep ventral groove in which the female lies during copulation in the definitive host. Schistosome eggs are released from the body of the definitive host via faeces or urine, depending on the schistosome species. Once in contact with freshwater, the eggs hatch to liberate the first larval stage, the miracidium. The miracidium is a ciliated organism that is incapable of long periods of independent survival and must quickly infect the correct species of snail for survival and continuation of the life cycle. The miracidium is thought to be attracted to the intermediate snail host by chemoattraction, where the larvae follow chemical gradients to find the host. Such behaviour has been reported by Haas *et al.* (1995),

where miracidia of *T. ocellata* and *S. mansoni* were found to swim towards their host snail both directly and with 'turnback swimming' where they follow both decreasing and increasing chemical gradients. The larvae (miracidia) of *S. mansoni* and *Schistosoma haematobium* are attracted to macromolecular glycoconjugates (with a molecular weight of >30,000) and it has been suggested that this chemoattraction allows the miracidia to achieve host snail specificity (Haberl *et al.*, 1995). Once contact is made, the miracidium penetrates the snail, loses its ciliated epidermis and subsequently transforms into a sporocyst. The sporocyst lacks a digestive system and has a syncytial, cytoplasmic outer surface for absorption of nutrients. Within the sporocyst, permanently embryonic cells divide and differentiate to form a second generation of sporocysts, which in turn divide and subsequently differentiate to form cercariae. This process of asexual division greatly enhances the schistosomes reproductive potential (Thèron, 1986). The cercaria is the final larval stage. Once it has emerged from the snail body and become free-living within the freshwater environment it is capable of infecting the definitive host. Schistosome cercariae are forked-tailed (furcocercous) and have been seen to hang from the surface film of water, detach, sink and then swim upwards again to the surface, thus increasing their chance of coming into contact with a suitable host (Haas *et al.*, 1994). Such behaviour has also been seen in other digenean species (for example, Rea & Irwin, 1995).

The definitive host is infected by direct cercarial penetration into the skin, which involves vigorous movement of the parasite and enzyme secretion. The enzymes involved in skin penetration are though to be cathepsin L and cathepsin B, which are stored in the post-acetabular glands of *S. mansoni* cercariae (Dalton *et al.*, 1997), although for *T. ocellata* the enzmes have not yet been described. Once inside the duck, the *T. ocellata* schistosomulum migrates to the lungs via the bloodstream within three days of infection, and also migrates to the liver, kidney and intestines (Haas & Pietsch, 1991). The life cycle of *T. ocellata* is illustrated in figure 1.3.1.

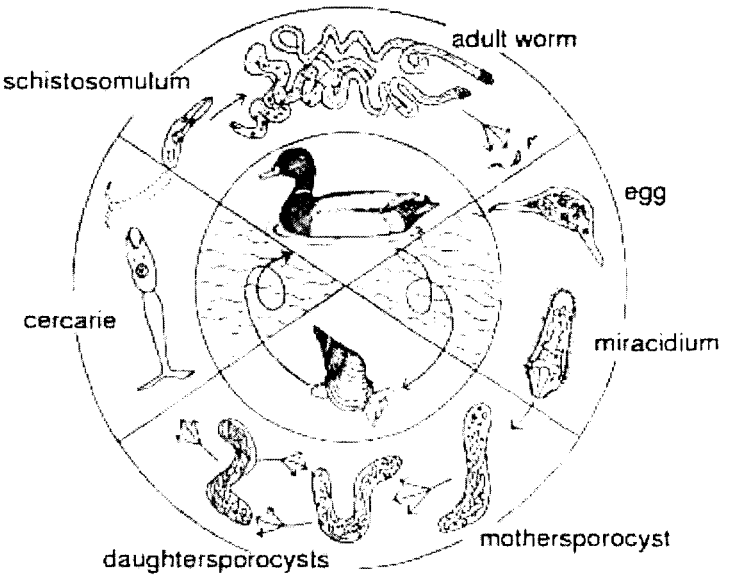

Figure 1.3.1. The *Trichobilharzia ocellata* life cycle. The parasite cycles between an intermediate molluscan host (*L. stagnalis*), where larval stages develop, and a definite vertebrate host, which for *T. ocellata* are ducks. If humans come into contact with the parasite, swimmers itch can develop, when *T. ocellata* cercaria penetrate into the skin. Taken from www.biologie.uni-erlangen.de/parasit/contents/research/tricho.html.

1.3.1.2 Immunity of the intermediate snail host to the schistosome parasite

The intramolluscan stages of the schistosome parasite possess a surface coat which comprises a carbohydrate-rich layer. The carbohydrates on the parasite coat have been studied using lectin-binding techniques, since each lectin binds specifically to only one or a few carbohydrate structures (see for example, Tateno *et al.*, 2004). This coat changes its carbohydrate composition according to the developmental stage of the parasite, possibly caused by the shedding of carbohydrate residues throughout development (Horak, 1995; Horak *et al.*, 1998; Nyame *et al.*, 2002). The change in schistosome carbohydrate composition throughout the development of *Trichobilharzia szidati* (which also has *Lymnaea stagnalis* as its intermediate host) larval stages has been studied, and fucose occurs in the glycocalyx in abundance during these stages (Horak *et al.*, 1995). This work also revealed that galactose, fucose and mannose were commonly found on many of the parasite stages with the exception of sporocysts which did not bind fucose-specific lectins. The carbohydrate composition of *T. ocellata and S. mansoni* intramolluscan stages are known to differ (Gerhardus *et al.*, 1991; Nyame *et*

al., 2002). Although little is known about the masking of the parasite to the host immune response, changes in surface saccharide composition could contribute to this phenomenon.

During penetration into the definitive host, changes also occur on the surface of the cercariae. During larval stages, the surface layer is known as the glycocalyx, and it predominantly comprises galactose and fucose. However, during penetration into the definitive host the glycocalyx is shed, the larvae can no longer survive in water, and the tegument is exposed as the outer membrane (Horak *et al.*, 1998). The tegument on the adult worm, also composed of glycoproteins including mannose, fucose, glucose and galactose, has been linked to the immune response towards the invading parasite (reviewed by Cummings & Nyame, 1999). Immunity to the adult worm involves both vertebrate innate and adaptive immune responses, with the innate immune response involving macrophage-mediated encapsulation which results in recruitment of the acquired immune response (reviewed by Allen & Maizels, 1996). Sugars comprising the adult worm tegument (fucose, mannose and galactose) have been shown to stimulate alveolar macrophage migration (Takata *et al.*, 1987) thus allowing encapsulation to occur.

When snails are infected with incompatible schistosome miracidia, the immune system attacks the larval body. In contrast to bacterial infection, when phagocytosis is sufficient to destroy the microbe, a combined effort by large populations of haemocytes results in encapsulation of the larvae. Humoral mechanisms of immunity also play an important part in snail defence upon infection. Lectins play the greatest role in the humoral immune response towards parasite larvae, since they act as recognition receptors for the carbohydrates on the glycocalyx of the parasite, thus mediating encapsulation by haemocytes (Richards & Renwrantz, 1991; Horak & van der Knaap, 1997; Loukas & Maizels, 2000). Plasma proteins also help host defence by facilitating cytotoxic reactions by the haemocytes towards larvae (Zelck *et al.*, 1995; Amen *et al.*, 1992a).

A host-parasite relationship that has been well studied is that between *B. glabrata* and *Schistosoma mansoni*, with *S. mansoni* being the most extensively studied member of the phylum Platyhelminthes. The biology of the *B. glabrata* immune response is often

studied with the *Biomphalaria glabrata* embryonic (Bge) cell line, derived from embryos of *B. glabrata* by Hansen (1976). Although this cell line was not derived from haemocytes, the morphology, function and biochemistry of Bge cells appear to be identical to that of primary haemocytes (Yoshino *et al.*, 1999). *B. glabrata* infected with *S. mansoni* larval stages exhibit changes in their behaviour, physiology and reproductive capability. Boissier *et al.* (2003) observed *B. glabrata* to be more attracted to *S. mansoni*-infected snails than to those that were uninfected, and it was thought that this effect may result in enhanced parasite transmission. Recently, studies into the relationship between *S. mansoni* and *B. glabrata* have taken place at a more molecular level (reviewed by Jones *et al.*, 2001).

Much research has taken place into how the schistosome profits from the stress it elicits on its molluscan host (reviewed by De Jong-Brink, 1995). One such molecular study has examined the NPY gene in *L. stagnalis*, and its activity when infection takes place (De Jong-Brink *et al.*, 1999). It was found that the *Lymnaea* NPY gene (LyNPY), known in vertebrates to be involved in energy budgeting, becomes upregulated when schistosome infection occurs (De Jong-Brink *et al.*, 1999), which proves to be highly advantageous to the parasite. The finding that the schistosome interferes with gene activity within its host offered possible explanations for previously unanswered questions concerning the morphological changes observed in the host snail following infection.

The survival of a schistosome in its intermediate snail host is partly due to host specificity, for example *T. ocellata* is capable of survival and development within *L. stagnalis* but not *B. glabrata*, although both snails are gastropods. This survival is at least in part due to avoidance of host immune defences by masking and mimicry at the larval surface. Excretory-secretory (E-S) products from *T. ocellata* have been extensively studied (Núñez *et al.*, 1997; Núñez & De Jong-Brink, 1997), and it is thought that these E-S factors play a critical role in determining compatibility between the snail and parasite. Immunological compatibility of the parasite and its host depends on three strategies: (i) physicochemical properties of both parasite and haemocyte surfaces, (ii) the capacity of the parasite to mimic/acquire host antigens and (iii) ability of the parasite to interfere with host defence mechanisms (for examples refer to De Jong-Brink, 1995; Van der Knaap & Loker, 1990). One of the key mechanisms of

defence employed by the mollusc during infection consists of a cytotoxic strategy via haemocytes (Amen *et al.*, 1991a; Amen *et al.*, 1991b; Nunez *et al.*, 1994). Interestingly, when *B. glabrata* haemocytes were exposed to E-S factors from *S. mansoni* miracidia, phagocytosis and haemocyte motility was suppressed (Lodes & Yoshino, 1990; Connors & Yoshino, 1990), demonstrating a down-regulation of host immune function by the parasite.

Núñez *et al.* (1997a) demonstrated that *T. ocellata* releases two E-S factors, one of which enhances *L. stagnalis* haemocyte activity and the other suppressing haemocyte function; these factors were of low (2kDa) and high (40kDa) molecular weights, respectively. Furthermore, the release of these factors appeared to differ between *T. ocellata* developmental stages, with the 2kDa E-S factor being secreted predominantly in early larval stages and the 40kDa factor being released in the latter stages of larval development (Nunez *et al.*, 1997a). Amen & De Jong-Brink (1992) and Nunez *et al.* (1997b) have studied the possible influence of ES products on host-parasite compatibility. In these studies *T. ocellata* E-S fractions released during transformation from miracidia to mother sporocysts suppressed *L. stagnalis* haemocyte activity, whilst *S. mansoni* E-S fractions upregulated *L. stagnalis* haemocyte function. It is thought that certain E-S components reduce phagocytic activity by blocking recognition receptors, thus preventing membrane-associated lectins from binding carbohydrates on the parasite surface (Van der Knaap & Loker, 1990).

Horák and co-workers have led investigations into the lectins of *L. stagnalis* and their association with carbohydrate moieties of *T. ocellata* (Horák *et al.*, 1998; Horák & Deme, 1998). Many of the studies investigating cell-bound and plasma lectins involve the addition of saccharides to haemocytes, with and without haemolymph. Saccharides commonly used in such studies are arabinose, fructose and mannose, either as free-monosaccharides or conjugated to albumin. In these studies, serum lectins of *L. stagnalis* were able to agglutinate bacteria and yeast, and this process could be inhibited by many mono- and oligosaccharides, including arabinose (van der Knaap *et al.*, 1982). Horák *et al.* (1998) also showed that arabinose and fructose inhibit phagocytosis of mouse red blood cells by *L. stagnalis* haemocytes. Both calcium-dependent and –independent lectins have been detected in the immune system of *L. stagnalis* (Horák & Deme, 1998).

1.4 INTRACELLULAR SIGNALLING

All cells have the ability to detect and alter their physiology to a wide range of mitogens and extracellular conditions. To respond to these extracellular signals, intracellular biochemical pathways are activated and deactivated accordingly, resulting in altered cell behaviour and often the translation of *de novo* proteins through gene transcription. It is still a puzzle to researchers how cells manage and coordinate numerous signalling pathways so effectively. The relatively recent development of research tools such as pharmacological inhibitors and antibodies to dissect intracellular pathways has made signal transduction a key area of research. Indeed, research is now focusing on the development of pharmacological drugs that target key signalling proteins involved in the progression of disease.

1.4.1 Concepts of signal transduction

An extracellular ligand, which binds to a membrane receptor, is commonly referred to as a 'first messenger'. The extracellular signal can be a growth factor, hormone or an immune related molecule which binds to a receptor on the surface of the cell, promoting the production of small molecules known as 'second messengers'. Second messenger production allows amplification to occur, causing a single binding event on the cell surface to result in many activated enzymes further down the signalling chain, so the end-result is a large effect on the cell. Examples of second messengers are cytosolic calcium ions, cAMP and phosphoinositides (Rachinsky & Tobe, 1996; Bruch, 1996; McAinsh *et al.*, 1997).

1.4.2 Protein kinases

Phosphorylation of an enzyme is the covalent addition of a phosphate group at specific amino acid residues by protein kinases, which often regulates enzyme activity (Hurley *et al.*, 1990). Addition of the phosphate group results in a conformational change of the protein (Barford *et al.*, 1991). During phosphorylation, the phosphate group is supplied by adenosine triphosphate (ATP), with the third phosphoryl group of the chain being transferred to the hydroxyl group of the acceptor amino acid and the subsequent release

of adenosine diphosphate (ADP). Protein kinases are grouped according to the amino acid sequences on which they act, for example serine/threonine kinases preferentially phosphorylate serine or threonine residues on the target protein. Indeed, the specificity of the kinase is determined by the specific amino acid sequence either side of the target amino acid residue due to receive the phosphoryl group (examples given by Stokoe *et al.*, 1993). Some proteins may be phosphorylated at a number of different sites (Korotchkina & Patel, 2001), allowing many different kinases to act on a single protein and the convergence of a number of different signalling pathways and crosstalk (bi-directional signalling between proteins). Although kinases are split into two groups depending on the amino acid residue they phosphorylate, the catalytic site of all protein kinases appears to be well conserved (reviewed by Hanks & Quinn, 1991). There appears to be two conserved regions in the catalytic domain of kinases, with the most prominent region being located N-terminally to the catalytic domain and thought to be involved in ATP binding. The central part of the catalytic domain is also highly conserved, containing an aspartic acid residue thought to be important in the catalytic activity of the protein kinase. Importantly, the sequences surrounding this central region of the catalytic domain appear to differ between the different groups of kinases.

1.4.2.1 Serine/threonine kinases

Protein kinases that phosphorylate downstream proteins on serine or threonine residues are termed serine/threonine kinases (reviewed by Hardie, 1999; Cantrell, 2003). Serine/threonine kinases include protein kinase C (PKC), protein kinase A (also known as cAMP dependent protein kinase; PKA), the calcium/calmodulin-dependent protein kinases, and the mitogen-activated protein kinases (MAPKs) which are discussed at length in section 1.5.

1.4.2.2 Tyrosine kinases

Protein kinases that add a phosphoryl group to tyrosine residues are much less common than serine/threonine kinases. This group of kinases is split into two general groups; those that are soluble and found in the cytosol, and those that are associated with the cell membrane (reviewed by Berk *et al.*, 1997). Receptor tyrosine kinases (RTK) possess a ligand-binding domain, a transmembrane domain and a cytoplasmic domain,

which possesses kinase activity. Binding of ligands to the extracellular portion of the receptor results in kinase activity in its cytosolic region, and can result in activation of various signalling proteins such as PI-3K or MAPKs. Existing as either homo- or heterodimers, dimerisation can be achieved by autophosphorylation, as seen, for example, in the EGF receptor, where tyrosine residues on the cytoplasmic portion accept phosphoryl groups either intra- or inter-molecularly. The intercellular portion of the RTK contains binding domains, the most important being the Src homology 2 (SH2) domain. Most members of the non-receptor tyrosine kinases are soluble and generally lack binding domains such as SH2. Examples of such kinases are members of the Src protein family and Janus family which are especially important in immune cell biochemistry as they associate with cytokine receptors.

1.4.2.3 Phosphatases

It is important for the effects of protein phosphorylation and activation to be transient, therefore dephosphorylation of proteins is key to this process. Dephosphorylation, mediated by phosphatases, is the removal of the phosphoryl group from the protein and results in the deactivation of the protein with the regeneration of the hydroxyl side-chain and release of orthophosphate (see, for example, the MAPK phosphatase; Haneda *et al.*, 1999). Interestingly, it has been found that the total cellular amount of serine/threonine phosphatases matches that of serine/threonine kinases. Tyrosine phosphatases are found in many more varieties than the serine/threonine phosphatases, and are either soluble in the cytoplasm or membrane-bound structures related to receptors. These phosphatases are very specific and fast-acting, ensuring that tyrosine phosphorylated proteins are at very low concentrations in the cell at any one time (reviewed by Burke *et al.*, 1998).

1.4.2.4 Other methods of control

The addition of a phosphoryl group is not the only method used by cells to regulate signals intracellularly. Farnesylation, the addition of a 15-carbon fatty acid farnesyl group donated from farnesyl pyrophosphate, is another way of controlling protein activity. Farnesylation causes a protein to be hydrophobic, resulting in the protein migrating to the cell membrane where subsequent phosphorylation may occur

(reviewed by Galichet & Gruissem, 2003). The most common protein whose activity relies on farnesylation is the monomeric G-protein Ras. Another method of activating intracellular proteins is by the addition of a 20-carbon fatty acid geranylgeranyl, a process termed geranylgeranylation. Both farnsylation and geranylgeranylation involves the addition of the fatty acid chain to the C-terminus of the protein. To enable the study of these processes in signalling farnesylation and geranylgeranylation inhibitors are commercially available (Huang *et al.*, 2004).

Other methods of regulating activity are adenylation, the addition of an adenosine group derived from ATP; palmitoylation, the addition of a palmityol group donated from palmitoyl CoA (Dunphy & Linder, 1998); and the addition of a myristoyl group donated by myristoyl CoA, termed myristoylation (Resh, 1999).

1.4.3 Adaptor proteins

Also termed scaffolding proteins or linker proteins, adaptor proteins do not have enzymatic activity, but function as linkers between proteins in a signalling chain. The specificity and regulation of signal transduction are increased by adaptor proteins, as only certain proteins are able to associate with binding domains within the adaptor proteins. Adaptor proteins are also important in the subcellular location of a specific pathway, since they are usually specific to a particular region of the cell (Horejsi *et al.*, 2004).

A common adaptor protein, important in Ras signalling, is the growth factor receptor binding protein, Grb2. Grb2 contains one SH2 domain and two SH3 domains. Many proteins have been described as having binding ability towards Grb2, including the EGF receptor, the phosphatase SH-PTP2 and another adaptor protein, Shc. The Grb2 protein is bound to the GTP-GDP exchange factor, Sos, which is important in its function, as this generates a coupling between the activated receptor tyrosine kinase and Ras (reviewed by Olson & Marais, 2000).

1.4.5 Signalling networks and crosstalk

Activation of one signalling pathway can lead to activity of other signalling pathways in a phenomenon known as crosstalk (Dumont *et al.*, 2001). This is due to the mutivalency of signalling proteins and the use of second messengers. The ability of proteins to covalently bind to other proteins belonging to other signalling pathways is also important in the creation of crosstalk. Crosstalk allows one pathway to influence other pathways in the cell, resulting in many effects on the cell from just one first messenger. However, some signalling pathways are currently thought to be linear in nature, and only possess the ability to interact with certain downstream proteins in the pathway. These proteins often have very simple structures and include members of the nuclear receptor family and the JAK/STAT proteins (Yamaoka *et al.*, 2004).

1.5 Mitogen-activated Protein Kinases

MAPKs are serine/threonine kinases and activation of these proteins often results in their translocation to the nucleus and the subsequent activation of transcription factors which are important in causing promoted growth and differentiation along with other effects on cellular activity. A diverse range of stimuli ranging from growth factors, cytokines, cell adherence and cellular stress activate MAPKs (Lenormand *et al.*, 1998; Suzuki *et al.*, 1999). All MAPK pathways consist of sequential phosphorylation of three proteins, with the final component of this hierarchy capable of translocating to the nucleus once activated. The necessity of dual phosphorylation for MAPK activation makes these enzymes unusual. Phosphorylation must occur at threonine and tyrosine residues before the enzymes are fully activated (Ray & Sturgill, 1988).

1.5.1 The SAPK/JNK, p38^{MAPK} and ERK 5 pathways

The c-Jun NH_2-terminal kinase (JNK) pathway was first identified in human cells in 1991 (Hibi *et al.*, 1993). This enzyme was found to be activated by stress (e.g. ultraviolet irradiation), with phosphorylation of c-Jun occurring at the NH2-terminal activating sites. The rat homolog of JNK is referred to as Stress-Activated Protein Kinase (SAPK) due to stress signals activating this pathway. Another MAPK pathway, the p38 MAPK pathway is activated upon ultraviolet irradiation, osmotic shock, lipopolysaccharide, cytokines and also activation of GPCRs (Moriguchi *et al.*, 1996; Hippenstiel *et al.*, 2000).

Upstream kinases that activate both JNK/SAPK and p38 MAPK through small GTPases include the MEKK1-4 proteins, the mixed lineage kinases (MLKs) and the 'thousand and one kinases' (TAO kinases), which are more involved in the activation of p38 MAPK (Tibbles *et al.*, 1996; Chen *et al.*, 2003). Both the JNK/SAPK and p38 MAPK pathways activate nuclear transcription factors including Elk-1 and c-Jun; they also target kinases which include the MAPK-activated protein kinases, MAPKAP-K2, K-3 and PRAK (Kyriakis *et al.*, 2001).

A third MAPK pathway, which is presently poorly understood and was not identified until very recently is the ERK 5 pathway, which has ERK 5 as its terminal kinase (Mody *et al.*, 2003).

1.5.2 Extracellular-signal Regulated Kinase (ERK) pathway

The most extensively researched MAPK pathway is the Extracellular-signal Regulated Kinase (ERK) pathway (fig. 1.5.1). ERK was first discovered in the early 1980s as a predominant 42kDa protein that was phosphorylated on tyrosine and was activated in a range of cells when stimulated with epidermal growth factors (Cooper *et al.*, 1982; Cooper *et al.*, 1983). Shortly after this discovery, it was found that this protein was one of three components, which undergo phosphorylation sequentially. When activated Raf (or MEK kinase (MEKK)) phosphorylates MEK (or MAPKK) which in turn phosphorylates ERK, the activated form of ERK is capable of translocating to the nucleus or actin filaments (Reszka *et al.*, 1995; Aplin *et al.*, 2001).

The activity of ERK requires the phosphorylation of both tyrosine and threonine residues, which are separated by a single amino acid. This dual phosphorylation is speculated to play a role in the regulation of activity, ensuring that only phosphorylation by MEK (MAPKK) results in the activation of ERK isozymes (Ray & Sturgill, 1988). Although there are many cytoplasmic proteins that have the potential to activate ERK 1/2, either directly or indirectly through crosstalk, protein kinase C (PKC), phosphoinositol-3-kinase (PI-3K) and protein kinase A (PKA) shall be discussed in detail as these are the most commonly studied pathways leading to ERK activation.

1.5.2.1 Protein kinase C pathway

The serine/threonine kinase, PKC, consists of many closely related protein kinases, which derive from the transcription of multiple genes, or from the alternative splicing of a single mRNA transcript. The first PKC enzymes to be cloned were the α, βI, βII and γ subspecies, with all these subspecies possessing four conserved regions and five variable regions (reviewed by Way *et al.*, 2000). Recently the δ, ε, ζ, η, θ and λ subspecies have been cloned, which appear to possess the same conserved regions.

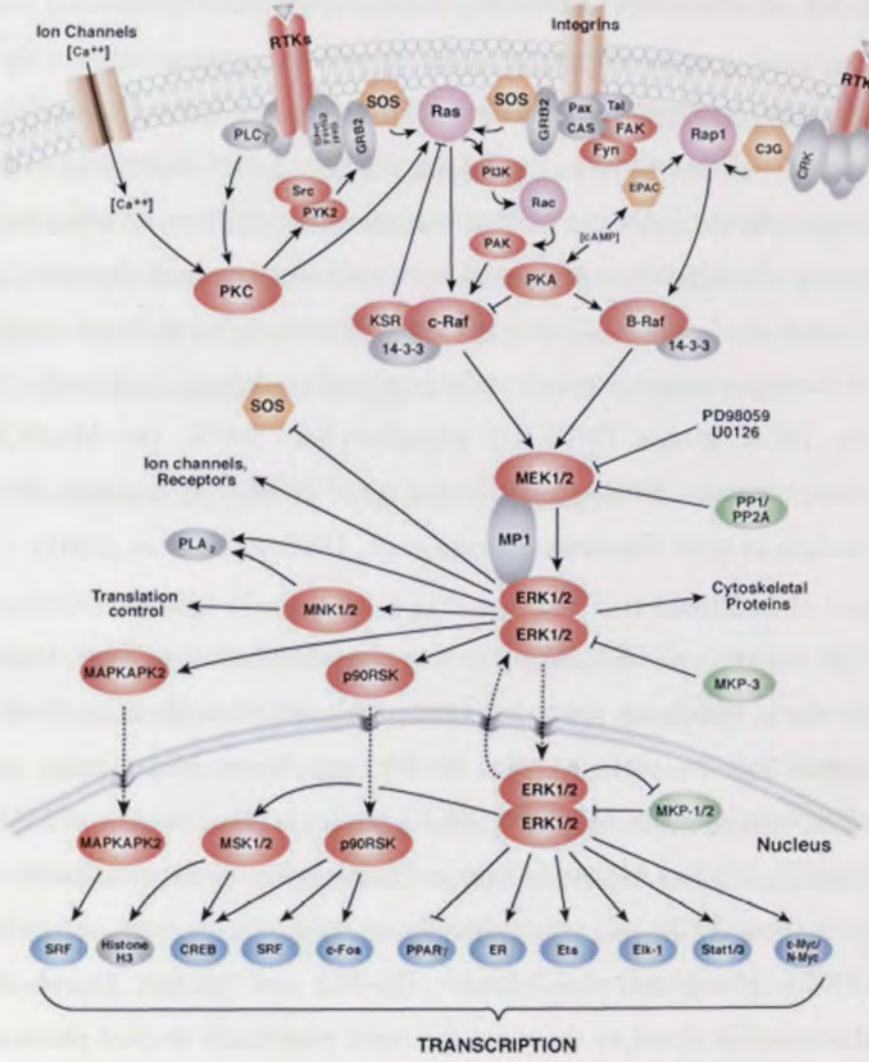

Figure 1.5.1. Diagram showing the MAPK/ERK pathway in mammalian cells. Signalling from receptor tyrosine kinases (RTKs) and integrins down to ERK 1/2 is shown. Inhibitors of MEK (U0126 and PD98059), along with the phosphatases associated with the ERK pathway (PP1/PP2A, MKP-3 and MKP-1/2), are included in the diagram. Solid arrows represent direct phosphorylation (activation), broken arrows represent indirect activation involving other signalling intermediates, whilst solid square arrows demonstrate inhibition. Diagram is adapted from www.cellsignal.com.

All PKC enzymes appear to be monomeric, possessing either approximately 592 or 737 amino acids and having molecular masses of 67 to 83 kDa (for example, Baier *et al.*, 1993). Many chemical stimulants are widely used to activate the PKC pathway in cells, such as phorbol 12-myristate 13-acetate (PMA) or 12-*O*-tetradecanoyl-phorbol-12-acetate (TPA). Both chemicals act as diacylglycerol (DAG) analogues, thus resulting in the activation of PKC. Physiological substances known to modulate PKC activity include thyroid hormones, prostaglandins, insulin and LPS (Alisi *et al.*, 2004; Li *et al.*, 2004b; Formisano *et al.*, 2000; Valledor *et al.*, 2000).

1.5.2.2 Protein kinase A (PKA) pathway

PKA comprises of four subunits, two of which are catalytic and two regulatory. Upon PKA activation, the catalytic subunits are released which phosphorylate phosphodiesterase, amongst other proteins. The activity of phosphodiesterase causes the hydrolysis of cAMP to AMP, which results in the PKA signal being reduced or terminated (reviewed by Skalhess & Tasken, 2000). Activity of PKA can be mediated by an increase in cAMP concentrations, or direct phosphorylation of the enzyme on serine and threonine residues.

PKA-dependent activation of ERK has been demonstrated in glial cells (Zanassi *et al.*, 2001) and neuronal cells (Bouschet *et al.*, 2003) amongst many other cell types. The activition of ERK by PKA occurs indirectly, involving Ras and PKC. However, ERK inhibition has also been reported in response to PKA activity in cultured fibroblasts (Dhillon *et al.*, 2002)

1.5.2.3 Phosphatidylinositol-3-kinase (PI-3K)

The adaptor protein, PI-3K, is unusual in that it contains catalytic and regulatory subunits. The possession of a catalytic subunit gives the protein kinase activity, enabling it to participate directly in signalling pathways. PI-3K phosphorylates many phosphatidylinositol derivatives, and importantly results in the production of PtdIns(3,4,5)P_3, which is responsible for many basic functions of the cell such as migration and growth (Liu *et al.*, 2004). The phosphatidylinositol pathway in general is thought to be highly important in the regulation of phagocytosis (Garcia-Garcia *et*

al., 2002; Lutz & Correll, 2003). PI-3K has been shown to regulate ERK activity in Swiss 3T3 fibroblasts (Kitamura *et al.*, 2001) and CHO cells (Takeda *et al.*, 1999).

1.5.2.4 Receptor tyrosine kinase signalling to ERK

It is widely known that receptor tyrosine kinase (RTK) activation commonly results in ERK activation. Signalling proteins that bind to activated RTK differ greatly in their structure and function, but most of these proteins appear to possess the highly conserved binding domains, SH2 and SH3 (reviewed by Hubbard, 1999). Such proteins (called linker proteins) that have SH2/SH3 domains serve to link proteins that lack binding domains to the activated RTK. Pathways that are downstream of RTKs are activated through Ras proteins, which belong to the large Ras superfamily of monomeric GTPases (Grosse *et al.*, 2000; Besset *et al.*, 2000). Ras proteins cycle between two distinct conformational states – active when GTP-bound and inactive when GDP-bound. Two classes of signalling proteins regulate Ras activity by influencing their transition between active and inactive states. GTPase-activating proteins (GAPs) increase the rate of hydrolysis of bound GTP by Ras so activating it, whilst guanine nucleotide releasing proteins (GNRPs) activate Ras by promoting the exchange of bound nucleotide by stimulating the loss of GDP and the subsequent uptake of cytosolic GTP. When active, RTKs can bind GAPs directly and GNRPs indirectly. However, the indirect binding to GNRP by RTKs often results in the activity of Ras. Ras activation occurs at the plasma membrane, but only after the protein has undergone farnesylation, which renders the protein hydrophobic. Relaying of the signal downstream requires the activation of kinases such as MAPKs.

The receptor for epidermal growth factor (EGFR) was the first tyrosine kinase receptor to be characterised, and binds EGF which initiates the proliferation of epidermal cells. The receptor is a single-pass transmembrane structure, with a large glycosylated, cysteine-rich extracellular binding region (Hsuan *et al.*, 1989). When EGF is bound to the extracellular region, dimerisation occurs and the intracellular tyrosine kinase domain is activated through autophosphorylation. EGFR activation of ERK has been shown in many cell types including myofibroblasts (Zhang *et al.*, 2003).

1.5.2.5 GPCR signalling to ERK

With over 100 G-protein coupled receptors (GPCRs) fully characterised from mammalian cells (reviewed by Wess, 1997), downstream signalling from these receptors is an ever-developing area of research. GPCRs are responsible for transducing the extracellular signals from a number of messengers including hormones, neurotransmitters and local mediators (e.g. immune associated proteins). The structure of GPCRs appears to be highly conserved, the NH2 terminus is extracellular, whilst the carboxyl terminus is intracellular along with binding domains for trimeric G-proteins to which the receptor is coupled; these G-proteins help create the downstream intracellular signal. When inactive, the G-protein exists as a trimer, but activation causes the α subunit to exchange GDP for GTP causing this subunit to dissociate from the β/γ subunits. GPCR-mediated activation of ERK mainly occurs through Ras, although Ras-independent mechanisms have also been identified. Studies with Chinese hamster ovary (CHO) cells indicate that GPCRs linked specifically to G_i (inhibitory G protein) are capable of activating MEK and ERK through a Ras-independent pathway involving PI-3K and PKC ζ (Takeda *et al.*, 1999). The α chain of G_o proteins have been specifically linked to ERK activation, regulating the activity of ERK through a Ras-independent, PKC and PI-3K-dependent mechanism (Antonelli *et al.*, 2000).

1.5.2.6 Integrin signalling to ERK

Ligation of integrin receptors has also been linked to the upregulation of ERK phosphorylation and activity. Members of the integrin family often comprise two covalently associated transmembrane glycoprotein subunits, α and β. Integrins are important in cellular adhesion, and both subunits are equally important in this function (reviewed by Aplin *et al.*, 1998). Binding of integrins to their ligands is dependent on calcium and magnesium being present in the extracellular environment, and the extracellular portion of the α chain possesses four divalent cation binding regions. The interaction of integrins with the cytoskeleton is extremely important in binding cells to the extracellular matrix. This cytoskeletal linkage occurs with the β-subunit associating with talin and actinin – proteins associated with the focal adhesion points (integrin linkage to actin filaments has been recently reviewed by Calderwood *et al.*, 2000). Indeed, the formation of focal adhesion points is thought to be initiated by this integrin-

focal adhesion protein linkage. Cytoplasmic signalling from integrins is an active area of research, with many groups showing that a wide array of signalling molecules are activated following integrin engagement (see for example, Haller *et al.*, 1998; Campos *et al.*, 2004). Recently, focal contacts were found to possess a protein with tyrosine kinase activity, which was later named focal adhesion kinase (FAK). This protein has been shown to be activated downstream of PI-3K and ERK *via* direct and indirect activation of c-Raf.

FAK signalling in cell motility is an evolving area of research, and was recently reviewed by Schlaepfer & Mitra (2004). Fincham *et al.* (2000) found that active ERK in rat embryo fibroblasts migrates to newly forming cell-matrix adhesions, and that this migration is triggered by integrin engagement and the linker protein, v-Src. Schlaepfer *et al.* (1998) have demonstrated that Grb2-mediated integrin-stimulated signalling pathways also result in ERK activation. Furthermore, upregulation of ERK activity through Grb2 was shown to involve FAK/Shc binding in fibroblast cells (Schlaepfer *et al.*, 1998), although FAK-independent mechanisms have recently been identified in fibroblasts (Barberis *et al.*, 2000). Schlaepfer *et al.* (1994) also showed that Ras is implemented in integrin-mediated signalling to ERK by showing that FAK phosphorylation (on Tyr^{925}) created an SH2-binding site for Grb2, which links integrin engagement to the Ras/ERK pathway. This study was furthered by Schlaepfer & Hunter (1996) who demonstrated that phosphorylation of FAK on Tyr^{397} recruits Src-family kinases, which results in FAK phosphorylation at Tyr^{925} and the formation of a Grb2 SH2-domain binding site, which provides a link to Ras and ERK. By overexpressing FAK in human 293 cells, Schlaepfer & Hunter (1997) found enhanced Ras-dependent integrin signalling to ERK 2 in particular through interactions involving c-Src. Although most studies implicate Ras activity in integrin-mediated signalling, Chen *et al.* (1996) have demonstrated a mechanism whereby integrin-mediated signalling is Ras-independent. This Ras-independent signalling leading from integrin-engagement could be, amongst other possibilities, a result of Rho activation of phosphatidylinositol 4,5-biphosphate production, or FAK acting directly on Raf (Chen *et al.*, 1996). Another study with fibroblasts also provides evidence that integrin-mediated activation of MAPK can occur independently of FAK (Lin *et al.*, 1997), providing further evidence that at least two integrin-mediated signalling pathways exist in fibroblasts. Direct activation of Raf by FAK has previously been demonstrated in

mouse fibroblasts by co-immunoprecipitation upon stimulation with EGF, which interestingly, gives this interaction a physiological relevance (Yujiri *et al.*, 2003). Indeed, Aplin & Juliano (1999) have shown that adhesion-mediated cytoskeletal organisation and focal adhesion complex formation are necessary for efficient EGF activation of ERK isozymes. PKC has also been associated with integrin-mediated signalling during muscle cell spreading (Disatnik *et al.*, 2002), which is thought to be via the phosphorylation of PKC substrates such as the myristoylated alanine-rich C kinase substrate (MARCKS) protein. Many cells, such as myocytes, also exhibit a requirement for PKC activity before phosphorylation of FAK can occur (Disatnik & Rando, 1999).

Nuclear translocation of ERK is extremely important, as this is where the effects of signalling pathways can be seen through the activation of transcription factors. Integrin-mediated adhesion of NIH 3T3 cells has been shown to regulate the translocation of active ERK to the nucleus, along with the phosphorylation of the downstream transcription factor, Elk-1 (Aplin *et al.*, 2001). This provides further evidence that integrin-mediated signalling can regulate the activity of ERK isozymes. A recent study with thyroid TAD-2 cells and the hepatoma cell line, Hep3B, showed that calcium/calmodulin-dependent protein kinase II can bind to Raf-1 and modulate integrin-mediated ERK activation (Illario *et al.*, 2003). Although many studies link FAK phosphorylation with integrin engagement, Achison *et al.* (2001) have found FAK phosphorylation on Tyr^{125} in human platelets to be integrin-independent when stimulated by collagen.

1.5.3 Interaction of MEK and ERK

Similarly to ERK, two isoforms of MEK exist and each is able to phosphorylate ERK. It has been found that MEK and ERK are present in equal concentrations in some cells (Ferrell, 1996), suggesting that unlike most other kinase interactions, this step in the ERK pathway is not providing amplification. MEK needs to be activated itself before it can act downstream on ERK since activated Raf is not capable of phosphorylating ERK directly (Xu *et al.*, 1995). It was speculated that a MEK 1 proline-rich insert was the site of Raf-MEK interaction. However, Dang *et al.* (1998) found that that although this insert provides modulation for the binding of MEK to Raf, it is not the actual site

of interaction. Indeed, the proline-rich insert was found to be essential for the efficient activation of ERKs by MEK (Dang *et al.*, 1998). Docking sites on MEK have been characterised, and have been found to be necessary for MEK/ERK complex formation and subsequent activation of ERK (Bardwell *et al.*, 2001).

