The involvement of PKD in Fcγ receptor activation of the NADPH oxidase in human neutrophils

by

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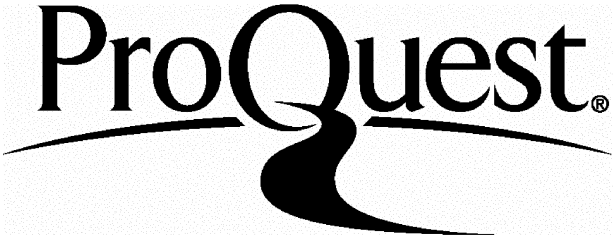

Abstract

NADPH oxidase is a membrane bound enzyme, crucial in the destruction of pathogens by neutrophils. We have studied the signalling cascade leading to the activation of the NADPH oxidase upon activation of the Fc receptor, with a particular focus on the involvement of protein kinases in this process.

Activation of the NADPH oxidase following Fcγ receptor stimulation was studied in human neutrophils using inhibitors of tyrosine kinases, phosphatidyl inositol 3-kinase and protein kinase C (PKC), which are all involved in this signalling cascade. Results with different PKC inhibitors showed unexpected results. While Gö 6976 inhibited activation completely, Ro 31-8220 caused only a 60% inhibition. Thus a Gö 6976-sensitive Ro 31-8220-insensitive component was suspected. Evidence from the literature suggest that Gö 6976 but not Ro 31-8220 inhibits an isotype of the PKC family, PKCμ/Protein kinase D (PKD), *in vitro*, indicating that PKD could be involved in NADPH oxidase activation. PKD antisense oligonucleotides inhibited NADPH oxidase activation, supporting an involvement of PKD. Kinase assays showed that PKD is activated upon Fc receptor ligation. Immunofluorescence techniques showed that PKD is present in the plasma membrane and appears in the phagosomal

membrane upon phagocytosis, further linking its functions to NADPH oxidase activation.

The NADPH oxidase contains the membrane bound proteins, $gp22^{phox}$ and $gp91^{phox}$ and the cytosolic components, $p47^{phox}$, $p40^{phox}$ and $p67^{phox}$, which translocate to the phagosomal membrane upon activation. Translocation of these cytosolic components is driven by phosphorylation. We have shown that PKD phosphorylates $p40^{phox}$ and $p47^{phox}$ but not $p67^{phox}$.

Mass spectrometry studies and 2 dimensional gel electrophoresis technology indicated that PKD may not only play a role in the activation of the NADPH oxidase, as described, but may also phosphorylate other proteins, including some involved in cytoskeletal function, signalling and metabolism. The further characterisation and the roles of these need investigation.

Thus PKD is present in neutrophils and is activated upon Fc receptor stimulation. It also plays a role in the signalling cascade that leads to NADPH oxidase.

Table of contents

List of Figures and Tables

Abbreviations

ATP	Adenosine 5'-triphosphate
CGD	Chronic Granulomatous Disease
DAG	Diacylglycerol
DMSO	Dimethyl sulphoxide
DTT	Dithiothreitol
ECL	Enhanced chemiluminescence
EDTA	Ethylenediamine tetra-acetic acid
EGF	Epidermal growth factor
EST	Express sequence tag
Fab	Fragment antigen binding
FAD	Flavin adenine dinucleotide
Fc	Fragment crystallisable
fMLP	N-formyl-methionyl-leucyl-phenylalanine
GDI	GDP-dissociation inhibitor
gp	Glycoprotein
GSCF	Growth colony stimulating factor
GST	Glutathione S-transferase
GTP	Guanosine 5'-triphosphate
HMP	Hexose monophosphate pathway
IEF	Isoelectric focusing
IgG	Immunoglobulin G
IP_3	Inositol triphosphate
ITAMS	Immunoreceptor tyrosine activating motif
kD	kilo Daltons
MALDI-TOF	Matrix assisted laser disorption/ionisation-Time of flight

MAP kinase	Mitogen-activated protein kinase
MDH	Malate dehydrogenase
MHC	Major histocompatibility complex
MLCK	Myosin-light chain kinase
MPO	Myeloperoxidase
NADPH	Nicotinamide adenine dinucleotide phosphate, reduced form
NBT	Nitroblue tetrazolium
OD	Optical density
PAGE	Polyacrylamide gel electrophoresis
PAK	p21-activated kinase
PBS	Phosphate-buffered saline
PDGF	Platelet derived growth factor
PH	Pleckstrin homology
Phox	Phagocyte oxidase
PI-3 kinase	Phosphatidylinositol-3 kinase
PKA	Protein kinase A
PKC	Protein kinase C
PKD	Protein kinase D
PLC	Phospholipase C
PLD	Phospholipase D
PMA	12-phorbol myristate 13-acetate
PMSF	Phenylmethylsulphonyl fluoride
PNS	Post nuclear supernatant
RNA	Ribonucleic acid
SDS	Sodium dodecyl sulphate
TBS	Tris-buffered saline
TCA	Trichloroacetic acid
TFA	Trifluoroactic acid
TLCK	N-α-p-tosyl-L-leucine chloromethyl ketone
TPCK	n-Tosyl-L-phenylalanine chloromethyl ketone

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Chapter 1

General Introduction

1.1 Blood

Blood. Known to be essential for life even before anything was understood about its function. If blood is shed life will cease; the bible uses the term "shed blood" meaning "to kill". (Wintrobe 1992) Historically and even in the present day blood is attached to emotions as well as health and disease. Some people faint at the sight of blood; in other contexts it is a sign of passion, of anger. It has been assigned with every human emotion. For example an emphasis of how angry you may be would be that "...it makes my blood boil", or fear "...it makes my blood run cold".

Medical dogma was for many years dominated by the doctrine of the four humours (blood, phlegm, black bile and yellow bile), first set out by Hippocrates (approx. 400 BC) and later systematised into a complex pattern by Galen (ca. AD 130-200). In this idea health and disease were seen as a consequence of either proper equilibrium or imbalance of these four components. Fahraeus, a Swedish pathologist, who devised the

erythrocyte sedimentation test, pointed out that the theory of the four humours was based on clinical observation and not solely on speculation. When blood is first extracted it appears as a homogeneous red fluid. However, if allowed to settle, clotting and clot retraction occurs and a clear yellow fluid can be observed. Below it another three layers appear: a deep red almost black jelly on the bottom, above that a thinner layer of red blood, followed by a pale green or whitish layer, which is enhanced if the donor is ill. The theory of the four humours remained a central doctrine in medicine for about 1400 years. So as early as medicine has been a practice, blood was recognised as being imperative for life and a predominant figure in diagnostics. (Craddock 1992)

In 1683/4 Robert Boyle wrote in his essay Humane Blood "it conveys nourifhment and vigour, and motion, and in a word health to the rest of the living engine". In modern times, The Encyclopaedia Britannica defines blood as the "fluid in multi-cellular animals that transports oxygen and nutrients to the cells and carries away waste products. In many species it also conveys hormones and disease-fighting substances". According to Dorland's medical dictionary "blood is the fluid that circulates through the heart, arteries, capillaries and veins, carrying nutriment and oxygen to the body cells". So, the definition in the last three hundred years has not changed much, but...what is blood?

1.2 Blood Composition

Blood consists of various major constituents: a pale yellow liquid called the plasma, a group of small cellular fragments called platelets and microscopically identifiable blood cells or leukocytes. Blood cells and platelets originate from a common progenitor cell, a pluripotent haematopoietic stem cell which gives rise to further progenitor cells: the erythroblast, the megakaryocyte, the common lymphoid progenitor and the myeloid progenitor cell.

The megakaryocyte gives rise to platelets, which are involved in blood clotting, by forming plugs to repair the vasculature.

Blood cells can be divided into two main groups, the white and the red blood cells. The red blood cells or erythrocytes, are small non-nucleated cells whose main function is the carriage of oxygen from the lungs to all other tissues in the body; they arise from the erythroblast.

White cells form a more diverse group but generally speaking their main function is in protection of the body. White cells are a part of the immune system. They "patrol" the host defending it from invading organisms. William Hewson first discovered these cells in the 18th century, long after the discovery of the red cells. Hewson noticed that the cellular content of lymph was nucleated, and called them "central particles"; he believed these structures were formed in the lymph nodes and carried into the blood.

White blood cells were first thought of as a group, and it is partly due to this, their origin, function and behaviour were not identified. Not until Paul Erlich, in the 19th century devised a way of differentially staining these cells and categorised them into separate groups could these cells be differentiated. The discovery by Elie Metchnikov, in 1882, of a process that he termed phagocytosis provided an early understanding of one of the functions of these cells.

White blood cells can be divided into lymphocytes and phagocytes, depending on function and origin.

The lymphocytes, which arise from the common lymphoid progenitor, are a group of cells, largely involved in memory immunity or adaptive immunity. (Hoffbrand 1993) Once exposed to a pathogen these cells will remember it, making a faster and more effective response when exposed to the pathogen again. (Roitt 1990) Lymphocytes are composed of two major groups, B and T cells, named after their "tissue of maturation".

B cells mature in the bone marrow, and hence are named B cells. These cells when activated by antigens differentiate into plasma cells, which secrete antibodies, and memory B cells. While the formers are cleared after antigen eradication, memory cells remain and enable a quantitatively and qualitatively superior secondary immune response to be mounted after a subsequent encounter with the same antigen. (Delves and Roitt 2000)

T cells, thymus derived lymphocytes, are divided into two main classes. The first group, the cytotoxic T cells, kill cells infected by viruses and other intracellular pathogens as they recognise the major histocompatibility complex (MHC) class I moiety, which presents antigen acquired intracellularly from an infection. The second group, helper and/or inflammatory T cells, activate other cells such as B cells in order to produce antibodies or drive an inflammatory reaction, their main function being the release of cytokines. (Delves and Roitt 2000; Delves and Roitt 2000)

Phagocytes form the first line of defence, and partake in innate immunity. The term innate immunity consists of all immune defences that lack immunologic memory. Therefore, a characteristic of this system is that it responds the same no matter how many times the antigen is encountered. The cells involved contain receptors that are encoded in their germ-line DNA and do not undergo somatic recombination as lymphocytes do. (Medzhitov and Janeway 1997; Medzhitov and Janeway 1998; Medzhitov and Janeway 1998; Medzhitov and Janeway 2000) The way phagocytes function is by ingesting and destroying the invading pathogen, hence their name, which comes from the Greek word *phagos*, to eat. Phagocytes can be divided into two groups: macrophages and granulocytes. While both originate from the myeloid precursor cells, the macrophage undergoes a two step differentiation.

Macrophages first leave the bone marrow where they are produced and circulate in the blood as monocytes. The latter then mature to

macrophages and may become resident in certain tissues, as specialised cells, which clear the host tissues of foreign material. Examples of tissue macrophages include Kupffer cells in the liver, dust cells in the alveoli or glial cells in the central nervous system. Macrophages are specialised in many other functions. These functions may differ depending on the tissue in which they set up residence. However jointly their functions range from antigen presentation to T cells, wound healing, tumour cell destruction, bone remodelling, as well as production of tissue and immune cell regulators by secreting cytokines and growth factors. (Roitt 1990)

The other group of phagocytes is jointly referred to as granulocytes because they contain dense staining granules within their cytoplasm; they are also sometimes called polymorphonuclear leukocytes because they have oddly shaped nuclei. There are three types of granulocytes: basophils, eosinophils and neutrophils. They are grouped together because of their morphological similarity and can be differentiated by their staining characteristics. Basophils stain with a basic stain, eosinophils with an acid stain and neutrophils with a neutral stain. Functionally, they are also different.

Basophils are found only within blood and are very rare, comprising less than 1% of the total white cell count in the circulation. Due to their low presence (0-0.1 x 10^9/L) (Hope 1998) it has been extremely difficult to characterise their function, however they are believed to be related to mast cells (a type of cell involved in allergic and immediate hypersensitivity

reactions). Like mast cells their granules contain histamine, serotonin and other components which can mediate allergic and inflammatory responses.

Eosinophils on the other hand are more abundant comprising of 1-7% of the total white cell count in the circulation. Their granules contain a number of enzymes that are toxic to parasites. In particular their granules contain a major basic protein which, when released onto the surface of parasites damages their cell membranes. Though eosinophils cannot *per se* phagocytose their large target, they surround the parasite and release their granular contents. (Mimms 1993)

The most abundant of all the white cells is the neutrophil. This cell type accounts for 40-65% of white blood cells, and is found in the blood at a range of 3-5 million/ml. (Edwards 1994) Peripheral blood, neutrophils exist in two pools: 60% are freely circulating while 40% marginate on the blood vessel walls. (Athens, Vodopick *et al.* 1967; Bishop, Athens *et al.* 1968; Orfanakis, Ostlund *et al.* 1970) Neutrophils have a short life span in the circulation, 8-20 hours; however they are constantly replaced by the bone marrow at a rate of approximately 5 $x10^{10}$ per day. Neutrophils are the first-line of defence of the body against bacterial and fungal infection. Their central role in the destruction of these pathogens can be seen in the susceptibility to infection shown by patients who have insufficient neutrophils (neutropenia) (Woolf 1998), or in diseases caused by defective neutrophil function.

Chediak-Higashi syndrome is characterised by recurrent pyogenic infections (Blume and Wolff 1972) due to defective neutrophil migration to the site of infection (chemotaxis) (Blume and Wolff 1972) and inability to release toxic enzymes (degranulation). (Clark and Kimball 1971) Chronic granulomatous disease (CGD) presents in a similar manner but the defect lies in the inability of the neutrophil to destroy the pathogen once phagocytosed. (Quie, Kaplan *et al.* 1968)

CGD was first recognised in 1950's however no causal relation was found. (Berendes 1957) It was later noticed to be a deficiency in leukocyte function, (Quie, White *et al.* 1967) and further a problem of superoxide production by the NADPH oxidase. (Quie, Kaplan *et al.* 1968, Baehner, 1968 #166); (Baehner and Karnovsky 1968) Subsequently it has been discovered that this disease is caused by specific mutation in genes encoding for components of the NADPH oxidase, and this, in many ways, has served as a basis for understanding and discovery of these components. (See sections 1.5 and 1.9)

1.3 The immune system

We are constantly exposed to all sort of pathogens in our daily lives. These pathogens may come in the form of chemicals, particulate material or microorganisms' and it is due to the immune system that we do not succumb to them. (Traves 1996)

Edward Jenner first noticed in 1796 that cowpox or vaccinia induced protection against small pox in humans. He observed that milkmaids were a predominant group free of this often-fatal disease. He postulated that their exposure to infected cows conferred immunity. In a bold experiment Jenner injected himself with material from the cowpox lesion, and thereby lost susceptibility to small pox infection. This was the birth of immunology and marks the first immunological experiment. (Traves 1996)

In 1890, Emil Von Boehring and Shibasaburo Kitasato notice that the serum of "vaccinated" individuals contain substances that specifically bound the relevant pathogen. They called these substances antikorpus (antibodies). Antibodies and their production play a central role in adaptive immunity. It is referred to as adaptive since it requires prior exposure to pathogens and the creation of an antibody in order to fight an infection caused by a specific pathogen. This, though unsuspected, was the principle behind Jenner's famous experiment. In many cases an adaptive response confers life long protection to re-infection by the same pathogen.

Innate immunity is distinguished from adaptive immunity in that it does not required previous exposure to pathogens. As mentioned above it is the phagocytes that play a central role in this function. These cells were first discovered by Russian immunologist Elie Metchnikoff who, while studying the digestive functions of certain amoebas, noticed that in transparent seafish larvae which do not have a digestive system, somatic cells fed by means of direct ingestion of particulate food. (Metchnikoff 1892; Hirsch

1982) Phagocytes are present in many different organisms and combat a wide variety of invading pathogens without prior exposure.
Originally there was a big divide between what we now know as innate and adaptive immunity, which ensued for many years. While both the discoveries of the phagocytic properties of certain cells and antitoxin capability of serum were substantial, the two mechanisms of immunity could not be reconciled as a single immune system. It was not until 1903-1905 that Almoth Wright and Captain Steward R. Douglas noticed that antibodies coated the particles and "prepared them to be eaten" (Greek-*opsonos*) by the phagocytes. (Douglas 1903) Hence the process of opsonisation enhances phagocytosis. Bernard Shaw in his satirical play, *The Doctor's Dilemma*, writes: "Opsonin is what you butter the disease germs with to make your white blood corpuscles eat them".

In summary, when a pathogen first enters the body it quickly elicits a phagocytic response. Initially neutrophils enter the site of infection in order to clear it. In a second wave macrophages will enter the site and either partake in the "bacterial feast" or, it is believed, ingest neutrophils which have become apoptotic; post phagocytosis the macrophage will process the pathogen and present a small fragment to a lymphocyte, in this case a T cell. The latter in turn will stimulate B cells to produce specific antibodies. (Roitt 1990; Traves 1996; Medzhitov and Janeway 1997; Delves and Roitt 2000; Delves and Roitt 2000; Medzhitov and Janeway 2000) Upon re-

infection the antibody binds to the pathogen and facilitates phagocytic uptake and quicker clearance of the infectious agent by neutrophils.

1.4 Neutrophil Function

Neutrophils patrol the body in what is a non-activated or resting state. They become activated by either physical or chemical stimuli. Upon infection of tissue the neutrophil will attach itself to the blood vessel wall (margination) and then squeeze through the gaps between endothelial cells (diapedesis). Once out of the circulation the neutrophil migrates to the site of infection (chemotaxis), where it encounters the invading pathogen and destroys it. Neutrophils kill their target pathogens by phagocytosis. (Cohen 1994)

Phagocytosis is a multi-faceted process by which extracellular matter is taken into cells. The neutrophil engulfs the bacteria, and within the vacuole (phagosome) created by this mechanism, it destroys the organism. The steps involved include cytoskeletal rearrangement, fusion of granular membranes to phagosomal membranes and activation of the NADPH oxidase. (Krause 2000)

Two systems execute the elimination of pathogens: non-oxidative mechanisms, which uses various microbicidal proteins stored in granules within the neutrophil cytoplasm; and oxidative-dependent killing, where reactive oxygen intermediates are produced

1.4.1 Non-oxidative microbicidal activity

Microbicidal proteolytic enzymes functioning under anaerobic conditions are the active components of this killing system. These are stored in three types of granules within the neutrophil cytoplasm. (Borregaard 1997; Gullberg, Andersson *et al.* 1997; Gullberg, Bengtsson *et al.* 1999) Upon activation the granules fuse with the phagosome, forming the phagolysosome, and release their contents in the vacuole.

The azurophilic granules, are the first to be synthesised during neutrophil maturation. There are approximately 1500 azurophilic granules in neutrophils and their contents are very heterogeneous. These granules contain myeloperoxidase, defensins, cathepsin G, azurocidin, Bactericidal permeability-inducing protein (BPI), hydrolases, elastases and collagenases. (Gabay 1994) Though their general functions are the same, their specific functions differ. For example defensins are particularly cationic proteins which appear to insert themselves into the bacterial membrane and form hydrophobic channels, which act as voltage dependent ion channels. Cathepsin G and elastase are serine proteases, which are particularly useful in the destruction of gram-negative organism and fungal infections. Mice deficient in elastase are more susceptible to infections with *Klebsiella pneumoniae* and *Escherichia coli* (Belaaouaj, McCarthy *et al.* 1998), whereas deletion of both proteases, increased susceptibility to *Candida albicans* and *Aspergillus fumigatus.* (Tkalcevic, Novelli *et al.* 2000) Myeloperoxidase (MPO) is another enzyme present in these granules.

MPO catalyses the reaction leading to the formation of hypochlorous acid (HOCl). MPO deficiency is prevalent in the human population (1:2000). (Segal personal communication) This deficiency has not been associated with an increase susceptibility to pathogenic infection in the majority of affected patients (Kutter 1998), though a correlation has been made between MPO-deficiency and a high incidence of malignant tumours. (Lanza 1998) However, reports exist of isolated cases in which a patient did show an increased susceptibility to fungal infections. (Edgerton 1999) Increased susceptibility to fungal infection was shown in transgenic mice lacking expression of MPO. While the mice matured and reproduced normally, upon challenge with *C. albicans* survival rates were lower than wild-type littermates. (Aratani, Koyama *et al.* 1999)

The second type of granules, also known as secondary or specific granules, is twice as abundant as the azurophilic granules in mature neutrophils. These granules include lysozyme, lactoferrin, and vitamin B12 binding protein. The importance of this group of granules can be seen in Specific Granule Deficiency, a disorder in which patients will present with recurrent infections. This deficiency seems to be mostly related to a lack of lactoferrin. (Breton-Gorius, Mason *et al.* 1980; Lomax, Gallin *et al.* 1989) Lactoferrin, an iron binding glycoprotein seems to be involved in inhibiting bacterial growth and in direct killing of such pathogens as *Streptococcus mutans* and *Vibrio cholera.* (Nuijens, van Berkel *et al.* 1996; Vorland 1999)

The final group of granules, the gelatinase-containing or tertiary granules, contain plasma membrane bound proteins and functions as storage for recycling receptors and other membrane bound proteins. These granules are formed by endocytosis of membrane and their receptors during a late stage of neutrophil maturation. (Borregaard, Lollike *et al.* 1993; Borregaard 1997)

The process by which granules are released, degranulation, can be induced by several stimuli and hence involve a number of intracellular pathways. Studies point to the involvement of p38 mitogen-activated protein kinase (MAP kinase). (Mocsai, Jakus *et al.* 2000; Smolen, Petersen *et al.* 2000) Other evidence suggests an involvement of increase in intracellular calcium on granular fusion to the phagolysosome. (Jaconi, Lew *et al.* 1990)

1.4.2 Oxidative-dependent microbicidal activity

The oxidative mechanism refers to the production of reactive oxygen intermediates driven by an enzyme system called the NADPH oxidase. (Iyer, Islam *et al.* 1961; Babior 1973; Babior 1982)

The NADPH oxidase is a multi-component enzyme, which functions as an electron pump, transferring electron from NADPH to oxygen inside the phagosome, and thereby forming superoxide. (Segal, Leto *et al.* 2000) (Figure 1.1)

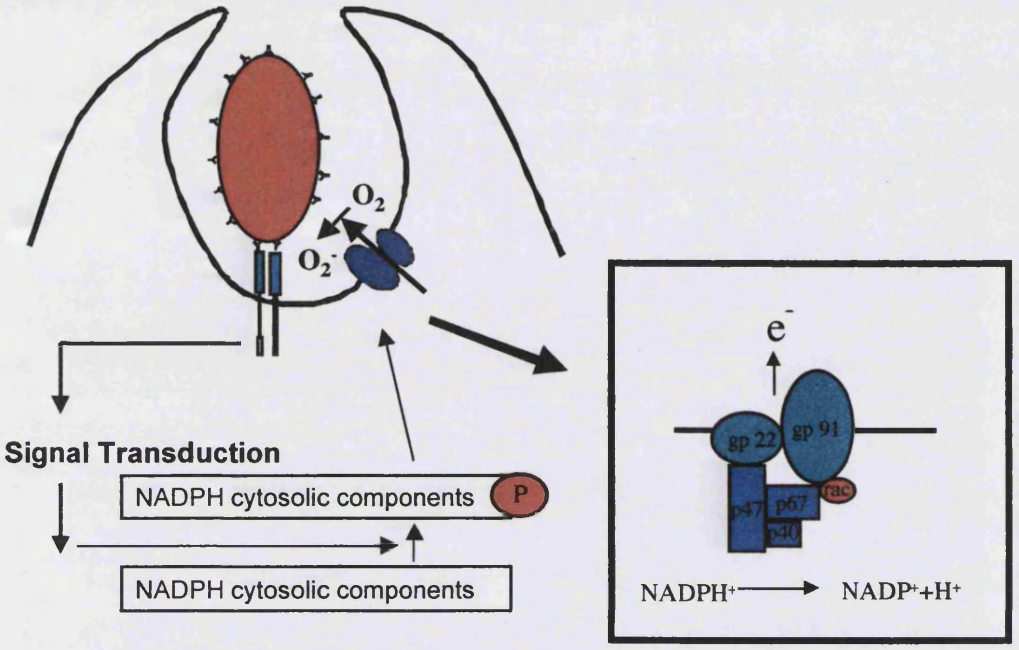


Figure 1.1 Model of NADPH oxidase activation

NADPH oxidase is activated through a signalling cascade which initiates on the plasma membrane and its transduced into the cells, where it leads to phosphorylation of the cytosolic components of the NADPH oxidase. Once phosphorylated these translocate to the phagosomal membrane. There they assemble with the membrane-bound components and the oxidase enzyme is activated.

Baldridge and Gerard first noticed that upon exposure of neutrophils to bacteria there was a surge of oxygen consumption by the cells. (Gerard 1933) At first the increase in oxygen consumption was attributed to increased energy consumption by the neutrophils upon the infectious challenge. By using inhibitors of mitochondrial respiration such as potassium cyanide, Sbarra and Karnovsky noticed that the respiratory burst was not due to an increase in mitochondrial metabolism but rather to other

mechanisms. (Sbarra and Karnovsky 1959) In the early 1960's it was shown that neutrophils produce hydrogen peroxide (a product of superoxide); it was then proposed that the oxygen consumed by activated phagocytes was used to destroy microorganisms by lethal oxidation. (Iyer, Islam *et al.* 1961) The process of reduction of oxygen to hydrogen peroxide via superoxide production, concurred with shunting of glucose through the hexose monophosphate pathway (HMP). (Sbarra and Karnovsky 1959; Iyer, Islam *et al.* 1961) One of the major products of the HMP is the reduction of $NADP^+$ to NADPH. (Stryer 1995) Further work supported the high turnover of NADPH upon phagocytosis, therefore the enzyme that was activated by phagocytosis came to be known as the NADPH oxidase. (Rossi and Zatti 1966; Patriarca, Cramer *et al.* 1971)

The NADPH oxidase is usually inactive until neutrophils are stimulated by various molecules that engage receptors on their cell surface. For example: fMLP (Lundqvist, Gustafsson *et al.* 1994), immunoglobins, complement (Hoogerwerf, Weening *et al.* 1990; Goodman and Tenner 1992) and cytokines (Ferrante 1992) are all known to activate superoxide production.

1.5 The NADPH oxidase

The NADPH oxidase has several components: a membrane bound flavocytochrome and three cytosolic factors, which come together on the phagosomal membrane in order for the enzyme to be active. (Figure 1.2)

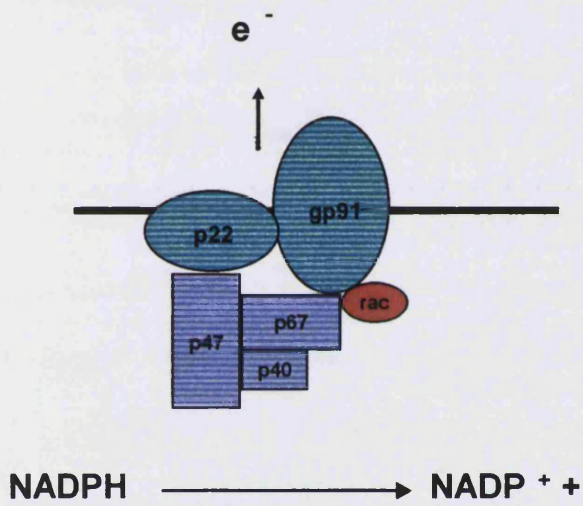


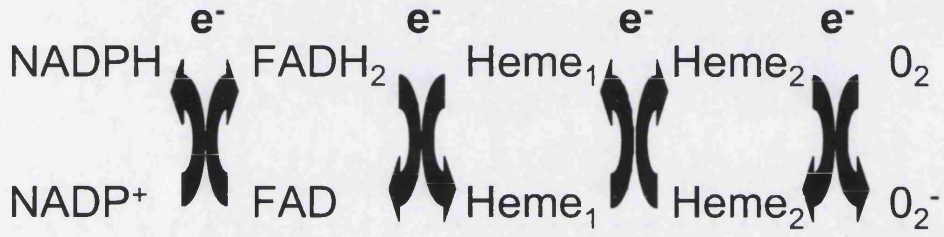
Figure 1.2 NADPH oxidase, a multi-component enzyme

The oxidase is has a membrane bound flavocytochrome, which is comprised of two sub units $p22^{phox}$ and $gp91^{phox}$, and three cytosolic components, $p47^{phox}$, $p67^{phox}$ and $p40^{phox}$. *Phox* denotes phagosome oxidase. Rac is a small GTPase associated with NADPH oxidase function

1.5.1 Membrane-bound components

The membrane bound unit, cytochrome b_{558}, is a heterodimer composed of the $gp91^{phox}$ and the $p22^{phox}$. (Segal and Jones 1979; Parkos, Allen *et al.* 1987; Wallach and Segal 1996) It has been designated cytochrome b_{558} due to its optical density absorbance at 558nm. The gp91 or β subunit of the cytochrome is heavily glycosylated and contains regions which bind flavin, two hemes, and its substrate NADPH. (Rotrosen, Yeung *et al.* 1992;

Segal, West *et al.* 1992; Wallach and Segal 1996) This structural information has helped in elucidating its specific function. The cytochrome b_{558} contains within it the above mentioned coenzymes (hemes and flavin) which help in the transfer of electrons to oxygen:



The order of the hemes was elucidated by Cross *et al.* While investigating an X-linked Chronic Granulomatous Disease (CGD) patient, it was noticed that the mid-point potentials for the two hemes differed. This led to the understanding that electrons are transferred in series, between FAD and two hemes, down a decreasing electrochemical potential gradient. (Cross, Rae *et al.* 1995)

The gene encoding for this protein is called CYBB and is located on the X chromosome at position Xq2.1. Mutations in this gene lead to X-linked CGD, and accounts for approximately two-thirds of total cases of this disease. (Roos, de Boer *et al.* 1996) These patients also present with the more severe clinical phenotype, and even heterozygous patients with partial oxidase activity, may present with at least classical disease. (Thrasher, Keep *et al.* 1994)

$p22^{phox}$, or α-subunit is encoded on chromosome 16q24 (gene CYBA). (Dinauer, Pierce et al. 1990) Mutations in this gene account for 5% of patients with CGD (Clark, Malech et al. 1989; Casimir, Chetty et al. 1992), and it is associated with autosomal recessive inheritance. Interestingly a mutation in this gene, which leads to a single amino acid change from a proline to a glutamine, gave indication of the function of this protein. This mutation is present in a proline rich region, which binds Src homology region 3 (SH3) domains. In these patients there was a failure of translocation of the cytosolic components of the oxidase to the membrane (see section 1.9) and hence activation of the enzyme. Therefore $p22^{phox}$ partakes in the overall assembly of the enzyme. (Dinauer, Pierce et al. 1991; Leusen, Bolscher et al. 1994) Furthermore, $p22^{phox}$ has 39% homology with a 31 amino acid stretch of polypeptide I of mitochondrial cytochrome C, which is a heme-coordinating domain, hinting at a role electron transfer for this protein in the NADPH oxidase. (Parkos, Dinauer et al. 1988)

There appears to be a stabilising link between the two subunits of the cytochrome, as they are both absent in certain forms of autosomal and X-linked of CGD. (Parkos, Dinauer et al. 1989)

On activation the total amount of cytochrome b_{558} seen in the plasma membrane appears to increase. Though this has no direct effect on the activation of the oxidase, it shows that a proportion of this component is

stored in the granular fraction of the neutrophil and is translocated to the membrane upon activation by phagocytic stimuli. (Garcia and Segal 1984)

1.5.2 Cytosolic components

It was observed that cytochrome b $_{558}$, isolated from non-stimulated cells was unable to produce superoxide. However, in the presence of NADPH, plus micromolar concentration of an amphiphile such as sodium dodecyl sulphate (SDS) or arachidonic acid, NADPH oxidase activity could be induced. (Bromberg and Pick 1985; Curnutte 1985; McPhail, Shirley *et al.* 1985; Curnutte, Kuver *et al.* 1987; Pick, Bromberg *et al.* 1987; Shpungin, Dotan *et al.* 1989) It was also noticed during this time that cytosol from patients who suffered from autosomal recessive CGD (AR-CGD) lacked the ability to reconstitute NADPH oxidase activity. (McPhail, DeChatelet *et al.* 1977; Curnutte, Berkow *et al.* 1988; Curnutte, Scott *et al.* 1989) This pointed towards the necessity of the cytosolic fraction for activation of the NADPH oxidase, and in turn opened the field for investigation of the actual cytosolic components.

Initial reports hinted at the possibility of two or more cytosolic cofactors involved in activation of the oxidase. (Pick, Kroizman *et al.* 1989) The absence of a phosphorylated protein at 48 kD, in patients with AR-CGD, upon activation with phorbol esters, gave other clues for identification of one of these components. (Segal, Heyworth *et al.* 1985; Hayakawa, Suzuki *et al.* 1986) This protein, p47^{phox} was cloned and showed to be able to

restore oxidase function in AR-CGD patients. (Lomax, Leto *et al.* 1989) Animal models have shown that mice, who lack the gene encoding $p47^{phox}$, develop lethal infections and granulomatous inflammation similar to those encountered in human CGD patients. (Jackson, Gallin *et al.* 1995) An antibody raised against a cytosolic fraction that could activate the oxidase in a cell-free system identified another cytosolic component $p67^{phox}$. (Volpp, Nauseef *et al.* 1988) Approximately 5% patients with CGD were shown to be lacking the $p67^{phox}$ protein, and its importance as a component of the NADPH oxidase became clear upon this. (Clark, Malech *et al.* 1989)

Activation of the cell-free system was augmented by guanosine triphosphate (GTP). (Aharoni and Pick 1990; Uhlinger, Burnham *et al.* 1991; Klinger and Aviram 1992) The necessity of GTP can be explained by the discovery of a third cytosolic protein required for NADPH oxidase activation, the small GTPase *rac*. In its active GTP bound form it becomes disassociated from the inhibitor GDP disassociation inhibitor (*rho* GDI) to which it is normally complexed. Subsequently, it translocates to the membrane where it possibly binds $p67^{phox}$. (Abo, Pick *et al.* 1991; Abo, Webb *et al.* 1994; Diekmann, Abo *et al.* 1994) The importance of *rac* for NADPH oxidase activity was shown in *rac* 2 deficient mice, which failed to produce equal levels of superoxide as wild types did. (Roberts, Kim *et al.* 1999)

By addition of recombinant cytosolic proteins and recombinant cytochrome b_{558} in the presence of the substrate NADPH, the oxidase could

be activated, implying that all subunits of the oxidase had been discovered. (Abo, Boyhan *et al.* 1992; Abo and Segal 1995)

However, it was observed that $p67^{phox}$ and $p47^{phox}$ if immunoprecipitated exist in a large complex of approximately 250kD. (Park, Ma *et al.* 1992; Someya, Nagaoka *et al.* 1993; Wientjes, Hsuan *et al.* 1993) This revealed a third component of 40kD, which was cloned and name $p40^{phox}$. (Wientjes, Hsuan *et al.* 1993) Someya and colleagues showed that $p40^{phox}$ is phosphorylated and translocates to the membrane compartment on activation. (Someya, Nagaoka *et al.* 1996; Someya, Nunoi *et al.* 1999) The specific function of this protein is not known, this is partially due to the fact that nature has not provided with a mutation that has a clear phenotype as the other components of the oxidase have. However work is being undertaken to create a $p40^{phox}$ -/- mouse, and try to elucidate specific $p40^{phox}$ function.

Translocation of all the above-mentioned cytosolic components to the membrane fraction and assembly with the cytochrome b_{558} is necessary to fully activate this enzyme.

1.6 Activation of the neutrophil/NADPH oxidase

The NADPH oxidase, which can be damaging to host tissues, must be very tightly regulated. It is activated *in vivo* by engaging certain receptors on the neutrophil plasma membrane. Receptors for chemoattractants such as the

f-methonyl-leucyl-phenylalanine (fMLP) or complement C5a are known to activate the oxidase. (Nauseef 1999) Opsonins enhance phagocytosis and bind specific receptors on the neutrophil leading to NADPH oxidase activity. Amongst them are the receptors for the complement fraction C3a and the receptors for immunoglobin, the Fc receptor. The following section concentrates on the Fc receptor, which was the topic of this investigation.

1.6.1 Fc receptors in neutrophils

There are different types of Fc receptors, each specifically recognising the Fc portion of different immunoglobins. (Raghavan and Bjorkman 1996; Daeron 1997; Ravetch 1997) There are four types of immunoglobins (antibodies), and they have the same general functions in protection of the host against invading pathogens. (Heyman 2000) These functions can be divided into three.

The first function is the *neutralisation* of pathogens. Viruses and intracellular bacteria need to enter cells in order to grow, they spread from cell to cell by binding to specific molecules on the surface of the target cell. Antibody surrounds the invading organism and in this way prevents its access to the human cell.

The second function is that of *activating complement cascade*. Complement is a series of plasma proteins composed of nine different fractions. Complement can either be activated by certain pathogens or by

the binding of antibody to an antigen (i.e. bacterial protein). Its roles include recruitment of phagocytes, opsonisation of pathogens or lysis of the same.

The third way that an antibody functions in immunity is by *opsonisation* of the pathogen (extracellular pathogens) as complement does. (Traves 1996)

Antibodies are Y-shaped molecules that circulate in the plasma. They contain two Fab (*fragment antigen binding*) regions that recognise and bind antigen, and a Fc (*fragment crystallisable*) portion, which is recognised by Fc receptors. (Figure 1.3)

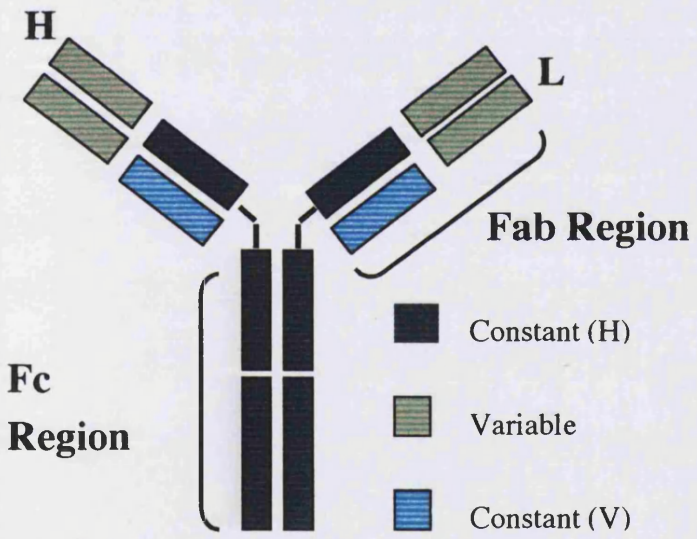


Figure 1.3 Schematic Representation of Antibody

Antibodies (Ab) or Immunoglobins are composed of four chains two heavy (H) and two light chains (L). Each chain is composed of variable (V) and constant (C) domains. The light chains contain one of each domain, whereas the heavy chains contain one light chain and three heavy chains. The variable chains determine the antigen specificity of the Ab, while the constant domains of the heavy chain determine the type of Ab it is ($\alpha,\varepsilon,\mu,\gamma$) and hence the receptor that will bind it Fc ($\alpha,\varepsilon,\mu,\gamma$).

There are different types of antibodies and correspondingly different types of Fc receptors, which bind the particular isotype. (Raghavan and Bjorkman 1996; Ravetch 1997; Heyman 2000) The neutrophil contains Fcγ receptors, which are specific for the IgG subfamily of immunoglobins. Neutrophils contain three types of Fcγ receptors, FcγRI, FcγRIIA and FcγRIIIB. (Figure 1.4) (McKenzie and Schreiber 1998) The FcγRI is a heavily glycosylated transmembrane protein. FcγRI, unlike the other two Fcγ receptors present in neutrophils, binds monomeric IgG with high affinity. This receptor is comprised of three immunoglobin like domains extracellularly, two of which share homology to the two extra-cellular domains of FcγRII and FcγRIII. The third extracellular domain of FcγRI is not homologous to the extracellular domains present in the other two receptors and may confer the ability of this receptor to bind monomers of IgG. (Unkeless, Scigliano *et al.* 1988) Though present during development (where it seems to have an important role), FcγRI is absent in mature neutrophils circulating in the blood stream. However, the receptor can be up regulated by exposure to certain cytokines such as γ-interferon and G-CSF. The FcγRI receptor contains two ITAMS in its intracellular domain, through which it transduces extracellular signals into the cell. (McKenzie and Schreiber 1998)

Immunoreceptor tyrosine activating motif (ITAM) is a motif depicted by a conserved sequence in which the tyrosine residue is phosphorylated upon activation. Upon phosphorylation the tyrosine residue provides a docking site for proteins containing SH2 domains.

The FcγRIIA is a transmembrane receptor present in a number of immune cells and expressed on the cell surface of neutrophils at approximately 1-2 x 10^4 receptors per cell. It binds monomeric IgG with very low affinity but has a high avidity for dimers, trimers and aggregates of IgG. It contains two extracellular immunoglobin domains and intracellularly it contains two ITAMS in a single intracellular chain. In the FcγRIIA receptor the ITAM motif is a slightly modified sequence consisting of Y*XX*L (where X denotes any amino acid repeat).

The FcγRIIIb receptor contains two extracellular chains but it does not cross the cellular membrane. Instead it is anchored to the membrane via a glycophosphoinositol chain and does not contain ITAMs. In human neutrophils there are 1-2 x10^5 per cell making it the most abundant Fcγ receptor in these cells. (Unkeless, Scigliano *et al.* 1988; Hulett and Hogarth 1994; Raghavan and Bjorkman 1996; McKenzie and Schreiber 1998)

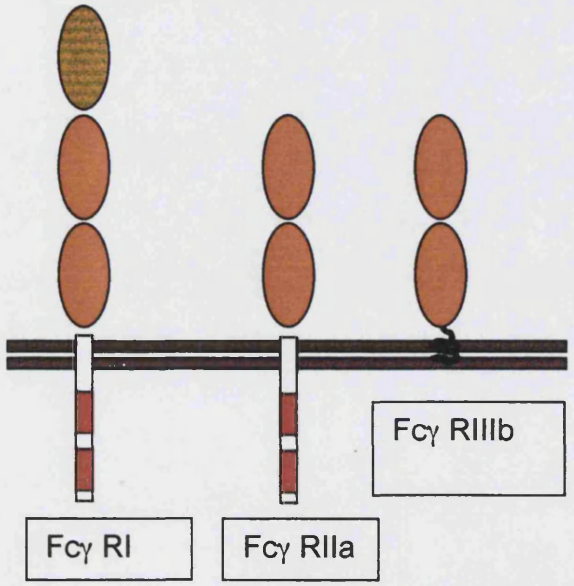


Figure 1.4 Schematic representation of Fcγ receptors in neutrophils

Three Fc receptors are present in human neutrophils. (see text). The IgG domains are orange except for the third domain in the Fcγ RI receptor which is green. Though the orange domains are homologous the green is not. ITAM regions are in red, and the glycophosphoinositol chain in the Fcγ RIIIb is black.

The generalised function of these receptors is the binding of opsonised particles. This triggers a series of intracellular cascades referred to as signal transduction, which will in turn activate the different mechanisms that depend on the phagocytotic stimulus. (Sanchez-Mejorada and Rosales 1998; Kwiatkowska and Sobota 1999) For example, it has been shown that transfection of the FcγIIa receptor will turn cells that are incapable of phagocytosing IgG-opsonised particles, into phagocytic cells. (Hunter, Kamoun *et al.* 1994)

1.6.2 Fcγ receptor signal transduction pathway

Activation of the Fcγ receptor requires dimerisation or oligomerisation; this was elucidated by studying a closely related receptor, the T-cell receptor. (Heldin 1995; Scott-Zaki, Purkall *et al.* 2000) This aggregation leads to phosphorylation of its ITAM, which initiates the signalling cascade. Fcγ receptors lacking ITAMs do not trigger cell activation. (Daeron 1997)

1.6.2a Tyrosine phosphorylation

Tyrosine phosphorylation plays a key role in initiation of Fcγ receptor signalling. As previously mentioned Fcγ receptor contain ITAMs on which tyrosine phosphorylation occurs upon receptor dimerisation. The specific process by which the ITAM on this receptor is phosphorylated is not entirely clear. One of the theories, is trans or cross phosphorylation. This theory implies that there is a constitutively associated kinase on the Fcγ receptor

intracytoplasmic tail whose activation is increased or becomes activated on dimerisation of the receptors. For example, stimulated Fcε receptors were shown to be able to phosphorylate the intracytoplasmic chain of another Fcε receptor after aggregation, hence it could transphosphorylate another Fc receptor once activated. (Pribluda, Pribluda *et al.* 1994) Another study showed that a member of the non-receptor tyrosine kinase Src family, Lyn, was associated with the cytoplasmic tail of the non-aggregated Fcγ receptor. (Ibarrola, Vossebeld *et al.* 1997)

Most studies implicate Syk, another non-receptor tyrosine kinase, as essential for Fcγ receptor signalling. Syk is strongly phosphorylated upon Fc receptor activation (Lofgren, Serrander *et al.* 1999) and in transgenic mice lacking the gene that encodes for Syk, activation of the NADPH oxidase, by IgG-opsonised particles, in neutrophils and macrophages was inhibited completely. (Kiefer, Brumell *et al.* 1998) Other studies using a pharmacological inhibitor of Syk, piceatannol, have shown a dose-related inhibition of phagocytosis. (Raeder, Mansfield *et al.* 1999)

Interestingly, enhancement of tyrosine phosphorylation by inhibition of tyrosine phosphatases is capable of activating the NADPH oxidase. (Bennett, Finan *et al.* 1995; Yamaguchi, Oishi *et al.* 1995) It has been shown that there is an increased and prolonged activation of superoxide production as a consequence of reactive oxygen accumulation, this is due to the fact that reactive oxygen is able to inactivate tyrosine phosphatases. (Brumell, Burkhardt *et al.* 1996; Pricop, Gokhale *et al.* 1999) These studies

show that inhibition of phosphatases is sufficient to activate the NADPH oxidase. Hence it could be postulated, that tyrosine kinases are constitutively active and loss of regulatory activity of phosphatases could be the method by which Fcγ receptor signalling is initiated.

Phosphorylated tyrosine residues form docking sites for Src homology 2 regions (SH2 domains). Many proteins contain these domains amongst them non-receptor tyrosine kinases. (Cohen, Ren *et al.* 1995) These kinases can in turn activate other proteins or form docking sites for other signal transduction proteins. Syk also functions as an anchor or link between the Fcγ receptor and other signalling proteins. (Sanchez-Mejorada and Rosales 1998) For example, it has been shown that there is a physical interaction between PI-3 kinase and Syk. (Ibarrola, Vossebeld *et al.* 1997)

1.6.2b Intracellular proteins

1.6.2b1.Ras signalling

Ras has been implicated in Fcγ receptor signalling by studies on the closely related receptor the FcγRI receptor. (Chu, Liu *et al.* 1998; Park, Erdreich-Epstein *et al.* 1999) In B-cells the membrane immunoglobin M (mIgM) receptor was shown to activate Shc/Grb/Sos complex, which activates the *ras/raf* cascade leading to MAP kinase activation. (Kumar, Wang *et al.* 1995) Activation of such pathways in neutrophils or related HL-60 cells is more confusing. Some reports suggest *ras* pathway activation by the

FcγRIIa receptor, this was concluded on the basis of phosphorylation of adaptor proteins, such as Shc, which are known to activate the *ras* pathway. (Rouard, Tamasdan *et al.* 1999) However other reports conflict in that the authors unequivocally state that MAP kinase activation (a downstream effector of the *ras/raf* pathway) certainly does not occur through *ras*. (Sanchez-Mejorada and Rosales 1998)

1.6.2b2. MAP kinases

On exposure of neutrophils to opsonised particles, in particular IgG-opsonised erythrocytes, MAP kinases were activated. (Suchard, Mansfield *et al.* 1997; McLeish, Klein *et al.* 1998; Raeder, Mansfield *et al.* 1999; Raeder, Mansfield *et al.* 1999; Hazan-Halevy, Seger *et al.* 2000) Some reports indicate that this activation is mediated by PKC. (Karimi and Lennartz 1998; Zhang, Garlichs *et al.* 1998) MAP kinase contributes to activation of the NADPH oxidase, specifically by phosphorylating $p47^{phox}$. (El Benna, Han *et al.* 1996)

1.6.2b3. Calcium signalling

Increases in intracellular calcium, due to influx from the extracellular milieu or release from intracellular stores are important in signal transduction. (Clapham 1995) In activation of the NADPH oxidase in neutrophils calcium has been implicated in translocation of the NADPH cytosolic components to the membrane. (Dusi, Della Bianca *et al.* 1993) Rises in intracellular

calcium occur on cross-linking of Fcγ receptors by soluble and insoluble immunocomplexes, and in such manner may aid in the activation of the NADPH oxidase. (Vossebeld, Kessler *et al.* 1995; Edwards, Watson *et al.* 1997; Watson, Gasmi *et al.* 1997; Lofgren, Serrander *et al.* 1999) Intracellularly increases in calcium level have also been shown to be important in modulation of phagocytosis by FcγII receptors. (Edberg, Lin *et al.* 1995) Calcium levels intracellularly may be mediated by several signalling intermediates. Phospholipase C, which is activated by Fcγ receptor in neutrophils, catalyses the formation of inositol triphosphate, which in turn stimulates release of calcium from intracellular stores, (see section 1.6.2b5) furthermore, calcium release from intracellular stores is sensitive to inhibition of PI-3 kinase. (Vossebeld, Homburg *et al.* 1997; Chuang, Sassaroli *et al.* 2000)

1.6.2b4. Phosphatidylinositol-3 kinase (PI-3 kinase)

PI-3 kinase was one of the first signalling proteins to be known in the activation of the NADPH oxidase. Baggiolini *et al* noticed that a fungal metabolite wortmannin had the capacity of inhibiting NADPH oxidase activity. (Baggiolini, Dewald *et al.* 1987) PI-3 kinase was also shown to be involved in regulation of phagocytosis, by allowing closure of the phagosome around the ingested particle. (Araki, Johnson *et al.* 1996; Crowley, Costello *et al.* 1997) PI-3 kinase is composed of a catalytic subunit p110 and a regulatory subunit p85, which may be phosphorylated

during activation of the kinase. The p85 regulatory sub-unit of PI-3 kinase has been shown to be tyrosine phosphorylated upon cross-linking of Fcγ receptors. (Ninomiya, Hazeki *et al.* 1994; Ibarrola, Vossebeld *et al.* 1997) In Syk deficient mice PI-3 kinase was not activated. (Crowley, Costello *et al.* 1997) In human neutrophils the link between Syk and PI-3 kinase was also seen by employing a Syk specific inhibitor, piacetannol, upon stimulation with IgG-opsonised particles there was an inhibition of PI-3 kinase as well as, ERK1 and ERK2 (members of the MAP kinase family) and PKCδ. (Raeder, Mansfield *et al.* 1999) This finding indicates that PI-3 kinase may form the link between Syk and the latter kinases. PI-3 kinase also regulates calcium signalling in neutrophils as mentioned above, and it has also been implicated in the regulation of phospholipase D (Bentz, Harvey *et al.* 1999) and AKT/protein kinase B (Tilton, Andjelkovic *et al.* 1997) upon Fc γ receptor stimulation. Therefore evidence exists for PI-3 kinase being an upstream regulator of many signalling proteins in Fcγ receptor signalling. In this manner PI-3 kinase may be a central molecule involved in diversion of Fcγ receptor signalling to different functions in neutrophils.

