Phosphorylation-dependent signalling mechanisms in human neutrophils

by

John H. Brumell

A thesis submitted in conformity with the requirements
for the degree of Doctor of Philosophy
Graduate Department of Biochemistry
University of Toronto

0-612-27612-0

Canada

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John H. Brumell

Doctor of Philosophy, 1997

Department of Biochemistry, University of Toronto

ABSTRACT

Neutrophil responses to invading microorganisms are controlled by a variety of cell surface receptors that bind to inflammatory ligands and initiate a number of intracellular biochemical events. In this thesis, I examined the control of protein phosphorylation in neutrophils following activation by exposure to chemoattractants.

Activation of the bacterial tripeptide receptor increased the activity of two renaturable serine/threonine kinases (PK65 and PK72) in parallel with tyrosine phosphorylation of proteins with similar molecular masses. While PK65 and PK72 were not phosphorylated on tyrosine, their activation was dependent on the activity of upstream tyrosine kinases.

Direct activation of the NADPH oxidase can initiate receptor-independent tyrosine phosphorylation of many cellular proteins, an event dependent on the production of reactive oxygen intermediates (ROI) by this enzyme. Activation of tyrosine kinases was observed in a manner dependent on their prior tyrosine phosphorylation. NADPH oxidase-derived ROI likely play an important role as auto- and paracrine signalling molecules.

The tyrosine phosphatase SHP-1 was phosphorylated on serine residues in response to inflammatory stimuli, concomitant with a decrease in its catalytic activity. Phosphorylation and inhibition of SHP-1 were blocked with an inhibitor of Protein Kinase C (PKC) isoforms and mimicked *in vitro* by PKC phosphorylation. These findings suggest a new link between PKC and the regulation of tyrosine phosphorylation.

Phosphorylation of another PKC substrate, pleckstrin, was also studied. Several lines of evidence suggest that pleckstrin is phosphorylated by a non-conventional isoform of PKC. Following phosphorylation, pleckstrin translocated from the cytosol to both membranes and

the cytoskeleton. Due to its demonstrated ability to interact with biologically active lipid and protein ligands, pleckstrin may act as an intracellular adaptor/targetting protein.

In addition to pleckstrin, other adaptor proteins were identified in neutrophils. Originally described in other systems, VAMP-2 and syntaxin 4 were localized to the secretory granules and plasma membrane of neutrophils, respectively. The interaction of these proteins (through a tripartite complex with soluble secretory factors such as NSF and SNAP) may play a role in regulating secretion of antimicrobial agents.

ACKNOWLEDGMENTS

I wish to thank my supervisor and mentor, Sergio Grinstein, for the many years of guidance and support that he has provided. Sergio has served as an excellent model of the successful scientist and has exposed me to a broad variety of scientific pursuits. In addition to his academic mentorship, I have greatly benefitted from Sergio's personal advice and support through many of the challenges I've faced during the last 4 years. I also wish to thank the members of my graduate committee, Drs. Mike Moran and Rheinhart Rheithmeier, for their guidance and constructive criticism.

Science has become more and more a field where collaboration is a crucial requirement for success. I wish to thank the following collaborators who have aided the work in this thesis and offered their support during the time of these studies: Drs. Amira Klip, Gregory Downey, Mike Tyers, Karen Craig, Katherine Siminovitch, Anne Burkhardt, Joeseph Bolen, and Niels Borregaard. I also wish to acknowledge both the technical and personal support that has been extended to me by the many members of the Sergio's laboratory, both past and present. In particular, I wish to thank Jeffrey Butler who assisted my first `baby steps to doing science' upon first arrival in Sergio's lab.

None of my academic pursuits would have been possible were it not for the unfailing love and support of many people. First and foremost were my parents who taught me to do what I love, never run from a problem and, when in doubt, go look it up in the library! I've been lucky to have studied under many excellent teachers over the years, including Adel Kamel. The positive influence of Adel and others like him has helped me to follow the path that I've taken. Always believing that there's no point in going to school if it isn't fun, I've enjoyed many good times with the company of cherished friends. Thanks for the memories!

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LIST OF ABBREVIATIONS

aPKC	Atypical PKC
BIM	*Bis*-indolylmaleimide I
BSA	Bovine serum albumin
cPKC	Conventional PKC
DiC8-eg	1,2 -dioctanoylethylene glycol;
DPI	diphenylene iodonium
DTT	Dithiothreitol
fMLP	Formyl-Met-Leu-Phe
G-proteins	GTP-binding proteins
GTPγS	guanosine 5'-O-(3-thiotriphosphate
H_2O_2	Hydrogen peroxide
MAP kinase	Mitogen-activated protein kinase
NAC	N-acetyl cysteine
NaV	Sodium orthovanadate
nPKC	Novel PKC
NSF	N-ethylmaleimide sensitive factor
OPZ	Opsonized zymosan
PBS	Phosphate-buffered saline
PI3K	Phosphatidylinositol 3'-kinase
PK65	Protein kinase of 65 kDa (renaturable)
PK72	Protein kinase of 72 kDa (renaturable)
PKC	Protein kinase C
PMSF	Phenylmethylsulfonyl fluoride
PTK	Protein tyrosine kinase
PTP	Protein tyrosine phosphatase
PTP-1B	Protein tyrosine phosphatase-1B
PVDF	Polyvinylidene diflouride

ROI	Reactive oxygen intermediates
SCAMP	Secretory carrier membrane protein
SDS PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
SH	Src Homology
SHP	SH2-containing tyrosine phosphatase
SNAP	Soluble NSF attachment protein
SNAP-25	Synaptosome-associated protein
SNARE	SNAP receptor
sv/pm	Secretory vesicle/plasma membrane fraction
TC-PTP	T-cell phosphatase
t-SNARE	Target membrane SNARE
TPA	12-O-tetradecanoylphorbol 13-acetate
VAMP	Vesicle-associated membrane protein
v-SNARE	Vesicular membrane SNARE

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Chapter 1:

Introduction

After a microbe has penetrated our physical barriers, only the immune system stands in the way of its rapid multiplication, movement throughout the body and ensuing tissue damage. An important weapon in the immune system's arsenal for fighting these invaders are the polymorphonuclear leukocytes, including neutrophils, eosinophils and basophils. Of these cellular defenders, the neutrophil is thought to be crucial for fighting infections, particularly those of bacteria and fungi. Indeed, neutrophils (also referred to as PMN) constitute 45-65% of leukocytes in the blood and their numbers rapidly increase in response to such infections. Being highly motile, the neutrophil is the first immune cell to arrive at sites of inflammation to combat microbial invaders. In fact, defects in the ability of PMN to kill their microbial targets result in serious and recurrent infections. For these reasons, the neutrophil has been hailed as the immune system's 'first line of defence' against microbial invasion (Edwards, 1994).

Neutrophils originate and mature in the bone marrow before being released into the bloodstream. There, they circulate freely and occasionally form weak interactions that allow 'rolling' along the endothelial surface. Exposure to chemoattractants, produced at sites of inflammation, causes these cells to increase the surface expression of adhesion molecules and interact tightly with the endothelium. Neutrophil chemoattractants include molecular components of the immune system (e.g.: complement factors and cytokines) as well as products of the microbial targets themselves (e.g.: formylated bacterial peptides). In response to such chemoattractants, neutrophils leave the bloodstream via trans-endothelial migration and, once in the extravascular milieu, migrate (up the chemoattractant gradient) to sites of inflammation by a process known as chemotaxis. In this way, chemoattractants produced at sites of infection act as a 'homing signal' for neutrophils to follow to their targets of action.

Having reached the site of infection, neutrophils attack and kill their microbial targets by a variety of biochemical means. This attack may be confined within the PMN itself by engulfment of the microbe into a membrane-bound compartment called the phagosome

Secretory Vesicles
-CD35
-alkaline phosphatase
-V-type H+ ATPase
-cytochrome b_{558}
-fMLP-R
-Mac-1

Tertiary Granules
-V-type H+ ATPase
-fMLP-R
-Mac-1
-gelatinase

Secondary Granules
-CD67
-lactoferrin
-lysozyme
-vit.B$_{12}$-binding protein
-collagenase

Chemotaxis

Primary Granules
-CD63
-V-type H+ ATPase
-myeloperoxidase
-cathepsins
-defensins
-elastase
-lysozyme

Secretion

Phago/lysosomal Fusion

Phagocytosis

Fig. 1.1   Neutrophils in action at sites of inflammation.

(Fig.1.1). Phagocytosis is initiated by recognition of the microbe by a number of cell surface receptors expressed exclusively in professional phagocytes. Phagocytic receptors may bind the unusual surface of the microbe itself or serum proteins that coat this surface. These serum proteins, called 'opsonins', include antibodies, complement factors and fibronectin and serve to make the phagocytic process more efficient.

Neutrophils possess two principal mechanisms by which they are able to kill pathogenic organisms. The first mechanism, termed degranulation, involves the exposure of microbes to a battery of antimicrobial substances contained within preformed secretory organelles. This exposure may occur by fusion of these secretory compartments with the phagosome, a process generically called phago/lysosomal fusion. Alternatively, the secretory compartments may fuse directly with the plasma membrane and release their contents into sealed off spaces (e.g.: beneath the adherent leukocyte) or into the extracellular environment. The second mechanism involves the production of toxic free radicals called reactive oxygen intermediates (ROI) by a multi-component enzyme complex, the NADPH oxidase.

Degranulation. Neutrophils contain at least 4 distinct types of secretory compartments, each possessing unique membrane components and matrix contents (Borregaard, et al., 1993; Borregaard, et al., 1993). First to appear during hematopoiesis are the primary (or azurophilic) granules which are identified by their exclusive content of myeloperoxidase (see Table 1.1). Following phagocytosis, primary granules preferentially fuse with the nascent phagosome, delivering a variety of matrix proteins that facilitate the destruction and degradation of foreign invaders (Tapper, 1996). These matrix components include cationic proteins such as bactericidal/permeability increasing protein (BPI) and defensins which permeabilize bacteria. Other primary granule contents are the neutral proteases of the serprocidin family, including cathepsin G, elastase and azurocidin. Serprocidins demonstrate potent microbicidal activity by an undefined mechanism that is often independent of their catalytic activity. Degradation of remaining material is accomplished by the actions of various hydrolytic enzymes (Levy, 1996).

Table 1.1   Components of neutrophil granules and secretory vesicles.   (adapted from "Granules and vesicles of human neutrophils. The role of endomembranes as source of plasma membrane proteins." by Borregaard et al., *Eur. J. Haematol.*, Vol 51, 1993)

Peroxidase-positive	Peroxidase-negative		
Primary granules	Secondary granules	Tertiary granules	Secretory vesicles

Membrane:

CD63	CD67	Mac-1	CD35
CD68	FMLP-R	FMLP-R	Mac-1
V-type H$^+$ ATPase	Fibronectin-R	V-type H$^+$ ATPase	FMLP-R
	G-protein α subunit		Alkaline phosphatase
	Laminin-R		Cytochrome b$_{558}$
	Mac-1		CD16 (FcγRIII)
	Rap1, Rap2		V-type H$^+$ ATPase
	Thrombospondin-R		
	TNF-R		
	Vitronectin-R		
	Cytochrome b$_{558}$		
	Annexin 3		

Matrix:

α1-antitrypsin	β2-microglobulin	Gelatinase	Albumin
α-mannosidase	Collagenase	Acetyltransferase	Tetranectin
Azurocidin	Gelatinase	Lysozyme	
BPI	Histaminase		
β-glucuronidase	Heparinase		
Cathepsins	Lactoferrin		
Defensins	Lysozyme		
Elastase	NGAL		
Lysozyme	Vit B$_{12}$-BP		
Myeloperoxidase	Sialidase		
Sialidase			
PLA$_2$			
Ubiquitin			

Abbreviations: BPI, bacterial permeability increasing protein;   NGAL, neutrophil gelatinase associated lipocalin;   PLA$_2$, phospholipase A$_2$;   V-type H$^+$ ATPase, vacuolar-type proton pump; Vit B$_{12}$-BP, vitamin B$_{12}$-binding protein

Primary granules (and other secretory compartments) also contain a membrane bound vacuolar-type H^+ ATPase (Nanda, et al., 1996) which mediates phagosomal acidification following phago/lysosomal fusion (Tapper and Sundler, 1995).

Twice as abundant as primary granules are the secondary (or specific) granules, which lack myeloperoxidase. In further contrast to the primary granule, secondary granules fuse preferentially with the plasma membrane and release their contents into the extracellular milieu following phagocytosis of opsonized pathogens (Tapper, 1996). These contents may have a bacteriostatic role (e.g.: lysozyme) or may facilitate chemotaxis by degradation of the extracellular matrix (e.g.: gelatinase, collagenase). In addition to delivering their matrix contents, secondary granules serve as endomembrane stores for a variety of receptors important for neutrophil functions (Borregaard, et al., 1993). Exocytosis exposes the adhesion molecule Mac-1 and allows tight adhesion of circulating neutrophils to the vascular endothelium. Also, increased surface expression of chemoattractant receptors allows for more sensitive detection of these molecules.

Tertiary granules are characterized by their high content of gelatinase and only recently became separable from secondary granules (Borregaard, et al., 1993). These secretory compartments also contain acetyltransferase and lysozyme. Similar to secondary granules, tertiary granules serve as receptor stores and contain fMLP receptors and the leukocyte adhesion molecule Mac-1.

Secretory vesicles are endocytic vesicles that can be rapidly translocated to the plasma membrane in response to chemoattractant. These vesicles contain serum proteins such as albumin and tetranectin in their matrix and latent alkaline phosphatase and CD35 (complement receptor 1) in their membrane.

The mechanisms that control the degranulation process are unclear. It has been suggested that proteins direct the interactions of secretory granules/vesicles with their target membranes. One family to be implicated in such a role is the Ca^{2+}-dependent, phospholipid binding family of annexins (Creutz, 1992). Members of this family are highly expressed in neutrophils and are present in both the cytosol (annexin I) and/or associated with secretory

granules (annexin III on secondary granules). *In vitro* studies have demonstrated the ability of annexin I to induce fusion of secondary granules with plasma membrane derived phospholipid vesicles in a Ca^{2+}-dependent manner (Meers, et al., 1993). No protein ligands for annexins have been found and the role of these proteins in neutrophil exocytosis remains speculative.

Recent progress has been made in the elucidation of the events that control vesicular fusion in other systems. Based primarily on results obtained using synaptic and Golgi membranes, as well as yeast secretion mutants, an attractive model involving specific docking proteins has been proposed (Bark and Wilson, 1994; Rothman, 1994; Schekman, 1992; Sudhof, et al., 1993). Briefly, a core exocytotic complex is believed to exist, composed of three intrinsic membrane proteins (see Fig. 1.2). These include the vesicular-bound synaptobrevin or VAMP (vesicle-associated membrane protein) as well as two proteins found in the plasma membrane, syntaxin and SNAP-25 (synaptosome-associated protein). These proteins are thought to form a tripartite complex, which in turn can interact with the soluble protein NSF (N-ethylmaleimide sensitive factor) through mediation of a connecting protein, SNAP (soluble NSF attachment protein). Complexation of the soluble proteins to the membrane-bound assembly confers ATP dependence to the docking and fusion event and renders it N-ethylmaleimide sensitive. The ability of the membrane-bound proteins to bind SNAP has led to their designation as SNAREs (SNAP receptors).

Inasmuch as NSF and SNAP are highly conserved and ubiquitous, it is believed that specificity during docking/fusion results from the existence of unique SNAREs in the donor or vesicular membrane (v-SNAREs) as well as the target membrane (t-SNARE) (Calakos, et al., 1994). Other proteins that are not part of the above mentioned core may participate in regulation of the fusion process. Suggested candidates include synaptotagmin (Jahn and Sudhof, 1994; Sollner, et al., 1993) and synaptophysin (Edelmann, et al., 1995). A new family of proteins referred to as secretory carrier membrane proteins (SCAMPs) (Brand and Castle, 1993; Brand, et al., 1991) could conceivably also fulfill a similar role.

It is presently not known whether the paradigm outlined above applies to the secretory events that occur in activated neutrophils. In fact, the presence of SNAREs or other proteins
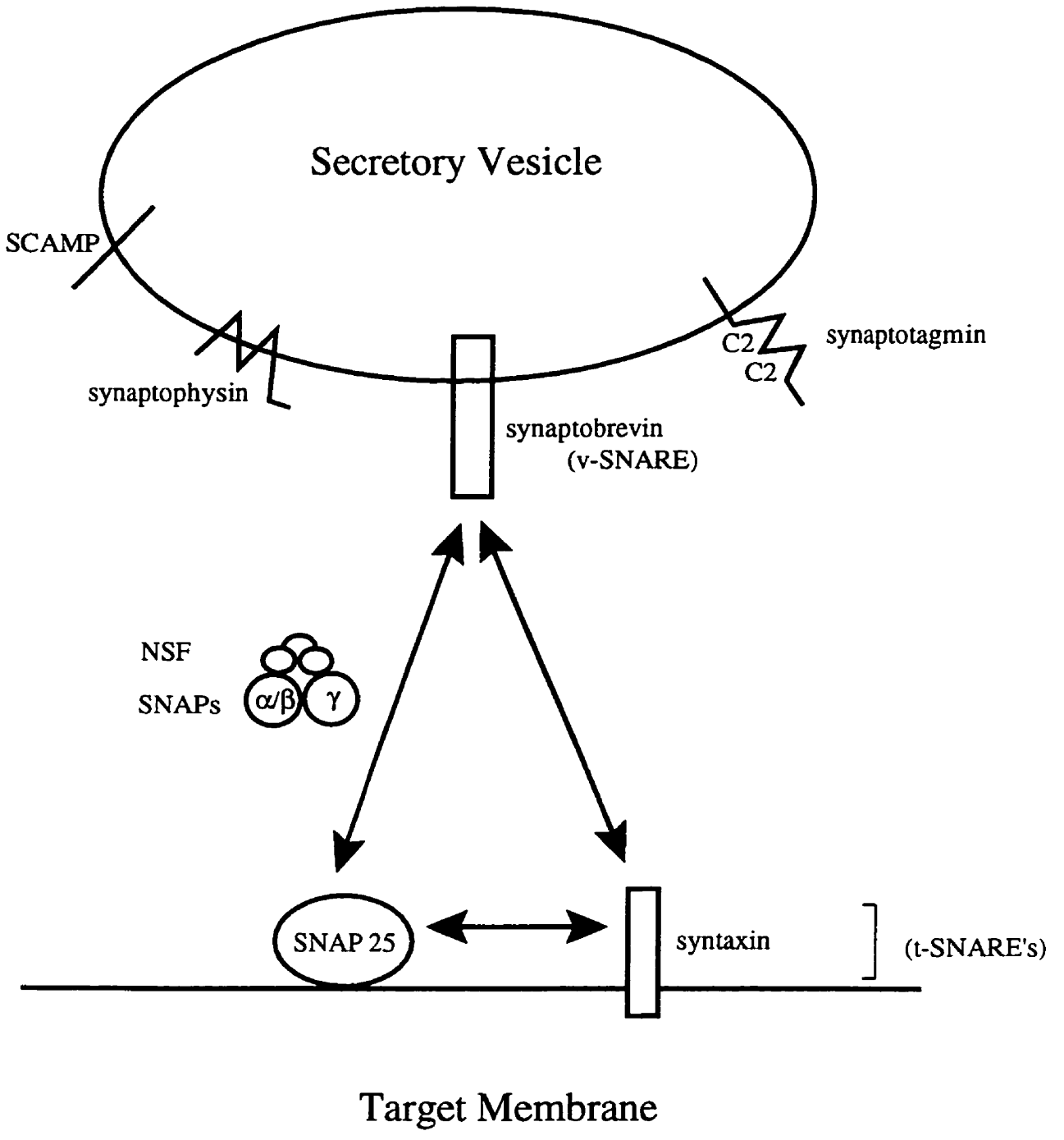
Fig. 1.2   Proposed model for the SNARE hypothesis.

relevant to the secretory pathway of these cells has not been documented. In this regard, neutrophils represent a unique and challenging system. Importantly, the rate and extent of secretion of each type of organelle are remarkably different and vary depending on the stimulus used for activation (Kjeldsen, et al., 1994). For example, conditions have been described that result in virtually complete exocytosis of secretory vesicles, with negligible release of primary granules (Sengelov, et al., 1993). Also, exocytosis of secretory vesicles occurs within seconds whereas release of primary and secondary granules requires many minutes for completion. These findings are suggestive of differences in the secretory machinery of individual organelles.

NADPH oxidase. The oxygen-dependent mechanism by which neutrophils destroy their targets is mediated by a multi-subunit enzyme complex, the NADPH oxidase (Segal, 1996). This complex catalyzes the transfer of one electron from cytosolic NADPH to molecular oxygen (O_2), producing superoxide (O_2^-):

$$NADPH + 2O_2 \rightarrow 2O_2^- + NADP^+ + H^+$$
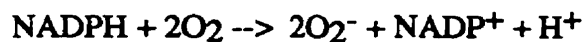
While O_2^- is a poor oxidant itself, it gives rise to a number of more potent oxidants by a series of spontaneous and enzyme catalyzed reactions. These include the dismutation of O_2^- to hydrogen peroxide (H_2O_2) which can occur spontaneously within the acidic environment of the phagosome or catalyzed by superoxide dismutase. The iron-catalyzed reaction of H_2O_2 with O_2^- generates hydroxyl radical ($OH\cdot$), one of the most potent oxidants known. Delivery of myeloperoxidase from primary granules to the phagosome allows it to interact with H_2O_2, producing hypochlorous acid (HOCl). Reaction of O_2^- with nitric oxide (NO·) yields peroxynitrite ($ONOO^-$) which decomposes into the radicals $NO_2\cdot$ and OH·. The latter two agents are substantially more toxic than either O_2^- or NO·. The importance of NADPH oxidase-derived oxidants is underlined by the fact that well defined genetic defects in the oxidase lead to life-threatening infections in patients with chronic granulomatous disease (CGD).

47   67

40   rac

rhoGDI

Cytosol

Rap

Membrane

p22

gp 91

Cytochrome b$_{558}$

Activation

NADPH $\longrightarrow$ NADP$^+$ + H$^+$

2e$^-$

P 40 P
47 67
Rap rac

rhoGDI

2O$_2$ 2O$_2^-$
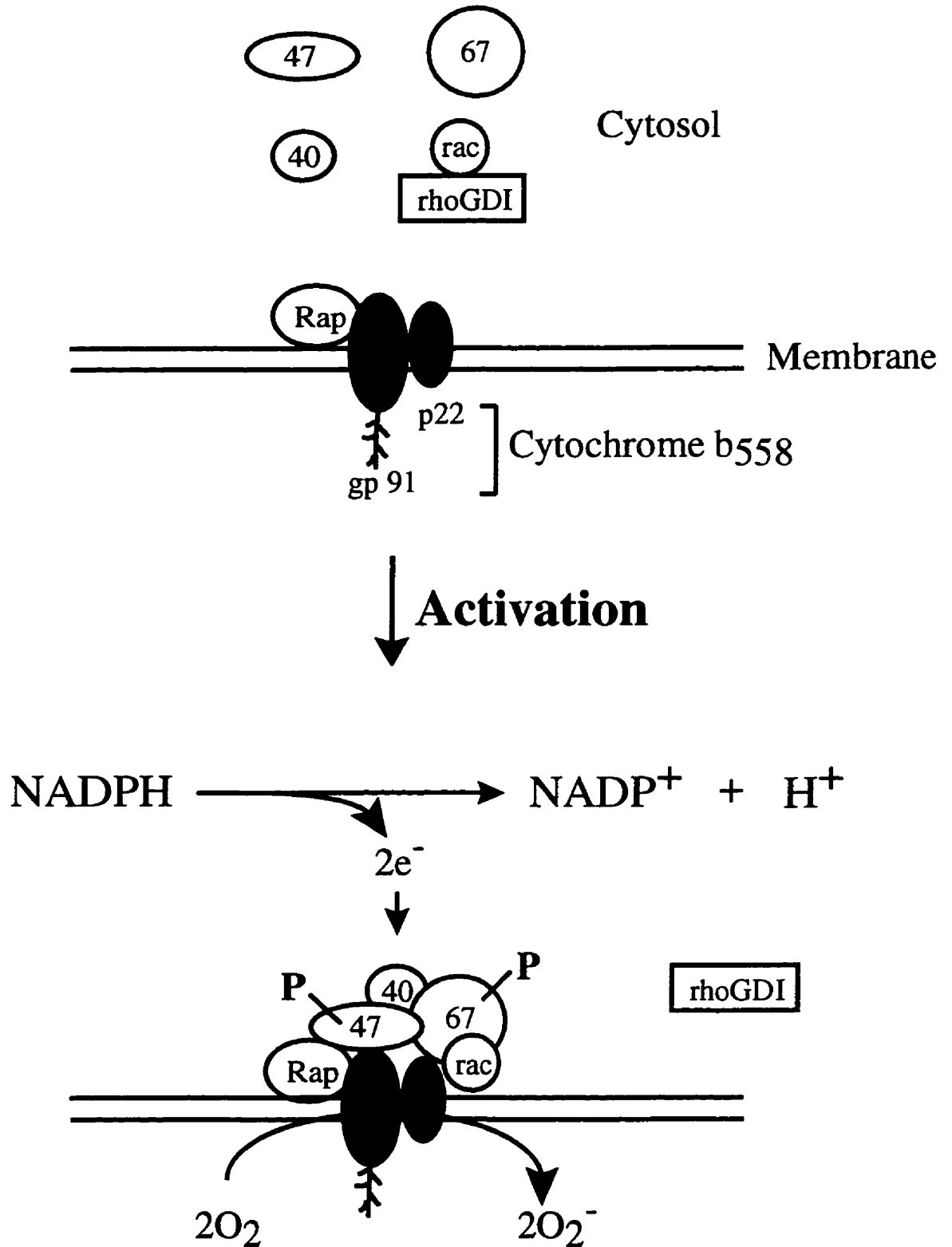
Fig. 1.3   Assembly and activation of the multi-component NADPH oxidase following neutrophil activation.

The NADPH oxidase consists of both cytosolic and membrane bound components (see Fig.1.3). The cytosolic proteins p47phox (for phagocyte oxidase) and p67phox were first identified by their affinity for immobilized guanosine triphosphate (GTP)(Volpp, et al., 1988). This observation, and the finding that the NADPH oxidase required GTP for its activity (Gabig, et al., 1987), led to the purification of a third cytosolic factor, the GTP-binding (G protein) Rac. In unstimulated cells, Rac is rendered soluble within the cytosol via the association of its isoprenylated C-terminus with the guanine nucleotide dissociation inhibitor (GDI) of its family member Rho (Quinn, 1995). The fourth cytosolic component, p40phox, has recently been isolated by taking advantage of its ability to bind p67phox (Wientjes, et al., 1993). Indeed, evidence suggests that all four cytosolic components interact stably and form a complex in quiescent cells, perhaps facilitating rapid activation of the oxidase (Iyer, et al., 1994; Park, et al., 1994).

The principal membrane-bound component of the NADPH oxidase is a novel heme-containing protein complex, cytochrome b_{558} (so called due to its absorption maximum in the reduced state). The heterodimeric cytochrome consists of a heavily glycosylated protein with an apparent molecular weight of 91 kDa (gp91phox) and a smaller integral membrane protein, p22phox (Jesaitis, 1995). Both proteins are intimately associated and required for oxidase activity: mutations that abolish the expression of one protein lead to the instability of the other protein and result in CGD (Parkos, et al., 1989). Cytochrome b_{558} acts as a flavocytochrome, binding flavin adenine dinucleotide (Doussiere, et al., 1995) and rendering the oxidase activity sensitive to flavoprotein inhibitors such as diphenylene iodonium (DPI) (Doussiere and Vignais, 1991). A second small molecular weight G protein, rap 1A , is associated with the NADPH oxidase (Bokoch and Quilliam, 1990). Unlike Rac, rap 1A is not localized in the cytosol of unstimulated neutrophils but rather associates constitutively with the plasma membrane and the membrane of secondary granules. While not obligatory for activation of the oxidase, rap1A has been shown to play a modulatory role over its activity (Maly, et al., 1994). All three membrane-bound components of the oxidase can be found in the plasma membrane but are predominantly localized to secondary granules and secretory vesicles in unstimulated

cells (Borregaard, et al., 1993). Degranulation of these compartments into either phagosomes or the plasma membrane targets this oxygen-dependent killing mechanism to its optimal site of action.

Activation of the NADPH oxidase requires translocation of the cytosolic components to the phagosomal and/or plasma membrane and assembly of the complete oxidase complex. Once assembled, the oxidase initiates electron transfer by an undetermined mechanism. This assembly process is mediated by numerous protein-protein interactions between and among cytosolic and membrane-bound components (De Leo, et al., 1996; Nauseef, et al., 1991). The details of how these interactions are regulated are incomplete but appear to involve two important events. The first event is the dissociation of RhoGDI from Rac, exposing its isoprenylated C-terminus and allowing it to translocate to the phagosomal and/or plasma membrane. As Rac has been found to interact directly with other cytosolic components of the oxidase (Iyer, et al., 1994; Park, et al., 1994), Rac translocation may represent an important event in the assembly process.

Phosphorylation of some cytosolic components may also represent a regulatory mechanism by which NADPH oxidase assembly is controlled. Indeed, p47^{phox} is rapidly phosphorylated following stimulation of neutrophils with a variety of soluble and particulate stimuli (Nauseef, et al., 1990). The highly basic C-terminus of p47^{phox} contains many potential sites of phosphorylation by Protein kinase C (PKC) and peptides derived from this region are efficiently phosphorylated *in vitro* with purified PKC. PKC regulation of oxidase assembly is suggested by the finding that treatment of cells with inhibitors of this kinase blocks oxidase activation (Nauseef, et al., 1991). In addition to PKC phosphorylation, p47^{phox} may be phosphorylated by proline-directed kinases of the mitogen-activated protein kinase (MapK) family and possibly other kinases (el Benna, et al., 1994).

Phosphorylation of p67^{phox} has been suggested to occur in activated neutrophils, however less is known about the role of this event (Dusi and Rossi, 1993). The phosphorylation sites and putative kinases which phosphorylate p67^{phox} remain to be determined. Phosphorylation of p47^{phox} and possibly p67^{phox} may induce a conformational

change in these proteins that facilitates their association with membrane-bound components of the NADPH oxidase.

Intracellular signalling through the chemoattractant receptors.

Each of the cellular responses described above, from endothelial adhesion to final destruction of the invading microbes, are controlled by a variety of receptors present on the neutrophil cell surface (see Table 1.2). Interaction of these receptors with their specific ligands leads to a number of intracellular biochemical events that serve to internalize the 'signal' and ultimately initiate anti-microbial responses. This section presents a brief review of the intracellular signalling events initiated by the fMLP receptor, the focus of my studies. Because of the breadth of the signal transduction field, I must apologize for any omissions or oversights in this review.

Initiation of bacterial protein synthesis requires the formylation of an N-terminal methionine residue. Processing of the nascent polypeptide often involves cleavage of this residue and several adjacent amino acids, generating a variety of formylated peptides which are released from the bacteria into its environment. During bacterial invasion, low concentrations of these formylated peptides serve as chemoattractants, guiding neutrophils and other leukocytes to sites of infection. Once at the site of infection, higher concentratations are inhibitory for chemotaxis but initiate degranulation and activation of the NADPH oxidase (Snyderman and Uhing, 1992).

The most potent peptide chemoattractant produced by bacteria is formyl-Met-Leu-Phe (fMLP) (Allen, et al., 1990). Using photoaffinity labelled derivatives of fMLP, the fMLP receptor was found to be a highly glycosylated integral membrane protein of approximately 50-70 kDa on denaturing gels. Deglycosylation of this protein by endoglycosidase treatment prior to electrophoresis revealed the true molecular mass of this receptor to be 35 kDa (Marasco, et al., 1984). Quiescent neutrophils possess ≈50,000 receptors on their cell surface, a number that can be upregulated in response to inflammatory stimuli via delivery of an internal receptor pool present in secretory granules/vesicles (Sengelov, et al., 1994).

Table 1.2   Regulation of neutrophil responses.

Receptors	Biochemical Effectors	Cellular Responses
chemoattractant -fMLP, LTB4, PAF, IL-8	G-protein activation -heterotrimeric & smg	shape changes
integrins -β1, 2 and 3 families	phospholipase activation PLA2, C, D	firm adhesion to endo/epithelium
selectins -L-selectin	increased [Ca²⁺]ᵢ	transmigration
complement -C1q, CR1, CR3-5a	phospholipid kinase act'n -PI3K	chemotaxis
antibody -FcγRII, FcγRIII	phosphorylation events -ser/thr & tyr phosph'n	phagocytosis
Others -GPI-linked e.g.: CD14 -CD45 (ligand?) -PDGF -Glucan (?)	production of ROI/NO	-secretion of granular/ vesicular contents -exposure of stored receptors
	cAMP, cGMP production	production of ROI/NO

Abbreviations: cAMP, cyclic AMP; [Ca²⁺]ᵢ, intracellular free calcium concentration; CD45, cluster of differentiation # 45; cGMP, cyclic GMP; CR1, complement receptor 1; fMLP, formyl-Met-Leu-Phe; GPI, glycosylphosphatidylinositol; IL-8, interleukin-8; LTB4, leukotriene B4; NO, nitric oxide; PAF, platelet activating factor; PDGF, platelet-derived growth factor; ROI, reactive oxygen intermediates,

Cloning of the fMLP receptor revealed it to be a member of the G protein-coupled receptor family (Boulay, et al., 1990). The more than 300 receptors of this family are widely expressed in mammalian tissues and are highly conserved from yeast to man (Neer, 1995). G protein-coupled receptors are characterized by their 7 transmembrane domain structure and their ability to initiate intracellular signalling through coupling to the heterotrimeric class of G proteins (Baldwin, 1994). This class of G proteins regulate a variety of biochemical effectors (enzymes, ion channels), so called because changes in their activity ultimately lead to cellular responses.

Heterotrimeric G proteins consist of 3 subunits (α, β and γ) and are classified according to their type of α subunit. The α subunit binds guanine nucleotides (GDP or GTP) and is often membrane-associated via lipid modifications such as myristoylation. The β and γ subunits form a stable dimer that only dissociates upon denaturation and can be regarded as a functional unit. Prenylation of the γ subunit is necessary for membrane attachment of the $\beta\gamma$ subunit. Most cells express a variety of the 20 known α subunits, 5 β subunits and 6 γ subunits. These different subunits can form heterotrimeric complexes with apparently little specificity for binding partners (Clapham and Neer, 1993).

In neutrophils, the fMLP receptor has been shown to couple to the G_{i2} heterotrimeric G protein via extensive contact between their intracellular domains (Bommakanti, et al., 1995; Schreiber, et al., 1994). This coupling event induces a conformational change in the receptor itself, increasing its affinity for ligand (see Fig. 1.4). Coupling of the fMLP receptor with its associated heterotrimeric G proteins can be blocked by pretreatment of cells with Pertussis toxin. This toxin catalyzes ADP-ribosylation of a conserved cysteine residue within G_{i2} α subunits and obliterates cellular responses to fMLP (Jiang, et al., 1996).

Binding of fMLP to its receptor converts the G_{i2} α subunit from its GDP-bound 'off' state to its GTP-bound 'on' state. This exchange facilitates release of the heterotrimeric complex from the receptor and, subsequently, the α subunit from the $\beta\gamma$ subunit. Signal transduction is then initiated by the direct interaction of both α and $\beta\gamma$ subunits with their signalling effectors and subsequent activation of downstream, indirect signalling effectors.
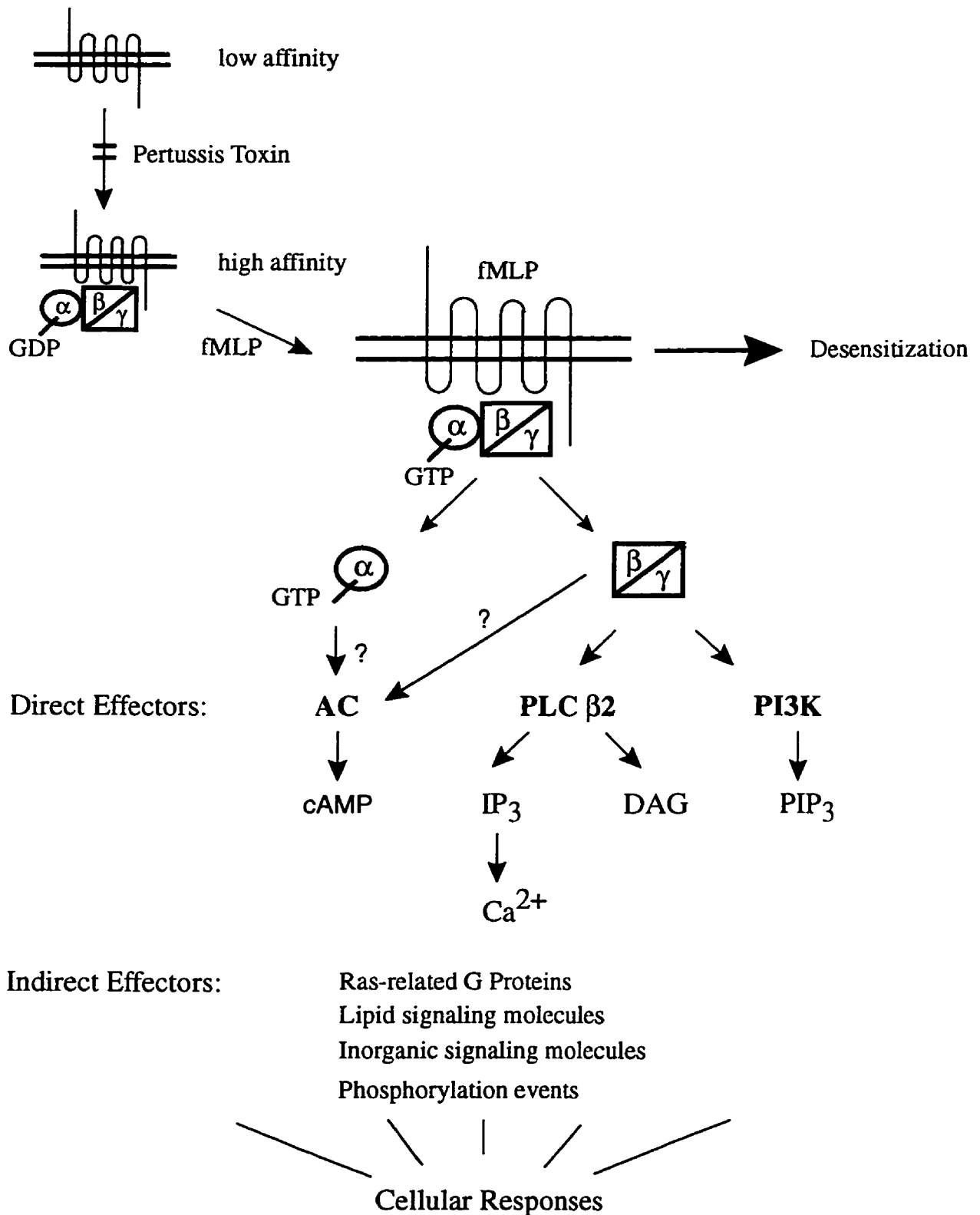
Fig. 1.4 Stimulus-response coupling through the fMLP receptor. Abbreviations: AC, adenylate cyclase; PLCβ2, phospholipase A_2; PI3K, phosphatidylinositol-3-kinase; IP_3, inositol trisphosphate; DAG, diacylglycerol; PIP_3, phosphatidylinositol trisphosphate.

Having initiated downstream signalling, the intrinsic GTPase activity of the α subunit mediates hydrolysis of bound GTP to GDP, becoming inactive. The GDP-bound α subunit is once again able to interact with the $\beta\gamma$ subunit and repeat the G protein cycle.

Inhibition of signal transduction through the fMLP receptor, a process called desensitization, occurs by a variety of mechanisms. These include segregation of the receptor within the plane of the plasma membrane to compartments where signalling is impaired, sequestration by endocytosis in clathrin-coated vesicles and down-regulation of the total number of receptors via lysosomal degradation (Klotz and Jesaitis, 1994). Some or all of these mechanisms may require prior phosphorylation of the receptor (Ali, et al., 1993) and/or covalent attachment of ubiquitin (Hicke and Riezman, 1996) to occur.

Effectors of G_{i2} activated in response to fMLP receptor signalling include adenylate cyclase (AC) (Iannone, et al., 1989). There are 10 known members of the adenylate cyclase family which are differentially regulated by both the α and $\beta\gamma$ subunits of heterotrimeric G proteins as well as by other factors (Sunahara, et al., 1996). Which adenylate cyclase isoforms are expressed in neutrophils is undetermined and the regulation of adenylate cyclase(s) may thus be through coupling to either the α, $\beta\gamma$ or both subunits.

Intracellular signalling through the fMLP receptor has also been shown to activate phospholipase $\beta2$ (PLC$\beta2$) through direct coupling with the $\beta\gamma$ subunit (Jiang, et al., 1996). Hydrolysis of phosphatidylinositol 4,5-bisphosphate (PIP$_2$) by PLC$\beta2$ leads to the generation of two important intracellular signalling molecules: inositol 1,4,5 -trisphosphate (IP$_3$) and diacylglycerol (DAG)(Snyderman and Uhing, 1992). Both molecules play important roles in mediating neutrophil responses to chemoattractant (see below).

Recent findings suggest that a novel isoform of phosphatidylinositol 3-kinase (PI3K-γ) couples to $\beta\gamma$ subunits of the fMLP receptor (Stephens, et al., 1994). While the importance of PI3K family members in fMLP receptor signalling has been well documented, the role of this novel isoform has yet to be determined. By analogy with other receptor signalling systems that utilize heterotrimeric G proteins, neutrophils are likely to express other signalling effectors that are activated upon exposure to fMLP (Clapham and Neer, 1993; Neer, 1995).

Directly activated effectors and their production of intracellular second messengers lead to the activation of other, secondary, signalling effectors throughout the neutrophil. These indirect effectors/mediators include the Ras superfamily of small G proteins, lipid signalling molecules, inorganic signalling molecules and phosphorylation events. The rest of this review will focus on what is known about these secondary signalling mechanisms downstream of the fMLP receptor.

Small G proteins of the Ras superfamily.

The Ras superfamily of GTP-binding proteins are small (20-35 kDa) polypeptides that regulate a great number of biological processes (Lowy and Willumsen, 1993). Sequence alignment of H-, K- and N-Ras reveal these proteins to be 85% identical to each other. Other members of the Ras superfamily are 50% or less identical to these G proteins (Bourne, et al., 1991). All Ras-related proteins share common structural motifs that impart their ability to bind GTP and catalyze its hydrolysis to GDP. Many Ras-related proteins undergo a number of post-translational modificactions at their C-terminus, including isoprenylation (farnesylation or geranyl-geranylation), which result in their association with target membranes and/or regulatory proteins (e.g.: the association of Rac with RhoGDI, described above).

The 50 known members of the Ras superfamily mediate their effects in a manner similar to that of heterotrimeric G proteins and the elongation and initiation factors of protein synthesis, acting as biological switches that are "on" when bound to GTP and "off" when bound to GDP. Cycling between these active and inactive states is controlled by positive and negative modulatory proteins which interact directly with the small G proteins (Boguski and McCormick, 1993). Positive modulators, called guanine nucleotide exchange factors, catalyze the dissociation of GDP and consequently allow GTP binding. Negative modulators include GTPase activating proteins (which stimulate the normally low intrinsic GTPase activity of small G proteins) and guanine nucleotide dissociation inhibitors, which bind to the GDP bound form small G proteins and prevent their activation.

The Ras superfamily of small G proteins can be divided into 6 families (Ras, Rho, Rab, Arf, Ran and Rad) based on primary sequence similarity (Quinn, 1995). These G proteins all share a similar structure, including a highly conserved catalytic domain, GTP-binding site and two 'switch' regions (Bourne, et al., 1991). The switch regions are within the nucleotide binding pocket and display different conformations depending on whether GDP or GTP is bound to the protein. In its GTP-bound conformation, the switch 1 region (amino acids 32-40) is the effector binding site which activates downstream biochemical effectors. Amino acid sequences of the switch 1 region are highly conserved within each Ras-related family but have low homology between different families (Bourne, et al., 1991). The unique structure of each G proteins' switch 1 region allows it to activate a distinct subset of biochemical effectors in its GTP-bound conformation. Examples of such effectors for the Ras and Rho families are shown in Table 1.3. A brief description of each Ras-related G protein family, their biochemical effectors and their putative roles in the regulation of neutrophil functions follows:

Ras family. Members of the widely expressed Ras family play an important role in the control of cellular growth and differentiation. Biochemical effectors that have been identified for Ras include the serine/threonine kinase Raf (see Table 1.3). Ras activation serves to translocate Raf from the cytosol to the plasma membrane where it becomes phosphorylated and activated by an unknown kinase (Stokoe, et al., 1994). Activated Raf initiates the mitogen-activated protein (Map) kinase signalling cascade, thought to be important for the regulation of many cellular processes (see below). Other Ras effectors include PI3K (Rodriguez-Viciana, et al., 1996) and PKC ζ (Diaz-Meco, et al., 1994). Activated Ras interacts with several Ras GAP's (including p120-RasGAP and neurofibromin) which may initiate downstream signalling pathways in addition to catalyzing GTP hydrolysis (Downward, 1996).

Rho family. Small G proteins of the Rho family play dynamic roles in the regulation of the actin cytoskeleton (Nobes and Hall, 1995), transcriptional control (Hill, et al., 1995) and cell cycle progression (Olson, et al., 1995). The best studied members of the Rho

Table 1.3 Biological roles of the Ras and Rho families of the Ras superfamily of small G-proteins.

| | Ras | Rho Family | | |
		Rho	Rac	Cdc42
Family members	H, N, K, R-Ras Rap-1A,B Ral-A,B TC21	Rho-A, B,C,G TC10	Rac-1,2	Cdc42Hs
Biological roles	cell growth differentiation secretion	actin polymer'n focal adhesions stress fibers transcription cell cycle chemotaxis degranulation endocytosis	actin polymer'n focal adhesions ruffling endocytosis pinocytosis oxidase act'n degranulation	actin polymer'n focal adhesions filopodia
Effectors	Raf PI3K Ral GEF PKC ζ RasGAP Rin Rsb	p160ROCK MBS citron PKN rhophilin rhotekin PIP5-kinase PLD	p160ROCK PAK65 MLK3 PI3K tubulin Myr5	p160ROCK PAK65 PI3K p120ACK WASP

Abbreviations: MBS, myosin-binding subunit; MLK3, mixed lineage kinase 3; Myr5, fifth unconventional myosin from rat; PAK65, p21-activated kinase of 65 kDa; PI3K, phosphatidylinositol 3-kinase; PIP5-kinase, phosphatidylinositol 4-phosphate 5-kinase; PKC ζ, protein kinase C ζ; PKN, protein kinase N; PLD, phospholipase D; p160ROCK, RasGap, Ras GTPase-activating protein; Rho activated coiled coil containing kinase of 160 kDa; Rin, Ras interacting protein; Rsb, Ras binding protein; WASP, Wiskott-Aldrich syndrome protein;

family are Rho, Rac and Cdc42, all of which initiate actin polymerization and focal complex formation (see Table 1.3). In addition to these functions, Rho itself appears to regulate stress fiber assembly (Ridley and Hall, 1992).

Numerous biochemical effectors mediate the actions of Rho family members on the actin cytoskeleton. They include the serine/threonine kinase p160ROCK which phosphorylates myosin light chain on Ser-19, the same residue phosphorylated by myosin light chain kinase (Kimura, et al., 1996). Activated, GTP-bound Rho may potentiate phosphorylation of this residue by binding to the myosin-binding subunit of myosin phosphatase and inhibiting its activity (Kimura, et al., 1996). Phosphorylation of myosin light chain on Ser-19 is thought to initiate myosin-actin interactions and thereby activate myosin ATPase activity. Thus, phosphorylation of myosin light chain by p160ROCK may account for, at least in part, the mechanism by which Rho regulates cell motility (Amano, et al., 1996). Another serine/threonine kinase activated upon GTP-Rho binding is protein kinase N, which is homologous to PKC within its kinase domain. The Rho-binding domain of protein kinase N is homolgous to that found in the Rho-binding proteins rhophilin and rhotekin, neither of which possess catalytic activity but may act as a structural proteins (Watanabe, et al., 1996). A 180 kDa coiled-coil containing protein (citron) with homology to p160ROCK has been isolated as a potential Rho target molecule that may act as a scaffolding protein to direct cytoskeletal rearrangements (Madaule, et al., 1995). Rho may also influence the cytoskeleton through activation of PIP5-kinase (by generation of PIP_2 which causes actin polymerization) (Chong, et al., 1994) and phospholipase D (Malcolm, et al., 1994).

Rac has been shown to regulate membrane ruffling (Ridley, et al., 1992), receptor-mediated endocytosis (Lamaze, et al., 1996) and pinocytosis (Renshaw, et al., 1996). In addition to activating p160ROCK (though Rac does not interact with the myosin binding subunit of myosin phosphatase), Rac induces the activation of other serine/threonine kinases including p21-activated kinase (PAK) (Knaus, et al., 1995) and mixed lineage kinase 3. The latter kinase initiates the stress kinase pathway via phosphorylation and stimulation of the stress-activated protein kinase activator, SEK-1 (Rana, et al., 1996). Like Ras, Rac has been

observed to bind and activate PI3K (Bokoch, et al., 1996). Interestingly, Rac activation by receptor tyrosine kinases is dependent on the activity of PI3K (Hawkins, et al., 1995), likely through the ability of phosphatidylinositol 3,4,5-trisphosphate to induce the dissociation of GDP-bound Rac from RhoGDI. Thus, the activities of Rac and PI3K may be functionally linked (Parker, 1995). Rac binding to tubulin (Best, et al., 1996) and to an unconventional myosin cloned from rat (Reinhard, et al., 1995) have also been described, possibly providing a direct link between Rac and the cytoskeleton.

Cdc42 regulates the formation of filamentous actin structures called filopodia (Nobes and Hall, 1995). Unique effectors of Cdc42 include the non-receptor tyrosine kinase p120ACK which has some similarity to focal adhesion kinase and inhibits the GTPase activity of Cdc42 (Manser, et al., 1993). Cdc42 has also been shown to bind the Wiskott-Aldrich syndrome protein which may regulate actin polymerization (Symons, et al., 1996). Cellular defects due to mutations in the Wiskott-Aldrich syndrome protein are limited to hematopoietic lineages and include cytoskeletal abnormalities of T cells, B cells and platelets (Ochs, et al., 1980).

Other Ras-related G protein families. The Rab family contains ≈30 G proteins that regulate vesicular traffic within cells (Novick and Brennwald, 1993). Also involved in the regulation of vesicular traffic is the ARF family, so named for its ability to enhance ADP-ribosylation of $G_{s\alpha}$ (Moss and Vaughan, 1995). While unique roles of the ran family have not been described in neutrophils, members of this family play an essential role in regulating protein import, mRNA export and maintenance of nuclear structures in other cell types (Gorlich and Mattaj, 1996). The recently discovered Rad family is composed of 29-35 kDa proteins which exhibit a core region with sequence homology to the guanine nucleotide-binding domains of Ras-related proteins but have extended regions at their N- and C-termini (Reynet and Kahn, 1993). The exact role of Rad family members remains unknown but may involve regulation of glucose transporters (Moyers, et al., 1996) or the control of cell proliferation (Maguire, et al., 1994).

Roles of small G proteins in regulating neutrophil functions. As Ras-related G proteins regulate many basic cellular processes (cytoskeletal rearragements, cell motility and secretion) in many different cell types, it is not surprising that these proteins play essential roles in the regulation of neutrophil responses to chemoattractants.

The cellular roles of Ras in neutrophils are not fully defined. Unlike its family member rap1A, Ras itself is not a component of the NADPH oxidase. Treatment of neutrophils with fMLP has been shown to activate Ras and initiate the Map kinase signalling pathway (Worthen, et al., 1994) but the ability of Ras to activate other downstream effectors in neutrophils has not been investigated. Microinjection of a constitutively active mutant of Ras was found to induce secretion in mast cells (Bar-Sagi and Gomperts, 1988), suggesting that Ras may also regulate secretion in other hematopoietic cells, including neutrophils.

A role for Rho in mediating neutrophil polarity and chemotaxis has been suggested by experiments using exoenzyme C3 from *Clostridium botulinum*. C3 catalyzes the ADP-ribosylation of Rho (Aktories, et al., 1989), causing cells to lose their actin stress fibers and round up (Ridley and Hall, 1992). Treatment of neutrophils with C3 was found to block chemoattractant-induced migration but had no effect on activation of the NADPH oxidase (Stasia, et al., 1991). The C3 exoenzyme has also been shown to inhibit chemoattractant-induced upregulation of surface adhesion molecules, a process that involves degranulation (Laudanna, et al., 1996). In support of this, microinjection of constitutively active Rho and Rac proteins into mast cells was found to initiate degranulation, suggesting that both Rho family members regulate secretion in hematopoietic cells (Price, et al., 1995).

In addition to its role as a component of the NADPH oxidase (see above), Rac regulates many important signalling pathways in neutrophils. These pathways include activation of the serine/threonine kinase PAK which is capable of phosphorylating $p47^{phox}$. Phosphorylation of $p47^{phox}$ by PAK occurs within a putative regulatory domain of this protein and may contribute to the regulation of NADPH oxidase assembly (Knaus, et al., 1995). Rac also initiates activation of PI3K in neutrophils, which is important in regulating many cellular processes (Bokoch, et al., 1996). In this context, it is of interest that treatment of neutrophils

with the fungal metabolite wortmannin (a potent and specific inhibitor of PI3K) induces periodic oscillations in actin assembly/disassembly that parallel changes in NADPH oxidase activity, suggesting that both events are coordinately regulated by Rac (Wymann, et al., 1989).

Neutrophils from patients with Wiskott-Aldrich syndrome display impaired chemotaxis (Ochs, et al., 1980). This suggests that the Wiskott-Aldrich syndrome protein is an important effector for activated Cdc42 in these cells.

The ability of Rab proteins to initiate leukocyte secretion is indicated by the finding that a peptide derived from Rab was able to stimulate mast-cell degranulation (Oberhauser, et al., 1992). Phospholipase D is an important effector for arf that may play a role in the regulation of vesicular traffic (Cockcroft, 1996). Arf-regulated phospholipase D has been demonstrated in neutrophils (Lambeth, et al., 1995) and is thought to mediate the activation of various responses to chemoattractants (see below). The roles of ran and Rad family G proteins in regulating neutrophil function await further definition.

Lipid signalling molecules.

Activation of the fMLP receptor leads to the generation of a number of lipid signalling molecules (LSM). These lipids can have biological effects on their own or can initiate further downstream signalling by the activation of other biochemical effectors. LSM are derived from both glycerolipid (e.g. PIP_2, phosphatidylcholine) and sphingolipid (e.g. sphingomyelin) components of cellular membranes (Liscovitch and Cantley, 1994). An overview of the LSM produced by chemoattractant-stimulated neutrophils is shown in Fig.1.5. The generation of LSM from both lipid classes, their cellular effects/effectors and their role in neutrophil signalling are briefly discussed below.

Phospholipase C. Central to the lipid signalling pathways initiated by the fMLP receptor is the hydrolysis of PIP_2 by PLC (see Fig. 1.5). Hydrolysis of PIP_2 is very rapid (within seconds) and generates two important signalling molecules, inositol 1,4,5 -trisphosphate (IP_3) and diacylglycerol (DAG). Soluble IP_3 diffuses throughout the cytosol
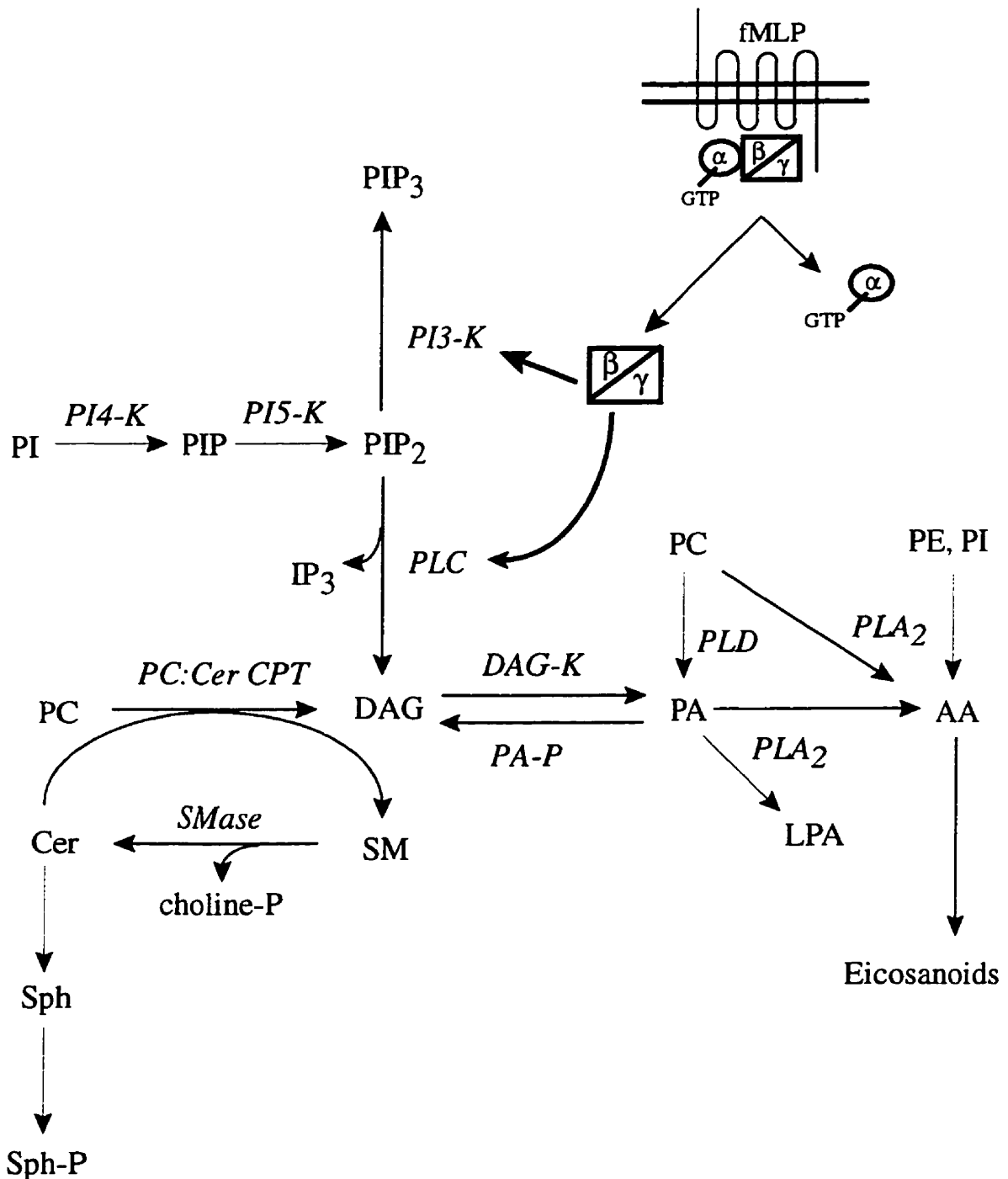
Fig. 1.5.   Lipid signalling molecules produced by neutrophils in response to the chemoattractant fMLP. Abbreviations: AA, arachidonic acid;   DAG, diacylglycerol; DAG-K, diacylglycerol kinase;   Cer, ceramide;   IP$_3$, inositol 1,4,5-trisphosphate;   PA, phosphatidic acid;   PA-P, phosphatidic acid phosphohydrolase;   PC, phosphatidylcholine; PE, phosphatidylethanolamine;   PI, phosphatidylinositol;   PLA$_2$, phospholipase A$_2$; PLC, phospholipase C;   PLD, phospholipase D.

while DAG remains associated with the plasma membrane. Binding of IP3 to receptors present on an internal Ca^{2+} storage compartment leads to increased cytosolic concentrations of Ca^{2+} (see below). Elevated intracellular Ca^{2+} has many cellular effectors including the co-activation, together with DAG, of conventional isoforms of PKC. The many roles of PKC isoforms in regulating neutrophil function has highlighted the importance of PLC activation and the production of DAG.