To enable MEK phosphorylation of ERK, these enzymes form a short-lived complex within the cytoplasm. MEK 1 and MEK 2 are found exclusively in the cytoplasm, which is where the dual phosphorylation of ERK occurs (Zheng & Guan, 1994). The cytoplasmic localisation of MEK is due to the presence of a nuclear export signal (NES) sequence near the N-terminal region of the protein (Fukuda *et al.*, 1996; Fukuda *et al.*, 1997a). ERK is located in the cytoplasm of unstimulated fibroblasts through its specific association with MEK. Furthermore, nuclear accumulation of ERK is coupled with the dissociation of cytoplasmic MEK/ERK complexes, thus MEK acts as cytoplasmic anchor for ERK due to the NES (Fukuda *et al.*, 1997). The dissociation of MEK/ERK complexes appears to be irrespective of the ERK phosphorylation status and Fukuda *et al.* (1997b) have speculated the MEK/ERK complex formation may be partially due to ERK phosphorylating MEK as part of a feedback loop.

1.5.4 Translocation of activated ERK

The entry of active ERK isozymes into the nucleus is dependent on the synthesis of short-lived proteins, which are induced by the activity of ERK 1/2. These proteins act like nuclear anchors, and could be MAPK-specific phosphatases, a set of transcription factors or a specific component of the ERK signalling cascade (Lenormand *et al.*, 1998).

ERK not only migrates to the nucleus but also translocates to actin filaments, that regulate cell shape changes. The ability of ERK to induce cell shape changes such as those seen in cell migration is often initiated by integrin engagement or growth factor stimulation. The association of ERK with the microtubule cytoskeleton has been reported in NIH 3T3 mouse fibroblasts, cardiac muscle cells and PC12 cells (Reszka *et al.*, 1995; Morishima-Kawashima *et al.*, 1996; Thorburn *et al.*, 1997). Indeed, Klemke *et al.* (1997) report a transcription-independent mechanism of enhanced cell migration upon the upregulation of ERK activity. Moreover, both ERK isozymes were found to

be capable of directly phosphorylating myosin light chain kinase *in vitro*, leading to enhanced myosin light chain phosphorylation (Klemke *et al.*, 1997).

1.6 INTRACELLULAR SIGNALLING IN MACROPHAGES AND HAEMOCYTES

1.6.1 Signalling in macrophages

Either primary or cultured cells have been used to study the signalling pathways within macrophages. Research using primary cultures has largely been achieved with macrophages obtained from the bone marrow of mice, whereas that done on cultured cells has generally been with RAW 264.7 or J774A.1 cell lines. Studies with primary macrophages can provide a more realistic picture of the signalling occurring *in vivo*. There have been many studies focusing on ERK signalling within bone marrow derived primary macrophages. Unlike the invertebrate immune system, the mammalian immune system is easier to study at the molecular level due to use of knockout mice, molecular tools such antibodies, and pharmacological inhibitors that have been developed to predominantly target mammalian forms of signalling enzymes and cell surface receptors.

1.6.1.1 Studies on primary macrophages

Winston *et al.* (1995a; 1995b) found the ERK pathway to be activated in a Raf-independent fashion when bone marrow-derived primary macrophages were stimulated with tumour necrosis factor-α (TNF-α). Indeed, these workers also found that it was ERK 2 (p42) that was preferentially activated by MEK 1. Subsequent studies revealed that p38 MAPK is also activated by TNF-α, via a pathway dependent on the upstream enzymes, mitogen-activated protein kinase kinase 3 (MKK3) and MKK4 (Winston *et al.*, 1997). SHP-1, a tyrosine specific phosphatase, is activated in macrophages via Ras during ERK signalling, and the activity of this phosphatase is indeed necessary for ERK activation (Krautwald *et al.*, 1996). Using bone marrow-derived primary macrophages deficient of Rac1 activity, Wells *et al.* (2003) found that macrophages had difficulty spreading and exhibited suppressed membrane ruffling. However, the absence of this GTPase did not appear to have a negative effect on cell migration, which indicates that this enzyme is principally involved in the regulation of macrophage shape (Wells *et al.*, 2003). PKC ζ-dependent activation of ERK 1/2 by LPS has been demonstrated in primary alveolar macrophages, and PKC ζ activation appeared to occur via PI-3K (Monick *et al.*, 2000). Primary peritoneal macrophages

do, however, appear to possess both PKC-independent and –dependent mechanisms for activating ERK (Qiu & Leslie, 1994). LPS has also been demonstrated to activate the ERK pathway in primary human monocytes (van der Bruggen *et al.*, 1999). Futhermore, these cells are able to phagocytose zymosan particles, *via* β_2 integrins, and p53 and p56 phosphoproteins, that are thought to mediate monocyte cytoskeletal arrangements necessary for internalisation (Zaffran *et al.*, 1995). Interestingly, G proteins can associate with CD14 (an LPS receptor), and this interaction has been shown to activate the ERK pathway in human monocytes (Solomon *et al.*, 1998). Moreover, the PI-3K/ERK pathway has been shown to play a role in NF-κB activation, but not phagocytosis, by cells of the THP-1 monocytic cell line (García-García *et al.*, 2001), and in the differentiation of monocytes to macrophages (García-García *et al.*, 2002). These studies have revealed the intricate nature of ERK signalling in primary macrophages and have played a key role in helping establish a function for this pathway in these cells.

1.6.1.2 Studies on cultured macrophage cell lines

Most studies with cultured cells utilise the RAW 264.7 macrophage cell line. Chen & Wang (1998) found that p38 MAPK, but not ERK, is involved in the production of inducible nitric oxide synthase (iNOS) following LPS stimulation in RAW 264.7 macrophages, which was supported by a more recent study confirming that ERK is not involved in either nitric oxide or interleukin production (Watters *et al.*, 2002). However, LPS does induce tyrosine phosphorylation in this cell line particularly in ERK (Weinstein *et al.*, 1992), and proline-directed kinases (Sanghera *et al.*, 1996). The association of active ERK with microtubules has been observed in RAW 264.7 cells, which were stimulated by LPS, suggesting a role for this enzyme in cytoskeletal dynamics (Ding *et al.*, 1996). Büscher *et al.* (1995) found that ERK can be activated by Ras-independent and –dependent mechanisms within RAW 264.7 macrophages. As macrophages are the main phagocytic cell of the innate immune system, many studies have aimed to decipher the role of signalling pathways in the phagocytic response. Phosphoinositol signalling is believed to play an important role in phagocytosis (reviewed by Gillooly *et al.*, 2001). Indeed, phosphatidylinositol-4,5-bisphosphate (4,5-PIP_2) is essential for phagocytosis, in that it is involved in the initial recruitment of

actin to the phagocytic cup (Botelho *et al.*, 2000). Furthermore, PKCα has also been implicated in phagocytosis in RAW 264.7 cells (Breton & Descoteaux, 2000).

Another macrophage cell line is the J774.A1 cell line which is of murine origin. Exposure of these cells to LPS results in the activation of p38 MAPK along with GTPases via the β2 integrin subunit (Schmidt *et al.*, 2001). This compound has also been shown to activate PI-3K, phospholipase D and ERK in the BAC-1.2F5 macrophage cell line (Procyk *et al.*, 1999). Studies with J774.A1 cells demonstrated that internalisation of the bacterium *Yersinia enterocolitica* deactivates all MAPK pathways (Ruckdeschel *et al.*, 1997), and also modulates the activity of the focal adhesion kinases, Cas, FAK and Pyk2 (Bruce-Staska *et al.*, 2002). Finally, Ras has been extensively studied in J774.A1 cells, and has been shown to be important in the regulation of cell growth and adhesion (Self *et al.*, 2001).

1.6.2 Signalling in haemocytes

In contrast to mammalian macrophages, the signalling pathways employed by haemocytes have been largely unstudied until recently. Given the similarities between haemocytes and macrophages in their morphology and function, it is possible that haemocytes possess similar signalling proteins to macrophages and transduce signals through similar intracellular pathways.

MAPKs have been identified in haemocytes from many invertebrate species. Probably most significantly, the *D. melanogaster* extracellular signal-regulated kinase homologue was fully characterised and its primary structure determined in the early 1990s (Biggs & Zipursky, 1992). In a similar way to mammalian ERK isozymes, activity of the *Drosophila* homologues requires dual phosphorylation at both tyrosine and threonine residues, with this phosphorylation following ligation of a RTK which is probably an LPS receptor (Biggs & Zipursky, 1992). ERK has also been identified, although not characterised in *M. galloprovincialis* (Canesi *et al.*, 2002b), and *C. capitata* (Foukas *et al.*, 1998; Soldatos *et al.*, 2003). Recently, Soldatos *et al.* (2003) found that ERK activity was upregulated in medfly haemocytes, when challenged with cell-free LPS, through two mechanisms, one involving Ras and the other involving PI-3K. Furthermore, the increased ERK activity caused changes in medfly haemocyte

morphology. Earlier studies with medfly haemocytes found that protein-tyrosine phosphorylation was necessary for internalisation of cell-free LPS (Charalambidis *et al.*, 1996b), and also for the exocytosis of recognition proteins from these haemocytes (Charalambidis *et al.*, 1995). An important study by Foukas *et al.* (1998), also on medfly haemocytes, showed that it was the activation of ERK 1/2 that was necessary for attachment and internalisation of *Escherichia coli*; this ERK activation was found to be Ras-dependent, and the β_3 integrin subunit was found to be important for the internalisation process. Finally the p38 MAPK, ERK and PI-3K signalling pathways have been detected within *M. galloprovincialis* haemocytes, with upregulation of all these signalling cascades occurring upon stimulation of haemocytes with *E. coli* (Canesi *et al.*, 2002a, 2002b).

A number of studies have been carried out on haemocyte signalling resulting from noradrenaline and adrenocorticotropin hormone (ACTH) stimulation. Although the function of these molecules is better known in mammalian systems, noradrenaline in particular is an important factor in invertebrate immune and stress responses (Lacoste *et al.*, 2001; 2002). Studies with *G. gigas* have shown that, when stressed, noradrenaline and dopamine are released into the circulation (Lacoste *et al.*, 2002) and Lacoste *et al.* (2001d) demonstrated that the neuropeptide ACTH induced significant noradrenaline release. ACTH also induces cell shape changes in haemocytes from *M. galloprovincialis*, and using pharmacological inhibitors it was found that ACTH causes these changes *via* adenylate cyclase/cAMP/PKA and PKC pathways (Sassi *et al.*, 1998). cAMP has also been shown to mediate cell shape changes in *M. galloprovincialis* haemocytes in a study by Malagoli *et al.* (2000), who suggested that inositol pathway may play a role in this process. Ottaviani *et al.* (1991) showed that by exposing haemocytes from the freshwater snail, *Planorbarius corneus*, to ACTH, phagocytosis of *Staphylococcus aureus* was significantly increased. Modifications of the haemocyte cytoskeleton from the gastropod, *Viviparus ater*, were also caused by the exposure of these cells to ACTH (Franchini & Ottaviani, 1994). These studies highlight the interplay that occurs between ACTH, signalling pathways and morphological changes in molluscan haemocytes.

The majority of studies looking at the effect of noradrenaline on haemocytes have utilised the oyster, *C. gigas*, and have been performed in the laboratory of Lacoste.

Exposure of *C. gigas* haemocytes to noradrenaline has been shown to result in decreased phagocytosis, which appears to be regulated via a β-adrenergic receptor/cAMP/PKA signalling pathway (Lacoste *et al.*, 2001b). Furthermore, *C. gigas* exposure to noradrenaline also results in suppressed reactive oxygen species production, which is of interest due to the importance of these intermediates in defence reactions (Lacoste *et al.*, 2001b). Moreover, this response appears to involve similar signalling pathways to those regulating phagocytosis (Lacoste *et al.*, 2001b). Noradrenaline and α-adrenergic signalling also induce the hsp70 gene promoter in *C. gigas* haemocytes, which, with the use of pharmacological inhibitors, was found to be dependent on the G-proteins/PLC/PKC and PI-3K (Lacoste *et al.*, (2001d). Apoptosis of mollusc immune cells was also investigated by Lacoste *et al.* (2002b), who found that noradrenaline induces haemocyte apoptosis probably via the β-adrenergic/Ras/ERK pathway. Catecholamines, including noradrenaline, have also been identified in larvae and juveniles of the gastropod, *Crepidula fornicata*, where they have been implicated as possible regulators of metamorphosis (Pires *et al.*, 2000).

Integrin receptors in haemocytes have been the focus of many recent studies. The integrin α subunit from haemocytes of the ascidian *H. roretzi* has recently been fully cloned and characterised (Miyazawa *et al.*, 2001). These haemocyte integrin receptors probably function as an ancestral form of complement receptors found in vertebrate systems, further suggesting that a primitive complement system exists in ascidians (Miyazawa *et al.*, 2001). Ballarin *et al.* (2002) recently confirmed the presence of integrin-like molecules on haemocytes from another ascidian, *Botryllus schlosseri*. Some time ago, Marcantonio & Hynes (1988) developed antibodies specific to the cytoplasmic domain of integrins, which recognise the β integrin subunit from a variety of vertebrates, invertebrates and fungi, demonstrating the high degree of conservation in this integrin domain. Cloning of the β_1-integrin subunit from the freshwater mollusc, *Biomphalaria glabrata* has also been achieved (Davids *et al.*, 1999). This study demonstrated a high degree of homology between mammalian and *B. glabrata* integrin structure. The integrin related kinase, FAK, has recently been shown to be expressed and phosphorylated in sea urchins (García *et al.*, 2004). Furthermore, the FAK/Src complex has been shown to play a specific role in the phagocytosis of *E. coli* by *C. capitata* haemocytes (Metheniti *et al.*, 2001). Interestingly, molecular characterisation has recently been carried out on a G protein-coupled receptor from a bivalve mollusc,

and the findings clearly indicate evolutionary links with vertebrate signalling (Herpin *et al.*, 2004).

Most studies on haemocyte signalling pathways to-date have employed monoclonal and polyclonal antibodies raised against human or rodent protein sequences, which are capable of recognising invertebrate proteins. Immunoreactivity with invertebrate signalling proteins is a consequence of these antibodies being developed to recognise highly conserved sequences, such as activation domains, in their target proteins. Furthermore, pharmacological inhibitors have also been successfully employed in many studies on invertebrates to block signalling components, allowing cellular pathways to be dissected with relative ease.

1.7 Summary and Aim of Study

Although molluscs are important intermediate hosts for many helminth parasites, the immune responses of molluscs are relatively understudied when compared to those of mammals. The main defence cell of molluscs is the haemocyte, which is functionally and morphologically similar to the mammalian macrophage. Although studies on haemocyte signalling pathways are relatively new, current evidence suggests that haemocytes share many biochemical pathways with vertebrate cells. Indeed, the ERK pathway has been shown to exist and be activated in the defence cells of a range of invertebrate groups including insects (e.g. Foukas *et al.*, 1998) and molluscs (e.g. Canesi *et al*, 2002a; 2002b), demonstrating that this is indeed an important pathway in phagocytosis cells, as it is in mammalian macrophages. However, studies in various vertebrate cell types have shown that phagocytosis can be either ERK-independent or –dependent (Weinstein *et al.*, 1992; Sanghera *et al.*, 1996; Ruckdeschel *et al.*, 1997; Procyk *et al.*, 1999; Monick *et al.*, 2000; Garcia-Garcia *et al.*, 2001).

This study aimed to clarify the role of the MAPK/ERK pathway in *L. stagnalis* primary haemocytes. Since the conditions for haemocyte culture were not yet optimised in our laboratory, the first task was to find the optimum temperature, saline solution and incubation periods for working with these cells. The ERK pathway was then characterised in these cells with the use of polyclonal antibodies, since ERK isozymes had not previously been reported in *L. stagnalis* haemocytes. Challenge of haemocytes with various endotoxins and compounds enabled the activation status of ERK isozymes to be determined following challenge, allowing a clearer picture of ERK signalling to be developed. Pharmacological inhibitors were also employed throughout the study to dissect the upstream processes that regulate ERK activation, and combined with phagocytosis assays, help clarify the role of ERK in phagocytosis by haemocytes. Integrin receptors and FAK have been implicated in the insect haemocyte phagocytic response, and the present study also aimed to elucidate a role for integrins in *L. stagnalis* haemocytes and demonstrate the presence of FAK or FAK activation in these cells.

Since *L. stagnalis* is an intermediate host for *T. ocellata*, carbohydrates that mimic schistosome coat components were introduced to the haemocytes to see whether or not

they modulated the molluscan immune response and haemocyte signalling pathways. These experiments were done in the presence and absence of haemolymph to assess the role of serum proteins in the molluscan defence response.

1.7.1 Experimental strategy

To fulfil the above aims, the experimental strategy outlined below was followed:

Preliminary studies with *L. stagnalis* haemocyte monolayers

- Determination of optimal conditions for the study of cell signalling in haemocytes
- Detection of ERK in haemocytes

Dissection and function of the ERK pathway in *L. stagnalis* haemocytes and activation of ERK isozymes

- Determine the effect of various compounds, (e.g. cell-free LPS, zymosan A, catecholamines, and heat-fixed *E. coli*) on the activity of the haemocyte ERK pathway
- Confirm that the immunoreactive ERK protein possesses kinase activity with the use of a non-radioactive kinase activity assay
- Dissection of upstream pathways leading to ERK activity using pharmacological inhibitors
- Using pharmacological inhibitors and FITC-conjugated *E. coli* determine the role of ERK and its upstream proteins in phagocytosis by haemocytes

The effect of albumin-linked monosaccharides on *L. stagnalis* haemocyte signalling

- Determine the effect of haemocyte exposure to BSA-conjugated galactose and fucose on ERK activity, PKC activity and phagocytosis, and assess the role of serum proteins in any identifiable responses

Detection of integrin subunits and FAK in *L. stagnalis* haemocytes

- Detect integrins on the haemocyte cell surface using integrin-mediated binding assays

- Detect FAK in haemocytes and determine the regulation of FAK phosphorylation with an integrin binding tetrapeptide
- Determine the role of both integrins and actin remodelling in haemocyte phagocytosis

CHAPTER TWO

MATERIALS AND METHODS

2.1 Materials

The anti-phospho p44/42 MAPK, anti-phospho MEK 1/2, anti-MEK 1/2, anti-p44/42 MAPK, anti-phospho Elk-1, anti-phospho FAK (Tyr^{925}) antibodies, and the MEK inhibitor U0126 were purchased from New England Biolabs (NEB; Hertfordshire, UK). Anti-active MAPK and anti-ERK 1/2 antibodies, PD98059 and goat-anti-rabbit HRP-conjugated secondary antibody were from Promega (Southampton, UK). Protogel [30% (w/v) acrylamide] was purchased from National Diagnostics (Hull, UK), whereas Hybond nitrocellulose membrane and ECL hyperfilm were from Amersham Biosciences (Amersham, UK). Phosphate Buffered Saline (PBS) tablets were from Oxoid (Hampshire, UK), the Opti-4CN substrate kit along with the DC protein assay kit were from BioRad (California, USA), and the anti-pan ERK antibody was from Transduction Laboratories (Oxford, UK). ECL developing reagent and fixing reagent were both from Kodak (Hemel Hempstead, UK) whilst Vectashield was purchased from Vector Laboratories (Peterborough, UK). The integrin-mediated binding assay kits were purchased from Chemicon International (California, USA). LY294002, KT5720, FTase inhibitor I, anti-phospho-FAK (Tyr^{397}) and Adrenocorticotropin Hormone (ACTH) were from Calbiochem (Nottingham, UK). Liquid PBS (supplemented with calcium and magnesium), RPMI 1640 and foetal bovine serum (FBS) were purchased from Gibco-Invitrogen Corporation (Paisley, UK). Sodium dodecyl sulphate (SDS), β-mercaptoethanol, glycerol, bromophenol blue, sodium chloride, tween-20, glycine and Tris-(hydroxymethyl)-methylamine were all obtained from VWR International Ltd (Poole, UK). FITC-conjugated BSA-fucose and BSA-fucose were purchased from EY Laboratories (Buckingham, UK). GF109203X, FITC-conjugated goat-anti-rabbit antibody, phosphatase and protease inhibitor cocktails, trypan blue, N,N,N',N'-tetramethylethylenediamine (TEMED), FITC-conjugated *E. coli* ("bioparticles"), FITC-conjugated BSA, FITC-conjugated BSA-galactose, zymosan A from *Saccharomyces cerevisiae*, *E. coli* lipopolysaccharides (LPS, serotype 0111:B4), ammonium persulphate (APS), bovine serum albumin (BSA), molecular weight markers (SDS-6H), dimethyl sulphoxide (DMSO), albumin-1-amido-1-deoxy-L-fucose, albumin-2-amido-2-deoxy-D-galactose, noradrenaline and all other chemicals were from Sigma (Poole, UK).

2.1 Lymnaea stagnalis *cultures*

Laboratory cultures of *L. stagnalis* were reared from eggs produced by adult snails purchased from Blades Biologicals (Edenbridge, UK). Eggs were collected and placed into water that had been filtered through a Brimak/carbon filtration unit (Silverline Ltd, Winkleigh, UK) and were left undisturbed until they had hatched. Once hatched, juvenile snails (shell length of 1-5mm) were transferred to larger aquaria to allow further development. These snails were kept at room temperature in tanks containing filtered water, until they reached a shell length of approximately 20-30mm. They were then transferred to an environmental chamber (Sanyo) and kept under a 12 h light-dark cycle at 20°C. Tanks within the environmental chamber had approximately 10 litres of filtered, continuously aerated water and each contained 12 snails. Tanks were cleaned every week and the water partially changed (retaining approximately 20% of the original water). This approach seemed to reduce snail mortality rates. Any dead snails were removed from the tanks on a daily basis, and were replaced with live snails each week. All snails were fed washed, fresh lettuce *ad libitum*. Only snails that had been in the incubator for at least ten days were used for experiments.

2.2 Haemolymph collection and monolayer preparation

Adult *L. stagnalis* were removed from the incubator and were washed with distilled water, and dried with tissue paper. The foot of the snail was then touched repeatedly with a pipette tip until the snail's body withdrew back into its shell and haemolymph was expelled through the haemal pore. This procedure is known as head-foot retraction (Sminia *et al*, 1972). Haemolymph was then collected from the shell cavity, transferred to a sterile tube and kept on ice where it was then diluted with an appropriate amount of Sterile Snail Saline (SSS; 3mM Hepes, 3.7mM NaOH, 36mM NaCl, 2mM KCl, 2mM $MgCl_2$, 4mM $CaCl_2$, pH 7.8, sterilised through a 0.22μm disposable filter) (1 part SSS : 3 parts haemolymph). Haemolymph from several snails was pooled to obtain sufficient haemolymph for each individual experiment.

To obtain total protein extracts, diluted haemolymph was centrifuged for 5 min at 800g, and the supernatant removed. The cell pellet was then either homogenised on ice in

100µl extraction buffer (50mM Tris-HCl (pH 7.4), 150mM NaCl, 1% (v/v) Triton X-100, 0.1% SDS, 0.25mM EDTA, 0.25% (w/v) deoxycholic acid, 0.25M sucrose) containing a protease and phosphatase inhibitor cocktail used at the manufacturers recommended strength, or was solubilised in boiling SDS-PAGE sample buffer (62.4mM Tris-HCl (pH 6.8), 2% (w/v) SDS, 2% (v/v) β-mercaptoethanol, 5% (v/v) glycerol, 0.003% (v/v) bromophenol blue). Protease inhibitors were used to prevent endogenous proteases from degrading proteins present in the cell lysate. The protease inhibitor cocktail contained 4-(2-aminoethyl) benzenesulfonyl fluoride hydrochloride (AEBSF), bestatin, *trans*-epoxysuccinyl-L-leucylamide-(4-guanidino) butane (E-64), leupeptin and aprotinin which is an ideal combination for the inhibition of serine, cysteine and metalloproteases. Phosphatase inhibitors were added to samples to prevent the dephosphorylation of phosphorylated proteins (this is important when probing cell extracts for phospho-proteins such as phosphorylated ERK). The phosphatase inhibitor cocktail comprised sodium orthovanadate, sodium molybdate, sodium tartrate and imidazole which are capable of inhibiting acid and alkaline phosphatases, as well as phosphate transferring enzymes.

When haemocyte monolayers were required for experiments they were prepared by allowing cells to adhere to individual wells of culture plates (Nunc) for 30 min at room temperature. When 6-well plates were used 500µl of diluted haemolymph was added per well, whereas when 24-well plates were used 200µl haemolymph was added. The diluted haemolymph contained approximately 3×10^6 viable cells per ml, determined by cell counting with a haemocytometer. Following the binding period, monolayers were washed three times with 200µl SSS (24-well plates) or 500µl SSS (6-well plates) prior to further treatment/equilibration at room temperature. Almost all of the haemocytes adhered during the 30 min binding stage since few cells were observed in the SSS that was removed at the first wash step.

2.4 Determination of optimum conditions for experiments with haemocyte monolayers

2.4.1 Use of RPMI, Alsever solution, anti-clumping buffer and SSS

Haemocyte monolayers were prepared in 6-well plates (Nunc) as described in section 2.3. Monolayers were either prepared in SSS, Alsever solution (27mM sodium citrate, 336mM sodium chloride, 115mM glucose, 9mM EDTA; pH 4.6), RPMI or anti-clumping buffer (20mM Tris, 45mM sodium chloride, 10mM EDTA, 50mM sodium citrate, 25mM sucrose; pH 7.3). At appropriate time points haemocyte morphology was observed using an inverted microscope (at magnification of x10 and x40). The degree to which the haemocytes appeared vacuolated, spread or adherent was then recorded. After the cells were observed, they were lysed with boiling SDS-PAGE sample buffer for 10 min and the soluble proteins were then electrophoresed immediately or were kept at -20°C for subsequent analysis (section 2.13).

2.4.2 Effect of temperature on L. stagnalis *haemocyte ERK 1/2*

Haemocyte monolayers were prepared in 6-well plates (Nunc) using SSS as described in section 2.3. During the 30 min binding period, plates were placed at 20°C, 30°C or ambient temperature (25°C). The cells were then lysed with 500µl extraction buffer on ice for 5 min and protein determination was performed on all samples as described in section 2.6. An appropriate amount of 5x SDS-PAGE sample buffer was then added to the extracts and they were boiled for 5 min. Samples were subsequently electrophoresed on an SDS-PAGE gel and Western blotted as described in section 2.13 with membranes subsequently probed the with anti-phospho p44/42 MAP kinase antibody.

2.5 Trypan blue cell viability assay

Haemolymph was collected as described in section 2.3 and haemocyte monolayers were prepared in wells of a 24-well plate. Haemocytes were subsequently washed with SSS three times and 150µl SSS was then added to half of the wells containing haemocyte monolayers. The remaining monolayers were then incubated with 150µl trypan blue for

5 min at room temperature. After this incubation period, 100 cells were counted at random in each well that contained trypan blue. Cells which had not taken up the dye (appearing as white) were classified as viable, whereas those which had taken up the dye (appearing as blue) were classified non-viable. This trypan blue exclusion test was repeated after 40 min with the monolayers that had been incubated in SSS; the SSS was removed and 150µl trypan blue added to each well prior to viability determination.

2.6 Protein determination

Protein assays were carried out on cell lysates that had been solubilised in extraction buffer to determine the protein concentration of samples in preparation for SDS-PAGE and Western blotting. Protein determination was done using the DC protein assay kit following the manufacturers microplate assay protocol. Briefly, 5µl of cell lysate was placed into a well of a 96-well microtitre plate and 25µl of working solution 'A' (containing 20µl of reagent S and 980µl of reagent A) was added prior to adding 200µl of solution B. Following 15 min incubation at room temperature, the absorbance of the samples was determined using a microplate spectrophotometer and a 750nm absorbance filter. All lysates were assayed in triplicate along with extraction buffer controls. A standard curve was produced for a range of BSA samples with known concentrations (0, 0.2, 0.4, 0.6, 0.8 and 1.0mg/ml) using the same protocol and the protein concentrations in the test samples were determined using the standard curve.

2.7 Challenge of L. stagnalis *haemocytes with bacterial and yeast components*

Haemocyte monolayers were prepared as described in section 2.3, in individual wells of 6-well or 24-well culture plate (Nunc). After washing, the cells were left to equilibrate for 15 min in SSS at room temperature. Once the various treatments had been applied (*E. coli* LPS, heat-killed *E. coli* and zymosan A; sections 2.7.1 – 2.7.3, below), cells were lysed on ice with extraction buffer, sample protein determinations were then done as described in section 2.6. Extracts were then transferred to microfuge tubes and either stored at -20°C or electrophoresed immediately as described in section 2.13.

2.7.1 Challenge with E. coli *LPS*

Escherichia coli LPS (1 - 10μg/ml in SSS) was incubated with haemocyte monolayers in 24-well culture plates (prepared as described in section 2.3) for various time periods (0-20 min) at room temperature. Control monolayers were exposed to SSS alone. The cells were then lysed with 200μl homogenisation buffer for 5 min on ice with agitation, protein determinations were done and the samples were processed for Western blotting.

2.7.2 Challenge with heat-killed E. coli

Cultures of *E. coli* were maintained in nutrient broth at 37°C, and the number of bacteria determined with a haemocytometer. An appropriate amount of nutrient broth (with the amount depending on the number of cells) was removed and placed at 80°C for 1 h to heat-kill the bacteria. To check that all the bacteria had been heat-killed, 100μl of the heated nutrient broth was spread onto nutrient agar (Oxoid) and checked for growth after an overnight incubation at 37°C. Haemocyte monolayers were then prepared as described in section 2.3, in individual wells of 6-well culture plates. While haemocytes were being incubated in SSS for 15 min after the binding period, 1 ml of heat-killed *E. coli* was centrifuged for 5 min at 3000*g*, the supernatent was removed and was replaced with SSS before a final cell count was done with the haemocytometer. Based on the assumption that 3 x 10^6 haemocytes are bound per ml of haemolymph (determined by previous cell counts), the amount of *E. coli* solution was adjusted (with SSS) so that the *E. coli* would be delivered in either a 20:1 or 40:1 ratio to the haemocytes. Cells were then exposed to *E. coli* for 2.5, 5, 10, 20 and 40 min before being lysed with 500μl extraction buffer for 10 min on ice. Protein determinations were then performed as described in section 2.6, and the samples were processed for Western blotting as described in section 2.13.

2.7.3 Challenge with Zymosan A

Haemocyte monolayers were prepared as in section 2.3, in individual wells of 24-well culture plates (Nunc). Zymosan A from *Saccharomyces cerevisiae* (0.2mg/ml in SSS) was then added to monolayers and incubated for various times (0-20 min) at room temperature (control monolayers contained SSS alone). Cells were lysed with

extraction buffer for 5 min on ice with agitation. Protein determination was then done and the samples were processed for Western blotting (section 2.13).

2.8 The effects of MEK inhibitors on ERK activation

Haemocyte monolayers were prepared as described in section 2.3 in individual wells of a 24-well culture plate (Nunc). After the cells were washed they were incubated with either U0126 (0-10μM), PD98059 (0-100μM) or the vehicle (methanol or DMSO, respectively) for 30 min at room temperature. After this 30 min incubation period, cells were challenged with *E. coli* LPS (1-10μg/ml) for 5 min at room temperature, in the presence of the inhibitor. U0126 was reconstituted in methanol, whilst PD98059 was resuspended in DMSO; final inhibitor concentrations were made by further dilution in SSS. Following challenge, cells were lysed with boiling SDS-PAGE sample buffer as described in section 2.7. Samples were then either processed immediately or were stored at -20°C prior to electrophoresis and Western analysis (section 2.13).

2.9 BSA-conjugated monosaccharides

Haemocyte monolayers were prepared as described in section 2.3 in individual wells of a 24-well culture plate (Nunc). After washing, the cells were equilibrated for 15 min in 200μl SSS which was removed before the addition of either albumin-1-amido-1-deoxy-L-fucose (0-800nM in SSS), albumin-2-amido-2-deoxy-D-galactose (0-800nM in SSS), a combination of the two sugars (1:1 i.e. 800nM + 800nM in SSS), or BSA control (800nM in SSS) to the wells for 1 hour. These carbohydrate conjugates comprised 15-25 sugar molecules per BSA molecule and concentrations used represent the final molarities of the conjugated BSA in the assays. Initially, control cells were incubated with BSA at all concentrations, or SSS, and under these conditions, the control cell levels of phospho-ERK were determined by Western analysis. Phosphorylation levels did not change significantly between BSA concentrations or SSS, and therefore the highest concentration (800nM) of BSA was used as a control for all subsequent experiments. The 1 h incubation period was determined from preliminary experiments in which four time periods over 90 min were tested (0, 15, 30, 60, 90 min). Although the effects on the phosphorylation levels observed were greatest after 60 min, there were no large differences between 15 – 90 min. Cells exposed to albumin-linked sugars in the

presence of haemolymph were not washed following binding, and the sugars were therefore diluted into the haemolymph. Following the 1 h incubation period, cells were lysed with 100µl of boiling SDS-PAGE sample buffer, and were sonicated and heated to 90°C (for 3 min each). Samples were then stored at -20°C or were electrophoresed immediately (section 2.13).

2.10 The effect of noradrenaline and Adrenocorticotropic Hormone (ACTH)

Haemocyte monolayers were prepared as described in section 2.3 in individual wells of a 24-well culture plate (Nunc). Cells were washed, then incubated with either noradrenaline (0-10µM; reconstituted in SSS) for 0-40 min, or with fragment 1-24 of human ACTH ($3x10^{-19}$ – $3x10^{-4}$M; reconstituted in 5% acetic acid and final concentrations made with further dilutions in SSS) for 2 h at room temperature. Control haemocytes were incubated with vehicle alone. Haemocytes were then lysed immediately with extraction buffer, or were challenged with *E. coli* LPS (1-10µg/ml) for a further 5 min prior to cell lysis. Protein determinations were performed as described in section 2.6 before sample buffer was added. Samples were transferred to microfuge tubes and electrophoresed immediately or stored at -20°C (section 2.13).

2.11 Levels of phosphorylated FAK in suspended and bound haemocytes

Haemocyte monolayers were prepared in 6-well culture plates (Nunc, 500µl diluted haemolymph per well) as described in section 2.3. Cells were left to bind to the plates for 30, 60 and 90 min before the haemolymph was removed and the haemocytes were lysed with boiling SDS-PAGE sample buffer. The collected cell lysates were then transferred to microfuge tubes and were sonicated for 3 min before heating to 90°C for a further 3 min in preparation for electrophoresis (section 2.13).

2.12 Effect of Arg-Gly-Asp-Ser (RGDS) on ERK and FAK activity

Haemocyte monolayers were prepared as described in section 2.3 and were washed three times with 200µl SSS after the 30 min binding period to remove dead and non-adherent cells. Haemocyte monolayers were then left to equilibrate in SSS for 30 min prior to the addition of RGDS (2mM) for various time periods (0-2 min for FAK and 0-

30 min for ERK activation). At the appropriate time points, the SSS/RGDS was removed and cells were lysed in boiling SDS-PAGE sample buffer. Cell lysates were then transferred to microfuge tubes, were sonicated for 3 min and heated to 90°C for 3 min. Samples were then electrophoresed immediately or were stored at -20°C (section 2.13).

2.13 SDS-PAGE and Western blotting of haemocyte proteins

Samples (containing equal amounts of protein where appropriate) were loaded onto a discontinuous SDS-PAGE slab gel. The resolving gel contained 10% acrylamide (375mM Tris-HCl (pH 8.8), 30% (v/v) acrylamide (30% stock solution), 0.1% (w/v) SDS, 0.25% (v/v) TEMED, 0.3% (w/v) APS), with the proportions of acrylamide and water changing accordingly for higher or lower gel concentrations. The stacking gel (125mM Tris-HCl (pH 6.8), 21.15% (v/v) acrylamide (30% stock solution), 0.1% (w/v) SDS, 0.25% (v/v) TEMED, 0.3% (w/v) APS) had the same concentration of acrylamide regardless of the acrylamide concentration in the resolving gel. Molecular weight markers (SDS-6H; 10µl per lane) comprising the proteins, myosin (from rabbit muscle; 205kDa), β-galactosidase (from *E. coli*; 116kDa), phosphorylase b (from rabbit muscle; 97.4kDa), bovine albumin (66kDa), egg albumin (45kDa) and carbonic anhydrase (from bovine erythrocytes; 29kDa) were also applied to the gel. After electrophoresis at 100V for approximately 60 min in running buffer (0.3% (w/v) Tris, 1.45% (w/v) glycine, 0.1% (w/v) SDS), proteins were electroblotted onto Hybond nitrocellulose membrane (0.45µm pore size), using a semi-dry transfer unit (BioRad) with blotting buffer (0.3% (w/v) Tris, 1.5% (w/v) glycine, 20% (v/v) methanol). Homogeneous transfer of proteins was confirmed by staining membranes with Ponceau S (working solution: 30% (w/v) trichloroacetic acid, 30% (w/v) sulfosalicylic acid, 2% (w/v) Ponceau S in distilled water). The position of the molecular weight markers was marked on the membrane with a pencil and Ponceau S stain was removed from the proteins by washing with Tris-buffered saline (150mMTris-HCl (pH 8.0), 1.5M NaCl) containing 0.1% (v/v) tween-20 (TTBS). Membranes were then blocked for 1 h at room temperature with 5% (w/v) non-fat dried milk in TTBS before being washed briefly in TTBS and incubated overnight at 4°C with the appropriate primary antibody (all diluted in TTBS):

Anti-phospho p44/42 MAP Kinase (NEB; 1/1000)
Anti-p44/42 MAP Kinase (NEB; 1/1000)
Anti-phospho MEK 1/2 (NEB; 1/1000)
Anti-phospho Elk 1 (NEB; 1/1000)
Anti-active MAPK (Promega; 1/5000)
Anti-ERK (Promega; 1/5000)
Anti-phospho MEK 1/2 (NEB; 1/1000)
Anti-phospho FAK (Tyr^{925}) (NEB; 1/800)
Anti-actin (Sigma; 1/5000)
Anti-α-tubulin (Sigma; 1/5000)

Membranes were washed with TTBS 3 times (5 min each wash) after primary antibody exposure. They were then incubated in secondary antibody (1:5000 in TTBS) for 1 h at room temperature with constant agitation and subsequently washed 3 times with TTBS (5 min each wash). The membrane was then incubated at room temperature in the Opti-4CN solution (Biorad) until the signal was fully developed (up to 30 min). Once the signal was obtained, membranes were rinsed in distilled water and allowed to air dry. In some cases, enhanced chemiluminescence (ECL) was required for signal detection. Briefly, after the membrane was sufficiently washed in TTBS it was transferred to ECL reagent and was incubated for 5 min in the dark at room temperature with constant rocking. The membrane was then wrapped in cling film and secured in a cassette (Sigma, UK) to keep the membrane in the dark. The membrane was then exposed to ECL hyperfilm for varying lengths of time, the film was immersed in developing reagent for 1 min, washed briefly with water, transferred to fixing solution for a further minute, washed once again with water and then air dried. The exposure and developing of the ECL film was performed in a photographic dark room. The blots (for Opti-4CN) or films (for ECL) were scanned and the images saved. Where appropriate, the intensity of the signal (either by Opti-4CN or ECL detection) was then analysed with Kodak 1D image analysis software as described in section 2.14.