1.6.2b5. Phospholipases

Evidence in the literature points to activation of phospholipase C (PLC) and phospholipase D (PLD) upon Fcγ receptor activation. (Della Bianca, Grzeskowiak *et al.* 1993, Lennartz, 1999 #246)

Certain groups have shown that upon phosphorylation of the cytoplasmic tail of the Fcγ receptor, PLC-γ translocates and becomes activated. (Ting, Einspahr *et al.* 1991; Liao, Shin *et al.* 1992) This has been linked to SH2 domains within PLCγ binding directly to the receptor or binding via Syk. (Poole, Gibbins *et al.* 1997; Gross, Melford *et al.* 1999) Activation of PLCγ requires tyrosine phosphorylation and this has been shown to follow Fcγ receptor dimerisation. Furthermore pharmacological inhibition of protein tyrosine kinases, blocked Fcγ receptor phosphorylation of PLC and the subsequent release of inositol phosphates. (Ting, Karnitz *et al.* 1992; Dusi, Donini *et al.* 1994; Hunter, Kamoun *et al.* 1994) PLC hydrolyses phosphatidyl inositol 4,5-bisphosphate (PIP_2), releasing diacylglycerol (DAG) and inositol triphosphate (IP_3). Other studies have shown that PLCγ is activated indirectly by the receptor through other kinases, such as PI3 kinase, (Vossebeld, Homburg *et al.* 1997) this could explain the result mentioned previously, which showed that calcium release was inhibited by PI-3 kinase inhibition.

PLD is also activated by Fc receptor activation. It has been reported that PLD activation is greatly enhanced by previous priming (preparing for action) of neutrophils. (Watson and Edwards 1998) However, activation of PLD by Fcγ receptor activation without prior priming has also been observed. This activation was shown to be tyrosine kinase dependent. (Gewirtz and Simons 1997; Kusner, Hall *et al.* 1999)

1.6.2b6. Protein Kinase C (PKC)

It is known that PKCs are activated upon Fcγ dimerisation in neutrophils (for review see: (Cohen 1994; Daeron 1997; McKenzie and Schreiber 1998; Sanchez-Mejorada and Rosales 1998; Kwiatkowska and Sobota 1999), most likely mediated via DAG. DAG is a main activator of classical and novel PKC. (Rink, Sanchez *et al.* 1983) The initial mechanism of DAG production was through agonist induced degradation of inositol lipids. One of the lipases involved in this process is phospholipase C (PLC). (See section 1.6.2b5) Both products of PLC activate PKC. DAG activates PKC directly by binding to the C1 domain and IP_3 does this indirectly by causing the release of calcium from intracellular calcium stores, which then binds to the C2 domain of cPKCs. (Berridge 1984; Nishizuka 1984) (See section 1.7) Furthermore, other sources of phospholipids can also be degraded to produce DAG, for example phosphatidyl choline via phospholipase D (PLD) and phosphatidic acid hydrolase, (Liscovitch 1996) in the absence of intracellular calcium release. Phorbol esters, DAG analogues, are used frequently to activate the NADPH oxidase. Phorbol ester is a potent

PLD cleaves phospholipids at the terminal phosphodiester bond. Upon PLD cleavage of phosophotidyl choline phosphatidic acid and choline are released, phosphatidic acid can be further modified by phosphatidic acid hydrolase, which removes the phosphate releasing diacylglycerol. This is another source of DAG involved in activation of PKC.

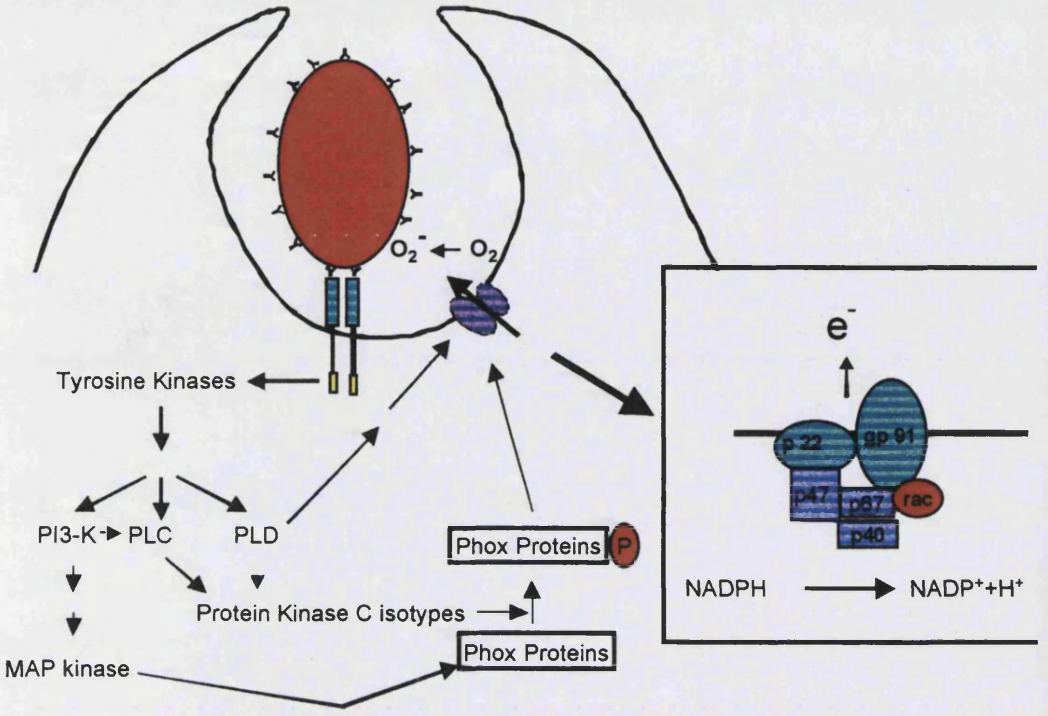


Figure 1.5 Summary of Fcγ receptor signalling upon activation of the NADPH oxidase

Schematic review of section 1.6, leading to phosphorylation of the NADPH oxidase components (see section 1.8)

1.7 PKC Superfamily of kinases

The PKC family consists of a large number of polypeptides with sequence homology in their catalytic domain.

All members of the PKC family contain a C-terminal catalytic domain and an N-terminal regulatory domain. There are three subfamilies based on divergence in the regulatory domain. Classical PKC were the first to be discovered. This subfamily contains the PKCα, PKC β and PKC γ, which were identified through low stringency screens of brain cDNA libraries.

(Coussens, Parker *et al.* 1986; Parker, Coussens *et al.* 1986) More stringent screening lead to the discovery of three further isoforms, namely PKCδ, PKCε and PKCζ. (Ono, Fujii *et al.* 1987; Ono, Fujii *et al.* 1989) Screening cDNA libraries from other tissues yielded other mammalian isoforms known to date as: PKCη, (Dekker, Parker *et al.* 1992; Osada, Mizuno *et al.* 1992), PKCι, (Selbie, Schmitz-Peiffer *et al.* 1993) PKCλ (Akimoto, Mizuno *et al.* 1994) and PKCμ/PKD (Johannes, Prestle *et al.* 1994; Valverde, Sinnett-Smith *et al.* 1994). The final PKC isoform discovered the PKCν this was done by performing a homology search on expressed sequence tag (EST) database. (Hayashi, Seki *et al.* 1999) The classical PKCs, named above form one subfamily, the other subfamilies are the novel PKCs (PKC δ, PKC ε, PKC η, and PKC θ) and the atypical PKCs (PKC ι, PKC ζ, and PKC λ).

One member of the PKC family, PKCμ, has a catalytic domain, which is divergent from the other PKCs. For this reason some investigators consider it a different type of kinase, PKD. (PKD will be discussed in section 1.7.1)

In the regulatory domain, classical PKCs contain 2 modules, C1 and C2. Functionally the C1 domain has been recognised as region where diacylglycerol and phorbol esters bind. The C1 domain has a characteristic tandem repeat of zinc finger like cysteine rich motifs that confer this binding. (Oancea, Teruel *et al.* 1998) The C2 domain on the other hand binds calcium. Due to the presence of these two domains, classical PKCs are

known to be activated by a combination of calcium and phorbol esters. (Rink, Sanchez *et al.* 1983) Novel PKCs also contain a C1 domain and are responsive to phorbol esters. However their C2 domain differs from the C2 domain of classical PKCs, as it does not bind calcium and hence it is called C2-like domain. The function of the C2-like domain is not entirely clear, however it may be involved in protein-protein interactions as shown by Dekker *et al* in nervous tissue. (Dekker and Parker 1997) The atypical group of PKCs do not contain a C1 domain and are unresponsive to phorbol ester activation, their C2 domain also does not bind calcium and hence their mode of activation is different from the other two sub families. (Dekker and Parker 1994)

1.7.1 PKCμ/PKD

PKCμ/PKD and PKCν are placed in a separate subfamily of the PKC superfamily or a different kinase family. Not much is known about PKCν, as it was only recently cloned. (Hayashi, Seki *et al.* 1999) PKCμ/PKD was initially cloned using PCR library screening technology. Different sets of oligonucleotides primers were employed for this identification. Johannes *et al* identified PKCμ by screening a library using primers targeted towards identification of ser/thr kinases. (Johannes, Prestle *et al.* 1994) Whilst, Valverde *et al* employed primers which were targeted towards the identification of tyrosine kinases. (Valverde, Sinnett-Smith *et al.* 1994) Though both groups identified the same protein they named it differently

PKCμ and PKD respectively. PKCμ will be referred to as PKD from now on. Structurally PKD contains a catalytic domain whose sequence shows closest homology to myosin light chain kinase (MLCK), thereby excluding it from the PKC family. On its N-terminus it contains a cysteine rich sequence which is homologous to the C1 domain in phorbol ester sensitive PKCs, although similar the sequence separating the cysteine rich domains are much longer (95 residues) than in PKCs (between 28 and 36 residues). (Valverde, Sinnett-Smith *et al.* 1994) In addition to a C1 domain PKD contains a putative transmembrane domain and a pleckstrin homology domain (PH domain). (Johannes, Prestle *et al.* 1994) PKD was shown to be activated by phorbol esters, compatible with the presence of a C1 PKC-like domain, however it is insensitive to calcium. (Johannes, Prestle *et al.* 1995; Van Lint, Sinnett-Smith *et al.* 1995; Dieterich, Herget *et al.* 1996)

The significance of the PH domain on this kinase is of interest. It is known that PH domains bind lipids, such as polyphosphoinositides, which are important second messengers in signal transduction. (Lemmon, Ferguson *et al.* 1995; Klarlund, Guilherme *et al.* 1997) These could therefore act as PKD activators, in a deletion study in which the PH domain of PKD was mutated, higher kinase activity of this enzyme was observed, leading to the conclusion that the PH domain may have a negative regulatory role. (Iglesias and Rozengurt 1998)

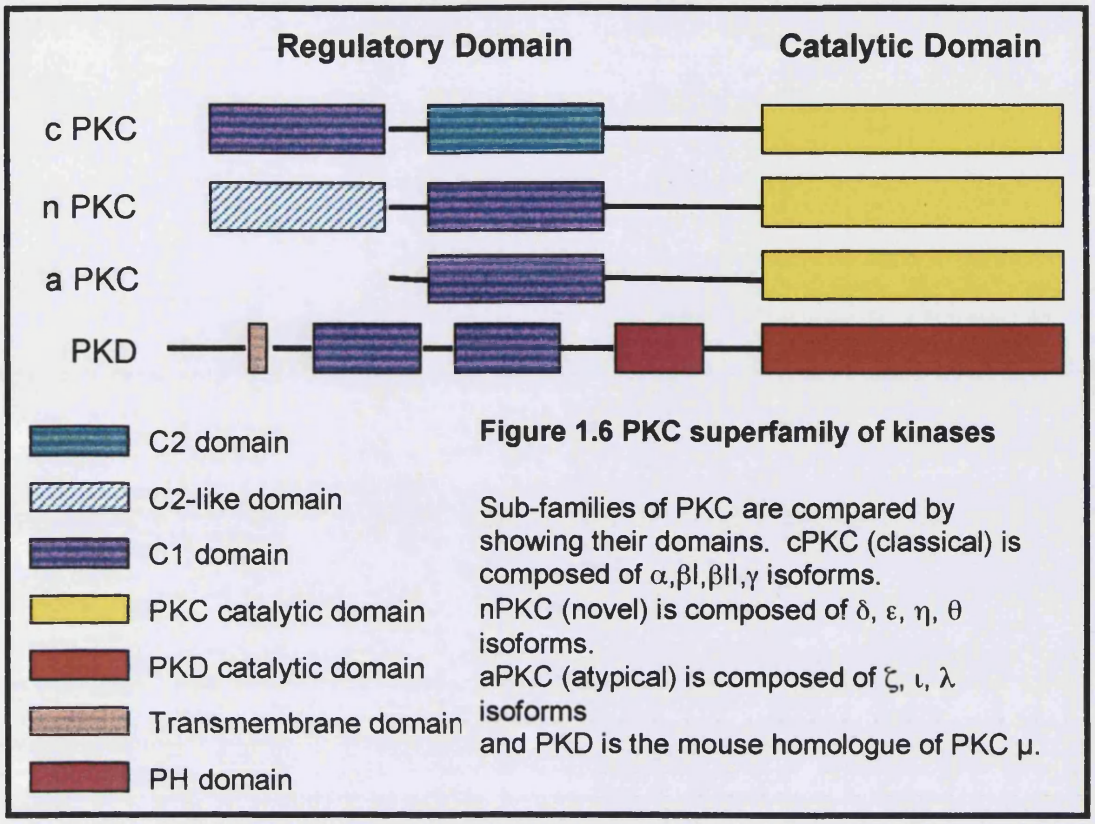


Figure 1.6 PKC superfamily of kinases

Sub-families of PKC are compared by showing their domains. cPKC (classical) is composed of α,βI,βII,γ isoforms. nPKC (novel) is composed of δ, ε, η, θ isoforms. aPKC (atypical) is composed of ζ, ι, λ isoforms and PKD is the mouse homologue of PKC μ.

Activation of PKD is confusing. As previously mentioned phosphoinositides may activate PKD however, other mediators have also been implicated. PKC was reported to activate PKD. It was shown that if PKC was inhibited in Swiss 3T3 cells so would activation of PKD. (Zugaza, Sinnett-Smith *et al.* 1996) Other experiments showed that PDGF receptor signalling leads to activation of PKD. Using a PDGF receptor mutant which could not bind phospholipase C, PKC activity and also PKD activity was low. Furthermore in this model system PKD could not be activated by addition of a DAG analogue in the presence of PKC inhibitor Bim I. (Van Lint, Ni *et al.* 1998)

In Swiss 3T3 cells it was shown that PKD was activated by lysophosphatidic acid, this activation was PKC as well as pertussis toxin sensitive. (Paolucci, Sinnett-Smith *et al.* 2000)

PKD has been shown to be expressed in different murine tissues, with thymus, lung and peripheral blood mononuclear cells expressing the highest levels. (Rennecke, Johannes *et al.* 1996) In B cells activation of the B cell receptor (BCR), a member of the immunoglobin super family, recruits Syk and PLC as well as a previously unidentified ser/thr kinase. This kinase was later identified as PKD (Sidorenko, Law *et al.* 1996; Johannes, Hausser *et al.* 1999) and its activation was dependent on Syk activation. Studies in B cells show translocation of PKD from the cytosol to the membrane upon activation. Once activated on the membrane PKD then translocates back to the cytosol where it remains active. Thus a possible role for PKD in B cells is to act as a physical transducer of B cell receptor signalling. (Matthews, Iglesias *et al.* 2000)

The spatial localisation of PKD may be important for its function in different tissues. PKD has been shown to be an integral part of the Golgi complex, where it may play a role in vesicular transport. (Prestle, Pfizenmaier *et al.* 1996) PKD colocalised with the βγ subunit of heterotimeric-G proteins in the Golgi complex. Heterotrimeric G-proteins are known to be involved in vesicular transport in the Golgi complex. (Pimplikar and Simons 1993) Gβγ subunit was found to activate PKD by binding to its PH domain, further substantiating the findings by Iglesias *et al*,

that the PH domain if not modified has a negative regulatory role on PKD activity. Other studies tried to further identify the role of PKD in vesicular transport. It was found in yeast that yeast homologues of lipid kinases such as phophatidylinositol-4-kinase (PtdIns 4-K) and phophatidylinositol-4-phosphate 5-kinase (PtdIns 4-P 5-K) are important in the regulating vesicular transport. (Stack, Horazdovsky *et al.* 1995; Hama, Schnieders *et al.* 1999) These lipid kinases were shown to co-immunoprecipitate with a ser/thr kinase. (Stack, Herman *et al.* 1993) This was investigated in a human cell line and PKD was found to co-immunoprecipitate with PtdIns 4-K and PtdIns 4-P 5-K, which were shown to be active and able to phosphorylate the substrates PIP and PIP2. (Nishikawa, Toker *et al.* 1998)

In T cells it has been found that 14-3-3 signalling proteins function as a negative feedback allosteric regulator of PKD activity. 14-3-3 proteins preferentially bind to phosphorylated serine residues present between the two cysteine clusters within the C1 domain and thereby quench PKD activity once activated. (Hausser, Storz *et al.* 1999)

1.7.2 PKC isoforms in neutrophils

Though the family of PKC is large, not all isoforms are expressed in every cell type. This suggests a certain functional specificity for these kinases. (Nishizuka 1988; Dekker and Parker 1994) The isoforms present in neutrophils are members of three subfamilies of PKC. From the classical family α and β isoforms were detected and from the novel and atypical

families δ and small quantities of ζ isoforms, respectively. (Kent, Sergeant *et al.* 1996; Sergeant and McPhail 1997) Studies from our own laboratory confirmed the presence of β and δ isoforms, ζ may be present but the data were not conclusive, however the α isoform could not be detected by any of the antibodies used. (Dekker personal communication)

1.8 Phosphorylation of the NADPH oxidase

Phosphorylation is an important means of cell regulation as can be seen by large number of protein kinase genes emerging from the genome project (estimated at approximately 2000). (Hunter 1995) Protein phosphorylation was at first thought to be a way to deliver phosphate to our body through our diet. However it is now recognised that this reversible phosphorylation is a dynamic process involved in the regulation of cellular processes. (Krebs and Beavo 1979; Cohen 1982; Krebs 1994) Activation of the NADPH oxidase involves many phosphorylation events. (Schneider, Zanetti *et al.* 1981; Hayakawa, Suzuki *et al.* 1986) The cytosolic components $p67^{phox}$ (Dusi and Rossi 1993), $p40^{phox}$ (Fuchs, Bouin *et al.* 1997) and $p47^{phox}$ (Segal, Heyworth *et al.* 1985) and both subunits of the cytochrome b_{558} (Garcia and Segal 1988) are all phosphorylated upon activation of the NADPH oxidase. Though different kinases have been identified as the possible phosphorylating agent in this process for the different subunits of

the oxidase, most evidence suggests PKC as the major player in this process. (Gennaro, Florio *et al.* 1986)

PKCs appear to activate the NADPH oxidase by direct phosphorylation of its cytosolic components. This has been shown, by using pharmacological or genetic mutation manipulations, in order to inhibit PKC function, and its activation by phorbol esters. Inhibitors such as staurosporin and H-7 have been widely used and these showed decrease in the activity of the NADPH oxidase, and phosphorylation of its sub-units. (Ohtsuka, Okamura *et al.* 1986; Sakata, Ida *et al.* 1987; Nauseef, Volpp *et al.* 1991; Combadiere, el Benna *et al.* 1993) However, they do not differentiate between isoforms. More recently PKC isoform specific inhibitors have been created, (Gordge and Ryves 1994; Goekjian and Jirousek 1999; Way, Chou *et al.* 2000) which may help in assigning different functions for different isoforms.

Genetic manipulations have also been used; using these methods specific isoforms can be deleted and its involvement in phosphorylation and activation of the oxidase can be shown more specifically. Dekker *et al* have shown in PKCβ transgenic mice that oxidase activation is reduced by approximately 50% upon Fcγ receptor stimulation. This coincided with inhibition by a specific β inhibitor Ly379196 in human neutrophils. (Dekker, Leitges *et al.* 2000) In other studies PKC β was depleted by directly inhibiting its synthesis (translation of protein). Promyelocytic cells (HL60) cells were incubated with PKCβ antisense. This treatment inhibited oxidase

activation induced by several stimuli including phorbol ester, fMLP and immune complexes. This was a consequence of reduction of $p47^{phox}$ phosphorylation and translocation and not depletion in the amount of NADPH oxidase components. (Korchak, Rossi *et al.* 1998) Both these studies highlight the importance and mechanism of this isoform on NADPH oxidase function, however other kinases are also involved.

1.8.1 $p47^{phox}$

Extensive studies on phosphorylation of $p47^{phox}$ have been done. It has been shown that $p47^{phox}$ is mostly phosphorylated on its C-terminus. (el Benna, Faust *et al.* 1994) At least seven phosphorylation sites have been identified in a region encompassing residues 303-379. Six of these residues appear in consensus sequence sites, which would favour PKC phosphorylation. Protein kinase A (PKA) consensus sites are also present and two other serines appear in canonical MAP kinase consensus sites. (el Benna, Faust *et al.* 1994) Phosphorylation of $p47^{phox}$ using the above mentioned kinases showed that while PKC could phosphorylate all the phosphosites except for the MAP kinase phosphosites *in vitro*. MAP kinase and PKA were restricted to distinctive sites. (El Benna, Faust *et al.* 1996)

Various phosphorylation sites on $p47^{phox}$ by different kinases would correlate with the great degree of regulation needed in activation of the NADPH oxidase. PKA phosphorylation does not activate the NADPH oxidase, but may negatively regulate oxidase activity. (Kramer, van der

Bend *et al.* 1988; Bengis-Garber and Gruener 1996) Growing evidence show the activation of MAP kinase in Fcγ receptor signal transduction in neutrophils (Liang and Huang 1995; Suchard, Mansfield *et al.* 1997; Vossebeld, Homburg *et al.* 1997; Coxon, Rane *et al.* 2000; Hazan-Halevy, Seger *et al.* 2000). MAP kinase, as mentioned previously, phosphorylates $p47^{phox}$, (El Benna, Han *et al.* 1996) this occurs at residues ser 345/348, however these phosphorylations were shown not to be essential for superoxide production. (Yu, Suchard *et al.* 1995) It would then appear that the MAP kinase, like PKA phosphorylation, play an indirect regulatory role on the function of $p47^{phox}$ and consequently activation of the NADPH oxidase

Upon activation of the NADPH oxidase, $p47^{phox}$ translocates to the membrane, concurrent with translocation $p47^{phox}$ is phosphorylated. (Nauseef 1993) It has been shown that phosphorylation leads to a conformational change in the structure of $p47^{phox}$ (Park and Park 1998; Park, Kim *et al.* 1999) and that the conformational change is the essential step for activation as it exposes certain domains in $p47^{phox}$ important in protein-protein binding.

The interplay between the different phosphorylation sites on $p47^{phox}$ have been studied by in site mutagenesis to try to elucidate their individual importance as well as the contribution by different kinases to overall NADPH oxidase activation. Mutations on ser 359 and ser 370 inhibited all other phosphorylations on $p47^{phox}$, signifying that these phosphorylations

precede and facilitate all others, possibly by exposing other relevant sites and making them accessible to kinases. (Johnson, Park *et al.* 1998) Translocation of $p47^{phox}$ to the membrane fraction appears to be driven by phosphorylation on ser 379. A mutated form of $p47^{phox}$ in which the serine 379 was replaced by an alanine failed to translocate to the membrane. (Faust, el Benna *et al.* 1995) However, mutations made on ser 303 and ser 304 to alanines could translocate to the membrane upon activation but were unable to sustain oxidase activity. (Inanami, Johnson *et al.* 1998) A different study showed that phosphorylation on residues ser 304 and ser 305 helped to unmask a protein binding domain Src homology 3 region (SH3 domain). SH3 domains bind poly-proline regions on other proteins. $P22^{phox}$ contains a poly-proline region, and unmasking the SH3 domain in $p47^{phox}$ would help in the interaction between $p47^{phox}$ and $p22^{phox}$, which is suggested as the rate limited step in oxidase activity. (Sumimoto, Hata *et al.* 1996; Ago, Nunoi *et al.* 1999; Huang and Kleinberg 1999) While other kinases such as p 21-activated kinase (PAK) have also been shown to phosphorylate $p47^{phox}$ the relevance of this phosphorylation is not understood. (Knaus, Morris *et al.* 1995)

The interactions of kinases and different phosphorylation sites closely regulate activity of certain proteins, in this case $p47^{phox}$. Though not all the functions of phosphorylation on $p47^{phox}$ have been elucidated it provides an excellent example of the effect of protein phosphorylation on protein function.

1.8.2 p67^{phox}

p67^{phox}, another of the cytosolic components of the oxidase is also essential for activation of the oxidase. This was noticed in patients with autosomal recessive CGD, who are both p47^{phox} and cytochrome b_{558} positive. (Nunoi, Rotrosen *et al.* 1988) Though the prevalence of this form of CGD is quite low (5% of all cases) it highlights the importance of p67^{phox} in NADPH oxidase activation. (Clark, Malech *et al.* 1989; Casimir, Chetty *et al.* 1992) It has been shown that p67^{phox} phosphorylation and translocation, like in the case of p47^{phox}, correlate with NADPH oxidase activation. (Dusi and Rossi 1993) It was shown that p67^{phox} was phosphorylated on the same peptide after PMA and fMLP stimulation. However it was also noted that a PKC inhibitor, Bim I, inhibited PMA but not fMLP phosphorylation of p67^{phox}. (Benna, Dang *et al.* 1997)

Forbes *et al.* shows that p67^{phox} is phosphorylated on a threonine residue at position 233. This was shown by site mutagenesis, in which substitution of alanine for threonine resulted in loss of phosphorylation. *In vivo* phosphorylation of Thr^{233} stimulated with serum-opsonised zymosan coincided with *in vitro* phosphorylation by MAP kinase. (Forbes, Truong *et al.* 1999) MAP kinase has been shown to be activated by Fc receptor stimulation and several studies have indicated that it is downstream of tyrosine kinases and PI-3 Kinase. (Section 1.6.2b and (Coxon, Rane *et al.*

2000) Other studies have shown that PAK phosphorylates $p67^{phox}$, allowing *rac* 1 or 2 to bind. (Ahmed, Prigmore *et al.* 1998)

1.8.3 $p40^{phox}$

The exact role of the third cytosolic component of the oxidase is unclear, since nature has not provided with a mutation on this gene to see the relevance of its absence on oxidase activation. However $p40^{phox}$ is phosphorylated and translocates to the plasma membrane in order for the NADPH oxidase to function. (Dusi, Donini *et al.* 1996; Someya, Nagaoka *et al.* 1996; Someya, Nunoi *et al.* 1999) It was also observed that a protein kinase C inhibitor, H-7 inhibited phosphorylation and translocation of p40phox. (Someya, Nunoi *et al.* 1999) Fuchs *et al* suggest that a different kinase, not involved in phosphorylation of $p47^{phox}$ is responsible for $p40^{phox}$ phosphorylation upon fMLP stimulation of HL-60 cells. ~~(Fuchs, Bouin *et al.*~~ 1997) Further studies found a total of 6 phosphosites. Two major phosphosites *in vivo* were identified as serine 315 and threonine 154. Some of the phosphorylated sites on $p40^{phox}$ were sensitive to a potent PKC inhibitor, Bim I. However, phosphorylation on other sites was not inhibited by Bim I, hence pointing towards the involvement of other kinases in this process. (Bouin, Grandvaux *et al.* 1998) Again, though the importance of phosphorylation in this protein is apparent by its need on activation of the NADPH oxidase, the exact role is not fully understood.

1.8.4 Cytochrome b $_{558}$

It was noticed in the late 1980's that upon PMA stimulation of oxidase activation both subunits of the oxidase were phosphorylated. (Garcia and Segal 1988) Since then, no identification of the kinase or site involved in phosphorylation of gp91^{phox} has taken place. However, a small GTP protein RAP 1A has been identified, as a negative regulator of oxidase activity by binding gp91^{phox}. This protein was found to be phosphorylated by PKA and upon its phosphorylation it disassociates from gp91^{phox} allowing it to be activated. (Bokoch, Quilliam *et al.* 1991)

The second component of the cytochrome, p22^{phox} has been shown to be phosphorylated on a threonine residue. The authors do not specify on which amino acid residue this phosphorylation takes place however they postulate that it is probably the threonine at position 147 due to the fact that this residue falls in a consensus homology for PKC phosphorylation. Both PKC (*in vitro*) and a novel phosphatidic acid activated protein kinase (cell-free assay) phosphorylated the same peptide. (Regier, Waite *et al.* 1999; Regier, Greene *et al.* 2000) *In vitro* it was shown that cPKCs phosphorylated p22^{phox}, but not novel or atypical PKC. However in a cell-free system DAG failed to activate p22^{phox}. A novel phosphatidic acid activated protein kinase was able to phosphorylate p22^{phox} in a cell free system, hinting that this is more likely to be the kinase involved in this process as the cell free system more closely resembles *in vivo* conditions.

(Regier, Waite *et al.* 1999; Regier, Greene *et al.* 2000) This finding shows the possible consequence of PLD activation upon Fc receptor stimulation.

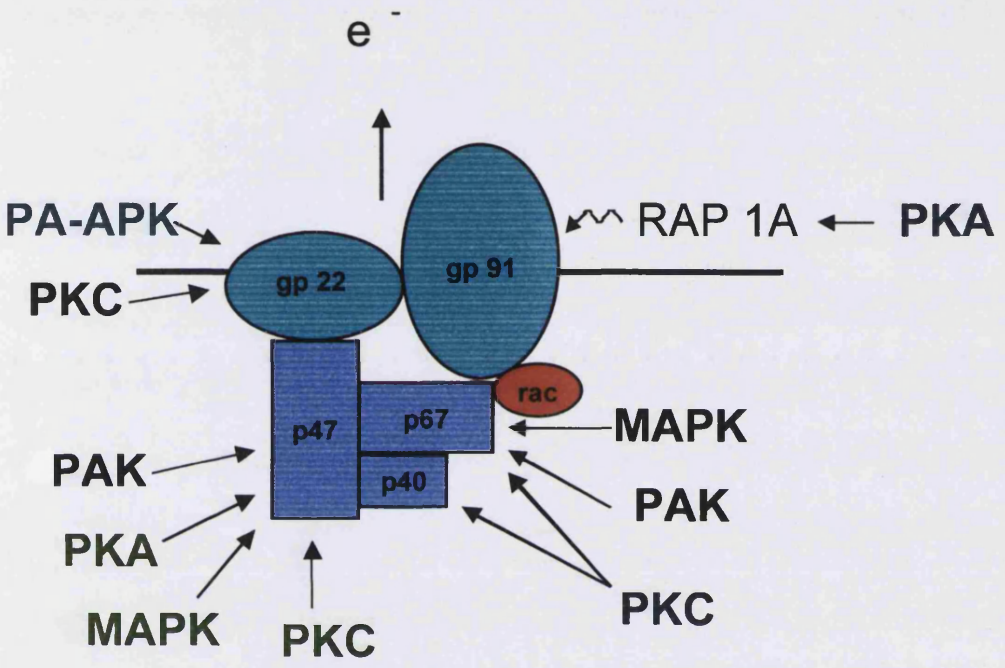


Figure 1.7 Summary of phosphorylation events on NADPH oxidase components

Kinases in dark blue have been shown to phosphorylate the appropriate NADPH substrate by *in vitro* kinase assay,in dark green are kinases that have been shown to phosphorylated the appropriate substrate both *in vivo* and *in vitro*. In dark grey PA-APK has been shown to phosphorylate $p22^{phox}$ in a cell-free reconstitution assay. (See text)

1.9 Aims of this thesis

The above summary (Figure 1.7) has tried to show some of the latest discoveries in elucidation of the activation of the NADPH oxidase, and its link to phosphorylation events. Many different extracellular signals activate this enzyme and different kinases are involved. The NADPH oxidase components themselves are known to be substrates for several protein kinases. Therefore, phosphorylation-dependent activation of the oxidase is a complex process involving a high level of regulation. Superoxide damage occurs in many diseases, such as rheumatoid arthritis and asthma while, on the other hand, its production is also of extreme importance in the clearance of bacterial infection by neutrophils. This is highlighted in the inherited disease CGD. It can therefore be inferred that superoxide production is a double-edged sword and needs to be kept under tight regulation-a role for phosphorylation.

Fcγ receptors bind IgG-opsonised bacteria and help activate the killing process by human neutrophils. The NADPH oxidase is crucial to this function. Many extracellular signals are transmitted into the cell by a series of enzymatic reactions that are collectively referred to as signal transduction. The large number of kinases and phosphatases involved in this process highlights the importance of phosphorylation in these reactions. One of the final steps in activation of the NADPH oxidase is the phosphorylation of its components on several sites by different kinases.

The protein kinase C family of serine/threonine kinases plays a major role on the activation of the oxidase, as seen by incubating neutrophils with PMA.

This project aimed to examine protein phosphorylation in neutrophils; to improve our understanding of the interrelationship of phosphorylation reactions that impinge on the NADPH oxidase. We set out to examine phosphorylation patterns under pharmacological manipulation of the NADPH oxidase. By employing these pharmacological agents, kinase activity not previously described in neutrophils was found to play a key role in activation of the NADPH oxidase, after Fcγ receptor stimulation. This kinase has been identified as PKD, a member of the protein kinase C family. We have determined its spatial location in the neutrophil in response to receptor activation and its substrates in oxidase activation.

Furthermore using proteomics technology we have found other cellular substrates for this kinase. Preliminary data shows that this kinase is a regulator of glucose metabolism, which is interesting, as the original studies performed on neutrophils and the discovery of the NADPH oxidase was largely based on studies of glucose metabolism. (See section 1.4.2)

Chapter 2

Materials and Methods

2.1 Materials

2.1.1 General Reagents

General reagents were purchased from Sigma, BDH and were of the highest grade available. More specific reagents used in this project are listed below:

Bufficoats	North London Blood transfusion service
α-cyano-4hydroxycinamic acid	Sigma
Duracryl (30% (w/v) acrylamide/ 0.8%(w/v) bisacrylamide)	Genomic Solutions
n-ethyl maleimide	Sigma
Ficoll Hypaque	Nycomed
Human IgG	Instituto Grifol, Spain

Immobiline™ Dry Strips (IEF)	Pharmacia
Lysostaphin	Sigma
Mowiol 4-88	Calbiochem
Pharmalyte 3-10	Amersham/Pharmacia
PKD antigenic peptide	Calbiochem
PKD recombinant enzyme	Calbiochem
RPMI 1640	Gibco BRL
Syntide	Calbiochem
TPCK (n-Tosyl-L-phenylalanine chloromethyl ketone) treated trypsin	Sigma
Trypsin (porcine)	Promega

2.1.2 Radioactive isotopes

D-[6-^{3}H]-glucose	Amersham
[γ-^{32}P]-ATP	Amersham
[^{32}P] orthophosphate	Amersham

2.1.3 Antibodies

PKC βI	SC 209	Santa Cruz
PKC βII	SC 210	Santa Cruz
PKC δ	SC 937	Santa Cruz
PKCμ/PKD	SC 935, SC 936	Santa Cruz
	34-290	Calbiochem

2.1.4 Protein kinase Inhibitors

Bisindolylmaleimide I	Calbiochem
Calphostin C	Sigma

Genistein	Sigma
Gö 6976	Calbiochem
Ly 294002	Eli Lilly
Ly 379196	Eli Lilly
Ro 31-8220	Calbiochem
Rottlerin	Calbiochem
Wortmannin	Sigma

2.2 Buffers

2.2.1 Electrophoresis buffer pH 1.9

2.5% (v/v) formic acid

7.8% (v/v) acetic acid

2.2.2 Equilibration buffer

6M Urea

30% (w/w) Glycerol

2% (w/v) SDS

0.05 M Tris-HCl pH 8.8

2.2.3 Isoelectric focusing (IEF) buffer

7M Urea

2M thio Urea

4% (w/v) CHAPS

1% (v/v) Triton X-100

0.8% (v/v) Pharmalyte 3-10

1% (w/v) dithiothreitol (DTT)

5mM Pefabloc

trace of bromophenol blue (BPB)

2.2.4 Kinase buffer

30mM Tris-HCl, pH 7.4

10mM $MgCl_2$

2.2.5 Laemmli sample buffer

62.5mM Tris-HCl, pH 6.8

2% (w/v) sodium dodecyl sulphate (SDS)

2.5% (v/v) glycerol

2.5mM DTT

trace of BPB

2.2.6 Lamberts break buffer

10mM PIPES, pH 7.1

100mM KCl

3mM NaCl

3.5mM $MgCl_2$

10mM Benzamidine

50mM NaF

10mM β-Glycerol Phosphate

2.2.7 Mowiol Mount

50mM Tris-HCl, pH 8.5

12% (v/v) glycerol

4.8% (w/v) Mowiol 4-88

Prior to use 2.5% (w/v) 1,4-diazabicyclo-[2.2.2] octane (DABCO) was added

2.2.8 Phosphatase inhibitors

100nM Mycrocystin

1μM Okadaic acid

200μM NaVanadate

2.2.9 Phosphate buffered saline (PBS)

10mM NaH_2PO_4

140mM NaCl

10mM KCl, pH 7.4

2.2.10 Protease Inhibitors

100μM Leupeptin

1μM Pepstatin A

100μM N-α-p-tosyl-L-lysine chloro methyl ketone (TLCK)

2μM Aprotinin

Complete™ protease inhibitors Pill (as per manufacturers recommendation)

0.2mM phenylmethylsulphonyl fluoride (PMSF)

1mM di-isopropyl fluorophosphate (DFP)

2.2.11 Thin layer chromatography buffer

7.5% (v/v) acetic acid

25% (v/v) pyridine

37.5% (v/v) n-butanol

2.2.12 Tris-buffered saline (TBS)

200mM NaCl

50mM Tris-HCl, pH 8.5

2.2.13 Triton lysis buffer

50 mM Tris-HCl, pH 7.4
1% Triton X-100

2.3 Methods

2.3.1 Preparation of neutrophils

Neutrophils were prepared from Bufficoats (North London Blood Transfusion Service), by Ficoll sedimentation and hypotonic lysis. Blood was diluted by adding 30% volume of normal saline (0.9% w/v NaCl), 5 units/ml of heparin and 1% dextran, which allows erythrocyte sedimentation. The upper layer containing the leukocyte enriched fraction was recovered and centrifuged through a cushion of 10% volume Ficoll Hypaque (Nycomed) at 2000 rpm (500 x g) for 10 minutes in a benchtop Mistral 3000i (MSE). The pellet was resuspended in H_2O for hypotonic lysis of the residual erythrocytes and equilibrated to normal saline by adding an equal volume of 2x normal saline (1.8% NaCl w/v). Subsequent centrifugation at 1500 rpm (300 x g) for 5 minutes at room temperature yielded a granulocyte pellet of >95% pure neutrophils. (Segal, Dorling *et al.* 1980) The cells were resuspended in RPMI 1640 buffer with or without phosphate depending on procedure undertaken thereafter. The neutrophils were counted using a microscope haemocytometer and diluted to the appropriate concentration.

2.3.2 Opsonisation of S. aureus with human IgG

An overnight culture of *Staphylococcus Aureus* in EZMix ™ LB Broth (Sigma) was incubated at 62°C for 15 minutes (in order to kill the bacteria) and centrifuged at 1000g for 25 minutes at room temperature. The resulting pellet was resuspended in 6ml 50mM Tris-HCl containing 50mg/ml (final concentration) of soluble human IgG and incubated at 37°C for a minimum of two hours up to overnight incubation. Bacteria were collected by centrifugation and washed twice in PBS and resuspended in RPMI 1640 buffer at a concentration of 3×10^{10} cells/ml. The bacterial concentration was determined by optical density (OD) measurements. At 600nm, 10^8 *S. aureus* have an OD of 0.17, which was taken as a standard against which the concentration of bacterial culture was calculated.

To prepare [^{3}H]-labelled *S. aureus* cells were grown in 10ml LB Broth as above in the presence of 25μCi/ml of D-[6-^{3}H]-glucose (Amersham, UK) as described by Segal AW *et al.* (Segal, Geisow *et al.* 1981) Opsonisation and bacterial count was carried out as for non-labelled *S. aureus*. Bacteria were resuspended in RPMI 1640 at a final concentration of 10^{10} cells /ml.

2.3.3 NADPH oxidase assay

NADPH oxidase activity was measured using a Clark type electrode (Rank Brothers, Cambridge, UK) which can measure oxygen levels in solution. As the active enzyme catalyses the reaction:

$$NADPH + O_2 \longrightarrow NADPH^+ + 2O_2^- + H^+$$

utilising the oxygen electrode would determine the amount and rate of oxygen consumption as an indication of superoxide production. (Segal and Coade 1978) The electrode was calibrated with sodium dithionite taking the oxygen concentration of water at 37°C as 230nmol/ml (Merck Index). The electrode polarising voltage was set to 0.6 volts, since plotting electrode output at given oxygen concentration against a polarising voltage gives a plateau between 0.4 and 0.8 volts. Therefore a polarising voltage within the plateau region gives a linear proportion between current output and oxygen tension in the cell suspension.

Typically one ml of neutrophils (5x 10^7 cells) were incubated for approximately 2 minutes at 37°C in the electrode chamber until oxygen consumption has stabilised (flat reading on a Lloyds 2000 dual pen chart recorder). The samples were then stimulated with either phorbol 12-myristate, 13-acetate (PMA; 0.1µg/ml final concentration or as stated in figure legends) or IgG-opsonised *S. aureus* (at concentrations stated in figure legends).

To test the effect of inhibitors, cells were pre-incubated for 15 minutes at room temperature in RPMI 1640 containing inhibitors at concentrations stated in text and figure legends, before being place in the electrode chamber.

2.3.4 Phagocytosis assay

Phagocytosis was measured as described previously. (Segal, Geisow *et al.* 1981) Isolated neutrophils (108 cells) were placed in RPMI 1640 in a stirring chamber at 37 °C for 5 minutes and stimulated by adding 500µl (5x 109 bacteria) of 3H-labelled IgG-opsonised S. aureus. A 100µl aliquot was taken and immediately at 30 second intervals and added to 1ml of ice-cold RPMI media containing 1mM n-ethyl maleimide to stop vesicle fusion and 100µg/ml lysostaphin to lyse non-phagocytosed bacteria. Samples were then incubated at 37°C for 15 minutes, set on ice and centrifuged in a micro-centrifuge at 7000 x g for four minutes. 100µl of the supernatant was mixed with Ultra GoldTM (Packard, Groningen, the Netherlands) and counted in a liquid scintillation counter. Bacterial uptake was assessed as the difference between total radioactivity added and counts in the supernatant post phagocytosis.

2.3.5 De novo protein biosynthesis assay

Neutrophils (108) were Incubated in the presence of 1mCi [35S]-methionine for 16 hours at 30°C. Cells were then collected by centrifugation and resuspended in 2 x Laemmli sample buffer and boiled at 95°C for 15 minutes. Samples were analysed by SDS-PAGE, dried and exposed to HyperfilmTM (Amersham Life Sciences). Films were developed in a Velopex MD 2000 automated developer.

2.3.6 Antisense experiments

Phosphorothiorated PKD sense (GCGATGAGCG CCCCTCCGGTC) and antisense (GACCGGAGGGGCGCT CATCGC) oligonucleotides were obtained from Alta Bioscience (Birmingham, UK). In a blast search no homology was found between these oligos and any human sequence other than PKD. Neutrophils were isolated as described above and 107 cells were incubated in 200µl RPMI 1640 medium in the presence of 10µM of the oligonucleotide for 16 hours at 30 °C. Cells were then diluted to one ml of RPMI 1640 medium and assayed for NADPH oxidase activity or phagocytosis as described above. Samples for Western blotting were prepared by resuspending the neutrophils in 2 x Laemmli sample buffer and boiling at 95°C for 15 minutes. The samples were analysed by SDS-PAGE, and blotted as described below.

2.3.7 PKC depletion

In order to deplete PKCs by prolonged exposure to PMA as described by Stabel S *et al* (Stabel, Rodriguez-Pena *et al.* 1987), neutrophils were incubated in the presence of 0.1µg/ml of PMA for four hours at 37°C. The cells were then resuspended in fresh RPMI medium as assayed for NADPH oxidase, phagocytosis or Western blotting as described above.

2.3.8 Subcellular cell fractionation

2.3.8a. Triton extraction

Neutrophils were resuspended in triton lysis buffer plus protease inhibitors and homogenised utilising a Dounce homogeniser for twenty strokes. The samples were allowed to sit on ice for 30 minutes. The triton soluble fraction was cleared by centrifugation, 15 minutes at 16,000 x g.

2.3.8b. Post-nuclear supernatant (PNS)

Neutrophils were resuspended in Lamberts break buffer plus protease inhibitors, and sonicated at 15 microns for 10 seconds in a Soniprep 150 sonicator (MSE). PNS was made by centrifuging the cell extra at 500 xg for 10minutes in a Mistral 3000i benchtop centrifuge (MSE).

2.3.8c. Sucrose gradient

Sucrose (w/w) was dissolved in Lamberts break buffer plus protease inhibitors at different concentrations ranging from 5-50% (w/w) sucrose. Gradient was made in 1ml cushions steps of 5% sucrose, and allowed to diffuse overnight at 4°C. Neutrophil PNS was loaded on the gradient, and centrifuged at 250,000 x g overnight, 4°C. The fractions were collected at 500µl intervals and analysed by SDS-PAGE and Western blotting.

centrifuged at 250,000 x g overnight, 4°C. The fractions were collected at 500µl intervals and analysed by SDS-PAGE and Western blotting.

2.3.8d. Isolation of phagosomal membrane

Neutrophils were stimulated using IgG-opsonised metallic beads (Superparamagnetic latex beads, Sigma), for five minutes at 37°C. The reaction was stopped by adding four times volume of ice-cold 1x saline (0.9% NaCl), centrifuged at 500 x g for 10 minutes, 4°C, and the pellet was resuspended in Lamberts break buffer plus protease inhibitors. The samples were homogenised with a Dounce homogeniser (Pestle A) for 20 strokes, and sonicated at 15 microns for 10 seconds (Soniprep 150, MSE). The sample was exposed to a magnet and the supernatant was removed, allowing the metallic beads surrounded by the phagosomal membrane to remain. The metallic beads were washed three times in Lamberts break buffer and resuspended in Laemmli sample buffer and analysed as for sucrose gradient fractions.

The supernatant removed was treated as described below for isolating membrane and cytosol fractions.

2.3.8e. Isolation of membrane and cytosol fractions

After treatment, cellular reactions were quenched by placing the cells in ice-cold 1 x saline, and placed on ice and centrifuged in a Mistral 3000i (MSE) by centrifugation at 500 x g for 10 minutes at 4°C. The pellet was

resuspended in Lamberts break buffer plus protease inhibitors and a post nuclear supernatant was made as described above. The supernatant was layered onto a 40% (w/w) sucrose cushion, in Lamberts break buffer plus protease inhibitors, and centrifuged at 100,000 x g in a Beckman benchtop centrifuge (Optima TLX Ultracentrifuge) for 30 minutes at 4°C. The supernatant and interface were taken and diluted using two volumes of Lamberts break buffer. The sample was centrifuged in a Beckman bench top centrifuge at 425,000 x g for 30 minutes in order to pellet the membrane fraction. The membrane fraction was washed twice in Lamberts break buffer and resuspended in Laemmli sample buffer and boiled. The cytosolic fraction was precipitated by adding 80% volume of Acetone, 10% tri-chloroacetic acid (TCA) and 20mM DTT and allowed to sit at –20 °C for one hour. Precipitated proteins were then pelleted in a microcentrifuge (Biofuge Fresco, Heraeus) by centrifugation at 16,000 x g for 15minutes at 4°C, and washed once with 100% acetone and 20mM DTT, in order to remove traces of TCA. Finally the proteins were resuspended in Laemmli sample buffer and boiled for five minutes at 95 °C.

2.3.9 Immunoprecipitation of cellular proteins

Neutrophils were treated and stopped by adding 4 x volume of ice cold 1x saline and placed on ice. Cells were centrifuged for 10minutes at 500 x g for 10minutes, 4°C. Pellet was resuspended in triton lysis buffer plus protease inhibitors and a triton soluble fraction was made as described

above (section 2.3.8a). The triton soluble supernatant was incubated, with appropriate antiserum, for one hour, followed by incubation with protein A sepharose (Sigma) for 1 hour. All incubations were performed at 4°C. Immunoprecipitates were collected by centrifugation and washed 3 times in triton lysis buffer and 3 times in kinase buffer.

2.3.9a. For assessment of in vivo phosphorylation

Radioactive immunoprecipitates were resuspended in 2x Laemmli sample buffer, and exposed to SDS-PAGE, and autoradiography.

2.3.9b. For kinase assays

Immunoprecipitate proteins were resuspended in 20 µl of kinase buffer and assayed for kinase activity.

2.3.9bi. Autokinase assay

Activity was measured by incubating the resuspended pellet with 100µM ATP and 10µCi [γ-^{32}P]-ATP for 10 minutes at 37°C. The reaction was terminated by addition of 4 x Laemmli sample buffer, and immunocomplexes were boiled for 10 minutes at 95°C and analysed by SDS-PAGE and autoradiography.

2.3.9bii. PKD substrate kinase

Activity was measured by incubating the resuspended pellet, with 100μM ATP and 4μCi [γ-^{32}P]-ATP and 10μg Syntide (Calbiochem) for 15 minutes at 37°C. The reaction mixture was spotted onto Whatmann P81 paper. The filter paper was washed three times for 20 minutes each in 30% Acetic acid and ^{32}P incorporation was determined by scintillation counting using Ultra Gold™ scintillant.

2.3.10 In-vitro kinase assay

Recombinant PKD purchased from Calbiochem was incubated, for different times as indicated in figure legends, with substrate tested. The substrate was combined with assay buffer (50mM Hepes, pH 7.5, 0.25mM EDTA, 12.5mM $MgCl_2$, 0.75mM $CaCl_2$, 50μg phosphatidyl-serine, 50ng PMA), and allowed to warm to 37°C for 5 minutes. ATP solution (100μM ATP and 4μCi [γ-^{32}P]-ATP) was added, and finally the kinase was added at concentration indicated in text or figure legends. The reaction was stopped by adding 5 x Laemmli buffer and boiling for 15 minutes.