Three classes of PLC have been described: the β family which is activated by interaction with $\beta\gamma$ subunits of heterotrimeric G proteins, the γ class which is regulated by tyrosine phosphorylation and the δ class, the regulation of which is currently undefined (Katan, 1996). In addition to the $\beta2$ isoform of PLC, neutrophils also contain PLC $\gamma2$. Expression of this γ isoform is thought to be important for immunoglobulin receptor (FcγRII and FcγRIII) and integrin ($\beta2$) signalling, as tyrosine kinase antagonists are capable of inhibiting phosphoinositide hydrolysis initiated by activation of these receptors (Dusi, et al., 1994; Hellberg, et al., 1996). By contrast, these pharmacologic agents have no effect on fMLP-mediated phosphoinositide hydrolysis and intracellular Ca^{2+} transients (Uings, et al., 1992). These findings suggest that PLC $\gamma2$ does not play a role in PIP2 hydrolysis initiated by the fMLP receptor. The expression of δ isoforms of PLC in neutrophils has not been documented.

PI3K. Also acting on the lipid precursor PIP2 is PI3K, which preferentially phosphorylates this phosphoinositide at the D-3 position to generate phosphatidylinositol (3,4,5)-trisphosphate (PIP3) (Traynor-Kaplan, et al., 1989). Approximately 20% of the decrease in PIP2 following fMLP stimulation is due to its conversion to PIP3 (Stephens, et al., 1991). Like PLC hydrolysis of PIP2, production of PIP3 is rapid (maximal within 15 seconds) and transient, correlating with a number of rapid responses of neutrophils (Stephens, et al., 1991).

The essential role of PI3K in mediating neutrophil responses to chemoattractant has been elucidated with the use of wortmannin, a potent and relatively specific inhibitor of this enzyme (Ui, et al., 1995). Wortmannin treatment of cells blocks production of PIP3 (Arcaro

and Wymann, 1993) and consequently inhibits many neutrophil responses to chemoattractant, including degranulation (Dewald, et al., 1988), phagocytosis (Ninomiya, et al., 1994) and activation of the NADPH oxidase (Arcaro and Wymann, 1993).

While cellular targets of PIP3 remain largely unknown, they likely include PKC. The lipid products of PI3K have been shown to activate members of both the atypical and novel families of PKC (Moriya, et al., 1996; Nakanishi, et al., 1993; Toker, et al., 1994). As members of both PKC familes are expressed in neutrophils (see below) it is likely that these serine/threonine kinases play a role in fMLP-mediated signalling processes.

Neutrophils possess two types of PI3K: a newly described enzyme (PI3Kγ) that is activated by direct interaction with the βγ-subunits of heterotrimeric G proteins (Stephens, et al., 1994) and the conventional heterodimeric form that consists of a p85 regulatory subunit and a p110 catalytic subunit (Fry and Waterfield, 1993). The latter enzyme can be activated by a number of mechanisms in various cell types. These include interaction with tyrosine phosphorylated growth factor receptors (Divecha and Irvine, 1995), binding to Src family tyrosine kinases (Pleiman, et al., 1994) and interaction with small G proteins of the Ras superfamily (Rodriguez-Viciana, et al., 1996).

The contributions of each PI3K family to chemoattractant signalling remains controversial. Because the fMLP receptor is known to couple to G proteins, one might expect the βγ-regulated form to predominate in chemoattractant-treated cells. Nevertheless, many of the responses of neutrophils to fMLP that are blocked by wortmannin are also blocked by tyrosine kinase antagonists (Berkow, et al., 1989; Kobayashi, et al., 1995), suggesting that activation of PI3K may require phosphotyrosine accumulation. Indeed, a PI3K activity is associated with immunoprecipitates of a tyrosine kinase, Lyn, from chemoattractant stimulated neutrophils. This PI3K activity is upregulated in a manner that correlates temporally with the very rapid activation/deactivation of Lyn (Ptasznik, et al., 1995). Moreover, the tyrosine kinase inhibitors genistein and radicicol abolished PIP3 production in response to fMLP, indicating that the tyrosine kinase signalling pathway accounts for the majority of this lipid signalling molecule (Ptasznik, et al., 1996). In conflict with these findings, other groups have

found little PI3K activity associated with tyrosine phosphorylated proteins following fMLP stimulation (Stephens, et al., 1993; Vlahos and Matter, 1992). While the contribution of each PI3K family remains controversial, the essential nature of PIP3 production to neutrophil responses has become well established.

Phospholipase D. Activation of the fMLP receptor leads to a biphasic accumulation of DAG (Reibman, et al., 1988), reflective of the fact that two distinct sources are utilized for its production (see Fig. 1.5). The first pathway involves the rapid hydrolysis of PIP2 by the β2 isoform of PLC, described above. Accumulation of DAG derived from PIP2 is transient due to its rapid metabolism by a number of mechanisms, including its conversion to phosphatidic acid (PA) by diacylglycerol kinase (Kanoh, et al., 1990). Inhibition of this metabolic pathway is accomplished with pharmacologic agents such as R59022 and dioctanoylethylene glycol. These agents have been shown to increase DAG levels following fMLP stimulation and potentiate activation of the NADPH oxidase, possibly via an enhanced activation of DAG-dependent PKC isoforms (Mege, et al., 1988).

Following PLC β2-mediated hydrolysis of PIP2, DAG is generated by a second mechanism termed the phospholipase D (PLD) pathway (Olson and Lambeth, 1996). This pathway involves the sequential actions of PLD and phosphatidic acid (PA) phosphohydrolase. PLD catalyzes the hydrolysis of phosphatidylcholine to form PA which is then dephosphorylated to DAG by the phosphohydrolase (see Fig.1.5). In contrast to its very rapid and transient production by PLC, DAG produced by the PLD pathway is sustained, keeping DAG levels elevated for periods greater than 1 hr.

Evidence suggests that PLD pathway derived products are important for the regulation of neutrophil responses to chemoattractant. Indeed, the PLD pathway is thought to be responsible for the majority of DAG produced following fMLP production (Billah, et al., 1989). Inhibition of PLD by treatment of cells with primary alcohols (eg: ethanol and butanol) has demonstrated the importance of this lipase to DAG production. These agents cause PLD to catalyze a transphosphatidylation reaction, whereby the alcohol replaces water in the hydrolysis

of phosphatidylcholine and subsequently blocks generation of PA. Treatment of neutrophils with primary alcohols inhibits many cellular responses to fMLP, including degranulation (Suchard, et al., 1994) and activation of the NADPH oxidase (Bonser, et al., 1989). Recent studies have also implicated PLD in phagocytosis mediated by complement receptors (Fallman, et al., 1992).

While DAG production is thought to modulate neutrophil functions via its activation of DAG-dependent PKC isoforms, several lines of evidence now suggest that PA is also an important signalling molecule in these cells (English, et al., 1996). First, levels of PA have been found to correlate within the onset of the NADPH oxidase activity (Korchak, et al., 1988). Second, inhibition of PA phosphohydrolase with propranolol has been shown to potentiate oxidase activity by increasing PA levels in response to fMLP (Rossi, et al., 1990). Finally, PA activates NADPH oxidase activity in a cell free, reconstitution assay (Agwu, et al., 1991). This effect of PA on O_2^- production was synergistic with added DAG, suggesting that both PLD products have distinct effectors that mediate activation of the NADPH oxidase complex (Qualliotine-Mann, et al., 1993).

A number of potential targets of PA have been identified that may mediate its actions in neutrophils (English, et al., 1996). These include: protein kinase C (Limatola, et al., 1994), other serine/threonine kinases (Ghosh, et al., 1996; McPhail, et al., 1995), phosphatidylinositol-4-phosphate 5-kinase (Moritz, et al., 1992) and phospholipase C (Jones and Carpenter, 1993). In addition, PA causes RhoGDI dissociation from Rac, possibly providing a mechanism for assembly and activation of the NADPH oxidase (Chuang, et al., 1993).

Regulation of PLD in neutrophils has been hampered by the fact that it has not been cloned in these cells and the reagents needed for its thorough study are unavailable. Complicating matters is the finding that neutrophils express at least two biochemically distinct PLD activities, present in different intracellular locations (Balsinde, et al., 1989). Because of these limitations, studies of PLD have provided a very complex and often conflicting picture of its regulation (Olson and Lambeth, 1996). Nonetheless, the use of pharmacologic and cell-free

systems (in which isolated membranes and cytosol are used to reconstitute PLD activity *in vitro*) have provided insight into the regulation of this important class of signalling molecule.

Many studies have demonstrated a Ca^{2+} requirement for PLD. Blocking fMLP-induced Ca^{2+} transients in whole cells by its omission from the extracellular medium and loading cells with the Ca^{2+}-chelating agent Quin 2 was found to block accumulation of PA (Gelas, et al., 1992). Similarily, electropermeabilized neutrophils stimulated in the absence of Ca^{2+} do not activate PLD, further supporting a requirement for this cation (Dennis, 1994). That Ca^{2+} alone was not sufficient to fully activate PLD activity in this study suggests that other regulatory mechanisms govern its activity.

Several lines of evidence implicate tyrosine phosphorylation as a possible regulatory mechanism for PLD. Treatment of cells with a potent tyrosine phosphatase inhibitor, vanadate, initiates phosphotyrosine accumulation in conjunction with activation of PLD (Bourgoin and Grinstein, 1992). Inhibiton of phosphotyrosine accumulation induced by vanadate (Bourgoin and Grinstein, 1992) or fMLP (Uings, et al., 1992) by prior incubation with tyrosine kinase inhibitors was found to block PLD activation. Following fMLP stimulation, PLD activity is associated with tyrosine phosphorylated proteins isolated by immunoprecipitation, indicating that direct phosphorylation of this phospholipase (or an associated regulatory protein) may regulate its activity (Gomez-Cambronero, 1995). Activation of PI3K by a tyrosine kinase-dependent mechanism may also regulate PLD activity, as wortmannin has been shown to inhibit elevation of PA levels following fMLP stimulation (Gelas, et al., 1992).

Phosphorylation of proteins on serine/threonine residues by PKC may also serve to regulate the activity of PLD in neutrophils. This is suggested by activation of DAG-dependent isoforms of this kinase by treatment of cells with phorbol esters such as 12-*O*-tetradecanoylphorbol 13-acetate (TPA). TPA activates PLD activity in intact neutrophils (Truett, et al., 1989) and in a cell free system (Lopez, et al., 1995). Cell-free activation of PLD was dependent on ATP and the phosphorylation of a membrane component, perhaps PLD itself (Lopez, et al., 1995). By contrast, an ATP-independent activation by PKC has also been

found to regulate PLD isolated from chinese hamster lung fibroblasts, though such a mechanism of PLD regulation has not been described in neutrophils (Conricode, et al., 1992).

Cell-free assays have demonstrated that PLD is activated by interaction with two small G proteins of the Ras superfamily, Rho and arf. Both G proteins activate PLD synergistically in the presence of an unidentified 50 kDa protein component of the cytosol (Bowman, et al., 1993; Kwak, et al., 1995; Lambeth, et al., 1995). In analogy with other systems, PLD may also be regulated by interaction with gelsolin, an actin-binding protein (Hwang, et al., 1996) and other unknown cytosolic proteins (Wright, et al., 1990).

Cloning of the first mammalian PLD isofom has recently been achieved (Hammond, et al., 1995). This enzyme displays many properties similar to those described in neutrophils, including its selectivity for phosphatidylcholine as a substrate and its activation by the small G protein arf. Molecular cloning of PLD isoforms in neutrophils should facilitate further study of this important signalling molecule.

Phospholipase A$_2$.   Upon stimulation, neutrophils release arachidonic acid (AA) and other fatty acids from cellular membranes. This is accomplished by phospholipase A$_2$ (PLA$_2$)-mediated hydrolysis of phospholipids at their sn-2 position. Production of AA can occur by a number of important pathways (see Fig. 1.5). These include: 1) hydrolysis of phosphatidylcholine, the preferred PLA$_2$ substrate, with concomitant production of lyso-platelet activating factor (a precursor of platelet activating factor, a potent inflammatory mediator), ii) hydrolysis of phosphatidylethanolamine and/or phosphatidylinositol, generating AA and a corresponding lysophospholipid and iii) hydrolysis of PA, producing AA and lysoPA. LysoPA has important signalling properties both within cells and extracellularly where it can activate cell surface receptors of the G protein-coupled receptor superfamily (Moolenaar, 1994).

While AA was originally thought to function mainly as a precursor of inflammatory mediators such as prostaglandins and leukotrienes (collectively termed eicosanoids), evidence suggests that this lipid may also serve itself as an intracellular signalling molecule (Cockcroft,

1992). AA generation is very rapid following fMLP stimulation and correlates with NADPH oxidase activity. Pharmacologic inhibitors of PLA_2 have been shown to block O_2^- production (Dana, et al., 1994; Henderson, et al., 1989). Interestingly, these inhibitors had no effect on the phosphorylation of $p47^{phox}$ and $p67^{phox}$ nor their translocation to the plasma membrane, suggesting that the target(s) of AA action may be subsequent to these events (Dana, et al., 1994). Indeed, direct activation of the NADPH oxidase in a cell-free system by addition of AA has been described (Steinbeck, et al., 1991). In addition to a role in regulating oxidase activity, *in vitro* studies suggest that PLA_2 may also promote degranulation (Blackwood, et al., 1996).

Many intracellular targets of AA have been described in a variety of cell types (Liscovitch and Cantley, 1994). These include isoforms of PKC (Shinomura, et al., 1991), the γ and δ isoforms of PLC (Hwang, et al., 1996) and sphingomyelinase (Jayadev, et al., 1994). Other fatty acids generated by PLA_2 may also activate other, unidentified serine/threonine kinases (Khan, et al., 1994).

Neutrophils, like other cells, express at least two biochemically distinct types of PLA_2 (Dennis, 1994). The first is a small (14 kDa), secretory PLA_2 that displays a high Ca^{2+} requirement (mM) and little specificity for fatty acid cleaved at the sn-2 position of its substrates. This PLA_2 is located within secretory granules (Rosenthal, et al., 1995) and plays an important role in bacterial killing (Wright, et al., 1990). Secretory PLA_2 appears to be the predominant source of AA-derived eicosanoids, which are released from the cell and serve to promote the inflammatory response (Barbour and Dennis, 1993; Marshall, et al., 1994).

A second type of PLA_2 ($cPLA_2$) plays an important role in mediating signal transduction within these cells (Clark, et al., 1991). This enzyme has a molecular mass of 85 kDa and is present in the cytosol of unstimulated cells. In response to increased cytosolic Ca^{2+} concentrations (μM), $cPLA_2$ translocates to the plasmalemma (Durstin, et al., 1994) and the nuclear membrane (Pouliot, et al., 1996), initiating AA release from these membranes. The catalytic activity of $cPLA_2$ is also regulated by phosphorylation by Map kinase (Durstin, et al., 1994).

Sphingomyelinase and ceramide signalling. Initially considered to be an inert constituent of cellular membranes, sphingomyelin has recently gained recognition as an important precursor for a number of signalling molecules in a variety of cell types (Hannun, 1994). The so-called 'sphingomyelin cycle' is initiated with its hydrolysis by a sphingomyelinase, releasing phosphocholine and ceramide. While ceramide has many important intracellular effectors itself (see below), it also gives rise to other important metabolites. First, ceramide can be de-acylated to form sphingosine. Both sphingosine and its phosphorylated product have important signalling functions within most cell types studied. Alternatively, ceramide can be utilized to regenerate sphingomyelin via the actions of phosphatidylcholine:ceramide cholinephosphotransferase. This reaction generates DAG, linking the sphingomyelin cycle directly to glycerophospholipid metabolism.

Intermediates of the sphingomyelin cycle activate a number of important signalling molecules. Ceramide has been shown to activate serine/threonine kinases, including Raf (Huwiler, et al., 1996), PKC ζ (Lozano, et al., 1994) and a novel renaturable kinase, ceramide-activated protein kinase (Liu, et al., 1994). In addition, ceramide regulates phosphorylation via activation of a serine/threonine phosphatase (Dobrowsky and Hannun, 1993). Interestingly, the structure of bacterial lipopolysaccharide (an important inflammatory mediator) has been found to closely resemble that of naturally occurring ceramides. Treatment of neutrophils with lipopolysaccharide activates ceramide-activated protein kinase (Joseph, et al., 1994). Thus, in addition to its receptor-driven signalling effects, lipopolysaccharide may also induce cellular effects by mimicking ceramide and activating ceramide-dependent sigalling pathways (Wright and Kolesnick, 1995). Sphingosine and its phosphorylated product likely have important signalling roles, including inhibition of PKC by the former lipid (Hannun, et al., 1986).

The roles of sphingolipids in regulating neutrophil responses to infection are currently undefined. In response to fMLP, ceramide elevation correlates with termination of O_2^- production by the NADPH oxidase (Nakamura, et al., 1994). Treatment of cells with soluble, synthetic analogs of ceramide (C2-ceramide) inhibited activation of the oxidase, further

supporting a role for ceramide as a negative modulator of this response (Wong, et al., 1995). A similar inhibitory effect of ceramide has been suggested to mediate the lag time witnessed following exposure of adherent neutrophils to the cytokine tumour necrosis factor prior to induction of cell spreading and activation of the NADPH oxidase (Fuortes, et al., 1996). In contrast to the inhibitory effects of ceramide elevation, sphingosine may serve to potentiate cellular responses. Sphingosine levels decrease following exposure of cells to stimulatory agents, including fMLP, and may diminute the inhibitory effect of sphingosine over PKC (Wilson, et al., 1988).

Inorganic signalling molecules.

Thus far, I have discussed how changes in the levels of certain organic molecules (eg: phospholipids) or their conformation (eg: small G proteins) can influence neutrophil functions. I will now focus attention on the ability of inorganic signalling molecules to regulate cellular responses to inflammatory stimuli.

Calcium. Ionized calcium (Ca^{2+}) is the most common signalling element in cells, acting on a number of intracellular systems (Clapham, 1995). Following exposure of neutrophils to chemoattractant, an elevation of the intracellular free Ca^{2+} concentration ($[Ca^{2+}]_i$) is one of the earliest events observed. Increased levels of $[Ca^{2+}]_i$ are achieved by two separate mechanisms (Krause, et al., 1990). The first is a rapid release of Ca^{2+} from an internal storage compartment under the control of IP_3-gated Ca^{2+} channels. Following PLC activation, IP_3 production and diffusion throughout the cytosol leads to the release of Ca^{2+} from this compartment. The second mechanism whereby $[Ca^{2+}]_i$ is elevated is Ca^{2+} influx through the plasma membrane (Demaurex, et al., 1994). Activation of the influx pathway occurs through depletion of intracellular stores, which causes the latter to release an unidentified, putative signalling molecule called the "calcium influx factor" (Clapham, 1993). Calcium influx factor activates a Ca^{2+} channel present on the plasma membrane by an unknown mechanism. The effect of depletion of the endomembrane stores on Ca^{2+} influx can

be mimicked by treatment of cells with thapsigargin, an inhibitor of the Ca^{2+}-ATPase present on these organelles (Thastrup, et al., 1990).

Much evidence has supported a role for Ca^{2+} in regulating neutrophil antimicrobial responses, though the exact mechanisms by which this occurs are unclear. Cell movement on surfaces coated with extracellular matrix proteins (fibronectin, vitronectin) appears to be strictly dependent on intracellular Ca^{2+} (Maxfield, 1993). Fluorescence microscopy imaging of neutrophils placed within an fMLP gradient has demonstrated a correlation between $[Ca^{2+}]_i$ and the speed of these cells as they undergo chemotaxis (Mandeville, et al., 1995). When $[Ca^{2+}]_i$ transients are inhibited in these cells, they cease to crawl due to an inability of the receptors required for chemotaxis to recycle to the front of the cell (Lawson and Maxfield, 1995).

Elevating $[Ca^{2+}]_i$ by treatment of cells with Ca^{2+} ionophores (such as A23187 or ionomycin), in the presence of extracellular Ca^{2+}, suffices to initiate the release of granular contents (Wright, et al., 1977). By varying the extracellular concentration of Ca^{2+}, experiments of this nature have determined the $[Ca^{2+}]_i$ requirement for degranulation of each of the four types of secretory organelles present in neutrophils. A rank order of sensitivity to $[Ca^{2+}]_i$ was obseved: secretory vesicles are the most Ca^{2+}-sensitive, followed by tertiary granules, secondary granules and primary granules (Sengelov, et al., 1993). While $[Ca^{2+}]_i$ elevation is clearly sufficient to induce degranulation under these conditions, the absolute necessity of this inorganic signalling molecule has been questioned. For instance, TPA is an effective secretagogue of some types of granules (Wright, et al., 1977) but has no effect on $[Ca^{2+}]_i$ (Korchak, et al., 1984). Thus, Ca^{2+}-dependent and independent mechanisms are likely to influence the secretory response of neutrophils.

The role of Ca^{2+} in regulating activation of the NADPH oxidase is controversial. Increased $[Ca^{2+}]_i$ correlates temporally with the onset of oxidase activation (Korchak, et al., 1984) and elevating $[Ca^{2+}]_i$ by treatment of cells with Ca^{2+} ionophores suffices to initiate O_2^- production (Pozzan, et al., 1983). In addition, lowering basal $[Ca^{2+}]_i$ and diminishing the fMLP-induced elevation of $[Ca^{2+}]_i$ by loading cells with intracellular Ca^{2+} chelators causes

inhibition of O_2^- production (Lew, et al., 1984). These findings all suggest an important role for $[Ca^{2+}]_i$ in the regulation of the NADPH oxidase.

In conflict with these findings, very low (chemotactic) concentrations of fMLP elicit the same increases in $[Ca^{2+}]_i$ as do higher concentrations of the chemoattractant but do not activate the NADPH oxidase (Korchak, et al., 1984). In addition, elevation of $[Ca^{2+}]_i$ by some agonists does not elicit activation of the oxidase (Apfeldorf, et al., 1985). The use of electropermeabilized neutrophils has been used to further characterize the Ca^{2+} requirement of O_2^- production by these cells (Grinstein and Furuya, 1988). With this system, cells are rendered permeable to small (<700 Da) molecules by electric discharges. This allows effective buffering of intracellular Ca^{2+} to a desired concentration, by suspending cells in solutions containing buffering agents and by depleting intracellular stores with Ca^{2+} ionophores. Under these conditions, fMLP was found to induce O_2^- production at a normal rate when $[Ca^{2+}]_i$ was buffered to basal values of 100 nM. By contrast, lowering $[Ca^{2+}]_i$ below 10 nM had an inhibitory effect, indicating that a minimal requirement for this cation existed. Interestingly, TPA-induced activation was not inhibited by lowering $[Ca^{2+}]_i$. It was concluded that activation of the NADPH oxidase, like initiation of degranulation, is regulated by both Ca^{2+}-dependent and -independent mechanisms. Thus, $[Ca^{2+}]_i$ elevation contributes to the activation of many antimicrobial responses by neutrophils but is not the sole mechanism for their regulation.

Nitric Oxide. Nitric oxide (NO) production has been described in a variety of cell types and NO has been recognized as an important mediator of various cellular processes. These include regulation of vascular tone, neurotransmission and cell-mediated immune responses. NO is produced by two types of NO synthase: one regulated by inducible expression and the other dependent on Ca^{2+}/calmodulin for activity. In the presence of molecular O_2 and required cofactors (NADPH, FAD and FMN), these enzymes catalyze the conversion of L-arginine to citrulline, yielding NO (Nathan and Xie, 1994).

NO is a highly reactive molecule with the ability to modify sulfhydryl groups, react with the metal centers of heme groups and covalently modify proteins on tyrosine residues. As a consequence, many intracellular targets for NO have been identified (Stamler, 1994). Thus, in addition to its established role in microbial killing (Malawista, et al., 1992), NO is likely to play an important signalling role in neutrophils.

Following exposure of neutrophils to chemoattractant, these cells produce NO at an approximate rate of 2-20 nmol/min/10^6 cells (Wright, et al., 1989). At low (10-100nM) concentrations, NO has a potentiating effect *in vitro* on many cellular responses, including adhesion to endothelial surfaces, chemotaxis (Van Uffelen, et al., 1996) and activation of the NADPH oxidase (Morikawa, et al., 1995). By contrast, higher concentrations of NO (5 μM) have inhibitory effects on these responses. Thus, low level NO production by neutrophils may serve to potentiate neutrophil migration to sites of inflammation and antimicrobial activities. To limit damage to surrounding tissues, higher rates of NO production by the vascular endothelium (Beckman, et al., 1990) and other cell types at sites of inflammation may serve to downregulate acute inflammatory reactions produced by neutrophils. In accord with this model for NO action, inhibition of endothelial NO production by venous perfusion with NO synthase inhibitors (eg: L-monomethyl-arginine) was found to increase both neutrophil attachment to vascular endothelium and emigration to extravascular tissues (Kubes, et al., 1991).

Reactive oxygen intermediates: In addition to their microbicidal role in phagocytes, ROI have been suggested to act as signalling molecules in other cells (Schreck and Baeuerle, 1991). Virtually all eukaryotic cells produce ROI, primarily as side products of electron transfer reactions in mitochondria and the endoplasmic reticulum (Halliwell and Gutteridge, 1985). In principle, ROI constitute good candidate signalling molecules since they are small, rapidly diffusible and highly reactive. Moreover, both intra- and extracellular concentrations of ROI can be rapidly scavenged by several enzymes, including superoxide dismutase, catalase and the glutathione peroxidase system, allowing tight control of ROI concentrations and rapid termination of signals.

Little is known about the role of ROI in signalling, but suggested targets include the transcription factor NFκB (Schreck, et al., 1991), tyrosine phosphatases (Fialkow, et al., 1994; Hecht and Zick, 1992), phospholipase A_2 (Zor, et al., 1993) and Ras (Lander, et al., 1995). While neutrophils are probably the most proficient source of ROI through activation of the NADPH oxidase, little is known about the potential role of these molecules in mediating signal transduction. The importance of ROI-dependent signalling mechanisms is suggested by previous findings which demonstrated a role for these molecules in potentiating a variety of important neutrophil antimicrobial responses, including upregulation of adhesion receptors (Simms and D'Amico, 1995), adhesion to endothelial cells (Fraticelli, et al., 1996), phagocytosis (Gresham, et al., 1988) and apoptosis (Watson, et al., 1996).

Phosphorylation events.

Protein phosphorylation is recognized as an important post-translational modification in the regulation of many cellular processes (Krebs, 1994). Phosphorylation can induce a variety of effects on a given protein, including changes in conformation, subcellular distribution and enzymatic activity. Transfer of phosphate from ATP to the side chains of target proteins is mediated by kinases, which were initially grouped into two classes based on the amino acids that they phosphorylated on their substrates: serine/threonine kinases and tyrosine kinases. More recently, dual specificity kinases have been described. In addition, phosphorylation of mammalian proteins on histidine and aspartate residues has also been demonstrated in mammals, though the relevance of these modifications are largely unknown (Crovello, et al., 1995).

Many lines of evidence suggest that protein phosphorylation is also important for initiation of antimicrobial responses in neutrophils: i) activation of many responses is dependent on cytosolic ATP (Grinstein and Furuya, 1988), ii) pharmacological inhibitors of protein kinases block these responses (Berkow, et al., 1989; Lambeth, 1988), iii) phosphorylation of many proteins is witnessed upon treatment with inflammatory stimuli, concomitant with cellular activation (Andrews and Babior, 1983) and iv) phosphorylation of

many effector proteins is required for their activity (eg: phosphorylation of $p47^{phox}$, see above). The enzymes that are thought to influence phosphorylation-dependent signalling events in neutrophils are briefly summarized below.

Protein kinase C   The PKC family of serine/threonine kinases are widely expressed and serve to transduce signals which promote lipid hydrolysis (Hug and Sarre, 1993). Members of this family are a single polypeptide, comprised of an N-terminal regulatory domain and a C-terminal catalytic domain. A bisecting hinge region allows folding of the enzyme and interaction between these two domains under quiescent conditions. In this conformation, catalytic activity is impaired by interaction of an N-terminal domain mimicking the consensus sequence motif of PKC substrates (the pseudosubstrate domain) with the catalytic core of the protein. Displacement of the pseudosubstrate domain from the catalytic core is an absolute requirement for activation of kinase activity and is a characteristic regulatory feature of all PKC family members (Newton, 1995).

To date, 11 PKC isoforms have been identified and are classified into 3 families based on their structure and cofactor requirements (Nishizuka, 1995). The first to be characterized were members of the conventional PKC family, including the α, βI, βII and γ isoforms. Cloning of the cPKC family revealed four conserved domains (C1-C4) which act as functional modules: the C1 domain contains 1-2 cysteine-rich motifs that bind DAG or phorbol esters such as TPA; the C2 domain binds acidic phospholipids (such as phosphatidylserine) in a Ca^{2+}-dependent manner; and the C3 and C4 domains which form the ATP and substrate-binding lobes of the kinase domain, respectively.

Cellular activation and generation of ligands for C1 and C2 domains serves to target cytosolic cPKC isoforms to the plasma membrane. Membrane binding causes the unfolding of these enzymes, removal of pseudosubstrate from the catalytic core and subsequent activation of kinase activity. Concomitant production of various LSM can also have an enhancing (eg: AA and other free fatty acids (Khan, et al., 1995)) or inhibitory (eg: sphingosine) effect on the activity of PKC isoforms (see table 1.4). Membrane-bound PKC isoforms are proteolytically

Table 1.4   Regulation of PKC family members.

| Family | Cofactor / Activator | | | | | | |
	PS	DAG	Ca^{2+}	AA/FFA	PA	PIP_3	Other
cPKC	+	+	+	+	+	-	Sph (-)
nPKC	+	+	-	+	+	+	Tyr-P (+)
aPKC	+	-	-	+	+	+	

Abbreviations: AA, arachidonic acid;   aPKC, atypical PKC;   cPKC, conventional PKC; DAG, diacylglycerol;   FFA, free fatty acids;   nPKC, novel PKC;   PA, phosphatidic acid; PIP3, phosphatidylinositol 3,4,5-phosphate;   PS, phosphatidylserine;   Sph, sphingosine;   Tyr-P, tyrosinephosphorylation.

labile within their hinge region. Proteolysis at this site causes the release of a constitutively active product (protein kinase M) which can phosphorylate targets throughout the cell.

While cPKC are dependent on elevated Ca^{2+} for activation, the two other PKC families lack a functional C2 domain and are thus unaffected by Ca^{2+} levels. First, the novel family of PKC (nPKC) is comprised of the δ, ϵ, η, θ and μ isoforms. In addition to their regulation by DAG, members of the nPKC family are also activated by other lipids including PA and PIP3 *in vitro* (Moriya, et al., 1996; Toker, et al., 1994). The finding that PKC δ can associate with PI3K suggests that these lipids are important regulatory factors over PKC activity following cellular activation (Ettinger, et al., 1996). Further regulation of nPKC isoforms may come from direct tyrosine phosphorylation, as has been described for PKC δ (Haleem-Smith, et al., 1995; Li, et al., 1994).

The least understood members of the PKC superfamily are the atypical kinases (aPKC), comprised of the ζ and λ isoforms. Members of this family lack both C1 and C2 domains and, as a consequence, are insensitive to both DAG and intracellular Ca^{2+}. Like their nPKC counterparts, aPKC are activated by LSM including PIP3 (Nakanishi, et al., 1993) and free fatty acids, including AA (Nakanishi and Exton, 1992). In addition, PA has been shown to be an important activator of PKCζ, binding tightly to this isoform following cellular activation (Limatola, et al., 1994).

Neutrophils have been found to express the α,βI and βII members of the cPKC family, while γ is not detectable (Smallwood and Malawista, 1992). Members of the nPKC (δ) and aPKC (ζ) are also represented in these cells (Dang, et al., 1994; Kent, et al., 1996). Following treatment with fMLP, all PKC isoforms are rapidly activated and translocate from the cytosol to the plasma membrane (Dang, et al., 1995; Deli, et al., 1987; Kent, et al., 1996; Wolfson, et al., 1985). The functional relevance of PKC is suggested by the fact that several components of the antimicrobial reaction can be initiated by direct activation of PKC with phorbol esters (Tauber, 1987) and by the finding that pharmacologic inhibition of PKC blocks many of the cellular responses induced by fMLP (Heyworth and Badwey, 1990; Lambeth, 1988;

Verhoeven, et al., 1993). Despite the importance of PKC in regulating neutrophil function, the substrates of this family of kinases are ill defined.

Substrates of PKC. The myristoylated, alanine-rich C kinase substrate (MARCKS) is a major PKC substrate in many different cell types, including neutrophils (Blackshear, 1993). In its unphosphorylated state, MARCKS binds to the plasma membrane via hydrophobic insertion of its myristate chain into the lipid bilayer and via electrostatic interactions of its cluster of basic residues with acidic phospholipids (Swierczynski and Blackshear, 1995; Swierczynski and Blackshear, 1996). Membrane associated MARCKS has several important functions, including the ability to bind and crosslink actin (Hartwig, et al., 1992), binding to calmodulin (Graff, et al., 1989) and sequestering PIP$_2$ in the plasma membrane (Glaser, et al., 1996). These functions are blocked by PKC-mediated phosphorylation within the basic region, blocking electrostatic interactions of this region with the membrane and causing the release of MARCKS into the cytosol (Thelen, et al., 1991). Dephosphorylation of MARCKS permits its rapid reassociation with the plasma membrane, allowing cycling between these two subcellular compartments to occur (Thelen, et al., 1991). The ability of MARCKS to couple the actin cytoskeleton to the membrane in a PKC-inhibitable fashion suggests a possible role for this protein in chemotaxis (Thelen, et al., 1991). In addition, MARCKS has recently been localized to nascent phagosomes in macrophages (Allen and Aderem, 1995) where it may act to permit fission from the plasma membrane (Glaser, et al., 1996).

Pleckstrin, a 47 kDa protein expressed exclusively in hematopoietic cells, was originally defined as a major substrate of PKC in platelets (Haslam and Lynham, 1977; Tyers, et al., 1988). Structurally, pleckstrin consists of two copies of a recently identified motif, the pleckstrin homology (PH) domain (Haslam, et al., 1993; Mayer, et al., 1993), bridged by a short region that includes PKC phosphorylation sites (Abrams, et al., 1995; Craig and Harley, 1996). To date, pleckstrin has not been found to display enzymatic activity and its role is unknown. Yet, because of its abundance and its susceptibility to rapid phosphorylation by PKC, pleckstrin is believed to play an important role in PKC-mediated activation. Expression

of pleckstrin is high not only in platelets, but also in HL-60 promyelocytes differentiated along the granulocytic line, where it constitutes 0.1% of the total protein (Tyers, et al., 1987). This finding suggests that pleckstrin may mediate some of the PKC-dependent microbicidal responses of neutrophils.

Map Kinase Cascades. The mitogen-activated protein (Map) kinase superfamily are a widely expressed and highly conserved group of serine/threonine kinases that initiate responses to many extracellular signals and physico-chemical stresses (Davis, 1994; Kyriakis and Avruch, 1996). Because of this signalling diversity, I will focus on the role of these kinases in mediating neutrophil responses to the chemoattractant fMLP (summarized in Fig. 1.6).

Three families of the Map kinase superfamily have been described to date. A unique feature of these kinases is their requirement to be phosphorylated on both threonine and tyrosine residues in order to become activated (Cobb and Goldsmith, 1995). This dual phosphorylation occurs within the conserved sequence TXY of subdomain VIII in the catalytic region. The amino acid at X is specific to each Map kinase family: TEY for the Extracellular signal-regulated kinase (Erk) family; TPY for the c-Jun N-terminal kinase (Jnk) family and TGY for the p38 family (Kyriakis and Avruch, 1996).

The first Mapk family to be discovered was the Erk family, with at least 3 isoforms identified to date. The Erk-1 and -2 isoforms are predominantly cytosolic proteins (of 44 and 42 kDa respectively) and have been described in many cell types, including neutrophils. These isoforms are rapidly activated upon stimulation of neutrophils with a variety of inflammatory stimuli, including fMLP, C5a, LTB4, TPA, tumour necrosis factor α and granulocyte-macrophage colony stimulating factor (Gomez-Cambronero, et al., 1993; Grinstein and Furuya, 1992; Okuda, et al., 1992; Thompson, et al., 1994). Little is known about the 62 kDa ERK-3 isoform which is constitutively localized to the nucleus in all cell types studied and does not appear to phosphorylate typical Erk family substrates (Cheng, et al., 1996).
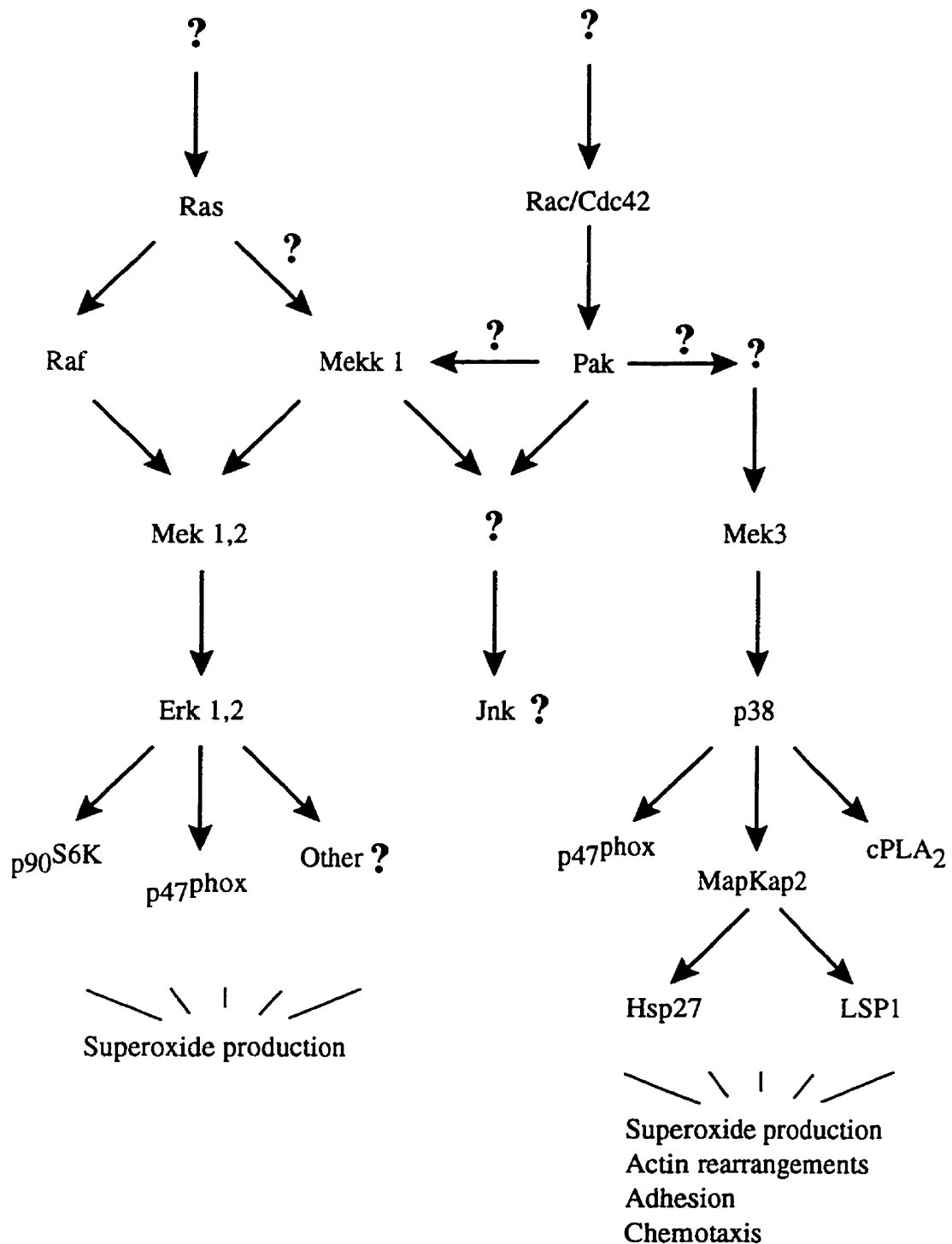
Fig. 1.6.   Map Kinase pathways in neutrophils. Abbreviations: cPLA$_2$, cyotosolic phospholipase A$_2$; Erk, extracellular-signal regulated kinase; Hsp27, heat shock protein 27; Jnk, c-Jun N-termimal kinase; LSP1, lymphocyte specific protein 1; MapKap2, mitogen activated protein kinase-activated protein kinase2; Mek, Map/Erk kinase; Mekk, Mek kinase; Pak, p21-activated kinase.

A number of cellular proteins have been shown to contain the Erk1 and -2 consensus phosphorylation motifs, PX(S/T)P or XX(S/T)P (Davis, 1993). In a variety of cell types, these substrates include transcription factors (eg: c-Fos, c-Jun), the epidermal growth factor receptor, and myelin basic protein (Davis, 1993). In neutrophils, important substrates of Erk1 and -2 include p90^{S6KII} (Huang, et al., 1994) and p47^{phox} (El Benna, et al., 1996).

Dual phosphorylation of Map kinases is catalyzed by the upstream activator Mek (for Map/Erk Kinase). To date, 6 members of the Mek family have been identified and subgrouped according to their ability to initiate downstream MapK signaling cascades (Han, et al., 1996). Both Mek1 and -2 phosphorylate Erk1 and -2, with Mek2 being the predominant Erk activator in human neutrophils in response to fMLP (Downey, et al., 1996). Inhibition of the Erk pathway can be accomplished with the Mek inhibitor PD98059 (Alessi, et al., 1995). Treatment of cells with this compound largely inhibits fMLP-mediated activation of Erk1 and -2 (60-70%), concomitant with an approximately 41% inhibition of superoxide production (Avdi, et al., 1996). These data suggest that the Erk kinases contribute to activation of the NADPH oxidase in response to fMLP.

Mek1 and -2 are activated by phosphorylation of two conserved serine/threonine residues (Zheng and Guan, 1994), mediated by at least two different kinases in neutrophils. The first is the 72 kDa Raf, a cytosolic protein in unstimulated cells. In other cell types, Raf is thought to be recruited to the plasma membrane by GTP-bound Ras and activated by an unknown kinase in response to mitogenic stimuli (Stokoe, et al., 1994). Following exposure to chemoattractants and other inflammatory stimuli, Ras-induced activation of Raf-1 participates in activation of the Erk pathway in neutrophils (Worthen, et al., 1994). The upstream activator(s) of Ras in these cells requires further definition.

In addition to Raf, upstream activation of Mek1 and -2 can be mediated by Mek kinase (Mekk). Mekk1 has been identified in neutrophils and is capable of activating both Mek1 and -2 following fMLP stimulation (Avdi, et al., 1996). Activation of Mekk1 may be dependent on the activation of Ras or a downstream effector of this G protein. Alternatively, Mekk1 may be activated downstream of the Ras-related small G proteins Rac and/or Cdc42 (Minden, et al.,

1995). Members of the p21-activated kinase (PAK) family are activated upon binding to GTP-bound Rac or Cdc42 and are thought to act as upstream activators of Mekk. Neutrophils express at least two members of the PAK family of serine/threonine kinases, suggesting that a similar pathway of Mekk1 activation also occurs in these cells (Knaus, et al., 1995). A novel Pak-related kinase, hematopoietic progenitor kinase 1, is widely expressed in hematopoietic cell lineages and has recently been proposed to act as a unique upstream activator of Mekk though this possibility remains to be tested in neutrophils (Hu, et al., 1996). In addition to Raf and Mekk, neutrophils may also express other activators of Mek1 and -2, including the Mos protooncogene product which is present in other systems (Posada, et al., 1993).

While initially identified as a specific activator of the Erk cascade, recent studies suggest that Mekk1 is a more potent upstream activator of Jnk and p38 (Xu, et al., 1996). Members of the Jnk family are important mediators of cellular responses to physico-chemical stimuli and inflammatory cytokines (Kyriakis and Avruch, 1996). Attempts to identify Jnk in human neutrophils have been unsuccessful to date (Nick, et al., 1997).

The third member of the MapK superfamily, p38, was initially isolated as the cellular target of the cytokine suppressive and anti-inflammatory drug SK&F 86002 (Lee, et al., 1994). Activation of p38 following lipopolysaccharide stimulation is required for translation of TNFα by macrophages (Han, et al., 1994). Like other MapK family members such as Erk and Jnk, p38 is activated by dual phosphorylation on threonine and tyrosine residues (but within a TGY consensus sequence not TEY as for the Erks) (Cobb and Goldsmith, 1995).

Following treatment of neutrophils with fMLP and other agonists, rapid activation of p38 has recently been observed (Krump, et al., 1997; Nahas, et al., 1996; Nick, et al., 1996). Upstream activation of p38 is mediated by Mek3 (Nick, et al., 1997) and possibly Mek6 (Han, et al., 1996). Upstream activators of these kinases are largely unknown but likely include Pak and Rac/Cdc42 (by analogy with other systems).

Many important p38 substrates have been described in neutrophils. These include p47phox, indicating that p38 may play a role in assembly and activation of the NADPH oxidase (El Benna, et al., 1996). While members of the Erk family were initially thought to

phosphorylate cPLA2 and contribute to its activation, recent studies have shown that p38 is actually the major cPLA2 activator in neutrophils and platelets (El Benna, et al., 1996; Kramer, et al., 1995). Another purported Erk target, MapK-activated protein kinase2 (MapKap2), is also activated by p38 in response to fMLP treatment (Krump, et al., 1997). Two important targets of MapKap2 are the heat shock protein Hsp27 (Krump, et al., 1997) and lymphocyte specific protein 1 (Huang, et al., 1997). When phosphorylated, Hsp27 can contribute to agonist-induced reorganization of the actin cytoskeleton (Landry and Huot, 1995). The ability of lymphocyte specific protein 1 to bind F-actin suggests that this p38 substrate also plays a role in regulating cytoskeletal dynamics (Huang, et al., 1997). Treatment of cells with the p38 inhibitor SK&F86002 was capable of inhibiting fMLP-mediated adhesion to fibronectin, chemotaxis and superoxide production (Nick, et al., 1997). Together, these studies suggest an important role for both the ERK and p38 members of the MapK superfamily of serine/threonine kinases.

Renaturable serine/threonine kinases. A family of chemoattractant-activated serine/threonine kinases has been described in rodent and human neutrophils (Ding and Badwey, 1993; Ding and Badwey, 1993; Grinstein, et al., 1993). Following fractionation by electrophoresis in denaturing polyacrylamide gels, these unique enzymes have the ability to renature and to phosphorylate themselves or exogenous substrates previously immobilized within the matrix of the gel. Four members of this renaturable serine/threonine kinase family have been identifed, with approximate molecular masses of 42, 49, 65 kDa and 72 kDa (Grinstein, et al., 1993). The 42 kDa protein has been identified as a member of the Erk family (Grinstein and Furuya, 1992). Subsequent to the studies presented in Chapter 3, the 65 and 72 kDa kinases were identified as members of the Pak family of kinases (Ding, et al., 1996). The identity of the 49 kDa renaturable kinase in neutrophils remains to be determined.

Tyrosine phosphorylation. One of the earliest biochemical events which follows receptor engagement is the attachment of phosphate to tyrosine residues of cellular

proteins. Increases in tyrosine phosphorylation can be initiated by a variety of soluble and particulate stimuli and temporally correlate with the appearance of cellular responses (Berkow and Dodson, 1990; Gomez-Cambronero, et al., 1989; Huang, et al., 1988). The importance of tyrosine phosphorylation to neutrophil function is further underlined by the finding that inhibitors of protein tyrosine kinases block many neutrophil responses, including adherence (McGregor, et al., 1994), chemotaxis (Gaudry, et al., 1992), phagocytosis (Kobayashi, et al., 1995) and production of reactive oxygen intermediates (Berkow, et al., 1989; Kusunoki, et al., 1992).

Tyrosine kinases. Phosphotyrosine accumulation is regulated by the competing activities of protein tyrosine kinases (PTK's) and protein tyrosine phosphatases (PTP's). Increased activity of tyrosine kinases has been demonstrated in neutrophils treated with chemotactic peptides (Ptasznik, et al., 1995), cytokines (Berton, et al., 1994; Corey, et al., 1993) and other ligands (Asahi, et al., 1993), and has been postulated to account for the increased tyrosine phosphorylation observed following stimulation with these agents. The subcellular localization of tyrosine kinases and their substrates may also play a role in regulating tyrosine phosphorylation. For example, neutrophils contain at least four types of secretory granules within their cytoplasm (Borregaard, et al., 1993), some of which have been shown to contain tyrosine kinases. Following stimulation, granular fusion with the plasma membrane and/or phagosome may allow associated kinases access to their substrates (Gutkind and Robbins, 1989; Mohn, et al., 1995). Some of the tyrosine kinases thought to play important roles in neutrophil signalling are discussed below.

Src family tyrosine kinases. The Src tyrosine kinase was identified as the cellular homologue of the prototype oncogene v-Src (Cantley, et al., 1991). Mutations in the Src gene, captured in the Rous sarcoma virus, give rise to a constitutively active tyrosine kinase that is capable of transforming infected cells. Since the discovery of Src, other closely related proteins have been identified, including Fyn, Yes, Fgr, Lyn, Hck, Lck, Blk and Yrk. While

their individual tissue distribution varies, tyrosine kinases of the Src family are represented in all mammalian cells, performing a great variety of signalling functions (Brown and Cooper, 1996).

Members of the Src family share a common structural organization, with each conserved region designated a Src-Homology (SH) domain (see Fig. 1.7). The N-terminus contains the SH4 domain which is myristoylated and targets the protein to cellular membranes. Reversible palmitoylation within this region has also been described for some family members (eg: Hck) and can serve to localize these kinases to cholesterol-rich compartments of the plasma membrane called caveolae (Robbins, et al., 1995). A region of 50-70 amino acids, unique to each Src family kinase, joins the SH4 domain with the SH3 domain. Both SH3 and SH2 domains act as functional units that mediate protein-protein interactions. SH3 domains bind to proline-containing peptides in a polyproline type-II (helical) conformation while SH2 domains bind polypeptide segments that contain a phosphotyrosine (Koch, et al., 1991). Expression of SH3 and SH2 domains is widespread and plays an important role in the regulation and function of many important signalling proteins. A short 'linker' region of 13 amino acids joins the SH2 domain to the functional catalytic unit, the SHI domain.

The modular nature of the Src structure provides the basis for regulation of its catalytic activity. In the basal state, kinase activity is inhibited by two distinct intramolecular interactions. The first, involves folding of the enzyme to allow intramolecular binding of the SH2 domain with a C-terminal tyrosine phosphorylated residue (Y527 in chicken Src). Phosphorylation of this residue is catalyzed by another kinase, C-terminal Src kinase, which displays a high basal activity within cells (Okada, et al., 1991). Several lines of evidence have demonstrated the importance of C-terminal phosphorylation in mediating autoinhibition of Src family members: i) dephosphorylation of Y527 increases Src kinase activity ii) mutations within the C-terminus that remove Y527, including those found in v-Src from the Rous sarcoma virus, lead to constitutive activation of kinase activity, and, iii) high affinity binding of the Src SH2 domain to tyrosine phosphorylated peptides increases catalytic activity (Brown and Cooper, 1996).

Src Family

Y416 Y527

N SH4 SH3 SH2 SH1 C

Unique Linker

Syk

N SH2 SH2 SH1 C

Btk

N PH TH SH3 SH2 SH1 C
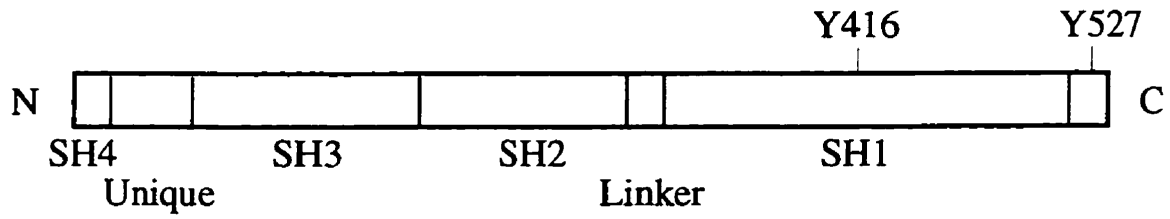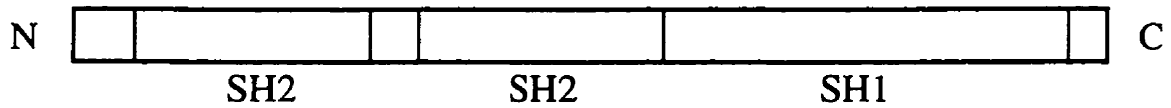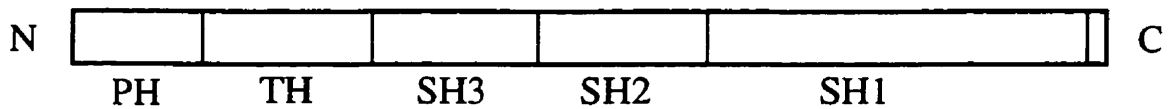
Fig. 1.7.   Structures of tyrosine kinases expressed in neutrophils. Abbreviations: PH, pleckstrin homology domain; SH, Src homology domain; TH, Tec homology domain.

Recent structural analyses have illuminated a second intramolecular interaction that acts to inhibit kinase activity of Src family PTK's in quiescent cells. Crystal structures of Src, phosphorylated at Y527, revealed tight associations between the SH3 domain and proline residues of the linker region (Xu, et al., 1997). In addition, two loops of the SH3 structure interacted with the lobes of the kinase domain, causing a conformational shift that is likely to inhibit catalysis. Solution of the autoinhibited Hck crystal structure provides evidence that SH3 domain-mediated inhibition of catalytic activity is common to all Src family members (Sicheri, et al., 1997).

Full activation of Src appears to require both Y527 dephosphorylation and autophosphorylation of a residue within the SH1 domain, namely Y416. Solution of the crystal structure of activated Lck (Y416 phosphorylated, Y527 dephosphorylated) suggests that Y416 may play a role in stabilizing active conformation of the catalytic fold within the SH1 domain (Yamaguchi and Hendrickson, 1996). Interestingly, partial activation of Src family kinases has been witnessed when they are doubly phosphorylated on Y416 and Y527 (Boerner, et al., 1996; D'Oro, et al., 1996). These findings suggest that the stabilizing effect of Y416 phosphorylation is capable of partially overcoming the inhibitory actions of intramolecular folding.

Neutrophils have been found to express members of Src family, including Lyn (Ptasznik, et al., 1995), Hck (Ziegler, et al., 1987), Fgr (Gutkind and Robbins, 1989)and Yes (Corey, et al., 1993). Expression of the other members of the Src family is undetectable (Mustelin, 1994). Activation of the Src family kinases expressed in neutrophil has been observed following treatment with a variety of inflammatory stimuli, including fMLP (Corey, et al., 1993; Gaudry, et al., 1995; Ptasznik, et al., 1995). The role of these kinases in mediating neutrophil functions has been studied with the use of transgenic mice deficient in their expression. Mice with targetted disruptions in the Hck gene displayed an impairment in phagocytosis (Lowell, et al., 1994). In addition, adherent neutrophils from mice lacking both Hck and Fgr are unable to produce ROI following stimulation with fMLP or tumour necrosis

factor α (Lowell, et al., 1996). These data suggest an important role for Src family kinases in mediating neutrophil responses to inflammatory stimuli.

Spleen tyrosine kinase. Spleen tyrosine kinase (Syk), so called because it was originally isolated from porcine spleen homogenates, is broadly expressed in many hematopoietic lineages (Taniguchi, et al., 1991). A unique feature of Syk (and the related kinase ZAP-70) is its tandem SH2 domain-containing structure (see Fig.1.7). These SH2 domains mediate the association of Syk with a variety of immune cell receptors which contain immunoreceptor tyrosine-based activation motifs (Chan and Shaw, 1995). Receptor phosphorylation within this motif allows binding of Syk, an event thought to increase Syk kinase activity and initiate downstream signalling (Kimura, et al., 1996).

Syk association with the B-cell receptor is thought to play an important role in B-cell development (Satterthwaite and Witte, 1996). Lymphoid cells lacking Syk expression displayed impaired differentiation of B-lineage cells (Cheng, et al., 1995). In addition, mice lacking Syk suffered severe hemorraging as embryos, indicating that Syk is involved in maintaining vascular integrity and wound healing during embryogenesis (Cheng, et al., 1995).

Syk also plays an important role in the initiation of phagocytosis via Fcγ receptors. Upon binding to IgG-coated microbial targets or particles, clustering of these receptors leads to their phosphorylation (or phosphorylation of an associated γ subunit) and association with Syk (Greenberg, et al., 1996). Activated Syk initiates many important downstream signalling events, including reorganization of the actin cytoskeleton around the nascent phagosome (Cox, et al., 1996).

Bruton's tyrosine kinase. Like Syk, Bruton's tyrosine kinase (Btk) plays an important role in B-cell development (Khan, et al., 1996). Mutations in the gene encoding Btk are responsible for X-linked agammaglobulinemia, a primary immunodeficiency characterized by a virtually complete absence of circulating B-lymphocytes. Btk belongs to the Tec family of PTK (comprising Btk, Tec and Itk), all of which share a common structure (Fig.1.7). Like the Src family, Tec-related kinases contain SH3 and SH2 domains in addition to the conserved

catalytic domain (SH1). Btk and its relatives also possess a PH domain and a conserved region (Tec homology domain) characterized by a proline-rich region.

Several models of Btk regulation have been proposed. Intramolecular association of the Btk SH3 domain with its own proline-rich region has been suggested to repress kinase activity in the basal state (Andreotti, et al., 1997). Following stimulation, ligand binding to Btk's SH3 and SH2 domains might then overwhelm this intramolecular interaction and unfold the kinase into an active state. Phosphorylation of Btk by Src family PTK's has also been shown to increase its activity (Rawlings, et al., 1996). Further regulation of Btk may come from the association of its PH domain with the $\beta\gamma$ subunits of G protein-coupled receptors (Langhans-Rajasekaran, et al., 1995).

Tyrosine Phosphatases. In addition to the effects of tyrosine kinases, decreases in the activity of protein tyrosine phosphatases (PTP's) may also lead to an increase in cellular tyrosine phosphorylation following exposure of neutrophils to inflammatory stimuli. In support of this notion, overall neutrophil phosphotyrosine phosphatase activity has been shown to decrease following stimulation with the chemoattractant fMLP or with phorbol esters, though the identity of the particular phosphatases responsible for this effect was not determined (Kansha, et al., 1993; Kraft and Berkow, 1987). Similarly, the inhibition of tyrosine phosphatases with vanadate or its peroxides has been shown to potentiate fMLP-induced superoxide production in whole cells (Lloyds and Hallet, 1994) and to activate a respiratory burst in electroporated cells, providing further evidence that a reduction of phosphatase activity may lead to anti-microbial responses in PMN (Grinstein, et al., 1990; Mitsuyama, et al., 1993).

While the spectrum of tyrosine phosphatases responsible for regulating neutrophil responses requires further definition, one PTP implicated in neutrophil signalling is CD45. A member of the transmembrane class of tyrosine phosphatases, CD45 has been found on secretory granules and the plasma membrane of neutrophils (Pulido, et al., 1989). Stimulation with a variety of agents has been shown to enhance the expression of CD45 on the plasma

membrane (Pulido, et al., 1992), a phenomenon thought to contribute to the desensitization of neutrophils following further stimulation with fMLP (Cui, et al., 1994). The intrinsic activity of CD45 is also modulated in neutrophils following production of reactive oxygen intermediates by the NADPH oxidase (Fialkow, et al., 1994). The latter finding is thought to reflect the oxidation of critical cysteine residues within the catalytic domain of CD45 (which is conserved in all tyrosine phosphatases, see (Fisher, et al., 1991)) and may represent a unique mode of auto/paracrine signalling (Schreck and Baeuerle, 1991). Inhibition of CD45 activity may also contribute to oxidant-induced activation of Erk (Fialkow, et al., 1994), an enzyme thought to represent a substrate of CD45 (Anderson, et al., 1990).