2.14 Image analysis

Where appropriate the intensity of the immunoreactive signal on individual blots was analysed using Kodak 1D Image analysis software. This procedure enabled any

increase or decrease in immuno-reactive signal following challenge to be determined. Values were calculated as a net difference in phosphorylation levels with treatment, compared to the control value for each replicate; hence, each control value was assigned a standardised value of 1. Changes were therefore represented as a fold-increase or decrease from control levels.

When actin, tubulin or total-ERK were used to confirm equal protein loading, intensities of all bands were quantified, and standardised to control values as described above. The experimental bands (e.g. phospho-ERK) were then further standardised to the protein loading values in order to compensate for any differences in the amounts of protein in the lanes.

2.15 Non-radioactive MAP Kinase activity assay

The MAP Kinase activity assay was performed with the 'Non-radioactive MAP Kinase activity assay kit' purchased from NEB. Haemocyte monolayers were prepared in individual wells of a six-well culture plate as described in section 2.3. After washing, monolayers were then either exposed to U0126 (10μM) for 30 min at room temperature before the addition of *E. coli* LPS (10μg/ml) for 5 min, not treated with U0126 but challenged with 10μg/ml LPS for 5 min, or incubated in SSS alone. Cells were then lysed with cell lysis buffer (20mM Tris (pH 7.5), 150mM NaCl, 1mM EDTA, 1mM EGTA, 1% (v/v) Triton X-100, 2.5mM sodium pyrophosphate, 1mM β-glycerolphosphate, 1mM sodium orthovanadate, 1μg/ml leupeptin), and placed on ice for 15 min with shaking before sonication (4 times, 5 s each). The cell lysates were then centrifuged at 4°C (800*g*) for 10 min to pelette larger cell debris, and the supernatent subsequently removed and kept on ice. The protein concentration of the supernatent was then determined with the Bio-rad DC protein assay kit as described in section 2.6. 200μg total protein was removed and added to a microfuge tube containing 15μl of resuspended immobilised Phospho-p44/42 MAP Kinase (Thr^{202}/Tyr^{204}) monoclonal antibody. Also, 200μg of protein from the control sample that had been incubated in SSS alone, was spiked with 20ng of purified active MAPK before the addition of immobilised phospho-p44/42 MAP Kinase monoclonal antibody. All samples were incubated overnight with gentle rocking at 4°C and were then microcentrifuged for 30 s to capture the immunocomplexes. The pellets were washed

twice with 500µl of cell lysis buffer, and a further two times with kinase buffer (25mM Tris (pH 7.5), 5mM β-glycerolphosphate, 2mM DTT, 0.1mM sodium orthovanadate, 10mM magnesium chloride). The pellet was finally resuspended in 50µl kinase buffer supplemented with 200µM ATP and 2µg Elk-1 fusion protein, and incubated at 30°C for 1 hour with gentle rocking. The reaction was finally terminated with 25µl 3X SDS-PAGE sample buffer, and samples were boiled for 5 min, vortexed and then microcentrifuged for 2 min. 30µl of each sample was loaded onto an SDS-PAGE gel and electrophoresed as previously described in section 2.13.

2.16 Immunocytochemistry

Haemocytes (100µl diluted haemolymph in SSS per coverslip) were allowed to adhere to coverslips for 30 min at room temperature, they were then washed 3 times with 1ml of SSS in a 6-well plate (Nunc). After equilibrating in SSS for 15 min, haemocytes were treated with or without various compounds at room temperature. *E. coli* LPS (0-10µg/ml in SSS; 5 min), or albumin-linked sugars (0-800nM; 1 h) were delivered to the cells by inverting the coverslips onto parafilm containing 100µl of the test compound. In some experiments U0126 (10µM) was incubated with the cells for 30 min prior to adding LPS. Albumin-linked sugars were delivered to the cells in the presence or absence haemolymph. For experiments done in the presence of haemolymph, cells were not washed and the sugars were diluted in the haemolymph serum with coverslips being inverted into the sugar-haemolymph solution. After exposure to the various compounds, haemocytes were fixed and permeabilised with fixing buffer (3.7% (v/v) formaldehyde, 0.18% (v/v) Triton X-100 in PBS) for 12 min at room temperature before being briefly washed once with 1ml PBS. The coverslips were then blocked with BSA (1% (w/v) in PBS) for a further 12 min at room temperature, before the blocking solution was drained onto filter paper. Cells were then incubated with the anti-phospho p44/42 MAP Kinase antibody (1:150 in blocking buffer; 3 hrs), followed by a FITC-conjugated goat-anti-rabbit secondary antibody (1:150 in blocking buffer; 45 min) and rhodamine phalloidin (0.1µg/ml in blocking buffer; 40 min). All incubations were performed at room temperature in humidified chambers, and cells were washed three times (5 min each wash) with PBS between incubations. Finally, coverslips were mounted onto slides with Vectashield, sealed with clear nail varnish (to give a semi-

permanent preparation) and observed with a Zeiss Axiophot 20 photomicroscope using a triple filter; excitation wavelengths were 410nm, 505nm and 585nm (with beamsplitters: 395nm, 485nm and 560nm; and barriers: 460nm, 530nm and 610nm respectively). Images were captured digitally using the Nikon DN100 camera, and the Nikon Eclipse Net Image analysis package.

2.17 Phagocytosis assays

Haemocyte monolayers were prepared as previously described (section 2.3) in individual wells (100µl diluted haemolymph per well) of 96-well culture plates (Nunc) and washed three times with 150µl SSS. Haemocytes were then pre-incubated with each of the following compounds, or with vehicle, for 30 min at room temperature prior to the addition of FITC-conjugated 'bioparticles' (6 x 10^6 per well), the test compounds remained in the wells throughout the experiment:

MEK inhibitors:	U0126 (0-100µM in methanol)
	PD98059 (0-100µM in DMSO)
PKA inhibitor:	KT5720 (10µM in DMSO)
PKC inhibitor:	GF109203X (10µM in DMSO)
PI-3-kinase inhibitor:	LY294002 (0-100µM in distilled water)
Farnesylation inhibitor:	FTase inhibitor I (0-100µM in distilled water)
Integrin blocking peptide:	Arg-Gly-Asp-Ser (RGDS) (0-2mM in distilled water)
Actin remodelling inhibitor:	Cytochalasin D (0-10µM in DMSO)
BSA-conjugated sugars:	Albumin–1-amido-1-deoxy-L-fucose (0-800nM in distilled water)
	Albumin–2-amido-2-deoxy-D-galactose (0-800nM in distilled water)
	Combined use of sugars (1:1; 0-800nM of each in distilled water)

After the haemocytes had been challenged with 'bioparticles' for 1 h at room temperature in a dark chamber, 'bioparticles' were removed and 100µl of 2% (w/v)

trypan blue was added to each well for 2 min in order to quench extracellular fluorescence. Intracellular fluorescence was then quantified using a Fluorstar Optima microplate spectrofluorometer (BMG Labtechnologies, Aylesbury, UK), with an excitation filter of 488nm, and emission filter of 530nm. For experiments in which cells were exposed to the BSA-conjugated sugars in the presence of haemolymph, haemocytes were not washed and the sugars were diluted straight into the haemolymph after binding.

2.18 Integrin-mediated cell adhesion assay

The integrin-mediated haemocyte adhesion assays were performed with the 'Integrin $\alpha_V\beta_3$-mediated cell adhesion kit' and the 'Integrin β_1-mediated cell adhesion kit' using the manufacturers recommended protocol. Briefly, the integrin antibody-coated (experimental) and –uncoated (control) strips were rehydrated with 200µl PBS (with and without Ca^{2+}/Mg^{2+}) per well for 10 min at room temperature. The PBS was removed and 100µl extracted haemolymph diluted in PBS (with and without Ca^{2+}/Mg^{2+}) was then placed into each of the wells and incubated at 37°C in a CO2 incubator for 2 h. After incubation, diluted haemolymph was removed from the wells and each well was washed 3 times with 200µl PBS (with and without Ca^{2+}/Mg^{2+}). After washing, 100µl of cell stain solution was added to each well and incubated for 5 min at room temperature. The cell stain was then removed and the wells were washed 5 times with PBS (with and without Ca^{2+}/Mg^{2+}) and allowed to air dry before the addition of 100µl extraction buffer. Samples were then incubated at room temperature on an orbital shaker until the cell stain was completely solubilised (5-10 min). The absorbance (at 570nm) of the samples was then determined using a microplate spectrophotometer. The presence/absence of integrins and Ca^{2+}/Mg^{2+}-dependency of adhesion was then determined by comparing the absorbance of the experimental wells to that of the control wells.

HL60 cells (leukaemic cells) were used as a negative control for the assays. Briefly, the HL60 cell line was grown in 20ml culture flasks in RPMI 1640 medium, containing 10% foetal bovine serum (FBS), at 37°C in an atmosphere containing 5% CO_2. All cell lines were passaged when cell densities reached $1x10^6$ cells/ml and were diluted to $1x10^5$ cells/ml, thereby maintaining a stock of cells that ranged from 10^5 to 10^6 cells/ml.

To harvest the cells, the media was transferred to a sterile tube and was centrifuged for 5 min at 800g. The cell pellet was then resuspended in 1 ml of PBS (containing Ca^{2+} and Mg^{2+}), and 100µl of the suspension was placed in both experimental and control wells. The wells containing the HL60 cells were then treated in a similar way to those containing the haemocytes.

2.19 Two-dimensional electrophoresis

For two-dimensional separation of haemocyte proteins, haemocyte monolayers were prepared in Petri dishes (2ml of diluted haemolymph per dish) as described in section 2.3. After the haemocyte monolayers had been washed three times (with 2ml SSS), 2ml SSS was added to two dishes and 2 ml of SSS containing 10µM U0126 solution was added to a third. The cells were then incubated for 30 min at room temperature. Haemocytes within one of the dishes containing only SSS were then lysed with 200µl of 2% SDS; the sample was then transferred to a microfuge tube and was sonicated for 2 min. Protein determination was carried out on this sample following the DC protein assay kit protocol described in section 2.6; this was done since it was necessary to extract the cells in the other SSS control dish and the dish exposed to U0126 with sample/rehydration buffer (8M urea, 2% 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS), 50mM dithiothreitol (DTT), 0.2% (w/v) Bio-Lyte® 3/10 ampholytes (pH 3 - pH 10) and bromophenol blue) which interfers with protein assays. Cells in the remaining dishes (i.e. control and U0126-exposed) were lysed with 200µl sample/rehydration buffer, and the cells scraped from the bottom of the dish with a sterile cell scraper. Once these samples had been transferred to microfuge tubes, they were then sonicated at room temperature for 2 min. Two IPG strips (pH 3 to 10) were taken from the -20°C freezer and left at room temperature for approximately 5 min, during which time the samples were also kept at room temperature (cooling of the samples would result in precipitation of urea). For two-dimensional gels, it is recommended to rehydrate the IPG strips with 185µl of sample containing a maximum of 250µg of total protein. 185µl of each sample was therefore pipetted into the middle of individual lanes of a disposable rehydration/equilibration tray so that the sample covered the whole lane except for 2 cm at each end. The plastic backing of each IPG strip was removed with tweezers and the strip carefully lowered onto the sample, gel side down, ensuring no air bubbles were trapped underneath the strip and none of the

sample was on top of the strip. After 1 h both IPG strips were overlayed with 2ml of mineral oil. The strips were then left overnight (11 – 16 h) for the passive rehydration process to continue.

After the overnight incubation the samples had been absorbed into the gel of the IPG strips, isoelectric focusing (IEF) was therefore performed. The strips were removed from the disposable tray and the mineral oil allowed to drain onto filter paper. Electrode wicks were placed over the cathodes and anodes of lanes the Protean IEF focusing tray, and 8µl of nanopure water was pipetted onto each wick. The strips were then carefully transferred into the focusing tray, ensuring the gel was touching each electrode. Once this was done, each strip was overlayed with 1ml mineral oil and the lid was placed on the focusing tray. The tray was transferred to the Protean IEF cell and isoelectric focusing was started at 250V (20 min), increasing to 8,000V (2.5 h) and finally 8,000V until 20,000V-hr was reached. The whole IEF process took approximately 5 h 20 min. During the electrophoresis procedure the strips were kept at 20°C, with a maximum current of 50µA/strip.

Once IEF had finished, the IPG strips were removed from the focusing tray, drained of mineral oil, and placed gel side up in a clean disposable rehydration/equilibrium tray. Each strip was then incubated in 2ml of equilibration buffer I (6M urea, 2% (w/v) SDS, 0.375M Tris-HCl (pH 8.8), and 20% (v/v) glycerol) on an orbital shaker for 10 min at room temperature. Equilibration buffer I was then removed by simply draining the buffer out of the lanes and flicking the tray to remove residual buffer. Strips were then incubated in 2 ml equilibration buffer II (6M urea, 2% SDS, 0.375M Tris-HCl (pH 8.8), 20% glycerol and 2.5% (w/v) iodoacetamide) for a further 10 min at room temperature on an orbital shaker. The pre-cast SDS-PAGE gels were unwrapped and washed with proteomic grade water and the overlay agarose containing bromophenol blue was heated in a microwave for approximately 45-50 sec. A 100ml graduated cylinder was filled with XT-MES electrophoresis running buffer and each strip was dipped into this buffer before being placed gel side up (with the lowest pH of the strip on the left-hand side of the SDS-PAGE gel), on the larger (front) plate of the SDS-PAGE gel cassette. The heated overlay agarose solution was then pipetted into the wells of the SDS-PAGE gels and the IPG strips were carefully pushed into the agarose ensuring they were it was straight and that no air bubbles were trapped between the strips and the SDS-PAGE

gels. Biotinylated molecular weight markers were heated to 95°C for 2-3 min and 10µl was pipetted into the marker well of each SDS-PAGE gel. Once the agarose had set, the second dimension of the electrophoresis procedure was performed, at 200V constant, until the bromophenol blue of the agarose had just run off the bottom of the gel. The separated proteins were then blotted onto nitrocellulose and Western blotting was performed as described in section 2.13. Nitrocellulose membranes were blocked with 5% non-fat dried milk, and incubated with an anti-phospho-threonine-proline primary antibody (1/1000 in TTBS) for 2 h. This antibody only recognises proteins phosphorylated on threonine residues followed by proline and therefore can be used to detect MAPK substrate proteins when used in combination with MAPK inhibitors. Membranes were then incubated in goat-anti-mouse secondary antibody (1/2000) and HRP-linked anti-biotin antibody (1/2000) for 1 h. Incubations were done at room temperature and the signal was detected by ECL as described in section 2.13.

2.20 Statistical analysis

Where appropriate, results were analysed with one-way analysis of variance (ANOVA) and post-hoc multiple comparison tests (Tukey), using the statistical software package SPSS. For studies with albumin-linked carbohydrates, two-way ANOVA was used to compare results obtained from the phagocytosis assays with and without haemolymph, and independent T-tests were also carried out between experiments with and without haemolymph at the specific concentrations.

CHAPTER THREE

RESULTS

SECTION 3.1

THE IDENTIFICATION OF ERK PATHWAY COMPONENTS IN *L. STAGNALIS* HAEMOCYTES AND THE DETERMINATION OF OPTIMAL CONDITIONS FOR HAEMOCYTE CULTURES

ERK pathway components have previously been identified in various invertebrates including the Mediterranean fruit fly *Ceratitis capitata* (Foukas *et al*, 1998), the bivalve mollusc *Mytilus galloprovincialis* (Canesi *et al*, 2002a; 2002b), and an embryonic cell line from the gastropod mollusc *Biomphalaria glabrata* (Humphries *et al*, 2001). The presence of an ERK homologue has also been reported in the *Drosophila* S2 embryonic cell line (Biggs & Zipursky, 1992). However, prior to this study, ERK pathway components were yet to be identified in *Lymnaea stagnalis* haemocytes. In this study, the detection of ERK-like proteins was achieved by Western blotting of haemocyte total protein extracts and by immunocytochemistry. In addition, before any experiments that relied on haemocyte incubations could be performed, the conditions to keep *L. stagnalis* primary haemocyte cultures had to be determined. Optimal environmental conditions for the culture of primary haemocyte monolayers were determined by incubating haemocytes for different periods of time in different saline solutions and media, and at temperatures. In these experiments haemocyte morphology was observed and the degree of ERK phosphorylation under the different incubation regimes was determined by Western blotting.

3.1.1 Detection of ERK 1/2 - like proteins by Western analyses

Western blotting was performed on total haemocyte protein extracts to identify ERK-like proteins in *L. stagnalis* haemocytes. To determine the protein concentration of the extracts, the BioRad detergent compatible (DC) protein assay kit was used with BSA as the standard protein (a BSA standard curve is shown in fig 3.1.1). The use of two different primary 'anti-phospho' antibodies, the anti-phospho p44/42 MAP kinase antibody (NEB) and the anti-active MAPK antibody (Promega), revealed the presence of ERK-like proteins in a phosphorylated state in unstimulated cells (fig. 3.1.2). Both antibodies recognise the ERK 1/2 isozymes when phosphorylated at Thr^{202}/Tyr^{204} residues and since phosphorylation is necessary for activation, these antibodies have been used in numerous studies to demonstrate ERK activity (see for example, Rossant *et al.*, 1999; Dang *et al.*, 1998). Rat pheochromocytoma 12 (PC12) cells were used as negative (untreated) and positive (NGF-treated) controls for Western blotting; as expected, active ERK was only present in the NGF-treated PC12 cells (fig 3.1.2).

The anti-active MAPK antibody (Promega) mainly identified just one single band that had migrated to an apparent molecular weight of 43kDa (fig 3.1.2), although when the proteins were allowed to migrate in the gel for longer two bands were occasionally seen. In contrast, the anti-phospho p44/42 MAP kinase antibody (NEB) often revealed two bands with approximate weights of 44 and 43kDa (fig 3.1.2). Since the anti-phospho p44/42 MAPK antibody seemed more effective at detecting both haemocyte ERK isozymes this antibody was used for all subsequent experiments.

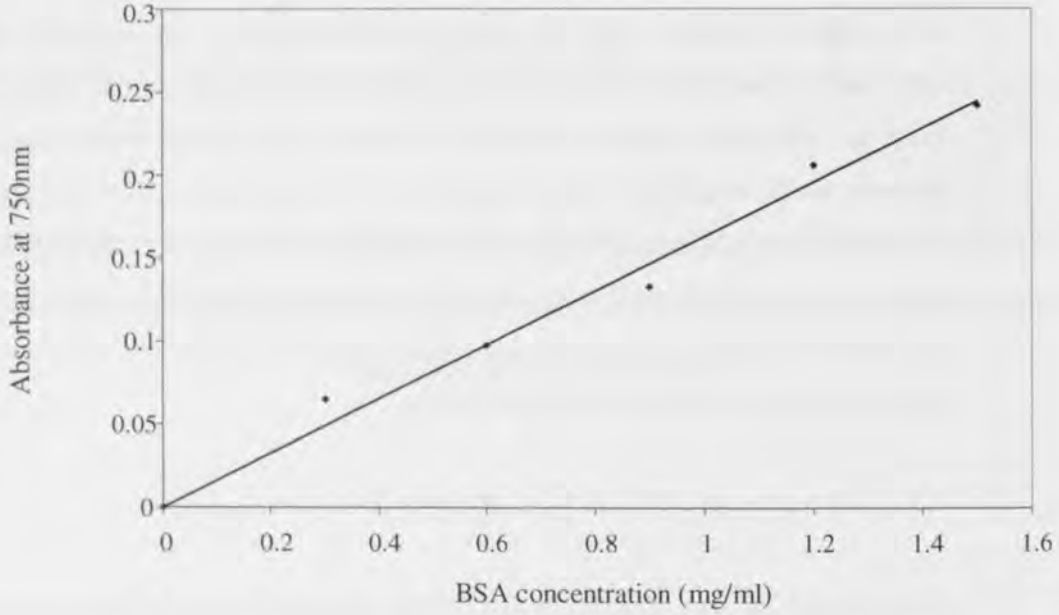

Figure 3.1.1. Example of a BSA standard curve used to calculate the protein concentrations of haemocyte lysates. The Bio-Rad detergent compatible (DC) protein assay kit was used, a modification of the traditional Lowry method. $R^2 = 0.9862$.

The anti-pan-ERK antibody was also used to probe total haemocyte protein extracts. This antibody is capable of detecting all ERK isoforms (p42, p44, p56 and p85) regardless of the enzyme's activation status, as shown by Keel *et al* (1995) in porcine granulosa cells. However, only proteins of approximately 44 and 43kDa were detected in *L. stagnalis* haemocytes (fig. 3.1.2). Using this antibody; bands of approximately 42, 44 and 85 kDa were detected in the positive control (RSV 3T3 cells) as expected. Since this antibody was generated towards a region in rat ERK 2, the results suggests that, if present, *L. stagnalis* p56 and p85 isoforms have differences in the sequence

homology when compared to rat ERK isoforms. With both phospho-specific antibodies, the ERK 1-like protein (44kDa) was always seen as the strongest band, whereas the pan-ERK antibody tended to show the ERK 2-like protein (42kDa in mammals, 43kDa in *L. stagnalis*) as the most abundant isozyme (fig. 3.1.2).

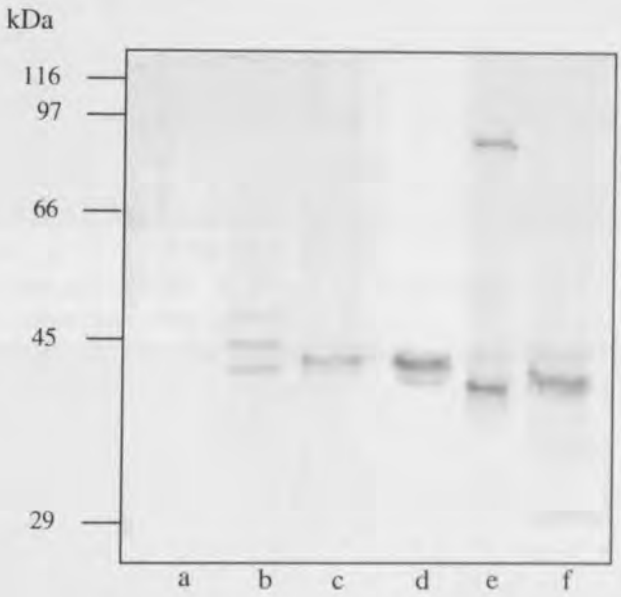

Figure 3.1.2. Detection of ERK 1/2-like proteins in unstimulated *L. stagnalis* haemocytes. The anti-active MAPK (Promega) antibody was used to detect phosphorylated ERK 1/2 homologues in haemocyte extracts (20µg) (c). Untreated (a) and NGF-treated (b) PC12 cells were used as controls. Haemocyte proteins (20µg) were also probed with the anti-phospho p44/42 MAP kinase antibody (NEB) (d). Haemocyte proteins (f, 20µg) were then probed for the presence of p42, p44, p56 and p85 ERK isoforms with the anti-pan ERK antibody (Transduction Labs), RSV-3T3 cells (e) were used as a positive control. Results shown are representative of three independent experiments.

Western analyses with the anti-p44/42 MAP kinase (NEB) and anti-ERK 1/2 (Promega) antibodies that detect total ERK protein levels, irrespective of their phosphorylation status, revealed 2 bands of approximately 44 and 43kDa in *L. stagnalis* haemocyte total protein extracts. Although the Promega anti-ERK 1/2 antibody, raised against a conserved peptide sequence in human/rat ERK 1, did reveal 2 bands (fig. 3.1.3), the antibody sometimes proved unreliable and did not always result in bands that could be scanned and analysed for relative band intensities. However, the anti-p44/42 MAP kinase (NEB) antibody, which was raised against a synthetic peptide

derived from rat ERK 2, revealed two bands of 44kDa and 43kDa regularly, therefore this antibody was used for subsequent experiments. In contrast to the phospho-specific antibodies, use of the total-ERK antibodies resulted in the ERK 2-like protein (42kDa) being the stronger band of the two (fig. 3.1.3).

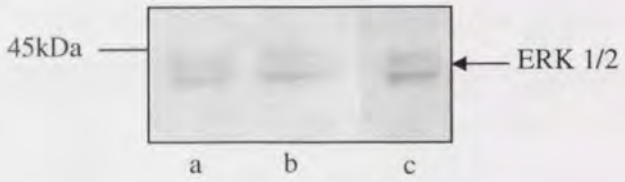

Figure 3.1.3. Detection of 'total' ERK proteins in *L. stagnalis* haemocytes. ERK isozymes in *L. stagnalis* haemocytes were detected using anti-ERK 1/2 (a, Promega, 20µg total protein; b, 30µg total protein), and anti-p44/42 MAPK (c, NEB; 15µg total protein) antibodies. These antibodies detect ERK 1/2 irrespective of their phosphorylation status. The blot shown is representative of two independent experiments for both antibodies.

3.1.2 Detection of ERK 1/2 – like proteins by immunocytochemistry

Detection of phosphorylated and non-phosphorylated ERK 1/2 by means of Western blotting revealed the molecular weights of the respective isozymes in *L. stagnalis* haemocytes and tested antibody reactivity by using positive and negative controls. However, immunocytochemical detection allows the cellular location of proteins to be determined in cells. As with the Western analyses, two phospho-specific primary antibodies were used (Promega and NEB) for the immunocytochemical detection of phosphorylated (activated) ERK 1/2-like proteins in *L. stagnalis* haemocytes; two total-ERK (Promega and NEB) antibodies were also used to detect ERK-like proteins irrespective of their phosphorylation status.

The use of both phospho-specific antibodies showed that, when haemocytes were unchallenged, phosphorylated ERK 1/2 was present throughout the haemocyte, and that it was often present at the periphery of the nuclear membrane (fig. 3.1.4 A, B and D).

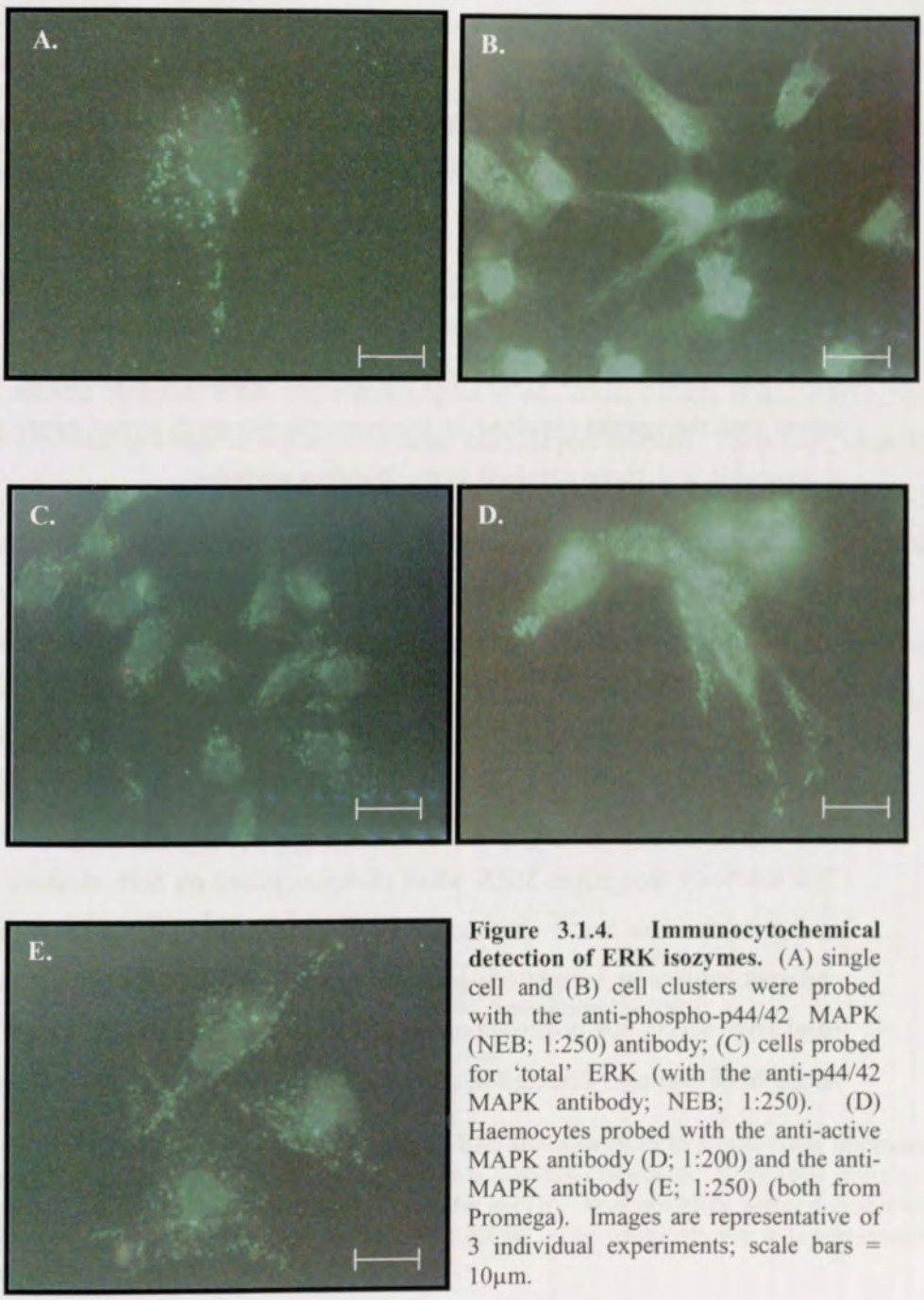

Figure 3.1.4. Immunocytochemical detection of ERK isozymes. (A) single cell and (B) cell clusters were probed with the anti-phospho-p44/42 MAPK (NEB; 1:250) antibody; (C) cells probed for 'total' ERK (with the anti-p44/42 MAPK antibody; NEB; 1:250). (D) Haemocytes probed with the anti-active MAPK antibody (D; 1:200) and the anti-MAPK antibody (E; 1:250) (both from Promega). Images are representative of 3 individual experiments; scale bars = 10μm.

When haemocytes were stained with the anti-p44/42 MAPK antibody (NEB) both active and non-active (i.e. total) ERK was found to be situated in the nuclear region of the cells, and as shown with the anti-phospho p44/42 antibody, it was particularly present at the periphery of the nuclear membrane (fig 3.1.4C). The anti-ERK antibody (Promega) showed total ERK proteins to be clustered throughout the cytosol with a clear presence in and around the nucleus (fig 3.1.4E). With this antibody, the nucleus was often much more defined when compared to the results obtained with the p44/42 MAPK NEB antibody. Overall, it was concluded that all antibodies worked well in immunocytochemistry with haemocytes, but as with the Western analyses the Promega antibodies did not always give the best images. The NEB 'phospho'- and 'total'-ERK antibodies were therefore used for all further immunocytochemical work; this also meant that the results obtained in immunocytochemistry experiments would be more comparable with those obtained in the Western analyses.

3.1.3 Detection of MAPK/ERK Kinase (MEK) by Western analysis

MEK 1/2 is known to be the activator of ERK in mammalian cells, but the presence of this signalling intermediate was yet to be reported in molluscan haemocytes. Using a phospho-specific MEK 1/2 ($Ser^{217/221}$) polyclonal antibody, active-MEK was detected in *L. stagnalis* haemocytes for the first time following Western blotting (fig 3.1.5). This antibody recognises MEK when phosphorylated on both residues or when singly phosphorylated at Ser^{217} (but not Ser^{221}). The MEK 1/2-like proteins possessed an apparent molecular weight of approximately 43kDa, and always appeared as a single band (fig. 3.1.5). A positive control (NGF-treated PC12 cells) was also used, and a single band was also detected in these cells at the same molecular weight (43kDa).

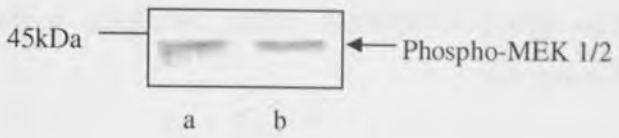

Figure 3.1.5. Detection of activated MEK-like protein in *L. stagnalis* haemocytes. NGF-treated human PC12 cells were used as a positive control (a; 10µg total protein) and haemocyte lysates are shown in (b; 30µg total protein). Blots were probed with the anti-phospho MEK 1/2 antibody (1/1000; NEB). The blot shown is representative of two independent experiments.

3.1.4 Detection of active Elk-1 by Western analysis

The nuclear target of ERK 1/2, Elk-1 (Aplin *et al.*, 2001; Sellers *et al.*, 2001), has not been reported in either insect or molluscan haemocytes to-date. Therefore, since MEK and ERK-like proteins were successfully detected in *L. stagnalis* haemocytes, haemocyte extracts were probed for the presence of this 62kDa transcription factor. Using an anti-phospho-Elk-1 (Ser^{383}) antibody a phosphorylated (active) Elk-1-like protein was detected in haemocyte total protein samples. The haemocyte Elk-like protein was found to have an approximate molecular weight of 62kDa (fig. 3.1.6), and a similar size protein was detected in NGF-treated PC12 cells which were used as a positive control.

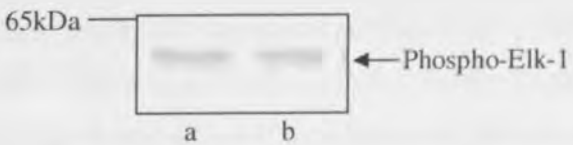

Figure 3.1.6. Detection of a phosphorylated Elk-1-like protein in *L. stagnalis* haemocytes. Human NGF-treated PC12 cells were used as a positive control (a; 10µg total protein) and 30µg total haemocyte lysate was loaded onto the gel (b). Western blots were probed with the anti-phospho Elk-1 primary antibody (1/1000; NEB). The image shown is representative of two independent experiments.

3.1.5 The effects of different saline solutions, media and incubation times on haemocytes

3.1.5.1 Cell morphology

Studies with molluscan haemocytes have often used sterile snail saline (SSS) as the saline for experimental procedures (Conte & Ottaviani, 1995; Horák & Deme, 1998; Horák *et al*, 1998). Initially, two solutions, SSS and RPMI, were used for monitoring haemocyte morphology and basal levels of ERK 1/2 activity over a 16 h post-binding period. RPMI is a media containing various amino acids, vitamins, inorganic salts and glucose. It is widely used in cell culture, and has been shown to support the growth of many cell types including human lymphocytes. The morphology of the cells in RPMI indicated that this was an inadequate media for the haemocyte monolayers to be kept in. Immediately, cells were non-adherent and rounded up (table 3.1.1). This is in comparison to cells in SSS, which were adherent and well spread for up to 16 hours after the initial binding period, with vacuoles only appearing in the cells approximately 3 hours after binding (table 3.1.1).

3.1.5.2 Phosphorylation of haemocyte ERK 1/2

The haemocytes incubated in SSS (for up to 16 h) were lysed and the basal levels of ERK 1/2 phosphorylation analysed by Western blotting. It was found that phosphorylation levels of the ERK isozymes decreased over the 16 hour period (fig. 3.1.7). Taken together with the morphological data this indicated that the cells were not unduly stressed and confirmed that SSS was an adequate saline for these cells to be kept in during future experimental procedures. As vacuoles were seen when haemocytes were kept in SSS for 3 h post-binding, a maximum incubation time of 2 hours was used in future experiments.

Time post binding period (h)	SSS	RPMI
0	Cells adherent with filopodia visible, some cell clumping.	Cells are rounded and non-adherent (floating in media).
1	Cells adherent with filopodia, more cell clumping evident.	-
2	Cells adherent with filopodia present, cells heavily clumped.	-
3	Cells still adherent and spread. Some cells with vacuoles.	-
4	Cells with more vacuoles, although still adherent with filopodia.	-
16	Cells heavily vacuolated, although still adherent with filopodia.	-

Table 3.1.1 Haemocyte morphology observed during incubation in SSS and RPMI over 16 h. Observations are based on triplicate monolayer preparations in 6-well culture plates with the aid of an inverted microscope.

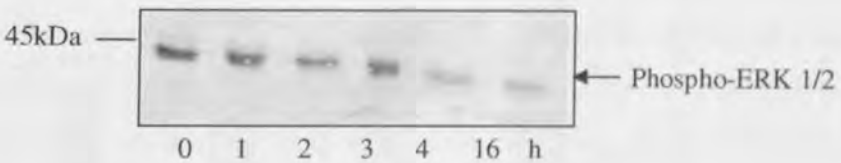

Figure 3.1.7. Haemocyte basal ERK activity over a 16 h period when incubated in SSS. ERK basal activity levels appear to decrease over time. Membranes were probed with anti-phospho p44/42 antibody, with 20µg total protein per well. The blot shown is representative of three independent experiments.

As the culture/equilibration period for haemocytes was to be kept to a maximum of 2 h, immunoblots of cells incubated in SSS for up to 4 h were analysed for relative ERK and MEK phosphorylation levels. Both ERK and MEK phosphorylation appeared to decrease with time (fig. 3.1.8). ERK and MEK phosphorylation levels were reduced to 53% and 47% ($P \leq 0.001$) of that of the control after 4 h incubation in SSS respectively (fig. 3.1.8C). Although following ANOVA the reductions in ERK phosphorylation did not prove to be significantly different at any time point, the decline in MEK activity proved to be significant at all time points studied (fig 3.1.8C).