2.3.11 Immunofluorescence

Protocols for immunofluorescence were adapted from Dekker LV *et al.* (Dekker, Leitges *et al.* 2000) Cells were resuspended in RPMI 1640 preincubated at 37 °C and stimulated as indicated in the figure legends. Cells were then treated differently in different experiments.

2.3.11a. Fixation in suspension

Cells were stimulated with IgG-*S. aureus* and stopped by adding 4 x volume of ice-cold 1 x saline. Cells were collected by centrifugation and resuspended in PBS plus 4.0% paraformaldehyde and 0.1% glutaraldehyde and incubated for 60 minutes at 22°C in order to fix the neutrophils. 100µl of fixed neutrophil were placed on the coverslip and allowed to sit for 15 minutes. The coverslips were then washed four times with PBS. For permeabilisation, the cells were treated with 0.5% Triton/PBS for 15 minutes at 22°C. This was followed by incubating twice for 15 minutes in 1mg/ml of Sodium Borohydride/PBS, four times washing with PBS, and incubation for 2 hours in 5% goat serum/PBS. The primary antibody (1:50 in 5% goat serum/PBS) was then added and incubated for 48 hours at 4°C and followed by an incubation for 1 hour at room temperature. After six washes with PBS, secondary antibody (1:200 in 5% goat serum PBS) was added and incubation was at room temperature for one hour. Subsequently, six washes were performed with PBS and two with H_20, coverslips were then blotted dry and sealed in Mowiol mount plus 2.5% (w/v) DABCO. Analysis was performed on a Leica Confocal Microscope.

2.3.11b. Fixation of adhered cells

For experiment with F- actin staining, by Rhodamine conjugated Phalloidin, the stimulatory reaction was quenched as described above. The cells were

pelleted and resuspended in 100µl of PBS and the whole solution was dropped onto a coverslip. Fixation was undertaken once the cells adhered to the coverslips as described above.

2.3.11c. Treatment of coverslips to increase cell adherence

In the PKD/p47^{phox} co-localisation experiments, coverslips were pre-treated as described by Kiley and Parker.(Kiley and Parker 1995) Briefly, clean coverslips were pre-treated with chromic-gelatin solution. Pig skin gelatin, 2% (w/v) (BDH Chemicals Ltd) was dissolved in boiling distilled water and cooled to room temperature. This solution was then mixed with an equal volume of 0.1% (w/v) chromic potassium sulphate, with 0.037% formalin added as a preservative. Coverslips were treated on one side with chromic gelatin for 30 seconds, excess gelatin was removed, and coverslips were dried in a warming oven (approximately 50°C) for 30 minutes. Cells were treated and the reaction was stopped as described above, the sample was then pelleted and resuspended in PBS plus 4.0% paraformaldehyde and 0.1% glutaraldehyde and incubated for 60 minutes at 22°C. The remaining procedures were performed as describe in the section, fixation of cells in suspension.

2.3.12 Protein gel electrophoresis and Western blotting

Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) was conducted using the standard Laemmli method. (Laemmli 1970)

Polyacrylamide gels used were composed of different final concentration of acrylamide between 7.5-15%, depending on the protein size we wanted to visualise and the resolution desired. Gels for visualisation of PKD were generally 7.5% acrylamide, where as resolution of $p40^{phox}$ and $p47^{phox}$ was done in a 12.5 or 15% gel. Hoefer Mighty Small II apparatus was used for electrophoresis. Low Molecular weight markers (Amersham Pharmacia) were used in all gels. For visualising protein gels were either stained with coomassie Blue or silver stained. Gels were dried on cellophane (Pharmacia) using a Biorad 543 gel dryer.

Immunoblotting was performed with a Transblot SD-Semi Dry Transfer Cell (Biorad) electroblotter using reinforced cellulose nitrate membrane (Schleicher and Schuell). Western blot transfer was performed at 100 mAmps/minigel for one hour as described by Towbin (Towbin, Staehelin *et al.* 1979) followed by Ponceau staining (Sigma). For antibody incubations non-specific antigenic sites were blocked by incubating for one hour at room temperature in TBS supplemented with 0.5% (v/v) Tween-20 (Sigma) and 5% fat-free powdered milk (Carnation). The concentration of antibody and time of incubation were determined empirically for each antibody used; however, all incubations were performed at 4°C in blocking solution. Blots were washed four times for 15 minutes each in TBS/0.05% (v/v) Tween-20, followed by incubation with species-specific antibodies against IgG (Amersham) in 1:1000 dilution for one hour at room temperature, and washed 3 times again. Blots were developed using enhanced

chemiluminescence method (ECL, Amersham) and exposed to Hyperfilm™. Films were processed in a Velopex MD2000 automated developer (Medivance). Quantification of Western blots was performed using NIH image computer software.

2.3.13 Two dimensional electrophoresis

2.3.13a Sample preparation

Neutrophils were isolated as described above and resuspended in RPMI 1640 buffer without sodium phosphate (Gibco, BRL) at a concentration of 10^8 cells /ml. One mCi/10^8 cells /ml of [^{32}P] orthophosphate (^{32}Pi, 10 mCi/ml aqueous solution, Amersham) was added and the cells were incubated for 1 hour at 30 °C. Where applicable, inhibitors were added, after 45 minutes incubation with [^{32}P], at the appropriate concentration and incubated for 15 minutes at 30 °C. Cells were then stimulated with *S. aureus*-IgG at a 1:100 ratio (neutrophils: opsonised bacteria), for the times indicated in figures and figure legends. The reaction was quenched as described above and cells were resuspended in Lamberts break buffer plus protease and phosphatase inhibitors. A post-nuclear supernatant was isolated. (Section 2.3.8b) Membrane and cytosol fractions were acquired as described above (section 2.3.8e), with the exception that samples were resuspended in IEF buffer instead of Laemmli buffer. In some experiments the triton soluble fraction was analysed (section 2.3.8a).

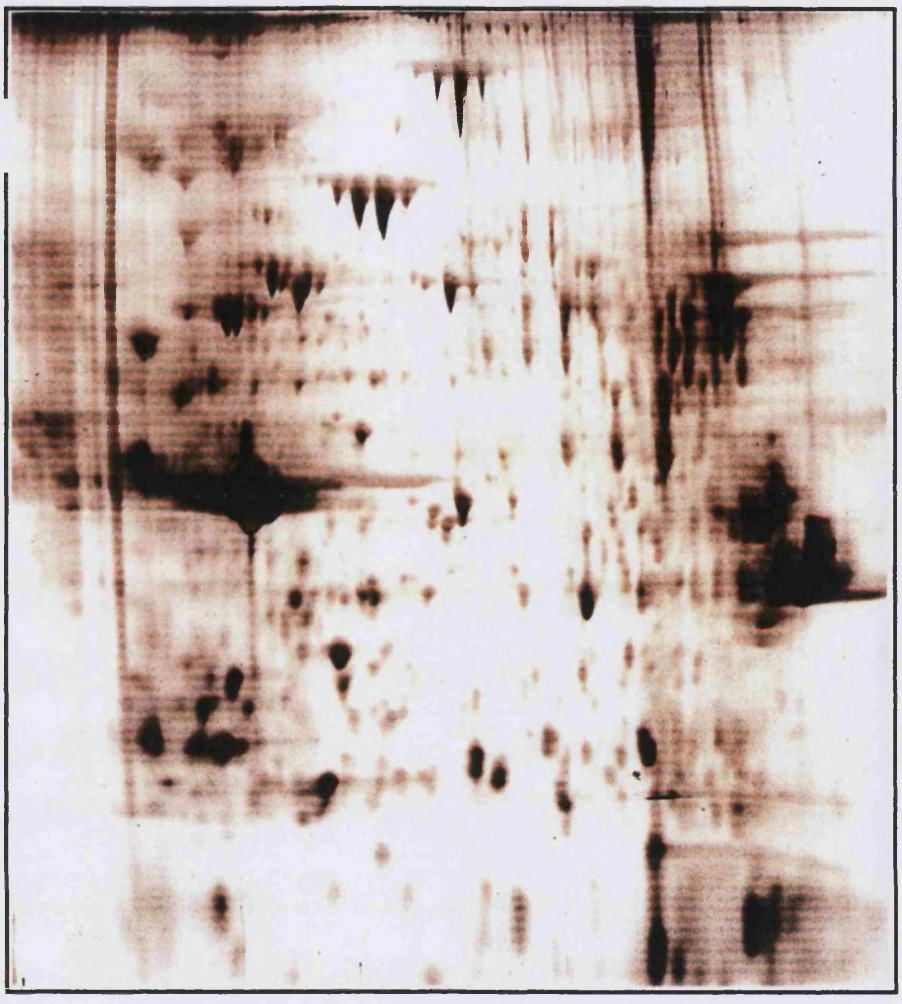


Figure 2.1 Example of two-dimensional gel electrophoresis

Direction of protein separation is indicated. Silver stained 10% SDS-PAGE gel. IEF separation was performed on a pH gradient of 3-10.

2.3.13b First dimension gel electrophoresis

2.3.13bi Principle

In the first dimension electrophoresis proteins are separated according to their isoelectric point. Proteins are amphoteric molecules, which are positively, negatively charged or zero-net charge depending on the pH surroundings. The net charge of the protein is the sum of all the amino acid side chains plus amino- and carboxy- termini. The isoelectric point is the specific pH at which the net charge of a specific protein is zero. Therefore, a positively charged protein will travel towards the cathode and become progressively less positively charged until it reaches its isoelectric point. The same principle applies for negatively charged proteins. In this manner proteins are separated on the basis of very small charge differences. (Figure 2.1)

2.3.13bii Protocols

Immobiline™ pH gradient strips were used, containing a 3-10 non-linear pH gradient. Two different set-ups were used to run the first dimension: 18cm strips were run on a Multiphor II system (Pharmacia) and 13cm strips were ran on an IPGphor system (Pharmacia).

18cm pH 3-10 non-linear strips protocol

IEF was performed at 18 °C, 1mAmp, and 5 Watts while the voltage changed in steps.

Step 1: 500 volts for 2600 Volt hours (Vhrs)

Step 2: 2500 volts for 52,000 Vhrs

Step 3: 3500 volts for 45,400 Vhrs

13cm pH 3-10 non-linear strips protocol

IEF was performed at 16 °C and 50μAmps/ strip, voltage was changed in steps

Step 1: 30 volts for 360 Vhrs

Step 2: 300 volts for 150 Vhrs

Step 3: 3000 volts for 3000 Vhrs

Step 4: 6000 volts for 3000 Vhrs

Step 5: 7500 volts for 55,000 Vhrs

Strips were received frozen and dry. They were allowed to rehydrate by incubation in the presence of sample plus IEF buffer (see under sample preparation). In this way the strip absorbed the buffer together with the sample to be analysed, resulting in the sample being homogeneously spread along the strip. Eighteen-cm strips were rehydrated at 22°C for 10 hours while 13cm strips were rehydrated under low current Step 1 in 13 cm strip protocol.

Strips were then removed from IEF and equilibrated for SDS-PAGE in the second dimension. This process involved incubating the IEF strip with equilibration buffer plus 10mg/ml DTT for 15 minutes followed by a second

incubation with equilibration buffer plus 48mg/ml Iodoacetamide also for 15 minutes, both incubations were performed at room temperature.

2.3.13c Second dimension

Post equilibration the strip was lightly washed with distilled water and excess water was blotted dry using Whatmann 3MM paper. The strip was then loaded onto a 10% SDS-PAGE gel and sealed in place by adding a mixture of heated 2 x Laemmli sample buffer and 2% (w/v) Agarose (Amersham/Pharmacia). The sample was analysed by 10% SDS-PAGE gel. 18 cm strips were run using the IsoDalt (Pharmacia) system at 50-100 mAmps/ gel and 13 cm strips were run on a Hoefer SE600 system at same current. In both cases electrophoresis was carried out at 5-10°C overnight.

2.3.13ci Staining

Proteins on the gels were visualised by either coomassie staining or silver staining.

2.3.13cii Drying

After staining the gels were treated with 4% (v/v) methanol, 30% (w/v) glycerol for 30 minutes. The gels were sandwiched in cellophane drying film (Pharmacia), assembled in an EasyBreeze Drying frame (Hoefer) and allowed to dry at room temperature. Radioactive gels were then exposed to BioMax MS film (Kodak) for autoradiography and developed as described above.

2.3.13c Analysis

Gels and autorads were scanned using a UMAX PowerLook III scanner through Adobe Photoshop imaging program. Images were analysed using Melanie II computer software (Biorad). Radioactive spots were quantified as percentage of volume (which is the intensity of the particular spot as a percentage of all detectable spot on a particular gel). Otherwise, if an internal standard was found to be consistent and equally phosphorylated in all conditions, this was taken as the standard by which all spots were compared to.

2.3.14 Two dimensional phosphopeptide mapping

A CBS Scientific Hunter Thin Layer Electrophoresis 7000 system was used followed by autoradiography or phosphoimaging.

Phosphorylated $p40^{phox}$ or $p47^{phox}$ was excised from a SDS-polyacrylamide gel. The gel slice was ground up and rehydrated in 50mM NH_4HCO_3, pH 7.3-7.6, for 5 minutes at room temperature. The protein was then eluted from the gel pieces by incubating twice in 5% (v/v) β-mercaptoethanol, 0.1% SDS, and 50mM NH_4HCO_3 pH 7.3-7.6, each time pelleting the gel pieces and extracting the supernatant. This was followed by precipitation of the protein in 100% TCA, and oxidating the methionine and cysteine residues with performic acid. Performic acid was made by mixing 9 parts of 98-100% formic acid with one part 30% hydrogen peroxide and allowing to sit on ice for one hour. Trypsin digestion was performed by

incubating twice with 10μg of 1mg/ml TPCK-treated trypsin for three to four hours at 37 °C. The reaction was stopped by adding deionised water and freezing in liquid nitrogen, followed by lyophilisation.

An RNAse carrier (20μg/ml) protein was incorporated in the sample prior to electrophoresis.

The protocol for two-dimension phosphopeptide mapping was adapted from the method described by Hunter. (van der Geer and Hunter 1994) Phosphopeptide maps were obtained by applying the sample onto a nitrocellulose coated glass plate purchased from Merck pharmaceuticals. Thin layer was carried out in electrophoresis buffer pH 1.9, 1500V for 30 minutes, followed by thin layer chromatography for 12 hours. Phosphorylated peptides were visualised by autoradiography.

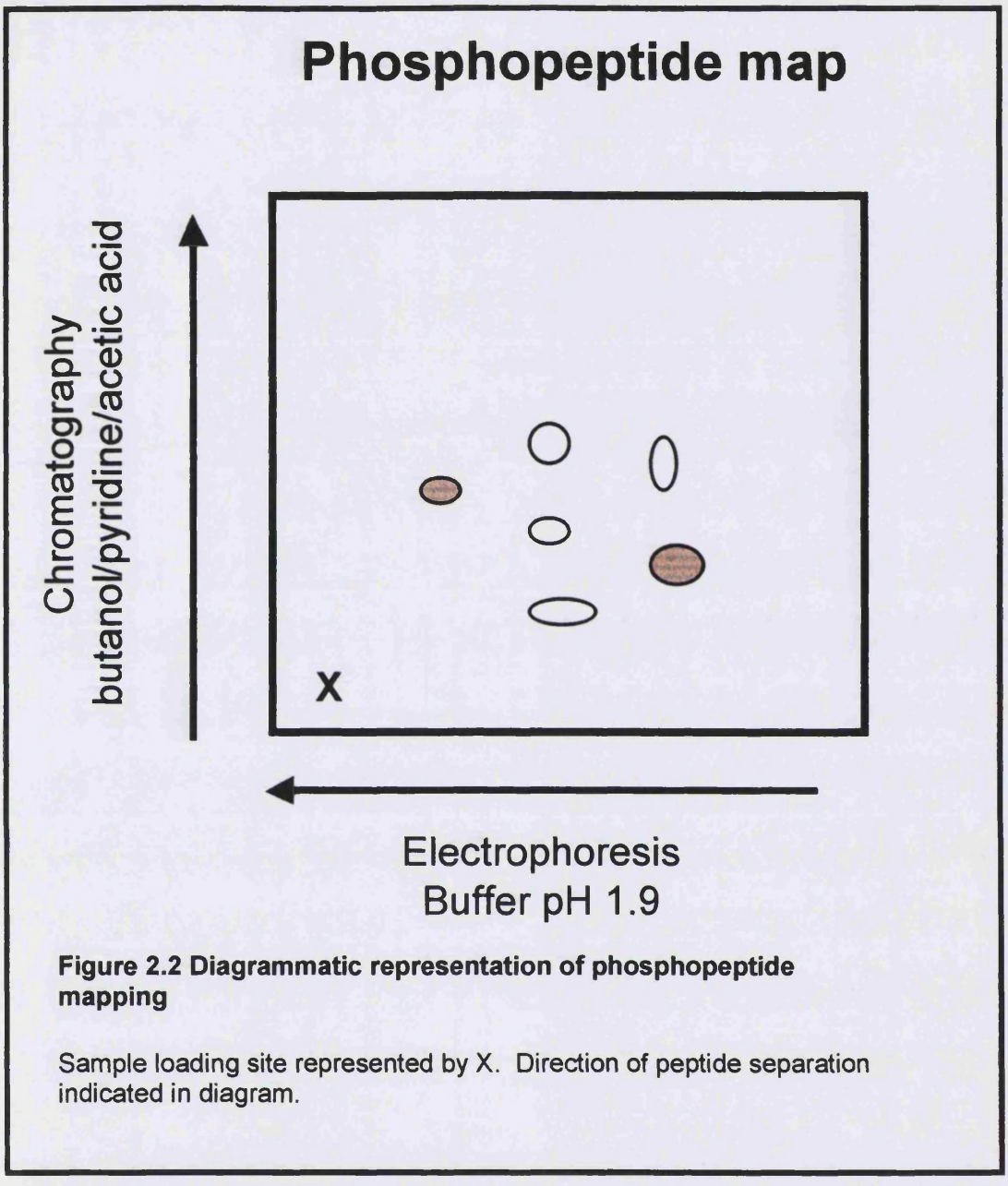


Figure 2.2 Diagrammatic representation of phosphopeptide mapping

Sample loading site represented by X. Direction of peptide separation indicated in diagram.

2.3.15 Mass spectrometry

2.3.15a Principle

Proteins can be cleaved at specific sites by employing proteases such as trypsin which cleave at the C-terminus of lysine and arginine residues.

Therefore trypsin digestion would produce a different series of peptide fragments for each protein, otherwise known as peptide fingerprinting. Mass spectrometry is an analytical technique, which separates these peptides on a mass-to-charge (m/z) ratio basis.

Tryptic digests are exposed to ionisation, which adds a positive charge to the peptides. Peptides are then accelerated down an electrical field, and allowed to travel through a vacuum tube (time of flight). The ions are detected by an electron multiplier. Peptide separation is achieved by the fact that the time of flight is proportional to the mass-to-charge ratio. (Figure 2.3)

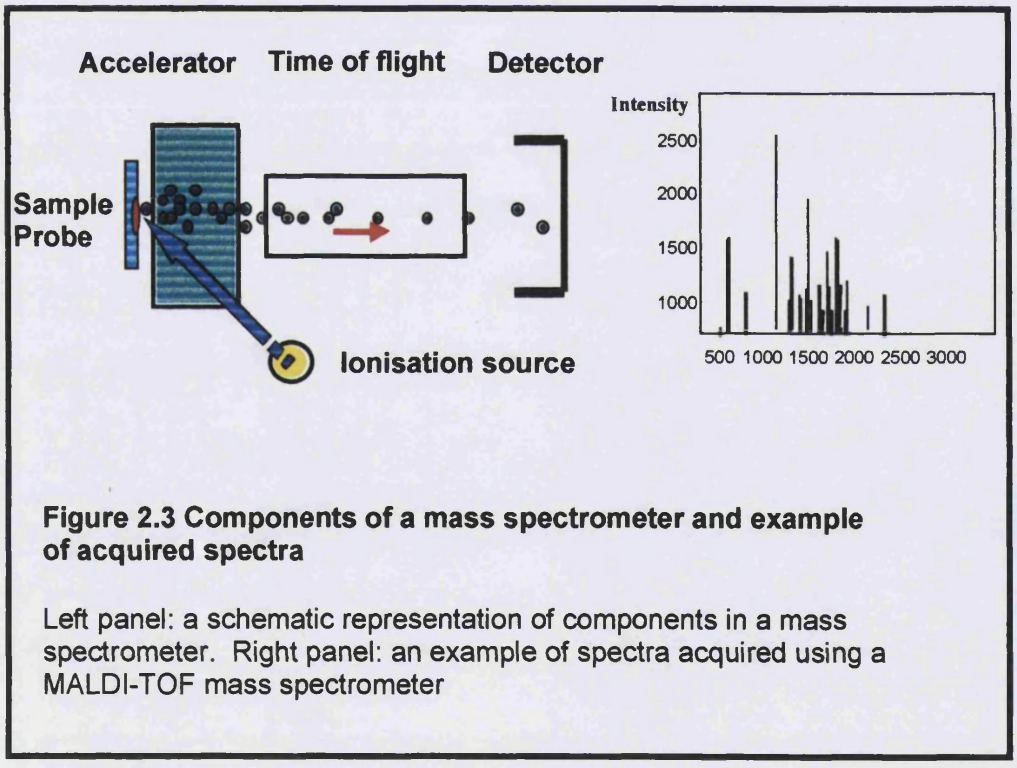


Figure 2.3 Components of a mass spectrometer and example of acquired spectra

Left panel: a schematic representation of components in a mass spectrometer. Right panel: an example of spectra acquired using a MALDI-TOF mass spectrometer

2.3.15b In gel tryptic digestion

Proteins of interest were cut out of a 2-D gel, allowed to de-stain in 50mM NH_4HCO_3, 40% (v/v) ethanol. The gel pieces was then soaked in 25mM NH_4HCO_3 twice for 15 minutes, in order to equilibrate the pH to 7-8 (optimal pH for trypsin activity).

At this point an extra step was added for silver stained proteins. If silver stained the gel piece was rehydrated in 100mM NH_4HCO_3, 10mM DTT for 30 minutes at 56°C, and lyophilised again.

In order to dehydrate the gel piece, it was suspended in 100% acetonitrile, three times for 10 minutes each, or until the gel piece turned white. Lyophilisation was done in a Savant Speed Vac Plus centrifuge.

Subsequently, the sample (whether silver or coomassie stained) was resuspended in 25mM NH_4HCO_3, 80ng/µl Trypsin (Promega) and incubated over night at 37°C. A sample was then taken (0.5µl) and spotted on the MALDI target.

2.3.15c MALDI (Matrix Assisted Laser Desorption/Ionisation)

Samples were applied to the target and allowed to air dry. Subsequently 0.5µl of matrix solution, (1% (w/v) α-cyano-4-hydroxysuccinamic acid in 50% acetonitrile, 0.1% (v/v) triflouroacetic acid (TFA)), was applied to the dried sample and again allowed to air dry. Spectra were obtained using a Bruker Biflex III MALDI time-of-flight mass spectrometer. The apparatus was calibrated using a mixture of six known peptides (angiotensin I, m/z

1046.54; angiotensin II, m/z 1296.68; Glu-fibrinopeptide, m/z 1570.67; renin substrate: angiotensinogen, m/z 1758.93; adrenocorticotropic hormone, m/z 2465.20; insulin chain B, m/z 3494.65) (Sigma) dissolved in 50% (v/v) acetonitrile, (0.1% (v/v) TFA.

2.3.15d Electrospray

MS/MS (mass spectrometry/mass spectrometry) analysis was performed on a Finnigan Mat LCQ ion-trap (Finnigan Corp.). Data acquired were processed using Bio Explore software and compared with known databases by employing Sequest software (Finnigan Corp.) Work done with this equipment was used to confirm data, acquired by MALDI mass spectrometry, that was not clear.

2.3.15e Analysis

For interpretation of MALDI spectra, a list of mass-to-charge ratio was acquired for each protein digest and processed using four different browsers (Mascot, Matrix Science; ProFound, Rockefeller Institute; MS-Fit, University of California-San Francisco; Peptident; ExpaSy), and analysed by comparison against three separate data bases (OWL, NCBI, Swiss Prot). Mass tolerance was limited to relative value of ± 200 ppm (parts per million), and proteins were matched according to probability scores deduced by each browser.

Chapter 3

Investigation of Fcγ receptor signalling intermediates in the activation of the NADPH oxidase

3.1. Aim

The aim of this study was to characterise some of the lipid and protein kinases involved in the signal transduction cascade that leads to the activation of the NADPH oxidase upon Fcγ receptor activation. The approach taken involved the measurement of oxidase activation by oxygen consumption and its response to pharmacological inhibition of signalling intermediates. As mentioned previously, Fcγ receptor engagement of opsonised bacteria leads to phagocytosis and consequent NADPH oxidase activation. In order to know at which level the pharmacological intervention took place assays measuring phagocytosis were performed in parallel to the measurement of the NADPH oxidase.

3.2. Introduction

3.2.1. Oxygen consumption as a measure of NADPH oxidase activity

Several methods for measuring NADPH oxidase activity have been developed. Indirect methods employ enzymes or products of secondary metabolic pathways, such as the hexose monophosphate shunt, that are stimulated in the process. Direct measurement of NADPH oxidase activation utilises the product of this enzyme, superoxide (O^{-}_2), or the products of superoxide dismutation, H_2O_2. Some of the methods utilised are the nitroblue tetrazolium test (NBT), the chemiluminescence, cytochrome C reductase assay or oxygen consumption assay.

NBT is an electron acceptor used to detect the production of superoxide. (Baehner and Karnovsky 1968) NBT is reduced to form formazan, an insoluble blue-black material that precipitates, and in this process converts O^{-}_2 to O_2. However, since patients who have CGD still showed a positive NBT test, the reliability of this test has been questioned. (Borregaard, Cross et al. 1983) NBT is known to form complexes with heparin, which are phagocytosed thereby stimulating the cells, and giving extra activation of the NADPH oxidase. (Hellum and Solberg 1973; Hohn and Lehrer 1974; Rothwell and Doumas 1975; Segal and Levi 1975) Since heparin is widely used to obtain blood samples, the NBT test is not always a good way to measure the oxidative burst.

Chemiluminescence employs the fact that in normal neutrophils the respiratory burst is associated with generation of light energy, which is dependent on singlet oxygen ($^1O_2$) production. This is produced when an unpaired electron is lifted to a higher orbit with an inversion of spin. Singlet oxygen is a product of the following reaction:

$$OCl^- + H_2O_2 \longrightarrow Cl^- + H_2O + {}^1O_2$$

This reaction requires the catalytic effect of myeloperoxidase (MPO), which forms hypochlorous acid (HOCl). Therefore the assay cannot be performed on MPO deficient patients. MPO deficiency has a high prevalence of 1:1000 in the overall population, though it does not seem to have a dominant phenotype. (Kutter 1998) It has been shown that MPO deficient donors have a low level of chemiluminescence production even though, super oxide production is normal. (Stevens, Winston *et al.* 1978)

Cytochrome C reductase assay is a good assay to follow kinetics of superoxide production by neutrophils (Dahlgren and Karlsson 1999). Cytochrome C is an iron containing enzyme which, when reduced to its ferrous form by electron scavenging, can be measured spectrophotometrically. (McCord and Fridovich 1969) However, cytochrome C is not membrane permeable, therefore superoxide detected by this method must be limited to extracellular superoxide, such as that

induced by PMA or large particles that lead to non-closure of the phagosome, (frustrated phagocytosis).

In the present study, in order to measure NADPH oxidase activation while approximating the most physiological conditions, an oxygen consumption assay was employed. The principle of measuring oxygen consumption as an indication of NADPH oxidase activation extends back to the first observation by Baldrige and Gerard in 1933, they observed an increase in oxygen consumption, above normal metabolic levels, upon exposure of neutrophils to bacteria. (Gerard 1933) This assay has the advantage that it can measure NADPH oxidase activity during normal phagocytosis, as it can measure intra and extracellular oxygen, and changes can be quantified upon neutrophil stimulation. Opsonised bacteria can therefore be used as a physiological stimulus in activating oxidase activity.

3.2.2 Inhibitors

3.2.2a Tyrosine kinases

Genistein, an isoflavone, is known to inhibit tyrosine phosphorylation. (Akiyama, Ishida *et al.* 1987) Tyrosine phosphorylation is an early and essential step in Fcγ receptor signalling. (Section 1.6.2a) Genistein has been shown to inhibit superoxide production by aggregated IgG stimulation and fMLP. (Kusunoki, Higashi *et al.* 1992; Liang and Huang 1995) Fcγ

receptor activation of superoxide production was inhibited by 62% after incubation of neutrophils with genistein. (Liang and Huang 1995) Since the effect of genistein on NADPH oxidase upon Fcγ receptor stimulation has been well characterised, it was also used in order to verify the specificity of the system employed, for measuring NADPH oxidase activity.

3.2.3b Phosphatidylinositol-3 kinase (PI-3 kinase)

Wortmannin, a fungal metabolite, has been shown to be a specific inhibitor of PI-3 kinase. (Arcaro and Wymann 1993) PI-3 kinase is activated upon serum-opsonised zymosan stimulation of neutrophils, and the activation has been shown to be inhibited by wortmannin. (Yamamori, Inanami *et al.* 2000); (Baggiolini, Dewald *et al.* 1987) Fcγ receptor activation of several processes in neutrophils such as superoxide production, increase in intracellular calcium, receptor capping and phagocytosis are wortmannin sensitive, thereby involving PI-3-kinase in Fcγ receptor signalling. (Ninomiya, Hazeki *et al.* 1994; Chuang, Sassaroli *et al.* 2000)

More recently another inhibitor for PI-3 kinase has been developed, Ly 294002, which competes with ATP at the binding site. (Vlahos, Matter *et al.* 1994) Ly 294002, has been tested on superoxide production in neutrophils and has shown to inhibit 80% of maximal production upon fMLP stimulation. (Ding, Vlahos *et al.* 1995; Vlahos, Matter *et al.* 1995) Fcγ receptor activation of calcium influx was also inhibited by this compound (Lang and Kerr 1997; Vossebeld, Homburg *et al.* 1997); however, no evidence in the

literature suggests that Ly 294002 inhibits Fcγ receptor activation of the NADPH oxidase. The involvement of PI-3 kinase in oxidase activation (Lang and Kerr 1997) as well as phagocytosis (Raeder, Mansfield *et al.* 1999) makes it an interesting kinase to study as it might provide information as to the point of diversion of two cellular processes activated by the Fcγ receptor.

3.2.2c. Protein kinase C (PKC)

DAG activates PKC, and it is known that analogues of the former, phorbol esters, are potent activators of PKC. (Cox, Jeng *et al.* 1985); (Castagna, Takai *et al.* 1982) Phorbol esters were known to activate NADPH oxidase activity even before it was known that this process occurred through PKC (Suzuki and Lehrer 1980; Tauber, Brettler *et al.* 1982; McPhail and Snyderman 1983). Several PKC inhibitors are available which were employed in the present study to investigate Fcγ receptor pathways.

Two of the inhibitors are highly specific for PKC but do not express high selectivity for any particular isoform or sub family. The inhibitors were:

1) GF 109203X, a bisindolylmaleimide, originally designed to be more PKC selective than the potent PKC inhibitor staurosporin. GF 109203X, referred to as Bim I in this study showed high selectivity for PKC as compared with other kinases. (Toullec, Pianetti *et al.* 1991) This compound was also shown to inhibit superoxide production by PMA stimulation

(Jacobson, Kuchera *et al.* 1995) or other stimuli such as serum opsonised zymosan. (Cabanis, Gressier *et al.* 1996)

2) Ro 31-8220, another bisindolylmaleimide compound, is highly selective for PKC but not specific for any particular isoform inhibiting all isoforms relatively equally. (Dieter and Fitzke 1991) Ro 31-8220 has been shown to inhibit superoxide formation by receptor and post receptor simulation (Twomey, Muid *et al.* 1990; Twomey, Muid *et al.* 1990) and phosphorylation of $p47^{phox}$, (Bengis-Garber and Gruener 1995) supporting a role for PKC in superoxide generation by several stimuli of the NADPH oxidase.

3) The final PKC inhibitor Gö 6976, an indolocarbazole compound which is quoted to be specific for Ca^{+} - dependent PKC isoforms, was used. (Georg Martiny-Baron 1993) Few studies employing Gö 6976 have been performed in neutrophils. One study reported that this inhibitor had no effect of superoxide production by PMA and fMLP. (Wenzel-Seifert, Schachtele *et al.* 1994) However, other studies have shown that genetic deletion of one of the isoforms, β, of the cPKC subfamily has a direct effect of Fcγ receptor activation of the NADPH oxidase. (Dekker, Leitges *et al.* 2000)

Studies investigating phosphorylation events in neutrophils have employed the different inhibitory profiles of Gö 6976 and Ro 31-8220. (Djafarzadeh and Niggli 1997) This study showed that a calcium-

independent (Ro 31-8220-sensitive and Gö 6976-insensitive) PKC partakes in dephosphorylation of cofilin in neutrophils.

Here the effect of different inhibitors on NADPH oxidase activation was tested, in order to assess temporal placement of kinases in Fcγ receptor activation of the NADPH oxidase.

3.3 Results

3.3.1 Activation of the NADPH oxidase

Activation of the NADPH oxidase by the PMA and IgG-opsonised *S. aureus* was recorded in an oxygen electrode (figure 3.1A). Neutrophils placed in the chamber were allowed to stabilise, the stimulus was added (2-3 x 10^9 IgG-opsonised *S. aureus,* medium or 0.1μg/ml PMA) and oxygen consumption increased. Post addition of the stimulus there was a lag period of approximately 30 seconds for both stimuli, after which the rate of oxygen consumption increased from 0nmol O_2/min/5x10^7 neutrophils (no stimulus) to 0.9 nmol O_2/min/5x10^7 neutrophils (100μl RPMI 1640 medium addition), 50 nmol O_2/min/5x10^7 neutrophils (IgG-opsonised *S. aureus* stimulus) and 66.4 nmol O_2/min/5x10^7 neutrophils (PMA stimulus). Figure 3.1B represents the oxygen consumption from 6 separate observations for each stimulus shown. IgG-opsonised *S. aureus* (3x10^9 bacteria) induced a rate of oxygen consumption of 39.6 ± 8.1 nmol O_2/min/5x10^7 neutrophils. Control buffer (RPMI 1640, 100μl) gave a response of 2.2 ± 0.4 nmol

O_2/min/5x10^7 neutrophils. PMA (0.1μg/ml) induced a rate of oxygen consumption of 40.5 ± 0.9 nmol O_2/min/5x10^7 neutrophils. The increase in oxygen consumption was dependent on the stimulus. Neutrophils, which were allowed to sit in the chamber with no stimulus, showed a negligible change in the rate of oxygen consumption.

Thus NADPH oxidase activation could be measured using this system. It was related to the stimuli used rather than increased metabolism by resting cells. The steady state oxygen consumption of resting neutrophils appeared to be constant for each experiment and reliable within each batch of neutrophils isolated on the same day.

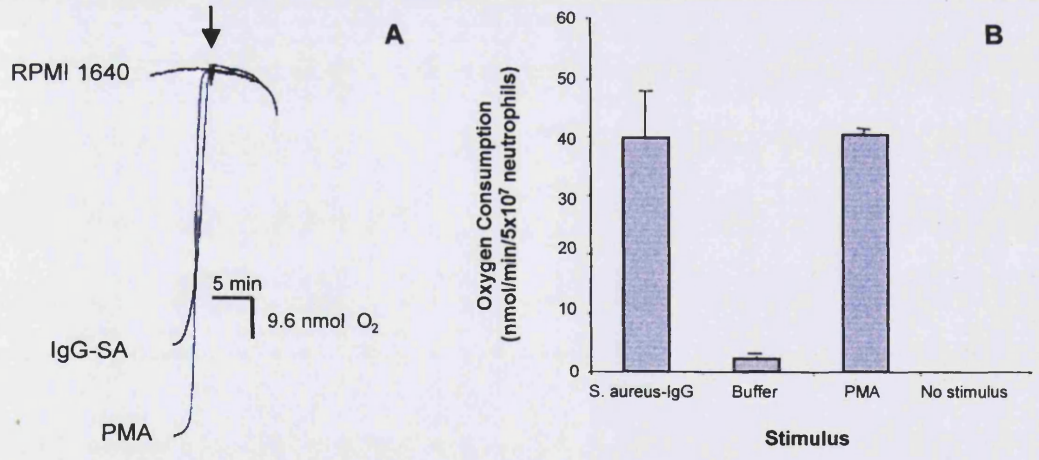


Figure 3.1 Measuring NADPH oxidase activity as rate of oxygen consumption

Panel A represents traces of oxygen consumption stimulated by three different stimuli. Neutrophils (10^8) were allowed to stabilise and subsequently stimulated (shown by arrow) with IgG-opsonised *S. aureus* (3x10^9 particles) PMA (0.1μg/ml) or RPMI 1640 (100μl). Panel B shows the average± standard error of 6 measurements as performed in panel A.

3.3.2. Rate of oxygen consumption is dependent on dose of the stimulus.

The dose dependence of NADPH oxidase activation by IgG-opsonised *S. aureus* or PMA was determined. (Figure 3.2) Figure 3.2A shows rate of oxygen consumption increasing in response to increasing amounts of IgG-opsonised *S. aureus*. The maximal rate of oxygen consumption in response to IgG-opsonised *S. aureus* was 72.7 ± 5.7 nmol O_2/min/5x10^7 neutrophils and for PMA maximal rate was 63.2 ± 7.5 nmol O_2/min/5x10^7. Half-maximal activation occurred at 5.75 ± 1.7 x 10^8 particles for IgG-opsonised *S. aureus* and 0.03 ± 0.01 μg/ml for PMA. (Figures 3.2B and C, respectively) It is interesting to note that, as the stimulus was increased, there was an increase in rate but also the total amount of oxygen consumed was increased. (Figure 3.2A)

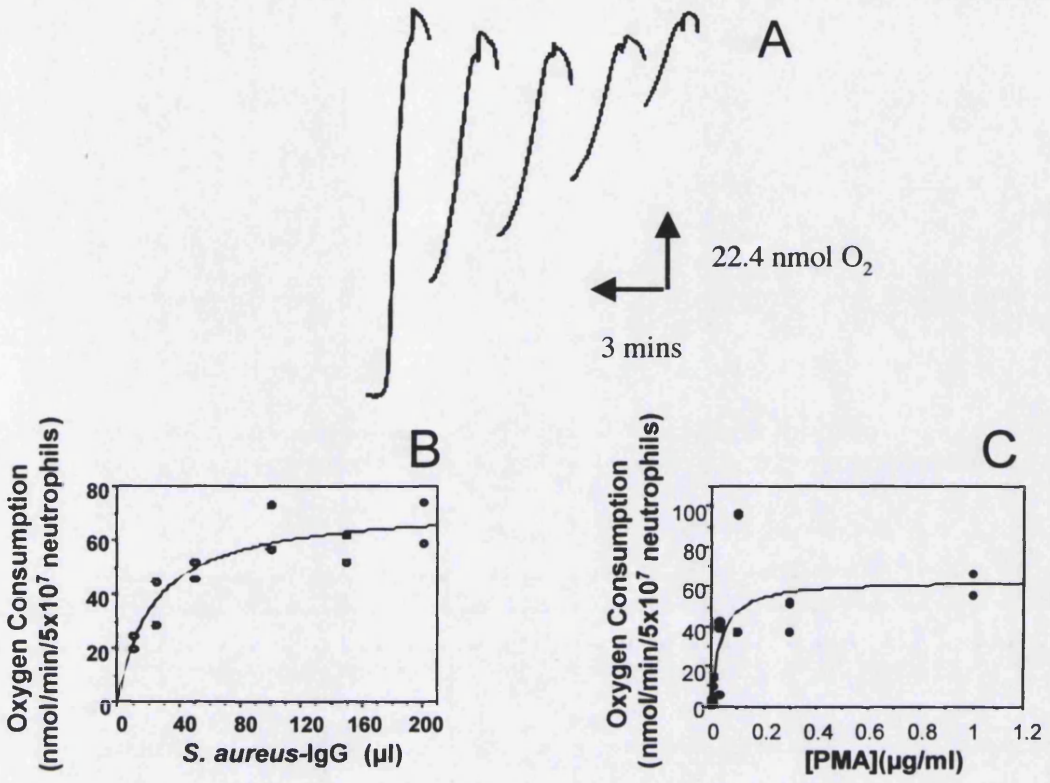


Figure 3.2 Dose dependent activation of NADPH oxidase

Panel A Shows increasing rate of oxygen consumption as stimulus is increased. From right to left 25µl, 50µl, 100µl, 150µl, and 250µl of IgG-opsonised *S. aureus* (3×10^{10} bacteria/ml) were added to a sample of 10^8 neutrophils. Rates of oxygen consumption increased from 39, 47, 64.5, 88.7 to 238 nmol O_2/min/5 x 10^7 neutrophils, respectively. Panel B and C NADPH oxidase dose response curves as calculated by GraFit software (Microsoft) for IgG- opsonised*S. aureus* and PMA, respectively. Equal number of neutrophils (5 x 10^7 cells) were used for each incubation, concentration of IgG-opsonised *S. aureus* was the same as for panel A.

3.3.3. Effect of protein and lipid kinase inhibitors on NADPH oxidase activation

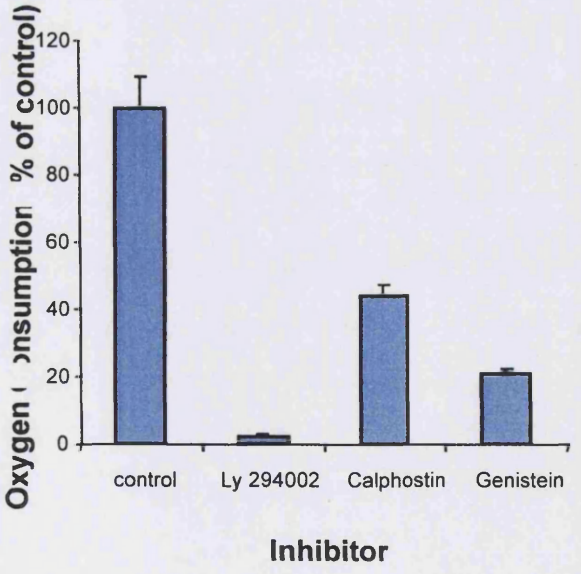
The oxygen consumption assay was used to measure the effect of inhibitors to different protein kinases believed to be involved in the signal transduction pathway that activates the NADPH oxidase upon Fc receptor

stimulation. Stimulation used for all experiments was performed at 90% of maximal stimulation that IgG-opsonised *S. aureus* could elicit per batch of neutrophils. This was assessed by performing a dose-response curve (figure 3.2) for every neutrophil preparation.

Inhibitors for tyrosine kinase and PI-3 kinase were used and their effects compared. (Figure 3.3) Genistein (200µM) reduced oxygen consumption to 21.8 ± 0.07 % of control. Ly 294002, the PI-3 kinase inhibitor, inhibited oxygen consumption completely, with a residual component similar to non-stimulated sample of 2.06 ± 1.25 % of control. (Compare with Figure 3.1) From this the importance that PI-3 kinase may play in the signalling cascade from Fcγ receptor to NADPH oxidase can be extrapolated.

Figure 3.3 Effect of tyrosine kinase, PI-3 kinase and PKC inhibitors on oxygen consumption

Neutrophils (5 x 10^7 cells) were incubated for 15 minutes at room temperature with inhibitor. Subsequently the cells were stimulated using 3 x 10^9 bacteria, and oxygen consumption was measured as described in section 2.3.3. Oxygen consumption was calculated as % of control (non-inhibited sample). Inhibitors were used at different concentration, which was determined empirically: Ly 294002 at 50µM, Calphostin at 10µM and Genistein at 200µM. Bars represent average± standard error. n=6 (Ly 294002); n=3 (calphostin); n=2 (genistein).



Wortmannin at high (10-100nM) concentrations inhibited *IgG-opsonised S. aureus* stimulation of the oxidase completely with an IC_{50} of 0.02 ± 0.01μM (table 3.2) similar to Ly 294002. Inhibition of the oxidase by wortmannin could be abrogated by ~~stimulation with~~ PMA (table 3.1), suggesting that PKC functions downstream of PI-3 kinase in this signalling cascade or perhaps in a parallel pathway. Similarly, the inhibition by genistein could also be rescued by activation with PMA (table 3.1), placing tyrosine kinase activation as an earlier event than PKC in this signalling process.

INHIBITOR	STIMULI	
	$3x10^9$ IgG-SA ⟶	PMA (0.1μg/ml)
-	100 %	ND
Genistein (200μM)	21.70 %	78.30 %
Wortmannin (100nM)	0 %	106.1 %

Table 3.1 Activation of NADPH oxidase by PMA stimulation post inhibition of Fcγ receptor induced oxidase activation

Neutrophils ($5x\ 10^7$ cells) were incubated with genistein or wortmannin for 15 minutes at room temperature, subsequently oxygen was measured as described in methods and materials. After 7-10 minutes of measurement the cells were stimulated with PMA (0.1μg/ml), and oxygen consumption measured. Oxygen consumption is expressed as percentage activation of equal numbers of neutrophils not exposed to inhibitors and stimulated with $3x10^9$ IgG-opsonised *S. aureus*.

3.3.4. PKC Inhibition

Three PKC inhibitors were tested for their ability to inhibit NADPH oxidase activated by IgG-opsonised *S. aureus* or by PMA. Gö 6976, a member of the indolocarbazole family of chemical compounds quoted to target Ca^+ dependent PKC isotypes, inhibited NADPH oxidase activity stimulated by both PMA and opsonised *S. aureus* with similar efficacy. The IC_{50} values were 2 ± 0.5 μM for PMA and 2.5 ± 0.3 μM for IgG-opsonised *S. aureus* (error represent Standard Error of the Fit, calculated using GraFit Software) (Table 3.2). Full inhibition was observed at high concentrations.

Ro 31-8220, a bisindolylmaleimide compound, targets a broader range of PKC isotypes. It inhibited NADPH oxidase activity stimulated by both PMA and IgG-opsonised *S. aureus* (IC_{50} 0.5 ± 0.1μM and 2.5 ± 2.8μM, respectively). Interestingly while Ro 31-8220 inhibition of PMA stimulation was total, IgG-opsonised *S. aureus* activation was only partially inhibited with a residual activity of approximately 40% remaining even at high concentration of inhibitor. (Table 3.2 and figure 3.4) Bim I, a bisindolylmaleimide compound, related to Ro 31-8220, showed a similar inhibitory profile as Ro 31-8220 (table 3.2). The inhibitors had no effect on oxygen consumption in the absence of stimuli (Gö 6976, 0.08 ± 0.04 nmol O_2/min (n=3) and Ro 31-8220, 0.07 ± 0.03 nmolO_2/min (n=3)). Pre-incubation of the cells with up to 10 μl of DMSO, the maximal amount of solvent used for these experiments, had no effect on the activity of the oxidase (87-100% of control activity remained). However at higher

concentrations (20 µl) DMSO started affecting the neutrophils (39% of control activity) (data not shown).

Inhibitor	IgG- *S. aureus Stimulus*		PMA Stimulus	
	range	IC_{50}	range	IC_{50}
Wortmannin	95.6 ± 7.9	0.02 ± 0.01	NA	NA
Bim I	82.2 ± 30	0.08 ± 0.6	87.5 ± 15.7	0.9 ± 0.3
Gö 6976	100.4 ± 7.2	2.5 ± 0.3	99.2 ± 13.1	2 ± 0.5
Ro 31-8220	62.9 ± 31.9	2.5 ± 2.8	97 ± 6.5	0.5 ± 0.1

Table 3.2 Properties of different kinase inhibitors

Inhibitors were used at different concentrations and dose dependent inhibition of the NADPH oxidase was calculated as described in section 2.3.3 and expressed as percentage of control (non-inhibited sample). The total range of observations and the inhibitory constant at 50% inhibition ± standard error of the mean were calculated using GraFit software (Microsoft) for each inhibitor.

A fourth PKC inhibitor tested on oxidase activation is calphostin C. Like Bim I and Ro 31-8220, it has an effect on a broad range of PKC isotypes. Similarly, calphostin did not inhibit the IgG-opsonised *S. aureus* activation of the oxidase fully but a resistant component remained. (Figure 3.3) However, PMA activation, for calphostin C was not tested.

In conclusion, while the more isotype specific inhibitor Gö 6976 was able to inhibit both stimuli with equal efficacy, three other inhibitors with a broader isotype specificity profile were efficient in inhibition of PMA stimuli but unable to inhibit IgG-opsonised *S. aureus* activation of the NADPH oxidase totally.

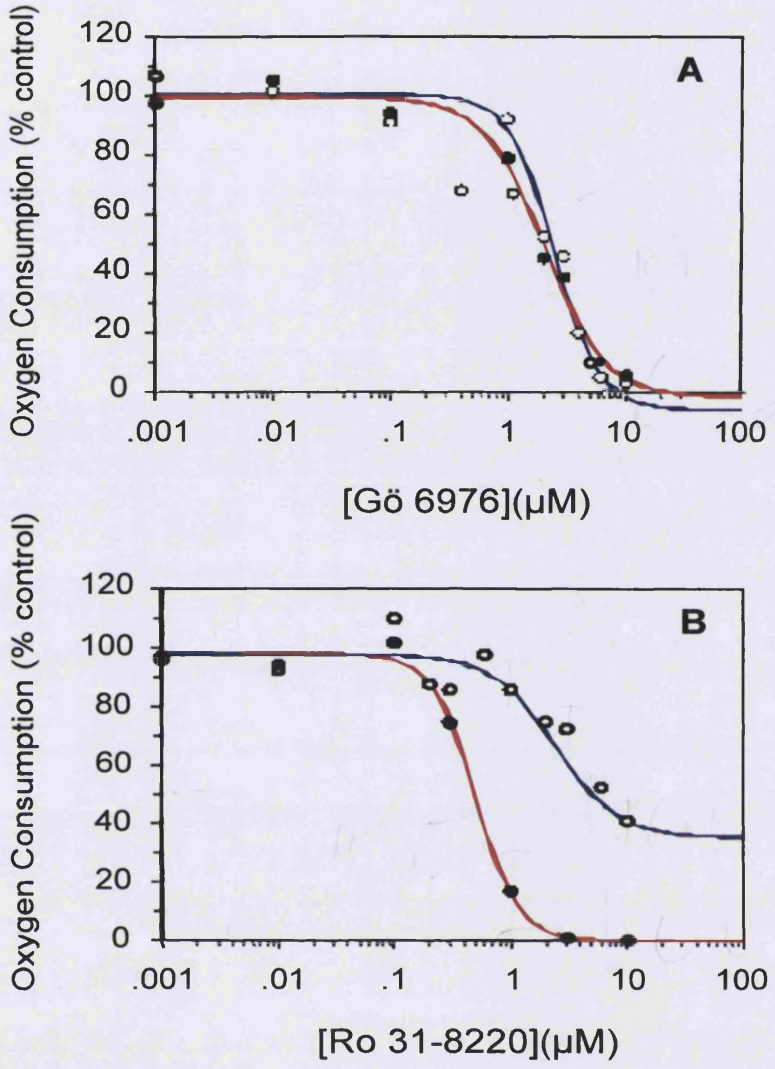


Figure 3.4 Comparison of the effect of Gö 6976 and Ro 31-8220 on PMA and IgG-opsonised *S. aureus* stimulation of the NADPH oxidase

Neutrophils (5×10^7) were pre-incubated with different concentrations of the inhibitors as indicated in the figure. Post stimulation rate of oxygen consumed was measured and expressed as % of control. Graphs were made using GraFit software and represent stimulation with PMA (0.1μg/ml) in red and IgG-opsonised *S. aureus* (3×10^9 bacteria) in blue. This is a compilation of 4-12 observations per concentration point.