In contrast to CD45, little is known about the role of soluble tyrosine phosphatases in neutrophil signal transduction. One such phosphatase, the SH2-containing tyrosine phosphatase-1 (SHP-1, also known as PTP1C, HCP, PTP-N6 and SHPTP-1), has been extensively studied as a potential regulator of the action of growth-promoters in haemopoietic cells (Okamura and Thomas, 1995). SHP-1 has been shown to dephosphorylate and inactivate the erythropoietin (Klingmuller, et al., 1995; Yi, et al., 1995), stem cell factor (Yi and Ihle, 1993), interleukin-3 (Yi, et al., 1993), interferon α (Yetter, et al., 1995) epidermal growth factor (Tomic, et al., 1995) and B-cell antigen receptors (Pani, et al., 1995). In addition, loss of function mutations in SHP-1 are known to represent the genetic defect responsible for serious autoimmune and immunodeficiency defects in *motheaten* mice (Bignon and Siminovitch, 1994; Kozlowski, et al., 1993; Shultz, et al., 1993; Tsui, et al., 1993).

Thesis summary.

Increasing evidence has suggested the importance of protein phosphorylation in the regulation of neutrophil responses to inflammatory stimuli. To further understand phosphorylation-dependent signalling mechanisms in these cells, the enzymes that control phosphorylation were examined. While neutrophils express a variety of cell surface receptors that often initiate unique signalling cascades, the work in this thesis will focus on the events downstream of the fMLP receptor.

Activation of the fMLP receptor increased the activity of two renaturable serine/threonine kinases, PK65 and PK72. These kinases have the unique ability to regain their catalytic activity following denaturation and electrophoresis in SDS PAGE gels. The rapid time course of activation of these kinases and their previously demonstrated ability to phosphorylate p47^{phox}, a subunit of the NADPH oxidase, suggested an important role in the initiation of signal transduction by the fMLP receptor. Activation of PK65 and PK72 paralleled the accumulation of tyrosine phosphate on proteins with similar molecular masses. Treatment of cells with tyrosine kinase inhibitors revealed that the renaturable kinases were dependent on the activity of tyrosine kinases. However, immunoprecipitation of tyrosine phosphorylated proteins demonstrated that PK65 and PK72 are not themselves phosphorylated on tyrosine. Thus, activation of tyrosine kinases is a required upstream event in the signal transduction cascade leading to the activation of PK65 and PK72. Subsequent to this study, the renaturable kinases were identified as members of the PAK family of serine/threonine kinases.

Recent studies have suggested an important role for reactive oxygen intermediates (ROI) in a variety of signal transduction systems. As neutrophils are capable of producing massive amounts of these inorganic molecules via the NADPH oxidase, the effect of endogenously produced ROI on neutrophil signalling was addressed. To induce endogenous production of ROI without other receptor-mediated signalling events, electropermeabilized cells were stimulated with non-hydrolyzable analogues of GTP. Direct activation of the NADPH oxidase in this manner was found to induce a massive accumulation of tyrosine phosphate on

many cellular proteins, in contrast to the characteristic pattern of tyrosine phosphorylation witnessed following treatment with bacterial tripeptide.

Phosphotyrosine accumulation is regulated by the competing activities of protein tyrosine kinases and protein tyrosine phosphatases. Earlier studies had suggested that ROI can inhibit the activity of tyrosine phosphatases by oxidation of a conserved cysteine residue within their catalytic domain. While the inhibition of tyrosine phosphatases may account for the elevated tyrosine phosphorylation induced by ROI, increased activity of tyrosine kinases could also conceivably contribute to the response. The role of tyrosine kinases in mediating accumulation of tyrosine phosphate was addressed by immunoprecipitation of the tyrosine kinases identified in neutrophils and measurement of their activity *in vitro*. Production of ROI was shown to activate several tyrosine kinases in a manner dependent on their prior tyrosine phosphorylation. It was concluded that oxidation of tyrosine phosphatases by oxidase-derived ROI mediates their inhibition, allowing tyrosine kinases to be phosphorylated on activating tyrosine residues. NADPH oxidase-derived ROI likely play an important role as auto- and paracrine signalling molecules, serving to enhance tyrosine kinase-dependent signalling functions.

To further delineate the role of tyrosine phosphatases in the initiation and/or modulation of neutrophil responses, I have studied the localization, activity and possible modes of regulation of SHP-1 in these cells. In addition to their inhibition by ROI, some tyrosine phosphatases are also regulated via their phosphorylation. This appears to be the case for SHP-1, which was phosphorylated on serine residues in response to inflammatory stimuli. Phosphorylation of SHP-1 was associated with a concomitant decrease in its catalytic activity and an accumulation of cellular tyrosine phosphate. Agonist-mediated phosphorylation and inhibition of SHP-1 were blocked by pretreatment of neutrophils with a potent and specific inhibitor of PKC isoforms, indicating that this kinase may regulate SHP-1 activity in cells. *In vitro* phosphorylation of SHP-1 with purified PKC inhibited its activity, mimicking the effect seen in intact cells. These findings suggest a new link between PKC and the regulation of tyrosine phosphorylation.

The role of PKC in activation of neutrophil responses to chemoattractants is still unclear. One PKC substrate thought to play an important role in hematopoietic cells is pleckstrin, which is highly expressed in these cells and rapidly phosphorylated in response to PKC activation. Using a variety of pharmacologic tools, the upstream regulation of pleckstrin phosphorylation was studied. Several lines of evidence suggested that pleckstrin is phosphorylated by a non-conventional isoform of PKC: phosphorylation was inhibited by tyrosine kinase and phosphatidylinositol 3-kinase (PI3K) antagonists, was independent of intracellular calcium transients and was prolonged by agents that elevated intracellular phosphatidic acid levels. As other PKC substrates (eg: MARCKS) have been demonstrated to change their subcellular distribution following phosphorylation, the subcellular localization of pleckstrin was also studied. Following phosphorylation, pleckstrin translocated from the cytosol to both membranes and the cytoskeleton. Phosphorylated pleckstrin may serve as an intracellular adaptor, targetting PH domain-associated molecules to these subcellular compartments in response to inflammatory stimuli.

In addition to pleckstrin, other adaptor proteins were identified in neutrophils. Originally described in other systems, VAMP-2 and syntaxin 4 were localized to the secretory granules and plasma membrane of neutrophils, respectively. The interaction of these proteins (through a tripartite complex with soluble secretory factors such as NSF and SNAP) may play a role in regulating secretion of antimicrobial agents.

All of the work described in this thesis has been published. To avoid repetition, experimental procedures have been consolidated into Chapter 2. All references can be found at the end of this document. The repetitive portions of introductory segments from each manuscript have been removed while the abstract, results and discussion remain largely untouched. Work presented in Chapter 5 was performed with the technical assistance of Chi Kin Chan. In Chapter 6, Fig. 6.4 was done with the help of Dr. Karen L. Craig while Donald Ferguson assisted with Figs. 6.2, 6.5 and 6.6. Chapter 7 was a collaboration with Allen Volchuk who contributed to Figs. 7.1, 7.2, 7.3, 7.4 and 7.5. Immunoelectron microscopy in Fig. 7.6 was performed by Drs. Anne-Marie Cieutat and Dorothy F. Bainton. A general

discussion of this work is found in the concluding chapter. The publications contained in this thesis are:

Chapter 3: Brumell, J.H. and Grinstein, S. (1994). Serine/threonine kinase activation in human neutrophils: relationship to tyrosine phosporylation. Am. J. Physiol. 267:C1574-C1581.

Chapter 4: Brumell, J.H., Burkhardt, A.L., Bolen, J.B. and Grinstein, S. (1996). Endogenous reactive oxygen intermediates activate tyrosine kinases in human neutrophils. J. Biol. Chem. 271:1455-1461.

Chapter 5: Brumell, J.H., Chan, C.K., Butler, J., Borregaard, N., Downey, G.P., Siminovitch, K.A. and Grinstein, S. (1997). Regulation of SHP-1 during activation of human neutrophils: Role of Protein Kinase C. J. Biol. Chem. 272:875-882.

Chapter 6: Brumell, J.H., Craig, K., Ferguson, D., Tyers, M. and Grinstein, S. (1997). Phosphorylation and subcellular redistribution of Pleckstrin in human neutrophils. J. Immunol. 158:4862-4871.

Chapter 7: Brumell, J.H., Volchuk, A., Sengelov, H., Borregaard, N., Cieutat, A-M., Bainton, D.F., Grinstein, S. and Klip, A. (1995). Subcellular distribution of docking/fusion proteins in neutrophils, secretory cells with multiple exocytic compartments. J. Immunol. 155:5750-5759.

Chapter 2:

Experimental Procedures

Materials. N-acetyl cysteine (NAC), aprotinin, EGTA, diamide, 1,2-dioctanoylethylene glycol, dithiothreitol (DTT), fibrinogen, fMLP, GTPγS, guanidine hydrochloride, HEPES, histone f2a (type VI-S from calf thymus), K-ATP, leupeptin, medium RPMI 1640, NADPH, Nonidet P-40 (NP-40), pepstatin A, phenylmethylsulfonyl fluoride (PMSF), phosphotyrosine, propranolol, sodium orthovanadate (NaV), and the 4β-form of 12-O-tetradecanoylphorbol 13-acetate (TPA) were from Sigma. Hydrogen peroxide (H_2O_2) was from Fisher. Ficoll 400, dextran T500, protein A-Sepharose, Sepharose beads and Ficoll-Paque were from Pharmacia LKB Biotechnology Inc. Centrifugation filters (Centricon) with a molecular weight cut-off of 30 kDa were from Amicon Corp. Ultra-pure acrylamide, alkaline phosphatase and bovine serum albumin (BSA) were from Boehringer-Mannheim. Calyculin A was from LC Services Corporation. Genistein was from BIOMOL. Erbstatin was the generous gift of Dr. K. Umezawa (Department of Applied Chemistry, Keio University, Japan). Bis-indolylmaleimide I (BIM, also known as GF 109203X), wortmannin, ionomycin and R59022 were purchased from Calbiochem. Diisopropylfluorophosphate (DFP) was obtained from Aldrich. The acetoxymethylesters of indo-1 and BAPTA were from Molecular Probes. Polyvinylidene difluoride (PVDF) filters were from Millipore. Okadaic acid, ^{32}P-H_2PO_4, 8-(N-morpholino) propanesulphonic acid and ^{32}P-γ-ATP were purchased from ICN. The BCA protein assay kit and desalting columns were from Pierce Chemical Co. Two-chain tetanus toxin was a kind gift from Dr. E. Habermann (Justus-Leibig Universtat, Giesen, Germany). Diphenylene iodonium (DPI) was synthesized in our laboratory as previously described (Collette, et al., 1956). Purified PKC from rat brain and the protein tyrosine phosphatase 1B (PTP-1B) pre-conjugated to agarose beads were obtained from Upstate Biochemicals Inc. Truncated T-cell phosphatase (TC-PTP) was the generous gift of Dr. C. Diltz (Department of Biochemistry, University of Washington, Seattle, WA, USA).

__Antibodies.__ Affinity-purified rabbit polyclonal antibodies to phosphotyrosine and a monoclonal antibody to SHP-1 were obtained from Transduction Laboratories. Monoclonal antibodies (4G-10 clone) to phosphotyrosine (free or conjugated to agarose beads) were from UBI. Affinity-purified rabbit polyclonal antibody to Raf-1 was purchased from Santa Cruz Biotechnology Inc. Anti-sera to the Src family tyrosine kinases as well as Syk, Zap-70 and Btk were provided by Bristol Myers Squibb (J. Bolen and A. Burkhardt). A GST-fusion protein of wild-type murine SHP-1 encompassing its two SH2 domains (amino acids 1-296) was generated as previously described (Kozlowski, et al., 1993). The recombinant protein was used to generate polyclonal antibodies to SHP-1 which were affinity purified and have been shown to be suitable for immunoblotting and immunoprecipitation (Kozlowski, et al., 1993; Pani, et al., 1995). A GST-fusion protein of wild-type pleckstrin was generated and the recombinant protein was used to generate polyclonal antibodies which were affinity-purified, as previously described (Craig and Harley, 1996). Rabbit polyclonal anti-VAMP-2 antiserum was raised to a GST-fusion protein encompassing residues 1-96 of the rat VAMP-2 sequence and was affinity purified. This antibody has been previously shown to react almost exclusively with VAMP-2 (Volchuk, et al., 1995). A polyclonal antibody (αSy4) to a GST-fusion protein encompassing residues 1-274 of rat syntaxin 4 was raised in rabbits and affinity-purified. This antibody does not react with any of the other recombinant syntaxin isoforms. An affinity-purified anti-cellubrevin antiserum (D204) (McMahon, et al., 1993) was generously provided by Dr. T. C. Sudhof (Howard Hughes Medical Institute, University of Texas, Dallas, TX). An affinity-purified polyclonal anti-VAMP-1 antibody and a monoclonal antibody to synaptophysin (clone SY38) were kind gifts of Dr. W. Trimble (University of Toronto, Toronto, Canada). A monoclonal antibody to SCAMP (SG7C12) was the kind gift of Dr. D. Castle (Department of Anatomy and Cell Biology, University of Virginia). Antibodies to both CD63 and CD67 were the generous gift of Dr. A. J. Verhoeven (Red Cross Blood Transfusion Centre, Central Laboratory of the Netherlands). A monoclonal antibody designated SPM-1 recognizing syntaxin 1A/1B was provided by Dr. T. Abe (University of Niigata, Niigata, Japan). A second monoclonal antibody to syntaxin 1A/1B (HPC-1) was from Sigma. Affinity

purified antibodies to the C3b receptor (CD35) were obtained from Dako (Denmark). Monoclonal anti-SNAP-25 (clone SMI 81) was purchased from Sternberger Monoclonals Inc.

Goat anti-rabbit and donkey anti-mouse secondary antibodies coupled to horseradish peroxidase used for immunoblotting were from Jackson ImmunoResearch and Amersham, respectively. The secondary antibodies used for immunofluorescence, FITC-conjugated donkey anti-rabbit and Texas Red-conjugated donkey anti-mouse, were from Jackson ImmunoResearch. Donkey serum used as a blocking agent for immunofluorescence was also obtained from Jackson ImmunoResearch.

Pharmacologic inhibitors of signalling molecules. An important tool in these studies was the tyrosine kinase inhibitor erbstatin, the chemical structure of which is shown in Fig. 2.1. Originally isolated from culture filtrates of *Streptomyces viridosporus*, erbstatin effectively competes with potential substrates for the active site of tyrosine kinases, possibly because of its structural similarities to tyrosine. Characterization of the effect of erbstatin on neutrophil responses to chemoattractant was performed by Naccache and colleagues (1990). In these studies, it was shown that pretreatment of cells with 10μg/mL erbstatin abolished both fMLP-induced tyrosine phosphorylation and superoxide production but had no effect on intracellular calcium transients. Unlike another tyrosine kinase inhibitor ST638, erbstatin had no effect on the ability of fMLP to bind its receptor. Superoxide production induced by TPA was unaffected by erbstatin pretreatment, confirming many previous reports that demonstrated the inability of this compound to affect PKC activity. These and other findings revealed the potent and specific nature of tyrosine kinase inhibition by erbstatin, making it an ideal compound to study the role of tyrosine kinases in neutrophil signalling pathways.

While many pharmacologic inhibitors of the PKC family have been identified, the specificity of these compounds has often been dubious at best. Staurosporine, the most potent PKC inhibitor described to date, demonstrates a nearly complete lack of specificity by competing with ATP for the ATP-binding sites of many serine/threonine and tyrosine kinases. In an effort to identify more selective inhibitors of PKC, Toullec et al. (1991) synthesized a

Erbstatin
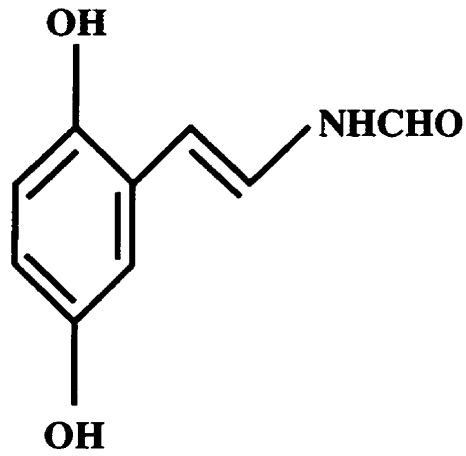
Bis-indolylmaleimide
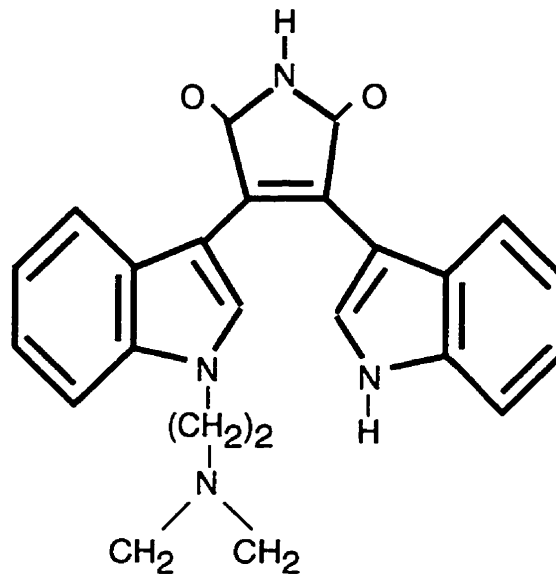
Wortmannin
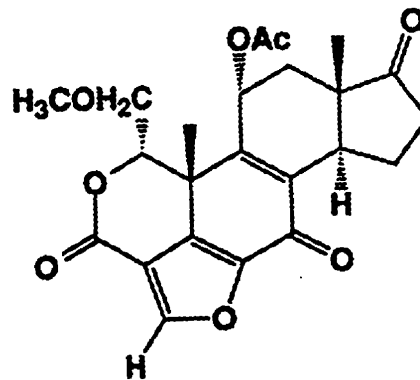
Fig. 2.1 Pharmacologic inhibitors used in this study.

variety of bis-indolylmaleimides (structurally similar to staurosporine) and assessed their ability to inhibit the activity of various kinases *in vitro*. Of the many structures tested, the most potent PKC inhibitor was GF 109203X (referred to as BIM throughout text), capable of inhibiting all PKC isoforms (see Fig. 2.1). Importantly, BIM inhibited PKC activity with IC_{50} values greater than 5000 times lower than that for tyrosine kinases such as the epidermal growth factor receptor. The effect of BIM on phosphorylation of platelet substrates following thrombin stimulation was also determined by Toullec and colleagues. Pretreatment with 5 μM BIM obliterated phosphorylation of pleckstrin with no effect on the phosphorylation of myosin light chain, suggesting that myosin light chain kinase was unaffected. Similarily, the activity of PKA *in vivo* appears to be unaffected by BIM since pretreatment of Swiss 3T3 cells with this compound had no effect on the phosphorylation of vimentin induced by agents that increased intracellular levels of cAMP. Thus, the demonstrated potency and relative specificity of BIM made it an ideal pharmacologic tool for the study of PKC signalling.

The study of PI3-kinases has been aided by the discovery of wortmannin, a fungal metabolite (reviewed by Ui et al., 1995). This compound is capable of irreversibly inhibiting PI3-kinases by covalently binding to the 110 kDa catalytic subunit of these enzymes, thereby obliterating their activity. The use of wortmannin as an inhibitor of PI3-kinases in neutrophils was first reported by Dewald et al. (1988). Treatment of neutrophils with 100 nM wortmannin abolished the production of PIP_3 induced by exposure to fMLP, in addition to inhibiting many cellular responses to the chemoattractant (see above). While wortmannin has been reported to inhibit the activities of phospholipase D and PI4-kinase in other systems, no inhibition of these enzymes was witnessed at the concentrations used during their study. This and later findings in a variety of systems have established wortmannin as a useful tool in the study of PI3-kinases.

Solutions. Bicarbonate-free RPMI 1640 medium was buffered to pH 7.3 with 25 mM Na-HEPES. Powdered PBS was obtained from Pierce. The Na^+-rich medium used for incubation of intact cells contained (in mM) 140 NaCl, 5 KCl, 10 glucose, 1 $MgCl_2$, 1 $CaCl_2$ and 10 Na-HEPES (pH 7.3). This medium was also prepared without Ca^{2+} by omission of

CaCl$_2$ and addition of 1 mM EDTA. Permeabilization medium contained 140 mM KCl, 10 mM glucose, 1 mM MgCl$_2$, 1 mM EGTA, 193 μM CaCl$_2$, 1 mM K-ATP and 10 mM K-HEPES (pH=7.0). All media were adjusted to 290 ± 5 mOsM with the major salt.

Cell isolation and manipulation. Neutrophils were isolated from fresh heparinized blood of healthy human volunteers by dextran sedimentation, followed by Ficoll-Hypaque gradient centrifugation. Contaminating red cells were removed by NH$_4$Cl lysis. Neutrophils were counted using a Model ZM Coulter Counter, resuspended in HEPES-buffered RPMI 1640 medium at 10^7 cells/mL and maintained in this medium at room temperature until use. To minimize proteolysis following cell lysis, the cells were pretreated with 2.5 mM diisopropylfluorophosphate for 30 min at room temperature. For electroporation, 1.5x 10^7 cells were sedimented and resuspended in 1 mL of ice-cold permeabilization medium. This suspension was transferred to a Bio-Rad Pulser cuvette and permeabilized with two discharges as described previously (Grinstein and Furuya, 1988). The cells were rapidly sedimented and resuspended in fresh, ice-cold permeabilization medium between discharges. Electroporated cells were used immediately. Where indicated, cells were pre-incubated for 2 min at 37°C in the presence of 5 μM diphenylene iodonium or 2 mM NAC prior to stimulation.

Treatment with tetanus toxin. Neutrophils (3x10^6) were resuspended in 30 μL of potassium glutamate buffer (138 mM potassium glutamate, 8 mM MgCl$_2$, 0.285 mM CaCl$_2$, 1 mM dithiothreitol and 20 mM HEPES, pH 7.15) containing a cocktail of protease inhibitors (1 mM PMSF, 10 μg/mL aprotinin, 10 μg/mL leupeptin and 1 μM pepstatin A) and boiled for 5 min. Boiled samples were briefly sonicated (3 bursts of 10 sec). Freeze-dried microsomes from 3T3-L1 adipocytes (40 μg) and brain homogenate (10 μg) were treated similarly in parallel. Samples were allowed to cool after boiling before addition of Triton X-100 (0.5% final) with or without 200 nM pre-activated tetanus toxin. Tetanus toxin was pre-activated by incubation with 10 mM dithiothreitol at 37°C for 1 h (Kistner, et al., 1993), and diluted 10-fold in potassium glutamate buffer. The reaction of membrane or cell lysate samples with toxin proceded for 1 h at 37°C under constant agitation, and was terminated by addition of 5x

concentrated Laemmli sample buffer, the samples boiled for 5 min and subjected to SDS-PAGE and immunoblotting.

Subcellular fractionation. Neutrophil subcellular fractionation was performed according to Kjeldsen et al. (Kjeldsen, et al., 1994). Briefly, neutrophils were disrupted by nitrogen cavitation in disruption buffer (100 mM KCl, 3 mM NaCl, 1 mM Na_2ATP, 3.5 mM $MgCl_2$, 0.5 mM PMSF, 5 mM NaF, 1 mM $NaVO_4$, 10 μg/mL aprotinin, 10 μg/mL leupeptin, 1 μM pepstatin and 10 mM PIPES, pH 7.2). Nuclei and unbroken cells were removed by centrifugation at 400g for 15 min and the resulting postnuclear supernatant was applied on top of a three-layer Percoll gradient (1.05/1.09/1.12 g/mL). Centrifugation at 37,000g for 30 min yielded 4 separable bands. Three of these correspond to the primary, secondary and tertiary granules. A fourth band contains a mixture of secretory vesicles and plasma membranes (sv/pm fraction). One mL fractions were collected from the gradient and each was assayed for markers of the above subcellular compartments. Myeloperoxidase (primary granules), lactoferrin (secondary granules), gelatinase (tertiary granules), HLA class I (plasma membrane) and albumin (secretory vesicles) were all measured by enzyme-linked assay as described (Borregaard, et al., 1992). Percoll was removed from the samples by centrifugation and the remaining biological material was mixed with boiling, 2x concentrated Laemmli sample buffer and equal amounts of protein from each fraction were subjected to SDS-PAGE and immunoblotting (see below).

Isolation of neutrophil membranes. Neutrophil suspensions were treated with or without stimuli, sedimented rapidly and resuspended (2.0×10^7 cells/mL) in disruption buffer (see above). The cells were then disrupted by sonication (3 bursts of 10 sec each with 5 min cooling between bursts) and nuclei and unbroken cells were removed by centrifugation at 14,000g for 5 min. A high speed pellet, referred to hereafter as "membranes" was isolated by centrifugation of the lysates at 100,000g for 30 min, washed 3x with disruption buffer and then boiled in Laemmli sample buffer. Remaining soluble (cytosolic) fractions were boiled in 2x Laemmli sample buffer and an identical number of cell equivalents (10^6) from both membrane

and cytosolic fractions were subjected to SDS-PAGE and immunoblotting (see below). Equal protein loading was confirmed by Coomassie staining.

Isolation of Triton X-100 insoluble ("cytoskeleton associated") proteins. Neutrophil suspensions were treated with or without stimuli, sedimented and then resuspended in lysis buffer (150 mM NaCl, 2 mM EDTA, 1 mM NaVO$_4$, 5 mM NaF, 1 mM PMSF, 10 µg/mL aprotinin, 10 µg/mL leupeptin and 50 mM Tris, pH 8.0) containing 1% Triton X-100. The samples were vortexed vigorously and left on ice for a minimum of 10 min to ensure complete lysis of cells. Insoluble (cytoskeleton-associated) proteins were isolated by centrifugation at 14,000g for 5 min, washed 3x with lysis buffer and boiled in Laemmli sample buffer. Triton-soluble proteins were boiled with 2x concentrated Laemmli sample buffer and an identical number of cell equivalents (10^6) from both Triton-soluble and -insoluble fractions were subjected to SDS-PAGE and immunoblotting (see below). Equal protein loading was confirmed by Coomassie staining.

SDS PAGE and immunoblotting. Samples were separated by SDS-PAGE (12% acrylamide) using a BioRad mini-gel apparatus and then transferred to PVDF membranes. After incubation in blocking buffer (0.25% gelatin, 10% ethanolamine and 0.1 M Tris, pH 9) for 2 h at room temperature, membranes were exposed to primary antibody in a buffer that contained 0.25% gelatin, 0.05% NP-40, 0.15 M NaCl, 5 mM EDTA and 50 mM Tris, pH 7.5 for 2 h at room temperature. Membranes were washed (3 times for 15 min) with the same buffer and then further incubated with donkey anti-rabbit or goat anti-mouse antibody conjugated to horseradish peroxidase. Membranes were again washed and detection was made using the enhanced chemiluminescence (ECL) system from Amersham Corp. Quantitation was performed by densitometry of exposed films using a Protein Databases (New York) model DNA 35 scanner with the Discovery series 1D gel analysis software.

To visualize the mobility shift of phosphorylated pleckstrin by immunoblotting, several important modifications were necessary for SDS PAGE of cell lysates. First, longer separation distances were allowed by using 18 x 20 cm gels, of which the stacking gel was no greater than 1 cm in height. Because of the larger size of these gels, complete polymerization was ensured

only after 8 h at 25°C. Finally, running gels overnight (14h at 125V) with cooling provided by a water jacket minimized diffusion and waviness of bands induced by excess heating.

For immunotitration, full-length GST-fusion proteins were electroeluted from SDS-PAGE gels, excess SDS was removed with a desalting column and the protein concentration was determined. By immunoblotting defined amounts of the fusion proteins alongside neutrophil lysates, the content of the respective wild type protein in neutrophils could be estimated. This was accomplished by scanning immunoblots (see above), plotting the optical densities and interpolating the band from cell lysates. Using an approximate cell volume of 350 10^{-15}L/cell (determined using a Coulter-Channelyzer), the cellular concentration of the protein in neutrophils could be estimated.

Kinase renaturation assay. The ability of renaturable kinases to phosphorylate an exogenous substrate, histone $f2_a$ (Sigma, type VI-S), was studied by the method of Kameshita and Fujisawa (Kameshita and Fujisawa, 1989). The application of this method to the study of neutrophils has been previously described elsewhere (Ding and Badwey, 1993; Grinstein, et al., 1993). Briefly, histone f2a (0.5 mg/mL) was dissolved in 12% SDS PAGE solution prior to polymerization. Small amounts of undissolved protein were removed by centrifugation. Following polymerization, the histone remains fixed within the matrix of the gel and does not affect the mobility of proteins that are separated within this gel. After electrophoresis of samples, the SDS was removed by treatment with 20% 2-propanol in 50 mM Tris (pH 8.0). Proteins were fully denatured with 6 M guanidine hydrochloride and then renatured with 0.04% Tween 40, both in a buffer containing 50 mM Tris, 50 mM β-mercaptoethanol. Renatured protein kinases were detected by incubation in kinase buffer (10 mM HEPES, 5 mM MgCl$_2$, 1 mM EGTA, 2 mM DTT) containing 25 μCi ^{32}P-γ-ATP for 60 min at 25°C. The contribution of kinase autophosphorylation is minimized in this assay by inclusion of Mg^{2+} and 25 μM non-radioactive ATP, revealing primarily the phosphorylation of the histone. Finally, gels were washed extensively with wash buffer (5.0 % trichloroacetic acid, 1.0 % sodium pyrophosphate). Negligible radioactivity is detected when the exogenous substrate is omitted (Grinstein, et al., 1993). Phosphorylation of the histone was visualized by

autoradiography of dried gels exposed to Kodak XAR film using an intensifying screen at -70°C for 16–48 h. Quantitation was performed with the use of either a Molecular Dynamics Phosphorimager using Imagequant software or by densitometry with a pdi model DNA 35 scanner with the Discovery series 1D gel analysis software.

Immunoprecipitations. Tyrosine phosphorylated proteins were immunoprecipitated following denaturation of cellular proteins. This was accomplished by resuspending cells (1.5 x 10^7) in 150 μl of denaturing lysis buffer (150 mM NaCl, 1 mM EGTA, 1 mM sodium orthovanadate (tyrosine phosphatase inhibitor), 10 mM NaF (serine/threonine phosphatase inhibitor), protease inhibitors (1 mM PMSF, 10 μg/mL aprotinin, 10 μg/ml leupeptin and 1 μM pepstatin), 10% glycerol and 50 mM Hepes-Na, pH 7.5) containing 1% SDS and boiling for 5 min. Next, 1.5 ml of ice-cold lysis buffer containing 1% NP-40 was added and the suspension was sonicated (three bursts of 5 sec). Lysates were centrifuged at 14,000x g for 5 min to remove insoluble debris and then pre-cleared with 50 μL of Sepharose beads. Antibodies to phosphotyrosine precoupled to agarose beads, previously blocked with 10% BSA in lysis buffer for >1 h, were incubated with these lysates for 2 h at 4°C while rotating end over end.

For immunoprecipitation of proteins from native cell lysates, neutrophil suspensions were treated with or without stimuli, sedimented rapidly and resuspended in ice-cold lysis buffer (1% Triton X-100, 150 mM NaCl, 2 mM EDTA, 1 mM NaVO₄, 5 mM NaF, 1 mM phenylmethylsulfonyl fluoride, 10 μg/mL aprotinin, 10 μg/mL leupeptin and 50 mM Tris, pH 8.0). Complete cellular lysis was ensured by vortexing the samples for 15 sec and incubation on ice for 10 min. Lysates were centrifuged at 14,000g for 5 min and the supernatants were then pre-cleared with 50 μL of Sepharose beads. Antibodies were incubated with these lysates for 2 h at 4°C while rotating end over end. Immune complexes were precipitated by addition of 100 μL of a 50% slurry of protein A-Sepharose beads, previously blocked with 10% BSA in lysis buffer, followed by incubation at 4°C for 2 h. The immunoprecipitates were washed 4–6 times with lysis buffer and then subjected to immune complex assays (see below) or SDS-PAGE and immunoblotting.

Immune complex kinase assay. The kinase activity of immune complexes was determined essentially as described (Burkhardt, 1993). In brief, immunoprecipitates from native lysates were washed with 1 mL of kinase buffer (5 mM $MnCl_2$, 20 mM 8-(N-morpholino) propanesulphonic acid, pH=7.0) and autophosphorylating activity was assayed by incubation with 25 μL of kinase buffer containing 12.5 μCi of [^{32}P]γ-ATP and 1 μM K-ATP. Where specified, 1 μg of rabbit muscle enolase was included as an exogenous substrate. Samples were incubated at 25°C in an Eppendorf Thermomixer and reactions were stopped by addition of boiling 2x concentrated Laemmli sample buffer. The samples were subjected to SDS-PAGE and the gels were stained with Coomassie blue and dried in gel wrap (Biodesign Inc). Dried gels were used for direct quantitation of radioactivity with a Molecular Dynamics Phosphorimager using Imagequant software, or were subjected to radiography with an intensifying screen.

To study the effect of oxidizing agents on the tyrosine kinase activity of Hck, immunoprecipitates of this kinase were isolated from untreated, electroporated cells. After washing, immune complexes were treated for 30 min with 1 mM diamide or 1 mM hydrogen peroxide at 30°C while shaking in a Thermomixer. As above, identical aliquots were used in parallel for immunoblotting and *in vitro* kinase assay. To study the effect of reducing agents on Hck, immune complexes from GTPγS-stimulated cells were treated with 20 mM NAC or 1 mM DTT for 30 min at 37°C and processed as above.

Immune complex tyrosine phosphatase assay. For immune complex phosphatase assays, immunoprecipitates from native lysates were washed once with assay buffer (0.5 mM EGTA, 25 mM HEPES, pH 7.0) and then incubated with 200 μL of assay buffer containing 10 mM p–nitrophenyl phosphate at 37°C for 4-16 hr while shaking. Reactions were stopped by addition of 800 μL of 0.2 M NaOH, beads were sedimented by brief centrifugation and phosphatase activity was assessed by measuring the absorbance of the supernatant at 420 nm.

In vitro **protein kinase C phosphorylation.** Neutrophil suspensions were treated with or without 10^{-7}M TPA for 10 min, sedimented rapidly and then resuspended in

ice-cold lysis buffer. SHP-1 was immunoprecipitated, as above, and immune complexes were washed with kinase assay buffer (1 mM β-mercaptoethanol, 1 mM $CaCl_2$, 1 mM $MgCl_2$ and 20 mM MOPS, pH 7.0). The beads were then incubated for 30 min at 30°C in kinase buffer in the presence of lipid activators (100 nM TPA, 100 μg/mL phosphatidylserine), purified PKC (1.6 μg/mL), 100 μM Na_2ATP and 400 μCi/mL ^{32}P-γ-ATP. Control experiments were performed in the absence of PKC or with immunoprecipitates obtained with non-immune sera. Where the phosphatase activity of SHP-1 was to be determined following PKC phosphorylation, treatments were performed in the absence of ^{32}P-γ-ATP and 1 mM Na_2ATP was present during the incubation of immunoprecipitates with PKC.

^{32}P-orthophosphate labelling and phosphoamino acid analysis. Neutrophil suspensions (2.0 x 10^7 cells/mL) were incubated for 3 h at 37°C in Na^+-rich medium containing 0.5% BSA in the presence of ^{32}P-labelled orthophosphate (2.0 mCi/mL). The cells were washed and treated with or without stimuli before lysis and immunoprecipitation, as above. The precipitates were subjected to SDS-PAGE and blotted onto PVDF membranes. Quantitation of phosphorylation was performed with a Molecular Dynamics phosphorimager, using the ImageQuant software.

Tryptic phosphopeptide analysis. Pleckstrin was immunoprecipitated from ^{32}P-orthophosphate labelled neutrophils or COS-1 cells transiently transfected with wild type pleckstrin. Immunoprecipitated samples were subjected to SDS-PAGE, transferred to nitrocellulose and, following exposure to film, the radioactive band was cut out and processed exactly as described (Craig and Harley, 1996). The TLC plates were exposed to a Phosphorimager screen or directly to film.

Tyrosine phosphatase treatments. For tyrosine phosphatase treatment of native cell lysates, cells were suspended in 375 μl of ice-cold lysis buffer (0.1% Triton X-100, 5% glycerol, 14.1 mM 2-mercaptoethanol, 1 mM PMSF, 1 μg/ml leupeptin, 1 μg/ml aprotinin, 1 μM pepstatin, 10 mM NaF, 1 mM EGTA and 10 mM imidazole, pH 7.2) with and without 1 mM sodium orthovanadate. The suspension was sonicated (three bursts of 5 sec) and then centrifuged for 5 min in an Eppendorf Microfuge at 4°C. Next, 10 μl of a 50% suspension of

the agarose-conjugated tyrosine phosphatase PTP-1B (1 μg total protein) was added to 100 μl aliquots of the supernatant and the mixture was incubated for 30 min at 37°C while tumbling. The beads were then removed by centrifugation, concentrated electrophoresis sample buffer was added to the supernatant and the solution boiled. Samples of phosphatase-treated extracts and the untreated, control extracts (1 x 10^6 cell equivalents) were loaded onto gels and assayed for kinase activity and phosphotyrosine content as described above. Whole cell samples (1 x 10^6 cell equivalents) were also loaded as a reference.

To prevent inactivation of the renaturable kinases by endogenous mechanisms, cell lysates were prepared under denaturing conditions, as described above for immunoprecipitation of tyrosine phosphorylated proteins. Following dilution of the denatured supernatant, 30 μl of a 50% suspension of the agarose-conjugated tyrosine phosphatase PTP-1B was added and the mixture was incubated for 30 min at 37°C while tumbling. The beads were then removed by centrifugation and the lysate was concentrated by centrifugation (4000g for 2.5 h) at 4°C on 30 kDa cut-off filters. Electrophoresis sample buffer was then added to the concentrated lysate and the solution boiled. Samples of the phosphatase-treated and untreated lysates were loaded onto gels and assayed for renaturable kinase activity and phosphotyrosine content as described above.

To study the role of tyrosine phosphorylation in tyrosine kinase activation, immunoprecipitates of Hck obtained from GTPγS-treated cells were incubated at 37°C for 30 min with or without 2 μg/mL of TC-PTP. Aliquots of the beads were used for kinase assays and for immunoblotting with anti-phosphotyrosine antibodies to confirm the effectiveness of dephosphorylation by TC-PTP.

Immunoelectron microscopy. Neutrophils were fixed in suspension using 2% paraformaldehyde and 0.05% glutaraldehyde in 0.1 M phosphate buffer (pH 7.4) for 30 min at room temperature. The cells were washed and then infiltrated in polyvinyl pyrrolidone/sucrose overnight, before freezing, sectioning with a Reicher-Jung Ultracut E microtome and labeling with the antibodies. The details of preparation of frozen ultrathin sections have been previously published (Stenberg, et al., 1985). A 1/100 dilution of affinity-purified rabbit anti-VAMP-2

was used. Goat anti-rabbit antibodies coupled to 10 nm gold particles (1:20 dilution, from Jansen) were used as a reporter. The grids were next stained and protected with 3% uranyl acetate and embedded in 1.5% methyl cellulose, according to the method of Tokuyasu (Tokuyasu, 1983). Control experiments were performed by replacing specific primary antibody with a comparable dilution of the corresponding pre-immune antisera that had been subjected to the affinity purification procedure.

Immunofluorescence microscopy. To study the localization of specific proteins in untreated cells, neutrophil suspensions (10^6 cells/mL) were allowed to adhere to glass coverslips for 4 min. Adherent cells were washed with PBS before fixation and permeabilization with methanol at -20°C for 10 min. To analyse the effects of stimulation, neutrophils were allowed to adhere to glass coverslips coated with fibrinogen (100 μg/ml), washed with Na$^+$-rich medium and then incubated at 37°C for 2-5 min in the presence or absence of 0.5 μM ionomycin in the same medium. Following incubation, coverslips were washed and then fixed with 2% paraformaldehyde in PBS for 40 min. Cells were permeabilized by treatment with a buffer containing 0.1% Triton X-100, 100 mM PIPES (pH 6.8), 5 mM EGTA, 100 mM KOH and 2 mM MgCl$_2$ for 15 min at room temperature. For both methods used, fixed cells were blocked with 5% donkey serum in PBS for 2-4 h, washed twice with PBS and incubated with primary antibody for 2 h in PBS containing 1% BSA. The samples were then washed three times with PBS and incubated with secondary antibody for 2 h, also in PBS containing 1% BSA. After washing three times with PBS, the samples were treated with Slow Fade (Molecular Probes) before mounting. Control experiments were performed by replacing specific primary antibodies by a comparable dilution of the corresponding pre-immune antisera that had been subjected to the affinity purification procedure. Samples were analyzed using a Zeiss LSM 410 Model laser confocal microscope with a 63x objective. Digitized images were cropped in Adobe Photoshop and imported to Adobe Illustrator for assembly and labelling.

Reverse transcriptase-polymerase chain reaction. To identify SHP-1 in neutrophils, total RNA was isolated from fresh cells and subjected to RT-PCR using the Gene

Amp RNA PCR kit from Perkin Elmer. The primers SHP-11002-5' (5'-CAGGAGAACACTCGTGTCAT-3') and SHP-11122-3' (5'-TGTATGGTATTGAACAAGGACC-3') were used to amplify a 120-bp mRNA fragment of SHP-1, as previously described (Kozlowski, et al., 1993). The PCR conditions used were: 5 min at 95°C followed by 25 cycles of 30 sec at 94°C, 50°C for 30 sec, 72°C for 2 min and 72°C for 7 min (last cycle). Samples of the final reaction mixture were then subjected to agarose gel electrophoresis and visualized by ethidium bromide staining. As a positive control, the amplification of a ribonucleotide sequence encoding IL-1α (pAW109 RNA, Perkin Elmer) was performed.

Other methods. Alkaline phosphatase treatment of denatured neutrophil lysates was performed as previously described (Brumell and Grinstein, 1994). For removal of lipids, neutrophil suspensions were sedimented rapidly and resuspended in acetone (-20°C). Samples were vigorously vortexed for several minutes and subjected to centrifugation at 14,000g for 5 min. Following aspiration, complete removal of acetone was achieved by drying under a stream of nitrogen and proteins in the pellet were solubilized in Laemmli sample buffer and analyzed by SDS-PAGE and immunoblotting. [Ca^{2+}]$_i$ was measured fluorimetrically using indo-1 and calibrated using ionomycin and Mn^{2+} as described previously (Grinstein and Furuya, 1988). Where specified, cells were loaded with BAPTA by incubation with 10 μM of the precursor acetoxymethlester.

Chapter 3

Chemoattractant-induced serine/threonine kinase activation in human neutrophils: relationship to tyrosine phosphorylation.

Summary

Tyrosine phosphorylation of several substrates is among the earliest responses of human neutrophils stimulated with chemotactic peptides. Some of these tyrosine phosphorylated proteins comigrate with serine/threonine kinases of 65 kDa and 72 kDa (PK65 and PK72), which are activated concomitantly by the chemoattractants. The present studies were designed to test whether tyrosine phosphorylation is required for activation of PK65 and PK72. Pretreatment of the cells with the tyrosine kinase inhibitors erbstatin or genistein prevented both phosphotyrosine accumulation as well as activation of PK65 and PK72. In nondenaturing lysates, PK65 and PK72 became spontaneously inactivated, in parallel with rapid endogenous tyrosine dephosphorylation. Spontaneous dephosphorylation and inactivation of PK65 and PK72 were prevented in denatured lysates. Under these conditions, dephosphorylation could be induced by exogenous phosphotyrosine phosphatase 1B. PK65 and PK72 activation persisted despite the virtually complete tyrosine dephosphorylation of the samples. Moreover, immunoprecipitation experiments indicated that PK65 and PK72 are not themselves tyrosine phosphorylated. I concluded that tyrosine phosphorylation is a necessary upstream event in the activation of the serine/threonine kinases. However, once the post-translational modification that renders PK65 and PK72 active has occurred, tyrosine phosphorylation is no longer required for maintenance of their kinase activity.

Introduction

A family of chemoattractant-activated serine/threonine kinases has been described in rodent and human PMN (Ding and Badwey, 1993; Ding and Badwey, 1993; Grinstein, et al., 1993). Following fractionation by electrophoresis in denaturing polyacrylamide gels, these unique enzymes have the ability to renature and to phosphorylate exogenous substrates previously immobilized within the matrix of the gel. Two members of the renaturable serine/threonine kinase family, with approx. molecular masses 65 kDa and 72 kDa, are rapidly and transiently activated in response to fMLP. I recently observed that activation of these kinases (referred to hereafter as PK65 and PK72), coincides and may even be preceded by tyrosine phosphorylation of proteins with similar molecular masses. This raised the possibility that activation of PK65 and PK72 involves tyrosine phosphorylation of the kinases themselves or of ancillary proteins. Experiments designed to test this hypothesis are the subject of the present communication.

Results

It has been previously demonstrated that increased tyrosine phosphorylation accompanies chemotactic stimulation of human PMN (Berkow, et al., 1989). A more detailed time course of this event is illustrated in Fig. 3.1A, where lysates obtained from cells stimulated for varying periods with fMLP were analyzed by immunoblotting with polyclonal anti-phosphotyrosine antibodies. In accord with earlier studies, fMLP was found to induce phosphotyrosine accumulation in proteins of 106, 44 and 42 kDa. The latter two bands are thought to correspond to the Erk-1 and -2 isoforms of MAP-kinase, respectively. Also phosphorylated in a very rapid and transient manner were a number of proteins in the 62-78 kDa region. Labelling of these proteins was transient, peaking after a 15 sec exposure to fMLP, the earliest time measured, and approaching baseline levels by 2 min. This rapid and transient pattern parallels the course of some of the biological responses to the chemoattractant, including actin polymerization and superoxide generation.

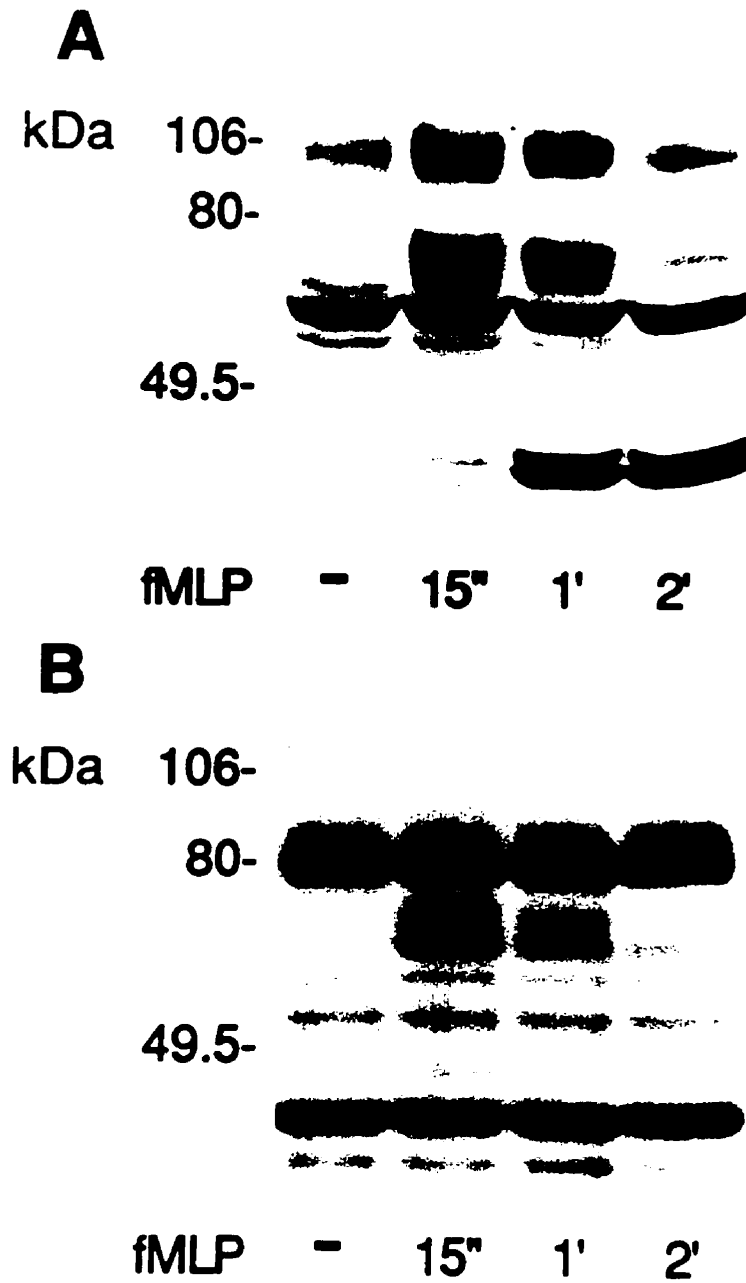Fig. 3.1. Time course of tyrosine phosphorylation and renaturable kinase activation induced by fMLP. Intact human neutrophils were treated with or without (-) 10^{-7} M fMLP for the indicated times, sedimented and prepared for SDS-PAGE. Samples were immunoblotted with polyclonal antiphosphotyrosine antibodies (A) or were assayed for renaturable kinase activity (B), as described in the Experimental Procedures section.

The course of activation of the kinases was studied by the method of Kameshita and Fujisawa (Kameshita and Fujisawa, 1989), using histone VI-S as the substrate (see Experimental section). This method takes advantage of the ability of certain kinases to renature within polyacrylamide gels following electrophoresis in the presence of SDS. Typical results are shown in Fig. 3.1B. Constitutively active kinases of approx. 85, 78, 54, 43 and 39 kDa were detected in unstimulated cells. Phosphorylation of these bands was somewhat variable and not affected by stimulation with fMLP. It is noteworthy that, despite the similar mobility, the 43 kDa band is unlikely to represent MAP-kinase, which has been shown to be essentially inactive in untreated PMN (Grinstein and Furuya, 1992). Two major kinases, PK65 and PK72 were reproducibly activated by fMLP. Stimulation was maximal after 15 sec, followed by rapid inactivation, declining towards baseline levels by 2 min. This pattern clearly parallels the course of tyrosine phosphorylation of bands with comparable mobility. Activation of Erk-1 and -2 by fMLP is not apparent in this assay because these enzymes are unable to use histone f2$_a$ as a substrate.

The possible relationship between tyrosine phosphorylation and the activation of PK65 and PK72 was investigated further using tyrosine kinase inhibitors. Pretreatment of the cells for 30 min with erbstatin (Fig. 3.2A) induced a modest increase in the tyrosine phosphorylation of unstimulated cells, by a mechanism that is unclear. More importantly, the inhibitor largely abolished the increase in phosphotyrosine accumulation induced by the chemotactic peptide. Phosphorylation of MAP-kinase was eliminated and that of the bands in the 62-78 kDa range was greatly diminished (Fig. 3.2A). In parallel, the activation of PK65 and PK72 was largely eliminated by erbstatin (Fig. 3.2B). In three separate experiments, the activation of PK65 and PK72 induced by fMLP was inhibited by 85.8 ± 7.0% (measuring PK65 and PK72 activities together). These findings further support a relationship between tyrosine phosphorylation and activation of PK65 and PK72.

A family of renaturable kinases was described recently in guinea pig PMN (Ding and Badwey, 1993; Ding and Badwey, 1993). Like those of human PMN, the rodent cells are activated by soluble agonists. The authors of these studies speculated that dephosphorylation

Fig. 3.2 Effect of erbstatin on tyrosine phosphorylation and renaturable kinase activation. Intact human neutrophils were incubated for 30 min at $25^{\circ}C$ in the presence (+) or absence (-) of the tyrosine kinase inhibitor erbstatin. The cells were then stimulated with 10^{-7} M fMLP for the indicated times, sedimented and prepared for SDS-PAGE. Samples were immunoblotted with polyclonal anti-phosphotyrosine antibodies (A) or were assayed for renaturable kinase activity (B).

of serine/threonine residues of the kinases was central to their activation, based on the observation that okadaic acid and calyculin antagonize the activation process (Ding and Badwey, 1993; Ding and Badwey, 1993). These agents are potent and specific blockers of phosphatases 1 and 2A (Cohen, et al., 1990). On the other hand, our studies in human cells appeared to indicate that phosphorylation of tyrosine residues is required for activation. In an attempt to reconcile these observations, I tested the effects of calyculin A and okadaic acid on activation of the human PK65 and PK72 and on the tyrosine phosphorylation induced by fMLP. Fig. 3.3B shows that, as in rodent PMN, calyculin A induced spontaneous activation of several kinases (e.g. 100, 60-62 and 55 kDa) in otherwise unstimulated cells. More importantly, the phosphatase blocker precluded the activation of PK65 and PK72. Similar results were obtained with okadaic acid (not illustrated).

Unexpectedly, I found that calyculin A (Fig. 3.3A) and okadaic acid (not shown) also profoundly affected the pattern of tyrosine phosphorylation in human PMN. The phosphatase antagonists induced the spontaneous phosphorylation of Erk-1 and -2, which accords with earlier reports of activation of this kinase in other cell types (Haystead, et al., 1990). In addition, calyculin and okadaic acid greatly inhibited tyrosine phosphorylation of the proteins in the 62-78 kDa region, which comigrate with PK65 and PK72 (Fig. 3.3A). It therefore appears that the extent of serine/threonine phosphorylation is an important determinant of the process of phosphotyrosine accumulation. These findings also suggest that control of the activity of the renaturable kinases by calyculin and okadaic acid may be indirect, possibly through alterations in tyrosine phosphorylation.

The observations summarized above prompted us to consider the possibility that PK65 and PK72 require phosphorylation on tyrosine residues for their activation. For this purpose, I assessed the effects of tyrosine dephosphorylation on the activity of the renaturable kinases, using the phosphotyrosine-specific phosphatase 1B (PTP-1B). The effectiveness of PTP-1B was monitored in parallel by immunoblotting with anti-phosphotyrosine antibodies. Typical results are shown in Fig. 3.4A. The increased tyrosine phosphorylation induced by fMLP persists in lysates prepared in non-denaturing detergent. However, when the same number of
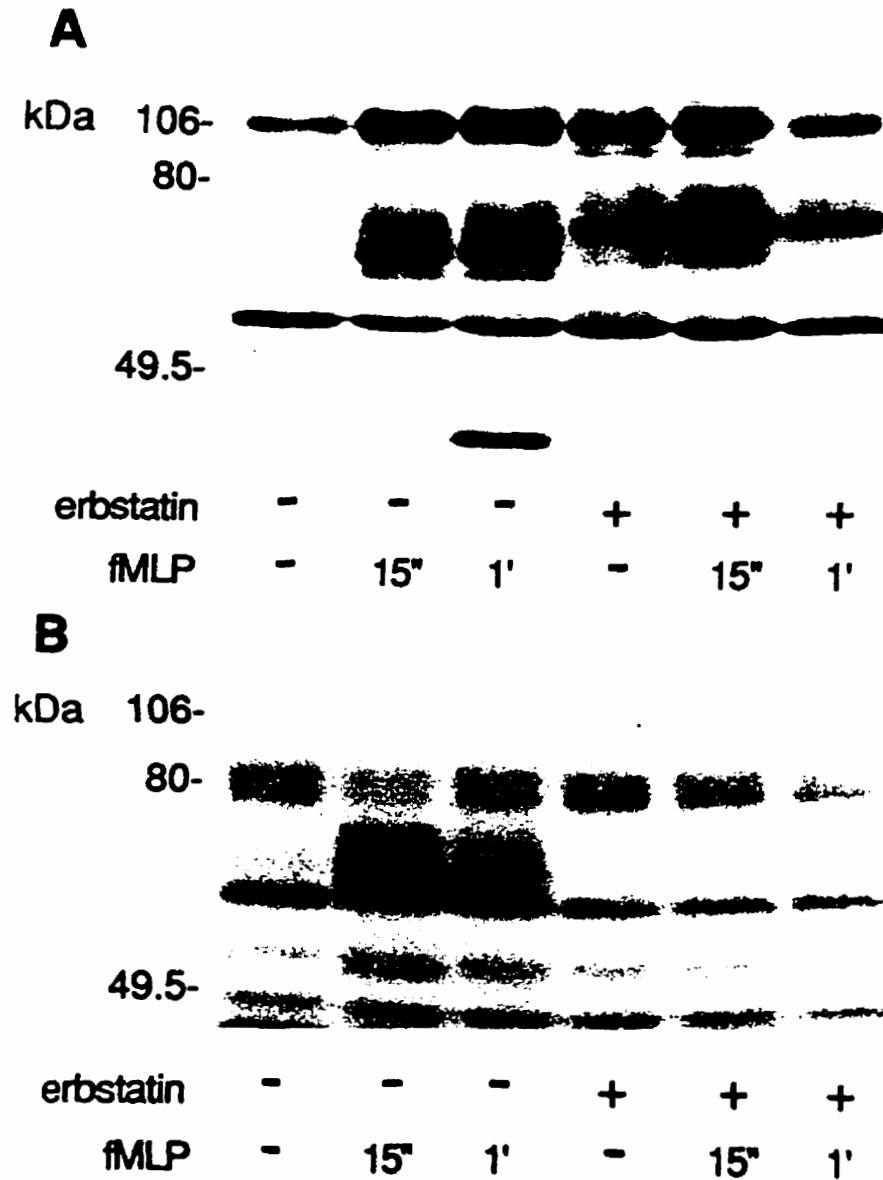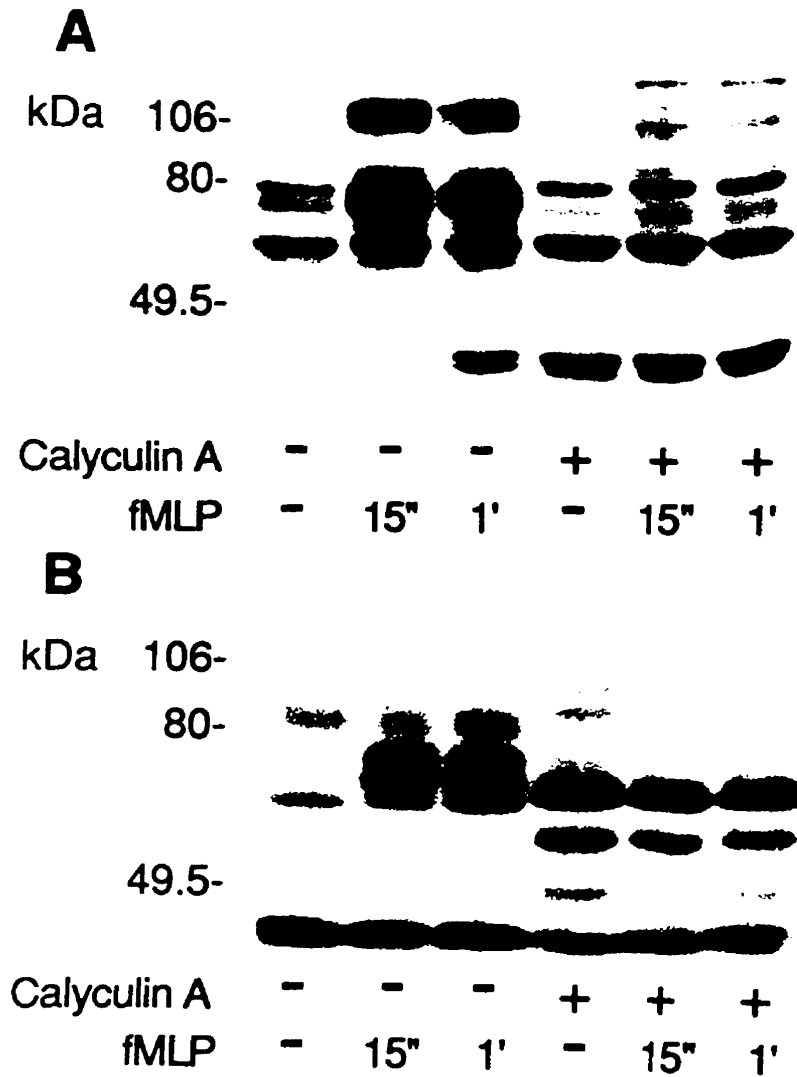
Fig. 3.3   Effect of calyculin A on tyrosine phosphorylation and renaturable kinase activation. Intact human neutrophils were incubated for 30 min at 25°C in the presence (+) or absence (-) of the serine/threonine phosphatase inhibitor calyculin A. The cells were then stimulated with 10^{-7} M fMLP for the indicated times, sedimented and prepared for SDS-PAGE. Samples were immunoblotted with polyclonal antiphosphotyrosine antibodies (A) or were assayed for renaturable kinase activity (B).

cell equivalents are compared, the phosphorylation of the lysate supernatant is lower than that of the whole cells (compare second and fifth lanes of Fig. 3.4A), suggesting that extraction of the phosphoproteins by the non-denaturing detergent is incomplete, or that partial dephosphorylation occurred during extraction. That the latter explanation is at least partially correct is suggested by the extensive reduction of tyrosine phosphorylated proteins noted to occur when the lysates were incubated for 30 min at 37°C (sixth lane). A further decrease was noted when PTP-1B was included during this incubation. I regard the occurrence of proteolysis as unlikely because: i) the cells had been pretreated with diisopropylfluorophosphate and the lysis buffer contained a cocktail of other protease inhibitors and ii) the Coomassie blue staining patterns of cells before and after the 30 min incubation were indistinguishable (not shown). The conclusion that dephosphorylation by endogenous phosphatases is responsible for the decreased phosphotyrosine accumulation was confirmed using vanadate (see the two rightmost lanes in Fig. 3.4A). This phosphotyrosine phosphatase inhibitor largely preserved the tyrosine phosphorylation effected by fMLP even after a 30 min incubation in the non-denaturing lysis buffer. Phosphorylation in stimulated lysates prepared with vanadate was comparable to that of whole cell preparations, indicating that endogenous tyrosine phosphatases, rather than proteases, were responsible for the spontaneous disappearance of phosphotyrosine.