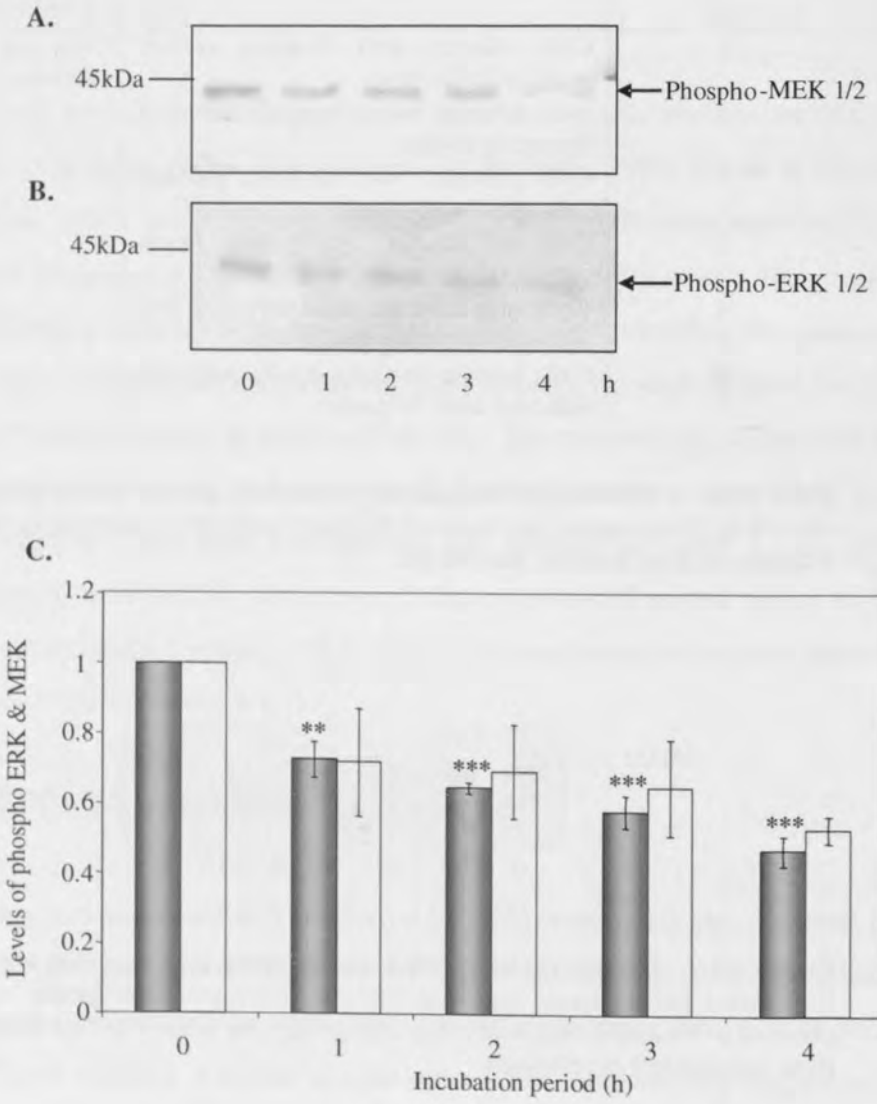

Figure 3.1.8. Levels of phosphorylated MEK and ERK over 4 hours culture. Western analysis demonstrates that levels of (A) phosphorylated MEK and (B) ERK isozymes decrease over 4 hours at room temperature in SSS. Blots were probed with anti-phospho MEK (1:800) and anti-phospho p44/42 MAPK (1:1000) antibodies (both from NEB). Blots were scanned (C; ± SEM) and then analysed, and the mean relative changes in MEK (shaded bars) and ERK (white bars) phosphorylation were calculated; (n=3; ** $P \leq 0.01$ and *** $P \leq 0.001$).

3.1.5.3 Anti-clumping buffers

Although SSS seemed to be an adequate saline for the incubation of haemocytes, the cells were still clumping to some extent. Since previous studies have shown that cell-to-cell contact results in increased ERK activity (Pece *et al*, 2000) the basal levels of ERK phosphorylation in haemocytes could be in part due to clumping of the cells. An anti-clumping buffer (Davids & Yoshino, 1998) and Alsever solution (Yip *et al*, 2001) were therefore used in an attempt to reduce cell-cell adhesion, but retain cell adhesion to the cell culture plates, thus enabling monolayer formation. Both solutions resulted in the complete loss of cell adhesion to the base of cell culture plates. When observed under the microscope, cells appeared rounded and floating in either media; these buffers were therefore deemed unsuitable for the preparation of haemocyte monolayers in experiments.

3.1.6 Effect of temperature on basal levels of phosphorylated ERK 1/2

Previous studies with haemocytes have shown that temperature can have an effect on haemocyte behaviour during the binding and incubation period of monolayers (Ravindranath, 1975; Anggraeni & Ratcliffe, 1991; Ellender *et al*, 1992). The effect of three temperatures on basal levels of haemocyte ERK phosphorylation were therefore determined over both a 30 min and 2 h incubation period with SSS as the saline. When haemocytes were incubated at 20°C for 30 min, a 5-fold increase in ERK phosphorylation was observed when compared to that of haemocytes incubated at room temperature, 25°C ($P \leq 0.001$; fig 3.1.9 A and C). This is in contrast to cells incubated for 2 h at this temperature, where no significant difference was recorded (fig 3.1.9 B and C). However, a 2 h incubation at 30°C did result in a slight but significant increase in ERK phosphorylation ($P \leq 0.05$), whilst 30 min at this temperature did not have an affect. Overall, the lowest basal phosphorylations were observed at room temperature and thus all future experiments were performed under these conditions.

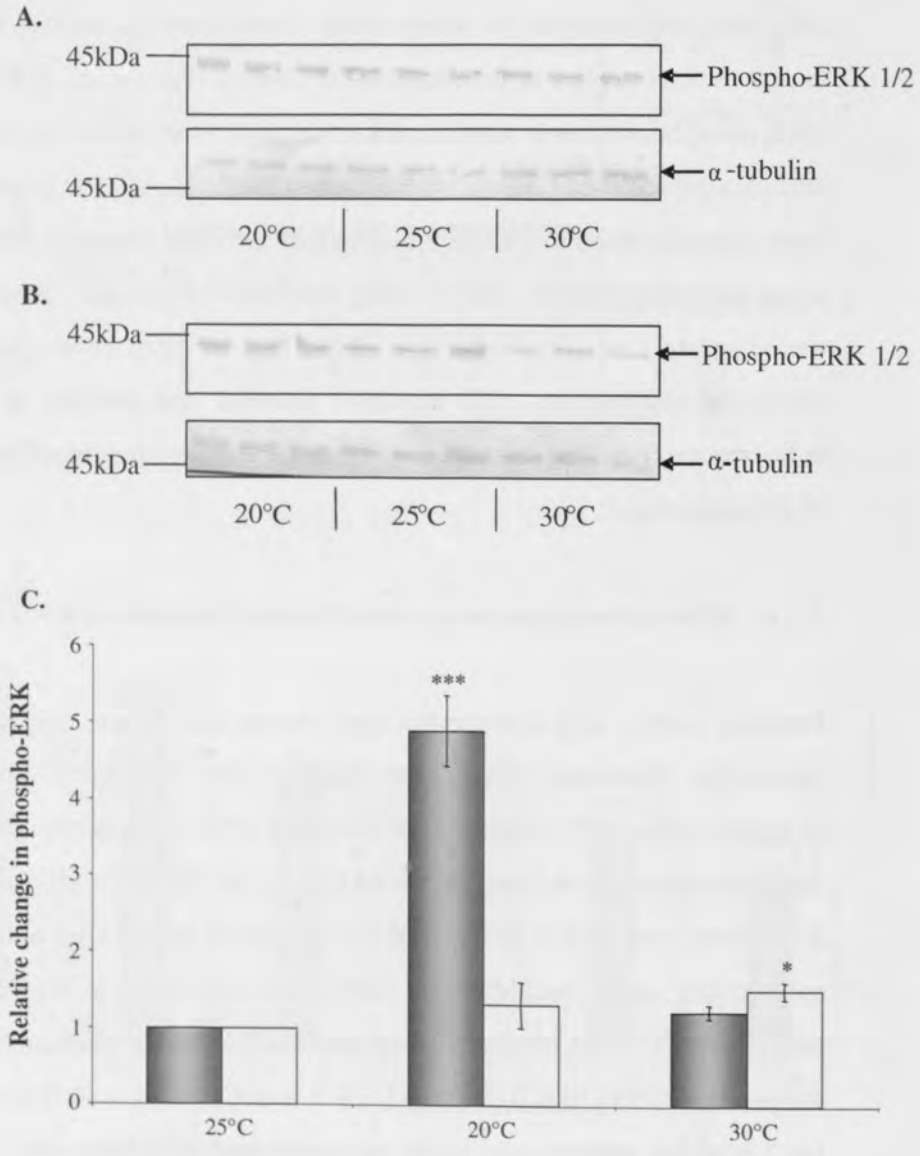

Figure 3.1.9. Levels of phosphorylated ERK in haemocytes cultured at different temperatures. Haemocytes were incubated for 30 min (A) and 2 h (B) in SSS at 20°C, ambient temperature (25°C) and 30°C. Membranes were probed with anti-p44/42 MAP kinase (1:1000) and anti-α-tubulin (1:5000) antibodies. The intensity of each band was analysed and normalised against α-tubulin values, the mean relative change in ERK phosphorylation levels (30 min: shaded bars; 2 h: white bars) were then determined (C; n=3; ± SEM; ** $P \leq 0.01$, *** $P \leq 0.001$ when compared to 25°C (control)).

3.1.7 Basal levels of phosphorylated ERK and MEK over 80 minutes

Although basal ERK phosphorylation was investigated over 4 h, a more detailed analysis of ERK phosphorylation was required before stimulation of the ERK pathway was attempted. Phosphorylation was therefore measured at 5, 10, 20, 40 and 80 min post-binding, so that any changes in phosphorylation seen following the addition of compounds could be attributed to pathway activities and not changes in basal phosphorylation levels. Western analyses revealed that the basal level of phosphorylated ERK 1/2 is fairly constant over an 80 min time period (fig 3.1.10). It was therefore decided to leave the cells for 15 min after the initial binding period before the addition of mitogens; this approach allowed extra time for the pre-incubation of inhibitors with haemocytes prior to stimulation where appropriate.

3.1.8 Trypan blue cell viability assay

Trypan blue was used to determine the number of viable cells present in the haemocyte monolayers immediately after monolayer washing, and again after a 40 min incubation in SSS. Random cell counts of 100 cells using an inverted microscope revealed that there was very little cell death during the 30 min binding period, with a mean viable cell count of 97.3 per 100 cells immediately after washing (table 3.1.2). After a 40 min incubation in SSS the mean viable cell count dropped to 96.6 cells out of 100, demonstrating that cell death is virtually nil during this time. Clearly, most non-viable cells will not have bound to the culture wells and will have been removed during monolayer washing.

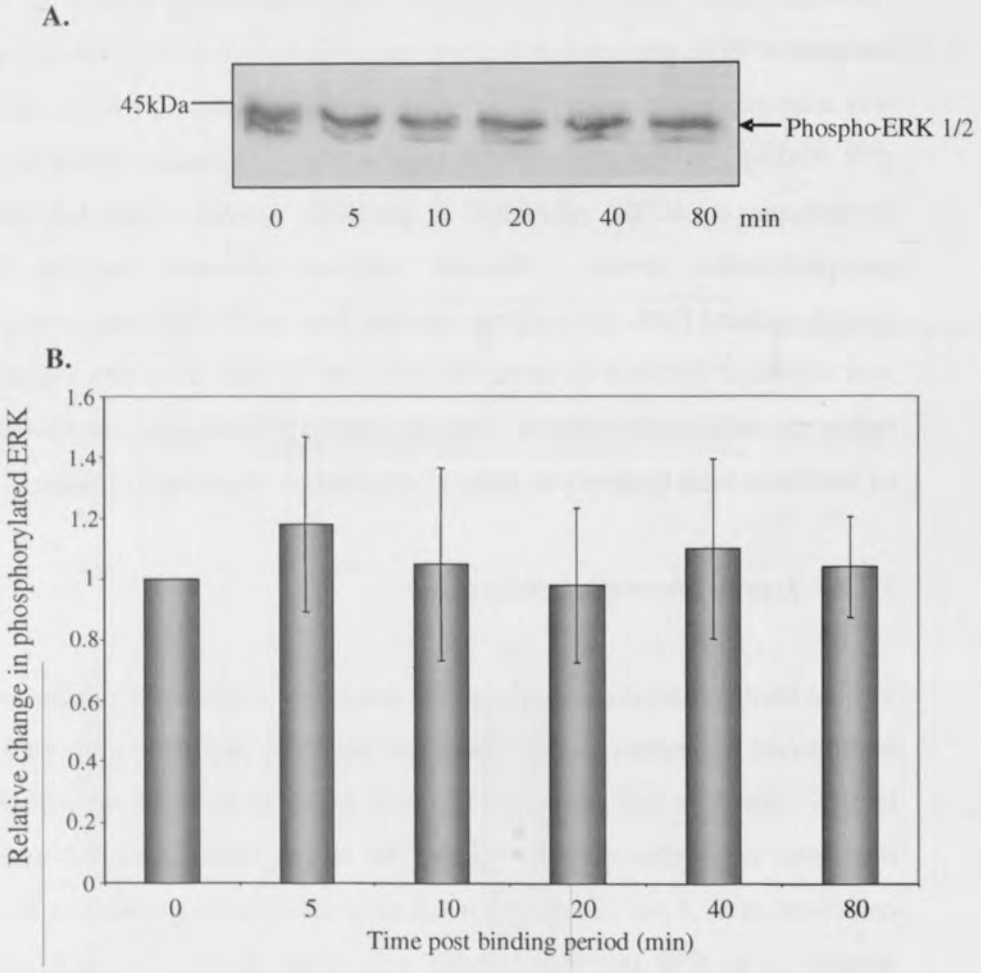

Figure 3.1.10. Basal levels of phosphorylated ERK in unchallenged haemocytes over an 80 min post-binding period. Membranes were probed with anti-phospho p42/44 MAP kinase antibody (1:1000; A), and band intensities on blots analysed. Values shown are mean changes in ERK phosphorylation levels (B; n=3; ± SEM). The blot shown is representative of three independent experiments.

	Immediately after washing		After 40 min	
	Viable	Non-viable	Viable	Non-viable
Well 1	98	2	97	3
Well 2	99	1	96	4
Well 3	95	5	97	3
Mean	97.3	2.7	96.7	3.3

Table 3.1.2. Viable and non-viable haemocyte numbers determined by the trypan blue cell viability assay. In each replicate, 100 cells were counted at random using an inverted microscope.

3.1.9 Summary of results

With the aid of anti-phospho-specific antibodies, the active (phosphorylated) forms of ERK 1/2, MEK 1/2 and Elk-1 have been identified in haemocyte cell lysates. ERK has also been detected with 'total' ERK antibodies which recognise phosphorylated and non-phosphorylated ERK and which will be used to monitor ERK 1/2 expression levels in subsequent experiments. Although the antibodies used in this study have been primarily raised against human or rat peptide sequences, they have been used successfully here for the detection of molluscan enzymes, demonstrating a high degree of sequence homology. The cellular distribution of phosphorylated ERK has also been observed by immunocytochemistry, which will prove to be a valuable tool for visualisating any re-location of activated ERK after haemocytes have been challenged with *E. coli* LPS.

By observing the morphology of haemocytes in RPMI and SSS, it has been concluded that SSS is the best medium for the culturing of *L. stagnalis* primary haemocyte monolayers. By incubating haemocytes at different temperatures, it was also concluded that the monolayers should be incubated at room temperature for 30 min to achieve lower basal phosphorylation levels, although morphological observations have shown cells to be viable for many hours post-binding. With a more detailed analysis of basal phosphorylation levels over 80 minutes post-binding, phosphorylation appeared to be most stable between 10 and 20 min. It was therefore concluded that the addition of mitogens should be done 15 min after the wash stage.

SECTION 3.2

ACTIVATION AND INHIBITION OF THE ERK PATHWAY

Since the identification of ERK pathway components in *L. stagnalis* haemocytes had been successful (section 3.1), various components were used to test their ability to modulate ERK pathway activity. Lipopolysaccharide (LPS; a major constituent of the outer cell envelope of Gram-negative bacteria) was used to challenge haemocytes, along with heat killed *E. coli*, zymosan A from *Saccharomyces cerevisiae*, adrenocorticotropin hormone (ACTH) and noradrenaline. All of these compounds havee been shown to modulate phagocytosis and immune activity in previous studies using haemocytes or mammalian macrophages (Ottaviani *et al.*, 1991a; Sassi *et al.*, 1998; Hiller & Sundler, 1999; Lacoste *et al.*, 2001b; 2001c; Canesi *et al.*, 2002b). The pharmacological inhibitors, U0126 and PD98059, were also used to dissect ERK pathway components and confirm whether or not MEK activated ERK in *L. stagnalis* haemocytes. The kinase activity of the ERK-like protein detected by Western analyses (in section 3.1) was tested using a non-radioactive MAP kinase activity assay.

3.2.1 Challenge of haemocytes with cell-free E. coli *LPS*

The exposure of mammalian macrophages to LPS can either lead to the activation or down-regulation of the ERK pathway (Weinstein *et al.*, 1992; Sanghera *et al.*, 1996; Foukas *et al.*, 1998; Procyk *et al.*, 1999; Monick *et al.*, 2000). To determine the effect of cell-free LPS on *L. stagnalis* haemocyte ERK pathway components, primary haemocyte monolayers were exposed to 1µg/ml of cell-free *E. coli* LPS (serotype 0111:B4). Cells were lysed after 2.5, 5, 10 and 20 min exposure to LPS to determine whether ERK, MEK or Elk-1 were activated or inhibited by LPS, and whether this activation (by phosphorylation) was transient or prolonged.

3.2.1.1 Activation of ERK

Western blot analyses revealed that the *L. stagnalis* ERK pathway is activated by cell free LPS. The ERK-like proteins show increased phosphorylation with time following LPS exposure, and this phosphorylation appears to be transient, with maximal activation occurring after 5 min and returning to basal levels after 20 min (fig. 3.2.1.A upper panel). Image analysis of blots followed by one way analysis of variance,

revealed that ERK phosphorylation significantly increased by an average of 70% after 5 min exposure to this compound ($P \leq 0.05$; fig. 3.2.1.B). After 20 min exposure, phosphorylation of ERK dropped considerably to 16% below baseline phosphorylation levels (fig 3.2.1A upper panel, and B), using the anti-phospho p44/42 MAPK antibody. Total-ERK 1/2 levels in the samples were also analysed and showed that the expression levels of ERK do not change when cells are challenged with LPS (fig. 3.2.1.A lower panel).

3.2.1.2 Activation of MEK and Elk-1

As LPS resulted in transient ERK phosphorylation, levels of phospho-MEK were also analysed after LPS exposure (1μg/ml) over a 20 min period. Since MEK is the upstream activator of ERK, it was thought that a similar time-dependent pattern of phosphorylation may exist. Western analyses showed that MEK phosphorylation (activation) is slightly upregulated in a similar fashion to that seen with ERK (fig 3.2.1.A), with levels of phospho-MEK being higher at 5 and 10 min (a mean of 13% and 17% higher than control values respectively) post exposure (fig. 3.2.1B). However following image analysis of blots, one-way analysis of variance revealed that the mean increases of MEK phosphorylation were not significantly different to control levels. In a similar fashion to ERK, MEK phosphorylation also fell towards control levels (0 min) after 20 min exposure to LPS (fig. 3.2.1.B).

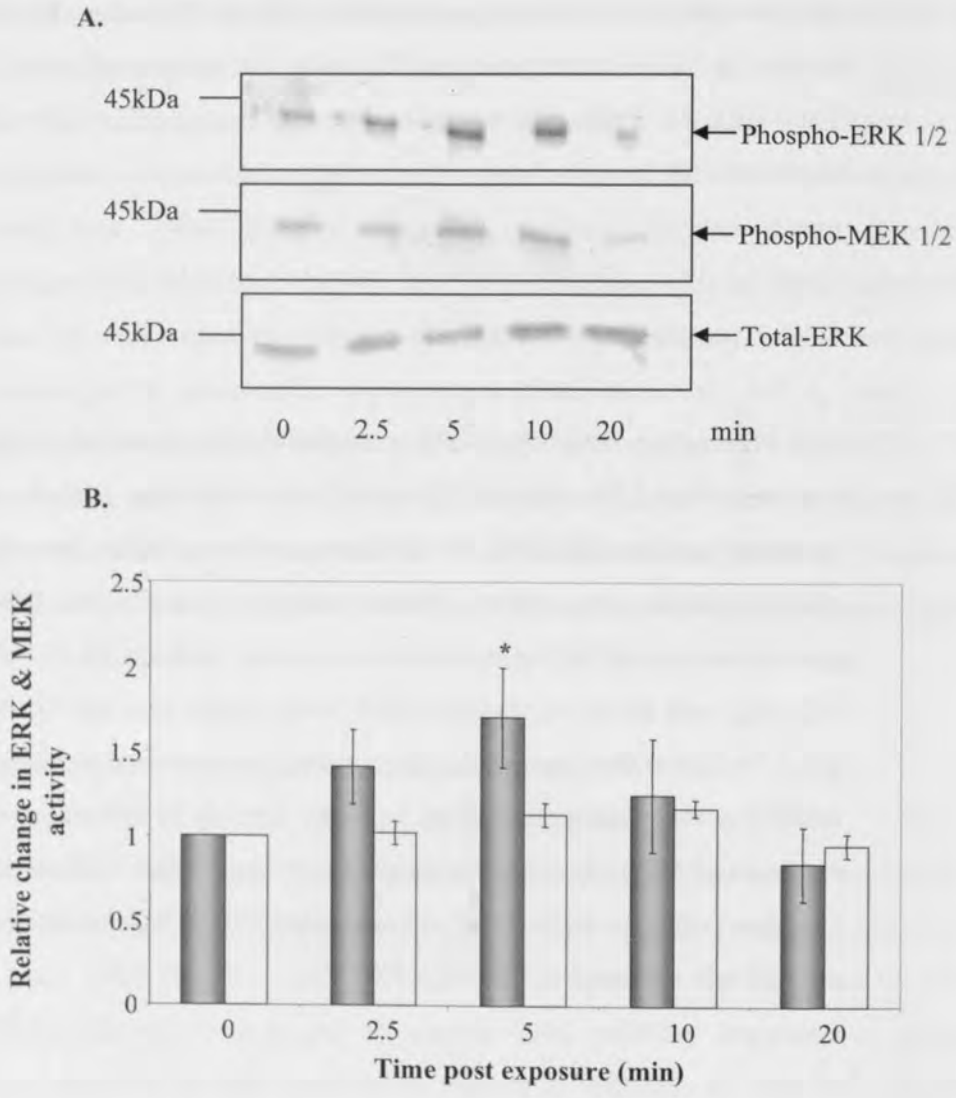

Figure 3.2.1. The effect of LPS (1µg/ml) on *L. stagnalis* ERK and MEK phosphorylation levels. (A) Equal amounts of protein were probed with the anti-phospho p44/42 MAP kinase antibody (1:1000) or anti-phospho MEK antibody (1:1000) to detect changes in ERK and MEK phosphorylation with time. Total levels of ERK were also determined using the anti-p44/42 MAPK antibody. (B) ERK immunoblots (n=6; shaded bars) and MEK immunoblots (n=2; white bars) were scanned and phosphorylation levels determined by image analysis; values shown are mean (± SEM; * $P < 0.05$ when compared to control (0 min) values; grey bars = ERK, white bars = MEK).

Western blotting with the anti-phospho Elk-1 antibody, demonstrated a clear increase in Elk-1 phosphorylation, and therefore activation, when haemocytes were exposed to 1μg/ml LPS for 5 min (fig. 3.2.2).

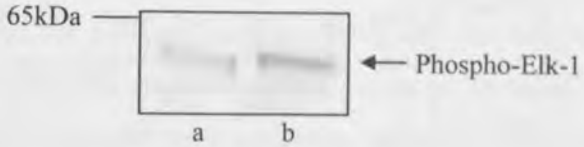

Figure 3.2.2. Activation of *L. stagnalis* haemocyte Elk-1 by *E. coli* LPS. Equal amounts of protein (30μg) were probed with the anti-phospho Elk-1 antibody (1:1000) to detect active Elk-1 in unchallenged haemocytes (a) and in haemocytes challenged with LPS (1μg/ml in SSS) for 5 min (b). The immunoblot is representative of two independent experiments.

3.2.2 Challenge of haemocytes with heat-killed E. coli

Live *E. coli* have been shown to activate the ERK pathway in mussel haemocytes over a 30 min time period in the presence of haemolymph serum when delivered at a ratio of 50:1 (bacteria : haemocytes; Canesi *et al.*, 2002b). *E. coli* were therefore used to challenge *L. stagnalis* haemocytes at a ratio of 20:1 and 40:1 (*E. coli* : haemocyte) to determine their effect on *L. stagnalis* ERK phosphorylation. Immunoblots of both experiments did not show a large increase in ERK phosphorylation in response to *E. coli* exposure (fig 3.2.3). Image analysis of blots revealed ERK phosphorylation levels to rise by only 14% and 24% from control levels after 20 min exposure to *E. coli* at the 20:1 and 40:1 ratios, respectively (fig. 3.2.3B). At 40 min exposure, phosphorylation levels fell to baseline levels, in a similar fashion to that seen with cell-free LPS.

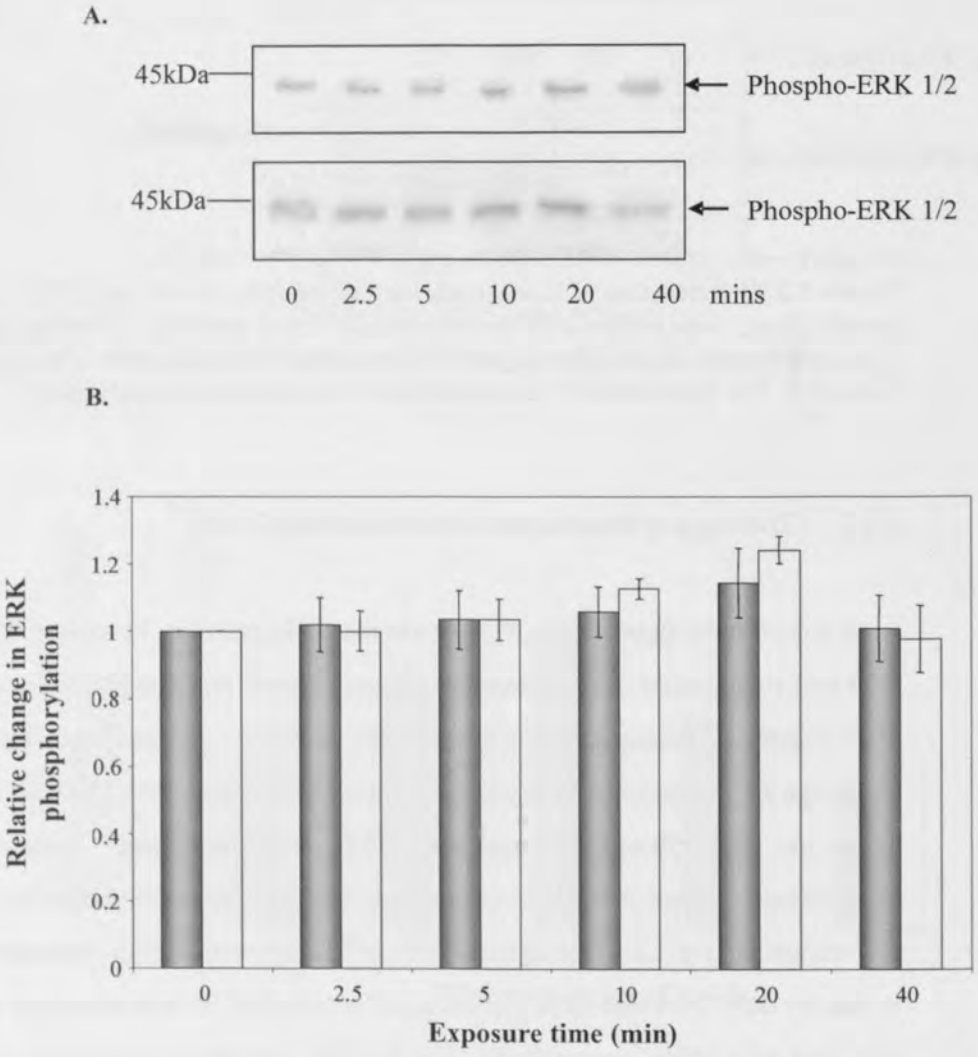

Figure 3.2.3. Haemocytes challenged with heat-killed *E. coli* do not possess significantly increased ERK phosphorylation. *E. coli* were applied to monolayers at ratios of 20:1 (*E. coli* : haemocytes; A, upper panel) and 40:1 (A, lower panel). Image analyses of immunoblots were performed (B; for both n=3; values are mean ± SEM; shaded bars, 20:1; white bars, 40:1) and analysis of variance did not reveal any significant increases in activity.

3.2.3 Challenge of haemocytes with zymosan A from S. cerevisiae

Hiller & Sundler (1999) showed ERK pathway activity to rise dramatically in macrophages exposed to zymosan A for 20 min. In the current study, zymosan A particles (0.2mg/ml in SSS) were used in the same manner as cell-free LPS to determine whether the ERK pathway in *L. stagnalis* haemocytes is phosphorylated by these particles, over 20 min. Western analyses revealed little change in ERK phosphorylation in response to zymosan A challenge, with the maximum increase in activity being only 7% higher than control levels after 10 min exposure (fig. 3.2.4). As expected, one-way ANOVA revealed that the observed changes were not significantly different from the control.

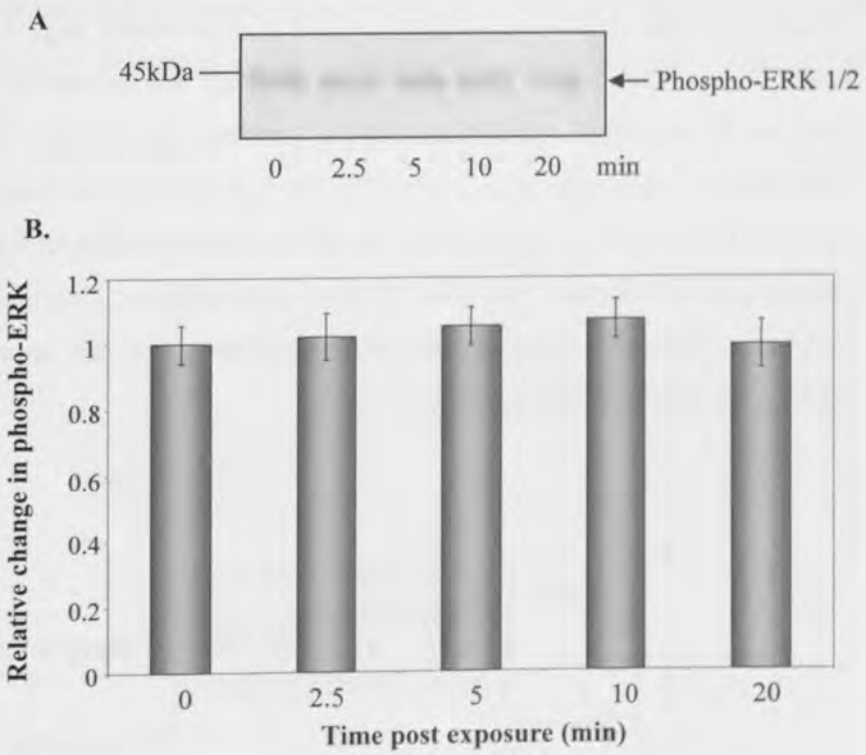

Figure 3.2.4. The effect of zymosan exposure (0.2mg/ml) on the phosphorylation of haemocyte ERK-like protein. (A) Blots were probed with anti-phospho p44/42 MAPK antibody to detect changes in levels of active ERK after zymosan exposure, before being scanned and analysed for relative band densities (B). Values shown are mean (n=3; values shown are mean ± SEM); one-way analysis of variance did not reveal any significant differences.

3.2.4 Adrenocorticotropin Hormone (ACTH)

Adrenocorticotropin hormone (ACTH) is widely known as a hormone of higher animals, being excreted by the anterior pituitary and stimulating the adrenal cortex. However, studies have shown that fragment 1-24 of human ACTH enhances phagocytosis by molluscan haemocytes (Ottaviani *et al.*, 1991a) and also cause dramatic cell shape changes in these cells (Sassi *et al.*, 1998). This part of the present study aimed to determine if ACTH could modulate *L. stagnalis* haemocyte ERK activity.

Initially, haemocytes were exposed to 10^{-4}M of human ACTH fragment 1-24 for up to 120 mins at room temperature. Results show that ACTH does not upregulate phosphorylation of haemocyte ERK over this time period (fig. 3.2.5A). However, levels of total ERK seemed to increase over the 120 min incubation period, suggesting that ACTH amplified expression of these proteins (fig 3.2.5B). The blots obtained from these experiments were a mix of those that showed two very distinct bands, or those that showed only one band; it was therefore not possible to analyse the bands for their relative intensities. However, the three blots obtained clearly showed that ACTH did not increase haemocyte ERK phosphorylation, but did appear to increase its expression after 120 min exposure.

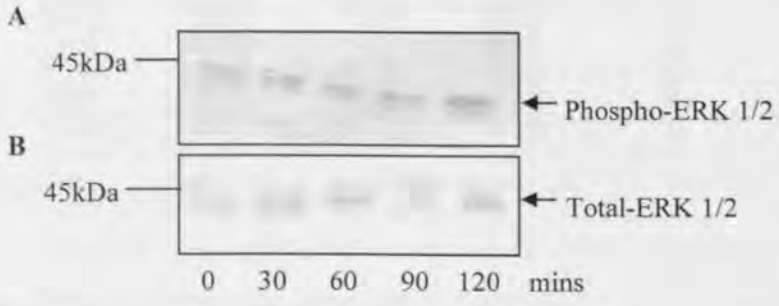

Figure 3.2.5. Exposure of *L. stagnalis* haemocytes to ACTH (10^{-4}M) over 120 min at room temperature does not modulate haemocyte ERK phosphorylation, but results in higher ERK 1/2 expression. Levels of phosphorylated ERK 1/2 (A) and total ERK (B) were determined in haemocyte lysates from cells exposed to 10^{-4}M ACTH (n = 3). Immunoblots were probed with anti-phospho p44/42 MAPK (1:1000) and anti-p44/42 MAPK (1:1000) antibodies respectively.

As total-ERK levels seem to increase when ACTH is present, LPS was added to see if the ERK that appeared to be pooled in the cells would be phosphorylated upon LPS challenge. Western analysis showed that levels of active ERK do not increase in response to combined use of ACTH and LPS, with activity decreasing from control levels (fig. 3.2.6). However, total-ERK increased by 253% at 10^{-4}M + LPS (fig. 3.2.6), although due to the large range in results this proved non-significant when ANOVA was applied. Total-ERK levels were at their highest when cells were exposed to 10^{-4}M, 10^{-7}M and 10^{-10}M all with LPS. (fig. 3.2.6), which is interesting as these concentrations are the highest reported in molluscs and only peak at these concentrations when the animal is under stress. The apparent pooling of ERK in the cells suggests that cells are preparing for a quick defence response by phosphorylating the already stored protein, although LPS may not be the correct mitogen to elicit this response. As ACTH was reconstituted with 2% acetic acid, control cells were exposed to 0.005% acetic acid and SSS, with no difference in ERK activity seen between the two control groups (data not shown).

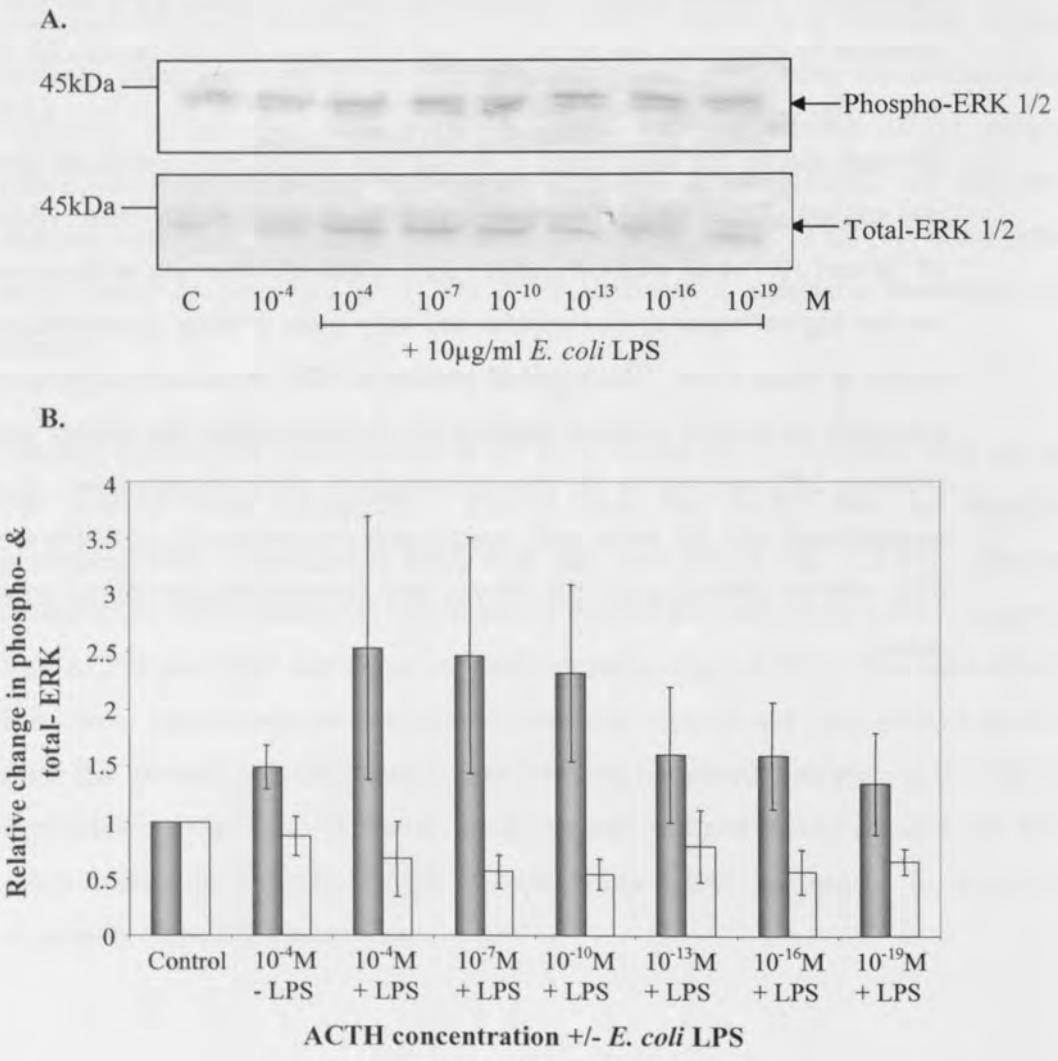

Figure 3.2.6. Haemocytes exposed to ACTH and LPS do not possess increased ERK activity, but have increased ERK expression. Immunoblots show level of phospho-ERK (A; upper panel) and total-ERK (A; lower panel) in lysates from haemocytes exposed to ACTH (10^{-19} – 10^{-4}M) for 150 min with LPS (10µg/ml) for 5 min (n = 3). Control cells were exposed to acetic acid (0.005%), with no difference seen between phospho-ERK levels in these cells and those exposed to SSS. Blots were then analysed for relative band densities for total-ERK (shaded bars) and phospho-ERK (white bars) (B; ± SEM).