Figure 3.4 shows a comparison between Gö 6976 and Ro 31-8220, in which the broad range PKC inhibitor, resistant component can be

observed.

3.3.5. Phagocytosis assays

The inhibition of the NADPH oxidase could be at the level of the oxidase itself or by affecting another part of the phagocytotic mechanism such as particle uptake. In order to differentiate between these possibilities phagocytosis was measured in the presence of several of the above mentioned inhibitors.

A time course was performed with different concentrations of bacteria ($2.5 \times 10^9 - 2.5 \times 10^{10}$) while maintaining the number of neutrophils constant at 1×10^8. (Figure 3.5) All the different concentrations of bacteria used could be ingested by the neutrophils, but the rate of uptake was different. At the lower concentration, bacteria were taken up faster than at higher concentration. This may be due to occupation and saturation of the Fc receptors present on the cells. From this study the concentration of bacteria chosen for further phagocytosis assay was 7×10^9 bacteria/ 1×10^8 neutrophils.

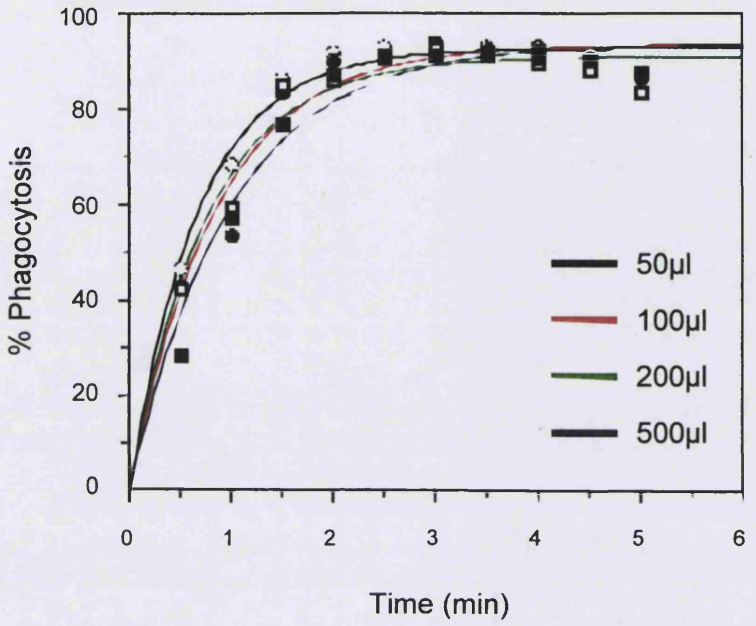


Figure 3.5 Time course of phagocytosis employing different concentrations of IgG-opsonised *S. aureus*

Neutrophils (10^8) were incubated with different concentration of [^{3}H]-labelled IgG opsonised bacteria (5 x 10^{10}/ml) for different times, and phagocytosis assay was performed as described in section 2.3.4. Phagocytosis represents bacterial uptake, expressed as percentage of total number of bacteria added.

Two PKC inhibitors, Gö 6976 and Ro 31-8220) employed above were tested in this system. (Figure 3.6A) Neither affected phagocytosis even at 10µM concentration, which had the greatest effect on activation of the respiratory burst. (section 3.3.3) As a control for inhibition, the effect of wortmannin on phagocytosis was tested. Previously this inhibitor has been reported to inhibit phagocytosis. (Ninomiya, Hazeki *et al.* 1994; Vlahos, Matter *et al.* 1995; Araki, Johnson *et al.* 1996) In our system it proved to be able to inhibit particle uptake by approximately 50%. (Figure 3.6B)

Other isotype specific inhibitors were tested and showed no inhibition of phagocytosis. A PKCδ specific inhibitor, Rottlerin (10μM) (Gschwendt, Muller *et al.* 1994) and PKC β specific inhibitor, Ly 379196 (0.1μM) (Dekker, Leitges *et al.* 2000) had no effect on phagocytosis. (Fig 3.6C) Thus the

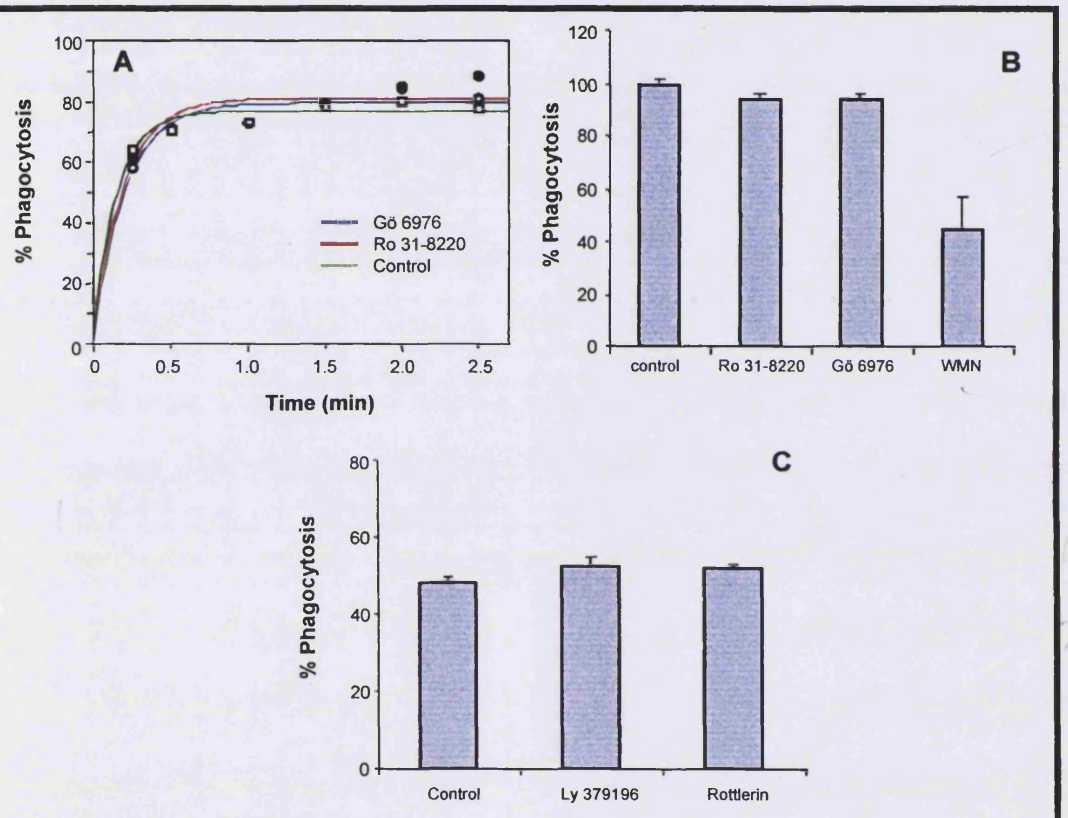


Figure 3.6 Effect of inhibitors on phagocytosis

Neutrophils (10^8 cells) were incubated for 15 minutes, with the respective inhibitor prior to phagocytosis. PKC inhibitors Ro 31-8220, Gö 6976 and rottlerin were all used at a concentration of 10μM. Ly 379196 was applied at a concentration of 0.1μM and wortmannin was used at 100nM. All figures express phagocytosis as percentage of total bacteria added, extrapolated as discussed in section 2.3.4. Panel A shows a time course of phagocytosis and the effect of Gö 6976 and Ro 31-8220 as compared to non-inhibitor control. Panel B is a compilation of % phagocytosis at 1.5 minutes. The graph shows average ± standard error of n=15 for PKC inhibitors and n=4 for wortmannin. Panel C shows the effect of PKC isoform specific inhibitors, Ly 379196 for β and rottlerin for δ, phagocytosis. Like panel B the graph is expressed as % phagocytosis, and each measure is the average and standard error for 4 individual measurements.

PKC inhibitors inhibit the NADPH oxidase activation directly and not through a reduced particle uptake.

3.4. Summary and discussion

In this part of the study, NADPH oxidase activity was measured by determining oxygen consumption with an oxygen electrode, and Fcγ receptor activation of the NADPH oxidase was characterised pharmacologically using kinase inhibitors. Tyrosine kinases, PI3 kinase and PKC are all involved in this signalling cascade. Both tyrosine kinase and PI-3 kinase inhibition can be reversed by direct activation of PKC by PMA. It has been previously shown that tyrosine kinase activity is a very early event in Fcγ receptor signal transduction. Therefore PKC is downstream of tyrosine kinase activity. It is possible that PKC is in a parallel pathway to PI-3 kinase or downstream of it. Reports have shown a tyrosine dependent functional coupling of PI-3 kinase to the intracytoplasmic tail of the Fcγ receptor (Ninomiya, Hazeki *et al.* 1994) showing that this is an early event in this signalling cascade, and therefore possibly upstream of PKC. The temporal relation between PI-3 kinase and tyrosine kinases was never tested. A summary of these findings is shown in figure 3.7.

In order to assess the PKC isoforms involved in this process, different PKC inhibitors were used. Gö 6976, an inhibitor which shows selectivity for

PKC-α and -β *in vitro*, was tested along with Ro 31-8220, which targets all PKC isotypes *in vitro*. Both inhibitors inhibited the NADPH oxidase activated by IgG-opsonised particles in a dose dependent way. Interestingly, while Gö 6976 gave full inhibition at 10 μM, full inhibition of the oxidase by Ro 31-8220 was never observed. This results is counterintuitive as Ro 31-8220 inhibits a broader range of PKC isotypes, which include isotypes inhibited by Gö 6976; however, this observation was seen in 12 individual observations. This concurred with inhibition by calphostin C and Bim I, which have similar inhibitory profiles of PKC isotypes. In contrast, all inhibitors completely inhibited NADPH oxidase activated by 0.1 μg/ml PMA, a direct activator of PKC. It therefore appears that a non-PKC component which is sensitive to Gö 6976 may be involved in NADPH oxidase activation.

At concentrations that inhibited the respiratory burst, the inhibitors did not affect the ingestion of opsonised particles suggesting that the effects of the inhibitors on NADPH oxidase activity are direct and not an effect on particle uptake.

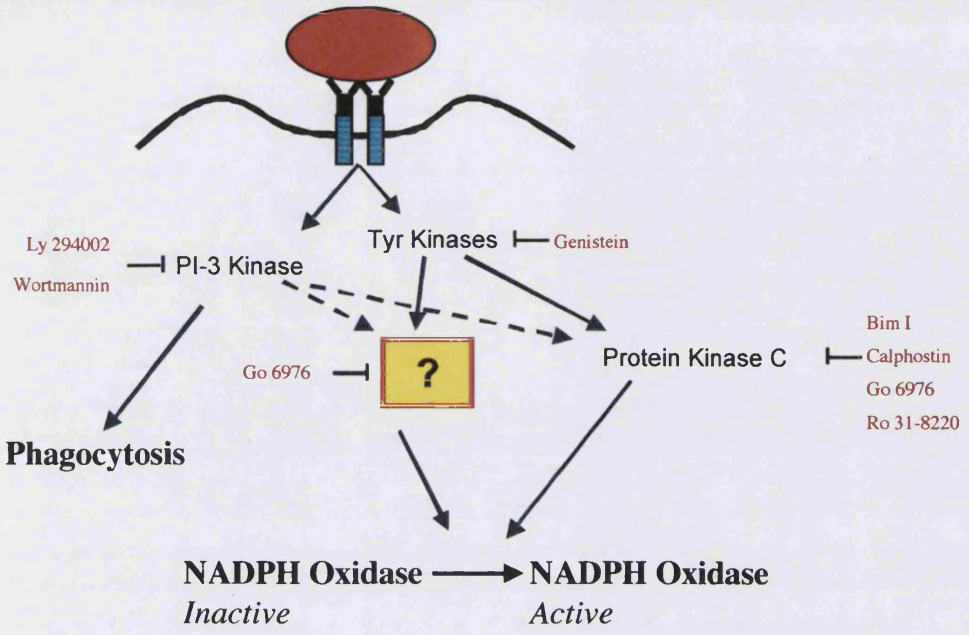


Figure 3.7 Diagrammatic representation of results

Findings in this chapter have allowed the temporal placement of the kinases investigated. It was shown that PKC is probably downstream or in a parallel pathway from tyrosine kinases and PI-3 kinase (dotted lines) in activation of the NADPH oxidase, but has no influence on the process of phagocytosis. The temporal relation between tyrosine kinases and PI-3 kinase in this pathway cannot be concluded from this study. However, the results have shown that a separate component (**?**) affected by Gö 6976 but not the other PKC inhibitors exists, and contributes to the activation of the NADPH oxidase. Analysis of this component is the topic of the following chapters.

We conclude that PKC activated by IgG-opsonised particles impinges directly on the NADPH oxidase and not through an effect on phagocytosis. Furthermore, the differences between Gö 6976 and Ro 31-8220 suggest the participation of a Gö 6976-sensitive, Ro 31-8220 insensitive component in the activation pathway. The following section of this study aims to characterise this component.

Chapter 4

Protein Kinase D invoivement in Fcγ receptor signal transduction pathway

4.1 Aim

The aim of this section was to characterise the Gö 6976-sensitive/Ro 31-8220-insensitive component of NADPH oxidase activation upon Fcγ receptor stimulation which was identified in chapter 3. Different approaches were used to separate the PKC contribution to NADPH oxidase activation (the component inhibitable by Ro 31-8220) and the residual component (non-inhibitable by Ro 31-8220). The component was identified as protein kinase Cμ (Protein kinase D, PKD), and its activation by the Fcγ receptor was shown. Using direct interference methods it was shown that this kinase is involved in Fcγ receptor activation of the NADPH.

4.2 Introduction

It has been reported that Gö 6976 inhibits protein kinase D activity, with a high specificity (IC_{50} 20nM) *in vitro*; whilst inhibitors Bim I and Ro 31-8220 were not very effective against it. (Gschwendt, Dieterich *et al.* 1996; Matthews, Pettit *et al.* 1997) This pattern of inhibition was very similar to the one observed for NADPH oxidase activation by Fcγ receptor engagement, as shown in the previous chapter.

PKD has been identified in lymphocytes (Sidorenko, Law *et al.* 1996; Matthews, Rozengurt *et al.* 1999; Matthews, Iglesias *et al.* 2000); but its presence in neutrophils is not known. PKD has been reported to be activated by antigen receptor engagement, including B and T cell receptor activation. A recent report shows the activation of PKD upon Fcε receptor activation in mast cells. (Sidorenko, Law *et al.* 1996; Matthews, Iglesias *et al.* 2000) PKD activation upon Fcγ stimulation is not known. Upon activation PKD auto-phosphorylates and/or is phosphorylated. (Iglesias and Rozengurt 1998; Matthews, Rozengurt *et al.* 1999; Vertommen, Rider *et al.* 2000) PKD activation can also be determined *in vitro* by its ability to phosphorylate a suitable substrate. Syntide a synthetic peptide derived from glycogen synthase, is an effective substrate for PKD. (Valverde, Sinnett-Smith *et al.* 1994; Dieterich, Herget *et al.* 1996)

The hypothesis in this chapter was that the Gö 6976-insensitive Ro 31-8220-sensitive component was PKD. First we report the presence of PKD

in neutrophils. Furthermore, PKD was found to be activated by Fcγ receptor engagement and this activation was partially regulated by both PKC and PI-3 kinase. Using antisense oligonucleotide interference, activation of PKD was shown to be directly linked to NADPH oxidase activation, on Fcγ receptor dimerisation.

4.3 Results

4.3.1 PKD is present in human neutrophils and activated upon Fcγ receptor activation

Figure 4.1A shows a Western blot of a neutrophil extract probed with a polyclonal PKD antibody (Santa Cruz, SC-935). An immunoreactive band of approximately 110 kD, the appropriate molecular weight for PKD, was detected, and this was competed with the antigenic peptide. In some cases, extra bands were observed. These may represent breakdown products of PKD, since they are the same molecular weight as the catalytic domain observed by others. (Vertommen, Rider *et al.* 2000) The 110 kD immunoreactive band co-migrated with a PKD standard on SDS-PAGE suggesting that it indeed represented PKD. (Figure 4.1B) Figure 4.1C shows a comparison of SC-935 antibody with second antibody raised against the N-terminus of PKD (Calbiochem, 34-290). The 110kD PKD band was observed with both antibodies. Thus PKD is present in neutrophils. Further evidence for the presence of PKD was obtained using

an *in vitro* immunocomplex kinase assay. PKD was immunoprecipitated and the immunoprecipitate was incubated with [γ^{32}P]-ATP. A 110 kD-radiolabelled band was observed in the immunoprecipitate. This band was not observed in control immunoprecipitates performed without PKD antibody, confirming the presence of PKD in the cells. (Figure 4.1D)

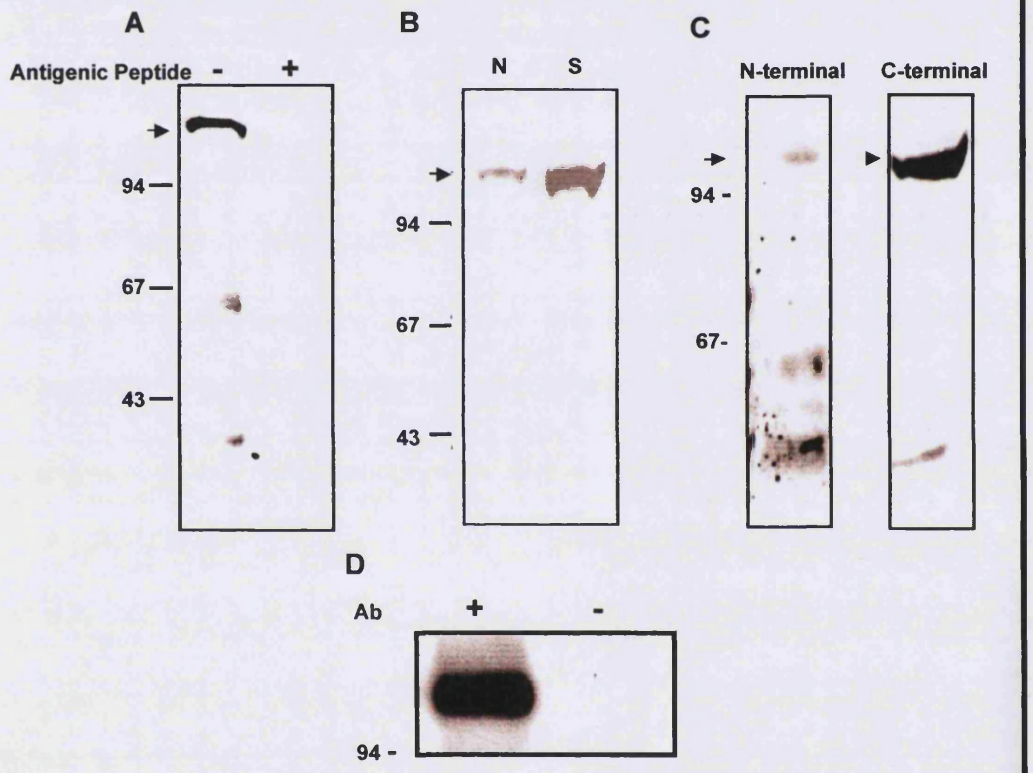


Figure 4.1 Identification of PKD in neutrophils

A: Western blots of neutrophil post-nuclear supernatants (PNS) were incubated with PKD antibody (SC 935) in the presence (+) or absence (-) of the appropriate antigenic peptide (SC 935p). B: Comparison of SC 935 immunoreactive band from neutrophil PNS (N) with recombinant PKD standard (Calbiochem). C: Two different antibodies raised to the C-terminal (SC 935) and N-terminal (34-290) elicited an immunoreactive band of equal molecular weight. D: PKD was immunoprecipitated as described in section 2.3.9, (+) represents addition of antibody (SC 935) was added and (-) no antibody control. To the left the position of molecular weight markers (*MW* in kD) is indicted as well as the 110 kD PKD immunoreactive band (arrows).

Activation of PKD upon Fcγ receptor stimulation was tested after the kinase had been identified in neutrophils. Figure 4.2A shows an autoradiograph of immunoprecipitated PKD in control and Fcγ receptor activation. Western blots of these types of experiments showed that equal amounts of PKD were present in all the extracts. (Data not shown) Upon activation of the Fcγ receptor by addition of IgG-opsonised *S.aureus*, PKD was capable of phosphorylating Syntide three-fold over non-stimulated immunoprecipitates. (Figure 4.2B) These experiments show the activation of PKD in response to stimulation of the Fcγ receptor.

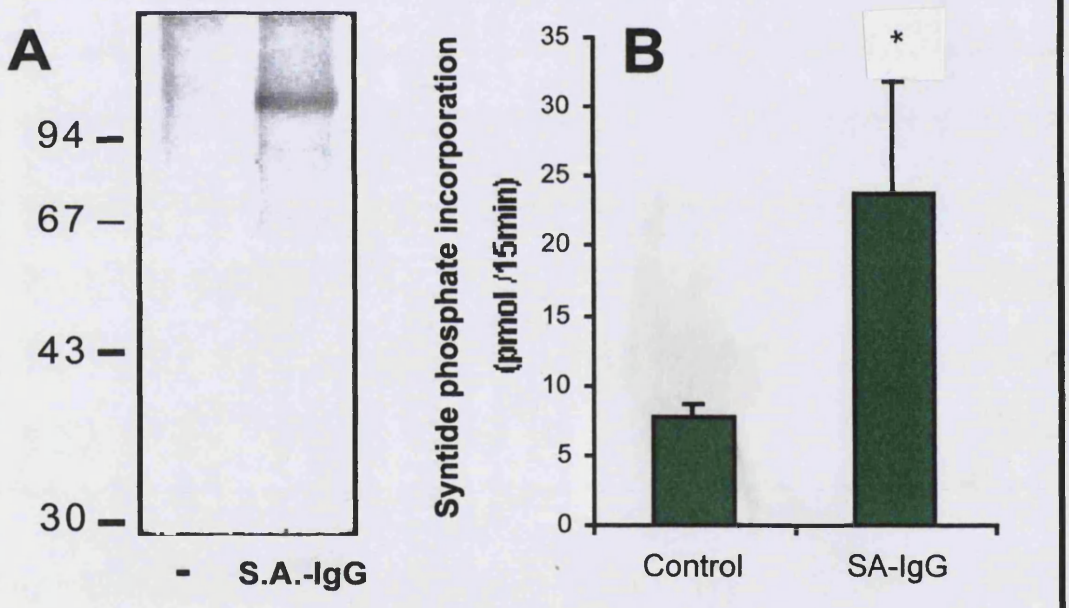


Figure 4.2 Activation of PKD by Fcγ receptor stimulation

Neutrophils (5 x 10^7 cells) were stimulated with IgG-opsonise *S. aureus* (3 x 10^9) for 2 minutes. PKD was immunoprecipitated as described in section 2.3.9, PKD autophosphorylation was measured as described in section 2.3.9b (panel A). Quantification of PKD activity by measuring phosphate incorporation in 10µg Syntide is shown in panel B, n=7 ± standard error; $p<0.05$. On the left of panel A molecular weights are indicated in kD.

* Significantly different from control ($p<0.05$, student T-test)

4.3.2 PKD is involved in NADPH oxidase activation

As shown in figure 3.4, activation of the NADPH oxidase by Fcγ receptor occupation is inhibited with Ro 31-8220, but a residual component remains. As suggested in section 4.3.1 this component could be PKD, and indeed PKD is present in neutrophils and is activated upon Fcγ receptor activation.

In order to investigate if PKD would mediate NADPH oxidase we made use of the fact that the different PKC isoforms can be depleted by prolonged treatment with PMA (Stabel, Rodriguez-Pena *et al.* 1987), but that PKD is not downregulated by this treatment. (Rennecke, Johannes *et al.* 1996) Neutrophils were incubated in the presence of PMA for four hours, and Western blot was performed using antibodies that recognised different isotypes of PKC. It can be seen that after 4 hours PKC isoforms βI, βII, and δ were all downregulated and no presence of these kinases could be detected. Furthermore, PKD was not downregulated and could still be detected by this method. (Figure 4.3)

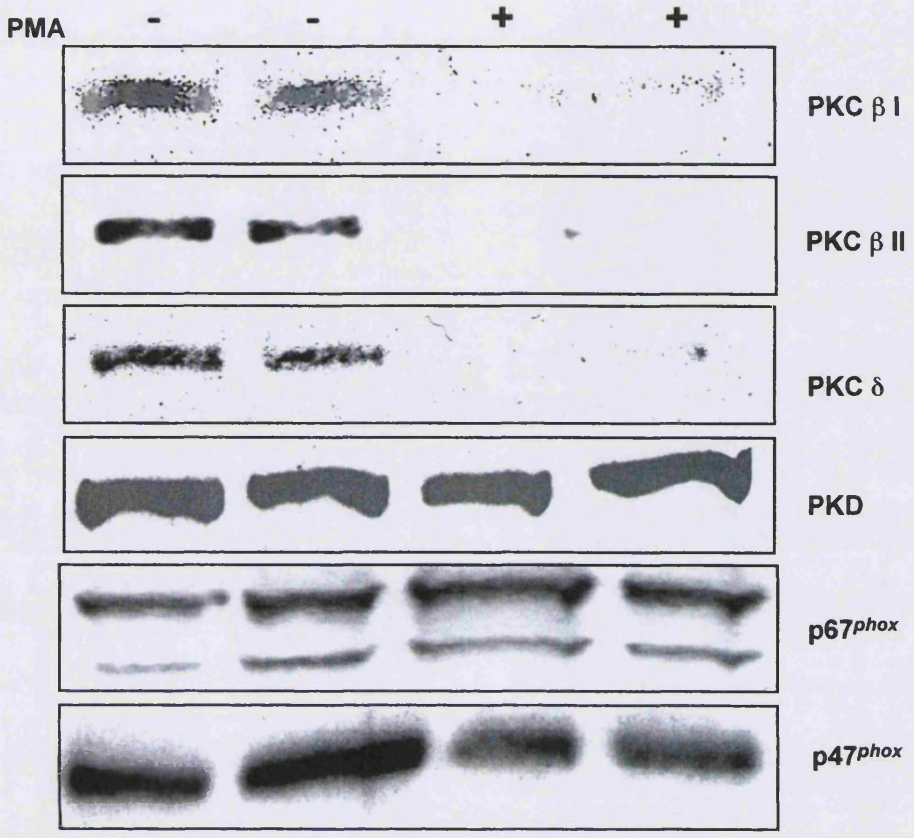


Figure 4.3 Effect of long term treatment, on neutrophils proteins, with PMA

Neutrophils (5×10^7) were pre-incubated with 0.1µg/ml PMA (+) of without PMA (-) for four hours. After 4 hours cells were extracted in Laemmli sample buffer (2.2.5) containing protease inhibitors (2.2.10) and analysed by SDS-PAGE and Western blotting for the presence of PKC βI, PKC βII, PKC δ, PKD, $p67^{phox}$ or $p47^{phox}$.

The effect on NADPH oxidase activation was tested following prolonged PMA treatment. First we analysed the effect of the down regulation on PMA induced NADPH oxidase. It was expected that if downregulation had been successful no activation would occur in response to acute PMA treatment, as no PKC should be present in the cells. Figure 4.4 shows that

this is indeed the case. Cells pre-incubated for four hours in the absence of PMA, in which PKC down regulation had not occurred, retained the response to acute PMA stimulation. (Figure 4.4) The loss of NADPH oxidase activation in PMA-pre-treated cells was not due to an effect of the actual PMA pre-treatment, on basal rates of oxygen consumption. For PMA pre-treated cells this was 0.1 ± 0.1 nmol/min/10^7 (n=10) and cells pre-incubated in the absence PMA basal consumption was 0.2 ± 0.1 nmol/min/10^7 (n=10). The four hours pre-incubation period itself did not change the inhibition characteristics of oxygen consumption; PMA-induced oxygen consumption, observed in cells pre-incubated for four hours in the absence of PMA, was completely inhibited by both Gö 6976 and Ro 31-

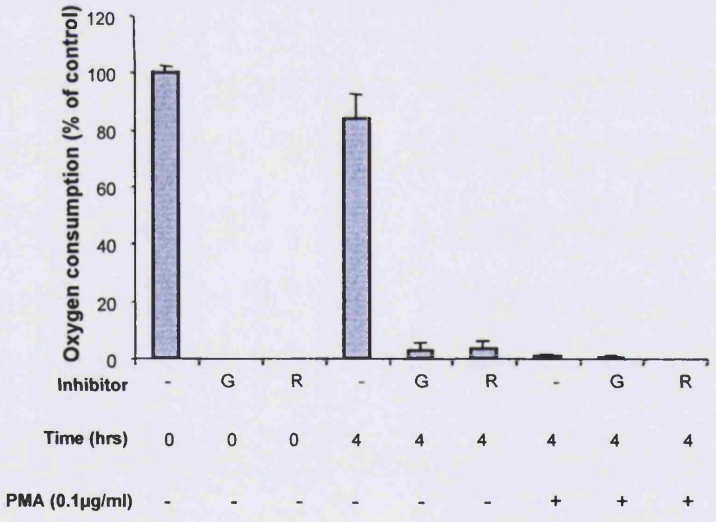


Figure 4.4 PMA stimulation post prolonged treatment with PMA

Neutrophils (5 x 10^7 cells) were pre-incubated with 0.1µg/ml PMA (+) or without PMA (-) for four hours at 37°C. After 4 hours, oxygen consumption was measured in response to 0.1µg/ml PMA in the absence or presence of 10µM Gö 6976 (G) or 10µM Ro 31-8220 (R) as indicated. Oxygen consumption was expressed as percentage of the activity at the start of the 4 hours incubation (26 ± 5 nmol/min/10^7 cells; n=18)

8220 as was observed in cells that had not been pre-incubated. (Figure 4.4 and 1.6) Thus PKC downregulation leads to a complete loss of NADPH oxidase activation by PMA.

Oxygen consumption induced by IgG-opsonised *S. aureus* was also reduced in PMA pre-treated neutrophils (Figure 4.5) when compared to cells incubated for four hours in the absence of PMA. However, a non-downregulated component remained even after four hours of PMA pre-treatment. This residual activity was inhibited by Gö 6976 but not by Ro 31-8220. The residual component after PMA pre-treatment (25.1 ± 6.3 % of control, n=15) was similar to the Ro 31-8220 resistant component in non-treated neutrophils (27.9 ± 0.5 % of control) and to the Ro 31-8220 resistant component in cells incubated for four hours in the absence of PMA (33.9 ± 10% of control, n=6). The change in oxygen consumption capacity by pre-treated neutrophils was related to PMA pre-treatment as neutrophils incubated for four hours in the absence of PMA could sustain an Fcγ receptor induced oxygen consumption of 87 ± 10 % (n=15) of non-incubated control. The basal levels of oxygen consumption in the absence of stimulus remained similar whether the neutrophils were pre-treated with or without PMA or no pre-incubation took place. (Figure 4.5) The effects seen of PMA pre-treatment on NADPH oxidase activity appear to be directly related to PKC depletion as components ($p47^{phox}$ and $p67^{phox}$) of the NADPH oxidase were unaffected by this pre-treatment. (Figure 4.3) In all

cases Gö 6976 was able to inhibit Fcγ receptor stimulation of the NADPH oxidase completely.

These experiments suggests that the Ro 31-8220 inhibitable component is PKC since treatment with Ro 31-8220 could no further decrease NADPH oxidase activation after depletion of PKC by PMA treatment. The Ro 31-8220 non-inhibitable component, down-regulation resistant activity was still sensitive to Gö 6976 treatment suggesting that a non PKC kinase participated in oxidase activation. All the evidence suggests that it is PKD.

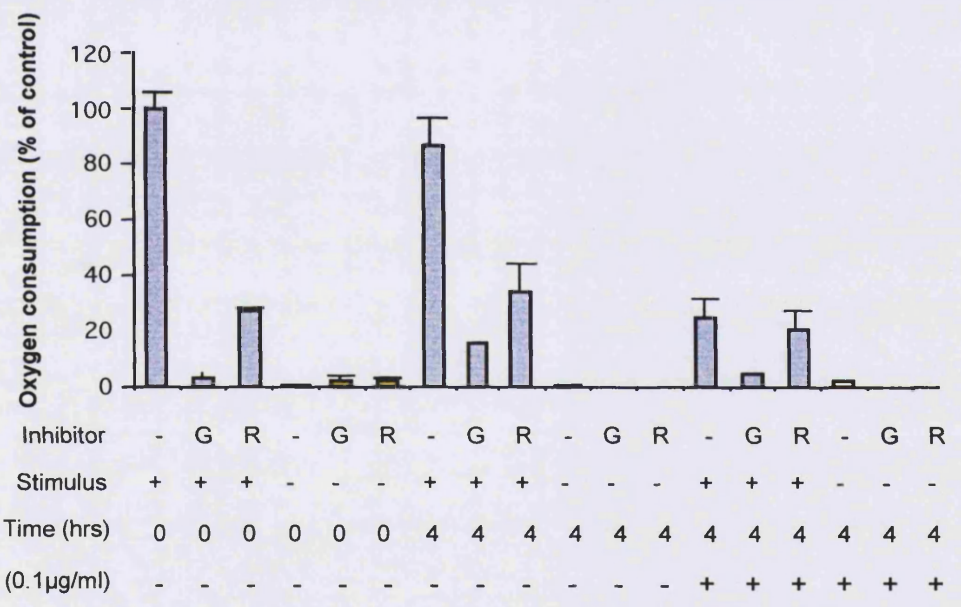


Figure 4.5 IgG-opsonised *S. aureus* stimulation post prolonged treatment with PMA

Neutrophils (5 x 10^7 cells) were pre-incubated in the presence (+) or absence (-) of 0.1µg/ml PMA for four hours at 37°C. Oxygen consumption was measured in response to IgG-opsonised *S. aureus* (3 x 10^9 bacteria) in the absence (-) or presence of 10µM Gö 6976 (G) or 10µM Ro 31-8220 (R). Oxygen consumption is expressed as percentage of activity at the start of the 4 hour incubation (18 ± 3 nmol/min/10^7 cells; n=15). Buffer (RPMI 1640) stimulation produced no oxygen consumption under any of the conditions used (yellow bars)

As discussed in section 3.3.4, the effects seen on NADPH oxidase inhibition could be a result of phagocytosis inhibition. Hence, the efficiency of phagocytosis by neutrophils pre-treated with and without PMA for four hours was tested. Figure 4.6 shows no effect of four hour PKC down regulation (PMA pre-treated cells) on the phagocytosis of ^{3}H-labelled IgG-opsonised *S. aureus* as compared to cells not pre-treated with PMA. This finding correlates with observations in figure 3.6, which shows no effect of a range of PKC inhibitors on phagocytosis.

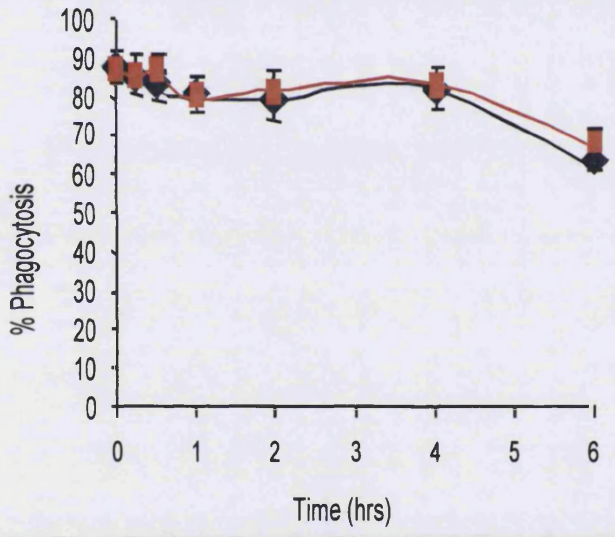


Figure 4.6 Effect of PKC down regulation on phagocytosis.

Neutrophils (5 x 10^{7} cells) were incubated with (blue) or without (red) 0.1µg/ml PMA for different times, as indicated, at 37°C. Phagocytosis was assayed as described in section 2.3.4. Phagocytosis is expressed as percentage of total bacteria presented to the cells (n=10 ± Standard error).

4.3.3 Antisense interference

In order to obtain direct evidence for the involvement of PKD in Fcγ receptor oxidase activation, antisense phosphothiorated oligonucleotides were used.

Neutrophils have long been thought to have no need or capacity for *de novo* protein biosynthesis. This belief arose from studies on the

morphology of these cells. Electron micrographs showed that neutrophils had little endoplasmic reticulum and few ribosomes. Furthermore, the condensed chromatin in its nuclei suggested that they were transcriptionally inactive. However, experiments performed in the 1960's by MJ Cline showed that RNA turnover occurred in neutrophils during phagocytosis. (Cline 1966) Other studies indicated that proteins can be newly synthesised by mature peripheral blood neutrophils in cell culture. (Granelli-Piperno, Vassalli *et al.* 1979)

To test whether protein synthesis happens in peripheral blood neutrophils, the incorporation of [^{35}S]-methionine into the protein pool was determined. Peripheral blood cell neutrophils were incubated at 30°C for 18 hours with [^{35}S]-methionine, after which the proteins were analysed. Figure 4.7 shows the result of the analysis of total protein in [^{35}S]-methionine labelled neutrophils.

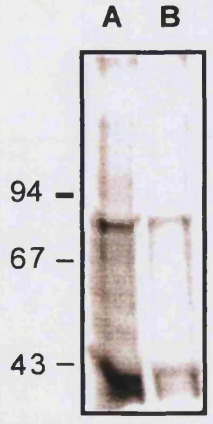


Figure 4.7 Protein synthesis in peripheral blood neutrophils

Neutrophils (10^7 cells) were incubated with 1mCi of [^{35}S] methionine at 30°C for 18 hours. Whole cell sample was extracted in Laemmli sample buffer (2.2.5) containing protease inhibitors (2.2.10) and analysed by SDS-PAGE and autoradiography. On the left of the figure molecular weight markers (kD) are shown.

Though the level of incorporation of radiolabelled methionine into proteins differs in the two lanes (A and B), it can be seen that many radioactive bands are present, indicating the presence of newly synthesised protein. It was concluded that protein synthesis occurs and therefore direct inhibition of PKD synthesis by using antisense PKD oligonucleotide could in principle be achieved. This was proven through Western blot analysis. Figure 4.8 shows that the levels of PKD are reduced in cells treated with antisense oligonucleotides.

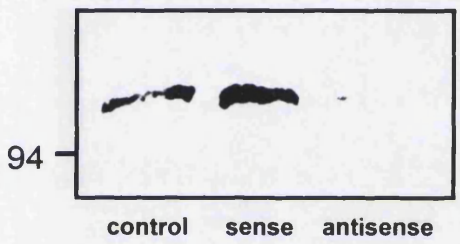


Figure 4.8 PKD depletion by using antisense oligonucleotides

Neutrophils (10^7 cells) were incubated for 18 hours at 30°C in the absence (control) or presence of 10μM sense or antisense, as indicated. Whole cell sample was extracted in Laemmli sample buffer (2.2.5) containing protease inhibitors (2.2.10) and analysed by SDS-PAGE and western blot for the presence of PKD (SC935). 94 kD marker indicated on the left. n = 10

Antisense treatment was used to test the involvement of PKD in NADPH oxidase activation. Typically, 10^7 cells stimulated with $3x10^9$ IgG-opsonise *S. aureus*, showed a rate of oxygen consumption of 32 ± 5 nmol O_2/min (n=9). After 16 hours of incubation in RPMI medium the rate of oxygen consumption was 26 ± 4 nmol O_2/min (n=15). Since these cells retained their oxidative burst after such a prolonged incubation in RPMI the effect of PKD depletion on oxidase activity could be determined. Neutrophils were incubated for 16 hours with antisense or sense PKD

oligonucleotides after which oxygen consumption was measured. Oxygen consumption in response to Fcγ receptor activation was reduced by 51% in cells treated with antisense oligonucleotide when compared with cells treated with sense oligonucleotide. (Figure 4.9) No significant difference was observed between sense-treated (22 ± 3 nmol/min/10^7 cells; n=15) and non-treated cells (26 ± 4 nmol/min/10^7 cells; n=15). The basal rate of oxygen consumption, seen as the response to buffer stimulation (non-stimulus) was 0.5 ± 0.2 nmol/min/10^7 cells (n=12) and no differences were observed between sense-treated, antisense-treated and non-treated cells.

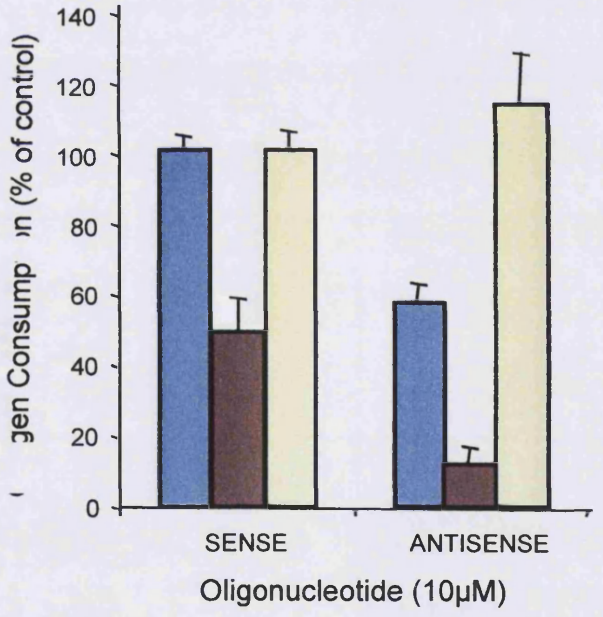


Figure 4.9 Effect of PKD antisense treatment on NADPH oxidase activation

Neutrophils (10^7 cells) were treated as described in figure 4.9. After treatment, oxygen consumption was measured in response to 0.1μg/ml PMA (yellow) of IgG-*SA* (10^9 bacteria) in the presence (red) or absence (blue) of 10μM Ro 31-8220. Oxygen consumption is expressed as percentage of sense-treated cells (22 ± 3 nmol/min/10^7 cells; n=15).

A number of reports suggest that PKD activity in the cells is controlled by PKC isotypes as reflected by PKD autophosphorylation and Syntide phosphorylation performed on *in vitro* immunoprecipitated kinase assays. (Zugaza, Sinnett-Smith *et al.* 1996; Van Lint, Ni *et al.* 1998; Paolucci and Rozengurt 1999; Matthews, Rozengurt *et al.* 2000; Vertommen, Rider *et al.* 2000) We therefore examine the effect of PKD depletion by antisense oligonucleotides on phorbol ester activation of the NADPH oxidase which goes through PKC. Treatment with PKD antisense had no effect on oxygen consumption induced by 0.1 μg/ml PMA. (Figure 4.9) Altogether these results show that PKD participates in activation of the NADPH oxidase upon Fcγ receptor stimulation of neutrophils, but not on PMA activation.

We next investigated if PKD represents the Ro 31-8220 insensitive component of oxidase activation, as suggested previously. As shown in figure 4.9, Ro 31-8220 inhibits NADPH oxidase activation in sense treated cells by 52%; this is comparable to the inhibition in freshly isolated cells treated with Ro 31-8220. (See figure 3.4 and table 3.2) The treatment of cells with antisense oligonucleotides along with Ro 31-8220 resulted in almost total inhibition of NADPH oxidase activity. Therefore it was concluded that the Ro 31-8220 insensitive component is PKD.

The stimulation of the NADPH oxidase by PKD was not caused by an effect of this kinase on phagocytosis, as treatment with antisense oligonucleotides did not inhibit phagocytosis of ^{3}H-labelled bacteria. (Figure 4.10) This finding concurs with the findings that the inhibitor, Gö 6976 did

not inhibit phagocytosis. (Figure 3.6) Since phagocytosis is the same in control, sense and antisense treated cells, the effect of the antisense oligonucleotides on NADPH oxidase appears to be specific and not related to an effect on other cellular processes, such as cell integrity and cell survival. Thus PKD is directly involved in the activation of NADPH oxidase by Fcγ receptors.

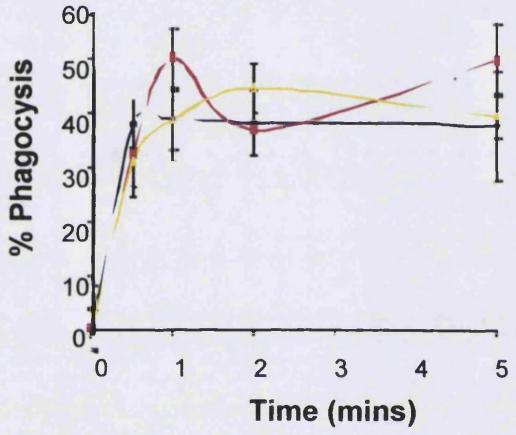


Figure 4.10 Effect of antisense PKD oligonucleotide on phagocytosis

Neutrophils were treated as described in figure 4.8, subsequently phagocytosis was assessed as described in section 2.3.4. Blue represents cells incubated in the absence of oligonucleotide (n=15), while violet, sense treated cells (n=11) and yellow antisense treated cells (n=12). Phagocytosis is expressed as percentage of total bacteria presented to the cells.

4.3.4 Regulation of PKD activity through PKC and PI-3 kinase in Fcγ receptor stimulation

The data above suggest that the Fcγ receptor engages PKD in order to regulate the NADPH oxidase. As previously mentioned PKD activation has been shown to be regulated by PKC. PKD activation has been shown for the antigen receptor such as the B cell receptor, T cell receptor and Fcε

receptor. (Matthews, Rozengurt *et al.* 2000) The different levels of inhibition and activation in several of our experiments led us to study the regulation of PKD in our system. As can be seen in figure 3.4 and table 3.2, maximal Ro 31-8220 inhibition was 62.9% of control leaving a residual component of approximately 40%. This result is not completely complementary to PKD antisense oligonucleotide pre-treatment which lead to an inhibition of 51%. The discrepancy could be due to the fact that Ro 31-8220 while selectively inhibiting PKC isotypes also has an effect on PKD by inhibiting the PKC contribution to activation of PKD. Therefore in order to test if PKC is involved in the activation of PKD by Fcγ receptor stimulation the PKC inhibitor Ro 31-8220 was again employed. Pre-treatment with 10μM inhibitor results in an inhibition of Fcγ receptor activation of PKD, measured by PKD autophosphorylation (figure 4.11A) compatible with an involvement of PKC. However, inhibition by Ro 31-8220 was not complete and some activation of PKD could be observed even in the presence on the PKC inhibitor, suggesting a PKC-independent PKD activation.

PKD contains a pleckstrin homology domain (PH domain). (Waldron, Iglesias *et al.* 1999) The PH domains have been shown to be important in regulation of activity in this kinase. (Iglesias and Rozengurt 1998; Waldron, Iglesias *et al.* 1999) It has been reported that PH domains binds phosphoinositides (Harlan, Hajduk *et al.* 1994; Lemmon, Ferguson *et al.* 1995), and the product of PI-3 kinase, PIP_3 (phosphoinositide-3,4,5-triphosphate) has been shown to signal through proteins containing PH

domains. (Klarlund, Guilherme *et al.* 1997) Therefore the PKC-independent contribution to PKD activation (figure 4.11), could be mediated by PI-3 kinase contribution. This was tested by assessing the effect of a PI-3 kinase inhibitor (Ly 294002) on PKD activation. Figure 4.11 (panel A and B) shows that Ly 294002 reduces PKD autophosphorylation. Incubation with both inhibitors showed a further reduction in autophosphorylation of PKD. Figure 4.10B shows graphically a compilation of three separate experiments, where the effect of inhibitors is expressed as percentage of un-inhibited cells. Autophosphorylation was determined by densitometry using NIH image software. Both PI-3 kinase inhibitor and PKC inhibitor were able to reduce autophosphorylation by approximately 50%, suggesting involvement of both kinases in regulation of PKD activity; combination of the inhibitors further suppressed PKD activation. For each experiment, total amount of PKD extracted from the cells was analysed by Western blotting. (Figure 4.11C)

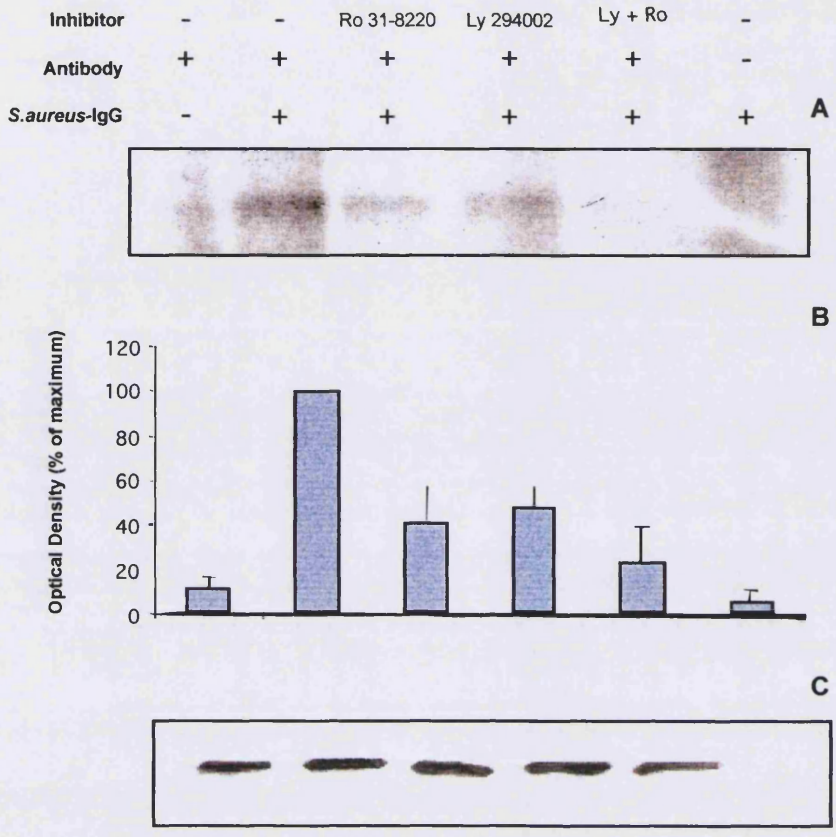


Figure 4.11 Regulation of PKD is via PKC and PI-3 kinase

Neutrophils were incubated for 2 minutes at 37 ˚C with IgG-opsonised *S. aureus* in the absence (-) or presence of inhibitors. Ly 294002 was employed at 50μM and Ro 31-8220 at 10μM. PKD was immunoprecipitated and autophosphorylation kinase assay was performed as described in section 2.3.9b. (panel A). Data was analysed using NIH image software and expressed as percentage of stimulated, non- inhibited sample (n=5± standard error). Panel C: prior to immunoprecipitation a sample of extract was taken and analysed by SDS-PAGE and Western blot for the presence of PKD.