Renaturable kinase activity in parallel samples was assessed in Fig. 3.4B. Comparison of the phosphorylation by PK65 and PK72 in whole cells vs. lysates indicates that extraction in non-denaturing detergent led to a sharp decrease in kinase activity, paralleling the decrease in tyrosine phosphorylation. Activity was further reduced by incubation of the stimulated lysate for 30 min at 37°C, both in the presence or absence of PTP-1B. Extracts prepared using lysis buffer containing vanadate yielded two interesting findings. First, the activity of PK65 and PK72 was markedly decreased in the extracts, despite the fact that tyrosine phosphorylation was comparable to that of whole cells (Fig.3.4A and B). Second, the kinases are further inactivated during the 30 min incubation (to $18.2 \pm 2.3\%$ of the activity at zero time for PK65 and $15.1 \pm 6.7\%$ for PK72, n=4) while tyrosine phosphorylation is virtually unaffected during
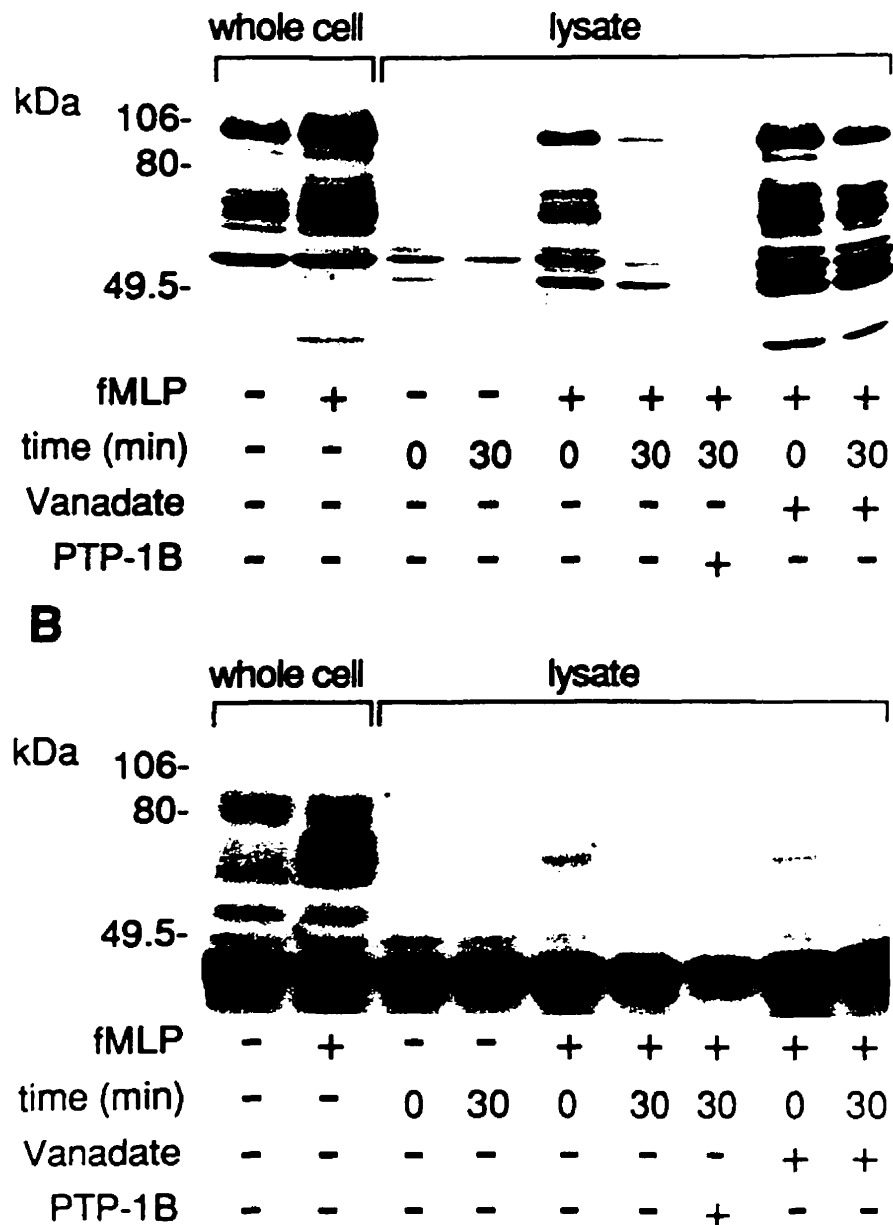
Fig. 3.4   Effect of PTP-1B and vanadate on tyrosine dephosphorylation and renaturable kinase activity.   Lysates from control and fMLP-stimulated (15 sec) neutrophils were prepared with a buffer that contained 1 mM sodium vanadate where indicated (+).   The lysates were then incubated at 37°C for the indicated times in the presence or absence of the tyrosine-specific phosphatase PTP-1B.   Samples of the lysate and whole cells (intact neutrophils directly solubilized in Laemmli buffer) were prepared for SDS-PAGE and either immunoblotted with polyclonal antiphosphotyrosine antibodies (A) or assayed for renaturable kinase activity (B).

this period. These observations strongly suggest that PK65 and PK72 can be inactivated during extraction by a mechanism other than dephosphorylation of tyrosine residues.

While it appears unlikely that tyrosine phosphorylation is the sole post-translational modification regulating the activity of PK65 and PK72, it is conceivable that dual phosphorylation on tyrosine and threonine (or serine) residues is required. It was therefore important to establish the effect of tyrosine dephosphorylation on kinase activity, without the confounding effects of endogenous inactivating mechanisms. This was accomplished by rapidly boiling control and fMLP-stimulated cells in a lysis buffer containing 1% SDS (see Experimental Procedures). After denaturing, the lysates were cooled and diluted to reduce the concentration of SDS to a level compatible with the activity of PTP-1B. The increased tyrosine phosphorylation induced by fMLP is clearly noticeable in extracts prepared in this manner (Fig. 3.5A). Importantly, stimulated lysates undergo only slight tyrosine dephosphorylation after a 30 min incubation at 37°C, particularly in the 62-78 kDa range, attesting to the efficacy of the denaturing lysis protocol to inactivate endogenous tyrosine phosphatases. Treatment of equivalent samples with exogenous PTP-1B, in contrast, eliminated all phosphotyrosine from the samples (rightmost lane, Fig. 3.5A).

This paradigm enabled us to specifically test the effect of tyrosine dephosphorylation on the activity of PK65 and PK72. As shown in Fig. 3.5B, stimulation of PK65 and to a lesser extent PK72 was detectable in extracts from fMLP-treated cells obtained under denaturing conditions. The reduced activity of these extracts compared to that of an equivalent amount of whole cells likely reflects incomplete solubilization, since protein aggregation and precipitation occurs upon boiling, despite the presence of 1% SDS. This phenomenon is likely also responsible for the modified phosphotyrosine pattern of the extract compared to the whole cells (Fig. 3.5A). Alternatively, rapid partial inactivation may have ocurred before total denaturation by SDS. As shown in Fig. 3.5B, incubation of the extract for 30 min did not reduce the activity of the kinases, indicating that boiling in SDS eliminated the endogenous inactivating factor(s). Under these conditions, the effect of PTP-1B could be readily assessed. As shown by the last lane of Figs. 3.5A and B, the renaturable kinase activity was largely preserved under
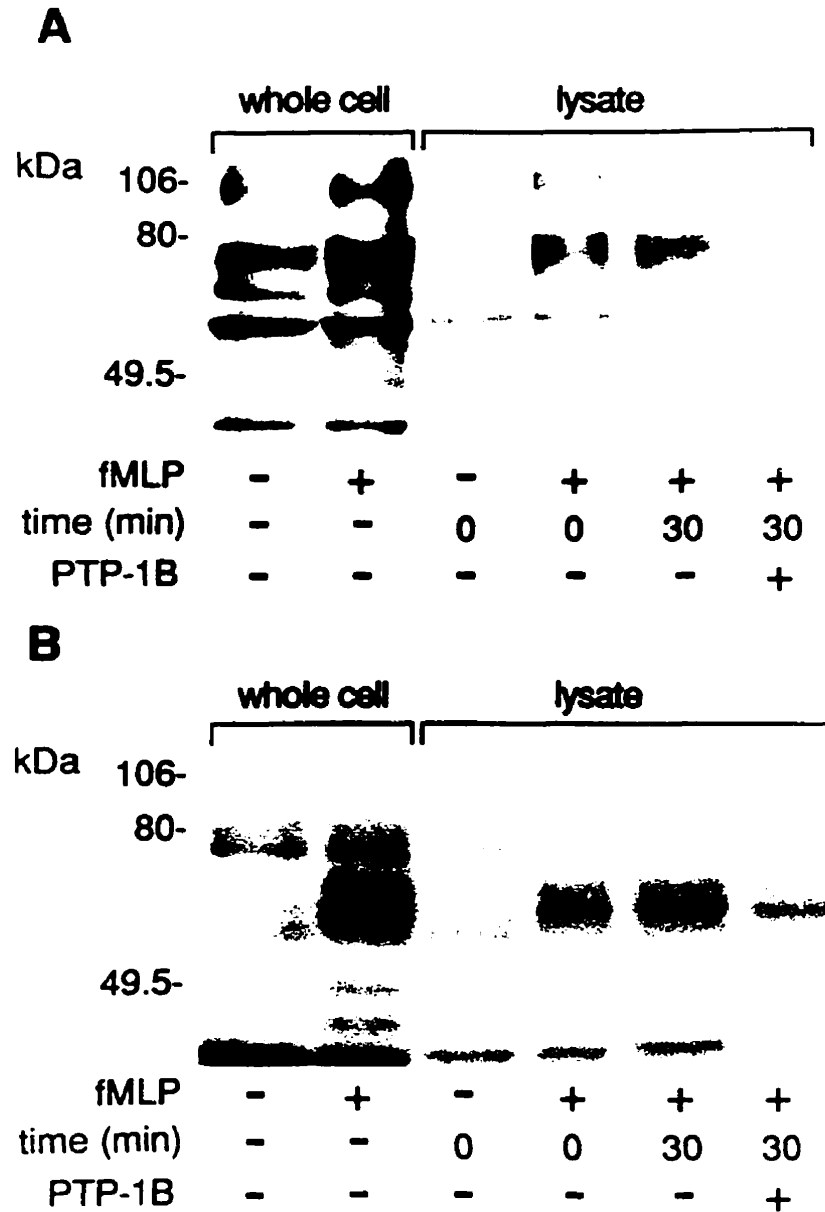
Fig. 3.5   Effect of PTP-1B on denatured cell lysates. Denatured lysates from control and fMLP stimulated (15 sec) neutrophils were prepared as described in Experimental Procedures. The lysates were then incubated for the indicated times in the presence or absence of the tyrosine-specific phosphatase PTP-1B. Samples of the lysate and of whole cells (intact neutrophils directly solubilized) were prepared for SDS-PAGE and either immunoblotted with polyclonal antiphosphotyrosine antibodies (A) or assayed for renaturable kinase activity (B).

conditions where tyrosine phosphorylation was essentially eliminated. PTP-1B treatment led to only a modest decrease in the renaturable activities of PK65 (32.3 ± 2.3%) and PK72 (21.6 ± 3.3%) as measured by densitometry. The combined results of Figs. 3.4 and 3.5 indicate that tyrosine phosphorylation of the 62-78 kDa bands is not required for the activity of PK65 and PK72.

While tyrosine phosphorylation is not essential for the catalytic activity of PK65 and PK72, phosphotyrosine residues on these proteins may play a modulatory or targetting role, perhaps by interaction with Src homology 2 (SH2) domains. It was therefore important to establish whether PK65 and PK72 are in fact phosphorylated on tyrosine residues. To this end, denatured cell lysates were prepared from stimulated cells and tyrosine phosphorylated proteins were immunoprecipitated using monoclonal anti-phosphotyrosine antibodies attached to agarose beads. The results of these experiments are illustrated in Fig. 3.6. The beads effectively precipitated tyrosine phosphorylated proteins, which were concomitantly depleted from the supernatant (Fig. 3.6A). The specificity of the precipitation protocol was confirmed by repetition of the experiment in the presence of an excess free phosphotyrosine during incubation with the beads. As shown in the two rightmost lanes of Fig. 3.6A, this prevented precipitation of tyrosine phosphorylated proteins, which remained in the supernatant. For technical reasons a lower amounts of cell equivalents was loaded in the supernatant lane, accounting for its reduced phosphotyrosine content compared to the whole cell extract (see Experimental section).

Kinase renaturation assays were performed in the immunoprecipitates and supernatants obtained in the presence and absence of excess phosphotyrosine. Fig. 3.6B illustrates a typical experiment. The extracted kinases were found to be quantitatively present in the supernatant, with no detectable activity in the immunoprecipitate, despite the large accumulation of tyrosine phosphorylated proteins in the 62-78 kDa region. A similar distribution of the kinase activity was detected from samples immunoprecipitated in the presence of phosphotyrosine. Therefore, PK65 and PK72 do not appear to be tyrosine phosphorylated in response to fMLP.
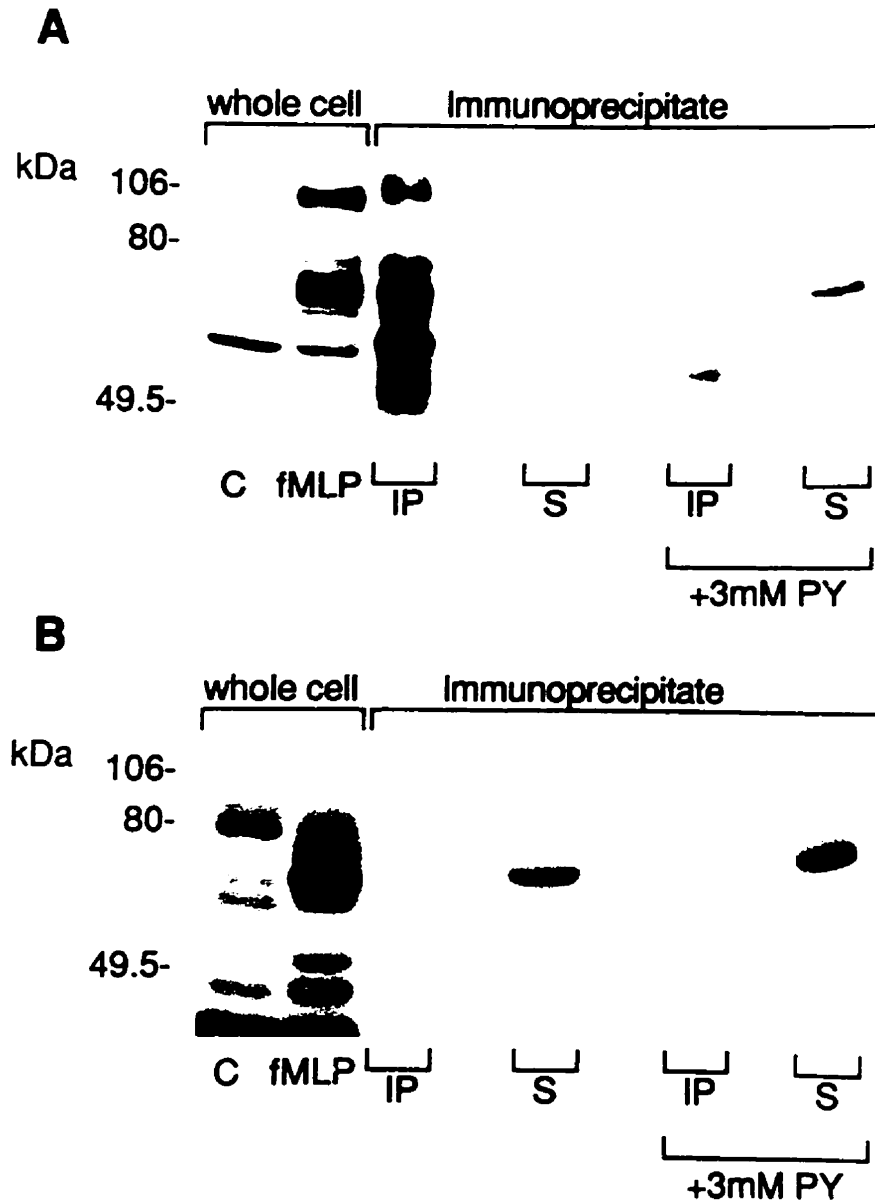
Fig. 3.6   Immunoprecipitation of tyrosine phosphorylated proteins. Agarose-conjugated anti-phosphotyrosine antibodies were used to precipitate tyrosine phosphorylated proteins from control (C) and fMLP stimulated (15 sec) neutrophils (see Experimental Procedures). Whole cell (intact neutrophils), immunoprecipitate (IP) and concentrated supernatant (S) samples were prepared for SDS-PAGE and either immunoblotted with polyclonal antiphosphotyrosine antibodies (A) or assayed for renaturable kinase activity (B). Where indicated, excess (3 mM) free phosphotyrosine was present during the immunoprecipitation to establish antibody specificity.

Discussion

In human PMN, activation of PK65 and PK72 by fMLP is temporally associated with the induction of extensive tyrosine phosphorylation. As determined by SDS-PAGE, some of the tyrosine phosphorylated substrates have very similar mobility to PK65 and PK72. These observations motivated the present studies, aimed at defining whether tyrosine phosphorylation was part of the mechanism leading to activation of the renaturable kinases and whether PK65 and PK72 themselves are phosphorylated on tyrosine residues.

Several observations are consistent with the notion that phosphotyrosine accumulation is involved in the process of kinase activation. First, the kinetics of activation and deactivation of PK65 and PK72 closely resemble the course of tyrosine phosphorylation/dephosphorylation of proteins in the 62-78 kDa region. Second, treatment with erbstatin not only inhibited the fMLP-induced tyrosine phosphorylation, but also blocked the activation of PK65 and PK72. Finally, calyculin A and okadaic acid similarly impaired both processes, by a mechanism that remains unresolved.

On the other hand, it is apparent that PK65 and PK72 are not themselves tyrosine phosphorylated, as indicated by the immunoprecipitation experiments of Fig. 3.6. I therefore conclude that tyrosine phosphorylation is a necessary upstream event in the activation of the serine/threonine kinases. However, once the post-translational modification that renders PK65 and PK72 active following renaturation has occurred, tyrosine phosphorylation is no longer required for maintenance of their kinase activity.

Unexpectedly, the chemoattractant-induced tyrosine phosphorylation of PMN was found to be reduced by the serine/threonine phosphatase inhibitors calyculin A and okadaic acid. This effect does not reflect a nonspecific inhibition of tyrosine phosphatases, as this would be anticipated to have the opposite effect, i.e. enhanced phosphotyrosine accumulation. Instead, serine/threonine phosphorylation is likely to modulate the activity of tyrosine kinases or phosphatases that are regulated by chemoattractants.

In summary, I conclude that PK65 and PK72 are not themselves tyrosine phosphorylated, but their activation is seemingly controlled by an upstream tyrosine kinase.

Since tyrosine kinase inhibitors effectively block several components of the microbicidal response and because the kinetics of tyrosine phosphorylation and of activation of PK65 and PK72 are compatible with a role in signalling activation, the establishment of their relationship and mode of action is of great interest.

Chapter 4

Endogenous Reactive Oxygen Intermediates Activate Tyrosine Kinases in Human Neutrophils.

Summary

In response to invading microorganisms, neutrophils produce large amounts of superoxide and other ROI by assembly and activation of a multi-component enzyme complex, the NADPH oxidase. While fulfilling a microbicidal role, ROI have also been postulated to serve as signalling molecules, since activation of the NADPH oxidase was found to be associated with increased tyrosine phosphorylation (Fialkow, et al., 1993). The mechanism whereby ROI induces phosphotyrosine accumulation was investigated using electroporated neutrophils stimulated with GTPγS, in order to bypass membrane receptors. *In vitro* immune complex assays and immunoblotting were used to identify five tyrosine kinases present in human neutrophils. Of these, Hck, Syk and Btk were activated during production of ROI. Interestingly, the *in vitro* autophosphorylation activities of Lyn and Fgr were found to decline with ROI production. The mode of regulation of Hck was explored in detail. Oxidizing agents were unable to activate Hck *in vitro* and, once activated *in situ*, reducing agents failed to inactivate it, suggesting that the effects of ROI are indirect. Tyrosine phosphorylation of Hck paralleled its activation and dephosphorylation *in vitro* reversed the stimulation. I therefore conclude that tyrosine phosphorylation is central to the regulation of Hck and likely also of Syk, which is similarly phosphorylated upon activation of the oxidase. As ROI have been shown to reduce the activity of tyrosine phosphatases, I suggest that this inhibition allows constitutively active kinases to auto/transphosphorylate on stimulatory tyrosine residues, leading to an increase in their catalytic activity. Enhanced phosphotyrosine accumulation would then result from the combined effects of increased phosphorylation with decreased dephosphorylation.

Introduction

Recent observations have suggested a role for ROI in neutrophil signal transduction (Fialkow, et al., 1993). Neutrophils stimulated to produce ROI were reported to undergo increased tyrosine phosphorylation of several proteins. Exogenous oxidants were able to mimic this response while anti-oxidants could block it. Several lines of evidence suggested that ROI generated by the NADPH oxidase were responsible for the effect, including the finding that the increased tyrosine phosphorylation failed to occur in neutrophils from patients with chronic granulomatous disease. Inasmuch as tyrosine phosphorylation is an important mediator in the regulation of anti-microbial responses, ROI may play an important role in the control of auto/paracrine signalling at sites of inflammation.

The extent of tyrosine phosphorylation is determined by the activity of two competing enzyme families, tyrosine kinases and phosphatases. Earlier *in vitro* (Hecht and Zick, 1992) and *in vivo* (Zor, et al., 1993) studies have suggested that ROI can inhibit the activity of certain tyrosine phosphatases by oxidation of a conserved cysteine residue within their catalytic domain. While the inhibition of tyrosine phosphatases may account for the elevated tyrosine phosphorylation induced by ROI, increased activity of tyrosine kinases could conceivably contribute to the response. Indeed, tyrosine kinases have been reported to be activated in lymphocytes by oxidizing agents (Bauskin, et al., 1991; Nakamura, et al., 1993). For these reasons, I investigated whether endogenous ROI, generated by the NADPH oxidase, affected the activity of tyrosine kinases in human neutrophils.

Results

The effect of ROI on tyrosine phosphorylation was studied in electropermeabilized cells stimulated with GTPγS. This approach was chosen for two reasons. First, direct stimulation of GTP-binding proteins bypasses cell surface receptors, circumventing possible direct effects of the latter on the kinases and obviating receptor down-regulation, which can greatly reduce the magnitude and duration of the respiratory burst (Klotz and Jesaitis, 1994). Using GTPγS, activation of the oxidase is sustained, resembling the physiological stimulation elicited by

phagocytic stimuli (Grinstein and Furuya, 1991). Second, equilibration of the permeabilized cells with EGTA-containing buffers precludes changes in cytosolic calcium concentration, which might alter tyrosine phosphorylation (Berkow and Dodson, 1990).

As shown in Fig. 4.1A, addition of GTPγS and NADPH to permeabilized cells induced the accumulation of phosphotyrosine on a number of proteins, as determined by immunoblotting (cf. lanes 1 and 4). Treatment of the electroporated cells with GTPγS or NADPH alone was found to have little effect (lanes 2 and 3). The stimulatory effect of GTPγS or NADPH was moderated by the presence of active tyrosine phosphatases. This is indicated by the pronounced enhancement in phosphotyrosine accumulation noted when vanadate, a phosphatase inhibitor, was included during stimulation (Fig. 4.1A). For this reason, 10 μM sodium orthovanadate was included routinely in subsequent assays to minimize dephosphorylation, thereby magnifying the responses. At the concentration used, vanadate itself had negligible effects on tyrosine phosphorylation (see lanes 1 and 2 in Fig. 4.1C), consistent with earlier findings (Bourgoin and Grinstein, 1992). Moreover, while vanadate increased the extent of phosphotyrosine accumulation, the phosphorylated substrates and the time course of phosphorylation were similar in the presence and absence of the phosphatase inhibitor. As illustrated in Fig. 4.1B, phosphotyrosine accumulation induced by GTPγS stimulation was rapid (evident after 1 min) and time dependent, with a maximal response seen after 10 min.

The effect of GTPγS on tyrosine phosphorylation was entirely dependent on the presence of NADPH. As shown in Fig. 1C (as well as in Fig. 4.1A), treatment of electroporated cells with GTPγS had little effect when the nucleotide was omitted (cf. lanes 3 and 4). This finding suggests that generation of superoxide by the NADPH oxidase is required for the increase in tyrosine phosphorylation following stimulation with GTPγS. In support of this hypothesis, it was found that NAC, a powerful anti-oxidant that has been shown to scavenge ROI and increase cytosolic levels of reduced glutathione (Halliwell and Gutteridge, 1985), effectively attenuated the tyrosine phosphorylation produced by GTPγS in the presence of NADPH. Moreover, DPI, an inhibitor of the flavoprotein component of the NADPH
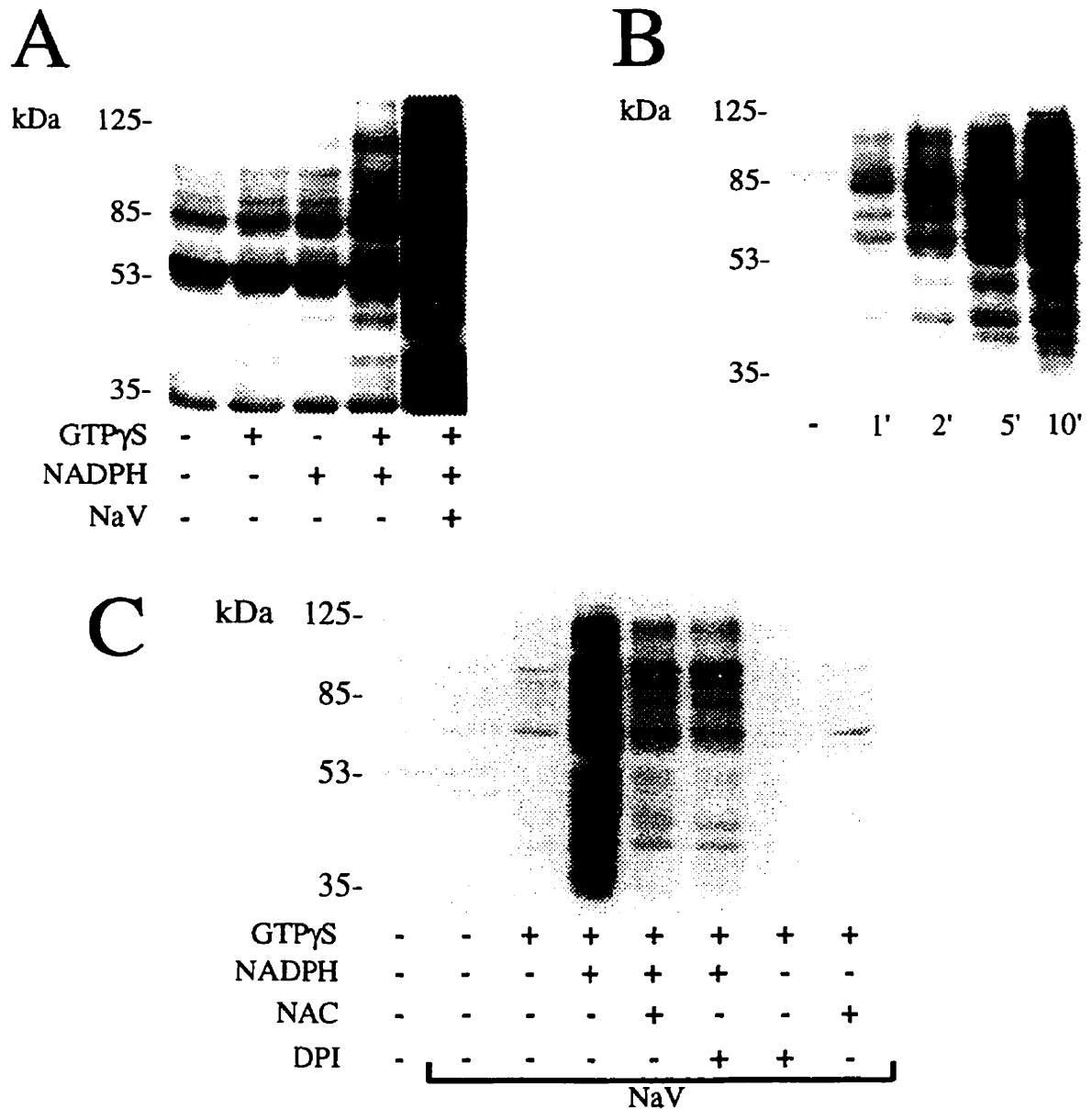
Fig. 4.1.   Effect of GTPγS on tyrosine phosphorylation. A. NADPH dependence and potentiation by sodium vanadate. Electroporated neutrophils were incubated at 37°C without (-) or with (+) the following agents for 5 min: 10 μM GTPγS, 2 mM NADPH and 10 μM Na-orthovanadate (NaV), as indicated. Cells were then rapidly sedimented, boiled in sample buffer and subjected to SDS-PAGE. Analysis was performed by immunoblotting with a monoclonal antibody to phosphotyrosine. B. Time course of phosphotyrosine accumulation. Electroporated neutrophils were treated without (-) or with 10 μM GTPγS, 2 mM NADPH and 10 μM NaV for the indicated time (min) and processed as in A.   C. Dependence of tyrosine phosphorylation on NADPH oxidase-derived ROI. Electroporated neutrophils were treated without (-) or with (+) 10 μM GTPγS and/or 2 mM NADPH for 5 min at 37°C. Where specified, the cells were treated with 5 μM DPI or 2 mM NAC for 2 min at 37°C prior to GTPγS stimulation. The presence of 10 μM NaV during treatment is indicated. Results shown are representative of three separate experiments.

oxidase (Ellis, et al., 1988), had a comparable effect (lane 6). These findings are in agreement with those of Fialkow et al. (Fialkow, et al., 1993) and indicate that NADPH oxidase-derived ROI promote tyrosine phosphorylation in neutrophils.

As an initial step in the study of the mechanism of action of ROI, I determined which of the known tyrosine kinases are present and active in GTPγS-stimulated[1] neutrophils. Electroporated cells were activated with the nucleotide and immediately solubilized for immunoprecipitation with one of a battery of antibodies to tyrosine kinases. The immune complexes were used for *in vitro* kinase assays and then subjected to SDS-PAGE and autoradiography. Of the 11 antibodies tested, 5 were found to immunoprecipitate active kinases detectable by their autophosphorylating activity, suggested by the close correspondence of the phosphorylated bands to the known molecular weight of the kinase immunoprecipitated (Fig. 4.2A). The active kinases included three Src family members; Lyn (53 and 56 kDa)[2], Hck (56 and 59 kDa) and Fgr (59 kDa). Also included were Syk (72 kDa) and Btk (77 kDa). In contrast, no significant activity was measurable in Src, Fyn, Yes, Blk, Lck, and Zap-70 immunoprecipitates or when rabbit non-immune serum (cont) was used. The presence of Lyn, Hck, Fgr, Syk and Btk in neutrophils had been reported previously (Asahi, et al., 1993; Gutkind and Robbins, 1989; Yamada, et al., 1993; Yamanashi, et al., 1987; Ziegler, et al., 1987).

In good agreement with the kinase assays of Fig. 4.2A, the presence of Lyn, Hck, Fgr, Syk and Btk in neutrophils was confirmed by immunoblotting whole cell lysates with the same antisera used for precipitation (Fig. 4.2B). Both the full length (72 kDa) Syk protein as well as its ~40 kDa degradation product were observed upon immunoblotting (Fig. 4.2B, closed arrowheads). A band of ~65 kDa was also recognized by the Syk anti-serum. It is not presently clear whether this polypeptide is related to Syk, or is merely a fortuitously cross-

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[1]Hereafter, 'GTPγS stimulation' refers to treatment of electroporated neutrophils with 10 μM GTPγS in the presence of 2 mM NADPH and 10 μM NaV at 37°C.

[2]We observed a phosphoprotein of about 60-62 kDa in *lyn* immunoprecipitates. *Lyn* has been extensively studied and only two isoforms have been identified, suggesting that the third band may be a separate protein. Several kinases have been shown to co-precipitate with *lyn* in other systems, including Syk (Sidorenko, et al., 1995), Btk (Cheng, et al., 1994) and a cell cycle regulatory protein, p34Cdc2 (Yuan, et al., 1996).

A — Kinase Assay

kDa cont. src fyn yes lyn blk hck fgr lck syk zap btk

200-

97-

68-

46-

35-

B — Immunoblot

Ab: lyn   hck   fgr   syk   btk

kDa 114-
      84-

      53-
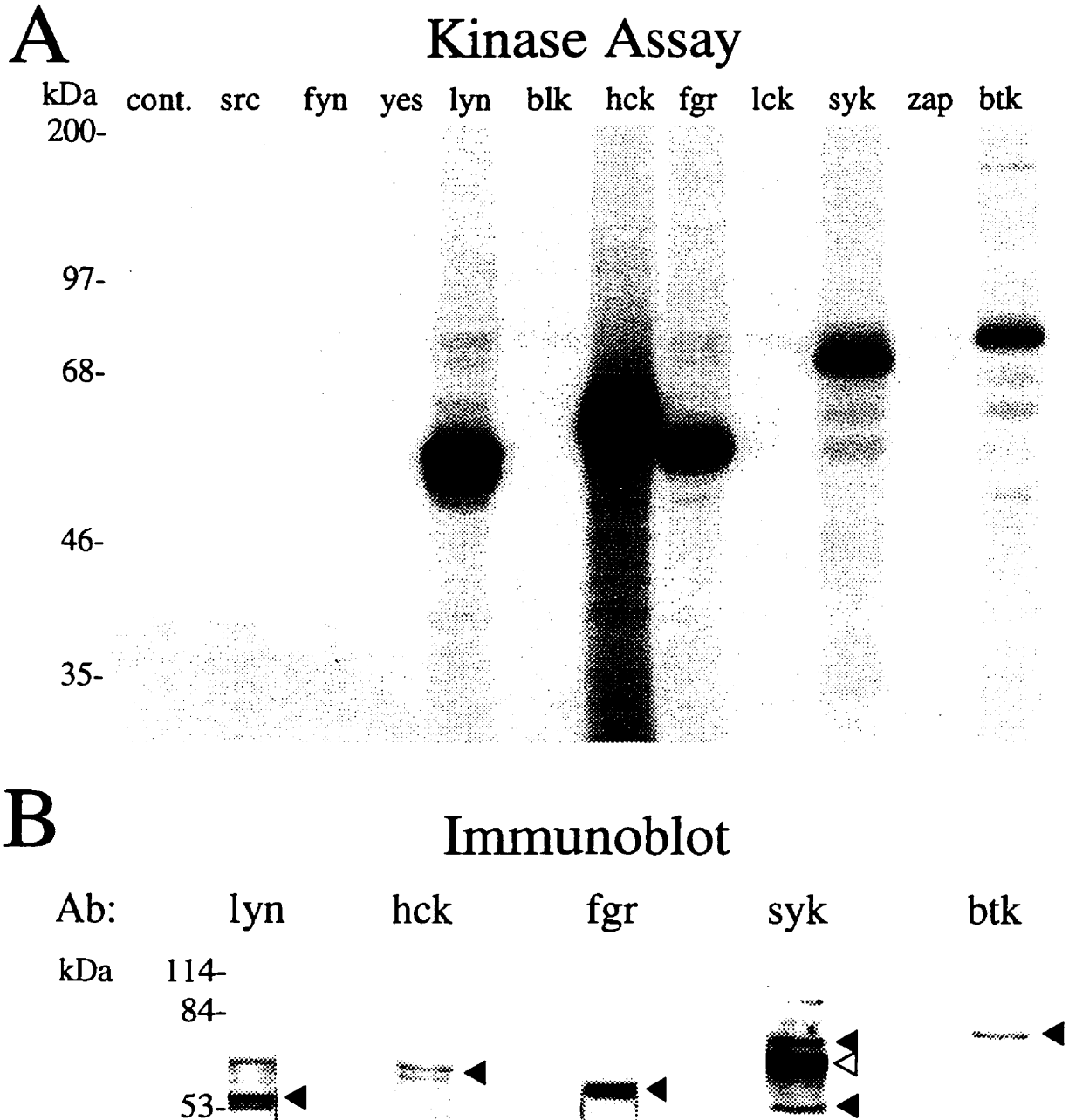
Fig. 4.2.   Identification of tyrosine kinases present in neutrophils. A. Immune complex kinase assays were performed *in vitro*, using immunoprecipitates of the tyrosine kinases indicated, prepared from lysates of electroporated neutrophils treated with 10 μM GTPγS, 2 mM NADPH and 10 μM NaV for 2 min. Kinase reactions were stopped and the material subjected to SDS-PAGE and autoradiography of the dried gel. The assay was also performed using a rabbit non-immune serum (cont.). B. Whole neutrophil lysates were immunoblotted with antisera to the tyrosine kinases indicated. Closed arrowheads point to the tyrosine kinase. The open arrowhead indicates an unidentified protein of ≈65 kDa that cross-reacts with the syk antibody.

reacting protein. It is noteworthy, however that a band of similar mobility was often found to be phosphorylated in Syk immune complex assays (Fig. 4.3A), suggesting that the 65 kDa polypeptide co-immunoprecipitates and can be phosphorylated by Syk.

The effect of ROI on neutrophil kinase activity was studied next. To this end, immunoprecipitates were prepared from control and GTPγS-stimulated cells using antisera to the kinases identified earlier in Fig. 4.2. As shown in Fig. 4.3A, the *in vitro* activity of Hck, Syk and Btk was noticeably increased following stimulation with GTPγS. The stimulation of Hck was investigated in more detail in Fig. 4.3B, where immune complexes obtained at various times after addition of GTPγS were assayed in the presence of the exogenous substrate enolase. Both the autophosphorylation of Hck (closed arrowhead in inset to Fig. 4.3B) and its ability to phosphorylate enolase (open arrowhead) followed a biphasic course, peaking between 1-5 min and declining thereafter. A similar increase in the ability of Syk and Btk to phosphorylate enolase was also observed (data not shown). It should be noted that qualitatively similar responses of Hck and Lyn were seen when NaV was ommitted during stimulation of electroporated cells (data not shown).

While production of ROI stimulated some tyrosine kinases, others were seemingly inhibited. The autophosphorylating ability of Lyn and Fgr was diminished (73 ± 14 and 48 ± 16 % of control activity, respectively; n=3) following 1 min of GTPγS stimulation (Fig. 4.3A). As for Hck, the detailed time course of the effects of ROI on Lyn activity was analysed with enolase as substrate (see Fig. 4.3C). Interestingly, quantitation of the auto- and enolase-phosphorylating activities of Lyn immune complexes revealed a discrepancy. Phosphorylation of the exogenous substrate was markedly increased, while autophosphorylation decreased. These findings suggest that non-radioactive phosphate is incorporated into Lyn in the cells, prior to immunoprecipitation, precluding subsequent incorporation of radiolabel into these sites. The reduced autophosphorylation is therefore an inaccurate indication of the enzymatic activity of Lyn, which is at least transiently stimulated by GTPγS.

The mechanism of tyrosine kinase activation by endogenous ROI was studied in detail for Hck. This kinase was chosen because it is virtually quiescent in unstimulated cells yet is
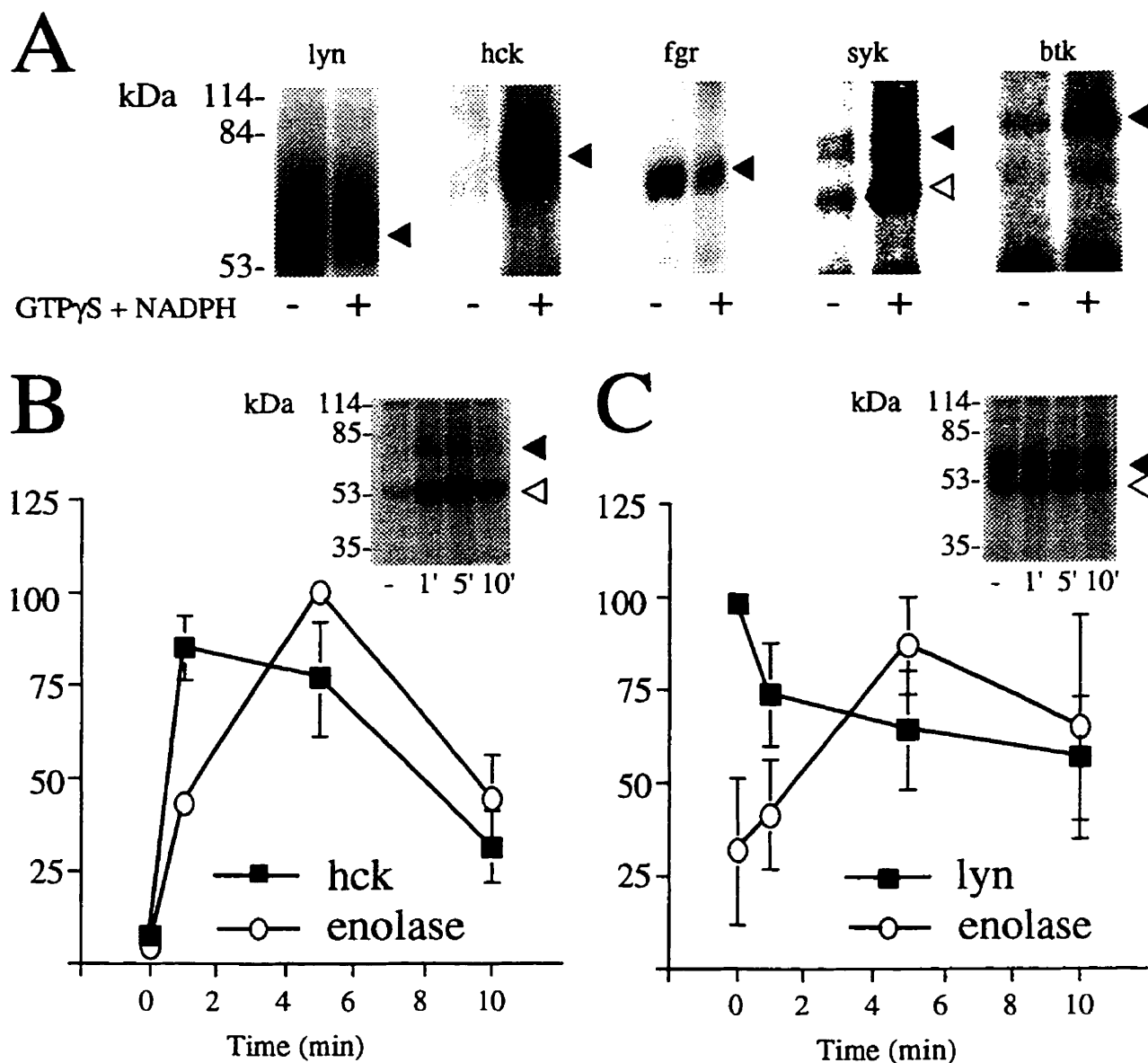
Fig. 4.3   Modulation of tyrosine kinase activity by ROI. A. Immune complex kinase assays were performed in vitro, using immunoprecipitates of the tyrosine kinases indicated, prepared from lysates of electroporated neutrophils treated without (-) or with (+) 10 μM GTPγS, 2 mM NADPH and 10 μM NaV for 1 min. B. Immunoprecipitates of Hck were prepared from lysates of electroporated neutrophils treated without (-) or with (+) 10 μM GTPγS, 2 mM NADPH and 10 μM NaV for the indicated time (min) and subjected to immune complex kinase assays in the presence of enolase. The kinase reactions were stopped and the samples were subjected to SDS-PAGE followed by autoradiography of the dried gels. A representative experiment is shown in the inset. Bands that correspond to autophosphorylation (closed arrows) and enolase phosphorylation (open arrows) were quantified with a phosphorimager and results are presented as the % maximal response in the main panel. C. In vitro autophosphorylation and enolase phsophorylation activities of Lyn were determined as in B for Hck. Data in B and C are means ± SE of 3 experiments.

the most active in immunoprecipitates from activated cells, providing an optimal signal-to-noise ratio. I first considered the possibility that activation of Hck by ROI resulted from direct oxidation of critical residues on the kinase. To test this notion, Hck immunoprecipitates obtained from unstimulated cells were treated *in vitro* with two strong oxidizing agents, diamide and H_2O_2. Comparable concentrations of these oxidants have been shown to promote phosphotyrosine accumulation when added to intact cells (Fialkow, et al., 1994). Neither diamide nor H_2O_2, however, were capable of activating isolated Hck in immune complexes (Fig. 4.4A). Conversely, reducing agents could not reverse the activation of Hck immunoprecipitated from lysates of GTPγS-treated cells. As shown in the rightmost lanes of Fig. 4.4A, when added directly to the immunoprecipitate neither NAC nor DTT diminished the activation of Hck. These findings contrast the preventive effect of NAC seen when added to permeabilized cells during the respiratory burst, described in Fig. 1B.

The inability of oxidants and reducing agents to affect Hck autophosphorylation was confirmed by immunoblotting the immunoprecipitates with anti-phosphotyrosine antibodies (Fig. 4.1B). The kinase was found to be tyrosine-phosphorylated only after stimulation of the cells with GTPγS, and the phosphotyrosine content was unaffected by oxidizing and reducing agents. Together, these results suggest that ROI do not directly activate Hck in GTPγS stimulated neutrophils.

Phosphorylation on tyrosine residues has been shown to be an important determinant of the activity of several tyrosine kinases, including those identified in neutrophils. Because tyrosine phosphorylation of Hck was detectable when this enzyme was precipitated from stimulated cells (Fig. 4.4B), I considered the possibility that ROI activate kinases in neutrophils indirectly, by mediating their phosphorylation on tyrosine residues. As an initial approach to test this hypothesis, I tested the kinase activity of immune complexes obtained from control and GTPγS-stimulated cells using anti-phosphotyrosine antibodies. The data in Fig. 4.5 demonstrate that phosphorylation was greater in precipitates from stimulated cells, suggesting that the relevant kinase activity may be associated with tyrosine phosphorylated

A Kinase Assay

GTPγS + NADPH — — — + + +

kDa 114-

85-

53-

35-

Treatment: none diamide H₂O₂ none NAC DTT

B Anti-PY Immunoblot

GTPγS + NADPH — — — + + +

kDa 114-

85-

53-

35-

Treatment: none diamide H₂O₂ none NAC DTT
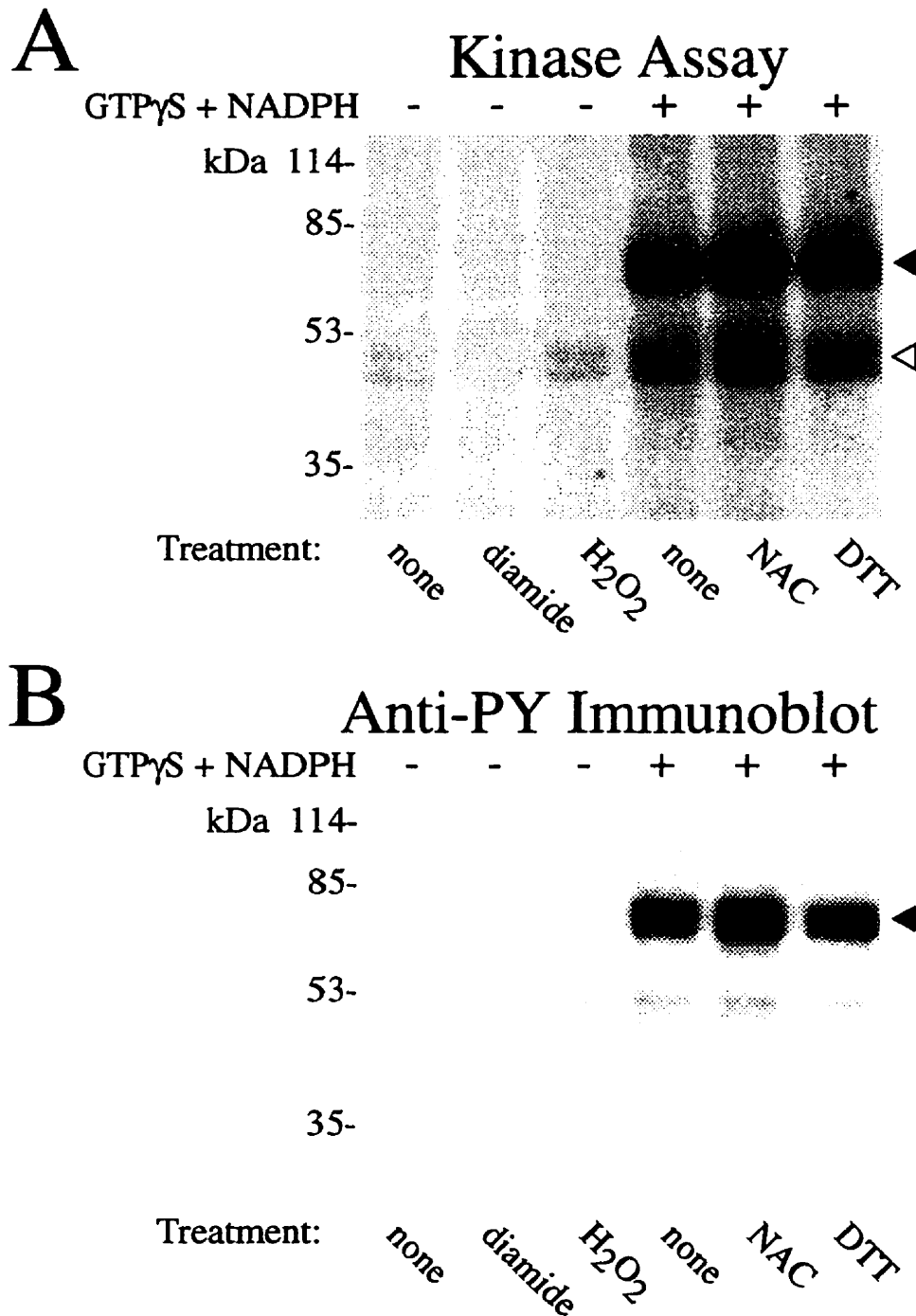
Fig. 4.4   Effect of oxidizing/reducing agents on Hck activity. Hck immunoprecipitates were obtained from lysates of electroporated neutrophils treated without (-) or with (+) 10 μM GTPγS, 2 mM NADPH and 10 μM NaV (5 min) were incubated at 30°C for 30 min with either 1 mM diamide, 1 mM H_2O_2, 20 mM NAC, 1 mM DTT or with buffer alone (none), as indicated. An aliquot of the immunoprecipitate was used for in vitro kinase assay (A) and another for anti-phosphotyrosine immunoblotting (B). Closed arrows indicate the position of immunoprecipitated Hck while the open arrow indicates the position of the exogenous substrate, enolase.

Kinase Assay

kDa 200-

97-

68-

46-

GTPγS + NADPH   -   +
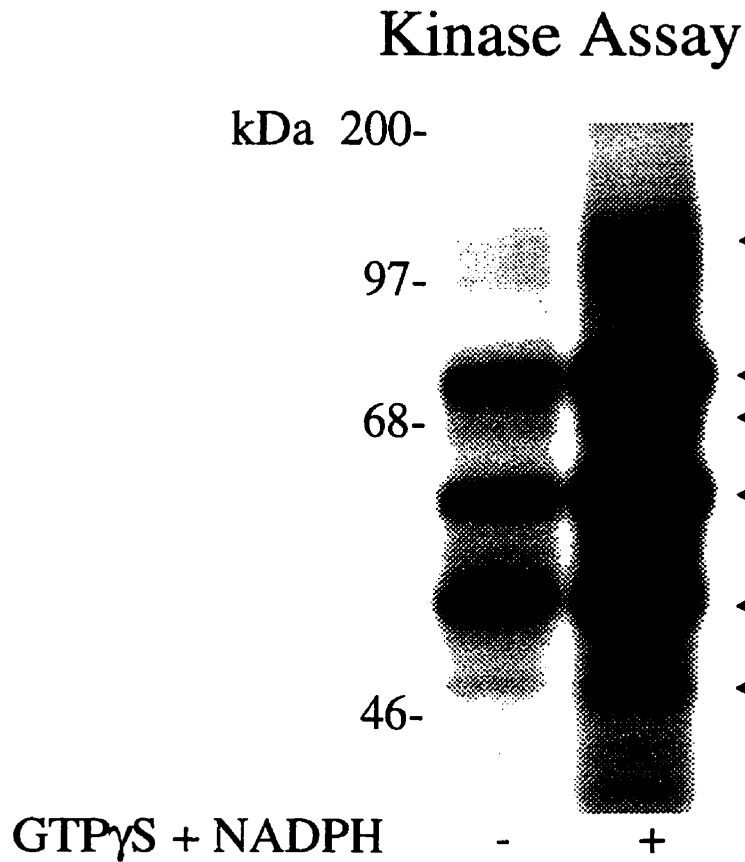
Fig. 4.5   Phosphotyrosine-associated kinase activity. Anti-phosphotyrosine immunoprecipitates were obtained from lysates of electroporated neutrophils treated without (-) or with (+) 10 μM GTPγS, 2 mM NADPH and 10 μM NaV (2 min) and used to perform *in vitro* kinase assays as described in Experimental Procedures. Closed arrowheads indicate bands which displayed a prominent increase in phosphorylation following stimulation. Results are representative of three separate experiments.

proteins. A number of prominent bands displayed increased phosphorylation *in vitro* (arrowheads in Fig. 4.5), with molecular masses of approx. 48, 54, 62, 68, 75 and 118 kDa. The size of some of the phosphoproteins in phosphotyrosine immunoprecipitates correspond to that of the active kinases detailed in Fig. 4.2. To establish more directly whether the active kinases are tyrosine phosphorylated, immunoprecipitates of Lyn, Hck, Fgr, Syk and Btk were prepared from control and GTPγS-treated cells and probed by immunoblotting with anti-phosphotyrosine antibodies. As illustrated in Fig. 4.6A, endogenous generation of ROI is accompanied by tyrosine phosphorylation of all the kinases studied (indicated with closed arrowheads), with the notable exception of Btk, which remained unaffected. The figure also shows that both the intact form of Syk as well as its 40 kDa proteolytic fragment (open arrowhead) were phosphorylated on tyrosine.

The correlation between the occurrence of tyrosine phosphorylation and the activation of the tyrosine kinases is further stressed by the similarity of the time courses of both events. In Fig. 4.6B, the degree of tyrosine phosphorylation was quantified in immunoprecipitates from cells stimulated for varying periods of time with GTPγS. All four kinases undergo rapid and progressive phosphorylation, which is detectable by 1 min and maximal between 5 and 10 min. This pattern closely resembles the time course of activation of Hck determined in Fig. 4.3C using enolase as the substrate. It therefore appears likely that tyrosine phosphorylation of the kinases regulates their activity.

This notion was directly addressed by treatment of immunoprecipitates with an active tyrosine phosphatase, the truncated T-cell phosphatase (TC-PTP). Hck was precipitated from GTPγS-stimulated neutrophils and incubated for 30 min in the presence or absence of TC-PTP. The effectiveness of the phosphatase was ascertained by immunoblotting the precipitates with anti-phosphotyrosine antibodies. Exposure to TC-PTP led to complete dephosphorylation of Hck (indicated by the solid arrowheads in Fig. 4.7A). Immunoblotting confirmed that equal amounts of Hck were present before and after treatment with TC-PTP (data not shown). The autophosphorylating (solid arrows) and exogenous kinase activity (open arrows) of stimulated
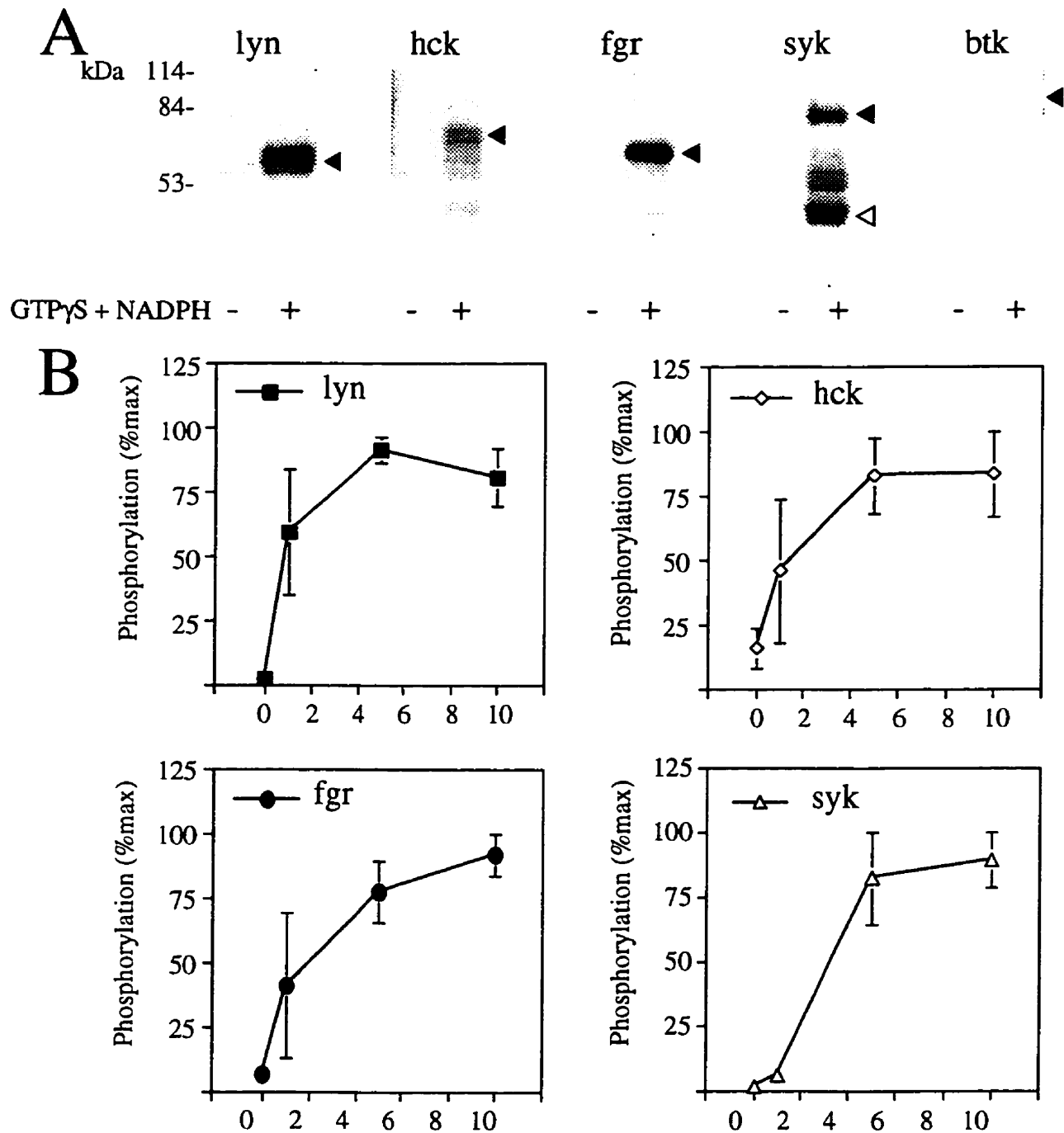
Fig. 4.6   Phosphorylation of tyrosine kinases *in situ*. Anti-phosphotyrosine immunoblotting was performed on immunoprecipitates of the specified tyrosine kinases, obtained from electroporated neutrophils treated without (-) or with (+) 10 μM GTPγS, 2 mM NADPH and 10 μM NaV for 5 min. Solid arrows indicate the position of the immunoprecipitated kinase. The open arrowhead indicates the 40 kDa proteolytic fragment of syk, also observed in anti-Syk immunoblots (see Fig. 2B). B. Time course of tyrosine phosphorylation. The extent of tyrosine phosphorylation was quantified at the times indicated by densitometry and is presented as % maximal. Data are means ± SE for three experiments.