3.2.5 Noradrenaline

Noradrenaline, a catecholamine produced by the neuroendocrine system and by immune cells in molluscs (Lacoste *et al.*, 2001a; 2002a), has been shown to increase levels of phosphorylated p44/42 MAPK in rat pinealocytes (Mackova *et al.*, 2000). In oyster haemocytes noradrenaline has resulted in increased phagocytosis and the production of reactive oxygen species (Lacoste *et al.*, 2001b; 2001c). In the current study, *L. stagnalis* haemocytes were exposed to 3 different doses of noradrenaline (0.1 – 10μM in SSS) for 0 - 20 min to determine the effects of this compound on ERK phosphorylation levels. It was found that noradrenaline alone does not enhance the phosphorylation of ERK isoforms in haemocytes (fig. 3.2.7 A-C), which was confirmed when the bands on the Western blots were analysed for their relative intensities (fig. 3.2.7D).

Noradrenaline (1μM) was also combined with 10μg/ml *E. coli* LPS in an attempt to upregulate haemocyte ERK phosphorylation. This higher concentration of LPS was used to try to elicit a stronger response by the haemocytes. The combined use of noradrenaline with LPS resulted in an increase in ERK phosphorylation at 40 min exposure to noradrenaline and a subsequent 5 min exposure to noradrenaline and LPS (fig. 3.2.8), with activity rising by 79% from control levels (fig. 3.2.8). ANOVA revealed that the observed differences were not statistically significant; this was due to the relatively large variability between the experiments.

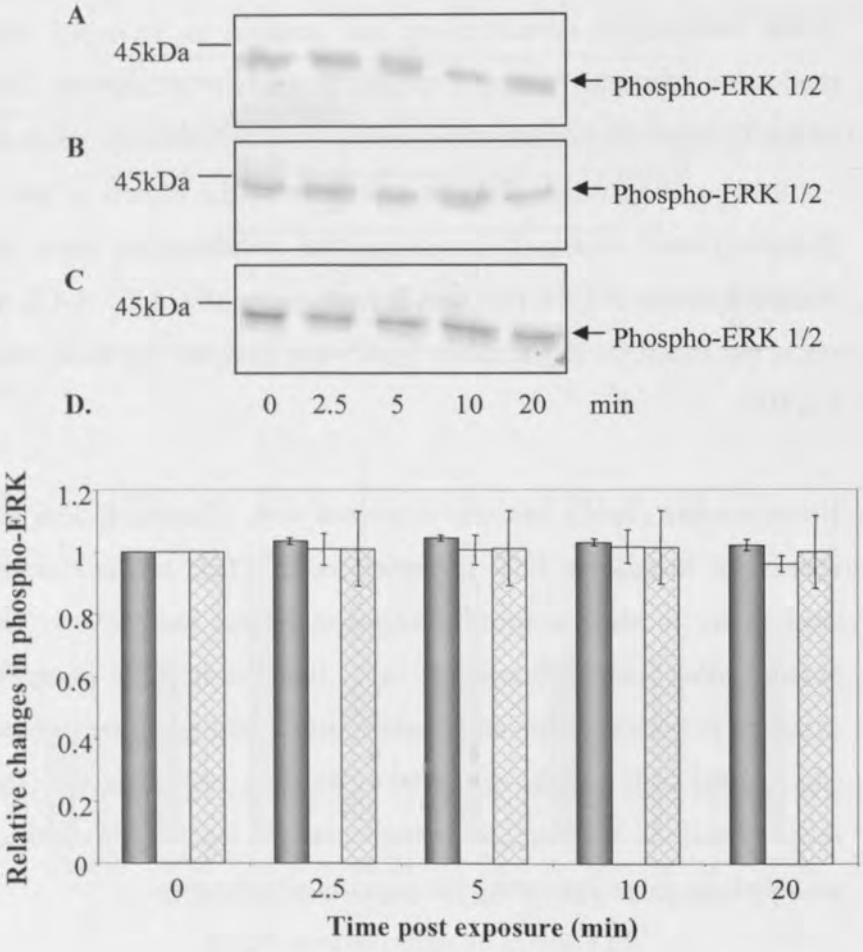

Figure 3.2.7. Haemocyte ERK activity is not modulated by noradrenaline. Haemocytes were exposed to 0.1μM (A), 1μM (B) and 10μM (C) noradrenaline for 0 – 20 min at room temperature. Image analysis was performed on scanned blots (D; n = 3; ± SEM; grey bars 0.1μM; white bars 1μM; hatched bars 10μM). Analysis of variance did not reveal any significant differences.

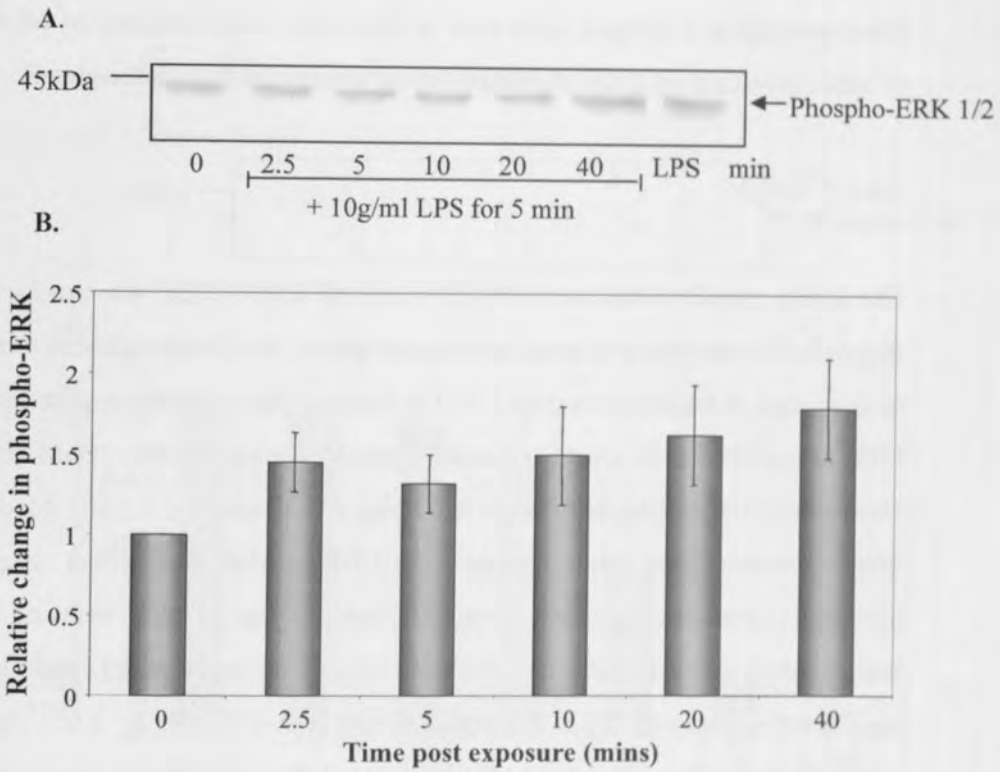

Figure 3.2.8. Mean haemocyte ERK 1/2 phosphorylation levels are increased by noradrenaline and LPS. Haemocytes were incubated with noradrenaline over 40 min with an additional 5 min exposure to both noradrenaline and *E. coli* LPS (A; 10µg/ml in SSS; n = 3). An LPS control (5 min in SSS; 10µg/ml) was also performed (A; LPS). Image analysis was performed on blots (B; ± SEM), analysis of variance did not reveal significant differences.

3.2.6 Blockade of MEK 1/2 phosphorylation

In mammalian cells, MEK lies directly upstream of ERK and activates it. To confirm that the MEK-ERK signalling module has been conserved in *L. stagnalis* haemocytes, pharmacological inhibitors were used to block the kinase activity of MEK. The effect of MEK blockade on ERK phosphorylation levels was then determined.

3.2.6.1 U0126

The highly selective inhibitor of MEK 1 and MEK 2, U0126, was pre-incubated with *L. stagnalis* haemocytes at a range of concentrations, for 30 min at room temperature prior to challenge of haemocytes with LPS for 5 min. This inhibitor resulted in a decrease in ERK phosphorylation levels in a dose-dependent manner, with 10μM resulting in total blockade of ERK phosphorylation following challenge (fig. 3.2.9). Methanol (at 0.1% final concentration), the vehicle for U0126, did not affect haemocyte ERK phosphorylation levels, since these remained similar to cells exposed to SSS alone. Image analysis followed by analysis of variance revealed that 0.1μM U0126 significantly reduced ERK phosphorylation by 45% ($P \leq 0.05$; fig. 3.2.9), and concentrations of 1 and 10μM U0126 reduced phosphorylation levels to 18% and 2% of control values respectively ($P \leq 0.001$; fig. 3.2.9).

Immunocytochemistry was also performed on haemocytes exposed to LPS and to 10μM U0126 for 30 min prior to LPS challenge. The results obtained confirm those seen with Western analyses (fig. 3.2.1); haemocytes exposed to LPS showed increased ERK phosphorylation compared to the control with an apparent clustering of ERK at the periphery of the nucleus (fig 3.2.10 A and B). When cells were treated with 10μM U0126, inhibition of ERK phosphorylation after LPS treatment was clearly seen (fig. 3.2.10C). A total blockade of ERK activation was not, however, seen with immunocytochemistry; this could be due to any 'remaining' active MEK that was phosphorylated before U0126 addition, acting on ERK. Control cells exposed to methanol (the vehicle for U0126), did not appear to have a different distribution or level of phosphorylated ERK when compared to cells incubated in SSS alone.

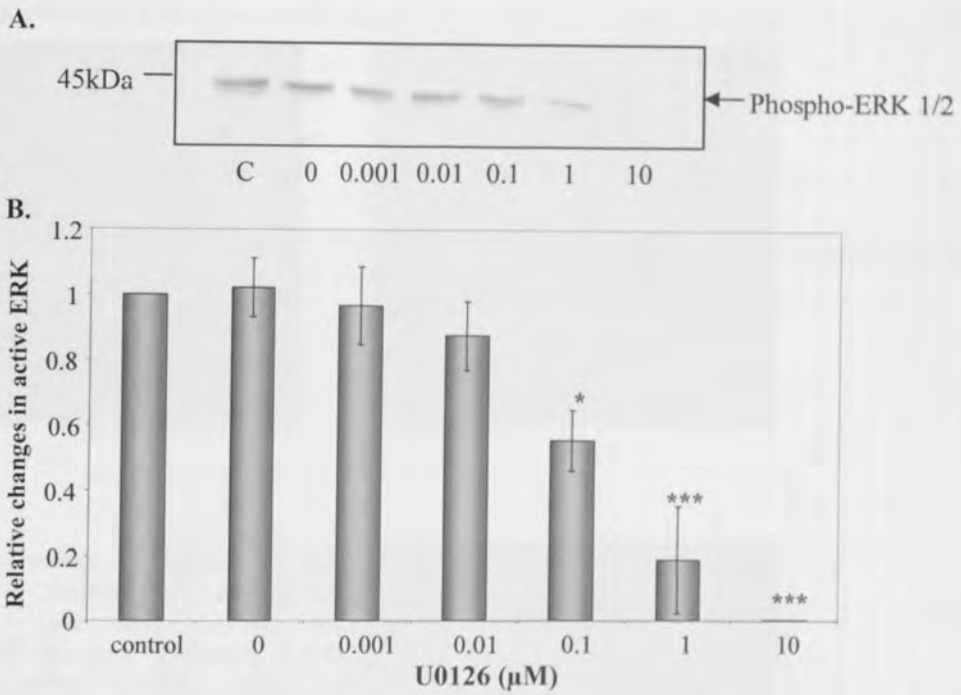

Figure 3.2.9. The MEK inhibitor, U0126, blocks ERK phosphorylation in a dose responsive manner. Cells were pre-incubated with U0126 (0.001 – 10μM), vehicle (control) or SSS only (0) for 30 min prior to challenge with 10μg/ml *E. coli* LPS. Equal amounts of protein were probed with anti-phospho p44/42 MAPK antibody dilution (A) and blots analysed for band intensities (B; n = 3; values shown are mean ± SEM; ANOVA * $P \leq 0.05$; *** $P \leq 0.001$).

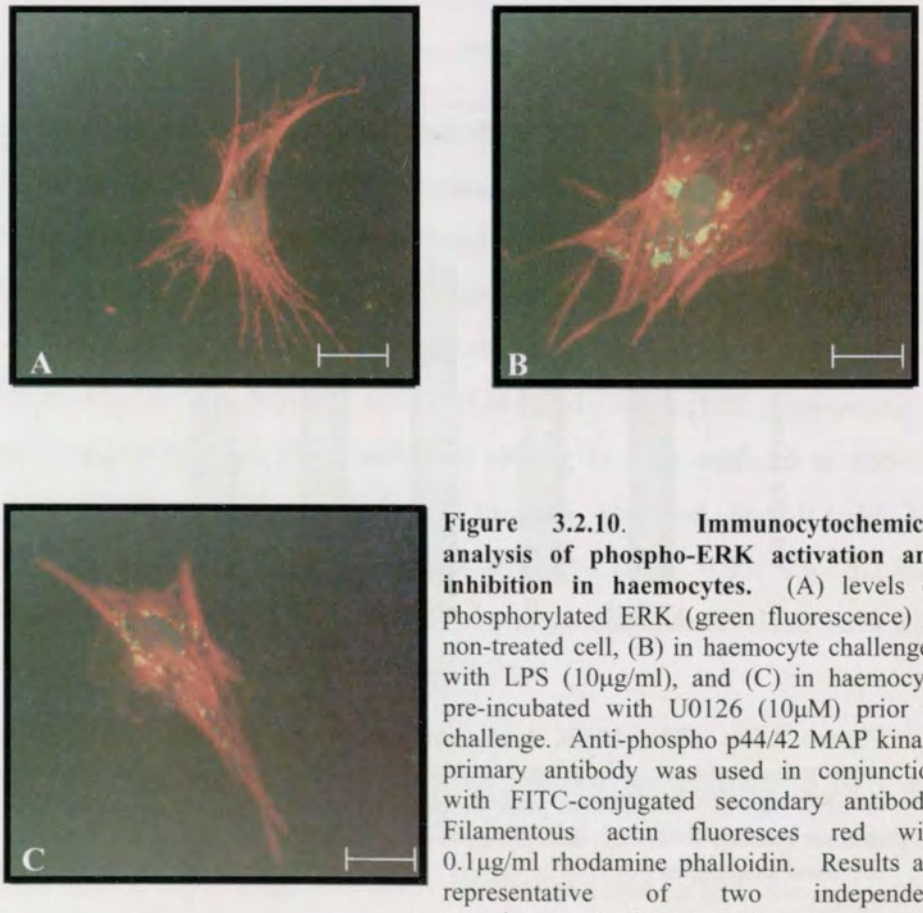

Figure 3.2.10. Immunocytochemical analysis of phospho-ERK activation and inhibition in haemocytes. (A) levels of phosphorylated ERK (green fluorescence) in non-treated cell, (B) in haemocyte challenged with LPS (10μg/ml), and (C) in haemocyte pre-incubated with U0126 (10μM) prior to challenge. Anti-phospho p44/42 MAP kinase primary antibody was used in conjunction with FITC-conjugated secondary antibody. Filamentous actin fluoresces red with 0.1μg/ml rhodamine phalloidin. Results are representative of two independent experiments. Scale bars = 10μm.

3.2.6.2 PD98059

Pre-incubation of haemocytes with the MEK 1 inhibitor, PD98059, for 30 min prior to LPS challenge resulted in reduced ERK activity in a dose-dependent manner (fig 3.2.11A). 100μM PD98059 significantly reduced ERK phosphorylation to 61% ($P \leq 0.05$; fig. 3.2.11B) that of the control. As PD98059 only inhibits the ability of MEK 1 to act on ERK, the high level of phosphorylated ERK remaining suggests that haemocytes rely heavily on MEK 2 activity for ERK phosphorylation.

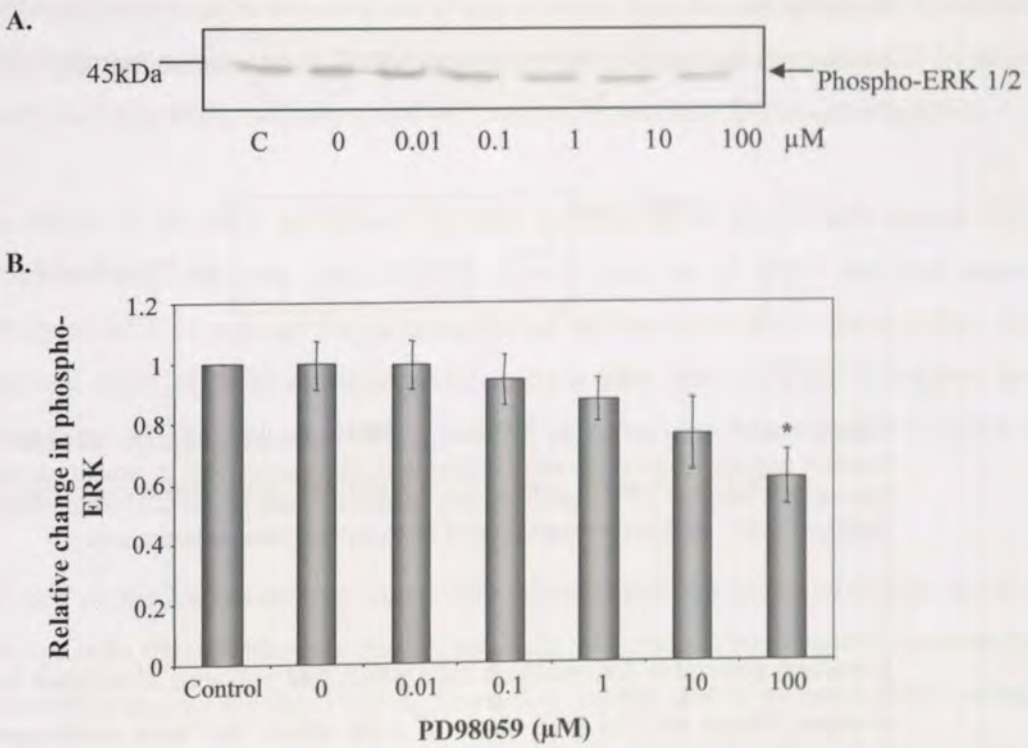

Figure 3.2.11. The MEK 1 inhibitor, PD98059, reduces ERK phosphorylation in *L. stagnalis* haemocytes. Cells were pre-incubated with PD98059 (0.01 – 100μM), vehicle (control) or SSS only (0) for 30 min prior to challenge with 10μg/ml *E. coli* LPS. Equal amounts of protein were loaded into the gel and blots were probed with anti-phospho p44/42 MAPK antibody (1:1000) (A). Blots were analysed and mean changes in ERK phosphorylation levels are shown (B; n = 3; ± SEM; ANOVA * $P \leq 0.05$).

3.2.7 Non-radioactive MAP kinase activity assay

Although haemocyte ERK-like proteins have been identified as described in section 3.1, it is important to demonstrate that the immunoreactive protein possesses kinase activity. This was done using a non-radioactive MAP kinase activity assay (developed by NEB). Briefly, phosphorylated ERK 1/2 was immunoprecipitated from haemocyte lysates, and the kinase activity of the immunoprecipitated proteins was tested by incubating it with an Elk-1 fusion protein and ATP. The samples were then electrophoresed and Western blots were probed for phosphorylated Elk-1, the target of ERK 1/2. Results obtained show that *L. stagnalis* ERK-like proteins isoforms do indeed possess kinase activity, due to the presence of phosphorylated Elk-1 in samples containing immunoprecipitated phospho-ERK from haemocytes (fig. 3.2.12).

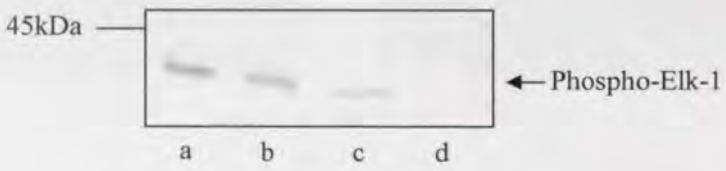

Figure 3.2.12. ***L. stagnalis*** **haemocyte ERK possesses kinase activity towards Elk-1.** Positive control samples (a) were spiked with 20ng pure ERK 2, unstimulated haemocyte cell lysates (b), 10μg/ml LPS stimulated cell lysates (c), and 10μM U0126 inhibited cell lysates (d) were prepared. Blot is representative of three independent experiments.

Immunoprecipitates derived from cells which had not been stimulated by LPS exhibited stronger kinase activity than those of cells which had been challenged (fig. 3.2.12). This does not fit in with the hypothesis that stimulated cells should have more phosphorylated ERK, and thus possess stronger kinase activity towards Elk-1. One explanation could be that active ERK in challenged cells could be associating with the cytoskeleton making it harder to extract and subsequently immunoprecipitate. However, the use of U0126 confirmed that it is only haemocyte ERK that is being precipitated and acting on Elk, since when the activation of ERK is blocked, so too is the activation of the Elk-1 transcription factor (fig. 3.2.12).

3.2.8 Summary of results

The results obtained in this section suggest that the most potent mitogen in activating *L. stagnalis* haemocyte ERK is cell-free LPS, which results in a transient phosphorylation over 20 min. The rapid phosphorylation of ERK, with maximal levels seen at 5 min post exposure to LPS, suggests that the haemocytes are able to produce a relatively quick response when potentially harmful organisms are present. It was surprising that heat-killed *E. coli* did not have a significant effect on ERK phosphorylation, as it is LPS on the outer surface of the bacteria that would be eliciting the haemocyte response. Although zymosan A, ACTH (fraction 1-24) and noradrenaline have all been shown to activate immune responses in gastropod molluscs, they do not appear to upregulate ERK pathway activity in *L. stagnalis* haemocytes. These compounds could of cause affect other signalling pathways, and this possibility warrants further investigation.

The ability of the MEK inhibitors, PD98059 and U0126, to significantly reduce ERK phosphorylation confirms that MEK is indeed upstream of ERK, and that kinase activity of MEK is required for the activation of the haemocyte ERK like proteins. The relatively high levels of remaining ERK activity after use of PD98059 suggest that haemocytes rely heavily on MEK 2 for ERK phosphorylation or that this inhibitor is less effective at inhibiting molluscan ERK 1 than mammalian ERK 1.

The use of the kinase activity assay demonstrated that the immunoreactive proteins detected with the anti-phospho MAPK antibody do possess kinase activity towards the conserved transcription factor Elk-1. Overall, it appears that as in mammalian systems the ERK signalling module in *L. stagnalis* functions as a kinase cascade involving MEK, ERK and Elk-1 like proteins.

SECTION 3.3

FUNCTION OF ERK-LIKE PROTEINS IN *L. STAGNALIS* HAEMOCYTES

To assess the function of the ERK-like proteins in *L. stagnalis* haemocytes, phagocytosis assays were performed with various pharmacological inhibitors. The aim of the study was to block the kinase activities of various upstream regulators of ERK and subsequently measure the phagocytic capability of the haemocytes. The signal from internalised fluorescent 'bioparticles' was measured with a spectrofluorometer after haemocytes had been incubated with bioparticles for 1 h; the fluorescence of any extracellular bioparticles' was quenched with trypan blue. Throughout the incubation period, pharmacological inhibitors remained in the wells, to ensure the effects of these inhibitors on the haemocytes lasted throughout the experiment.

3.3.1 The effects of MEK 1/2 inhibitors on phagocytosis

In previous experiments, U0126 (10μM) was shown to reduce ERK phosphorylation levels significantly to 2% of control values (ANOVA $P \leq 0.001$; fig. 3.2.9), by blocking the kinase activity of MEK 1/2. A dose-responsive reduction in phagocytosis of 'bioparticles' was observed with this inhibitor, with 100μM U0126 reducing phagocytic activity by 80% ($P \leq 0.001$; fig. 3.3.1A). Since ERK phosphorylation was seen to be blocked by 10μM U0126 (fig 3.2.9), it was surprising that this dose did not result in a large decrease in phagocytosis; phagocytic activity was only reduced by 13.5% (fig. 3.3.1A). A possible reason for this could be that the incubation period in U0126 for phagocytosis assays was 1 h longer than that of previous experiments involving Western blotting, so cells may have recovered from the effect of this inhibitor, therefore, a higher concentration may have been required for a longer lasting effect.

3.3.2 The effects of MEK 1 inhibitors on phagocytosis

In contrast to U0126, PD98059 only inhibits MEK 1. Western analysis showed this inhibitor to block ERK activity in a dose-responsive manner, with the greatest concentration (100μM) reducing activity to 61% of control levels ($P \leq 0.05$; fig. 3.2.11). As expected, this dose of the inhibitor did not reduce phagocytic activity towards the 'bioparticles' as dramatically as U0126, and only inhibited phagocytosis by

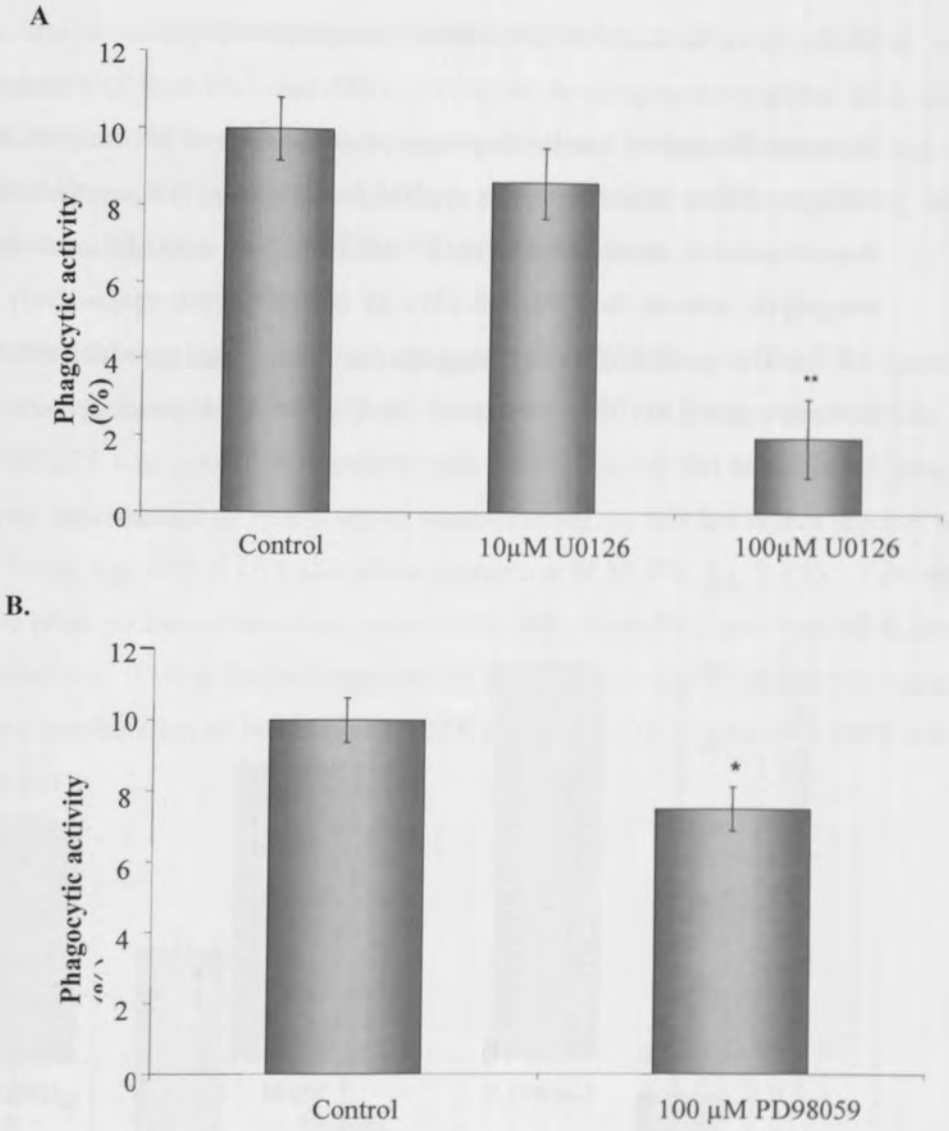

Figure 3.3.1. Inhibition of haemocyte phagocytic activity with MEK inhibitors, (A) U0126 and (B) PD98059. Haemocytes were pre-incubated in each inhibitor for 30 min prior to the addition of *E. coli* 'bioparticles'. Values shown are mean (± SEM; n = 6). * $P < 0.05$ and ** $P < 0.001$, when compared to control values.

25% ($P \leq 0.05$; fig. 3.3.1B). This result further suggests that haemocytes rely on MEK 2 kinase activity to mount an efficient immune response.

3.3.3 The effects of Ras inhibition on phagocytosis

To assess the role of Ras in the phagocytic responses of haemocytes, the farnesylation inhibitor, FTase inhibitor I, was applied to the cells. Phagocytosis was reduced in a dose-responsive manner by the FTase inhibitor, with 50 and 100μM reducing phagocytic activity to 75% and 61% of control levels, respectively ($P \leq 0.05$; fig. 3.3.2). This partial inhibition suggests that both Ras-dependant and Ras-independent pathways lead to ERK activation and subsequent phagocytosis in *L. stagnalis* haemocytes.

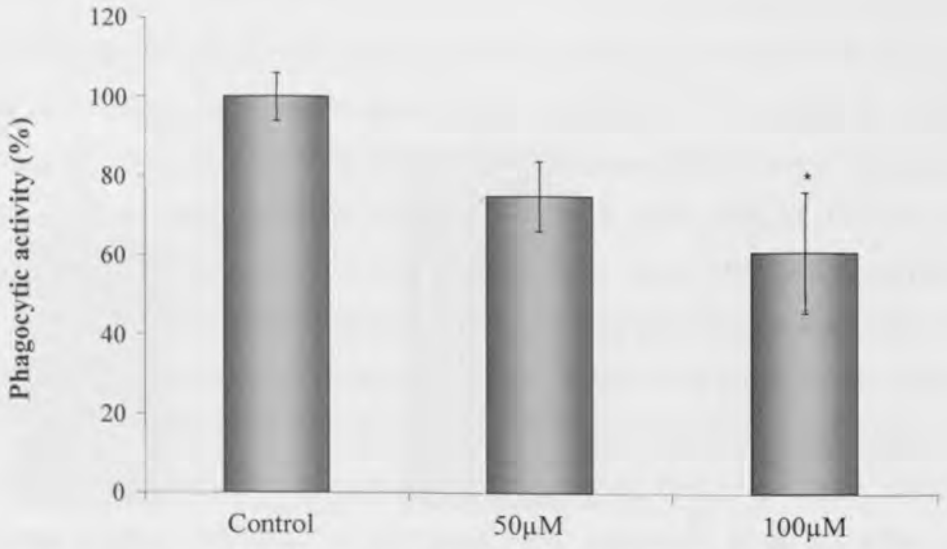

Figure 3.3.2. The inhibition of phagocytosis by blockade of Ras farnesylation. Haemocytes were pre-incubated with FTase inhibitor I for 30 min prior to the addition of *E. coli* 'bioparticles'. Values are means (± SEM; n = 6; * $P \leq 0.05$, when compared to control values).

3.3.4 The effects of Protein Kinase C inhibition on phagocytosis

It was previously demonstrated that 10μM of the PKC inhibitor GF109203X inhibited *L. stagnalis* haemocyte PKC phosphorylation significantly (Walker & Plows, 2003), but the role of PKC in haemocyte phagocytic responses was not known. This study revealed that GF109203X (10μM) significantly reduced phagocytosis of 'bioparticles'

by 44%, confirming that phagocytosis can occur, but not as efficiently, in the absence of PKC phosphorylation and, therefore, activity (fig. 3.3.3).

The inhibitors GF109203X and PD98059 were used in conjunction to test the importance of both PKC and MEK activity to the phagocytic response of *L. stagnalis* haemocytes. The combined use of the two inhibitors (10μM GF109203X and 100μM PD98059) resulted in a 56% reduction in phagocytosis ($P \leq 0.05$; fig. 3.3.3), which is greater than that seen with just GF109203X (10μM) alone.

GF109203X was also used in conjunction with U0126 to assess the haemocytes requirement for activity of both PKC and the two MEK isozymes. When 10μM GF109203X was used in conjunction with a high dose of U0126 (100μM), phagocytic activity was reduced to 36.6% which was surprising, as this is a higher level of activity than using just U0126 (100μM) alone (reduction of 80.9%; fig. 3.3.1). This could be due to other pathways becoming active in the cells when PKC and both MEK isozymes are inhibited. This is further supported by the relatively low reduction in phagocytosis when a combination of 10μM GF109203X and 10μM U0126 was used (39% reduction; $P \leq 0.001$).

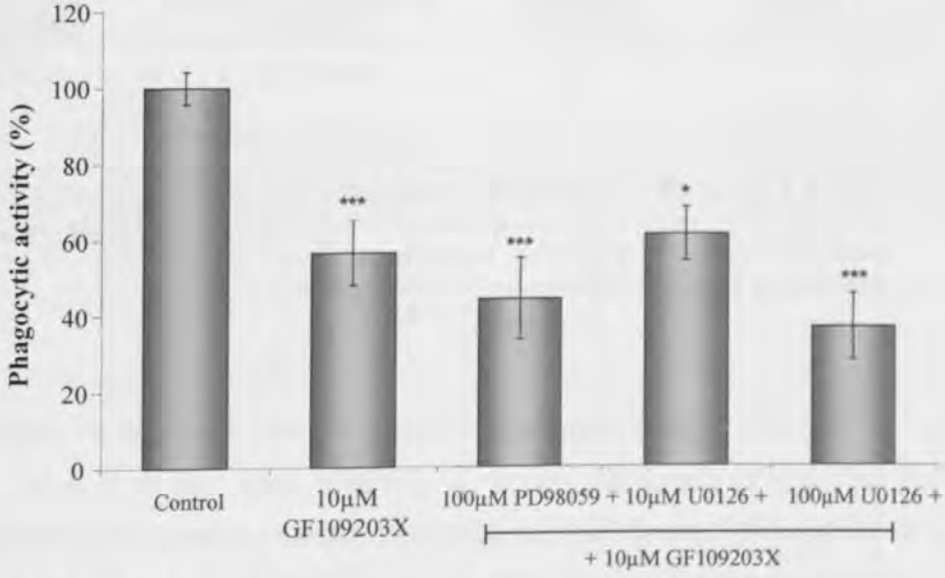

Figure 3.3.3. The effect of blocking PKC and MEK 1/2 activity on phagocytosis by *L. stagnalis* haemocytes. Haemocytes were pre-incubated with U0126 or PD98059 in combination with GF109203X for 30 min prior to addition of *E. coli* 'bioparticles'. Values shown are mean (± SEM; n = 6). * $P \leq 0.05$ and *** $P \leq 0.001$ when compared to control values.

3.3.5 The effect of Protein Kinase A inhibition on phagocytosis

The effect of the highly selective Protein Kinase A (PKA) inhibitor, KT5720, on the phagocytic response of haemocytes was tested. At concentrations of 50 and 100nM KT5720 did not have a significant effect on phagocytosis, since the fluorescence signal from internalised bioparticles was only reduced by 8.9 and 13.3%, respectively (fig. 3.3.4). This suggests that PKA does not play a major role in haemocyte phagocytic activity, and therefore confirms that PKA does not activate the ERK pathway in these cells when challenged with bioparticles.

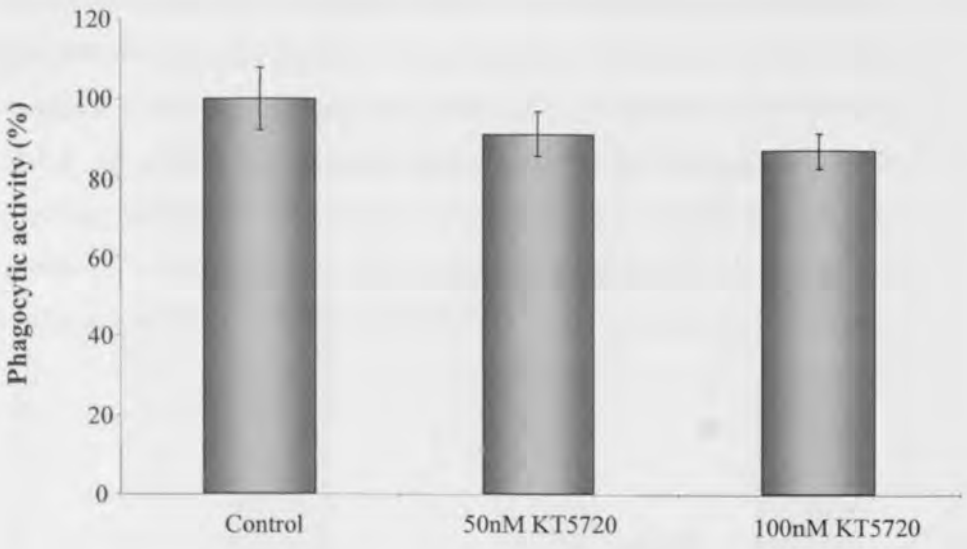

Figure 3.3.4. Lack of inhibition of haemocyte phagocytic activity with the PKA inhibitor, KT5720. Haemocytes were pre-incubated with KT5720 (50 or 100nM) for 30 min before the addition of *E. coli* 'bioparticles'. Mean phagocytic activity is shown (± SEM; n = 6). ANOVA revealed no significant differences between treatments.

3.3.6 The effect of phosphatidylinositol-3-kinase inhibition on phagocytosis

Phosphatidylinositol-3-kinase (PI-3-K) is known to play a key role in phagocytosis by macrophages (reviewed by Gillooly *et al.*, 2001), but its role in haemocyte phagocytic activity has not yet been investigated. *L. stagnalis* haemocytes were therefore exposed to the selective PI-3K inhibitor, LY294002, to determine whether or not PI-3K inhibition effects the phagocytic response. Haemocytes exhibited reduced

phagocytosis in a dose-dependent manner in response to this inhibitor (fig. 3.3.5). Moreover, at the highest concentration LY294002 (10μM) reduced 'bioparticle' internalisation significantly by 62% ($P \leq 0.001$; fig 3.3.5). Lower doses of LY294002 also resulted in highly significant reductions in haemocyte phagocytic activity, with 0.1μM and 1μM reducing phagocytosis by 22% and 43% respectively ($P \leq 0.001$; fig 3.3.5). These findings demonstrate that PI-3K plays an important role in haemocyte phagocytosis.