4.4 Summary and discussion

In this section it has been shown that PKD is present in neutrophils, and activated by Fcγ receptor activation. PKD activation was shown by

autophosphorylation and by Syntide phosphate incorporation. PKD was identified as the residual NADPH oxidase activity insensitive to Ro 31-8220, but sensitive to Gö 6976.

Depletion of PKC by long term pre-treatment with PMA showed that indeed the Ro 31-8220-sensitive component was indeed PKC.

Pre-treatment with PMA decreased IgG-opsonised *S. aureus* induced oxygen consumption by 75%, this could further be reduced by Gö 6976 to 4.7 ± 2% of maximal stimulation; however, it was completely insensitive to Ro 31-8220 under which a residual component, of 20.9 ± 6 % of maximal stimulation, still remained. Confirmation that this resistant component is PKD was performed by employing antisense oligonucleotides in order to directly interfere with PKD in the cells.

It was shown that incubating the cells with antisense oligonucleotides targeted against PKD could deplete kinase protein levels in neutrophils. Furthermore, depletion of PKD by antisense decreased oxygen consumption by 49% of maximal activity, and the remaining activity was Ro 31-8220-sensitive.

Initially it had been shown that Ro 31-8220 could limit Fcγ receptor activation of the NADPH oxidase to 40% of maximal stimulation, while depletion of PKC by long term treatment with PMA decreased oxygen consumption by 75%, but direct depletion of PKD by employing antisense decreased oxygen consumption by 49%. Therefore, there appears to be cross talk between PKC and PKD. PKD activation in the presence of PKC

inhibitor was tested, and shown to be decreased; this was further decreased by the presence of a PI-3 kinase inhibitor Ly 294002, placing both PKC and PI-3 kinase upstream of PKD as regulations of the latter; though a residual component remained in the activation of PKD, implying that other factors may contribute to PKD activation.

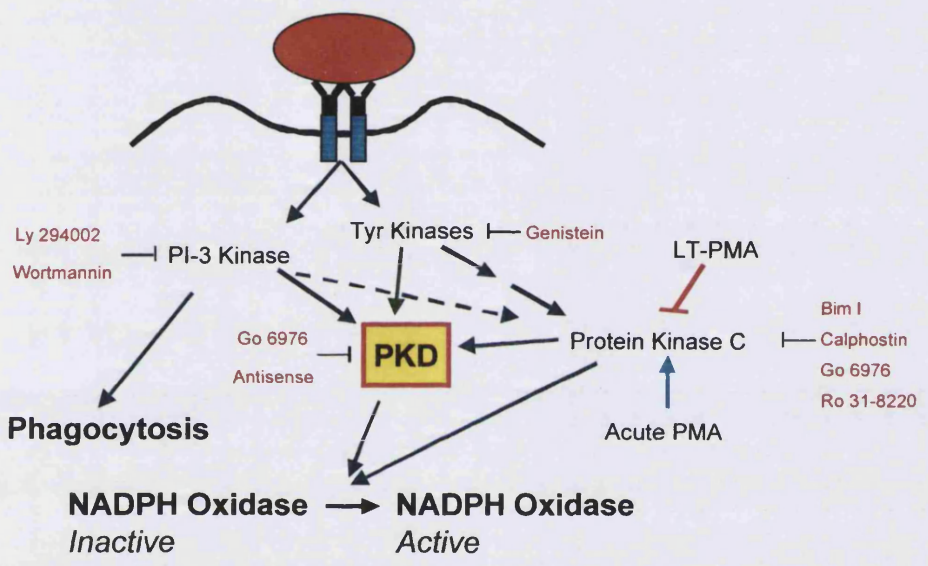


Figure 4.12 Summary of findings

PKD is placed in the signalling cascade leading to NADPH oxidase activation upon Fcγ receptor stimulation. It has been shown that PI-3 kinase and PKC both contribute to its regulation. Whether tyrosine kinases regulate PKD directly was not tested, and tyrosine phosphorylation sites on PKD have not been determined. The arrow connecting PKD and tyrosine kinases is green as this may be the third component that regulates PKD activity in this signalling cascade.

In summary, PKD was placed in the signal transduction cascade leading to activation of NADPH oxidase by Fcγ receptor stimulation. It was also show that PKD is regulated by PKC and PI-3 kinase independently, each contributing approximately to 50% of its activity. We, however, do not rule

out that other regulatory mechanisms might be used. How PKD contributes to the activity of the NADPH oxidase in not clear at this point and will be the subject of later experiments in this study. (Figure 4.12)

Chapter 5

Subcellular localisation of PKD

5.1 Aim

To further evaluate the role of PKD in neutrophils, its subcellular localisation was determined. The effect of the Fcγ receptor stimulation on PKD localisation was investigated as well as co-localisation with $p47^{phox}$. It was observed that PKD appears mostly in the plasma membrane and upon phagocytosis it concentrates in the phagosome. This was confirmed by observing that PKD co-localised with $p47^{phox}$ in phagosomal membrane.

5.2 Introduction

NADPH oxidase activation requires translocation and assembly of its components on the phagosomal membrane. (Heyworth, Curnutte *et al.* 1991; Garcia, Whitaker *et al.* 1992) In the previous chapter we showed that PKD plays a role in NADPH oxidase activation. (Section 4.3.3) Several components of the NADPH oxidase are known to be phosphorylated upon

activation. (Section 1.10) PKD could play a direct role in activation of the NADPH oxidase by phosphorylating one or more of these components, and if this is the case PKD would be expected to be present in the phagosomal membrane upon activation. This principle has been shown for PKC β, which is involved in NADPH oxidase activation. (Dekker, Leitges *et al.* 2000)

The regulatory region of PKD contains domains that would target the kinase to the membrane fraction. In its regulatory domain, PKD contains a cysteine rich domain (C1), a pleckstrin homology (PH) domain and an N-terminal hydrophobic region. (Waldron, Iglesias *et al.* 1999) In other proteins these domains are important in sub-cellular localisation. For example, PKC translocation to membrane fractions is driven by its cysteine rich domain (C1 domain) (Oancea, Teruel *et al.* 1998), whereas proteins that contain PH domains are associated with membrane surfaces. (Lemmon, Ferguson *et al.* 1995)

The C1 domain of PKD binds phorbol esters with high affinity (Valverde, Sinnett-Smith *et al.* 1994), and it mediates PKD translocation to the membrane fraction in RBL 2H3 mast cells in response to phorbol esters. (Matthews, Iglesias *et al.* 1999)

The PH domain, however, appears to play more of a regulatory role. Some studies have shown that it is a negative regulatory domain within PKD (Iglesias and Rozengurt 1998), while others have shown that it may play a positive regulatory role by interacting with the Gβγ sub-unit of

heterotrimeric G-proteins in regulation of Golgi complex organisation. (Jamora, Yamanouye *et al.* 1999) The latter coincides with the finding that in hepatocyte carcinoma cell line, HepG2, PKD is localised in the Golgi complex. (Prestle, Pfizenmaier *et al.* 1996) For the hydrophobic or transmembrane N-terminal tail of PKD, a definitive role has not yet been defined.

In immune cells PKD is activated by antigen receptors (Sidorenko, Law *et al.* 1996; Johannes, Hausser *et al.* 1999); furthermore it has been shown to translocate to the membrane upon such activation. (Matthews, Iglesias *et al.* 1999; Matthews, Iglesias *et al.* 2000)

We aimed to explore the localisation of PKD within human neutrophils and see the effect of Fcγ receptor stimulation upon its subcellular localisation. Based on the fact that PKD is activated upon Fcγ receptor stimulation and that it plays a role in NADPH oxidase activation, it was hypothesised that PKD might be present in the phagosomal membrane upon stimulation.

5.3 Results

5.3.1 PKD resides mostly in the membrane fraction of resting neutrophils

In order to determine the sub-cellular localisation of PKD, sucrose density gradient experiments were performed. The gradients were

characterised using the following markers for subcellular fractions: $p47^{phox}$ as a cytosolic marker, lactoferrin as a marker for specific granules and myeloperoxidase (MPO) as a marker for azurophilic granules. A post-nuclear supernatant (PNS) was made from 1 x 10^8 neutrophils and loaded onto a 5 – 50% sucrose (w/w) gradient. After centrifugation, 0.5 ml fractions were collected and analysed by Western blot using $p47^{phox}$, lactoferrin and MPO antibodies. (Figure 5.1)

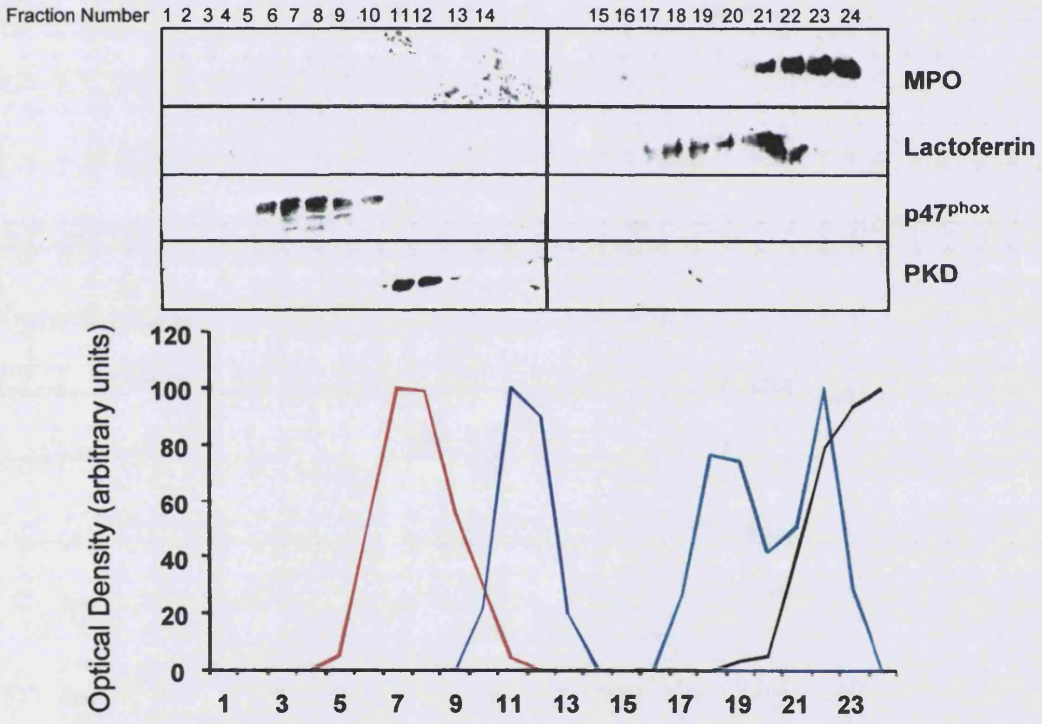


Figure 5.1 Sucrose density gradient fractionation of neutrophils

Post nuclear supernatant was extracted in *Lambert* break buffer (section 2.2.6) containing protease inhibitors (section 2.2.10) from neutrophils (10^8 cells), and fractionated in a 5-50% (w/v) sucrose gradient. Fractions were collected in 500µl intervals and analysed by SDS-PAGE and Western blot. Blots were probed for myeloperoxidase (MPO), lactoferrin, $p47^{phox}$ and PKD (top figure). Bottom panel: graphical representation of signal analysed by NIH image software. (Red=$p47^{phox}$; blue=PKD; green=lactoferrin; black=MPO)

For this study the most obvious choice as a membrane marker would have been gp91^{phox}, but due to lack of availability this was excluded.

p47^{phox} was found in fraction 5 through 10, which therefore represented the cytosol. An immunoreactive band with anti-lactoferrin antibody appeared in fractions 16 to 22, and MPO antibody in fractions 20 to 26. These fractions therefore contained the respective intracellular granules. By exclusion it was determined that fractions 10 to 14 were the plasma membrane, as it is known to migrate through this density gradient between the cytosolic and granular fractions. (LV Dekker personal communication) A protein band, which reacted to the anti-PKD antibody, was found in fraction 10 to 14, indicating that PKD in resting neutrophils resides predominantly in the plasma membrane.

5.3.2 Total PKD increases in the membrane fraction upon Fcγ receptor stimulation.

The sub-cellular profile for PKD, upon stimulation with IgG-opsonised *S. aureus*, was determined. Neutrophils ($5x10^7$ cells) were stimulated with IgG-opsonised *S. aureus* ($3x\ 10^9$) for different times as shown in figure 5.2. A post-nuclear supernatant was prepared and centrifuged over a 40% (w/w) sucrose cushion in order to remove granular fraction along with un-lysed cells. The supernatant and floating membrane were removed and ultracentrifuged to separate the cytosol from the membrane. Cytosol and membrane were analysed by SDS-PAGE and immunoblotted for different

proteins. As can be seen in figure 5.2, p47^{phox} is not present in membrane but upon stimulation there is a time dependent translocation to that fraction. It can also be noticed that gp91^{phox} is present in the membrane fraction and not in the cytosol and that there is a slight increase upon stimulation. PKC βI and PKC δ are present in control cytosol, and decrease upon stimulation. Concomitantly, there is an increase of the two PKC isoforms in the membrane fractions. As seen in figure 5.1, PKD is present in control plasma membrane and absent from the cytosol. Upon stimulation the PKD immunoreactive signal increases in the membrane fraction. Coomassie stained gels showed that protein loading were similar for all lanes, and this would imply that increase and decrease of protein levels in the different fractions are not by-products of extraction errors.

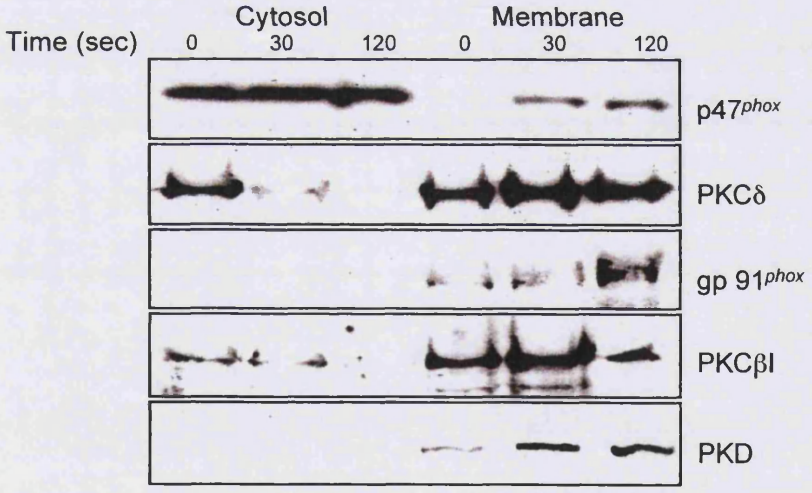


Figure 5.2 Behaviour of PKD upon Fcγ receptor stimulation in neutrophils.

Neutrophils (5 x 10^7 cells) were stimulated with IgG-*SA* (3 x 10^9 bacteria) for different times, as indicated. Membrane and cytosol fractions were extracted as described in section 2.3.8e. Cell fractions were analysed by SDS-PAGE and Western blot and probed for p47^{phox}, PKC δ, gp 91^{phox}, PKC βI, and PKD.

Though PKD was not detected in any other fraction except for the plasma membrane in unstimulated cells, upon activation its signal increases in the membrane, thus hinting at the possibility that PKD is located in another subcellular location from which it translocates.

5.3.3 PKD is present in the phagosomal membrane upon phagocytosis

Since NADPH oxidase activation takes place at the phagosome, we tested whether PKD is present in the phagosomal membrane. Neutrophils (10^9) were incubated with and without IgG-opsonised magnetic beads (50% slurry in RPMI 1640) for two minutes at 37°C. The cells were then homogenised twenty times using a dounce homogeniser. Phagosomal membrane was recovered and washed three times. After extraction of the phagosomal membrane, the cytosol and membrane of both stimulated and unstimulated neutrophils were isolated. All fractions were analysed by SDS-PAGE and Western blotting using PKD antibody (Santa Cruz, SC 935). Figure 5.3 shows that there is a presence of PKD in the cytosolic fraction, and this could probably now be seen due to the large amount of cells used in these experiment. Such cell numbers had to be used as phagosomal membrane recovery is not very efficient. However, as seen previously, PKD is predominantly in the membrane, much more than in the cytosol, and upon stimulation with IgG-opsonised particles, PKD levels are increased. Importantly, PKD is present in the phagosomal fraction

indicating its presence in the cell where activation of the NADPH oxidase takes place. (Figure 5.3)

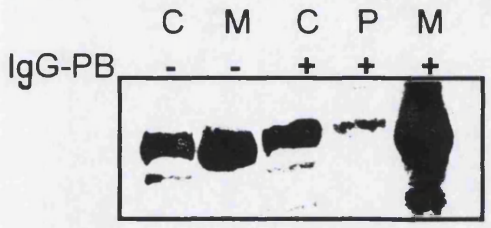


Figure 5.3 Presence of PKD in the phagosomal membrane

Neutrophils (10^9) were stimulated using opsonised magnetic beads (PB). After extraction of the phagosome (section 2.3.8d), the cytosol and membrane fractions were isolated as described previously (section 2.3.8e). The samples were analysed by SDS-PAGE and Western blot. Blots were probed with anti-PKD antibody (SC 935).

5.3.4 Immunofluorescence of PKD in neutrophils

In order to verify the presence of PKD in the phagosomal membrane, immunofluorescence was employed. Neutrophils ($1\text{-}2x10^6$) were stimulated with IgG-opsonised *S. aureus* (1:100 ratio neutrophils: *S. aureus*) for the indicated times and the cells were fixed in suspension. Figure 5.4 show a time course of IgG-opsonised *S. aureus* stimulation. In control non-stimulated cells (time 0), most PKD appears in a punctuate manner surrounding the cell. Upon stimulation with IgG-opsonise *S. aureus* the signal becomes polarised towards one end of the cell, most likely the phagocytosing end. Maximal polarisation occurs at 1 minute and 2 minutes of stimulation. By five minutes PKD is no longer aggregated in large clusters but becomes more dispersed, as seen in non-stimulated cells. The

process of PKD aggregation coincides with time of NADPH activation and phagocytosis. (Sections 3.3.1 and 3.3.4, respectively) PKD immunofluorescence was specific since it was not seen in the absence of antibody. (bottom panel)

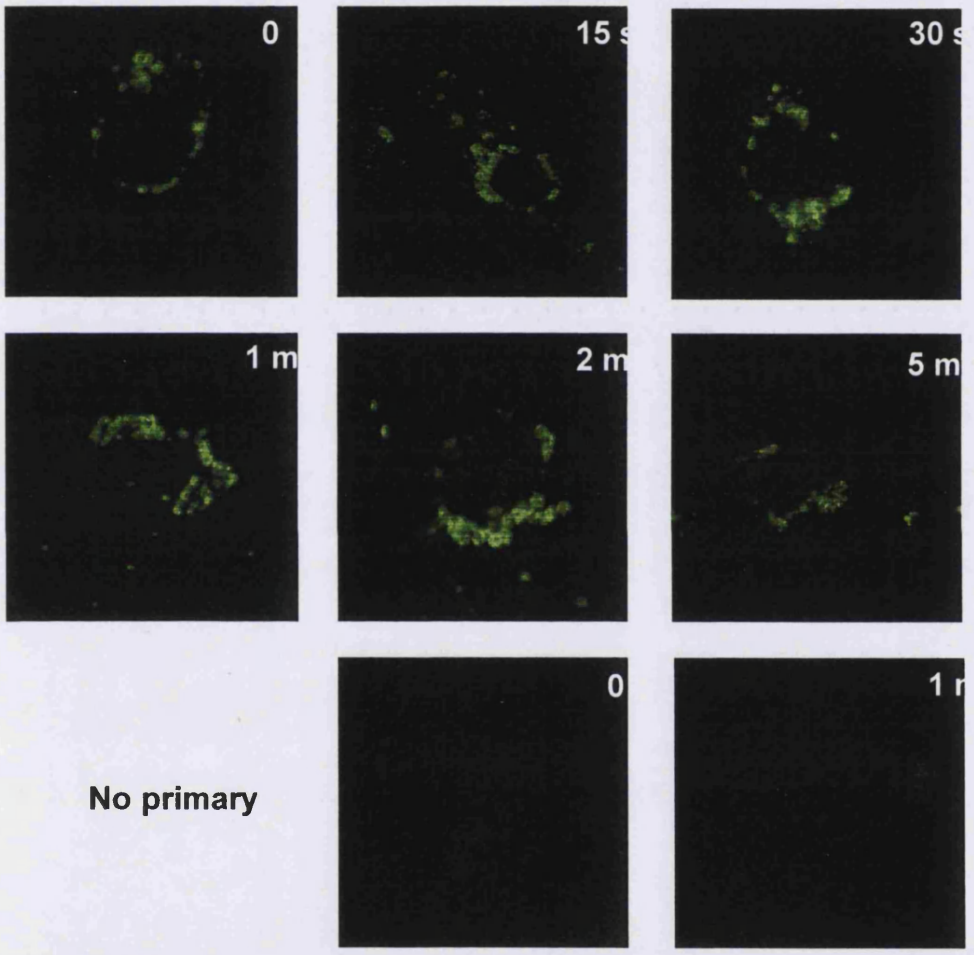


Figure 5.4 PKD aggregates into vesicular like formations upon stimulation with IgG-opsonised bacteria

Neutrophils (1-2x10^6) were stimulated with IgG-opsonised *S. aureus* for times indicated (0, 15 seconds, 30 seconds, 1 minute and 2 minutes). The reaction was quenched and the cells were fixed in suspension and treated as described in section 2.3.11a. Neutrophils were visualised by confocal microscopy. Bottom panels represent antibody control of cells treated in the same manner, but incubated in the absence of primary antibody.

In order to verify if the vesicular structures were actually phagosomes, *S. aureus* was opsonised using rhodamine labelled human IgG in addition to the normal IgG. This would allow the phagocytosed bacteria to be detected through the red channel and in this way identify the phagosomal particle. Neutrophils (1-2x10^6 cells) were stimulated with rhodamine-labelled IgG-opsonised *S. aureus* (ratio of 1:100 neutrophils: *S. aureus*) and fixed in solution. Figure 5.5 shows a single cell phagocytosing rhodamine labelled *S. aureus* at the 30 second time point. The vesicle like structures are again polarised to one end in the neutrophil membrane. The presence of red spherical staining patterns suggests that these structures indeed represent phagocytosed bacterial particles residing inside the cell. It can be seen that PKD immunoreactivity (green) surrounds the red staining, as expected if PKD is present in the phagosomal membrane. Amplification of two of these vesicular structures shows in more detail that PKD (green) forms the vesicle, which surrounds a phagocytosed bacterium (red). In the absence of antibody to PKD, the green channel showed no staining.

Thus from these experiments it can be concluded that PKD is localised at the phagosomal membrane during phagocytosis.

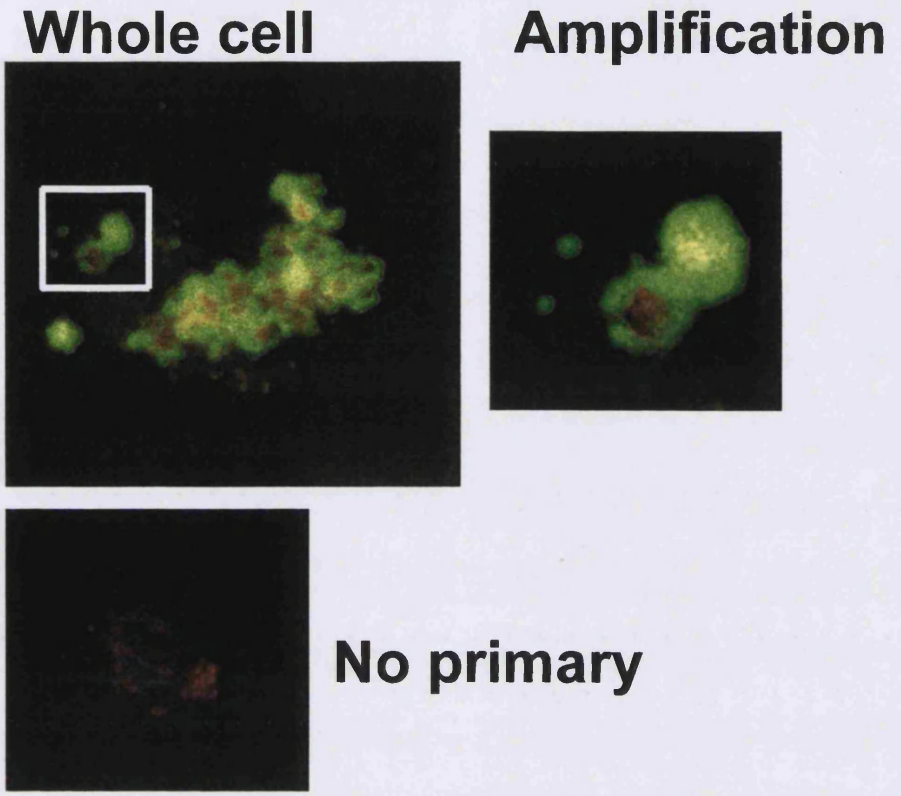


Figure 5.5 PKD sourrounds phagocytosed *S. aureus*

Neutrophils (1-2x10^6 cells) were incubated with rhodamine labelled IgG-opsonised *S. aureus* for 30 seconds. The sample was treated as described in figure 5.4. Top left figure: Neutrophil phagocytosing *S. aureus*, the phagosomes can be observed, mostly in one pole of the cell. Top right figure: magnification of phagosomes. Bottom panels represent antibody control, were primary antibody was not added.

5.3.5 Colocalisation of PKD with actin and p47^{phox}

Figure 5.5 shows the aggregation of the phagosomes, but it is difficult to view the whole cell in relation to the cluster of phagosomes. It is known that phagocytosis requires actin assembly and pseudopod extension to occur. (Greenberg 1999) Staining of filamentous actin with rhodamine-conjugated phalloidin has previously been employed in order to visualise the

phagosome. (May, Caron *et al.* 2000) Actin was therefore chosen to visualise the whole cell as well as the phagosome.

Neutrophils were first allowed to adhere on glass coverslips prior to stimulation and fixation. The cells were then permeabilised and probed, using both rhodamine-conjugated phalloidin and anti-PKD antibody. A short time course of IgG-opsonised *S. aureus* stimulation was performed. (Figure 5.6) Non-stimulated cells show the presence of PKD on the membrane and in addition diffuse staining can be seen in the cytosplasm. Actin is distributed in the membrane surrounding the neutrophil. At two minutes a circular structure can be observed at the polarised end of the cell. This structure contains actin as well as PKD, and it may represent sites where phagocytosis is taking place.

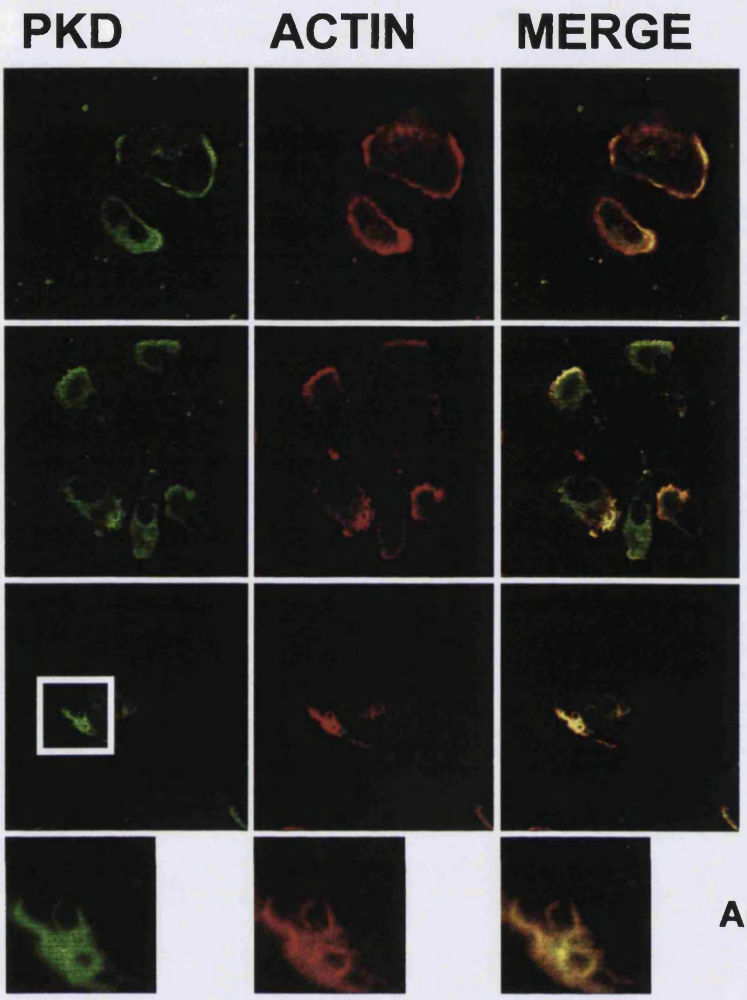


Figure 5.6 Definition of the phagosome by visualisation of the actin cytoskeleton

Neutrophils (1-2x10^6 cells) were stimulated with IgG-opsonised *S. aureus* for times indicated in the figure. Cells were subsequently treated as described in section 2.3.11b. PKD was visualised by fluorescein-labelled secondary antibody (green). Actin was visualised by rhodamine-labelled phalloidin (red). Merge represents visualisation of both channels.

Next, the colocalisation of $p47^{phox}$ with PKD was investigated. Neutrophils were fixed in suspension post stimulation and adhered onto gelatin treated coverslips, as described by Kiley SC and Parker PJ. (Kiley and Parker 1995) Cells were then incubated with rabbit anti-PKD antibody

used previously and with a goat anti-p47^{phox} antibody. Both antibodies recognised a single band by Western analysis. (Figure 5.2) Control (unstimulated) cells show the diffused staining of PKD in the cell periphery. p47^{phox} appears localised in the cytosol and there is no colocalisation with PKD. As the neutrophils are exposed to opsonised bacteria the circular structure can again be seen, which stain for both PKD and p47^{phox}. It is likely that this reflects phagosomal localisation of PKD and p47^{phox}.

Interestingly, it can be observed that after 30 second stimulation most of the p47^{phox} is in the phagosome; however, PKD, while present in the phagosome, some signal is still dispersed within the plasma membrane. After 2 minutes stimulation both p47^{phox} and PKD are totally localised in the phagosomal membranes. This result suggests that there is a temporal translocation of these proteins into the phagosome, p47^{phox} appears first followed by PKD.

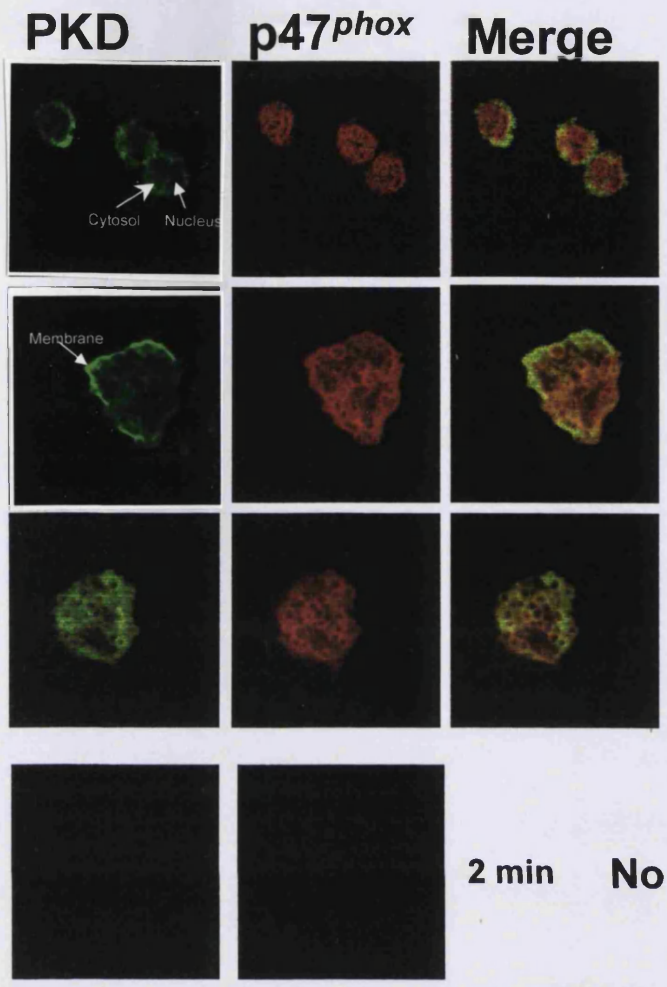


Figure 5.7 Colocalisation of PKD and p47^{phox} upon IgG-opsonised *S. aureus* stimulation

Neutrophils (1-2x10^6, cells) were stimulated with IgG-opsonised *S. aureus* for times indicated in the figure. The reaction was stopped and the cells were treated as described in section 2.3.11c. PKD was visualised with flourescein-labelled secondary antibody and p47^{phox} was visualised with rhodamine-labelled secondary antibody (red). Merge represents visualisation of both channels. Bottom panels show no antibody control for both antibodies.

5.4 Summary and discussion

In this section it has been shown that PKD is present mostly in the membrane in neutrophils. This was determined using immunobloting techniques as well as immunocytofluorescence confocal microscopy.

On a sucrose density gradient of unstimulated neutrophils PKD appears solely on the membrane; however, upon activation by Fcγ receptor stimulation, levels in the membrane increase, hence this kinase is not solely membrane bound but might be compartmentalised elsewhere.

Some groups have shown that PKD translocates to the plasma membrane from the cytosol, (Matthews, Iglesias *et al.* 1999; Matthews, Iglesias *et al.* 2000) while others have shown that PKD is located in the Golgi complex. (Prestle, Pfizenmaier *et al.* 1996) In neutrophils it could be that a smaller pool of PKD is present within the cytosol, which then translocates to the membrane upon stimulation. When greater numbers of cells are used (figure 5.3) a PKD immunoreactive band appears in the cytosol which is approximately 2-3 times less intense than in the membrane. In figures 5.1 and 5.2 cell numbers used for these experiments were approximately 200 fold less, and therefore the amount of PKD in the cytosol could be below a detection level for the method employed. The presence, of cytosolic PKD can also be seen in cells that were adhered to glass prior to fixation. (Figure 5.6)

Immunocytofluorecence techniques show that PKD is present in the membrane, possibly in membrane rafts which could explain the punctate appearance in non-stimulated cells, and polarises in vesicle-like formation

on the membrane upon Fcγ receptor dimerisation. It was shown that these vesicles contained rhodamine-labelled *S. aureus*, which were encircled by PKD. Furthermore, using rhodamine-conjugated phalloidin we showed that these vesicles contained F-actin. It was then shown that PKD colocalises with $p47^{phox}$. Thus it appears that these structures represent phagosomal vesicles and that PKD is present in the phagosomes upon Fcγ receptor stimulation. This hints towards a direct involvement of PKD in the activation of the NADPH oxidase activation, which will be discussed in the following chapter. The fact that there appears to be a temporal difference in the translocation of $p47^{phox}$ and PKD to the phagosomes is interesting. (Figure 5.7) It could be that PKD assists in the phosphorylation and translocation of NADPH oxidase cytosolic proteins to the membrane, or it possibly arrives at the phagosome post NADPH oxidase activation and prolongs its activity by phosphorylating some of the NADPH oxidase components.

It is important to mention that different techniques were employed to adhere the neutrophils onto glass coverslips for visualisation by confocal microscopy. The different techniques showed differences in PKD localisation in control (unstimulated) neutrophils. Adherence to glass post fixation showed a much more punctate peripheral distribution, whereas adherence pre-fixation to glass or post fixation to gelatin treated cover-slips showed a much more diffuse staining which included staining in the cytosol. We have not been able to determine a reason for this; however, regardless of the process of fixation and adherence to the glass coverslips, PKD

aggregated in the membrane in vesicle like structures, which we determined to be the phagosome.

We therefore conclude that PKD is present in neutrophils and upon Fcγ receptor stimulation aggregates in the phagosome.

Chapter 6

PKD phosphorylates two cytosolic components of the NADPH oxidase

6.1 Aim

Following the finding that PKD is involved in Fcγ receptor stimulation of the NADPH oxidase, (chapter 4) and its identification of PKD in the phagosomal membrane (chapter 5) the possibility that PKD phosphorylates components of the NADPH oxidase was investigated.

It was observed that *in vitro* PKD was able to phosphorylate $p47^{phox}$ and $p40^{phox}$ but not $p67^{phox}$. For *in vivo* phosphorylation of these components by PKD, parameters which were previously determined (Ro 31-8220-insensitive and Gö 6976-sensitive) were employed. Though it was observed that Gö 6976 had an effect on $p40^{phox}$ and $p47^{phox}$ phosphorylation, the actual phosphorylation sites by PKD could not be determined.

6.2 Introduction

Phosphorylation of NADPH oxidase components is essential for activation of the overall enzyme complex (section 1.8;(DeLeo, Allen *et al.* 1999). It has been shown for $p47^{phox}$ that phosphorylations can occur even after its translocation. (Rotrosen and Leto 1990) Hence it could be conceivable that a kinase like PKD which resides in the membrane could be involved in the phosphorylation of the NADPH oxidase components post translocation, or PKD could be activated which would then phosphorylate cytosolic $p47^{phox}$ which then translocates to the membrane. PKC, like PKD, translocates and therefore is in the membrane fraction upon activation by Fcγ receptor dimerisation. (Sergeant and McPhail 1997; Matthews, Iglesias *et al.* 2000) PKC has also been implicated in phosphorylation of several of the NADPH oxidase components, which may occur in the cytosol or post translocation. (See section 1.8)

It has been shown that PKD is localised in the Golgi complex and thereby involved in Golgi organisation. (Prestle, Pfizenmaier *et al.* 1996; Jamora, Yamanouye *et al.* 1999) Following this principle, and the fact that PKD has been found to localise in the phagosomal membrane, we investigated if its presence on the phagosome (chapter 5), and its involvement in NADPH regulation (chapter 4) coincides with phosphorylation of one or more of the components of the NADPH oxidase.

In this chapter PKD was shown to be involved in phosphorylation of some of the NADPH oxidase components, namely $p40^{phox}$ and $p47^{phox}$ *in vitro*. Furthermore, through phosphopeptide mapping several phosphopeptides were observed, suggesting that PKD's contribution is *via* phosphorylation of several sites on each of these two NADPH oxidase components. It was determined that $p40^{phox}$ and $p47^{phox}$ phosphorylation by PKD also occurs *in vivo;* identification of the specific phosphorylation sites, in order to determine the direct role of PKD in the activation and possible regulation of the oxidase, was attempted but was unsuccessful.

6.3 Results

6.3.1 *Parameters for PKD in-vitro kinase assays*

In order to determine possible substrates for PKD in neutrophils, *in vitro* kinase assays were performed using a recombinant PKD (Calbiochem 539671). Optimal assay conditions for the PKD *in vitro* kinase assay were determined by employing Syntide, which is known to be a good substrate for PKD (see chapter 4). First the PKD concentration was determined at a constant assay time (four minutes) and amount of substrate (10μg). Figure 6.1A shows that increasing the concentration of PKD in the assay increases phosphate incorporation in Syntide. Though kinase activity increased with increased concentration of the recombinant enzyme, it reached a plateau after 5μl. This could be an effect of depletion of substrate or the depletion

of ATP used. Figure 6.1B shows the time dependence of the phosphorylation reaction, at three µl (18.3pmol/µl) of added enzyme. The reaction time within the time spectrum used was not limiting as activity increased in a linear manner. From this experiment we opted for using 55pmol (three µl) of PKD and to carry out the kinase reactions for five minutes.

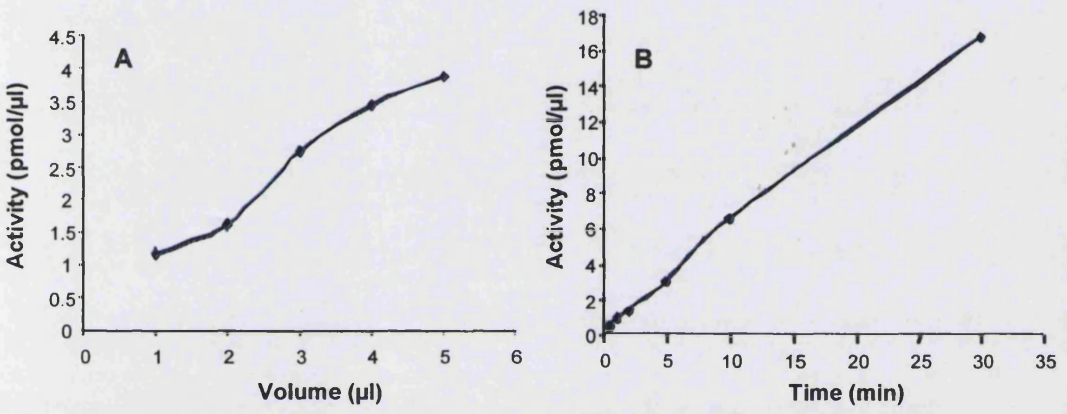


Figure 6.1 *In vitro* kinase assay using recombinant PKD and Syntide

Recombinant PKD was used at different concentrations (see text) and incubated with Syntide (10µg) for 4 minutes at 37 °C. (panel A) After determining an appropriate concentration of recombinant enzyme to used phosphate incorporation was measured in a time dependant manner. (panel B) Phosphate incorporation was determined as described in section 2.3.9bii.

6.3.2 PKD phosphorylation of NADPH oxidase components in vitro

Figure 6.2 shows the results of *in vitro* kinase assay of recombinant NADPH oxidase components. *In vitro* kinase assay were performed on $p40^{phox}$, $p47^{phox}$ and $p67^{phox}$. All these proteins were recombinant proteins made in Prof. Segal's laboratory from human sequences. Approximately

1μg of recombinant NADPH oxidase proteins replaced Syntide as the substrate in the *in vitro* kinase assays; the kinase reaction was started by adding recombinant PKD in the assay mixture (see section 2.3.10) and stopped after 5 minutes in Laemmli sample buffer. Panel A shows an autoradiograph from which it can be seen that *in vitro* PKD phosphorylates $p40^{phox}$ and $p47^{phox}$ but not $p67^{phox}$. The double band in the $p47^{phox}$ lane represents a breakdown product in which the bottom band has a small c-terminal deletion. (Reeves, Dekker *et al.* 1999) $p47^{phox}$ was raised as a N-terminal GST-fusion protein, and when eluted from the purification column two bands of different size appeared leading to the conclusion that the bottom band is a breakdown product. This is quite interesting as PKC and other kinases have been found to phosphorylate $p47^{phox}$ on its C-terminus. Here we see a situation in which the top and bottom densities appear very similar indicating that perhaps PKD is phosphorylating $p47^{phox}$ on its N-terminus. Panel B is a graphical representation of fmol of phosphate incorporation into the substrates. The exact amount of phosphate incorporation per nmol of protein could not be determined, as the exact amount of oxidase protein added was not known, though equal amounts of protein were loaded as visualised by coomassie staining. $p67^{phox}$ as mentioned before was not phosphorylated by PKD and this can also be seen by comparing phosphate incorporation in $p67^{phox}$ versus background, which was measured by counting a blank piece of gel.

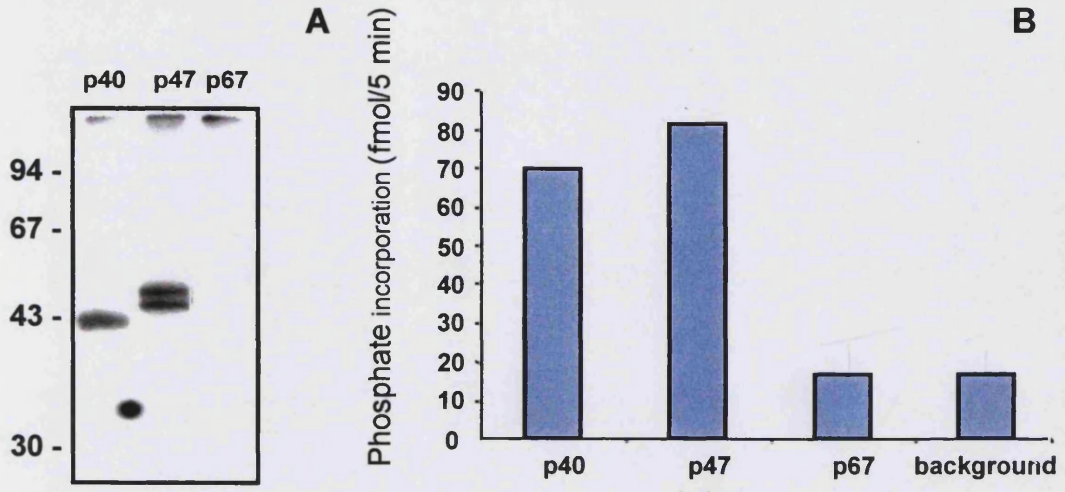


Figure 6.2 Phosphorylation of NADPH oxidase components by PKD

Recombinant *phox*-proteins were used as substrates for *in vitro* kinase assay by PKD, as described in section 2.3.10. Panel A: Samples were analysed by SDS-PAGE and autoradiography. Panel B: Proteins bands were cut out of the gels and phosphate incorporation was measured in a scintillation counter. Data are expressed as fmol of phosphate incorporation. n = 3

6.3.3 PKD phosphorylation sites on p47^{phox} and p40^{phox}

It has been shown in figure 6.2 that PKD can phosphorylate p40^{phox} and p47^{phox} *in vitro*. In order to identify the actual phosphorylation sites on these two proteins, tryptic phosphopeptide maps were performed. Recombinant proteins, phosphorylated *in vitro* were eluted from an acrylamide gel after SDS-PAGE analysis, and exposed to tryptic digestion as described by van der Geer and Hunter. (van der Geer and Hunter 1994) The peptides were then separated in two dimensions by electrophoresis and thin layer chromatography. (Figure 6.3) It can be seen that for p40^{phox} there are four major phoshopeptides while for p47^{phox} nine were observed. It cannot be conclusively determined if these represent different phosphosites as more

than one peptide could migrate to the same site, or due to missed tryptic cleavages one phosphosite can occur in several peptides which would then be seen as different spots on a phosphopeptide map. Work is being undertaken to determine the PKD phosphorylation sites, on the different phosphopeptides.

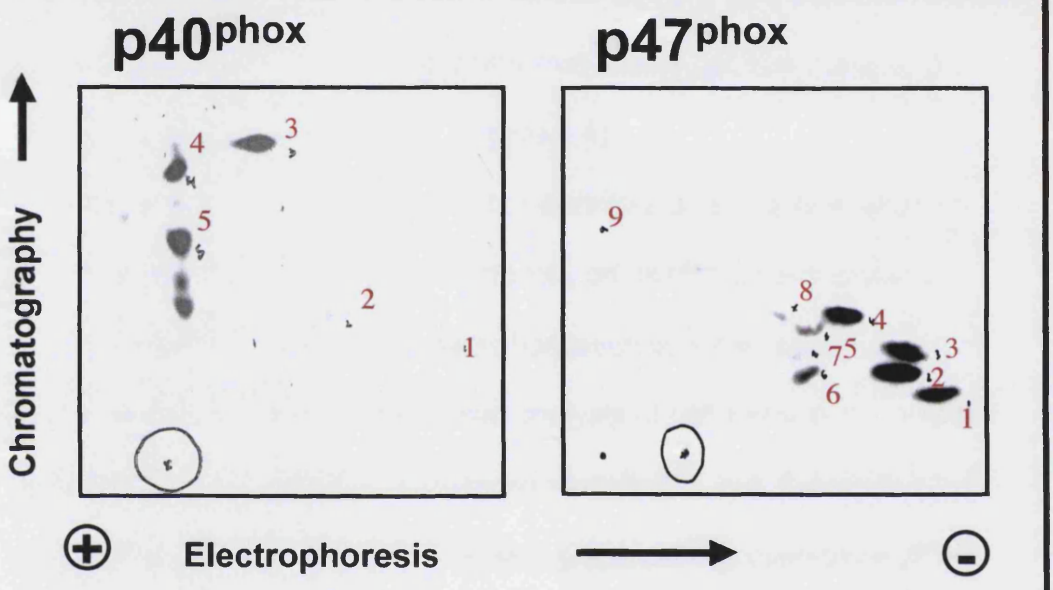


Figure 6.3 Phosphopeptide maps of p40^{phox} and p47^{phox} phosphorylation by PKD

2 dimensional phosphopeptide maps of p40^{phox} (left) and p47^{phox} (right). Proteins were treated as described in section 2.3.14 and direction of peptide separation is indicated in the figure. Numbers (red) represent the different phosphopeptides.

6.3.4 In vivo phosphorylation of p47^{phox} and p40^{phox}

It has been proposed in chapter 3 and 4 that Gö 6976 sensitive and Ro 31-8220 insensitive phosphorylations may be induced by PKD. Following this

premise, we have tried to elucidate the involvement of PKD in phosphorylation of $p47^{phox}$ and $p40^{phox}$ *in vivo,* by comparing the effect of these inhibitors on $p47^{phox}$ and $p40^{phox}$ phosphorylation, in the cell.

Cells ($5x10^7$ neutrophils) were pre-incubated with the different inhibitors and activated with IgG-opsonised *S. aureus* ($3x10^9$) for 3 minutes, followed by $p47^{phox}$ and $p40^{phox}$ immunoprecipitation then a gel was made and phosphorylation was measured. (Figure 6.4)

Figure 6.4 panel A shows effect of inhibitors on phosphorylation of $p40^{phox}$ and panel B the effect of inhibitors on $p47^{phox}$ phosphorylation.