A   Anti-PY Immunoblot

kDa   114-
      85-

      53-

      35-

TC-PTP   -   +

B   Kinase Assay

kDa   114-
      85-

      53-

      35-

TC-PTP   -   +
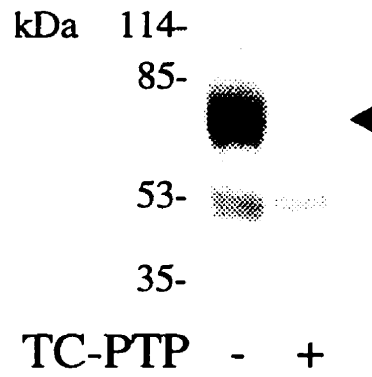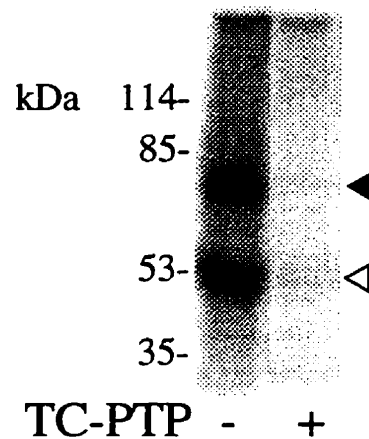
Fig. 4.7   Effect of tyrosine dephosphorylation on Hck activity.   Hck was immunoprecipitated from lysates of neutrophils treated with 10 μM GTPγS, 2 mM NADPH and 10 μM NaV for 5 min. Immunoprecipitates were then treated for 30 min without (-) or with (+) recombinant T-cell phosphatase (TC-PTP) at 30°C while shaking. An aliquot of the immunoprecipitated material was subjected to anti-phosphotyrosine immunoblotting (A) while another was used to perform the *in vitro* kinase assay (B).   Closed arrowheads indicate immunoprecipitated Hck while the open arrow indicates the location of the exogenous substrate, enolase.   Results shown are representative of three separate experiments.

and dephosphorylated Hck was next compared. Fig. 4.7B demonstrates that treatment with

TC-PTP eliminated the kinase activity of Hck stimulated by ROI.

Discussion

In this report, I analyzed the mechanism leading to increased phosphotyrosine accumulation following ROI production in neutrophils. In electroporated cells treated with GTPγS, I detected an elevated activity of several kinases, measured *in vitro*. The activation of these kinases was rapid and correlated well with the increase in tyrosine phosphorylation observed under these conditions.

Kinases of three separate families were found to be activated by ROI, as determined by autophosphorylation and phosphorylation of an exogenous substrate, enolase. Hck, a member of the Src family of tyrosine kinases and highly expressed in granulocytes and macrophages (Ziegler, et al., 1987), displayed little activity in untreated cells but was rapidly stimulated following addition of GTPγS. Syk, which belongs to a separate family of kinases, also displayed increased activity following ROI production. In contrast, the closely related ZAP-70 tyrosine kinase, thought to be important in B- and T-cell receptor signalling (Sefton and Taddie, 1994) was not detectable in active neutrophils using our immune complex kinase assay. Btk, a member of the Tec family of tyrosine kinases, is expressed in cells of myeloid and lymphoid lineage (Yamada, et al., 1993) and was also activated by ROI. To our knowledge, activation of Btk in neutrophils had not been reported previously.

While ROI production led to the activation of some tyrosine kinases, it appeared to have an opposite effect on the activity of others, when estimated from autophosphorylation in immune complex kinase assays. Thus, Lyn and Fgr displayed high activities in untreated, electroporated neutrophils, which decreased following GTPγS stimulation. However, at least in the case of Lyn, the apparent decrease in activity likely reflected occupancy of substrate sites by non-radioactive phosphate, which may have occurred *in situ*, prior to immunoprecipitation. Indeed, the ability of the enzyme to phosphorylate exogenous substrates was *increased* following stimulation of the respiratory burst. Therefore, caution must be exercised when equating the autophosphorylating and catalytic activities of tyrosine kinases.

None of the other tyrosine kinases tested were found to be activated following generation of ROI. These included Yes, which is reported to be present in neutrophils, where

it can be stimulated by GM-CSF (Corey, et al., 1993). Clearly, though 11 different antisera were used, our survey was incomplete, since other tyrosine kinases are likely to exist in neutrophils.

The mechanism underlying the activation of the kinases by ROI was explored in some detail using Hck as a prototype. While ROI production *in situ* appeared to activate Hck, oxidizing agents could not mimic this effect when applied to Hck immunoprecipitates *in vitro*. Moreover, reducing agents failed to reverse the activation of Hck isolated from GTPγS-treated cells. It is concluded that Hck activity is not regulated directly by ROI, but rather by some other post-translational modification. Though not tested directly, I suggest by extension that activation of the other kinases is similarly indirect.

Because tyrosine phosphorylation of Lyn, Hck, Fgr, and Syk was found to occur upon stimulation by GTPγS, this post-translational modification was considered as a possible mechanism of regulation. This notion was evaluated using TC-PTP to dephosphorylate activated Hck. This procedure was found to eliminate the activity of the kinase, suggesting that tyrosine phosphorylation mediates the effect of ROI on hck activation.

Tyrosine kinase activity of Src family members is thought to be suppressed by phosphorylation of a C-terminal residue, conserved among family members (Cooper, 1988; Cooper and Howell, 1993; Liu, et al., 1993). Dephosphorylation of this residue has been shown to increase the activity of Src family kinases (Cooper and King, 1986), in apparent conflict with our findings with Hck, where complete dephosphorylation of the enzyme led to its inactivation. However, recent evidence has questioned this simple model of regulation. This includes the finding that a T-cell line lacking CD45 (the phosphatase that activates the Src family member Lck) was found to have higher Lck activity even though its inhibitory C-terminal tyrosine residue was hyperphosphorylated (Burns, et al., 1994). While dephosphorylation of the C-terminus may be important for the de-repression of Src family member, a number of unique tyrosine residues have been reported to be phosphorylated upon activation. These include the so-called autophosphorylation site within the kinase domain (Patschinsky, et al., 1982; Smart, et al., 1981) and sites within the N-terminal domain of some

Src family members (Soula, et al., 1993). Evidence exists that phosphorylation of these residues is essential for kinase activity, possibly by stabilization of the active kinase (Mustelin, 1994; Veillette and Fournel, 1990). Recent evidence has also implicated serine phoshorylation in the regulation of Src family members (Watts, et al., 1993; Winkler, et al., 1993). While the regulation of Src family kinases remains incompletely understood, our findings imply that tyrosine phosphorylation is necessary to maintain the activity of Hck following activation by endogenous ROI.

The steps that follow ROI generation and lead to kinase phosphorylation are unknown, but some insight is provided by recent reports that: a) critical conserved cysteine residues exist in the catalytic domain of many tyrosine phosphatases (Fisher, et al., 1991) and b) that both exogenous (Fialkow, et al., 1994; Zor, et al., 1993) as well as endogenous oxidants can inactivate tyrosine phosphatases, likely by targetting their critical sulfhydryl moieties. In view of these considerations, the following scenario can be envisaged. Under basal conditions, the accumulation of tyrosine phophoproteins and the autophosphorylation and stimulation of tyrosine kinases, some of which are constitutively active, are prevented by the offsetting action of tyrosine phosphatases. This delicate balance can be disrupted when ROI diminish the rate of dephosphorylation by reaction with sulfhydryl side chains in the catalytic domain of one or more tyrosine phosphatases. Indeed, in neutrophils, CD45 has been shown to be susceptible to inactivation by oxidants (Fialkow, et al., 1994), and other phosphatases present in these cells are likely to be similarly affected.

It is noteworthy that modulation of tyrosine kinase activity has been reported in lymphoid cells exposed to H_2O_2 (Schieven, et al., 1993). Treatment with the oxidant was found to activate Syk but not Lyn, resembling our observations in neutrophils. Like most other cells, lymphocytes can potentially generate ROI by electron transfer reactions in mitochondria and the endoplasmic reticulum. However, the magnitude of the oxidative response is far greater in phagocytes, which express high levels of the NADPH oxidase (see Introduction). In this regard, it is important that in the present experiments activation of phosphorylation was elicited by *endogenously* generated ROI, lending credence to the

physiological significance of the observations. It is possible to envisage that stimulation of the NADPH oxidase, one of the earliest effectors of neutrophils, could promote phosphotyrosine accumulation by the combined inhibition of phosphatases and activation of kinases. This could in turn have important consequences on more slowly developing responses such as phagocytosis and degranulation (Gresham, et al., 1988). In this context, Hck has been suggested to have a role in phagocytosis (Lowell, et al., 1994) and Syk has also been proposed to be essential to the anti-microbial response (Asahi, et al., 1993). It is also conceivable that ROI secreted by neutrophils may have paracrine effects, stimulating neighboring quiescent neutrophils, or other cells present in the inflammatory milieu, including lymphocytes and macrophages.

Chapter 5

Regulation of SHP-1 during activation of human neutrophils: role of protein kinase C.

Summary

The tyrosine phosphorylation of several proteins induced in neutrophils by soluble and particulate stimuli is thought to be crucial for initiating anti-microbial responses. While activation of tyrosine kinases is thought to mediate this event, the role of tyrosine phosphatases in the initiation and modulation of neutrophil responses remains largely undefined. I investigated the role of SHP-1 (also known as PTP1C, HCP, PTP-N6 and SHPTP-1), a phosphatase expressed primarily in haemopoietic cells, in the activation of human neutrophils. SHP-1 mRNA and protein were detected in these cells and the enzyme was found to be predominantly localized to the cytosol in unstimulated cells. Following stimulation with neutrophil agonists such as phorbol ester, chemotactic peptide or opsonized zymosan, a fraction of the phosphatase redistributed to the cytoskeleton. Agonist treatment also induced significant decreases (30-60%) in SHP-1 activity which correlated temporally with increases in the cellular phosphotyrosine content. Phosphorylation of SHP-1 on serine residues was associated with the inhibition of its enzymatic activity, suggesting a causal relationship. Accordingly, both the agonist-evoked phosphorylation of SHP-1 and the inhibition of its catalytic activity were blocked by treatment with bis-indolylmaleimide I, a potent and specific inhibitor of protein kinase C (PKC) activity. Immunoprecipitated SHP-1 was found to be phosphorylated efficiently by purified PKC in vitro. Such phosphorylation also caused a decrease in the phosphatase activity of SHP-1. Together, these data suggest that inhibition of SHP-1 by PKC-mediated serine phosphorylation plays a role in facilitating the accumulation of tyrosine phosphorylated proteins following neutrophil stimulation. These findings provide a new link between the PKC and tyrosine phosphorylation branches of the signalling cascade that triggers anti-microbial responses in human neutrophils.

Introduction

Phosphotyrosine accumulation is regulated by the competing activities of protein tyrosine kinases and protein tyrosine phosphatases (PTPs). In addition to the effect of increased tyrosine kinase activities, decreases in the activity of protein tyrosine phosphatases (PTP's) may also lead to an accumulation in cellular tyrosine phosphorylation following exposure of neutrophils to inflammatory stimuli. In support of this notion, overall neutrophil phosphotyrosine phosphatase activity has been shown to decrease following stimulation with the chemoattractant fMLP or with phorbol esters, though the identity of the particular phosphatases responsible for this effect was not determined (Kansha, et al., 1993; Kraft and Berkow, 1987). The tyrosine phosphatase SHP-1 has been shown to act as a negative modulator of many receptor signalling systems in other hematopoietic cell types, suggesting that it may also play a role in neutrophils. To further delineate the role of tyrosine phosphatases in the initiation and/or modulation of neutrophil responses, I have studied the localization, activity and possible modes of regulation of SHP-1 in these cells.

Results

While previous studies have demonstrated SHP-1 expression in a variety of haematopoietic cells and cell lines (Plutzky, et al., 1992; Shen, et al., 1991; Yi, et al., 1992), the presence of SHP-1 in neutrophils has not been documented. To address this issue, reverse transcriptase-polymerase chain reaction (RT-PCR) was employed. Reactions were performed using RNA purified from neutrophils and oligonucleotide primer pairs designed for amplification of the SH2 domain-containing region of the SHP-1 cDNA (Kozlowski, et al., 1993). As shown in Fig. 5.1A, results of this analysis revealed amplification of a 120 bp mRNA fragment of SHP-1 from neutrophil RNA (lane 3). No products were seen in the absence of either reverse transcriptase or neutrophil RNA (lanes 1 and 2, respectively), ensuring the specificity of these reactions.

The presence of SHP-1 protein was confirmed by immunoblotting, using a polyclonal antibody raised to a GST fusion protein encompassing residues 1-296 of the phosphatase (Fig.
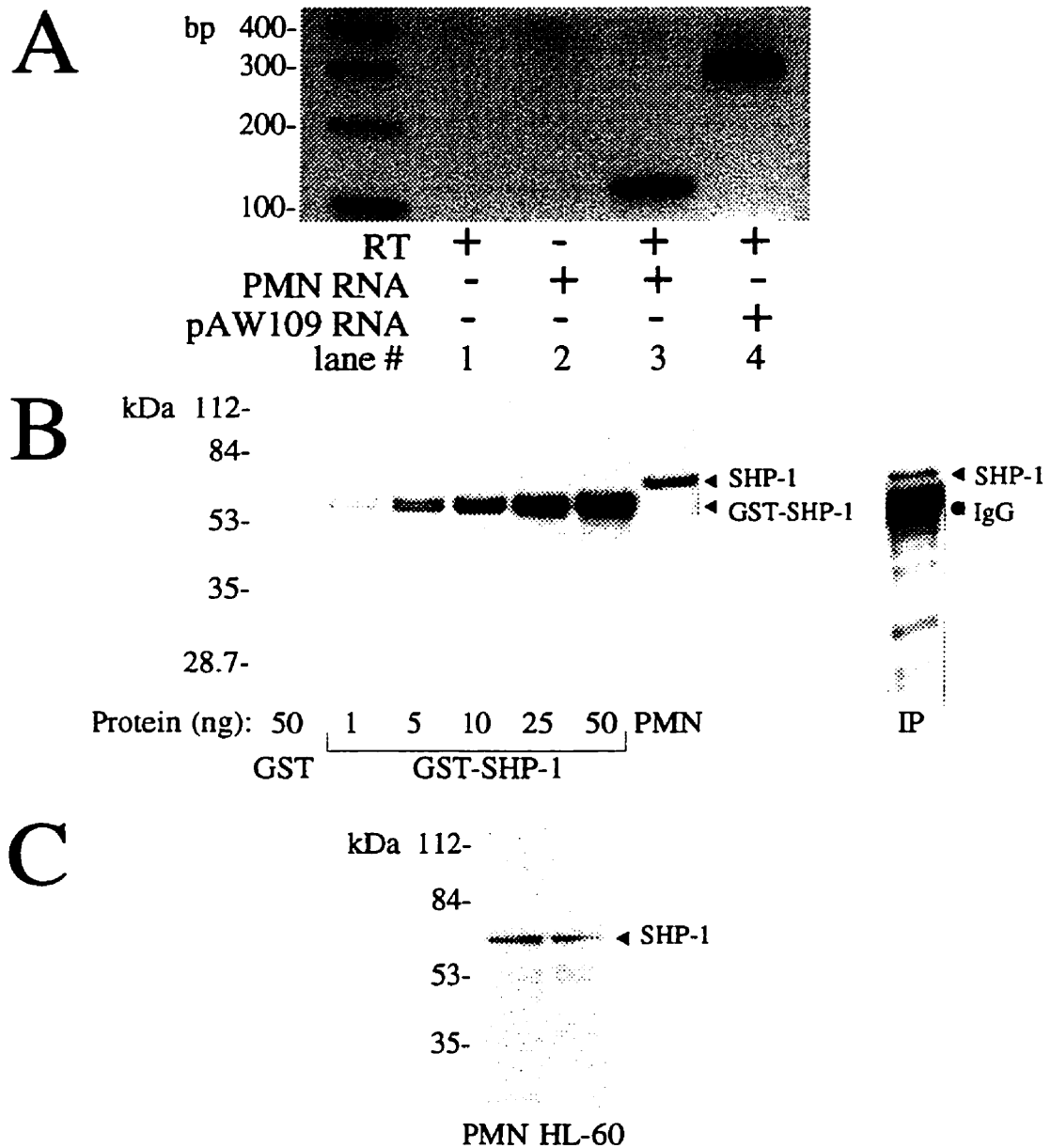
Fig. 5.1.   Identification of SHP-1 in human neutrophils. A. RT-PCR was performed using primers designed to amplify a 120 bp sequence of SHP-1 (lanes 1-3), or interleukin 1-a (lane 4).   Reactions were performed in the presence (+) or absence (-) of reverse transcriptase (RT), freshly isolated RNA from human neutrophils (PMN RNA) or RNA encoding interleukin-1a (pAW109RNA, from Perkin Elmer). Samples were subjected to agarose gel electrophoresis and visualized by ethidium bromide staining. B. Quantitation of SHP-1 in human neutrophils by immunoblotting. GST (50 ng), increasing amounts (in ng) of GST-SHP-1, whole neutrophil lysate (PMN; 10^6 cell equivalents) and a SHP-1 immunoprecipitate (IP) were loaded onto SDS-PAGE gels and subjected to immunoblotting with affinity-purified antibodies to SHP-1. C. Comparison of the mobility of SHP-1 in neutrophils and HL60 cells.   Whole-cell extracts of human neutrophils (PMN) and undifferentiated HL-60 cells were analysed by immunoblotting with affinity-purified polyclonal antibodies to SHP-1. The position of the a splice variant of SHP-1 is indicated.

5.1B). A single immunoreactive band of approximately 65 kDa is apparent in neutrophil cell lysates and the same band was identifiable in immunoprecipitates obtained with the SHP-1 antibody. This corresponds to the reported molecular mass of one of the splice variants of SHP-1 (Kozlowski, et al., 1993; Uchida, et al., 1993). To quantify the level of SHP-1 expression, increasing amounts of the fusion protein were loaded into SDS-PAGE gels along with neutrophil lysates and subjected to immunoblotting. By interpolation of the optical density of these bands, it was determined that neutrophils contain 12 ± 5 ng (n=3) of SHP-1 per 10^6 cells. SHP-1 accounts for 0.024% of the total cellular protein of neutrophils, at an intracellular concentration of approximately 530 nM (based on a volume of 350 fL/cell, determined using the Coulter-Channelyzer).

Two variants of SHP-1 have been identified, generated by the alternative splicing of 39 amino acids within the C-terminal SH2 domain of SHP-1 (Tsui, et al., 1993). To determine which splice variant is expressed in human neutrophils, whole-cell extracts were analyzed by immunoblotting with affinity-purified antibodies to SHP-1 and compared with HL-60 cells, a promyelocytic cell line found earlier to express only the higher molecular weight, α splice variant of SHP-1 (Uchida, et al., 1993). As shown in Fig. 5.1C, the SHP-1 immunoreactive band of neutrophils comigrated precisely with that seen in HL-60 lysates. Similar results were obtained using polyclonal and monoclonal antibodies to SHP-1. I conclude that neutrophils express only the α splice variant of SHP-1.

Immunofluorescence staining of untreated neutrophils fixed in suspension revealed a diffuse, predominantly cytosolic localization of SHP-1 (Fig. 5.2A, left panel). In these cells, weak nuclear staining and a variable degree of punctation in the cytosol was also noted, while no staining was seen in fixed cells stained with an affinity-purified non-immune serum (right panel). To confirm that SHP-1 was predominantly cytosolic in resting cells, subcellular fractions obtained by Percoll gradient centrifugation were analyzed by immunoblotting (see Experimental Procedures). As shown in Fig. 5.2B, the results of this analysis also revealed SHP-1 to be almost exclusively located in the cytosolic fraction. Minute amounts of the phosphatase were occasionally found in the combined secretory vesicle/plasma membrane

A

Anti-SHP-1   Non-Immune

B

Anti-SHP-1

◄ SHP-1

1°   2°   3°   sv/pm cyto   PMN
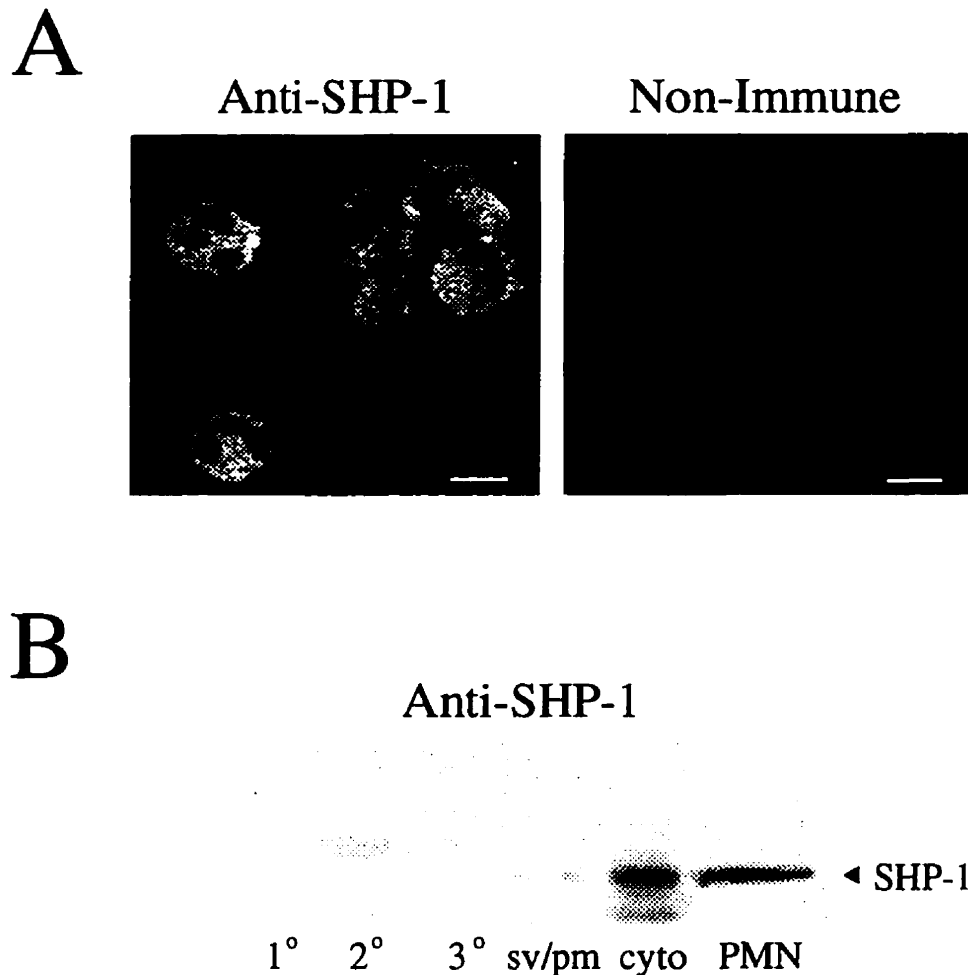
Fig. 5.2.   Subcellular localization of SHP-1 in human neutrophils. A. Unstimulated neutrophils were fixed in suspension with paraformaldehyde, allowed to adhere to poly-lysine coated coverslips, permeabilized with a buffer containing 0.1% Triton X-100 and blocked with 5% donkey serum. Fixed cells were then stained with antibodies to SHP-1 (left) or an affinity-purified non-immune serum (right). Samples were analyzed using a Zeiss laser confocal microscope. Size bars indicate 5 nm. B. Subcellular fractionation of human neutrophils was performed by nitrogen cavitation followed by Percoll gradient centrifugation (see Experimental Procedures). Fractions displaying the maximal activity of marker protein for primary (1°), secondary (2°) and tertiary granules (3°), the combined secretory vesicles/plasma membrane fraction (sv/pm) and the cytosol (cyto) were selected, and an equal amount of protein (25 μg) from each fraction was analyzed by immunoblotting with affinity-purified antibodies to SHP-1. For reference, 25 μg of a whole neutrophil lysate (PMN) was also loaded.
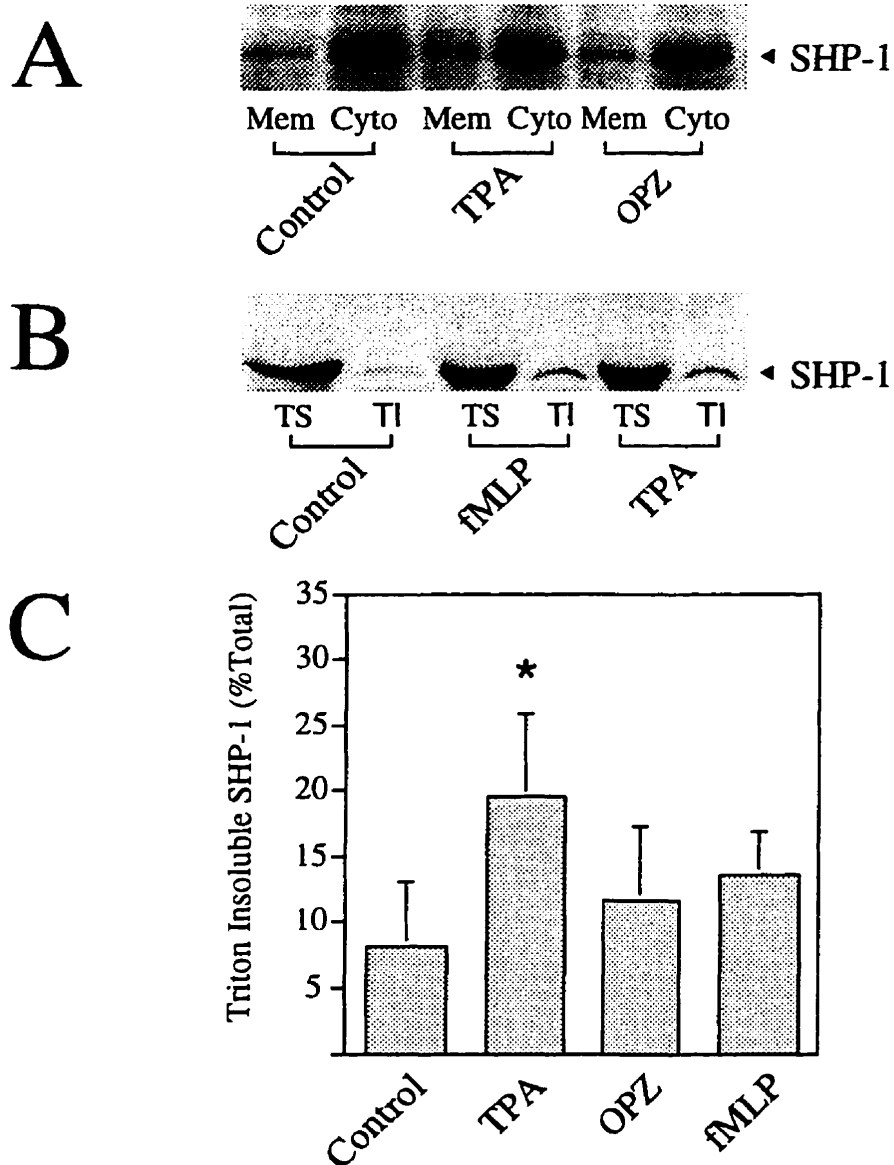
Fig. 5.3.   A. Neutrophil suspensions were treated without (control) or with phorbol ester (TPA) or opsonized zymosan (OPZ) for 10 min prior to the isolation of membranes (Mem). Both membranes and the remaining cytosolic (Cyto) fraction were resuspended in sample buffer and aliquots (10^6 cell equivalents) were subjected to SDS-PAGE and immunoblotting with affinity-purified antibodies to SHP-1. B. Neutrophil suspensions were treated without (control) or with phorbol ester (TPA) or opsonized zymosan (OPZ) for 10 min or with chemoattractant peptide (fMLP) for 2 min and then rapidly sedimented and resuspended in a lysis buffer containing 1% Triton X-100. Triton soluble (TS) and insoluble (TI) proteins were isolated by centrifugation of lysates at 14,000g for 5 min and equal number of cell equivalents (10^6) of each analyzed as in A. C. Immunoblots from three experiments like that in B were analyzed by densitometry and the amount of SHP-1 isolated in the Triton-insoluble fraction is shown as a percentage of the total (soluble + insoluble) immunoreactive protein (mean ± SE of 3 experiments). The asterisk indicates $p < 0.05$.

fraction, which is known to trap cytosolic components upon resealing of vesicles. No SHP-1 was detected in fractions containing primary, secondary or tertiary granules.

To determine whether SHP-1 translocates between cellular compartments upon neutrophil stimulation, SHP-1 localization was examined by separating the high speed pellet and supernatant (i.e. whole membrane and cytosolic fractions) from cells stimulated with the following agents: a chemotactic peptide (fMLP), which activates a GTP-binding protein-coupled receptor, a phorbol ester (TPA), which directly activates PKC, and opsonized zymosan (OPZ), a particulate stimulus that signals via Fcγ and CR3 receptors. Immunoblotting of these fractions revealed minimal amounts of SHP-1 to be associated with membranes (high speed pellet) in unstimulated cells and this amount was not substantially altered following stimulation (Fig. 5.3A).

While SHP-1 could not be detected to associate with membranes during stimulation, a fraction of this phosphatase was found to associate with the Triton X-100 insoluble residue of activated cells. The Triton-insoluble material, thought to represent mostly cytoskeletal components, bound little SHP-1 before activation (<10%), but significant amounts were associated following stimulation with either fMLP or TPA (Fig. 5.3B). Quantitation by densitometry revealed that stimulation by TPA induced a 2-3 fold increase in cytoskeletal-associated SHP-1 (Fig. 5.3C). A more modest increase in the cytoskeletal association of SHP-1 was noted following stimulation with OPZ and fMLP (Fig. 5.3C), though this change was not statistically significant. These findings suggest that SHP-1 may play a role in the cytoskeletal remodeling that occurs during neutrophil activation, by dephosphorylating cytoskeletal-associated proteins.

Agonist-induced activation of neutrophils is known to be associated with increased cellular tyrosine phosphorylation. This phenomenon is illustrated in the experiment in Fig. 5.4A, where whole-cell lysates from control and stimulated cells were immunoblotted with anti-phosphotyrosine antibody. This effect can be mimicked by treatment with oxidizing agents such as diamide, which have been shown to directly inhibit tyrosine phosphatases. Inhibition of the phosphatases suffices to induce a massive accumulation of phosphotyrosine on
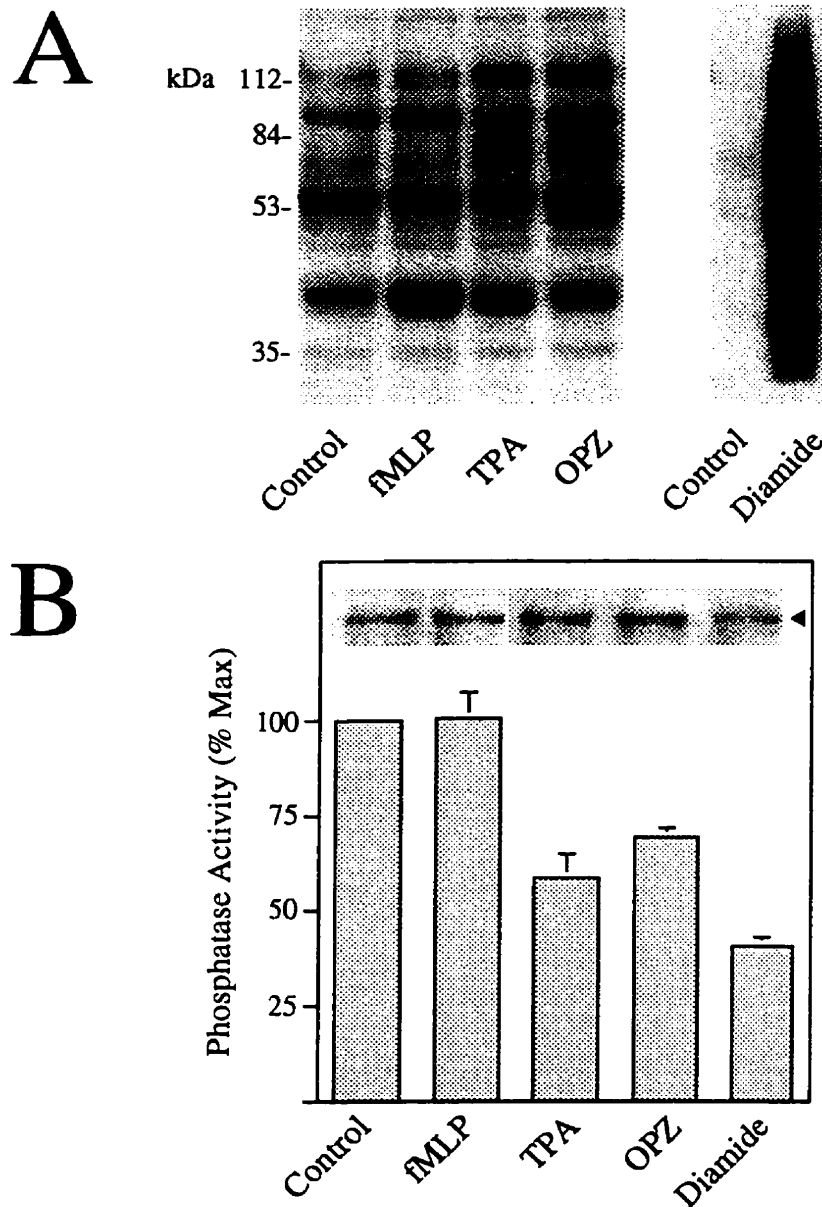
Fig. 5.4.   Inhibition of SHP-1 activity by agents that induce tyrosine phosphorylation. A.
Neutrophil suspensions were treated without (control) or with the indicated agents for 10
min, except fMLP stimulation which was for 2 min. Following treatment, cells were
resuspended in boiling Laemmli sample buffer and subjected to SDS-PAGE and
immunoblotting with a monoclonal antibody to phosphotyrosine. B. Neutrophil suspensions
were treated as in A with the indicated stimulus and the activity of SHP-1 was determined in
vitro following immunoprecipitation (see Experimental Procedures). Results (mean ± SE of
3 experiments) are presented as the % maximal activity, which was always found in
untreated cells.   Immunoblotting of these immunoprecipitates confirmed that equivalent
amounts of SHP-1 were immunoprecipitated following stimulation with each agent (see
inset; SHP-1 is indicated with an arrowhead).
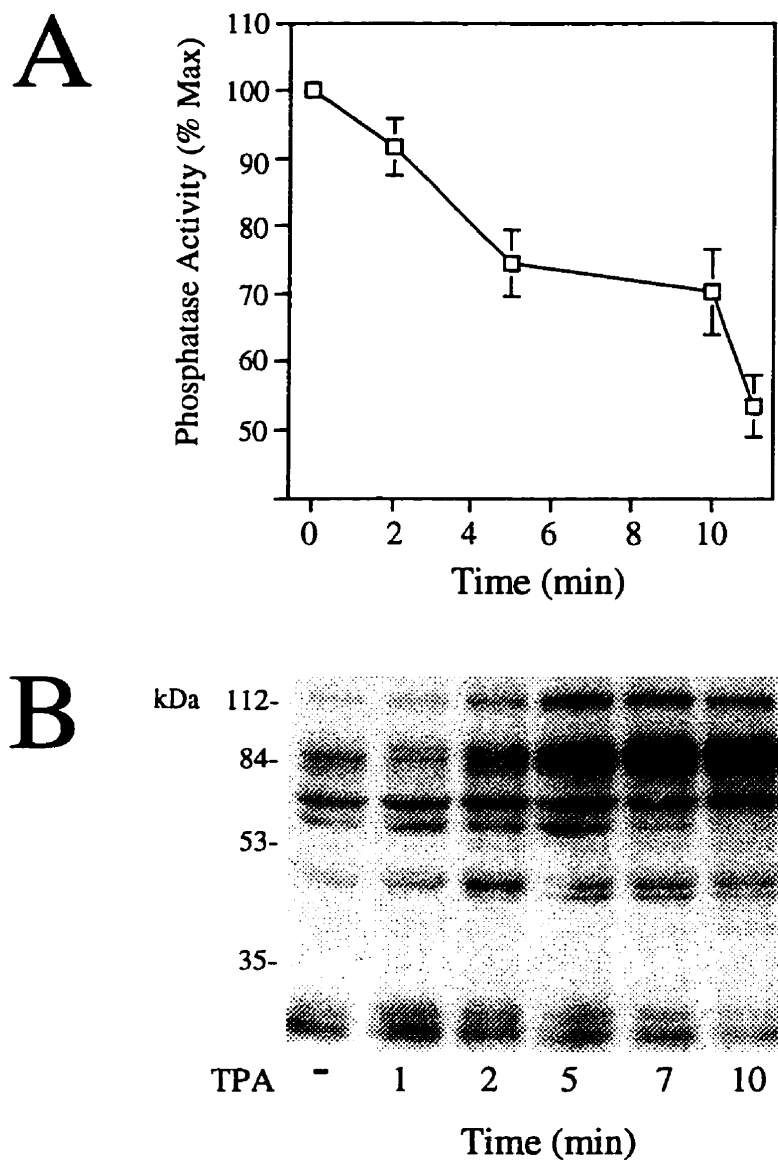
Fig. 5.5.   Inhibition of SHP-1 activity by agents that induce tyrosine phosphorylation. A. Neutrophil suspensions were treated with 10^{-7}M TPA for the indicated time (min) and the activity was then determined in vitro. Means ± SE of 3 experiments are shown. B. Neutrophil suspensions were treated with 10^{-7}M TPA for the indicated time (min). Following treatment, cells were resuspended in boiling Laemmli sample buffer and subjected to SDS-PAGE and immunoblotting with a monoclonal antibody to phosphotyrosine.

numerous cellular proteins (rightmost lane in Fig. 5.4A; the latter two lanes were underexposed relative to the lanes in left panel, due to the high intensity of the diamide lane).

In view of these data, I considered the possibility that inhibition of SHP-1 contributed to phosphotyrosine accumulation in cells stimulated with physiological agonists. Some of the stimuli that induced a net increase in tyrosine phosphorylation were also found to inhibit the activity of SHP-1, as determined *in vitro* by immune-complex phosphatase assays (see Experimental Procedures). Shown in Fig. 5.4B, immunoprecipitable SHP-1 activity was inhibited by 42± 5.9% and 31± 2.1% following treatment with TPA or OPZ, respectively (n=3). Diamide treatment of neutrophils prior to immunoprecipitation produced the greatest inhibition of SHP-1 (59.8 ± 2.7%, n=3), while fMLP did not have a significant effect at any of the time points examined[3]. Treatment of cells with TPA for varying times revealed a correlation between the extent of SHP-1 inhibition (Fig. 5.5A) and the accumulation of cellular protein phosphotyrosine (Fig. 5.5B). Together, these results suggest that decreased SHP-1 activity can contribute to increased tyrosine phosphorylation following stimulation of human neutrophils.

Oxidants have been shown to inhibit the activity of tyrosine phosphatases *in vitro* (Hecht and Zick, 1992) and *in vivo* (Zor, et al., 1993). It was previously demonstrated that activation of the NADPH oxidase, an endogenous source of reactive oxygen species in neutrophils, can lead to inhibition of CD45 and concomitant increased tyrosine phosphorylation (Fialkow, et al., 1993; Fialkow, et al., 1994). I therefore tested whether inhibition of SHP-1 was similarly mediated by oxidation. However, under conditions that greatly inhibit CD45, SHP-1 was minimally affected, implying that these phosphatases are differentially regulated.

Phosphorylation has been shown to alter the activity of several phosphatases, including SHP-1 (Li, et al., 1995; Trachman, et al., 1995; Uchida, et al., 1994). I therefore examined the phosphorylation state of this phosphatase in resting and activated neutrophils. As shown in

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[3]The activity of SHP-1 was examined after fMLP treatment for 15 sec-20 min. A statistically significant decrease in activity was not observed within this period.
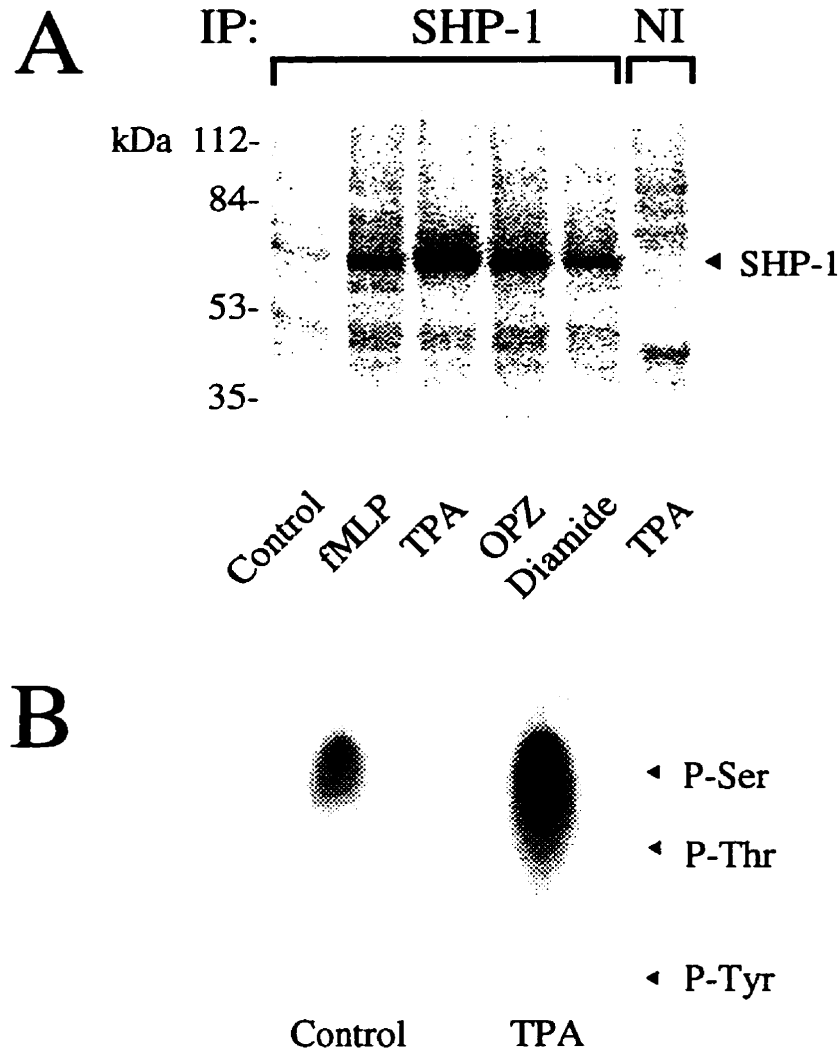
Fig. 5.6. Phosphorylation of SHP-1 following neutrophil activation. A. Neutrophil suspensions were loaded with ^{32}P-orthophosphate (see Experimental Procedures) and then treated without (control) or with the indicated agents for 10 min, with the exception of fMLP treatment, which was for 2 min. Following stimulation, SHP-1 was immunoprecipitated (as indicated) and subjected to SDS-PAGE, transferred onto polyvinylidene difluoride membranes and counted with a Molecular Dynamics phosphorimager. Immunoprecipitates obtained using an affinity-purified non-immune serum (NI) were also isolated from TPA stimulated neutrophils. The position of SHP-1 is indicated. B. Phosphoamino acid analysis was performed on SHP-1 immunoprecipitates isolated from ^{32}P-orthophosphate-labelled neutrophils treated without (control) or with 10^{-7}M TPA for 10 min. The mobility of the phosphoamino acid standards is shown. A and B are representative of at least three experiments.
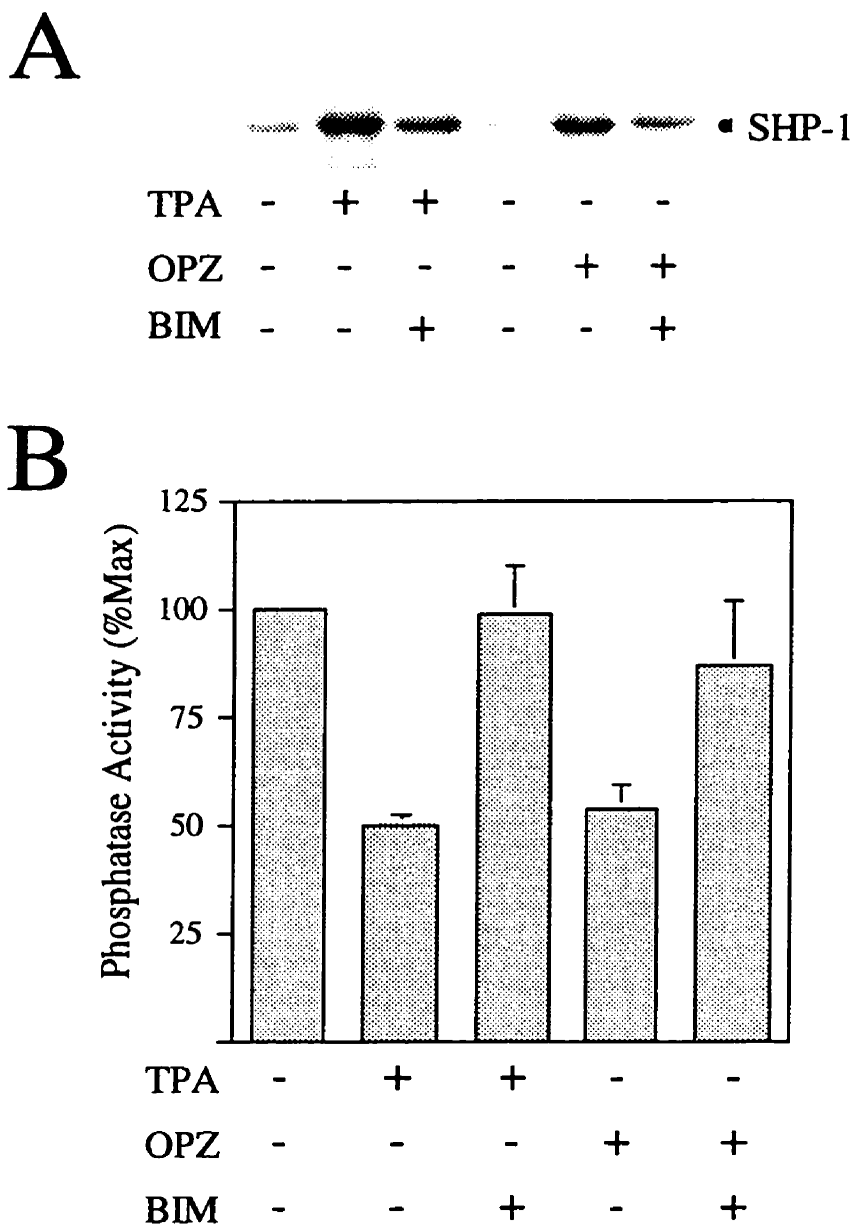
Fig. 5.7.   Role of PKC in SHP-1 regulation. A. Neutrophil suspensions were loaded with ^{32}P-orthophosphate (see Experimental Procedures) and then incubated in the absence (-) or presence (+) of 2 μM bis-indolylmaleimide (BIM) for 30 min at 37°C. Cells were then treated without (control) or with the indicated stimulus for 10 min and SHP-1 was immunoprecipitated and subjected to SDS-PAGE and autoradiography. The position of SHP-1 is indicated. B. Neutrophil suspensions were incubated in the absence (-) or presence (+) of 2 μM BIM for 30 min at 37°C. The cells were then treated without (control) or with the indicated stimulus for 10 min, subjected to immunoprecipitation of SHP-1 and measurement of its activity *in vitro* . Data are means ± SE of 3 experiments.

Fig. 5.6A, immunoprecipitation of SHP-1 from ^{32}P-orthophosphate labelled cells revealed a low but detectable amount of phosphorylation in untreated cells. By contrast, phosphorylation was markedly increased by treatment of cells with the agonists. Immunoprecipitates from stimulated cells obtained using non-immune serum (NI) displayed no signal (rightmost lane in 5.6A). As TPA induced the largest increase in phosphorylation, phosphoamino acid analysis was then performed on immunoprecipitates of SHP-1 from neutrophils treated with or without this PKC agonist. Illustrated in Fig. 5.6B, basal and TPA-induced phosphorylation of SHP-1 was found to be primarily on serine residues. Anti-phosphotyrosine immunoblotting of SHP-1 immunoprecipitates confirmed that neither TPA nor any of the other agonists studied were capable of inducing tyrosine phosphorylation of this phosphatase (not shown).

Activation of PKC is known to occur following stimulation by either fMLP or opsonized zymosan. As both of these agents, as well as the direct PKC activator TPA induced phosphorylation of SHP-1, I next examined the possibility that phosphorylation of this phosphatase is mediated by PKC. As demonstrated in Fig. 5.7A, pretreatment of ^{32}P-orthophosphate labelled neutrophils with 2 μM *bis*indolylmaleimide I (BIM), a potent and specific inhibitor of PKC (Toullec, et al., 1991), inhibited both TPA- and OPZ-induced phosphorylation of SHP-1. However, SHP-1 phosphorylation was not entirely abrogated by BIM treatment, suggesting that PKC was not fully inhibited or that other kinases also contribute to SHP-1 phosphorylation[4]. By contrast, prior treatment with BIM fully prevented the agonist-induced decrease in SHP-1 activity (Fig. 5.7B). Thus, it appears that PKC plays a significant role in regulating SHP-1, apparently through a phosphorylation-dependent mechanism.

Finally, I wished to determine whether PKC was responsible for direct phosphorylation and inhibition of SHP-1 or was instead acting upstream of the regulatory kinase(s). To this end, immunoprecipitates of SHP-1 were prepared from resting or TPA-treated neutrophils, washed extensively and subjected to *in vitro* phosphorylation using PKC purified from rat

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[4]While MAP kinase has been proposed to phosphorylate and inhibit the activity of the related tyrosine phosphatase, SHP-2, we observed no effect on the phosphorylation of SHP-1 following TPA treatment in the presence of 50 μM PD09589, a potent and specific inhibitor of MAP kinase kinase (MEK1).

120

A   IP: SHP-1   NI

◄ SHP-1

TPA pretreatment -   -   +   -

PKC -   +   +   +

lane # 1   2   3   4

B

PKC -   +   -

TPA pretreatment -   -   +
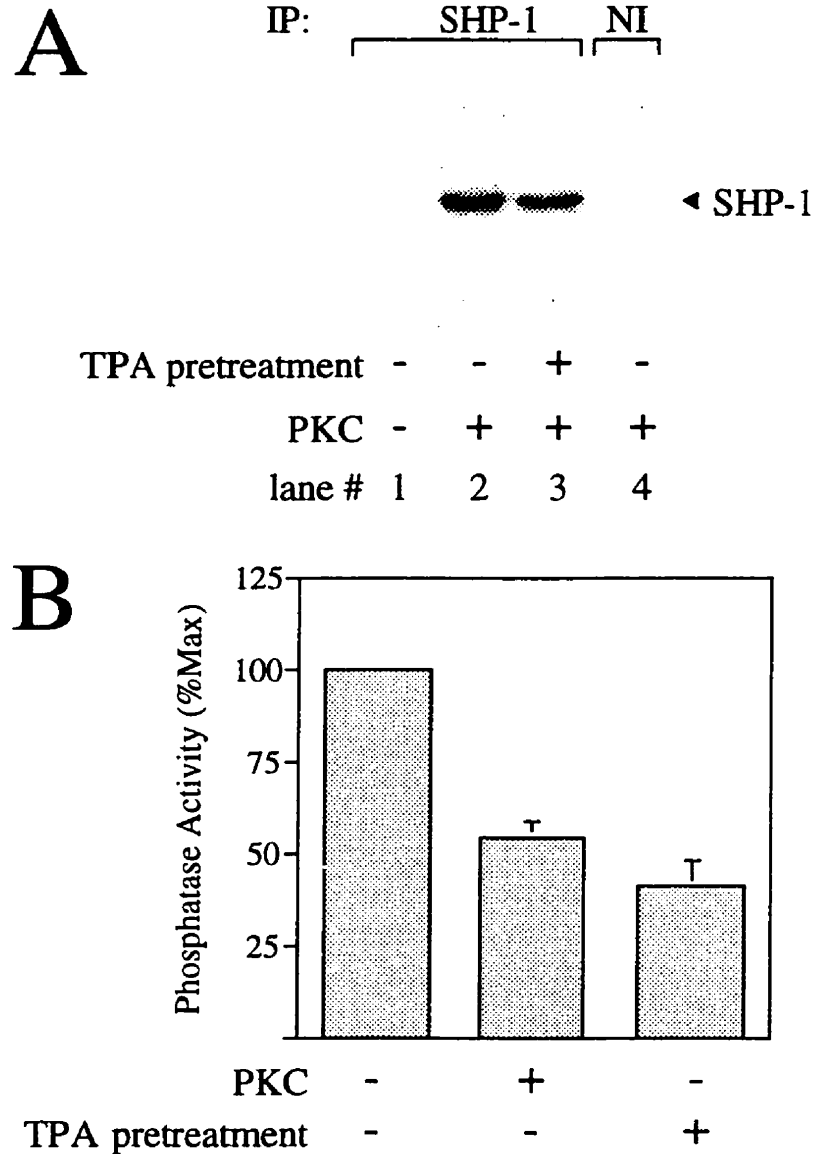
Fig. 5.8.   *In vitro* phosphorylation and inhibition of SHP-1 by PKC. A. Neutrophil suspensions were treated without (-; lanes 1,2 and 4) or with (+; lane 3) 10^{-7}M TPA prior to immunoprecipitation of SHP-1. Immunoprecipitates were subjected to *in vitro* phosphorylation in the absence (-; lane 1) or presence (+; lanes 2-4) of purified PKC, with added TPA, phosphatidylserine and ^{32}P-labelled ATP. Controls were performed using immunoprecipitates obtained with an affinity-purified non-immune serum (NI; lane 4). The location of SHP-1 is indicated. B. Neutrophil suspensions were treated without (-) or with (+) 10^{-7} M TPA prior to immunoprecipitation of SHP-1. After washing extensively, SHP-1 immunoprecipitates were subjected to *in vitro* phosphorylation in the absence (-) or presence (+) of purified PKC as in A, and the phosphatase activity of SHP-1 was then determined. Immunoprecipitates obtained with an affinity-purified non-immune serum or with beads alone displayed phosphatase activities that were consistenly less than 25% the activity of SHP-1 immunoprecipitates from untreated cells. Data are means ± SE of 3

brain. As shown in Fig. 5.8A, immunoprecipitated SHP-1 was readily phosphorylated by PKC (lane 2). Moreover, stimulation of cells with TPA prior to immunoprecipitation decreased the amount of *in vitro* PKC-mediated SHP-1 phosphorylation (lane 3 in Fig. 5.8A), presumably due to the incorporation *in situ* of non-radioactive phosphate into sites on SHP-1 that are substrates of PKC. Treatment of these immunoprecipitates with ^{32}P-γ-ATP alone, in the absence of PKC, did not result in phosphorylation of SHP-1 (lane 1), confirming that phosphorylation of SHP-1 in the *in vitro* assay is not mediated by co-precipitating kinases. Similarly, no PKC-induced phosphorylation was evident in experiments performed with immunoprecipitates of non-immune serum (lane 4).

The effect of *in vitro* phosphorylation on the activity of SHP-1 was determined. Immunoprecipitates of the phosphatase were subjected to phosphorylation by purified PKC, as in Fig. 5.8A, and assayed for activity. *In vitro* phosphorylation by PKC was found to inhibit the activity of SHP-1 to 54 $\pm$ 3.9% (3 experiments with duplicate determinations) of the control level (Fig. 5.8B). This inhibition of phosphatase activity was comparable to that induced by TPA pretreatment of intact cells prior to immunoprecipitation of SHP-1. Together, these results suggest that direct phosphorylation of SHP-1 on serine residues by PKC mediates, at least in part, the inhibition of SHP-1 phosphatase activity following neutrophil stimulation.

Discussion

Little is known about the role of tyrosine phosphatases in regulating neutrophil antimicrobial responses. In this report, I established that SHP-1 is expressed in human neutrophils and that the concentration of SHP-1 in these cells is approximately 530 nM. In contrast to other haemopoietic cell types which can express alternatively spliced forms (66 and 62 kDa) of SHP-1 (Kozlowski, et al., 1993; Uchida, et al., 1993), only the α splice variant of SHP-1 was found to be expressed in human neutrophils. While the molecular basis for this observation is unclear, the preferential expression of one splice variant may be of functional relevance, as has been suggested for the related tyrosine phosphatase, SHP-2 (Mei, et al., 1994).

In unstimulated cells, SHP-1 was found to be predominantly in the cytosol, although minimal amounts were found to be associated with the nucleus, the plasma membrane and the cytoskeleton. Stimulation with TPA, and to a lesser extent with OPZ and fMLP, induced an increase in the amount of SHP-1 associated with the cytoskeleton. Cytoskeletal association of SHP-1 has been demonstrated in platelets stimulated with thrombin and this translocation was postulated to mediate dephosphorylation of cytoskeletal-associated substrates (Ezumi, et al., 1995). By analogy, I suggest that the functional effects of SHP-1 on neutrophil function reflect its action on both cytoskeletal and cytosolic targets.

Agents that induce tyrosine phosphorylation of neutrophil proteins, such as OPZ and TPA were found to inhibit the activity of SHP-1. Furthermore, the time course of SHP-1 inhibition following stimulation paralleled that of cellular tyrosine phosphate accumulation. These results suggest that inhibition of SHP-1 contributes to phosphotyrosine accumulation and may play a role in the regulation of anti-microbial responses. Stimuli that inhibited the activity of SHP-1 were also found to induce its phosphorylation on serine residues. TPA-induced serine phosphorylation of SHP-1 had been reported in HL-60 cells, but inhibition of catalytic activity was not described (Zhao, et al., 1994). The increased SHP-1 expression induced by TPA in these cells may have masked the inhibitory effect of phosphorylation on enzyme activity (Uchida, et al., 1993; Zhao, et al., 1994). PKC-dependent phosphorylation of

SHP-1 has been demonstrated in human thymocytes and this phosphorylation event was shown to inhibit its activity (Trachman, et al., 1995). That phosphorylation by PKC is responsible for the functional inhibition of SHP-1 in human neutrophils is suggested by the experiments using BIM. This PKC antagonist diminished phosphorylation of the phosphatase, while simultaneously precluding the inhibition of its catalytic activity. It is noteworthy that concentrations of BIM that inhibited phosphorylation incompletely resulted in almost complete reversal of the functional inhibition. This can be interpreted to mean that phosphorylation at multiple sites is required for inhibition of catalytic activity. This interpretation would also account for the observation that, while inducing phosphorylation of SHP-1, fMLP failed to significantly inhibit its activity. In accord with this notion, phosphorylation of SHP-1 on multiple sites has been described in stimulated platelets (Li, et al., 1995).

In the current study, tyrosine phosphorylation of SHP-1 was not observed with any of the agonists used. Nevertheless, tyrosine phosphorylation of SHP-1 has been observed in other cell types (Bouchard, et al., 1994; Yeung, et al., 1992) and, contrary to our findings of serine phosphorylation-mediated inhibition, is thought to increase the activity of this phosphatase (Li, et al., 1995; Uchida, et al., 1994). The apparent lack of phosphotyrosine-mediated regulation of SHP-1 in human neutrophils may reflect the absence of specific tyrosine kinases capable of phosphorylating this protein. In this regard it is noteworthy that Lck, which is primarily responsible for SHP-1 phosphorylation in lymphoid cells (Lorenz, et al., 1994), is not detectable in human neutrophils (Brumell, et al., 1996).