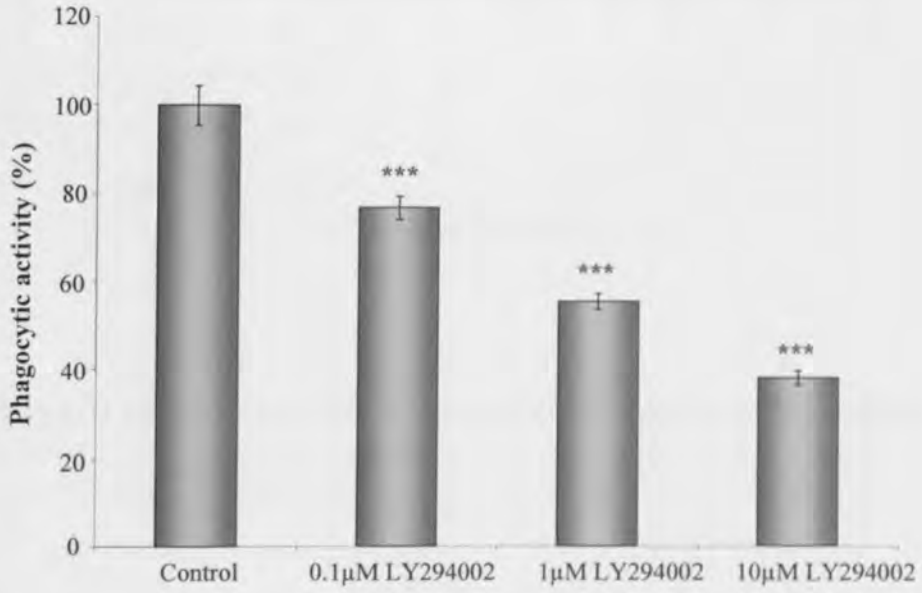

Figure 3.3.5. Reduction in 'bioparticle' phagocytosis as a result of PI-3K inhibition. Haemocytes were pre-incubated with LY294002 (0.1 – 10μM) for 30 min before the addition of *E. coli* 'bioparticles'. Mean phagocytic activity is shown (± SEM; n = 3; *** $P \leq 0.001$, when compared to control values).

3.3.7 Summary of results

The use of pharmacological inhibitors to identify which pathways regulate phagocytic activity has been extremely successful in *L. stagnalis* haemocytes. By blocking MEK 1/2 activity (with U0126), the lowest level of phagocytosis was recorded, confirming that ERK phosphorylation is required for this defence response. The inhibitor PD98059 (a MEK 1 inhibitor) only partially reduced phagocytosis, which suggests that MEK 2 activity is vital for ERK phosphorylation and subsequent phagocytosis. Results

with the farnesylation inhibitor (FTase inhibitor I) suggest that haemocyte phagocytosis relies on Ras-dependent and Ras-independent mechanisms, which could have involved PKA activity. However, by using the PKA inhibitor, KT5720, this possibility was discounted, since blocking PKA activity did not have a significant affect on phagocytosis. One protein found to be heavily involved in phagocytosis was phosphatidylinositol-3-kinase. By blocking the activity of this enzyme, phagocytosis was significantly reduced, which not only implies the presence of PI-3-K in haemocytes, but also demonstrates that the function of this protein has been conserved in both mammalian macrophages and molluscan haemocytes.

SECTION 3.4

EFFECT OF MONOSACCHARIDES ON ERK, PKC AND PHAGOCYTOSIS

A study was carried out with albumin-linked monosaccharides to assess how they affect ERK and PKC activity and phagocytosis by haemocytes. The monosaccharides, galactose and fucose, were used since these sugars are key components of the outer coat of helminth larvae and adults (the glycocalyx). To determine whether haemolymph components could also alter the responses of haemocytes to sugars, studies were carried out in the presence or absence of haemolymph.

3.4.1 Effect of sugars on ERK and PKC activity

3.4.1.1 Effect on ERK and PKC in the absence of haemolymph

In the absence of haemolymph, exposure of haemocytes to either BSA- galactose or BSA-fucose resulted in reduced levels of ERK phosphorylation (Fig. 3.4.1A). This effect was most prominent when haemocytes were exposed to 400nM BSA-galactose or 800nM BSA-fucose, since at these concentrations ERK phosphorylation was significantly reduced by 54% and 50%, respectively ($P \leq 0.05$, Fig. 3.4.3A). Moreover, the inhibitory effect of albumin-linked fucose on ERK phosphorylation appeared to be dose-dependent. The combined use of albumin-linked sugars also resulted in a dose-responsive decline in ERK phosphorylation (Fig. 3.4.1A) with 800nM of BSA-galactose/BSA-fucose reducing phosphorylation by 67% ($P \leq 0.01$; Fig. 3.4.3A). The use of 800nM galactose/fucose gave the largest overall reduction in ERK activity. One-way analysis of variance did not reveal any significant differences within the sugar groups (i.e. 200nM fucose was not significantly different to 400 or 800nM). For controls, haemocytes were incubated in SSS or BSA (800nM) and no differences in ERK phosphorylation were observed in cells incubated in either solution.

PKC phosphorylation levels were also reduced when haemocytes were exposed to sugars in the absence of haemolymph (Fig. 3.4.2A). The effect of BSA-galactose was dose-responsive with phosphorylation levels being significantly reduced by 56% and 73% at 400nM and 800nM, respectively ($P \leq 0.01$ and $P \leq 0.001$; Fig. 3.4.3B). Analysis of variance between galactose concentrations revealed that the phosphorylation levels seen with 200nM were significantly different to those seen with 800nM ($P \leq 0.05$). Phosphorylation of haemocyte PKC was also reduced in the presence of BSA-fucose, but at the higher doses, the inhibitory effects were less than

seen with galactose (Fig. 3.4.2A). Interestingly, the combined use of BSA-galactose and BSA-fucose gave highly significant reductions in PKC phosphorylation at all three concentrations (Fig. 3.4.2A), with sugars at 200nM, 400nM, and 800nM suppressing phosphorylation levels to 36%, 24% and 8% of the control respectively ($P \leq 0.001$; fig 3.4.3B). As for phospho-ERK experiments, controls were performed with SSS and BSA (800nM), with no differences in the phosphorylation status of PKC were observed following incubations in these sugars.

3.4.1.2 Effect on ERK and PKC activity in the presence of haemolymph

In contrast to results obtained in the absence of haemolymph, with haemolymph present, haemocyte ERK phosphorylation was not significantly reduced when sugars were either delivered independently or in combination (Fig. 3.4.1B), suggesting that haemolymph components may play a role in sugar recognition. Image analysis of individual blots revealed that fucose had in some cases increased ERK phosphorylation, although overall no significant increase was found (Fig. 3.4.3A), neither were there any significant differences within sugar groups.

In contrast to ERK phosphorylation, PKC phosphorylation levels were reduced in the presence of haemolymph when BSA-sugars were delivered either separately or in combination (Fig. 3.4.2B). Whereas BSA-galactose only caused a significant reduction (45%) in PKC phosphorylation at 800nM ($P \leq 0.001$; Fig. 3.4.3B), BSA-fucose resulted in significant reductions of PKC phosphorylation at all concentrations (Fig. 3.4.3B). Exposure to albumin-linked fucose at 200nM, 400nM and 800nM resulted in PKC phosphorylation levels being reduced to 68% ($P \leq 0.05$), 65% ($P \leq 0.05$) and 36% ($P \leq 0.001$) of control levels, respectively (Fig. 3.4.3B). The effect of fucose proved highly dose-responsive, with the effects of 200nM being significantly different to those of 800nM ($P \leq 0.05$). When used in combination, BSA-galactose and BSA-fucose inhibited PKC phosphorylation more than ERK phosphorylation, with all concentrations of sugars mediating significant effects ($P \leq 0.001$; fig. 3.4.3B). As for experiments without haemolymph, controls were performed with SSS and BSA (800nM), and these different solutions did not have any different effects on ERK phosphorylation.

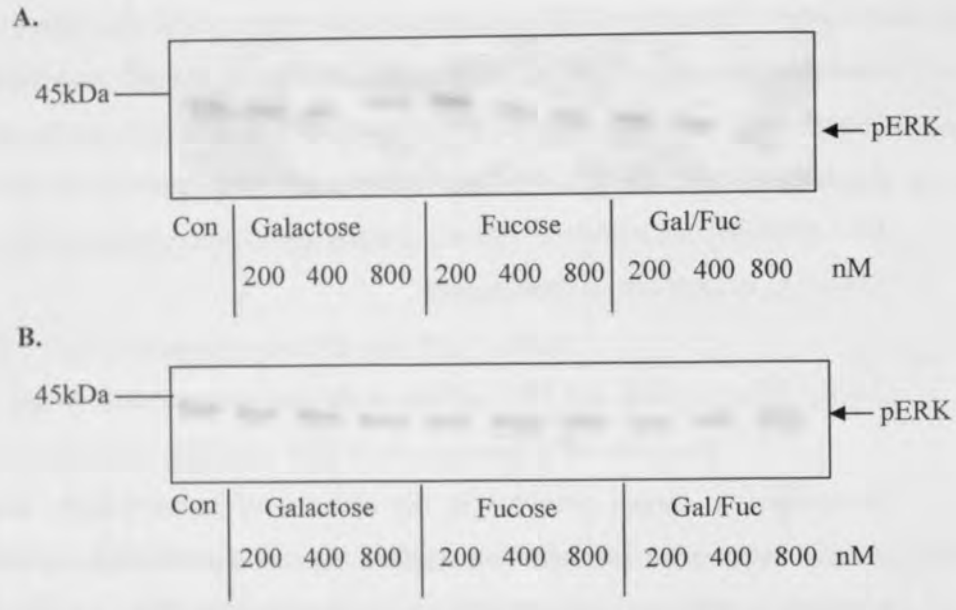

Figure 3.4.1. Western blots showing ERK phosphorylation after haemocyte exposure to albumin-linked monosaccharides. Haemocytes were exposed to the relevant sugar, or BSA (control) for 1 h at room temperature in the absence (A) or presence (B) of haemolymph. Membranes were probed with anti-phospho p44/42 MAPK antibody (1:1000); blots shown are representative of three independent experiments.

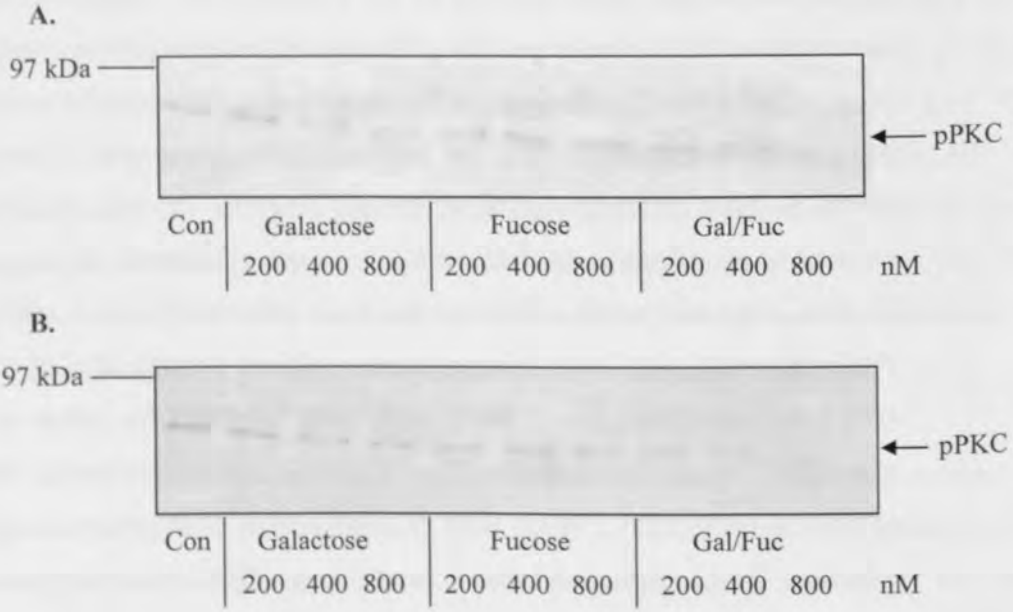

Figure 3.4.2. Levels of phospho-PKC decline in a dose-responsive effect with exposure to sugars with and without haemolymph present. Haemocytes were exposed to sugars, or BSA (control), for 1 h in the absence (A) and presence of haemolymph (B). Membranes were exposed to anti-phospho PKC (pan) antibody; blots shown are representative of three independent experiments.

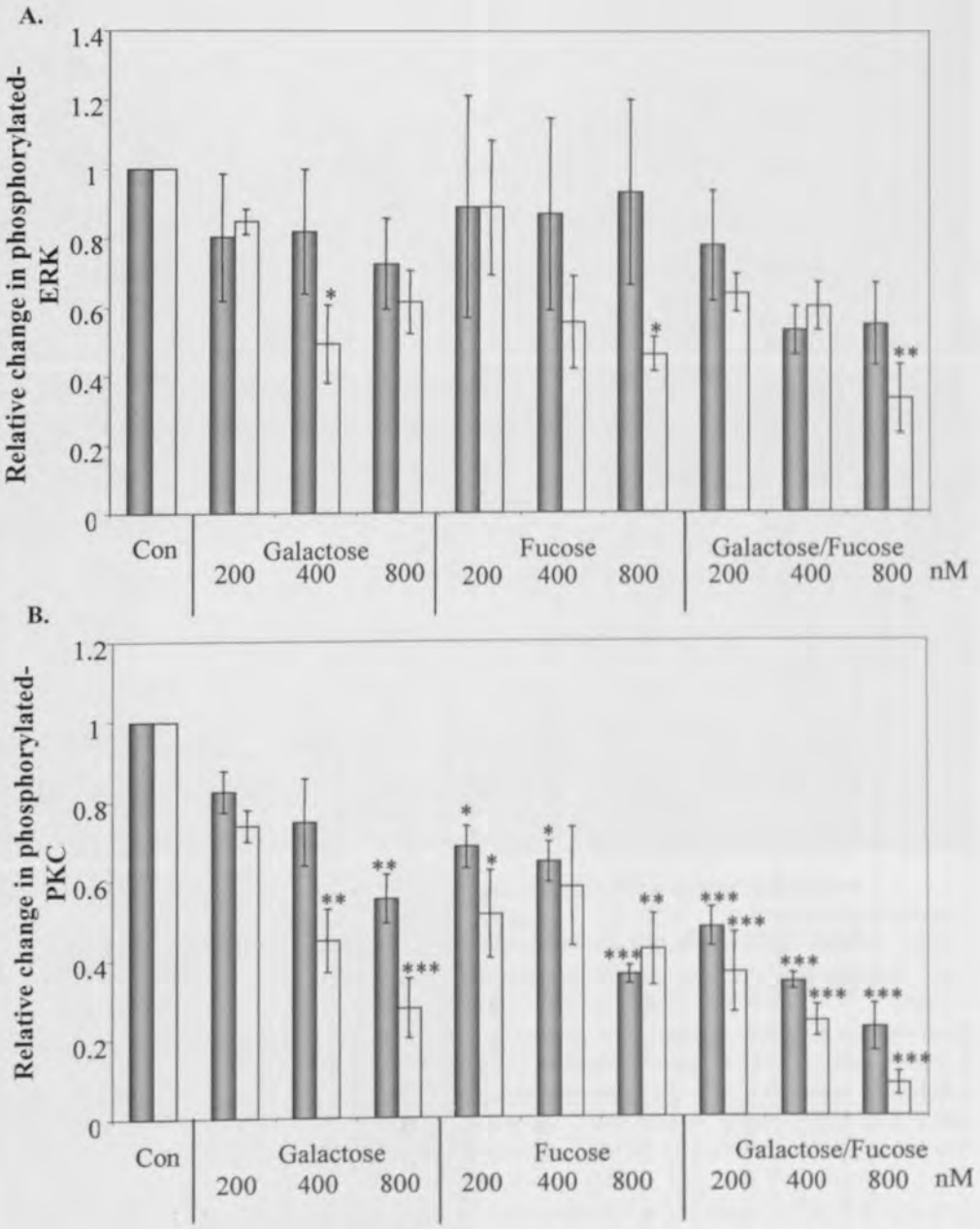

Figure 3.4.3. Analysis of ERK and PKC immunoblots to determine the effects of sugar exposure on phosphorylation levels. ERK and PKC immunoblots (fig. 3.4.1 and 3.4.2) were analysed for relative band intensities. Values shown are means (n = 3; ± SEM) of ERK phosphorylation (A) and PKC phosphorylation (B); white bars = without haemolymph, grey bars = with haemolymph. * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$ when compared to control values (BSA).

3.4.1.3 Changes in the cellular location of ERK when exposed to sugars

Western analyses of cells exposed to sugars allowed the levels of active ERK to be monitored. However, immunocytochemistry was applied to these cells in hope of monitoring the cellular distribution of active ERK isozymes after haemocytes were incubated with the corresponding sugar for 1 h with or without haemolymph. With LPS it was observed that active ERK redistributes to the nucleus, with clustering of the enzymes in particular at the periphery of the nuclear membrane (fig. 3.2.10).

Haemocytes exposed to BSA (control; fig. 3.4.4A) had phospho-ERK equally distributed in the cytoplasm, with clustering at the periphery of the nuclear membrane (thus making this membrane highly visible). In contrast, haemocytes exposed to BSA-galactose (800nM) in the absence of haemolymph had active ERK isozymes predominantly at the cell periphery of the cells (fig. 3.4.4B). This was also true of those cells exposed to galactose (800nM) with haemolymph (fig. 3.4.4C), although the effect is much more marked with haemolymph present. Fucose (800nM) did not have such a noticeable effect when applied to the cells without haemolymph (fig. 3.4.4E). Active ERK appeared to be solely in the nuclear region of the haemocytes, with hardly any fluorescence from ERK appearing in the cytoplasm. However, when haemocytes were incubated with fucose and haemolymph it is highly apparent that the phospho-ERK isozymes are located in the cytoplasm at the cell periphery of the cells (fig. 3.4.4D).

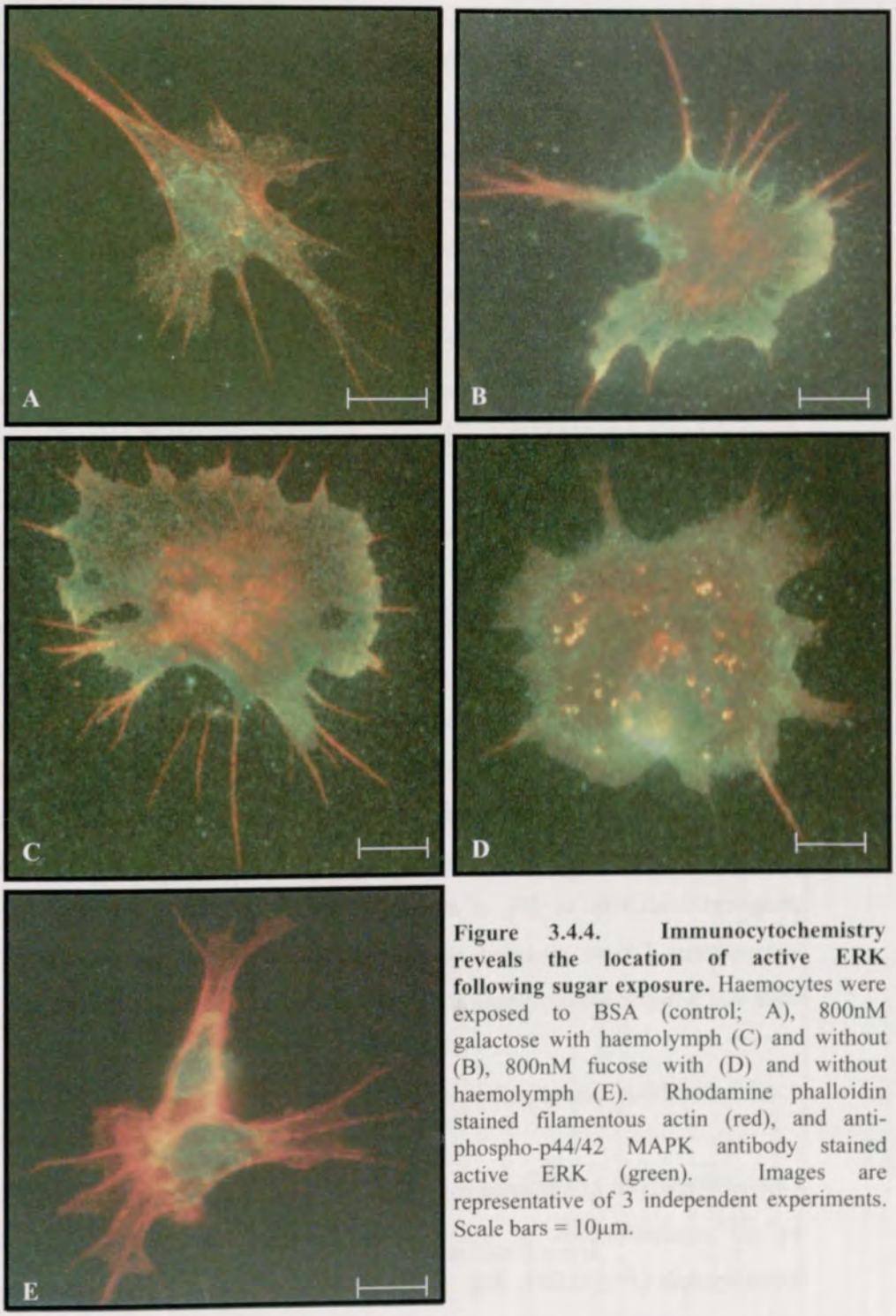

Figure 3.4.4. Immunocytochemistry reveals the location of active ERK following sugar exposure. Haemocytes were exposed to BSA (control; A), 800nM galactose with haemolymph (C) and without (B), 800nM fucose with (D) and without haemolymph (E). Rhodamine phalloidin stained filamentous actin (red), and anti-phospho-p44/42 MAPK antibody stained active ERK (green). Images are representative of 3 independent experiments. Scale bars = 10μm.

3.4.2 Effect of sugars on phagocytosis by haemocytes

3.4.2.1 In the absence of haemolymph

Haemocytes exposed to BSA-fucose in the absence of haemolymph displayed significantly reduced phagocytic activity ($P \leq 0.001$; fig. 3.4.5A), with 800nM BSA-fucose resulting in the lowest level of 'bioparticle' internalisation (9% of control levels; fig. 3.4.5A). Phagocytosis was also reduced following exposure to BSA-galactose (fig. 3.4.5B). In the absence of haemolymph, phagocytosis was significantly reduced to 12% of control levels when haemocytes were exposed to 800nM BSA-galactose, with lower concentrations of BSA-galactose also mediating significant effects ($P \leq 0.001$).

3.4.2.2 In the presence of haemolymph

In contrast to in the absence of haemolymph, in the presence of haemolymph, the highest concentration of BSA-fucose (800nM) significantly increased haemocyte phagocytic activity by 77% ($P \leq 0.05$; Fig. 3.4.5A). As expected, two-way analysis of variance revealed that there was a significant effect of haemolymph on the phagocytic activities following sugar exposure ($P \leq 0.001$; fig 3.4.5A & B). Interestingly, when haemolymph was present, the highest dose of galactose (800nM) reduced haemocyte phagocytic activity to 2% of control levels ($P \leq 0.001$; fig. 3.4.5B). In addition, independent T-tests revealed significant differences between levels of phagocytosis with and without haemolymph following galactose exposure ($P \leq 0.01$).

3.4.2.3 Combined use of galactose and fucose

One-way analysis of variance revealed that phagocytosis was also significantly reduced by all concentrations of the combined sugars, in the presence or absence of haemolymph ($P \leq 0.001$; Fig. 3.4.5C). The effect of combined sugar exposure on phagocytic activity was however reduced when haemolymph was present, such that haemolymph preserved the phagocytic activity to approximately 4-5 times that seen in

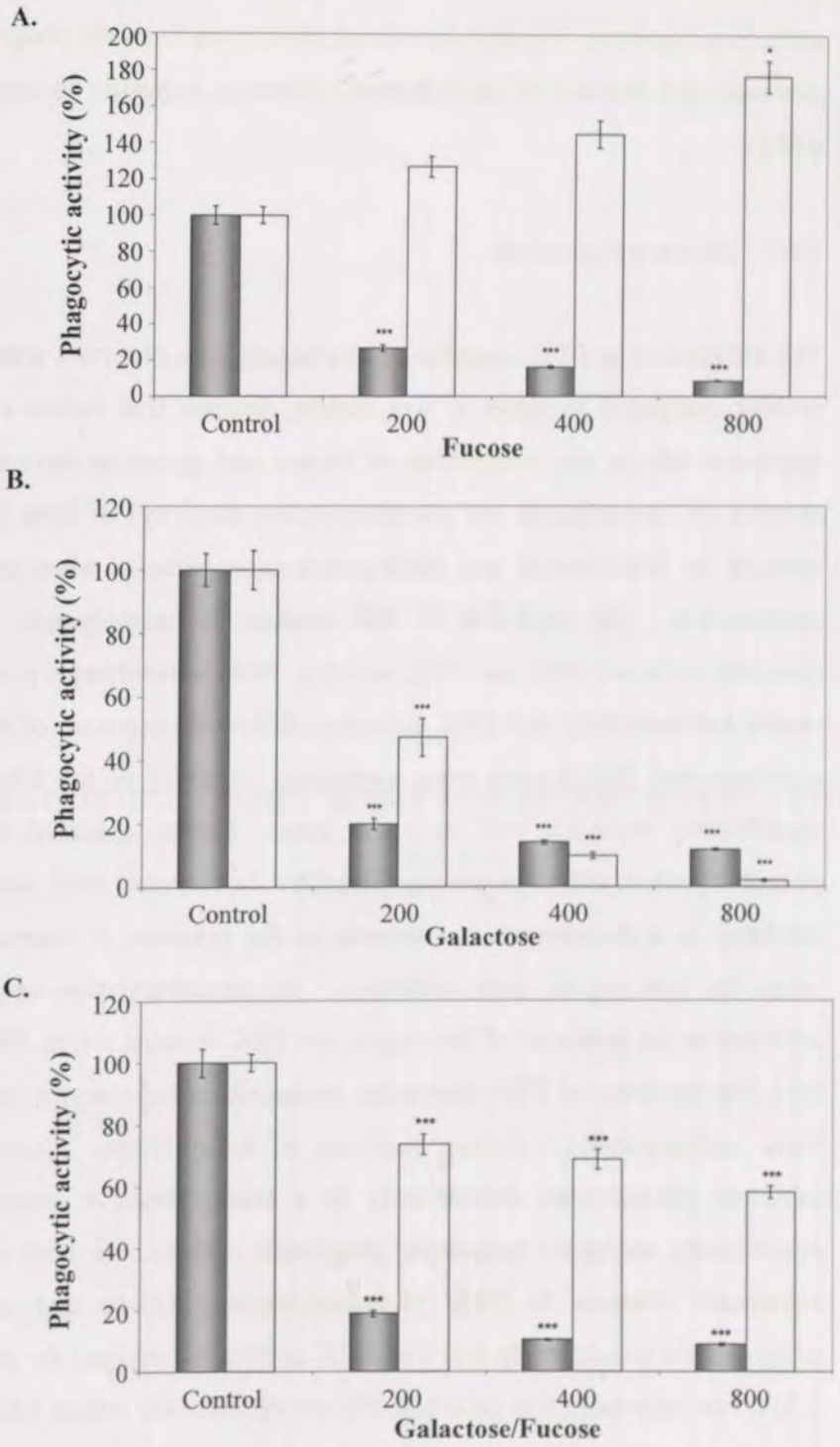

Figure 3.4.5. Phagocytic activity of haemocytes exposed to BSA-conjugated fucose (A), galactose (B) or combined sugars (C) in the absence (grey bars) and presence (white bars) of haemolymph. Values shown are mean phagocytic activities (n = 6; ± SEM). * $P < 0.05$, *** $P < 0.001$ when compared to control (BSA) values.

the absence of haemolymph, at all sugar concentrations (Fig. 3.4.5C). Two-way analysis of variance revealed significant differences between phagocytic activity in the presence and absence of haemolymph following exposure to combined sugars ($P \leq 0.001$).

3.4.3 Summary of results

The differences in ERK signalling and phagocytosis observed when haemolymph was present compared to when it was absent, suggest that serum components play an important role in the recognition of fucose and galactose monosaccharides. In the absence of haemolymph the phosphorylation (activity) of both ERK and PKC was reduced by BSA-fucose and BSA-galactose, especially when sugars were used in combination. As expected, in the absence of haemolymph, phagocytic activity generally reflected ERK and PKC activity. With haemolymph present, the contrasting results between PKC and ERK activation following exposure of haemocytes to BSA-galactose and BSA-fucose were surprising. Activity of the ERK pathway was not significantly reduced, and in some cases, fucose appeared to upregulate ERK phosphorylation, although not significantly. In contrast, PKC activity appeared to be inhibited in a dose-responsive manner in the presence of haemolymph, particularly when the two sugars were combined. As phosphorylation of PKC appears to be inhibited in the presence of the sugars, but ERK remains active, PKC does not seem to be a key mediator of ERK-dependent responses in haemocytes following exposure to these carbohydrates. In the presence of haemolymph, albumin-linked galactose inhibited phagocytosis dramatically in a dose-responsive manner, whereas fucose significantly increased haemocyte phagocytic activity. In both cases, there were no significant changes in ERK phosphorylation. Given that galactose suppresses phagocytosis considerably and that ERK activity is required for phagocytosis (section 3.3) it was surprising that galactose did not significantly reduce ERK activation.

SECTION 3.5

DETECTION OF HAEMOCYTE INTEGRIN RECEPTORS AND A FAK-LIKE PROTEIN

To date the presence of integrins on the cell surface of *L. stagnalis* haemocytes and the presence of FAK in these cells had not yet been identified. In this part of the study, integrin-mediated binding was tested with the aid of an integrin mediated cell adhesion kit, and the presence and stimulation of a FAK-like protein was demonstrated with an anti-phospho FAK (Tyr^{925}) monoclonal antibody.

3.5.1 Integrin-mediated haemocyte binding

To determine whether or not *L. stagnalis* haemocytes use integrin-dependent mechanisms for binding to extracellular substrates, 'integrin-mediated binding assays' were done. Two anti-integrin antibody based binding assays were tested, which targeted the $\alpha_V\beta_3$ and β_1 integrins.

With both antibody based binding assays, the control wells contained no antibody and the experimental wells were coated with the respective anti-integrin antibodies. Results obtained with the $\alpha_V\beta_3$-integrin binding assay suggest that *L. stagnalis* haemocytes rely on engagement of this integrin heterodimer for binding. The experimental wells had a mean absorbance of 0.414 which was significantly greater than that of the control wells which had a mean absorbance of 0.172 ($P \leq 0.001$; fig. 3.5.1A). Furthermore, it is evident that haemocytes need calcium and magnesium in the extracellular environment for successful integrin engagement, since significantly less cells were bound in the absence of Ca^{2+}/Mg^{2+} salts ($P \leq 0.001$ when compared to experimental wells in the presence of salts; fig. 3.5.1A). The non-adherent human leukeumic HL60 cell line was used as a negative control. Indeed, the lack of integrins on these cells resulted in very low levels of binding, with control and experimental wells possessing a mean absorbance of 0.08 and 0.07 respectively (fig. 3.5.1A).

As with the $\alpha_V\beta_3$-integrin experiments, haemocytes were incubated in PBS with and without Ca^{2+}/Mg^{2+} to assess the involvement of β_1 integrin in haemocyte binding. The mean absorbance of the experimental wells (0.21) was significantly greater than that of the control wells (0.15) in the presence of calcium and magnesium demonstrating that β1 integrin mediates haemocyte binding ($P \leq 0.001$; fig 3.5.1B). Similar to $\alpha_V\beta_3$ integrin-mediated binding, cellular adhesion via the β_1 subunit is reliant on the presence

of extracellular salts as demonstrated by the significant reduction in binding in wells lacking Ca^{2+}/Mg^{2+} ($P \leq 0.05$, fig. 3.5.1B). HL60 cells were also utilised for the anti-β_1 integrin binding assay as a negative control. As expected for these cells, the mean absorbance levels in the control and experimental wells were low and were significantly less than those seen with the haemocytes in the presence of salts ($P \leq 0.001$).

3.5.2 Role of integrins in phagocytosis

To determine the importance of haemocyte integrins in phagocytosis, cells were exposed to soluble RGDS peptide (0.02 – 2mM) or vehicle (DMSO) in the presence of *E. coli* 'bioparticles'. Engagement of integrins with RGDS resulted in a dose-responsive reduction in phagocytic activity, with the greatest concentration (2mM) reducing phagocytosis by 88%, when compared to controls ($P \leq 0.001$; fig. 3.5.2A). Lower concentrations of RGDS also resulted in significant reductions in 'bioparticle' internalisation compared to the DMSO control (75.7% ($P \leq 0.05$) and 57% ($P \leq 0.001$) for 0.02 and 0.2mM respectively).

Blockade of haemocyte actin polymerisation, with cytochalasin D (0.1 – 10μM) resulted in a dose-dependent reduction in phagocytosis of *E. coli* 'bioparticles' by haemocytes ($P \leq 0.001$). The highest dose of this inhibitor (10μM) significantly suppressed phagocytic activity to 11% of control levels ($P \leq 0.001$; fig. 3.5.2B). Lower concentrations also resulted in significantly lower 'bioparticle' phagocytosis when compared to control (84% ($P \leq 0.01$) and 35% ($P \leq 0.001$) remaining activity in cells exposed to 0.1 and 1μM cytochalasin D respectively). Phagocytic activity in cells exposed to 0.1 and 1μM cytochalasin D proved to be significantly different to phagocytosis by cells exposed to 10μM ($P \leq 0.001$).

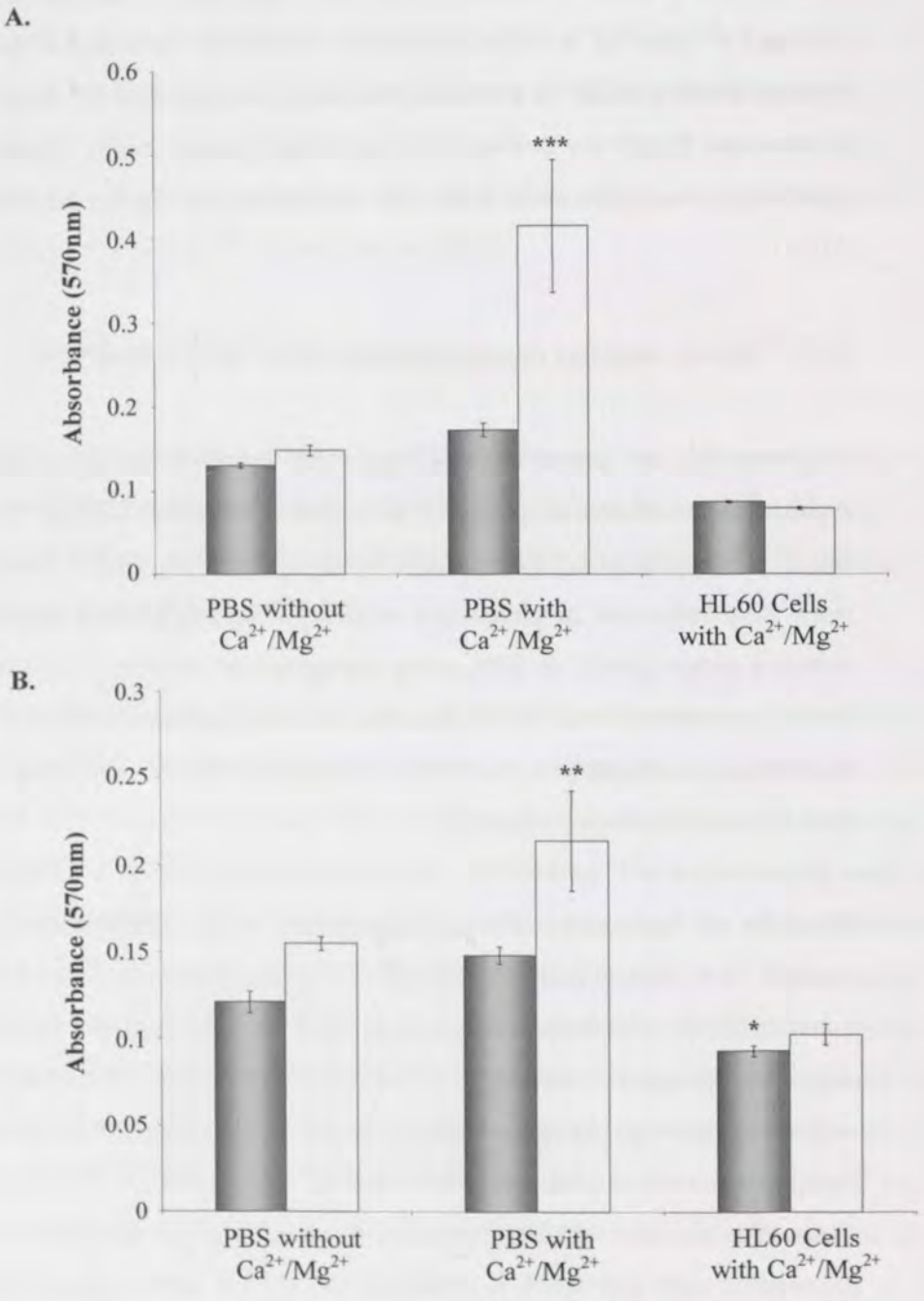

Figure 3.5.1. Graph showing haemocyte requirement for calcium and magnesium in the extracellular environment for $\alpha_V\beta_3$-integrin (A) and β_1-integrin (B) mediated binding. PBS with and without calcium and magnesium was used to incubate haemocytes in experimental (white bars) and control (grey bars) wells of the 'integrin-mediated cell adhesion assay' kit. Binding occurred at 37°C for 1 h, and absorbance was read at 570nm with a spectrophotometer. Graphs show mean absorbance values (n = 7; ± SEM; * $P \leq 0.05$ compared to control without salts, ** $P \leq 0.01$ and *** $P \leq 0.001$, when compared to control with salts).