The effect of the inhibitors phosphorylation is not a consequence or fault in protein loading. Western blot analysis of cell extracts showed the amount of extract subjected to immunoprecipitation was the same in all cases. (Figure 6.4C) Figure 6.4 gives a graphical representation of phosphorylation of $p40^{phox}$ and $p47^{phox}$ immunoprecipitated following Fcγ receptor activation (n=3). $p40^{phox}$ has a lower basal phosphorylation level than $p47^{phox}$. Upon activation by IgG-opsonised *S. aureus* the levels of phosphorylation were increased by three fold, while for $p47^{phox}$ the levels increased two fold. Gö 6976 inhibits phosphorylation of both proteins by approximately 70% of maximal (non-inhibited sample) reducing $p40^{phox}$ phosphorylation to its basal level, and $p47^{phox}$ to half its basal level. Ro 31-8220 also reduces the levels of phosphorylation of both proteins; in this case the phosphorylation levels of both proteins are reduced to below basal levels, thereby having a greater effect on $p40^{phox}$ than $p47^{phox}$. Effects on

the latter are similar to that of Gö 6976. The PI-3 kinase inhibitor Ly 294002 has a partial effect on phosphorylation of both proteins leading to a decrease of approximately 35-40% of maximum. The combination of the PKC (Ro 31-8220) and PI-3 kinase (Ly 294002) did not have a cumulative effect on inhibition of phosphorylation levels, though levels for both proteins were below basal state. Inserts in each panel depict a representative example of phosphorylation profiles in the presence of inhibitors for each protein.

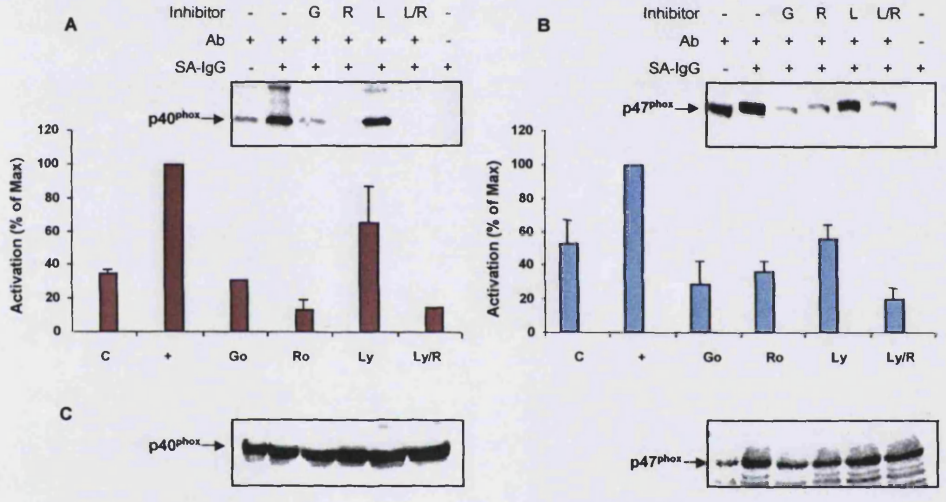


Figure 6.4 Effect of inhibitors on Fcγ receptor phosphorylation of p40^{phox} and p47^{phox}

Neutrophils (5 x 10^7 cells) were incubated with 0.5 mCi [^{32}P] for 40 minutes at 30˚C. Subsequently, the samples were incubated with different inhibitors as indicated, 10μM Gö 6976 (G), 10μM Ro 31-8220 (R) and/or 50μM Ly 294002 (L) for 15 minutes at room temperature. Cells were then stimulated for 2 minutes with IgG-*SA* (3 x 10^9 bacteria) and p40^{phox} (A) or p47^{phox} (B) were immunoprecipitated as described in section 2.3.9. Immunoprecipitates were analysed by SDS-PAGE and autoradiography. Signal intensity from autoradiography was analysed using NIH image software. Graphs of signal intensity are expressed as percentage of maximal signal (stimulated non-inhibited sample) (n=3). Inset an example of an autoradiograph; no antibody controls did not give a signal for each immunoprecipitation. Panel C shows Western blots of protein extraction.

6.4 Summary and discussion

In this section we have tried to investigate the mechanism by which PKD may mediate NADPH oxidase activation following Fcγ receptor stimulation. Employing recombinant PKD, we first attempted to optimise our system for *in vitro* kinase assay. Cytosolic NADPH oxidase components were selected as substrates following the findings in chapter 5, which indicated that PKD was present on the phagosomal membrane post phagocytic stimulus. It was noticed that *in vitro* PKD was capable of phosphorylating two out of the three cytosolic oxidase components tested. Phosphopeptide mapping revealed nine and four phosphopeptides for $p47^{phox}$ and $p40^{phox}$, respectively. Recently a report demonstrated that substrate selectivity for PKD and the isoforms of PKC family are different. (Nishikawa, Toker *et al.* 1997) This further points to a possibility of different phosphorylation sites for PKD than those phosphorylated by PKC, which is known to phosphorylate $p47^{phox}$, on its C terminus, and perhaps $p40^{phox}$ *in vivo*. (section 1.8) The fact that the C-terminal breakdown in the $p47^{phox}$ and the whole protein recombinant products were similarly phosphorylated by PKD indicates that the PKD and PKC phosphosites may differ. To strengthen this point a comparison should be made between PKD and PKC phosphosites. So far attempts to elucidate the phosphorylation sites have failed, but efforts are being undertaken to elucidate these.

As described in the introduction (see section 1.8) different kinases have been identified which potentially phosphorylate these proteins and contribute to their regulation. From the literature and the results seen in figure 6.4 it can be postulated that PKD may be involved in this phosphorylation, since there is an effect of Gö 6976 on the level of phosphorylation. Furthermore it has been argued that PKD is regulated upstream by PKC and PI-3 kinase (section 4.3.4) and in this experiment we have seen a greater effect on combining inhibitors to both these kinases than on inhibiting each kinase individually. (Figure 6.4) The data, however, are not conclusive. Gö 6976 has an effect on phosphorylation levels of both proteins, which for $p47^{phox}$ are very similar to the effects of Ro 31-8220. Employing this method to dissect the different contribution made by each kinase through inhibitory profiles of the inhibitors is difficult, and conclusive evidence could only be seen by comparison of *in vivo* phosphorylation sites with *in vitro* results. However experiments to compare *in vivo* phosphorylation sites profiles and effect of inhibitors with *in vitro* phosphorylation sites have been hard to perform due to the limitations of different techniques attempted, for example extraction of enough phosphorylated sample from *in vivo* activation has not been successful. New techniques to compare these sites are being tested and results are pending.

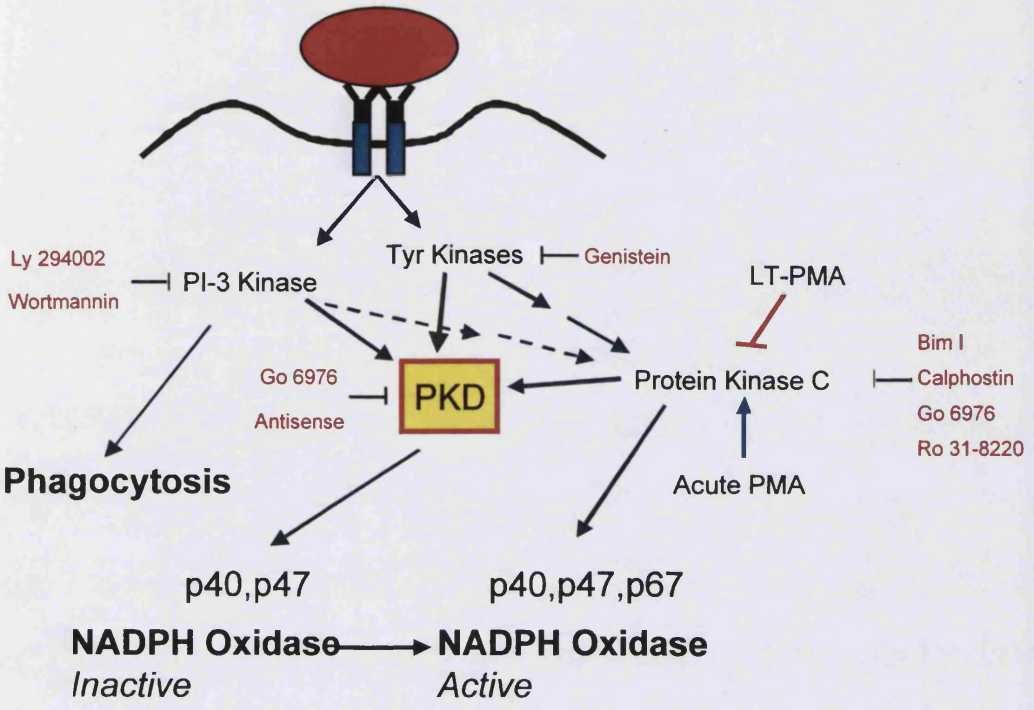


Figure 6.5 Placement of PKD in Fcγ receptor signalling cascade leading to activation of the NADPH oxidase

PKD was placed in this signalling cascade in chapter 4; the resulsts from this chapter show more directly how it contributes to NADPH oxidase activation, by phosphorylating $p40^{phox}$ and $p47^{phox}$.

In conclusion these data indicate that the contribution of PKD on activation of the NADPH oxidase is a direct effect. By phosphorylating two cytosolic components, $p47^{phox}$ and $p40^{phox}$, *in vitro* and possibly *in vivo*, PKD directly pitches in the regulation and activation of the oxidase. In figure 6.5 PKD has been placed in the overall signalling cascade explored in this project.

Chapter 7

Identification of PKD substrates in human neutrophils

7.1 Aim

Chapters 4 and 6 described that the NADPH oxidase is a target for PKD, which directly phosphorylates two of its cytosolic components, $p40^{phox}$ and $p47^{phox}$.

This chapter focuses on the identification of further potential substrates for PKD. By exploiting inhibitory profiles identified in chapter 1 in combination with proteomics approach, five substrates were identified *in vivo*. *In vitro* kinase assays, utilising recombinant PKD, confirmed two of these to be direct PKD substrates.

7.2 Introduction

Many studies have been devoted to determining the regulation of PKD. Most show that PKD is regulated via PKC. (Zugaza, Sinnett-Smith *et al.* 1996; Van Lint, Ni *et al.* 1998; Paolucci and Rozengurt 1999; Waldron, Iglesias *et al.* 1999; Matthews, Rozengurt *et al.* 2000; Waldron and Rozengurt 2000) However, less information is available on PKD function, and its targets.

PKD is implicated in regulation of cell growth and proliferation. In MDA-MB-231 breast cancer cells, PKD has been shown to co-express in invadopodia (membrane protrusions, associated with active extracellular degradation) with cortactin and paxillin; presence of this complex directly correlated with the invasive potential of the cancer, which could be mediated by an effect of PKD on actin rearrangement. In fibroblasts and keratinocytes, PKD was selectively expressed during tissue proliferation, in this case, PKD appears to partake in DNA synthesis; incubation, of mouse keratinocytes, with PKD inhibitor Gö 6976 lead to a decrease in DNA synthesis. Concurrent with this idea, it was shown that overexpression of PKD in NIH3T3 fibroblasts lead to enhanced cell proliferation. (Bowden, Barth *et al.* 1999; Rennecke, Rehberger *et al.* 1999)

Several intracellular signalling cascades have been shown to be regulated by PKD. PKD was shown to regulate adenylyl cyclase in macrophages, however this data is controversial as the authors failed to recognise that Gö 6976 inhibits PKD. (Lin and Chen 1998) In fibroblasts PKD is activated by the platelet-derived growth factor (PDGF) receptor.

This results in cross phosphorylation of the epidermal growth factor (EGF), which is a direct PKD substrate. PKD phosphorylates the EGF receptor on a threonine residue leading to downregulation of EGF-induced transcriptional activity. In this manner PKD forms the link in the cross talk between PDGF and EGF receptors and partakes in the regulation of cell proliferation. (Bagowski, Stein-Gerlach *et al.* 1999)

In COS-7cells, it has been shown that the Na^+/H^+ exchanger is partly under regulation of PKD. PKD was shown to phosphorylate the Na^+/H^+ exchanger. Transfection of a kinase mutant PKD into these cells lead to an increase in acid efflux, indicating that native PKD functions as a negative regulator of the exchanger. (Haworth, Sinnett-Smith *et al.* 1999) Interestingly, the Na^+/H^+ exchanger is also activated upon Fcγ receptor stimulation of neutrophils (Fukushima, Waddell *et al.* 1996) although it is not clear if this is mediated by PKD.

In this chapter we tried to identify PKD substrates in neutrophils upon Fcγ receptor activation. It was argued in chapter 3 and 4 that inhibitory profiles of Gö 6976 and Ro 31-8220 differ, and while PKD is sensitive to Gö 6976 it is insensitive to Ro 31-8220. By employing the inhibitory profile of these kinases, five proteins were identified, as PKD substrates. Gö 6976 inhibited phosphorylation of L-plastin, transaldolase, malate dehydrogenase, moesin and rab GDI β, however Ro-31-8220 did not. *In vitro* kinase assay using recombinant PKD further confirmed that transaldolase and moesin were phosphorylated by PKD.

These results suggest that PKD may be involved in other cellular functions in neutrophils, such as cytoskeletal rearrangement and control of glucose metabolism.

7.3 Results

7.3.1 Testing two dimensional gel electrophoresis and identification of location markers

In order to test and become familiarised with the 2 dimensional electrophoresis system and mass spectrometry, neutrophil (10^8 cells) membranes were isolated and processed as described in section 2.3.13a. Proteins were separated by two-dimensional gel electrophoresis, and visualised by coomassie staining. (Figure 7.1A) Several spots were cut out, indicated by numbers, and identified using MALDI-TOF mass spectrometry. These protein spots were selected in order to establish if proteins could be directly sequenced from the gel. The larger spots (1, 3, 4, 7, 9, 12, 14, 15, 20, 21, 22, 23, 25 and 27) were later used as location markers in order to relate smaller spots in gels of further experiments. Proteins that were identified by mass spectrometry are shown in table 7.1. The identification of the proteins was based on the number of peptides out of the total, matched, as well as the percentage sequence of the protein covered. (Table 7.1) Proteins are grouped by known function, for example proteins known to be involved in regulation of actin or the cytoskeleton are first (1-

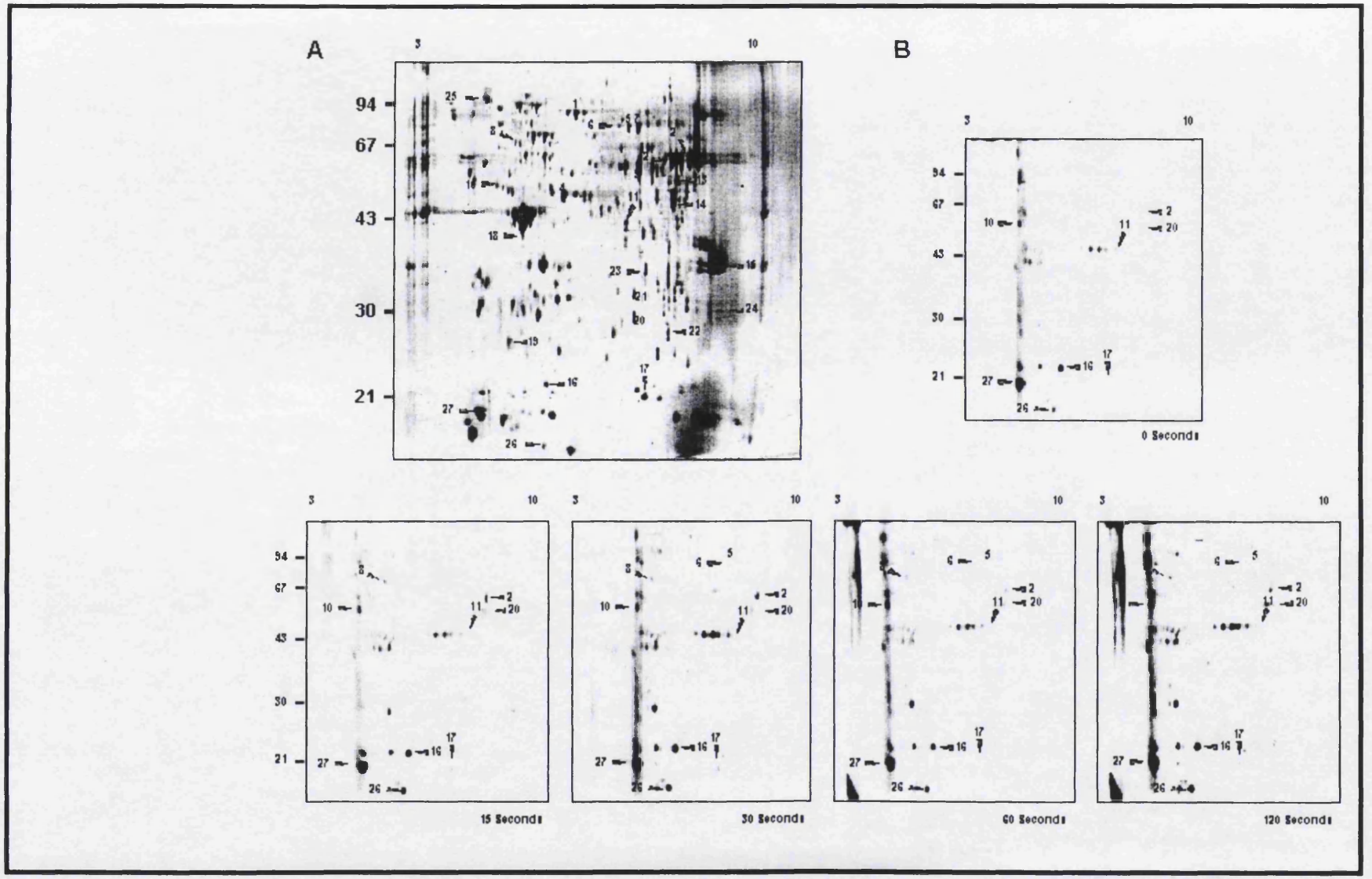
10); enzymes identified are second (11-15) this is followed by small G-proteins involved in signalling (16-18), then proteins involved in proteolysis (19-22) and finally a miscellaneous group (23-27).

7.3.2 Time course of protein phosphorylation upon Fcγ receptor stimulation

The temporal effect of Fcγ receptor stimulation on phosphorylation was then studied. Neutrophils (10^8) were incubated with 1mCi of [^{32}P] for 40 minutes and stimulated with IgG-opsonised *S.aureus* for 15, 10, 60, and 120 seconds. The reaction was quenched and the plasma membrane was extracted as described in section 2.3.8e. Proteins were analysed by two dimensional gel electrophoresis as in section 7.3.1, and gels were exposed to radiography in order to visualise the phosphorylated proteins. (Figure 7.1B) Phosphorylation patterns of the phosphorylated spots (2, 5, 6, 8, 11, 16, 17 and 26) was mapped (figure 7.2), and the proteins identified by mass spectrometry. (Table 7.1) The proteins identified concurred with some of the proteins identified in the previous section. (Section 7.3.1)

Figure 7.1 Two dimensional gel electrophoresis of neutrophil membrane and time course of phosphorylation events post Fcγ receptor stimulation

Neutrophils (10^8 cells/ml) were incubated with [^{32}P] (1mCi/ml) for 1 hour at 30°C. The cells were stimulated with IgG-opsonised *S. aureus* for times indicated in figure. The plasma membrane was extracted as described in section 2.3.8e, and analysed by 2 dimensional gel electrophoresis as described in section 2.3.13. Panel A: Shows coomassie stained gel of neutrophil plasma membrane after 2 dimensional gel electrophoresis separation. Panel B: Autoradiographs of Fcγ receptor stimulation and effects on protein phosphorylation of plasma membrane proteins. Proteins identified by MALDI-TOF are indicated by numbers.


A
B
3
10
94
67
43
30
21
0 Seconds
15 Seconds
30 Seconds
60 Seconds
120 Seconds

Spot	Identified Protein	Swiss Prot entry	Mass (kDa)	pI	No. of Peptides	%Sequence
1	*Gelsolin*	P06396	85.7	5.9	6/9	10
2	*CAP 1*	Q01518	52	8.07	15/32	30
3	*Actin β*	P02571	41.6	5.31	17/21	44.7
4	*Actin like protein 3 (ARP 3)*	P32391	47.3	5.6	15/21	41
5	*Moesin*	P26038	67.8	6.07	26/37	44
6	*Moesin*	P26038	67.8	6.07	5/7	14
7	*Moesin*	P26038	67.8	6.07	13/16	24
8	*L-Plastin*	P13796	70.2	5.2	6/9	11
9	*Coronin like protein p 57*	P31146	51.0	6.25	8/8	16
10	*Basp 1*	P80723	22.5	4.6	6/11	50
11	*Pyruvate Kinase*	P14618	57.8	7.5	10/15	27
12	*Catalase*	P04040	59.8	6.9	11/13	29
13	*Leukocyte elastase inhibitor*	P30740	42.7	5.9	5/9	18
14	*α-enolase*	P06733	47.2	7.0	11/19	35
15	*Glyceraldehyde-3 phosphate dehydrogenase*	P04406	36.1	8.6	6/9	21

16	*Ras related protein (Rap 1B)*	P09526	20.8	5.65	5/9	22.1
17	*Ras related protein (Rap 1A)*	P10113	20.6	6.4	7/11	36
18	*Adenylyl cyclase inhibitor protein G-I*	P04899	40.4	5.3	8/12	29
19	*Proteosome chain 7 precursor*	P28065	23.3	4.9	6/10	28
20	*Proteasome Iota chain*	P34062	27.4	6.4	4/4	19
21	*Proteasome component C2*	P25786	30	6.2	8/24	37
22	*Proteasome component C3*	P25787	25.6	6.9	9/17	38
23	*Annexin I*	P04083	38.7	6.6	50	15/26
24	*Insulin like GF binding protein*	P24593	30.6	8.6	6/11	18
25	*Integrin β-3*	P05106	87.2	5.0	11/23	16
26	*Calgranulin B*	P06702	13.2	5.7	5/11	37
27	*Myosin Regulatory light chain 2*	P19105	19.6	4.6	7/11	41.8

Table 7.1 Identification of protein spots, by MALDI-TOF, from neutrophil membrane separated by two-dimensional gel electrophoresis

Phosphorylation patterns of these proteins differed. While in some proteins phosphorylation was transient (spot 2, 6, 8 and 11), in others it increased and remained high (spot 16 and 26); one spot changes were very slight (spot 17) and finally one protein appeared to be dephosphorylated (spot 5). (Figure 7.2)

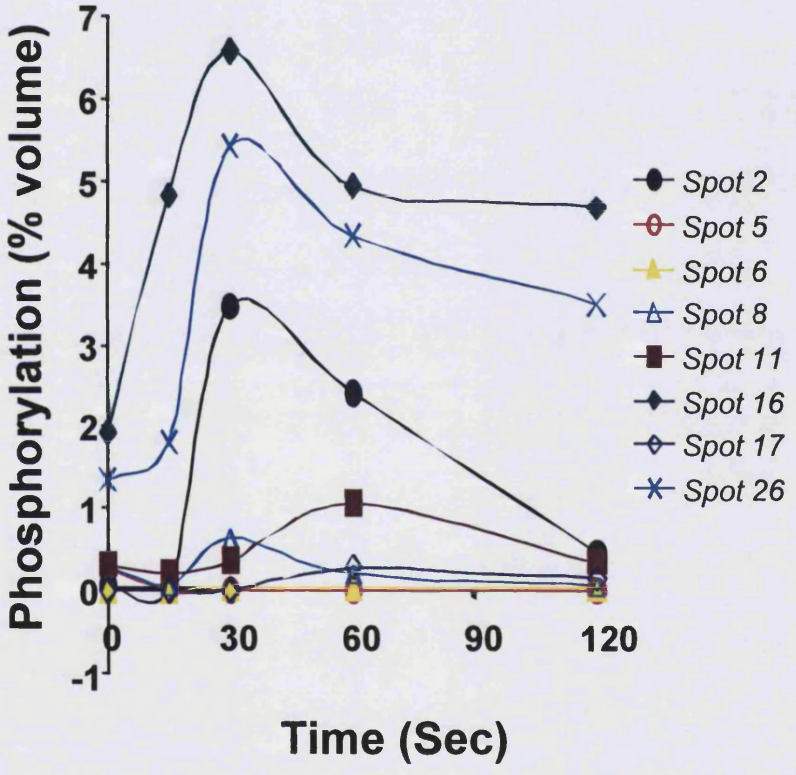


Figure 7.2 Phosphorylation of proteins over time post Fcγ receptor stimulation

Autoradiographs of gels in figure 7.1 were scanned and analysed using Melanie II software. Signal density per protein spot is expressed as percent volume (this signifies optical density/area of each spot as percentage of overall phosphorylation of the sample).

These proteins were identified; spot 2 is CAP 1 (cyclic AMP associated protein 1), this protein is phosphorylated 3 fold within 30 seconds of Fcγ receptor ligation and within 2 minutes phosphorylation returns to basal state. CAP 1 has been associated with ras activation of adenylyl cyclase in yeast (Nishida, Shima *et al.* 1998; Shima, Okada *et al.* 2000), in mammalian cells it has been shown to bind actin and profilin and thereby take part in actin rearrangement. (Hubberstey, Yu *et al.* 1996; Zelicof, Protopopov *et al.* 1996)

Spot 16 and 26, Rap (ras related protein) 1B and Calgranulin B, respectively, are phosphorylated approximately 3 fold within 30 seconds, both proteins remain highly phosphorylated and within the time course tested they do not return to basal level. Another member of the Rap family Rap 1A (spot 17) was also observed to be phosphorylated, though to a lesser degree than Rap 1B. Rap proteins have been shown to be phosphorylated by PKA in neutrophils. Rap 1A is a negative regulator of NADPH oxidase activity, it binds to the cytochrome b_{558} and inhibits its function. Upon stimulation of the NADPH oxidase Rap 1A becomes phosphorylated and disassociates from the cytochrome, allowing its activation. (Bokoch, Quilliam *et al.* 1991; Gabig, Crean *et al.* 1995)

Calgranulin B (spot 26) is a calcium binding protein very abundant in neutrophils. It is secreted during fMLP and C5a stimulation, though no evidence is present for its secretion upon Fc receptor stimulation. (Hetland, Talgo *et al.* 1998) Studies have shown that it performs a bactericidal

function by chelating zinc in infected tissues. (Brandtzaeg, Gabrielsen *et al.* 1995; Clohessy and Golden 1995) Interestingly, Calgranulin B was heavily phosphorylated upon Fcγ receptor stimulation, and has been previously been shown to be phosphorylated upon fMLP and PMA stimulation. (Dekker and Paclet personal communication) (Bengis-Garber and Gruener 1993) Phosphorylation of this protein may relate to its calcium binding properties. Calcium is known to be an important second messenger in signal transduction (section1.6.2b3), Calgranulin could therefore partake in signalling by chelating calcium and not making it accessible to other proteins.

L-plastin (spot 8) phosphorylation is quick and transient, phosphorylation is increased two fold within 30 seconds but returns to basal levels within a minute. L-plastin is an actin binding protein, which is phosphorylated upon neutrophil activation by Fcγ receptor. (Rosales, Jones *et al.* 1994; Jones and Brown 1996) Phosphorylation of L-plastin has been shown to be mediated by PKC, PKA and PI-3 kinase. (Jones, Wang *et al.* 1998; Lin, Lau *et al.* 1998; Wang and Brown 1999) L-plastin's functions have been related to control of neutrophil adhesion and calcium signalling. For example, inhibiting phosphorylation of L-plastin impeded integrin-β mediated adhesion of neutrophils. (Jones, Wang *et al.* 1998) Moreover, L-plastin binding to bromophenacyl bromide (BPB), an alkylating agent, which inhibits leukocyte degranulation, adherence, and phagocytosis, inhibited IgG-stimulated increases in intracellular calcium. L-plastin may couple Fcγ

receptor to calcium induced rearrangement of the cytoskeleton, and assist in calcium signalling. (Rosales, Jones *et al.* 1994) Furthermore, it has recently been shown that L-plastin interacts with Grancalcin, a calcium binding protein, and suggestions have been made that this confers a calcium dependency to L-plastin. (Jia, Han *et al.* 2000)

Spots 5 and 6 were identified as moesin. Moesin was dephosphorylated upon activation (spot 5), at the same time another spot (6), also identified as moesin, was phosphorylated. The relation between phosphorylation and dephosphorylation of moesin is graphically depicted in figure 7.3.

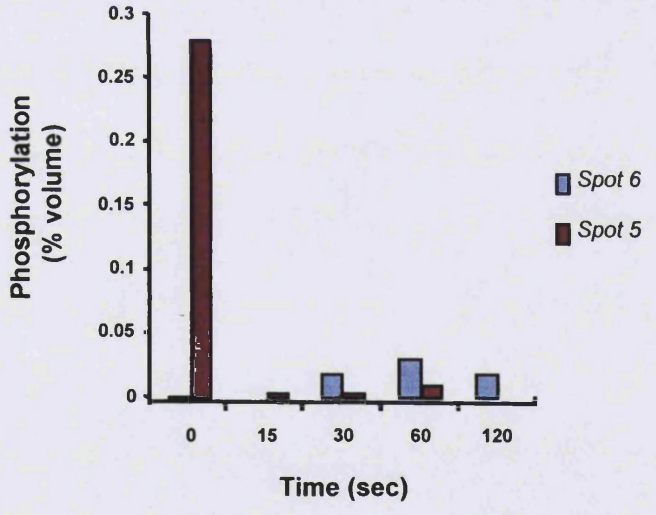


Figure 7.3 Moesin phosphorylation and dephosphorylation

As is figure 7.2 spot 5 and 6, identified as moesin were cut out of a two-dimensional. Phosphorylations were analysed using Melanie II software, and protein signals are expressed as percent volume.

It should be noted that overall a decrease in phosphorylation of moesin was observed. Proteins shift towards the anode when phosphorylated; therefore it is possible that as phosphorylation of moesin increases, its isoelectric point shifts, hence the intensity of the signal would increase in spot 6 and decline in spot 5. However on coomassie stained gel, protein shift in isoelectric focusing was not seen. Moesin forms an anchor between the cytoskeleton and the plasma membrane and in this way assists in the formation of filopodia. Through this action moesin is essential for morphological changes and migration of cells. (Pestonjamasp, Amieva *et al.* 1995; Simons, Pietromonaco *et al.* 1998) Moesin is activated by phosphorylation, which induces a conformational change in the protein, exposing binding sites for the plasma membrane and F-actin. (Huang, Wong *et al.* 1999) PKC(theta)ϕ has been implicated in this phosphorylation. (Pietromonaco, Simons *et al.* 1998; Simons, Pietromonaco *et al.* 1998)

Pyruvate kinase (spot 11) was also shown to undergo phosphorylation within one minute and then return to basal levels by 2 minutes. Pyruvate kinase activity is decreased by phosphorylation. (Eigenbrodt, Reinacher *et al.* 1992; Stryer 1995) It is known that the hexose monophosphate pathway (HMP) is activated during phagocytosis in neutrophils (see section 1.5) hence inactivation of pyruvate kinase could help shunt glucose-6 phosphate through the HMP.

It was exciting to note that some of our findings correlated with the literature, thereby giving proof that this system functioned.

7.3.3 PKD substrates- inhibition by Gö 6976 but not Ro 31-8220

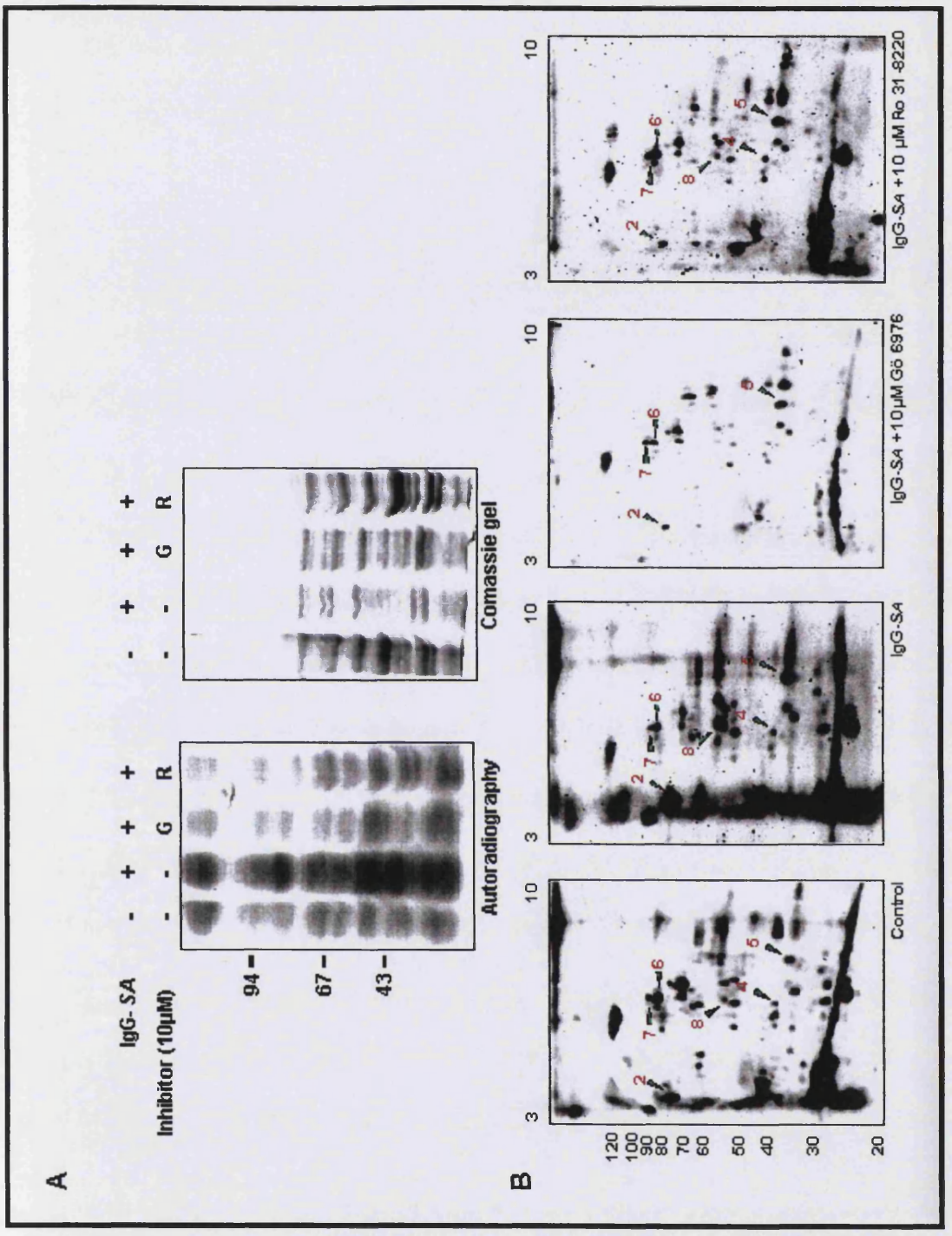
In the previous section several proteins were identified and shown to change in phosphorylation state upon Fcγ receptor stimulation. Most changes occurred between 30 seconds and one minute, therefore the one-minute stimulation point was chosen for the following experiments.

The differential effects of Gö 6976 and Ro 31-8220 on PKD activity were employed in order to identify substrates for PKD. A Triton-soluble fraction was used as it encompasses a greater cellular fraction, comprising of both cytosol and membrane proteins.

Figure 7.4A (left panel) shows the effect of Gö 6976 and Ro 31-8220 on phosphorylation of proteins induced by Fcγ receptor activation. It can be seen that overall phosphorylation increases upon stimulation with IgG-opsonised *S. aureus*, however this is reduced by the effect of the inhibitors. The pattern of phosphorylation in each inhibitor lane is different. Interestingly, the protein pattern also differs implying that the inhibitors have an effect also on translocation of proteins between the Triton-soluble and -insoluble fractions.

Figure 7.4 Effects of inhibitors as indication of PKD substrates *in vivo*

Neutrophils (10^8/ml) were labelled for 45 minutes with 1mCi/ml [^{32}P], followed by incubation with inhibitor, as indicated in figure, for 15 minutes at room temperature. Subsequently the cells were stimulated with IgG-opsonised *S. aureus* (1:100 dilution of cells to bacteria). Triton soluble fraction was extracted as described in section 2.3.8a. The sample was then analysed by SDS-PAGE (panel A) and visualised by coomassie staining or autoradiography as indicated. The samples were also analysed by 2 dimensional gel electrophoresis as described in section 2.3.13, and autoradiography. (Panel B)



It was difficult to distinguish individual proteins in a one-dimensional gel, hence 2 dimensional gels were run in order to clarify phosphorylation differences. In figure 7.4B shows a series of autoradiographs of

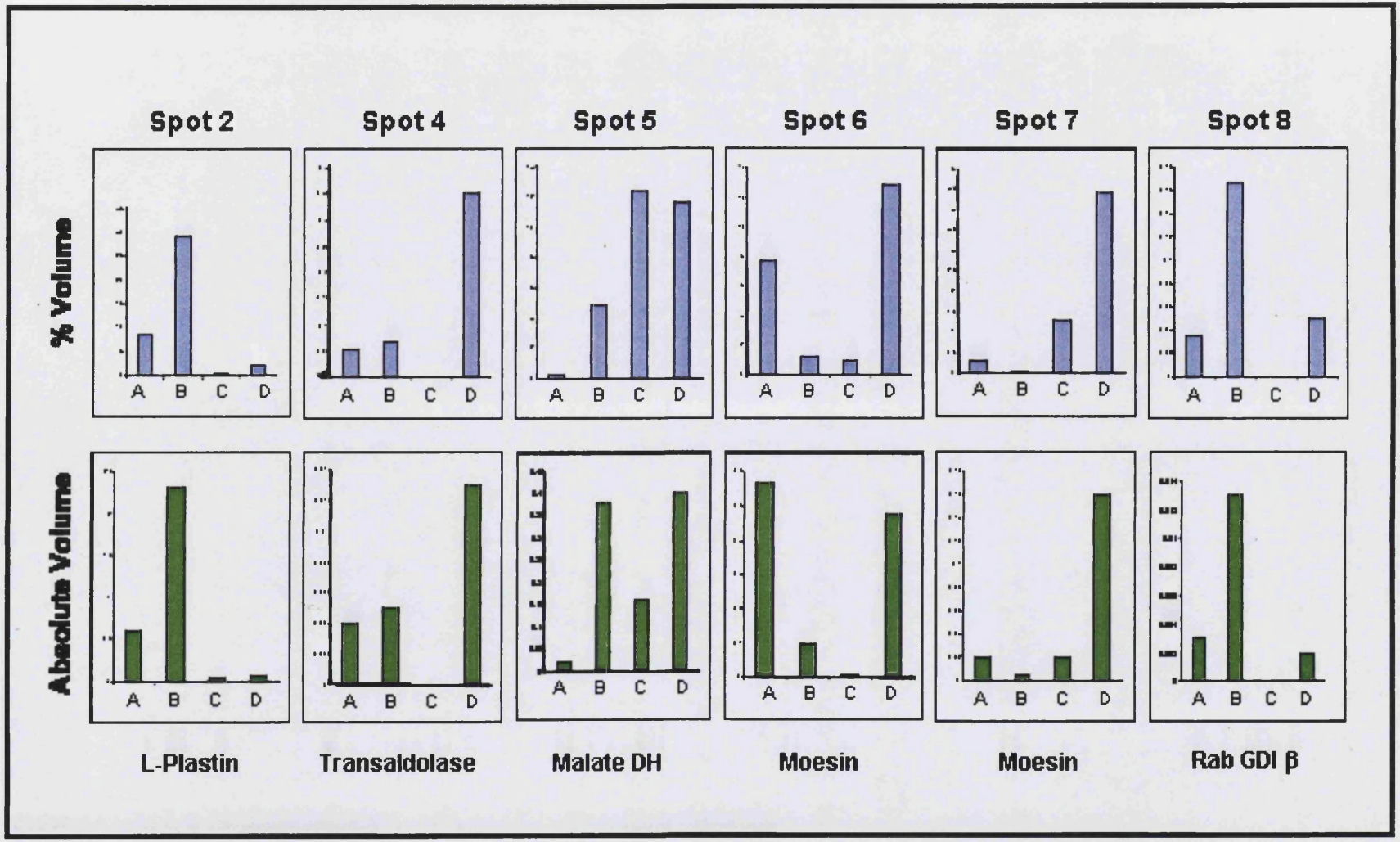
phosphorylated proteins. Proteins were separated by two-dimensional gel electrophoresis post treatment with inhibitors and exposure to IgG-opsonised bacteria. Phosphorylation of several spots differed by the effects of the inhibitors. Eight spots were sequenced and the proteins were identified. As mentioned previously, big protein spots were sequenced in order to ascertain that separation of proteins and sequencing was reliable. In this case catalase was sequenced as the standard (table 7.1 spot 12 and table 7.2 spot 3). Six proteins were identified as being differentially phosphorylated under the influence of Gö 6976 and Ro 31-8220 (table 7.2). Quantification of phosphorylation per protein was performed using Melanie II software, and the results are graphically depicted in figure 7.5. Due to the differences in overall phosphorylation, phosphospots are expressed as percentage volume. Expressing values in this way controls for small differences in overall protein loading per gel. In this manner the phosphorylation level of a single protein is related to overall protein phosphorylation in the particular sample. As a comparison the actual volume of the phosphorylated spot (bottom panel figure 7.5) was compared to the percentage volume (top panel figure 7.5). It can be observed that the relation between the percent and absolute volume is not very different implying that protein loading was similar. This was confirmed by visual inspection of coomassie stained gels (not shown) and therefore the effect of phosphorylation differences is a direct effect of inhibitors. In figure 7.5 it

can also be observed that phosphorylation for the proteins selected are Gö 6976 but not Ro 31-8220 sensitive.

The proteins identified in this section can be divided into two groups: proteins that are involved in glucose metabolism and proteins that regulate cytoskeletal rearrangement. The former group comprises of transaldolase and malate dehydrogenase, and the latter group moesin, rab GDI β and L-plastin.

Figure 7.5 Diagrammatic representation of effects of inhibitors on phosphorylation

Gels in figure 7.4 were analysed using Melanie software (BioRad), and phosphorylation intensity of different proteins (spot numbers in figure 7.4) was compared under different treatment. Top row represents comparison of phosphospot as percent volume, as analysed by software. Bottom row compares absolute volume of phosphorylated spot. (See text) A: control cells (non-stimulated) B: Stimulated cells (IgG-opsonised *S. aureus* $1x10^9$) C: Stimulated cells pre-incubated with 10μM Gö 6976. D: Stimulated cells pre-incubated with 10μM Ro 31-8220.


Spot 2
Spot 4
Spot 5
Spot 6
Spot 7
Spot 8
% Volume
Absolute Volume
A B C D
L-Plastin
Transaldolase
Malate DH
Moesin
Moesin
Rab GDI β

Spot	Identified Protein	Swiss Prot entry	pI	Mass (kDa)	No. of Peptides	%Sequence
1	*Glucose related protein Precursor*	P11021	5.0	72.21	23/58	42
2	*L-Plastin*	P13796	5.2	70.84	31/66	50
3	*Catalase*	P04040	6.9	59.8	11/34	28
4	*Transaldolase*	P37837	6.4	37.7	15/31	34
5	*Malate Dehydrogenase*	P40925	6.9	36.6	12/52	40
6	*Moesin*	P26038	6.1	67.9	33/34	45
7	*Moesin*	P26038	6.1	67.9	30/36	41
8	*RAB GDI β*	P50395	5.9	51.1	18/71	35

Table 7.2 Identification of phosphorylated spots differentially affected by Gö 6976 and Ro 31-8220

7.3.4 In vitro kinase assay with recombinant PKD

To confirm that the proteins identified in section 7.3.2 are in fact substrates for PKD, *in vitro* kinase assays were performed using cytosol and membrane fractions of neutrophils as the substrate. Neutrophils (2×10^8) were isolated and divided into two batches. One batch of cells was not stimulated, while the second batch was stimulated with IgG-opsonised *S. aureus* for one minute and, after quenching the reaction, the cytosolic and membrane fractions were extracted from batches of cells as described in section 2.3.8e. The samples of cytosol and membrane fractions were further divided into two and while one of the samples was incubated with recombinant PKD as described in section 2.3.10, the other was incubated in the same manner excluding the enzyme. Phosphorylated proteins were analysed by SDS-PAGE.

Figure 7.6A shows autoradiographs from results of the *in vitro* kinase assay. No difference could be noted in membrane fractions between the samples in which PKD had been added or not added, and this was irrelevant to incubation with IgG-opsonised *S. aureus*. In the stimulated cytosol fraction, the difference was also negligible, though addition of PKD gave a stronger signal, no major difference in phosphorylation pattern were observed. This could be due to high numbers of activated kinases in the membrane and IgG-opsonised *S. aureus* stimulated cytosol and upon addition of lipid cofactors and ATP, used for the assay, their activity would overwhelm any phosphorylation performed by PKD. Using non-activated

cytosol as a substrate gave the most interesting results; it could be observed that at least four new bands appeared upon addition of PKD.

The protein gel showed differing results. While changes in cytosolic protein patterns pre- and post- IgG-opsonised *S. aureus* stimulation were quite uneventful, the membrane fractions gave quite interesting results. In this fraction activation clearly changed the protein pattern of these fractions. (Figure 7.6B).

Two-dimensional gels, of the non-stimulated cytosol fractions, were

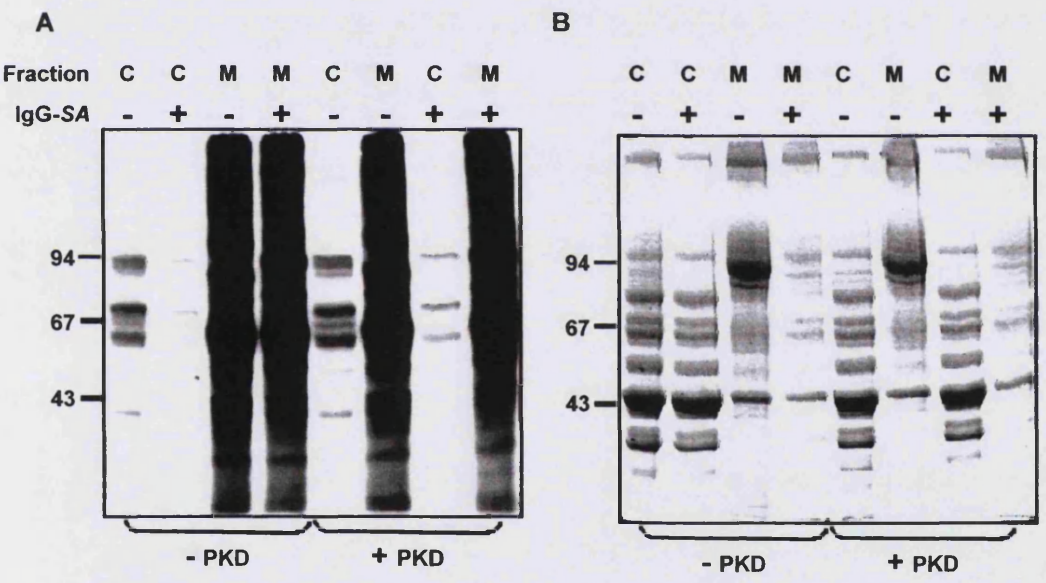


Figure 7.6 *In vitro* kinase assay, PKD phosphorylation of neutrophil proteins.

Neutrophils (10^8 cells) were incubated in the presence and absence of IgG-opsonised *S. aureus* (3×10^9) for 2 minutes at 37°C. The plasma membrane (M) and cytosol (C) fraction where isolates as described in section 2.3.8e, subsequently, half of each fraction was incubated with 92pmols of recombinant PKD and half was not, as described in section 2.3.10. Proteins were then analysed by SDS-PAGE and autoradiography. Panel A: shows radiography of phosphorylated proteins. Panel B coomassie stained gel of same fractions. On the left of each gel molecular weight are indicated in kD.

subsequently run. Figure 7.7 shows a comparison of phosphorylated proteins upon addition of PKD and control, in which no PKD was added.

Figure 7.7A1 and B1 shows the one dimension separation of phosphorylated non-stimulated cytosolic proteins. Over all phosphorylation is increase in panel B1 (PKD added) as compared to A1 (no PKD added), more interestingly is the appearance of extra bands in sample B1 were PKD was added. Further separation of these proteins in a two-dimensional gel, showed the appearance of nine new phosphorylated spots upon addition of PKD. These spots were cut out of the gel and sequenced by MALDI-TOF. Only two of these proteins were identified. (Table 7.3)

It is exciting to note that the proteins identified, moesin and transaldolase, were also identified as PKD substrates in *in vivo* experiments. (Section 7.3.2)

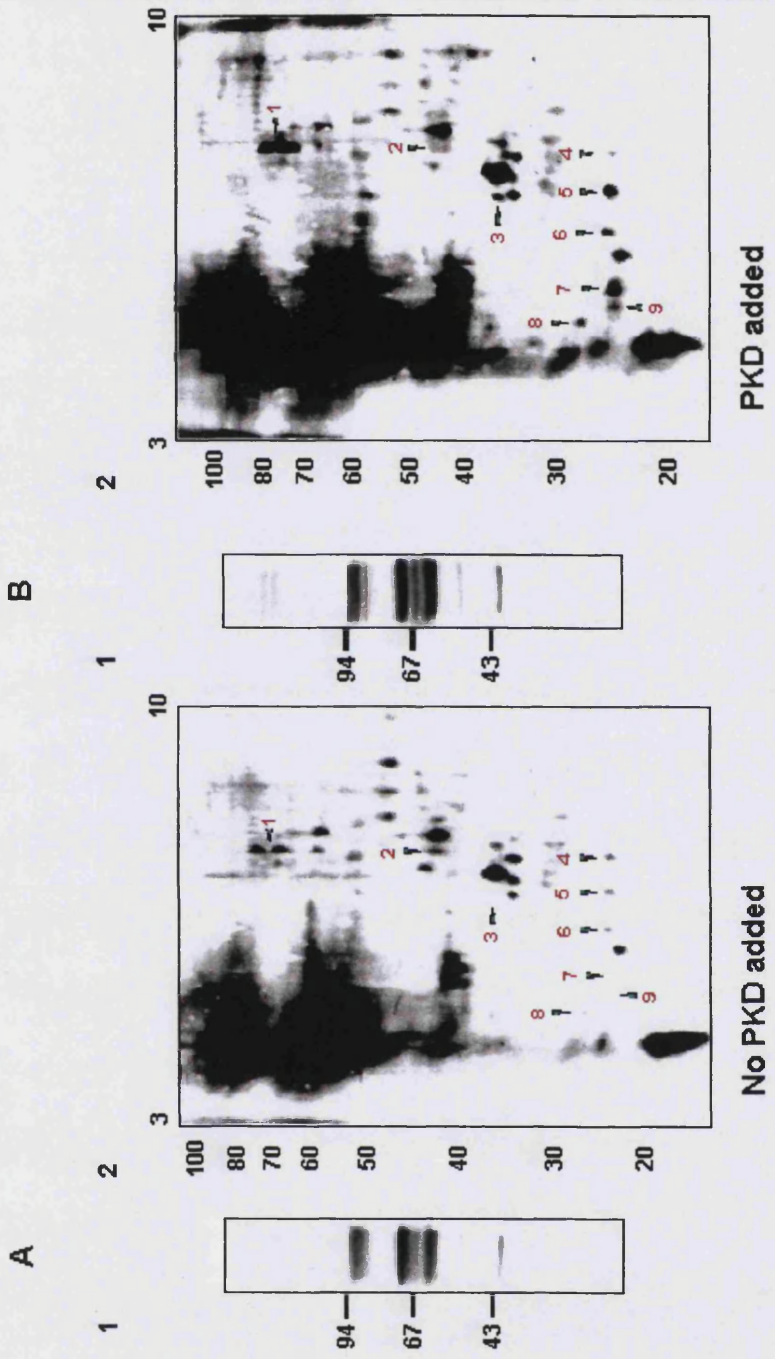


Figure 7.7 *In vitro* PKD phosphorylation of neutrophil cytosol

Neutrophils were treated as described in figure 7.6. The unstimulated cytosol fractions after treatment with (B) or without (A) PKD was analysed by SDS-PAGE and autoradiography (1). Samples were exposed to isoelectric focus, prior to SDS-PAGE, as described in section 2.3.13 and autoradiography (2). Numbers refer to identified proteins.