In conclusion, our findings suggest that SHP-1 plays an important role in regulating the balance of protein tyrosine phosphorylation in neutrophils. While the substrates for SHP-1 in these cells have not been defined, our data indicate a role for this enzyme in dephosphorylating both cytosolic and cytoskeleton-associated proteins. In addition, the finding that PKC acts to inhibit SHP-1 activity following neutrophil stimulation provides a new link between the serine/threonine and tyrosine phosphorylation branches of the signalling cascade that triggers anti-microbial responses in human neutrophils.

Chapter 6

Phosphorylation and Subcellular Redistribution of Pleckstrin in Human Neutrophils

Summary

Pleckstrin, originally described as a major substrate of protein kinase C (PKC) in platelets, was found to be highly expressed in human neutrophils (intracellular concentration ≈ 15 μM). As PKC isoforms play an important role in mediating neutrophil anti-microbial responses, I studied the regulation of pleckstrin phosphorylation in response to inflammatory stimuli. Following treatment of neutrophils with fMLP, TPA or opsonized zymosan, pleckstrin was rapidly phosphorylated, which resulted in a shift in its electrophoretic mobility. Several lines of evidence suggest that pleckstrin is phosphorylated, in part, by a non-conventional PKC following stimulation by fMLP: 1) chelation of intracellular Ca^{2+} had only a partial inhibitory effect; 2) the phosphatidic acid phosphohydrolase antagonist propranolol extended it and 3) wortmannin and erbstatin blocked the phosphorylation of pleckstrin. These results suggest that non-conventional PKC isoforms, possibly δ or ζ, mediate phosphorylation of pleckstrin. Both PKC δ or ζ are expressed in human neutrophils. Increased association of pleckstrin with both microsomes and with the cytoskeleton was observed in stimulated cells. These findings suggest that phosphorylation by non-conventional PKC isoforms induces a conformational change in pleckstrin that promotes its interaction with membranes and/or with the cytoskeleton. Such a translocation may serve to target proteins or lipids recognized by pleckstrin homology (PH) domains to sites where they can contribute to the microbicidal response.

Introduction

Despite the importance of PKC in regulating neutrophil function, the substrates of this family of kinases are ill defined. One such substrate, pleckstrin, is highly expressed in plateletes and is rapidly phosphorylated in response to thrombin stimulation of these cells. In an effort to further define the sequence of events that follow activation of PKC in stimulated neutrophils, the expression and phosphorylation of pleckstrin in human cells treated with fMLP was evaluated. Our results suggest that pleckstrin is phosphorylated in human neutrophils by both conventional and non-conventional PKC isoforms and that this modification induces an association with membranes and with cytoskeletal components. Phosphorylated pleckstrin may serve as an intracellular adaptor, targetting PH domain-associated molecules to these subcellular compartments in response to inflammatory stimuli.

Results

To detect pleckstrin in human neutrophils, I immunoblotted whole-cell lysates with an affinity-purified antibody to pleckstrin (Fig. 6.1A). The major immunoreactive band in neutrophil lysates had a mobility of $\approx$46 kDa (PMN lanes in Fig. 1A). A weakly reactive band of $\approx$24 kDa was frequently noted in neutrophil lysates and may correspond to a proteolytic fragment of pleckstrin. Defined amounts of purified recombinant GST-pleckstrin were used to quantify the pleckstrin content of neutrophils. By scanning immunoblots, plotting the optical density and interpolation of the cell lysate bands, its was determined that neutrophils express approx. 250 ng of pleckstrin per 10^6 cells (mean of 3 determinations). Based on a total cell volume of 350×10^{-15} L/cell (determined electronically using the Coulter-Channelyzer), the cellular concentration of pleckstrin in neutrophils is calculated to be approximately 15 µM.

The subcellular localization of pleckstrin in neutrophils was explored by immunofluorescence. As shown in Fig. 6.1B (left side), staining of fixed cells with anti-pleckstrin antibodies revealed a diffuse, predominantly cytosolic localization of pleckstrin. Some punctation was evident in the cytosol, and a compact structure was also stained in the nuclear compartment (indicated with arrows). Under similar conditions, pre-immune serum

A

kDa 80-

◄ GST-Pleckstrin

50-

◁ Pleckstrin

33-
29-

100 150 200 250 300 400 500   Con fMLP

GST-Pleckstrin (ng)   PMN (10^6)

B

Anti-Pleckstrin   Non-Immune
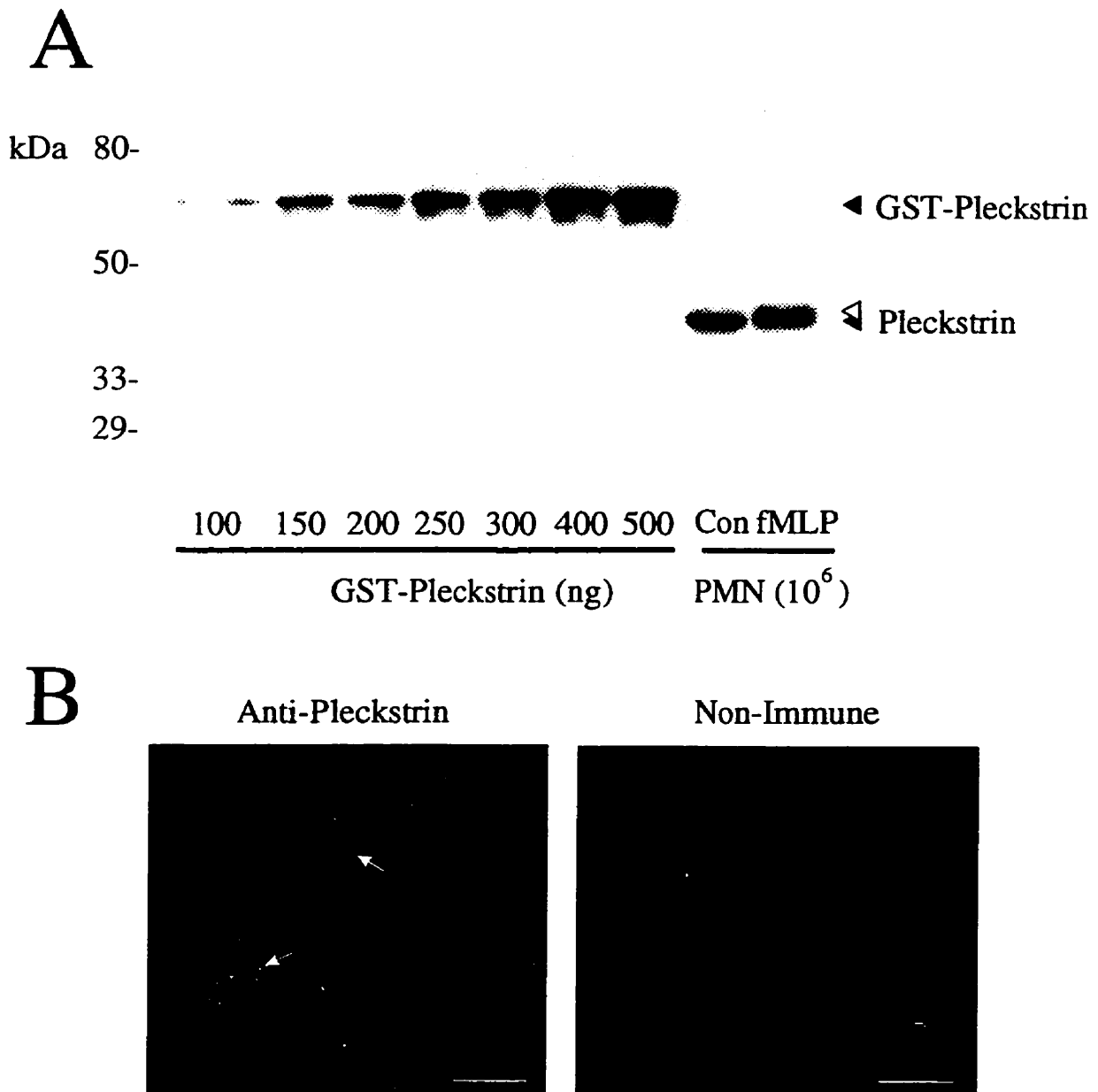
Fig. 6.1   Expression and localization of pleckstrin in human neutrophils. A. Indicated amounts of GST-pleckstrin (in ng) were analyzed by SDS-PAGE along with lysates from human neutrophils (10^6 cell equivalents) treated without (Con) or with fMLP (10^{-7} M) for 15 sec (fMLP). Samples were then immunoblotted with affinity-purified antibodies to pleckstrin. Treatment with fMLP was found to induce an upward shift in the mobility of pleckstrin, as indicated with an open arrowhead. B. Neutrophils were stained with affinity-purified antibodies to pleckstrin (Anti-Pleckstrin) or with non-immune serum (Non-Immune). Samples were analyzed using a Zeiss laser confocal microscope. Size bars = 10 nm. Data are representative of three separate experiments.

subjected to the affinity purification procedure used for the immune serum did not yield detectable staining (right panel, Fig. 6.1B). The subcellular distribution of pleckstrin was confirmed by nitrogen cavitation and fractionation of neutrophils on Percoll gradients (Brumell, et al., 1995). Immunoblotting of isolated fractions indicated that pleckstrin was found almost exclusively in the cytosolic fraction, with minimal amounts in the combined secretory vesicle/plasma membrane fraction. No immunoreactivity was detectable in primary, secondary or tertiary granules. Thus, in unstimulated neutrophils, pleckstrin is localized predominantly in the cytosol, resembling the results obtained in platelets (Imaoka, et al., 1983; Lyons and Atherton, 1979).

The rightmost lane in Fig. 6.1A shows that, while stimulation of the cells with fMLP prior to lysis did not affect the immunoreactivity of pleckstrin, it caused a decrease in the mobility of a fraction of the protein on SDS-PAGE (open arrowhead). Induction of the slower migrating form of pleckstrin was very rapid and transient following fMLP stimulation (Fig. 6.2A). Shifts in the mobility of proteins during SDS-PAGE can be caused by several post-translational modifications, including phosphorylation. To test whether pleckstrin was phosphorylated following stimulation of neutrophils with fMLP, cells were metabolically labelled with ^{32}P-orthophosphate, treated with or without the chemoattractant and then subjected to immunoprecipitation. As shown in Fig. 6.2B, pleckstrin phosphorylation in response to fMLP was coincident with its shift in electrophoretic mobility, peaking between 15 sec and 1 min and decaying rapidly thereafter. No phosphorylation was apparent in immunoprecipitates obtained from cells treated with fMLP for 15 sec using non-immune serum (NI; rightmost lane in Fig. 6.2A).

I next sought to determine if phosphorylation was responsible for the fMLP-induced mobility shift of pleckstrin. For this, denatured neutrophil lysates was treated with alkaline phosphatase. Samples were then analyzed by SDS-PAGE and pleckstrin immunoblotting and representative results are shown in Fig. 6.3A. As before, lysates from untreated neutrophils displayed only a single immunoreactive form of pleckstrin (first lane, solid arrowhead) that did not change in intensity or mobility following incubation with exogenous alkaline phosphatase.

A

◁ Pleckstrin

- .25 1 2 5 7

Time (min)

B

IP: Anti-Pleckstrin   NI

◀ Pleckstrin

fMLP: ⁻ .25 1 2 5 7 10 .25

Time (min)
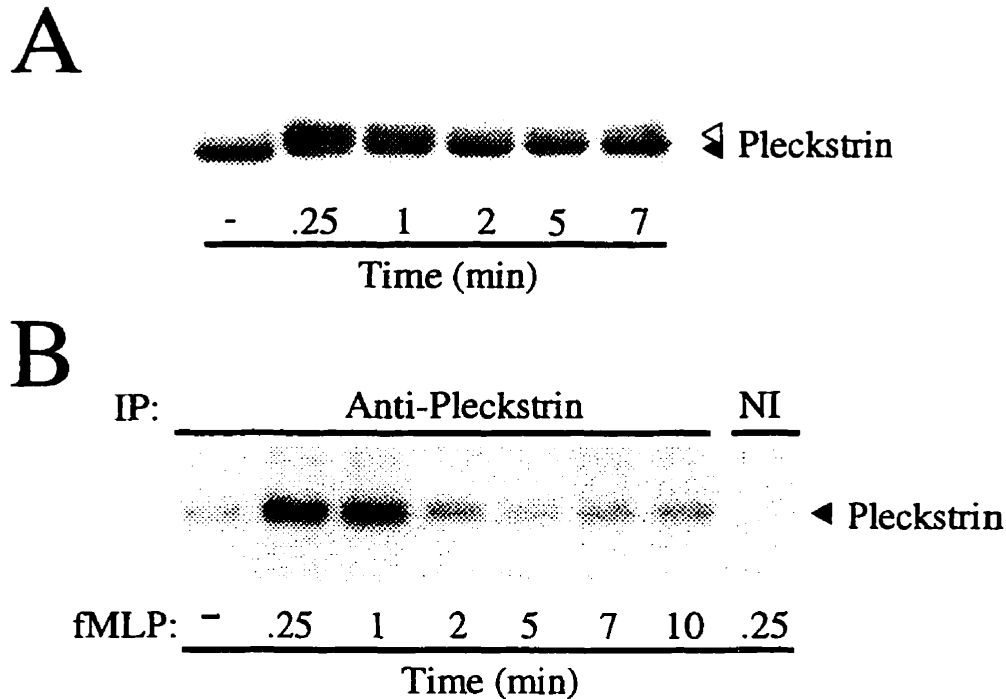
Fig. 6.2   Phosphorylation induces a shift in the mobility of pleckstrin. A. Neutrophil suspensions were treated with or without (-) 10^{-7} M fMLP for the indicated time (in min). Cells were rapidly sedimented, resuspended in boiling sample buffer and samples (10^6 cell equivalents/lane) were subjected to SDS-PAGE and immunoblotting with affinity-purified antibodies to pleckstrin. The slower (open arrowhead) and faster (solid arrowhead) migrating forms of pleckstrin are indicated. B. *In situ* phosphorylation was determined by immunoprecipitation of pleckstrin (Anti-Pleckstrin) from neutrophils that had been labeled with ^{32}P-orthophosphate and then treated without (-) or with 10^{-7} M fMLP for the time shown (min), as described in Experimental Procedures. Non-immune serum (NI) was used for immunoprecipitation of a sample stimulated for 15 sec. The position of pleckstrin is indicated (arrowhead).

As in Fig. 6.2A, treatment of cells with fMLP induced the appearance of a slower migrating form of pleckstrin (fourth lane in Fig. 6.3A, open arrowhead), which was distinct from the faster migrating species seen in control cells. Treatment of denatured lysates from fMLP-stimulated cells with alkaline phosphatase eliminated the slower migrating form of pleckstrin (sixth lane) while a comparable incubation in the absence of phosphatase had no effect (fifth lane). The latter finding indicates that the denaturing protocol effectively inactivated endogenous proteases and other enzymes potentially capable of altering pleckstrin during the 30 min incubation at 37°C and that reversal of the mobility shift was indeed due to the action of alkaline phosphatase. These data imply that phosphorylation of pleckstrin is the post-translational modification responsible for the mobility shift induced by fMLP.

Tight binding of certain lipids has been shown to cause a decrease in the electrophoretic mobility of several proteins, including PKC ζ (Limatola, et al., 1994) and albumin (Tigyi, et al., 1990). Since pleckstrin has been shown to associate with lipids (Lemmon, et al., 1995), it was conceivable that phosphorylation promoted lipid binding, thereby contributing to the mobility changes. To test this notion, I extracted resting and fMLP-treated neutrophils with acetone and subjected the de-lipidated proteins to SDS-PAGE and immunoblotting with anti-pleckstrin antibodies. As shown in Fig. 6.3B, though retrieval of proteins after acetone extraction was incomplete, the fMLP-induced mobility shift of pleckstrin was still evident following this de-lipidation protocol. Considering these data, the mobility shift of pleckstrin can be most simply explained by a conformational change induced directly by phosphorylation of the protein.

The mobility shift on SDS-PAGE provided a convenient, non-isotopic means to monitor the phosphorylation of pleckstrin. I took advantage of this property to compare the time course of pleckstrin phosphorylation induced by fMLP by other soluble and phagocytic stimuli of neutrophils. As shown in Fig. 6.3C, treatment of cells with a direct PKC agonist, TPA, caused a shift of most of the pleckstrin which was rapid (fully apparent by 15 sec) and sustained (lasting for up to 30 min; not shown). Opsonized yeast cell wall particles (zymosan), which signal primarily via Fcγ and CR3 receptors, induced a more modest, progressive

A

Pleckstrin

fMLP	−	−	−	+	+	+
Incubation	−	+	+	−	+	+
AP	−	−	+	−	−	+

B

| Acetone | − | − | + | + |

Pleckstrin

| fMLP | − | + | − | + |

C

Pleckstrin

| − | .25 | 1 | 2 | 5 | 7 |

Time (min)
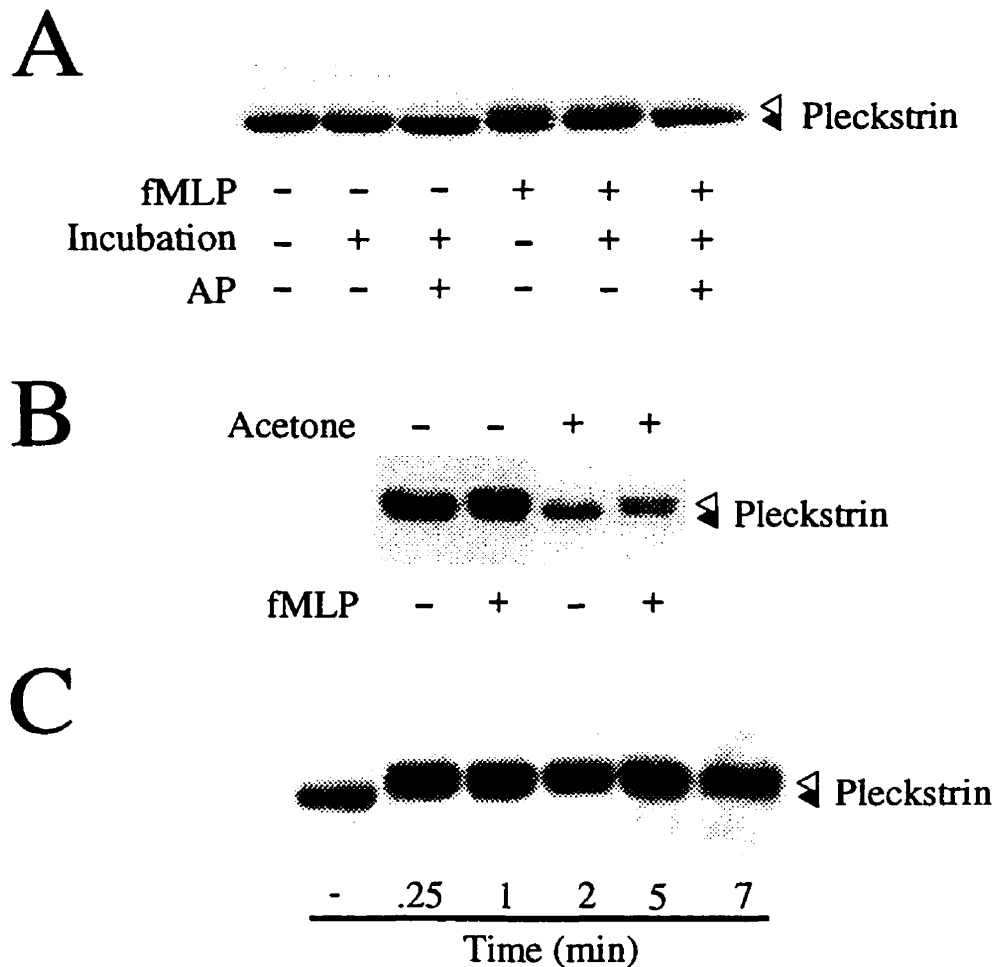
Fig. 6.3. Phosphorylation induces a shift in the mobility of pleckstrin. A. Denatured neutrophil lysates were prepared from cells treated without (-) or with (+) 10^{-7} M fMLP for 15 sec (see Materials and Methods). Aliquots of these lysates were either boiled immediately with an equal volume of 2x concentrated sample buffer (no incubation) or following incubation for 30 min at 37°C in the presence (+) or absence (-) of alkaline phosphatase (250 Units/ml). B. Neutrophil suspensions were treated without (-) or with (+) 10^{-7} M fMLP for 15 sec and either subjected to SDS-PAGE and immunoblotting immediately or after lipid extraction with acetone at -20°C, as indicated. C. Neutrophil suspensions (10^{7} cells/ml) were treated without (-) or with 10^{-7} M TPA for the indicated time (min). Cells were rapidly sedimented, resuspended in boiling Laemmli buffer and samples (10^{6} cell equivalents/lane) were subjected to SDS-PAGE and immunoblotting with affinity-purified antibodies to pleckstrin. The mobilities of phosphorylated (open arrowheads) and unphosphorylated pleckstrin (solid arrowheads) are indicated.

increase in pleckstrin phosphorylation. Thus, pleckstrin phosphorylation is common to the signal transduction pathways of soluble and particulate stimuli and may conceivably play a role in the initiation of the antimicrobial response.

The sites phosphorylated on pleckstrin in activated neutrophils were examined. Sequence alignment comparisons of pleckstrin with known kinase substrates suggest a number of potential PKC phosphorylation sites. These include Ser-40, Ser-43 and Thr-73 within the N-terminal PH domain and Ser-113, Thr-114 and Ser-117 located in the sequence intervening between the two PH domains. Proteolytic digest analysis of pleckstrin isolated from platelets previously localized *in vivo* phosphorylation to the latter domain (Abrams, et al., 1995), which is illustrated in Fig. 6.4A. By analysis of ectopically expressed pleckstrin in fibroblast cell lines, one of us (K. C.) recently determined that Ser-113 and Ser-117 (indicated with asterisks in Fig. 6.4A) are the primary targets of PKC, with phosphorylation occurring almost exclusively at these sites (Craig and Harley, 1996). This conclusion was based on tryptic phosphopeptide analysis of pleckstrin mutants transfected into COS-1 cells and phosphorylation of synthetic peptides corresponding to this region. A typical phosphopeptide map of wild type pleckstrin isolated from stimulated COS-1 cells is shown in Fig. 6.4B, with the 5 major phosphopeptide species indicated. Mutational analysis revealed the identity of these individual phosphopeptide species: mutation of Ser-117 to Ala abolished the formation of peptide 1, while mutation of Ser-113 to Ala blocked the generation of peptides 2-5 (Craig and Harley, 1996). Multiple phosphopeptides can arise upon phosphorylation of Ser-113 due to alternate tryptic cleavage sites (the potential trypsin cleavage sites in the 109-120 linker region of pleckstrin are indicated with arrows in Fig. 6.4A). Using the same method, tryptic phosphopeptide analysis of pleckstrin immunoprecipitated from neutrophils was performed. As shown in Fig. 6.4C, pleckstrin isolated from unstimulated cells displays a low but detectable level of phosphorylation on peptide 1, corresponding to Ser-117. Treatment of neutrophils with fMLP led to increased phosphorylation of peptide 1 and induced the appearance of phosphopeptide species 2-5 (Fig. 6.4D). No other major phosphopeptide species were observed. These results suggest that in neutrophils, as in platelets and transfected

A

↓↓ ↓↓ ↓
-FARKSTRRSIRL-
109   *   *   120
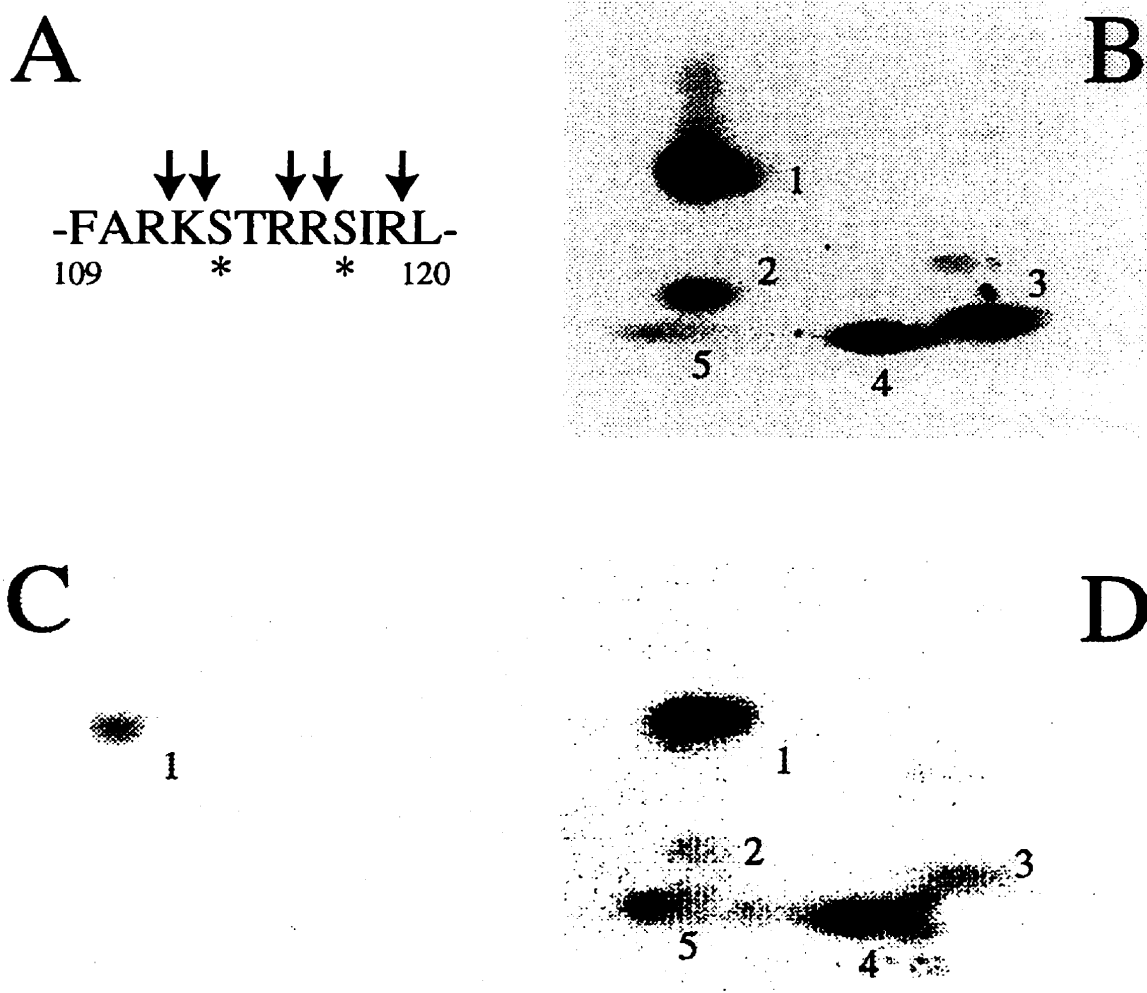
Fig. 6.4.   Tryptic phosphopeptide analysis of pleckstrin phosphorylation. A. Amino acid sequence of intervening sequence (residues 109-120) that joins the two PH domains of pleckstrin. Potential trypsin cleavage sites are indicated by arrows. The primary phosphorylation sites of pleckstrin, Ser-113 and Ser-117 are indicated with asterisks. B. *In vivo* phosphorylated pleckstrin from transiently transfected Cos-1 cells was immunoprecipitated and subjected to tryptic phopshopeptide analysis as described in Experimental Procedures. The major phosphopeptide species are indicated. C. *In vivo* phosphorylated pleckstrin from human neutrophils was immunoprecipitated and subjected to tryptic phosphopeptide analysis, as described in B. D. *In vivo* phosphorylated pleckstrin from neutrophils treated with 10^{-7}M fMLP (30 sec) was immunoprecipitated and subjected to tryptic phosphopeptide analysis, as described in B. These data were provided by Dr. Karen L. Craig.

fibroblast cell lines, pleckstrin is phosphorylated on Ser-113 and Ser-117. Accordingly, I was unable to detect phosphotyrosine residues in pleckstrin in fMLP-activated neutrophils by immunoblotting with anti-phosphotyrosine antibodies (not shown). Similar results were obtained with TPA-stimulated neutrophils.

Binding of fMLP to its receptor and the resulting activation of heterotrimeric GTP-binding proteins initiate downstream signalling pathways that include the activation of phospholipase C, which cleaves phosphatidylinositol 4,5 bisphosphate to form inositol trisphosphate and diacylglycerol (Snyderman and Uhing, 1992). In turn, soluble inositol trisphosphate induces the release of Ca^{2+} from intracellular stores and, as a consequence, an influx of extracellular Ca^{2+} across the plasma membrane (Krause, et al., 1990). Increased intracellular Ca^{2+} and diacylglycerol production synergistically activate members of the conventional class of protein kinase C (cPKC). To ascertain the involvement of cPKC isoforms in pleckstrin phosphorylation by chemoattractant-activated neutrophils, I examined the influence of the individual activators of this class of PKC. The role of Ca^{2+} was assessed by preloading cells with the chelating agent BAPTA and stimulating cells in a Ca^{2+}-free medium. That this protocol effectively buffered the intracellular Ca^{2+} transient elicited by fMLP is shown in Fig. 6.5A. The large increase in cytosolic $[Ca^{2+}]$ triggered by the chemoattractant was virtually eliminated by the combined effects of the chelator and the omission of external Ca^{2+}. As shown in Fig. 6.5B, while partial inhibition of the pleckstrin mobility shift was noted, the slower migrating form of the protein (open arrowhead) is readily apparent following stimulation with fMLP under conditions where intracellular $[Ca^{2+}]$ remains unaltered (fourth lane). The existence of a Ca^{2+}-independent component of the stimulation of pleckstrin was confirmed by immunoprecipitation of ^{32}P-orthophosphate labelled cells (Fig. 6.5C). BAPTA buffering of intracellular Ca^{2+} release led to a ≈50% inhibition of pleckstrin phosphorylation, consistent with the effect of chelation on the mobility shift. Together, these findings indicate that the fMLP-induced Ca^{2+} transient is not an obligatory requirement for pleckstrin phosphorylation, suggesting that Ca^{2+}-independent, non-conventional isoforms of PKC or other kinases contribute to this event. Two classes of non-conventional, Ca^{2+}-
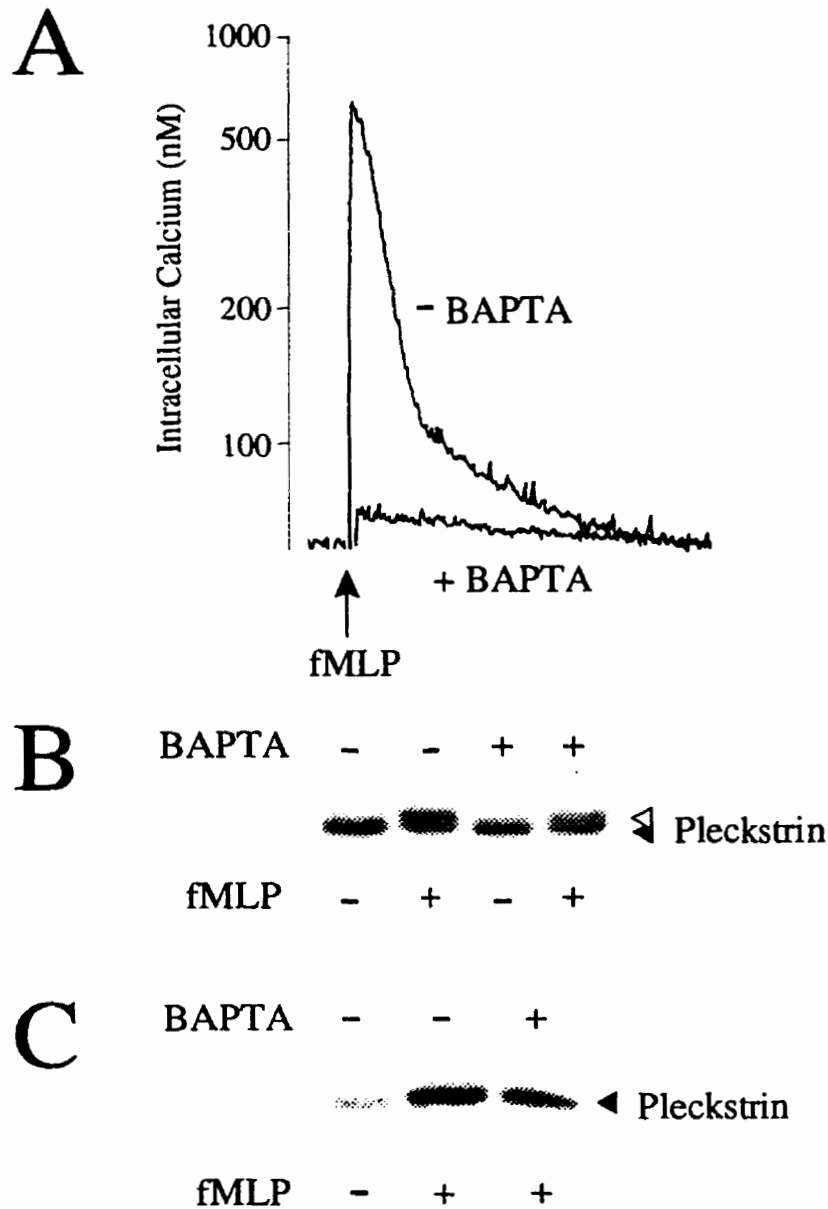
Fig. 6.5.   Role of conventional PKC in pleckstrin phosphorylation.   A. Neutrophil suspensions were loaded with indo-1 with or without BAPTA, as indicated.   The cells were then suspended in Ca^{2+} -free medium and $[Ca2+]_i$ was determined fluorimetrically. Where indicated, the cells were stimulated with 10^{-7} M fMLP.   B. Neutrophil suspensions (10^7 cells/ml) were loaded without (-) or with (+) BAPTA and suspended in Ca^{2+}-free medium. Where indicated, the cells were next stimulated with 10^{-7} M fMLP (15 sec). The cells were immediately sedimented, resuspended in boiling Laemmli buffer and aliquots (10^6 cell equivalents/lane) were subjected to SDS-PAGE and immunoblotting with affinity-purified antibodies to pleckstrin.   The mobilities of phosphorylated (open arrowheads) and unphosphorylated pleckstrin (solid arrowheads) are indicated.   C. *In situ* phosphorylation was determined by immunoprecipitation of pleckstrin from ^{32}P-orthophosphate labelled neutrophils that had been pre-loaded without (-) or with (+) BAPTA, as above, prior to stimulation with 10^{-7} M fMLP for 15 sec, where indicated. Pleckstrin is indicated.

independent PKC isoforms have been described to date (Newton, 1995). They include the novel PKC family (nPKC) consisting of 5 isoforms (δ, ϵ, η, θ and μ) and the atypical PKC family (aPKC: ζ and λ isoforms). Members of both non-conventional PKC families, specifically the δ (Lopez, et al., 1995) and ζ (Dang, et al., 1994) isoforms, have been identified in neutrophils and may play a role in pleckstrin phosphorylation.

Phosphatidic acid is generated by neutrophils following fMLP stimulation and has been shown to activate PKCζ (Limatola, et al., 1994). Catabolism of phosphatidic acid to diacylglycerol is performed by phosphatidic acid phosphohydrolase, an enzyme that is inhibited by treatment of cells with propranolol. This agent, which has been shown to enhance and prolong the increase in phosphatidic acid produced following fMLP stimulation (English and Taylor, 1991), should serve to distinguish the contribution of PKCζ. As illustrated in Fig. 6.6A, treatment with propranolol was found to prolong the effect of the chemotactic peptide on pleckstrin: the band with slower mobility was still present after 2.5 min in cells treated with the phosphohydrolase inhibitor, while it was undetectable at this time in untreated cells. These results suggest that phosphatidic acid generation plays an important role in pleckstrin phosphorylation, possibly by activating PKCζ.

Phosphatidylinositol (3,4,5)-phosphate and possibly other phosphoinositides generated by activation of phosphatidylinositol 3'-kinase (PI3K) are also thought to stimulate PKCζ (Nakanishi, et al., 1993). In permeabilized platelets, addition of exogenous phosphoinositides was in fact sufficient to induce phosphorylation of pleckstrin (Toker, et al., 1995; Zhang, et al., 1995). Because PI3K is activated rapidly in fMLP-stimulated neutrophils (Stephens, et al., 1991; Traynor-Kaplan, et al., 1989), I considered the role of this enzyme in the phosphorylation of pleckstrin. Cells were pretreated with or without wortmannin, an inhibitor of PI3K, prior to fMLP stimulation and lysates were subjected to immunoblotting. As shown in Fig. 6.6B, pretreatment with wortmannin prevented the appearance of the slower migrating, phosphorylated form of pleckstrin (open arrowhead). By contrast, inhibition of p70^{S6k}, a kinase located downstream of PI3K (Chung, et al., 1994), by pretreatment of cells with rapamycin (Chung, et al., 1992) had no effect (Fig. 6.6B). These data suggest that in addition

A

Untreated ◄ Pleckstrin

Propranolol ◄ Pleckstrin

fMLP   −   .25   1   1.5   2   2.5

Time (min)

B

Untreated ◄ Pleckstrin

Wortmannin ◄ Pleckstrin

Rapamycin ◄ Pleckstrin

fMLP   −   .25   1   1.5   2   2.5

Time (min)
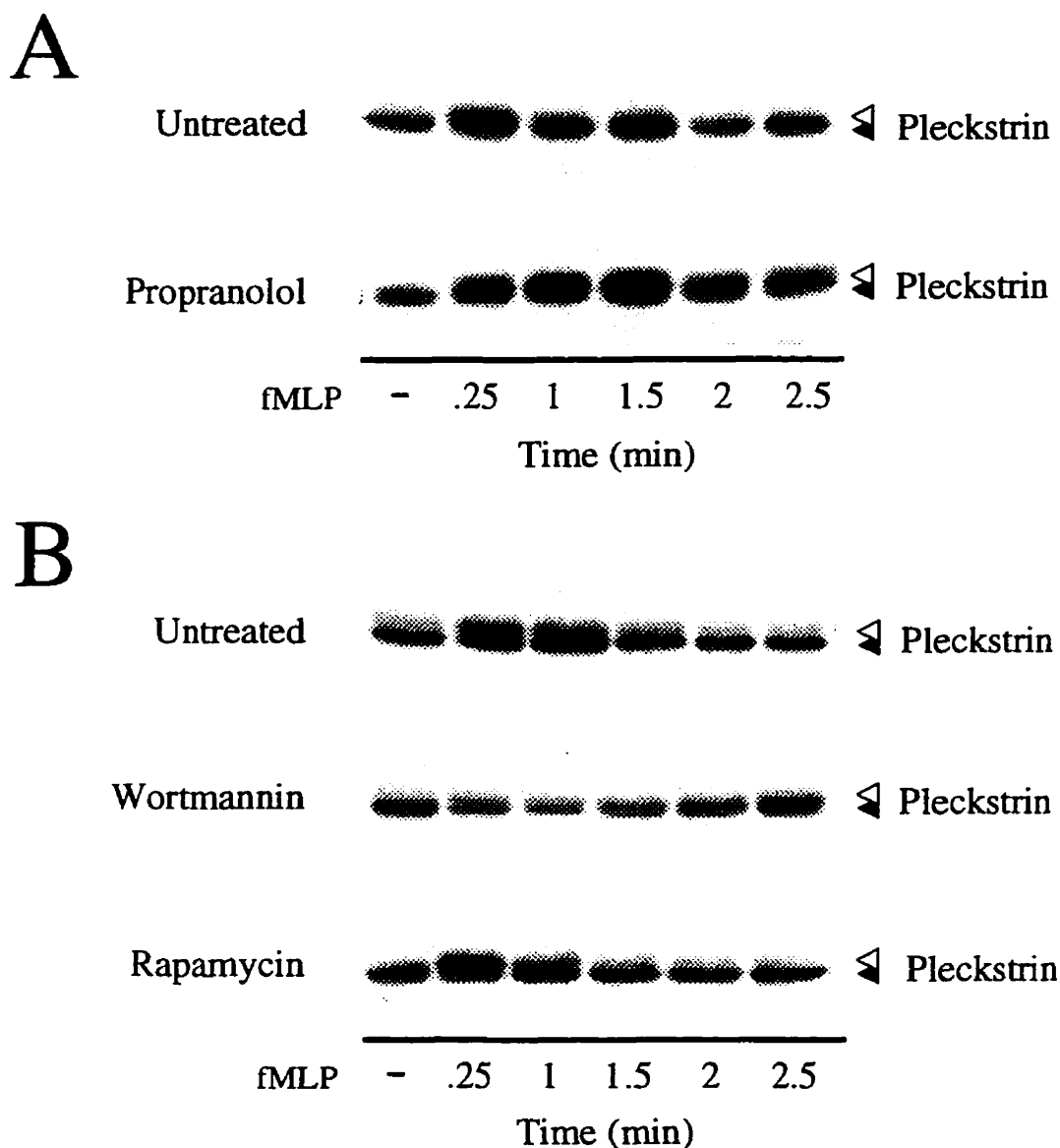
Fig. 6.6.   Role of activators of non-conventional PKC isoforms in pleckstrin phosphorylation. A. Neutrophil suspensions (10^7 cells/ml) were treated without (untreated) or with 150 mM propranolol for 5 min at 37°C prior to stimulation with 10^{-7} M fMLP for the time indicated (in min). Cells were rapidly sedimented, resuspended in boiling Laemmli sample buffer and aliquots (10^6 cell equivalents/lane) were subjected to SDS-PAGE and immunoblotting with affinity-purified antibodies to pleckstrin. The mobilities of phosphorylated (open arrowheads) and unphosphorylated pleckstrin (solid arrowheads) are indicated. B. Neutrophil suspensions (10^7 cells/ml) were treated without (untreated) or with 100 nM wortmannin or 50 ng/ml rapamycin for 30 min at 37°C prior to stimulation with 10^{-7} M fMLP for the time indicated (in min) and samples were analyzed for pleckstrin phosphorylation, as in A. Data are representative of at least three experiments of each type.

A

◁ Pleckstrin

fMLP (min)	-	.25	.5	1.0	-	.25	.5	1.0
erbstatin	-	-	-	-	+	+	+	+

B

erbstatin	-	-	+
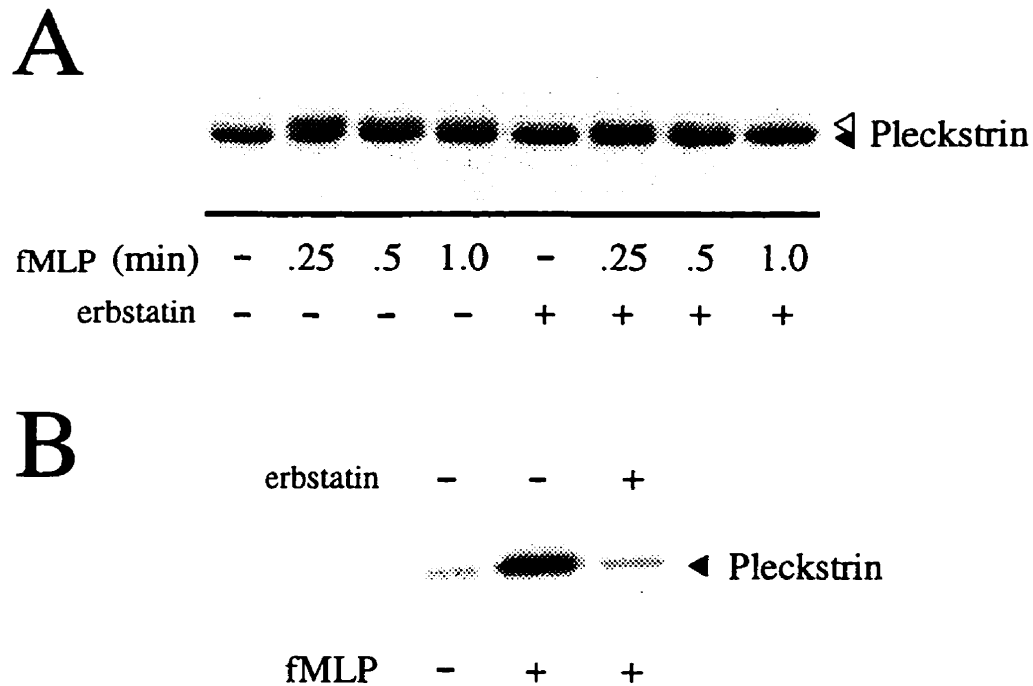
◀ Pleckstrin

fMLP	-	+	+

Fig. 6.7.   Role of tyrosine kinases in pleckstrin phosphorylation. A. Neutrophil suspensions (10^7 cells/ml) were treated without (-) or with (+) 10 µg/ml erbstatin for 30 min at 37°C prior to stimulation with 10^{-7} M fMLP for the times indicated (in min). Cells were rapidly sedimented, resuspended in boiling Laemmli sample buffer and aliquots (10^6 cell equivalents/lane) were subjected to SDS-PAGE and immunoblotting with affinity-purified antibodies to pleckstrin. The mobilities of phosphorylated (open arrowheads) and unphosphorylated pleckstrin (solid arrowheads) are indicated. B. In situ phosphorylation was determined by immunoprecipitation of pleckstrin from ^{32}P-orthophosphate-labelled neutrophils treated without (-) or with (+) 10 µg/ml erbstatin for 30 min at 37oC prior to stimulation with 10^{-7} M fMLP for 15 sec, where indicated (+). The position of pleckstrin is indicated. Representative of three experiments. representative of at least three experiments of each type.

to phosphatidic acid, phosphoinositides are also important activators of the kinase(s) that phosphorylate pleckstrin in human neutrophils and further support a role for non-conventional PKC isoforms in mediating this event.

Tyrosine phosphorylation of a number of substrates has been observed upon treatment of neutrophils with fMLP. The functional importance of this event is suggested by the finding that inhibitors of tyrosine kinases block many of the cellular responses to the chemoattractant (Berkow, et al., 1989; Kobayashi, et al., 1995). It was therefore of interest to establish whether tyrosine phosphorylation was similarly required for pleckstrin phosphorylation. Neutrophils were pretreated with the tyrosine kinase inhibitor, erbstatin, prior to fMLP stimulation. This treatment greatly inhibited phosphorylation of pleckstrin, as determined by the mobility shift of phosphorylated pleckstrin (Fig. 6.7A). Inhibition of pleckstrin phosphorylation by erbstatin was corroborated by directly measuring the incorporation of radiolabelled phosphate *in situ* (Fig. 6.7B). As pleckstrin is itself not tyrosine phosphorylated in neutrophils (see above), these results imply that tyrosine kinases regulate pleckstrin phosphorylation one or more steps upstream of the ser/thr kinases that directly phosphorylate it.

Pleckstrin has no identifiable catalytic activity. On the other hand, the presence of two PH domains, which are thought to mediate association with other proteins or lipids, suggests that pleckstrin may function as a PKC-regulated adaptor. Association of pleckstrin with other cellular components upon activation by either fMLP or TPA was studied by cell fractionation and immunoblotting. Cellular membranes were separated by differential centrifugation (Fig. 6.8.A&B), whereas a cytoskeleton-enriched fraction was obtained as a Triton X-100-insoluble pellet (Fig. 6.8C&D). Consistent with the results described above, immunoblotting revealed minimal amounts of pleckstrin to be associated with membranes and the cytoskeleton in unstimulated cells (<5%). However, stimulation with fMLP and TPA induced a 2-3 fold increase in its association with membranes (Fig. 6.8A) and an even greater increase was noted in the case of the cytoskeleton (Fig. 6.8C). The fMLP-induced association of pleckstrin in both cases was transient and correlated temporally (Fig. 8B and D) with the phosphorylation of
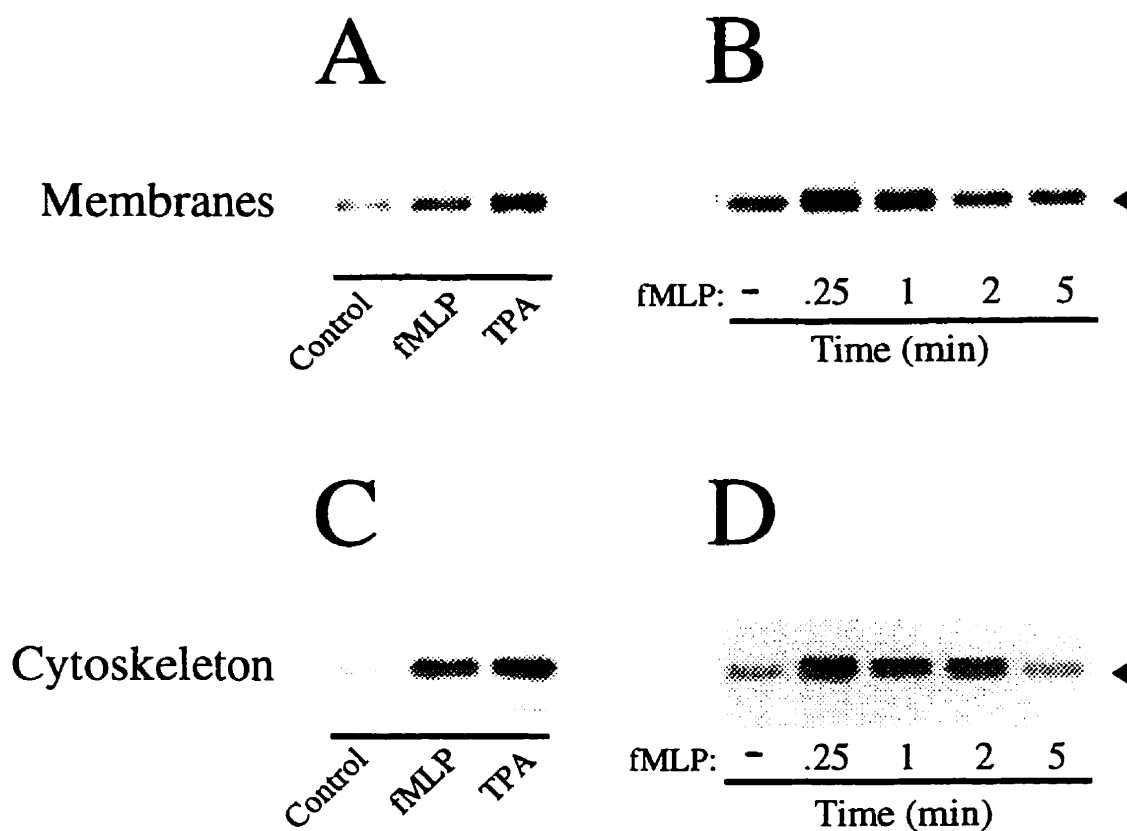
Fig. 6.8.   Subcellular redistribution of pleckstrin following cell stimulation. A. Neutrophil suspensions (2 x 10^7 cells/ml) were treated without (control) or with 10^{-7} M fMLP for 15 sec or with 10^{-7} M TPA for 1 min prior to the isolation of membranes. Membranes were resuspended in Laemmli sample buffer and aliquots (2 x 10^6 cell equivalents/lane) were subjected to SDS-PAGE and immunoblotting with affinity-purified antibodies to pleckstrin. B. Membranes were isolated from cells stimulated without (-) or with 10^{-7} M fMLP for the indicated times (in min) and analyzed as in A. C. Neutrophil suspensions (2 x 10^7 cells/ml) were treated without (control) or with 10^{-7} M fMLP for 15 sec or with 10^{-7} M TPA for 1 min prior to isolation of cytoskeleton-associated proteins. Cytoskeletal-associated proteins were dissolved in Laemmli sample buffer and aliquots (2 x 10^6 cell equivalents/lane) were subjected to SDS-PAGE and immunoblotting with affinity-purified antibodies to pleckstrin. D. Cytoskeleton-associated proteins were isolated from cells stimulated without (-) or with 10^{-7} M fMLP for the times indicated (in min) and analyzed as in C.

A

BIM: ____-____ ____+____

Membranes   ◄ Pleckstrin

Control *TPA* *Control* *TPA*

B

BIM: ____-____ ____+____

Cytoskeleton   ◄ Pleckstrin

Control *TPA* *Control* *TPA*
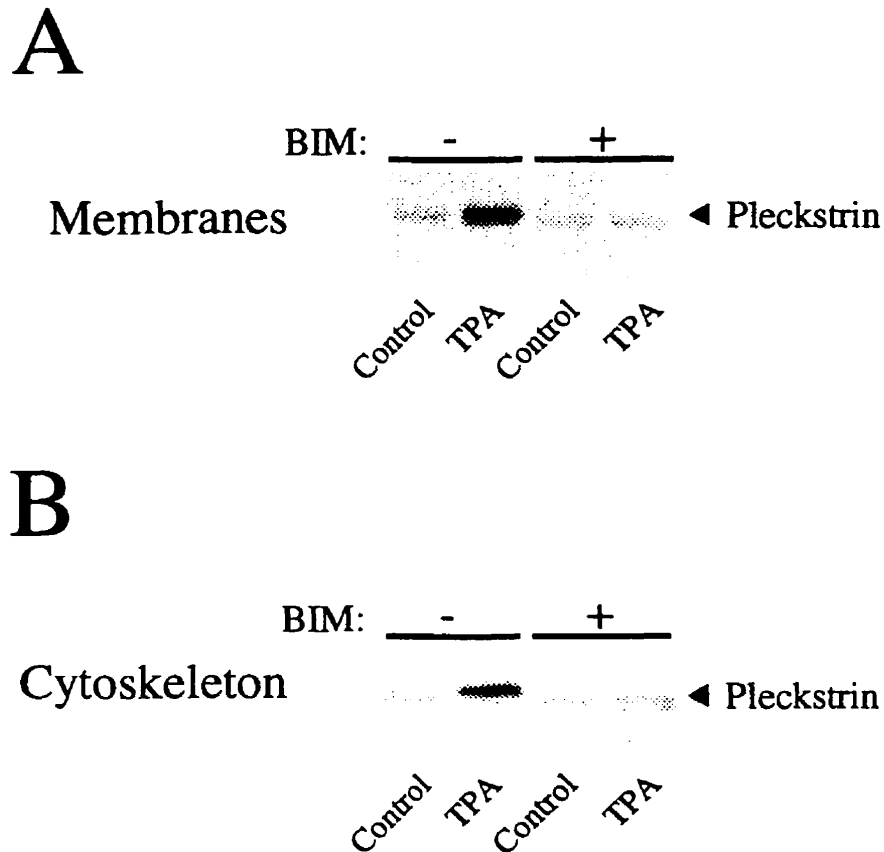
Fig. 6.9.   Subcellular redistribution of pleckstrin following cell stimulation: dependence on PKC activity. A. Membranes were isolated from neutrophils incubated without (-) or with (+) 5 µM bis-indolylmaleimide (BIM) prior to treatment with 10^{-7} M TPA for 1 min, where indicated. Membranes were resuspended in Laemmli sample buffer and aliquots (2 x 10^6 cell equivalents/lane) were subjected to SDS-PAGE and immunoblotting with affinity-purified antibodies to pleckstrin. The position of pleckstrin is indicated. B. Cytoskeleton-associated proteins were isolated from cells treated without (-) or with (+) 5 µM bis-indolylmaleimide (BIM) prior to treatment with 10^{-7} M TPA for 1 min, where indicated, and analyzed as in A. The position of pleckstrin is indicated.

pleckstrin determined in Fig. 6.2B, suggesting that this modification might be necessary for translocation. In accordance with this notion, pretreatment of cells with the potent and specific PKC inhibitor, *bis*-indolylmaleimide (BIM), prevented both TPA-induced phosphorylation of pleckstrin (not shown) and its association with membranes (Fig. 6.9A) and the cytoskeleton (Fig. 6.9B). Thus, pleckstrin redistributes within neutrophils in a phosphorylation-dependent manner.

Discussion

The study of pleckstrin has been largely confined to platelets, where the protein was originally identified. By comparison, very little is known about pleckstrin in leukocytes. Gailani and colleagues (Gailani, et al., 1990) proposed that a 47 kDa phosphoprotein in neutrophils was pleckstrin. However, the ≈47 kDa phosphoprotein detected by these authors could have been p47^{phox}, a subunit of the NADPH oxidase known to become phosphorylated upon neutrophil stimulation (Morel, et al., 1991). In this report, it was established that pleckstrin is indeed expressed in human neutrophils, at a comparatively high intracellular concentration (≈15 μM). In unstimulated cells, pleckstrin was localized predominantly to the cytosol, with minimal amounts associated with membranes, the cytoskeleton and the nucleus. In response to chemoattractant, pleckstrin was rapidly and transiently phosphorylated and underwent a partial redistribution to membranes and the cytoskeleton. The magnitude of this association could have been underestimated, to the extent that rapidly reversible interactions may have dissociated during the course of fractionation. In any event, the observed redistribution may signify that pleckstrin, which lacks known catalytic activity, may function as an intracellular adaptor, perhaps by bridging proteins and/or lipids via its dual PH domains.

Using ionic manipulation and pharmacological tools, I attempted to identify the isoforms of PKC that phosphorylate pleckstrin in response to fMLP. Blocking the fMLP-induced increase in cytosolic Ca^{2+} had only a partial inhibitory effect, indicating that Ca^{2+}-independent PKC isoforms (or other kinases) contribute to the phosphorylation of pleckstrin. Furthermore, propranolol was found to extend the duration of pleckstrin phosphorylation. These observations suggested that phosphatidic acid is an important lipid mediator, possibly acting on PKC ζ. In accordance, PI3K activation was found to be necessary for fMLP-induced pleckstrin phosphorylation. The lipid products of PI3K have been shown to activate members of both the atypical (ζ) and novel families of PKC (Moriya, et al., 1996; Nakanishi, et al., 1993; Toker, et al., 1994) and, when added to platelets, suffice to induce phosphorylation of pleckstrin (Toker, et al., 1995; Zhang, et al., 1995). These data support a

role for non-conventional PKC family isoforms in mediating pleckstrin phosphorylation in human neutrophils.

Neutrophils possess two types of PI3K: the conventional heterodimeric form that is thought to be activated by tyrosine phosphorylation and a recently identified enzyme that is directly stimulated by the βγ-subunits of G proteins (Stephens, et al., 1994). Because the fMLP receptor is known to couple to G proteins, one might expect the latter form to predominate in chemoattractant-treated cells. Nevertheless, many of the responses of neutrophils to fMLP are blocked by both tyrosine kinase antagonists (Berkow, et al., 1989; Kobayashi, et al., 1995) and by wortmannin (Dewald, et al., 1988; Okada, et al., 1994; Thelen, et al., 1994) suggesting that activation of PI3K may require phosphotyrosine accumulation. Indeed, a PI3K activity is associated with immunoprecipitates of a tyrosine kinase, Lyn, from chemoattractant stimulated neutrophils. This PI3K activity is upregulated in a manner that correlates temporally with the very rapid activation/deactivation of Lyn (Ptasznik, et al., 1995). These findings prompted us to test the effects of erbstatin. This tyrosine kinase antagonist was found to obliterate pleckstrin phosphorylation. This finding suggests that the classical type of PI3K may be responsible for activation of non-conventional forms of PKC. Alternatively, tyrosine kinase inhibitors may block activation of phospholipase D (Bourgoin and Grinstein, 1992; Uings, et al., 1992), which could participate in PKC activation by generating phosphatidic acid. However, I regard this possibility as unlikely since pretreatment of cells with 1% ethanol, which precludes the formation of phosphatidic acid (Billah, et al., 1989), had little effect on the kinetics or extent of pleckstrin phosphorylation (unpublished observations). Finally, tyrosine kinases can directly phosphorylate PKCδ, an isoform known to be present in neutrophils (Lopez, et al., 1995). Tyrosine phosphorylation has been shown to directly influence the activity and/or substrate specificity of this isoform (Haleem-Smith, et al., 1995; Li, et al., 1994) and may have contributed to the phosphorylation of pleckstrin.