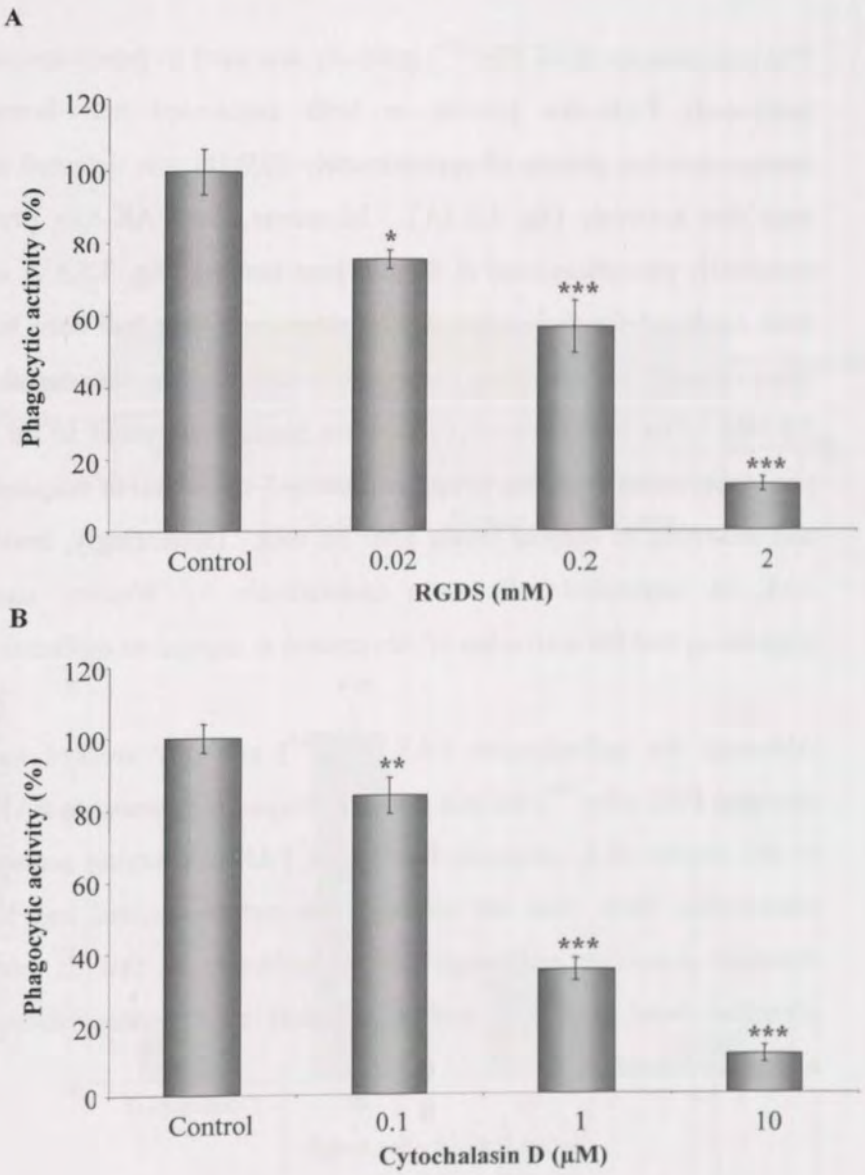

Figure 3.5.2. Suppression of haemocyte phagocytic activity in a dose responsive manner by RGDS (0.02 - 2mM; A) and cytochalasin D (0.1 - 10µM; B). Cells were pre-incubated with the respective inhibitor for 30 min before addition of 'bioparticles'. Values shown are mean values (n = 12; ± SEM; ANOVA * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$).

3.5.3 Haemocyte binding and phosphorylation of FAK

The anti-phospho FAK (Tyr^{925}) antibody was used to detect levels of a phosphorylated (activated) FAK-like protein in both suspended and bound haemocytes. An immunoreactive protein of approximately 125kDa was detected in haemocyte extracts with this antibody (fig 3.5.3A). Moreover, the FAK-like protein appeared to be maximally phosphorylated at 30 min post binding (fig. 3.5.3 A and B). When bands were analysed for their relative intensities, cells that had been bound for 30 min had approximately 8 times more phosphorylated FAK than suspended cells ($P \leq 0.001$; fig. 3.5.3B). The activation of FAK upon binding appeared to be transient, with FAK phosphorylation dropping to approximately 3 times that of suspended cells after 60 min and returning to control levels after 90 min. Interestingly, levels of phosphorylated FAK in suspended cells were undetectable by Western analyses (fig. 3.5.3A), suggesting that the activation of this protein is dependent on haemocyte binding.

Although the anti-phospho FAK (Tyr^{925}) antibody worked successfully, the anti-phospho FAK (Tyr^{397}) did not detect *L. stagnalis* haemocyte FAK. This could be due to this region of *L. stagnalis* haemocyte FAK not having sequence homology to the mammalian form, thus the antibody has not recognised and bound to the protein. Another possibility, although highly unlikely, is that *L. stagnalis* FAK is not phosphorylated on Tyr^{397}, and relies solely on phosphorylation at Tyr^{925} or another amino acid residue.

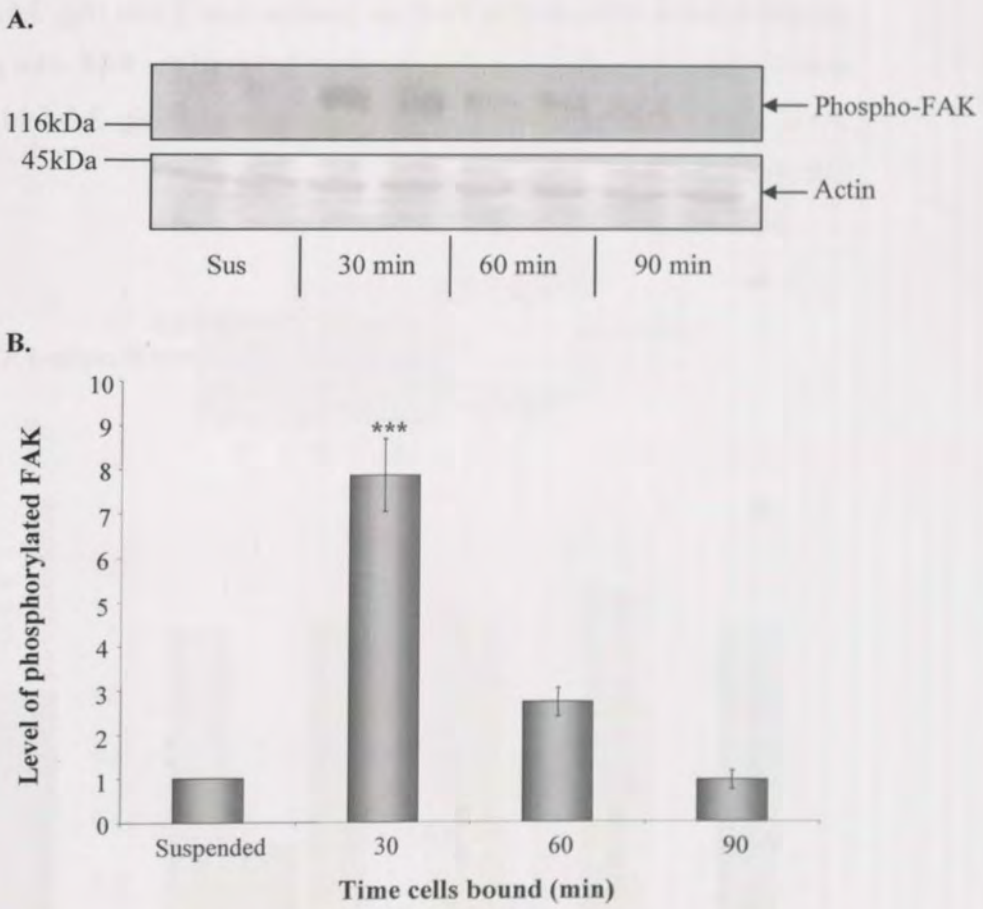

Figure 3.5.3. Increased FAK phosphorylation in bound cells. Haemocytes were bound for 30, 60 and 90 min or left in suspension (sus; control; A). Cells were then lysed with boiling sample buffer and membranes probed with anti-phospho FAK (Tyr^{925}; A upper panel) or anti-actin antibodies (A; lower panel). The blot shown is representative of three independent experiments (two of which are shown in figure). Bands were analysed for relative intensities (B; n = 3; ± SEM; *** $P \leq 0.001$ when compared to suspended cells).

3.5.4 Effect of the RGDS peptide on FAK and ERK phosphorylation

To assess whether or not integrin engagement by the tetrapeptide, RGDS (2mM), resulted in altered FAK phosphorylation in haemocytes, RGDS was delivered to the haemocytes after a 60 min equilibration period. This equilibration period allowed FAK phosphorylation to return to near basal levels as determined from previous experiments (fig 3.5.4A). Western analyses showed that RGDS treatment resulted in increased phosphorylation of haemocyte FAK on tyrosine over 2 min (fig. 3.5.4A). Analyses of blots for band intensities revealed phosphorylation of the FAK-like protein to increase 2-fold after 1 min exposure to RGDS, and approximately 3.5-fold after 2 min ($P \leq 0.05$; fig. 3.5.4B).

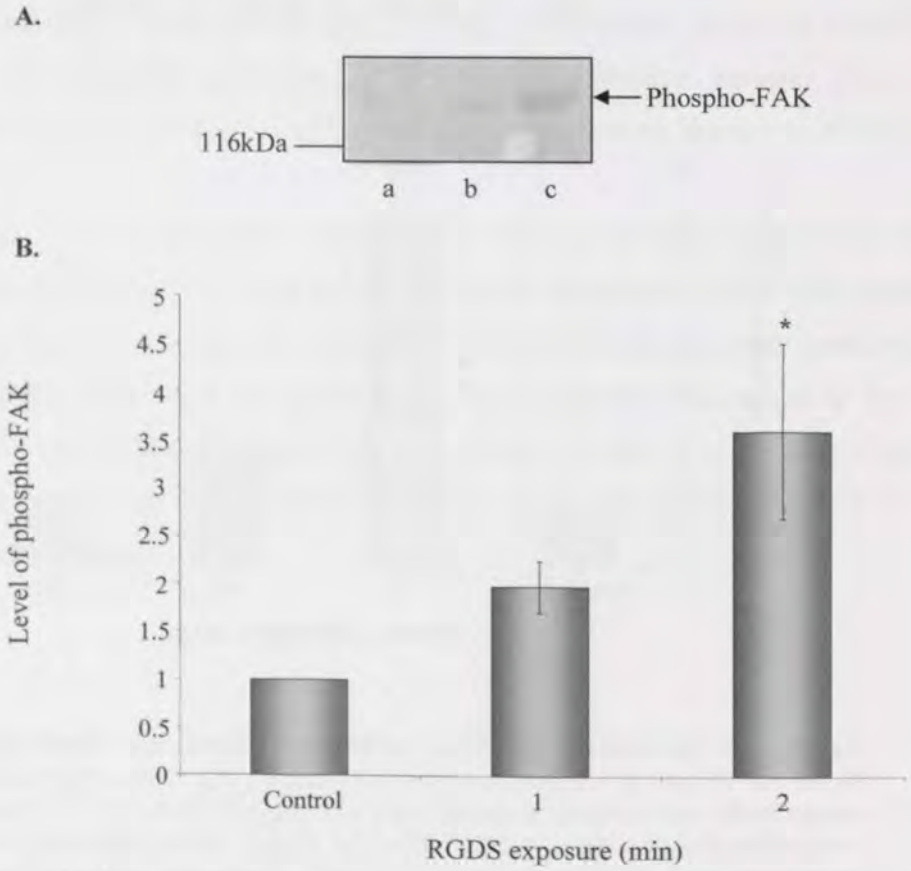

Figure 3.5.4. Haemocytes exposed to RGDS have increased FAK phosphorylation. Cells were either exposed to RGDS (2mM) for 1 (b) and 2 (c) min or vehicle (control; a) and lysed with boiling sample buffer. Membranes were probed with anti-phospho FAK (Tyr^{925}) and anti-actin antibodies. Blots were subsequently scanned and analysed for relative band intensities (B). * $P \leq 0.05$ when compared to control levels. The blot shown is representative of three independent experiments.

In contrast to FAK phosphorylation, ERK phosphorylation levels did not change significantly when haemocytes were exposed to RGDS, for short (up to 4 min) or long (30 min) periods (fig 3.5.5). Image analysis of blots showed that ERK activity was highest at 4 min exposure to RGDS, although it was not significantly different to control levels when analysed with ANOVA.

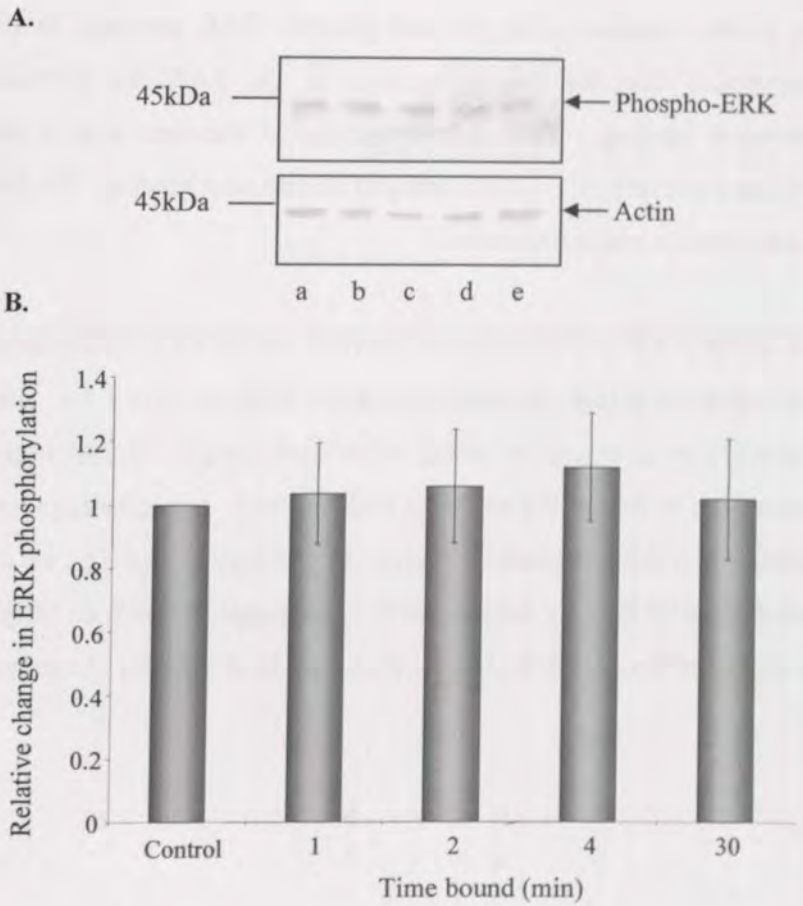

Figure 3.5.5. RGDS exposure does not result in increased haemocyte ERK activity. Cells were exposed to RGDS for 1, 2, 4, and 30 min (A; lanes b – e) or vehicle (A; control; lane a). Membranes were probed with anti-phospho p42/44 MAPK and anti-actin antibodies. The blots shown are representative of three independent experiments. Immunoblots were analysed for band intensities and the mean values are shown (B; ± SEM; n = 3). No significant differences were seen between treatments following analysis by one-way ANOVA.

3.5.5 Summary of results

The presence of integrins and a FAK-like protein in *L. stagnalis* haemocytes has been confirmed with the use of antibodies in binding assays and Western analyses. Integrin-mediated binding assays showed that haemocytes rely on $\alpha_V\beta_3$ and β_1 integrin subunits for binding, which is similar to many other cells. In addition, these assays confirmed that haemocytes need calcium and magnesium in the extracellular environment for integrin-mediated binding to occur.

The results obtained using the anti-phospho FAK antibody in binding experiments demonstrate that the phosphorylation of the FAK-like protein is dependent on haemocyte binding. This phosphorylation is transient over a 90 min period, with maximum phosphorylation occurring at 30 min post binding. No FAK phosphorylation was detected in suspended cells.

The integrin-binding tetrapeptide, RGDS, raised FAK phosphorylation significantly when applied to the haemocytes, which suggests that FAK activation is integrin-mediated (as observed in many other cell types). Surprisingly, the exposure of haemocytes to RGDS did not raise ERK activity. Integrin engagement by RGDS also resulted in a dose-dependent decline in phagocytic activity, as did use of the actin remodelling inhibitor, cytochalasin D. This suggests that both integrin engagement and the ability of the cytoskeleton to remodel are both vital for phagocytosis.

SECTION 3.6

DETECTION OF PHOSPHO-THREONINE-PROLINE HAEMOCYTE PROTEINS BY TWO-DIMENSIONAL SEPARATION

One-dimensional SDS-PAGE followed by Western blotting is the traditional method for the immuno-detection of proteins. However, two-dimensional protein electrophoresis is becoming increasingly used for the 'mapping' of proteins and as a more informative method of protein analyses. The first dimension of separation is isoelectric focusing, which separates the proteins to their respective pH where the overall charge of the protein is neutral, and the second dimension separation being SDS-PAGE. Usually followed by mass spectrometry, two-dimensional analyses allow for protein 'spots' to be excised from the gel and the amino-acid sequence of the protein determined. To date, haemocyte proteins have not yet been separated by two-dimensional analyses.

In this study, haemocytes were pre-incubated in U0126 (10μM) and vehicle (control) prior to two-dimensional separation and Western blotting. Membranes were then probed with an anti-phospho threonine-proline antibody to detect proteins phosphorylated on these adjacent residues. It is known that activated ERK phosphorylates downstream proteins on these sites, so theoretically these proteins should not be phosphorylated in the presence of U0126.

Cells pre-incubated in U0126 (10μM) displayed fewer protein 'spots' than control cells, and many of the larger proteins were not detected in the central area of the gel (pH 5-6; approximately 40-100 kDa). These proteins (some of which are circled on fig. 3.6.1A) are therefore presumed to be targets of MEK-mediated ERK phosphorylation. Interestingly, cells pre-incubated in U0126 exhibited many proteins phosphorylated on threonine-proline residues which were not seen in control cells, which could be due to other signalling pathways being upregulated in response to MEK 1/2 inhibition.

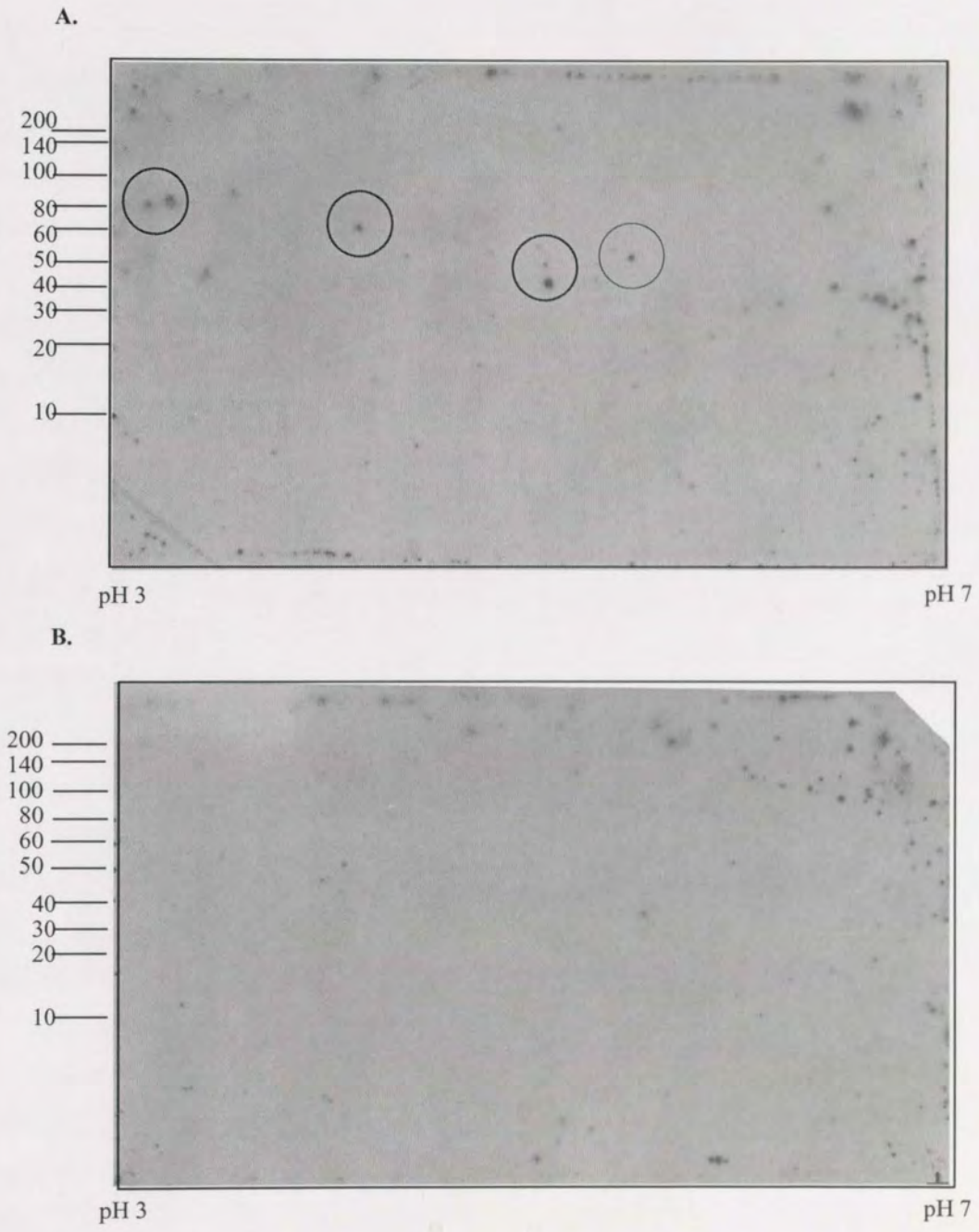

Figure 3.6.1. Two-dimensional separation of haemocyte proteins followed by Western blotting with anti-phospho threonine-proline antibody. Haemocytes were incubated in SSS (control; A), or 10μM U0126 for 30 min (B) prior to isoelectric focusing (pH 3-7) followed by SDS-PAGE on a gradient acrylamide gel (4-12%). Blots are representative of three individual experiments.

CHAPTER 4

DISCUSSION

Intracellular signalling pathways of invertebrate haemocytes have only recently become topic for research by comparative immunologists and biochemists. This recent interest has probably been fuelled by the large amount of research that has been done over the previous decade on signalling pathways of mammalian cells, particularly those of leukocytes.

This study set out to answer the following questions:

- Is the ERK pathway present in *L. stagnalis* haemocytes and is it upregulated by immune challenge?
- What is the function of the ERK pathway in *L. stagnalis* haemocytes?
- Do carbohydrate moieties that mimic those found on the surface coat of schistosomes modulate ERK pathway activity?
- Do *L. stagnalis* haemocytes possess integrin receptors and are they involved in the immune response?

The successful use of biochemical approaches that include Western blotting, kinase assays and the use of pharmacological inhibitors in *L. stagnalis* haemocytes has enabled these questions to be answered.

4.1 IDENTIFICATION, ACTIVATION AND DISSECTION OF THE ERK PATHWAY IN *L. STAGNALIS* HAEMOCYTES

4.1.1 Identification of ERK pathway components

Before the signalling pathways of haemocytes were studied in detail, the optimal conditions for the maintainance of haemocyte monolayers had to be determined. Although culture conditions are known for insect haemocytes and other molluscan species (Foukas *et al.*, 1998; Canesi *et al.*, 2002), only a few studies have been published describing work that relies on the maintenance of primary haemocytes from *L. stagnalis* (Horak *et al.*, 1998a; 1998b). The conditions required for the short-term culture of haemocytes following extraction were established by monitoring haemocyte morphology and basal ERK phosphorylation (activition) levels. Therefore, to do these experiments, anti-active and anti-total ERK antibodies were initially used to detect *L. stagnalis* haemocyte ERK. ERK pathway components have previously been identified in invertebrates including the medfly, *C. capitata* (Foukas *et al.*, 1998), the bivalve mollusc *M. galloprovincialis* (Canesi *et al.*, 2002b), the fruit fly *Drosophila melanogaster* (Pace *et al.*, 2002) and *Biomphalaria glabrata* embryonic (Bge) cells (Humphries *et al.*, 2001). Western blotting revealed that phosphorylated ERK 1/2-like proteins were present in unstimulated haemocytes from *L. stagnalis*, and that these proteins appeared to be approximately 44 and 43 kDa, respectively. This is the first study to report the identification of ERK-like proteins in *L. stagnalis* haemocytes, and show that the molecular weight of *L. stagnalis* ERK 2 (approximately p43) is slightly different to that of the mammalian isoform (p42).

A transcription factor downstream of ERK, Elk-1 in mammalian cells, was also successfully identified in *L. stagnalis* haemocytes with an anti-phospho Elk-1 antibody. The identified protein had an apparent molecular weight of 62kDa. Although other studies have identified haemocyte ERK-like proteins (Foukas *et al.*, 1998; Canesi *et al.* 2002) by Western blotting, this is the first study that has measured the kinase activity of the identified proteins by means of a MAP kinase activity assay. This assay relied on the ability of immunoprecipitated ERK 1/2 to phosphorylate the Elk-1 transcription factor. MAP kinase activity was measured in unstimulated and LPS-stimulated

haemocytes. Since LPS-challenged *L. stagnalis* haemocytes had been found to possess increased ERK phosphorylation in previous experiments, it was thought that these cells would have higher kinase activity levels. However, in contrast, results showed that unstimulated haemocytes possessed higher ERK activity than those cells which had been challenged with LPS. One possible explanation for this could be that following LPS challenge, activated ERK translocates to the cytoskeleton, a known phenomenon in fibroblasts (Reszka *et al.*, 1995), PC12 cells (Morishima-Kawashima *et al.*, 1996) and carcinoma cells (Klemke *et al.*, 1997). Once translocated to the cytoskeleton, phospho-ERK may become tightly bound to the cytoskeletal filaments, thus making it harder to immunoprecipitate ERK 1/2 prior to the activity assay. The MAP kinase activity assay did, however, confirm that, as seen in mammalian cells, the *L. stagnalis* ERK-like protein(s) phosphorylate(s) the transcription factor, Elk-1. Moreover, by using the highly specific MEK 1/2 inhibitor U0126 to block ERK phosphorylation by MEK, phosphorylated Elk-1 was not detected in the activity assay. This finding confirms that MEK is the upstream activator of ERK-like proteins in *L. stagnalis* haemocytes, and that MEK-directed activation of ERK leads to phosphorylation of the Elk-1 transcription factor as it does in mammalian systems (Aplin *et al.*, 2001).

Phosphorylated MEK-like proteins were also identified in *L. stagnalis* haemocytes, with use of an anti-phospho-MEK 1/2 antibody. Although two MEK isozymes are known to be present in mammalian cells (Xu *et al.*, 1995), only one band was seen following Western analysis of total haemocyte proteins, which had an apparent molecular weight of 43kDa. This protein was detected using anti-rabbit antibodies, which demonstrates that sequence homology exists between the phosphorylation site of mammalian and molluscan MEK.

To study downstream targets of activated ERK in *L. stagnalis* haemocytes which are phosphorylated on proline-threonine residues, the technique of two-dimensional electrophoresis was applied to total haemocyte proteins from cells exposed to LPS alone, or U0126 with LPS. This is the first study which has employed this technique for haemocyte proteins, and has showed that U0126 blocked proline-threonine phosphorylation of proteins by ERK. Surprisingly, some proteins seemed to become phosphorylated with the addition of U0126, which may be due to other pathways becoming activated resulting from the downregulation of ERK activity.

Owing to the ERK pathway being, in part, a stress-activated signalling pathway, the phosphorylation status of *L. stagnalis* ERK-like proteins was monitored at different temperatures and incubation times, along with cell morphology, to determine the optimal conditions for the short-term maintenance of haemocyte monolayers. The haemocytes were also incubated in two different media, SSS and RPMI, and SSS was found to be the more appropriate media for *L. stagnalis* haemocytes since the cells remained adherent and were less vacuolated for up to 16 h after binding. This media has also been used by other workers (Horak *et al.*, 1998a; Horak & Deme, 1998). The basal levels of ERK phosphorylation in *L. stagnalis* haemocytes were found to be lowest when the cells were incubated for 30 min at room temperature. Although the basal phosphorylation levels of ERK were lower if cells were incubated for longer time periods (for up to 4 h), the cells showed some cytoplasmic vacuoles after approximately 3 hours of incubation in SSS. Therefore, for all subsequent experiments, cells were incubated for 30 min, washed and then incubated for a further 15 min, all in SSS.

The basal levels of haemocyte ERK-like protein phosphorylation were high after the 30 min binding period. This basal activity could be due to the cells binding to the cell culture plate, but also may result from cell-cell contacts which have previously been found to result in high ERK phosphorylation levels (Pece & Gutkind, 2000). To try to limit cell-cell contact and therefore cell clumping, two anti-clumping media were tested: Alsever solution (Yip *et al.*, 2001) and anti-clumping buffer (Davids & Yoshino, 1998). Both solutions resulted in complete loss of haemocyte binding, and therefore monolayer formation. Torreilles *et al.* (1999) have demonstrated that Modified Alsever Solution (MAS) inhibits cell clumping by chelating calcium ions, and that this medium is not suitable to maintain *M. galloprovincialis* haemocyte cultures. Although it proved difficult to reduce the basal levels of ERK phosphorylation for short-term culture, it was still possible to determine any changes in the phosphorylation levels after challenge, or pathway inhibition, and compare these to the control phosphorylation levels.

4.1.2 Activation of the ERK pathway

4.1.2.1 Cell-free E. coli *LPS*

Previous studies with mammalian macrophages have shown that LPS activates the ERK pathway (Weinstein *et al.*, 1992; Procyk *et al.*, 1999; Monick *et al.*, 2000). Studies with invertebrate haemocytes have also shown that this compound can stimulate ERK, thus demonstrating that LPS-mediated activation of ERK may be conserved in phagocytes (Soldatos *et al.*, 2003). In the present study, *L. stagnalis* haemocytes were challenged with 1µg/ml LPS for up to 20 min. Maximal activation of the ERK-like protein occurred at 5 min post exposure to LPS, which is a reasonably rapid response to the endotoxin. A similar rapid activation of the ERK pathway in response to LPS has been reported in vascular endothelial cells (Arditi *et al.*, 1995) and canine smooth muscle cells (Luo *et al.*, 2000). A longer exposure was found to be required by primary human monocytes for maximal activation of ERK by LPS (which occurred at 20 min post exposure; Van der Bruggen *et al.*, 1999), RAW 264.7 macrophages (10 min; Watters *et al.*, 2002) and macrophages of the BAC-1.2F5 cell line (10 min; Büscher *et al.*, 1995). The activation of proline-directed kinases upon LPS challenge of RAW 264.7 macrophages has also been reported, which include ERK isozymes (Sanghera *et al.*, 1996). The finding that LPS results in the activation of ERK signalling in both macrophages and *L. stagnalis* haemocytes, suggests that these cells have similar signalling machinery that may enable their functional responses to be co-ordinated in a similar manner.

The location of active ERK-like proteins in *L. stagnalis* haemocytes was also visualised by immunocytochemistry. After 5 min exposure to LPS, the active-ERK proteins appeared to translocate towards the nucleus of the cell, and were particularly clustered at the periphery of the nuclear membrane. Clustering of phosphorylated ERK also appeared in the cytoplasm of the haemocytes, which could be due to movement of ERK to focal adhesion points. The clustering of ERK around membrane focal adhesion sites is discussed further in section 4.4.

After 20 min exposure of haemocytes to LPS, the phosphorylation status of ERK-like proteins was below baseline levels, demonstrating the existence of a negative feedback

loop or activation of phosphatases. The negative feedback of ERK to MEK was first discovered in hamster lung fibroblast cells, where ERK was seen to retrophosphorylate, and subsequently inhibit, MEK *in vitro* (Brunet *et al.*, 1994). Negative feedback loops are a common feature in intracellular pathways as a way of preventing overstimulation of the pathway (see for example Lynch & Daly, 2002). Negative control of the ERK pathway upon LPS stimulation has been reported in primary murine macrophages, where MAP kinase phosphatase-1 (MKP-1) is produced upon LPS challenge (Valledor *et al.*, 2000), thus downregulating ERK pathway activity.

Challenge of *L. stagnalis* haemocytes with 1μg/ml LPS also seemed to result in a transient increase in the phosphorylation status of haemocyte MEK-like proteins with time, although activation did not prove to be statistically significant. Upregulation of haemocyte MEK activity could be expected, due to MEK being the only upstream kinase able to directly activate ERK on both threonine and tyrosine residues (Ray & Sturgill, 1988). In the present study, MEK was shown to be maximally stimulated at 5 and 10 min post-exposure to LPS, whereas ERK was shown to be maximally stimulated after 5 min exposure. Although earlier activation of MEK would be expected in the haemocytes to result in the high ERK activity at 5 min (as demonstrated by Hipskind *et al.*, 1994), a similar transient pattern of MEK activity is seen in primary macrophages in response to LPS, although over a 30 min period with maximal activity at 15 min (Beinke *et al.*, 2004).

Although an anti-phospho-Raf antibody was used in an attempt to detect Raf when phosphorylated in *L. stagnalis* haemocytes, Western analysis of total haemocyte proteins with the anti-phospho Raf primary antibody was unsuccessful (purchased from NEB; unreported data). This finding is most likely to be due to *L. stagnalis* haemocyte Raf possessing a different peptide sequence to the mammalian form given that the antibody was raised to a peptide sequence derived from rabbit Raf. However, it would be interesting to study the effect of LPS on *L. stagnalis* haemocyte Raf, as LPS signalling has been reported to be Raf-independent in monocytes (Guthridge *et al.*, 1997) and primary macrophages (Monick *et al.*, 2000), but Raf-dependent in smooth muscle cells (Luo *et al.*, 2000). The lack of success in detecting phosphorylated Raf in *L. stagnalis* haemocytes after LPS challenge could also be due to Raf-independent

signalling to ERK following LPS exposure, as seen in monocytes and primary macrophages.

Many workers have used LPS to challenge insect and mollusc haemocytes to study aspects of immune function, but have not studied the signalling pathways modulated by such challenge (Lorenzon *et al.*, 1999; Sritunyalucksana *et al.*, 1999; Cao *et al.*, 2004). Covalent associations between *E. coli* LPS, a cell-free protein exocytosed from haemocytes (p47), and a membrane associated protein (mp47), are required for the subsequent internalisation of the LPS molecule into *C. capitata* haemocytes (Charalambidis *et al.*, 1995; Charalambidis *et al.*, 1996b). Its possible that membrane bound p47 could be a pattern recognition receptor, and act similarly to the mammalian LPS binding protein or cluster of differentiation (CD) proteins found on the macrophage surface. Studies with the crustacean, *C. gigas*, have shown LPS to cause a decrease in the number of circulating haemocytes *in vivo* (Lorenzon *et al.*, 1999), suggesting that LPS has a cytotoxic effect on the cells. This phenomenon has previously been demonstrated with rat epithelial cells and murine macrophages, where increased iNOS production and apoptosis was observed in response to LPS (Lakics & Vogel, 1998; Lamarque et al., 2000). It would be advantageous to determine the signalling pathways involved in this LPS-mediated apoptotic response, and to determine whether or not LPS has cytotoxic activity towards *L. stagnalis* haemocytes.

4.1.2.2 Heat-killed E. coli *and other compounds*

Since cell-free LPS activated *L. stagnalis* haemocyte ERK significantly, cell-associated LPS should in theory modulate a similar effect. Surprisingly, challenging *L. stagnalis* haemocytes with heat-killed *E. coli* did not significantly alter the activity of haemocyte ERK-like proteins. Live *E. coli* have been used to challenge *M. galloprovincialis* haemocytes by Canesi *et al.*, (2002a), which resulted in upregulation of ERK 2 activity within 5 min of exposure to the bacteria. This response lasted for up to 30 min, however it was p38 MAPK that was most significantly affected when the haemocytes were challenged. p38 MAPK has recently been identified in *L. stagnalis* haemocytes (A. Walker; unpublished results), so the activity of this kinase could easily be monitored by means of Western blotting in response to live *E. coli*; such a study may reveal similarities in p38 MAPK signalling between *L. stagnalis* and *M.*

galloprovincialis haemocytes. *Salmonella typhimurium* challenge resulted in the activation of ERK isozymes in BAC-1.2F5 macrophages within 5 min of exposure (Procyk *et al.*, 1999), with PI-3K being identified as one of the proteins involved in the signalling to ERK. Hiller & Sundler (1999) found that by challenging macrophages with *Prevotella intermedia*, ERK 1/2 activity was modulated reasonably quickly, with an increase in phosphorylation seen within 5 min. Studies done to date with macrophages and haemocytes clearly suggest that live *E. coli* activate different MAPK pathways, and this is an area of research which warrants further investigation.

Modulation of MAPK pathways by LPS and *E. coli* in macrophages and haemocytes, could be a way of producing reactive nitrogen and oxygen intermediates, compounds which are important in defence responses and aid in the destruction of pathogens. Indeed, the p38 MAPK pathway plays a key role in the production of nitric oxide synthase, an enzyme required for the synthesis of nitric oxide, in RAW 264.7 macrophages via NF-κB (Chen & Wang, 1998). The challenge of phagocytes with live or heat-killed *E. coli* could result in up-regulation of p38 MAPK and the subsequent production of nitric oxide synthase, which has been shown to be ERK-independent (Chen & Wang, 1998). However, studies performed in our laboratory with *L. stagnalis* haemocytes have shown that U0126 blocks the synthesis of nitric oxide, thus suggesting a role for ERK in the production of reactive nitrogen intermediates (unpublished data; B. Wright).

Cell-free LPS and cell-associated LPS (heat-killed *E. coli*) appeared to elicit different effects on *L. stagnalis* haemocyte ERK. In agreement with this observation, internalisation of cell-free LPS and cell-associated LPS (heat-killed *E. coli*) appear to involve different cellular signalling mechanisms in *C. capitata* haemocytes, with only cell-associated LPS requiring cytoskeletal rearrangement for internalisation (Foukas *et al.*, 1998).

Zymosan A, an insoluble preparation of yeast *Saccharomyces cerevisiae* cells, has previously been shown to be internalised by mammalian macrophages and insect haemocytes (Carballal *et al.*, 1997; Girotti *et al.*, 2004). In this study, challenge with zymosan A did not result in an upregulation of haemocyte ERK activity. However, in peritoneal macrophages, zymosan has been shown to mediate the upregulation of ERK

1/2 significantly (Qiu & Leslie, 1994; Hiller & Sundler, 1999). Zymosan also increases the association of tyrosine phosphorylated proteins with the cytoskeleton in human monocytes (Zaffran *et al.*, 1995). Even though Western analyses did not reveal up-regulation of *L. stagnalis* ERK phosphorylation upon zymosan A challenge, basally activated ERK could have migrated to the cytoskeleton, as seen with human monocytes.