Spot	Identified Protein	Swiss Prot entry	pI	Mass (kDa)	No. of Peptides	%Sequence
1	*Moesin*	P26038	6.1	67.9	15/58	47
2						
3	*Transaldolase*	P37837	6.4	37.7	8/33	26

Table 7.3 Identification of proteins phosphorylated by PKD *in vitro*

7.4 Summary and discussion

In this section it was observed that proteins were phosphorylated as part of the signalling cascade activated upon Fcγ receptor stimulation by IgG-opsonised *S. aureus*. Twenty-seven proteins were identified in the membrane fraction of neutrophils of which eight were phosphoproteins. Many more proteins can be observed to undergo phosphorylation changes upon Fcγ receptor stimulation. This experiment, as in subsequent experiments, presented a frustrating difficulty, many of the proteins whose phosphorylated state could be detected through autoradiography were below detection of the protein staining protocols used for SDS-PAGE, making it impossible to reliably select the actual protein spot. When it was attempted to simply cut the gel area below the phosphorylated spot visualised by autoradiography, no reliable signal was received from mass fingerprinting by MALDI-TOF. Hence the detection levels used limited determination of many phosphorylated proteins.

Some of the functions of the proteins identified in the stimulation time course are presented above (section 7.3.1). *In vivo* identification of PKD substrates elicited eight candidates.

The function of moesin and L-plastin has been discussed, however the possible relation of these protein to PKD is not clear. It has been shown that moesin is activated by PIP_2, a substrate of PI_4P_5 kinase (Matsui,

Yonemura *et al.* 1999), it has also been shown that PKD associated with PI_4P_5 kinase in COS-7 cells, and this association was dependent on activation of PKD. (Nishikawa, Toker *et al.* 1998) Activation or subcellular distribution of PI_4P_5 kinase could involve PKD in regulation of moesin activity in neutrophils.

L-plastin was also identified as a possible substrate for PKD. L-plastin becomes phosphorylated by PMA implicating PKC, however no specific isoform of this kinase family has been identified, this may suggest a role for PKD.

Rab proteins have been shown to partake in vesicular transport in cells, it mediates fusion of lysosome to endosomes in brain tissue. (Mullock, Bright *et al.* 1998) This process requires the dissociation of Rab GDI from Rab in order to translocate to the membrane. (Ullrich, Stenmark *et al.* 1993) Dissociation requires phosphorylation of the GDI component (Steele-Mortimer, Gruenberg *et al.* 1993) though the kinase involved in this has not been identified; PKD may be a candidate.

By phosphorylating moesin, L-plastin and Rab GDI, PKD could be implicated in the regulation of the cytoskeleton and granule phagosome fusion in neutrophils.

The discovery of the NADPH oxidase was largely based on studying glucose metabolism in neutrophils. It was noticed that glucose was shunted through the HMP and NADPH was being produced and consumed in large amounts leading to the naming of the oxidase (see section 1.5). Two of the

proteins identified as PKD substrates are enzymes which catalyse glucose metabolism.

Transaldolase is a key enzyme in the HMP, it catalyses the reversible reaction which converts sedoheptulose 7-phosphate and glyceraldehyde 3-phosphate to erythrose 4-phosphate and fructose 6-phosphate. Both glyceraldehyde 3-phosphate and fructose 6-phosphate and intermediates of glycolysis, in this manner transaldolase forms a reversible link between glycolysis and HMP. The manner by which transaldolase is regulated is not known, and no reports were found of it being phosphorylated. However, if regulated by phosphorylation, PKD could be influential in salvaging glycolysis intermediates from conversion to ribose 5-phosphate (one of the final products of the HMP) or commit these to the production of NADPH necessary for oxidase function.

Malate dehydrogenase (MDH) was the second metabolic enzyme identified as a possible substrate for PKD. This enzyme catalyses the reversible reaction which converts malate to oxaloacetate. Two isoforms of this enzyme exist a mitochondrial form and a cytosolic form. The former partakes in citric acid cycle (TCA), while the latter helps in transport of acetyl-CoA from the mitochondria to the cytosol. MDH has been shown to be phosphorylated in yeast, which induces inactivation of the enzyme. (Minard and McAlister-Henn 1994) In humans phosphorylation of this enzyme has also been observed (Weller, Jaffe *et al.* 1996), but the effect of this post translation modification is less clear. If phosphorylation would lead

to inactivation of MDH it could be argued that momentary inhibition of the TCA cycle is necessary for further diverting glucose towards the HMP pathway. However if by phosphorylation MDH is activated, this leads to a different hypothesis. Firstly in the TCA cycle, enhanced enzymatic activity would lead to more ATP being produce, which is important as more energy is being consumed by the extra challenge of phagocytosis. (Segal personal communication) The cytosolic isoform, which was the isoform identified by Weller *et al* as the phosphorylated form of MDH, could be activated as well. This enzyme converts oxaloacetate, used as the carrier of acetyl-CoA, back to malate. Interestingly the following step in this metabolic pathway is the conversion of malate to pyruvate by malic enzyme. Malic enzyme uses as a cofactor $NADP^+$ that is reduced to NADPH this in turn could be used by the NADPH oxidase. Equally pyruvate is shifted back into the mitochondria were it is converted to oxaloacetate and the cycle is completed. So presented here is a new way of producing NADPH which could be utilised in neutrophils to replenish depleting NADPH stores during phagocytosis and NADPH oxidase activation.

In vitro kinase assay confirmed that upon addition of recombinant PKD to neutrophils cytosol, transaldolase and moesin were phosphorylated, further confirming some of the finding from *in vivo* phosphorylation assay. Other proteins were observed to be preferentially phosphorylated upon addition of recombinant PKD but the protein could not be identified.

Many other studies should be conducted in order to verify these findings, however this technology provides an exciting tool for identification of kinase substrates.

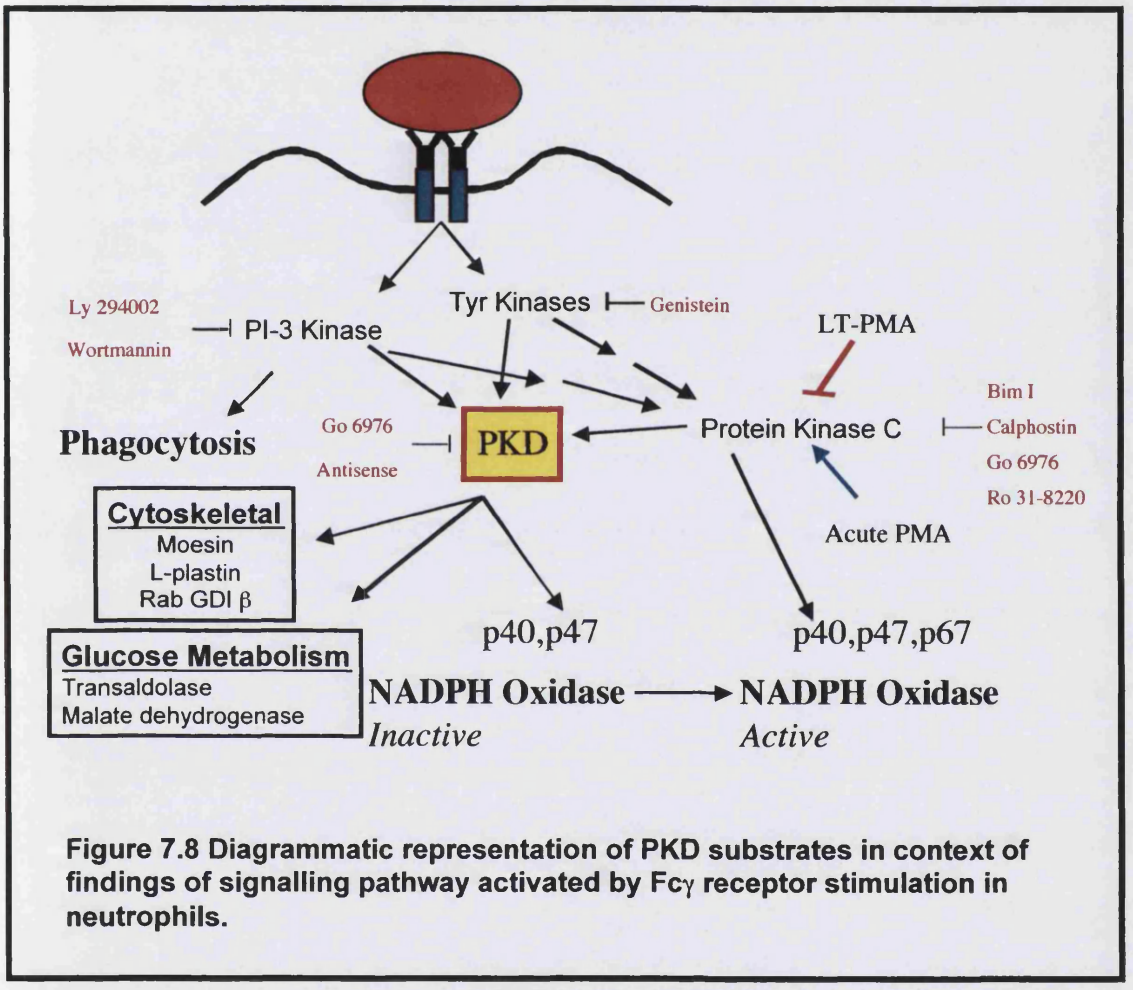


Figure 7.8 Diagrammatic representation of PKD substrates in context of findings of signalling pathway activated by Fcγ receptor stimulation in neutrophils.

In conclusion, five proteins were identified as PKD substrates *in vivo*; MDH, transaldolase, moesin, L-plastin and Rab GDI, two of these were also phosphorylated *in vitro* by PKD. This links PKD to other functions in neutrophils such as cytoskeletal rearrangements and glucose metabolism. (Figure 7.8)

Chapter 8

General Summary and Discussion

Neutrophils are the most abundant phagocyte in the body, and neutrophil dysfunction and neutropenia highlight its importance in the clearance of infections. Neutrophils act by taking up bacteria and inactivating them. Opsonisation of particles facilitates and greatly speeds up uptake and clearance of invading pathogens. Opsonised particles bind Fcγ receptors on the neutrophil plasma membrane, which initiates the process of phagocytosis, through a zippering mechanism.

During phagocytosis several cellular mechanisms are activated, amongst them cytoskeletal rearrangement, degranulation and activation of the NADPH oxidase. These mechanisms are activated through a series of biochemical events, which couple the Fcγ receptor to the activity required. These biochemical events are collectively referred to as signal transduction, and they include activation of phospholipases and lipid kinases, generation of second messengers, and activation of protein kinases and phophatases. It is known that the state of phosphorylation of proteins is essential their

activity and many signal transduction cascades lead to the activation of kinases that regulate this process.

The role of kinases in the signalling process leading to activation of the NADPH oxidase in human neutrophils upon Fcγ receptor stimulation was the focus of this thesis.

Activity of the NADPH oxidase was measured by changes in rates of oxygen consumption in neutrophils. By employing pharmacological agents which are known to selectively inhibit specific kinase function, the involvement of several kinases in activation of the NADPH oxidase was shown. Tyrosine kinases, PI-3 kinase and PKC are all directly involved in this signalling process. (Section 3.3) Physiologically, activation of the NADPH oxidase occurs concurrently with phagocytosis, and while it was shown that PI-3 kinase is involved in particle uptake as well as NADPH oxidase activation, PKC affected only the NADPH oxidase. (Chapter 3) Furthermore, it was shown that PKC may be downstream of PI-3 kinase or in a pathway parallel to it. These findings would suggest a central role for PI-3 kinase as a point of divergence in Fcγ receptor signalling cascade.

PKC is not a single kinase but a family of kinases. It comprises of 11 isoforms subdivided into 3 groups. Neutrophils contain members of 2, or possibly 3 subfamilies (Section 1.7). Two isoforms, PKCβ and PKC δ are most abundant. In order to examine the contribution of the different isoforms in activation of the NADPH oxidase, various PKC inhibitors were used. While Gö 6976, inhibits the classical subfamily (in the case of

neutrophils the PKCβ isoform), Ro 31-8220, calphostin C and Bim I has a lower isoform specificity and inhibit all PKCs. Contradictory to what was expected it was noticed that the less specific inhibitor, Ro 31-8220, had a lower inhibitory effect, the more isoform-specific inhibitor, Gö 6976, was able to inhibit NADPH oxidase activity completely upon Fcγ receptor stimulation. (Figure 3.4 and table 3.2)

This inhibitory profile, Gö 6976 sensitive and Ro 31-8220 insensitive, correlated with previous studies by a German group, which reported that, *in vitro,* a recently identified PKC isoform, PKCμ/PKD fit this profile. PKDs presence in neutrophils had previously not been reported.

We showed that PKD is present in neutrophils and activated upon Fcγ receptor ligation in human neutrophils (section 4.3.1). Using PKD antisense oligonucleotides it was determined that PKD partakes in the activation of the NADPH oxidase, and contributes between 40 to 50% of activation. (Figure 3.6 and 4.9) It was shown that PKD is regulated by two kinases upstream. It was observed that by inhibiting PKC, PKD activity was 50% of total, and inhibition of PI-3 kinase gave a similar result. (Figure 4.11) Combination of inhibitors did not produce an additive inhibition implying that a third regulatory mechanism might be involved.

PKD subcellular localisation gave a further hint to its function in NADPH oxidase activation. In non-stimulated cells, PKD presence was predominantly in the plasma membrane, and upon activation with IgG-opsonised *S. aureus,* it became localised in the phagosome. (Chapter 4)

Here it colocalised with p47^{phox}, a component of the NADPH oxidase. (Figure 5.7) Furthermore it was shown that two of the cytosolic components of the NADPH oxidase, p40^{phox} and p47^{phox} are direct substrates for PKD (chapter 6), thus explaining the mechanism by which PKD may exert its influence on superoxide formation during the respiratory burst in neutrophils. Further work is currently being carried out to identify the PKD phosphorylation sites in these proteins and hereby characterise its specific targets. Preliminary results suggest that PKD may be phosphorylating a site on the N-terminus of p47^{phox} *in vitro*, this is quite exciting as no sites on this end of the protein have ever been described. Though it has been shown that phosphorylation of p47^{phox} on the C-terminus is sufficient to maintain NADPH oxidase activation. Therefore, the possible PKD target(s) on the N-terminus of p47^{phox} could mediated protein-protein interactions for p47^{phox} and thereby reveal further potential functions for some of the cytosolic NADPH oxidase components.

In searching for further PKD substrates in neutrophils 5 different proteins were identified as potential candidates. On this basis PKD could possibly regulate morphological changes in these cells through its substrate moesin or intracellular vesicular movement by regulating *rab* GDI. Furthermore, PKD may regulate glucose metabolism through its effect on malate dehydrogenase and transaldolase. This is of particular interest as initial identification of the NADPH oxidase in was largely based on

observations of glucose metabolism. Further work on the identification of PKD substrates in neutrophils need to be undertaken.

In summary, some aspects of the Fcγ receptor signalling cascade were identified and PKD was placed in this cascade. (See figure 7.8) Furthermore PKD is placed as one of the kinases that regulate NADPH oxidase activity. (Figure 8.1)

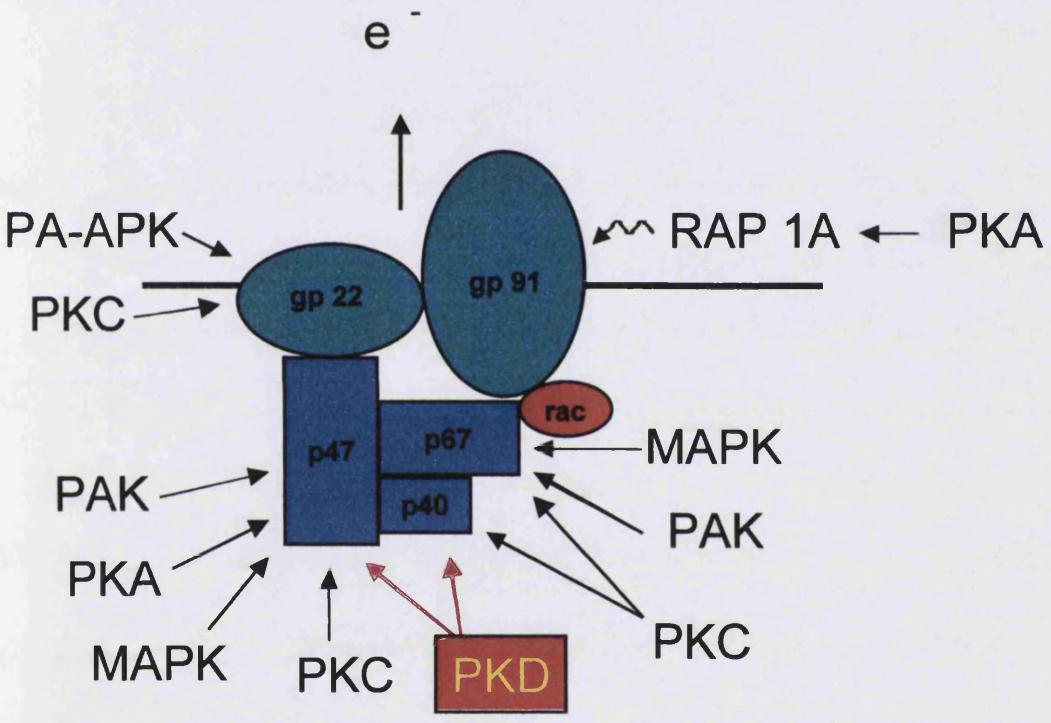


Figure 8.1 Diagrammatic representation of kinases known to phosphorylate components of the NADPH oxidase

PKD is placed in the scheme of kinases that phosphorylate NADPH oxidase components and thereby assist in regulation of superoxide production. (See figure 1.7, section 1.8 and chapter 6)

Chapter 9

References

Abo, A., A. Boyhan, I. West, A. J. Thrasher and A. W. Segal (1992). "Reconstitution of neutrophil NADPH oxidase activity in the cell-free system by four components: p67-phox, p47-phox, p21rac1, and cytochrome b-245." J Biol Chem **267**(24): 16767-70.

Abo, A., E. Pick, A. Hall, N. Totty, C. G. Teahan and A. W. Segal (1991). "Activation of the NADPH oxidase involves the small GTP-binding protein p21rac1." Nature **353**(6345): 668-70.

Abo, A. and A. W. Segal (1995). "Reconstitution of cell-free NADPH oxidase activity by purified components." Methods Enzymol **256**: 268-78.

Abo, A., M. R. Webb, A. Grogan and A. W. Segal (1994). "Activation of NADPH oxidase involves the dissociation of p21rac from its inhibitory GDP/GTP exchange protein (rhoGDI) followed by its translocation to the plasma membrane." Biochem J **298 Pt 3**: 585-91.

Ago, T., H. Nunoi, T. Ito and H. Sumimoto (1999). "Mechanism for phosphorylation-induced activation of the phagocyte NADPH oxidase protein p47(phox). Triple replacement of serines 303, 304, and 328 with aspartates disrupts the SH3 domain-mediated intramolecular interaction in p47(phox), thereby activating the oxidase." J Biol Chem **274**(47): 33644-53.

Aharoni, I. and E. Pick (1990). "Activation of the superoxide-generating NADPH oxidase of macrophages by sodium dodecyl sulfate in a soluble cell-free

system: evidence for involvement of a G protein." J Leukoc Biol **48**(2): 107-15.

Ahmed, S., E. Prigmore, S. Govind, C. Veryard, R. Kozma, F. B. Wientjes, A. W. Segal and L. Lim (1998). "Cryptic Rac-binding and p21(Cdc42Hs/Rac)-activated kinase phosphorylation sites of NADPH oxidase component p67(phox)." J Biol Chem **273**(25): 15693-701.

Akimoto, K., K. Mizuno, S. Osada, S. Hirai, S. Tanuma, K. Suzuki and S. Ohno (1994). "A new member of the third class in the protein kinase C family, PKC lambda, expressed dominantly in an undifferentiated mouse embryonal carcinoma cell line and also in many tissues and cells." J Biol Chem **269**(17): 12677-83.

Akiyama, T., J. Ishida, S. Nakagawa, H. Ogawara, S. Watanabe, N. Itoh, M. Shibuya and Y. Fukami (1987). "Genistein, a specific inhibitor of tyrosine-specific protein kinases." J Biol Chem **262**(12): 5592-5.

Araki, N., M. T. Johnson and J. A. Swanson (1996). "A role for phosphoinositide 3-kinase in the completion of macropinocytosis and phagocytosis by macrophages." J Cell Biol **135**(5): 1249-60.

Aratani, Y., H. Koyama, S. Nyui, K. Suzuki, F. Kura and N. Maeda (1999). "Severe impairment in early host defense against Candida albicans in mice deficient in myeloperoxidase." Infect Immun **67**(4): 1828-36.

Arcaro, A. and M. P. Wymann (1993). "Wortmannin is a potent phosphatidylinositol 3-kinase inhibitor: the role of phosphatidylinositol 3,4,5-trisphosphate in neutrophil responses." Biochem J **296**(Pt 2): 297-301.

Athens, J. W., H. A. Vodopick, G. E. Cartwright and M. M. Wintrobe (1967). "The use of radiosulfate to evaluate myelocyte kinetics in the dog." Haematol Lat **10**(1): 117-22.

Babior, B. M. (1982). The Production of Superoxide by Neutrophils. Phagocytosis-Past and Future. M. L. K. a. L. Bolis. New York, Academic Press: 157-166.

Babior, B. M., Kipnes, R. S., Curnutte, J. T. (1973). "Biological defense Mechanisms The production by leukocytes of superoxide, a potential bactericidal agent." The Journal of Clinical Medicine **52**: 741-744.

Baehner, R. L. and M. L. Karnovsky (1968). "Deficiency of reduced nicotinamide-adenine dinucleotide oxidase in chronic granulomatous disease." Science **162**(859): 1277-9.

Baggiolini, M., B. Dewald, J. Schnyder, W. Ruch, P. H. Cooper and T. G. Payne (1987). "Inhibition of the phagocytosis-induced respiratory burst by the fungal metabolite wortmannin and some analogues." Exp Cell Res **169**(2).

Bagowski, C. P., M. Stein-Gerlach, A. Choidas and A. Ullrich (1999). "Cell-type specific phosphorylation of threonines T654 and T669 by PKD defines the signal capacity of the EGF receptor." Embo J **18**(20): 5567-76.

Belaaouaj, A., R. McCarthy, M. Baumann, Z. Gao, T. J. Ley, S. N. Abraham and S. D. Shapiro (1998). "Mice lacking neutrophil elastase reveal impaired host defense against gram negative bacterial sepsis." Nat Med **4**(5): 615-8.

Bengis-Garber, C. and N. Gruener (1993). "Calcium-binding myeloid protein (P8,14) is phosphorylated in fMet-Leu-Phe-stimulated neutrophils." J Leukoc Biol **54**(2): 114-8.

Bengis-Garber, C. and N. Gruener (1995). "Involvement of protein kinase C and of protein phosphatases 1 and/or 2A in p47 phox phosphorylation in formylmet-Leu-Phe stimulated neutrophils: studies with selective inhibitors RO 31-8220 and calyculin A." Cell Signal **7**(7): 721-32.

Bengis-Garber, C. and N. Gruener (1996). "Protein kinase A downregulates the phosphorylation of p47 phox in human neutrophils: a possible pathway for inhibition of the respiratory burst." Cell Signal **8**(4): 291-6.

Benna, J. E., P. M. Dang, M. Gaudry, M. Fay, F. Morel, J. Hakim and M. A. Gougerot-Pocidalo (1997). "Phosphorylation of the respiratory burst oxidase subunit p67(phox) during human neutrophil activation. Regulation by protein kinase C- dependent and independent pathways." J Biol Chem **272**(27): 17204-8.

Bennett, P. A., P. M. Finan, R. J. Dixon and S. Kellie (1995). "Tyrosine phosphatase antagonist-induced activation of the neutrophil NADPH oxidase: a possible role for protein kinase C." Immunology **85**(2): 304-10.

Bentz, M., K. Harvey, D. J. Burtschi, D. English and R. A. Siddiqui (1999). "Induction of neutrophil calcium mobilization by phosphatidic acid-enriched plasma membranes." IUBMB Life **48**(2): 179-85.

Berendes, H., Bridges, R.A., Good, R.A. (1957). "A fatal granulomatous disease." Minnesota Medicine(May): 309-312.

Berridge, M. J. (1984). "Inositol trisphosphate and diacylglycerol as second messengers." Biochem J **220**(2): 345-60.

Bishop, C. R., J. W. Athens, D. R. Boggs, H. R. Warner, G. E. Cartwright and M. M. Wintrobe (1968). "Leukokinetic studies. 13. A non-steady-state kinetic evaluation of the mechanism of cortisone-induced granulocytosis." J Clin Invest **47**(2): 249-60.

Blume, R. S. and S. M. Wolff (1972). "The Chediak-Higashi syndrome: studies in four patients and a review of the literature." Medicine (Baltimore) **51**(4): 247-80.

Bokoch, G. M., L. A. Quilliam, B. P. Bohl, A. J. Jesaitis and M. T. Quinn (1991). "Inhibition of Rap1A binding to cytochrome b558 of NADPH oxidase by phosphorylation of Rap1A." Science **254**(5039): 1794-6.

Borregaard, N. (1997). "Development of neutrophil granule diversity." Ann N Y Acad Sci **832**: 62-8.

Borregaard, N., A. R. Cross, T. Herlin, O. T. Jones, A. W. Segal and N. H. Valerius (1983). "A variant form of X-linked chronic granulomatous disease with normal nitroblue tetrazolium slide test and cytochrome b." Eur J Clin Invest **13**(3): 243-8.

Borregaard, N., K. Lollike, L. Kjeldsen, H. Sengelov, L. Bastholm, M. H. Nielsen and D. F. Bainton (1993). "Human neutrophil granules and secretory vesicles." Eur J Haematol **51**(4): 187-98.

Bouin, A. P., N. Grandvaux, P. V. Vignais and A. Fuchs (1998). "p40(phox) is phosphorylated on threonine 154 and serine 315 during activation of the

phagocyte NADPH oxidase. Implication of a protein kinase c-type kinase in the phosphorylation process." J Biol Chem **273**(46): 30097-103.

Bowden, E. T., M. Barth, D. Thomas, R. I. Glazer and S. C. Mueller (1999). "An invasion-related complex of cortactin, paxillin and PKCmu associates with invadopodia at sites of extracellular matrix degradation." Oncogene **18**(31): 4440-9.

Brandtzaeg, P., T. O. Gabrielsen, I. Dale, F. Muller, M. Steinbakk and M. K. Fagerhol (1995). "The leucocyte protein L1 (calprotectin): a putative nonspecific defence factor at epithelial surfaces." Adv Exp Med Biol **371A**: 201-6.

Breton-Gorius, J., D. Y. Mason, D. Buriot, J. L. Vilde and C. Griscelli (1980). "Lactoferrin deficiency as a consequence of a lack of specific granules in neutrophils from a patient with recurrent infections. Detection by immunoperoxidase staining for lactoferrin and cytochemical electron microscopy." Am J Pathol **99**(2): 413-28.

Bromberg, Y. and E. Pick (1985). "Activation of NADPH-dependent superoxide production in a cell-free system by sodium dodecyl sulfate." J Biol Chem **260**(25): 13539-45.

Brumell, J. H., A. L. Burkhardt, J. B. Bolen and S. Grinstein (1996). "Endogenous reactive oxygen intermediates activate tyrosine kinases in human neutrophils." J Biol Chem **271**(3): 1455-61.

Cabanis, A., B. Gressier, C. Brunet, T. Dine, M. Luyckx, M. Cazin and J. C. Cazin (1996). "Effect of the protein kinase C inhibitor GF 109 203X on elastase release and respiratory burst of human neutrophils." Gen Pharmacol **27**(8): 1409-14.

Casimir, C., M. Chetty, M. C. Bohler, R. Garcia, A. Fischer, C. Griscelli, B. Johnson and A. W. Segal (1992). "Identification of the defective NADPH-oxidase component in chronic granulomatous disease: a study of 57 European families." Eur J Clin Invest **22**(6): 403-6.

Castagna, M., Y. Takai, K. Kaibuchi, K. Sano, U. Kikkawa and Y. Nishizuka (1982). "Direct activation of calcium-activated, phospholipid-dependent

protein kinase by tumor-promoting phorbol esters." J Biol Chem **257**(13): 7847-51.

Chu, J., Y. Liu, G. A. Koretzky and D. L. Durden (1998). "SLP-76-Cbl-Grb2-Shc interactions in FcgammaRI signaling." Blood **92**(5): 1697-706.

Chuang, F. Y., M. Sassaroli and J. C. Unkeless (2000). "Convergence of Fc gamma receptor IIA and Fc gamma receptor IIIB signaling pathways in human neutrophils." J Immunol **164**(1): 350-60.

Clapham, D. E. (1995). "Calcium signaling." Cell **80**(2): 259-68.

Clark, R. A. and H. R. Kimball (1971). "Defective granulocyte chemotaxis in the Chediak-Higashi syndrome." J Clin Invest **50**(12): 2645-52.

Clark, R. A., H. L. Malech, J. I. Gallin, H. Nunoi, B. D. Volpp, D. W. Pearson, W. M. Nauseef and J. T. Curnutte (1989). "Genetic variants of chronic granulomatous disease: prevalence of deficiencies of two cytosolic components of the NADPH oxidase system." N Engl J Med **321**(10): 647-52.

Cline, M. J. (1966). "Phagocytosis and synthesis of ribonucleic acid in human granulocytes." Nature **212**: 1431-3.

Clohessy, P. A. and B. E. Golden (1995). "Calprotectin-mediated zinc chelation as a biostatic mechanism in host defence." Scand J Immunol **42**(5): 551-6.

Cohen, G. B., R. Ren and D. Baltimore (1995). "Modular binding domains in signal transduction proteins." Cell **80**(2): 237-48.

Cohen, M. S. (1994). "Molecular events in the activation of human neutrophils for microbial killing." Clin Infect Dis **18 Suppl 2**: S170-9.

Cohen, P. (1982). "The role of protein phosphorylation in neural and hormonal control of cellular activity." Nature **296**(5858): 613-20.

Combadiere, C., J. el Benna, E. Pedruzzi, J. Hakim and A. Perianin (1993). "Stimulation of the human neutrophil respiratory burst by formyl peptides is primed by a protein kinase inhibitor, staurosporine." Blood **82**(9): 2890-8.

Coussens, L., P. J. Parker, L. Rhee, T. L. Yang-Feng, E. Chen, M. D. Waterfield, U. Francke and A. Ullrich (1986). "Multiple, distinct forms of bovine and

human protein kinase C suggest diversity in cellular signaling pathways." Science **233**(4766): 859-66.

Cox, J. A., A. Y. Jeng, N. A. Sharkey, P. M. Blumberg and A. I. Tauber (1985). "Activation of the human neutrophil nicotinamide adenine dinucleotide phosphate (NADPH)-oxidase by protein kinase C." J Clin Invest **76**(5): 1932-8.

Coxon, P. Y., M. J. Rane, D. W. Powell, J. B. Klein and K. R. McLeish (2000). "Differential mitogen-activated protein kinase stimulation by Fc gamma receptor IIa and Fc gamma receptor IIIb determines the activation phenotype of human neutrophils." J Immunol **164**(12): 6530-7.

Craddock, C. G. (1992). Defenses of the Body: The Initiators of Defense, The Ready Reserves, and The Scavengers. Blood Pure and Eloquent- A Story of Discovery, of people, and of Ideas. M. M. Wintrobe. New York, McGraw-Hill Book Company: 417-454.

Cross, A. R., J. Rae and J. T. Curnutte (1995). "Cytochrome b-245 of the neutrophil superoxide-generating system contains two nonidentical hemes. Potentiometric studies of a mutant form of gp91phox." J Biol Chem **270**(29): 17075-7.

Crowley, M. T., P. S. Costello, C. J. Fitzer-Attas, M. Turner, F. Meng, C. Lowell, V. L. Tybulewicz and A. L. DeFranco (1997). "A critical role for Syk in signal transduction and phagocytosis mediated by Fcgamma receptors on macrophages." J Exp Med **186**(7): 1027-39.

Curnutte, J. T. (1985). "Activation of human neutrophil nicotinamide adenine dinucleotide phosphate, reduced (triphosphopyridine nucleotide, reduced) oxidase by arachidonic acid in a cell-free system." J Clin Invest **75**(5): 1740-3.

Curnutte, J. T., R. L. Berkow, R. L. Roberts, S. B. Shurin and P. J. Scott (1988). "Chronic granulomatous disease due to a defect in the cytosolic factor required for nicotinamide adenine dinucleotide phosphate oxidase activation." J Clin Invest **81**(2): 606-10.

Curnutte, J. T., R. Kuver and P. J. Scott (1987). "Activation of neutrophil NADPH oxidase in a cell-free system. Partial purification of components and characterization of the activation process." J Biol Chem **262**(12): 5563-9.

Curnutte, J. T., P. J. Scott and L. A. Mayo (1989). "Cytosolic components of the respiratory burst oxidase: resolution of four components, two of which are missing in complementing types of chronic granulomatous disease." Proc Natl Acad Sci U S A **86**(3): 825-9.

Daeron, M. (1997). "Fc receptor biology." Annu Rev Immunol **15**: 203-34.

Dahlgren, C. and A. Karlsson (1999). "Respiratory burst in human neutrophils." J Immunol Methods **232**(1-2): 3-14.

Dekker, L. V., M. Leitges, G. Altschuler, N. Mistry, A. McDermott, J. Roes and A. W. Segal (2000). "Protein kinase C-beta contributes to NADPH oxidase activation in neutrophils." Biochem J **347**(Pt 1): 285-9.

Dekker, L. V. and P. J. Parker (1994). "Protein kinase C--a question of specificity." Trends Biochem Sci **19**(2): 73-7.

Dekker, L. V. and P. J. Parker (1997). "Regulated binding of the protein kinase C substrate GAP-43 to the V0/C2 region of protein kinase C-delta." J Biol Chem **272**(19): 12747-53.

Dekker, L. V., P. J. Parker and P. McIntyre (1992). "Biochemical properties of rat protein kinase C-eta expressed in COS cells." FEBS Lett **312**(2-3): 195-9.

DeLeo, F. R., L. A. Allen, M. Apicella and W. M. Nauseef (1999). "NADPH oxidase activation and assembly during phagocytosis." J Immunol **163**(12): 6732-40.

Della Bianca, V., M. Grzeskowiak, S. Dusi and F. Rossi (1993). "Transmembrane signaling pathways involved in phagocytosis and associated activation of NADPH oxidase mediated by Fc gamma Rs in human neutrophils." J Leukoc Biol **53**(4): 427-38.

Delves, P. J. and I. M. Roitt (2000). "The immune system. First of two parts." N Engl J Med **343**(1): 37-49.

Delves, P. J. and I. M. Roitt (2000). "The immune system. Second of two parts." N Engl J Med **343**(2): 108-17.

Diekmann, D., A. Abo, C. Johnston, A. W. Segal and A. Hall (1994). "Interaction of Rac with p67phox and regulation of phagocytic NADPH oxidase activity." Science **265**(5171): 531-3.

Dieter, P. and E. Fitzke (1991). "RO 31-8220 and RO 31-7549 show improved selectivity for protein kinase C over staurosporine in macrophages." Biochem Biophys Res Commun **181**(1): 396-401.

Dieterich, S., T. Herget, G. Link, H. Bottinger, K. Pfizenmaier and F. J. Johannes (1996). "In vitro activation and substrates of recombinant, baculovirus expressed human protein kinase C mu." FEBS Lett **381**(3): 183-7.

Dinauer, M. C., E. A. Pierce, G. A. Bruns, J. T. Curnutte and S. H. Orkin (1990). "Human neutrophil cytochrome b light chain (p22-phox). Gene structure, chromosomal location, and mutations in cytochrome-negative autosomal recessive chronic granulomatous disease." J Clin Invest **86**(5): 1729-37.

Dinauer, M. C., E. A. Pierce, R. W. Erickson, T. J. Muhlebach, H. Messner, S. H. Orkin, R. A. Seger and J. T. Curnutte (1991). "Point mutation in the cytoplasmic domain of the neutrophil p22-phox cytochrome b subunit is associated with a nonfunctional NADPH oxidase and chronic granulomatous disease." Proc Natl Acad Sci U S A **88**(24): 11231-5.

Ding, J., C. J. Vlahos, R. Liu, R. F. Brown and J. A. Badwey (1995). "Antagonists of phosphatidylinositol 3-kinase block activation of several novel protein kinases in neutrophils." J Biol Chem **270**(19): 11684-91.

Djafarzadeh, S. and V. Niggli (1997). "Signaling pathways involved in dephosphorylation and localization of the actin-binding protein cofilin in stimulated human neutrophils." Exp Cell Res **236**(2): 427-35.

Douglas, S. A. W. a. S. R. (1903). An Experimental Investigation of the Role of the Blood Fluids in Connection with Phagocytosis. Milestones in Immunology- A Historical Exploration. D. J. Bibel. Madison WI, Sciemce Tech Publishers: 174-177.

Dusi, S., V. Della Bianca, M. Grzeskowiak and F. Rossi (1993). "Relationship between phosphorylation and translocation to the plasma membrane of

p47phox and p67phox and activation of the NADPH oxidase in normal and Ca(2+)-depleted human neutrophils." Biochem J **290**(Pt 1): 173-8.

Dusi, S., M. Donini, V. Della Bianca and F. Rossi (1994). "Tyrosine phosphorylation of phospholipase C-gamma 2 is involved in the activation of phosphoinositide hydrolysis by Fc receptors in human neutrophils." Biochem Biophys Res Commun **201**(3): 1100-8.

Dusi, S., M. Donini and F. Rossi (1996). "Mechanisms of NADPH oxidase activation: translocation of p40phox, Rac1 and Rac2 from the cytosol to the membranes in human neutrophils lacking p47phox or p67phox." Biochem J **314**(Pt 2): 409-12.

Dusi, S. and F. Rossi (1993). "Activation of NADPH oxidase of human neutrophils involves the phosphorylation and the translocation of cytosolic p67phox." Biochem J **296**(Pt 2): 367-71.

Edberg, J. C., C. T. Lin, D. Lau, J. C. Unkeless and R. P. Kimberly (1995). "The Ca2+ dependence of human Fc gamma receptor-initiated phagocytosis." J Biol Chem **270**(38): 22301-7.

Edgerton, M. (1999). "Myeloperoxidase deficiency associated with atypical oral candidiasis: A clinical report." J Prosthet Dent **82**(3): 263-5.

Edwards, S. W. (1994). Biochemistry and physiology of the neutophil. Cambridge, Cambridge University Press.

Edwards, S. W., F. Watson, L. Gasmi, D. A. Moulding and J. A. Quayle (1997). "Activation of human neutrophils by soluble immune complexes: role of Fc gamma RII and Fc gamma RIIIb in stimulation of the respiratory burst and elevation of intracellular Ca2+." Ann N Y Acad Sci **832**: 341-57.

Eigenbrodt, E., M. Reinacher, U. Scheefers-Borchel, H. Scheefers and R. Friis (1992). "Double role for pyruvate kinase type M2 in the expansion of phosphometabolite pools found in tumor cells." Crit Rev Oncog **3**(1-2): 91-115.

el Benna, J., L. P. Faust and B. M. Babior (1994). "The phosphorylation of the respiratory burst oxidase component p47phox during neutrophil activation.

Phosphorylation of sites recognized by protein kinase C and by proline-directed kinases." J Biol Chem **269**(38): 23431-6.

El Benna, J., R. P. Faust, J. L. Johnson and B. M. Babior (1996). "Phosphorylation of the respiratory burst oxidase subunit p47phox as determined by two-dimensional phosphopeptide mapping. Phosphorylation by protein kinase C, protein kinase A, and a mitogen-activated protein kinase." J Biol Chem **271**(11): 6374-8.

El Benna, J., J. Han, J. W. Park, E. Schmid, R. J. Ulevitch and B. M. Babior (1996). "Activation of p38 in stimulated human neutrophils: phosphorylation of the oxidase component p47phox by p38 and ERK but not by JNK." Arch Biochem Biophys **334**(2): 395-400.

Faust, L. R., J. el Benna, B. M. Babior and S. J. Chanock (1995). "The phosphorylation targets of p47phox, a subunit of the respiratory burst oxidase. Functions of the individual target serines as evaluated by site-directed mutagenesis." J Clin Invest **96**(3): 1499-505.

Ferrante, A. (1992). "Activation of neutrophils by interleukins-1 and -2 and tumor necrosis factors." Immunol Ser **57**: 417-36.

Forbes, L. V., O. Truong, F. B. Wientjes, S. J. Moss and A. W. Segal (1999). "The major phosphorylation site of the NADPH oxidase component p67phox is Thr233." Biochem J **338**(Pt 1): 99-105.

Fuchs, A., A. P. Bouin, T. Rabilloud and P. V. Vignais (1997). "The 40-kDa component of the phagocyte NADPH oxidase (p40phox) is phosphorylated during activation in differentiated HL60 cells." Eur J Biochem **249**(2): 531-9.

Fukushima, T., T. K. Waddell, S. Grinstein, G. G. Goss, J. Orlowski and G. P. Downey (1996). "Na+/H+ exchange activity during phagocytosis in human neutrophils: role of Fcgamma receptors and tyrosine kinases." J Cell Biol **132**(6): 1037-52.

Gabay, J. E. (1994). "Antimicrobial proteins with homology to serine proteases." Ciba Found Symp **186**: 237-47.

Gabig, T. G., C. D. Crean, P. L. Mantel and R. Rosli (1995). "Function of wild-type or mutant Rac2 and Rap1a GTPases in differentiated HL60 cell NADPH oxidase activation." Blood **85**(3): 804-11.

Garcia, R. C. and A. W. Segal (1984). "Changes in the subcellular distribution of the cytochrome b-245 on stimulation of human neutrophils." Biochem J **219**(1): 233-42.

Garcia, R. C. and A. W. Segal (1988). "Phosphorylation of the subunits of cytochrome b-245 upon triggering of the respiratory burst of human neutrophils and macrophages." Biochem J **252**(3): 901-4.

Garcia, R. C., M. Whitaker, P. G. Heyworth and A. W. Segal (1992). "Okadaic acid produces changes in phosphorylation and translocation of proteins and in intracellular calcium in human neutrophils. Relationship with the activation of the NADPH oxidase by different stimuli." Biochem J **286**(Pt 3): 687-92.

Gennaro, R., C. Florio and D. Romeo (1986). "Co-activation of protein kinase C and NADPH oxidase in the plasma membrane of neutrophil cytoplasts." Biochem Biophys Res Commun **134**(1): 305-12.

Georg Martiny-Baron, M. G. K., Harald Mischak, Peter M. Blumberg, Georg Kochs, Hubert Hug, Dieter Marme, and Christoph Schachtele. (1993). "Selective Inhibition of Protein Kinase C Isozymes by the Indolocarbazole Go 6976." The Journal of Biological Chemistry **268**(13): 9194-9197.

Gerard, C. B. a. R. (1933). "The Extra Respiration of Phagocytosis." American Journal of Physiology: 235-236.

Gewirtz, A. T. and E. R. Simons (1997). "Phospholipase D mediates Fc gamma receptor activation of neutrophils and provides specificity between high-valency immune complexes and fMLP signaling pathways." J Leukoc Biol **61**(4): 522-8.

Goekjian, P. G. and M. R. Jirousek (1999). "Protein kinase C in the treatment of disease: signal transduction pathways, inhibitors, and agents in development." Curr Med Chem **6**(9): 877-903.

Goodman, E. B. and A. J. Tenner (1992). "Signal transduction mechanisms of C1q-mediated superoxide production. Evidence for the involvement of temporally distinct staurosporine- insensitive and sensitive pathways." J Immunol **148**(12): 3920-8.

Gordge, P. C. and W. J. Ryves (1994). "Inhibitors of protein kinase C." Cell Signal **6**(8): 871-82.

Granelli-Piperno, A., J. D. Vassalli and E. Reich (1979). "RNA and protein synthesis in human peripheral blood polymorphonuclear leukocytes." J Exp Med **149**(1): 284-9.

Greenberg, S. (1999). "Modular components of phagocytosis." J Leukoc Biol **66**(5): 712-7.

Gross, B. S., S. K. Melford and S. P. Watson (1999). "Evidence that phospholipase C-gamma2 interacts with SLP-76, Syk, Lyn, LAT and the Fc receptor gamma-chain after stimulation of the collagen receptor glycoprotein VI in human platelets." Eur J Biochem **263**(3): 612-23.

Gschwendt, M., S. Dieterich, J. Rennecke, W. Kittstein, H. J. Mueller and F. J. Johannes (1996). "Inhibition of protein kinase C mu by various inhibitors. Differentiation from protein kinase c isoenzymes." FEBS Lett **392**(2): 77-80.

Gschwendt, M., H. J. Muller, K. Kielbassa, R. Zang, W. Kittstein, G. Rincke and F. Marks (1994). "Rottlerin, a novel protein kinase inhibitor." Biochem Biophys Res Commun **199**(1): 93-8.

Gullberg, U., E. Andersson, D. Garwicz, A. Lindmark and I. Olsson (1997). "Biosynthesis, processing and sorting of neutrophil proteins: insight into neutrophil granule development." Eur J Haematol **58**(3): 137-53.

Gullberg, U., N. Bengtsson, E. Bulow, D. Garwicz, A. Lindmark and I. Olsson (1999). "Processing and targeting of granule proteins in human neutrophils." J Immunol Methods **232**(1-2): 201-10.

Hama, H., E. A. Schnieders, J. Thorner, J. Y. Takemoto and D. B. DeWald (1999). "Direct involvement of phosphatidylinositol 4-phosphate in

secretion in the yeast Saccharomyces cerevisiae." J Biol Chem **274**(48): 34294-300.

Harlan, J. E., P. J. Hajduk, H. S. Yoon and S. W. Fesik (1994). "Pleckstrin homology domains bind to phosphatidylinositol-4,5- bisphosphate." Nature **371**(6493): 168-70.

Hausser, A., P. Storz, G. Link, H. Stoll, Y. C. Liu, A. Altman, K. Pfizenmaier and F. J. Johannes (1999). "Protein kinase C mu is negatively regulated by 14-3-3 signal transduction proteins." J Biol Chem **274**(14): 9258-64.

Haworth, R. S., J. Sinnett-Smith, E. Rozengurt and M. Avkiran (1999). "Protein kinase D inhibits plasma membrane Na(+)/H(+) exchanger activity." Am J Physiol **277**(6 Pt 1): C1202-9.

Hayakawa, T., K. Suzuki, S. Suzuki, P. C. Andrews and B. M. Babior (1986). "A possible role for protein phosphorylation in the activation of the respiratory burst in human neutrophils. Evidence from studies with cells from patients with chronic granulomatous disease." J Biol Chem **261**(20): 9109-15.

Hayashi, A., N. Seki, A. Hattori, S. Kozuma and T. Saito (1999). "PKCnu, a new member of the protein kinase C family, composes a fourth subfamily with PKCmu." Biochim Biophys Acta **1450**(1): 99-106.

Hazan-Halevy, I., R. Seger and R. Levy (2000). "The requirement of both extracellular regulated kinase and p38 mitogen-activated protein kinase for stimulation of cytosolic phospholipase A(2) activity by either FcgammaRIIA or FcgammaRIIIB in human neutrophils. A possible role for Pyk2 but not for the Grb2-Sos-Shc complex." J Biol Chem **275**(17): 12416-23.

Heldin, C. H. (1995). "Dimerization of cell surface receptors in signal transduction." Cell **80**(2): 213-23.

Hellum, K. B. and C. O. Solberg (1973). "Influence of anticoagulants on the nitroblue tetrazolium test." Scand J Infect Dis **5**(1): 67-70.

Hetland, G., G. J. Talgo and M. K. Fagerhol (1998). "Chemotaxins C5a and fMLP induce release of calprotectin (leucocyte L1 protein) from polymorphonuclear cells in vitro." Mol Pathol **51**(3): 143-8.

Heyman, B. (2000). "Regulation of antibody responses via antibodies, complement, and Fc receptors." Annu Rev Immunol **18**: 709-37.

Heyworth, P. G., J. T. Curnutte, W. M. Nauseef, B. D. Volpp, D. W. Pearson, H. Rosen and R. A. Clark (1991). "Neutrophil nicotinamide adenine dinucleotide phosphate oxidase assembly. Translocation of p47-phox and p67-phox requires interaction between p47-phox and cytochrome b558." J Clin Invest **87**(1): 352-6.

Hirsch, J. G. H. a. B. I. (1982). Metchnikoff's Life and Scientific Contributions in Historical Perspective. Phagocytosis-Past and Future. M. L. K. a. L. Bolis. New York, Academic Press.

Hoffbrand, A. V. P., J.E. (1993). Essential Haematology. Oxford, U.K., Blackwell Science.

Hohn, D. C. and R. I. Lehrer (1974). "Mechanism of the heparin effect on the nitroblue-tetrazolium slide test." Infect Immun **10**(4): 772-5.

Hoogerwerf, M., R. S. Weening, C. E. Hack and D. Roos (1990). "Complement fragments C3b and iC3b coupled to latex induce a respiratory burst in human neutrophils." Mol Immunol **27**(2): 159-67.

Hope, R. A. L., J.M.; McManus, S.K.; Wood-Allum, C.A. (1998). Oxford Handbook of Clinical Medicine. Oxford, U.K., Oxford University Press.