The role of pleckstrin in agonist-stimulated neutrophils remains undetermined. Phosphorylation of pleckstrin correlated temporally with previously described, rapid responses to soluble and particulate stimuli, though the influence of pleckstrin on these responses is

unclear. Phosphorylation was found to induce a shift in the electrophoretic mobility of the protein on SDS-PAGE gels, likely due to a stable conformational change in pleckstrin. It is conceivable that this phosphorylation-dependent conformational change causes an alteration in the interaction(s) of pleckstrin with protein and/or lipid ligands via association with its two PH domains. In this regard, PH domains from pleckstrin and/or other signalling molecules were found to bind phosphoinositides such as phosphatidylinositol 4,5 bisphosphate (Harlan, et al., 1994), βγ subunits of heterotrimeric G-binding proteins (Touhara, et al., 1994) and all three classes of PKC (Konishi, et al., 1994; Konishi, et al., 1995). Association of pleckstrin with these ligands may be dependent on its prior phosphorylation and may mediate subcellular redistribution of this protein in neutrophils following stimulation with chemoattractants or with phagocytic stimuli. In accord with this hypothesis, inhibition of pleckstrin phosphorylation by treatment of cells with a PKC inhibitor was found to block its redistribution upon stimulation.

In summary, these data establish that pleckstrin is highly expressed in human polymorphonuclear leukocytes and is phosphorylated following stimulation of these cells with a variety of soluble and particulate stimuli. Phosphorylation was found to be mediated in part by non-conventional PKC isoforms and dependent on the activities of unidentified tyrosine kinases and PI3Kinase. Phosphorylation of pleckstrin was associated with a partial redistribution of the protein to the membrane and cytoskeleton. While no enzymatic function for this protein has been described, I speculate that pleckstrin acts as an intermolecular adaptor that targets interacting molecules to the membrane and/or cytoskeleton following activation.

Chapter 7

Subcellular distribution of docking/fusion proteins in neutrophils, secretory cells with multiple exocytic compartments.

Summary

Neutrophils contain at least four distinct types of secretory organelles, which undergo exocytosis during infection and inflammation. The signalling pathways leading to secretion of individual granules and their kinetics of exocytosis vary greatly, causing temporal and regional differences in docking and fusion with the plasma membrane. As a step towards understanding the processes underlying differential granular secretion in neutrophils, I assessed the presence and distribution of a number of proteins reported to be involved in vesicular docking and/or fusion in other systems. Specific antibodies were used for immunoblotting of cells fractionated by density gradients and free-flow electrophoresis, and for localization by confocal immunofluorescence and electron microscopy. Syntaxin 1, VAMP-1, SNAP-25, synaptophysin and cellubrevin were not detectable in human neutrophils. In contrast, syntaxin 4, VAMP-2 and the 39 kDa isoform of SCAMP were present. SCAMP was found mainly in secondary and tertiary granules and in a fraction containing secretory vesicles, but was virtually absent from the primary (lysosomal) granules. This profile is consistent with the proposed "post-Golgi" distribution of SCAMP. VAMP-2 was largely absent from primary and secondary granules, but concentrated in tertiary granules and secretory vesicles. This pattern of distribution parallels the increasing sensitivity of these exocytic compartments to intracellular free calcium. Accordingly, ionomycin induced translocation of VAMP-2 towards the plasma membrane. Syntaxin 4 was found almost exclusively in the plasma membrane, and it accumulated especially in lamellipodia. This regional accumulation may contribute to localized secretion into the phagosomal lumen.

Introduction

Polymorphonuclear leukocytes (neutrophils) present one of the earliest lines of defense against infection. Upon detection of bacterial products or inflammatory metabolites, neutrophils migrate to the site of infection, where they activate a series of microbicidal processes, including generation of reactive oxygen metabolites and release of lytic enzymes (Edwards, 1994). Virtually all the steps in this sequence depend on the timely occurrence of exocytic events. Thus, polarized appearance of receptors during chemotaxis (Sengelov, et al., 1994; Sengelov, et al., 1994), efficient assembly of the superoxide generating NADPH oxidase (Morel, et al., 1991) and secretion of microbicidal enzymes (Borregaard, et al., 1993; Borregaard, et al., 1993) all involve exocytic events. Despite the obvious importance of degranulation in neutrophils, the molecular mechanisms underlying this process remain poorly understood.

As an initial step towards understanding the mechanisms underlying secretion in neutrophils, I have assessed the presence and subcellular distribution of SNAREs, SCAMP and synaptophysin, using a combination of biochemical, immunological and morphological approaches.

Results

The presence in neutrophils of proteins reported to participate in membrane docking and/or fusion was initially assessed by immunoblotting whole cell extracts with specific antibodies. To ensure the efficacy of the blotting procedure, samples of tissues known to express these proteins were analyzed in parallel. Fig. 7.1A compares the expression of five proteins reported previously to be present in the brain. In agreement with earlier findings, synaptophysin, syntaxin 1A/1B, SNAP-25, VAMP-1 and VAMP-2 were readily detectable in samples of brain homogenate. Of these, only VAMP-2 was detectable in neutrophils, despite the fact that greater amounts of protein were loaded (50 μg vs. only 10 μg of brain). Negative results in neutrophils were also obtained using a second anti-syntaxin 1A/1B antibody (SPM-1), which effectively reacted with the brain protein. Failure to detect synaptophysin, syntaxin
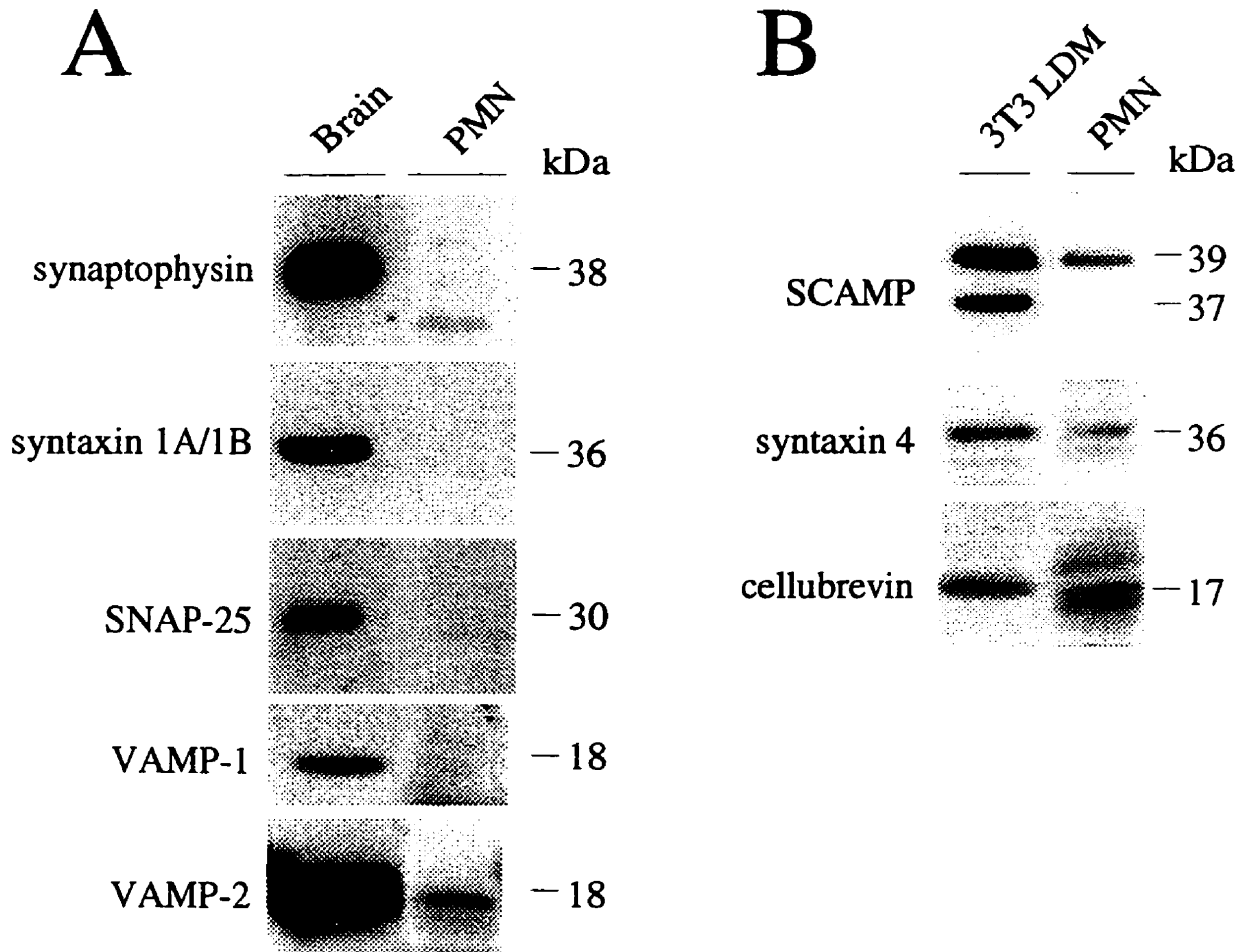
A. Expression of neuronal SNAREs in human neutrophils. Crude rat brain homogenate (10 μg protein) and whole neutrophil lysates (PMN, 10^6 cell equivalents) were resolved by SDS-PAGE and immunoblotted for the indicated proteins. **B.** Detection of syntaxin 4, cellubrevin, and SCAMPs in human neutrophils. Light density microsomes from 3T3-L1 adipocytes (3T3 LDM, 25 μg protein) and whole neutrophil lysates (PMN, 10^6 cell equivalents) were separated by SDS-PAGE and immunoblotted for the indicated protein. The molecular weight of each band in A and B is indicated (kDa). Results shown are representative of three separate experiments.

1A/1B, SNAP-25 and VAMP-1 is not likely due to species specificity of the antibodies, since: a) the synaptophysin and syntaxin 1A/1B (SPM-1) antibodies used have been reported to recognize the human homologs (Alford, et al., 1994) and b) for both VAMP-1 and SNAP-25 the primary sequences of the antigenic regions of the human and rodent homologs are identical (Trimble, 1993; Zhao, et al., 1994) .

The presence in neutrophils of other docking/fusion-related proteins, expressed in tissues other than the brain, was examined in Fig. 7.1B. In this instance, light-density microsomes from 3T3-L1 adipocytes were used as a reference. Two different SCAMPs of molecular weight 39,000 and 37,000 were recognized in the microsomes by the monoclonal antibody used (SG7C12). Only the 39 kDa form was readily evident in neutrophil lysates. Syntaxin 4, a SNARE of ≈36 kDa was also detectable in neutrophils. Finally, the anti-cellubrevin antibody, which recognizes a polypeptide of ~17 kDa in 3T3-L1 microsomes, reacted with a doublet of slightly lower molecular weight in neutrophils (Fig. 7.1B).

The vesicular SNAREs, VAMP-2 and cellubrevin, are susceptible to proteolytic cleavage by specific clostridial toxins (Niemann, et al., 1994). This property was utilized to confirm the identity of the immunoreactive bands detected in neutrophils. As shown in Fig. 7.2, exposure of the brain homogenate to tetanus toxin produced marked degradation of VAMP-2. Similarly, the immunoreactive 18 kDa band of neutrophils was extensively cleaved by treatment of cell lysates with the toxin, confirming its identity as VAMP-2. As reported previously (Volchuk, et al., 1995), the cellubrevin present in 3T3-L1 cells was susceptible to degradation by tetanus toxin. Importantly, the two immunoreactive bands of similar molecular weight identified in neutrophils were unaffected by the toxin, suggesting non-specific interaction of these polypeptides with the antibody. Indeed, these bands comigrate with two abundant granular proteins of neutrophils that are prominent in Coomassie-stained gels, and with non-specific bands often seen in immunoblots using other antibodies (e.g. the tetanus toxin-insensitive bands detectable in the VAMP-2 immunoblot of Fig. 7.2). I therefore tentatively conclude that cellubrevin is not the protein detected by the antibody in neutrophils in

TeTx   -   +   -   +   -   +   -   +

VAMP-2   cellubrevin
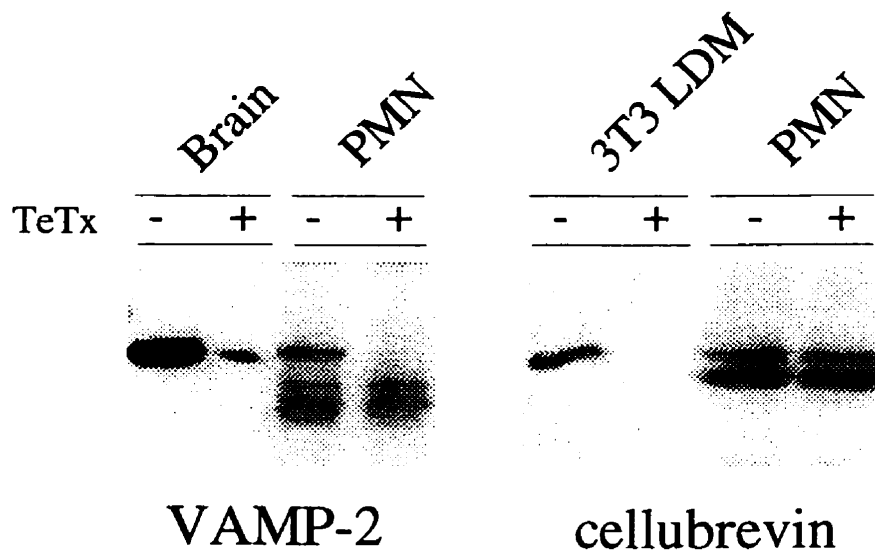
Fig. 7.2.   Susceptibility to tetanus toxin cleavage. Human neutrophils (PMN, 4 x 10^6), crude brain homogenate or microsomes from 3T3-L1 cells were denatured by boiling in K-glutamate buffer and these lysates were treated in vitro with (+) or without (-) reduced two-chain tetanus toxin (see Experimental Procedures). Reactions were terminated by addition of 5x concentrated Laemmli sample buffer, the samples were fractionated by SDS-PAGE and immunoblotted for VAMP-2 (left) or cellubrevin (right).

Figs. 7.1B and 7.2. While its presence cannot be conclusively ruled out, it is apparent that cellubrevin is not an abundant component of neutrophils.

The subcellular localization of the proteins positively identified in neutrophils was investigated next. For this purpose, cells were disrupted gently by nitrogen cavitation and the lysate was subjected to fractionation by sedimentation through a Percoll density gradient. Individual fractions were collected and analyzed for the activity of specific markers of individual subcellular compartments. Primary (azurophilic) granules, which migrate near the bottom of the gradient were identified by their high myeloperoxidase content. Secondary (specific) granules were characterized by the presence of lactoferrin, while tertiary granules were rich in gelatinase. The lightest organellar fraction contains a mixture of secretory vesicles and plasma membranes (sv/pm fraction). The latter were identified by the presence of HLA class I, while the secretory vesicles contained trapped albumin and were endowed with latent alkaline phosphatase. A cytosolic fraction that remains on top of the gradient was also collected and analyzed. A typical fractionation profile is illustrated in Fig. 7.3A.

Samples bracketing the peak of the individual organelles were pooled and comparable amounts of protein were subjected to electrophoresis and immunoblotting. As illustrated in Fig. 7.3B, minute amounts of the 37 kDa SCAMP were detected in some purified fractions, particularly in the sv/pm fraction. The 39 kDa SCAMP was clearly detectable in all but the cytosolic fraction, with increasing abundance in the order primary granules<secondary granules<tertiary granules<sv/pm fraction. By contrast, syntaxin 4 was only present in the combined sv/pm fraction. Finally, VAMP-2 was negligible in primary granules and only marginally present in secondary granules, but was readily detectable in tertiary granules and especially in the sv/pm fraction. None of the proteins was found in the cytosol. Purified fractions were also probed for proteins that gave negative reactions in whole cell lysates (i.e. synaptophysin, syntaxin 1, SNAP-25, VAMP-1 and cellubrevin, see Fig. 7.1). In all cases these proteins were also undetectable in the purified subcellular fractions (not illustrated).

The results of three similar experiments are summarized in Fig. 7.4. Data are normalized to the fraction containing the highest density of the individual protein. It is
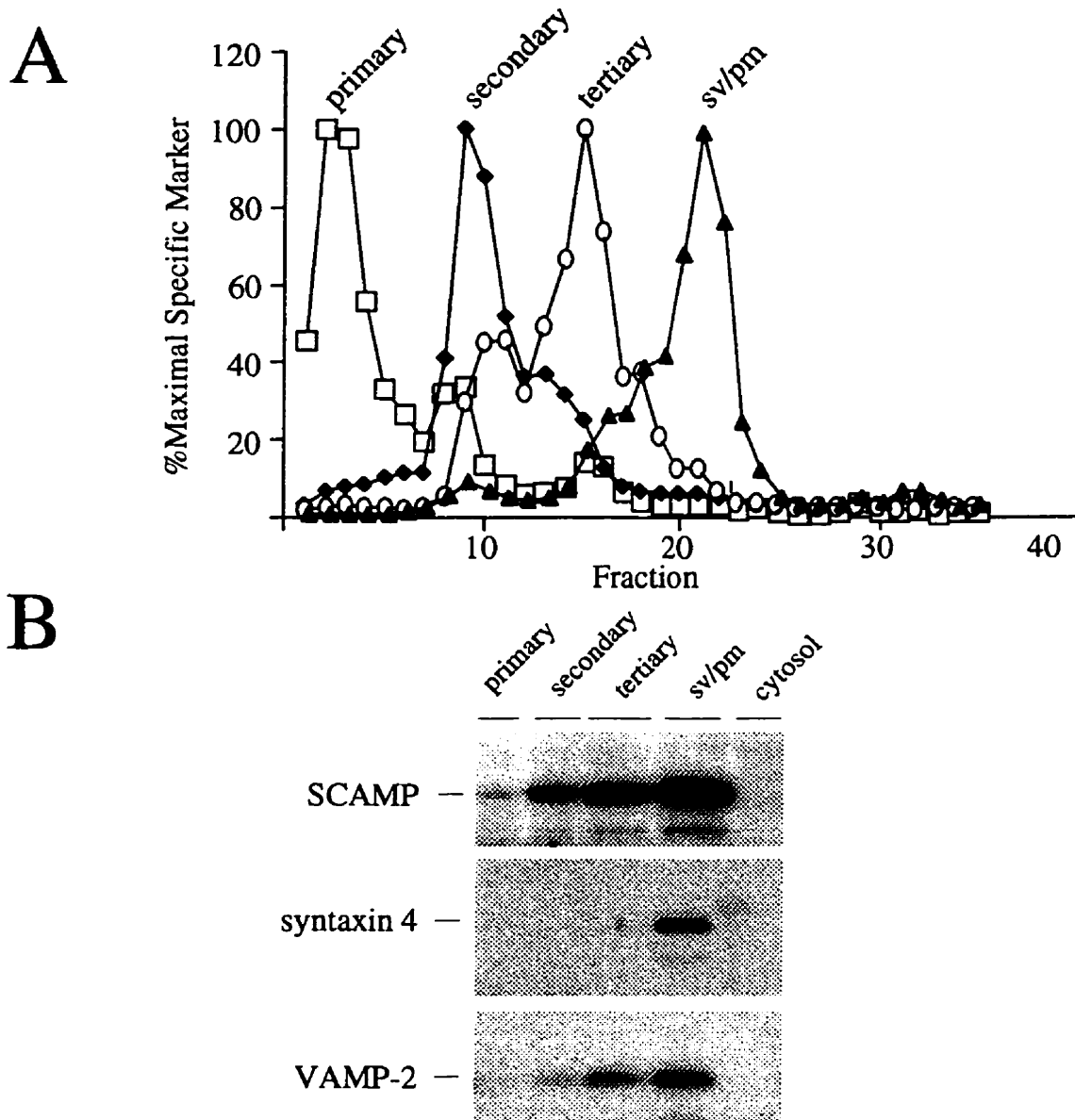
Fig. 7.3.   A. Subcellular fractionation of human neutrophils. Neutrophils were lysed by nitrogen cavitation and subjected to Percoll gradient fractionation. Fractions were collected and analyzed for the activity of specific organellar markers, which are plotted normalized to the fraction with maximal activity. The following markers were assayed: primary granules: myeloperoxidase (open squares), secondary granules: lactoferrin (closed diamonds), tertiary granules: gelatinase (open circles),   secretory vesicles and plasma membrane (sv/pm): albumin (closed triangles).   B. Subcellular distribution of SCAMP, syntaxin 4 and VAMP-2 in human neutrophils. Percoll was removed from fractions such as those in A that contained maximal amounts of the specific markers for individual compartments and the biological material was boiled with 2x concentrated sample buffer. The fractions (25 µg protein) were then subjected to SDS-PAGE and immunoblotted with the indicated antibodies. Cytosolic proteins (25 µg), collected from the top of the gradient, were also subjected to similar treatment. Subcellular fractionation was performed by Henrik Sengelov.
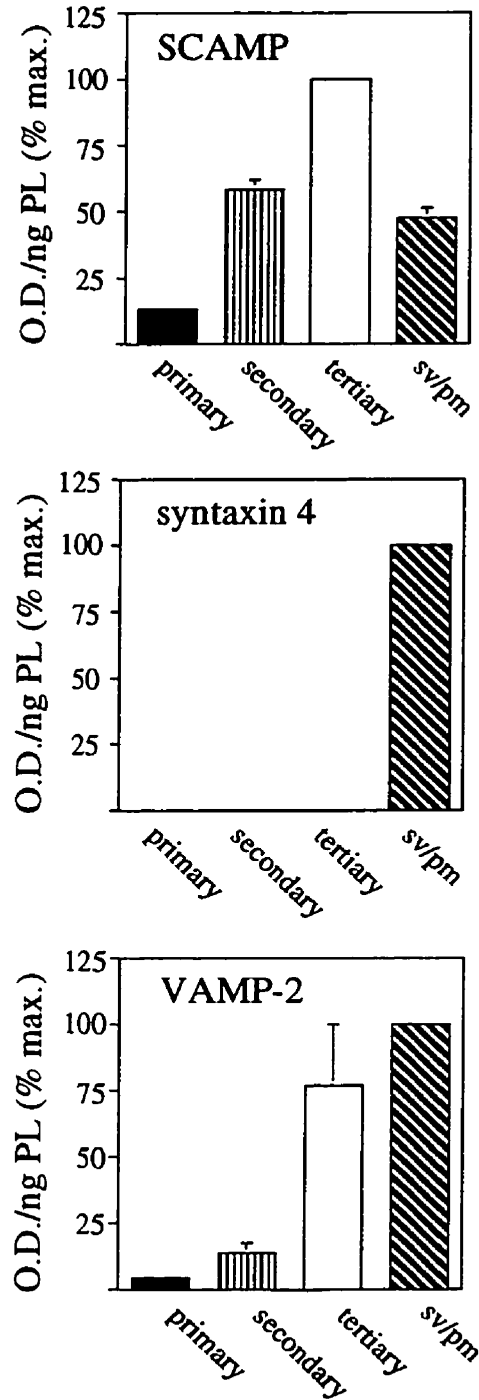
Fig. 7.4. Relative subcellular distribution of SCAMP, syntaxin 4 and VAMP-2 in human neutrophils. Quantitation of immunoblots from three experiments like that in Fig. 7.3B was performed by densitometry. For each protein, the optical density (O.D.) was determined for each fraction and is normalized per unit phospholipid (PL). Values are expressed relative to the fraction containing the most amount of each protein (% Max). Top: SCAMP. Middle: syntaxin 4. Bottom: VAMP-2. sv/pm: combined secretory granule/plasma membrane fraction. Results shown are means ± SE of three separate experiments.

noteworthy that, as is customary, identical amounts of protein were used for immunoblotting analysis in experiments like that in Fig. 7.3. On the other hand, different organelles vary greatly in their ratio of membranous to content (luminal) proteins. This is readily apparent by inspection of electron micrographs, such as that in Fig. 7.6, that reveals large disparities in the density of the granular lumen. Because SCAMP, syntaxin 4 and VAMP-2 are all integral membrane proteins, normalization of their amount per total protein content fails to reflect their density on the membrane. To remedy this situation, the samples were normalized per unit phospholipid, rather than per protein, using phospholipid to protein ratios of the individual fractions determined earlier (Bjerrum, et al., 1989). When using this correction, SCAMP was found to be most abundant in the tertiary granules and VAMP-2 in the sv/pm (Fig. 7.4). The conclusions regarding syntaxin 4, which is only detectable in the sv/pm, remained unaffected.

As described above, the density of secretory vesicles and the plasma membrane is similar, precluding separation of these organelles by sedimentation. On the other hand, it is possible to isolate these components by treatment of the combined sample with neuraminidase, followed by free-flow electrophoresis (Sengelov, et al., 1992). A representative electrophoretic profile is shown in Fig. 7.5A. Fractions containing the highest amounts of HLA class I, indicative of the plasmalemma (pm), were convincingly segregated from fractions containing peak amounts of latent alkaline phosphatase containing secretory vesicles (sv).These isolated fractions were next used to define the precise localization of syntaxin 4 and VAMP-2, both of which were found to be enriched in the combined sv/pm fraction. Fig. 7.5B demonstrates that the distribution of these proteins is clearly divergent: syntaxin 4 is present almost exclusively in the plasma membrane, while VAMP-2 is most abundant in the secretory vesicles, with just trace amounts noted in the membrane. Though readily observable in the combined sv/pm fraction (Fig. 7.3), SCAMP was not detectable in the isolated fractions. I speculate that the epitope is lost or damaged during the additional steps involved in separating secretory vesicles from plasma membranes.

It is conceivable that the elaborate procedures required for fractionation of membranes and secretory vesicles might have altered the distribution or immunogenicity of the SNAREs.
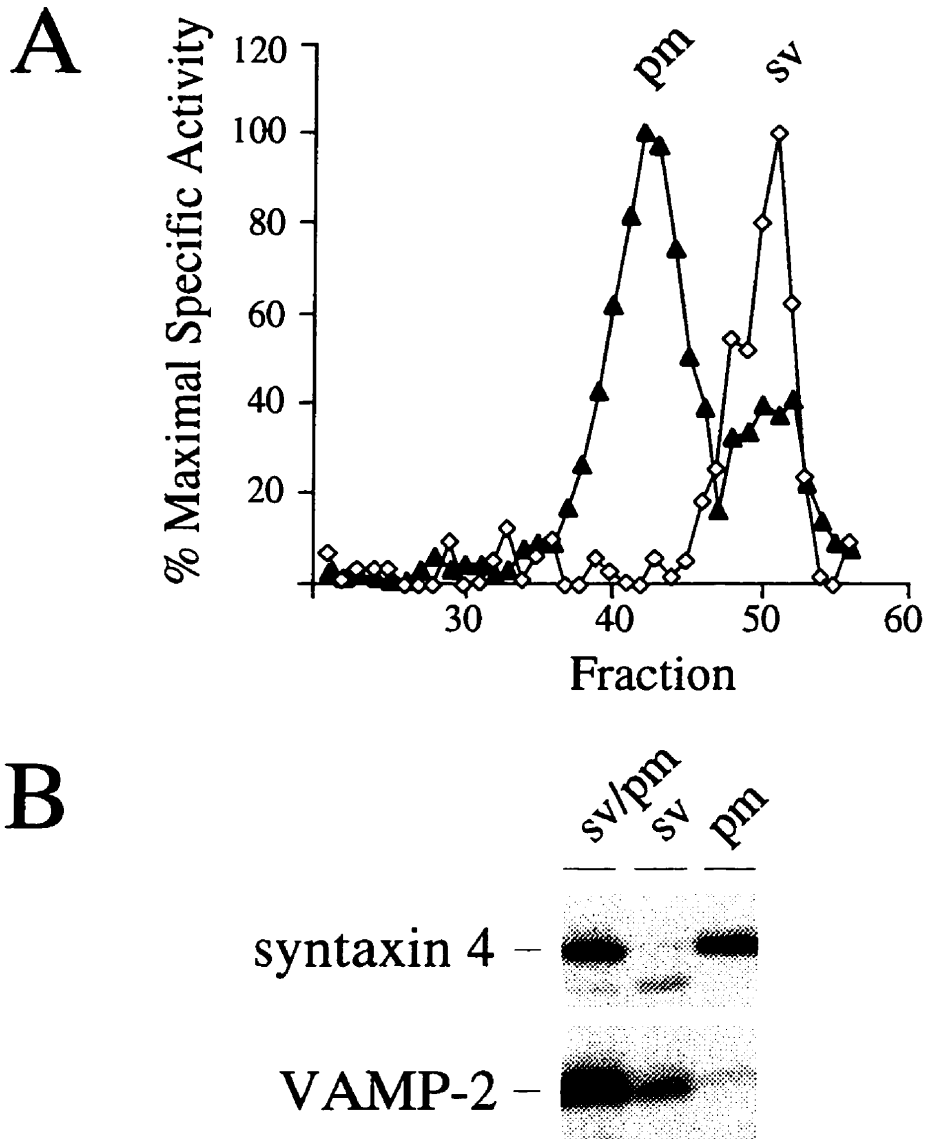
Fig. 7.5. A. Separation of specific vesicles from the plasma membrane. Secretory vesicles (sv) were separated from plasma membranes (pm) by free-flow electrophoresis of sv/pm fractions collected by Percoll fractionation (see Experimental Procedures). Fractions were collected and analyzed for the activity of markers specific for plasma membrane (HLA class I, closed triangles) or secretory vesicles (latent alkaline phosphatase, open circles). B. Localization of syntaxin 4 and VAMP-2. Fractions containing maximal activities of sv or pm enzymatic markers were boiled with 2x concentrated sample buffer, subjected to SDS-PAGE and immunoblotted with the indicated antibody. A sample of the original (unfractionated) sv/pm fraction (25 μg) is shown, as well as fractions containing isolated sv and pm (10 μg each). Free flow electrophoresis was performed by Henrik Sengelov.

Fig. 7.6.   Immunoelectron microscopy of VAMP-2 in human neutrophils. Neutrophils were fixed with 2% paraformaldehyde and 0.05% glutaraldehyde, infiltrated in polyvinyl pyrolidone/sucrose and frozen before ultracryosectioning and labelling with affinity-purified, polyclonal antibodies to VAMP-2 (a and b) or with preimmune serum (c). Secretory vesicles (v), specific granules (s), azurophilic or primary granules (a), small dense granules (g), the nucleus (Nu) and the plasma membrane (PM) are identified. The small dense granules (g) may be tertiary granules or secondary granules cut in cross-section. Arrows point to immunogold particles.   Size bar represents 200 nm.   Data provided by Anne-Marie Cieutat and Dorothy Bainton.

To circumvent this potential difficulty, I sought to confirm the distribution of syntaxin 4 and VAMP-2 by immunoelectron microscopy. Freshly isolated neutrophils were fixed, sectioned and stained with antibodies to both proteins, followed by gold-labelled secondary antibodies.

A modest amount of VAMP-2 was detected on the plasmalemma, but the majority of the label was found associated with vesicles (v) and small granules (Fig. 7.6A and B), which possibly correspond to secretory vesicles and tertiary granules. Labeling of primary and secondary granules was infrequent. Samples exposed to pre-immune antibody (Fig. 7.6C) or to the secondary antibody alone did not display any significant labeling. These data are in good agreement with the fractionation experiments, suggesting that VAMP-2 is found in secretory vesicles and tertiary granules, with only trace amounts found in the membrane. Unfortunately, our antibodies to syntaxin 4 were incompatible with the fixation and sample preparation technique used. Hence, I was unable to confirm the localization of syntaxin 4 by immunoelectron microscopy.

The distribution of VAMP-2 and syntaxin 4 was further explored by confocal microscopy of samples stained by immunofluorescence. Cells were allowed to adhere to glass coverslips, fixed, permeabilized and stained. Representative samples are shown in Fig. 7.7. At lower magnification, syntaxin 4 appeared diffusely distributed, particularly in cells that remained rounded. However, at higher magnification a punctate staining and distinct margination were noted, particularly in cells that spread on the substratum. In symmetrical, seemingly stationary cells, syntaxin 4 was distributed throughout the periphery. In contrast, Fig. 7.7A (rightmost panel) demonstrates that this protein was very prominent in the lamellipodium of asymmetric, migrating cells.

VAMP-2 staining was also punctate, but appeared more centrally located, consistent with labelling of endomembrane vesicles. This notion was confirmed by experiments where VAMP-2 and individual granule markers were simultaneously analyzed using dual staining (Fig. 7.8). In these experiments, the optimal focal plane for the individual granule markers was chosen for illustration. Primary granules were identified using CD63, a granular membrane marker earlier found exclusively in these organelles (Kuijpers, et al., 1991). There
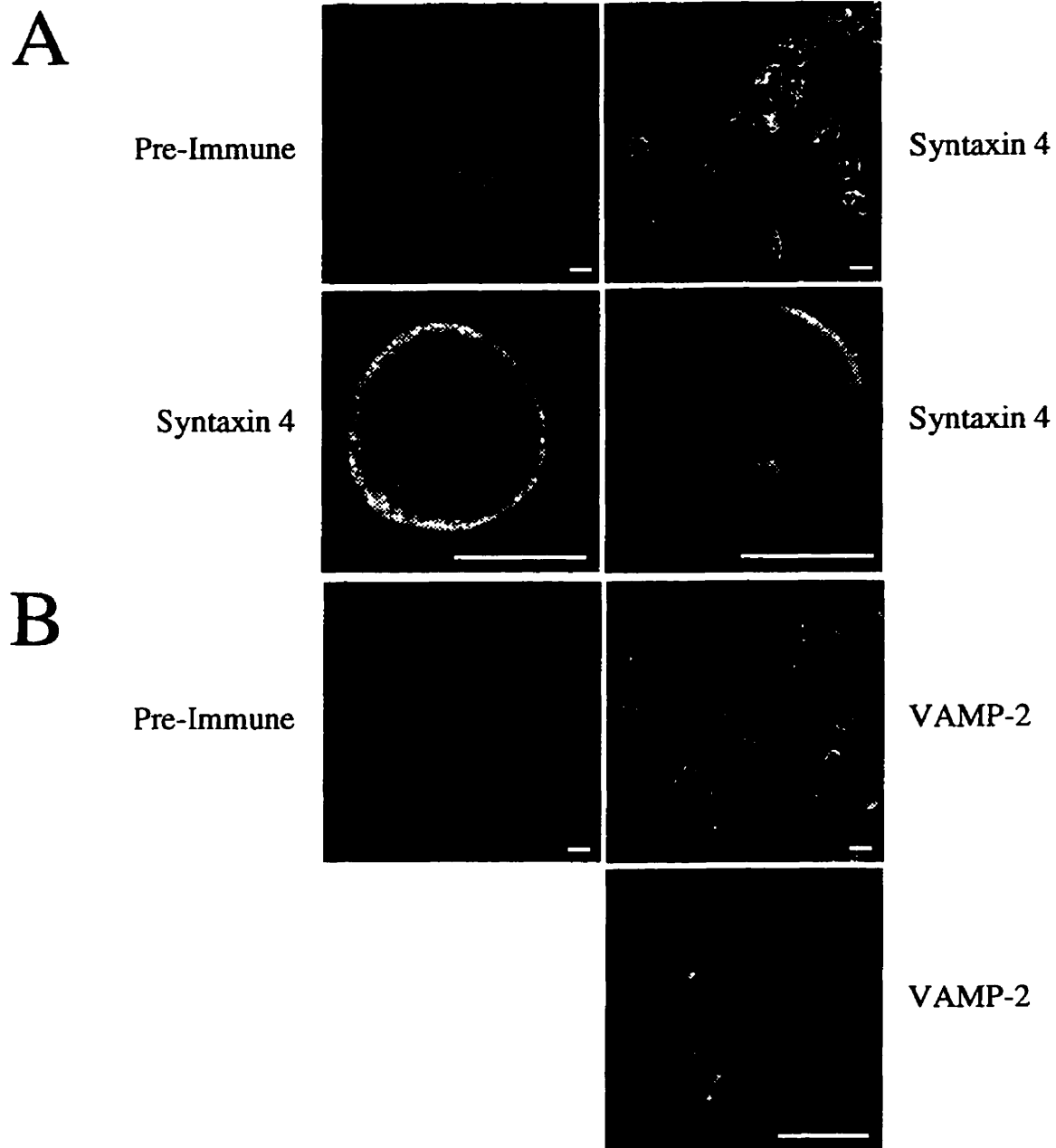
Fig. 7.7.   Immunofluorescence microscopy of syntaxin 4 and VAMP-2 in neutrophils. Neutrophils adherent to glass coverslips were fixed and permeabilized with methanol, then blocked with 5% donkey serum (see Materials and Methods). Fixed cells were incubated with affinity-purified, polyclonal antibodies to syntaxin 4 (A) or VAMP-2 (B). Rabbit pre-immune sera subjected to the affinity purification protocol were used as controls (leftmost panels). The samples were then stained with fluoresceinated donkey anti-rabbit antibodies and analyzed using a Zeiss laser confocal microscope. Size bars indicate 10 nm. Data shown are representative of 3 separate experiments.
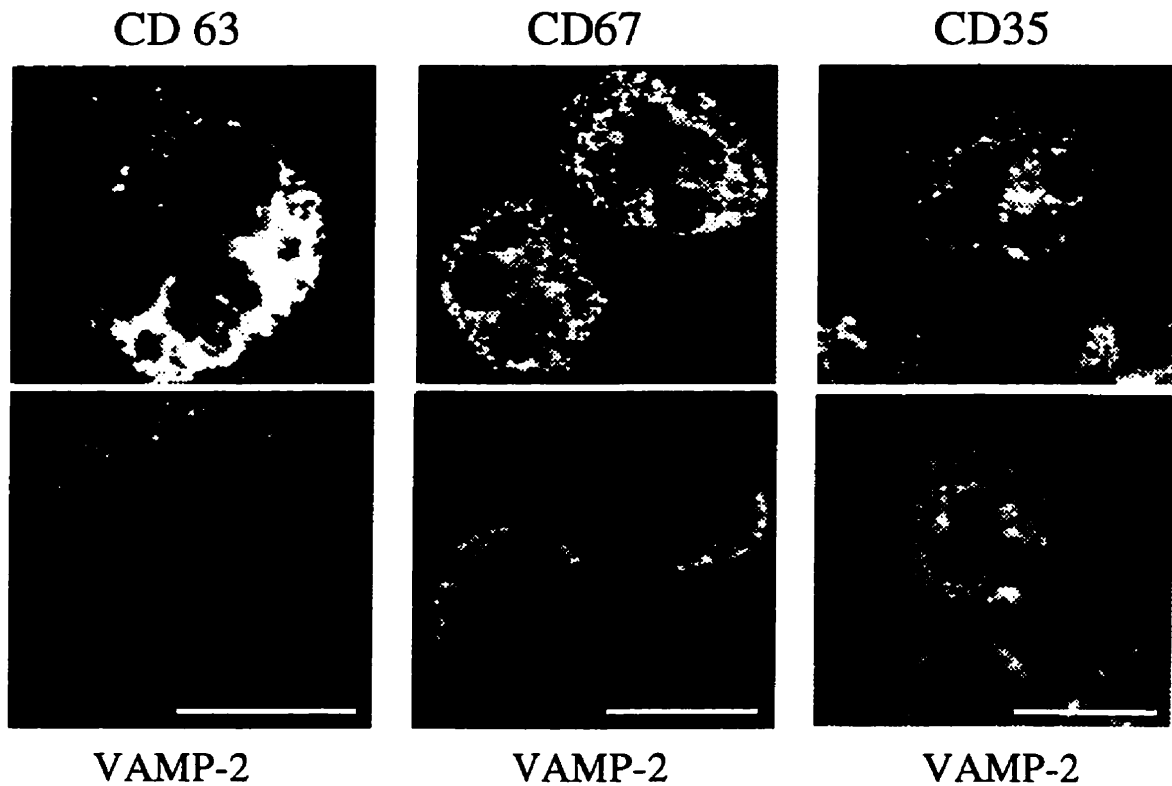
Fig. 7.8. Localization of VAMP-2 and granular markers in neutrophils. Neutrophils adherent to glass coverslips were fixed and permeabilized with methanol, then blocked with 5% donkey serum (see Experimental Procedures). Fixed cells were dually stained with affinity-purified, polyclonal antibodies to VAMP-2 (lower half of each panel), as well as monoclonal antibodies to marker proteins characteristic of specific subcellular compartments (top half of each panel). CD63 (left panel) was used as a marker for primary granules, CD67 (middle panel) for secondary granules and CD35 (left panel) for secretory vesicles. Detection of VAMP-2 and the specific markers was performed by incubation with fluoresceinated donkey anti-rabbit antibodies and donkey anti-mouse antibodies coupled to Texas Red, respectively. Samples were analyzed using a Zeiss laser confocal microscope. Size bars indicate 10 nm. Data shown are representative of 3 separate experiments. Note that the focal plane was chosen for optimal illustration of the specific granular markers, even when VAMP-2 staining was clearer at a different plane.

Ionomycin: -   2 min   5 min

CD 35
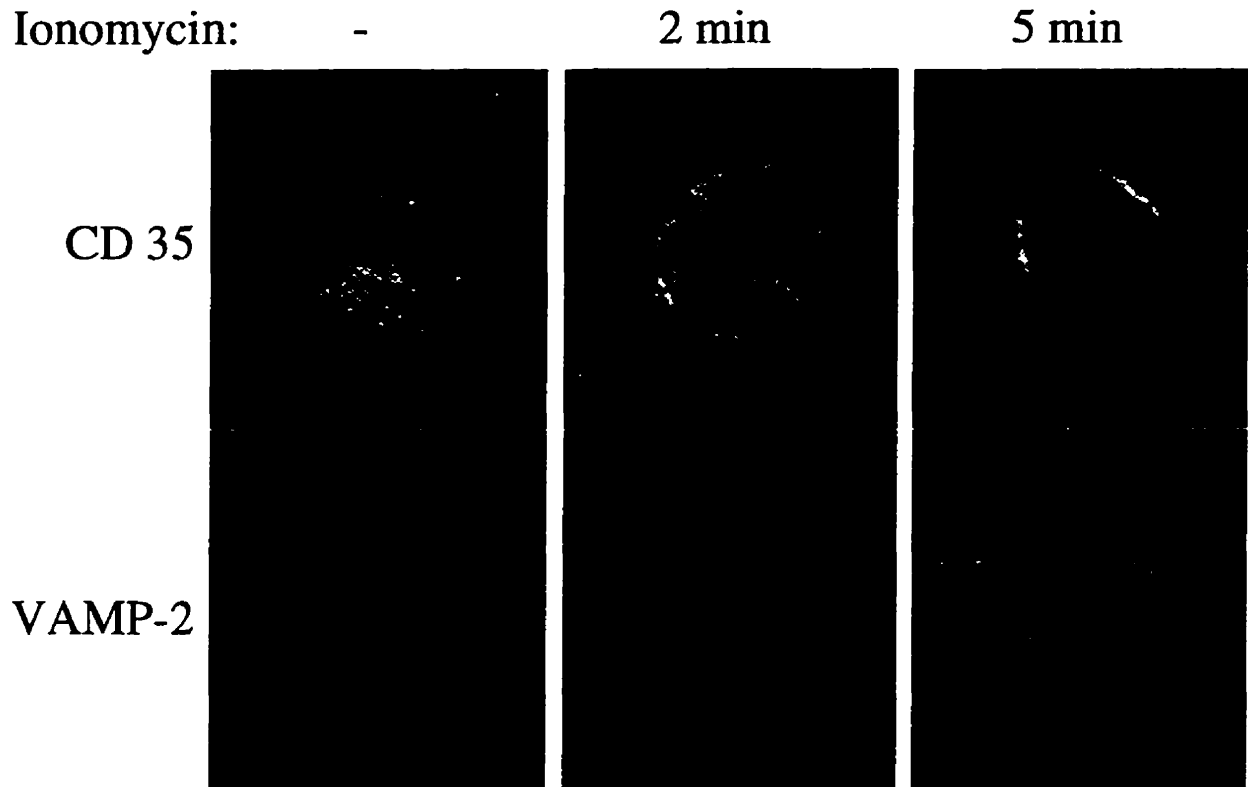
VAMP-2

Fig. 7.9.   Translocation of VAMP-2 upon stimulation. Neutrophils adherent to fibrinogen-coated coverslips were treated without (-) or with 0.5 µM ionomycin for the time indicated. The cells were then fixed with paraformaldehyde, permeabilized with a buffer containing 0.1% Triton X-100 and blocked with 5% donkey serum (see Materials and Methods). Fixed cells were dually stained with monoclonal antibodies to a marker protein for secretory vesicles, CD35 (upper half of each panel), and with affinity-purified antibodies to VAMP-2 (lower half of each panel). Detection of VAMP-2 and CD35 was performed by incubation with fluoresceinated donkey anti-rabbit antibodies and donkey anti-mouse antibodies coupled to Texas Red, respectively. Samples were analyzed by confocal microscopy. Data are representative of 3 separate experiments.

was little overlap between the CD63 and VAMP-2 labelled structures. Similarly, the distribution of VAMP-2 differed from that of CD67, a secondary granule marker (Jost, et al., 1991). However, a greater degree of coincidence was found in samples dual-labelled with anti-VAMP-2 and anti-CD35 antibodies. CD35 is thought to be a good indicator of secretory vesicles (Sengelov, et al., 1994). Notice that CD35 also stained the surface membrane in spread, adherent cells. This likely results from the induction of exocytosis of the labile secretory vesicles during spreading. It is also noteworthy that the number of organelles labelled with VAMP-2 exceeds that of CD35 labelled structures. The excess VAMP-2 positive vesicles may represent tertiary granules. I was unable to test this assumption, due to the lack of an adequate immunological label for tertiary granule membranes.

The distribution of VAMP-2 was also assessed in cells that were stimulated with agents known to induce secretion. For this, cells adherent to fibrinogen-coated coverslips were treated with the Ca^{2+} ionophore, ionomycin at concentrations expected to induce exocytosis. Following fixation, the localization of CD35 and VAMP-2 was compared in control and stimulated cells. As shown in Fig. 7.9, both CD35 and VAMP-2 were diffusely distributed throughout the cytosol in cells adherent to fibrinogen. Treatment with the ionophore induced rapid translocation of the markers towards the plasma membrane, consistent with exocytosis of secretory granules and vesicles. These results indicate that vesicles containing VAMP-2 undergo translocation during activation and suggest a functional role for this protein in exocytosis in neutrophils.

Discussion

To establish if there are similarities between the mechanisms underlying docking/fusion of synaptic vesicles and granular secretion in neutrophils, I explored whether common proteins exist in both systems. For simplicity, I will discuss vesicular and target membrane SNAREs separately.

Vesicular and granular proteins in neutrophils. Synaptic vesicles contain a series of well defined proteins, some of which have been proposed to participate in vesicular exocytosis. One of the earliest discovered was synaptophysin, which has been suggested to participate in synaptic transmission, since microinjection of neutralizing antibodies was found to alter the pattern of neurosecretion (Alder, et al., 1992). While the particular biochemical function of synaptophysin is not known, it has been hypothesized to contribute to the formation of the fusion pore between the synaptic vesicle and the plasma membrane, based on its similarity to a gap-junctional pore protein and on its ability to form ion channels in lipid bilayers (Thomas, et al., 1988; Trimble, et al., 1991). Monoclonal antibodies to synaptophysin failed to detect an equivalent protein in human neutrophils. This and other antibodies used in the present work have been shown to cross-react with the human homologs and/or were raised to regions of the proteins that are fully conserved among different species. It therefore seems that neutrophils lack synaptophysin but could conceivably express a non-neuronal isoform, perhaps the serologically related granulophysin or leukophysin (Abdelhaleem, et al., 1991). The latter is a protein found in lymphocytes, U937 monocytes and neutrophils which cross-reacts with anti-granulophysin antibodies (Abdelhaleem, et al., 1991).

VAMP-1, one of the synaptobrevins, is a SNARE believed to be involved in synaptic vesicle docking/fusion (Rothman, 1994). This protein was readily detected by immunoblotting in rat brain homogenates, but not in cell extracts of human neutrophils. These findings suggest that VAMP-1 is absent in the leukocytes. The results are consistent with the notion that expression of VAMP-1 is restricted to neuronal and neuroendocrine tissues (Trimble, 1993). Indeed, while present in rat brain and in PC12 cells (a neuroendocrine line of rodent origin)

(Chilcote, et al., 1995), VAMP-1 was not detected in mouse fibroblasts, rat adipocytes or rat skeletal muscle (Volchuk, et al., 1994; Volchuk, et al., 1995).

Unlike VAMP-1, another synaptobrevin, cellubrevin, is expressed in a variety of tissues (McMahon, et al., 1993) yet is conspicuously absent from neurons (Chilcote, et al., 1995). This protein was recently found to be present in recycling endosomes of fibroblastic cells and has been suggested to be a marker of the recycling endocytic pathway (Galli, et al., 1994; McMahon, et al., 1993; Volchuk, et al., 1995). Surprisingly, cellubrevin was not detected in neutrophils by an antibody that reacted positively with fibroblasts, adipocytes, muscle and other cells. The absence of cellubrevin from human neutrophil granules and secretory vesicles suggests that these entities do not participate in constitutive recycling. This may reflect the terminal nature of the secretory events of neutrophils, which have an extremely short biological half-life that is further shortened upon activation (Borregaard, et al., 1992; Sengelov, et al., 1995). In this situation, endocytic recycling of secretory granules and vesicles would appear unnecessary.

A third synaptobrevin, VAMP-2, is an integral membrane protein originally detected in synaptic vesicles, where it exposes most of its mass to the cytosolic space (Elferink, et al., 1989; Sudhof, et al., 1989). In vitro, the protein forms complexes with plasma membrane proteins and can be isolated from bovine brain as part of the 20S docking protein complex (Sollner, et al., 1993). VAMP-2 has recently been found to be expressed in a variety of non-neuronal tissues with active secretory activity, such as endocrine ß cells (Braun, et al., 1994; Gaisano, et al., 1994) and chromaffin cells (Baumert, et al., 1989) but also in non-secretory cells with regulated exocytic membrane traffic such as adipocytes (Cain, et al., 1992; Volchuk, et al., 1995), and rat skeletal muscle (Ralston, et al., 1994; Volchuk, et al., 1994). Using an antibody raised to the rat VAMP-2, I observed specific reactivity with a single band of 18 kDa in human neutrophils, which co-migrated with the rodent brain VAMP-2 (Figs. 7.1 and 7.2). That the reacting polypeptide in human neutrophils is a synaptobrevin was further confirmed by its susceptibility to proteolysis by tetanus toxin (Fig. 7.2).

In neutrophils VAMP-2 was found predominantly in secretory vesicles, with significant labeling also in tertiary granules and considerably less in secondary and primary granules. This distribution was confirmed biochemically (Fig. 7.4) as well as morphologically (Figs. 7.6 and 7.8). It is noteworthy that the relative density of VAMP-2 in the different compartments follows the same rank order as the reported kinetics of degranulation and the sensitivity of the granules to calcium (i.e. secretory vesicles>tertiary granules>secondary granules>>primary granules). This correlation seems to point to an active role of VAMP-2 in the secretory process. In keeping with this notion, stimulation of the cells with either ionomycin (Fig. 7.9) or phorbol esters promoted migration of VAMP-2 towards the plasma membrane.

A novel type of protein, termed SCAMPS, was recently found on the surface of a variety of granules and vesicles in several cell types but to be excluded from lysosomes (Brand, et al., 1991). These proteins, with molecular masses of 39 kDa and 37 kDa, have been proposed as general markers of post-Golgi vesicular structures (Brand and Castle, 1993). In neutrophils only one isoform (39 kDa) was readily visible (Fig. 7.1), with minute amounts of the 37 kDa detectable in some fractions. The 39 kDa species was most abundant in the secondary and tertiary granules and in the sv/pm fraction. The virtual absence of SCAMPs from primary granules of human neutrophils is in agreement with the lysosomal nature of these granules. As in other cells, the function of SCAMPs in intracellular traffic in neutrophils remains unknown. Also, the significance of the differential distribution of the two SCAMPs needs to be determined.

t-SNAREs in neutrophils. The interaction between synaptic vesicles and their target is believed to be mediated by SNAP-25 and syntaxin 1 on the pre-synaptic membrane. Using antibodies specific to the rat form of these proteins, the rodent homologues were easily detectable in brain homogenates but neither protein could be detected in human neutrophils. The results are in line with the notion that the expression of SNAP-25 and syntaxin 1 is restricted to neuronal tissues. Accordingly, syntaxin 1A, syntaxin 1B and SNAP-25 transcripts are not detectable in the non-neuronal rat tissues tested thus far (Bark, et al., 1995; Bennett, et al., 1993).

In vitro studies have demonstrated the formation of a tripartite complex between the vesicular protein VAMP-2 and the plasmalemmal proteins syntaxin 1 and SNAP-25. Given the absence of the latter two proteins from human neutrophils, it is plausible that these cells utilize non-neuronal isoforms of syntaxin and SNAP-25 for docking of secretory vesicles and granules. Indeed, the syntaxins are a growing family of related proteins (Bennett, et al., 1993). While only syntaxin 1 has been identified as a member of the complex that VAMP-2 forms with NSF and SNAP, other syntaxins are likely to fulfill the same role in non-neuronal tissues. To date, five different isoforms of syntaxin have been described by Bennett and colleagues (Bennett, et al., 1993). In their study, transcripts of syntaxins 2 and 5 were found to be ubiquitously expressed. In contrast, mRNAs encoding syntaxins 3 and 4 have a more restricted tissue expression: syntaxin 3 is expressed abundantly in spleen, lung and kidney, while syntaxin 4 was detected in heart, spleen and skeletal muscle. Syntaxin 5 is unlikely to mediate v-SNARE docking and fusion during degranulation of neutrophils since, when transfected into COS cells, this isoform was found to be localized to the *cis* cisternae of the Golgi (Bennett, et al., 1993), where it has been implicated in vesicular traffic between the endoplasmic reticulum and the Golgi complex (Dascher, et al., 1994). In addition, only syntaxins-1 and -4, but not syntaxins 2 or 3, can associate with VAMP-2 *in vitro* (Calakos, et al., 1994). Because two separate antibodies showed syntaxin 1 to be absent from neutrophils, I concentrated on syntaxin 4 as a possible target for the VAMP-2 detected in secretory vesicles, tertiary and secondary granules.

Neutrophil lysates were probed with an antibody raised to the cytoplasmic domain of syntaxin 4 (Figs. 7.1 and 7.3). Immunoblotting experiments revealed a single polypeptide of 36 kDa, which corresponds to the predicted size of syntaxin 4. This protein was predominantly found in the fraction containing secretory vesicles and plasma membranes (sv/pm) and was virtually undetectable in primary, secondary or tertiary granules. Upon further purification of the sv/pm fraction, syntaxin 4 was found to reside almost exclusively in the plasma membrane. In agreement with these findings, immunofluorescence studies

localized the protein predominantly at the cell surface. In view of the absence of syntaxin 1, together with the reported inability of syntaxins 2 and 3 to bind VAMP-2, I tentatively favor syntaxin 4 as the t-SNARE that interacts with granular and vesicular VAMP-2 in human neutrophils.

While syntaxin 4 appeared randomly distributed in freshly plated cells, a distinct polarization became apparent in asymmetric cells, which have been identified as undergoing migration (Roos, et al., 1987). In such cells, syntaxin 4 appeared to accumulate at or near the lamellipodium, which constitutes the leading edge of chemotaxing cells (*e.g.* Fig. 7.7). It is noteworthy that this is the region of the cell that becomes involved in formation of the phagosome, where secretion of organellar contents occurs preferentially. It is tempting to speculate that redistribution of syntaxin 4 contributes to the polarization of secretion into the phagocytic vacuole. To our knowledge, this is the first instance where acute, functional redistribution of syntaxin 4 has been reported. The molecular mechanisms responsible for syntaxin 4 accumulation at the lamellipodium remain to be defined, but interaction with the underlying cytoskeleton is an attractive possibility currently under investigation.

Chapter 8

Discussion

At the outset of these studies, the importance of phosphorylation-dependent signalling mechanisms in neutrophils was well established. The details of how phosphotransfer mechanisms control the responses of these cells to chemoattractant, however, required further definition. The work presented in this thesis has focussed on the regulation of the kinases and phosphatases that influence neutrophil behavior via changes in their activity and/or subcellular localization. A discussion of the findings presented in this thesis is presented below.

Upstream Regulation and Potential Roles of the Renaturable Serine/Threonine Kinases (PAKs).

The procedure used to identify renaturable protein kinase activity in protein samples following electrophoresis on SDS PAGE gels was first described by Harrison and colleagues (Geahlen, et al., 1986). Using this technique, the authors identified 3 major protein kinases present in a mixture of proteins from red blood cell membranes that were capable of renaturation and phosphorylation of casein. Subsequent modification of this technique by Kameshita and Fujisawa allowed them to study the activity of calmodulin-dependent protein kinase II from cellular samples (Kameshita and Fujisawa, 1989). Using microtubule associated protein 2 in their gels, it was found that numerous unidentified kinases were also capable of phosphorylating this substrate, in addition to calmodulin-dependent kinase II.

Utilizing the method of Kameshita and Fujisawa, renaturable kinases have also been identified in rodent and human neutrophils (Ding and Badwey, 1993; Grinstein, et al., 1993). Treatment with fMLP was found to increase the activities of at least 4 renaturable kinases, namely PK41, PK49, PK65 and PK72. The rapid time course of activation of PK65 and PK72 (detectable after 5 sec stimulation) made them good candidates for regulating early responses to chemoattractant. In addition, studies also demonstrated that PK65 and PK72 were capable of phosphorylating a number of important substrates *in vitro*, including

MARCKS and a peptide derived from p47^{phox} (Ding, et al., 1993). Though the identity of PK65 and PK72 was not determined, use of the renaturable kinase assay allowed for studies of their activation and upstream regulation.

Since members of the PKC family had been found to initiate a variety of signalling cascades, the role of these enzymes was explored (Grinstein, et al., 1994). While fMLP was capable of rapidly activating PK65 and PK72, phorbol esters such as TPA had no effect on their activity. TPA is thought to activate cPKC and nPKC, but has also been shown to cause activation of PKC ζ in neutrophils, possibly by an unknown feedback mechanism (Dang, et al., 1994). A role for Ca^{2+}-dependent kinases, such as members of the cPKC family, was also explored. Abolition of fMLP-induced Ca^{2+} transients by removing this cation from the extracellular milieu and loading cells with Ca^{2+} buffering agents had no effect on activation of the renaturable kinases. Furthermore, inhibitors of PKC activity had no effect on activation of the renaturable kinases. These findings suggested that members of the PKC family were not involved in the upstream regulation of PK65 and PK72 (Grinstein, et al., 1994).

Treatment of cells with inhibitors of PI3Ks (wortmannin or LY294002) blocked activation of PK65 and PK72 by fMLP (Ding, et al., 1995). A correlation was noted between the degree of PI3K inhibition, inhibition of PK65 and PK72 and attenuation of NADPH oxidase activity, further suggesting the importance of the renaturable kinases. In addition to PI3K inhibitors, pretreatment of cells with 1-butanol (a PLD inhibitor) or inhibitors of serine/threonine phosphatases PP1 and PP2A (okadaic acid, calyculin) was found to attenuate activation of PK65 and PK72 (Ding and Badwey, 1993; Ding and Badwey, 1994).

The study presented in Chapter 3 extended our understanding of PK65 and PK72 regulation by demonstrating a role for tyrosine kinases. A temporal association between tyrosine phosphorylation of proteins that comigrated with activated PK65 and PK72 initially lead us to suspect that these renaturable kinases might themselves be regulated by phosphorylation on their tyrosine residues. This hypothesis was further supported by the finding that tyrosine kinase inhibitors such as erbstatin and genistein were capable of blocking both cellular tyrosine phosphorylation and activation of PK65 and PK72. Two lines of

evidence, however, demonstrated that direct tyrosine phosphorylation was not responsible for the latter event: 1) PK65 and PK72 activity persisted after complete tyrosine dephosphorylation of cellular lysates and, 2) immunoprecipitation of tyrosine phosphorylated proteins revealed that PK65 and PK72 are not themselves phosphorylated on tyrosine following fMLP treatment. It was concluded that activation of tyrosine kinases is a necessary upstream regulatory event leading to the activation of PK65 and PK72. It was later demonstrated that phosphorylation of PK65 and PK72 on serine/threonine residues likely mediates their activation since dephosphorylation with alkaline phosphatase was capable of abolishing their activity in lysates from fMLP stimulated cells (Ding and Badwey, 1993; Grinstein, et al., 1993).