Noradrenaline and ACTH are known to be important in the stress responses of bivalve molluscs, with both being released upon exposure to temperature extremes, light and pathogens (Ottaviani *et al.*, 1992; Lacoste *et al.*, 2002a). ACTH (fragment 1-24) is also known to modulate cell shape changes by activating PKA and PKC in *M. galloprovincialis* haemocytes (Malagoli *et al.*, 2000). As PKC and PKA are known to act upstream of ERK 1/2 in mammalian cells, it is possible that ACTH exerted its effects on the cells via the ERK pathway in invertebrate haemocytes. *Lymnaea stagnalis* haemocytes were therefore exposed to ACTH at the physiological concentrations known to be present in both stressed and non-stressed bivalve molluscs. The levels of phosphorylated ERK in *L. stagnalis* haemocytes did not differ between control, or those treated with 'non-stressed' or 'stressed' ACTH concentrations, suggesting that this pathway is not modulated by the presence of extracellular ACTH. However, total-ERK levels in *L. stagnalis* haemocytes increased in a dose-dependent manner to ACTH, which suggests that expression of the ERK protein was up-regulated by the compound, but that it was not subsequently phosphorylated. To test this, haemocytes were exposed to the highest concentration of ACTH over 120 min, in conjunction with LPS, to try to phosphorylate *de novo* produced unphosphorylated ERK. However, ERK phosphorylation levels did not increase over time in response to LPS challenge. It is not known why total-ERK levels increased during haemocyte exposure to ACTH; this phenomenon has not previously been reported in other cells, mammalian or invertebrate.

ERK phosphorylation has been shown to be up-regulated in rat pinealocytes in response to noradrenaline (Mackova *et al.*, 2000). This catecholamine also known to increase phagocytosis and reactive oxygen species production in oyster haemocytes (Lacoste *et al.*, 2001a; Lacoste *et al.*, 2001c). Exposure of *L. stagnalis* haemocytes to noradrenaline alone did not result in increased phosphorylation of ERK-like proteins.

However, by pre-incubating haemocytes with noradrenaline and subsequently stimulating the cells with LPS for 5 min, an increase in phosphorylated ERK was observed. This enhanced activity could be due to the effect of LPS alone, since LPS was found to significantly enhance ERK-like protein phosphorylation. More work needs to be done in this area as little is known about the effects of catecholamines generally in gastropod molluscs.

4.1.3 Inhibition of ERK

In mammalian cells, MEK 1/2 is known to phosphorylate (activate) ERK isozymes on threonine and tyrosine residues (Andersen *et al.*, 1990). Although both proteins had been detected in *L. stagnalis* haemocytes in the preliminary experiments, it was not known whether this pattern of sequential activation occurred in *L. stagnalis*.

Two different MEK inhibitors, U0126 and PD98059, were used to block the activation of *L. stagnalis* haemocyte ERK-like proteins by MEK. U0126 blocks the kinase activity of both MEK 1/2 isozymes, whilst PD98059 only blocks the kinase activity of MEK 1. It was clearly seen that MEK activates ERK, since when haemocytes were pre-treated with U0126 and then stimulated with LPS no phosphorylated ERK was detected. However, the use of PD98059 suggested that MEK 2 is important in the phosphorylation of haemocyte ERK-like proteins, since this inhibitor only reduced ERK phosphorylation to 50% of the control phosphorylated ERK levels. U0126 and PD98059 have both been successfully used to block LPS-induced ERK phosphorylation in medfly (*C. capitata*) haemocytes (Soldatos *et al.*, 2003).

The phosphorylation of the transcription factor, Elk-1, by phosphorylated ERK and its inhibition by U0126 was demonstrated in the MAP kinase activity assay and was discussed earlier in this section.

4.2 THE FUNCTIONAL ROLE OF THE ERK PATHWAY IN *L. STAGNALIS* HAEMOCYTES

To examine the role of cell signalling pathways in *L. stagnalis* haemocyte phagocytosis haemocytes were exposed to *E. coli* fluorescent 'bioparticles', in the presence or absence of pharmacological inhibitors which target signalling intermediates. The extent to which 'bioparticles' were internalised by phagocytosis was determined using a spectrofluorometer, and the fluorescence from any extracellular 'bioparticles' which had not been internalised was quenched with trypan blue. Although the trypan blue was used at a high concentration (2%), it was only in contact with the cells for 2 min before removal, and subsequent measurement of fluorescence signal. Although this study used *E. coli* 'bioparticles' as the target for phagocytosis, other studies focusing on phagocytosis by haemocytes from other species have employed yeast cells (Ishikawa *et al.*, 2000), latex beads (Fryer & Bayne, 1996), live *E. coli* (Foukas *et al.*, 1998) and fluorescent-labelled latex beads (Lacoste *et al.*, 2001b).

To test whether *L. stagnalis* ERK-like proteins were involved in phagocytosis, haemocytes were pre-incubated with the MEK inhibitors, U0126 and PD98059, before the introduction of *E. coli* 'bioparticles'. U0126, which inhibits MEK 1 and 2, was the most potent inhibitor of haemocyte phagocytosis, reducing phagocytic activity by 80%. This suggests that haemocyte phagocytic activity is highly dependent on ERK activity, particularly given that Western blotting showed that U0126 completely blocked ERK phosphorylation. Although 10μM U0126 was sufficient to block ERK phosphorylation, as determined by Western analyses, 100μM was required for the blockade of phagocytosis. The higher dose required for inhibition of phagocytic activity may be due to the longer incubation period that was required for the phagocytosis assay. The MEK 1 inhibitor, PD98059, only partially inhibited *L. stagnalis* haemocyte phagocytic activity, which is in accordance with the results gathered from Western analysis which shows that this inhibitor is less effective at blocking ERK phosphorylation. This further suggests that haemocyte ERK, and thus phagocytic activity, rely heavily on the activity of the MEK 2 isoform. Canesi *et al.* (2002a; 2002b) found that PD98059 only had a limited effect on haemocyte phagocytosis of live *E. coli* by *M. galloprovincialis* haemocytes, and their results are in accordance with those in this study.

To investigate which signalling pathways, known to be upstream of ERK in mammalian systems, were involved in haemocyte phagocytic activity, inhibitors of PKC, PKA, PI-3K and Ras were all used in an attempt to block signalling to *L. stagnalis* haemocyte MEK. ERK pathway signalling is dependent on PI-3K and PKA activation in many cells including human fibroblasts (Duca *et al.*, 2002). The PKC inhibitor, GF109203X, blocked phagocytosis by approximately 50%, while a combination of GF109203X and PD98059 reduced phagocytosis by approximately 40%. The combination of 100μM U0126 and 10μM GF109203X resulted in the greatest reduction in phagocytic activity, with remaining phagocytosis reduced to below 40%.

PKC and MAPK signalling is known to occur via Ras (see for example, Cai *et al.*, 2002; Grewal *et al.*, 2003; Zhang *et al.*, 2004), and the involvement of Ras in pahgocytosis was tested by using the Ras farnesylation inhibitor, FTase inhibitor I. By pre-incubating *L. stagnalis* haemocytes with FTase inhibitor I, phagocytosis was reduced by approximately 50%, suggesting that both Ras-dependent and –independent pathways are involved in phagocytosis by these cells. Foukas *et al.* (1998) have previously reported Ras-dependent internalisation of *E. coli* by insect haemocytes, whereas Lamprou *et al.* (2004) have recently demonstrated Ras to be involved in activation of p38 MAPK and subsequent phagocytosis by insect haemocytes.

Since some phagocytic activity remained with PKC, MEK 1/2 and Ras activity blocked, other pathways may have been involved in phagocytosis by *L. stagnalis* haemocytes. These pathways could be Ras-independent pathways that involve PKA or PI-3K signalling. Using pharmacological inhibitors, the involvement of these Ras-independent pathways in *L. stagnalis* haemocyte phagocytosis was assessed.

When the PI-3K inhibitor LY294002 was used, a dose-responsive reduction in phagocytosis was seen, with the highest concentration (10μM) resulting in a 60% reduction in phagocytic activity. This indicated that PI-3K plays an important role in phagocytosis by *L. stagnalis* haemocytes. PI-3K has also been found to play an important role in phagocytosis by ascidian haemocytes (Ishikawa *et al.*, 2000). In addition, PI-3K has recently been implicated in the modulation of medfly haemocyte cell shape changes and LPS endocytosis (Soldatos *et al.*, 2003), and this enzyme is also

activated during phagocytosis of *E. coli* by *M. galloprovincialis* haemocytes (Canesi *et al.*, 2002). The phosphoinositide signalling pathway is known to be a potent modulator of immune responses in mammalian cells (reviewed by Frumen & Cantley, 2002; Stephens *et al.*, 2002), and PI-3K activity is greatly enhanced by LPS challenge of primary alveolar macrophages (Monick *et al.*, 2000). Furthermore, Monick *et al.* (2000) showed PI-3K to activate PKC ζ, which then subsequently activates ERK in a Raf-independent manner. Clearly a similar sequence of events could be occurring in *L. stagnalis* haemocytes.

PKA phosphorylation can lead to either inhibition or activation of the ERK pathway in many cell types (Hafner *et al.*, 1994; Schmitt *et al.*, 2002; Miggin *et al.*, 2002; Dhillon *et al.*, 2002; Cancedda *et al.*, 2003). Pre-incubation of *L. stagnalis* haemocytes with the PKA inhibitor, KT5720, did not result in either the reduction or enhancement of 'bioparticle' internalisation, suggesting that this enzyme is not involved in *L. stagnalis* phagocytosis. This appears to be in contrast to phagocytosis in primary mammalian macrophages, which is modulated by intracellular cAMP concentrations, suggesting an involvement of PKA (Rossi *et al.*, 1998). Noradrenaline has been shown to inhibit oyster haemocyte phagocytosis through a cAMP pathway, which subsequently leads to increased PKA activity (Lacoste *et al.*, 2001b). Therefore, use of the PKA inhibitor in this study may block any negative regulation of ERK via PKA, thus resulting in increased/stable phagocytic activity of *L. stagnalis* haemocytes.

Phagocytosis involves cell migration (chemotaxis), attachment to the 'foreign' particle and its subsequent internalisation. Foukas *et al.* (1998) and Lamprou *et al.* (2004) have both shown that ERK pathway activation, via Ras, is required for the internalisation of heat-killed *E. coli* and LPS by medfly haemocytes. From the present study it appears that *L. stagnalis* and medfly haemocytes both require ERK activation for efficient phagocytosis, although *L. stagnalis* haemocytes also possess Ras-independent mechanisms of phagocytosis. Recently, the phagocytosis of apoptotic cells by macrophages has also shown to be mediated by ERK (Kurosaka *et al.*, 2003), demonstrating conservation between macrophages and haemocytes.

4.3 EFFECT OF SACCHARIDES ON *L. STAGNALIS* HAEMOCYTES

Since molluscs are important intermediate hosts for schistosome parasites that possess carbohydrate-rich surface coats, it is surprising that the effects of such carbohydrates on mollusc immune cells have been largely overlooked at a molecular level. The effect of *T. ocellata* infection of *L. stagnalis* has been shown to cause a general net activation of the *L. stagnalis* internal defence system (increased number of circulating haemocytes and cell spreading) *in vivo* (Amen *et al.*, 1991a).

With haemolymph absent, the activity of both ERK and PKC was reduced by BSA-fucose and BSA-galactose, especially when sugars were used in conjunction. Also, in the absence of haemolymph, phagocytic activity generally reflected ERK and PKC activity; this was expected given the results from the phagocytosis assays that employed pharmacological inhibitors. Whilst other researchers have reported the effects of sugars, such as arabinose and fructose, on haemocyte phagocytic activity (Horak *et al.*, 1998), the present study is the first to demonstrate that key intracellular signalling pathways can be modulated by such sugars leading to downstream effects on immune function.

With haemolymph present, the contrasting results between PKC and ERK activation following exposure of haemocytes to BSA-galactose and BSA-fucose were surprising. Activity of the ERK pathway was not significantly reduced, and in some cases, fucose appeared to upregulate ERK activation, although not significantly. In contrast, PKC activity appeared to be inhibited in a dose-responsive manner in the presence of haemolymph, particularly when the two sugars were combined. Taken together these results suggest that within snails, haemocyte signalling mechanisms can be differentially regulated by carbohydrate exposure. As phosphorylation of PKC appears to be inhibited in the presence of the sugars, but ERK remains active, PKC does not seem to be a key mediator of ERK-dependent responses in haemocytes following exposure to these carbohydrates. ERK can, however, be activated by numerous other signalling components including Protein Kinase A (PKA) (Schmitt and Stork, 2002; Miggin and Kinsella, 2002; Cancedda *et al.*, 2003), Phosphatidylinositol-3-kinase (PI-3-K) (Versteeg *et al.*, 2000) and integrin binding which may involve Focal Adhesion

Kinase (FAK) (Irigoyen and Nagamine, 1999; Yu and Basson, 2000; Barberis *et al.*, 2000; Yujiri *et al.*, 2003).

The phagocytic responses of haemocytes in the presence of haemolymph differed following BSA-galactose and BSA-fucose exposure. Albumin-linked galactose inhibited phagocytosis dramatically in a dose-responsive manner, whereas fucose significantly increased haemocyte phagocytic activity by 77%. In both cases, there were no significant changes in ERK phosphorylation levels. The differing effects of BSA-galactose and BSA-fucose on phagocytosis could be due to haemolymph components and fucose acting in concert as an opsonin, thus upregulating the phagocytosis of 'bioparticles'. This is plausible since molluscan serum lectins are known to be able to act as opsonins, as discussed by Horak and Van der Knaap (1997). Clearly, further research into *L. stagnalis* haemolymph lectins needs to be undertaken to gain further insight into the enhancing effect of fucose on phagocytosis.

Given that galactose suppresses phagocytosis by haemocytes considerably and ERK activity has been shown to be vital for efficient phagocytosis, it was surprising that galactose did not significantly reduce haemocyte ERK activation. Levy *et al.* (2003) have recently reported that galectins (galactose-specific lectins) can bind to integrins on the membrane of Chinese Hamster Ovary (CHO) cells. Integrins are known to be part of the cellular machinery used for phagocytosis in insect haemocytes (Foukas *et al.*, 1998), and galactose specific lectins have been isolated from many invertebrate species, including the fruit fly *D. melanogaster* and the earthworm *Lumbricus terresteris* (Hirabayashi *et al.*, 1998; Mann *et al.*, 2000; Chiou *et al.*, 2000; Pace *et al.*, 2002). Integrin activation has been linked to increased ERK activity in a number of cell types including thyroid TAD-2 cells, Hep-3B cells (Illario *et al.*, 2003), neuronal, gonadal and Swiss-3T3 cells (Barberis *et al.*, 2000). Therefore, the low levels of phagocytosis and high levels of active ERK in our system may be due to galectins binding to galactose and subsequent engagement of integrins. The presence of integrins in *L. stagnalis* haemocytes and their potential role in defence reactions is discussed further in section 4.4.

Immunocytochemistry revealed that following exposure of haemocytes to BSA-galactose, in the presence or absence of haemolymph, phosphorylated ERK was located

mainly at the cell periphery of haemocytes, but was also diffuse throughout the cytoplasm. The presence of active ERK at the cell periphery following carbohydrate exposure further suggests integrin engagement/activation or ERK binding to focal adhesion sites associated with integrins. These sites are comprised of enzymes such as Focal adhesion Kinase (FAK), an activator of Raf in mammalian fibroblasts (Yujiri *et al.*, 2003). Clearly, the possibility that integrins may be involved in carbohydrate recognition and downstream signalling in molluscan haemocytes needs further investigation.

BSA-galactose and BSA-fucose were chosen for use in this study since they have been shown to modulate molluscan immune responses (Hahn *et al.*, 2000), and because galactose and fucose are major components of the schistosome antigens LacdiNac (LDN) and fucosylated LacdiNac present on intramolluscan stages of the parasite (Nyame *et al.*, 2002). Results obtained in the presence of haemolymph using a combination of sugars may be more representative of a helminth infection, since haemocytes will be exposed to more than one sugar, and snail serum components will also be present. Interestingly, with haemolymph present and both sugars combined, phagocytosis was significantly reduced suggesting a possible mechanism of host immune evasion by schistosome larvae.

Although phagocytosis and encapsulation are different biological responses, the initial recognition events and their effects on downstream signalling pathways are likely to be similar. Our results suggest that parasite-mediated down-regulation of signalling events to facilitate immune evasion could be a strategy employed by extracellular parasites. It is well established that intracellular parasites interfere with host immune function by altering signalling pathway activities and recently *Leishmania* has been shown to secrete phosphatases to switch off ERK signalling in macrophages, thus increasing its chance of survival (Martiny *et al.*, 1999).

This study has demonstrated that fucose and galactose, sugars commonly found on surface coats of schistosome larval stages, are capable of causing down-regulation of ERK and PKC signalling in *L. stagnalis* haemocytes. These sugars also affect the phagocytic activity of haemocytes, an effect that is at least in part due to the modulation of ERK and PKC signalling pathways. The differences in ERK signalling

and phagocytosis observed when haemolymph was present compared to when it was absent, suggest that serum components may play an important role in the recognition of fucose and galactose monosaccharides.

Clearly, further research is needed to fully understand the mechanisms of carbohydrate recognition by haemocytes. It would be interesting to challenge haemocytes with sporocyst surface coats to see if they affect PKC and ERK signalling and modulate behavioural responses in a similar way to BSA-fucose and BSA-galactose. Further characterisation of *L. stagnalis* serum and cell-surface lectins is also required to fully understand the role of humoral immunity in the molluscan immune response. Such research would help elucidate how lectin-carbohydrate binding to the haemocyte surface results in the modulation of intracellular signalling pathways. BSA-conjugated fucose, mannose and galactose have previously been shown to modulate the generation of reactive oxygen species in *B. glabrata* haemocytes (Hahn et al., 2000). It would be very interesting to investigate whether or not this is true for *L. stagnalis* haemocytes, as this could be one of the mechanisms used for killing the larvae of intramolluscan parasites.

4.4 IDENTIFICATION OF INTEGRINS AND FAK-LIKE PROTEIN IN *L. STAGNALIS* HAEMOCYTES

The FAK/Src complex has been shown to play a role in phagocytosis by insect haemocytes (Metheniti *et al.*, 2001), and more recently, a phosphorylated form of FAK has been identified in sea urchin embryos (Garcia *et al.*, 2004). Integrins have also been identified in cells of many invertebrates such as *Drosophila* (Marcantonio & Hynes, 1988; Holmblad *et al.*, 1997; Miyazawa *et al.*, 2001; Lavine & Strand, 2003). However, our knowledge of molluscan integrins is still relatively poor and is largely restricted to the cloning of a β-integrin subunit from an embryonic cell line derived from the mollusc *B. glabrata* (Davids *et al.*, 1999).

Integrins are known to play a key role in cell adhesion, and as such are particularly important in immune defence mechanisms. With the aid of integrin-based cell adhesion kits, binding of *L. stagnalis* haemocytes was found to be integrin-mediated and to be dependent on the presence of calcium and magnesium in the extracellular environment. Since integrin clustering is known to activate FAK in mammalian cells (Naik *et al.*, 2003), we set out to determine levels of phospho-FAK in both suspended and adherent haemocytes using an anti-phospho FAK (Tyr^{925}) antibody. Activation of FAK has been reported in skeletal muscle cells upon binding to fibrinogen coated plates (Disatnik *et al.*, 2002), and cell spreading and migration have been found to be enhanced by inducing FAK expression in FAK-null mouse embryo cells (Owen *et al.*, 1999). In the present study, the FAK-like protein in haemocytes that were allowed to bind for 30 min in plastic cell-culture plates had an 8-fold increase in phosphorylation when compared to suspended cells. The phosphorylation of this FAK-like protein was transient, with levels of phosphorylation reduced to near basal levels after 90 min of binding. Moreover, haemocytes in suspension did not appear to have any detectable levels of the phosphorylated FAK-like protein, which further suggests that FAK activity is reliant on cell adhesion in *L. stagnalis* haemocytes. A similar result was reported by Schlaepfer *et al.* (1998), who showed that tyrosine phosphorylated FAK is found in cells that are fibronectin bound but not in those that are suspended. Also, cells that are cultured on fibronectin-coated plates possess FAK activity 20 min after plating and this activation is prolonged for up to 6 hours (Schlaepfer *et al.*, 1998). Integrin-

specific adhesion of NIH 3T3 cells is also known to activate all components of the ERK pathway (Raf-1, MEK and ERK) for up to 1 hour (Chen *et al.*, 1996).

Using an anti-phospho FAK antibody, Metheniti *et al.* (2001) have previously demonstrated a specific role for the FAK/Src complex in phagocytosis by insect haemocytes, with FAK being phosphorylated on Tyr^{397} in the presence of *E. coli*. In the present study, use of an anti-phospho Tyr^{397} antibody proved unsuccessful. The fact that the anti-phospho Tyr^{397} antibody did not work with *L. stagnalis* haemocyte extracts suggests that the sequence homology around this region of the FAK-like protein in *L. stagnalis* is not sufficiently homologous to that of mammalian or insect FAK. Since both integrins and FAK have shown to be involved in haemocyte phagocytic activity (Foukas *et al.*, 1998; Metheniti *et al.*, 2001), phagocytosis assays were performed with RGDS. RGDS is an integrin blocking tetrapeptide that has been shown to inhibit phagocytosis in alveolar macrophages (Currie *et al.*, 2000) and fish haemostatic cells (Hill & Rowley, 1998). RGDS has also been used to block integrin signalling in RAW cells (Monick *et al.*, 2002). The use of RGDS in the present study confirmed the involvement of integrin receptors on the surface of haemocytes since this tetrapeptide reduced phagocytic activity. Moreover, blocking actin remodelling with cytochalasin D resulted in a similar effect demonstrating a crucial role for the actin cytoskeleton in phagocytosis by haemocytes. When used at similar concentrations to those used in this study, cytochalasin D has been shown to inhibit FAK activity in NIH 3T3 cells (Aplin & Juliano, 1999). Such inhibition of FAK can also result in amplified signalling to ERK *via* Shc in fibroblasts (Barberis *et al.*, 2000).

Western analysis showed that RGDS binding to adherent haemocytes resulted in the rapid phosphorylation of the haemocyte FAK-like protein as early as 1 min following exposure. Interestingly, however, haemocyte ERK did not seem to be activated following exposure to this tetrapeptide. Integrin engagement has previously been linked to ERK activation in NIH 3T3 and thyroid TAD-2 cells (Aplin & Juliano, 1999; Aplin *et al.*, 2001; Illario *et al.*, 2003), and FAK activation has also been linked to ERK activation in mouse fibroblast cells (Illario *et al.*, 2003). However, it has also been reported that integrin-mediated activation of MAP kinase can be independent of FAK in fibroblasts (Lin *et al.*, 1997). Furthermore, integrin-mediated activation of FAK in fibroblasts is not necessarily required for signalling to ERK following growth factor

stimulation (Barberis *et al.*, 2000). In contrast, a study with NIH 3T3 cells demonstrated that integrin-mediated activation of MAP kinase can occur, but that it appears to be via a pathway independent of Ras (Chen *et al.*, 1996). FAK over-expression can enhance fibronectin stimulated activation of ERK by approximately 4 fold in human 293 cells, where FAK-mediated association and activation of c-Src was found to be essential for maximal signalling to ERK 2 (Schlaepfer & Hunter, 1997). Although the mechanisms for ERK activation following integrin clustering often seem complex, the results obtained in this study suggest that integrin/FAK downstream signalling is Ras-independent, similarly to that found with fibroblasts, since ERK activity is not upregulated following integrin engagement by RGDS.

Taken together, this study has revealed the presence of integrin-like molecules in *L. stagnalis* haemocytes and has shown that they play a role in phagocytosis of *E. coli* "bioparticles". In addition, integrin engagement results in FAK phosphorylation in these cells. Clearly, it would be interesting to identify the downstream targets of FAK in *L. stagnalis* haemocytes and other proteins recruited to the focal adhesions by integrin engagement. FAK activity also appears to be reliant on Ras (Kwong *et al.*, 2003), and also intracellular calcium and PKC activity (Achison *et al.*, 2001) when cells are stimulated with collagen. PKC has been implicated in integrin-mediated muscle cell spreading (Disatnik *et al.*, 2002), and has also been shown to be a key intermediary in integrin signalling to FAK in many cell types (Vuori & Ruoslahti, 1993; Disatnik & Rando, 1999; Miranti *et al.*, 1999). Phosphatidylinositol-3-kinase has also recently been implicated in FAK-independent signalling from the β_1 integrin subunit (Armulik *et al.*, 2004). It would be interesting to see if these pathways integrate integrin-mediated cell signalling events in *L. stagnalis* haemocytes.

4.5 CONCLUSIONS AND FUTURE STUDIES

Many studies on the defence systems of molluscs have focused on bivalve haemocytes, since these animals are important economically due to them being a major food source. Cell signalling in gastropod haemocytes has, however, been largely overlooked, and this study on *L. stagnalis* haemocytes has significantly expanded our knowledge of gastropod haemocyte signalling pathways. Diagram 4.1 summarises the protein pathways found in this study, and shows the proposed interactions of the pathways given the results using pharmacological inhibitors. This is the first study to report the detection and stimulation by LPS of the ERK pathway in *L. stagnalis* haemocytes. The effect of ACTH and noradrenaline on *L. stagnalis* haemocytes requires further investigation, as results obtained this study were largely inconclusive. Indeed, it would be interesting to see if ACTH and noradrenaline are produced by *L. stagnalis* haemocytes during stress, as seen in other mollusc species (Lacoste *et al.*, 2002a). This is also the first study to report *L. stagnalis* haemocyte integrin-mediated binding and the presence of a FAK-like protein, which is transiently phosphorylated upon haemocyte binding. Although other studies have identified a tyrosine phosphorylated protein at the correct molecular weight of FAK in insect haemocytes (Metheniti *et al.*, 2001), this is the first to use an anti-phospho FAK antibody specific for the tyrosine^{925} phosphorylated protein, in invertebrates. Dissection of the signal transduction pathways that are affected by integrin engagement in molluscan haemocytes may result in a better understanding of haemocyte survival, gene transcription and cell migration, as well as enhancing our knowledge of the molecular events regulating phagocytosis.

Using pharmacological inhibitors, the pathways involved in phagocytosis by *L. stagnalis* haemocytes were investigated. It can be concluded that phosphorylation of *L. stagnalis* haemocyte ERK-like proteins is vital for efficient phagocytosis of *E. coli* 'bioparticles'. Furthermore, phagocytic activity relies on both Ras-dependent and –independent signalling pathways, with PKC and PI-3K both being important for haemocyte phagocytosis. PKA does not appear to modulate phagocytic activity in *L. stagnalis* haemocytes. Presently, this is also the first study to identify and dissect the signalling pathways involved in *L. stagnalis* haemocyte phagocytosis. Although inhibitors have been used to dissect the signalling pathways involved in phagocytosis, it would be interesting to introduce activators of phagocytosis to the bioparticle assays,

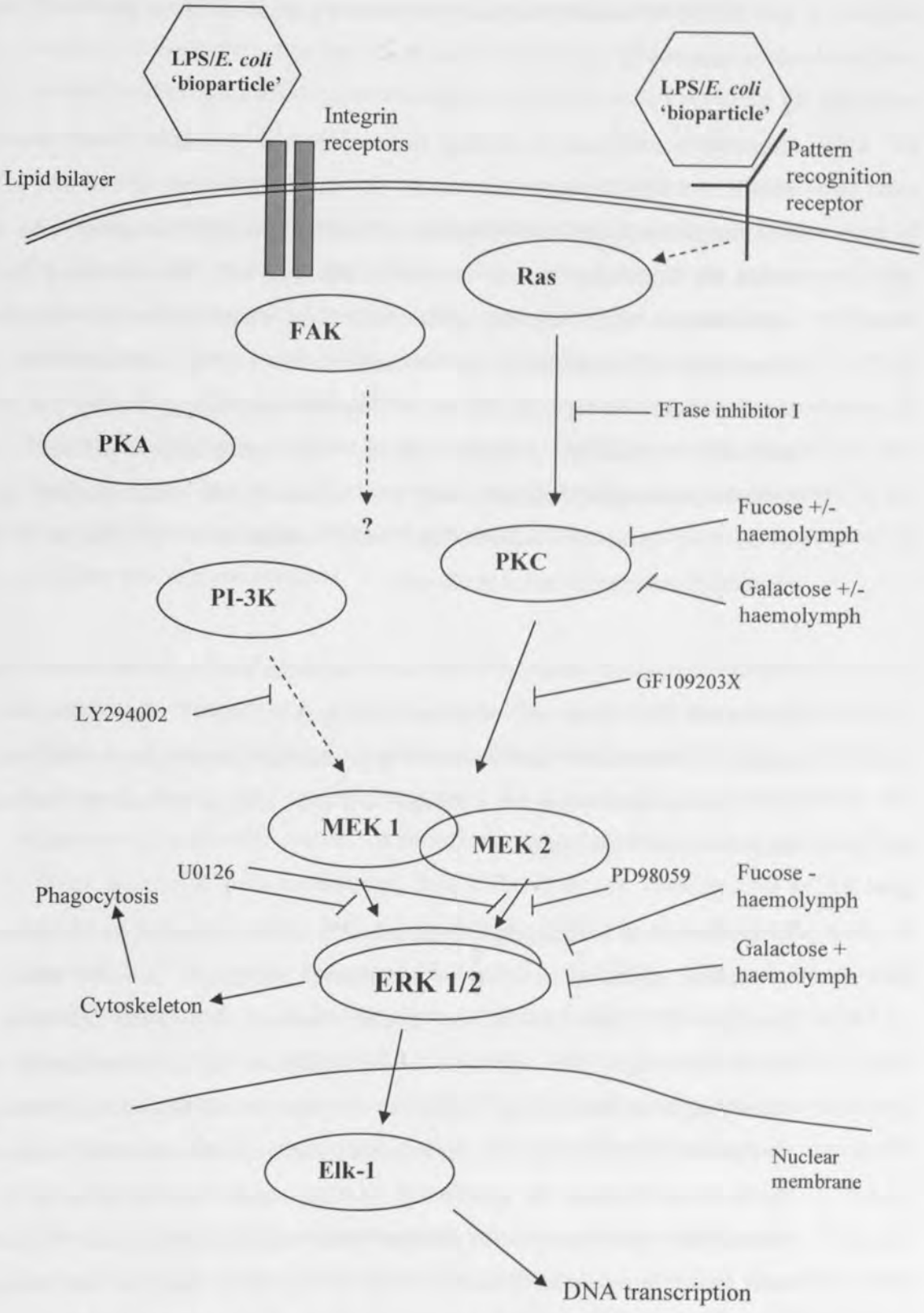

Figure 4.1. Diagram summarising ERK pathway signalling in *L. stagnalis* haemocytes as found in this study. Solid arrows demonstrate phosphorylation (activation), whereas dotted arrows demonstrate indirect activation through protein intermediates. As the actions of FAK are unknown in *L. stagnalis* haemocytes, a question mark has been used to represent signalling proteins lying downstream of FAK

such as ACTH which has shown to activate phagocytosis by gastropod haemocytes (Ottaviani, 1992).

Recently, a study on *C. capitata* haemocytes was published which identified p38 MAPK and ERK as important proteins for the internalisation of cell-free LPS, whilst the internalisation of *E. coli* required p38 MAPK and JNK (Lamprou *et al.*, 2004). It would be interesting to see if p38 MAPK and JNK are necessary for particle internalisation by *L. stagnalis* haemocytes. This would involve the identification of these proteins in haemocytes and blockade of their activity with inhibitors. It would also be of interest to, with the aid of confocal microscopy, analyse the location of activated signalling proteins during the phagocytic process. Although immunocytochemistry has been used with success in this study, confocal microscopy would give improved resolution and would enable better elucidation of the exact location of activated proteins in the cell.

A study has recently been published which identifies PKC to be involved in phagosome maturation using proteomic techniques (Hing *et al.*, 2004). This is the first study to apply two-dimensional electrophoresis to *L. stagnalis* haemocyte proteins, and it would be of interest to develop this technique to further analyse haemocyte proteins perhaps even after sub-fractionation.

In the absence of haemolymph, exposure of *L. stagnalis* haemocytes to albumin-linked saccharides appeared to result in a decreased phagocytic activity, which reflected decreased ERK and PKC activity levels. Presently, this is the first study to show intracellular signalling pathways to be modulated by BSA-conjugated sugars in macrophages or haemocytes. Results of the present study complement those gathered by Hahn *et al.* (2000), who has demonstrated that these albumin-linked sugars stimulate the immune response by generating reactive oxygen intermediates in *B. glabrata* haemocytes. Moreover, results gathered in this study in the presence of serum suggest that serum components (lectins) have an important role in the recognition of carbohydrate moieties. Indeed, previous studies on the *L. stagnalis* defence system have shown that lectin binding can alter the immune response considerably, for example, lectin binding has resulted in increased haemocyte phagocytosis (Horák & Van der Knaap, 1997; Horák *et al.*, 1998; Horák & Deme, 1998). In order to further

investigate *L. stagnalis* immune responses in the presence of carbohydrates, it would be useful to characterise serum and cell-associated lectins of this snail. This has been done in other studies using affinity chromatography, which involves capturing the lectins on to carbohydrate coated beads (used by Gerlach *et al.*, 2004; Sultan *et al.*, 2004). To determine *L. stagnalis* defence responses in response to infection by schistosomes more accurately, it would be interesting to expose haemocytes to the intramolluscan stage of the parasite, the sporocyst, and examine the biochemistry of the haemocytes after exposure. A number of studies have used sporocysts to challenge molluscan haemocytes (Bayne *et al.*, 1980; Lodes & Yoshino, 1990; Amen *et al.*, 1991b), however, none of these studies have examined the intracellular pathways modulated in haemocytes following exposure to sporocysts. Overall, the results presented in this study have implications for host immunity to schistosomes and ongoing work in the laboratory hopes to further define the molecular interactions that occur between snails and their schistosome parasites.

As *L. stagnalis* genomic data become available, many more molecular techniques can be applied to the study of *L. stagnalis* haemocytes. Instead of using pharmacological inhibitors, double stranded RNA-mediated RNA interference (RNAi) (reviewed by Scherer & Rossi, 2004) could be applied to *L. stagnalis*, to down-regulate the expression of a specific protein. Although the use of pharmacological inhibitors has proved successful with haemocytes, it is difficult to use them in vivo or for long periods of time. RNAi has been successfully applied to *L. stagnalis* for the study of neuronal NOS in feeding behaviour (Korneev *et al.*, 2002). The technique would require whole snails to be injected with synthetic ds-RNA molecules. It would be interesting to carry out adenoviral transfections with the haemocytes, which could result in upregulation of a specific protein. This technique has not yet been employed in haemocyte studies, and could further the available knowledge of haemocyte signalling considerably, especially concerning the functional aspects of the cells. Although the use of immunocytochemistry has shown that a proportion of *L. stagnalis* haemocyte phosphorylated ERK translocates to the nucleus upon LPS stimulation, and kinase activity assays have shown phosphorylated ERK to activate Elk-1, other techniques could provide us with a more detailed picture of the actions of ERK in the nucleus. To demonstrate that ERK activation of Elk-1 results in Elk-1–DNA binding, electrophoretic mobility shift assays (EMSA) could be performed, which would give

qualitative data regarding the levels of free or DNA-bound transcription factor (for examples refer to Darville *et al.*, 2001). This technique could not only be applied to Elk-1, but other transcription factors known to be downstream of ERK in mammalian cells, such as c-Jun. Polymerase chain reaction (PCR) could also be applied to *L. stagnalis* haemocytes to provide qualitative data of RNA transcripts upon ERK activation, whilst real-time PCR would provide semi-quantitative data (for examples refer to Jurstrand *et al.*, 2005).

Overall, this study has provided much needed data on the molecular control of *L. stagnalis*, and indeed molluscan, defence reactions. It is anticipated that this study will provide a framework on which future studies can be built to further explore the intricacies of signalling in invertebrate defence cells.

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APPENDICES

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Abbreviations

$4,5\text{-}PIP_2$	Phosphatidylinositol-4,5-bisphosphate
ACTH	Adrenocorticotropin hormone
ADP	Adenosine diphosphate
AMP	Adenosine monophosphate
ANOVA	One-way analysis of variance
APS	Ammonium persulphate
Arg	Arginine
Asp	Aspartine
ATP	Adenosine triphosphate
BSA	Bovine serum albumin
cAMP	Cyclic adenosine monophosphate
CD	Cluster of Differentiation
DAG	Diacylglycerol
DC	Detergent compatible
DMSO	Dimethyl sulphoxide
Ds-RNA	Double stranded ribonucleic acid
ECL	Enhanced chemiluminescence
EGF	Epidermal growth factor
EGFR	Epidermal growth factor receptor
EMSA	Electrophoretic mobility shift assay
ERK	Extracellular-signal Regulated Kinase
FAK	Focal adhesion kinase
FITC	Fluoroisothiocyanate
FTase	Farnesyltransferase
GalNAc	N-acetylgalactosamine
GAP	GTPase activating protein
GDP	Guanine diphosphate
GlcNAc	N-acetylglucosamine
Gly	Glycine
GNBP	Gram negative bacteria binding protein
GNRP	Guanine nucleotide releasing factor

GPCR	G protein coupled receptor
GTP	Guanine triphosphate
GTPase	Guanine triphosphatase
HPO	Haemocyte-producing organ
HRP	Horseradish peroxidase
IFN	Interferon
IL	Interleukin
iNOS	Inducible nitric oxide synthase
JNK	c-Jun NH_2-terminal kinase
LBP	Lipopolysaccharide binding protein
LPS	Lipopolysaccharide
MAPK	Mitogen-actived Protein Kinase
MARCKS	Myristolyated alanine-rich C kinase substrate
MEK	Mitogen-activated Protein Kinase Kinase
MGD1	*Mytilus galloprovincialis* defensin 1
MKK	Mitogen-activated protein kinase kinase
MKP	MAP kinase phosphatase
NF-κB	Nuclear factor κ B
NGF	Nerve growth factor
NK Cell	Natural killer cell
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PI	Phosphatidylinositol
PI-3K	Phosphatidylinositol 3 kinase
PKA	Protein kinase A
PKC	Protein kinase C
PLC	Phospholipase C
proPO	Prophenoloxidase
proPO-AS	Prophenoloxidase-activating system
RGDS	Asganine-Glycine-Aspartine-Serine
RNA	Ribonucleic acid
RNAi	Ribonucleic acid interference
RTK	Receptor tyrosine kinase

SAPK	Stress activated protein kinase
SDS	Sodium dodecyl sulphate
SDS-PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
SEM	Standard error of the mean
Ser	Serine
SH	Src homology
SSS	Sterile snail saline
TBS	Tris buffered saline
TEMED	N,N,N',N'-tetramethylethylenediamine
TGF	Transforming growth factor
Thr	Threonine
TLR	Toll-like receptor
TNF	Tumour necrosis factor
TTBS	Tris buffered saline – Tween 20
Tyr	Tyrosine

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