Huang, J. and M. E. Kleinberg (1999). "Activation of the phagocyte NADPH oxidase protein p47(phox). Phosphorylation controls SH3 domain-dependent binding to p22(phox)." J Biol Chem **274**(28): 19731-7.

Huang, L., T. Y. Wong, R. C. Lin and H. Furthmayr (1999). "Replacement of threonine 558, a critical site of phosphorylation of moesin in vivo, with aspartate activates F-actin binding of moesin. Regulation by conformational change." J Biol Chem **274**(18): 12803-10.

Hubberstey, A., G. Yu, R. Loewith, C. Lakusta and D. Young (1996). "Mammalian CAP interacts with CAP, CAP2, and actin." J Cell Biochem **61**(3): 459-66.

Hulett, M. D. and P. M. Hogarth (1994). "Molecular basis of Fc receptor function." Adv Immunol **57**: 1-127.

Hunter, S., M. Kamoun and A. D. Schreiber (1994). "Transfection of an Fc gamma receptor cDNA induces T cells to become phagocytic." Proc Natl Acad Sci U S A **91**(21): 10232-6.

Hunter, T. (1995). "Protein kinases and phosphatases: the yin and yang of protein phosphorylation and signaling." Cell **80**(2): 225-36.

Ibarrola, I., P. J. Vossebeld, C. H. Homburg, M. Thelen, D. Roos and A. J. Verhoeven (1997). "Influence of tyrosine phosphorylation on protein interaction with FcgammaRIIa." Biochim Biophys Acta **1357**(3): 348-58.

Iglesias, T. and E. Rozengurt (1998). "Protein kinase D activation by mutations within its pleckstrin homology domain." J Biol Chem **273**(1): 410-6.

Inanami, O., J. L. Johnson, J. K. McAdara, J. E. Benna, L. R. Faust, P. E. Newburger and B. M. Babior (1998). "Activation of the leukocyte NADPH oxidase by phorbol ester requires the phosphorylation of p47PHOX on serine 303 or 304." J Biol Chem **273**(16): 9539-43.

Iyer, G., M. Islam and J. Quastel (1961). "Biochemical aspects of phagocytosis." Nature **192**(4802): 535-541.

Jackson, S. H., J. I. Gallin and S. M. Holland (1995). "The p47phox mouse knock-out model of chronic granulomatous disease." J Exp Med **182**(3): 751-8.

Jacobson, P. B., S. L. Kuchera, A. Metz, C. Schachtele, K. Imre and D. J. Schrier (1995). "Anti-inflammatory properties of Go 6850: a selective inhibitor of protein kinase C." J Pharmacol Exp Ther **275**(2): 995-1002.

Jaconi, M. E., D. P. Lew, J. L. Carpentier, K. E. Magnusson, M. Sjogren and O. Stendahl (1990). "Cytosolic free calcium elevation mediates the phagosome-lysosome fusion during phagocytosis in human neutrophils." J Cell Biol **110**(5): 1555-64.

Jamora, C., N. Yamanouye, J. Van Lint, J. Laudenslager, J. R. Vandenheede, D. J. Faulkner and V. Malhotra (1999). "Gbetagamma-mediated regulation of Golgi organization is through the direct activation of protein kinase D." Cell **98**(1): 59-68.

Jia, J., Q. Han, N. Borregaard, K. Lollike and M. Cygler (2000). "Crystal structure of human grancalcin, a member of the penta-EF-hand protein family [In Process Citation]." J Mol Biol **300**(5): 1271-81.

Johannes, F. J., A. Hausser, P. Storz, L. Truckenmuller, G. Link, T. Kawakami and K. Pfizenmaier (1999). "Bruton's tyrosine kinase (Btk) associates with protein kinase C mu." FEBS Lett **461**(1-2): 68-72.

Johannes, F. J., J. Prestle, S. Dieterich, P. Oberhagemann, G. Link and K. Pfizenmaier (1995). "Characterization of activators and inhibitors of protein kinase C mu." Eur J Biochem **227**(1-2): 303-7.

Johannes, F. J., J. Prestle, S. Eis, P. Oberhagemann and K. Pfizenmaier (1994). "PKCu is a novel, atypical member of the protein kinase C family." J Biol Chem **269**(8): 6140-8.

Johnson, J. L., J. W. Park, J. E. Benna, L. P. Faust, O. Inanami and B. M. Babior (1998). "Activation of p47(PHOX), a cytosolic subunit of the leukocyte NADPH oxidase. Phosphorylation of ser-359 or ser-370 precedes phosphorylation at other sites and is required for activity." J Biol Chem **273**(52): 35147-52.

Jones, S. L. and E. J. Brown (1996). "FcgammaRII-mediated adhesion and phagocytosis induce L-plastin phosphorylation in human neutrophils." J Biol Chem **271**(24): 14623-30.

Jones, S. L., J. Wang, C. W. Turck and E. J. Brown (1998). "A role for the actin-bundling protein L-plastin in the regulation of leukocyte integrin function." Proc Natl Acad Sci U S A **95**(16): 9331-6.

Karimi, K. and M. R. Lennartz (1998). "Mitogen-activated protein kinase is activated during IgG-mediated phagocytosis, but is not required for target ingestion." Inflammation **22**(1): 67-82.

Kent, J. D., S. Sergeant, D. J. Burns and L. C. McPhail (1996). "Identification and regulation of protein kinase C-delta in human neutrophils." J Immunol **157**(10): 4641-7.

Kiefer, F., J. Brumell, N. Al-Alawi, S. Latour, A. Cheng, A. Veillette, S. Grinstein and T. Pawson (1998). "The Syk protein tyrosine kinase is essential for

Fcgamma receptor signaling in macrophages and neutrophils." Mol Cell Biol **18**(7): 4209-20.

Kiley, S. C. and P. J. Parker (1995). "Differential localization of protein kinase C isozymes in U937 cells: evidence for distinct isozyme functions during monocyte differentiation." J Cell Sci **108**(Pt 3): 1003-16.

Klarlund, J. K., A. Guilherme, J. J. Holik, J. V. Virbasius, A. Chawla and M. P. Czech (1997). "Signaling by phosphoinositide-3,4,5-trisphosphate through proteins containing pleckstrin and Sec7 homology domains [see comments]." Science **275**(5308): 1927-30.

Klinger, E. and I. Aviram (1992). "Involvement of GTP in cell-free activation of neutrophil NADPH oxidase. Studies with GTP analogues." Biochem J **285**(Pt 2): 635-9.

Knaus, U. G., S. Morris, H. J. Dong, J. Chernoff and G. M. Bokoch (1995). "Regulation of human leukocyte p21-activated kinases through G protein--coupled receptors." Science **269**(5221): 221-3.

Korchak, H. M., M. W. Rossi and L. E. Kilpatrick (1998). "Selective role for beta-protein kinase C in signaling for O-2 generation but not degranulation or adherence in differentiated HL60 cells." J Biol Chem **273**(42): 27292-9.

Kramer, I. M., R. L. van der Bend, A. J. Verhoeven and D. Roos (1988). "The 47-kDa protein involved in the NADPH:O2 oxidoreductase activity of human neutrophils is phosphorylated by cyclic AMP-dependent protein kinase without induction of a respiratory burst." Biochim Biophys Acta **971**(2): 189-96.

Krause, K. H. (2000). "Professional phagocytes: predators and prey of microorganisms." Schweiz Med Wochenschr **130**(4): 97-100.

Krebs, E. G. (1994). "The growth of research on protein phosphorylation." Trends Biochem Sci **19**(11): 439.

Krebs, E. G. and J. A. Beavo (1979). "Phosphorylation-dephosphorylation of enzymes." Annu Rev Biochem **48**: 923-59.

Kumar, G., S. Wang, S. Gupta and A. Nel (1995). "The membrane immunoglobulin receptor utilizes a Shc/Grb2/hSOS complex for activation

of the mitogen-activated protein kinase cascade in a B-cell line." Biochem J **307**(Pt 1): 215-23.

Kusner, D. J., C. F. Hall and S. Jackson (1999). "Fc gamma receptor-mediated activation of phospholipase D regulates macrophage phagocytosis of IgG-opsonized particles." J Immunol **162**(4): 2266-74.

Kusunoki, T., H. Higashi, S. Hosoi, D. Hata, K. Sugie, M. Mayumi and H. Mikawa (1992). "Tyrosine phosphorylation and its possible role in superoxide production by human neutrophils stimulated with FMLP and IgG." Biochem Biophys Res Commun **183**(2): 789-96.

Kutter, D. (1998). "Prevalence of myeloperoxidase deficiency: population studies using Bayer-Technicon automated hematology." J Mol Med **76**(10): 669-75.

Kwiatkowska, K. and A. Sobota (1999). "Signaling pathways in phagocytosis." Bioessays **21**(5): 422-31.

Laemmli, U. K. (1970). "Cleavage of structural proteins during the assembly of the head of bacteriophage T4." Nature **227**(259): 680-5.

Lang, M. L. and M. A. Kerr (1997). "Neutrophil Fc receptors signal through PI 3-kinase to trigger a respiratory burst." Biochem Soc Trans **25**(4): S603.

Lanza, F. (1998). "Clinical manifestation of myeloperoxidase deficiency." J Mol Med **76**(10): 676-81.

Lemmon, M. A., K. M. Ferguson, R. O'Brien, P. B. Sigler and J. Schlessinger (1995). "Specific and high-affinity binding of inositol phosphates to an isolated pleckstrin homology domain." Proc Natl Acad Sci U S A **92**(23): 10472-6.

Leusen, J. H., B. G. Bolscher, P. M. Hilarius, R. S. Weening, W. Kaulfersch, R. A. Seger, D. Roos and A. J. Verhoeven (1994). "156Pro-->Gln substitution in the light chain of cytochrome b558 of the human NADPH oxidase (p22-phox) leads to defective translocation of the cytosolic proteins p47-phox and p67-phox [see comments]." J Exp Med **180**(6): 2329-34.

Liang, L. and C. K. Huang (1995). "Activation of multiple protein kinases induced by cross-linking of Fc gamma RII in human neutrophils." J Leukoc Biol **57**(2): 326-31.

Liang, L. and C. K. Huang (1995). "Tyrosine phosphorylation induced by cross-linking of Fc gamma-receptor type II in human neutrophils." Biochem J **306**(Pt 2): 489-95.

Liao, F., H. S. Shin and S. G. Rhee (1992). "Tyrosine phosphorylation of phospholipase C-gamma 1 induced by cross- linking of the high-affinity or low-affinity Fc receptor for IgG in U937 cells." Proc Natl Acad Sci U S A **89**(8): 3659-63.

Lin, C. S., A. Lau and T. F. Lue (1998). "Analysis and mapping of plastin phosphorylation." DNA Cell Biol **17**(12): 1041-6.

Lin, W. W. and B. C. Chen (1998). "Distinct PKC isoforms mediate the activation of cPLA2 and adenylyl cyclase by phorbol ester in RAW264.7 macrophages." Br J Pharmacol **125**(7): 1601-9.

Liscovitch, M. (1996). "Phospholipase D: role in signal transduction and membrane traffic." J Lipid Mediat Cell Signal **14**(1-3): 215-21.

Lofgren, R., L. Serrander, M. Forsberg, A. Wilsson, A. Wasteson and O. Stendahl (1999). "CR3, FcgammaRIIA and FcgammaRIIIB induce activation of the respiratory burst in human neutrophils: the role of intracellular Ca(2+), phospholipase D and tyrosine phosphorylation." Biochim Biophys Acta **1452**(1): 46-59.

Lomax, K. J., J. I. Gallin, D. Rotrosen, G. D. Raphael, M. A. Kaliner, E. J. Benz, Jr., L. A. Boxer and H. L. Malech (1989). "Selective defect in myeloid cell lactoferrin gene expression in neutrophil specific granule deficiency." J Clin Invest **83**(2): 514-9.

Lomax, K. J., T. L. Leto, H. Nunoi, J. I. Gallin and H. L. Malech (1989). "Recombinant 47-kilodalton cytosol factor restores NADPH oxidase in chronic granulomatous disease [published erratum appears in Science 1989 Nov 24;246(4933):987]." Science **245**(4916): 409-12.

Lundqvist, H., M. Gustafsson, A. Johansson, E. Sarndahl and C. Dahlgren (1994). "Neutrophil control of formylmethionyl-leucyl-phenylalanine induced mobilization of secretory vesicles and NADPH-oxidase activation: effect of an association of the ligand-receptor complex to the cytoskeleton." Biochim Biophys Acta **1224**(1): 43-50.

Matsui, T., S. Yonemura and S. Tsukita (1999). "Activation of ERM proteins in vivo by Rho involves phosphatidyl-inositol 4-phosphate 5-kinase and not ROCK kinases." Curr Biol **9**(21): 1259-62.

Matthews, S., T. Iglesias, D. Cantrell and E. Rozengurt (1999). "Dynamic re-distribution of protein kinase D (PKD) as revealed by a GFP- PKD fusion protein: dissociation from PKD activation." FEBS Lett **457**(3): 515-21.

Matthews, S. A., T. Iglesias, E. Rozengurt and D. Cantrell (2000). "Spatial and temporal regulation of protein kinase D (PKD)." Embo J **19**(12): 2935-2945.

Matthews, S. A., G. R. Pettit and E. Rozengurt (1997). "Bryostatin 1 induces biphasic activation of protein kinase D in intact cells." J Biol Chem **272**(32): 20245-50.

Matthews, S. A., E. Rozengurt and D. Cantrell (1999). "Characterization of serine 916 as an in vivo autophosphorylation site for protein kinase D/Protein kinase Cmu." J Biol Chem **274**(37): 26543-9.

Matthews, S. A., E. Rozengurt and D. Cantrell (2000). "Protein kinase D. A selective target for antigen receptors and a downstream target for protein kinase C in lymphocytes." J Exp Med **191**(12): 2075-82.

May, R. C., E. Caron, A. Hall and L. M. Machesky (2000). "Involvement of the Arp2/3 complex in phagocytosis mediated by FcgammaR or CR3." Nat Cell Biol **2**(4): 246-8.

McCord, J. M. and I. Fridovich (1969). "The utility of superoxide dismutase in studying free radical reactions. I. Radicals generated by the interaction of sulfite, dimethyl sulfoxide, and oxygen." J Biol Chem **244**(22): 6056-63.

McKenzie, S. E. and A. D. Schreiber (1998). "Fc gamma receptors in phagocytes." Curr Opin Hematol **5**(1): 16-21.

McLeish, K. R., J. B. Klein, P. Y. Coxon, K. Z. Head and R. A. Ward (1998). "Bacterial phagocytosis activates extracellular signal-regulated kinase and p38 mitogen-activated protein kinase cascades in human neutrophils." J Leukoc Biol **64**(6): 835-44.

McPhail, L. C., L. R. DeChatelet, P. S. Shirley, C. Wilfert, R. B. Johnston, Jr. and C. E. McCall (1977). "Deficiency of NADPH oxidase activity in chronic granulomatous disease." J Pediatr **90**(2): 213-7.

McPhail, L. C., P. S. Shirley, C. C. Clayton and R. Snyderman (1985). "Activation of the respiratory burst enzyme from human neutrophils in a cell-free system. Evidence for a soluble cofactor." J Clin Invest **75**(5): 1735-9.

McPhail, L. C. and R. Snyderman (1983). "Activation of the respiratory burst enzyme in human polymorphonuclear leukocytes by chemoattractants and other soluble stimuli. Evidence that the same oxidase is activated by different transductional mechanisms." J Clin Invest **72**(1): 192-200.

Medzhitov, R. and C. A. Janeway, Jr. (1997). "Innate immunity: impact on the adaptive immune response." Curr Opin Immunol **9**(1): 4-9.

Medzhitov, R. and C. A. Janeway, Jr. (1997). "Innate immunity: the virtues of a nonclonal system of recognition." Cell **91**(3): 295-8.

Medzhitov, R. and C. A. Janeway, Jr. (1998). "An ancient system of host defense." Curr Opin Immunol **10**(1): 12-5.

Medzhitov, R. and C. A. Janeway, Jr. (1998). "Innate immune recognition and control of adaptive immune responses." Semin Immunol **10**(5): 351-3.

Medzhitov, R. and C. A. Janeway, Jr. (2000). "How does the immune system distinguish self from nonself? [In Process Citation]." Semin Immunol **12**(3): 185-8.

Metchnikoff, E. (1892). Comparative Pathology of Inflammation. Milestones in Immunology- A Historical Exploration. D. J. Bibel. Madison WI, Science Tech Publishers: 170-173.

Mimms, C. A., Playfair, J.H.L., Roitt, I.M., Wakelin, D., Williams,R. (1993). Medical Microbiology. Oxford U.K., Mosby Europe Ltd.

Minard, K. I. and L. McAlister-Henn (1994). "Glucose-induced phosphorylation of the MDH2 isozyme of malate dehydrogenase in Saccharomyces cerevisiae." Arch Biochem Biophys **315**(2): 302-9.

Mocsai, A., Z. Jakus, T. Vantus, G. Berton, C. A. Lowell and E. Ligeti (2000). "Kinase pathways in chemoattractant-induced degranulation of neutrophils: the role of p38 mitogen-activated protein kinase activated by Src family kinases." J Immunol **164**(8): 4321-31.

Mullock, B. M., N. A. Bright, C. W. Fearon, S. R. Gray and J. P. Luzio (1998). "Fusion of lysosomes with late endosomes produces a hybrid organelle of intermediate density and is NSF dependent." J Cell Biol **140**(3): 591-601.

Nauseef, W. M. (1993). "Cytosolic oxidase factors in the NADPH-dependent oxidase of human neutrophils." Eur J Haematol **51**(5): 301-8.

Nauseef, W. M. (1999). "The NADPH-dependent oxidase of phagocytes." Proc Assoc Am Physicians **111**(5): 373-82.

Nauseef, W. M., B. D. Volpp, S. McCormick, K. G. Leidal and R. A. Clark (1991). "Assembly of the neutrophil respiratory burst oxidase. Protein kinase C promotes cytoskeletal and membrane association of cytosolic oxidase components." J Biol Chem **266**(9): 5911-7.

Ninomiya, N., K. Hazeki, Y. Fukui, T. Seya, T. Okada, O. Hazeki and M. Ui (1994). "Involvement of phosphatidylinositol 3-kinase in Fc gamma receptor signaling." J Biol Chem **269**(36): 22732-7.

Nishida, Y., F. Shima, H. Sen, Y. Tanaka, C. Yanagihara, Y. Yamawaki-Kataoka, K. Kariya and T. Kataoka (1998). "Coiled-coil interaction of N-terminal 36 residues of cyclase-associated protein with adenylyl cyclase is sufficient for its function in Saccharomyces cerevisiae ras pathway." J Biol Chem **273**(43): 28019-24.

Nishikawa, K., A. Toker, F. J. Johannes, Z. Songyang and L. C. Cantley (1997). "Determination of the specific substrate sequence motifs of protein kinase C isozymes." J Biol Chem **272**(2): 952-60.

Nishikawa, K., A. Toker, K. Wong, P. A. Marignani, F. J. Johannes and L. C. Cantley (1998). "Association of protein kinase Cmu with type II

phosphatidylinositol 4-kinase and type I phosphatidylinositol-4-phosphate 5-kinase." J Biol Chem **273**(36): 23126-33.

Nishizuka, Y. (1984). "The role of protein kinase C in cell surface signal transduction and tumour promotion." Nature **308**(5961): 693-8.

Nishizuka, Y. (1988). "The molecular heterogeneity of protein kinase C and its implications for cellular regulation." Nature **334**(6184): 661-5.

Nuijens, J. H., P. H. van Berkel and F. L. Schanbacher (1996). "Structure and biological actions of lactoferrin." J Mammary Gland Biol Neoplasia **1**(3): 285-95.

Nunoi, H., D. Rotrosen, J. I. Gallin and H. L. Malech (1988). "Two forms of autosomal chronic granulomatous disease lack distinct neutrophil cytosol factors." Science **242**(4883): 1298-301.

Oancea, E., M. N. Teruel, A. F. Quest and T. Meyer (1998). "Green fluorescent protein (GFP)-tagged cysteine-rich domains from protein kinase C as fluorescent indicators for diacylglycerol signaling in living cells." J Cell Biol **140**(3): 485-98.

Ohtsuka, T., N. Okamura and S. Ishibashi (1986). "Involvement of protein kinase C in the phosphorylation of 46 kDa proteins which are phosphorylated in parallel with activation of NADPH oxidase in intact guinea-pig polymorphonuclear leukocytes." Biochim Biophys Acta **888**(3): 332-7.

Ono, Y., T. Fujii, K. Ogita, U. Kikkawa, K. Igarashi and Y. Nishizuka (1987). "Identification of three additional members of rat protein kinase C family: delta-, epsilon- and zeta-subspecies." FEBS Lett **226**(1): 125-8.

Ono, Y., T. Fujii, K. Ogita, U. Kikkawa, K. Igarashi and Y. Nishizuka (1989). "Protein kinase C zeta subspecies from rat brain: its structure, expression, and properties." Proc Natl Acad Sci U S A **86**(9): 3099-103.

Orfanakis, N. G., R. E. Ostlund, C. R. Bishop and J. W. Athens (1970). "Normal blood leukocyte concentration values." Am J Clin Pathol **53**(5): 647-51.

Osada, S., K. Mizuno, T. C. Saido, K. Suzuki, T. Kuroki and S. Ohno (1992). "A new member of the protein kinase C family, nPKC theta, predominantly expressed in skeletal muscle." Mol Cell Biol **12**(9): 3930-8.

Paolucci, L. and E. Rozengurt (1999). "Protein kinase D in small cell lung cancer cells: rapid activation through protein kinase C." Cancer Res **59**(3): 572-7.

Paolucci, L., J. Sinnett-Smith and E. Rozengurt (2000). "Lysophosphatidic acid rapidly induces protein kinase D activation through a pertussis toxin-sensitive pathway." Am J Physiol Cell Physiol **278**(1): C33-9.

Park, H. S., I. S. Kim and J. W. Park (1999). "Phosphorylation induces conformational changes in the leukocyte NADPH oxidase subunit p47(phox)." Biochem Biophys Res Commun **259**(1): 38-42.

Park, H. S. and J. W. Park (1998). "Conformational changes of the leukocyte NADPH oxidase subunit p47(phox) during activation studied through its intrinsic fluorescence." Biochim Biophys Acta **1387**(1-2): 406-14.

Park, J. W., M. Ma, J. M. Ruedi, R. M. Smith and B. M. Babior (1992). "The cytosolic components of the respiratory burst oxidase exist as a M(r) approximately 240,000 complex that acquires a membrane-binding site during activation of the oxidase in a cell-free system." J Biol Chem **267**(24): 17327-32.

Park, R. K., A. Erdreich-Epstein, M. Liu, K. D. Izadi and D. L. Durden (1999). "High affinity IgG receptor activation of Src family kinases is required for modulation of the Shc-Grb2-Sos complex and the downstream activation of the nicotinamide adenine dinucleotide phosphate (reduced) oxidase." J Immunol **163**(11): 6023-34.

Parker, P. J., L. Coussens, N. Totty, L. Rhee, S. Young, E. Chen, S. Stabel, M. D. Waterfield and A. Ullrich (1986). "The complete primary structure of protein kinase C--the major phorbol ester receptor." Science **233**(4766): 853-9.

Parkos, C. A., R. A. Allen, C. G. Cochrane and A. J. Jesaitis (1987). "Purified cytochrome b from human granulocyte plasma membrane is comprised of two polypeptides with relative molecular weights of 91,000 and 22,000." J Clin Invest **80**(3): 732-42.

Parkos, C. A., M. C. Dinauer, A. J. Jesaitis, S. H. Orkin and J. T. Curnutte (1989). "Absence of both the 91kD and 22kD subunits of human neutrophil

cytochrome b in two genetic forms of chronic granulomatous disease." Blood **73**(6): 1416-20.

Parkos, C. A., M. C. Dinauer, L. E. Walker, R. A. Allen, A. J. Jesaitis and S. H. Orkin (1988). "Primary structure and unique expression of the 22-kilodalton light chain of human neutrophil cytochrome b." Proc Natl Acad Sci U S A **85**(10): 3319-23.

Patriarca, P., R. Cramer, S. Moncalvo, F. Rossi and D. Romeo (1971). "Enzymatic basis of metabolic stimulation in leucocytes during phagocytosis: the role of activated NADPH oxidase." Arch Biochem Biophys **145**(1): 255-62.

Pestonjamasp, K., M. R. Amieva, C. P. Strassel, W. M. Nauseef, H. Furthmayr, E. J. Luna, J. Wang and E. J. Brown (1995). "Moesin, ezrin, and p205 are actin-binding proteins associated with neutrophil plasma membranes Immune complex-induced integrin activation and L-plastin phosphorylation require protein kinase A." Mol Biol Cell **6**(3): 247-59.

Pick, E., Y. Bromberg, S. Shpungin and R. Gadba (1987). "Activation of the superoxide forming NADPH oxidase in a cell-free system by sodium dodecyl sulfate. Characterization of the membrane-associated component." J Biol Chem **262**(34): 16476-83.

Pick, E., T. Kroizman and A. Abo (1989). "Activation of the superoxide-forming NADPH oxidase of macrophages requires two cytosolic components--one of them is also present in certain nonphagocytic cells." J Immunol **143**(12): 4180-7.

Pietromonaco, S. F., P. C. Simons, A. Altman and L. Elias (1998). "Protein kinase C-theta phosphorylation of moesin in the actin-binding sequence." J Biol Chem **273**(13): 7594-603.

Pimplikar, S. W. and K. Simons (1993). "Regulation of apical transport in epithelial cells by a Gs class of heterotrimeric G protein." Nature **362**(6419): 456-8.

Poole, A., J. M. Gibbins, M. Turner, M. J. van Vugt, J. G. van de Winkel, T. Saito, V. L. Tybulewicz and S. P. Watson (1997). "The Fc receptor gamma-chain

and the tyrosine kinase Syk are essential for activation of mouse platelets by collagen." Embo J **16**(9): 2333-41.

Prestle, J., K. Pfizenmaier, J. Brenner and F. J. Johannes (1996). "Protein kinase C mu is located at the Golgi compartment." J Cell Biol **134**(6): 1401-10.

Pribluda, V. S., C. Pribluda and H. Metzger (1994). "Transphosphorylation as the mechanism by which the high-affinity receptor for IgE is phosphorylated upon aggregation." Proc Natl Acad Sci U S A **91**(23): 11246-50.

Pricop, L., J. Gokhale, P. Redecha, S. C. Ng and J. E. Salmon (1999). "Reactive oxygen intermediates enhance Fc gamma receptor signaling and amplify phagocytic capacity." J Immunol **162**(12): 7041-8.

Quie, P. G., E. L. Kaplan, A. R. Page, F. L. Gruskay and S. E. Malawista (1968). "Defective polymorphonuclear-leukocyte function and chronic granulomatous disease in two female children." N Engl J Med **278**(18): 976-80.

Quie, P. G., J. G. White, B. Holmes and R. A. Good (1967). "In vitro bactericidal capacity of human polymorphonuclear leukocytes: diminished activity in chronic granulomatous disease of childhood." J Clin Invest **46**(4): 668-79.

Raeder, E. M., P. J. Mansfield, V. Hinkovska-Galcheva, L. Kjeldsen, J. A. Shayman and L. A. Boxer (1999). "Sphingosine blocks human polymorphonuclear leukocyte phagocytosis through inhibition of mitogen-activated protein kinase activation." Blood **93**(2): 686-93.

Raeder, E. M., P. J. Mansfield, V. Hinkovska-Galcheva, J. A. Shayman and L. A. Boxer (1999). "Syk activation initiates downstream signaling events during human polymorphonuclear leukocyte phagocytosis." J Immunol **163**(12): 6785-93.

Raghavan, M. and P. J. Bjorkman (1996). "Fc receptors and their interactions with immunoglobulins." Annu Rev Cell Dev Biol **12**: 181-220.

Ravetch, J. V. (1997). "Fc receptors." Curr Opin Immunol **9**(1): 121-5.

Reeves, E. P., L. V. Dekker, L. V. Forbes, F. B. Wientjes, A. Grogan, D. J. Pappin and A. W. Segal (1999). "Direct interaction between p47phox and protein kinase C: evidence for targeting of protein kinase C by p47phox in

neutrophils [published erratum appears in Biochem J 2000 Feb 1;345 Pt 3:767]." Biochem J **344 Pt 3**: 859-66.

Regier, D. S., D. G. Greene, S. Sergeant, A. J. Jesaitis and L. C. McPhail (2000). "Phosphorylation of p22phox is Mediated by Phospholipase D-dependent and -independent Mechanisms: Correlation OF NADPH Oxidase Activity and p22phox Phosphorylation." J Biol Chem.

Regier, D. S., K. A. Waite, R. Wallin and L. C. McPhail (1999). "A phosphatidic acid-activated protein kinase and conventional protein kinase C isoforms phosphorylate p22(phox), an NADPH oxidase component." J Biol Chem **274**(51): 36601-8.

Rennecke, J., F. J. Johannes, K. H. Richter, W. Kittstein, F. Marks and M. Gschwendt (1996). "Immunological demonstration of protein kinase C mu in murine tissues and various cell lines. Differential recognition of phosphorylated forms and lack of down-regulation upon 12-O-tetradecanoylphorphol-13- acetate treatment of cells." Eur J Biochem **242**(2): 428-32.

Rennecke, J., P. A. Rehberger, G. Furstenberger, F. J. Johannes, M. Stohr, F. Marks and K. H. Richter (1999). "Protein-kinase-Cmu expression correlates with enhanced keratinocyte proliferation in normal and neoplastic mouse epidermis and in cell culture." Int J Cancer **80**(1): 98-103.

Rink, T. J., A. Sanchez and T. J. Hallam (1983). "Diacylglycerol and phorbol ester stimulate secretion without raising cytoplasmic free calcium in human platelets." Nature **305**(5932): 317-9.

Roberts, A. W., C. Kim, L. Zhen, J. B. Lowe, R. Kapur, B. Petryniak, A. Spaetti, J. D. Pollock, J. B. Borneo, G. B. Bradford, S. J. Atkinson, M. C. Dinauer and D. A. Williams (1999). "Deficiency of the hematopoietic cell-specific Rho family GTPase Rac2 is characterized by abnormalities in neutrophil function and host defense." Immunity **10**(2): 183-96.

Roitt, I. M. (1990). Essential Immunology. Oxford, U.K., Blackwell Scientific Publishers.

Roos, D., M. de Boer, F. Kuribayashi, C. Meischl, R. S. Weening, A. W. Segal, A. Ahlin, K. Nemet, J. P. Hossle, E. Bernatowska-Matuszkiewicz and H. Middleton-Price (1996). "Mutations in the X-linked and autosomal recessive forms of chronic granulomatous disease." Blood **87**(5): 1663-81.

Rosales, C., S. L. Jones, D. McCourt and E. J. Brown (1994). "Bromophenacyl bromide binding to the actin-bundling protein l-plastin inhibits inositol trisphosphate-independent increase in Ca2+ in human neutrophils." Proc Natl Acad Sci U S A **91**(9): 3534-8.

Rossi, F. and M. Zatti (1966). "Effect of phagocytosis on the carbohydrate metabolism of polymorphonuclear leucocytes." Biochim Biophys Acta **121**(1): 110-9.

Rothwell, D. J. and B. T. Doumas (1975). "The effect of heparin and EDTA on the NBT test." J Lab Clin Med **85**(6): 950-6.

Rotrosen, D. and T. L. Leto (1990). "Phosphorylation of neutrophil 47-kDa cytosolic oxidase factor. Translocation to membrane is associated with distinct phosphorylation events." J Biol Chem **265**(32): 19910-5.

Rotrosen, D., C. L. Yeung, T. L. Leto, H. L. Malech and C. H. Kwong (1992). "Cytochrome b558: the flavin-binding component of the phagocyte NADPH oxidase." Science **256**(5062): 1459-62.

Rouard, H., S. Tamasdan, W. H. Fridman and J. L. Teillaud (1999). "Vav and SLP-76 recruitment by cross-linking of FcgammaRIIa1 in promyelocytic HL-60 cells." Immunol Lett **68**(2-3): 347-53.

Sakata, A., E. Ida, M. Tominaga and K. Onoue (1987). "Arachidonic acid acts as an intracellular activator of NADPH-oxidase in Fc gamma receptor-mediated superoxide generation in macrophages." J Immunol **138**(12): 4353-9.

Sanchez-Mejorada, G. and C. Rosales (1998). "Fcgamma receptor-mediated mitogen-activated protein kinase activation in monocytes is independent of Ras." J Biol Chem **273**(42): 27610-9.

Sanchez-Mejorada, G. and C. Rosales (1998). "Signal transduction by immunoglobulin Fc receptors." J Leukoc Biol **63**(5): 521-33.

Sbarra, A. and M. Karnovsky (1959). "Metabolic changes during ingestion of particles by polymorphonuclear leukocytes." J Biol Chem **234**(6): 1355-62.

Schneider, C., M. Zanetti and D. Romeo (1981). "Surface-reactive stimuli selectively increase protein phosphorylation in human neutrophils." FEBS Lett **127**(1): 4-8.

Scott-Zaki, P., D. Purkall and S. Ruddy (2000). "Neutrophil chemotaxis and superoxide production are induced by cross- linking FcgammaRII receptors." Cell Immunol **201**(2): 89-93.

Segal, A. W. and S. B. Coade (1978). "Kinetics of oxygen consumption by phagocytosing human neutrophils." Biochem Biophys Res Commun **84**(3): 611-7.

Segal, A. W., J. Dorling and S. Coade (1980). "Kinetics of fusion of the cytoplasmic granules with phagocytic vacuoles in human polymorphonuclear leukocytes. Biochemical and morphological studies." J Cell Biol **85**(1): 42-59.

Segal, A. W., M. Geisow, R. Garcia, A. Harper and R. Miller (1981). "The respiratory burst of phagocytic cells is associated with a rise in vacuolar pH." Nature **290**(5805): 406-9.

Segal, A. W., P. G. Heyworth, S. Cockcroft and M. M. Barrowman (1985). "Stimulated neutrophils from patients with autosomal recessive chronic granulomatous disease fail to phosphorylate a Mr-44,000 protein." Nature **316**(6028): 547-9.

Segal, A. W. and O. T. Jones (1979). "Identification of a previously undescribed cytochrome b in human neutrophils and its relationship to phagocytosis-induced oxidase activity [proceedings]." Biochem Soc Trans **7**(1): 187-8.

Segal, A. W. and A. J. Levi (1975). "Factors influencing the entry of dye into neutrophil leucocytes in the Nitroblue Tetrazolium test." Clin Sci Mol Med **48**(3): 201-12.

Segal, A. W., I. West, F. Wientjes, J. H. Nugent, A. J. Chavan, B. Haley, R. C. Garcia, H. Rosen and G. Scrace (1992). "Cytochrome b-245 is a

flavocytochrome containing FAD and the NADPH-binding site of the microbicidal oxidase of phagocytes." Biochem J **284**(Pt 3): 781-8.

Segal, B. H., T. L. Leto, J. I. Gallin, H. L. Malech and S. M. Holland (2000). "Genetic, biochemical, and clinical features of chronic granulomatous disease." Medicine (Baltimore) **79**(3): 170-200.

Selbie, L. A., C. Schmitz-Peiffer, Y. Sheng and T. J. Biden (1993). "Molecular cloning and characterization of PKC iota, an atypical isoform of protein kinase C derived from insulin-secreting cells." J Biol Chem **268**(32): 24296-302.

Sergeant, S. and L. C. McPhail (1997). "Opsonized zymosan stimulates the redistribution of protein kinase C isoforms in human neutrophils." J Immunol **159**(6): 2877-85.

Shima, F., T. Okada, M. Kido, H. Sen, Y. Tanaka, M. Tamada, C. D. Hu, Y. Yamawaki-Kataoka, K. Kariya and T. Kataoka (2000). "Association of yeast adenylyl cyclase with cyclase-associated protein CAP forms a second Ras-binding site which mediates its Ras-dependent activation." Mol Cell Biol **20**(1): 26-33.

Shpungin, S., I. Dotan, A. Abo and E. Pick (1989). "Activation of the superoxide forming NADPH oxidase in a cell-free system by sodium dodecyl sulfate. Absolute lipid dependence of the solubilized enzyme." J Biol Chem **264**(16): 9195-203.

Sidorenko, S. P., C. L. Law, S. J. Klaus, K. A. Chandran, M. Takata, T. Kurosaki and E. A. Clark (1996). "Protein kinase C mu (PKC mu) associates with the B cell antigen receptor complex and regulates lymphocyte signaling." Immunity **5**(4): 353-63.

Simons, P. C., S. F. Pietromonaco, D. Reczek, A. Bretscher and L. Elias (1998). "C-terminal threonine phosphorylation activates ERM proteins to link the cell's cortical lipid bilayer to the cytoskeleton." Biochem Biophys Res Commun **253**(3): 561-5.

Smolen, J. E., T. K. Petersen, C. Koch, S. J. O'Keefe, W. A. Hanlon, S. Seo, D. Pearson, M. C. Fossett and S. I. Simon (2000). "L-selectin signaling of

neutrophil adhesion and degranulation involves p38 mitogen-activated protein kinase." J Biol Chem **275**(21): 15876-84.

Someya, A., I. Nagaoka, H. Nunoi and T. Yamashita (1996). "Translocation of guinea pig p40-phox during activation of NADPH oxidase." Biochim Biophys Acta **1277**(3): 217-25.

Someya, A., I. Nagaoka and T. Yamashita (1993). "Purification of the 260 kDa cytosolic complex involved in the superoxide production of guinea pig neutrophils." FEBS Lett **330**(2): 215-8.

Someya, A., H. Nunoi, T. Hasebe and I. Nagaoka (1999). "Phosphorylation of p40-phox during activation of neutrophil NADPH oxidase." J Leukoc Biol **66**(5): 851-7.

Stabel, S., A. Rodriguez-Pena, S. Young, E. Rozengurt and P. J. Parker (1987). "Quantitation of protein kinase C by immunoblot--expression in different cell lines and response to phorbol esters." J Cell Physiol **130**(1): 111-7.

Stack, J. H., P. K. Herman, P. V. Schu and S. D. Emr (1993). "A membrane-associated complex containing the Vps15 protein kinase and the Vps34 PI 3-kinase is essential for protein sorting to the yeast lysosome-like vacuole." Embo J **12**(5): 2195-204.

Stack, J. H., B. Horazdovsky and S. D. Emr (1995). "Receptor-mediated protein sorting to the vacuole in yeast: roles for a protein kinase, a lipid kinase and GTP-binding proteins." Annu Rev Cell Dev Biol **11**: 1-33.

Steele-Mortimer, O., J. Gruenberg and M. J. Clague (1993). "Phosphorylation of GDI and membrane cycling of rab proteins." FEBS Lett **329**(3): 313-8.

Stevens, P., D. J. Winston and K. Van Dyke (1978). "In vitro evaluation of opsonic and cellular granulocyte function by luminol-dependent chemiluminescence: utility in patients with severe neutropenia and cellular deficiency states." Infect Immun **22**(1): 41-51.

Stryer, L., Ed. (1995). Biochemistry. New York, WH Freeman and Company.

Suchard, S. J., P. J. Mansfield, L. A. Boxer and J. A. Shayman (1997). "Mitogen-activated protein kinase activation during IgG-dependent phagocytosis in human neutrophils: inhibition by ceramide." J Immunol **158**(10): 4961-7.

Sumimoto, H., K. Hata, K. Mizuki, T. Ito, Y. Kage, Y. Sakaki, Y. Fukumaki, M. Nakamura and K. Takeshige (1996). "Assembly and activation of the phagocyte NADPH oxidase. Specific interaction of the N-terminal Src homology 3 domain of p47phox with p22phox is required for activation of the NADPH oxidase." J Biol Chem **271**(36): 22152-8.

Suzuki, Y. and R. I. Lehrer (1980). "NAD(P)H oxidase activity in human neutrophils stimulated by phorbol myristate acetate." J Clin Invest **66**(6): 1409-18.

Tauber, A. I., D. B. Brettler, E. A. Kennington and P. M. Blumberg (1982). "Relation of human neutrophil phorbol ester receptor occupancy and NADPH-oxidase activity." Blood **60**(2): 333-9.

Thrasher, A. J., N. H. Keep, F. Wientjes and A. W. Segal (1994). "Chronic granulomatous disease." Biochim Biophys Acta **1227**(1-2): 1-24.

Tilton, B., M. Andjelkovic, S. A. Didichenko, B. A. Hemmings and M. Thelen (1997). "G-Protein-coupled receptors and Fcgamma-receptors mediate activation of Akt/protein kinase B in human phagocytes." J Biol Chem **272**(44): 28096-101.

Ting, A. T., K. J. Einspahr, R. T. Abraham and P. J. Leibson (1991). "Fc gamma receptor signal transduction in natural killer cells. Coupling to phospholipase C via a G protein-independent, but tyrosine kinase-dependent pathway." J Immunol **147**(9): 3122-7.

Ting, A. T., L. M. Karnitz, R. A. Schoon, R. T. Abraham and P. J. Leibson (1992). "Fc gamma receptor activation induces the tyrosine phosphorylation of both phospholipase C (PLC)-gamma 1 and PLC-gamma 2 in natural killer cells." J Exp Med **176**(6): 1751-5.

Tkalcevic, J., M. Novelli, M. Phylactides, J. P. Iredale, A. W. Segal and J. Roes (2000). "Impaired immunity and enhanced resistance to endotoxin in the absence of neutrophil elastase and cathepsin G." Immunity **12**(2): 201-10.

Toullec, D., P. Pianetti, H. Coste, P. Bellevergue, T. Grand-Perret, M. Ajakane, V. Baudet, P. Boissin, E. Boursier, F. Loriolle and et al. (1991). "The

bisindolylmaleimide GF 109203X is a potent and selective inhibitor of protein kinase C." J Biol Chem **266**(24): 15771-81.

Towbin, H., T. Staehelin and J. Gordon (1979). "Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications." Proc Natl Acad Sci U S A **76**(9): 4350-4.

Traves, C. A. J. a. P. (1996). ImmunoBiology-The immune System in Health and Disease. London, Current Biology Limited.

Twomey, B., R. E. Muid and M. M. Dale (1990). "The effect of putative protein kinase C inhibitors, K252a and staurosporine, on the human neutrophil respiratory burst activated by both receptor stimulation and post-receptor mechanisms." Br J Pharmacol **100**(4): 819-25.

Twomey, B., R. E. Muid, J. S. Nixon, A. D. Sedgwick, S. E. Wilkinson and M. M. Dale (1990). "The effect of new potent selective inhibitors of protein kinase C on the neutrophil respiratory burst." Biochem Biophys Res Commun **171**(3): 1087-92.

Uhlinger, D. J., D. N. Burnham and J. D. Lambeth (1991). "Nucleoside triphosphate requirements for superoxide generation and phosphorylation in a cell-free system from human neutrophils. Sodium dodecyl sulfate and diacylglycerol activate independently of protein kinase C." J Biol Chem **266**(31): 20990-7.

Ullrich, O., H. Stenmark, K. Alexandrov, L. A. Huber, K. Kaibuchi, T. Sasaki, Y. Takai and M. Zerial (1993). "Rab GDP dissociation inhibitor as a general regulator for the membrane association of rab proteins." J Biol Chem **268**(24): 18143-50.

Unkeless, J. C., E. Scigliano and V. H. Freedman (1988). "Structure and function of human and murine receptors for IgG." Annu Rev Immunol **6**: 251-81.

Valverde, A. M., J. Sinnett-Smith, J. Van Lint and E. Rozengurt (1994). "Molecular cloning and characterization of protein kinase D: a target for diacylglycerol and phorbol esters with a distinctive catalytic domain." Proc Natl Acad Sci U S A **91**(18): 8572-6.

van der Geer, P. and T. Hunter (1994). "Phosphopeptide mapping and phosphoamino acid analysis by electrophoresis and chromatography on thin-layer cellulose plates." Electrophoresis **15**(3-4): 544-54.

Van Lint, J., Y. Ni, M. Valius, W. Merlevede and J. R. Vandenheede (1998). "Platelet-derived growth factor stimulates protein kinase D through the activation of phospholipase Cgamma and protein kinase C." J Biol Chem **273**(12): 7038-43.

Van Lint, J. V., J. Sinnett-Smith and E. Rozengurt (1995). "Expression and characterization of PKD, a phorbol ester and diacylglycerol-stimulated serine protein kinase." J Biol Chem **270**(3): 1455-61.

Vertommen, D., M. Rider, Y. Ni, E. Waelkens, W. Merlevede, J. R. Vandenheede and J. Van Lint (2000). "Regulation of protein kinase D by multisite phosphorylation. Identification of phosphorylation sites by mass spectrometry and characterization by site-directed mutagenesis." J Biol Chem **275**(26): 19567-76.

Vlahos, C. J., W. F. Matter, R. F. Brown, A. E. Traynor-Kaplan, P. G. Heyworth, E. R. Prossnitz, R. D. Ye, P. Marder, J. A. Schelm, K. J. Rothfuss and et al. (1995). "Investigation of neutrophil signal transduction using a specific inhibitor of phosphatidylinositol 3-kinase." J Immunol **154**(5): 2413-22.

Vlahos, C. J., W. F. Matter, K. Y. Hui and R. F. Brown (1994). "A specific inhibitor of phosphatidylinositol 3-kinase, 2-(4-morpholinyl)-8-phenyl-4H-1-benzopyran-4-one (LY294002)." J Biol Chem **269**(7): 5241-8.

Volpp, B. D., W. M. Nauseef and R. A. Clark (1988). "Two cytosolic neutrophil oxidase components absent in autosomal chronic granulomatous disease." Science **242**(4883): 1295-7.

Vorland, L. H. (1999). "Lactoferrin: a multifunctional glycoprotein." Apmis **107**(11): 971-81.

Vossebeld, P. J., C. H. Homburg, R. C. Schweizer, I. Ibarrola, J. Kessler, L. Koenderman, D. Roos and A. J. Verhoeven (1997). "Tyrosine phosphorylation-dependent activation of phosphatidylinositide 3-kinase

occurs upstream of Ca2+-signalling induced by Fcgamma receptor cross-linking in human neutrophils." Biochem J **323**(Pt 1): 87-94.

Vossebeld, P. J., J. Kessler, A. E. von dem Borne, D. Roos and A. J. Verhoeven (1995). "Heterotypic Fc gamma R clusters evoke a synergistic Ca2+ response in human neutrophils." J Biol Chem **270**(18): 10671-9.

Waldron, R. T., T. Iglesias and E. Rozengurt (1999). "Phosphorylation-dependent protein kinase D activation." Electrophoresis **20**(2): 382-90.

Waldron, R. T., T. Iglesias and E. Rozengurt (1999). "The pleckstrin homology domain of protein kinase D interacts preferentially with the eta isoform of protein kinase C." J Biol Chem **274**(14): 9224-30.

Waldron, R. T. and E. Rozengurt (2000). "Oxidative stress induces protein kinase D activation in intact cells. Involvement of Src and dependence on protein kinase C." J Biol Chem **275**(22): 17114-21.

Wallach, T. M. and A. W. Segal (1996). "Stoichiometry of the subunits of flavocytochrome b558 of the NADPH oxidase of phagocytes." Biochem J **320**(Pt 1): 33-8.

Wang, J. and E. J. Brown (1999). "Immune complex-induced integrin activation and L-plastin phosphorylation require protein kinase A." J Biol Chem **274**(34): 24349-56.

Watson, F. and S. W. Edwards (1998). "Stimulation of primed neutrophils by soluble immune complexes: priming leads to enhanced intracellular Ca2+ elevations, activation of phospholipase D, and activation of the NADPH oxidase." Biochem Biophys Res Commun **247**(3): 819-26.

Watson, F., L. Gasmi and S. W. Edwards (1997). "Stimulation of intracellular Ca2+ levels in human neutrophils by soluble immune complexes. Functional activation of FcgammaRIIIb during priming." J Biol Chem **272**(29): 17944-51.

Way, K. J., E. Chou and G. L. King (2000). "Identification of PKC-isoform-specific biological actions using pharmacological approaches." Trends Pharmacol Sci **21**(5): 181-7.

Weller, J. L., H. Jaffe, P. H. Roseboom, M. J. Zylka and D. C. Klein (1996). "2D-PAGE analysis: adrenergically regulated pineal protein AIP 37/6 is a phosphorylated isoform of cytosolic malate dehydrogenase." Brain Res **713**(1-2): 8-16.

Wenzel-Seifert, K., C. Schachtele and R. Seifert (1994). "N-protein kinase C isoenzymes may be involved in the regulation of various neutrophil functions." Biochem Biophys Res Commun **200**(3): 1536-43.

Wientjes, F. B., J. J. Hsuan, N. F. Totty and A. W. Segal (1993). "p40phox, a third cytosolic component of the activation complex of the NADPH oxidase to contain src homology 3 domains." Biochem J **296**(Pt 3): 557-61.

Wintrobe, M. M. (1992). Milestones on the Path of Progress. Blood Pure and Eloquent- A Story of Discovery, of people, and of Ideas. M. M. Wintrobe. New York, McGraw-Hill Book Company: 1-31.

Woolf, N. (1998). Pathology *Basic and Systemic*. London, U.K., W.B. Saunders Company Ltd.

Yamaguchi, M., H. Oishi, S. Araki, S. Saeki, H. Yamane, N. Okamura and S. Ishibashi (1995). "Respiratory burst and tyrosine phosphorylation by vanadate." Arch Biochem Biophys **323**(2): 382-6.

Yamamori, T., O. Inanami, H. Nagahata, Y. Cui and M. Kuwabara (2000). "Roles of p38 MAPK, PKC and PI3-K in the signaling pathways of NADPH oxidase activation and phagocytosis in bovine polymorphonuclear leukocytes." FEBS Lett **467**(2-3): 253-8.

Yu, H., S. J. Suchard, R. Nairn and R. Jove (1995). "Dissociation of mitogen-activated protein kinase activation from the oxidative burst in differentiated HL-60 cells and human neutrophils." J Biol Chem **270**(26): 15719-24.

Zelicof, A., V. Protopopov, D. David, X. Y. Lin, V. Lustgarten and J. E. Gerst (1996). "Two separate functions are encoded by the carboxyl-terminal domains of the yeast cyclase-associated protein and its mammalian homologs. Dimerization and actin binding." J Biol Chem **271**(30): 18243-52.

Zhang, H., C. D. Garlichs, A. Mugge and W. G. Daniel (1998). "Involvement of tyrosine kinases, Ca2+ and PKC in activation of mitogen-activated protein (MAP) kinase in human polymorphonuclear neutrophils." J Physiol (Lond) **513**(Pt 2): 359-67.

Zugaza, J. L., J. Sinnett-Smith, J. Van Lint and E. Rozengurt (1996). "Protein kinase D (PKD) activation in intact cells through a protein kinase C-dependent signal transduction pathway." Embo J **15**(22): 6220-30.