Subsequent to the studies in Chapter 3, it was demonstrated that neutrophils express members of the Pak family of serine/threonine kinases (Knaus, et al., 1995). Both Pak1 and -2 were identifed by immunoblotting neutrophil lysates with isoform specific antibodies to these proteins. Binding of both Paks to GTP-bound Rac or Cdc42 was found to initiate autophosphorylation and induction of their activity *in vitro*. Pak1 and -2 were activated rapidly and transiently in response to fMLP (but not TPA), with a time course identical to that determined for PK65 and PK72. In addition, recombinant Pak1 was able to catalyze phosphorylation of a peptide derived from p47^{phox} *in vitro* (Knaus, et al., 1995). The authors of this study proposed that members of the Pak family were the renaturable kinases which are activated by fMLP stimulation. Experiments performed by Ding et al. (1996) confirmed this hypothesis. Immunoprecipitation of Paks from fMLP stimulated neutrophils was capable of removing the activated renaturable kinases from cellular lysates. Addition of GTP-bound Rac and ATP to immunoprecipitates of Pak1 from unstimulated cells was sufficient to activate both renaturable kinase activities. Thus, it is concluded that the renaturable kinases PK65 and PK72 identified in neutrophils are indeed members of the Pak family of serine/threonine kinases (Ding, et al., 1996).

The identification of PK65 and PK72 as members of the Pak family allows us to apply previously acquired knowledge regarding upstream regulation of the renaturable kinases to the regulation of the Paks in activated neutrophils. In doing so, an apparent conflict arises with the

simple model of Pak activation by binding to GTP-bound Rac/Cdc42 and autophosphorylation/activation. This conflict comes from previous findings which demonstrated the ability of TPA to initiate activation of the NADPH oxidase concomitant with translocation of Rac to the plasmalemma (Quinn, et al., 1992). The inability of TPA to activate Paks in neutrophils suggests that additional factors also serve to regulate these kinases in response to fMLP.

What are the additional factors that regulate Pak activation in addition to binding GTP-bound Rac/Cdc42? Previous studies of the renaturable kinases PK65 and PK72 have demonstrated the essential role of PI3Ks for Pak activation in response to chemoattractant stimulation. Inhibiton of PI3K activity was found to abolish Pak activation in response to chemoattractant (Ding, et al., 1995). With respect to the discussion above, it is important that PI3Ks are activated by fMLP but not TPA (Traynor-Kaplan, et al., 1989). Hence, PI3Ks are able to modulate the activity of Paks by an unknown mechanism, in addition to their regulation by binding to Rac/Cdc42. Because PI3Ks have been shown to initiate many downstream signals, including the activation of other kinases (eg: Akt (Franke, et al., 1997)) and non-conventional PKC isoforms (Toker, et al., 1994)), the exact nature of this modulation is unknown. The recent availability of purified PIP3 (eg: see Toker, et al., 1995) will allow examination of the effect of this lipid, and its downstream targets, on the activity of Paks in neutrophils.

Interaction of Paks with other proteins may also regulate their activity *in vivo*. Interaction of Pak1 with the protooncogene Nck has been described in Swiss 3T3 and COS-7 cells (Bokoch, et al., 1996). The primary sequence of Nck contains one SH2 domain and three SH3 domains but has not been found to possess any enzymatic activity (Lehmann, et al., 1990). As such, Nck is thought to act as an adaptor protein, coupling activated cell surface receptors to various signalling pathways. Association with Nck (or possibly other adaptor molecules) in neutrophils may serve as a means to link Pak to various upstream signalling components in addition to Rac/Cdc42.
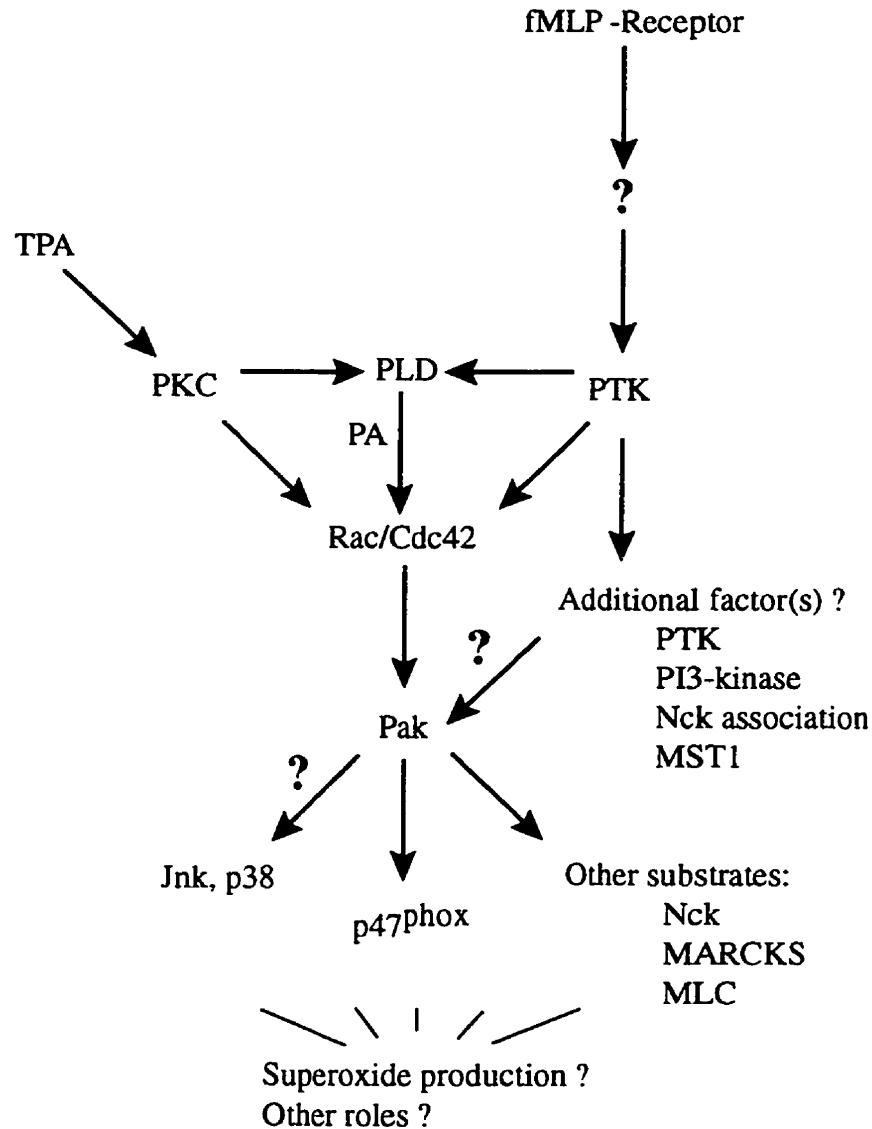
Fig. 8.1. Model of upstream regulation of the Pak family of serine/threonine kinases in response to chemoattractant stimulation. Abbreviations: fMLP, formyl-Met-Leu-Phe; Jnk, Jun N-terminal kinase; MARCKS, myristoylated alanine-rich C-kinase substrate; MLC, myosin light chain; MST1, mammalian sterile twenty-like kinase 1; p47phox, 47 kDa subunit of the phagocyte oxidase; PA, phosphatidic acid; Pak, p21-activated kinase; PI3-Kinase, phosphatidylinositol 3-kinase; PKC, protein kinase C; PLD, phospholipase D; PTK, protein tyrosine kinase(s); TPA, 12-O-tetradecanoylphorbol 13-acetate.

Inhibition of the renaturable kinases by 1-butanol implicates the actions of phospholipase D upstream of Pak activation (Ding and Badwey, 1994). The role of PLD, however, appears not to be additional to the effect of Rac/Cdc42 activation, as TPA has been found to activate this lipase (Truett, et al., 1989) without having an effect on the activity of Paks. The role of PLD may reside in the ability of phosphatidic acid to initiate release of RhoGDI from Rac, allowing it to exchange GTP for GDP and translocate to the plasma membrane (Bokoch, 1993). Hence, the model of Pak activation in Figure 8.1 depicts PLD as an upstream activator of Rac/Cdc42, but with no additional influence over Pak activation. PLD activation is depicted as being downstream of tyrosine kinases since 1) tyrosine kinase inhibitors block PLD activation (Uings, et al., 1992) and, 2) antiphosphotyrosine immunoprecipitates contain phospholipase D activity (Gomez-Cambronero, 1995).

Activation of Paks by fMLP was blocked by pretreatment of cells with inhibitors of serine/threonine phosphatases of the PP1 and PP2A class. This effect may be through the contributions of a recently described serine/threonine kinase, MST1, to Pak regulation. MST1 is ubiquitously expressed and phosphorylated on serine/threonine residues in quiescent cells. Dephosphorylation by PP2A was found to increase the kinase activity of MST1 by 3-4 fold both *in vivo* and *in vitro* (Creasy and Chernoff, 1995). A role for MST1 in the regulation of Paks would be suggested if the activity and/or subcellular distribution of this kinase was found to be affected by chemoattractant stimulation of neutrophils. This might easily be determined by immunoprecipitation of MST1 and assessment of its activity using an *in vitro* kinase assay. The ability of MST1 to phosphorylate recombinant Pak1 as a substrate in this reaction would further suggest that the effect of serine/threonine phosphatase inhibitors over Pak activation was due to an effect on phosphotransfer reactions.

Which tyrosine kinases mediate Pak activation in response to fMLP? The identity of the tyrosine kinase(s) that mediates activation of Paks in response to fMLP has not been determined, nor the mechanism by which this occurs. It is possible that the necessary tyrosine phosphorylation event(s) is/are upstream of Rac in the signal transduction sequence leading to

Pak activation. This notion is supported by the finding that translocation of Rac to the plasma membrane is blocked by treatment of cells with tyrosine kinase antagonists (Dorseuil, et al., 1995). Alternatively, tyrosine kinases may be required to activate the additional factors necessary for Pak activation (that are not activated by TPA treatment).

Candidate kinases include members of the Src family (eg: Lyn) which display rapid activation (maximal after 15 sec) following fMLP stimulation (Ptasznik, et al., 1995). PI3K activity is associated with immunoprecipitates of Lyn from chemoattractant stimulated neutrophils. This PI3K activity is upregulated in a manner that correlates temporally with the very rapid activation/deactivation of Lyn (Ptasznik, et al., 1995). Moreover, the tyrosine kinase inhibitors genistein and radicicol abolished PIP3 production in response to fMLP, indicating that the tyrosine kinase signalling pathway accounts for the majority of this lipid signalling molecule (Ptasznik, et al., 1996). Thus, in addition to contributing to translocation of Rac (through PLD and possibly other mechanisms), tyrosine kinases may also contribute to Pak activation by the induction of phosphotyrosine-dependent PI3Ks in response to fMLP (Fig. 8.1).

Neutrophils also express Btk, a member of the Tec family of tyrosine kinases (Brumell, et al., 1996). Activation of Btk by $\beta\gamma$ subunits of heterotrimeric G proteins (via interaction with the PH domains of Btk) has recently been described (Langhans-Rajasekaran, et al., 1995; Tsukada, et al., 1994). This suggests that Btk, in addition to PLCβ and PI3K γ, may also be a direct downstream effector of $\beta\gamma$ subunits in response to fMLP receptor activation. While Btk activation in response to fMLP remains to be demonstrated, the availability of antibodies to this kinase will facilitate assessment of its activity *in vitro* following immunoprecipitation from cells. Should Btk prove to be activated upon fMLP stimulation, its role in Pak activation may be assessed by studies of neutrophils from patients with X-linked agammaglobulinemia, which lack functional expression of this kinase.

Receptor engagement by concanavalin A treatment has been shown to activate Syk in human neutrophils (Asahi, et al., 1993). A role for this kinase in mediating downstream signalling through G protein-coupled receptors has also been suggested. Activation of Mek2

by either m1 (Gq-coupled) or m2 (Gi-coupled) muscarinic acetylcholine receptors was dependent on the expression of Syk in DT40 cells, an avian B cell line (Wan, et al., 1996). Use of a novel tyrosine kinase inhibitor that is selective for Syk, piceatannol, may serve to determine if this kinase plays a role in Pak activation (Oliver, et al., 1994).

The upstream mechanisms that activate tyrosine kinases in response to fMLP are almost completely unknown. A recently described tyrosine kinase, Pyk2, may play an important role in this capacity. Activation of Pyk2 can be accomplished by a wide variety of signals, including membrane depolarization, tumour necrosis factor, osmotic shock, and ultraviolet light (Lev, et al., 1995; Tokiwa, et al., 1996). Stimulation of G protein-coupled receptors (including the pertussis toxin-sensitive LPA receptor) also activates Pyk2 activity. LPA stimulation of PC12 cells was found to activate Pyk2, causing its autophosphorylation on Tyr-402 (Dikic, et al., 1996). Phosphorylation of this residue caused association of Pyk2 with Src, causing activation of the latter kinase (as determined by Src phosphorylation on Tyr-416, see Introduction). Importantly, overexpression of Pyk2 mutants lacking tyrosine kinase activity, or in which Tyr-402 was replaced with phenylalanine, blocked the ability of LPA to activate Src (Dikic, et al., 1996). Because Pyk2 appears to play a role in transducing a wide variety of signals, and because of its demonstrated role in activating Src in response to G protein-coupled receptor activation, it is tempting to speculate that Pyk2 (or perhaps other isoforms of this newly described kinase) also plays a role in activating the Src-related kinases expressed in neutrophils in response to fMLP. This notion may be addressed through studies of neutrophils from transgenic mice lacking expression of Pyk2, currently under development.

What are the roles of Paks in activated neutrophils? Earlier studies of the renaturable kinases PK65 and PK72 suggested that these enzymes were important for activation of the NADPH oxidase. Their rapid activation paralleled or preceded superoxide production and pharmacologic inhibitors (eg: wortmannin, tyrosine kinase inhibitors) that blocked one event also blocked the other to a similar degree (Brumell and Grinstein, 1994; Ding, et al., 1995). The renaturable Paks were also capable of phosphoryalting a peptide derived from a region of

p47phox that contained 6 serine residues known to be phosphorylated in cells in response to fMLP (Ding, et al., 1993). Later identification of the renaturable kinases as Paks allowed for further study of the substrate specificity of these enzymes. Knaus et al. (1995) employed recombinant Pak1 and a series of small peptides from p47phox that contained only single phosphorylatable serine residues as substrates for *in vitro* kinase reactions. Pak1 was capable of phosphorylating only one peptide derived from p47phox, containing Ser-328 in the wild type protein. This residue is within a consensus PKC-phosphorylation sequence and is known to be phosphorylated in neutrophils in response to both TPA and fMLP stimulation (el Benna, et al., 1994). The importance of the region of p47phox containing Ser-328 has previously been suggested. A peptide derived from residues 323-332 was capable of inhibiting p47phox phosphorylation, translocation and superoxide production using a cell-free assay (Nauseef, et al., 1993). Thus, phosphorylation of Ser-328 by members of both the PKC and Pak families may provide alternative means of activation of the NADPH oxidase in response to inflammatory stimuli.

In other cell types, Pak plays an important role in the activation of Jnk and p38 (Bagrodia, et al., 1995; Zhang, et al., 1995). Jnk is not detetable in neutrophils but p38 has been shown to play an important role in mediating activation of the NADPH oxidase and adhesion to fibronectin (Nick, et al., 1996). While upstream regulation of p38 in neutrophils has not been determined, activation by Paks would provide an indirect mode of NADPH oxidase activation in addition to direct phosphorylation of p47phox.

Activation of Tyrosine Kinases by Endogenously Produced Reactive Oxygen Intermediates.

The studies in Chapter 4 confirmed previous findings which demonstrated a role for endogenously produced ROI in inducing tyrosine phosphorylation in neutrophils (Fialkow, et al., 1993). Treatment of electropermeabilized neutrophils with non-hydrolyzable analogues of GTP (eg: GTPγS) was found to induce tyrosine phosphorylation of numerous cellular proteins. This effect was dependent on the presence of NADPH in the stimulation medium, suggesting

involvement of the NADPH oxidase. Tyrosine phosphorylation was blocked by treatment of cells with a powerful antioxidant, N-acetylcysteine, or by direct inhibition of the NADPH oxidase with diphenyleneiodonium. Previous findings have also demonstrated that GTPγS stimulation of neutrophils from CGD patients does not yield tyrosine phosphorylation. Together, these findings suggest an important role for NADPH oxidase-derived ROI in neutrophil signalling processes.

The mechanism by which ROI induce tyrosine phosphorylation in electropermeabilized cells was investigated. Utilizing *in vitro* immune complex assays of the tyrosine kinases identified in neutrophils, superoxide production was found to increase the activities (as measured by autophosphorylation) of at least three kinases (Hck, Syk and Btk). Interestingly, the autophosphorylation of two other kinases, Lyn and Fgr, appeared to decrease following GTPγS stimulation. This may have been due to incorporation of 'cold' phosphate *in situ*, which would conceivably prohibit further incorporation of radiolabelled phosphate *in vitro*. In support of this notion, the ability of Lyn to phosphorylate an exogenous substrate (enolase) was found to increase upon activation of the NADPH oxidase. Thus, with respect to their ability to phosphorylate other proteins, at least 4 tyrosine kinases are activated in electropermeabilized neutrophils following production of ROI.

The mode of regulation of Hck was explored in detail. Oxidizing agents were unable to activate Hck *in vitro* and, once activated *in situ*, reducing agents failed to inactivate it. These experiments suggested that the effects of ROI are not direct, but rather they influence the activity of tyrosine kinases by an indirect mechanism. Tyrosine phosphorylation of Hck paralleled its activation, and dephosphorylation *in vitro* reversed the stimulation. It was concluded that tyrosine phosphorylation is central to the regulation of Hck and likely also of Syk and Lyn, which are similarly phosphorylated upon activation of the oxidase. As ROI have been shown to reduce the activity of tyrosine phosphatases by oxidation of a critical sulfhydryl residue within the catalytic domain, it is suggested that this inhibition allows constitutively active kinases to auto/transphosphorylate on stimulatory tyrosine residues, leading to an

increase in their catalytic activity. Enhanced phosphotyrosine accumulation would then result from the combined effects of increased phosphorylation with decreased dephosphorylation.

Exposure of a human T-cell line to H_2O_2 was also found to activate a member of the Src family of tyrosine kinases, namely Lck (Hardwick and Sefton, 1995). Mutational analysis of Lck demonstrated the requirement of phosphorylation at Tyr-394 (the autophosphorylation site) for increased activity, consistent with a recent structural model for the activated protein (Yamaguchi and Hendrickson, 1996). In addition, H_2O_2 treatment was capable of inducing phosphorylation at Tyr-394 in cells expressing a catalytically inactive mutant and lacking endogenous Lck. These results demonstrated that a kinase other that Lck was capable of phosphorylating its autophosphorylation site, potentially activating Lck upon exposure to ROI such as H_2O_2. Thus, in addition to autophosphorylation and activation, Src family kinases in neutrophils may also be regulated by unidentified tyrosine kinases which are activated by endogenously produced ROI upon activation of the NADPH oxidase.

While the experiments presented in Chapter 4 were performed exclusively with suspended cells, a recent study by Yan and Berton demonstrates a signalling role for ROI in neutrophils adherent to extracellular matrix proteins (Yan and Berton, 1996). Following adhesion and spreading of cells onto fibrinogen, the activities of Lyn and Fgr were found to increase several-fold in vitro, as determined by both autophosphorylation and phosphorylation of enolase. Partial inhibition of this effect was accomplished by pretreatment of cells with diphenyleneiodonium and could be mimicked by treatment of cells with H_2O_2. This suggested that activation of the NADPH oxidase by engagement and clustering of integrins (and possibly other cell surface receptors) was at least partially responsible for the increased tyrosine kinase activity of Lyn and Fgr. In support of this hypothesis, adhesion-stimulated activities of these kinases was greatly reduced in neutrophils from patients with CGD. By contrast, Syk was activated in CGD cells to the same degree as it was in oxidase-competent cells. These studies suggest that endogenously produced ROI are capable of differentially affecting the activity of various kinases in adherent neutrophils (Yan and Berton, 1996). The differences between my results in Chapter 4 and those by Yan and Berton may reflect an influence of the cytoskeleton

over tyrosine kinase activities or perhaps the proximity of the NADPH oxidase to the signalling targets influenced by ROI production.

A comparison of fMLP and ROI-induced tyrosine phosphorylation. Neutrophils display a characteristic pattern of tyrosine phosphorylation that appears rapidly (maximal after 15 sec) following treatment with the chemoattractant fMLP (see Fig. 3.1A). Rapid termination (within 2 min) of this response is due to receptor downregulation and desensitization. In contrast, GTPγS treatment of electropermeabilized cells caused phosphorylation of a greater number of cellular proteins (see Fig. 4.1A,B,C). This event was much slower to develop than that witnessed for fMLP, with a maximal response seen after 10 min. These differences in both the pattern and kinetics of tyrosine phosphorylation reflects the different mechanisms by which these agents function. In the case of GTPγS, activation of the NADPH oxidase was shown to be essential. Following production of endogenous ROI, the combined effects of phosphatase inhibition and kinase activation mediate phosphotyrosine accumulation. In the case of fMLP, tyrosine kinase activation has also been demonstrated (Ptasznik, et al., 1996). This event, however, is independent of ROI production. This was determined previously by comparison of neutrophils from patients with CGD to cells from normal controls (Fialkow, et al., 1993). Phosphotyrosine accumulation in response to fMLP treatment was identical in both cases, despite the fact that CGD neutrophils lack a functional NADPH oxidase. The inability of endogenous ROI to influence fMLP-mediated responses is likely due to the fact that the amount of ROI produced in response to this agent is 20-fold less than that elicited by GTPγS stimulation of electropermeabilized cells (Fialkow, et al., 1993). Activation of the NADPH oxidase by fMLP is also a transient event since, as mentioned above, receptor signalling is rapidly terminated. It has been proposed that fMLP and other receptor agonists promote phosphotyrosine accumulation, in part, by the inhibition of tyrosine phosphatases (Kraft and Berkow, 1987). Because ROI do not appear to influence fMLP-induced tyrosine phosphorylation, ROI-independent mechanisms are likely to mediate this effect.

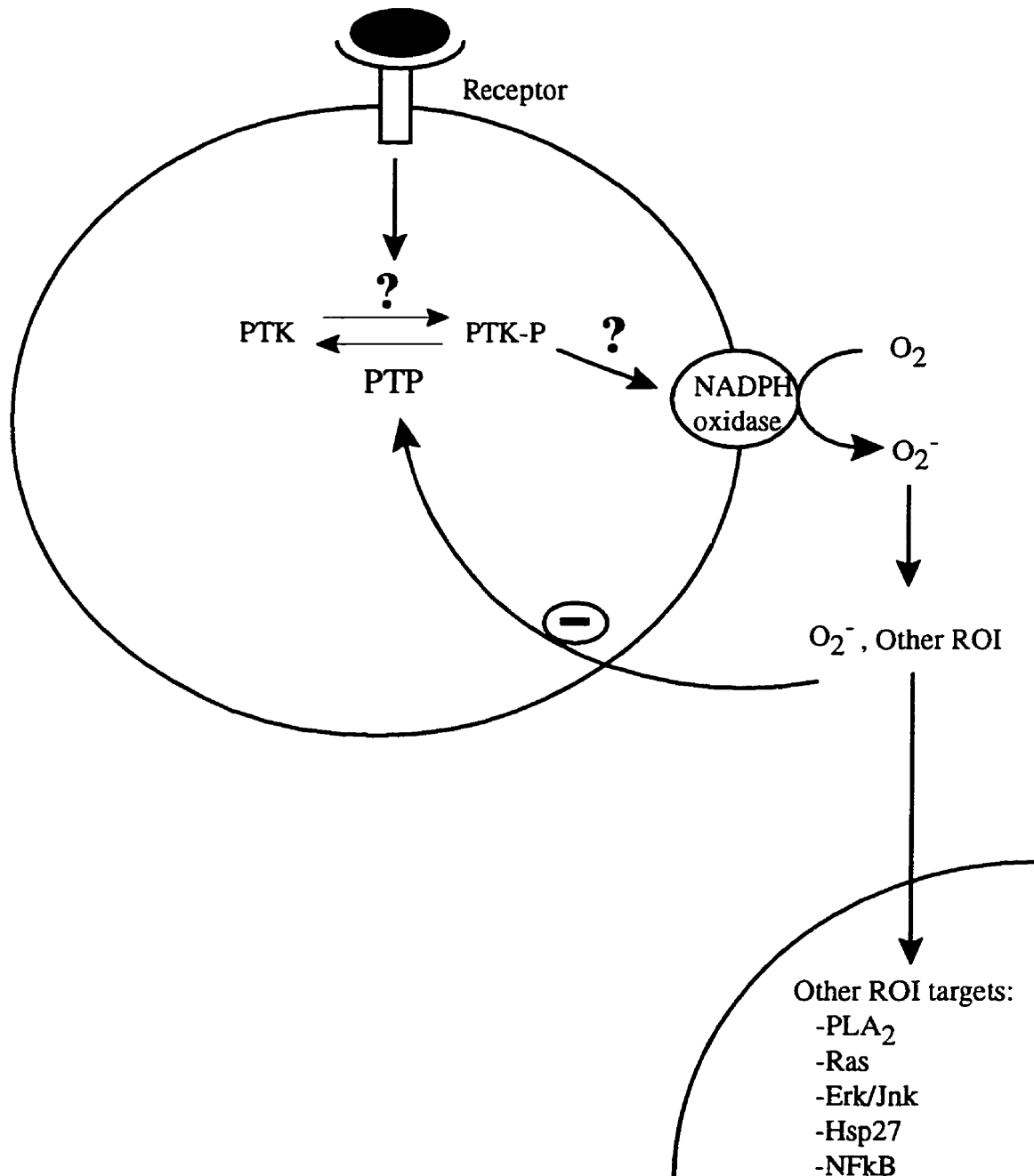Fig. 8.2. Model for ROI-mediated auto/paracrine signalling at sites of inflammation. Abbreviations: Erk, extracellular signal-regulated kinase; Hsp27, heat shock protein 27; Jnk, c-Jun N-terminal kinase; NFκB, nuclear factor κ B; O_2, molecular oxygen; O_2^-, superoxide; PLA_2, phospholipase A_2; PTK, protein tyrosine kinase; PTP, protein tyrosine phosphatase; ROI, reactive oxygen intermediates.

Model for ROI-mediated auto/paracrine signalling. A model for ROI-mediated auto/paracrine signalling at sites of inflammation is shown in Figure 8.2. Following engagement of a variety of cell surface receptors (eg: chemotactic receptors, integrins, phagocytic receptors), intracellular signalling cascades that include the activation of tyrosine kinases initiate activation of the NADPH oxidase (in addition to other antimicrobial responses). Subsequent production of ROI allows for the efficient killing of invading microorganisms. These molecules are capable of diffusion through cellular membranes, where they also influence a variety of signalling molecules including tyrosine phosphatases. Inhibition of the latter allows for phosphorylation-dependent activation of tyrosine kinases and potentiation of phosphotyrosine-dependent cellular processes (see Introduction). This auto/paracrine effect of ROI allows for significant potentiation of various neutrophil responses to inflammatory stimuli, including adhesion to endothelial surfaces (Fraticelli, et al., 1996), and phagocytosis of microbial targets (Gresham, et al., 1988).

Regulation of SHP-1 during Neutrophil Activation. Possible link between PKC and Tyrosine Phosphorylation.

In addition to their inhibition by ROI, the study presented in Chapter 5 demonstrates that tyrosine phosphatases are also regulated by phosphorylation-dependent mechanisms. Agonist treatment induced significant decreases (30-60%) in SHP-1 activity which correlated temporally with increases in the cellular phosphotyrosine content. Phosphorylation of SHP-1 on serine residues was associated with the inhibition of its enzymatic activity, suggesting a causal relationship. Accordingly, both the agonist-evoked phosphorylation of SHP-1 and the inhibition of its catalytic activity were blocked by treatment with *bis*-indolylmaleimide I, a potent and specific inhibitor of PKC activity. Immunoprecipitated SHP-1 was found to be phosphorylated efficiently by purified PKC *in vitro*. Such phosphorylation also caused a decrease in the phosphatase activity of SHP-1. Together, these data suggest that inhibition of SHP-1 by PKC-mediated serine phosphorylation plays a role in facilitating the accumulation of tyrosine phosphorylated proteins following neutrophil stimulation. These findings provide a

new link between PKC and tyrosine phosphorylation in the signalling cascade that triggers anti-microbial responses in human neutrophils.

Further elucidation of the mechanism by which serine phosphorylation inhibits SHP-1 activity will require definition of the exact residue(s) which is/are phosphorylated by PKC *in situ*. The primary sequence of SHP-1 contains several candidate serine residues within PKC consensus phosphorylation motifs. Transfection of hematopoietic cell lines with SHP-1 mutants lacking individual serine residues (mutated to alanine) and subsequent peptide mapping of these SHP-1 mutants following phosphorylation by PKC may provide this information.

Inhibitory role of SHP-1 in the regulation of neutrophil antimicrobial responses. Previous studies have suggested a role for SHP-1 in turning off phosphotyrosine-dependent signals through a wide variety of cell surface receptors present on hematopoietic cells (Okamura and Thomas, 1995). Consistent with this model, the data presented in Chapter 5 suggest that receptor-mediated inhibition of SHP-1 is an important mechanism by which phosphotyrosine accumulation can occur. Recent studies of motheaten (*me*) mice, which lack expression of SHP-1, have determined the role of this phosphatase in mediating neutrophil responses to inflammatory stimuli (Butler, et al., 1995). In response to TPA or opsonized zymosan particles, tyrosine phosphorylation of some cellular proteins was enhanced in neutrophils from *me* mice compared to their littermate controls. Importantly, activation of the NADPH oxidase in cells from the *me* mice occurred with a decreased lag time, a higher maximal rate of superoxide production and was prolonged in duration. In addition, further studies have determined that cellular adhesion to extracellular matrix proteins is enhanced in neutrophils from *me* mice[5]. Together, these studies suggest an important role for SHP-1 as a negative regulator of receptor-mediated responses of neutrophils to inflammatory stimuli. Furthermore, because many soluble and particulate stimuli can activate PKC, SHP-1 inhibition may play a

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[5]J. Butler and G. Downey, personal communication.

common role in mediating accumulation of phosphotyrosine, in addition to the activation of tyrosine kinases.

Possible role of SHP-1 as an intracellular adaptor protein. Following stimulation with neutrophil agonists such as phorbol ester, chemotactic peptide or opsonized zymosan, a fraction of SHP-1 redistributed to the cytoskeleton. Translocation may allow interaction of SHP-1 with cytoskeleton-associated substrates. Cytoskeletal association of SHP-1 has been demonstrated in platelets stimulated with thrombin and this translocation was postulated to mediate the observed dephosphorylation of cytoskeletal-associated proteins (Ezumi, et al., 1995). Alternatively, SHP-1 may serve as an intracellular adaptor by targetting interacting proteins to the cytoskeletal compartment. In support of this possibility, SHP-1 has been found to associate with many important signalling molecules in other hematopoietic cell types. These include Src (Falet, et al., 1996), a recently identified SH2 domain-containing protein called SLP-76 (Mizuno, et al., 1996) and the Abl tyrosine kinase (Kharbanda, et al., 1996). SHP-1 has also been found to associate with Vav, Grb2 and mSos, three proteins that play a role in initiating guanine nucleotide exchange in Ras (Kon-Kozlowski, et al., 1996). Association of SHP-1 with Ras activators supports previous findings that suggested a role for this phosphatase in mediating Erk activation under some conditions (Krautwald, et al., 1996; Su, et al., 1996). Thus, while SHP-1 appears to act as a negative regulator of phosphotyrosine-dependent events in neutrophils, it may also play a role as an adaptor protein in regulating other signalling events. The possibility that SHP-1 acts as an adaptor protein may be addressed with the use of chemical crosslinkers to search for associated proteins in metabolically labelled neutrophils. Alternatively, use of SHP-1 in a yeast two hybrid screen may also identify proteins from a neutrophil cDNA library that interact with this phosphatase.

Phosphorylation and Subcellular Redistribution of Pleckstrin in Human Neutrophils.

A major substrate of PKC in platelets, pleckstrin was also found to be highly expressed in neutrophils with an intracellular concentration calculated to be approximately 15 μM (or ≈ 0.5 % of the total cellular protein). Upon stimulation with fMLP, opsonized zymosan or TPA, rapid phosphorylation of pleckstrin was observed. Phosphopeptide mapping revealed that phosphorylation of pleckstrin occurred on residues Ser-113 and Ser-117, the same residues phosphorylated in platelets and transfected Cos-1 cells.

The upstream regulation of pleckstrin phosphorylation was examined. A role for non-conventional PKC isoforms was suggested by several findings: 1) chelation of intracellular Ca^{2+} had only a partial inhibitory effect; 2) the phosphatidic acid phosphohydrolase antagonist propranolol extended it and 3) wortmannin and erbstatin blocked the phosphorylation of pleckstrin. These results suggest that non-conventional PKC isoforms, possibly δ or ζ which are expressed in neutrophils, contribute to phosphorylation of pleckstrin in response to both soluble and particulate stimuli.

While pleckstrin is primarily localized to the cytosol of unstimulated cells, agonist treatment was found to induce a subcellular redistribution of this protein. Increased association with both membranes and the triton-insoluble fraction (thought to represent cytoskeletal-associated proteins) was observed. This event could be blocked by pretreatment of cells with a potent PKC antagonist, suggesting that prior phosphorylation of pleckstrin was necessary for its translocation.

A recent study by Ma et al. has also demonstrated phosphorylation-dependent association of pleckstrin with the plasma membrane (Ma, et al., 1997). In this study, Cos-1 cells (which do not express endogenous pleckstrin) were transfected with wild type pleckstrin or various mutants of this protein. Transfection of cells with mutants in which the three phosphorylatable residues (Ser-113, Thr-114 and Ser-117) were replaced with glutamic acid residues, the so called 'pseudophosphorylated' mutant of pleckstrin, caused the protein to be constitutively associated with the plasma membrane. Interestingly, pseudophosphorylated pleckstrin (or the endogenously phosphorylated wild type protein) associated with membrane projections at the cell surface, possibly causing their formation. A pleckstrin mutant in which

the phosphorylatable residues were mutated to glycine was localized exclusively to the cytosol and did not redistribute upon TPA treatment. Mutation of three critical lysines within the N-terminal PH domain that have been shown to mediate PIP_2 binding of this region was found to block membrane association of pleckstrin. This suggested that membrane association of phosphorylated pleckstrin was due to its association with PIP_2 and not other PH domain ligands such as the $\beta\gamma$ subunits of heterotrimeric G proteins. In support of this notion, overexpression of $\beta\gamma$ heterodimers had no effect on pleckstrin's localization at the membrane (Ma, et al., 1997).

The culmination of many studies on a variety of PH domain-containing proteins has led to the current view that these protein modules act universally as membrane adaptor domains (Hemmings, 1997). In the case of pleckstrin, the requirement of phosphorylation prior to its membrane association suggests that an unknown event must take place before association can occur. Because membrane association appears to be mediated by PIP_2 binding, it is especially obvious that other factors influence translocation since the levels of PIP_2 are rapidly decreasing during the time that pleckstrin phosphorylation and translocation occurs (Stephens, et al., 1991). In support of an intramolecular event, the electrophoretic mobility of pleckstrin on SDS PAGE gels was found to change upon its phosphorylation. This shift represents a stable conformational change that is capable of withstanding denaturation and electrophoresis and may reflect conformational changes that occur *in situ*, allowing subcellular redistribution of pleckstrin. Structural analysis of pleckstrin and its pseudophosphorylated mutant may provide insight into the mechanism by which phosphorylation mediates translocation of pleckstrin in neutrophils.

Phosphorylated pleckstrin inhibits the Ca^{2+}-mobilizing signal initiated by PLC activation. Transfection of Cos-1 cells with pleckstrin has been shown to inhibit the activation of PLC and subsequent production of IP_3, an important Ca^{2+}-mobilizing signal in all cells (Abrams, et al., 1995; Luttrell, et al., 1995). Phosphorylation appeared to play a role in this effect since pseudophosphorylated pleckstrin mutants displayed a greater effect than the wild type protein.

Inhibition of phosphoinositide hydrolysis was accomplished following stimulation of either growth factor receptors or G protein-coupled receptors, suggesting that the effect of pleckstrin was due to its interaction with the substrate of PLC, PIP_2, and not due to its binding to $\beta\gamma$ subunits of heterotrimeric G proteins (Abrams, et al., 1995).

In addition to PIP_2 binding, pleckstrin may also terminate the Ca^{2+}-mobilizing signal by association with other proteins. Platelet pleckstrin was recently found to bind in a stoichiometric complex with inositol polyphosphate 5-phosphatase I (5-phosphatase I) (Auethavekiat, et al., 1997). Substrates of 5-phosphatase I include IP_3 and IP_4, which both contribute to Ca^{2+} release from internal stores. Pseudophosphorylated pleckstrin was capable of activating 5-phosphatase I activity *in vitro*, while non-phosphorylated pleckstrin had no effect. Thus, pleckstrin termination of the Ca^{2+}-mobilizing signal may occur by inhibiting the availability of the substrate of PLC and by acceleration of the metabolism of its bioactive products.

Pleckstrin inhibits activation of PI3Kγ. PIP_2 binding may also underlie the ability of pleckstrin to inhibit the activity of PI3Kγ isolated from human platelets (Abrams, et al., 1996). Inhibition was limited to PI3Kγ phosphorylation of PIP_2, consistent with this hypothesis. Only phosphorylated pleckstrin (or its pseudophosphorylated mutant) was capable of inhibition.

Inhibitory feedback model for pleckstrin. The role of pleckstrin in neutrophils requires further definition. Its high expression in hematopoietic cells and the finding that only a small percentage of platelet pleckstrin is present in a complex with 5-phosphatase I suggests that other protein ligands may interact with neutrophil pleckstrin. Use of the yeast two-hybrid system has identified the partial cDNA sequence of a novel protein that interacts specifically with the N-terminal PH domain of pleckstrin[6]. Isolation of the complete cDNA for this interacting protein, dubbed PHi-1 (for PH domain interactor-1), will shed further light onto the role of pleckstrin in hematopoietic cells.

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[6]K. Craig and M. Tyers, personal communication.
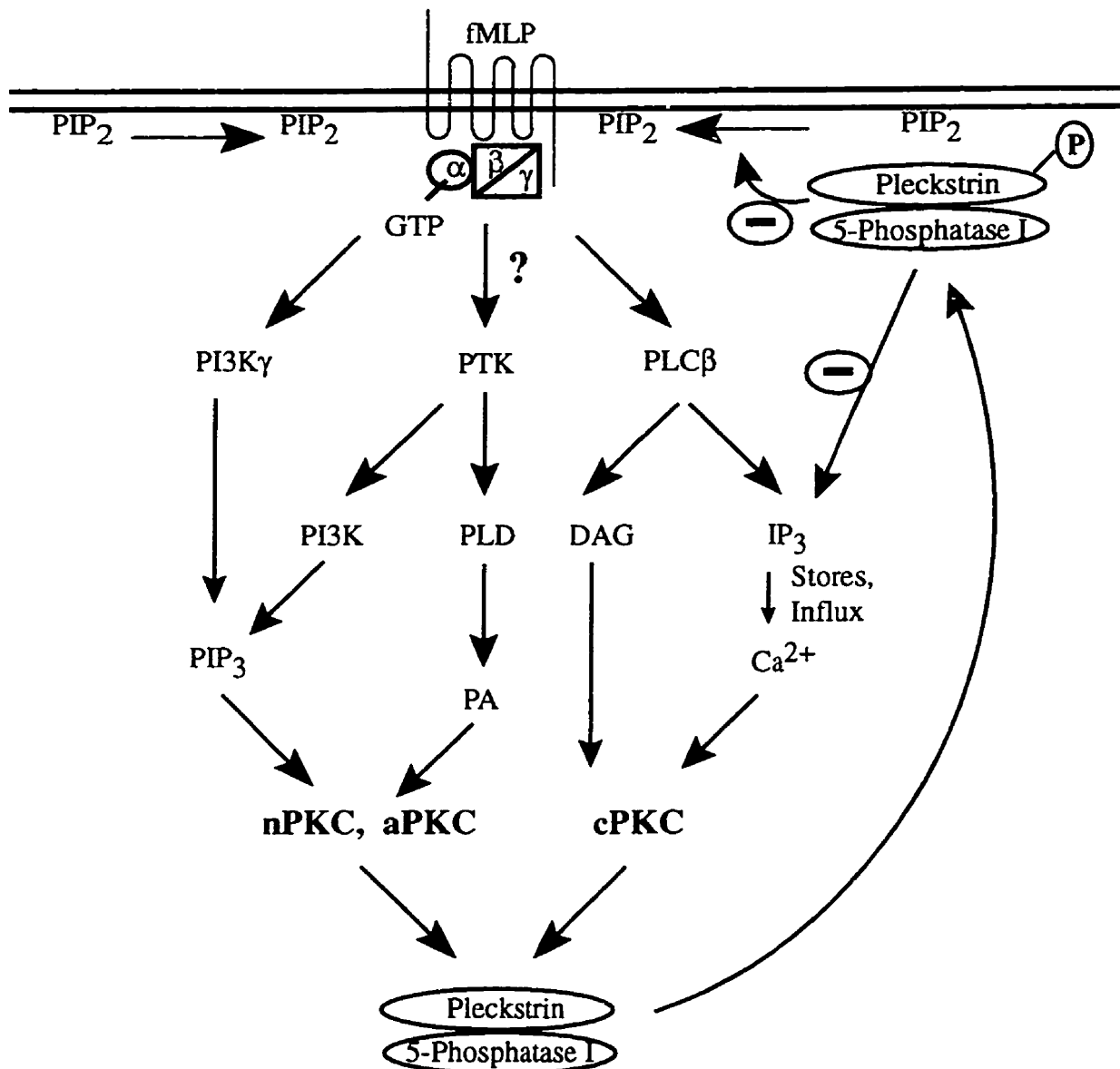
Fig. 8.3. Inhibitory feedback model for phosphorylated pleckstrin. Abbreviations: aPKC, atypical PKC; cPKC, conventional PKC; DAG, diacylglycerol; IP$_3$, inositol (1,4,5)-trisphosphate; nPKC, novel PKC; PA, phosphatidic acid; PI3K, phosphatidylinositol 3-kinase; PIP$_2$, phosphatidylinositol (4,5)-bisphosphate; PIP$_3$, phosphatidylinositol (3,4,5)-trisphosphate; PLCβ, phospholipase β; PLD, phospholipase D; PKC, protein kinase C; PTK, protein tyrosine kinase.

Incorporating recent data concerning pleckstrin phosphorylation in both transfected Cos-1 cells and platelets, I propose the following model for the function of pleckstrin in hematopoietic cells, as shown in Figure 8.3. Activation of the fMLP receptor initiates rapid phosphorylation of pleckstrin by both conventional and non-conventional isoforms of PKC. A phosphorylation-induced conformational change allows association of pleckstrin (and interacting proteins) with the plasmalemma. Binding and sequestration of PIP_2 prevents its hydrolysis by PLC and its phosphorylation by PI3Ks. In addition, the basal activity of phosphopleckstrin-bound 5-phosphatase I is enhanced severalfold, further attenuating the Ca^{2+}-mobilizing signal initiated by PLC. Inhibitory feedback through phosphorylated pleckstrin may provide a threshold that receptor stimulation must overcome to induce cellular responses to inflammatory stimuli. The suggestion that phosphorylated pleckstrin is capable of producing membrane protrusions in Cos-1 cells also suggests a role for this protein in regulating membrane dynamics in activated cells (Ma, et al., 1997).

Presence and Subcellular Distribution of Docking/Fusion proteins in Human Neutrophils.

Neutrophils contain at least four distinct types of secretory organelles, which undergo exocytosis during infection and inflammation. The signalling pathways leading to secretion of individual granules and their kinetics of exocytosis vary greatly, causing temporal and regional differences in docking and fusion with the plasma membrane. As a step towards understanding the processes underlying differential granular secretion in neutrophils, I assessed the presence and distribution of a number of proteins reported to be involved in vesicular docking and/or fusion in other systems.

Using specific antibodies for each protein, syntaxin 4, VAMP-2 and the 39 kDa isoform of SCAMP were identified in neutrophils. SCAMP was found mainly in secondary and tertiary granules and in a fraction containing secretory vesicles, but was virtually absent from the primary (lysosomal) granules. This profile is consistent with the proposed "post-Golgi" distribution of SCAMP. VAMP-2 was largely absent from primary and secondary

granules, but concentrated in tertiary granules and secretory vesicles. This pattern of distribution parallels the increasing sensitivity of these exocytic compartments to intracellular free calcium. Accordingly, ionomycin induced translocation of VAMP-2 towards the plasma membrane. Syntaxin 4 was found almost exclusively in the plasma membrane, and it accumulated especially in lamellipodia. This regional accumulation may contribute to localized secretion into the phagosomal lumen. In support of this notion, recent studies have demonstrated the presence of syntaxins 2,3 and 4 on the phagosomal membrane of both murine and human macrophages (Hackam, et al., 1996).

A key component of the tripartite docking/fusion complex in neuronal cells, SNAP-25, was absent in neutrophil lysates. Subsequent to the study presented in Chapter 7, a non-neuronal isoform of this t-SNARE, namely SNAP-23, was identified (Ravichandran, et al., 1996). Immunoblotting whole cell lysates with an affinity purified antibody to SNAP-23 reveals expression of this protein in a variety of hematopoietic cell types, including neutrophils (see Appendix 1.A following this discussion). Immunoblotting neutrophil subcellular fractions reveals SNAP-23 to be largely localized to the combined secretory vesicle/plasma membrane fraction (Appendix 1.B). SNAP-23, in conjunction with syntaxin 4, may play an important role in mediating secretory events in activated neutrophils. Future analysis by immunofluorescence will determine if SNAP-23, like the syntaxins, preferentially redistributes within the plasmalemma during chemotaxis or phagocytosis. Further proof of the role of SNARE proteins in mediating secretory responses of hematopoietic cells to inflammatory stimuli may come from microinjection of macrophages (neutrophils are unamenable to microinjection) with clostridial toxins which can cleave these proteins.

Recent findings have suggested that phosphorylation-dependent signalling mechanisms can influence secretory events at the level of docking/fusion proteins. Subjection of various recombinant synaptic proteins to *in vitro* kinase reactions revealed that many are efficiently phosphorylated by three kinases: casein kinase II, calmodulin-dependent kinase II and protein kinase A (Hirling and Scheller, 1996). Of significance, phosphorylation of αSNAP by protein kinase A decreased the affinity of this protein for the core docking/fusion complex by 10 fold.

In addition to phosphorylation by protein kinase A and calmodulin-dependent kinase II, PKC-mediated phosphorylation of SNAP-25 has been described in PC12 cells (Shimazaki, et al., 1996). Following phosphorylation, the amount of co-immunoprecipitatable syntaxin was decreased, suggesting that PKC-dependent phosphorylation of SNAP-25 modifies the interaction of SNARE proteins. Our unpublished results support a model of phosphorylation-dependent regulation of SNARE complex formation in neutrophils. Following subcellular fractionation of neutrophils treated with or without TPA, an upward shift in the mobility of VAMP-2 was observed (Appendix 1.C). Further definition of docking/fusion protein phosphorylation and the role of this post-translational modification in regulating neutrophil secretion are required.

Future directions

The study of signal transduction in neutrophils, as in all systems, has revealed a surprising complexitiy by which cells control their differentially prescribed functions. Through the course of these studies, an important role for phosphorylation-dependent signalling mechanisms has emerged. Prior to the studies presented in this thesis, a great deal of attention was paid to the role of PKC family members in the control of neutrophil responses to chemoattractants and other inflammatory stimuli. Later, it became evident that many of the same functions that were attributed to PKC regulation were also influenced by the activity of tyrosine kinases (protein phosphorylation) and PI3-kinases (lipid phosphorylation). Thus, the antimicrobial responses of neutrophils appeared to be regulated by multiple signalling pathways.

While initially considered separate signalling systems, my studies have revealed a relationship between signalling components of the PKC family and those of both PI3-kinases and tyrosine kinases. In Chapter 5, I demonstrate a model by which PKC phosphorylation of the tyrosine phosphatase SHP-1 can inhibit its activity. By this manner, PKC activation may contribute to the observed increase in phosphotyrosine content upon cellular stimulation. In turn, the activity of at least some PKC family members appears to be influenced by tyrosine phosphorylation and the lipid products of PI3-kinases (see Chapter 7). Pharmacologic inhibition of the latter was found to block phosphorylation of pleckstrin, a known PKC substrate. Thus, while considering signalling mechanisms downstream of the fMLP receptor, one must recognize that a significant amount of cross-talk (and possibly feedback) is occurring following receptor engagement.

The findings of Chapter 7 demonstrated a major role for non-conventional members of the PKC family in mediating pleckstrin phosphorylation, with a minimal contribution by the Ca^{2+}-dependent, conventional isoforms. These findings point to an important issue that will need to be addressed in future studies, namely which kinases are responsible for controlling which functions. Having addressed the question "what are the functions of tyrosine kinases", it is now appropriate to ask "which tyrosine kinase(s)" control those functions. The

advancement of several technologies available to the researcher will make this more achievable. These include the development of enzyme-specific pharmacologic inhibitors (e.g.: PD98059, the Mek inhibitor), transfectable leukocyte cell lines and the use of transgenic technology. Considering the fact that only a small percentage of the human genome has been sequenced (and studied) to date, it is certain that many more important signalling molecules will be identified in the future. These new faces of signal transduction will require assimilation into the increasingly complex and interconnected pathways that are rapidly being delineated for the control of antimicrobial responses.

Having addressed which kinases and phosphatases control cellular phosphorylation cascades in response to chemoattractants and other inflammatory stimuli, it will be important to determine the role of the phosphorylated substrates downstream. It has been witnessed in the body of this thesis that phosphorylation can invoke a change in the catalytic activity, subcellular distribution or possibly the inter/intramolecular interactions of the phosphorylated target. Determining the individual effects of each phosphorylation step of the signalling cascade will eventually provide a solid link between activation of cell surface receptors and the initiation of cellular responses.

The study of signal transduction will increasingly move to a three dimensional realm, as it becomes more important to ask the question "where are signalling events occurring" and not just "when". These spatial considerations are limited somewhat by our understanding of the architecture of cells. A case in point would be the recent appreciation of the role of caveolae, which are cholesterol-rich subdomains of the plasma membrane. These detergent-resistant compartments have been found to sequester both cell surface receptors (e.g.: growth factor receptors) and many of their downstream signalling targets (e.g.: Src family PTK). While caveolae (or homologous membrane subdomains) have yet to be been demonstrated in neutrophils, their presence in leukocytes (RBL-2H3 mast cells) has been previously demonstrated (Field et al., 1995). Other important signalling compartments will likely be recognized, possibly including the nucleus. In this thesis I have demonstrated the presence of two important signalling molecules (SHP-1 and pleckstrin) within this organelle. While the

significance of these findings remains currently unknown, it is tempting to speculate that the nucleus, as well as other, perhaps undefined regions of the cell, will become the focus of future studies of signal transduction.

A. Whole cell lysates (10^6 cell equivalents) of neutrophils, monocytes and lymphocytes were subjected to SDS PAGE and immunoblotting with an affinity purified antibody to SNAP-23.

B. Neutrophils were subjected to subcellular fractionation by Percoll gradient centrifugation.

C. Neutrophils were treated with or without 10^{-7}M TPA.
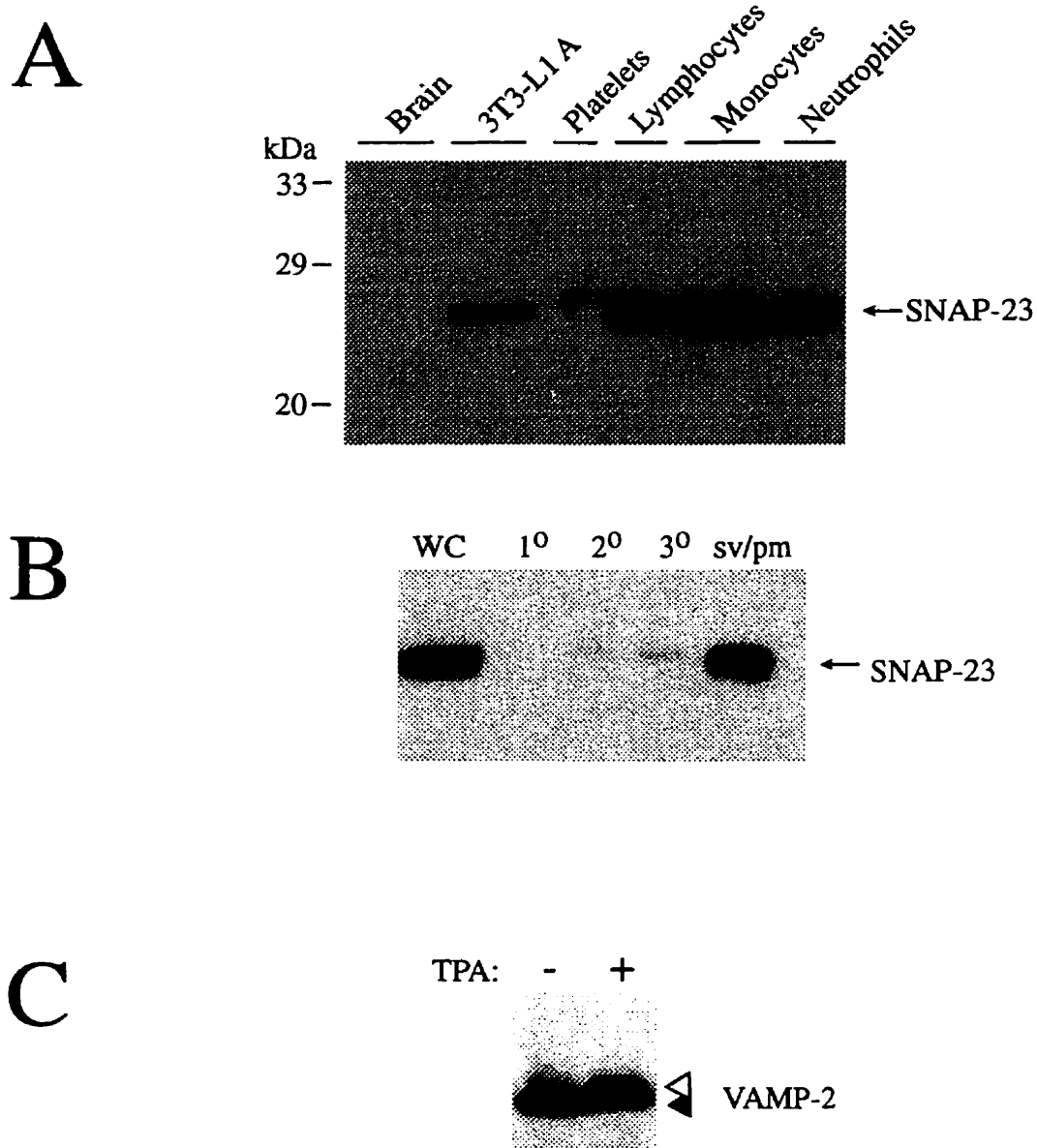
Appendix 8.1.   A. Whole cell lysates (10^6 cell equivalents) of neutrophils, monocytes and lymphocytes were subjected to SDS PAGE and immunoblotting with an affinity purified antibody to SNAP-23. Also analyzed were platelets (5×10^6 cell equivalents), 3T3-L1 adipocytes ($50\mu g$) and rat brain homogenate ($30\mu g$). B. Neutrophils were subjected to subcellular fractionation by Percoll gradient centrifugation and samples were analyzed by SDS PAGE and immunoblotting with an affinity purified antibody to SNAP-23. A whole cell lysate was also analyzed (WC). C. Neutrophils were treated with or without 10^{-7}M TPA for 10 min prior to subcellular fractionation and analysis as in B with an affinity purified antibody to VAMP-2. The mobilities of VAMP-2 in untreated (solid arrowhead) and TPA treated (open arrowhead) are indicated.

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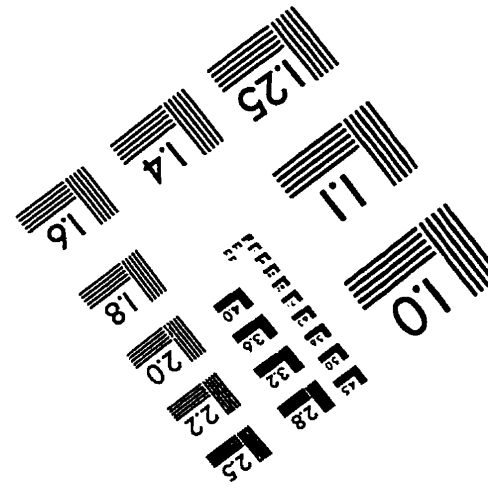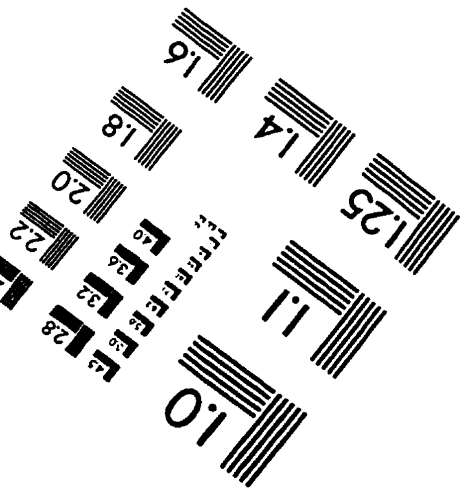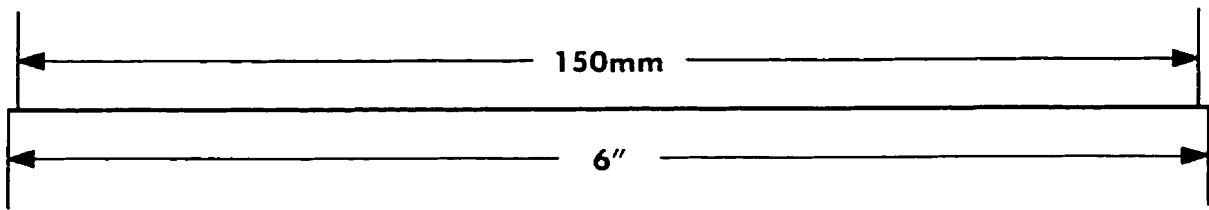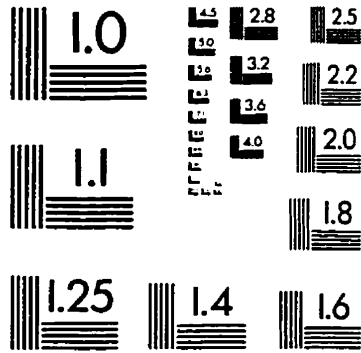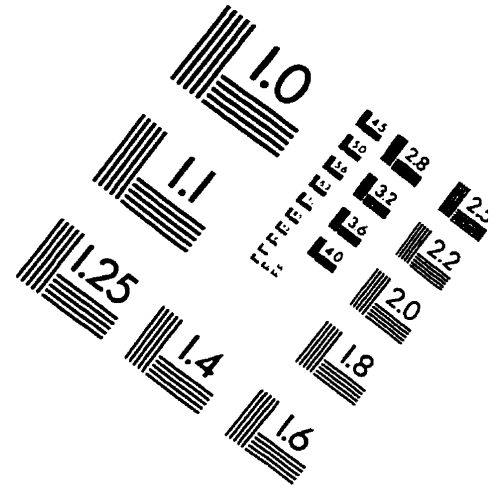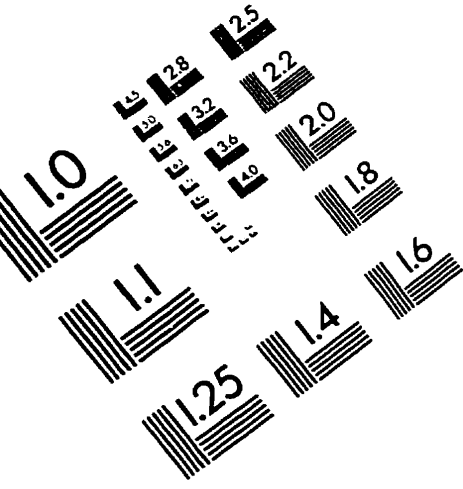
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IMAGE EVALUATION
TEST TARGET (QA-3)

150mm

6"